HISTONE mRNA TRANSLATION IN METAZOANS: SLIP1 AS THE BRIDGING FACTOR BETWEEN THE 5' AND 3' UTRs OF THE HISTONE mRNA

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ABSTRACT

NIHAL GULSEREN CAKMAKCI: Histone mRNA translation in metazoans: SLIP1 as the bridging factor between the 5' and 3' UTRs of histone mRNA
(Under the direction of William F. Marzluff)

Histone proteins packages newly synthesized DNA into higher organizations. During DNA synthesis in metazoans, there is a high demand for biosynthesis of histone proteins. Strict regulation of histone protein expression in the S phase is achieved by unique structure of histone mRNA. Unlike other eukaryotic mRNAs, histone mRNAs end with a stem-loop, rather than a polyA tail. The stem-loop binds the stem loop binding protein (SLBP), a novel RNA binding protein that is conserved among metazoans. *Xenopus laevis* has two forms of SLBP. xSLBP1 is the homolog of hSLBP and N-terminus of the both proteins are shown to stimulate translation of histone mRNA. xSLBP2, the oocyte specific form, maintains high similarity to hSLBP only in the RBD. xSLBP2 does not stimulate histone mRNA translation, but instead represses histone mRNAs during early oogenesis. xSLBP2 is degraded during oocyte maturation, and then xSLBP1 binds histone mRNAs coinciding with the timing of translational activation. I have characterized a motif in the N-terminus of xSLBP1 and hSLBP which is responsible for translational activity. The motif, $DWX_{(3-4)}VEE$, is highly conserved among vertebrates. I demonstrated that this motif interacts with a novel factor, named as SLBP-Interacting Protein 1 (SLIP1). Interestingly, SLIP1 stimulates translation of histone mRNA to higher levels in the presence of SLBP. SLBP mutants that can not

stimulate histone mRNA translation are also incapable of interacting with SLBP.

Previously, it had been reported that eukaryotic polyadenylated mRNAs were translationally stimulated by circularization via protein-protein interactions. I showed that SLIP1 interacts with translation initiation factors IF4GI and II. Possibly, histone mRNA forms a closed loop similar to polyadenylated mRNAs during translation. Together with Jeremy Kupsco and Bob Duronio, I genetically investigated the role of SLBP and SLIP1 in *Drosophila melanogaster*. We identified a region in the N-terminus of dSLBP which has an essential post-processing function in *Drosophila* during early development. We demonstrated that dSLIP1 interacts with dSLBP. We characterized the SLIP1 interaction motif in the N-terminus of SLBP, ($KFX_{(2-3)}VEKE$), which is similar to $DWX_{(3-4)}VEE$ motif in vertebrates. Both *dSLBP* and *SLIP1* null mutant flies are embryonically lethal, probably due to a defect in translation of histone mRNAs. We conclude that the SLIP1-SLBP interaction is crucial for viability of *Drosophila* during early development.

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ABREVIATIONS

SLBP: Stem-loop binding protein

RBD: RNA-binding domain, especially referred to RBD of SLBP in my proposal

SL: Stem loop

TL: Tetra loop which has conserved Us in the loop mutated

HDE: Histone downstream element

dSBLP: *Drosophila* SLBP

hSLBP: Human SLBP

xSLBP: *Xenopus* SLBP

suSLBP: Sea urchin SLBP

SLIP1: SLBP-interacting protein 1

eIF4GI/ II: Eukaryotic translation initiation factor gamma I or gamma II

eIF4E: Eukaryotic translation initiation factor 4E, cytoplasmic cap binding protein

PABP: PolyA tail binding protein

Ternary complex: eIF2·GTP·met-tRNA

43S ribosomal complex: 40S ribosomal subunit + eIF2·GTP·met-tRNA + eIF3·eIF1·eIF1A

48S ribosomal complex: mRNA + 43S ribosomal complex

CAF1: Chromosome assembly factor 1

RRM: RNA recognition motif

mRNPs: mRNA ribonucleoproteins

Ternary complex: eIF2·GTP·Met-tRNA$_i$

IRES: Internal ribosome entry sites

ORF: Open reading frame

Luc-SL: Luciferase open reading frame ending with wild type histone stem loop

Luc-TL: Luciferase open reading frame ending with mutated loop

RT: Room temperature

SDS: Sodium dodecyl sulphate

IPTG: Isopropyl-beta-D-thiogalactopyranoside

CHAPTER I

INTRODUCTION

Chromatin

In eukaryotes, the genomic DNA is packed in to nucleosome by wrapping around an octamer core consisting of two molecules of histone H2A, H2B, H3, and H4. 146 bp of DNA winds around each nucleosome molecule twice forming a bead-like structure (Figure 1). Nucleosomes are further compacted into a higher organization, called chromatin, with internucleosomal interactions by linker histone, H1 and non-histone proteins. The histone and non-histone proteins mediate compaction of DNA, allowing smallest 14 mm long DNA to fit into microscopically observable nucleus. This chromatin structure is the template for DNA replication, repair, and recombination and as well as for transcription in eukaryotes.

Histone proteins are composed of two domains: the globular C-terminal domain that provides the contact surface for the DNA and the N-terminal domain that extends outside of the nucleosome core. The positively charged N-terminal domain binds and neutralizes the phosphate backbone of the DNA. Biological function of histones is regulated through posttranslational modifications. The core histones are heavily modified within their first 40 amino acids in N-terminus which are highly conserved despite playing no structural role. Acetylation of N-terminus allows DNA to gain an accessible conformation by transcription factors, so is indicative of active transcription sites. In contrast, deacetylation process of nucleosomes is usually associated with gene silencing in the cell

(Braunstein et al. 1993; Jeppesen and Turner 1993). Double stranded DNA wraps around the nucleosome more tightly when core histones are hypoacetylated. Tight interaction between DNA and nucleosome renders sequence recognition by transcription factor and chromatin remodeling factors more difficult. Acetylated histones bind DNA more loosely, so factors mentioned above can have easy access to DNA. However, Acetylation status of histones can affect assembly of linker H1 into nucleosomes and therefore affects higher order organization of chromosome. Histones are also methylated by histone methytransferases such as Suv30h. Suv30h methylates histone H3 9 lysine residues and mouse embryonic stem cells lacking Suv30h display an altered DNA methylation profile at pericentric satellite repeats (Lehnertz et al. 2003). Reciprocally, human DNA methytransferases 1 (DMT 1) is required to maintain the histone H3 modification pattern (Espada et al. 2004). Human cancer cells lacking DMT 1 had decreased level of H3 9 lysine dimethylation and trimethylation, but an increase in acetylation status of histones was observed. Interdependently, those activities are involved in preservation of the correct organization of large heterochromatic regions. In summary, histones with their different modifications play critical role in gene expression by controlling accessibility of DNA to transcription factors and chromosome remodeling factors (Chen et al. 1999; Forrester et al. 1999; Lorincz et al. 2000) and organization of nuclear architecture (Espada et al. 2004). The interdependent modifications may generate a histone code that is deciphered by the cell for conformational changes in the chromatin structure and status of gene expression.

Significance of Studying Histone mRNA Translation

Eukaryotic proliferating cells double their DNA content during S phase and histone proteins are incorporated into newly synthesized DNA. This process also demands high level of histone proteins during cell cycle progression. Histone mRNA level increases during S phase correlating with DNA replication. Inhibition of DNA replication results in cessation of histone protein expression and rapid degradation of histone messages (Heintz et al. 1983; Sittman et al. 1983; Graves and Marzluff 1984). Eukaryotic cells have evolved many surveillance mechanisms to maintain genomic integrity. Any defects in one of those mechanisms may lead to increased incidence of mutations, genomic instability, thus cell cycle arrest, cell death, and more dramatically into oncogenesis in human beings. After a failure in regulation of replication, transcription, condensation into chromatin or damage in DNA, cell cycle arrest occurs due to check point activation to provide additional time for efficient repair (Weinert et al. 1994). Cell cycle progression resumes after the damage has been repaired. There are some reports correlating the histone protein level and surveillance mechanisms in cell cycle arrest, cell death, DNA damage sensitivity, chromosome loss phenotypes and oncogenesis. (Gunjan and Verreault 2003) have shown that Rad53 in S. cerevisiae is required for degradation of excess histones that are not incorporated into chromatin. As a result, *rad53* mutants accumulate abnormally high amounts of soluble histone proteins. Mutant cells become highly sensitive to histone over expression showing remarkable DNA damage sensitivity, slow growth, and chromosome loss phenotypes. Disruption of one of the two loci encoding histones H3/H4 significantly suppressed *rad53* mutant phenotypes (Gunjan and Verreault 2003). Their results argue that those phenotypes are partly due to excess histone proteins. Chromatin aggregation and inhibition of

transcription were observed after a slight stoichiometric increase in histone protein level over DNA in *in vitro* assays (Steger and Workman 1999). In *Drosophila melanogaster*, strong *dSLBP* alleles result in zygotic lethality late in development and yield in production of stable histone mRNA that accumulates in non-replicating cells (Sullivan et al. 2001). Like replication-independent histones, those mRNAs are cytoplasmic and have poly (A) tail. Also, mutations that cause high level of histone mRNAs lead to defects in oogenesis (Berloco et al. 2001). Incidence of mitotic chromosome loss increases with overexpression of histone proteins in yeast (Meeks-Wagner and Hartwell 1986). In contrast, hypomorphic *dSLBP* alleles support zygotic development with female sterility and eggs from these females have reduced level of histone mRNA (Sullivan et al. 2001). Mutant embryos will not reach adulthood due to incomplete syncytial embryonic cycles (Sullivan et al. 2001) . The reason for this phenotype is partly because of a failure in chromosome condensation at mitosis leading to inhibition of chromosome movements to poles during anaphase. Probably, reduced level of histone proteins result in low incidence rate of their incorporation into newly synthesized DNA and thus cause defects in chromosome condensation as a consequence.

Cell Cycle Regulation and Early Development in Xenopus Laevis

The large size of the *Xenopus* oocytes and egg (1.2 mm in diameter) (Gerhart and Keller 1986) and its ease of experimental manipulation make it a favored system for investigation of cellular processes, such as translation, protein modifications including phosphorylation and acetylation, cascades of extracellular signaling, and export/import of molecules and so on. Fully grown oocytes contain large pools of the components required for

protein synthesis during early embryogenesis including maternal mRNAs, ribosomes, and tRNAs (Davidson 1986). In my thesis, I concentrated on translation of histone mRNAs and role of factors involved in this process by taking advantage of *Xenopus* oocytes.

Oogenesis

The unfertilized *Xenopus* egg has an inherent asymmetry which consists of a pigmented animal hemisphere and an unpigmented, yolk rich, vegetal hemisphere. Dark color of animal pole (brownish) is given by a layer of melanin granules near the plasma membrane (Gerhart and Keller 1986). Animal pole contains organelles and cytosolic materials, and the metaphase II spindles with chromosomes reside at its pole. The yellowish vegetal pole encompasses relatively smaller amounts of organelles and cytosol but contains the bulk of nutrient reserves of the egg, mainly membrane-bound yolk platelets (Gerhart and Keller 1986). A single sperm enters into the egg anywhere in the animal pole, leaving a dark pigmented sperm entry point. After fertilization, extra sperm entry is prevented due to changes in the egg's plasma membrane initiated by entering sperm. These alterations block polyspermy (Grey et al. 1974; Grey et al. 1976). Fertilization competent eggs are produced through six consecutive stages that are characterized by oocyte diameter (Dumont 1972). The smallest oocyte is designated as stage I and the largest as the stage VI (Figure 2). Progression from stage I to stage VI requires at least 8 months. Since oocytes do not grow synchronously, at the end of 8 months, a frog ovary will contain all different stages.

Unfortunately, it is difficult to distinguish stage V from stage VI by simple observation through a stereomicroscope. Stage V oocytes are 1000-1200 μm in diameters,

whereas stage VI oocytes have diameters larger than 1200 μm (Dumont 1972). However, significant metabolic differences between late stage IV, stage V and stage VI are observed (Cicirelli and Smith 1987; Taylor and Smith 1987). These differences can exert major effect on the capacity of oocytes to translate injected mRNAs. So, ocular micrometer can be utilized to size the oocytes, thus some of the variability in the experiments can be reduced.

When oocytes are removed from the frog, their development is interrupted and oocytes can not progress to the next stage. Stage VI oocytes are arrest in prophase I and can escape the arrest by progesterone treatment that results in maturation of oocytes (Davidson 1986; Smith 1989). Maturation is a simple cell cycle that consists of G2 → M transition. During maturation, the unfertilized egg completes the first meiotic division and is again arrested at metaphase II until the egg is fertilized by a sperm (Maller 1985). Induction of maturation comprise a number of morphological and biochemical changes. One of the external indications of maturation is the breakdown of the oocyte nucleus or germinal vesicle (GVBD) that appears as a white spot in animal pole. GVBD in stage VI oocyte occurs at greater than 6-8 hours after progesterone exposure (Smith 1989). A decrease in the level of cAMP is observed during maturation and as a consequence, a decrease in the level of cAMP-dependent kinase is both necessary and sufficient to trigger oocyte maturation (Maller and Krebs 1977; Huchon et al. 1981). However, permeability of oocyte membrane is reduced, and rate of protein synthesis is doubled at maturation (Smith 1989). Upon fertilization, meiosis is completed and embryogenesis starts, including M → S transition. Just after maturation, translation of most of the mRNAs is activated, but transcription is still inactive.

Early Embryogenesis

During the early periods of embryogenesis in *Xenopus*, fertilized single cell embryo undergoes 12 rapid synchronous cell divisions (4000 cells) and DNA synthesis, lacking G1 and G2 phases. The first cell division takes place 100 minutes after fertilization, followed by consecutive divisions with 30 minutes intervals up to 13^{th} cell division. During those rapid divisions, multicellular embryo demands high amount of histone proteins to package newly synthesized DNA into chromatin. Interestingly, there is no transcription, if any, during these first cell divisions. High amounts of histone proteins are provided by the activated translation of maternally stored histone mRNAs right after maturation of the oocyte (Figure). In fact, most of the proteins are regulated like histone proteins during those early cell cycles, being translated from stored mRNAs accumulated during oogenesis, rather than having elevated levels of transcription (Newport and Kirschner 1982b; Dworkin and Dworkin-Rastl 1990). Rapid synchronous cleavages are then followed by a period of slower asynchronous divisions more typical of somatic cells (Satoh 1977; Newport and Kirschner 1982a). This alteration in cell cleavage is termed as (MBT) (Gerhart 1980) when the rate of cell cycle slows, cell cycle becomes asynchronous, maternal mRNAs are degraded, and zygotic transcription resumes (Newport and Kirschner 1982a; Ballantyne et al. 1997). Timing of the MBT depends on reaching a critical ratio of nucleus to cytoplasm. Newport and Kirschner suggested that MBT occurs due to titration of some substance, originally present in the egg, by exponentially increasing nuclear material.

Status of Messenger RNA and Protein Synthesis during Early Development

Xenopus oocytes contain large reserves of stored maternal mRNAs. During the entire period of oogenesis, oocytes have very active chromosomes that display transcription rates higher than typical somatic cells by several orders of magnitude (Anderson and Smith 1977; Anderson and Smith 1978). Unlike somatic cells, most of newly synthesized RNA is not exported into cytoplasm. Nevertheless, amount of polyA mRNAs that enters the cytoplasm is quiet high due enormous rate of RNA synthesis in the nucleus and there is no difference in that amount between stage III and stage VI (Dolecki and Smith 1979). Though, this is still small amount of all mRNAs in the oocytes and only 10% of the newly synthesized cytoplasmic transcripts associate with polysomes (Dolecki and Smith 1979). Rest of the mRNAs is stored in translationally, mRNPs in the cytoplasm. There is a 100 fold increase in the rate of overall protein synthesis through the progression of oogenesis from stage I to stage VI (Smith et al. 1991). In parallel, the content of the ribosomal RNA increases. However, the fraction of translationally active ribosomes remains constant at about 2% throughout oogenesis. Protein synthesis is stimulated up to 2-fold when oocyte maturation is induced (Wasserman et al. 1982).

Stage VI oocytes are an advantageous system to study translation of many exogenous mRNAs, since injected mRNAs successfully competes with endogenous mRNAs for translational machinery of the oocyte (Laskey et al. 1977). The rate of protein synthesis in stage IV oocytes is about one third that in stage VI oocytes (Smith et al. 1991). Levels of protein synthesis in stage IV can be elevated to the similar levels observed in stage VI by injection of increasing amounts of mRNAs (Taylor et al. 1985). This observation demonstrated that stage IV oocytes definitely have spare translational capacity.

Early development of *Xenopus* is directed by maternally inherited mRNAs that are synthesized during the very early period of oogenesis (Pique et al. 2006). Translationally inactive mRNAs usually possess short polyA tails and are stored as mRNPs that prevent ribosomal recruitment. When *Xenopus* oocytes are matured by meiosis-inducing hormone progesterone, polyA tails of those mRNAs are elongated, and the mRNAs are shifted into polysomes. The cytoplasmic polyadenylation element (CPE) in the 3' UTR of the mRNA directs this cytoplasmic polyadenylation. Translational activation of specific mRNAs occurs in a sequential manner during meiosis and early development (Pique et al. 2006).

The importance of a polyA tail for translation has been one of the greatest interests of researcher. A group of studies, mainly involving injection of mRNA into *Xenopus* oocytes, has indicated that a poly A tail may serve to increase the message stability and to stimulate translational efficiency (Drummond et al. 1985; Soreq 1985; Galili et al. 1988; Jackson and Standart 1990). First time, Galili et al. (1988) showed that polyadenylated mRNAs were translated 10-15-fold more efficiently after injection into stage VI oocytes when compared with mRNAs lacking polyA tail. Additionally, reinitiation of terminating ribosomes on polyadenylated mRNAs was more efficient than non-polyadenylated messages in oocytes.

Translation Initiation in Eukaryotes

Eukaryotic organisms developed a rapid response mechanism to manage gene expression in order to control cell growth, proliferation and development (Conlon and Raff 1999; Gingras et al. 1999). Since initiation is the rate-limiting step in formation of a competent translation initiation complex, therefore regulation occurs predominantly at

initiation. The initiation step involves many translation factors and adaptor proteins directing an ordered multistage process of ribosome recruitment to mRNA (Gingras et al. 1999).

Eukaryotic mRNAs possess two untranslated fundamental structures. 5'UTR carries a 7'mGpppN cap [where m is methyl group and N is any nucleotide, (Shatkin 1976)] and the 3'UTR is elongated with a polyA tail consisting of 50-200 nucleotides in length (Hershey et al. 1996). Methylation of the 2'-O-ribose on the cap requires the polyA tail during development of amphibian embryos (Kuge and Richter 1995) These cis-acting structures of the mRNA are critical for efficient translation and are sites for specific recognition by proteins (Table 1). Through those interactions, cap and polyA tail allows mRNA to have concerted interactions with numerous components during the first steps of translation initiation (Pain 1996), leading to stimulation of translation synergistically *in vivo* and *in vitro* (Gallie 1991; Iizuka et al. 1994; Tarun and Sachs 1995). However, the length, nucleotide composition, and structure of the 5' UTR, as well as sequences in the 3' UTR, contribute to the translational efficiency of mRNAs (Hershey and Merrick 2000).

The cap structure is bound by eIF4E, a 24kDa cap binding protein, in a complex called eIF4F (Sonenberg et al. 1979). eIF4E interaction with cap facilitates ribosome recruitment to mRNA (Conlon and Raff 1999). eIF4F is composed of eIF4E, eIF4A, and eIF4G. eIF4A, 46kDa polypeptide, is a bidirectional RNA-dependent helicase and ATPase (Ray et al. 1985; Rozen et al. 1990). eIF4A is thought to unwind the secondary structures in the 5'UTR. Helicase activity is stimulated by initiation factor eIF4B. eIF4G is a scaffold protein which interacts with eIF4E, 4A, eIF3 and recruits 40S small ribosomal subunit via interaction with eIF3 (Sachs et al. 1997; Gingras et al. 1999). The interaction between eIF4G and eIF4E is RNA-independent (Tarun and Sachs 1996). There two homolog of eIF4Gs in

human: 4GI and 4GII are 46% identical at amino acid level and overall identity is 56% (Gradi et al. 1998) and are exclusively cytoplasmic protein. Saccharomyces cerevisiae also has two genes expressing corresponding human eIF4G homologs, *TIF4631* and TIF4632 (Goyer et al. 1993). eIF4Gs have two binding sites for e IF4A, one located in the central third and the other in the carboxy third portion of the protein (Imataka and Sonenberg 1997). The consensus eIF4E binding site on e IF4G is YXXXXLΦ, where Φ is usually L, but may also be M or F at position 572-578 in human eIF4GI N-terminus (Mader et al. 1995; Johannes and Sarnow 1998; Johannes et al. 1999).

In humans, eIF4GI has five isoforms translated from the same mRNA with alternative start sites in the N-terminus. Additionally, in human, there are two homologs; eIF4GI, the abundant form and eIF4GII, tissue specific form. A second species of eIF4A (46.3 kDa) is encoded by a different gene. Since eIF4AI and 4AII are functionally equivalent, as are eIF4GI and eIF4GII, in the table they are referred as eIF4A and eIF4G, respectively (Table 1).

A 636-amino acid protein was named polyA binding protein (PABP) in mammalian cells and PAB1 in yeast binds to polyA tail at 3'UTR and required at least 12 As to bind with maximal affinity (Sachs et al. 1986; Sachs 2000). PolyA tail can simultaneously associate with many PABPs. PABP is phylogenetically conserved and is essential in *Saccharomyces cerevisiae* growth. N-terminal piece, containing the RNA binding sites, can replace the full length protein for polyA tail binding activities and yeast cell viability (Sachs et al. 1987). Human PABP contains four N-terminal RNA recognition motifs (RRM) that are required for mRNA binding (Sachs et al. 1987; Burd et al. 1991), and a proline-rich C-terminus containing an approximately 75 amino acids conserved domain (PABP-C). PABP C-terminus

is involved in protein-protein interactions. The C-terminal half of PABP binds eRF3 (Hoshino et al. 1999), Paip1 (Gray et al. 2000; Roy et al. 2002), Paip2A (Khaleghpour et al. 2001a; Khaleghpour et al. 2001b) , Paip2B (Berlanga et al. 2006), Pbp1p (Mangus et al. 1998), and a viral RNA polymerase (Wang et al. 2000). PABP forms homodimers through its C-terminal domain that is also required for nuclear shuttling (Kuhn and Pieler 1996; Afonina et al. 1998). Importantly, the RRM1 and RRM2 domains of PABP together interact with N-terminus of eIF4G in mammals (Imataka et al. 1998; Kessler and Sachs 1998), plants (Le et al. 1997), and yeast (Tarun and Sachs 1996; Imataka et al. 1998). Yeast homologs TIF4631 and tif4632 of eIF4G interacts with PAB1 in an RNA-dependent manner *in vivo* and at least in a polyA tail-dependent manner *in vitro* (Tarun and Sachs 1996) in contrast to interaction observed between plant homologs of these proteins (Le et al. 1997; Wei et al. 1998). By using atomic force microscopy, it was shown that those protein-protein interactions result in a circular conformation, thus bringing the ends of the mRNA in close proximity (Wells et al. 1998). So, eIF4G is a key protein that bridges the 5' and 3' ends of the message by interactions with eIF4E at the 5' end and with PABP at 3'end.

Translation initiation is catalyzed by these proteins together with some other factors. Initiation commences with dissociation of terminating 80S ribosome. The first step in translation initiation is the formation of eIF2·GTP·Met-tRNA$_i$ ternary complex. Upon each completed round of translation initiation, eIF2 hydrolyzes GTP to GDP and leaves the initiation complex. Since eIF2 has approximately 100-fold higher affinity for GDP than GTP (Kapp and Lorsch 2004), recycling eIF2·GDP to GTP-bound form is facilitated by eIF2B. eIF3 binds to 40S ribosomal subunit in addition to eIF1 and 1A. Ternary complex binding to 40S ribosomal subunit occurs with assistance of eIF3, eIF1 and eIF1A, resulting in the

formation of 43S complex. eIF4F complex, which contains eIF4G as a scaffolding protein, assembles on the 5' end of the message through interaction of eIF4E with cap structure, while PABP binds to 3'UTR on polyA tail.

Secondary structures in the 5'UTR are likely to be removed by the helicase activity of eIF4A interacting with eIF4G. eIF4G binds eIF3 which then associates with 43S complex and thus ribosomal subunit is recruited to mRNA 5'UTR. The 43S complex starts scanning down the message for the initiation codon in the 5' to 3' direction. The scanning process may require ATP hydrolysis and although an ATPase has not been identified yet. Once 43S complex finds the start codon, usually the first AUG, codon-anticodon bases pairing between the initiation codon and the initiator tRNA takes place. eIF1 also helps in identifying the initiation codon. Then, GTP is hydrolyzed to GDP by eIF2 and GTPase activating protein eIF5, leading to release of Met-tRNA$_i$ into the P site of the 40S subunit and dissociation of eIF2·GDP from the complex. Also, eIFs 1, 1A, 3 and 5 may dissociate at this stage. After dissociation of eIFs 1, 1A, 2, 3, 5, 60S ribosomal large subunit joining is catalyzed by eIF5B. 60S association with 40S results in 80S ribosome formation with hydrolysis of GTP by eIF5B. eIF5B·GDP has low affinity for ribosome, so it dissociates from the complex. Now, ribosome is free to synthesize the peptides, and enters the elongation phase of translation.

Closed Loop Model

As mentioned above, all eukaryotic mRNAs originated from nucleus possess an m7GPPPN cap structure at 5' UTR and contain a polyA tail at 3' UTR with some exceptions like histone mRNAs. The cap structure and polyA tail act synergistically to stimulate

translation. This phenomenon has been observed in several systems derived from yeast extract, yeast, plant and mammalian cell lines (Gallie 1991; Iizuka et al. 1994; Michel et al. 2000). Cap and polyA synergy led to a model, originally hypothesized by Jacobson, in which mRNA is circularized by protein-protein interactions (Jacobson and Favreau 1983). This circularization presumably promotes enhanced translation. In fact, detection of circular polysomes in electron microscopy images (Christensen et al. 1987) supports the idea of circularization of the mRNA during translation initiation.

How does the polyA tail participate in translation? PABP binds to polyA tail and was shown to mediate translational effect of polyA tail on translation (Sachs et al. 1986). Cap is bound by eIF4E and eIF4F can exert its translational effect through that interaction. The bridge between 4E and PABP is formed by scaffold protein eIF4G which interacts with both proteins (Figure). eIF4E-eIF4G–PABP interaction was proposed to be key event for this circularization and thus to stimulate translation (Sachs 2000). In *Xenopus* oocytes, an e IF4GI mutant defective in PABP binding reduces polyA-dependent translation and importantly inhibits progesterone-induced maturation, underscoring the biological importance of the eIF4G–PABP interaction (Wakiyama et al. 2000).

Still, these results do not explain the how the cap, polyA tail and those specific interactions, which result in circularization, lead to stimulation of translation initiation. There may be several mechanisms rather than one mechanism: (1) recycling of ribosomes (2) enhancement in the affinity of eIF4F, hence stronger interactions between eIF4E and cap structure due to conformational changes, as well as stimulation of 40S ribosomal subunit recruitment. (3) stimulation of 60S ribosome joining. Early studies *in vitro* yeast translation system showed that PABP stimulated 40S ribosomal subunit joining (Tarun and Sachs 1995).

However, (Searfoss et al. 2001) demonstrated that either the absence of eIF5B (fun12p) or a defect in eIF5, which are involved in 60S ribosomal subunit joining, specifically reduces translation of polyadenylated mRNAs in yeast, but not mRNA without a polyA tail. The most recent experiments by Sonenberg and his laboratory allowed us to seek for possible mechanisms in a more direct way. They showed that PABP enhances 60S subunit joining through its interaction with eIF4GI in Krebs-2 cell-free translation extracts (Kahvejian et al. 2005). However, PABP M161A, which is incapable of interacting with eIF4GI, failed to restore translation and 80S ribosome association with a radioactively labeled mRNA. Also, this mutant could not restore both 40S and 60S ribosomal subunit joining to mRNA. On the other hand, eIF4G–PABP interaction stimulated eIF4E and eIF4A association with capped mRNA in a polyA-dependent manner and as a consequence, 4A enhanced 4B joining to the message (Kahvejian et al. 2005). Additionally, PABP exerts its effect by increasing the affinity of eIF4G for eIF4E and 4E affinity for cap structure. Likewise, it is true that eIF4E enhances the affinity of eIF4G for PABP. Stronger binding affinities of translation factors for each other may result in translationally more stable mRNA-protein complexes or induce conformational changes in either translation factors or the mRNA structure. Altogether, those studies suggest that circularization of 5' and 3' ends of RNA may act via multiple mechanisms to stimulate translation.

The length of the polyA tail also contributes to translational stimulation. Strikingly, polyadenylation of maternal mRNAs after meiotic maturation or fertilization coincides with translational activation of those same mRNAs in *Drosophila*, mouse and *Xenopus* early development (Hershey et al. 1996). Although polyadenylation-translational activation seems to be logical by allowing multiple PABP binding and more efficient interaction with 5' end

proteins, some mRNAs undergo deadenylation after they are translationally activated (Hershey et al. 1996).

HISTONE mRNAs

Biosynthesis of histone messages is tightly coupled to DNA replication. During S phase where DNA is replicated, there is a high demand for histone proteins. Then, newly synthesized DNA will be efficiently packaged into chromosomes by wrapping around a histone octamer. A higher order organization can be achieved by linker histone, H1, which is responsible for internucleosomal interactions. When DNA replication ceases, due to no need for histones to package DNA, histone proteins are no longer required. To control the need of histones, accumulation of histone mRNAs occurs only during S phase and coupled to ongoing DNA replication in metazoans (Schumperli 1986; Osley 1991).

Replication-dependent histone mRNAs are the only eukaryotic mRNAs without a polyA tail. Metazoan replication-dependent histone mRNAs possess a highly conserved stem-loop structure at the 3' end (Figure 5) (Dominski and Marzluff 1999). In contrast, histone mRNAs of plants, yeast, fungi and protists end with a polyA tail. However, the replication-independent class of histone mRNAs encoding histone variants H3.3, H2a.Z, H3-cid is polyadenylated (Marzluff 2005). Histone mRNAs unlike polyadenylated mRNAs do not have any introns (Dominski and Marzluff 1999).

Replication-dependent histone mRNAs are bound by a unique class of RNA-binding proteins, stem-loop binding proteins (SLBP) (Wang et al. 1996; Martin et al. 1997). SLBP has three distinct domains: N-terminus, RNA-binding domain (RBD), and C-terminus. RBD of SLBP, a 70 amino acid region, is not similar to any other known protein domain. SLBP

present in both nucleus and cytoplasm on polysomes (Whitfield et al. 2004; Erkmann et al. 2005b). SLBP associates with histone pre-mRNA taking a role in processing of the 3' end of the message to form a mature histone mRNA.

The biosynthesis of a mature histone mRNA requires only one-step endonucleolytic cleavage reaction (Dominski and Marzluff 1999). At the 3' end, stem-loop and histone downstream element (HDE) are the *cis* elements that are necessary for this cleavage process. U7 snRNP, which is essential for histone message processing, binds HDE by base-pairing of 5' end of U7 snRNA with the 3' end of HDE (Figure 6) (Schaufele et al. 1986). Stable base-pairing between U7 and HDE is essential for histone 3' end formation *in vivo* and *in vitro* (Bond et al. 1991).

Histone mRNA is exported into cytoplasm with SLBP bound to stem loop (Erkmann et al. 2005b). In general, SLBP likely stays bound to histone mRNA throughout its life span. Not surprisingly, SLBP participates in multiple aspects of histone mRNA metabolism. The stem loop at the 3' end of histone mRNA is necessary and sufficient for regulation of histone mRNA stability, and the correlation of histone mRNA levels with the cell cycle (Pandey and Marzluff 1987). When stem loop composed of approximately 30 nucleotides is replaced after globin coding sequence, those chimeric mRNAs are subjected to cell cycle-dependent regulation similar to natural histone mRNAs.

Knock down of SLBP in the mammalian cells results in an S phase defect including prolonged progression time through S phase, although the levels of histone mRNAs are not dramatically altered, as much as the amount of histone protein biosynthesis. These results suggest that SLBP must play an essential role other than in histone pre-mRNA processing.

Regulation of Histone mRNA Translation during Early Development in Xenopus Laevis

Early developmental stages in *Xenopus*, as well as in *Drosophila melanogaster* and *C. elegans* do not maintain the strict cell cycle regulation of histone mRNAs unlike somatic cells. Levels of histone mRNAs are not coupled to DNA replication during early stages. Although there are high rates of DNA replication and desire for histone proteins to incorporate newly synthesized DNA into chromosome structure, there is a switch from cell cycle regulation to *default* standard mechanism for histone mRNAs and histone protein levels. Histone mRNAs and protein are continuously present in oocytes. Those histone mRNAs still end with a stem-loop and can be bound by SLBP, suggesting that it is a mechanistic switch rather than only a structural change. For example, nucleoplasmin, N1 and N2, binds to histone proteins in *Xenopus* (Dilworth et al. 1987) and then stores histones through oogenesis (Woodland and Adamson 1977). In contrast, somatic cells have little capacity to store histone proteins which are incorporated into chromatin just after DNA synthesis ceases (Marzluff and Duronio 2002). Oogenesis of *Xenopus* involves 6 different one cell stages which need approximately 8 months to reach a full growth and to be ready for fertilization. Histone mRNAs in *Xenopus* are transcribed until stage III and then stored in a translationally inactive form until maturation (Van Dongen et al. 1983) as in *Drosophila* (Anderson and Lengyel 1980) (Figure 3). *In vitro*, fully-grown stage VI oocytes can be artificially induced to undergo maturation after progesterone treatment. Stage VI oocytes are arrested in prophase II and completes first meiosis upon maturation. Also, SLBP1 protein levels increase during oogenesis and reaches its peak at maturation. So, translational activation and highest level in SLBP1 matches to the same time during early oogenesis. Then oocyte is arrested in metaphase II until fertilization occurs. Upon release from arrest in

metaphase II when egg is fertilized, embryo undergoes 13 consecutive divisions without gap phases and cell growth, including only S and M phases. Since stored histone proteins are not enough to supply the high demand for histones, translation of stored maternal mRNA is reactivated, but still transcription is inactivated. 70% of histone proteins are supplied by this way until midblastula transition (MBT). After stage 7 during early embryogenesis, zygotic transcription resumes at MBT to achieve full supply of histone proteins which counts for 30% of the whole histone protein pool (Newport and Kirschner 1982b).

In *Xenopus* oocytes, there are two stem-loop binding proteins named as SLBP1 and SLBP2 which can bind to stem-loop with similar affinities (Wang et al. 1999). Those two proteins are only similar in RBD. SLBP1 is the human SLBP homologue and able to stimulate histone mRNA translation (Sanchez and Marzluff 2002) and required for pre-mRNA processing (Ingledue et al. 2000). During oogenesis, maternal histone mRNAs are added a short poly (A) tail after stem-loop structure and bound by SLBP2. SLBP2 is observed in high levels during early stages of oogenesis, by stage II, and rapidly degraded during oocyte maturation (Wang et al. 1999). Sanchez and Marzluff demonstrated that SLBP2 and short oligo (A) tail play active role in translational repression of histone mRNAs during oogenesis (Sanchez and Marzluff 2004). SLBP2 degradation and oligo (A) tail removal occurs at maturation when translation is activated. Additionally translational repression is coupled to transcription in *Xenopus* oocytes. We do not know if SLBP degradation is enough for SLBP1 binding to stem-loop or if there is a more active mechanism which interplays between SLBP1 and SLBP2 during transition from repression to translational activation.

Translation of Histone mRNAs

Efficient translation of polyadenylated mRNAs is achieved by the communication between the 5' end and 3' end of the message via protein-protein interactions. Since histone mRNAs are not polyadenylated, instead ends with a conserved stem-loop structure, translational mechanism of histone mRNAs may use different components than poly A-dependent translational mechanism. Our lab showed that stem-loop (Gallie et al. 1996) and SLBP (Ling et al. 2002; Sanchez and Marzluff 2002; Gorgoni et al. 2005) are required for efficient translation of histone mRNAs. The 3' end is also important for histone mRNA localization to polyribosomes (Sun et al. 1992). In addition, SLBP co-purifies with polysome-associated histone mRNAs (Whitfield et al. 2004) .

Sanchez and Marzluff demonstrated that deletion of the entire C-terminus or replacement of *Xenopus* RBD with SLBP2 RBD does not affect the translational activity of xSLBP. A 13 amino acid sequence in the N-terminus of SLBP is required to enhance the translation (Sanchez and Marzluff 2002). In a subsequent study, fusion of only the SLBP N-terminus to MS2 yields the same amount of the stimulation of histone mRNA translation as full length protein does (Gorgoni et al. 2005). After detailed analysis, those studies demonstrated that N-terminus is responsible for the stimulation of histone mRNA translation. Furthermore, a region in N-terminus positioned between amino acids 68 and 83 was identified as essential for this activity of xSLBP1 (Sanchez and Marzluff 2002).

A Xenopus laevis oocyte-specific stem-loop binding protein, SLBP2 (Wang et al. 1999), represses the translation of reporter mRNAs in oocytes, while human homologue of *Xenopus* SLBP1 stimulates the translation (Sanchez and Marzluff 2002). However mammals only have one SLBP which is the ortholoque of xSLBP1 and is involved in both processing

and translational activation. Thus SLBPs may perform the function in translation similar to that PABP does for polyadenylated mRNAs. It is not known yet whether SLBP interacts with eIF4F complex or other translation components like PABP. The goal of my thesis research has been to discover the other factors that may interact with SLBP and the 5' end of the message during translation of histone mRNAs.

Figure 1. EM visualization of reconstituted DNA-histone complexes. Core histones were reconstituted with col.E1 DNA circles at histone : DNA ratios of 1.5:1 and sedimented in sucrose gradient. Peak fractions were fixed and examined by direct mounting with spermidine on thin carbon supports by tungsten rotary shadowing (Dunn and Griffith 1980).

Nucleosome
H1
H2b
H4
H2a
H3
DNA
Core of histone proteins
A Nucleosome Unit
DNA

Figure 2. Xenopus oocytes. Oogenesis emcompasses six different stages characterized according to the sizes of oocytes. Dark colored pole is the animal hemisphere and light colored pole is the vegetal hemisphere (Adapted from Dumont et al. 1972).

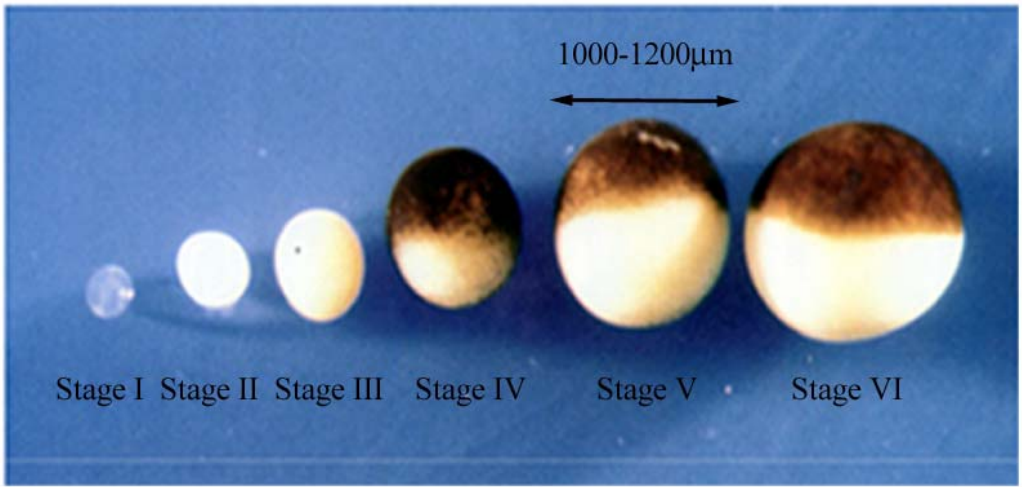

1000-1200μm
Stage I
Stage II
Stage III
Stage IV
Stage V
Stage VI

Figure 3. Oogenesis and blastulation. Most of the maternal mRNAs are transcribed and translated by the end of stage III during oogenesis and then mRNAs are stored in translationally inactive complexes. Proteins translated here will be the main source since there will be little translation for most of the proteins until maturation. Oocytes can be induced to mature by progesterone. Mature oocytes resume the translation and waits for fertilization by a single sperm. The first cleavage occurs at about 100 minutes after fertilization. Subsequent cleavages are observed with 30 minutes intervals. Until cell cycle 13, cell division is synchronous and composed of only alternating S and M phases. At midblastula transition by stage 8, cell cycle slows down and becomes asynchronous. Then G1 and G2 gap phases are introduced to cell cycle and zygotic transcription starts.

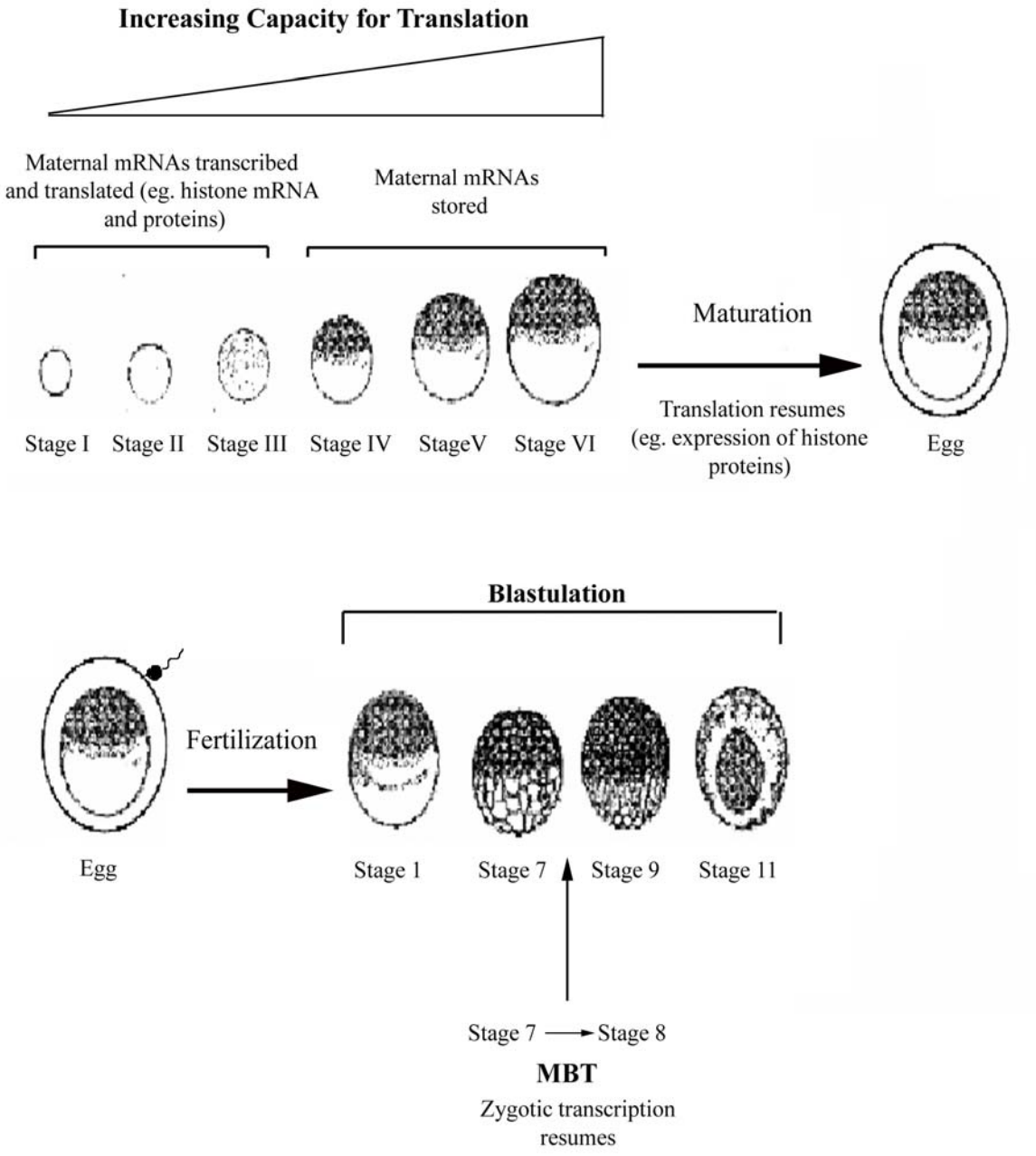
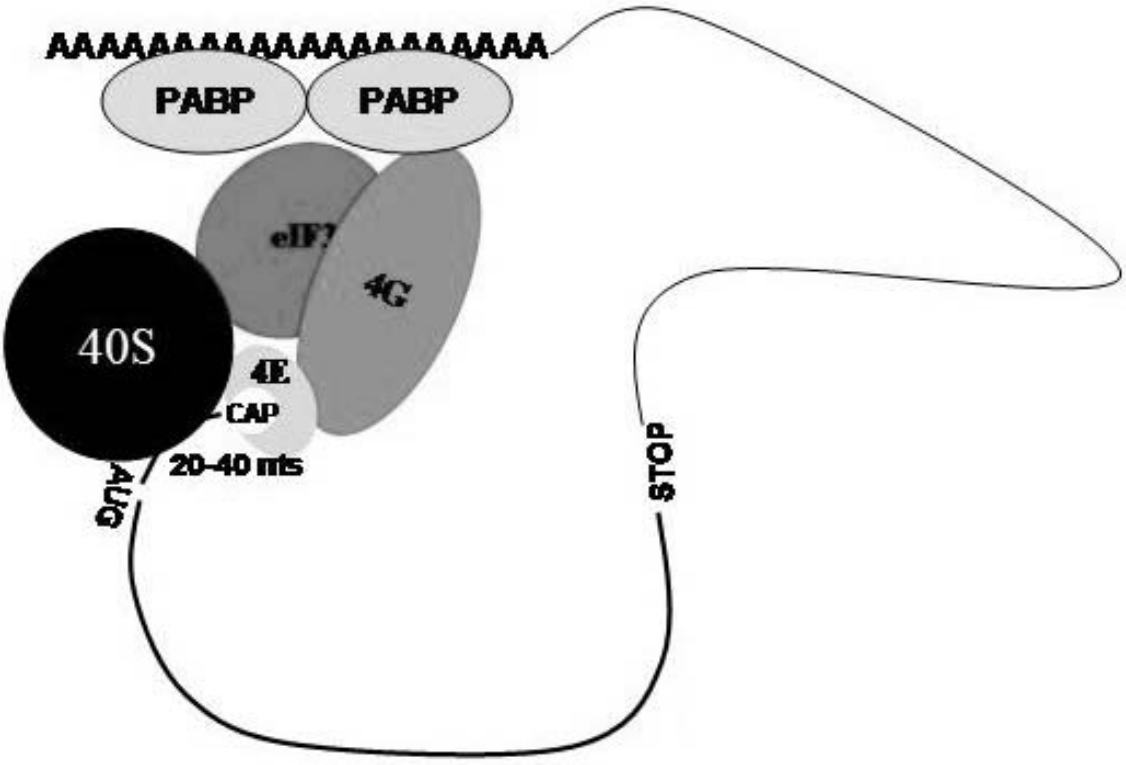

Increasing Capacity for Translation
Maternal mRNAs transcribed and translated (eg. histone mRNA and proteins)
Maternal mRNAs stored
Stage I
Stage II
Stage III
Stage IV
StageV
Stage VI
Maturation
Translation resumes (eg. expression of histone proteins)
Egg
Blastulation
Fertilization
Egg
Stage 1
Stage 7
Stage 9
Stage 11
Stage 7 → Stage 8
MBT
Zygotic transcription resumes

Table 1. Mammalian translation initiation factors.

Name	Number of Subunits	Size (kDa)	Function
eIF1	1	12.6	Promotes ribosome scanning for the initiation codon; destabilization of aberrant initiation complexes; monitor the fidelity of codon-anticodon interaction;
aIF1A	1	16.5	Promotes binding of met·$tRNA_i$ to 40S ribosomal subunit; helps ribosomal scanning with eIF1
eIF2	3	126	Forms eIF2·GTP·met·$tRNA_i$ ternary complex and catalyzes binding of ternary complex to 40S subunit in a codon independent manner; GTPase
eIF2B	5	261	Guanine nucleotide exchange factor which catalyzes exchange of eIF2·GDP to eIF2·GTP; thus required for eIF2 recycling
eIF3	12	~730	Contribute to all steps of initiation involving 40S subunit recruitment to mRNA via its interaction with eIF1, eIF4G, eIF5 and 40S subunit
eIF4A	1	44	RNA-dependent ATPase; RNA helicase activities depended on ATP hydrolysis and eIF4B; interacts with eIF4G
eIF4B	1	69.2	Promotes RNA helicase activity of eIF4A
eIF4E	1	26	Binding to 7'mGpppN cap structure; interaction with eIF4G
eIF4G	1	171.6	Function as scaffold protein; binding to RNA in sequence independent manner, PABP, eIF4E, eIF4A, eIF3; helps eIF3 to load 40S subunit to mRNA
eIF4F	1		Heterotrimer composed of eIF4E, eIF4A, eIF4G which binds to cap structure
eIF5	1	49	GTPase-activating protein that promotes hydrolysis of GTP to GDP in the ternary complex following the recognition of the start codon
eIF5B	1	139	GTPase activity; required for 60S ribosomal subunit joining

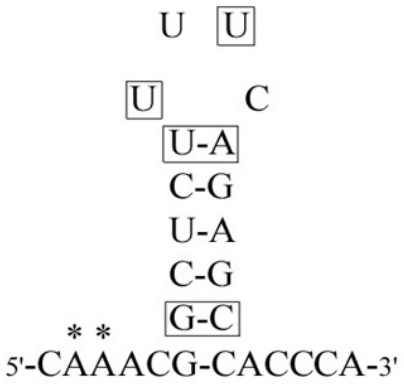
Figure 4. Closed Loop Model for Polyadenylated mRNAs. 7'mGpppN cap structure binds eIF4E and multiple PABP proteins simultaneously bind polyA tail. 5’ and 3’ ends of the mRNA are brought together by eIF4G via its interaction both with eIF4E and PABP. eIF4G also associate with eIF3 and eIF4A. eIF3-bound 40S ribosomal subunit will be recruited to mRNA and start scanning for the initiation codon in 5' to 3' direction. Possibly, eIF4A removes any secondary structure on the mRNA during the scanning process. Once initiation codon is reached, 60S ribosomal large subunit associates 40S small ribosomal subunit forming 80S ribosome which then engages in protein synthesis.



Polyadenylated mRNA

Figure 5. Mature histone 3' stem-loop structure. A) Human histone 3' end possess a conserved stem-loop flanked by five nucleotides. Highly conserved amino acid residues in the stem and loop are shown in blocks and stars present the consensus residues in the 5' flanking region. Those conserved residues are required for the binding of a specific factor, SLBP. **B)** *Xenopus* histone mRNA 3' stem-loop during oogenesis is followed by a short oligoA tail which will be removed at oocyte maturation yielding exact same structure as human histone mRNA stem-loop. Arrow shows where the degradation of the oligoA tail will stop.

A.


U U
U C
U-A
C-G
U-A
C-G
G-C
* *
5'-CAAACG-CACCCA-3'

B.

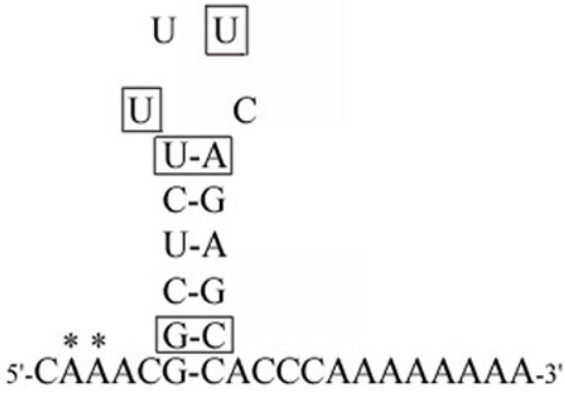
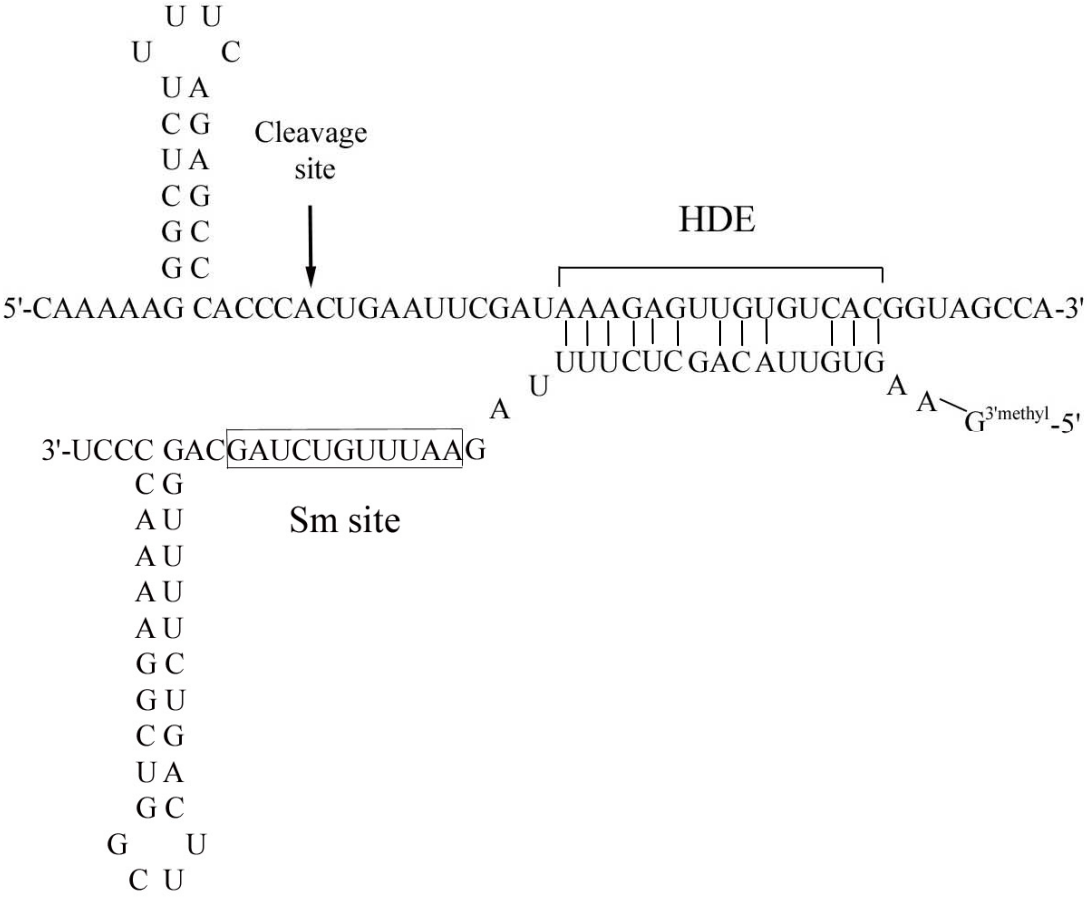

U U
U C
U-A
C-G
U-A
C-G
G-C
* *
5'-CAAACG-CACCCAAAAAAAA-3'

Figure 6. Histone H2a 614 pre-mRNA. Mouse histone H2a pre-mRNA consists of two cis-acting elements, involving one stem loop and histone downstream element (HDE). Stem loop binds SLBP and HDE hybridize with 3' end of U7 snRNA, altogether they will promote cleavage reaction. This reaction will yield to a translationally potent mRNA ending in stem loop structure.


U U
U C
U A
C G
U A
C G
G C
G C
Cleavage
site
HDE
5'-CAAAAAG CACCCACUGAAUUCGAUAAAGAGUUGUGUCACGGUAGCCA-3'
UUUCUC GAC AUUGUG
U
A
A
A
G3'methyl-5'
3'-UCCC GAC GAUCUGUUUAA G
C G
A U
A U
A U
A U
G C
G U
C G
U A
G C
G U
C U
Sm site

CHAPTER II

IDENTIFICATION OF A TRANSLATIONAL ACTIVATION MOTIF IN THE SLBP N-TERMINUS

Introduction

Efficient translation of polyadenylated mRNAs requires both the cap and the polyA tail (Gallie 1991). The role of the polyA tail in translation is reasonably well understood in both mammals and yeast. The cap is bound by the factor eIF4E which interacts with the large scaffold protein, eIF4G. In mammals the polyA tail is bound by PABP in the cytoplasm, and PABP also interacts directly with eIF4G (Imataka et al. 1998). Direct biochemical experiments using purified yeast PAB1, eIF4E and eIF4G demonstrate that these three factors can effectively circularize the mRNA (Wells et al. 1998). eIF4G also binds the factors eIF4A (Imataka and Sonenberg 1997), a helicase which assists in scanning the 5' UTR, and eIF3, a multi-subunit factor that also binds to the 40S ribosomal subunit, resulting in recruitment of the other factors required for initiation of translation.

Metazoan replication-dependent histone mRNAs differ from other eukaryotic mRNAs in that they do not end in a polyA tail. Instead these mRNAs end in a conserved 26 nucleotide sequence that contains a stem loop (Dominski and Marzluff 1999). The structural difference between the 3' end of histone mRNAs and polyadenylated mRNAs led our lab to investigate whether translation of histone mRNA occurs via a similar mechanism like

polyadenylated mRNAs. Since SLBP replaces PABP in the 3' UTR of histone mRNAs, our lab investigated translational involvement of proteins that bind to 3' UTR of histone mRNA.These studies showed that the protein that specifically binds to the stem loop, stem loop binding protein (SLBP), is required for histone mRNA translation in the cytoplasm (Sanchez and Marzluff 2002).

Sanchez and Marzluff identified a 15 amino acid region in the amino terminal domain of xSLBP1 which is essential for activation of histone mRNA translation (Sanchez and Marzluff 2002). This region lies between amino acids 68 and 83 in the N-terminus. Deletion or mutation of this sequence completely abolished the activity of xSLBP1 in translation. A region of xSLBP1 containing this sequence fused to xSLBP2 converted xSLBP2 to a translational activator, and an MS2-SLBP fusion protein activated translation of a reporter mRNA ending in an MS2 site, indicating that the RNA binding domain (RBD) of xSLBP1 was not required for translation (Sanchez and Marzluff 2002; Gorgoni et al. 2005).

In this chapter I further characterize the critical amino acids in xSLBP1 between amino acids 68 and 83 in the N-terminus required for translation as well as its position relative to the RBD that is important for translational activity of xSLBP1. I also report that similar results are obtained with hSLBP mutants, leading me to hypothesize the presence of a highly conserved motif among vertebrates: the proclaimed motif is DWX_3EE in vertebrates.

Materials and Methods

Plasmid Constructions

Wild type xSLBP1 and hSLBP were cloned into a modified p64T vector pXFRM as previously described (Wang et al. 1999; Dominski et al. 2001). The xSLBP2 clone was obtained from Richardo Sanchez (Sanchez and Marzluff 2002). Single or double amino acid substitutions to alanine or phenylalanine or Glycine (xD72A, xW73A, xW73F, xGS74-75A_2, xV77A, xV77G, xE78A, xE79A, xE78-79A_2 for xSLBP1 and hW75A, hE80-81A_2 for hSLBP) were generated by site directed mutagenesis in PCR reactions with either xSLBP1 or hSLBP as the template. Nucleotide sequences of the mutated amino acids were centered in the middle of the forward and reverse primers with 10-12 flanking nucleotides. PCR reactions were performed according to QuikChange® II Site-Directed Mutagenesis Manual from Stratagene. xSLBP1 internal deletions Δ14 and Δ37 were also generated by PCR with 5-P tagged primers as SLBP1 being the template.

Chimeric mutant xSLBP1 (Δ37) + N2(37) was generated by deletion of 37 amino acids between 89th and 126th amino acids in the N-terminus of wt xSLBP1 and fusion of 35 amino acids of xSLBP2 (90th and 126th aa) at a matching location. xSLBP1 in pXFRM vector was used as the template in the PCR reaction with primers introducing 5' and 3' NdeI sites to delete 35 amino acids. 35 amino acids from xSLBP2 were amplified with primers encoding 5' and 3' NdeI sites by PCR. After digestion with NdeI and treatment with calf intestinal phosphatase (CIP), xSLBP1 Δ35 and PCR products were ligated. The NdeI sites added an extra two amino acids resulting in 37 amino acid introduction into xSLBP1 N-terminus. xSLBP2 (Δ89) + N1 (1-89) chimera was generated using similar approach. xSLBP2 in pXFRM vector served as the template to delete amino acids 1 through 89 from the N-

terminus of xSLBP2 with primers introducing 5'NcoI and 3'NdeI. Amino acids 1-89 of xSLBP1 was amplified using primers containing the same restriction enzyme sites in a PCR reaction. After digestion, both products were ligated. xSLBP2 (Δ37) + N1 (1-89) deletion chimera was generated in a two-step cloning. First, xSLBP2 N-terminus was deleted adding 5' and 3'NcoI sites by PCR. Ligated products had 1 shift in the open reading frame. Sequenced DNA was then used as the template to clone xSLBP1 (1-89) N-terminus. 5' and 3'NcoI and 1 nucleotide 5' to start codon of N1 (1-89) were introduced with the primers by PCR. Digested products were ligated. The sequences of all clones were verified for the insertions and the orientations by automated sequencing.

Firefly luciferase constructs ending either with stem loop (Luc-SL) or tetra loop (Luc-TL) or poly A_{50} tail (Luc-polyA_{50}) and renilla stem loop (Ren-SL) under the control of T7 promoter have been described previously (Gallie et al. 1996; Sanchez and Marzluff 2002). Also, cloning of polyadenylated chloramphenicol acetyltransferase (CAT-A_{50}) was described in Sanchez and Marzluff (2002).

In Vitro Transcription

Luc-SL, Luc-TL, Luc-polyA_{50} and CAT-A_{50} bearing plasmids were transcribed with T7 RNA polymerase in the presence (for oocyte experiments) or absence (for reticulocyte lysate experiments) of a cap analogue as previously described (Sanchez and Marzluff 2002). pXFRM vectors encoding hSLIP1, SLBP and SLBP mutants were linearized with EcoRI or AflII prior to transcription and transcribed with Sp6 RNA polymerase in the presence of a cap analogue (for oocyte experiments). Uncapped transcripts were transcribed according to

the manual (Sanchez and Marzluff 2002) while capped transcripts were generated by using MESSAGE mMACHINE™ Sp6 or T7 Kit from Ambion. Transcription reactions were treated with RQ1 DNAse (Promega) and mRNAs were purified on G-50 micro columns (Ambion), followed by phenol-chloroform extraction. Precipitated mRNAs were dissolved in dH_2O.

In Vitro Translation

MS2-hSLBP, MS2-hSLIP1, and SLBP variants were expressed from SP6 promoter in pXFRM vector in a micrococcal nuclease-treated reticulocyte lysate reaction (TNT Sp6 RRL system, Promega) containing ^{35}S-methionineand Sp6 polymerase at 30^{o}C in 90 minutes. *In vitro* translation assay was employed as in Sanchez et al. (Sanchez and Marzluff 2002). One third of the *in vitro* translation reactions were resolved by SDS-12% polyacrylamide gel electrophoresis (PAGE) and protein synthesis was monitored on a Storm 840 PhosphorImager screen. The remainder was mixed with fresh reticulocyte lysate and then used for testing the ability of the extracts to translate reporter mRNAs as described by Sanchez and Marzluff (2002).

In Vivo Translation Assay

Stage VI oocytes were collected from adult female *Xenopus Laevis*, African Clawed frogs (NASCO) and treated for 2 hours in 0.2% collagenase in OR-2 buffer at 27^{o}C. OR2 buffer contains 4.82g NaCl, 0.19g KCl, 0.15g $CaCl_2.2H_2O$, 0.20g $MgCl_2.6H_2O$, 1.19g Hepes,

0.12g $Na_2HPO_4.7H_2O$, 86.5 µl pyruvic acid (Sigma, P-1656), 1 ml 50mg/ml gentamycin and the pH was adjusted to 7.7 with NaOH tablets. After removal of the collagenase and several washes in OR-2 buffer, the oocytes were incubated at 18°C for about 16-18 hours to allow them recover. Stage VI oocytes were injected in the cytoplasm with 30nl of capped SLBP mRNAs (100 ng/µl) or/and hSLIP1 mRNA (100 ng/µl) and incubated in OR-2 solution for 16 hours at 18°C. Control oocytes were injected with 30 nl water. Then, oocytes were re-injected with 30 nl of capped Luc-SL, Luc-TL, or Luc-polyA mRNAs (100 ng/µl) mixed with 30 nl of capped Ren-SL mRNA. 16 hours later, 12 oocytes per test condition in 60 µl lysis buffer [20 mM Tris pH 7.5, 1 mM PMSF, 1X protease inhibitor cocktail (Sigma)] were harvested by sonication. Cleared lysates by centrifugation for 5 minutes at 4°C at 5000 rpm were used for luciferase assays and western blotting. For long-term samples are stored at -80°C and for short-term at -20°C

Luciferase Assay

Fire fly luciferase activities from *in vitro* and *in vivo* translation assays were measured with a Monolight 2010 luminometer in 1X luciferase passive lysis buffer (Luciferase Kit, Promega E1501) as described previously (Sanchez and Marzluff 2002). Activities of different SLBPs and hSLIP1 were calculated like that: The ratio of Luc-SL over Luc-TL in the water injected samples was set to 1. Activities of SLBPs were normalized by calculating the ratio of the value measured for Luc-SL over the value measured for Luc-TL. Normalized values were divided by buffer value resulting in the fold activation.

Western Blot Analysis

The lysates from the equivalent of four oocytes were diluted in 500µl 20 mM Tris (pH 7.5)-1 mM PMSF and centrifuged for 5 minutes at 14,000 rpm. The cleared supernatants were precipitated in 1 ml acetone at -20°C for 30 minutes and then centrifuged for 20 minutes at 14,000 rpm at 4°C. The pellets were resuspended in 1% SDS sample buffer and boiled for 5 minutes after a brief sonication. Proteins from one oocyte equivalent per sample were resolved on 12% SDS-polyacrylamide gel (PAGE) and transferred to nitrocellulose membrane. Membranes were blocked 1hr at room temperature (RT) with 1X PBST including 5% non-fat dry milk and 0.1% Tween 20 (PBSTM). Then membranes were incubated with appropriate antibodies diluted in 1X PBSTM for 2 hrs at RT and then were incubated with peroxidase-coupled secondary antibodies diluted in 1X PBSTM for 1 hr at RT. Detection of antibodies performed with ECL reagent (Amersham Pharmacia) and exposure to X-ray film (Kodak). Dilutions of antibodies were as follows: rabbit polyclonal anti- xSLBP1, anti-xSLBP2, anti-hSLBP, rabbit polyclonal anti-hSLIP1 1:500; horseradish peroxidase-conjugated either anti-rabbit or anti-mouse IgG 1:10000.

Results

Amino Acids Between 83 and 126 in The N-terminus of SLBP Serve as a Spacer Region

Initial experiments by Richardo Sanchez in our lab had defined a 15 amino acid region of xSLBP1 (amino acids between 68 and 83) that was required for translation (Figure 7). Deletion analysis in the N-terminal domain of xSLBP1 demonstrated that the amino terminal 68 amino acids were not essential for translation (Sanchez and Marzluff 2002). He

did not test whether there might also be a role for amino acids between 83 and 126, located between the translation activation region and the start of the RNA binding domain (RBD). I created mutants in xSLBP to address whether this region also plays a role in translation using *Xenopus* oocytes.

To investigate the possible roles of SLBP mutants in histone mRNA translation, I used two reporter mRNAs, shown in figure 8. The reporters ended either in the histone stem loop (Luc-SL) containing invariant nucleotides in the loop or in a mutated loop (Luc-TL). Luc-TL has the invariant nucleotides Us at positions 1 and 3 replaced by G and C, and this mutant can not bind SLBP anymore. The mRNAs are transcribed *in vitro* in the presence of a cap analog for in *vivo* micro-injection experiments. I determined the effect of various SLBP expressions on translation of those reporter luciferase mRNAs in *Xenopus* oocytes.

A schematic of the *in vivo* translation assay is shown in figure 9A. Stage VI oocytes were injected in the cytoplasm with capped mRNAs encoding SLBPs or with only buffer (dH_2O) as a control (T_1). Oocytes were incubated at 18^oC for 16 hours to allow them to express SLBPs. Then same oocytes were injected with equimolar amounts of either capped Luc-SL or capped Luc-TL mRNAs (T_2). 16 hours later (T_3), 12 oocytes per test condition were pooled and harvested. The deletion and/or chimeric mutants of xSLBPs are depicted in figure 10B. The luciferase activities were measured by luminometry and SLBP over-expressions were monitored by western blotting (Figure 9C and 9D). There was variability in the magnitude of the activation observed among different batches of oocytes from different frogs, but the qualitative effects of various SLBPs were still reproducible.

Figure 9B shows the SLBP proteins that I designed and used in those experiments. I deleted 14 or 37 amino acids starting at amino acids 89 from xSLBP1. The deletion of 37

amino acids brings the translation activation domain close to the RBD, which starts at amino acid 126. I also fused the first 89 amino acids of xSLBP1 to the RBD of xSLBP2 and to a site containing 37 amino acids before the RBD of xSLBP2. Finally I substituted the 37 amino acids immediately before the RBD of xSLBP2 for the 37 amino acids located in this position in xSLBP1 (Figure 9B). All of these proteins were expressed in the *Xenopus* oocyte (Figure 9C), after injection of the appropriate mRNA.

Deletion of 14 amino acids (89-102) from xSLBP1 had no affect on translation activity, but deletion of 37 amino acids (89-125) from xSLBP1 just after the translation activation region (xSLBP1 Δ37), effectively moving the translation activation region next to the RBD, abolished translation activity. Without any protein expression, there is a small activation of the Luc-SL reporter which we attribute to endogenous xSLBP1 in the oocyte. Expression of a protein that can bind the stem loop but not activate translation, competes for the binding of the endogenous SLBP1 and acts as a dominant negative. Indeed xSLBP1 Δ37, which still binds the stem loop normally, inhibited translation compared to expression of no protein. A similar result was obtained with xSLBP2 (Sanchez and Marzluff, 2002; Figure 9D). These results suggested that specific sequences in SLBP between amino acids 83 and 126 were not required, but a minimal appropriate space between the RBD and the translation activation region at amino acids 68-83 was important for translation activation (Figure 9D).

To confirm this interpretation, I constructed proteins that contained the SLBP2 RBD and C-terminal region with different portions of the xSLBP1 N-terminal region. Previously we had shown that a protein with the N-terminal of xSLBP1 fused to the RBD of SLBP2 (1-2-2) activated translation as effectively as xSLBP1 (Figure 7) (Sanchez and Marzluff 2002). I also fused the first 89 amino acids of xSLBP1 to the RBD of xSLBP2 [xSLBP2(Δ37) +

N1(1-89) mutant) or to 37 amino acids of xSLBP2 before the RBD [xSLBP2 +N1(1-89)]. The xSLBP2 (Δ37) + N1(1-89) mutant was inactive in translation (acted as a dominant negative), while xSLBP2 +N1(1-89) did activate the translation (Figure 9D).

To confirm the interpretation that a non-specific spacer region is required between the RBD and the translation activation region, I took the advantage of amino terminal sequence differences between xSLBP1 and xSLBP2. Those two proteins have significant sequence similarity only in their RBDs, and are totally different in their N-termini. I created chimeric mutants of xSLBP1 by replacing 37 amino acids with its equivalent sequence from xSLBP2 N-terminus [xSLBP1 (Δ37)N2] and of xSLBP2 by placing first 89 amino acids from xSLBP1 in front of xSLBP 37 amino acid followed by xSLBP2 RBD and C-terminus [xSLBP2 + N1(1-89)]. My aim with those two chimeric mutants was to analyze the sequence specificity of 37 amino acids. I reasoned that if specific sequences are required, then 37 amino acids from xSLB2 should block the translational activity of both of the mutants. Otherwise, this region may behave as a linker between translational activation region and RBD. My results demonstrated that those two mutants were able to stimulate translation as efficient as wild type xSLBP1 (Figure 9D). This result proves that the translation activation region must be separated from the RBD, but that there is not a requirement for specific sequences in this region. Since deleting 14 amino acids from this region resulted in an active protein, I can conclude that the distance between the RBD and the translation activation region, does not need to be a fixed length, but likely of sufficient length to allow SLBP to simultaneously bind both the 3' end of the mRNA and translation initiation factors.

Specific Residues between Amino Acids 68-83 of xSLBP1 are Essential for Translation

Comparison of the SLBP sequences from vertebrates reveals that there is extensive conservation of much of the SLBP sequence throughout the protein (Wang et al. 1996). However, when we included sea urchins and *Ciona* (sea squirt) in the comparison, then the similarity among the SLBPs is only in the RBD and in the region we previously identified as important for translation. The sequences in this region for human, mouse, *Xenopus*, sea urchin and *Ciona* are compared in Figure 10A. Extending the comparison to more evolutionarily distant organisms (*Drosophila* and *C.elegans*) results in loss of obvious similarity among the SLBPs other than in the RBD, and the *Drosophila* SLBP is not active in translation in *Xenopus* oocytes (Sanchez and Marzluff, unpublished). Sequence divergency between lower eukaryotes and higher eukaryotes likely accounts for the fact *Drosophila* SLBP does not work in our translation system. Unlike dSLBP, hSLBP stimulates the translation in *Xenopus* oocytes to some extent, but not as efficient as xSLBP1 (Figure12). So, those results suggest that eukaryotic proteins from vertebrates can at least partially execute translational activities in different vertebrate systems.

The sequence comparison suggests that the consensus core sequence required for translation between 68th and 83rd amino acids is: <u>DW</u>X_3<u>VEE</u> with the invariant amino acids in higher vertebrates underlined (Figure 10A). Based on this comparison, I made a number of point mutants in the consensus sequence in xSLBP1 (Figure 10C) and tested them for their activity in translation (Figure 10D and E). Initial analysis of some of the mutants was performed in a test-tube based *in vitro* translation assay as explained in Sanchez and Marzluff (Sanchez and Marzluff 2002). A schematic of *in vitro* assay is shown in Figure 10B. I used

the same luciferase reporters in the *in vitro* assay as I did in the *in vivo* micro-injection assays (Figure 8).

I determined the ability of some of the mutants to support translation of the reporter mRNAs in reticulocyte lysates (Figure 10). To measure the activity of the stimulatory activity of SLBP in reticulocyte lysates, we expressed the SLBPs from pXFRM plasmids, labeled with ^{35}S- methionine in the TNT reticulocyte lysate (RRL). The lysates containing SLBPs were mixed with fresh RRL and uncapped luciferase reporter mRNAs ending in either with stem loop or the tetra loop and translation assay was performed in the presence of ^{35}S-methionine to also label newly expressed reporters (Sanchez and Marzluff, 2002). A control polyadenylated uncapped mRNA that encoded CAT (CAT-A_{50}) was included in all assays as a non-specific competitor RNA and internal control to monitor the integrity of the translation machinery. The proteins labeled with ^{35}S-methionine were detected with a PhosphorImager and luciferase activity was also measured by luminometry (Figure 10 D and E).

Initial analysis of xSLBP1 mutants W73A, W73F, E78A, E79A, and EE7879AA and xSLBP2 as negative control *in vitro* demonstrated that these mutants all have a detrimental effect on translational activity xSLBP1 (Figure 10D and E). The results were visually observed by PhosphorImager analysis (Figure 10D) and quantified by luminometry (Figure 10E). xSLBP1 stimulates the translation up 3 fold over buffer levels (Figure 10D, compare lanes 3 and 4 to lanes 1 and 2 and Figure 10E). I did not observe any difference for expressions of Luc-SL and Luc-TL in buffer samples in the absence of any SLBP. Strikingly, the W73A mutation completely abolished activity in this assay, and the W73F mutation also reduced the translational activity. Similarly, mutation of either glutamic acid to alanine

(E78A and E79A) or both glutamic acids to alanines (EE7879AA) significantly reduced the activity of the xSLBP1 in the reticulocyte lysate. This initial *in vitro* analysis suggested that W73 residue was particularly important for translational activation.

I also tested the abilities of the mutant xSLBP1s to enhance translation of the reporter luciferase mRNAs ending either with histone stem loop or tetra loop in *Xenopus* oocytes and extended my mutational analysis to include the invariants xD72 and V77 as well as testing the xGS7475AA mutant where the G and S are not conserved (Figure 10C). Synthetic mRNAs encoding xSLBP1 mutants were injected into stage VI oocytes to express the SLBP. Control oocytes were injected with buffer or with mRNA encoding xSLBP2 which is inactive in translation. 16 hours later, the oocytes were injected with a luciferase mRNA ending in either a histone stem loop or tetra loop (see Figure 9A for the schematic of the *in vivo* assay). Luc-TL can not bind SLBP, thus allows us to measure the basal level of translation. Luciferase activities were measured 16 hours later (Figure 11A). Expression of all the proteins was monitored by Western blotting (Figure 11B) and they were expressed at similar levels.

Similar to the results in reticulocyte translation system, point mutations in the conserved residues reduced or abolished translational activity of xSLBP1 *in vivo* (Figure 11A). Luc-TL can not bind SLBP, thus allowing us to measure the basal level of translation. Again there is a small stimulatory affect of Luc-SL over Luc-TL in buffer injected samples, since oocytes contain free xSLBP1. Mutation of the conserved aspartic acid to alanine (xD72A), the tryptophan to phenylalanine or alanine (xW73F and xW73A), the valine to glycine or alanine (xV77G and xV77A), or the conserved glutamic acid amino acids to alanines (xE78A, xE79A, and xEE7879AA) abolished the translational stimulatory activity

of xSLBP1. Those amino acid residues are 100 % conserved in higher eukaryotes such as in human, *Xenopus*, and mouse (Figure 11A). In contrast, mutation of the non-conserved amino acids glycine and serine to alanine (xGS7475AA) did not effect the ability of SLBP to enhance translation of the stemloop reporter mRNA. xGS7475 is naturally replaced by alanine and serine in human and mouse SLBPs. These results demonstrate that each of the conserved amino acids was essential in oocyte and reticulocyte lysate systems. Variant amino acids do not contribute to translational activity of xSLBP1. Expression of xSLBP2 also does not support this activity as shown previously (Sanchez and Marzluff 2002). My results suggest that previously identified translation activation region bears a novel translation motif: DWX_3VEE.

A Uniform Translational Motif is Conserved among Higher Eukaryotes

The translational activation region of human SLBP is the same as *Xenopus* one, except G74 is replaced by an A. Our lab previously observed that hSLBP is also capable of stimulating translation of reporter mRNAs ending in stem loop in reticulocyte lysate system and in oocytes (Sanchez and Marzluff 2002). I also investigated the activities of hW75A and hE8081AA mutants *in vivo* to explore if the critical amino acid residues identified in *Xenopus* SLBP1 serve as a uniform translational motif in vertebrates.

Although, I am able to measure significant amount of translational stimulatory affect of hSLBP in oocytes, hSLBP can not stimulate the translation of reporter mRNAs to the similar levels as xSLBP1 does (Figure 12B, compare ~2.5 fold activity of hSLBP to ~6 fold activity of xSLBP in figure 9D and figure 11A). This might be due to lower capacity of

hSLBP to interact with the rest of the *Xenopus* translation machinery. However, hSLBP mutants W75A and EE8081AA did not stimulate the translation of reporter mRNAs ending in stem loop (Figure 13B). hW75A acted as a dominant negative probably due to its competition with endogenously already present xSLBP1, because the activity measured from those oocytes were below the activity measured from buffer injected oocytes. A similar result was obtained for *Xenopus* W73A mutant (see Figure 11A). The hEE8081AA has significantly reduced amount of translational activity. These results are parallel with what I observed for *Xenopus* SLBP mutants W75A and EE7879AA. Based on those observations, I conclude that the motif **DWX$_3$VEE** is a conserved uniform translational motif in vertebrates.

Discussion

There are two sets of cellular mRNAs in metazoans that are classified by their 3' UTRs. The majority of the eukaryotic mRNAs terminates in a poly A tail whose length varies between 50 and 200 adenosines (Hershey et al. 1996). At least 12 adenosine stretches in polyA tail are required to bind PABP and several PABPs may simultaneously associate with a polyA tail. The second set of mRNAs contains cell cycle-dependent histone mRNAs, which end in a highly conserved stem loop (Dominski and Marzluff 1999). The stem loop binds SLBP (Wang et al. 1996), and thus replaces PABP in the mRNP. SLBP likely acts like PABP to stimulate histone mRNA translation (Sanchez and Marzluff 2002). Sanchez and Marzluff demonstrated that a 15 amino acid region in the N-terminus of xSLBP lying between 68th and 83rd positions was responsible for the stimulatory effect of SLBP *in vivo* and *in vitro*. Although they demonstrated the evidence that those 15 amino acid stretch was important,

they did not test whether the region between 89th and 126th (just near RBD) is required for the stimulatory effect of the SLBP or identify which specific residues were important.

In this chapter, I first provide evidence that there is a specific spacing between translation activation region (starting from 68-83) and RBD (starting at amino acid 126). The spacing is not sequence dependent and can be as small as twenty amino acids. Secondly, I identified the critical amino acids in the previously identified translation activation region and my results demonstrate the presence of a uniform translation activation motif between amino acids 68th and 83rd as follows: DWX_3VEE.

A Specific Spacing is Required between Translation Activation Region and RBD

Luc-TL mRNA allowed me to measure the basal level of translation since it can not bind any SLBP with its mutated stem loop. When I introduced SLBPs that bound to Luc-SL 3' end, I was able to observe the translational effect of those SLBPs on Luc-SL mRNAs and compare it to basal level of translation measured from Luc-TL. Since there is some endogenous xSLBP1 in the oocyte, the Luc-SL reporter is activated a small amount compared to the Luc-TL reporter in the absence of any exogenous SLBP. Expression of an inactive SLBP which can bind the stem loop results in inhibition of Luc-SL and hence these proteins act as dominant negatives. Note that xSLBP2, which does not stimulate translation of histone mRNAs, represses it down to the level of the Luc-TL (Figure 9D, xSLBP2 bar) (Sanchez and Marzluff 2002). This is due that xSLBP2 has the same binding affinity for stem loop as endogenous xSLBP1 (Wang et al. 1999). Since I over-expressed xSLBP2 from

exogenous mRNA in the oocyte, xSLBP2 competed for binding to stem loop due to being in excess amount, preventing endogenous xSLBP1 from activating the Luc-SL reporter.

My mutational analysis showed that translational activation region in the N- terminus of SLBP must be separated from the RBD by as much as 25 amino acids to exert its stimulatory effect on translation of the Luc-SL reporter. Immediate fusion of first 89 amino acids from xSLBP1 containing activation region to RBD in xSLBP1 and xSLBP2 resulted in full loss of translational activity *in vivo* and those proteins acted as dominant negatives[Figure 9D, xSLBP1 Δ37 and xSLBP2 (Δ37) +N1(1-89)]. This was surprising, because first deletion of identified 15 amino acid region (between 68^{th} and 89^{th} amino acids) in the full length xSLBP resulted in total loss of translational activity; secondly deletion of first 68 amino acids in the N-terminus of xSLBP did not affect the translation activity while deletion of further 13 amino acid (Δ89) did; third whole N-terminus fusion to xSLBP2 RBD and C-terminus resulted in full stimulation of reporters ending in stem loop (Sanchez and Marzluff 2002), suggesting that those 15 amino acid was sufficient for stimulation of histone mRNA translation. In fact, the 15 amino acid region (68-83) only needs to be spaced from RBD and any sequence can serve as a linker since replacement of 37 amino acids between 89^{th} and 126^{th} by 37 amino acids from xSLBP2 did not affect the translational activity [Figure 9D, xSLBP1 (Δ37)N2 and xSLBP2 +N1(1-89) bars]. This spacing can be minimized to 23 amino acids (Fgure 9D, xSLBP1 Δ14 bar).

It is likely that the spacing is essential, since the SLBP must be bound to the mRNA through RBD-SL interaction. Since the translation activation region is likely to bind initiation factors at the 5' end of the mRNA, the spacing may provide both the appropriate space, as well as flexibility to allow SLBP to function in translation. I suggest that this flexibility may

help SLBP to easily interact with the translation machinery and to find its target proteins involved in activated translation of histone mRNAs.

A Highly Conserved Motif is Required for Translation Activation by Vertebrate SLBPs

I used *in vitro* assay in addition to the *in vivo* assay to identify the critical amino acids in previously described translational activation region (Sanchez and Marzluff 2002). The *in vitro* assay (Reticulocyte lysate system) was established to have synthetic mRNAs competing for initiation factors, making it more similar to cellular environment, and allowing me to measure the contribution of the 3' end to the translational activity. Since the reticulocyte lysate is enriched for eIF4E activity which enhances the translation of messages in cap-polyA dependent manner (Iizuka et al. 1994; Michel et al. 2000), I used uncapped mRNAs to only measure the effect of 3' stem loop with its bound proteins. In additional to my reporter mRNAs, I included CAT-A_{50} mRNA as a competitor and internal control to monitor the integrity of the translation machinery in reticulocyte lysate. To ensure that all the SLBP molecules were active in RNA binding activity, I synthesized the various SLBPs in the reticulocyte lysate prior to *in vitro* translation assay.

Both the i*n vitro* and *in vivo* systems demonstrated that each invariant amino acids in the translation activation region among higher vertebrates (Figure 10A) were necessary for translational activity of xSLBP, including amino acids xD72, xW73, xV77, xE78, and xE79 (Figure 10E and 11A). In contrast, mutations of invariant amino acids such as GS7475 did not affect the stimulatory effect of xSLBP1 (Figure 11A). In parallel we observed similar effect for human mutants in the same region (Figure 12B), suggesting the presence of a

uniform translation activation motif among vertebrates in the previously identified region. This motif consists of DWX_3EE. Not surprisingly, xSLBP2 does not have any sequence similar to this motif in its N-terminus, since the sequence of whole xSLBP2 N-terminus being so different than xSLBP1. That may explain why xSLBP2 does not activate the translation of the reporters ending in stem loop in our experimental systems. This sequence is also not present in *Drosophila* SLBP which is not active in stimulating the Luc-SL reporter in the *Xenopus* oocyte and the reticulocyte lysate system (Ricardo Sanchez, personal contact).

How does the translation activation region function to stimulate translation of histone mRNA? One possibility is that this region of SLBP interacts directly with one of the initiation factors, such as eIF4GI, and stimulates translation in much the same way PABP does. Alternatively, this region could bind another novel protein specific to histone mRNA translation, and this complex could then stimulate translation. In the next chapter, I show that the translation activation motif does indeed interact with another functionally novel protein to promote histone mRNA translation.

Figure 7. Translational activities of different SLBPs. SLBPs contain three domains, including N-terminal, RBD, and C-terminal domain. xSLBP1 and xSLBP2 are also labeled as 1-1-1 and 2-2-2 referring to each domain, respectively. Sanchez and Marzluff demonstrated that SLBP N-terminus is responsible for translational activity in a mutational analysis (Sanchez and Marzluff 2002). However Gorgoni et al. 2005 showed that N-terminal domain could be separated from the rest of the protein and its fusion with MS2 protein yields the similar translational affect as full length xSLBP on a luciferase reporter ending with MS2-binding sequence at the 3' end (Gorgoni et al. 2005).

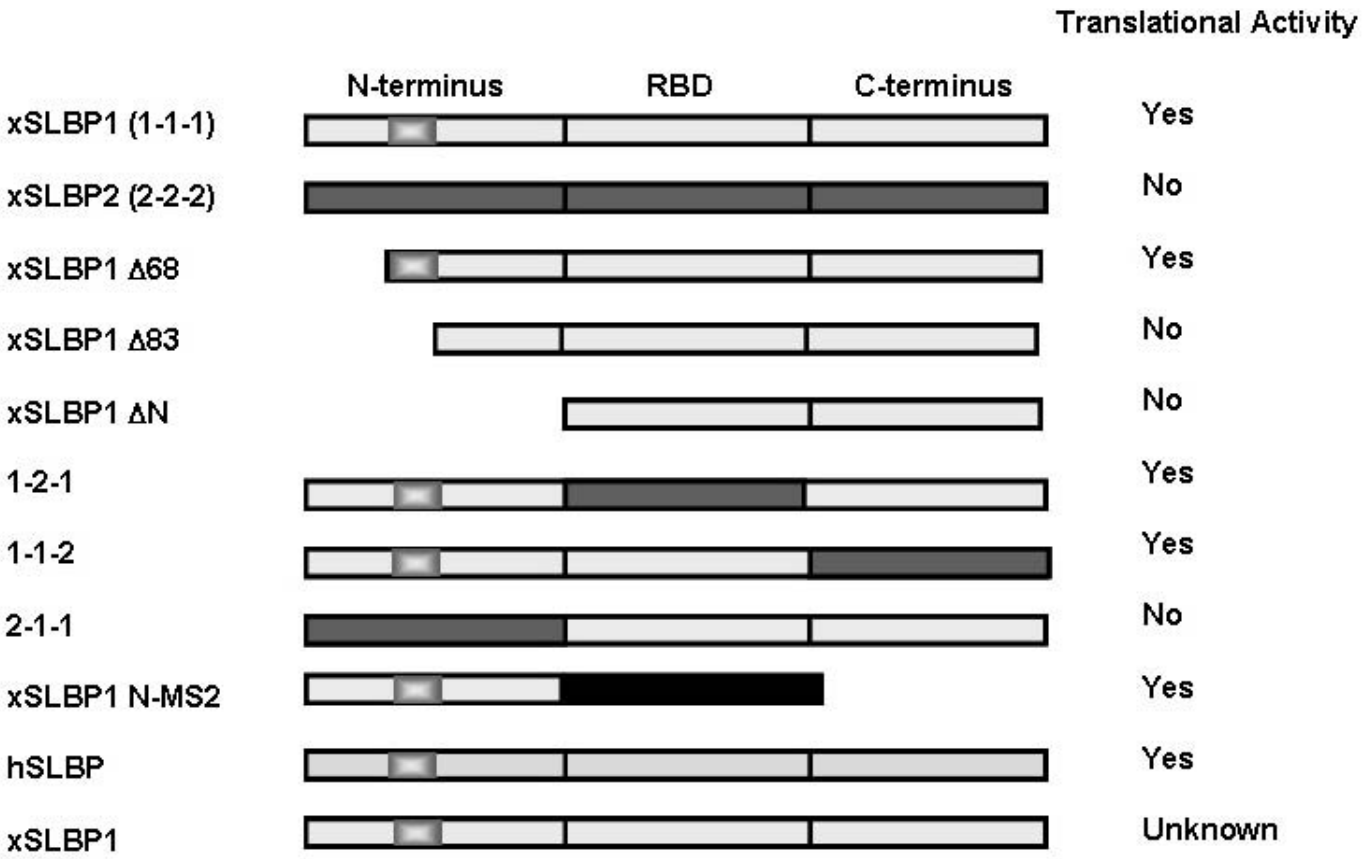

Translational Activity
N-terminus
RBD
C-terminus
xSLBP1 (1-1-1)
Yes
xSLBP2 (2-2-2)
No
xSLBP1 Δ68
Yes
xSLBP1 Δ83
No
xSLBP1 ΔN
No
1-2-1
Yes
1-1-2
Yes
2-1-1
No
xSLBP1 N-MS2
Yes
hSLBP
Yes
xSLBP1
Unknown

Figure 8. The structure of the 3' end of the luciferase reporter mRNAs. The Luc-SL mRNA ends with the highly conserved histone stem loop that specifically binds SLBP. Luc-TL is almost same as Luc-SL, except in the loop. Luc-TL has invariant nucleotides mutated in the loop, so this reporter can no longer bind SLBP and helps me to measure basal level of translation in the oocytes. Luc-polyA has 50 adenines at the 3' end. At least 12 A stretches is required for PABP binding.

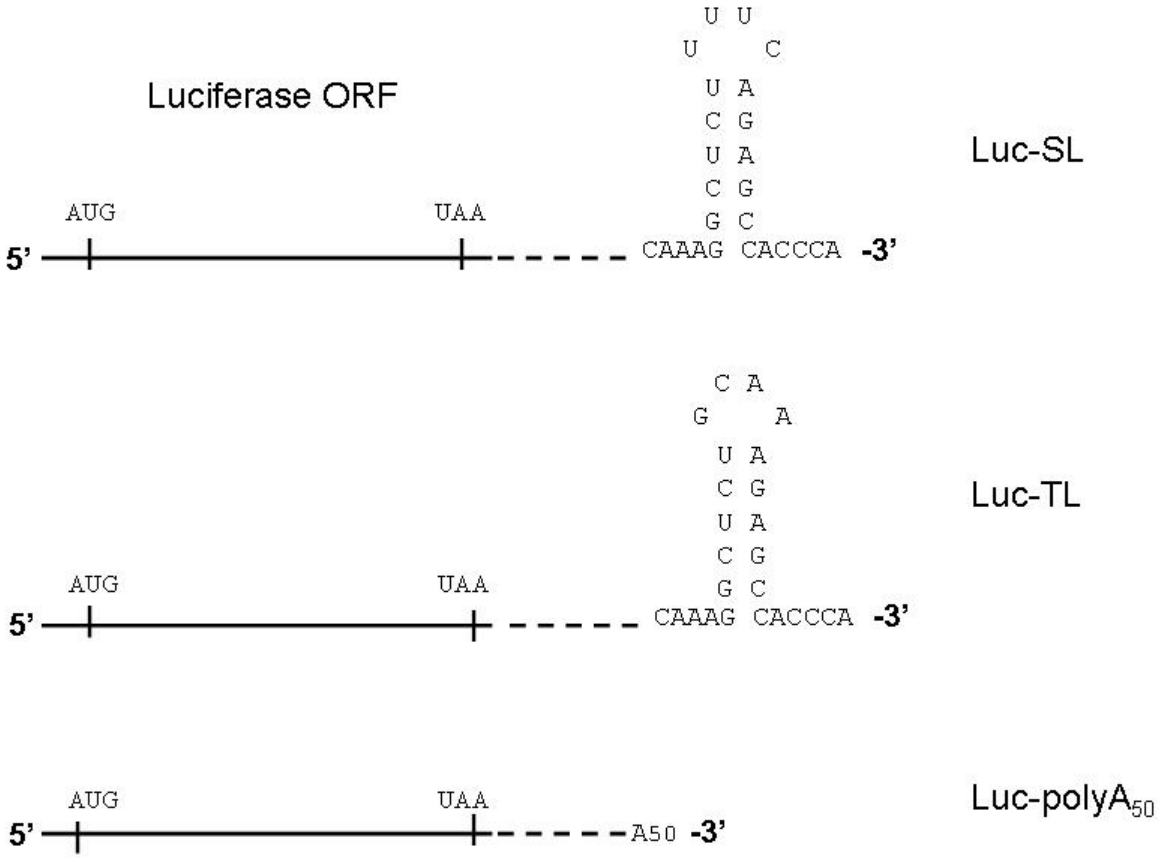
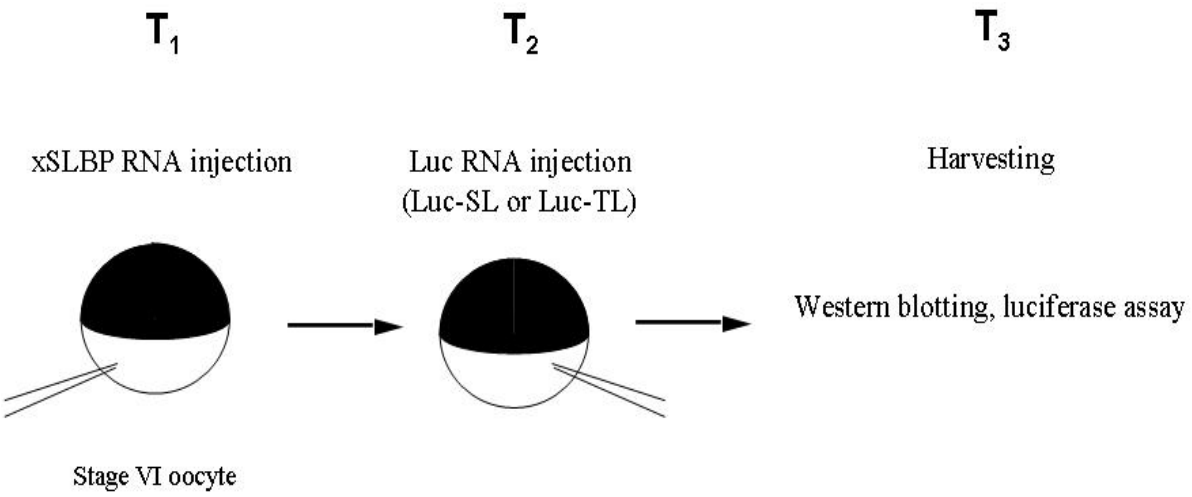
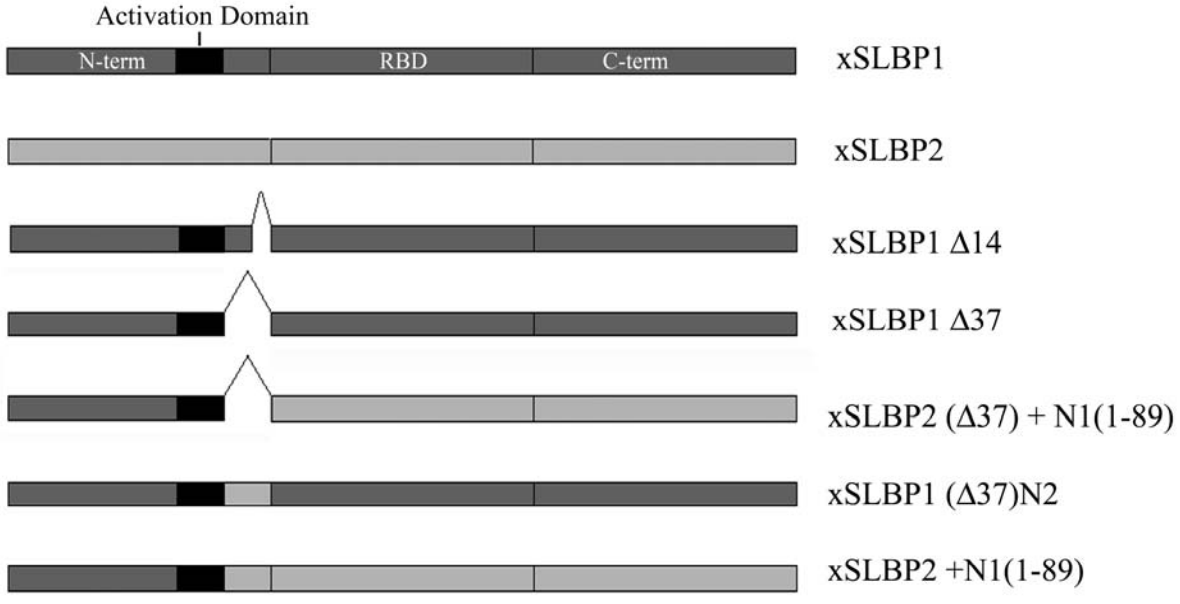

Luciferase ORF
AUG
UAA
5'
CAAAG CACCCA -3'
Luc-SL
Luc-TL
Luc-polyA50
A50 -3'

Figure 9. The region between amino acids 89 and 126 in the xSLBP required as a linker for translational activity. A) A schematic of the *in vivo* assay is shown. Stage VI *Xenopus* oocytes were injected either with buffer or 30 nl 100 ng/μl mRNAs encoding various SLBPs at T_1. 16 hours later at 18^{o}C (T_2), same oocytes were injected with one of the reporter, either with Luc-SL or Luc-TL (30 nl, 100 ng/μl). After incubation for an additional 16 hours at 18^{o}C (T_3), 12 oocytes per sample were pooled and harvested by brief sonication in lysis buffer. Same samples were assayed for both luciferase activities and for SLBP over-expressions by western blotting. **B)** Schematic of the SLBP mutants tested for translational activities. Several deletion and chimeric mutants were generated in PCR based reactions. **C)** Western blotting for SLBP mutants. xSLBP1 mutants were blotted with α-xSLBP1 and xSLBP2 mutants were blotted with α-xSLBP2 with a ratio of 1:1000. **D)** Translational activities of various SLBP mutants. Luciferase activities were measured by luminometry from three independent experiments. The ratio of the activities of Luc-SL and Luc-TL (Luc-SL/ Luc-TL) measured from buffer injected samples were set to 1. Then ratios of Luc-SL over Luc-TL from each test condition were adjusted according to that buffer ratio to calculate the fold activation:
[(Ratio of Luc-SL/Luc-TL from SLBP injected sample) / (Ratio of Luc-SL/Luc-TL from buffer injected samples)].
Averages of measurements from three independent experiments with their standard deviations are shown on the graph.

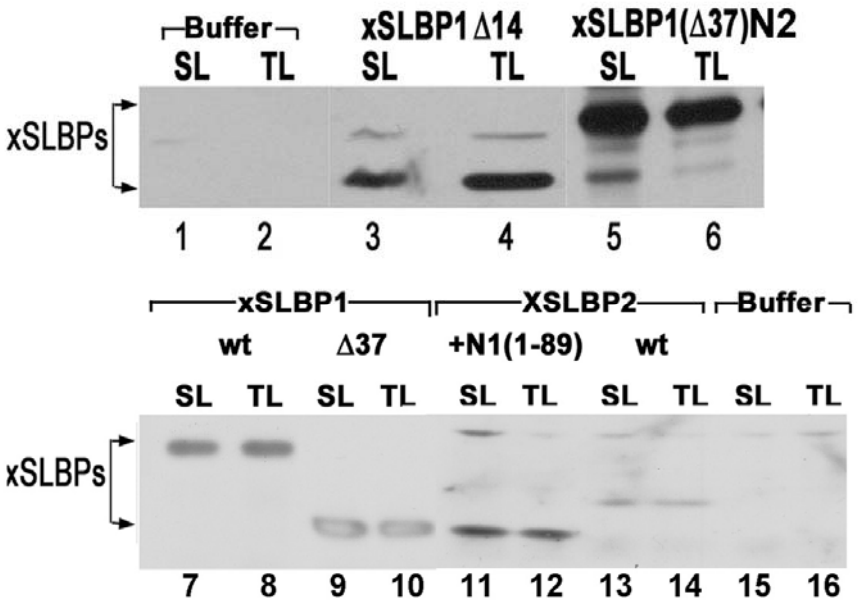
A


T1
T2
T3
xSLBP RNA injection
Luc RNA injection
(Luc-SL or Luc-TL)
Harvesting
Western blotting, luciferase assay
Stage VI oocyte

B


Activation Domain
N-term
RBD
C-term
xSLBP1
xSLBP2
xSLBP1 Δ14
xSLBP1 Δ37
xSLBP2 (Δ37) + N1(1-89)
xSLBP1 (Δ37)N2
xSLBP2 +N1(1-89)

C


Buffer
xSLBP1 Δ14
xSLBP1(Δ37)N2
SL TL SL TL SL TL
xSLBPs
1 2 3 4 5 6
xSLBP1
XSLBP2
Buffer
wt Δ37 +N1(1-89) wt
SL TL SL TL SL TL SL TL SL TL
xSLBPs
7 8 9 10 11 12 13 14 15 16

D

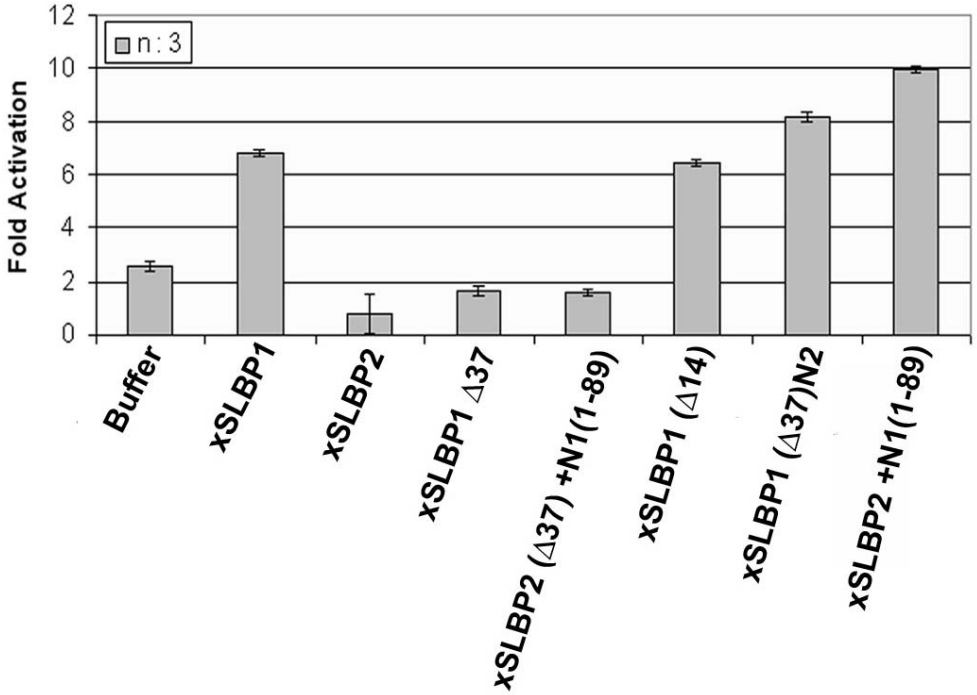
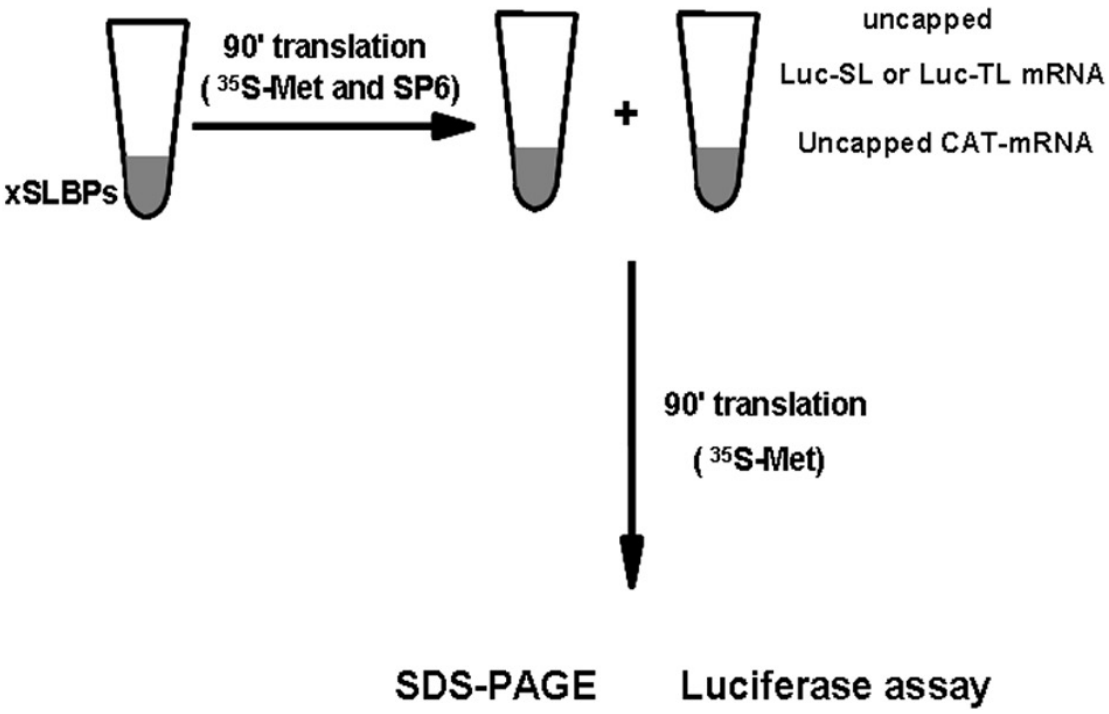

n: 3
Fold Activation
0
2
4
6
8
10
12
Buffer
xSLBP1
xSLBP2
xSLBP1 Δ37
xSLBP2 (Δ37) +N1(1-89)
xSLBP1 (Δ14)
xSLBP1 (Δ37)N2
xSLBP2 +N1(1-89)

Figure 10. A highly conserved motif in the N-terminus of SLBP is responsible for translational activity *in vitro*. A) Sequence comparison of SLBP N-termini in the translational activation region among different organisms. Sequence alignments were done by using Clustal X program. h stands for human, m for mouse, x for *Xenopus*, s. urchin for sea urchin , and d for *Drosophila* melanogaster SLBPs. Color code depicts the individually mutated amino acid residues in xSLBP1. **B)** A schematic of the *in vitro* translation assay is shown. An aliquot of rabbit reticulocyte lysate (RRL) was incubated either with buffer (dH_2O) or with one of the plasmid DNAs encoding SLBPs in the presence of SP6 polymerase and ^{35}S-methionine for 90 minutes at 30^{o}C to express SLBPs. Then reticulocyte-expressed SLBPs were mixed with 5 µl fresh RRL and either uncapped 100 ng/100 ng Luc-SL/CAT-A_{50} or Luc-TL/CAT-A_{50} mRNAs in the presence of ^{35}S-methionine. Translation of CAT-A_{50} was used as an internal control to monitor the integrity of the whole translation machinery and as competitor mRNA similar to competition observed within the cell. After incubation for 90 minutes at 30^{o}C, reactions were analyzed by SDS-PAGE and *in vitro* synthesized proteins were detected by autoradiography. An aliquot of the reactions were assayed for luciferase activities by luminometry. **C)** This panel represents the point mutants in the previously identified translation activation region. Mutants were generated by site directed mutagenesis method. Light grey letters shows the substituted amino acid residue(s) by alanine(s) if otherwise is not mentioned. **D and E)** The effect of xSLBP1 and various xSLBP1 mutants on the translation of mRNAs in the reticulocyte lysate was analyzed by first expressing the indicated SLBPs from supercoiled DNA in pXFRM in the reticulocyte lysate and then adding fresh lysate plus the uncapped reporter Luc-SL or Luc-TL mRNA together with the uncapped CAT-A_{50} RNA (Sanchez and Marzluff 2002). The products were analyzed by electrophoresis on 12% SDS-PAGE and detected by autoradiography (panel D) and the luciferase activity quantified by luminometry (panel E).

A

hSLBP	PRSR-CSDWASAVEEDE-MRTRVNK
mSLBP	PRSR-CSDWASAVEEDE-MRTRVNK
xSLBP1	PVAR-CKDWGSAVEEDEQLREKVDQ
s.urchin SLBP	KVSESSTDWAVQVEEFEEEEARAKR
ciona SLBP	DRKASSSDWFDQVEKSNELEEEAIK
dSLBP(1)	WAQEVRAEFGHS---DEASSSLNSS
dSLBP(2)	LDGVNEVKFERLVKEEKLKTPYKRR
dSLBP(3)	RNSKKSGNFRAHK-EEKRVRHNSYT

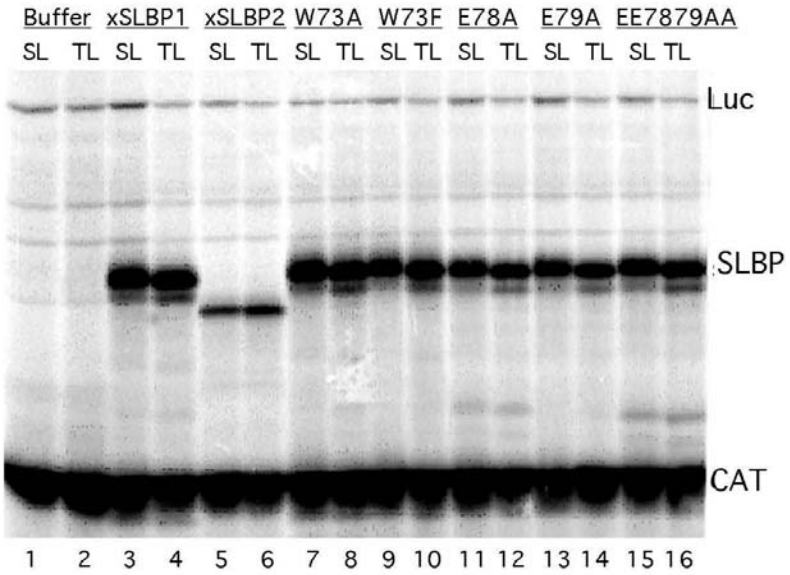
B



C

xSLBP1 wt	CKDWGSAVEEDEQ
xSLBP1 D72A	CKAWGSAVEEDEQ
xSLBP1 W73A	CKDAGSAVEEDEQ
xSLBP1 W73F	CKDFGSAVEEDEQ
xSLBP1 GS7475AA	CKDWAAAVEEDEQ
xSLBP1 V77A	CKDWGSAAEEDEQ
xSLBP1 V77G	CKDWGSAGEEDEQ
xSLBP1 E78A	CKDWGSAVAEDEQ
xSLBP1 E79A	CKDWGSAVEADEQ
xSLBP1 EE7819AA	CKDWGSAVAADEQ

D



E

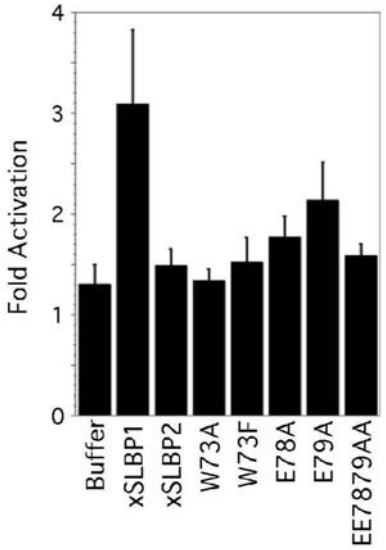
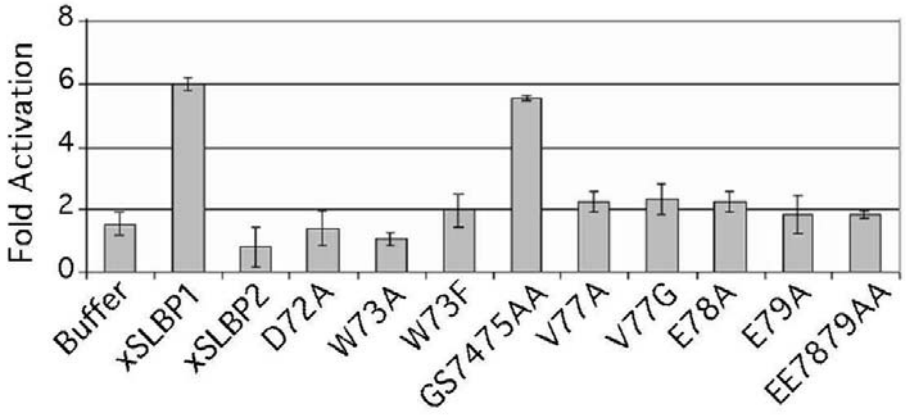


Figure11. Identification of the critical amino acid residues in translation activation region *in vivo.* A) Wild-type xSLBP1, xSLBP2 or mutants of xSLBP1 with the indicated amino acids changes (Figure 11C) were expressed in *Xenopus* oocytes by injection of synthetic capped mRNAs. 16 hours later the oocytes were injected with a reporter capped luciferase mRNA ending either in the stem loop or a tetra loop. 16 hours later the oocytes were harvested. Results are expressed relative to the expression of the Luc-TL mRNA which was set at one. Oocytes injected with buffer were the control, and they have higher activity than 1 because of the endogenous xSLBP1 in the oocyte. For each point mutant, 12 oocytes were pooled. The results are an average of three independent experiments. **B)** Proteins from the equivalent of 0.5 oocytes that had been injected with either the Luc-SL or Luc-TL reporter were resolved on 12% SDS-PAGE. The amount of wild type xSLBP1 and xSLBP1 mutants was determined by Western blot analysis. α- xSLBP1 was used with 1:1000 ratios.

A


Fold Activation
8
6
4
2
0
Buffer
xSLBP1
xSLBP2
D72A
W73A
W73F
GS7475AA
V77A
V77G
E78A
E79A
EE7879AA

B

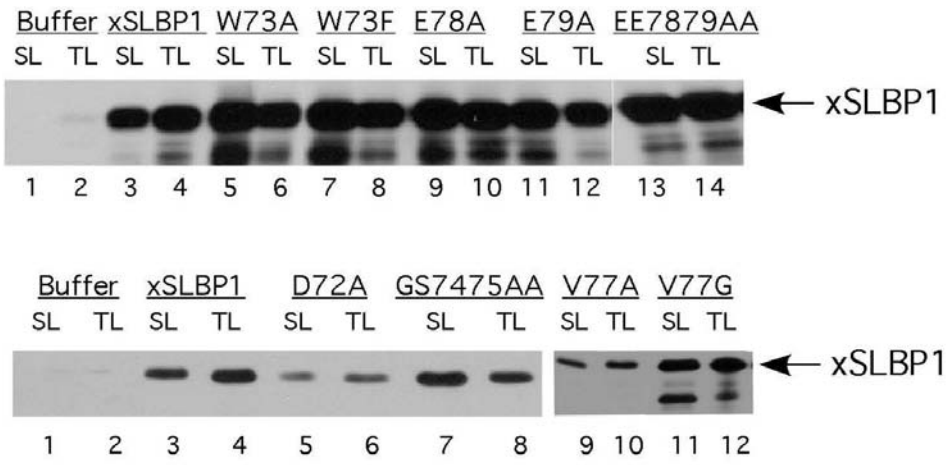
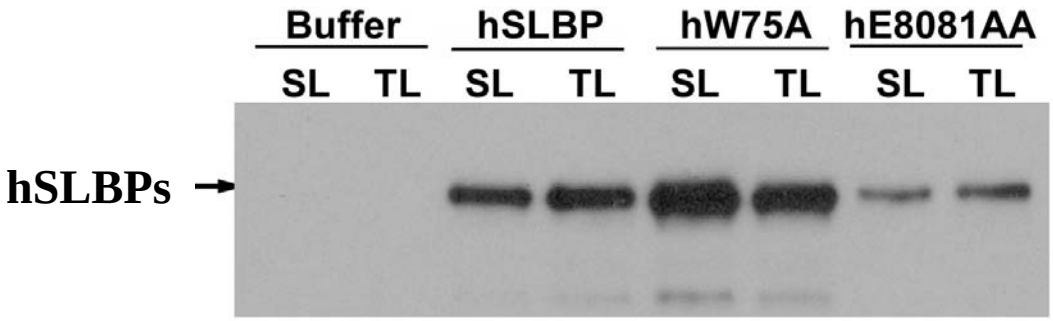

Buffer xSLBP1 W73A W73F E78A E79A EE7879AA
SL TL SL TL SL TL SL TL SL TL SL TL SL TL
xSLBP1
1 2 3 4 5 6 7 8 9 10 11 12 13 14
Buffer xSLBP1 D72A GS7475AA V77A V77G
SL TL SL TL SL TL SL TL SL TL SL TL
xSLBP1
1 2 3 4 5 6 7 8 9 10 11 12

Figure 12. Human SLBP mutants do not support translation *in vivo*. A) Western blotting. Lysates from 0.5 oocyte equivalent were resolved on 12% SDS-PAGE and blotted with α-hSLBP with a ratio of 1:1000. **B)** Stage VI oocytes were injected with 3 ng capped mRNAs expressing either wild type hSLBP or represented mutants on the figure (30 nl was injected from 100 ng/μl). 16 hours later, same oocytes were injected either with 3 ng capped Luc-SL or Luc-TL mRNAs and let to go another 16 hours. Then, they were harvested to be used in western blotting (A) and to measure luciferase activities by luminometry (B).

A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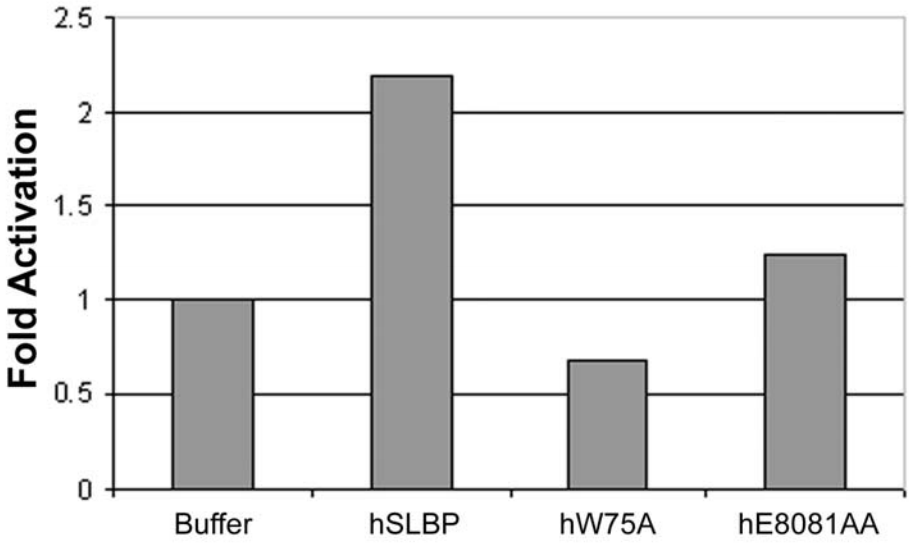
hSLBP
hW75A
hE8081AA
SL
TL
SL
TL
SL
TL
SL
TL
hSLBPs

B


Fold Activation
2.5
2
1.5
1
0.5
0
Buffer
hSLBP
hW75A
hE8081AA

CHAPTER III

SLBP-INTERACTING PROTEIN 1 (SLIP1) AS AN ADAPTOR FACTOR BETWEEN 5' AND 3' ENDS FOR HISTONE mRNA DURING ITS TRANSLATION

The majority of eukaryotic mRNAs consist of a cap structure at 5'end and polyA tail at the 3' end. Each of these structures synergistically contributes to the translation of polyadenylated mRNAs (Iizuka et al. 1994; Tarun and Sachs 1995). The cap is bound by the factor eIF4E which interacts with the large scaffold protein, eIF4G. In mammals the polyA tail is bound by PABP in the cytoplasm, and PABP also interacts directly with eIF4G (Imataka et al. 1998). Yeast PAB1, eIF4E and eIF4G can effectively circularize the mRNA (Wells et al. 1998). eIF4G also binds the factors eIF4A (Imataka and Sonenberg 1997), a helicase which assists in scanning the 5' UTR, and eIF3, a multi-subunit factor that also binds to the 40S ribosomal subunit, resulting in recruitment of the other factors required for initiation of translation.

The structurally unique histone mRNAs ends in a stem loop structure rather than a poly A tail and 3' stem loop binds SLBP. Although our lab demonstrated that SLBP can act as a PABP orthologue during translation by stimulating the translation of histone mRNAs (Sanchez and Marzluff 2002) and this activity is observed at the initiation step of the translation in cap-dependent manner (Gorgoni et al. 2005). There is currently no evidence whether histone mRNAs may circularize during translation initiation or utilize a similar translational mechanism to polyadenylated mRNAs. Attempts to demonstrate any

factors such as eIF4E, eIF4G, eIF3, and eIF4A failed (Ling et al. 2002; Gorgoni et al. 2005) have not been successful . In this chapter, I will introduce a functionally novel factor that interacts with both eIF4G and SLBP, thus allows the communication between 5' and 3' ends of histone mRNAs. This novel factor, SLBP interacting protein 1 (SLIP1) stimulates translation in the presence of SLBP. My results suggest that SLIP1 is an essential factor for cellular survival, which localizes to both the cytoplasm and nucleus. Thus SLIP1 may have additional functions in addition to its function in histone mRNA translation.

Methods and Materials

Plasmid Constructions

To generate yeast-two hybrid vectors encoding SLBPs, xSLBP1, xSLBPW73A, xSLBPΔ68-123, hSLBP, hSLBP SAVEE→A_5 were amplified in PCR reactions with primers introducing 5'EcoRI and 3' NcoI sites. Digested products were cloned into pGBT vector. hSLIP1 was cloned in frame just after GAL4-activation domain with the same strategy into pGAD10, using 5'EcoRI and 3' XhoI sites. Those constructs were used in directed yeast two-hybrid assays.

Full length eIf4GI and eIF4GII were generous gifts from Dr. M. Coldwell (Sussex, England) and Dr. N. Sonenberg (McGill University, Montreal). Different fragments of eIF4GI and eIF4GII were generated by PCR and 5' NcoI and either 3'NcoI (for 27-420 amino acids fragment) or 3'XmaI were introduced. Following digestion, eIF4G fragments were cloned into new version of pGBT, named as pGBTK7 which has a myc tag and T7 promoter 5' to cloning sites. eIF4G fragments were expressed from T7 promoter in the

presence of ^{35}S-methionine to test them in GST pull down experiments *in vitro*. To investigate whether those interactions were direct or mediated by another factor(s), fragments of eIF4GI encoding amino acids between either 27 and 170 or 164 and 420 were amplified by PCR with primers introducing 5' NcoI and 3'XhoI which was missing stop codon. Those fragments were cloned into pet28a+ vector in frame with 6 histidine residues at the C-terminus. eIF4GI 27-170 and 164-420 fragments tagged with histidine in pet28a+ were used to express them in E.coli and his tag was used to purify those protein through Ni+ column.

The Luc-polyA$_{50}$, Luc-SL, Luc-TL and CAT-A50 constructs have been previously described in Chapter 2.

hSLIP1 was cloned into pXFRM vector in a PCR reaction with 5' and 3' NcoI sites as well as being cloned into pGEX vector to generate GST-hSLIP1. Also, HA-hSLIP1 was generated by cloning hSLIP1 into pcDNA3 with primers encoding 5'HindIII and 3'XbaI sites by PCR. To generate the siRNA#1 resistant form of HA-hSLIP1 in pcDNA3.HA, we used those primers to introduce silent mutations: Forward primer 5'-GCTTGATGGGGGAGCCCTCTCGTGAAGAGTATAAAATCCAGTCC-3' and reverse primer 5' GGACTGGATTTTATACTCTTCACGAGAGGGCTCCCCCATCAAGC-3'. Amplified hA-hSLIP1 with silent mutations in the vector was treated with 1 μl DpnI enzyme at 37^{o}C for 30 minutes to digest maternal template DNA. Then, agarose gel-purified PCR products were transformed into DH5α cells and resistant form of HA-hSLIP1 clone was verified by automated sequencing.

The cloning of LacZ, myc-ZFP100 and HA-SLBP in pcDNA3 were described previously (Wagner and Marzluff 2006; Wagner et al. 2006)

Directed Yeast Two-Hybrid Experiments

Yeast transformations were carried out according to the standard yeast protocol, as described in Dominski et al 2001 (Dominski and Marzluff 2001). CG-1945 yeast strain was transformed with the pGBT bait plasmids encoding wild type and mutant forms of SLBPs under *TRP1* selection. Transformed yeast cells were then transformed with target plasmid pGAD10 bearing hSLIP1 under *TRP1* and *Leu* selection. Four individual colonies for each tested interaction were grown on the SD minimal media lacking tryptophan, leucine, and histidine with 20 mM 3-aminotriazole (3-AT). Same colonies were also analyzed for the protein expression by western blotting.

Western blot analysis

Proteins from HeLa cell lysates in Np40 lysis buffer [50 mM Tris.Cl pH 7.5, 150 mM NaCl, 0.5% Np40, 50 mM NaF, 1 mM PMSF, 1 mM DTT, 1X protease inhibitor cocktail (Sigma, P8340)] were directly used in western blot analysis. To prepare protein samples from yeast cells, transfected CG-1945 yeast cells were grown in 20 ml YPD media to an OD_{595} of 1.0 at 27°C at 220 rpm. Pelleted cells were resuspended in 0.5 ml lysis buffer [1M sorbitol, 0.1 M EDTA, 150 µg lyticase (Sigma L-5263) per 0.5 ml buffer] and incubated at 37°C for 90 minutes. Then, cells were pelleted and resuspended with 300 µl cracking complete buffer prepared according to BD Bioscience Yeast Manual. Resuspended Cells were incubated at 70°C for 15 minutes and then lysates were centrifuged at 13K rpm for 5 minutes at 4°C. Finally supernatant was added with 1 Mm PMSF and 100 µl 2X SDS-Sample buffer. 100 µl of protein samples from yeast were run on 12% SDS-PAGE. Proteins were transferred to a

nitrocellulose membrane. Membranes were blocked 1 hour at RT with 1X PBST including 5% non-fat dry milk and 0.1% Tween 20 (PBSTM). Then membranes were incubated with appropriate antibodies diluted in 1X PBSTM for 2 hours at RT and then were incubated with peroxidase-coupled secondary antibodies diluted in 1X PBSTM for 1 hour at RT. Detection of antibodies performed with ECL reagent (Amersham Pharmacia) and exposure to X-ray film (Kodak). Dilutions of antibodies were as follows: rabbit polyclonal anti- xSLBP1, anti-hSLBP, anti-eIF4GI 1:1000; rabbit polyclonal anti-hSLIP1 1:500; rabbit polyclonal anti-PTB 1:2000; mouse monoclonal anti-HA 1:1000 (Covance), anti-His 1:1000; horseradish peroxidase-conjugated either anti-rabbit or anti-mouse IgG 1:10000.

Protein preparations from *in vivo* translation assay following micro-injections into oocytes were previously described in chapter 2. Over-expressions of proteins were monitored by western blotting with α-xSLBP1, α-hSLBP, and α-hSLIP1.

In Vitro Translation

1-2 μg supercoiled pXFRM plasmids encoding xSLBP1, xD72A, xW73A, xGS7475AA, xE78A, xE79A, xEE7879AA, and hSLIP1 were incubated with 2 μl Sp6 polymerase for 90 minutes at 30°C in a 50 μl translation reaction which also contained 1 μl RNAsin**,** 6 μl 35S-methionine, 1 μl amino acid mixture (-methionine), 25 μl reticulocyte lysate (TNT kit, Promega). Expressed proteins were used in the GST pull down assays and observed by autoradiography.

In Vivo Translation Assay

Luciferase reporters ending in SL, TL, or polyA$_{50}$, as well as xSLBP variants and hSLBP variants were previously described in chapter 2. Capped mRNAs were transcribed and translation assays in oocytes were performed as described in chapter 2.

In vitro Translation Assay

In vitro translation assay was described in chapter 2. Here, MS2-hSLIP1 and MS2-hSLBP were tested for their translational activity in this assay. Luciferase measurements were performed as explained in chapter 2.

Protein expression and purifications

Expression and purification of hSLBP-his, GST-hSLBP-his, GST-hSLBPΔ1-68-his, GST-hSLBPΔ1-81-his, GST-hSLBPΔ1-126-his in Sf9 insect cells were previously described (Dominski et al. 1999; Ingledue et al. 2000). Glutathione S-transferase (GST)-fused hSLIP1 and GST were expressed from pGEX vector in BL21(λDE3). Bacteria were grown to an OD_{595} of 0.4 at 27^{o}C and induced overnight at 24^{o}C or 20^{o}C with 0.7 mM IPTG. Bacteria were pelleted and resuspended in lysis buffer [20 mM Tris.Cl pH 7.5, 10% Glycerol, 10 mM DTT, 150 mM NaCl, 1 mM PMSF, 1X Protease inhibitor cocktail (Sigma, P8340)], followed by sonication and centrifugation for 30 minutes at 15K rpm in a Sorvall RC-SB centrifuge. The clarified supernatant was incubated for 2 hours at 4^{o}C with 1 ml slurry Glutathione-Sepharose beads per 1 lt starting culture which is equilibrated with lysis buffer. After

washing three times with 1X PBS, proteins were eluted with 5 mM glutathione (Sigma G4251) dissolved in 50 mM Tris.Cl pH 8.0 at 4°C. Proteins were quantified by SDS-PAGE using coomasive staining, or a Bio Rad Assay in which concentrations were calculated according to BSA standards.

eIF4GI fragments 27-170-his and 164-420-his were expressed from pet28a+ in BL21(λDE3) with a similar strategy as explained above. Overnight induction was done with 0.5 mM IPTG at 18°C. Pelleted bacteria were lysed in Buffer A containing (20 mM Tris, pH 8.0, 200 mM NaCl, 10 mM imidazole, 10 mM β-ME, 1 mM PMSF, and 0.1% Triton; 30 ml per 1 liter of starting culture) and sonicated for 30 seconds 3 times on ice. The clarified supernatant was incubated with 600 ml slurry Ni+ beads (equilibrated in lysis buffer without PMSF, triton, and β-ME) per 600 ml starting culture for 3 hours at 4°C. After washing three times with Buffer B **(**20 mM Tris, pH 8.0, 300 mM NaCl, 20 mM imidazole), beads were transferred to column and then 200 µl fractions up to total 3 ml were collected with elution buffer containing 20 mM Tris, pH 8.0, 300 mM NaCl, and 250 mM imidazole. Beads were finally wiped off residual protein with 500 mM imidazole in elution buffer. Concentrations were calculated as written above.

GST Pull down Assay

8 µg E.coli expressed GST-hSLIP1 or 4 µg E. coli expressed GST was incubated with 8 µl ^{35}S-methionine labeled xSLBP proteins on ice for 30 minutes. Protein mixtures were then added with 40 µl (50% v/v) glutathione 4B sepharose beads (Amersham) equilibrated in 1X PBS and incubated for an additional 30 minutes on ice. Beads were washed three times

with 1X PBS supplemented with 150 mM NaCl. Proteins bound to beads were recovered in 40 µl 2X SDS-Sample buffer. Reciprocal experiments were performed with the same strategy as above in which 3-4 µg GST-hSLBP-his and its mutants expressed in baculovirus system (Sf9 insect cells) or 2 µg GST were utilized to pull down hSLIP1 expressed and labeled with ^{35}S-methionine *in vitro*. Pulled proteins were detected with PhosphorImager. Directed pull down of recombinant his-hSLBP (4 -5 µg) by recombinant GST-hSLIP1 was analyzed by western blotting with α-hSLBP. Pull downs of ^{35}S-methionine labeled eIF4GI, eIF4GII and their fragments were carried out with E.coli expressed GST-hSLIP1 as described above.

Direct interactions between eIF4GI fragments (27-170-his and 164-420-his) and GST-hSLIP1 or GST-hSLBP-his were analyzed with recombinant proteins in GST pull down assay. eIF4GI fragments and GST-hSLBP-his were expressed in E. coli and Sf9 cells, respectively and purified through Ni+ column. E. coli expressed GST-hSLIP1 was purified with glutathione 4B sepharose beads. 5-6 µg eIF4GI fragments were pulled down either by 5-6 µg GST-hSLIP1 or GST-hSLBP-his or 2-3 µg GST. Pulled eIF4GI fragments and also GST-hSLBP-his were monitored by western blotting with α-his. GST and GST-SLIP1 were observed by Coomassie staining.

Cell culture and transient transfection

Culturing 293T cells and transfection of cDNAs were carried out as previously described in Wagner et al. (Wagner and Marzluff 2006). Cell lines stably expressing HA-SLIP1 were isolated by first transfecting cells with HA-SLIP1 cloned into pcDNA3, and a stable population resistant to G418 (Gentamycin) was selected as described. The cells were

plated at low density and single clones were isolated. These clones were expanded and the cells expressing HA-SLIP1 were identified by western blotting with both α-HA and α-hSLIP1 and by immunofluorescence, and cultures with 100% of the cells expressing SLIP1 were utilized in the experiments. Immunofluorescence experiments were performed as previously described (Erkmann et al. 2005b).

Dual Immunostaing (IF)

HeLa cells (200.000 cells/well) grown on coverslips in 6-well plate were washed briefly with 1X PBS and fixed with 4% Formaldehyde in 1X PBS for 10 minutes at RT. Cells were permeabilized with 0.5% Triton X-100 in 1X PBS for 5 minutes at RT and then washed 3 times with 1X PBS. Coverslips were blocked for 30 minutes with 300 µl of 10% Normal Goat Serum diluted in 1X PBS at 37°C humidifier prior to incubation for 30 minutes at 37°C humidifier with both anti-hSLIP1 and anti-TRAP antibodies diluted 1:100 and 1:500 in 1X PBS, respectively. After washing coverslips 3 times with 1X PBS for 5 minutes at RT, coverslips were incubated with anti-rabbit TritC (red) and anti-mouse Gregon Green with a ratio of 1:500 in 1X PBS at 37°C humidifier for 30 minutes. Washed coverslips were then counterstained with 0.1 µg/ml 4, 6-diamidino-2-phenylindole (DAPI, Sigma-Aldrich) for 10 minutes at RT. Finally, coverslips were mounted with anti-fade and sealed. Anti-hSLIP1 antibody was raised in rabbit, so red represented hSLIP1 in dual IF. Anti-TRAP antibody was a generous gift from Dr. Christopher Nicchitta (Department of Cell Biology, Duke University, NC) and Green represented TRAP in dual IF.

Expression of the GFP-SL reporter

293T cells were transfected with either plasmids encoding LacZ (control) or myc-tagged ZFP100 using Lipofectamine 2000 (Invitrogen, CA) according to manufacturer's protocols. The following day, 2 μg of GFP-SL reporter was co-transfected with increasing amounts of plasmid encoding SLIP1 and decreasing amounts of plasmid encoding LacZ using Lipofectamine reagent (Invitrogen, CA). Note that there was a total of 4 μg of plasmid present in all transfections, thus allowing equivalent transfection efficiencies. Two days after the second transfection, cells were harvested and measured by FACS analysis and analyzed by using Summit Software (Fort Collins, CO). Total cellular RNA was analyzed for reporter mRNA by S1 nuclease protection assay as previously described (Wagner et al. 2006) or by Northern blot analysis of cellular RNA (2 μg) that was separated by electrophoresis using a 6% denaturing acrylamide gel.

Immunoprecipitation

HeLa cells stably expressing HA-hSLIP1 or regular HeLa cells were grown to 70-80% confluency on 150 mm plates containing Dulbecco's modified Eagle medium (DMEM) including 10% FBS, 1% Antibiotics, and 1% Geneticin. 4-6 plates were used for each experiment. Cells were cultured with no drug or 5 mM hydroxyurea was added to the cells 1 hour prior to lysis or 10 mg/ml RNAse A was included during incubation with the beads when stated in the experiments. Cells were lysed on ice for 15 minutes in NP-40 Lysis Buffer. Lysates were cleared by centrifugation at 13K rpm at 4^{o}C for 10 minutes. Lysates were then incubated with either α-HA (Covance) or α-Myc (Invitrogen) for stable HA-

hSLIP1 cell line and α-hSLIP1 for HeLa cells at 4°C for 3 hours. Protein G beads were equilibrated with PBS including 0.07% NP40 and resuspended in equal volume of PBS. 50 µl protein G beads were added to lysates and incubated for 1 hour at 4°C. The beads were washed with lysis buffer and resuspended in 2X SDS-sample buffer and the proteins were detected by western blotting.

Transfection with siRNAs and measurement of cell growth

Sequences of two different siRNAs against hSLIP1 purchased from Dharmacon, Inc. were as follows: siRNA#1 UUAUACUCCUCUCUACUGGdTdT; siRNA#2 AUGAUGGCGUAGCACAUGCdTdT. Control siRNA (C2) and hSLBP siRNA were previously described (Wagner et al. 2005). $1x10^5$ hela cells/well were plated into 24-well plate. siRNA transfections were performed with Lipofectamine 2000 (Life Tech.) according to manufacturer's instructions, using a final concentration of 90 nM of indicated siRNAs. Cells were harvested 48 hours after transfection. Levels of the proteins were determined by Western blot analysis (Figure 21A, left).

For rescue experiments and growth analysis, $2.5x10^5$ HeLa cells/well in 6 well-plate were co-transfected with the combinations of 180 nM control C2 siRNA or hSLIP1 siRNA#1, and 500 ng of siRNA#1 resistant form of HA-hSLIP1*, or GFP expression constructs. Transfections were performed with Lipofectamine Regular (Life Tech.) according to manufacturer's instructions. At 24, 48, and 72 hours post-transfection, cells were collected by trypsinization and viability determined by trypan blue exclusion. 90 µl cells from each time point were mixed with 10µl 0.4% trypan blue and analyzed under the microscope. Blue

cells were accepted as dead and percentages of live cells were calculated as follows: number of unstained cells divided by the number of whole cells counted and multiplied with 100. Additionally microscopic observations were done to confirm the reduction of cell numbers in the wells treated with hSLIP1 siRNAs, as compared to C2 siRNA treated cells. Western blotting with anti-hSLIP1was performed 48 hours later following the transfections.

Results

Identification of SLIP1 as a Factor That Binds Translation Activation Region in the N-terminus of SLBP

Lianxing Zheng, a former graduate student in the Marzluff lab, performed a yeast-2-hybrid screen with human SLBP lacking the last 27 amino acids as the bait (the last 27 amino acids contain an intrinsic trans-activation activity), against a HeLa cell cDNA library. One of the clones isolated, termed SLIP1 (Stem-Loop binding protein Interacting Protein 1), encoded a functionally novel polypeptide. Comparison of this clone within the EST database and the mouse genome suggested that it encoded a full-length polypeptide plus an extensive portion of the 5' UTR which remained in frame with the initiator AUG (Figure 13A). The human SLIP1 protein is a 222 amino acid protein that has not previously been described, but which is conserved in metazoans. Our lab developed an antibody against full-length hSLIP1 and this antibody recognizes both the 25 kDa protein in the mammalian cells and E. coli expressed recombinant hSLIP1.

The protein is structurally most similar to a number of translation factors, and contains a number of HEAT repeats (computer based modeling, unpublished data, Marzluff

Lab) which are similar to those found in the middle domain of eIF4G. eIF4GII is composed of 5 pairs of antiparallel α-helices, forming 5 HEAT repeats that are responsible for interacting with eIF4A and picornaviral IRESs (Marcotrigiano et al. 2001). Surprisingly, our computer based modeling predicts that hSLIP may also contain 10 α-helices arranged as 5 HEAT repeats that stacked over each other (Figure 13B). However, the *Danio rerio* orthologue of hSLIP1 (PDB accession numbers 2I2OA and 2I2OB) has been shown to contain 6 HEAT repeats, consisting of 12 helices. *D.rerio* orthologue is only 3 amino acids longer and shares 71% amino acid identity, suggesting that predicted SLIP1 HEAT repeats may be biologically addressing the conserved structure of SLIP1 among eukaryotes.

I confirmed the interaction of SLBP and SLIP1 and mapped the region on SLBP that SLIP1 associates in both directed yeast two-hybrid experiments by testing *Xenopus* SLBP1 and human SLBP mutants as baits against wild type hSLIP1 as the target and in the binding experiments using a GST pull down assay *in vitro*. The yeast two-hybrid assay is based on controlled ON/OFF transcription of histidine gene that is under the control of GAL4 promoter and GAL4 activation domain. A schematic of yeast two-hybrid assay is represented in Figure 14A. Baits are fused to GAL4 DNA-binding domain, so SLBP fusions will be able to interact with GAL4 promoter. The plates lacked tryptophan (Trp, W), and leucine (Leu, L) to force yeast to express those missing amino acids from the exogenously introduced vectors, each one of them also expresses GAL4-SLBPs with Trp and Leu with hSLIP1 that is fused to Gal4-activation domain. Thus constitutive expressions of fusion proteins were assured. If SLBPs interact with hSLIP1 which is fused to GAL4-activation domain, activation domain will be in the close proximity of the promoter and thus will turn on the transcription of his3 gene and express histidine. So, yeast can grow on the media lacking

histidine (His, H) and the growth is the indicator of the interaction between proteins. During the experiment, I included 20 mM 3-AT on the plates to repress leaky transcription of histidine gene. Both hSLBP and xSLBP1 interacted with human SLIP1 in the directed yeast two-hybrid assay (Figure 14B, top panels). A mutant of xSLBP1 which has amino acids between 68 and 123 deleted (xSLBP1Δ68-123) in the N-terminus, also lacking translation activation motif, did not interact with human SLIP1 (Figure 14B, bottom right panel). Similarly, single point mutant xSLBP1W73A, which can not stimulate the translation of reporters ending in stem loop (Figure 11A), can not interact with hSLIP1 (Figure 14B, middle left panel). In support of this important observation, a human mutant SLBP with the core SAVEE sequence substituted by five alanines (hSLBP SAVEE→A_5), another translationally inactive form of SLBP (Sanchez and Marzluff 2002), also did not interact with hSLIP1 (Figure14B, middle right panel). The different bait and target fusion proteins were expressed at the detectable levels by western blotting, demonstrating that the loss of interaction (incapacity to grow) was not due to the absence or the degradation of the proteins in yeast (Figure 14C). I also observed that yeast expressing hSLIP1 and hSLBP grew faster than yeast expressing hSLIP1 and xSLBP1 on plates containing 50 mM 3-AT, suggesting that hSLBP may have a higher affinity for hSLIP1 than xSLBP1 does (not shown). My results from this study demonstrated that hSLIP1 interacts with SLBP in its translation activation region, and the interaction required specific amino acids in the conserved motif identified in Chapter 2.

I tested the ability of recombinant GST-SLIP1 expressed in *E.coli* to pull down radioactively labeled xSLBP1 *in vitro*. In the reticulocyte lysate, wild type xSLBP1 and its mutants in pXFRM vector were expressed from the Sp6 promoter in the presence of ^{35}S-

methionine prior to GST pull down assay. The bound fractions were resolved on SDS-PAGE and observed by PhosphorImager. The interaction of GST with SLBPs was used to measure non-specific binding. The xSLBP1 bound to hSLIP1 and very little background binding was observed (Figure 15A, lane 2 and 3). The invariant amino acids in the translation activation motif xD7A, xW73A, xE78A and xE7879AA did not bind specifically to GST-hSLIP1, showing similar binding to GST (Figure 15A, compare lanes 6, 9, 18, and 24 to lanes 5, 8, 17, and 23). Thus by this criterion these mutants did not interact with hSLIP1. xW73F and xE79A mutant bound to GST-hSLIP1 more weakly than wild-type xSLBP1 (Figure 15A, compare lanes12 and 21 to lane 3). The xGS7475AA, which still stimulates the translation of reporter mRNAs ending in stem loop, interacts with hSLIP1 as strong as wild type xSLBP1 (Fig. 15A, lane 15). Those results are first consistent with yeast two-hybrid results; and secondly suggest that hSLIP1 interacts with xSLBP1 in the translation activation motif, DWX_3EE.

By using similar experimental strategy explained above using a deletion analysis, I confirmed hSLIP1 interaction with hSLBP and showed that interaction region of hSLBP with hSLIP1 again maps to translation activation region (Figure 15B). GST fusion proteins containing various N-terminal deletions of hSLBP were used in the GST pull down assay with radioactively labeled hSLIP1. In these experiments, hSLIP1 in pXFRM vector was expressed from Sp6 promoter in the presence of ^{35}S-methionine in reticulocyte lysate prior to pull down assay. Recombinant GST-tagged hSLBP and the deletion mutants were expressed in baculovirus system. Both the full-length human SLBP and the hSLBPΔ1−68 protein bound hSLIP1 with equal efficiency (Figure 15B, lanes 2 and 3), while hSLBPΔ1−81 and hSLBPΔ1−126, which have the entire translation activation motif deleted, pulled down

hSLIP1 to a much less extent (Figure 15B, compare lanes 4 and 5 to lanes 2 and 3). We did not observe any non-specific binding of hSLIP1 to neither GST nor beads (Figure 15B, lanes 6 and 1). These results are consistent with a critical region of hSLBP required for the interaction lying between amino acids 68 and 81.

Purified recombinant his-tagged hSLBP also interacted with purified recombinant GST-hSLIP1 *in vitro*, demonstrating that there was no dependence on other proteins or RNAs for this interaction (Figure 15C). Altogether, my results strongly suggest that hSLIP1 interacts with translation activation motif, DWX_3VEE, in the SLBP N-terminus and that this interaction is direct.

hSLIP1 Cooperates with SLBP to Stimulate Translation of a Reporter mRNA Ending in the Histone Stem Loop in Xenopus Oocytes

To test whether hSLIP1 plays a role in translation of histone mRNAs, I injected synthetic mRNAs encoding xSLBP1, hSLBP or hSLIP1 into oocytes followed by injection of a luciferase reporter mRNA ending either in a wild type histone stem loop (Luc-SL) or mutated loop (Luc-TL). We compared the effect of expressing SLBPs with the effect of co-expression of SLBPs and hSLIP1 (Figure 16A). xSLBP1 stimulated expression approximately 6-fold, and hSLBP stimulated expression 4-fold, not as much as xSLBP1, as previously reported (Sanchez and Marzluff 2002). Expression of hSLIP1 alone had little effect on the expression of the Luc-SL reporter (Figure 16A, last bar), suggesting that in the presence of limiting amounts of endogenous xSLBP1 present in the oocyte, xSLIP1 is not limiting. However, expression of hSLIP1 together with xSLBP1 resulted in an increase in

total activity, to approximately 9-fold activation. Moreover, expression of hSLIP1 together with hSLBP resulted in a larger increase in translational activity, up to the same level as seen with xSLBP1 and hSLIP1 (Figure 16A). I propose that the lower activity of hSLBP in oocytes compared to xSLBP1 may be due to the fact that hSLBP may not interact as well with xSLIP1 as xSLBP1. Thus the hSLIP1-hSLBP combination activated translation to the same extent as xSLBP1-hSLIP1. I was not able to test hSLIP1 activity on reticulocyte lysate system-based *in vitro* translation assay due to the presence of large amounts of rabbit SLIP1 in the lysate, which was readily detected by Western blotting (not shown). I conclude that hSLIP1 together with SLBP stimulates translation of mRNAs ending in the histone 3' stem loop.

Western blots confirmed that the hSLIP1 (top panel), xSLBP1 (middle panel) and hSLBP (lower panel) proteins were expressed in the oocytes with comparable levels in each test conditions and that protein levels did not vary with co-injection of two different mRNAs or injection of reporter mRNAs (Figure 16B). The hSLIP1 antibody, which was raised against the full-length hSLIP1 protein, also detected a protein in *Xenopus* oocytes migrating at about 36 kDa (Figure 16B, top panel, question mark), in addition to hSLIP1 which migrates at about 30 kDa. This 36 kDa protein may be the *Xenopus* SLIP1 orthologue (Accession number AAH48773), or it may be a cross-reacting protein.

I also compared the effect of hSLIP1 on the translation of Luc-SL mRNA and a polyadenylated luciferase reporter mRNA (Luc-polyA$_{50}$). Expression of hSLIP1 together with xSLBP1 again stimulated translation compared with injection of xSLBP1 alone, but hSLIP1 had no effect on translation of the Luc-polyA mRNA (Figure 16C). Thus the effect of SLIP1 is specific for the Luc-SL mRNA when compared to Luc-polyA$_{50}$ mRNA in oocyte

system. However, this result does not rule out the possibility that other mRNAs may be affected by hSLIP1.

hSLIP1 Activates Translation of Histone mRNAs in Mammalian Cells

To investigate whether hSLIP1 also affects translation of mRNAs ending in histone stem loop in mammalian cells, we utilized a GFP reporter gene containing both a histone promoter and ending in histone stem loop (Wagner and Marzluff 2006). HeLa cells were transfected with the plasmid carrying that reporter which undergoes all the processes that endogenous histone genes do, such as transcriptional control and processing of the mRNA prior to export from the nucleus and translation. We quantified the expression of GFP by FACS analysis (Wagner and Marzluff 2006). We compared expression of the GFP-SL gene to the GFP-polyA gene. Transfection of SLBP specifically stimulated expression of the reporter as previously reported (Wagner and Marzluff 2006) (Figure 17A). Transfection of hSLIP1 stimulated expression of the GFP-SL reporter 4-fold but had a much smaller effect (1.5 fold stimulation) on translation of GFP-polyA reporter (Figure 17). Expression of both SLIP1 and hSLBP resulted in a similar increase in GFP expression as expression of either protein alone.

The production of substantial amounts of GFP-SL mRNA from this reporter requires the expression of ZFP100, a U7 snRNP protein that is limiting for processing of histone mRNA (Wagner and Marzluff, 2006). Correctly processed histone mRNAs are transported to the cytoplasm (Erkmann et al. 2005a) and then engaged in translation while misprocessed mRNAs are targeted for degradation (should be Pandey et al., 1994). To further assess the

role of SLIP1 in translation in mammalian cells, we transfected cells with ZFP100 to express high amounts of GFP reporter mRNAs. 24 hours later, we co-transfected cells with increasing amounts of SLIP1 and GFP-SL reporter. Then, we analyzed the levels of GFP-SL mRNA by Northern blot and the levels of GFP protein by using FACS 48 hours later (Figure 17). SLIP1 increased the expression of the GFP protein up to 5-fold in a concentration dependent manner (Figure 17B).

To rule out the possibility that SLIP1 increased GFP-SL mRNA levels either by enhancing processing or increasing stability, we determined whether the expression of SLIP1 altered the amount of total GFP mRNA or processed GFP mRNA ending in the stem loop. We measured the amount of GFP mRNA processed at the histone 3' end as described previously (Wagner and Marzluff 2006) by using S1 nuclease mapping (Figure 17C, left panel). There was no change in the amount of processed reporter mRNA or of read-through reporter mRNA with transfection of increasing amounts of SLIP1. We also performed Northern blot analysis for total GFP mRNA and 7SK RNA (as an internal control) on the same samples (Figure 17C, right panel). Expression of hSLIP1 stimulated GFP expression but did not increase the levels of GFP-SL mRNA in either of these assays, indicating that the increased GFP levels are due to increased translation of the GFP-SL mRNA due to SLIP1 activity. SLIP1 may be limiting in the cultured cells (SLBP may be limiting in the *Xenopus* oocytes, accounting for the inability of increased SLIP1 levels to stimulate translation of the reporter mRNA in *Xenopus* oocytes (compare figure 16A to figure 17). Another reason for that observation may be due to the fact that hSLBP in mammalian system is present in excess over histone mRNA (Erkmann et al. 2005b), so a pool of hSLBP would be available in mammalian cells.

SLIP1 interacts with the amino terminus of eIF4G1 and eIF4G2 in vitro

It is likely that translation of histone mRNA requires that the 5' and 3' ends of the mRNA must be in close proximity to form a translationally competent initiation complex. It is hypothesized that SLIP1 might fulfill the function of PABP, bridging between the ends of histone mRNA, possibly by interacting with eIF4G and SLBP. PABP and eIF4E both interact with the N-terminal of eIF4G. In Figure 18A, there is a schematic of eIF4G showing the position of the PABP and eIF4E binding sites. I also show a comparison of eIF4G1 and eIF4G2 sequences in the region around the PABP binding site.

I synthesized full-length ^{35}S-eIF4GI and eIF4GII in the reticulocyte lysate and tested the ability of GST-hSLIP1 to pull down the labeled eIF4Gs. GST-hSLIP1 interacted with both full-length eIF4GI and eIF4GII in this assay (Figure 18B, left lane 3). I then tested N-terminal fragments of eIF4GI for their ability to interact with hSLIP1. The numbering of the fragments were based on Imataka et al (Imataka et al. 1998). A fragment containing amino acids 27-420 bound to hSLIP1. Amino acids 27-420 contains the region of eIF4GI that interacts with PABP, but does not contain the binding site for eIF4E (Figure18A, bottom panel). We tested fragments containing portions of the first 420 amino acids of eIF4GI to determine the SLIP1-binding region. A fragment containing amino acids 27-129 of eIF4GI bound to hSLIP1, while a fragment containing amino acids 129-420 showed very little binding (Figure 18B, center panel, lane 6). The eIF4GI fragment composed of 27 through 129, which lacks the PABP-binding site (Figure 19A, underlined, bottom), contains the predicted SLIP1 binding site.

I also determined which region of eIF4GII binds to hSLIP1. The fragment containing amino acids 31-420 of eIF4GII bound to hSLIP1 (Figure 18B, right panel, lane 9). A

fragment containing amino acids 101-420 did not bind to hSLIP1, although it contained the PABP-binding site (Figure 18B, right panel), suggesting that SLIP1-binding site is located between amino acids 31 and 100 on eIF4GII. The largest region of identity between eIF4GI and II among the first 180 amino acids corresponds to the site that binds to PABP (Figure 18A, underlined, bottom). About 40 amino acids prior to this site there is a second conserved region, which is 80% identical over 16 amino acids. The rest of the amino terminal sequences are much less similar, whereas two proteins in the middle domain are 79% identical. Based on my alignment and interaction results, it is predicted that SLIP1-binding site may be located in the second highly conserved region in the N-terminus of mammalian eIF4GI and eIF4GII (Figure 18A, second underlined region, top).

SLIP1 interacts with both SLBP and eIF4GI in vivo

To determine whether SLIP1 interacts with eIF4G1 and SLBP *in vivo*, I produced a HeLa cell line stably expressing HA-tagged hSLIP1. Lysates from these cells were immunoprecipitated with anti-HA or anti-myc antibodies, and the immunoprecipitates analyzed for the presence of HA-hSLIP1, eIF4GI and hSLBP. The anti-HA antibody efficiently precipitated HA-hSLIP1, and also co-precipitated eIF4GI and SLBP (Figure 18C, lane 3). HA-SLIP1 and eIF4GI were not present in the anti-myc immunoprecipitates, although there was a small amount of SLBP that was non-specifically immunoprecipitated (Figure 18C, lane 2). These results demonstrate that hSLIP1 interacts with eIF4GI and SLBP *in vivo.*

It is not possible from this data to determine if there is a ternary complex containing the three proteins in the cell. However previous studies (Ling et al. 2002) demonstrated that eIF4GI can be immunoprecipitated with anti-SLBP from cultured mammalian cells. Direct interactions between SLBP and eIF4G have not been observed with the mammalian proteins despite extensive attempts to test for this interaction (Gorgoni et al. 2005). Together these results are consistent with the possibility that there is a ternary eIF4G, SLIP1, SLBP complex on the histone mRNA during translation in mammalian cells.

When DNA synthesis is inhibited, histone mRNAs are rapidly degraded and SLBP relocalizes from the cytoplasm to the nucleus (Whitfield et al. 2004). We tested whether SLIP1 interaction with SLBP depends on histone mRNA presence in the cell after inhibition of DNA synthesis. The HA-hSLIP1 expressing cells were treated with hydroxyurea for 1 hour, and whole cell lysates were immunoprecipitated with anti-HA or anti-myc antibodies. The anti-HA antibody precipitated HA-SLIP1, and the immunoprecipitates also contained SLBP regardless of HU treatment (Figure 18D, lanes 3 and 6). However only anti-HA immunoprecipitates from the untreated cells contained eIF4G1 (Figure 18E, lane 3 top).

By following the similar approach above, I further investigated the ability of endogenous hSLIP1 to co-precipitate hSLBP and eIF4GI. I used an anti-hSLIP1 antibody directed against the C-terminal peptide to pull down hSLIP1 from HeLa cell lysates (Figure 18F). Endogenous hSLIP1 co-precipitated hSLBP and this interaction was not RNA-dependent (Figure 18F, lanes 3 and 5). In contrast, the hSLIP1 interaction with eIF4GI was sensitive to RNAse A treatment, and RNase treatment abolished the pull down (Figure 18F, lanes 3 and 5).

I verified that there was a direct interaction between eIF4GI and hSLIP1 in a GST pull down assay. Fragments of eIF4GI 27-129 and 123-420, C-terminally tagged with his, were expressed in *E.coli* and recombinant proteins were purified through Ni^{+} column. *E.coli* expressed hSLIP1 or baculovirus expressed GST-hSLBP-his were tested for their ability to interact with recombinant eIF4GI fragments (Figure 18G and H). eIF4GI 27-129 bound hSLIP1 (Figure 18G, lane 6) while eIF4GI 123-420 did not (Figure 18G, lane 3), in parallel with the observations *in vitro* (Figure 18B, center panel). However, hSLBP did not bind to any fragment of eIF4GI (Figure 18H), once more confirming the results from Gorgoni et al (Gorgoni et al. 2005). Together, those results suggest that hSLIP1 directly interacts with eIF4GI and hSLBP, and forms a bridge between eIF4GI and SLBP. Thus the 5' and 3' ends of the message comes into close proximity to enhance the translation similar to what is suggested for translation of polyadenylated mRNA.

Localization of hSLIP1

I examined the localization of SLIP1 in cells using immunofluorescence. Using a clone of HeLa cells expressing HA-hSLIP1, I showed that SLIP1 is present in both the nucleus and cytoplasm in the cells (Figure 19A, top). The distribution of SLIP1 is similar in all cells, and the protein levels do not change during the cell cycle (not shown). This is in contrast to the localization of SLBP, which is cell cycle regulated and whose localization changes during the cell cycle (Erkmann et al. 2005b). Note that when I observed a uniform staining for hSLIP1, hSLBP was detected in only some of the cells (Figure 19A, compare bottom panel to top panel), which are the cells in S-phase as reported previously (Erkmann et

al. 2005b). SLBP is present only in late G1 and S-phase cells (Whitfield et al. 2000; Zheng et al. 2003), and is present in the cytoplasm only in S-phase cells (Erkmann et al. 2005b). SLBP is stable after treatment of cells with HU, and relocalizes to the nucleus after HU treatment (Whitfield et al. 2004). Thus, based on my immunoprecipitation results that were not affected with HU treatment (Figure 18D), it is possible that SLIP1 associates with SLBP in the nucleus and may be a component of the mature mRNP as it exits the nucleus.

I also compared hSLIP1 localization with exclusively cytoplasmic eIF4GI and mainly ER-localized protein TRAP (Figure 19B and C). As reported previously, eIF4GI (Coldwell et al. 2004) was exclusively cytoplasmic (Figure 19B, center panel) and TRAP (Nicchitta et al. 2005) was mainly around the ER (Figure 19C, second panel). On the other hand hSLIP did not have any preferential localization in the cellular compartments. I suggest that hSLIP1 associates with SLBP regardless of the presence of histone mRNA. In contrast, the interaction between hSLIP1 and eIF4G1 was not strong enough to withstand the washing conditions in the absence of RNA.

SLIP1 is an essential protein in HeLa cells

To establish whether SLIP1 is an essential gene, I used RNA interference (RNAi) to knockdown hSLIP1 in HeLa cells (Figure 20). As a control, I also used RNAi to knock down SLBP, which has been shown to cause delays during S-phase progression in the cell (Wagner et al. 2005), without affecting viability (Figure 20A, left). Two different siRNAs against hSLIP1 were used and siRNA#1 targeted a sequence in the terminus while siRNA#2 targeted a sequence in the C-terminus. Both of the siRNAs effectively depleted both HA-tagged

hSLIP1 expressed from a transfected gene (Figure 20B, top, lanes 4-6) as well as the endogenous hSLIP1 (Figure 20B, middle, lanes 4-6). A Western blot against the PTB protein was used as a loading control (Figure 21BB, bottom). Depletion of hSLBP or usage of C2 siRNA as a control did not affect the levels of hSLIP1 (Figure 20B, lanes 2, 3). HeLa cells treated with either siRNA#1 or 2 against hSLIP1 underwent a rapid depletion of hSLIP1 protein and cell death, with 50% of the cells dying within 24 hours and over 70% dying by 72 hours (Figure 20A, left). HeLa cells treated with a control siRNA, C2 that does not target any sequence in the genome (Wagner et al. 2005), were not affected. In contrast, knocking down SLBP by RNAi resulted in a cell growth arrest in S-phase (Wagner et al. 2005), although there is no detectable loss of cell viability over 72 hours (Figure 20A). Thus, unlike SLBP, hSLIP1 is essential for viability of HeLa cells and other cell lines that we tested (not shown).

To demonstrate that cell death was a result of knocking down hSLIP1, I created a gene expressing an RNAi-resistant form of hSLIP1 mRNA by mutating the siRNA1 target site. Expression of the siRNA#1 resistant form of hSLIP1 (hSLIP1*) both rescued cell death (Figure 20A, right) and restored the expression of SLIP1 protein (Figure 20C, lane 8), in cells treated with siRNA1 but not siRNA2 (not shown), confirming that the cell death phenotype was due to SLIP1 depletion. Because SLIP1 depletion results in cell death while SLBP depletion does not, those data suggests that SLIP1 may function in other important cellular processes in addition to histone mRNA translation and/or may contribute to translation of a subset of mRNA that I could not detect under my experimental conditions.

The MS2-SLIP fusion protein activates translation of Luc-MS2 mRNA

To determine whether SLIP has to bind to SLBP or simply physically associated with the mRNA to activate translation, I constructed a fusion protein that had the entire hSLIP1 fused to the MS2 protein (MS2-hSLIP1). Previously, our lab also constructed a luciferase reporter gene that had a binding site for the MS2 protein at the 3′ end of the mRNA (Luc-MS2). I expressed the MS2-hSLBP, the hSLBP, and the MS2 protein as controls as well as MS2-hSLIP and hSLIP1 in the reticulocyte lysate and tested each for the ability to activate translation of the Luc-MS2, Luc-SL, or Luc-TL mRNA (Figure 21). The MS2-hSLBP fusion protein activated translation of both the Luc-MS2 and Luc-SL mRNAs up to 2.5 fold conforming the previous reports (Sanchez and Marzluff 2002), whereas it had no effect on the translation efficiency of the Luc-TL mRNA. Surprisingly, MS2-hSLIP1 stimulated translation of Luc-MS2 mRNA up to 2.5 fold and did not activate translation of Luc-SL mRNA. However, SLIP1 and hSLBP did not have any effect on neither Luc-MS2 nor Luc-TL, although hSLBP, not hSLIP1, activated Luc-SL mRNA translation nearly 2.5 fold. Control protein MS2 failed to activate translation of any of the reporter mRNAs tested (Figure 21). Thus, the MS2-hSLIP1 can activate translation when tethered to mRNA with a RNA-binding protein (MS2), even though the SLBP was not included in the translation reactions. If SLIP1 is not tethered to mRNA with such a RNA-binding domain, then probably SLIP1 uses SLBP as a landing platform to exert its translational activity over histone messages. This experiment also suggests that SLBP is only required to recruit SLIP1, and it is disposable for other functions, if there is any, during translation.

Discussion

All eukaryotic cellular mRNAs, with the exception of the metazoan replication-dependent histone mRNAs, are polyadenylated. Efficient translation of polyadenylated mRNAs requires both the 5' end cap and 3' end polyA tail of the mRNAs. The cap at the 5' end is bound by eIF4E and the polyA tail at the 3' end bound by PABP. These two proteins bind to eIF4G and this allows eIF4G to bind and recruit eIF3 and the small ribosomal subunit resulting in initiation of translation.

The rotavirus viral genome mRNA does not end in a polyA tail and is translated in mammalian cells. Its translation is mediated by a viral protein, NSP3, that binds to both the 3' end of viral mRNA and to the same region on eIF4G as PAB1 binds, likely circularizing the viral RNA (Piron et al. 1998; Vende et al. 2000), while at the same time preventing access to eIF4G by cellular polyadenylated mRNAs. Many viral mRNAs which infect plants are also not polyadenylated, ending instead in a complex RNA pseudoknot (Leathers et al. 1993). The 3' end of these mRNAs is also required for their translation, although there is no evidence of specific proteins that bind the 3' end of these mRNAs.

A novel protein, SLIP1, stimulates histone mRNA translation

The 3' end of histone mRNA is required for efficient translation of histone mRNA both in mammalian cells (Gallie et al. 1996) and in *Xenopus* oocytes (Sanchez and Marzluff 2002). SLBP stimulates translation of reporter mRNAs ending in the histone 3' end both in *Xenopus* oocytes and in a rabbit reticulocyte lysate (Sanchez and Marzluff 2002). We identified a 15 amino acid region in SLBP essential for translation that includes a highly

conserved motif, DWX_3EE, and identified a novel protein, SLIP1, that interacts with this sequence. Point mutations in the conserved motif in the translation activation region of SLBP prevent activation of translation of the Luc-SL reporter (Figure 11) and also inhibit binding of SLBP to SLIP1 (Figure 15), strongly supporting a role for SLIP1 in histone mRNA translation.

SLIP1 is a 25.4 kDa protein that is not similar to any other proteins in the database, but is predicted to contain multiple HEAT domains. In BLAST searches the most closely related proteins include several translation factors, including the middle domain of eIF4G, all of which contain multiple HEAT domains. There is a putative orthologue of SLIP1 in all metazoans, but no obvious orthologue in plants or fungi. Plants and fungi express polyadenylated histone mRNAs and thus wouldn't need SLIP1 for histone mRNA translation. SLIP1 is not cell-cycle regulated (R.Lerner, unpublished) and is present in both the nucleus and the cytoplasm as judged by immunofluorescence of HA-SLIP1 using anti-HA antibodies, as well as measurement of endogenous SLIP1 by Western blotting after cell fractionation (L.X. Zheng, Ph.D. thesis).

SLIP1 stimulates translation of a reporter mRNA ending in a stem loop when it is expressed together with either hSLBP or xSLBP1 in *Xenopus* oocytes, although expression of hSLIP1 has little effect on the translation of the reporter RNA when only endogenous levels of xSLBP1 and xSLIP1 are present in *Xenopus* oocytes (Figure 16). SLIP1 also has little effect on the translation of a polyadenylated reporter mRNA in either *Xenopus* oocytes or mammalian cells, demonstrating that it does not affect the translation of all mRNAs (Figure 16C; Figure 17A). Human SLBP has a lower activity than xSLBP1 in oocytes, suggesting that it does not interact optimally with the *Xenopus* translation factors (Figure

16A). However, expression of hSLIP1 together with human SLBP resulted in maximal activation of the reporter (Figure 16A). SLIP1 also activates expression of GFP from a reporter mRNA ending in the stem loop in mammalian cells, while it had little effect on expression of GFP from a polyadenylated reporter mRNA (Figure 17A). Importantly there was no change in the levels of the GFP-SL mRNA after SLIP1 expression, while there was a 5-fold increase in GFP protein, consistent with a role of SLIP1 in translation.

SLIP1 is an essential protein in cultured mammalian cells

Knocking down hSLIP1 in both HeLa cells (Figure 20) and U2OS cells (data not shown) by RNA interference results in rapid cell death. This phenotype is a result of the hSLIP1 knockdown, since expression of RNAi resistant hSLIP1 rescues cell viability. This phenotype was unexpected, since knocking down other proteins required for histone mRNA metabolism, SLBP (Zhao et al. 2004; Wagner et al. 2005), ZFP100, Lsm10 or Lsm11 (Wagner and Marzluff 2006) does not affect cell viability, but rather causes cell cycle arrest. hSLIP1 is not cell-cycle regulated and is also present in reticulocyte, which do not contain nuclei. These results suggest that SLIP1 likely has additional function(s) in addition to participating in histone mRNA translation.

Polyadenylated mRNAs require that both PAB1 and eIF4E bind to the 3' and 5' end of the mRNA respectively, and each of these proteins then bind to eIF4G to circularize the mRNA. Several proteins affect the translation of polyadenylated mRNAs by interacting with PAB1. PAIP1 and PAIP2 both interact with PAB1, with PAIP1 stimulating translation while

PAIP2 inhibits translation. These two proteins have been characterized using reporter mRNAs, and it is not known if they affect only a subset of mRNAs in the cell.

Mechanism of histone mRNA translation

These results strongly suggest that SLIP1 is a critical factor in histone mRNA translation. It is likely that the mechanism of translation of polyadenylated mRNAs and histone mRNAs is similar, and that the role of SLIP1 is to help bring the 5' end 3' ends of histone mRNAs together (Figure 22). Consistent with this hypothesis, SLIP1 interacts with a region in the amino terminus of eIF4G1 and eIF4G2, which is located just before the region that binds PABP (Figure 18). Thus this region of eIF4G likely plays a critical role on activation of eIF4G for effective translation initiation. Binding of PAB1 to its binding site on eIF4G results in an increase of the affinity of eIF4G for the eIF4E-cap complex (Haghighat and Sonenberg 1997). This region is also the target for binding of PAIP2 which binds to eIF4G preventing PABP binding (Karim et al. 2006). Recent experiments showed that PAB1 protein enhances the transfer of ribosomes that are terminating translation to the 5' UTR from the 3' end increasing the rate of translation initiation complex formation. Additionally, a closed loop conformation may enhance the formation of more stable complexes on the nascent mRNA by enhancement of eIF4E affinity for mRNA 5' cap structure by eIF4G1 whose affinity for eIF4E is increased by PABP (Kahvejian et al. 2005). Probably, the interaction of SLIP1with both SLBP and eIF4G allows formation of a similar closed loop structure, thus mimicking the translation mechanism for polyadenylated mRNAs (Figure 22). It will be of interest to see if there are subsets of mRNAs that can also recruit SLIP1 and

whether SLIP1 and PAB1 can bind simultaneously to eIF4G, resulting in a stronger activation of translation of a subset of cellular mRNAs.

Every attempt to demonstrate any biologically significant interaction between SLBP and one of the core translation factors such as eIF4E, eIF4G, eIF3, and eIF4A failed (Ling et al. 2002; Gorgoni et al. 2005). Ling et al. concludes that SLBP interacts with eIF4G in yeast and does not require any other factor between SLBP and eIF4G. The reason for this misleading conclusion is that the experiment was performed in yeast which lacks the cell cycle dependent histone mRNAs and SLBP. Also, the yeast translation machinery does not require any accessory factors to stimulate the translation, only needs core translation factors. So this system is naturally artificial experimental system to study translation of cell cycle-dependent histone mRNAs. Because of their early conclusion based on yeast studies, they did not consider co-purification of an unknown factor that may mediate the interaction between SLBP and eIF4G in their mammalian pull down experiments. In contrast to Ling et al., no interaction between SLBP and eIF4G was identified in the directed yeast two-hybrid analysis (Gorgoni et al. 2005). This study suggested that SLBP and IF4GI do not directly interact even though they may co-purify in mammalian system. However, Gorgoni et al. observed interaction between SLBP and eIF3 subunit h (p40) like our lab (L.X. Zheng, Ph.D. thesis) and PAIP1 (Gorgoni et al. 2005). The interaction domain in SLBP required for binding to the eIF3 subunit p40, was mapped to the RBD(eIF3h) or the C-terminus (PAIP1), respectively (Gorgoni et al. 2005). Deletion of the whole C-terminus (Sanchez and Marzluff 2002) or tethering only the N-terminus of SLBP fused to MS2 (Gorgoni et al. 2005) does not reduce the translational activity of SLBP, showing that RBD and C-terminus of SLBP is not required for the stimulatory effect of SLBP on translation. Thus the interactions of eIF3h and

Paip1 with SLBP cannot be essential to stimulate translation in *Xenopus* oocytes. These interactions could be secondary interactions that help stabilize the translation complex on histone mRNAs.

Figure 13. Functionally novel factor SLIP1 A) The predicted protein sequence of the hSLIP1 protein. **B)** Predicted Structure of hSLIP1. hSLIP1 contains multiple heat domains similar to some of the translation factors such as eIF4GI middle domain. SLIP1 dimerizes. Top figure shows the ribbon like structure, brown and blue represent two different molecules of SLIP1 that dimerized. Below is the predicted protein like structure of dimerized SLIP1 (Courtesy of Brenda Temple, UNC-CH).

A

MGEPSREEYKIQSFDAETQQLLKTALKDP
GAVDLEKVANVIVDHSLQDCVFSKEAGRM
CYAIIQAESKQAGQSVFRRGLLNRLQQEY
QAREQLRARSLQGWVCYVTFICNIFDYLR
VNNMPMMALVNPVYDCLFRLAQPDSLSKE
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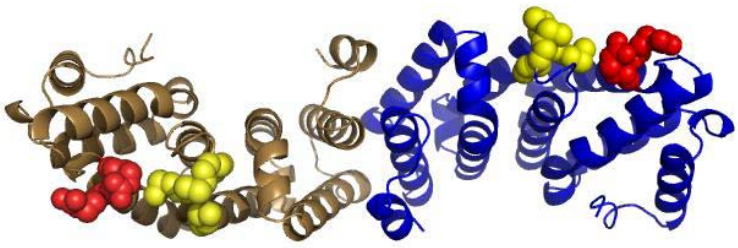
B



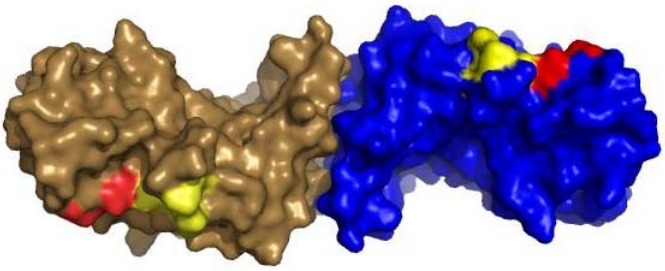
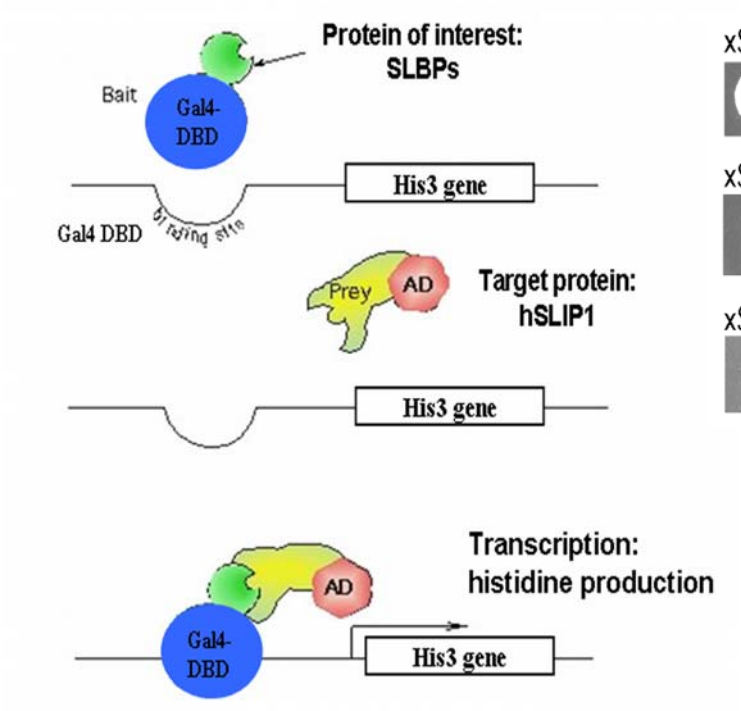
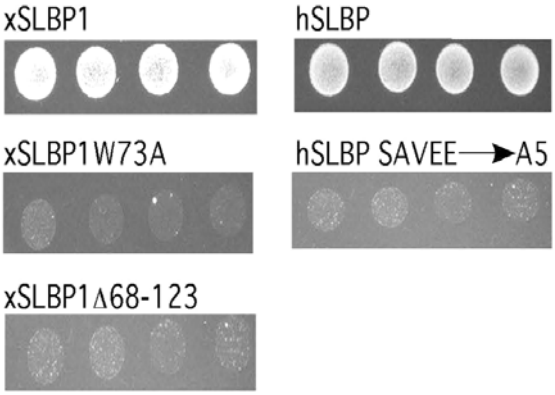


Figure 14. SLIP1 interacts with the translation activation motif in the SLBP N-terminus. A) A schematic of directed yeast two-hybrid experiment. SLBPs are fused to GAL4 DNA binding domain and hSLIP1 is fused GAL4-activation domain. If those two proteins interact, the activation domain will be in close proximity of the promoter, thus will turn on the transcription of histidine 3 gene. As a result, yeast that is deficient to express histidine without that activation will survive on the plates lacking histidine. So the growth is used as the indicator of the protein interactions. **B)** Directed yeast two-hybrid assays between xSLBP1 or hSLBP and hSLIP1. Four independent yeast colonies containing the two hybrid vectors were spotted on plates lacking L, W, H in the presence of 20 mM 3-aminotriazole (3-AT) and the growth was observed for 3 days. The xSLBP1 mutants used were single point mutant xSLBP1W73A and xSLBP1Δ68-123 that was an internal deletion of amino acids between 68 and 123 which also deletes the whole translation activation motif. The hSLBP mutant had the SAVEE sequence mutated to AAAAA (hSLBP SAVEE→A_5). **C)** Western blotting of fusion proteins expressed in yeast. Protein extracts from yeast transformed with GAL4 vectors to express GAL4-SLBPs and AD-hSLIP1 were prepared as explained in the methods and materials part. Since α-xSLBP1 cross-reacts with hSLBP, it was used to detect xSLBP and its mutants as well as hSLBP and its mutants (Top panel). 3 different colonies were tested for their ability to express hSLIP1 by using α-hSLIP1 (Bottom panel). Expressions were compared to yeast cells (UnT yeast) which were transformed with empty vector. GAL4 represents DNA-binding domain and AD stands for activation domain of GAL4 system.

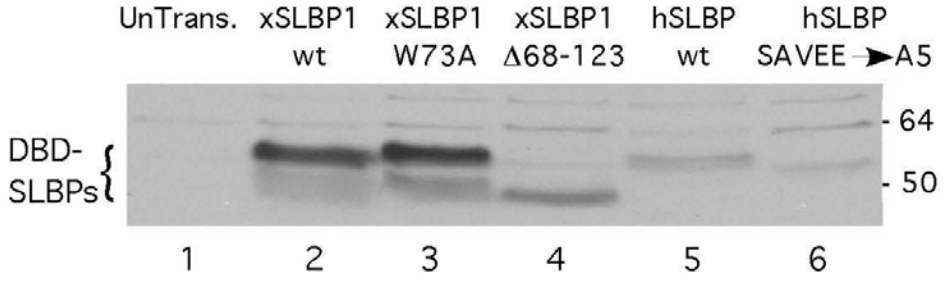
A


Protein of interest:
SLBPs
Bait
Gal4-
DBD
His3 gene
Gal4 DBD
Prey
AD
Target protein:
hSLIP1
His3 gene
AD
Gal4-
DBD
Transcription:
histidine production
His3 gene

B


xSLBP1
hSLBP
xSLBP1W73A
hSLBP SAVEE→A5
xSLBP1Δ68-123

C


UnTrans.
xSLBP1 wt
xSLBP1 W73A
xSLBP1 Δ68-123
hSLBP wt
hSLBP SAVEE→A5
DBD-SLBPs
64
50
1
2
3
4
5
6

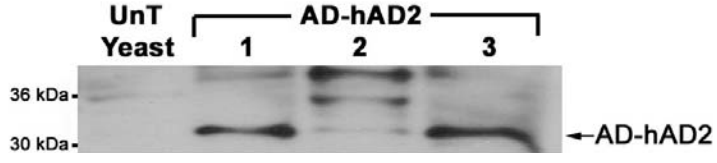
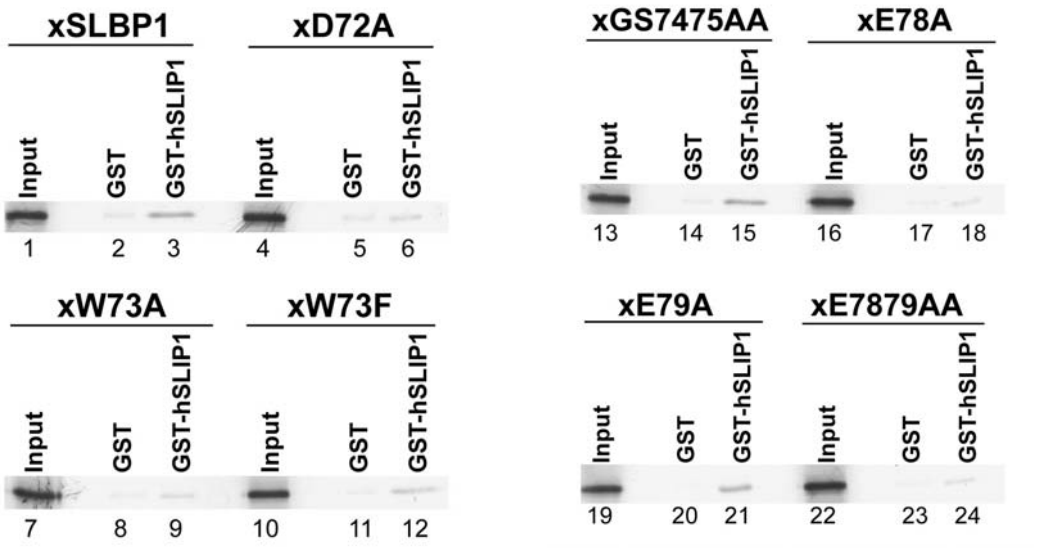
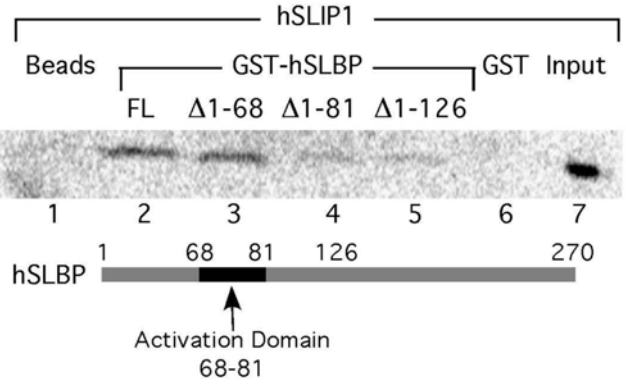

UnT Yeast
AD-hAD2
1
2
3
36 kDa
30 kDa
←AD-hAD2

Figure 15. SLIP1 interacts with the translation activation motif, DWX_3VEE, in the N-terminus of SLBP. **A)** Full-length xSLBP1 and its point mutants xD72A, xW73A, xGS747AA, xE78A, xE78A, and xEE7879AA were labeled with ^{35}S-methionine by *in vitro* translation in reticulocyte based TNT system. 8 µl TNT expressed SLBPs were mixed with either 4 µg E. coli expressed recombinant GST (lanes 2, 5, 8,11, 14, 17, 20, and 23) or 8 µg GST-SLIP1 (lanes 3, 6, 9, 12, 15, 18, 21, and 24) and incubated for 30 minutes on ice prior to addition of glutathione agarose beads (40 µl per tube). Final reactions were incubated on ice for an additional 30 minutes. To prevent non-specific binding, 1X PBS (contains 143 mM KCL) as washing buffer was supplemented with 150 mM NaCL, so final salt concentration during washing was ~300 mM salt. The bound proteins were resuspended in 30 µl 2X SDS-Sample Buffer and resolved by 12% SDS-PAGE. The xSLBPs were detected using a PhosphorImager. Lanes 1, 4, 7, 10, 13, 16, 19, and 22 are 10% of the input used in the pull down assays. **B)** All the human SLBP mutants as well as the wild type were N-terminally tagged with GST and C-terminally with six histidines. Those proteins were expressed in baculovirus system and purified through Ni+ column. GST was expressed in E. coli and purified through GS-sepharose beads. Human SLIP1 was labeled with ^{35}S-methionine by *in vitro* translation and GST or the indicated GST-hSLBP-his fusion proteins added and the rest of the experiment was performed as explained in Figure 16A. Lane 7 is the input for hSLIP1. hSLIP1 was also incubated with only beads as a second control (Lane1). A schematic of the full length human SLBP showing the translation activation domain is below the figure. **C)** Recombinant hSLBP-his was incubated either with GST (lane 2) or recombinant GST-SLIP1 (lane3) and the proteins bound to the glutathione agarose beads were detected by Western blotting using the anti-SLBP antibody. The same amount of human SLBP was included in all reactions, and the input is shown in lane 1.

A


xSLBP1
xD72A
xGS7475AA
xE78A
xW73A
xW73F
xE79A
xE7879AA
Input
GST
GST-hSLIP1
1 2 3 4 5 6
13 14 15 16 17 18
7 8 9 10 11 12
19 20 21 22 23 24

B


hSLIP1
Beads
GST-hSLBP
GST
Input
FL
Δ1-68
Δ1-81
Δ1-126
1 2 3 4 5 6 7
hSLBP
1
68
81
126
270
Activation Domain
68-81

C

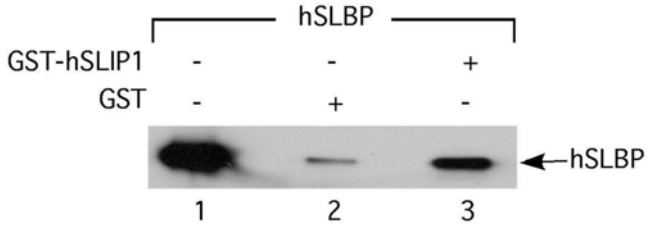
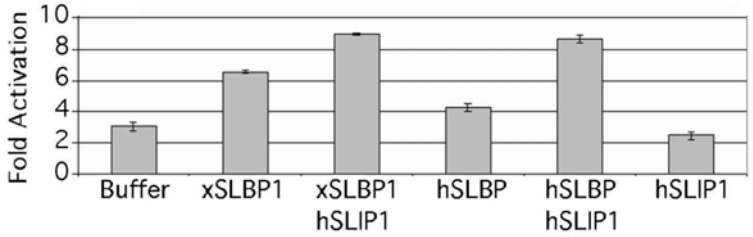
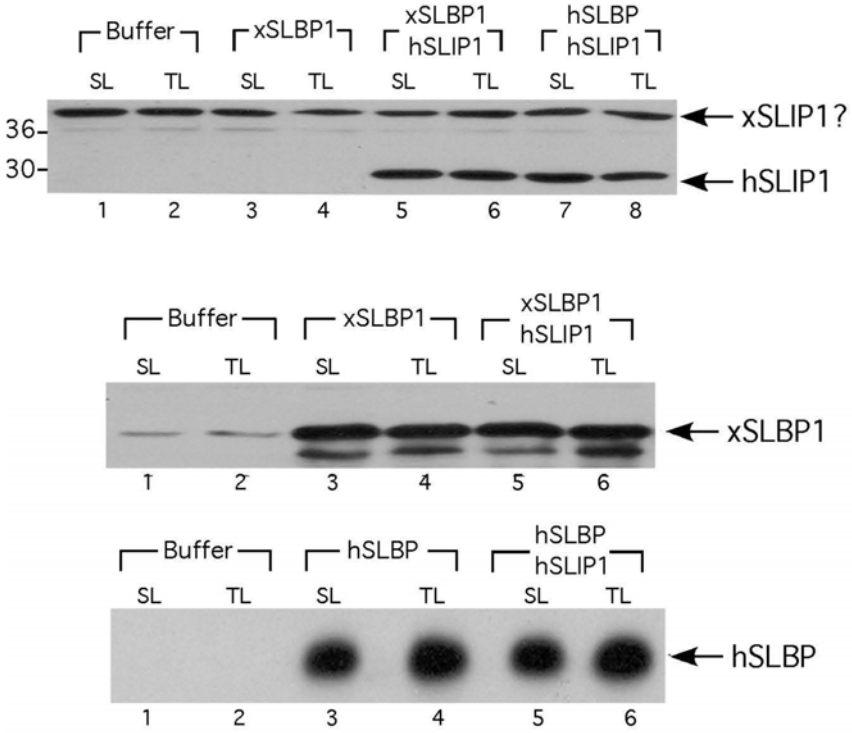

hSLBP
GST-hSLIP1 - - +
GST - + -
hSLBP
1 2 3

Figure 16. Stimulation of translation of luciferase reporter ending in stem loop by SLIP1 in oocytes. A) *Xenopus* oocytes were injected with buffer or with equimolar amount of capped synthetic mRNAs encoding xSLBP1, hSLBP, hSLIP1, xSLIP1/hSLIP1 and hSLBP/hSLIP1 16 hours prior to injection of Luc-SL or Luc-TL mRNA. Details of this assay were explained in chapter 2. As described previously, results were expressed relative to the translational activities measured from Luc-TL mRNA. Average of three independent experiments is shown in the graph. **B)** Over-expressions of hSLIP1 (top panel), xSLBP1 (middle panel), and hSLBP (bottom panel) in oocytes were detected with appropriate antibodies by western blotting. The hSLIP1 antibody detected a *Xenopus* protein slightly larger than hSLIP1, which is likely the *Xenopus* orthologue of hSLIP1 (top panel). **C)** A similar experiment was performed as in figure 17A, except that the oocytes expressing the various proteins were injected with either Luc-SL or Luc-polyA reporter mRNAs. The relative expression of the Luc-SL or Luc-polyA in the oocytes injected with buffer was set at 1 and fold activations of questioned proteins were calculated according to buffer injected oocytes.

A


Fold Activation
10
8
6
4
2
0
Buffer
xSLBP1
xSLBP1 hSLIP1
hSLBP
hSLBP hSLIP1
hSLIP1

B


Buffer
xSLBP1
xSLBP1 hSLIP1
hSLBP hSLIP1
SL TL SL TL SL TL SL TL
36
30
xSLIP1?
hSLIP1
1 2 3 4 5 6 7 8
Buffer
xSLBP1
xSLBP1 hSLIP1
SL TL SL TL SL TL
xSLBP1
1 2 3 4 5 6
Buffer
hSLBP
hSLBP hSLIP1
SL TL SL TL SL TL
hSLBP
1 2 3 4 5 6

C

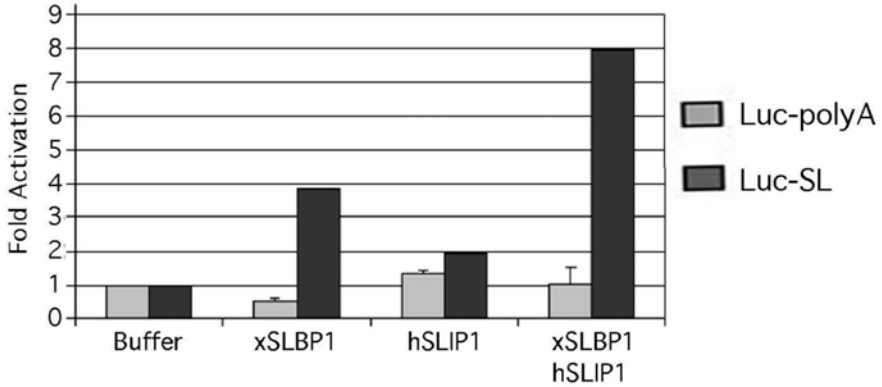
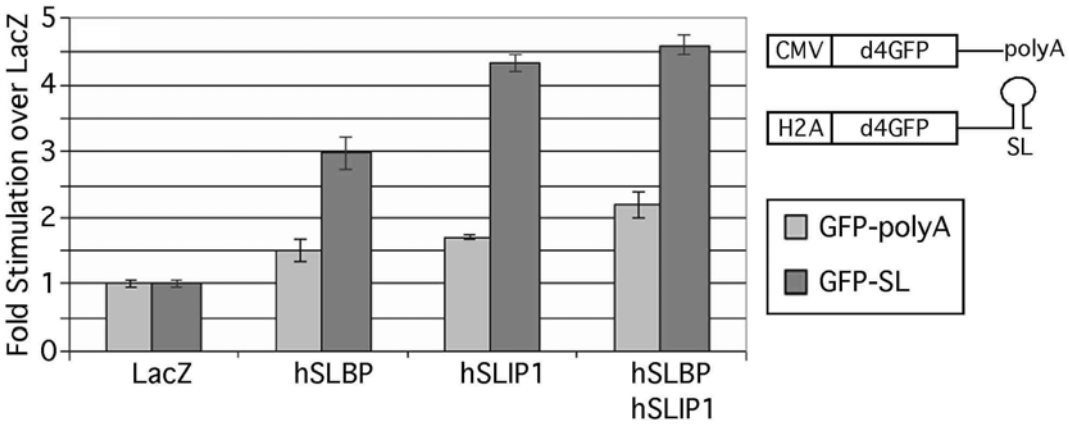
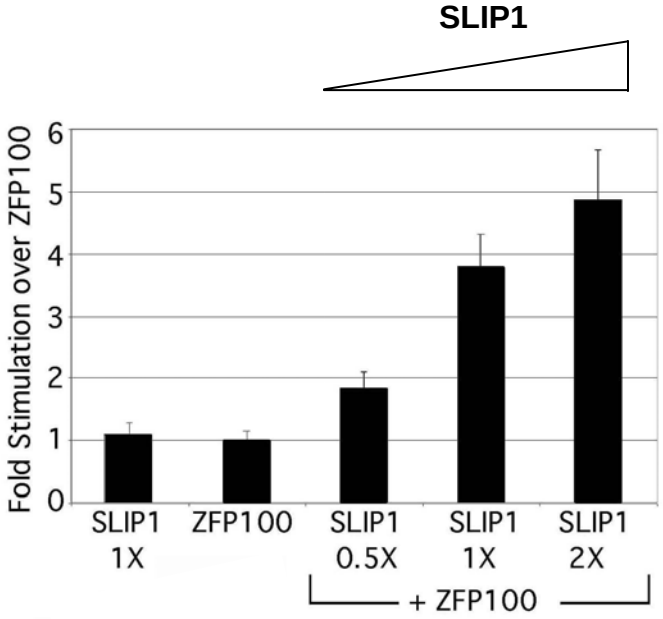

Fold Activation
9
8
7
6
5
4
3
2
1
0
Buffer
xSLBP1
hSLIP1
xSLBP1 hSLIP1
Luc-polyA
Luc-SL

Figure 17. Human SLIP1 activates translation of GFP-SL reporter in mammalian cells. **A)** A reporter DNA encoding GFP followed by the histone stem-loop and processing signals or a reporter DNA encoding GFP followed by a polyadenylation signal (Wagner and Marzluff 2006) were transfected into 293T cells together with plasmids expressing the indicated HA-tagged proteins. 48 hours later the cells were harvested and the amount of GFP expression quantified by flow cytometry as previously described (Wagner and Marzluff 2006). **B)** A similar experiment was performed as in figure 18A, except that the ZFP100 plasmid was transfected into the cells together with the reporter construct for 24 hrs to promote the expression of the processed GFP-SL mRNA (Wagner and Marzluff, 2006). The hSLIP1 plasmid was then transfected into cells and the cells harvested 48 hrs later and the expression of GFP quantified. **C)** Total cellular RNA from the 293T cells tested in figure 18B was analyzed using an S1 nuclease protection assay as described previously (Wagner and Marzluff 2006). That allows us to detect the mRNAs processed at the 3' end and the mRNAs resulting from read-through past the 3' end. The band labeled P represents the probe. The RNAs were from cells transfected with ZFP100 (lane 1) and with ZFP100 plus increasing amounts of SLIP1 (lanes 2-4). Additionally, stabilities of total amount of GFP mRNA was monitored by Northern blotting performed on the same RNA samples and 7SK snRNA present in total cellular RNAs was used as loading control (lanes 5-8).

A


Fold Stimulation over LacZ
0
1
2
3
4
5
LacZ
hSLBP
hSLIP1
hSLBP
hSLIP1
CMV
d4GFP
polyA
H2A
d4GFP
SL
GFP-polyA
GFP-SL

B


SLIP1
Fold Stimulation over ZFP100
0
1
2
3
4
5
6
SLIP1
1X
ZFP100
SLIP1
0.5X
SLIP1
1X
SLIP1
2X
+ ZFP100

C

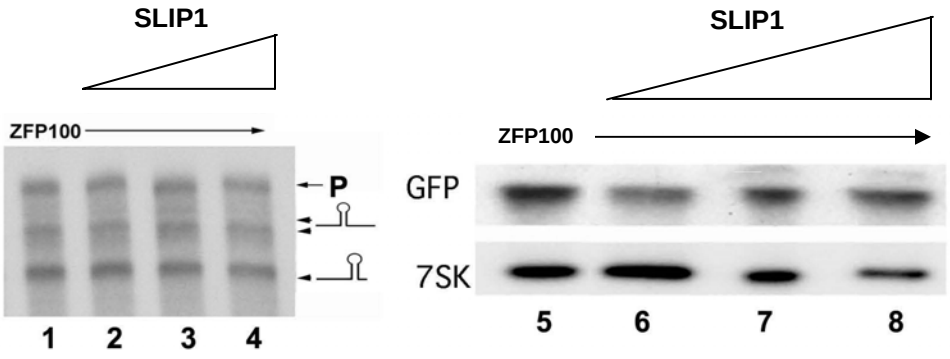
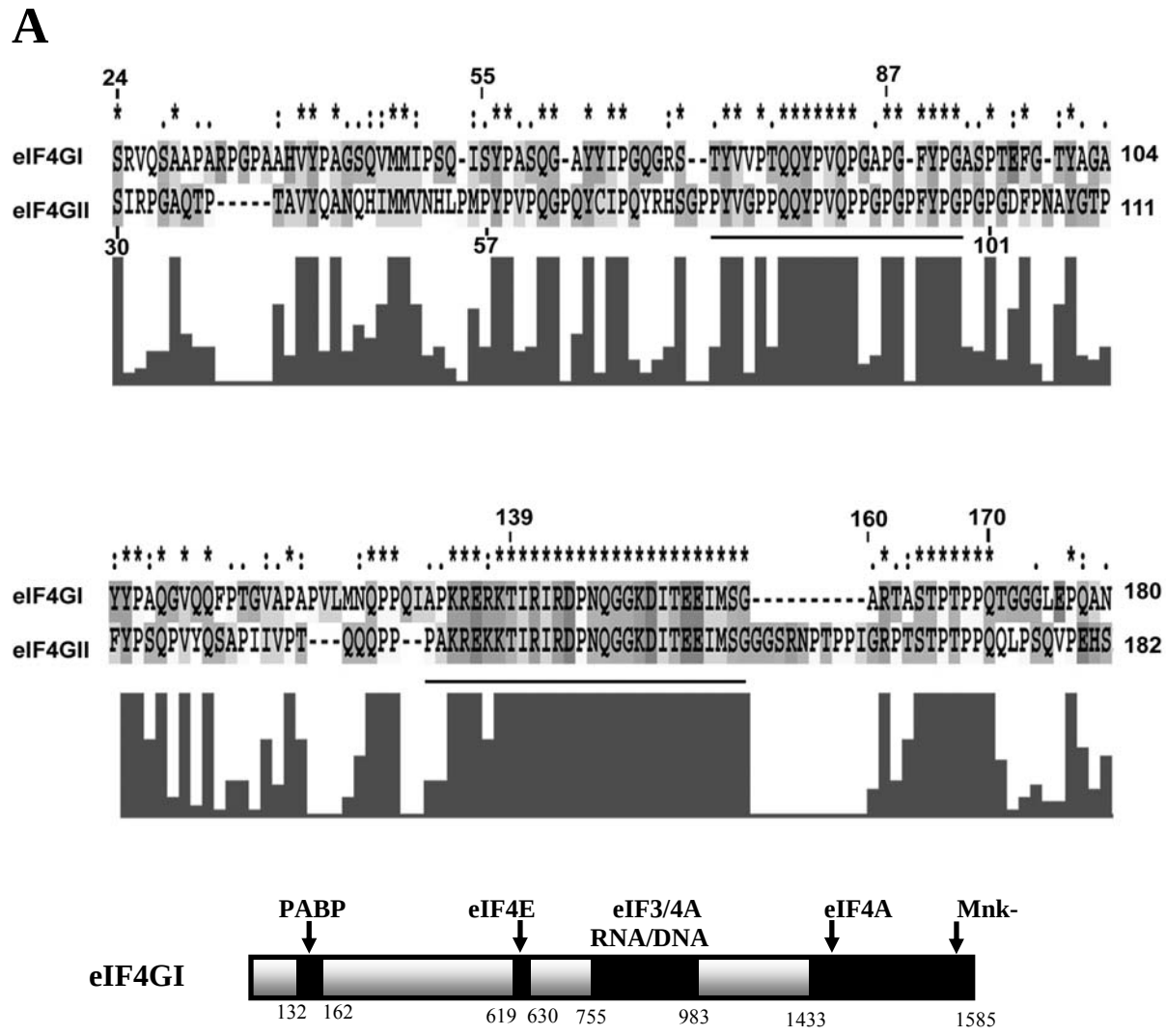
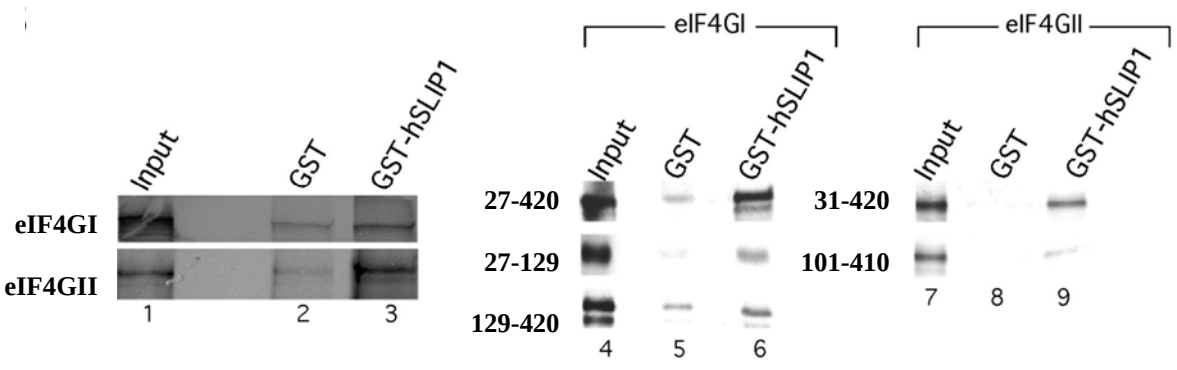

SLIP1
ZFP100
P
1
2
3
4
SLIP1
ZFP100
GFP
7SK
5
6
7
8

Figure 18. SLIP1 interacts with eIF4G *in vitro* and *in vivo*. A) The sequence alignment of eIF4GI and eIF4GII containing the amino acids between 24-180 and 30-182 in the N-terminus, respectively. Clustal X (1.81) program was used to align N-terminal sequences of eIF4GI and eIF4GII. Numbering is based on the published data in Imataka et al. (Imataka et al. 1998). PABP-interacting region is underlined that is between amino acids132 and 159 on eIF4GI and 134 and 161 on eIF4GII. Second underlined region is predicted to be the possible SLIP1-binding region on eIF4G due to being the only highly conserved region between amino acids 27-420 and 31-420 on eIF4GI and II, respectively. Asterisks indicate identical amino acids in eIF4G1 and eIF4G2. **B)** Interaction between hSLIP1 and eIF4G *in vitro* by GST pull down assay. Full-length eIF4G1 or eIF4G2 (lanes 1-3), indicated fragments of eIF4G1 (lanes 4-6) or eIF4G2 (lanes 7-9) were labeled with ^{35}S-methionine by *in vitro* translation. The lysates were incubated with either GST (lanes 2, 5, 8), or GST-SLIP1 (lanes 3, 6, 9) and the bound proteins were resolved by 12% SDS-PAGE and detected by autoradiography. 10% of the input protein was analyzed in lanes 1, 4, 7. **C)** *In vivo* co-immunoprecipitation of HA-hSLIP1 with hSLBP and eIF4GI. Lysates were prepared from HeLa cells stably expressing HA-tagged hSLIP1 and incubated with either 10 of anti-myc or anti-HA antibody. The bound proteins were resolved by electrophoresis and detected by Western blotting using the anti-HA antibody, hSLBP antibody or the eIF4G1 antibody. Lane 1 contains 10% of the protein subjected to immunoprecipitation. **D and E.** Hydroxyurea effect on immunoprecipitation of HA-hSLIP1. Lysates were prepared from exponentially growing HeLa cells stably expressing HA-hSLIP1 or from cells treated with hydroxyurea for 1 hour. The lysates were immunoprecipitated with either anti-myc (lanes 2 and 5) or anti-HA (lanes 3 and 6) antibodies and the bound proteins resolved by electrophoresis were detected by Western blotting. In panel D, lysates from control cells is shown in lanes 1-3 and from HU treated cells is shown in lanes 4-6. Lane 1 shows 10% of the lysate subjected to immunoprecipitation. Figure E shows the analysis of the same lysates with eIF4G1 antibody. **F)** Endogenous hSLIP1 also interacts with hSLBP and eIF4GI in a RNA independent manner. Lysates from exponentially growing HeLa cells were incubated with either affinity purified anti-hSLIP1 (10 μl) or anti-myc antibody (10 μl). Besides, same amount of lysates were treated with 10 μg/ml RNAse A prior to immunoprecipitation. Then, bound proteins were analyzed by western blotting with anti-hSLIP1, anti-hSLBP, or anti-eIF4GI antibodies. Lanes 1 show the 10% input of lysates subjected to immunoprecipitation. **G)** Direct interaction between hSLIP1 and eIF4GI. E. coli expressed ant C-terminally his tagged recombinant eIF4GI fragments 27-129 and 129-420 were incubated with E. coli expressed GST-hSLIP1 for 30 minutes on ice prior to incubation with 40 ml glutathione-Sepharose beads for another 30 minutes on ice. Beads were washed in 1X PBS supplemented with 100 mM NaCl. Eluted proteins in 2X SDS-sample buffer were resolved by electrophoresis and subjected to western blotting with anti-His antibody. **H)** hSLBP does not interact with eIF4GI fragment. GST pull down assay was performed with GST-hSLBP-his to pull down recombinant eIF4GI fragments as explained in figure G. Input lane shows 35% of the eIF4GI 27-129 used in the pull down. Notice that in the same western blotting with anti-his antibody, both eIF4GI 27-129 and hSLBP could be detected since both of them were his-tagged.


A
24
55
87
eIF4GI SRVQSAAPARPGPAAHVYPAGSQVMMIPSQ-ISYPASQG-AYYIPGQGRS--TYVVPTQQYPVQPGAPG-FYPGASPTEFG-TYAGA 104
eIF4GII SIRPGAQTP-----TAVYQANQHIMMVNHLPMPYPVPQGPQYCIPQYRHSGPPYVGPPQQYPVQPPGPGPFYPGPGPGDFPNAYGTP 111
30
57
101
139
160
170
eIF4GI YYPAQGVQQFPTGVAPAPVLMNQPPQIAPKRERKTIRIRDPNQGGKDITEEIMSG----------ARTASTPTPPQTGGGLEPQAN 180
eIF4GII FYPSQPVYQSAPIIVPT---QQQPP--PAKREKKTIRIRDPNQGGKDITEEIMSGGGSRNPTPPIGRPTSTPTPPQQLPSQVPEHS 182
PABP
eIF4E
eIF3/4A
RNA/DNA
eIF4A
Mnk-
eIF4GI
132 162 619 630 755 983 1433 1585


B
Input
GST
GST-hSLIP1
eIF4GI
eIF4GII
1 2 3
eIF4GI
eIF4GII
27-420
27-129
129-420
31-420
101-410
4 5 6 7 8 9

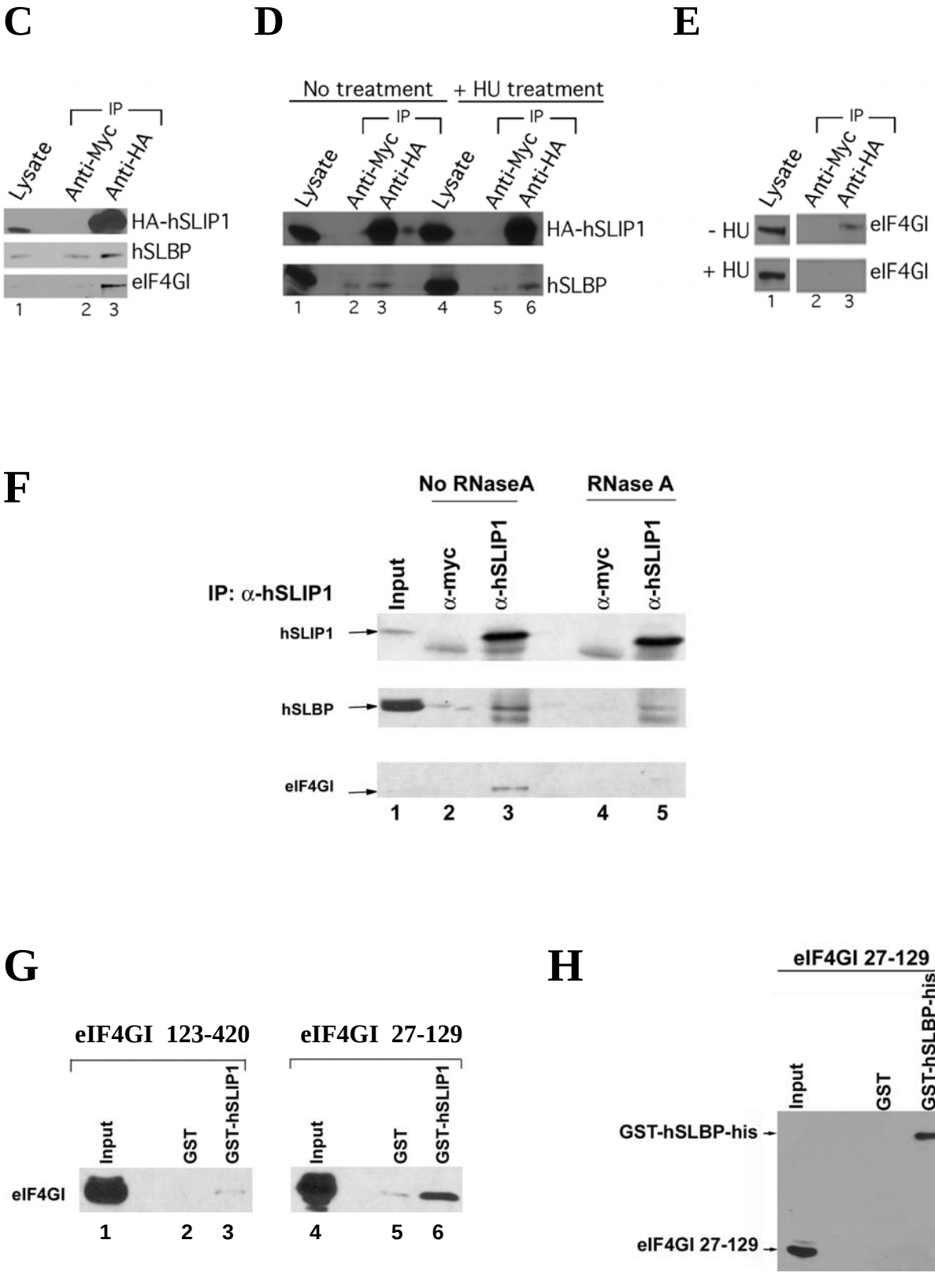
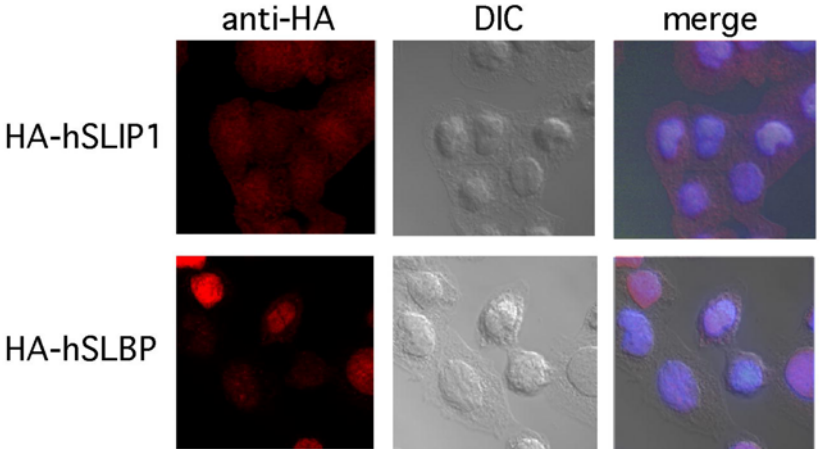
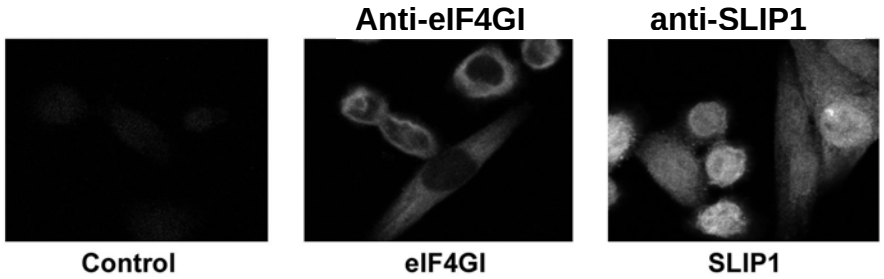

C
IP
Lysate
Anti-Myc
Anti-HA
HA-hSLIP1
hSLBP
eIF4GI
1 2 3
D
No treatment
+ HU treatment
IP
Lysate
Anti-Myc
Anti-HA
HA-hSLIP1
hSLBP
1 2 3 4 5 6
E
IP
Lysate
Anti-Myc
Anti-HA
- HU
+ HU
eIF4GI
1 2 3
F
No RNaseA
RNase A
IP: α-hSLIP1
Input
α-myc
α-hSLIP1
hSLIP1
hSLBP
eIF4GI
1 2 3 4 5
G
eIF4GI 123-420
eIF4GI 27-129
Input
GST
GST-hSLIP1
eIF4GI
1 2 3 4 5 6
H
eIF4GI 27-129
Input
GST
GST-hSLBP-his
GST-hSLBP-his
eIF4GI 27-129

Figure 19. Localization of SLIP1. A) HeLa cells stably expressing either HA-SLIP1 or HA-SLBP were processed for immunofluorescence as previously described(Erkmann et al. 2005b). Cells were stained with anti-HA antibody to show both SLIP1 and SLBP localization in the respective cell lines, and DAPI to show nuclei. Left panels show anti-HA staining in red, middle panels show DIC images, and right shows merged anti-HA and DIC images in addition to nuclear staining by DAPI in blue. **B)** Comparison of hSLIP1 localization with eIF4GI. HA-SLIP1 cell line was stained either with anti-HA (right panel) or anti-eIF4GI (middle panel) antibodies. Control cell (Left panel) are not treated with anti-HA or anti-eIF4GI antibodies, but only treated with secondary antibody (Rabbit). **C)** Localization of endogenous SLIP1 by dual immunofluorescence. Anti-hSLIP and anti-TRAP antibody was used with a ratio of 1:100 and 1:500 on the same HeLa cells and the protocol for dual immunofluorescence was explained in methods and materials section. First panel shows DIC image. Green color represents TRAP staining and red represents SLIP1. Last panel shows the merged images of TRAP and SLIP1 stainings with DIC.

A


anti-HA
DIC
merge
HA-hSLIP1
HA-hSLBP

B


Anti-eIF4GI
anti-SLIP1
Control
eIF4GI
SLIP1

C

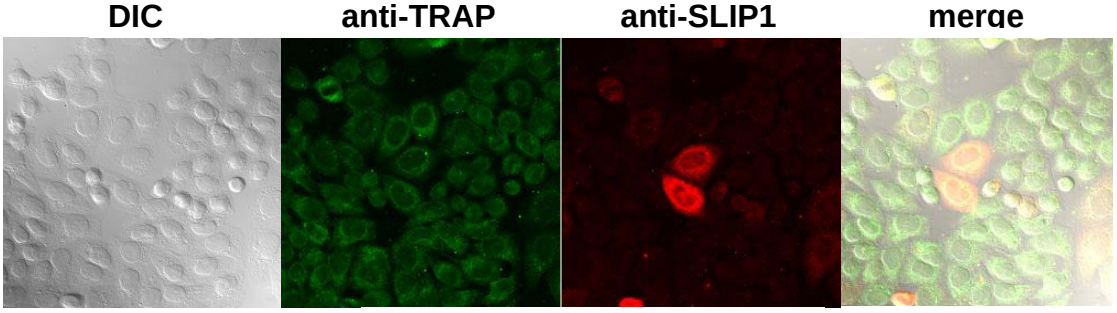
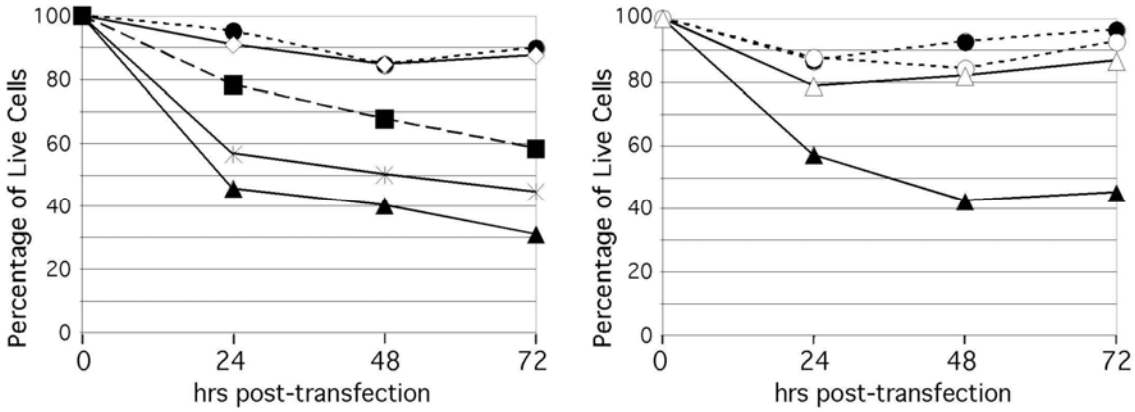
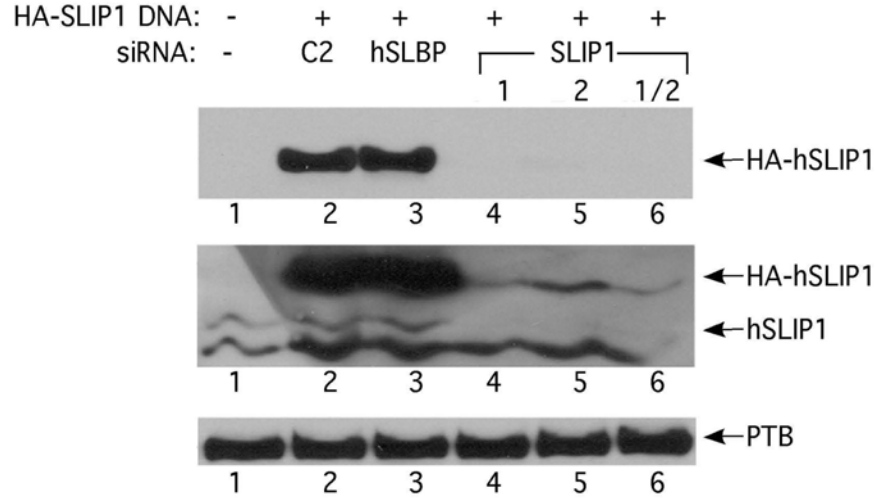

DIC
anti-TRAP
anti-SLIP1
merge
TRAP
hSLIP1

Figure 20. Knockdown of SLIP1 results in the death of HeLa cells. A) (Left) Cells were transfected with two different siRNAs against SLIP1 (siRNA #1, closed triangle; siRNA #2, closed square; co-transfection of siRNA #1 and #2, star), an siRNA against SLBP (open diamond), and a control siRNA (closed circle) with Lipofectamine 2000 according to manual. Final concentrations of the siRNAs were 90 nM in the reactions. (Right) Cells were transfected with siRNA#1 against human SLIP1 (closed triangle) or a control siRNA (closed circle), together with either a plasmid expressing GFP (SLIP1 siRNA + GFP, closed triangle; control siRNA + GFP, closed circle) or a plasmid expressing an RNAi resistant form of SLIP1 (SLIP1*) against siRNA#1 (SLIP1 siRNA + HA-SLIP1*, open triangle: control siRNA + HA-SLIP1*, open circle) with Lipofectamine Regular according to the manual. Total amount of the siRNAs and DNA constructs were 180 nM and 500 ng, respectively. siRNA resistant form of SLBP (HA-SLIP1*) was constructed by mutating the third nucleotides of the codons in the region targeted by the siRNA#1. But this HA-SLIP1* is not resistant to siRNA#2 since second siRNA targets a different sequence in SLIP1. The viability of the cells was determined daily for consecutive 3 days. There were fewer total cells in the siRNA treated cultures without the RNAi resistant SLIP1, suggesting that cell growth had also been affected. **B)** A duplicate of siRNA treated cells used in A (left) was tested for the knock down efficiencies 48 hours after the first transfection. The ability of the two different siRNAs to knock down endogenous SLIP1 protein and exogenously expressed SLIP1 (HA-tagged SLIP1) in HeLa cells (lanes 4, 5) and a combination of the two SLIP1 siRNAs (lane 6) as well as C2 siRNA (lane 2) and hSLBP siRNA (lane 3) treated cells were tested. Equal amounts of proteins resolved by gel electrophoresis were analyzed by Western blotting using an anti-HA antibody (top), anti-hSLIP1 antibody (middle) or an antibody against polypyrimidine tract binding protein (PTB, bottom) as a loading control (Wagner and Garcia-Blanco 2001). **C)** A duplicate of siRNA treated cells in A (right) were tested for the knock down efficiency of SLIP1 by western blotting. Cells were treated with either the control siRNA C2 (lanes 2-4) or the indicated SLIP1 siRNA (lanes 5-8), together with either a plasmid expressing HA-tagged SLIP1 (lanes 3, 6, 7) or a plasmid expressing HA-SLIP1 resistant to siRNA#1, but not siRNA2 (lanes 4 and 8). Anti-hSLIP1 antibody was used to simultaneously detect both exogenously expressed HA-SLIP1 and endogenously expressed SLIP1.

A


Percentage of Live Cells
100
80
60
40
20
0
0
24
48
72
hrs post-transfection
Percentage of Live Cells
100
80
60
40
20
0
0
24
48
72
hrs post-transfection

B


HA-SLIP1 DNA: - + + + + +
siRNA: - C2 hSLBP SLIP1
1 2 1/2
HA-hSLIP1
1 2 3 4 5 6
HA-hSLIP1
hSLIP1
1 2 3 4 5 6
PTB
1 2 3 4 5 6

C

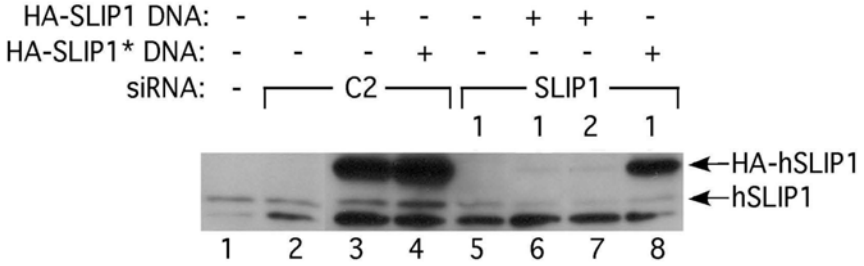

HA-SLIP1 DNA: - - + - - + + -
HA-SLIP1* DNA: - - - + - - - +
siRNA: - C2 SLIP1
1 1 2 1
HA-hSLIP1
hSLIP1
1 2 3 4 5 6 7 8

Figure 21. MS2-hSLIP1 activates translation of Luciferase mRNA ending with MS2-binding site. hSLIP1 sequence was C-terminally fused to the sequence encoding MS2 protein in pXFRM vector (MS2-SLIP1) and a luciferase mRNA ending in MS2-binding site (Luc-MS2) was manipulated to investigate the translational activity of hSLIP1 in tethering assay. SLIP1 binding to the reporter was mediated by MS2, since hSLIP1 can not bind to stem loop or MS2-binding site itself (unpublished data, Marzluff Lab). The *in vitro* translation assay and calculation of fold activations were previously described in chapter 2. As a control, MS2-hSLBP and MS2 were used in the assay for their activities as reported previously (Sanchez and Marzluff 2002).

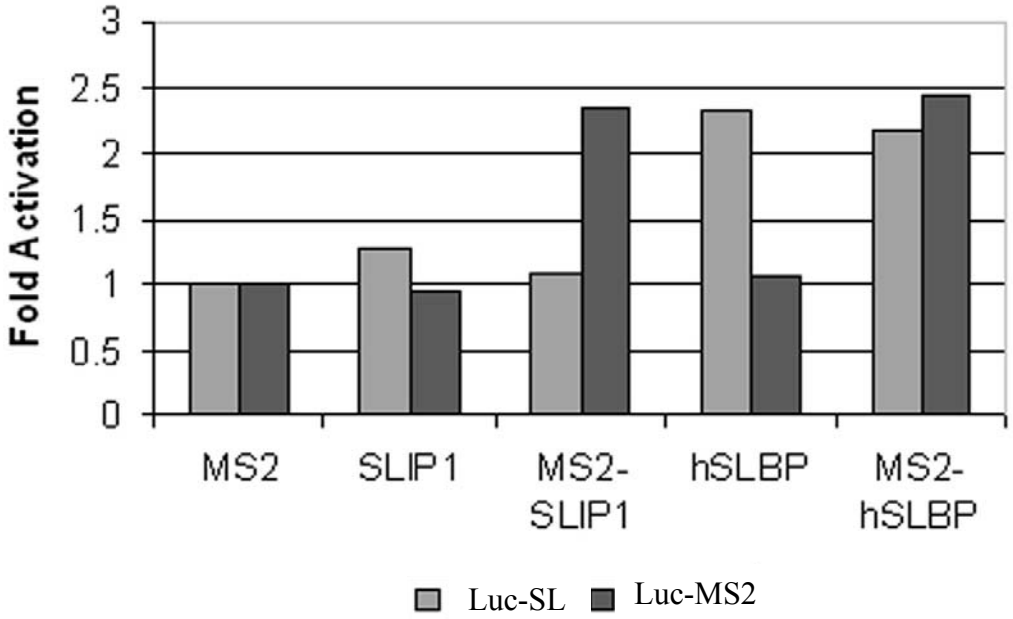
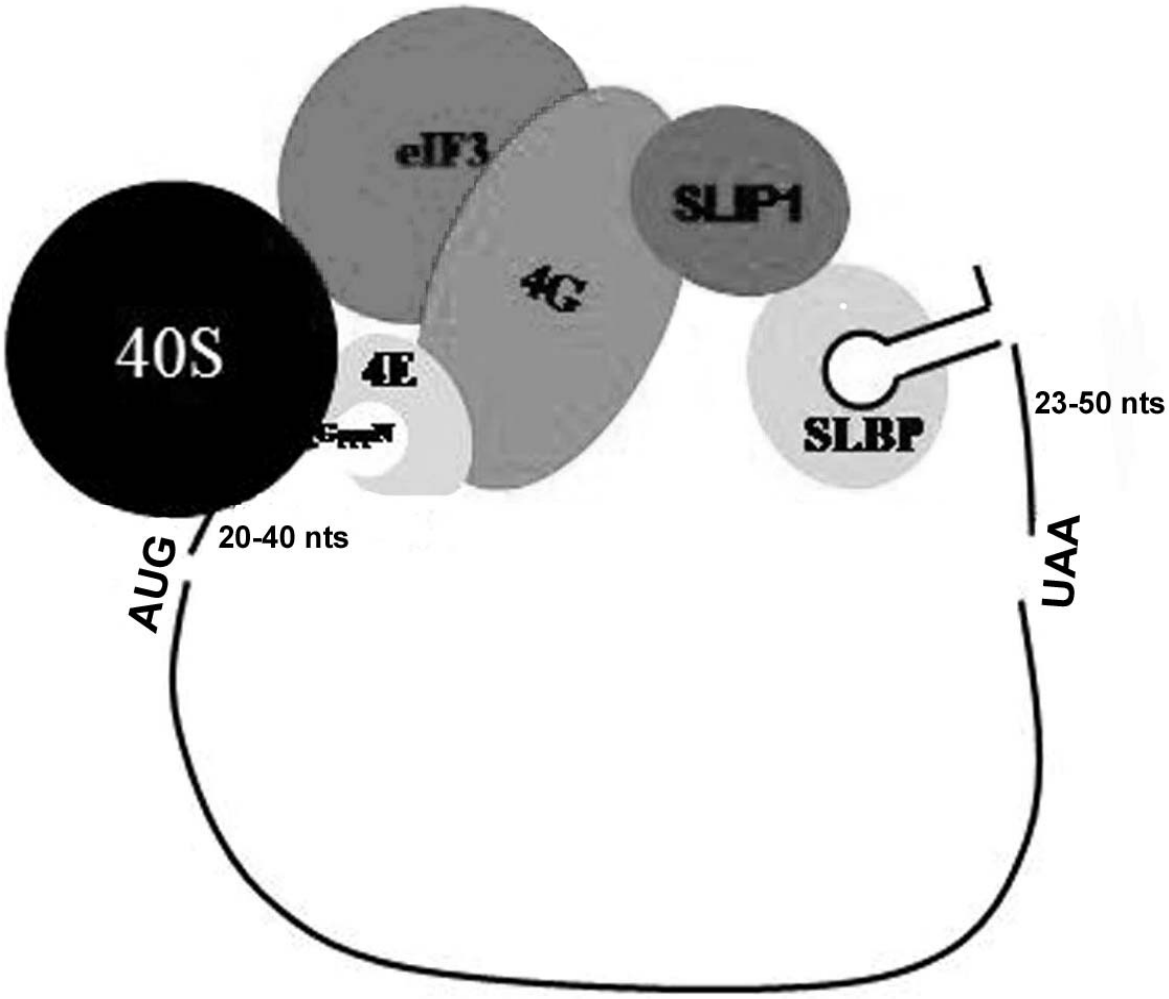

Fold Activation
3
2.5
2
1.5
1
0.5
0
MS2
SLIP1
MS2-SLIP1
hSLBP
MS2-hSLBP
Luc-SL
Luc-MS2

Figure 22. Translation initiation model for histone mRNA. SLBP binds to the stem loop at the 3' end of the histone mRNA. 5' end of the messaged is capped and bound by eIF4E that interacts with eIF4G. SLIP1 spontaneously binds to eIF4GI and SLBP, thus circularizing the mRNA. eIF4GI binds eIF3, in turn ribosomal small subunit 40S is recruited to the mRNA. Then 40S subunit starts to search first initiator methionine codon.


eIF3
SLIP1
4G
40S
4E
SLBP
23-50 nts
20-40 nts
AUG
UAA

CHAPTER IV

DETERMINATION OF MINIMAL SEQUENCE OF SLBP REQUIRED FOR VIABILITY IN *D. MELANOGASTER*

Introduction

In all metazoans, histone mRNAs end in a conserved stem-loop rather than a poly (A) tail (Dominski and Marzluff 1999). The stem loop is bound by a 220 amino acid protein called stem loop binding protein (SLBP). SLBP participates in several aspects of histone mRNA metabolism, including processing to create a mature mRNA, transport of the message from the nucleus to cytoplasm and translation of histone mRNAs.

Drosophila melanogaster has a single set of replication-dependent histone genes that are encoded in a 5-kb repeat present in approximately 100 tandem copies near the centromere on the left arm of chromosome 2 (Lanzotti et al. 2002). Each repeat unit contains one copy of each of the five histone genes. Like the histone genes in other metazoans, these genes in *Drosophila* encode non-polyadenylated mRNAs, ending in stem loop.

In *D. melanogaster*, DNA replication occurs rapidly in early development, creating a massive demand for histone proteins during the first 13 syncytial cell cycles. This demand is met by the stored histone proteins deposited in the *Drosophila* egg. Expression of zygotic histone mRNA, essential for cell division, is initiated after cycle 11. SLBP in *D. melanogaster* has been demonstrated to be required for production of properly processed histone mRNA *in vivo* (Lanzotti et al. 2002). SLBP mutant flies do produce histone mRNAs

but these mRNAs are polyadenylated due to read through passed the stem loop. Null mutant flies for SLBP allele do not survive dying in the late larval or pupal stage (Lanzotti et al. 2002). Thus properly processed histone mRNA is essential for viability.

The null mutant of SLBP in *Drosophila* can be rescued by expression of a full-length SLBP, which restored both processing and viability. However, expression of a mutant SLBP that only has the last 110 amino acids of SLBP including last 17 amino acids from the N-terminus, RBD, and C-terminus can efficiently process the histone mRNA *in vivo*, but does not support viability (Lanzotti et al. 2002). This region of dSLBP is sufficient for processing histone pre-RNA *in vitro* (Dominski et al. 2002). Those observations suggested that there must be a second essential function(s) of SLBP in addition to its processing activity, and that region must in in the first 180 amino acids of dSLBP.

The N-terminus of vertebrate SLBP was previously shown to be involved in histone mRNA translation (Sanchez and Marzluff 2002). Sanchez and Marzluff have identified a 15 amino acid region in the N-terminus of xSLBP that is required to stimulate translation of reporters ending in histone stem loop (Sanchez and Marzluff 2002). Whether this activity in stimulating translation of histone mRNA is essential for viability in vertebrates and *Drosophila* is not known.

Based on all those observations, I postulate that dSLBP is likely involved in the translation of histone mRNA in *Drosophila*, and this is an essential function of dSLBP during development. Thus the essential function of the N-terminal region of dSLBP is to promote histone mRNA translation, which is necessary for the survival of *D. melanogaster*. While the minimal region required for translation of histone mRNA has been discovered in several vertebrates, there is no significant homology outside of the RBD between vertebrates

and *D. melanogaster*. Therefore, the translational activation region in the dSLBP N-terminus could not be identified by sequence comparison. Moreover, *Drosophila* SLBP does not function in *Xenopus* oocytes and reticulocyte lysate system in either processing or translation of histone mRNAs (unpublished data). So, there is not a system in which one can study the factors required for *Drosophila* histone mRNA translation *in vivo* or *in vitro*. Therefore, the translational activation region in the dSLBP N-terminus could not directly be identified in our systems.

To identify the minimal region required for the viability in *D. melanogaster*, in collaboration with Jeremy Kupsco in Bob Duronio's lab, we constructed transgenic flies containing N-terminal deletions in dSLBP and tested these for their ability to rescue the viability. All these deletion mutants were able to properly process histone pre-mRNA. A deletion mutant that fails to complement lethality in a SLBP mutant background but is able to rescue histone pre-mRNA processing must contain the region that plays an essential role other than processing.

In this chapter, I demonstrate that the second critical region for viability in dSLBP is located between amino acids 86 and 139 in the N-terminus. This 53 amino acid region contains a motif with similarity to that found to be critical for translation in vertebrates. I identified a point mutant in this region that does not bind the *Drosophila* orthologue of the vertebrate SLBP-Interacting Protein 1 (SLIP1) and this mutant can not rescue the viability. Based on those observations, I hypothesize that the region required for viability may be responsible for translational activation of histone mRNA.

Methods and Materials

Construction of Transgene Strains Expressing SLBP Mutations

The HA-tagged dSLBP genomic locus in the pRD121 fly vector was used as a template to amplify dSLBP sequences using PCR reaction which were then cloned into the pXFRM vector with SphI at 3' end and PstI at 5'end. This clone was then used to create serial deletions in the N-terminus of dSLBP. Each mutant lacks one of the putative translation activation regions in the N-terminus. Deletions start at the first amino acid and continue down to the stated amino acid number: Δ35, Δ87, and Δ139. The mutants in pXFRM vector were digested with SphI and PstI and ligated back into the fly vector. The cloning of Δ179 has been previously reported (Lanzotti et al. 2002). Those clones were injected into fly embryos and were used to generate transgenic fly strains which also contain the wild type *dSLBP* allele. dSLBP K99F100V104E105→AAAA mutant was first created in pXFRM vector by site directed mutagenesis and then cloned back into pRD121 fly vector.

Jeremy Kupsco carried out appropriate crosses with the dSLBP null mutant to create heterorozygote flies that were then crossed to give embryos where the only SLBP gene is the transgene. He evaluated the ability of the transgenes to rescue viability. Since all the embryos were viable until the third larval instar, Jeremy was also able to determine whether the transgene was able to restore histone pre-mRNA processing.

In Vitro Expression Constructs

dSLBP truncation mutants Δ86, and Δ139 were amplified in PCR reaction with primers phosphorylated at the 5' end by using dSLBP without its intron in pXFRM as a

template. Blunt ends were ligated with T4 DNA ligase and transformed into DH5α cells. Using the same dSLBP template in pXFRM vector, dSLBP K99F100→AA mutant was generated by site directed mutagenesis according to manufacturer's protocol. Then PCR products were treated with 1.5 μl DpnI enzyme (NEB) for 30 minutes at 37°C. Purified products were transformed into DH5α cells. Sp6 promoter preceding the cloned sequences was used to translate Δ86, Δ139, and K99F100→AA in reticulocyte lysate system.

Cloning of dSLIP1

dSLIP1 was identified by sequence homology to the human SLIP1 and the cDNA cloned into a pOT7 vector was obtained from Steve Crews' Lab (UNC-CH). This clone was used as a template to amplify the dSLIP1 sequence with 5' and 3' SpeI sites without a stop codon in the 3' reverse primer by PCR. Purified PCR products and pGEX-GST-6his vectors were digested with SpeI restriction enzyme. Digested PCR products and vectors were mixed in a ratio of 3:1, respectively, in a ligation reaction. The amplified fragments of dSLIP1 were cloned into the pGEX vector and N-terminally tagged with GST and C-terminally with 6 histidines. Thus, dSLIP1 was cloned to generate GST-dSLIP1-His. The sequences were confirmed by automated sequencing.

Expression of GST-dSLIP1-his in E. Coli

The GST-SLIP1-his in the pGEX vector was transformed into BL2lRL+ cells. These cells were grown at 27°C until OD_{595} was 0.6. Protein expression was induced with 5 mM

IPTG overnight at 20°C. Induced cells were harvested at 4°C at 5000 rpm in a Sorvall centrifuge. 10 ml of lysis buffer containing 20 mM Tris pH 7.5, 150 mM NaCl, 10% Glycerol, 10 mM DTT, 1 mM PMSF, and 1X Protease Inhibitor Cocktail (Sigma-Aldrich), was used to lyse the pelleted cells. Sonication was performed on these cells on ice. The lysate was centrifuged at 10,000 rpm for 15 minutes at 4°C. GST-dSLIP1-his fusion protein was purified through Ni+ column as explained in chapter 3 to ensure that the full length protein was purified. Expression was verified by with Coomassie blue staining. Expression of GST in pGEX vector was also described in chapter 3.

The dSLBP-his protein was expressed in Baculovirus system according to manufacturer's manual, and purified by chromatography on Ni+ agarose..

GST Pull Down Experiments

To test the interaction between dSLIP1 and dSLBP, 3 μg of purified GST-dSLIP1-his was mixed with 2-3 μg of purified dSLBP. The mixture was incubated on ice for 30 minutes. Next, 50 μl of GSH-beads, which had been washed 3 times with 1X PBS, was added to the protein mix and incubation on ice continued for an additional 30 minutes. The mixture was then washed 3 times with 1X PBS supplemented with 100 mM NaCl. Bound proteins were re-suspended in 40 μl of 2X SDS-sample buffer. The mixture was analyzed on a 12% SDS-PAGE and bound dSLBP was detected by western blotting.

Pull down assays between GST-dSLIP1-his and either wild type dSLBP, Δ86, Δ139, or dSLBP K99F100→AA were performed as explained above, except dSLBPs were

expressed in reticulocyte system (TNT, Promega) and labeled with ^{35}S-methionine according to manufacturer's manual.

Western Blot Analysis

For protein extraction, 0-12 hour embryos or adult female flies were collected from homozygous transgenic lines. The embryos were dechorionated in 50% bleach and ground in a solution of lysis buffer (50 mM Tris [pH 7.5], 150 mM NaCl, 0.5% NP-40, 50mM NaF) and protease inhibitors (1:1000 leupeptin, 1:100 pepsin, and 1:100 PMSF). SDS containing sample buffer was then added. The extracts were then boiled for 5 minutes and spun for 30 seconds at 4,000 rpm. 100 μg of protein from each extract were loaded and run on a 12% polyacrylamide gel. The proteins were transferred to a nitrocellulose membrane, and blocked 1 hour with 5% milk diluted in 1X PBS containing 0.05% Tween 20 (PBST) at RT. The membranes were incubated for 1 hour at RT with mouse monoclonal anti-HA (Covance, Denver CO) diluted in 5% milk-PBST to a ratio of 1:500. The membranes were then incubated for 1 hour at RT with anti-mouse secondary antibodies diluted in 5% milk-PBST to a ratio of 1:10000. As loading control, blots were probed with mouse anti-beta tubulin (Amersham Biosciences) with a ratio of 1:2000. Detection of antibodies was performed with ECL reagents and exposure to X-ray film (Kodak).

dSLBP-his protein was detected by anti-dSLBP antibody by using the same technique explained above.

Northern Blot Analysis

Total cellular RNAs for wild type, Δ35, Δ86, Δ139, and Δ179 were isolated from 0adult female flies using TRIzol Reagent (Gibco). For analysis of histone mRNAs, 2 μg of RNA per lane denatured with 7 M urea were subjected to electrophoresis in 1x TBE in a 1-1.5% agarose gel containing 0.5 M MOPS (pH 7.0) and 2.2 M formaldehyde. Once separated, the RNA was transferred in cold 0.5x TBE to an N+ nitrocellulose membrane (Amersham) using a Genie Blotter (Idea Scientific). The RNA was crosslinked with UV irradiation to the nitrocellulose membrane with a Stratalinker UV Crosslinker (Stratagene). For detection of the RNA, a probe was made from DNA containing the coding region of H3, or rp49 which were labeled with $\alpha[^{32}P]$-dCTP using a random primer labeling kit (Stratagene). The hybridizations were performed at 60°C for H3 and rp49 or U1 overnight in QuickHybe (Stratagene).

Determination of the Ability of SLBP Transgenes to Rescue Viability

Mutant lines that expressed dSLBP transgene were selected for phenotypic rescue crosses. The lines used were as follows: T271, T279, and T280 (Δ35 deletion), T268 and T270 (Δ86 deletion), and T287 and T288 (Δ139 deletion) on either chromosome 2 or X, except Δ86 strains. The transgenes for T268 and T270 (Δ86) were located on chromosome 3, the same chromosome in which the *SLBP* gene is located. Therefore, a crossing scheme involving a recombination event was completed for both lines before the rescue crosses. Four recombinant lines were created for the T268 and T270 mutants. They were named T333 and T334 (T270), and T335 and T336 (T268).

For the phenotypic rescue crosses, a series of three crosses were performed so that the transgene was on one of the second chromosome (lines T268, T270, T271, and T288) or X chromosome (lines T279, T280, and T287). Also, either *SLBP*^{10} or *SLBP*^{15}, and *SLBP*^{12} were on homologous third chromosomes. *SLBP*^{12} is a null, lethal mutation; *SLBP*^{10}, is a sterile hypomorphic mutation, and *SLBP*^{15} is a lethal mutation (Sullivan et al. 2001). Croses were as follows:

A.

Cross 1: ♀ p[Δ *Slbp*]/p[Δ *Slbp*] ; +/+ X ♂ +/+ ; *D*/TM3 *Sb, Ser*

↓

Cross 2: ♀ p[Δ *Slbp*]/+ ; +/ TM3 *Sb, Ser* X ♂ +/+ ; *Slbp*$^{10\ or\ 15}$/TM3 *Sb [act-GFP]*

↓

Cross 3: ♀ p[Δ *Slbp*]/+ ; *Slbp*$^{10\ or\ 15}$/TM3 *Sb, Ser* X ♂ +/+ ; *Slbp*^{12}/TM3 *Sb [kr-GFP]*

↓

Products: ♀ and ♂ p[Δ *Slbp*]/+ ; *Slbp*$^{10\ or\ 15}$/ *Slbp*^{12}

B.

Cross 1: ♀ p[Δ *Slbp*]/ p[Δ *Slbp*] ; +/+ X ♂ +/Y ; *D*/TM3 *Sb, Ser*

↓

Cross 2: ♂ p[Δ *Slbp*]/Y ; +/TM3 *Sb, Ser* X ♀ +/+ ; *Slbp*$^{10\ or\ 15}$/TM3 *Sb[act-GFP]*

↓

Cross 3: ♀ p[Δ *Slbp*]/+ ; *Slbp*$^{10\ or\ 15}$/TM3 *Sb, Ser* X ♂ +/Y ; *Slbp*^{12}/TM3 *Sb [kr-GFP]*

↓

Products: ♀ p[Δ *Slbp*]/+ ; *Slbp*$^{10\ or\ 15}$/ *Slbp*^{12} and ♂ p[Δ *Slbp*]/Y ; *Slbp*$^{10\ or\ 15}$/*Slbp*^{12}

C.

Cross 1: ♀ p[Δ *Slbp*], *Slbp*^{12}/TM3 *Sb* X ♂ *Slbp*$^{10\ or\ 15}$/TM3 *Sb, Ser*

↓

Products; ♀ and ♂ p[Δ *Slbp*], *Slbp*^{12}/ *Slbp*^{15}

A. Phenotypic rescue crossing scheme for transgene on chromosome 2. B. Phenotypic rescue crossing scheme for transgene on X chromosome. C. Phenotypic rescue crossing scheme for transgene on chromosome 3. Wild type chromosomes are indicated by "+" and "p" indicates a p-element insertion of the transgene.

Later, same mutant strains were tested for their ability to rescue viability when they were two copies on the same chromosome, except Δ86 as one copy since it was on the same chromosome as wild type dSLBP.

These experiments were done in collaboration with an undergraduate research student (Andrew Courson) under my direction, who made many of these constructs, and together we characterized the expression of the HA-SLBPs in the resulting transgenic flies. Jeremy Kupsco in Bob Duronio's lab carried out the viability studies and the analysis of the histone mRNA by Northern blotting.

Results

Amino Acids Between 86 and 139 in The N-terminus of dSLBP is Required For Survival During Early Embryogenesis

SLBP function is required maternally and zygotically to achieve a complete development and then to form an adult fly (Sullivan et al. 2001). dSLBP null mutants, $SLBP^{12}$ and $SLBP^{15}$, do not support survival of zygotic development and result in production of stable histone mRNAs that accumulate in non-replicating cells and terminate with polyA tail after stem loop due to a cryptic polyadenylation signal, suggesting a deficiency in processing of histone pre-mRNA. Those results suggested that processing activity of SLBP is at least partially necessary for survival, but does not rule out any other possible functions of SLBP. However, the hypomorphic mutant allele, $dSLBP^{10}$, supports zygotic development and healthy adult flies are produced, but the females are sterile (Sullivan et al. 2001). These embryos demonstrated chromosome condensation defects at mitosis during syncytial stage,

thus blocking anaphase. Failure of DNA condensation suggests that histone proteins are present at lower levels than the levels in wild type eggs. The levels of histone mRNAs in the eggs are also reduced about 10-fold in the $SLBP^{10}$ mutant, likely resulting in a reduction of histone proteins deposited in the eggs. So I postulate that SLBP may have other functions to support survival in addition to its processing activity, possibly translation of histone mRNAs.

Previously, it has been shown that the SLBP N-terminus in vertebrates is necessary for stimulation of histone mRNA translation (Sanchez and Marzluff 2002; Gorgoni et al. 2005). In addition I represented the evidence for the presence of a highly conserved translation activation motif in the N-termini of *Xenopus* and human SLBPs (Chapter 2, Cakmakci et al., 2007 submitted).

To investigate the N-terminal region of dSLBP for candidate translation activation motif, I aligned N-termini of human, mouse, *Xenopus* and *Drosophila* SLBPs. Unfortunately, there is no significant homology outside of the RBD between vertebrates and *D. melanogaster*. Therefore, the translational activation region in the dSLBP N-terminus could not be identified by sequence comparison with a high accuracy unlike vertebrates. Figure 23A shows a diagram of the dSLBP. Note that the RBD is close to the C-terminus and hence there is a long N-terminal region of 188 amino acids prior to the region required for processing. Based on the nature of the amino acids in vertebrate translation motif, DWX_3VEE, we predicted three possible motif regions in dSLBP with some similarity to vertebrate motif: a polar residue followed by an aromatic residue, separated by 3 amino acids from an acidic region (Figure 23B). We created serial deletions in the N-terminus of dSLBP, each one excluding one of the candidate motifs (Figure 23C).

First, those mutants Δ35, Δ86, Δ139 and Δ179 mutants were tested for their ability to support viability during *Drosophila* development. For this purpose, mutant transgenes and a wild type transgene for dSLBP were injected into flies and crossed into an $SLBP^{15}$ null background, except for the Δ86 transgene which is in a background of $SLBP^{15}/SLBP^{12}$ (the Δ86 transgene exists as a recombinant chromosome with the $SLBP^{12}$, a null deletion allele of *SLBP*). Then, the ability for each deletion to complement the lethality associated with loss of SLBP protein was measured. The wild type transgene as well as Δ35 and Δ86 mutants were able to fully rescue viability in an *SLBP* mutant background, while the Δ139 and Δ179 deletions were not able to rescue viability in an *SLBP* mutant background to any extent (Table 2). These results demonstrate that the region between amino acids 86th and 139th is required for the viability. To demonstrate that the inability of Δ139 and Δ179 to rescue viability in a *SLBP* mutant background was not due to the lack of transgene expression, expression of each HA-tagged protein was verified by western blotting with anti-HA antibody. Figure 24A shows the expression from all the generated mutant strains. Note that the Δ86 mutant had the lowest expression level and the Δ35 and D179 mutants were expressed at a much higher level. Figure 24B shows the expression from the strains tested for viability in table 2. Expression of all the transgenes could be readily detected, except for the Δ86 deletion which was not detected in this experiment due to the low amount of protein loaded. Also note that the Δ179 and the wild type transgenes each had 3 HA-tags while the other deletions only had a single tag. Since the Δ86 transgene is able to genetically rescue viability in an *SLBP* mutant background, the low level of expression must be sufficient to rescue the null mutant. More importantly, the transgenes that failed to rescue viability were expressed at levels similar to the levels of wild type transgene, indicating that the inability of

these transgenes to rescue viability in an *SLBP* mutant background is not due to a low level of transgene expression (Figure 24B). Our initial analysis in the fly thus suggests that the 53 amino acid region lying between 86 and 139 positions in the N-terminus of dSLBP contains a sequence that is required for the viability during development. In addition to restoring viability the Δ86 transgene also resulted in males and females that were fertile, although the female fertility was slightly reduced (data not shown).

dSLBP Has Another Function in Addition to Its Processing Activity

This result does not rule out the possibility that the early death of the flies is due to the defects of histone pre-mRNA processing. Next, we determined whether processing of histone mRNA was separate from the essential activity between amino acids 86 and 136. Dominski et al., 2002 have demonstrated that the RBD of dSLBP with its 17 amino acid long C-terminus is necessary and sufficient to carry out histone pre-mRNA processing in the *in vitro* processing extracts (Dominski et al. 2002). However these studies did not determine if the delineated region is sufficient to support processing of histone pre-mRNA *in vivo*. So, it is possible that there are additional requirements *in vivo* for processing that are not needed in the *in vitro* system. Therefore, we determined the processing efficiencies of those mutant flies over an *SLBP* mutant background using *in situ* hybridizations with the H3-ds probe, which only recognizes mis-processed H3 ending with polyA tail after stem loop (Sullivan et al. 2001), and by Northern blot analysis of histone mRNAs present in mutant 3rd instar larva. The experiments were performed on $SLBP^{15}$ homozygous embryos that carry two copies of each transgene, except for the Δ86 transgene which is in an $SLBP^{15}/SLBP^{12}$ trans

heterozygous background and only carries one copy of the Δ86 transgene due to presence of the transgene on a recombinant chromosome with *SLBP*^{12}. The wild type dSLBP transgene over *dSLBP*^{15} background was included in this study for comparison to estimate the efficiencies of the rescue of the mis-processing by each mutant transgene.

The wild type flies should not have expression of mRNAs containing the downstream region (Figure 25A), while the SLBP^{15} mutant flies showed extensive expression throughout the embryo (Figure 25B). The wild-type dSLBP transgene extensively rescued histone pre-mRNA processing in the central nervous system (Figure 25C, arrow), although there was some mis-processed H3 mRNA present in the gut endocycling cells. The Δ86 line also rescued processing in the central nervous system (Figure 25D, arrow) and gave an overall phenotype similar to the wild-type transgene (Figure 25C). Strikingly in both the Δ139 and Δ179 embryos, there was complete rescue of processing in the nervous system (Figure 25E and F, arrows), and some residual staining in the gut, similar to that seen in both embryos either with wild type or Δ86 transgenes (Figure 25C and D). Since the Δ139 and Δ179 transgenes did not rescue viability but did rescue processing we can conclude that there must be an essential function of dSLBP other than histone pre-mRNA processing.

To precisely examine the amount of rescue of histone pre-mRNA processing by the mutants, Northern analysis was performed on total RNAs extracted from 3rd instar larvae containing each of the transgenes. The wild type embryos showed no longer mRNAs, while in the *SLBP*^{15} mutants all the RNAs were mis-processed (Figure 26, lanes 1 and 2). The full-length SLBP transgene rescued processing about 40% as did the Δ179 transgene (Figure 26, lanes 3 and 7). The Δ35 transgene completely rescued processing, likely a result of the high expression of this gene, while both the Δ86 and Δ139 transgenes rescued processing to a

greater extent that the wild type transgene (Figure 26, lanes 4-6). These results are consistent with the results in embryos analyzed by in situ hybridization (Figure 25). Importantly, both Δ139 and Δ179 transgenes, that failed to rescue the viability, rescued processing at least as well as the wild type dSLBP transgene. Furthermore, the Δ179 protein is the minimal protein required for processing *in vitro*. The ability of this protein to rescue processing confirms the *in vitro* result that only the RBD and C-terminus is required to efficiently process histone pre-mRNAs (Dominski et al. 2002). These results strongly suggest that the inability of Δ139 and Δ179 to rescue viability is not due to their inability to process histone mRNA, but must be due to some other essential function of the SLBP N-terminus.

dSLBP Interacts with dSLIP1.

Since we demonstrated that in addition to its processing activity, SLBP has another function that is important for the viability of *D. melanogaster*, we postulated that this function may be related to translation of histone mRNAs. dSLBP did not work in the translation assays that were used to analyze human and *Xenopus* SLBPs for their translational activities in the previous chapters in my thesis (R. Sanchez, unpublished data). So, we followed an indirect approach to test translational activities of dSLBP and its mutants. Previously I showed that SLBP and hSLIP1 cooperate in stimulation of histone mRNA translation (Chapter 3). Mutants in translation motif of SLBP, that were incapable of stimulating the translation, also could not bind hSLIP1.

There is a potential SLIP1 homologue present in all the metazoan genomes sequenced thus far, also including *Drosophila* and *C.elegans*. We reasoned that if SLIP1 and SLBP

interaction is conserved for the translation of histone mRNAs in very divergent organisms, the interaction abilities of wild type dSLBP, Δ86 and Δ139 would allow us to understand the contribution of histone mRNAs translation to the viability of *Drosophila*. Thus, I tested the ability of dSLIP1 to bind dSLBP, point mutants in the putative translation activation domain 2 as well as deletion mutants (Figure 23B). Point mutants were also tested for their ability to rescue viability.

dSLIP1 was cloned out of POT7 vector obtained from the *Drosophila* SLIP1 collection. Figure 27 shows the sequence comparison of dSLIP1 with human SLIP1. dSLIP1 is composed of 510 amino acids and has an extended N-terminus compared to hSLIP1. The hSLIP1 is homologous to the last 210 amino acids in the C-terminus of dSLIP1 (Figure 27). The dSLIP1 is also predicted to contain HEAT repeats like hSLIP1.

I used an *in vitro* GST pull down assay to determine whether dSLIP1 could interact with dSLBP, and with the different dSLBP deletion mutants. GST-dSLIP1-his was expressed in E.coli and purified through Ni+ column. To test for direct interaction between the two proteins, his-dSLBP expressed in baculovirus was used. GST was used to measure the nonspecific binding of dSLBP. dSLBP bound to dSLIP1 was detected by western blotting with anti-dSLBP antibody. dSLBP binding with dSLIP1 was enriched over the background binding to GST, suggesting that dSLIP1 interacts with SLBP directly (Figure 28A, compare lane 3 to 2).

We tested the deletions mutants of dSLBP using a different pull-down assay. All the mutants were expressed in a reticulocyte lysate and labeled with ^{35}S-methionine. The lysates were incubated with the *E.coli* expressed GST-dSLIP-his. Radioactively labeled dSLBPs were detected by PhosphorImager and visualized by autoradiography. Both the wild-type and

the Δ86 dSLBP interacted with dSLIP1 to similar levels and showed very little binding to GST (Figure 28B). In contrast, Δ139 mutant that did not support the viability did not bind to dSLIP1 (Figure 28B). Those results suggest that there is a binding site for dSLIP1 between amino acids 86 and 139 in the N-terminus of SLBP.

There might be a conserved motif similar to vertebrate translation motif that is responsible to bind SLIP1. In figure 23B, all the possible motif regions were shown. Our analysis demonstrated that dSLBP (2) region might contain the translation motif, and hence the region essential for viability. To test if this is true, I created a double point mutant of full length dSLBP by substituting KF at positions 99 and 100 with alanines. dSLBP K99F100→AA was expressed and labeled with ^{35}S-methionine *in vitro* and was tested for its binding activity to dSLIP1. Strikingly, just two amino acid changes abolished the ability of the protein to interact with dSLIP1 (Figure 28B), reminiscent of the effect of the human and *Xenopus* point mutant SLBPs (Figure 15A, chapter 3). I also created a four amino acid point mutant in dSLBP, K99F100 and V104E105→AAAA in the transgene vector, and Jeremy Kupsco tested its ability to rescue the *SLBP*^{15}. This mutant did not support viability although it was expressed at similar levels as the wild-type SLBP transgene and was able to rescue the processing of histone pre-mRNA (not shown). This result is consistent with the possibility that a second essential function of SLBP is to support histone mRNA translation.

Discussion

In vertebrates, recent studies have demonstrated that the N-terminus of SLBP is necessary and sufficient and necessary for the efficient translation of histone mRNA

(Sanchez and Marzluff 2002; Gorgoni et al. 2005). In contrast, there is no report claiming any specific function that belongs to N-terminus of *Drosophila* SLBP. The last 110 amino acids, consisting of the RBD and short C-terminus are required to process histone pre-mRNA *in vitro* (Dominski et al. 2002). In this chapter, I provide the evidence showing that a 53 amino acid region between position 86 and 139 in the N-terminus of dSLBP is required for viability. The loss of this 53 amino acid region does not disrupt processing activity of dSLBP *in vivo,* demonstrating that this region of the N-terminus has an essential function for viability in the fly that is not related to the processing activity of dSLBP. Additionally we confirmed that the 53 amino acid region contains a motif similar to the vertebrate translation motif that is required to interact with dSLIP1 *in vitro*.

Amino Acids Between 86 and 139 in dSLBP N-terminus is Required for Viability

By using fly genetics, we were able to determine the region in the dSLBP responsible that is sufficient for viability and fertility of *Drosophila* embryos during development *in vivo*. The critical region lies in the N-terminus of dSLBP between amino acids 86 and 139 (Table 2). However, this 53 amino acid stretch is dispensable for the activity of dSLBP in histone pre-mRNA processing *in vitro* and *in vivo*, indicating that this region is responsible for some other processes that is required for the viability of the organism.

SLBP does not bind any RNAs other than histone mRNA 3' stem loop (Townley-Tilson et al. 2006). So it is very likely that mutations of dSLBP only affect the histone mRNA metabolism. However we still can not exclude any potential role for dSLBP outside of histone metabolism which may not require RNA binding activity of SLBP. Based on the

data from mammalian and *Xenopus* systems, it is most likely that this region of SLBP might also contain a motif that is required for the efficient translation of histone transcripts, and that this activity might be essential for viability.

dSLIP Interacts with the N-terminus of dSLBP between Amino Acids 86 and 139 in vitro

We identified a single *Drosophila* homolog of hSLIP1 in fly database. Fly data base ID for this protein is CG13124 and we have named it dSLIP1. dSLIP1 is longer than hSLIP1, but the last 210 amino acids of dSLIP1 is 26% identical to hSLIP1. The N-terminal of dSLIP1 is not homologous to any human protein. Because fly histone mRNAs end in stem loop, contain SLBP homolog and a SLIP1 homolog, we hypothesized that the mechanism of translation of histone mRNAs might have been conserved between vertebrates and very distantly related organisms, such as *Drosophila*.

Vertebrate SLBP interacts with SLIP1 *in vivo* (Figure 18C) and *in vitro* (Figure 15) and the interaction region maps to the translational activation motif, DWX_3VEE (Figure 14 and 15). We hypothesized that this interaction might also be preserved in *Drosophila*. dSLBP not only binds to dSLIP1, but the interaction region mapped to the region between amino acids 86 and 139 (Δ86 mutant), the region required for viability (Figure 28B; Table2). We identified a short sequence in this region which had some similarity to the vertebrate SLBP translation activation motif by amino acid sequence comparison with human, *Xenopus* and mouse SLBPs. This motif, KFX_3VKEE, contains a charged amino acid followed by an aromatic amino acid, and after a space of 3 amino acids, a V followed by an acidic region. This is similar to the motif responsible for translation activation in vertebrates, DWX_3VEE

defined in Chapter 2. A two amino acid mutation in the motif K99F100→AA, abolished binding of full-length dSLBP to dSLIP1 (Figure 28). Similarly a four amino acid mutation in the full length dSLBP (K99F100 V104E105→AAAA, the only mutant tested) was not capable of rescuing the *SLBP*^{15} mutant. Thus this motif is responsible for the interaction with dSLIP1 as it is true for vertebrate SLBP translation motif, and essential for viability. These studies strongly argue that KFX_3VKEE may be the translation motif required for stimulation of translation of histone mRNA through its interaction with dSLIP1 as in the vertebrate translation system. If this is true, then the second essential function of SLBP is to support translation of histone mRNA.

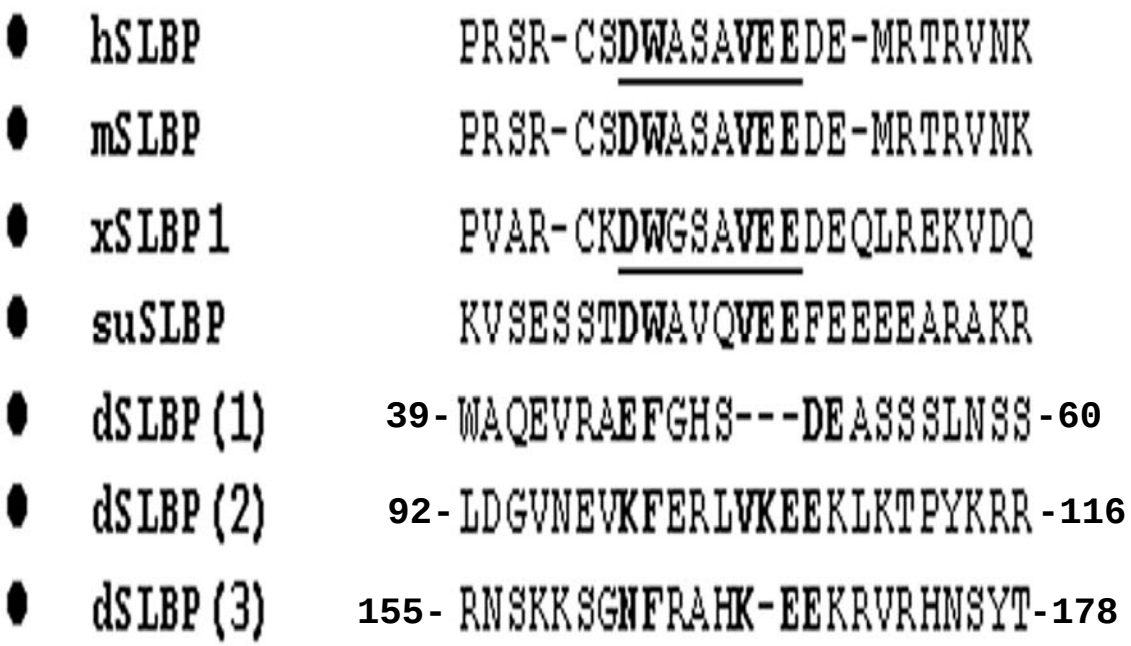
The motif KFX_3VKEE, differs substantially from the DWX_3KVEE motif in vertebrates. Substitution of *Xenopus* W to F killed the translational activity of SLBP (Figure 11, chapter 2), demonstrating that aromatic W can not be replaced with another aromatic residue like F and we know that the W to F mutation in vertebrates is inactive in translation. Hence it is not surprising that dSLBP does not stimulate translation in our vertebrate translation systems. The *Drosophila* motif also has a basic amino acid rather than an acidic amino acid adjacent to the aromatic residue. Thus dSLBP is not able to interact with hSLIP1 or xSLIP1 in oocyte and reticulocyte lysate based translation systems. In the future, it will be useful to develop a translation system to test translational activities of motif mutants of dSLBP together with dSLIP1. Several attempts to develop a system like that have proved unsuccessful (R. Sanchez, personal communication).

Figure 23. Sequence comparison of various SLBPs. A) Schematic of dSLBP. dSLBP is composed of 276 amino acids, consisting of an extended N-terminus, 71 amino acid long RBD that is 75% identical with vertebrate RBD of SLBP, and a 17 amino acid long C-terminus. Possible translation motif regions in the N-terminus are also numbered as 1, 2, and 3. **B)** Multiple sequence alignment of the region around the 13 amino acids required for translation in mSLBP, hSLBP, xSLBP1, suSLBP, and possible *D. melanogaster* (dSLBP) sequences. The underlined residues represent the essential core of the translation activation region with bold letters showing the invariant amino acids. Three possible regions in dSLBP that could be involved translation are shown with bold letters to be likely the most critical amino acids. dSLBP(1) is located between amino acids 39^{th} and 60^{nd}; dSLBP(2) between 92^{nd} and 116^{th}; dSLBP(3) between 155^{th} and 178^{th}. su stands for sea urchin SLBP. **C)** Schematic of SLBP N-terminal deletions. All deletion constructs have an N-terminal HA tag. The RBD box delineates the RNA binding domain of SLBP starting at amino acid 188 and ending at amino acid 259. Δ86 excludes the first [dSLBP(1)], Δ139 excludes the second [dSLBP(2)] and Δ179 excludes the third [dSLBP(3)] possible translation activation motif. Δ35 have amino acids between 1 and 35 deleted which do not contain any significant possible motif similarity to vertebrate translation motif.

A


1
189
259
276
1
N-Term
2
3
RBD
C-term

B


hSLBP PRSR-CSDWASAVEEDE-MRTRVNK
mSLBP PRSR-CSDWASAVEEDE-MRTRVNK
xSLBP1 PVAR-CKDWGSAVEEDEQLREKVDQ
suSLBP KVSESSTDWAVQVEEFEEEEARAKR
dSLBP(1) 39-WAQEVRAEFGHS---DEASSSLNSS-60
dSLBP(2) 92-LDGVNEVKFERLVKEEKLKTPYKRR-116
dSLBP(3) 155-RNSKKSGNFRAHK-EEKRVRHNSYT-178

C

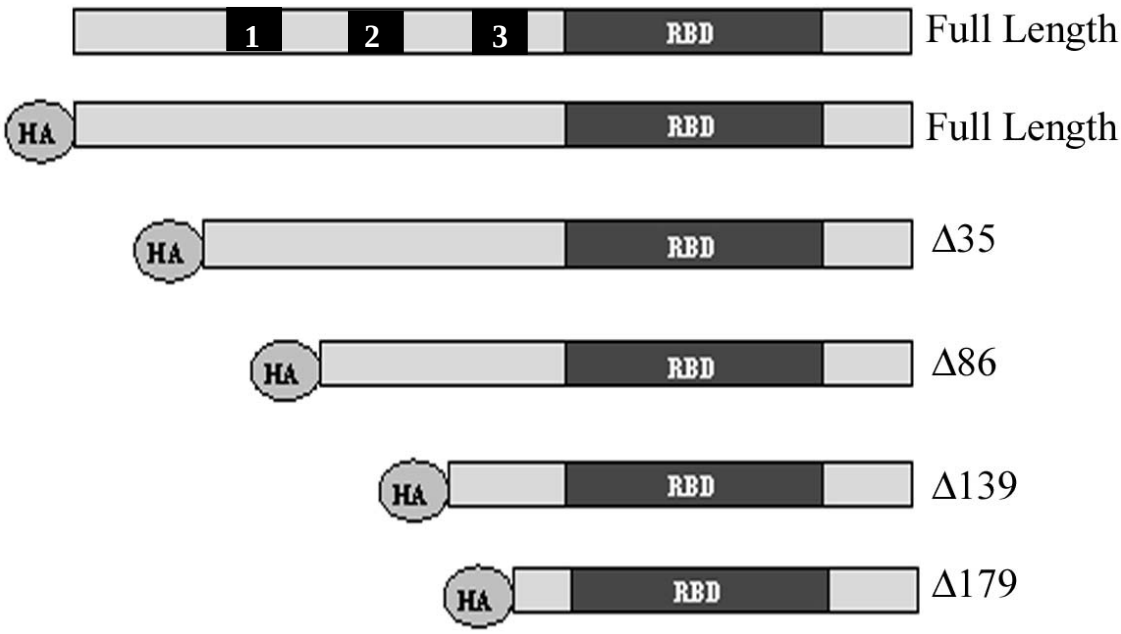
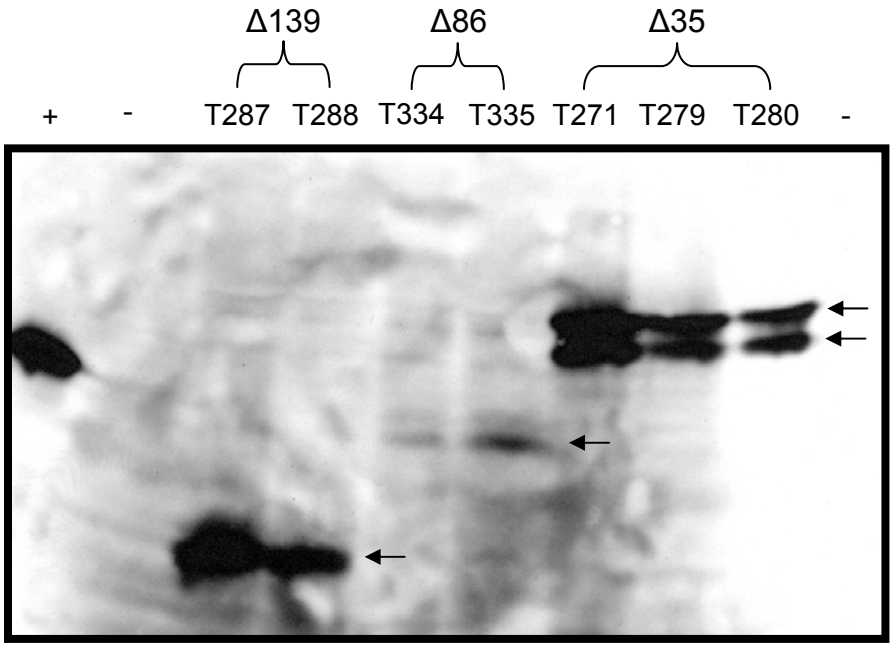

1
2
3
RBD
Full Length
HA
RBD
Full Length
HA
RBD
Δ35
HA
RBD
Δ86
HA
RBD
Δ139
HA
RBD
Δ179

Table 2. Ability of N-terminal deletions to rescue viability in an *SLBP* mutant background. Results of phenotypic rescue crosses are shown over the background of *Slbp*^{15} or *SLBP*^{12}

Transgene	Rescues Viability?
WT-SLBP	YES
Δ35	YES
Δ86	YES
Δ139	NO
Δ179	NO

Figure 24. Western analysis of SLBP transgenic fly embryos. A) Anti-HA antibody was used to detect wild type dSLBP and its mutant forms expressed from the transgenes. Protein extracts were prepared from 0-12 hours embryos. The positive control, indicated by "+", was HA tagged human SLIP1 exogenously expressed in Hela cells. The negative controls, indicated by "-", were from an extract of wild type *D. melanogaster* embryos which did not contain any HA tagged SLBP transgene. Arrows indicate the bands corresponding to the three deletion mutants of SLBP, Δ35, Δ86, and Δ139. All strains have two copies of the transgene, except Δ86 which is only one copy. **B)** Western blot showing expression of N-terminal deletion constructs in 3rd instar larvae. Jeremy Kupsco prepared protein extracts from 3rd instar larvae and blotted with anti-HA. Lane one shows wt SLBP expression without HA-tag from wt larvae. Asterisks in all lanes denote the tagged protein. The Δ179 deletion in lane 6 runs at a higher mobility than the Δ139 deletion in lane 5 due to containing 3HA tags on the N-terminus versus a single HA tag for Δ139. Expression of the Δ86 construct could not be detected in lane 4, despite being able to rescue viability. Beta-tubulin was blotted with anti-β–tubulin as a loading control.

A


Δ139
Δ86
Δ35
+
-
T287
T288
T334
T335
T271
T279
T280
-

B

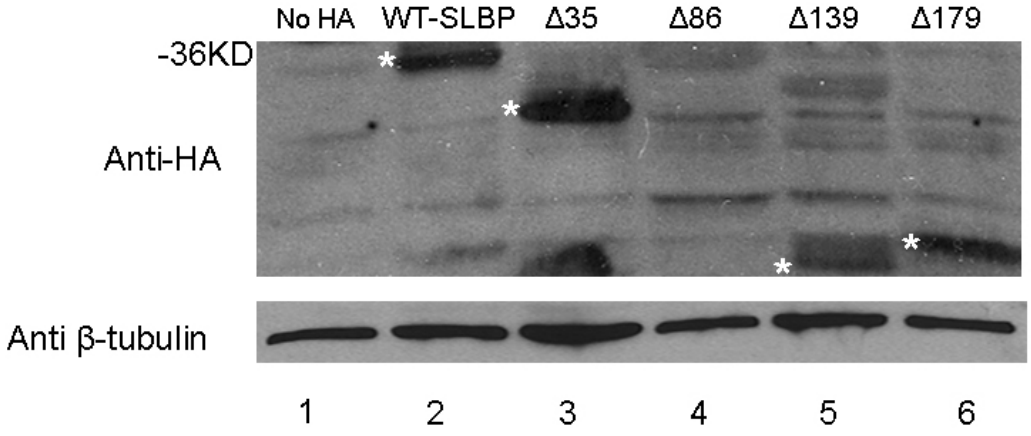

No HA
WT-SLBP
Δ35
Δ86
Δ139
Δ179
-36KD
Anti-HA
Anti β-tubulin
1
2
3
4
5
6

Figure 25. *In situ* hybridizations on stage 13 embryos with the H3-ds probe. A) In wild type embryos without any transgenes, no mis-processed H3 was detected as previously reported (Sullivan et al. 2001). H3-ds probe only detects mis-processed mRNA with polyA tail after stem loop, thus indicates the deficiency in processing of histone mRNA. **B)** Large amounts of mis-processed H3 can be detected in an $SLBP^{15}$ mutant embryo. **C)** The wild type dSLBP transgene is considerably able to rescue processing of H3 mRNA in the central nervous system when crossed into an $SLBP^{15}$ mutant background, but not with 100% efficiency. Some mis-processed H3 was still observed in the inferior gut and hind gut (red arrows). **D, E, F** represents the Δ86, Δ139, Δ179 mutants, respectively. All those deletions appear to rescue some of the H3 mis-processing when crossed into an SLBP mutant background, predominantly in the CNS. Arrows in C, D, E, and F show the central nervous system (Jeremy Kupsco, Duronio Lab).

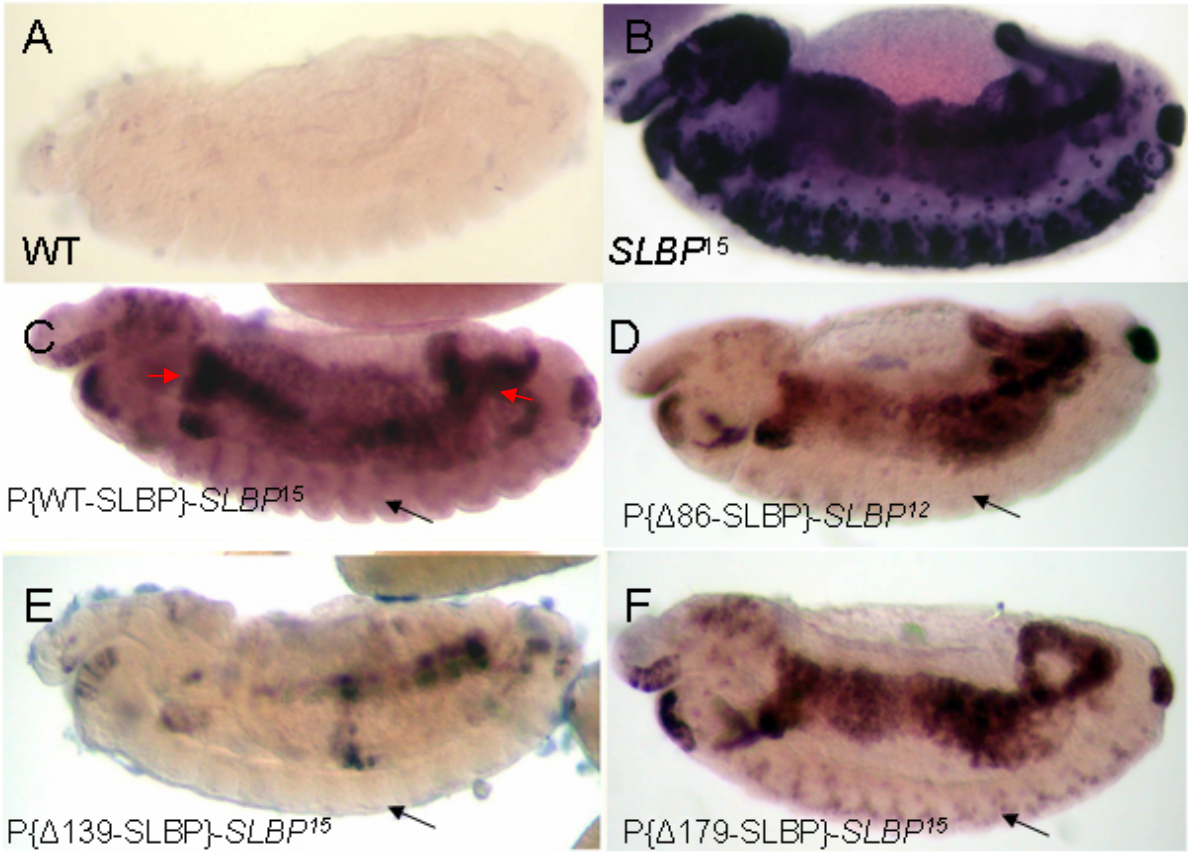

A
WT
B
SLBP15
C
P{WT-SLBP}-SLBP15
D
P{Δ86-SLBP}-SLBP12
E
P{Δ139-SLBP}-SLBP15
F
P{Δ179-SLBP}-SLBP15

Figure 26. Northern blot analysis. A) RNA was extracted from *SLBP* mutant 3rd instar larvae that contained two copies of the indicated deletion transgenes, except for Δ86 of which only one copy was present. The blot was probed with H3 that detects both correctly processed and miss-processed mRNA. Notice that all deletion transgenes are able to rescue processing as well as the wild type dSLBP transgene in lane 3; even the Δ139 and Δ179 transgenes in lanes 6 and 7 which fail to rescue viability have more processed H3 mRNA. The U1 snRNA was probed as a loading control. **B)** Percentages of amounts of misprocessed and processed mRNAs were calculated to quantitatively compare processing efficiencies of Δ86, Δ139, and *dSLBP*^{15} mutants to wild type (Jeremy Kupsco, Duronio Lab).

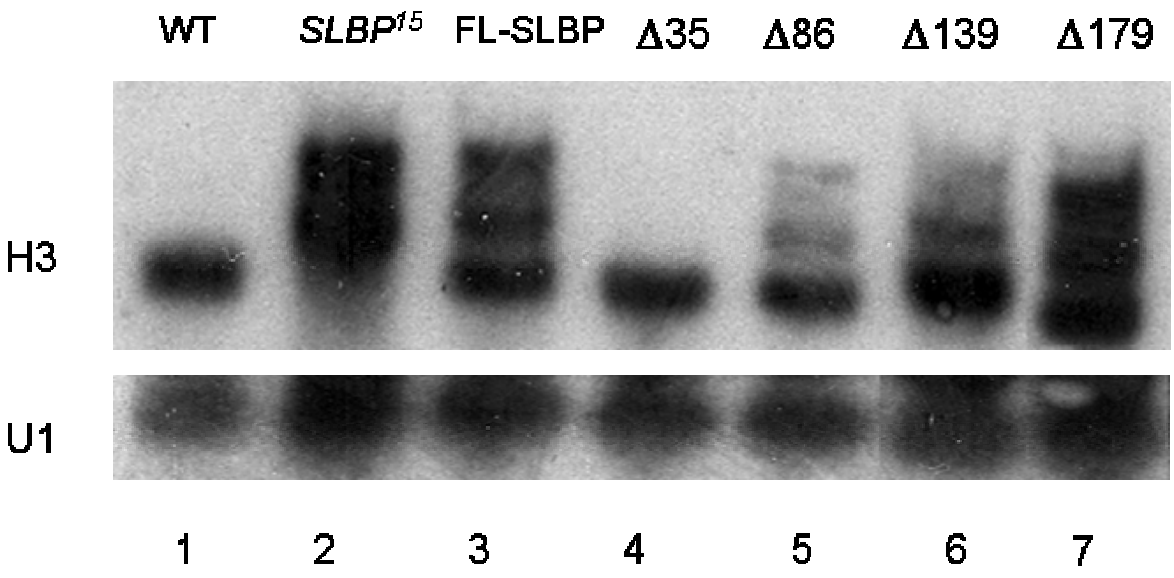

WT
SLBP15
FL-SLBP
Δ35
Δ86
Δ139
Δ179
H3
U1
1
2
3
4
5
6
7

Figure 27. Amino acid sequence alignment of SLIP1 from human and *Drosophila*. To identify the potential homologue of dSLIP1, hSLIP1 amino acid sequence was used as the search template by using fly database (http://www.flybase.org). According to the fly database, dSLIP1 is located on chromosome 2L. The reference identity is AAF52823 and fly equivalent gene product is CG13124. *Drosophila* SLIP1 has an extended N-terminus compared to hSLIP1 and most of the similarities between two proteins are observed in the C-terminus. Alignment was done by using Clustal X program.

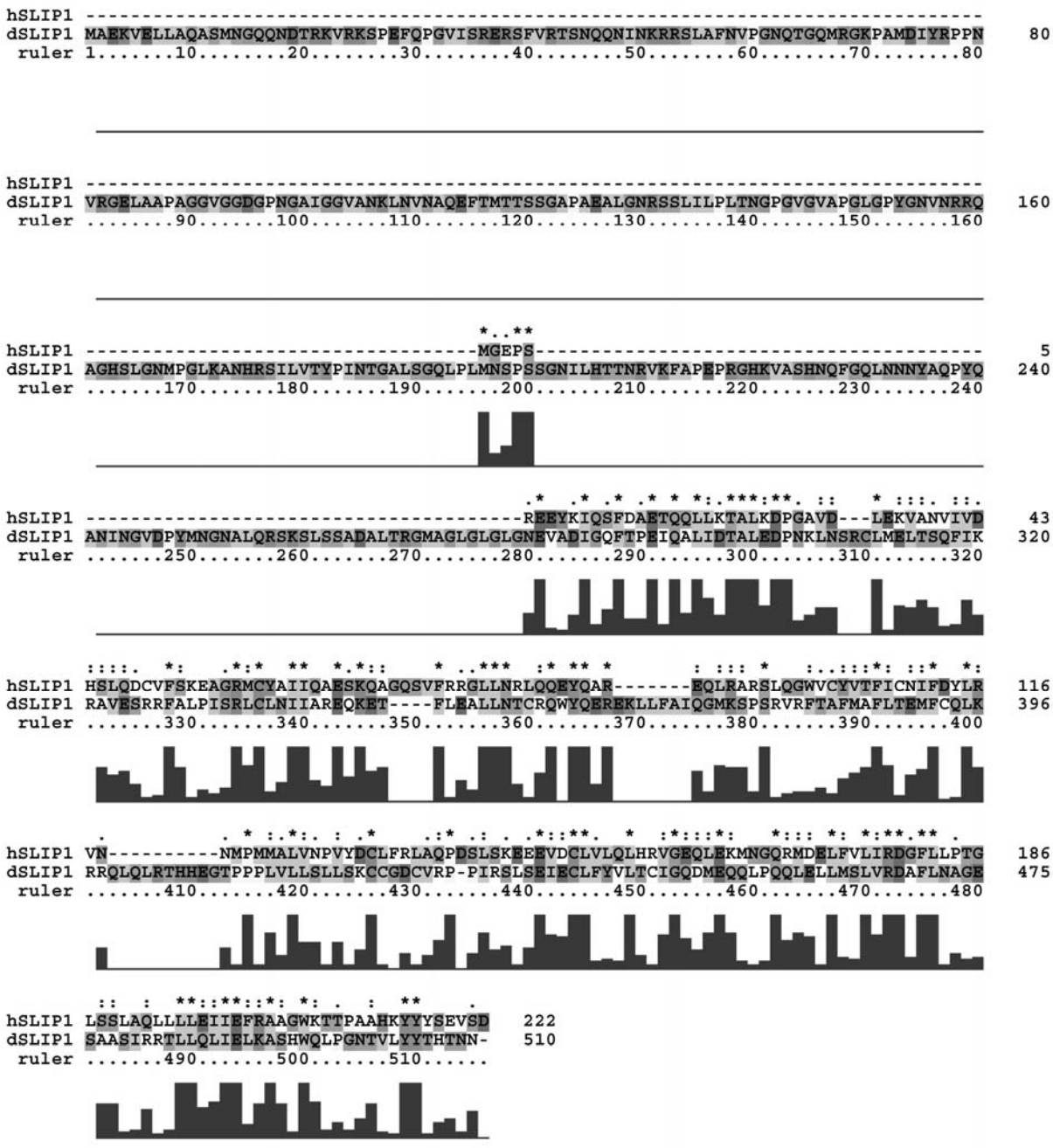
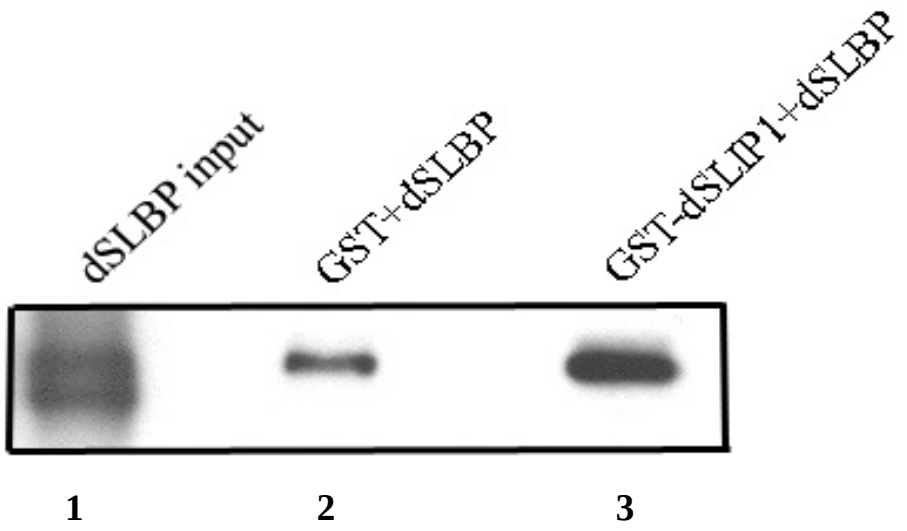
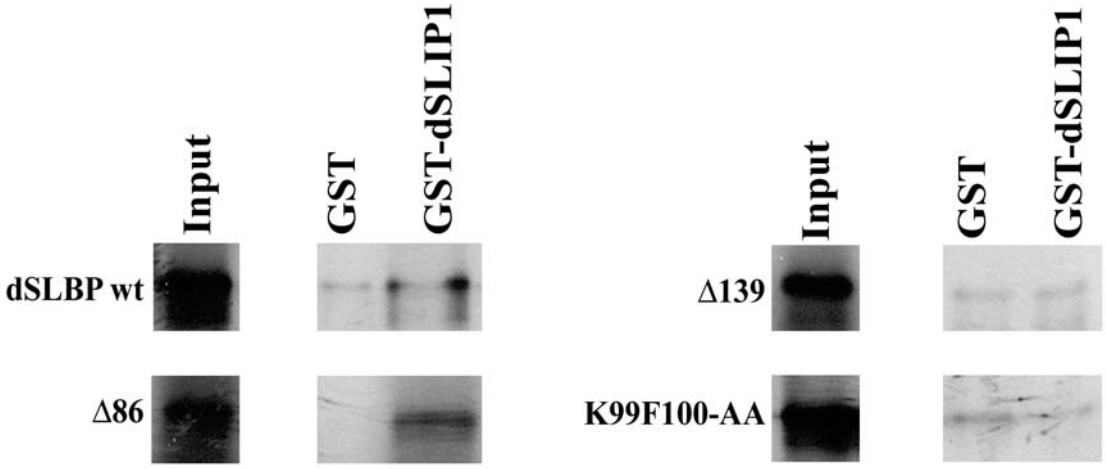

hSLIP1 ----------
dSLIP1 MAEKVELLAQASMNGQQNDTRKVRKSPEFQPGVISRERSFVRTSNQQNINKRRSLAFNVPGNQTGQMRGKPAMDIYRPPN 80
ruler 1.....10.....20.....30.....40.....50.....60.....70.....80
hSLIP1 ----------
dSLIP1 VRGELAAPAGGVGGDGPNGAIGGVANKLNVNAQEFTMTTSSGAPAEALGNRSSLILPLTNGPGVGVAPGLGPYGNVNRRQ 160
ruler .....90.....100.....110.....120.....130.....140.....150.....160
hSLIP1 ----------MGEPS---------- 5
dSLIP1 AGHSLGNMPGLKANHRSILVTYPINTGALSGQLPLMNSPSSGNILHTTNRVKFAPEPRGHKVASHNQFGQLNNNYAQPYQ 240
ruler .....170.....180.....190.....200.....210.....220.....230.....240
hSLIP1 ----------REEYKIQSFDAETQQLLKTALKDPGAVD---LEKVANVIVD 43
dSLIP1 ANINGVDPYMNGNALQRSKSLSSADALTRGMAGLGLGLGNEVADIGQFTPEIQALIDTALEDPNKLNSRCLMELTSQFIK 320
ruler .....250.....260.....270.....280.....290.....300.....310.....320
hSLIP1 HSLQDCVFSKEAGRMCYAIIQAESKQAGQSVFRRGLLNRLQQEYQAR-------EQLRARSLQGWVCYVTFICNIFDYLR 116
dSLIP1 RAVESRRFALPISRLCLNIIAREQKET----FLEALLNTCRQWYQEREKLLFAIQGMKSPSRVRFTAFMAFLTEMFCQLK 396
ruler .....330.....340.....350.....360.....370.....380.....390.....400
hSLIP1 VN----------NMPMMALVNPVYDCLFRLAQPDSLSKEEEVDCLVLQLHRVGEQLEKMNGQRMDELFVLIRDGFLLPTG 186
dSLIP1 RRQLQLRTHHEGTPPPLVLLSLLSKCCGDCVRP-PIRSLSEIECLFYVLTCIGQDMEQQLPQQLELLMSLVRDAFLNAGE 475
ruler .....410.....420.....430.....440.....450.....460.....470.....480
hSLIP1 LSSLAQLLLLEIIEFRAAGWKTTPAAHKYYYSEVSD 222
dSLIP1 SAASIRRTLLQLIELKASHWQLPGNTVLYYTHTNN- 510
ruler .....490.....500.....510.....

Figure 28. dSLBP interacts with dSLIP1. A) GST-dSLIP1-his was expressed in E.coli while dSLBP-his was expressed in baculovirus system. Both proteins were purified through Ni+ column. Both proteins were incubated on ice for 30 minutes prior to incubation with 40 ml slurry GS-sepharose beads. Then protein mixtures bound to the beads were washed 3 times with 1X PBS supplemented with 100 mM NaCl. Proteins were eluted with 2 X-SDS sample buffers and resolved on 12 % SDS-PAGE. dSLBP was detected by western blotting with anti-dSLBP. **B)** All the dSLBPs were expressed in reticulocyte lysate from Sp6 promoter in pXFRM vector and labeled with ^{35}S-methionine. GST-dSLIP1-his recombinant protein was used to pull down dSLBPs in GST pull down assay as explained in A, except bound dSLBPs were monitored by radiography.

A


dSLBP input
GST+dSLBP
GST-dSLIP1+dSLBP
1
2
3

B


Input
GST
GST-dSLIP1
dSLBP wt
Δ86
Input
GST
GST-dSLIP1
Δ139
K99F100-AA

CHAPTER V

DISCUSSION

In eukaryotes, DNA is packaged into chromosome by wrapping twice around histone core composed of histone proteins H2A, H2B, H3 and H4 and this smallest unit is called nucleosome. This structure is compacted to higher organization by linker histone protein, H1, which brings adjacent nucleosome units together. Synthesis of histone protein in somatic cells is controlled at the mRNA level, mainly through post-transcriptional regulation of histone mRNA and its specific binding factor SLBP. To obtain translationally active histone mRNAs (mature mRNA) requires only one-step processing reaction which involves the cleavage after a unique 3' end structure. Since histone mRNAs do not contain any introns, they do not undergo any splicing unlike most of the eukaryotic mRNAs. The mature histone mRNA terminates with a 26 nucleotide long stem loop structure, rather than a polyA tail. The stem loop binds a specific factor, SLBP. In vertebrates, stem loop and SLBP were shown both to be required for processing (Dominski and Marzluff 1999; Dominski et al. 1999) and to stimulate translation of histone mRNAs (Ling et al. 2002; Sanchez and Marzluff 2002; Gorgoni et al. 2005).

In my thesis I have provided evidence for a mechanism of histone mRNA translation, showing how translational of histone mRNAs may work in a similar fashion to the translation of polyadenylated mRNAs. There are obviously still many questions that remain to be answered in the future.

Spacing between the Translation Activation Domain and the RBD is Essential for Translational Activity of SLBP

xSLBP1 and hSLBP were been shown to activate translation of reporter mRNAs ending in stem loop and the stimulation only requires sequences in the N-terminus of SLBP (Sanchez and Marzluff 2002; Gorgoni et al. 2005). Tethering SLBP or only the entire N-terminus of SLBP fused to another RBD domains like the MS2 coat protein were enough to stimulate translation, demonstrating that the RBD and C-terminus are not required.. Additionally, by mutational analysis, Sanchez and Marzluff, 2002, demonstrated that the responsible region for this activity lies between amino acids 68 and 89 in the N-terminus of SLBP (Sanchez and Marzluff 2002). However, up to now, no experiments have addressed the question of whether the amino acid region between 89 and 126 (at the start of the RBD) is required for translational activity of SLBP. I showed that the translation activation region must be separated from the RBD to exert its stimulating function over histone mRNAs, but there is no requirement for a specific sequence in this region. This region could be replaced with an unrelated 37 amino acid sequence from translation repressing factor, xSLBP2. Also, the length of this region could be reduced to 24 amino acids without affecting the translational activity of xSLBP1, suggesting that a short, non-sequence specific linker region is necessary. The linker may allow the translation activation region to be flexible to prevent interference between the RBD and the translation initiation factors. If the translation activation region is involved in protein-protein interaction, it is likely that the fusion of it immediately to RBD may occlude that region preventing its protein interactions. It is probable that fusion to RBD may result in a total conformational change in the N-terminus of SLBP which renders its recognition difficult by the target protein(s) that are also involved in

the translation of histone mRNAs. For example placing 20 amino acids containing the 13 amino acid long translation activation region into xSLBP2 N-terminus at the same position as it is in xSLBP1, did not result in a protein that was capable of activating translation (R. Sanchez, unpublished data), although xSLBP1 N-terminus fusion to RBD2 and C2 resulted in translationally active chimera (Sanchez and Marzluff 2002). xSLBP2, oocyte specific repressor, is essentially different than xSLBP1 at the amino acid level, except in the RBD. These results suggest that the translation activation region has some conformational requirements that the relatively unstructured N-terminus of SLBP can accommodate.

Identification of Critical Residues in a Conserved Motif between Amino Acids 68 and 83 in the SLBP N-Terminus

The N-termini of vertebrate SLBPs are conserved, and those similarities are very high in previously defined translation activation region between amino acids 68 and 83 (Sanchez and Marzluff 2002). Based on the conserved similarity, I postulated that there might be an extensively conserved motif in this region responsible for translational activation of histone mRNAs. This motif in the vertebrates is DWX_3VEE. When we extend our research for this motif outside of vertebrates to more distantly related organisms such as *Drosophila*, we can not find a similar region by simple amino acid comparison. However, we observe the presence of three possible slightly conserved motifs in the N-terminus of dSLBP, composed of a charged amino acid followed by an aromatic, followed after a space by an acidic region. In our studies, one of these motifs, KFX_3VKEE, has been identified as a candidate for the translation activation domain. The vertebrate motif is critical to stimulate the translation

of histone mRNAs *in vivo* and *in vitro*. Unfortunately, we could not test dSLBP motif due to the lack of a translation system to analyze its function *in vivo* or *in vitro*. Wild type dSLBP does not activate translation of luciferase reporters ending in stem loop, probably due to the heterologous systems (reticulocyte lysate, *Xenopus* oocytes, and HeLa cells) that I have been used. It is certainly of potential interest to develop such a system in the future. There are reports of *Drosophila in vitro* translation systems that reproduce some aspects of regulation of translation of maternal mRNAs (Gray et al. 1996).

Identification of a translational motif in the SLBP N-terminus still does not answer how the histones are translated or why this motif is important in the context of translation of histones. So, next topic of this chapter discusses this issue.

A Functionally Novel Factor, SLIP1, Activates Translation of Histone mRNAs

SLIP1 was identified in a yeast two hybrid assay in which hSLBP was used as the bait against a human cDNA library. Interaction of SLIP1 with SLBP N-terminus made it a possible candidate to be involved in histone mRNA translation, since SLBP N-terminus is essential to stimulate translation of histone mRNAs. SLIP1 activates translation of histone mRNAs in the presence of SLBP *in vivo*. Strikingly, SLBP point mutants that can not activate translation were also incapable of interacting with SLIP1, demonstrating that the translation motif DWX_3VEE is responsible to bind SLIP1. Those results suggest a mechanism about why motif mutants of SLBP can not activate translation. When the interaction between SLIP1 and SLBP is disrupted, SLIP1 cannot be recruited to the histone mRNA, and thus the mRNA is not efficiently translated. Possibly the sole function of SLBP

in translation is to simply tether SLIP1 to the mRNA. In support of this hypothesis, I observed that MS2-SLIP1 fusion protein was able to activate translation from reporter mRNAs ending in stem loop *in vitro* to the similar levels as SLBP and MS2-hSLBP (Figure 21).

It is still possible that SLBP binds SLIP1 and also directly assists in the stimulation of translation. For example in the reticulocyte lysate, one measures largely initiation, while in the fog oocyte one measures the incorporation of mRNA into polyribosomes resulting in multiple rounds of translation. It is possible that there will be a different effect of the MS2-SLIP1 fusion protein in oocytes.

It will be very useful to identify the region on SLIP1 that is responsible for binding SLBP. Since SLIP1 is a compact single domain protein this can not be done by deletion analysis but by making mutants in conserved residues on the surface and testing for their ability to bind SLBP (and to stimulate translation). Expression of SLIP1 mutants that are unable to interact with SLBP can help us to understand the translational function of SLIP1 in detail, particularly if the mutant SLIP1 retains it ability to interact with eIF4G.

Recent reports strongly suggest that bringing the 5' and 3' ends of the polyadenylated mRNAs close to each other (closed loop model) facilitates the translation efficiency, increasing the rate of protein synthesis. This is obviously very important for synthesis of large amounts of proteins, such as histone proteins in S-phase, rapidly. There are several hypotheses how this might occur. One possible mechanism is that ribosomes coming of the 3' end might be easily transferred to 5' end of the message without completely dissociating into subunits, thus eliminating the need to wait for another 40S ribosomal subunit to bind to the mRNA. Another theory is that 5' and 3' end-bound proteins increase their binding

affinity to the message when they interact with eIF4G. These stable multi-protein complexes may help translation initiation, at least to recruit 40S ribosomal subunit (Haghighat and Sonenberg 1997; Kahvejian et al. 2001).

SLIP1 Interacts with eIF4G

Based on translational mechanism of polyadenylated messages, we theorize that interaction of SLIP1 and SLBP forms the similar basis, and probably their interactions with either eIF4G or eIF3 or eIF4E leads to closed loop structure. Extensive studies aimed at demonstrating an interaction between SLBP and one of the core translation factors such as eIF4E, eIF4G, eIF3, and eIF4A have failed (Gorgoni et al. 2005). However, Ling et al., did conclude that SLBP interacts with eIF4G in yeast and does not require any other factor between SLBP and eIF4G (Ling et al. 2002). This artificial system involved expressing SLBP in yeast and transfecting stem loop RNAs into yeast. The yeast translation machinery does not have any accessory factors, such as PAIP2, but only needs core translation factors. In their mammalian studies, they showed that SLBP and eIF4G co-precipitated from cells in their pull down experiments. It is certainly possible that this pull-down required additional factors bridging the SLBP and eIF4G which would not have been detected in their experiments.

However, Gorgoni et al. observed interaction between SLBP and the eIF3h subunit (p40), as we did (L. Zheng, Ph.D. thesis) and PAIP1 (Gorgoni et al. 2005). Unfortunately, the interaction domain on SLBP mapped to RBD and C-terminus for eIF3h and PAIP1, respectively (Gorgoni et al. 2005). Deletion of the whole C-terminus (Sanchez and Marzluff

2002) or tethering only the N-terminus of SLBP fused to MS2 (Gorgoni et al. 2005) does not reduce the translational activity of SLBP, showing that RBD and C-terminus of SLBP is not required for the stimulatory effect of SLBP. Thus the interactions of eIF3h and Paip1 with SLBP do not correlate with N-terminal domain of SLBP as previously described to stimulate translation in *Xenopus* oocytes. So the interactions of eIF3h and PAIP1 with SLBP are dispensable for translational activity of SLBP. Additionally, I did not detect any significant interaction between recombinant proteins SLBP and eIF4GI in my directed GST pull down assay (Figure 18H). All those studies mentioned here raised the question: Is SLBP interaction with the translation machinery at the 5' end of the mRNA mediated by an unknown factor?

SLIP1 interacts with eIF4G *in vivo* and *in vitro*, and this interaction is likely to be direct. Also SLIP1 co-purifies with SLBP. I cannot conclude that both eIF4G and SLBP can be co-isolated with the same molecule of SLIP1 from those studies. SLIP1 crystallized as a dimer, so it is possible that each one of the SLIP1 molecule is responsible for interaction with either SLBP or eIF4G or one molecule may simultaneously interact with both of the proteins.

SLIP1 interacts with eIF4G close to the PABP-interacting region in the N-terminus. This raises an additional question. Can SLIP1 and PABP bind to the same molecule of eIF4G, or is one of the proteins' binding exclusive for the other? In the future, this question is going to be answered by pull down assays. By sequence comparison between eIF4GI and eIF4GII, we predicted a region on eIF4G N-terminus that is highly rich for proline residues. It is possible that SLIP1 and PABP may simultaneously associate with the same molecule of eIF4GI. This prediction can not be verified without direct measurements.

Translation Model for Histone mRNAs

Capped 5' end of the histone mRNAs will bind eIF4E (Gorgoni et al. 2005) while 3' end will bind SLBP (Sanchez and Marzluff 2002; Gorgoni et al. 2005). eIF4E will associate with scaffold protein eIF4G (Gorgoni et al. 2005). SLIP1 will then unite the 5' and 3' ends of histone mRNA via its interaction with both SLBP and eIF4G (Cakmakci et al. 2007), thus forming a similar closed loop structure to polyadenylated mRNAs (Figure 22). The structure does not need to be necessarily a complete circularized message; probably bringing the 5' and 3' ends to close proximity is sufficient to stimulate translation of histone mRNAs. Then eIF3 will recruit 40S small ribosomal subunit to the 5' end of the message through its interaction with eIF4G. Finally, 1st step of translation initiation will be accomplished. As explained previously for polyadenylated messages, the circularized structure will facilitate the rate of protein synthesis, at least by affecting faster formation of a potential translation complex on the mRNA.

Why does the SLIP1 Knockdown Kill the Mammalian Cells?

When we knock down SLIP1 in HeLa cells by RNAi interference, those cells starts to die in 24 hours and only 30% survive until 72 hours. In contrast, SLBP knock down by RNAi does not kill the cells, only causes a delay during S-phase progression (Wagner et al. 2005). Those two different outcomes suggest that SLIP1 may not be only involved in histone mRNA metabolism. The knockdown of SLBP is greater than 95% and causes an S-phase arrest (Wagner et al. 2005), likely due to a failure to synthesize sufficient amounts of histone protein. Since histone mRNA levels are only slightly reduced, it is likely the defect in histone

production is due to failure to efficiently translate the mRNA (R. Lerner, personal communication). If the only function of SLIP1 was to help translate histone mRNA, then we would expect that the absence of SLIP1 would result in similar delays during S-phase, rather than killing the cells.. However, we tried to find a range for SLIP1 knock down that did not kill the cells. Unfortunately, those cells treated with different concentrations of SLIP1 siRNA all eventually died, suggesting that SLIP1 may have other function(s) in addition to its translational activity for histone mRNAs. So, SLIP1 function(s) may include stimulating translation of other mRNAs that are essential for cell viability.

What are possible roles for SLIP1 in the cell? One possibility involves the translation of a subset of mRNAs. SLIP1 absence may affect the translation of those yet to be identified mRNAs, thus results in cell death. A microchip RNA assay to identify those mRNAs is also planned for the future. Additionally, the possible proteins that interact with SLIP1 will be tested in a yeast two hybrid assay by using a human cDNA library. Those studies may allow us to predict what kind of mRNAs may be influenced by SLIP1 and also to predict what kind of function(s) SLIP1 may have. On the other hand, SLIP1 may be involved in totally different cellular processes, such as programmed cell death, cell signaling…etc.

An Essential Function for SLBP Other than Pre-mRNA Processing

SLBP functions in the processing of the histone mRNA (Dominski et al. 1999; Dominski et al. 2002), and is an essential gene. *SLBP* null flies die as pharate adults or 3rd instar larvae, although replication-dependent histone mRNAs are present during all stages of development in amounts that are equal to or slightly greater than that found in wild type flies,

except in the germ line. The majority of histone mRNAs are mis-processed from a cryptic polyadenylation signal located after stem loop, and it is likely that overexpression of histone protein synthesis results in failure to successfully undergo metamorphosis.

The SLBP null mutant can be rescued by expression of an HA_tagged SLBP transgene (Lanzotti Ph.D thesis). Dave Lanzotti showed that an SLBP with the first 179 amino acids deleted was not capable of rescuing the SLBP mutant, although it contained all the sequences necessary for histone pre-mRNA processing in vitro (Dominski et al. 2002). Jeremy Kupsco and I determined that a region in the N-terminus of dSLBP between amino acids 86 and 139 is also essential to rescue viability. More importantly, we demonstrated that all the mutants, including the Δ179 mutant had similar processing efficiencies regardless of that whether they supported the viability. Thus the lethality is not due to failure to process histone pre-mRNA, but must be due to another function(s) of SLBP required after the processing of histone mRNAs.

A Possible Role for Drosophila SLBP in Translation of Histone mRNAs

Based on the growing evidence from vertebrates, it is intriguing to think that the mechanism of histone mRNA translation may be conserved among very distantly related organisms, such as in *Drosophila melanogaster*, and that translation of histone mRNA stimulated by SLBP and SLIP1 might be essential. Although there is very little conservation between vertebrate SLBPs and *Drosophila* SLBP outside of the RBD, a similar motif of vertebrate translation activation motif, DWX_3VEE, is found in the N-terminus of dSLBP between amino acids 86 and 139. The identity of this motif, KFX_3VKEE, has maintained the

aromatic residue (W→F) and charged nature of the amino acid stretch, but converted to a mixture of negative and positive charges (Ks and Es) rather than only negative charges (only Es) as found in vertebrate motif. Strikingly, the Drosophila homolog of SLIP1, dSLIP1 (CG13124) does interact with dSLBP. I demonstrated that a region between amino acids 86 and 139 of dSLBP was also required to bind dSLIP, and a point mutant in this region, K99F100 mutated to alanines, abolished binding, did not rescue viability. Furthermore, a dSLBP mutant fly that had K99F100V104E105 mutated to alanines, also did not rescue viability.

So it is intriguing to think that this region might be involved in translation of Drosophila histone mRNAs in similar manner to the translation activation motif of vertebrate SLBPs, and that stimulation of histone mRNA translation is an essential function of dSLBP. The details of how SLIP1 and SLBP activate translation will be excellent topics to study in the future. In addition, we need to understand if SLIP1 plays a role in translation of other subsets of mRNAs, or whether it has a totally different function in the cell.

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