Biochemical and Biophysical Research Communications 426 (2012) 122–128

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Cancer cell growth suppression by a 62nt AU-rich RNA from C/EBPβ 3′UTR through competitive binding with HuR

Da-Quan Sun, Ying Wang, Ding-Gan Liu *

State Key Laboratory of Molecular Biology, Institute of Biochemistry and Cell Biology, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, China

ARTICLE INFO

Article history:
Received 7 August 2012
Available online 16 August 2012

Keywords:
Distinct AU-rich RNA
C/EBPβ 3′UTR
Tumor suppression
HuR
Competitive binding

ABSTRACT

AU-rich elements are functional motifs in the 3′untranslated region of mRNA and are binding sites for the RNA binding protein HuR, an mRNA stabilizer and translation enhancer implicated in carcinogenesis. It is not clear whether, and, if so, how the AU-rich elements function in cells when they are separated from their mRNA and form an independent RNA species. Here, we show that a short RNA with AU-rich elements derived from C/EBPβ 3′UTR suppressed growth in a human liver cancer cell line. It specifically bound HuR, and it competed with C/EBPβ mRNA in order to bind to HuR. Our results provide evidence that the cancer cell growth suppression by this 62nt RNA containing AU-rich elements may be due to competitive binding to HuR. This work may open new options for the development of novel anti-cancer drugs.

1. Introduction

Eukaryotic mRNA consists of several functional elements including the 5′untranslated region, the coding region, and the 3′untranslated region. These functional elements contain many shorter motifs, such as AU-rich stretches [1]. These functional elements and motifs have been studied extensively as integral parts of mRNA e.g. [2–4]. The AU-rich elements in mRNAs have been found to bind to HuR, a ubiquitous RNA binding protein, in order to regulate mRNA stability [4–8]. For example, in adipocytes, HuR binds to the AU-rich region of C/EBPβ mRNA [7]; and in ALK-transformed cells HuR binds to the C/EBPβ mRNA 3′untranslated region in order to stabilize it [9]. It has recently been found that, in primary fibroblasts, the C/EBPβ coding region plus 3′UTR inhibited oncogenic ras-induced cellular senescence, an action which required the AU-rich motif in the 3′UTR and the HuR protein that bound the motif [4]. HuR increases the stability of mRNAs by binding to them, and therefore accelerates cell growth, a condition that favors tumor growth. Indeed, HuR is a determinant of carcinogenesis in several cancer types, including liver cancer [10,11].

Recently, it was found that the 3′untranslated region RNAs exist separately from the other parts of mRNA molecules as a distinct RNA species in at least human, mouse, and fly cells. They arise from post-transcriptional processing of the mRNAs, and are regulated differently from their original mRNAs [12]. This interesting fact suggests that some functional elements of eukaryotic mRNA may exert their functions independently, and in that situation they may play roles which are more or less different from their roles as an integral part of mRNA. In fact, RNAs from the 3′untranslated regions of several mRNAs, as separate and distinct RNA species, were found to be tumor suppressors [13–18].

An important statement of the "RNA World" hypothesis is that modern RNAs may have been derived from many smaller components during the molecular evolution [19]. Although this statement has yet to be verified experimentally, it allows us to imagine that at least some portion of the motifs which have conserved their functions in contemporary mRNA molecules may be derived from ancestors which were independent RNA function molecules. If this is true, we can guess that some small functional motifs or elements in modern eukaryotic mRNA may function *in vivo* even after they are separated from their mother molecules and become a distinct RNA species.

In 1991, our group discovered that the 3′untranslated region of C/EBPβ (also known as NF-IL6) acted as a tumor suppressor [13,14]. While investigating the molecular mechanism of the tumor suppression function of this isolated, distinct C/EBPβ 3′UTR RNA [13,20,21], we found that the deletion of a short sequence containing AU-rich elements near the 3′-terminus of this RNA significantly lowered its tumor suppression activity [21], indicating that this short sequence was important in tumor suppression. As AU-rich elements are generally conserved [22], we posited that this AU-rich sequence might be functional even once it was separated from the 3′UTR RNA to become a distinct RNA species. We discovered that the independent AU-rich motif, a 62nt RNA (named R62), did indeed exert a cell growth suppression effect when introduced into a human hepatoma cell line SMMC-7721. Here we show our results in detail.

* Corresponding author. Address: Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Institute of Biochemistry and Cell Biology, 320 Yueyang Road, Shanghai 200031, China. Fax: +86 21 54921011.
E-mail address: dgliu@sibs.ac.cn (D.-G. Liu).

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http://dx.doi.org/10.1016/j.bbrc.2012.08.049

2. Materials and methods

2.1. Materials and reagents

SMMC-7721 cells were obtained from the Cell bank of the Chinese Academy of Sciences, Shanghai, China. 1-ethyl-3-(3-dimethyl-aminopropyl)carbodiimide hydroxide (EDC) and *N*-hydroxylsuccinimide (NHS) were obtained from Aladdin Co., Shanghai, China. The molecular beacon for R62 (5′Hex-CAGCGCTCGCCAGCTTC CTAATAGATTATTGCGCTG-dabcyl 3′) was synthesized in Shinegene Biotechnologies, Co., Shanghai, China. The Apoptosis Inducers Kit was obtained from Beyotime Institute of Technology, Jiangsu, China. The chemical reagents were made in China and were of the analytical reagent grade of purity. The RiboMax™ Large Scale RNA Production System-SP6 and RNasin were purchased from Promega, Beijing, China. The PrimeScript™ RT-PCR kit was obtained from Takara, Dalian, China. The Lipofectamine™ 2000, Dynabeads® M-270 Amine and pOTB7 vector were from Life Technologies, CA, USA. The cell counting kit-8 (CCK-8) was from the Dojindo Chemical Institute, Kumamoto, Japan. The antibodies were obtained from Santa Cruz, CA, USA. The protease inhibitors cocktail was obtained from Sigma–Aldrich, Munich, Germany. The Biotin Luminescent Detection Kit was obtained from Roche, Basel, Switzerland.

2.2. RNA transcription in vitro

Plasmids pSP64/0.28 [14] and pSP64/62 (Fig. 1) were linearized with EcoRI, and pOTB7 with Xho I, and were then transcribed with SP6 RNA polymerase or T7 RNA polymerase respectively *in vitro*. The transcripts were purified by passing through Sephadex G-50 columns and ethanol-precipitation.

2.3. Transient RNA transfection

Transient RNA transfection was performed using lipofectamine 2000 according to the manufacturer's instructions. Twenty-four hours before transfection, the cells were replated, and 1 h before transfection, the complete medium (10% newborn calf serum in RPMI-1640) was replaced by DMEM medium without any serum. Six hours post-transfection the transfection medium was once more replaced with the complete medium mentioned above.

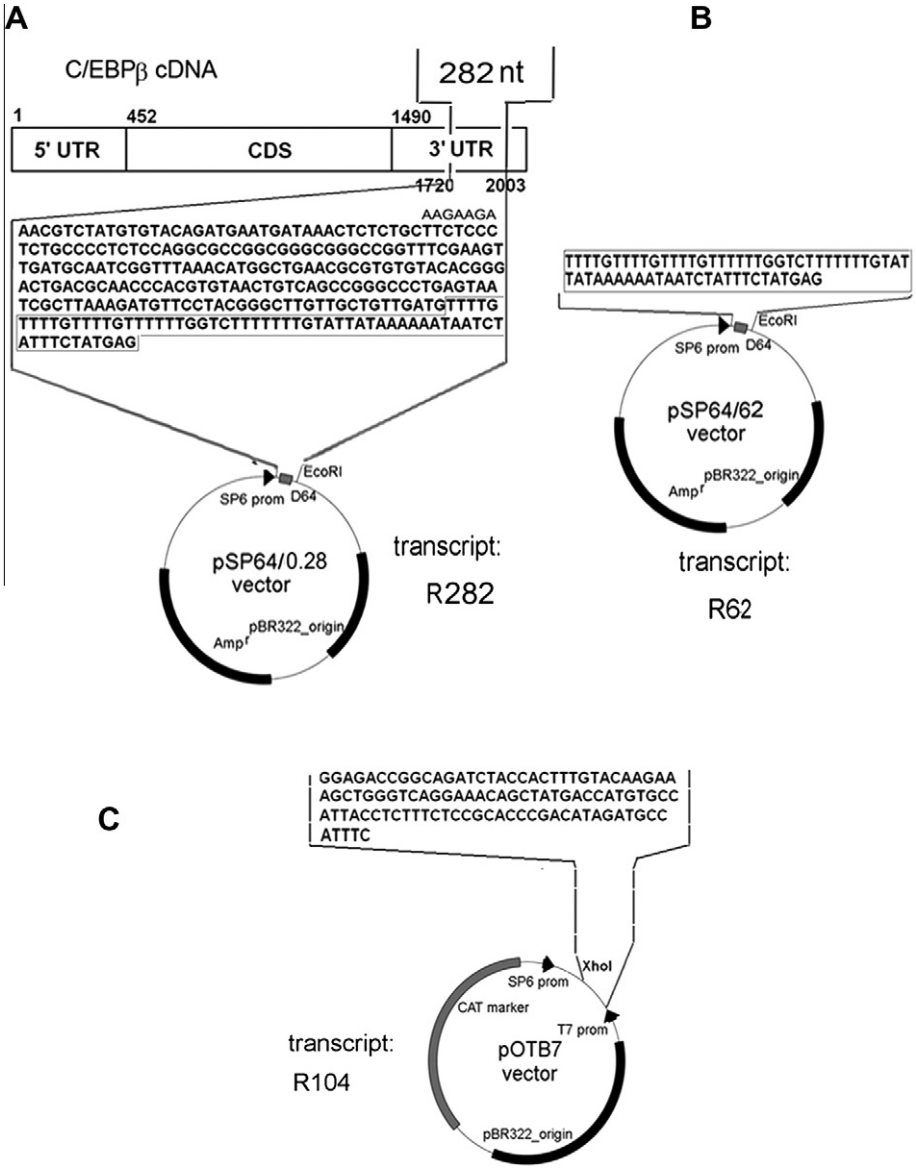

Fig. 1. Plasmids and RNA transcripts. (A) Plasmid pSP64/0.28 and R282 (i.e. C/EBPβ 3′UTR RNA). (B) Plasmid pSP64/62 and R62. (C) Plasmid pOTB7 and Control R104.

2.4. Proliferation assay and flow cytometry

The RNA-transfected cells were cultured in a 96-well plate for proliferation assay using a CCK-8 kit according to the manufacturer's instructions. After 48 h of RNA transfection, the cells were trypsinized, washed three times with PBS (pH 7.2), stained with propidium iodide (PI) and FITC-annexin V, and subjected to flow cytometry.

2.5. Molecular beacon hybridization and immunocytochemistry

The RNA-transfected cells were cultured on coverslips in 24-well plates. Twenty-four hours post-transfection, the cells were washed three times with PBS and fixed with 4% paraformaldehyde (in PBS, pH 7.2) for 40 min at room temperature. 0.5% Triton X-100 (in PBS, pH 7.2) was added to the cells in order to increase cell membrane permeability. For hybridization with the molecular beacon for R62, a 5% blocking reagent (from the Biotin Luminescent Detection Kit) was added and incubated for 1 h at room temperature. After washing three times with 0.1 M maleic acid pH 7.2/0.15 M NaCl/0.3% Tween 20 [v/v], 1 μM molecular beacon for R62 (in 5% blocking reagent) was incubated with the cells in a humid box at 75 °C for 15 min, and then the temperature was gradually lowered to room temperature over the course of 1 h. For immunocytochemistry, the cells were incubated with 5% skim milk at 37 °C for 1 h, antibodies were added, and incubation was continued at 4 °C for more than 8 h. The stained coverslips were mounted on microscopic glass plates and were observed through a laser confocal microscope.

2.6. Immobilization of RNA on Dynabeads® M-270 amine magnetic beads

The immobilization of RNAs on Dynabeads® M-270 amine magnetic beads was performed according to the manufacturer's instructions, with certain modifications. 200 μl of Dynabeads® M-270 amine was washed three times with 1 ml H_2O, centrifuged to remove the supernatant, and 90 μl RNA(1 μg/μl) and 30 μl of 1 M 2-(*N*-morpholino) ethanesulfonic acid (MES, pH 6.5)was added. 20 mg of EDC and 31 mg of NHS in 100 μl H_2O were immediately added to the mixture. 80 μl H_2O was then added and the mixture was rotated at 25 °C for 3 ~ 4 h. After incubation, the suspension of RNA-crosslinked magnetic beads was washed, diluted by adding glycerol to 50%, and stored at −70 °C.

2.7. Affinity isolation and identification of RNA-binding proteins

7×10^6 cells, cultured in serum-free PRMI 1640 medium 48 h before extraction, were re-suspended in 1 ml of cell lysis buffer (25 mM HEPES, pH 7.2/100 mM KCl/5 mM $MgCl_2$/1 mM EGTA/15% glycerin/40 μl protease inhibitors cocktail/100 U RNasin/0.5% Triton X-100), and sonicated for 20 min at 4 °C. The cell lysate was centrifuged for 10 min at 12000 r/min and 4 °C, and the supernatant was divided into 100 μl aliquots, into each of which 40 U RNasin was added. 20 μl of RNA-crosslinked magnetic beads (blocked by a blocking solution of 10 mg/ml heparin sodium/0.8% polyvinylpyrrolidone/0.8% ficoll 400/40 μg/ml tRNA/40 U/ml RNasin) were added to the cell lysate, which was then rotated at 4 °C overnight. The magnetic beads were washed three times with 25 mM HEPES pH 7.2/100 mM KCl/5 mM $MgCl_2$/1 mM EGTA/15% glycerin, boiled at 100 °C for 5 min in 1 × SDS loading buffer (25 mM Tris–Cl, pH 6.8/10% glycerin [v/v]/2% SDS [w/v]/1% mercaptoethanol [v/v]), centrifuged, and then the supernatant was separated by an SDS–PAGE. The gel was fixed for more than 1 h in a fixing solution (50% methyl alcohol [v/v]/10% acetic acid [v/v]). The liquid was changed twice. Staining was performed with silver-staining. The bands were cut off and subjected to MS.

2.8. Western blotting

This was performed using routine procedures. Cell proteins or immunoprecipitates were separated via an SDS–PAGE. The protein bands on the gel were transferred onto a PVDF membrane in a CAPS buffer (pH 11.0) containing 10% methanol. This membrane containing the protein bands was blocked with 5% skim milk in TBST, incubated at 4 °C with a primary antibody for 12 h, and then with a secondary antibody at 4 °C for 6 h. The hybridization bands were detected through chemoluminescence.

2.9. Competitive RNA immunoprecipitation

7×10^6 cells were lysed by sonication in 1 ml protein extraction buffer (25 mM HEPES pH7.2/1 mM EDTA/0.5% Triton X-100/0.1% sodium deoxycholate/40 μL/ml protease inhibitors cocktail) at 4 °C. Salt solution (final concentration: 25 mM $MgCl_2$, 5 mM $CaCl_2$, 100 mM KCl, 40 mM NaCl) was added and the mixture was centrifuged at 12000 r/min and 4 °C for 20 min. The supernatant was transferred into a new tube and 160 U RNasin was added. The supernatant was divided into 100 μl per tube, the competitor R62 RNA was added in different concentrations along with 2 μl of the anti-HuR antibody. The mixtures were rotated at 4 °C for 4 h, 1 μl of secondary antibody per tube was added, and then the rotation was continued overnight. The immunoprecipitates were collected by centrifugation for 25 min at 12000 r/min and 4 °C. The object RNAs were extracted from the immunoprecipitates and were quantified with qPCR. In brief, the mRNAs were reverse transcribed with a PrimeScript™ RT-PCR kit. The real time PCR conditions were: preheating at 95 °C for 20 s followed by 40 cycles of: denaturation at 95 °C for 20 s; annealing at 55 °C for 30 s; and elongation at 72 °C for 30 s. The C/EBPβ primers were: 5′-CCGGTTTCGAAGTTGATGCAATCGG-3′; 5′-CCGGCTGACAGTTACACGTGGGTT-3′.

3. Results

3.1. Experimental system

We had previously investigated the tumor suppression effects of the independent C/EBPβ 3′UTR RNA in SMMC-7721 cells. SMMC-7721 is a hepatocarcinoma cell line derived from surgically-dissected specimens of a Chinese liver cancer patient [23]. SMMC-7721 is used extensively in molecular biological research in China and the results obtained from using it have been published in numerous international scientific journals. In this research, we used SMMC-7721 to check for the effects of the independent RNA containing AU-rich elements. We constructed pSP64/62 plasmid, which contained a 62-basepair cDNA encoding the AU-rich sequence, through the deletion mutation of plasmid pSP64/0.28 vector. Then we prepared the 62nt RNA (henceforth called R62) *in vitro* by transcription with SP6 RNA polymerase, using the SP6 Large scale RNA production kit (Promega) (Fig. 1B). As controls, the C/EBPβ 3′UTR RNA (henceforth called R282) was transcribed by the same kit. Another (negative) control, named R104, was prepared by transfection of a 104nt segment on the pOTB7 plasmid backbone sequence using T7 RNA polymerase in the reverse direction (Fig. 1A, C). Theoretically, the ideal negative control would be a 62nt long RNA segment with a scrambled sequence. However, it is impossible to scramble the R62 sequence with neither removing existing bases nor introducing a new AU stretch, because there are 38 U residues and 13 A residues in the sequence. Therefore, we were forced to select a sequence which

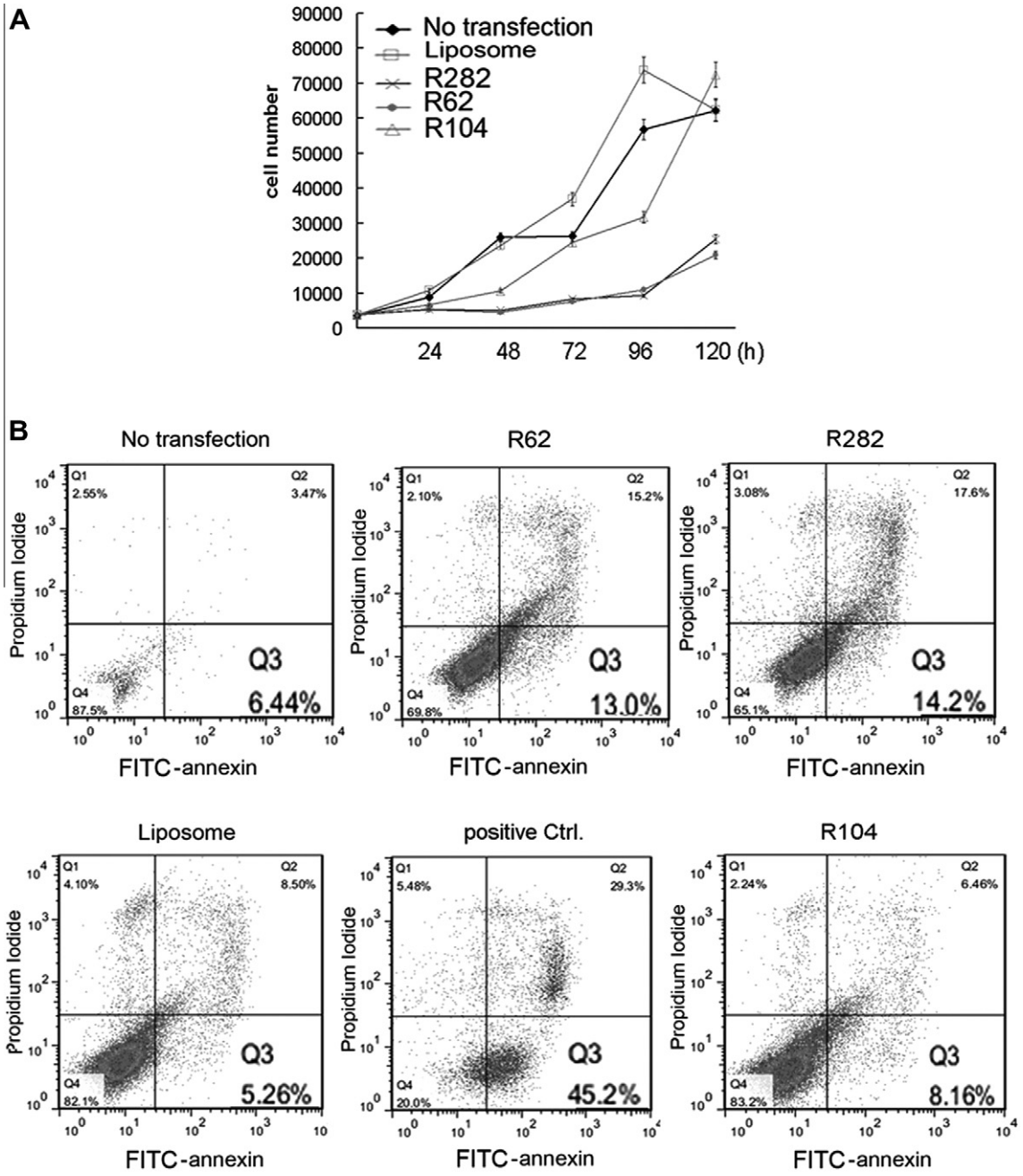

Fig. 2. R62 transfection into SMMC-7721 cells suppresses cell growth rate and induces apoptosis. (A) Growth suppression by R62 transfection. The cell growth rates were determined in multiwell plates using a CCK-8 kit. (B) Flow cytometry showing that apoptosis increased in R62-transfected SMMC-7721 cells. Cells transfected with various RNAs or the "positive control for apoptosis" substance, a proprietary Beyotime product, and the non-transfected cells, were stained with PI and FITC-annexin V before running flow cytometry. The legends to Q3 were magnified.

is known to have no independent functions and which was reverse-transcribed from a plasmid backbone, although this sequence is longer (104nt) than the R62.

3.2. R62 suppresses growth and induces apoptosis in SMMC-7721 cells

Our previous investigations had arrived at the conclusion that the deletion of a short segment containing AU-rich elements near the 3′ terminus of R282 (i.e. R62) decreased the tumor suppression activity of R282 [21]. In this investigation, we transfected groups of SMMC-7721 cells with R62, as well as with the controls R282 and R104. We then observed the growth states of the cells daily for 5 consecutive days. Results in Fig. 2A indicate that, as expected, the cells transfected with R282, i.e. C/EBPβ 3′UTR RNA, grew significantly more slowly than the controls (cells transfected with R104; with liposome only; and without transfection). The cells transfected with R62 also grew more slowly than the controls, in a similar fashion to R282. Moreover, while observing the proliferation of the cells, we noted that some of cells transfected with R62 and R282 were in apoptosis. Flow cytometry was performed to verify this. The two-dimensional flow cytometry graph (Fig. 2B) shows that the proportions of apoptotic cells (percentage of cells in the 3rd quadrant – Q3) in R62- and R282-transfected cells were markedly higher than those of the controls, except for the positive control. This indicated a strong degree of apoptosis. Therefore, our results indicated that R62, in a similar fashion

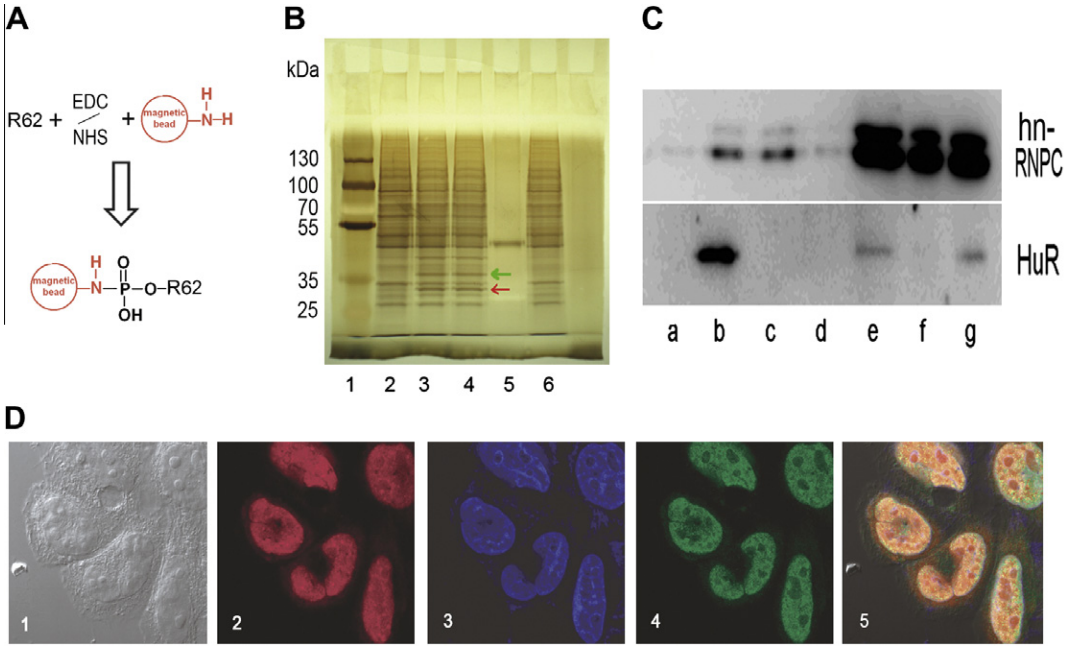

Fig. 3. R62 RNA specifically bound with HuR protein. (A) Immobilization of R62 RNA on magnetic beads through a condensation reaction using EDC and NHS. (B) Silver staining of a 10% SDS–polyacrylamide gel containing proteins eluted from R62-immobilized magnetic beads. (1) MW. (2) Beads. (3) and (4) Beads containing R62. (5) Blocking solution. (6) Beads containing control RNA (R104). Red arrow, HuR as determined by MS. Green arrow, hnRNPC as determined by MS. (C) RNA affinity chromatography and Western blots for hnRNPC and HuR, respectively. (a) Beads without RNA. (b) Beads containing R62. (c) and (d) Beads containing control RNA. (e) Cell lysate precipitate. (f) Immunoprecipitation supernatant. (g) Supernatant from cell lysate not treated with beads. (D) R62 co-localized with HuR in the nuclei and cytoplasm of hepatoma cell line SMMC-7721, as detected by confocal microscopy. (1) Phase contrast. (2) Molecular beacon for R62. (3) DAPI. (4) Anti-HuR antibody. (5) Merge. The red fluorescence arises from the molecular beacon for R62, the blue fluorescence is from DAPI, and the green fluorescence is from an HuR antibody. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

to R282, inhibited cell growth and induced apoptosis in SMMC-7721 cells.

3.3. In vitro and in vivo experiments suggest that R62 specifically binds HuR

As mentioned above, previous literature has indicated that the AU-rich regions in RNAs can bind HuR, the RNA binding protein [4–8]. *In vitro* binding experiments were conducted to confirm whether our independent RNA, R62, also bound HuR. R62 RNA was covalently immobilized on the surface of Dynabeads M270 amine by chemical cross-linking. Then RNA affinity chromatography was conducted using these derivatized magnetic beads. We found two proteins which were prominent in binding R62 (Fig. 3A, B). The two relevant bands were cut from the gel and subjected to mass spectrometry (MS) for identification. MS results unambiguously showed that the protein of molecular weight less than 35 kDa was HuR (red arrow, Fig. 3B), and the larger protein (green arrow, Fig. 3B) was hnRNPC. To clarify whether the binding of these two RNA binding proteins was specific, Western blots were performed. The results (Fig. 3C) showed that the hnRNPC also bound control R104, while HuR did not bind the control. Thus the interaction (binding) between HuR and R62 was specific.

To investigate the situations of HuR and R62 *in vivo*, a molecular beacon for R62 (red fluorescence) and an anti-HuR antibody (green fluorescence) were used in confocal microscopy observations of R62-transfected SMMC-7721 cells. The observation results showed that, in these cells, R62 and HuR were co-localized in the nuclei and cytoplasm (Fig. 3D). Although the co-localization of two biomolecules does not necessarily mean that they are bound together, it indicates that *in vivo* they are spatially very close, so there is a larger opportunity for them to interact.

Therefore, our experiments *in vitro* and *in vivo* suggest that R62 specifically binds HuR.

3.4. Molecular mechanism of the suppression of SMMC-7721 cell growth by R62 may be competitive binding to HuR

It is known that HuR can promote gene expression at the translational level by stabilizing mRNAs which contain AU-rich elements in the 3′untranslated regions. This stabilization is believed to be conducted through competition with destabilizing binding proteins whose mRNAs contain AU-rich elements [24]. In our investigation, the results suggested that the independent R62 RNA specifically bound HuR in R62-transfected SMMC-7721 cells, and this RNA also exerted cell growth-suppression and apoptosis-inducing activity. Therefore, we believe that it is probable that R62 competes with mRNAs which contain AU-rich elements in order to bind to HuR. Thus the latter become less stable, leading to a decrease in gene expression in these mRNAs.

To examine this proposition, we performed a competitive RNA-immunoprecipitation, in which the competitor, R62 RNA, was added to the SMMC-7721 cell lysates in increasing amounts, before the antibody for HuR was added. We observed the influence of the exogenously added R62 on the C/EBPβ mRNA content of this immunoprecipitate. After adding the secondary antibody and incubation, the precipitate was subjected to qPCR to determine the C/EBPβ mRNA content. The results are shown in Fig. 4. It is clear that the C/EBPβ mRNA content of the anti-HuR immunoprecipitate was inversely correlated with the amounts of R62 added. Since we have shown that the binding of R62 with HuR is specific (see above), the competition displayed here is also highly likely to be specific. This result indicates that the HuR-bound C/EBPβ mRNA decreased in amount as the amounts of exogenously added R62 increased. We believe that this means that the R62 we added prevented C/EBPβ mRNA from binding to HuR.

Based on these facts, we propose that the cell growth suppression effect of exogenous R62 may be exerted though competitive binding to HuR protein, leading to a decrease in stability of the

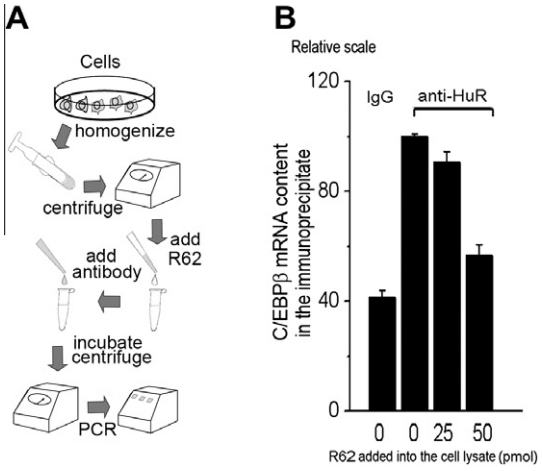

Fig. 4. R62 reduces the amount of C/EBPβ mRNA in SMMC-7721 cell lysate. (A) Experiment procedures for competitive RNA immunoprecipitation. SMMC-7721 cells were homogenized and centrifuged, the supernatant was aliquoted and increasing amounts of competitive (R62) RNA were added into each aliquot. Antibodies to HuR and to IgG were then added to the mixtures. After incubation at 4 °C, the mixtures were centrifuged and the precipitates were subjected to qPCR to determine the amounts of C/EBPβ mRNA. (B) Experimental results, showing that the contents of C/EBPβ mRNA were inversely correlated with the amounts of R62 added.

mRNAs that would otherwise be bound to HuR, resulting in a down-regulation of their expression.

4. Discussion

In this investigation, we have provided evidence that an independent and distinct short RNA derived from the AU-rich region of 3′untranslated region (3′UTR) of C/EBPβ mRNA (R62) exerted a cell growth suppression effect in a human hepatocarcinoma cell line, SMMC-7721. To our knowledge, this is the first report on the function *in vivo* of a distinct AU-rich short RNA.

It has been generally accepted that eukaryotic mRNA is an integral biomolecule, which consists of several connected functional parts such as the 5′untranslated region, the coding region and the 3′untranslated region, and that these parts (or motifs) perform their own functions merely as a part of the biomolecule. Many years ago, it was found that several 3′UTR RNAs which had been separated from some mRNAs functioned as tumor suppressors. Unfortunately, apart for one individual article [15], these findings do not seem to have evoked the attention of the biochemical community. Recently, it has been found that the RNAs of 3′UTR can exist independently and can perform their own important functions [12]. This has led to a deeper consideration of the functions of mRNA motifs. Our work is another (along with [13]) attempt to reveal whether a separated RNA motif can perform a specific function in living cells. Our results show that at least some portion of the motifs in an integral mRNA can function when isolated from their mother molecule.

The functions of mRNA motifs as parts of integral mRNAs have been studied extensively. However, we still need to investigate in detail what functions they have providing they can exist as independent molecules. The possibility should not be excluded that the functions of independent RNA motifs, providing they exist, may be different from their functions as integral parts of an mRNA. For example, Basu et al. [4] found that in primary fibroblasts C/EBPβ 3′UTR RNA attached after the C/EBPβ coding region promotes the expression of cancer-related genes, and that this effect requires the participation of HuR. These are functions performed by the C/EBPβ 3′UTR as an integral part of the C/EBPβ mRNA. It is therefore not surprising that those results differ from the results of investigations into C/EBPβ 3′UTR RNA as an independent RNA molecule. In addition, Basu et al. results were obtained in primary fibroblasts, a cell type whose gene composition and intracellular microenvironment are very different from a hepatoma cell. Further investigations into this area should be very interesting.

The significance of our investigation lies in the possibility that, once further studies of the possible functions of independent functional motifs found within mRNA progress, short RNAs with therapeutic values (e.g. for cancer treatment) may be discovered. These could potentially become the theoretical basis for the development of novel drugs. Therefore, new options may be discovered in our long-lasting struggle against cancer and other diseases which harm human health.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgments

The authors thank Prof. Fu-Kun Zhao for the mass spectrometry analysis; Yan Wang, Yang Yu and Yan-Qiong Jiang for help in using the laser confocal microscope and qPCR thermocycler. This work was supported by Grants No. 30970585 and 31170722 from the State Natural Science Foundation of China, and by a grant from the State Key Laboratory of Molecular Biology for its open research teams.

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