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Reassessing the role of a 3′-UTR-binding translational inhibitor in regulation of circadian bioluminescence rhythm in the dinoflagellate *Gonyaulax*

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Abstract

The nightly bioluminescence of the dinoflagellate *Gonyaulax* is a circadian rhythm caused by the presence in cells of specialized bioluminescent organelles, termed scintillons, containing the reaction catalyst luciferase, the substrate luciferin and a luciferin-binding protein (LBP). LBP levels increase at the start of the night phase because of increased protein synthesis rates *in vivo*, and this regulation has been ascribed to circadian binding of an inhibitory protein factor binding to the 3′ untranslated region (UTR) of *lbp* mRNA at times when LBP is not normally synthesized. To purify and characterize the binding factor, the electrophoretic mobility shift assays and UV crosslinking experiments used to first characterize the factor were repeated. However, neither these protocols nor binding to biotinylated RNA probes confirmed the presence of a specific circadian RNA-binding protein. Furthermore, neither RNA probe screening of a cDNA library expressed in bacteria nor three-hybrid assays in yeast were successful in isolating a cDNA encoding a protein able to bind specifically to the *lbp* 3′UTR. Taken together, these results suggest that alternative mechanisms for regulating *lbp* translation should now be examined.

Keywords: bioluminescence; circadian rhythm; dinoflagellate; luciferin-binding protein; translational control.

Introduction

The unicellular marine dinoflagellate *Gonyaulax polyedra* (now *Lingulodinium polyedrum*) has been a model for experimental characterization of the circadian clock for over 50 years. In particular, the wealth of different biological rhythms that are available for study is quite remarkable. These rhythms include daily vertical migration (Eppley et al., 1968), the daily photosynthesis rhythm (Hastings et al., 1961), the nightly bioluminescence rhythm (Hastings and Sweeney, 1958) and the circadian gating of mitosis to just after dawn (Sweeney and Hastings, 1958). Since *Gonyaulax* is not yet amenable to transformation, analysis of such rhythms has involved two approaches, one in which the physiological characteristics of the rhythms have been documented and one in which the biochemical underpinnings of the rhythms have been addressed.

The most spectacular rhythm, which is also the best studied, is that of bioluminescence. This rhythm is of high amplitude, with the amount of light produced varying approximately 50- to 100-fold between day and night. The light-producing reaction occurs when the enzyme luciferase adds oxygen to the substrate luciferin, producing a photon of blue-green light. This luciferin is oxygen-sensitive *in vitro* (Nakamura et al., 1989), and in extracts of *Gonyaulax* is found primarily bound to a substrate-binding protein (luciferin-binding protein, LBP; Morse et al., 1989b). All three components are found together in discrete light-producing organelles called scintillons (Johnson et al., 1985; Desjardins and Morse, 1993) whose number within the cell varies in phase with bioluminescence (Fritz et al., 1990). The only proteins detected in purified scintillons are luciferase and LBP (Desjardins and Morse, 1993), and cellular levels of luciferase (Johnson et al., 1984), LBP (Morse et al., 1989a) and luciferin (Bode et al., 1963) all vary over the course of the daily cycle. Changes in both enzyme and substrate levels thus constitute biochemical correlates to the physiological rhythm.

To test the possibility that transcriptional control might underlie the circadian changes in protein levels, the amount of RNA encoding luciferase and LBP was examined over the course of the circadian cycle. The half-life of LBP RNA is at least 12 h (Rossini et al., 2003), and the amount of RNA encoding both proteins does not change (Morse et al., 1989a; Mittag et al., 1998), suggesting that transcription is not involved. In addition, LBP RNA can be translated efficiently in a heterologous translation system (Milos et al., 1990), and the amount of protein produced is independent of the time at which the RNA was extracted. These observations, together with the finding that LBP translation *in vivo* is only observed during the early hours of the night (Morse et al., 1989a), suggest that it is translation of LBP RNA that is under clock control. The mechanism for this was proposed to involve binding of a ca. 45-kDa protein to a U(U)G repeat region in the 3′ untranslated region (UTR) of LBP RNA, as demonstrated by electrophoretic mobility shift assay (EMSA; Mittag et al., 1994). The increase in protein binding found using daytime extracts suggested that the factor might thus act as a translational inhibitor. Interestingly, a factor binding to a UG repeat region has been isolated and characterized from the green alga *Chlamydomonas* (Zhao et al., 2004), and this factor may be involved in the clock mechanism in this organism (Iliev et al., 2006).

The goal of the work presented here was to exploit EMSA as an assay to aid in purification of the protein factor binding to the 3′-UTR of LBP RNA. Unfortunately, we were unable to confirm specific protein binding to the 3′-UTR sequence by EMSA, by crosslinking to LBP RNA *in vivo* or *in vitro*, by RNA binding to a cDNA library expressed in bacteria, or by yeast three-hybrid assays. We conclude that a reexamination of protein binding to different regions of the LBP RNA is now required.

Results

Protein extracts prepared from *Gonyaulax* cultures at two different times under the LD cycle were prepared for EMSA. LD14 corresponds to the time of maximum LBP synthesis *in vivo*, while LD2 was selected as a time at which no detectable synthesis occurs (Morse et al., 1989a). Both extracts were tested over a range of different concentrations with an RNA derived from the LBP 3′-UTR. Both extracts exhibited modest mobility shifts and degradation of the RNA probe at high protein levels, but no repeatable difference could be discerned between the two (Figure 1A). The mobility shift observed here appears to be non-specific, as it cannot compete with unlabeled RNA at concentrations up to 400-fold greater than the radioactive substrate (Figure 1B). All these effects were observed with both XbaI (Figure 1B) and BglI (Figure 1A) probes, which differ essentially in the presence and absence of a poly(A) tail, respectively. Furthermore, the binding pattern was not altered by addition of phosphatase inhibitors (data not shown), indicating that our untreated extracts had not lost binding affinity due to dephosphorylation. These results were observed for dozens of different protein extractions. The experiment was also performed with several different protein extracts prepared by ammonium sulfate precipitation (10–70% cut), but no differences were observed in the pattern of bound proteins.

As a complement to EMSA, the UV crosslinking method previously described was also tested (Mittag et al., 1994). This was expected to reveal the presence of a ~25-kDa protein on SDS-PAGE that had become radioactive following binding of the radiolabeled BglI probe *in vitro*. In the absence of protein extracts, RNase-treated samples contain little free RNA probe alone, and the radiolabeled RNA was completely digested, as expected (Figure 2A, lane 1). However, neither the LD2 nor the LD14 protein extracts were able to protect any of the radiolabeled probe from digestion (Figure 2A, lanes 2 and 3). As an extension to this technique, *in vivo* binding was also tested, in which intact cells were exposed to UV irradiation to crosslink endogenous LBP RNA to its

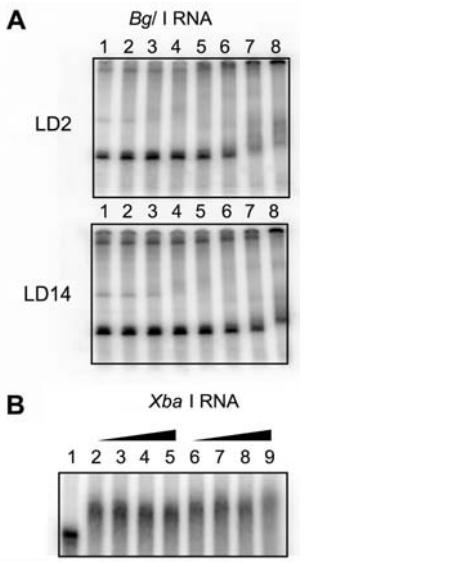

Figure 1 Electrophoretic mobility shift assay (EMSA) of *Gonyaulax* protein extracts does not show circadian differences. (A) Autoradiograms of EMSA with 20 000 cpm radiolabeled BglI fragment (145 nt) from the LBP 3′-UTR either alone (lane 1) or after incubation with extracts prepared from both LD2 and LD14 (lanes 2–8). The reaction mixtures contain 0.44, 0.87, 1.75, 3.5, 7, 14 or 28 μg (lanes 2–8, respectively) of *Gonyaulax* cell extract. (B) EMSA with 20 000 cpm radiolabeled XbaI fragment (263 nt) either alone (lane 1) or after incubation of 28 μg of protein extract from LD2 (lanes 2–5) or LD14 cell cultures (lanes 6–9). A non-radioactive specific transcript was added to the reactions as a competitive inhibitor at a concentration of 2× (lanes 3 and 7), 20× (lanes 4 and 8) or 400× (lanes 5 and 9) that of the radiolabeled fragment.

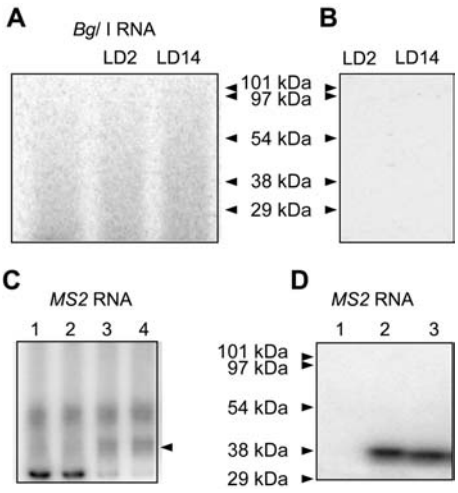

Figure 2 Protein extracts are not crosslinked to the LBP 3′-UTR.
(A) Autoradiogram of an *in vitro* UV crosslinking experiment using a radiolabeled BglI transcript either alone (lane 1) or in the presence of 28 μg of protein extract from either LD2 (lane 2) or LD14 (lane 3) phase cells. Samples were RNase-treated, then subjected to denaturing SDS-PAGE. The position of molecular mass markers is shown.
(B) Autoradiogram of an *in vivo* UV crosslinking experiment in which intact cells were exposed to UV, extracted proteins subjected to denaturing SDS-PAGE, transferred to nitrocellulose and incubated with a random-primed LBP probe containing the entire coding sequence and the 3′-UTR. Cells were treated at either LD2 or LD14. (C) Autoradiogram of control electrophoretic mobility shift assays using 20 000 cpm radiolabeled MS2 RNA either alone (lane 1), with 14 μg boiled protein extracts from YBZ1 yeast expressing a LexA-MS2 coat protein fusion, or with 14 μg (lane 3) or 28 μg (lane 4) non-boiled yeast protein extract. (D) Autoradiogram of an *in vitro* UV cross-linking experiment using a radiolabeled *MS2* RNA alone (lane 1) or in the presence of 14 μg or 28 μg protein extract from YBZ1 yeast. Samples were RNase-treated then electrophoresed using denaturing SDS-PAGE as in (A).

potential binding partner. Following extraction and electrophoresis of the protein fraction by SDS-PAGE, the sample was transferred to a membrane and challenged with an antisense LBP probe to test for potential *lbp* RNA binding to a protein. Once again, no binding could be detected using either LD2 or LD14 protein extracts (Figure 2B). As a control, MS2 phage RNA and the LexA-MS2 coat fusion protein expressed in YBZ1 yeast was tested and found to produce a modest EMSA gel shift (Figure 2C). Complete binding of all added probe, even at the lowest amount of yeast extract tested, is presumably due to a high level of LexA-MS2 fusion protein expression in the YBZ1 strain. More importantly, *in vitro* crosslinking between radiolabeled MS2 phage RNA and the LexA-MS2 fusion protein showed a strong signal at the expected size of ca. 40 kDa (Figure 2D).

As an alternative to direct biochemical demonstration of factor binding to the 3′-UTR, two different screening methods were carried out to isolate a binding factor expressed from a cDNA. In one technique, the 3′-UTR probe was used to screen a bacterial expression library, whereby proteins found in phage plaques transferred to nitrocellulose were challenged with the probe. In these tests, while 16 potential signals were observed during a first round of screening (Figure 3, left panel), none persisted through a second round of screening (four illustrated in Figure 3, right panels).

In a second technique, the yeast three-hybrid assay was used to detect interactions between the LBP 3′-UTR and a protein expressed in yeast from a *Gonyaulax* cDNA library. Similar to the standard two-hybrid version, the three-hybrid assay employs a DNA-binding (DB) protein fusion construct and a GAL4 activation domain (AD) fusion construct. Three-hybrid assays utilize the same AD-cDNA fusion constructs as standard two-hybrid screens, but the DB fusion contains an MS2 phage coat RNA-binding protein. The third part of the system, an RNA fusion construct combining MS2 RNA and the LBP 3′-UTR, thus allows reconstitution of a transcription factor when the cDNA expresses a protein able to bind LBP RNA. This screening resulted in a total of eight cDNAs that were able to reproducibly complement the *His3* mutation after purification and retransformation (Table 1). All eight had identical sequences and were identified as the *Gonyaulax* homolog of the yeast *His3* gene by BLAST searches.

Table 1 Three-hybrid screening.

Step	Colonies
Transformants	840 000
HIS3+	58
Homogeneous culture[a]	11
Recomplementation	8
LBP 3′-UTR-binding protein	0

[a] Only cultures that did not flocculate during growth in liquid culture were taken for further analysis.

In a final attempt to identify factors potentially binding to the 3′-UTR, approximately 1 mg of *Gonyaulax* protein extract was allowed to bind *in vitro* to a biotin-labeled BglI probe linked to Streptavidin-coated paramagnetic beads. After 2-h incubation, specific fractions were eluted with 100 mM, 500 mM and 1 M NaCl, and the products were analyzed on SDS-PAGE (Figure 4). Although some protein bands were observed following elution with 500 mM NaCl, a ca. 25-kDa band was not among them. Furthermore, none of the bands visible by Coomassie Blue staining could be identified by protein microsequencing as a potential RNA-binding protein (Table 2).

Discussion

The experiments described here were initiated to purify and characterize the protein factor whose circadian binding to the 3′-UTR of the LBP RNA by EMSA had been reported previously (Mittag et al., 1994). The probes used in this study (BglI and XbaI) were identical to those employed previously, and we have tested, among others, the same buffers, protein concentrations and methods of sample preparation. Our results do show some similarity

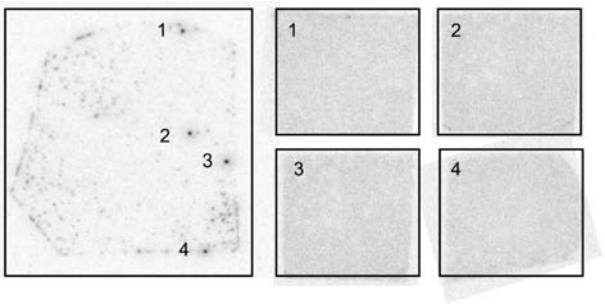

Figure 3 RNA probe binding to bacterially expressed cDNAs. Autoradiograms of nitrocellulose membranes carrying phage protein hybridized with a radiolabeled BglI RNA transcript. First round screening (left) produced several candidate phages, but none reproducibly bound radiolabeled transcript after phage selection and replating (panels 1–4, right).

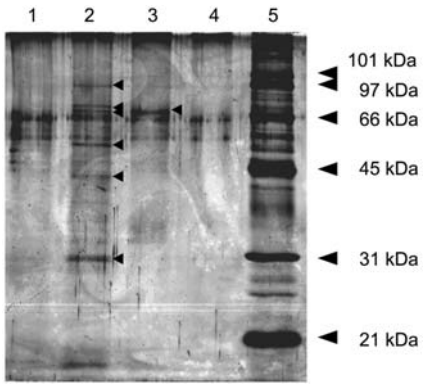

Figure 4 Purification of RNA-binding protein by Streptavidin affinity chromatography.
SDS-PAGE analysis of protein fractions eluted from a Streptavidin affinity column with 100 mM NaCl (lane 1), 500 mM NaCl (lane 2) or 1 M NaCl (lane 3) visualized using the fluorescent dye Flamingo. The column was loaded with 1 mg of *Gonyaulax* protein extract preincubated with a biotin-labeled BglI RNA transcript and 10% of the eluate was run on the gel. Bands marked in lanes 2 and 3 were excised from a Coomassie Brilliant Blue-stained gel run in parallel with the remaining 90% of the protein fractions for microsequencing. Molecular mass markers (lane 5) and sample buffer alone (lane 4) are also shown.

Table 2 Microsequence identification of proteins binding to biotin-labeled BglI RNA.

Apparent MW (kDa)	Proteins identified	Species	Coverage[a] (%)	Length (amino acids)
97[b]	EF-1α-like protein	*Heterocapsa*	15	474
74[b]	Unidentified			
72[b]	Unidentified			
55[b]	Mitochondrial F1-ATPase	*Toxoplasma*	15	560
	Rubisco	*Gonyaulax*	12	547
42[b]	Unidentified			
31[b]	Plastid ferredoxin $NADP^+$ reductase	*Heterocapsa*	14	416
	Glyceraldehyde-3-P dehydrogenase	*Gonyaulax*	11	419
72[c]	Luciferin-binding protein	*Gonyaulax*	24	668

[a] Percentage of identified protein for which the corresponding peptide sequence was recovered.
[b] Elution with 500 mM NaCl.
[c] Elution with 1 M NaCl.

to previous observations, in particular the general appearance of the slowly migrating probe in the presence of a protein extract. However, our results differ markedly in an inability to confirm a circadian difference in factor binding. The EMSA experiments described here have also been performed independently elsewhere, and there were no circadian differences in factor binding, although specific shifts were observed (T. Fagan, personal communication). The differences observed between the different laboratories suggest that circadian factor binding to the 3′-UTR of *lbp* mRNA may not be the mechanism employed to regulate circadian LBP synthesis *in vivo*.

Despite the similarities, there are some notable differences between the experiments reported here and those previously described. The principal difference lies in the cell strain used, with previous experiments employing a strain isolated in 1970 and ours using a newer strain isolated in 1998. However, the bioluminescence rhythm is as robust in the new strain as in the previous isolate, and it seems unlikely that different strains would use completely different mechanisms to regulate LBP translation. Another difference lies in the light regime, as our cultures are grown at approximately one-third of the light intensity used previously. Once again, it seems unlikely that this difference would result in lack of circadian protein binding.

One potential complication in all EMSA techniques is that post-translational modifications of the factor itself might influence binding to the probe. We addressed this issue by comparing extracts prepared in the absence of phosphatase inhibitors to extracts containing phosphatase inhibitor cocktails. We did not observe any difference in probe mobility using the two types of extract, suggesting that differential phosphorylation states of the protein factor are also unlikely to have influenced our experiments. We are aware that we have not controlled explicitly for other forms of post-translational modification, so this remains a formal possibility. However, since we have tested the same protocol used in the previous study, differential modification is unlikely to be the cause of our inability to observe specific protein binding.

With respect to the screening methods, there are two different caveats that must be noted. First, any potential protein-binding factors expressed from a single cDNA will only function correctly if the protein is a monomer or homopolymer. Secondly, should the factor require post-translational modification for activity, it is unlikely that such modifications will be faithfully reproduced following expression in either yeast or bacteria. Thus, potential involvement of a heteromeric protein in RNA binding would provide a facile explanation for our inability to recover cDNAs encoding binding proteins, either in bacteria or during the yeast three-hybrid screen. Nonetheless, we note that our screening results are consistent with the other experimental approaches used.

As a general conclusion to these experiments, it seems logical to propose that alternative regulation mechanisms for translation of LBP in *Gonyaulax* now be explored. In this regard, it may be worthwhile to reexamine the 5′-UTR. Although the 5′-UTR sequence derived from a genomic clone was previously tested by EMSA (Mittag et al., 1994), recent studies have demonstrated that dinoflagellate messages are modified post-transcriptionally by addition of a *trans*-sliced leader sequence (Zhang et al., 2007). It is well within the realm of possibility that an additional sequence in the 5′-UTR of *lbp* mRNA may be required for factor binding. Alternatively, it is also possible that probes prepared from a fusion construct of the 5′- and 3′-UTRs might display altered behavior; constructs containing both untranslated regions have never been tested.

Materials and methods

Cell culture

Gonyaulax polyedra (now *Lingulodinium polyedrum*; strain 1936) was obtained from the Provasoli-Guillard Culture Center for Marine Phytoplankton (Boothbay Harbor, ME, USA) and grown in a modified seawater medium (f/2; Guillard and Ryther, 1962) at constant temperature (18±1°C) in 12-h light/12-h dark cycles using cool white fluorescent light at an intensity of 50 μmol photons m^{-2} s^{-1}. The beginning of the light period was defined as time 0 (LD0), and the beginning of the dark period as LD12. Cultures were grown to a cell density of 12–14 000 cells/ml.

Protein extraction

Cell cultures were filtered through a Whatman 541 filter, frozen in liquid nitrogen and stored at -80°C until use. Frozen cells were thawed on ice in either Mittag buffer [40 mM NaCl, 10 mM Tris, pH 7.4, 0.1 mM EDTA, 2 mM dithiothreitol (DTT) and 5% v/v glycerol; Mittag et al., 1994] or Munro buffer (10 mM HEPES, pH

7.6, 40 mM KCl, 3 mM $MgCl_2$, 1 mM DTT, and 5% v/v glycerol; Coughlin et al., 2000) supplemented with 0.1% Nonidet P-40, 300 μM phenylmethylsulfonyl fluoride, 3 μg/ml leupeptin, 25 mM NaF, 80 mM β-glycerophosphate and 0.2 mg/ml yeast RNA. Cells were broken by three 1-min treatments in a bead beater with zirconium beads. Insoluble cell debris was removed by centrifugation at 16 000 *g* for 12 min at 4°C, and the supernatant was retained. Protein concentrations were measured using the Bradford assay (Bio-Rad, Montreal, Canada). For some experiments, 1% phosphatase inhibitor cocktail 1 and 2 (Sigma, Oakville, Canada) was included in the extraction buffer.

Probe preparation

RNA probes were transcribed from the pMM3 plasmid (Mittag et al., 1994), generously provided by Dr. M. Mittag, using a commercial kit (Promega Biotech, Montreal, Canada). Plasmids linearized with XbaI gave rise to 263-nt transcripts that contain the entire 3′-UTR, as well as the poly(A) tract of the cDNA, while plasmids linearized with BglI produced 145-nt-long transcripts that do not contain a poly(A) and lack the last 30 nt of the ~150-nt LBP 3′-UTR. The reaction buffer contained 40 mM Tris-HCl (pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine, 10 mM NaCl, 10 mM DTT, 1 U/μl porcine RNAGuard RNase inhibitor (Pharmacia, Montréal, Canada), 0.5 mM rATP, 0.5 mM rCTP, 0.5 mM rGTP, 12 μM rUTP, 50 ng/μl linearized plasmid pMM3 (Mittag et al., 1994), either 6.8 or 2.5 μCi/μl [α-^{32}P]-rUTP (3000 Ci/mmol) and 1 U/μl T7 RNA polymerase. After 1-h incubation at 37°C, the DNA template was digested with 1 U of RQ1 RNase-free DNase per μg DNA plasmid. Proteins were removed by extraction with one volume of chloroform/isoamyl alcohol (24:1) and unincorporated nucleotides were removed by filtration using a Microcon column (Millipore, Billerica, MA, USA). Radioactivity was measured using a scintillation counter and probe quality was assessed by electrophoresis on 8 M urea-polyacrylamide gels before use. In some experiments, probe RNA was purified after electrophoresis from urea-containing 6% polyacrylamide gels; however, as this purification had no impact on the electrophoretic mobility shift patterns, it was not routinely performed. All RNA probes were heated at 70°C for 10 min, then allowed to cool slowly to room temperature before use.

Biotin-labeled probes were prepared similarly, except that all nucleotides were used at 0.5 mM concentration and rCTP was replaced by CTP/biotin-11-rCTP (Enzo Life Sciences Inc., Farmingdale, NY, USA) in a 2:1 ratio. This transcript was linked to 500 μl of Streptavidin-coated paramagnetic beads (Spherotech Inc., Lake Forest, IL, USA) by 1-h incubation at room temperature in a total volume of 200 μl of phosphate-buffered saline (PBS) with shaking. The beads were then washed four times in 500 μl of PBS to remove non-bound transcripts, after which the beads were equilibrated in 500 μl of Munro buffer. The protein extract (1 mg) was preincubated for 20 min on ice with 1 mg of yeast RNA as a non-specific inhibitor, then incubated with the biotin-labeled beads for 2 h at 4°C. Non-bound proteins were removed by four washes with 500 μl of Munro buffer before elution of bound protein in 200 μl of 1 M NaCl for 45 min at room temperature.

UV crosslinking

For *in vitro* crosslinking, protein samples were incubated with radiolabeled probe as described for protein binding to magnetic beads charged with transcript (see above). Following incubation, samples were exposed to UV radiation of 2000 μW/cm^2 for 30 min on ice as previously described (Waltenberger et al., 2001). Excess RNA was removed by 30-min incubation with 2 μl of RNase A (10 mg/ml) at 37°C. The reaction was then prepared for SDS-PAGE using standard conditions and electrophoresed at 150 V for 1 h on a 10% w/v acrylamide/bis-acrylamide (37.5:1) resolving gel using a 5% stacking gel. The gels were dried and subjected to autoradiography.

For *in vivo* crosslinking, 25 ml of cell culture was exposed to UV radiation of 2000 μW/cm^2 for 30 min on ice (Waltenberger et al., 2001). Cells were sedimented by centrifugation at 1000 *g* for 1 min, resuspended in Munro buffer and broken in a bead beater with zirconium beads. Following 30-min incubation at 37°C with 2 μl of RNase A (10 mg/ml), samples were prepared for SDS-PAGE and electrophoresed as above. Proteins were then transferred electrophoretically to nitrocellulose Hybond C membranes (Amersham, Piscataway, USA) that were then incubated at 65°C with a random primed LBP probe containing the entire coding sequence and the 3′-UTR.

Electrophoretic techniques

For EMSA, 100 pM radiolabeled RNA was typically mixed with 28 μg of protein extract preincubated for 20 min with 28 μg of yeast RNA as a non-specific inhibitor, and incubated for a further 30 min at room temperature. No difference was observed if poly(G) was used in place of the yeast RNA as a non-specific inhibitor, so the latter was typically used owing to cost considerations. An aliquot of 1 μl of 98% glycerol containing 1% bromophenol blue and 1% xylene cyanol was added to a 20-μl reaction mixture prior to electrophoresis. Samples were electrophoresed in 0.5× Tris-borate-EDTA buffer (TBE) at 200 V for 4 h at 4°C on 6% (w/v) non-denaturing 20:1 acrylamide/bisacrylamide gels containing 0.5× TBE and 10% glycerol. Following electrophoresis, gels were dried and exposed to either autoradiography film or Phosphorimager screens.

Protein samples for SDS-PAGE analysis were prepared and electrophoresed using standard techniques (Wang et al., 2005). Proteins excised from Coomassie Blue-stained gel were extensively destained and subjected to trypsin digestion, and the tryptic fragments were sequenced by mass spectroscopy. Tryptic fragment preparation and sequence analysis were performed at the Institut de Recherche en Immunologie et en Cancérologie (Montreal, Canada).

Yeast three-hybrid screening

A recently described three-hybrid screening protocol was chosen for our analyses (Hook et al., 2005). This method uses the yeast strain YBZ1 of genotype *MAT**a**, ura3-52, leu2-3, 112, his3-200, trp1-1, ade2, LYS2::*(*LexAop*)*-HIS3, ura3::* (*lexAop*)*-lacZ, LexA-MS2 coat* (*N55K*), which expresses a LexA (DNA-binding domain)-MS2 (RNA-binding domain) fusion protein. The RNA fusion plasmid pIIIA was designed to express a LBP 3′-UTR-MS2 RNA hybrid, and was constructed by cloning a PCR-amplified LBP 3′-UTR into the SmaI and SphI sites of the pIIIA MS2-2 plasmid. This plasmid carries the ADE2 and URA3 auxotrophic markers. The plasmids containing the activation domain fusion proteins were prepared previously as a cDNA library in the pAD-GAL4 vector (Stratagene, La Jolla, CA, USA; Bertomeu and Morse, 2004). This vector carries the LEU2 auxotrophic marker and an ampicillin resistance gene.

For screening, the YBZ1 yeast strain was transformed first with the pIIIA plasmid containing the LBP-MS2 fusion construct (Gietz and Woods, 2002) and maintained on SC-Ura selective media. This transformed strain was retransformed with the cDNA library and grown on SC-Leu-His selective media. Clones surviving after 1 week were grown in SC-Leu liquid culture, and any clones causing aggregation were discarded. Plasmids were purified from the remaining clones and used to transform XL-1 Blue MRF′ *E. coli* (Stratagene). Plasmids purified from the bacteria were retransformed into YBZ1 yeast containing the pIIIA plasmid with the LBP-MS2 fusion construct to confirm growth

on SC-Leu-His media. All clones allowing growth were identified by a BLAST search of public domain databases using the nucleic acid sequence.

Expression library screening in *E. coli*

XL-1 Blue MRF′ *E. coli* cells were transformed with a previously constructed cDNA library in the Stratagene lambda ZAP vector (Chaput et al., 2002) and plated at a phage density of 2×10^4 pfu on ten 150-mm Petri plates. Nitrocellulose membranes impregnated with 20 mM isopropyl β-D-thiogalactoside were then applied to the surface of the Petri plates and the bacteria were allowed to grow overnight at 37°C. The plaque lifts were washed four times in screening buffer (15 mM HEPES, pH 7.9, 50 mM KCl, 0.1% Ficoll, 0.1% polyvinyl pyrollidone, 0.01% Nonidet P-40, 0.1 mM $MnCl_2$, 0.1 mM $ZnCl_2$, 0.1 mM EDTA and 0.5 mM DTT in DEPC-treated water) then incubated for 2 h in screening buffer containing 50 μg/ml yeast RNA and a radiolabeled BglI RNA transcript as previously described (Sagesser et al., 1997). Non-specifically bound probe was removed by washing four times in screening buffer and the membranes were dried and exposed to film. Plaque purification for the second round of screening was performed as described by Sambrook et al. (1989).

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