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Natural antisense transcripts in Dictyostelium discoideum

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Abstract

Endogenous RNAs with antisense (as) orientation towards protein coding genes have been found in various organisms. The aim of the project was to study a small RNA from an extensive cDNA library complementary to the protein coding gene rcc1 in the model organism *Dictyostelium discoideum*. The following questions were asked (i) does this small asRNA derive from a longer transcript, (ii) does the expression of the asRNA and its mRNA vary during development and (iii) does the expression change in mutant strains lacking various RNAi related proteins? The results showed that a longer antisense transcript towards the gene *rcc1* most likely exists, and that asRNA and mRNA are developmentally regulated. Furthermore, the asRNA expression could not be detected in growing AX4 mutant strain lacking the RNA dependent RNA polymerase homolog, RrpC, which suggests that this protein might be involved in the asRNA production using an mRNA as a template.

Keywords

RNA, RNA interference, Natural antisense transcript, siRNA, gene regulation, *D. discoideum*

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Monika Hodik

Sammanfattning

Endogena RNA med antisense orientering mot protein-kodande mRNA, även kallade naturliga antisense transkript (NAT), har hittats i ett flertal organismer. Man har spekulerat om att dessa RNA skulle kunna kontrollera uttrycket av sina motsvarande mRNA genom t ex RNA interferens (RNAi). Dokumenterade studier i t ex *Arabidopsis thaliana, Xenopus laevis*, *Homo sapiens*, *Drosophilia melanogaster* samt *Dictyostelium discoideum* har alla visat att ett antisense-medierat mRNA uttryck förekommer. Under mitt examensarbete har jag valt att studera ett litet RNA (20 nt) med motsatt orientering (antisenseorientering) till ett mRNA som kodar för ett hypotetiskt protein med en RCC1 domän i modell organismen *D. discoideum*. Detta utvalda RNA kommer från ett omfattande cDNA-bibliotek som tagits fram med storskalig sekvensering av RNA från denna organism, i samarbete med Prof. Victor Ambros, Dartmouth Medical School, USA. I biblioteket kan man hitta ytterligare ett femtiotal små RNA (17-24nt) i antisense riktning mot mRNA. De frågor jag ville ha svar på i mitt projekt är: (i) Kommer detta lilla antisense RNA (asRNA) från ett längre transkript, (ii) är uttrycket av detta transkript och dess motsvarande mRNA utvecklingsreglerat (dvs skiljer sig uttrycket av RNA under amöbans svältcykel), samt (iii) skiljer sig uttrycket i stammar där man tagit bort olika RNAi-relaterade gener? För att få svar på den första frågan användes Reverse Transcription-PCR (RT-PCR) och på de två övriga frågorna Northern Blot-analyser. Vi gjorde även ett försök att anrika små RNA molekyler. Detta gjordes med hjälp av en YM-50 kolonn som bara släpper igenom nukleinsyror som är mindre än 125 baser långa. Våra resultat visar att asRNAt mot *rcc1* med stor sannolikhet kommer från ett längre transkript, men p.g.a. kontamination i den negativa kontrollen, så måste försöket upprepas innan man kan säga detta med säkerhet. Vidare visade Northern Blot-analysen att dessa asRNA och mRNA är utvecklingsreglerade. asRNA kunde dessutom inte detekteras i en av de mutanta stammarna som saknar det RNA-beroende RNA polymeraset, RrpC. Detta leder till spekulationen att RrpC kanske kan vara inblandad i generering av asRNA genom att t.ex. använda mRNA som templat.

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CONTENT

ABBREVIATIONS

asRNA	antisense ribonucleic acid
dsRNA	double stranded ribonucleic acid
ncRNA	Non-coding RNA
ORF	Open Reading Frame
rRNA	Ribosomal RNA
tRNA	Transfer RNA
snRNA	Splicesosomal RNA
snoRNA	Small nucleolar RNA
siRNA	Small interfering RNA
miRNA	Micro RNA
NAT	Natural antisense transcript
RT-PCR	Reverse Transcripion Polymerase Chain Reaction
THRA	Thyroid hormone receptor alpha
RISC	RNA induced silencing complex
RdRP	RNA dependent RNA polymerase
cAMP	Cyclic adenosine monophosphate

CHAPTER 1: INTRODUCTION

1.1 Non-coding RNA

Non-coding RNAs (ncRNAs) are defined as RNA molecules that have a biological function in the cell other than being a template for protein translation. They usually lack an extensive Open Reading Frame (ORF) and have other features like many stop-codons [1]. Just a couple of decades ago the only known ncRNAs were the ribosomal RNAs (rRNAs), transfer RNAs (tRNAs) spliceosomal RNAs (snRNAs) and small nucleolar RNAs (snoRNAs). This list has radically expanded during the past years and new classes of RNAs have successively been added like, for example, small interfering RNAs (siRNAs), micro RNAs (miRNAs) as well as new members of old classes [1,2,3]. It is believed that about 40 % of the human genome is transcribed into RNA. About 2 % of these transcripts are thought to be further translated into proteins, whereas the majority of the remaining 38 % are believed to become ncRNAs [2]. Biological functions connected with ncRNAs have been reported to involve RNA cleavage and ligation, gene silencing, DNA imprinting and chromatin structure dynamics, just to mention a few. Furthermore, ncRNAs have been connected to different types of diseases, like e.g. cancer [3]. This all together confirms that ncRNAs have a bigger importance than earlier anticipated.

Based on their size, ncRNAs are usually divided into three classes: 18-25 nt ncRNA which consists of so called microRNAs (miRNAs) and small interfering RNA (siRNAs), <300 nt ncRNA and ncRNA up to and larger than 10 000 nts (e.g. Xist which is involved in X-chromosome silencing [4]).[3]. The ncRNAs do usually not carry out their functions by themselves, but interplay with several proteins [2].

In this degree project I focused on siRNAs, or more specifically siRNAs derived from natural antisense transcripts (NATs).

1.2 Natural Antisense Transcript (NAT)

Natural antisense transcripts (NATs) are endogenous RNAs (both protein coding and non-coding) that are complementary to other RNA transcripts. They are usually divided into two groups: *cis*-NATs that are transcribed from the opposite DNA-strand of a locus and *trans*-NATs that are transcribed at a different location. *Cis*-NATs show perfect sequence complementarity to their target, while *trans*-NATs can base-pair imperfectly and thus target a larger number of transcripts. NATs have been found in viruses, prokaryotes and eukaryotes. Examples of organisms in which NATs have been documented include mice, rats, chickens, *Drosophila melanogaster*, nematodes, rice, yeast, *Arabidopsis thaliana*, as well as humans [5]. Based on cDNA and EST libraries that have been compared to genomic sequences, it has been estimated that 4%-9% of human genes, up to 22% of *D. melanogaster* genes and 10 % of *A. thaliana* genes overlap in a *cis*-NAT manner [6].

Cis NATs, can further bee divided into three classes, depending on how they overlap: head-to-head (5' overlaps), tail-to-tail (3' overlaps) or "internal" overlaps which means that one transcript completely covers the other transcript [5].

1.2.1 Biological Function of NATs

The biological function of NATs is still very obscure, but they seem to act through several gene regulation mechanisms. Four of these are presented below:

1. **Transcriptional interference**: This phenomenon was first observed in the late 70s when Ward and Murray saw that convergent transcription between *trp* and λP_L promoters in the λ*trp* transducing vectors of a bacteriophage resulted in impaired expression of both genes. They suggested that two RNA II polymerases, transcribing in opposite directions, might sterically collide and thereby terminate the transcription [7].

2. **RNA masking**: NATs could bind to a splice site and mask that region, making it "invisible" for the spliceosome and thereby creating different protein isoforms. One known case is the human thyroid hormone receptor alpha (THRA), where the antisense transcript (RevErbAα) is able to mask the transcript in a way that either TRα1- or TRα2 isoform is formed [8].

3. **RNA interference**: The NATs can potentially bind to its complementary sequence and form a dsRNA. If the duplex strand is long enough, gene silencing by RNA interference (RNAi) might occur, giving rise to so called nat-siRNAs [8]. One example of this type of gene regulation was recently found in *A. thaliana,* in which two overlapping mRNAs (one induced upon exposure of the plant to elevated salt concentrations) were processed by the RNAi machinery, giving rise to siRNAs [6].

4. **Chromatin remodelling**: Several NATs, such as the mammalian Xist RNA are believed to transcriptionally silence genes by recruiting histone modifying enzymes [4,5].

1.3 RNA Interference (RNAi)

RNA interference (RNAi) is defined as "a process initiated by a double stranded RNA which inhibits gene expression in a sequence specific fashion" [9]. It is not only a mechanism for cells to defend themselves against viruses carrying dsRNA, keeping transposons under control and maintaining heterochromatin, but also a powerful research tool[10]. The phenomenon was first described by Andrew Fire and Craig Mello in 1998. They noted that, when microinjecting a long dsRNA into the nematode *Caenorhabditis elegans*, one strand being in sense direction of an RNA coding for a muscle protein and the other one in the antisense direction, the worm developed a twitching phenotype, identical to the knockout phenotype of the same gene. They also noted that the mRNA encoding the protein disappeared. Encouraged by these remarkable observations they went on and tested dsRNA with sequence specificity towards other genes, and intriguingly they observed repression also of these genes [11]. Both scientists were awarded the Nobelprize in Medicine and Physiology in 2006 for their extraordinary finding.

A more recent study, which also was performed on C. *elegans,* has suggested that the dsRNA that enters the cell exogenously shares a similar RNAi pathway as the one that originates from within the cell (e.g. from overlapping genomic sequences) [12].

1.3.1 Mechanism of RNAi

1.3.1.1 The actors

The mechanism of RNAi is found in basically all eukaryotes. There are so far two small ncRNAs that are known to be involved in the in the RNAi machinery. One is the so called small interfering RNAs (siRNA), which originate from longer dsRNAs that has entered the cell either exogenously (e.g. with a virus) or from NATs. The other one is the related microRNA (miRNA), which derives endogenously from longer pre-cursors in the genome, so called pre-miRNA. So far miRNA have only been reported in plants and animals. A schematic picture of the RNAi pathway is shown in Figure 1. In addition, there are several co-players (proteins) of equal importance to miRNAs and siRNAs. These are: (i) the ribonuclease III-like protein, Dicer, (ii) all the proteins involved in the RNA induced silencing complex (RISC-complex) of which the Argonaute-Piwi protein has a prominent role and (iii) the RNA dependent RNA polymerases (RdRPs). RdRPs are proteins that are able to make double stranded RNA from RNA templates (sometimes using a primer and sometimes not) and are believed to amplify the silencing effect of RNAi. They are present in some organisms like nematodes and plants, but have not been found in mammals or flies [13,14,15].

1.3.1.2 siRNA

When a dsRNA enters the cell, the structure is recognized by the ribonuclease III-like protein, Dicer, which cleaves it into approximately 21-25 nt long sequences with a 2-nt long 3' overhang. The short dsRNAs (siRNAs) are, in turn, incorporated into the RISC complex which is able to unwind the duplex. A part of the RISC complex, the so called Argonaute-Piwi protein, will remain bound to one RNA strand. Eventually this RNA-protein complex finds a complementary RNA strand, base-pairs to it and induces endonucleolytic cleavage. The cleaved RNA can no longer serve as a template for protein synthesis, and the expression of the gene is thereby "silenced". Huge numbers of siRNAs have been found in various eukaryotes, but the biological function of the majority of these siRNAs is still unknown [13,14,15].

1.3.1.3 miRNA

miRNAs are found in multicellular eukaryotes and are processed from long imperfectly base-paired hairpin precursors, so called pre-miRNA. The pathway is otherwise similar as for the siRNA. Because animal miRNAs can bind in the 3' untranslated region with just a few numbers of base-pair complementarity it is believed that approximately one third of the human gene expression is regulated by miRNAs [14,15].
Since the first miRNA was found in *C. elegans* in 1993, there has been an explosion of articles of observed miRNAs in various organisms and the number is steadily increasing [13,14,15].

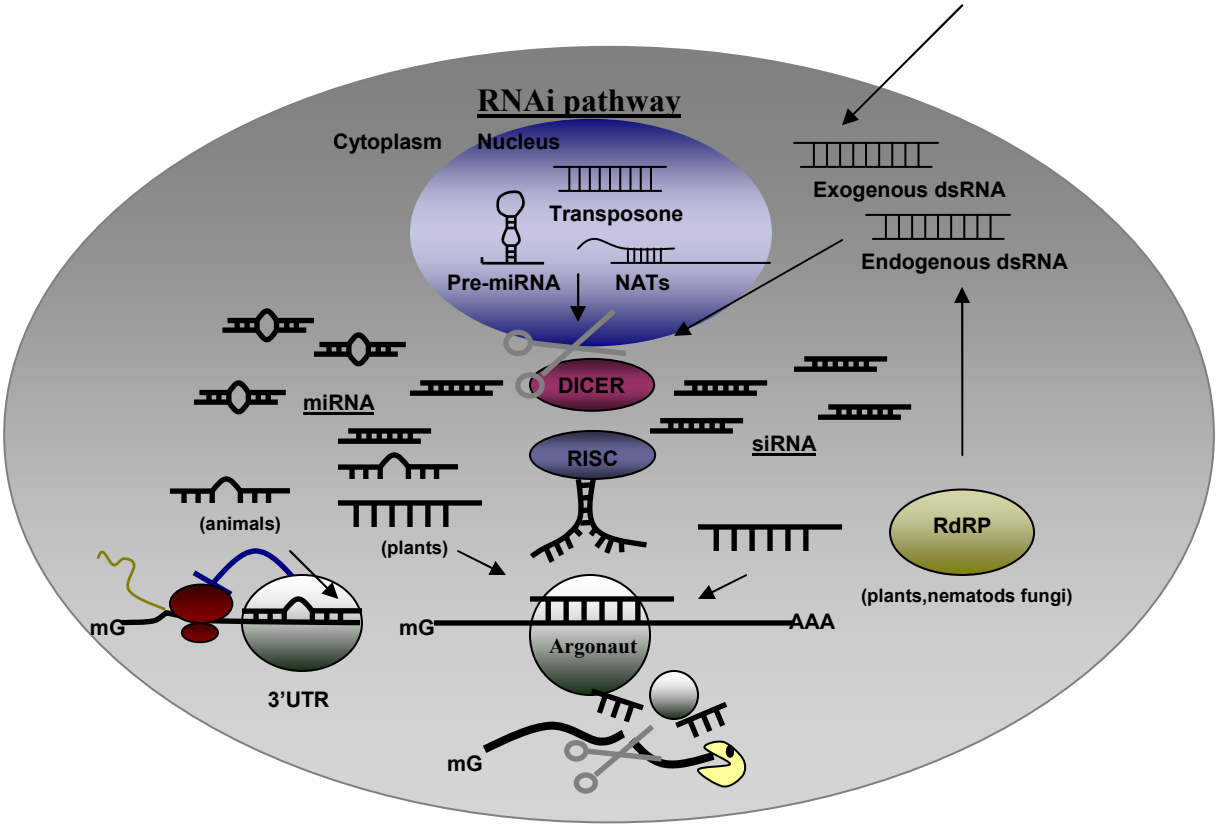


Figure 1 General model of the RNAi pathways for siRNA and miRNA. Double stranded RNA is recognized by the DICER protein which chops it into smaller fragments. These fragments are in turn separated by a protein complex termed RISC. Furthermore the small, separated fragment can bind to a complementary mRNA which can either lead to blocking of translation or endonucleotic cleaving of the mRNA. In some organisms there exists a RdRP protein which is believed to be able to make a double stranded RNA from a single stranded RNA and thereby amplifying the silencing effect.

1.4 *D. discoideum* – a model organism

D. discoideum is the best-studied member of a group of species called social amoebas or slime molds and has provided valuable knowledge about e.g. cell motility, signalling and interaction [16].

It was first described from a finding in a forest in North Carolina in 1935, but the organism can be found in the soil all over the world [17]. As an amoeba, it belongs to the mycetozoa on the phylogenetic branch, after plants and before fungi (Figure 2) [18].

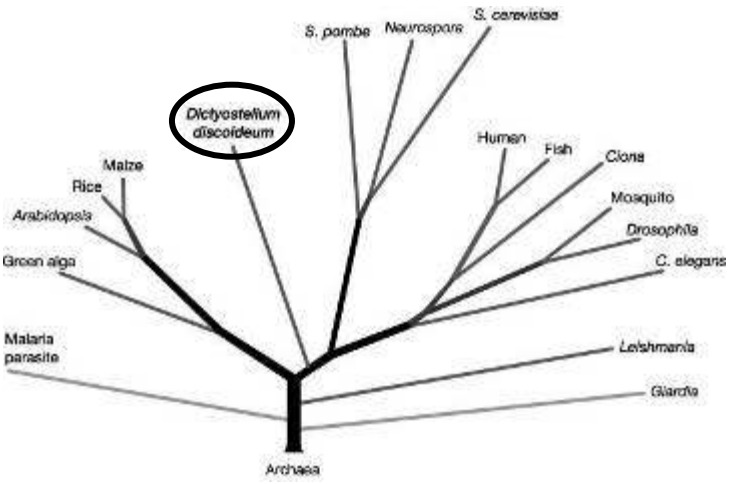


Figure 2 On the phylogenetic tree, *D. discoideum* is placed after the plant branch and before the division between fungi and animals [22].

1.4.1 Developmental stages at starvation

Dictyostelium discoideum cells live independently in the soil where they feed on bacteria and yeast. When the food is consumed the amoeba's life cycle dramatically changes, leading to an onset of several starvation pathways. One of these starvation pathways leads to the production of the second messenger molecule cyclic adenosine monophosphate (cAMP) which is secreted from the cell in pulses about every six minutes. These molecules are recognized by the receptors of surrounding amoebas and together they start to move towards a gradient of higher cAMP concentration, a process called chemotaxis. When about 100 000 cells have assembled they form a relatively flat structure called a loose aggregate. This process usually takes 8 hours under laboratory conditions. During the following sixteen hours the amoeba undergoes several developmental changes which eventually lead to a fruiting body. The different stages during *D. discodium* are shown in Figure 3. The first step after the loose aggregate is the tight aggregate, or mound. This is followed by a structure called the tip. The cells in this complex show an organized polarity. The front part, or tip, consists of cells that will later become prestalk cells and they control the movement of the cells behind them that will later become prespore cells. It is approximated that about 20 % of the cells will become prestalk cells and the remaining 80 % prespore cells.

The tip can further elongate itself creating a so called finger or standing slug. This structure can then either continue in the developmental cycle or fall over, becoming a slug or pseudoplasmodium. Slugs are motile and are able to move towards light and shallow heat

gradients. Eventually the slug rises up again in a standing position and continues its last part of the developmental cycle, also called culmination. In these stages the prestalk cells successively move down an axis, become vacuolated as they make cellulose and die. The prespore cells, on the other hand, move upwards, where they eventually become encapsulated. The cells can then stay encapsulated for a very long time. When sensing bacteria, or more precisely folates secreted by the bacteria, the ball of spores bursts open, releasing the ameobas and allowing them to feast on the prey [16].

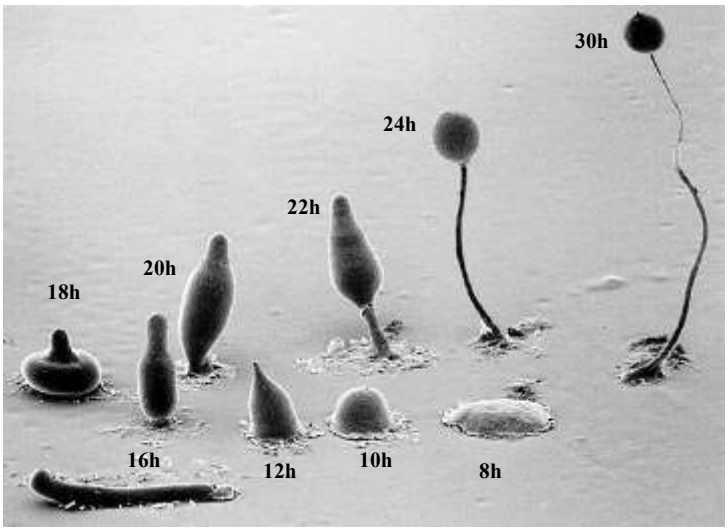


Figure 3 SEM of the structure and time-point of developmental stages of *D. discoideum* at starvation. 8h: Loose aggregate, 10h: Tight aggregate (mound), 12h: Tip, 16h: Slug or finger or pseudoplasmodium (standing slug), 18h: Mexican hat, 20-30h: Culmination steps leading to a fruiting body [16].

The extraordinary feature of the slime mold to swap between unicellularity and multicellularity has made it an attractive model organism. Furthermore it is relatively easy to handle in the laboratory and several genetic tools, that are available in other model organisms, are also available in *D. discoideum*. One example of a genetic tool is homologous recombination which has been used to create several knockout strains where specific gene(s) have been disrupted [19].

1.4.2 Genome

The complete *D. discoideum* AX4 genome assembly was released in 2005. The physical distance spans 33,817 kb distributed over six chromosomes. The number of protein encoding genes is estimated to around 12500. The genome is very (A+T)-rich (overall 77.7%), especially in the intergenic regions [18].

1.4.3 RNA interference

The *D. discoideum* genome encodes several proteins that are homologous to components of the RNAi machinery. These include two Dicer-like proteins, DrnA and DrnB and three RNA-dependent RNA polymerases, RrpA, RrpB and RrpC. Among these proteins, only RrpA is required for transgenic RNAi [19]. Interestingly a helicase domain, which is may be important for unwinding dsRNA has been found in all three of the RdRPs and not in any of the Dicers which in other organisms usually have this domain. Other RNAi-related genes in the amoeba are *helF-1,* which also appears to have a helicase domain [20]. All the mentioned RNAi related proteins have been successfully knocked out by molecular tools like e.g. homologous recombination allowing the study of the RNAi mechanism in this organism. No RISC complex has yet been identified, but five genes and one partial gene has been found that are predicted to encode proteins with Piwi-domains which are a part of the RISC complex [21].

1.4.4 Gene regulation by natural antisense transcripts

So far only one gene, *psvA*, has been reported to be regulated by a natural antisense transcript in *D. discoideum*. This gene is constitutively transcribed both in growing and developing cells but only starts to accumulate during aggregation and prespore/prestalk assembly. During the growth phase a 1.8 kb non-coding RNA in antisense orientation has been found to be transcribed on the opposite strand and seems to decrease the stability of the mRNA [22].

Antisense RNAs towards three more genes have been investigated: *hatA*, *rsmF* and *DDB0230011*. Both the antisense RNAs and their mRNAs seemed to be developmentally regulated in the mentioned study. Furthermore, longer antisense transcripts could be detected for all three genes with RT-PCR, but only for *hatA* with Northern Blot analysis. Unfortunately *hatA* is, if not identical, at least very closely related to two other genes (*hatB* and *hatC*), which makes it difficult to know which of the genes is actually targeted during the hybridization [23].

CHAPTER 2: MATERIALS AND METHODS

2.1 **Chemicals and reagents**

All chemicals and reagents are purchased from Invitrogen if not otherwise stated.

2.2 **Oligonucleotides**

DNA oligonucleotides (Invitrogen) used in this study are listed in Table 1.

Table 1 Oligonucleotides used in different applications and their respective melting temperature (T_m) calculated by a program from CyberGene AB. The sequences are shown in 5'-3' direction. NB indicates use of oligo in Northern Blot-analysis.

Name	Primer	T_m(C°)
rcc1_forw	**TGGTCGATTTCTCATCATATGA**	57.8
rcc1_rev	**GGTACAATTGTGATACCATTCT**	57.8
oAH1	**GAACTCTCACGACTCATACTCATACTTATA**	66
oAH5	**TGTTACCATCCTCAATTCTTGGTATA**	62
M13_forw	**CAGGAAACAGCTATGAC**	49,8
M13_rev	**GTAAAACGACGGCCAG**	48.2
T7 - pCR2.1TOPO forward	**GGTAATACGACTCACTATAGGG-** **-CAGTGTGATGGATATCTGCA**	75.3
T7-pCR2.1TOPO reverse	**GGTAATACGACTCACTATAGGG-** -GAGCTCGGATCCACTA**GTAA**	75.3
Ddsi1	**ACCTCGATTGGAGTCAATGGA**	57.6

Name	Analysis
rcc1_forw	RT-PCR, Semi-colony PCR
rcc1_rev	RT-PCR
oAH1	RT-PCR, Semi-colony PCR
oAH5	NB
M13_forw	Semi-colony PCR
M13_rev	Semi-colony PCR
T7 - pCR2.1TOPO forward	*In vitro* transcription (NB)
T7-pCR2.1TOPO reverse	*In vitro* transcription (NB)
Ddsi1	RT-PCR

2.3 Growth and development of *D. discoideum*

The AX4 strain of *D. discoideum* was initially grown on a lawn of *Klebsiella aerogenes.* The cells were then transferred to HL5 medium with additional Penicillin-Streptomycin (GIBCO) and put on a shaker at 22°C for continuous growth. For synchronous development, 5 x 10^7 cells were collected, centrifuged at 300 g at 22ºC for 5 minutes. The supernatant was discarded and the cells were washed with room temperate Phosphate-Buffered Salines (PBS), followed by another centrifugation. After yet another washing and centrifugation, the cells were spread on a nitrocellulose filter that had been moistened in advance with PBS. The cells

were then kept in a 22°C moist chamber until they reached the desired developmental stage [24].

2.4 Total RNA preparation and DNase treatment

2.4.1 Cell harvesting

10^8 growing cells ("0h") or ~5 x 10^7 developed cells ("16h" or "24h") were collected and centrifuged at 300 g at 4°C for 5 minutes. Next, the cells were washed in cold PBS and the centrifugation step was repeated, followed by another wash. Subsequently the pellet was resuspended in 1 mL TRIzol and stored in the -80ºC freezer before continuing with the RNA extraction.

2.4.2 RNA extraction

200 µl chloroform was added to the TRIzol treated cells from the previous step to separate the organic phase from the aqueous. This was followed by vortexing, incubation for 3 minutes in room temperature and centrifugation for 15 minutes at 16523 g. The aqueous phase (upper phase) was transferred to a new tube and the RNA was allowed to precipitate in 500 µl isopropanol for 10 minutes in room temperature. Next, the sample was centrifuged at 17 900 g for 10 minutes. The supernatant was discarded and the pellet washed in 1 mL 70% EtOH, followed by another centrifugation at 17 900 g for 5 minutes. The pellet was left to air dry in room temperature for five minutes and was then dissolved in RNase free water. The amount of water depended on the size of the pellet. Eventually the RNA concentration was measured in a spectrophotometer (NanoDrop).

2.4.3 RNA Precipitation

To concentrate the RNA and remove any left-over chemicals from the RNA extraction, precipitation was usually necessary. 0.1 vol 3M NaOAc and 2-3 vol ice cold 95% EtOH was added to the RNA sample, which was then left to incubate for at least 30 minutes or overnight at -20ºC. If the pellet was small an additional 1 µg glycogen was also added to visualize the precipitate. The sample was subsequently centrifuged at 17 900 g in 4 ºC for 30 minutes. The supernatant was removed and the pellet was washed with 70% EtOH (about the same amount as previous total volume), followed by centrifugation at 17 900 g at 4 ºC for 5 minutes. The pellet was, as previously, left to air dry in room temperature for 5 minutes before dissolving it in RNase free water.

2.4.4 DNase Treatment

DNase treatment was performed to remove all genomic DNA from the RNA sample prior to RT-PCR. 100 units FPLCpure DNase I (Amersham) was added to every 50 µg RNA in 40mM Tris HCl with pH 7.5 and 6 mM $MgCl_2$ and subsequently incubated at 37 ºC for 10 minutes. 300 µl water and 400 µl phenol was added to the 100 µl DNase treated solution. The mixture was vortexed for 15 seconds and centrifuged at 17 900 g for 5 minutes in room temperature. 350 µl of the upper phase was transferred to a new tube. 50 µl water and 400 µl chloroform was added to the 350 µl solution from the phenol extraction and centrifuged as previously described. The DNase treatment was ended with an RNA precipitation (see section 2.4.3).
DNase treatment was subsequently repeated.

2.5 Reverse Transcription PCR (RT-PCR)

2.5.1 First Strand cDNA-synthesis

2 µg 2xDNase treated RNA was mixed with 1µl sense or antisense primer (20pmol/µl), 2µl dNTP (10mM) and ddH_2O up to 12µl. The sample was incubated at 65°C for 5 minutes. Next, an 8µl mastermix was added containing 4µl 5x cDNA buffer, 1µl DTT (0.1M), 5µl RNA Guard™ (25.2U/µl), 1µl ddH_2O and 1 µl ThermoScript™ RNase H^- (15U/µl). For the negative control, ThermoScript™ was replaced with ddH_2O. The sample was incubated at 55°C for 20 minutes, 60°C for 20 minutes and at 65°C for 20 minutes. The transcriptase was inactivated by heating at 85°C for 5 minutes. Next the sample was cooled on ice and spun down. To remove any RNA remnants 1µl RNase H (2U/µl) was added and the sample was incubated at 37°C for 20 minutes and then put in a -20°C freezer, until continuing with the PCR reaction.
All incubations during cDNA synthesis as well as the PCR reaction were carried out in a thermal cycler (Techne).

2.5.2 PCR

1µl cDNA template from the previous step was mixed with 2.5 µl 10xTaq buffer, 0.5 µl dNTP (10mM), 0.75 µl of the rcc1_rev primer (20pmol/µl), 0.75 µl of the rcc_forw primer (20pmol/µl) and 0.335µl Taq-polymerase (5U/µl). As controls 0.14 µg genomic DNA (with thr rcc_1 primers) and 0.5 ng of the unrelated PA28REG plasmid (with primers oAH1 and oAH2) was used. The PCR conditions were as follows: 95°C for 1 minute (denaturation step), 53°C or 58°C for 1 minute (primer annealing step) and 60°C for 2 minutes (extension step). The number of PCR cycles was 35. The PCR reaction was terminated by a final extension step at 60°C for 7 minutes, allowing newly synthesized fragments to fill up.
The PCR products were eventually analyzed on a 2 % agarose gel.

2.6 pCR® TOPO® 2.1-cloning

2.6.1 Adding extra A nucleotides to the 3' ends of PCR-product

To incorporate the PCR-product into the vector, an extra A nucleotide at the 3' ends of the PCR product was required. The overhang was achieved by mixing 15 µl of the PCR product with 2 µl ddH_2O, 2 µl 10xTaq buffer, 0.5 µl dNTP (10mM) and 0.5 µl Taq-polymerase (5U/µl) and incubating it at 72°C for 30 minutes.

2.6.2 Cloning

2 µl PCR-product from sense cDNA was mixed with 1 µl salt solution (provided with the kit), 0.3 µl pCR® TOPO® 2.1 vector and ddH_2O up to 6 µl. The samples were then incubated at room temperature for 30 minutes and cooled on ice.

2.7 Transformation

25 µl competent DH5α cells were thawed on ice for 5 minutes. They were then mixed with 2 µl of the ligation mix (see section 2.6.2). Next the cells were heat-shocked at 42°C for 30 seconds, allowing the vectors to be transferred across the membrane, followed by cooling on ice for two minutes. The samples were diluted in 200 µl Luria-Bertani (LB) medium. 10 µl from this solution was then further diluted in 200 µl LB medium and plated out on pre-warmed (37°C) LA-plate containing 50µg/µl ampicillin. The remaining cells were spread on a separate plate. As a positive control 10ng a PA28 plasmid was used, which is easily transferred across the membrane. The negative control consisted of water. The plates were put in a 37°C incubation chamber overnight for colony growth.

2.8 Semi-colony PCR

Cells from one colony were picked with a sterile tooth pick and transferred to a Falcon tube with 2 mL LB-medium and 50 µg/mL ampicillin. The tube was then put in a shaking chamber at 37°C to allow cell growth. After 2 hours 50 µl of the cell-culture was transferred to a PCR tube, incubated for 10 minutes at 95°C and subsequently cooled in ice water. Next, 1 µl of the cell extract was mixed with 0.5 µl dNTP (10mM), 2.5 µl 10 x Taq buffer, 0.25 µl M13_forw primer (20pmol/µl), 0.25 µl M13_rev primer (20pmol/µl), 20.3 µl ddH_2O and 0.25 µl Taq Polymerase (5U/µl). The PCR cycle that followed had the parameters: 94°C 2 minutes and (94°C 45 seconds, 55°C 45 seconds, 72°C 2 minutes) x 30. 10 µl of each PCR-product was mixed with 2 µl DNA dye (0.025% bromphenol blue, 0.025% xylene cyanol, 30% glycerol) and analyzed on a 2 % agarose gel.

2.9 Plasmid Preparation and Sequencing

The plasmid purification was performed according to QIAprep Miniprep Handbook. 350ng of each plasmid along with 4pmol of M13_rev primer was sent to Rudbeck laboratory (Uppsala) for sequencing.

2.11 Northern Blot

2.11.1 Gel preparation

Total RNA was prepared as described earlier.10 µg RNA from each sample was subsequently separated on a 1.2 % agarose gel containing 1 x MOPS buffer and formaldehyde (11%, pH 4) and then transferred to a Hybond N+ membrane (Amersham) overnight. To immobilize the RNA on the membrane, UV-crosslinking was carried out at 150mJ. An RNA (High Range, Fermentas) size marker was also separated on the same gel, cut out and bathed in ethidium bromide.

2.11.2 PCR and in vitro transcription

To generate templates for Northern blot 20 ng plasmid from the plasmid preparation (see section 2.9) was mixed with 0.5 µl dNTP (10mM), 2.5 µl 10 x Taq buffer, 0.25 µl Taq polymerase (5U/µl), 0.25 µl T7_rev (20pmol/µl) and Rcc1_rev (20pmol/µl) or 25 µl T7_forw (20pmol/µl) and 25 µl Rcc1_forw (20pmol/µl) and water up to 25 µl. As positive control the same mix was but with 25 µl Rcc1_forw (20pmol/µl) and Rcc1_rev primers (20pmol/µl).As negative control the Taq polymerase was replaced by water. The same PCR cycle was used as described under the PCR-step (see section 2.5.2).

The templates were pre-purified with a G-50 sephadex Quickstep column (see handbook from manufacture). 2 µl of respective template was mixed with 2 µl T7-polymerase (20U/µl, Ambion), 2.5 µl [P^{32}]- α-UTP (20µCi/µl), 2 µl 1 x transcription buffer, 0.5 µl ATP/CTP/GTP, 1 µl UTP (0.1mM) and water up to 20 µl. The samples were then incubated at 37ºC for 60 minutes. Any DNA leftovers were removed by addition of 10 units DNAseI followed by 15 minute incubation at 37ºC. The transcription procedure was finished with yet another G-50 purification step to remove unincorporated nucleotides. 1 µl of the labelled transcript was mixed with RNA dye (91.6 % formamid, 17mM EDTA, 0.025% brom phenol blue, 0.025 % xylene cyanol), and separated on an 8 % polyacrylamide gel to test the quality of the probes.

2.11.3 Pre-hybridization, hybridization and washing

The membrane was pre-hybridized in Church-buffer (7% SDS, 0.5M $NaPO_4$ pH 7.2, 1mM EDTA, 1% BSA) for about one hour at 65ºC to block any unspecific binding sites. The radiolabeled probes were then added and left to hybridize overnight at the same temperature. Next day the membrane was rinsed in 2xSSC/0.1% SDS and then washed in the following order: 2x5 minutes 2xSSC/0.1% SDS, 2x10 minutes 1xSSC/0.1% SDS and 2x5 minutes 0.5xSSC/0.1% SDS. All washes were carried out at 65 ºC. Eventually the membrane was exposed on an imaging plate and analyzed using Phosphorimager and the ImageQuantNT software (Molecular Dynamics).

2.12 Purification of small RNAs

2.12.1 Isolation of small RNAs

To purify small RNAs, an YM-50 centrifugal filterdevice was used. It only allows nucleic acids smaller than 125 bases to pass through its membrane. 400 µl (0.720 µg/µl) RNA extract from growing AX4 cells was briefly denaturated at 95ºC and applied on the filter device. The device was then centrifuged at 14 000g in the cold room for 15 minutes and small RNAs collected in a plastic tube. To obtain the larger RNAs, the membrane was turned upside into a new tube and centrifuged at 3000 g for 3 minutes. The small RNA fraction was ethanol precipitated prior to Northern blot analysis.

2.12.2 Probe labeling

0.4 µl oligo 66 (20pmol/ µl) against a 21 nt small DIRS-1 RNA was mixed with 5 µl γ-ATP (10 µCi/µl), 2 µl buffer A (Fermentas), 1 µl T4-PNK (10 u/ µl, Fermentas) and water up to 20 µl. The sample was incubated at 37 ºC for 30 minutes, followed by G-50 purification.

2.12.3 Northern Blot

0.35 µg small RNA, 10 µg large RNA and 10 µg total RNA was mixed with RNA dye and resolved on a 12% denaturating polyacrylamide gel together with a 5' end-labeled pUCmix8 DNA-ladder (Fermentas). The gel was put in an electroblotter and the RNA was transferred to a Hybond N+ membrane (Amersham) overnight. The membrane was, after UV cross-linking, pre-hybridized as previously described and subsequently hybridized with the radiolabelled probe against small DIRS-1. All the hybridizations were carried out at 42 ºC. Washing was performed as previously described (see section 2.11.3), as well as the development of the image.

CHAPTER 3: RESULTS

3.1 Choosing a small RNA with antisense orientation towards a mRNA

Recently, three small RNAs in antisense orientation to mRNAs were discovered in *D. discoideum* [26]. All of these genes produce longer antisense RNAs. However, these genes are not optimal for expression analysis due to e.g. genes with highly similar relatives making hybridization detection difficult to distinguish. Therefore, when choosing a small RNA from the high-through-put sequence library (based on 454 sequencing of 0h, 16h and 24h RNA) the aim was to cover as many of the following criteria as possible: The small RNA should ideally be (i) 17-25 nts long and (ii) antisense only towards one gene in the genome. Its corresponding mRNA should ideally (iii) encode a protein with a known domain (iv) have a known expression pattern and also (v) known knockout phenotype. Furthermore, the mRNA and asRNA should preferable be detectable by Northern blot, facilitating analysis.

Using these criteria as guidelines we decided to further investigate a 20 nt long RNA, with antisense orientation to the gene *DDB0235388* which encodes a hypothetical protein with an RCC1 (regulator of chromosome condensation) domain. There are presently 21 annotated proteins in *D. discoideum* with this domain, although the isolated RNA is only complementary to *DDB0235388*. The small antisense RNA represented in the 454 library was derived from cells developed for 16h.

DDB0235388, which I from now on will call *rcc1*, can be found on chromosome 3, consists of one exon and exists only in one copy (http://www.dictybase.org). The structure of the *rcc1* gene, along with the position of the isolated small RNA and existing sequence tags (ESTs) is shown in Figure 4.

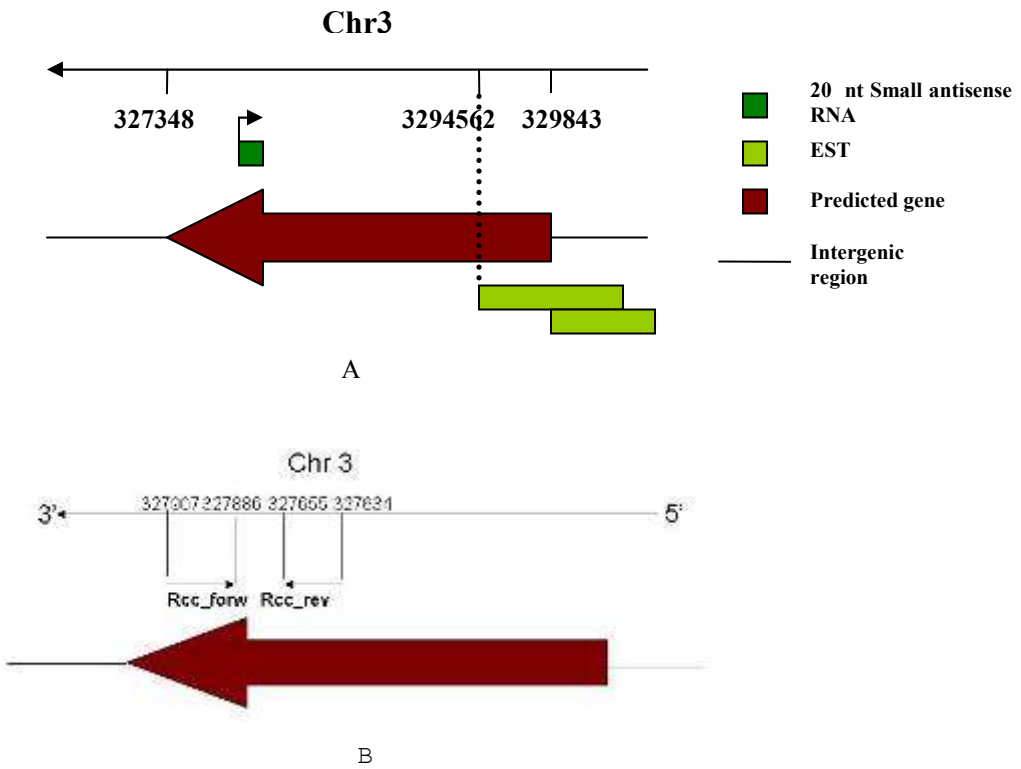


Figure 4 A The predicted gene *DDB0235388* lies on chromosome 3 at the positions 329843-327348 and is believed to encode a protein with an RCC1 domain. B Orientation of the primers used in the study relative to the *rcc1* coding region.

3.1.1 Proteins with RCC1 domains

Proteins with RCC1 domains are chromatin binding proteins. They are the only known guanine nucleotide exchange factors for the Ran GTPase, promoting uptake of Ran GDP and releasing Ran GTP. The released Ran GTPs are, in turn, involved in a various events in the cell, like nucleo-cytoplasmic transport, mitosis and nuclear envelope assembly. It has been demonstrated that when the interaction between RCC1 and the chromatin is disrupted, the cell can not form a mitotic spindle assembly [25]. A multiple alignment comparing the RCC1 domain from *DDB0235388* with RCC1 domains from various organisms is shown in Figure 6.

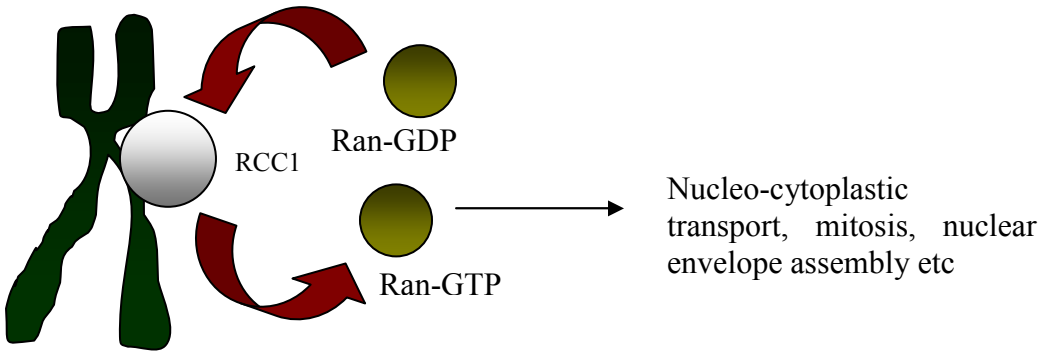


Figure 5 Proteins with RCC1 domains bind to the chromatin. They are the only known guanine nucleotide exchange factors for Ran GTPase.

```
C.auranticus       GGAHTCALTGSGGVMCWGGNDFGQLGDGTPTTRSTPGAVSG--LPSGVTAIAAGNYHTCA 118
M.xanthus          GDAHSLALGADGSVWTWGGNSSGQLGDGTTTDRATPMRVAG--LDS-VVAVAAGDFHSLA 173
H.sapiens          GESHSLALSDRGQLFSWGAGSDGQLGLMTTEDSVAVPRLIQKLNQQTILQVSCGNWHCLA 151
X.laevis           GEAHTLALNDKGQVFSWGHAAHGQIGVSIAEDYIRVPRNIKSLSDIQIVQVACGHHHSLA 151
D.melanogaster     GSRHSLALSDWGQVLSWGDNDCGQLGHATDKEIVQLPKVVRQLVTKTVVQIACGNNHSLA 157
D. discoideum       GNDHSIALTSSSQVLTWGSSSLGRLGQGKTLAIYQPKLVPGLSNIESVYAGGASSAAISK 160
                   *  *: **   . :  **    *::*                    :   ...

C.auranticus       LTGSGGVRCWGANYSGQLGNGL----TFGRSTPGAVSGLPSGVTAIAAGGDHTCALMSSG 174
M.xanthus          LREDGTVWAWGTNFHGQLGRGH----TQPGLTPEQVPGLN-GVVALAAGFDFTLAVLEDG 228
H.sapiens          LAADGQFFTWGKNSHGQLGLGK--EFPSQASPQRVRSLEGIPLAQVAAGGAHSFALSLSG 209
X.laevis           LSKESNIYSWGQNQYGQLGLGS--EKRKESAPRHIKSLSGIPFAHIAAGGGHSFALTISG 209
D.melanogaster     LTSCGELYSWGSNIYGQLGVNSPNDLTHCNYPLRLTTLLGIPLAAIACGGNHSFLISKSG 217
D. discoideum       DTVQRKLYTWGYNKYGQLGINN---QTDQYTPFRCVFFDNIGIKTLAIGDRHMGAIDLKG 217
                         .  ** *  **** .          .          .  :* *  .   :  .*

C.auranticus       GVMCWGDNYYGQLGDGTTTNRRTPVAVSGLP------------------------------ 205
M.xanthus          TVRAWGSNGSGQLGDGTSTQRLSPVKVSELSGITEVRAGTYHALALGKDGGVWTWGSNAS 288
H.sapiens          AVFGWGMNNAGQLGLSDEKDRESPCHVKLLRT------------------------------ 241
X.laevis           AIFGWGRNKFGQLGLNDETDRSDPALLKSLRS------------------------------ 241
D.melanogaster     AVFGWGRNNCGQLGLNDETNRSYPTQLKTLRT------------------------------ 249
D. discoideum       ELYTWGMNEEYQLGDGSSFNKSLPIPIKVKSF-----------------------------
                    :  ** *  .***      .
```

Figure 6. Multiple alignment of various protein sequences with RCC1 domain (parts of) from different organisms. The protein from *D. discoideum* corresponds to the hypothetical protein *DDB0235388.* Stars ("*") indicate identical residues in all organisms and dots correspond to conserved substitutions (":"=conserved substitution, "."=semi-conserved substitution). (ClustalW)

3.2 Does the Small RNA Originate from a Longer Transcript?

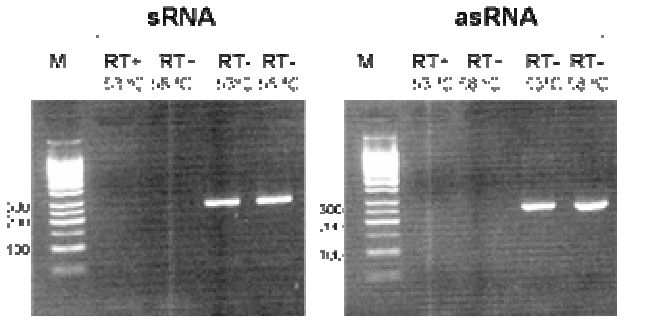
To test if the small antisense RNA, identified in the 454 sequencing, derives from a longer transcript, RT-PCR was performed. The primers were designed to amplify a 274 nt region, including the region corresponding to the small antisense RNA (see Figure 6 below).

To amplify any potential asRNA transcripts, Rcc_forw was first added to create cDNA, followed by PCR using both primer Rcc_forw and primer Rcc_rev. To detect mRNA, Rcc_rev was added to create cDNA followed by the same procedure as for the asRNA.

The amplified RNA sample was a mix between equal amounts of RNA from growing cells, 16h developed cells and 24h developed cells.

3.3 Contamination Problems

During the first RT-PCR analysis, different annealing temperatures in the PCR reaction were tested. At the temperatures 53ºC and 58ºC bands at expected sizes could be seen both in the mRNA and asRNA reactions only in the negative controls (see Figure 7A and 7B). The results indicated that there must have been a mix-up and the RT-PCR was therefore repeated. Unfortunately, in the following experiments, which were carried out using a new batch of DNase treated-RNA, the PCR samples appeared to be contaminated by genomic DNA since bands appeared in all lanes, including the lanes where negative controls were loaded (Figure 7 C). The reason for the contamination could be e.g. bad DNase I. Additional bands could also be seen in the asRNA, which indicates that unspecific binding of the primers had occurred. As positive controls, the unrelated PA28reg plasmid (P) and genomic DNA (G) were used. The product corresponding to the rcc1 mRNA from the second RT-PCR experiment (Figure 7C) was cloned and sequenced, confirming the genomic sequence of this gene.



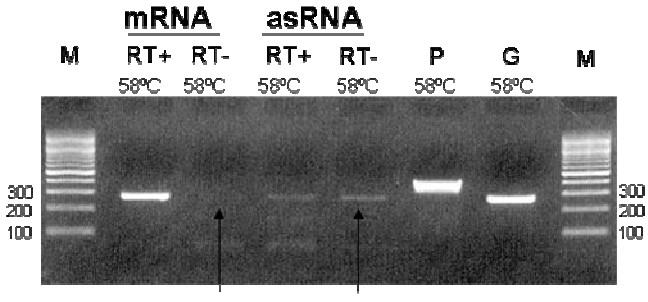


Figure 7 RT-PCR analysis of RNA extracted after 0,16 and 24h development after induced starvation (A) mRNA at annealing temperature 53ºC and 58ºC (B) asRNA at annealing temperature 53ºC and 58ºC (C) mRNA, asRNA, PA28reg (P) and genomic DNA (G) at annealing temperature 58 ºC. RT+ and RT- designate presence and absence of reverse transcriptase.

3.4 asRNA and mRNA Expression During Growth and Development

In order to analyze the expression of asRNA and mRNA during growth and development, RNA from growing AX4:4 cells (0 hours) and developed AX4:4 cells at 16 respectively 24 hours were extracted. 10 µg RNA from each time point was then separated on a 1.2 % agarose gel and transferred to a membrane as described in Material and Methods. Radioactively labelled probes for hybridization were generated by *in vitro* transcription and analyzed by PAGE (Figure 8A). The probe for the sense RNA showed one major band as expected, whereas the probe for the asRNA showed several strong bands, which could lead to unspecific binding during hybridization. The multiple bands have probably derived from the amplification product, which for some reason could not be fully transcribed.

Figure 8B and C demonstrate the results from the Northern Blot-analysis. The first picture (8B) shows the result with the asRNA probe hybridization. The probe appears to have cross-hybridized to rRNA (28s = 3224 nt, 17s =1874 5.8 and 5s=125-162nt), which makes it difficult to interpret the results. However, one band around 800 nt appears to be the result of specific binding. This band is only visible in the lane with growing cells ("0h"), but an earlier Northern Blot showed that the same band also appeared in 16h developed cells (data not shown). It is important to note that the RNA is unevenly loaded in the different lanes in Figure 8B and 8C, with much less RNA in the lane of 16h developed cells than in the 0h and 24h lanes. This is most likely the explanation for the lack of asRNA signal at 16h.

Figure C visualizes the hybridization pattern of the mRNA. A band around 3000 nt in growing cells and 24 h developed cells and one band >3224 nt could be seen in 24 h developed cells.

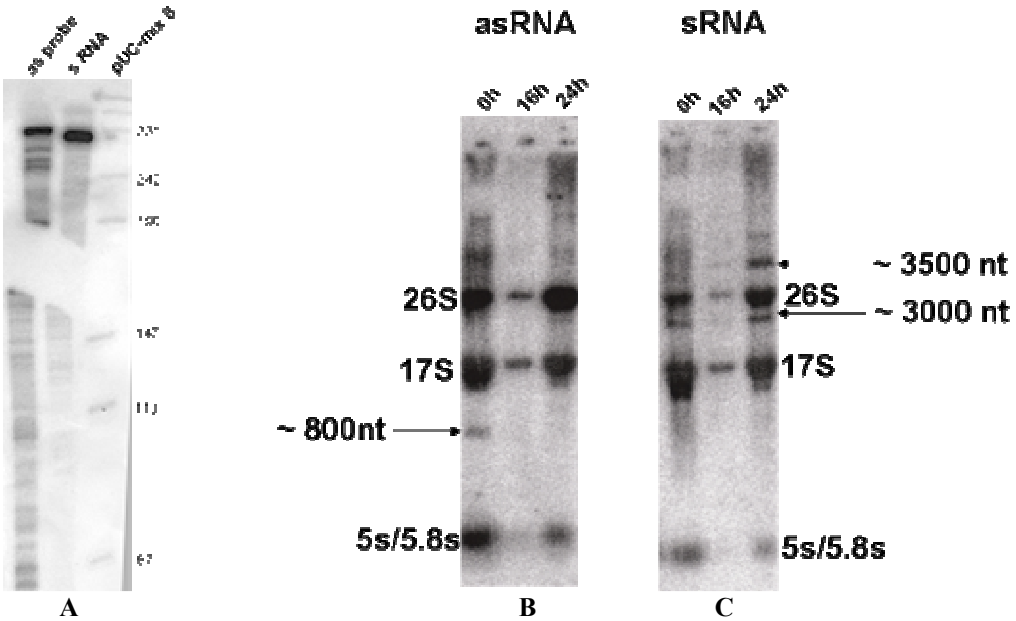


Figure 8 (A) Radiolabeled probes for mRNA and asRNA. (B) Membrane hybridized with the probe against antisense RNA. (C) Membrane B stripped and rehybridized with probe against mRNA. The three most prominent bands are most likely rRNA (28S=3241 nt,17S=2141 nt and 5S=125nt and 5.8S=162nt rRNA) and the sizes were estimated from these RNAs.

3.5 asRNA and mRNA Expression in Growing AX2, AX4 and RNAi Knockout Strains

Northern Blot was also made with the purpose of investigating the possible connection between RNAi and the expression pattern of the rcc1 asRNA and mRNA. RNA was extracted from the following strains: AX2 (0h), drnA$^-$, drnB$^-$, rrpA$^-$, rrpB$^-$, rrpC$^-$, helF$^-$, AX4 (0h, same as described previously). All the RNA from the mutant strains also came from growing cells (0h). AX2 is a wildtype strain that is closely related to AX4. There are some significant differences though, e.g. different gene copy numbers that might have impact on how the expression pattern looks. All the mutant strains derive from AX2 except for rrpC$^-$ which originates from AX4. Figure 8A shows the probes used during hybridization (same as previously). Figure 8B shows the results for the asRNA. A more or less strong band could be detected around 800 nt in all strains except for drnA$^-$ and rrpC$^-$, in which there appeared to be no signal. The lack of signal in drnA$^-$ is probably due to low amount of RNA in that lane whereas the absence of detectable asRNA in the rrpC$^-$ strain seems to be significant, since the rRNA band in this lane is clearly visible.

Figure 9C shows the results for the mRNA. One band is visible in AX4 cells around 3000 nt. (same as noted earlier), but only weak or not at all in any of the other RNAs.

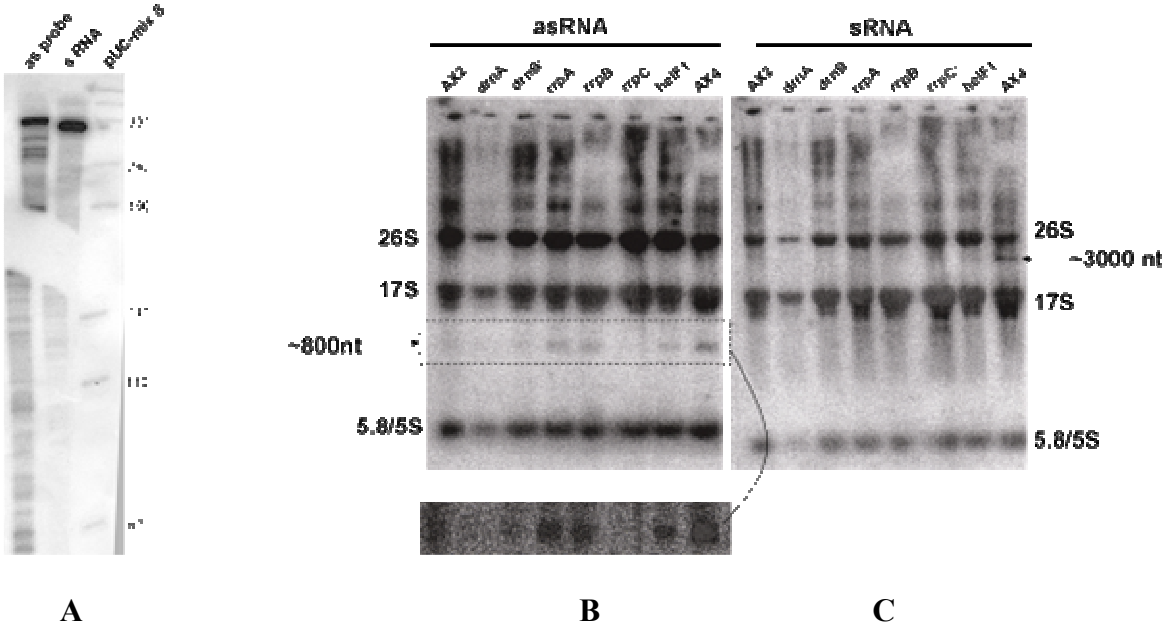


Figure 9 Norhtern Blot analysis (A) Radiolabelled probes for as and mRNA. (B) Membrane hybridized with the asRNA probe. Arrow indicates probable specific binding at around 800 nt (C) Membrane hybridized with the mRNA probe. Arrow indicates probable specific binding at around 3000 nt.

3.6 Purfication of small RNAs

To prove that RNAi is involved in the regulation process it is crucial to be able to detect small RNAs. However, this has proven difficult for rare small RNAs such as asRNAs (Hinas *et al.*, manuscript in preparation). To enrich for small RNAs, a YM-50 centrifugal filter device was used which only allows nucleic acid less than 125 nt pass. About 29 µg total RNA was applied on the device. From this we were able to obtain 0.35 µg small RNAs and 273 µg large RNAs. All of the small RNAs, 10µg of large RNAs and 10 µg of the total (input) RNA were resolved, along with a DNA-marker on a 12% denaturating polyacrylamide gel and blotted on to a membrane with an electroblotter. The membrane was subsequently hybridized with a

radiolabelled probe against an abundant small (21nt) RNA derived from the DIRS-1 retrotransposon. The result from this hybridization can be seen in figure 11 below. Bands around 21 nt in all three RNA containing lanes can be observed. The band in the lane containing the small RNA is somewhat stronger than the other two, demonstrating a small RNAs indeed pass through the filter. However, the fraction that is supposed to contain only large RNAs also shows a band of ~21nt which means that a substantial amount of small RNAs remain in the fraction supposed to only contain large RNAs.

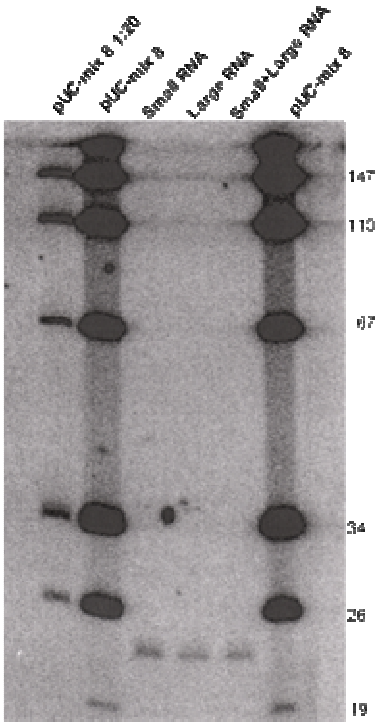


Figure 10 Northern blot showing enrichment of small RNA. The small RNAs were enriched using a Y-50 centrifugal filter device which only lets nucleic acids smaller than 125 nt pass. The hybridization was carried out with a radiolabelled probe against a small (21nt) RNA derived from the abundant retrotransposon DIRS-1.

CHAPTER 4: DISCUSSION

4.1 Northerna Blot analysis

The Northern Blot-analysis showed that there appears to exist an approximately 800nt long RNA in antisense orientation towards the hypothetical *rcc1*. RT-PCR also indicated expression of such an antisense RNA. Furthermore, this asRNA seems to be developmentally regulated, visible only in growing (0h) AX4 cells and potentially in 16h AX4 cells. There was a very weak expression in the AX2 strain as well. Differences in expression of other genes between the two wildtype strains have been observed earlier (Hinas *et al.*, manuscript in preparation). The mRNA, on the other hand, seemed to be expressed in growing AX4 cells, 24 hour developed cells and potentially 16 hour cells, but not in the growing AX4 cells. The size of the rcc1 ORF is 2495 nt according to http://www.dictybase.org. It is unknown where the transcript begins or ends, but I would approximate that the total mRNA (including the untranslated regions and poly-A tail) should be around 3000 nt, which strengthens the idea that the band found at 3000 nt in AX4 growing cells and developed cells is really our targeted mRNA. Interestingly, in cells developed for 24h, an additional band was visible around 3500-4000 nt. One explanation to this longer band could be that the asRNA is somehow involved in the regulation of the mRNA processing or transcription. When the asRNA is absent, like in the 24h cells, this might give rise to an alternative, longer mRNA transcript.

4.2 Northern Blot analysis in mutant strains

The expression of the asRNA was visible in all mutant strains, except for AX2 mutant strain lacking the Dicer protein, DrnA, and AX4 mutant strain lacking the RdRP protein, RrpC. The absence of the expression in drnA$^-$ is most likely because of the relatively low amount of RNA loaded on the gel for this particular strain. The reason why the expression cannot be detected in the rrpC$^-$ could e.g. be that this RdRP is specifically required to synthesize asRNA using the complementary mRNA as template. One detail that contradicts this hypothesis is that no clear mRNA band could be seen in the Northern Blot analysis for the rrpC$^-$ mutant. On the other hand, there might be expressions, which we were unable to detect, “hidden” behind the cross-hybridized rRNA,that could serve as templates for asRNA synthesis. The results from the Northern Blot analysis must be looked upon with certain carefulness though, due to the high extent of cross-hybridizations. The bands we think result from specific hybridization might actually be binding of rRNAs or other RNAs, although stringent hybridization and washing conditions were used. In order to confirm the results, it would be recommended to repeat the analysis with e.g. different probes and hybridization buffer.
If we assume that the results are correct, then possible dsRNA could be formed by the asRNA and mRNA found in AX4 growing cells and potentially 16h cells. It should be noted that, except for rrpC$^-$, the disrupted genes in this study were in an AX2 background, in which both rcc1 mRNA and asRNA expression was low. It would be interesting to knockout the RNAi related genes in AX4 and study the effect on expression of respective RNA.

4.3 Detection of small RNA

To verify that the RNAi pathway is involved in an asRNA/mRNA interaction, it is necessary to detect accumulating siRNAs. The detection procedure could either be solution hybridization, Northern Blot, Real Time-PCR, Microarray or some other method. Before detection can be made, the small RNAs have to be enriched in order to remove background noise that could interfere with the results. This is a rather difficult task. One method is using a filter device, like we did in this project. Even though we managed to enrich some small RNAs a substantial amount was lost during isolation. If we would like to detect siRNA, which is not

as abundant as the small DIRS1, it might me necessary to pool the small RNA. This is quite an expensive and time consuming procedure though.

CHAPTER 5: FUTURE PROSPECTIVE

The first step would be to repeat all the analyses in this study, which would hopefully lead to results without any disturbing contaminations and unspecific hybridizations. If the results are the same as speculated in the discussion chapter, it would be exciting to look at the expression in AX4 mutant strains, in which different RNAi related proteins have been knocked out, both at 0 hours and 16 hours. It would also be interesting to know where the mRNA and asRNA exactly starts and ends, e.g. by using RACE analysis, to know at which positions the two transcripts might overlap. Finally, it would be nice to see were the mRNA and the asRNA (as well as the remaining RNAi related proteins, especially RrpC) are located in the cell with the help of an *in situ* technique like FISH.

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I would moreover like to thank UGSBR for giving me the opportunity to do three different and very interesting projects – I have learned a lot during this past year and also had the luck of meeting some people with a rare blend of kindness, humour and optimism. Hopefully I will continue seeing them in the future!

Last but not least I would like to give a big hug to my family and friends for all the love and support they have shown me, not only during this last year, but for all the years past!

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