INVESTIGATION OF tRNA-DERIVED SMALL RNAs IN *Drosophila melanogaster*

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ABSTRACT

INVESTIGATION OF tRNA DERIVED SMALL RNAs IN *Drosophila melanogaster*

Types of small RNAs considered as crucial players of regulation of gene expression increase gradually in number. Improvements in cloning and sequencing strategies and technologies resulted in identification of tRNA derived small RNAs which are highly expressed in the cell just like other small RNAs such as microRNAs, endo-siRNA, and Piwi interacting RNAs.

Depending on stress and changing physiological conditions, tRFs are originated from different positions, in different frequency and different tRNAs. However, their functions and the complexes they interact with remain largely unknown.

In this thesis study, one of the aims is to characterize tRNA derived small RNAs expressed during embryonic development in *Drosophila melanogaster* by in-vitro and in-vivo experiments. This study also aimed to demonstrate the differences between embryonic tRFs and stress induced tRNA derived small RNAs. Lastly, it was aimed to obtain some clues about their biogenesis, mechanism and functions.

It was shown that the tRFs expressed in 1-hour and 8-h *Drosophila* embryos are different from stress induced tRNA derived small RNAs in terms of both position and length. The other important result is that embryonic tRFs are associated with complexes in mRNP and 60S fractions and they are expressed temporally and selectively during *Drosophila* embryogenesis.

ÖZET

Drosophila melanogaster' DE tRNA KAYNAKLI KÜÇÜK RNA'LARIN ARAŞTIRILMASI

Gen ifadesinin düzenlenmesinde hayati oyuncular olarak düşünülen küçük RNA çeşitleri giderek sayıca artmaktadır. Klonlama ve sekanslama strateji ve teknolojilerindeki gelişmeler, mikro RNA, endojen küçük susturucu RNA ve Piwi etkileşimli RNA'lar gibi diğer küçük RNA'lara benzer olarak hücre içinde yüksek oranda ifade edilen tRNA kaynaklı küçük RNA'ların tanımlanması ile sonuçlanmıştır.

Stres ve değişen fizyolojik şartlara bağlı olarak, tRF'lerin farklı tRNA lardan farklı pozisyonlarda farklı sıklıkta orjinlendikleri deneysel olarak gösterilmiştir. Buna rağmen fonksiyonları ve etkileştikleri kompleksler büyük oranda bilinmemektedir.

Bu tez çalışmasında hedeflerden biri, *Drosophila melanogaster'* de embriyonik gelişim sırasında ifade edilen tRNA kaynaklı küçük RNA'ların in-vitro ve in-vivo deneylerle karakterize edilmesidir. Ayrıca bu çalışmada, embryonic tRF'ler ile stres şartlarında üretilen tRNA kaynaklı küçük RNA'lar arasındaki farklılığın ortaya konması hedeflenmiştir. Son olarak, tRF'lerin biyogenezlerine, mekanizmalarına ve fonksiyonlarına ilişkin ipuçlarının elde edilmesi amaçlanmıştır.

Sonuç olarak 1 ve 8 saatlik embryolarda ifade edilen tRNA kaynaklı küçük RNA'ların stres koşullarında üretilen küçük RNA'lardan pozisyon ve uzunluk bakımından farklı oldukları ortaya çıkarılmıştır. Diğer önemli sonuç ise tRNA kaynaklı küçük RNA'ların oluşturdukları komplekslerin translasyonun mRNP ve 60S fraksiyonlarında yoğunlaştıkları ve *Drosophila* embriyonik gelişiminde geçici ve seçici olarak ifade edildikleridir.

To people never giving up...

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LIST OF ABBREVIATIONS

AGO……..Argonaute
DEPC……Diethylpyrocarbonate
DGCR8,……Di George Critical Syndrome Region 8
Endo siRNA…… Endogenous Small Interfering RNA
miRNA……. microRNA
PAGE……... Polyacrylamide Gel Electrophoresis
PAZ……...Piwi Argonaute Zwille
piRNA…….. Piwi Interacting RNAs
Pre-miRNA……Precursor MicroRNA
Pri-miRNA……Primary MicroRNA
RISC……. RNA Induced Silencing Complex
tRFs……...tRNA derived RNA Fragments
UTR……...Untranslated Region

CHAPTER 1

INTRODUCTION

1.1. Small RNAs

In the human genome, 20,500 protein-coding genes have been identified by International Human Genome Sequencing Consortium in 2004 but there are more than 2 million functional proteins regulating a great number of functions in the whole body (Clamp, *et al*. 2007). This situation raised some questions about the real definition of a eukaryotic gene and the total number of genes. These intriguing issues could be explained by the presence of alternative splicing and many of non-coding small RNAs in eukaryotic transcriptomes. Both the genome and the transcriptome are kept under extensive surveillance by small-coding RNAs. The functions of small RNAs range from heterochromatin formation to mRNA destabilization and translational control (Filipowicz, *et al*. 2008). Because they have ability to impact genome and transcriptome extensively, they are involved in a great deal of biological processes including cell differentiation, apoptosis, cell proliferation, developmental timing, metabolic control, transposon silencing, anti-viral defense and so on.

Recent progress in high throughput sequencing has revealed the astounding scopes regarding small RNAs. Small RNAs are classified into three groups based on their biogenesis mechanism and the type of the Argonaute protein that they are associated with (Lu, *et al*. 2005; Margulies, *et al*. 2005). These small RNAs are microRNAs, endogenous small interfering RNAs (endo-siRNAs) and Piwi interacting RNAs (piRNAs). But recently, the newest small RNA group is falling under the small RNA family as tRNA derived small RNAs having common and different characteristics with the other small RNAs in terms of biogenesis, mechanisms and function revealed recently (Lee, *et al*. 2011).

1.2. MicroRNAs

miRNAs are single stranded RNAs (ssRNAs) about 22 nt in length which are generated from endogeneous hairpin-shaped transcripts mediated by RNA polymerase II in general. The primary transcripts (pri-miRNAs) are usually several kilobases long and contain local stem loop structures (Lee, *et al*. 2002). In nucleus these primary transcripts are processed by the nuclear RNase III type protein Drosha to produce precursor miRNAs (pre-miRNAs) (Lee, *et al*. 2003). Drosha requires a cofactor called DiGeorge Syndrome Critical Region Gene 8 (DGRC8) in humans and Pasha in *D. melanogaster.* The association of Drosha and Pasha or DGRC8 is named microprocessor complex which is 500 kDa in *Drosophila melanogaster* and 650 kDa in humans (Han, *et al*. 2004; Denli, *et al*. 2004; Gregory, *et al*. 2004; Wang, *et al*. 2007). Following nuclear processing, pre-miRNAs are transported to cytoplasm mediated by exportin 5 (EXP5) which is a member of the nuclear transport receptor family (Lund, *et al*. 2004; Yi, *et al*. 2005; Bohnsack, *et al*. 2004). The next step is that pre-miRNAs are cleaved by Dicer, which is a higly conserved protein in almost all eukaryotic organisms, releasing about 22 nt miRNA dublexes. After then Dicer cleavage, the RNA dublex is loaded onto an Ago protein so as to generate effector complex, RISC. One strand of this RNA dublex is selected in terms of thermodynamic stabilities of strands and the selected strand called as mature miRNA remains in RISC complex while the other strand is degraded in the cytoplasm (Figure 1.1).

After incorporation into RISC complex, miRNAs direct the RNAi machinery to their target mRNAs by the formation of imperfect hybrids with 3' UTR sequences of target mRNA. By the means of this imperfect match, squence specific repression of productive translation or mRNA decay comes about (Ambros, *et al*. 2004; Bartel, *et al*. 2004; Zamore and Haley, 2005). At first, general consideration about miRNA- mediated repression mechanism is merely inhibition of target mRNA translation (Olsen and Ambros, 1999; Seggerson, *et al*. 2002). Subsequently, studies have indicated that miRNAs can also induce rapid decay of target mRNAs (Bagga, *et al*. 2005; Lim, *et al*. 2005; Behm-Ansmant, *et al*. 2006; Giraldez, *et al*. 2006; Wu, *et al*. 2006; Eulalio *et al*. 2007). A recent report about this issue shows that destabilization of target mRNAs is the predominant reason for reduced protein output (Guo, *et al*. 2010). Thus, there are at least two general modes of miRNA-mediated downregulation of targets in metazoan cells: miRNA-mediated translational repression and miRNA-mediated RNA decay. miRNA-mediated translational repression

includes inhibition of initiation of translation resulting in prevention of ribosome association with target mRNA and inhibition of translation post-initiation resulting in premature ribosome drop off, slowed or stalled elongation and co-tranlational protein degredation (Notrott, *et al.* 2006; Petersen, *et al.* 2006; Humphreys, *et al.* 2005). Run-off of ribosomes results in aggregation of the repressed ribosome-free mRNA into P-bodies for either storage or degradation (Bhattacharyya, *et al.* 2006; Pillai, *et al.* 2005; Liu, *et al.* 2005). miRNA-mediated RNA decay includes de-adenylation and de-capping of target mRNAs bringing about destabilization of target mRNAs (Behm-Ansmant, *et al.* 2006; Giraldez, *et al.* 2006; Wu, *et al.* 2006.).

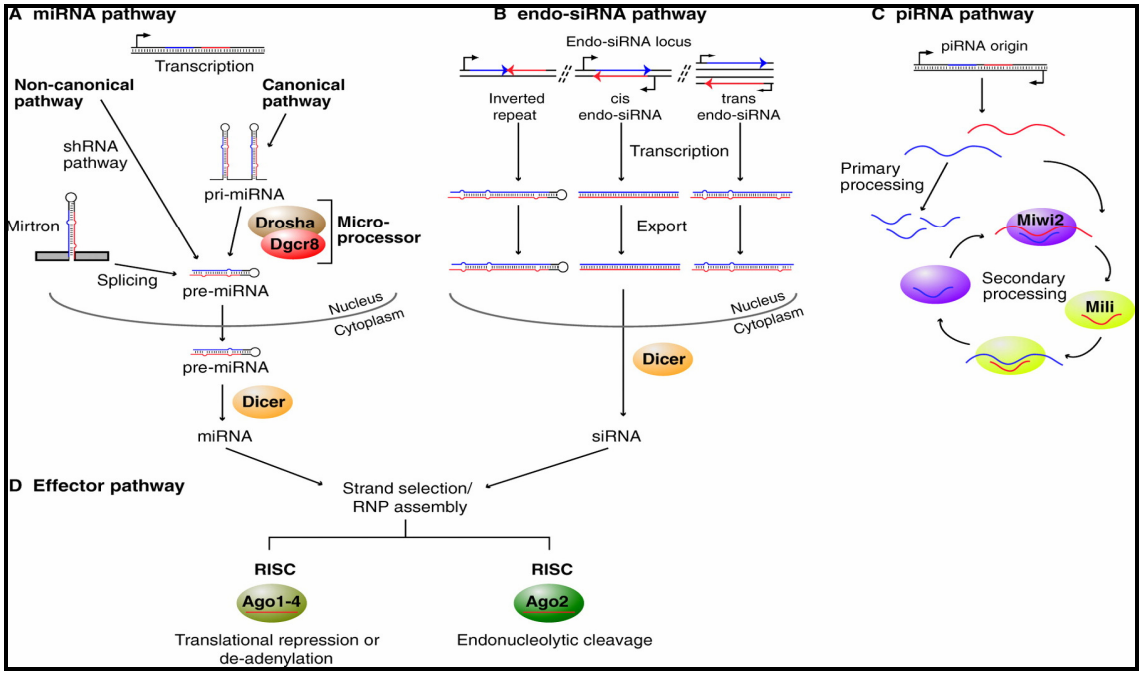

Figure 1.1. Biogenesis of small RNAs
(Source: Suh and Bleloch, 2011)

1.3. Small Interfering RNAs

The second small RNAs group is small interfering RNAs which are 21 bp in length and have perfect match with their targets. They can be classified into two groups as endogenous small interfering RNAs and exogenous interfering RNAs based on their origin.

Exo-siRNAs are derived from experimentally introduced dsRNAs or viral RNAs by cellular transfections, microinjections, feeding, or from genetic material of invading viruses and can be processed from these precursor RNAs (Fire, *et al.* 1998; Timons and Fire, 1998; Elbashir, *et al.* 2001; Wilkins, *et al.* 2005).

Endogenous small interfering RNAs are originated from transposon transcripts, sense- antisense transcripts pair and long stem-loop structures (Figure 1.1), (Okamura, *et al*. 2008; Kawamura, *et al.* 2008). The processing of endo-siRNAs is dependent on Dicer 2 rather than Dicer 1(Ruby, *et al.* 2006.) Also, endo-siRNAs are derived form long stem-loop based on Laquacious (LOQS) which functions in miRNA biogenesis (Forstemann, *et al.* 2005 and Saito, *et al.* 2005). esiRNAs are loaded onto AGO1-4 in order to acquire the ability of transcriptional repression, translational block, or mRNA cleavage. 21 nt in length endo-siRNAs binds their target with perfect base pairing. Therefore, they induce clearly degredation of their target mRNAs (Czech, *et al*. 2008).

1.4. Piwi-interacting RNAs

Piwi-interacting RNAs responsible for transposon silencing and regulating during embryonic development are derived from intergenic repetitive elements, transposons or large piRNA clusters (Aravin, *et al*. 2001; Aravin, *et al.* 2003). They are processed form single-stranded RNA precursor and not dependent on either Drosha or Dicer. Processing based on nuclease activity is carried out by Piwi proteins (Cox, *et al.* 1998). piRNA biogenesis contains primary and secondary processing mechanisms (Figure 1.1). Because Piwi proteins which are MIWI and MILI are localized in the cytoplasm, primary process and loading occur in there (Aravin, *et al*. 2006). Factors involving in primary process are not known yet. The secondary process is 5' processing of precursor by MILI and MIWI2. The nuclease responsible for 3' processing of piRNAs is also not known (Brennecke, *et al*. 2007; Gunawardane, *et al*. 2007).

1.5. tRNA-derived Small RNAs

tRNAs are basic and fundamental component of translation machinery and until a decade, they were considered as quite stable RNA structures. Over the last decade, through improvement of techniques and instruments regarding cloning and sequencing, in various organisms, it has been noticed and revealed that tRNAs are the source of some fragments 17-55 bp in length originating from different parts of tRNAs in the cell (Thompson, *et al*. 2008; Hsieh, *et al*. 2009; Cole, *et al*. 2009).

In microbes, *Streptomyces coelicolor, Aspergillus fumigatus, Giardia lamblia, Tetrahymena thermophila, Saccharomyces cerevisiae* have 30–35, 36–39, 44–49, 33–42, and 35–50 nt in length respectively. tRNA derived small RNA originated from anticodon loops of tRNAs under starvation and oxidative stress (Haiser, *et al*. 2008; Jochl, *et al*. 2008; Li, *et al*. 2008; Lee, *et al*. 2005; Thompson, *et al*. 2008; Thompson, *et al*. 2009). In *Drosophila melanogaster,* 16-26 nt in length tRNA derived fragments were identified without positions originated from in which parts of tRNAs during developmental process (Aravin, *et al*. 2003). In plants, *Cucurbita maxima* (pumpkin) has 31-68 nt in length tRNA derived small RNA originated from anticodon and D loop of tRNAs especially in phloem sap tissue (Zhang, *et al*. 2009). Studies in *Arabidopsis thaliana* have revealed that under oxidative stress conditions, 48-55 nt in lenght tRNA derived small RNAs are produced position in anticodon loop (Thompson, *et al* 2008). Under phosphate starvation in *Arabidopsis thaliana* 30-40 nt in length small RNAs are produced from D and anticodon loop of tRNAs in root tissues (Hsieh, *et al*. 2009). In human data, tRFs have been reported 35-45 nt in length under oxidative stress, nutrition deficiency heat shock, hypothermia, hypoxia, UV irridation originated from anticodon. Also, in HeLa and proliferating cancer cell line 17-26 nt in length derived from D and T loop and 3' end of tRNA precursor tRFs have been identified (Thompson, *et al*. 2008; Fu, *et al*. 2009; Yamasaki, *et al*. 2009; Cole, *et al*. 2009; Lee, *et al.* 2009; Haussecker, *et al*. 2010).

Up to now, findings about biogenesis of tRFs are based on two different patterns. One of them is the biogenesis of type I tRFs which require Dicer activity but not Drosha activity (Figure 1.2) (Cole, *et al*. 2009; Haussecker, *et al*. 2010). Second of them is type II tRFs for which biogenesis process is based on RNase Z nuclease family activity depending on RNA polymerase III termination for maturation of 5' and 3' ends respectively. (Figure 1.2) (Haussecker, *et al*. 2010). Also Rny I and angiogenin are 2 nucleases responsible for

the cleavage of tRNA molecules in yeast and mammalian cells respectively. (Thompson, *et al*. 2009 and Fu, *et al*. 2009.). In order to define which RNAi related proteins are associated with tRFs, it has been demonstrated because 3' terminal ribose of tRNA derived fragments are modified, tRNA fragments are not efficiently incorporated into Ago complexes. (Cole, *et al*. 2009). But, in immunoprecipitation experiments, tRNA derived small RNAs have relative preference for Argonaute 3 and 4 association. (Haussecker, *et al*. 2010).

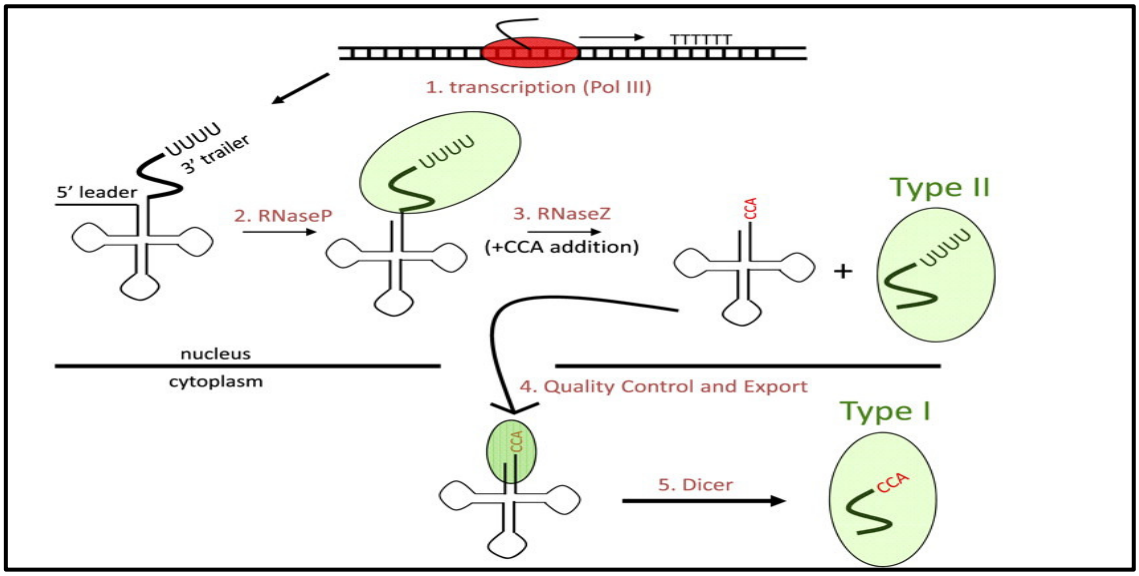

Figure 1.2. Model for tRFs Biogenesis: RNA polymerase III (Pol III) generates a precursor tRNA (*1*). The 5′ leader and 3′ trailers are removed by RNaseP (*2*) and Z (*3*), respectively. The mature tRNA is then exported into the cytoplasm (*4*). There, Dicer recognizes some, potentially misfolded tRNAs to produce Type I tsRNAs (*5*). The small RNA produced by nuclear RNaseZ cleavage and Pol III termination is a Type II tsRNA. Based on the near-exclusive cytoplasmic localization of type II tsRNAs, it is possible that a cytoplasmic pool of RNaseZ is responsible for the processing into type II tsRNAs of immature tRNAs have evaded nuclear quality control (Source : Haussecker *et al*. 2010).

The potential roles of newly discovered tRFs in gene regulation are largely unknown. First group of tRFs identified during stress conditions called stress induced tRNAs have several potential mechanisms by which cleaved or nicked tRNAs might inhibit mRNA function. For example, nicked tRNAs might activate a stress response or stall elongation by interacting with the translation machinery. tRNA fragments could interact either with an unknown general repression complex or with known complexes such as Argonaute or Piwi

to inhibit translation. tRNA fragments may also direct cleavage of specific mRNAs in conjunction with tRNA processing or Argonaute complexes (Figure 1.3).

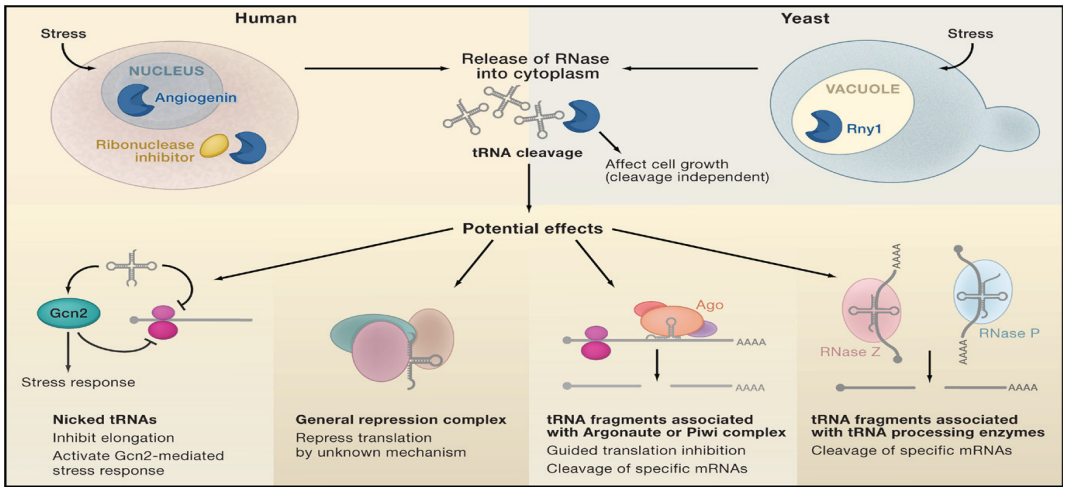

Figure 1.3. Stress induced tRNA cleavage and potential mechanisms
(Source: Thompson and Parker, 2009)

Due to the involment of Dicer in tRF biogenesis, it may be speculated that tRFs could compete with pre-miRNAs for Dicer. By this way, they affect the modulating of the miRNA homeostasis. (Cole, *et al*. 2009). Although there is no any direct link related with abundance of miRNAs and tRFs levels, tRFs were also proposed to modulate the silencing activities of microRNAs and siRNAs (Haussecker, *et al*., 2010).

The most known tRFs in function is tRF-1001 which was shown to be essential for cell proliferation in human prostate cancer cell line (Figure 1.4). Biogenesis of tRF-1001, and most likely all short RNAs of the tRF-1 series, is catalyzed by ELAC2, which had been identified originally as a candidate prostate cancer susceptibility gene (Tavtigian, *et al*. 2001) and was revealed later as an endonuclease for 3' trimming of pre-tRNA (Takaku, *et al*. 2003). tRF-1001 as well as its pre-tRNA was detected exclusively in the cytoplasm. It is believed that 5' and 3' trimming of pre-tRNA to produce the mature tRNA occurs in the nucleus. pre-tRNA transcripts from a given tRNA gene may have two fates: rapid cleavage in the nucleus for biogenesis of mature tRNA, or export to the cytoplasm for generation of tRF-1 series of short RNA. The pretRNA for tRF-1001 was not detected in the nucleus, possibly because processing to mature tRNA or export to the cytoplasm is too fast to detect a steady-state level of nuclear pre-tRNA. In contrast, the cytoplasmically exported pre-tRNA that serves to generate tRF-1001 is more stable and can be detected easily. A

cytoplasmic location of the tRF-1001 precursor pre-tRNA is consistent with its susceptibility to siRNA, since the siRNA machinery is believed to poorly target nuclear RNAs. Interestingly, ELAC2, the enzyme that trims the pretRNA to generate tRF-1001, is localized predominantly in the cytoplasm (Korver, *et al*. 2003).

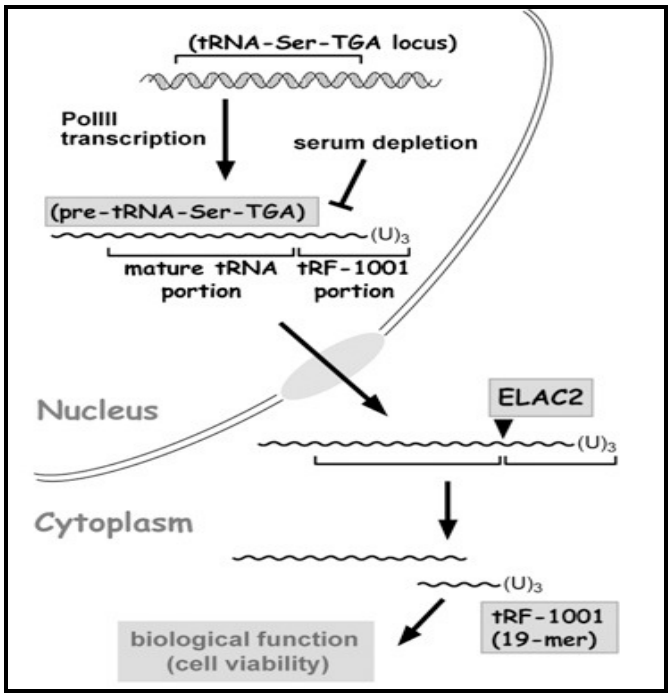

Figure1.4. A model for biogenesis and regulation of tRF-1001
(Source : Lee, *et al* .2009)

1.6. Aims of the Study

In this study, it was aimed to investigate tRNA-derived from small RNAs regulating gene expression at the level of translation in 1 hour and 8 hours *Drosophila* embryos. Also it was aimed to confirm Deep sequence results obtained from fractionated total RNA of 1 hour and 8 hours embryos tRFs by in-vitro and in-vivo experiments. Finally it was aimed to compare these tRFs and other tRNA derived fragments documented in literature.

CHAPTER 2

MATERIAL AND METHODS

2.1. *Drosophila melanogaster* Maintenance and Embryo Collection

Drosophila melanogaster lines were grown at 25ºC on yeast-sucrose-agar medium prepared as follows. 30 gram agar and 60 gram yeast were dissolved and boiled in 1,8 L dH2O and also in different cage, 110 gram corn meal and 96 gram sucrose were dissolved and mixed in 1,5 L dH2O. Then sucrose and corn meal mixture were added into boiling yeast-agar mixture. The total mixture was boiled for 20 minutes and after then cooling 25 ml propionic acid (Merc) and 25 ml nipagyn were added to the mixture to prevent mold growing. Medium was changed every two weeks. When *Drosophila* population reached sufficient density, the whole population was transferred to a common and large cage. Embryo collection plates were prepared as follows. 22.5 g agar was boiled in 700 mL dH2O, 94 g sucrose was dissolved in 150mL dH2O and mixed with 330 mL fruit juice. 0-1h embryos was collected by placing embryo collection plates in a large cage and waiting for an hour. 8 hour embryos were collected by incubating the plate in an incubator at 25ºC for 7 hours after the plate incubated for one hour in the large *Drosophila* case. Embryos were immediately washed with 0,7% NaCl and 0.1% Triton-X and then stored at -80 ºC until use.

2.2. *Drosophila* Schneider 2 Embryonic Stem Cell Culture

2.2.1. *Drosophila* Schneider 2 Embryonic Stem Cell Maintenance

Drosophila Schneider 2 embryonic stem cells are maintained in *Drosophila* Schneider medium (Invitrogen) supplemented with L-Glutamine and 10% FBS and (GIBCO), 2% penicillin-streptomycin (Biochrom AG) at 25ºC without CO_2. Passages of cells were performed twice a week.

2.2.2. RNA Transfection

$5x10^6$ S2 cells were seeded in each well of a 6-well plate and incubated in complete medium containing 10% FBS and 2% penicillin-streptomycin 24 hours before transfection. Calcium-Phosphate Transfection Kit (Invitrogen) was used to transfect the tRNA fragments. Two reactions were prepared in two different micro centrifuge tubes. The first reaction comprised 18 µl 2 M CaCl2 and 2 µg biotin labelled tRNA fragments in 150 µl steril water. The second one comprised 150 µl HBS (Hepes Buffered Saline). Using a pasteur pipette, first solution was slowly added dropwise to the second solution while bubbling air through second solution with another pipette. This is a slow process which was completed over 1 or 2 minutes. Then, the mixture was incubated for 30 minutes and dropped on the cells gently. The medium was replaced with fresh medium 24 hours post transfection.

2.2.3. Stress Induction

In order to induce stress-mediated tRNA fragmentation, S2 cells were exposed to oxidative and heat stress conditions. Oxidative stress was applied by incubating cells in a medium containing 1%, 10% and 20% hydrogen peroxide for 2 hours and heat shock stress condition was applied to cells by incubating cells at 37 ºC and 42 ºC for 2 and 4 hours.

2.4. RNA Isolation

2.4.1. RNA Isolation by Sucrose Density Gradient Fractionation System

2.4.1.1. Sucrose Gradient Preparation

Sucrose gradients were prepared by a combination of 5% and 70% sucrose solutions including 100mM NaCl2, 10mM MgCl2, 30mM Tris-HCl (pH 7), 200U Superase RNase Inhibitor (Ambion) via Density Gradient Fractionation System Gradient Making Program (ISCO) in a polyallomer tube (Beckman). At the end of the procedure, the

density of sucrose through gradient decreases from the bottom (70%) to top (5%) of the polyallomer tube. Gradients were kept at +4 C for overnight.

2.4.1.2. RNA Fractionation

0,2 gram *Drosophila* embryo or $50x10^6$ S2 cells were homogenized in lysis buffer containing 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). After 8 minutes incubation on ice, homogenates were centrifuged at 12.000xg for 8 min at 4ºC in order to eliminate cell nuclei and debris. 2 ml of supernatant including RNAs were loaded on to top of the sucrose gradients and were centrifuged at 27.000 rpm for 2h 55 minutes at 4ºC in a Beckman SW28 rotor. Gradients including RNAs, genomic DNA and proteins were fractionated by ISCO Density Gradient Fractionation System at 254 nm absorbance. Fractions were collected based on their A_{254} readings as four different subgroups presenting translation levels which are mRNP, 40S, monosome, and polysome. Fractions were made to 150mM NaCI2 and 0,5% SDS (Applichem).

2.4.1.3. Phenol-Chloroform Extraction and Ethanol Precipitation

Total RNA was extracted with an equal volume of phenol-chloroform-isoamylalcohol 25:24:1 (pH: 4-5 Applichem). The mixture was vortexed for 1,5 minutes and centrifuged at 3000 RPM for 5 minutes at room temperature. The clear upper phase was transfered into a fresh falcon tube and an equal volume of phenol-chloroform-isoamylalcohol 25:24:1 was added and the previous step was repeated. The upper aqueus phase was transferred into fresh tube then equal volume of chloform (Merck) was added and vortexed 1 minute and centrifuged at same condition for once to eliminate phenol. After picking up aqueous phase including only RNAs, 1/10 volume of 3M NaOAC (ph: 7) and two volumes of 100% ethanol were added into this aqueous phase and incubated at -20ºC overnight. Next day, 100% and 70% ethanol precipitation were applied to RNA in clear tubes of Beckman adaptable for SW28 rotor of Beckman Ultracentrifuge. RNA was pelleted at 12.000xg for 20 min at 4ºC. Pellet was dissolved in 50µL DEPC treated water.

Concentration (260/280) potential contamination of proteins and other organic molecules (260/230) were measured by Nanodrop ND UV-Vis Spectrophotometer.

2.4.2. RNA Isolation By Trizol Reagent

After 48 hours post- transfection, cells were harvested and rinsed with PBS two times. The pelleted cells were lysed with 1 ml of Trizol by repetitive pipeting. The homogenized cells were incubated for 5 minutes at room temperature to permit the complete dissociation of nucleoprotein complexes. Then, 0,2 ml chloroform was added to lysate and shaken vigorously by hand for 15 seconds and mixture was incubated at room temperature for 2 to 3 minutes. Following centrifugation at 12000xg for 15 minutes at 4 ºC, the mixture separated into a lower red, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. The aqueous phase including RNA was transferred into a fresh tube and 0,5 ml isopropyl alcohol was added and incubated for 10 minutes at room temperature. RNA was precipitated at 12000xg for 10 minutes at 4 ºC. After centrifugation, the pellet was washed with 1 ml 75% RNase free, dried for 20 minutes at room temperature and dissolved in 50 µl RNase free water. The concentration of RNA was measured by Nanodrop ND UV-Vis Spectrophotometer.

2.4.3. Small RNA Isolation

The total RNA isolated from mRNP, 40S, monosome and polysome fractions were further purified by a mirVANA miRNA isolation kit (Ambion) to remove RNAs longer than 200nt. 5 volumes of lysis/binding buffer and then 1/10 volume of miRNA homogenate additive were added to 100µg RNA and incubated for 10 min on ice. After 1/3 volume of 100% ethanol was added into the RNA mixture, 700 µL was applied to a filter 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. The mixture was applied onto a new filter and centrifuged at 5000xg for 1. 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 12 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 were measured by Nanodrop ND UV-Vis Spectrophotometer.

2.5. RNA Quality Control

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.

2.6. Small RNA Deep Sequencing Analysis

Fractionated total RNAs obtained from mRNP, 60S, monosome and polysome fractions and total RNA itself of 0-1 hour and 8 hours embryos were pyrosequenced for small RNAs using Illumina Genome Analyzer (Fasteris, Switzerland). 0-1 hour and 8 hour, fractionated and total small RNA profiles were compared and contrasted by bioinformatic tools.

2.7. Northern Blotting

2.7.1. *In Vitro* Transcription

6 µl from 100 µM commercial linear sense and anti-sense DNA primers containing T7 polymerase promoter were mixed and incubated at 95 ºC for 5 minutes in order to form double stranded template for *in-vitro* transcription. Then, 1 µl of ATP (10 mM),CTP (10 mM), GTP (10 mM) (Ambion Maxiscript *In-vitro* Transcription kit) and biotin-labeled UTP (10 mM) (Epicentre) and 2 µl RNA polymerase (Ambion) and 2 µl 10X transcription buffer (Ambion) were added to DNA template and mixture was incubated at 37 ºC for 1 hour. RNase free DNase was added to the mixture to eliminate the DNA template and incubated at 37 ºC for 15 minutes.

2.7.2. Denaturing Polyacrylamide Gel Electrophoresis

In order to visualize the biotin-labeled tRNA fragments or non-transfected naturally produced tRNA fragments in S2 cells, the total RNA isolated from S2 cells was run on 12% polyacrylamide gel containing 8M urea [10X TBE (0.9M Tris base, 0.9M Boric acid, 20mM EDTA pH 7), 40% Acrylamide (19:1) (Applichem)]. To prepare 90 ml denaturing PAGE , 43,2 gram urea (Ambion), 9 ml 10X TBE and 27 ml %40 Acrylamide-Bisacrylamide (19:1) were mixtured and the volume was completed to 90 mL with DEPC-treated water. The mixture was heated and stirred until urea completely dissolved. 320µL APS (Applichem), and 43 µL TEMED (Applichem) were added to 40 ml gel mixture and the mix was immediately poured into the glass plate set. After polymerization, the gel was placed into the tank and pre-run at 300V for 15min. RNA samples were mixed with an equal volume of 1X gel loading buffer (Ambion) and heated at 95ºC for 5 minutes. Samples were then kept on ice. Running was performed at 350 V for 3 hour.

2.7.3. RNA Blotting

In order to transfer RNAs separated on denaturing gel to positively charged Nylon membrane (Ambion Brightstar-Plus), Thermo OWL HEP1 semidry electroblotter system was used. The gel piece of interest was cut and sandwiched in between Whatman 3M Chromatography papers. The membrane and 3M paper were wet by 1X TBE. The transfer was performed at 400 mA for 1 hour. The transferred RNAs were cross-linked by incubating the filter at 80 ºC for 20 minutes.

2.7.4. RNA Imaging

The membrane cross-linked with RNA was pre-hybridized in 25 ml hybridization buffer at 37 ºC for at least 30 minutes in a hybridization oven (If biotin-labeled RNA was used, pre-hybridization and hybridization step were skipped). Then 7 µl probe synthesized by *in vitro* transcription was diluted in 10 ml hybridization buffer and dropped on membrane and incubated for overnight in the hybridization oven at 37ºC after discarding the pre-hybridization buffer. About 12-24 hour later, the membrane was washed with

~50ml pre-warmed SSPE wash buffer (2X SSPE, 0.1% SDS) for three times each for 10 minutes. The membrane was then washed twice in 50 ml washing buffer for 5 min. To prevent non-specific binding, membrane was incubated twice with blocking buffer for 5 minutes and once for 30 minutes. 1 µl Streptavidin-conjugated HRP (0,5 µg /ml) was diluted in 10 ml blocking buffer and incubated with the membrane for 30 minutes followed by incubation in 50 ml blocking buffer to remove the excess secondary antibody. Following three rounds of washes in 50 ml washing buffer for 10 minutes, the membrane was incubated with substrate (Thermo luminol enhancer) and reaction buffer (Thermo peroxide buffer). Before addition of components, the membrane was incubated with 1X Assay buffer for 2 minutes. Having added the substrate and buffer of HRP, the membrane was incubated at dark for 5 minutes. At the end of the reaction, the chemical light was measured by chemiluminescence camera of Versa-Doc Imaging System (3X3 gain, 4X4 bin) (Biorad).

2.8. Gel Shift Assay (Electromobilitic Shift Assay)

In order to determine the proteins associated with tRFs under *in-vitro* conditions, electromobilitic shift assay was performed. EMSA consisted of four steps, which were isolation of cytoplasmic total protein by lysis of S2 cells, incubation proteins with biotin labeled tRNA fragments in EMSA reaction buffer, running non denaturing gel and imaging or staining protein and RNA complex. 10 million S2 cells were lysed with 100 µl lysis buffer containing 0,5% triton, 50mM Hepes, 150 mM NaCl, 50mM Tris, phosphatase inhibitor and protease inhibitor 1 vial (Pierce), 10% Glycerol. Cell membranes, organels, nucleus and debris were eliminated by centrifugation at 13000 g at 4 ºC for 10 minutes. 5µg protein was incubated with 20nM biotin labeled tRNA fragments (26 bp) in EMSA buffer containing 10 nM Tric-HCl (pH:8), 50 mM KCl, 1mM EDTA,(pH:8) , 5 mM $MgCl_2$, 5 mM DTT in 100 µL total reaction volume for 30 minutes. Unlabelled tRNA fragments were used as specific competitors. 20 µl of total reaction in 50% glycerol was loaded on 8% native (non-denaturing) gel. At 250V for 2 h , gel was run and transferred to membrane and visualized tRNA pieces and associated proteins as explained in the sections 2.7.3 and 2.7.4 on page 16.

2.9. *In Vivo* Croslinking

S2 cells were plated in 6 well plate and transfected with biotin labeled tRNA fragments as explained in the sections 2.2.1 and 2.2.2. After 48 hours, cells were rinsed with 1X PBS two times and $5x10^6$ cells in 3 mL 1X PBS were exposed to 86 mJ/cm^2 UV light in UV box for 10 minutes. Then cells were lysed in lysis buffer (0,5% triton, 50mM Hepes, 150 mM NaCl, 50mM Tris, Phosphatase inhibitor and protease inhibitor 1 vial (Pierce), 10% Glycerol) and 10 µg total protein was run in 8% native PAGE gel as explained in the EMSA procedure.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Polysome Profile of 0-1Hour and 8 Hours Embryos

By Polysome profiling, cytoplasmic mRNP complexes can be fractionated based on their translational status such as mRNP, 60S, monosome and polysome. There is no ribosomal subunits. 40S or 60S status represent that mRNPs bounded with small ribosomal subunits in the mRNP fractions. At monosome or 80S levels, messenger RNAs are bounded with only one full ribosome. Polysomes contains at least more than one ribosomes. Polysome profiles of 0-1 and 8 h embryos were obtained successfully with an increasing size and volume (Figure 3.1).

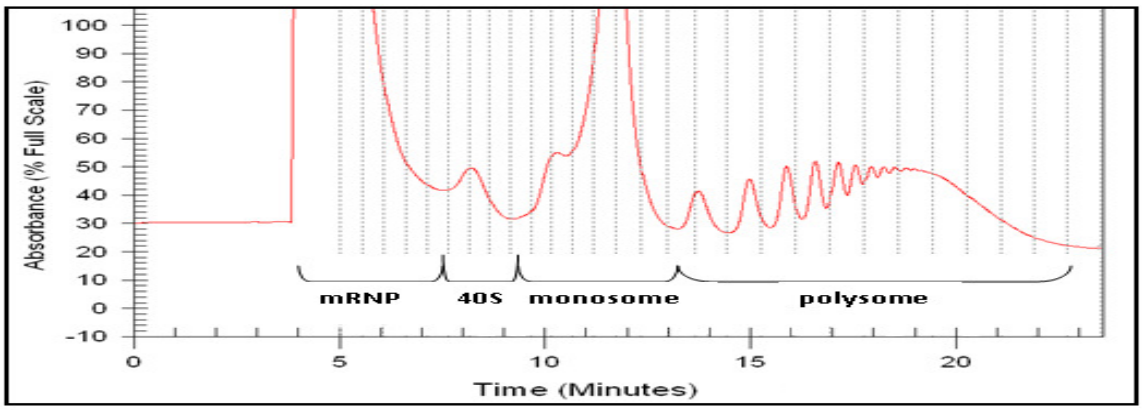

Figure 3.1.Polysome profilings of 1 and 8 hour embryos. The lysate of embryos was centrifuged and fractionated by Density Sucrose Gradient Fractionation (DSGF) system. The first highest peak represents mRNP status and second one represents 60S subunits of ribosome and in the middle of the profile, the highest peak represents monosome. Polysome is heaviest fraction relative to monosome, 60S and mRNP. Each increasing peak presents the ribosome number on mRNA and polysome volume.

3.2. RNA Quality Control by Bioanalyzer

RNA quality was measured by bioanalyzer (Agilent 2100) and reference points are sharpness and intactness of 18S and 28S peaks and flat baseline of RNA electropherogram. RNAs used for deep sequence analysis were controlled by chip base analysis to eliminate degradation products. As a result, it was seen that the total and small RNAs isolated had high quality based on sharpness of their 18S and 28S peaks (Figure 3.2).

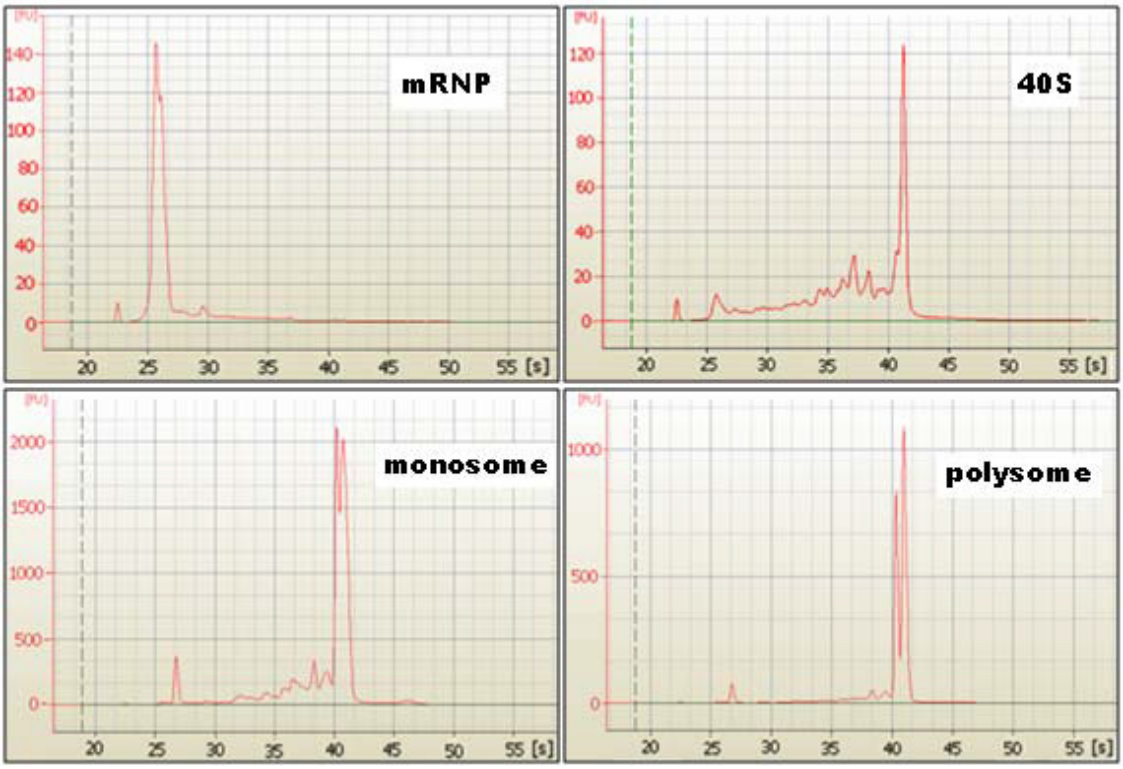

Figure 3.2. Agilent 2100 Bioanalyzer electropherograms of mRNP, 60S, monosome and polysome RNAs. Quality of fractionated RNAs depending on their translational status were measured with the .reference of 18S and 28S peaks and flat baseline.

3.3. Deep Sequence Analysis

Three replicates from unfractionated total RNAs and fractionated mRNP, 60S, monosomal and polysomal RNAs of 0-1 hour and 8 hour embryos were mixed in equal amounts and sequenced using Illumina Genome Analyzer by Fasteris (Switzerland).

The number of unique sequence in each sample ranged from 45,685 to 687,558 accounting approximately for 20,2-48,29% of total reads for each sample (Table 3.1)

Table 3.1. All unique sequences analysis in the total sequence read in each fraction.

	mRNP		60S		MONOSOME		POLYSOME		TOTAL		ALL
	1h_em	**8h_em**	**1h_em**	**8h_em**	**1h_em**	**8h_em**	**1h_em**	**8h_em**	**1h_em**	**8h_em**	
Unique seq	478116	119109	391938	396700	316214	299926	157426	45685	687588	113458	3006160
Total seq	1646432	589616	811581	1101742	838995	1071315	334176	101883	2424271	435539	9355550
%	29,039	20,201	48,293	36,006	37,689	27,9960	47,108	44,840	28,3626	26,050	32,132

The analysis of origins of the all sequences revealed that 9% of the all sequences stem from tRNAs (Figure 3.3). In addition, a great majority of these tRNA-derived small RNAs present in mRNP status of 1h and 8h embryos (Figure 3.4).

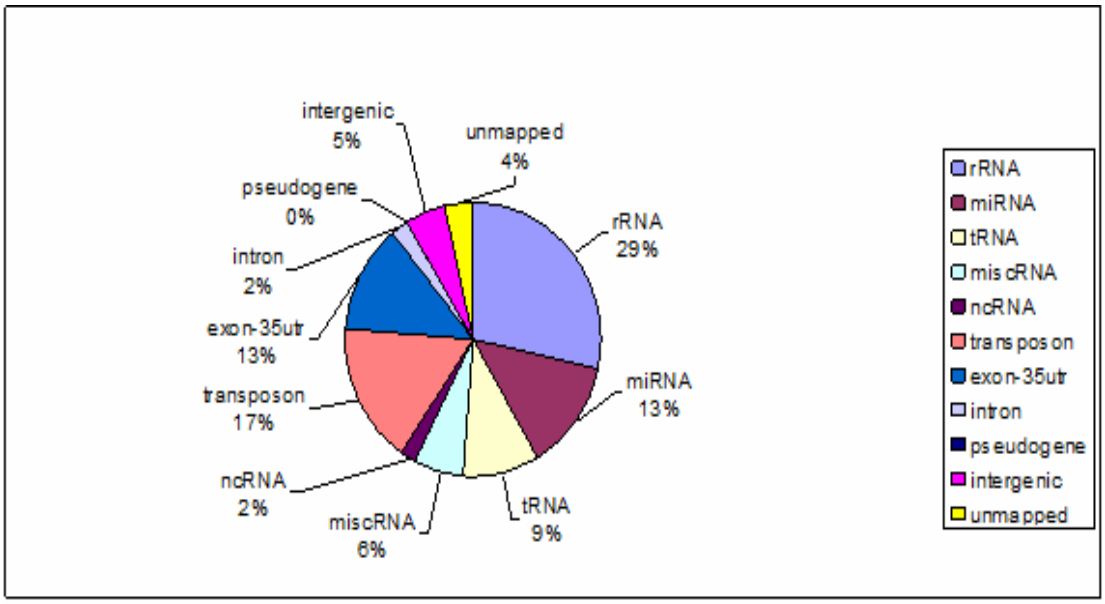

Figure 3.3. Classification of small RNAs in all sequences.

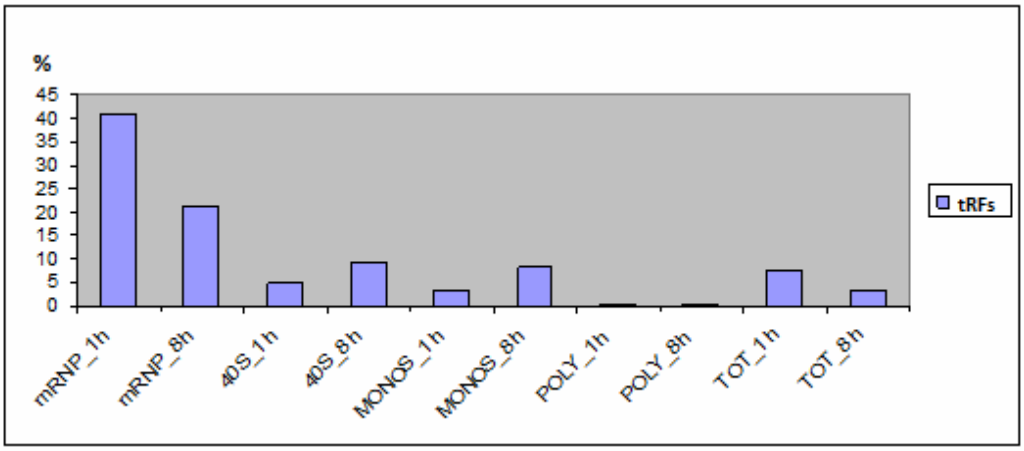

Figure 3.4. Distrubition of tRNA-derived small fragments in fractions of 1h and 8h embryos. The great majority of tRNA-derived small fragments present in mRNP status in both 1h and 8h embryos.

When all tRNA derived sequences were imposed onto a single tRNA, more than 90% of reads matched to the 5' ends of tRNAs flanking the nucleotides 1 and 26. When tRNA derived reads were blasted to individual tRNAs, some reads specifically matched to the 3' ends without any significant matches to the 5' ends (Figure 3.5).

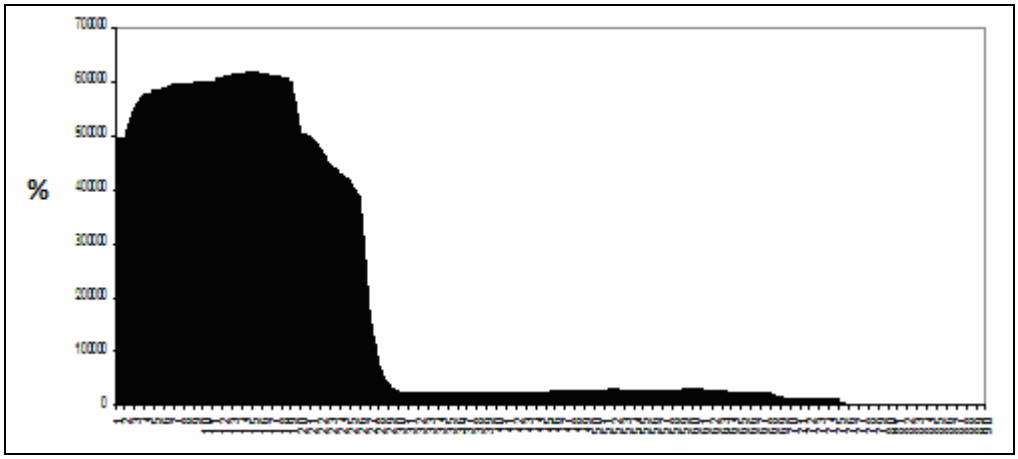

Figure 3.5. Blast results of tRNA derived sequences imposing to tRNAs. %96 of tRNA derived sequences match with 5' ends of tRNAs flanking the nucleotides 1 and 26.

In order to determine the 3' of processing site of tRFs, all tRFs were blasted to individual tRNA . The majority of 3' processing site was 19, 26 or 27 nucleotide downstream from the 5' end (Figure 3.6)

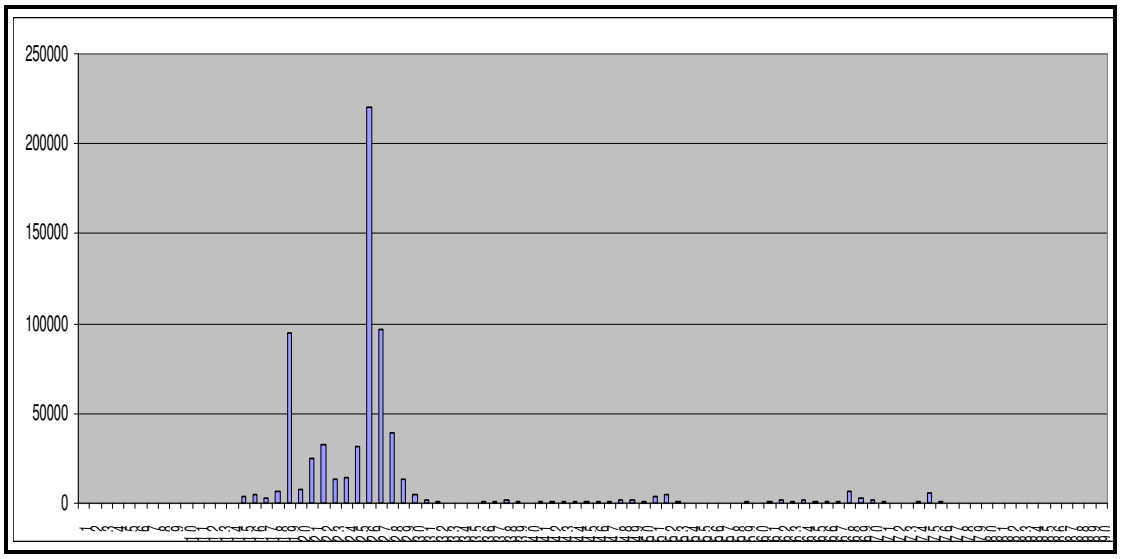

Figure 3.6. Analysis of 3' processing site of tRNA derived sequences. The 3' trimming site of tRNA derived sequences are nucleotides 26,27,19 in density respectively.

In order to determine the location of 3' processing site on folded tRNAs, 12 coordinates were assigned on folded tRNAs and aligned all tRNA-derived sequences relative to each coordinate. By means of this analysis, it was revealed that the 3' processing site of tRFs is towards the 5' stem of anticodon loop closer to the D arm (Figure 3.7)

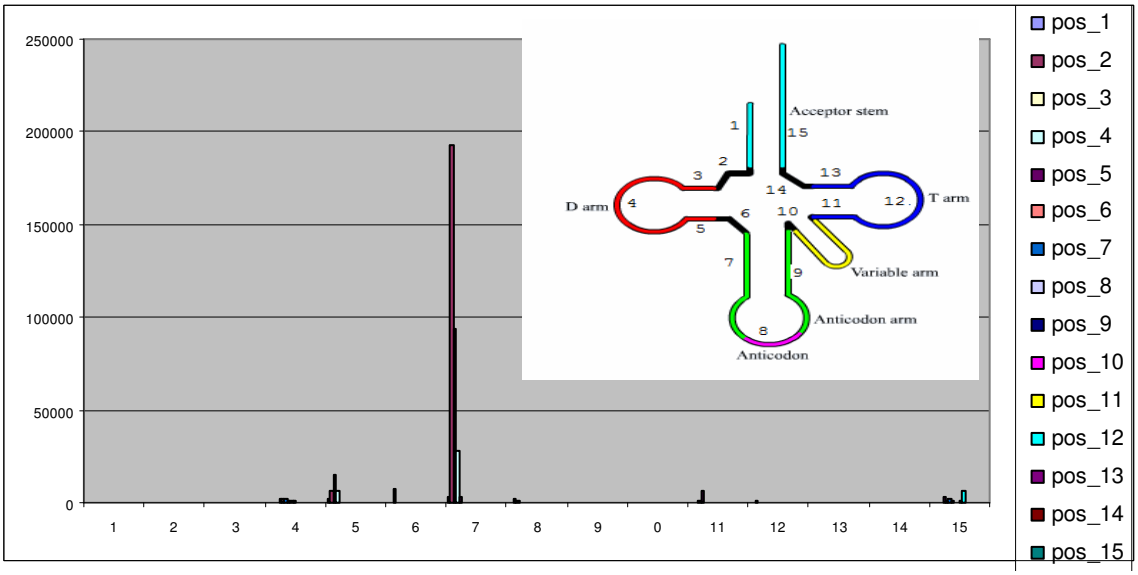

Figure 3.7. Analysis of mapping site of tRNA derived small RNAs on folding tRNA

According to blast results, although sequences have the same length distribution sequences enrichment are in length of 23 to 26 bp (Figure 3.8).

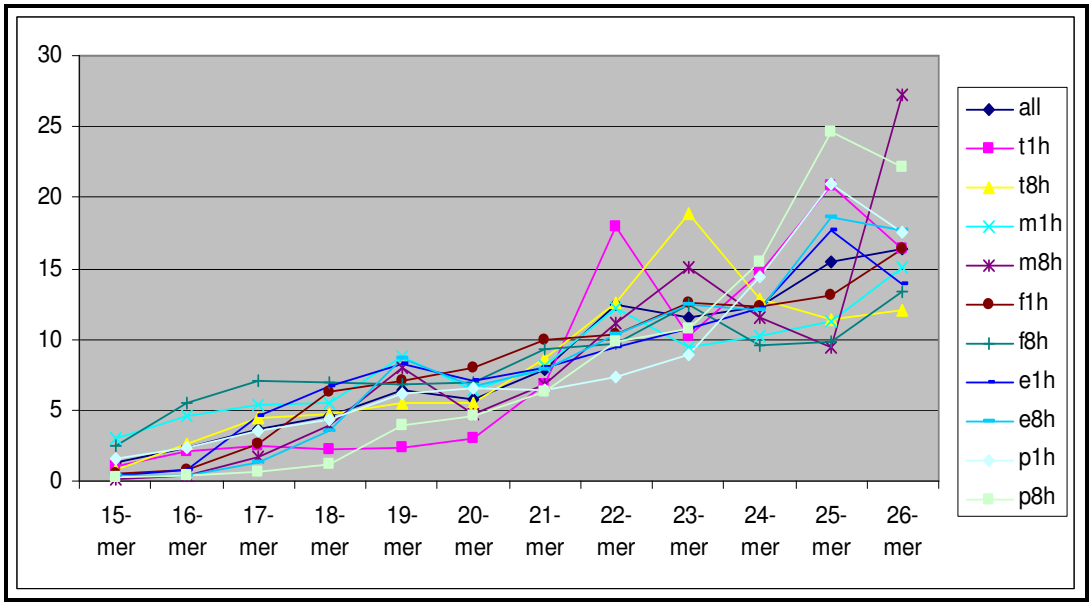

Figure 3.8. Analysis of length distrubition of all small RNA sequences.

The quantitative analysis of expression levels of tRFs in both fractionated and unfractionated 1 and 8 hour embryo total RNAs revealed several interesting points. Firstly, certain tRFs appear to be over-expressed in 1 hour embryos while some others are down-regulated. At 1h, tRFs derived from tRNA proline, histidine, valine are over expressed while at 8h, those from tRNA glycine and mitochondrial Serine are overexpressed (Table 3.2). Secondly, tRFs are generated not from all but a selective subset of tRNAs. In some case , this selectivity is originated from tRNAs carrying same aminoacid but possessing a different anticodon. For examples, tRFs derived from tRNA Proline with TGG anticodon are expressed about 10-fold more than tRFs derived from tRNA Proline with CGG anticodon at 1h embryos (Table 3.2). Also, mitochondrial tRNA seem to be processed less frequently relative to cytosolic tRNA.

Table 3.2. Cloning frequency of tRNA derived sequences in fractionated and unfractionated 1 hour and 8 hour total RNAs.

	1h					8h				
tRNA Name	mRNP	40S	MON	POLY	TOT	mRNP	40S	MON	POLY	TOTAL
tRNA:G:GCC:AE002638-a	49890	4293	1494	1188	2288	110188	9901	4320	4260	12214
tRNA:G:GCC:AE002690-c	49890	4293	1494	1188	2288	110188	9901	4320	4260	12214
tRNA:G:GCC:AE002638-a	32628	0	0	0	264	0	0	0	0	0
tRNA:G:GCC:AE002690-c	32628	0	0	0	264	0	0	0	0	0
mt:tRNA:S:AGY	26019	313	4872	473	24	31546	1372	21690	3082	1635
mt:tRNA:S:AGY	10542	26	311	66	36	1231	93	982	432	223
mt:tRNA:S:AGY	6535	9	91	9	16	90	6	120	20	25
tRNA:P:TGG:AE002708-e	5100	0	0	0	141	0	0	0	0	0
tRNA:H:GTG:AE002787-a	4839	884	418	126	17	1035	1599	224	128	62
mt:tRNA:K	3789	335	145	30	6	23986	826	341	304	106
mt:tRNA:K	3739	755	441	185	54	10651	2004	921	1865	994
tRNA:P:AGG:AE002602-c	2197	0	0	0	440	0	0	0	0	0
tRNA:G:TCC:AE002708-a	886	5	2	24	48	107	3	0	108	44
tRNA:P:CGG:AE002593	658	35	89	12	111	2074	52	165	79	9
tRNA:K:CTT:AE002787-a	475	22	14	6	75	690	60	18	20	248
tRNA:K:TTT:AE002699-a	405	6	2	3	30	39	5	6	10	16
tRNA:S:CGA:AE002593-c	372	9	7	6	1	3	0	0	0	0
tRNA:Q:TTG:AE002602	372	9	7	6	1	3	0	0	0	0
tRNA:Q:TTG:AE002566	372	9	7	6	1	3	0	0	0	0
tRNA:Q:CTG:AE002690	372	9	7	6	1	3	0	0	0	0
tRNA:Q:CTG:AE002690	338	9	1	3	3	0	1	0	0	0
tRNA:Y:GTA:AE002708-b	256	12	14	15	0	2	1	1	0	0
tRNA:A:AGC:AE002708-f	256	6	39	1014	119	7	54	6	108	16
tRNA:A:AGC:AE002708-d	256	6	39	1014	119	7	54	6	108	16
tRNA:G:TCC:AE002575-b	250	5	0	27	7	19	1	0	20	14

(Continued on next page)

Table 3.2. (Continued)

tRNA:R:TCG:AE002593-e	213	11	12	6	4	0	0	0	0	0
tRNA:R:TCG:AE002708-b	213	11	12	6	4	0	0	0	0	0
tRNA:A:TGC:AE002787-a	202	27	38	69	223	0	555	54	0	23
tRNA:A:TGC:AE002787-b	202	27	38	69	223	0	555	54	0	23
tRNA:A:TGC:AE002787-a	180	63	44	21	24	31	38	31	10	129
mt_tRNA_A	178	0	6	0	5	12	24	0	29	14
mt:tRNA:R	149	1	7	12	6	14	8	19	0	0
tRNA:Y:GTA:AE002708-b	138	10	4	3	1	0	1	0	0	0
tRNA:Q:CTG:AE002787	138	10	4	3	1	0	1	0	0	0
tRNA:S:AGA:AE002593-a	132	32	25	3	49	87	51	32	0	177
tRNA:S:CGA:AE002593-c	132	32	25	3	49	87	51	32	0	177
mt_tRNA_H	132	6	2	0	1	34	15	12	29	7
mt_tRNA_N	126	2	0	0	1	54	11	5	0	2
tRNA:W:CCA:AE002787-a	117	9	18	0	1	0	0	0	0	2
tRNA:W:CCA:AE002638	117	9	18	0	1	0	0	0	0	2
tRNA:A:CGC:AE002575-a	108	0	0	0	0	0	0	0	0	0
tRNA:G:TCC:AE002575-b	102	0	0	0	110	2	0	0	0	0
tRNA:A:AGC:AE002708-f	80	505	438	132	616	32	219	226	147	792
tRNA:A:AGC:AE002708-d	80	505	438	132	616	32	219	226	147	792
tRNA:T:AGT:AE002787-b	77	0	0	0	19	0	5	0	0	0
tRNA:T:AGT:AE002708-b	77	0	0	0	19	0	5	0	0	0
tRNA:TΨ:AGT:AE002602	77	0	0	0	19	0	5	0	0	0
tRNA:TΨ:AGT:AE002602	74	1	4	0	5	0	0	0	0	0
tRNA:E:CTC:AE002584-e	56	46	29	15	270	7	19	28	20	276
tRNA:R:TCG:AE002681	47	4	0	0	1	0	3	0	0	2
tRNA:S:AGA:AE002593-e	44	18	5	0	0	2	5	1	0	0
tRNA:S:AGA:AE002638-a	44	18	5	0	0	2	5	1	0	0

(Continued on next page)

Table 3.2. (Continued)

tRNA:S:GCT:AE002708-c	36	1	2	0	2	0	3	0	0	0
tRNA:S:GCT:AE002708-e	36	1	2	0	2	0	3	0	0	0
tRNA:V:CAC:AE002708-c	36	0	0	0	0	0	0	0	0	0
mt:tRNA:P	34	9	17	0	0	29	20	16	20	7
tRNA:TΨ:AGT:AE002602	30	0	0	0	0	0	0	0	0	0
tRNA:R:ACG:AE002708-b	29	25	27	0	0	0	3	3	0	0
tRNA:TΨ:AGT:AE002602	21	1	1	0	0	0	6	0	0	0
tRNA:TΨ:AGT:AE002602	20	9	4	3	0	0	0	0	0	0
tRNA:S:CGA:AE002593-c	20	9	4	3	0	0	0	0	0	0
tRNA:SeC:TCA:AE002787	20	1	1	0	0	0	0	0	0	0
tRNA:S:CGA:AE002593-c	16	2	0	0	3	2	4	0	0	2
tRNA:L:CAA:AE002787-b	16	2	0	0	3	2	4	0	0	2
tRNA:M:CAT:AE002787-c	16	0	1	0	0	195	8	13	0	5
tRNA:S:CGA:AE002593-c	13	1	1	3	1	0	0	0	0	0
tRNA:S:CGA:AE002593-c	13	1	1	3	1	0	0	0	0	0
tRNA:S:CGA:AE002593-c	13	0	0	0	0	0	0	0	0	0
tRNA:S:CGA:AE002593-c	13	0	0	0	0	0	0	0	0	0
tRNA:S:CGA:AE002593-c	13	10	19	0	21	2	7	9	0	2
mt:tRNA:I	13	118	250	9	1	7	36	142	0	7
mt:tRNA:M	12	10	7	0	0	10	18	14	0	0
mt:tRNA:Y	12	10	2	9	182	3	27	12	0	0
tRNA:V:AAC:AE002602-a	10	2	1	9	26	0	6	0	0	57
mt_tRNA_L_CUN	10	292	4	3	1	5	254	3	0	0
tRNA:N:GTT:AE002769-d	10	16	5	6	0	2	2	1	0	0
tRNA:F:GAA:AE002566-a	9	79	5	6	2	2	13	3	0	11
tRNA:V:CAC:AE002708-a	7	4	4	0	0	32	10	7	0	0
tRNA:V:AAC:AE002708-a	7	1	0	3	4	0	0	0	0	2

(Continued on next page)

Table 3.2.(Continued)

tRNA:E4:53A	6	36	25	0	153	2	21	15	0	69
tRNA:E4:53A	6	36	25	0	153	2	21	15	0	69
mt:tRNA:G	5	1	1	0	0	15	23	0	0	0
tRNA:S:CGA:AE002593-c	5	2	12	0	3	0	3	8	0	0
tRNA:S:CGA:AE002593-c	4	0	0	0	1	0	0	0	0	0
tRNA:Q:TTG:AE002566	4	81	119	6	1	2	8	34	0	0
tRNA:Y:GTA:AE002620-b	4	73	7	3	7	31	97	11	0	71
tRNA:Y:GTA:AE002638-b	4	73	7	3	7	31	97	11	0	71
tRNA:Y:GTA:AE002708-b	4	73	7	3	7	31	97	11	0	71
tRNA:S:CGA:AE002593-c	4	0	0	0	0	0	0	0	0	0
tRNA:S:CGA:AE002593-c	3	1	0	0	0	0	0	0	0	0
tRNA:S:CGA:AE002593-c	3	4	1	0	0	0	0	0	0	2
tRNA:S:CGA:AE002593-c	3	0	0	0	0	0	0	0	0	0
tRNA:E4:53A	3	531	51	6	42	14	218	51	39	99
mt:tRNA:Y	3	531	51	6	42	14	218	51	39	99
tRNA:P:CGG:AE002566	2	28	253	18	5	17	16	156	0	5
tRNA:S:CGA:AE002593-c	2	0	0	0	0	0	0	0	0	0
tRNA:S:CGA:AE002593-c	2	0	0	0	0	0	0	0	0	0
mt:tRNA:Y	2	28	253	18	5	17	16	156	0	5
mt_tRNA_C	1	0	0	0	0	473	18	7	0	0
tRNA:E:TTC:AE002787-c	1	352	27	0	2	3	146	8	0	5
tRNA:S:CGA:AE002593-c	1	1	0	0	2	0	0	0	0	2
tRNA:E4:53A	1	352	27	0	2	3	146	8	0	5
tRNA:E4:53A	1	153	6	0	0	59	553	11	0	7
mt:tRNA:T	1	9	0	0	0	0	10	1	0	0
mt:tRNA:Q	1	1072	5	0	0	7	563	4	0	9
mt_tRNA_D	0	4	0	0	0	0	73	0	0	0

3.4. Northern Blotting Analysis for Fractionated Total RNA

Before transfection of biotin labeled tRNA fragments, Northern Blotting analysis of fractionated RNAs from both *Drosophila* embryos and S2 cells confirmed the data as in deep sequence results. As it can be seen in figure 3.9, tRNA derived small RNAs are found in mRNP and 60S fractions.

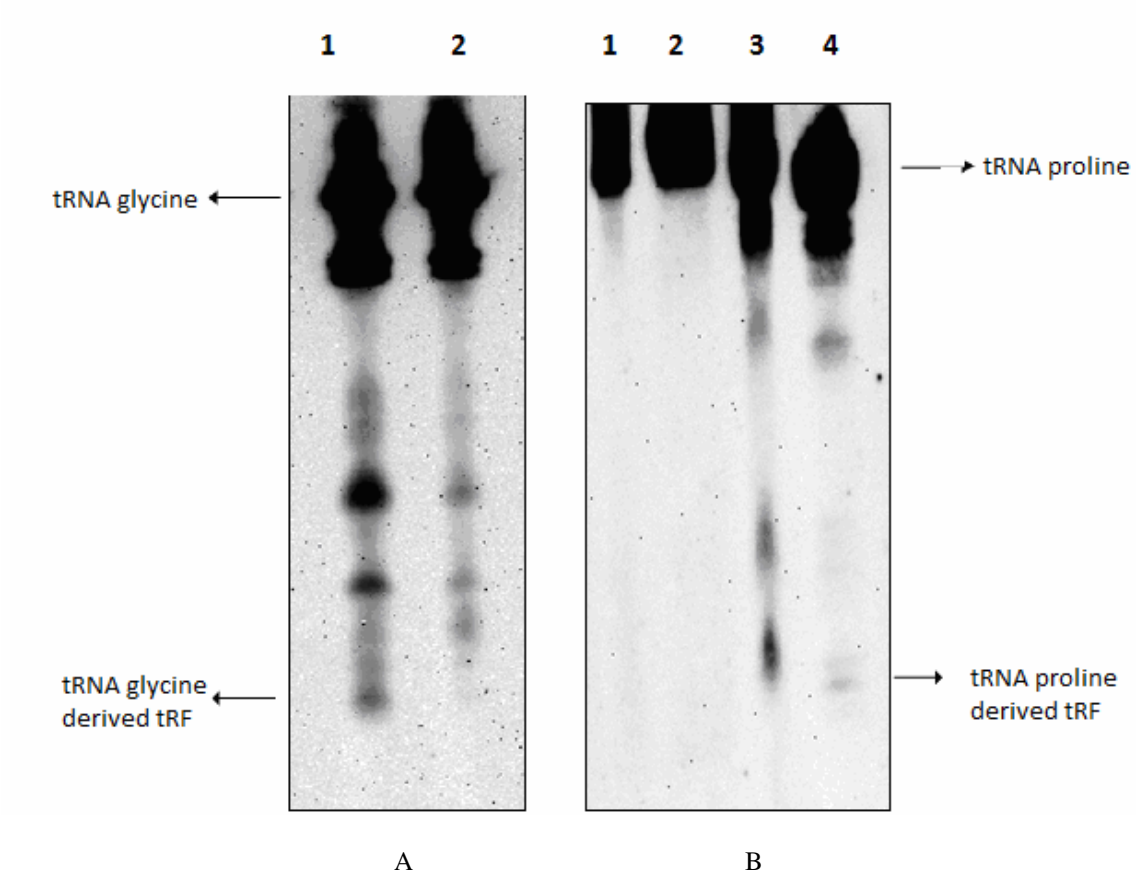

Figure 3.9. Northern Blotting Analysis of tRNA derived small RNAs in *Drosophila* embryos and S2 cells. **A)** Northern Blotting of tRNA glycine derived small RNAs in 1 hour and 8 hour *Drosophila* embryos. **1.** mRNP total RNA of 8 hour embryos (5 µg), **2.** mRNP total RNA of 1 hour embryos (5 µg). **B)** Norhern Blotting of tRNA Proline derived small RNAs in fractionated total RNA of S2 cells. **1.** Polysome total RNA (50 µg), **2.** Monosome total RNA (50 µg), **3.** 60S total RNA (27 µg), **4**. mRNP total RNA (5 µg).

3.5. Demonstration of Differences from Stress Induced tRNA-derived Small RNA

In order to reveal differences between tRFs obtained from deep sequencing analysis and stress-induced tRNA derived small RNAs in the literature, S2 cells were exposed to Hidrogen peroxide (H_2O_2) and heat shock to induce stress response resulting in tRNA degradation. Stress-induced small RNAs were produced in a range of 40-60 bp which are longer than tRNA derived small RNAs produced in *Drosophila* embryos (Figures 3.10, 3.11 and 3.12)

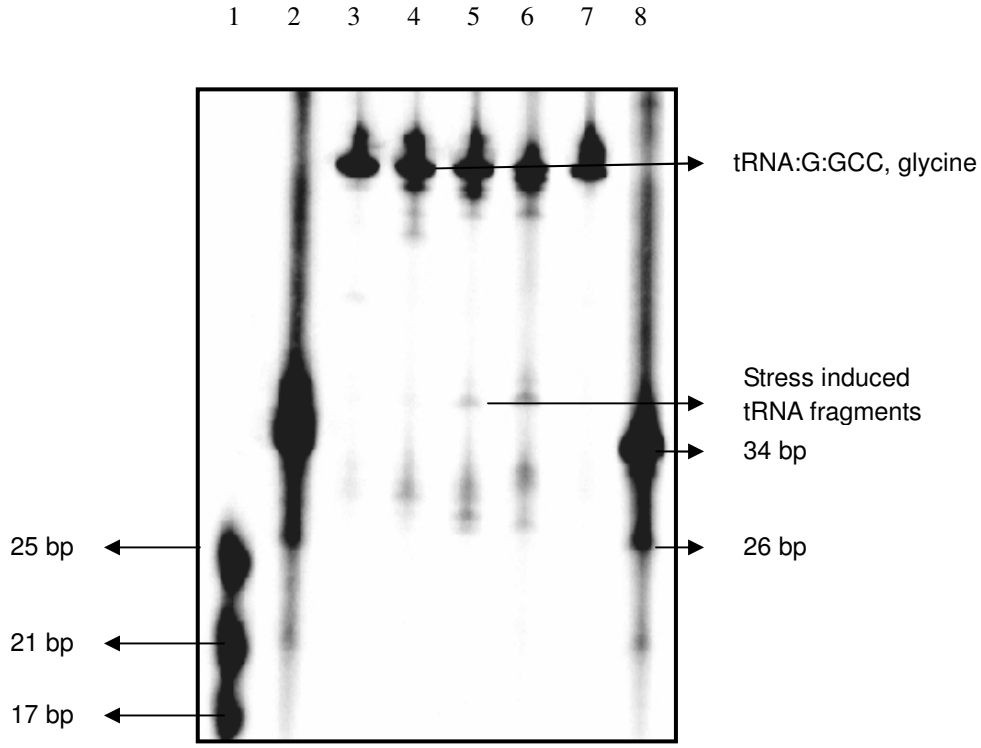

Figure 3.10. Northern analysis of stress induced tRNA Glycine fragmentation in S2 cells. **1**. MicroRNA marker, **2**. Biotin labeled tRFs as marker 26 bp (5 ng) and 34 bp (10 ng), **3**. 10 µg total RNA of S2 cells incubated with medium containing 1mM H_2O_2 for 3 hour at RT, **4**. 10 µg total RNA of S2 cells incubated with medium containing 20 mM H_2O_2 for 3 hour at RT. **5.** 10 µg total RNA of S2 cells incubated at 37ºC for 3h (heat stress), **6.** 10 µg total RNA of S2 cells incubated at 42°C for 3h (heat shock condition), **7.** 10 µg total RNA of S2 cells not exposed to any stress condition as control group, **8.** Biotin labeled tRFs as marker 26 bp(5 ng) and 34 bp (10 ng).

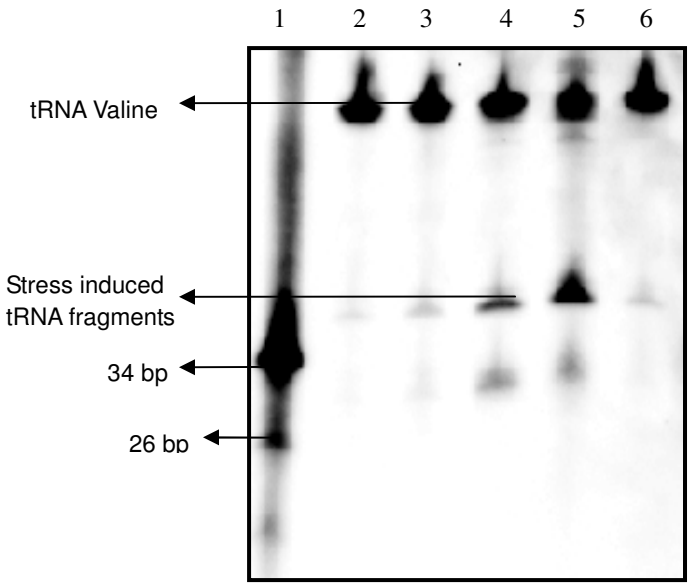

Figure 3.11. Northern analysis of stress induced tRNA Valine fragmentation in S2 cells, **1**. Biotin labeled tRFs as marker 26 bp (5 ng) and 34 bp (10 ng), **2**. 10 µg total RNA of S2 cells incubated with medium containing 1mM H_2O_2 for 3 hour at RT, **3**. 10 µg total RNA of S2 cells incubated with medium containing 20 mM H_2O_2 for 3 hour at RT. **4.** 10 µg total RNA of S2 cells incubated at 37ºC for 3h (heat stress), **5.** 10 µg total RNA of S2 cells incubated at 42ºC for 3h (heat shock condition), **6.** 10 µg total RNA of S2 cells not exposed to any stress condition as control group,

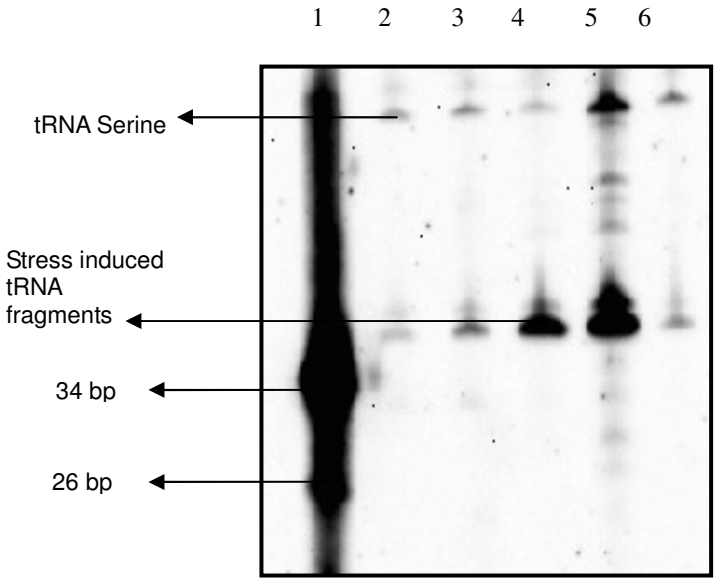

Figure 3.12. Northern analysis of stress induced mitochondrial tRNA Serine fragmentation in S2 cells, **1**. Biotin labeled tRFs as marker 26 bp (5 ng) and 34 bp (10 ng), **2**. 10 µg total RNA of S2 cells incubated with medium containing 1mM H_2O_2 for 3 hour at RT, **3**. 10 µg total RNA of S2 cells incubated with medium containing 20 mM H_2O_2 for 3 hour at RT. **4.** 10 µg total RNA of S2 cells incubated at 37ºC for 3h (heat stress), **5.** 10 µg total RNA of S2 cells incubated at 42ºC for 3h (heat shock condition), **6.** 10 µg total RNA of S2 cells not exposed to any stress condition as control group.

In the result obtained from three different tRNAs degradation induced by different stress conditions, stress induced tRNA derived small RNAs which are 40-60 bp in range are different from tRNA derived small RNAs produced at 0-1 and 8h *Drosophila* embryos.

3.6. Polysome Analysis of Transfected Biotin Labeled tRFs

In order to further verify the mRNP-association of tRFs, one of the biotin-labelled tRFs (5' of tRNA glycine) was transfected into S2 cells. The transfected tRF (5' of tRNA glycine) was co-sedimented in mRNP and 60S fractions (Figure 3.13). This was in agreement with deep sequence analysis and tRFs apparently interact with 60S of ribosomal subunit suggesting a potential involvement during pre-translational status of target mRNAs.

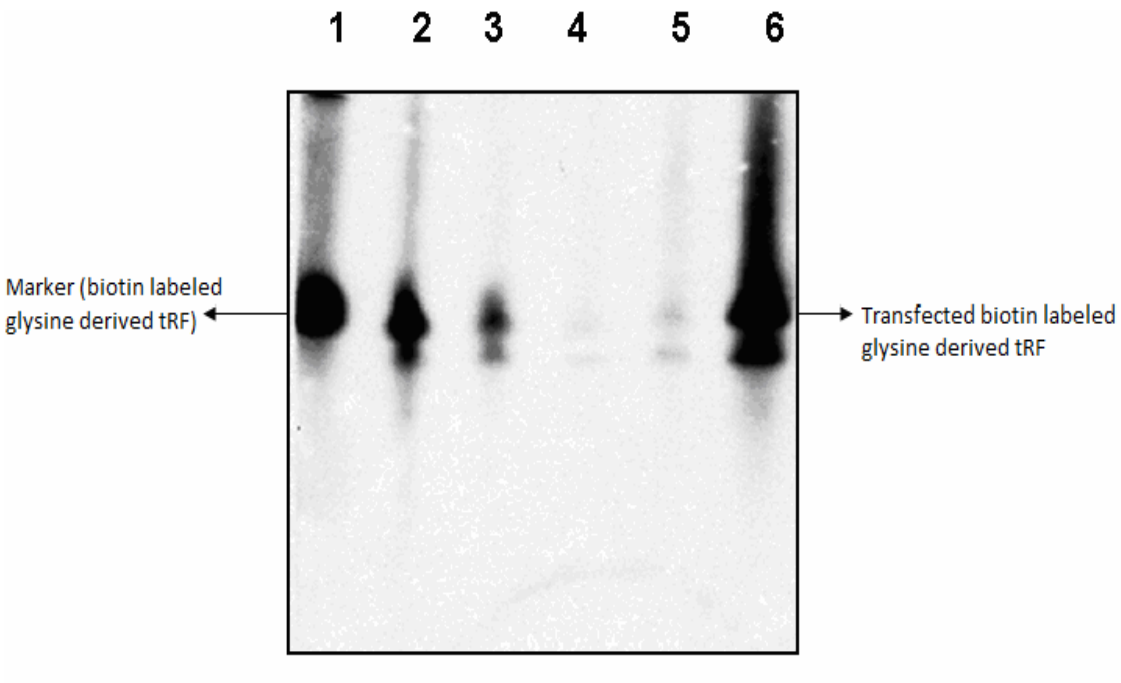

Figure 3.13. Northern Blotting of Polysome Analysis. **1.** Biotin labeled tRF itself as a marker 26 bp (10 ng), **2.** mRNP total RNA (2,5 µg). **3.** 60S total RNA(3 µg), **4.** Monosome total RNA (15 µg), **5.** Polysome total RNA (15µg), **6.** Total RNA (10 µg).

3.7. Determination of Proteins That Associate With tRFs

After determination where candidate tRF complex act as a regulator during translation, the next step was to reveal which proteins are associated with the tRF complex. Initially, biotin labeled tRF (5' of tRNA glycine) was incubated with fresh S2 cell lysate including cytoplasmic proteins under *in vitro* conditions. As a negative control 3' of tRNA glycine was also incubated with S2 cell lysate. As a specific competitor, same RNA fragments without biotin mark were used. As it can be seen in Figure 3.14, there was an association between some proteins and 5' of tRNA. An association of a complex related with the negative control 3' of tRNA was also observed. This association probably originated from different conditions in S2 cells or could be non-specific bindings.

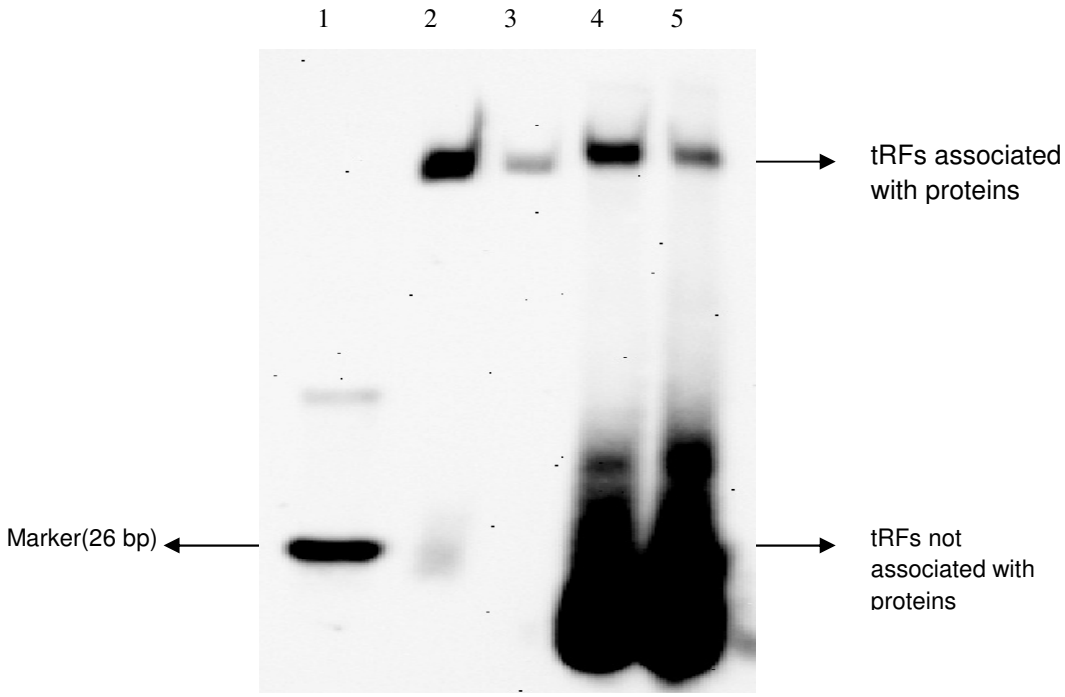

Figure 3.14. Electromobilitic Shift Assay of 5' and 3' of tRNA glycine. **1.** Biotin labeled tRF (5' of tRNA glycine) itself as a marker 26 bp (10 ng), **2.** S2 cell lysate (5 µg) and 20 nM biotin labeled 5' of tRNA glycin e, **3.** S2 cell lysate (5 µg) and 20 nM biotin labeled 5' of tRNA glycine and 400 nM of unlabelled 5' of tRNA glycine as specific competitor, **4.** S2 cell lysate (5 µg) and 20 nM biotin labeled 3' of tRNA glycine, **5.** S2 cell lysate (5 µg) and 20 nM biotin labeled 3' of tRNA glycine and 400 nM of unlabelled 3' of tRNA glycine as specific competitor.

Following *in vitro* association of proteins and tRNA derived fragments, biotin labeled tRNA derived fragments were transfected to S2 cells to examine interactions *in vivo.* The same pattern about 5' of tRNA derived fragments were observed that is, there was a complex association (Figure 3.15). Interestingly the complex observed under *in vitro* condition regarding 3' of tRNA derived fragment disappeared. It can be inferred the forming complex associated with 3' of tRNA was non-specific association which is absent under *in- vivo* conditions.

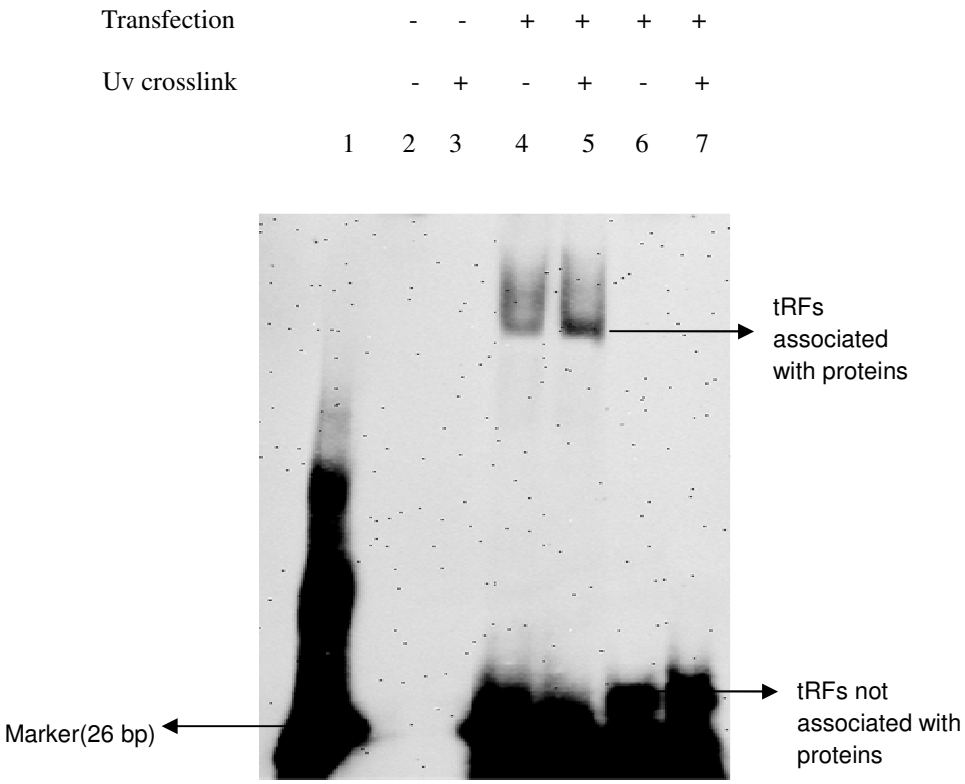

Figure 3.15. *In-vivo* crosslinking analysis. **1.** Biotin labeled tRF (5' of tRNA glycine) itself as a marker 26 bp (10 ng), **2.** Transfection and UV negative cells' total cell lysate (30 µg), **3.** Transfection negative UV positive cells' total lysate (30 µg), **4.** Transfected with biotin labeled 5' of tRNA glycine and UV negative cells, **5.** Transfected with biotin labeled 5' tRNA glycine and UV positive cells, **6.** Transfected with biotin labeled 3' of tRNA glycine and UV negative cells, **7.** Transfected with biotin labeled 3' of tRNA glycine and UV positive cells.

CHAPTER 4

CONCLUSION

For the first time, it was shown that tRNA derived small RNAs are temporally and selectively expressed in 1 hour and 8 hour *Drosophila* embryos. The tRFs generated from 5' end of tRNAs are associated with primarily non-polysomal fractions. The findings in S2 cells were in parallel to those in embryos.

Based on the importance of the cell death and proliferation in development and stress response, the temporal and selective expression of embryonic tRFs represent biological and metabolic roles modulating apoptosis and stress response processes during embryonic development.

The tRFs demonstrated here are different from the tRNA-derived small fragments generated during stress response (Thompson *et al*. 2009b). Stress-induced fragments were about 40 nt in length longer than about 26 nt in length embryonic tRFs. In addition, stress-induced tRNA derived small RNAs are originated from the vicinity of anti-codon loop of tRNAs. However, *Drosophila* embryonic tRFs are produced from upstream from the anti-codon loop, nearly at the stem of the loop. Moreover, the tRFs are different form stress induced tRNA derived small fragments in terms of selectivity in the type and amount of tRFs up on developmental stage. Our data put forward that selectivity exists not only among the tRNAs carrying different aminoacids but also those carrying same aminoacid anti-codon.

Our data also suggest that tRFs may have function differently from miRNAs. Though miRNAs are selectively associated with all of the four fractions (mRNP, 60S, Monosome, Polysome) in our experimental settings, tRFs are found in extremely low levels in polysomes. tRFs particularly co-localize with complexes matching the size of 60S ribosomal subunit and mRNPs. This shows tRFs act as a regulator away from actively translation machinery. According to our data it can be speculated that tRFs may potentially interact with 40S, 43S, 48S, or 60S ribosomal subunits to interfere with association of these subunits with other subunits or proteins needed for forming active translation machinery.

Much more work is needed to understand how tRNAs are selectively processed, and what their biological function is. It will be quite exciting and challenging to investigate regulatory small fragments derived from tRNAs, which adds another layer of complexity to gene regulation in eukaryotes.

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