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Three-Hybrid Strategy Reveals a Peptide Segment That Specifically Binds to the 3′-Untranslated Region of NF-IL6 mRNA

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The 3′UTR of eukaryotic mRNA is an important regulation region, on which many *trans* factors act. In recent years, a series of 3′UTRs were shown to have tumor suppressor function, including the 3′UTR of the human nuclear factor for interleukin-6 (NF-IL6 3′UTR). To understand molecular basis for this function, we have tried to isolate genes encoding protein factors acting on the RNA of NF-IL6 3′UTR. Here we show that, by using a yeast three-hybrid system, a cDNA fragment was successfully isolated. This cDNA was allowed to express in *E. coli,* and its expression product, a polypeptide of ca. 70 amino acids long, was shown to specifically bind to the NF-IL6 3′UTR RNA. A search in GenBank did not reveal homologous sequences. Therefore, this cDNA fragment may be a part of the gene of a novel NF-IL6 3′UTR specific binding protein.

Key Words: **NF-IL6 3′UTR; RNA-binding protein; yeast three-hybrid system.**

The 3′ untranslated region (3′UTR) of eukaryotic mRNA plays important roles in diverse cellular processes, including early development, cell cycle control, cell growth and differentiation. Among other functions, it is very interesting that 3′UTR of some cellular genes, when separated from their coding regions and introduced by transfection into malignant cells, induced lowering in tumorigenicity, i.e., exerted tumor suppression function.

To date, to our knowledge, at least six 3′UTRs were found to have the potential of suppression of malignant phenotype. They are: 3′UTR of human nuclear factor for interleukin-6 (NF-IL6) mRNA (1, 2); 3′UTR of α-tropomyosin mRNA (3); 3′UTRs of mRNAs of ribonucleotide reductase R1 and R2 (4); 3′UTR of prohibitin mRNA (5); and 3′UTR of p15INK4b, a cell cycle inhibitor (6). All these 3′UTRs share a common characteristic, i.e., induction of suppression of tumorigenicity of those cells, in which the 3′UTRs were introduced cloned in certain eukaryotic expression plasmids. For example, NF-IL6 3′UTR suppressed the malignant phenotype of a part of transfectants of DT, a malignant cell line transformed by active *ras* oncogene (1), and of some human liver cancer cell line (unpublished); 3′UTR of α-tropomyosin mRNA induced tumor suppression in the NMU2 cells (3); etc. These facts indicate that the 3′UTR of some growth- and differentiation-related genes are involved in the regulation of cellular phenotype.

At present, the exact molecular mechanism of tumor suppression of those 3′UTRs remains obscure. The tumor suppressive activity of α-tropomyosin mRNA 3′UTR was assigned to the activation of a double-strand RNA-dependent protein kinase by the 3′UTR (7). However, we think it is difficult to explain, by using this hypothesis, why 3′UTRs without stable double-stranded structure also exerted tumor suppressor activity. On the other hand, it was proposed that 3′UTR of certain differentiation-specific RNAs were *trans*-acting regulators that inhibit cell division and promote differentiation (8). Consistent with this hypothesis, a reversion-related protein species was found in cells transfected with NF-IL6 3′UTR clone; the amount of this protein species was positively related to the extent of reversion of those cells, and it specifically bound to the RNA expressed from NF-IL6 3′UTR clone (2). Therefore, we imagined that the tumor suppressor activity of the 3′UTR of these genes might be exerted through direct interactions between their 3′UTRs and some *trans* factors with protein nature; and these pro-

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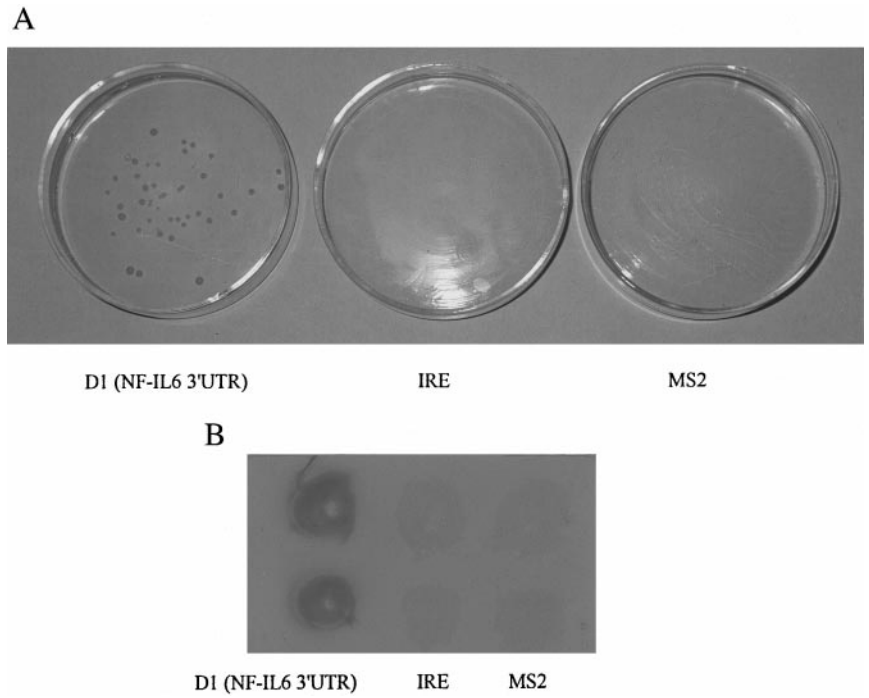

FIG. 1. (A) The test of the abilities of different RNA baits to reconstitute the transcription of the report gene *His.* Different RNA bait plasmids (D1, IRE, MS2) were introduced into the colony deprived of RNA plasmid, then transformants were selected on SD-Leu,his/3AT (synthetic minimal medium lacking leucine and histidine, 3AT was added at 5 mM). Only D1 (NF-IL6 3′UTR) bait reconstituted the transcription of the report gene *His.* (B) The test of the abilities of different RNA baits to reconstitute the transcription of *LacZ,* another report gene The cDNA plasmid was transformed with different RNA bait plasmids into L40-coat and β-galactosidase activity was tested. Only D1 (NF-IL6 3′UTR) bait reconstituted the transcription.

tein factors might in turn form a "traverse" signal transduction chain, which transfers signal from the 3′UTRs over to their target genes, thus resulting in the regulation of the phenotypes of the cells.

Based on this hypothesis, we have tried to study the molecular mechanism of tumor suppression of NF-IL6 3′UTR in an effort to find genes encoding cellular factors which specifically interacted with the RNA expressed from this 3′UTR. As it was already found that this 3′UTR was active in human liver cancer cells, we chose a human normal liver cDNA library to screen the cDNA for any possible specific NF-IL6 3′UTR RNA binding protein. For screening, we selected the yeast three-hybrid system, which was developed recently and was proved very useful for isolation cDNAs of RNA-binding proteins (9). Here we describe the successful isolation cDNAs of a cDNA fragment, which encoded a polypeptide that specifically bound to the RNA transcribed from NF-IL6 3′UTR.

MATERIALS AND METHODS

Yeast three-hybrid selection and screening. All yeast three-hybrid system plasmids and the yeast strain *L40-coat* (9, 10) were a gift of Dr. Marv Wickens, University of Wisconsin at Madison, U.S.A. Human liver two-hybrid cDNA library was a gift of Professor Yuan Wang, Shanghai Institute of Biochemistry. The RNA bait, namely NF-IL6 3′UTR bait, was constructed as follows. First, the 3′UTR fragment was PCR amplified from a clone of it, named pSP64/0.28 (2). The primers used were 5′-AGACCCGGGAAGAAGAAACG-TCTATG-3′ and 5′-AATCCCGGGACTTCGAAACCGGCCCGC-3′.

Then the amplified 3′UTR fragment, called D1, was cut by *Sma*I and inserted into the *Sma*I site of the RNA plasmid pIIIA/MS2-2 (10), forming pIIIA/D1-MS2. Plasmids were transformed into yeast cells by LiAc/PEG method (11). In library screening, the colony color assay was combined with the 5-FOA (5-fluoroorotic acid) negative selection (12). This combination effectively eliminated overwhelming majority of the false RNA-independent positive clones. For RNA plasmid deprivation, the primary his^+ colonies were first streaked onto SD-Leu (synthetic minimal medium lacking leucine) plate. After 2–3 days' growth, single colonies were picked out and streaked onto SD-Leu plate containing 0.1% 5-FOA. β-Galactosidase assay was done according to Bartel (13).

GST-fusion peptide expression and purification. The cDNA insert of the putative RNA-binding protein gene was cut by *Eco*RI and inserted into the *Eco*RI site of a GST (glutathione *S*-transferase) fusion vector, pGEX-6P-1 (Pharmacia). The correct orientation was confirmed by DNA sequencing. Then the recombinant GST fusion plasmid was transformed into *E. coli* strain *BL21 (DE3).* To induce expression of fusion protein, isopropyl-β-D-thiogalactoside (IPTG) was added into the culture medium to a final concentration of 1.0 mM when the cell density was about OD_{600} = 0.5-0.9, and the incubation was continued for additional 3–5 h at 37°C. Cells were harvested by centrifugation and were broken by sonication, then the lysate was loaded onto a prepacked glutathione Sepharose 4B column (Pharmacia). 5–10 mM of reduced glutathione was used to elute

the specifically bound protein. GST protein, the expression product of the blank vector pGEX-6P-1, was purified by the same way for control.

In vitro preparation of transcripts. Plasmid pSP64/0.28 was linearized by *Eco*RI. The plasmid pGMET-200 (gift of Dr. Ying Xiong), which encodes 200bp 3′UTR of CTP:phosphocholine cytidylyltransferase β2 (CCT) isoform (14), was used as control, and was linearized by *Nco*I. Radiolabeled NF-IL6 3′UTR RNA was produced by the *in vitro* transcription using Promega SP6 Ribroprobe *in vitro* Transcription System, and 50 μCi [α-^{32}P]UTP was included in the transcription reaction. The radioactive RNA was purified by electrophoresis on a 5% acrylamide/7 M urea denaturing gel, followed by the modified purification method of Maxam and Gilbert (15). Nonradiolabeled competitors, CCT 3′UTR and NF-IL6 3′UTR RNAs, were produced by *in vitro* transcription using AmpliScribe SP6 High Yield Transcription Kit (Epicentre).

Gel shift analyses. Gel-purified ^{32}P-Labeled NF-IL6 3′UTR RNA (about 5 ng) was mixed with the purified protein samples in 10 μl binding buffer containing 20 mM triethanolamine, pH 7.6/2.5 mM EDTA/100 mM NaCl/2 mM DTT/10% glycerol, and the mixture was incubated for 10 min at 0°C, then 5 min at room temperature. In the competition experiments, nonradiolabeled control RNAs or tRNA were added into the above reactions. RNA–protein complexes were separated at 4°C by 5% nondenaturing polyacrylamide gels. The gels were dried and autoradiographed at −70°C.

RESULTS

Screening of RNA-Binding Protein cDNA with Yeast Three-Hybrid System

The yeast three-hybrid system, that was developed in recent years, presents a valuable tool for detecting and analyzing RNA–protein interaction *in vivo.* This system derives from the yeast two-hybrid system, and utilizes the interaction between an RNA bait and an RNA-binding protein encoded by cDNA clones of a certain cDNA library, to reconstitute the Gal4 transcription activation, thus allowing expression of the report gene (*His, LacZ*), changing the phenotype of the yeast cells. This system has been successfully used in analyzing RNA–protein interaction, and several novel RNA-binding proteins have been isolated by this system since it was developed (10, 16–18). However, a major drawback of this system is the frequent occurrence of false-positive clones, due to RNA-independent activation of the reporter gene. In the first attempt, we used the colony color assay developed by Zhang *et al.* (10) to eliminate the false RNA-independent positives. However, even in the uniformly white colonies, we actually found more than 90% clones were still RNA-independent. This may be due to the very low frequency of spontaneous loss (about 0.01% per generation) of 2 μm DNA of the RNA plasmid (19). Then we combined this color assay with 5-FOA counterselection in later library screening, and found that this combination effectively eliminated a very large number of bait RNA-independent false positives, greatly improving the screening efficiency. We screened about 2×10^6

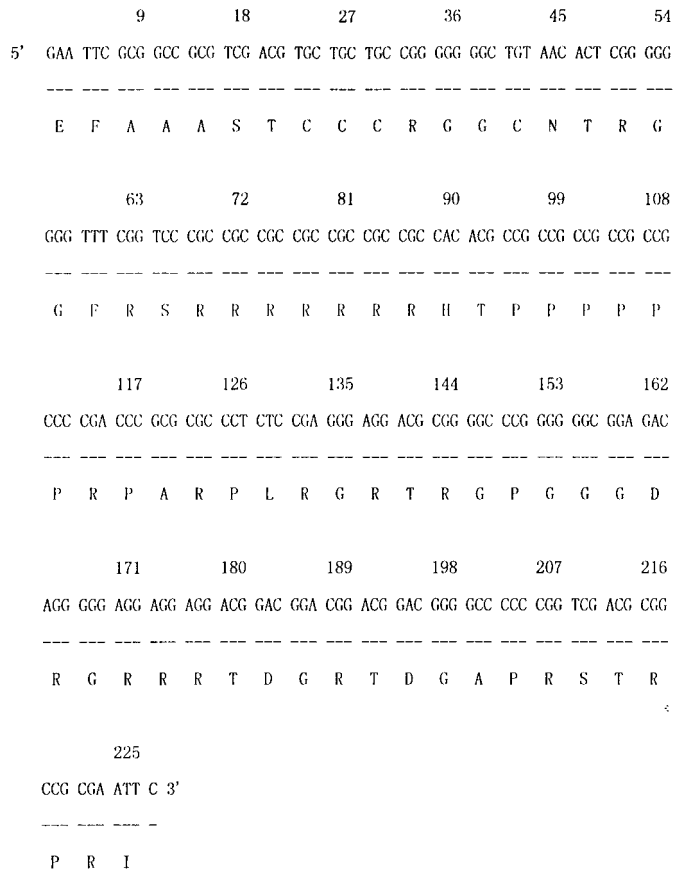

FIG. 2. DNA sequence and the predicted amino acid sequence of the cDNA plasmid of the putative RNA-dependent positive clone.

transformants, and found one colony met all the following criteria for RNA-dependent positives: it turned uniformly white in the medium lacking histidine and leucine; it could not survive in the medium containing 5-FOA and turned blue in the β-galactosidase assay. Then we deprived this colony of its RNA plasmid, and found that the RNA-deprived colony could not grow on the plate lacking histidine. We then reintroduced three different RNA bait plasmids, pIIIA/D1-MS2 (harbors NF-IL6 3′UTR D1 fragment), PIIIA/IRE-MS2 (harbors an iron response element, IRE) and PIIIA/MS2-1 (harbors MS2 binding sequence only), into this RNA-deprived colony, respectively. Only D1 fragment reconstituted the transcription of the report gene *His,* as shown in Fig. 1A. We also extracted cDNA plasmid of this RNA-dependent colony and reintroduced this cDNA plasmid, and different RNA plasmids, into the yeast strain L40-coat, respectively, then tested another report gene *LacZ.* As shown in Fig. 1B, only D1 fragment reconstituted the transcription.

We sequenced the cDNA plasmid of this RNA-dependent colony, and found that it encoded a 75 amino acid peptide, as shown in Fig. 2. No poly(A) tail

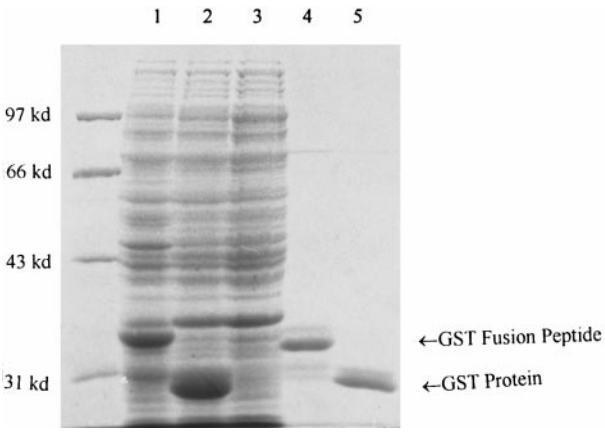

FIG. 3. Expression and purification of GST-fusion peptide. Lanes 1–3, total protein of induced cells containing GST-fusion peptide, GST and control respectively; lane 4, purified GST-fusion peptide; lane 5, purified GST protein.

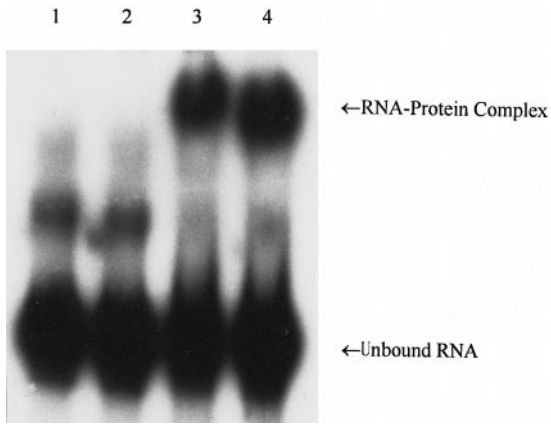

FIG. 4. Gel shift analysis of the binding of GST-fusion peptide with NF-IL6 3′UTR RNA. Purified protein sample (about 2 μg) was incubate with 5 ng radiolabeled NF-IL6 3′UTR RNA. Lane 1, RNA probe; lane 2, GST protein; lane 3, GST-fusion peptide; lane 4, GST-fusion peptide with tRNA added to 0.2 μg/μl.

was found at the 3′terminus of it. This indicated that this 70 amino acid peptide might come from the N-terminus or middle region of a large protein. We searched this sequence in GenBank by visiting the web site of NCBI, U.S.A., using the BLAST program, and found no homologous sequences. This suggested that this cDNA fragment may be a part of a novel specific binding protein gene of NF-IL6 3′UTR, i.e., its RNA recognition motif (RRM).

Expression of the cDNA Fragment in E. coli and Verification of Specific Binding of Expression Product to NF-IL6 3′UTR RNA

To test whether this cDNA encoded peptide really bound to NF-IL6 3′UTR RNA *in vitro,* this cDNA insert was cloned into the plasmid pGEX-6P-1 as a fusion protein gene, and was allowed to be expressed in *E. coli* strain *BL21(DE3)* cells. The expressed GST-fusion peptide was affinity-purified by Glutathione Sepharose 4B column from sonicated cells (Fig. 3).

The purified GST-peptide fusion protein was incubated with the purified radiolabeled NF-IL6 3′UTR RNA probe in the binding buffer. RNA–protein complex was resolved in 5% native gels. As shown in Fig. 4, GST-peptide fusion protein really bound to the labeled NF-IL6 3′UTR RNA, while the control, GST protein (without fusion with any insert), did not bind to the RNA probe. tRNA showed no significant competition. To show the dose-response effect, purified GST-peptide fusion protein, at a series of concentrations, was added in the binding buffer, respectively. As shown in Fig. 5, GST-peptide binds to all radiolabeled RNA probes when protein concentrations was above ~0.5 μg/μl. To test the specificity, non-radiolabeled CCT RNA and NF-IL6 3′UTR RNA were added into reaction mixture at a series of concentrations, as competitors, respectively. As shown in Fig. 6, CCT RNA showed no clear competition; however, NF-IL6 3′UTR RNA showed significant competition. Therefore, these facts confirmed the ability of this polypeptide to specifically bind to the RNA expressed from NF-IL6 3′UTR.

DISCUSSION

We have isolated a cDNA clone, which encoded a polypeptide that showed specific binding ability to the RNA expressed from the NF-IL6 3′UTR. And this polypeptide may be a part, namely the RNA binding motif, of a novel, probably larger protein that specifi-

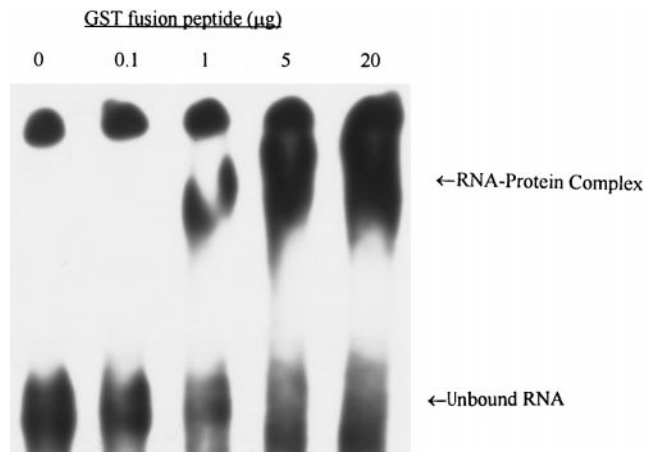

FIG. 5. The dose–response effect of purified GST-peptide fusion protein binding to NF-IL6 3′UTR probe.

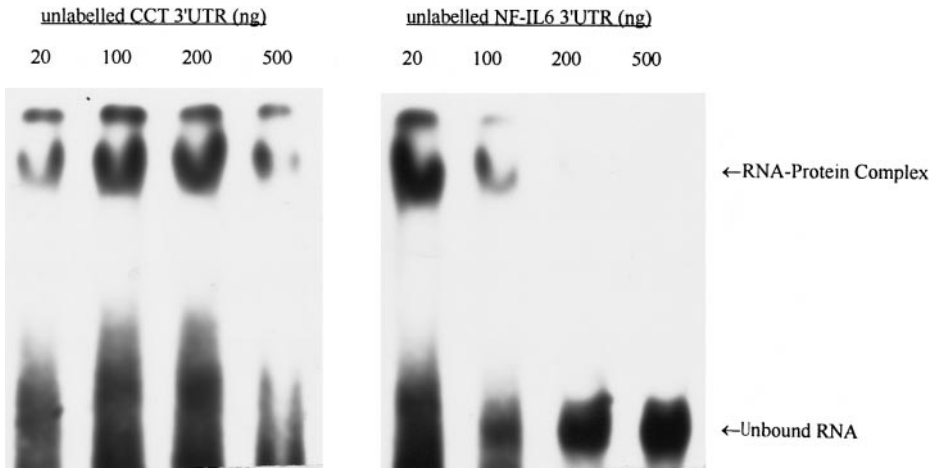

FIG. 6. Competition for unlabeled CCT 3′UTR and NF-IL6 3′UTR.

cally binds this 3′UTR. As mentioned above, it is proposed that 3′UTRs of mRNA may act as *trans* factors that convey signals to their targets; this should be done, of course, through direct interactions of them with other *trans* protein factors. Therefore, it is possible that the protein, a part of which is our 70 amino acid polypeptide, plays a role in such signal transduction. In our case, the 3′UTR of NF-IL6 is a tumor suppressor. This may mean that this specific binding protein, in turn, may be the product of a novel tumor suppressor gene, which has not been discovered yet.

On the other hand, the specific binding protein of NF-IL6 3′UTR may also be the first component of a putative signal transduction chain. Whether this chain exists, and what role does it play in cells at physiological conditions, are all interesting problems. We will continue our efforts.

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REFERENCES

1. Liu, D. G., Wang, D., Chen, Z. Z., Li, Z. P., Noda, M., and Ikawa, Y. (1991) A cDNA clone with antioncogene activity. *Sci. China (Ser. B)* **7,** 730–737 [Chinese]; (1992) *Sci. China (Ser. B)* **35,** 822–833 [English].
2. Liu, D. G., Li, Z. P., Akira, S., and Kishimoto, T. (1995) Overexpression of a reversion-related protein in the revertant RR cells. *Sci. China (Ser. B)* **25,** 372–378 [Chinese]; (1996) *Sci. China (Ser. C)* **39,** 300–309 [English].
3. Restinejad, F., Conboy, M. J., Rando, T. A., and Blau, H. M. (1993) Tumor suppression by RNA from the 3′untranslated region of α-tropomyosin. *Cell* **75,** 1107–1117.
4. Amara, F. M., Smith, G. M., Kuschak, T. I., Takeuchi, T. L., and Wright, J. A. (1996) A *cis–trans* interaction at the 3′-untranslated region of ribonucleotide reductase mRNA is regulated by TGF-beta 1, TGF-beta 2 and TGF-beta 3. *Biochem. Biophys. Res. Commun.* **228,** 347–351.
5. Jupe, E. R., Liu, X. T., Kiehlbauch, J. L., McClung, J. K., and Dell'Orco, R. T. (1996) The 3′untranslated region of prohibitin and cellular immortalization. *Exp. Cell Res.* **224,** 128–135.
6. Malumbress, M., Perez de Castro, I., Santos, J., Fernandez Piqueras, J., and Pellicer, A. (1999) Hypermethylation of the cell cycle inhibitor p15INK4b 3′-untranslated region interferes with its transcriptional regulation in primary lymphomas. *Oncogene* **18,** 385–396.
7. Davis, S., and Watson, J. C. (1996) *In vitro* activation of the interferon-induced, double-stranded RNA-dependent protein kinase PKR by RNA from the 3′untranslated regions of human α-tropomyosin. *Proc. Natl. Acad. Sci. USA* **93,** 508–513.
8. Restinejad, F., and Blau, H. M. (1993) Genetic complementation reveals a novel regulatory for 3′untranslated region in growth and differentiation. *Cell* **72,** 903–917.
9. SenGupta, D. J., Zhang, B., Kraemer, B., Pochart, P., Fields, S., and Wickens, M. (1996) A three-hybrid system to detect RNA–protein interaction *in vivo. Proc. Natl. Acad. Sci. USA* **93,** 8496–8501.
10. Zhang, B., Gallegos, M., Puoti, A., Durkin, E., Fields, S., Kimble, J., and Wikens, M. P. (1997) A conserved RNA-binding protein that regulates sexual fates in the *C. elegans* hermaphrodite germ line. *Nature* **390,** 477–484.
11. Gietz, D., St. Jean, A., Woods, R. A., and Schiestl, R. H. (1992) Improved method for high efficiency transformation of intact yeast cells. *Nucleic Acids Res.* **20,** 1425.
12. He, Y., Bao, L., and Liu, D. G. (2000) Colony color assay coupled with 5-FOA negative selection greatly improves yeast three-hybrid system library screening efficiency. *Chinese Sci. Bull.* (accepted).
13. Bartel, P. L., Chien, C.-T., Sternglanz, R., and Field, S. (1993) Using the two-hybrid system to detect protein-protein interactions. *In* Cellular Interactions in Development: A Practical Approach (Hartley, D. A., Ed.), pp. 153–179, Oxford Univ. Press, Oxford.
14. Xiong, Y., Wang, Y., Liu, X., and Du, Y. (1999) Cloning of a gene

in rat brain induced to be expressed differently by AVP_{4-8} with diferential display PCR. *Acta Biochim. Biophys. Sinica* **31,** 509–512.

15. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
16. Wang, Z. F., Whitfield, M. L., Ingledue, T. C., III, Dominski, Z., and Marzluff, W. F. (1996) The protein that binds the 3′ end of histone mRNA: A novel RNA-binding protein required for histone pre-mRNA processing. *Genes Dev.* **10,** 3028–3040.
17. Jan, E., Motzny, C. K., Graves, L. E., and Goodwin, E. B. (1999) The STAR protein, GLD1, is a translational regulator of sexual identity in *Caenorhabditis elegans. EMBO J.* **18,** 258–269.
18. Park, Y. W., Wilusz, J., and Katze, M. (1999) Regulation of eukaryotic protein synthesis: Selective influenza viral mRNA translation is mediated by the cellular RNA-binding protein GRSF-1. *Proc. Natl. Acad. Sci. USA* **96,** 6694–6699.
19. Toh-e, A., and Wickner, R. B. (1981) Curing of the 2μ DNA plasmid from *Saccharomyces cerevisiae. J. Bacteriol.* **145,** 1421–1424.