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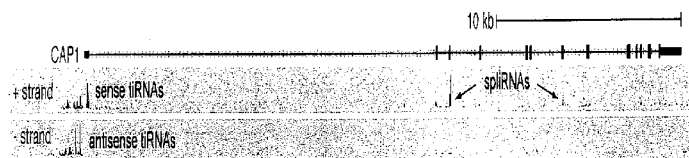


FIG. 3

(57) Abstract: The invention relates to a class of small RNA molecules involved in gene regulatory activities. More particularly, this invention relates to non-protein-coding, splice-site associated small RNA molecules linked to gene regulatory activities and uses thereof.

TITLE

SMALL RNA MOLECULES AND METHODS OF USE

FIELD OF THE INVENTION

5 THIS INVENTION relates to RNA molecules. More particularly, this invention relates to non-protein-coding, splice-site associated small RNA molecules linked to gene regulatory activities and uses thereof.

BACKGROUND OF THE INVENTION

10 In recent years, numerous small RNAs with interesting biological roles have emerged. These small RNAs, which are present in all kingdoms of life, are thought to be involved in many, if not most, fundamental cellular processes (Chu and Rana, 2007; Mattick and Makunin, 2005). For example, the best-studied class of small regulatory RNAs, micro RNAs (miRNAs), are predicted to regulate
15 thousands of eukaryotic genes (Pillai *et al.*, 2007; Vasudevan *et al.*, 2007) and their dysregulation is associated with various diseases and conditions (Rooij and Olson, 2007; Zhang *et al.*, 2007).

 More recently, advances in RNA sequencing techniques have led to the detection of new members of established classes of small RNAs (Ghildiyal &
20 Zamore, 2009; Malone & Hannon, 2009), and to the discovery and characterization of several classes of promoter-proximal species including 5' capped promoter-associated small RNAs (PASRs) (Fejes-Toth *et al.*, 2009), TSSa-RNAs (Seila *et al.*, 2008), and transcription initiation RNAs (tiRNAs) (Taft *et al.*, 2009 (I)).

25

SUMMARY OF THE INVENTION

 It has previously been established that small RNA molecules (*e.g.*, miRNAs, piwi-interacting RNAs (piRNAs) and tiRNAs) are widespread in mammalian genomes. However, it has remained unclear as to whether there are as
30 yet unidentified classes of small non-coding RNAs involved in regulating transcription and developmental pathways in mammalian and other genomes. The present invention has arisen from the inventors' unexpected discovery of a new class of small RNA molecules involved in gene regulatory activities that are

distinguishable from any previously identified class of small RNA molecules, including tRNAs.

In a first aspect, the invention provides an isolated substantially single-stranded RNA molecule that comprises a nucleotide sequence comprising no
5 more than 26 contiguous nucleotides that correspond to a genomic DNA sequence associated with gene regulation, wherein said nucleotide sequence comprises a nucleotide sequence corresponding to a 3' nucleotide sequence of an internal exon of said genomic DNA sequence.

Typically, the isolated RNA molecule comprises a nucleotide sequence
10 that corresponds to a sense strand of the internal exon of the genomic DNA sequence.

In one preferred form, said isolated RNA molecule terminates in a 3' nucleotide sequence that corresponds to the 3' end of the internal exon of the genomic DNA sequence.

15 In another preferred form, said isolated RNA molecule consists of a nucleotide sequence that corresponds to the 3' end of the internal exon of the genomic DNA sequence.

In one preferred form, said isolated RNA molecule comprises a nucleotide sequence comprising 14-26 contiguous nucleotides.

20 In another preferred form, said isolated RNA molecule comprises a nucleotide sequence comprising 14-20 contiguous nucleotides.

In yet another preferred form, said isolated RNA molecule comprises a nucleotide sequence comprising 16-18 contiguous nucleotides.

In still yet another preferred form, said isolated RNA molecule comprises
25 a nucleotide sequence comprising 17 or 18 nucleotides.

Typically, although not exclusively, the isolated RNA molecule comprises a nucleotide sequence that is located in, or obtainable from, a cell nucleus.

Typically, the isolated RNA molecule comprises a nucleotide sequence that corresponds to a nucleotide sequence that is located at or near a 5' splice site
30 of the internal exon of the genomic DNA sequence.

Preferably, the isolated RNA molecule consists of a nucleotide sequence that corresponds to a nucleotide sequence that is located at the 5' splice site of the internal exon of the genomic DNA sequence.

It will be appreciated that the terms "*3' end of an internal exon*" and "*5' splice site of an internal exon*" refer to the same location in the genomic DNA sequence and may hereafter be used interchangeably.

Preferably, the genomic DNA sequence is of or obtainable from a eukaryote.

More preferably, the genomic DNA sequence is of or obtainable from a metazoan.

Even more preferably, the genomic DNA sequence is of or obtainable from a vertebrate or a mammal.

Advantageously, the genomic DNA sequence is of or obtainable from a human.

In certain embodiments, the nucleotide sequence of the isolated RNA molecule is GC enriched.

This aspect of the invention also provides a modified, isolated RNA molecule, a fragment of an isolated RNA molecule and/or an RNA or DNA molecule at least partly complementary to said isolated RNA molecule.

In a second aspect, the invention provides a genetic construct which comprises or encodes one or a plurality of:

- (i) an isolated RNA molecule according to the first aspect;
- (ii) a fragment of the isolated RNA molecule according to the first aspect;
- (iii) a modified RNA molecule according to the first aspect; and/or
- (iv) an at least partly complementary RNA or DNA molecule according to the first aspect.

In one particular embodiment, the genetic construct is an expression construct comprising a DNA sequence complementary to one or a plurality of the isolated RNA molecules of the first aspect operably linked or connected to one or more regulatory nucleotide sequences.

In a third aspect, the invention provides a method of identifying the isolated RNA molecule of the first aspect, said method including the step of isolating one or more of said isolated RNA molecules from a nucleic acid sample obtained from an organism.

5 In a fourth aspect, the invention provides a method of identifying the isolated RNA molecule of the first aspect, said method including the step of identifying a DNA sequence in a genome of an organism which is complementary to the nucleotide sequence of said one or more isolated RNA molecules.

In a fifth aspect, the invention provides a computer-readable storage
10 medium or device encoded with data corresponding to one or more of:

- (i) an isolated RNA molecule according to the first aspect;
- (ii) a fragment of the isolated RNA molecule according to the first aspect;
- (iii) a modified RNA molecule according to the first aspect;
- 15 and/or
- (iv) an at least partly complementary RNA or DNA molecule according to the first aspect;

In a sixth aspect, the invention provides a method of identifying a regulatory region in a genome, said method including the step of identifying an
20 isolated RNA molecule according to the first aspect to thereby identify said regulatory region.

In some particular embodiments, said regulatory region is associated with one or more regulatory activities selected from the group consisting of mRNA splicing, transcription, epigenetic modification, epigenetic regulation, chromatin
25 modification and nucleosome positioning.

In a seventh aspect, the invention provides a method of determining whether a mammal has, or is predisposed to, a disease or condition associated with one or more regulatory regions of a genome, said method including the step of determining whether said mammal comprises one or more isolated RNA
30 molecules according to the first aspect, wherein the or each nucleotide sequence of said one or more isolated RNA molecules corresponds to a genomic DNA sequence associated with said disease or condition.

In some particular embodiments, said regulatory region is associated with one or more regulatory activities selected from the group consisting of mRNA splicing, transcription, epigenetic modification, epigenetic regulation, chromatin modification and nucleosome positioning.

5 Preferably, the mammal is a human.

In an eighth aspect, the invention provides a nucleic acid array comprising a plurality of isolated RNA molecules according to the first aspect (or their DNA equivalents), immobilized, affixed or otherwise mounted to a substrate.

In a ninth aspect, the invention provides an antibody which binds:

- 10 (i) an isolated RNA molecule according to the first aspect;
(ii) a fragment of the isolated RNA molecule according to the first aspect;
(iii) a modified RNA molecule according to the first aspect;
and/or
15 (iv) an at least partly complementary RNA or DNA molecule according to the first aspect.

In a tenth aspect, the invention provides a kit comprising one or more isolated RNA molecules according to the first aspect, or one or more isolated nucleic acids respectively complementary thereto, and/or an antibody according to the ninth aspect, and one or more detection reagents.

In an eleventh aspect, the invention provides a method of treating a disease or condition in a mammal, said method including the step of administering to the mammal a therapeutic agent selected from the group consisting of:

- 25 (i) an isolated RNA molecule according to the first aspect;
(ii) a fragment of the isolated RNA molecule according to the first aspect;
(iii) a modified RNA molecule according to the first aspect;
(iv) an at least partly complementary RNA or DNA molecule
30 according to the first aspect; and/or
(v) an antibody according to the ninth aspect;

to thereby treat said disease or condition.

In one non-limiting embodiment, said disease or condition is associated with aberrant regulatory activity of one or more genes.

In another non-limiting embodiment, said disease or condition is associated with aberrant mRNA splicing of one or more genes.

5 In yet another non-limiting embodiment, said disease or condition is associated with aberrant transcriptional activity of one or more genes.

In still another non-limiting embodiment, said disease or condition is associated with aberrant epigenetic modification and/or regulation of one or more genes.

10 In still another non-limiting embodiment, said disease or condition is associated with aberrant chromatin modification.

In still yet another non-limiting embodiment, said disease or condition is associated with aberrant nucleosome positioning.

Preferably, the mammal is a human.

15 In a twelfth aspect the invention provides a pharmaceutical composition comprising a therapeutic agent selected from the group consisting of:

- (i) an isolated RNA molecule according to the first aspect;
- (ii) a fragment of the isolated RNA molecule according to the first aspect;
- 20 (iii) a modified RNA molecule according to the first aspect
- (iv) an at least partly complementary RNA or DNA molecule according to the first aspect; and
- (v) an antibody according to the ninth aspect

and a pharmaceutically acceptable carrier, diluent or excipient.

25 In one embodiment, the pharmaceutical composition is for treating a disease or condition, such as but not limited to a disease or condition associated with one or more aberrant regulatory activities of one or more genes.

Typically, although not exclusively, said one or more aberrant regulatory activities is selected from the group consisting of mRNA splicing, transcription,
30 epigenetic modification, epigenetic regulation, chromatin modification and nucleosome positioning.

Throughout this specification, unless the context requires otherwise, the words “comprise”, “comprises” and “comprising” will be understood to imply the inclusion of a stated integer or group of integers but not the exclusion of any other integer or group of integers.

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BRIEF DESCRIPTION OF THE FIGURES

FIGURE 1. Human spliRNA sequences from THP-1 cells (n=16,298).

FIGURE 2. (A) Mouse spliRNAs derived from (*wild type*) mouse primary granulocyte nuclei (n=200); (B); Embryonic *Drosophila* spliRNAs (n=200) (C); *C. elegans* spliRNAs (n=200).

FIGURE 3. Features of nuclear-localized very small RNAs. (a) A schematic showing an example of nuclear small RNAs density at the human CAP-1 locus. tiRNAs are present downstream of the CAP1 TSS, and antisense and upstream, consistent with bidirectional promoter activity. Splice-site RNAs (spliRNAs), small RNAs whose 3' ends map to the exon 3' end (*i.e.*, the 5' splice site) are expressed at exons 4 and 7.

FIGURE 4. Splice site RNAs are conserved across metazoa. The position of small RNA 3' ends is plotted with respect to the 5' splice site, *i.e.*, the 3' end of the exon. Schematics at the top of the image depict the position of spliRNAs and their strand orientation with respect to exon-exon junctions. (a) Small RNAs, dominantly ~17-18 nt, are >35-fold enriched at the 5' splice site in THP-1 nuclei compared to either the background or (b) cytosolic THP-1 small RNAs. SpliRNA expression is conserved in species representative of all major metazoan lineages, including (c) mouse (see also **Figs. 10-12**) (d) the fruit fly *Drosophila melanogaster* (see also **Figs. 14, 16**), (e) the nematode worm *Caenorhabditis elegans* (see also **Figs. 13, 15**), and (f) the marine sponge *Amphimedon queenslandica* (see also **Fig. 17**). The data presented in (c), (d), (e) and (f) are derived from the publicly available NCBI GEO series GSE1251, GSE11624, GSE11738 and GSE12578, respectively. See **Table 7** for further detail.

FIGURE 5. Quantitative real time PCR validation of the expression of (A) H/ACA snoRNA SNORA19, (B-D) three snRNAs, and (E-G) three tRNAs in nuclear, cytoplasmic, and total RNA fractions from cultured human THP-1 cells which were subsequently interrogated by deep sequencing.

5

FIGURE 6. Northern validation of cytoplasmic and nuclear THP-1 RNA fractions. (A) Validation of the nuclear expression of C/D snoRNA SNORD77, which has been previously shown to generate an ~30 nt sdRNA, and H/ACA snoRNA SNORA19. (B) Validation of the cytoplasmic localization of tRNA Lys (AAG) and tRNA His (CAY).

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FIGURE 7. The density of (A) nuclear, and (B) cytoplasmic THP-1 small RNAs across exon-intron boundaries. Note that (A) and (B) have vertical axes on different scales.

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FIGURE 8. The density of (A) nuclear, and (B) cytoplasmic THP-1 small RNA that multimap (2-5x) across exon-exon boundaries. Note that (A) and (B) have vertical axes on different scales.

20 **FIGURE 9.** The density of total THP-1 small RNAs at (A) exon-exon junctions, or (B) exon-intron junctions.

FIGURE 10. The density of small RNAs in primary mouse granulocyte nuclei at (A) exon-exon, or (B) exon-intron junctions. See **Fig. 11** and the **Methods** for
25 more information about the isolation and sequencing of these small RNAs.

FIGURE 11. Mouse primary granulocyte purification. (A-C) Flow cytometric analysis of FACS purified granulocytes showing percentage of sorted cells in each gate (>95% purity). Granulocytes were defined as (A) lineage negative (B220, CD19, CD3, Sca-1 negative), side scatter high (SSC-A), (B) CD34 negative, c-kit negative and (C) Gr-1 high, CD16/32 positive. (D) Granulocyte morphology was confirmed by May-Grunwald Giemsa staining of FACS purified
30

cells. (E) The purity of whole cells (cell lysate) and nuclear fractions (nuclear lysate) was determined by SDS-PAGE and Western blot for β -actin (42kDa; both nuclear and cytoplasmic) and GAPDH (36kDa; cytoplasmic).

- 5 **FIGURE 12.** The density of small RNAs in mouse embryonic stem (ES) cells lacking the micro RNA processing enzymes (A) Dicer or (B) Dgcr8 across exon-exon junctions. spliRNA biogenesis is not affected by the loss of known components of the RNAi pathway in mice.
- 10 **FIGURE 13.** The density of *C. elegans* small RNAs across exon-exon junctions. spliRNAs are expressed in (A) Fog-2, (B) Glp-4, and (C) Prg-1 mutant animals, indicating that spliRNA production is not abolished in response to mutations affecting the development of the germline.
- 15 **FIGURE 14.** The density of *Drosophila* small RNAs across exon-exon junctions. spliRNAs are expressed in (A,B) early embryonic development and (C,D) cultured S2 and KC cells.
- FIGURE 15.** The density of *C. elegans* small RNAs across exon-exon junctions. spliRNAs are expressed in (A) embryonic worms, and (B-E) developing larvae. (F) This pattern is consistent in a mixed stage library.
- 20 **FIGURE 16.** The density of *Drosophila* small RNAs across exon-exon junctions in various anatomical regions. spliRNAs are expressed in adult (A) male and (C) female heads but are not apparent in (B) male or (D) female bodies. spliRNAs are weakly expressed compared to background in (E) imaginal discs.
- 25 **FIGURE 17.** The density of *Amphimedon queenslandica* (marine sponge) small RNAs across exon-exon junctions. Although spliRNAs are expressed in both (A) embryonic and (B) adult sponge, their relative expression compared to background is ~2 fold higher in embryo.
- 30

FIGURE 18. The relationship between spliRNA and gene expression. (A) Genes with spliRNAs are significantly more highly expressed than those without in THP-1 cells. (B) Genes with spliRNAs are generally more highly expressed than those without throughout *Drosophila* early embryonic development. **p-value < 2.2e-16 and **p-value < 0.05 by Wilcoxon rank sum test with continuity correction.

FIGURE 19. The density of small RNAs from *Arabidopsis* and two budding yeast (*S. cerevisiae* and *S. castellii*) across exon-exon junctions. spliRNA expression is not evident in (A) *Arabidopsis* or (B,C) budding yeast.

FIGURE 20. spliRNA sequence logos. Sequence logos of (A) 18 nt, (B) 17 nt, and (C) 16 nt nuclear-localized THP-1 spliRNAs are shown.

FIGURE 21. The correlation between GRO-seq density and spliRNAs. GRO-seq mappings were obtained from NCBI GEO (see Methods) and their density was plotted against all Refseq exons (excluding annotations on the haplotype, random, or mitochondrial chromosomes), or against the subset of exons that show spliRNAs in the THP-1 nuclear dataset. The spliRNA average density shown is derived from the THP-1 nuclear dataset. A local minimum of GRO-seq density correlates with the presence of spliRNAs, consistent with a model of spliRNA biogenesis dependent on RNAPII backtracking and TFIIIS-mediated cleavage.

FIGURE 22. The relationship between spliRNAs and downstream intron size. The size distribution of all introns in the human knownGene dataset, up to 1.2kb, is shown as blue bars. The size distribution of introns immediately upstream or downstream of an exon with a THP-1 spliRNA is shown in orange and green, respectively. Short introns are twofold more common immediately downstream of exons with spliRNAs in THP-1 cells.

FIGURE 23. The relationship between spliRNAs and exon size. The size distribution of all exons in the human knownGene dataset, up to 900 bp, is shown

as red bars. The size distribution of exons associated with spliRNAs is shown as blue bars. spliRNAs are ~2-fold less abundant at very short exons, which do not have positioned nucleosomes.

5 **FIGURE 24.** Small RNAs from wild type *S. cerevisiae* exhibit characteristics similar to tRNAs and spliRNAs. (A) *S. cerevisiae* small RNA average density is plotted with respect to the average density of nucleosomes downstream of all annotated Refgene transcription start sites. Peak small RNA density coincides with local nucleosome density minima. (B) Consistent with previous analysis, *S.*
10 *cerevisiae*, which lack the miRNA and siRNA pathways, have a distribution of small RNAs that peaks at ~17-18 nucleotides. These small RNAs exhibit a random distribution of 5' end nucleotides. (C) Like tRNAs and spliRNAs, *S. cerevisiae* small RNAs show a purine 3' nucleotide bias.

15 **FIGURE 25.** KEGG pathway enrichment analysis for genes with spliRNAs in normal adjacent tissue.

FIGURE 26. KEGG pathway enrichment analysis for genes with spliRNAs in DCIS tissue.

20

FIGURE 27. Splice-site RNA expression comparison per gene in the normal adjacent (*i.e.*, control) versus the DCIS (*i.e.*, cancer) tissues. Note the number of genes with differentially highly expressed spliRNAs in the DCIS tissue.

25 **FIGURE 28.** Genes with greater than twofold greater spliRNA expression in the DCIS sample are significantly associated with breast cancer pathways in GeneSigDB.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENT

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To investigate splice-site associated small RNAs in detail, the present inventors analysed the relationship between 5' splice-sites and small RNAs present in deep sequencing libraries from human and mouse cell lines, mouse

primary granulocyte nuclei, a variety of nematode worm *Caenorhabditis elegans* (*C. elegans*), fruit fly *Drosophila melanogaster* (*D. melanogaster*) and marine sponge *Amphimedon queenslandica* (*A. queenslandica*) primary tissues.

The present invention arises from the finding of a novel class of “splice-site” RNA molecules (spliRNAs) that may be associated with gene regulatory mechanisms including, but not limited to, mRNA splicing, transcription, epigenetic modification, epigenetic regulation, chromatin modification and nucleosome positioning. The present inventors have surprisingly found that these spliRNAs may be distinguished from any previously identified class of small RNAs based on their location in the genome and the features they are associated with. In a particular embodiment, the spliRNA may be characterized as comprising a nucleotide sequence that (i) corresponds to a nucleotide sequence that is located at or near a 5' splice-site of an internal exon of a genomic DNA sequence, and (ii) corresponds to a 3' nucleotide sequence of the internal exon of the genomic DNA sequence.

It will be appreciated that these small RNA molecules exhibit different characteristics to the small non-coding RNA molecules (e.g., miRNAs, piRNAs and tiRNAs) previously identified. The present invention is based on the inventors' identification of spliRNA molecules, the manipulation of these spliRNAs and the use of spliRNAs to characterize their role and function in cells. The invention also concerns methods and compositions for identifying spliRNAs, arrays comprising spliRNAs (spliRNA arrays) and use of spliRNAs for diagnostic, therapeutic and prognostic applications in mammals, particularly humans.

For the purposes of this invention, by “isolated” is meant present in an environment removed from a natural state or otherwise subjected to human manipulation. Isolated material may be substantially or essentially free from components that normally accompany it in its natural state, or may be manipulated so as to be in an artificial state together with components that normally accompany it in its natural state. The term “isolated” also encompasses terms such as “enriched”, “purified”, “synthetic” and/or “recombinant”.

The term “*nucleic acid*” as used herein designates single- or double-stranded mRNA, RNA, cRNA, RNAi and DNA inclusive of cDNA and genomic DNA. Nucleic acids may comprise naturally-occurring nucleotides or synthetic, modified or derivatized bases (*e.g.*, inosine, methyinosine, pseudouridine, methylcytosine *etc.*). Nucleic acids may also comprise chemical moieties coupled thereto to them. Examples of chemical moieties include, but are not limited to, biotin, locked nucleic acids (LNAs), peptide nucleic acids (PNAs), cholesterol, 2'-O-methyl, Morpholino, and fluorophores such as HEX, FAM, Fluorescein and FITC.

According to a first aspect, the invention provides an isolated substantially-single stranded RNA molecule (referred to herein as a “*splice-site RNA*” or “*spliRNA*”) that comprises a nucleotide sequence comprising no more than 26 contiguous nucleotides that correspond to a genomic DNA sequence associated with gene regulation, wherein said nucleotide sequence comprises a nucleotide sequence corresponding to a 3' nucleotide sequence of an internal exon of said genomic DNA sequence.

In this context, “*corresponding to*” and “*corresponds to*” means that the *spliRNA* molecule has a nucleotide sequence of, or a sequence complementary to, a genomic DNA nucleotide sequence. It will be appreciated that this definition should take into account that RNA uses a U instead of a T, as found in DNA.

Typically, the *spliRNA* comprises a nucleotide sequence that corresponds to a sense strand of the internal exon of the genomic DNA sequence.

In one preferred form, said *spliRNA* molecule terminates in a 3' nucleotide sequence that corresponds to the 3' end of the internal exon of the genomic DNA sequence.

In another preferred form, said isolated RNA molecule consists of a nucleotide sequence that corresponds to the 3' end of the internal exon of the genomic DNA sequence.

In one preferred form, the *spliRNA* molecule comprises a nucleotide sequence comprising 14-26 contiguous nucleotides.

In another preferred form, the *spliRNA* molecule comprises a nucleotide sequence comprising 14-20 contiguous nucleotides.

In yet another preferred form, the spliRNA molecule comprises a nucleotide sequence comprising 16-18 contiguous nucleotides.

In still yet another preferred form, the spliRNA molecule comprises a nucleotide sequence comprising 17 or 18 contiguous nucleotides.

5 It will be appreciated that the length of the spliRNA molecule may vary depending on the genome that it is located in and/or derived from. For example, a spliRNA molecule of a human genome may comprise on average 17 or 18 nucleotides while a spliRNA molecule of a different eukaryotic genome (e.g., mouse and *Drosophila*) may comprise on average 17 nucleotides, although
10 without limitation thereto.

Typically, the isolated RNA molecule comprises a nucleotide sequence that corresponds to a nucleotide sequence that is located at or near a 5' splice site of the internal exon of the genomic DNA sequence.

Preferably, the isolated RNA molecule consists of a nucleotide sequence
15 that corresponds to a nucleotide sequence that is located at the 5' splice site of the internal exon of the genomic DNA sequence.

Typically, the spliRNA does not encode a peptide or a protein encoded by a genome. Accordingly, the spliRNA comprises a nucleotide sequence that is referred to herein as "non-coding".

20 The spliRNAs are typically not linked to the pathways that produce miRNAs, siRNAs and piRNAs. Thus, in contrast to other small non-coding RNA molecules (e.g., miRNAs, siRNAs and piRNAs), spliRNAs do typically not require a dicer and/or a Dgcr8 for their processing and/or production.

It will be appreciated that the spliRNAs are expressed in most tissues and
25 developmental stages in eukaryotes such as, but not limited to, humans, mice, *Drosophila* and *C. elegans*. In some cases, the spliRNAs are enriched, increased or otherwise more prevalent in actively differentiating and/or undifferentiated tissues. For example, the expression of a spliRNA may be higher in a *Drosophila* head compared to in a *Drosophila* body. It will also be appreciated that the
30 expression of the spliRNA may be upregulated, induced, elevated or otherwise increased in an embryonic sponge compared to in an adult sponge (e.g., the marine sponge *A. queenslandica*).

It will also be appreciated that genes associated with spliRNAs may be more highly expressed compared to genes that lack or have a low abundance of spliRNAs.

While in one embodiment the spliRNA molecule has a nucleotide
5 sequence transcribed from the corresponding DNA sequence, it will be appreciated that said spliRNA molecule may be chemically-synthesized *de novo*, rather than transcribed from a DNA sequence.

Chemical synthesis of RNA is well known in the art. Non-limiting examples include RNA synthesis using TOM amidite chemistry, 2-
10 cyanoethoxymethyl (CEM), a 2'-hydroxyl protecting groups and fast oligonucleotide deprotecting groups.

As hereinbefore described, the nucleotide sequence of a spliRNA molecule is typically GC rich. By this is meant, that the percent GC content of the nucleotide sequence is substantially greater than the average GC content of the
15 genome from which the spliRNA is derived. This GC content also differs from that of miRNAs.

Preferably, the GC content of spliRNAs is greater than about 50-70%, or greater than about 51-60%.

Typically, the GC content of spliRNAs is greater than about 51%, greater
20 than about 52%, greater than about 53%, or greater than about 54% compared to about 50% for miRNAs and compared to about 70% for tiRNAs.

It will be appreciated that this comparison is organism dependent hence the actual GC content will vary for spliRNAs of each different organism.

In some embodiments, a spliRNA may be is associated with the
25 maintenance and/or initiation of nucleosome positioning.

In certain embodiments, a spliRNA may be located at a region of a genome with (i) RNA polymerase II binding and/or (ii) transcription elongation factor II (TFIIS) activity.

In some cases, spliRNA expression may be at least partly upregulated,
30 elevated or otherwise increased at exons located upstream of short introns.

Thus, spliRNA expression at an exon located upstream of an intron that comprises 60-120 nucleotides or less may be, for example, 25%, 50%, 75%,

100% 150%, 200%, 250%, 300%, 350%, 400%, 450% and up to about 500% increased compared to spliRNA expression at an exon located upstream of an intron that comprises 121 nucleotides or more.

In some cases spliRNA expression may be at least partly reduced, decreased or otherwise down-regulated at short exons. Consequently, spliRNA expression may be, for example, 25%, 50%, 75%, 100% 150%, 200%, 250%, 300%, 350%, 400%, 450% and up to about 500% reduced at an exon comprising 60 nucleotides or less compared to spliRNA expression at an exon comprising 61 nucleotides or more.

Typically, although not exclusively, spliRNA molecules do not form secondary structures, such as stem and loop structures. Accordingly, spliRNA molecules are substantially free of internal base-pairing. In this context, by “*substantially free*” is meant fewer than 3, 2 or 1 internal base pairs.

Therefore, in one particularly preferred embodiment, the invention provides an isolated substantially single-stranded spliRNA molecule, wherein said isolated spliRNA molecule comprises a nucleotide sequence that:

- (i) consists of 17 or 18 contiguous nucleotides that correspond to a sense strand of an internal exon of a mammalian genomic DNA sequence located at a 5' splice site;
- (ii) corresponds to a 3' end of the internal exon of the mammalian genomic DNA sequence;
- (iii) comprises a GC content greater than 54%; and
- (iv) is substantially free of internal base-pairing.

Preferably, the mammalian genomic sequence is of, or obtainable from, a human genome.

Non-limiting examples of the isolated spliRNA molecules of the invention are set forth in SEQ ID NOs:1-6884 (**Fig. 1** (human)) and SEQ ID NOs:6885-16,898 (**Fig. 2 A-C** (mouse, *C. elegans*, and *Drosophila*)).

Typically, although not exclusively, the isolated spliRNA molecule comprises a nucleotide sequence that is located in, or obtainable from, a cell nucleus.

It will also be appreciated that the invention contemplates nucleic acid molecules (e.g., RNA or DNA) complementary to or at least partly complementary to the spliRNA molecules of the invention. Complementary or at least partly complementary nucleic acid molecules may be in DNA or RNA form.

5 By “*at least partly complementary*” is meant having at least 60%, at least 70%, at least 75%, at least 80%, at least 90%, or at least 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% sequence identity with a nucleotide sequence of a spliRNA molecule.

The invention also provides a modified spliRNA molecule.

10 A modified spliRNA may be altered by, complexed, labeled or otherwise covalently or non-covalently coupled to one or more other chemical entities. In some embodiments, the chemical entity may be bonded, linked or otherwise attached directly to the spliRNA, or it may be bonded, linked or otherwise attached to the spliRNA via a linking group (e.g., a spacer).

15 Examples of such chemical entities include, but are not limited to, incorporation of modified bases (e.g., inosine, methylinosine, pseudouridine and morpholino), sugars and other carbohydrates such as 2'-O-methyl and locked nucleic acids (LNA), amino groups and peptides (e.g., peptide nucleic acids (PNA)), biotin, cholesterol, fluorophores (e.g., FITC, Fluorescein, Rhodamine, 20 HEX, FAM, TET and Oregon Green) radionuclides and metals, although without limitation thereto (Fabani and Gait, 2008; You *et al.*, 2006; Summerton and Weller, 1997). A more complete list of possible chemical modifications can be found at <http://www.oligos.com/ModificationsList.htm>.

In one particular embodiment, the modified spliRNA is an “*antisense inhibitor*”. By “*antisense inhibitor*” is meant a nucleic acid sequence that is 25 either complementary to or at least partly complementary to the spliRNA molecule (Dias and Stein, 2002; Kurreck, 2003; Sahu *et al.*, 2007). The antisense inhibitor pairs with the spliRNA and interferes with interactions such as, but not limited to, spliRNA-mRNA and spliRNA-DNA interactions. Experiments 30 showing sequence-specific inhibition of small RNA function have previously been demonstrated both *in vitro* (Meister *et al.*, 2004; Hutvagner *et al.*, 2004) and *in vivo* (Krützfeldt *et al.*, 2005).

In another particular embodiment, the modified spliRNA is a "*point mutant*". By "*point mutant*" is meant a spliRNA molecule where 1 or 2 nucleotides have been removed, substituted or otherwise altered. Point mutants of spliRNAs or their targets can be employed to study the function of spliRNAs in disease or to increase the affinity of spliRNAs to variant targets. Small RNA molecules involved in disease processes, including spliRNAs, may have "*seed-sequences*". By "*seed-sequences*" is meant nucleic acid sequences that comprise 2-7 nucleotides and are involved in target recognition (Lewis *et al.*, 2003; Lewis *et al.*, 2005). Increasing the mismatch in these sequences is predicted to significantly decrease the gene regulation function of spliRNAs. This approach may be applicable for partial inhibition of spliRNA targets.

In yet another particular embodiment, the modified spliRNA molecule is a "*spliRNA sponge*". By "*spliRNA sponge*" is meant a genetically encoded competitive spliRNA inhibitor that may be stably expressed in a cell, such as a eukaryotic cell. The spliRNA binds to the spliRNA thereby preventing it from binding its mRNA target in a technique called "*sponging*". The spliRNA sponges may be produced using methods such as the ones described in Cohen, 2009, Ebert *et al.*, 2007, Hammond, 2007 and Rooij *et al.*, 2008. It will be appreciated that a spliRNA sponge may bind to, soak up and/or inhibit a specific spliRNA and/or a family of spliRNAs.

In still yet another particular embodiment, the modified spliRNA is a "*spliRNA mimic*". A "*spliRNA mimic*" is a single-stranded RNA oligonucleotide that is complementary to or at least partly complementary to the spliRNA. The spliRNA mimic may inactivate pathological spliRNAs through complementary base-pairing. It will also be appreciated that chemical modification to LNA, PNA or morpholino and conjugation to cholesterol may stabilize the spliRNA mimic molecule and facilitate delivery of single-stranded RNA molecules to targets following intravenous administration (Rooij and Olson, 2007).

The invention also provides a fragment of a spliRNA of the invention. By "*fragment*" is meant a portion, domain, region or sub-sequence of a spliRNA molecule which comprises one or more structural and/or functional characteristics of a spliRNA molecule. By way of example only, a fragment may comprise at

least 5, at least 6, at least 7, at least 8, at least 9, at least 10, at least 11, at least 12, at least 13, at least 14, at least 15, at least 16 or at least 17 nucleotides of a spliRNA molecule.

It will be appreciated that the spliRNA molecules can be chemically
5 modified to facilitate penetration into cells. Examples of such modifications include, but are not limited to, conjugation to cholesterol, Morpholino, 2'-O-methyl, PNA or LNA (Partridge *et al.*, 1996; Corey and Abrams, 2001; Kos *et al.*, 2003).

Modified spliRNA molecules also include "variants" of the spliRNA
10 molecules of the invention. Variants include RNA or DNA molecules comprising a nucleotide sequence at least 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98% or 99% identical to a nucleotide sequence of a spliRNA molecule such as described in Fig. 1 and Fig. 2. Such variants may include one or more point mutations, nucleotide substitutions, deletions or additions.

15 According to another aspect, there is provided a genetic construct comprising or encoding one or a plurality of the same or different spliRNA molecules, modified spliRNA molecules, at least partly complementary DNA or RNA molecules, or fragments thereof.

It will be appreciated that said spliRNA molecules may be oriented in
20 tandem repeats or with multiple copies of each spliRNA sequence.

As used herein, a "genetic construct" is any artificially constructed nucleic acid molecule comprising heterologous nucleotide sequences.

A genetic construct is typically in DNA form, such as a phage, plasmid, cosmid, artificial chromosome (*e.g.*, a YAC or BAC), although without limitation
25 thereto. The genetic construct suitably comprises one or more additional nucleotide sequences, such as for assisting propagation and/or selection of bacterial or other cells transformed or transfected with the genetic construct.

In one particular embodiment, the genetic construct is a DNA expression
30 construct that comprises one or more regulatory sequences that facilitate transcription of one or more spliRNA molecules, modified spliRNA molecules or fragments thereof.

Such regulatory sequences may include promoters, enhancers, polyadenylation sequences, splice donor/acceptor sites, although without limitation thereto.

Suitable promoters may be selected according to the cell or organism in which the spliRNA molecule is to be expressed. Promoters may be selected to facilitate constitutive, conditional, tissue-specific, inducible or repressible expression as is well understood in the art. Examples of promoters are T7, SP6, SV40, PolIII, U6, H1 and 7SK, although without limitation thereto.

It will be appreciated that the spliRNA molecule may be provided as an encoding DNA sequence in an expression construct that, when transcribed, produces the spliRNA molecule as a transcript.

It will also be appreciated that spliRNA molecules appear to be a hitherto unknown form of small, single stranded RNA molecules that occur throughout evolution. Accordingly, spliRNA molecules may be isolated, identified, purified or otherwise obtained from a number of different organisms.

Preferably, the organism is a eukaryote.

More preferably, the organism is a metazoan inclusive of all multi-celled animals ranging from marine sponge to insects and vertebrates.

Even more preferably, the organism is a vertebrate, inclusive of mammals, avians such as chickens and ducks and aquaculture species such as fish, although without limitation thereto.

Even more preferably, the organism is a mammal.

Mammals include humans, livestock such as horses, pigs, cows and sheep, domestic animals such as cats and dogs, although without limitation thereto.

In further aspects, the invention therefore provides methods of identifying, purifying or otherwise obtaining a spliRNA molecule.

Broadly, such methods may include analysis of nucleic acid samples obtained from an organism, and/or bioinformatic analysis of genome sequence information.

Preferably, the nucleic acid samples are derived from the genome of a eukaryote.

More preferably, the nucleic acid samples are derived from the genome of a metazoan inclusive of marine sponge, insects and vertebrates.

Even more preferably, the nucleic acid samples are derived from the genome of a vertebrate, inclusive of mammals, avians such as chickens and ducks
5 and aquaculture species such as fish, although without limitation thereto.

Even more preferably, the nucleic acid samples are derived from the genome of a mammal.

Mammals include humans, livestock such as horses, pigs, cows and sheep, domestic animals such as cats and dogs, although without limitation thereto.

10 Preferably, methods for analyzing a nucleic acid sample to identify a spliRNA include "*deep sequencing*" and mapping strategies that consider exon-exon and/or exon-intron boundaries and multi-mapping deep sequencing reads. Examples of specific deep sequencing technologies employed for the identification of exon boundaries and spliRNAs include, but are not limited to,
15 454TM-, Helicos-, PacBio-, Solexa/Illumina- and SOLiD-sequencing.

In particular embodiments that relate to bioinformatic analyses of genome sequence information, the invention provides a computer-readable storage medium or device encoded with structural information of one or more spliRNA molecules.

20 The structural information may be nucleotide sequence, sequence length, GC content and/or proximity to a 5' splice site of an internal exon, although without limitation thereto.

A computer-readable storage medium may have computer readable program code components stored thereon for programming a computer (*e.g.*, any
25 device comprising a processor) to perform a method as described herein. Examples of such computer-readable storage media include, but are not limited to, a hard disk, a CD-ROM, an optical storage device, a magnetic storage device, a ROM (Read Only Memory), a PROM (Programmable Read Only Memory), an EPROM (Erasable Programmable Read Only Memory), an EEPROM
30 (Electrically Erasable Programmable Read Only Memory) and a Flash memory. Further, it is expected that one having ordinary skill in the art, notwithstanding possibly significant effort and many design choices motivated by, for example,

available time, current technology, and economic considerations, when guided by the concepts and principles disclosed herein will be readily capable of implementing the invention by generating necessary software instructions, programs and/or integrated circuits (ICs) with minimal experimentation.

5 Typically, the computer-readable storage medium or device is part of a computer or computer network capable of interrogating, searching or querying a genome sequence database.

In one example, a bioinformatic method may utilize a high performance computing station which houses a local mirror of the UCSC Genome Browser.

10 One further aspect of the invention provides antibodies which bind, recognize and/or have been raised against a spliRNA of the invention, inclusive of fragments and modified spliRNA molecules.

Antibodies may be monoclonal or polyclonal. Antibodies also include antibody fragments such as Fc fragments, Fab and Fab'2 fragments, diabodies and ScFv fragments. Antibodies may be made in a suitable production animal
15 such as a mouse, rat, rabbit, sheep, chicken or goat.

The invention also contemplates recombinant methods of producing antibodies and antibody fragments. For example, antibodies to RNA molecules have been produced by a method utilizing a synthetic phage display library approach to select RNA-binding antibody fragments (Ye *et al.*, 2008).
20

As is well understood in the art, antibodies may be conjugated with labels selected from a group including an enzyme, a fluorophore, a chemiluminescent molecule, biotin, radioisotope or other label.

Examples of suitable enzyme labels useful in the present invention include
25 alkaline phosphatase, horseradish peroxidase, luciferase, β -galactosidase, glucose oxidase, lysozyme, malate dehydrogenase and the like. The enzyme label may be used alone or in combination with a second enzyme in solution or with a suitable chromogenic or chemiluminescent substrate.

Examples of chromogens include diaminobenzidine (DAB), permanent
30 red, 3-ethylbenzthiazoline sulfonic acid (ABTS), 5-bromo-4-chloro-3-indolyl phosphate (BCIP), nitro blue tetrazolium (NBT), 3,3',5,5'-tetramethyl benzidine (TNB) and 4-chloro-1-naphthol (4-CN), although without limitation thereto.

A non-limiting example of a chemiluminescent substrate is Luminol™, which is oxidized in the presence of horseradish peroxidase and hydrogen peroxide to form an excited state product (3-aminophthalate).

Fluorophores may be fluorescein isothiocyanate (FITC),
5 tetramethylrhodamine isothiocyanate (TRITC), allophycocyanin (APC), Texas Red (TR), Cy5 or R-Phycoerythrin (RPE), although without limitation thereto.

Radioisotope labels may include ¹²⁵I, ¹³¹I, ⁵¹Cr and ⁹⁹Tc, although without limitation thereto.

Other antibody labels that may be useful include colloidal gold particles
10 and digoxigenin.

In other aspects, the invention provides a method of identifying a spliRNA expression profile as a quantitative or qualitative indicator or measure of gene regulation. These methods may be particularly, although not exclusively, relevant to diagnosis of diseases and conditions associated with differential gene
15 regulation.

In one particular embodiment, said spliRNA expression profile is an indicator and/or measure of mRNA splicing activity.

In another particular embodiment, said spliRNA expression profile is an indicator of particular exon inclusion as a result of mRNA splicing activity.

20 In another particular embodiment, said spliRNA expression profile is an indicator and/or measure of gene transcriptional activity.

In yet another particular embodiment, said spliRNA expression profile is an indicator and/or measure of epigenetic modulatory and/or regulatory activity.

25 In still another particular embodiment, said spliRNA expression profile is an indicator and/or measure of chromatin modification activity.

In still yet another particular embodiment, said spliRNA expression profile is an indicator and/or measure of nucleosome positioning activity.

In one embodiment, the method uses a "nucleic acid array" (spliRNA array).

30 By "nucleic acid array" is meant a plurality of nucleic acids, preferably ranging in size from 10, 15, 20 or 50 bp to 250, 500, 700 or 900 kb, immobilized, affixed or otherwise mounted to a substrate or solid support. Typically, each of

the plurality of nucleic acids has been placed at a defined location, either by spotting or direct synthesis. In array analysis, a nucleic acid-containing sample is labeled and allowed to hybridize with the plurality of nucleic acids on the array. Nucleic acids attached to arrays are referred to as "*targets*" whereas the labelled
5 nucleic acids comprising the sample are called "*probes*". Based on the amount of probe hybridized to each target spot, information is gained about the specific nucleic acid composition of the sample. The major advantage of gene arrays is that they can provide information on thousands of targets in a single experiment and are most often used to monitor gene expression levels and "*differential*
10 *expression*".

"*Differential expression*" indicates whether the level of a particular spliRNA in a sample is higher or lower than the level of that particular spliRNA in a normal or reference sample.

The physical area occupied by each sample on a nucleic acid array is
15 usually 50-200 μm in diameter thus nucleic acid samples representing entire genomes, ranging from 3,000-32,000 genes, may be packaged onto one solid support. Depending on the type of array, the arrayed nucleic acids may be composed of oligonucleotides, PCR products or cDNA vectors or purified inserts. The sequences may represent entire genomes and may include both known and
20 unknown sequences or may be collections of sequences such as miRNAs. Using array analysis, the expression profiles of normal and diseased tissues, treated and untreated cell cultures, developmental stages of an organism or tissue, and different tissues can be compared.

In one embodiment, gene profiling, such as but not limited to using a
25 spliRNA array, is used to identify mRNAs whose expression shows a positive or inverse correlation with the expression of a specific spliRNA.

It will be appreciated that an absence of spliRNA expression could correlate with a presence of mRNA expression, or *vice versa*. Alternatively, a presence of spliRNA expression could correlate with a presence of mRNA
30 expression or an absence of spliRNA expression could correlate with an absence of mRNA expression. Furthermore, a level of spliRNA expression could correlate with a level of mRNA expression, whether directly or inversely. It will be

appreciated that a level of expression may be measured as a quantitative or a relative expression level.

In another embodiment, gene profiling allows the identification of regulators of disease processes and potential therapeutic targets.

5 Examples of diseases and conditions that show differential gene regulation include but are not limited to Crohn's disease, Alzheimer's disease, Parkinson's disease, schizophrenia, infertility, rheumatoid arthritis, myocardial infarction, diabetes, congenital developmental disorders, coronary heart disease, and cancer such as breast cancer, lymphoma, leukemia, colorectal cancer, gastric cancer,
10 ovarian cancer, aggressive metastatic brain cancer, and pituitary tumors (McKaig *et al.*, 2003; Grünblatt *et al.*, 2007; Liang *et al.*, 2008; Lübke *et al.*, 2008; Ridker, 2007; Zecchini *et al.*, 2008; Perrin *et al.*, 2007; Zumodio *et al.*, 2008).

It will be appreciated that said gene regulation may refer to aberrant gene transcription, aberrant mRNA splicing, aberrant epigenetic modification, aberrant
15 epigenetic regulation, aberrant chromatin modification and/or aberrant nucleosome positioning.

Further, spliRNAs may be associated with aberrant regulatory activity of oncogenes or tumor suppressors (Zhang *et al.*, 2006) and may therefore become useful biomarkers for cancer diagnostics.

20 It will be appreciated that said aberrant regulatory activity may in some embodiments refer to activities such as transcription, mRNA splicing, epigenetic modification, epigenetic regulation, chromatin modification and/or nucleosome positioning.

In one particular embodiment, the spliRNAs may be associated with
25 oncogenes such as *myc**, *Bcl-2* and *-3**, *myb**, *mdm2**, *mdmx**, and *ras*.

In another particular embodiment, the spliRNAs may be associated with a tumour suppressor gene, for example, p21 and/or p53 (see, *e.g.*, Sotos-Reyes & Recillas-Targa, 2010).

In another particular embodiment, the spliRNAs may be linked to aberrant
30 mRNA splicing of genes associated with diseases and conditions such as, *Dnmt1** and *Dnmt3** associated with cancer progression, and *APP* and beta amyloid in Alzheimer's disease.

In yet another particular embodiment, the spliRNAs may be linked to aberrant epigenetic modification and/or regulation of genes associated with various cancers, α -Thalassaemia, and Prader-Willi, ATR-X, Fragile X, ICF, Angelman's, and Rett syndromes.

5 Other methods of the invention, including but not limited to the herein mentioned spliRNA array, relate to diagnostic applications of the claimed nucleic acid molecules. For example, spliRNAs may be detected in biological samples in order to determine and classify certain cell types or tissue types or spliRNA-associated pathogenic disorders which are characterized by differential expression
10 of spliRNA molecules or spliRNA molecule patterns. Further, the developmental stage of cells, organs and/or tissues may be classified by determining spatial and/or temporal expression patterns of spliRNA molecules.

In another aspect, the invention provides a method of treating a disease or condition in an animal, said method including the step of administering to the
15 animal a therapeutic agent selected from the group consisting of:

- (i) an isolated spliRNA molecule;
- (ii) a fragment of the isolated spliRNA molecule;
- (iii) a modified spliRNA molecule;
- (iv) an at least partly complementary RNA or DNA molecule;
- 20 and/or
- (v) an antibody that binds any one of (i)-(iv);

to thereby treat said disease or condition.

Accordingly, the aforementioned therapeutic agents may be suitable for prophylaxis and/or therapy of animals, including mammals such as humans. For
25 example, the therapeutic agents may be used to treat diseases, conditions, developmental processes and/or disorders associated with developmental dysfunctions. Certain spliRNAs may function as tumour-suppressors and thus expression or delivery of these spliRNAs or *spliRNA mimics* to tumor cells may provide therapeutic efficacy.

30 In one embodiment, the use of chemically modified spliRNAs to target either a specific spliRNA or to disrupt the binding of a spliRNA and its specific

mRNA target *in vivo* may provide a potentially effective means of inactivating pathological spliRNAs.

Alternatively, spliRNAs may be administered to potentiate the effects of natural spliRNAs by promoting the expression of beneficial gene products such as tumour suppressor proteins (Rooij and Olson, 2007).

Therapeutic agents may be delivered to an animal in the form of a pharmaceutical composition comprising a pharmaceutically acceptable carrier diluent or excipient.

Accordingly, the invention provides a pharmaceutical composition comprising a therapeutic agent selected from the group consisting of:

- (i) an isolated spliRNA molecule;
- (ii) a fragment of the isolated spliRNA molecule;
- (iii) a modified spliRNA molecule;
- (iv) an at least partly complementary RNA or DNA molecule and/or
- (v) an antibody that binds any one of (i)-(iv);

and a pharmaceutically acceptable carrier, diluent or excipient.

By "*pharmaceutically-acceptable carrier, diluent or excipient*" is meant a solid or liquid filler, diluent or encapsulating substance that may be safely used in systemic administration. This includes carriers, diluents or excipients suitable for veterinary use.

Depending upon the particular route of administration, a variety of carriers, well known in the art may be used. These carriers may be selected from a group including sugars, starches, cellulose and its derivatives, malt, gelatine, talc, calcium sulfate, vegetable oils, synthetic oils, polyols, alginic acid, phosphate buffered solutions, emulsifiers, isotonic saline and salts such as mineral acid salts including hydrochlorides, bromides and sulfates, organic acids such as acetates, propionates and malonates and pyrogen-free water.

A useful reference describing pharmaceutically acceptable carriers, diluents and excipients is Remington's Pharmaceutical Sciences (Mack Publishing Co. N.J. USA, 1991).

Any safe route of administration may be employed for providing a patient with the composition of the invention. For example, oral, rectal, parenteral, sublingual, buccal, intravenous, intra-articular, intra-muscular, intra-dermal, subcutaneous, inhalational, intraocular, intraperitoneal, intracerebroventricular, transdermal and the like may be employed. Intra-muscular and subcutaneous injection is appropriate, for example, for administration of immunotherapeutic compositions, proteinaceous vaccines and nucleic acid vaccines. In the case of gene therapy, which contemplates the use of electroporation or liposomal transfection into tissues, the drug may be transfected into cells together with the DNA.

Dosage forms include tablets, dispersions, suspensions, injections, solutions, syrups, troches, capsules, suppositories, aerosols, transdermal patches and the like. These dosage forms may also include injecting or implanting controlled releasing devices designed specifically for this purpose or other forms of implants modified to act additionally in this fashion. Controlled release of the therapeutic agent may be achieved by coating the same, for example, with hydrophobic polymers including acrylic resins, waxes, higher aliphatic alcohols, polylactic and polyglycolic acids and certain cellulose derivatives such as hydroxypropylmethyl cellulose. In addition, the controlled release may be achieved by using other polymer matrices, liposomes and/or microspheres.

Compositions of the present invention suitable for oral or parenteral administration may be presented as discrete units such as capsules, sachets or tablets each containing a pre-determined amount of one or more therapeutic agents of the invention, as a powder or granules or as a solution or a suspension in an aqueous liquid, a non-aqueous liquid, an oil-in-water emulsion or a water-in-oil liquid emulsion. Such compositions may be prepared by any of the methods of pharmacy but all methods include the step of bringing into association one or more agents as described above with the carrier which constitutes one or more necessary ingredients. In general, the compositions are prepared by uniformly and intimately admixing the agents of the invention with liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product into the desired presentation.

The above compositions may be administered in a manner compatible with the dosage formulation, and in such amount as is pharmaceutically-effective. The dose administered to a patient, in the context of the present invention, should be sufficient to achieve a beneficial response in a patient over an appropriate period of time. The quantity of agent(s) to be administered may depend on the subject to be treated inclusive of the age, sex, weight and general health condition thereof, factors that will depend on the judgement of the practitioner.

Methods and compositions may be used for treating diseases or conditions in any animal. Animals include and encompass fish, avians (*e.g.*, chickens and other poultry) and mammals inclusive of humans, livestock, domestic pets and performance animals (*e.g.*, racehorses), although without limitation thereto.

So that the invention may be readily understood and put into practical effect, reference is made to the following non-limiting example.

15

EXAMPLES

Example 1 – Nuclear-localized tiny RNAs are associated with transcription initiation and splice sites in metazoans

Methods

THP-1 RNA isolation

THP-1 cells were grown in suspension culture according to previously published methods (Taft *et al.*, 2009 (I)); Suzuki *et al.*, 2009) and harvested. Growth media was aspirated and the pellets were resuspended and washed twice in equivalent volumes of ice-cold PBS. The cells were then split into two equal volumes for the extraction of total RNA and the extraction of nuclear and cytoplasmic RNA. Total RNA was extracted using TRIzol (Invitrogen), according to the manufacturer's instructions.

Nuclear and cytoplasmic RNA was isolated as previously described (Hwang *et al.*, 2007), except that each wash was carried out using 1ml of wash buffer and tween-40 was substituted for tween-20 in the final wash. Additionally, to ensure complete clearing of any intact cells or nuclei, the cytoplasmic fraction was subjected to an additional centrifugation step at 1000 X g for 5 minutes at 4°C, after which the supernatant was transferred to clean tubes for RNA

extraction. To validate the integrity of the cytoplasmic fraction an aliquot was aspirated and inspected under an inverted light microscope to confirm that it was free from intact cells or nuclei. The nuclear fraction was similarly confirmed free from intact cells under an inverted light microscope by comparing an aliquot of nuclei after the final wash step to an aliquot of intact cells. RNA was extracted using the TRIzol (Invitrogen) according to the manufacturer's instructions and the resulting RNA pellets were resuspended in equal volumes of ultra-pure water to obtain cell-equivalent concentrations.

10 *Validation of THP-1 nuclear and cytoplasmic RNA fractionation*

Seven RNA species were assessed by qPCR on cDNA prepared from total, cytoplasmic, and nuclear RNA fractions (for a list of targets, primers and results, see **Table 5** and **Fig. 5**. A total of 6ul from each cell-equivalent fraction was used (0.336ug from nuclear fraction, 1.74ug from cytoplasmic fraction and 3ug from the total fraction). Each 6ul fraction was DNase treated using TURBO DNase (Ambion) according to manufacturer's instructions and then reverse transcribed using the SuperScript III Reverse Transcriptase kit (Invitrogen). Reverse transcription was primed using random hexamers, which were added at a concentration of 250ng per 5µg RNA. RT negative reactions were carried out in parallel. PCR amplicons were generated from each of the qPCR targets and extracted from a 2% agarose gel using the Wizard SV Gel and PCR Clean-Up System kit (Promega) and quantified using a Nanodrop spectrophotometer. Each amplicon was then diluted to a concentration of 2ng/ul and used as templates for generating qPCR standard curves as a 1/10 serial dilution series.

25 Quantitative PCR of the cDNA targets was carried out using the SYBR Green PCR Master Mix (Applied Biosystems) in 20ul reactions, using 2ul of cDNA, 4ul of primers (2uM), 4ul water and 10ul 2X master mix per reaction. The reactions were carried out in 96 well plates on an Applied Biosystems 7500 QPCR System in triplicate and also included both RT negative controls and water controls. Mean cDNA concentrations and standard deviations were assessed using the Applied Biosystems Sequence Detection Software v1.4.

Northern blots were completed by EDC cross-linking as previously described (Pall *et al.*, 2007). Successful separation of cytoplasmic and nuclear THP-1 RNA fractions was assessed using probes against two snoRNAs and two tRNAs (Table 6, Fig. 6). Nuclear enrichment of miR-15 was assessed using a probe spanning the 5' 16 nucleotides (Table 6), which facilitated detection of both miR-15a and miR-15b without any increase in background. MicroRNA-16 was detected using a northern probe spanning its entire length (Table 6).

THP-1 small RNA deep sequencing

Prior to deep sequencing 2.4×10^{-6} pmol and 2.4×10^{-7} pmol of three 21mer and three 18mer synthetic RNA spike-ins with 5' phosphate and 3' hydroxyl groups, respectively, were added to the cytoplasmic, nuclear, and total RNA pools in equal concentrations (Table 2). Spike-ins were designed so that they would not map to the human genome. Deep sequencing of the THP-1 small RNA samples was performed by GeneWorks (Adelaide, Australia) on the Illumina GAII. Library preparation and sequencing was completed according to the manufacturer's instructions with one modification. Sample isolation from the PAGE gel after adaptor ligation was performed with a modified set of size markers to facilitate sequencing of small RNAs greater than or equal to 15 nt, allowing detection and quantification of tRNAs and other very small RNA species.

Mouse granulocyte nuclei preparation, isolation and small RNA sequencing

C57BL/6J mice were obtained from the Animal Resource Centre (Perth, Australia), with all animal experiments performed in a pathogen free facility according to national and institutional guidelines. Bone marrow was harvested from the femur, tibia and spine using a mortar and pestle in PBS supplemented with 2% fetal calf serum (FCS) as previously described (Holst *et al.*, 2006). Bone marrow cells were passed through a 70 μ m filter to ensure a single cell suspension, and incubated with lineage specific antibodies (B220, CD19, CD3, Sca-1; BioLegend), conjugated to biotin, together with anti-Gr-1-fluorescein isothiocyanate (FITC; Biolegend), anti-cKit-phycoerythrin (PE; Becton

Dickinson), anti-CD34-Alexafluor 647 (eBioscience) and anti-CD16/32-PerCP/Cy5.5 (Becton Dickinson) antibodies. Cells were washed twice in PBS with 2% FCS and incubated with streptavidin-APC-Cy7 (Biolegend). Control stains for FITC, PE, PerCP/Cy5.5, Alexafluor 647 and APC-Cy7 were used to determine compensation settings and gating for each population. Mature granulocytes were purified as shown previously (Guibal *et al.*, 2009), using fluorescence activated cell sorting (FACS) on a Becton Dickinson Aria II. After purification, a small sample of the cells was reanalyzed for purity by flow cytometry, and a separate sample was stained using May-Grünwald Giemsa following a cytospin. Purification of the nucleus from sorted cells was carried out using the PARIS kit (Ambion) with modifications to minimise RNA degradation. In order to confirm the purity of the nuclear fraction, SDS-PAGE and Western blots were carried out to detect the known cytoplasmic protein GAPDH, and the ubiquitous protein β -actin, on whole cell and nuclear lysates to ensure absent cytoplasmic contamination of the nuclear fraction. RNA was extracted from the nuclear fraction using Trizol prior to deep sequencing. Deep sequencing of nuclear small RNAs was performed by GeneWorks (Adelaide, Australia) on the Illumina GAII. Library preparation and sequencing was completed according to the manufacturers' instructions.

20

Other small RNA deep sequencing datasets

Small RNA datasets from mouse (Babiarz *et al.*, 2008), *Drosophila* (Chung *et al.*, 2008), *C. elegans* (Batista *et al.*, 2008), *A. queenslandica* (Grimson *et al.*, 2008) and budding yeast (Drinnenberg *et al.*, 2009) were obtained from the NCBI Gene Expression Omnibus (NCBI GEO). See **Table 7** for a complete list of the datasets and their corresponding identifiers. Human GRO-seq data (Core *et al.*, 2008) was obtained through NCBI GEO (GSE13518). The summary 'aligned' BED files provided by the authors, and available at the GEO website, were used for all analyses. Arabidopsis small RNA datasets were obtained from the Arabidopsis SBS database, available at http://mpss.udel.edu/at_sbs/ (Nakano, 2006), and pooled.

30

Reference genome and annotation sources

Human (hg18, NCBI Build 36.1), mouse (mm9, NCBI Build 37), *Drosophila* (dm3, BDGP Release 5), *C. elegans* (ce6, WS190), and *S. cerevisiae* (sacCer2, SGD June 2008) genome sequences, and gene and genome feature annotations, were obtained from a local mirror of the UCSC genome browser (Kuhn *et al.*, 2009). Human and mouse Refseq genes were obtained from the respective refGene databases. *Drosophila* Flybase, *C. elegans* Sanger, and *S. cerevisiae* SGD gene annotations were obtained from the dm3.flyBaseGene, ce6.sangerGene, and sacCer2.sgdGene databases, respectively. *A. queenslandica* contig sequences and gene annotations were obtained from the University of Queensland sponge genome sequence repository. The *S. castellii* genome sequence and gene annotations were obtained from the Yeast Gene Order Database, available at <http://wolfe.gen.tcd.ie/ygob/> (Byrne & Wolfe, 2005). We used the Arabidopsis TAIR8 genome sequence (ftp://ftp.arabidopsis.org/home/tair/Sequences/whole_chromosomes/) and the TAIR8 Ensemble gene annotations (Poole, 2007).

CD4+ T-cell nucleosome modification data (Barski *et al.*, 2007; Wang *et al.*, 2008) was downloaded directly from the authors' website, and is available at <http://dir.nhlbi.nih.gov/papers/lmi/epigenomes/hgtcell.aspx> and <http://dir.nhlbi.nih.gov/papers/lmi/epigenomes/hgtcellacetylation.aspx>. The summary bed files provided by the authors were used as the basis for all analyses (see below for more details). Control CD4+ T-cell nucleosome datasets were obtained from the NCBI Sequence Read Archive (SRR000711 - SRR000720), and processed to obtain nucleosome-length fragments as described previously (Nahkuri *et al.*, 2009) (more below). *S. cerevisiae* combined H3 and H4 nucleosome data was obtained from <http://atlas.bx.psu.edu/yeast-maps/yeast-index.html> (Mavrich *et al.*, 2008).

Bioinformatic analyses

All bioinformatic analyses were done on a local high-performance computer that houses a mirror of the UCSC Genome Browser. For most analysis we used a suite of in-house AWK, Perl, and Python scripts or backend tools

inherent to the UCSC mirror. Small RNA datasets, raw CD4+ nucleosome data, and *S. cerevisiae* H3 and H4 nucleosome data were mapped to the appropriate genome using ZOOM (Lin *et al.*, 2008). Small RNA, GRO-seq, chromatin modification and nucleosome density distributions were accomplished by
5 converting mapped tag positions (*i.e.*, BED coordinates) to genome-wide wiggle density plots and averaging these densities across all loci of interest (*e.g.*, Refgene TSSs) using a set of in-house Perl scripts.

CD4+ T-cell nucleosome data (Barski *et al.*, 2007; Wang *et al.*, 2008) were processed to facilitate high-resolution bioinformatic queries. The signals
10 from the plus and minus strands associated with the same nucleosome are typically ~150 bp apart, because the sequence tags are derived from the ends of the strands rather than over their whole length. Therefore, to obtain accurate ChIP-seq nucleosome profiles from the publicly available deep sequencing data we extended the genomic matches of all uniquely mapping tags *in silico* in the 3'
15 direction so that they reached a total length of 150 nt, consistent with the expected length of nucleosome associated DNA, as described previously (Nahkuri *et al.*, 2009; Schmid & Bucher, 2007). To compute the 'wiggle profile' we summed the distribution of all tags across the genome downstream of the TSS, and then computed the average based on the number of TSSs queried. This resolves into a
20 distinct single curve representing peak nucleosome density. Pol II ChIP-seq fragments were also summed across all genes with tiRNAs and, like the nucleosome data, resolved to a single high-resolution curve.

The abundance of THP-1 nuclear and cytoplasmic small RNA datasets were normalized by the relative expression of spike-ins 2 and 6 (**Table 2**).
25 Bioinformatic queries against spike-ins were performed without mismatches to ensure accurate quantification and normalization. Identification and analysis of THP-1 nuclear tiRNAs was performed as previously described (Taft *et al.*, 2009 (I)). Briefly, tags that mapped to known small RNA loci, repeat elements, or other potential confounding features were removed and small RNAs with a modal
30 length of ~18 nt that map sense and proximal (generally -60 to +120 nt) to TSSs (defined by Refgene annotations) were identified.). Analysis of the expression of genes with tiRNAs derived from the THP-1 nuclear, cytoplasmic, or total RNA

fractions was accomplished using gene expression data from undifferentiated THP-1 cells (<http://fantom.gsc.riken.jp/4/>) (Suzuki *et al.*, 2009) as described previously Taft *et al.*, 2009 (I). Refgenes with high tiRNA abundance (>8) or low tiRNA abundance (1) were obtained and regions -60 to +300 relative to the TSS were assessed for chromatin mark densities, as described above. 'Unannotated' 18mers were identified after eliminating all canonical tiRNAs, and then further filtering to exclude those proximal to any knownGene TSS or within a knownGene boundary (*i.e.*, within the bounds defined by the transcription start and stop sites). Enrichments at chromatin marks were accomplished using loci with chromatin mark tag densities (relative scores) two standard deviations higher than the mean for that mark across the entire genome. These loci were then collapsed down to single unique entities using the UCSC backend tool, featureBits. Loci located near TSSs (within 200 nt) or that mapped to known small RNA annotations were excluded from the analysis. The relative enrichment of nuclear small RNAs at each chromatin mark or protein binding site was assessed using an in-house bootstrapping program over 1000 iterations. All chromatin marks and binding sites were high-confidence – the loci examined were required to have ChIP-seq scores greater than 2 standard deviations above the mean for that sample/chromatin mark. Bootstrap control experiments at 50, 100, and 500 iterations showed no significant difference in enrichment profiles, suggesting that small RNA enrichments at specific chromatin marks are robust.

Splice site RNAs are defined as small RNAs less than or equal to 26 nt, dominantly 17-18 nt, whose 3' ends are precisely coincident any exonic splice donor site (*i.e.*, the 3' end of an exon), including donor sites at both non-protein-coding and canonical protein-coding genes. Splice site RNAs also include small RNAs less than or equal to 26 nt, dominantly 17-18 nt, whose 3' ends map to the 3' end of internal exons, that is, exons that are bounded on both sides by an intron and/or exons that are exclusively protein encoding. To ensure the observed spliRNA enrichment was not influenced by the failure to map tags across splice site junctions, small RNAs were mapped to both the genome and a library of splice site junctions for each organism. Dips of small RNAs just across the splice site in some organisms may reflect poor gene annotations (*i.e.*, incorrectly

annotated or missed exons). Analysis of the expression of genes with spliRNAs in human and *Drosophila* was accomplished using gene expression data from undifferentiated THP-1 cells (<http://fantom.gsc.riken.jp/4/>) (Suzuki *et al.*, 2009) and a *Drosophila* developmental time course (Arbeitman *et al.*, 2002), as described previously (Taft *et al.*, 2009 (I)). Sequence logos were generated using Weblogo (Crooks *et al.*, 2004) To examine the association of spliRNAs with alternative and constitutive exons UCSC knownGene exon annotations were used to derive the splicing status of exons with spliRNAs versus exons without spliRNAs in the same genes. The prevalence of four different alternative splicing events (see **Table 4**) in both data sets was assessed, and the statistical significance of the observed difference was calculated using the Fisher's exact test. The analysis was performed using in-house C and Perl programs that are available on request.

We used annotations from miRbase Version 12 (Griffiths-Jones *et al.*, 2008) to assess THP-1 nuclear and cytoplasmic micro RNA expression. To ensure accurate miRNA expression values we included both uniquely mapping and multimapping tags in our analysis. However, multimapping tags were only analysed if they multimapped amongst miRNA loci. Relative micro RNA expression was calculated as the sum of the normalized abundance of all tags that mapped to any particular pre-miRNA. We defined micro RNA-offset RNAs as any RNA tag that covered the most 5' or 3' ends of a pre-miRNA annotation.

Results

Investigation of human Refseq exon boundaries revealed more than 5,000 THP-1 genes with small RNAs whose 3' termini map precisely to the splice donor site (*i.e.*, the 3' end of the exon), are approximately 35-fold enriched in the nuclear deep sequencing library (**Fig. 4a,b**), and present at internal exons regardless of gene length or exon number (**Table 3**). These splice-site RNAs (spliRNAs) are detectable using mapping strategies that consider exon-exon or exon-intron boundaries and multi-mapping deep sequencing reads (**Figs. 7-9**).

We also found that spliRNAs are nuclear localized in primary mouse granulocyte nuclei (**Figs. 10 and 11**), and are detectable in a diverse set of

evolutionarily distant animals. Splice-site RNAs are expressed in mouse embryonic stem (ES) cells (Fig. 4c), a wide range of *Drosophila melanogaster* (Fig. 4d) and *Caenorhabditis elegans* (Fig. 4e) tissues, and in one of the most basal multicellular animals, the marine sponge *Amphimedon queenslandica* (Fig. 4f). They have a modal length of 17-18 nt in human THP-1 cells, and a modal length of 17 nt in all other species examined. Their expression is not affected by the loss of Dicer or DGCR8 in mouse ES cells or *C. elegans* germline mutants (Figs. 12 and 13), indicating that spliRNA biogenesis is not intimately connected with the pathways that produce miRNAs or siRNAs. Indeed, with few exceptions, spliRNAs are expressed in most tissues and developmental stages in *D. melanogaster* and *C. elegans* (Figs. 14 and 15). However, spliRNAs are more enriched compared to background in *Drosophila* heads compared to bodies, are almost undetectable in imaginal discs, and are less abundant in adult sponge compared to embryo (Figs. 16 and 17), suggesting that spliRNAs may be connected with high gene expression in actively proliferating or undifferentiated tissues. Indeed, THP-1 and *Drosophila* genes with spliRNAs are more highly expressed than those without (Fig. 18). To investigate if spliRNAs are present outside the animal kingdom, small RNA distributions at splice donor sites in the flowering plant *Arabidopsis thaliana* and the budding yeasts *Saccharomyces castellii* and *Saccharomyces cerevisiae* were investigated. No evidence of spliRNAs was detected in yeast or plants (Fig. 19).

Overall, spliRNAs are weakly expressed (the median abundance in THP-1 nuclei is 1) and show a strong enrichment for 3'-terminal guanines, which is likely, however, driven by the consensus splice site sequence (Fig. 20). Additionally, although spliRNAs are statistically more common at constitutive splice sites, we also observed a mild but statistically significant enrichment of spliRNAs at alternative first exons (Table 4). To query the relationship between RNAPII activity and spliRNAs we examined the recently described GRO-seq (Core *et al.*, 2008) dataset, which captures the position, amount and orientation of transcriptionally engaged RNA polymerases. We found a local GROseq minimum at the splice donor site (Fig. 21), which aligns with the position of spliRNAs and may be consistent with a model of spliRNA biogenesis dependent on cleavage of

the 3' end of the nascent transcript. Consistent with this hypothesis, short introns were twofold enriched downstream of exons expressing spliRNAs, which could promote RNAPII pausing and backtracking due the proximity of the downstream exon-associated nucleosome (**Fig. 22**), and spliRNAs are approximately 2-fold
5 less frequent at exons < 60 nt (**Fig. 23**), which generally lack positioned nucleosomes (Anderson *et al.*, 2009).

Discussion

Taken together, these data suggest that there is a wide diversity of small
10 RNAs localized to, and abundant in, the metazoan nucleus. We propose that many of these species are involved in regulating epigenomic modifications and transcription. Transcription initiation RNAs and spliRNAs may have a common origin and a common function, possibly associated with the positioning of nucleosomes. If this is so, our preferred hypothesis is that this is an evolved
15 capacity of RNAPII backtracking and TFIIS activity (Taft *et al.*, 2009 (II)) that allows efferent signals to be produced in parallel with transcription elongation to mark the position for future reference. Indeed, 31% of THP-1 genes with spliRNAs also have tiRNAs. However, two alternative, but not mutually
20 exclusive, possibilities are that spliRNAs are linked to, or are by products of, splicing or result from post-transcriptional cleavage of longer capped RNAs (Fejes-Toth *et al.*, 2009).

The absence of tiRNAs and spliRNAs in yeast and plants may reflect different systems of nucleosome positioning, chromatin marking, or the criteria used to define these small RNA classes. For example, small RNAs derived from
25 wild type *S. cerevisiae* (Drimmenberg *et al.*, 2009), which lacks RNAi, are dominantly ~17-18 nt, have a 3'-terminal nucleotide purine (*i.e.*, adenine) bias, and are phased such that local small RNA maxima coincide with minima of nucleosome density (**Fig. 24**). Therefore, although these small RNAs do not meet the criteria we have used to define tiRNAs and spliRNAs in metazoans, they
30 exhibit many similar characteristics, suggesting that very small RNAs are a basal feature within the eukaryotic lineage that may have been coopted to specific genomic positions, and into specific roles, in animals.

Example 2 – Splice site RNAs differentiate normal and breast cancer tissue

To test if spliRNAs are differentially expressed in, and therefore diagnostic of, cancer, we assessed small RNA deep sequencing data obtained from normal adjacent and ductal carcinoma *in situ* (DCIS, stage 2) human breast tissue from a single patient. These data show that spliRNAs are highly expressed in both normal and DCIS tissue, but are nonetheless significantly different. Indeed, a Gene Ontology enrichment analysis showed that while genes with spliRNAs in the normal adjacent tissue are strongly enriched for terms associated with terminal differentiation (**Table 8**), including three morphogenesis-associated Gene Ontology terms, they are completely absent in the breast carcinoma sample (**Table 9**). Likewise, KEGG pathway analysis revealed that enrichment for the Cell Cycle and Focal Adhesion pathways was specific to genes with spliRNAs in the normal adjacent tissue (**Fig. 25**). These enrichments were lost in DCIS tissue, and replaced with enrichments for the spliceosome and the RNA degradation pathway (**Fig. 26**)

Additionally, a differential expression analysis showed that a subset of more than 170 genes had spliRNA expression values twofold or more up-regulated in the DCIS sample compared to normal (**Fig. 27**). Querying GeneSigDB revealed that these genes are significantly associated with gene expression signatures previously linked to breast cancer, specifically those with ERCC2 and BRCA1 mutations (**Fig. 28**). Likewise, querying the GSEA MSigDB revealed significant overlap of genes with highly expressed spliRNAs in the DCIS sample with gene sets previously associated with a number of breast cancer subtypes and responses to treatments (**Table 10**). Overall, this data indicates that spliRNAs are differentially expressed between normal and cancer tissue, and are as good a diagnostic as much larger gene sets. This strongly suggests that spliRNAs may be a better diagnostic and prognostic indicator than traditional gene profiling.

30

Throughout this specification, the aim has been to describe the preferred embodiments of the invention without limiting the invention to any one

embodiment or specific collection of features. Various changes and modifications may be made to the embodiments described and illustrated herein without departing from the broad spirit and scope of the invention.

5 All computer programs, algorithms, patent and scientific literature referred to in this specification are incorporated herein by reference in their entirety.

41
TABLES

Table 1. THP-1 small RNA deep sequencing library statistics

	Cytoplasmic	Nuclear	Total
Total reads	6226629	6579778	5820445
Total reads minus 5' and 3' adaptor contamination	6057957	6270629	5089263
% of reads that map uniquely w/o mismatches	23	24	23
% of reads that map to the genome any number of times w/o mismatches	58.1	67	58
Average Illumina GAI v1.0 quality scores*	36.3	32.8	36.4

*Quality scores are the average of all quality values given in the Illumina FASTQ files for each library.

Table 2. Synthetic RNA spike-ins and library normalization

Spike-in	Cytoplasmic abundance	Nuclear abundance	Total RNA abundance	Fold Change (Nuc v. Cyto)
ATCAGCTGTTATAAGCCGGCC	3	12	14	-
AGTAACTCTAGCGGCTTAGTC	1836	2741	3010	1.49
AACCTATGGTTGCGCTACGAC	6	14	25	-
ATCGTGCAATCGCGCATA	2	4	12	-
ACTCTATACGCGGTACGA	10	15	37	-
ATACGTCGACACGGTTCA	263	476	580	1.8
Average Fold Change (Nuc v. Cyto)				1.645

*All values listed above are total sequence counts (*i.e.*, total sequence abundance in a given library).

Table 3. Number of unique spliRNAs per exon in human THP-1 cells

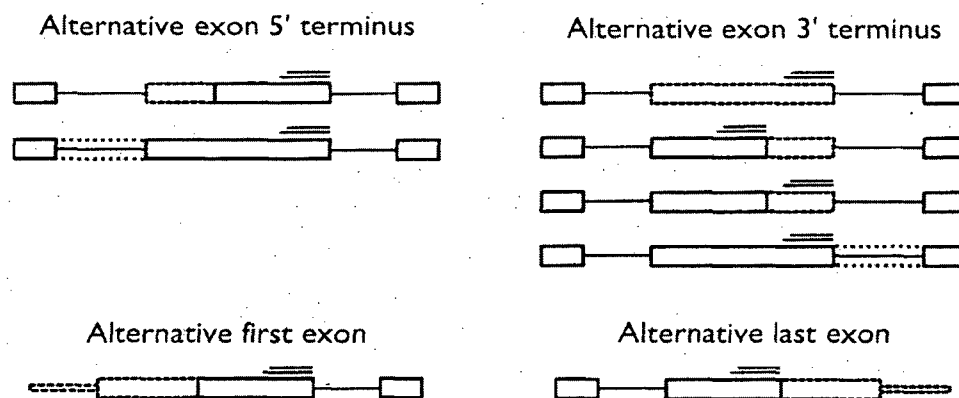
	1st exon	2nd exon	3rd exon	4th exon	5th exon	6th exon	7th exon	8th exon	9th exon	10th exon	11th exon	12th exon	13th exon	14th exon	15th exon	16th exon	17th exon	18th exon	19th exon
two	56	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
three	50	89	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
four	49	77	76	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
five	67	77	93	89	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
six	77	95	101	98	94	-	-	-	-	-	-	-	-	-	-	-	-	-	-
seven	45	79	75	102	86	85	-	-	-	-	-	-	-	-	-	-	-	-	-
eight	48	61	76	68	96	121	79	-	-	-	-	-	-	-	-	-	-	-	-
nine	45	59	71	80	66	85	85	81	-	-	-	-	-	-	-	-	-	-	-
ten	63	80	66	101	78	81	51	82	75	-	-	-	-	-	-	-	-	-	-
eleven	52	77	78	63	59	81	69	68	83	55	-	-	-	-	-	-	-	-	42
twelve	31	57	72	69	47	41	51	62	59	53	47	-	-	-	-	-	-	-	-
thirteen	40	36	71	73	51	49	59	39	52	52	40	54	-	-	-	-	-	-	-
fourteen	26	43	34	47	47	33	42	39	38	41	39	38	38	-	-	-	-	-	-
fifteen	28	37	30	39	55	49	30	58	39	46	43	29	28	30	-	-	-	-	-
sixteen	23	16	20	33	53	27	65	33	57	27	21	32	27	36	23	-	-	-	-
seventeen	13	18	11	24	39	24	29	35	27	30	28	25	33	31	29	14	-	-	-
eighteen	14	21	15	20	33	21	29	25	19	18	36	28	31	14	30	18	16	-	-
nineteen	13	14	21	21	25	19	30	19	29	10	31	28	32	18	23	19	17	10	-
twenty	13	23	7	12	24	8	22	26	21	12	14	24	28	24	17	20	10	25	11

*Note: Human Refseq genes with various numbers of exons are shown as rows. For example 'three' refers to genes with three exons. Columns show the number of spliRNAs (*i.e.*, small RNAs that map to 5' splice sites) counted at each exon in that gene. SpliRNAs may be counted more than once since they may map to exons present in more than one mRNA isoform. spliRNAs are not preferentially positioned at any one exon, but do appear to be generally enriched at internal exons. Overall, 7.6% of exons within genes with spliRNAs show evidence of spliRNA expression.

Table 4. Splice-site RNA occurrence by exon type

Splicing class	Frequency (%) at spliRNA exons	Frequency (%) at other exons in the same genes	Right P value (characteristic to spli exons)	Left P value (characteristic to other exons)
Constitutive exons	78.3	74.7	1×10^{-6}	0.99
Alt exon 5' terminus	3.2	2.9	0.17	0.85
Alt exon 3' terminus	12.6	17.5	1	6.1×10^{-14}
Alt first exon	3.4	2.4	2.6×10^{-4}	1.0
Alt polyA site	3.6	3.7	0.57	0.47

*Note that all percentages and statistics were calculated by examining exons amongst genes with spliRNAs.



*Diagrammatic representation of the exon types analysed in **Table 4**, above.

Table 5. qRT-PCR primers

Species	Forward Primer	Reverse Primer
tRNA Lys (AAG)	CGGCTAGCTCAGTCGGTAGA	CCAACGTGGGGCTCGAAC
tRNA SeC (TGA)	GGATGATCCTCAGTGGTCTG	CGAAAGGTGGAATTGAACC
tRNA His (CAY)	GCCGTGATCGTATAGTGGTTAG	CCGTGACTCGGATTCTGAAC
U1 snRNA	CTTACCTGGCAGGGGAGATA	GCAGTCGAGTTTCCACATT
U4 snRNA	CGTGCCAATGAGGTTTATCC	AAAATTGCCAATGCCGACTA
U3 snoRNA	AACCCCGAGGAAGAGAGGTA	CACTCCCAATACGGAGAGA
SNORA19	GCACATTTTCATTGACCTGCTT	GAAGGAGACTGGCAGCATTAC

Table 6. Northern blot probes

Target	Probe sequence (5' to 3')
SNORD77	CATCAGACAGATAGTACATCTCTTCATGAT
SNORA19	AAGCAGGTCAATGAAATGTGC
tRNA Lys (AAG)	TCTACCGACTGAGCTAGCCG
tRNA His (CAY)	CTAACCCTATACGATCACGGC
miR-16	CGCCAATATTACGTGCTGCTA
miR-15	GTAAACCATGATGTGC

Table 7. GEO small RNA deep sequencing datasets

GEO identifier	Library identifier	Description
<i>Mus musculus</i> (mouse)		
GSE12521	GSM314552	WT ES cells
	GSM314553	Dicer ^{-/-} ES cells
	GSM314557	DGCR8 ^{-/-} ES cells
<i>Drosophila melanogaster</i> (fruit fly)		
GSE11624	GSM286613	0-1h embryo
	GSM286605	2-6h embryo
	GSM286606	6-10h embryo
	GSM240749	Female heads
	GSM286603	Female bodies
	GSM286601	Male heads
	GSM286602	Male bodies
	GSM275691	Imaginal discs
	GSM272651	S2 and KC cells
	GSM272653	KC cells
<i>Caenorhabditis elegans</i> (nematode)		
GSE11738	GSM297742	Embryo
	GSM297743	L1 larvae
	GSM297744	L2 larvae
	GSM297745	L3 larvae
	GSM297746	L4 larvae
	GSM297749	Glp-4 mutant
	GSM297750	Mixed stage
	GSM297751	Adult
	GSM297752	Prg-1 mutant
	GSM297753	Fog-2 mutant
<i>Amphimedon queenslandica</i> (marine sponge)		
GSE12578	GSM315551	Embryo
	GSM315553	Adult
Budding yeast		
GSE17872	GSM447740	Wild type <i>Saccharomyces castellii</i>
	GSM447747	Wild type <i>Saccharomyces cerevisiae</i>

Table 8. Gene Ontology enrichment analysis – normal adjacent tissue

GO ID	Description	P value
GO:0003159	morphogenesis of an endothelium	0.006
GO:0015803	branched-chain aliphatic amino acid transport	0.006
GO:0015820	leucine transport	0.006
GO:0015827	tryptophan transport	0.006
GO:0060356	leucine import	0.006
GO:0060440	trachea formation	0.006
GO:0061047	branching involved in lung morphogenesis	0.006
GO:0061154	endothelial tube morphogenesis	0.006
GO:0030388	fructose 1,6-bisphosphate metabolic process	0.005
GO:0015801	aromatic amino acid transport	0.015
GO:0014009	glial cell proliferation	0.042
GO:0070062	extracellular vesicular exosome	0.042
GO:0050775	positive regulation of dendrite morphogenesis	0.04
GO:0048593	camera-type eye morphogenesis	0.009
GO:0016278	lysine N-methyltransferase activity	0.006
GO:0016279	protein-lysine N-methyltransferase activity	0.006
GO:0018024	histone-lysine N-methyltransferase activity	0.006
GO:0016571	histone methylation	0.011
GO:0042054	histone methyltransferase activity	0.01
GO:0004003	ATP-dependent DNA helicase activity	0.011
GO:0051291	protein heterooligomerization	0
GO:0008094	DNA-dependent ATPase activity	0.01
GO:0003678	DNA helicase activity	0.045
GO:0004386	helicase activity	0
GO:0016568	chromatin modification	0
GO:0016887	ATPase activity	0.011
GO:0005524	ATP binding	0.042
GO:0032559	adenyl ribonucleotide binding	0.049

Table 9. Gene Ontology enrichment analysis – breast carcinoma sample

GO ID	Description	P value
GO:0015803	branched-chain aliphatic amino acid transport	0
GO:0015820	leucine transport	0
GO:0015827	tryptophan transport	0
GO:0060356	leucine import	0
GO:0015801	aromatic amino acid transport	0.004
GO:0001786	phosphatidylserine binding	0.003
GO:0005544	calcium-dependent phospholipid binding	0.015
GO:0004386	helicase activity	0
GO:0008026	ATP-dependent helicase activity	0
GO:0070035	purine NTP-dependent helicase activity	0
GO:0042623	ATPase activity, coupled	0
GO:0016887	ATPase activity	0
GO:0005524	ATP binding	0.005
GO:0032559	adenyl ribonucleotide binding	0.007
GO:0030554	adenyl nucleotide binding	0.015

Table 10. GSEA MsigDB query

Description	P value
Genes down-regulated in non-metastatic breast cancer tumors having type 1 amplification in the 20q13 region.	1.24E-05
Genes down-regulated in PaCa44 and CFPAC1 cells (pancreatic cancer) after treatment with decitabine.	1.54E-05
Genes down-regulated by ESRRA [Gene ID=2101] only.	2.36E-05
DNA repair genes whose promoters contain putative ZNF143 [Gene ID=7702] binding sites.	2.97E-05
Upregulated by induction of exogenous BRCA1 in EcR-293 cells.	5.22E-05
Genes regulated by ESRRA [Gene ID=2101] in MCF-7 cells (breast cancer).	5.60E-05
Genes up-regulated in hepatoblastoma samples compared to normal liver tissue.	6.11E-05

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CLAIMS

1. An isolated substantially single-stranded RNA molecule, wherein said isolated RNA molecule comprises a nucleotide sequence:
 - (i) comprising no more than 26 contiguous nucleotides that correspond to a genomic DNA sequence associated with gene regulation; and
 - (ii) comprising a nucleotide sequence corresponding to a 3' nucleotide sequence of an internal exon of the genomic DNA sequence.
2. The isolated RNA molecule of Claim 1, wherein said isolated RNA molecule comprises a nucleotide sequence that corresponds to a sense strand of the internal exon of the genomic DNA sequence.
3. The isolated RNA molecule of Claim 1 or 2, wherein said isolated RNA molecule terminates in a 3' nucleotide sequence that corresponds to the 3' end of the internal exon of the genomic DNA sequence.
4. The isolated RNA molecule of any one of Claims 1-3, wherein said isolated RNA molecule consists of a nucleotide sequence that corresponds to the 3' end of the internal exon of the genomic DNA sequence.
5. The isolated RNA molecule of any one of Claims 1-4, wherein said isolated RNA molecule comprises a nucleotide sequence that corresponds to a nucleotide sequence that is located at or near a 5' splice site of the internal exon of the genomic DNA sequence.
6. The isolated RNA molecule of Claim 5, wherein said isolated RNA molecule consists of a nucleotide sequence that corresponds to a nucleotide sequence that is located at the 5' splice site of the internal exon of the genomic DNA sequence.
7. The isolated RNA molecule of any one of Claims 1-6, wherein said isolated RNA molecule consists of 14-26 contiguous nucleotides.
8. The isolated RNA molecule of Claim 7, wherein said isolated RNA molecule consists of 14-20 contiguous nucleotides.
9. The isolated RNA molecule of Claim 8, wherein said isolated RNA molecule consists of 16-18 contiguous nucleotides.

10. The isolated RNA molecule of Claim 9, wherein said isolated RNA molecule consists of 17 or 18 nucleotides.
11. The isolated RNA molecule of any one of Claims 1-10, wherein said isolated RNA molecule comprises a nucleotide sequence that is located in or obtainable from a cell nucleus.
12. The isolated RNA molecule of any one of Claims 1-11, wherein the genomic DNA sequence is of or obtainable from a eukaryote.
13. The isolated RNA molecule of any one of Claims 1-12, wherein the genomic DNA sequence is of or obtainable from a metazoan.
14. The isolated RNA molecule of any one of Claims 1-13, wherein the genomic DNA sequence is of or obtainable from a vertebrate or a mammal.
15. The isolated RNA molecule of any one of Claims 1-14, wherein the genomic DNA sequence is of or obtainable from a human.
16. The isolated RNA molecule of any one of Claims 1-15, wherein the isolated RNA molecule comprises a nucleotide sequence that is GC enriched, preferably comprising a GC content of >54%.
17. An isolated substantially single-stranded RNA molecule comprising a nucleotide sequence:
 - (i) consisting of 17 or 18 nucleotides that correspond to a sense strand of an internal exon of a mammalian genomic DNA sequence located at a 5' splice site;
 - (ii) corresponding to a 3' end of the internal exon of the mammalian genomic DNA sequence;
 - (iii) comprising a GC content of >54%; and
 - (iv) that is substantially free of internal base-pairing.
18. The isolated RNA molecule of any one of Claims 1-17, wherein said isolated RNA molecule comprises a nucleotide sequence selected from any one of the nucleotide sequences set forth in SEQ ID NOs:1 to 16,898, or a nucleotide sequence at least partly complementary thereto.

19. A modified RNA molecule comprising the isolated RNA molecule any one of Claims 1-18, or a nucleotide sequence at least 70% identical thereto.
20. The modified RNA molecule of Claim 19 comprising a chemical entity selected from the group consisting of: a modified base, a carbohydrate, a peptide, a biotin, a cholesterol molecule, a fluorophore, a radionuclide, and a metal.
21. The modified RNA molecule of Claim 19 comprising a chemical modification selected from the group consisting of an LNA, a PNA, a 2'-O-methyl, and a morpholino.
22. The modified RNA molecule of Claim 19, wherein said modified RNA molecule is selected from the group consisting of an antisense inhibitor, a point mutant, a spliRNA mimic, and a spliRNA sponge.
23. A fragment of the isolated RNA molecule of any one of Claims 1-18, wherein said fragment comprises at least 5 nucleotides of said isolated RNA molecule.
24. A genetic construct comprising or encoding one or a plurality of:
 - (i) the isolated RNA molecule of any one of Claims 1-18;
 - (ii) the modified RNA molecule of any one of Claims 19-22; or
 - (iii) the fragment of Claim 23.
25. The genetic construct of Claim 24, wherein said genetic construct is an expression construct comprising a DNA sequence complementary to one or a plurality of the isolated RNA molecules of any one of Claims 1-18, the modified RNA molecule of any one of Claims 19-22, or the fragment of Claim 23, operably linked or connected to one or more regulatory nucleotide sequences.
26. The genetic construct of Claim 24 or the expression construct of Claim 25, wherein said genetic construct or said expression construct is selected from the group consisting of a phage, a plasmid, a cosmid, and an artificial chromosome.
27. A host cell comprising the genetic construct of Claim 24 or 26, or the expression construct of Claim 25 or 26.

28. A method of identifying the isolated RNA molecule of any one of Claims 1-18, or the fragment of Claim 23, said method including the step of isolating one or more of the isolated RNA molecules from a nucleic acid sample.
29. The method of Claim 28, wherein said nucleic acid sample is of or obtainable from a human.
30. A method of identifying one or more of the isolated RNA molecules of any one of Claims 1-18, or the fragment of Claim 23, said method including the step of identifying a genomic DNA sequence which is complementary to the nucleotide sequence of said one or more isolated RNA molecules.
31. The method of Claim 30, wherein said genomic DNA sequence is of or obtainable from a human.
32. A method of identifying a regulatory region in a genome, said method including the step of identifying one or more of the isolated RNA molecules of any one of Claims 1-18, or the fragment of Claim 23, to thereby identify said regulatory region.
33. The method of Claim 32, wherein said regulatory region is associated with one or more regulatory activities selected from the group consisting of mRNA splicing, transcription, epigenetic modification, epigenetic regulation, chromatin modification and nucleosome positioning.
34. The method of Claim 32 or 33, wherein said genome is of a human.
35. The method of any one of Claims 28-34, wherein said method is undertaken using a deep sequencing technology selected from the group consisting of 454TM-, Helicos-, PacBio-, Solexa/Illumina- and SOLiD-sequencing.
36. A computer-readable storage medium or device encoded with data corresponding to one or more of:
 - (i) the isolated RNA molecule of any one of Claims 1-18;
 - (ii) the modified RNA molecule of any one of Claims 19-22; or
 - (iii) the fragment of Claim 23; and

- (iv) an at least partly complementary RNA or DNA molecule of the isolated RNA molecule of any one of Claims 1-18 or the fragment of Claim 23
37. A method of determining whether a mammal has, or is predisposed to, a disease or condition associated with one or more regulatory regions of a genome, said method including the step of determining whether said mammal comprises one or more of the isolated RNA molecules of any one of Claims 1-18, or the fragment of Claim 23, wherein the or each nucleotide sequence of said one or more isolated RNA molecules or said fragment corresponds to a genomic DNA sequence associated with said disease or condition.
38. The method of Claim 37, wherein said regulatory region is associated with one or more regulatory activities selected from the group consisting of mRNA splicing, transcription, epigenetic modification, epigenetic regulation, chromatin modification and nucleosome positioning.
39. The method of Claim 37 or 38, wherein said mammal is a human.
40. A nucleic acid array comprising a plurality of the isolated RNA molecules of any one of Claims 1-18, the modified RNA molecule of any one of Claims 19-22, the fragment of Claim 23, or one or more isolated nucleic acids respectively complementary thereto, immobilized, affixed or otherwise mounted to a substrate.
41. An antibody which binds:
- (i) the isolated RNA molecule of any one of Claims 1-18;
 - (ii) the modified RNA molecule of any one of Claims 19-22; or
 - (iii) the fragment of Claim 23; and
 - (iv) an at least partly complementary RNA or DNA molecule of the isolated RNA molecule of any one of Claims 1-18 or the fragment of Claim 23.
42. A kit comprising one or more of the isolated RNA molecules of any one of Claims 1-18, the modified RNA molecule of any one of Claims 19-22, the fragment of Claim 23, or one or more isolated nucleic acids

respectively complementary thereto, and/or the antibody of Claim 41, and one or more detection reagents.

43. A method of treating a disease or condition in a mammal, said method including the step of administering to the mammal a therapeutic agent selected from the group consisting of:

- (i) the isolated RNA molecule of any one of Claims 1-18;
- (ii) the modified RNA molecule of any one of Claims 19-22;
- (iii) the fragment of Claim 23;
- (iv) an at least partly complementary RNA or DNA molecule of the isolated RNA molecule of any one of Claims 1-18 or the fragment of Claim 23; and/or
- (v) the antibody of Claim 41;

to thereby treat said disease or condition.

44. The method of Claim 43, wherein said disease or condition is associated with aberrant regulation of one or more activities selected from the group consisting of transcription, mRNA splicing, epigenetic modification, epigenetic regulation, chromatin modification and nucleosome positioning.

45. The method of Claim 43 or 44, wherein the mammal is a human.

46. A pharmaceutical composition comprising a therapeutic agent selected from the group consisting of:

- (i) the isolated RNA molecule of any one of Claims 1-18;
- (ii) the modified RNA molecule of any one of Claims 19-22;
- (iii) the fragment of Claim 23;
- (iv) an at least partly complementary RNA or DNA molecule of the isolated RNA molecule of any one of Claims 1-18 or the fragment of Claim 23; and/or
- (v) the antibody of Claim 41.

and a pharmaceutically acceptable carrier, diluent or excipient.

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TTGTGAAGGACCAGGAG
TTGTGGAAATGTGGCG
TTGTGGACTGTGACGG
TTGTGGGAGCAGAAGTTG
TTGTGGGCCAGACAAG
TTGTTCGGGAAAACAG
TTGTGACCGGCTAC
TTGTTTAGTGTGGACAT
TTTAATATTAAGGAAGAG
TTTACTGCAAGCATCCAG
TTTACTGGGGCTGAGGG
TTTATCTGTGTGACACC
TTTCACAGACTGGAAGAT
TTTCATCAGTGAGATTG
TTTCCACCATGCGCACGG
TTTCCAGGATGAAGAAG
TTTCCATTCTTACCTG
TTTCCCTGGACTGCAG
TTTCTCTTACTGGGCGCG
TTTCTGCAGCGGAACG
TTTCTGCTTTGGCTGAGGGTAGT
TTTCTGGGAGTGACGTT
TTTGAAGAGGAGGACTTT
TTTGAAGGCATTGGAATG
TTTGAAGTGGCGCTCAG
TTTGACAGCGAGCGGGG
TTTGACTGATGACGAAG
TTTGAGCCTTTTGAAGA
TTTGATGCCGGCACTGT
TTTGCACGGGACCTGGG

TTTGCTCTGGAACAGCAG
TTTGGACAACATGACAG
TTTGGACCAGAATATGAG
TTTGGATCCGTCGTGAAT
TTTGGTATTGCAGATGAC
TTTGTGAGCTCATCAG
TTTGTGGACCAGCCAG
TTTTCAAAGATGGTAGAG
TTTTCTGGGTGGACACG
TTTTGATCGGCAGAAAA
TTTTGATCTTGTGCCTTG
TTTTGATGGACAGGAG
TTTTTGTCTGGATTAAG

CAGGATAAGCTAGAAT	AGATACACACAAACCTAAACCTCTGGG
TGCTGGTGGTCTGAAAG	TTGGACAGACTGGAGGAG
GACTGTAGAGGCGCTTCCTA	CCATCATGAACTACCAG
TTTTCTGGAGAACGAAG	GCCATTAAGTCTTAGGATTCACGTGG
CGACACAAGAAGCGGATCCAG	CCCAAGCCCATTTCATTTGAAT
AGGACCACGTGGACAAAGG	CGTGGCTGAGATGGAGGA
GTTGTTAATTGTAGACC	TCTCACCCCAACAAGCAG
ATGGTGTCCCTTTGGATG	GGACTCGCCTTCAGAAG
CGGCGGGGAGCTTGAACG	GAGTTTGTGGACAAGCAG
ATGTTGAAAAGATTGAAG	GTCTTTCTGAACAGGAG
CCGAGGCTTTTGTGGAG	TTTTTCGTTCACTTACTTAGG
TCCTGAAAGTCTCTGCAG	CGGCCCATGTGGAAGCAG
ATGTCTGACGAGGAAAC	AAACCATTAGTCATACAG
AAGAACATGGGCGCTCGCAG	AGAACGAGAATCAGAGAGCGGAG
AGCCCTTGTAGAAAGAAG	GTCTACTTCGGATTAAGCG
TCCTGTGCGAGAATCTGAG	CTTCAGTCCAACTACAG
TACCTGATCCAGGACGG	AAAGAGTGCTGGAAACAAGGACAG
TGTATGAAATGTTGGCTGGCCAG	TGTGAGAACTACATCAG
CAAGGAAGTCACTCATCCG	AATGTCTGTTGGAACAG
AAGTCACCAGATGTTTCAAG	AGACATCTCTGGAAGCAT
ATTCTCCATCAAGATGAAAG	ATGACATCATTTGCCTTGTTTCATG
ACCTTTGCTGAACTCAG	CTGTTTATTGGGCAAGAG
GAAACTGGGAACTAACAT	ATCAGCCTCAAAGCTCTGGAG
CTCAAGCGATTTCTTCAGGAG	GATGGAGGAATTTATGCC
GGGGGTCTTAGGAGGCATCT	CTCATCACCAGCGTGGAG
CCTGACTGTACTGAGAG	GGACACCACCAAAATGAG
ATCTGAACGTAGGGGGCAAGCG	GGGCGACTCGTCAGTGCGAG
CACTGTATGAGCTGGAG	ATATTTAGATTTATCTATG
CGTGGACAACTTGAAATCCG	GAAACTGCAGCTGAGAAG
TTTTTGCTGACTCTAAG	CCAGAGTTAAAGTGAACGTCTACTCGG
CCGATATAGATGAATCTG	ATTGTGTGAACAGACCAGTGAAG
CTCAACTTCCAACGTCAG	TCAGTGGATATAAAGGTTCAAG
GCCGGGACAAGAAGTCG	GTCATCCAGTTTGGTGTGCGCG
CTACGGGACCAAAGAGC	GCTACTACTCTGATAACGG
CACCAAGTGCCACAAAG	CATTGAGCGTCTCTTAAAGCTGGCG
CTGGTGGATGTAGAGAAG	TTATTTGACGAAAACG
TGATGTCCGGCGTCCTCTCTACAAG	AGAACAGCACCAGTAGCAG
GACTTGAAGGATACTCC	AAGACTGCAGCTGGACCG
TGCTTAGCAGACCAAAAG	GGAGTTTATGGATCAGTGAAC
ACTGTCTGGAACCGGACTGCAGAGAAA	GAGGACTTCCTGGTGCATGAG
GCCGGAGCCTGGGAGACG	AAGATATTGGTGGGAAAAG
CGCATTGGTGACCTTG	TCAGGGATCCGGAAGTAGT
AATTCAAGTGATGTAGAG	TTCAGAGACGTTCTGAGGGCTGTGGAG
GGAACCTTGTGCAATCGAG	ATCGTTGTTGGGATTCTGGTAGCTCAG
ATTTGACTTTGGGTCTGATGGCAAG	TCCCGCGGCTGCCTGAG
TGCCCAGGGATCCAGCGTGAG	GCATTTCTGTTGTGCCAG
ACATCCATACAGAACCTGAATAAAA	CAGCTCTTCTCAGCTCTGCAGCATCAG
AGGGGACTCTAACCAAG	GGACAACCTGGATGAGCAAGCAGGAG
TGACAGAAAAATCTGAG	TCTCATTGGATACTTGG
TGAACATCACTGAGGAGAAG	CTTGAGGAACCTATGACTTATTCAAG

FIG. 2A

GATAAACAGGAACTTGAG
ACATGTCAGGACTGAGG
AATCAATCGGAAGGAAG
CTGAACGCCATCATTGGG
CAAACCAGGGGATTAAAG
CTACTGAGCCGACTGCAGAAGCCCCAG
CCTATCGACGGAGACAACCG
CTGATAAGACTGCCAAG
TCACTTCTGTAAGTATAG
GGAATTGGATTTCGGAACATTGAAGAG
ATGTGCTACTGGCCCGG
CTGGAGCATCTGTGCAG
AACGGTTTTAGTTAAAG
GATACACCTTTACAGAGTCCCTGAG
GAAGATCCCGTCAGCTGTG
TTTATCACCTGCTTCAAAG
CGAGAAAGTGAGATAAAG
GAGGACGTCCAGAACAGCTG
GGAACAGCGGAACCTGAATGAACAG
GGAGAATTCAGAAACAG
CAGAACATTGCCCGCCAG
ACGTAAGTCACTCTGCAG
GCCTTTACTACTTTGAATACACCAG
GACCGCATTCTGCCAAG
TGGCTCGCACTGCAGGGACAACCCACAC
TGCTAGTGCTGTTGGCAG
GACTATCCTCGATGCCAG
CGGCCAACGAGCAGGCTG
GTGGAATGGCAGCAAAAG
TCGGAAAAGAACAAGACGGTTGTGTG
GAAGGAAGGGCTCGGAGCGGAG
CCATACGGCTGAGGAAGAAGGAAGAGG
TGCAGAGGAAGGTTGAAGTCTCTGAG
TTGATGACCTGATAGCAG
CAGTCTGTGGAAGAAGAG
AACACTGATCCGGAACG
CCAGCAGCCATGTACAG
TCACTGTGGAAGCTCCTACCACTTCAG
TCCGCATGGAACCTGACGAGACG
GTACTTCACGGCCTCT
GTTTCATGAAGCTCTGGAG
TTGGACGTTTGAGAAG
GTGCGAGAAGAACTCAG
CACTGCAGTACTGTTAAG
CTTCTCCACGTAGAACCCAAGCGGAAG
GTCCGGAACCTATGAATAG
CGAACTCACCTACAACCTAGAGAAG
GTTCCGTCTTGTAGAAAAG
AACGAAAAGACTAAAGA
AAGAAATACTGAATACAAAACCTTGACAG

AACGGATATTAAAAAG
AGAGAGACCGGATGGACAAG
CCAAAAAGGACATAGAAG
ATTATTGAAGATCTGGAG
TAGCTACTACTCTGATAACGG
ATCGGAGATGAAACAAAG
CTTGGATGTGGAGAAGG
AACAAATTCTGAAATACGATTGAG
GGAGAAAGCCTTCTGGGGTACTCG
AGTGATCGTCCCTGCAG
TCTGCTCACACCCCATCGCTGCTTCAG
TGAACGGAGAGAGGAGG
ATGGTGGCTTGTGGGAAG
CGGCTGGACCCTGGAGCAG
TCTCATGGATCTACAG
ATTTGCTGCACCTTGAG
TGAGACCATCTCCAACCTGGGCCTGG
ATGGACATGAGCACAATCAAG
GAGGAGGAAGATGATGACGATGG
CTGTGCTGGACTTACGA
ATCAAGATCCGTCTGACAG
CTGGACTTTGAGAAAGCAAAAG
CGCAGCGCTCGGTGAGGATTG
GGGAACCTTTGGCCAGCAAG
TGAATGAAACAACCTGCAG
AGAACCAAAGTGACTTGAAG
TGGAACCTACTGAAAGCAGGG
AGCGGCCCGACCTTGTTTT
ATGAGTGACCTGTTGAG
TGTGAACAGACCAGTGGAAG
ACGTGCGGACCCTGGAG
TGAATGCCTGTGGGATCAATTGCAGTG
GCGGAACAGCGGAACCTGAATGAACAG
AAATTTTCATGGCAATG
GAAGAAGGGATGAGAGTCGG
GAAGAGGACCACCCTGG
GAGTGAACCAAGAGAAGAG
CCCTGCTGTGTTGTTGG
CTCTTTGGAGGTTATGTCGCTAAGG
TTGTCAAAGAACCTGATG
GTGGGGACTCCGGCGGCAG
ACGTGAACCTCCAGCAGCCGGCGGACG
CTCTTCTCAGCTCTGCAGCATCAG
CTGCTGAAGCAGAGACATGTCCAG
AAGTTACTGTGAACATTGGCCAG
AAAGAAAGGGGTGTGAAG
TTACTATCTCTGGATGT
TTTACACGAAGAATGGACATAGTTTAG
CCTGAATCTGAATAAAGTG
CATTTGAGGACTCTGATG

CTGGTCAACTATCCAGAC (*Drosophila*)
CCATGGAACAGATGCCG
CCCCATTGCCAACGACG
ACATGGCGCAACTACA
TGGAGTGGTACCACAGT
GGAACCATGGCGGAGG
AGCTCGCCGACCATGCCA
CACCGCACATCAAAGAG
GTGCTGTCTTTCTCAAT
GAGTGCAATGGAATG
CCACCGATGAAGTTAAG
TCGAAGCACAGGAAGCG
CGTTCCGCGTTGCAAAG
GGGCTGCCACACTAACG
CCAGATTGGCAAGGATG
CAGGAGGTTTCTAAGCTG
GTGCGTAATCAAACGAAG
GCCAGGACTCGCTACA
CGCCACCGAAGAGTCACAAA
CTATGGACGAATGCGATGTAGACAAG
GCTGCAACGACATATCAG
CACAGGAATCTTAAAG
TTGTGATCACCATGCTGCCGAACA
ATGCGCTGCACACCCCCAAAG
TGCAATGGGCGATGACG
TAGCAAGGACTTCCGGCAG
CTCCATACACAGCAGCG
GGCCTCATCCGCCTGCA
CTATTCTACATAGAAA
TAGCTCTCAAAATGGTTAACATAGTG
ATGAGGAGCCACCTACG
GAGTTCGAAGATGCGCG
TCTACGAGGTGCCCGCTG
TGGAAGGTGGCGTCAAGTTGG
GGGCTTATGCAACACGA
ATGAGAAGTTGGGCGAG
GGGATAGCTCTGTATTG
CTCAGGTGTGAGGATCA
GAAGTGGAGCCGGAATTGCACAAG
GGCCGAGGCTGCAACAG
ACACCGACGCGCAATCTTATC
CGCAAATTGCTGCTGG
AGTGCTGGTTCGTGCAG
CCCGCCTGCGATATAA
TGTCGTGGGTCCAGAAACG
ACGTGCCCGTCAGTAAG
AATCGTCGACAATCCAG
CCTTCCAGCGCTGCGGAG
GACGATGTCGCCGTCACC
CCTCTCCGAGGGCGTCAAATAAAG

TGCGAGATCCCAGTCAG
CGCTTCTGGTTTCCGGCAAG
CTCGCTAACGAGCAA
CACGATGGCCAAAGAG
CCAATTTGGTGGAGCAGCAAAGCCG
CAAGGCCTCCAAGGGCAGCAGCGAA
TCACGCCGTGAGCAAAAG
CTATTGGAGCAGACAA
CTCTCTGTGTTGGCACTGCGAG
ATCATGGATGTGGCCGACTTC
CTGGCGGGGTGTCAATTG
CTGCGATAAAGATCAG
AGCTGCGACTGCGACAAG
AGCCAAGCAAATAAGG
TGAAACATGCAGGAGCTCAAG
GACTCGGAGCGCACGAA
CCGATTGATTTGCCCATG
CGATGTGCGCGTCACC
ACTTTGCCAGAACCCG
AGCAACTACGAAGTGAAG
CTACAGTGTGCCAATG
GTGCATCTCGTTACAG
TTCGAAACGCAGCCAG
AGACGCTGCAGAGCGAG
CATTGTCAGCCGACGAAG
CCAACGTGGTGGCCCCG
ACCAGGGCATGCGATAT
CTTTCTGTGGCATGAAAGC
TCGCCCTCACAAGCCATG
GACGCTTGCGAAGATGA
TAAAGACCTACAAGCACATCAACGAG
TGGAGATGTCGTTCCGT
CCAGGAAAGAGCGACCGA
GTGCTTGTCTTCTTTGTG
TCGACGTATTTGCGAT
AATGGTCAGCGAATTC
TCTACGATCCCACTAG
AGACGGTCGCATTGAG
CACCTCGTCGTCTTTGTGCTGG
AAAGCATGGAACAACCG
GTTCAAGGTGTTGCCACG
AGTCGAGCGCTCAAGG
AACCAGCCGCCACCG
CACAGGCACCAAGTGGG
GCCATGGAGAGCGGAAG
GAGGCGCCCGAAGAGCTAT
CACCAGCAGGCGCAGCAGCAATTG
CACCCCGTCCACCACAG
GAGGAAGCCGAAGATAAG
TCACGCGTCACCTGAAG

FIG. 2B

AGCCGCCGGAGATTCA
ACAAGAATCACAGCAAG
GACGTGCCCGTCAGTAAG
GTTCCGGACGCTACAG
ACCCACGTGCCATTCAAG
GGCCATGAACGGCAGCAC
CAGCCCCAAGCGATGG
GAAGCAGCAAGGACGC
GCACCGCAGTGCATCAA
AACTCCTTGCTGACGG
TGATGCCAGGCGATCAG
GCCGAATTGATACATAAG
CTGGACCCCTGCCTTGG
CCAAGGAGTCTTACAAC
AGCGATGTGCCCCACAG
TCCCGGCGATGCCTATGCCATTTACA
GCTGATGGGTCTGTTTAG
GGCGGTGGACTACAAGG
TCCGTGGCGTGTTGCAT
CGGCGCCCATCGTAGC
GCGGCTTTACGACCAAG
AAGCAGCCGCAGCAAAAGC
CTTCTGGAACGCAAAGA
GCCCCTGCGAGCCAAG
GCGAGTGCAGCAAAATAG
AGGACTCAATCGAAAG
CAGCCAGACAACTTAAG
GCAGTCCGCCATCTAAG
AGGCGGAGGATTTCTTTGA
CAAAACGCAGAGGCATCCGG
CTGGAAATCGAGACCC
GAGATGATCAGGACGATGG
GCCTGGATGTGGACACG
GGTTTCCCATGTAAGCG
CCGCCCGACATGCAGAC
CGCTTTTCCAGTTCAGC
CCCCGATTCTACGACGAG
CGAGTGCAATGGAAATG
ATTCCAATGCTAGCGGG
ACTACTCCGATGCCAG
TCCAGTCAAAGATTTTAAG
ATCAAGGAGATCCGCGAT
CGGTGGAATTGGACGAG
AGGAGGCGGCCTACAAG
ATGGAAGGCCCGAAAA
CTGGGTGGCTCTAGTCA
GGACGGTCTGCTGCTGG
ATTGACGACGGCACAG
AGGTGGAGCGAACTAAG
CGCCCTCCAGGTTACAG

TGGATGTCCGAGTCTAAG
GAGGATGATGAGTCAG
CTGGATAGTGCCTGG
TAGCGATTTGAGCCAGA
AATGCAGAGCCACAAG
CAAAGACCCTAGTAAACA
TGATGGTGCATCTCGTTACAG
AAGAGCAGAAGAGCGAG
GGAGCACATGTTTCAG
CCTTTGTTGCTGGTCTG
GTTGAGGACATGGCTACG
TTCCTGGGCACCTGGGAG
GCGGCGCCAATGTAACAG
CCTGGCTGCGCGTAAAAA
GTCGCGGATGTCGGAAG
CCATGGAGAGCGGAAG
GAGCACATGGTGCGTATG
ATTGGTCGCAGCGATGCGGATAATGGG
CGTGCCCGTCAGTAAG
GCGCATTGTGGAAATG
AAGCACCAGTGTGCAAG
CCAAGGGCCGTAAAGATA
TGCCAGTGCCCATGAGTTCAACA
ACCAGGATTTGGAGGAG
CGCTGAACAAGGTTAAT
ACGACGGCCACAACAG
AGAAGGATTAGGATTTCT
TTAAGGAGCTGCTCGAAAAG
CTGCAACGACATATCAG
AATCGATTGTGCGAAATA
CTCCACCTACAAGGAGG
CGTGTATCCCATTCATG
TGCTGTGTGATAAAAA
GCACGGGTGACACTCTG
ACCATGGTCAAGATCGATGGCAAG
GCGAGGCGGTTGCTTG
TTGCTGCCCCGAACTG
CGACGGGCAACAGCAAT
CCTGGATGATAATATG
GCTCGCCGACCATGCCA
TGTTGTGCTGCGCTGGAGCTGATAG
GCGATGCTTTCAACAAG
GCTAGCCGTGCAAGAG
GGACATGTGCTTGAAAG
AGGGGTTTCTTACAG
CAACGAGCACAAGTTGA
AAGGATAAATTGGAAAG
CTGGCTGCGCGTAAAAA
AGGTCTACGGCAAAAAA
GCACCCACTCACTGGAC

CGCCGCTAGAACGCTTTTAG (*C. elegans*)
 AGAATGAGGATGTTGAG
 ATGGCCTTATTGATATT
 TGGATAGATTCGGAAGG
 TGCGAAAGTGGATCAGAGT
 TTTTCTTGGAGAACAAGACTTGCGGG
 GATGGAAGACTACTACTTGGAGTTCG
 GCCAACTCGACAGTACTTAGAA
 ATGAAGGAATCGCCGAT
 TCCTACTCTCCAGACATGTCCG
 GAATTGTCAATGAGAACTTG
 TTTCCAGTGTAAGAAG
 AATGGGGAACCTGGGTTGATTTTGAG
 ACAACAAACTCCGACAACATG
 AGTGGAATAATTCGAGAG
 CGTATATTGCAGATCCAAATAGG
 TTAGATACCGAGCTTGAT
 AAATTTCGATTTGACTGC
 AAAAGTACATGACAAAG
 ATCAAATCGGTTCTGAAG
 GATGATGTGAAGAAGAGTGAGAG
 TTTTACAGTGCCGAGGT
 AAGATTCTGCTGAATTGAA
 CATGCCCAAAAGTGAATC
 AGTCGGCATGGTCAAAGAAA
 TGCTGACGAAGAGGAAGTGCT
 GATGAGATGCAATTCTGCAAG
 ATTTGAAATGAAAAGTGACATG
 AGTCGGTTAGGAGTTTAAATAG
 CATGATTGTTGCCACGT
 ATTCGATAAAAAGGAGCG
 GTCTTTTCTTCTGTGGAAT
 ACAAAATGGTCGACGGATGTGCTGAG
 GATATGATCAAGCTTCGAGAT
 GACTGGAATTCCAGCAGTTTCAGAG
 TTTTGGGACAACTGAAG
 AGAATAGCTTTGAATTCGAG
 TCATCAAGAAGCCTATGG
 AGGACCAATGGGAGCTAT
 AGATGCTGCCAAGACTC
 ATTACCGAAGCTGAAG
 TTGAATAAGAAGGACGTTGTG
 ATATGATAGAAAAGTTTAGG
 CCTGAATGTACAAGCAG
 GGAAGGACAGCCAGAG
 TACGGATGTAGATCAA
 CACTGTGGAATCAACAAG
 GTACATGAAAACACGGA
 TAGATGAAGAACTTCAGAGAC
 GTGCTCGAAGATCCAG
 ATTTGCCAGAGGATGTG
 CTGATTGGCGTTTACAG
 CTGTGCGTCGTTTACAG
 CAGCAGTTGAATCAGAT
 CATGGACGAGGCCAAGTATGGAGCCG
 TTTGGGCAGATTGTTGC
 GATCGGATCATTCCAG
 AATCGGACCAAATTTGAT
 TCAAAGAGTAAGAGTCCG
 GAAACCGATTACTGCAAG
 GAGTCCGGAGATATTGT
 TATGGAATCTACTCTCAAG
 TCGACATGAGATCTTGG
 ACGTGTTGCTAGAGATG
 AAGTAACTTGATTTTGAC
 GACTTTTTCGGAAATGG
 ACAACGATTCCGAAAAAG
 GTTGAGTATAAAGTTGGTTGTAAG
 CTATAATGGATTTGCTCCGG
 AACAGCTTCTTGCTCTGCGGG
 GGTTGAACTTGATAGAAAG
 TTGTGATTCGATAAAAGGAGCG
 ATGGAATGTTGAGCAAG
 ACTCAAGGATCGTTGGGGAGTG
 TGATGATAAAGCTAGA
 AAATTAAATTTGACATG
 GAAAAATTAATTATGGAT
 ATAAGGTCGTCACGATAATGAG
 GTTCGAGAAGGAGGCTCAGGAG
 CCGGATGATCTGAAAAAG
 GGAATGGAAGAAAGG
 GAAAATGATAGTAGATCGGCCGAG
 CTGTTGGAAAGATTGATCAAGATCAG
 TGAAAGTCGTGCTCCGCGAGTTTAA
 GTTAAGCGTTGAAGCGC
 CAACTCGGATGATTTCA
 TTTGATCCAAGATTCGAG
 AATTGGAAAGATACTCACAGCTGAAG
 AACTCTTCACAATTGAT
 CTCCGTCTTCGTGGAG
 AGTGAGACGAAAAGCTCAAACGTAGA
 ACTCTTGGTGCAGATATTCCG
 AGCTTCTGGCGTAAAT
 CGAAGAACACATCAGA
 CCAGTTTGCTGGATCAAC
 CTAGAGACAAAAAGAAG
 GACAATCATTGATCCAAAG
 CTTGGCGGAAGTATTAAG
 CAAGGAGCCATCTCGTG
 CCGCCAACTCTTGCCAAG

FIG. 2C

GAGTCTGCCGTCGACAG
CAGAGGCAGAATTCCAG
ATGCGTTAGGTGTACGG
TTAGAGAACCGATTGAAAATGGAGAG
CTCAAAGGACAACGTTGAG
AGCCACATTTCGAGAG
CAAACCTGGATTTCGATTC
AGGACGAAAAGCCGATTTTCAGTGAT
AGGACGAGAATGAGGATGTTGAG
CAGCCGGGATCGAAAAG
AATCACAGATGCAAAGC
AAGATGAGAGCATAGAG
GCTTCTTGCTCTGCGGG
TCTTGATCCAAACATGGAAG
ACAGAAAGTGCTCTCCAGG
ATGAAGTAACACCGCCTGGA
ATGGGAACAACTAGTGCTTAAG
AGACCATTGAGGACGAGAAG
ACTCCGGCTATAGTGAG
CAAAGAGAAAAGAGAAAGTG
TCACTTTATGGATCAAG
GTGAAAAATTGGCGGGA
AGGATCAAGCTAATGAGCAGCAG
TGGACAATTGGAGACACAAAAATT
CGTTAGAAATGCAAAAAG
CGAGCGGGTGTGTCTTACG
GAAGACGCCGCTGAAGAT
GAGATGATATTTCGGCAAG
ATGCTAGTTGTGAAAAA
AAACTGATCGAGTCAAAG
GAAACAGGATCAAGATG
CGTTGAAAAGGAACCAAATG
CTTCTAGTTCTCAAAAT
AGGACCAAATTGTCAATTTG
ACGTGACCGCAAGAAGGTCAAGAACG
AACGAGGGGAGAATCAG
GACAAGGATCGATCTTTT
AGGCGATATGGGCCAAAG
TGATGACCATGACTAAT
GTTGATCGGCCGCCCGGATCTA
GATTCAACCATATCGG
GAATACTCGCGCGAAACG
TGTACCAATTGTTGAGCCAGAG
TATCAGCAGTATAATCAG
CTGTTATCCAAATGCTG
AATACGATCGCTTTTAA
GAACAAGAGATGTTGATAAA
TATGAAGAGAAATCGAACTTTG
AATCGATAGCATTATTCATTGGTG
ATCATGGATGGACACAAAA

AAGTTCCAGATGGAAGG
GATAAGGCGTGTATAAAG
CCCTTGATCTCTTGAAG
ATACCGGAGATAATAAG
GTGGACATCAGTCAAG
GTTTGGAAGATTTTGAGATTCCCAAG
GGTTAATATTCGTGTTGA
TGATTTGCTGAAGGAG
GTTCTATTGCCGATATCCTTC
CCGATCAAAATAATGTGTTTTATCAG
CTTTGCAGGAGCTAATG
CGACGAAGAGAAACAGGAGATG
TTTTCATCGTTATGGAG
AAGGAAACATGTGTCATG
CGGCGCTGTTGAAAATG
TGATCGCTCACACTCAG
ACATATTTAGAGGAAG
AGTCGGAGCGAGTAAAG
ATTTGATCAATAAAGAGA
ACAATGAGATGACTGTG
GAGGAATACAGTGGAAGAGTTCATG
ATGGACAAGTCAATTATGAGG
GTTCAATTTGGAGCGCTTTCATGTAG
TGGACTTCAGGAGGCCAAGAAG
CTCTTGCAATGAGAACAGGAGCAAGA
ATAAGAATCCGGATAAG
TGGCGAAGAATCAACGATCTG
CAACTGGATTAGTACTTCAG
CTGGACAAAACAAGTCGTGT
TTAACGCAGGCGGCACAAG
GTTTCAGTTGGAAGCAG
TGGTGACAGATTAAAGATA
GCGAGAACAGAGAAGGAGAGG
TTTGATCGATATCGCCCG
AACCGTCGATTTTGAGAA
GATACCTGAAGGAAAAG
CACCGGAGTCAAGCTCCACGGA
CGGAATAATTGACAAG
TGTTGGCATGTATATG
ATAATAATACTGATGCGTCTGAAG
CTACATCTATGATCCAAC
ATGCCATGATGTTGGGCG
TCAAGATGATGGGAGGCTTCTGTCAG
AATGAGAGGAGTTTTAC
TCAGCTTACTGTACTTG
AATGTCCTGTGGAACCT
AATCATTCCCGCGATGCAACATGCTG
CAGCATCAAAGAAGAATATTGTGAAG
AACGAACAACGAGCACAG
AAACTACCGGTAGTTGTTG

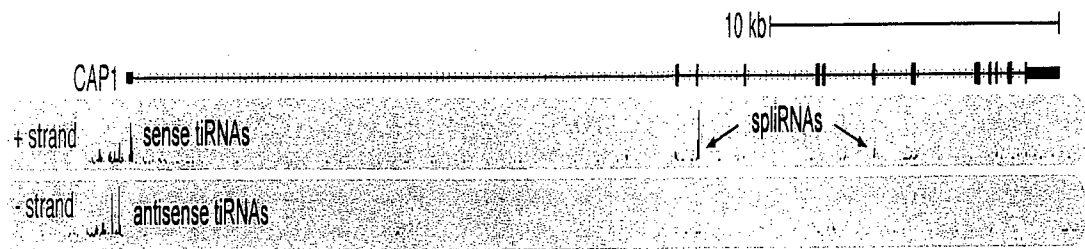


FIG. 3

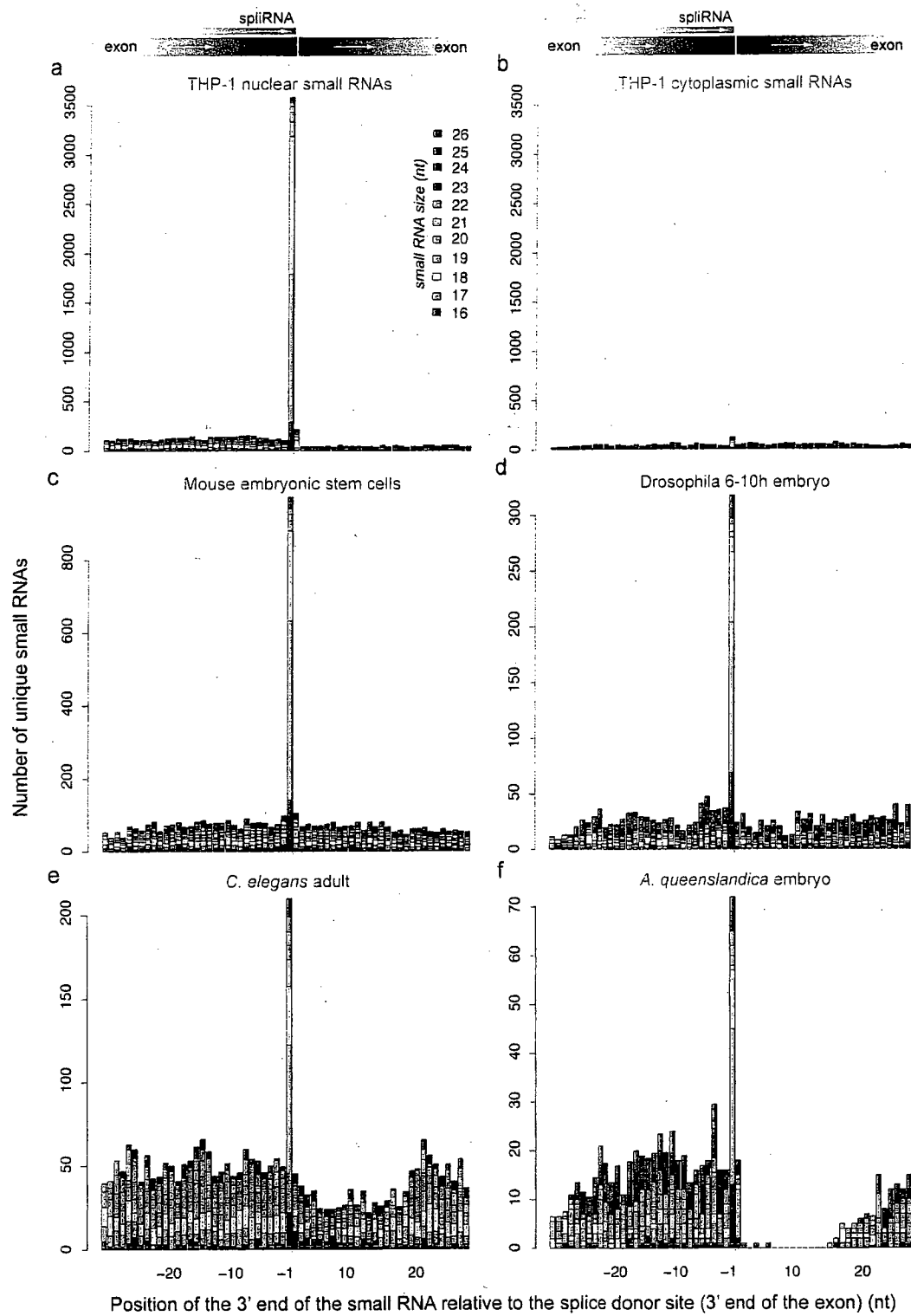


FIG. 4

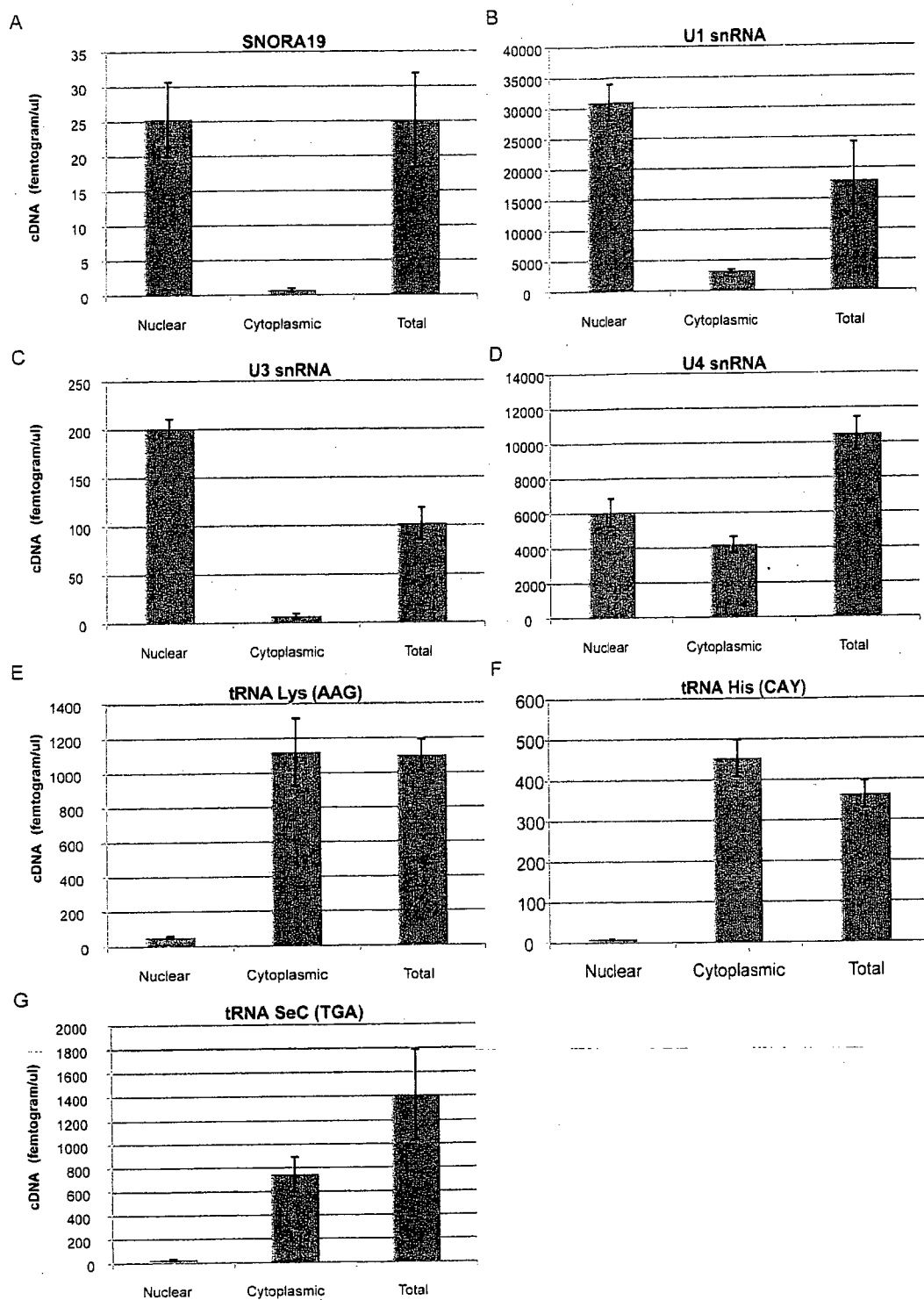


FIG. 5

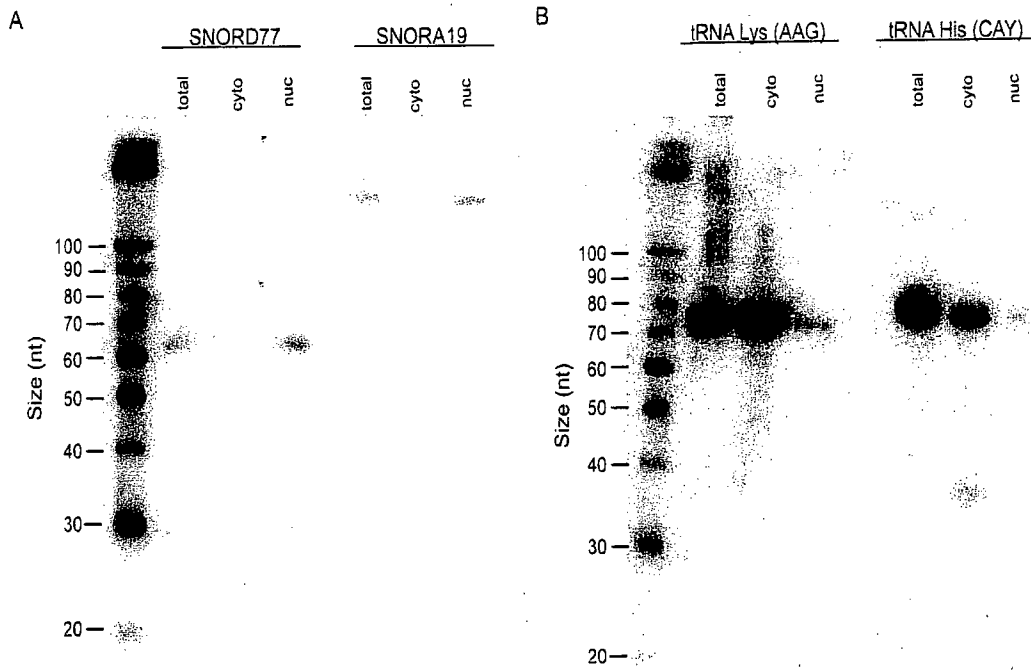


FIG. 6

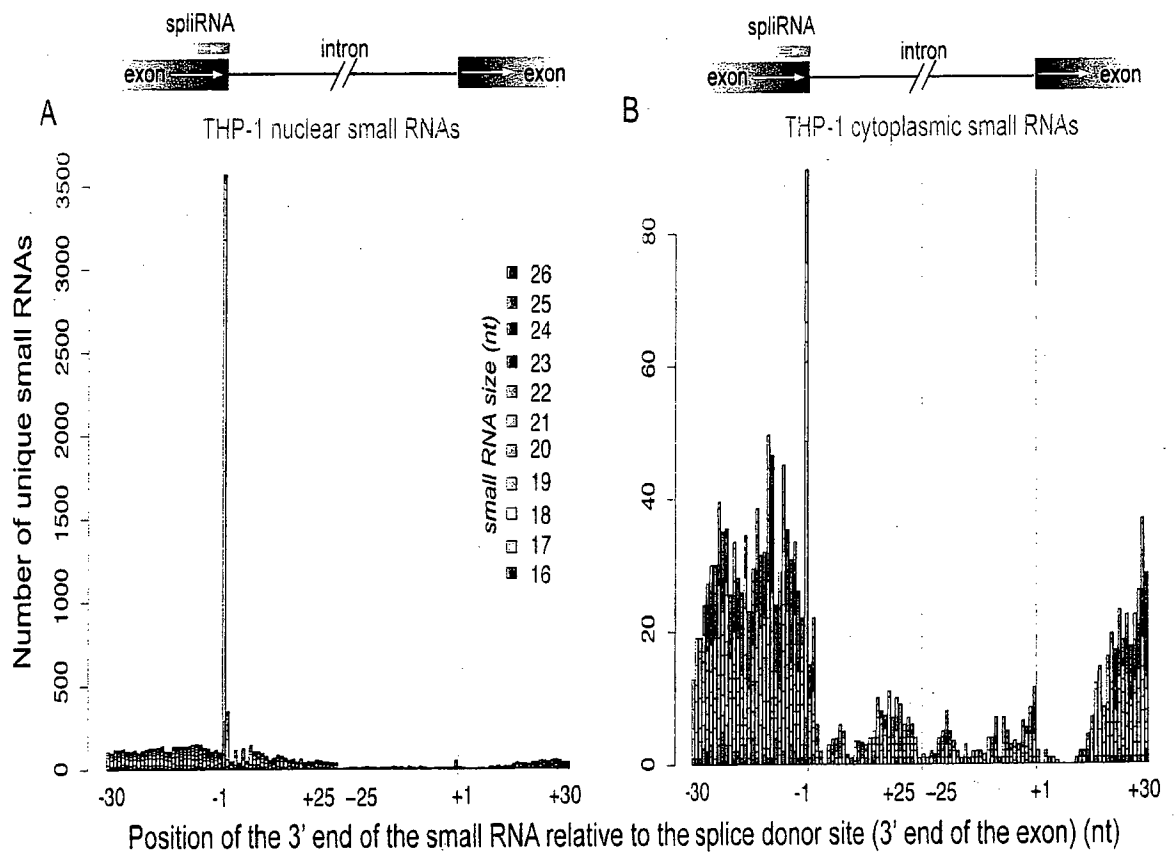


FIG. 7

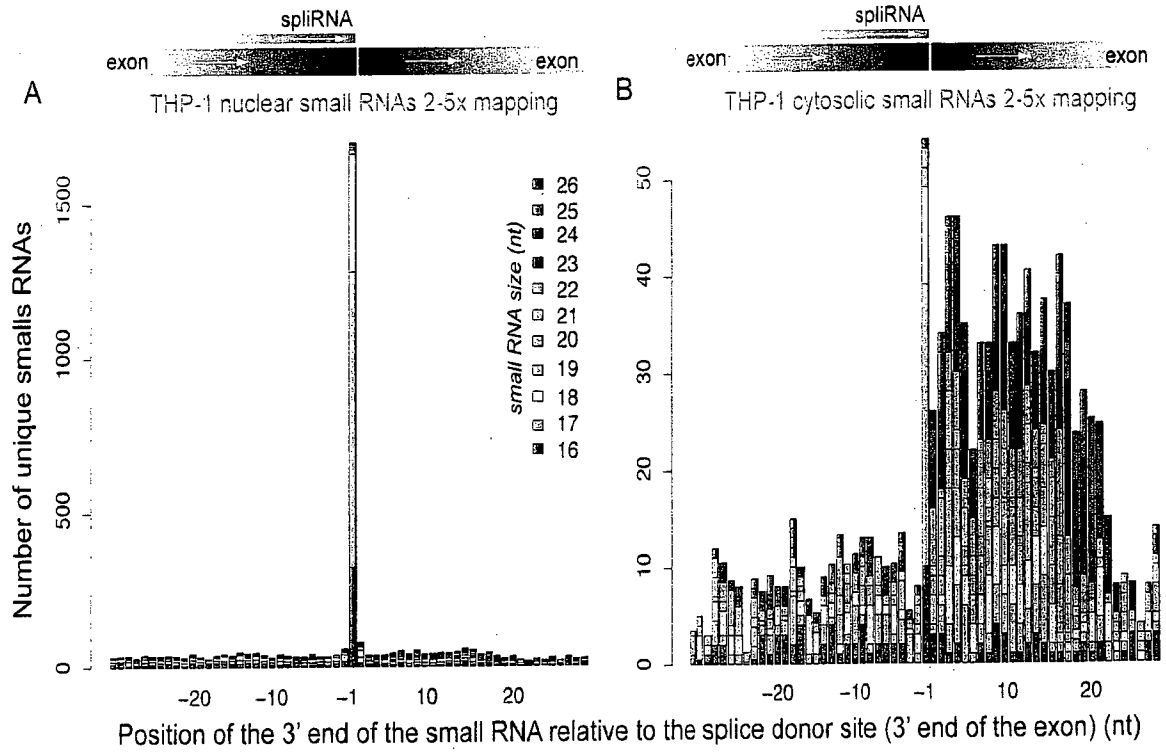


FIG. 8

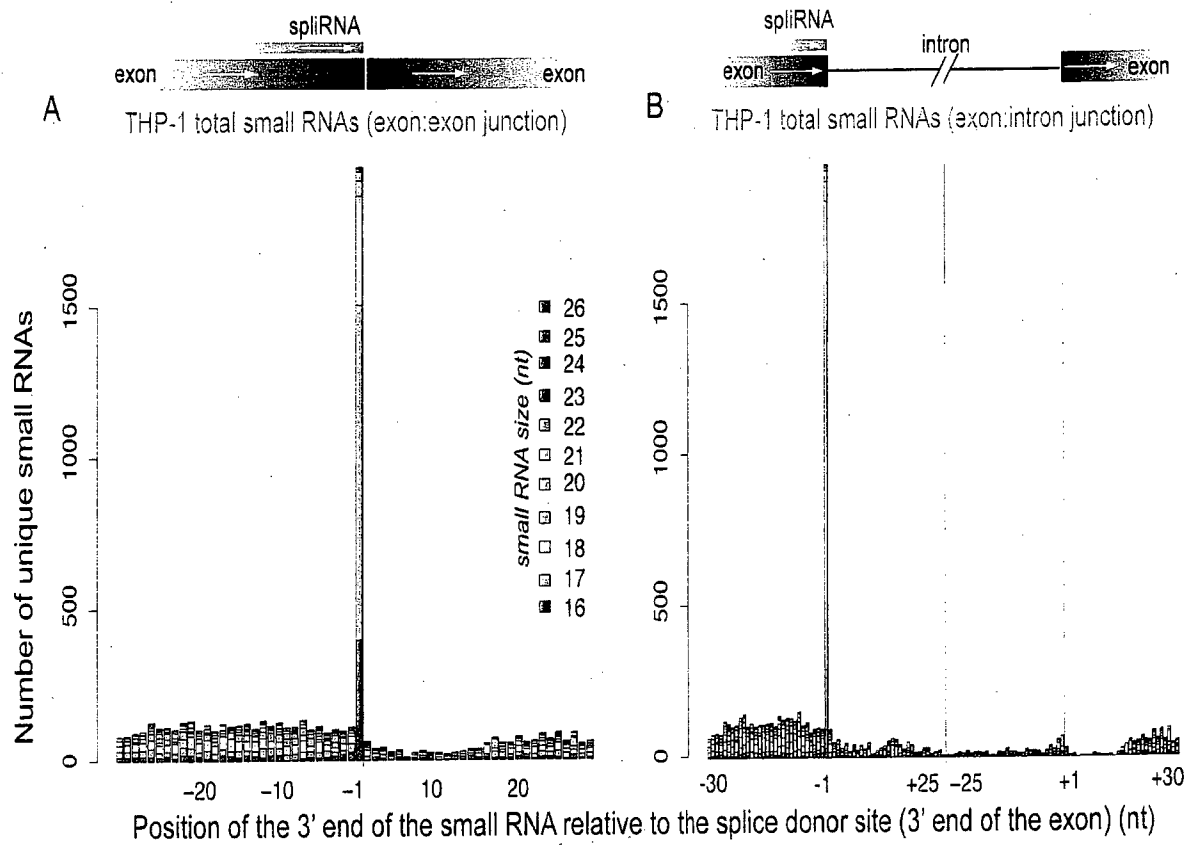


FIG. 9

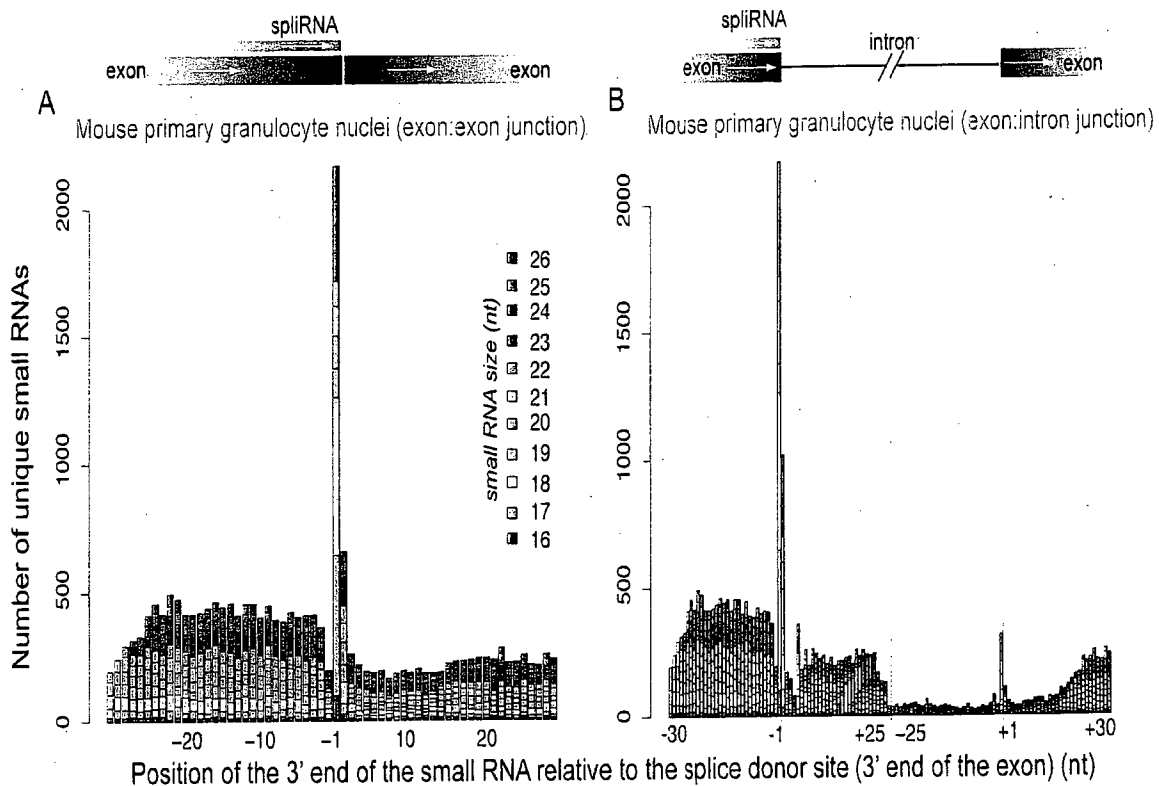


FIG. 10

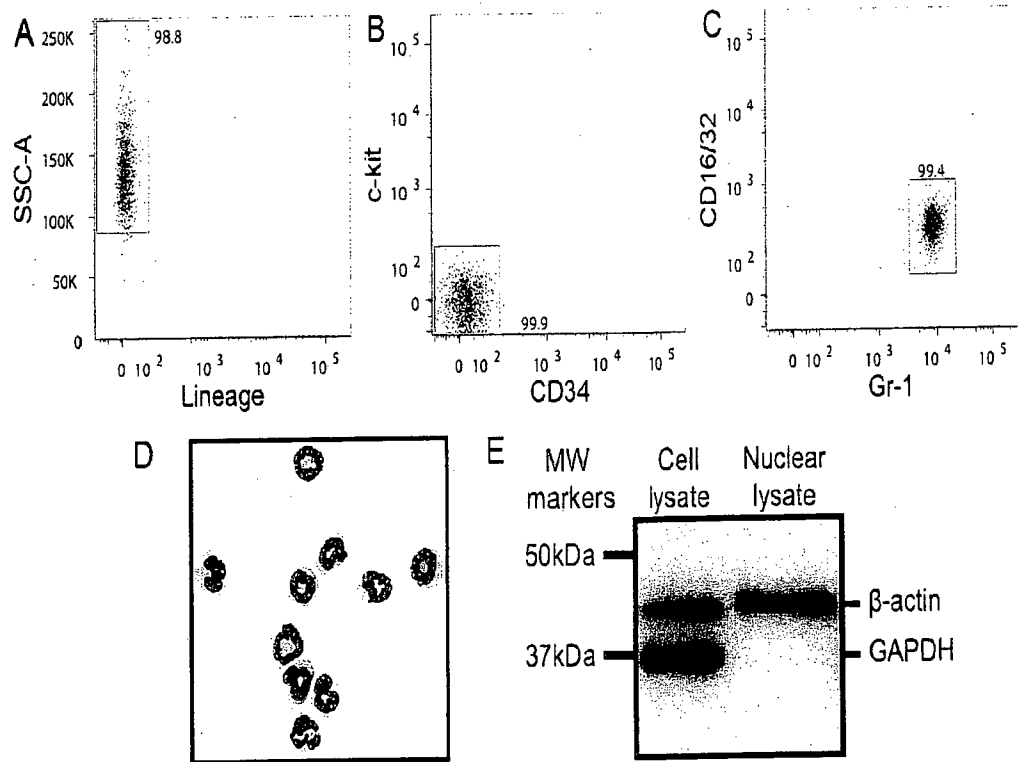


FIG. 11

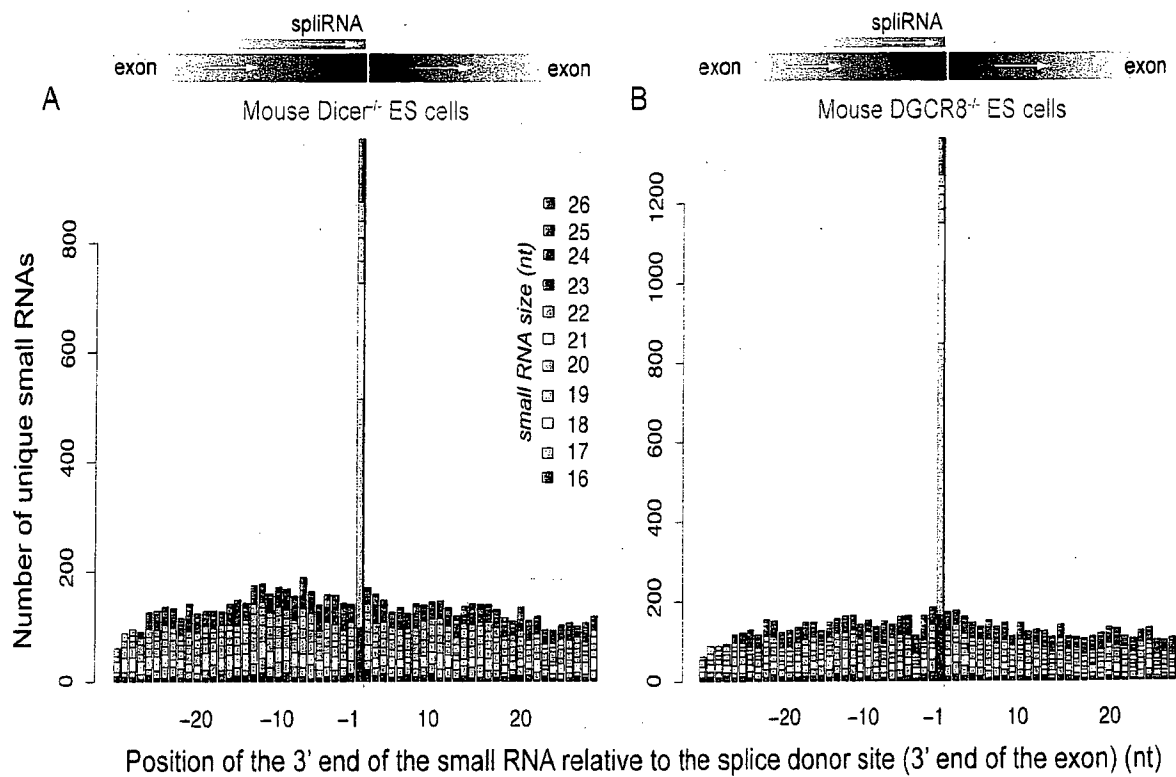


FIG. 12

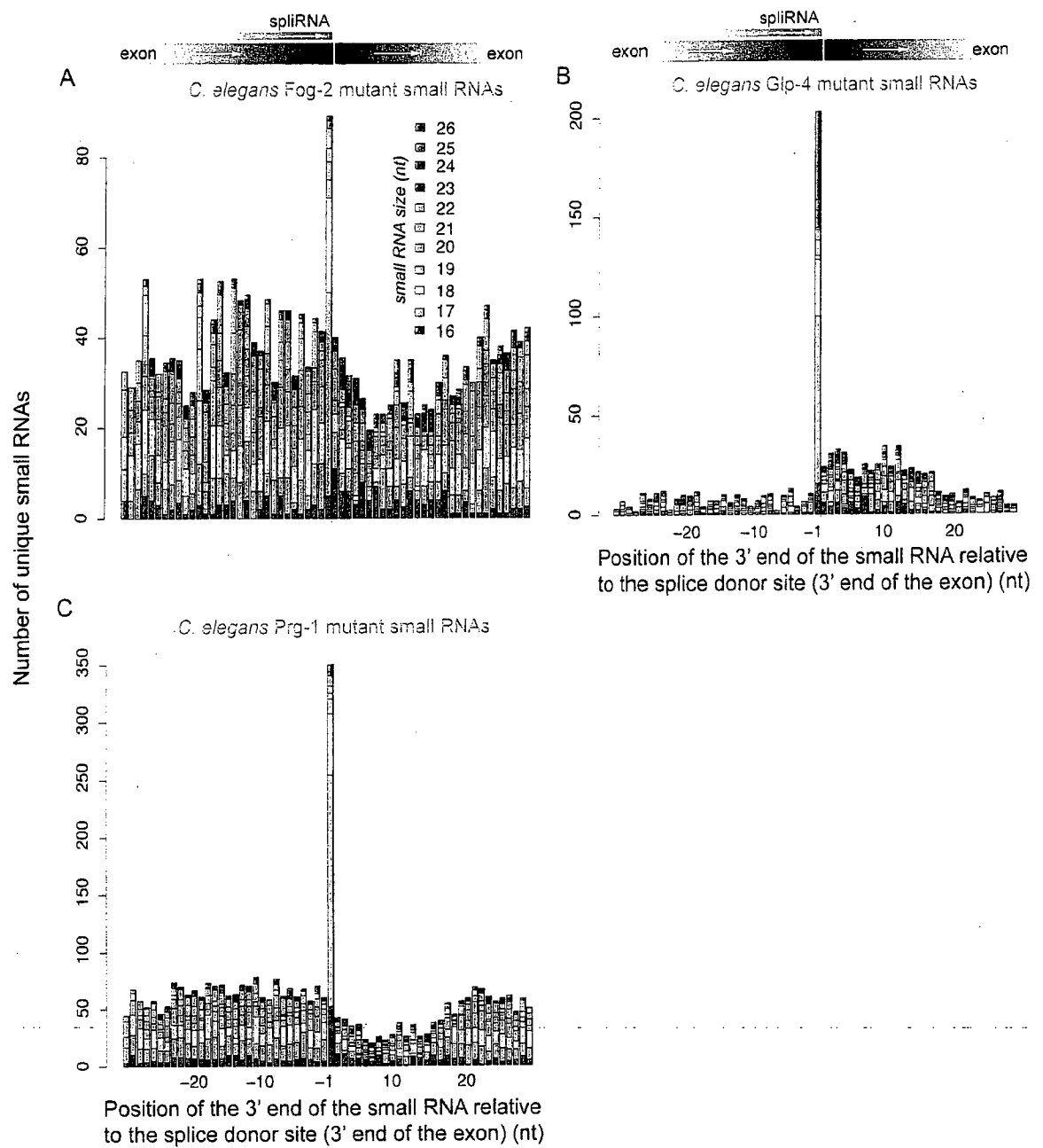


FIG. 13

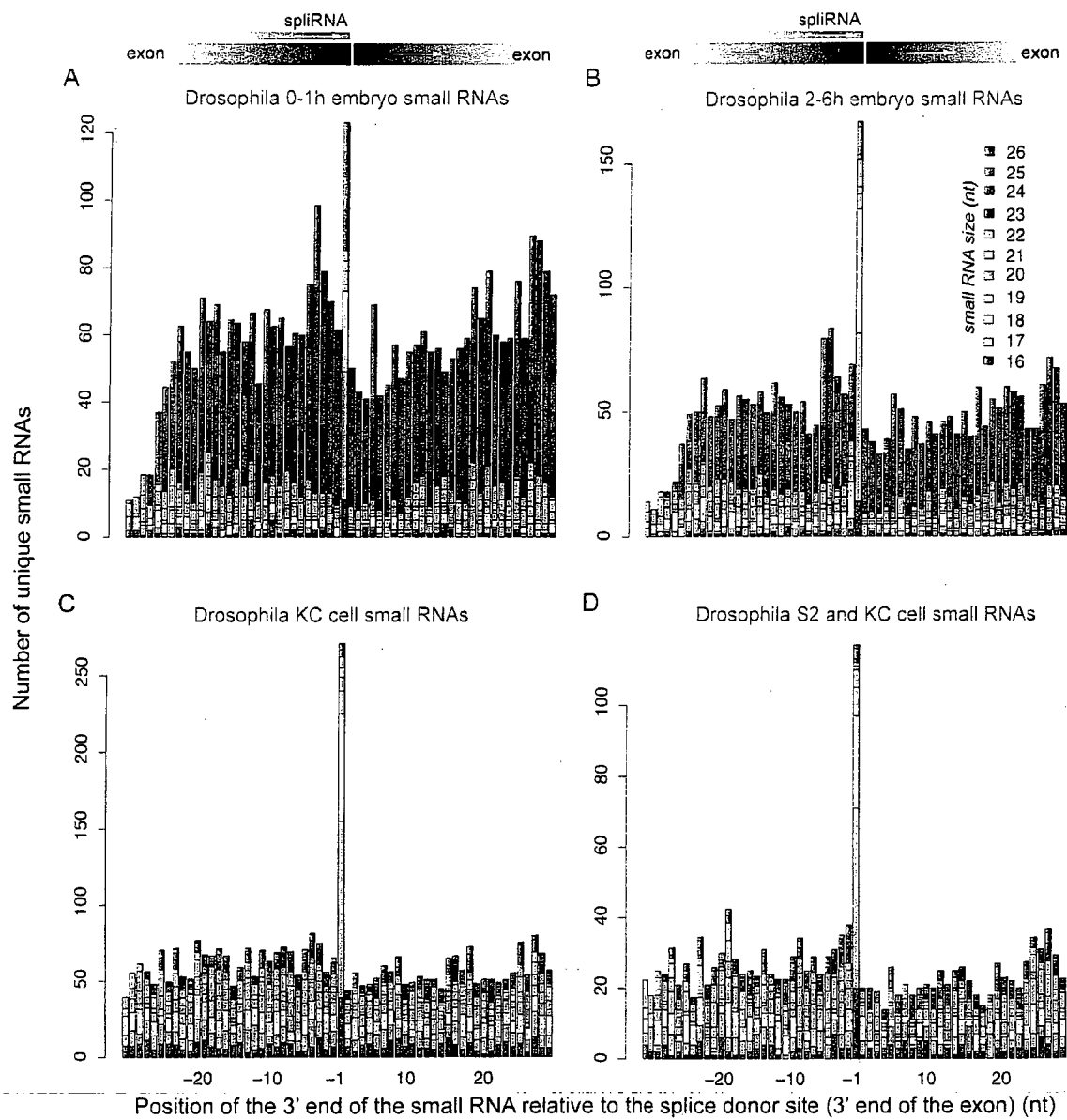


FIG. 14

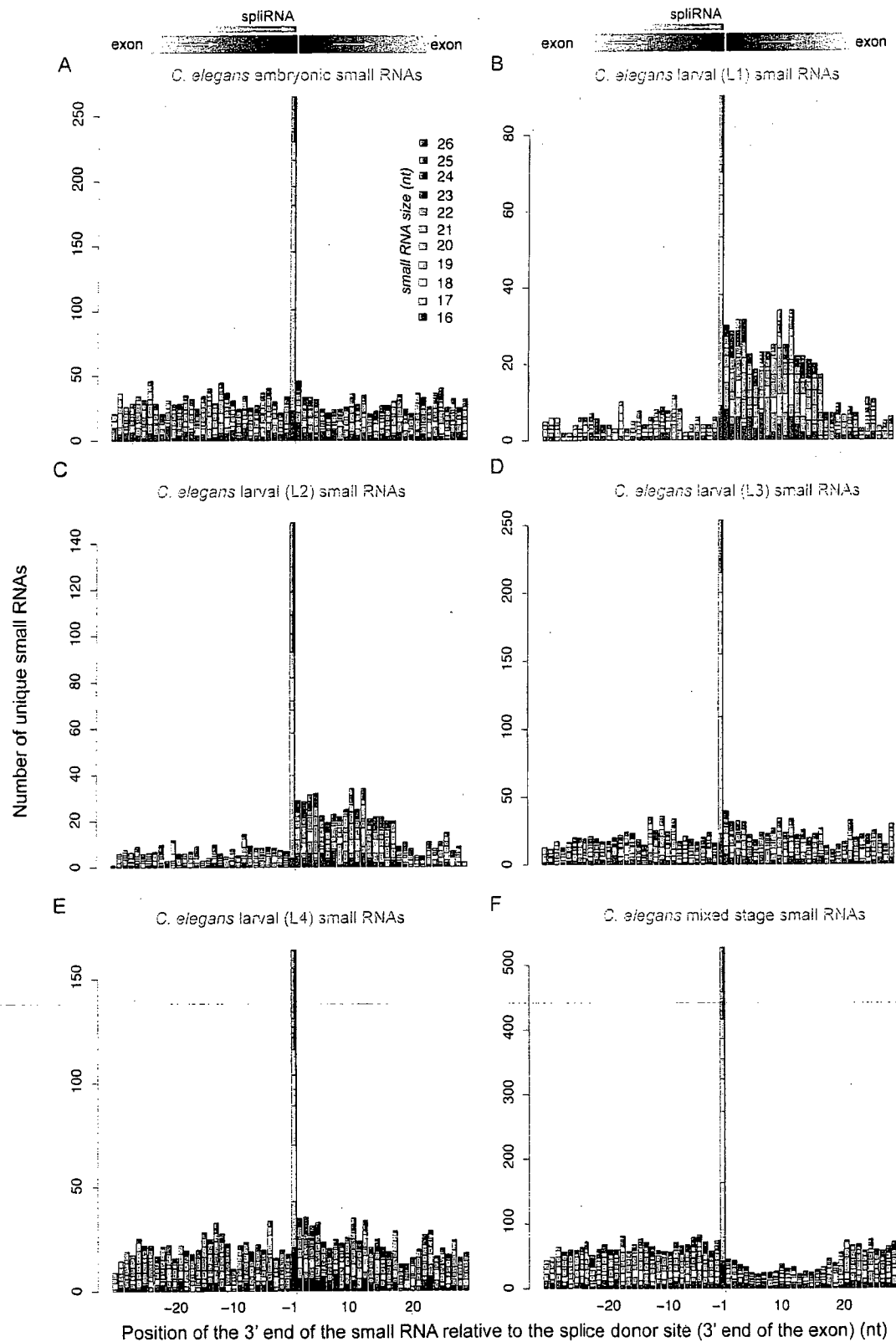


FIG. 15

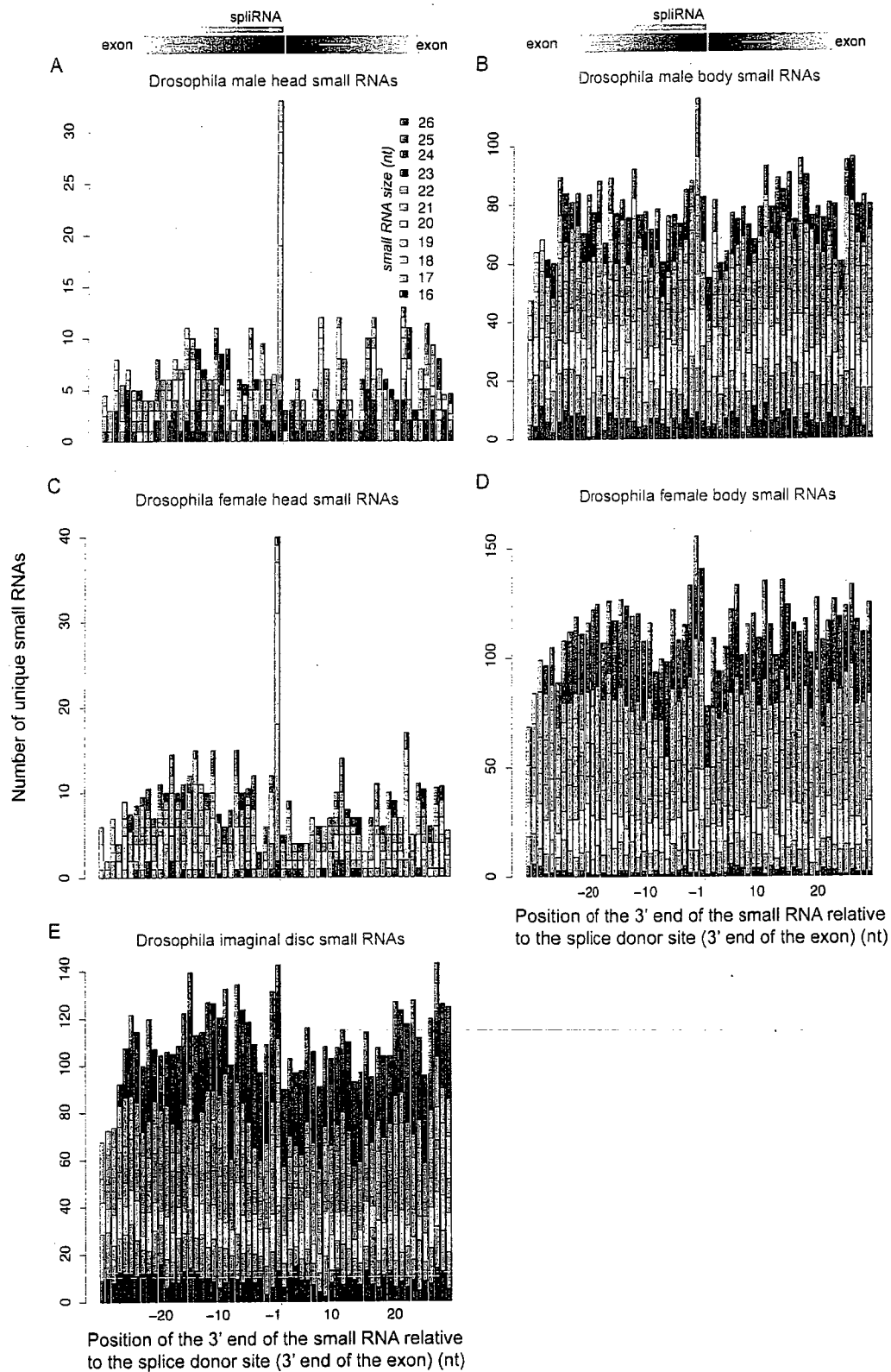


FIG. 16

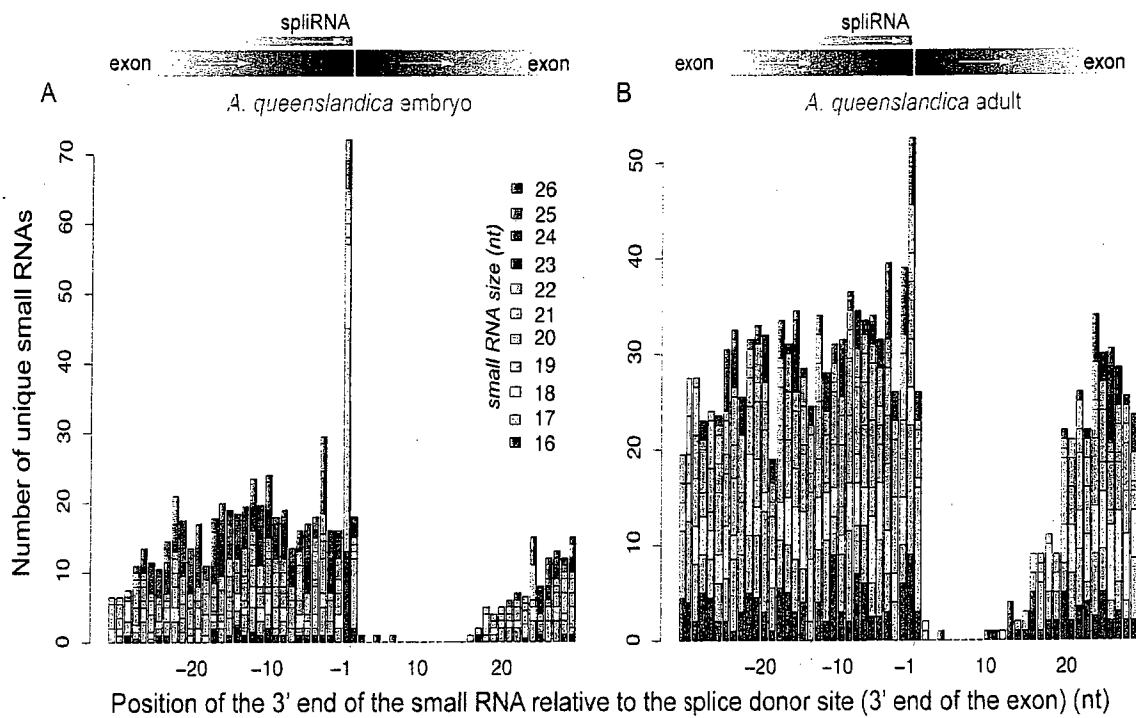


FIG. 17

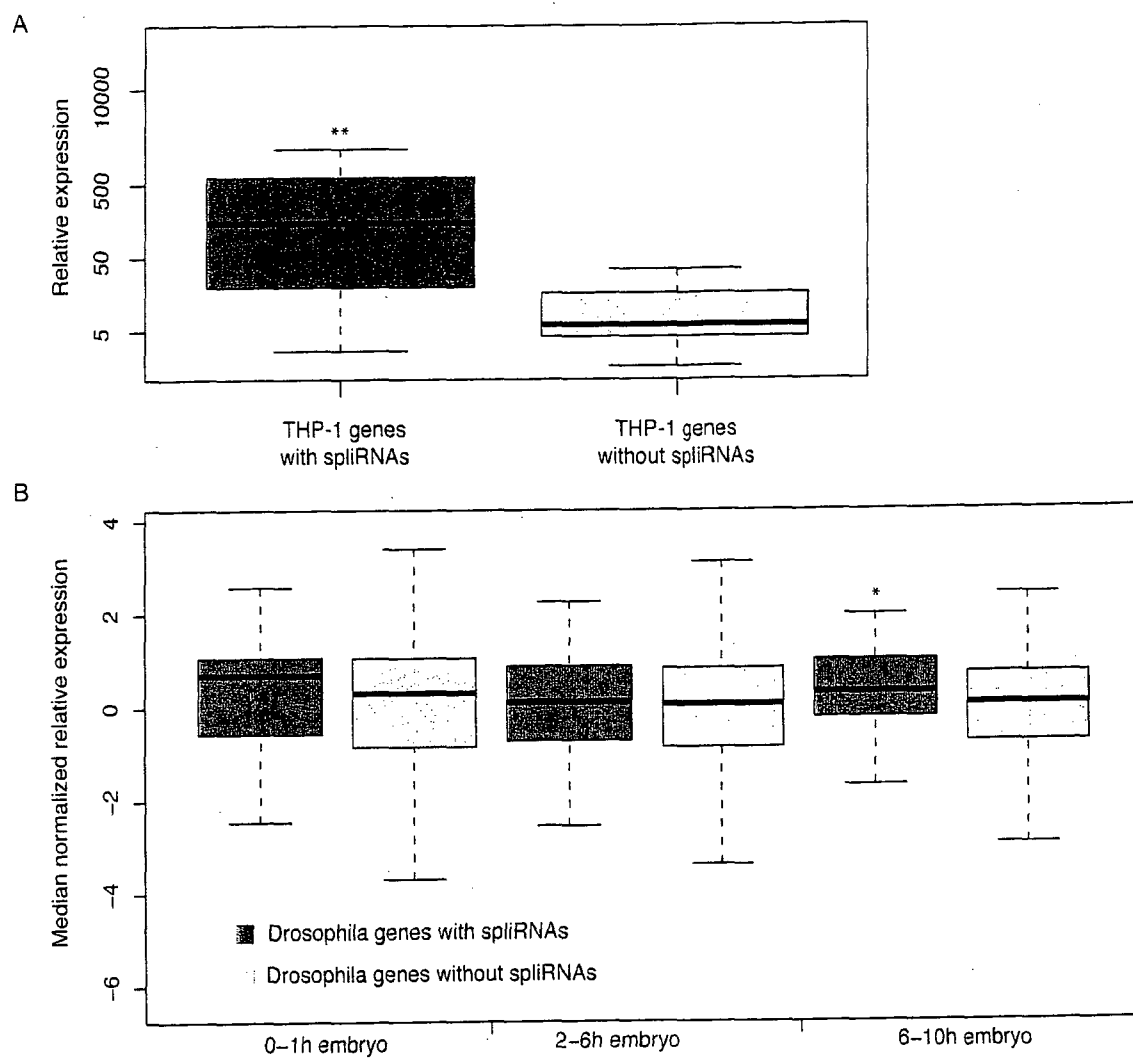


FIG. 18

157

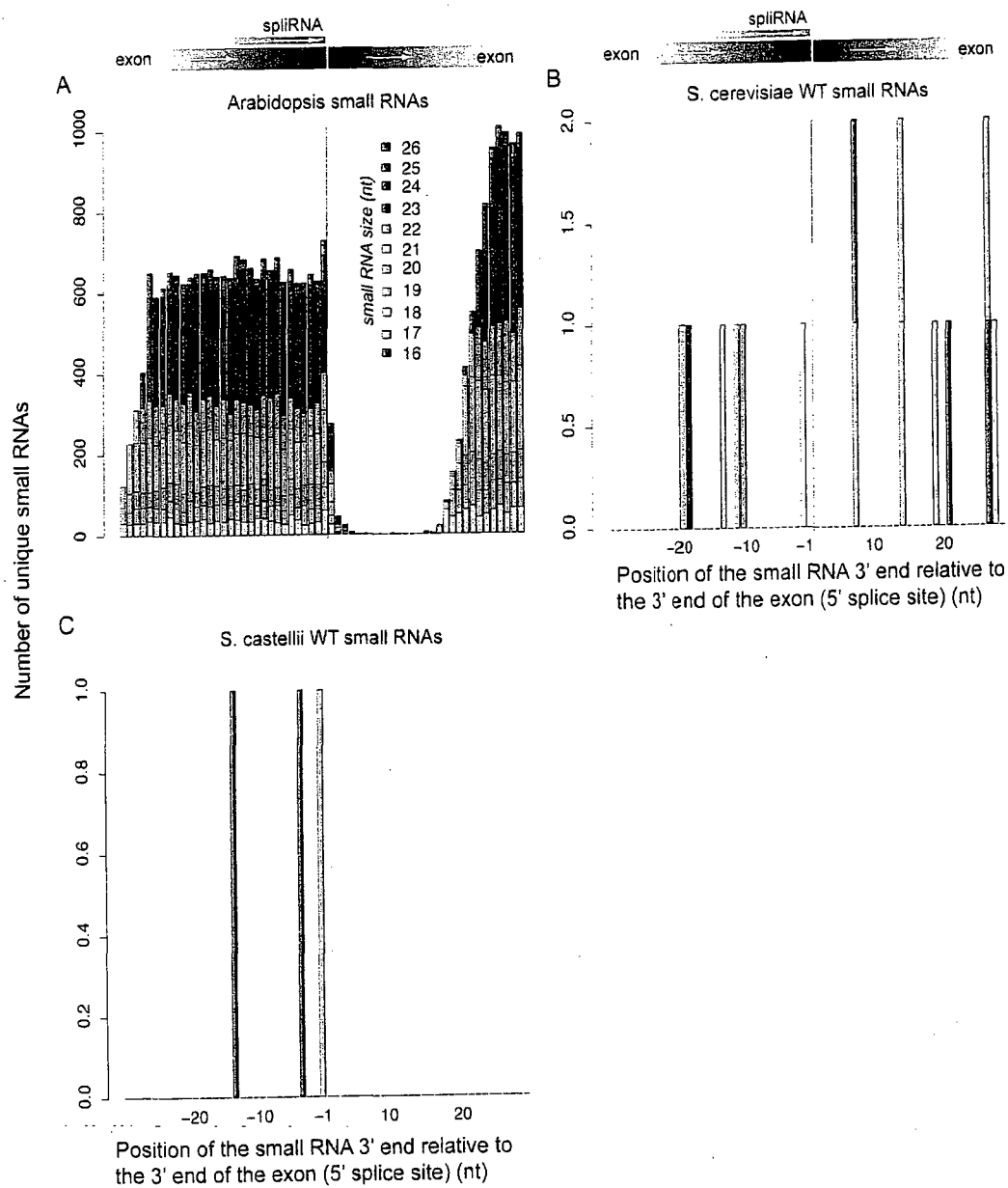


FIG. 19

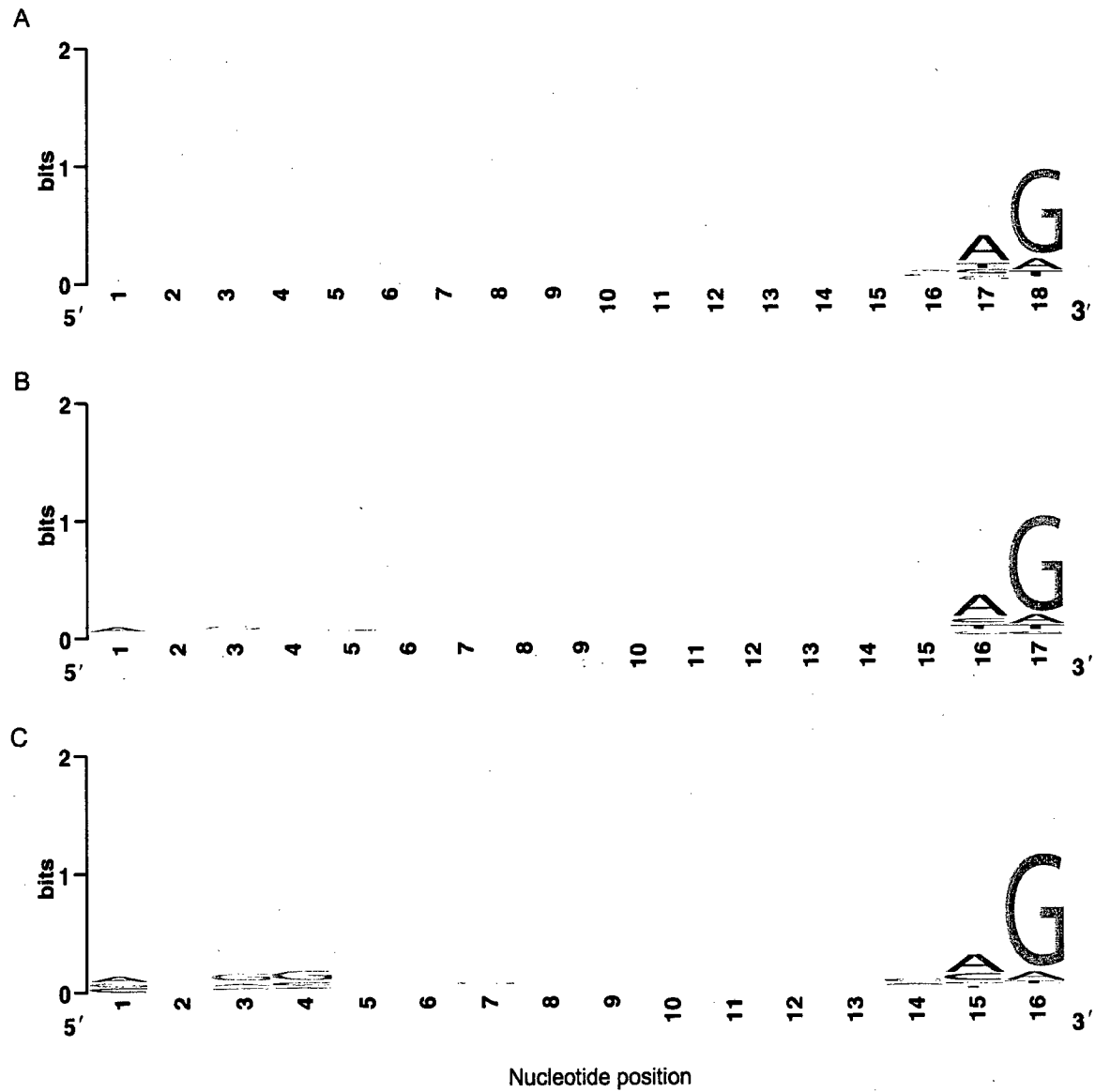


FIG. 20

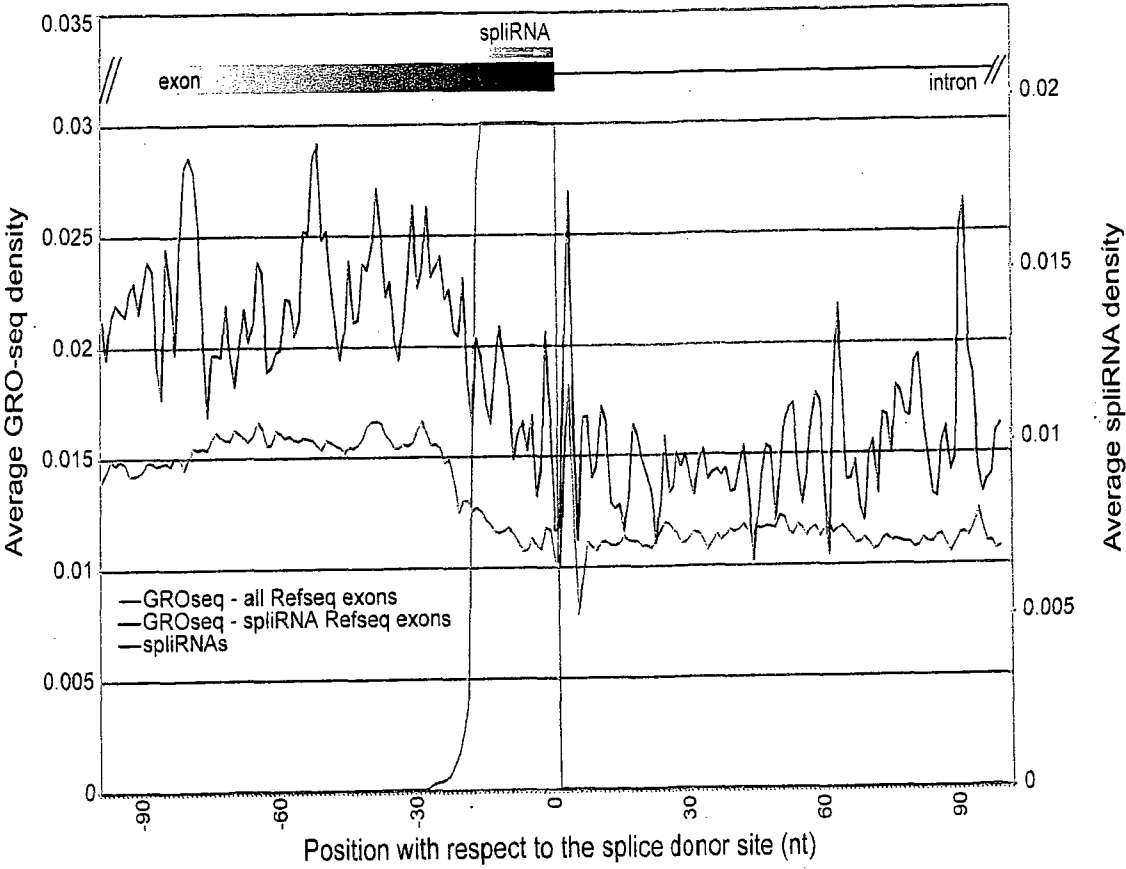


FIG. 21

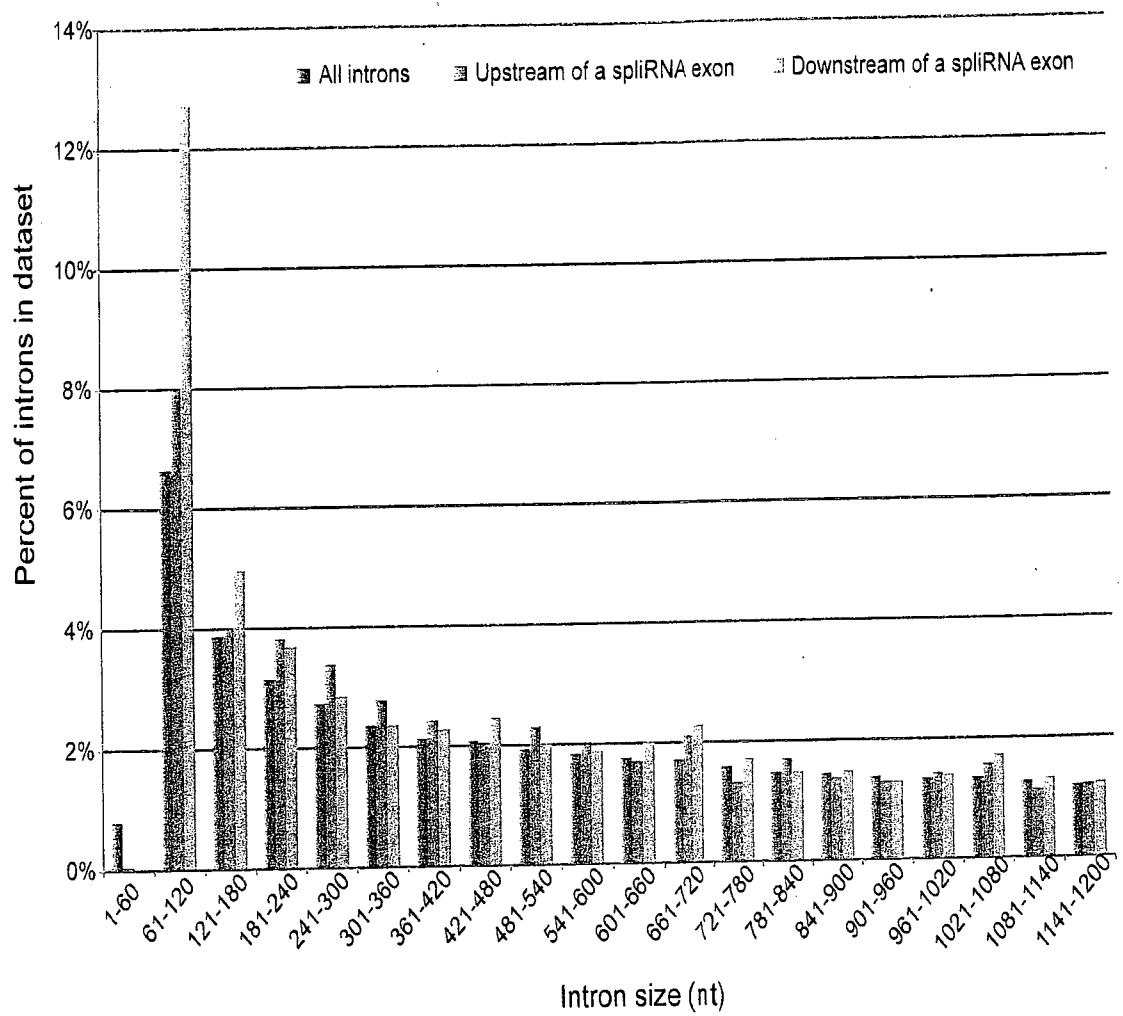


FIG. 22

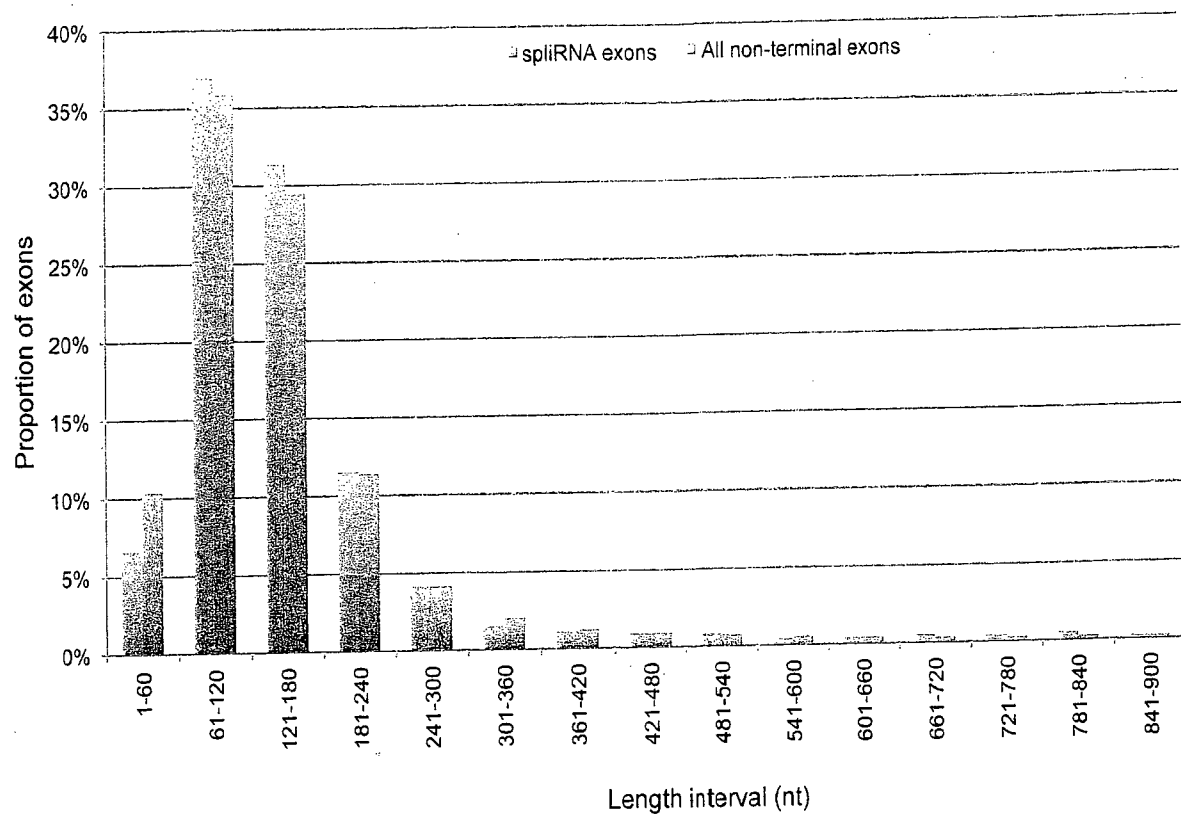


FIG. 23

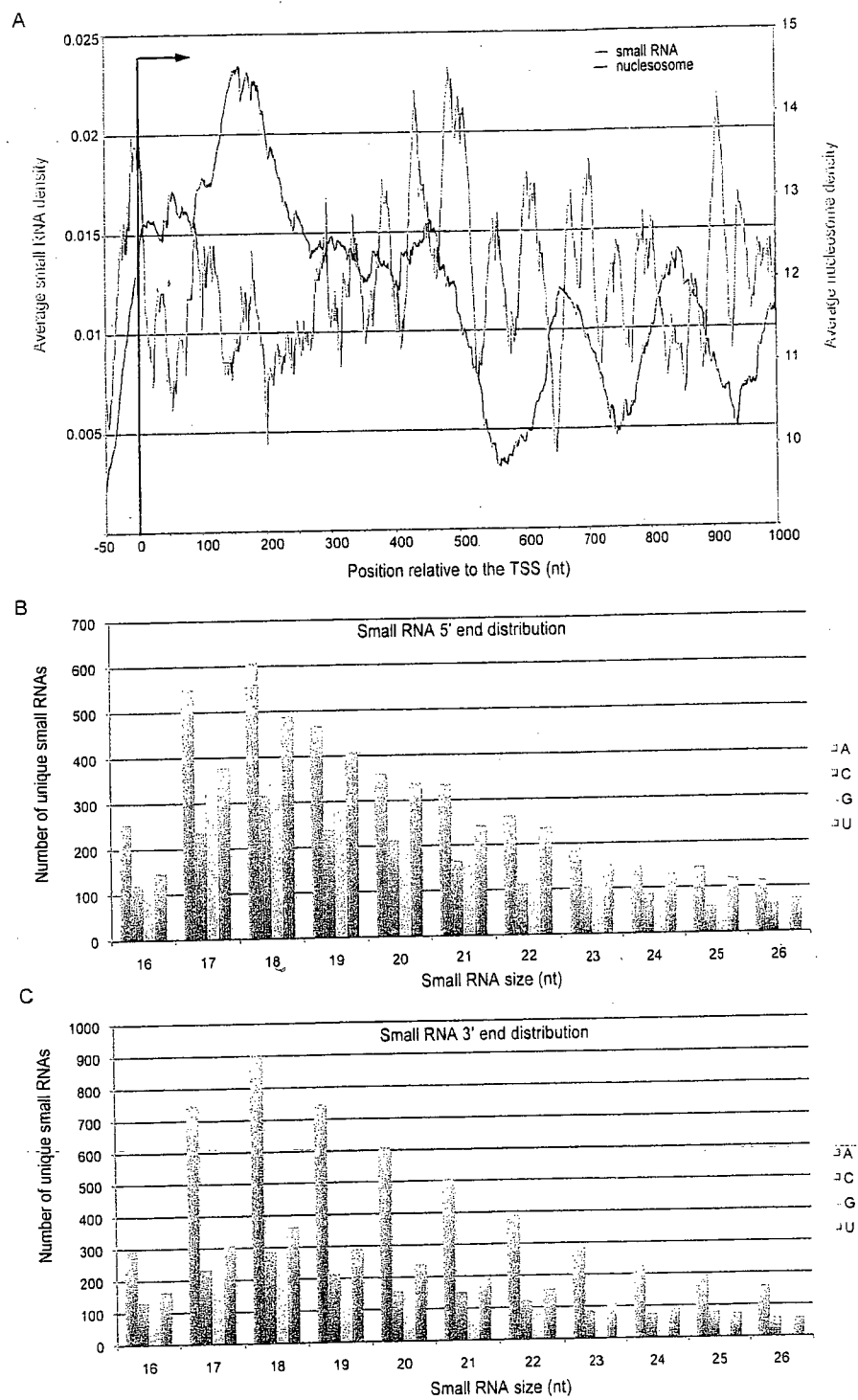


FIG. 24

KEGG

KEGG significant terms (pvalue<0.05) : significant_kegg_0.05.txt (download as EXCEL)

Term	Term size	Term size (in genome)	Term annotation % per list	Annotated ids	Odds ratio (log _e)	pvalue	Adjusted pvalue
Pentose phosphate pathway (hsa00030)	29	26	As1 1: 0.44% As1 2: 0.09%	As1 1: NM_000034.NM_00... As1 2: ENSG00000007350.ENS...	1.6039	0.0004069	0.01329
Fructose and mannose metabolism (hsa00051)	38	34	As1 1: 0.59% As1 2: 0.12%	As1 1: NM_000034.NM_00... As1 2: ENSG00000003987.ENS...	1.5926	0.00004995	0.003382
Cell cycle (hsa04110)	123	119	As1 1: 1.18% As1 2: 0.44%	As1 1: NM_000051.NM_00... As1 2: ENSG00000004897.ENS...	0.9892	0.00006933	0.003397
Focal adhesion (hsa04510)	220	207	As1 1: 1.67% As1 2: 0.83%	As1 1: NM_000227.NM_00... As1 2: ENSG00000002330.ENS...	0.708	0.0003337	0.01308
Small cell lung cancer (hsa05222)	94	90	As1 1: 0.89% As1 2: 0.34%	As1 1: NM_000227.NM_00... As1 2: ENSG00000005884.ENS...	0.964	0.0006762	0.01742
Insulin signaling pathway (hsa04910)	147	139	As1 1: 1.43% As1 2: 0.53%	As1 1: NM_000294.NM_00... As1 2: ENSG00000002330.ENS...	1.0045	0.0000103	0.002019
mTOR signaling pathway (hsa04150)	54	51	As1 1: 0.59% As1 2: 0.19%	As1 1: NM_000368.NM_00... As1 2: ENSG000000017427.ENS...	1.1501	0.00136	0.02666
GnRH signaling pathway (hsa04912)	103	99	As1 1: 0.93% As1 2: 0.38%	As1 1: NM_000516.NM_00... As1 2: ENSG00000004152.ENS...	0.9181	0.0007858	0.01742
ErbB signaling pathway (hsa04012)	93	88	As1 1: 0.84% As1 2: 0.34%	As1 1: NM_001614431.NM... As1 2: ENSG00000002330.ENS...	0.9063	0.001608	0.02864
Fc epsilon RI signaling pathway (hsa04684)	88	83	As1 1: 0.79% As1 2: 0.32%	As1 1: NM_001014431.NM... As1 2: ENSG000000010671.ENS...	0.8994	0.002302	0.03471

prev next page 1 of 2

FIG. 25

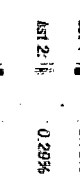
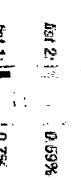
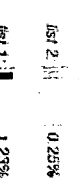
Term	Term size	Term size (in genome)	Term annotation % per list	Annotated ids	Odds ratio (log e)	pvalue	Adjusted pvalue
Pentose phosphate pathway (hsa00030)	30	26	 Lst 1: 0.47% Lst 2: 0.1%	Lst 1: NM_000034.NM_00... Lst 2: ENSG000000350.1.ENS...	1.5718	0.000604	0.02877
Insulin signaling pathway (hsa04910)	144	139	 Lst 1: 1.46% Lst 2: 0.53%	Lst 1: NM_000232.NM_00... Lst 2: ENSG00000002390.1.ENS...	1.0289	0.000024	0.002352
mTOR signaling pathway (hsa04150)	54	51	 Lst 1: 0.64% Lst 2: 0.19%	Lst 1: NM_000388.NM_00... Lst 2: ENSG0000017427.1.ENS...	1.2209	0.001174	0.02817
Adipocytokine signaling pathway (hsa04920)	79	77	 Lst 1: 0.82% Lst 2: 0.29%	Lst 1: NM_000594.NM_00... Lst 2: ENSG0000004680.1.ENS...	1.0487	0.001149	0.02877
Inositol phosphate metabolism (hsa00562)	56	54	 Lst 1: 0.64% Lst 2: 0.2%	Lst 1: NM_000832.NM_00... Lst 2: ENSG00000061405.1.ENS...	1.1754	0.001605	0.03485
Phosphatidylinositol signaling system (hsa04070)	78	76	 Lst 1: 0.76% Lst 2: 0.29%	Lst 1: NM_000832.NM_00... Lst 2: ENSG000000011405.1.ENS...	0.975	0.002895	0.04891
Aminoacyl-tRNA biosynthesis (hsa00970)	43	42	 Lst 1: 0.56% Lst 2: 0.16%	Lst 1: NM_001014457.1.NM... Lst 2: ENSG000000001376.1.ENS...	1.3902	0.0006564	0.02877
Spliceosome (hsa03040)	154	152	 Lst 1: 1.23% Lst 2: 0.59%	Lst 1: NM_001039465.1.NM... Lst 2: ENSG0000000051596.1.ENS...	0.7403	0.002722	0.04849
RNA degradation (hsa03018)	68	66	 Lst 1: 0.7% Lst 2: 0.25%	Lst 1: NM_001134477.1.NM... Lst 2: ENSG0000000039123.1.ENS...	1.0438	0.002618	0.04849
Erbb signaling pathway (hsa04012)	93	88	 Lst 1: 1.23% Lst 2: 0.32%	Lst 1: NM_001146166.1.NM... Lst 2: ENSG0000000002330.1.ENS...	1.3567	0.000001526	0.0002992

FIG. 26

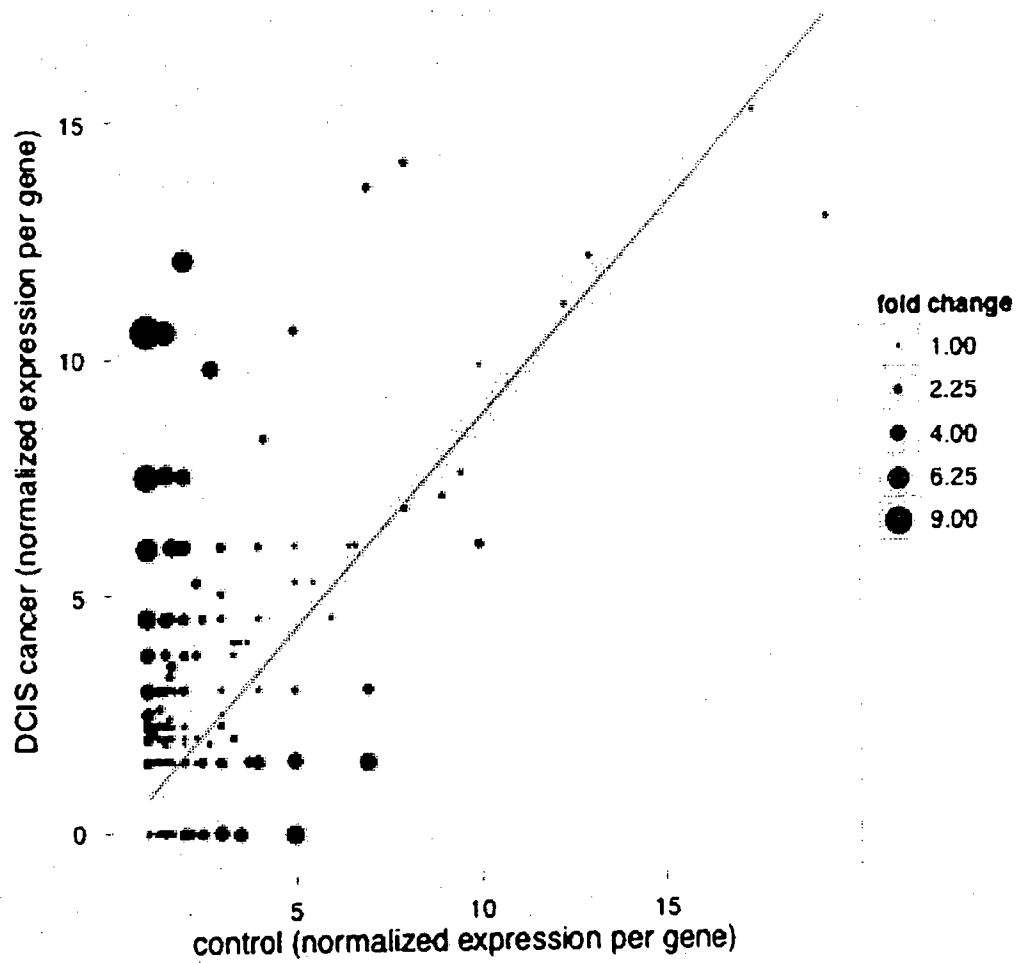


FIG. 27

GeneSigDB Curated Gene Signatures

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Analyzably Genes

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Fisher Exact Test

Fisher Exact Result

Show entries

Showing 1 to 25 of 597 entries

2 3 4 5 Next Last

Gene Signature	Signature Name	GeneSigDB Gene Signature Size	Your Gene List Size	Overlap	Background	P Value
15735024-SuppTable2	Breast_Reyal05_3373genesSuppTable2SuppTable2	3257	170	37	35339	0.00000000
16835340-Table1	Colon_Glebov06_173genes	40	170	4	35339	0.00000000
18662380-S3-ERBB2	Breast_Wirapat08_20genes_ERBB2_Module	20	170	3	35339	0.00010000
17565661-Table1	Breast_Mackay07_1284GenesDownRegulated	1054	170	15	35339	0.00020000
16651414-Supp2	Breast_Bertucci06_2537genes	1741	170	20	35339	0.00030000
12610208-Figure1-2	Breast_Hedenfalk03_38gene_down_subsetBRCAx	28	170	3	35339	0.00030000

FIG. 28

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU2011/000380

A. CLASSIFICATION OF SUBJECT MATTER																						
Int. Cl.																						
C12Q 1/68 (2006.01)																						
According to International Patent Classification (IPC) or to both national classification and IPC																						
B. FIELDS SEARCHED																						
Minimum documentation searched (classification system followed by classification symbols)																						
Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched																						
Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)																						
WPI, EPODOC, MEDLINE CAPLUS, BIOSIS. Keywords splice RNA, 3' exon and like terms																						
C. DOCUMENTS CONSIDERED TO BE RELEVANT																						
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.																				
P,X	TAFT, R. J. <i>et al.</i> , 'Nuclear-localized tiny RNAs are associated with transcription initiation and splice sites in metazoans', Nature Structural and Molecular Biology, August 2010, vol. 17, no. 8, pages 1030-1035	1-35, 37-42, 46																				
A	Affymetrix ENCODE Transcriptome Project/Cold Spring Harbor Laboratory ENCODE Transcriptome Project, 'Post-transcriptional processing generates a diversity of 5'-modified long and short RNAs', Nature, 2009, Vol. 457, No. 7232, pages 1028-1032																					
A	WO 2009/124341 A1 (THE UNIVERSITY OF QUEENSLAND) 15 October 2009																					
☐ Further documents are listed in the continuation of Box C ☒ See patent family annex																						
* Special categories of cited documents: <table border="0"> <tr> <td>"A"</td> <td>document defining the general state of the art which is not considered to be of particular relevance</td> <td>"T"</td> <td>later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</td> </tr> <tr> <td>"E"</td> <td>earlier application or patent but published on or after the international filing date</td> <td>"X"</td> <td>document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone</td> </tr> <tr> <td>"L"</td> <td>document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</td> <td>"Y"</td> <td>document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art</td> </tr> <tr> <td>"O"</td> <td>document referring to an oral disclosure, use, exhibition or other means</td> <td>"&"</td> <td>document member of the same patent family</td> </tr> <tr> <td>"P"</td> <td>document published prior to the international filing date but later than the priority date claimed</td> <td></td> <td></td> </tr> </table>			"A"	document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention	"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone	"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art	"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family	"P"	document published prior to the international filing date but later than the priority date claimed		
"A"	document defining the general state of the art which is not considered to be of particular relevance	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention																			
"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone																			
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art																			
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family																			
"P"	document published prior to the international filing date but later than the priority date claimed																					
Date of the actual completion of the international search 27 May 2011		Date of mailing of the international search report 31 MAY 2011																				
Name and mailing address of the ISA/AU AUSTRALIAN PATENT OFFICE PO BOX 200, WODEN ACT 2606, AUSTRALIA E-mail address: pct@ipaaustralia.gov.au Facsimile No. +61 2 6283 7999		Authorized officer Julie Christie AUSTRALIAN PATENT OFFICE (ISO 9001 Quality Certified Service) Telephone No: +61 2 6283 2463																				

INTERNATIONAL SEARCH REPORT

International application No.

PCT/AU2011/000380

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 36
because they relate to subject matter not required to be searched by this Authority, namely:
This claim is directed towards a computer readable storage medium characterised by the information contained thereon. As indicated at paragraph 9.11 of the PCT Search and Examination Guidelines this is a presentation of information hence excluded subject matter.
2. ☒ Claims Nos.: 1-35, 37-46 (all partially)
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
See Supplemental Box I
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a)

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
- ☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- ☐ No protest accompanied the payment of additional search fees.

Supplemental Box I

(To be used when the space in any of Boxes I to VIII is not sufficient)

Continuation of Box No: II.2

Claims 1-35 and 37-46 lack clarity and full support to such an extent as to render these claims unsearchable over their entire scope as follows:

Claims 1-35 and 37-46 (all partially) do not meet the requirements of Articles 5 and 6 of the PCT.

These claims are directed towards single-stranded isolated RNA molecules defined solely by a combination of unusual parameters including (i) consisting of no more than 26 contiguous nucleotides; (ii) corresponding to 3' nucleotide sequence of an internal exon of genomic DNA; and (iii) having an average GC content that is greater than 54%.

It is considered that the claimed subject matter is not supported by the specification in a manner sufficiently clear and complete for the invention to be carried out by a person skilled in the art over the entire scope of the claim (see paragraphs 5.54 and 5.58 of the PCT GL). Furthermore, the claimed invention lacks clarity because the claims contain so many options, variables, and possible permutations, that the claims are rendered unclear and inconcise to the extent that it is not in compliance with Article 6 and Rule 6 (see 9.25 PCT GL).

The above observations are particularly relevant to claims 18 and 19. These claims lack support, and are unclear for the reasons stated above. Furthermore, a compounding lack of clarity and lack of support pertains to these claims. Firstly, claim 18 is directed to any sequence showing at least a partial degree of complementarity to SEQ ID Nos: 1-16,898. It is considered that the person skilled in the art would not be able to determine all of the possible sequences which are encompassed by these claims. Secondly, with respect to claim 19, this claim is directed towards any sequence with at least 70% identity to sequences in appended claims which have not been defined by their sequence. As such, it would be impossible for the person skilled in the art to determine a sequence of 70% identity to a sequence which has not been specified clearly in a claim.

Pursuant to the above lack of clarity and lack of full support from the specification, the ISA considered that the claims are unsearchable over their entire scope. As such the search report is limited to the overall concept of the invention as defined in claims 1 and 17.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/AU2011/000380

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report		Patent Family Member			
WO	2009124341	AU	2009235941	EP	2268813
Due to data integration issues this family listing may not include 10 digit Australian applications filed since May 2001.					
END OF ANNEX					