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Noncoding RNAs of the Mammalian Testis: The Meiotic Transcripts *Nct1* and *Nct2* Encode piRNAs

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Abstract

In eukaryotic cells, the vast majority of transcribed sequences are extragenic with no known functions. Translin is a DNA/RNA-binding protein involved in mRNA transport and translation in postmeiotic male germ cells. In an effort to identify meiotic target RNAs of Translin, reversible RNA protein cross-linking and immunoprecipitations with an affinity purified antibody to Translin were performed. Four new meiotically expressed mRNAs and one noncoding RNA with Translin binding sites were identified. Following sequencing, the noncoding RNA, *Nct1*, was 100% identical to a site on mouse chromosome 2. A second partially homologous sequence, *Nct2*, was detected nearby. *Nct1* and 2 contained sequences identical to piRNAs. *Nct1* and 2 appear to be male germ cell-specific transcripts and are predominantly detected in pachytene spermatocytes. Focusing on the abundant single-copy PIWI-interacting RNA (piRNA), germline small RNA (gsRNA10) (the gsRNA10 sequence is identical to 29 nt in *Nct1*), we find that gsRNA10 increases greatly as spermatogenesis proceeds with concomitant decreases in *Nct1* and 2. The piRNA gsRNA10 binds to the germ cell-specific Y-box protein, MSY2, but not to Translin. Although the size of the primary transcript(s) encoding the piRNAs in the locus on chromosome 2 is not known, we propose that *Nct1* and 2 are part of a piRNA precursor.

Keywords

spermatogenesis; testis; meiosis; gene regulation; male germ cells; piRNAs; gsRNAs; noncoding RNAs

Introduction

Transcripts from genes that encode translatable open reading frames (proteins) represent a very small percentage (less than 5%) of the total transcripts of eukaryotic cells, suggesting that a disproportionate amount of research effort has been focused on a minor RNA population. In practice, the vast majority of transcribed sequences is extragenic and encode ribosomal RNAs, transfer RNAs, antisense sequences, small interfering RNA (siRNA), small nucleolar RNAs, microRNAs, and chromatin-associated RNAs.¹ In addition, testes appear to uniquely transcribe another group of small noncoding RNAs, the Piwi-interacting RNAs/germline small RNAs (piRNAs/gRNAs).^{2–8} The recently discovered piRNAs are especially abundant (>57,000 copies), are believed to be solely expressed in the germ cells of the testis, are evolutionarily conserved but not sequence conserved, and are predominantly sense sequences. With the exception of noncoding RNAs such as the transfer and ribosomal RNAs, the functions

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of most of the mammalian noncoding RNAs are unknown, leading investigators to propose numerous exciting but speculative regulatory functions for this diverse population of RNA.

Our laboratory has had a long-standing interest in two DNA/RNA-binding proteins, Translin and MSY2. Translin, also known as testis brain RNA-binding protein (TB-RBP), binds to breakpoint junctions of chromosomal translocations as a DNA-binding protein.⁹ As an RNA-binding protein, Translin binds to a group of mRNAs first transcribed in postmeiotic male germ cells by the transcription factor, CREM-tau.¹⁰ Although Translin is primarily a cytoplasmic protein in spermatids, it shuttles between cellular compartments and transports mRNA encoding proteins such as AKAP4 and the protamines intracellularly and intercellularly in a complex containing VCP (previously called TER ATPase) and the kinesin, KIF17 (previously called KIF 17b).^{11,12} Following the release of the mRNA from the RNA-protein complex, the mRNAs are translated, suggesting an involvement of Translin in the movement, stability, and translational suppression of specific mRNAs in haploid male germ cells. Movement of Translin between the nucleus and cytoplasm is facilitated by its association with a related protein, Translin-associated factor X.^{13,14} Translin has a functional nuclear export signal, while Translin-associated factor X contains a functional nuclear localization signal.

A second testicular DNA/RNA-binding protein, MSY2, is a Y-box protein that is solely expressed in male and female germ cells.¹⁵ It binds to both DNA and RNA through a highly conserved cold-shock domain. MSY2 recognizes a consensus highly conserved 12-nt Y-box DNA sequence found in the promoters of many genes expressed in germ cells. In the nuclei of germ cells MSY2 functions as a coactivator of transcription and selectively binds to specific transcripts.^{16,17} This selection is based on MSY2 binding to the DNA Y-box sequence, thereby marking specific mRNAs for subsequent stabilization and translational repression in the cytoplasm. Many germ cell-specific transcripts, such as the protamines, transition proteins, and GAPDS, are bound by MSY2 and from its abundance (about 2% in oocytes and 0.7% in male germ cells), we consider MSY2 to be a global suppressor/stabilizer of mRNAs.^{17,18} Until recently, all of the known target RNAs of Translin and MSY2 were mRNAs encoding proteins. A screen for meiotic RNA targets of Translin detected the novel noncoding RNA described below.

In 2006 several laboratories reported on a previously uncharacterized highly abundant and puzzling class of small RNAs of ~26–30 nt.^{2–8} Because many of these small RNAs bind to murine members of the PIWI protein family, these RNAs are commonly called PIWI-interacting RNAs (piRNAs), although others have named them germline small RNAs (gsRNAs). (For the sake of simplicity we will use the term piRNAs to describe this variable class of small RNAs.) PIWI proteins are members of the Argonaute protein family involved in germline-specific events such as germline stem-cell maintenance and meiosis.^{19,20} The piRNAs map to defined genomic regions in 20–90 kilobase (kb) clusters, and similar piRNAs have been identified in syntenic regions of mice, humans, and rats. They generally distribute along one genomic strand, but when they are on both strands, they generally appear not to overlap. Subpopulations of piRNAs are associated with polysomes and are in the ribonucleoprotein fraction. The first nucleotide of most of the piRNAs is uridine, they contain a 5' phosphate and a 3' hydroxyl group, and the majority is detected in testes from mice of 3 weeks to adults. To increase complexity, the piRNAs cloned from 15-day-old mice appear to be a few nucleotides shorter than those from adults and may represent a population of piRNAs expressed in earlier stages of germ cells. Little is known of the biogenesis pathway of piRNAs, but the extreme strand bias and lack of stem-loop structures suggests it will differ from that of the miRNAs and siRNAs. Although speculation abounds that piRNAs are processed from very large primary transcripts (up to 60 kb) in meiotic-stage germ cells, data are lacking.

Although Translin is primarily found in the cytoplasm in postmeiotic male germ cells, in pachytene spermatocytes it is primarily a nuclear protein. Reversible RNA protein cross-linking and immunoprecipitations with an affinity purified antibody to Translin were performed to identify RNA targets of Translin in meiotic cells.²¹ Four new meiotically expressed mRNAs and one noncoding RNA with Translin binding sites were selectively precipitated from testis extracts of 17-day-old (and adult) mice. Parallel immunoprecipitations with testis extracts from Translin-null mice did not precipitate the mRNAs or noncoding RNA, confirming the specificity of the precipitation. RNA gel shift assays confirmed the specificity of interaction of the five meiotic transcripts. Here we will focus on the meiotically expressed noncoding RNA, which we named *Nct1* and demonstrate that it is transiently present in male germ cells and contains sequences identical to piRNAs.

Materials and Methods

RNA Preparation and Northern Blotting

Total RNA was extracted from mouse testes using TRIzol Reagent (Invitrogen, Carlsbad, California). To detect piRNAs, total small RNA-enriched preparations were extracted from mouse testes using the *mirVana* miRNA Isolation Kit (Applied Biosystems, Foster City, California). RNA (10 µg) was electrophoretically separated on 15% denaturing polyacrylamide gels and tRNAs were visualized by ethidium bromide staining to ensure the quality of the RNA. The RNAs were transferred to Hybond-N+ membrane using a semidry Transblot electrophoresis apparatus (Bio-Rad, Hercules, California) and UV cross-linked to membranes. DNA oligonucleotide probes (sequences in Table 1A) were 5'-end labeled with γ -[³²P]-ATP, and hybridization was carried out using Perfect-HybPlus Hybridization buffer (Sigma-Aldrich, St. Louis, Missouri) following the manufacturer's instructions. After washing, the membranes were imaged with the STORM Phosphorimager (model 860) (Molecular Images, Mountain View, California). Sequences of each probe were complementary to the corresponding piRNA sequence.

For 5' labeling, aliquots of the isolated nucleic acids were first subjected to dephosphorylation with calf intestinal alkaline phosphatase (New England Biolabs, Ipswich, Massachusetts). After phenol/chloroform extraction and ethanol precipitation, the RNAs were labeled with γ -[³²P]-ATP by T4 polynucleotide kinase and resolved by a 15% denaturing PAGE along with radioactive oligoribonucleotide-size markers.

Real-time Quantitative Polymerase Chain Reaction

Total RNA (1 µg) was converted to cDNA using the SuperScript III kit (Invitrogen). Syber green-based real-time quantitative polymerase chain reaction (qPCR) was performed using the ABI 7900 HT Sequence detection system (Applied Biosystems). All qPCR data were normalized to β -actin. The primer sequences are described in Table 1B.

RNase Protection Assays

Two DNA oligonucleotides, one containing a sequence corresponding to a piRNA followed by a complementary sequence for the T7 promoter and a second containing the T7 promoter, were mixed and made double-stranded by PCR (95°C for 3 min, 5 cycles of 95°C for 30 s; 50°C for 30 s; 72°C for 10 s). The oligonucleotide sequences used are listed in Table 1C. Radiolabeled RNA probes complementary to each piRNA were generated with α -[³²P]-CTP using double-stranded DNA by *in vitro* transcription using the Maxiscript Kit (Applied Biosystems). Probes were purified by electrophoresis in 15% urea-polyacrylamide gels and used in ribonuclease protection assays following hybridization to samples of the small-RNA enriched total RNA (0.8 µg) using the *mirVana* miRNA Detection Kit following the manufacturer's protocol (Applied Biosystems).

Immunoprecipitation

Four adult CD-1 mouse testes were extracted with TBS-T buffer (20 mM Tris·HCl, pH 7.4, 137mM NaCl, 0.1% Tween 20, 0.1% Empigen BB, protease inhibitor, and RNasin) and precleared with 400 μ L of Protein A agarose beads for 2 h at 4°C. After centrifugation, the supernatants were incubated with anti-MSY2 or anti-Translin (100 μ g) for 1 h and 200 μ L of protein A agarose beads overnight at 4°C. Following centrifugation (400 $\times$ g for 5 min), the supernatants were washed four times with 5 mL of TBS-T buffer, and small RNAs were purified and hybridized as described earlier.

RNA Gel Shift Assays

Recombinant His-tagged MSY2 protein (0.3 μ g) was incubated with 10,000 cpm of commercially synthesized single stranded RNA probes that had been 5'-end labeled with γ -[³²P]-ATP (IDT, Inc., Coralville, Iowa; sequences in Table 1D) for 15 min, followed by RNase T1 digestion for 5 min. For supershift assays, anti-MSY2 (1 μ g) was added to the assay mixture before RNase T1 digestion and incubated for 15 min. The RNA-protein complexes were separated by 6% PAGE at 4°C and detected with the STORM Phosphorimager. All experiments were performed at room temperature except where noted.

Results and Discussion

Following its sequencing, a BLAST search using the 178-nt sequence of *Nct1* in the databases available in 2004 revealed the *Nct1* transcript to be 100% identical to a site on mouse chromosome 2. Using RLM-RACE, we were able to extend the *Nct1* sequence to 685 nt.²¹ Three hundred sixty nucleotides downstream of *Nct1* was a second partially homologous sequence that we named *Nct2*. *Nct2* showed identity in 120 of the 178 nt of *Nct1*. Unlike all the other RNAs known to bind to Translin, we concluded that *Nct1* (and *Nct2*) are nonprotein coding genes, because they contain multiple stop codons in all frames and in both directions. Neither *Nct1* nor *Nct2* contain introns. Sequence analysis of the genomic DNA between *Nct1* and 2 identified a GGA triplet repeated 16 times, a motif often associated with recombination hot-spot sites immediately upstream of *Nct2*. *Nct1* and 2 appear to be male germ cell-specific transcripts, because they are not detectable by RT-PCR in RNAs from brain, heart, intestine, kidney, liver, lung, spleen, ovary, or KIT w/KIT^{w-v} testes (the latter contain somatic cells and spermatogonia, but lack meiotic and postmeiotic germ cells). Most notably, *Nct1* and 2 are readily detected by RT-PCR with RNAs isolated from STAPUT-purified pachytene spermatocytes, but not from populations of similarly purified round spermatids, suggesting a rapid degradation and/or processing of the *Nct1* and 2 transcripts as the germ cells pass from meiosis into spermiogenesis.

With the identification, sequencing, and sequence availability of piRNAs/gsRNAs in 2006, we made a startling discovery. Numerous piRNAs mapped to the *Nct1* and 2 genomic region on chromosome 2. As of March 2007, the region encodes at least 42 of the piRNA registered in the NCBI database (Fig. 1). Assuming that each piRNA is not a product of individual primary short transcripts, but is derived from a longer primary transcript(s), we wondered whether *Nct1* and 2 could be part of a longer precursor noncoding RNA that encoded multiple piRNAs.

To gain insight into the relationship between *Nct1* and 2 and piRNAs, we focused our efforts on one abundant single-copy piRNA, *gsRNA10* (*piR-108205*, -9097, -119800). The sequence of this piRNA, present in the *Nct1* transcript, is found as a single-copy RNA in the mouse genome. By Northern blotting *gsRNA10* is not detectable in RNA from brain, heart, intestine, kidney, liver, lung, or spleen, suggesting it may be testis-specific (Fig. 2A). Analyzing testis RNAs from 17-day, 22-day, and adult mice with RNase protection assays, *gsRNA10* and three other piRNAs that map in the *Nct1* and 2 region all increase substantially as spermatogenesis

proceeds (Fig. 2B). Not only do these four piRNAs increase in testes between prepuberal and adult mice, but a similar general increase is seen when total piRNA levels are quantitated (Fig. 2C). We do not understand the variations in amount of piRNAs from presumably the same parental *Nct1* and 2 transcripts. This may reflect selective degradation of some piRNAs or multiple transcripts containing subpopulations of the piRNAs in the chromosomal locus.

Our ability to detect *Nct1* and 2 in RNA prepared from purified pachytene spermatocytes (or in testis from 17-day-old mice), but not in round spermatids, indicates a rapid decrease in *Nct1* and 2, coincident with increases in the piRNAs. To examine this further, qPCR was used to measure the amounts of *Nct1* and 2 in 17-, 21-, 28-, and 42-day-old mice (Fig. 3). The highest levels of *Nct1* and 2 are detected in the testes of 17-day-old mice with transcript decreases in adult mice. Could *Nct1* and 2 be serving as precursors for the piRNAs they encode? We do not know yet, but the decrease in *Nct1* and 2 at the time of increasing levels of piRNAs suggests yes. Preliminary studies with mice lacking *Nct1* and 2 indicate a reduction of piRNAs both within and external to the deleted region.

The ~30-nt RNAs of the testis have been named piRNAs, because of their association with the protein PIWI. Because Translin and MSY2 both bind DNA and specific subpopulations of RNA and MSY2 are present in the chromatoid body, we used affinity purified anti-Translin and anti-MSY2 for immunoprecipitations to determine if piRNAs are detectable in RNA-protein complexes with either protein (Fig. 4).²² Anti-MSY2, but not anti-Translin, selectively immunoprecipitated a group of ~30-nt RNAs from both ribonucleoprotein and polysomal fractions from adult mouse testis extracts (Fig. 4A). Assaying piRNAs that are in the *Nct1* and 2 region, we discovered by Northern blotting that *gsRNA10* was immunoprecipitated by anti-MSY2, but not by anti-Translin. This specific interaction between *gsRNA10* and MSY2 was confirmed by RNA gel shift assays using recombinant MSY2 and a radiolabeled probe for *gsRNA10* (Fig. 4B). Although several other piRNAs whose sequences are detectable in *Nct1* and 2 are not detected in the immunoprecipitates, they are present at much lower levels than *gsRNA10* and their “absence” could be due to our inability to detect them. These findings indicate that at least one piRNA, *gsRNA10*, whose sequence is found in *Nct1*, binds to the germ cell-specific Y-box protein, MSY2. Although the size of the primary transcripts encoding the piRNAs on chromosome 2 is not yet known, it is intriguing that one testis RNA-binding protein, Translin, selectively binds the *Nct1* noncoding transcript and a second germ cell-specific RNA-binding protein, MSY2, binds to at least one piRNA, *gsRNA10*, whose single-copy sequence is encoded in *Nct1*. Clearly, much remains to be discovered about the mechanism of biogenesis of piRNAs and of the functions of mammalian piRNAs and noncoding RNAs in general.

Acknowledgments

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FIGURE 1.

piRNA sequences are in *Nct1* and 2. As of March 2007, 64 piRNAs that are 100% identical to sequences in the *Nct1* and 2 genomic locus were registered in the NCBI database. The piRNAs are indicated by lines below *Nct1* and 2. *Filled boxes*, similar sequences in other chromosomes. *Open boxes*, piRNAs that map to this region. *Asterisk* indicates the site of piRNAs containing at least 26 nt of the *gsRNA10* sequence.

FIGURE 2.

RNA expression patterns of piRNAs in diverse tissues and prepuberal and adult mouse testes. (A) RNA (10 µg) was hybridized to a radiolabeled probe complementary to *gsRNA10* and later with a probe complementary to *let7 a-1* as a control to demonstrate the presence of RNA in all lanes. (B) RNase protection assays for four piRNAs present in the *Nct1* and 2 genomic locus were performed using RNA isolated from the testes of 17-, 21-, 28-, and 42-day-old (adult) mice. The *piR-118612* sequence maps to consensus region of *Nct1* and 2. The *gsRNA10* sequence maps to *Nct1*. The *piRNA-int* sequence maps between *Nct1* and 2. The *piR-132236* sequence maps to ~0.45 kb downstream of the 3' terminus of *Nct2*. (C) Small RNA-enriched

total RNAs from testes of 17-, 21-, 28-, and 42-day-old mice were ^{32}P -end-labeled and analyzed by electrophoresis in a 15% denaturing polyacrylamide gel.

FIGURE 3.

RNA levels of *Nct1* and *2* were quantitated in testes from prepuberal and adult mice by qPCR. The fold differences were determined by comparing the delta Ct of each gene normalized to β -actin. Clusterin (*filled squares*) was used as the control showing the decrease concomitant with mouse age, because of its Sertoli cell-specific expression. *Diamonds* indicate relative RNA amounts of the *Nct1* and *2* consensus region.

FIGURE 4.

gsRNA10 forms an RNA–protein complex with MSY2. (A) *gsRNA10* immunoprecipitates from adult mouse testis extracts with anti-MSY2, but not with anti-TSN. (B) ^{32}P -labeled RNA probes for *gsRNA10* and a 29 nt negative control containing a polyA signal from *Rnf139* were used for gel shift assays with recombinant MSY2 (rMSY2). Super shift bands were detected with anti-MSY2 (arrowheads).

TABLE 1
Probes and primers

A. DNA oligonucleotides used for Northern blotting	
<i>gsRNA10</i>	GTT CTA TAC CAT CAC TCC CTA TTT ATT GA
<i>let7 a-1</i>	ACT ATA CAA CCT ACT ACC TCA
B. Primers used for qPCR	
<i>Nct1/2</i>	Fw CTA GGC CAA AAT AGA AGA GGA TAG Rv GGA GTC TCT CGG ACC CCA AAA TG
<i>Clusterin (Clu)</i>	Fw ACC GGC AGC AGA GCC AAG TCC Rv GTC ATC TTC ACC TTC TCT TAA G
C. DNA oligonucleotides used for the RNA protection assay	
<i>piR-118612</i>	CCT AGA TCT CAG AGC ATA CAG CAC TTT CAG GTT TTC CTA TAG TGA GTC GTA TTA
<i>gsRNA10</i>	GCT CAA TAA ATA GGG AGT GAT GGT ATA GAA CGA TCC TAT AGT GAG TCG TAT TA
<i>piRNA-int</i>	GAT AGG ATG GAT GTG GAA AAG GGA CTA GGT TCC TAT AGT GAG TCG TAT TA
<i>piR-132236</i>	CAT CTG TTT GAT GTT TGT GAG TTG GGA AGT CCT ATA GTG AGT CGT ATT A
D. RNA probes used for RNA gel shifts	
<i>gsRNA10</i>	UCA AUA AAU AGG GAG UGA UGG UAU AGA AC
<i>Rnf139</i>	ACA AUA AAA AGG UGG ACA UUG UUA ACU AA