Viral mediated stabilization of AU-rich element containing mRNA contributes to cell transformation

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Abstract

E4orf6 is one of the oncogene products of adenovirus and it also plays an important role for transportation of cellular and viral mRNA during the late phase of virus infection. We previously revealed that E4orf6 controls the fate of AU-rich element (ARE) containing mRNA by perturbing the CRM1-dependent export mechanism. Here, we show that E4orf6 stabilizes ARE-mRNA through the region required for its oncogenic activity and ubiquitin E3 ligase assembly. Cells that failed to stabilize ARE-mRNA after HuR knockdown were unable to produce colonies in soft-agar, even when E4orf6 was expressed. Furthermore, the stabilized ARE-mRNA induced the transformation of rodent immortalized cells. These findings indicate that stabilized ARE-mRNA is necessary, if not all, for the oncogenic activity of E4orf6 and has the potential to transform cells at least under a certain condition.

Keywords: Adenovirus/ AU-rich element/E4orf6/transformation/stabilization,

Introduction

Research on DNA tumor viruses has been conducted in connection with numerous biological events of host cells. These viruses encode proteins that are capable of controlling the mRNA transport systems of host cells in order to replicate their genome and propagate their virus particles. Generally, these proteins achieve their functions by interacting with cellular proteins.

Adenovirus E4orf6 promotes the nuclear export of viral late mRNA and inhibits the bulk of cellular mRNA export during the late phase of adenovirus infection (Dobner and Kzhyshkowska 2001; Shenk 2001). It also cooperates with E1A and E1B to transform primary baby rat kidney (BRK) cells, and the expression of E4orf6 markedly enhances the ability of BRK and human 293 cells to form tumors in nude mice (Moore *et al*, 1996; Nevels *et al*, 1997). E4orf6 forms a complex with adenovirus E1B55k (Sarnow *et al*, 1984), and this complex assembles into E3 ubiquitin ligase through the domain of E4orf6 that binds with elongins B and C (Querido *et al*, 2001). It is suggested that most if not all of the functions of the E1B55k/E4orf6 complex, including the control of mRNA export, are achieved through the degradation of target proteins by E4orf6 via its E3 ligase activity (Woo and Berk, 2007; Blanchette *et al*, 2008).

AU-rich elements (ARE) are present in many proto-oncogene, growth factor, and cytokine mRNA sequences and target ARE-mRNA for rapid degradation (Chen and Shyu 1995; Brennan and Steitz 2001). HuR, a member of the embryonic lethal abnormal vision (ELAV) family of RNA-binding proteins, binds to ARE to protect ARE-mRNA from rapid degradation (Hinman and Lou 2008; Brennan and Steitz 2001). Although HuR is localized predominantly in the nucleus, it can shuttle between the nucleus and cytoplasm and the stabilization of ARE-mRNA by HuR is believed to be linked to its localization in the cytoplasm (Hinman and Lou 2008; Lopez de Silanes *et al*, 2005).

The export of HuR is mediated by its association with transportin 1 (Trn1) and Trn2 (Rebane *et al*, 2004; Gallouzi and Steitz 2001b) via the shuttling sequence in its hinge region, which is termed HNS (Fan and Steitz, 1998), and by its association with pp32 and APRIL, which includes the nuclear export signal recognized by the export receptor chromosome maintenance region 1 (CRM1) (Brennan *et al*, 2000; Gallouzi *et al*, 2001a). Usually, HuR is exported into the cytoplasm of cancer cells, and cytoplasmic HuR expression has been implicated in the malignancy of several types of carcinomas, such as colon cancer and has also been postulated to contribute to the cancerous malignant phenotype (Lopez de Silanes *et al*, 2005).

We previously found that pp32 associates with E4orf6 (Higashino *et al*, 2005) and that E4orf6 induces the cytoplasmic accumulation of ARE-mRNA in a manner independent of CRM1. Here, we show that E4orf6 stabilizes ARE-mRNA via two distinct functional domains, which are required for its oncogenic activity and its assembly into the E3 ligase. HuR knockdown cells failed to stabilize ARE-mRNA and make colonies in soft-agar, even when E4orf6 is expressed in the cells. Furthermore, rodent cells containing the stabilized ARE-mRNA were able to form colonies. These results indicate that E4orf6 stabilizes ARE-mRNA to exert its oncogenic activity, and this study provides the first evidence that the stabilization of ARE-mRNA induces malignant transformation.

Results

Stabilization of ARE-mRNA by E4orf6

The stabilization of ARE-mRNA was examined using transformed baby rat kidney (BRK) cells. The amounts of *c-fos*, *c-myc*, and *COX-2* mRNA in the BRK E1+E4 cells, which were transformed with the adenovirus *E1* (*E1A* and *E1B*) and *E4orf6* genes, were estimated by quantitative real-time RT-PCR and compared with those in BRK E1 cells, which were transformed with the *E1* gene. The amount of *c-fos* mRNA that accumulated in the BRK E1+E4 cells was about 4.0 times higher than that in the BRK E1 cells (Figure 1A). The BRK E1+E4 cells also accumulated approximately 5.5- and 4.2-fold greater levels of *c-myc* and *COX-2* mRNA, respectively, than the BRK E1 cells (Figure 1A).

To eliminate the possibility that the stabilization was induced by the transcriptional activation of each gene, we examined their half-lives. The cells were treated with actinomycin D (act D), and the quantity of each mRNA species in BRK cells was measured by quantitative real-time RT-PCR at 0, 60, and 120 minutes after treatment. The half-lives of *c-fos*, *c-myc*, and *COX-2* mRNA in BRK E1+E4 cells were about 141, 144, and 165 minutes, respectively. These values were significantly longer than those of the BRK E1 cells (50, 50, and 73 minutes, respectively) (Figure 1B).

To confirm the stabilization of ARE-containing mRNA by E4orf6, we constructed pGL3-based luciferase reporter plasmids with and without the 3'-UTR of *c-fos* mRNA including the ARE (luciferase-ARE$^{c\text{-}fos}$; Figure 1C left), and these plasmids were introduced into the BRK cells to examine the stabilization of the fusion gene via luciferase activity. Luciferase activity was downregulated in the BRK E1+E4 cells when the luciferase reporter without ARE$^{c\text{-}fos}$ (pCMVGL) was used (Figure 1C right). On the other hand, the luciferase activity derived from pCMVGL-ARE$^{c\text{-}fos}$ was significantly activated in BRK E1+E4 cells compared with that in BRK E1 cells (Figure 1C right). These results suggest that luciferase-ARE$^{c\text{-}fos}$ mRNA was stabilized in the E4orf6 expressing cells in an ARE dependent manner, indicating that the ARE allows mRNA to be stabilized by E4orf6.

Since act. D suppresses the synthesis of all mRNA, the effect of E4orf6 on the ARE-mRNA stabilization described above might be due to secondary effects. To eliminate this possibility, we constructed 293 cells harboring the luciferase-ARE$^{c\text{-}fos}$ gene (Figure 1C left), the expression of which was controlled by the Tet-Off system, which can stop the expression of single genes. Constitutively expressed luciferase-ARE$^{c\text{-}fos}$ mRNA was stopped by adding doxycycline, and the half-life of the mRNA was calculated. The half-life of luciferase-ARE$^{c\text{-}fos}$ mRNA was extended from 56 to 147 minutes when E4orf6 was present in 293 cells (Figure 1D). Taken together, we conclude that E4orf6 stabilizes ARE-containing mRNA.

The alpha helix structure of E4orf6 is required for its interaction with pp32

We analyzed the region of E4orf6 that is required for the stabilization of ARE-mRNA. In our previous report, we identified that the carboxyl terminal region of E4orf6 (the oncodomain; see Figure 2A) including the arginine-faced amphipathic alpha helix is required for its interaction with pp32 and the cytoplasmic accumulation of ARE-mRNA (Higashino *et al*, 2005). This alpha helix structure is known to be essential for the oncogenic activities of E4orf6, including cooperative focus formation with E1A and the promotion of tumor growth in nude mice (Nevels *et al*, 2000). In addition, the same structure is also required for E4orf6 to assemble into the E3 ubiquitin ligase complex with E1B55k (Blanchette *et al*, 2004). To analyze this region further, we produced additional E4orf6 mutants, E4orf6 L245P and E4orf6 R248E (Figure 2A), which possess amino acid substitutions in their alpha helices, and then examined their *in vivo* interactions with pp32. The E4orf6 L245P mutant has a disrupted alpha helical structure (Orlando and Ornelles 1999) and fails to display oncogenic activity (Nevels *et al*, 2000). On the other hand, the E4orf6 R248E mutation was reported to interfere with the nuclear retention of E4orf6, but did not affect its oncogenic activity (Dobbelstein *et al*, 1997; Nevels *et al*, 2000). As shown in Figure 2B, the E4orf6 L245P mutant failed to interact with pp32, although the E4orf6 R248E mutation did not have any influence on pp32 binding. These findings indicate that the alpha helix structure of E4orf6 is necessary for pp32 binding and that the interaction of E4orf6 with pp32 plays an important role in the oncogenic activity of the viral gene product.

The alpha helix structure and BC-box motif of E4orf6 are required for the stabilization and the cytoplasmic localization of ARE-mRNA

To examine the effect of the alpha helix structure of E4orf6 on its ability to stabilize ARE-mRNA, we repeated the same experiment as shown in Figure 1. No increases in the amount of ARE-mRNA were seen in the E4orf6 dl210-294 or E4orf6 L245P expressing BRK cells (Figure 2C). The half-lives of *c-fos*, *c-myc,* and *COX-2* mRNA expressed in BRK dl210-294 and BRK L245P cells (38, 37, and 56 minutes and 39, 46, and 38 minutes, respectively) were shorter than those in BRK E1+E4 cells (141, 144, and 165 minutes)(Figure 2D). Thus, these ARE-mRNA were not stabilized in the cells expressing the oncodomain mutants of E4orf6.

The same results were obtained from experiments examining the half-life of luciferase-ARE$^{c\text{-}fos}$ fusion mRNA using Tet-Off system. The half-life of luciferase-ARE$^{c\text{-}fos}$ mRNA in 293 cells expressing the E4orf6 L245P mutant was 68 minutes (Figure 2E). This is shorter than that in the cells expressing wild-type E4orf6 (147 minutes). From these results, we conclude that the alpha helix structure of E4orf6 is required for the stabilization of ARE-mRNA and that this stabilization is related to the oncogenic activity of E4orf6.

As shown previously, stabilized ARE-mRNA was detected in the cytoplasm of cells that had been transformed by E4orf6 with its associated proteins such as HuR and pp32 (Higashino *et al*, 2005). If the export of ARE-mRNA is a prerequisite for its stabilization, the oncodomain of E4orf6 including the alpha helix may be required for the cytoplasmic accumulation of ARE-mRNA. To confirm this, we observed the interaction of *c-fos* mRNA in the cytoplasm with its associated proteins using an RNP-immunoprecipitation (RIP) assay. Bands for *c-fos* mRNA appeared in both the nucleus and cytoplasmic fractions precipitated with anti-HuR and -pp32 antibodies using the lysate from BRK cells expressing wild-type E4orf6 (Figure 2F). This data indicates that *c-fos* mRNA bound to HuR and pp32 exists in the nucleus and cytoplasm of cells expressing wild-type E4orf6. On the other hand, there was no such band in the cytoplasm of the cells expressing the E4orf6 oncodomain mutants, whereas mRNA for these proteins was present in the nucleus (Figure 2F). The stabilization of the ARE-mRNA in the cytoplasm of the E4orf6 derivative expressing 293 cells was confirmed using luciferase-ARE$^{c\text{-}fos}$ mRNA. In the cells expressing E4orf6 mutants, the half-life of the mRNA (34 minutes) was shorter than that of the wild-type E4orf6 expressing 293 cells (65 minutes; Figure 2G). In this case, the half-lives of luciferase-ARE$^{c\text{-}fos}$ mRNA were different from those in Fig. 1D and Fig. 2F, which were calculated by the data obtained from tet-off system. These data strongly suggest that HuR and pp32 are associated with ARE-mRNA and that the complex is exported from the nucleus and stabilized in the cytoplasm in cells that express E4orf6.

Since the alpha helix structure is also thought to be important for E3 ubiquitin ligase activity mediated by E4orf6 (Orlando and Ornelles, 2002; Blanchett *et al*, 2004), we examined whether another E4orf6 mutant that has lost the ability to assemble into the E3 ligase complex is able to stabilize ARE-mRNA. In order to confirm this, we constructed the E4orf6 (BC-) mutant by introducing specific point mutations in BC boxes 1 and 2 (Figure 2A), which are necessary for E4orf6 to associate with Cullin5, a complex containing E3 ubiquitin ligase bound with E1B55k, using mutant adenovirus H5pm4139 (a generous gift from T. Dobner) as a template (Blanchett *et al*, 2008). We confirmed whether this mutant is functional by measuring its ability to degrade p53 (Supplementary Figure 1). The levels of accumulated *c-fos* and *COX-2* mRNA expressed in the cytoplasm of 293 cells expressing wild-type E4orf6 were upregulated compared to those of the control 293 cells, which were transfected with a parent plasmid. On the other hand, their levels were not increased in the cells possessing E4orf6 L245P or BC (-) mutants (Figure 3A). The level of the control *GAPDH* mRNA was not increased by the expression of wild-type E4orf6. These results suggest that the ability of E4orf6 to assemble into E3 ligase is required for the cytoplasmic expression of ARE-mRNA. Similar results were obtained when we examined the stabilization of ARE-mRNA using total cell lysates from each cell type (Figure 3B). As in the case of the cytoplasmic mRNA, the E4orf6 L245P and BC (-) mutants were not able to stabilize *c-fos* or *COX-2* mRNA. These findings suggest that E4orf6 mediated E3 ligase activity is important for the export and stabilization of ARE-mRNA.

The stabilization of ARE-mRNA mediated by HuR is required for the oncogenic activity of E4orf6

From the above results, we concluded that ARE-mRNA is stabilized in cells expressing E4orf6. Then, we examined whether this stabilized ARE-mRNA contributes to the oncogenic activity of E4orf6. To achieve this, we constructed cells that failed to stabilize ARE-mRNA even when E4orf6 is expressed. Since HuR has been shown to interact with ARE directly, HuR knockdown is expected to inhibit the stabilization of ARE-mRNA, as we have shown in oral cancer cells (Kakuguchi *et al*, 2010). To make stable HuR-knockdown BRK E1+E4 cells, HuR shRNA was introduced using pSUPER, which directs the synthesis of small interfering RNA (siRNA) in mammalian cells (Brummelkamp *et al*, 2002) (Figure 4A). The cytoplasmic accumulation of *c-fos* mRNA in the BRK E1+E4 cells was reduced in the HuR-silenced BRK cells (data not shown). Concomitantly, the half-life of the mRNA in the BRK E1+E4 cells (approximately 136 minutes) was shortened to 33 minutes by HuR knockdown (Figure 4B). Furthermore, a shortening of the half-life was also seen when 293 cells that had been transiently transfected with E4orf6 were used. The half-life of *c-fos* mRNA in E4orf6 transfected 293 cells was 71 minutes, whereas it was shortened to 27 minutes when HuR siRNA was transfected into the cells (Figure 4C). These results suggest that HuR knockdown inhibits the cytoplasmic expression and stabilization of ARE-mRNA, even when E4orf6 is expressed.

In order to examine the contribution of ARE-mRNA stabilization to the oncogenic activity of E4orf6, stable HuR-silenced BRK cells were subjected to a soft-agar colony formation assay. The BRK E1+E4 cells produced many large colonies in soft-agar, whereas the BRK E1 cells expressing only the *E1* (*E1A* and *E1B*) gene did not produce many colonies (Figure 4D). This is reasonable because the BRK cells transformed with the *E1* and *E4orf6* genes produced tumors in nude mice, whereas BRK E1 cells did not (Moore *et al*, 1996). On the other hand, the HuR-silenced BRK cells produced almost no colonies in soft-agar (Figure 4D). These results suggest that the HuR-silenced cells lost their ability for anchorage-independent growth in conditions in which ARE-mRNA was not stabilized, even when E4orf6 was present in the cells. Taken together, these results suggest that the HuR-mediated stabilization of ARE-mRNA is one of the major causes of the oncogenic activity of E4orf6.

Although ARE-mRNA is stabilized in many cancer cells, there is no evidence that confirmed whether the stabilized ARE-mRNA really transforms cells. To address this question, ARE-mRNA expression constructs, such as those for *c-myc*, *c-fos* and *COX-2*, were introduced into rat embryonal fibroblast (REF) cells together with expression vectors for several viral oncogenes. As shown in Figure 4 E, whereas *c-myc*, *c-fos* or *COX-2* mRNA did not have the potential to produce many colonies (15, 11.5 or 0 colonies), cotransfection of the *E1* and *E4orf6* expression vector induced to produce a number of colonies (171.5, 129.5 or 143.5 colonies), and those colony numbers are much higher than that produced only by E1 + E4orf6. On the other hand, the expression of the *E4orf6 L245P* or *BC (-)* mutant, which were not able to stabilize ARE-mRNA (Figure 2 and Figure 3), could not produce so many colonies. These findings suggest that these viral proteins enhance the ability of *c-myc*, *c-fos* and *COX-2* mRNA to transform rodent cells and that the stabilization of ARE-mRNA induces cell transformation, at least under certain conditions.

Discussion

We demonstrated here that the adenovirus E4orf6 stabilizes ARE-mRNA via an HuR mediated pathway. The alpha helix structure and the BC box motif within the E4orf6 protein are required for ARE-mRNA stabilization. Furthermore, E4orf6-mediated transformed cells could not produce colonies in soft agar when the stabilization of ARE-mRNA was inhibited by HuR knockdown. The stabilized ARE-mRNA appeared to have the potential to induce the formation of transformed colonies. These findings indicate that E4orf6 transforms cells at least in part by stabilizing system of ARE-mRNA.

Since elucidating the mechanisms of action of viral oncoproteins has often led to insights into human oncogenesis, we hypothesize that the stabilization of ARE-mRNA plays an important role in the generation of human cancer. The export of ARE-mRNA and HuR are evident in a number of human cancers and cytoplasmic HuR expression has been implicated in the malignancy of these carcinomas (Lopez de Silanes *et al.,* 2005). Indeed, the stabilization of ARE-mRNA is reported in several cancers such as oral carcinoma (Hasegawa *et al.,* 2009). Furthermore, in oral cancer cells, ARE-mRNA was exported to the cytoplasm of cells in a manner independent of CRM1 just like the case of transformed cells mediated by E4orf6. These data indicate further that the oncogenic mechanism through stabilization of ARE-mRNA shown in this report is general oncogenic process.

Although the stabilization of ARE-mRNA has been observed in cancer cells, there was no evidence that ARE-mRNA stabilization has the potential to induce oncogenic transformation. This is due to the fact that there was no constitutive system for exporting HuR or ARE-mRNA into the cytoplasm, whereas some stresses like heat shock can export them to the cytoplasm transiently. As confirmed in this report, ARE-mRNA was constitutively stabilized in the cells transformed with E4orf6, such as the BRK E1+E4 cells, we think this is one of the best systems for examining the effects of ARE-mRNA stabilization on cell transformation. Indeed, the cells expressing *c-fos*, *c-myc* and *COX-2* mRNA with *E4orf6* produced a lot of colonies compared to the control (Figure 4E). Although these are the data using only three ARE-mRNAs, the results indicate that the stabilization of ARE-mRNA can transform cells at least in certain condition. Again, these results support our hypothesis that the stabilization of ARE-mRNA is general oncogenic mechanism.

We found that the BC boxes 1 and 2 mutant of E4orf6 failed to export and stabilize ARE-mRNA (Figure 3). This indicates that not only the alpha helix structure, which is the region essential for the pp32 binding, but also the BC box motif is required for stabilizing ARE-mRNA. Since BC box is binding site of elongin C (Blanchette *et al.,* 2004), which is one of the subunits of E3 ubiquitin ligase, the data suggest that the E3 ubiquitin ligase complex including E4orf6 plays an important role for the stabilization of ARE-mRNA. As far as we know, this is a new function of the E4orf6 ubiquitin ligase. So far several proteins were identified as a target of this ubiquitin ligase such as p53 (Querido *et al.,* 2001), Mre11 (Stracker *et al.,* 2002), DNA ligase IV (Baker *et al.,* 2007) and integrin α3 (Dallaire *et al.,* 2009), we have no evidence about which protein or additional another protein is required as a substrate for the stabilization of ARE-mRNA. Since the E4orf6 E3 ubiquitin ligase activity was reported to be necessary for the export of viral late mRNAs (Woo and Berk, 2007; Blanchette *et al*, 2008), this activity is involved in the regulation both of cellular and viral mRNAs.

The stabilization of ARE-mRNA is also interesting with regard to viral infection. So far several virus gene products, for example HSV UL41 (Esclatine *et al*, 2004) and KSHV KaposinB (McCormick and Ganem 2001), have been shown to have the potential to control the fate of ARE-mRNA. In the late phase of adenovirus infection, although the export of the bulk of cellular mRNA is inhibited, ARE-mRNA is exported into the cytoplasm (Higashino *et al*, 2005). Since viral late mRNA is exported into the cytoplasm at the same time, it is possible that ARE-mRNA and late mRNA share the same export pathway.

Materials and Methods

Cells and plasmids

Human embryonal kidney 293 cells, baby rat kidney cells (BRK E1+E4 and BRK E1; Higashino *et al*, 2005), and rat embryonic fibroblast (REF) cells were cultured in DMEM containing 10% fetal bovine serum with antibiotics. To produce BRK L245P and BRK dl210-294 cells, BRK E1 cells were transfected with pcDNAE4orf6 L245P and pcDNAE4orf6 R248E (see below), respectively, and the clones were selected in medium containing 300 μg/ml G418.

To create E4orf6 substituted mutants (pcDNAE4orf6 L245P, pcDNA-FALG-E4orf6 L245P, pcDNAE4orf6 R248E, and pcDNA-FLAG-E4orf6 R248E), the leucine and the arginine at amino acids 245 and 248, respectively, were changed to proline and glutamic acid by site-directed mutagenesis (Quickchange, Stratagene), as described previously (Dobbelstein *et al*, 1997; Orlando and Ornelles, 1999), using pcDNA3-E4orf6 and pcDNA3-FLAG-E4orf6 (Higashino *et al*, 2005) as templates. E4orf6 dl210-294, pCMVGL, and pCMVGL-ARE$^{c\text{-}fos}$ were described previously (Higashino *et al*, 2005). The cDNA of the luciferase-ARE$^{c\text{-}fos}$ fusion gene was inserted into the pTRE vector to produce a Tet-Off system according to the manufacturer's (Clontech) instructions. pcDNA-E4orf6 BC(-) was made by amplifying segments of the E4orf6 coding region using the primer 5'-cgggatcccatgactacgtccggcgtt-3', together with second primer 5'-gaattcctacatgggggtagagtcata-3' using mutant adenovirus H5pm4139 (a generous gift from T. Dobner) as a template (Blanchett *et al*, 2008). The PCR-amplified fragments were inserted into the BamHI/EcoRI site of pcDNA3. All mutants were confirmed by nucleotide sequencing. *COX-2* expression plasmid (pCMV-sport-COX-2) was purchased from Invitrogen (NIH Mammalian Gene Collection).

mRNA stability analysis

To examine the accumulation of ARE-mRNA in total cell lysate, the cells were treated with TRI Reagent (Sigma) to acquire total RNA, and then the cells were treated with actinomycin D (final concentration 10 μg/ml) or doxycycline (1 μg/ml) to elucidate the half-life of ARE-mRNA. After treatment, total RNA was isolated and subjected to reverse transcription using Rever Tra Ace (TOYOBO).

For quantitative real-time RT-PCR analysis, PCR amplification was performed in DNA Engine Opticon 2 (MJ Research) with SYBR Green PCR Master Mix (DyNAmo SYBR Green qPCR kit, MJ Research). The primers used in this assay were as follows: for *c-fos*, 5'-tggacctgtctggttccttc-3' and 5'-ctcggggtaggtgaagacaa-3' ; for *c-myc*, 5'-atcactgtacgccccaagac-3' and 5'-cctttggtggaacgacactt-3' ; for *COX-2*, 5'-ctgaggggttaccacttcca-3' and 5'-tgagcaagtccgtgttcaag-3' ; for *GAPDH*, 5'-caactacatggtctacatgttc-3' and 5'-cgccagtagactccacgac-3'. The Student's t-test was used to assess statistically significant differences.

To examine the cytoplasmic transportation of ARE-mRNA, the cells were separated into nuclear and cytoplasm fractions (Higashino *et al*, 2005), and the amounts of mRNA in the cytoplasm were measured by quantitative real-time RT-PCR.

For the luciferase assay, BRK cells were transfected with the pCMVGL3 or pCMVGL-ARE reporter plasmid using the FuGENE6 transfection reagent (Roche), and luciferase assays were performed 20 hours later according to the manufacturer's instructions.

Protein analysis

Western blot analysis and immunoprecipitation were performed, as described previously (Aoyagi *et al*, 2003). The antibodies used were specific to HuR, pp32 (Santa Cruz), and β-actin M2 (anti-FLAG) (SIGMA). The secondary antibody was horseradish peroxidase-conjugated IgG (Jackson ImmunoResearch laboratories).

RNP-immunoprecipitation (RIP) assay

BRK cells were treated with phosphate buffered saline containing 1% formaldehyde (Higashino *et al*, 2005). After the cells had been separated into nuclear and cytoplasmic fractions, each lysate was immunoprecipitated with mouse IgG, anti-HuR, or -pp32 antibody. The pellet and supernatant were incubated at 70˚C for 45min to reverse cross-linking, the isolated RNA were subjected to reverse transcription, and PCR amplification for *c-fos* was performed using primers as described above.

HuR knockdown

pSUPER (Brummelkamp *et al*, 2002) including the sequence for HuR shRNA (5'-uuaccaguuucaauggucauuuucaagagagaaugaccauugaaacugguaa-3') was introduced into BRK cells by calcium phosphate-mediated transfection, and stable transfectants were selected with 300μg/ml G418.

Soft-agar colony formation assay

The soft-agar colony formation assay was performed as described previously (Kakuguchi *et al*, 2010). Single-cell suspensions of $3X10^4$ cells were plated in 60-mm culture dishes in 3ml of DMEM containing 10% FBS and 0.36% agar on a layer of 5ml of the same medium containing 0.75% agar. Three weeks after plating, the colonies were stained with 0.04% crystal violet-2% ethanol in PBS, and photographs were taken of the stained colonies.

Transformation assay

REF cells on 6-well dishes were transfected with a total 1.5 μg DNA (0.5 μg of pXhoIC, 0.5 μg of pEF-c-myc, pSG5-c-fos or pCMV-sport-COX-2, and 0.5 μg of plasmids encoding E4orf6 variants; the total amount of DNA was adjusted with pUC118) using Hilymax (DOJINDO Laboratories) according to the manufacturer's instructions and maintained in DMEM with 7.5 % fetal bovine serum. Two weeks after transfection, the plates were stained with crystal violet, and the number of foci was counted.

Conflict of interest

The authors declare no conflict of interest.

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Figure 1 E4orf6 stabilizes ARE-mRNA. (**A**) The accumulation of *c-fos*, *c-myc*, and *COX-2* mRNA in BRK E1 and BRK E1+E4 cells was measured by quantitative real-time RT-PCR. Data are shown as the mean ±SEM of three independent experiments. (**B**) The same cells were treated with act D, and the amounts of each mRNA were estimated at 60 and 120 minutes after treatment by real-time RT-PCR. The plot shows the mean of three independent experiments. $t^{1/2}$ indicates the half-life time (minutes). The half-lives in (E1+E4) cells are calculated values. (**C**) pGL3 based luciferase reporters with (pCMVGL-ARE$^{c\text{-}fos}$) or without (pCMVGL) the ARE of *c-fos* mRNA (*left*) were transfected into BRK E1 or BRK E1+E4 cells, and luciferase activity was measured 20 hours after transfection (*right*). Data are shown as the mean ±SEM of three independent experiments. (**D**) The half-life of the luciferase-ARE$^{c\text{-}fos}$ mRNA was estimated in pcDNA-E4orf6 transfected 293 cells using the Tet-Off system. The synthesis of the fusion gene was stopped by adding doxycycline (dox), and the quantities of the gene were estimated at 60 and 120 minutes after treatment by real-time RT-PCR. The plot shows the mean of three independent experiments. $t^{1/2}$ indicates half-life time (minutes). The half-life in (+E4) cells is calculated value.

Figure 2 The alpha helix structure of E4orf6 is required for the stabilization of ARE-mRNA. (**A**) Schematic diagram of E4orf6 mutants. BC1, BC2, and α-helix indicate BC boxes 1 and 2 (Blanchett *et al*, 2004) and the amphipathic alpha helix region (Orlando and Ornelles 1999), respectively. The bar indicates the oncodomain (Nevels *et al*, 2000). (**B**) 293 cells were transfected with the indicated FLAG-tagged E4orf6 derivatives, and the extracts were subjected to an M2 affinity column followed by immunoblotting for pp32. The immunoblots shown below confirm the expression of FLAG-E4orf6 derivatives and pp32 in each transfected cell. (**C**) The accumulation of *c-fos*, *c-myc*, and *COX-2* mRNA expression in BRK E1, BRK E1+E4, BRK dl210-294, and BRK L245P cells was measured by quantitative real-time RT-PCR. $^{*}P < 0.05$; $^{**}P < 0.01$; values are expressed as the mean ±SEM of three independent experiments. (**D**) The same cells as were shown in *C* were treated with act D, and the amounts of each mRNA were estimated at 0 and 60 minutes after treatment by real-time RT-PCR. The plot shows the mean of three independent experiments. $t^{1/2}$ indicates the half-life time (minutes). (**E**) The half-life of the luciferase-ARE$^{c\text{-}fos}$ mRNA was estimated as described in Figure 1D using pcDNA-E4orf6 or pcDNA-E4orf6 L245P transfected 293 cells. (**F**) *c-fos* associated with HuR and pp32 was isolated by RIP analysis using the nuclear and cytoplasmic fractions of BRK cells expressing E4orf6 derivatives. Mouse IgG (mIgG) was used as a control for the antibodies. P and S indicate the pellet and supernatant produced by immunoprecipitation, respectively. (**G**) The half-life of the luciferase-ARE$^{c\text{-}fos}$ mRNA in the cytoplasm of 293 cells transfected with indicated E4orf6 derivatives. The cells were treated with act D, and the amounts of each mRNA in the cytoplasm were estimated at 30, 60, and 120 minutes after treatment by real-time RT-PCR. The plot shows the mean of three independent experiments. $t^{1/2}$ indicates half-life time (minutes).

Figure 3 The BC-box motif of E4orf6 is required for the stabilization of ARE-mRNA. The accumulation of *c-fos*, *COX-2,* and *GAPDH* mRNA in the cytoplasm (**A**) and the (**B**) total of 293 cells expressing indicated E4orf6 derivatives were measured by quantitative real-time RT-PCR. Data show the mean ±SEM of three independent experiments.

Figure 4 Stabilized ARE-mRNA is required for the oncogenic activity of E4orf6. (**A**) HuR shRNA was introduced into BRK E1+E4 cells, and the amounts of HuR and β-actin in each BRK cells were estimated by Western blot analysis. (**B**) The HuR-knockdown BRK cells were treated with act D, and the amounts of *c-fos* mRNA were estimated at 60 and 120 minutes after treatment by quantitative real-time RT-PCR. The plot shows the mean of three independent experiments. $t^{1/2}$ indicates half-life time (minutes). (**C**) The half-life of *c-fos* mRNA was estimated using 293 cells that had been transfected with pcDNA-E4orf6 and HuR siRNA as described in *B*. (**D**) The BRK cells described in *A* were subjected to a soft-agar colony formation assay. Photographs of the stained colonies were taken three weeks after transfection. (**E**) REF cells were transfected with the expression constructs of the indicated genes. The number of colonies was counted 2 weeks after transfection and is shown as a histogram. Data represent the mean ±SEM of three independent experiments.

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Figure 1. E4orf6 stabilizes ARE-mRNAs

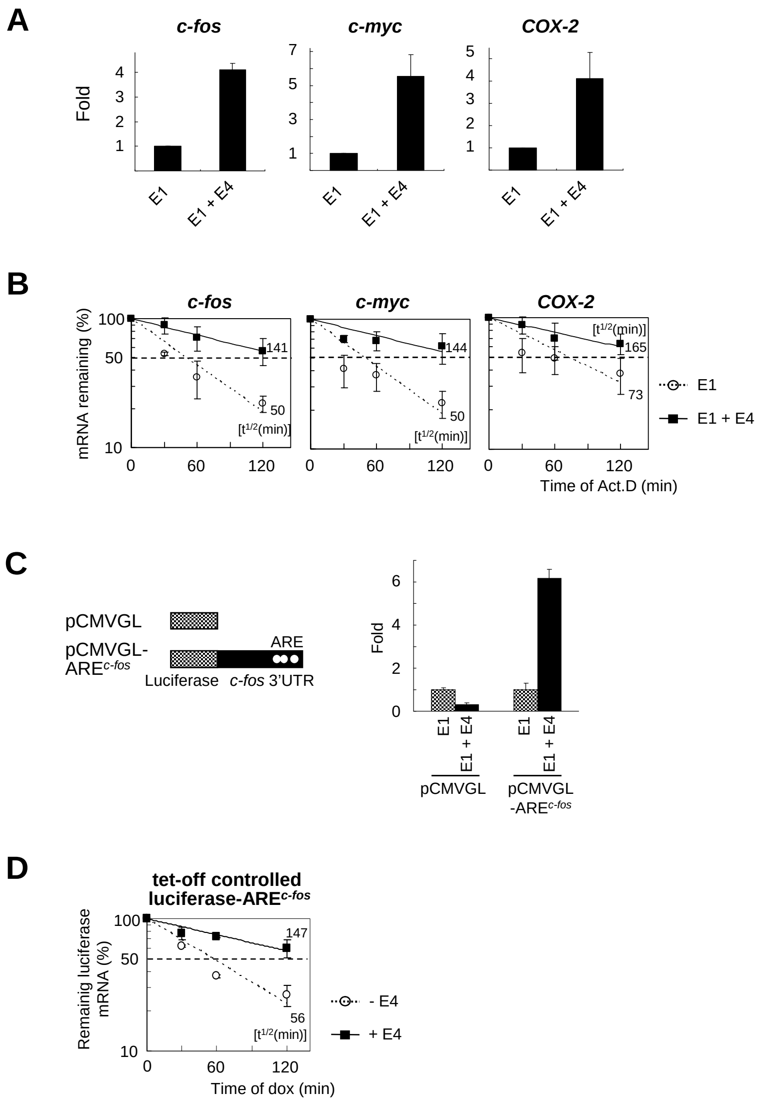

Figure 2. The alpha helix structure of E4orf6 is required for the stabilization of ARE-mRNAs.

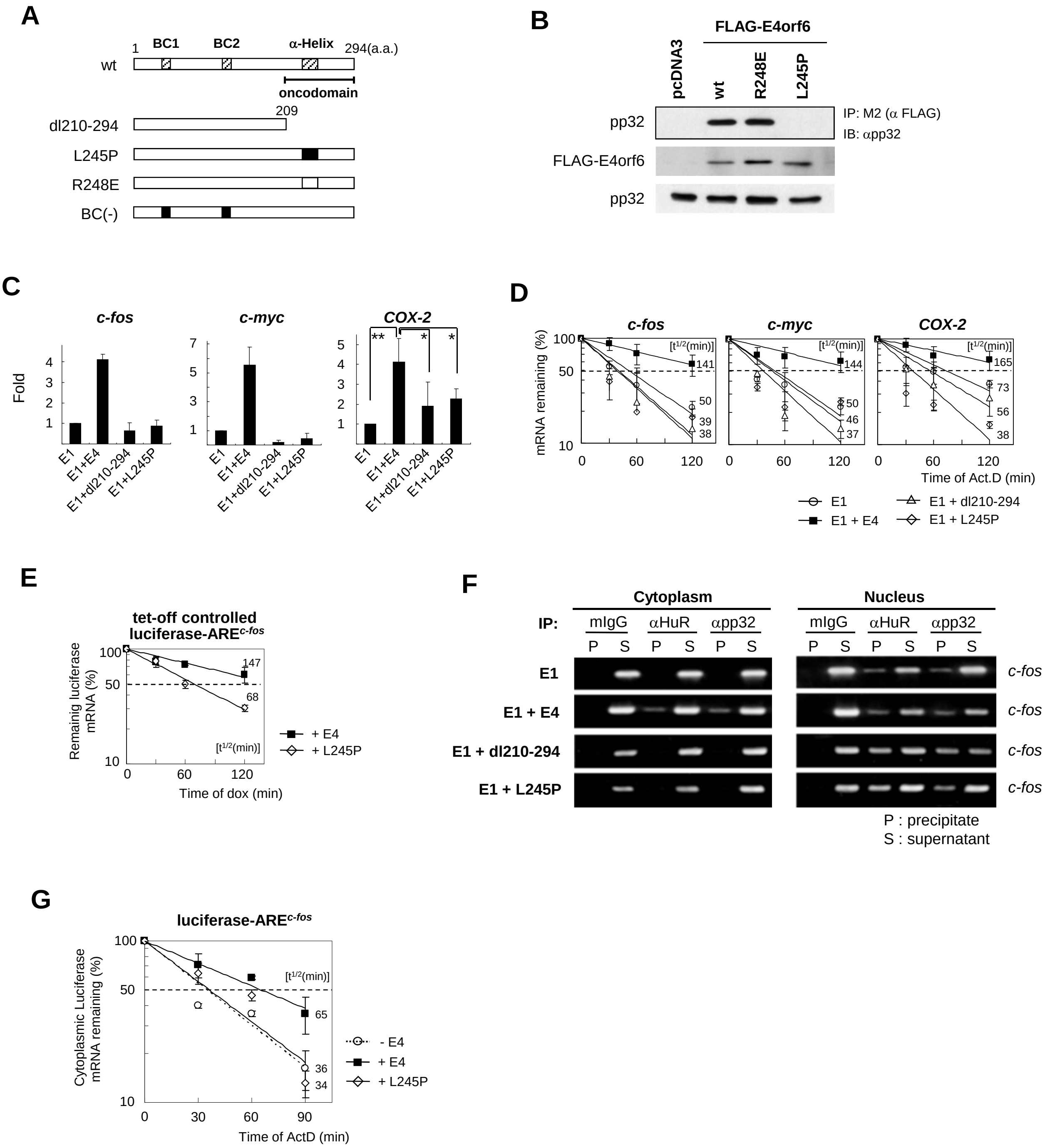

Figure 3. The BC-box motif of E4orf6 is required for the stabilization of ARE-mRNAs.

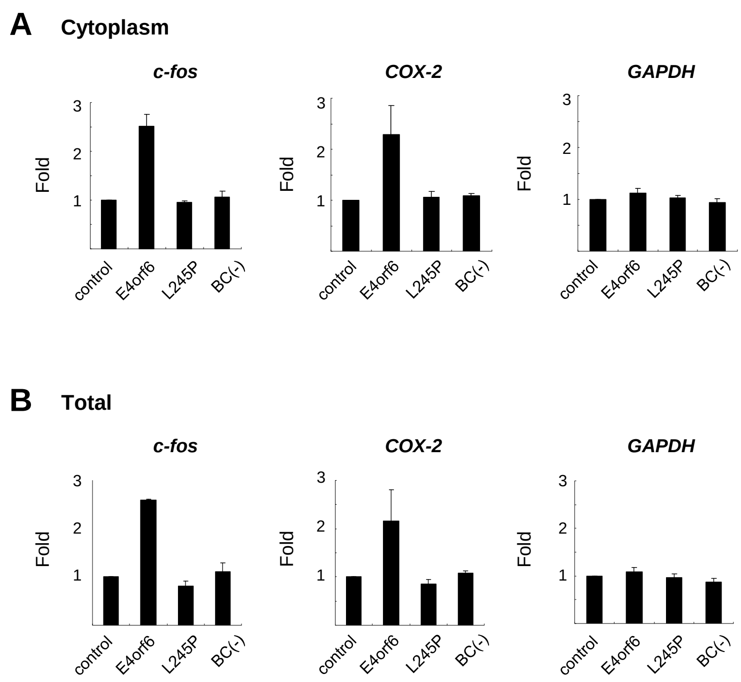

Figure 4. The stabilized ARE-mRNA is required for the oncogenic activity of E4orf6.

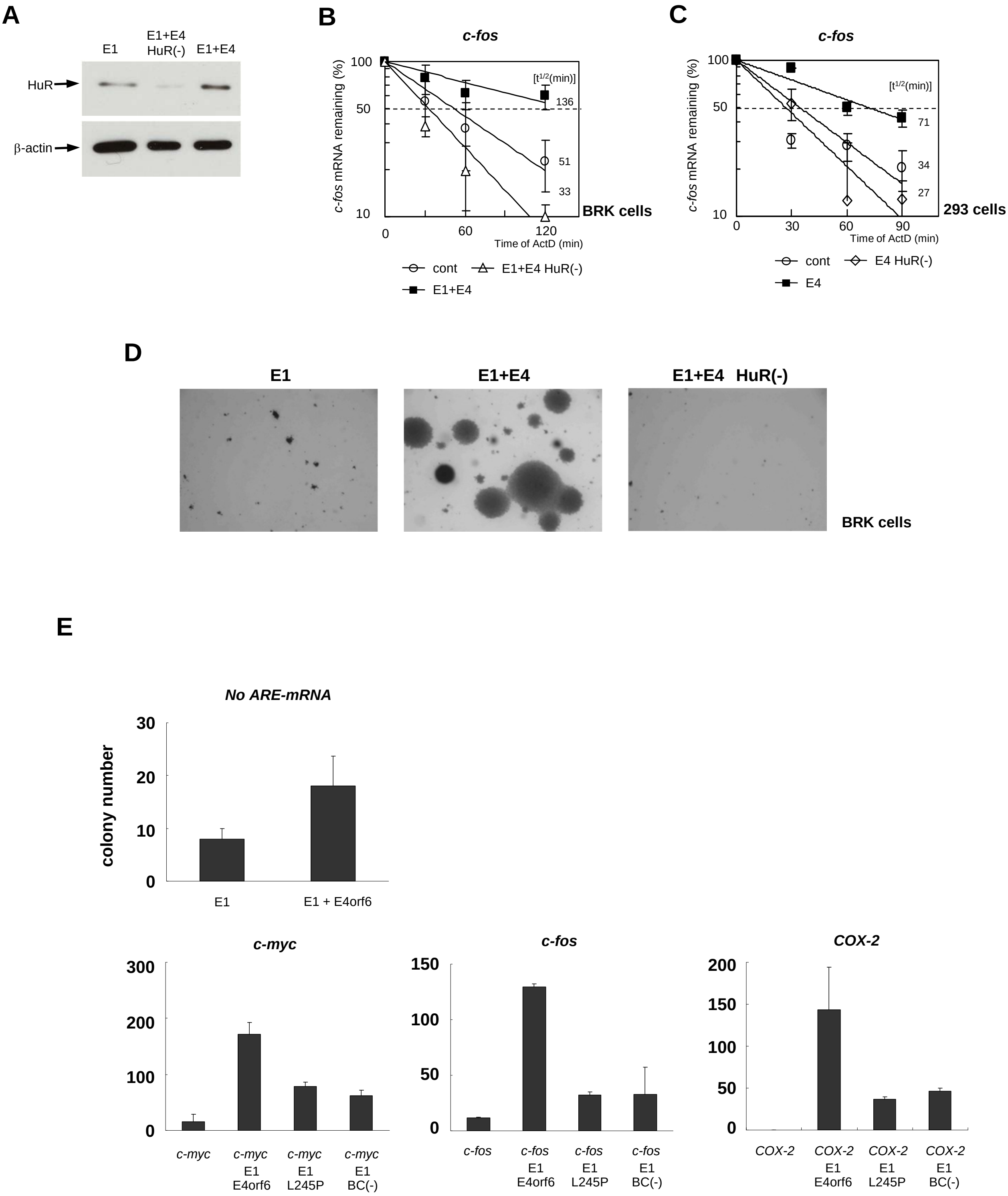

Figure S1. Degradation of p53 by E4orf6 mutants