RNA aptamers as effective protein antagonists in a multicellular organism

HUA SHI, BRYAN E. HOFFMAN, AND JOHN T. LIS*

Department of Molecular Biology and Genetics, Cornell University, Ithaca, NY 14853

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ABSTRACT RNA aptamers selected against proteins can be used to modulate specific protein function. Expression of such reagents in cells and whole organisms could provide a means of dissecting and controlling molecular mechanisms in vivo. We demonstrate that Drosophila B52 protein can be specifically inhibited in vitro and in vivo by a multivalent RNA aptamer. This inhibitory aptamer RNA binds B52 avidly and inhibits B52-stimulated pre-mRNA splicing. It can be expressed in cultured cells and whole animals in a stable form that accumulates up to 10% of total mRNA. It binds B52 in vivo and suppresses all phenotypes caused by B52 overexpression. The strategies presented here should prove general in design and expression of functional and therapeutic RNAs.

Multiprotein assemblies drive a variety of highly regulated biological processes. To better understand and control such processes, novel reagents are needed to modulate functions of specific proteins in cells and whole organisms. An ideal reagent would, (i) like an antibody, be made to order specifically for a particular protein, (ii) like a small organic molecule, be able to rapidly target a specific protein domain within cells, and (iii) like a conditional allele, be able to exert its effect in whole organisms, but (iv) also be targetable to specific tissues, cells, or stages of development.

Although many strategies exist for inactivating genes or gene products, use of RNA aptamers (1) presents several compelling advantages. Extremely rare RNAs that have high affinity for specific proteins can be selected in vitro (SELEX) (2, 3). Genetically controlled induction of such high affinity RNA aptamers could provide a means of rapidly inactivating a specific domain of a protein and thereby assessing its primary function and mechanism of action in vivo. Alternatively, continuous expression of specific RNA aptamers after stable gene transfer could achieve a long-term alteration in the activity of a target protein. In yeast, the constitutive expression of an aptamer against RNA polymerase II caused cell growth defects under conditions in which the RNA polymerase level was artificially reduced (4). Although this study demonstrated the potential of RNA aptamers as inhibitors of protein function in vivo, several advances are needed to apply this approach to multicellular organisms.

Previously, we selected and characterized RNA aptamers to B52, a regulator of RNA splicing in Drosophila melanogaster. B52, also known as SRp55, is a member of the Drosophila SR protein family, a group of nuclear proteins that are essential for pre-mRNA splicing and influence splice site choice (5, 6). B52 contains two RNA recognition motifs in its N-terminal half and a domain rich in serine-arginine dipeptide repeats in its C-terminal half (7). RNA aptamers that bind B52 with high affinity (Kd = 20–50 nM) and specificity were selected from a large pool of RNAs (8). The B52 binding sites (BBS) of members of this nonclonally derived family of aptamers not only have a “conserved” consensus sequence but also have a virtually identical hairpin loop structure as predicted by a computer program using free energy minimization and confirmed by structure-specific enzymatic probing. Both RNA recognition motifs of the protein are required for interaction with the RNA aptamer.

Here, we design a multivalent “inhibitory aptamer RNA” (iaRNA) molecule for B52 based on the sequence and structure of our previously selected RNA aptamers, and configure a regulated expression system to produce this iaRNA in cells and in flies. We visualize the iaRNA–B52 interaction in cell nuclei and demonstrate the efficacy of this iaRNA as a B52 antagonist in whole animals. This strategy for the construction and controlled expression of iaRNA should be generally applicable to other protein targets in both basic research and therapeutic intervention.

MATERIALS AND METHODS

Construction of the iaRNAB52 and Its Expression System. The monomeric template of the pentavalent iaRNA, BBS (5.1), was constructed with synthetic oligonucleotides (sequences are published as supplemental data on the PNAS web site, www.pnas.org) and was cloned in-between the XhoI and SalI sites of the pSP73 vector (Promega). This cloned monomeric unit was further ligated into polyomers (the longest being a dodecamer generated by two rounds of ligation) with a head-to-tail arrangement (9) and was cloned again. To construct the HicBBS series and the MtnBBS plasmid series, the transcriptional templates of pre-iaRNA were lifted from the pSP73 vectors as XhoI-SalI fragments and were cloned into the SalI site of the Hic-L vector (10) and the XhoI site of the pMtnEX vector (gift from K. Hirayoshi, Kyoto University). The iaRNA gene (including its promoter) was moved from the Hic-BBS (5.12) plasmid as a BamHI-EcoRV fragment to the BamHI-HpaI site of the pW8 vector (11) to generate the pW8-ia-BBS (5.12) plasmid. To generate the pUsBBS series of plasmids, the transcriptional templates of iaRNA of different length, as XhoI-SalI fragments, were cloned into the XhoI site of the pUAST vector (12).

Binding and Splicing Assays. B52 protein was prepared by using the baculovirus expression system, and the avidity of the iaRNA to B52 was examined by a band shift assay, both as described (8). The inhibitory RNA was prepared in vitro with the T7-MEGAscript in vitro transcription kit (Ambion) and was purified on a 5% polyacrylamide gel with 7 M urea. The fts pre-mRNA was produced by run-off transcription from XhoI linearized plasmid pGEM2 V61 S/B (13). S100 extracts were made from Kc cells (14). In vitro splicing reactions were assembled essentially as described in ref. 13 and were carried out at 20°C for 90 minutes.

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*To whom reprint requests should be addressed at: Department of Molecular Biology and Genetics, Cornell University, 417 Biotechnology Building, Ithaca, NY 14853. E-mail: jtl10@cornell.edu.

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Cell Transfection. Each 60-mm plate of S2 cells (initially 5 × 10⁶) was transfected with 2.5 μg of plasmid DNA by using Lipofectin (Life Technologies, Rockville, MD) according to the manufacturer’s instructions. Transfection efficiency was measured to be 10% by β-gal expression from the plasmid pCMV-SPORT-βgal (Life Technologies). The genes were induced 24 hours later by either heat shock at 36.5°C for 90 minutes or adding CuSO₄ to final concentration of 0.5 mM for 24 hours. The half-life of the iaRNA was measured by treating the cells with actinomycin D (Life Technologies, 35 μl, 1 mg/ml) immediately after heat shock and harvesting cells at 0, 2, 4, and 8 hours thereafter. The total RNA samples from transfected cells were quantitated by UV absorbance, and the abundance of iaRNA in these samples was determined by comparing to the standards in a RNase protection assay with the IMAGEQUANT software (Molecular Dynamics).

Drosophila Genetics. Drosophila germ line transformation was performed essentially as described (15). The double transgenic fly lines were synthesized by manipulating the second and the third chromosome with an additional line (CUX), whose genotype is In(2LR)O, Cy; TM2, Ubx130/ T(2.3)ap6K. For more information about the Drosophila genetics see the supplemental data at www.pnas.org.

RNase Protection Assay. The RNase protection assay was performed by using the HybSpeed RPA protocol (Ambion). To determine the abundance of the iaRNA, the internally labeled antisense transcript of part of the monopentameric iaRNA sequence was used as a probe. In each assay, 4 μg of the RNA samples from transfected cells or 1–2 μg of RNA samples from larvae, both DNase treated, were used. The linear range of the assay was determined by serial dilution of gel-purified iaRNA (Fragment B) produced by in vitro transcription.

In Situ Hybridization and Immunofluorescence. The RNA probe was internally labeled with ChromaTide Texas Red-5-UTP (Molecular Probes). Hybridization of the probe to whole, formaldehyde-fixed salivary gland tissue was performed at 60°C overnight in solution containing 50% formamide, 5× standard saline citrate (SSC), 100 μg/ml yeast RNA, 50 μg/ml heparin, and 0.1% Tween-20. The glands were subsequently washed at 60°C for 3–4 hours in eight changes of solution in which the hybridization buffer is gradually displaced by the PBT buffer (Drosophila PBS plus 0.1% Tween-20). Polytenic chromosome spreads were prepared from salivary glands of late third instar larvae as described (7). The anti-B52 antibody was described in ref. 16. Immunofluorescence was performed as described in ref. 7.

RESULTS AND DISCUSSION

Design and Construction of the B52 iaRNA and Its Expression System. In an effort to generate an RNA that would have enhanced avidity for B52, and hence increased potency as a B52 antagonist, we designed a suite of genes that encode a pentavalent inhibitory aptamer RNA (iaRNA) comprising five tandemly arranged BBS aptamer sequences, each of them forming a hairpin loop structure (Fig. 1). Correct folding of each BBS in an array is critical because sequences in both the loop and a portion of the stem are known to be important for binding B52, and a BBS sequence trapped in a contiguous duplex is not functional (8). To ensure the stable pairing of sequences in the stem of each individual aptamer in the pentavalent iaRNA, we reinforced and/or elongated the stem of some BBS units with different sequences. In addition, we engineered a structurally stable tetra-loop near the 3’ end of the iaRNA molecule to stabilize the RNA against degradation by 3’ exonucleases and to serve as a nucleation site for folding (17).

To increase the efficiency of iaRNA production from each transcription cycle, we generated homopolymers of 2, 4, 8, and 12 pentavalent units by “head-to-tail” ligation of synthetic DNA fragments. Such tandemly arrayed sequences are known to be relatively stable in bacteria (18) and can persist for many generations in transgenic fly lines (9, 19). A hammerhead ribozyme sequence (20) is incorporated at the 3’ end of each unit, to allow self-cleavage of the long multimeric pre-iaRNA transcript to yield the mature pentavalent iaRNA. This self-cleavage produces three different types of RNA fragments, A, B, and C (Fig. 1). Of these, Fragment B is the canonical iaRNA molecule, and its molar fraction among the cleavage products should increase in proportion to the number of pentavalent units contained in the template. The 5' and 3' ends of B should pair to produce an “S35” motif, which has been shown to increase >100-fold the accumulation of a tRNA-ribozyme chimeric transcript in cells (21).

We constructed a set of genes with different length and orientation of the transcriptional template under the control of different promoters, to produce the iaRNA not only in vitro, but also in a controlled manner in vivo in either cultured cells or whole animals. The gene name indicates the promoter and the number of BBS aptamers contained in the transcriptional template. For example, HcBBS (5.12) is an iaRNA gene containing 60 BBSs in 12 pentavalent units and driven by a heat shock promoter.

Avidity of iaRNA to B52 and Its Inhibitory Effect on B52-Stimulated Splicing. The iaRNA transcriptional templates were fused to a T7 promoter to produce RNAs in vitro to test their avidity to B52 and effects on B52 function. The self-cleavage of the iaRNA by the internal ribozymes allowed use of a circular template to achieve higher transcriptional efficiency (22). Ribozyme cleavage of a single unit pre-iaRNA [BBS (5.1)] yielded equimolar amounts of Fragments A and C. Because Fragment C contains no binding site, it served as an internal loading control in a band shift assay to assess the avidity of the pentavalent construct (Fig. 2A). During the
Exons are represented by boxes, introns by lines. Enhances in vitro when provided in cis within the pre-mRNA. We too find that been shown to function as synthetic splicing enhancers (23, 24). The formation of splicing products and intermediates (Fig. 2) shows that splicing of a pre-mRNA inhibited splicing reactions indicates that B52, like some other SR proteins, acts at an early step in the splicing process (5, 6). To confirm the specificity of this inhibition, we restored the splicing activity suppressed by the iaRNA by adding additional amounts of B52 to the suppressed assay mixture (Fig. 2B, lanes 10–12). These results support the hypothesis that binding of the iaRNA to the RNA recognition motifs of B52 would prevent its interaction with the pre-mRNA.

The Temporally and Spatially Regulated Expression of iaRNA in Vivo. Genes expressing various inhibitor RNAs, such as antisense RNAs and ribozymes, have been generated by modifying small RNA transcription units that normally produce tRNAs (25), small nuclear RNAs (26), or small viral RNAs (27). Although in some cases high levels of the inhibitor RNA have accumulated, a major disadvantage of such transcription units is the limited ability to regulate their expression. Also, because tRNA promoters have intragenic promoter elements, the RNA transcripts would carry additional tRNA sequences, which may affect the folding of the adjoining functional RNA moiety. To overcome these limitations, we used strong, yet tightly controllable, RNA polymerase II promoters to drive the expression of multimeric templates. For example, a BBS dodecapentamer transcribed from an induced heat shock promoter in Drosophila would yield ~10^18 B52 binding sites in 1 minute, which would build up an intranuclear concentration to >100 nM in ~10 minutes. This is calculated by using the diameter of a Drosophila nucleus (measured to be 2 μm (28)) to give a nuclear volume of 3.4 × 10^-12 liters, and with the known density of RNA polymerase II (one per 80 bp on an induced gene) and elongation rate (1.2 kb/min) (29).

To evaluate the expression and stability of the iaRNA in vivo, we initially introduced BBS-expressing genes into cultured Drosophila S2 cells. Transient expression of the iaRNA was measured by a quantitative RNase protection assay with in vitro transcribed iaRNA as standards. Two strongly inducible promoters were used to drive BBS transcriptional templates of different lengths: a metallothionein promoter (in the mBBS constructs), which can be induced by Cu^{2+} in a few hours, and a heat shock promoter (in the HisBBS constructs), which becomes fully active within minutes. As shown in Fig. 3A, both promoters resulted in similar levels of iaRNA accumulation after induction, when an identical template was transcribed. By comparing to in vitro transcribed iaRNA standards, we estimated that iaRNA transcribed from a dodecapentameric template can accumulate to a level equivalent to 0.1% of total RNA or 10% of total mRNA in S2 (Fig. 3A) and Kc cells (data not shown). The iaRNA is stable with a half-life of ~4 hours.

To express the iaRNA in flies, two systems were compared. First, a heat shock promoter was used to directly control the expression of the iaRNA in the HisBBS strains, and second, a yeast GAL4 regulatory region (UASs) was used to drive expression in flies that produce the GAL4 transcription factor (12). The heat shock promoter-driven iaRNA gene [HisBBS (5.12)] (Fig. 3B, lanes 9 and 10) provides precise temporal control and was used for cytological experiments. Because B52 is a nuclear protein, we designed the iaRNA to be retained inside the nuclei by having a cis-acting hammerhead ribozyme sequence cleave off the downstream polyadenylation signal transcribed from the vector (30, 31). Fig. 3C demonstrates the exclusiveness of nuclear retention of the accumulated iaRNA by in situ hybridization with whole mount salivary gland tissue. The co-compartmentalization of iaRNA with its target protein not only facilitated their encounter but also concentrated the iaRNA in a relevant region within the cell.

In Drosophila, the polyclene chromosomes provided an ideal venue to visualize the in vivo interaction between B52 protein and BBS iaRNA, as the distribution of B52 protein on the polytenic chromosomes has been well characterized (7). We mapped the locus of transgene insertion (Fig. 3D Left) by
polytene in situ hybridization and, with a similar technique, visualized the expression of the iaRNA in the heat shock-induced chromosome puff at this locus (Fig. 3D Center). Immunofluorescence with an anti-B52 antibody showed massive recruitment of B52 on heat shock to this puff that contains the expressed HscBBS (5.12) transgene (Fig. 3D Right). This colocalization of B52 protein with its iaRNA indicates an interaction between them in vivo. Notably, this B52 recruitment to the site of nascent iaRNA synthesis far exceeded that at the native heat shock loci, which are normally the strongest sites labeled during heat shock. Also, in contrast to its puff-bracketing pattern observed at the native heat shock loci (7), B52 covers the entire puff at the transgene’s insertion site.

To further enhance the accumulation of iaRNA and to achieve spatial control of expression in different tissues, we constructed UAS-driven BBS transgenes that are activated by a GAL4 (12). When GAL4 expression was controlled by a heat shock promoter (hsGAL4), an additional step of amplification in the iaRNA expression was achieved, as shown in Fig. 3B. When identical templates were used, indirect heat shock induction via the GAL4-UAS system resulted in a severalfold increase in iaRNA accumulation (Fig. 3B, lane 8 vs. 10). Even without heat shock, the basal level transcription from the heat shock promoter provided sufficient GAL4 to sustain a steady-state iaRNA level in the heterozygous hsGAL4-UASBBS (5.12) flies comparable to that in the homozygous HscBBS (5.12) flies right after heat treatment (Fig. 3B, lane 7 vs. 10). In addition to hsGAL4, many transgenic fly lines that produce GAL4 in developmentally regulated patterns exist and can be used to provide correspondingly specific patterns of iaRNA expression (12).

The in Vivo Efficacy of the iaRNA as B52 Antagonists. Previous genetic studies had shown that the level of B52 is critical to Drosophila development. While a B52 deletion resulted in lethality (32), overproduction of B52 also caused lethality or morphological defects (16). To appraise the efficacy of the iaRNA as an inhibitor of B52, we first examined the phenotype caused by the expression of the iaRNA in transgenic flies. We designed a genetic test in which flies carrying different copy numbers of either the hsGAL4 or UASBBS (5.12) transgene can be identified and counted (see the supplemental data at www.pnas.org). We found that heat-induced, high level expression of iaRNA in transgenic flies caused up to a 50% reduction in survival to adulthood. We also noticed that even without heat treatment the homozygous double transgenic line hsGAL4-UASBBS (5.12) produced less progeny than wild-type strains whereas the homozygous single transgenic hsGAL4 line produced progeny at the level of wild type. Because the effect of a protein antagonist in vivo is realized by reducing the concentration of the functional form of the protein asymptotically to zero, and this is a dynamic process even at a steady state, a complete null phenotype was not anticipated. While our findings are consistent with the lethality caused by B52 deletion (32), the aptamer approach also allows examination of the requirement for B52 at developmental stages that were not accessible in a deletion mutant. The overall morphology of the surviving animals appeared normal, agreeing with Ring and Lis (32) that flies harboring the B52 deletion were ostensibly normal in general morphology until they arrest developmentally.

For a more rigorous verification of the iaRNA’s mechanism of action and a quantitative assessment of its efficacy, we generated transgenic fly lines containing genes that can overexpress both B52 (UASB52) and its iaRNA (UASBBS). Both genes have a promoter that is strongly activated by the GAL4 activator of yeast that also has been introduced to transgenic fly lines (12). The GAL4 gene itself can be controlled by a heat shock promoter or by various developmental enhancers. Because there are many different GAL4-expressing lines, different UASBBS lines that contain different numbers of BBS, and different lines that carry UASB52, we selected one representative from each suite to synthesize three fly lines that contain two transgenes: UASB52-UASBBS, hsGAL4-UASB52, and hsGAL4-UASBBS. Each of these then was mated with a series of single transgenic lines containing, respectively, the GAL4, UASBBS, or UASB52 transgene. This composite cross scheme generated three different groups of progeny that each contained three transgens, and, in each group, one of the transgenes was varied. This allowed us (i) to use different GAL4 source strains to test the suppression of all phenotypes caused by B52 overexpression (Fig. 4A), (ii) to investigate the dosage response to BBS on two quantitative phenotypes (Fig. 4B), and (iii) to verify the results with different B52 transgenic
The expression level of both B52 and BBS (iaRNA) in these heterozygous triple transgenic flies was verified by a RNase protection assay (Fig. 4C).

Crosses that place the GAL4 gene and the UASB52 gene in the same strain can produce five distinctive phenotypes depending on the pattern and level of GAL4 expression (ref. 16 and Fig. 4A). Remarkably, the introduction of an UASBBS gene into a strain that has this B52 overproduction rescued all of these phenotypes. One dramatic phenotype of B52 overexpression is the absence of larval salivary glands. This salivary gland development is largely restored by the coexpression of iaRNA (Fig. 4A). In addition, bristles of the adult notum are missing in a line that overexpresses an UASB52 gene. Here, too, the bristles are largely restored to their normal number by coexpression of iaRNA (Fig. 4A). Abnormal phenotypes of wings and abdominal sternites, as well as the lethality caused by B52 overexpression, were also all suppressed in the presence of iaRNA [summarized in the Table in Fig. 4A (see supplemental data for details)]. We also examined the effect of iaRNA dosage in quantitatively rescuing lethality and bristle development by using the cross scheme of Fig. 4B, in which the dose of iaRNA was varied by expressing iaRNA genes containing different numbers of pentavalent iaRNA units (see also Fig. 3B). With greater production of iaRNA in the transgenic flies, the two B52 overexpression phenotypes became less severe and approached wild type, indicating a quantitative dosage response. These results demonstrate that the iaRNA is able specifically to reverse or alleviate all of the phenotypes specifically caused by B52 overexpression, thus strongly supporting the hypothesis that the iaRNA can inhibit B52 function in vivo.

**Future Prospects.** In this study, we demonstrated that RNA aptamers against B52 can be selected and modified to function as effective B52 antagonists both in vitro and in vivo. Our approach has the combined advantages of (i) antibodies, in that reagents against specific protein targets can be selected, (ii) small organic molecules, in that the reagents can access intracellular locations, and (iii) conditional alleles, in that the effects of the reagent can be evaluated in living whole organisms. Additional advantages over alternative methods of perturbing gene function include the precise control, both temporal and spatial, of the iaRNA production in vivo, coupled
with its direct targeting to protein, making it very attractive for kinetic, mechanistic, and developmental studies.

Other RNA reagents, such as antisense molecules and ribozymes, exist and act as “code blockers” to inactivate gene function. Mechanistically, both of them are expected to achieve specific binding in the first step of their action by forming a stable duplex (or triplex) with a target nucleotide sequence. However, this mechanism remains to be demonstrated. Furthermore, a wide variety of unexpected non-antisense effects have come to light (33). Although some of these side effects may have clinical value, the use of antisense compounds as research reagents has documented limitations (33).

During the last year, double-stranded RNA has emerged as a mediator of gene-specific silencing of expression in some organisms (34–36). Although its mechanism is still unclear, this RNA interference is remarkably efficient. However, potent and specific as it is, its temporal persistence, which may extend to the next generation, and its spatial “spreading,” which usually leads to a strong systemic interference phenotype, make it less tractable than iaRNA for certain applications. In addition, the targets of all RNA reagents mentioned above are thought to be nucleic acids that code for proteins, rather than proteins per se, thereby limiting their use in kinetic analyses or the determination of primary functions of targeted proteins.

Recent reports (37, 38) have demonstrated that peptide aptamers can be expressed in vivo as protein inhibitors in a multicellular organism. Although both approaches act directly against protein targets and allow powerful selection of specific inhibitors, the iaRNA approach offers three significant advantages for at least some applications. (i) Although in vitro selections with RNA routinely explore combinatorial libraries containing 10^{13}–10^{15} different molecules, the isolation of peptide aptamers is usually done with two-hybrid or phage display technology, which have an upper limit at the order of 10^{8} transformants or 10^{9} phages. Because it is estimated to take 10^{6}–10^{11} different molecules to cover the molecular shape space (39), the large searching capacity of in vitro selection methods may provide more efficiency in generating aptamers. (ii) The induction of iaRNA encoding genes leads to a direct control over the production of inhibitors in the nucleus, but aptamer peptides require additional translation and subcellular transportation. (iii) In higher eukaryotes, RNA is less likely to evoke an immune response than peptides.

We have been trying to develop a method that is general in its utility. B52 is an abundant protein (16), and SR proteins have functional redundancy (5). A less abundant protein with lower elasticity in its cellular demand should be a much easier target (4). To direct the iaRNA to different subcellular compartments, functional RNA sequences or structures, such as the Constitutive Transport Element of the type D retrovirus (40), can be incorporated into the iaRNA constructs. Our results, coupled with many studies showing that RNA aptamers can be isolated for a variety of protein targets (3), suggest that RNA aptamers should find broad use in both basic research, as tools in the dissection of mechanisms of sophisticated macromolecular machines and regulatory circuits, and in therapeutic applications, as inhibitors of specific disease causing proteins. Many diseases are known to be caused by either overexpression of an endogenous gene (such as an oncogene in cancer) or expression of exogenous genes (as in a virus infection). As shown here with the reversal of severe phenotypes caused by B52 overexpression, the regulated in vivo expression of RNA aptamers that target disease-inducing proteins has the potential to avert or ameliorate the morbidity and mortality caused by these proteins.

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