CLONING OF POLYSOME-ASSOCIATED SMALL RNAs IN *Drosophila melanogaster* EMBRYOS

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ABSTRACT

CLONING OF POLYSOME-ASSOCIATED SMALL RNAs IN *Drosophila melanogaster* EMBRYOS

Genome-encoded regulatory small RNAs are classified into 3 groups; microRNAs (miRNAs), endogeneous small interfering RNAs (endo siRNAs) and piwi interacting RNAs (piRNAs). miRNAs, 17-21 nucleotide in size, are involved in posttranscriptional gene regulation via precise or imprecise base pairing with target mRNAs resulting in either target mRNA degradation or translational inhibition. Endo siRNAs ,on the other hand, may function transposon regulation but their precise regulatory function and mechanism have not been elucidated yet. piRNAs are mainly involved in transposon silencing in spermatogenesis.

Despite their discovery, biological roles and modes of functions of small RNAs remain to be elucidated. The aim of this thesis was to identify polysome-associated small RNAs in *Drosophila melanogaster* embryos by deep sequencing and investigate their role in translational regulation. Deep sequencing and microarray results determined stage and fraction specific distribution of genome encoded small RNAs. Surprisingly, the results implied that mRNAs may be posttranscriptionally regulated by antisense transcripts in polysome.

ÖZET

Drosophila melanogaster EMBRİYOLARINDA POLİZOMUN BİR PARÇASI OLAN KÜÇÜK RNALARIN KLONLANMASI

Genom tarafından kodlanan düzenleyici küçük RNA'lar üç gruba ayrılır; mikroRNAlar (miRNA), endojenik küçük RNAlar (endo siRNA), piwi etkileşimli RNAlar (piRNA). 17-21 nükleotitlik mikroRNAlar hedef mRNA ile tam yada tam olmayan baz eşleşmesi yoluyla hedef mRNA'nın parçalanmasına yada translasyonun baskılanmasına sebep olurlar. Endo siRNAların transposon regulasyonunda fonksiyon görebileceği öne sürülmesine rağmen kesin olarak fonksiyonları ve mekanizmaları bulunamamıştır. piwi-etkileşimli RNAlar spermatogenez sırasında transposonların baskılanmasında rol alırlar.

Keşfedilmelerine rağmen, düzenleyici RNAların biyolojik rolleri ve fonksiyon mekanizmaları aydınlatılamamıştır. Bu tez çalışmasının amacı *Drosophila melanogaster* embriyolarında bulunan, polizomun bir parçası olan küçük RNAları ayrıntılı sekans kullanılarak in vivo koşullarda tanımlanması ve translasyonel düzenlemedeki rollerinin araştırılmasıdır. Sekans ve mikroarray sonuçları genom tarafından kodlanan küçük RNAların fraksiyon ve zamana bağlı olarak dağılımını belirlemiştir. Sonuçlar ilginç bir şekilde mRNAların polizomlarda posttranskripsiyonel olarak antisense transkripler tarafından regule edilebileceğini göstermiştir.

TABLE OF CONTENTS

LIST OF FIGURES viii

LIST OF TABLES ix

ABBREVIATIONS x

CHAPTER 1. INTRODUCTION 1

CHAPTER 2. MATERIALS AND METHODS 9

2.1. *Drosophila melanogaster* Maintanence and Embryo Collection 9

2.2. 5-70% Sucrose Gradient Preparation 9

2.3. RNA Isolation by Sucrose Density Gradient System 10

2.4. RNA Quality Control by Bioanalyzer 11

2.5. Small RNA Isolation 11

2.6. Denaturing Gel Electrophoresis (PAGE) of RNAs 12

2.7. Small RNA Cloning 12

2.7.1. cDNA Preparation and PCR Amplification 12

2.7.2. Concatamerization of PCR Products 15

2.7.3. Ligation of PCR Products into pGEM® T Easy Vector 16

2.8. Small RNA Deep Sequencing 17

2.9. Small RNA Microarray 17

CHAPTER 3. RESULTS 18

3.1. Polysome Profile of 0-1 and 8h Embryos 18

3.2. RNA Quality Control by Bioanalyzer 19

3.3. Small RNA Isolation 20

3.4. Small RNA Cloning 21

3.5. Sequencing of PCR-Amplified Small RNAs 23

3.6. Deep Sequencing of RNAs 23

3.7. Microarray Analysis of Endogeneous Small Interfering RNAs 24

CHAPTER 4. DISCUSSION 27

CHAPTER 5 CONCLUSION 28

REFERENCES 29

LIST OF FIGURES

Figure		Page
Figure 1.1.	MicroRNA Biogenesis.	4
Figure 1.2.	Mechanism of Translation Initiation.	5
Figure 2.1.	Size-fractionated Cloning of Small RNAs.	15
Figure 2.2.	Detailed Map of pGEM T Easy Vector	16
Figure 3.1.	Polysome Distribution of 0-1 hour Stage *Drosophila* Embryos	18
Figure 3.2.	Agilent 2100 Bioanalzer Electropherogram of mRNP, 40S, Monosome and Polysome RNAs	20
Figure 3.3.	12% Denaturing Page of Isolated RNAs	21
Figure 3.4.	2% Agarose Gel Electrophoresis PCR Products	21
Figure 3.5.	12% Non-denaturing Page of Isolated RNAs	22
Figure 3.6.	2% Agarose Gel Electrophoresis of Eco*RI* Digested Products	23
Figure 3.7.	Percentage of siRNA Distribution	25
Figure 3.8.	Percentage of endo siRNA-target mRNA Matching Region	26

LIST OF TABLES

Table		Page
Table 3.1.	Average RNA Concentration and Enrichment.	19
Table 3.2.	The Blast Results of PCR-amplified Small RNAs	23
Table.3.3.	The Microarray Results of Endo siRNA Expression	25

ABBREVIATIONS

AGO	Argonaute
DEPC	Diethylpyrocarbonate
DGCR8	Di George Critical Syndrome Region 8
Endo siRNA	Endogenous Small Interfering RNA
eIFs	Eukaryotic Translation Initiation Factors
miRNA	microRNA
PABP	Poly (A) Binding Protein
PAGE	Polyacrylamide Gel Electrophoresis
PAZ	Piwi Argonaute Zwille
PCR	Polymerase Chain Reaction
piRNA	Piwi Interacting RNAs
Pre-miRNA	Precursor MicroRNA
Pri-miRNA	Primary MicroRNA
rasiRNA	Repeat Associated Small Interfering RNAs
RISC	RNA Induced Silencing Complex
TRBP	The Human Immunodeficiency Virus Trans-activating Response RNA-binding Protein
UTR	Untranslated Region

CHAPTER 1

INTRODUCTION

1.1. Small RNAs

Genome-encoded small RNAs are classified into three groups according to their size and protein partners; microRNAs (miRNAs), endogenous small interfering RNAs (endo-siRNAs) and piwi-interacting RNAs (piRNAs) (Kim, et al. 2008). miRNAs are the member of noncoding RNAs that regulate gene expresion post-transcriptionally through translational inhibition or mRNA degradation via imperfect or perfect base pairing with the 3' untranslated region (UTR) of its target mRNA. They are approximately 17-22 nt in length. These small RNAs are found either in exon or intron of protein coding sequences (Bartel 2004). Firstly discovered miRNAs are *lin4* and *let7* regulating developmental timing in *Caenorhabditis elegans* (Lee, et al. 1993). Hundreds of members have been discovered in worms, flies, plants and mammals. 152 miRNAs were identified in *Drosophila melanogaster*. Recent findings suggest that miRNAs might regulate more than 30 % of protein coding genes. Target prediction studies demonstrate that one miRNA is capable of binding more than 100 target mRNAs (Lai and Flynt 2008).

Biochemical and genetic studies indicate that miRNAs have a regulatory role in diverse cellular processes such as synaptic development, apoptosis and cell differentation. Schratt *et* al. showed that a brain specific miRNA, miR-134, is found in synaptodendritic part of rat hippocampus and maintains the size of dendritic spine by inhibiting target LIMK1 protein translation (Schratt, et al. 2006). Another important physiological event, apoptosis, is induced by miR-15 and miR-16 which repress the translation of anti-apoptotic protein B cell lymphoma 2 (bcl2) in human (Cimmino, et

al. 2005). One of the intriguing roles of miRNAs is that miR-181 was discovered to specify the hemapoietic cell differentiation in mouse bone marrow (Chen, et al.2004).

After virus infection, cells process exogenous double strand RNAs into exogenous siRNA for protection. Intriguingly, genome-encoded siRNAs were discovered in *Drosophila melanogaster* and named as endogenous siRNA (Ghildiyal, et al. 2008). They are ~21 nt in length and derived from retrotransposons, bidirectional transcription and stem loop structures (Okamura, et al. 2008, Kawamura, et al. 2008, Czech, et al. 2008). Their roles have not been revealed yet.

The other class of genome-encoded RNAs is piRNAs originally discovered in *Drosophila* genome and firstly named as repeat-associated small interfering RNAs (rasi-RNAs). Due to the relationship between piwi proteins and rasi-RNAs, they were renamed as piRNAs (Kim, et al. 2008). piRNAs are ~30nt in length and longer than miRNAs, and endo-siRNAs. They are mainly derived from retrotransposons and expressed during spermatogenesis (Grivna, et al. 2006).

1.2. Small RNA Biogenesis

miRNA biogenesis is a two-step process that gives rise to mature miRNAs; 1) excision of ~80nt precursor miRNAs from several hundreds in length primary miRNAs, 2) generation of mature miRNAs from precursor miRNAs (Gregory, et al. 2004).

In the first step, most of miRNA genes are transcribed in the nucleus by RNA Polymerase II into capped, polyadenylated primary microRNAs (pri-miRNA) which are several hundred nucleotides in length (Cai, et al. 2004). These pri-miRNAs are recognized by RNAse III enzyme Drosha to yield an approximately 70nt precursor microRNA (pre-miRNA) carrying a 2-nucleotide 3' overhang that is a characteristic of RNase III cleavage. Pri-miRNA processing is a crucial event as it identifies mature miRNA sequences in long pri-miRNAs (Han, et al. 2006). Studies on Drosha cleavage revealed that double strand binding proteins Di George Critical Syndrome Region 8 (DGCR8) in human and its homolog Pasha in *Drosophila* are required for efficient processing (Gregory, et al. 2004, Han, et al. 2006). Gregory *et* al. has demonstrated that

recombinant human Drosha exhibits non-specific RNAse activity without protein partner DGCR8 on whereas Drosha effieciently cleavages pri-miRNAs after joining of DGCR8. Additionally, depletion of Drosha and DGCR8 cause pri-miRNA accumulation in the cell (Gregory, et al. 2004). The Drosha and DGCR8 or Pasha complex is named as "microprocessor" (Gregory, et al. 2004, Denli, et al. 2004). After microprocessing, pre-miRNA is transported from the nucleus into the cytoplasm by the RanGTP-dependent Exportin5 receptor (Bohnsack, et al. 2004, Yi, et al. 2003). Exportin5 specifically binds to the 2-nt 3' overhang of pre-miRNA in a sequence-independent manner and recognizes a stem structure >14nt of pri-miRNAs (Bohnsack, et al. 2004, Zeng and Cullen 2004).

In the cytoplasm, the second RNAse III cleavage occurs by Dicer to cut the hairpin structure and to generate a double-strand RNA duplex that consists of both the mature miRNA and its antisense strand (He and Hannon 2004). Dicer functions with its double-strand binding protein partner TRBP (the human immunodeficiency virus trans-activating response RNA-binding protein) in human (Chendrimada, et al. 2005). After dicing, Argonaute (AGO) protein is recruited to Dicer, TRBP and ~22nt double strand miRNA:miRNA* complex (Gregory, et al. 2005) (Figure 1.1). In *Drosphila,* this complex is generated by Dicer-2, AGO1 and Loquacious (Saito, et al. 2005). The guide strand with a lower stability at the 5' end is chosen to function in the RNA-Induced Silencing Complex (RISC) whereas the passenger strand is degraded (Schwarz, et al. 2003).

The well-characterized proteins in the RISC are Argonaute family proteins possessing two characteristic domains. The first one is the Piwi Argonaute Zwille (PAZ) domain found in the amino terminus and binds specifically to the 3' end of the guide strand. The other is Piwi Protein (PIWI) domain at the carboxy terminus and exhibits a RNAse H-like tertiary structure (Song, et al. 2004). *Drosophila melanogaster* has five AGO proteins that can be grouped into two families; piwi proteins including Piwi, Aubergine and AGO3 and Argonaute class consisting of AGO1 and AGO2 (Kim, et al. 2008).

After joining to RISC, miRNA binds to the 3' UTR of target mRNA by imperfect base pairing in animals causing translational repression. For an efficient microRNA-target mRNA interaction, 2-8 nt of miRNA referred to as "seed region" at the 5' end should match perfectly with mRNA. In addition, a bulge must be included in

the middle of mature microRNA in not to endonucleolytically degrade target mRNAs (Filipowicz, et al. 2008).

The biogenesis pathway of retrotransposons, transcripts of bidirectional transcription and stem loop structure derived endo siRNAs has not been detailed yet. Clues about pathway comes from Dicer2, Loquacious and mutant *Drosophila* lines. Results indicate that endo siRNA production requires Dicer-2 and miRNA partner Loquacious (Czech, et al. 2008). During biogenesis, 3' terminus of endo siRNA may be methlylated by RNA methlytransferase (Kawamura, et al. 2008). Immunoprecipitation studies showed that endo siRNAs bind to AGO2 and they may target protein coding gene and transposons (Kawamura, et al. 2008). Another model proposes that intermediate cleavage products of long mRNAs are capped and function as small RNAs (Fejes-Toth, et al. 2009).

Intergenic repetitive-originated piRNAs are produced through a Dicer independent pathway. They interact with piwi proteins Piwi, Aubergine and AGO3. Roles of these protein are suggested in biogenesis (Klattenhoff and Theurkauf 2008).

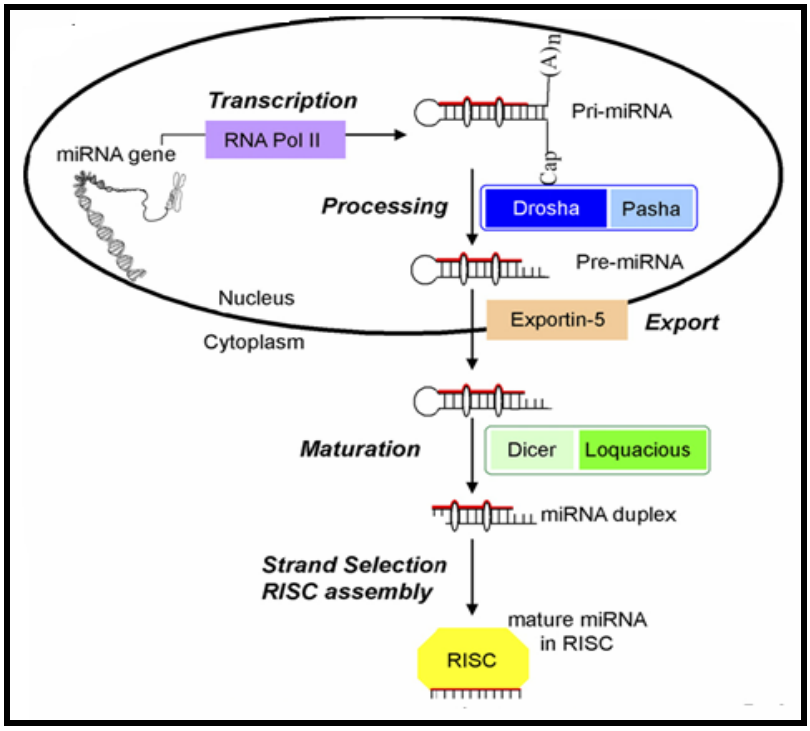

Figure 1.1. MicroRNA biogenesis. pri-miRNAs are transcribed by RNA Pol II and subsequently processed by Drosha in the nucleus. After transportation by exportin5, the hairpin structure of pre-miRNAs are removed by Dicer. Guide strand is selected and joined into RISC to function (Source: Jaubert, et al. 2007).

1.3 Mechanism of Translation

Translation is the process by which genomic information is converted into protein structure. This process is divided into three steps; initiation, elongation and termination. Among them, initiation is the most strictly regulated step (Hershey and Merrick 2000). Initiation begins with binding of eukaryotic initiation factors (elFs) 3, 1, 1A, 5, methionine loaded tRNA and eIF2 to 40S the small ribosomal subunit to yield 43S pre-initiation complex. This complex interacts with the mRNA by association between eIF3 and eIF4G that is the member of eIF4F protein, the cap-binding complex. eIF4G functions as a scaffold protein. It also interacts with poly A binding protein (PABP) facilitating mRNA circulation and increased translational efficiency. The eIF4F complex also contains eIF4E and eIF4A. eIF4E binds to the 7mG cap structure with a high specificity. eIF4A which is the member of DEAD-Box family protein with an ATP binding domain. It is responsible for unwinding of secondary structures in the 5' UTR (Gebauer and Hentze 2004, Hershey and Merrick 2000).

After recognition of eIF4G by the 43S complex, the 5' UTR of the mRNA is scanned through 5'⟶ 3' direction to find initiator codon, AUG, and generates the 48S complex. The 60S large ribosomal subunit associates with the 48S complex, forming a 80S ribosome, a monosome (Figure 1.2). Subsequently, several ribosomes bind to mRNA forming translationally active structure, polysome (Beilharz and Preiss 2004).

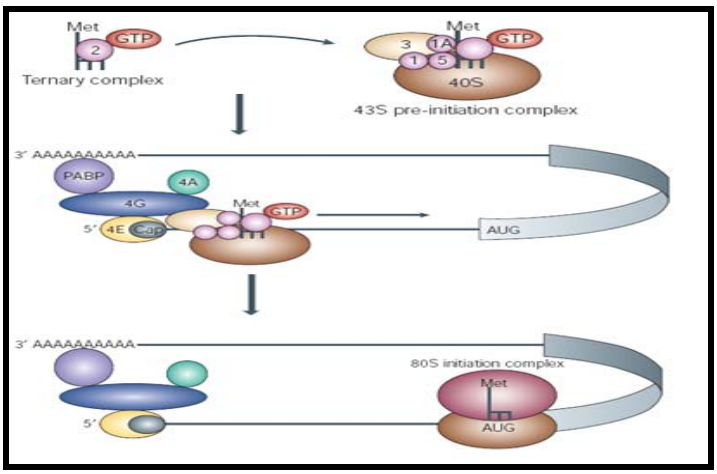

Figure 1.2.Mechanism of translation initiation. 43S complex is generated by translation initiation factors. Cap structure is recognized and the 60S large ribosomal subunit is recruited. After 80S initiation complex, translation starts. (Source: Hershey and Merrick 2000).

1.4 Translational Regulation by Small RNAs

There are four hypotheses on miRNA-mediated translational repression although none of them is well established. Some studies claim that repression occurs either at the initiation or post initiation step. This hypothesis is supported by interactions among cap structure, initiation factors and miRNP. Studies on using reporter mRNAs with an either m^7G cap or non-functional cap structure in synthetic miRNA-transfected HeLa cells revealed that translation of capped reporter mRNAs was repressed (Humphreys, et al. 2005). In contrast, there was no effect on translation of the reporter mRNA carrying a non-functional cap. This suggests that microRNAs regulate translation at the initiation step by interfering with cap binding protein eukaryotic initiation factor 4E (Humphreys, et al. 2005). The side chains of two tryptophan residues of eIF4E interact with m7G by π- π bonds (Kiriakidou, et al. 2007). Kiriakidou *et* al. determined that middle domain Ago proteins has two phenylalanine to interact with cap. This suggests that miRNA-mediated repression may occur by mimicking eIF4E, which results in initiatian inhibition (Kiriakidou, et al. 2007). Additionally, polysome gradient analysis of let -7 transfected Hela cells demonstrated a strong shift of reporter mRNA from polysome to lighter fractions. This implies that ribosome loading cannot be achived due to inbition of initiation (Pillai, et al. 2005). Studies on structural requirement for initiation repression underline the importance of 5' phosphate as the target mRNA lacking a 5' phosphate.

The other proposed mechanism involves ribosome assembly. eIF6 binds to the 60S subunit of ribosome and prevents the association with 40S. Ribosome assembly does not occur as a result. Identification of eIF6 in RISC may propose its potential function in microRNA-mediated regulation (Chendrimada, et al. 2007). Additionally, Wang *et* al. presents the evidence that miRNA repressed-mRNAs posses 40S subunit. This may be the results of preventing 60S from ribosome assembly (Wang, et al. 2008).

To investigate miRNA-mediated post-initiation regulatory events, studies generally examine the polysome association of target mRNA. In *C. elegans,* lin-4 miRNAs regulate lin-14 protein synthesis for proper development. Polysome profile analysis showed lin-4-mRNA-polysome association during miRNA repression (Olsen and Ambros 1999). Maroney *et* al confirmed the association of microRNAs with

polysome and it was found that miRNAs interact with actively translated mRNAs in human cells (Maroney, et al. 2006). A similar study was perfomed on human let-7a miRNA transfected Hela cells in which miRNAs were shown to be associated with actively translating polysome and Ago (Nottrott, et al. 2006).

Repression after initiation may occur by ribosome drop-off. In this model, disassociation rate of ribosome is faster than ribosome loading during miRNA repression and it does not affect ribosome loading (Petersen, et al. 2006). Some viral mRNAs do not posses cap structure and translation starts from internal ribosome entry site(IRES) in a cap-independent manner. The repressive effect of miRNAs was observed on reporter mRNA constructed by modelling IRES carrying mRNA (Petersen, et al. 2006).

Pyrosequencing revealed that most of endo siRNAs have complementarity to the 3' UTR of neighbouring genes (Czech, et al. 2008). The only existing model on endo siRNA function proposes that the inhibitory effect of AGO2-RISC complex prevents the interaction between eIF4E and eIF4G. The detail of the mechanism remains to be elucidated (Iwasaki, et al. 2009).

1.5 The Methods of Small RNA Identification

Studies on small RNAs started with cloning, identification and profiling. The first studies included forward and reverse genetic techiques. However they were not efficient because of small size of RNAs. In order to discover novel small RNAs new cloning strategies have been developed known as sequence-size fractionated cDNA libraries. In this method, linkers are added to the two ends of unknown RNAs. Size fractionation by polyacrylamid gel provides elimination of long RNAs and cDNA libraries are generated (Berezikov, et al. 2006). The cloning strategy is combined with pyrosequencing called as deeep sequencing. It is the most powerful technique to analyze the whole small RNA population. The technology has the capacity to sequence RNAs smaller than 50nt (Kong, et al. 2009).

1.6 Specific aim

Recent experiments have revealed the presence of genome encoded regulatory small RNAs more than previously appreciated. The functions and mechanism of them have not been elucidated yet.

Based on literature and small RNA identification strategies, the aim of this study is to identify polysome-associated regulatory small RNAs in *Drosophila melanogaster* embryos by deep sequencing *in vivo* and characterize them based on their translational status.

CHAPTER 2

MATERIALS AND METHODS

2.1. *Drosophila melanogaster* Maintanence and Embryo Collection

Drosophila melanogaster has important characteristic properties as a model organism. Handling is easy in laboratory condition. It has a short generation time and high reproducibility. Importantly, the genome of *Drosophila* has been sequenced facilitating genetic manipulation. (Roberts 1997)

P2 *Drosophila melanogaster* line was grown at 25°C on yeast-sucrose-agar medium. 107g yeast, 37g agar and 3.5 L distilled H_2O (dH_2O) were boiled. 480g sucrose and 120g corn meal were boiled separately in 1 L dH_2O and added into the mixture. They were mixed by a magnetic shaker without heating. 40 mL of 10% nipagine solution and 40mL propionic acid (Applichem) combination was added to suppress mold growth. Stocks were transferred into fresh medium every two weeks. After stocks reached enough number, they were transferred into a large cage. Embryo collection plates were prepared as follows 22.5 g agar was boiled in 700 mL dH_2O. 94 g sucrose was dissolved in 150mL dH_2O and mixed with 330 mL grape juice. Two embryo collection medium was placed into the cage. 0-1h and 8h embryos were chosen as a model. Because in the first 2 hours of development, there is no zygotic transcription and development proceeds by maternal mRNA translation (Qin, et al. 2007) It is well-known that this translationally active state is highly regulated by known and unknown factors. To collect 1h *Drosophila* embryos, the plates were kept in the cage for 1h and washed with 0,7% NaCl and 0.1% Triton-X. To collect 8h *Drosophila* embryos, the plates were kept in the cage for 1h and incubated in an incubator in the absence of flies for 7 hours. The same washing procedure was applied and samples were stored at -80°C.

2.2. 5-70% Sucrose Gradient Preparation

Gradients included 5% gradient and 70% sucrose solutions in 100mM $NaCl_2$, 10mM $MgCl_2$, 30mM Tris-HCl (pH 7), 200U Superase RNase Inhibitor (Ambion). 5-70(w/v) gradients was prepared in a polyallomer tube (Beckman) by using Density Gradient Fractionation System Gradient Making Program (ISCO).

2.3. RNA Isolation by Sucrose Density Gradient System

0,2 g embryo was homogenized in 5mL lysis buffer [(100mM $NaCI_2$, 10mM $MgCl_2$, 30mM Tris-HCl (ph 7), 1% Triton-X, 1% NaDOC, 100µg/mL cycloheximide (Applichem) and 30U/mL Superase RNase Inhibitor (Ambion)] (Akgül and Tu 2006) and transferred into 1.5mL eppendorf tubes for 8min incubation on ice. The homogenates were centrifuged at 12.000xg for 8 min at 4ºC. 2 mL supernatant was loaded directly onto 5-70% (w/v) gradient and centrifuged at 27.000rpm for 2h 55min at 4ºC in a Beckman SW28 rotor. Fractions were collected from the top of the gradient using an ISCO density gradient system while monitoring absorbance at 254 nm. Fractions were then pooled into 4 subgroups based on their A_{254} readings; mRNP, 40S, monosome and polysome. Subsequently, extraction started by adding 150mM $NaCI_2$ and 0,5% SDS (Applichem) as final concentration. Then DEPC treated water was added to reach two volume. RNA was extracted with an equal volume of phenol/chloroform/isoamylalcohol 25:24:1 (pH 4.5, Applichem). After vigorously vortexing for 2min, falcon tubes were centrifuged at 3000 rpm for 5 min at 24ºC to separate the aqueous and organic phase. The upper phase (aqueous) was withdrawn without disturbing the organic phase containing phenol/chloroform/isoamylalcohol 25:24:1 and transferred into a fresh falcon tube. An equal volume of phenol/chloroform/isoamylalcohol 25:24:1 was added to the solution and re-centrifuged as before. The aqueous phase was extracted once with an equal volume of chloroform (Merck) 1/10 volume of 3M NaOAC (ph: 7) and two volumes of 100% ethanol were

added into recovered RNA and stored at -20ºC overnight. RNA samples were centrifuged at 12.000xg for 20 min at 4ºC in 30mL Corex tube, in a Beckman Avanti JE JS13.1 rotor. After washing with 70% ethanol, the pellets were dried and RNAs were suspended in 50µL DEPC treated water. Concentration, 260/280 and 260/230 values of mRNP, 40S, monosome and polysome RNAs were measured by Nanodrop ND UV-Vis Spectrophotometer.

2.4. RNA Quality Control by Bioanalyzer

RNA integrity was checked by 2100 bioanalyzer using Agilent RNA 6000 Nano Kit and Agilent Small RNA Kit based on manufacturer instructions. Agilent 2100 bianalyzer software was used to asses the results.

2.5. Small RNA Isolation

To remove RNAs longer than 200nt, isolated mRNP, 40S, monosome and polysome RNAs were purified by a mirVANA miRNA isolation kit (Ambion). 5 volumes of lysis/binding buffer and then 1/10 volume of miRNA homogenate additive were added to 100µg RNA. It was incubated for 10 min on ice. After 1/3 volume of 100% ethanol was was added into the RNA mixture, 700 µL was applied to a filter supplied by kit and centrifuged at 5000xg for 1 min at room temperature. For greater volumes, centrifugation was repeated with the same filter and filtrate including small RNAs was collected. The filter trapped longer RNAs. 2/3 volume 100% ethanol was added to the filtrate and the mixture was applied onto a new filter and centrifuged at 5000xg for 1. At this step, small RNAs bounded to the filter. The filter was washed with 700µL miRNA wash solution and centrifugated at 5000xg for 1 min. Two times of 500 µL wash solution 2/3 was applied. After centrifugation at 10.000xg for 1 min, 50 µL

pre-heated elution buffer was loaded onto the center of filter and centrifuged at 10.000xg for 1 min. Concentration, 260/280 and 260/230 values of RNAs smaller than 200nt were measured by Nanodrop ND UV-Vis Spectrophotometer.

2.6. Denaturing Polyacylamide Gel Electrophoresis (PAGE) of RNAs

RNAs isolated by sucrose density and mirVANA kit were run on a denaturing polyacyrlamide gel (PAGE) which is a very sensitive method to visualize RNAs. Because of secondary structure, RNAs must be converted into a linear structure. In the gel formation, urea is used as a denaturing agent that eliminates secondery structures of RNAs. For 45mL of 12% 8M urea containing denaturing gel, 21.6g urea (Ambion), 4.5mL 10X Tris-borate-EDTA buffer (TBE, 0.9M Tris base, 0.9M Boric acid, 20mM EDTA pH 7,Ambion) and 13.5 mL 40% acylamide acryl:bis acryl= 19:1 (Ambion) and DEPC treated water were mixed. The mixture was heated and stirred until urea completely dissolved. Then, 360µL APS (Applichem), 48 µL TEMED (Applichem) were added and the mix was immediately poured into glass plate set. After polymerization, gel was placed into the tank and pre-runned at 300V at 15min. RNA samples were mixed with an equal volume of 1X gel loading buffer (Ambion) and heated at 95ºC for 5min. Samples were then kept on ice. Running was performed at 300 V for 1.5h. The gel was visualized by 0.5µg/mL ethidium bromur (Applichem) in 300 mL 1XTBE buffer.

2.7. Small RNA Cloning

2.7.1. cDNA Preparation and PCR Amplification

Cloning procedure required 50µg small RNA as an initial material and included 6 steps. RNA samples were run on 12% PAGE stained with RNA staining solution (Abnova). 2 bands of interests and small RNA region under 30nt 2S rRNA from mRNP were cut and transferred into 1.5mL eppendorfs. 3 to 4 volumes of RNA exraction buffer (Abnova) was added and it was shaken at 400 rpm overnight at 4ºC. Then phenol-chloroform exraction and ethanol precipitation were performed. An equal volume of TE-saturated phenol (pH 4.5, Applichem) was mixed with the buffer containing gel pieces. After vortexing, it was centrifuged at 13.000 rpm for 10 min. The upper phase was transferred into a new tube. The same volume of chloroform (Applichem) was added and centrifuged at 10.000rpm for 2 min. Then, 1/10 volume of 3M sodium acetate (NaOAc, ph 5.2) , 1/50 volume of 5µg/µL glycogen (Ambion) and 2 volume of 100% ethanol were added. The tubes were stored at -80ºC at least for 2 h. RNA mixture was then centrifugated at 14.000 rpm for 10 min at 4ºC. The pellets were washed by 70% ethanol and dried.

In dephosphorylation step, 5' phosphate of small RNA was removed to prevent circularization. 40 µL reaction was set up by resuspension of precipitated RNA in DEPC treated water, 0,6u/µL bacterial alkaline phosphatase (Fermentas) and 1X phosphatase buffer. The reaction was incubated at 37ºC for 1h. Process carried on with phenol/chloroform and ethanol precipitation. To reduce RNA amount during this process, 110µL DEPC treated water was added to the reaction and phenol-chloroform extraction and ethanol precipitation were repeated.

The subsequent step involved ligation of 17nt 3' linker sequence, 5' CTG TAA CTC TCA AT 3'(Integrated DNA Technology, IDT) to small RNAs. The 5' end of the 3' linker was modified to prevent 5' linker - 3' linker ligation. Dephosphorylated RNAs were resuspended in 15µL DEPC treated water. Reactions were set up by 17µL dissolved RNA, 1µL 100mM 3' linker, 2µL 10u/ µL T4 RNA Ligase (Fermentas), 4µL

10X T4 RNA ligase buffer and 16µL polyethlylenglycol (PEG,45%, Applichem). Phenol/chloform extraction and ethanol precipitation were performed as described before. Ligated RNAs were dissolved in 10µL DEPC treated water and run on 12% denaturing polyacrylamide gel. After staining with RNA staining solution , ~57 nt and 67 nt long RNAs were excised from the gel. RNA elution, phenol-chloroform extraction were repeated as previously mentioned.

To activate 5' ends of RNAs, 3' linker ligated RNAs were phosphorylated by T4 Polynucleotide Kinase (PNK, Fermantas). 40µL reaction was set up with dissolved small RNAs from the previous step, 10u T4 PNK, 1X PNK buffer and 100mM ATP and incubated at 37ºC for 30 min. 110µL DEPC treated water was added to the reactions and phenol-chloroform extraction and ethanol precipitation were repeated.

The next step involved the ligation of the 5' linker. The reaction included RNAs resuspended in DEPC-treated water, 100mM 5' linker, 5' rArUrCrGrUrUrCrGrGrGrArUrGrArArArA 3' (IDT), 20u T4 RNA ligase, 1X T4 RNA ligase buffer and 9% PEG solution were added. Incubation was at 15ºC for 1 h. 110µL DEPC-treated water was added to the reactions and phenol-chloroform extraction and ethanol precipitation were applied. At the end of this step, known 5' linker + unknown small RNA sequences + known 3' linker sequences were generated. To remove background, reactions were run on 12% denaturing polyacrylamide gel. After staining with RNA staining solution, ~74 nt and 84 nt long RNAs were excised and RNA elution, phenol-chloroform extraction and ethanol precipitation were repeated.

The next step included cDNA reaction by reverse transcription and amplification by PCR using primers matching the 5' and 3' adapters. Gel purified products were dissolved in DEPC-treated water. 50 µM 3' reverse transcription (RT) primer, 5' ATT GAC CCG AGT TAC AG 3' (MWG), was added and heated at 70ºC for 3 min, cooled at room temperature for 2 min and put on ice to anneal the primer. Reverse transcription reactions were set up by primer+RNA mixture, 100U M-MuLV reverse transriptase (Fermentas), 2X reverse transcriptase buffer, 1mM dNTP and 3µL DEPC treated water . Incubation temperatures were at 37ºC for 10 min, 42 ºC for 1 h and 85ºC for 5 min. To amplify cDNA, 25µL PCR reaction was set by 1µL cDNA, 1U DNA Taq Polymerase (Fermentas), 1X Taq polymerase buffer, 3mM $MgCl_2$, 1µM 3' RT primer, 1µM 5' primer synthesized by MWG, 0,2mM dNTP, cycler. PCR programme included initial denaturation at 94ºC for 1 min, 25 cycle for initiation at 94ºC for 1 min, for annealing

at 50 ºC for 1 min, for elongation at 72 ºC for 1 min and final extention at 72 ºC for 10 min. The PCR products were run on 2% agarose gel and 12% non-denaturing polyacrylamide gel.

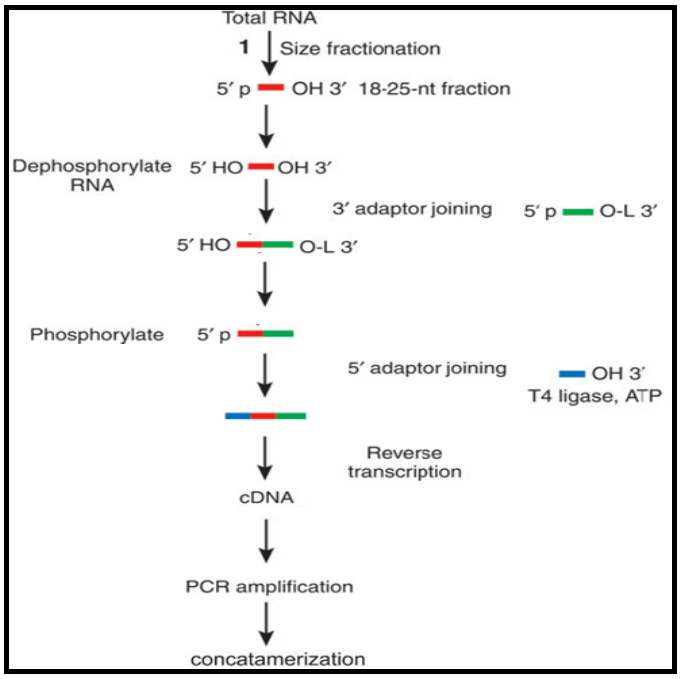

Figure 2.1.Size-fractionatated cloning of small RNAs. Procedure included dephosphorylation of RNAs to inactivate 5' end. 3' end of RNAs were ligated with linker and phosphorylation then was performed. After 5' linker ligation, reverse transcription reaction was set. cDNA was ampilified. Procedure was carried on concatamerization into pGEM T easy vector (Source:Berezikov, et al. 2006).

2.7.2 Concatamerization of PCR Products

The aim of concatamerization was to ligate several DNA fragment to each other and then to ligate a vector. For ligation to each other, sticky ends must be compatible. So primers were designed to carry the same AvaI (Fermentas) restriction recognition site.

The PCR products, ~74 nt, 84 nt, and 55nt were extracted from 2% agarose gel by using agarose gel extraction kit (Fermentas). The recovered DNA was measured by Nanodrop and following reaction was set by AvaI to generate sticky ends. Phenol-chloroform and ethanol precipitation were performed. Precipitated product was

dissolved in dH_2O and ligation reaction was set as described; 600u T4 DNA ligase(Fermentas), 1X ligase buffer, 25µg DNA and in 20 µL at 15ºC for 4 h. Ethanol precipitation and phenol-chloroform were performed.

After ligation, extension was performed by using 25u DNA taq polymerase, 1X taq buffer, 1,5mM $MgCl_2$, 10mM dNTP and 350ng DNA in 20µL reaction volume. Samples were run on 2% agarose and extracted by DNA extraction kit (Fermentas).

2.7.3 Ligation of PCR Products Into pGEM T Easy Vector

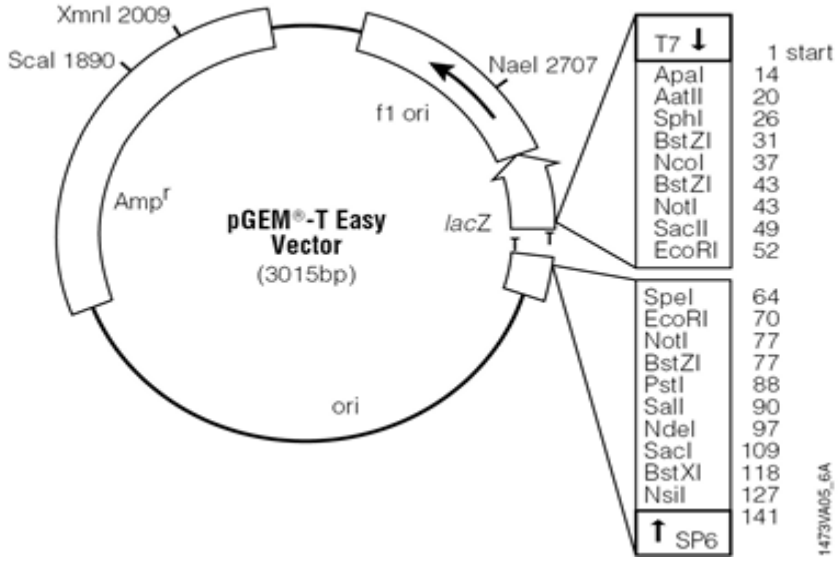

Figure 2.2.Detailed map of pGEM T easy vector. Multiple cloning site and ampicillin resistance site are shown.

Concatamerization reaction was set using 3U T4 DNA ligase, 1X T4 DNA ligase buffer, 50ng pGEM T easy vector (Promega) and 4,5ng DNA in 10 µL at room temperature for 1 h. Vector containing concatamerized DNA was transformed into DH5α *E. coli* competent cells (Invitrogene). Transformants were plated on agar plates including 100 µg/ml ampicilin/X gal/IPTG at 37ºC for overnight. Insert containing white colonies were selected that is known as blue-white selection. They were inoculated into LB liquid medium including 100 µg/ml ampicillin. After 16h, plasmids

were isolated by quick prep plasmid isolation kit (Fermentas). To check the concatamerization and insert, EcoRI restriction reaction was applied to plasmids. The clones were sequenced by RefGen (Turkey).

2.8. Small RNA Deep Sequencing

mRNP, 40S, monosome, polysome RNAs and total RNA of 0-1 h and 8 embryos were sequenced using Illumina Genome Analyzer by Fasteris (Switzerland).

2.9. Small RNA Microarray

mRNP, 40S, monosome, polysome RNAs and total RNA of 0-1 h and 8 embryos were profiled by microarray by Febit Biomed GMBH (Germany)

CHAPTER 3

RESULTS

3.1. Polysome Profile of 0-1h and 8 h Embryos

Polysome profile analysis makes it possible to seperate mRNP complexes based on translational status. mRNP and 40S fractions do not include any translating ribosomes. Translation is just initiated in monosome fractions. In polysome fractions, mRNAs are in complex with ribosomes at the elongation phase. Polysome profile of 0-1 and 8h embryos that was explained in 2.4 showed well separated fractions with an increasing polysome size and volume (Figure 3.1).

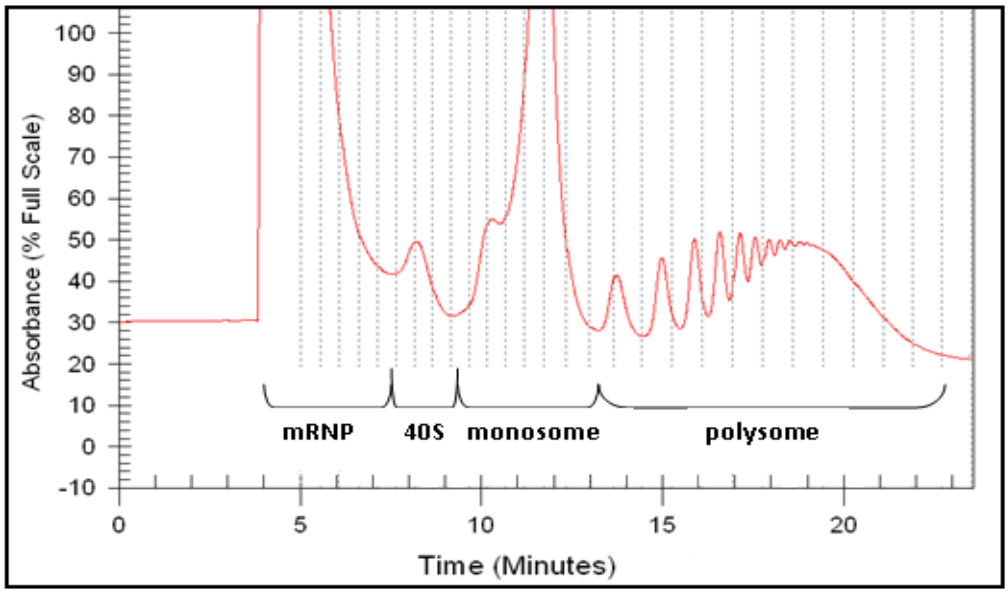

Figure 3.1.Polysome distrubution of 1h stage *Drosophila* embryos. Embryo lysates were fractionated by sucrose gradient centrifugation and collected into fourty eppendorf tubes including 30 drops at A_{254}. The top of the gradient is on the left and peaks representing the mRNP, 40S ribosomal subunit and monosome. Polysome is heaviest fraction relative to monosome, 40S and mRNP. Each increasing peak presents the ribosome number on mRNA and polysome volume.

Another aspect of RNA isolation by sucrose density method is the enrichment of various type of RNAs. This isolation collects same RNA molecules in a same pool in terms of their role in translation. It provides cloning of rarely expressed small RNAs that have a role in translation regulation. Average nanodrop results of RNA isolation and small RNA enrichment are given in Table 1.

Table 3.1.Average RNA concentration and enrichment folds. Enrichment of 300µg RNA are given relative to total RNA concentration.

Fraction	RNA concentration	Enrichment	260/280	260/280
mRNP	10 µg	30X	1,90	2,0
40S	10 µg	30X	1,8	2,1
monosome	100 µg	3X	2,2	2,3
polysome	180 µg	1.6X	2,0	2,1

The purified RNAs had an ideal 260/280 ratio that reflects no protein contamination with a ratio between 2.2 and 1.8. 260/230, which is an indicator of ethanol contamination, was between 1.8 and 2.2 as standart value.

3.2. RNA Quality Control by Bioanalyzer

A more accurate measure of RNA quality is determined by the sharpness of 18S and 28S rRNA peaks and flat baseline of RNA electropherogram. 0-1h embryo RNA that was used for cloning and deep sequencing had high quality as shown in Figure 3.2. In Figure 3.2,baseline of 40S fraction is not flat however it is not degraded. 8h embryo had the same quality (not shown).

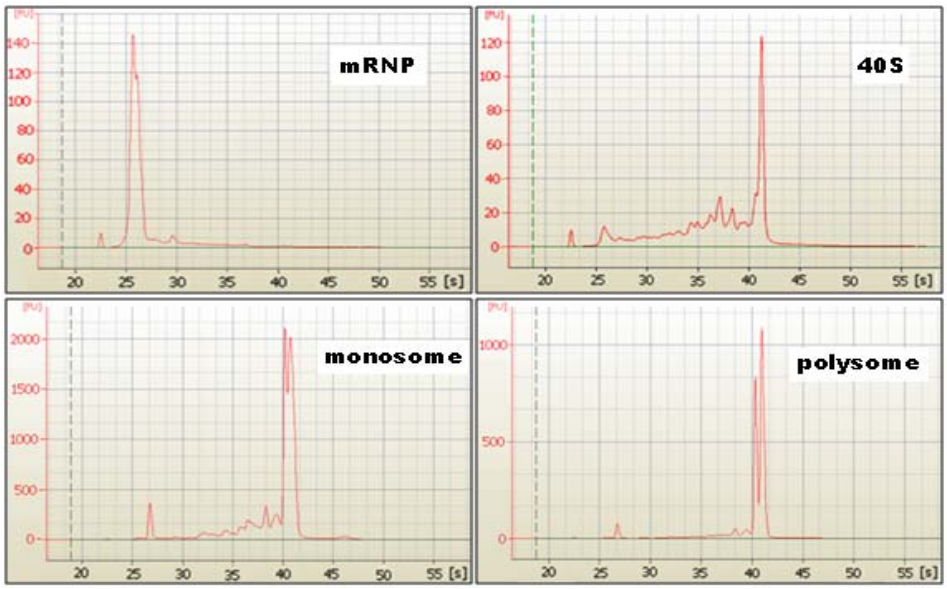

Figure 3.2.Agilent 2100 Bioanalyzer electropherograms of mRNP, 40S, monosome and polysome RNAs. Samples were isolated from 0-1 h embryo by density gradient. Chip based analysis detected RNA integrity by comparing isolated RNAs with 18S and 28S rRNA. All samples have a flat baseline, which confirmed integrity. The quality of isolated RNAs were considerably good.

3.3. Small RNA Isolation

4µg small RNA was isolated from 100 µg of total RNAs from fractions. The typical total RNA includes 85% rRNA, 10% tRNA and small RNAs, 1-5% mRNA population. That is the reason of low yield from initial 100µg total RNA. When isolated RNAs were run on 12% PAGE, two interesting RNA bands, ~40nt and 50nt in size, were visualized in mRNP fraction (Figure 3.4). It was decided to be cloned because literature search did not give any information about them. Small RNAs including siRNA and microRNAs which are smaller than 30nt 2S rRNA of mRNP fraction were also used for cloning.

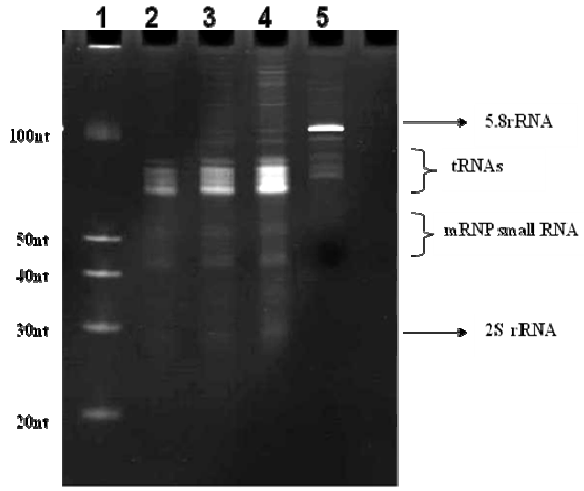

Figure 3.3.12% Denaturing PAGE of isolated RNAs. 1.small RNA marker, 2.mRNP (0,25 ug), 3.mRNP (0,5 ug), 4.mRNP (1 ug), 5.Polizom (0,25 ug**).** The presence of 40nt and 50nt is clear.

3.4. Small RNA Cloning

~40nt-50nt small RNAs and small RNAs including siRNAs and microRNAs were successfully cloned as described in 2.7. The size of PCR product of ~40nt-50nt small RNAs was expected between 67nt-111nt marker. MicroRNA-siRNA band was longer than 37 nt, however 2% agarose gel electrophoresis did not give exact result for miRNA-siRNA cloning.

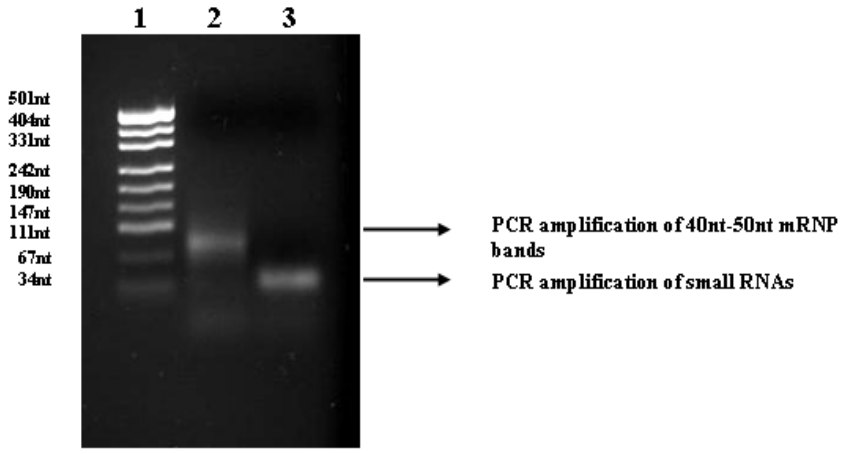

Figure 3.4.2% Agarose gel electrophoresis of PCR products. 1. pUC 19 DNA marker, 2. Linker ligated 40nt-50nt small , 3.linker ligated ~20nt small RNA cloning. Both two product had expected size.

To fractionate the PCR products from the cloning of siRNAs and miRNAs more precisely, %12 non-denaturing polyacrylamide gel electrophoresis was performed. As seen in Figure 3.6 (4th and 5th lanes) the cloning products had the expected size. Surprisingly, 1st and 2nd wells consist of bands between 34nt and 67 nt. The expected size of miRNAs and siRNAs is near 60 nt. Strong background is observed, which may stem from cloning of minor RNA degradation products

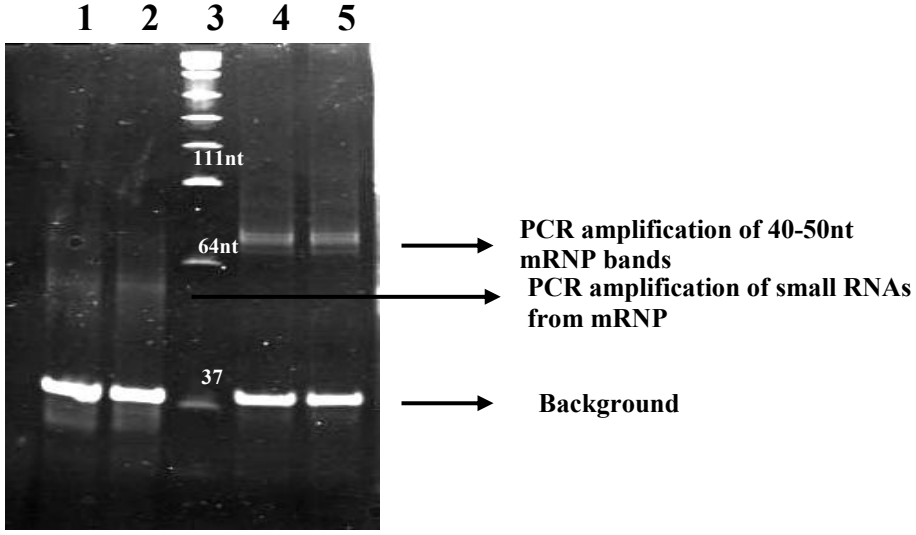

Figure 3.5.12% non-denaturing polyacrylamide gel electrophoresis of PCR amplification. 1th and 2nd lanes include small RNA that are smaller than 30 nt amplification. 4th and 5th present 40-50 nt small RNAs in mRNP.

The PCR amplified small RNAs were then concatamerized and ligated into pGEM T Easy vector to faciliate sequencing of multiple inserts in a single clon. When pGEM T Easy vector were cut by *EcoRI*, different size of inserts confirmed concatamerization and ligation (Figure 3.6)

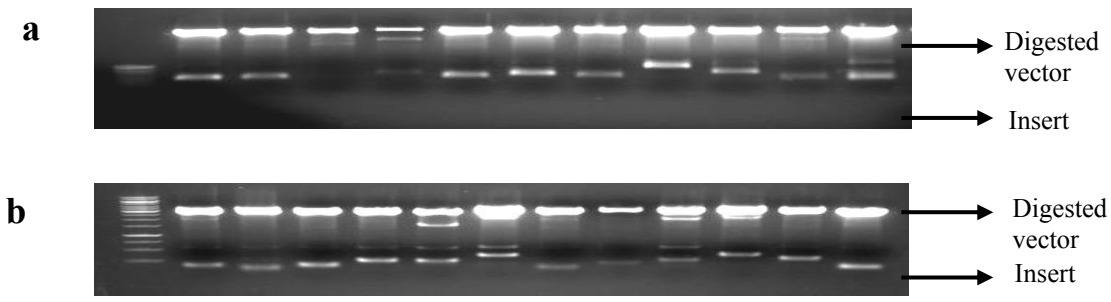

Figure 3.6.2% Agarose gel electrophoresis of *EcoRI* digested products. Panel a shows the restriction reaction of 40-50nt small RNAs. Panel b presenst the restriction reaction of 20nt small RNAs. Restriction digestion gives different size of insert, which reflects the different number of ligated DNA fragments to each other.

3.5. Sequencing of PCR-amplified Small RNAs

Totally 115 plasmid were sequenced from ~40nt-50nt (75 plasmid) and ~20 nt (40 plasmid) from mRNP fraction of 0-2 h embryo. The results were assessed using Flybase database. Among them, 76 plasmid (54 plasmid from ~40nt-50nt and 22 plasmid from ~20 nt small RNA) including 92 sequences of inserts gave meaningful results and matched with tRNAs; tRNA:D:96A(14), tRNA:CR31494, tRNA:CR30238, tRNA:CR30238, tRNA:CR30407, tRNA:D2:69 F, tRNA:N5:42Ah, tRNA:N5:84F, 28S rRNA, 18S rRNA, 5S rRNA snRNA,snoRNA,7SL RNA and a part of mRNAs. Table 3.2 shows the results of sequencing. Total 35 sequences from 22 clone of 20nt small RNAs were analyzed. Total 57 sequences from 54 clone of 40nt-50nt RNAs were analyzed.

Table 3.2. The blast results of PCR-amplified small RNAs.

20 nt		~40nt-50nt		~40nt-50nt	
28S rRNA	2	tRNA:D:96A	14	28S rRNA	6
18S rRNA	7	tRNA:CR31494	2	18S rRNA	7
5S rRNA	3	tRNA:CR30238	1	5S rRNA	5
7SL RNA	5	tRNA:CR30407	2	7SL RNA	0
snRNA	1	tRNA: D2:69 F	1	snRNA	4
a part of mRNAs	16	tRNA: N5:84F	1	a part of mRNAs	10
intron	1	intron	4	no match	5
		transposon	1		

Interestingly, 14 sequences (24,6%) from ~40nt-50nt RNAs were matched with either 3’ or 5’ part of tRNA:D:96A The whole sequence of tRNA:D:96A and matched sequences are given below.

tRNA:D:96A

5’TCCTCGATAGTATAGTGGTTAGTATCCCCGCCTGTCAC**GCGGGAGACCGGGGTTCAATTCCCCGTCGGGGAGCCA3’**

MIGRL-54

5’TCCTCGATAGTATAGTGGTTAGTATCCCCGCCTGTCAC3’

MIGRL-94

5’CGCCTGTCACGCGGGAGACCGGGGTTCAATTCCCCGTCGGGGAGCCA3’

Another important result was that intronic RNA was cloned from 20 nt and 40-50 nt of mRNP fraction.

3.6. Microarray Analysis of siRNAs

We took advantage of the present RNA samples to profile the recently discovered endegenous siRNAs, which are not reported to have any *in vivo* gene regulatory function yet. 0-2h and 8 h embryo RNAs were isolated by sucrose density and analyzed usind microarray by Febit Biomed GMBH, Germany. For endo-siRNA, 1255 probes were designed. Among them, 476 endo siRNAs were present in fractions. Based on their expression profile, 476 siRNAs generated 8 groups as shown Table 3.3 and Figure 3.7;

1. 146 endo siRNAs are expressed at both 40S and monosome in only 8hour (8h 40S+monosome).
2. 108 endo siRNAs are expressed at monosome in only 8hour (8h monosome).
3. 101 endo siRNAs are expressed at 40S in only 8hour (8h 40S).
4. 25 endo siRNAs are expressed at polysome in 2h and mRNP, 40S and dominantly monosome in 8 hour (2h polysome+8h).
5. 20 endo siRNAs are expressed at both mRNP and 40S in only 8hour (8h mRNP+40S).
6. 16 endo siRNAs are expressed at mRNP, 40S and monosome in only 8hour (8h).
7. 8 endo siRNAs are expressed at mRNP in only 8hour (8h mRNP).
8. Remaining siRNA generated small groups and all of them were collected under the other group.

Table 3.3.The microarray result of endo siRNA expression. Fractionated RNAs which were isolated from 0-2h and 8h *Drosophila melanogaster* embryos to their translational status by sucrose gradient centrifugation were analyzed. Expressed endo siRNA were grouped into eight groups. The results present that large percentage of endo siRNAs are found in 8h. However 25 endo siRNAs were found in polysome in 2 h.

Groups	Number of siRNAs	Percentage of siRNAs
No expression	777	62
8h 40S+monosome	146	11,6
8h monosome	108	8,6
8h 40S	101	8
2h polysome+8h	25	2
8h mRNP+40S	20	1,6
8h (all fractions)	16	1,3
8h mRNP	7	1,5
others	55	4,4

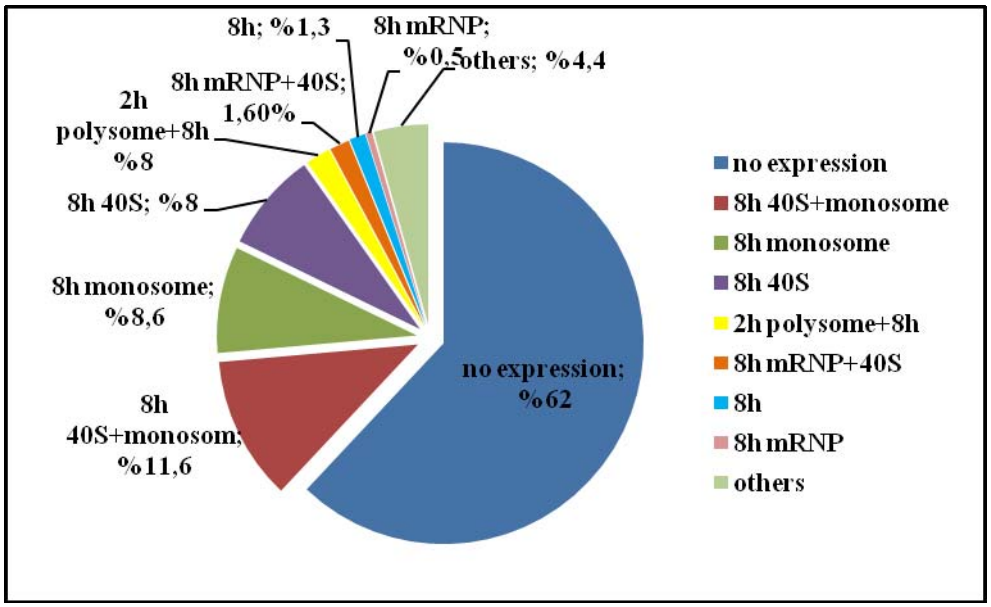

Figure 3.7.Percentage of siRNA distribution. Endo-siRNAs are dominantly localized at 40S and monosome in 8h.

The most interesting group included siRNAs which are associated with polysome in 2h developmental stage but are switched to 40S and monosome at the 8h stage. To find siRNA binding region on target mRNA, flybase database was used. Endo siRNA members of 4 groups were analyzed; 8h 40S, 8h monosome, 8h

40S+monosome, 2h polysome+8h. The results showed that 68% of siRNA found in polysomes have putative binding sites in the 3' untranslated region of target mRNAs (Figure 3.8). siRNAs in other fractions rather than polysome mainly matched with exons.

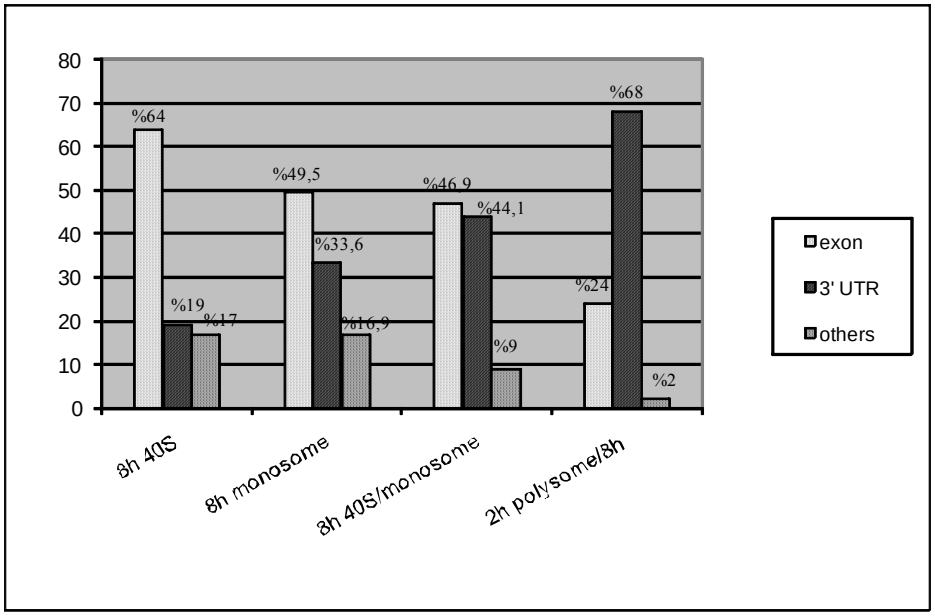

Figure 3.8.Percentage of endo siRNA-target mRNA matching region. Using Flybase database, endo siRNA sequences were matched with their target mRNAs and compared to their matching site. 3 groups that were expressed different fractions in 8h mainly matched with exon region of target mRNA. On the other hand 2h polysome+8h showed interesting matching which 68% of endo-siRNAs were matched with 3' UTR of target mRNA.

CHAPTER 4

DISCUSSION

Cloning 40nt-50nt small RNAs from mRNP fraction gave interesting results. 36,8% of 57 insert match tRNAs and among them 24,6% insert have a part of tRNA:D:96A. If the cloning frequency was considered, the value is high. This may be hypothesized as a biogenesis or degradation pathway of tRNAs. To find out the details, the presence of part of tRNA:D:96A should be confirmed. The clone of ~20 nt small RNAs mainly included rRNA fragments.

Cloned intronic 23nt small RNA may be microRNA candidate. Studies on biogenesis determined intronic origin of miRNAs.

Microarray results of endo siRNAs gave interesting clues about their roles. The most interesting group included siRNAs which are associated with polysome in 2h developmental stage but are switched to 40S and monosome at the 8h stage. This switch may have an important role in development. In addition, if siRNAs are associated with polysome, they may regulate translation. When the role of miRNAs in translation regulation by binding 3' UTR of target mRNA is examined, endogenous siRNAs may function as miRNA.

When the orientation of endo siRNAs found in 2h polysome+8h were considered, 13(52%) of 25 siRNAs were antisense. 20% of human and 15% of *Drosophila* genome have overlapping transcripts. However in eukaryotes, the roles of antisense transcripts have not been revealed. The micrroarray data of endo siRNAs may suggest that antisense endo siRNA found in polysome post-transcriptionally regulate gene expression by binding 3'UTR of target mRNA.

Assesment of deep sequencing results may reveal small RNA population and provide insight into their possible mechanism.

CHAPTER 5

CONCLUSION

Microarray results proved the presence of the polysomal sense and antisense endo siRNAs. To confirm the microarray results, validation of antisense and sense endo-siRNA presence in wild type but not in AGO2 mutant will be done. Importantly, siRNAs switch from 2h polysome to 8h mRNP, 40S, monosome. This stage specific switch may be important for proper development. To observe chances in target mRNA level during endo-siRNA switches in wild type, their target mRNAs will be quantified.

Polysome associatiated endo siRNAs matched with 3' UTR of their target mRNAs. It suggests the role of endo siRNAs at posttranscriptional regulation. Based on this speculation, their target mRNAs in AGO2 mutant *Drosophila melonogaster* line and wild type will be quantified.

Other intriguing result of microarray is the association of short sense endo siRNAs with polysome. Antisense endo siRNA may bind the complementarity region of target mRNAs and regulation occurs. On the other hand, sense endo siRNAs cannot bind to their target mRNAs because of sequence similarity. Possible speculative model may involve antisense endo siRNA regulation by sense endo siRNAs.

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