A RecA-mediated exon profiling method

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ABSTRACT

We have developed a RecA-mediated simple, rapid and scalable method for identifying novel alternatively spliced full-length cDNA candidates. This method is based on the principle that RecA proteins allow to carry radioisotope-labeled probe DNAs to their homologous sequences, resulting in forming triplexes. The resulting complex is easily detected by mobility difference on electrophoresis. We applied this exon profiling method to four selected mouse genes as a feasibility study. To design probes for detection, the information on known exonic regions was extracted from public database, RefSeq. Concerning the potentially transcribed novel exonic regions, RNA mapping experiment using Affymetrix tiling array was performed. As a result, we were able to identify alternative splice variants of Thioredoxin domain containing 5, Interleukin1β, Interleukin 1 family 6 and glutamine-rich hypothetical protein. In addition, full-length sequencing demonstrated that our method could profile exon structures with >90% accuracy. This reliable method can allow us to screen novel splice variants from a huge number of cDNA clone set effectively.

INTRODUCTION

It is known that most mammalian genes are subjected to alternatively splicing, probably to maximize the diversity of proteins from the unexpectedly small number of genes encoded by the genome (1–4). Many alternative splicing transcripts have been reported to play critical roles in living cells, e.g. in disease cascades (5,6). Conventional, pairwise comparison of full-length cDNAs (7–12) and expressed sequence tags (ESTs) have been used to identify alternative transcripts. Although these methods are powerful tools to help us to identify individual alternative exons, it is still difficult to determine the entire exon structure including the information on exon boundaries, which is indispensable for functional analysis. From this aspect, collecting physical cDNAs is still the only solution for acquiring the whole structure of transcripts.

As a tool for expression profiling analysis, DNA microarray was innovated (13) and has been a widely used tool in finding responsible genes for various diseases (14). Recently, exon junction arrays (15) and oligonucleotide tiling arrays (10,16–19) have also been employed to study alternatively spliced transcripts. More recently, the newly developed exon arrays promise to identify alternative exons genome-wide. Among these methods are powerful tools to help us to identify individual alternative exons, it is still difficult to determine the entire exon structure including the information on exon boundaries, which is indispensable for functional analysis. From this aspect, collecting physical cDNAs is still the only solution for acquiring the whole structure of transcripts.

To shed light on potential alternative splice variants within the huge cDNA clone set, the most accurate and reliable way is to determine the entire sequence of all transcripts by sequencing. However, it is time-consuming and cost-consuming procedure and far from the reality. At present, $100 000 and $1000 genome projects are ongoing to develop next-generation type sequencers. Some pioneering sequencers are getting available (24), but these sequencers usually take shotgun-like sequencing strategy and would not be suitable

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors

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for full-length cDNA sequencing since it requires the correspondence of each clone to each sequence data. Therefore, if a novel and simple method for exon profiling is available, it would be useful to screen alternatively spliced transcripts from the huge cDNA clone set.

To this end, we have developed a simple RecA-mediated exon profiling method to investigate exon structures without sequencing. In this method, the sequence-specificity of homologous recombinase, RecA protein, was utilized (25–27). Radioisotope-labeled ‘probe’ oligonucleotides were first carried to their homologous sequences by RecA protein in a sequence-specific manner, resulting in pairing at the same sequences between the single-stranded (ss) and double-stranded (ds) DNA molecules. Although there is no stable triple-stranded DNA formation since RecA filaments within the strands are already exchanged (28), we call the paired DNA complexes as ‘triplex’. The triplexes were detected by mobility difference on electrophoresis. Full-length sequencing demonstrated that our method was able to detect exonic regions with high accuracy. Moreover, several novel splice variants were successfully identified with this method. Here we report the principle of the simple and reliable RecA-mediated method for exon profiling.

MATERIALS AND METHODS

The sequence information on fully sequenced cDNAs in this study has been deposited to DDBJ with the library name, RMEP. DDBJ accession nos = AK224911-AK225023.

Optimization of RecA-mediated exon profiling conditions

Oligonucleotides and target DNA. Oligonucleotides with identical and non-identical sequences to pUC19 (‘Target’ DNA) were designed from 10mer to 50mer with the increment of 10 bases. The pUC19 was used as a model to optimize the detection conditions. The oligonucleotides for detection are defined as probes in this study. After designing probes, a BLAST search (29) was performed to investigate whether unintended homologous sequences were present in the target DNA sequence. To avoid cross hybridization, the most specific probes to the target sequences were carefully selected. The probe sequences are listed in Supplementary Table S1. The pUC19 DNA was purified with Mini Prep kit according to the manufacture’s protocol (Qiagen). Oligonucleotides were purchased from Operon Biotechnology, Inc.

Labeling of oligonucleotides. Oligonucleotides were labeled at the 5’-terminus with T4 polynucleotide kinase (TaKaRa-Bio) in the presence of [γ-32P]ATP (3000 Ci/mmol; GE Healthcare Bio-Sciences Corp.) by incubating for 30 min at 37°C. To terminate the labeling reaction and to purify, 50 μl of resultant mixture was then, mixed with 1 μl of 0.5 M EDTA, 1 μl of 10% SDS, and 1 μl of 20 mg/ml Proteinase K and incubated for 30 min at 45°C followed by phenol-chloroform extraction. Then, the purified labeled DNA solution was applied to Microspin S-400 HR columns (GE Healthcare Bio-Sciences Corp.) to remove excess of [γ-32P]ATP.

The RecA-mediated triplex formation analysis. The probe DNAs were diluted to ~1 pmol/l and the triplex formation reaction was performed in 20 μl of the solution containing 25 mM Tris–acetate, 5 mM Magnesium acetate, 1 mM DTT and 0.1 mM ATPγS (Roche Diagnostics), 3 μg of Escherichia coli RecA protein (New England Biolabs) and 50 ng of pUC19 plasmid. The reaction mixture was incubated for 1 h at 37°C. The reaction was terminated by placing on ice and adding 6× loading dye, which is composed of 10 mM Tris–HCl (pH 7.6), 0.03% bromophenol blue, 0.03% xylene cyanol FF, 60% glycerol and 60 mM EDTA; and then it was followed by 1.2% agarose gel electrophoresis in 1× TAE buffer. The gel was dried with a gel drier (Bio-Rad Laboratories), and exposed to an IP sheet (Fuji Photo Film Co., Ltd) for 3–5 h. The exposed sheet was analyzed by BAS2500 (Fuji Photo Film Co., Ltd).

The RecA-mediated exon profiling analysis using RIKEN clone sets

Selection of drug-related genes from the RIKEN Clone Bank. The RIKEN clone sets were used for this analysis. Four human genes, which were related to commercially available drugs, were randomly selected. With the sequences of these four drug-related genes, the mouse orthologs to these genes were searched with HomoloGene (http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=homologene). The RIKEN cDNA clones corresponding to these mouse orthologs were computationally identified using their gene locus information. When necessary, Gene Ontology information was used to select the RIKEN cDNA clones that included similar functional domains to drug-related genes. The E.coli carrying the selected cDNA sequences in plasmids were cultivated, and the plasmids were purified with Qiagen Mini preparation kit (Qiagen) for further analyses.

RNA mapping experiment with tiling arrays. To obtain the information on novel exon candidates, we used the GeneChip Mouse Tiling Array ver. 1.0 (Affymetrix). The RNAs, which were used for cDNA library construction in the RIKEN mouse encyclopedia project, were utilized for microarray-based RNA mapping. A total of 66 different RNA samples with the same amount were mixed and 10 μg of the mixed RNA were used for hybridization. The tissue/cell names of the mixed 66 RNAs are provided in Supplementary Table S2. The first-strand cDNA was synthesized with random primers using SuperScript II (Invitrogen). The resulting cDNAs were then used as template for the second-strand cDNA synthesis under conditions described in the manufacture’s protocol. After the second-strand synthesis, the remaining RNAs were degraded using a combination of RNaseA/T1 cocktail (Ambion) and RNaseH (Ambion). The dscDNAs were then purified with PCR purification kit (Qiagen, Inc.) and fragmented with DNase I, and end-labeled with biotin as described previously (10). One set of whole genomic tiling array comprises 16 separate chips. For each chip, ~4 μg of the end-labeled cDNA samples were applied. The labeled DNAs were hybridized to the array chips for 16–18 h at 45°C. The array chips were washed as per the manufacture’s protocol, and scanned using a GeneChip Scanner 3000 7G (Affymetrix, Inc). The hybridization experiments were carried out in duplicate.
by joining positive probes (signal intensity > significant level of expression was detected, were identified by INTEGRATED GENOME BROWSER software. The signal intensity was given on log2 scale as shown in Figure 3. The median generated by the Wilcoxon test was assigned as an estimate of the signal intensity to the probe position. The signal intensity was given on log2 scale as shown in Figure 3.

The graphs from these processed data were generated by INTEGRATED GENOME BROWSER software (Affymetrix, Inc.). The transfrags, genomic regions where a significant level of expression was detected, were identified by joining positive probes (signal intensity >7.4) separated by less than a certain distance (maxgap = 100 bp) and selecting regions with an extension of ≥30 bp (minrun).

### Probe design and exon profiling analysis
Oligonucleotide probes were designed according to the following work order: (i) Extraction of known and candidate exonic regions for the drug-related genes from RefSeq and transfrag data (transcriptional fragments data from tiling arrays). (ii) Designing of all possible combinations of 30mer oligonucleotide sequence probes within each extracted exonic region. (iii) Comparison of each 30mer oligonucleotide sequence with the cloning vector sequence by BLAST. The most specific oligonucleotide sequences for detection were chosen. All oligonucleotides were purchased from Operon Biotechnology, and the probes for RIKEN clone sets were prepared as described above. Exon profiling for RIKEN clone sets was performed as described above, except that 96-well plates were used (Abgene, Inc.).

### Full-length cDNA sequence determination
All cDNA clones clustered within the Tnxdc5 locus and all alternative splice variant candidates in IL-1β, IL-1F6 and glutamine-rich hypothetical protein (GR) were fully sequenced by using an ABI3700 capillary sequencer (Applied Biosystems) and Big Dye terminator sequencing kit (Applied Biosystems). For sequencing, primers were designed for every 300 bp on the sequence of fully sequenced representative clones. Their sequences were base-called by Phred (30) and assembled by Phrap (31).

Further characterization of newly identified splice variants. The sequences of splice variant candidates were compared to sequences in genetic databases; GeneBank, the Mouse Genome Database and TIGR databases. The comparison was performed using the nucleotide-to-nucleotide sequence local alignment algorithm, BLASTN. In addition, deduced amino acid sequences for longest ORF were computationally determined. Finally, the splice variant candidates were analyzed with the SCOP (32), Pfam (33) and InterPro (34,35) programs for identifying potential functional domains. SOSUI program (36) was also applied for transmembrane protein prediction.

### RESULTS

#### Principle of RecA-mediated exon profiling method
In this study, radioisotope-labeled ss oligonucleotides were defined as probes, and dsDNAs containing their homologous sequences were called as targets. The principle of our simple exon profiling method utilizing RecA protein is described in Figure 1. At the first step, radioisotope-labeled oligonucleotide probes were incubated with RecA protein and Adenosine 5'-O-(3-thio)triposphosphate (ATPγS), which stabilizes the interaction of a short ss oligonucleotide with dsDNA (37,38). The resulting complex was further incubated with dsDNA to pair with its homologous sequence. And then, the paired complex, ‘triplex’ formation was evaluated by mobility difference on agarose electrophoresis and by autoradiography.

#### Examination of probe length in RecA-mediated exon profiling method
Our computational analysis using the FANTOM cDNA clone set showed that out of 126,322 internal exons ~93% of the exons were longer than 50 bp and 99.3% of the exons were longer than 30 bp (S. Kondo, unpublished data), indicating that the detection for exons shorter than 50 bp is required. To determine the minimum probe length in our method, various probes in length were examined using pUC19 as a target dsDNA.
Each probe DNA labeled with $^{32}$P at the 5' end was incubated with RecA, ATPyS and pUC19 DNA, as described in Materials and Methods. The resultant products were electrophoresed, and signals were detected by autoradiography. As shown in Figure 2, the RecA-mediated triplex formation corresponding to the presence of probe sequence was detected with mobility difference on electrophoresis. Although the signals derived from triplexes were detected down to 10mer probes, the signals using 20mer and 10mer probes were faint and seemed not to be reliable (Figure 2, lanes 7 and 9). As for non-homologous probes, no mobility change was detected (Figure 2, lanes 2, 4, 6, 8 and 10), indicating that triplex formation with pUC19 DNA occurred in a sequence-specific manner. In addition, it was also verified that the triplex formation took place in a RecA protein-dependent manner (Figure 2, lane 11). Altogether, we decided to utilize 30mer probes for further analyses, and it means that our method is able to detect >99% of internal exons theoretically.

**Selection of gene loci for evaluation**

To evaluate the RecA-mediated exon profiling method, we next selected four drug-related gene loci for exon profiling from the RIKEN clone bank: Thioredoxin domain containing 5 (Txndc5), Interleukin 1 beta (IL-1$b$), Interleukin 1 family, member 6/IL-1F6 and glutamine-rich hypothetical protein. Through our FANTOM activity, all collected cDNA clones were clustered with their EST information on their genomic loci, intending to encompass all overlapping transcripts derived from the same strand of a single gene locus (21). These clusters are called as transcriptional units (TUs) (21). All cDNA clones clustered within the four selected TUs were computationally identified. As shown in Table 1, a total of 139 clones were clustered into the four loci, and the Txndc5, IL-1$b$,

<table>
<thead>
<tr>
<th>Table 1. Summary of exon profiling</th>
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<tr>
<td>Annotated name/Gene ID/symbol</td>
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<tr>
<td>-----------------------------------</td>
</tr>
<tr>
<td>Thioredoxin domain containing 5 (Txndc5)/105245/Txndc5</td>
</tr>
<tr>
<td>Interleukin 1 beta (IL-1$b$)/16176/Il1b</td>
</tr>
<tr>
<td>Interleukin 1 family, member 6/ (IL1-F6)/54448/Il1f6 glutamine-rich hypothetical protein (GR)/73332</td>
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<tr>
<td>Total</td>
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**Extraction of known exons and candidate exons**

To design probes for exon profiling, all exons were divided into two categories: known exons and candidate exons. The known exons were extracted in silico from RefSeq for the four gene loci. As for the candidate exons, RNA mapping using Affymetrix mouse whole genome tiling array was performed. The RNAs from 66 different tissues/cells were mixed and used for hybridization. We generated the signal intensity using the standard tiling array protocol implemented in the Affymetrix software, TAS (17). The transcription fragments (transfrags) with signal intensity above 7.4 on four different loci were generated as potentially transcribed regions. The transfrags, RefSeq and the RIKEN FANTOM representative loci were compared (Figure 3). The transfrags which did not overlap with any known exonic regions were considered as novel exon candidates (striped boxes).
candidate exons. Number of extracted candidate exons on each gene is summarized in Table 1.

Although RNA mapping data using tiling arrays can include false positive transfrags, all transfrag data were used for designing probes because the purpose of this study was to identify alternatively splice variants as many as possible. Within the selected four gene loci, the number of the known exons was 45, and that of novel exon candidates was 36. In this study, one 30mer probe was designed for each 100 bp. If an exon exceeded 100 bp in length, each probe was designed near the ends of exons to improve the efficiency of detection for different exon length. For the four gene loci, 58 probes and 36 probes were designed for known exons and novel candidate exons, respectively.

**Evaluation of RecA-mediated exon profiling**

As the first step, we evaluated our RecA-mediated exon profiling method by using the RIKEN cDNA clones clustered within Txndc5 locus. In this locus, 95 RIKEN cDNA clones were entered (Supplementary Table S7). For exon profiling, 13 probes for 10 known exons and 3 probes for 3 novel candidate exons were designed with following the rule described above. The probe sequences are represented in Supplementary Table S3. In total, 1520 assays (=95 cDNA clones x 16 probes) were performed with the RecA-mediated method. One typical result is shown in Figure 4b. To evaluate the method precisely, the full-length sequences of the 95 cDNA clones were determined by sequencing and were compared with the results from our method. Out of 95 cDNA clones, 25 clones were alternative splice variants.

In this study, the specificity was defined as ‘the number of assays in which the presence/absence of probe sequences was correctly detected by our method’ over ‘the number of total assays’. Concerning the sensitivity, it was defined as ‘the number of assays in which triple-stranded complex formations were detected by RecA-mediated method’ over ‘the number of assays in which triplex formations should be detected due to full-length sequencing’. With these definitions, the specificity and the sensitivity of our RecA-mediated method were calculated as 93.3% [(1520) – (15 + 86)]/(1520) and 91.9% (976/1062), respectively. Result is summarized in Table 2.

**Identification of alternative splice variants on three selected gene loci**

Since the reliability of this method could be confirmed as above, we further applied this method to three other selected gene loci, IL-1β, IL-1F6 and glutamine-rich hypothetical protein. As for these three gene loci, only splice variant candidates which were selected by the RecA-mediated method were fully sequenced. The result of the exon profiling on these three loci was summarized in Table 3.

In IL-1β locus, 12 probes for known exons and 2 probes for novel exon candidates were designed (Figure 5a), and 14 cDNA clones were subjected to exon profiling. The probe sequences and the list of clone IDs are provided in Supplementary Tables S4 and S8, respectively. The exon profiling experiment suggested that three clones were alternative splice variants. In one of these splice variant candidates, triplex formation was detected for the probe 3, which was designed on a predicted novel exon (Figure 5b, lane 13). Following full-length sequencing demonstrated that an intron retention phenomenon was successfully detected (clone 13 in Figure 5b–d), and that this cDNA clone (P830203M10) was confirmed to be a novel alternative splice variant. It should be a significant example for the combination of RNA
mapping with RecA-mediated exon profiling method. Concerning the remaining two candidates, the probe (Probe 5) designed on the known exon (Exon 4) did show no triplex formation, implying the lack of the corresponding exon (Figure 5a and c, lanes 2 and 4). However, sequencing data showed that one of the clones (I830007B19) was wrongly selected by the RecA-mediated exon profiling method. The probe 5 was designed on a known exon (exon 4). (d) Exon structures of three alternative splice candidate transcripts (clones 2, 4 and 13). Closed boxes and lines indicate exons and introns, respectively. The clone IDs for the clones 2, 4 and 13 are indicated at the bottom.

In IL-1F6 locus, 11 probes on 6 known exons and 3 probes on 3 transfrags, and 17 cDNA clones were employed to exon profiling (Supplementary Tables S5 and S9). Out of 17 clones, 2 clones were detected as alternatively splice candidates with lacking the sequence of probe 6 designed on exon 4. Full-length sequence demonstrated that the two clones (Supplementary Figure S2, lanes 1 and 11) lacking the exon 4 were identical to each other even at the nucleotide level. Compared with the sequence of the known cDNAs, these two were proved to be novel splice variants (Figure 6b).

Concerning the glutamine-rich hypothetical protein locus, 13 cDNA clones were clustered into this locus, and 22 probes on 22 known exons and 28 probes on 28 novel exon candidates were designed for exon profiling (Supplementary Tables S6 and S10). In this locus, eight different splice patterns were already reported. The RecA-mediated exon profiling suggested that seven clones had different splice patterns from the reported ones. Out of these seven clones, four clones were bona fide novel splice variants. Two of the four clones showed the same splice pattern each other, resulting in identifying three novel splice patterns (Supplementary Figure S3).

Altogether, RecA-mediated exon profiling analysis could identify eight alternative splice variants from two, two and four clones of IL1β, IL-1F6 and glutamine-rich hypothetical protein, respectively.
Characterization of functionally altered variants

Following the confirmation of newly identified variants by sequencing, we characterized them with several analyses.

The InterPro analysis indicated that the known Txndc5 cDNA has a Thioredoxin domain 2 (IPR: 006663), expanding between exon 1 and exon 9. Out of the 25 clones clustered in this locus, on the other hand, 21 clones lacked the exon 1 or used an alternative exonic region as exon 1 (5'-most exon), implying the alteration of function for novel splice variants. Consistent with this observation, the SOSUI program predicted that the cDNA containing the known exon 1 carried a signal peptide and was an insoluble protein, but the splice variant lacking the exon 1 became soluble without the signal peptide.

IL-1β is a well-known cytokine with various important biological functions. It has been reported to be a central mediator of inflammation and connective tissue destruction in rheumatoid arthritis. The IL-1β activates articular chondrocytes to produce matrix metalloproteinase-1 (MMP-1), which is capable of dismantling the collagen scaffold of articular cartilage (39). Within the IL-1β locus, two novel splice patterns were identified as mentioned above. One of these lacked first 4 exons, implying a truncated clone. To make sure whether it is a truncated cDNA or not, the sequence of this clone was compared with the information on transcription start sites derived from CAGE and GIS tags (40, 41). As a result, two CAGE tags corresponding to the position of 5' end of splice variant, suggested that this clone should be a full-length cDNA. In addition, the InterPro analysis indicated that this short clone lacked the IL-1β domain and had an 'unintegrated transmembrane region' instead (Figures 6a and 7).

IL-1F6 is involved in a wide array of biological activities that initiate and promote the host response to injury or infection by activating a set of transcription factors (42). From our analysis, we found that two clones, which were identical each other, lack a cytokine IL-1-like domain (IPR: 008996). The cytokine IL-1 domain is known to have a function in stimulating mRNA and protein synthesis level of insulin-like growth factor 1 (IGF-1), possibly promoting the intimal hyperplasia (43). The novel splice variant, lacking the cytokine IL-1-like domain may lead to alteration of transcriptional level or even translational level of IGF-1.

DISCUSSION

In this study, we have developed an effective exon profiling method by utilizing RecA protein. This method allows us to screen alternatively spliced transcripts/cDNAs from a cDNA clone set with high accuracy. With this method, we successfully identified splice variants from four independent gene loci.

Recent cDNA projects largely contributed to understand the complexity of transcriptome to describe its detailed picture. However, limited number of cDNAs was fully sequenced, and most of collected cDNAs were clustered into TUs with their EST information and stored without being fully sequenced, implying that many of novel splice variants remain to be sequenced. In our RIKEN cDNA clone bank, a few million of cDNAs were clustered into 28 163 TUs, and 53% (14 994 TUs) of all TUs include more than two clones within each TU. In addition, 18% (5019 TUs) of all TUs contain >10 clones/TUs, and the number of clones for 163 TUs is >100 entries. In this study, we used 96-well plates for exon profiling, and were able to profile all exon structures of 95 clones easily. This fact strongly suggests that our RecA-mediated exon profiling method is applicable to most TUs of RIKEN clone bank. In other words, our method should be a considerable option for the first screening to identify novel splice variants, especially in the case that one looks for splice variants of certain genes.

To consider exon profiling, PCR technique can be another possible option. When sequence information about known exons and candidate exons are available, exon profiling can be carried out by PCR with primers corresponding to exonic regions. Designing primers for PCR, however, requires considering the melting temperature of primers and/or careful selection to avoid mis-priming of primers. In addition, ends of exonic regions tend to be conserved (44), and this fact might cause difficulties on designing primers for PCR. In contrast, our detection method using RecA protein allows us to design probe DNAs more flexibly because of its little sequence restriction. Moreover, regardless of probe sequences, RecA reaction can be simply performed with incubation mixture at 37°C. This flexibility and easiness of the RecA-mediated method should be advantageous to profile exon structures.

It should be noted that the RecA-mediated method has some limitations for exon profiling. One possible limitation is the difficulty on designing probes for unknown/novel exons. In our analysis, novel exon candidates were experimentally extracted from the microarray-based RNA mapping since the RNAs utilized for cDNA library construction were available. This successfully led us to identify a novel splice variant of IL-1β. In contrast, full-length sequencing for Txndc5 gene showed that two novel splice variants harboring unknown exons were not detected by the RecA-mediated method because the corresponding exons were not predicted as novel exons through RNA mapping. In addition, out of total 36 novel exon candidates, the sequences of 35 candidates could not be found within any fully sequenced cDNAs.

Figure 7. Alignment of known transcript, newly identified splice variant and CAGE tags. CAGE tags are represented with closed arrowheads. Boxes and lines represent exons and introns, respectively. Closed boxes indicate the region of protein-coding sequence. Tissue names of origin are indicated.
from four selected genes. It implies the possibility that some rare transcripts were diluted by mixing RNAs and signals for these transcripts were below the detection limit of microarray. Better prediction for novel exon candidates is required. In the case that target tissues and genes are determined, the number of RNA pools to mix can be kept as less as possible, it would help to improve the prediction for novel exon candidates. However, this might increase the risk of cross hybridization instead. To compensate, exon prediction programs such as GENSCAN (45) and Exoniphy (46) should be utilized for predicting novel exon candidates.

Another possible limitation is the difficulty on detecting exons, which are different in length. It has been known that many splice variants carry exons, especially 5′- and 3′-most exons, which are different in length. In our analysis, one probe per 100 bp was designed, and our method failed to detect the difference of exon length for the 5′-most and 3′-most exons in Txndc5 at 6% (12/190). To detect this kind of exons more precisely, probes should be designed more frequently with the help of statistical model (47).

SUPPLEMENTARY DATA
Supplementary Data are available at NAR Online.

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Conflict of interest statement. None declared.

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