The existence of a putative post-transcriptional regulatory element in 3′-UTR of drosophila antibacterial peptide diptericin's mRNA

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Abstract Antibacterial peptides' genes are rapidly and transiently expressed on immune stimulation, which is the characteristic of immediate early genes. It implies post-transcriptional regulation is an important pathway in antibacterial peptides' gene expression. In a search of putative post-transcriptional regulatory elements, we found a segment of an AU-rich sequence in 3′-untranslated region (UTR) of drosophila diptericin mRNA. 3′-UTR of diptericin mRNA can be specifically bound with Elav and this binding can be competed with the typical AU-rich element (ARE) of c-fos mRNA. These results suggest that the AU-rich sequence in the 3′-UTR of diptericin mRNA may be a *cis*-acting element and involved in post-transcriptional regulation.

Key words: Antibacterial peptide; Diptericin; Immediate early gene; Post-transcriptional regulation; *cis*-Acting element; Elav

1. Introduction

Insects possess an efficient immunity system against pathogens [1]. The induced synthesis of antibacterial peptides plays an important role in this system [2]. Antibacterial peptides' genes are rapidly and transiently expressed [3]. Some of the transcriptional regulatory mechanisms involved in induced expression of these genes are relatively well understood [4–6] and similar at a molecular level to those in mammalian acute phase responses [7]. The rapidity and transiency of antibacterial peptides' gene expression are the characteristics of immediate early genes [8], and many immediate early mRNAs are targeted for post-transcriptional regulation by virtue of *cis*-acting elements in their 3′-untranslated region (UTR) [9–13]. So this suggests that the post-transcriptional regulation may be an important pathway in the antibacterial peptides' gene expression.

Diptericins are inducible 9-kDa antibacterial peptides and the drosophila diptericin gene is strongly expressed 1–2 h after bacterial challenge [14,15]. Two decameric nuclear factor (NF)-κB-related sequences [16] and motifs similar to interferon-sensitive response elements from mammalian genes [17–19] are located upstream of the TATA-box in the diptericin promoter. In addition to transcriptional regulation, post-transcriptional regulation may contribute to the diptericin gene expression. However, no report on characterization of diptericin *cis*-acting elements involved in post-transcriptional regulation is present. In a search of putative *cis*-acting elements in drosophila antibacterial peptides' mRNA, we found a segment of an AU-rich sequence in 3′-UTR of diptericin mRNA, which is similar with AU-rich elements (AREs). AREs are typical *cis*-acting elements that have been identified in many mRNAs [20–23]. We anticipate that this AU-rich segment in diptericin mRNA may be a *cis*-acting element. In order to characterize this element, RNA binding protein Elav from drosophila [24] was used for RNA–protein interaction analysis, since Elav-like proteins can bind to ARE with high affinity and selectivity [25–28]. The biological relevance of these interactions has come to light when in vivo studies showed that HuR, a member of Elav-like proteins, participates in stabilization of the VEGF transcript [29].

2. Materials and methods

2.1. Cloning and construction of diptericin in vitro transcription plasmids

Total RNA was extracted from the wild-type Berlin flies using Trizol (Gibco BRL) and reverse transcribed with oligo(dT)18 primer. The following oligodeoxynucleotides were then used to amplify the diptericin gene: P_dptF, 5′-GGGAATTCGAGAACAACTGAGATGC-3′ and P_dptR, 5′-CTGGATCCGCTAGACTCGGATACC-3′. The following oligodeoxynucleotides were used to amplify the diptericin coding sequence with 5′-UTR: P_dptF and P_dpt-codR, 5′-GCGGATCCTTCGGAAATCTGTAGG-3′. The following oligodeoxynucleotides were used to amplify diptericin 3′-UTR: P_dpt-3utrF, 5′-CTGAATTCCACCTACAGATTTCCGA-3′ and P_dptR. All polymerase chain reaction (PCR) products were digested with *Bam*HI/*Eco*RI and subcloned into *Bam*HI/*Eco*RI-digested pGEM-3Z vector (Promega). The resultant plasmids were called pGEM-dpt, pGEM-dpt_cod and pGEM-dpt_3utr, respectively.

In vitro transcription plasmid pGEM-dptΔAU encoding the diptericin gene minus the AU-rich segment (sequence from residue 3 to 27 downstream of the diptericin stop codon) was constructed as follows: pGEM-dpt was used as the template and the oligodeoxynucleotides P_dpt-loss5, 5′-CTTCACCAAATATTATCTCGATTGG-3′ and P_dpt-loss3, 5′-GCTTAGAAATTCGGAAATCTGTAGGTGTATCC-3′ were used as primers for PCR; the PCR product was phosphate labeled using T4 kinase and ligated to itself using T4 DNA ligase.

2.2. Construction of a glutathione S-transferase (GST)-Elav fusion protein plasmid

A cDNA encoding residues 2–483 of Elav was generated using PCR with upstream primer P_elavF, 5′-GACCCGGGAATGGCCTTTAT-

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TATGGCAAATACCGG-3′ and downstream primer P_elavR, 5′-CTGAATTCCTACTTGGCTTTGTTGGTCTTGAAG-3′. The resultant product was digested with *Sma*I/*Eco*RI and subcloned into *Sma*I/*Eco*RI-digested pGEX-2T (Pharmacia). The resultant construct was called pGEX-Elav.

2.3. Purification of GST-Elav protein

0.5 ml overnight culture of *Escherichia coli* BL21, transformed with pGEX-Elav, was diluted in 50 ml Luria–Bertani (LB) medium and incubated at 37°C for 2 h. The culture was induced with isopropyl thiogalactose (IPTG) (0.04 mM). After 4 h of further growth at 30°C, *E. coli* cells were spun down and suspended in 5 ml buffer A (50 mM Tris (pH 8.0), 200 mM NaCl, 1 mM ethylenediamine tetraacetic acid (EDTA)). The cells were lysed by adding lysozyme to a final concentration of 0.2 mg/ml and Triton X-100 to 1%. The lysate was centrifuged at 12 000×*g* for 30 min. The resultant supernatant was mixed with 0.15 ml glutathione Sepharose 4B and incubated with gentle agitation at room temperature for 1 h. The suspension was centrifuged at 1000×*g* for 5 min. After washing the pellet three times with 1.5 ml buffer A, the bound protein was eluted with elution buffer (50 mM Tris (pH 8.0), 10 mM glutathione).

2.4. Preparation of RNA transcripts

Plasmids were digested with the appropriate restriction enzymes and transcribed using MAXIscript kit (Ambion). For the synthesis of biotin-labeled RNA, 50% ribocytidine triphosphate (rCTP) were replaced by biotin-14-CTP (Invitrogen). pAUFL [30] was linearized with *Hin*dIII and transcribed with T3 RNA polymerase, yielding a transcript (called AUFL) of 251 nucleotides of 3′-UTR of c-fos mRNA. pSP65Hγ [26] was linearized with *Sau*3AI and transcribed with SP6 RNA polymerase, yielding 165 nucleotides containing 80 nucleotides of the coding sequence and 85 nucleotides of 3′-UTR of human γ-globin. pGEM-dpt was linearized with *Bam*HI and transcribed with T7 RNA polymerase, yielding a transcript of 391 nucleotides of diptericin mRNA. pGEM-dpt_cod was linearized with *Bam*HI and transcribed with T7 RNA polymerase, yielding a transcript (called dpt_cod) of 327 nucleotides containing 314 nucleotides of the coding sequence and 13 nucleotides of 5′-UTR of diptericin mRNA. pGEM-dpt_3utr was linearized with *Bam*HI and transcribed with T7 RNA polymerase, yielding a transcript (called dpt_3utr) containing 57 nucleotides of 3′-UTR of diptericin mRNA. pGEM-dptΔAU was linearized with *Bam*HI and transcribed with T7 RNA polymerase, yielding a transcript of 366 nucleotides of diptericin mRNA minus the AU-rich segment (called dptΔAU).

A gene fragment (called dpt-mut) was amplified by PCR as follows: P_T7, 5′-GTAATACGACTCACTATAGGG-3′ and P_dpt-mut, 5′-CGGATACC AATCGAGATAATATTTGGTGAAGTTTTTTACA-ATGCGATATTTATGAAGC-3′ were used as primers and pGEM-dpt was used as the template. The amplified dpt-mut DNA fragment was transcribed with T7 RNA polymerase, yielding a transcript of 391 nucleotides of the dpt-mut RNA.

2.5. Gel retardation assay

Reaction mixtures (0.02 ml) contained 50 mM Tris (pH 7.0), 150 mM NaCl, 0.25 mg/ml tRNA, 0.25 mg/ml bovine serum albumin, RNA and protein as indicated. The mixture was incubated at 37°C for 10 min. Following incubation, 2 μl of a dye mixture (50% glycerol, 0.025% bromophenol blue, 0.025% xylene cyanol) was added. The reaction mixture was immediately loaded on a 1% agarose gel in TBE buffer and electrophoresed at 70 V for 45 min.

2.6. Detection of the biotin-labeled RNA and quantification of its optical density

The RNA was transferred from gel to nylon membrane Zeta-Probe (Bio-Rad) using capillary transfer [31]. After being exposed to 302 nm radiation on a ultraviolet (UV) transilluminator for 3 min, the membrane was incubated in the block solution (0.1 M Tris, pH 7.5, 0.1 M NaCl, 2 mM $MgCl_2$, 3% bovine serum albumin fraction V) at 30°C for 1 h and then incubated at 30°C with 0.85 μg/ml alkaline phosphatase-linked streptavidin (Promega) in 10 ml AP7.5 buffer (0.1 M Tris pH 7.5, 0.1 M NaCl, 2 mM $MgCl_2$) for 10 min. After being washed twice with AP7.5 buffer and once with AP9.5 buffer (0.1 M Tris pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$), the membrane was incubated with 2.5 mg nitroblue tetrazolium (NBT) and 1.25 mg 5-bromo-4-chloro-3-indolyl phosphate (BCIP) in 7.5 ml AP9.5 buffer at room temperature. After 30 min, TE was added to stop the reaction.

After the color development reaction of biotin-labeled RNA, the nylon membrane was scanned by a scanner. The optical density of each band was quantified using ImageQuant version 5.2 software (Molecular Dynamics). The integrated density of all the pixels in the area of each band was quantified and adjusted by a background subtraction of density in nested blank area of the same size. This value represents the integrated optical density of the band.

3. Results

3.1. The diptericin mRNA can form RNA–protein complex with Elav protein

In gel retardation assay, purified GST-Elav can convert the diptericin mRNA to a stable RNA–protein complex that migrates more slowly on agarose gel than diptericin mRNA alone (Fig. 1, lane 1 vs. lane 2). The complex formation is specific, since no reaction was observed with 3′-UTR of γ-globin mRNA (Fig. 1, lane 3 vs. lane 4).

3.2. The diptericin mRNA can be bound with Elav protein in a concentration-dependent pattern

The amount of the formed RNA–protein complex increased when the concentration of GST-Elav increased from 25 to 800 nM (Fig. 2A, lanes 3–8). No complex formation was observed with high concentrations (1000 nM) of purified GST (Fig. 2A, lane 1 vs. lane 2). Thus Elav specifically binds to the diptericin mRNA.

In order to make quantitative analysis on the interaction between diptericin mRNA and Elav, we need to estimate the quantity of biotin-labeled diptericin mRNA on the nylon membrane. We used a series of biotin-labeled diptericin mRNA samples whose quantities are 2.42, 7.26, 21.8, 65.4 and 196 fmol and measured their corresponding optical densities after electrophoresis. The optical density of biotin-labeled diptericin mRNA versus the logarithm of diptericin mRNA quantity reveals a straight line within this range. The diptericin mRNA quantity and optical densities measured in all experiments lie in this linear range. With this standard curve, the biotin-labeled diptericin mRNA quantity can be

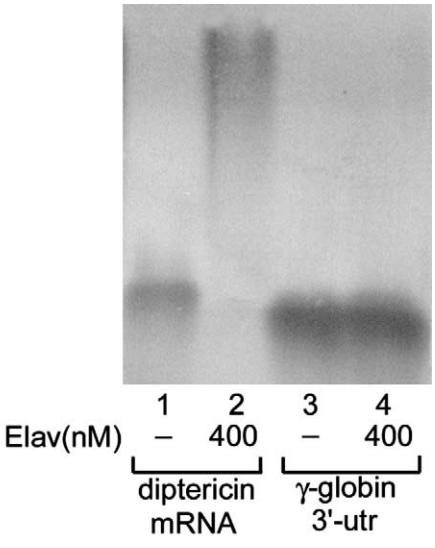

Fig. 1. The binding of diptericin mRNA to Elav. 5 nM biotin-labeled diptericin mRNA or 5 nM γ-globin mRNA (3′-UTR) was incubated with the indicated concentrations of Elav. Following incubation at 37°C for 10 min, the reaction mixture was resolved by gel electrophoresis on a 1% agarose gel.

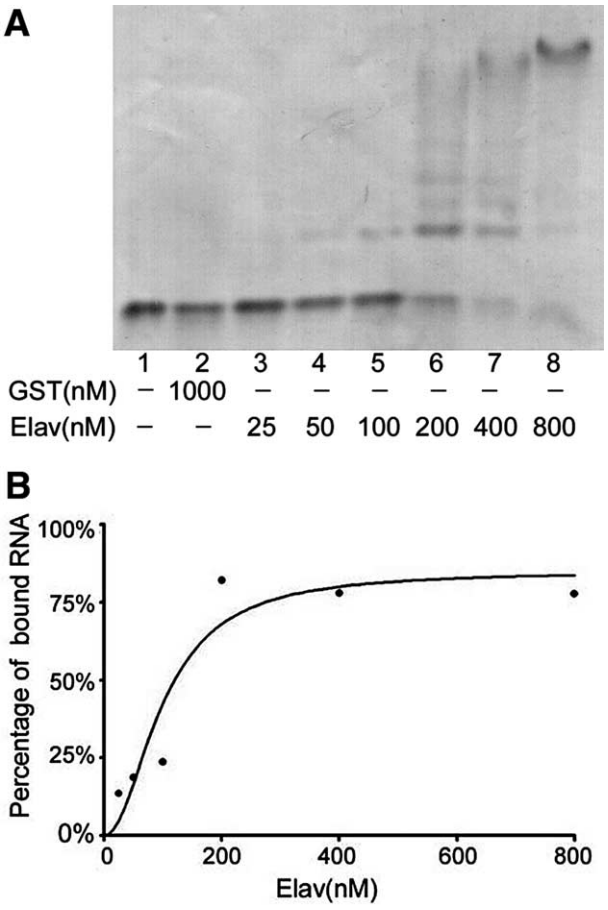

Fig. 2. The diptericin mRNA can be bound with Elav in a concentration-dependent pattern. A: 5 nM biotin-labeled diptericin mRNA was incubated with the indicated concentrations of GST or GST-Elav. Following incubation at 37°C for 10 min, the reaction mixture was resolved by gel electrophoresis on a 1% agarose gel. B: Plot of percentage of bound RNA versus Elav concentration.

calculated according to the optical density of biotin-labeled diptericin mRNA within the linear range.

We employ this quantitative method to measure and analyze lanes 3–8 of Fig. 2A and calculate the quantities of the unbound diptericin mRNA and bound diptericin mRNA (retarded). The relationship between percentage of bound diptericin mRNA and the concentration of Elav is shown in Fig. 2B, which is in accordance with S-shape binding curve. When Elav is about 130 nM, 50% mRNA was converted into RNA–protein complex. Thus the apparent dissociation constant (K_d) of Elav–diptericin interaction is about 1.3×10^2 nM.

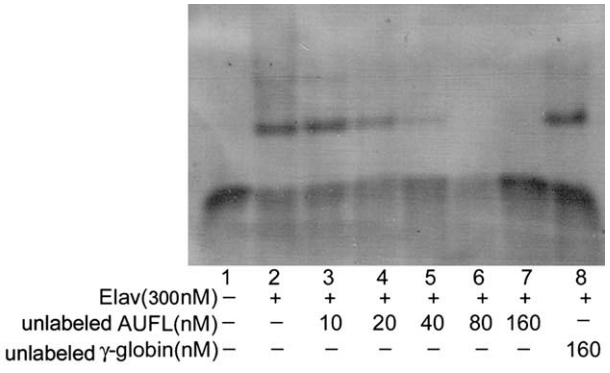

Fig. 3. The Elav–diptericin complex is displaced by AUFL RNA. 5 nM biotin-labeled diptericin mRNA was incubated with 300 nM Elav protein. The indicated unlabeled AUFL RNA or unlabeled γ-globin mRNA (irrelevant RNA) was added into the reaction mixture. After 10 min of incubation at 37°C, the reaction mixture was resolved on 1% agarose gel.

3.3. *Diptericin mRNA binding to Elav can be competed by AUFL RNA*

We next determined whether the Elav–diptericin interaction

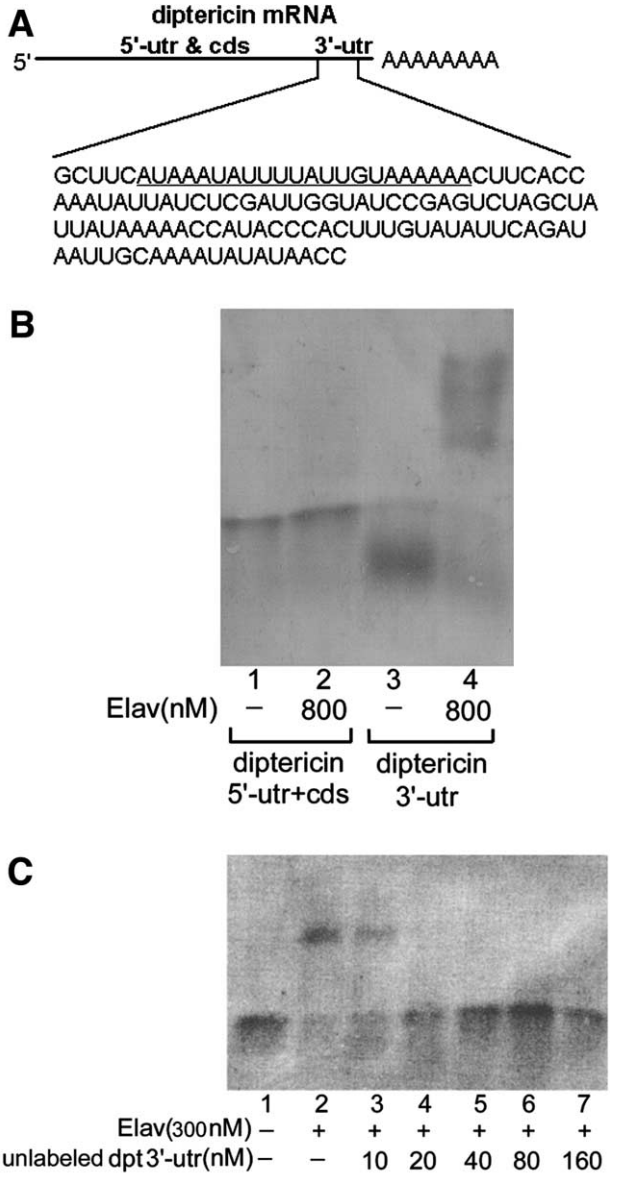

Fig. 4. Elav binds to the 3′-UTR of diptericin mRNA. A: The sequence of 3′-UTR of the diptericin mRNA. The segment of AU-rich sequence is underlined. B: 5 nM biotin-labeled diptericin 5′-UTR with coding sequence or 5 nM diptericin 3′-UTR was incubated with the indicated concentrations of Elav. Following incubation at 37°C for 10 min, the reaction mixture was resolved by gel electrophoresis on a 1% agarose gel. C: 5 nM biotin-labeled diptericin mRNA was incubated with 300 nM Elav protein. The indicated unlabeled diptericin 3′-UTR RNA was added into the reaction mixture. After 10 min of incubation at 37°C, the reaction mixture was resolved on 1% agarose gel.

has similarity with the typical interaction between ARE and Elav-like proteins. The competition experiment was carried out to check whether diptericin mRNA would be displaced by AUFL RNA for bound with Elav. As shown in Fig. 3, from lanes 2 to 5, Elav–diptericin complex formation decreased when the AUFL RNA quantity increased. When 80 nM or more AUFL RNA was added, no Elav–diptericin formation existed (Fig. 3, lanes 6, 7). Elav–diptericin complex can be eliminated by AUFL RNA, and no inhibition was observed when 160 nM γ-globin mRNA was added (Fig. 3, lane 8). So AUFL RNA can effectively compete with diptericin mRNA for Elav binding; diptericin mRNA and ARE may have similarity in the binding site or interaction pattern with Elav.

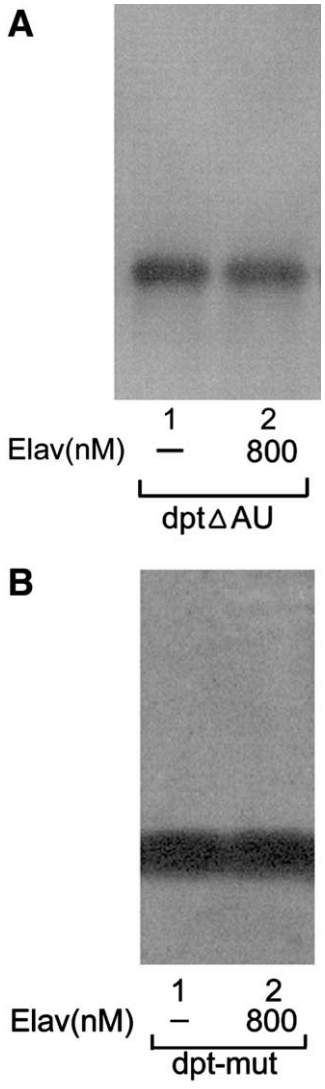

Fig. 5. Mutated 3′-UTR of diptericin mRNA could not bind to Elav. A: 5 nM biotin-labeled dptΔAU RNA was incubated with the indicated concentrations of Elav. Following incubation at 37°C for 10 min, the reaction mixture was resolved by gel electrophoresis on a 1% agarose gel. B: 5 nM biotin-labeled dpt-mut RNA was incubated with the indicated concentrations of Elav. Following incubation at 37°C for 10 min, the reaction mixture was resolved by gel electrophoresis on a 1% agarose gel.

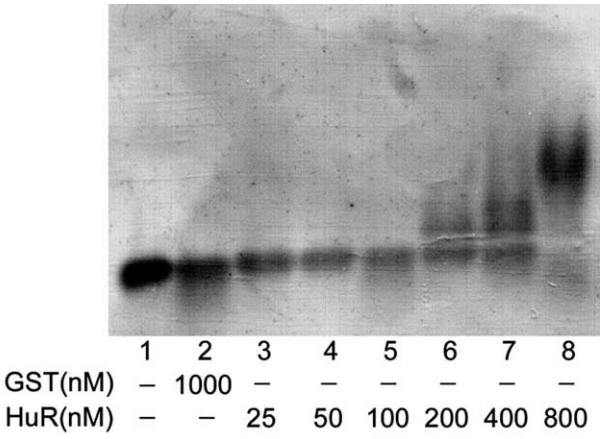

Fig. 6. Complex formation of diptericin mRNA with Elav-like protein HuR. 5 nM biotin-labeled diptericin mRNA was incubated with the indicated concentrations of GST or GST-HuR. Following incubation at 37°C for 10 min, the reaction mixture was resolved by gel electrophoresis on a 1% agarose gel.

3.4. *Elav binds to the 3′-UTR of diptericin mRNA*

We next located the Elav binding part on diptericin mRNA in more detail. Two transcripts were synthesized: dpt_cod which contained the coding sequence with 5′-UTR and dpt_3utr which contained 3′-UTR of diptericin mRNA (sequence shown in Fig. 4A). Gel retardation reveals that Elav binds to the 3′-UTR of diptericin mRNA (Fig. 4B, lanes 3, 4) while no binding was observed with the coding sequence with 5′-UTR in the presence of 800 nM Elav (Fig. 4B, lanes 1, 2). And as shown in Fig. 4C, diptericin 3′-UTR can effectively compete with diptericin mRNA for Elav binding.

3.5. *Mutational analysis of AREs in 3′-UTR of diptericin mRNA*

Transcript dptΔAU has a deletion of the AU-rich segment (sequence from residues 3 to 27 downstream of the diptericin stop codon), and transcript dpt-mut RNA differs from diptericin mRNA in the AU-rich region from residues 13 to 16 downstream of the diptericin stop codon: UCGC for dpt-mut and UUUU for diptericin. Agarose gel retardation revealed that no complex formation was observed with dptΔAU RNA (Fig. 5A, lane 1 vs. lane 2) or dpt-mut RNA (Fig. 5B, lane 1 vs. lane 2) at 800 nM Elav.

3.6. *Complex formation of diptericin mRNA with ARE binding protein HuR*

We next determined whether the diptericin mRNA could be bound with HuR, an ARE binding protein. As shown in Fig. 6, the amount of the formed RNA–protein complex increased when the concentration of GST-HuR increased from 25 to 800 nM (Fig. 6, lanes 3–8). No complex formation was observed with high concentrations (1000 nM) of purified GST (Fig. 6, lane 1 vs. lane 2). Thus the diptericin mRNA can be bound with ARE binding protein HuR.

4. Discussion

In this paper, our results demonstrate that 3′-UTR of dip-

tericin mRNA can be bound by Elav with specificity and this binding can be effectively competed by AUFL (3′-UTR of c-fos mRNA). It suggests that 3′-UTR of the diptericin mRNA contains a *cis*-acting element, which is very likely to play a role in the post-transcriptional regulation. The post-transcriptional regulation of the diptericin mRNA can be a very effective molecular adaptation that enables the cell to maintain a translatable transcript without embarking upon transcription and respond rapidly to extrinsic or intrinsic immunity stimuli.

We found out the similarity between the Elav–diptericin interaction and the Elav–AUFL interaction. The Elav–diptericin interaction can be competed by AUFL. The ARE of AUFL has been functionally defined by deletion analysis. Moreover, HuR [25], which specially binds to the ARE of AUFL, can form RNA–protein complex with diptericin mRNA. The apparent K_d of HuR with diptericin is about 210 nM which is comparable with but slightly higher than that of Elav. Since HuR is an ARE binding protein from human, it shows typical binding feature and capability to ARE containing mRNA. According to these results, we anticipated that Elav binds to ARE of the diptericin 3′-UTR.

It was reported that the AU-rich sequence in the 3′-UTR of sarcotoxin II (a kind of antibacterial peptide from Sarcophaga) mRNA can cause instability of mRNA [32] and a protein that can bind to this sequence was purified from Sarcophaga pupal extract. The molecular mass of this protein was estimated to be 39 kDa. Moreover, this protein has thiolase activity [33]. However, according to the data from this paper, this RNA binding protein showed little binding specificity with the AU-rich sequence; its affinity with tRNA or other RNA was similar to the AU-rich sequence. This protein is 'broad spectrum' regarding its ability of binding RNA. The RNA–protein complex was observed in the absence of tRNA as a non-specific competitor. In our results we use an excess amount of tRNA as non-specific competitor to assure the specificity of ARE–protein interaction. We assume that Elav-like proteins and thiolase should be different in the specificity and functionality of interacting with RNAs. They play different roles in RNA–protein interaction.

Further study on the mode of Elav–diptericin interaction is needed. The binding site of Elav within the diptericin 3′-UTR will be further located, and mutations of the diptericin ARE can be synthesized to check out the sequence motif required for binding. Moreover, the diptericin ARE or its mutations can be inserted downstream of the reporter gene to examine the influence and effectiveness on the post-transcriptional regulation of the reporter gene in vivo. These studies will help to understand the critical structural features and function of the ARE that may be involved in the ARE-directed diptericin gene regulation at the post-transcriptional level, such as RNA processing, translation and RNA degradation. Since in vivo studies show that HuR, a human Elav-like protein, regulates the stability of ARE containing mRNA [29], it is likely that 3′-UTR regulatory element may affect the diptericin mRNA stability by interaction with Elav in the post-transcriptional regulation.

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