A Multiprotein Complex That Mediates Translational Enhancement in Drosophila

Received for publication, August 1, 2007, and in revised form, September 20, 2007. Published, JBC Papers in Press, September 21, 2007, DOI 10.1074/jbc.M706363200

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Modulating the efficiency of translation plays an important role in a wide variety of cellular processes and is often mediated by trans-acting factors that interact with cis-acting sequences within the mRNA. Here we show that a cis-acting element, the Hsp83 degradation element (HDE), within the 3′-untranslated region of the Drosophila Hsp83 mRNA functions as a translational enhancer. We show that this element is bound by a multiprotein complex, and we identify components using a novel affinity-based method called tandem RNA affinity purification tagging. Three proteins (DDP1, Hrp48, and poly(A)-binding protein) are components of the HDE-binding complex and function in translational enhancement. Our data support a model whereby the HDE is composed of several cis-acting subelements that represent binding sites for trans-acting factors, and the combined action of these trans-acting factors underlies the ability of the HDE to stimulate translation.

Regulated translation plays an essential role in a wide variety of cellular processes. Although translational regulation is likely to function in virtually all eukaryotic cell types, these controls are particularly important in cells where transcriptional regulation is not an option. For example, maturation of mammalian red blood cells occurs after the nucleus is extruded and thus is driven by previously synthesized mRNAs. Similarly, in early metazoan embryos, the zygotic genome is transcriptionally silent, and maternally deposited mRNAs control early development. Translational regulation is also very important in large cells, such as neurons, where correct spatial and temporal expression of proteins cannot be achieved through transcriptional controls alone.

Regulation of specific transcripts is often mediated by cis-acting elements within the 5′- or 3′-untranslated region (UTR) of an mRNA (1). These elements can act as binding sites for trans-acting factors that either directly or indirectly contact the translational machinery. Some of the best characterized mechanisms involve repressing protein expression, but mechanisms that stimulate protein production also exist. In principle, these positively acting events can be divided into two different classes. The first acts on transcripts that are translationally repressed. Translational stimulation is achieved by blocking the repressive mechanism (i.e. enhancement results from relief of repression). The second class of stimulatory events acts on mRNAs that are not repressed. In these cases, an mRNA is better able to recruit the basic translation machinery and is, therefore, expressed at a higher level. This latter type of mechanism is likely to be particularly important when a component of the translation machinery is limited and, consequently, transcripts must compete for access to the translational apparatus.

Many viral RNAs contain elements that aid in preferential expression in infected cells. For example, the 5′-UTR of the tobacco mosaic virus RNA contains a cis-acting element, Ω, that is bound by Hsp101, which in turn recruits the eIF4F translation initiation complex to the RNA to enhance its translation (2, 3). Examples of cellular mRNAs that carry cis-acting translational enhancers have also been described, but, for the most part, the molecular mechanisms involved are poorly understood. One well characterized example involves metazoan histone mRNAs, which terminate in a stem-loop structure and do not carry a poly(A) tail. The stem-loop is bound by the stem-loop-binding protein which interacts directly with the translation initiation factors, eIF4G and eIF3 (4). Stem-loop-binding protein has an analogous function to poly(A)-binding protein (PABP); both bind to the 3′ end of the transcript and interact with the translation initiation machinery to facilitate protein translation.

The abbreviations used are: UTR, untranslated region; PABP, poly(A)-binding protein; HDE, Hsp83 degradation element; TRAP, tandem RNA affinity purification; hnRNP, heterogeneous nuclear ribonucleoprotein.
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synthesis (1). PABP can also bind to cis-acting sequences that are outside the poly(A) tail. The mammalian YB-1 RNA, for example, carries a PABP binding sequence within its 3′-UTR (5). By binding to this element, PABP enhances translation of YB-1 RNA in a poly(A) tail-independent manner.

Previous work provided indirect evidence that the Drosophila Hsp83 mRNA contains a translational enhancer in its 3′-UTR that functions in the early embryo (6). The Hsp83 transcript is regulated through multiple mechanisms. The RNA is localized to the posterior of the embryo by selective degradation of the transcript in the bulk cytoplasm of the embryo, whereas transcripts present in the germ plasm at the posterior are protected. A region of the 3′-UTR, termed the Hsp83 degradation element (HDE), was originally identified through its ability to direct the degradation of transgenic mRNAs. Expression of one of the mRNAs results in embryonic defects, which were enhanced when the HDE is present in the reporter mRNA 3′-UTR, suggesting that the HDE might stimulate translation.

To obtain direct evidence that the HDE functions as a translational enhancer and to dissect the molecular mechanisms involved, we developed a microinjection-based assay that recapitulates HDE-mediated translational enhancement in Drosophila embryos. Deletion analysis demonstrated that the HDE contains multiple subelements capable of mediating translational enhancement. To identify trans-acting factors that act through the HDE, we developed a widely applicable method to identify proteins that bind to RNA sequences of interest, which we call tandem RNA affinity purification (TRAP) tagging. Using several approaches, including TRAP tagging, we identified three proteins (DDP1, Hrp48, and PABP) that bind to the HDE as part of a protein complex. We show that both DDP1 and Hrp48 function in translational enhancement. Although previous work indicated that Hrp48 functions as a translational repressor, our data suggest that Hrp48 is a bifunctional modulator of translation and that the ability of Hrp48 to act as a repressor or activator is regulated by other proteins that are recruited to the target mRNA. We propose that the HDE is a modular element composed of several cis-acting sequences representing binding sites for various factors that regulate translation. Thus, HDE-mediated translational enhancement is a read-out of the combined action of its associated trans-acting factors.

**EXPERIMENTAL PROCEDURES**

**RNA Injection Assay**—Luciferase RNAs were generated as described previously (7). Injection experiments were performed as described by Nelson et al. (8) with the following modifications. Wild-type embryos were collected from w1118 mothers, and hrp48 germ line clones were generated for hrp4802647 and hrp4802307 mutant alleles (9) using established techniques (10, 11). Firefly luciferase and Renilla luciferase RNAs were injected at a concentration of 200 and 50 ng/μl, respectively. Injected embryos were harvested 3.0–3.5 h after egg laying. Translational enhancement for RNAs bearing 3′-UTR inserts were expressed as its corrected value divided by the corrected value from firefly RNA carrying no insert. For example, the translational enhancement of luc-HDE-(1–97) RNA = (firefly activity of luc-HDE-(1–97)/Renilla activity)/(firefly activity of luciferase RNA carrying no insert/Renilla activity).

**Embryo Extract Preparation**—All experiments employed extract prepared by collecting embryos 0–2 h after egg laying from w1118 animals. Embryos were disrupted in a minimal volume of 150 mM KCl, 20 mM Hepes-KOH (pH 7.4), 1 mM MgCl2, 1 mM 4-(2-aminoethyl)-benzenesulfon fluoride, 2 mM benzamidine, 2 μg/μl leupeptin, 2 μg/μl pepstatin, and 1 mM dithiothreitol. After centrifugation, the supernatant was supplemented with glycerol to a final concentration of 10% (v/v) and stored at −80 °C.

**RNA-binding Assays**—Gel electromobility shift reactions contained 10 μg of tRNA, 0.1 μg of heparin, 1 μl of RNasin (Promega), 4 μl of 150 mM KCl, 20 mM Hepes-KOH, pH 7.4, 1 mM MgCl2, 20% glycerol, 2.5 mM dithiothreitol, 1 × 106 cpm probe RNA, and 1 μl of embryo extract. Reactions were incubated for 10 min at room temperature and run on a 4% native polyacrylamide gel in 0.5 × TBE at 4 °C. UV cross-linking assays employed 2.5 × 106 cpm of probe RNA, 0.5 μl of embryo extract, 10 μg of tRNA, 10 μg of heparin, 4.5 μl of 40 mM KCl, 20 mM Hepes-KOH, pH 7.4, 43% glycerol, all in a final volume of 10 μl. Reactions were incubated for 10 min at room temperature and UV-irradiated as previously described (12), followed by the addition of 5 μg of RNase A (Fermentas) and incubation for 15 min at room temperature. Proteins were then electrophoresed on an 8% SDS-polyacrylamide gel, and labeled proteins were visualized by phosphorimaging. Where indicated, gel shift reactions were immunoprecipitated with anti-DDP1 antibody, which was raised in guinea pigs by Cedarlane Laboratories Ltd. against residues 1–650 of the DDP1 protein, anti-PABP antibody (13), anti-Hrp48 antibody (9), or anti-Smaug antibody (7). For gel shift assays that were to be followed by UV cross-linking, gel shift reactions were performed, and the shifted complex was cross-linked as described above. Gel slices were incubated at 37 °C for 30 min in 150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 0.1 mg/ml RNase A (Fermentas). Nonidet P-40, sodium deoxycholate, and SDS were then added to final concentrations of 1, 1, and 0.1%, respectively, and incubated at 30 °C overnight.

**TRAP**—TRAP-tagged RNAs, containing a 5′ S1 aptamer (14, 15), followed by two MS2 coat protein binding sites (16) and the bait sequence of interest, were generated via in vitro transcription using T7 RNA polymerase. 40 μg of TRAP-tagged RNA was mixed with 40 μl of streptavidin resin (Sigma) in 100 μl of TBP (150 mM KCl, 20 mM Hepes-KOH, pH 7.4, 1 mM MgCl2, 0.1% Triton X-100, and 10% glycerol) for 1 h at 4 °C. The resin was washed extensively with TBP. Embryo extract was supplemented with Triton X-100 to 0.1% and 5 μg of soluble Avidin (Sigma)/mg of protein and then held on ice for 20 min. The extract was centrifuged, and the supernatant was removed and mixed with RNA/streptavidin resin for 2 h at 4 °C. After extensive washing with TBP, RNA was eluted with 100 μl of TBP plus 5 mM biotin. The eluate was mixed for 2 h at 4 °C with 20 μl of glutathione resin carrying 2 mg/ml GST-MS2 coat protein. The resin was then washed extensively with TBP, and proteins were eluted in 35 μl of TBP + 0.25 μg of RNase A/T1 mix (2 mg/ml and 5000 units/ml, respectively) (Fermentas) for 1 h at 4 °C. Proteins were separated by SDS-PAGE and visualized with sil-
RESULTS

The HDE Functions as a Translational Enhancer in Drosophila Embryos—To prove that the HDE functions as a translational enhancer and to investigate the mechanism involved, we developed a convenient microinjection-based assay that recapitulates translational enhancement in a manner similar to assays previously developed to study translational repression (8, 17). We reasoned that insertion of the HDE into a reporter RNA would enhance its translation relative to that of the reporter lacking the HDE. The capped reporter RNAs contained the firefly luciferase open reading frame and a 30-nucleotide-long poly(A) tail. To control for variables such as the amount of RNA injected and the efficiency of embryo homogenization, an unregulated RNA encoding Renilla luciferase was co-injected with each firefly luciferase reporter RNA. The two luciferase enzymes have different substrate requirements, allowing us to distinguish, quantify, and compare the activities of each enzyme in the same sample. The level of firefly activity was thus normalized to that of the Renilla enzyme.

When the reporter RNA carried an intact 97-nucleotide-long HDE (luc-HDE-(1–97)), it directed a 14-fold increase in normalized luciferase expression relative to reporter RNA carrying no insert (Fig. 1). Nucleotides 1–250 and 347–404 of the Hsp83 3′-UTR, which account for the rest of the 3′UTR, failed to enhance luciferase expression, mapping translational enhancer function uniquely to the HDE.

To confirm that the increase in luciferase expression occurred at the level of translation, semiquantitative RT-PCR was performed on RNA recovered from injected embryos to assess the stability of each firefly reporter RNA relative to the control, Renilla RNA. The ratio of firefly luciferase/Renilla RNA from four independent injection experiments was 0.34 ± 0.11, whereas the ratio for luc-HDE-(1–97)/Renilla RNA for three independent experiments was 0.27 ± 0.15. These values are not significantly different as judged by Student’s t test (p = 0.5), and thus the HDE does not have a significant effect on the stability of the reporter RNA. We conclude that the increased luciferase expression observed for the luc-HDE-(1–97) RNA occurs at the level of translation. Since our microinjection assay recapitulates only the translational enhancer function of the HDE, we were ideally positioned to specifically study translational enhancement.

The HDE Can Be Subdivided into Multiple Translational Regulatory Elements—To map regions within the HDE required for translational enhancement, the effects of various deletions on the ability of the HDE to stimulate translation were assessed using the microinjection assay (Fig. 1). Deletion of the first 25 nucleotides of the HDE gave a 35-fold enhancement of luciferase activity, more than twice that generated by the intact HDE. This suggests the presence of a repressor element in the first 25 nucleotides of the HDE. Deletion of the last 25 nucleotides of the HDE, on the other hand, reduced translational enhancement by 80% (from 14- to 2.8-fold), suggesting the presence of positively acting sequences within this region. Simultaneous deletion of the first 25 nucleotides and the last 25 nucleotides gave 4.3-fold enhancement, whereas fusion of the first 25 nucleotides directly to the last 25 nucleotides gave a 6.4-fold enhancement. Given the presence of a repressive element within the first 25 nucleotides, it was possible that the last 25 nucleotides on their own might stimulate translation above levels seen for the fusion of the first 25 to the last 25 nucleotides. This is not, however, the case, since the last 25 nucleotides alone enhanced expression over the empty reporter by only 4.0-fold.

In summary, the HDE contains multiple subelements that are able to modulate translation, including one that can dampen HDE-mediated enhancement and several that contribute positively. The net effect of the HDE thus represents the combined action of all of these elements.

The HDE Is Bound by a Single Protein Complex—The existence of multiple subelements within the HDE suggests that several trans-acting factors interact with the HDE and that the combined action of all of these factors, both stimulatory and inhibitory in function, is responsible for the magnitude of translational stimulation. To identify these trans-acting factors, we performed an electromobility shift assay to detect embryonic

FIGURE 1. The HDE mediates translational enhancement in injected Drosophila embryos. A, Drosophila embryos derived from wild-type mothers were injected with control Renilla luciferase RNA and firefly luciferase RNAs carrying the indicated inserts. Injected embryos were aged, and firefly and Renilla enzyme levels were assayed in embryo extracts. After correcting the levels of firefly enzyme activity using the levels of Renilla activity as a control, translational enhancement was calculated as described under “Experimental Procedures.” Results are the average of at least four independent experiments, and error bars indicate the S.D. B, schematic representation of the different HDE constructs, the associated -fold translational enhancement, and the results of t tests comparing the translation of each RNA construct to that carrying no insert. Note that translation of reporter RNAs carrying nucleotides 1–250 and 347–404 of the Hsp83 3′-UTR were not significantly different from the RNA carrying no insert, as judged by a t test (p = 0.34 and 0.36, respectively).
Using crude embryo extract and radiolabeled HDE RNA, followed by immunoprecipitation with either an anti-DDP1 antibody or nonimmune serum (Fig. 2C). The UV cross-linked protein that migrates at 170 kDa is specifically immunoprecipitated by the anti-DDP1 antibody but not by nonimmune serum. These results demonstrate that DDP1 is an HDE-binding protein and that DDP1 contacts the RNA directly.

Hrp48 Interacts with the HDE—To facilitate the identification of additional HDE-binding proteins, we attempted to more precisely map their binding sites within the HDE, reasoning that smaller RNAs would be more useful in the TRAP-tagging method described below. To carry out this mapping, we used 25-nucleotide-long overlapping fragments of the HDE as probes in UV cross-linking reactions. Only the triplet at 55 kDa was able to bind efficiently to these small RNA probes (supplemental Fig. 2). Although the upper band bound to each probe, the lower two bands only bound to sequences within the 5’ and 3’ 25 nucleotides of the HDE.

To identify the 55-kDa HDE-binding proteins, we developed an affinity-based purification method that we refer to as “TRAP tagging.” The TRAP protocol involves generation via in vitro transcription of an RNA that carries the sequence of interest along with two RNA affinity tags. The RNA is incubated with extract, and the RNA along with bound proteins is purified via the tags. The first tag used in this purification is an S1 aptamer that binds streptavidin resin and may be specifically eluted by soluble biotin (14, 15). This gentle elution preserves binding of protein to the RNA, allowing for a second round of purification via the second affinity tag, which is composed of two tandemly arrayed hairpins that bind to the bacteriophage MS2 coat protein (16). The RNA-protein complexes eluted from streptavidin resin are captured on glutathione S-transferase-MS2 coat protein fusion immobilized on glutathione resin. Bound proteins are then eluted by treatment with RNases.

Our bait RNA for TRAP tagging carried three copies of HDE nucleotides 73–97. Three copies of HDE nucleotides 49–72 were used as the negative control, since this region showed little binding to the 55-kDa proteins (supplemental Fig. 2). Proteins purified via the TRAP tag method were separated via SDS-PAGE and visualized by silver staining (Fig. 3A). A band that specifically co-purified with nucleotides 73–97 of the HDE and migrated at ~55 kDa was excised from the gel and identified as the Drosophila heterogeneous nuclear ribonucleoprotein (hnRNP), Hrp48, using mass spectrometry. Hrp48 is a member of the hnRNP A/B family of proteins that has previously been shown to function in RNA splice site selection, RNA localization, and translational repression (9, 22–25).

To confirm that Hrp48 is the 55-kDa HDE-binding activity, we performed a UV cross-linking reaction with radiolabeled HDE RNA followed by immunoprecipitation with an anti-Hrp48 antibody. The doublet at 55 kDa was immunoprecipitated by the anti-Hrp48 antibody but not by nonimmune serum (Fig. 3B), indicating that the 55-kDa HDE binding activity is indeed Hrp48.

PABP Is an HDE-binding Protein—Our initial UV cross-linking experiments indicated that proteins additional to DDP1 and Hrp48 bind directly to the HDE (Fig. 2B). To identify additional proteins that are capable of interacting with the intact HDE. We observed a single shifted band, suggesting that a single protein or a single complex of proteins interacts with the HDE (Fig. 2A). This complex binds specifically, since excess unlabeled HDE RNA competed efficiently for binding, whereas an unlabeled, irrelevant RNA did not. Interestingly, the inclusion of different amounts of specific competitor revealed distinct shifted complexes. This suggests that the complex formed in the absence of competitor represents multiple proteins with different affinities for the HDE and hence different competition profiles. Additional evidence, presented below, indicates that the HDE is indeed bound by a multiprotein complex.

We next employed a UV cross-linking assay to detect proteins that directly contact the HDE (Fig. 2B). Several of the labeled proteins, including species at 170, 110, and 70 kDa and three bands at ~55 kDa, bound specifically, since unlabeled HDE RNA competed away their binding more efficiently than did an irrelevant RNA. These HDE-binding proteins are candidate mediators of translational enhancement in early embryos.

DDP1 Interacts with the HDE—Biochemical purification of the 170-kDa HDE-binding protein allowed its identification via mass spectrometry as the Drosophila dodeca-satellite protein 1 (supplemental Fig. 1). DDP1 contains 15 “K-homology” domains (18) and belongs to a highly conserved family of proteins collectively called “vigilins,” several of which are known to function as post-transcriptional regulators (19–21).

To confirm the identification of DDP1 as the 170-kDa HDE-binding protein, we performed a UV cross-linking reaction using radiolabeled HDE RNA and embryo extract (Fig. 2A) was subjected to immunoprecipitation (IP) with anti-DDP1 antibody or nonimmune serum.
HDE-binding proteins, we again used TRAP tagging. UV cross-linking reactions with fragments of the HDE indicated that most of the HDE-binding proteins require longer sequences to bind to the RNA. Therefore, we used TRAP-tagged full-length HDE as bait in this particular purification.

We found that several proteins co-purify with TRAP-tagged full-length HDE but not with an irrelevant negative control RNA of similar length (Fig. 3C). One of these proteins was identified as PABP. An anti-PABP antibody, but not nonimmune serum, specifically immunoprecipitated the 72-kDa protein present in UV cross-linked material, confirming the identification (Fig. 3D).

We note that PABP co-purified with the full-length HDE, that Hrp48 co-purified with the TRAP-tagged 3x (73–97) subelement but not TRAP-tagged full-length HDE, and that DDP1 co-purified with neither bait but was, in fact, purified by more conventional methods. One possible interpretation of these data is that the interaction of DDP1 and Hrp48 proteins is not sufficiently stable for them to co-purify with the full-length HDE during TRAP purification. If so, our results highlight the utility of using both multimerized subelements and the full-length element as bait.

**DDP1, Hrp48, and PABP Are Components of the HDE-binding Complex**—Having shown that DDP1, Hrp48, and PABP each bind directly to the HDE and that there is a single HDE-binding complex (Fig. 2A), we next investigated whether all three proteins are present in the complex and therefore could function together in translational enhancement. To do this, we performed a gel mobility shift assay with radiolabeled HDE probe, excised the shifted complex, and exposed the gel slice to UV radiation to cross-link HDE-binding proteins to the probe. Proteins were eluted from the gel in the presence of RNase A, the eluate was subjected to immunoprecipitation with the appropriate antibody or nonimmune serum, and radiolabeled immunoprecipitated protein was detected via phosphorimaging following SDS-PAGE. Labeled DDP1, Hrp48, and PABP were each detected in the eluate from the complex, whereas another RNA-binding protein, Smaug, which does not interact with the HDE (data not shown), was not (Fig. 4). These results suggest that that DDP1, Hrp48, and PABP interact directly and simultaneously with the HDE to form a single RNP complex.

**DDP1 Functions in HDE-mediated Translational Enhancement**—Although a role for PABP in translational stimulation is well documented, the same cannot be said for DDP1 or Hrp48. We therefore decided to focus our efforts on establishing roles for DDP1 and Hrp48 in HDE function. We were unable to obtain mutations in the HDE that specifically blocked binding of only one of these proteins and so could not use mutation of their binding sites within the HDE to assess their individual roles. For DDP1, a genetic approach was not feasible, since we found that females expressing significantly reduced levels of DDP1 lay fragile eggs that are not suitable for injection. As an alternative approach to assessing the role of DDP1 in translational enhancement, we asked what effect recruitment of DDP1 to a reporter mRNA would have on its translation. The *Xenopus* DDP1 homolog, Vigilin, has been shown to bind to RNAs greater than 55 nucleotides long carrying repeats of the sequence A_{15}CU (26), and a single match to this consensus was found within the HDE. A 62-nucleotide-long RNA carrying three tandem copies of this sequence, AAAAAACU, was used as a probe in a UV cross-linking reaction followed by immunoprecipitation with an anti-DDP1 antibody or nonimmune serum. The anti-DDP1 antibody specifically immunoprecipitated the 170-kDa DDP1 band, whereas nonimmune serum did not (supplemental Fig. 3A). An unlabeled version of the RNA

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**FIGURE 3.** TRAP tagging identifies Hrp48 and PABP as HDE-binding proteins. A, TRAP tag purification was carried out using RNAs carrying three copies of nucleotides 73–97 or 49–72 of the HDE. Proteins eluted in the final step of the purification were resolved via SDS-PAGE and stained with silver. The ~50-kDa protein that specifically purified with RNA carrying nucleotides 73–97 was identified as Hrp48. B, a UV cross-linking reaction using radiolabeled HDE RNA and embryo extract (starting material) was subjected to immunoprecipitation (IP) with anti-Hrp48 antibody or nonimmune serum. C, TRAP tag purification was carried out using RNAs carrying the intact HDE or a similarly sized fragment of the firefly luciferase open reading frame, and proteins eluted in the final step of the purification were resolved via SDS-PAGE and stained with silver. The ~60-kDa protein that specifically purified with RNA carrying the HDE was identified as PABP. D, a UV cross-linking reaction using radiolabeled HDE RNA and embryo extract (starting material) was subjected to immunoprecipitation with anti-PABP antibody or nonimmune serum.
carrying these three repeats was able to compete for binding to DDP1 in a UV cross-linking assay. In contrast, mutation of the C residue to a G within each repeat prevented an unlabelled RNA from competing for DDP1 binding (supplemental Fig. 3) but had no effect on binding of other proteins, arguing that any difference in the function of the wild-type and mutant sequences reflects a specific difference in DDP1 binding.

Firefly luciferase reporter RNAs were then generated carrying either the wild-type or mutant DDP1-binding sequence inserted into the firefly luciferase 3′-UTR and injected into embryos. The wild-type element enhanced translation 3.5-fold relative to firefly luciferase RNA carrying no insert, and the mutant element reduced translational enhancement by more than 50% (Fig. 5). These results suggest that DDP1 by itself directs modest translational enhancement and that additional HDE sequences and their cognate binding proteins (such as Hrp48 and PABP) are required for high level enhancement. It is also possible that additional, unknown proteins interact with and contribute to the ability of the synthetic DDP1-binding sequence to stimulate translation.

**Hrp48 Functions in HDE-mediated Translational Enhancement**—Next we analyzed the role of Hrp48 in HDE-mediated translational enhancement using our microinjection assay in embryos that express reduced amounts of functional Hrp48. 

Microinjection experiments showed that the intact HDE stimulated translation in these mutant embryos to the same extent as in wild type (data not shown). We reasoned that this might have resulted from the fact that the activity of the HDE reflects the combined action of several proteins that interact with the HDE and that a partial loss of only Hrp48 would thus not have a significant effect. This hypothesis is consistent with the fact that subregions within the HDE were each able to mediate some level of translational enhancement (Fig. 1). We reasoned that a mutant version of the HDE that is impaired in its ability to stimulate translation might be more sensitive to loss of Hrp48. When such clones are homozygous for strong hrp48 mutations, no eggs are produced, necessitating the use of weaker alleles to produce embryos for microinjection.

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DISCUSSION

Affinity Purification of RNA-binding Proteins—The TRAP procedure described here is a novel method for identification of proteins that interact with a given RNA sequence. The TRAP procedure is adaptable to a wide variety of bait sequences; here we have employed tagged RNAs generated in vitro to purify proteins from a crude extract. In principle, tagged RNAs could also be expressed in vivo, allowing for the purification of native endogenous RNA complexes. This will be particularly important when the recruitment of a complex to an RNA is influenced by earlier regulatory events. Although techniques for analysis of genomes and proteomes are well established, methods to study the ribonome (i.e. the sum total of all RNAs and their interacting proteins within the cell) lag behind. The TRAP tagging system provides a new method to employ in such efforts.

Translational Enhancement in Drosophila—Although our previous work provided genetic evidence that the HDE is a multifunctional cis-acting element capable of stimulating both translation and transcript degradation, here we have conducted biochemical experiments that demonstrate that the HDE is a bona fide translational enhancer. These experiments identified DDP1, Hrp48, and PABP as HDE-binding proteins, and our injection assay provided evidence that both DDP1 and Hrp48 contribute to the ability of HDE to stimulate translation. Since, in the context of the injection assay, translational stimulation occurs in the absence of transcript degradation, these two post-transcriptional processes are likely to be mediated by separable mechanisms. This is consistent with our recent results, which show that the major RNA destabilization element resides in the Hsp83 open reading frame, whereas the HDE functions as an auxiliary degradation element.5

Evolutionary Conservation of the Translational Enhancement Machinery—Our data, taken together with data of others, suggest that all three of the HDE-binding proteins identified here play conserved roles in translational enhancement. In mammals, PABP enhances translation of YB-1 mRNA in a poly(A) tail-independent manner by binding to an element located in the transcript’s 3′-UTR (5). Likewise, our data showing that PABP interacts with the HDE suggests that recruitment of PABP to elements internal to a transcript’s 3′-UTR may be a conserved mechanism for translational enhancement. We note, however, that we do not yet know whether translational enhancement via the HDE is poly(A) tail-independent.

DDP1 is a member of the vigilin family of proteins. These proteins have been shown to function in diverse processes, including centromeric silencing, chromosome segregation, and control of mRNA stability and translation. The yeast homolog of DDP1, Scp160p, binds RNA, is present on polysomes, and associates with specific mRNAs in vivo (20, 21). One of these mRNAs, DHH1, shows decreased polysome association in an scp160 deletion strain, providing indirect evidence that Scp160p may enhance DHH1 translation. Here we have shown that DDP1 participates in translational enhancement by binding to the HDE, suggesting a conserved function for this family of proteins in translational control.

Hrp48 is a member of the hnRNP A/B family (27). A mammalian homolog, hnRNP A2, stimulates translation of myelin basic protein mRNA in oligodendrocytes through an hnRNP A2 response element (A2RE) in the transcript’s 3′-UTR (28). Interestingly, the A2RE can also mediate translational repression when hnRNP A2 recruits hnRNP E1 to the transcript (29). Therefore, hnRNP A2 can mediate both translational repression and enhancement via the A2RE.

Drosophila Hrp48 was first identified based on its role in splice site selection (9, 22). More recently, it has been shown play a role in the translational repression and localization of mRNAs in Drosophila oocytes (23–25). Here we have shown that Hrp48 binds the HDE and mediates translational enhancement. The ability of Hrp48 to function in both translational repression and stimulation suggests that its function is influenced by other trans-acting factors that are known to be bound to that element (e.g. DDP1 and PABP) as well as other, unknown proteins present in the HDE-binding complex.

A Combinatorial Model for Translation Enhancement—In addition to playing a conserved role in translational enhancement, our results show that DDP1, Hrp48, and PABP function together in this process. Stable formation of the DDP1-Hrp48-PABP complex on the HDE may involve cooperative interactions between individual proteins, either before or after they are bound to the mRNA. Consistent with this model, we were unable to identify mutations that block the binding of one of these proteins to the HDE without inhibiting binding of the others. Whether these proteins can form a stable complex in the absence of the HDE and subsequently bind RNA or whether they assemble on the HDE in a stepwise manner, will await further investigation. Different proteins within the complex could, for example, serve different functions, such as transcript recognition, complex stabilization, and interaction with the basal translational apparatus.

With respect to the structure and function of cis-elements that mediate translational enhancement, our results support a model whereby the HDE is composed of binding sites for several proteins, each of which functions in translational control. Thus, the ability of the HDE to stimulate translation reflects integration of the individual action of each of its bound proteins. Consistent with this model, we find that no individual deletion within the HDE completely disables its ability to stimulate translation. Instead, these deletions enhance or diminish the extent of translational stimulation. Modulation of the output of the HDE could be achieved in different cell types and at different developmental stages by changing the complement of HDE-binding proteins that are present. By extension, the expression of mRNAs other than Hsp83 is likely to be fine tuned via cis-acting elements similar to the HDE, which are composed of different combinations of binding sites for proteins that mediate translational enhancement and repression.

Acknowledgments—We thank P. Lasko, D. Rio, and T. Schüpbach for Drosophila melanogaster stocks and reagents.

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