Investigating the role of 3' readenylation in facilitating 3'-5' mRNA decay in Aspergillus nidulans

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Investigating the role of 3' readenylation in facilitating 3'-5' mRNA decay in *Aspergillus nidulans*

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Abstract

Cytoplasmic tagging of the 3' end of mRNA with non-canonical U and C nucleotides has emerged as a novel mechanism that appears to play an important role in determining the fate of mRNA. In *Aspergillus nidulans,* expression of genes involved in utilisation of poor nitrogen sources are regulated at different levels, including at the level of regulated transcript stability. Here changes occurring on the 3' ends of genes involved in nitrate utilization, *niaD* and *niiA,* were analysed by Sanger sequencing of clones obtained after the 5' adenylated (5rApp-) adaptor was ligated to the 3' end of transcripts followed by RT, PCR and cloning into the TA vector system. It was found that partially degraded 3' ends of the *niiA* and *niaD* transcripts contained sequences of adenosine nucleotides, suggesting that readenylation may be required for 3'-5' mRNA decay. Furthermore, the addition of poly/oligo (A) tails to the 3' end of mRNA was found to be diminished in strains depleted for either *cutA* or *cutB* and abolished in the absence of both enzymes. These findings strongly suggested that the two nucleotidyltransferases, CutA and CutB, which are responsible for the cytoplasmic C/U tagging in *A. nidulans,* may have an additional role in mRNA turnover. These findings argued that readenylation of eukaryotic transcripts represents a mechanism which resembles both the degradation of ρ-independent mRNAs in prokaryotes and re-uridylation dependent 3'-5' degradation of the human cell-cycle regulated histone transcripts.

1. Literature Review

1.1 Introduction

Gene expression is a multistep process where the genetic information is decoded into the two final products, namely proteins and non-coding RNAs (ncRNAs). Gene expression revolves around RNA molecules that produce and regulate protein synthesis. The biogenesis, function and turnover of RNA in eukaryotic organisms is determined by the primary sequences and the structures at the 5' and 3' termini. For instance, the process of eukaryotic mRNA biogenesis (transcription, splicing, 3' end formation and transport to the cytoplasm), function (translation), and turnover (degradation) involve the 5' cap and 3' poly (A) tail structures. In the cytoplasm, both the 5' and 3' ends of mRNA become targets for decay machinery, ribosomes and translational factors. Although the key steps of gene expression are conserved between prokaryotes and eukaryotes, there are significant differences between the two kingdoms.

One of the main differences is that in eukaryotic cells, the nucleus is enclosed by a double membrane which separates transcription from translation in time and space. Prokaryotic cells do not have a membrane bound nucleus, so transcription and translation are coupled. Eukaryotic and prokaryotic transcripts need to ensure resistance from degradation machinery to be translated, thus the level of mRNA is defined by the rate of transcription and the rate of degradation. For a long time, it was thought that prokaryotes and eukaryotes developed opposite mechanisms to degrade their mRNA. For instance, in bacteria adenylation of the 3' end of ρ-independent transcripts signals for degradation, while in eukaryotes the poly (A) tail stabilises mRNA and facilitates translation.

However, it was later found that this lack of structure of adenosine nucleotides, depending upon its length, appears to be the major determinant of decay and

stabilisation. Thus, the poly (A) tail may have just acquired an additional role for stabilisation in eukaryotes. For the majority of eukaryotic transcripts, destabilisation is initiated by degradation of the poly (A) tail in the cytoplasm. However, it has been known that the poly (A) tail can be added to the 3' end of translationally silent mRNA in the cytoplasm, to activate translation during development.

Other types of nucleotides have been discovered to be used in the cytoplasm to extend 3' ends of transcripts in eukaryotes, which include uracil and cytosine nucleotides. The addition of C/U nucleotides results in the appearance of a short single stranded platform which lack structure as in the case of adenosine nucleotide addition in bacteria. Consistent with the role of a short oligo (A) tail being a signal for degradation in bacteria and in the nucleus in the eukaryotes, the addition of oligo C/U nucleotides in the cytoplasm has been found to have a similar role in degradation in the eukaryotes. Recent reports indicate that it is not just C/U nucleotides which can be added to the 3' end of mRNA in the cytoplasm of eukaryotes, but a short stretch of A nucleotides can also be added to the 3' end of partially degraded mRNA in the cytoplasm which may have implications on mRNA degradation. It appears that the addition of single stranded unstructured nucleotide platforms of U/C/A nucleotides to the 3' end of eukaryotic mRNAs in the cytoplasm is a conserved eukaryotic mechanism, which is involved in mRNA stability and translation. However, the enzymes involved in the addition of these modifications are yet to be fully elucidated.

1.2 The role of mRNA stability in gene expression

The stability of mRNA involves setting the basal level of the transcript in the cell, in the adaptive response to environmental signals in order to allow for rapid alterations in gene expression, in defence mechanisms against viruses and in quality control (Stoecklin *et al,* 1994; Morozov *et al,* 2001; Schmidt *et al,* 2011; Decker and Parker 2002; Caddick *et al,* 2006; Pérez-Ortín *et al,* 2013). The stability of eukaryotic mRNAs primarily depends upon two co-transcriptional modifications, namely the N^7-methylguanylate cap at the 5' end and the poly (A) tail at the 3' end (Shatkin 1976; Banerjee 1980; Colgan and Manley 1997). The 5′ cap consists of a guanine nucleotide connected to mRNA via an unusual 5′ to 5′ triphosphate linkage. This guanosine becomes methylated on the 7th position directly after capping *in vivo* by a methyltransferase, referred to as a N^7-methylguanylate cap (^{m7}G) (Shatkin 1976; Banerjee 1980; Sonenberg *et al,* 1998; Marcotrigiano *et al,* 1997). In the nucleus, the capping process is initiated before the completion of transcription as the nascent pre-mRNA is being synthesized (Guhaniyogi and Brewer 2001). The poly (A) tail consists of multiple adenosine nucleotides which are added by a nuclear poly (A) polymerase (PAP) up to around 200 nucleotides in mammals and 70-100 nucleotides in fungi. This polyadenylation process happens in combination with the cleavage polyadenylation specificity factor (CPSF), which recognises the canonical polyadenylation signal and varies among species (Edmonds 2002). Furthermore, as the poly (A) tail is synthesised it interacts with poly (A) binding proteins (PABPs) which protect it against degradation machinery (Sonenberg and Hinnebusch 2009; Tarun *et al,* 1996; Kapp *et al,* 2004; Caponigro and Parker 1995). Generally, the 5' cap and the 3' poly (A) tail are added in the nucleus of a cell, and are important for nuclear transport, stability and translation whilst also protecting the mRNA against degradation (Guhaniyogi and Brewer 2001). Destabilisation of mRNA requires removal of these stability determinants and most, if not all, mRNAs in eukaryotes can be degraded by two major pathways. This includes

the poly (A) dependent 5'-3' decay pathway catalysed by Xrn1 and the 3'-5' pathway which is catalysed by the exosome (Hsu *et al,* 1993; Muhlrad *et al,* 1994; Stevens, 2001). These two pathways were initially characterised in *Saccharomyces cerevisiae* and have been found to be conserved among eukaryotes (Meyer *et al,* 2004; Parker and Song 2004; Stoecklin *et al,* 2006; Bönisch *et al,* 2007; Morozov *et al,* 2006; Morozov *et al,* 2010b).

1.3 Polyadenylation

Poly (A) tails play multiple roles in RNA biogenesis, function and degradation in all kingdoms of life and their length appears to play an integral role. It also seems that the evolutionary function of poly (A) tails has become more diverse in complex organisms. The major function of adenylation in prokaryotes is in destabilising RNA and promoting degradation, which appears to be irrespective of the length (Mohanty *et al,* 2004; Folichon *et al,* 2003). In eukaryotes adenylation plays a much more diverse role. In the nucleus, adenylation with short oligo (A) sequences promotes degradation of maturing RNAs; the addition of a long poly (A) tail promotes stability and export of mRNAs (and some long ncRNAs) to the cytoplasm (Slomovic *et al,* 2006; West *et al,* 2006; Guhaniyogi and Brewer 2001). In the cytoplasm, the major function of poly (A) tails is to control mRNA turnover and translation by either promoting or inhibiting RNA decay machinery (Parker and Song 2004; Meyers *et al,* 2004; Parker 2012). In this section the diverse function of adenylation/ poly (A) tails will be described in both prokaryotes and eukaryotes.

1.3.1 The role of adenylation in prokaryotes

It was long believed that a major difference between the bacterial and eukaryotic mRNAs was the presence of poly (A) tails at the 3' end of eukaryotic mRNAs. Later it was established that the poly (A) tail is involved in the regulation of bacterial gene expression (Sarkar 1997; Dreyfus and Régnier 2002). Surprisingly, the first poly (A) polymerase enzyme purified was from *Escherichia coli* however, very few efforts were made to understand bacterial adenylation *in vivo* until the late 1970's (August *et al,* 1962; Modak and Srinivasan 1973; Sarkar *et al,* 1978). It was initially identified that *E. coli* mRNAs are adenylated by poly (A) polymerase 1 (PAPI) (Cao and Sarkar 1992). Later, another poly (A) polymerase was characterised in *E. coli* (PAPII) to have adenylation activity and several bacterial mRNAs were being shown to possess oligo (A) tails (Cao and Sakar 1992; Cao *et al,* 1996; Hajnsdorf *et al,* 1995; Haugel-Nielsen

et al, 1996; O'Hara *et al,* 1995; Xu and Cohen 1995). Furthermore, adenylation in bacteria was found to be regulated by Hfq (sm-like protein) and it was shown that by binding to the A-tails, Hfq enhances the processivity of PAP1 (Mohanty *et al,* 2004; Filichon *et al,* 2003). Post transcriptional addition of short A tails (around 5 nucleotides) have been associated with destabilisation of bacterial mRNA, and in particular the conserved 3' end step-loom, the results of Rho-independent transcription termination (Coburn and Mackie 1996; Hajnsdorf *et al,* 1995; O'Hara *et al,* 1995; Xu and Cohen, 1995). Deletion of the *pcnB* gene (structural gene of PAP1) resulted in enhanced stability of bacterial mRNAs (Xu *et al,* 1993; He *et al,* 1993; O'Hara *et al,* 1995; Hajnsdorf *et al,* 1995). Bacterial mRNA decay was found to be generally initiated via a 5' end endonucleolytic cleavage by RNase E which is part of the bacterial degradasome, along with PNPase which is a 3'-5' exonuclease, enolase and Rh1B, a DEAD box helicase (Carpousis 2007; Donovan and Kushner 1986; Kushner 2002) Most mRNAs in *E. coli* contain a stable stem-loop structure on 3' end and a very short 3' single stranded extension, which results from the Rho-independent transcription termination (Peters *et al,* 2011; d'Aubenton Carafa *et al,* 1990; Ray-Soni *et al,* 2016; Gusarov and Nudler 1999). The bacterial degradasome is generally inefficient in degradation of double stranded mRNAs, e.g. stem-loop structures (Blum *et al,* 1997). Endonucleolytic cleavage by RNase E generates a 3' end mRNA product of degradation containing 5' phosphate which becomes subject to rapid 3'-5' exonucleolytic decay (Perwez and Kushner 2006). Stem-loop structures block 3'-5' exoribonucleases which are overcome by short stretches of adenosine nucleotides (1-5 nucleotides) (O'Hara *et al,* 1995; Mohanty and Kushner 1999; Cao and Sarkar 1992). These short (A) tails act as a toe hold for the substrates of the PNPase component of the degradasome to bind (O'Hara *et al,* 1995; Mohanty and Kushner 1999; Cao and Sarkar 1992). Once bound, PNPase ensures rapid deadenylation and removal of one 3' end nucleotide of the stemloop structure (Blum *et al,* 1997). Several rounds of adenylation and deadenylation can occur at the bacterial 3' end to overcome the stem-

loop structure (O'Hara *et al,* 1995; Mohanty and Kushner 1999; Cao and Sarkar 1992). Additionally, RNase R and RNase II are hydrolytic processive 3'-5' exonucleases which can also rapidly degrade polyadenylated mRNAs in bacteria (Mohanty and Kushner 2000; Cheng and Deutscher 2005). Hfq inhibits the PNPase and RNase II, possibly to allow for the addition of the oligo (A) toe-hold to occur (Folichon *et al,* 2003). Interestingly, *in vitro* studies in *E. coli* demonstrated that oligo (G) and oligo (U) tails do not stimulate degradation by the degradasome, suggesting that these oligo G/U sequences are inaccessible for the exoribonucleases (Blum *et al,* 1997). This was from a study that showed that bacterial RNAs generated from genes with a Rho-independent termination mechanism, ending with an oligo (G/U) tail were found to be more stable than transcripts ending with an oligo (A) tail at the 3' end (Blum *et al,* 1997). It therefore appeared that adenylation of the 3' end of bacterial mRNA with adenosine nucleotides signals for degradation and the addition of oligo G/U nucleotides signals for stability. These findings demonstrated how different single stranded unstructured nucleotide platforms can regulate transcript stability in bacteria.

1.3.2 The role of nuclear adenylation in eukaryotes

Eukaryotic transcription occurs in the nucleus and requires a number of steps to mature RNA. Nuclear adenylation appears to be a part of the nucleus quality control mechanism where 95% of eukaryotic transcripts are degraded. The addition of a short (A) tail (between 1 and 5 nucleotides) signals for degradation similar to bacterial transcripts (Slomovic *et al,* 2008; West *et al,* 2006). Nuclear addition of short (A) tails (oligoadenylation) depends upon the TRAMP complex, which is a conserved polyadenylation complex (reviewed by Houseley *et al,* 2006). The TRAMP complex contains non-canonical poly (A) polymerases Trf4 and Trf5, a zinc-knuckle RNA binding protein (Air1p or Air2p) and an RNA helicase, Mtr4p (Thompson and Parker 2007; LaCava *et al,* 2005). The Trf4/ Trf5 enzymes provide poly (A) polymerase activity

and are activated by the RNA binding proteins Air1 and Air2, which result in the addition of the adenosine nucleotides to the 3' end of target transcripts (Thompson and Parker 2007; LaCava *et al,* 2005; Vanacova *et al,* 2005; Wyers *et al,* 2005). Mtr4p controls the number of adenosine nucleotides added and seems to limit them to only 3-5 nucleotides (Jia *et al,* 2011). Oligoadenylation in turn promotes the 5'-3' degradation pathway by the nuclear 5'-3' exoribonuclease Xrn2 and/ or 3'-5' decay by the exosome (Bonneau *et al,* 2009; Egecioglu *et al,* 2006; Li *et al,* 2005; West *et al,* 2006). Long poly (A) tails are added by poly (A) polymerases (a homolog of the bacterial PAP) in the nucleus, which promote stability and export from the nucleus to the cytoplasm. Thus, adenylation in the nucleus appears to have two roles, where the addition of a short (A) tail signals for degradation and a long (A) tail signals for stability and export.

1.3.3 Role of poly (A) tails in the cytoplasm in eukaryotes

Eukaryotic poly (A) tails in the cytoplasm play multiple roles in mRNA function and stability which are all dependent upon their length. All eukaryotic mRNAs have a maximum length for their poly (A) tails. For example, plant RNAs have poly (A) tail lengths of around 50 nucleotides, yeast around 30 nucleotides and for mammals 70-90 nucleotides (Subtenly *et al,* 2014). Poly (A) tails can function in maintaining the basal level eukaryotic transcripts because throughout their life time, (during every round of translation) the poly (A) tail is degraded until shortened to a threshold length of around 15-25 nucleotides, depending on the organism, which results in degradation of the mRNA body (Parker and Song 2004; Meyers *et al,* 2004). Poly (A) tail shortening rates vary in different mRNA therefore, their degradation rates are regulated (Parker and Song 2004; Meyers *et al,* 2004). Poly (A) tails can also function in stabilising transcripts and stimulating translation initiation from their interaction with PABPs. Poly (A) tails above the threshold length are able to bind to a sufficient number of PABPs

which have a footprint of around 15-25 nucleotides (Kuyumcu-Martinez 2002). The PABP is involved in formation of a closed loop structure which also comprises of eIF4G, the bridge protein which binds to both PABP and eIF4E, resulting in formation of the eIF4F complex which brings both the 5'- and 3' ends in close proximity (Wakiyama *et al*, 2000; Rissland 2017). This structure protects the 5' cap from access to the decapping machinery and promotes another round of translation initiation (Wakiyama *et al*, 2000). It is thought that shortening of the poly (A) tail to the threshold length or shorter triggers degradation, namely deadenylation, which leads to PABP dissociation. The dissociation of the PABPs means that interactions with eIF4G and eIF4E is broken, which results in the loss of the closed-loop structure. Loss of the closed-loop structure exposes mRNA to 5'-3' degradation machinery.

The Ccr4-Caf1-Not complex is the major deadenylase and is conserved among eukaryotes (Parker and Song 2004; Balagopal *et al,* 2012; Wiederfold and Passmore 2010). Ccr4 and Caf1 are the two deadenylases which play different roles in degradation of mRNA. Ccr4 is a conserved general deadenylase which appears to be dispensable in yeast; nonetheless in *Aspergillus nidulans* and mice it functions as a regulated deadenylase (Morozov *et al,* 2010; Bianchin *et al,* 2005; Denis *et al,* 2003; Collart *et al,* 2013). The Not proteins (1-5) full biological relevance is not well understood yet however; one possible role of these proteins could be in adapting the deadenylase complex to different mRNAs through regulatory proteins. Another major deadenylase complex was identified in eukaryotes which consists of Pan2 and Pan3 (Sachs and Deardorff 1992; Uchida *et al,* 2004). Pan 2 was found to be the catalytic subunit during deadenylation and Pan3 the regulator of Pan2 (Boeck *et al,* 1996; Mangus *et al,* 2004; Uchida *et al,* 2004; Brown *et al,* 1996). Unlike the Ccr4-Caf1-Not complex, Pan2/3 is characterised by its requirement for the presence of PABP for its deadenylation activity. Yeast studies demonstrated that Pan2 is activated by binding to PABPC1 through its interactions with Pan3 (Mangus *et al,* 2004; Uchida *et al,* 2004).

Although both the Ccr4-Caf1-Not and Pan2/3 complexes appear to have very similar activities, their functional differences with regards to cytoplasmic deadenylation remain unclear. It is postulated that Pan2/3 is involved in the nucleus quality control mechanisms and trims the newly synthesised poly (A) tail to a certain length which allows for mRNA to be exported to the cytoplasm. It is also thought that in the cytoplasm, Pan2/3 acts to edit down the poly (A) tail to a certain length and the Ccr4-Caf1-Not completes the deadenylation process (Sachs 1993; Yamashita *et al,* 2005). This hypothesis is supported by the requirement of Pan2/3 complex to interact with the PABP (yeast studies) while Ccr4-Caf1-Not is inhibited its presence (Sachs 1993; Mangus *et al,* 2004; Uchida *et al,* 2004). Furthermore, co-immunoprecipitation studies show that Pan2 interacts with Caf1 which supports the hypothesis of a two-step deadenylation process, where the complexes act sequentially in a co-ordinated manner (Zheng *et al,* 2008). Interestingly, a new and currently accepted model suggests that when the poly (A) tail is degraded to a length of around 15-25 nucleotides, it becomes a target for cytoplasmic nucleotidyltransferase such as Cid1 from *Schizosacchromyces pombe* and its homologues in other organisms (Mullen and Marzluff 2008; Rissland and Norbury 2009; Morozov *et al,* 2010; Morozov *et al,* 2012; Chang *et al,* 2014).

It has been found that enzymes add, to the 3' end of mRNA, non-canonical (not encoded in DNA) C/U nucleotides in the cytoplasm, namely 3' end tagging (Mullen and Marzluff, 2008; Rissland and Norbury 2009; Morozov *et al,* 2010a; Morozov *et al,* 2012; Chang *et al,* 2014). Tagging is a conserved mechanism across all eukaryotes and its disruption retards transcript decay (Morozov *et al,* 2010; Mullen *et al,* 2008; Rissland and Norbury 2009) while coinciding with decapping. Once these non-templated nucleotides are added, it is thought that they increase the affinity of Lsm 1-7 heptamer thus, recruiting Pat1 to the 3' end of the mRNA and hence inhibiting translation and stimulating decapping by Dcp1/Dcp2 (Tharun *et al,* 2000 Van Dijk *et al,* 2002; Steiger *et al,* 2003). Dcp2 cleaves the cap structure to release m^7GDP, leaving a 5'

monophosphate mRNA while Dcp1 interacts with Dcp2 to promote its catalytic activity (She *et al,* 2004; She *et al,* 2008; Deshmukh *et al,* 2008). Following decapping, mRNAs are degraded in a 5'-3' direction by the Xrn1 nuclease, which targets mRNA with a 5' monophosphate and/or by 3'-5' degradation by the exosome (Hsu and Stevens 1993; Muhlrad *et al,* 1994; Stevens 2001 Mullen and Marzluff 2008). In many eukaryotes there is another deadenylase called poly (A)-specific ribonuclease (PARN) which is functionally different from both Ccr4-Caf1-Not and Pan2-Pan3 because it requires the 5' cap for it to be activated (Yamashita *et al,* 2005). Therefore, in the cytoplasm, eukaryotic transcripts long poly (A) tails function to stabilise target mRNA which results in them being translated, whereas shortened poly (A) tails function in their destabilisation thus leading to degradation in an intricate process which involves many proteins.

1.4 Tagging of cytoplasmic mRNA in eukaryotes

There are over 100 post-transcriptional modifications that can affect mRNA structure and hence, function, localisation and stability (e.g. Wang and He 2014; Roundtree and He 2016; Fu *et al,* 2014; Chen *et al,* 2016). RNA modifications are important for the recruitment of different sets of proteins to RNA and therefore influence the RNP structure and subsequently their function (Mitchell and Parker 2014). This makes RNA modifications important to regulatory processes by altering the transcript RNP structure in response to developmental and environmental cues (Fu *et al,* 2014; Chen *et al,* 2016; Licht and Jantseh 2016; Meyer and Jaffrey 2014; Haussmann *et al,* 2016). RNA modifications can be divided into two main subgroups. The first subgroup includes chemical modifications of nucleosides, which are very diverse and represent the majority of mRNA modifications. Examples of nucleoside modifications include N^6-methyladenosine, N^1-methyladenosine, pseudouridine or 5-hydroxymethylcytosine modifications (e.g. Wang and He 2014; Roundtree and He 2016; Dominissini *et al,* 2016; Delatte *et al,* 2016; Li *et al,* 2016 Hussmann *et al,* 2016). The second subgroup of RNA modifications includes tagging of the 3' ends of RNA, which encompasses adenylation, uridylation, cytidylation and guanylation, which are termed 3' end tagging (Shen and Goodman 2004; Mullen and Marzluff 2008; Rissland and Norbury 2009; Morozov *et al,* 2010; Morozov et al, 2012; Chang *et al,* 2014; Lim *et al,* 2016). Although the tagging of uridine nucleotides has been known since the late fifties and early sixties, its significance was clearly underestimated. Daniel and Littauer, (1963) found uridine residues incorporated in the 3' ends of rat liver RNA chains. Klemperer (1963) described an enzyme that adds uridine residues to the 3' terminal positions of RNA, which is now known to be a nucleotidyltrasferase (Rissland *et al,* 2007; Rissland and Norbury 2009). Wilkie and Smellie (1968a) found an increased uptake of uridine residues for 5' dephosphorylated RNA at the 3' hydroxyl ends in comparison to phosphorylated RNA. Although this research appeared to describe tagging with uridine

nucleotides to occur at the 3' ends of RNA, they did not define its significance or biological function. In this chapter 3' end tagging mainly with U and C nucleotides and their effects on the fate of mRNA will be discussed.

1.4.1 Tagging of RISC-cleaved mRNAs

In early 2000's a link between tagging with uridine nucleotides and mRNA decay was initially noticed when 5' fragments of mRNA cleaved by the RNA-induced silencing complex (RISC), were found to be modified by U-rich tails in *Arabidopsis thaliana* (Shen and Goodman 2004). Adaptor ligation reverse-transcription PCR revealed a stretch of 9 non-canonical uridines added to the 3' end of cleavage sites on the 5' end mRNA fragment in the cytoplasm (Shen and Goodman 2004). The non-canonical uridine nucleotides appeared to be somehow involved during degradation of the mRNA fragments. Consistent with these results, it was shown that in mouse and human cells that non-template stretches of uridine nucleotides can be added to the 3' end of the cleavage sites of the 5' mRNA degradation intermediates (Yekta *et al,* 2004; Xu *et al,* 2016). Enzymes involved in the tagging of these uridine nucleotides to the 5' RISC cleaved fragments were identified in *A. thaliana* (HESO1) and humans both of which belong to the nucleotidyltrasferase family (Ren *et al,* 2014; Xu *et al,* 2016). It was later found that tagging of the 5' decay intermediate fragments coincides with decapping and 5'-3'decay by Xrn1 (Shen and Goodman 2004; Song and Kiledjian 2007). Tagging of RISC cleaved mRNA fragments represents the first example of these modifications being linked to degradation.

1.4.2 Tagging of replication dependent human histone mRNAs

Tagging with uridine nucleotides has been found to elicit degradation of replication-dependent histone mRNAs in humans (Mullen and Marzluff 2008). Unlike the majority

of eukaryotic mRNAs, histone replication dependent transcripts end with a conserved stem–loop structure. Histones are essential for proper chromatin formation, and their expression is tightly regulated and linked to the cell cycle (Pandey and Marzluff 1987; Kaygun and Marzluff 2005). Upon inhibition of DNA replication, at the end of S-phase, the expression of histone genes is shut down and degradation of their mRNAs is triggered, which represents a major regulatory step (Marzluff *et al,* 2008). This degradation process is initiated by tagging of uridine nucleotides which subsequently triggers both the 5'-3' and 3'-5' degradation pathways (Mullen and Marzluff 2008). Cell cycle regulated histone transcripts, being unique in their lack of 3' poly (A) tail, form a mRNP complex with the stem-loop binding protein (SLBP) and Eri-1 which are bound to the 5' and 3' part of the stem-loop, respectively (Tan *et al,* 2013). (Hoefig and Heissmeyer 2014; Marzluff *et al,* 2008). The interaction of the SLBP and translation initiation factors is critical for histone mRNA translation. The switch from translation to degradation of histone mRNA, in response to replication arrest, is signalled by phosphorylation of the RNA helicase Upf1 (an important nonsense mediated decay (NMD) factor (Behm-Ansmant *et al,* 2006). Recruitment of Upf1 has been found to result in disruption between translation initiation factors and the SLBP, most likely during translation termination which initiates degradation (Choe *et al,* 2014). It was proposed that Upf1 recruits TUTases (Terminal Uridyl Transferases) resulting in tagging of the 3' ends (Mullen and Marzluff 2008) and this occurs co-translationally. Tagging in histones has been found to promote binding of the decapping factor, Lsm1-7 which interacts with Eri-1, thus recruiting 3'-5' exonuclease (Hoefig *et al,* 2013; Slevin *et al,* 2014). Eri1 is a single-strand dependent nuclease which degrades only single stranded RNA and not double stranded structures (Hoefig *et al,* 2013; Slevin *et al,* 2014; Mullen and Marzluff 2008;). Tagging in histones could be acting as a single stranded platform for Eri1 to bind therefore, as it degrades the uridine nucleotides it will also degrade some canonical nucleotides in a similar mechanism to bacterial systems (Hoefig *et al,* 2013; Slevin *et al,* 2014). In support of this hypothesis, multiple

rounds of tagging by TUTases and removal of the uridine nucleotides by Eri1, has been found to be required for complete degradation of the stem-loop structure (Choe *et al,* 2014; Slevin *et al,* 2014). Each cycle of tagging and degradation was found to remove one or two nucleotides from the 3' end of the stem-loop which ultimately led to the destruction of the stem-loop and the subsequent degradation of histone mRNAs by RRP6 (PM/Scl-100) (a member of the exosome complex) (Slevin *et al,* 2014). High-throughput sequencing studies revealed that TUTase 7 and TUTase 4 are the main nucleotidytrasferases that catalyse the addition of these uridine nucleotides to the 3' end of cell-cycle regulated histone mRNAs (Schmidt *et al,* 2011; Lim *et al,* 2014; Lackey *et al,* 2016). Uridine tagging of histone mRNA represented the next major breakthrough towards realising that it is an integral step of mRNA decay.

1.4.3 Tagging of polyadenylated mRNAs

Tagging was not only found to occur on non-adenylated transcripts but also on polyadenylated mRNA in species such as the fission yeast, *S. pombe* and filamentous fungus *A. nidulans* (Rissland and Norbury 2009; Morozov *et al,* 2010). In *S. pombe*, circularised RT-PCR analysis revealed that six functionally diverse transcripts *act1, adh1, gar2, hcn1, pof9 and urg1* were subject to tagging with uridine nucleotides (Rissland and Norbury 2009). Tagging in *S. pombe* is largely catalysed by the TUTase Cid1 that adds 1-2 uridines at the 3' end of the target mRNAs (Rissland and Norbury 2009). Tagging in *S. pombe* was found to occur concurrently with decapping, which resulted in 5'-3' degradation because *urg1* mRNA levels increased in the *ΔCid1* background and uridylated mRNAs accumulated when *dcp1* and *lsm1* decapping factors were deleted (Rissland and Norbury, 2009). It was also found that this tagging occurred independently of poly (A) tail shortening (Rissland and Norbury 2009). Irrespective of fission yeast mRNA being polyadenylated, the reason for this deadenylation independent decapping could be due to its short poly (A) tails, in

general, compared to other eukaryotes (Subtelny *et al,* 2014; Chang *et al,* 2014). Also, mRNA tagging in *S. pombe* was also found to promote the 3'-5' decay pathway by Dis3L2 (Malecki *et al,* 2013). *adh1* mRNA was recognised for decay by exoribonuclease Dis3L2 (Malecki *et al,* 2013) which belongs to the RNase II family proteins which form the catalytic subunit of the exosome (Dziembowski *et al,* 2007; Malecki *et al,* 2013). In humans, there are three characterised paralogues of this enzyme which are Dis3, Dis3L and Dis3L2 (Staals *et al,* 2010; Tomecki *et al,* 2010; Astuti *et al,* 2012). Dis3 and Dis3L interact with the exosome ring facilitated by the N-terminal PIN and CR3 domains (Schaeffer *et al,* 2012; Makino *et al,* 2013). However, Dis3L2 functions independently of the exosome, most possibly due to the lack of the N-terminal PIN and CR3 domains (Schaeffer *et al,* 2012; Makino *et al,* 2013). Although Dis3L2 has been found to function independently of the exosome it is known to be involved in degrading human tagged mRNA, in a similar manner as in *S. pombe* (Lubas *et al,* 2013; Ustianenko *et al,* 2013; Labno *et al,* 2016). The role of Dis3L2 in 3'-5' mRNA decay has been further supported by its role in apoptosis-induced decay. Apoptosis induced decay in humans involves tagging with uridine nucleotides of decay intermediates and subsequent degradation by Dis3L2 (Thomas *et al,* 2015). Knockdown of Dis3L2 was found to remove U tags at the 3' end which inhibits apoptotic mRNA decay, translation arrest and cell death (Thomas *et al,* 2015).

In contrast to *S. pombe,* deadenylation to oligo (A) 15-25 nucleotides has been found to precede tagging in *A. nidulans*, *Arabidopsis thaliana* and humans which implies that tagging can only occur on shorted poly (A) tails (Morozov *et al,* 2010a; Morozov *et al,* 2012; Chang *et al,* 2014; Sement *et al,* 2013; Zuber *et al,* 2016). In *A. nidulans,* polyadenylated *gdhA* and *uaZ*$^{+}$ transcripts were found to be tagged with the addition of nucleotides carrying a CUCU consensus (Morozov *et al,* 2010a; Morozov *et al,* 2012). These C and U nucleotides were added when the poly (A) tail was degraded to around 15 nucleotides which is the length of poly (A) tail at which decapping was found

to occur (Decker and Parker 2002; Morozov *et al,* 2010a; Morozov *et al,* 2012). Nucleotidyltransferase family enzymes, CutA and CutB, were found to be required for these C and U nucleotide modifications in *A. nidulans* (Morozov *et al,* 2010a; Morozov *et al,* 2012). Deletion of either CutA or CutB resulted in a significant reduction in the amount of C/U tagged transcripts observed and deletion of both led to total loss of tagging (Morozov *et al,* 2010; Morozov *et al,* 2012). It was proposed in *A. nidulans* that these cytoplasmic C/U nucleotide modifications are associated with decapping as seen in *S. pombe* (Rissland and Norbury 2009; Morozov *et al,* 2010a). In humans, genome-wide associated studies showed that tagging with uridine nucleotides preferentially targets oligo (A) tails of a threshold length of around 25 nucleotides (Lim *et al,* 2014). Using the Tail-seq method TUTase 4 and 7 nucleotidyltrasferases were the enzymes identified to catalyse mRNA uridylation in humans (Lim *et al,* 2014). TUTase 4 and TUTase 7 were found to have an intrinsic preference for shorter A tails because the cytosolic PABPC1 prevented uridylation on mRNAs with poly (A) tails longer than 25 nucleotides (Lim *et al*, 2014). Depletion of either TUTase4 or TUTase7 resulted in a loss of tagging and an increase in the half-lives of their mRNAs implicating these nucleotidyltraferases in catalysing the addition of these modifications (Lim *et al*, 2014). From these results it became apparent that tagging by nucleotidyltrasferases may form an integral part of eukaryotic mRNA decay (with the exception of *S. cerevisiae*) and this was supported by global analysis of uridine tagging eukaryotes (Chang *et al,* 2014). The deep sequencing method, Tail-seq, designed to analyse both poly (A) tail length and any potential 3' end modifications at a transcriptome wide level, demonstrated that U tagging occurs at a global scale when the poly (A) tails are shortened to a threshold length (Chang *et al,* 2014). Furthermore, when mRNA decay factors, such as Xrn1, Dcp1 and Lsm1 were depleted it resulted in an accumulation of tagged mRNAs which was consistent with initial results found in *S. pombe* (Chang *et al*, 2014). Altogether these studies established that tagging of mRNA is a global phenomenon that elicits both 5'-3' and 3'-5' degradation in eukaryotes (Shen and

Goodman 2004; Mullen and Marzluff 2008, Morozov *et al,* 2010a; Morozov *et al,* 2012; Rissland and Norbury 2009; Lim *et al,* 2014; Chang *et al,* 2014).

1.4.4 Tagging of plant mRNAs

In *A. thaliana* at least two enzymes were identified as being involved in this tagging. Uridine tags of the circadian-regulated transcript, *CCR2,* were found to occur on natively decapped transcripts when the poly (A) tail was degraded to an oligo (A) tail, implicating their role facilitating 5'-3' degradation (Morozov *et al*, 2012). Nucleotidyltransferase enzyme HESO1 was proposed as a possible candidate for uridine tagging favoured degradation in *A. thaliana* (Ren *et al,* 2012; Zhao *et al,* 2012). On the other hand, Tail-seq was instrumental in defining an additional role for uridine tagging in plants. This additional role was in repairing deadenylated mRNAs by nucleotidyltransferase enzyme URT1, which was identified as one that targets readenylating mRNAs (Zuber *et al*, 2016; Sement *et al,* 2013). URT1 was demonstrated to repair deadenylated mRNAs with uridine nucleotides (13-15 nucleotides) to restore size distribution of tails centred at 16 nucleotides (Zuber *et al,* 2016). *In vitro* data indicated that this size distribution reflected the PABP footprint. URT1-mediated tagging and PABP binding may slow down deadenylation however, it does not prevent 3'-5' shorting of the oligo (A) tail. From these results it appears that uridine tagging in plants has either a stabilising or destabilising function depending upon the enzyme that is used to catalyse the addition of these modifications.

1.4.5 Translational control by uridylation

The addition of C and U nucleotides in *A. nidulans* was thought to negatively regulate the translation of nucleus encoded mRNAs during NMD (Morozov *et al,* 2012). C/U tagging was found to induce poly (A) independent decapping and favour transcript

dissociation from the translating ribosome for transcripts with premature stop codons (Morozov *et al,* 2012). The effects of C/U nucleotide addition on ribosome dissociation were determined by deleting CutA and CutB which resulted in an increase in the proportion of PTC-containing mRNAs associated with ribosomes (Morozov *et al,* 2012). This addition of C/U nucleotides is dependent on Upf1 which reduces levels of C/U tagging (Morozov *et al,* 2012). Furthermore, a clear translation inhibition function of tagging was observed in *Xenopus* oocytes. Tethering of TUTase7 led to translation prevention of target mRNAs but had no effect on stability (Lapointe and Wickens 2013). 96% of cyclin B mRNAs stored in starfish oocytes were found to be tagged with uridine nucleotides which triggered trimming and poly (A) tail extension following meiotic re-initiation by hormonal stimulation (Ochi and Chiba 2016). Further studies will be required to elucidate the function of tagging in relation to translation inhibition.

1.5 Uridylation in viruses

Viruses are small infectious agents that replicate only inside living cells of other organisms. It has been known for a long time that RNA of many DNA and RNA viruses, that infect eukaryotic cells, undergo polyadenylation thereby generating 3' poly (A) tails (Dreher 1999; Barr *et al*, 2010). These poly (A) tails are used to maintain stability as well as being important for translation initiation of viral transcripts (King *et al,* 2012; Dreher *et al,* 2010; Barr and Fearns 2010). Tagging with uridine nucleotides in viruses has recently been characterised for hepatitis C virus (HCV), Sindbis virus (SIV), Beet necrotic yellow vein virus (BNYVV) and Coxsackievirus B3 (CVB3). Their RNAs, following removal of 3' poly (A) tails regain (A) tails containing U-rich or A/U-rich linkers which appear to trigger degradation (Jupin *et al*, 1990; Raju *et al,* 1999; Van *et al*, 2006a; Van *et al,* 2006b). Recent studies revealed that a broad array of RNA viruses including mycoviruses, plant viruses and animal viruses are found to bear oligo(U) or 'U' rich tails (Huo *et al,* 2016). These viruses ranged from positive stranded, to negative stranded or double stranded RNA viruses (Huo *et al,* 2016). It could be postulated that this tagging in viruses is a conserved mechanism in virus host interactions though the function of this modification still remains unknown.

1.6 Key Features of nucleotidyltransferases

RNA tagging emerges as a generic feature involved in mRNA decay making it important to understand the nature of the enzymes which are involved in catalyzing these modifications. The central actors of uridylation are enzymes belonging to the polymerase *β*-like nucleotidyltransferase superfamily (Martin and Keller 2007). These enzymes are template independent which covalently add nucleotides to the 3' ends of mRNA (Martin and Keller 2007). Proteins belonging to this family are namely RNA-specific nucleotidyltransferases (rNTrs) and they can be divided into three sub-groups. The first is the canonical group of enzymes, corresponding to nuclear poly (A) polymerases *α, β* and *γ* which were initially characterized and isolated from yeast and mammals (Lingner *et al,* 1991; Raabe *et al,* 1991; Wahle *et al,* 1991). Poly (A) polymerases *α, β* and *γ* are all found in eukaryotes and share similar RNA binding and enzymatic domains. The second sub-group comprises of a variety of non-canonical rNTrs that are able to target diverse RNA substrates, these include the Gld2, Trf4/5- and Cid1-type of poly (A) or poly (U) polymerases, the 2'-5'-oligo (A) synthetases, and the trypanosomal terminal uridylil transferases (reviewed by Munoz-Tello *et al*, 2015). This group of enzymes share the same catalytic domain with canonical rNTrs however, they contain a different nucleotide base-recognition motif (Reviewed by Martin and Keller 2007). The third sub-group is one of the CCA-adding enzymes that mature tRNAs in eukaryotes and some bacteria. A hypothesis was made which stated that these enzymes have rapidly and independently diverged from a common ancestor, which presented general and nonspecific nucleotidyltransferase activity. Subsequently, different family members would have acquired distinct functional domains in order to occupy vacant evolutionary niches (Aravind and Koonin 1999). In this section only the second sub-group of non-canonical rNTrs will be reviewed because they are the enzymes responsible for 3' mRNA tagging.

1.6.1 The core catalytic domain of non-canonical nucleotidyltransferases

Every member of the non-canonical rNTrs group is defined by two main domains which form the core catalytic domain (CCD). This CCD defines the minimal catalytic organisation present in all non-canonical rNTrs. One of these is the pol *β* nucleotidyltransferase domain which is the enzymatic domain. This catalytic domain is made up of 5 *β*-strands which are backed by two α helixes forming a *αβ* two-layer sandwich. One of the main features of the pol *β* nucleotidyltransferase domain, which differs between organisms, is a single or double helical turn between strands one and two (Balbo *et al,* 2005). The second *β*-strand contains two catalytic aspartate or glutamate residues which are separated by one hydrophobic amino acid (DxD or DxE respectively; aspartate “D” or glutamate “E” residues with a hydrophobic residue “x”) (Balbo *et al,* 2005; Balbo *et al,* 2007; Bard *et al,* 2000). A third aspartate or glutamate residue is found on the third *β*-strand of the catalytic domain, which runs parallel to the second strand (Balbo *et al,* 2005; Balbo *et al,* 2007). The poly (A) polymerase-associated domain forms the central domain of the CCD. This PAP-associated domain has evolved to bind UTP rather than ATP (Deng *et al,* 2005). It contains a type II nucleotide recognition motif (NRM) (Munoz-Tello *et al,* 2012). This NMR corresponds to a 10-15 amino acid loop which forms one end of the nucleotide triphosphate binding pocket (Munoz-Tello *et al,* 2012). There are residues located in the NRM which are able to stabilise any substrate of the nucleotide triphosphate through water mediated and/ or direct hydrogen bonds on their side chain atoms (Deng *et al,* 2005; Stagno *et al,* 2010; Munoz-Tello *et al,* 2012). Catalytic reactions for these enzymes include a nucleophilic attack on the *α* phosphate of the bound nucleotide triphosphate by the RNA 3’ hydroxyl group. Subsequently, the three aspartate/ glutamate residues interact with the incoming RNA and two metal ions, which are necessary to stabilise the reaction intermediate (reviewed by Steitz 1998). A few non-canonical rNTrs contain an

RNA-binding domain (RBD) found inserted into the catalytic domain or at the N or C terminus. The RBD activity could be restricted to specific RNAs through a protein partner which may explain its absence on many non-canonical rNTrs (Zhelkovsky *et al,* 1995; Martin and Keller 1996). This RBD has a structural homology with the RNA-recognition motif protein family (RRM). It has been postulated that the function of RRM in non-canonical rNTrs is likely in binding to RNA substrates in a non-specific manner (Martin and Keller 1996; Zhelkovsky *et al,* 1995).

1.6.2 Complex and diverse domain architectures of some non-canonical rNTrs uridylating mRNAs

The minimal CCD corresponds to Cid1 in *S. pombe*. Cid1 does not have a dedicated RBD adjacent to the catalytic module. Cid1 binds to RNA substrates through interactions with three patches, which are distributed on the surface of the enzyme (reviewed in De Almeida *et al,* 2018). Cid1 seems to have no interacting partners but rather acts as a standalone enzyme (reviewed in Almeida *et al,* 2018). Unlike Cid1, most other non-canonical rNTrs have diverse domain architectures with domains or regions mediating protein-protein interactions or protein-RNA binding (Yates *et al,* 2012). Examples of these include CutA and CutB enzymes found in *A. nidulans*. CutA and CutB's CCDs are surrounded by intrinsically disordered regions (IDRs) which are thought to aid specific RNA-protein binding (IDRs are predicted using DISOPRED) (Jones and Cozzetto 2015; Calabretta and Richard 2015; Castello *et al,* 2012; Castello *et al,* 2016). *A. thaliana* TUTases URT1 and HESO1 also contain IDRs but only on the N terminus for URT1 and the C terminus for HESO1. Human TUT4, TUT7 and *Xenopus* TUT7 contain two CCDs although, only the C terminus domain is found to be active (Blahna *et al,* 2011; Faehnle *et al,* 2017). The inactive CCD is still required for structural functions albeit independent of catalysing any RNA 3' tailing (Blahna *et al,* 2011; Faehnle *et al,* 2017). Other features of TUT4/TUT7 and *Xenopus* TUT7 include

C2H2-type zinc finger and C2HC-type zinc finger motifs. These zinc finger motifs are located upstream of the inactive CCD and on both sides of the active one. These zinc finger motifs can promote protein-protein interactions and RNA binding (Brown 2005; Loughlin and Mackay 2006; Loughlin *et al*, 2011). The last two C2HC-type zinc finger motifs surround stretches basic rich lysine and arginine residues (BR), conserved from *Xenopus* to humans, which promote RNA binding (Lapointe and Wickens 2013; Jarvelin *et al,* 2016). IDRs are predicted to be in the N terminal, middle and C terminal regions of human and *Xenopus* TUTases. Albeit the presence of large IDRs is common across these enzymes their diversity in size and position is what largely contributes to the variability of the enzyme organisation across these organisms. Many functions have been proposed for IDRs outside of promoting RNA-protein interactions. IDRs are thought to be able to mediate protein-protein interactions with effectors involved in RNA substrate recognition or in downstream consequences of tagging. IDRs are also thought to be able to influence the localisation of these enzymes to P-bodies and stress granules which are known to store many enzymes involved in mRNA turnover. This hypothesis of localisation is supported by CutA and URT1 being found to be present in P-bodies and stress granules, respectively (Sement *et al,* 2013; Morozov *et al,* 2010).

1.7 Readenylation of cytoplasmic mRNAs

For over 30 years, it was widely accepted that the addition a long of poly (A) tail to the 3' end of mRNA has a stabilising function thus triggering export from the nucleus and promoting translation and the shortening of poly (A) tails signal for degradation both in the nucleus and the cytoplasm. Studies now demonstrate that poly (A) tails can be added in the cytoplasm in a regulated manner (Ibrahim *et al,* 2006; Slomovic *et al,* 2010; Harnisch *et al,* 2016). This transient addition of A tails in the cytoplasm, to date, has been accredited to non-canonical poly (A) polymerases. Current research indicates that these cytoplasmic born (A) tails have a destabilising function (except during development), similar to what has been observed in bacteria and the nucleus, namely readenylation.

1.7.1 Readenylation during development

Cytoplasmic readenylation has been known to be an important step in oogenesis and early development for many animal species. During development, mRNA produced in the nucleus can be stored in the cytoplasm for later use which is a feature that occurs at different stages of development. Once translation of mRNA is required, cytoplasmic elongation of the poly (A) tail regulates translation of the targeted mRNAs. This process was initially discovered about 30 years ago in oocytes and early embryos of clams, worms, frogs and mice (Paris and Richter 1990; Rosenthal *et al,* 1983; Vassalli *et al,* 1989; Fox *et al,* 1989; McGrew *et al,* 1989). mRNA acquires a long poly (A) tail in the nucleus which is subsequently shortened in the cytoplasm (Huarte *et al,* 1992). These transcripts with short poly (A) tails (20-40 nucleotides) are regarded as being translationally repressed but are not subject to degradation. It is thought that most mRNAs are kept at this translationally repressed state while the oocyte is growing. Upon oocyte maturation, or after fertilisation, the poly (A) tails of the translationally repressed mRNA are elongated to longer poly (A) tails of usually about 80-250

nucleotides in length thus, stimulating translation (reviewed by Radford *et al,* 2008). It is important to note that there is no threshold length for the poly (A) tail during development, it appears that the longer the poly (A) tail the more likely it will be translated. Most of the molecular details regarding poly (A) tail regulated translation have been elucidated from *Xenopus* oocyte mRNA such as those encoding *mos* and cyclin B1. These mRNAs have two 3' UTR *cis* acting elements which are involved in regulating polyadenylation in the cytoplasm. This includes the cytoplasmic polyadenylation element (CPE) and the polyadenylation hexanucleotide AAUAAA/ AUUAAA (Fox *et al,* 1989; McGrew *et al,* 1989; Simon *et al,* 1992; Fox *et al,* 1992; McGrew and Richter 1990). Other machinery shown to be essential components of the cytoplasmic polyadenylation machinery, associated with the cytoplasmic polyadenylation element binding protein CPEB containing complex, include PARN (which is a 5' cap dependent exoribonuclease) which is responsible for the cytoplasmic poly (A) tail shortening and is part of the RNP complex (Paris and Richter 1991; Hake and Richter 1994). Other complexes shown to be essential components for cytoplasmic polyadenylation include Gld2 (ribonucleotidyltransferase) which constitutes the cytoplasmic polyadenylation and symplekin which acts as a scaffold whereby the polyadenylation machinery is assembled (Barnard *et al*, 2004; Copeland and Wormington 2001; Kim and Richter 2006; Juge *et al*, 2002). Interestingly, it was shown that both PARN and Gld2 are active in the cytoplasm therefore actively catalysing polyadenylation and deadenylation simultaneously (Kim and Richter 2006). However, PARN activity was shown to be more robust which means it removes most of the poly (A) tail, while it is being added by Gld2, keeping the transcript with a relatively short poly (A) tail that is translationally dormant (Kim and Richter 2006). During oocyte maturation/fertilisation PARN is expelled from the RNP complex which involves an intricate process, allowing Gld2 to polyadenylate the RNA by default which makes the transcript translationally active. Although the activities of Gld2 and PARN appear to be energetically wasteful, it is thought that this continuous addition and

removal of the poly (A) tail is to ensure that the oocytes are not translated prematurely and hence may represent a development quality control mechanism (Kim and Richter 2006). Premature polyadenylation may lead to precocious translation which may result in those eggs not being fertilised. Furthermore, this process of polyadenylation and deadenylation is important in controlling gene expression at both spatial and temporal levels during development.

1.7.2 Readenylation in Chlamydomonas reinhardtii

The role of cytoplasmic adenylation in promoting mRNA decay was initially identified in *Chlamydomonas reinhardtii* (Ibrahim *et al,* 2006). In this study it was documented that adenylation stimulates degradation of RISC generated 5' RNA fragments in a similar way to what was found with regards to uridylation (Ibrahim *et al,* 2006; Shen and Goodman 2005). The non-canonical nucleotidyltransferase MUT68 was found to be responsible for the addition of oligo (A) on *MAA7* mRNA in *C. reinhardtii* (Ibrahim *et al,* 2006). 45% of examined *MAA7* clones contained oligo (A) tails (A1-5) and deletion of the *MUT68* locus resulted in partially degraded mRNAs at very discrete sites but with no non-templated nucleotides (Ibrahim *et al,* 2006). It was proposed that cytoplasmic adenylation in *C. reinhardtii* facilitates 3'-5' decay by acting as a single stranded platform for the exosome to bind, however the exact mechanisms of this will need to be further elucidated. Taking into consideration the functionally analogous pathway described in *A. thaliana* and the findings from *C. reinhardtii*, this implies that depending on the organism, mRNA decay intermediates, arising after cleavage carried out by RISC, may undergo either oligouridylation or oligoadenylation (Ibrahim *et al,* 2006; Shen and Goodman 2004). Both of these non-canonical modifications are able to facilitate degradation but in opposite directions (U tagging facilitates 5'-3' and A tagging facilitates 3'-5' decay) (Ibrahim *et al*, 2006; Shen and Goodman 2004). *In vitro* assays demonstrated that MUT68 uses ATP and UTP as substrates (Ibrahim *et al,*

2010). UTP reference was observed in siRNA and miRNA, where MUT68 was found to be able to add U residues and stimulate degradation of the RNA by the RRP6 exosome catalytic subunit (Ibrahim *et al,* 2010). It can be postulated from these findings that the nucleotide specificity of MUT68 is somehow dictated by the nature of the RNA substrate however the mechanisms underlying the differences of MUT68 activity towards mRNAs and small regulatory RNAs remains unknown. Furthermore, it appears that cytoplasmic tagging of A nucleotides may facilitate 3'-5' RNA decay.

1.7.3 Readenylation in Human HeLa cells

Transient addition of adenylated tails to mRNAs were also reported in human HeLa cells, for 28S rRNA and *β actin* mRNA degradation intermediates (Slomovic *et al,* 2010). Oligo dT (RT-PCR) was performed on 28S and *β actin* and the results displayed cytoplasmic readenylated sequences (up to 58 A's) at different sites along the rRNA (Slomovic *et al,* 2010). Some sequences were heteropolymeric so were found to contain some U, G or C nucleotides (Slomovic *et al,* 2010). To ensure that these added A tails were cytoplasmic and not intermediates of a nuclear process which could leak into the cytoplasm *in vivo* or during the experiment, a cytoplasmic specific virus (vaccinia virus) was used (Slomovic *et al,* 2010). The vaccinia virus belongs to the pox family of viruses and contains a double stranded DNA genome and does not enter the nucleus through its life cycle (reviewed by Broyles, 2003). Viral RNAs were purified from infected HeLa cells and analysis revealed that they also contained homopolymeric and heteropolymeric A tails, providing additional support that these modifications are cytoplasmic in humans (Slomovic *et al,* 2010). RNAi silencing of exosome catalytic subunits Dis3 and Dis3L resulted in an accumulation of truncated adenylated rRNA and mRNA in the cytoplasm, leading to the conclusion that these RNAs are degraded in the 3'-5' direction (Slomovic *et al,* 2010). The enzymes

responsible for the addition of these A nucleotides in the cytoplasm still remain unknown in humans.

1.7.4 Readenylation in *Drosophila* S2 cells

Studies on Hsp70 mRNAs in *Drosophila* S2 cells further supported the findings of the possibility of the 3'-5' decay pathway requiring 3' oligoadenylation of mRNAs (Harnisch *et al,* 2016). Degradation of Hsp70 mRNA in *Drosophila* mainly occurs through the 5'-3' decay pathway via Ccr4-Caf1-Not dependent deadenylation (Bönisch *et al,* 2007). Degradation through the 3'-5' direction by the exosome only becomes apparent when the 5'-3' decay pathway is inhibited (Bönisch *et al,* 2007 Harnisch *et al,* 2016). 5-10% of Hsp70 mRNAs in Drosophila were found to be oligoadenylated with a mean length of 2-3 nucleotides (Harnisch *et al,* 2016). This oligoadenylation facilitates the 3'-5' decay pathway by the exosome as the depletion of the exosomal components increased the abundance of adenylated degradation intermediates, while down regulation of Dcp2 leads to the opposite effect indicating that 5'-3' decay pathway is disfavoured (Harnisch *et al,* 2016). This oligo (A) addition was found to be dependent upon the non-canonical Trf4-1- poly (A) polymerase (member of the TRAMP complex) and knock-down of this enzyme led to a lack of oligoadenylation thus inhibiting 3'-5' decay (Harnisch *et al,* 2016). This enzyme was initially identified to be of a nuclear localisation however fluorescence microscopy revealed that it is able to shuttle to the cytoplasm (Harnisch *et al*, 2016). Furthermore, these mRNA decay intermediates co-fractionated with polysomes consistent with the cellular localisation of Trf4-1. These results supported the finding that oligoadenylation may facilitate exosome-mediated 3'-5' mRNA decay in the cytoplasm in *Drosophila* (Harnisch *et al,* 2016). Also, readenylation may help to overcome dsRNA regions as seen in bacteria and cell cycle regulated histone mRNAs (Mullen and Marzluff 2008; Cao and Sakar 1992; Cao *et al,*

1996; Hajnsdorf *et al,* 1995; Haugel-Nielsen *et al,* 1996; O'Hara *et al,* 1995; Xu and Cohen 1995).

1.8 RNA degradation in *A. nidulans*

The analysis of RNA degradation in *A. nidulans* was initially focused on the GATA transcriptional factor, AreA (Lowry and Atchley 2000; Caddick and Arst 1998). AreA is a principal transcriptional factor which is essential in determining nitrogen utilisation in the cell (Caddick 2004). Nitrogen is a vital component to all biological systems that is available in various forms in the environment. Many organisms have a hierarchy in the utilisation of nitrogenous compounds and in *A. nidulans*, a wide range of nitrogen sources are able to be utilised (Caddick 1994). AreA was probably one of the first eukaryotic regulatory genes to be characterised as encoding for a transcriptional factor (Wiame *et al,* 1985; Caddick 1994). In *A. nidulans areA* regulates hundreds of genes, involved in the utilisation of poor nitrogen sources, in the absence of the prime nitrogen sources, ammonium and glutamine (Wiame *et al,* 1985; Cove 1979; Unkles *et al,* 2001; Caddick 2004). Under nitrogen limitation, the activity of *areA* increases, resulting in the expression of many structural genes involved in utilisation of poor nitrogen sources (Caddick 2004). Under sufficient nitrogen regimes (in the presence of ammonium or glutamine) AreA activity is downregulated. Among the best characterised poor nitrogen sources are those of the nitrate assimilation pathway (Strauss *et al*, 1998). Four genes are known to be involved in the nitrate assimilation pathway; these include two transporters (*crnA/nrtA* and *nrtB*), nitrate reductase (*niaD*) and nitrite reductase (*niiA*) (Cove 1979; Unkles *et al,* 2001). *areA* and its targeted genes can be regulated at the level of transcription (nitrogen metabolite repression by AreA) and induction by nitrate or nitrite by pathway specific transcription factor (NirA) and at the level of transcript stability (Caddick 2004; Caddick *et al,* 2006; Platt *et al,* 1996). The modulation of *areA* activity in response to the quality and quantity of available nitrogen sources was localised in three regions (Platt *et al,* 1996). Two of these regions were located within the coding region and one was found 200 nucleotides within the 3' UTR of the *areA* transcript (Platt *et al,* 1996; Morozov *et al,* 2000). The sequence within the 3' UTR of

areA is one of the most conserved non-coding sequences within *Aspergilli* and is enough to determine regulated transcript stability of target mRNA (Galagan *et al,* 2005; Morozov *et al,* 2000; Platt *et al,* 1996). It was shown that in the presence of ammonium or glutamine, transcripts of *areA* and nine other genes involved in the utilisation of poor nitrogen sources became destabilised (Caddick *et al,* 2006). Furthermore, in the presence of nitrate, *niaD* and *niiA* transcripts were stabilised. The combined effects of glutamine and nitrate demonstrated that the nitrate stabilisation signal was dominant, thus overcoming glutamine or ammonium signalled destabilisation (Caddick *et al,* 2006).

Investigating the underlying mechanism of regulated transcript stability in these transcripts determined that mRNA decay is generally initiated by shortening of the poly (A) tail that is then rapidly degraded primarily via the decapping-dependent pathway (Caddick *et al,* 2006; Morozov *et al,* 2010a). The Ccr4-Caf1-Not complex was shown to be the deadenylase responsible (Morozov *et al,* 2010a; Morozov *et al,* 2010b). In *A. nidulans*, Caf1 and Ccr4 are functionally distinct deadenylases *in vivo*. Caf1 is required for the regulated degradation of specific transcripts and Ccr4 is required for basal degradation (Morozov *et al,* 2010a). It was found that the regulated degradation of specific transcripts involved in poor nitrogen source utilisation, in response to glutamine, correlated with their deadenylation rates (Caddick *et al,* 2006; Morozov *et al,* 2000). This correlation of degradation and deadenylation rates was lost in strains deleted for *caf1*, indicating that this deadenylase is required for accelerating degradation in response to the glutamine signal (Morozov *et al,* 2010b). Importantly, the rate of deadenylation in *A. nidulans* is variable among different mRNAs and can be regulated (Morozov *et al,* 2012). A key RNA binding protein involved in glutamine signalled decay is RrmA, which can bind specifically to the 3' UTR of *areA* (Krol *et al*, 2013). Work to characterise poly (A) tail length determined that deadenylation to a threshold length of 15 nucleotides triggered decapping and rapid degradation of the

mRNA in *A. nidulans* (Morozov *et al,* 2010a; Morozov *et al,* 2012). In this work it was also demonstrated that at this threshold length, C/U nucleotides were added to the transcript's 3' end and the ribonucleotidyltransferase enzymes, CutA and CutB, were responsible (Morozov *et al,* 2010a; Morozov *et al,* 2012). Work in a wide range of organisms have supported the notion that 3' tagging of C or U nucleotides plays an important role in mRNA decay, as mentioned previously. Furthermore, it has been demonstrated in other organisms that the addition of adenosine nucleotides can also facilitate decay (mentioned previously). Could this addition of A nucleotides be involved in decay in *A. nidulans* as seen with C/U nucleotides? Furthermore, it was demonstrated that the non-canonical poly (A) polymerase is involved in the re-addition of adenosine nucleotides in the cytoplasm therefore, could CutA and CutB be able to add C/U and A nucleotides in the cytoplasm? Furthermore, do A nucleotides trigger the same degradation pathways (5'-3') as C/U nucleotides in *A. nidulans*?

1.9 Project aims

Cytoplasmic modifications of 3’ extremities are emerging as novel mechanisms that play a role in determining the fate of mRNA. Recent studies have revealed an additional role for poly (A) tails in not only stabilising eukaryotic transcripts and promoting their translation but also in degrading them. This study aimed to characterise whether readenylation facilitates degradation in *A. nidulans* and what enzymes are involved in these modifications. Additionally, this study aimed to identify how this readenylation process is regulated. *A. nidulans* was used as a model eukaryote because it is amenable to molecular and genetic analysis with a well characterised genome. Importantly, it has proved to be great model system for studying RNA decay mechanisms especially with respect to 3’ end modifications of mRNA, which appears more typical than the major model, *S. cerevisiae*.

2. Materials and Methods

This chapter gives a detailed description of the procedures and methods used during this present study.

2.1 *Aspergillus nidulans* growth and strains

2.1.1 *Aspergillus nidulans* strains

The *A.nidulans* strains used for this study were constructed using standard genetic crosses supplied by Mark X Caddick and they had the following genotypes: Wild Type:veA+; *ΔcutA riboB$_2$ pabaA$_1$; ΔcutB pyroA$_4$; ΔcutA cutB pyroA$_4$.* (Morozov *et al,* 2010; Morozov *et al,* 2012).

2.1.2 Media and supplements used for *Aspergillus nidulans* growth.

A. nidulans growth media and incubation conditions used were as described by Clutterbuck 1974. *A. nidulans* strains were grown in either solid/ liquid minimal medium (as and when required). Minimal growth medium was prepared with 1% glucose (w/v), 2% (v/v) *Aspergillus* salt solution made up of 349mM KCl, 105mM $MgSO_4$. $7H_2O$, 559mM KH_2PO_4, 0.2% chloroform and 5% trace elements solution (stock trace elements solution containing 64mM $CuSO_4$, 121mM $FePO_4$, 15mM $Na_2[B_4O_5(OH)_4]\cdot 8H_2O$, 121mM $MnSO_4$, 165mM Na_2MoO_4, 1.3mM $ZnSO_4$). The pH of the minimal medium was adjusted to about 6.5 using NaOH and in the case of solid media 1% of technical agar was added. 10mM of ammonium tartrate or 10mM nitrate were added as a nitrogen source and vitamin supplements were added to the minimal medium, as previously described in published papers (shown on appendix 1) (Morozov et al, 2010; Morozov et al, 2012). All media and supplements were sterilised by autoclaving prior to being used. A list of all supplements is shown on appendix 1.

2.1.3 Maintenance and growth of *A. nidulans* strains

A. nidulans stock cultures were kept as conidiated mycelia using premade glycerol stocks from the Protect microorganisms preservation system at -80°C (Technical Service Consultant Ltd).

2.1.4 Mycelia growth and preparation

For the preparation of conidial suspensions to inoculate into liquid cultures, strains were grown on minimal media containing 1% agar (w/v), 10mM NH_4^+ (w/v) as a nitrogen source, and appropriate vitamin growth supplements (appendix 1, chapter 2.1.1 and 2.1.2). This was followed by incubation at 37˚C for three days for the growth and maturation of conidia, as described by Clutterbuck 1974. Harvested conidia from such plates were suspended in 5ml H_2O and inoculated in 1L flasks containing 200ml minimal medium supplemented with 10mM% NO_3^- (w/v) as a nitrogen source, and appropriate vitamin supplements listed on appendix 1 (chapter 2.1.1 and 2.1.2). Flasks were incubated at 30˚C for 16-18 hours with shaking at 250rpm (until conidia begin to germinate). Mycelia were harvested by filtration through Miracloth (Calbiochem Corp), washed with cold water, dried by blotting with a paper towel and wrapped in foil. The mycelium was transferred to liquid nitrogen for quick freezing (below -190˚C) of which point conidia could be kept at -80˚C until ready for RNA extraction.

2.1.5 Mycelia growth for time-course of mRNA degradation analysis

For time course of mRNA degradation analysis, mycelia were grown over night in 200ml minimal media, washed in 1L minimal media at 30˚C with no nitrogen source but with appropriate supplements for different strains (appendix 1, chapter 2.1.1 and 2.1.2). Mycelia from three 1L flasks were transferred into two 1L flasks with 250ml minimal media with no nitrogen source but with appropriate supplements for different strains (appendix 1, 2.1.1 and 2.1.2) and incubated at 30˚C for 1 hour shaking at 250rpm for nitrogen starvation. Following 1 hour of nitrogen starvation 40µg/ml

proflavine was added directly to the culture and this was incubated for 10 minutes at 30˚C and shaking at 250rpm for the inhibition of transcription prior to the commencement of the mRNA degradation analysis (Platt *et al,* 1996; Morozov *et al,* 2000; Cybis and Weglenski 1972). Where appropriate to assess the differential effect of specific nitrogen sources on transcript stability, NH_4^+, Gln or NO_3^- was added at T_0 to a final concentration of 40mM, 10mM and 10mM respectively (Caddick *et al,* 2006). Mycelia were harvested by filtration (described 2.1.4) at T_0, T_5, T_{10} and T_{20} minutes and around 60 ml aliquots of the culture were harvested at each time point.

2.2 Molecular techniques for the manipulation of RNA

To minimise degradation of RNA by ribonucleases all consumables were autoclaved twice. Disposable gloves were always worn during the preparation of material and solutions and during the extraction and handling of RNA. RNAse free microcentrifuge tubes (Fisher Scientific) and pipette tips (Sarstedt) were used throughout the experiment also. Diethyl pyrocarbonate (DEPC) H_2O was prepared and used for solutions. For all enzyme reactions 40U of RNasin, which is an RNase inhibitor, was added (Promega).

2.2.1 RNA preparation from *A. nidulans*

For RNA extraction frozen mycelia were ground using mortar and pestle with the use of liquid nitrogen to maintain frozen temperatures to minimise RNA degradation. When cells had been ground to a fine power they were transferred to 700µl of phenol and 700µl of lysis buffer (100mM TrisCl pH 8.0, 600mM NaCl, 10mM EDTA, and 5% SDS). Cells were vortexed vigorously and centrifuged at 16,000g for 15 minutes thus producing three phases (upper aqueous phase which contains the RNA/DNA, middle phase containing denatured proteins and a lower organic phase). About 500µl of the upper aqueous phase was transferred and mixed with equal volumes of phenol and chloroform. This was followed by 15 minutes of centrifugation at 16,000g and two phases were formed, with the upper phase containing RNA. 500µl of the upper phase was transferred to equal volumes of 5M LiCl, incubated in 4˚C for 1 hour and centrifuged for 20 minutes. The resulting RNA pellet was washed with 180µl 70% ethanol, dried and re-suspended in 360µl DEPC treated H_2O followed by ethanol precipitation. Samples were centrifuged for 20minutes at 16,000g; the supernatant was discarded; the RNA pellet was washed twice with 180µl 70% ethanol and left to dry.

RNA stop solution (5mM EDTA and 1% SDS) was then added to each sample and the amount added was dependent on the size of the pellet and the concentration of RNA was measured. At this point RNA could have been stored at -20^{O}C until when needed. All centrifugation steps were carried out in room temperature at maximum speed of 16,000g. RNA concentration was treated with DNase1 (Thermo Fisher scientific) followed by chloroform extraction and re-precipitated with ethanol before being dissolved in DEPC-treated H_2O for subsequent analysis.

2.2.2 Nucleic acid quantification

The quantity and quality of RNA was measured with the NanoDrop-1000 spectrophotometer (Thermo Fisher Scientific) using a 2 µl sample per measurement.

2.2.3 Phenol- Chlorophorm purification following enzymatic reactions

To purify RNA following any enzymatic reactions equal volumes of phenol and Chloroform (1:1) was added to samples, centrifuged for 10 minutes (maximum speed of 16,000g) and the upper phase (containing RNA) was subject to ethanol precipitation.

2.2.4 Ethanol Precipitation of RNA

For the precipitation of RNA using ethanol 1/10th volume of 3M NaAc pH 5-5.5 and 2.5 volumes of ethanol were added to the RNA. This was followed by incubation at -20°C for at least 2 hours (RNA can be stored under ethanol for long term storage).

2.2.5 RNA clean-up (Qiagen)

The RNA clean-up and concentrator kit was used for the concentration of RNA adaptor ligation enzymatic reaction. This kit uses specialised RNeasy MinElute spin columns

based on silica-membrane technology. For RNA purification, sample volume was adjusted to 100µl using RNase-free H_2O provided with the kit and mixed well with 350µl of lysis buffer RLT and 250µl of 100% ethanol. The sample was transferred to an RNeasy MinElute spin column placed in a 2ml collection tube and centrifuged at 12,000g for 15 seconds. The flow through was discarded and the RNeasy MiniElute spin column with the sample inside was added to a new 2ml collection tube. 500µl of wash buffer RPE, which removes any trace salts, was added to the sample and centrifuged for 15 seconds at 12,000g. The flow through was discarded and 500µl 80% ethanol was added to the sample for washing, then centrifuged for 2 minutes. The RNeasy MiniElute spin column with the sample was transferred to a new 2ml collection tube and centrifuged at maximum speed for 5 minutes to dry the membrane. The RNase MiniElute spin column with the sample was transferred to a 1.5ml microcentrifuge tube and 14µl of RNase-free water was added to the sample and centrifuged for 1 minute at maximum speed of 16,000g to elute the RNA.

2.2.6 DNase I treatment of RNA (Thermo Fisher Scientific)

DNase I degrades DNA but is RNase free. All RNA samples were treated with 6U DNase, 1x DNase I buffer (100mM TrisCl pH 7.5, 25mM $MgCl_2$ and 5mM $CaCl_2$) for 30 minutes at room temperature. RNA was subject to extraction phenol/ chloroform extraction followed by ethanol precipitation as shown in section 2.2.3 and 2.2.4

2.2.7 Alkaline Phosphatase treatment of RNA (Roche)

Alkaline phosphatase enzyme is used to dephosphorylate the 5’ ends of RNA by removing the 5’ phosphoryl group. 25µg of DNase I treated RNA was treated with 3U of alkaline phosphatase and 1x Tris EDTA buffer (50mM Tris-Cl pH 8.5 and 0.1mM

EDTA) for 30 minutes at room temperature. RNA was subject to phenol/ chloroform extraction followed by ethanol precipitation (2.2.3 and 2.2.4).

2.2.8 Tobacco Acid Pyrophosphatase treatment (New England Biolabs)

For the analysis of capped mRNA, the tobacco acid pyrophosphatase enzyme was used which cleaves the pyrophosphate bond of the 5′ cap of mRNA. Complete hydrolysis results in removal of the β and γ phosphates, leaving only the α phosphate attached. The resulting 5′-monophosphorylated terminus may be ligated to a 3′-hydroxylated terminus using T4 RNA Ligase. 10µg capped alkaline phosphatase treated RNA was treated with 2.5U of tobacco acid pyrophosphatase, and 1x Tris-EDTA buffer (10mM Tris-Cl pH 8.5 and 0.1mM EDTA) followed by incubation for 1 hour at 37°C. RNA was purified using phenol/ chloroform followed by ethanol precipitation (chapter 2.2.3 and 2.2.4).

2.2.9 Xrn1 treatment of RNA (New England Biolabs)

For the analysis of capped RNA, Xrn1 was also used which is a highly processive exoribonuclease that required a 5'monophosphate to initiate degradation of RNA. For this reaction 4µg of DNase I treated RNA was mixed with 2U Xrn1 and 1x buffer (100mM NaCl pH 7.9, 50mM Tris-Cl, 10mM $MgCl_2$, 1mM dithiothreitol). This reaction mixture was incubated for 2 hours at 37°C and 10 minutes at 70°C to denature the enzyme.

2.2.10 Circularisation of RNA (New England Biolabs)

Circularisation of RNA involves ligation of 5' phsphoryl-terminated nucleic acid donors to a 3' hydroxyl-terminated nucleic acceptor through the formation of a 3'-5' phosphodiester bond. For circularisation, 5µg of capped or decapped RNA was treated with 10U of T4 RNA ligase, 10mM ATP and 1x RNA ligase buffer (50mM Tris-Cl pH

7.5, 10mM $MgCl_2$, 1mM DTT) in a volume of 400µl. This reaction was incubated at 16˚C for 16 hours and subject to phenol/chloroform and ethanol precipitation and resuspended in 12 µl DEPC H_2O (chapter 2.2.3 and 2.2.4).

2.2.11 Adaptor primer ligation to RNA (Bioo Scientific)

A custom adenylated primer was ligated to DNase I treated RNA using a truncated version T4 RNA ligase 2 (AIR Ligase). This enzyme specifically ligated the adenylated 5' end of the adaptor primer to the 3' end of targeted RNA. This truncated AIR Ligase only contains the first 249 amino acids of T4 RNA ligase 2 and cannot ligate the phosphorylated 5' end (end that is not pre-adenylated) of the targeted RNA to the 3' end. For adaptor ligation 1µg of RNA was treated with 5µM adenylated adapter primer, 1X Air Ligase buffer (20mM Tris-Cl pH 7.5, 50mM NaCl, 0.1mM EDTA, 0.1 mM DTT), 10% PEG (8000MW) (New England Biolabs) for 30 seconds at 95˚C. The reaction mixture was transferred into ice for 1 minute and 400U of AIR ligase enzyme was added and incubated at 25˚C for 2 hours. The adaptor ligated RNA was purified using the RNA clean and concentrator kit (Qiagen).

2.2.12 cDNA synthesis (Bioline)

RNA was converted to complementary DNA using a reverse transcription protocol. Generated cDNA in this work was used for PCR and other downstream applications. For reverse transcription 5µg of RNA was mixed with 1µM primer (different primers used for different downstream applications as mentioned in chapter 2.3), 2.5µM dNTPs and H_2O. This reaction mixture was heated to 65˚C for 5 minutes to open up the secondary structures of the RNA and then put in ice for 10 minutes. 1X reverse transcriptase buffer (50mM Tris-Cl pH 8.3, 75mM KCl, 3mM $MgCl_2$) and 10mM DTT (reduces the disulfide bonds of proteins) was then added to the mixture and heated to

20˚C for 2mins. 200U of reverse transcriptase was added and the reaction mixture, incubated at 42˚C for 90 minutes, 95˚C for 5 minutes (denature the enzyme) and stored at -20˚C.

2.3 Polymerase chain reaction methods

2.3.1 circularised RT-PCR

Many techniques that are used to characterise individual RNA molecules can potentially alter the original transcript sequence or its post-transcriptional modifications, such as polyadenylation (Slomovic and Schuster 2013). Circularised reverse transcription coupled with PCR (cRT-PCR) preserves the original 5' and 3'ends of the transcript and any post-transcriptionally added extensions (Slomovic and Schuster 2013). Additionally, posttranscriptional modifications such as adenylation or uridylation can influence transcript longevity, stability and translatability; these can be elucidated using cRT-PCR. Before carrying out cRT-PCR, it was important to consider certain things about the target RNA molecule. For example, since RNA ligase-based-circularisation is the foundation of cRT-PCR it was imperative to know if the target RNA was 5'capped or triphosphorylated, since these modifications can hinder circularisation (ligation of 5' and 3' ends) (Slomovic and Schuster 2013). cRT-PCR can be coupled with a variety of downstream applications such as cloning.

Before carrying out the cRT-PCR technique DNase I treated RNA 3' and 5' ends were ligated together using T4 RNA ligase I (2.2.10) (for capped RNA treatment with alkaline phosphatase and tobacco acid phosphatase prior to ligation was done). Following ligation RNA was reverse transcribed using a gene specific reverse primer, R1 found in figure 2.3.1. This would result in a cDNA comprising of the adjoined 5' and 3' ends. Two rounds of PCR were done; the first was classed as non-nested PCR which used gene specific R1 and F1 primers as shown in figure 2.3.1 and the second round was classed as a nested PCR using F2 and R2/R1 primer depending on the gene, which allowed for more gene specificity (figure 2.3.1). For PCR 1µg cDNA, 1µM reverse primer (R1/R2), 1µM forward primer (F1/F2) (figure 2.3.1) and 1x PCR master mix (0.025U *Taq* DNA polymerase (Roche), 0.2mM dNTPs, 2mM $MgCl_2$) and reaction

buffer (20mM Tris-Cl pH 8.4, 50mM KCl) was used. A temperature of 95˚C for 4 minutes served as a heat activation step for the enzyme DNA polymerase. Cycles consisted of 95˚C for 30 seconds for denaturation of DNA by disrupting hydrogen bonds, 55˚C for 30 seconds for the annealing of the primers to the single stranded DNA template and 72˚C for 1 minute for the synthesis of the new DNA strand complementary to the DNA template, for 30 cycles. Finally, samples were left at 72˚C for 10 minutes to ensure that any remaining single stranded DNA was fully elongated. At this point samples were analysed using gel electrophoresis, purified and cloned for sequencing.

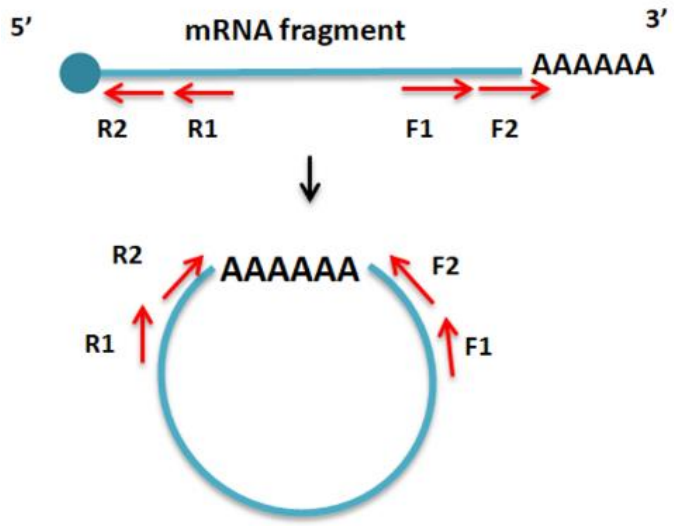


Figure 2.3.1 showing mRNA circularisation using the cRT-PCR approach. From this figure the approximate position of non-nested (F1 and R1) and nested primers (F2 and R2) that were designed are shown.

Table 2.3.1 showing the sequence of primers used for cRT-PCR

(Sequence of primers in 5'-3' direction)

Gene	Forward	Reverse
areA	F1:CAGGAATGGGAGTGGTTG F2:GAGCAGGCGTTTGGTGAT	R1:TTCCCACCTCGCATGATTTC R2:ACGGTCATGTCTGGGTTG
niaD	F1:GACACCGGATAGAGAGAC F2:ACGGACTGGGTATGGTGT	R1:ACTTCCCAGATCAGGGTC R2: GACCCTGATCTGGGAAGT
nirA	F1:TTCAGCGATAGCAGGCCT F2:TAGGCAGTTGGTGGACTC	R1:ACCTCACACGAAGTCGTC R2:CATGCGCTGTCTTGGTCT
niiA	F1:CACTCGGTCTCCTTCAAA F2:CTGGTAGACTGACGAGGA	R1:CACACTTCGAGGCGACAT R2:GAGACTGTCACTGCCAGT
meaA	F1:AGGTATCAGGCGTGCAAG F2:TCCTCTGGTGCATCCACT	R1:CATGGCCTCTGAAGGCAT R2:CACAACCAGGACTCCTCT

These primer sequences were manually designed with the use of gene sequences given by the *Aspergillus* Genome Database (Aspergillus genome database 2018) and were purchased from Invitrogen™ or Sigma (UK).

2.3.2 Adaptor ligation RT-PCR

3’ adaptor ligation is a technique that involves the ligation of a 5’ adenylated adaptor primer to the 3’ end of DNase I treated mRNA. This primer allows for any posttranscriptional modifications occurring at the 3’ end of mRNA to be elucidated. The adaptor primer used in this study was custom made (adaptor sequence: GACTGGAATTCTCGGGTGCCAAGGC). It was an active adenylated oligomer which eliminates the need for ATP when being ligated to the RNA therefore, allowing for specific and clean ligations. The linker was also designed with a modified dideoxy C (ddC) terminal to prevent self-ligation.

For the analysis of capped transcripts, mRNA was treated with Xrn1 prior to ligation (2.2.1). 5’ adenylated adaptor was ligated to RNA using a truncated T4 RNA ligase enzyme (AIR Ligase) followed by purification of RNA using a RNA clean and concentrator kit. After purification, reverse transcription of the adaptor ligated RNA was done using a gene specific R1 reverse primer that is complementary to the adaptor oligomer (figure 2.3.2). One or two rounds of PCR were conducted with the first being classed as a non-nested PCR which amplifies using R1 and F1 primers (figure 2.3.2). The second round was classed as a nested PCR which amplifies using R1 and F2 primers. For PCR 72ng cDNA, 1µM reverse primer (R1), 1µM forward primer (F1/F2) (figure 2.3.2) and 1x PCR master mix (0.025U *Taq* DNA polymerase (Roche), 0.2mM dNTPs, 2mM $MgCl_2$ and reaction buffer (20mM Tris-Cl pH 8.4, 50mM KCl) were mixed together. For PCR an initial temperature of 95˚C for 4 minutes served as a heat activation step. Cycles consisted of 95˚C for 30 seconds, 55˚C for 30 seconds and 72˚C for 1 minute for 30 cycles. Samples were left at 72˚C for 10 minutes to ensure that any remaining single stranded DNA was fully elongated. At this point samples were analysed using gel electrophoresis, purified and cloned for sequencing.

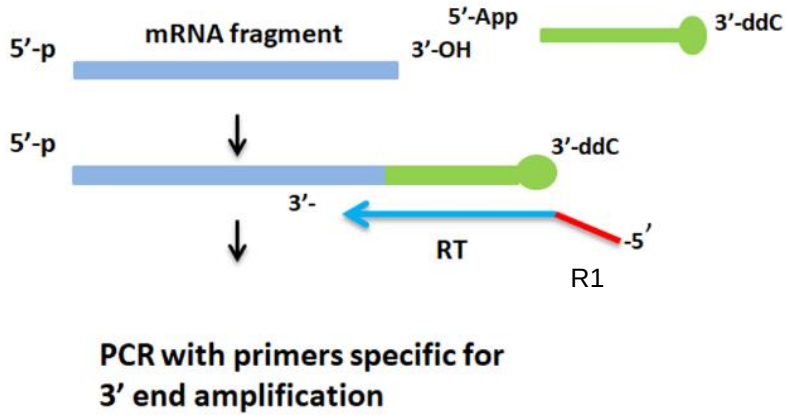


Figure 2.3.1 showing the mRNA 3' adaptor ligation approach. From this figure, the adaptor primer (in green) is ligated on to the 3' end mRNA (in blue) and the mRNA fragment amplified on the 3'- adaptor primer junction.

Table 2.3.2 showing the sequence of primers used for adaptor ligation RT-PCR (Sequence of primers in 5'-3' direction)

Gene	Forward	Reverse
areA	F1:CAGGAATGGGAGTGGTTG F2:GAGCAGGCGTTTGGTGAT	-
niiA	F1:CACTCGGTCTCCTTCAAA F2:CTGGTAGACTGACGAGGA	-
niaD	F1:GACACCGGATAGAGAGAC F2:ACGGACTGGGTATGGTGT	-
Adaptor primer	-	R1:GCCTTGGACCCGAGAATT

These primer sequences were manually designed with the use of gene sequences from the *Aspergillus* Genome Database (Aspergillus genome database 2018). The adaptor the primer was designed manually based on its sequence. These primers were purchased from Invitrogen™ or Sigma (UK).

2.3.3 Real-Time PCR

Quantitative PCR involves monitoring the amplification of a targeted DNA molecule during PCR in real-time. The main method used for the detection of PCR products produced in real time is a fluorescent dye called SYBR-green which intercalates with any double-stranded DNA. As the number of gene copies increases during the reaction so does the fluorescence of the SYBR-green because the number of copies of DNA it is intercalating with is also increasing. The cycle threshold value is the number of cycles required for the fluorescent signal to cross the threshold that has been set. It is important to set a cycle threshold value because there is a lot of background fluorescence during qRT-PCR which will need to be bypassed in order to extrapolate data only for the genes of interest. Thus, the C_t value is a line in the graph produced at the end of PCR that represents a level above background fluorescence and at the beginning of the exponential phase of the PCR reaction. The C_t value is inversely proportional to the amount of target nucleic acid in the sample because the more the product to begin with then the lower the Ct value.

In this study qRT-PCR was used to measure the level of degradation of target genes under different nitrogen conditions over a time course. For qRT-PCR analysis the reaction mixture contained 1µg cDNA 1µM reverse primer, 1µM forward primer and 1X Hi-ROX sensi-FAST PCR master mix (containing all components necessary for real-time PCR including SYBR Green I dye) (Bioline). For PCR an initial temperature of 95˚C for 2 minutes served as a heat activation step for the enzyme DNA polymerase. Cycles consisted of 95˚C for 5 seconds for, 57˚C for 10 seconds and 72˚C for 10 seconds, for 40 cycles. Primers used for qPCR analysis are shown on table 2.3.3.

Table 2.3.3 showing the sequence of primers used for qPCR

(Sequence of primers in 5'-3' direction)

Gene	Forward	Reverse
areA	AGGGAGAGTGAGTTCGGA	CTAGCTCATGACCCGGAT
niiA	GTCATGATGCCAAGGGGA	GACCGCAACAAATGGGTG
meaA	TGGGGTTACTCGCTTGCT	TTCCCCTTGGCTGACAGT
niaD	TGGCGTTCACCCGTTCAA	GAATGTCCTCGTCGCGAA

These primer sequences were manually designed with the use of gene sequences given from the *Aspergillus* Genome Database (Aspergillus genome database 2018) and were purchased from Invitrogen™ or Sigma (UK).

2.4 Agarose gel electrophoresis

For gel electrophoresis 1% agarose and 40ml TBE buffer (tris-Cl/borate/EDTA) was used at all times. 1X TBE buffer was produced by mixing 1.8% Tris-CL, 0.55% boric acid and 0.5M Na_2EDTA and to be adjusted to pH 8.0 using HCl. 0.25mg/ml Gel red was used as a DNA staining dye on each agarose. This dye was added into the melted agarose and left to set in the electrophoresis tank with a gel electrophoresis comb added (Thermo fisher). After setting, 1X TBE buffer was poured in to the tank until the gel was completely covered and the gel electrophoresis comb was removed. For loading different volumes of PCR samples were added depending upon the required use (chapters where gel electrophoresis was used include: 2.3.1, 2.3.2 and 2.6.4) and 1x loading dye (New England Biolabs) was used ran at about 100 volts for about 30-45 minutes.

2.5 DNA purification methods

2.5.1 PCR Purification (Qiagen)

This was performed according to the manufacturer's instructions and all centrifugation steps were carried out at 16,000g.

. For PCR purification 11µl PCR reaction mixture was used. This was dissolved in 5x the volume of buffer PB, which was used as a binding buffer in DNA clean up procedures. This PCR reaction and PB buffer mixture was placed in a mini elute column supplied with a 2ml collection tube, centrifuged for 1 minute and the flow was discarded. Following this 750µl of buffer PE was added, which serves as a wash buffer and contains ethanol. Samples were centrifuged for 1 minute twice and the flow was discarded in-between the centrifugation steps. The mini elute column was placed in a 1.5ml micro centrifuge tube and 10µl H_2O was added, left to stand for 1 minute and centrifuged for another minute. At this point the flow would contain the purified DNA therefore, the mini elute column would not be required at this point. The purified DNA concentration was measured using a nano-drop spectrophotometer (chapter 2.2.2) and stored at -20^{o}C until required for further analysis.

2.5.2 Gel Purification (Qiagen)

This was performed according to the manufacturer's instructions and all centrifugation steps were carried out at 16,000g.

This was required for samples that had been separated an agarose gel and yielded DNA fragment of a desired size. These DNA fragments were excised from the agarose gel using a sterile sharp scalpel. The gel slice was weighed in a colourless tube and 3x the volume of buffer GQ to 1 volume of gel were added (100mg gel equal to 100µl volume). Buffer GQ is a solubilisation and binding buffer which also contains a pH indicator. Samples were to be incubated at 50˚C for about 10 minutes or until the gel

has completely dissolved, with vortexing every 2-3 minutes to help dissolve the gel. The colour of the mixture was checked to see if it was still yellow (similar to Buffer QG without dissolved agarose). If the colour of the mixture turned orange or violet, 10µl 3 M sodium acetate, pH 5.0, would need to be added and mixed to make the colour of the mixture return to yellow. When the gel had dissolved, 1 x the total volume of isopropanol was added, and all the contents placed in a spin column with a 2ml collection tube and centrifuged for 1 minute. The flow through was discarded and 500µl of buffer QG was added again. This was followed by centrifugation again for 1 minute and the flow discarded. DNA on the column was washed with 750µl buffer PE, centrifuged twice for 1 minute and the flow discarded in between. The collection tube was discarded at this point and replaced with a 1.5ml microcentrifuge tube. DNA on the column was resuspended in 20µl H_2O, left to stand for about 4 minutes to increase the yield of purified DNA and centrifuged for 1 minute so the DNA will collect in the 1.5ml microcentrifuge tube.

2.6 Cloning methods

2.6.1 T/A cloning (Promega)

TA cloning was used as it is a subcloning technique that avoids the use of restriction enzymes and is easier and quicker than traditional subcloning. The technique relies on the ability of adenine (A) and thymine (T) (complementary base pairs) on different DNA fragments to hybridize and, in the presence of ligase, become ligated together. T/A cloning vectors are linearized vectors with a single 3´-terminal thymine at both ends. The T-overhangs at the insertion site greatly improve the efficiency of ligation of PCR products by preventing recircularization of the vector and providing a compatible overhang for PCR products generated by certain thermostable polymerases. PCR products are usually amplified using Taq DNA polymerase which preferentially adds an adenine to the 3' end of the product. Such PCR amplified inserts are cloned into linearized vectors that have complementary 3' thymine overhangs. pGEM®-T Easy Vectors were used in this study and they are high-copy-number vectors containing T7 and SP6 RNA polymerase promoters flanking a multiple cloning region within the α-peptide coding region of the enzyme β-galactosidase. Insertional inactivation of the α-peptide allows identification of recombinants by blue/white screening on indicator plates. If β-galactosidase is produced, X-gal is hydrolysed to form 5-bromo-4-chloro-indoxyl, which spontaneously dimerizes to produce an insoluble blue pigment called 5,5'-dibromo-4,4'-dichloro-indigo. The colonies formed by non-recombinant cells therefore appear blue in colour while the recombinant ones appear white. Furthermore, isopropyl β-D-1-thiogalactopyranoside (IPTG) is used along with X-gal for blue-white screening

PCR products were ligated into the pGEM®-T Easy Vectors by adding to a 0.5ml microcentrifuge tube 1x T4 DNA ligase rapid ligation buffer, 25ng pGEM®-T Easy Vector, 3.5µg PCR product DNA (either PCR purified or gel purified from 2.5.1 or 2.5.2) and 3U T4 DNA ligase and incubated at 20°C for 16 hours.

2.6.2 Transformation of plasmids in *E. coli* strain DH5-alpha (Invitrogen)

An aliquot of 5µl of extracted plasmid (2.6.1) was mixed gently with 50µl of *E. coli* strain DH5-alpha competent cells and incubated on ice for 30 minutes. A brief heat shock was carried out at 42˚C for 30 seconds which changes the fluidity of the bacterial membrane. Once the fluidity changes, DNA can then enter the bacteria at an efficient rate perhaps by cell surface invagination (Lodish 2000). After heat shock for 30 seconds, the mixture was immediately placed on ice for 1-2 minutes before adding culture medium in order for the bacteria to recover. 700µl of ampicillin free LB medium was added to the bacteria with plasmid and incubated for 1 hour with slight shaking (about 150rpm). Transformed cells were plated out on LB agar supplemented with 100mg/ml ampicillin and 50mg/ml X-gal and incubated over night at 37˚C for approximately 16 hours. If the plates were to be left at 37^{O}C much longer than this, satellite colonies would begin to appear which would hamper the screening of individual colonies.

2.6.3 Screening of clones for plasmid isolation (Qiagen)

Plasmid extraction was done according to the manufacturer's instructions and centrifugation steps were carried out at 16,000g

To isolate plasmid DNA from positive white colonies (chapter 2.6.1), 5ml of LB-amp was prepared by adding 100µg/ml ampicillin to LB medium (1% Tryptone, 1% NaCl and 0.5% Yeast Extract). White colonies were inoculated into the prepared LB-amp mixture and incubated at 37˚C overnight. 3ml of the overnight culture was spun at 16,000g for 3 minutes to pellet the cells. The medium was discarded, and the bacterial pellet was resuspended in 200µl resuspension buffer P1. This was followed by the addition of 200µl buffer P2 which acts as an alkaline lysis buffer as it contains NaOH

and SDS. After adding buffer P2 the samples were mixed by inverting 4-6 times (harsh mixing can shear genomic DNA, resulting in chromosomal DNA contamination in the final recovered plasmid DNA). The lysis reaction was not allowed to exceed more than 5 minutes because prolonged alkaline lysis may permanently denature supercoiled plasmid DNA. 350µl buffer N3 was added to the mix, which acts as a neutraliser for the alkaline lysis reaction. Samples were then centrifuged for 10 minutes and the supernatant containing the plasmid was transferred to a QIAprep spin column supplied with a 2ml collection tube, centrifuged for 1 minute and the flow discarded. 500µl wash buffer PB was added, which acts as a binding buffer, centrifuged for 1 minute and the flow discarded. 750µl of buffer PE was added, which is a second wash buffer containing ethanol, centrifuged twice for 1 minute and the flow discarded in between. The QIA prep column was placed on a clean 1.5 ml micro-centrifuge tube and 50µl of H_2O was added to the centre of the column, left to stand for at least 1 minute and centrifuged for another minute. The plasmid DNA would have been suspended in the H_2O and have moved through the column to the micro-centrifuge tube and this could be stored at -20˚C ready to be sent off for Sanger sequencing. The concentration of plasmid DNA was also measured using a nanodrop spectrophotometer when required.

2.6.4 Restriction Digest (New England Biolabs)

A restriction digest was carried out on the plasmid DNA in order to confirm the presence of DNA of interest. Around 200ng of plasmid DNA was added to 1x EcoR1 buffer (100mM Tris-HCl pH 7.5, 50mM NaCl, 10mM MgCl2, 0.025% Triton® X-100), 10U EcoR1 enzyme. This mixture was incubated at 37˚C for about 2 hours and run on 1% agarose gel for about 30-40 minutes (chapter 2.4).

2.6.5 Growing of colonies for Sanger sequencing (GATC)

White were selected as described in chapter 2.6.5, and each colony was placed in 96 well plates with each well having LB agar and ampicillin 100µg/ml. Plates were grown overnight and send to GATC for sequencing.

2.7 Computational analysis

2.7.1 Databases

Databases used in this work include the *Aspergillus* genome database to obtain sequences for different genes (Aspergillus genome database 2018) for designing primers and data analysis. The National Center for Biotechnology Information (NCBI) database (nucleotide blast) was also used for searching for unknown sequences (https://blast.ncbi.nlm.nih.gov/).

2.7.2 Online Tools

For the analysis of sequences produced the Lalign website was used (https://embnet.vital-it.ch/software/LALIGN_form.html). For the analysis of secondary structures UNAfold RNA software was used (http://unafold.rna.albany.edu).

3. Results

3.1 Regulated transcript stability

It has been previously shown that regulated transcript stability is one of the mechanisms that is essential for the expression of genes involved in the utilisation of poor nitrogen sources in *A.nidulans* (Caddick *et al,* 2006). In *A.nidulans,* ammonium and glutamine serve as prime nitrogen sources and are utilised preferentially (Caddick 2004; Morozov *et al,* 2000; Morozov *et al,* 2001). This signalling pathway involves the co-ordinated expression of hundreds of genes, many of which depend upon the GATA transcriptional factor AreA (Arst *et al,* 1973; Caddick 1994). AreA is required for the utilisation of poor nitrogen sources in the absence of ammonium or glutamine (Caddick 2004). Under nitrogen limitation, the activity of *areA* increases, resulting in the expression of many structural genes involved in the expression of poor nitrogen sources (Caddick 2004). On the other hand, when there is sufficient nitrogen (in the presence of ammonium or glutamine), AreA activity is downregulated. When AreA activity is down regulated, it is unable to activate the expression of poor nitrogen sources.

Among the best characterised pathway for these poor nitrogen sources are those of the nitrate assimilation pathway. This pathway is composed of two transporters (NrtA and NrtB), nitrate reductase (NiaD) and nitrite reductase (NiiA) (Unkles *et al*, 2001). The respective genes are subject to regulation at the level of transcription, including nitrogen metabolite repression mediated by AreA and induction by nitrite or nitrate, mediated by the pathway specific transcription factor NirA (Caddick 2004; Caddick *et al,* 2006). Both transcriptional factors act synergistically to regulate all four structural genes when nitrogen is limiting and either nitrate or nitrite is available (Narendja *et al,* 2002).

It has been found that in the presence of sufficient intracellular ammonium or glutamine, deadenylation of the *areA* transcript is triggered, which leads to decapping and subsequent degradation (Morozov *et al,* 2000). It was later shown that stability of other transcripts (including *niiA, niaD* and *meaA* (ammonium permease), including those involved in nitrate assimilation, are also regulated by glutamine or ammonium dependent deadenylation, resulting in subsequent degradation (Caddick *et al,* 2006). In the presence of nitrate, the *niiA* and *niaD* transcripts are stabilised (Caddick, *et al,* 2006) and surprisingly nitrate favoured stabilisation was observed for *niaD* and *niiA* in the presence of both nitrate and glutamine, hence preventing accumulation of toxic nitrate and nitrite in the cell.

From published data, nitrogen metabolism serves to be a good model to use to study regulation of C/U modifications at the 3' end (Caddick *et al,* 2006; Morozov *et al,* 2010a). Thus, in this study a repeat of some of the published data to investigate regulation of the genes involved in nitrogen metabolism in response to nitrogen availability was performed using qRT-PCR, as all studies performed in published data were done using Northern blot analysis (Caddick *et al,* 2006).

Both qRT-PCR and northern blot can be used for analysis gene expression at the level of RNA, however there are a few advantages and disadvantages of using either analysis technique. The advantages of using qRT-PCR are that it is more sensitive than northern blot hence requires less starting material of 50-100ng against 10-20 µg for northern blot (Bustin 2000; Chuaqui *et al*, 2005; Gachon *et al*, 2004). qRT-PCR is highly sequence specific as it uses fluorescence-based detection methods and has a large dynamic quantification range compared to northern blot (Bustin 2000; Chuaqui *et al*, 2005; Gachon *et al*, 2004; Wong and Medrano 2005; Van Guilder *et al,* 2008; Exposito-Rodrihuez *et al*, 2008; Ahmed 2002). Furthermore, with qRT-PCR there is little/ no post-amplification processing and it does not require special safety arrangements, because unlike northern blot no radiation is used for qRT-PCR. One of

the disadvantages of using qRT-PCR include that it requires quality control assurance (melting curve analysis) to ensure the same DNA region is amplified (Wong and Medrano 2005; Nolan *et al,* 2006). Northern blot requires gel electrophoresis, followed by gene specific probe dependent detection of target mRNA. The amount of mRNA can be quantified and directly compared between multiple samples on a single membrane, however the number of transcripts to be analysed via the same membrane is limited to 3-4 (Van Guilder *et al,* 2008). The advantages of using northern blotting include that it is less probable for non-specific amplification to occur hence reduces the possibility of false positive results occurring and it gives the ability to be able to detect RNA size (Maderazo *et al,* 2003; Lee *et al,* 2005). A DNase treatment step is also not required during northern blot as any potential DNA contaminants will be at the top of the gel/membrane thus will not be interfering with hybridisation of the probe with the target (Maderazo *et al,* 2003; Lee *et al,* 2005; Van Guilder *et al,* 2008). Other disadvantages of using northern blot include that it is time consuming compared to qRT-PCR (Maderazo *et al,* 2003; Lee *et al,* 2005; Van Guilder *et al,* 2008). Although using qRT-PCR or northern blot have their advantages and disadvantages both analysis methods have been shown to produce consistent results when analysing gene expression or RNA degradation (Van Guilder *et al,* 2008). In this work the regulation of *areA, niiA, niaD* and *meaA* in response to nitrogen availability were analysed using qRT-PCR. This was followed by time-course degradation analysis of the Sanger sequencing data to investigate regulation of the C/U tagging in response to nitrogen availability. An independent sample *t-test* was used to determine statistical significance between data sets.

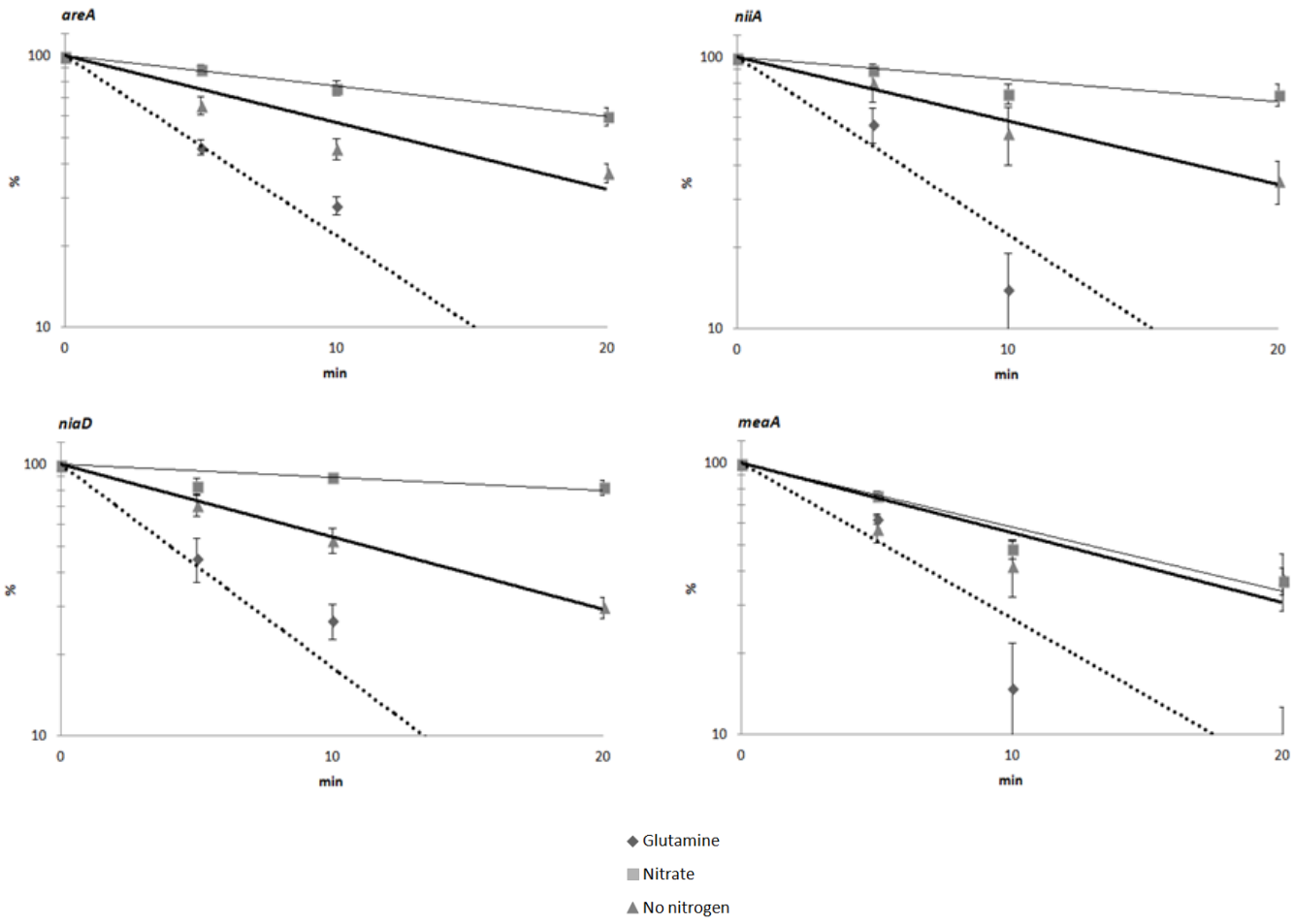
Four genes known to be regulated in response to nitrogen availability were selected (*areA, niiA, niaD, meaA*) for qRT-PCR analysis. The qRT-PCR results are shown in figure 3.1.1 and they were consistent with the northern blot data published previously (Caddick *et al,* 2006). Regulated degradation of the tested transcripts in response to

nitrogen availability was observed. For *areA*, treatment with glutamine resulted in a significant destabilisation of the gene thus having the lowest half-life, of 4.4 minutes, compared to samples under nitrogen starvation (*t test* = 0.0198). In the in the presence of nitrate there was no significant stabilisation (*t test* = 0.099) of the *areA* transcript albeit there was an increase in the half-life (half-life of 51 minutes) compared to samples under nitrogen starvation, which was consistent with published results (Caddick *et al,* 2006). For *niiA,* destabilisation of the transcript in the presence of glutamine and stabilisation in the presence of nitrate was observed, which was also consistent with published northern data. Under no nitrogen treatment the *niiA* transcript was found to have a half-life of 13.2 minutes, which significantly increased to 43.9 minutes in the presence of nitrate (*t test* = 0.001) and significantly reduced to 4.9 minutes in the presence of glutamine (*t test* = 0.0142), compared to samples analysed under nitrogen starvation. The pattern of the results found for *niiA* were consistent with published northern data, although slight differences with the half-lives compared to published data could have been a result of different analysis method being used for this study (Caddick *et al,* 2006). For *niaD,* under no nitrogen treatment the half-life of the transcript was 11.5 minutes. There was significant destabilisation of the *niaD* transcript in the presence to glutamine (*t test*= 0.0142), with a half-life of 3.9 minutes. Significant stabilisation of the *niaD* transcript in the presence of nitrate was found to occur when the half-life increased to 72.1 minutes, compared to samples analysed under nitrogen starvation (*t test* = 0.0096). Similar to *niiA*, the half-lives found in this work for *niaD* were different to published data. However, they had a similar pattern in showing nitrate signalled stabilisation and glutamine signalled destabilisation. For both *niaD* and *niiA* the rate of degradation increased in the presence of glutamine because it is utilised preferentially as it is a prime nitrogen source in the cell, as shown in published northern data. Furthermore, regulated transcript stability in response to nitrogen availability was confirmed by another technique, namely qRT-PCR (Caddick *et al,* 2006).

For *meaA*, which is an ammonium permease, in the presence of glutamine it was quickly degraded with a half-life similar to *areA, niiA* and *niaD* of 5.8 minutes (*t test* = 0.028 compared to *meaA* transcripts analysed under nitrogen starvation). However, there was no significant nitrate stabilisation of the *meaA* transcript compared to samples not treated with any nitrogen source (*t test* = 0.963), which was consistent with published results.

This data was sufficient to confirm that the qRT-PCR analysis reflected findings observed with previously published Northern analysis methods and confirmed that nitrogen metabolism was a good model to use in this study.

A.)



B.)

Transcript Half-lives (mins)

	areA	***niiA***	***niaD***	***meaA***
Glutamine	4.4	4.9	3.9	5.8
Nitrate	51.0	43.9	72.1	13.9
No nitrogen	14.0	13.2	11.5	14.1

Figure 3.1.1 Time course of degradation of the *areA, niiA, niaD* and *meaA* mRNA under glutamine, nitrate and no nitrogen.

Transcription was inhibited by using proflavin (Morozov et al 2012) prior to harvesting at T_0 (chapter 2). Transcript degradation was monitored over a 20-minute time-course at 30^{o}C after the addition of a required nitrogen source at T_0 (chapter 2.1.4). 18s rRNA was used as a normalisation control for qRT-PCR (chapter 2.3.3). Two biological and two technical repeats of qPCR experiments were quantified. The mean of the values was therefore represented graphically (±standard error [SE]) and used to determine the transcript half-lives ($T_{1/2}$) relative to the rRNA standard. These results were taken at T_0, T_5, T_{10} and T_{20} minutes for all the transcripts under the different nitrogen regimes, with the *y* axis being a logarithmic plot of the percentage of transcript remaining (3.1.1a). Figure 3.1.1b is a representation of the average of the extrapolated half-lives, derived by regression analysis of all three transcripts under the different nitrogen regimes.

3.2 Evaluation of genes suitable for cRT-PCR

3' end mRNA modifications are emerging as important features that are involved in determining transcript stability in the cytoplasm. In *A. nidulans,* it has been found that modifying the 3' end of wild-type mRNA with C/U nucleotides, once the poly (A) tail is shorten to 15 nucleotides, coincides with 5' end decapping of target transcripts (Morozov *et al,* 2010a). The initial aim of this study was to investigate regulation of C/U tagging in response to nitrogen availability. Differential mRNA degradation in response to nitrogen availability was found to occur during nitrogen metabolism in *A. nidulans* (chapter 3.1, Caddick *et al*, 2006).

To investigate the role of 3' end tagging under different nitrogen regimes, circularised reverse transcription coupled with PCR (cRT-PCR) was proposed to be used. cRT-PCR was previously used to study tagging in *A. nidulans, S. pombe* and mammalian histone mRNAs (Rissland and Norbury 2009; Mullen and Marzluff 2008; Morozov *et al,* 2010a; Morozov *et al,* 2012). This technique includes ligation of the 5' and 3' ends by using T4 RNA ligase I and this technique ideally preserves the original 5' and 3' ends of the transcript and post-transcriptionally added extension, i.e. tagging, for sequencing (Slomovic and Schuster 2013). Initial steps of this study were to optimise the circularisation/ ligation technique.

For preliminary tests the *areA, niaD, niiA,* and *meaA* transcripts were selected. Isolation of the transcripts of interest could be done using one (non-nested) or two rounds (nested) of PCR (methods shown in chapter 2.3.1). Furthermore, results were confirmed by the Sanger sequencing of the cRT-PCR products. For all the genes analysed, circularisation was only successful for *areA, niiA* and *meaA* for both non-nested and nested PCR primers (figure 3.2.1). All the transcripts successfully circularised were found to have 3'UTRs and 5'UTRs of different lengths (figure 3.2.1). 3'UTRs not degraded beyond 50 nucleotides from the full 3UTR length found in this work (chapter 3.5) were not classed as being partially degraded, because deviation

within 50 nucleotides of the 3'UTR could be a result of possible fluctuation in the formation of the 3' end during transcription. Figure 3.2.1 represents the gel electrophoresis results of the PCR products that were sequenced. Transcripts with longer 3'UTR and 5'UTRs may not have been visible in the gel (PCR product longer than 1000bps) images shown in figure 3.2.1. This may have been because the cRT-PCR approach appeared to be biased towards transcripts with shorter 3'UTR and 5'UTR lengths from these results.

For *areA,* (figure 3.2.1 and 3.2.2a) the published full 5'UTR is -495 nucleotides and 3'UTR is 526 nucleotides which would produce a full product length of 1021 nucleotides (Aspergillus genome database 2018). Theoretically *areA* would have been a complicated transcript to work with using the cRT-PCR approach because it had the longest 3'UTR and 5'UTR compared to the other transcripts analysed. In order to overcome this potential issue, the nested PCR primers were designed within the 5'UTR and 3'UTR regions to produce a full PCR product of 706 nucleotides (Chapter 2.3.1). The non-nested PCR primers represented the whole 5'UTR and 3'UTRs thus, producing a full PCR product of 1063 nucleotides (Chapter 2.3.1). From the results in figure 3.2.1b, which represent the nested PCR results for *areA*, a range between 150 to around 600bps was seen for the cRT-PCR product on the agarose gel, indicating that many transcripts may have been partially degraded. However, transcripts with long 5'UTRs and 3'UTRs may not have been visible from the gel image due to them being at low concentrations; these transcripts were also purified by gel extraction (chapter 2.5.2) for Sanger sequencing albeit not visible on the gel image (chapter 2.6.2). From figure 3.2.1a, non-nested PCR samples were shown for all transcripts analyses and a large smear was produced which was an expected result. Non-nested PCR samples were purified by PCR purification and subject to T/S cloning (chapter 2.5.1, 2.6.1, 2.6.2, 2.6.3. 2.6.4 and 2.6.5). For the sequencing data shown in figure 3.2.2a, only nested PCR samples were successfully sequenced and analysed. The average 5'UTR length

was -371 nucleotides with a maximum length of -692 nucleotides. This maximum 5'UTR length exceeded the published 5'UTR length for *area,* which has been found to be around -495 nucleotides (Aspergillus genome database 2018). The average sequenced 3'UTR length for *areA* was 410 nucleotides with a maximum length of 522 nucleotides, indicating that some transcripts were partially degraded (published 3'UTR length is 526 nucleotides) (Aspergillus genome database 2018). The maximum sequenced 3'UTR length for *areA* was consistent with the published length of 526 nucleotides (Aspergillus genome database 2018). Poly (A) tail lengths remained relatively short, with a maximum length of 11 nucleotides. It appeared that both 5'-3'and 3'-5' decay pathways were both involved in degrading the *areA transcript,* as there were some transcripts which were found to have completely degraded 5'UTRs but 3'UTRs remaining (17%) intact and completely degraded 3'UTRs with 5'UTRs (17%) remaining intact. No C/U tags were for all *areA* transcripts sequenced.

For *niiA* the published full 3'UTR is 120 nucleotides. However, the length of 5'UTR has not yet been published (Aspergillus genome database 2018) therefore, primers were designed downstream from AUG for the cRT-PCR approach (Chapter 2.3.1). The 5'UTR length for *niiA* was predicted to be around -50 nucleotides based on the 5'UTR length for *niaD,* because these genes work synergistically together and are found within the same gene cluster (Amaar and Moore 1998; Aspergillus genome database 2018). Non-nested *niiA* PCR fragments produced a range of around 75-300 bps from the agarose gel image (figure 3.2.1a), indicative for the presence of full and partially degraded transcripts. From the nested *niiA* PCR (figure 3.2.1b) products a range of around 50-300bps was found on the gel image. Only nested PCR fragments were able to be successfully sequenced and analysed for *niiA* (figure 3.2.2b). The average 5'UTR length was -35 nucleotides, which is similar to the length of the 5' UTR of *niaD* of -37 nucleotides (Aspergillus genome database 2018). The maximum 3'UTR length for *niiA* was around 147 nucleotides and the average full 3'UTR length was 135 nucleotides,

which was longer than the published 3'UTR length of 120 nucleotides (Aspergillus genome database 2018). For transcripts with long 3'UTRs (<50 nucleotide degradation from 135 nucleotides) the poly (A) tail remained relatively short with maximum length of adenosine nucleotides being found at 14 nucleotides. 25% of all transcripts were found to have partially degraded 3'UTRs (3'UTR degraded beyond 50 nucleotides from 135 nucleotides). These results were indicative of the 3'-5' degradation pathway being more active in degrading the *niiA* transcripts compared to the 5'-3' decay pathway, because transcripts were found to have partially degraded 3'UTRs but with full 5'UTRs. Two of the sequences analysed, with partially degraded 3'UTRs, were found to be readenylated with 1 and 14 adenosine nucleotides because they had partially degraded 3'UTRs with adenylated ends. Surprisingly no C/U tags were observed for *niiA*. 92% of all *niiA* transcripts analysed were sequenced and from this data therefore, the cRT-PCR method was successful for nested PCR approach.

For *meaA* the published full 3'UTR length is 495 nucleotides and 5'UTR is -128 nucleotides. From the primers designed (Chapter 2.3.1), non-nested PCR product sizes were expected to be a maximum of 732 nucleotides. For nested PCR products, the expected maximum size was 535 nucleotides based on the primers designed (chapter 2.3.1). From figure 3.2.1a, a visible PCR product produced for *meaA* ranged from 50 to around 300 nucleotides for the non-nested PCR. Larger PCR product sizes may not have been visible on the gel image due to the PCR reaction being more biased towards amplifying shorter/ partially degraded transcripts. From figure 3.2.1b, for nested- PCR products, visible PCR bands were seen at 75, 150 and around 500 nucleotides. From the sequencing data shown in figure 3.2.2c, only the nested PCR products were able to be sequenced for *meaA* and 31% of those were analysed. All of the sequenced transcripts were found to have no 5'UTRs and only partial degraded 3'UTRs ranging from 93 to 299 nucleotides. These results could be indicative of the 5'-3' decay pathway being more active in degrading *meaA.* One sample with a 3'UTR

degraded to 93 nucleotides, was found to be readenylated with 3 nucleotides in similar manner to *niiA* (figure 3.1.2b). It seemed that the cRT-PCR approach was very biased towards partially degraded or shorter transcripts for *meaA* thus, it was concluded that cRT-PCR method was not feasible to analyse 3' end modifications for *meaA*.

To conclude, from the cRT-PCR data it appears that the nested cRT-PCR approach was successful in analysing *areA* and *niiA* mRNA. The *niiA* transcript had the highest percentage of sequences which were able to be sequenced and successfully analysed. This was most likely a result of *niiA* having much shorter 3'UTR and 5'UTR compared to *areA* and *meaA*. The cRT-PCR approached appeared to be biased towards transcripts with shorter 5'UTRs and 3'UTRs and this was the representative for *meaA.* On the other hand, *areA* samples sequenced were found to have both long 3'UTRs and 5'UTRs demonstrating that this method could be used to analyse much larger transcripts.

Surprisingly short poly (A) tails, generally less than 15 nucleotides, were observed for all the samples analysed and no C/U tags were observed. The lack of any C/U tags indicated that these modifications may not be involved in degradation of these genes. However, the lack of C/U tags could have been a result of the cRT-PCR approach not being a suitable method of for detection. Either way it could not be ruled out that C/U tagging was involved in facilitating degradation of these transcripts. Thus, to investigate to possible function of C/U tagging in facilitating degradation, in *A. nidulans*, further analysis of *areA* and *niiA* mRNA was made (chapter 3.3). Furthermore, 2 *niiA* transcripts and 1 *meaA* transcript was found to be readenylated, when the 3'UTR was partially degraded. These readenylated transcripts resembled what has been found in bacteria, where short stretches of adenosine nucleotides were found to facilitate the 3'-5' decay pathway (Cao and Sakar 1992; Cao *et al,* 1996; Hajnsdorf *et al,* 1995; Haugel-Nielsen *et al,* 1996; O'Hara *et al,* 1995; Xu and Cohen 1995). It would also be

interesting to investigate the function of this readenylation process further in *A. nidulans*.

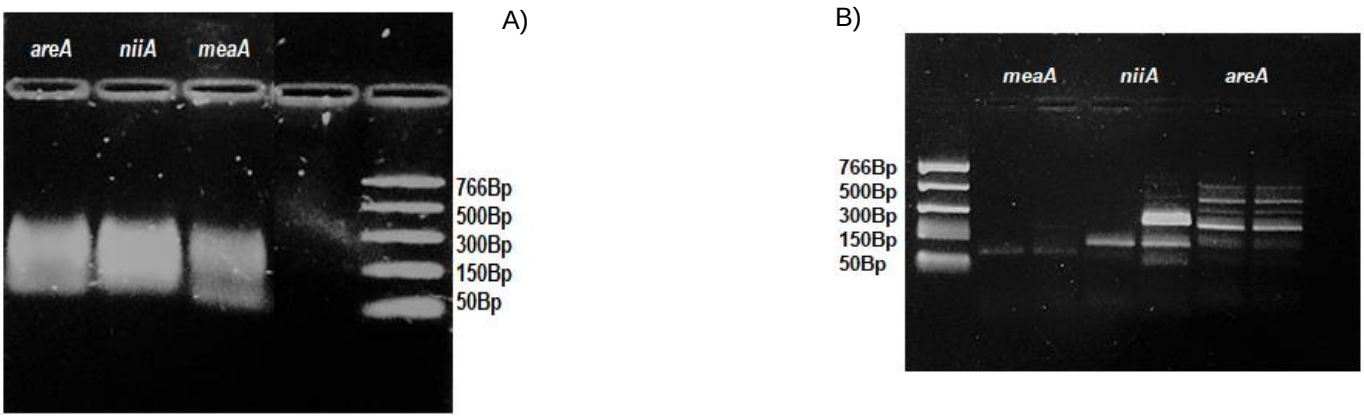


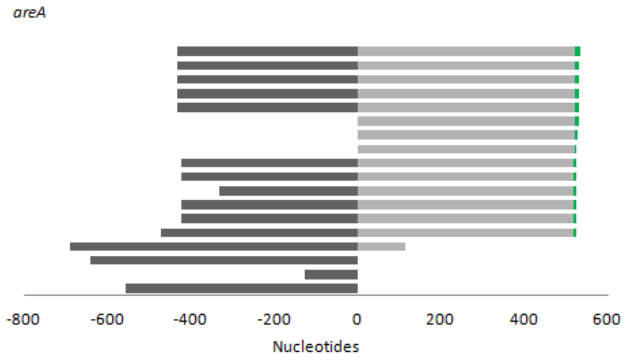
Figure 3.2.1 Gel electrophoresis of results cRT-PCR products of natively decapped *areA, niiA, niaD* and *meaA* mRNA in the wild-type background.

A.) PCR based validation using 1% agarose gel for non-nested PCR products for *areA*, *niiA*, and *meaA* transcripts confirming that circularisation of their mRNAs was successful. Non-nested PCR primers were used (described in chapter 2.3.1). From the image a large smear was produced, for a range of sizes for all genes, demonstrating the different size transcripts which were circularised. These products were purified using Qiagen PCR purification method described in 2.5.1 and cloned (chapter 2.6.1) for sequencing.

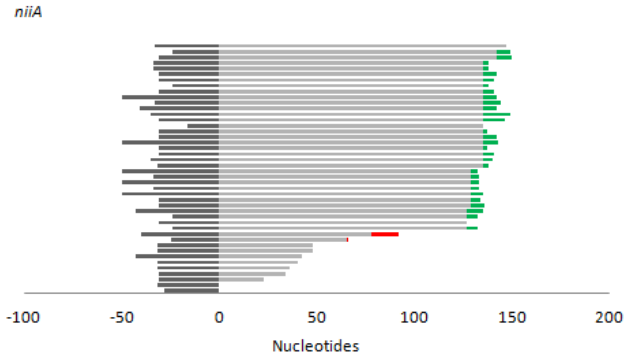
B.) PCR based validation using 1% agarose gel of nested *areA, niiA,* and *meaA* transcripts from the first PCR (as shown in figure 3.2.1a). Nested PCR primers were used (described in chapter 2.3.1). From the image more distinct PCR products were seen compared to the non-nested PCR products (figure 3.2.1a). For *areA,* PCR products are shown ranging from around 600Bp to 150Bps. For *niiA* PCR products are ranging from around 400Bps to 50Bps and for *meaA* PCR products are ranging from

300Bps to around 100Bps. PCR products were purified using the Qiagen Gel purification method described in 2.5.2 and cloned (chapter 2.6.1).

A).


areA
-800
-600
-400
-200
0
200
400
600
Nucleotides

B).


niiA
-100
-50
0
50
100
150
200
Nucleotides

C).

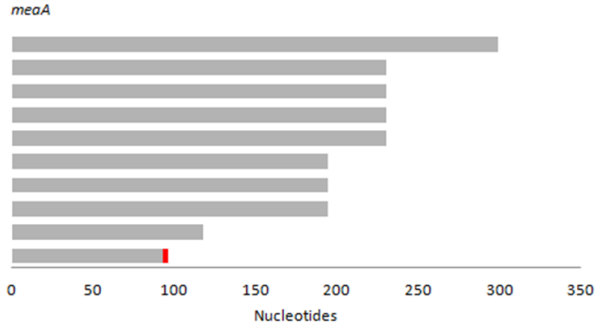

meaA
0
50
100
150
200
250
300
350
Nucleotides

Figure 3.2.2 A schematic presentation of nested cRT-PCR analyses of natively decapped *areA, niiA* and *meaA* transcripts in the wild-type background.

Total RNA was extracted from wild-type strains and subject to cRT-PCR as described in chapter 2.3.1. The products were cloned and sequenced to confirm that the genes of interest could be analysed using the cRT-PCR method and also to determine the position of 5' and 3' ends. Samples (cloned colonies) were randomly picked for sequencing analysis. The results are displayed indicating the length of the 5'UTR (dark grey lines), 3'UTR (light grey lines) and poly/oligo (A) tail (green lines). Red lines indicate what appears to be readenylation (on 3'UTR degraded beyond 50 nucleotides). 5'UTR is +1 upsteam from AUG and 3'UTR is +1 downstream from stop codon.

A.) *areA* cRT-PCR results are shown for 18 transcripts. 5'UTR lengths ranged from -0 to -692 nucleotides in length, 3'UTR lengths ranged from 0 to 522 nucleotides in length and poly/oligo (A) tail lengths ranged from 0 to 11 nucleotides in length for different transcripts.

B.) *niiA* cRT-PCR results are shown for 44 *niiA* transcripts. 5'UTR lengths ranged from -28 to -33 nucleotides in length, 3'UTR lengths ranged from 0 to 147 nucleotides in length and poly/oligo (A) tail lengths ranged from 0 to 14 nucleotides in length. Two samples appeared to be readenylated when the 3'UTRs were degraded to 65 and 78 nucleotides with 1 and 14 adenosine tails respectively.

C.) *meaA* cRT-PCR results are shown for 10 sequencing samples. All samples analysed had 5'UTRs completely degraded, 3'UTR lengths ranging from 93 to 299 nucleotides and one sample appeared to be readenylated with 3 nucleotides when the 3'UTR was degraded to 93 nucleotides.

3.3 Determining the regulation of 3' mRNA tagging in response to environmental signals

3' mRNA C/U tagging has been associated with destabilising transcripts in *A. nidulans* (Morozov *et al,* 2010a; Morozov *et al,* 2012). Transcript stability is fundamental for gene expression in determining the cytoplasmic abundance which is a product of both the rate of synthesis and degradation. The mRNA primary stability determinants are the two co-transcriptional modifications, being the 5' cap and 3' poly(A) tail, with the exception of mammalian histone mRNA which contain a 3' end conserved stem-loop (Parker 2012; Mullen *et al*, 2008). Once the 5' cap and 3' poly (A) tail are added to mRNA they protect it against degradation, stimulate export from the nucleus to the cytoplasm and play a key role in stability and translation. In the cytoplasm, degradation of polyadenylated transcripts is generally initiated by shortening of the poly (A) tail, namely deadenylation. Recent studies in *A. nidulans* revealed that when the poly(A) tail is degraded to a threshold length of approximately 15 nucleotides the transcript is subject to tagging with C/U nucleotides (Morozov *et al,* 2010a; Morozov *et al,* 2012). C/U tags were mainly found on decapped transcripts thus, it was postulated that this tagging triggers decapping and subsequent 5'-3' degradation (Morozov *et al,* 2010a; Morozov *et al,* 2012). It was found in *A. nidulans,* that the nucleotidyltransferase enzymes, namely CutA and CutB, were responsible for the C/U modifications, their disruption leading to an increase in stabilisation of mRNA and no tagging (Morozov *et al,* 2010a; Morozov *et al,* 2012). Disruption of *xrn1*, *dcp1* and *Lms1* resulted in an accumulation of tagged mRNAs with partially degraded poly (A) tails (Chang *et al,* 2014; Rissland and Norbury 2009). These findings led to the proposed model that during degradation of polyadenylated transcripts, the poly (A) tail is degraded to 15-25 nucleotides depending on the organism resulting in the 3' oligo (A) tail being tagged with C/U nucleotides (most studied organisms are tagged with U nucleotides except

for *A. nidulans*) (Morozov *et al,* 2010a; Morozov *et al,* 2012; Rissland and Norbury 2009; Mullen and Marzluff 2008; Chang *et al,* 2014; Lim *et al*, 2014). This C/U tagging was proposed to increase the affinity of Lsm1-7 which recruits Pat1 thus subsequently triggering decapping in an intricate process involving multiple factors, including decapping (Dcp1/Dcp2, decapping complexes), surveillance and translation factors (Morozov *et al,* 2010a; Morozov *et al,* 2012; Rissland and Norbury 2009; Mullen and Marzluff 2008; Chang *et al,* 2014; She *et al,* 2004; She *et al,* 2008; Deshmukh *et al,* 2008). The main aim of this study was to determine if 3' C/U tagging is regulated in response to environmental signals using nitrogen signaling in *A. nidulans* as a model system.

For *niiA*, the regulation of 3' mRNA tagging was studied over a 20-minute time course in the presence of nitrate, glutamine and under nitrogen starvation (figure 3.1.1). From figure 3.3.1, all *niiA* transcripts analysed under all the nitrogen conditions were found to have no C/U tags. Instead low levels of what appeared to be readenylation of partially degraded transcripts was identified. Under nitrogen starvation (figure 3.3.1a) decapped *niiA* mRNA analysed at T_0 appeared to have 3'UTRs which were not degraded, with an average 3'UTR of 135 nucleotides. 60% of *niiA* transcripts analysed at T_0 had a 3'UTR of 135 nucleotides, confirming the findings from chapter 3.2 (Fig. 3.2.2b) where 3'UTR lengths for *niiA* did not support the published length 120 nucleotides. The *niiA,* 5'UTR for most transcripts analysed appeared to be the full length. One sample had a 5'UTR which was -16 nucleotides upstream from AUG, indicative of being partially degraded. The average length of the *niiA* 5'UTR excluding the one that appeared partially degraded was -31 nucleotides. 70% of *niiA* transcripts analysed at T_0 had 5'UTRs with length of 31 nucleotides, which could be the possible maximum length for 5'UTR for *niiA*. The poly (A) tail length remained relatively short with a maximum length of 8 nucleotides and an average of 5 nucleotides overall.

For *niiA* transcripts analysed at T_5 under nitrogen starvation, two samples had partially degraded 3'UTRs of 73 and 74 nucleotides (degraded beyond 50 nucleotides from a full 3'UTR of 135 nucleotides). One of these *niiA* transcripts with a partially degraded 3'UTR, was found to be readenylated with 2 nucleotides. Two *niiA* transcripts had 3'UTR lengths of 92 and 114 nucleotides which could have been partially degraded. However, they were not considered degraded as the length of their 3'UTRs were not over the 50 nucleotides baseline. The *niiA* transcript with a 3'UTR of 92 nucleotides was found to have a poly (A) tail of 26 nucleotides, which could have been a canonical poly (A) tail or cytoplasmic readenylation. Apart from this, the maximum poly (A) tail length for *niiA* transcripts analysed at T_5, which did not have partially degraded 3'UTRs, was 11 nucleotides with an average of 5 nucleotides. For 5'UTR lengths at T_5 (figure 3.3.1a), 1 sample appeared to not have been degraded and 1 sample had a 5'UTR that was completely degraded but had a full 3'UTR indicating that the transcript would have been degraded through the 5'-3' decay pathway. The average 5'UTR length was -35 nucleotides excluding the sample that was completely degraded. Interestingly, the sample that was classed as being readenylated, with a 3'UTR of 72 nucleotides, had what appeared to be a partially degraded 5'UTR degraded to a length of -24 nucleotides upstream from AUG, indicative that both the 5'-3' and 3'-5' decay pathways were involved in the degradation of the *niiA* transcript.

For *niiA* transcripts analysed at T_{20} under nitrogen starvation, 100% of samples analysed were able to be sequenced. All *niiA* transcripts analysed at T_{20} appeared to be have full 3'UTRs averaging at 135 nucleotides, which was longer than the published 3'UTR length of 120 nucleotides for *niiA*. Poly (A) tail lengths remained short with a maximum length of 14 nucleotides and an average length of 3 nucleotides. 5'UTR lengths analysed at T_{20} (figure 3.3.1a) appeared to not be partially degraded apart from one sample having a completely degraded 5'UTR implicating the 5'-3' decay pathway. The average 5'UTR length was -36 nucleotides. From these results no C/U tagging

was found for all *niiA* transcripts analysed over the time-course under nitrogen starvation. However, some transcripts with partially degraded 3'UTRs were found to be readenylated.

Under nitrate treatment, for the analysis of decapped *niiA* transcripts, 92% of T_0 samples sequenced were able to be analysed (figure 3.3.1b). 72% of these transcripts had 3'UTRs that were completely or partially degraded, with two of them being found to be readenylated with 1 and 14 nucleotides when the 3'UTR was degraded to 65 and 78 nucleotides respectively. The average full 3'UTR was 132 nucleotides (excluding transcripts that were partially or fully degraded). The average poly (A) tail length was 4 nucleotides for transcripts classed as having full 3'UTRs, which was shorter than expected, because under nitrate treatment *niiA* is known to be stabilised. At T_0 under nitrate treatment for *niiA,* the average 5'UTR length was -32 nucleotides, with all samples appearing to not be degraded. From these results, transcripts appeared to have been degraded through the 3'-5' decay pathway and readenylation may have been facilitating this degradation.

For *niiA* transcripts under nitrate treatment, analysed at T_5, 83% of samples sequenced were able to be analysed (figure 3.3.1b). Two transcripts found to have partially degraded 3'UTRs were readenylated with 3 and 4 nucleotides respectively. The average full 3'UTR at T_5 was 128 nucleotides for *niiA,* under nitrate treatment. One transcript with a 3'UTR of 114 nucleotides could have also been partially degraded. All but one sample had full 5'UTRs averaging at -34 nucleotides. The sample which had a partially degraded 5'UTR had 2 nucleotides left and this sample had a full 3'UTR and a poly (A) tail of 7 nucleotides.

For *niiA* transcripts analysed at T_{20}, under nitrate treatment, 67% of samples sequenced were able to be analysed (figure 3.3.1b). 75% of these samples had partially or completely degraded 3'UTRs, with 25% of these transcripts being found to have full 3'UTRs averaging at 128 nucleotides, implicating the 3'-5' decay pathway.

One of these transcripts, with full a 3'UTR, had an oligo (A) tail of 4 nucleotides. Interestingly, one sample with completely degraded 3'UTR and a full 3'UTR of -28 nucleotides was found to be readenylated with 8 nucleotides. The average 5'UTR at T_{20} under nitrate treatment was -37 nucleotides, excluding one transcript which was completely degraded but had a full 3'UTR length of 135 nucleotides implicating the 5'-3' decay pathway for this sample. From this data no C/U tags were found for all *niiA* transcripts analysed under nitrate treatment but instead readenylation was found. Also, most partially degraded transcripts appeared have to been degraded through the 3'-5' decay pathway with the 5'UTR for *niiA* appearing to be protected over the time course under nitrate treatment.

In response to glutamine, 66% decapped *niiA* transcripts analysed at T_0 were able to be sequenced and analysed (figure 3.3.1c). The average 3'UTR length was 133 nucleotides excluding 3 transcripts that had partially degraded 3'UTRs. The average poly (A) tail length for *niiA* transcripts analysed at T_0, under glutamine treatment, was 6 nucleotides. All transcripts analysed at T_0, with partially degraded 3'UTRs, were not found to be readenylated and the average 5'UTR was -36 nucleotides. It appears that most transcripts were degraded through the 3'-5' decay pathway however, it is unclear why all transcripts appeared to have relatively short poly (A) tails.

For *niiA* transcripts analysed at T_5 under glutamine treatment, 81% of all sequences were able to be analysed (figure 3.3.1c). The average 3'UTR length was 133 nucleotides, with the exception of two transcripts which were found to be partially degraded. The average poly (A) tail length was 8 nucleotides. 4 transcripts were found to have long poly (A) tails between 18 to 30 nucleotides and these had both full 3'UTRs and 5'UTRs. The average 5' UTR length was -36 nucleotides. One samples was found to have a partially degraded 3'UTR but a 5'UTR length of -110 nucleotides, which is longer than the predicted length of maximum 5'UTR length of 50 nucleotides. For *niiA* transcripts analysed at T_{20} under glutamine treatment, 81% of all seqeunces were able

to be analysed (figure 3.3.1c). The average 3'UTR length was 133 nucleotides with the exception one sample which was found to be partially degraded to 66 nucleotides and readenylated with 12 nucleotides. The average poly (A) tail length was 4 nucleotides, thus remaining relatively short. However, 5 of the transcripts analysed did not have poly (A) tails but had full 3'UTRs. The average 5'UTR length was -34 nucleotides, with the exception of two transcripts with fully degraded 5'UTRs and one with a partially degraded 5'UTR, implicating the 5'-3' decay pathway for these samples. Under glutamine treatment for *niiA,* no C/U tags were found, with one transcript being found to be readenylated over the time course and transcripts appeared to have generally protected full 3'UTRs and 5'UTRs.

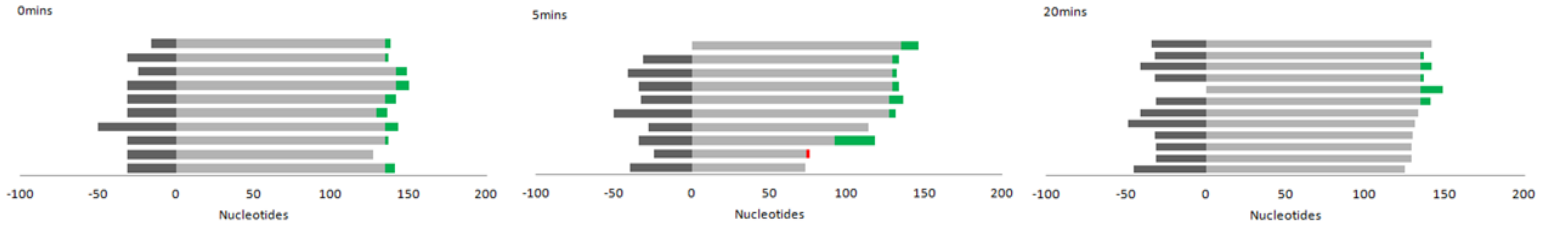
Decapped *areA* mRNA samples treated with glutamine, shown in figure 3.3.2, were analysed over a 20-minute time-course and the sequences are shown for T_0, T_5 and T_{20} samples. For transcripts sequenced at T_0, 43% of samples analysed were able to be sequenced successfully. The average 3'UTR length was 520 nucleotides (published 3'UTR length is 526 nucleotides) and an average poly (A) tail length was 8 nucleotides. The average 5'UTR was -424 nucleotides. Three of the *areA* transcripts analysed at T_0 were found to have completely degraded 5'UTRs and all but one sample which was found have a partially degraded with 5'UTR (published 5'UTR length for *areA* is -495 nucleotides) (Aspergillus genome database 2018).

For *areA* transcripts analysed at T_5 under glutamine treatment, 69% of all samples analysed were able to be sequenced (figure 3.3.2). The average 3'UTR was 521 nucleotides and all transcripts were not found to be partially degraded with an average poly (A) tail length of 15 nucleotides. The average 5'UTR length was -137 nucleotides but 41% of the samples had completely degraded 5'UTRs and rest were partially degraded, implicating the 5'-3' decay pathway as being more active in degrading these transcripts. For *areA* transcripts analysed at T_{20} under glutamine treatment, 38% of the sequences were able to be analysed (figure 3.3.2). The average 3'UTR length was

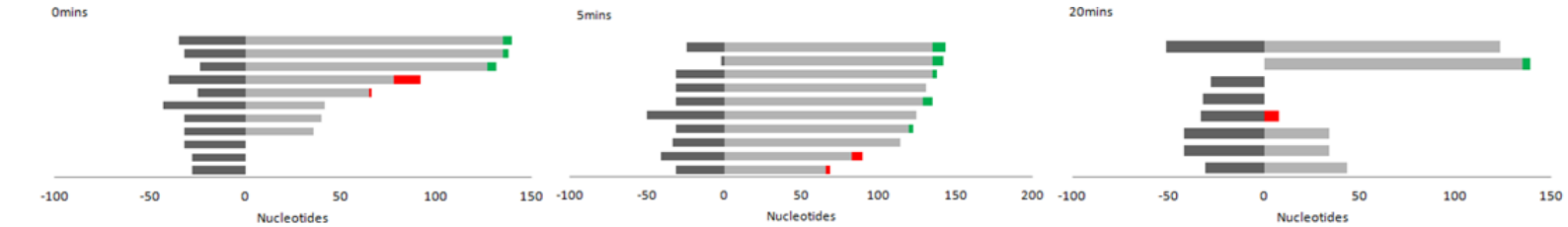
520 nucleotides, with no samples being found to be partially degraded and the average poly/ oligo (A) tail length was 17 nucleotides. The average 5'UTR length was -213 nucleotides with 42% having completely degraded 5'UTRs and the rest being partially degraded. From these results the 5'-3' decay pathway appeared to be more active in degrading the *areA* transcripts in response to Gln signal. As seen for *areA* transcripts analysed at T_5, some samples with poly (A) tails longer than 15 nucleotides, were found to have completely degraded or partially degraded 5'UTR, indicative of a poly (A) tail dependent decapping mechanism.

No readenylation or C/U tags were observed for all *areA* transcripts analysed. The difficulty in finding any readenylated transcripts for *areA* could have been a result of its large 3'UTR and 5'UTR length. On the other hand, for *niiA,* readenylation observed appeared to be involved in degrading target transcripts. Based on these findings further characterisation for the function of 3' readenylation was made using adaptor ligation RT-PCR (chapter 2.3.2). Adaptor ligation RT-PCR focused mainly on 3' end modification as it seemed that readenylation did not facilitate 5'-3' mRNA decay pathway from these results and some published data (mentioned in chapter 1.7).

A.)


0mins
5mins
20mins
-100
-50
0
50
100
150
200
Nucleotides

B.)


0mins
5mins
20mins
-100
-50
0
50
100
150
200
Nucleotides

C.)

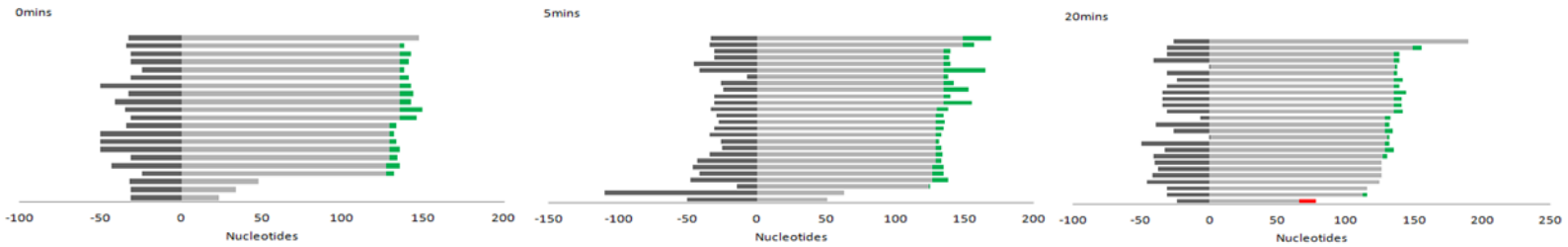

0mins
5mins
20mins
-150
-100
-50
0
50
100
150
200
250
Nucleotides

Figure 3.3.1. Nested cRT-PCR analysis of the natively decapped *niiA* mRNA under no nitrogen (a), nitrate (b) and glutamine (c) treatment in the wild-type background.

Cultures were treated with proflavin to inhibit transcription as described in chapter 2.1.5 prior to time course degradation analysis. The required nitrogen source was added at T_0 in the amounts described in chapter 2.1.5. Samples were harvested at T_0, T_5, and T_{20} for RNA extraction. RNA was extracted as described in chapter 2.2.1. Total RNA was subject to nested cRT followed by PCR as described in chapter 2.3.1. The products were cloned in the TA-vector (Promega) and sequenced as described in chapter 2.6.1. The results are displayed indicating the length of the 5'UTR (dark grey lines), 3'UTR (light grey lines) and poly/oligo (A) tail (green lines). Red lines indicate what appears to be readenylation (3'UTR degraded beyond 50 nucleotides). 5'UTR is +1 upstream from AUG and 3'UTR is +1 downstream from stop codon.

A.) cRT-PCR results of decapped samples for *niiA* under no nitrogen treatment at T_0, T_5 and T_{20} following transcription inhibition. At T_0, 10 samples were analysed. 3'UTR lengths ranged from 127 nucleotides to 142 nucleotides and 5'UTR lengths ranged from -16 to -50 nucleotides in length. Poly/oligo (A) tails ranged from 0 to 8 nucleotides for. At T_5, 10 samples were analysed. 3'UTR lengths ranged from 73 to 135 nucleotides, 5'UTR lengths ranged from -0 to -50 nucleotides and poly/oligo (A) tails ranged from 0 to 26 nucleotides with one sample appearing to be readenylated with 2 nucleotides. At T_{20} 12 samples were analysed. 3'UTR lengths ranged from 125 to 142 nucleotides, 5'UTR lengths ranged from -0 to -49 nucleotides and poly/oligo (A) tails ranged from 0 to14 nucleotides.

B.) cRT-PCR results of decapped samples for *niiA* under nitrate treatment at T_0, T_5 and T_{20} following transcription inhibition. At T_0, 11 samples were analysed. 3'UTR lengths ranged from 0 to135 nucleotides, 5'UTR lengths ranged from -24 to -43 nucleotides and poly/oligo (A) tail lengths ranged from 0 to14

nucleotides with two samples appearing to be readenylated with 1 and 14 nucleotides. At $T_{5,}$ 10 samples were sequenced. 3'UTR lengths ranged from 66 to 135 nucleotides, 5'UTR lengths ranged from -2 to -50 nucleotides and poly/oligo (A) tail lengths ranged from 0 to 9 nucleotides with two samples appearing to be readenylated with 3 and 7 nucleotides. At T_{20}, 8 samples were sequenced and able to be analysed. 3'UTR lengths ranged from 0 to 135 nucleotides, 5'UTR lengths ranged from -0 to -51 nucleotides and poly/oligo (A) tail lengths ranged from 0 to 8 nucleotides in length.

C.) cRT-PCR results of decapped samples for *niiA* under glutamine treatment at $T_{0,}$ T_5 and T_{20} following transcription inhibition. At T_0, 21 samples were analysed. 3'UTR lengths ranged from 23 to 147 nucleotides, 5'UTR lengths ranged from -24 to -50 nucleotides and poly/oligo (A) tails ranged from 0 to14 nucleotides. At T_5, 26 samples were able to be analysed. 3'UTR lengths ranged from 51 to 149 nucleotides, 5'UTR lengths ranged from -8 to -111 nucleotides and poly/oligo (A) tails ranged from 0 to 30 nucleotides. At T_{20}, 26 samples were able to be analysed. 3'UTR lengths ranged from 66 to 190 nucleotides, 5'UTR lengths ranged from -0 to -51 nucleotides and poly/oligo (A) tails ranged from 0 to 12 nucleotides with one sample appearing to be readenylated with 12 nucleotides.

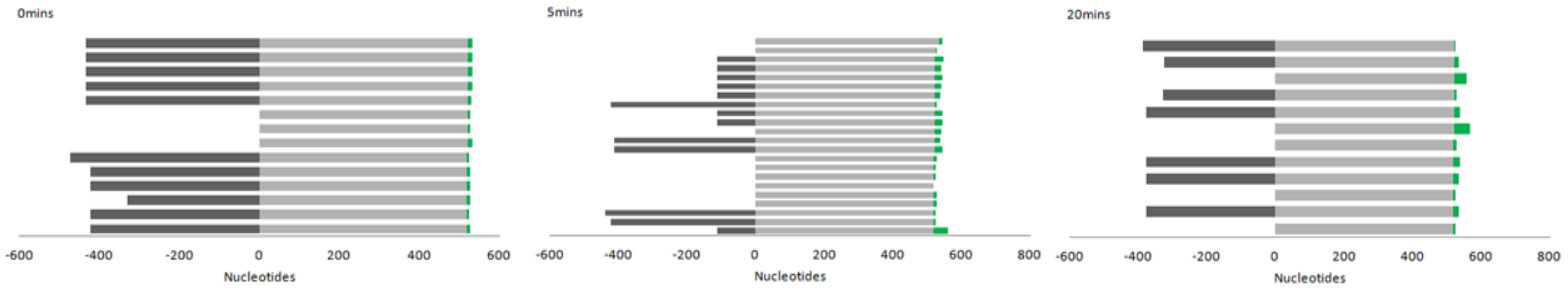


Figure 3.3.2. Nested cRT-PCR analysis of decapped *areA* mRNA in the presence of Glutamine in the wild-type background.

cRT-PCR results are shown for *areA* in response to Gln at T_0, T_5, and T_{20} following transcription inhibition with proflavin. At T_0, 14 samples were sequenced and analysed. 3'UTR lengths ranged from 518 to 522 nucleotides, 5'UTR lengths ranged from -0 to -473 nucleotides and poly/oligo (A) tail lengths ranged from 6 to 11 nucleotides. At T_5, 22 samples were sequenced and analysed. 3'UTR lengths ranged from 517 to 534 nucleotides, 5'UTR lengths ranged from -0 to -440 nucleotides and poly/oligo (A) tail lengths ranged from 0 to 45 nucleotides. At T_{20}, 12 samples were sequenced and analysed. 3'UTR lengths ranged from 518 to 522 nucleotides, 5'UTR lengths ranged from -0 to -386 nucleotides and poly/ oligo (A) tails ranged from 2 to 45 nucleotides. The results are displayed indicating the length of the 5'UTR (dark grey lines), 3'UTR (light grey lines) and poly/oligo (A) tail (green lines). Red lines indicate what appears to be readenylation (3'UTR degraded beyond 50 nucleotides). 5'UTR is +1 upstream from AUG and 3'UTR is +1 downstream from stop codon.

3.4 3’ end decay intermediates of *niaD* and *niiA* transcripts can be readenylated

Readenylation has been associated with the degradation of bacterial mRNA to help overcome any stable 3’ secondary structures (Coburn *et al,* 1996; Hajnsdorf *et al,* 1995; O’Hara *et al,* 1995; Xu and Cohen 1995; Cao and Sakar 1992; Cao *et al,* 1995; Haugel-Nielsen *et al,* 1996; Xu *et al,* 1996). 3’ secondary structures in bacteria have been found to block 3’-5’ exoribonucleases. Thus, short stretches of about 5 adenosine nucleotides, added to the 3’ end of the secondary structure, allow the bacterial 3’-5’ exoribonuclease to bind by acting as a ‘toe hold’ for attachment (Coburn *et al,* 1996; Hajnsdorf *et al,* 1995; O’Hara *et al,* 1995; Xu and Cohen 1995; Cao and Sakar 1992; Cao *et al,* 1995; Haugel-Nielsen *et al,* 1996; Xu *et al,* 1996). Once bound, rapid deadenylation and removal of the adenosine nucleotides, occurs along with a few nucleotides from the 3’ end of the secondary structure. Several rounds of readenylation and degradation have been found to be required for complete removal of the 3’ secondary structures in bacteria. In eukaryotes, a similar mechanism has been found to occur during the degradation of mammalian cell cycle regulated histone mRNAs, with the exception of different nucleotides being used (Mullen and Marzluff 2008). Histone transcripts are not polyadenylated in their 3’ terminus but contain a conserved stem-loop structure. During the degradation of histone transcripts, tagging with uridine nucleotides by TUTase 7 and TUTase 4 has been associated with facilitating degradation of their mRNAs through both the 3’-5’ and the 5’-3’ decay pathways simultaneously (Mullen and Marzluff 2008; Hoefig *et al,* 2013). For the stimulation of the 3’-5’ decay pathway, tagging of histone transcripts has been found to promote binding of the decapping factor Lsm1-7 which interacts with Eri-1 thus recruiting 3’-5’ exonucleases (Hoefig *et al,* 2013; Slevin *et al,* 2014). Once bound, exonucleases degrade the uridine tags along with some canonical nucleotides from the stem-loop. Furthermore, in eukaryotes cytoplasmic readenyation has been found to facilitate 3’-5’

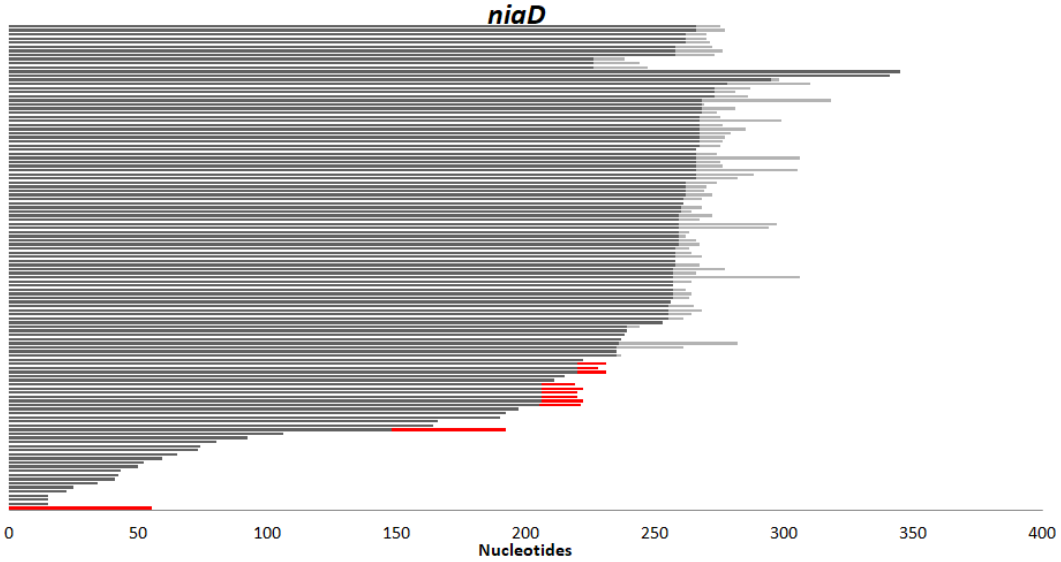
degradation of *Chlamydomonas reinhardtii* mRNA by the non-canonical nucleotidyltransferase MUT68 (Ibrahim *et al,* 2006). Readenylation was found to be involved during the degradation human HeLa cells however, enzymes responsible were not characterised (Slomovic *et al,* 2010). Readenylation has also been found to be involved during the degradation of *Drosophila* Hsp70 mRNAs, catalysed by the nuclear Trf4-1- poly (A) polymerase (member of the TRAMP complex), which has been found to shuttle to the cytoplasm (Harnisch *et al,* 2016). From this work, it now appears that readenylation is involved in facilitating 3'-5' mRNA decay in some *A. nidulans* transcripts. To characterise this mechanism further, adaptor ligation-RT-PCR for wild-type T_0 samples were sequenced for *meaA, niiA* and *niaD,* to analyse changes occurring on the 3' end of transcripts. Only *niiA* and *niaD* transcripts were able to be successfully sequenced and analysed.

For *niaD* (figure 3.4.1b), 70% of transcripts analysed were found to have full 3'UTRs ranging from 107 to 151 nucleotides, with an average 3'UTR length of 145 nucleotides, which correlated with the polyadenylation start site characterised for *niaD* in this work (figure 3.5.1a) but not with the published data (Aspergillus genome database 2018). 30% of samples were found to have fully degraded and partially degraded 3'UTRs. Degradation was found to proceed to the mRNA body downstream of the primer. 30% of the partially degraded *niaD* transcripts were found to be readenylated and the sites of readenylation were at 105, 91, 90, and 33 nucleotides downstream of the stop codon, within the 3'UTR (figure 3.4.1b). Hotspots for readenylation were found when the 3'UTR was degraded to 91 and 105 nucleotides. A transcript was found to have a 3'UTR which was completely degraded while being readenylated upstream of the stop codon thus, implicating this modification in facilitating the degradation of *niaD* mRNA within the open reading frame (ORF).

For *niiA* (Figure 3.4.1a), 80% of transcripts were classed as having full 3'UTRs with lengths ranging from 95 to 151 nucleotides and average length of 131 nucleotides,

which correlated with the polyadenylation start site characterised for *niiA* (figure 3.5.1c). 20% of *niiA* transcripts analysed were found to have partially degraded 3'UTRs, with 33% of those being found to be readenylated. No specific hot-spots for readenylation were identified for *niiA*, as readenylation was found at different sites along the 3'UTR. These results confidently confirmed that readenylation is a modification which facilitates 3'-5' decay pathway in *A. nidulans* for *niaD* and *niiA*. These findings gave an additional function for the poly (A) tail in not only stabilising their mRNA but in also facilitating their degradation in a length dependent manner. The next question was to determine what enzymes are involved in re-adding these adenosine nucleotides.

A.)


niaD
0
50
100
150
200
250
300
350
400
Nucleotides

B.)

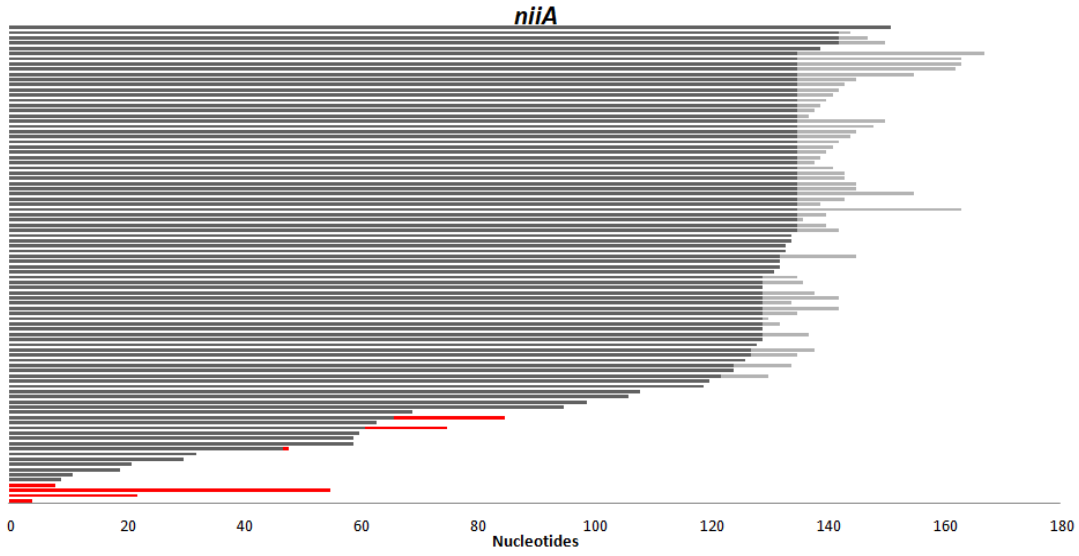

niiA
0
20
40
60
80
100
120
140
160
180
Nucleotides

Figure 3.4.1 Adaptor ligation RT-PCR analysis for decapped and capped *niaD* (a) and *niiA* (b) under no nitrogen treatment in the wild-type back ground.

Cultures were treated with proflavin to inhibit transcription as described in chapter 2.1.5. RNA was extracted (chapter 2.2.1) for T_0 samples after 1 hour of nitrogen starvation. A 5' adenylated adaptor (adaptor sequence: GACTGGAATTCTCGGG TGCCAAGGC) was ligated on to the 3' end of mRNA as described in chapter 2.3.2 and the 3' junction of *niiA* and *niaD* mRNA was amplified using gene specific primers. PCR products were cloned and sequenced. The results displayed indicate the length of 3'UTR (PCR product for *niaD*) (dark grey lines), poly/oligo (A) sequences (light grey lines) and those classed as readenylation (red lines) as they are poly/ oligo (A) sequences found on partially degraded 3'UTRs (>50 nucleotides 3'UTR degradation). 0 is 1+ downstream from the stop codon.

A.) Capped and decapped adaptor ligation RT-PCR results at T_0 are shown for *niiA* under no nitrogen treatment. 92 samples were sequenced. 3'UTR lengths ranged from 0 to 151 nucleotides, poly/oligo (A) tail lengths ranged from 0 to 32 nucleotides with 7 samples appearing to be readenylated with 1 to 55 nucleotides.

B.) Capped and decapped adaptor ligation RT-PCR results at T_0 are shown for *niaD* under no nitrogen treatment. 118 samples were sequenced and analysed. 3'UTR lengths ranged from -115 to 151 nucleotides (up to 266 nucleotides for PCR product). Poly/oligo (A) tail lengths ranged from 0 to 50 nucleotides with 11 samples appearing to be readenylated with 8 to 55 nucleotides.

3.5 Major polyadenylation start sites for *niiA* and *niaD*

Polyadenylation is an essential step during the maturation of most eukaryotic mRNAs and it is a tightly coupled two-step process. For polyadenylated transcripts, the first step involves endonucleolytic cleavage at the poly (A) site which is generally catalysed by the cleavage polyadenylation specificity factor (CPSF) and this is followed by direct addition the poly (A) tail (Beinroth *et al,* 1993; Davila Lopez and Samuelsson 2007). The endonucleolytic cleavage site is usually found around 10-35 nucleotides upstream from the polyadenylation site and it is the site where the polyadenylation signal is generally found (Beaudoing *et al,* 2000; Venkataraman *et al,* 2005; Millevoi *et al,* 2006). The polyadenylation signal, AAUAAA, has been found in about 50% of mammalian transcripts and is the most highly conserved signal (Beaudoing *et al,* 2000). However, this polyadenylation signal has been found to be more diverse in other eukaryotes, such as plants and fungi (Tanaka *et al,* 2011). Currently no experimental data exist regarding polyadenylation signals for *A. nidulans*. In *A. oryzae,* filamentous fungi, 3' polyadenylation signals where identified for different transcripts, such as AAUGAA, AAUGA and AUAUGUA (Tanaka *et al,* 2011).

In the work done in chapter 3.2, 3.3 and 3.4, different potential polyadenylation start sites were identified for both *niaD* and *niiA,* which did not support published data (Aspergillus genome database 2018). To support these findings from this work, the position of the polyadenylation start site was determined using a reference from one of the polyadenylation signals identified in *A. oryzae*, namely AUUAUA (Tanaka *et al,* 2011). The AUUAUA found in *A. oryzae* was the only polyadenylation signal found in both niiA and niaD genes (Tanaka *et al,* 2011). This polyadenylation signal analysis was done on capped mRNA with long poly (A) tails (>15 nucleotides). Only transcripts with long poly (A) tails (>A15) were analysed to reduce the chances of any partially degraded 3'UTRs being analysed. This was followed by predicting the polyadenylation

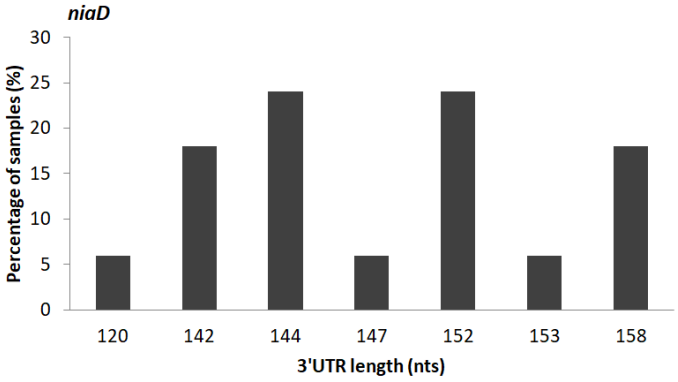
signal for both *niiA* and *niaD*. An independent sample *t test* was used to determine statistical significance.

For *niaD,* the published 3'UTR length is 200 nucleotides (Aspergillus genome database 2018). The major polyadenylation start sites identified in this work varied for *niaD*; transcripts were found with 3'UTR lengths of 120 (6%), 142 (18%), 144 (24%), 147 (6%), 158 (18%), 153 (6%), and 152 (24%) nucleotides (Fig. 3.5.1a). From this work the major 3'UTR lengths were found to be 144 and 152 nucleotides. All transcript were found with shorter 3'UTRs compared to the published length of 200 nucleotides (Aspergillus genome database 2018). It appeared that the 3'UTR length for *niaD* varied because the percentage of transcripts found with 3'UTR lengths of 142 and158 nucleotides were not significantly less than the number of transcripts found to have 3'UTR lengths of 144 and 152 nucleotides (*p value* <0.05). Thus, it appeared that the polyadenylation signal for *niaD* could range between 142-158 nucleotides. To further support the polyadenylation start sites found in this work, the polyadenylation signal was predicted. A polyadenylation signal of AUUAUA (highlighted in yellow), was found 24 and 32 nucleotides upstream of the major polyadenylation start sites of 144 and 152 nucleotides respectively. The distance of the polyadenylation signal from the polyadenylation start sites was within the published distance found in mammals of 10 to around 35 nucleotides (Beaudoing *et al,* 2000). Based on the distance of the polyadenylation signal to the polyadenylation start site was unlikely that the polyadenylation signal for *niaD* was 200 nucleotides because that would make the polyadenylation signal found 80 nucleotides form the polyadenylaion start site. Also, *niaD* did not seem to have another polyadenylation signal further downstream within the 3'UTR, from bioinformatics analysis. It is important to note that the distance of the polyadenylation signal to the polyadenylation start site has not been studied in fungi and may be different compared to mammals.

For *niiA*, the published 3'UTR length is 120 nucleotides from the *Aspergillus* genome database (Aspergillus genome database 2018). Full 3'UTR lengths found for *niiA* in this work were 127 (11%), 129 (6%), 130 (6%), 135 (65%) and 149 (11%) nucleotides (Fig. 3.5.1c). Hence, the major (*p value* <0.05 compared to other 3'UTR lengths) 3'UTR length appeared to be 135 nucleotides with 65% of capped *niiA* samples being found to have 3'UTRs of this length, which was 15 nucleotides longer than the published 3'UTR length. All capped polyadenylated *niiA* samples analysed were found to have longer 3'UTR lengths than the published length of 120 nucleotides. To further support the 3'UTR length found in this work for *niiA,* the position of polyadenylation signal was predicted. A signal of AUUAUA (highlighted in yellow), the same sequence found for *niaD,* was found for *niiA* and this polyadenylation signal was based on polyadenylation signal found in *A. oryzae* (Tanaka *et al*, 2011). The *niiA* polyadenylation signal was 11 nucleotides upstream from the major polyadenylation start site of 135 nucleotides. Interestingly, the same sequence of AUUAUA (highlighted in blue) was found 21 nucleotides upstream from the main candidate thought to be the polyadenylation signal (highlighted in yellow). This polyadenylation signal (highlighted in blue) was found to be 97 nucleotides downstream from the stop codon making it 23 nucleotides upstream from the published polyadenylation start site of 120 nucleotides for *niiA* (Aspergillus genome database 2018). If the polyadenylation signal was AUUAUA, then it appears that *niiA* may have two polyadenylation signals, the first (highlighted in blue) being specific to 3'UTR length of 120 nucleotides and the second (highlighted in yellow) being specific to 3'UTR length of 135 nucleotides. It was found in plants that some genes can have more than one polyadenylation signal therefore, it cannot be ruled out that the *niiA* gene has two polyadenylation signals as well (Tian and Graber, 2011). The analysis in this work suggests that the *niiA* transcript was being polyadenylated relative to the second, downstream, polyadenylation signal (highlighted in yellow). From this work it was determined that for both *niiA* and *niaD*, the published polyadenylation start sites were different to the published polyadenylation start sites.

For *niiA* the major polyadenylation start site was found to be 135 nucleotides and for *niaD* the major polyadenylation start site appeared to be between 142 -158 nucleotides. These results were confirmed by finding the most likely polyadenylation signal, of AUUAUA, which happened be the same for both genes.

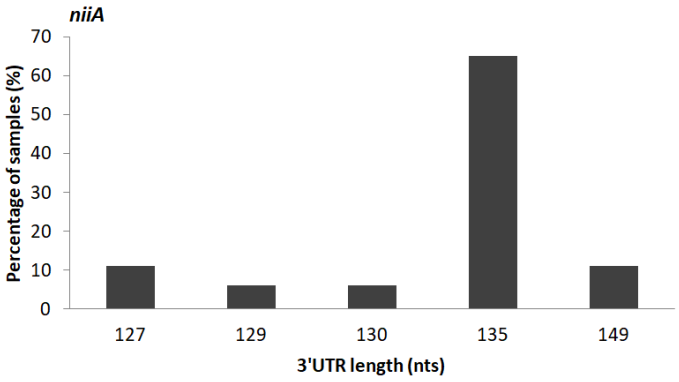
A.)



B.) *niaD* 3'UTR section

2970TAGACGGGTTCGCATAGGTTTGGGGTTGTATCTTGGCGTTGGGACGGACT
GGGTATGGTGTTTCTTTTGGATATATGACATGATATGTACACGGCCGTGAATCTT
TAACTTTATATCATTATAGAAATGCACTTGCACATTTCAACACGCTGCGAGCAGA
ATCTCGAAGATTGTTCCGCAAGTATTAGATCATGAGAGCATTT**3173**

C.)



D.) *niiA* 3'UTR section

3706TAG
ACTGACGAGGATACGTTTTGCGATGTGATATTAGTATGGTGGACATGCTTATTG
GTTTGCATGGCGTTTTTCTATTCAGGCGGTTCTATGCATTATACCTAGTGTTAAA
CAATCTATGATTATACTATACTCGA ATCGGTAACAGTCCA**3858**

Figure 3.5.1 *niaD* (a) and *niiA* (c) 3'UTR lengths and polyadenylation signal sites for *niaD* (b) and *niiA* (d).

The analysis of capped *niaD* and *niiA* mRNA was made. The *niaD* RNA was treated with Xrn1 which degrades all decapped, 5' phosphorylated mRNA samples (Chapter 2.2.9). A 5' adenylated adaptor (adaptor sequence: GACTGGAATTCTCGGGTGCC AAGGC) was ligated on to the 3' end of mRNA as described in chapter 2.3.2. The 3' junction of *niaD* mRNA was amplified using PCR and the analysis of 3'UTR lengths for transcripts with long poly (A) tails (15> nucleotides) was made and represented in figure 3.5.1a. This was followed by determination of the polyadenylation signal in figure 3.5.1b. For *niiA,* RNA was analysed using cRT-PCR as described in chapter 2.3.1. The 5'-3' junction was sequenced and the analysis of 3'UTR lengths of transcripts with long poly (A) tails was made and represented in figure 3.5.1c. This was followed by determining the polyadenylation signal in figure 3.5.1d.

A.) The figure shows the distribution of capped transcripts classed as having full 3'UTRs. The percentage fully polyadenylated capped *niaD* transcripts at different 3'UTR lengths is shown. 6% of fully polyadenylated samples were found to have 3'UTR lengths of 120, 147 and 153 nucleotides. 18% of fully polyadenylated transcripts were found to have 3'UTR lengths of 142 and 158 nucleotides. 24% of samples were found to have 3'UTR lengths of 144 and 152 nucleotides.

B.) A selected sequence (from 2970 to 3173 nucleotides) of the 3'UTR of *niaD* from the *Aspergillus* genome database is shown. The predicted polyadenylation signal for *niaD* (AUUAUA) is highlighted in yellow and the poyladenylation start site of when the 3'UTR length is 144 nucleotides is highlighted in green. The polyadenylation start site of the 3'UTR is 158 nucleotides is highlighted in red.

C.) The distribution of capped transcripts classed as having full 3'UTRs is shown. The percentage of fully polyadenylated capped *niiA* transcripts with different 3'UTR lengths is shown. 6% of fully polyadenylated transcripts were found to have 3'UTR lengths of 129 and 130 nucleotides. 11% of fully polyadenylated transcripts were found to have 3'UTR lengths of 127 and 149 nucleotides. 65% of fully polyadenylated transcripts were found to have 3'UTR lengths of 135 nucleotides.

D.) A selected sequence from (3706 to 3858 nucleotides) of the 3'UTR of *niiA* from the *Aspergillus* genome database is shown. The predicted polyadenylation signals for *niiA* (AUUAUA) are highlighted in yellow and blue. The poyladenylation start site for when the 3'UTR is a length of 120 nucleotides is under lined and highlighted in yellow and the polyadenylation start site for when the 3'UTR is 135 nucleotides is highlighted in green.

3.6 CutA and CutB nucleotidytransferases dependent readenylation of 3' end decay intermediates for *niiA* and *niaD* transcripts.

A current degradation model suggests that the 3' end of adenylated transcripts can be tagged with cytoplasmic non-canonical C/U nucleotides by terminal nucleotidyltrasferase enzymes, namely CutA and CutB in *A. nidulans* (Morozov *et al,* 2010a; Morozov *et al,* 2012). However, *in vitro* data argues that CutA and CutB are able to add adenosine nucleotides to the 3' end of mRNA (Caddick, personal communication). Furthermore, these nucleotidyltrasferases have been associated with readenylating histone mRNA (Morozov and Caddick personal communication). Readenylation may be associated with facilitating 3'-5' degradation for *niiA* and *niaD* in *A. nidulans* (chapter 3.1, 3.3 and 3.4). It was therefore proposed that CutA and CutB may possibly be involved in readenylating *niiA* and *niaD* transcripts. Furthermore, associating these enzymes as being responsible for readenylating *niiA* and *niaD* mRNA, would indicate for this readenylation process is a cytoplasmic mechanism because these enzymes have been found to be predominantly cytoplasmic, although CutB has also been located in the nucleus (Morozov *et al,* 2010a; Morozov *et al,* 2012). To test whether these enzymes are involved in readenylating *niiA* and *niaD* transcripts some Sanger sequencing data for wild-type, Δ*cutA,* Δ*cutB,* and Δ*cutA*Δ*cutB* strains under no nitrogen treatment at T_0 was compared. The Fisher exact chi-squared statistical test was used to determine statistical significance.

For wild-type *niaD* mRNA shown in figure 3.6.1, 3'UTR lengths ranged from 0 (24 nucleotide PCR product) to151 (266 nucleotide PCR product) nucleotides in length. 70% of all wild-type *niaD* transcripts analysed in the wild-type background were found have full 3'UTRs ranging from 107 to 151 nucleotides, with an average length of 145 nucleotides. 30% of the samples were found to have partially degraded 3'UTRs ranging from 0 (15 nucleotide PCR product) to 105 nucleotides. 30% of all *niaD*

transcripts in the wild-type background, found to have partially degraded 3'UTRs, were readenylated. One partially degraded transcript was found to be modified with two uridine nucleotides when the 3'UTR was degraded to 42 nucleotides. For *niaD* transcripts in the Δ*cutA* background, 3'UTR lengths ranged from 0 (19 nucleotide PCR product) to 158 nucleotides. 60% of all *niaD* transcripts in the Δ*cutA* background were found with full 3'UTRs, ranging from 117 to 158 nucleotides with an average of 146 nucleotides in length. Among the 40% of *niaD* transcripts in the Δ*cutA* background with partially degraded 3'UTRs, 6% were found to be readenylated. This was a 24% reduction in the number of transcripts found to be readenylated for *niaD* transcripts in the Δ*cutA* background compared to *niaD* transcripts in the wild-type background, however it was not statistically significant (*p value* = 0.26).

. For *niaD* transcripts in the Δ*cutB* background, 3'UTR lengths ranged from 0 (15 nucleotide PCR product) to 153 nucleotides. 30% of all *niaD* transcripts in the Δ*cutB* background were found with relatively full 3'UTRs ranging from 108 to 153 nucleotides with an average length of 147 nucleotides. 70% of transcripts were found with partially degraded 3'UTRs, with 4% of samples being found to be readenylation. This was a 26% reduction in the number of transcripts being found to be readenylated for *niaD* samples in the Δ*cutB* background compared to wild-type. 2 deadenylated *niaD* transcripts out of 26 in the Δ*cutB* background, with partially degraded 3'UTRs, were found to be modified with uridine nucleotides, similar to what was seen in the wild-type background (1 out of 35 partially degraded transcripts). 3' end modifications with uridine nucleotides could have a function in degrading *niaD* in similar to readenylation. Unlike *niaD* transcripts in the Δ*cutA* background, the increase in the number of *niaD* transcripts in the Δ*cutB* background being found to be partially degraded compared to wild-type was statistically significant (*p value* = 0.0001). Furthermore, the decrease in the percentage of *niaD* transcripts in the Δ*cutB* background found to be readenylated was also statistically significant compared to *niaD* transcript in the wild-type background (*p value* = 0.02). From this data, it appeared that *cutB* was more active in

modifying the 3' end target transcripts for degradation, because the percentage of partially degraded and readenylated mRNA in its absence was significantly increased. For *niaD* mRNA in the Δ*cutA*Δ*cutB* background, 3'UTR lengths ranged from 0 (14 nucleotide PCR product) to 197 nucleotides in length. 42% of all *niaD* transcripts in the Δ*cutA*Δ*cutB* background were found to have relatively full 3'UTRs ranging from 114 to 197 nucleotides and an average length of 144 nucleotides. 58% of samples were found to have partially degraded 3'UTRs with no samples being found to be readenylated. Readenylation was significantly (*p value* = 0.0001) abolished for the *niaD* transcript when both *cutA* and *cutB* enzymes were deleted, arguing that these enzymes readenylate transcripts *in vivo*. It is not clear why the percentage of partially degraded transcripts found for Δ*cutA*Δ*cutB* was significantly greater than for the wild-type transcripts (*p value* = 0.0001) however, this could have been a result of a lack of very active 3'-5' decay pathway. This is because if there were no enzymes involved in actively readenylating any 3' decay intermediates, transcripts would appear partially degraded.

For *niiA* mRNA in the wild-type background, 3'UTR lengths ranged from 9 to 151 nucleotides. 84% of all wild-type transcripts analysed were found to have relatively full 3'UTRs ranging from 95 to 151 nucleotides and having an average of 131 nucleotides. U tags were found on two transcripts with full 3' UTRs (tagged at 3'UTR length of 131 and 127 nucleotides) with two transcripts being tagged when the poly (A) tail was completely degraded and a U tag being found on a sample when the poly (A) tail was degraded to 11 nucleotides. It is not clear what the function of tagging with uridine nucleotides has however, it could have a similar function to readenylation. 16% of *niiA* transcripts in the wild-type background analysed were found to have partially degraded 3'UTRs ranging from 9 to 69 nucleotides. 21% of *niaD* transcripts in the wild-type background, with partially degraded 3'UTRs, were found to be readenylated.

For *niiA* transcripts in the Δ*cutA* background, 3'UTR lengths ranged from 11 to 139 nucleotides. 67% of *niiA* transcripts in the Δ*cutA* background were found to have relatively full 3'UTRs ranging from 114 to 139 nucleotides with an average of 132 nucleotides in length. A single uridine tag was found on a transcript with a long 3'UTR with no poly (A) tail, supporting the possibility that the addition of uridine nucleotides could also be involved in facilitating 3'-5' mRNA decay pathway, in a similar mechanism as readenylation. 33% of *niiA* transcripts in the Δ*cutA* background were found to have partially degraded 3'UTRs ranging from 11 to 48 nucleotides with no samples being found to be readenylated. The number *niiA* transcripts in the Δ*cutA* background found to be partially degraded and readenylated was not significantly greater or less (*p value* = 0.06 and 0.26 respectively) than wild-type samples respectively.

For *niiA* transcripts in the Δ*cutB* background, 3'UTR lengths ranged from 11 to 142 nucleotides. 65% of samples were found with relatively full 3'UTRs ranging from 123 to 142 nucleotides with an average of 131 nucleotides in length. Not RNA samples were tagged with uridine nucleotides or readenylated, similar to the *niiA* transcripts in the Δ*cutA* background. 35% of *niiA* transcripts in the Δ*cutB* background were found to be partially degraded, which was significantly higher (*p value* = 0.04) than *niiA* transcripts in the wild-type background and is also similar to the results found for *niaD* transcripts in the Δ*cutB* background. Furthermore, the decrease of *niiA* transcripts in the Δ*cutB* background being found to be readenylated was significant (*p value* = 0.003) compared to *niaD* transcripts in the wild-type background. For *niiA* it appeared that *cutB* was more active modifying the 3' end of target transcripts compared to *cutA,* in a similar manner as what was found for the *niaD* transcript.

For *niiA* transcripts in the Δ*cutA*Δ*cutB* background, 3'UTR lengths ranged from 9 to 222 nucleotides. 82% of samples were found with long 3'UTRs ranging from 92 to 22 nucleotides, with an average of 133 nucleotides. No *niiA* transcripts in the Δ*cutA*Δ*cutB* background were found to be readenylated (*p value* = 0.02 compared to wild-type

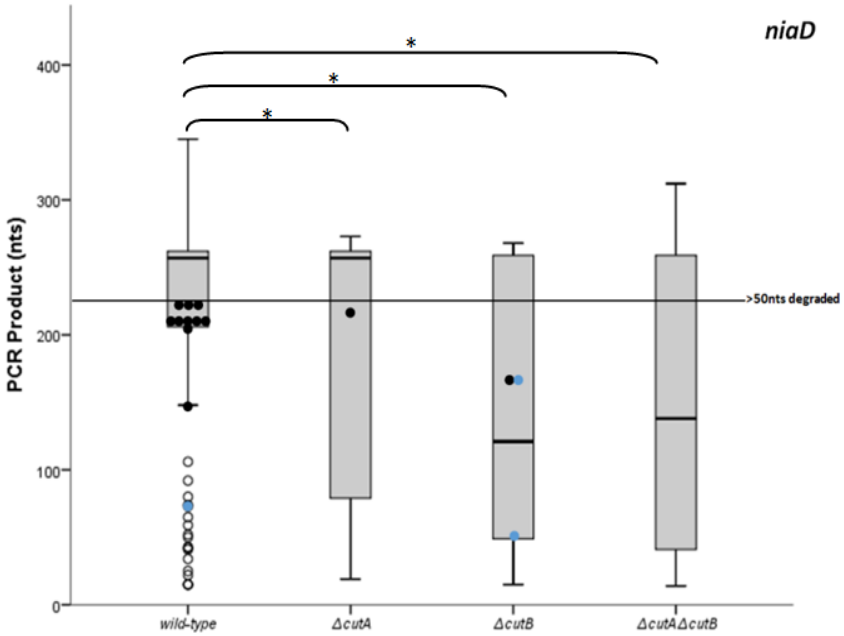
transcripts) or tagged with uridine nucleotides yet again as seen for the *niaD* transcript. These results argue that these enzymes are involved in readenylating *niiA* mRNA as seen for *niaD* transcripts. Furthermore, the percentage of transcripts found with short 3'UTRs was 18%, comparable with the wild-type samples (*p value* = 1).

These results were very interesting because the reduction in the presence of partially degraded mRNA for the *niiA* transcripts in the Δ*cutA*Δ*cutB* background, may be a result of secondary structures (discussed in chapter 4) being present in much longer positions of the 3'UTR. The lack of enzymes present to catalyse the readenylation process at these potential secondary structure sites would halt degradation. This intriguing possibility can be confirmed by the presence of non-templated U nucleotides found on deadenylated transcripts containing long 3'UTRs.

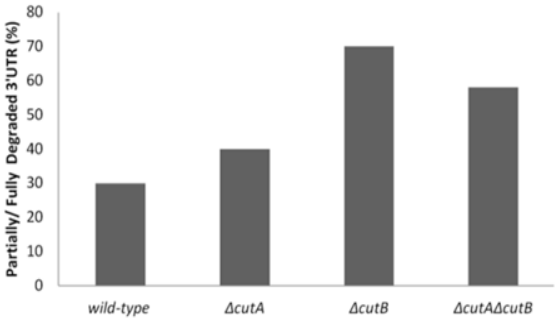
From these results, readenylation of the 3' end of the *niaD* transcript significantly reduced in the strains depleted for either *cutA* or *cutB*. Readenylation was completely abolished for the *niaD* transcript when both the *cutA* and *cutB* genes were deleted. 3' end readenylation of the *niiA* transcript in the Δ*cutA,* Δ*cutB* or Δ*cutA*/Δ*cutB* background was completely abolished. This data argued that these enzymes can readenylate transcripts *in vivo*. Uridine tagging was also found on deadenylated transcripts with both and long and shortened 3'UTRs, implying that the addition of U and not just A nucleotides may facilitate *niiA* and *niaD* transcript degradation. These uridine tags may be catalysed by CutA and CutB as shown previously but with a different function in degrading *niiA* and *niaD* (Morozov *et al*, 2010a; Morozov *et al,* 2012). It was more efficient to detect the addition of uridine nucleotides on transcripts with much longer (<50 nucleotides 3'UTR degradation) 3'UTRs supporting the possibility of secondary structures being present on transcripts which were not classed as being partially degraded, because the 3'UTR lengths were not degraded beyond 50 nucleotides. The ability of CutA and CutB to facilitate 3'-5' decay by readenylation and uridylation was

consistent with the *in vitro* data (unpublished data from Mark Caddick's lab, Liverpool University UK).

A.)



B.)



C.)

(*niaD*) Strain	Total Number of transcripts analysed	Number of transcripts with shortened 3'UTR (>50nts)	3' non-encoded nucleotides	3' non-encoded nucleotides on long 3'UTR
Wild-type	117	35 (30%)	10x 11-44As 1x UU	0
Δ*cutA*	45	17 (40%)	1x A	0
Δ*cutB*	37	26 (70%)	1x A 2x U	0
Δ*cutA*Δ*cutB*	78	46 (58%)	0	0

Figure 3.6.1 Adaptor ligation RT-PCR analysis for decapped and capped *niaD* mRNA in wild-type, Δ*cutA,* Δ*cutB,* and Δ*cutA*Δ*cutB* strains

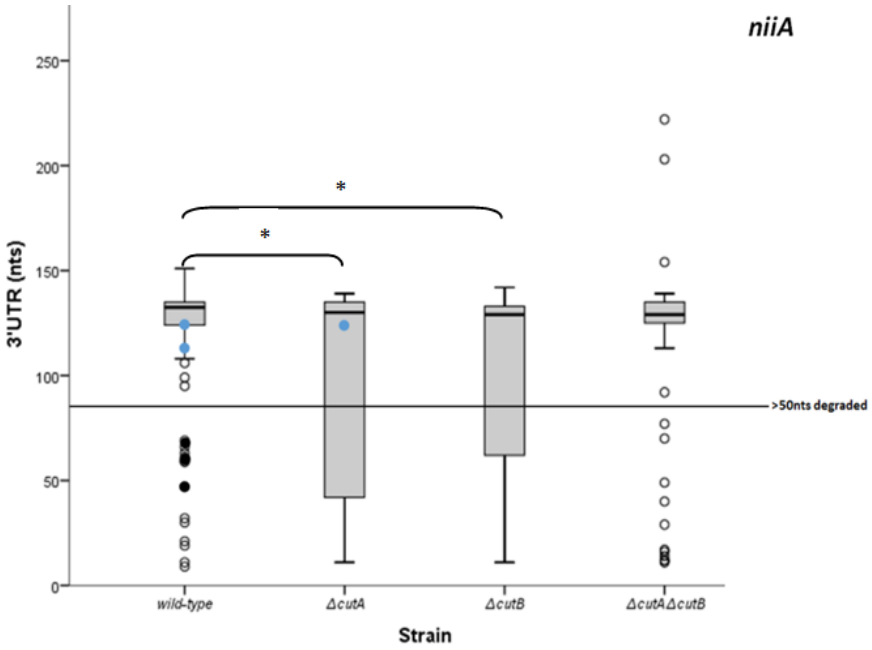
Wild-type, Δ*cutA,* Δ*cutB,* and Δ*cutA*Δ*cutB* strains were treated with proflavin to inhibit transcription as described in chapter 2.1.5. RNA was extracted (chapter 2.2.1) for T_0 samples under nitrogen starvation (-N) and a 5' adenylated adaptor (adaptor sequence: GACTGGAATTCTCGGGTGCCAAGGC) was ligated on to the 3' end of mRNA as described in chapter 2.3.2. The 3'-adaptor primer junction for the *niaD* mRNA was amplified using RT-PCR. PCR products were cloned and sequenced.

A.) The distribution of PCR product lengths with the forward primer (Chapter 2.3.2) being 94 nucleotides upstream from the stop codon, in Wild-type, Δ*cutA,* Δ*cutB,* and Δ*cutA*Δ*cutB* backgrounds, is displayed using box plots where the top and the bottom of the box represents upper and lower quartiles. The median of the boxplot is indicated by the horizontal line which lies within the boxes. All outliers (o) represent transcripts with 3'UTRs degraded beyond 50 nts. Projected on this box plot is the distribution of transcripts which are readenylated (●) and tagged (●) (Figure. 3.6.1a).

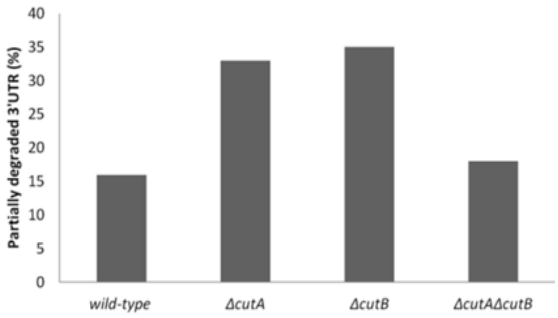
B.) A bar chart showing the percentage of *niaD* transcripts with partially (≥50 nts) degraded 3' UTRs in Wild-type, Δ*cutA,* Δ*cutB,* and Δ*cutA*Δ*cutB* background. The percentage of samples with 3'UTRs being degraded beyond 50 nts is 30% for wild-type, 40% for Δ*cutA* 70% for Δ*cutB* and 58% for Δ*cutA*Δ*cutB*

C.) Figure 3.6.1c shows the number of transcripts analysed, those that have shortened 3'UTRs and those which are readenylated and tagged with U nucleotides in Wild-type, Δ*cutA,* Δ*cutB,* and Δ*cutA*Δ*cutB* background.

A.)



B.)



C.)

(*niiA*) Strain	Total Number of transcripts analysed	Number of transcripts with shortened 3'UTR (>50nts)	3' non-encoded nucleotides	3' non-encoded nucleotides on long 3'UTR
Wild-type	88	14 (16%)	3x 1-18 As	2x U
Δ*cutA*	30	10 (33%)	0	1x U
Δ*cutB*	34	12 (35%)	0	0
Δ*cutA*Δ*cutB*	73	13 (18%)	0	0

Figure 3.6.2 Adaptor ligation RT-PCR analysis for decapped and capped *niiA* mRNA in wild-type, Δ*cutA,* Δ*cutB,* and Δ*cutA*Δ*cutB* strains

Wild-type, Δ*cutA,* Δ*cutB,* and Δ*cutA*Δ*cutB* strains were treated with proflavin to inhibit transcription as described in chapter 2.1.5. RNA was extracted (chapter 2.2.1) for T_0 samples under nitrogen starvation (-N) and a 5' adenylated adaptor (adaptor sequence: GACTGGAATTCTCGGGTGCCAAGGC) was ligated on to the 3' end of mRNA as described in chapter 2.3.2. The 3'-adaptor primer junction for the *niaD* mRNA was amplified using RT-PCR. PCR products were cloned and sequenced.

A.) The distribution of 3'UTR lengths, for Wild-type, Δ*cutA,* Δ*cutB,* and Δ*cutA*Δ*cutB,* is displayed using box plots where the top and the bottom of the box represents upper and lower quartiles. The median of the box plot is indicated by the horizontal line which lies within the boxes. All outliers (o) represent transcripts with 3'UTRs degraded beyond 50 nts. Projected on this box plot is the distribution of transcripts which are readenylated (●) and tagged (●) (Figure. 3.6.1b).

B.) A bar chart showing the percentage of transcripts with partially (≥50 nts) degraded 3' UTRs for Wild-type, Δ*cutA,* Δ*cutB,* and Δ*cutA*Δ*cutB* backgrounds. Percentage of *niiA* transcripts analysed found with partially degraded 3'UTRs are 16% for wild-type, 33% for Δ*cutA* 35% for Δ*cutB* and 18% for Δ*cutA*Δ*cutB.*

C.) Figure 3.6.1c shows the number of transcripts analysed, those that have shortened 3'UTRs and those and which are readenylated and tagged with U nucleotides for Wild-type, Δ*cutA,* Δ*cutB,* and Δ*cutA*Δ*cutB* backgrounds.

3.7 Is 3' mRNA readenylation a default or regulated process facilitating 3'-5' decay?

To determine if readenylation is a regulated process, a time-course degradation analysis was performed under nitrogen starvation, nitrate and glutamine regimes. Chapters 3.4 and 3.6 have demonstrated that readenylation occurs in *A. nidulans* for *niiA* and *niaD* with *cutA* and *cutB* nucleotidyltransferases being involved in the addition of adenosine nucleotides to the 3' end. The readenylation process found in this work appears to be a cytoplasmic process because the enzymes that are involved are mainly cytoplasmic enzymes (Morozov *et al,* 2010a; Morozov *et al,* 2012). In this study, the regulation of 3' readenylation was studied over a 20-minute time course however, not enough sequencing data could be retrieved under nitrate treatment for both *niiA* and *niaD* thus, only results for samples analysed under glutamine treatment and nitrogen starvation are shown in figures 3.7.1 and 3.7.2.

From these results it could not be determined if readenylation is regulated in response to nitrogen availability (glutamine signalled destabilisation and nitrate signalled stabilisation) albeit readenylation was found to occur on different transcripts over the 20-minute time course for *niiA* and *niaD*. The Fisher exact chi-squared statistical test was used to determine if differences in the percentage of partially degraded transcripts and readenylation over the time courses were statistically significant.

Under no nitrogen treatment for *niiA,* the analysis of both capped and decapped mRNA is shown over a 20-minute time course in figure 3.7.1a. The same T_0 transcripts for *niiA* were used for both no nitrogen and glutamine treated samples shown in figure 3.7.1. For T_0, 84% of transcripts were found to have 3'UTR lengths ranging from 95 to 151 nucleotides in length with an average of 131 nucleotides, being consistent with the full 3'UTR length found for capped samples (chapter 3.5) of 135 nucleotides. The poly (A) tail lengths varied for all samples with full 3'UTRs ranging from 0 to 28 nucleotides

with an average of 7 nucleotides. 16% of transcripts analysed at T_0 were found to have partially degraded 3'UTRs with 21% of those samples being found to be readenylated. There appeared to be no hot-spot for readenylation, as this modification occurred at different sites of the 3'UTR.

For transcripts analysed at T_5, 81% of samples were found to have relatively full 3'UTRs ranging from 105 to 135 nucleotides in length with an average of 129 nucleotides. Poly (A) tail lengths ranged from 0 to 21 nucleotides, with an average of 5 nucleotides. The percentage of transcripts analysed at T_5 found to have partially degraded 3'UTRs was 19%; 13% of the partially degraded transcripts were found to be readenylated. The percentage of partially degraded transcripts found at T_5 was not significantly greater compared to transcripts sequenced at T_0 (*p value* = 0.63). Furthermore, although the percentage of readenylated transcripts found for T_5 transcripts compared to T_0 was reduced, this was also not significant (*p value* = 1).

For *niiA* transcripts analysed at T_{20}, 69% of samples were found to have relatively full 3'UTRs, ranging from 110 to 178 nucleotides, with an average of 133 nucleotides. Poly (A) tail lengths ranged from 0 to 32 nucleotides with an average of 4 nucleotides. The percentage of transcripts sequenced at T_{20} found to be partially degraded was 31%; 49% of the partially degraded transcripts were found to be readenylated. The percentage of transcripts found to be partially degraded increased for transcripts sequenced at T_{20} compared to samples sequenced at T_0 and T_5 although this was not a significant increase (*p value* = 0.07 and 0.23 respectively). The percentage of transcripts found to be readenylated also increased for T_{20} samples compared to T_0 and T_5 however, this was also not a significant increase (*p value* = 0.43 and 0.35 respectively).

From these time course degradation results, for *niiA* under nitrogen starvation, the amount of readenylated and partially degraded transcripts remained relatively consistent throughout the time-course because there were no significant differences found.

Under nitrogen sufficient conditions (glutamine), the analysis of capped and decapped mRNA for *niiA* over a 20-minute time course is shown in figure 3.7.1b. The same T_0 samples were used for *niiA,* no nitrogen and glutamine treated samples shown in figure 3.7.1. For *niiA* transcripts analysed at T_0, 84% of transcripts were found to have 3'UTR lengths ranging from 95 to 151 nucleotides in length with an average of 131 nucleotides, being consistent with the full 3'UTR length found for capped samples (chapter 3.5) of 135 nucleotides. Poly (A) tail lengths varied for all transcripts found with full 3'UTRs ranging from 0 to 28 nucleotides, with an average of 7 nucleotides. 16% of transcripts analysed at T_0 were found to have partially degraded 3'UTRs with 21% of those samples being found to be readenylated. There appeared to be no hot-spot for readenylation, as this modification occurred at different sites of the 3'UTR.

For *niiA* transcripts analysed at T_5, 76% of samples were found to have relatively full 3'UTR lengths ranging from 109 to 186 nucleotides, with an average of 133 nucleotides, which was close to the full 3'UTR length found for capped data (chapter 3.5). Poly (A) tail lengths varied for all samples found with full 3'UTRs, ranging from 0 to 54 nucleotides, with an average length of 8 nucleotides. 24% of *niiA* transcripts analysed at T_5 were found to have partially degraded 3'UTRs and 8% of those were to be readenylated. Although there was an increase in the percentage of samples found to be partially degraded for T_5 samples compared to T_0, this was not significant (*p value* = 0.27). There was a decrease in the samples found to be readenylated for T_5 samples compared to T_0 however, this was also not a significant decrease (p value = 0.6), indicating that the level of readenylation remained relatively consistent over the time-course under glutamine treatment.

For *niiA* transcripts analysed at T_{20}, 94% of samples were found to have relatively full 3'UTR lengths ranging from 105 to 149 nucleotides with an average length of 129 nucleotides. Poly (A) tail lengths varied for all samples, ranging from 0 to 33 nucleotides, with an average length of 3 nucleotides. The percentage of partially

degraded transcripts found was 6% and 30% of those were readenylated. There was a significant decrease in percentage of samples found partially degraded for T_{20} samples compared T_5 transcripts (*p value* = 0.02) however, this was not a significant decrease compared to transcripts analysed at T_0 (*p value* = 0.17). Although there was an increase in the percentage of transcripts found to be readenylated for T_{20} samples compared to T_0 and T_5, this was not a significant increase (*p value* = 1 and 0.45 respectively). From these results it appears that level of readenylation did not increase over the 20-minute time-course under glutamine treatment for *niiA,* as there were no significant differences. Therefore, from this data readenylation did not seem to be regulated in response to nitrogen availability for *niiA*. Interestingly, for *niiA,* during nitrogen starvation, there was a gradual increase of percentage of partially degraded transcripts observed (figure 3.7.1). However, under glutamine treatment, for *niiA,* there was a gradual increase of partially degraded transcripts from T_0 to T_5 then a significant decrease from T_5 to T_{20} (figure 3.7.1). This significant decrease in the percentage of partially degraded transcripts under glutamine could be a result of an increased rate of degradation thus, a possible increased rate of readenylation unable to be detected using this analysis approach.

Under no nitrogen treatment for *niaD*, the analysis of both capped and decapped mRNA was done over a 20-minute time course and is shown in figures 3.7.2a and 3.7.2c. The same T_0 samples were used for *niaD*, both no nitrogen and glutamine treated samples shown in figure 3.7.2. For *niaD* transcripts analysed at T_0, 70% of samples were found to have relatively full 3'UTR lengths ranging from 107 to 151 nucleotides with an average of 145 nucleotides similar to the full 3'UTR length found for capped *niaD* mRNA in chapter 3.5. Poly (A) tail lengths varied ranging from 0 to 49 nucleotides, with an average length of 12 nucleotides. 30% of *niaD* transcripts analysed at T_0 were found to be partially degraded, with 29% of those samples being

found to be readenylated. There appeared to be hot spots for readenylation as this modification occurred at specific sites in the 3'UTR.

For *niaD* transcripts sequenced at T_5, 53% of samples were founds to have relatively full 3'UTR lengths ranging from 112 to 178 nucleotides with an average length of 145 nucleotides. Poly (A) tail lengths ranged from 0 to 29 nucleotides, with an average of 7 nucleotides. 47% of T_5 samples were found to have partially degraded 3'UTRs, of different lengths, and 14% of those were found to be readenylated. The percentage of partially degraded transcripts increased for T_5 samples compared to T_0 however, this increase was not significant (*p value* = 0.09). The percentage of transcripts found to be readenylated reduced for T_5 samples compared to T_0 however, this reduction was also not significant (*p value* = 0.47), indicating that the readenylation rate remained consistent for *niaD*. For *niaD* transcripts sequenced at T_{20}, 44% of them were found to have full 3'UTR lengths ranging from 107 to 177 nucleotides with an average length of 135 nucleotides, which was less than the full 3'UTR characterised for capped *niaD* mRNA of 145 nucleotides (chapter 3.5). It is important to note that some of the transcripts that were characterised as having full 3'UTR lengths (3'UTR not degraded beyond 50 nucleotides) could have also been partially degraded particularly because some transcripts were found to either not have poly (A) tails or very short A tails of 1 to 3 nucleotides which could have been degradation intermediates. However, it was difficult to confirm if these short A tails, on 3'UTRs which were not classed as being partially degraded, were degradation intermediates using the adaptor ligation RT-PCR approach, because it was hard to distinguish if these modifications were canonical or non-canonical. 56% of *niaD* transcripts analysed at T_{20} were found to be partially degraded with no samples being found to be readenylated. As seen for *niiA* samples there was a gradual increase over the time course of samples being found to be partially degraded for *niaD* however this increase was not significant *(p value* for T_{20} compared to T_0 and T_5 = 0.01 and 0.61 respectively). However, there was a decrease of transcripts being found to be readenylated for T_{20} samples compared to and T_5 and

this decrease was not significant (*p value* = 0.18). On the other hand, the decrease of readenylated T_{20} transcripts was significantly different to T_0 (p value = 0.02). From these results for *niaD*, it appeared that readenylation significant decreased over the time-course, however it is not clear what these results represented biologically.

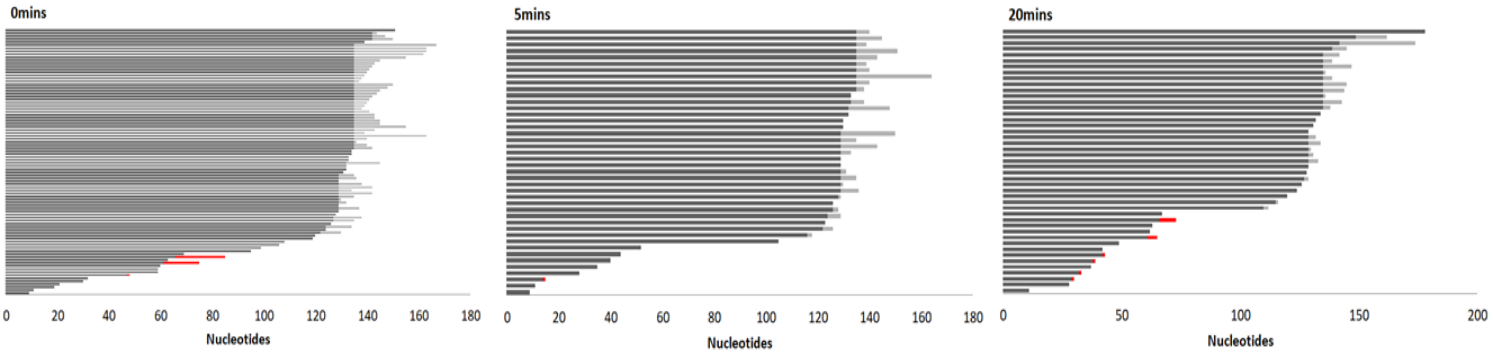
For *niaD* transcripts analysed under glutamine treatment the analysis of both capped and decapped mRNA, is shown in figure 3.7.2b and 3.7.2c over a 20-minute time course. The same T_0 samples were used for *niaD,* both no nitrogen and glutamine treated samples shown in figure 3.7.2. For *niaD* transcripts analysed at T_0, 70% of samples analysed were found to have relatively full 3'UTR lengths ranging from 107 to 151 nucleotides with an average of 145 nucleotides similar to the full 3'UTR length for capped *niaD* mRNA in chapter 3.5. Poly (A) tail lengths varied ranging from 0 to 49 nucleotides, with an average length of 12 nucleotides. 30% of *niaD* transcripts analysed at T_0 were found to be partially degraded, with 29% of those samples being found to be readenylated. There appeared to be hot spots for readenylation as this modification occurred at different sites of the 3'UTR.

For *niaD* transcripts sequenced at T_5, 78% of them were found to have relatively full 3'UTR lengths ranging from 116 to 173 nucleotides, with an average of 147 nucleotides which was similar to the capped *niaD* mRNA analysis (chapter 3.5). Poly (A) tail lengths varied, ranging from 0 to 65 nucleotides, with an average of 22 nucleotides. 22% of *niaD* transcripts analysed at T_5 samples were found to be partially degraded with 13% of them being found to be readenylated. Although there was a decrease in the percentage of transcripts being found to be partially degraded for T_5 samples compared to T_0, it was not significant (*p value* = 0.40). The decrease in readenylation was also not significant (*p value* = 0.66) for T_5 samples compared to T_0, indicating that this modification remained relatively consistent over the time-course.

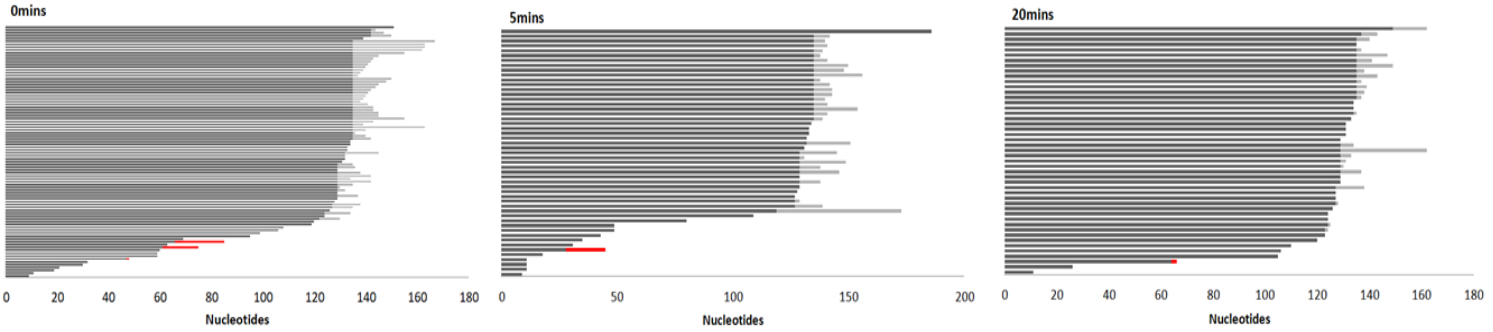
For *niaD* transcripts sequenced at T_{20}, 55% of them were found to have relatively full 3'UTR lengths ranging from 115 to 194 nucleotides with an average of 145 nucleotides,

similar to the capped *niaD* mRNA analysis (chapter 3.5). Poly (A) tail lengths remained relatively short ranging from 0 to 11 nucleotides, with an average of 5 nucleotides. The percentage of T_{20} samples found to be partially degraded was 45% with 6% of those being found to be readenylated. Although the percentage of transcripts being found to be readenylated decreased for transcripts sequenced at T_{20} compared to T_0 and T_5, this was not a significant reduction (p value = 0.14 and 1 respectively). Also, there was a significant increase in the percentage of transcripts being found to be partially degraded for transcripts sequenced at T_{20} compared to T_5 (p value = 0.03). This increase in the percentage of transcripts being found to be partially degraded for *niaD,* under glutamine treatment could be a result of an increased rate of degradation therefore a higher number of degradation intermediates would be found. From these findings readenylation did not seem to be regulated in response to nitrogen availability for both *niiA* and *niaD,* as it was very difficult to make any conclusions with this data. However, these findings further support that readenylation is involved in facilitating 3'-5' transcript degradation, possibility as a default process.

A.)


0mins
0 20 40 60 80 100 120 140 160 180
Nucleotides
5mins
0 20 40 60 80 100 120 140 160 180
Nucleotides
20mins
0 50 100 150 200
Nucleotides

B.)


0mins
0 20 40 60 80 100 120 140 160 180
Nucleotides
5mins
0 50 100 150 200
Nucleotides
20mins
0 20 40 60 80 100 120 140 160 180
Nucleotides

C.)

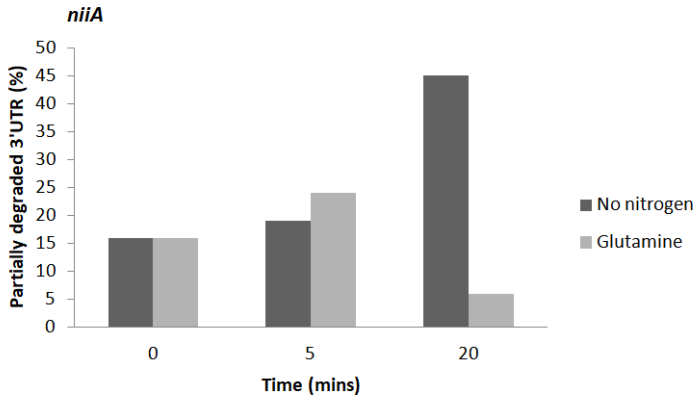

niiA
Partially degraded 3'UTR (%)
50
45
40
35
30
25
20
15
10
5
0
0
5
20
Time (mins)
No nitrogen
Glutamine

Figure 3.7.1 Adaptor ligation RT-PCR analysis of natively decapped and capped *niiA* mRNA under nitrogen starvation (a) and glutamine (b) regimes and a graph to show percentage of partially degraded transcripts (c) (degradation beyond 50 nucleotides), all in the wild-type background over a 20-minute time course

Cultures were treated with proflavin to inhibit transcription prior to time course degradation analysis as described in chapter 2.1.5. The required nitrogen source was added at T_0 in the amounts described in chapter 2.1.5. Samples were harvested at T_0, T_5, and T_{20} for RNA extraction. RNA was extracted as described in chapter 2.2.1, an adenylated adaptor was ligated on to the 3' end of mRNA is described in chapter 2.3.2 and the 3' junction of *niiA* mRNA was amplified using PCR. PCR products were cloned and sequenced. The results displayed in figures 3.7.1a and 3.7.1b indicate the length of 3'UTR (dark grey lines), poly/ oligo (A) sequences (light grey lines) and those classed as readenylation (red lines) as they are poly/ oligo (A) sequences found on partially degraded 3'UTRs (>50 nucleotides degradation). 0 is 1+ downstream from the stop codon. Figure 3.7.1c represents the percentage of partially degraded transcripts analysed over the time course for both glutamine treated samples and those that were not treated with a nitrogen source.

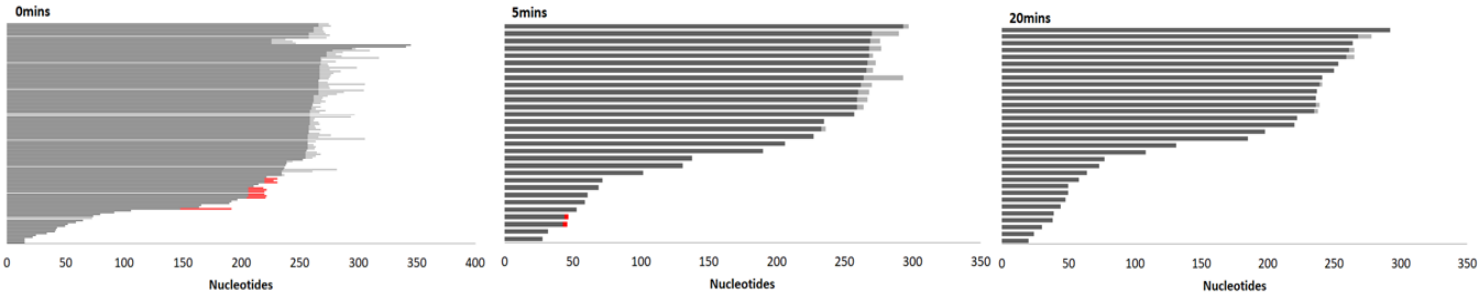
A.) Adaptor ligation RT-PCR results are shown for *niiA* under no nitrogen treatment at T_0, T_5 and T_{20}. At T_0, 88 samples were able to be successfully sequenced and analysed. 3'UTR lengths ranged from 9 to 151 nucleotides and poly/ oligo (A) tails ranged from 0 to 28 nucleotides in length, with 3 samples appearing to be readenylated with 1 to 19 nucleotides. At T_5, 42 samples were able to be successfully sequenced and analysed. 3'UTR lengths ranged from 9 to 135 nucleotides and poly/ oligo (A) tails ranged from 0 to 29 nucleotides, with 1 sample appearing to be readenylated with 1 nucleotide. At T_{20}, 45 samples were successfully sequenced and analysed. 3'UTR lengths ranged from 11 to 178 nucleotides and poly/ oligo (A) tail

lengths ranged from 0 to 32 nucleotides, with 6 samples appearing to be readenylated with 1 to 7 nucleotides.

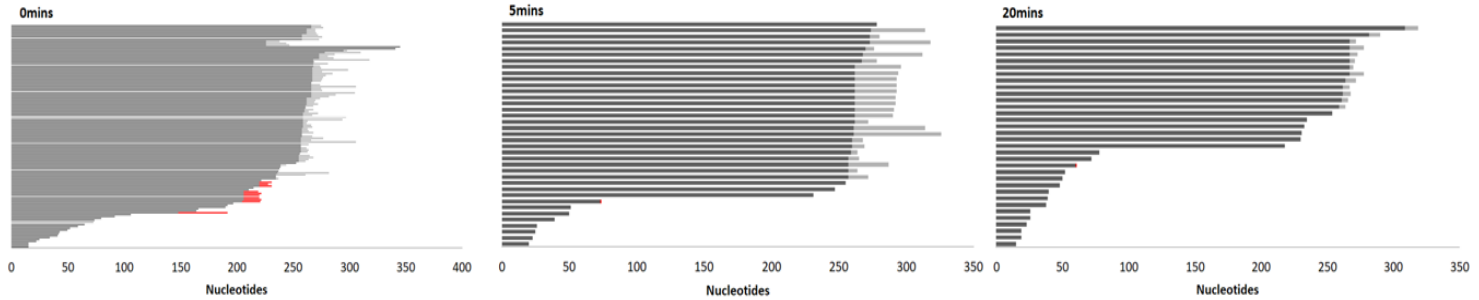
B.) Adaptor ligation RT-PCR results are shown for *niiA* under glutamine treatment at T_0, T_5 and T_{20}. At T_0, 88 samples were able to be successfully sequenced and analysed. 3'UTR lengths ranged from 9 to 151 nucleotide and poly/ oligo (A) tails ranged from 0 to 28 nucleotides in length, with 3 samples appearing to be readenylated with 1 to 19 nucleotides (>50 3'UTR nucleotide degradation). At T_5, 51 samples were able to be successfully sequenced and analysed. 3'UTR lengths ranged from 9 to 186 nucleotides and poly/ oligo (A) tail lengths ranged from 0 to 54 nucleotides with 1 sample appearing to be readenylated with 28 nucleotides. At T_{20}, 47 samples were able to be successfully sequenced and analysed. 3'UTR lengths ranged from 11 to 149 nucleotides and poly/ oligo (A) tails ranged from 0 to 33 nucleotides, with one sample appearing to be readenylated with 2 nucleotides.

C.) Figure shows percentage of *niiA* transcripts found with shortened 3'UTRs from figure 3.7.1a and 3.7.1b for T_0, T_5 and T_{20}. For samples not treated with a nitrogen source percentage of partially degraded transcripts was 16% at T_0, 19% at T_5, and 31% at T_{20}. For samples treated with glutamine, the percentage of transcripts found with partially degraded 3'UTRs was 16% at T_0, 24% at T_5 and 6% at T_{20}.

A.)


0mins
5mins
20mins
0 50 100 150 200 250 300 350 400
0 50 100 150 200 250 300 350
0 50 100 150 200 250 300 350
Nucleotides
Nucleotides
Nucleotides

B.)


0mins
5mins
20mins
0 50 100 150 200 250 300 350 400
0 50 100 150 200 250 300 350
0 50 100 150 200 250 300 350
Nucleotides
Nucleotides
Nucleotides

C.)

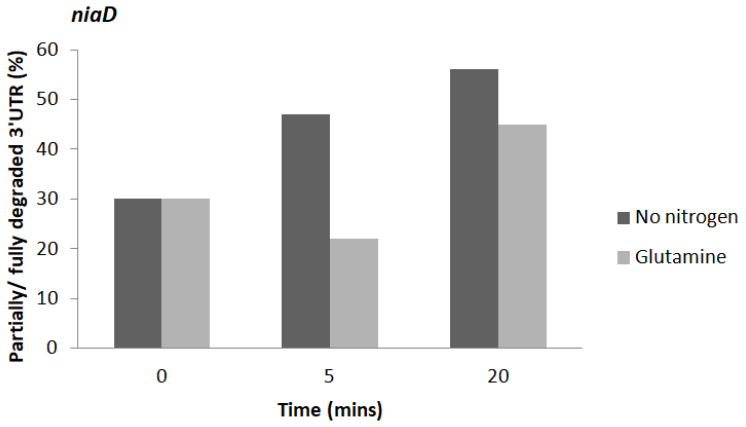

niaD
Partially/ fully degraded 3'UTR (%)
60
50
40
30
20
10
0
0
5
20
Time (mins)
No nitrogen
Glutamine

Figure 3.7.2 Adaptor ligation RT-PCR analysis of decapped and capped *niaD* mRNA under nitrogen starvation (a), glutamine treatment (b) and a graph to show percentage of partially degraded transcripts (c) (degradation beyond 50 nucleotides), all in the wild-type background over a 20 minute time course.

Cultures were treated with proflavin to inhibit transcription as described in chapter 2.1.5 prior to time course degradation analysis. The required nitrogen source was added at T_0 in the amounts described in chapter 2.1.5. Samples were harvested at T_0, T_5, and T_{20} for RNA extraction. RNA was extracted as described in chapter 2.2.1, an adenylated adaptor was ligated on to the 3' end of mRNA is described in chapter 2.3.2 and the 3' junction of *niiA* mRNA was amplified using PCR. PCR products were cloned and sequenced. The results displayed in figures 3.7.1a and 3.7.1b indicate the length of 3'UTR (dark grey lines), poly/ oligo (A) sequences (light grey lines) and those classed as readenylation (red lines) as they are poly/ oligo (A) sequences found on partially degraded 3'UTRs (>50 nucleotides degradation). 0 is 1+ downstream from the stop codon. Figure 3.7.1c represents the percentage of partially degraded transcripts analysed over the time course for both glutamine treated samples and those that were not treated with a nitrogen source.

A.) Adaptor ligation RT-PCR results are shown for *niaD* under no nitrogen treatment at T_0, T_5 and T_{20}. At T_0, 117 samples were able to be successfully sequenced and analysed. 3'UTR lengths ranged from 0 (15 nucleotide sized PCR product) to 151 nucleotides (266 nucleotide sized PCR product) and poly/ oligo (A) tail lengths ranged from 0 to 40 nucleotides, with 10 samples appearing to be readenylated with 8 to 44 nucleotides. At T_5, 30 samples were able to be successfully sequenced and analysed. 3'UTR lengths ranged from 0 (28 nucleotide sized PCR product) to 178 nucleotides (293 nucleotide sized

PCR product) and poly/ oligo (A) tail lengths ranged from 0 to 29 nucleotides, with 2 samples appearing to be readenylated with 3 nucleotides. At T_{20}, 32 samples were able to be successfully sequenced and analysed. 3'UTR lengths ranged from 0 (20 nucleotide sized PCR product) to 177 nucleotides (292 nucleotide sized PCR product) and poly/ oligo (A) tail lengths ranged from 0 to 10 nucleotides.

B.) Adaptor ligation RT-PCR results are shown for *niaD* under glutamine treatment at T_0, T_5 and T_{20}. At T_0, 117 samples were able to be successfully sequenced and analysed. 3'UTR lengths ranged from 0 (15 nucleotide sized PCR product) to 151 nucleotides (266 nucleotide sized PCR product) and poly/ oligo (A) tail lengths ranged from 0 to 40 nucleotides, with 10 samples appearing to be readenylated with 8 to 44 nucleotides. At T_5, 37 samples were successfully sequenced and analysed. 3'UTR lengths ranged from 0 (20 nucleotide sized PCR product) to 163 nucleotides (178 nucleotide sized PCR product) and poly/ oligo (A) tail lengths ranged from 0 to 65 nucleotides, with 1 sample appearing to be readenylated with 1 nucleotide. At T_{20}, 34 samples were successfully sequenced and analysed. 3'UTR lengths ranged from 0 (15 nucleotide sized PCR product) to 194 nucleotides (309 nucleotide sized PCR product) and poly/ oligo (A) tail lengths ranged from 0 to 11 nucleotides, with 1 sample appearing to be readenylated with 1 nucleotide.

C.) Figure shows percentage of *niaD* transcripts found with shortened 3'UTRs from figure 3.7.2a and 3.7.2b at T_0, T_5 and T_{20}. For samples not treated with a nitrogen source, the percentage of partially degraded transcripts was 30% at T_0, 47% at T_5, and 56% at T_{20}. For samples treated with glutamine, the percentage of transcripts found with partially degraded 3'UTRs was 30% at T_0, 22% at T_5 and 45% at T_{20}.

4. Discussion

This thesis details a number of studies aimed at determining the role of readenylation in facilitating 3'-5' mRNA decay in *A. nidulans* and what enzymes are involved catalysing this mechanism. This work has involved a range of approaches including circularisation RT-PCR, adaptor ligation RT-PCR, qRT-PCR and Sanger sequencing in *A. nidulans*. In this work, readenylation was found to be involved in facilitating 3'-5' mRNA decay for *niiA* and *niaD*. CutA and CutB nucleotidyltransferases were characterised as being the primary candidates for catalysing readenylation for *niiA* and *niaD.* It has been proposed based on structural predictions (Figure 4.4.1), that readenylation may be involved in helping to overcome potential stable secondary structures to facilitate 3'-5' degradation, which appears to be a conserved mechanism across different kingdoms of life.

4.1 Readenylation in *A. nidulans* facilitates 3'-5' mRNA decay

Readenylation was initially identified in bacteria, were it was found to be associated with mRNA degradation (Coburn *et al,* 1996; Hajnsdorf *et al,* 1995; O'Hara *et al,* 1995; Xu and Cohen 1995; Cao and Sakar 1992; Cao *et al,* 1995; Haugel-Nielsen *et al,* 1996; Xu *et al,* 1996). Post transcriptional addition of short A tails (around 5 nucleotides) were associated with 3'-5' degradation of bacterial mRNA by poly (A) polymerase 1 and 2. Readenylation in bacteria was shown to help overcome the conserved the 3' end stem-loop structure of bacterial mRNAs, which is formed as during the ρ-independent transcription termination process (Cao and Sakar 1992; Cao *et al,* 1996; Hajnsdorf *et al,* 1995; Haugel-Nielsen *et al,* 1996; O'Hara *et al,* 1995; Xu and Cohen 1995). This stem-loop structure was found to block the access of 3'-5' exoribonucleases with single-strand specificity, which degrade one-two nucleotides at a time (PAP1 and PAP2) (Perwez and Kushner 2006). Thus, readenylation of the 3' end of the stem-loop structure provided access for these enzymes to degrade target mRNA in the 3' to 5' direction (O'Hara *et al,* 1995; Mohanty and Kushner 1999; Cao and Sarkar 1992).

Multiple rounds of readenylation were found to be required to degrade this conserved stem-loop structure (Dreyfus and Regnier 2002). It is important to note that in bacteria there is no 5'-3' exonuclease (a homologue of the eukaryotic *xrn1*) thus, mRNA can only be degraded through the 3'-5' decay pathway. A similar mechanism was found to occur during the degradation of human cell cycle regulated histone mRNA at the end of S-phase, in the cytoplasm (Mullen and Marzluff 2008; Lim *et al,* 2014). Multiple rounds of tagging with uridine nucleotides instead of adenosines, were found to be required to degrade the 3' end stem-loop structure, a mechanism which resembles the bacterial degradation of ρ-dependent mRNAs (Lim *et al,* 2014). Similar to bacteria, each cycle of tagging and degradation was found to remove one or two nucleotides from the 3' end of the histone mRNA stem-loop which ultimately led to the destruction

of the stem-loop and subsequent degradation (Slevin *et al,* 2014). TUTase 4 and 7 were found to be the main enzymes involved in this tagging process in histones and they belong to the nucleotidyltransferase family (Schmidt *et al,* 2011; Lim *et al,* 2014; Lackey *et al,* 2016). Later, cytoplasmic readenylation was found to occur during the 3'-5' degradation of *MAA7* mRNA in *C. reinhardtii*, human HeLa cells and in Drosophila *S2* cells (Ibrahim *et al,* 2006; Slomovic *et al,* 2010; Harnisch *et al,* 2016). However, the biological relevance of readenylation has not yet been fully characterised in eukaryotes.

In efforts to study the regulation of C/U tagging in *A. nidulans*, using nitrogen metabolism as a model system, no C/U tagging was found in all (*niiA, niaD, meaA and areA*) transcripts analysed but instead readenylation was observed. In this work readenylation appeared to be involved in facilitating the 3'-5' decay pathway for *niiA* and *niaD* mRNA. 3' end sequences of the *niiA* and *niaD* mRNA were analysed by 3' end adapter ligation (Thomas *et al,* 2015). For *niaD*, 30% of transcripts analysed in the wild-type background were found to have partially degraded 3'UTRs with 30% of those were found to be readenylated (chapter 3.4). Some transcripts were readenylated with relatively long poly (A) tails (>15 nucleotides) and it is not clear why such long stretches of adenosine nucleotides were required for readenylation. For *niiA,* 20% of transcripts analysed in the wild-type background were found to have partially degraded 3'UTRs with 33% of those being found to be readenylated (chapter 3.4). As seen for *niaD* transcripts, a diverse length of stretches of adenosine nucleotides was detected on the 3' end of *niiA* mRNA, with adenosine stretches ranging from 1 to 55 nucleotides. From published data for eukaryotes it appeared that only short stretches of adenosine nucleotides of around 1-5 nucleotides were required for readenylation (Ibrahim *et al,* 2006; Slomovic *et al,* 2010; Harnisch *et al,* 2016). It was postulated that this short stretch of adenosine nucleotides would most likely be important in preventing PABP binding thus, promoting translation of target transcripts. However, in *A. nidulans*, it

appears that this trend of short stretches of adenosine nucleotides to facilitate 3'-5' mRNA degradation is not always applicable. It seems that a much longer stretches of adenosine nucleotides can be used for readenylation in *A. nidulans*. The length of oligo (A) tail added in the cytoplasm may depend upon the properties of the *Aspergillus* 3'-5' exonuclease, such as homologues to rrp44/Dis3l which are shown to degrade 3' U-tags in mammals (Chang *et al*, 2014). Understanding the detail mechanism of the removal of non-canonical oligo (A) sequences and characterisation of their properties may help to explain the length of the readenylated sequences. Hot spots for readenylation were found for *niaD* when the 3'UTR was degraded to 91 and 105 nucleotides, which could be indicative of 3' secondary structures being present at these sites of readenylation. Readenylation, for *niaD* and *niiA*, may be helping to overcome 3' secondary structures and by providing a single stranded platform for the exosome to bind, as seen for bacteria and histone mRNA (discussed further in chapter 4.4) (Mullen and Marzluff 2008; Cao and Sakar 1992; Cao *et al,* 1996; Hajnsdorf *et al,* 1995; Haugel-Nielsen *et al,* 1996; O'Hara *et al,* 1995; Xu and Cohen 1995).

4.2 Alternative polyadenylation start sites identified for *niiA* and *niaD*

The 3' end of eukaryotic transcripts can be diverse in length; having a wide range within 50 nucleotides. Interestingly, different polyadenylation start sites were found for both *niiA* and *niaD* in this work. The major polyadenylation starts for both these genes was mapped by analysing capped polyadenylated transcripts and predicting the position of the polyadenylation signal (AUUAUA). The polyadenylation signal for *niaD* and *niiA* was predicted based on different polyadenylation signals found in the closely related filamentous fungus, *A. orzyae* (Tanaka *et al,* 2011). Polyadenylation signals have not been extensively studied in filamentous fungi however, it was determined in *A. orzyae* that the major polyadenylation signal, found in around 50% of mammals (AAUAAA), does not occur as frequently in (Tanaka *et al,* 2011).

Many potential polyadenylation signals were identified to occur in different genes in *A. orzyae* which were similar to the polyadenylation signal that was identified for *niaD* and *niiA* (chapter 3.5). The major polyadenylation start site for *niaD* was found to be between 142 and 158 nucleotides downstream from the stop codon (Chapter 3.5). The published 3'UTR for the *niaD* transcript is 200 nucleotides downstream from the stop codon, while the 3'UTR length found in this work was at least 48 nucleotides shorter than the published length (Aspergillus genome database 2018).

The discrepancies in the length of the 3'UTR may be a result of the different methodological approaches used to the study changes happening in the 3' end. The published data was obtained through the 3' end RACE approach which can detect the major 3'UTR length. In this work a more sensitive technique, namely cRT-PCR and adaptor-ligation RT-PCR, which can amplify low abundant transcripts converted into cDNA was used. The position of the major polyadenylation start site found in this work was supported by the predicted polyadenylation signal (AUUAUA) for *niaD*, which was between 24-32 nucleotides upstream from the polyadenylation start site. The distance of the polyadenylation signal to the polyadenylation start site correlated with the

published distance of around 10 to 35 nucleotides, found in mammals (Beaudoing *et al,* 2000; Venkataraman *et al,* 2005; Millevoi *et al,* 2006). The major polyadenylation start site for *niiA* was found to be at 135 nucleotides downstream from the stop codon (Chapter 3.5), which was 15 nucleotides longer than the published 3'UTR length of 120 nucleotides (Aspergillus genome database 2018). The predicted polyadenylation signal found for *niiA* was the same as for *niaD* (AUUAUA), which was 11 nucleotides upstream from the polyadenylation start site of 135 nucleotides. Interestingly the same polyadenylation signal (AUUAUA) sequence for *niiA* was found 43 nucleotides upstream from the polyadenylation start site of 135 nucleotides; this was a greater distance than the published polyadenylation signal distance from the polyadenylation start site. However, this second polyadenylation signal was only 28 nucleotides upstream from the published 3'UTR length for *niiA* of 120 nucleotides, which is within the published distance for the polyadenylation signal (Beaudoing *et al,* 2000; Venkataraman *et al,* 2005; Millevoi *et al,* 2006). It is possible that *niiA* may have two polyadenylation signals, thus meaning that this gene could have two polyadenylation start sites, which is known to be a regulatory mechanism of mRNA stability in higher eukaryotes (Shen *et al,* 2008; Tian *et al,* 2005; Danckwardt *et al,* 2008).

The ability for a gene to have alternative polyadenylation start sites has been extensively studied in humans and mice. In a large-scale analysis of human and mouse genes, alternative polyadenylation start sites were identified in around 54% and 32% of transcripts respectively (Tian *et al,* 2005). Alternative polyadenylation start sites have not been well studied in filamentous fungi however, it has been shown in *A. orzyae* that some genes have alternative polyadenylation start sites (Tanaka *et al,* 2011). The alternative polyadenylation start sites found in *A. orzyae* are at least 30 nucleotides apart, similar to *niaD* (Tanaka *et al,* 2011). Therefore, it could be possible that *niiA* and *niaD* contain alternative polyadenylation start sites in *A. nidulans*. It is unclear why alternative 3'UTRs have evolved although, it has been postulated that because 3'UTRs mediate RNA-protein interactions, alternative 3'UTRs may function

in facilitating the formation of alternative 3'UTR RNP complexes which can be a regulatory mechanism to regulate mRNA stability in response to different signals, such as nitrogen availability in *A. nidulans* (e.g. Caddick *et al,* 2006) and possibly translation. It has been found that around 15-30% of alternative 3'UTRs have significantly different half-lives in humans and mice, which could contribute to the transcriptome diversity in single cells (Tian *et al,* 2005). It would be interesting to further identify the presence of alternative polyadenylation start sites on a large scale in *A. nidulans* and characterise their function.

4.3 CutA and CutB nucleotidyltransferases are responsible for readenylation in *A. nidulans*

CutA and CutB are Cid1 related enzymes that belong to the second sub-group of the nucleotidyltransferase family, comprising of a variety of non-canonical enzymes which are able to target diverse RNA substrates. This group of enzymes is known have the same catalytic domain as canonical rNTrs although, they contain a different nucleotide base-recognition motif (Martin and Keller 2007). The current accepted function for this group of enzymes is in catalysing the addition of C/U nucleotides to the 3' end of polyadenylated and non-polyadenylated eukaryotic coding and non-coding RNA, to facilitate degradation and maturation of ncRNA as well as degradation and translation termination of mRNAs (reviewed by Munoz-Tello *et al*, 2015). In *A. nidulans*, 3' end tagging by CutA and CutB was found to occur when the poly (A) tail was degraded to around 15 nucleotides (Morozov *et al,* 2010a; Morozov *et al,* 2012). This tagging was found to be a conserved eukaryotic mechanism (with the exception of *S. cerevisiae*) that precedes decapping thus, triggering 5'-3' degradation (Morozov *et al,* 2010a). These findings in *A. nidulans* and other eukaryotes led to the current accepted degradation model, which suggests that tagging increases the affinity of the Lsm1-7 complex which recruits Pat1 and stimulates decapping, thus repressing translation and activating subsequent degradation (Rissland and Norbury 2009; Mullen and Marzluff 2008; Morozov *et al,* 2010a; Morozov *et al,* 2012; Lim *et al,* 2014; Chang *et al,* 2014; Nissan *et al,* 2010). Tagging with C/U nucleotides in *A. nidulans* was also associated with dissociation of transcripts containing premature stop codons from translating ribosomes, during NMD (Morozov *et al,* 2012). When studying the regulation of tagging in *A. nidulans,* using genes involved in nitrogen metabolism as a model system, no tagging was found but instead what appeared to be cytoplasmic readenylation (Chapter 3.4). We proposed that this readenylation process was being catalysed by CutA and CutB, based on some *in vitro* data which argued that both of these enzymes

can add adenosine nucleotides to the 3' end of mRNA (unpublished data). To test this, strains deleted for either or both *cutA* and *cutB* were analysed using adaptor ligation RT-PCR (Δ*cutA,* Δ*cutB,* Δ*cutA/*Δ*cutB)*.

Readenylation on the 3' end of *niaD* was reduced in the strains depleted for CutA or CutB compared to wild-type. No readenylated transcripts were found for the *niiA* transcript in Δ*cutA* or Δ*cutB* strains. In the absence of both CutA and CutB readenylation was abolished for both *niiA* and *niaD* arguing that these enzymes can readenylate transcripts *in vivo* and work together (*p values* = 0.0001 for *niaD* and 0.025 for *niiA*). Interestingly, 3' end uridylation of transcripts with partially degraded 3'UTRs, lacking A tails, was found to occur in strains depleted of CutB for the *niaD* transcript. This addition of uridine nucleotides appeared to function in a similar mechanism as readenylation and was most likely catalysed by CutA and CutB because, once these enzymes were deleted, no uridylated transcripts were found for *niaD*.

From these findings it appeared that both the CutA and CutB enzymes were able to add both uridine and adenosine nucleotides to facilitate 3'-5' decay by readenylation and uridylation, which was consistent with the *in vitro* data (unpublished data).

For *niiA*, 3' end readenylation in Δ*cutA,* Δ*cutB* or Δ*cutA/*Δ*cutB* mutants was completely abolished compared to wild-type transcripts, in support of these nucleotidyltrasferases being involved in catalysing this readenylation process. Interestingly, in strains depleted of either *cutA* or *cutB*, readenylation was completely abolished which could be indicative of these enzymes working together during the degradation of *niiA* mRNA. On the other hand, possibly the method of analysis used in this work may not have been sensitive enough to analyse all readenylated transcripts especially when either *cutA* or *cutB* were deleted, because the readenylation process would have been depleted. Interestingly, U tags were found for *niiA* in both wild-type and Δ*cutA* backgrounds with full length 3'UTRs. Some of these uridine tags were on transcripts without any adenosine nucleotides (Chapter 3.6). The uridine tagging process, like for

niaD, may be facilitating 3'-5' degradation in a similar mechanism as readenylation. Also, these uridine tags were found on transcripts classed as not being partially degraded, indicating that even these essentially intact transcripts could have been readenylated. This is because the same sites within the 3'UTR found to be tagged with uridine nucleotides were also the sites found with short stretches of adenosine nucleotides, albeit not classed as readenylation. This uridine tagging for *niiA* was found to also be completely abolished in the absence of both *cutA* and *cutB*, being consistent with the involvement of these enzymes in C/U tagging.

The novel substrate specificity for CutA and CutB in adding adenosine, cytosine and uridine nucleotides has not yet been fully elucidated. However, several crystal structures of Cid1 have provided insight into the catalytic mechanism for adding uridine nucleotides in *S. pombe* (Lunde *et al,* 2012; Munoz-Tello *et al,* 2012; Yates *et al,* 2012; Munoz-Tello *et al,* 2014; Yates *et al,* 2015). Studies have shown that, within the nucleotide recognition motif (NRM) of Cid1, a hydrogen bond formed between a histidine, H336 and the nucleobase is crucial for UTP selectivity (Lunde *et al,* 2012; Munoz-Tello *et al,* 2012; Yates *et al,* 2012). In fact, Cid1's specificity towards UTP was found to be 30-fold higher than towards ATP within this special histidine in its NRM (Lunde *et al*, 2012; Yates *et al,* 2012). It was shown that substituting the H336 with Asp or Ala led to a significant increase in the ability for Cid1 to utilise ATP as a substrate (Lunde *et al,* 2012; Yates *et al,* 2012). Biochemistry studies have not yet been undertaken for CutA or CutB to determine the substrate specificity of these enzymes in the addition of A/U/C nucleotides. It is possible that different unidentified isoforms of CutA and CutB may exist in *A. nidulans,* which have different NRM and are able to add either A/U/C nucleotides depending on which isoform is recruited. In support of this possibility a recent study demonstrated that a CutA protein which had specificity towards CTP could be partially changed to have PAP or PUP specificity by rational mutagenesis within the NRM (Kobylecki *et al,* 2017). It was also suggested in

this study that a short cluster of amino acids within the NRM is most likely to be the main determinant for nucleotidyltransferase substrate preference allowing for the prediction of their specificity (Kobylecki *et al,* 2017). Another possibility for differences in substrate specificities for these enzymes could be dependent upon the degradation pathway being used for decay; other proteins or ncRNAs (trans-acting factors) may influence CutA and/ or CutB's specificity (structure).

In this work it was found that CutA and CutB are involved in readenylating *niiA* and *niaD* transcripts. In a similar mechanism, these enzymes are able to modify the 3' end of degrading transcripts with uridine nucleotides thus, implicating these modifications in also facilitating 3'-5' degradation. It would be interesting to further explore the nucleotide specificity of these enzymes and why they use different nucleotides to modify 3' ends of target transcripts.

4.4 Readenylation by CutA and CutB helps to overcome 3' secondary structures in *A. nidulans*.

Unlike DNA, mRNA does not have a complementary strand, but left in its natural environment, it tend to fold into intricate 3 dimensional structures. These structures are determined by specific base paring mechanisms encoded within the primary sequence, leading to the formation of secondary structures (Buratti *et al*, 2004; Brierley *et al,* 2007; Cooper *et al*, 2009; Cruz and Westhof 2009; Sharp 2009). It is thought that the main function of the formation of these secondary structures is in the recruitment of different proteins, which form various RNP structures (Mitchell and Parker 2014).

Most information regarding secondary structures is known for non-coding RNA. For example, rRNA must form intricate secondary structures to enable formation of proper functional ribosomes (Trappl and Polacek 2011). With mRNA, studies have suggested that eukaryotic pre-mRNA molecules need to fold into precise secondary structures during the mRNA maturation process (i.e. splicing and polyadenylation) (Buratti *et al,* 2004; Cooper *et al,* 2009; Cruz and Westhof 2009). Work in *S. cerevisiae* showed a higher percentage of secondary structures in coding regions compared to untranslated regions within mRNA but, the reason for this remains predominately unknown (Kertesz *et al,* 2010). Studies in plants (Arabidopsis) demonstrated that increasing the presence of secondary structures *in vitro*, reduced the abundance of the mRNA (Goodarzi *et al,* 2012). Thus, the same mRNA with a low level of secondary structures was found to be more abundant within the transcriptome (Goodarzi *et al,* 2012).

This reduction in abundance with an increase of secondary structure within a transcript was confirmed by qRT-PCR, where highly folded mRNAs were found to be degraded more frequently than less structured transcripts (Goodarzi *et al,* 2012). Work in Arabidopsis suggested that RNA secondary structures had an intrinsically destabilising function for mRNA (Goodarzi *et al,* 2012). The mechanisms by which these secondary

structures are being degraded has not been as well studied in eukaryotes as it has been in bacteria.

In this work, readenylation in *A. nidulans* was found to be associated with 3'-5' degradation. Multiple rounds of readenylation during the degradation of target transcripts in *A. nidulans* were found to be required in facilitating 3'-5' mRNA decay. We postulated that this readenylation process in *A. nidulans* is required to help overcome any 3' secondary structures within the targeted transcript. 3' readenylation has been associated with helping degrade bacterial mRNA which form strong 3' secondary structures (Coburn *et al,* 1996; Hajnsdorf *et al,* 1995; O'Hara *et al,* 1995; Xu and Cohen, 1995; Cao and Sakar; 1992; Cao *et al,* 1995; Haugel-Nielsen *et al,* 1996; Xu *et al,* 1996; Dreyfus and Regnier 2002). Additionally, multiple rounds of uridylation have been found to be required to help overcome the conserved strong stem-loop structure found on mammalian cell cycle regulated histone mRNAs (Hoefig *et al*, 2013; Mullen and Marzluff 2008). In both bacteria and mammals, the addition of multiple rounds of a single stranded platform, namely oligo (A) and oligo (U) respectively, to a structural 3' end of a transcript ensures degradation of local double stranded regions formed within the 3' ends. This is because single stranded exonucleases, involved in 3'-5' degradation, cannot degrade double stranded structures but can remove 1-2 nucleotides from the stem-loop, due to their processivity properties.

Consistent with these published findings, degradation of potentially structured ends of *niiA* and *niaD* transcripts were found to be readenylated. Interestingly, bioinformatics analysis of the secondary structure of the truncated 3'UTRs of the *niiA* and *niaD* transcripts (UNAfold RNA software) correlated with potential double stranded sites for readenylation (figure 4.1). The nucleotidyltransferase enzymes, CutA and CutB, were found to be involved in the readenylation process in *A. nidulans* (Chapter 3.6). Interestingly, in the absence of CutB there was a significant increase in the percentage

of partially degraded transcripts in both *niiA* and *niaD*, compared to wild-type mRNA (*niaD* = 30% for wild-type, and 70% for *ΔcutB* (*p value* = 0.0001)); (*niiA* = 16% for wild-type, 33% for *ΔcutB* 35% for *ΔcutB* (*p value* = 0.04)) (chapter 3.6). However, a significant increase in the percentage of partially degraded transcripts in the absence of CutA was not observed for both *niaD* and *niiA*. These findings indicated that CutB is more active in readenylating partially degraded transcripts than CutA. This is because, following the deletion of either *cutA* or *cutB,* theoretically readenylation would be slowed down because there would only be one enzyme present and actively readenylated targeted transcripts. This would result in an increase in the presence of partially degraded and non-adenylated transcripts compared to wild-type strains. Truncated sites of readenylation were analysed for the presence of secondary structures using UNAfold RNA software (figure 4.1). These truncated sites correlated with the positions of secondary structures (figure 4.1).

Overall the results from this work demonstrated very interesting findings, namely nucleotidyltransferase enzymes appear to be involved in helping to overcome any stable 3' secondary structures through readenylation. This is the first example of cytoplasmic readenylation by nucleotidyltransferases of mRNAs. Also, these findings were consistent with readenylation being a universal (in both prokaryotes and eukaryotes) process of facilitating 3'-5' degradation via degrading double stranded local structures. Little is known about the function of these secondary structures within mRNA molecules and whether they are found in the same positions for mRNAs from the same gene. It would be interesting to determine if CutA and CutB are involved in degrading all secondary structure sites in the 3' UTRs or if they only act at sites that are very stable and difficult to degrade. Based on the secondary structure site predictions from figure 4.1, it appears that new secondary structures could be forming as the target transcript is being degraded. However, it is difficult to predict what is happening in the cell during transcript degradation. Furthermore, it appears the CutA

and CutB enzymes may be involved in different processes during the degradation of different transcripts in *A. nidulans*, by promoting 5'-3' mRNA decay by tagging and 3'-5' mRNA degradation by readenylation and facilitating the release of ribosomes from premature stop codons (Morozov *et al,* 2010a; Morozov *et al,* 2012).

4.5 Future plans

This study has produced a number of interesting findings in relation to characterising readenylation in *A. nidulans* and identifying the enzymes that are involved in doing that. In this work we also characterised the presence of alternative polyadenylation start sites for the *niiA* and *niaD* transcripts. Although C/U tagging by CutA and CutB has been found to be an important modification in facilitating 5'-3' mRNA degradation and NMD, this work showed that this was not the only function for these enzymes. It would be interesting to determine if readenylation is a regulated process during the degradation of transcripts involved in nitrogen metabolism, using the RNA-seq approach because there were limitations with using adaptor ligation RT-PCR, with regards to generating a large dataset to see any small regulatory changes. The RNA-Seq approach based on next generation sequencing to investigate the prevalence and quantity of RNAs which are expressed at a given time (Chu and Corey 2012; Wang *et al,* 2009; Levin *et al,* 2010). Through the use of RNA-Seq, a larger dataset and a greater resolution of the dynamic nature of the transcript can be achieved (Chud and Corey 2012; Wang *et al,* 2009; Levine *et al,* 2010)

It would also be important to confirm the function of readenylation in helping to overcome any stable 3' secondary structures within the 3'UTR. Furthermore, it was shown *in vitro* that more secondary structures are found within the coding region of *S. cerevisiae* transcripts, thus it would be interesting to determine if this was also the case for *A. nidulans* and if readenylation was required for the degradation of these structures within the ORF. CutA and CutB appear to be able to add C/U/A nucleotides in *A. nidulans,* based on both *in vitro* and *in vivo* data. It would be interesting to determine how these enzymes use different single stranded platforms to modify the 3' ends of target transcripts. Lastly, it would be useful to confirm the major polyadenylation signal in *A. nidulans.*

Figure 4.1 Showing different secondary structure sites for both *niiA* and *niaD*

These images represent different fold predictions for the presence of potential secondary structures for *niaD* and *niiA*. UNAfold RNA software was used (http://unafold.rna.albany.edu) for the analysis of secondary structures (chapter 2.7.2). Transcript sequences where uploaded in to the UNAfold RNA software and RNA structures with the lowest energy levels were used for analysis. Annotated on these secondary structures are the different sites of readenylation found (results from chapter 3.4, 3.6 and 3.7) which are indicated by the numbered arrows.

Figure 4.1.a.) Showing the full 3'UTR structure including for *niiA* and *niaD*.

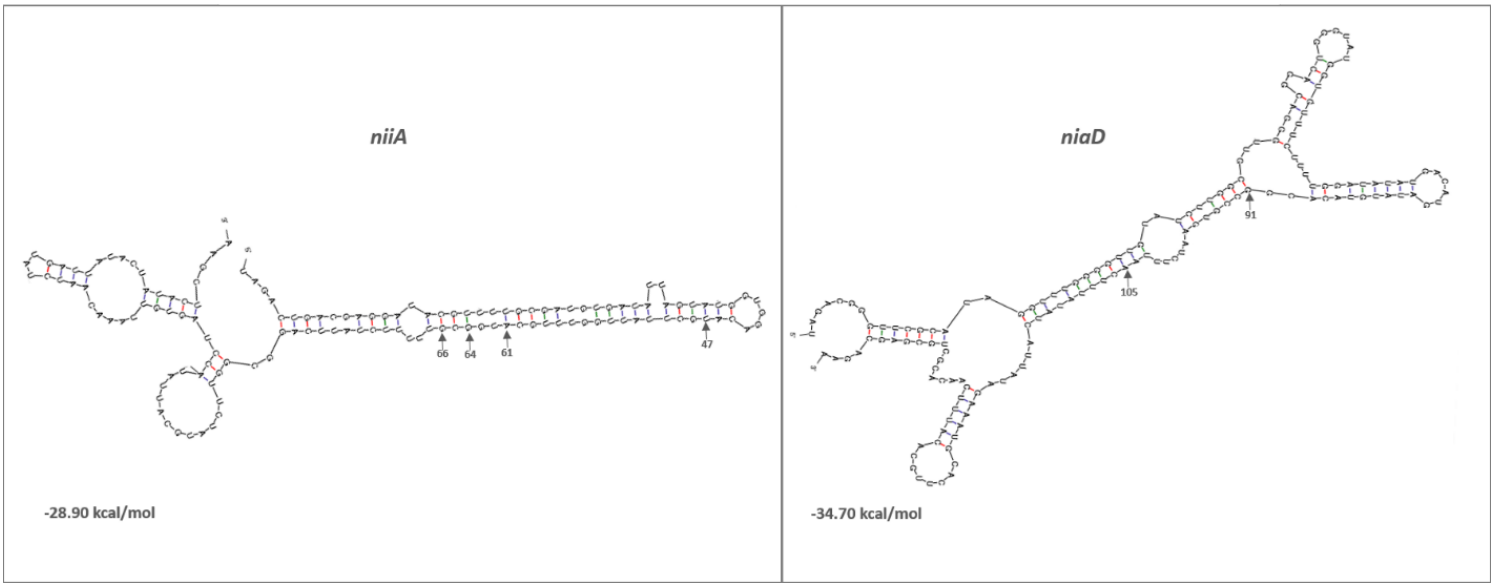


Figure 4.1.b.) Showing partially degraded 3'UTR structures for *niiA* (66, 56, and 36 nucleotides +1 from the stop codon respectively)

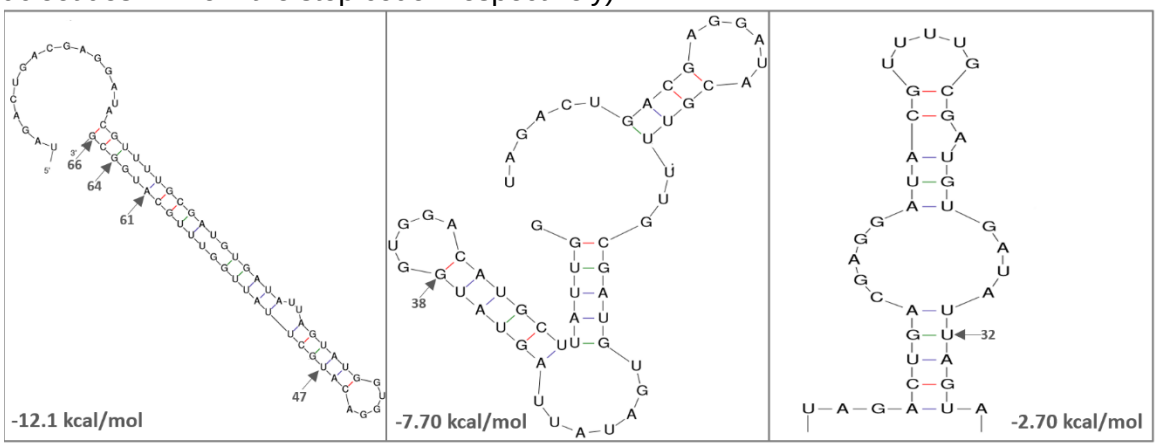


Figure 4.1.c). Showing partially degraded 3'UTR structures for *niiaD* (105, 43, and 49 nucleotides +1 from the stop codon respectively)

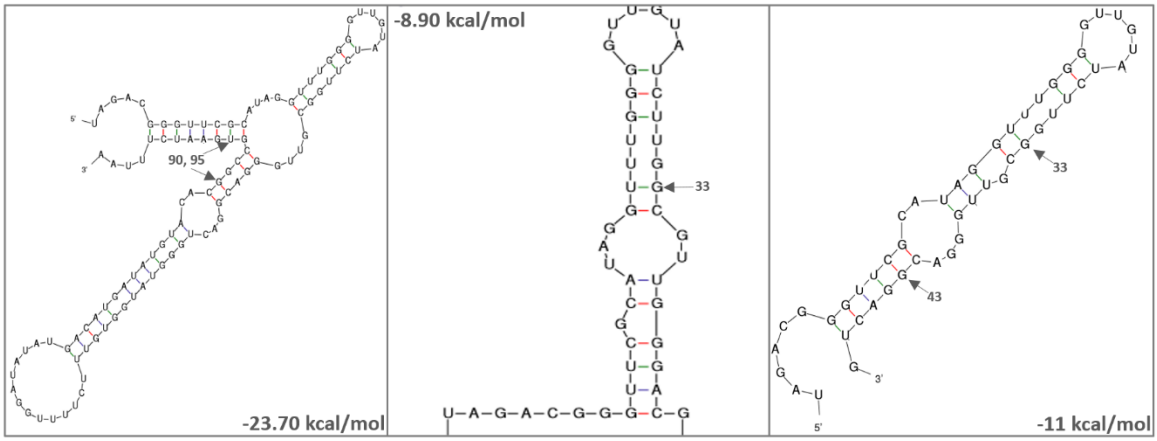


Figure 4.1.d). Showing 800 nucleotide structures of the *niiA* transcript (including the whole and 66 nucleotides of the 3'UTR respectively)

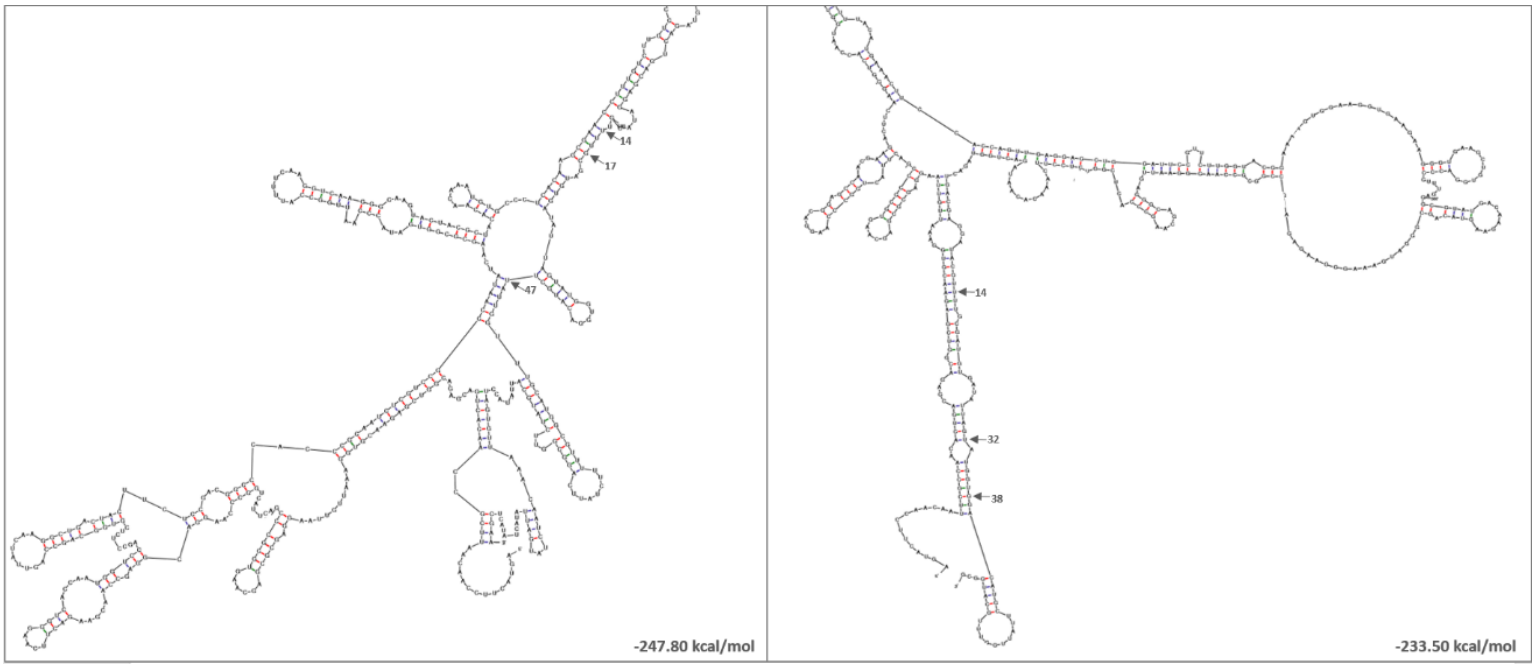
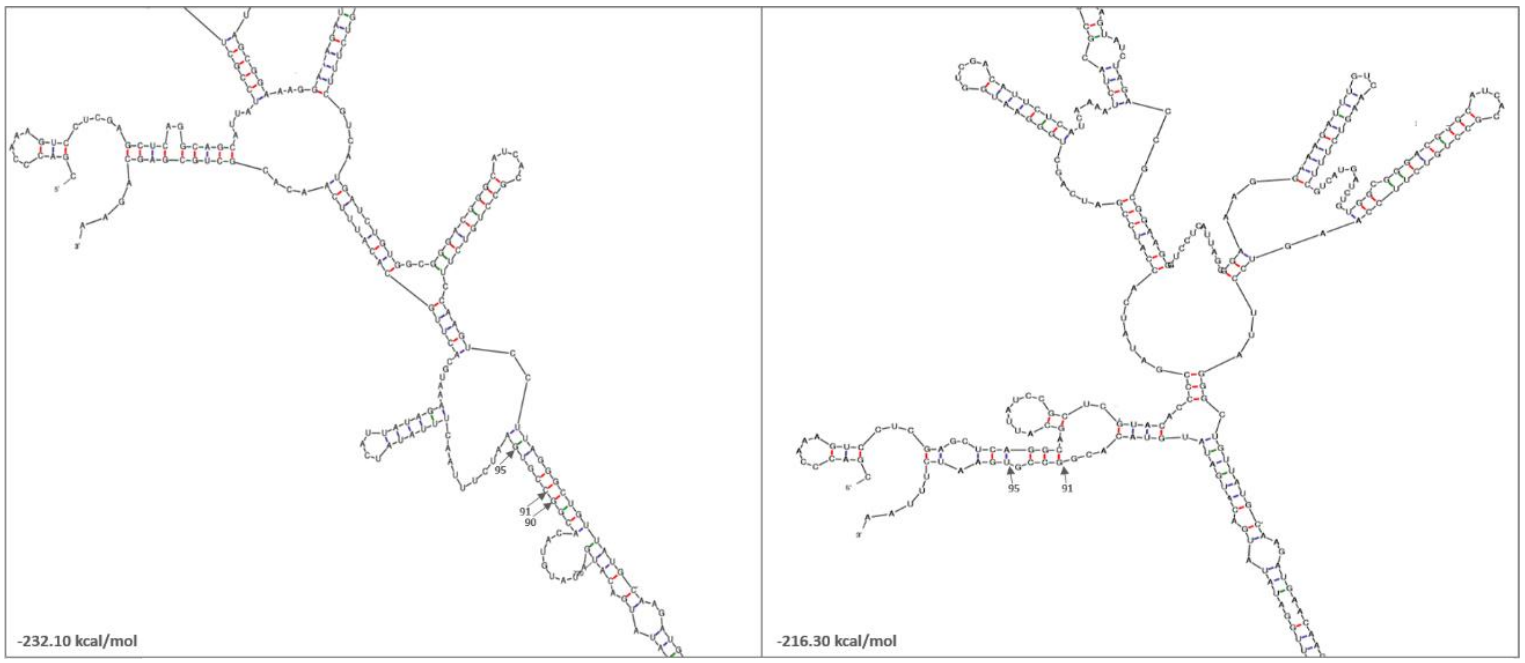


Figure 4.1.e.) Showing 800 nucleotide structures of the *niaD* transcript (including the whole and 105 nucleotides of the 3'UTR respectively)



Appendix 1

All vitamin supplements were made in a 100x stock and 1x was used in media and agar preparations

Inositol (myo-inositol)
12 mg/ml

Panto (Calcium (+) pantothenate *OR* D-pantothenic acid)
60mg/ 100ml

Pyroglutamic acid
2mg/100ml

PABA (p-amino benzoic acid) or (4-amino benzoic acid)
400mg/L

Riboflavin
250mg/L

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