In Vivo Recognition of an RNA Aptamer by Its Transcription Factor Target†

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ABSTRACT: In vitro-selected RNA aptamers are potential inhibitors of disease-related macromolecules. Our laboratory previously isolated an RNA aptamer that specifically binds to the human transcription factor NF-κB. This RNA aptamer competitively inhibits DNA binding by NF-κB in vitro. In the study presented here, this aptamer was tested for binding to the p50 homodimer form of NF-κB ($p50_2$) in eukaryotic cells using a yeast three-hybrid system. We show that the α-p50 RNA aptamer selectively binds recombinant $p50_2$ expressed in yeast, demonstrating in vivo recognition of an in vitro-selected RNA aptamer by its protein target. This result suggests that RNA decoys might be used to inhibit the function of DNA-binding proteins in vivo.

In recent years, the advent of in vitro selection systems has led to an explosion in the discovery of potential nucleic acid ligands for target proteins. Using the technique of systemic evolution of ligands by exponential enrichment [SELEX[1] (*1*, *2*)], our laboratory has isolated an RNA ligand to the p50 homodimer form ($p50_2$) of the human transcription factor NF-κB from a pool of $\sim 10^{14}$ randomized, 60-nucleotide RNA sequences (*3*). The in vitro-selected aptamer, here termed "α-p50", bound $p50_2$ with an affinity identical to that of κB DNA ($K_d \sim 1$ nM) (*3*). Although selected for binding to $p50_2$, α-p50 was shown to bind equally well in vitro to the p50−p65 form of NF-κB (*3*). Inhibition of NF-κB activity in cells by controlled expression of such an RNA aptamer could potentially have important therapeutic consequences since NF-κB is involved in inflammatory processes, prevention of apoptosis, and HIV transcription (*4*). Indeed, inhibition of NF-κB by other approaches has been shown to reduce myocardial damage following ischemia and reperfusion (*5*), potentiate tumor cell killing by tumor necrosis factor (TNF) and radio- and chemotherapies (*6*), and cause spontaneous apoptosis in Epstein-Barr virus-transformed lymphoblastoid cells (*7*).

In vitro selections continue to offer exciting leads for inhibition of disease-related factors. However, the selective pressures during in vitro selection often differ from actual in vivo conditions. For example, specificity issues are a major concern for in vitro-selected RNA aptamers. During the selection process, RNA aptamers are chosen solely for their capacity to bind the target protein and not for their ability to discriminate against other potential interaction partners as are encountered in vivo. The ability of an in vitro-selected RNA aptamer to bind its target protein in vivo also depends on aptamer stability, proper folding, and correct subcellular localization.

To explore the ability of the in vitro-selected α-p50 aptamer to bind its protein target in the eukaryotic cell, we utilized a yeast three-hybrid system (Figure 1). This extremely sensitive assay was developed by Wickens, Fields, and colleagues to test RNA−protein interactions (*8*) and provides an ideal tool for detecting RNA aptamer−target interactions in vivo. The yeast three-hybrid system utilizes reporter genes whose expression depends on the interaction of a specific RNA and protein in the yeast nucleus. Using the yeast three-hybrid system, we present evidence that the α-p50 RNA aptamer selected in vitro is specifically recognized by the $p50_2$ form of NF-κB in vivo.

MATERIALS AND METHODS

Hybrid RNAs. DNA oligonucleotides encoding various forms of α-p50, flanked by sequences for ligation to (and inactivation of) *Sma*I and *Sph*I restriction sites, were synthesized by phosphoramidite methodology using an Applied Biosystems model 380B DNA synthesizer. Oligonucleotides were annealed and cloned between the *Sma*I and *Sph*I sites of the shuttle vector pIIIA/MS2-2 (*8*), upstream of two tandem MS2 sequences, to encode various hybrid RNAs. For in vitro transcription, PCR was conducted with primers that flanked the full hybrid RNA transcript sequence to append a 5′ T7 RNA polymerase promoter. Templates were transcribed in vitro using the AmpliScribe T7 High Yield Transcription Kit (Epicentre, Madison, WI), and the hybrid RNA products were purified by electrophoresis and quantitated. The IRE/MS2 control hybrid RNA used in yeast three-hybrid assays was encoded by pIIIA/IRE-MS2-2, which contains the IRE RNA sequence upstream of two tandem MS2 sequences in the pIIIA/MS2-2 vector.

Hybrid Protein Constructs. The GAL4AD/p50 hybrid protein yeast expression plasmid encodes p50 amino acid

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[1] Abbreviations: SELEX, systemic evolution of ligands by exponential enrichment; TNF, tumor necrosis factor; IRE, iron response element; GAL4AD, yeast GAL4 transcriptional activation domain; 3-AT, 3-amino-1,2,4-triazole; IRP, iron regulatory protein; PCR, polymerase chain reaction; RLU, relative light units; TE, Tris-EDTA.

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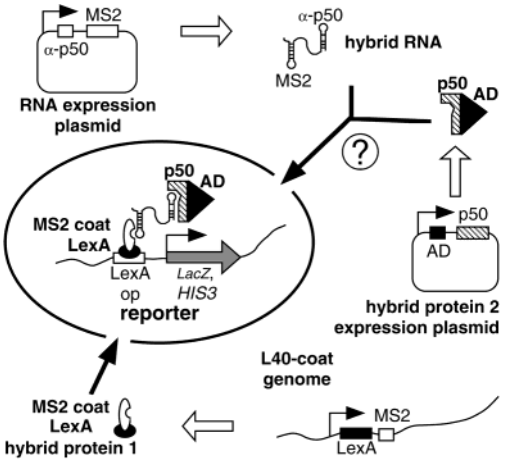

FIGURE 1: Yeast three-hybrid system. Yeast strain L40-coat harbors chromosomal copies of *LacZ* and *HIS3* reporter constructs (circled), as well as the gene encoding hybrid protein 1, a LexA/MS2 coat fusion protein (lower right). The LexA portion of hybrid protein 1 (lower left) binds to the LexA operator upstream of the reporter genes (circled), while the MS2 coat protein domain binds to the MS2 sequence in the hybrid RNA (upper right). The RNA sequence of interest, in this case the in vitro-selected α-p50 RNA aptamer, comprises a second domain of the hybrid RNA. Hybrid protein 2 is likewise introduced into the yeast cell on an expression plasmid (middle right) and consists of the yeast GAL4 transcriptional activation domain fused to the protein to be tested, p50 in these experiments. In vivo binding of the α-p50 RNA aptamer to its intended $p50_2$ target should result in expression of the *LacZ* and *HIS3* reporter genes.

residues 1−460 downstream of the yeast GAL4 transcriptional activation domain sequence in the yeast shuttle vector pGAD424 (Clontech, Palo Alto, CA). Plasmid pGAD424 encodes the yeast GAL4 transcriptional activation domain (GAL4AD/− −), and pGAD53m (Clontech) encodes the mouse p53 protein downstream from the GAL4AD sequence in pGAD424 (GAL4AD/p53). pAD-IRP1 encodes the rabbit iron regulatory protein (IRP) downstream of the GAL4AD in the yeast shuttle vector pACTII (GAL4AD/IRP).

In Vitro Decoy Activity of Hybrid RNAs. In vitro-transcribed hybrid RNAs (∼300 nucleotides) were tested for the ability to compete with radiolabeled κB duplex DNA for binding to recombinant, purified $p50_2$ (*3*) in an electrophoretic mobility shift assay (EMSA). Duplex κB DNA (*3*) was radiolabeled using the Klenow fragment of DNA polymerase I to fill in recessed 3′ termini with [α-^{32}P]dATP in the presence of dGTP, dTTP, and dCTP (each at 0.1 mM). The EMSA was conducted as described previously (*3*), using a 1- or 5-fold molar excess (relative to labeled probe) of the indicated competitor RNA or DNA. Complexes were detected and analyzed by storage phosphor technology.

Yeast RNA Isolation and Northern Blot Analysis. Yeast total RNA was extracted as described previously (*9*). RNA (20 μg) from yeast strain L40-coat (*8*) expressing various hybrid RNAs and hybrid proteins was extracted and electrophoresed on a 6% denaturing polyacrylamide gel. RNA was transferred by electroblotting to a GeneScreen hybridization transfer membrane (NEN, Boston, MA) and cross-linked by UV irradiation. Hybridization was conducted utilizing radiolabeled oligonucleotide probes complementary to the RNaseP RPR1 leader (5′-AGCAC_2ACAGCGTAC_2ATGT) and to the U14 snRNA (5′-C_2TAC_2GTG_2A_3CTGCGA_2).

Yeast One-Hybrid Assays. A yeast dual reporter strain containing three tandem κB sites upstream of the integrated *HIS3* and *LacZ* reporter genes was constructed in host strain YM4271 as described previously (*10*). A control strain containing three tandem p53 recognition sequences upstream of the *HIS3* and *LacZ* reporter genes was similarly constructed. Appropriate hybrid protein expression constructs were then introduced into the reporter strains via lithium acetate-mediated transformation (*11*). *HIS3* growth assays and *LacZ* color and chemiluminescent assays were conducted as described previously (*10*−*12*). For the *HIS3* growth assay, cell suspensions were plated on medium lacking histidine and leucine and containing 0−10 mM 3-amino-1,2,4-triazole (3-AT). For the *LacZ* color assay, cell patches transferred to nitrocellulose filters were lysed with liquid N_2 and incubated in Z buffer (*11*) containing 1 mg/mL X-gal for 30 min at 30 °C. Three independent chemiluminescent *LacZ* assays were conducted for each strain using the Clontech Galacton-Star liquid assay (Clontech), and light emission (RLU) was recorded as a 5 s integral on a Turner Designs model TD-20e luminometer.

Yeast Three-Hybrid Assays. A positive control strain, the test strain, and six negative control strains were constructed by transformation of the host yeast strain L40-coat (*8*) using simultaneous lithium acetate-mediated transformation as described previously (*11*). *HIS3* growth assays and *LacZ* color and chemiluminescent assays were conducted as described above.

RESULTS AND DISCUSSION

Proper Folding of Hybrid RNAs in Vitro. Our first objective was to ensure that the α-p50 RNA aptamer would adopt the correct conformation for $p50_2$ binding when presented in the context of the α-p50/MS2 hybrid RNA required for the yeast three-hybrid system. To this end, we tested the ability of α-p50 (**1**, Figure 2A) and three variants (**2**−**4**, Figure 2A) to bind $p50_2$ in vitro. Variant **2** contained a stabilizing tetraloop in place of the 7-nucleotide unpaired distal loop of the α-p50 aptamer (Figure 2A). This modification was introduced in an attempt to stabilize the α-p50 hairpin structure within the hybrid RNA. α-p50 variant **3** contained a 13 bp GC-rich clamp ("GC clamp") appended to the α-p50 aptamer structure in an attempt to thermodynamically "lock" the aptamer into the correct conformation (Figure 2A). α-p50 variant **4** contained both a GC clamp and a tetraloop. As an example, the predicted folded structure of the α-p50/MS2 hybrid RNA containing sequence **3** is shown in Figure 2B. After cloning, the four versions of the RNA sequence shown in Figure 2B were synthesized by in vitro transcription and purified, and the resulting RNAs were tested for the ability to compete with κB DNA for $p50_2$ binding in vitro.

As shown in Figure 2C, RNAs **3** and **4** competed with the radiolabeled κB DNA duplex for binding to $p50_2$. In contrast, RNAs **1** and **2** or a version of **1** with a scrambled α-p50 sequence did not compete. These results are quantitated in Figure 2D. Thus, the addition of a GC clamp was found to be essential for maintaining the α-p50 secondary structure within the context of the α-p50/MS2 hybrid RNA. According to thermodynamic predictions provided by the RNA folding algorithm MULFOLD (*13*−*15*), the GC clamp may serve to drive the correct folding of the α-p50 aptamer within the hybrid RNA. In addition, the clamp may facilitate

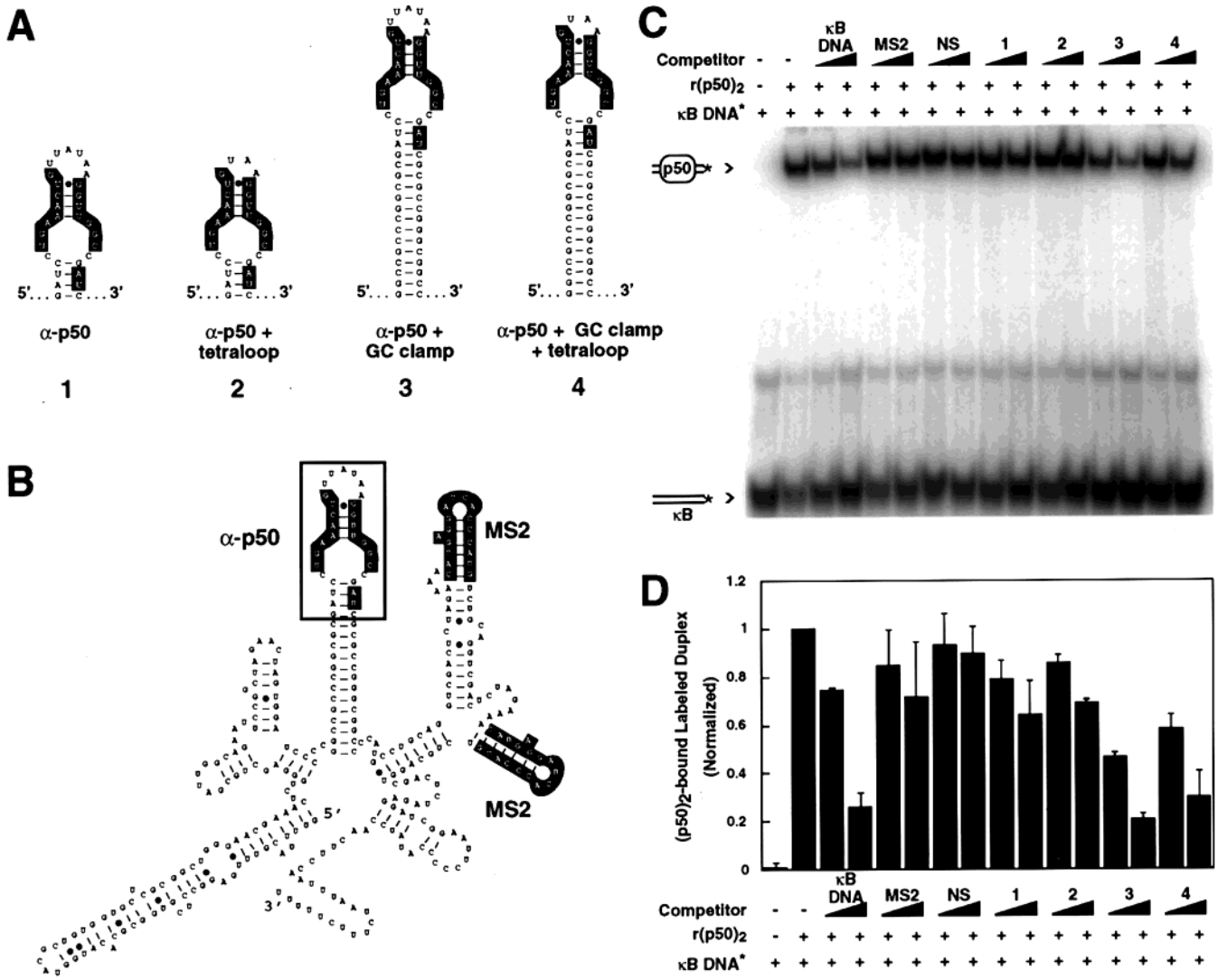

FIGURE 2: Addition of an extended stem structure facilitates binding of the α-p50/MS2 hybrid RNA to $p50_2$ in vitro. (A) Predicted secondary structures of the α-p50 RNA aptamer domain and three variants. RNAs **1**−**4** are α-p50/MS2 hybrid RNAs (total lengths of 292−317 nucleotides) containing the indicated aptamer domains. Nucleotides previously shown to be important for $p50_2$ binding are highlighted (*3*). (B) Predicted secondary structure of RNA **3**. Again, the α-p50 RNA aptamer domain is boxed, with nucleotides previously shown to be important for $p50_2$ binding highlighted (*3*). MS2 sequences are also highlighted. (C) Competition gel shift experiment assessing RNA aptamers as competitors for $p50_2$ binding to labeled *κ*B DNA. Unlabeled *κ*B DNA was used as a positive control competitor, while - -/MS2 (MS2; 271 nucleotides) and scrambled α-p50 + GC clamp/MS2 (NS; 317 nucleotides) were used as negative control competitors. $r(p50)_2$ represents the recombinant, purified $p50_2$ protein; *κ*B DNA* represents ^{32}P-labeled *κ*B duplex DNA. (D) Quantitation of competition gel shift assay results. Values were normalized to the signal of $r(p50)_2$-bound, labeled DNA duplex in the absence of competitor and represent the mean ± standard deviation based on three experiments.

presentation of the α-p50 aptamer projecting away from the body of the hybrid RNA for optimal interaction with $p50_2$. RNA **3** appeared to compete efficiently with the radiolabeled *κ*B DNA duplex for binding to $p50_2$, since addition of 1- and 5-fold excess competitor reduced the level of binding of the probe to $p50_2$ by ∼50 and ∼83%, respectively (Figure 2D). Therefore, the construct encoding RNA **3** was utilized for subsequent experiments.

Hybrid RNA Expression in Yeast. Upon transformation of the three-hybrid system host yeast strain L40-coat with the construct encoding RNA **3**, a Northern blot of total yeast RNA was conducted to assess accumulation of this RNA containing the α-p50 aptamer. The blot was simultaneously probed for the RNaseP RPRI leader sequence (common to all hybrid RNAs and also found endogenously in yeast cells) and the U14 snRNA sequence (as an internal loading control). As shown in lane 3 of Figure 3, RNA **3** was abundantly expressed in yeast cells relative to the endogenous yeast small RNAs RNaseP RPRI and U14 snRNA. Lane 2 of Figure 3 shows total RNA from a yeast strain expressing the iron response element (IRE)/MS2 hybrid RNA (*8*), simultaneously probed for comparison.

Recombinant NF-κB p50 Homodimers Function in Yeast. We next sought to demonstrate that the hybrid protein component of our yeast three-hybrid assay was functionally expressed in yeast. This was accomplished using a yeast one-hybrid system (*16*) (Figure 4A). A *β*-galactosidase colony color assay was conducted to confirm the transcriptional activation and DNA-binding competence of a GAL4AD/p50 hybrid protein utilized in our studies (Figure 4B). The only strains to test positive for a protein−DNA interaction were the positive control strain (GAL4AD/p53 hybrid protein expressed with a reporter containing p53 binding sites upstream of *LacZ*) and the test strain expressing the

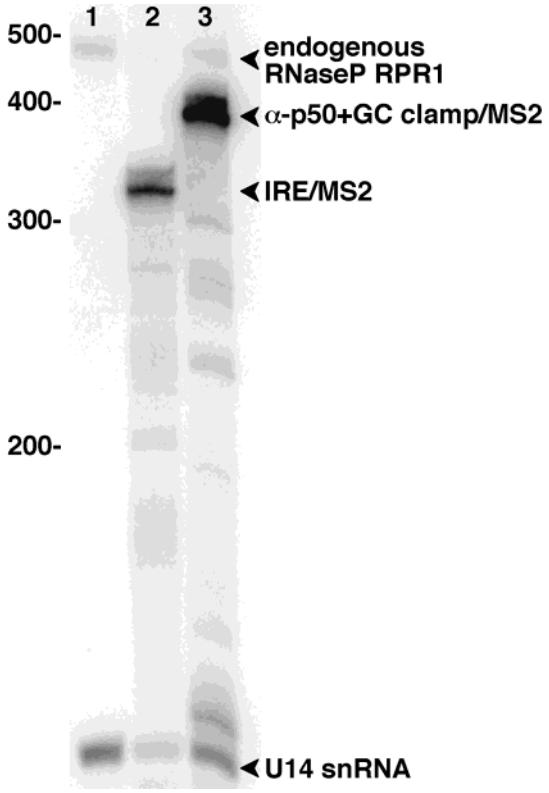

FIGURE 3: RNA **3** accumulates in yeast three-hybrid strain L40-coat. Yeast total RNA from L40-coat-based strains expressing the GAL4AD/p50 hybrid protein (lane 1), the GAL4AD/IRP1 hybrid protein with IRE/MS2 hybrid RNA (lane 2), and the GAL4AD/p50 hybrid protein with RNA **3** (lane 3) was extracted and assayed by Northern blotting, probing with radiolabeled oligonucleotides complementary to the RNaseP RPR1 leader and to the U14 snRNA. Mobilities of DNA size markers are indicated. The expected length of the α-p50 + GC clamp/MS2 hybrid RNA is 317 nucleotides. Phosphorimager analysis showed that RNA **3** accumulated to 60 ± 8% of the IRE/MS2 hybrid RNA levels, based on normalization to the RNaseP RPR1 and U14 snRNA controls.

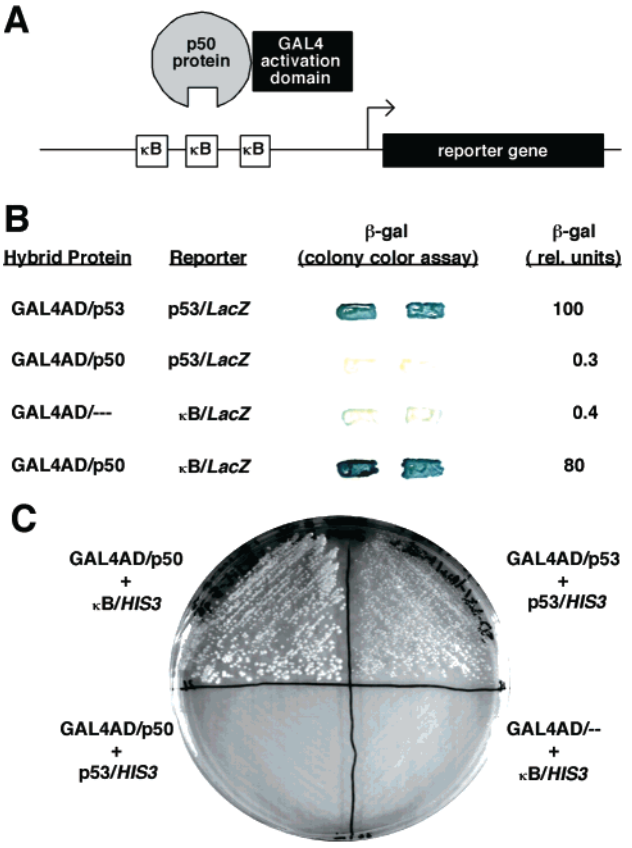

FIGURE 4: GAL4AD/p50 fusion protein expressed and functional in yeast. (A) The yeast one-hybrid system was employed to verify the activation and DNA binding competence of the GAL4AD/p50 hybrid protein. Three κB sites were inserted upstream of the *LacZ* and *HIS3* reporter genes (κB/*LacZ* and κB/*HIS3*, respectively). A control plasmid was likewise constructed containing three p53 recognition sequences upstream of reporter genes (p53/*LacZ* and p53/*HIS3*). Plasmids harboring these reporter constructs were integrated into the genome of yeast strain YM4271. Plasmids encoding the hybrid proteins were then introduced. (B) *LacZ* reporter gene expression was assayed by a colony color assay and direct measurement of enzyme activity. Two colonies of each strain were patched onto medium lacking leucine, and a colony color assay for *LacZ* reporter gene expression was performed as described in Materials and Methods. At least three independent color assays were conducted in duplicate for each strain. For the direct assay of enzyme activity, values were normalized to the p53 positive control strain (signal set at 100 arbitrary units). Data represent the mean of three independent assays conducted in triplicate. (C) *HIS3* reporter gene expression was assayed by spreading cell suspensions on plates lacking histidine and leucine and containing 3 mM 3-amino-1,2,4-triazole (3-AT). 3-AT is an inhibitor of the *HIS3* gene product, allowing discrimination of true positives.

GAL4AD/p50 hybrid protein with a reporter containing κB sites upstream of *LacZ*. The negative control strain expressing the GAL4AD alone with a reporter containing κB sites upstream of *LacZ* exhibited very weak activation of the reporter gene (faint blue in Figure 4B). A chemiluminescent liquid β-galactosidase assay was conducted to quantitate the relative *LacZ* expression levels in the different strains. As shown in the final column of Figure 4B, only the positive control strain and the strain expressing the GAL4AD/p50 hybrid with a reporter containing κB sites upstream of *LacZ* yielded detectable β-galactosidase activity. These results indicate that the GAL4AD/p50 fusion protein is a functional site-specific transcription factor in yeast.

To confirm these data, a growth assay for expression of the *HIS3* reporter gene was conducted. In the presence of 3 mM 3-AT, only the positive control strain (expressing the GAL4AD/p53 hybrid with a reporter containing p53 binding sites upstream of *HIS3*) and the test strain (expressing the GAL4AD/p50 hybrid with a reporter containing κB sites upstream of *HIS3*) were able to grow (Figure 4C). Neither the negative control strain (expressing GAL4AD alone with a reporter containing κB sites upstream of *HIS3*) nor an unmatched strain (expressing the GAL4AD/p50 hybrid with a reporter containing p53 sites upstream of *HIS3*) was able to grow detectably in the presence of 3 mM 3-AT. Taken together, these results confirm that the GAL4AD/p50 fusion protein is functional in yeast.

Three-Hybrid Assay of the α-p50 RNA Aptamer−p50$_2$ Interaction in Yeast. Having validated the necessary components of the yeast three-hybrid system, we assayed strains expressing the appropriate combinations of hybrid RNAs and hybrid proteins for *LacZ* and *HIS3* reporter gene activation. The positive control strain (GAL4AD/IRP + IRE/MS2) utilizes a naturally occurring eukaryotic RNA/protein interaction: the iron regulatory protein (IRP) binds to the iron response element (IRE) in the 5′ untranslated region of ferritin mRNA to inhibit translation of the mRNA encoding this iron storage protein (*17, 18*). As shown in Figure 5A, only the positive control strain (GAL4AD/IRP + IRE/MS2)

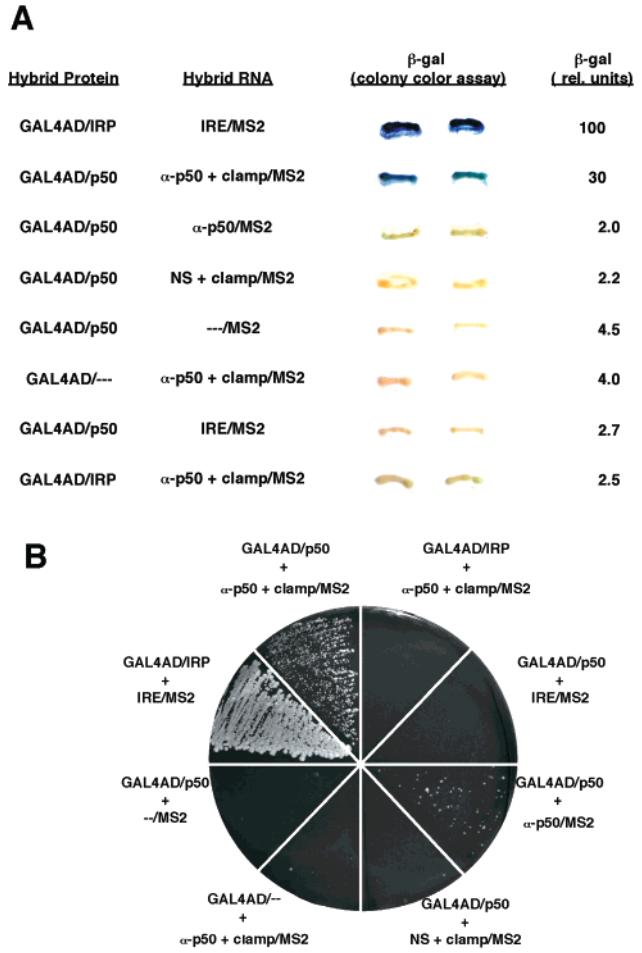

FIGURE 5: α-p50 RNA aptamer binds its $p50_2$ target protein in vivo. (A) Expression of *LacZ* reporter gene in three-hybrid strains. The yeast strain L40-coat was transformed with plasmids encoding the indicated hybrid proteins and RNAs. Transformants were assayed for *LacZ* reporter gene expression by a colony color assay and measurement of *β*-galactosidase activity as described in the legend of Figure 3. "α-p50 + clamp/MS2" is identical to RNA **3** in Figure 2. (B) Three-hybrid system growth assay for expression of the *HIS3* reporter gene. The yeast strain L40-coat was transformed with the indicated hybrid proteins and RNAs and plated on medium lacking uracil and leucine. After 3 days, individual colonies were suspended in TE buffer and streaked on plates lacking uracil, leucine, and histidine and containing 1 mM 3-AT.

and the test strain [GAL4AD/p50 + (α-p50 + clamp)/MS2] strain displayed *LacZ* expression in the colony color assay. A chemiluminescent liquid *β*-galactosidase assay was conducted to quantitate the relative *β*-galactosidase activity (Figure 5A, final column). These results provide the first evidence of an in vivo interaction between the α-p50 RNA aptamer and its $p50_2$ target. A *HIS3* growth assay was conducted to confirm this finding. As shown in Figure 5B, only the positive control strain (GAL4AD/IRP + IRE/MS2) and the test strain [GAL4AD/p50 + (α-p50 + clamp)/MS2] were able to grow appreciably on medium containing 3-AT. Interestingly, a strain expressing the GAL4AD/p50 hybrid with a hybrid RNA containing an unclamped version of the α-p50 aptamer (RNA **1** in Figure 2A) exhibited weak growth in 1 mM 3-AT. This strain was able to grow on plates containing up to 2 mM 3-AT, whereas the strain expressing the GAL4AD/p50 hybrid and α-p50 + clamp/MS2 was able to grow on plates containing up to 3 mM 3-AT. These results indicate that the in vitro-selected α-p50 RNA aptamer binds its $p50_2$ target in vivo and confirm the in vitro results depicted in Figure 2 which suggest incorporation of a GC clamp enhances recognition of p50 by the α-p50 aptamer. The three-hybrid data demonstrate that the GC clamp assists aptamer presentation but is not itself recognized by the target protein. Moreover, the data rule out recruitment by the hybrid RNA of endogenous yeast transcriptional activators as has recently been reported in another three-hybrid study (*19*).

Implications. Converging evidence suggests that RNA regulation of transcription factors may constitute a previously underappreciated mechanism for controlling gene expression. Our interest in RNA decoys is based on the example of a naturally occurring RNA decoy for a transcription factor provided by *Xenopus* 5S RNA. Upon transcription of the 5S gene by RNA polymerase III, excess RNA product can bind the transcription factor TFIIIA and sequester it from DNA. Consequently, the level of 5S gene transcription is reduced through an autoregulatory mechanism (*20*). Another fascinating example is provided by the TSU (trophoblast STAT utron), a small, untranslated RNA that appears to bind the transcription factor STAT1 to repress major histocompatibility complex antigen expression in human trophoblasts (*21*). The TSU appears to bind to STAT1 by virtue of STAT-binding promoter-like motifs occurring within the RNA sequence (*21*). This observation raises the intriguing possibility that regulator RNAs may recognize transcription factors through the formation of pseudoduplexes that mimic the factor's natural binding site. The α-p50 RNA aptamer characterized in our studies shows no obvious sequence homology to the NF-κB DNA binding site; however, the possibility remains that the RNA structure forms a pseudoduplex, perhaps through the formation of noncanonical base pairs, that is uniquely suited for recognition by the NF-κB transcription factor.

The mounting evidence for RNA regulation of proteins bespeaks the possibility of imitating such naturally occurring interactions to artificially regulate gene expression. As in our studies, in vitro selections may serve as starting points for identifying potential RNA ligands for transcription factors. The yeast three-hybrid system will serve as an important tool for optimizing our α-p50 RNA aptamer: $p50_2$ interaction with the ultimate goal of inhibiting NF-κB function in cells.

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