Targeting a KH-domain protein with RNA decoys

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ABSTRACT

RNA-binding proteins are involved in the regulation of many aspects of eukaryotic gene expression. Targeted interference with RNA–protein interactions could offer novel approaches to modulation of expression profiles, alteration of developmental pathways, and reversal of certain disease processes. Here we investigate a decoy strategy for the study of the α CP subgroup of KH-domain RNA-binding proteins. These poly(C)-binding proteins have been implicated in a wide spectrum of posttranscriptional controls. Three categories of RNA decoys to α CPs were studied: poly(C) homopolymers, native mRNA-binding sites, and a high-affinity structure selected from a combinatorial library. Native chemistry was found to be essential for α CP decoy action. Because α CP proteins are found in both the nucleus and cytoplasm, decoy cassettes were incorporated within both nuclear (U1 snRNA) and cytoplasmic (VA1 RNA) RNA frameworks. Several sequences demonstrated optimal decoy properties when assayed for protein-binding and decoy bioactivity in vitro. A subset of these transcripts was shown to mediate targeted inhibition of α CP-dependent translation when expressed in either the nucleus or cytoplasm of transfected cells. Significantly, these studies establish the feasibility of developing RNA decoys that can selectively target biologic functions of abundant and widely expressed RNA binding proteins.

Keywords: α CP; decoys; IRES; polio RNA; poly(C)-binding proteins; posttranscriptional control; translation control

INTRODUCTION

Targeted control of eukaryotic gene expression has been attempted using a wide variety of transcriptional and posttranscriptional strategies. Posttranscriptional controls over mRNA stability, translation efficiency, and subcellular localization present specific opportunities and challenges in this field. These posttranscriptional controls are mediated via sequence-specific RNA–protein interactions (Hazelrigg, 1998). Such interactions can, in theory, be blocked or modified by appropriately expressed and structured nucleic acid decoys. The possibility of using molecular decoys to block *trans*-factor binding and functions was first suggested over a decade ago (Baltimore, 1988). Despite early attempts in this area, only a limited number of model systems have been explored in any detail. This slow pace has been dictated to some extent by the need to define RNP complexes that constitute appropriate and functionally important targets for decoy action.

A nucleic acid decoy strategy may be used to inhibit either transcriptional or posttranscriptional controls. Tran-

scriptional controls can be modified by presenting DNA sequences in the nucleus that compete with endogenous promoter or enhancer elements for limiting concentrations of specific protein factors. Such approaches have been used to target transcription factors E2F-1 (Ishizaki et al., 1996) and NF- κ B (Lebruska & Maher, 1999), and to modulate expression of estrogen receptor (Penolazzi et al., 1998, 2000) and renin genes (Tomita et al., 1999). Decoy approaches can also target cytoplasmic events. The most extensive studies of this kind have focused on inhibiting human immunodeficiency virus (HIV) replication by expressing RNA sequences that mimic binding sites for the HIV *trans* activation response (TAR) element or the Rev response element (RRE) (Bohjanen et al., 1997; Konopka et al., 2000). Surprisingly, this decoy approach, although meeting with limited success, has not been more fully explored and extended to additional posttranscriptional events.

Here we investigate the potential of decoys to target a set of abundant RNA-binding proteins that mediate well-defined posttranscriptional controls. The α CP subgroup of KH-domain RNA-binding proteins are encoded in mouse and humans by four dispersed loci and encode five major protein isoforms (Makeyev & Liebhabe, 2000, 2002). The most abundantly expressed

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α CP isoforms are α CP-1, α CP-2, and α CP-2KL. α CPs can be detected in both the nucleus and cytoplasm and are likely to shuttle between the two compartments (Kiledjian et al., 1995; Funke et al., 1996; our unpubl. results). These α CP proteins, along with the nuclear hnRNP K, constitute the major poly(C)-binding proteins in the mammalian cell (Makeyev & Liebhaber, 2002).

The α CP proteins serve important roles in mRNA stabilization and translational control (reviewed in Makeyev & Liebhaber, 2002). Three specific functions of α CPs have been described in detail: (1) posttranscriptional enhancement of human (h) α -globin mRNA levels in terminally differentiating erythroid cells (Kiledjian et al., 1995; Wang et al., 1995), (2) translational silencing of 15-lipoxygenase (LOX) mRNA in a defined window of erythroid development (Ostareck et al., 1997), and (3) enhancement of internal ribosome entry site (IRES) mediated translation of polio viral mRNA (Blyn et al., 1997; Gamarnik & Andino, 1997). In each case, α CPs appear to mediate their function by binding to a pyrimidine-rich and usually C-rich determinant. In all three cases, the structure of this α CP-binding site has been described in detail and mutational analyses have confirmed the function of the corresponding RNP complex (Gamarnik & Andino, 1997; Holcik & Liebhaber, 1997a; Ostareck et al., 1997). Enhancement of α -globin expression appears to reflect stabilization of α -globin mRNA via α CP binding to a C-rich motif within the α 3' UTR (Chkheidze et al., 1999). Formation of this " α -complex," or a closely related complex, has been identified in additional highly stable mRNAs encoding α 1(I) collagen (Stefanovic et al., 1997), tyrosine hydroxylase (Paulding & Czyzyk-Krzeska, 1999), and erythropoietin (Czyzyk-Krzeska & Bendixen, 1999). These studies suggest that the α -complex constitutes a general determinant of mRNA stabilization (Holcik & Liebhaber, 1997a). The roles for α CP in translational silencing of LOX mRNA and translational enhancement of polio viral mRNA may represent more generalized functions of α CP proteins as well (Collier et al., 1998; Walter et al., 1999). Thus, interactions of α CPs with target mRNAs have clear and varied actions. Selectively blocking α CP binding to native mRNA targets by expression of decoy RNAs is predicted to interfere with the respective control mechanisms. Such targeted interference may expand insights into the varied aspects of α CP function in nuclear and cytoplasmic compartments and may suggest novel avenues for therapeutic interventions.

RESULTS

Design of RNA decoys

An RNA decoy can be composed of two parts, the decoy cassette that binds the targeted protein and the structural framework in which the decoy is presented in the cell. Three categories of sequences were utilized

as α CP decoy cassettes: a homopolymer, a native binding target, and a sequence generated by in vitro SELEX (Fig. 1). α CP proteins were initially defined by their high affinity and binding specificity to poly(C) homopolymers. To determine the minimal effective poly(C)-binding site for α CP, a series of synthetic poly(dC) and poly(rC) oligonucleotides of 7 to 17 bases were incubated with mouse erythroleukemia (MEL) cell cytosolic extract.

A. Homopolymer

B. Native target

C. SELEX sequence

FIGURE 1. Three categories of α CP decoy cassettes. **A:** Poly(rC). The number of bases is not indicated because a range of sizes were studied. **B:** Native target. The full-length α 3' UTR of the h α -globin mRNA is shown. The structure is as defined previously (Thisted et al., 2001). Watson-Crick base pairing is indicated by a single line between bonded bases. The three C-rich patches involved in α -complex formation are in large font and protected region (PR) is bracketed. **C:** SELEX sequence. The specific SELEX sequence used in the current study, R7 α 1, is shown. The secondary structure is as previously defined (Thisted et al., 2001). The three C-patches involved in high affinity interaction with α CP are displayed in large font. The two horizontal lines indicate the borders between the random sequence selected in SELEX procedure and the constant sequence flanking arms used in the amplification procedure (for details, see Thisted et al., 2001).

TABLE 1. Apparent dissociation constants for oligos used in this study.

Oligo	Apparent K_d (nM)
poly(dC) ₁₇	6.9
poly(rC) ₁₇	6.4
S-rC ₁₇	212
OMe-rC ₁₇	229–595
S-OMe-rC ₁₇	663–676
$\alpha 3'$ UTR	0.5
α PR	2.0
R7 α 1 SELEX	0.05*

*Measured as a relative value with respect to 3' UTR.

Complex formation between the oligonucleotides and α CP was detected by a filter-binding assay and by analysis of RNP complexes on native gels (electrophoretic mobility shift assay (EMSA)). The complexes formed by these interactions comprised a single band on EMSA analysis. This single complex represents a binary interaction between a single molecule of α CP and the nucleic acid target (Chkheidze et al., 1999). An apparent dissociation constant (K_d) was determined for the interaction of α CPs with each length of RNA or DNA oligonucleotide. The data revealed that the high affinity α CP-RNA interaction was equivalent for deoxyribo- and ribo-poly(C) (Table 1). It was not significantly affected by the size of the poly(C) until the length decreased below 11 bases (Fig. 2). Below this critical size, the apparent K_d increased dramatically. When interactions with poly(C) oligos longer than 17 were studied, a ladder of RNP complexes with mobilities slower than the binary α -complex was observed (data not shown). Although a stoichiometry of these larger complexes was not determined, it is likely that they reflect binding of multiple α CP molecules.

The second candidate decoy was the native h $\alpha 3'$ UTR (Fig. 1B). The apparent K_d for the $\alpha 3'$ UTR sequence interacting with α CP (0.5 nM) is significantly lower than that measured for the interaction of α CP with the poly(C) oligonucleotides (Table 1; Russell et al., 1998). A 32-nt subsegment of the $\alpha 3'$ UTR corresponding to the region of the $\alpha 3'$ UTR protected by α -complex against RNase digestion (α -protected region; α PR) was also tested (Fig. 1B, bracketed). This α PR segment is sufficient for α CP binding. The binding affinity of the α PR for α CP is intermediate to the full-length $\alpha 3'$ UTR and the poly(C) oligomers (Table 1).

A final category of decoy was generated by in vitro selection from a random and highly complex library of 50-nt RNAs. Seven cycles of binding selection to the most abundantly expressed α CP isoform, α CP2-KL, generated a set of tightly binding RNAs (Thisted et al., 2001). A representative sequence from this group of α CP SELEX RNAs was selected for further testing (R7 α 1; Fig. 1C). The R7 α 1 SELEX RNA binds α CPs

FIGURE 2. Relationships of poly(rC) and poly(dC) lengths to α CP binding affinities. The binding constant (apparent K_d) in each case is determined for the interaction of the respective oligonucleotide with native α CP in an S100 MEL cell cytosolic extract (see Materials and Methods for details). The vertical bars represent standard errors calculated from a minimum of three independent measurements.

with dissociation constants 10-fold lower than that of the native $\alpha 3'$ UTR (Table 1; Thisted et al., 2001).

Effects of oligonucleotide chemistry on α CP binding

Various modes of decoy delivery were considered and tested. Isolated decoy cassettes in the form of synthetic oligonucleotides can be directly introduced into cells in culture or in whole animals. However, nuclease sensitivity of oligonucleotides with native phosphodiester backbones can limit their in vivo stability and effectiveness in such settings. This problem can be substantially reduced by the introduction of stabilizing sulfur or methyl groups for nonbridging oxygen in the phosphodiester backbone or by modifying the 2'OH of the ribose with methyl groups (Helene & Toulme, 1990; Miller, 1991). In the context of decoy design, a potential problem with such chemical modifications is that they may interfere with protein binding (Cheng et al., 1997). To assess this variable, three commonly used chemical stabilization modifications were incorporated in the C₁₇ oligomer and the resulting probes were compared for their ability to bind α CP. The binding data revealed that each of the tested modifications resulted in a dramatic decrease in α CP binding (Table 1). These results suggested that chemically modified and stabilized oligonucleotides would not be useful as α CP decoys.

Assembly of chimeric decoy expression vectors and verification of protein binding to their expressed RNAs

An alternative approach to RNA decoy delivery is to express the decoy cassettes *in vivo* in the context of a defined RNA framework from appropriately designed vectors. It is important to note that the α CP proteins are present in both the nucleus and cytoplasm and shuttle between the two compartments (Kiledjian et al., 1995; Funke et al., 1996; our unpubl. results). For this reason, both nuclear and cytoplasmic expression vectors were selected for α CP decoy delivery and functional analysis. An U1 snRNP RNA expression vector (Montgomery & Dietz, 1997) was chosen for nuclear expression. In this plasmid, the U1 snRNA gene is under the control of its potent and constitutively active promoter. The adenoviral VA1 RNA expression vector (Ilves et al., 1996) was chosen for cytoplasmic expression (Fig. 3). The polymerase III-driven adenovirus VA1 transcription unit ubiquitously expresses high intracellular levels of its RNA. These encoded RNAs contain highly ordered secondary structures believed to enhance transcript stability and functional activity (Conn & Draper, 1998).

The aforementioned expression vector systems were selected because they have been shown to express their respective RNAs at high levels and in the correct compartment of the cell. We took advantage of these self-contained systems and inserted the decoy cassettes into the U1 and VA1 transcription units so that native structures important for folding and localization were still intact and the sequences important for α CP binding could be recognized (Fig. 3). The decoy cassettes included poly(C₃₂), α 3' UTR, α PR, α Δ PR, and R7 α 1. The decoy insertions in the VA1 vector were positioned within stem-loop IV of adenoviral VA1 RNA (Fig. 3A). Insertion within this loop prevents the VA1 RNA from interacting with the cellular p68 kinase, thereby eliminating the cellular toxicity of this RNA transcript (Katze et al., 1987; Galabru et al., 1989; Ghadge et al., 1991). In the case of the U1 vector, each decoy cassette was substituted for the short Sm protein-binding site located between the two stable hairpin loops (Fig. 3B). Although the Sm-binding site is essential to nuclear accumulation of snRNAs, it has been shown that it is highly tolerant of mutations (Jones & Guthrie, 1990). The sequence at the 5' ends of the inserted decoys maintained the Sm consensus site [PuA(U)nGPu, Pu = A or G]; similar alterations have been shown to maintain accumulation of U1 chimeric transcripts within the nuclear compartment (Montgomery & Dietz, 1997).

When inserting the decoy cassette into a larger RNA framework, it is possible that structures essential for effective decoy action may be altered or masked. Although secondary structure predictions can be computer generated, directly testing for effects of the RNA framework on decoy function is essential to detect such

FIGURE 3. Two expression systems and RNA frameworks selected to express decoy RNAs in the cytoplasmic and nuclear compartments. **A:** Adenoviral VA1. The site of transcription initiation is indicated by the angled arrow. The transcription unit is shown along with the site of decoy RNA insertion. The predicted secondary structure of the framework RNA is as described (Barcellini-Couget et al., 1998). The boxed segment of the framework RNAs is replaced with the decoy cassettes. **B:** U1 snRNA; as in **A**. The structure of the framework is as described (Montgomery & Dietz, 1997).

problems. For this reason, the activity of each chimeric decoy RNA was assessed by an EMSA-competition assay (Fig. 4). Incubation of [³²P] α 3' UTR (3' UTR) with MEL cell extract generates the well-characterized α -complex composed of a single molecule of α CP bound to the RNA target (Chkheidze et al., 1999). The ability of each decoy to block assembly of this α -complex is a direct reflection of its ability to bind α CP. Each of the isolated decoy cassettes demonstrated full decoy action (Fig. 4A, B). As expected, the native VA1 and U1 framework transcripts lacked decoy activity, as did the VA1- Δ PR transcript, which is the α 3' UTR cassette with the α CP-binding site (PR) deleted (Fig. 4A). These results emphasized the specificity of the decoy/protein interaction. The VA1-C₃₂ and VA1-R7 α 1 transcripts were

FIGURE 4. In vitro α CP-binding activity of candidate decoy RNAs. **A:** Tests of oligonucleotides and of VA1/decoys. α -complexes formed between $[^{32}\text{P}]\alpha$ 3' UTR and MEL cell cytoplasmic extract were assessed for sensitivity to decoy action. **B:** Tests of oligonucleotides and of U1/RNA decoys; as in **A**; nuclear extracts were used for α -complex formation. Each of the indicated RNAs ("Competitors") was synthesized in vitro and tested for ability to inhibit α CP binding to a $[^{32}\text{P}]\alpha$ 3' UTR probe. The position of the radiolabeled α -complex formed by the association of $[^{32}\text{P}]\alpha$ 3' UTR with α CP is indicated to the left of the autoradiograph and the identity of each of the candidate decoys is listed below its respective lane. The candidate decoy sequences were tested either alone or in the framework of the VA1 and U1 vector RNA. The sequences of VA1- Δ PR and U1- Δ PR represent VA1-3' UTR and U1-3' UTR transcripts in which the α CP binding site had been deleted.

fully effective in α CP binding. In contrast the VA1-3' UTR was only moderately effective, and the VA1-PR had little appreciable action. The U1/decoy RNAs had a higher frequency of success in α CP-binding activity; the U1-R7 α 1, U1-C32, and U1-3' UTR each demonstrated full binding activity whereas the U1-PR was lacking in this function (Fig. 4B). It is assumed, but not proven, that the chimeric decoy transcripts with diminished decoy activity compared to their corresponding isolated cassettes had undergone interactions between the decoy cassette and the framework RNA that interfered with the α CP binding site. However, for the majority of the cassettes, the structures of the framework RNAs were self-contained and did not interfere with decoy action.

α CP decoy activity in a cell-free system

The in vitro binding studies detailed above were followed by direct analysis of decoy function using an in vitro translation assay. α CP-1 and α CP-2 bind to two defined structures within the 5' UTR of the poliovirus RNA; the 5'-terminal cloverleaf and stem-loop IV, which is centrally located within the IRES (Gamarnik & Andino, 2000). Binding of α CPs to stem-loop IV enhances IRES translational efficiency (Blyn et al., 1997; Walter et al., 1999). Translational enhancement by α CP can be modeled in an in vitro rabbit reticulocyte lysate (RRL) by linking the IRES to a luciferase reporter ORF (Gamarnik & Andino, 1998). When we use this system, an effective α CP decoy should repress IRES activity with a commensurate decrease in luciferase synthesis.

To validate the use of the RRL model for the purpose of studying α CP-IRES interaction, it was necessary to document the presence of α CPs in the RRL extract. Incubation of $[^{32}\text{P}]\text{dC}_{32}$ in RRL resulted in the formation of two major complexes on EMSA (Fig. 5, α -Complexes I and II). These two complexes contain α CP-1 and α CP-2, as demonstrated by antibody supershifts. Both complexes were competed by poliovirus stem-loop IV RNA. These studies verified characteristics of the RRL that are critical to its use as a model of decoy action on polio viral mRNA IRES function.

The RRL translation system was used to assess bioactivity of α CP decoys. The decoy reporter mRNA contained a firefly luciferase (fLuc) open reading frame

FIGURE 5. Detection of α CP-1 and α CP-2 in rabbit reticulocyte lysate. **A:** EMSA-supershift analysis of RRL for α CP. $[^{32}\text{P}]\text{dC}_{32}$ was incubated with RRL, digested with RNase T1, and electrophoresed on a native gel. The complexes formed are indicated. Complexes I and II are selectively supershifted when incubated with epitope-specific antisera to α CP1 and α CP2, respectively. The position of the free $[^{32}\text{P}]\text{dC}_{32}$ probe is seen in the first lane. **B:** Domain IV of poliovirus 5' UTR ("Domain IV") competes with α -complex formation (Complexes I and II) in the RRL. The sequence of the neutral RNA corresponds to a segment of the h α -globin coding sequence.

(ORF) linked 3' to the polio IRES (Fig. 6A). In vitro-transcribed Polio-fLuc RNA was efficiently translated in RRL. As expected from prior studies (Walter et al., 1999),

the poly(C₃₂) decoy inhibited translation of the Polio-fLuc reporter transcript (Fig. 6B). In contrast, poly(C₃₂) had no appreciable effect on a control capped *Renilla*

FIGURE 6. Decoy action in a RRL translation system. **A:** Diagram of the decoy functional assay. A diagram of the polio IRES/Luc reporter RNA is shown. The Luc ORF is linked to the IRES and its translation is dependent on IRES function. In the absence of decoy, the abundant αCP in the RRL binds to the IRES and enhances luciferase synthesis. In the presence of decoy, levels of αCP available for IRES interaction are decreased, resulting in a corresponding decrease in Luc activity. **B:** Specificity of the reporter system and decoy action. Translation of firefly luciferase is dependent on the function of the poliovirus 5' UTR IRES, whereas translation of *Renilla* luciferase is cap dependent. The ribo and dexoyribo poly(C) oligonucleotides are equally effective in decoy action with a selective repression of firefly Luc and no appreciable effect on the internal control *Renilla* Luc. **C–F:** Measurements of I₅₀ for different decoy sequences using polio-luciferase reporter RNA. Decoy RNAs comprised chemically synthesized oligonucleotides or in vitro transcribed chimeric U1/decoy or VA1/decoy sequences. Vertical bars represent standard errors calculated from at least three independent measurements.

TABLE 2. Decoy activity.

Decoy	I_{50} (μ M)
rC ₁₇	3.5–4.0
dC ₃₂	3.5–4.0
U1-C ₃₂	3.0–3.5
3' UTR	1.5–2.0
U1 UTR	2.0–2.5
PR	4.0
R7 α 1	1.0–1.5
VA1-R7 α 1	1.0–1.5
U1-R7 α 1	1.0–1.5

luciferase (rLuc) mRNA. The effectiveness of each candidate decoy was then tested as an isolated cassette and in the context of the U1 or VA1 framework RNA. Neither the native U1 nor the native VA1 framework RNAs affected translation of Polio-fLuc RNA (Fig. 6C, F, respectively). In contrast, each of the U1-decoy RNAs, with the exception U1-PR RNA, substantially repressed Polio-fLuc translation (Fig. 6C, D, and E). This activity profile paralleled the *in vitro* α CP binding activity of the corresponding decoy RNAs in the EMSA competition study (Fig. 4B). Similarly, both the R7 α 1 cassette and the VA1-R7 α 1 RNA bound α CP strongly in the EMSA study (Fig. 4A), and effectively repressed IRES function in the RRL translation assay (Fig. 6F). The VA1-C₃₂ RNA were also able to repress translation of the Polio-fLuc, whereas VA1-PR and VA1-3' UTR RNAs had no effect on IRES function (data not shown).

The decoy action of each of the tested transcripts was quantified by establishing the I_{50} of decoy action (Fig. 6C–F). Of note, the I_{50} s of active U1-decoys were indistinguishable from the I_{50} s of their corresponding decoy cassettes. Moreover, the relative I_{50} values for these decoys correlated remarkably well with their relative α CP-binding affinities (compare values in Tables 1 and 2); the rank order of decoy strength was R7 α 1 > α 3' UTR > poly(C₃₂).

Expression and subcellular localization of VA1-R7 α 1 and U1-R7 α 1 chimeric decoy RNAs in transfected cells

The U1 and VA1 expression systems were chosen to express decoys in the nucleus (U1) or the cytoplasm (VA1) of an intact cell. As an initial step in evaluating the activities of these decoy RNAs *in vivo*, the localization of the transcripts encoded by the U1-based and VA1-based vectors was determined. Due to its optimal activities in the *in vitro* assays, the R7 α 1 SELEX decoy was selected for these *in vivo* studies. The vectors were transfected into HeLa cells and RNAs were isolated 48 h later from nuclear and cyto-

FIGURE 7. Intracellular localization of VA1-R7 α 1 and U1-R7 α 1 RNAs. **A:** Cytoplasmic expression from the pVA1-R7 α 1 vector. RNA was extracted from the nuclear (N) and cytoplasmic (C) fractions of HeLa cells 12 h posttransfection with the pVA1-R7 α 1 vector. RNA analysis was carried out by RT/PCR; + and - indicate the presence or absence of reverse transcriptase in the RT reaction. Based on a set of preliminary studies, the PCR was carried out with a limited number of cycles (15 cycles) appropriate to the linear range of detection. The experiment was repeated twice with identical results. The positions of the amplified fragments corresponding to the endogenous U6 RNA, vector expressed VA1 RNA, and vector expressed chimeric VA1-R7 α 1 are each indicated. **B:** Nuclear expression from the pU1-R7 α 1 vector. As detailed in **A**, above. The analysis was carried out for 20 cycles (appropriate to the linear range of detection) and the position of the endogenous U1 RNA and vector expressed U1-R7 α 1 RNA are indicated.

plasmic fractions. The adequacy of the cell fractionation was supported by localization of endogenous U6 snRNA predominantly in the nuclear fraction, and endogenous GAPDH mRNA predominantly in the cytoplasmic fraction (Fig. 7A; data not shown). When primers specific to the VA1 framework were used, both the framework VA1 RNA and the chimera decoy VA1-R7 α 1 RNA were found to be predominantly localized in the cytoplasmic fraction (Fig. 7A). In contrast, when primers specific to the U1 vector were used, the control framework U1 RNA and the chimera U1-R7 α 1 RNA were enriched in the nuclear compartment (Fig. 7B). These observations were reproducible over several independent assays and were confirmed by similar analyses of additional pVA1-decoy and pU1-decoy vectors (data not shown).

In vivo action of α CP decoys

Decoy transcripts expressed from the U1- and VA1-based vectors were tested for bioactivity in HeLa cells. A dicistronic reporter mRNA containing the Polio-fLuc ORF (see Fig. 6A) inserted 3' to the rLuc ORF was specifically designed to accurately quantify decoy action in the transfected cells (Fig. 8A). This dicistronic construct was inserted in a eukaryotic expression vector (pRL-TK; Promega). Translation of the rLuc ORF will reflect normal capped mRNA translation whereas

translation of the fLuc ORF will depend on IRES function. Thus the effectiveness of decoy action in suppressing IRES function will be directly reflected by a decrease in the fLuc:rLuc ratio.

Initial experiments were performed to determine the levels of dicistronic reporter plasmid that would optimize the expression of firefly luciferase activity. Decoy action was then tested by varying the ratios of pVA1-R7 α 1 or pU1-R7 α 1 to the dicistronic reporter plasmid in the transfection mix, keeping the dicistronic plasmid concentration at a constant optimal level (Fig. 8B). The

FIGURE 8. Assessment of decoy action in cell culture. **A:** Dicistronic reporter used to detect selective decoy action on polio viral IRES-dependent translation. The dicistronic luciferase reporter plasmid (pDL) contains a 5' cap-dependent *Renilla* (r) ORF and 3' IRES-dependent firefly (f) ORF. IRES function is dependent on α CP binding and this interaction should be selectively blocked by decoy action. **B:** In vivo silencing of IRES-mediated translation by expression of nuclear and cytoplasmic decoys. The dicistronic reporter was transfected into HeLa cells either alone or in combination with 5, 10, or 20 μ g of the VA1-R7 α 1 or U1-R7 α 1 decoy vectors. Levels of *Renilla* and firefly luciferase activity generated in each cell transfection were determined. Individual luciferase activity levels are plotted in the top set of histograms and the ratios of firefly:*Renilla* enzymatic activity are plotted in the bottom set of histograms. Vertical bars represent standard errors calculated from three independent measurements. **C:** Specificity of in vivo decoy action. The dicistronic reporter was transfected into HeLa cells along with vectors expressing the VA1 framework RNA (pVA1), the VA1-R7 α 1 decoy (pVA1-R7 α 1), or a test decoy RNA in which the α CP binding site had been deleted (pVA1- Δ PR; see text for details). The quantity of each decoy vector cotransfected with the dicistronic reporter is noted above the respective set of histogram bars. Details of analysis and histograms are as in **B**.

decoy vectors had no appreciable effect on rLuc activity. In contrast, increased concentrations of decoy vectors in the transfection mix resulted in a progressive and selective decrease in fLuc activity (Fig. 8B, lower panels). The VA1-R7 α 1 and U1-R7 α 1 transcripts both appeared to be approaching saturation in their decoy effects at the highest levels of transfected plasmid (20 μ g). The maximal levels of repression of fLuc translation relative to expression of fLuc in cells transfected with framework expression plasmids was 55% and 37%, respectively. The slightly higher activity of the VA1-based decoy was reproducible over three independent experiments and was statistically significant (Fig. 8B). Transfection with a mixture of 10 μ g of each decoy vector, pVA1-R7 α 1 and pU1-R7 α 1, gave the same effect as 20 μ g of each alone (Fig. 8B). The specificity of decoy action was further demonstrated by expressing the VA1- Δ PR chimeric RNA. Graded amounts of VA1- Δ PR decoy plasmid mixed with the dicistronic reporter plasmid were transfected into HeLa cells. The VA1- Δ PR decoy had no effect on rLuc or fLuc translation, even at the highest levels of transfected plasmid (Fig. 8C). These results demonstrated significant and specific decoy action on a targeted biologic function of endogenous α CP proteins.

DISCUSSION

This study explores the feasibility of generating RNA decoys that target a set of abundant RNA-binding proteins. The α CP proteins have been linked to a number of well-described posttranscriptional controls, and, as such, represent reasonable decoy targets. The approach to decoy design was to analyze three categories of nucleic acids that are high affinity targets of α CP: (1) homopolymers poly(rC) and poly(dC), (2) a natural α CP-binding site (α 3' UTR) and its α CP-binding PR subregion, and (3) artificial RNA SELEX aptamers. The impact of modifications in RNA size, chemistry, and structure on α CP binding was assessed by in vitro binding studies. Activities of candidate decoys as isolated cassettes and presented within defined vector RNA frameworks were determined by in vitro studies and in intact cells using model reporters. These studies support the conclusion that rational design and testing can result in successful decoy development with physiologic consequences.

Poly(C) oligomers were attractive as an initial decoy model as they were the first identified α CP targets. In addition, it may be possible to increase the number of α CPs that can bind these homopolymers by simply expanding their size (our unpubl. data). If binding of multiple copies of α CP to the elongated homopolymer were to occur with cooperative kinetics, such multiprotein binding would make this a particularly attractive characteristic. However, the use of poly(C) sequences as decoys has the disadvantage that they also interact

with the other subset of poly(C)-binding KH-domain proteins, hnRNP K/J (Matunis et al., 1992).

The α 3' UTR may offer a higher level of specificity than poly(C). This α 3' UTR sequence was the initial native target to be identified for α CP (Kiledjian et al., 1995; Wang et al., 1995). The α 3' UTR has a higher affinity for α CP than poly(C) and does not bind effectively to hnRNP K (Chkheidze et al., 1999). A potential disadvantage of α 3' UTR as an α CP decoy when compared to the poly(C) is that it only binds a single molecule of α CP (Chkheidze et al., 1999). However, it may be difficult to predict whether binding affinity or capacity is the more important parameter for effective decoy function. In the present case, the cell-free translation assays revealed that the decoy I_{50} for α 3' UTR was significantly lower than for C₃₂, suggesting an advantage of α 3' UTR's high affinity over its lower capacity (Fig. 6).

The full-length α 3' UTR sequence used as a decoy is 110 nt. This size may exceed limitations of vectors optimized for expression of small, highly abundant transcripts. Theoretically, this problem could be overcome by using the subsegment of the 3' UTR corresponding to the α CP binding site (Fig. 1B). However, this PR sequence has a lower affinity than the full-length α 3' UTR for α CP (Table 1) and this α CP-binding activity shows a further decrease when placed in the VA1 or U1 RNA frameworks (Figs. 4 and 6D). Thus, a balance must be achieved between decoy cassette size and the retention of sufficient higher-order structure to optimize decoy presentation. In the case of the α CP decoys, this latter point is clearly important because the C-patches must be situated in a highly exposed single-stranded configuration to be maximally effective as binding sites (Thisted et al., 2001).

The need for generating decoys with high affinity and binding specificity suggests that an optimal binding site may be generated by targeted in vitro selection of RNA aptamers from a randomized RNA library. Using a combinatorial library of RNA molecules and iterative in vitro selection technique, RNA aptamers that bind tightly to proteins and inhibit their function have been successfully isolated (Ellington & Szostak, 1990; Tuerk & Gold, 1990; for reviews, see James, 2001; White et al., 2000). RNA aptamers have been recently raised against α CPs (Thisted et al., 2001). These aptamers contain three short "C-patches" in a highly exposed loop bracketed by a strong stem structure. One of these SELEX RNAs, R7 α 1, was used for the present decoy study (Fig. 1C). The binding affinity of R7 α 1 to α CP is 10-fold greater than that of the α 3' UTR. In addition, this SELEX sequence has a significantly higher affinity for α CP than for hnRNP K (Thisted et al., 2001). Consistent with these properties, R7 α 1 demonstrated the lowest I_{50} in the cell-free translational test of decoy function (Fig. 6E, F). Based on the combination of high affinity and specificity, R7 α 1 was chosen for subsequent in vivo studies.

Decoy chemistry was addressed as a design parameter. Homo-C polymers with deoxyribose and ribose sugars in the phosphodiester backbone had equivalent binding activities (Fig. 2; Table 1). This was also found to be the case for oligonucleotides with more complex sequence compositions such as PR and R7 α 1 (our unpubl. data). However, substitutions of sulfur or methyl groups for nonbridging oxygen in the phosphodiester bond and the substitutions of 2-O-Me for 2'-OH on the sugar moieties of RNA oligonucleotides resulted in dramatic decreases in α CP binding affinities (Table 1). In addition to their practical utility, these observations can be used to infer how the KH domains interact with their nucleic acid targets.

When considering applications of decoys to in vivo settings, expression via episomal or integrated plasmids offers several advantages over the introduction of synthetic oligonucleotide cassettes. First, it appears that chemical modifications designed to prolong the life of synthetic oligonucleotides result in dramatic loss of protein binding. Synthetic oligodeoxynucleotides can also demonstrate unexpected effects on immune stimulation and complement activation (reviewed by Agrawal, 1996). In contrast, in situ expression from the vectors used in this study provides the cell with a continuous source of decoy sequences in a highly structured and stable carrier RNA framework (Ilves et al., 1996; Bertrand et al., 1997). In vivo expression also allows for specific targeting of decoy expression to particular cell types and to particular cell compartments via appropriate combinations of promoter/enhancer and RNA frameworks. The U1 snRNA and the VA1 expression systems contain many structural elements that are important for expressing their RNAs at high levels in the appropriate compartments of the cell (Fig. 7A, B). In the present study, the U1 snRNA was used to protect the decoy from nucleases and present the U1-decoy chimera at high levels in the nucleus of the cell, whereas the VA1 system targeted the majority of the decoy RNA to the cytoplasm in the VA1 RNA framework (Fig. 7). Finally, in vivo decoy synthesis can be rendered conditional by employing available promoter modifications (for review, see Lewandoski, 2001). This would allow for the temporal control of decoy synthesis in somatic tissues while decreasing toxicity resulting from excessive decoy expression.

A potential problem in using expression vectors to generate decoy RNAs is that hybrid RNAs may not maintain the protein binding capacity of their encompassed cassettes. However, the well-characterized and stable secondary structures of pVA1 and pU1 frameworks (Fig. 3) appear to limit their interactions with the inserted decoy cassettes. Some interference can, however, be noted even with these optimized vectors. For example, the VA1-3' UTR RNA is less effective than the isolated 3' UTR cassette in α CP binding (Fig. 4A). The same loss of binding is seen when the U1-PR is

compared with the α PR cassette (Fig. 4B). These data demonstrate the difficulty in predicting whether an RNA framework will interfere with an effective decoy cassette. In this regard, it is useful to note that in vitro α CP binding of candidate decoys, as determined by a simple EMSA-competition study (Fig. 4), predicts with reasonable accuracy the corresponding decoy activity (Fig. 6).

Expression of α CP decoys in transfected HeLa cells resulted in repression of IRES-dependent translation (Fig. 8B). This activity was dose dependent and appeared to be reaching a plateau at 37–55% repression. Further increases in decoy plasmid concentration or increases in the ratio of decoy to reporter plasmid in the transfection mix failed to further decrease reporter activity. RNA quantification showed that this saturation did not result from a plateau in decoy RNA accumulation (data not shown). The reason for this apparent saturation of decoy function will be important to define. A major unknown in this regard relates to details of the reporter model. Although studies from other laboratories have clearly demonstrated a role for α CP in achieving full levels of IRES function, a baseline level of translational activity from the IRES may be α CP independent (for review, see Andino et al., 1999; Gamarnik & Andino, 2000). The level of α CP-independent IRES function may be difficult to determine by creating mutant IRES elements because mutations that eliminate α CP-binding site could also affect overall RNA secondary structure with consequences on interactions with other protein factors that are important for translation. A more informative approach may involve depletion of α CP from cell extracts. In this regard, it is remarkable that HeLa cell extracts fully depleted of α CP sustain significant residual levels of polio viral translation (Blyn et al., 1997). Thus, it is difficult to firmly conclude at this point whether the residual level of firefly luciferase activity observed in HeLa cells at high levels of decoy expression reflects a basal, α CP-independent level of IRES function or reflects an inability of the decoys to fully compete with the target sequences.

The apparent decoy-resistant level of IRES translational activity may also relate to a technical aspect of the study. Because the reporter and the decoy plasmids were introduced into the HeLa cells by cotransfection, the subpopulation of cells that took up the reporter plasmid but not the decoy plasmid would contribute to continued reporter translation. Attempts were made to keep this fraction of cells to a minimum by using a high ratio of decoy to reporter plasmids in the transfection mix (see Materials and Methods). However, this is unlikely to be fully effective. Despite these caveats, it is possible to conclude from the data that the R7 α 1 decoy cassette in the U1 and VA1 RNA frameworks had a significant and specific effect on α CP function in an in vivo setting.

Successful inhibition of endogenous targets depends not only on the decoy expression level, but also on the ability of the decoy to colocalize with its target (Bertrand et al., 1994, 1997). An unexpected finding from the cell transfection studies was that the nuclear-targeted decoy was only slightly less effective than the cytoplasmic targeted decoy in suppressing IRES-dependent translation (Fig. 8B). This may reflect the shuttling behavior of the α CPs. Whereas the cytoplasmic decoy can directly block association of the IRES with α CP in the cytoplasm, the nuclear decoy may be working indirectly by depleting the cellular pool of shuttling α CP. This may occur either by trapping the shuttling α CP in the nucleus or by binding to this protein in the nucleus and then traveling with it as it reenters the cytoplasm. The absence of a major increase in repression by coexpressing a combination of nuclear and cytoplasmic decoys is consistent with the premise that both decoys are targeting a common α CP pool. It will be of scientific interest and of practical importance to further explore the mechanisms of nuclear versus cytoplasmic decoy actions.

The presently reported studies demonstrate the feasibility of designing decoys using in vitro assays and applying them to in vivo settings. The SELEX-generated RNA aptamers that showed greatest decoy action against α CP function in the present study could now be further optimized to increase their effectiveness and to place their expression under more precise temporal and spatial control. Decoy effects on endogenous processes regulated by α CPs can then be further investigated and their applicability to disease states can be addressed in appropriately designed animal models.

MATERIALS AND METHODS

Construction of expression and reporter plasmids

U1/decoy and VA1/decoy expression vectors were generated from the previously described pU1-RTB (a kind gift of Dr. Robert A. Montgomery, Johns Hopkins University; Montgomery & Dietz, 1997) and pGEM7Z-VA1-PL (a kind gift of Dr. Gabor Veres, Systemix Inc., Palo Alto, California; Ilves et al., 1996) vectors, respectively. All plasmids were constructed using standard techniques (Maniatis et al., 1982). When cloning involved synthesized oligonucleotides (C_{32} , G_{32} , or PR) or PCR products ($\alpha 3'$ UTR, R7 α 1, or Δ PR), restriction sites to *Eco*RI and *Spe*I (for pU1-RTB U1 vector recombination) or *Kpn*I and *Bam*HI (for pGEM7Z-VA1-PL vector recombination) were incorporated in corresponding oligonucleotides or primers.

pRLuc-31 reporter plasmid was a kind gift of R. Andino, University of California, San Francisco (Gamarnik & Andino, 1998). The dicistronic reporter plasmid, pDL was derived from pRL-TK plasmid (Promega) by insertion of the polio 5' UTR-luciferase sequence from pRLuc-31 into the unique *Xba*I site 3' to the coding region of *Renilla* luciferase. The identity and orientation of all inserts were verified by sequencing.

In vitro transcription and translation in a cell-free system

DNA templates for the synthesis of framework (VA1 and U1) and hybrid (VA1/decoys and U1/decoys) RNAs were generated by PCR with corresponding expression vectors as templates. The sequences of the primers were as follows: T7VA1: 5'-TAATACGACTCACTATAGGGCACTCTTCCGTGGTC-3' (sense); VA1: 5'-CGCTCCCCCGTTGTCTG-3' (antisense); T7U1: 5'-TAATACGACTCACTATAGGGAGATACTTACCTG GCAGGGGAGA-3' (sense); U1: 5'-CAGGGGAAAGCGC GAA-3' (antisense). T7 promoter sequence was incorporated in the 5' primer in each set (T7VA1 or T7U1). For in vitro synthesis of reporter RNAs, pRLuc-31 and pRL-TK plasmids containing T7 promoter sequences were linearized with *Mlu*I or *Bam*HI, respectively. DNA templates or linearized plasmids were then transcribed in vitro with T7 RNA polymerase using MegaScript and MegashortScript Kits (Ambion) according to the manufacturer's instructions.

In vitro translations were carried out in micrococcal nuclease-treated RRL (SpeedRead Lysate 2; Novagen). A typical reaction mix contained 0.5 μ g reporter RNA, in the absence or in the presence of varying concentrations of decoy RNAs, in a total volume of 25 μ L. Translations were incubated at 30 °C for 60 min and the lysate was then directly measured for luciferase activities.

Gel mobility shift and supershift assays

Cytoplasmic and nuclear extracts used in the RNA EMSA were prepared as described previously (Dyer & Herzog, 1995) and EMSA was carried out as previously detailed (Holcik & Liebhaber, 1997b). Briefly, ^{32} P-labeled probe was heated to 100 °C for 5 min. The probe (50,000 cpm) was incubated with 25 μ g of extract in a total volume of 30 μ L at room temperature for 30 min. Binding buffer was 10 mM Tris-Cl, pH 7.4, 150 mM KCl, 1.5 mM $MgCl_2$, and 0.5 mM dithiothreitol. RNase T1 (1 U/ μ L) was then added, and the mixture was incubated at room temperature for 10 min. Complexes were subsequently resolved on a 5% nondenaturing polyacrylamide gel in 0.5 $\times$ Tris-borate-EDTA buffer. Bands were visualized by autoradiography of the dried gel. For EMSA with ^{32} P-labeled synthetic poly(C) oligonucleotides, 20,000 cpm of probe was mixed with 50 μ g of protein extracts, incubated, and then gel analyzed as detailed above except that RNase T1 digestion was omitted. Supershift assays were performed with polyclonal rabbit antisera against α CP-1 and α CP-2 (lab designations FF1 and FF3, respectively; Chkheidze et al., 1999). Antibodies were added to the reaction mixture at a concentration of 1 μ g/20 μ L.

Estimation of apparent K_d values

Cytosolic extracts (S100) from mouse erythroleukemia cells were prepared as described (Holcik & Liebhaber, 1997b). Poly(C) DNA oligonucleotides, unmodified phosphodiester poly(C) RNA oligomers, and their phosphorothioate and 2'-O-methyl derivatives were synthesized by the University of Pennsylvania Nucleic Acid Core Facility and were 5'-end labeled by using T4 polynucleotide kinase (New England Biolabs, Beverly, Massachusetts) and [γ - ^{32} P]ATP (Amersham).

A fixed amount of S100 extract (5 μ g) was then incubated for 30 min with ^{32} P-labeled oligos in concentrations ranging from 0.1 to 250 nM, and the resulting RNP complexes were resolved on a 5% native polyacrylamide gel. Bound and free probes were quantified by PhosphorImager (Molecular Dynamics) with ImageQuant software. The values of apparent equilibrium K_d were calculated by linear regression in a double-reciprocal plot for the one-binding-site hyperbola (Feldman, 1972).

Cell transfection, cellular RNA extraction, and analysis

HeLa cells were cultivated in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum and 1% (w/v) penicillin/streptomycin and were grown to approximately 60% confluency at the time of transfection. Cells were trypsinized, washed several times with phosphate buffer saline (PBS), resuspended in MEM at 10^6 cells/mL, and electroporated at 220 V, using a Bethesda Research Laboratories System II Porator as previously detailed (Weiss et al., 1990; Weiss & Liebhaver, 1994). Twelve hours post electroporation, the media was removed and replaced with new complete DMEM media. Reporter gene expression was analyzed 24 h after the start of transfection. Maximal luciferase expression was achieved when 10^6 cells were transfected with 10 μ g of the dicistronic reporter plasmid; further increases in DNA uptake did not result in proportional increases in luciferase activity. Based on these data, 10 μ g of the dicistronic reporter plasmid per 10^6 cells was used as a standard concentration in subsequent analysis of decoy action.

Cytoplasmic and nuclear RNA fractions were prepared from HeLa cells as follows. Cells were lysed in RLN buffer (50 mM Tris HCl, pH 8.0, 140 mM NaCl, 1.5 mM $MgCl_2$, 1 mM DTT, 0.5% Nonidet P-40, RNase Inhibitor) at 4°C. Pelleted nuclei were homogenized using QIAshredder columns (Qiagen). Cytoplasmic and nuclear RNAs were then isolated using RNeasy kit (Qiagen) according to the manufacturer's instructions. All samples were treated with DNase I (Ambion) in digesting buffer (50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 5 mM DTT, 1 mM EDTA, and 2 U of RNase Inhibitor (Eppendorf-5 Prime)).

In the reverse transcription (RT)-PCR analysis, first-strand cDNA was synthesized with 10 U of avian myeloblastosis virus reverse transcriptase (Promega), using the total cytoplasmic or nuclear preparation RNA and specific VA1 or U1 primers (see "In vitro transcription" above). Double-stranded DNA was then generated by PCR amplification with Taq DNA polymerase (Perkin-Elmer), using a ^{32} P-end-labeled antisense primers (VA1 or U1) and an unlabeled sense primer (T7VA1 or T7U1, respectively). The primers used were designed to coamplify the pU1-derived transcripts and endogenous U1 snRNA (primers T7U1 and U1) or the pVA1-derived transcripts (primers T7VA1 and VA1). Conditions for PCR were as follows: preheating at 95°C for 1 min, denaturation at 94°C for 30 s, annealing at 55°C for 1 min, and extension at 72°C for 1 min. Samples taken during the linear phase of the PCR amplification (cycles 15 to 25) were applied to 6% acrylamide gels. The dried gels were analyzed with a PhosphorImager (Molecular Dynamics), and the radioactivity in each band was determined by using ImageQuant software.

Measurement of luciferase activity

Cell lysates were prepared by manually scraping the cells from culture dishes with a disposable rubber policeman in the presence of Passive Lysis Buffer (Promega). The lysates were cleared of residual cell debris by sedimentation for 30 s in a refrigerated microcentrifuge. The activities of the firefly and *Renilla* luciferases were measured sequentially in the same sample. The firefly luciferase reporter was measured first by adding the RRL to Luciferase Assay Reagent II (Promega). The reaction was added directly to the scintillation vial and quantified using a Beckman LS6500 scintillation counter. The substrate for *Renilla* Luciferase was then added directly to the firefly luciferase reaction. The addition of the Stop & Glo reagent (Promega) quenched the firefly luciferase luminescence and activated *Renilla* luciferase. The luminescence from the *Renilla* luciferase was then quantified. A linear relationship between luciferase concentration and counts per minute was determined by calculating the square root of measured counts per minute minus background counts per minute.

Computer and statistical analysis

Secondary structure of RNAs was analyzed by Mfold version 3.0 (Zuker et al., 1999). All experimental values are expressed as mean $\pm$ SEM. Student's *t* tests were used to determine the significance of differences in multiple comparisons. $P < 0.05$ was considered to be statistically significant.

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