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Cells, plasmids, and cell fractionation

E4orf6 deletion mutants (E4orf6 dl210-294 and E4orf6 dl110-294) were constructed by amplifying segments of the E4orf6 coding region using the primer 5′-ggtggatccatgactacgtccggcgttcc-3′ together with second primers 5′-taagaattcctagctgtatccaaagctcatggc-3′ or 5′-taagaattcctacgtcatgatatcaatgttggc-3′. The PCR-amplified fragments were inserted into the BamHI–EcoRI site of pcDNA3. All mutants were confirmed by nucleotide sequencing. To produce BRK #9, BRK dl210-294 and BRK NES (−) BRK E1 cells were transfected with the plasmids described above and clones were selected in medium containing 300 mg/ml G418 or 1 μg/ml puromycin.

To generate pGL3-based reporter plasmids, cytomegarovirus (CMV) promoter region from pcDNA3 was cloned into the BglII–HindIII site of pGL3-basic (Promega) to produce pCMVGL. Amplified 3′-untranslated region of *c-fos* cDNA was inserted into the XbaI–BamHI site of pCMVGL to create pCMVGL-ARE.

Cell fractionation was performed as follows. Cells were separated into cytoplasmic and nuclear fractions as described previously (Weigel and Dobbelstein, 2000). In brief, cells were harvested and resuspended in fractionating buffer (10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1.5 mM $MgCl_2$, 0.5% NP-40, and protease inhibitor cocktail), were subjected to vigorous shaking for 5 min, and were centrifuged at 12,000 rpm for 30 s. The supernatant was used as the cytoplasmic fraction. To estimate the accuracy of cell fractionation, cytoplasmic (β-tubulin) and nuclear (PARP) proteins were detected by immunoblotting.

In situ hybridization

BRK cells were fixed in cold 3% formaldehyde and permeabilized with cold 0.5% Triton X-100. In situ hybridization was performed overnight at 37°C in 20 μl of a mixture containing 2.5 μg tRNA, 10 μg of salmon sperm DNA, 2 × SSC, 0.2% BSA, 1 mM vanadyl ribonucleoside complexes, 50% formamide, 10% dextran sulfate, and 10–30 ng of 3′ digoxigenin-labeled antisense deoxyoligonucleotide probe for *c-fos* or *c-myc* or *COX-2* mRNA. The coverslips were washed three times in 2 × SSC at 37°C and then 1 × SSC at RT. The coverslips were incubated for 60 min at RT with 1:50 dilution of anti-DIG fluorescein Fab fragments (Roche) in 0.2% Triton X-100/PBS containing 1% BSA. The coverslips were washed twice in 0.2% Triton X-100/PBS and then twice in PBS. The probes were complementary to nucleotides 288 to 328 for *c-fos*, to nucleotides 6278 to 6311 for *c-myc*, or to nucleotides 1417 to 1460 for *COX-2*. Some cells were treated with heat shock or LMB (5 ng/ml). Nuclei were stained using DAPI.

Quantitative real-time RT-PCR

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′. To generate standards for relative quantification, dilutions (triplicates of 10^{-1}, 2×10^{-2}, 10^{-2}, 5×10^{-3}; these numbers were designated as dilution factors) of a cDNA solution made from 3 μg of total or cytoplasmic RNA of BRK, 293, and HeLa cells were PCR amplified. The value of the threshold cycle (Ct: the point during cycling when the PCR product is first detected) obtained from the PCR reaction and the dilution factors were used to determine the standard curves for *c-fos*, *c-myc*, *COX-2*, and *GAPDH* mRNA. The value representing *c-fos*, *c-myc*, and *COX-2* mRNA expression was obtained from the Ct value using the standard curve and divided by that of *GAPDH* for normalization. To determine expression level of the cytoplasmic *c-fos*, *c-myc*, and *COX-2* mRNAs, the cytoplasmic fraction of each ARE-mRNA relative to the total RNA level was calculated by using the normalized value, and then compared these values between the cells with and without expressing E4orf6. Some cells were treated with heat shock (45°C, 1 h) or LMB (5 ng/ml).

To evaluate the half-lives of cytoplasmic ARE-mRNAs, BRK cells were treated with 5 μg/ml Actomyosin D for 15, 30, or 60 min. The cytoplasmic fractions were separated from the cells and the samples were subjected to quantitative real-time RT-PCR.

RNP immunoprecipitation assay

BRK cells were treated with PBS containing 1% formaldehyde (Niranjanakumari et al., 2002). After the cells were separated into nuclear and cytoplasmic fractions by sonication, each lysate was immunoprecipitated with mouse IgG, anti-HuR, -pp32/LANP -E4orf6, or-E1B-55kD antibody. The pellets and supernatants were incubated at 70°C for 45 min to reverse the cross-links, the isolated RNA was subjected to reverse transcription, and PCR amplification for *c-fos* was performed using the primers as described above.

Northern blot analysis

RNA (~15 μg) from each cell sample was separated electrophoretically on a 1% agarose-2.2 M formaldehyde gel. The gel was rinsed in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) buffer, and the RNA was transferred to a positively charged nylon membrane by a TurboBlotter (Schleicher & Schuell). The HindIII–XbaI fragment from pGL3 basic was labeled with AP according to the manufacturer's protocol (AlkPhos Direct; GE Healthcare) to yield AlkPhos-labeled probe. AP activity was visualized fluorescently by using CDP-Star chemiluminescence reagent.

References

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