Assaying RNA chaperone activity *in vivo* using a novel RNA folding trap

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**In the absence of proteins, RNAs often misfold *in vitro* due to alternative base pairings which result from the molecule being trapped in inactive conformations. We identify an *in vivo* folding trap in the T4 phage *td* gene, caused by nine base pairs between a sequence element in the upstream exon of the *td* gene and another at the 3' end of the intron. During translation, the ribosome resolves this interaction; consequently the intron folds correctly and splicing occurs. The introduction of a stop codon upstream of this base pairing prevents resolution of the inactive structure so that splicing cannot proceed. We have used this folding trap to probe for RNA binding proteins which, when overexpressed, either resolve the misfolded structure or impede its formation *in vivo*. We distinguish between proteins which recognize the intron structure and those which bind non-specifically and apparently ignore the intron. The first class, e.g. *Neurospora crassa* CYT-18, can rescue the exonic trap and intron mutants which cause a structural defect. However, known RNA chaperones such as *Escherichia coli* StpA and S12 and the HIV protein NCp7, only resolve the exonic trap without suppressing intron mutations. Thus, this structural trap enables detection of RNA chaperone activity *in vivo*.
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Introduction

RNAs have two fundamental folding problems. The first arises from the tendency to form alternative base pairings which then kinetically trap the molecule in inactive conformations. The second problem is due to the difficulty of some RNAs to specify a single tertiary structure which is thermodynamically strongly favoured over other possible three-dimensional (3D) structures. These problems can be solved with the aid of RNA binding proteins. Two classes of such proteins, based on their binding specificities, have been identified.

RNA chaperones represent the first class of these RNA binding proteins. According to Dan Herschlag, they are defined as proteins that aid in the process of RNA folding by preventing misfolding or by resolving misfolded structures (Herschlag, 1995). They are thought to possess non-specific RNA binding activity; thus, they recognize

neither a distinct sequence nor a specific structure (Munroe and Dong, 1992; Portman and Dreyfuss, 1994; Darlix *et al.*, 1995; Weeks, 1997; Rein *et al.*, 1998).

The other group of RNA binding proteins is thought to solve the problem of the thermodynamically unstable 3D structure by specifically recognizing the correct tertiary structure and subsequently stabilizing it. In the case of the 3D folding of group I introns, two proteins, CYT-18 and CBP-2, have been shown to act in this manner (Garriga and Lambowitz, 1986; Gampel and Cech, 1991; Guo and Lambowitz, 1992; Weeks and Cech, 1995; Caprara *et al.*, 1996b).

The best method of distinguishing between non-specific RNA binding chaperones and specific RNA binding proteins is that once the correct RNA fold has been achieved, the RNA chaperone may be digested by protease treatment without affecting the correct RNA structure. In contrast, proteolysis of a protein stabilizing an RNA structure eliminates the positive effect on the correct 3D fold (Zhang *et al.*, 1995).

At present, RNA chaperone activity *in vitro* can be monitored in several ways. Assays include the measurement of RNA annealing activity, of strand exchange activity and of ribozyme turnover stimulation (Herschlag *et al.*, 1994; Zhang *et al.*, 1995). These assays have revealed RNA chaperone activity *in vitro* to be present in several proteins, for example: (i) a peptide derived from the nucleocapsid protein of HIV (NCp7) and the hnRNP A1 protein catalyse the annealing of a short RNA substrate to the hammerhead ribozyme, and they accelerate turnover by stimulating product dissociation from the ribozyme (Tsuchihashi *et al.*, 1993; Herschlag *et al.*, 1994); (ii) the *Escherichia coli* protein StpA was isolated as multicopy repressor of a splicing defective T4 *td* intron mutant (Zhang *et al.*, 1995). Subsequently, Zhang *et al.* (1996) could also show that besides its activity as a transcriptional repressor, the StpA protein can also act as an RNA chaperone *in vitro*; (iii) several ribosomal proteins, most prominently S12, facilitate *in vitro* and *in vivo* splicing of the T4 phage *td* intron and enhance the activity of the hammerhead ribozyme (Coetzee *et al.*, 1994; Semrad and Schroeder, 1998). Furthermore, (iv) cold-shock proteins from *Bacillus subtilis* have been suggested to exert RNA chaperone activity by facilitating the initiation of translation (Graumann *et al.*, 1997; Jiang *et al.*, 1997); and (v) a specific RNA–RNA annealing activity was purified from yeast and its gene (*YRA1*) isolated; the *YRA1* gene product is postulated to play a role in pre-mRNA metabolism (Portman *et al.*, 1997). More recently, RNA chaperone activity has been reported for the hepatitis Delta antigen peptides (Huang and Wu, 1998) and for the yeast La autoantigen (Pannone *et al.*, 1998).

At present, RNA chaperone activity can only be demonstrated with the above-mentioned *in vitro* assays; to

investigate the *in vivo* roles of a putative RNA chaperone, an *in vivo* assay is required.

Here we identify a specific 9 bp interaction between the upstream exon of the T4 *td* gene and the 3' terminal intron, representing an RNA folding trap in the absence of translation. We use the resolution of this folding trap as an *in vivo* assay to detect RNA chaperone activity. Using this method we are able to determine whether a protein, which has been shown to exert RNA chaperone activity *in vitro*, can resolve this folding trap or can impede its formation *in vivo*. Furthermore, we take advantage of the fact that intronic mutations are available which disrupt the 3D structure of the intron, and whose phenotype can be suppressed by specific RNA binding proteins (such as CYT 18) that stabilize and restore the correct 3D structure. We define an RNA chaperone activity as one which resolves the exonic trap, but without significantly suppressing the *cis*-acting intronic mutations.

Results

An exon–intron interaction traps the pre-mRNA in a splicing incompetent conformation

We observed previously that the presence of stop codons in the upstream exon inhibits splicing of the T4 phage *td* intron *in vivo* (Semrad and Schroeder, 1998). The inability of the stop codon mutants to splice could, however, be suppressed by point mutations in the region –80 to –60 upstream from the 5' splice site, suggesting that this sequence interfered with folding of the intron. Computer analysis (Zuker, 1989) identified a potential 9 bp interaction between the –81/–73 region of the upstream exon and the 3' terminal sequences of the intron (Figure 1). To prove the existence of this interaction, we first introduced, in addition to a stop codon at position –82 (mutant *td* KS-82), a point mutation in the exon (mutant *td* KS-82/A-78C), which disrupts this pairing and finally a third, compensatory mutation in the intron, which restores the pairing (mutant *td* KS-82/A-78C/U1012G; Figure 2A).

The optimal compensatory mutation was obtained by testing in the 3' end of the intron several single point mutations, which disrupt the proposed exon–intron pairing. Interestingly, these mutations (G1007A, A1009U, A1013G) resulted *per se* in splicing deficiency *in vivo*, suggesting that this intron sequence is involved in 3D interactions, as proposed by Jaeger *et al.* (1993; data not shown). These positions could therefore not be used to test the proposed exon–intron interaction. Only the single point mutation U1012G showed just a 2-fold reduction in splicing activity *in vivo* compared with the wild type, and was thus deemed suitable for the demonstration of the exon–intron interaction (Figure 2B and C).

The introduction of a stop codon at position –82 upstream from the 5' splice site results in very low splicing activity (~1%). Additional introduction of a point mutation at position –78, which destabilizes the proposed interaction, restored splicing to about half of the wild-type activity. However, on introduction of a third compensatory mutation which restores the proposed interaction, namely the U1012G mutation described above, splicing returns to the level of the initial single mutation, the stop codon mutant. This clearly demonstrates the existence of the

Fig. 1. An exon–intron interaction, which traps the pre-mRNA in a splicing incompetent conformation. The secondary structure of the *td* intron with stems P1–P9 is shown (Michel and Westhof, 1990; Cech *et al.*, 1994). The 3' end of the intron is shown interacting with the exon sequence, forming the folding trap. Exon sequences are in black, intron sequences in magenta. 5'SS and 3'SS represent 5' and 3' splice sites, respectively. Boxed nucleotides in green indicate mutations C870U and C873U.

suggested exon–intron interaction, which traps the pre-mRNA in a splicing incompetent conformation.

The RNA chaperone *StpA* rescues the effect of the stop codon in the upstream exon, but not that of intronic mutations

The exon–intron interaction, which forms in the absence of translation, represents an ideal system to detect RNA chaperone activity *in vivo* since correct pre-mRNA folding correlates directly with a high splicing efficiency, an easily measured parameter. To test our system we examined the *in vivo* RNA chaperone activity of the *StpA* protein which has previously been shown to exert RNA chaperone activity *in vitro* (Zhang *et al.*, 1995). We used the stop codon mutant at position –82 and two splicing deficient intron mutations, C870U and C873U, shown in green in Figure 1 (Belfort *et al.*, 1987; Schroeder *et al.*, 1991). All these mutants are severely defective in splicing (~1%). Their splicing activities in the absence and presence of overexpressed *StpA* are shown in Figure 3A and C. As can be seen, *StpA* very efficiently rescues splicing activity of the exonic stop codon mutant, but only very slightly that of the intron mutants. This suggests that *StpA* acts preferentially on the exonic trap, but does not recognize nor stabilize the intron structure. Therefore, according to our definition, *StpA* exerts RNA chaperone activity *in vivo*.

Fig. 2. Identification of the RNA folding trap. (A) Mutations disrupting and restoring the exon-intron interaction. Exon sequences are in upper case, intron sequences in lower case. Mutated bases are in bold face and boxed. A negative number indicates the distance to the 5' splice site. Positive numbers indicate positions in the intron. (B) Splicing activity of the mutants assayed by primer extension. Reverse transcription of mRNA with the NBS2 primer complementary to exon 2 sequences results in a primer extended by 16 nucleotides in the presence of excess ddTTP. Reverse transcription of cryptic and pre-mRNAs results in primer + 8 and primer + 5 products, respectively. (C) Splicing activities were quantified using a phosphoimager (see Materials and methods).

Surprisingly however, StpA was originally isolated as an *in vivo* suppressor of the C873U mutation. Nevertheless the suppression was very weak and was based on the total amount of thymidylate synthase produced from this rescued mutant, which was very low but probably just sufficed to ensure growth. Furthermore, overexpression of StpA also results in slightly increased concentrations of the *td* RNA in the cell (Zhang *et al.*, 1995; Cusick and Belfort, 1998).

The CYT-18 protein rescues the effect of intronic mutations as well as that of the stop codon in the upstream exon

Two proteins, CBP2 and CYT-18, have been shown to interact specifically with group I intron RNA (Garriga and Lambowitz, 1986; Gampel and Cech, 1991; Weeks and Cech, 1995). CYT-18 rescues many structurally defective mutants in the T4 *td* intron *in vivo* (Mohr *et al.*, 1992) and suppresses the effect of stop codon mutants in the upstream exon of the *td* gene (Semrad and Schroeder,

Fig. 3. Effects of proteins (A) StpA and (B) CYT-18 on exonic and intronic mutants of the *td* gene. RNA species were identified as in Figure 2. (C) Comparison of the effects of StpA and CYT-18 by quantification using a phosphoimager. Activities on the bar chart are average values of three to seven independent experiments.

1998). Here we compared the activity of the CYT-18 and the StpA proteins, upon overexpression in *E. coli* in rescuing exonic and intronic mutations of *td*. The constructs were those used to test StpA. Figure 3B shows that CYT-18 rescued all mutants, whether exonic or intronic. The C873U intron mutant was rescued less efficiently, but nevertheless still significantly. Figure 3C compares quantitatively the effects of StpA and CYT-18. While StpA only rescued the exonic mutant, CYT-18 significantly rescued both exonic and intronic mutants.

Cryptic splicing is much more pronounced in rescued mutants than in the wild type

Correct splicing occurs when the 5' splice site is embedded in a stem termed P1, which docks into the catalytic core of the ribozyme (Figure 4A). An alternative folding occurs when another sequence base pairs with the internal guide sequence of the intron, resulting in a cryptic splice product (Chandry and Belfort, 1987; Figure 4B). Interestingly, the cryptic splice product is more prominent in the mutants that are rescued by the proteins than in the wild type (Figures 3 and 4C). Usually, the ratio of mRNA to cryptic RNA in the wild type construct is ~10. When the stop

Fig. 4. Secondary structure of the *td* intron with (A) correct P1 pairing with exon sequences in blue and (B) cryptic P1 pairing with exon sequences in magenta. Intron sequences are black. 3D interactions between P1 (cryptic P1) and the intron core are shown in green. 5'SS and 3'SS represent 5' and 3' splice sites, respectively; crypt.5' SS stands for the cryptic splice site. (C) Bar chart showing amounts of mRNA (blue) and cryptic RNA (magenta) in the wild type (left), in the stop codon mutant KS-82 rescued by StpA (middle) and by CYT-18 (right).

Fig. 5. Ability of ribosomal protein S12 to act as an RNA chaperone *in vivo*. (A) Effect of S12 on exonic and intronic mutants of the *td* gene. RNA species were identified as in Figure 2. (B) Bar chart showing phosphoimager quantification of the results obtained from (A). Results are an average of three independent experiments.

codon mutants are rescued by StpA or CYT-18, this aberrant mRNA species increases and the ratio of mRNA to cryptic mRNA is reduced to 2–4 (Figure 4C). This clearly indicates that these proteins can neither resolve nor impede the formation of the cryptic P1 stem (Figure 4B). It is known that CYT-18 binds the group I intron core from the side opposite to the P1 stem (Caprara *et al.*, 1996a). Therefore it is not surprising that it cannot distinguish between cryptic and correct P1 stems.

The ribosomal protein S12 is a weak RNA chaperone *in vivo*

In a search for proteins able to stimulate *cis*- and *trans*-splicing of the *td* intron *in vitro*, the ribosomal protein S12, among others, was purified (Coetzee *et al.*, 1994). In addition to stimulating splicing, the protein S12 also enhanced a hammerhead ribozyme reaction. These *in vitro* results suggested that S12 can act as an RNA chaperone. Therefore we assayed overexpression of this protein in our *in vivo* system. Figure 5A shows that the KS-82 stop codon mutant is only rescued 2-fold and the intronic mutants splice to the same extent as when co-expressed with StpA (Figures 4A and 5B). From these results we

Fig. 6. RNA chaperone activity of viral single-strand RNA binding proteins. (A) Polyacrylamide gels of the primer extension assays. Labelling as in Figure 2. (B) Relative splicing activities (wild-type splicing in the absence of co-expressed protein = 1) of the *td* gene in the absence (blue) and in the presence of influenza nucleoprotein NP (cyan) or of HIV nucleocapsid protein derived NCp7 (magenta).

conclude that in our system S12 exerts, if at all, only a very weak RNA chaperone activity *in vivo* showing that the *in vitro* activity does not necessarily correlate with that *in vivo*. Our assay might be suitable to detect only a subset of potential RNA chaperones.

Assaying RNA chaperone activity of viral single-strand RNA binding proteins

To provide a first mechanistic framework for RNA chaperone activity, we tested two single-strand RNA binding proteins: (i) the NCp7 domain of the nucleocapsid protein of HIV for which RNA chaperone activity has been demonstrated *in vitro* (Tsuchihashi *et al.*, 1993) and (ii) the influenza A virus nucleoprotein NP which is a single-strand RNA binding protein which has not been shown to exert RNA chaperone activity *in vitro* (Galarza *et al.*, 1992). We co-expressed both viral ssRNA binding proteins with the wild-type *td* gene, with the KS-82 stop codon mutant and with the intronic *td* C870U mutant (Figure 6). Splicing of the wild type is significantly increased when NCp7 is co-expressed (2.7-fold) but not in the presence of NP. The stop codon mutant KS-82 is also significantly rescued in the presence of NCp7, but again not in the presence of NP. Neither protein influenced splicing of intronic *td* C870U mutant. According to our definition, NCp7 has RNA chaperone activity *in vivo*. In contrast, the influenza A virus nucleoprotein NP is not able to increase splicing either of the exonic or of the intronic mutants nor of the wild type. From these results, we

conclude that non-specific single-strand RNA binding activity *per se* is not sufficient for a protein to exert RNA chaperone activity *in vivo*.

Discussion

We observed previously that the presence of stop codons in the upstream exon of the *td* gene was detrimental to splicing. The inability of the pre-mRNA to splice in the absence of translation was due to an alternative exon–intron interaction which led to incorrect pre-mRNA folding (Semrad and Schroeder, 1998). In this report, we prove that this proposed exon–intron interaction exists and that in the absence of the ribosome, it traps the pre-mRNA in a splicing incompetent conformation.

The crucial property of the folding trap identified in this work is that it leads to splicing deficiency. Thus, the resolution of the trap can be efficiently measured by monitoring *in vivo* splicing instead of probing for RNA structures. In addition to the kinetically trapped folding intermediates, intronic mutations which disturb the thermodynamic stability of the 3D structure represent a second type of splicing deficient mutants. The two types of mutant add strength to our assay as they allow us to distinguish between the non-specific RNA binding activity of RNA chaperones (like StpA) and proteins such as CYT-18, which specifically recognize and stabilize the 3D structure of the intron. This is the first demonstration that a protein (StpA) that exerts RNA chaperone activity *in vitro* acts similarly *in vivo*.

The molecular mechanism underlying RNA chaperone activity is unknown. It is currently thought that proteins with RNA chaperone activity lower the energy barrier for the breakage and reformation of base pairs, thus avoiding non-native conformations, which represent kinetic traps. Elucidation of this mechanism will include sequence analyses, investigation of structural requirements, as well as determination of stoichiometry and turnover by the RNA chaperone.

Which structures do RNA chaperones resolve? StpA resolves the exon–intron trap efficiently but resolves neither the cryptic P1 stem nor the correct P1 stem. The exon–intron interaction is a long range pairing of 9 bp and P1 contains only 7 bp, suggesting that this parameter of the pairing is not the only critical determinant. In contrast, a major difference between the exon–intron trap and the P1 stem is that the latter is stabilized by 3D interactions with the intron core (red lines in Figure 4A and B), such that the P1 stem docks into the catalytic core of the ribozyme via hydrogen bonds between P1 and bases in J8/7 and loop 4/5 (Pyle *et al.*, 1992; Strobel and Cech, 1993, 1995; Strobel *et al.*, 1998). However these additional 3D contacts only increase the stability of P1 by the equivalent of three or four hydrogen bonds, which correspond to ~2 bp (Pyle *et al.*, 1992; I.Hoch and R.Schroeder, unpublished). Thus, thermodynamic stability of a secondary structure element cannot be the discriminative determinant for the chaperone. Since RNA chaperones do not hydrolyse ATP, it is not unexpected that thermodynamic parameters do not regulate the specificity of the proteins. The unwinding of RNA duplexes coupled with ATP hydrolysis is performed by RNA helicases (Schmid and Linder, 1992; Bird *et al.*, 1998; Staley and Guthrie, 1998).

We assume that 3D interactions, which condense the RNA in more compact folds, possibly indicate a final conformation to the RNA chaperone. Another possibility is that a compact 3D structure does not leave unfolded single-stranded regions, which might be required to initiate unfolding. Helicases, for instance, require 3' or 5' flanking single-stranded regions for activity (Lohman and Bjornson, 1996). RNA chaperones might, in analogy to helicases, require single-stranded regions to bind, and there are no such accessible single-stranded segments in the correct 3D structure of the group I intron.

To test whether a non-specific single-strand RNA binding activity is sufficient to resolve or impede formation of the exon-intron trap, we tested two viral ssRNA binding proteins. For the NC protein of HIV several chaperone-like activities have been demonstrated including the formation of the genomic RNA dimer and annealing of the tRNA on the primer binding site (Feng *et al.*, 1999). In contrast, influenza A virus NP has not been reported to exert RNA chaperone activity. NP interacts with the homopolymers poly(A), poly(G), poly(U) and poly(C) (Albo *et al.*, 1995). In our folding trap assay only NCp7 showed activity; NP had no detectable effect on the splicing activity. Additional single-strand RNA binding proteins will have to be assayed before we can definitely exclude that single-strand RNA binding activity is sufficient to resolve the folding trap. We propose that RNA chaperone activity is not equivalent to single-strand RNA binding activity.

The folding of large RNAs is often a multi-step process which includes intermediate organizational levels of the RNA chain, creating a hierarchical folding pathway (Brion and Westhof, 1997). Such a folding pathway might contain transient interactions which are essential for regulation and catalysis of the correct RNA fold (Powers *et al.*, 1993). A major role for RNA chaperones might be to facilitate formation and resolution of intermediate conformations on the way to the correct fold.

Materials and methods

Escherichia coli strains and growth media

Escherichia coli strains used in this study were derivatives of thymine⁻ C600 strain [F⁻, *supE44*, *thi-1*, *thr-1*, *leuB6*, *lacY1*, *tonA21*, *thy*⁻]. Complete medium TB-YE (+thymine) has been described earlier (Belfort *et al.*, 1983; Salvo *et al.*, 1990). Bacto agar (15%) was added for solid media. Antibiotics were used at the following concentrations: ampicillin (100 mg/l), chloramphenicol (25 mg/l), kanamycin (50 mg/l). Thymine was added to 50 mg/l.

Plasmids

All *td* constructs are in the context of the vector pTZ18U-*td* Δ P6-2 (Salvo *et al.*, 1990). The stop codon mutant in exon 1 (*tdKS-82*), the intron mutant *tdU1012G*, the exon mutant *tdKS-82/A-78C* and the compensatory mutant *tdKS-82/A-78C/U1012G* were generated by *in vitro* mutagenesis (Kunkel *et al.*, 1987) using the following mutagenic oligonucleotides (the mutagenic residue is underlined):

KS-82: 5'-ATAGAACATATGTCAAGGCGGTAAT-3'
U1012G: 5'-AACGGTAGCATTCTGTTTCAGATAAG-3'
KS-82/A-78C: 5'-CTGATAGAACAGATGTCAAGGCGGTAA-3'.

For the intronic mutants, the mutant *tdC870U* was described previously (Schroeder *et al.*, 1991) and the mutant *tdC873U* is equivalent to the SC34 mutant described in Belfort *et al.* (1987) but in the context of the pTZ18-*td* Δ P6-2 plasmid. Vector pAZ205 is a pSU18 derivative and contains the *E. coli* *stpA* gene under the control of the *E. coli* *lac* promoter (Zhang *et al.*, 1995). The vector pA550 is a derivative of pACYC and carries the *trp* tRNA synthetase (*Cyt-18*) gene from *Neurospora crassa*

(Mohr *et al.*, 1992). The vector pWKS 130.S12 is a low copy pACYC derivative, carrying the ribosomal protein S12 under *lac* promoter control (Wang and Kushner, 1991). The pSU20/NCp7 vector was constructed by ligating a PCR amplified fragment from the vector pHIVp7his1 (Tsuchihashi and Brown, 1994) into the *EcoRI* and *SalI* sites of plasmid pSU20 (Bartolome *et al.*, 1991) using the following primers: 5'-TCG AGG GGT CGA CAT GCA GAG AGG CAA TTT TAG GAA C-3' and 5'-ATA CGA GAA TTC TTA AAA ATT CCC TGG CCT TCC CTT-3'. The vector pSU20/NP was constructed by ligating a PCR amplified fragment from plasmid pHMG-NP (Pleschka *et al.*, 1996) into the *EcoRI* and *SalI* sites in the vector pSU20 using the following primers: 5'-TCG AGG GGT CGA CAT GGC GTC CCA AGG CAC CAA AGG GTC T-3' and 5'-AAT ACG AGA ATT CTT AAT TGT CGT ACT CCT CTG CAT TGT C-3'.

In vivo RNA isolation

For the isolation of cellular RNA, the lysozyme freeze-thaw method was used as described in Belfort *et al.* (1990) with the following modifications. First, cells were grown with IPTG added to a final concentration of 1 mM. Secondly, in the extraction protocol, RNA precipitations were performed in the absence of Mg(OAc)₂ but in addition contained 5 mM EDTA.

Poisoned primer reaction to determine splicing of *td*

The poisoned primer reaction is a primer extension assay and was described previously (Mohr *et al.*, 1992). We used the primer NBS-2 (5'-GACGCAATATTAACGGT-3'). It was performed with the following modifications: reactions were annealed in a 90°C water bath for 1 min and contained 10 µg total RNA (maximal volume of 2.5 µl), 1 ng ³²P-labelled primer (1 µl) and 1 µl hybridization buffer (50 mM Tris-HCl pH 8.4, 60 mM KCl). Samples were allowed to cool to 45°C. For the extension reaction, 2.2 µl extension mix [1 µl H₂O, 0.6 µl extension buffer (500 mM Tris-HCl pH 8.4, 50 mM MgCl₂, 50 mM DTT), 0.3 µl poisoned mix (3 µl 10 mM dCTP, 3 µl 10 mM dATP, 3 µl 10 mM dGTP, 25 µl 5 mM ddTTP) and 0.3 µl AMV reverse transcriptase (4 U/µl)] were added. The reaction was incubated at 42°C for 45 min. After precipitation, formamide loading dye was added and half of the reaction was loaded on a 8% polyacrylamide gel (20:1 acrylamide: bis-acrylamide-7 M urea). Quantification was done with a phosphorimager and average values were calculated from three to six independent experiments. Absolute splicing values vary between different experiments but relative ratios between constructs are constant. Therefore each experiment was performed for all constructs in parallel.

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