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Physical Interaction between Hepatitis C Virus NS4B Protein and CREB-RP/ATF6β

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Abbreviations used: aa, amino acids; ATF6α, activating transcription factor 6α; ATF6β, activating transcription factor 6β; bZIP, basic leucine zipper; CREB-RP; cyclic AMP-response-element-binding protein-related protein; ER, endoplasmic reticulum; HCV, hepatitis C virus; NS4B, nonstructural protein 4B; PBS, phosphate-buffered saline.

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ABSTRACT

By using a yeast two-hybrid assay, cyclic AMP-response-element-binding protein-related protein (CREB-RP), also called activating transcription factor 6β (ATF6β), was identified as a cellular protein that interacts with the NS4B protein of hepatitis C virus. An N-terminal half of NS4B and a central portion of CREB-RP/ATF6β containing the basic leucine zipper (bZIP) domain were involved in the interaction. The interaction between NS4B and CREB-RP/ATF6β was demonstrated also in mammalian cells by co-immunoprecipitation and confocal microscopic analyses using specific antibodies. The bZIP domain of ATF6α, which shares high sequence similarity with CREB-RP/ATF6β, was also shown to interact with NS4B in yeast although the interaction was weaker than that between NS4B and CREB-RP/ATF6β. CREB-RP/ATF6β and ATF6α are known as endoplasmic reticulum (ER) stress-induced transcription factors. Collectively, our results imply the possibility that NS4B modulates certain cellular responses upon ER stress through the physical interaction with CREB-RP/ATF6β and ATF6α.

Key words: hepatitis C virus; NS4B protein; cyclic AMP-response-element-binding protein-related protein (CREB-RP); activating transcription factor 6β (ATF6β)□ activating transcription factor 6α (ATF6α)□ basic leucine zipper (bZIP); protein-protein interaction; endoplasmic reticulum (ER) stress.

INTRODUCTION

Hepatitis C virus (HCV), a member of the *Flaviviridae* family, is an important human pathogen that can cause acute and chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (1, 2). HCV is currently classified into 6 genotypes, with each genotype being further classified into a number of subtypes, such as subtype 1a (HCV-1a), HCV-1b and HCV-1c (3-5). Geographic distribution and viral characteristics, such as hepatopathogenicity and interferon sensitivity, differ with different subtypes (6-8). The HCV genome, single-stranded, positive sense RNA of ca. 9.6 kb, has a long open reading frame that encodes a polyprotein of about 3,000 amino acids (aa). The polyprotein is cleaved by the cellular signal peptidase and virally encoded two proteases into at least 10 mature proteins; core, envelope glycoprotein 1 (E1), E2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A and NS5B (9, 10).

NS4B is a hydrophobic protein with an apparent molecular mass of 27 kDa, which is present only in the cytoplasm, particularly in the perinuclear region (11, 12) and is thought to be an integral endoplasmic reticulum (ER) membrane protein (13). NS4B has been reported to co-localize with the other HCV nonstructural proteins (13) and form a nonionic detergent-stable complex with NS4A (14). NS4B was shown to activate intracellular signaling pathways that involve NF-κB, AP-1 and serum responsive element (SRE) (15). Park et al. (12) demonstrated that NS4B enhanced H-*ras* oncogene-mediated malignant transformation of NIH3T3 cells. Little is known, however, about a cellular protein(s) that directly interacts with NS4B. By using a yeast two-hybrid system, we screened a human cDNA library to identify a possible cellular target(s) for NS4B. We report here that NS4B physically interacts with cyclic AMP-response-element-binding protein-related protein (CREB-RP) (16, 17). CREB-RP has recently been proposed as activating transcription

factor 6β (ATF6β) and, therefore, is referred to as CREB-RP/ATF6β□□□□□□□□□□□□□□□. We also report that NS4B interacts to a lesser extent with ATF6α, another ER stress-associated transcription factor, which shares high sequence similarity with CREB-RP/ATF6β (18, 19).

MATERIALS AND METHODS

Plasmid construction. A cDNA fragment encoding the full-length NS4B (NS4B-F) of HCV-1b was amplified from the plasmid M094AJ (20) by polymerase chain reaction (PCR) with primers NS4B-1 and NS4B-2 (Table 1). The amplified fragment was digested with ◀ Table 1
*Eco*RI, subcloned into the unique *Eco*RI site in-frame to the LexA DNA-binding domain of pHybLex/Zeo (Invitrogen) to generate pHybLex/NS4B-F for expression in yeast. The same fragment was subcloned into pcDNA3.1(-) vector (Invitrogen) to generate pcDNA/NS4B-F for expression in mammalian cells. Sequences encoding various deletion mutants of NS4B were amplified by PCR using appropriate sets of primers (Table 1 and Fig. 1) and subcloned ◀ Fig. 1
into pHybLex/Zeo and pcDNA3.1(-).

An expression plasmid for the full-length CREB-RP/ATF6β tagged with the FLAG peptide was constructed as described below. A 250-bp sequence of CREB-RP/ATF6β (from the ATG initiation codon to the *Bgl*II site) was amplified by PCR from pcDNA3.1/CREB-RP (21; a generous gift from Dr. K. Mori, Kyoto University, Kyoto, Japan) using primers CREB-rp-Flag-1 and -2 (Table 1). The amplified fragment was digested with *Bam*HI and *Bgl*II. The remaining coding sequence of CREB-RP/ATF6β □□□□obtain□□□from pcDNA3.1/CREB-RP by digestion with *Bgl*II and *Not*I. A vector plasmid, pcDNA3.1/N-FLAG, was constructed by introducing the coding sequence of the FLAG peptide into the unique *Kpn*I and *Bam*HI sites of pcDNA3.1(+). The above two fragments

were subcloned by 3-piece ligation into *Bam*HI-*Not*I-treated pcDNA3.1/N-FLAG, so that the FLAG peptide was fused to the N-terminus of CREB-RP/ATF6β in-frame to it. The resultant plasmid was designated as pcDNA3.1/N-FLAG-CREB-RP and used for expression in mammalian cells.

An expression plasmid for ATF6α was also constructed. A 348-bp cDNA fragment encoding a portion of ATF6α (aa 294 to 409) was amplified by PCR from pcDNA-ATF6α (21; a generous gift from Dr. K. Mori) using primers ATF6α-294S/B42 and ATF6α-409R (Table 1), digested with *Eco*RI and subcloned into the unique *Eco*RI site in-frame to the B42 activation domain of pYESTrp2 (Invitrogen). This plasmid was designated as pYESTrp2-ATF6α(294-409) and used in yeast two-hybrid assay.

Yeast two-hybrid assay. Yeast two-hybrid assay was performed using Hybrid Hunter kit (Invitrogen) to identify a cellular protein(s) that interacts with NS4B. The plasmid pHybLex/NS4B-F was used as a bait, and the pYESTrp cDNA library prepared from HeLa cells (Invitrogen) was used as a source of prey genes. An L40 strain carrying pHybLex/NS4B-F was transformed with the HeLa cDNA library by lithium acetate method. Transformants were screened for growth on YC-WHUKZ300 plates lacking tryptophan, histidine and uracil. Resultant colonies were tested for β-galactosidase (β□Gal) activity with a filter assay according to the manufacture's protocol. Plasmid DNAs in the positive clones were rescued, amplified in *Escherichia coli* (strain HB101), and their nucleotide sequence determined by using a Model 377 DNA Sequencer (Applied Biosystems).

Cell culture and protein expression. For transient expression experiments, vaccinia virus T7 hybrid expression system was used, as reported previously (22). In brief, HeLa cells, maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10%

heat-inactivated fetal bovine serum, were infected with recombinant vaccinia virus vTF7-3 expressing T7 RNA polymerase. After 1h, the cells were co-transfected with pcDNA3.1/NS4B-F and pcDNA3.1/N-FLAG-CREB-RP using Lipofectin (GIBCO) and incubated overnight in serum-free DMEM. Protein expression was analyzed as described below.

Double-staining immunofluorescence analysis. HeLa cells transiently expressing NS4B-F and FLAG-tagged CREB-RP/ATF6β were washed with phosphate-buffered saline (PBS) and fixed with 2% paraformaldehyde at room temperature for 15 min, and then with 100% methanol at –20°C for 20 min. Primary antibodies used were a patient serum that strongly reacted to NS4B and mouse monoclonal antibody against the FLAG peptide (M2; Sigma-Aldrich Corp.). FITC-conjugated goat anti-human IgG and Texas Red-conjugated sheep anti-mouse IgG (MBL, Co., Ltd., Nagoya, Japan) were used as secondary antibodies. The stained cells were observed with a laser scanning confocal microscope (Bio-Rad).

Co-immunoprecipitation analysis. Co-immunoprecipitation analysis was performed as described previously with slight modifications (23). In brief, HeLa cells transiently expressing NS4B-F and FLAG-tagged CREB-RP/ATF6β were harvested and lysed in lysis buffer (1% NP-40, 150 mM NaCl, 10 mM Tris-HCl [pH 7.5], 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and 0.5 mM dithiothreitol). The lysates were centrifuged at 15,000 rpm for 20 min to remove cell debris and the supernatants were preincubated with protein G-sepharose (CN Biosciences) at 4°C for 30 min to eliminate nonspecific binding. The supernatants were incubated for 3 h at 4°C with anti-FLAG monoclonal antibody (to immunoprecipitate CREB-RP/ATF6β) or anti-p21/Waf1 monoclonal antibody (as a control), followed by incubation with 10 μl of protein G-sepharose for 30 min. After being washed

five times, the immunoprecipitated proteins were analyzed by immunoblotting, as described below. The immunoprecipitates were dissolved in a solution consisting of 50 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue and 10% glycerol, subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and electrophoretically blotted onto a polyvinylidene difluoride filter (Bio-Rad). After blocking in PBS containing 5% nonfat dry milk, the filters were incubated with a patient serum that strongly reacted to NS4B. The filters were then washed five times with PBS containing 0.5% Tween 20, and incubated with peroxidase-labeled goat anti-human IgG (MBL). After being washed five times, the protein bands were visualized by an enhanced chemiluminescence method (ECL; Amersham Biosciences).

RESULTS

Identification of CREB-RP/ATF6β as an NS4B-interacting protein in yeast. To identify a human protein(s) that physically interacts with NS4B, we screened HeLa cell cDNA library by the yeast two-hybrid assay. The L40 transformant harboring pHybLex/NS4B-F was transformed with the pYESTrp-based HeLa cDNA library and screened for growth on YC-WHUKZ300 plates lacking tryptophan, histidine and uracil. Out of a total of 2 x 10^6 yeast transformants screened, 49 clones grew on the YC-WHUKZ300 selection plates. Screening for β-Gal activity identified 7 positive clones; 5 clones were tested positive in 30 min and the other 2 clones in 3 h. DNA sequence analysis revealed that all of the 5 clones that exhibited strong β-Gal activity contained an identical sequence that matched a reported sequence for a portion of CREB-RP/ATF6β (aa 313 to 428; see Fig. 1) (DDBJ accession numbers X98054 and U31903) (16, 17) while the other 2 clones with moderate β-Gal activity contained cDNA for another human protein. In this study we

focused on the analysis of CREB-RP/ATF6β. The cloned cDNA fragment of the partial CREB-RP/ATF6β sequence was designated as CREB-RP(313-428).

To confirm the positive interaction between NS4B and CREB-RP/ATF6β in yeast, the parental (naive) L40 strain was transfected with various combination of the expression plasmids [Lex-NS4B-F and B42-CREB-RP(313-428)] and tested for the abilities to grow on the YC-WHUK Z300 plates and β-Gal activity. The results obtained demonstrated specific interaction between NS4B and CREB-RP/ATF6β in yeast (Table 2). ← Table 2

In order to determine which region of NS4B was involved in the interaction with CREB-RP/ATF6β, a series of truncated forms of NS4B were cloned into pHybLex/Zeo in frame to the B42 activation domain (Fig. 1) and co-transfected into the parental L40 with pYESTrp-CREB-RP(313-428), the plasmid that had been cloned in the initial screening. The yeast transformants containing C-terminally truncated forms of NS4B, such as NS4B(1-195) and NS4B(1-133), grew on the YC-WHUKZ300 plates and exhibited β-Gal activity as did NS4B-F. Further C-terminal truncation of NS4B, as observed with NS4B(1-63), abolished the ability to interact with CREB-RP/ATF6β. On the other hand, N-terminal truncation by 14 residues or more, as observed with NS4B(15-133) and NS4B(41-133), diminished the CREB-RP/ATF6β-binding ability considerably, and N-terminal truncation by 62 residues, NS4B(63-133), abolished the activity completely . A C-terminal half of NS4B, NS4B(134-261), did not show any sign of positive interaction with CREB-RP/ATF6β.

Interaction between NS4B and CREB-RP/ATF6β in mammalian cells. Possible interaction between NS4B-F and FLAG-tagged CREB-RP/ATF6β in mammalian cells was tested. Anti-FLAG antibody co-immunoprecipitated NS4B-F from lysates of HeLa cells expressing NS4B-F and FLAG-tagged CREB-RP/ATF6β, but not from lysates of cells

← Fig. 2

expressing NS4B-F alone (Fig. 2A). A control antibody (anti-p21/Waf1) did not immunoprecipitate NS4B from lysates of the co-expressing cells. These results suggested that NS4B-F formed a complex with CREB-RP/ATF6β□ N-terminal deletion of NS4B by 40 residues [NS4B(41-261)] considerably impaired the complex formation with FLAG-tagged CREB-RP/ATF6β (Fig. 2B). Further N-terminal deletion resulted in more reduction in the complex formation with CREB-RP/ATF6β, and NS4B(134-261) was shown to have completely lost the ability to interact with CREB-RP/ATF6β.

We then tested possible co-localization of NS4B-F with CREB-RP/ATF6β in HeLa cells by double-staining immunofluorescence analysis using a confocal laser microscope. Both NS4B-F and CREB-RP/ATF6β were detected in the cytoplasm, especially in the perinuclear region (Fig. 3, A and B). Co-localization of NS4B-F and CREB-RP/ATF6β was demonstrated by the yellow-color staining in the perinuclear region (Fig. 3C). ← Fig. 3

Interaction of NS4B with ATF6α in yeast. The NS4A-binding region of CREB-RP/ATF6β (aa 313 to 428) shows strikingly high homology with the corresponding region of ATF6α (aa 294 to 409) (Fig. 4). The overall sequence identity in this region between them is 50% (58/116). Moreover, most residues that differ from each other are homologous residues with similar characteristics. Therefore, we were interested to test whether or not NS4B interacts with ATF6α as well. Yeast two-hybrid assay using pHybLex-NS4B-F and pYESTrp2-ATF6α(294-409) revealed specific interaction between NS4B-F and ATF6α(294-409) (Table 3). The degree of the interaction between NS4B-F and ATF6α, as represented by β-Gal activity, was weaker than that between NS4B and CREB-RP/ATF6β. ← Fig. 4 ← Table 3

DISCUSSUION

By means of yeast two-hybrid assay, we identified CREB-RP/ATF6β as a cellular target for NS4B (Table 2). An N-terminal half of NS4B (aa 1 to 133) was required, with an N-terminal quarter (aa 1 to 63) being insufficient, for the full degree of interaction (Fig. 1). An N-terminal deletion by 14 aa (aa 15 to 133) diminished drastically the degree of the interaction. It was recently proposed that N-terminal 46 residues of NS4B was localized in the cytoplasm while regions spanning aa 47 to 64, 69 to 86, 91 to 108, 113 to 133, and 138 to 155 were transmembrane domains (24). Collectively, these results suggest the possibility that appropriate conformation created by the N-terminal cytoplasmic region and four, but not the first one alone, of the five transmembrane domains is necessary for NS4B to interact maximally with CREB-RP/ATF6β. As for CREB-RP/ATF6β, a central portion (aa 313 to 428) containing the basic leucine zipper (bZIP) domain was shown to be involved in the interaction with NS4B. Evidence for physical interaction between NS4B-F and FLAG-tagged CREB-RP/ATF6β in mammalian cells was demonstrated by co-immunoprecipitation analysis (Fig. 2A). Consistent with the results obtained with yeast two-hybrid assay, N-terminal deletion of NS4B by 40 residues significantly impaired the interaction with CREB-RP/ATF6β and further deletion almost completely abolished it (Fig. 2B). Confocal microscopic analysis revealed co-localization of NS4B-F and CREB-RP/ATF6β in ER-like perinuclear structures (Fig. 3).

CREB-RP/ATF6β is a member of the CREB/ATF subfamily of the bZIP superfamily and shares high sequence homology with ATF6α (18, 19). It was reported that CREB-RP/ATF6β suppressed transcription of ER stress-inducible genes while ATF6α enhanced it (18, 19, 21). In response to ER stress, CREB-RP/ATF6β□and ATF6α each are cleaved by a cellular protease and their N-terminal portions (aa 1 to 392 and 1 to 373, respectively), which contain the bZIP domain, are transported to the nucleus to

trans-modulate expression of ER stress-induced genes (18, 19, 25-28). It would be interesting to determine whether or not NS4B modulates such transcriptional regulatory functions of CREB-RP/ATF6β and ATF6α upon ER stress. It is expected that the apparent effect(s) of NS4B might vary with different cell types and different causes of ER stress because activation of CREB-RP/ATF6β and ATF6α may occur differentially depending on the different conditions. In this connection, it should be noted that HCV replication itself also activates ATF6α, as demonstrated by using HCV subgenomic replicons (29).

Many oncogenic viruses possess viral proteins that bind to the bZIP class of transactivators, which is thought to be responsible, at least partly, for cellular transformation by the viruses. For example, HBx protein of hepatitis B virus has been reported to interact with the bZIP class of transcription factors (30). Similarly, the core protein of HCV was shown to bind to LZIP, a member of the bZIP superfamily, to inhibit its function, which correlated with cellular transformation (31). Since NS4B was reported to transform NIH3T3 cells in cooperation with the H-*ras* oncogene (12), it is intriguing to speculate that, in addition to the core protein, NS4B plays an important role in carcinogenesis by HCV through the interaction with CREB-RP/ATF6β. Further study is needed to clarify the issue.

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FIGURE LEGENDS

FIG. 1. Schematic representation of CREB-RP/ATF6β and NS4B. (A) CREB-RP/ATF6β□ A thick line indicates the region (aa 313 to 428) that has been identified to interact with NS4B. bZIP, basic region/leucine zipper domain; TM, transmembrane domain. (B) Full-length and various deletion mutants of NS4B, and summary of possible interaction with CREB-RP/ATF6β in yeast. Thick lines depict the full-length (NS4B-F) and various deletion mutants of NS4B. The numerals along the lines indicate aa positions. Indicators for the positive interaction between NS4B and CREB-RP/ATF6β (growth on selection plates and β-Gal activity) are shown on the right.

FIG. 2. Complex formation between NS4B and CREB-RP/ATF6β in mammalian cells. (A) NS4B-F was expressed in HeLa cells without (lanes 1 and 3) or with FLAG-tagged CREB-RP/ATF6β□□□□□□□□□□□□□□□□□□□□□. Cell lysates were immunoprecipitated with anti-FLAG (lanes 3 and 4) or anti-p21 antibodies (lane 5; served as a negative control). The immunoprecipitates or cell lysates without immunoprecipitation (lanes 1 and 2) were probed with a patient serum that strongly reacted to NS4B. (B) NS4B-F and N-terminally deleted mutants, such as NS4B(41-261), NS4B(63-261) and NS4B(134-261), were each expressed in HeLa cells with (lanes 1 to 4, 6, 8, 10 and 12) or without FLAG-tagged CREB-RP/ATF6β (lanes 5, 7, 9 and 11). Cell lysates were immunoprecipitated with anti-FLAG antibody (lanes 5 to 12) and probed with a patient serum that strongly reacted to NS4B. The cell lysates were directly (without immunoprecitated with anti-FLAG antibody) probed with a patient serum that strongly reacted to NS4B to verify a comparable degree of expression of NS4B-F and each of the N-terminally deleted mutants (lanes 1 to 4).

FIG. 3. Co-localization of NS4B and CREB-RP/ATF6β in mammalian cells. HeLa cells co-expressing NS4B-F and FLAG-tagged CREB-RP/ATF6β were analyzed by confocal laser microscopy. (A) Cells were stained with a patient serum that strongly reacted to NS4B and FITC-conjugated goat anti-human IgG. (B) Cells were stained with anti-FLAG mouse monoclonal antibody and Texas Red-conjugated sheep anti-mouse IgG. (C) The fluorescent images of (A) and (B) were merged to assess co-localization. The yellow color represents co-localization of NS4B and CREB-RP/ATF6β.

FIG. 4. Amino acid sequence alignment between CREB-RP/ATF6β and ATF6α. The NS4B-binding region of CREB-RP/ATF6β□□aa 313 to 428□ and the corresponding region of ATF6α (aa 294 to 409) are aligned. Dashes in the ATF6α sequence indicate residues identical to those of CREB-RP/ATF6β. Identical and homologous residues are written in boldface letters. Basic region, leucine zipper and transmembrane domains are shown by dashed, solid and chained lines, respectively.

(Table 1) (Table 2) (Table 3) (Fig. 1) (Fig. 2) (Fig. 3) (Fig. 4)

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An expression plasmid for the full-length ATF6α tagged with the FLAG peptide was also constructed as follows. A 522-bp region of pcDNA3.1/ATF6α (20; a generous gift from Dr. K. Mori, Kyoto University, Japan) encoding the N-terminal 174 aa residues of ATF6α was amplified using primers ATF6α-1S and ATF6α-174R (Table 1), and digested with *Bam* HI and *Eco*RI. This fragment was ligated with *Bam*HI-*Xho*I-treated pcDNA3.1/N-FLAG together with a ~1500-bp fragment of pcDNA3.1/ATF6α encoding a C-terminally deleted ATF6α (aa 175 to 670) that was flanked with *Eco*RI and *Xho*I sites at the 5'- and 3'-terminus, respectively. This plasmid was designated as

pcDNA3.1/N-FLAG-ATF6α. and used for expression in HeLa cells.

An expression plasmid for a C-terminally deleted mutant of CREB-RP/ATF6β was constructed by replacing an internal 1.5-kb *Sac*I-*Not*I fragment of pcDNA3.1/N-FLAG-CREB-RP with a short stretch of sequence (5'-AAGTTAGGGTCTTAG-3'; underline indicates a stop codon) flanked with *Sac*I-*Not*I adapter sequence. This plasmid was designed to express a CREB-RP/ATF6β mutant (aa 1 to 392) and referred to as pcDNA3.1-FLAG-CREB-RP(1-392).

Analysis of possible effects of NS4B on trans-activating function of CREB-RP/ATF6β. HuH-7 human hepatoma cells were transiently transfected with pcDNA3.1-CREB-RP(1-392) in the presence or absence of pcDNA/NS4B-F using FuGENE 6 (Roche Diagnostics). After 24 h, the cells were treated with tunicamycin (2 μg/ml) or left untreated for another 24 h. The cells were then lyzed in a buffer consisting of 50 mM Tris-HCl (pH 6.8), 100 mM dithiothreitol, 2% SDS, 0.1% bromophenol blue and 10% glycerol, and the cell lysates were subjected to immunoblot analysis using anti-KDEL (lysine-aspartic acid-glutamic acid-leucine) mouse monoclonal antibody (SPA-827; StressGen Biotech., Victoria, BC, Canada) that recognizes ER stress-inducible proteins, such as glucose-regulated proteins 78 (GRP78), as reported previously (18, 19, 21, 25). The same filters were also probed with anti-actin goat polyclonal antibody (sc-1615, Santa Cruz) to verify an equal amount of sample loading in each lane.

Inhibition of CREB-RP/ATF6β function by NS4B in mammalian cells.

CREB-RP/ATF6β(1-392) was reported to mediate transcriptional activation on expression of ER stress-inducible proteins, such as GRP78 (18, 19, 21, 25). Using this system, we analyzed possible inhibitory effects of NS4B on CREB-RP/ATF6β function in mammalian cells. HuH-7 cells transiently expressing either CREB-RP(1-392) alone or CREB-RP(1-392) *plus* NS4B-F were analyzed for GRP78 expression. CREB-RP/ATF6β(1-392) significantly induced GRP78 expression in the absence of tunicamycin treatment (Fig. 4A, lane 3). On the other hand, CREB-RP/ATF6β(1-392)-mediated GRP78 induction was considerably inhibited by co-expressed NS4B (Fig. 4A, lane 5). Expression of β-actin and CREB-RP/ATF6β(1-392) was not affected by co-expressed NS4B under this experimental condition Fig. 4B, C). Collectively, these results suggested that NS4B inhibited *trans*-activating function of CREB-RP/ATF6β in mammalian cells.

FIG. 4. *Inhibition of CREB-RP/ATF6β function by NS4B in mammalian cells.* (A) HuH-7 cells were transiently transfected with pcDNA3.1 vector alone (lanes 1 and 2), pcDNA3.1-CREB-RP(1-392) *plus* pcDNA3.1 vector ((lanes 3 and 4) or pcDNA3.1-CREB-RP(1-392) *plus* pcDNA/NS4B-F (lanes 5 and 6). After 24 h, the cells were treated with tunicamycin (2 μg/ml) (lanes 1, 3 and 5) or left untreated (lanes 2, 4 and 6) for another 24 h. The cells were lyzed and analyzed by immunoblotting using anti-KDEL antibody. (B) The same filter was probed with anti-actin antibody to verify an equal amount of sample loading in each lane. (C) The same filter was probed with anti-FLAG antibody.

TABLE 1

Primers Used in This Study

Name	Polarity	Sequences
NS4B-1	Sense	5'-GGCGGAATTCGCC**ATG**GCTTCGCACCTCCCTTACAT-3'
NS4B-2	Antisense	5'-CCGCGAAT**TCA**ACATGGCGTGGAGCAGTC-3'
NS4B-133R	Antisense	5'-CCGCGAAT**TCA**GAGGCCTATGCTGCCAAC-3'
NS4B-195R	Antisense	5'-CCGCGAAT**TCA**CACATGCCGACGCAGTAT-3'
NS4B15F	Sense	5'-TATAGAATTCATC**ATG**GAGCAATTCAAGCAG-3'
NS4B41F	Sense	5'-TATAGAATTCATC**ATG**TCCAAGTGGCGA-3'
NS4B63F	Sense	5'-TATAGAATTCATC**ATG**TACTTAGCAGGCTT-3'
NS4B134F	Sense	5'-TATAGAATTCATC**ATG**GGGAAGGTGCTTGT-3'
CREB-rp Flag-1	Sense	5'-CGTGGATCC**ATG**GCGGACCTG-3'
CREB-rp Flag-2	Antisense	5'-CTGAAGATCTGGGAAGATCGG-3'
ATF6α-294S/B42	Sense	5'-CCAAGAATTCAAAGTACCATGAGAAAT-3'
ATF6α-409R	Antisense	5'-CCAAGAATT**CTA**CACACTAGGGTTCAT-3'

The translation initiation codon and complementary sequences of stop codons are shown in boldface letters. The enzyme recognition sites are underlined.

TABLE 2

Interaction between NS4B and CREB-RP/ATF6β in Yeast

pHybLex/Zeo (bait plasmid))	pYESTrp2 (prey plasmid)	β -Gal activity
Lex-vector	B42-CREB-RP(313-428)	–
Lex-NS4B-F	B42-vector	–
Lex-NS4B-F	B42-CREB-RP(313-428)	+ (~30 min)
Lex-Lamin[a]	B42-CREB-RP(313-428)	–
Lex-Lamin[a]	B42-Jun[b]	–
Lex-Fos[c]	B42-Jun[b]	+ (~30 min)

[a] Negative control for bait plasmid.

[b] Positive control for prey plasmid.

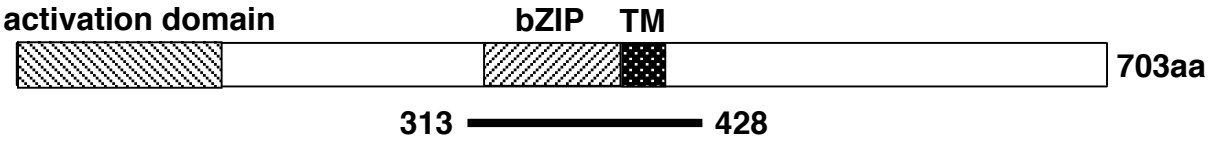
[c] Positive control for bait plasmid.

TABLE 3

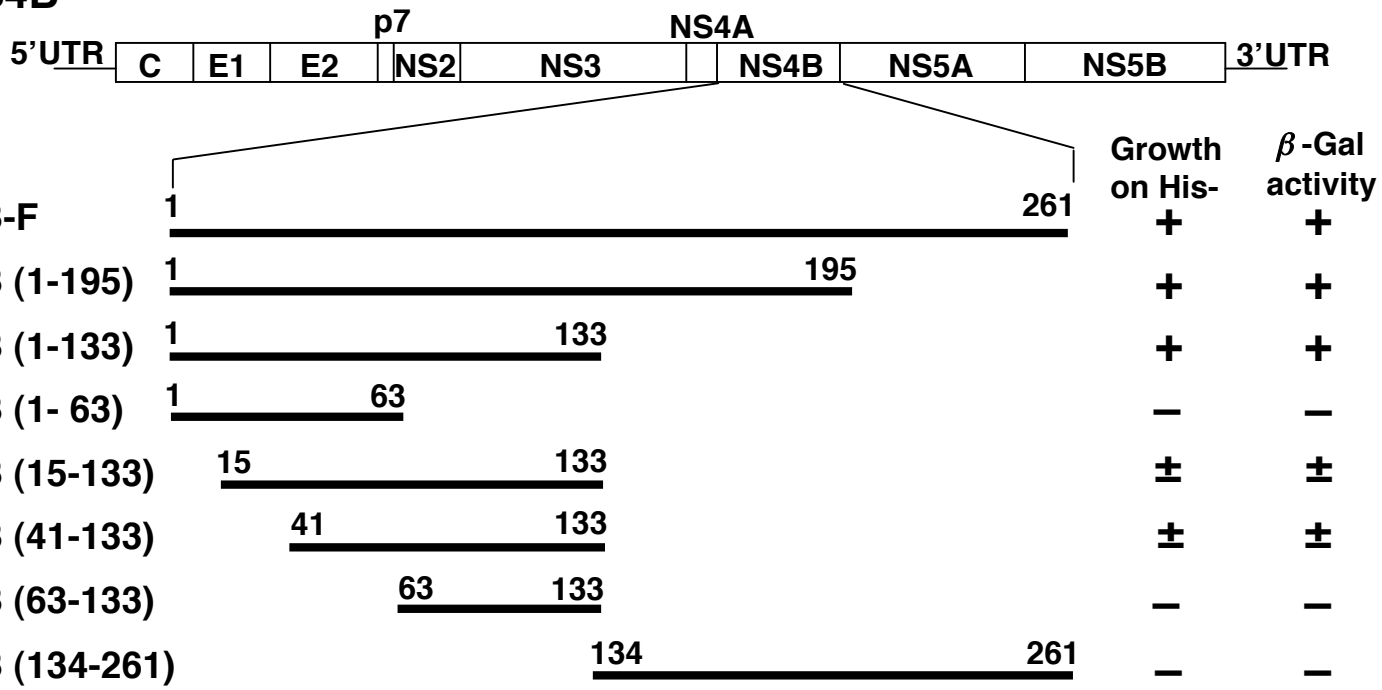
Interaction between NS4B and ATF6α in Yeast

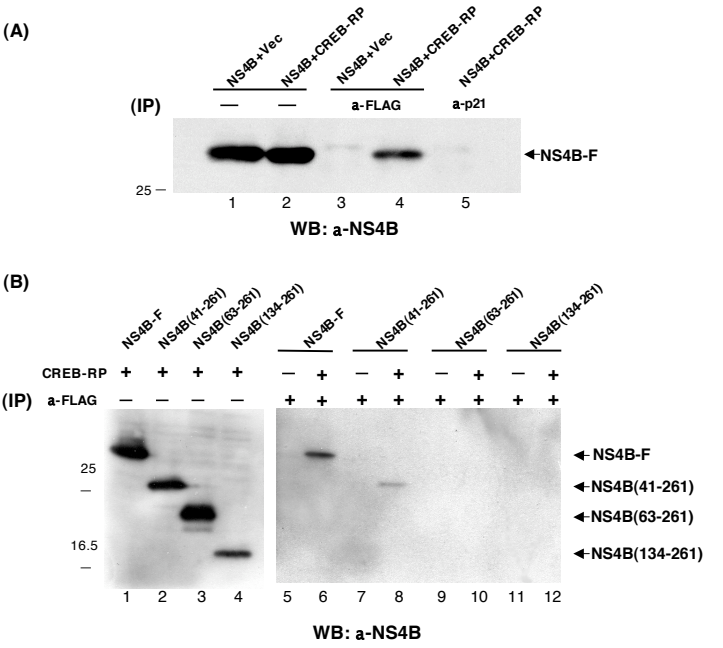
pHybLex/Zeo (bait plasmid))	pYESTrp2 (prey plasmid)	β -Gal activity
Lex-vector	B42- ATF6α(294-409)	–
Lex-NS4B-F	B42-vector	–
Lex-NS4B-F	B42- ATF6α(294-409)	+ (~3 h)
Lex-NS4B-F	B42-CREB-RP(313-428)	+ (~30 min)

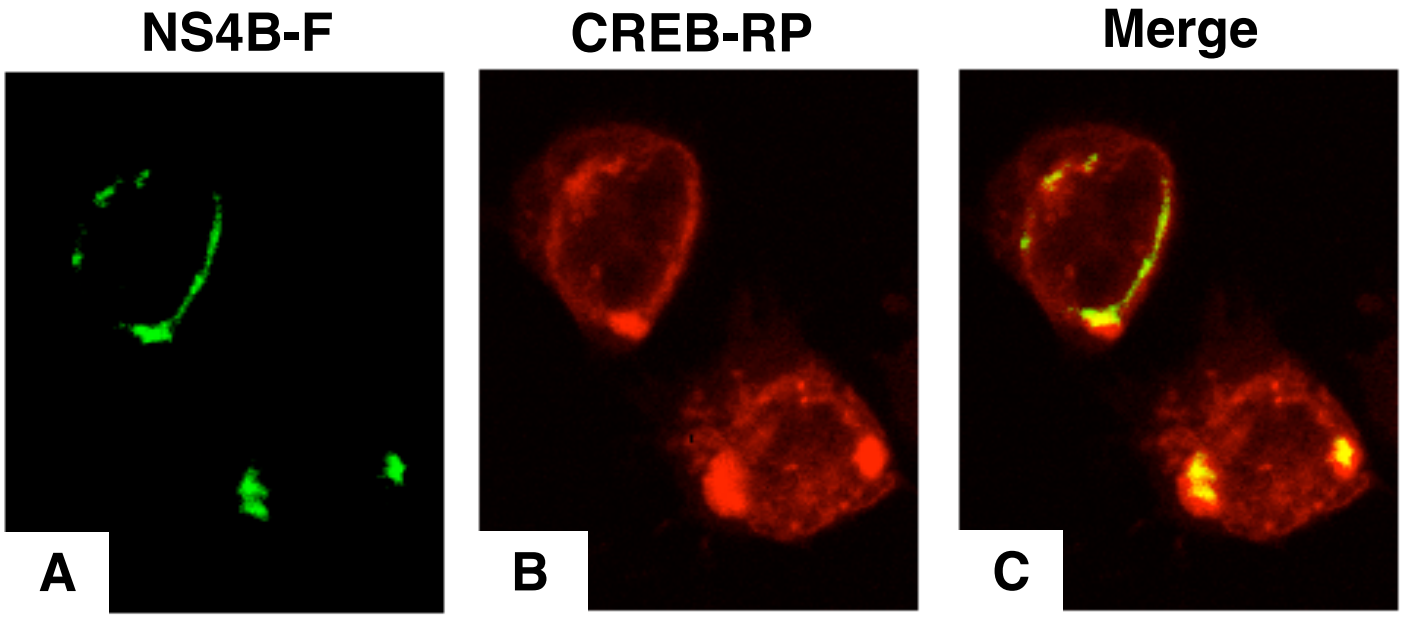
(A) CREB-RP/ATF6β

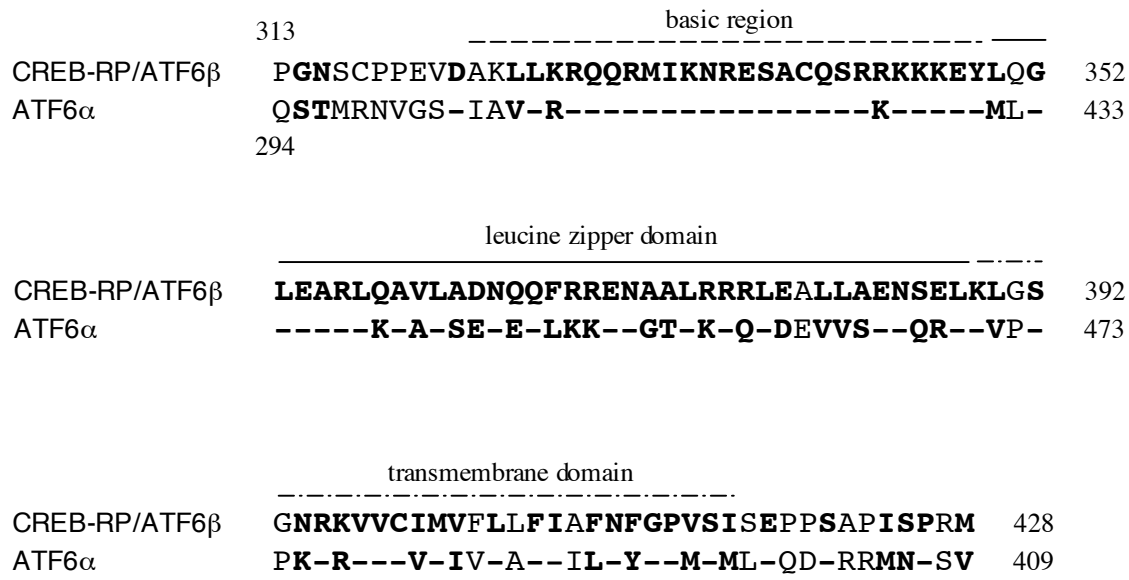


(B) NS4B




(A)
NS4B+Vec
NS4B+CREB-RP
NS4B+Vec
NS4B+CREB-RP
NS4B+CREB-RP
(IP)
—
—
a-FLAG
a-p21
NS4B-F
25
1
2
3
4
5
WB: a-NS4B
(B)
NS4B-F
NS4B(41-261)
NS4B(63-261)
NS4B(134-261)
NS4B-F
NS4B(41-261)
NS4B(63-261)
NS4B(134-261)
CREB-RP + + + + — + — + — + — +
(IP) a-FLAG — — — — + + + + + + + +
25
16.5
NS4B-F
NS4B(41-261)
NS4B(63-261)
NS4B(134-261)
1 2 3 4 5 6 7 8 9 10 11 12
WB: a-NS4B


NS4B-F
CREB-RP
Merge
A
B
C


basic region
313
CREB-RP/ATF6β PGNSCPPEVDAKLLKRQQRMIKNRESACQSRRKKKEYLQG 352
ATF6α QSTMRNVGS-IAV-R----------K-----ML- 433
294
leucine zipper domain
CREB-RP/ATF6β LEARLQAVLADNQQFRRENAALRRRLEALLAENSELKLGS 392
ATF6α -----K-A-SE-E-LKK--GT-K-Q-DEVVS--QR--VP- 473
transmembrane domain
CREB-RP/ATF6β GNRKVVCIMVFLLFIAFNFGPVSISEPPSAPISPRM 428
ATF6α PK-R---V-IV-A--IL-Y--M-ML-QD-RRMN-SV 409