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Identification of a New Antizyme mRNA + 1 Frameshifting Stimulatory Pseudoknot in a Subset of Diverse Invertebrates and its Apparent Absence in Intermediate Species

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The expression of eukaryotic antizyme genes requires +1 translational frameshifting. The frameshift in decoding most vertebrate antizyme mRNAs is stimulated by an RNA pseudoknot 3' of the frameshift site. Although the frameshifting event itself is conserved in a wide variety of organisms from yeast to mammals, until recently no corresponding 3' RNA pseudoknot was known in invertebrate antizyme mRNAs. A pseudoknot, different in structure and origin from its vertebrate counterparts, is now shown to be encoded by the antizyme genes of distantly related invertebrates. Identification of the 3' frameshifting stimulator in intermediate species or other invertebrates remains unresolved.

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Introduction

Ornithine decarboxylase (ODC) is a rate-limiting enzyme in the biosynthesis of polyamines. Ornithine decarboxylase antizyme (or simply antizyme) binds to, and inhibits, ODC and subsequently tags it for ubiquitin-independent proteolysis by the 26 S proteasome.^{1,2} In addition, antizyme can inhibit cellular uptake of polyamines.^{3,4} It is a critical regulator of intracellular polyamine levels. High intracellular levels of free polyamines induce synthesis of antizyme protein, thus closing an autoregulatory loop. Several studies have addressed the importance of antizyme,^{5–7} and an analysis of the phenotype of knock out mice is in progress.

All known antizyme genes have two partially overlapping open reading frames (ORFs). The longer, ORF2, which is known to encode all relevant biochemical activities of antizyme, is in the +1 reading frame relative to ORF1.^{8–10} Synthesis of full-length antizyme protein requires a +1 ribosomal frameshift at a specific sequence

at the end of ORF1.^{11–13} The frameshifting efficiency is responsive to the level of free intracellular polyamines, with more of the negative regulator, antizyme, synthesized at high levels of polyamine. Therefore, the antizyme frameshift site can be viewed as a biosensor for intracellular free polyamines.

The core sequence of the frameshift site is highly conserved from yeast to mammals, indicating the ancient nature of this regulatory event.¹⁴ From work done on mammalian and yeast antizyme genes, it is known that mRNA sequences flanking the core frameshift site can act as frameshift stimulators.^{10,11,15–18} Both 5' and 3' stimulators have been identified. These stimulators are often conserved within related antizyme genes but are often not conserved between distant relatives. There is strong evidence for multiple emergence/evolution of antizyme +1 frameshift stimulators. One of the known 3' stimulators is an RNA pseudoknot present in the orthologs of vertebrate antizymes 1 and 2 (though not antizyme 3).^{19,20} Although a number of RNA pseudoknot structures are known to stimulate –1 ribosomal frameshifting events, the pseudoknot in vertebrate antizyme 1 and 2 was the first and is still the only, such structure known to stimulate +1 frameshifting. Despite an intensive search, a similar RNA pseudoknot could not be identified as being encoded in any of

Abbreviations used: ODC, ornithine decarboxylase; ORF, open reading frame; IBV, infectious bronchitis virus.

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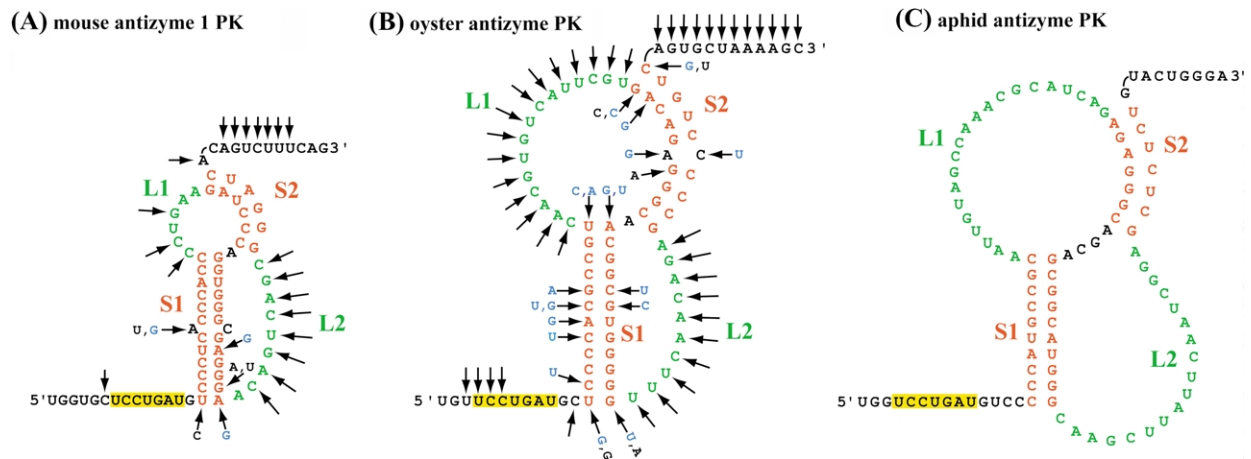


Figure 2. Two-dimensional structure of antizyme RNA pseudoknots involved in stimulation of +1 frameshifting. Black arrowheads represent substitutions deduced from phylogenetic comparison. Non-compensatory changes in the stems are shown in black letters; compensatory changes are shown in blue letters. The frameshift site is highlighted in yellow. (A) Structure of the mRNA pseudoknot present in orthologs of vertebrate antizyme 1; the actual sequence is that of *Mus musculus* antizyme 1. (B) Structure of newly discovered invertebrate antizyme pseudoknot using the *Crassostrea gigas* sequence as a template. (C) Structure of the putative pseudoknot in *Populus tremula* (the actual species is unknown but is most likely an aphid).

the invertebrate antizyme genes. Here, we report the discovery of a substantially different RNA pseudoknot in a subset of distantly related metazoan, but non-vertebrate, organisms. The newly discovered structure appears to have evolved independently, and is older than the previously known antizyme frameshifting pseudoknot stimulator.

Results

Alignments and phylogenetic analysis

Visual analysis of a recently deposited Expressed Sequence Tag (EST) sequence corresponding to the *Crassostrea gigas* (Pacific oyster) antizyme gene suggested the possibility for a stem-loop or pseudoknot structure 3' of the frameshift site. The nucleotide sequence of this gene was compared to all available antizyme sequences. After small adjustments in alignment, a total of seven antizyme genes coming from six different species showed significant nucleotide similarity in the same region (Figure 1). These seven antizyme sequences come from *C. gigas*, *Lumbricus rubellus* (an annelid roundworm with two paralogous antizyme genes), *Amblyomma variegatum* (an ixodid tick), *Argopecten irradians* (bay scallop), *Trichinella spiralis* (the nematode causative agent of trichinosis) and *Dugesia japonica* (a planaria). To assist with visualization of the phylogenetic data, the RNA sequence 3' of the frameshift site of oyster antizyme was drawn in the form of the putative pseudoknot with the position of alternative nucleotides in related pseudoknots indicated by an arrow, to generate a "phylogenetic probing" (i.e. in form similar to chemical probing). The results, shown

in Figure 2(B), demonstrate that 25 of 25 (100%) nucleotides in the putative pseudoknot "loop" regions are mutated in at least one of the six species examined. By contrast, only 17 of 44 (39%) nucleotides in "stem" regions differ. Moreover, in 13 of these 17 stem alterations the change is complementary (maintaining base-pairing). In three additional positions the change is at least sometimes complementary. Taken together, these data argue strongly in favor of functional significance of the proposed RNA pseudoknot.

To analyze the role of the newly identified pseudoknot in antizyme +1 frameshifting, the *C. gigas* sequence was subjected to site-directed mutagenesis analysis. Since *C. gigas* could not be utilized readily for molecular analysis, the experiment was performed in a human kidney cell line using transfections. The cassette was inserted between two different fused luciferase genes and the level of frameshifting was measured by the dual luciferase assay.²¹ The "wild-type" cassette in this analysis consists of the last six sense codons of ORF1 and 79 nucleotides downstream of the frameshift site. Since the +1 frameshifting produced by this construct is substantial, 13.5%, even without added polyamines, the working assumption in subsequent experiments is that the mammalian system recapitulates fairly closely the events occurring in translation of the oyster antizyme gene. The results from these experiments are shown in Figure 3. Significantly, complete deletion of the proposed pseudoknot region (construct 3'/del) leads to a fourfold reduction of frameshift efficiency, demonstrating the presence of a 3' RNA stimulator.

Consistent with the phylogenetic data, changing (inverting) the sequence of either loop 1 or loop 2 of the proposed RNA pseudoknot has no

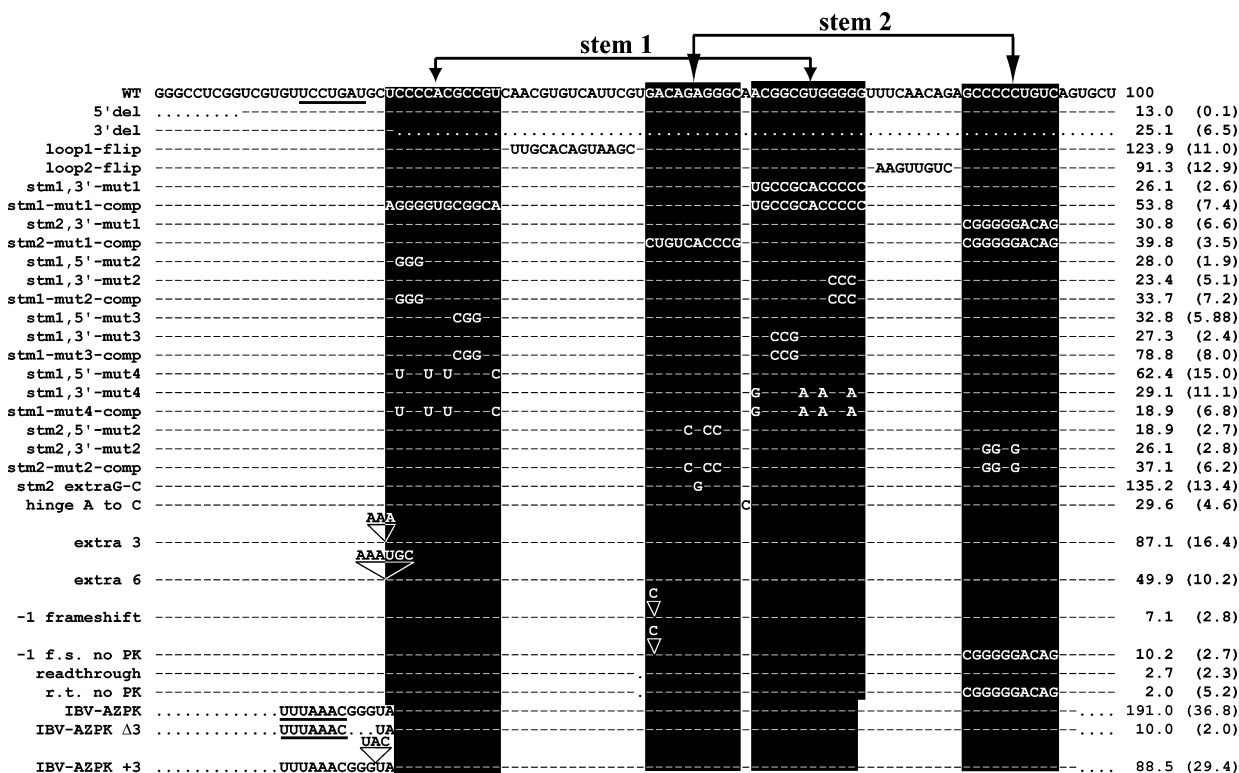


Figure 3. Results from site-directed mutagenesis of the *Crassostrea gigas* antizyme frameshift cassette. The sequence of the wild-type cassette is shown in the top line. Each construct's name is on the left side next to a line that displays the sequence alteration to which it corresponds. Unaltered nucleotides are represented by a dash. Deleted nucleotides are represented by ".". Regions that are involved in base-pairing are shown by shading. The percentage frameshifting relative to wild-type is shown on the right side of each line followed in parentheses by the standard deviation expressed as % relative to wild-type frameshifting. The numbers next to the last three lines are relative to IBV wild-type frameshifting. The absolute level of +1 frameshifting for wild-type is 13.52% for oyster antizyme and 4.0% for -1 IBV frameshifting (see Discussion). The frameshift sites of oyster antizyme and IBV are underlined.

discernable negative effect on the frameshifting efficiency (Figure 3 loop1-flip; loop2-flip). In the case of loop 1, it even leads to increased frameshifting (to 16.8%). By contrast, replacing the 3' region of either stem 1 or stem 2 of the pseudoknot with their complementary sequences leads to the same reduction of frameshifting efficiency as seen with the construct missing the entire sequence 3' of the frameshift site (Figure 3 stem1,3'-mut1; stem2,3'-mut1). Though not without precedent in other 3' frameshifting pseudoknots, making complementary changes to the original stem disruptions leads to only partial restoration of wild-type frameshifting (Figure 3 stem1,3'-comp; stem2,3'-comp). To further investigate this finding, additional stem-disrupting mutations were made, introducing smaller reciprocal changes, which were then tested separately. Alternatively, two reciprocal (complementary) changes together comprising four separate sets were made. In one case, construct stm1,5'-mut4, the changes introduced are naturally occurring variations in the 5' region of stem 1 inferred from the phylogenetic analysis, though they do not occur naturally in the combination used here. When all eight unilateral (non-complementary) mutations were tested for their effect on +1 frameshifting, the one with the naturally occur-

ring changes was the only one that caused a minimal reduction in frameshifting. This result can be explained by the fact that three of the four individual nucleotides in the construct do not disrupt base-pairing but change C-G base-pairs to U-G. The other seven unilateral constructs, stm1,5'-mut2, stm1,3'-mut2, stm1,5'-mut3, stm1,3'-mut3, stm1,3'-mut4, stm2,5'-mut2, and stm2,3'-mut2, support much reduced frameshifting comparable to a complete 3' deletion (3'del). Three of the four bilateral (complementary) constructs, stm1-mut2-comp, stm2-mut2-comp and stm1-mut4-comp,—the latter incorporates the naturally occurring changes in stem 1, show only minimal restoration relative to wild-type. The construct stm1-mut3-comp, in which three nucleotides near the top of stem 1 were swapped, shows a significant restoration of frameshifting activity, though the level achieved is less than that of wild-type.

Additional experiments were performed to dissect further aspects of the RNA pseudoknot structure in oyster antizyme mRNA. The A-C mismatch in stem 2 was changed to a G-C base-pair (construct stm2 extraG-C). This results in a frameshifting level, 18.3%, which is 1.3-fold higher than that of wild-type, 13.5%. Repositioning the pseudoknot relative to the frameshift site by inserting

three or six nucleotides in the spacer, constructs extra 3 and extra 6, has a modest effect, 87% and 50% of wild-type frameshifting, respectively. Replacing the hinge A between stem 1 and stem 2 with a C results in an almost threefold reduction of frameshifting.

To investigate the possibility of a 5' stimulator in oyster antizyme mRNA and, if present, the extent to which it affects frameshifting in decoding oyster antizyme mRNA, a construct containing only the last three codons of ORF1, 5' of the shift site, was made. The nine nucleotides omitted from this construct are conserved both among vertebrate and invertebrate antizyme genes. Deletion of the nine nucleotides (construct 5'del) results in an even greater reduction (nearly eightfold) of frameshift efficiency than achieved by deleting the entire 3' RNA pseudoknot-containing region, thus confirming the presence of a 5' stimulatory sequence.

In order to test the specificity of the oyster RNA pseudoknot, its sequence was replaced with that of the infectious bronchitis virus (IBV, a coronavirus) pseudoknot, which is known to stimulate -1 frameshifting by as much as 15-fold.²² The spacing between the end of the frameshift site and the beginning of the pseudoknot in this construct is four nucleotides compared to two nucleotides in the wild-type oyster antizyme gene. As shown above, increasing the spacing in the oyster cassette to five nucleotides has little effect on the level of shifting. Replacement of the oyster antizyme pseudoknot with the IBV pseudoknot leads to a level of frameshifting (3.8%, data not shown) that is essentially the same as having no 3' stimulator at all (3.4% compared to 13.5% for wild-type), i.e. the IBV pseudoknot does not stimulate $+1$ frameshifting in the oyster antizyme context. A reciprocal attempt was made to test if the oyster antizyme pseudoknot is capable of simulating -1 frameshifting on the IBV frameshift site. First, the minimum wild-type sequence of IBV necessary for efficient frameshifting was inserted into the dual luciferase vector. The spacing between the frameshift site and the pseudoknot in this construct is six nucleotides. Two companion constructs were made. One is an in-frame control and in the second, the U-UUA-AAC frameshift site is altered to U-UUA-UAC to simulate a negative control: -1 frameshifting with the wild-type IBV sequence was 4.0% (the same result was obtained with multiple constructs; data not shown), which is significantly less than previously reported by others in both reticulocyte lysates and in transfection experiments (see Discussion). Importantly, frameshifting was essentially abolished, down to 0.6% (which is the same as background in this vector), in the construct with altered frameshift site. A hybrid construct, IBV-AZPK, was made combining the IBV frameshift site fused to an appropriately spaced oyster antizyme pseudoknot (Figure 3). In this hybrid, the pseudoknot starts five nucleotides 3' of the shift site. This sequence supports a -1 frameshifting level of 7.5%, which is almost two-

fold higher than that with the wild-type IBV pseudoknot. The length of the spacer in the hybrid separating the frameshift site from the RNA pseudoknot was changed by either deleting (construct IBV-AZPK $\Delta 3$), or inserting (construct IBV-AZPK $+3$), three nucleotides, as was done in the original work on the IBV pseudoknot.²² When the distance was shortened to two nucleotides, frameshifting was effectively abolished (down to 0.4%). When the distance was increased by three nucleotides, frameshifting was reduced to 3.6%, which, however, is still comparable to the 4.0% frameshifting with the wild-type IBV sequence.

In another set of experiments, the frame specificity of the oyster antizyme mRNA frameshifter pseudoknot was assessed; it was tested for its possible ability to induce -1 frameshifting (and/or stop codon "readthrough"), in addition to $+1$ frameshifting. Prior *in vitro* experiments showed that under certain conditions, the mammalian antizyme 1 mRNA frameshift context could support significant levels ($\sim 10\%$) of readthrough (or $+3$ "frameshifting", the two being indistinguishable under the testing conditions).¹⁶ Two sets of constructs were made. In one set (see Figure 3) the pseudoknot is present (-1 frameshift and readthrough). In the other set it is disrupted (-1 frameshift no PK and readthrough no PK). No recoding difference was observed in either the 0 or -1 frames, for the constructs containing, or lacking, the 3' pseudoknot. The oyster antizyme frameshift cassette supports only 0.35% readthrough (essentially background levels) and 1.0% -1 frameshifting; more than background, but almost 14-fold less than $+1$ frameshifting.

While examining all available antizyme nucleotide sequences, we noticed that a sequence obtained from *Populus tremula* (European aspen) cDNA library exhibits some similarity to the newly discovered antizyme RNA pseudoknot. Plants are not known to have antizyme genes. On the basis of this and other information, we concluded that this clone is most likely a contaminant from the animal kingdom. While this manuscript was in preparation, the general identity of this animal was revealed when a nearly identical sequence was found in *Toxoptera citricida* (a citrus aphid). The sequence 3' of the frameshift site of this aphid antizyme can be folded into a putative RNA pseudoknot, the structure of which is shown in Figure 2(C). Stem 1 of this putative pseudoknot is nearly identical with stem 1 in the newly discovered RNA pseudoknot in oyster antizyme. The sequence of stem 2 is less similar. More significantly, other components of the pseudoknot are also different. In particular, there are four bases between what appears to be the boundaries between stem 1 and stem 2, three more than in any other antizyme pseudoknot previously identified. Loops 1 and 2 are also longer than usual. To test if the second stem of this putative aphid pseudoknot is functional, two constructs were made and tested as above. Both constructs have 5'

sequence, as far as the ORF1 stop codon, identical with that of the constructs described above. However, they differed in the sequence 3' of the frameshift site. One included the 86 nucleotide wild-type sequence 3' of the frameshift site of *P. tremula* antizyme mRNA. The second introduces an inversion in the 3' half of the putative stem 2, resulting in complete non-complementarity in the same region. Although frameshifting with the wild-type 3' sequence of *P. tremula* antizyme mRNA is significantly less than with the 3' sequence of *C. gigas* (4.3% versus 13.5%), introducing the mutation in the putative stem 2 of the aphid pseudoknot results in significant reduction in frameshifting (from 4.3% to 1.7% or 2.5 fold reduction, data not shown).

Discussion

The data show that a subset of invertebrates use a 3' pseudoknot to stimulate the frameshifting required for expression of their antizyme mRNAs. The features of the newly discovered invertebrate pseudoknots will first be contrasted to their vertebrate counterparts, and to the pseudoknots known to stimulate -1 frameshifting.

One of the striking, and perhaps counter-intuitive, features of the pseudoknots that promote the +1 frameshifting required for the synthesis of vertebrate antizymes 1 and 2, is their proximity (2–3 nt) 3' of the shift sites (pseudoknots that stimulate -1 frameshifting are commonly 4–9 nt 3' of the shift site). This feature is present in the invertebrate pseudoknots reported here. Expression of mammalian antizyme 1 mRNAs in reticulocyte lysates showed that extending the spacing between the shift site and the pseudoknot by 3 or 6 nt decreased the efficiency of frameshifting to 77% and 56% of wild-type, respectively.¹⁶ At least as judged by this *in vitro* experiment, where the pseudoknot effect is not as great as in cells, the pseudoknot does not have a direct effect in positioning the shift site within the ribosome, which has been suggested as one possible mechanism of pseudoknot stimulation of frameshifting. Consistent with this, moving the oyster pseudoknot 3 or 6 nt further 3' had modest and graduated effect on frameshift levels, 87% and 50% of wild-type, respectively, at least as assayed in transfected mammalian cells. This is remarkably similar to the results obtained with the mammalian antizyme 1 gene mentioned above, again negating a direct effect in positioning the shift site within the ribosome. Interestingly, when the same pseudoknot was tested for its ability to stimulate -1 frameshifting on an IBV shift sequence, reducing the spacing to 2 nt completely abolished frameshifting, very similar to the result with endogenous IBV pseudoknot.²² (Spacing effects have been described in other cases of frameshifting.^{22–26})

The new pseudoknots are also similar to their mammalian antizyme mRNA counterparts in that

the 5' end of stem 1 is C-rich. In contrast, the corresponding region of the known equivalent pseudoknots that function to stimulate -1 frameshifting, especially those in IBV and SRV-1 mRNAs^{26,27} and the most efficient retroviral shift sites, namely the *gag-pro* rather than *pro-pol* sites, tend to be G-rich. However, there was little effect of swapping the sides of stem 1 in IBV,²⁸ or in HTLV-2, provided the full stem was inverted²⁹ and only a 50% effect in the case of equine infectious anaemia virus, a lentivirus.³⁰ So, it is not obvious that this distinction is significant. Of importance, however, is their shared property of having a strongly paired bottom part of stem 1. Consistent with this, increasing the GC content of stem 1 of a pseudoknot, that did not evolve to support frameshifting and that does so only very weakly, made it a much better frameshifting stimulator.³¹ The relevance of strong pairing at the start of stem 1 for stimulation of frameshifting may be augmented by a loop 2 strand being in close vicinity and influencing strand unwinding.^{32,33} Earlier *in vitro* results with the rat antizyme 1 pseudoknot have shown that changes in any number of nucleotides in either stem do not significantly reduce frameshifting efficiency so long as compensatory changes are made on the opposite side.¹⁶ By contrast, none of the six sets of compensated stem alterations of the oyster pseudoknot resulted in wild-type levels of frameshifting. Only one, which swaps three G-C base-pairs near the top of stem 1, comes close, at 79% of wild-type. These results set apart the antizyme pseudoknots in vertebrate and invertebrates, and indicate that in the oyster class pseudoknots what is important for function is the base-pairing in stem 1 and 2, and at least some of the nucleotide identities; perhaps for some currently unknown intra- or intermolecular interaction. In either case, such a result suggests that the antizyme mRNA pseudoknots in vertebrates and invertebrates function somewhat differently in exerting their effects on the efficiency of ribosomal frameshifting.

Several different types of pseudoknots that stimulate -1 frameshifting are well known. One distinction between them is based on the length of stem 1. An important property of stem 1 of the IBV frameshifter pseudoknot is that it is at least 11 nt long.²⁶ Topology is likely to be the key characteristic. Shorter stems 1, predicted to have equal or higher, thermodynamic stability are unable to stimulate frameshifting. In addition, while the ribosome pausing caused by natural frameshift stimulatory pseudoknots may well be important for switching to an alternative frame, certain other pseudoknots or stem-loops that cause an equal pause, do not stimulate frameshifting.³⁴ Stem 1 of the oyster and related invertebrate antizyme pseudoknots, has 11 or 12 uninterrupted Watson-Crick pairs, whereas stem 1 of vertebrate antizyme 1 and 2 pseudoknots has at least one apparent A.C mismatch in its center. A stem 1 of 11 or 12 bases is equal to or more than, the minimal of 11 for one turn of an A-form helix. This feature is not

sufficient by itself to account for the +1 frameshifting properties of the oyster pseudoknot, as the coronaviral IBV pseudoknot, which also has 11 bp in stem 1 is unable to stimulate +1 ribosomal frameshifting in the oyster antizyme mRNA context. However, the oyster antizyme pseudoknot is able to stimulate -1 frameshifting on the IBV slippery sequence perhaps even better than the IBV pseudoknot itself (at least with the cell type and vector used in our analysis). The reason for the anomalously lower level of IBV frameshifting in the present study is being investigated. This result suggests the possibility that the oyster antizyme +1 pseudoknot has all the features necessary for stimulation of -1 frameshifting, although those features on their own are not sufficient for stimulation of +1 shifting, since the IBV pseudoknot obviously lacks them.

One feature common to all frameshift-stimulatory pseudoknots whose detailed structure is known,^{35,36} and especially in the luteoviral pseudoknots, is the presence of extensive tertiary interactions involving nucleotides from loops 1 and 2.³⁷ Some of these interactions are undoubtedly required for recoding but others may be related to the fact that compact structures with extensive tertiary interactions are easier to crystallize and thus might be over-represented among the solved pseudoknot structures. Loop 1 of the oyster and related pseudoknots is much larger (15–22 nt) than its vertebrate counterpart, which in turn is larger than that of several -1 frameshifter pseudoknots e.g. IBV, Mouse Mammary Tumor Virus (MMTV) *gag-pro* and Simian Retrovirus-1, but not that of others e.g. Rous sarcoma virus (RSV) *gag-pol*.³⁸ The oyster loop 1 may have internal structure but if so, its form is not important for frameshift stimulation, since inverting its sequence leads to no reduction in frameshifting efficiency. The same appears to be the case with loop 2 of the pseudoknot.

A class of pseudoknots, typified by the MMTV *gag-pro* pseudoknot, has a stem 1 of around 5 bp but a wedge base, generally A, between the stems and a characteristic bent shape rather than co-axial stacking of the stems. Removal of the wedge base substantially reduces frameshifting^{39–41} and the change is also destabilizing.^{31,33,42} This class of frameshifter pseudoknots, and constructed mimics, has short loops that are important for its bent conformation.⁴³ In contrast, there is no wedge base between the stems of the IBV pseudoknot that features the long stem 1. However, the oyster and other related pseudoknots described here do have a potential wedged nucleotide between stems 1 and 2. In all species where we have identified this pseudoknot, the identity of this wedge nucleotide is adenosine. Changing it to cytosine leads to severe reduction in frameshifting. This is in contrast to the antizyme pseudoknots in vertebrates. Though these pseudoknots almost always have a potential wedged A at the corresponding position, the A can, nevertheless, be changed to any other

nucleotide, including C, without significantly reducing effectiveness for frameshifting *in vitro*.¹⁶ The significance of the distinction is not clear but it suggests that the seeming homology of the potential wedged A bases might be misleading, and the two classes of antizyme pseudoknots may behave differently.

As described above, while examining all known antizyme coding sequences for any homology to the pseudoknots of the oyster class, one additional potential structure with striking resemblance was identified in two clones belonging to a cDNA library of *P. tremula*. Both likely belong to a contaminant closely related to the aphid *T. citricida*. The sequence 3' of this aphid antizyme frameshift site can be folded in a two-dimensional pseudoknot structure shown in Figure 2(C). Stem 1 is nearly identical with the antizyme pseudoknots of the oyster class but stem 2 is highly divergent. Perhaps more importantly, there appear to be more nucleotides between stems 1 and 2. Our preliminary experiments suggest that this RNA pseudoknot stimulates antizyme frameshifting. However, it may belong to a separate subclass.

Interestingly, the six species in which the newly discovered oyster class pseudoknot occurs are not expected to be closely related. The evidence supporting the great evolutionary distance among the antizyme genes with the newly discovered pseudoknot includes the distant relationship of their antizyme protein sequence as well as the accumulation of large number of nucleotide variations in the loop regions of the antizyme mRNA pseudoknot. According to the current "tree of life",⁴⁴ the last common ancestor of the six organisms that have an antizyme pseudoknot homologous to that in oyster lived very early in the Bilateria lineage (possibly in the last common ancestor of extant Bilateria), before the radiation that led to Acoelomorphs (planaria, *Dugesia japonica*), Ecdyzoa (nematodes, *Trichinella spiralis*; arthropods, *Amblyomma variegatum*) and Lophotrochozoa (annelids, *Lumbricus rubellus*; bivalves, *C. gigas* and *Argopecten irradians*). Barring horizontal gene transfer (and we can find no evidence in this case), these observations argue in favor of a very ancient origin of the newly identified antizyme mRNA pseudoknot, more than 550 million years ago. In contrast, the previously known antizyme pseudoknot is present only in vertebrates (but not in lower chordates) and is thus no older than 510 million years and perhaps as recent as 410 million years. If, as currently believed, Acoelomorphs are the most divergent branch of Bilateria, the common ancestor of many other higher eukaryotes, e.g. nematodes, insects and vertebrates, contained this type of antizyme pseudoknot stimulator. However, many of the branches have since lost it. What was happening as this was occurring is obscure, but identification of the putative stimulators in descendent species will likely be informative. At least for vertebrates, the identity of its (ultimate)

replacement is known to be a different type of pseudoknot.^{11,14}

An unusual evolutionary feature is seen in *T. spiralis*. The 5' half of stem 1 of the pseudoknot in its antizyme mRNA is present in a different reading frame compared to that of the other six sequences. This would require simultaneous or nearly simultaneous, deletion of one nucleotide immediately preceding it and insertion of one nucleotide in loop 1 following it. Another distinctive feature present in one of the seven newly identified genes is the AUU UGA (U) shift site in the planarian instead of the more common UCC UGA (U). An investigation of this will be reported separately.

Perhaps the most enigmatic *cis*-acting stimulator of +1 frameshifting in antizyme genes is the so called 5' stimulator.^{11,16} Current evidence suggests that there is more than one 5' stimulatory element in at least some of the antizyme mRNAs. An argument has been made that the 5' stimulator in vertebrates is modular and comprised of at least three distinct elements with different evolutionary history.¹⁴ On the basis of sequence comparison, the antizyme mRNAs containing the newly discovered oyster class pseudoknots have the two modules proximal to the frameshift site. The sequence of the third more distant module is less conserved among them. Deletion of just 9 nt of what should be the middle 5' stimulatory module of oyster antizyme mRNA leads to nearly eightfold reduction of frameshifting in mammalian cells. This is a significantly greater reduction than when the same region is deleted from mammalian antizyme mRNA and assayed for frameshifting *in vitro* in reticulocyte lysates.¹⁶ Perhaps this particular segment of the 5' stimulator is more important for the frameshifting of the oyster antizyme, at least when assayed in mammalian cells. In any case, this experiment clearly demonstrates that a 5' frameshift stimulator is indeed present in oyster antizyme. The 5' stimulator of mammalian antizyme 1, at least when tested in a heterologous system, *Schizosaccharomyces pombe*, appears to work through its primary nucleotide sequence rather than through the sequence of the nascent peptide encoded by it.⁴⁵ However, how it actually exerts its potential is less clear. Mostly likely, it somehow interacts with a component, perhaps rRNA, of translating ribosomes.

Materials and Methods

Bioinformatics

The following EST entries were used to deduce the composite sequence of each of the antizyme pseudoknot sequences presented in this study: for *C. gigas*, BQ426812; for *T. spiralis*, BG232823; for *A. variegatum*, BM293331, BM290266, BM290106, BM289905, and BM289964; for the first antizyme in *L. rubellus*, BF422516 and CA036221, and for the second, CA036210 and CA036028; for *A. irradians*, CF197657; for *Populus tremula*,

BQ426812 and BU826038. In the case of *C. gigas* and *P. tremula* the clones corresponding to BQ426812, BQ426812 and BU826038 were obtained and resequenced for validation.

Constructs and site-directed mutagenesis

The vector, p2Luc, used in the present study has been described.^{18,21} Briefly, the assayed sequences are inserted between the Sall and BamHI sites of p2Luc. An extra A nucleotide is added after Sall to correct the reading frame. Mutations were made by introducing the desired changes into PCR primers, except for the two long deletions, which were made by directly ligating DNA oligonucleotides between the cloning sites. In-frame constructs were made by deleting the U of the UGA stop codon of ORF1. The sequence of each variant of oyster antizyme frameshift cassette presented in this study is shown in Figure 3. The IBV pseudoknot-containing construct was made as follows. The first 24 nucleotides of the wild-type oyster cassette shown in Figure 3 (from the 5' end through three nucleotides downstream of the ORF1 stop codon) were placed 5' of the IBV pseudoknot sequence listed below:

```
ACGGGGTAGCAGTGAGGCTCGGCTGATACCCCTT
GCTAGTGGATGTGATCCTGATGTTGTAAAGCGAG
CC
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The wild-type IBV frameshift construct was made by inserting the following sequence between the two cloning sites:

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TTTAAACGGGTACGGGGTAGCAGTGAGGCTCGG
CTGATACCCCTTGCTAGTGGATGTGATCCTGATGTT
GTAAAGCGAGCCTT
```

For the in-frame control an extra A was inserted between the Sall site and the sequence listed above.

In the construct containing the aphid (*P. tremula*) pseudoknot, the ORF1 sequence of *C. gigas* antizyme was fused to the sequence of *P. tremula* starting with the first nucleotide 3' of the stop codon of ORF1 through three nucleotides past the end of stem 2 of the pseudoknot shown in Figure 2(C).

Cell culture and transfections

The human embryonic kidney cell line HEK 293 was obtained from ATCC and maintained as described¹⁸ in the absence of antibiotics. Cells used in these studies were subcultured at 70% confluence and used between passages 7 and 15. Cells were transfected using Lipofectamine 2000 reagent (Invitrogen), using the one-day protocol in which suspension cells are added directly to the DNA complexes in 96-well plates. DNA (25 ng) and 0.2 μ l of Lipofectamine 2000/well in 25 μ l of Opti-Mem (Gibco) were incubated and plated in opaque 96-well half-area plates (Costar). Cells were trypsinized, washed and added at a concentration of 4×10^4 cells/well in 50 μ l of Dulbecco's modified Eagle's medium (DMEM), 10% (v/v) fetal bovine serum (FBS). Transfected cells were incubated overnight at 37° in 5% (v/v) CO₂, then 75 μ l of DMEM, 10% FBS was added gently to the existing medium and the plate incubated for an additional 48 hours.

Dual luciferase assays of frameshifting and stop codon readthrough

Firefly and renilla luciferase activities were determined using the Dual Luciferase Reporter Assay System

(Promega). Relative light units were measured on an MLX microplate luminometer (Dynex). Transfected cells were lysed in 12.5 μ l of lysis buffer and light emission was measured following injection of 50 μ l of luminescence buffer. Frameshifting (and readthrough) efficiency was calculated as described,¹⁸ by comparing firefly to renilla luciferase ratios of experimental constructs to those of control (in-frame) constructs. Two control constructs were made by deleting the U from the UGA stop codon of ORF1, one corresponding to the wild-type oyster sequence (i.e. WT-construct in Figure 3) the other to the oyster 3' deletion construct (i.e. construct del3' in Figure 3). Since both produced identical ratios of firefly to renilla luciferase activities only one, that corresponding to WT, was used for all data presented here.

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