Function and Biogenesis of small RNAs in *Dictyostelium discoideum*

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Abstract
Advancements in DNA sequencing technology have led to whole genome studies, which revealed that only 2% of entire mammalian genome is transcribed into RNAs coding for proteins while the remaining 98%, may be transcribed into RNAs (ncRNAs) that do not code for any proteins. Non-coding RNAs have diverse functions, for example: transfer RNAs (tRNAs), ribosomal RNAs (rRNAs) and small nuclear RNAs (snRNAs). These RNAs are known as housekeeping RNAs and are essential for cellular processes. Small RNAs like microRNAs (miRNAs) and small interfering RNAs (siRNAs) provide an unanticipated level of gene regulation in both metazoans and plants.

In recent years many aspects of miRNAs and siRNAs including their function, biogenesis and mechanism of action have been studied in various organisms. Recently, miRNAs and siRNAs were discovered in D. discoideum. The main objective of this study was to determine the function and biogenesis of small RNAs that have been identified in this organism. In evolution, D. discoideum is placed between plants and animals and it was therefore interesting to see whether small RNA biogenesis in this organism is similar to plants or animals. By studying the composition of small RNA ends their biogenesis can be determined. In order to study the composition of small RNAs, 5’ and 3’ end analyses were performed and I found that biogenesis of DIRS-I siRNA and mir1177 (miRNA) is similar to animals.

Small RNAs localization studies were performed, to know where they localize in the cell; by this study mir1177 was found to be localized to cytoplasm.
1. Introduction

1.1 Small non-coding RNAs

About ten years ago, only little was known about small non-coding RNAs. After the discovery of RNA interference (RNAi) significant developments have been observed in this area\(^1\). Non coding RNAs are functional RNA molecules that do not code for any functional protein. They include long non coding RNAs and small non coding RNAs (Figure 1). Large number of non-coding RNAs has been identified both in prokaryotes and eukaryotes. They have diverse functions in transcriptional regulation, translational inhibition, processing and modification of other RNAs\(^2\). Non-coding RNAs regulate gene expression either in cis or in trans. They induce gene silencing through sequence specific base pairing with the target molecules, and can serve as an experimental tool in gene knock down experiments\(^3\).

![Figure 1: Different types of RNAs. Coding RNAs code for proteins, whereas non-coding RNAs are functional molecules that do not code for proteins\(^4\).](image-url)
1.2 Biogenesis of small non-coding RNAs

Small RNAs are produced mostly from double-stranded RNAs (dsRNAs). Biogenesis of small non-coding RNAs can be classified into Dicer dependent and Dicer independent pathways. In the case of Dicer dependent pathway, dsRNAs are processed to form small RNAs through the action of an enzyme called Dicer. The substrate for the Dicer cleavage may be either an intramolecular fold back RNA structure (hair-pin structure) which gives rise to micro (mi)RNAs (Figure 2) or a dsRNA formed as a result of bidirectional transcription which gives rise to endogenous small interfering (si)RNAs (endo-siRNAs) (Figure 2)\(^1\). In animals, the biogenesis of miRNAs requires an enzyme called Drosha and its binding partner called Pasha in flies and DGCR8 in humans. These two enzymes which reside in the nucleus, convert the the pri-miRNAs, which are transcribed from the genome\(^2\) into the pre-miRNAs. The pre-miRNAs are then exported into the cytoplasm where they are further processed into mature miRNAs by Dicer.

![Figure 2: Biogenesis of small RNAs.](image)

**Figure 2: Biogenesis of small RNAs.** microRNAs (miRNAs) are encoded endogenously and are derived by processing short RNA hairpins. miRNAs may cause either the translational repression of target mRNA or its degradation. Small interfering RNAs (siRNAs) derived from long dsRNAs (double stranded RNAs) cause destruction of target mRNA\(^5\).
In some cases, viral infection of a cell can also be a source of dsRNAs, as many viruses transcribe RNAs in both sense and antisense polarity\(^1\). In the case of Dicer independent pathway, for example, in organisms like \textit{Caenorhabditis elegans} (\textit{C. elegans}), RNA-dependent RNA polymerase (RdRP) can generate siRNAs\(^6\).

**1.3 Mechanisms of gene regulation**

The first experimental proof of gene regulation by miRNAs came from the animal \textit{C. elegans}. In this organism, it has been shown that the regulation by miRNA can take place post-transcriptionally as the abundance of miRNA-regulated mRNAs was not substantially changed, but the abundance of proteins encoded from them was significantly reduced\(^7\). In animals, the targets of miRNAs are recognized through the seed pairing of seven nucleotides at the 5’ end of miRNA (from #2 to #8) with the 3’-UTRs of the target mRNAs\(^8\) whereas, in plants, miRNAs show perfect or nearly perfect complementarity to a site in the target mRNA\(^9\). In both plants and animals, precursor miRNAs are processed into mature ds miRNA complex by Dicer (Figure 2). Within ds miRNA complex, the strand (guide strand) which is less strongly base paired at 5’ end is incorporated into miRISC (RNA induced silencing complex) and the other strand (passenger strand) is subsequently degraded (Figure 2). This is the widely accepted model of the RISC loading with small RNAs\(^10\). In the similar way, siRNAs are loaded into RISC\(^11\). Argonaute proteins (AGO) are the functional components of RISC. Some of them (like AGO2) possess endonuclease activity and are responsible for target mRNA cleavage\(^12\).

Interestingly, in \textit{Drosophila}, the RISC formation may proceed in a slightly different way. Here, both the guide and passenger strands of certain ds miRNA complexes are loaded into miRISC AGO1 and miRISC AGO2 respectively, and these complexes act on two different target mRNAs\(^13\). The binding of miRISC to the target mRNA may cause translational inhibition or premature ribosome drop off or stalled elongation or co-translational protein degradation\(^14\). However, recently it has been shown that the main effect of miRNA induced silencing is due to mRNA degradation through the de-adenylation of the polyA tail and subsequent mRNA degradation (Figure 2). miRNAs and siRNAs use similar mechanisms to regulate their target mRNAs (Figure 2)\(^15\).
1.4 Amplification of silencing by RdRPs

The initiator RNA for RNA dependent RNA polymerase (RdRP) amplification of small RNAs is a dsRNA, which is cleaved by Dicer into siRNA or miRNA (also known as primary silencing RNAs). These single stranded RNAs of 21-to 25 nucleotides bind to Arganoute proteins and target their target mRNA. The target mRNA acts as template for secondary siRNA production by RdRPs and the produced secondary siRNAs thereby act on the target mRNA. In this way silencing signal is amplified\(^6\). RdRPs are the enzymes that catalyze the formation of complementary RNA strands from single stranded RNAs. The resulting dsRNAs are then processed into the secondary siRNAs via Dicer dependent or Dicer independent small RNA pathways\(^6\). In the Dicer dependent pathway (the right panel in the figure 3) RdRPs produce long dsRNAs, which are then cleaved by Dicer into ds secondary siRNAs and one of their strands is loaded into Argonaute and acts on the target mRNA. RNA products synthesized by the Dicer dependent pathway possess one phosphate at their 5’ end. This type of mechanism is commonly observed in plants. For instance, Axtell et al. showed that in plants there is an efficient production of secondary siRNAs, and Argonaute cleaves the targeted RNA\(^6\). In plants there are six RdRPs that are involved in different gene silencing mechanisms whereas in humans hTERT (human telomerase reverse transcriptase) is a functional homologue for RdRP\(^6\).

**Figure 3: Production of secondary siRNAs by RdRPs.** RdRPs mediate Dicer dependent (in plants) and Dicer independent (in C. elegans) functional small RNA synthesis\(^6\).
In the Dicer independent pathway (the left panel in the figure) short antisense RNAs are synthesized \textit{de novo}, which are then cleaved by an unknown endonuclease. RNA products synthesized by Dicer independent pathway possess three phosphates at their 5' end. This type of mechanism is commonly observed in \textit{C. elegans}\textsuperscript{6}.

\textbf{1.5 About \textit{Dictyostelium discoideum}}

\textit{Dictyostelium discoideum} is a unicellular eukaryotic model organism first discovered by German mycologist Oskar Bredfeld in horse dung\textsuperscript{17}. It has a haploid genome of 34Mb size and six chromosomes that codes for 12,500 proteins\textsuperscript{18}. It shares many features with multicellular organisms\textsuperscript{19}. Normally, it grows at 22\textdegree{} to 24\textdegree{}C\textsuperscript{20}. Interesting features of \textit{D. discoideum} genome are a high A/T content (77\%) and a high abundance of transposable elements.

\textit{D. discoideum} has a unique life cycle. During the growth cycle, \textit{Dictyostelium} cells grow solitarily by feeding on soil living bacteria. The switch to the developmental cycle occurs during starvation (Figure 4). Starvation initiates the synthesis of glycoproteins and adenylyl cyclase\textsuperscript{21}. The glycoproteins are responsible for cell-cell contact whereas adenylyl cyclase produces cAMP, a secondary messenger to attract other \textit{Dictyostelium} cells nearby. Around 100,000 cells form an aggregate by chemotaxis and enter into the developmental cycle\textsuperscript{22}. After 16 h of starvation the cells form a slug\textsuperscript{19}. The slug has a sausage shape and is up to a few mm long. It migrates towards light and humidity\textsuperscript{23}. After 24 h of starvation, in the culmination phase (Figure 4), the prestalk and prespore cells differentiate into stalk and spore cells forming the mature fruiting body\textsuperscript{20}. The walls of the stalk and spores contain cellulose\textsuperscript{22}. Since \textit{D. discoideum} has a haploid genome, it is easy to study gene knock outs and it is easy to work with \textit{D. discoideum} as it divides every 2-8 h. It contains several genes that are homologous to higher eukaryotes. Recently, large numbers of small silencing RNAs have been identified in \textit{D. discoideum} in addition to proteins involved in small RNA biogenesis and functions i.e. two Dicers (drnA, drnB)\textsuperscript{24}, five Arganoute proteins\textsuperscript{25} and three RdRPs\textsuperscript{24}. 

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Figure 4: Life cycle of *Dictyostelium discoideum*. In the presence of nutrients *D. discoideum* grows solitarily by feeding on bacteria (vegetative cycle) whereas in the absence of nutrients (starvation) it divides sexually by aggregation of cells (developmental cycle)\textsuperscript{21}.

1.6 Non coding RNAs identified in *D. discoideum*

Hinas A. \textit{et al} were the first who have identified developmentally regulated miRNAs (mir1177, mir1176) and siRNAs derived from DIRS-I retrotransposons in *D. discoideum*\textsuperscript{26}. Apart from this, small RNAs that are antisense to mRNAs were also identified indicating RdRP synthesis of longer antisense RNA molecules. DIRS-I is the most abundant retrotransposon in *D. discoideum* that codes for 4.5 kb mRNA and a 900 nt antisense RNA\textsuperscript{27}. Small RNAs were identified along entire retrotransposon.

Figure 5: DIRS-I retrotransposon of *D. discoideum*. The left asterisk indicates the location of DIRS-I siRNA used in this study whereas the right asterisk indicates the location of another DIRS-I siRNA which was studied by Hinas A \textit{et al.}, 2007.
In evolution, *D. Discoideum*, which belongs to the Amoeboza kingdom, is placed in between animals and plants, hence it shares features of either plants or animals or both.

![Evolutionary position of D. discoideum](image)

**Figure 6: Evolutionary position of *D. discoideum***

Biogenesis of small RNAs in this organism has until now not been studied. It is interesting to see whether small RNA biogenesis in *D. discoideum* is similar to the biogenesis in plants or in animals.
1.7 Aim of this project

Recently, a large number of small RNAs such as miRNAs and siRNAs were identified in *D. discoideum* by our research group, but the function and biogenesis of the identified small RNAs have not yet been studied. The aim of my project was to determine the function and biogenesis of certain small RNAs which had been identified in this organism. In order to determine the biogenesis of small RNAs in *D. discoideum*, I performed 5’ and 3’ end analysis of small RNAs. To study the 5’ ends of small RNAs, total RNA or enriched small RNA fraction was treated with different enzymes to determine the number of phosphates at their 5’ ends. To study the 3’ ends of small RNAs total RNA was subjected to chemical treatment. I have also investigated the cellular localization of mir1177 to understand where in the cell it functions. I have also studied the expression profile of miRNAs during development.
2. Materials and methods

2.1 Culturing of *D. discoideum*

Wild type strain (AX2 wt) and different microRNA over expressing AX2 strains (AX2 mir1177, AX2 miR-can1) of *D. discoideum* were used in this study. The strains were spread on SM-plates containing a lawn of *Klebsiella aerogenes* (used as a nutrient source for *D. discoideum*). The SM-plates with *D. discoideum* were incubated at 22 °C, until plaques appeared. The vegetative cells from the outer part of the plaques were scraped off and inoculated in 2 ml HL5 medium (10 g Peptic peptone, 5 g Yeast extract, 0.35 g Na$_2$HPO$_4$, 0.34 g KH$_2$PO$_4$, 950 ml H$_2$O, pH 6.4) together with PenStrep solution (dilution 1:100) in a sterile round-bottom glass tube at 22°C on a shaker (NEW BRUNSWICK) at 170 rpm. After 2-3 days of incubation, 2 ml of the culture were added to 25 ml HL5 medium together with PenStrep (dilution 1:100) in a sterile 100 ml Erlenmeyer flask and incubated at 22 °C on a shaker at 150 rpm. The cell cultures could be kept for one month provided the cell concentration was monitored and the medium were changed whenever the cells reached a concentration of 4x10$^6$ cells/ml. The cell concentration was calculated by counting the cells using a hemocytometer (Bright line® Hemacytometer) under light microscope (ZEISS) (growth and development of *D. discoideum*)$^{28}$.

2.2 Total RNA extraction

Approximately 10$^8$ cells of *D. discoideum* were harvested by centrifugation at 500 g for 5 min at 4 °C in a swing-out rotor (Multifuge 3 S-R Heraeus). The cell pellet was resuspended in cold PDF (20 mM KCl, 5 mM MgCl$_2$.6H$_2$O, 20 mM KPO$_4$. H$_2$O to 1 litre, pH 6.2, sterile filtrate) with a volume equal to the culture volume and centrifuged at 500 g for 5 min. The cell pellet was then resuspended in 1 ml of TRIzol reagent (Invitrogen), vortexed and incubated for 5 min at room temperature (RT). Then 200 µl of chloroform was added, vortexed and incubated for 3 min at RT, and was then centrifuged for 15 min at 12000 rpm at RT in a microcentrifuge (Biofuge pico Heraeus). The upper (aqueous) phase (~600 µl) was taken into a fresh eppendorf tube and mixed with 500 µl of isopropanol, incubated 10 min at RT and centrifuged for 10 min at 13,000 rpm at RT. The supernatant was discarded and the pellet was washed with 1 ml of 70 % ethanol and centrifuged for 5 min at 13,000 rpm at RT. The supernatant was discarded and the pellet was air dried and dissolved in RNase free water.
If the pellet was hard to dissolve, then it was incubated at 65 °C for 5-10 min. The RNA concentration was measured using a Nanodrop (Thermo scientific, NANODROP 1000 spectrophotometer).

2.3 Small RNA enrichment

120 µg - 150 µg of total RNA was dissolved in 200 µl of distilled water (dH2O) after which 200 µl of PEG/NaCl (20 % PEG 8000, 2 M NaCl) precipitation solution was added. The resulting solution was mixed and kept on ice for at least one hour. The mixture was centrifuged at 13000 rpm for 15 min at 4 °C. The supernatant containing small RNA fraction was transferred to a new eppendorf tube. The pellet which consisted of large RNA fraction was washed with 70 % ethanol and centrifuged for 5 min at 13,000 rpm at 4 °C. The previous step was repeated and the pellet was air dried and dissolved in 50 µl of dH2O. The small RNA fraction was precipitated with 2.5 volumes of 99 % ethanol and kept overnight at -20 °C. The sample was centrifuged at 13,000 rpm for 30 min at 4 °C. The pellet was resuspended in 1 ml of 70 % ethanol and centrifuged for 5 min at 13,000 rpm at 4 °C. The previous step was repeated and the pellet was air dried and dissolved in 15 µl of dH2O.

2.4 Nuclear RNA extraction

Approximately, 10^8 cells were pelleted out for 5 min at 300 g at 4 °C. The supernatant was discarded and the cells were resuspended in 1 ml ice-cold nuclei buffer (40 mM Tris-Cl, pH 7.8, 1.5 % sucrose, 0.1 mM EDTA, 6 mM MgCl2, 50 mM KCl, 5 mM DTT, 0.4 % NP40, H2O to 500 ml) for lysis for 3-6 min. The nuclei were pelleted at 500 g for 5 min at 4 °C. The supernatant was discarded and the pellet was resuspended in 1 ml ice cold nuclei buffer for 2-3 min. The nuclei were then centrifuged at 500 g for 5 min at 4 °C. The supernatant was discarded and the pellet was resuspended in 1 ml Trizol (Invitrogen) and subsequently total RNA was extracted.

2.5 Filter development of D. discoideum

AX2 cells over expressing specific miRNAs (mir1177, miR-can1) were grown in HL5 medium until they reached a concentration of ~ 3x10^6 cells/ml. Then ~5x10^7 were harvested and centrifuged at 500 g for 5 min. A filter pad was placed in a 15 mm petri dish and 1 ml
PDF (20 mM KCl, 5 mM MgCl\textsubscript{2}.6H\textsubscript{2}O, 20 mM KPO\textsubscript{4}, H\textsubscript{2}O to 1 litre, pH 6.2 and sterile filtrate) was added to wet the pad. A nitrocellulose filter was placed on the top of the pad. The cells were washed by pouring media from the pellet. The pellet was knocked loose and resuspended in PDF and the wash was repeated. The cells were resuspended to a concentration ~10\textsuperscript{8} cells/ml in PDF. 500 µl of the resuspended cells were poured onto the filter and spread evenly. 500 µl of PDF was added to completely saturate the filter. The cells were incubated in a moist chamber at 22 °C.

2.6 Agarose gel electrophoresis

After isolation of RNA, its size and purity were analyzed using agarose gel electrophoresis. Depending on the size of the fragments, gel concentrations of 0.8% (to view RNA of 0.8 kb) or 1.2% (to view RNA of 0.5 kb) were prepared by dissolving 0.8 g or 1.2 g of agarose powder in 100 ml 0.5 X TBE buffer, respectively. Ethidium bromide (0.5 µg/µl) was added to the agarose gel solution to visualize the RNA fragments under a UV illuminator fitted with a camera (Gel photo system GFS 1000). The gels were run at 100 V and 60 mA. Depending on the size of the fragments to be analyzed DNA ladders of 1 kb or 100 bp (Fermentas) were used.

2.7 Northern blot analysis

To 15 % polyacrylamide solution (sometimes 10%) containing 7 M urea and 1 X TBE, 1% APS (1:100) and 0.1% TEMED (1:1000) were added. The gel was cast between 20 X 20 cm glass plates and polymerized for one hour. Then the gel was pre-run for one hour. 20 µg of total RNA or 2 µg of enriched small RNA were mixed in the 1:1 ratio with 2 X RNA loading dye. [\gamma-p32]-ATP end labelled pUC19/MspI (Fermentas) and [\gamma-p32]-ATP end labelled Decade marker (Ambion) were used as DNA and RNA ladders respectively. The RNA samples and RNA ladder were denatured for 5 min at 95 °C on a heating block (Techne DR1-Block) and chilled on ice. The wells in the gel were rinsed before RNA samples were loaded. The gel was run until bromophenol blue and xylene cyanol dyes reached the bottom and middle of the gel, respectively.
2.7.1 Electroblotting

Four whatman paper sheets and one nylon membrane (Amersham Hybond-N+ GE Healthcare) were cut in the same size as the gel and soaked in 1 X TBE. Two whatman paper sheets were first placed on top of the gel, then the gel was flipped on to other side and the nylon membrane was placed first, then the two remaining whatman sheets were placed. The entire setup was kept in between two pads, then in the blotting device (BioRad TransBlot Cell) and blotted at 20 V for 16 h in 1 X TBE buffer at 4 °C (overnight).

2.7.2 UV-cross linking

The RNA transferred onto the membrane was immobilised by UV-crosslinking (UVC 500 crosslinker, Amersham Biosciences) at 150 mJ.

2.7.3 Oligo labelling and hybridization

The membrane was pre-hybridized in a hybridization tube (Amersham Biosciences) containing 20-30 ml of church buffer (0.5 M NaPO₄ buffer, pH 7.2, 7 % SDS, 1 mM EDTA, 5 g BSA, H₂O to 0.5 L, sterile filtrate) for one hour in hybridization chamber (Hybridization chamber, GFL, 7601). 8 pmol of the DNA oligonucleotide was end labelled by 1 µl of T4PNK (Fermentas) in the presence of 5 µl γ-ATP (50 µCi), 2 µl T4 PNK buffer A (Fermentas) and 11.6 µl H₂O. The reaction was incubated for 30 min at 37 °C and the unincorporated nucleotides were removed using a G-50 column (ProbeQuant™ G-50 Microcolumns, GE Healthcare). Then labelled oligo was denatured at 95 °C for 5 min and chilled on ice for 3-5 min. Before the addition of labelled oligo into the hybridization tube, fresh pre-warmed church buffer was added and hybridization of the oligo took place overnight at 42 °C. The next day, membrane was washed at 42°C; 2×5 min with 2 X SSC/0.1% SDS, 2×10 min with 1 X SSC/0.1 % SDS and 2×5 min with 0.5 X SSC/0.1 % SDS. The membrane was sealed in a kapak heat seal bag and kept in the exposure cassette (Molecular Dynamics) for exposure. After few days or hours, the hybridization signal on the membrane was analyzed by phosphor imager (Molecular Dynamics). In the case of reprobing or stripping the membrane, it was boiled twice in 0.1 X SSC/0.1 % SDS for 45 min.
2.8 Oligos used

Table 1: List of oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Lab designation</th>
<th>Oligo name</th>
<th>Purpose</th>
<th>Sequence</th>
</tr>
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<tbody>
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<td>18</td>
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<td>NB</td>
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<tr>
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<td>U6 RNA</td>
<td>NB</td>
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<td>U5 RNA</td>
<td>NB</td>
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<tr>
<td>312</td>
<td>mir1177</td>
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<tr>
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<td>DIRS-1 siRNA</td>
<td>NB</td>
<td>GGCCTAATCTCCACTACTGG</td>
</tr>
<tr>
<td>623</td>
<td>miR-can1</td>
<td>NB</td>
<td>TTATCCAATTGTACAGATCC</td>
</tr>
</tbody>
</table>

NB: Northern Blot

2.9 Phenol extraction of RNA

1:1 volumes of RNA solution and phenol were vortexed for 15 sec and centrifuged at 16000 g for 5 min at room temperature (RT). The upper phase was placed in a new eppendorf tube and diluted with 20 µl of dH2O. Equal volume of chloroform was mixed, vortexed for 15 sec and centrifuged for 5 min at 16000 g at RT. The supernatant was then precipitated in ethanol as described below.

2.10 Ethanol precipitation

1 volume of RNA was mixed with 0.1 volume of 3 M NaOAc (pH 5.2) and 3 volumes of ice cold 99 % ethanol and incubated at -20 °C for 30 min and centrifuged at 16000 x g for 30 min at 4 °C. The supernatant was discarded and the pellet was washed twice with 70 % ethanol (same as the initial volume) and centrifuged at 16000 x g for 10 min. The supernatant was carefully removed and the pellet was air dried and dissolved in RNase free water.
2.11 Preparation of radioactively-labelled DNA ladder

A reaction mixture consisting of 1-20 pmol of 5’-termini, 10 X reaction buffer B for T4 polynucleotide kinase (γ-P\textsuperscript{32} or γ-P\textsuperscript{33}-ATP (40 pmol), 24 % (w/v) PEG 6000 solution, 19 µl of nuclease free water and 1 µl T4 polynucleotide kinase) was incubated at 37 °C for 30 min. 1 µl of 0.5 M EDTA (pH 8.0) was added and heated at 75 °C for 10 min. The labelled DNA was separated from unincorporated label by gel filtration on a G-50 column (ProbeQuant™ G-50 Microcolumns, GE Healthcare).

2.12 Labelling of pUC19/MspI marker

A mixture containing 2 µl of pUC19/MspI (Fermentas, 1 µg/µl), 1 µl of γ-ATP, 15 µl of H\textsubscript{2}O, 2 µl of 10 X buffer A (Fermentas) and 1 µl of T4 PNK (Fermentas, 10 U/µl) was incubated at 37 °C for 30 to 60 min. The mixture was later passed through a G-50 column (ProbeQuant™ G-50 Microcolumns, GE Healthcare) to remove the unincorporated γ-ATP and mixed with equal volume of 2 X RNA dye and stored at -20 °C.

2.13 Labelling of RNA decade marker

A mixture containing 1 µl of Decade marker (Applied Biosystemes, 0.1 µg), 1 µl of γ-ATP, 6 µl of H\textsubscript{2}O, 1 µl of 10 X T4 PNK reaction buffer (Ambion) and 1 µl of T4 PNK (Ambion, 10 U/µl) was incubated at 37 °C for 30 to 60 min. 8 µl of water and 2 µl of 10 X cleavage reaction were added to the mixture and incubated at room temperature for 5 min. 20 µl of 2 X RNA loading dye was added to the mixture.

2.14 6X DNA loading dye

To 300 µl of glycerol, 25 µl of 1 % bromo phenol blue, 25 µl of 1 % xylene cyanol and 650 µl H2O were added.

2.15 2X RNA loading dye

To 916 µl of formamide, 34 µl of 0.5 M EDTA, 25 µl of 1 % bromo phenol blue, and 25 µl of 1 % xylene cyanol were added.
2.16 Over-expressing miRNA strains

mir1177 precursor and miR-can1 precursor were cloned separately into pDM 304 vectors carrying constitutive promoter (actin promoter) and were transformed into D. discoideum, to produce mir1177 and miR-can1 over-expressing strains respectively.

2.17 End analysis of small RNAs

2.17.1 5’ end analysis using Ap and T4PNK

A “starter” tube containing 24 µl of total RNA (3.321 µg/µl) from AX2 cells over expressing mir1177 miRNA was prepared.

(A) Control tube: 6 µl (20 µg) of total RNA from the starter tube, dissolved in 194 µl H₂O was extracted by phenol and then precipitated with ethanol.

(B) Treatment of total RNA with Alkaline phosphatase (Ap): First, 6 µl (20 µg) of total RNA from the starter tube was dissolved in 74 µl H₂O, then mixed with 10 µl of Ap buffer (Fermentas), 10 µl of Ap (Fermentas, 1U/µl), 0.5 µl RiboGuard (Fermentas, 40U/µl) and incubated for 30 min at 37 °C. The reaction mixture was heated at 75 °C for 5 min and spun down. Then 100 µl of H₂O and 5 µg of glycogen were added, after which total RNA was extracted with phenol and precipitated with ethanol. Finally, the RNA pellet was washed twice with 70 % ethanol and resuspended in 10 µl H₂O.

(C) Treatment of total RNA with Ap and then with T4Polynucleotide kinase (T4PNK): Firstly, 6 µl (20 µg) of total RNA from the starter tube was treated with Ap (as described in (B)). Then, 10 µl of RNA from the Ap reaction was mixed with 2.5 µl of 10mM rATP, 5 µl of 10 X Reaction buffer A (Fermentas), 5 µl of T4 PNK (Fermentas, 30U/µl), 0.5 µl RiboGuard (Fermentas, 40U/µl), 27 µl H₂O and incubated for 30 min at 37 °C. The reaction was inhibited by adding 2.5 µl of 0.5 M EDTA, pH 8.0. RNA was then precipitated with ethanol and pelleted. RNA pellet was washed twice with 70 % ethanol and resuspended in 10 µl of H₂O.

2.17.2 5’ end analysis using TE and TAP

A “starter” tube containing 12 µl enriched small RNAs (1 µg/µl) from AX2 cells over expressing mir1177 miRNA was prepared.
2.17.3 3’ end analysis (β-elimination assay)

Total RNA of 54 µl (1.709 µg/µl) from AX2 cells over-expressing mir1177 miRNA was taken in one eppendorf tube “1” and 36 µl (2.599 µg/µl) of total RNA from AX2 wild type cells was taken in another eppendorf tube “2”. 18 µl of total RNA from eppendorf tube “1” were taken into another eppendorf tube labelled with “tube A” and diluted by adding 5.5 µl of H20. 12 µl of total RNA from eppendorf tube “2” were taken into another eppendorf tube labelled with “tube B” and diluted by adding 11.5 µl of H20. The following steps were carried out separately for both “tube A” and “tube
B”. 4 µl of 5 X 300 mM borax/boric acid buffer pH 8.6 (150 mM borax + 150 mM boric acid) and 2.5 µl 200 mM NaIO₄ (sodium meta-periodate) were added. The reaction was incubated for 10 min at room temperature in dark and 2 µl of glycerol were added to quench unreacted NaIO₄, then the reaction was vacuum dried (Speed vac concentrator, savant) for 25 min. Then the pellet was dissolved in 50 µl 1 X borax/boric acid buffer pH 9.5 (the pH was adjusted using NaOH) and incubated for 90 min at 45 °C. Then 3 µl 5M NaCl, 150 µl 99 % ethanol and 1 µl glycogen were added and incubated for two hours on ice. After that the reaction was centrifuged at 16,000 x g for 30 min and the pellet was washed twice with 80 % ethanol and dissolved in 10 µl H₂O. Then 10 µl 2 X RNA loading dye were added and the sample was loaded on 10% PAGE³¹.
3. Results

3.1 Small RNAs enrichment

The procedure of RNA enrichment is described in the section 2.3 (Small RNA enrichment). This treatment predominantly removes large RNA molecules (ribosomal RNAs, mRNAs) enriching the sample with RNA molecules ranging between 10 - 200 nt, thereby increasing the sensitivity of subsequent small RNAs detection by northern blot analysis. Since there is a low expression of miRNAs in *D. discoideum*, cells over expressing certain miRNAs were used in this study. Small RNAs were enriched from total RNA extracted from *D. discoideum* AX2 cells overexpressing mir1177. The quality and integrity of enriched small RNA fraction and total RNA fraction was verified by agarose gel. Figure 7 shows that the separation of small RNA fraction from total RNA preparation was successful.

![Small RNA enrichment](image)

**Figure 7: Small RNA enrichment.** 1µg of total RNA from mir1177 miRNA overexpressing strains was loaded in the lane A. After small RNA enrichment, 1µg of RNA from large RNA and small RNA fractions were loaded in the lanes B and C respectively, whereas 1kb DNA marker was loaded in the lane M (0.8 % agarose).

3.2 End analysis of small RNAs

3.2.1 5’ end analysis using Ap and T4PNK

As mentioned earlier, RNAs processed by Dicer contain one phosphate at the 5’end while in *C. elegans* there are some secondary siRNAs that carry three phosphates at their 5’ end. The 5’ ends of small RNAs were first studied by Alkaline phosphatase (Ap) and T4
Polynucleotide kinase (T4PNK) treatment (Figure 8). Initially, total RNA was extracted from AX2 cells over expressing mir1177. Then, total RNA sample without any enzymatic treatment was loaded in lane A (untreated), total RNA sample treated with Ap was loaded in lane B and total RNA sample first treated with Ap, and then with T4PNK was loaded in lane C. Alkaline phosphatase catalyzes the release of all 5’ phosphate groups from DNA, RNA and protein (Appendix). Hence, RNA samples treated with Ap were expected to migrate a bit slower (due to loss of all phosphates at 5’ end of RNA) when compared to the untreated RNA sample (no loss of phosphates at 5’ end). Figure 8 shows that it was indeed the case (compare lane A and lane B in figures 8a and 8b).

**Figure 8: Northern blot analysis of DIRS-1 and mir1177 small RNAs using Fast Ap and T4PNK treatment.** (15% PAGE) 20 µ g of total RNA were loaded in each lane (A, B and C). A: Untreated total RNA; B: Total RNA treated with Fast AP; C: Total RNA first treated with Fast AP, then treated with T4 PNK; M: RNA decade marker. In (a) the membrane was probed for DIRS-I siRNA. (b) The same membrane was stripped and probed for mir1177.

T4 Polynucleotide kinase (PNK) catalyzes the transfer of gamma-phosphate from ATP to a free 5’OH group of single or double stranded DNA or RNA molecules (Appendix). In the case of RNA sample first treated with Ap and then with T4 PNK, the resulting RNA after this treatment contains only one phosphate at its 5’ end. If the untreated RNA molecule had one phosphate at its 5’ end, then RNA loaded in lane A and lane C should have equal gel mobility. If the untreated RNA molecule had three phosphates at its 5’ end, then RNA loaded in lane A should have faster gel mobility (due to three phosphates at its 5’ end) when
compared to RNA loaded in lane C (which has only one phosphate at its 5’ end). The membrane was first probed for DIRS-I siRNA and analyzed after three days of exposure. The signal pattern on the membrane indicates that DIRS-1 siRNA contains only one phosphate at its 5’ end, since RNA loaded in lane A and lane C have equal gel mobility (figure 8a). Then the membrane was stripped and probed for mir1177 and analyzed after two days of exposure. Based on the signal pattern on the membrane (figure 8b), we believe that mir1177 might also contain one phosphate at its 5’ end. To confirm that both DIRS-1 siRNA and mir1177 RNAs indeed contain one phosphate at their 5’ end we used a different approach. Here, enriched small RNAs were treated with Terminator Exonuclease (TE) and Tobacco Acid Pyrophosphatase (TAP). TE is a processive 5’ to 3’ exonuclease that degrades RNAs with 5’ monophosphate but not RNA containing 5’ triphosphate, 5’ cap or 5’ OH group (Appendix). TAP is an enzyme that converts RNA containing three phosphates at its 5’ end into 5’ mono phosphate (Appendix); TAP can act even on 5’ capped RNAs.

**Figure 9: Northern blot analysis of DIRS-1 and mir1177 small RNAs using TE and TAP treatments.** (15% PAGE) 2 µg of enriched small RNA was loaded in each lane (A, B, C and D). A: Untreated enriched small RNA, B: Enriched small RNA treated with TE, C: Enriched small RNA treated with TAP, D: Enriched small RNA first treated with TAP, then with TE, M: RNA decade marker. In (a) the membrane was probed for DIRS-I siRNA. (b) Another membrane probed for mir1177. U5 RNA was used as loading control.

Enriched small RNA fraction without any treatment with TE or TAP was loaded in lane A (untreated), enriched small RNA fraction treated with TE was loaded in lane B (TE), enriched small RNA fraction treated with TAP was loaded in lane C (TAP) and enriched small RNA fraction first treated with TAP, then with TE was loaded in lane D (TAP+TE) (Figure 9). If
the original RNA molecule had three phosphates at its 5’ end, then no RNA degradation would be expected in the TE treated RNA samples (lane B), since TE does not degrade RNA containing 5’ triphosphates. The observed degradation indicates again that the RNA has one phosphate at its 5’ end. In the last lane (TAP+TE), RNA samples were first treated with TAP, which ends up with RNA molecules containing 5’ mono phosphate (as TAP converts RNA containing 5’ triphosphate to 5’ monophosphate) and then treated with TE, which results in the degradation of all RNA molecules (as TE degrades RNA containing 5’ monophosphate): hence no signal was expected to be seen in this lane. This lane (TAP+TE) was used as a control lane for the TE treated RNA samples.

The membrane shown in the figure 9a was probed for DIRS-I siRNA and analyzed after three days of exposure. Based on the signal pattern on the membrane, it is clear that DIRS-1 siRNA contains only one phosphate at its 5’ end, since no signal was observed in the lane B (TE treated RNA) and signals in lane A (untreated) and lane C (TAP treated RNA) were observed. As a loading control, the membrane was probed for U5 RNA, (Figure 9a) which is a primary transcript containing a 5’ cap structure. As expected, a signal was observed in the lane B (TE treated RNA) since TE does not act on the cap structure. By this analysis, it is clear that DIRS-I siRNA has one phosphate at its 5’ end.

Another membrane (figure 9b) was probed for mir1177 and analyzed after two days of exposure. Figure 9b confirms that mir1177 has one phosphate at its 5’ end, as no signal was observed in the lane B (TE) and signals in lane A (untreated) and lane C (TAP) were observed. As a control, the same membrane was probed for U5 RNA.

3.2.2 3’ end analysis

In certain organisms the 3’ end of small RNAs is protected by methylation. To determine whether 3’ ends of certain small RNAs were modified or not in *D. discoideum*, the 3’ end analysis (β-elimination assay) was performed. For this, total RNA was treated with periodate (NaIO₄). Periodate is used to open saccharide rings between vicinal "diols", which are then oxidized into aldehydes. The β-elimination reaction is initiated by the base treatment of aldehydes. It results in the removal of the β-hydrogen and subsequent cleavage of one nucleotide from the 3’ end of RNA molecule, hence a shift was expected in the lane where periodate treated RNA was loaded compared to the untreated RNA lane.
Figure 10: Northern blot analysis of DIRS-1 and mir1177 small RNAs using periodate treatment. (15% PAGE) In (a) 30 µg of total RNA from AX2 wild type cells, untreated and periodate treated were loaded in the lanes A and B respectively and 30 µg of total RNA from AX2 cells over expressing mir1177, untreated and periodate treated were loaded in the lanes C and D respectively. The membrane was then probed for mir1177. In (b) 20 µg of total RNA from AX2 cells over expressing mir1177, untreated and periodate treated were loaded in the lanes A and B respectively. The membrane was then probed for DIRS-I siRNA. M: DNA ladder, N: RNA ladder. U6 RNA was used as loading control.

RNA molecules containing 3’ end modifications lack vicinal diols and such RNA molecules are not sensitive to periodate treatment. In figure 10a, total RNA extracted from AX2 wild type cells treated without and with periodate were loaded in the lanes A and B respectively, whereas total RNA extracted from AX2 cells over expressing mir1177 treated without and with periodate were loaded in the lanes C and D, respectively. By comparing the lanes C and D in the figure 10a, the observed shift in periodate treated RNA compared to untreated RNA suggests that the 3’ end of mir1177 is not modified. Unfortunately, no signal was observed in the lanes A and B even after one week of exposure. In figure 10b, total RNA extracted from AX2 cells over expressing mir1177 treated without and with periodate was loaded in the lanes A and B respectively. By comparing the lanes A and B in the figure 10b, a shift in periodate treated RNA molecules compared to untreated RNA molecules suggest that DIRS-I siRNA has no modification at their 3’ ends.
3.3 Subcellular localization of small RNAs

In order to study the subcellular localization of certain small RNAs in *D. discoideum*, total and nuclear RNAs were extracted from AX2 cells over expressing mir1177 and the purity of nuclear RNA separation was checked by agarose gel (Figure 11).

![Agarose gel electrophoresis of total and nuclear RNA preparations](image)

Figure 11: (0.8%) Agarose gel electrophoresis of total and nuclear RNA preparations. 1 µg of total RNA and nuclear RNA from mir1177 over expressing cells were loaded in lanes A and B respectively. M: DNA ladder.

Instead of cytoplasmic RNA, total RNA was compared to nuclear RNA, as it is difficult to remove all nuclei from the cytoplasm. Absence of tRNAs and 5.8 S rRNAs (present only in the cytoplasm) and the presence of larger ribosomal RNA precursors (as they are processed in the nucleus) in the lane B (nuclear RNA lane) (Figure 11) confirm the purity of nuclear RNA preparation. Then 3.7 µg of nuclear RNA and 20 µg of total RNA preparation (quantification from Hinas, A., unpublished) were loaded on 10 % PAGE, followed by northern blot analysis. The membrane was probed for mir1177 and also for U2 RNA as a nuclear RNA purity control (U2 RNA is localized to the nucleus) (Figure 12). If the small RNAs were localized to the cytoplasm, then the signal should be seen only in the total RNA lane, whereas if the small RNAs are localized to the nucleus, then signal with the same intensity should be
seen in both the total RNA and nuclear RNA lanes. After 3 days of membrane exposure, signal was observed only in the total RNA lane (Figure 12) indicating that mir1177 might be localized to the cytoplasm.

Figure 12: Northern blot analysis of subcellular localization of mir1177 in *D. discoideum*. (10% PAGE) 20 µg of total RNA was loaded in the lane A and 3.7 µg of nuclear RNA were loaded in the lane B. The membrane was then probed for mir1177. U2 RNA was used as a nuclear RNA control. M:DNA ladder, N:RNA ladder.

3.4 Expression of microRNAs during development of *D. discoideum*

In wild type cells of *D. discoideum* there is a very low expression of miRNAs. As it is difficult to study them, *Dictyostelium* cells over expressing certain miRNAs were prepared by placing miRNA precursor (procedure described in section 2.16) behind an actin promoter. In order to see whether cloned miRNAs are expressed at all stages of development, this study was performed. In figures 13a and 13b *Dictyostelium* cells over expressing mir1177 and miR-can1 were used, respectively. Total RNA isolated from growing cells (0 h), 16 h developed cells (slugs) and 24 h developed cells (fruiting bodies) were loaded in the lanes A, B and C respectively (figures 13a and 13b).
Figure 13: Northern blot analysis of small RNAs during development of *D. discoideum*. (15% PAGE) In (a) 20 µg of total RNA from AX2 cells overexpressing mir1177 isolated from growing cells (0 h) and from developed cells (16 h and 24 h) were loaded in the lanes A, B and C respectively. The membrane was probed for mir1177. In (b) 20 µg of total RNA from AX2 cells overexpressing miR-can1 isolated from growing cells (0 h) and from developed cells (16 h and 24 h) were loaded in the lanes A, B and C respectively. The membrane was then probed for miR-can1. U6 RNA was used as loading control. M: DNA ladder, N: RNA ladder.

The membranes in the figures 13a and 13b were probed for mir1177 and miR-can1 respectively. Based on the signal pattern observed on the membrane, it is clear that mir1177 and miR-can1 are expressed during all developmental stages.
4. Discussion

Biogenesis of certain small RNAs in *Dictyostelium discoideum*

Some clues about the biogenesis of small RNAs can be gleaned from the identification of their 5’ and 3’ ends. In plants, siRNAs and miRNAs have one phosphate at their 5’ ends and their 3’ ends are methylated, whereas in *C. elegans*, secondary siRNAs have three phosphates at their 5’ ends, and the status of the 3’ ends is unknown. Based on the 5’ and 3’ end composition of small RNAs in *D. discoideum*, their biogenesis can be related either to small RNA biogenesis in plants or in animals. In this study, 5’ ends of certain small RNAs were studied by different enzymatic treatments (Ap, T4PNK, TE, TAP) and 3’ ends were studied by treatment with periodate.

5’ end analysis of small RNAs

It has been shown that RNAs treated with Ap migrate slower than untreated RNAs (figures 7a and 7b). The signal in lane A (untreated RNA) appears to be in the same position as in lane C (Ap+T4PNK treated RNA) which indicates the presence of one phosphate at the 5’ ends of DIRS-1 siRNA and mir1177. To confirm that DIRS-1 siRNA and mir1177 have indeed one phosphate at their 5’ ends, enriched small RNAs were treated with another set of enzymes (TE, TAP). TE does not degrade RNA containing three phosphates at its 5’ end, whereas TAP converts RNA with 5’-triphosphate to 5’-monophosphate. From the figures 8a and 8b, presence of the signal in lanes A (untreated RNA) and C (TAP treated RNA) and absence of signal in lane B (TE treated RNA), confirms the presence of one phosphate at the 5’ ends of DIRS-1 siRNA and mir1177 (Table 2). Other studies in our group have shown that DIRS-1 siRNA is Dicer B independent but to some extent requires the presence of RdRP (data not shown). The presence of one phosphate at the 5’ end of DIRS-1 siRNA suggests that RdRP might be involved in the production of a longer antisense RNA molecule which is then further processed by Dicer A. This is analogous to the situation in plants.

3’ end analysis of DIRS-1 siRNA and mir1177 (β-elimination assay)

In plants 3’ ends of siRNAs and miRNAs are modified, whereas in *Drosophila* only siRNAs are modified at their 3’ ends. The reason for this modification is due to the addition of a 2’-O-methyl group by Hen1, an S-adenosyl methionine dependent methyl transferase. The
function of the 3’ end modification in plants is to protect siRNAs and miRNAs against 3’-terminal degradation, but in *Drosophila*, the function remains unknown. To study 3’ ends of small RNAs in *D. discoideum*, β-elimination assay was performed. Periodate is a chemical that only reacts with RNAs containing vicinal “diols” (adjacent 2’ and 3’ hydroxyl groups) which are further oxidized to aldehydes and upon treated with borax/boric acid buffer results in the β-elimination, i.e. in the cleavage of one nucleotide from their 3’ end (Figure 14).

**Figure 14: β-elimination assay.** RNA containing vicinal “diols” upon treatment with periodate undergoes oxidation to aldehyde and subsequent treatment with base leads to β-elimination.

From figures 10a (lanes C and D) and 10b (lanes A and B) it was clear that mir1177 and DIRS-I siRNA have no modification at their 3’ ends, because of the shift in periodate treated RNA samples (due to loss of one nucleotide) compared to untreated RNA samples.
Table 2: Comparison of 5’ and 3’ ends of small RNAs in different organisms.

<table>
<thead>
<tr>
<th>Organism</th>
<th>5’ end</th>
<th>3’ end</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plants</td>
<td>one phosphate</td>
<td>methylated</td>
<td>(Bin Yu, Liu Bi, et al., 2010)</td>
</tr>
<tr>
<td>Animals (secondary siRNAs in C. elegans)</td>
<td>three phosphates</td>
<td>not known</td>
<td>(Bailon-Morcombe DC., 2007)</td>
</tr>
<tr>
<td>Animals (siRNAs in Drosophila)</td>
<td>one phosphate</td>
<td>no modification</td>
<td>(Diarness, Zamore PD, et al., 2002)</td>
</tr>
<tr>
<td>D. discoideum (DIRS-I siRNA and mir1177)</td>
<td>one phosphate</td>
<td>no modification</td>
<td>(From this work)</td>
</tr>
</tbody>
</table>

The biogenesis of DIRS-I siRNA and mir1177 in *D. discoideum* is more similar to animals (Table 2).

**Localization studies**

In order to understand where in the cell small RNAs function, cellular localization studies were performed. For example, if small RNAs are localized within the nucleus, it may indicate that they are involved in transcriptional regulation. From figure 11, the presence of signal only in the total RNA lane and absence of signal in the nuclear lane suggests that mir1177 might be localized to the cytoplasm. When the membrane was probed for U2 RNA, the signal intensity was stronger in the total RNA lane than in the nuclear RNA lane (Figure 12) indicating that gel loading concentrations of nuclear RNA and total RNA should be further quantified.

**Filter development of *D. discoideum***

In order to see whether miRNAs (mir1177 and miR-can1) precursors cloned into different pDM 304 vectors carrying constitutive promoter are expressed during all developmental stages of *D. discoideum*, AX2 cells over expressing specific miRNAs (mir1177, miR-can1)
were developed for 16 h and 24 h. The samples from 0, 16 and 24 h were loaded in the lanes A, B and C respectively on 15 % PAGE (Figures 13a and 13b). From the figures, it was clear that mir1177 and miR-can1 were expressed during all stages of development indicating that actin promoter is active in all developmental stages. The time span of the project has not allowed me to work with other miRNAs (mir1176, miR-can2) precursors.

**Significance of this study**

It has recently been realized that small RNAs play a significant role in the regulation of gene expression in many eukaryotes during their development. This led to an increased interest in the identification and characterization of small RNAs in different organisms. Until recently, very little was known about small RNAs in *D. discoideum*. Our research group was the first to identify small RNAs in this organism. However, their function and biogenesis in *D. discoideum* are yet unknown. In evolutionary perspective, *D. discoideum* is placed between plants and animals. This project represents therefore the first step towards answering the question whether small RNA biogenesis in this organism is more similar to plants or animals.
5. Acknowledgements

I am extremely thankful to Fredrik Söderbom for giving me an opportunity to work in his group and for his excellent guidance and timely suggestions. I extend my gratitude to Lotta Avesson for her excellent supervision throughout my project and for all the help in clarifying my doubts regarding project. Many thanks to Åsa Fransson for being my friend and for her motivation and valuable suggestions to plan my future research career. I also want to thank my parents and my near and dear for their moral support and good wishes. Lastly, I would like to thank all people in the department for being so kind and good to me and also thankful to M. Grimson, R. Blanton Texas Tech University for the title page picture.
6. References

7. Appendix

(1) Treatment of RNA with Alkaline Phosphatase (AP)

\[
\begin{align*}
\text{RNA with one phosphate at its 5'} \rightarrow & \quad \text{AP} \rightarrow \text{RNA with 5'} \text{hydroxyl group} \\
\text{RNA with three phosphate at its 5'} \rightarrow & \quad \text{AP} \rightarrow \text{RNA with 5'} \text{hydroxyl group}
\end{align*}
\]

(2) Treatment of RNA with T4 Polynucleotide Kinase (T4 PNK)

\[
\begin{align*}
\text{ATP} & \quad \xrightarrow{T4PNK} \quad \text{RNA with 5'} \text{hydroxyl group} \\
\text{RNA with 5'} \text{hydroxyl group} + & \quad \text{T4PNK} \rightarrow \text{RNA with one phosphate at its 5'} \text{end}
\end{align*}
\]

(3) Treatment of small RNA with Terminator Exonuclease (TE)

\[
\begin{align*}
\text{RNA with one phosphate at its 5'} \rightarrow & \quad \text{TE} \rightarrow \text{Cleaved RNA} \\
\text{RNA with three phosphate at its 5'} \rightarrow & \quad \text{TE} \rightarrow \text{Un cleaved RNA}
\end{align*}
\]

(4) Treatment of small RNA with Tobacco acid pyrophosphatase (TAP)

\[
\begin{align*}
\text{RNA with three phosphate at its 5'} \rightarrow & \quad \text{TAP} \rightarrow \text{RNA with one phosphate at its 5'} \text{end}
\end{align*}
\]