Processing of naturally occurring sense/antisense transcripts of the vertebrate Slc34a gene into short RNAs

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Antisense RNAs have an established regulatory role in epigenetic phenomena such as imprinting or X chromosome inactivation (1, 16, 18, 25). For example, the long antisense transcripts Air and Kcnq1ot1 are essential for parental imprinting of the cognate gene clusters (28, 30). In addition, the noncoding transcript Xist and the related antisense transcript Tsix play a critical role in orchestrating X chromosome inactivation. However, the molecular processes involved in imprinting and X chromosome inactivation were found not to rely on sense/antisense hybrid formation (24, 28, 30). These examples may therefore not be representative for the bulk of natural antisense RNAs.

We have studied a bidirectionally transcribed locus from zebrafish that shows important hallmarks of natural sense/antisense RNA pairs such as exonic overlap and tissue-specific expression patterns. The sense transcript encodes a transport system for inorganic phosphate (P_i) that overlaps in exonic regions with a fully processed noncoding antisense transcript. The solute transporter is instrumental in maintaining P_i homoeostasis in vertebrates including mammals, fish, and amphibians (21). Zebrafish expresses at least two isoforms of the protein, Slc34a2a and Slc34a2b, with only the gene encoding Slc34a2a showing antisense transcription (22). Overlapping transcription is conserved in selected isoforms of Slc34a3 in fish and mammals (35). We have used Slc34a2-related transcripts to investigate the consequences of sense and antisense RNA coexpression. We found evidence for the processing of coexpressed transcripts into short RNAs after injection in Xenopus oocytes. Slc34a2-related short RNAs were also detected in RNA preparations from zebrafish embryos that coexpress both sense and antisense RNAs.

IN MOUSE, up to 72% of all genomic loci show evidence of sense and antisense transcription, and comparable numbers have been suggested for other organisms (13, 37). The majority of these natural antisense transcripts (NATs) are fully processed and overlap in exonic regions. NATs are underrepresented on mouse and human X chromosomes compared with autosomes (4, 14). This bias, however, is far less pronounced if the transcripts lack exonic overlaps (i.e., the potentially overlapping sequences are removed during splicing). These findings imply that antisense transcripts included in these studies are subjected to comparable evolutionary restrictions and may be processed by related mechanisms; furthermore, they indicate that the formation of RNA-RNA hybrids seems essential for the processing of these NATs (33). The biochemical function of NATs has been assessed in various organisms, and evidence for RNA interference (2), RNA editing (38), RNA splicing (11), transcriptional interference (23), or direct RNA-protein interaction (19) has been reported. These important findings have been reviewed in detail (16, 17). However, the extent to which these mechanisms contribute to the phylogenetic drive to accumulate antisense transcripts in eukaryotic genomes is a matter of debate.

MATERIALS AND METHODS

Animals. Zebrafish (Danio rerio) embryos were bred in house. Animals were killed by hypothermia in ice water. Frogs were obtained from the African Xenopus Facility (South Africa). Xenopus were anesthetized by immersion into ice-cold tricaine solution (1 g/l; Sigma) and killed by decapitation before the removal of the oocytes.

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according to registered procedures. The oocytes were surgically removed and rinsed in ORII solution (mM: 82.5 NaCl, 2 KCl, 1 MgCl₂, 5 HEPEs pH 7.4), followed by collagenase treatment (2 mg/ml in ORII) to remove the follicular cell layer. Oocytes were stored in modified Barth’s solution (mM: 88 NaCl, 1 KCl, 0.82 MgSO₄, 0.41 CaCl₂, 0.33 Ca(NO₃)₂), 10 HEPEs-Tris pH 7.5, 2.4 NaHCO₃ at 18°C. All animal procedures were licensed and controlled by the UK Home Office.

Expression in Xenopus oocytes. cRNA was produced from HindIII- or EcoRV-linearized plasmids with the mMESSAGE mMACHINE kit (Ambion). RNA was purified by LiCl precipitation, and concentration was determined by spectrophotometry. Routinely, between 2 and 10 ng of cRNA in 50 nl of water was injected per oocyte. The oocytes were kept at 18°C in modified Barth’s solution up to 3 days before the assays. Radiotracer flux measurements were performed with [3²P] (Amersham) as a tracer. If the expressing oocytes were used for RNA extraction, functional activity was determined by two-electrode voltage-clamp technique as described by Graham et al. (9).

All injections were performed with oocytes from at least three different frogs with comparable results.

PCR and cloning. PCR-related techniques were used for expression analysis, cloning purposes, and probe generation. To detect Na-Pi transporter-related sense or antisense transcripts, 0.1–0.5 µg of total RNA was reverse transcribed with a kit from Qiagen. The RNA in 5 µl was denatured at 70°C for 5 min in the presence of 0.1 µg/µl oligo(dT) primer and cooled to 37°C. Fifteen microinjectors of RT mix was added containing buffer, nucleotides (250 µmol each), 4 U of reverse transcribease (Qiagen), and 20 U of RNase inhibitor (Promega). After 60-min incubation at 37°C enzymes were denatured at 95°C. For PCR the supplier’s protocol was downscaled, and 2 µl of the RT reaction was used for hot-start PCR with Taq polymerase and reagents from Qiagen. The final volume was 25 µl. All reverse transcription (RT)-PCR experiments were performed three or more times, often by two different researchers. Representative experiments are shown. For 3’ rapid amplification of cDNA ends (RACE) an oligo(dT) adaptor primer (Sigma) was used to prime the reverse transcription. A first PCR was done as above including the gene-specific primer F1 and the adaptor primer. A second, nested reaction was performed with 1 µl of the 1/2,000 diluted first reaction, a second specific primer F2, and the adaptor primer. PCR conditions were modified according to the annealing temperature of the primers and the length of the expected fragments. For 5’ RACE a kit from Invitrogen was used, and the protocol was strictly followed. The sequences of the primers (R1-R3) are given in Supplemental Table S1. The two DNA fragments from 3’ and 5’ RACE were separated on agarose gels and purified. The entire fragment was amplified by overlapping PCR. One microinjector of each fragment was added to a PCR reaction (Qiagen). After four cycles at a low annealing temperature (50°C) to allow hybridization of the overlapping DNA ends and extension of the full fragment, the two adaptor primers were added and the cDNA was amplified. The resulting fragment was cloned into pCR2.1-TOPO with the TOPO cloning kit from Invitrogen according to the standard protocol. The cDNA was verified by sequencing.

Isolation of nucleic acids. Mouse tissues (<100 mg) were ground to powder in liquid nitrogen and added to 1 ml of TRI reagent (Sigma). Homogenization was completed by passing the slurry repeatedly through 21-gauge and 25-gauge injection needles. RNA extraction was then completed according to the supplier’s protocol. The integrity of the RNA was checked by denaturing gel electrophoresis, and concentration was determined as above. Total RNA was used for all PCR and cloning steps. Single oocytes were quickly homogenized in 100 µl of TRI reagent with disposable plastic pestles. Thereafter, the supplier’s protocol was followed. The centrifugation times were increased for precipitation of nucleic acids if short RNAs were to be detected.

Nonradioactive detection of nucleic acid. Assessment of RNA from injected oocytes was done with denaturing formaldehyde agarose gels (1.2%). RNA from single oocytes was separated and blotted by capillary force onto nylon membranes (Roche). After UV cross-linking the membranes were prehybridized (>1 h) and hybridized overnight in digoxigenin (Dig) EasyHyb solution (Roche). The Dig-labeled probes were generated by in vitro transcription with the mMESSAGE mACHINE kit (Ambion) and Dig-labeled nucleotides (Roche). Hybridization was carried out at 50°C. Membranes were washed at a final stringency of 1× SSC-0.1% SDS at 58°C following the detection of chemiluminescence (ECL, Roche). Short RNAs were separated on 15% Tris-HCl polyacrylamide gel with Tris-borate-EDTA (TBE) as a running buffer and blotting buffer. This combination gave superior results compared with TBE-urea gels. Gels were prerun for 1 h before loading of the samples. Blotting was performed at a constant 100 mA for 50 min. All steps were performed with precast gels and a Criterion midi gel system (Bio-Rad). Hybridization was essentially done as above; however, the stringency was reduced. Hybridization was performed at 37°C, and final washes were at room temperature in 0.1× SSC-0.1% SDS. Batches of oocytes from at least two different frogs were used for a specific set of experiments, and RNA from six or more single oocytes was blotted.

Accession numbers. Public database accession numbers of the Na-Pi-related transcripts are as follows: zebrafish: Slc34a2a, AF121796; Slc34a2b2, NM_131624; antisense 1, AY308479; antisense 2, AY308480; Bufo bufo: Slc34a2, AY427791.

RESULTS

We have cloned two splice forms of an antisense transcript related to the zebrafish Na-Pi cotransporter. The fully processed RNAs, AS1 and AS2, overlap with the sense transcript in several exons. AS2 potentially hybridizes to the 1st, 10th, and 13th exons of the sense transcript and represents the predominant isoform (Fig. 1A). Expression of the Slc34a2-related transcripts was assessed by RT-PCR in RNA preparations from whole zebrafish embryos (Fig. 1B). The antisense transcript was already detectable 2 h postfertilization (hpf) and preceded the expression of the sense transcript, which was first detected at 48 hpf. A window around 48 hpf exists in which robust coexpression of sense and antisense RNA occurs in whole zebrafish embryos. If PCR cycle numbers were increased, the antisense transcript remained detectable at low levels during later embryonic stages and in adult fish (22).

We used the Xenopus laevis oocyte system to investigate the impact of antisense RNA on the expression of the transporter-encoding sense transcript. The considerable size of the oocytes allows direct injection of message into the nucleus or the cytoplasm. Under standard conditions 2.5–5 ng of in vitro-synthesized RNA representing the fully processed sense and antisense transcripts was injected. The RNA was extracted from single oocytes after incubation for 24–48 h. Injection of Slc34a2a sense RNA into the cytoplasm resulted in 10- to 20-fold increase in Na-Pi transport activity (10). Transport was not inhibited significantly in the presence of equimolar amounts of antisense RNA. At higher concentrations AS2 caused a slight inhibition of transport (<40%), whereas AS1 had no effect. Comparable functional results were obtained with electrophysiology and radiotracer flux assays (Supplemental Fig. S1). Extraction of the RNA from single oocytes revealed that both sense and antisense transcripts were stable over 3 days (Supplemental Fig. S1). This confirmed findings

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from other groups that exogenous RNA is relatively stable in the cytoplasm of Xenopus oocytes even if sense/antisense hybrids can occur (26, 27). We then performed similar experiments but injected the RNA into the nucleus of the oocytes. In this case, sense and antisense RNAs alone remained stable, whereas coinjected RNAs were rapidly degraded within 4 h (Fig. 2, A, lane 6, and C, lanes 7–9). To test the specificity of the reaction we used homologous sense RNAs from zebrafish (Slc34a2b) and toad (Bufo bufo) (9, 20). Both transcripts cross-reacted with the Northern probe, but they have no more than 14 consecutive identical bases. As shown in Fig. 2B control RNAs failed to induce degradation; both sense RNAs and the antisense transcript remained detectable (lanes 13 and 15). To summarize, coinjection of Slc34a2a sense and antisense RNA induced rapid degradation of both transcripts. This reaction only occurred in the nucleus and required extended regions of perfect complementarity (>14 bp).

The most plausible explanation for these findings would be that short RNAs are produced by an RNA interference-related mechanism. Other possibilities include extensive RNA editing or complete degradation of the transcripts. We tested the first hypothesis by examining whether key enzymes of RNA interference or microRNA processing are expressed in Xenopus oocytes. Fragments related to dicer, dorsha, and DGCR-8 were amplified from single-oocyte RNA preparations (Supplemental Fig. S2). We then performed Northern blots to visualize the short RNAs directly. Slc34a2a-related oligonucleotides of ~23 nt size were present in RNA preparations from nuclear sense/antisense-injected oocytes but not in control preparations from cytoplasmatically injected oocytes (Fig. 3, lanes 2–5). Short RNAs were also detected in total RNA preparations harvested from zebrafish embryos at developmental stages in which coexpression of sense and antisense transcripts had been detected (at 48 hpf; Fig. 3, lanes 10 and 11). Remarkably, only one strand of the original RNA duplex could be detected and short RNAs with different orientation prevailed at 48 hpf and 72 hpf. At 48 hpf a strong signal of short RNAs complementary to the sense transcript was detected, whereas at 72 hpf an antisense complementary signal was found (Fig. 3). The exact sequences of the short sense/antisense RNAs remain to be established. These results indicate that the processing of natu-

**Fig. 2.** Expression of Slc34a2 sense and antisense transcripts in Xenopus laevis oocytes. Combinations of protein encoding sense and antisense transcripts were injected into Xenopus oocytes nuclei or cytoplasm. After incubation (routinely 48 h) total RNA was extracted from single oocytes and assayed by Northern blotting. A: degradation of sense and antisense RNA coinjected in the nucleus. The RNA of each oocyte was used for 3 parallel blots and assayed for sense (top), antisense (middle), and actin (bottom), respectively, with single-stranded RNA probes. In the nucleus, the sense/antisense combination was efficiently degraded (lane 6, S+AS). B: specificity of the nuclear degradation process. In addition to the sense transcript (Slc34a2a, lanes 1, 2 and 9, 10) another protein coding isoform from zebrafish (Slc34a2b2, expressed in kidney, lanes 4, 5 and 12, 13) and from another species (Bufo, lanes 6, 7 and 14, 15) was tested. C: time dependence of nuclear processing of the sense/antisense transcripts. Oocytes were injected with the overlapping transcripts as indicated, and RNA was extracted after 1, 4, and 24 h. Single-stranded probes were used as indicated.
Natural sense/antisense transcript pairs into short RNAs is not restricted to (Xenopus) oocytes but also occurs in zebrafish.

We next tested whether these RNA oligonucleotides were biologically active. If so, we would expect that Slc34a2a-related reporter transcripts injected into the cytoplasm of Xenopus oocytes would become targets for degradation if short RNAs were coexpressed. We coinjected in vitro-synthesized sense and antisense RNA in the nucleus to produce short RNA fragments and reinjected the oocytes after 12 h with Slc34a2a sense/antisense transcripts or homologous control transcripts in the cytoplasm, respectively. Both the Slc34a2a sense and antisense transcripts were efficiently degraded (Fig. 4A, lanes 5–9), whereas Slc34a2b sense and the homolog from toad remained unaffected (not shown). To test the biological activity of short RNAs from zebrafish embryos we injected Slc34a2a sense and antisense reporter transcripts and reinjected these oocytes 12 h later with total RNA extracted from 24 hpf and 48 hpf embryos. RNA from 24 hpf embryos had no effect on the stability of the Slc34a2a-related reporter RNAs. Injection of the 48 hpf RNA resulted in complete degradation of the Slc34a2a-related RNAs in both nucleus and cytoplasm. Nuclear processing was more efficient than cytoplasmic processing: in the nucleus both sense and antisense transcripts were degraded within 1 h, whereas cytoplasmic processing took up to 4 h (Fig. 4B, lanes 9–12).

**DISCUSSION**

We investigated the biochemical mechanism that targets a natural sense/antisense transcript pair from zebrafish with the Xenopus laevis expression system. Our results suggest that overlapping sense and antisense transcripts are processed in the nucleus and result in short RNAs that are competent to induce degradation of a reporter RNA. We also provide evidence that a comparable mechanism may be active in vivo by detecting sense/antisense-related short RNAs on Northern blots from zebrafish embryos.

We interpret and discuss our results from a genomic, rather than a physiological, angle. This approach was prompted by the simple observation that in winter flounder (seawater habitat), zebrafish (freshwater habitat), and mouse (terrestrial) Slc34a transporters play a pivotal role in maintaining Pi homeostasis (34). Important hallmarks of Pi regulation in these species are alternate apical/basolateral sorting to mediate Pi secretion or absorption in flounder (7, 15); the expression of two isoforms with different substrate binding affinities in zebrafish (9); and hormonal regulation as well as the expression of two functionally divergent isoforms in mouse (21). All of these species express an antisense transcript related to Slc34a (35). Given the phenotypic diversity of the three species, it seems unlikely that the antisense transcription common to all plays an essential role in regulating Pi homeostasis.

Natural antisense transcripts in eukaryotes are fully processed RNAs that are expressed in a development- and tissue-specific manner (36). The prevalence of genes with complementary transcripts approximately correlates with the complexity of the organism and exceeds 50% in mice and humans (4, 13, 37). Homologous antisense transcripts are poorly conserved with respect to their genomic organization (5, 35).
Antisense transcription per se and the formation of exonic overlaps between the sense and the antisense transcripts seem to be the key features required for phylogenetic conservation.

The putative biochemical functions of antisense transcripts have been reviewed in great detail (16, 17, 31, 32). We will focus our discussion on aspects of RNA interference that relate directly to our work. There are several papers that interrogate the involvement of RNA interference in relation to natural antisense transcripts, two of which are particularly relevant to our findings (2, 8). Borsani et al. (2) investigated the stress-related gene P5CDH in plants (Arabidopsis thaliana) that overlaps with a gene of unknown function (SRO5). On salt-induced stress both transcripts are induced; concomitantly, a short RNA of 24 nt is formed and further processed into a 21-mer. The authors suggest that a similar process might apply to complementary RNAs in other organisms. The mechanism involves the plant-specific RNA-dependent RNA polymerase 6 (RdRP 6) and RNA polymerase IV (NRPD1A), indicating that the biochemical function of antisense transcripts in plants and vertebrates may follow a similar strategy but show crucial mechanistic differences. The second paper by Faghihi and Wahlestedt (8) argues against (cytoplasmic) sense-antisense duplex formation and concomitant short RNA formation. Their approach involved the transient transfection of mammalian cell lines with constructs that mimic natural sense-antisense pairs [thymidylate synthase and hypoxia-inducible transcription factor (HIF)]. Short RNAs were produced in transfected HeLa cells when a hairpin construct of sense and antisense transcripts or a mix of in vitro-transcribed RNAs was used but not on cotransfection of sense and antisense transcript encoding DNA plasmids, respectively. An essential difference between our experiments and the above-mentioned report is that we include in vivo samples, whereas Faghihi and Wahlestedt relied exclusively on transfected cells. There results may therefore reflect the incompatibility of plasmid expression and a putative sense/antisense processing mechanism rather than a situation in which “RNA interference is not involved in natural antisense mediated regulation of gene expression in mammals,” as the authors conclude.

Our results suggest that overlapping sense and antisense transcripts are processed in the nucleus and result in short RNAs that are competent to degrade a reporter construct in the Xenopus oocyte expression system. With so many antisense transcripts in vertebrate cells, why have sense/antisense-derived short RNAs remained largely unnoticed? A number of reasons may apply. First, the sense and antisense transcriptomes are independently regulated, and overlapping transcripts may be coexpressed only during a short time period (3, 13, 36). Second, antisense transcripts are usually expressed at low levels. Third, algorithms detecting microRNAs that are of similar size to the short RNAs we detected have so far relied on the characteristic hairpin structure of pre-microRNAs that is not a feature of sense/antisense hybrids. Finally, the short RNAs have probably been missed from large-scale cloning screens because they cannot be discriminated from degradation products of normal cellular mRNAs. Interestingly, a recent paper has documented an enrichment of short RNAs at transcript boundaries (12); these results support our findings because most antisense transcripts overlap with the corresponding sense transcripts at either the 3′ or the 5′ end (12, 29).

Our experiments yielded two key findings that may become instrumental in exploring a general biological role of NATs. First, we found significant coexpression of sense and antisense transcripts to be restricted to a small developmental window. The occurrence of SLC34A2-derived short RNAs within the same time window indirectly shows that both transcripts are coexpressed in the same cell types. The extent to which other cell populations exist within the developmental window that express either sense or antisense transcript but not both cannot be predicted from our experiments. The production of short RNAs, however, provides strong indirect evidence that coexpression and hybridization of sense and antisense transcripts occur in the same cells. The observed expression pattern of the RNAs could reflect an initial widespread transcriptional activity to shape the transcriptional landscape. The low level of antisense transcripts at later stages could reflect stochastic transcriptional activation that results in maintaining the status quo. The second important finding represents the developmentally regulated strand selection of the short RNAs. The short RNAs of alternate orientation potentially recognize different targets and could therefore be used to direct a downstream response toward either sense or antisense. Interestingly, during early development, when the sense-encoded protein is not expressed (the kidney in zebrafish embryos only starts filtering around 48 hpf; Ref. 6), the antisense transcript is expressed. In the early phase of sense/antisense coexpression the short RNAs complementary to the sense transcript are selected. Later, strand selection is reversed: the sense transcript is predominantly expressed and the short RNAs complementary to antisense prevail. To our knowledge, this is the first observation of this kind and may hold important clues to decipher the importance of antisense transcription.

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REFERENCES

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