METHOD

A proximity-dependent assay for specific RNA–protein interactions in intact cells

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ABSTRACT

The proximity ligation assay (PLA) is an immune staining method that detects protein–protein interactions in fixed cells. We describe here RNA–PLA, a simple adaptation of this technology that allows the detection of specific RNA–protein interactions in fixed cells by using a DNA oligonucleotide that hybridizes to a target RNA in combination with an antibody that recognizes the protein bound to the target RNA. Stable and transient RNA–protein interactions can be readily detected by generation of a fluorescent signal in discrete compartments in intact fixed cells with high specificity. We demonstrate that this approach requires the colocalization of the binding protein and its RNA target in the same cellular compartment, use of an oligonucleotide complementary to the target RNA, and the presence of a binding site for the protein in the target RNA.

Keywords: PLA; RNA–protein complex; small nuclear RNA; RNP; gene expression

INTRODUCTION

RNA-binding proteins associate with RNA molecules in cells to form ribonucleoprotein (RNP) complexes that regulate the post-transcriptional activity of RNAs by affecting their processing, subcellular localization, translation, or stability (Lunde et al. 2007; Glisovic et al. 2008). The study of RNA–protein interactions has primarily relied on the use of biochemical techniques, such as RNA electrophoretic mobility shift assays, pull-down assays, and RNase H assays (Zurla et al. 2016). Although powerful methods have been developed to visualize individual engineered RNAs in cells (Raj et al. 2008; Paige et al. 2011; Strack and Jaffrey 2015), it is difficult to detect and localize specific RNA–protein complexes within cells. Sequential detection of RNA and protein by fluorescence in situ hybridization (FISH) and immunofluorescence can be used for this purpose, but this method is time consuming and technically challenging. In addition, colocalization analyses are sometimes compromised by the relative signal-to-noise ratio and relatively low resolution of the images.

The proximity ligation assay (PLA) is a sensitive tool for examining protein–protein interactions in cells (Soderberg et al. 2006). In PLA, a pair of interacting proteins of interest are recognized by different primary antibodies and specific corresponding secondary antibody probes conjugated to unique short DNA oligonucleotides. If the target proteins are in close proximity (nominally within 40 nm), the oligonucleotides can hybridize to two additional oligonucleotides, bridging them to form a DNA circle. Following ligation and rolling-circle amplification (RCA) using one of the antibody-conjugated oligonucleotides as a primer, hybridization of a fluorescently labeled oligonucleotide probe to the amplification product generates punctate fluorescent signals as an indication of protein pairs in close proximity.

Modified PLA protocols have been developed to study RNA–protein interactions in cells (Jung et al. 2013a,b; Weibrecht et al. 2013). However, these methods are relatively complex and involve manipulations such as localized cDNA synthesis using a primer containing locked nucleic acid-modified bases followed by visualization with padlocked probes and target-primed amplification. Alternatively, the construction of peptide- and dye-conjugated chemically modified RNA–DNA oligonucleotide chimeras and their

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delivery into cells by streptolysin O-mediated permeabilization can be used to detect RNA–protein interactions in cells.

Here, we report a simple PLA method that can detect interactions between an unmodified endogenous or exogenous RNA and a binding protein in situ in fixed cells. In this RNA–PLA protocol, one of the primary antibody/probe pairs in standard PLA is replaced by a DNA oligonucleotide containing a 40–50 nucleotide (nt) segment complementary to the RNA of interest, 20–35 adenylates as a linker segment, and a common 25-nt segment that serves as one of the PLA hybridization/ligation arms (PLUS probe) (Fig. 1). A protein bound to the targeted RNA is recognized by a primary antibody and a secondary antibody probe conjugated to the other PLA hybridization/ligation arm (MINUS probe). When the protein is bound to the target RNA, the two probes will hybridize to two other oligonucleotides that form a closed DNA circle after ligation. The PLUS probe then serves as a primer for RCA as in classic PLA, and the signal is observed as spots after hybridization with a fluorescently labeled oligonucleotide. In an earlier report, we used this method to detect a transient interaction between a viral transcript and its cellular processing enzyme (Xie et al. 2015). Here, we provide further details and documentation that this method can be used to detect interactions between native viral and cellular small nuclear RNAs (snRNAs) as well as mRNAs and their corresponding binding proteins at the single-cell and subcellular level.

RESULTS

Detection of complexes between proteins and small nuclear RNAs

We first assessed the RNA–PLA method by testing known nuclear RNA–protein interacting pairs. U1 and U2 snRNAs

associate with Sm proteins to form the small nuclear ribonucleoproteins (snRNPs) of the spliceosome and reside primarily within the nucleus (Seraphin 1995). To determine whether RNA–PLA can detect these interactions, HeLa cells were fixed, permeabilized, and incubated with anti-Sm antibody (and a corresponding commercially available oligonucleotide-conjugated secondary antibody probe) and an RNA–PLA oligonucleotide probe containing a 5' segment sense or antisense to U1 or U2 RNA. After the samples were processed for PLA, specific green fluorescent nuclear signals were detected in samples incubated with the oligonucleotides antisense U1 or U2 RNA, but not with the sense oligonucleotides (Fig. 2A,B). These data show that RNA–PLA detects endogenous stable nuclear RNA–protein interactions.

To further explore the specificity of RNA–PLA, we detected the association of the Lupus autoantigen (La) protein with Epstein-Barr virus (EBV)-encoded RNA 2 (EBER2), a small noncoding RNA that is abundantly expressed in the nucleus of EBV-infected cells (Lerner et al. 1981; Glickman et al. 1988). La is a nuclear protein that associates with the 3' ends of RNA polymerase III transcripts to promote their maturation (Wolin and Cedervall 2002; Fok et al. 2006). EBER2 interacts with La protein to form RNP complexes that bind to the terminal repeat sequences of the EBV genome (Lerner et al. 1981; Wolin and Cedervall 2002; Lee et al. 2015). BJAB is an EBV-negative human Burkitt lymphoma B-cell line and BJAB-B1 is a clonal BJAB derivative harboring a latent EBV genome that expresses EBER2 (Fresen and zur Hausen 1976). We performed RNA–PLA on both cell lines using a primary antibody against La protein and an RNA–PLA oligonucleotide probe for EBER2. RNA–PLA signals were observed in the EBV-positive BJAB-B1 cells when the antisense oligonucleotide, but not the sense oligonucleotide, was used (Fig. 2C). As expected, the signals were present

FIGURE 1. Schematic of RNA–PLA. See text for details.

FIGURE 2. Specific detection of stable nuclear RNA-protein interactions by RNA-PLA. HeLa cells were fixed, permeabilized, and incubated with anti-Sm antibody and an oligonucleotide sense or antisense to U1 (A) or U2 (B) RNA. PLA was then performed to detect the interaction of Sm protein and the U RNA (green); nuclei were stained with DAPI (blue). In each column, a confocal image of the same Z-plane is displayed for the PLA signal (top) and the merged signal with DAPI (bottom). Representative results of three independent experiments are shown. Scale bar, 20 μ m. (C) As described in panels A and B, RNA-PLA was used to detect the interaction of La protein and EBER2 in BJAB-B1 (EBV+) cells by using anti-La antibody and an oligonucleotide sense or antisense to EBER2. (D) BJAB (EBV-) and BJAB-B1 (EBV+) cells were fixed, permeabilized, and incubated with the antisense oligonucleotide to EBER2 and the anti-La or the anti-Sm antibody. PLA was then performed as described above. A representative result of three independent experiments is shown. The total RNA-PLA fluorescence intensity per cell in each sample (minimum 150 cells each condition) was measured by Blobfinder software. The numbers at the bottom show the average results ($\pm$ standard error of the mean [SEM]) for three experiments relative to the La/EBER2 signal in BJAB-B1 cells.

exclusively in the nucleus, where La and EBER2 localize. In contrast, little signal was observed when La/EBER2 RNA-PLA was performed in BJAB cells lacking EBV, or in EBV-infected BJAB-B1 cells if the anti-La antibody was replaced with an anti-Sm antibody (Fig. 2D). Although Sm protein is abundant in nucleus, it does not bind EBER2, which lacks an Sm protein-binding site (Moss et al. 2014). Thus, a robust RNA-PLA signal requires coexpression of the interacting protein and its target RNA, as well as the use of an antisense RNA-PLA oligonucleotide probe.

Detection of complexes between protein and mRNA in discrete cellular compartments

The RNA-PLA assay can also be used to localize mRNA-protein interactions in various cell compartments. HuR (Human antigen R) is an RNA-binding protein that interacts with specific AU- or U-rich elements (AREs) in certain mRNAs (Darnell 1996; Gallouzi et al. 2000; Lopez de Silanes et al. 2004). This interaction is important for regulating mRNA stability and translational efficiency (Dai et al. 2012). HuR is predominantly localized in the nucleus, but treatment with the transcription inhibitor actinomycin D (ActD) causes

translocation of HuR into the cytoplasm (Supplemental Fig. S1; Gallouzi et al. 2000; Lopez de Silanes et al. 2004). Cytoplasmic HuR can bind to AREs in the 3'-untranslated region (3' UTR) of mRNAs, affecting mRNA stability (Dormoy-Raclet et al. 2007).

We used RNA-PLA to investigate whether HuR interacts with a specific mRNA in different subcellular compartments. We chose to investigate β -actin mRNA, which binds to HuR via a 3' U-rich sequence (Lopez de Silanes et al. 2004; Dormoy-Raclet et al. 2007; Jung et al. 2013b). HeLa cells were treated with 5 μ g/mL ActD or vehicle control for 1 h, fixed and processed for RNA-PLA using a primary antibody recognizing HuR and a β -actin mRNA-specific RNA-PLA oligonucleotide probe. In cells without ActD treatment, we observed weak PLA signals exclusively in the nucleus when the antisense probe was used and no signals with the sense probe, suggesting that HuR interacts with the low levels of β -actin transcripts in the nucleus (Fig. 3A, left two panels). As shown in the right two panels of Figure 3A, ActD treatment results in the generation of a significant cytoplasmic RNA-PLA signal with the antisense, but not the sense β -actin oligonucleotide probe, due to HuR translocation to the cytoplasm where it interacts with the much more abundant cytoplasmic β -actin

FIGURE 3. Detection of mRNA-protein interactions in subcellular compartments by RNA-PLA. (A) HeLa cells were treated with ActD (+) or vehicle control (–) for 1 h, fixed, permeabilized, and incubated with an anti-HuR antibody and an oligonucleotide sense or antisense to β-actin mRNA. PLA and confocal microscopy were performed as described in Materials and Methods and displayed as in Figure 2. The PLA signal was quantified and is shown as the average results (±SEM) for three experiments relative to the HuR/β-actin mRNA signal with the antisense probe in the ActD-treated cells. (B) HeLa cells were treated with ActD for 1 h, fixed and processed for PLA with an oligonucleotide that is antisense to β-actin mRNA and anti-HuR antibody, anti-TGN46 antibody, or anti-β-tubulin antibody, as indicated. PLA and confocal microscopy were then performed as described above.

mRNA. FISH confirmed that only the antisense RNA-PLA β-actin probe detects cytoplasmic β-actin mRNA (Supplemental Fig. S2). These data demonstrate that the RNA-PLA assay can be used to track mRNA-protein interactions spatially.

We carried out additional specificity controls in ActD-treated cells to confirm that HuR specifically recognizes β-actin mRNA. RNA-PLA failed to generate a robust PLA signal for the association of β-actin mRNA with TGN46 (an irrelevant trans-Golgi network marker) (Fig. 3B). In contrast, RNA-PLA showed that β-actin mRNA strongly associates with β-tubulin in the cytoplasm, consistent with previous reports that the cytoskeleton is involved in β-actin mRNA transport (Hesketh and Pryme 1991; Condeelis and Singer 2005; Lifland et al. 2011; Jung et al. 2013a).

RNA-PLA detection requires a binding site for the protein partner on the target RNA

Previous biochemical studies showed that the insertion of the ARE sequence from c-fos or GM-CSF (granulocyte-macrophage colony-stimulating factor) into the 3' UTR of rabbit β-globin mRNA allows HuR binding (Fan et al. 1997), result-

ing in rapid degradation of the mRNA. To confirm that ARE sequences are required for the interaction between HuR and target mRNA detected by RNA-PLA, we tested the association of HuR and native rabbit β-globin mRNA, which lacks an endogenous ARE, or rabbit β-globin mRNA containing an exogenous ARE (Fig. 4A; Fan et al. 1997). HeLa cells were transfected with pBBB expressing rabbit β-globin mRNA or with plasmids expressing β-globin mRNA containing a foreign ARE, treated with 5 μg/mL ActD for 1 h, fixed and processed for RNA-PLA using anti-HuR antibody and an RNA-PLA oligonucleotide probe specific for rabbit β-globin mRNA. In cells transfected with pBBB, which expresses β-globin mRNA lacking an ARE, minimal RNA-PLA signal was detected. In contrast, we observed strong RNA-PLA signals in samples transfected with a β-globin construct containing a c-fos or GM-CSF ARE when the antisense but not the sense oligonucleotide probe was used (Fig. 4B). Thus, the absence of the binding site on the RNA substantially reduces the signal, showing that the target protein and RNA must physically interact for RNA-PLA to generate a robust signal. Because insertion of an ARE into β-globin mRNA greatly reduces its stability (Fan et al. 1997), the ARE-containing RNAs are less abundant than control β-globin mRNA in transfected cells (Fig. 4C), even though a higher amount of DNA was used for transfection of the GM-CSF and c-fos constructs (compared to pBBB) in an attempt to achieve higher β-globin mRNA expression levels in cells expressing these transcripts. The much higher RNA-PLA signals in cells receiving the ARE-containing transcripts, despite the lower absolute β-globin mRNA levels in these cells, further demonstrates the sensitivity and specificity of this assay.

DISCUSSION

In this study, we modified the PLA procedure to detect the specific association of RNAs with their binding proteins inside single cells. We detected a strong RNA-PLA signal indicative of stable nuclear complexes between two U-class cellular snRNAs and the Sm protein, and between viral EBER2 RNA and the La protein. As expected, RNA-PLA probes for an interaction between EBER2 and Sm generated only a weak RNA-PLA signal, even though these molecules are both abundantly expressed in the nucleus. The weak signal generated by this probe set presumably represents low-level background staining, which is in general <10% as intense as an authentic RNA-PLA signal generated by complex formation. We also showed that we can detect specific interactions between an RNA binding protein and its target mRNA in discrete intracellular compartments and that a strong RNA-PLA signal requires a binding site for the protein on the target mRNA. In previously published work, we used RNA-PLA to detect a transient interaction in 293T cell nuclei between an unusual bipartite Herpesvirus saimiri (HVS) primary-microRNA (pri-miRNA) and the Int9 subunit of the Integrator complex, which processes this RNA (Xie et al.

FIGURE 4. RNA-PLA detection requires a protein-binding site on the RNA. (A) Schematic diagram of the β -globin reporter constructs. pBBB does not contain an ARE. The GM-CSF and c-fos constructs contain the c-fos and GM-CSF AREs, respectively, inserted into the 3' UTR of the β -globin reporter plasmid. (B) HeLa cells were transfected with the β -globin constructs, treated with ActD, fixed and processed for PLA using anti-HuR antibody and an oligonucleotide antisense or sense to β -globin mRNA, as indicated. PLA and confocal microscopy were then performed as described above. The total PLA fluorescence intensity per cell in each sample (minimum 150 cells each condition) was measured using the Blobfinder software and is shown as the average results ($\pm$ SEM) for three experiments relative to the signal in the sample transfected with the c-fos ARE/ β -globin construct and processed for RNA-PLA with the antisense probe. Results of a representative experiment are shown. Similar results were obtained in three independent experiments. (C) Northern blot analysis of β -globin RNA containing or lacking an ARE. Total RNA was prepared from HeLa cells transfected with empty vector or plasmids expressing the indicated rabbit β -globin constructs. After agarose gel electrophoresis and UV crosslinking, 28S ribosomal RNA was detected by staining the membrane with methylene blue. β -Globin RNA was detected by hybridization to a β -globin RNA-specific antisense oligonucleotide.

2015). As expected, no RNA-PLA signal was detected in cells incubated with anti-Drosha antibody, even though the Drosha RNA processing enzyme, which does not process the HVS pri-miRNA, is highly abundant in the nucleus.

Compared to other PLA-based methods that detect RNA-protein interactions (Lifland et al. 2011; Jung et al. 2013b), our protocol is simple, inexpensive, time-efficient, uses commercially available reagents, and requires minimal cell manipulation and no insertion of foreign sequences into the RNA. In most cases, we designed active RNA-PLA oligonucleotide probes by using a simple on-line prediction tool, but it is also possible to experimentally map accessible sites on RNA. Upon addition of DNA oligonucleotides to cell lysates, DNA-RNA hybrids form at accessible sites, resulting in cleavage at those sites by endogenous RNase H, which can be detected by Northern blot analysis (Lee et al. 2015). This approach was used to identify the EBER2 probe. However, we have not systematically studied the impact of oligonucleotide probe position within the target RNA. Such studies are likely to provide additional guidance regarding probe design.

RNA-PLA should be valuable to confirm that RNA-protein interactions initially detected by biochemical approaches do, in fact, occur in cells. Because the oligonucleotide probes that anneal to the target RNAs are delivered to cells after membrane permeabilization and fixation, we are able to

detect RNA-protein complexes in specific intracellular compartments. Thus, RNA-PLA may be especially useful for exploring RNA-protein interactions that are induced or occur in different intracellular locations under certain physiological conditions or in response to various treatments. In addition, it may be possible to combine RNA-PLA with standard immunofluorescence staining of differentiated cell markers to allow the detection of RNA-protein complexes in specific cell types in heterogeneous cell populations or tissues. Finally, although we have used RNA-PLA to detect and analyze known RNA-protein interactions, it may also be possible to use this approach to identify novel RNA-protein interactions. For example, to detect RNAs that are bound by a specific RNA binding protein, a panel of RNA-PLA oligonucleotide probes that hybridize to different candidate target RNAs could be tested individually for their ability to generate a PLA signal with an antibody probe that recognizes the protein of interest. However, because of the complex, folded structure of many RNAs, we do not believe RNA-PLA can be used to assess the distance between the oligonucleotide binding site in the RNA and the bound protein.

We can also envision a number of modifications of RNA-PLA that will make it suitable for additional applications. A previous publication successfully detected c-Myc/Max/RNA polymerase II interactions by modified protein PLA, in

which three pairs of primary antibodies/proximity probes conjugated with short DNA oligonucleotides recognize each of the three proteins in a complex (Soderberg et al. 2006). Similarly, it may be possible to use an RNA–PLA strategy to detect the coexistence of two proteins bound to an RNA molecule. It may also be possible to modify the technique to detect specific RNA–RNA interactions or DNA–protein interactions in intact cells.

MATERIALS AND METHODS

Cell culture

HEK293T and HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), 1% (v/v) penicillin-streptomycin, and 2 mM L-glutamine. BJAB and BJAB-B1 cells (Fresen and zur Hausen 1976) were maintained in RPMI medium containing the same supplements. 10^5 cells were plated on 12 mm glass coverslips in a 24-well plate and incubated at 37°C overnight. To improve adhesion of 293T cells, coverslips were coated with poly-L-lysine according to manufacturer's instructions (Sigma-Aldrich). In experiments in Figures 3 and 4, cells were treated as indicated with 5 µg/mL actinomycin D (Sigma-Aldrich) or 0.25% DMSO vehicle control for 1 h at 37°C prior to RNA–PLA.

RNA–PLA probe design

The RNA–PLA probes targeting the RNA of interest are ultramer DNA oligonucleotides purchased from IDT DNA Technologies. A typical PLA probe consists of three regions from 5' to 3': (A) a 40–50 nucleotide (nt) sequence complementary to (or sense to) the RNA of interest. This sequence was designed based on RNA FISH probes in the literature or targeted an unstructured region as determined by RNase H assays; (B) 20–35 adenylates that serve as a linker; and (C) a 25-nt sequence that bridges the DNA circle and serves as a primer for rolling-circle amplification during PLA detection (Soderberg et al. 2006). Regions B and C are identical for all probes. We suggest using online FISH probe design resources (e.g., <http://prober.cshl.edu/>) to identify region A for each target RNA. The sequences of all oligonucleotides used in this study are shown in Supplemental Table S1.

Fixation, permeabilization, and blocking for PLA

Cells were washed once with phosphate-buffered saline (PBS) and fixed in 4% formaldehyde (v/v) in PBS on ice for 30 min. The samples were permeabilized with 1% saponin (w/v) (Sigma-Aldrich) for 1 h at room temperature and washed three times with PBS. The samples were then blocked with blocking buffer (10 mM Tris-acetate, pH 7.5, 10 mM magnesium acetate, 50 mM potassium acetate, 250 mM NaCl, 0.25 µg/µL bovine serum albumin [BSA], and 0.05% Tween 20) in the presence of 20 µg/mL sheared salmon sperm DNA (ssDNA) at 4°C for 1 h. One hundred nanomolar specific oligonucleotide probe was added to fresh blocking buffer, heated at 70°C for 3 min, and incubated with fixed/permeabilized cells at 37°C for another hour or at 4°C overnight. Subsequently, the cells were washed three times with PBS and blocked in PBS with Tween 20 (PBST [137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄,

1.8 mM KH₂PO₄, and 0.1% Tween-20]) containing 1% (v/v) BSA and 20 µg/mL ssDNA at room temperature for 1 h.

Primary antibody, RNA probes, and PLA probes

The samples were washed once with PBS, once with 300 mM NaCl, 30 mM sodium citrate buffer, pH 7.0 (2× SSC) containing 0.1% (v/v) Tween 20, once with PBS, and incubated with the appropriate primary antibody in PBST at room temperature for 1 h. The source of antibodies and dilutions are shown in Supplemental Table S2. The probe solution was prepared by diluting the corresponding species-specific minus PLA probe (Olink Bioscience) 1:5 into PBST containing 20 mg/mL ssDNA and allowing the mixture to sit for 20 min at room temperature. After three washes with PBS, the coverslips were transferred to a prewarmed humidified chamber (Lipovsky et al. 2015) and incubated with the probe solution for 1 h at 37°C.

Ligation, amplification, and labeling

The subsequent PLA ligation and amplification steps were performed according to the manufacturer's instructions (Olink Bioscience). Briefly, the PLA probe solution was aspirated from the cells and samples were washed twice with wash buffer A for 5 min each under gentle agitation. Cells were incubated with freshly prepared ligation mix for 30 min at 37°C. Samples were then washed twice with wash buffer A for 2 min each with gentle agitation and incubated with freshly prepared amplification mix for 100 min at 37°C. To enhance the signal intensity, the amplification step can be extended to 2–4 h if needed. Cells were washed twice with wash buffer B for 10 min with gentle agitation and stained with 4',6-diamidino-2-phenylindole (DAPI). After a final wash with 0.01× wash buffer B for 1 min, samples were mounted onto glass slides with FluorSave reagent (EMD Millipore).

Microscopy and image analysis

Images were captured by Leica TCS SP5 fluorescent microsystems using appropriate filters. All images were taken with the same exposure time and the same threshold to allow subsequent quantitative analysis in the respective channel. At least 150 nuclei per sample were imaged and the images were processed with ImageJ. Statistical analyses were then done by Blobfinder software (available for download from the Centre of Image Analysis at Uppsala University, <http://www.cb.uu.se/~amin/BlobFinder>) to determine the total fluorescence intensity per cell (Lipovsky et al. 2015). Experiments were repeated three times and averaged to obtain mean ± SEM. There is a low, variable background in these experiments, which is typically <10% of the signal deemed positive.

Fluorescence in situ hybridization

Cells were fixed, permeabilized, and blocked as described for the PLA assays. The samples were hybridized overnight at 37°C with 50 nM digoxigenin (DIG)-labeled oligonucleotide probes that recognize sequences in the 3' UTR of β-actin mRNA (see Supplemental Table S1). Sense and antisense oligonucleotides (purchased from IDT DNA Technologies) were labeled with DIG-dUTP using the 3' DIG Oligonucleotide Tailing kit, 2nd generation (Roche),

according to the manufacturer's instructions. After hybridization, slides were washed twice for 10 min each in $2\times$ SSC at 37°C followed by two 10-min washes in $1\times$ SSC at 37°C and a final wash with $0.5\times$ SSC for 10 min at room temperature. Cells were fixed again in 4% formaldehyde/PBS and washed three times in PBS; hybridized probes were detected using a 1:250 dilution of anti-DIG antibody conjugated to fluorescein (green; Roche) in blocking buffer at room temperature for 1 h.

Immunofluorescence

HeLa cells grown on glass coverslips were treated with $5\text{ }\mu\text{g/mL}$ ActD or vehicle control for 1 h, fixed with 4% paraformaldehyde in PBS, and permeabilized with 1% saponin in PBS. After incubation in blocking buffer (DMEM containing 10% FBS) for 1 h at room temperature, samples were stained with a 1:1000 dilution of anti-HuR mouse antibody followed by incubation with 1:200 dilution of the AlexaFluor 488 goat anti-mouse antibody (Molecular Probes). Both antibodies were diluted in blocking buffer. The nuclei were stained with DAPI (blue). The images were taken by Leica TCS SP5 fluorescent microsystems and processed with ImageJ.

Plasmids, transfection, and Northern blotting

Plasmids described elsewhere express rabbit β -globin mRNA (pBBB) or the same mRNA with a c-fos or GM-CSF ARE inserted into the β -globin mRNA 3' UTR (Fan et al. 1997). HeLa (JW36) cells in six-well plates were transfected with $0.5\text{ }\mu\text{g}$ of pBBB plus $1.5\text{ }\mu\text{g}$ pBluescript or with $2\text{ }\mu\text{g}$ of pB-ARE (c-fos) or pB-ARE (GM-CSF) containing an inserted ARE. Lipofectamine 2000 (Invitrogen) was used for transfection according to the manufacturers' instructions. Twenty-four hours later, 10^5 transfected cells were seeded on glass coverslips in a 24-well plate for another 24 h. The cells were treated with $5\text{ }\mu\text{g/mL}$ ActD for 1 h, fixed, and processed for PLA. Alternatively, after 72 h, total RNA was harvested using TRIzol reagent (Invitrogen), and $25\text{ }\mu\text{g}$ total RNA was resolved in a 1.4% agarose (w/v)/6.5% formaldehyde (v/v) gel and transferred to a Zeta-Probe GT blotting membrane (Bio-Rad) via upward capillary transfer with $20\times$ SSC. The membrane was crosslinked with 254 nm UV light at 120 mJ/cm^2 using Stratalinker UV crosslinker 2400 (Agilent genomics). To detect 28S rRNA, the membrane was stained with 0.3% methylene blue (v/v) in 0.3 M sodium acetate, pH 5.2, for 2–3 min until the 28S rRNA bands were clearly visible. Images were taken with the Gel Doc system. Fifty picomoles of rabbit β -globin DNA oligonucleotide probe was $5'-[^{32}\text{P}]$ labeled and hybridized in 10 mL of ExpressHyb Hybridization Solution (Clontech) at 42°C overnight. The membrane was washed with $1\times$ SSC, 0.1% SDS (20 min at room temperature), $0.5\times$ SSC, 0.1% SDS (20 min at room temperature) and analyzed by PhosphorImager.

SUPPLEMENTAL MATERIAL

Supplemental material is available for this article.

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