VERSITA

CELLULAR & MOLECULAR BIOLOGY LETTERS
http://www.cmbl.org.pl

Received: 18 May 2007
Revised form accepted: 23 October 2007
Published online: 16 January 2008

Volume 13 (2008) pp 303-311
DOI: 10.2478/s11658-008-0002-0
© *2008 by the University of Wrocław, Poland*

Short communication

hnRNP-R REGULATES THE PMA-INDUCED *c-fos* EXPRESSION IN RETINAL CELLS

JIA HUANG[1], SHU-JING LI[1], XIAN-HUA CHEN[2]*, YU HAN and PING XU[2]*
[1]Laboratory of Genomic Physiology and [2]State Key Laboratory of Medical Neurobiology, Fudan University, 138 Yixueyuan Road, Shanghai, 200032, P. R. China

Abstract: This study focused on the function of hnRNP-R in the regulation of *c-fos* expression. We demonstrated that hnRNP-R accelerated the rise and decline phases of *c-fos* mRNAs and Fos proteins, allowing PMA to induce an augmented pulse response of *c-fos* expression. Then, we examined the role of the *c-fos*-derived AU-rich element (ARE) in hnRNP-R-regulated mRNA degradation. Studies with the ARE-GFP reporter gene showed that hnRNP-R significantly reduced the expression of GFP with an inserted ARE. Moreover, immunoprecipitation-RT-PCR analysis demonstrated that in R28 cells and rat retinal tissues, the *c-fos* mRNA was co-immunoprecipitated with hnRNP-R. These findings indicate that hnRNP-R regulates the *c-fos* expression in retinal cells, and that the ARE of *c-fos* mRNAs contributes to this regulation.

Key words: hnRNP-R, Retina, *c-fos*, mRNA turnover, ARE

INTRODUCTION

The immediate-early gene (IEG) *c-fos* encodes the protein Fos, which mediates light-elicited cellular activities in the retina by controlling the expression of its downstream genes encoding neurotransmitters or neuromodulators [1, 2]. The mechanisms regulating *c-fos* expression in the retina are not completely

* Authors for correspondence; e-mail: matibuck@yahoo.com or xhchen@fudan.edu.cn, tel: 086-21-54237094, fax: 086-21-54237094

Abbreviations used: ARE – AU-rich element; DMSO – dimethyl sulfoxide; GFP – green fluorescence protein; PMA – phorbol 12-myristate 13-acetate; SMA – spinal muscular atrophy; UTR – 3'-untranslated region

understood. One mechanism is attributed to the regulation of its mRNA degradation [3, 4] by *cis*-elements [2, 5, 6] including an AU-rich element (ARE) located in the 3'-untranslated region (UTR) of the *c-fos* mRNA [5, 7]. Heteronuclear RNA-binding proteins (hnRNPs) are predominantly nuclear RNA-binding proteins involved in many cellular activities, including transcription and pre-mRNA processing [8]. Two of the hnRNPs, hnRNP-Q and hnRNP-D, were recently shown to enhance the stability of ARE-contained mRNAs, indicating the importance of hnRNPs in regulating the *c-fos* mRNA degradation [6].

hnRNP-R is another member of the hnRNP family, and it is important in normal neural function and neural disease SMA [9-11]. The biochemical similarities between hnRNP-R and hnRNP-Q [9, 12] and the known function of hnRNP-Q in regulating the *c-fos* mRNA turnover process [6] suggest that hnRNP-R may act as a new protein component in the regulation of *c-fos* expression. The results of our studies support this possibility.

MATERIALS AND METHODS

Cell culture

Immortalized R28 retinal precursor cells (a gift from Dr. Seigel) were cultured and transfected as described previously [13]. PMA (100 ug/ml in DMSO) with a final concentration of 100 ng/ml was added to induce *c-fos* expression in the cells. Cells were harvested at different time points (15, 30, 45, 60, 120, 240 min) after PMA addition for time-course analysis, or at 60 min for other analyses. Actinomycin (ActD, 5 μg/ml) was used to inhibit transcription. In PMA-induced R28 cells, ActD was added 45 min after the addition of PMA.

Preparation of DNA constructs

The DNA construct for hnRNP-R expression (pcDNA-R) was prepared as described previously [13]. To prepare the ARE-green fluorenscence protein (GFP) reporter gene, a cDNA fragment containing the ARE located at the 3'-UTR of *c-fos* mRNA (1844~2026 bp, GenBank No. X06769) was generated using reverse transcription (RT)-PCR with the primers: ARE-GFP forward, 5'-ATT CTC GAG AGC GTC CAT GTT CAT TGT-3'; and ARE-GFP reverse, 5'-TCG GGA TCC CGA AAG ACC TCA GGA TAG-3'. The ARE cDNA was then inserted into pEGFP-C2 vectors (Clontech) after XhoI/BamHI digestion.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

RT-PCR analysis of *c-fos* mRNAs was performed as described previously [13]. The following primers were used: for *c-fos* mRNA (GenBank No. X06769), 5'-AGC GTC CAT GTT CAT TGT CAT-3' (forward), 5'-CGA AAG ACC TCA GGA TAG AAA -3' (reverse); and for GAPDH (as a control, GenBank No. M17701), 5'-TGA TGA CAT CAA GAA GGT GGT GAA G-3' (forward), 5'-TCC TTG GAG GCC ATG TGG GCC AT-3' (reverse). The reactions were performed with 30 cycles for *c-fos* and 20 cycles for GAPDH.

Western blot analysis
Cell lysates were separated by 9% SDS-PAGE for further Western blot analysis as described previously [13]. Rabbit anti-hnRNP-R (1:1000, [13]), monoclonal mouse anti-Fos (1:5000) or anti-GAPDH (as a control, 1:5000) antibodies were used as primary antibodies and AP-labeled goat anti-rabbit or horse anti-mouse IgG (1:1000) as secondary antibodies.

Immunoprecipitation-RT-PCR analysis
The immunoprecipitation of hnRNP-R antibodies with R28 cell pellets or rat retinal tissues was performed following Esnaults' protocol [14]. Preimmune rabbit serum was used as a negative control. Precipitated samples were then split, with 40% dissolved in TriReagent (Watson, China) for the purification of RNA according to the manufacturer's recommendations, and 60% dissolved in SDS-PAGE loading buffer for Western blot analysis. The RT-PCR analysis for *c-fos*, tra2β and β-actin were performed as described above or previously [13, 15].

Statistical analysis
The experiments were performed in triplicate, and repeated at least three times independently. The data is presented as mean ± SE. The difference was determined as significant by Student's t-test at $P < 0.01$ (**) or $P < 0.05$ (*).

RESULTS

hnRNP-R regulates the *c-fos* expression in retinal R28 cells
To study whether hnRNP-R regulates *c-fos* expression, we examined the effect of hnRNP-R overexpression on the level of *c-fos* expression induced by PMA in retinal R28 cells [16]. By comparison to the time-course of the *c-fos* expression induced by PMA in cells transfected with pcDNA plasmids (control), we demonstrated that hnRNP-R overexpression not only accelerated the rise, but also the decline of the levels of *c-fos* mRNAs and Fos proteins, as shown in Figs 1A and B, indicating a "dual effect" of hnRNP-R on PMA-induced *c-fos* expression [3, 4]. Figs 1A and B also showed a delayed increase in Fos compared with the increase in *c-fos* mRNA after the transfection, which is consistent with the known delay effect between *c-fos* mRNA and Fos protein expression [3, 4]. The transfection efficiency (~30%) was monitored by co-expression of EGFP. The overexpression of hnRNP-R was determined by immunobloting with hnRNP-R antibodies (data not shown). This dual effect resulted in an increased peak expression level or pulse expression of *c-fos* in response to PMA induction. To observe the effect of hnRNP-R on *c-fos* mRNA degradation, we used the transcription inhibitor ActD after PMA induction. As shown in Fig. 1C, in the presence of ActD, hnRNP-R increased the *c-fos* mRNA degradation rate, suggesting that hnRNP-R accelerated the *c-fos* mRNA degradation.

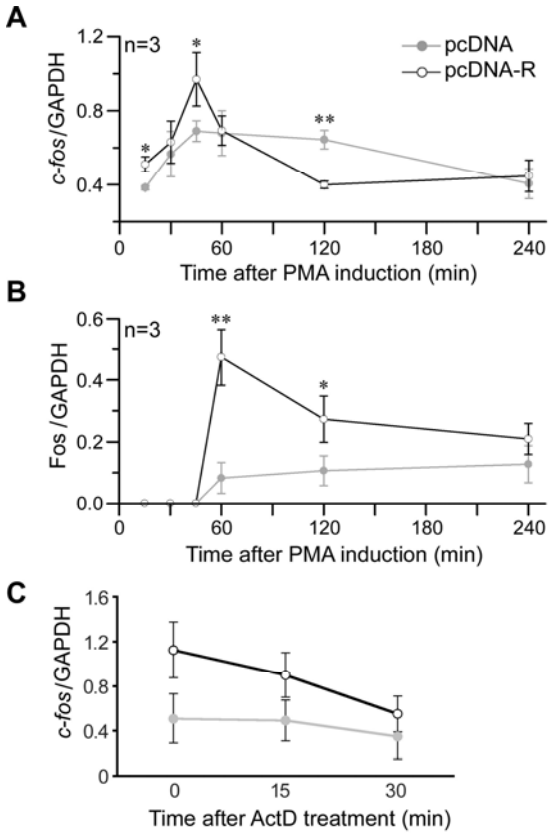

Fig. 1. The effect of hnRNP-R on PMA-induced *c-fos* expression in R28 cells. A and B – The time-courses of *c-fos* mRNA and Fos protein expression in R28 cells with (pcDNA-R) or without (pcDNA) hnRNP-R overexpression. C – The time-courses of *c-fos* mRNA levels in R28 cells with (pcDNA-R) or without (pcDNA) hnRNP-R overexpression in the presence of ActD. Error bar = ± SE.

hnRNP-R reduces the expression of the ARE-GFP reporter gene

To examine the role of the ARE in hnRNP-R-accelerated *c-fos* mRNA degradation without the interference of hnRNP-R-increased *c-fos* transcription, we prepared a reporter gene (ARE-GFP) by inserting a *c-fos*-derived ARE at the 3'-UTR of the full-length GFP cDNA. The results demonstrated that the GFP mRNA and protein levels were significantly reduced in the cells transfected with ARE-GFP plasmids, compared to the control (GFP) (Fig. 2A). Then, we used this reporter gene to test whether the ARE mediates the effect of hnRNP-R on mRNA degradation. As shown in Fig. 2B, hnRNP-R overexpression significantly reduced the ARE-GFP mRNA levels, but not the GFP levels (Fig. 2C), indicating that the function of hnRNP-R requires the presence of the ARE. The result supports the idea that the *c-fos*-derived ARE may contribute to the accelerated *c-fos* mRNA degradation by hnRNP-R.

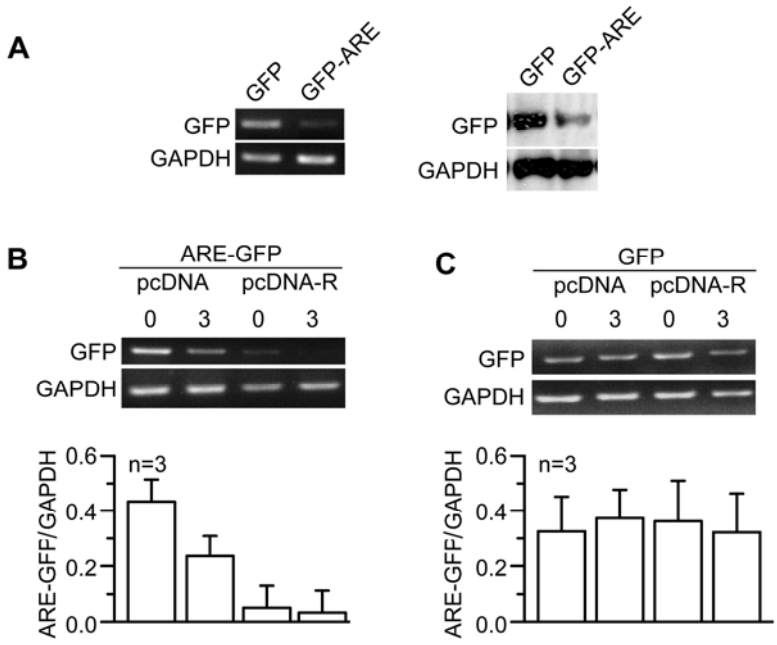

Fig. 2. The effect of *c-fos*-derived ARE on GFP expression (A) and hnRNP-R on ARE-mediated GFP expression (B and C). A – GFP mRNA (left) and protein (right) expression in R28 cells transfected with ARE-GFP or GFP plasmids (same dosages). B – Time courses of ARE-GFP expression in R28 cells without (pcDNA) or with (pcDNA-R) hnRNP-R overexpression after ActD treatment (0 h, 3 h). C – GFP expression in R28 cells without or with hnRNP-R overexpression after ActD treatment (0 h, 3 h).

The exogenous *c-fos*-derived ARE reduces the decline rate of endogenous *c-fos* mRNA in the presence of the overexpressed hnRNP-R

To further examine whether the ARE contributes to the accelerated *c-fos* mRNA degradation by hnRNP-R, the ARE-GFP was used as an exogenous *c-fos*-derived ARE, with the GFP as a control, to study its effect on PMA-induced *c-fos* mRNA expression in R28 cells. We assumed that if the ARE is involved in hnRNP-R-regulated *c-fos* mRNA degradation *in vivo*, then the ARE-GFP competes with the endogenous ARE and thereby attenuates the hnRNP-R-accelerated *c-fos* mRNA degradation, resulting in an augmentation of hnRNP-R-promoted *c-fos* expression. All the experiments were performed in the presence of ActD to inhibit the *c-fos* transcription. Fig. 3A showed that in R28 cells transfected with ARE-GFP plasmids, the decline rate of the *c-fos* mRNA level was significantly decreased, compared to that in the control (Fig. 3B), suggesting that the ARE contributes to the *c-fos* mRNA degradation regulated by either endogenous or overexpressed hnRNP-R.

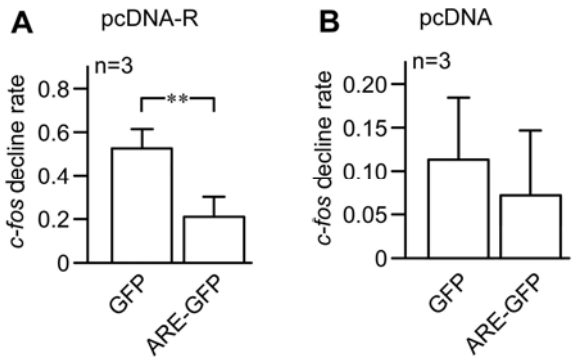

Fig. 3. The effect of ARE-GFP or GFP on the *c-fos* mRNA decline rate in R28 cells with (A) or without (B) hnRNP-R overexpression. ActD was added 45 min after the addition of PMA to the R28 cells. The *c-fos* mRNA level at 0 min (L_0) and 30 min (L_{30}) after ActD treatment was measured and normalized according to the GAPDH mRNA level. The decline rate of *c-fos* mRNA was calculated by the equation: $(L_0-L_{30})/L_0$.

The *c-fos* mRNA is co-imunoprecipitated with hnRNP-R proteins

We determined whether the hnRNP-R is associated with *c-fos* mRNAs *in vivo* using RT-PCR analysis of *c-fos* mRNAs in the hnRNP-R immunopreciciptates in R28 cells and rat retinal tissues using a pair of primers specific to *c-fos*-ARE. Fig. 4 showed that the *c-fos*-ARE was present in the hnRNP-R immunoprecipitate, but not in the control (preimmune). In the experiments, the β-actin mRNAs known to bind with hnRNP-R proteins [11] were detected as a positive control. In addition, the immunoprecipitation with an antibody specific to another RNA binding protein, Tra2-β, was performed as an unrelated control. The results demonstrated that hnRNP-R was associated with the *c-fos* mRNA, indicating the involvement of hnRNP-R in regulating the *c-fos* mRNA degradation *in vivo*.

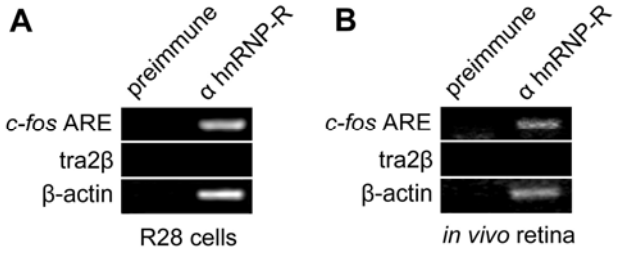

Fig. 4. The *c-fos* mRNA was co-immunoprecipitated with hnRNP-R in cells induced with PMA to express *c-fos* R28 cells (A) and rat retinal tissues (B).

DISCUSSION

The *c-fos* plays a critical role in mediating the biological rapid responses to various stimuli, such as diurnal responses in the retina [17]. The rapid response is tightly controlled by multiple mechanisms, especially highly regulated mRNA degradation [6, 18]. Two *cis*-elements, mCRD (the major protein-coding-region determinant of instability) and ARE (the AU-rich element), are known to act as key elements in regulating *c-fos* mRNA degradation. In addition, the mRNA degradation process requires multiple factors acting cooperatively. Therefore, identifying additional candidate factors regulating *c-fos* mRNA degradation is an important issue for understanding the mechanisms of mRNA degradation. hnRNP-Q has been shown to stabilize ARE-containing mRNA and regulate *c-fos* expression [6]. In this study, we examined the function of hnRNP-R, a newly identified member of the hnRNP family that binds to mRNAs, in regulating the *c-fos* expression in retinal R28 cells. Because *c-fos* is an inducible gene with an extremely low basal expression, we used the PMA-induced *c-fos* expression in R28 cells as a model [2, 16]. We showed that hnRNP-R significantly accelerates the rise and decline phases of *c-fos* expression, suggesting that hnRNP-R promotes *c-fos* transcription and mRNA degradation [19]. This is consistent with the results of previous studies showing that *c-fos* expression is tightly controlled by transcription and mRNA degradation mechanisms [2, 6, 18]. This "dual effect" results in a transient or pulse increase in *c-fos* expression, which is crucial for *c-fos* to function as an IEG in acute responses to stimuli [2].

In view of the known function of hnRNP-Q to enhance the stability of ARE-contained mRNAs [6], in this study, we focused on the possibility that hnRNP-R, which is highly homologous to hnRNP-Q, may accelerate the *c-fos* mRNA degradation via an ARE-mediated mechanism. To eliminate the potential interference of the *cis*-element mCRD, an ARE-GFP reporter gene was used. We demonstrated that regulation of GFP expression by hnRNP-R requires the attachment of *c-fos*-derived ARE to the GFP, suggesting the requirement of the ARE for hnRNP-R to regulate the mRNA degradation. We further examined the role of the ARE and hnRNP-R in regulating the *c-fos* mRNA degradation *in vivo*, and showed that the exogenous ARE decreases the *c-fos* mRNA decline rate and that the *c-fos*-derived ARE is co-immunoprecipitated with hnRNP-R. Together, the results suggest that hnRNP-R regulates *c-fos* expression via an ARE-mediated mechanism.

In contrast to the function of hnRNP-Q as a stabilizer for ARE-contained mRNAs [6], our results indicate that hnRNP-R may function as de-stabilizer in regulating the *c-fos* mRNA degradation via an ARE-mediated mechanism. Our results do not exclude the possibility that hnRNP-R may regulate the *c-fos* mRNA degradation by cooperating with other members of hnRNPs, such as hnRNP-Q [6]. In addition, the importance of mCRD in hnRNP-R-regulated *c-fos* expression is unknown. In order to fully understand how hnRNP-R regulates the *c-fos* mRNA degradation, these issues will be studied in the future.

Acknowledgements. We would like to thank Dr. Seigel for providing the R28 cells. This study was supported by the National Foundation of Natural Sciences of China (grants No. 30070242, No. 30470383, No. 30470535), the Shanghai Leading Academic Discipline Project (No. B111) and the Shanghai Key project (No. 07DJ14005).

REFERENCES

1. Sheng, M. and Greenberg, M.E. The regulation and function of *c-fos* and other immediate early genes in the nervous system. **Neuron** 4 (1990) 477-485.
2. Kovacs, K.J. c-fos as a transcription factor: a stressful (re)view from a functional map. **Neurochem. Int.** 33 (1998) 287-297.
3. Rahmsdorf, H.J., Schonthal, A., Angel, P., Litfin, M., Ruther, U. and Herrlich, P. Posttranscriptional regulation of *c-fos* mRNA expression. **Nucleic Acids Res.** 15 (1987) 1643-1659.
4. Sariban, E., Luebbers, R. and Kufe, D. Transcriptional and posttranscriptional control of *c-fos* gene expression in human monocytes. **Mol. Cell Biol.** 8 (1988) 340-346.
5. Mitchell, P. and Tollervey, D. mRNA turnover. **Curr. Opin. Cell Biol.** 13 (2001) 320-325.
6. Grosset, C., Chen, C.Y., Xu, N., Sonenberg, N., Jacquemin-Sablon, H. and Shyu, A.B. A mechanism for translationally coupled mRNA turnover: interaction between the poly(A) tail and a *c-fos* RNA coding determinant via a protein complex. **Cell** 103 **(**2000) 29-40.
7. Chen, C.Y. and Shyu, A.B. Au-Rich Elements - Characterization and importance in messenger-RNA degradation. **Trends Biochem. Sci.** 20 (1995) 465-470.
8. Krecic, A.M. and Swanson, M.S. hnRNP complexes: composition, structure, and function. **Curr. Opin. Cell Biol.** 11 (1999) 363-371.
9. Mourelatos, Z., Abel, L., Yong, J., Kataoka, N. and Dreyfuss, G.. SMN interacts with a novel family of hnRNP and spliceosomal proteins. **EMBO J.** 20 (2001) 5443-5452.
10. Rossoll, W., Kroning, A.K., Ohndorf, U.M., Steegborn, C., Jablonka, S. and Sendtner, M. Specific interaction of Smn, the spinal muscular atrophy determining gene product, with hnRNP-R and gry-rbp/hnRNP-Q: a role for Smn in RNA processing in motor axons? **Hum. Mol. Genet.** 11 (2002) 93-105.
11. Rossoll, W., Jablonka, S., Andreassi, C., Kroning, A.K., Karle, K., Monani, U.R. and Sendtner, M. Smn, the spinal muscular atrophy-determining gene product, modulates axon growth and localization of beta-actin mRNA in growth cones of motoneurons. **J. Cell Biol.** 163 (2003) 801-812.
12. Kim, T.D., Kim, J.S., Kim, J.H., Myung, J., Chae, H.D., Woo, K.C., Jang, S.K., Koh, D.S and Kim, K.T. Rhythmic serotonin N-acetyltransferase mRNA degradation is essential for the maintenance of its circadian oscillation. **Mol. Cell Biol.** 25 (2005) 3232-3246.

13. Huang, J., Chen, X.H., Wu, K. and Xu, P. Cloning and expression of a novel isoform of heterogeneous nuclear ribonucleoprotein-R. **Neuroreport** 16 (2005) 727-730.
14. Esnault, S. and Malter, J.S. Hyaluronic acid or TNF-alpha plus fibronectin triggers granulocyte macrophage-colony-stimulating factor mRNA stabilization in eosinophils yet engages differential intracellular pathways and mRNA binding proteins. **J. Immunol.** 171 (2003) 6780-6787.
15. Chen, X., Guo, L., Lin, W. and Xu, P. Expression of Tra2beta isoforms is developmentally regulated in a tissue- and temporal-specific pattern. **Cell Biol. Int.** 27 (2003) 491-496.
16. Amemiya, T., Kambe, T., Fukumori, R. and Kubo, T. Role of protein kinase C beta in phorbol ester-induced *c-fos* gene expression in neurons of normotensive and spontaneously hypertensive rat brains. **Brain Res.** 1040 (2005) 129-136.
17. Humphries, A. and Carter, D.A. Circadian dependency of nocturnal immediate-early protein induction in rat retina. **Biochem. Biophys. Res. Commun.** 320 (2004) 551-556.
18. Schiavi, S.C., Wellington, C.L., Shyu, A.B., Chen, C.Y., Greenberg, M.E. and Belasco, J.G. Multiple elements in the *c-fos* protein-coding region facilitate mRNA deadenylation and decay by a mechanism coupled to translation. **J. Biol. Chem.** 269 (1994) 3441-3448.
19. Panchision, D.M., Gerwin, C.M., DeLorenzo, R.J. and Jakoi, E.R. Glutamate receptor activation regulates mRNA at both transcriptional and posttranscriptional levels. **J. Neurochem.** 65 (1995) 969-977.