Supporting Information

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SI Materials and Methods

Construction of Bicistronic Plasmids. To generate bicistronic plasmids that contain the c-Jun 5′UTR (pR5′UTRF) or fragments thereof (pR1-562F, pR1-397F, pR549-974F, pR1-277F, pR271-577F, pR1-141F, and pR28-203F), the 5′UTR and the corresponding fragments were recovered by PCR from the previously generated pj5′HAjun3′ plasmid (1), using the oligonucleotide primers shown in Table S1. The PCR products were ligated to pGEM T easy (Promega), and clones with fragments at the correct orientation were selected. The fragments were excised by digestion with HindIII and SpeI and were ligated to the pR-F vector also digested with HindIII and SpeI. Bicistronic plasmids with deletions in the first 277 bases, pRΔ53-120F and pRΔ145-191F, were generated using two sets of primers for each (Table S1). For pRΔ145-191F, one PCR product was cleaved with BglII and EcoRI and the other was cleaved with EcoRI and HindIII. For pRΔ53-120F, one PCR product was cleaved with NcoI and EcoRI and the other was cleaved with EcoRI and HindIII. For each plasmid, the two cleaved fragments were ligated, subcloned, and recovered by PCR using primers corresponding to the ends of the ligated fragment. The PCR products were ligated to pGEM T easy and cloned in pR-F, as described above. To generate bicistronic plasmids with a hairpin structure at the transcription start site (pLR1-397F and pLR1-277F), the excised 1–397 and 1–277 fragments were ligated to the pLRp27F vector (2), which was cleaved by HindIII and SpeI. In all plasmids, the cloned region was confirmed by sequencing. Plasmid DNA was prepared using the NucleoBond PC500 (Macherey–Nagel) plasmid preparation kit.

Rat Primary Glia Cultures. Cerebral cortices of 1- to 2-d-old Sprague–Dawley rat pups were isolated, and all meninges and large vascular vessels were removed with forceps. Tissues were chopped into small pieces and incubated with trypsin (Trypsin EDTA Solution B; Biological Industries) for 15 min at 37 °C, followed by addition of DMEM containing 10% FBS and 100 μg/mL DNase I (Sigma). Cells were triturated, filtered through a sterile 100-μm cell strainer, and centrifuged at 1,000 × *g* for 7 min. The pellet was then completely resuspended in warm DMEM supplemented with 10% FBS and filtered again through a sterile 100-μm cell strainer. Cells were plated in 75-cm^2 flasks (2 brains per flask) coated with poly L-Ornithine (0.01% solution; Sigma) and kept at 37 °C in a humidified atmosphere containing 5% CO_2. Medium was replaced after 24 h and after 7 d. The mixed glial culture was confluent and ready for analysis after 10–12 d.

Northern Blot Analysis and Quantitative RT-PCR Assay. For Northern blot analysis, RNA [30 μg or 70 μg for Firefly luciferase (FL) detection] was denatured by heating at 60 °C for 10 min in 2.2 M formaldehyde/50% (vol/vol) formamide and fractionated by electrophoresis in 1.2% (wt/vol)agarose gels containing 2.2 M formaldehyde and MOPS buffer [40 mM MOPS (pH 7), 10 mM NaOAc, 1 mM EDTA]. The fractionated RNA was transferred to a nitrocellulose filter, and the filter was incubated for 2 h at 42 °C with prehybridization buffer [50% (vol/vol) formamide, 5× SSC, 5× Denhardt's solution, 0.1% SDS (770 mM Na_2HPO_4 – 20 mM NaH_2PO_4 [pH 7.4], 0.1 mg/mL salmon sperm DNA)], to which a [^{32}P]dCTP-labeled DNA probe specific for the c-Jun, GAPDH, or FL gene was added for overnight hybridization. Probes were prepared using Random DNA labeling mix (Biological Industries). The c-Jun and GAPDH probe templates were made as previously described (3). The levels of hybridization were visualized by autoradiography. As a loading control, rRNA was stained with ethidium bromide incorporated in the gel.

For real-time RT-PCR analysis, RNA was digested with RNase-free DNase (MBI Fermentas) to remove residual DNA and purified with phenol-chloroform. First-strand cDNA synthesis was performed using the Verso cDNA synthesis kit (Thermo Fisher Scientific) according to the manufacturer's instructions. Quantitative real-time PCR was performed on a LightCycler (Takara Bio USA). A cDNA template (2 μL), 0.5 μL (20 mM) of forward and reverse primers, and 10 μL of SYBR Premix Ex Taq (Takara, Saint-Germain-en-Laye, France) in a total of 20 μL were applied to the following PCR program: 30 s at 95 °C (initial denaturation); 20 °C/s temperature transition rate up to 95 °C for 15 s, 3 s at 68 °C, and 5 s at 72 °C; 86 °C single-acquisition mode, repeated 40 times (amplification). The PCR assay was evaluated by melting curve analysis and by checking the PCR products on 2% (wt/vol) agarose gels. β-actin was amplified to ensure cDNA integrity and to normalize expression.

The following oligonucleotide primers were used for the c-Jun transcript: 5′-AAGTAAGAGTGCGGGAGGCA3′ (forward) and 5′-GGGCATCGTCATAGAAGGTCG-3′ (reverse).

The following oligonucleotide primers were used for the β-actin transcript: 5′-CTACGTCGCCCTGGACTTCGAGC-3′ (forward) and 5′-GATGGAGCCGCCGATCCACACGG-3′ (reverse).

1. Polak P, et al. (2006) The cytoskeletal network controls c-Jun translation in a UTR-dependent manner. *Oncogene* 25:665–676.
2. Kullmann M, Göpfert U, Siewe B, Hengst L (2002) ELAV/Hu proteins inhibit p27 translation via an IRES element in the p27 5′UTR. *Genes Dev* 16:3087–3099.
3. Knirsh R, et al. (2009) Loss of E-cadherin-mediated cell-cell contacts activates a novel mechanism for up-regulation of the proto-oncogene c-Jun. *Mol Biol Cell* 20:2121–2129.

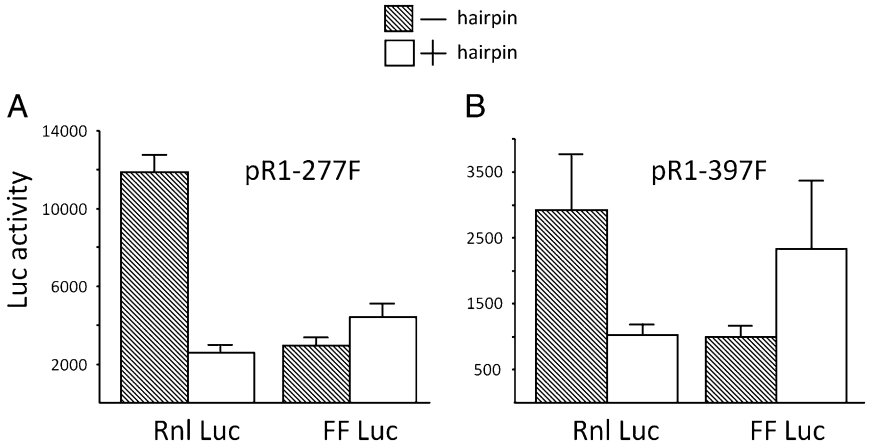

Fig. S1. Presence of a hairpin structure at the transcription start site inhibits Renilla luciferase (Rnl Luc) but not Firefly luciferase (FF Luc) expression. U87 cells were transfected with bicistronic reporter plasmids that contain the first 277 (pR1-277F) (*A*) or 397 (pR1-397F) (*B*) nucleotides with or without a hairpin structure at the transcription start site. Rnl Luc and FF Luc activities (per 10^6 cells) were determined. The data shown are the mean ± SEM of three separated experiments.

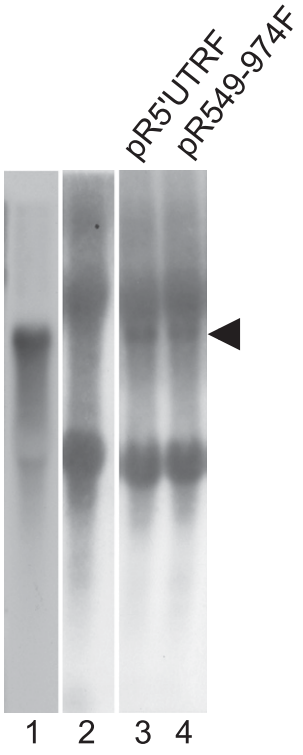

Fig. S2. Northern blot analysis of RNA from transfected U87 cells. RNA samples (70 μg) from U87 cells, untransfected (lanes 1 and 2) or transfected with pR5′ UTRF (lane 3) or pR549-974F (lane 4) were analyzed using a probe specific to FL. As a size marker, in vitro transcribed pR1-277F RNA was added to the cellular RNA in lane 1. Lane 1 was exposed for 10 min, whereas lanes 2–4 were exposed for 24 h. Arrowhead indicates bicistronic RNA.

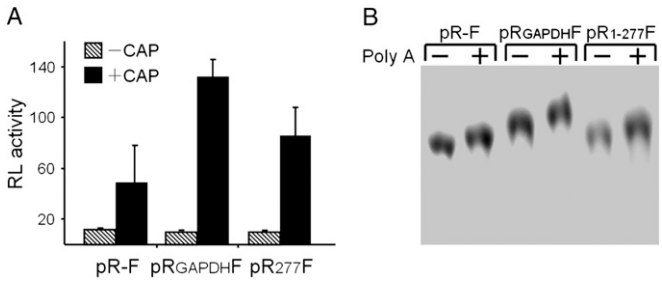

Fig. S3. Analysis of the in vitro-transcribed bicistronic RNA. (*A*) Equal amounts of capped or uncapped RNA were transfected into U87 cells and assayed for Renilla luciferase (RL) activity. RL activity obtained with an uncapped construct was given the arbitrary value of 1 and used to normalize results obtained with the capped construct. The results are the mean ± SD of two separate experiments. (*B*) Capped transcripts (1 μg), subjected (+) or not subjected (–) to polyadenylation, were analyzed on 1.2% (wt/vol) denaturing formaldehyde agarose gel.

Table S1. Primers used for cloning of the bicistronic plasmids

	Nucleotide sequence	
Name	Forward primer	Reverse primer
5′UTR	5′-AGATCTGAGTTGCACTGAGTGTGG-3′	5′-AAGCTTAGAACAGTCCGTCACTTC-3′
1–562	5′-AGATCTGAGTTGCACTGAGTGTGG-3′	5′-AAGCTTCCGACGACTTGTCCCCT-3′
1–397	5′-AGATCTGAGTTGCACTGAGTGTGG-3′	5′-AAGCTTAAGACGCAGGAAAGGCTTG-3′
549–974	5′-AGATCTGAGGGGACAAGTCGTCGG-3′	5′-AAGCTTAGAACAGTCCGTCACTTC-3′
1–277	5′-AGATCTGAGTTGCACTGAGTGTGG-3′	5′-AAGCTTGGGGTACCGCTGCTTT-3′
271–577	5′-TTAGATCTACCCACGCGCCCGCTG-3′	5′-TTTAAGCTTGGCCGCCCGGACTC-3′
1–141	5′-AGATCTGAGTTGCACTGAGTGTGG-3′	5′-TATAAGCTTAGAGAGAAGGTGAAAAGAAAATAAG-3′
28–203	5′-TGGATCCGAGGCGGGAGTGGAGG-3′	5′-TATAAGCTTCAAGGCTCTCTGGACACTCCCG-3′
Δ145–191	5′-AGATCTGAGTTGCACTGAGTGTGG-3′	5′-GAATTCAGTTAGAGAGAAGGTGAAAAGAAAATAAG-3′
	5′-AGAATTCAGAGAGCCTTGTCTCCAGCCG-3′	5′-AAGCTTGGGGTACCGCTGCTTT-3′
Δ53–120	5′-CTCCATGGCGACGGGTG-3′	5′-TGAATTCCGCGCACCTCCACTC-3′
	5′-TGAATTCTTTTCACCTTCTCTCTAACTGCCCAG-3′	5′-AAGCTTGGGGTACCGCTGCTTT-3′