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A General Method for Isolating Targets of RNA and DNA Binding Proteins

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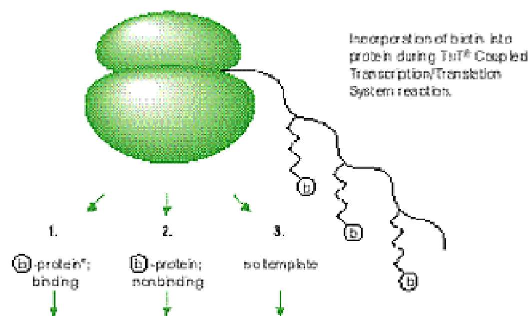
To isolate and characterize in vivo *targets of RNA and DNA binding proteins, our laboratory used a novel strategy in which biotinylated protein produced using TNT® Coupled Transcription/Translation System*[(a)] *is bound to a SoftLink™ Soft Release Avidin*[(b)] *solid support and used to capture either mRNA (from total RNA) or DNA. Bound nucleic acids are eluted from the solid support and then amplified by differential display RT-PCR*[(c)] *(DDRT-PCR). Bands specific for the target protein are reamplified and cloned into the pGEM®-T Vector*[(d,e)] *for subsequent sequencing. Cloned products can then be used to define the binding motif in the target using a sensitive biotinylated protein capture assay.*

Introduction

RNA and DNA binding proteins mediate a diverse array of cellular processes including transcription (1), translation (2), RNA processing (3,4), RNA transport (5), RNA localization (6), RNA storage (7) and mRNA stability (8). Binding occurs at specific protein domains (9,10), or sets of domains, to both primary and higher order structure within the target nucleic acid (11-13). In many cases, a particular binding protein may bind specifically to multiple targets (14,15), affecting complex regulatory schemes. We have developed a system based on programmed incorporation of biotin into the RNA or DNA binding protein of interest that permits affinity selection of the nucleic acid targets of these proteins.

Isolating RNA or DNA binding protein targets

Figure 1 outlines a three-step scheme for isolating targets of DNA- or RNA-binding proteins using the latter as the example. Unless otherwise stated, all manipulations were performed at 4°C in the presence of 1u/µl RNasin® Ribonuclease Inhibitor and a protease inhibitor cocktail (16). First, a biotinylated binding protein or protein binding domain is synthesized by incorporating biotinylated lysines in a coupled transcription/translation reaction using the TNT® System (17) programmed with plasmid DNA. At least one lysine residue is required for biotinylated protein synthesis. In the unlikely event of no naturally occurring lysine residues, a lysine tag can be engineered at the end of the protein or peptide fragment. Next, the biotinylated binding protein is coupled to a SoftLink™ Soft Release Avidin solid support (18) making an affinity column. Total RNA or poly(A)+ RNA (19) is then incubated with the affinity resin in TBS buffer, allowing the protein/target RNA interaction to occur. Unbound RNA is removed batchwise by centrifugation at 18,500 x *g* for 30 seconds. After extensive washing in TBS buffer, the bound RNA is eluted under strong denaturing conditions. This RNA, which contains the entire subpopulation of RNA specifically bound to the biotinylated protein, must then be fractionated in a way that allows the identification of individual messages. Three techniques for doing this are currently available: differential display RT-PCR (DDRT-PCR), subtractive hybridization and reverse Northern blotting. Each of these techniques is equally valid, provided the proper controls are performed. In the experiments described here, DDRT-PCR amplification (20) followed by T-vector cloning (21) and sequencing was used to identify individual target mRNAs. An analogous procedure (without cDNA synthesis) can be performed using genomic DNA to identify DNA binding protein targets.



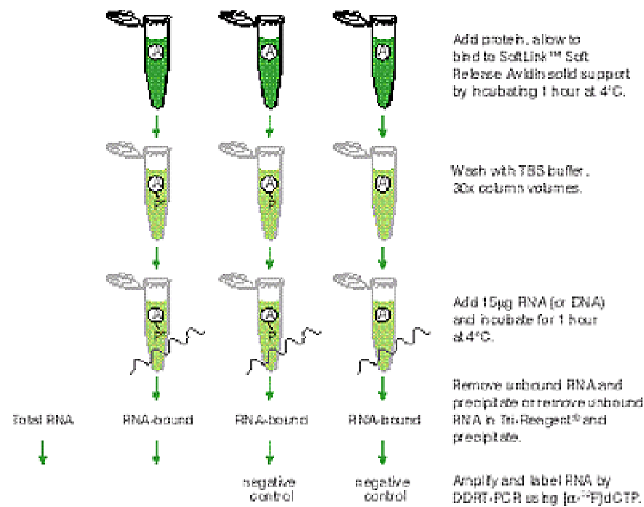
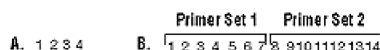


Figure 1. Experimental paradigm for isolating the targets of RNA and DNA-binding proteins. Biotinylated RNA or DNA binding proteins were produced in a 25µl TNT® Coupled Transcription/Translation System reaction (17) in the presence of 2µl Transcend™ tRNA (18) and were bound to 100µl of SoftLink™ Soft Release Avidin Resin at 4°C. Fifteen micrograms of total RNA or genomic DNA were added and allowed to bind for 1 hour. Unbound nucleic acid was removed with 30 column washes using TBS buffer. Bound nucleic acid was then eluted in Tri-Reagent® (Molecular Research Center; 19), and purified by ethanol precipitation. One-third of the purified products were converted to complementary ssDNA using one of the 3´-DDRT-PCR primers. A fraction (1/25th) of the complementary ssDNA sample was then amplified by DDRT-PCR in the presence of 2µCi [alpha-^{32}P]dCTP (20). The products were sized on denaturing polyacrylamide gels and select bands were cloned (21) and sequenced. To demonstrate specificity, two control reactions were run in parallel with the protein of interest, an unrelated control protein (column 2) and transcription/translation reaction without plasmid DNA (column 3).

To differentiate between RNAs specifically bound to biotinylated proteins and RNAs nonspecifically bound to the column resin or to proteins in the TNT® System lysate, two control batch-columns were run in parallel with the column containing the biotinylated protein (Figure 1, column 1). One control column (Figure 1, column 2) contained an unrelated nonbinding protein (the bacterial luciferase control supplied with the TNT® System). The other control (Figure 1, column 3) was a mock reaction column, containing all of the reagents for coupled transcription/translation except for the plasmid DNA used to produce the nucleic acid binding protein. RNAs from each of these three columns were then amplified by DDRT-PCR and subjected to side-by-side comparison on denaturing polyacrylamide gels. The only bands considered as potential target cDNAs were those present in the DDRT-PCR amplification from material eluted from column 1 and absent from the DDRT-PCR control reactions from columns 2 and 3.

DDRT-PCR often produces false positives, so a procedure designed to minimize this occurrence was followed (20). Each DDRT-PCR amplification was performed in duplicate using two different reverse transcriptases. DDRT-PCR amplifications were performed using 0.5-1.0µg of RNA from each column, a random 5´-15mer, and one of twelve 3´-22mers. The 3´-primers were of the following composition: 5´-GCGCAAGCT$_{12}$XN-3´ (where X is A, C or G, and N is any base). The DDRT-PCR products were analyzed on denaturing polyacrylamide sequencing gels. Only bands amplified in both enzyme reactions and specific to the target biotinylated protein of interest (column 1) were chosen for further analysis. The areas on the dried gels corresponding to these bands were carefully excised and rehydrated in 200µl of deionized distilled water. Fifteen microliters were then used for reamplification using the same cycling conditions as the DDRT-PCR protocol (20). The reamplified bands were subcloned into the pGEM®-T Vector (21). Clones containing the reamplified insert were identified by restriction digestion and sequenced. Figure 2 shows the results of a typical experiment using this scheme. In Panel A, DDRT-PCR amplification of unbound RNA from the wash fractions of an RNA binding protein column (lane 2), a putative non-RNA binding protein (lane 3) and mock transcription translation reaction mix (lane 4) were compared with an equivalent amount of total RNA before it was applied to the column (lane 1). The results clearly show that RNA binding occurs only in the presence of biotinylated protein (compare lanes 2 and 3 to lane 1). Further, the amount of binding is a function of the particular biotinylated protein (compare lanes 2 and 3). This experiment shows that the SoftLink™ Soft Release Avidin Resin does not bind many RNAs.



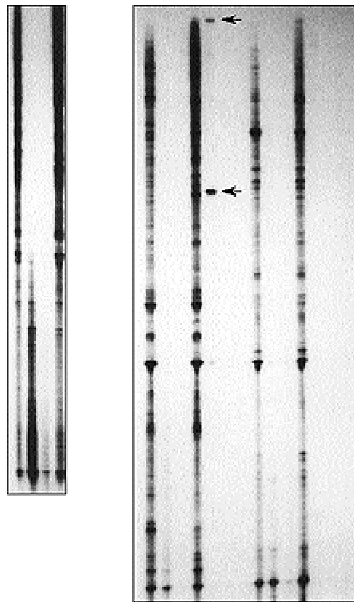


Figure 2. DDRT-PCR analysis of RNAs. Panel A: DDRT-PCR was performed using 15µg of total human brain RNA (lane 1), unbound RNAs obtained after passing 15µg of brain RNA through 100µl of a biotinylated protein-SoftLink^{TM} Avidin solid support (lane 2), a biotinylated negative control protein-SoftLink^{TM} Avidin solid support (lane 3) or a SoftLink^{TM} Avidin solid support (lane 4). **Panel B:** Specific brain cDNAs amplified from RNA bound to biotinylated protein. DDRT-PCR was performed using PCR primer set 1 (lanes 1-7) or primer set 2 (lanes 8-14). Lanes 1 and 8: DDRT-PCR products from total human brain RNA. Lanes 2-4 and 9-11: DDRT-PCR products from unbound RNA obtained from biotinylated target protein column washes (lanes 2 and 9), negative control column washes (biotinylated nonbinding protein, lanes 3 and 10; no template column, lanes 4 and 11). Lanes 5-7 and 12-14: DDRT-PCR products from RNAs bound to the biotinylated target protein column (lanes 5 and 12), negative control columns (biotinylated nonbinding protein, lanes 6 and 13; no template column, lanes 7 and 14). Arrows indicate cDNAs obtained from RNAs bound to the biotinylated protein SoftLink^{TM} Avidin solid support. One DDRT-PCR amplification using a single reverse transcriptase is shown.

Figure 2, Panel B shows that specific RNAs within the subpopulation of RNA bound to the biotinylated protein of interest, can be recovered by DDRT-PCR. These fragments were significantly enhanced (lane 5) compared to a similar amount of total RNA that was not bound to the affinity resin (lane 1) and were not present in the bound RNA of the negative control columns (compare lane 5 with lanes 6 and 7). In some cases, specific primer pairs do not result in the amplification of specific RNAs (lane 12). However, using a complete set of 165 DDRT-PCR primer combinations (22) should enable the majority of nucleic acid targets of a particular binding protein to be found since both weak binding and strong binding targets have been isolated. Figure 3, showing the effect of ionic strength on the biotinylated protein/nucleic acid target binding, illustrates this point.

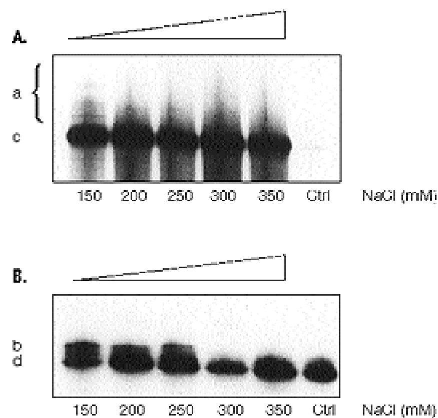


Figure 3. Effect of salt concentration on binding of RNA to biotinylated protein. DDRT-PCR of RNAs bound to the biotinylated protein-SoftLink^{TM} Avidin solid support at different salt concentrations reveals four levels of RNA binding. **Panel A:** (a) those that are weakly bound and (c) those that are independent of salt concentration but require biotinylated protein. **Panel B:** (b) those that are more tightly bound and (d) those nonspecifically bound to the SoftLink^{TM} Avidin solid support. Panels A and B represent magnified images of small regions of a single DDRT-PCR gel.

ANALYSIS OF TARGET:RNA BINDING PROTEIN INTERACTIONS

To confirm that the putative target mRNAs isolated using this technique specifically bound to the proteins of interest, solution binding

assays between ^{32}P-labeled *in vitro* transcribed RNAs (23) and the biotinylated binding protein (18), coupled with SoftLink™ Soft Release Avidin capture (24) of the RNA/binding protein complex, were performed. Using this procedure the only known mRNA target of the Fragile X RNA-binding protein FMR1P, namely FMR1 mRNA, was isolated (data not shown).

Once an RNA target has been identified, the site of its interaction with the binding protein can be mapped using a similar strategy. Figure 4 is a schematic diagram illustrating the gross localization of a target RNA binding motif. Solution binding assays between *in vitro* transcribed RNAs (23) and the biotinylated binding protein (18), coupled with SoftLink™ Soft Release Avidin capture (24) of the RNA/binding protein complex were performed on a series of ^{32}P-labeled 3´-end truncated target RNAs (Figure 4). Progressive truncation of the 3´-untranslated region of the mRNA was used to delineate the RNA-binding motif to a sequence spanning the 3´-end of the longest RNA that does not bind and to the 5´-end of the shortest truncated RNA that does bind. Fine structure mapping can then be performed once these endpoints are known.

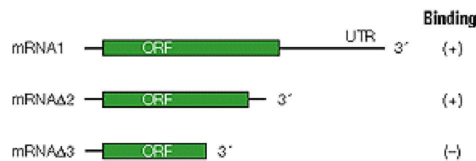


Figure 4. Human brain mRNAs isolated by DDRT-PCR bind biotinylated protein in a sequence-specific manner. One microgram of plasmid DNA containing full-length cDNA of a DDRT-PCR clone was linearized at three points within the putative target mRNA. ^{32}P-labeled mRNA1, mRNAdelta2 and mRNAdelta3 were generated by *in vitro* transcription using the RiboMAX™ Large Scale RNA Production System[(f)] (23). The schematic shows progressive truncation of the 3´-untranslated region (UTR) of mRNA1 relative to the end of its open reading frame (ORF). Labeled RNA was bound in solution to ~2ng of biotinylated protein (18). Bound protein-RNA complexes were captured using 10µl SoftLink™ Avidin Resin (24). Unbound RNA was removed by three washes (300µl each) with TBS buffer. The remaining bound RNA was quantified by liquid scintillation counting. Binding of mRNA is indicated by (+); nonbinding mRNA is indicated by (-).

Testing known RNAs with known nucleic acid binding proteins

Solution binding/affinity capture can also be performed with putative targets of a particular biotinylated nucleic acid binding protein without using the scheme in Figure 1 if an appropriate clone is available. In such cases, it is especially important to conduct control binding/affinity capture reactions using a mock reaction mixture to ensure that any observed binding is specific to the biotinylated protein of interest.

Summary

This article outlines a strategy to retrieve and characterize the *in vivo* targets of nucleic acid binding proteins. These methods should greatly aid researchers in understanding post-transcriptional gene regulation.

Acknowledgements

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References

1. Ghisolfi, L. *et al*. (1992) *J. Biol. Chem*. **267**, 2955.
2. Stripecke, R. *et al*. (1994) *Mol. Cell. Biol*. **14**, 5898.
3. Greenleaf, A. (1993) *TIBS* **18**, 117.
4. Reddy, R. and Busch, H. (1988) In: *Structure and Function of Major and Minor Small Nuclear Ribonucleoprotein Particles*, Birnstel, M., ed. Springer-Verlag, NY
5. Michael, W. *et al*. (1995) *Cold Spring Harbor Symp. Quant. Biol*. **60**, 633.
6. Ainger, K. *et al*. (1997) *J. Cell. Biol.* **138**, 1077.
7. Curtis, D., Lehmann, R. and Zamore, P. (1995) *Cell* **81**, 171.
8. Sachs, A.B. (1993) *Cell* **74**, 413.
9. Takimoto, M. *et al*. (1993) *J. Biol. Chem*. **268**, 18249.
10. Dubnau, J. and Struhl, G. (1996) *Nature* **379**, 694.
11. Zaidi, S.H., Denman, R. and Malter, J.S. (1994) *J. Biol. Chem*. **269**, 24000.
12. Harford, J. (1993) In: *Control of Messenger RNA Stability*, Belasco, J. and Brawerman, G., eds., Academic Press, NY.
13. De Guzman, R.N. *et al*. (1998) *Science* **279**, 384.
14. Serin, G. *et al*. (1997) *J. Biol. Chem*. **272**, 13109.
15. Sioud, M. and Jespersen, L. (1996) *J. Mol. Biol*. **257**, 775.

16. *Complete Cocktail,* Boehringer Mannheim Biochemicals.
17. *TNT® Coupled Wheat Germ Extract Systems Technical Bulletin* #TB165, Promega Corporation.
18. *Transcend™ Non-Radioactive Translation Detection Systems Technical Bulletin* #TB182, Promega Corporation.
19. *Tri-Reagent® RNA/DNA/Protein Isolation Reagent Technical Manual*, Molecular Research Center, Inc.
20. Sung, Y.-J. and Denman, R.B. (1997) *BioTechniques* **23**, 462.
21. *pGEM®-T and pGEM®-T Easy Vector Systems Technical Manual* #TM042, Promega Corporation.
22. Linskens, M.H.K. *et al*. (1995) *Nucl. Acids Res.* **23**, 3244.
23. *RiboMAX™ Large Scale RNA Production Systems-SP6, T3, T7 Technical Bulletin* #TB166, Promega Corporation.
24. Ashley, C.T. *et al*. (1993) *Science* **262**, 563.

Ordering Information

Product	Size	Cat.#
pGEM®-T Vector System I	20 reactions	A3600
RiboMAX™ Large Scale RNA Production System - T7		P1300
RNasin® Ribonuclease Inhibitor	2,500u	N2111
	10,000u	N2115
SoftLink™ Soft Release Avidin Resin	1ml	V2011
	5ml	V2012
TNT® T7 Coupled Reticulocyte Lysate System		L4610
TNT® T7 Quick Coupled Transcription/Translation System		L1170
Transcend™ Biotinylated tRNA	30µl	L5061

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