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METHOD

StreptoTag: A novel method for the isolation of RNA-binding proteins

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ABSTRACT

We describe a fast and simple one-step affinity-purification method for the isolation of specific RNA-binding proteins. An in vitro-transcribed hybrid RNA consisting of an aptamer sequence with high binding specificity to the antibiotic streptomycin and a putative protein-binding RNA sequence is incubated with crude extract. After complex formation, the sample is applied to an affinity column containing streptomycin immobilized to Sepharose. The binding of the in vitro-assembled RNA–protein complex to streptomycin-Sepharose is mediated by the aptamer RNA and the specifically bound proteins are recovered from the affinity matrix by elution with the antibiotic. Employing two well-characterized RNA–protein interactions, we tested the performance of this new method. The spliceosomal U1A protein and the bacteriophage MS2 coat protein could be isolated via their appropriate RNA motif containing hybrid RNA from crude yeast extracts in high yield and purity after only one round of affinity purification. As the purification principle is independent of the extract source, this new affinity chromatography strategy that makes use of an in vitro-selected antibiotic-binding RNA as a tag, "StreptoTag," should be applicable to extracts from other organisms as well. Therefore, we propose StreptoTag to be a versatile tool for the isolation of unknown RNA-binding proteins.

Keywords: affinity chromatography; antibiotic; aptamer; protein purification; RNA–protein complex

INTRODUCTION

RNA–protein interactions are fundamental to the assembly of complex catalytic machineries performing essential processes like translation or splicing. A wide variety of RNA-binding proteins that stabilize, protect, or package RNA or control key steps in the posttranscriptional regulation of gene expression are known. Cutting, unwinding, modifying, or replicating RNA needs the action of proteins interacting with RNA. RNA-binding proteins are involved as *trans*-acting factors in establishing asymmetries during cell division or development by localizing and anchoring mRNAs to distinct subcompartments of the cell (Siomi & Dreyfuss, 1997; Lipshitz et al., 1998; Krecic & Swanson, 1999; Minvielle-Sebastia & Keller, 1999). In addition to well-known RNA-binding motifs (e.g., the RNP domain, dsRBD, or KH domain, reviewed in Nagai, 1996), new RNA-binding folds are being discovered, reflecting the various functions of RNA–protein complexes in the cell (Cusack, 1999).

Therefore, a major goal for understanding the molecular basis of cellular processes performed by ribonucleoprotein particles is to identify the involved RNA–protein interactions. This requires efficient purification methods for the isolation, characterization, and identification of the components of these RNA–protein complexes. For example, a pure preparation of an RNA-binding protein could be directly used for analysis and identification of the protein by mass spectroscopy (Kuster & Mann, 1998).

Existing affinity purification methods for the isolation of in vitro formed RNA–protein complexes that use poly-U Sepharose (Neupert et al., 1990) or streptavidin-beads (Rouault et al., 1989) often copurify unspecific proteins.

In this study, we present a new variation of affinity purification based on the use of an in vitro-selected streptomycin-binding RNA as a tag for the isolation of RNA-binding proteins. We established the new purification method with two different well-characterized RNA–protein interactions, the binding of spliceosomal protein U1A to U1 snRNA and the binding of MS2 coat protein to the MS2 replicase mRNA. We show that both high affinity RNA-binding proteins can be isolated with

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striking purity from crude yeast extracts in only one round of affinity purification. Importantly, the MS2 coat protein could be isolated with a mutant RNA motif with a binding affinity in the low millimolar range, demonstrating that StreptoTag is able to detect weak RNA–protein interactions.

RESULTS AND DISCUSSION

Experimental strategy and expression of functional RNA-binding proteins in *Saccharomyces cerevisiae*

A schematic drawing of a hybrid RNA containing a putative protein-binding site (RNA X) and the streptomycin-binding sequence is shown in Figure 1A. The streptomycin-binding RNA aptamer was isolated via SELEX and binds to the antibiotic with a dissociation

constant (K_d) of around 1 μM and only in the presence of magnesium ions (Wallace & Schroeder, 1998). Different strategies for the construction of the DNA templates for in vitro transcription were used and are shown in Figure 1B. The templates of hybrid RNAs containing the aptamer and the MS2 coat protein binding site (wild-type and mutant, MS2(wt)-apt or MS2(mt)-apt, respectively) were generated by polymerase chain reaction (PCR) from the aptamer-clone with oligodeoxynucleotides containing the respective protein-binding motif (RNA-X, compare strategy (i)). For generating the template of the hybrid RNA U1stII-apt, harboring the aptamer and stem-loop II of U1 snRNA, a single-stranded oligodeoxynucleotide consisting of the T7 RNA polymerase promoter, the U1A protein binding motif, and the aptamer was converted to a double stranded template by asymmetric PCR (see strategy (ii)). A third possible strategy is outlined in strategy (iii), which employs an aptamer-containing
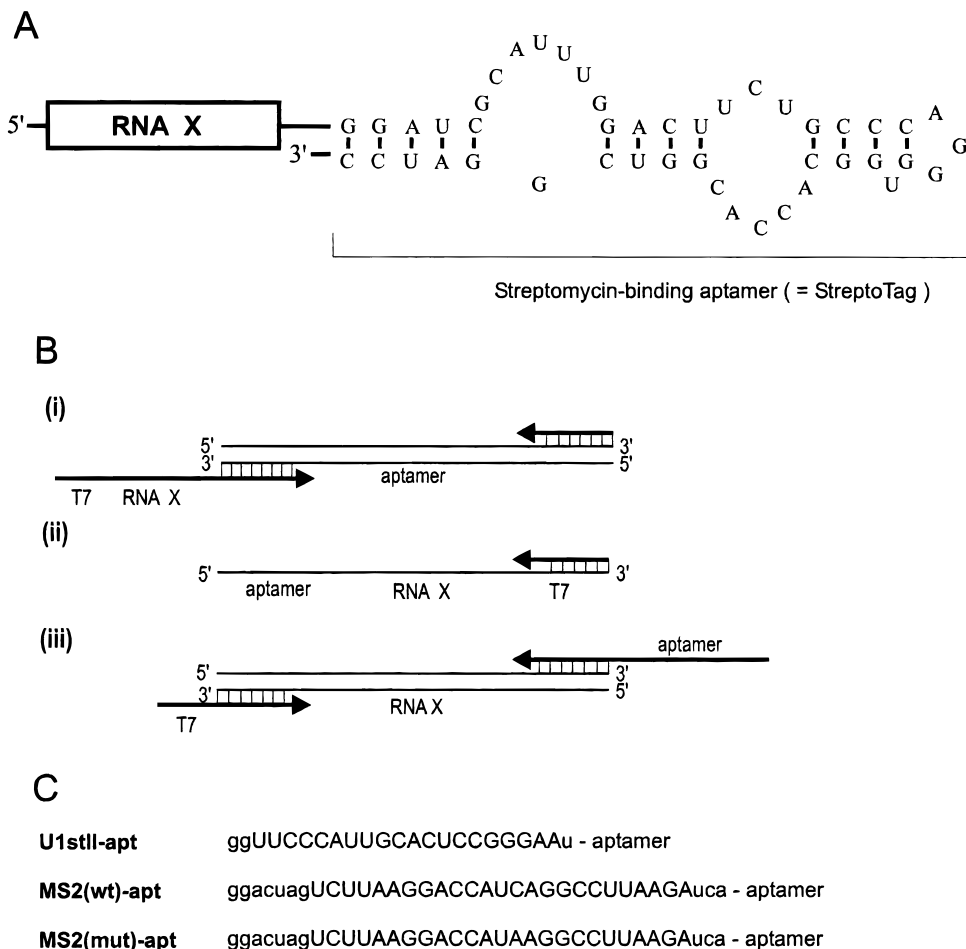

FIGURE 1. **A**: Schematic drawing of a hybrid RNA containing an RNA of interest (RNA X) and the streptomycin-binding aptamer (StreptoTag). **B**: Different PCR strategies for the construction of DNA templates to generate hybrid RNAs. **C**: Sequences and nomenclature of hybrid RNAs used for the purification procedure. The protein-binding sites are indicated in capital letters. A single mutation in MS2(mut)-apt RNA (underlined) decreases the binding affinity two orders of magnitude.

primer and an RNA X-containing template for PCR. The sequence and nomenclature of the hybrid RNAs can be seen in Figure 1C. Approximately two-thirds of the aptamer RNA is elutable from streptomycin affinity columns (Wallace & Schroeder, 1998; results not shown). We tested the binding and elution properties of several in vitro-transcribed hybrid RNAs containing sequences of different length and structure as the RNA X part. The addition of up to 100 nt to the aptamer did not influence the specific elution of hybrid RNAs (results not shown). The insertion of the aptamer into a group I intron (approximately 250 nt) even improved the elution capability (results not shown). This could be due to the strong three-dimensional structure formation of the intron RNA forcing the aptamer into the correct folding.

The MS2 coat protein was expressed under the control of the PGK/GAL fusion promoter from YCpCP (Stripecke et al., 1994). We generated several myc epitope-tagged versions of a fragment of the human U1A protein containing the N-terminal 102 amino acids, with or without a nuclear localization signal (NLS) expressed under the control of different promoters (see Materials and Methods). The in vitro binding of yeast-expressed MS2 coat protein and U1A-myc6 protein to their corresponding hybrid RNA under column conditions was confirmed by gel retardation assays (results not shown).

Purification of the U1A protein

Stem-loop II of U1 snRNA in the hybrid RNA U1stII-apt RNA is sufficient to interact with a fragment of the U1A protein containing residues 1–102 with a $K_d < 10^{-9}$ (Hall & Stump, 1992). For the in vitro assembly of the RNA–protein complexes, 150 pmol U1stII-apt RNA were mixed with 50 μg crude yeast extract expressing U1A-myc6, incubated for 20 min under column buffer conditions at room temperature, followed by a 10-min incubation in 2 μg/μL heparin. Subsequently, the RNA–protein mixture was applied to the affinity column. After three washes for the removal of unspecific RNA-binding proteins, specific RNA–protein complexes were eluted with 10 μM streptomycin. Elution and washing fractions were pooled and analyzed by silver-stained SDS-PAGE. Figure 2A shows the purification of U1A-myc6 protein by StreptoTag. A single polypeptide corresponding to the U1A-myc6 protein could be isolated with almost no background of nonspecific proteins (Fig. 2A, lane 2). The identity of the U1A protein was confirmed by Western blotting with α-myc antibodies (results not shown). As expected, in the absence of U1A-myc6 protein (Fig. 2A, lane 4) or with a hybrid RNA without the U1A protein binding motif (apt-RNA; Fig. 2A, lane 6), no U1A-myc6 protein was detectable. Lanes 1, 3, and 5 in Figure 2A show an aliquot of the pooled washing fractions.

Efficiency of StreptoTag purification

To evaluate the efficiency of StreptoTag-based protein purification we isolated U1A protein expressed at different levels in yeast (i) or from diluted U1A-myc6 protein containing extract (ii) and by varying the hybrid RNA concentration for in vitro complex formation (iii).

(i)

Several U1A protein constructs that express the protein at different levels were transformed into yeast and extracts were prepared and used for affinity purification. A NLS-U1A-myc9 fusion protein was expressed under the control of GAL1 promoter or a shortened GAL1 promoter (GAL S; Mumberg et al., 1994); the expression of full-length human U1A protein from YCpU1A was driven by the GAL/PGK fusion promoter (Stripecke et al., 1994). The purification procedures were performed as described in Figure 2A except that 100 μg of the different crude extracts were used for in vitro complex formation and one additional washing step was performed. The purification of U1A protein, expressed at the different levels, is shown in Figure 2B. As expected, both U1A fusion proteins (U1A-myc6 and NLS-U1A-myc9) expressed under the control of the GAL1 promoter were isolated with identical efficiency (Fig. 2B, lanes 1 and 2). The small amount of purified U1A protein (Fig. 2B, lane 3), expressed under the control of the GALS promoter, directly correlates with the weak expression rate that was shown to be $\sim$15-fold decreased compared to expression driven by the original GAL1 promoter. Full-length U1A protein expressed under the control of the GAL/PGK fusion promoter could also be purified (Fig. 2B, lane 4).

(ii)

Crude extracts expressing U1A-myc6 protein under the control of the strong GAL1 promoter were diluted 20-fold with extract expressing no U1A protein and used for affinity chromatography. The purification of U1A-mcy6 protein from 1 mg diluted extract was performed as described above except for washing the columns with eight column volumes of buffer (Fig. 2C, lanes 4–8). Diluting the extract did not change the purification efficiency when compared to U1A-myc6 protein purification from undiluted extract (Fig. 2C, lanes 3 and 5). The U1A protein could even be isolated when we used 8 mg of 80-fold diluted yeast extract for in vitro complex formation (Fig. 2C, lane 8). Both approaches (i), the expression from the weak GAL S promoter, and (ii), the 80-fold dilution of the amount of specific RNA-binding protein, approximate the range of weakly expressed genes (Iyer & Struhl, 1996). Convincingly, the highly increased amount of extract resulted in only a slightly increased background of unspecific RNA-binding pro-
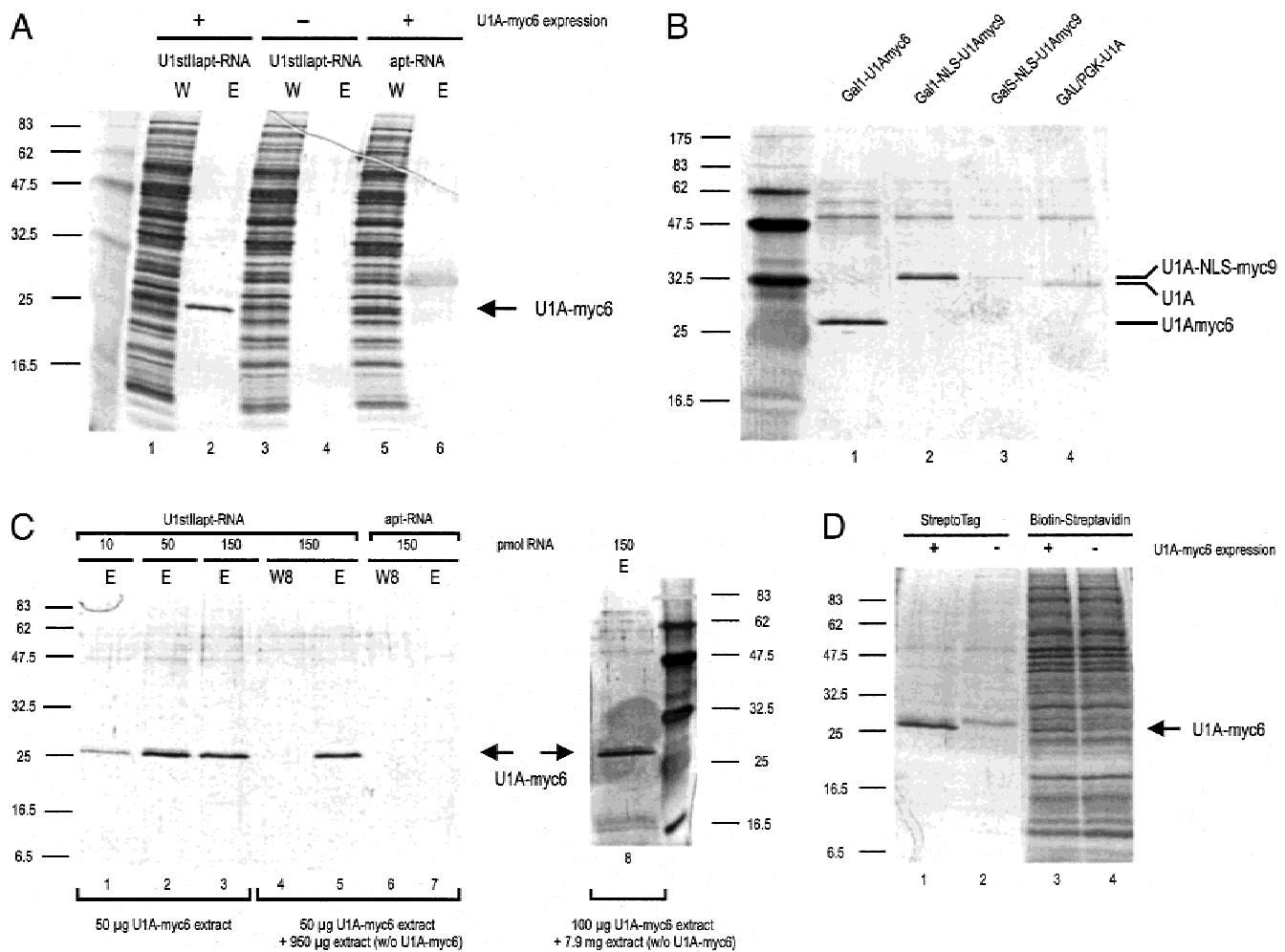
M. Bachler et al.

FIGURE 2. Purification of U1A protein with StreptoTag. **A:** By SDS-PAGE (silver-stained) separation of the pooled elution fractions (E) from streptomycin-columns and aliquots of the total washing fractions (W). The U1A-myc6 protein is indicated by an arrow. + or − indicates extract with or without U1A-myc6 protein expressed, respectively. U1stII-apt RNA: hybrid RNA containing the StreptoTag and the U1A protein binding site; apt-RNA: StreptoTag alone. **B:** Purification of U1A protein expressed at different levels. Shown are the elution fractions after purification. Different U1A fusion proteins were expressed under the control of GAL1 (strong promoter), GALS (very weak promoter) or PGK/GAL promoter as indicated in the figure. The isolated U1A proteins are indicated on the right. **C:** Purification of U1A-myc6 protein with the use of different amounts of hybrid RNA (lanes 1–3) or from U1A-myc6 protein extracts diluted 20-fold (lanes 4–7) or 80-fold (lane 8) with extract containing no U1A protein. E: eluted fraction; W8: eighth washing fraction. **D:** Comparison of the purification efficiency of StreptoTag and the biotin-streptavidin-system. 3′ biotinylated U1stII-apt RNA was used for both purification procedures. Shown are the elution fractions after purification. + or − indicates extract with or without U1A-myc6 protein expressed, respectively. The arrow points to U1A-myc6 protein enriched by StreptoTag (lanes 1 and 2) in comparison to biotin-streptavidin affinity purification (lanes 3 and 4).

teins and indicates that the method can be easily downscaled or upscaled.

(iii)

Next we titrated different amounts of hybrid RNA into identical amounts of protein extract. U1A-myc6 protein was isolated from 50 μg U1A-myc6 containing extract with 10 pmol, 50 pmol, or 150 pmol U1stII-apt RNA. By comparing the silver-staining intensity of the specific U1A-myc6 protein band in Figure 2C, lane 1, with a protein standard (results not shown) we estimated the

amount of purified U1A-myc6 protein to be in the low nanogram range. This indicates a roughly equimolar amount of isolated protein and input hybrid RNA. The use of a fivefold amount of hybrid RNA increased the efficiency of U1A-myc6 protein purification accordingly (Fig. 2C, lane 2). Performing the in vitro complex formation with 150 pmol hybrid RNA did not further improve the purification efficiency, possibly indicating a saturation of the affinity matrix or limited amount of protein. We conclude from our results that RNA degradation is not a severe problem at the conditions tested; however, addition of carrier RNA does not influence the

streptomycin–aptamer interaction (results not shown) and could prevent possible RNA degradation.

Comparison of StreptoTag and biotin-streptavidin based protein purification

Next we compared the quality of StreptoTag purification to affinity purification based on the biotin-streptavidin interaction. Both strategies were performed in parallel to isolate U1A-myc6 protein from crude yeast extract. Three hundred picomoles of U1stII-apt RNA were biotinylated at the 3′ end and incubated with 1 mg U1A-myc6 extract (fivefold diluted with extract expressing the empty vector) for 20 min under column conditions. After incubation in 2 μg/μL heparin, the in vitro formed complexes were split into two parts and applied to the streptomycin affinity column or mixed with streptavidin-coated magnetic beads. Both purification procedures were performed under identical buffer conditions and eight rounds of washes. The elution fractions of both purification strategies were analyzed by silver-stained SDS-PAGE. Figure 2D shows the isolation of U1A-myc6 protein with the two different methods. Again, the StreptoTag-purified U1A-myc6 protein can be seen together with a very low amount of unspecific proteins (Fig. 2D, lane 1). The only additional weak band migrating above the U1A-myc6 protein in Figure 2D, lanes 1 and 2, is most likely the input RNA, because a similar band appeared when we loaded hybrid RNA not incubated with proteins on a silver-stained SDS-PAGE (results not shown). By employing the streptavidin-coated magnetic beads, the U1A-myc6 protein could be isolated too, but with a much higher background of unspecific proteins (Fig. 2D, lanes 3 and 4). These copurifying proteins could either be in vivo biotinylated proteins (Lindqvist & Schneider, 1996) or could result from unspecific binding proteins that are released during elution upon heating of the beads at 95 °C in SDS-boiling buffer.

Purification of the MS2 coat protein

The bifunctional MS2(wt)-apt RNA comprises the streptomycin-binding aptamer and the wild type hairpin structure taken from the MS2 replicase mRNA interacting with MS2-CP with an affinity in the low nanomolar range (Lowary & Uhlenbeck, 1987; Witherell et al., 1991). The purification procedure was performed as described above with 1 mg extract and eight rounds of washes. The purification of MS2 coat protein can be seen in Figure 3. We isolated MS2-CP to almost homogeneity (Fig. 3, lane 1). As expected, MS2-CP could not be detected when extract expressing the empty vector was used for the in vitro complex formation (Fig. 3, lane 2). To verify that the new purification procedure allows the isolation of low affinity RNA-binding
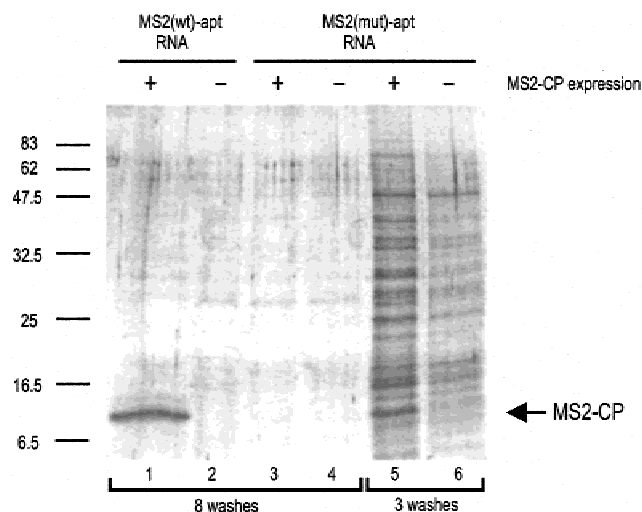

FIGURE 3. Affinity purification of MS2 coat protein (MS2-CP) with wild-type (MS2(wt)-apt RNA) or a mutant RNA (MS2(mut)-apt RNA) harboring a low affinity protein-binding motif. Shown are the elution fractions after purification, separated on a silver-stained SDS-PAGE. The arrow points to the purified MS2-coat protein (lanes 1 and 5). The mutant RNA with a K_d in the low millimolar range (Lowary & Uhlenbeck, 1987; Witherell et al., 1991) allowed purification of the MS2 coat protein only at less stringent conditions (lanes 5), resulting in a higher background (lanes 5 and 6); no effort was made to improve this purification.

proteins as well, we used a hybrid RNA containing a mutant MS2-CP binding site, termed MS2(mut)-apt RNA. This mutant protein-binding motif is reported to form a very weak RNA–protein complex with an affinity in the low millimolar range (Witherell et al., 1991). Applying the above described conditions to the purification procedure with MS2(mut)-apt RNA, no MS2-CP was detectable in a silver-stained SDS-PAGE (Fig. 3, lanes 3 and 4). When we decreased the number of washing steps and left out the heparin incubation, the MS2-CP could be purified (Fig. 3, lanes 5 and 6). By comparing lanes 5 (in vitro complex formation with MS2-CP) and 6 (in vitro complex formation with crude yeast extract containing no MS2-CP), an additional band, corresponding to MS2-CP, becomes visible in lane 5. As expected, fewer washes of the column resulted in a higher background of unspecific binding proteins; yet, we have not tried to improve this purification. Binding affinities of RNA–protein interactions vary within a broad range. Unknown RNA–protein complexes are often disrupted during conventional purification procedures and are therefore difficult to detect. Our results suggest that StreptoTag facilitates the isolation of uncharacterized physiologically relevant RNA-binding proteins as we successfully enriched a low affinity RNA-binding protein with a K_d in the low millimolar range.

In summary, StreptoTag offers a versatile approach for the detection and purification of RNA-binding proteins. Our silver-staining protocol detects less than 5 ng protein (results not shown), supporting the high

purity of StreptoTag-isolated proteins. Streptomycin affinity chromatography was successfully performed under buffer conditions ranging from 50–300 mM NaCl or KCl, 2–5 mM MgCl$_2$, pH 6.8–7.9, at room temperature or at 4 °C. As magnesium ions are essential for the streptomycin-aptamer interaction, the elution can be carried out by the addition of EDTA (in molar excess to magnesium ions), alternatively to 10 μM streptomycin (results not shown) to avoid a possible interference of the antibiotic with subsequent assays. The mode of generating the chimeric DNA templates for in vitro transcription of hybrid RNAs (as shown in Fig. 1C) does not affect purification, as all hybrid RNAs transcribed from the diversely constructed chimeric DNA templates were successfully used to purify the corresponding RNA-binding proteins. However, it is probably advisable to minimize the length of the RNA to be fused to the aptamer RNA, because its folding could impair correct structure formation of the aptamer, which is the basis for binding to the antibiotic on the column. In vitro-selected RNAs have been used for different applications, for example, translational control, drug resistance, or fluorescence tag technology (Gold et al., 1997; Holeman et al., 1998; Werstuck & Green, 1998). Our new method expands the capacity of aptamers to biochemical protein purification.

MATERIALS AND METHODS

Construction of hybrid RNAs

The DNA templates for synthesizing the various hybrid RNAs were generated by PCR from the streptomycin-binding aptamer clone #128 (Wallace & Schroeder, 1998; presented in Fig. 1A) with appropriate primers containing the various protein-binding sequences as shown in Table 1. Oligo U1stII-apt could be used as a single-stranded template; however, converting it into double-stranded DNA by asymmetric PCR under standard conditions improved the efficiency of the transcription reaction.

All PCR templates were phenolized and precipitated with ethanol. In vitro-transcription reactions were carried out in a 100-μL volume and contained 3–5 μg PCR template DNA,

5 mM NTPs, 1 mM dithiothreitol (DTT), 40 mM Tris-HCl (pH 7.5), 26 mM MgCl$_2$, 3 mM Spermidine, 40 U of human placenta RNAse inhibitor, and 4 μL T7 RNA-polymerase (80 U). Reactions were incubated at 37 °C for 3 h. Subsequently DNAse I (10 U) was added and incubated for 30 min. All RNAs were purified by electrophoresis in 5–8% denaturing polyacrylamide-7 M urea gels, overnight elution in a buffer containing 250 mM NaCl, 10 mM Tris-HCl (pH 7.5), 2 mM EDTA, and 0.1% sodium dodecyl sulfate (SDS), followed by extraction with phenol-chloroform and chloroform and ethanol precipitation.

Plasmid construction

For the expression of the MS2 coat protein we used YCpCP (Stripecke et al., 1994). To express U1A protein, we generated p415 GAL1 U1A-myc6, which expresses a C-terminal myc6-epitope tagged version of a 102-amino-acids fragment of the human U1A protein under the control of the GAL1 promoter. A *Spe*I-*Bam*HI U1A protein fragment (amplified by PCR with restriction enzyme sites (underlined) containing primers 5′-CATGTA*ACTAGTATG*GCAGTTCCCGAG-3′ and 5′-TAGGAT*GGATCCTTA*TCTAGACTCCACGAAGGTGCC TTTC-3′ was inserted into the *Xba*I-*Bam*HI sites of p415 GAL1, a CEN-based, single copy number plasmid (Mumberg et al., 1994). A *Xba*I site before the stop codon of the U1A coding region was then used to insert a *Spe*I-cut myc6-epitope cassette to generate p415 GAL1 U1A-myc6.

For the expression level comparison, shown in Figure 2B, the NLS from SV40 large T antigen was added at the N-terminus of a U1A fragment (102 amino acids) and a myc9-epitope tag was fused to the C-terminus to generate p415 GAL1 NLS-U1A-myc9 and p415 GALS NLS-U1A-myc9. Full-length U1A protein was expressed from plasmid YCpU1A (Stripecke et al., 1994).

Yeast culture conditions and preparation of protein extracts

The *S. cerevisiae* haploid strain W303 was grown at 28 °C in YPD medium (1.1% yeast extract, 2.2% peptone, 2% glucose) or YPD-plates. Transformants were selected on minimal media containing plates lacking uracil or leucine. Overnight inocula were grown in selective media containing 2% raffinose as the only carbon source. Induction of protein expres-

TABLE 1. Sequences of oligodeoxynucleotides used for the generation of DNA templates by PCR. T7 RNA polymerase promoter sequences are underlined.

RNA	Sense primer (5′–3′)	Antisense primer (5′–3′)
MS2(wt)-apt	TAATACGACTCACTATAGGACTAGTCTTAAGACCATCA GGCCTTAAGATCAGGATCGCATTTGGAC	ACTGGAATTCGGATCCGACCGTGG
MS2(mut)-apt	TAATACGACTCACTATAGGACTAGTCTTAAGACCATAA GGCCTTAAGATCAGGATCGCATTTGGAC	ACTGGAATTCGGATCCGACCGTGG
apt	TCTAATACGACTCACTATAGGAGCTCAGCCTTCACTGC	GTGGATCCGACCGTGGTGCC
U1stII-apt	TAATACGACTCACTATAGG	GGATCCGACCGTGGTGCCACCCTGGGCAG AAGTCCAAATGCGATCCATTCCCGGAGT GCAATGGGAACCTATAGTGAGTCGTATTA

sion was performed by diluting the cultures to an OD_{600} of 0.1–0.2 in YP media containing 2% galactose, and grown to an OD_{600} of 0.5–0.7. Yeast protein extracts were prepared as follows: yeast cultures of approximately 80 mL were harvested by centrifugation at room temperature. The pellet was washed once with 40 mL of cold sterile water and resuspended in 1 mL cold lysis buffer (150 mM NaCl, 50 mM Tris-Cl (pH 7.5), 3 mM $MgCl_2$). The cell suspension was briefly centrifuged and resuspended in 200 μL lysis buffer containing 1 mM phenylmethylsulfonyl fluoride, 0.5 μg/mL leupeptine, and 1 mM DTT. Cells were disrupted with glass beads at 4 °C by 4 cycles of 2 min vortexing on a vibrax and 1 min incubation on ice, followed by centrifugation for 5 min at 2,200 g at 4 °C. The supernatant was centrifuged again for 10 min at 15,000 g at 4 °C, transferred into a fresh tube, and kept on ice until incubation with RNA or stored at −80 °C. Protein concentrations were determined with the Bio-Rad protein assay kit and were in the range of 15–25 μg/μL.

Coupling of streptomycin to Sepharose 6B

The swelling of epoxy-activated Sepharose 6B (Pharmacia) was done according to the manufacturer's recommendations. Because the coupling reaction occurs in 10 mM NaOH and streptomycin undergoes irreversible hydrolysis under such high pH conditions, dihydrostreptomycin (Sigma) was used. To obtain a final antibiotic concentration of 1 mM on the matrix, and assuming a 33% coupling efficiency, the coupling reaction was done in 3 mM dihydrostreptomycin. For coupling the ligand 5 vol of 10 mM NaOH, pH 10, containing 3 mM dihydrostreptomycin were added per gram dry weight and shaken overnight at 37 °C. The solution was applied to a sintered filter and washed four times with 50 mL 10 mM NaOH, pH 10. The dihydrostreptomycin-Sepharose was washed with 3 cycles of alternating pH starting with pH 4 washing buffer (0.1 M CH_3COONa, 0.5 M NaCl) and pH 8 washing buffer (0.1 M $NaHCO_3$, 0.5 M NaCl). Dihydroxystreptomycin-Sepharose was washed four times with 50 mL double distilled H_2O and suspended in 5 vol of double distilled H_2O per gram Sepharose dry weight containing 1 mM EDTA and 0.05% sodium azide. The coupled matrix was stored in the dark at 4 °C for a maximum of 4 weeks.

Affinity purification

One milliliter dihydrostreptomycin-coupled Sepharose per column was equilibrated with 10 mL column buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 3 mM $MgCl_2$) at room temperature. In vitro-transcribed RNA (150 pmol) was renatured (incubated at 65 °C for 3 min in H_2O, and for 10 min at 37 °C in column buffer) in a 15-μL volume. The U1stII-apt RNA was mixed with yeast extracts ($\pm$ U1A-myc6 expression). The MS2-hybrid RNAs were mixed with 1 mg yeast extract ($\pm$ MS2-CP expression). The in vitro complex formation reactions were undertaken in 100−400 μL volumes under column buffer conditions and 0.25 mM DTT at room temperature. After the addition of 2 μg/μL heparin and continued incubation for 10 min, the mixtures were applied to equilibrated columns. Depending on the amount of yeast extract used for the in vitro complex formation, different numbers of washes

were carried out (e.g., 3 column volumes for 50 μg, 8 column volumes for 1 mg extract). Subsequently, the proteins were eluted with 4 column volumes of buffer containing 10 μM streptomycin (Sigma). One-milliliter fractions were collected and precipitated with trichloracetic acid (final concentration 5%). Washing and elution fractions were pooled in SDS-boiling buffer. The entire elution and one-fourth of the washing fraction (if not indicated otherwise) were analyzed on 13% SDS-PAGE and stained with silver.

Silver-staining of SDS-PAGEs

Proteins separated by SDS-PAGE were fixed by placing the SDS-gel in a mixture of ethanol/acetic acid/water (40/10/50) for at least 1 h or overnight. After 5 min of washing the gel with water, it was soaked in a cold (4 °C) solution containing 1% glutaraldehyde and 0.5 M sodium acetate. Subsequently, the gel was washed three times for 10 min with cold water. The gel was stained for 30 min in silver nitrate solution, prepared as follows: 0.6 g silver nitrate was dissolved in 3 mL water. Sixteen milliliters of double distilled water, 1 mL 25% NH_3 solution, and 500 μL 3 N NaOH were agitated in a graduated cylinder. The concentrated silver solution was gently added to the ammoniac/NaOH solution under constant stirring. After it cleared, double distilled water was added to a total volume of 100 mL. After staining, the gel was washed four times for 4 min in water and developed in a solution containing 0.005% citric acid and 0.1% formaldehyde. When the required development had been achieved, the gel was immersed in stopping solution (5% Tris-base and 2% acetic acid).

Biotin-streptavidin affinity purification

Coupling of biotincarbamoylhydrazide (Sigma) to the 3′ end of periodate-treated U1stII-apt RNA was carried out as described (von Ahsen & Noller, 1995).

Washing of streptavidin-coated magnetic beads (Dynabeads M-280, Dynal A.S., Oslo, Norway) was done according to the recommendations of the manufacturer. Fifty-microliter beads were equilibrated by incubating in 200 μL column buffer (150 mM NaCl, 50 mM Tris-HCl (pH 7.5), 3 mM $MgCl_2$) for 10 min with several buffer changes. One hundred microliters of in vitro-formed complexes were mixed with beads and incubated for 15 min at room temperature. Nonspecifically bound proteins were removed with eight rinses of 200 μL column buffer and specific proteins were eluted in SDS-boiling buffer and analyzed on 13% SDS-PAGE and stained with silver.

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