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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

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

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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	–

Table 3 Sequences of primers used for gene-specific RT and PCR

Primer[1)]	Sequence (from 5′→ 3′)	Product length	Tm (°C)
M-*β-actin*	F: TCCGGCATGTGCAAAGC R: TCTTCTCCATGTCGTCCCAGT	214 bp cDNA (301 bp DNA)	56
C-*β-actin*	F: TCATTGTGCTAGGTGCCA R: CCTCTTCCAGCCATCTTT	197 bp cDNA (503 bp DNA)	53
M-*Tsix* (Xist antisense RNA)	F: TAGTCCTCTGCGGCTTCC R: TGCTGATCGTTTGGTGCT	350 bp	57
C-*LXN* (latexin)	F: AAAGAAAGCAGGATGAAATATGGAG R: ATGCGAGAATGACTGTGGAATGAC	205 bp	55

[1)] M, mouse; C, chicken.

2.4. S1 endonuclease treatment of RNA and conventional cDNA

The obtained RNA and conventional cDNA were respectively treated with 0.1 μL S1 endonuclease (TaKaRa, Dalian, China) at 23°C for 30 min, followed by 5 min of heat at 95°C. The reaction mixture was listed in Table 4.

Table 4 Reaction components of the RNA and conventional cDNA treated with S1 endonuclease

Components	Additive amount (μL)	Concentration
ddH_2O	17	–
10× S1 buffer	2	–
S1 nuclease	0.1	180 U μL^{-1}
RNA (or conventional cDNA)	1	1 200 ng μL^{-1}

2.5. Sense or antisense transcript detection

The gene transcription orientation was detected *via* PCR with the F-cDNA, R-cDNA or S1 endonuclease treated cDNAs as templates. The PCR program included 95°C for 5 min, followed by 34 cycles of 94°C for 30 s, annealing for 30 s, 72°C for 40 s, and a final extension at 72°C for 10 min. The PCR products were separated by electrophoresis on agarose gels and stained with ethidium bromide.

2.6. The endogenous RNA:asRNA pairs detection

The S1 endonuclease-treated RNA was transcribed by the use of specific primer at 50°C for 30 min with M-MLV reverse transcriptase (TaKaRa, Dalian, China). The obtained cDNAs were performed PCR according to the above program.

2.7. Sequence confirmation

All the obtained PCR products were submitted to sequence by Sangon Biotech Company (Guangzhou, China).

3. Results

3.1. DNA contamination detection

Mouse and chicken *β-actin* genes were used to detect DNA contamination in the obtained cDNA. The PCR products of 214 bp (chicken, 197 bp) electrophoretic bands were identified when the individual mouse (chicken) cDNA was used as the template; 301 bp (chicken, 503 bp) bands were identified when the control DNA was used as the template, which confirms that there was no genomic DNA contamination of the cDNA samples (Fig. 1).

3.2. Strand-specific PCR for sense or antisense transcription analysis

The transcript mechanisms of the mouse *β-actin* and *Tsix* genes have been clearly demonstrated (Ghosh *et al.* 2008; Navarro *et al.* 2009). Thus, both genes were selected to establish the transcriptional orientation detection method. PCR was conducted with the strand-specific transcription F- or R-cDNA. Electrophoretic detection indicated that a 214-bp band was detected when F-cDNA was the template, whereas no PCR products were detected when R-cDNA was used as the template (Fig. 2-A). These findings indicated that the negative strand, but not the positive strand, of the mouse *β-actin* gene was transcribed, whereas for the mouse *Tsix* gene, the opposite results were obtained (Fig. 2-B). Sequencing the obtained PCR products and nucleotide sequence alignments confirmed both genes.

3.3. Further verification of strand-specific PCR results

To validate the previously described strand-specific PCR results of both genes, we treated the conventional cDNA with S1 endonuclease, which was followed by PCR analysis. S1 endonuclease is a single-strand-specific endonuclease and can specifically degrade single-strand cDNA. Thus, if one gene was transcribed from both strands, the sense and antisense transcripts formed a duplex and could not be recognized by the S1 endonuclease. If one gene was transcribed from one orientation, the cDNA would

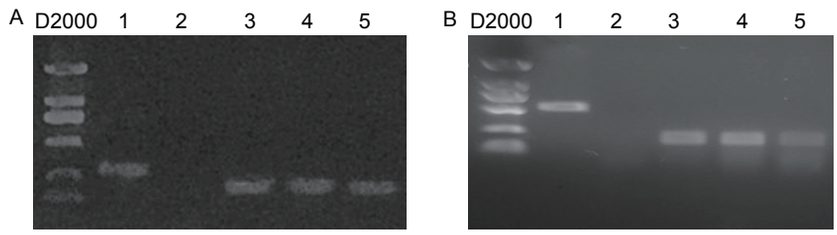

Fig. 1 Mouse and chicken *β-actin* genes were used for DNA contamination detection. None of the cDNA utilized in this study yielded 301 bp (A, mouse) or 503 bp (B, chicken) products, which confirms the effectiveness of the DNase treatment and the absence of cross-contamination. D2000, DNA marker, which the bands represent 2 000, 1 000, 750, 500, 250 and 100 bp; lane 1 was PCR product using genomic DNA as template; lane 2 was no template control; lanes 3–5 were PCR products using cDNA as template.

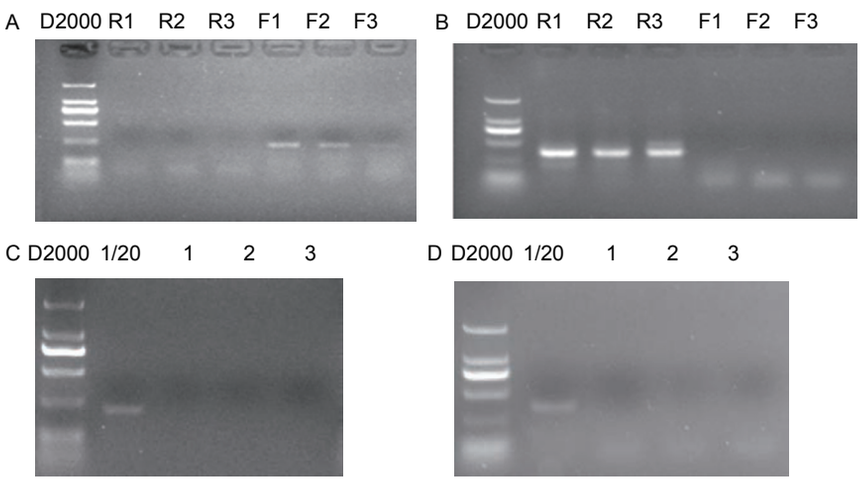

Fig. 2 Mouse *β-actin* and *Tsix* gene transcriptional orientation detection. Mouse R-cDNA (R1–R3) and F-cDNA (F1–F3) from three individuals were used as PCR templates. *β-actin* PCR products were detected when F-cDNA was used (A), whereas *Tsix* genes were detected when R-cDNA was used (B). No products of both *β-actin* (C) and *Tsix* (D) genes were detected when S1 endonuclease treated cDNA was as template, with the exception of the control cDNA diluted 20 times (1/20) with ddH_2O.

be recognized and degraded. Electrophoretic analysis demonstrated that no PCR products of these genes were detected when the S1 endonuclease treated cDNA was used as the templates; however, the positive control cDNA diluted 20 times with ddH_2O was detected, which indicates that both the mouse *β-actin* and *Tsix* genes were transcribed from a single orientation (Fig. 2-C, D). Combined with the strand-specific PCR results, we conclude that the *β-actin* gene possessed an antisense transcript while *Tsix* exhibited a sense transcript.

3.4. Comparison of the obtained results with the gene annotations in GenBank

The published gene transcription orientations (http://www.ncbi.nlm.nih.gov/gene/) demonstrated that the mouse *β-actin* gene was transcribed along the direction of the negative strand (Fig. 3-A), whereas the *Tsix* and *Xist* genes were transcribed from both directions (Fig. 3-B). Mouse *Xist*, which is related to X chromosome inactivation, cannot be transcribed during mouse postnatal development (McCarrey *et al.* 2002; Navarro *et al.* 2009). Thus, the transcription orientations of the mouse *β-actin* and *Tsix* genes detected in this study were the same as the orientations annotated on the website, which indicates that the method used to detect sense and antisense transcripts was reliable.

3.5. Orientation detection of genes transcribed from both directions

We further analyzed if this method could be used to detect genes transcribed from both directions. Gene annotations in GenBank indicated that the chicken *LXN* and *GFM1* genes were on the same DNA region; however, they were transcribed from the opposite directions (Fig. 4-A). Thus, we first performed strand-specific RT and conventional RT, respectively, followed by strand-specific PCR and S1 endonuclease treatment according to the previous manipulation.

The primers used for the strand-specific RT and PCR were located in the overlapping region. The strand-specific RT-PCR demonstrated that both products of F- and R-cDNA can be detected, which indicates that the DNA region was transcribed from both directions (Fig. 4-B). Furthermore, PCR product sequencing confirmed the target genes. PCR detection using S1 endonuclease treated cDNA as a template also confirmed the results (Fig. 4-C). These findings indicate that this method is valid for the analysis of genes transcribed from both directions.

3.6. The endogenous RNA:asRNA hybrids detected by S1 endonuclease

Many sense-antisense transcript hybrids have been detected by S1 endonuclease protection assay (Yu *et al.* 2008; Zghidi-Abouzid *et al.* 2011). The cDNAs duplexes produced during RT suggested the RNA:asRNA could hybridize *in vivo*. This question was examined by treating total RNA with the single-strand specific nucleases S1. PCR detection confirmed the RNA hybrids existed which indicated that the overlapping region of *LXN* and *GFM1* genes transcribed from both directions (Fig. 5). The result shows that the proposed strategy to detect sense-antisense pairs works.

4. Discussion

With the development of high-throughput sequencing, an increasingly greater number of genes have been demonstrated to be transcribed from both strands (Chen *et al.* 2004; Engstrom *et al.* 2006; Lapidot and Pilpel 2006). An easy approach was needed to detect and validate these complicated RNAs. In the current study, a technique was developed, which took advantage of the strand specificity

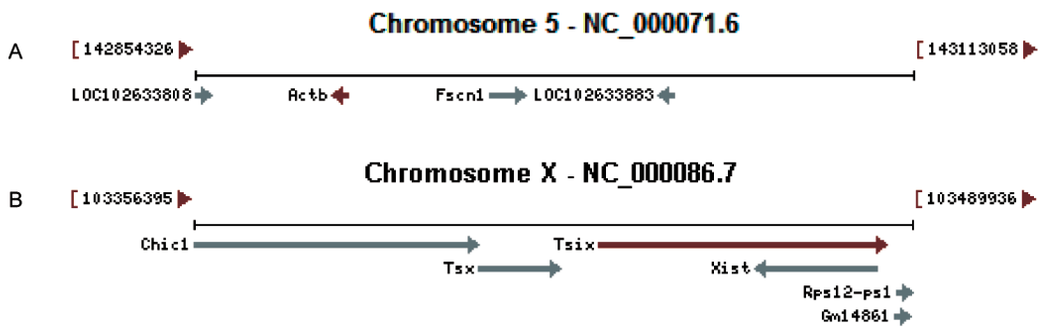

Fig. 3 Transcription orientations of the mouse *β-actin* and *Tsix* genes annotated on the website (the figure was cited from http://www.ncbi.nlm.nih.gov/gene/). The black arrows represent the transcriptional directions of the mouse *β-actin* (A) and *Tsix* (B) genes.

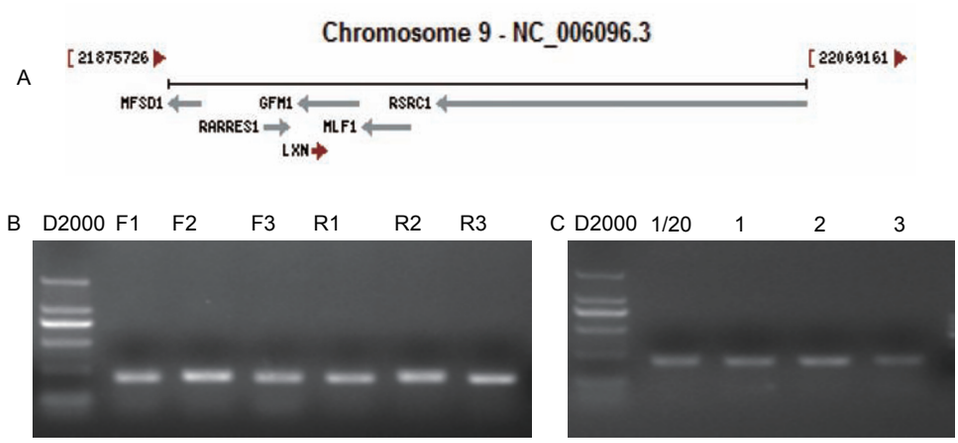

Fig. 4 Transcriptional orientation detection of the chicken *LXN* (latexin) and *GFM1* (G elongation factor, mitochondrial 1) genes. A, chicken *LXN* and *GFM1* genes transcribed from opposite directions in the same DNA region (the figure was cited from http://www.ncbi.nlm.nih.gov/gene/). The arrows represent the transcriptional orientations of the *LXN* and *GFM1* genes. B and C, the results of the strand-specific PCR (B) and S1-treated experiment (C) indicated the overlapping DNA regions of the *LXN* and *GFM1* genes were transcribed from opposite directions. 1/20, the control cDNA, diluted 20 times with ddH_2O, was used as PCR template. F1–F3 and R1–R3 in B represented the three F-cDNA and the three R-cDNA were used as PCR template, respectively.

of RT-PCR and the single-strand specificity of S1 endonuclease to analyze sense and antisense transcripts. Four known genes, including mouse *β-actin*, *Tsix*, chicken *LXN* and *GFM1*, were used to establish the method. The results indicated that the method can easily distinguish sense, antisense and sense-antisense transcriptional pairs.

To date, many techniques and methods, such as RNA-seq, Northern blotting and strand specificity PCR, can be used to detect gene transcriptional orientation and the antisense transcript. Our method has three advantages compared with other ones. Firstly, high-throughput sequencing has substantial costs and was adapted for whole transcriptome analysis (Yang *et al.* 2011). While our method can be mainly used in analyzing single gene and was low cost. Secondly, Northern blotting, strand-specific PCR and our method can primarily be used to analyze single gene. However, the Northern blotting protocol is complicated, RNA degradation can easily occur, and it involves a time-consuming process; furthermore, it lacks sensitivity and requires large amounts of RNA sample (Josefsen and Nielsen 2011). In contrast, our method exhibited a simple and easy operation. Thirdly, In comparison with the previously established strand-specific PCR (Haddad *et al.* 2007), our method integrated strand-specific RT-PCR with S1 endonuclease, which can improve the accuracy of detection.

cis-NATs can be classified as head-to-head (5′ to 5′), tail-to-tail (3′ to 3′), or partially or fully overlapping with the sense RNA according to their orientations and degrees of overlap (Lapidot and Pilpel 2006). S1 endonuclease exhibits a single-strand recognizing peculiarity that can help to deduce the double or single-strand transcription regions. Thus, it is important to locate the PCR primer positions when the method is used to verify an existing natural antisense transcript. Additional pairs of primers can be attempted, when necessary.

During strand-specific reverse transcription, temperature control is important. Firstly, all reagents should be placed on ice and all reactions should occur on ice until treatment on the machine. Secondly, reverse transcription should be performed at higher temperatures than usual, and the lowest temperature should be 50°C according to different genes. This high reaction temperature can improve the specificity of the reverse-transcriptase and increase the efficiency *via* the disruption of RNA secondary structures (Haddad *et al.* 2007). Thirdly, a reverse transcription mixture should be incubated at 95°C for 10 min at the end of reverse transcription to inactivate the reversetranscriptases.

Whether the reverse transcription is used with gene-specific primers or random oligomer primers, AMV reverse transcriptases, but not M-MLV reverse transcriptases, should be used. The intrinsic RNase H^+ activity of the AMV reverse transcriptase can gradually degrade the RNA as it is converted into cDNA, whereas the M-MLV transcriptase does not have this function (Konishi *et al.* 2012). The RNA-cDNA duplex transcribed by M-MLV transcriptase cannot be completely digested by S1 endonuclease, which can thus lead to misleading results.

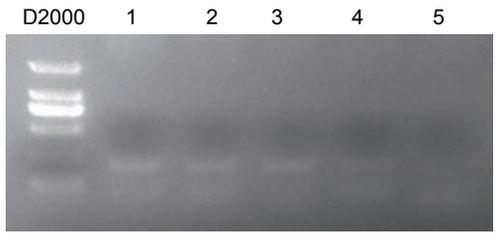

Fig. 5 RT–PCR amplification of the overlapping regions of *LXN* and *GFM1* genes after digesting RNA with nuclease S1. Lanes 1–4 were PCR products using chicken cDNA as template and lane 5 was no template control.

Recent reports have demonstrated the post-transcriptional process was an important regulation, especially for lncRNA (Ballarino *et al.* 2015; Wang *et al.* 2015). Sense and antisense transcripts, such as *Wrap53* and *p53*, can interact through their complementary regions, which results in double-stranded RNA structures that regulate the stability, transport, and/or translation of the sense transcript (Mahmoudi *et al.* 2009). Further, single strand recognition enzymes such as S1 endonuclease and RNae A have been used in sense/antisense RNA interaction research (Yu *et al.* 2008). Thus, our method could also be used to analyze the regulatory mechanisms between two molecules *via* the formation of duplex or other ways.

5. Conclusion

We developed an easy method to detect the antisense transcript of a single gene. This method can be used to verify the results of high-throughput sequencing, as well as to analyze the regulatory mechanisms between RNAs.

Acknowledgements

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(Managing editor ZHANG Juan)