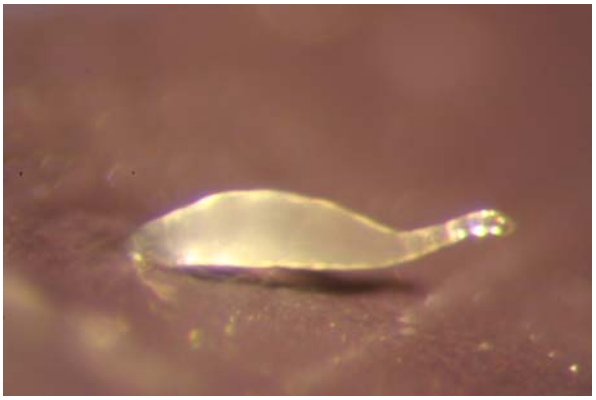
# mRNA and natural endogenous antisense RNA in *Dictyostelium discoideum*



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# ABSTRACT

The aim of this Master's Thesis was to investigate the presence and regulation of RNAi in the model organism *Dictyostelium discoideum*. The background for the project is a cDNA library representing 18-25nt RNAs (9000 clones) from *Dictyostelium discoideum* that recently was constructed. The result revealed a number of small RNAs with antisense complementary to mRNAs and from these we chose three genes for further investigation; *hatA*, *rsmF*, and *DDB0230011* (*kielin*). We also studied two "relatives" to *hatA*, namely *hatB* and *hatC* to see if the genes are similar to each other in the setup of both an mRNA and asRNA. The first aim was to find both mRNA and asRNA for the genes which was done with RT-PCR. We found that all genes have both an mRNA and an asRNA, except for *hatC* which lacks an asRNA. By performing a Northern blot analysis of *hatA* mRNA and asRNA during development and in some RNAi knockout strains we found that both RNAs are is down regulated during development and also in some of the knockout strains.

*hatA* has previously been reported to have a role in osmotic stress. We decided to investigate this effect in two normal *Dictyostelium* (Ax4:4 and Ax2) strains and two knockout strains, rrpC$^-$ and drnA$^-$ (done in Ax4:4 and Ax2 respectively). The results revealed that the asRNA and mRNA is down regulated due to stress and that it also differs in gene expression between untreated cells and cells treated with a starvation buffer. The theory based on our results is that the RNA expression goes down during development which is initiated by the starvation buffer and when the hyper osmotic buffer is added the expression goes down even more.

The mRNA predicted to encode a Kielin homolog has previously been proven to be located in the tip of structure that has been allowed to develop for 16 hours and therefore we wanted to study the location of both mRNA and asRNA in this type of structure, also known as a slug. By using Fluorescent *In situ* hybridization we were able to detect both mRNA and asRNA, where the mRNA seems to be located in the entire structure while the majority of the asRNA can be found in the outer cell layer in one end of the slug.

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# SAMMANFATTNING

RNA interference (RNAi) är ett ämne som under hösten 2006 kom upp på tapeten tack vare att Nobelpriset i Medicin och fysiologi tillägnades forskarna Craig Mello och Andrew Fire i just detta ämne. Principen innebär att när dubbelsträngat RNA kommer in i en cell känns det igen av ett protein som kallas för Dicer som klipper ner RNAt i mindre bitar varpå en bit fäster till ett protein komplex (RISC). RISC komplexet snurrar upp den dubbelsträngade RNA biten och delar den i två delar och använder därefter den ena delen som en mall för att hitta bitar som matchar. När komplexet hittar matchande sekvenser på andra RNA molekyler klyvs dessa och därigenom tystas den gen som skulle ha bidragit till ett protein, till exempel ett virus protein.

I mitt examensarbete har jag studerat RNAi hos modellorganismen *Dictyostelium discoideum*, en amöba som lever i det översta jordlagret och som har en intressant livscykel. *Dictyostelium* celler lever som encelliga organismer och äter jästsvampar och bakterier, men när svält inträffar skickas en signal substans ut (cykliskt AMP, cAMP) vilket gör att de encelliga organismerna samlar ihop sig och går ihop till en multicellulär organism. Denna organism genomgår därefter ett flertal olika livsstadier innan den slutligen når en struktur där en sporboll sitter högst upp på en lång stjälk, ett utseende som påminner om blomman och frökapseln hos björnmossa.

Bakgrunden till experimenten är ett cDNA bibliotek över små RNA (siRNA) som nyligen konstruerades och där man lyckades hitta några asRNAn (asRNA) som är komplementära till messenger RNA (mRNA). Utifrån dessa valdes tre ut och genom att använda RT-PCR lyckades vi påvisa både asRNA och mRNA från generna. Vi analyserade därefter RNA från celler som utvecklats under olika lång tid för att se om genuttrycket för genen *hatA* förändras under utvecklingen. Vi studerade även RNA från RNAi knockout stammar för att se om det finns någon skillnad i genuttryck mellan dessa. Genom att använda analysmetoden Northern blot lyckades vi se att genen *hatA* nedregleras under utvecklingen samt att den är reglerad i vissa av knockout stammarna. *hatA* kodar för proteinet hisactofilin, ett protein som är viktigt för cellmembranet, och man har i tidigare studier sett att *hatA* spelar en roll vid hyperosmotisk stress. Celler utsattes först för en svältbuffert och därefter för en buffert innehållandes en hög sockerhalt. När vi sedan analyserade RNA från celler behandlade med enbart svältbuffert och celler behandlade med osmosbufferten och jämförde dessa mot RNA från helt obehandlade celler såg vi att det var skillnad i mRNA och asRNA uttrycket av *hatA* mellan dels de olika RNA proverna men även mellan de olika cellstammarna som vi använde oss av. Svältbufferten fick cellerna att börja utvecklas och därför gick uttrycket av mRNA och asRNA ner redan där, och när vi därefter tillsatte osmosbufferten gick uttrycket ner ännu mer i vissa av stammarna.

Min hypotes som jag grundar på mina resultat är att *hatA* nedregleras i och med det att cellerna utvecklas på grund av att hisactofilinet helt enkelt inte behövs i

den multicellulära organismen. Den encelliga organismen behöver hisactofilin i sitt membran för att kunna skydda sig mot miljön runt om kring sig, men i den multicellulära organismen blir det yttre membranet tillräckligt tjockt att nivån av hisactofilin kan nedregleras eller tas bort helt.

Vi studerade även genen *kielin* och koncentrerade oss här på att hitta var i den multicellulära organismen som genen uttrycks. Detta gjordes med hjälp av fluorescerande *in situ* hybridisering, en teknik som går ut på att mRNAt och asRNAt detekteras med hjälp av självlysande komplementära bitar som fäster till mRNAt eller asRNAt i organismen. Vi studerade så kallade slugs, en struktur som uppkommer efter 16 timmar av utveckling, och resultatet visade att mRNA finns i hela strukturen medan asRNAt finns i spetsen/framänden på strukturen.

RNAi är ett hett område inom den molekylärbiologiska och medicinska forskningen idag där ökad kunskap inom detta område skulle kunna leda till att man lär sig kontrollera exempelvis sjukdomsalstrande gener och gener som orsakar cancer. Man skulle alltså teoretiskt sett kunna stänga av en cancerframkallande gen och därigenom se till att cancer inte bryter ut och att man slipper de långa och påfrestande behandlingar som finns. RNAi används redan inom andra tillämpningsområden inom biologi och molekylärbiologi, och därigenom hjälper oss att lösa en del av livets gåtor.

# INTRODUCTION

**RNA interference and small RNAs**

Molecular biology and gene regulation has been a popular topic of interest in the area of life science for a long time. The thriving after knowledge in how life is regulated has resulted in many groundbreaking findings. A big jump forward in science was the discovery of the base-pairing rules that was described by Watson and Crick in 1953, a discovery that was rewarded with the Nobel prize in Medicine or Physiology in 1962 (Zamore et al 2005, nobelprize.org). In 1990 a phenomenon called co-suppression was introduced to the world by a group of scientists. They had tried to over express an enzyme involved in pigment synthesis in flowers with the aim of producing deep purple petunia flowers. The flowers turned out to be white instead of purple, something that is caused by repression of the transgenic and endogenous genes (Napoli et al. 1990, van der Krol et al. 1990).

In 1995 Guo and Kemphues tried to suppress gene expression of *par*-1 in *Caenorhabditis elegans* (*C.elegans*, worm) by using RNA complementary to the *par*-1 mRNA, a technique also known as "antisense-mediated silencing". Although, the results created some new question marks since the gene was silenced in both antisense and in the sense control, something that was thought to be impossible since the mRNA should not be able to silence an identical mRNA sequence (Guo et al., 1995). The question marks was later solved by Fire et al. who in 1998 published an article in Nature describing their findings in gene regulation where they showed that double stranded RNA (dsRNA) triggers specific mRNA destruction by using the dsRNA sequence to determine which mRNA to destroy, a phenomenon that they called RNA interference, also known as RNAi. They had also seen that this type of regulation could be distributed to other tissues in the animal and that it could be transferred to the germ line and thereby making it inheritable to the following generations through the egg and sperm (Fire et al., 1998). Andrew Fire and Craig Mello were rewarded with the Nobel Prize in Medicine and Physiology in 2006 for their findings in the area of gene regulation and RNAi (nobelprize.org).

The findings by Mello and Fire set the ground for RNAi research and some year's later scientists had found that RNAs, and especially small RNAs, control animal and plant gene expression. These small RNAs (21-30nt) can be divided into distinct classes where they are distinguished by origin and not by their functions. The different classes are: microRNAs (miRNAs), small interfering RNAs (siRNAs), and repeat associated small interfering RNAs (rasiRNAs), where the miRNAs are predicted to regulate at least one third of all human genes (Lewis et al., 2005, Hutvágner et al., 2005). It has also been shown that small RNAs have a very important role in several biological pathways, where one has seen that stem

cells and brain and muscle cells that lack certain RNAs, fail to develop properly, if they even develop at all. In plants the small RNAs play a part in the development of flowers, directing the shape of the flower and enhancing the plants ability to combat viral infections that otherwise could have been lethal for the plant. When it comes to the shapes of the flowers, researchers have seen that this is an important factor for the plant to be recognised by pollinating agents like bees (Zamore et al., 2005).

When double stranded RNA enters a cell (fig. 1), it is recognized by Dicer (RNase III-like enzyme) which chops it into small fragments between 21-25 base pairs in length. These short RNA fragments (called small interfering RNAs, siRNAs) bind to the RNA-induced silencing complex, RISC. The double stranded RNA fragment is unwound and the strands are separated from each other. The siRNA strands subsequently guide the RISC complex to complementary mRNA molecules. They target mRNA is subsequently cleaved by Slicer, a protein in the RISC complex. Once the mRNA has been cleaved, it can no longer be translated into a functional protein (Alberts et al, 2002).

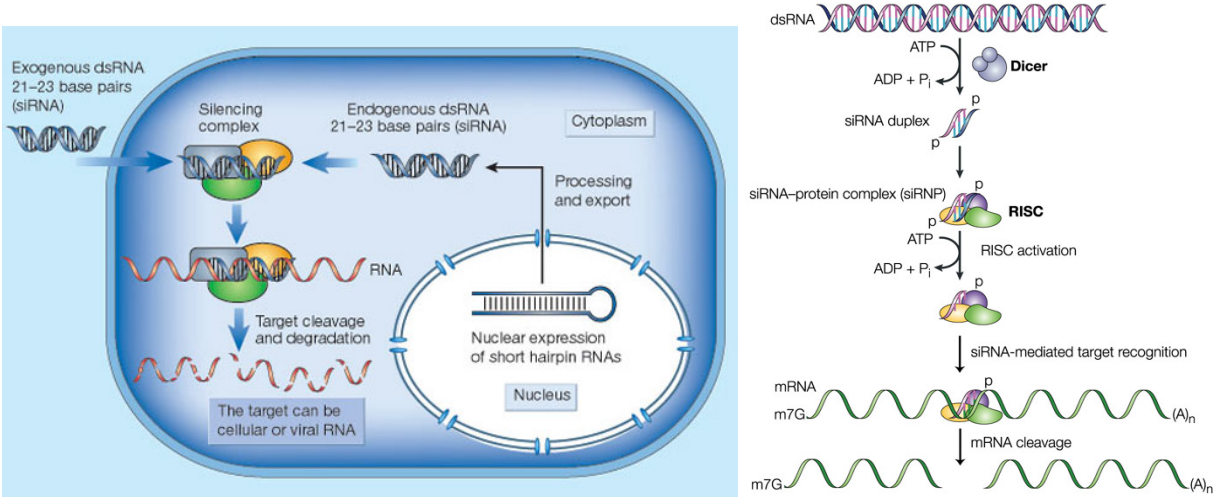


**Figure 1.** RNA interference. Both pictures show gene silencing by RNAi, but they display it from two different angles, the picture to the right is more detailed, but the left picture shows how it works in the cell. Here we can see the dsRNA that is exogenous or endogenous and how it is assembled with Dicer which cuts the dsRNA in smaller pieces before presenting it to the RISC complex. The dsRNA strands is unwound and separated and after that used by the RISC complex to find complementary sequences on mRNA in the surroundings. When a matching sequence is found on an mRNA strand, the mRNA is cleaved and the gene is thereby silenced. (**www.nature.com**)

## RNAi in *Dictyostelium discoideum*

### Genes with a predicted function

A cDNA library was constructed with the aim to locate small RNAs (18-25nt long) in the *Dictyostelium* genome (Kuhlman et al. 2005, Hinas et al. manuscript in preparation). The results revealed a number of small RNAs with antisense complementary to mRNA, and three of these were selected for further studies. The three genes have all different predicted functions in *Dictyostelium*, and one of them has a homolog in another model organism, *Xenopus*.

#### *hatA* – hisactophilin I

Hisactophilin I (*hatA*) is a histidine-rich (26%) actin binding protein known to be associated with the inner surface of the plasma membrane as well as being present as a soluble protein in the cytoplasm of *Dictyostelium* cells where it binds to actin in a strictly pH-dependent matter (Sheel et al. 1989). Motile amoebas need mechanisms for coupling of their cytoskeleton to the plasma membrane, and for the cell to be able to move it is very important to regulate this coupling locally at the front of the cell and also at the contact areas with the underlying surface to which the cell adheres (Hanakam et al. 1994). *Dictyostelium* contains two isoforms of hisactophilin; both genes are independently transcribed and carry a short intron at the same position of the coding region. Hisactophilin II (*hatB*) showes the same biochemical functions as *hatA* and it is pH-dependent when it binds to F-actin (Stoeckelhuber et al. 1996). There is a third hisactophilin encoding gene, *hatC*, which lacks the intron that both *hatA* and *hatB* have (www.dictybase.org).

#### *rsmF*

Ras Superfamily member F (RsmF) is a small GTPase. Small GTPases is a family of proteins (20-25KDa) that binds to guanosine triphosphate (GTP) with the aim to control protein activity. The small GTPase family is homologous to another family called the Ras Superfamily GTPases which contains more than a hundred proteins. GTP binds tightly to the protein which thereby activates the protein. GTP is then hydrolysed into an inactive conformation, GDP, a reaction that is catalyzed by the protein itself. This type of on-off switch, which is regulated by the presence of GTP is crucial for cellular functions in all types of organisms. Small GTPases regulate a wide range of processes and functions in a cell, including growth, cellular differentiation, cellular movement and lipid vesicle transport (Alberts et al., 2002).

#### *DDB0230011* – *Kielin*-like

*DDB023011* (will further be called *kielin*) has a homolog in *Xenopus* which is a popular model organism in molecular biology research. Matsui et al. (2000) reported their findings on *Xenopus* kielin as a dorzaling factor in the embryonic

development. Kielin resembles chordin by having multiple cystein rich repeats and is induced in the ectoderm and mesoderm by nodal-related genes. Kielin has also some similarity to von Willebrand factor and Fraser syndrome protein (Matsui et al., 2000).

**RNAi knockout strains**

When it comes to test gene expression it is very useful to use RNAi knockout strains where a specific gene has been silenced. *drnA* (Martens et al. 2002) and *drnB* (Kuhlmann et al. 2005) are genes that encode a Dicer like protein and the knockouts are both done in the *Dictyostelium* Ax2 strain. *rrpA*, *rrpB* and *rrpC* (Martens et al. 2002) all encodes RNA-directed RNA polymerases that are very important in posttranscriptional gene silencing. Knockouts for the genes have been done in *Dictyostelium* strain Ax4 ($rrpC^-$) and in Ax2 ($rrpA^-$ and $rrpB^-$).

## A social amoeba by the name *Dictyostelium discoideum*

*Dictyostelium discoideum* is an organism that has gone from a simple life in the forest floor to the research laboratories around the world where it has become a well studied model organism. This little social amoeba is especially interesting since it has two cellular stages, unicellular and multicellular. *Dictyostelium* lives in the soil were it eats bacteria and yeast which it tracks by chemotaxis, movement towards chemicals in the environment. The organism lives in a constant battle when it comes to food and its enemies are other amoebas and fungi, bacteria and the nematode *C. elegans*. During this battle it also has to defend itself against toxins and predators as for example *C. elegans*. The nematode is not only a dangerous predator and a competitor for bacterial food; it is also a possible dispersal agent for *Dictyostelium* spores (Kessin et.al, 1996). During growth and development *Dictyostelium* display motility that resembles the one in human leukocytes. This feature makes this organism an interesting model when it comes to immunological and cytoskeleton research (Noegel et.al, 2000). The survival of *Dictyostelium* in its habitat in the soil is dependent on its ability to track down and eat the bacteria in its surroundings. This is done with the help from various advanced systems for chemotaxis and phagocytosis. Unicellular *Dictyostelium* cells forms a multicellular organism due to starvation, a process which is possible thanks to chemotactic aggregation (Eichinger et.al, 2005).

Distinct cell types have also evolved and the ability to regulate the proportion and morphogenesis of these cells. A study of the proteins that are playing a crucial role in the development towards a multicellular organism showed that certain characteristics like cell adhesion and signalling that are usually associated with animals have been conserved in *Dictyostelium*. On the other hand, some structural components that can be found in the fruiting body and other terminally differentiated cells have been found to originate from plants.

Unicellular *Dictyostelium* cells develop as a multicellular organism due to starvation. It goes through several steps (fig. 2) which includes a motile slug which after some hour of development and minor steps turns in to a fruiting body which consists of a cellular, celluloid stalk with a bolus of spores at the top (Madigan et al., 2003). It has also evolved differentiated cell types and the ability to regulate their proportions and morphogenesis. A study of the proteins that are playing a crucial role in the development towards a multicellular organism showed that *Dictyostelium* has retained cell adhesion and signalling modules that is normally associated exclusively with animals. It was also shown that the structural elements of the fruiting body and terminally differentiated cells derived from the control of cellulose disposition and metabolism that is now associated with plants.

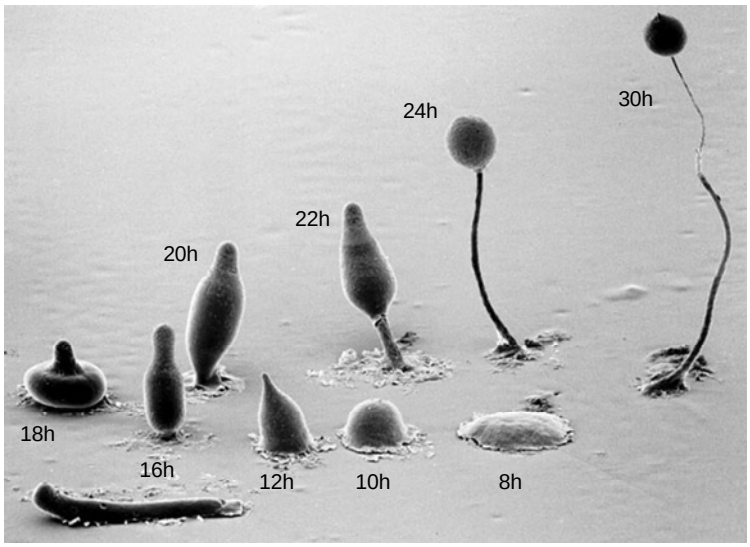


**Figure 2.** This beautiful picture shows the different stages during the development that starts due to starvation. (Mark Grimson and Larry Blanton)

## Genome and phylogeny

The genome of *Dictyostelium* was published in 2005 by Eichinger and coworkers and the data revealed six chromosomes, a relative high number of protein coding genes (12,500). The genes are distributed approximately uniformly across the genome which also contains few and short introns. The genome is very A+T rich (77,57%). The size of the genome is 34 mega base pairs (Mb) (Eichinger et al., 2005).

When it comes to phylogeny and the phylogenetic tree of the eukaryotes, all of the organisms diverged from the same ancestor and has kept some of the features that characterise a eukaryotic cell. Their genomes have been edited by chromosomal deletions and duplications which have resulted in for example genes with new functions (Kellis et.al, 2004 and Duijon et al, 2004). In the past, scientists looked

at morphological criteria but were then unsure to which group *Dictyostelium* should belong to, protozoan or fungi. With today's technologies in molecular science, it has been suggested that they belong to the amoebozoans and that this group diverged from line leading to animals at almost the same time as the plants (Loomis et.al, 1995 and Baldauf et al, 1997).

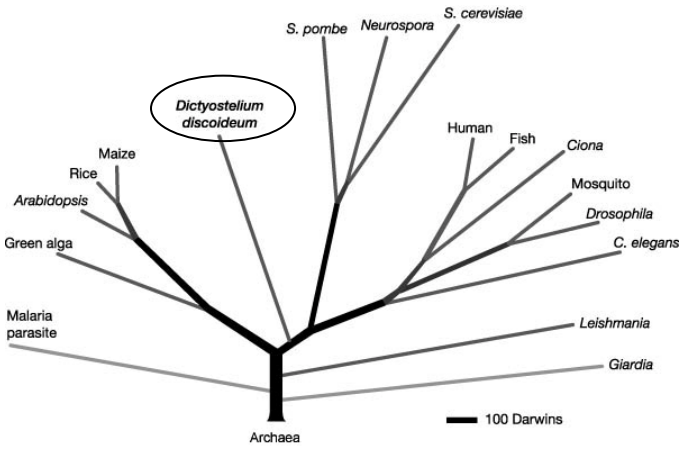


**Figure 1.** The phylogenetic three of eukaryotic life based on protein sequences from the proteome. As can be seen *Dictyostelium discoideum* branched out from the stem between plants and animals (Eichinger et al. 2005).

# AIM OF THE PROJECT

The background for the degree project is a large-scale cloning of small RNAs in *Dictyostelium discoideum* that was done in collaboration between Fredrik Söderbom, Swedish University of Agricultural Sciences and Prof. Victor Ambros, Dartmouth Medical School, USA. The cloning yielded a cDNA library with 9000 sequences and among these some small RNAs antisense to mRNAs. In my degree project I have been focusing on three of these genes, all with annotated functions. Further characterization of the identified antisense transcripts as well as searching for more antisense transcripts was done for all the genes. *hatA* was studied further since its protein product has a known function as a pH-dependent acting-binding protein in the cytoskeleton. We therefore studied the reaction to hyper osmotic stress in several strains, of which some had been depleted of certain RNAi-related proteins by gene disruption. The aim was to see if the mRNA and asRNA levels are regulated due to the stress and if proteins involved in RNAi are involved in this response. *Kielin* has a homolog in *Xenopus* that plays a part in the developmental stage where the orientation of the organism is decided, and therefore we decided to study regulation and localization of *kielin* mRNA and asRNA in *Dictyostelium* with fluorescent *in situ* hybridization. Our aim was to locate the mRNA and asRNA in the structure and to see if they are expressed differently from each other.

# MATERIALS AND METHODS

## Design of oligonucleotides

DNA oligonucleotides (Invitrogen) used during PCR and RT-PCR are listed in table 1.

**Table 1.** Gene specific primers used during RT-PCR and PCR, T7-polumerase and primer used for sequencing.

| | **primer** | **sequence** |
|---|---|---|
| | | |
| ***DDB0230011*** | DDB0230011_forward 1 | CACAAGTACCAGTTGGTGAATGT |
| | DDB0230011_forward 2 | AGCTGTTCAATGTCTTGTTCCAA |
| | DDB0230011_reverse 1 | CTTCACATACTGGCATGGCACA |
| | DDB0230011_reverse 2 | CAAGGGTGCAGTCTTGTTTACAT |
| | | |
| ***hatA*** | hatA_forward 1 | TAATATAAATACAATGGGTAACA |
| | hatA_forward 2 | AGCGCTGAAGGCGAAGCTGTA |
| | hatA_reverse 1 | CCATGATGATCAGCGGAAATGT |
| | hatA_reverse 2 | AATGTAGTGGTGGTGATGACCT |
| | | |
| ***hatB*** | hatB_forward | AGCGCTGAACACGATCACGTC |
| | hatB_reverse | CATGATGATCGTGGTGATGGGA |
| | | |
| ***hatC*** | hatC_forward | TGCCATAAGAACTCACGCCAA |
| | hatC_reverse | ATCATGGTGATGGTGGTGAGTA |
| | | |
| ***rsmF*** | rsmF_forward | TAGTTTATTCATTAACCGATAGA |
| | rsmF_reverse | CTTTGGCACTGGCTTCAATGT |
| | | |
| **Sequencing** | M13 forward | GTAAAACGACGGCCAG |
| | M13 reverse | CAGGAAACAGCTATGAC |
| | | |
| **T7 polymerase** | T7_forward oligo | ggtaatacgactcactataGGG-CAGTGTGATGGATATCTGCA |
| | T7_reverse oligo | ggtaatacgactcactataGG-GAGCTCGGATCCACTAGTAA |

## Growth and development

*Dictyostelium discoideum* strain Ax4 was grown in HL5 medium. For development, $5 \cdot 10^7$ cells were harvested, washed, spread on nitrocellulose membranes and allowed to develop in a moist chamber at 22°C. (Sussman M, 1987)

## Total RNA extraction and DNase treatment

*Dictyostelium discoideum* Ax4 cells were harvested, washed, and mixed with TRIzole reagent (Invitrogen) and stored at -80°C. DNase I (100U), DNase I buffer and water was mixed together with 75µg RNA (concentration measured by a

Nanodrop spectrophotometer) and incubated for 10 minutes at 37°C. The volume was after that diluted to 400µl with water and an additional 400µl of phenol was added. The tubes were vortexed and centrifuged for 5 minutes at 13.000 rpm, the supernatant transferred to a new tube and diluted to 400µl. 400µl chloroform was added and the tubes vortexed and centrifuged as in the previous step. The supernatant was once again transferred to a new tube and EtOH precipitated by adding 0,1vol 3M NaOAc (pH 5,2), 3vol ice cold 99% EtOH and storing the solution at -20°C for 30 minutes and after that centrifuged at 16 000 x g for 30 minutes. The supernatant was removed and the pellet washed with 70% EtOH (same volume as total volume in the previous step) and centrifuged for 30 minutes at 16 000 x g. The supernatant was once again removed and the pellet air dried at room temperature for a couple of minutes before dissolved in RNase free water. The RNA was treated with DNase and the following steps as described above twice to make sure that no traces of DNA would remain in the RNA sample. The RNA quality was analysed on an agarose gel.

## RT-PCR

DNase treated RNA (2µg/tube and 2 tubes per gene), 1,6mM dNTP, gene specific primer (20pmol) (see table 2) and water (up to 12µl) was incubated at 65°C for 5 minutes. The tubes were then divided into +RT and –RT and treated similar but with the difference that 15U Thermoscript RNase $H^-$ (Invitrogen) was added to the +RT tubes and a similar amount of water to the –RT tubes. 0,1M DTT, 40U RNase inhibitor (RNA guard$^{TM}$ (porcine), Invitrogen), 5x cDNA synthesis buffer (Invitrogen) and water was added to the tubes which were then incubated in the PCR-machine (Techne) at 55°C for 20 minutes, 60°C for 20 minutes, 65°C for 20 minutes before inactivation of the Thermoscript which was done at 85°C for 5 minutes. The samples were put on ice and 2U RNase H (*E.coli*, 2U/µl, Invitrogen) was added and the samples incubated one more time at 37°C for 20 minutes before stored in the freezer at -20°C. The PCR reactions contained water, AmpliTaq buffer (Roche), 2,55mM $MgCl_2$, 0,2mM dNTP, 0.6U AmpliTaqGold (Roche, 5U/µl) gene specific primers (0.6pmol) (se table 2 in results) and 0,5µl of the +RT or –RT reactions.

**Table 2.**The table is showing the different oligos that were used for each one of the RT-PCR reactions. The specific oligo sequence can be seen in table 1.

| **Gene** | **Primer 1** | **Primer 2** |
|---|---|---|
| *hatA* | hatA_forward1 | hatA_reverse1 |
| *hatB* | hatB_forward | hatB_reverse |
| *hatC* | hatC_forward | hatC_reverse |
| *rsmF* | rsmF_forward | rsmF_reverse |
| *DDB02300* (*kielin*) | DDB0230011_forward2 | DDB0230011_reverse2 |

The PCR-program (hotstart, touchdown): 95°C 9 minutes, 5x (95°C 30 seconds, 55-50°C 40 seconds, 72°C 40 seconds), 35x (95°C 30 seconds, 50°C 40 seconds, 72°C 40 seconds), 72°C 10 minutes. Different annealing temperatures were used for the different genes (se table 3).
The PCR products were analysed on 2% agarose gels and photographed under UV-light.

**Table 3.** Different annealing temperatures had to be used to match the different oligos. The list is showing these temperatures for each one of the genes.

| Gene | Annealing temperature |
|---|---|
| *hatA* | 55-50°C |
| *hatB* | 55-50°C |
| *hatC* | 55-50°C |
| *rsmF* | 55-50°C |
| *DDB00230011* | 59-54°C |

## Cloning, plasmid purification and sequencing

PCR fragments were cloned into a PCR2.1-TOPO vector (Invitrogen) and transformed into competent DH5α cells (see below). Plasmids were purified using Qiagen QIAprep spin miniprep (microcentrifuge) and sequenced using an ABIPRISM 3700 DNA Analyser (Applied Biosystems). Sequencing was done to check the inserts and their direction which then could be used to determine which clones that should be used for *in vitro* transcription and Northern blot hybridization.

## Competent cells

Frozen DH5α cells were spread on to an agar plate whereupon twelve large colonies were isolated and grown in SOB media (Sambrook et al. 2001) at 18°C for approximately 48 hours. The cells were put on ice for 10 min, pelleted (10 min at 4°C, 2500 x g) and washed with ice-cold TB-buffert (10mM Pipes, 55mM $MnCl_2$, 15mM $CaCl_2$, 250mM KCl)). The cells were then resuspended in 20ml TB and DMSO was added to a final concentration of 7% before aliquoted into sterile eppendorf tubes (50µl/tube). The cells were quickly frozen in liquid nitrogen and stored at -80°C.

## Hyper osmotic shock

Hyper osmotic shock was done according to a protocol described by Schuster et.al (1996) and later on Pintch et.al (2002) but with minor modifications as in the number of cells required.
Cells from the strains Ax4:4, *rrpC*$^-$, Ax2 and *drnA*$^-$ were grown in HL5 media whereupon $4x10^7$ cells were harvested at 300xg and washed in Soerensen phosphate buffer (SPB: 2mM $Na_2HPO_4$, 14,6mM $KH_2PO_4$, pH 6,0). The cells

were resuspended in SPB and incubated for 1 hour at 22°C in a shaker (150rpm). 10µl cells from each tube were collected, diluted (1:100 and 1:1000 which makes about 20 cells/ml), mixed with 350µl *Klebsiella aerogenes* and spread on SM plates. These plates served as "before induction" plates in a viability test. To the rest of the cells 0,5ml 2M sorbitol/SPB or an equal amount of SPB was added and the mixture incubated for 2 hours under the same conditions as previous. 12,5µl of cells were after that collected, diluted (as previous), mixed with *K. aerogenes* and spread on SM plates. These plates served as "after induction" plates, with or without hyper osmotic stress. The rest of the cells were harvested and washed in SPB and after that treated according to the TRIZOLE method for RNA preparation. RNA was also prepared from the remaining untreated starting culture. The RNA was later on loaded on to a PAGE gel and used for northern analysis.

## Northern blot

RNA (20µg) was denatured for 5 minutes at 95°C together with loading buffer (46% formamide, 8,5mM EDTA, 0,013% bromphenol blue and 0,013% xylene cyanol), loaded on to a 6% polyacrylamid gel (7M urea, 1xTBE) together with a radioactively labelled ladder (pUCMIX and pUC19, Fermentas). The ladders were labelled as follows: 0,5µg pUC19/MspI (Fermentas) was incubated at 37°C for 30 minutes together with 10µCi γ-ATP, 10xRX buffert, 15U T4 PNK (Amersham) and water. The ladder was then purified through a G50 column (Probe Quant, Amersham Pharmacia) and stored in the freezer at -20°C. The RNA were separated on the gels at 22A for about 3-5 hours and after that transferred to Hybond-$N^+$ nylon membrane (Amersham Biosciences) by electroblotting over night. The membranes were then UV crosslinked at 150mJ to immobilize the RNA.

Hybridization probes with a T7-promotor sequence were *in vitro* transcribed according to MAXIscript in vitro transcription kit (Ambion) with a $^{32}$P-labeled nucleotide. Briefly, PCR product from a PCR reaction with T7 polymerase was mixed together 10x transcriptionbuffer, 0,5mM ATP, 0,5mM CTP, 0,5mM GTP, 5µM UTP, 12,5µCi, T7 enzyme mix and water. The mixture was incubated for 45 minutes at 37°C before 10U DNase was added and the mixtures incubated once again at 37°C for 15 minutes. 1µl 0,5M EDTA was then added and the sample purified through a G50 column (Probe Quant, Amersham Pharmacia). As a control for the probes and to see that no degradation hade occurred during the *in vitro* transcription or in earlier steps, the probes were analysed on an 8% PAGE, the gel set to dry and after that expose it in a cassette which could then be developed in a Phosphor imager (Molecular dynamics).

The probe for SRP (signal recognition particle RNA) was done by mixing 8ρmol DdR-20 (SRP) oligo (Aspegren et al. 2004), 50µCi γ-ATP, 10xbuffer (A-buffer, Fermenta), 15U T4 PNK and water and incubating it at 37°C for 30 minutes. Purification was done with a QIAquick nucleotide removal kit (Qiagen) and the

probe was eluted in 100µl elution buffer. The probe was denatured at 95°C and put on ice before hybridizing it to the membranes.

The membranes were pre hybridized and finally hybridized (5µl $P^{32}$-labelled *in vitro* transcribed probe) with a modified version of G&C buffer (10mM EDTA, 0,5M $Na_2HPO_4$, 0,5M $NaH_2PO_4$, 7% SDS). The tubes were placed in an oven with a spinning wheel over night with the temperature set on 42°C or 65°C depending on the probe (lower temperature for the SRP-probe). The next day the membranes were washed (2x 5min 2xSSC/0,1% SDS, 2x 10min 1xSSC/0,1% SDS and 2x 5min 0,5xSSC/0,1% SDS) at the same temperature. Membranes were as a final step wrapped in plastic and placed in a cassette for 1-3 days and the signals were after that detected by a Phosphor Imager (Molecular dynamics).

## Semi-colony PCR

Colonies were isolated and grown in 2ml LB and 2µl Kanamycine at 37°C for 2 hours. 50µl from each culture were heat denatured at 95°C for 10 minutes and put on ice water. 1µl were used as template in a PCR reaction with M13 forward and M13 reverse as oligonucleotides (see oligo list). PCR-program that was used was: 94°C 2 min, 30 cycles(94°C 45sec, 55°C 45sec), 72°C 2 min. The products were analysed on a 1,5% agarose gel.

## Fluorescent *In situ* hybridization

The probe for the *in situ* hybridization was *in vitro* transcribed and the template that was used in this experiment were PCR product containing inserts from kielin together with a T7 promotor sequence for mRNA and asRNA detection. 0,5µg template was mixed together with 10x transcription buffer, water, 60mM ATP/GTP/CTP-mix, 2,5mM UTP, 15mM labelled UTP (5'(3 aminoallyl)-UTP, Ambion), 0,25M DTT, 40U RNase inhibitor (RNAguard™, Invitrogen) and 20U T7 RNA polymerase (Ambion). The mixtures were incubated at 37°C for 2 hours before addition of 10U DNaseI (FPLCpure™, Amersham/GE) and an additional incubation for 15 minutes at the same temperature. The volume was diluted to 50µl and purified through a G50 column (Probe Quant, Amersham Pharmacia). The concentration was measured by a Nanodrop spectrophotometer and the samples concentrated by EtOH precipitation followed by a new measurement of the concentration. 5µl of the *in vitro* transcribed RNA was mixed together with 3µl sodium bicarbonate (25mg/ml) before adding it to the fluorofore tube(Alexa fluor 488, Invitrogen) to which 2µl 100% dimetyl sulfoxide (DMSO) had been added. The fluorofore tube, now containing the *in vitro* transcribed RNA was then incubated in the dark for 1 hour and after that G50 purified and EtOH precipitated as in earlier steps. The concentration and incorporation of the fluorofore was measured by a Nanodrop spectrophotometer.
Dictyostelium cells were set to develop for 16 hours and were after that washed down into an eppendorf tube. The cells were washed twice in cold 90% methanol

and fixed by replacing the methanol with 3,7% formaldehyde in PDF and placed at room temperature with gentle rocking for 2,5 hours. The cells were then washed for 5 minutes three times with PDF and after that incubated with 20µg/ml Proteinase K for 30 minutes at 37°C. After a 5 minute long wash with PDF, fixing the cells once again but this time for 20 minutes the cells were pre-hybridized for 3 hours at 42°C with pre-hybridization buffer (4xSSC, 1x Denhardt's, 100µg /ml sonicated salmon sperm DNA, 60% formamide). The buffer was changed to hybridization buffer (4xSSC, 0,5mg/ml sonicated salmon sperm DNA, 0,25mg/ml yeast RNA, 60% formamide) and the fluorescent *in vitro* transcribed probe (1µg/ml) was added and set to hybridize for 16 hours at 42°C. The cells were washed with 2xSSC, 1xSSC and 0,5xSSX for 30 minutes each, rinsed once in PDF for 5 minutes at room temperature before mounting them in VectaShield mounting medium (Vectorlabs).

By comparing the labelling efficiency of the asRNA and mRNA probes with each other we could see that the base:dye ratio for asRNA probe is 5.9 ($Abs_{260}$:0.069) and 3.2 ($Abs_{260}$:0.065)for the mRNA probe.

# RESULTS

## The presence of mRNA and its antisense RNA in *Dictyostelium discoideum*

A cDNA library representing 18-25nt RNAs (9000 clones) from *Dictyostelium discoideum* was recently constructed (Kuhlmann et.al 2005, Hinas et.al, manuscript in preparation). Among these were a number of small RNAs with antisense complementary to mRNAs. Three of the corresponding genes were selected for further analysis: *hatA* (encodes hisactophilin I, an actin binding protein), *rsmF* (encodes a small GTPase) and *DDB0230011* (kielin-like, will further on be referred to as *kielin*). A BLASTN search against expressed sequence tag (EST) libraries at www.dictybase.org revealed longer antisense RNAs (asRNAs) for *hatA* and *kielin*. To confirm the presence of these asRNAs, RT-PCR was performed yielding products that correspond to the mRNA and the asRNA for all three genes. All fragments were subsequently cloned and sequenced and the sequences were analyzed by a BLAST-tool to make sure that the right fragments, mRNA and asRNA for each of the genes, were successfully cloned.

Genomic PCR and RT-PCR for *hatA* (fig. 3b) yielded a longer fragment for the genomic DNA (gDNA) than for the mRNA and asRNA for the gene. As the primers used in this experiment were located in different exons (fig. 3) this indicated that the asRNA lacks the intron part of the gene. This was also confirmed by sequencing, which showed that the asRNA was perfectly complementary to the mature mRNA. *hatA* has two more members in its family, *hatB* and *hatC* and RT-PCR analysis was also carried out for these two.
RT-PCR analysis of the *hatB* locus (fig. 3c) demonstrated the presence of both mRNA and asRNA for the gene. We would not be able to see a difference in size between the gDNA, mRNA and asRNA since the primers are located in the same exon.
Except for small sequence variations, the main difference between *hatA*, *hatB* and *hatC* is that *hatC* does not have an intron, which both *hatA* and *hatB* have. Also, no ESTs (sense or antisense) corresponding to *hatC* is present in the EST libraries. The results from the RT-PCR (fig. 3d) showed expression of the *hatC* mRNA but not of a corresponding asRNA. The small RNA that is present for *hatA*, is not fully complementary to *hatB* or *hatC* (fig. 3).

For *rsmF*, RT-PCR analysis revealed both an mRNA and an asRNA, although the asRNA do not have an EST in the library. The gene is shorter than both *hatA* and *kielin*, but just as the other two it contains an intron which can be seen in the gene map in figure 3e. As in the case for *hatB*, the reason why we do not see a difference in size between the gDNA, mRNA and asRNA is because of the location of the primers, which are located in the same exon.

RT-PCR analysis of *kielin* (figure 3a) showed the presence of both an asRNA and an mRNA, but as in the case of *rsmF* and *hatB* we would not be able to see the intron because of the locations of the primers. The gene map for kielin shows the three exons that are present and the location of the small RNA which can be found in exon 3.

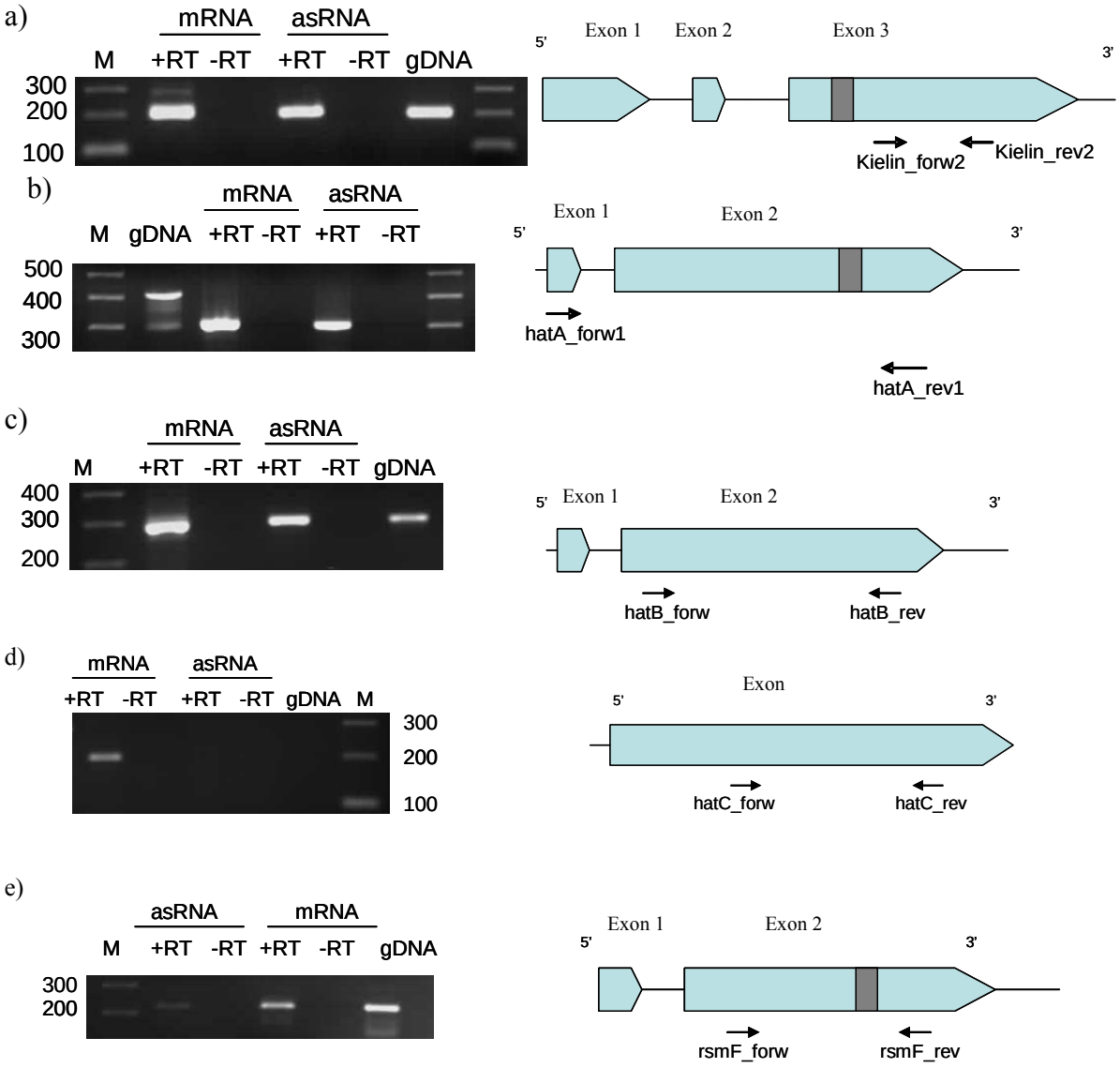


**Figure 3.** RT-PCR analysis of mRNA and asRNA and gene structure with EST and primer location. The gray area in some of the structures shows the locations of the small RNAs, which *hatB* and *hatC* both lack. gDNA designates genomic PCR. The difference between for asRNA and mRNA is only the primer used for reverse transcription. +/-RT indicates presence or absence of reverse transcriptase.

A) The RT-PCR for *kielin* shows that an mRNA and asRNA is present. B) *hatA* contains a intron which can be seen as the difference in size between the gDNA and the asRNA and mRNA. C) RT-PCR for *hatB* viewes the presence of both mRNA and asRNA for the gene. The locations of the

primers can be seen in the gene map to the right of the gel photo. D) The RT-PCR revealed the presence of an mRNA and the lack of an asRNA for *hatC*. E) *rsmF* shows a weak band in the mRNA lane. We can also see that *rsmF* has an antisense RNA, indicated by the band in the +RT lane for the antisense. In the gene map one can see that *rsmF* contains a intron, but as in the case for *hatB*, we would not be able to see the difference in size since the location of both of the primers are in the same exon.

## Development of *Dictyostelium discoideum*

To illustrate the different life stages, in this case 16 and 24 hours of development, a photo session was set up with a digital camera attached to a microscope. The cells were allowed to developed and after that placed on a glass slide attached to a tripod, making it possible to put the cells in an appropriate angle and thereby take nicer pictures. The upper picture in figure 4b shows a typical slug, a structure after 16 hours of starvation and development. The lower picture shows a structure after 24 hours of development where it has reached it final conformation, a ball of spores on the top of a stalk. All life stages can be seen in figure 2 in the introduction.

## Expression of hatA mRNA and asRNA during growth and development

To investigate the developmental expression pattern for the *hatA* mRNA and asRNA, we analysed the RNA from *D. discoideum* after 16 hours of development and from growing cells, by Northern blot analysis (fig. 4a). Here we could see that the asRNA expression goes down during development since there is a weaker signal after 16 hours than for growing cells. The same down regulation can be seen in the expression of mRNA, where the expression almost disappears completely after 16 hours of development. The SRP (signal recognition particle RNA) is a control for equal loading and to see that the RNA has not been degraded.

The interesting down regulation of the asRNA and mRNA for *hatA* set the ground for further investigation where we wanted to see how these two RNAs are regulated during stress. Since *hatA* encodes Hisactophilin, we chose to investigate the gene regulation during hyper osmotic stress, something that has been studied by others as well (Schuster et al. 1996, Pintch et al. 2002)

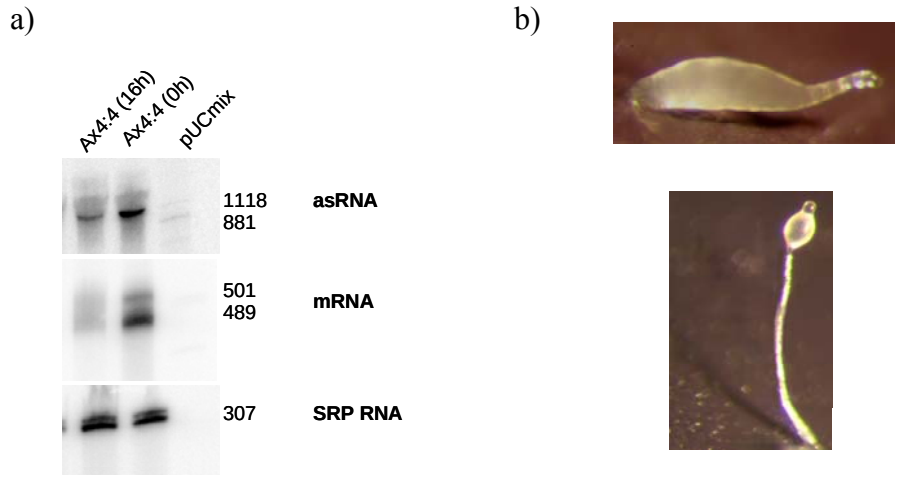


**Figure 4.)** a) Northern analysis showed that both mRNA and asRNA expression seems to be down regulated after 16 hours of development. b) Photographs taken of developed cells after 16 hours (the upper picture) and 24 hours (the picture at the bottom) of development.

## Expression of hatA mRNA and asRNA in RNAi knockout strains

To investigate the possible role of the RNAi pathway in regulation of the *hatA* mRNA and asRNA, we performed Northern blot analysis using RNA isolated from growing cells from a number of RNAi machinery knockout strains: *drnA*$^-$ (Kuhlman et al., 2005), *drnB*$^-$ (Nellen W., unpublished), *rrpA*$^-$, *rrpB*$^-$, *rrpC*$^-$ (Martens et al., 2002) and *helF*$^-$ (Popova et al., 2006). The membrane was hybridized with $P^{32}$-labelled *in vitro* transcribed probes directed against mRNA and later on asRNA (figure5).

If we compare the *hatA* expression patterns between Ax2 and the RNAi knockout strains *drnA*$^-$, *drnB*$^-$, *rrpA*$^-$, and *rrpB*$^-$, we can see that the expression of mRNA is higher in the Ax2 than in the knockouts. The knockout strain *helF*$^-$ although, revealed an asRNA expression that was comparable in strength to the signal for the Ax2 strain, while the mRNA expression was lower than the one for the Ax2, just like for the other knockouts. If we compare the knockouts with each other we can see that the mRNA expression in *drnA*$^-$ is much lower than for the rest and that the asRNA expression in this knockout is very low as well. Otherwise it is very hard to distinguish between the different strains since the expression is quite low in all of them.

When looking at the Ax4:4 strain and the knockout strain *rrpC*$^-$, we can see that the mRNA expression is lower in the knockout than in Ax4:4, and the same goes for the asRNA expression. If we compare the mRNA and asRNA expression between Ax4:4 and Ax2 we can see that the level of expression is higher in the Ax4:4 strain than for the Ax2.

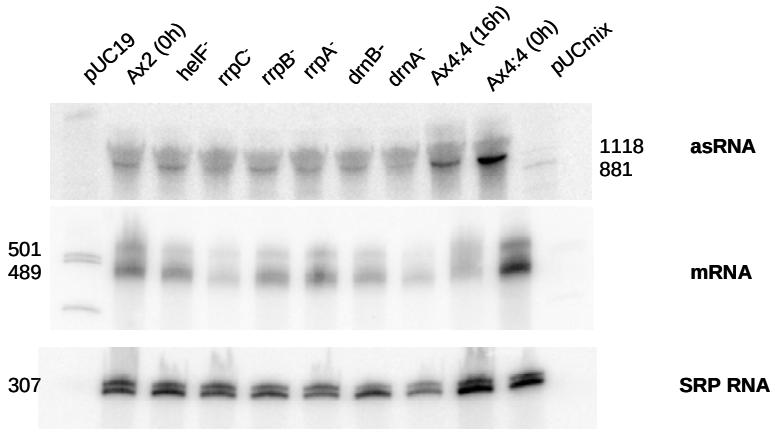


**Figure 5.** Northern blot for hatA in different developmental stages and strains. The common trend seems to be that there is a higher signal for the asRNA in all strains but it Ax2. Another interesting thing is also that the mRNA expression seems to be down regulated in the *rrpC*$^-$ and *drnA*$^-$ strains.

To investigate the down regulation of *hatA* expression in the two knockout strains *drnA*$^-$ and *rrpC*$^-$ further, we selected these two for a hyper osmosis test where we wanted to see how the *hatA* expression was effected by the hyper osmotic condition.

## Regulation of *hatA* expression due to hyper osmotic stress

It has previously been shown by Schuster et.al (1996) and later on Pintch et.al (2002) that the protein encoded by *hatA*, hisactophilin, plays a crucial part during hyper osmotic shock. We decided to study the mRNA and asRNA regulation in Ax4 and Ax2 and in the knockouts *rrpC*$^-$ and *drnA*$^-$ , which are done in the Ax4 and Ax2 respectively. We wanted to see how the four strains respond to hyper osmotic stress and if the asRNA and mRNA expression is affected by the stress. Cells were grown, harvested, washed and exposed to sorbitol for 2 hours to induce hyper osmotic shock. The cells were then mixed with *K. aerogenes* and plated on to SM plates. These plates served as a test for the viability and showed that the viability is down regulated in the *Ax4:4, Ax2, rrpC*$^-$ strains if we compare before and after shock, while the viability in the *drnA*$^-$ strain is up regulated (table 4).

**Table 4.** Viability test. The table is showing the number of plaques that could be seen four days after the hyper osmotic stress experiment. In the case for Ax2 after shock (-sorbitol), the *Klebsiella* had grown poorly which resulted in that the *Dictyostelium* did not grow at all and therefore no placks could be counted.

| Strain | before shock | after shock (+ sorbitol) | before shock | after shock (- sorbitol) |
|---|---|---|---|---|
| Ax4:4 | small: 58<br>large: 39<br>total: 97 | small: 62<br>large: 2<br>total: 64 | small: 45<br>large: 24<br>total: 69 | small: 55<br>large: 50<br>total: 105 |
| *rrpC*$^-$ | small: 206<br>large: 9<br>total: 215 | small: 67<br>large: 8<br>total: 75 | small: 45<br>large: 11<br>total: 56 | small: 100<br>large: 6<br>total: 106 |
| Ax2 | small: 75<br>large: 7<br>total: 82 | small: 41<br>large: 41<br>total: 50 | small: 70<br>large: 27<br>total: 97 | small: -<br>large: -<br>total: - |
| *drnA*$^-$ | small: 26<br>large: 2<br>total: 28 | small: 57<br>large: 0<br>total: 57 | small: 35<br>large: 4<br>total: 39 | small: 60<br>large: 35<br>total: 95 |

RNA was isolated from the cells that hade been exposed to osmotic shock and from the cells to which only buffer had been added instead of the hyper osmotic solution. RNA was also isolated from growing cells without any treatment. Northern blot analysis showed the presence and the levels of mRNA and asRNA in the different strains and how they were affected by the osmotic shock (figure 6).

The results for Ax4:4 showed that the asRNA and the mRNA expression is down regulated during the osmotic shock, but there is also difference between the untreated cells (Ax4:4 RNA) and the cells that were treated with only SPB buffer (-osmos). The asRNA is almost gone after the osmotic shock. In the asRNA for the sorbitol treated Ax4:4 cells we can also see a double band that appears around 800nt, something that also appears in all of the lanes in the *drnA*$^-$ and *Ax2* strains.

Ax2 have a higher mRNA expression in the buffer treated cells (-osmos) than in the sorbitol treated cells (+sorbitol), which can be seen in figure 6b. The mRNA expression in the untreated cells (Ax2 RNA) is higher than in the buffer treated cells, a pattern that also can be seen for the asRNA expression. Although, between sorbitol treated and buffer treated cells we do not see a difference in the asRNA expression patterns.

*drnA*$^-$ have a similar expression pattern for the mRNA as for the Ax2 mRNA, with higher expression in the untreated cells (*drnA*$^-$ RNA) than in the sorbitol treated or buffer treated ones. If we compare the mRNA expression in the buffer

treated and the sorbitol treated cells, we can see a higher expression in the buffer treated cells. asRNA expression is down regulated in the buffer treated cells compared to the untreated ones, but for the sorbitol treated ones we instead see a up regulation of the expression.
For the knockout *rrpC*$^-$ it seems like the asRNA is slightly up regulated after osmotic shock compared to the expression in the untreated cells (*rrpC*$^-$ RNA) and those treated with only buffer. The mRNA expression is up regulated in the buffer treated cells compared to the untreated ones, but is down regulated in the sorbitol treated cells compared to both the buffer treated and untreated cells.

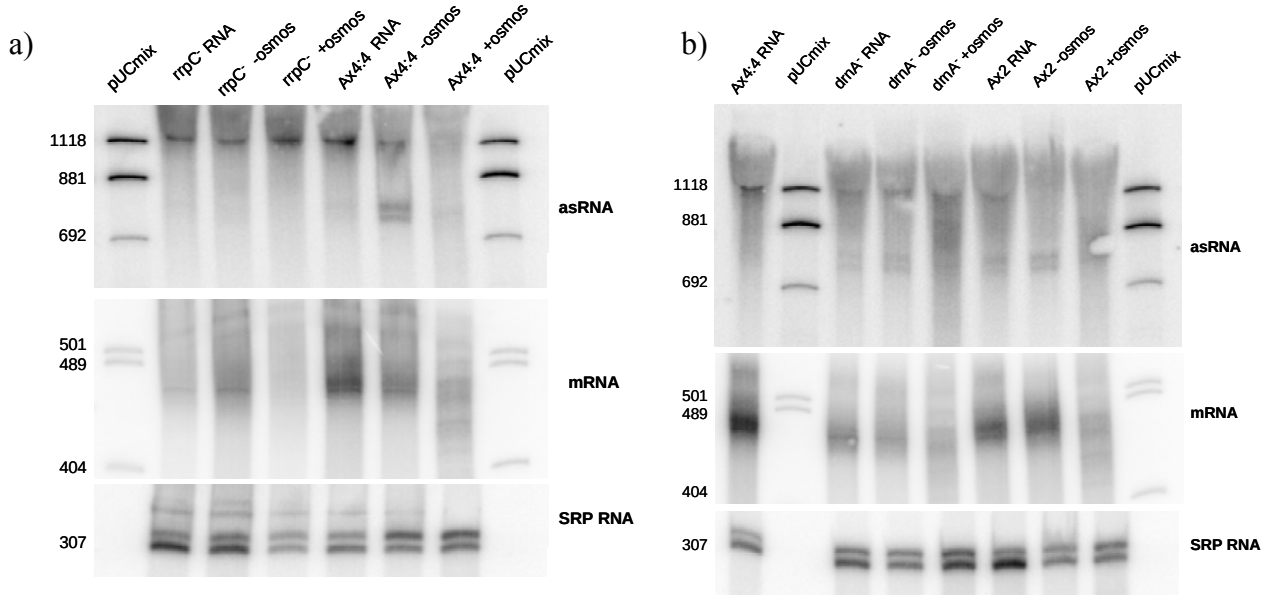


**Figure 6.** Northern analysis of RNA from cells exposed to osmotic shock. Ax4:4 is present on both membranes as a control. The differences between the two membranes are the different strains, Ax2 and *drnA*$^-$ on the a) membrane and *rrpC*$^-$and Ax4:4 on the b) membrane. The membranes have been treated equal and hybridized with the same probes. The SRP RNA is a control for the RNA quality and equal loading of the RNA. +osmos is RNA from cells treated with hyper osmotic buffer, -osmos is cells treated with buffer (starvation buffer) and lanes marked with RNA contains RNA from untreated cells.

To check the RNA quality and equal loading, the membranes where hybridized with a probe directed against SRP RNA (Aspegren et.al, 2004)

## Detection of mRNA and asRNA for *kielin* by using Fluorescent *In Situ* hybridization

Since we could detect mRNA and asRNA for *kielin* by RT-PCR, we wanted to analyse these further. In an article written by Matsui and co-workers (2000) they were able to locate *kielin* mRNA in *Xenopus* by using fluorescent *in situ* hybridization (FISH). In 2003 Maeda and co-workers found that *kielin* is located in the front of the *Dictyostelium*. To visualize the expression pattern of the *kielin* asRNA and mRNA in *Dictyostelium*, we performed FISH on cells developed for 16 hours (slugs). The structures were hybridized with fluorescently labelled *in vitro* transcribed probes directed against asRNA and mRNA respectively and observed by fluorescence microscopy (figure 7).

a) b)

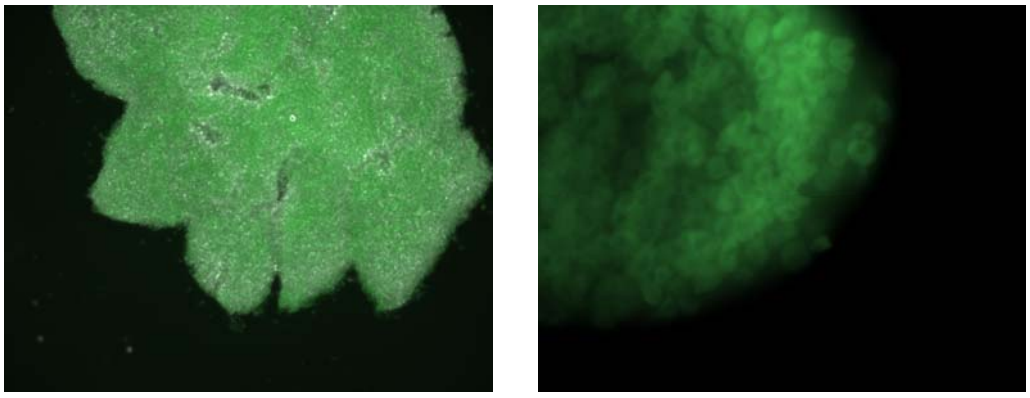

**Figure 7.** Fluorescent *in situ* hybridization with probes directed against asRNA and mRNA for *kielin*. a) Structure hybridized with a fluorescent probe directed against mRNA for *kielin*. b) asRNA is detected by a fluorescent probe directed against asRNA for *kielin*. The asRNA is located in the outer layer of the structure while the mRNA is located in the entire structure.

Here we can see a difference in location between the asRNA and the mRNA. The asRNA is located in the outer cell layer, while the mRNA can be found in the entire slug. The asRNA seems to be located on one side of the structure in its outer layer.

# DISCUSSION

## Revealing the presence of mRNA and asRNA in *Dictyostelium*

RT-PCR was done with the aim to find mRNA and complementary asRNA for three selected genes (*hatA, rsmF, kielin*) in *Dictyostelium* cells. The genes had been selected from a cDNA library representing 18-25nt RNAs, where the mRNA had shown to have a small antisense RNA complementary to its mRNA.

RT-PCR, and later on sequencing of the PCR products of these three genes, confirmed the presence of both an mRNA and asRNA. Interestingly the hisactophilin I (*hatA*) asRNA lack the intron. We also studied two genes within the same "gene family" as *hatA*, *hatB* and *hatC*, and here we found the mRNA for *hatC* but we also saw that it lacks an asRNA and that the *hatC* does not contain any intron. For the *hatB* gene we were able to find both an mRNA and an asRNA. *hatB* does contain an intron, but here we decided to place the primers in the same exon instead and therefore we do not see the intron in the PCR product. In the case of *kielin* and *rsmF* we found both asRNA and mRNA for the two genes.

The aim with the studies that have been done was to investigate the expression pattern of *hatA* mRNA and asRNA during development, in RNAi knockout strains and also how the expression responds to hyper osmotic stress.

## *hatA* asRNA and mRNA expression is regulated in *Dictyostelium discoideum* during development.

By looking at the expression levels of *hatA* mRNA and asRNA in *Dictyostelium discoideum* during development, we could see a trend that the expression of both mRNA and asRNA goes down during development. The reason for this could be that the gene is simply not needed when the structure goes through its developmental stages. We only looked at growing cells (0h) and structures that had been starving and developing for 16 hours. My belief is that if we had studied structures after 24 hours of development, we would have seen an even lower expression of both mRNA and asRNA compared with the expression levels after 16 hours or in growing cells.

A question that one could possibly ask is why *hatA* is necessary in growing cells but not in structures that have been starving. One possible answer is that due to starvation and when the cells aggregate and starts to develop into different stages, the outer surface is strong enough to stand against the different conditions in the environment. A single cell can not protect itself against for example osmotic changes. To test the response towards osmotic stress we ran a hyper osmosis-test where we wanted to study the regulation of the *hatA* mRNA and asRNA expression due to the stress (see below).

## *hatA* is regulated in RNAi knockouts

By comparing the expression of *hatA* mRNA and asRNA in some RNAi knockout strains (*drnA*$^-$, *drnB*$^-$, *rrpA*$^-$, *rrpB*$^-$, *rrpC*$^-$ and *helF*$^-$) with the two wild type strains Ax4:4 and Ax2, we could see that there is a significant difference in expression. The expression in *rrpC*$^-$ is down regulated if we compare its asRNA and mRNA expression with the one in Ax4:4, in fact, the mRNA expression almost seems to disappear. One possible explanation for this could be that *hatA* mRNA is dependent on the presence of the rrpC. If we look at the other knockout strains and compare their expression with the Ax2 strain we can see a similar pattern in the *drnA*$^-$ strain where the mRNA expression almost seems to disappear, and here we can use the same theory as for the *rrpC*$^-$ knockout. When it comes to the other knockouts (*drnB*$^-$, *rrpA*$^-$, *rrpB*$^-$ and *helF*$^-$) we only see small differences in the levels of expression if we compare them to the Ax2 strain.

## Response towards hyper osmotic stress in *Dictyostelium* wildtype and RNAi knockout strains

Hisactophilin has previously been shown to have a role in osmo-protection in the *Dictyostelium* cell and therefore we decided to investigate this phenomenon. The results from the Northern blot analysis revealed both up and down regulation of *hatA* mRNA and asRNA expression due to osmotic stress. A viability test was performed with the aim to see if the survival skills are affected due to the osmotic stress. If we compare the results from the viability test we see a down regulation in the number of counted placks in some of the strains after shock (*Ax4:4, Ax2, rrpC*$^-$) and an up regulation in one of the knockout strains (*drnA*$^-$) after osmotic shock.

The results from the northern blot analysis revealed a down regulation of the mRNA in Ax4:4 strain due to the osmotic stress, and here we also saw the highest expression level in the untreated RNA. The same trend can be seen in the asRNA for the same strain. If we compare these results with the viability test we can draw the conclusion that the ability to survive together with the expression of both mRNA and asRNA goes down due to the stress. Since we see a difference between the untreated RNA and the RNA from cells treated with a starvation buffer (-osmos) we can say that the cells are affected even before the osmotic solution is added. The cells prepare to enter development due to starvation and maybe this can be a normal down regulation of *hatA* that occurs when the cells starts too develop, a phenomenon which has been seen in previous experiments (Schuster et.al 1996, Pintch et.al 2002). Simply, hisactophilin is no longer needed in the cell membrane to protect the cells from the environmental changes that occur. When we then treat the cells with an osmotic buffer the *hatA* expression is lowered even more. An interesting notation is the double band in the -osmos lane in the asRNA blot. Why this fragment appear is hard to tell, but one thing is

interesting, and that is that it appears in all the lanes for the Ax2, *drnA*$^-$ strains and in the –osmos lane for Ax4:4 but for the *rrpC*$^-$ we do not se this fragment at all.
For the *rrpC*$^-$ strain we see another expression pattern than the one for Ax4:4. The *hatA* mRNA is up regulated in the –osmos compared to both the untreated RNA and the +osmos RNA. Maybe this has something to do with the initiation of development that the starvation buffer causes. The asRNA has its highest expression in the +osmos RNA while the –osmos and untreated RNA have almost the same level of expression. The osmotic stress causes an up regulation in asRNA for *hatA*, indicating a role for RRpC in the down-regulation of *hatA* asRNA observed in the wildtype strain.
The expression of mRNA and asRNA in the Ax2 strain is both down regulated due to the stress. The expression level of asRNA is low compared to the asRNA patterns in the other strains, and since even the untreated RNA is low this indicates that the asRNA in the Ax2 strain is low due to another factor than the stress that the osmotic or starvation buffer causes. Why *hatA* asRNA expression is low in the Ax2 strain is hard to tell but one suggestion that Ax2 simply have a low level of *hatA*. The mRNA is down regulated and it has its highest expression in untreated cells and in cells treated with starvation buffer (-osmos). Cells that have been treated with the hyper osmotic buffer shows a low expression of *hatA* mRNA compared to the other Ax2 RNA samples. The expression level of the mRNA is comparable to the level that can be seen in the Ax4:4 strain. All three Ax2 RNA samples contained a double band at the same size as the one in the Ax4:4 strain. These double bands also appeared in *drnA*$^-$ where we saw an up regulation in asRNA and a down regulation in mRNA due to the stress. mRNA expression is lower than the one in Ax4:4 and Ax2, while the asRNA expression is comparable with Ax2.
The pattern can be explained by the theory that *hatA* mRNA is not needed after the step where unicellular *Dictyostelium* aggregate to a multicellular structure and go through development. A theory could be that the outer membrane becomes thick enough to resist changes in the environment, a function that *hatA* encodes for in the unicellular organism.

We have in two experiments (RNAi knockouts and hyper osmosis) seen that there is a difference in the expression levels of *hatA* mRNA and asRNA in the wild types Ax4:4 and Ax2, but we do not know why we see this difference.

## Fluorescent in situ hybridization reveals the location of mRNA and asRNA for kielin in *Dictyostelium* slug

For kielin we were interested to find the location of asRNA and mRNA in a 16 hours structure, also called a slug. The results revealed that it seems like that the mRNA is present in the entire slug, while the asRNA seems to be located in the outer area of the structure. Why *kielin* mRNA and asRNA have these separate

locations in the structure is hard to tell, but a theory could be that because of some unknown reason, the asRNA silences the mRNA expression in the outer area of the structure. *Kielin* has been found at the front of the slug in previous research by others (Maeda et al. 2003) and so may be the case in our experiment as well. One suggestion could be that the mRNA is silenced by the asRNA at the front of the structure preventing it from becoming to large to handle by first regulating the slug phase and after that directing the phase where a upper cup is created.

The method and the handling of the structures on the other hand might have played a part in the fade results. Errors may have occurred in for example the fixation step where we lost a lot of cells since they adhered to the tube. A solution for this problem might be tubes with a teflon-coating on the inside or glass tubes. Another mistake that was done, which explains the picture with the mRNA and the crushed appearance, is that the structures might have been crushed when they were fixated on a glass plate. If these errors disturbs the results or not is hard to tell, since we only did the experiment once. A refinement of the method and a more gentile hand while handling the structures should give better pictures.

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