ISSN 1672-9145 **Acta Biochimica et Biophysica Sinica** 2006, **38**(1): 58–62 **CN** 31-1940/Q

Construction and Activity Assay of the Activating Transcription Factor 3 Reporter Vector pATF/CRE-luc

Jun-Qing XU[1,2], Jing-Lan DENG[1], You-Sheng WU[2], Han-Yan FU[2], Rui-Hua WANG[2], Jian ZHANG[2], Fan LU[2]*, and Zhong-Liang ZHAO[2]*

[1] *Department of Nuclear Medicine, Xijing Hospital, Fourth Military Medical University, Xi'an 710032, China;*
[2] *Department of Biochemistry and Molecular Biology, Fourth Military Medical University, Xi'an 710032, China*

Abstract Activating transcription factor 3 (ATF3), a member of the activating transcription factor/cAMP responsive element binding protein (ATF/CREB) family of transcription factors, is induced by many physiological stresses. To investigate the activity of ATF/CREB in cells with physiological stresses, we developed a practical reporter vector, the plasmid pATF/CRE-luc, bearing activating transcription factor/cAMP responsive element (ATF/CRE) binding sites. This plasmid was constructed by inserting three repeats of the ATF/CRE binding element into the plasmid pG5luc, replacing the GAL-4 binding sites. The plasmids pACT/ATF3 and pATF/CRE-luc were transfected into HeLa and NIH3T3 cells, respectively, and the results showed that the expression of luciferase was increased in a dose-dependent manner on plasmid pACT/ATF3. The data suggested that the plasmid pATF/CRE-luc could be used as a sensitive and convenient reporter system of ATF3 activity.

Key words ATF/CRE; luciferase gene; pG5luc; reporter system

The activating transcription factor/cAMP responsive element binding protein (ATF/CREB) family of transcription factors in mammals represents a large group of basic region-leucine zipper (bZip) proteins, which were originally defined in the late 1980s because of their ability to bind to the consensus AP-1 or "TGACGTCA" of the activating transcription factor/cAMP responsive element (ATF/CRE) site [1]. ATF3, a member of the ATF/CREB family of transcription factors, was isolated from HeLa cells treated with tetradecanoylphorbol acetate [2]. ATF3 is expressed at very low levels in normal quiescent cells, but can be rapidly and highly induced in different types of cells by multiple and diverse extracellular signals including mitogens (e.g., serum and epidermal growth factor) [3], cytokines (e.g., interferon and interleukin-4) [4] and genotoxic agents (e.g., ionizing radiation and ultraviolet light) [5,6]. *In vivo*, ATF3 is highly expressed in situations of cellular growth or stress, such as liver regeneration, brain seizure, ischemia-reperfusion of the heart and nerve damage [7–10]. It appears to function in the regulation of the cellular stress response or in cell proliferation by forming homo- and selective-heterodimers with certain other bZip proteins [5,11]. The expression of ATF3 and other immediate-early genes is followed by the sequential expression of a set of delayed-early genes and the onset of DNA synthesis [12]. Several genes have been implicated to be the targets, including *gadd153*/*Chop10* [13,14], *E-selectin* [15,16] and *phosphoenolpyruvate carboxykinase* [17].

Although there is strong evidence that this transcription factor plays an important role in the regulation of responses to stress stimuli, little is known about the modulation and physiological significance of ATF3 induction. Clearly, in order to understand the significance of ATF3 induction by stress signals, it is important to elucidate how it is induced by extracellular signals and what target genes it regulates. So far, the main clue for the physiological

Received: June 29, 2005 Accepted: August 29, 2005
This work was supported by the grants from the National Natural Science Foundation of China (No. 30271457 and No. 30470874)
*Corresponding authors:
Zhong-Liang ZHAO: Tel/Fax, 86-29-83374537; E-mail, zlzhao@fmmu.edu.cn
Fan LU: Tel/Fax, 86-29-83374537; E-mail, lufan@fmmu.edu.cn

DOI: 10.1111/j.1745-7270.2006.00122.x

function of ATF3 has come from studies of its expression pattern, not its activity.

In the present study, we developed a plasmid bearing the sequence of ATF/CRE, which could be driven by the binding of ATF3, used as a reporter plasmid to detect the activity of ATF3. Using transient transfection and luciferase activity assay, this plasmid was confirmed to be functional because it could be induced to express luciferase by ATF3 in a dose-dependent manner. It is reasonable to expect that the application of the ATF3 reporter plasmid would then provide a more convenient tool for the research of the physiological function of ATF3.

Materials and Methods

Bacterial strain and growth conditions

Plasmid cloning was carried out in the *Escherichia coli* DH5α strain [18]. Procedures for preparation of *E. coli* competent cells and transformation of target DNA into competent cells were as described previously [19].

DNA procedures and plasmids

Basic DNA manipulations and molecular techniques were employed as described in Sambrook *et al*. [19]. Extraction of DNA from agarose gels was done with a QIAEX II gel extraction kit (Qiagen, Valencia, USA). Nucleotide sequence determination was performed by Sunbiotech (Beijing, China).

The pBIND vector (Promega, Madison, USA; GenBank accession No. AF264722) is a 6.3 kb eukaryotic high-copy-number plasmid bearing the *renilla* luciferase gene preceded by the SV40 early promoter and a growth hormone intron. In this study, the plasmid pBIND was used as a reference to normalize transfection efficiency.

The pACT vector (Promega; GenBank accession No. AF264723) is a 5.5 kb eukaryotic high-copy-number plasmid in which the CMV immediate-early promoter drives expression of the herpes virus VP16 activation domain (amino acids 411–456). The coding sequence of the *ATF3* gene was obtained by reverse transcription-polymerase chain reaction with primers GCAGGATCCTGATGCTTCAACACCCAGG (underline indicating the *Bam*HI site) and TCGACGCGTGCTTAGCTCTGCAATGTTCC (underline indicating the *Mlu*I site). Then the sequence was inserted into the *Bam*HI/*Mlu*I sites of pACT to construct pACT/ATF3.

pG5luc (Promega; GenBank accession No. AF264724) is a 4.9 kb eukaryotic high-copy-number plasmid bearing the resistance gene for ampicillin, and five GAL-4 binding sites upstream of a minimal TATA box, which in turn is upstream of the firefly luciferase gene (*luc*$^+$). This plasmid was used as the vector of our reporter plasmid. Plasmid pATF/CRE-luc was constructed as follows: a 77 bp DNA fragment containing three repeats of the ATF/CRE binding site (TGACGTCA) was chemically synthesized and cloned into the *Kpn*I/*Nhe*I sites of pG5luc (121 bp) using standard cloning techniques. Thus the fragment replaced the GAL-4 binding sites of pG5luc completely. Plasmid pATF/CRE-luc could be driven by the binding of ATF3 to the ATF/CRE site and could be used as the ATF3 reporter plasmid.

The ligation products were used to transform competent bacteria. Plasmid DNA of several randomly selected colonies was isolated by the endotoxin-free ultrapure plasmid DNA mini-prep kit (V-gene Biotechnology, Hangzhou, China). Plasmid pACT/ATF3 was digested with *Bam*HI/*Mlu*I; plasmid pATF/CRE-luc was digested with *Kpn*I/*Nhe*I. The size of the DNA fragment obtained on agarose gels gave a strong indication of the probable positive clone.

Cell culture

HeLa and NIH3T3 cells were grown in 10% fetal calf serum/Dulbecco's modified Eagle's medium (Promega). Cultures were maintained at 37 ºC in an incubator with 5% CO_2. The cells grew in a confluent monolayer. When they were at 80%–90% confluence, they were transferred into a 24-well plate. Twenty-four hours after plating, the cells were serum-starved for another 24 h to synchronize cultures into quiescence to be used for transfection experiments.

Transient transfections and luciferase activity assays

Following serum starvation of the cells, the ATF3 reporter plasmid pATF/CRE-luc and/or pACT/ATF3 were transfected into HeLa and NIH3T3 cells with Lipofectamine 2000 reagent (Invitrogen, Grand Island, USA). For each transfection, 50 ng pBIND was added to normalize the transfection efficiency. Every sample was transfected with the same quantity of reporter system pATF/CRE-luc and a different dose of pACT as a balanced plasmid, to ensure every sample was transfected with the same amount of DNA (**Table 1**). The cells were harvested and lysed at 48 h post-transfection, and the firefly luciferase and *renilla* luciferase were detected using the Dual-luciferase reporter assay system (Promega) according to the manufacturer's instructions. All transfection experiments were performed three times.

Table 1 Amount of plasmid DNA used in transfection experiments

Group	pACT (ng)	pACT/ATF3 (ng)	pATF/CRE-luc (ng)	pBIND (ng)
Group 1	450	0	300	50
Group 2	325	125	300	50
Group 3	200	250	300	50
Group 4	120	330	300	50

This study is divided into four groups according to the quantity of transfected ATF3 to observe whether ATF3 could stimulate the expression of the luciferase gene controlled by ATF/CRE both in cells and whether the effect is dose-dependent. The pBIND was used as a reference to normalize transfection efficiency.

Results

Construction of plasmid pACT/ATF3 and reporter plasmid pATF/CRE-luc

After digestion by *Bam*HI/*Mlu*I, pACT/ATF3 was divided into two fragments, and the size of the smaller fragment was expected to be 546 bp [**Fig. 1(A)**]. The ATF3 cDNA present in pACT-ATF3 was sequenced to verify the sequence integrity (GenBank accession No. NM_001674.1). The sequence showed two mutations at position A001C and position G501A [**Fig. 1(B)**], but the first mutation had no effect on the resulting translation product and the second was silent.

A schematic representation of the construction of pATF/CRE-luc is shown in **Fig. 2(A)**. After digestion with *Kpn*I/*Nhe*I, pATF/CRE-luc was cut into two fragments, and the size of the smaller fragment was expected to be 77 bp [**Fig. 2(B)**]. After being treated with *Kpn*I/*Nhe*I, pG5luc was cut into two fragments. The size of the smaller fragment was expected to be 121 bp [**Fig. 2(B)**]. The result of sequencing of ATF/CRE (TAGCTCTCTCTC-TGACGTCAGCCAACGCGTCTCTCTCTGACGTCAG-CCAATCTCTCTCTGACGTCAGCCAAGGTACCT; underlined nucleotides symbolize the ATF/CRE sites) was consistent with our design.

Luciferase activity correlates with the quantity of transfected ATF3

To assess whether the plasmid pATF/CRE-luc functions effectively, transient transfection assays were performed in both HeLa and NIH3T3 cells. Analysis of the activity of luciferase after cotransfection of the plasmids pBIND, pACT/ATF3 and pATF/CRE-luc indicated that ATF3 could stimulate the expression of the luciferase gene controlled by ATF/CRE both in HeLa and NIH3T3 cells (**Fig. 3**). The results indicated that the activating effect of ATF3 could be dependent on the binding of ATF3 to the ATF/CRE site, suggesting that the plasmid pATF/CRE-luc did work. Moreover, this activation effect was dose-dependent on the amount of transfected ATF3 (**Fig. 3**). In these experiments, the basal activity of the ATF3 in cells was low in HeLa cells and NIH3T3 cells, which may reflect the lack of some important transcription factors for basal ATF3 transcription in these cells.

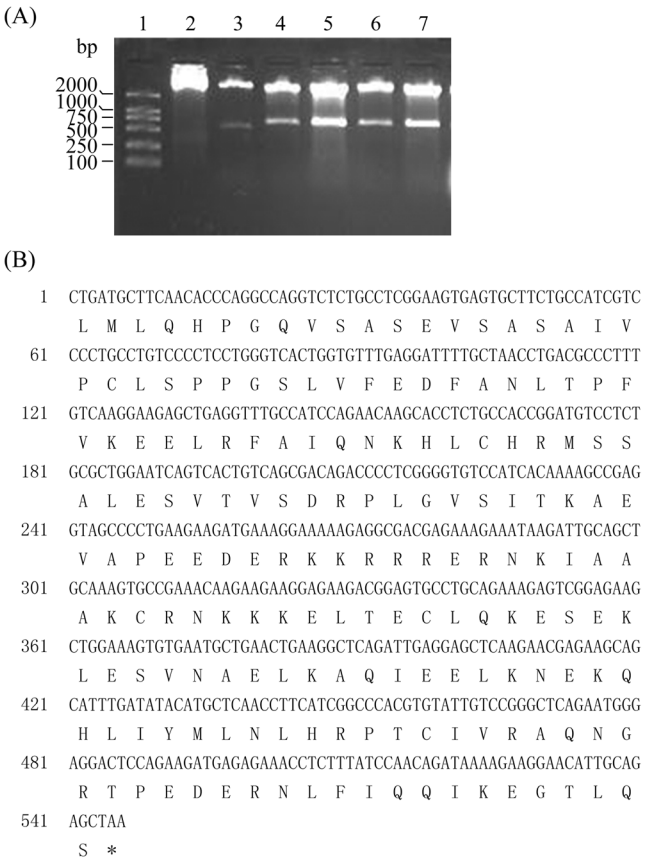

Fig. 1 Agarose gel electrophoresis of pACT/ATF3 and sequence of the activating transcription factor 3 (*ATF3*) gene
(A) 1, molecular weight marker; 2, vector pACT/ATF3 alone; 3–7, pACT/ATF3 vectors digested by *Bam*HI/*Mlu*I. (B) Sequence of the *ATF3* gene. The corresponding sequence of protein is also indicated, the translation beginning at the first in-frame leucine of the open reading frame. Underlined nucleotides symbolize the mutations highlighted by sequencing of the *ATF3* cDNA.

Discussion

Although a lot of evidence has confirmed that ATF3 is a stress-inducible transcriptional factor, little was known

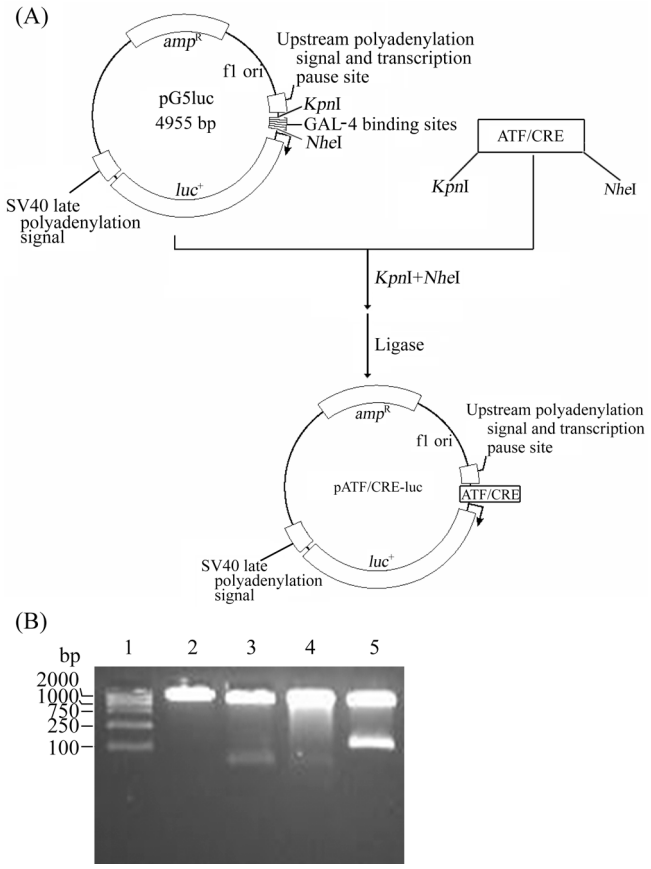

Fig. 2 Construction of pATF/CRE-luc and agarose gel electrophoresis analysis

(A) Construction of pATF/CRE-luc. The arrowheads point in the direction of transcription. (B) Agarose gel electrophoresis analysis of ligation products of pATF/CRE-luc. 1, molecular weight marker; 2, pATF/CRE-luc alone; 3 and 4, pATF/CRE-luc digested by *Kpn*I/*Nhe*I; 5, pG5luc digested by *Kpn*I/*Nhe*I.

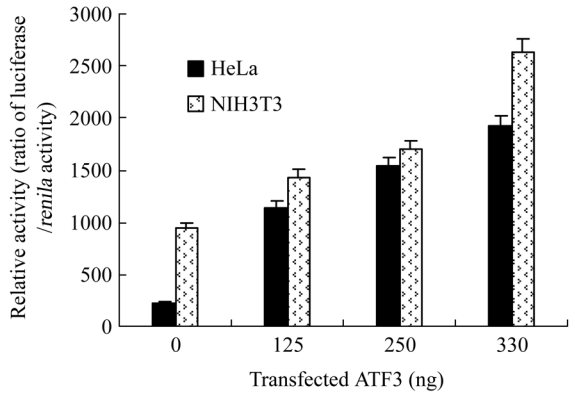

Fig. 3 Assays of luciferase expression in transfected HeLa and NIH3T3 cells

Transient transfection assay to evaluate the effectiveness of activating transcription factor 3 reporter. The count unit is light unit which was normalized by reference luciferace value (*renilla* luciferase) and so all the data in the figure were normalized at the same transfection efficiency. Data are presented as the mean±SD (n=3).

about the physiological significance of ATF3. It was not clear how the activity of ATF3 changes with different cell types and different stresses. Furthermore, there must be chemical factors or proteins in the cells, which could affect the activity of ATF3. To answer these questions, we need a method to detect the activity of ATF3 *in vivo*. One very useful technique is an ATF3 reporter system.

In this study, a new integrative ATF3 reporter plasmid was constructed and proved to be an efficient reporter of the activity of ATF3 in HeLa and NIH3T3 cells. To construct this plasmid we utilized the mammalian two-hybrid system (Promega), which was designed for detecting protein interactions *in vivo*. This system was very beneficial for the construction of a reporter system of transcriptional factors because it was a dual-luciferase system. The pBIND vector, which carried a *renilla* luciferase gene, could be used as a reference to normalize transfection efficiency. The pG5luc vector carried the firefly luciferase gene (luc^+) controlled by five GAL-4 binding sites with a minimal TATA box, so we could replace the GAL-4 binding site with the ATF/CRE site.

To construct the ATF3 reporter vector, the GAL-4 binding sites in the pG5luc vector were replaced by ATF/CRE. To ensure that this reporter system functions effectively, the repeat number of ATF/CRE sites and the distance between two ATF/CRE sites were investigated. Finally, the reporter vector carrying three repeats of ATF/CRE were effective. Our data showed that the quantity in the group of cotransfection of ATF3 and ATF/CRE was much higher than that in the group of transfection of only ATF/CRE in HeLa and NIH3T3 cells. This means that the plasmid pATF/CRE-luc could use the ATF3 reporter successfully. This reporter system could help us to understand the change of activity of ATF3 in different cells and under different stresses. From these results, it could also be deduced that ATF3 is expressed at very low levels in cells under normal conditions, even in tumor cells. This could be very important to our future study.

In conclusion, our successful construction of an ATF3 reporter has offered a convenient system to study the physiological significance of ATF3 induction, and to investigate what influence different extracellular signals have on the activity of ATF3. This work also gave rise to the idea of constructing a practical reporter system of transcriptional factors.

References

1 Landschulz WH, Johnson PF, McKnight SL. The leucine zipper: A hypo-

thetical structure common to a new class of DNA binding proteins. Science 1988, 240: 1759–1764
2 Hai T, Liu F, Coukos WJ, Green MR. Transcription factor ATF cDNA clones: An extensive family of leucine zipper proteins able to selectively form DNA-binding heterodimers. Genes Dev 1989, 3: 2083–2090
3 Weir E, Chen Q, DeFrances MC, Bell A, Taub R, Zarnegar R. Rapid induction of mRNAs for liver regeneration factor and insulin-like growth factor binding protein-1 in primary cultures of rat hepatocytes by hepatocyte growth factor and epidermal growth factor. Hepatology 1994, 20: 955–960
4 Drysdale BE, Howard DL, Johnson RJ. Identification of a lipopolysaccharide inducible transcription factor in murine macrophages. Mol Immunol 1996, 33: 989–998
5 Hai T, Wolfgang CD, Marsee DK, Allen AE, Sivaprasad U. ATF3 and stress responses. Gene Expr 1999, 7: 321–335
6 Abe T, Oue N, Yasui W, Ryoji M. Rapid and preferential induction of ATF3 transcription in response to low doses of UVA light. Biochem Biophys Res Commun 2003, 310: 1168–1174
7 Hsu JC, Laz T, Mohn KL, Taub R. Identification of LRF-1, a leucine-zipper protein that is rapidly and highly induced in regenerating liver. Proc Natl Acad Sci USA 1991, 88: 3511–3515
8 Chen BP, Wolfgang CD, Hai T. Analysis of ATF3, a transcription factor induced by physiological stresses and modulated by gadd153/Chop10. Mol Cell Biol 1996, 16: 1157–1168
9 Tsujino H, Kondo E, Fukuoka T, Dai Y, Tokunaga A, Miki K, Yonenobu K *et al*. Activating transcription factor 3 (ATF3) induction by axotomy in sensory and motoneurons: A novel neuronal marker of nerve injury. Mol Cell Neurosci 2000, 15: 170–182
10 Takeda M, Kato H, Takamiya A, Yoshida A, Kiyama H. Injury-specific expression of activating transcription factor-3 in retinal ganglion cells and its colocalized expression with phosphorylated c-Jun. Invest Ophthalmol Vis Sci 2000, 41: 2412–2421
11 Hai T, Hartman MG. The molecular biology and nomenclature of the activating transcription factor/cAMP responsive element binding family of transcription factors: Activating transcription factor proteins and homeostasis. Gene 2001, 273: 1–11
12 Lanahan A, Williams JB, Sanders LK, Nathans D. Growth factor-induced delayed early response genes. Mol Cell Biol 1992, 12: 3919–3929
13 Wolfgang CD, Chen BP, Martindale JL, Holbrook NJ, Hai T. gadd153/Chop10, a potential target gene of the transcriptional repressor ATF3. Mol Cell Biol 1997, 17: 6700–6707
14 Fawcett TW, Martindale JL, Guyton KZ, Hai T, Holbrook NJ. Complexes containing activating transcription factor (ATF)/cAMP-responsive-element-binding protein (CREB) interact with the CCAAT-enhancer-binding protein (C/EBP)-ATF composite site to regulate Gadd153 expression during the stress response. Biochem J 1999, 339: 135–141
15 Chen BP, Liang G, Whelan J, Hai T. ATF3 and ATF3 delta Zip. Transcriptional repression versus activation by alternatively spliced isoforms. J Biol Chem 1994, 269: 15819–15826
16 Nawa T, Nawa MT, Cai Y, Zhang C, Uchimura I, Narumi S, Numano F *et al*. Repression of TNF-α-induced E-selectin expression by PPAR activators: Involvement of transcriptional repressor LRF-1/ATF3. Biochem Biophys Res Commun 2000, 275: 406–411
17 Allen-Jennings AE, Hartman MG., Kociba GJ, Hai T. The roles of ATF3 in glucose homeostasis. A transgenic mouse model with liver dysfunction and defects in endocrine pancreas. J Biol Chem 2001, 276: 29507–29514
18 Hanahan D. Studies on transformation of *Escherichia coli* with plasmids. J Mol Biol 1983, 166: 557–580
19 Sambrook J, Fritsch EF, Maniatis T. Molecular Cloning: A Laboratory Manual, 2nd ed. New York: Cold Spring Harbor Laboratory Press 1989

Edited by
Xiang-Fu WU