

Direct isolation of specific RNA-interacting proteins using a novel affinity medium

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ABSTRACT

Isolation of proteins that specifically interact with a given RNA or RNA regulation element is essential for studies on the molecular mechanisms of gene expression. Here, a novel method for direct isolation of such interacting proteins is described. It uses an affinity medium that consists of an interacting RNA with an artificially added 'tail', which is annealed to one end of a DNA 'arm', the other end of which is fixed covalently on the surface of aminosilanized glass powder. Thus the RNA itself is fully suspending, facilitating its interactions with proteins in its natural conformation. The proteins bound on the interacting RNA are eluted and subjected to SDS-PAGE, and the Coomassie-stained protein bands are cut and subjected to mass spectrometry (MS) analysis. Using this method, three proteins specifically interacting with the C/EBP β 3'-untranslated region (3'-UTR) RNA were isolated and identified. This method is simple and convenient, and the DNA-glass powder medium can be used repeatedly.

INTRODUCTION

RNA-protein interactions underlie the most important intracellular biological processes, such as all the steps of gene expression and its regulation (1) and cellular resistance to viral infection (2). Therefore, studies on RNA-protein interactions are essential for understanding the mechanisms of various life activities within the cell. For this purpose, isolation and characterization of the proteins specifically interacting with various RNAs are the first step of most of these studies.

The methods for isolation of RNA-interacting proteins and/or their genes or cDNAs are well documented. Screening of a cDNA expression library with a labeled RNA probe is a common and effective method (3). Besides, many others specific for certain protein factors that interact with RNA in their

functions are available (4–8). A very valuable *in vivo* method is the yeast three-hybrid system (9). It is unique in that it has created an intracellular mechanism for the interaction of specific proteins with their target RNAs, leading to expression of a reporter gene, which changes the phenotype of the yeast cell that harbors the cDNA of the protein. These methods are all useful and meet specific requirements and conditions of various studies.

The achievements of the Human Genome Project, and the advent of the protein mass spectrometry (MS) analysis and microsequencing (10) have provided new strong tools for research workers to find specific RNA-interacting proteins. As little as nanomole, or even less, amounts of protein samples can be analyzed using MS; therefore, the specific RNA-interacting proteins can be identified successfully, provided that they can be obtained in these amounts.

Here, a simple and effective method is described for the direct isolation of proteins that specifically interact with RNA. The principle of this method is to isolate RNA-interacting proteins by using an affinity medium, i.e. a protein-interacting RNA with its additional 'tail' annealed to an end of a DNA 'arm', which is immobilized through its other end on a glass base, and then to separate the bound proteins by SDS-PAGE. The protein bands in stained gels are subjected to MS analysis. The 3'-untranslated region (3'-UTR) of the mRNA of C/EBP β , a transcription factor currently under our studies, is used as an example.

MATERIALS AND METHODS

Cell line and extraction of cellular proteins

The SMMC7721 human hepatocarcinoma cell line (11) was provided by the Cell Bank of the Chinese Academy of Sciences (Shanghai) and kept in RPMI1640 medium with 10% newborn calf serum (top grade, Si Ji Qing Biotechnological Materials Co. Hangzhou, China, or an equivalent from Invitrogen) and antibiotics supplemented. The cytoplasmic proteins (C-fraction, or mixed C- and E-fractions) were extracted according to literature (12).

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***In vitro* transcription of RNA**

The recombinant plasmid pSP64/0.28, which harbors the cDNA of the C/EBP β 3'-UTR mRNA (0.28 kb fragment, with the poly A sequence deleted), was constructed by our group (13). It was digested with PvuII before *in vitro* transcription with the RiboMax large scale RNA production kit (Promega) according to the manufacturer's instructions.

Affinity medium preparation

Preparation of aminosilanized glass powder. Common glass powder (50–100 mesh, made in Shanghai, China) was cleaned thoroughly, dried, and allowed to react with a 1:1 (v/v) solution of 3-aminopropyltriethoxysilane (Sigma) in acetone at room temperature for 30 min. Then the glass powder was washed thoroughly with acetone and dried at 50°C.

Activation of DNA 'arms'. Plasmid pSP65 (Promega) was cut to its linear form with EcoRI. The linear plasmid (1 mg), dissolved in 100 μ l of 0.1 M MES (pH 6) and 0.5 M NaCl, was added into a tube containing 0.4 mg of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) (Sigma) and 1 mg of N-hydroxysuccinimide (Shanghai, China). The mixture was incubated at room temperature for 30 min. The reaction was stopped by the addition of 2 μ l of 2-mercaptoethanol, and the DNA was precipitated, washed and dissolved in 0.1 M MES (pH 6) and 0.5 M NaCl.

Mounting DNA 'arms' on glass powder surface. The solution of activated pSP65 plasmid DNA was mixed with 0.5–1 g of aminosilanized glass powder and allowed to react for 2 h at room temperature, with shaking. The glass powder was washed thoroughly with TE buffer. The mounting efficiency was estimated by the mixing of about 10^5 c.p.m. of the 32 P-labeled pSP65 with the activated pSP65 and measuring the immobilized radioactivity. Before using, the DNA-glass powder was treated with HindIII (100 U/g) for 2–4 h, and then washed thoroughly with TE buffer.

Annealing RNA on the DNA 'arms'. The *in vitro* transcribed C/EBP β 3'-UTR RNA (0.5 mg) was dissolved in the annealing buffer [90 mM NaCl, 2 mM EDTA, 10 mM HEPES (pH 7.8)] and mixed with the HindIII-treated DNA-glass powder. The mixture was incubated at 70°C for 10 min, cooled slowly (2–3°C/min) to 37°C, incubated again at 37°C for 2 h with shaking, and then cooled slowly to 4°C. The RNA–DNA-glass powder was washed thrice with the annealing buffer at 0–4°C, and stored at 4°C or frozen. The annealing efficiency was estimated by including about 10^5 c.p.m. of 32 P-labeled C/EBP β 3'-UTR RNA in the mixture, and counted the radioactivity remained on the glass powder.

Affinity capture of specific C/EBP β 3'-UTR RNA-interacting proteins

The cytoplasmic fraction(s) of the SMMC7721 human hepatocarcinoma cell extract (1 ml, about 10^7 cells) was mixed at 0°C with 0.25 ml of cooled 5 \times binding buffer [200 mM KCl, 15 mM MgCl₂, 50 mM HEPES (pH 7.8), 5 mM DTT, 15% v/v glycerol, 50 mg/ml heparin sodium and 200 μ g/ml yeast tRNA], and added to 0.5 g of 0°C ice-cold RNA–DNA-glass powder medium. The tube with the mixture was fixed to the rotation axle of a Pynus 70YN-2CT120G micromotor

with speed regulator (Pynus Micromotor Factory, Shanghai), and rotated at 1 r.p.m. at 0–4°C for 4 h or overnight. Subsequently, the RNA–DNA-glass powder was washed at 0–4°C with 1 \times binding buffer 5 times, 1 ml each time, and then with annealing buffer thrice, 1 ml each time. Finally, the RNA–DNA-glass powder was mixed with 1 \times protein loading buffer, heated at 80°C for 5 min, and the eluted proteins were electrophoresed on an 8% SDS–polyacrylamide gel. The Coomassie-stained protein bands were cut and analysed by MS.

Re-use of the DNA-glass powder

After elution, the affinity medium was heated at 80°C for 15 min in a large volume (3–4 ml) of TE with intermittent shaking, and then washed several times with TE at room temperature. The washed DNA-glass powder can be re-used for annealing with newly prepared RNA, at least for two additional uses.

Binding and UV-crosslinking of specific interacting proteins with C/EBP β 3'-UTR RNA

The reaction mixtures contained 10 μ l of cytoplasmic extract, 4 μ l of 5 \times binding buffer, 5 μ l of H₂O and 1 μ l of 32 P-labeled C/EBP β 3'-UTR RNA probe. The mixtures were incubated at room temperature (20–26°C) for 10 min, followed by UV irradiation with two 15 W UV tubes at 0°C for 30 min. The horizontal distance between UV tubes was 5 cm. The sample tubes (0.5 ml Eppendorf tubes), laid on a sheet of Saran Wrap covered over an ice-cold water surface, were mounted just beneath the UV tubes at a distance of about 10 cm. After UV treatment, each sample was digested with 1 μ l of 10 mg/ml RNase A (Promega) at 37°C for 20 min, and then separated by SDS–PAGE as before. The gels were fixed, dried and autoradiographed.

For target removal experiments, 10 μ l of cytoplasmic extract were incubated with 1 μ l of appropriate antibodies (kindly provided by Rong Zeng and Kan Liao; Santa Cruz) at 4°C for 2 h, and then incubated with 2 μ l of protein A-Sepharose (kindly provided by Xue-Jun Zhang and Mu-Jun Zhao) for 2 h. The mixtures were centrifuged and the supernatants were used for binding and UV-crosslinking experiments as above. The mouse anti-IgG antibody was used as a control.

RESULTS

Principle of the method

The principle of this method is illustrated in Figure 1. The interacting RNA is transcribed *in vitro* from the coding strand (+) of the recombinant pSP64 plasmid, which has been cut to linear form by the restriction enzyme PvuII; thus transcript contains a segment of RNA that is complementary to the vector sequence between the polycloning site and PvuII site, i.e. a 'tail', at the 3' end of the RNA. On the other hand, the plasmid pSP65, which shares the plasmid sequence with pSP64 except for the polycloning sites in reverse orientation, is cut with EcoRI. The linear pSP65 has phosphorylated 5'-termini, which can be activated by the water-soluble carbodiimide (EDC) and then linked covalently to an amino group on the aminosilanized glass. After HindIII treatment and heating, the strand of pSP65 identical to the (+) strand

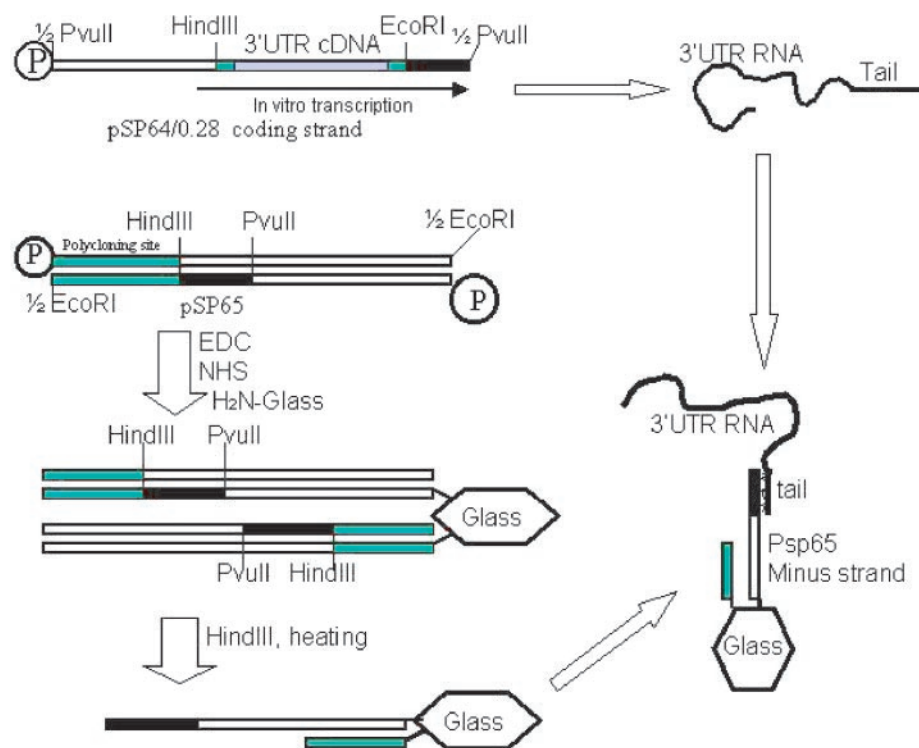


Figure 1. Construction of the affinity medium. The C/EBP β 3'-UTR RNA is transcribed from the coding strand of pSP64/0.28 plasmid cut with PvuII, so that a segment of the vector sequence is also transcribed forming a 'tail' at the 3' end of the RNA. The pSP65 plasmid was cut with EcoRI, so that, after EDC activation, linking to amino-glass surface, cutting with HindIII and heating, the minus strand of it remains linked on the glass with the sequence complementary to the 'tail' suspending. Therefore, the RNA is easily annealed to the DNA strand through its 'tail', leaving the RNA itself freely suspending, facilitating its interactions with proteins. The sequence complementary to 'tail' is shown in black.

of the pSP64/0.28 was removed. So, only the strand with the sequence complementary to the 'tail' of the interacting RNA remains linked to the glass, with the complementary sequence at the end that suspends in the microenvironment. Therefore, the interacting RNA is easy to anneal to that end. This annealing does not affect to the RNA itself, which at least theoretically should keep its natural conformation and bind its specific interacting proteins normally.

The amount of pSP65 DNA linked covalently on the amino glass was estimated to be 2 mg/g glass powder or more, and the C/EBP β 3'-UTR RNA which can be bound on the DNA-glass powder was about 50–100 μ g/g glass powder.

Direct isolation and identification of C/EBP β 3'-UTR RNA-specific interacting proteins by using the RNA–DNA-glass medium

Previously, we found that the C/EBP β 3'-UTR RNA specifically interacts with several cytoplasmic proteins (Figure 2). The method described above was used to isolate and identify them. Accordingly, a C/EBP β 3'-UTR RNA affinity medium was used for binding the specific cellular interacting proteins, with the DNA-medium without RNA as control. By using Coomassie blue BB250 staining, three apparent protein bands were detected reproducibly, whereas no band was found in corresponding positions in the lane to eluate from the control medium (Figure 3). These three bands were cut and sent to the National University of Singapore for MALDI-TOF-MS

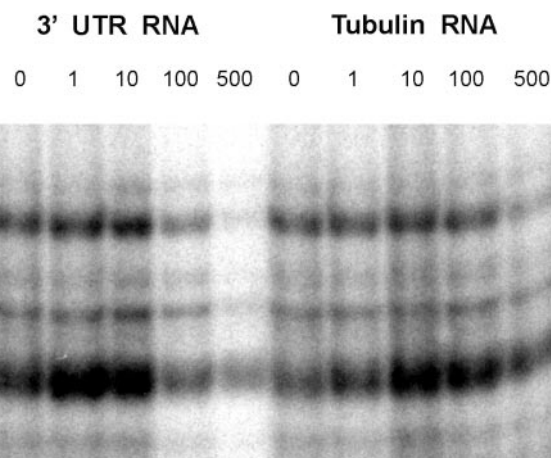


Figure 2. Binding and UV-crosslinking of cytoplasmic proteins to the 32 P-labeled C/EBP β 3'-UTR RNA probe in the absence and presence of a specific RNA competitor (C/EBP β 3'-UTR RNA) or a non-specific RNA competitor (tubulin RNA). Numbers indicate approximate molar ratios between competitors and the probe. PhosphorImager image is shown.

analysis. Results indicated that these proteins were vimentin, cytokeratin 8 and cytokeratin 18, respectively.

To confirm these results we performed a gel shift assay. However, when the antibodies to vimentin, cytokeratins 8 and 18 were allowed to interact with the cytoplasmic extract, and the binding was performed, almost all the radioactivity

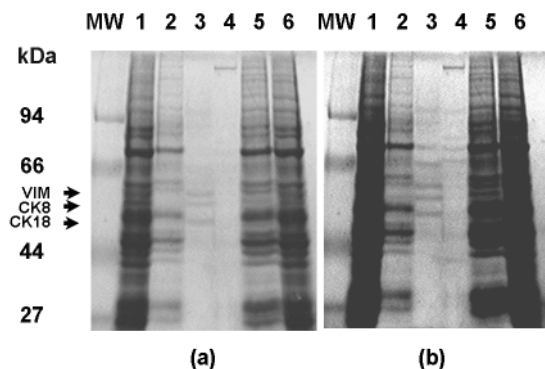


Figure 3. Analysis by PAGE of the proteins bound on the C/EBP β 3'-UTR RNA-DNA-glass powder medium. Cytoplasmic extract before reaction with the affinity medium (lanes 1 and 6). Cytoplasmic extract that was not bound to the C/EBP β 3'-UTR RNA-DNA-glass powder medium (lane 2). Eluate from the C/EBP β 3'-UTR RNA-DNA-glass powder medium (lane 3). Eluate from the control DNA-glass powder medium (without RNA) (lane 4). Cytoplasmic extract that was not bound to the control DNA-glass powder medium (lane 5). Arrows indicate bands of vimentin (VIM), cytokeratin 8 (CK8) and cytokeratin 18 (CK18), respectively, in lanes 3. (a), Staining was with Coomassie brilliant blue R-250. (b), Silver staining of the gel that was stained with Coomassie brilliant blue R-250.

remained in the sample wells and could not enter the gel. Considering that these proteins might be interacting with each other (14), efforts were made to do the immune reactions under denaturing conditions, but still failed (data not shown). Therefore, a 'target removal' strategy was used in the identification, i.e. to remove the proteins to be checked from the cellular extract before binding with radiolabeled RNA. Should this strategy work, some bands on the autoradiograph, representing the protein bound by a certain antibody, would become weaker. The result is shown in Figure 4. Although, this is not clear-cut, it is discernible that several bands are weaker compared with the control as well as with other bands, showing the likely positions of the three proteins. Therefore, these three proteins existed in the pool crosslinked to C/EBP β 3'-UTR RNA. The observation that one antibody often caused weakening of more than one band, was perhaps due to the interactions between those proteins.

DISCUSSION

This method was designed to meet our needs, that is, to investigate the proteins specifically interacting with C/EBP β 3'-UTR RNA. Our group has found that this 3'-UTR suppressed the malignant phenotype of the SMMC7721 human hepatocarcinoma cells (15), indicating that the interactions between this 3'-UTR and *trans*-acting proteins should play some role in the cellular phenotypic reversion. Efforts made to isolate those proteins led to develop this direct affinity method.

By using this method, three proteins interacting with C/EBP β 3'-UTR RNA were identified. The bands of the proteins captured by the RNA-DNA-glass powder were very reproducible, and their amounts were enough for MS analysis. Moreover, the glass powder sediments very quickly, the washing could be performed without centrifugation.

The key point for success of this method is that the capturing should be done at low temperature (0–4°C). At such temperatures, the amounts of bound proteins were not significantly

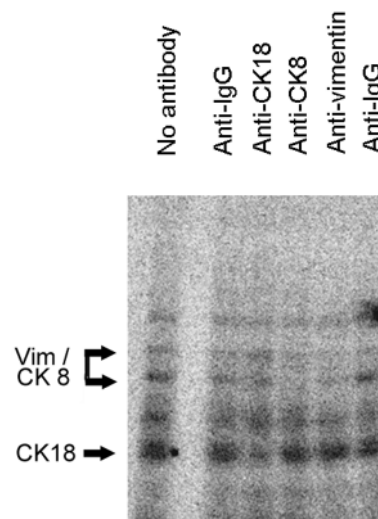


Figure 4. 'Target removal' experiment to identify proteins that specifically interact with C/EBP β 3'-UTR RNA. Various antibodies were used to remove their respective target proteins from the cytoplasmic extract before binding and UV-crosslinking with the C/EBP β 3'-UTR RNA probe. The control was an anti-IgG antibody. Vim, vimentin; CK8, cytokeratin 8; CK18, cytokeratin 18. PhosphorImager image with the contrast enhanced.

different with incubations of 4 h or overnight, a sign that the RNA was not degraded markedly, even in the absence of added RNase inhibitor. At higher temperatures (e.g. 25°C), RNase inhibitors (e.g. RNasin) must be added to protect RNA integrity. The ProtectRNA™ RNase inhibitor (Sigma), however, should be used with care, because it may prevent the binding of proteins to RNA.

The main disadvantage of this method is that less abundant or weakly interacting proteins may be more difficult to be captured. Furthermore, for different proteins and RNAs, the composition of the binding buffer may need modification.

In our work, three cytoskeleton proteins were found to interact with the C/EBP β 3'-UTR RNA. Despite the cytoskeletal proteins being components of the cellular architecture, recent data indicate that the cytoskeleton proteins, especially the intermediate filament components such as cytokeratins, are dynamic and motile within the cell (16). Therefore, we are inclined to think that they may also play the role of *trans*-acting factors, and interact with their target RNAs to perform some functions yet to be determined.

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Conflict of interest statement. None declared.

REFERENCES

1. Larson, D.E. and Sells, B.H. (1987) The function of proteins that interact with mRNA. *Mol. Cell. Biochem.*, **74**, 5–15.
2. Stanton, G.J., Weigent, D.A., Fleischmann, W.R., Jr, Dianzani, F. and Baron, S. (1987) Interferon review. *Invest. Radiol.*, **22**, 259–273.
3. Sagesser, R., Martinez, E., Tsagris, M. and Tabler, M. (1997) Detection and isolation of RNA-binding proteins by RNA-ligand screening of a cDNA expression library. *Nucleic Acids Res.*, **25**, 3816–3822.
4. Lax, S.R., Lauer, S.J., Browning, K.S. and Ravel, J.M. (1986) Purification and properties of protein synthesis initiation and elongation factors from wheat germ. *Methods Enzymol.*, **118**, 109–128.
5. Graham, D.A., Deb, J.K. and Busch, H. (1984) Purification of p53/55 kinase from nuclear ribonucleoproteins of Namalwa cells. *Mol. Cell. Biochem.*, **63**, 39–52.
6. Dreyfuss, G. (1986) Structure and function of nuclear and cytoplasmic ribonucleoprotein particles. *Annu. Rev. Cell. Biol.*, **2**, 459–498.
7. Stark, B.C. (1995) (1995–1996) Early studies of native ribonuclease P, including discovery of its essential RNA component. *Mol. Biol. Rep.*, **22**, 95–97.
8. Jurica, M.S. and Moore, M.J. (2003) Pre-mRNA splicing: awash in a sea of proteins. *Mol. Cell.*, **12**, 5–14.
9. Hook, B., Bernstein, D., Zhang, B. and Wickens, M. (2005) RNA-protein interactions in the yeast three-hybrid system: affinity, sensitivity, and enhanced library screening. *RNA*, **11**, 227–233.
10. Wysocki, V.H., Resing, K.A., Zhang, Q. and Cheng, G. (2005) Mass spectrometry of peptides and proteins. *Methods*, **35**, 211–222.
11. Dong, R.C., Zhou, R.H., Lu, F.D. and Tao, W.Z. (1980) Establishment of SMMC7721, a human hepatocarcinoma cell line, and observations on its biological characteristics. *Journal of 2nd Military Medical University (Shanghai)*, **1**, 5–9 (in Chinese).
12. Bronfman, M., Loyola, G. and Koenig, C.S. (1998) Isolation of intact organelles by differential centrifugation of digitonin-treated hepatocytes using a table Eppendorf centrifuge. *Anal. Biochem.*, **255**, 252–256.
13. Liu, D., Li, Z., Akira, S. and Kishimoto, T. (1996) Overexpression of a reversion-related protein in the revertant RR cells. *Sci China C Life Sci.*, **39**, 300–309 (The original paper in Chinese published in *Sci. China (ser.B)*, **25**, 372–378, 1995).
14. Tolstonog, G.V., Sabasch, M. and Traub, P. (2002) Cytoplasmic intermediate filaments are stably associated with nuclear matrices and potentially modulate their DNA-binding function. *DNA Cell Biol.*, **21**, 213–239.
15. Liu, D.G., Jiang, Q.H., Wei, Y.Y., Sun, L., Fu, B.B., Zhao, F.K. and Zhou, Q. (2003) Gene expression profile favoring phenotypic reversion: a clue for mechanism of tumor suppression by NF-IL6 3'UTR. *Cell Res.*, **13**, 509–514.
16. Helfand, B.T., Chang, L. and Goldman, R.D. (2004) Intermediate filaments are dynamic and motile elements of cellular architecture. *J Cell Sci.*, **117**, 133–141.