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RESEARCH ARTICLE

An efficient and rapid method to detect and verify natural antisense transcripts of animal genes

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Abstract

High-throughput sequencing has identified a large number of sense-antisense transcriptional pairs, which indicates that these genes were transcribed from both directions. Recent reports have demonstrated that many antisense RNAs, especially lncRNA (long non-coding RNA), can interact with the sense RNA by forming an RNA duplex. Many methods, such as RNA-sequencing, Northern blotting, RNase protection assays and strand-specific PCR, can be used to detect the antisense transcript and gene transcriptional orientation. However, the applications of these methods have been constrained, to some extent, because of the high cost, difficult operation or inaccuracy, especially regarding the analysis of substantial amounts of data. Thus, we developed an easy method to detect and validate these complicated RNAs. We primarily took advantage of the strand specificity of RT-PCR and the single-strand specificity of S1 endonuclease to analyze sense and antisense transcripts. Four known genes, including mouse *β-actin* and *Tsix* (Xist antisense RNA), chicken *LXN* (latexin) and *GFM1* (G elongation factor, mitochondrial 1), were used to establish the method. These four genes were well studied and transcribed from positive strand, negative strand or both strands of DNA, respectively, which represented all possible cases. The results indicated that the method can easily distinguish sense, antisense and sense-antisense transcriptional pairs. In addition, it can be used to verify the results of high-throughput sequencing, as well as to analyze the regulatory mechanisms between RNAs. This method can improve the accuracy of detection and can be mainly used in analyzing single gene and was low cost.

Keywords: natural antisense transcripts, transcription orientation, detection method, RNA sequencing, long non-coding RNA

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1. Introduction

Natural antisense transcripts (NATs) are widespread in the mammalian transcriptome and consist of both coding and non-coding regulatory RNAs. It is estimated that at least 22–40% of genes have an antisense partner (Chen *et al.* 2004; Katayama *et al.* 2005; Engstrom *et al.* 2006). Recently, RNA sequencing (RNA-seq) technology has also

identified overlapping transcripts and double-stranded RNA (Lapidot and Pilpel 2006), which indicates that an increasing number of NATs are transcribed from the opposite DNA strand of genes, such as the NATs of *MHC* (myosin heavy chain) (Haddad *et al.* 2006), *ApoE* (apolipoprotein E) (Seitz *et al.* 2005), *p15* (cyclin-dependent kinase inhibitor 2B) (Yu *et al.* 2008) and *p53* (tumor protein p53) (Mahmoudi *et al.* 2009) genes. These NATs possess partially or completely complementary sequences with the sense transcripts and can interact with sense RNA through their complementary regions (Yu *et al.* 2008; Mahmoudi *et al.* 2009). A more accurate method is needed to detect and distinguish the sense-antisense RNA pairs transcribed from the same DNA region; otherwise, a false result may be obtained because of their complementary sequences.

Mouse (*Mus musculus*) *β-actin* and *Tsix* (X-inactive specific transcript (Xist) antisense RNA) genes have been well studied. The orientation and expression features of their transcripts have been identified (Ghosh *et al.* 2008; Navarro *et al.* 2009). Mouse *β-actin* was transcribed along with the negative strand of the DNA while *Tsix* was obtained along with the positive strand of the DNA during mouse postnatal development (McCarrey *et al.* 2002; Navarro *et al.* 2009). In addition to that, our group conducted a strand-specific-transcriptome sequencing and a digital gene expression (DGE) sequencing on chicken liver, we found that many transcripts were transcribed in the same DNA region but in opposite directions including chicken (*Gallus gallus*) *LXN* (latexin) and *GFM1* (G elongation factor, mitochondrial 1) genes (Zhang *et al.* 2015). Also, the two genes annotated in GenBank (http://www.ncbi.nlm.nih.gov/gene/) were transcribed from opposite directions. Thus, using these four genes, we developed an easy and systematic method to detect natural sense, antisense and sense-antisense paired transcripts from the same DNA region. This approach can be widely used in verification tests and helps to avoid incorrect conclusions when both the sense and antisense RNAs are simultaneously expressed.

2. Materials and methods

2.1. Ethics statement

The Animal Care Committee of South China Agricultural University (Guangzhou, China) approved this study (approval number SCAU#0017). The animals involved in this study were humanely sacrificed as necessary to ameliorate suffering.

2.2. Animals and samples

Three BALB/c female mice and three hens (White Recessive Rock chicken, WRR) at the age of 7 weeks were used in this study. The animals were euthanized, and their livers were isolated and then rapidly frozen in liquid nitrogen. The livers were stored at –80°C until DNA and RNA extraction. Genomic DNA samples were isolated from mouse and chicken livers using a phenolic extraction protocol (TaKaRa, Dalian, China); the samples were used for DNA contamination detection. Total RNA was extracted from the livers using Trizol (TaKaRa, Dalian, China) following the manufacturer's protocol. Genomic DNA and total RNA were tested *via* agarose electrophoresis.

2.3. Reverse transcription with random oligomers or gene-specific primers

1 μg of extracted total RNA was DNase-treated at 37°C for 30 min using 5 U of RNase-free DNase I (Promega, Beijing, China), followed by the addition of 1 μL EDTA (50 mmol L^{-1}) at 65°C for 10 min (Table 1). The strand specificity of sense or antisense cDNA was established by the use of specific primer. The R-cDNA of the sense RNA was synthesized by priming with the reverse PCR primer, whereas the F-cDNA of the antisense RNA was transcribed with the forward PCR primer in the reverse transcription (RT) reaction (Tables 2 and 3). Both RT reactions were performed at 50°C for 30 min using Amv reverse transcriptase (Promega, Beijing, China), followed by heating for 15 min at 95°C. In addition, the conventional cDNA without strand choices was obtained using oligo$(dT)_{15}$ and random primers. The RT reactions were performed at 42°C for 30 min using AMV reverse transcriptase, followed by heating for 15 min at 95°C. All obtained cDNAs were stored at –20°C.

Table 1 Reaction mixture of genomic DNA removed from total RNA

Reagents	Additive amount (μL)	Concentration
Nuclease-free water	6.75	–
10× reaction buffer with $MgCl_2$	1	–
RNase inhibitor	0.25	20 U μL^{-1}
DNase I, RNase free	1	5 U μL^{-1}
Total RNA	1	1 000 ng μL^{-1}

Table 2 Reverse transcription with gene-specific primers or random oligomers

Reagents	Additive amount (μL)	Concentration
$MgCl_2$	4	25 mmol L^{-1}
Reverse transcription 10× buffer	2	–
dNTP Mix	2	10 mmol L^{-1}
RNase inhibitor	0.1	20 U μL^{-1}
F or R primer or random oligomers	0.1	10 μmol L^{-1}
Amv reverse transcriptase	0.25	20 U μL^{-1}
Nuclease-free water	0.55	–