FUNCTIONAL CHARACTERIZATION OF DED1P, A DEXD/H BOX RNA HELICASE, IN SACCHAROMYCES CEREVISIAE

DISSertation

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By

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The yeast DED1 protein (Ded1p) belongs to an evolutionarily conserved DExD/H-box protein family. Members of this family are involved in essentially all aspects of RNA metabolism, including pre-mRNA splicing, ribosomal biogenesis, RNA transport, and translation. They are often regarded as RNA helicases, or RNA unwindases, because some of them can couple ATP hydrolysis to unwinding short RNA duplexes \textit{in vitro}. However, recent results suggest that, \textit{in vivo}, DExD/H box proteins may dissociate specific RNA-binding proteins to remodel ribonucleoprotein complexes. Our lab previously found that Ded1p is a novel essential translation factor. However, Ded1p’s mechanistic role in translation remains to be defined.

To examine Ded1p’s role further, I sought to identify its interacting proteins. In the first part of this dissertation (Chapter 1 to 4), I describe the unexpectedly finding that Ded1p binds to yeast L-A virus particles and accelerates the rate of L-A’s negative-strand RNA synthesis \textit{in vitro}. Viruses are intracellular parasites that must use the host machinery to multiply, because their genomes are very small. Thus, identification of the host factors
that perform essential functions in viral replication is of crucial importance to the understanding of virus-host interactions. The findings in this dissertation and the fact that Ded1p is also required for translating the brome mosaic virus RNA2 in yeast thus raise an intriguing possibility that Ded1p is one of the key host factors favored by several evolutionarily related RNA viruses including the human hepatitis C virus.

In the second part of this dissertation (Chapter 5), I show that Ded1p is associated with active spliceosomes and inactivation of Ded1p affects spliceosome formation. Moreover, data from our lab demonstrates that introns accumulate in \textit{ded1} mutant strains after being shifted to non-permissive temperature. These findings indicate that Ded1p is also involved in pre-mRNA splicing. A growing body of evidence has suggested that many proteins can function in different nuclear processes in gene expression, implying a close inter-dependent relationship among these processes. The observations in this dissertation and the fact that Ded1p is a translation factor suggest a possible intimate coordination in gene expression between the nucleus and the cytoplasm.
Dedicated to my parents
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CHAPTER 1

INTRODUCTION

All viruses are in effect cellular parasites. To successfully reside and replicate in their hosts, viruses need to overcome two fundamental obstacles. First, viruses must develop successful strategies to immediately combat host’s defense or “discrimination” mechanisms. This is in particular urgent for RNA viruses, because their RNA genomes and newly synthesized transcripts are often lacking a 5’ cap and/or poly(A) tail, two intrinsic features of host mRNAs that serve to enhance their stability, translatability, and other important functions. Second, owing to their gene poor nature relative to their hosts, viruses must exploit the vast number of cellular proteins and recruit useful components for assembling their replication machinery. This act of
“borrowing” represents an unending co-evolution process between viruses and their hosts and is a critical area for research to understand virus-host interactions, which may lead to development of means of viral interventions. In this thesis work, I describe a case in which DED1 protein (Ded1p), an evolutionarily conserved translation factor, is potentially recruited by the yeast double-stranded RNA (dsRNA) virus, L-A, and exploited by the L-A virus to its own replication advantage.

In this Chapter, I will begin by introducing the yeast dsRNA viruses and other relevant RNA viruses to this work. This is to be followed by a brief overview of some well-known host factors and an introduction of DExD/H-box protein family, to which Ded1p belongs. Finally, I will provide a short summary of previous experimental findings related to Ded1p, from which this thesis study is originated.

1.1 Yeast RNA virus

The budding yeast *Saccharomyces cerevisiae* contains several virus-like elements, including the well-studied intracellular dsRNA viruses, L-A and its satellite M₁ (Wickner, 1996a; Wickner, 1996b). Importantly, studies on the L-A and M₁ virus have provided great insights into aspects of viral RNA
transcription and replication, viral RNA packaging, virus structure, mRNA decapping, and translation of poly (A)⁺ mRNA.

1.1.1 The L-A virus

The L-A virus in *Saccharomyces cerevisiae* transmits only upon mating and budding of the yeast cells. It contains a single linear 4.6-kb dsRNA segment packaged in a coat made by 120 copies of the 80-kDa major coat protein (Gag) and two copies of a minor 180-kDa Gag-Pol fusion protein produced by -1 ribosomal frameshift translation (Dinman et al., 1991). Gag alone is sufficient to form virus particles morphologically similar to normal viral particles (Fujimura et al., 1992). The pol domain of Gag-pol has a RNA-dependent RNA polymerase activity and is also necessary for the binding of the viral genome (Fujimura et al., 1992; Fujimura and Wickner, 1988a).

The Gag-Pol protein uses the L-A negative-strand RNA of a dsRNA as a template to synthesize the positive-strand RNA, which extrudes from virus particles and serves as mRNA for translation to produce both Gag and Gag-Pol (Figure 1.1) (Fujimura and Wickner, 1987). These proteins then assemble with a L-A positive-strand RNA to form a new particle (Fujimura and Wickner, 1987). Subsequently, these newly assembled virus particles utilize the
positive-strand RNA as a template to synthesize the negative-strand RNA to form mature virus particles.

Recently, the structure of L-A virus particle has been determined by X-ray crystallography at 3.4 Å resolution. It was found that L-A virus particle is 400 Å in diameter and contains a single protein shell of 60 asymmetric dimers of Gag (Naitow et al., 2002). The structure also reveals that L-A virus particle has 18 Å diameter openings, providing a portal for the entry of nucleotide triphosphates and exit of the newly synthesized positive-strand RNA (Naitow et al., 2002).

1.1.2 The M₁ virus

The M₁ virus contains two 1.8-kb dsRNA segments packaged by both Gag and Gag-Pol produced by the L-A virus, so it is called the L-A satellite virus (Esteban and Wickner, 1986; Wickner, 1996a). The M₁ virus undergoes the same replication cycle as that of the L-A virus, except that the second dsRNA segment is synthesized after completion of the first segment in virus particles (Figure 1.1). The genome of the M₁ virus only encodes a 32-kDa killer toxin/immunity precursor protein, which is subsequently processed and secreted as mature toxin (Woods and Bevan, 1968). Yeast cells without the M₁ virus are susceptible to the viral toxin. The toxin works by activating the
potassium channel, leading to excess potassium flux and cell death (Sesti et al., 2001). Because yeast is amenable to reverse genetics, the unique phenotype of the M virus has become useful tool for discovering many cellular factors affecting viral replication (Toh et al., 1978; Uemura and Wickner, 1988).

1.1.3 MAK and SKI genes affect yeast virus propagation

A number of host genes that are involved in maintenance, expression, or replication of the L-A virus and the M virus have been identified. For example, MAK genes are required for maintenance of the killer phenotype. In contrast, mutations in SKI genes cause the "superkiller" phenotype. Thus, the wild-type SKI genes function in controlling (or repressing) virus replication.

By screening mutants which fail to kill the sensitive cells, over 30 MAK genes have been found to be necessary for propagation of the M virus (Wickner, 1996a). Recent studies have shown that many mak mutations not only affect M virus propagation but also decrease the total amount of the L-A virus. Interestingly, several MAK genes turn out to encode proteins involved in translation, such as MAK7, MAK8, GCD1, GCD10, GCD11, and GCD13 (Wickner et al., 1982). A subset of MAK genes has been studied. Interestingly, many mak mutations caused a deficiency of free 60S ribosomal
subunits (Ohtake and Wickner, 1995). Thus, it appears that the deficiency selectively inhibits translation of poly(A)$^+$ mRNAs, such as L-A mRNA.

Yet, the Mak$^-$ phenotype may result from more than one mechanism. For example, MAK3 encodes an N-acetyltransferase that modifies the N-terminus of the Gag protein, which is necessary for viral assembly (Polevoda and Sherman, 2001; Tercero and Wickner, 1992). Another example is that mutations in RPS28B, which encodes a 40S subunit protein, also resulted in the Mak$^-$ phenotype (Wickner et al., 1982). Finally, MAK10 affects virus maturation, although its function is still not known (Fujimura and Wickner, 1987).

On the other hand, the SKI genes were identified as recessive mutations that increase killer toxin production (Ridley et al., 1984; Toh et al., 1978). Notably, Ski1p (Xrn1p) was found to be a major 5$\rightarrow$3$'$ exonuclease (Johnson, 1997). Recent studies further indicated that Ski2p, Ski3p, and Ski8p exist in the same complex and function with the exosome, the major 3$'$→5$'$ cellular RNA decay machinery (Brown et al., 2000; Jacobs et al., 1998).

Normally, a cellular mRNA is degraded by gradual removal of its 3$'$ poly(A) tail, which is immediately followed by removing the 5$'$ cap by the
decapping enzyme (van Hoof and Parker, 1999). The uncapped and non-poly(A) mRNA is then ready for degradation by Ski1p. Because the L-A transcript lacks both the 5’ cap and 3’ poly (A) tail, it is expected to be a good substrate for degradation. Loss of functions of SKI genes may allow viral transcripts to survive in the cell, resulting in more effective toxin production to cause the "superkiller" phenotype.

The L-A virus itself has also evolved another strategy to overcome the problem of lacking the 5’ cap structure. It has been shown that the Gag protein of L-A could covalently attach to the 5’ cap of cellular mRNAs, and as a result, remove the cap to yield a large number of decapped cellular mRNAs (Blanc et al., 1994). It is believed that the L-A virus uses this strategy to produce 5’-uncapped decoys for protecting its own uncapped L-A transcripts from degradation by Ski1p (Masison et al., 1995).

1.1.4 The in vitro system for studying yeast virus replication

The discovery of the MAK and SKI genes allows one to understand how viral replication is impacted by the host environment. Nevertheless, it does not provide mechanistic insights as to how the L-A virus replication machinery synthesizes viral RNAs and how virus particles are assembled (Wickner,
Therefore, the development of an *in vitro* system was needed for a detailed analysis of yeast virus replication machinery.

To better understand how the L-A virus synthesizes the positive-strand and the negative-strand RNAs, Fujimura and coworkers developed an *in vitro* system (Fujimura et al., 1986). They used the CsCl-density-gradient centrifugation method to purify L-A virus particles containing the positive-strand RNA or dsRNA from either log-phase cells or stationary-phase cells individually (Fujimura et al., 1986). It was found that the dsRNA-containing virus particles could synthesize the positive-strand RNA, and the positive-strand-RNA-containing virus particles could synthesize the negative-strand RNA *in vitro* (Fujimura et al., 1986). The positive-strand-RNA-containing virus particles could also produce the positive-strand RNA, but only after the negative-strand RNA was synthesized. This indicates that the same virus particle contains activities of synthesizing the negative-strand and the positive-strand RNA.

Later, it was found that dialysis of the dsRNA-containing virus particles in a low-salt solution inhibits their abilities to synthesize the positive-strand RNA, apparently because the dsRNAs are released (Fujimura and Wickner, 1988b). Importantly, the empty virus particles from the now “opened” virus
particles can synthesize the negative-strand or the positive-strand RNA in a reaction containing exogenously added L-A positive-strand RNA or dsRNA as templates. However, this reaction requires the addition of an 0—50% ammonium sulfate fraction prepared from the crude cell extract. This observation thus suggests that host factors are important for the replication and transcription activities (Fujimura and Wickner, 1988b; Fujimura and Wickner, 1989).

The development of the \textit{in vitro} system also permits a detailed analysis of the functional requirement of the template sequences. It turns out that the correct three bases at the 3’ end of the positive-strand RNA is required for the \textit{in vitro} replication (Esteban et al., 1989). A stem-loop structure near the 3’ end of the positive-strand RNA proves to enhance the replication activity (Esteban et al., 1989). Furthermore, a region about 400 nucleotides upstream of the 3’ end is required for the binding of empty virus particles and this defines the RNA packaging site (Fujimura and Esteban, 2000). The \textit{in vitro} studies also reveal that the pol region of Gag-pol is responsible for viral RNA binding (Fujimura and Esteban, 2000; Fujimura et al., 1992; Fujimura and Wickner, 1988a).
1.2 RNA viruses in other species

RNA viruses can be divided into positive-strand, negative-strand, and double-stranded RNA (dsRNA) viruses (Noueiry and Ahlquist, 2003; Whitcomb and Hughes, 1992). Despite the fact that all three classes of RNA viruses have their own strategies to infect and replicate in their host cells, it has been found that they share some evolutionarily similarities in replication. For example, the structure of the RNA-dependent RNA polymerase of bacteriophage ø6, a dsRNA RNA virus (Butcher et al., 2001) is highly similar to that of hepatitis C virus (HCV), a positive-strand RNA virus, providing an evolutionary link between dsRNA and positive-strand RNA viruses. Additionally, the study of the Brome mosaic virus (BMV) replication complex revealed that all RNA viruses use related mechanisms for nucleic acid replication and may have evolved from common ancestors (Schwartz et al., 2002). Therefore, studies of one RNA virus replication often provide information for understanding other viruses’ replication. In the next section, I will introduce two examples of RNA viruses related to this dissertation.

1.2.1 Brome mosaic virus

Brome mosaic virus (BMV) infects many crop cereals, although it does not cause significant economical losses in crop production. However, BMV
has emerged as a model system for RNA virus replication, because of its high virus yield and manipulatable genome, which have facilitated detailed investigation of BMV replication processes, recombination, and virion assembly (Janda and Ahlquist, 1993; Noueiry and Ahlquist, 2003).

BMV has three genomic RNAs (Figure 1.2). RNA1 and RNA2 encode proteins 1a and 2a essential for viral RNA synthesis. The C-terminal domain of protein 1a is a DEAD-box-like helicase (see below) and the N-terminal domain has been implicated in RNA capping. Protein 2a contains a central domain conserved among RNA-dependent RNA polymerases. Proteins 1a and 2a interact with each other and also with some incompletely characterized host factors to form a membrane-bound viral RNA replication complex associated with the endoplasmic reticulum (ER). RNA3 of BMV encodes protein 3a and the RNA itself is used as a template for making a subgenomic mRNA, RNA4, which is then translated into viral coat protein. Protein 3a and the coat protein are involved in the spread of BMV infection, but are dispensable for RNA replication (Noueiry and Ahlquist, 2003).

1.2.2 Host factors involved in Brome mosaic virus replication

Because yeast is amenable to genetics and replication of all RNA viruses occur at a single cell level, yeast has been successfully used to
replicate BMV for setting up a genetic screening system to identify host factors (Ishikawa et al., 1997). In this system, yeast express proteins 1a and 2a from DNA plasmids to replicate RNA3 for producing subgenomic RNA4. The region on RNA3 expressing coat protein was substituted with a reporter gene (Ishikawa et al., 1997). This yeast system reproduces essentially all known features of BMV RNA replication that occur in natural cells, including the dependence of RNA3 replication on proteins 1a, 2a, and localization of proteins 1a, 2a to the ER (Diez et al., 2000).

By using this system, many host factors have been identified to affect BMV replication. One notable example is that mutations in the $DED1$ gene selectively inhibit translation of RNA2, but not cellular mRNA translation. Because inhibition of RNA2 translation is dependent on the 31-nucleotide sequence in the 5’ non-coding region of RNA2, it was thought that strong stem-loop structures might form in the region to inhibit translation of RNA2 and Ded1p functions in unwinding the secondary structures (Noueiry et al., 2000). Alternatively, the 5’ non-coding region of RNA2 may bind undefined cellular proteins, resulting in inhibiting translation and Ded1p may function in dissociating the binding proteins from RNA2.
A second example is that mutations in the *OLE1* gene inhibit BMV replication in steps between template recognition and RNA synthesis, but do not inhibit the association of proteins 1a and 2a to the ER membrane (Lee et al., 2001). Because the *OLE* gene encodes Δ9 fatty acid desaturase required for synthesis of unsaturated fatty acids, this finding suggests that viral RNA synthesis is highly sensitive to lipid composition on membranes.

A third example is that alterations in the Lsm1p-Lsm7p/Pat1p complex, which is required for mRNA decapping, affect the translation of BMV RNAs (Diez et al., 2000; Noueiry et al., 2003). This inhibition of BMV RNA translation is again selective, because there is no effect on general cellular translation. Moreover, high-resolution sucrose density gradient analysis showed that the amount of viral RNA associated with ribosomes was not changed in mutant yeast. This suggests that defects in the Lsm1p-7p/Pat1p complex inhibit BMV RNA translation primarily by stalling or slowing the elongation of ribosomes along the viral open reading frame. Therefore, the Lsm1p-7p/Pat1p complex functions not only in mRNA decapping, but also in translation (Noueiry et al., 2003).
1.2.3 Hepatitis C virus

Hepatitis C virus (HCV) is a positive-stranded RNA virus (Choo et al., 1989). Approximately 170 million people worldwide are infected by HCV, more than four times the number infected by HIV. It is estimated that during the next decade, the number of annual U.S. deaths resulting from HCV-related liver damage and cancer may higher than the number of the deaths caused by HIV infection (Alter, 1995; Mansell and Locarnini, 1995).

Despite the successful cloning of the HCV genome in 1989, HCV has not being purified or propagated in the laboratory; nor is there any non-primate animal model available for its study (Choo et al., 1989). As a result, the models of HCV initial infection, replication, and subsequent persistent infection have remained mostly unknown. It was not until recently, that the HCV replication system of the HCV subgenomic RNA has been developed in hepatoma cell lines (Blight et al., 2000; Lohmann et al., 1999). The systems thus may provide the basis for detailed molecular studies of HCV and the development of antiviral drugs.

The HCV virus transcript is not capped and its translation occurs via an internal ribosome entry site (IRES) at the 5' end of the viral RNA. This IRES functions in directly recruiting the 40S ribosomes and eukaryotic initiation
factor 3 (eIF3) (Kieft et al., 2002). The HCV transcript is translated into a polyprotein, which is then cleaved by both host and viral proteases into several smaller polypeptides (Selby et al., 1993). The center and middle regions of the polyprotein consist of the nonstructural proteins (NS), such as NS2, NS3, NS4A, NS4B, NS5A, and NS5B (Hijikata et al., 1991). Nonstructural proteins are considered to have enzymatic activities that are important for virus replication (Bartenschlager and Lohmann, 2000). The N-terminal region of the polyprotein consists of the structural proteins, including the core protein and two envelope glycoproteins, E1 and E2 (Hijikata et al., 1991).

The HCV core protein functions to package viral RNAs. However, many studies indicate that the HCV core protein is closely related to the development of HCV-associated liver lesions (Ray and Ray, 2001). This suggests that the HCV core protein may have other regulatory roles in host cells. Recently, the HCV core protein was found to bind to human DDX3 (or DBX) by three independent labs (Mamiya and Worman, 1999; Owsianka and Patel, 1999; You et al., 1999). DDX3 is the homolog of yeast Ded1p, which was shown to be essential for translation (Chuang et al., 1997) (see below). Additionally, it was found that the HCV core protein binds to lymphotoxin-receptor, tumor necrosis factors, and transcription factors (Hsieh et al., 1998;
Matsumoto et al., 1997). Altogether, it is now thought that the core protein may affect cellular mRNA translation in infected cells and/or regulate cellular proteins to enhance its own replication.

1.3 Examples of known host factors recruited by viruses

Several examples of how viruses utilize or regulate host factors to facilitate their own replication have long been documented. A classic example is the RNA bacteriophage Qβ, which incorporates the host translation elongation factors (EFs) Ts and Tu and the ribosomal protein S1 into its replicase (Brown and Gold, 1996). Another notable example is that the polymerase of vesicular stomatitis virus (VSV) binds strongly to EF-1 (homologous to EF-Tu), which in turn binds EF-1β and 1 (both homologous to EF-Ts), and that all three host proteins are required for viral replication in vitro (Das et al., 1998). A third example is that purified replicase of poliovirus, a positive-strand RNA virus, requires terminal uridylyl transferase for replication in vitro (Andrews et al., 1985). In these cases, the recruitment of host proteins may or may not serve the same functions in viral replication as they do in the host cells.
Viral transcripts are considered to be discriminated in translation. Because they lack a 5' cap and/or 3' poly(A) tail, they are poor substrates for translation (Belsham and Sonenberg, 2000; van Hoof and Parker, 2002). To overcome this problem, viruses have evolved different strategies to efficiently translate their transcripts. The first strategy is that viruses produce proteases to cleave cellular proteins, especially translation factors, to greatly inhibit host translation efficiency, so viral transcripts can be translated more effectively. For example, picornaviruses, a positive-strand RNA virus, use a virally encoded protease to cleave the translation factor eIF4G (Ali et al., 2001). The second strategy is that viral proteins bind translation factors and recruit them to viral transcripts for translation. A notable example is that rotavirus, a dsRNA virus, uses its viral protein NSP3A to bind both viral transcripts and eukaryotic initiation factor 4GI (eIF4GI), which then recruits 40S ribosomal subunits through eukaryotic initiation factor 3 (eIF3) to initiate translation (Piron et al., 1998).

1.4 DExD/H box proteins

The DExD/H-box (where x can be any amino acid) proteins are evolutionarily conserved from viruses to humans (Schmid and Linder, 1992; Tanner and Linder, 2001). These proteins belong to ATPase superfamily II.
and possess a number of conserved motifs, including the DExD/H sequence (Figure 1.3). After several years of studies, important functions for the different conserved motifs have been assigned (Linder et al., 1989). For examples, the motif I (AXXGXGKT) binds to ATP. The motif II (DExD/H) is critical for ATPase activity, but is not essential for ATP binding. The motif III (SAT) links ATP hydrolysis with RNA unwinding activity (Figure 1.4) (Tanner and Linder, 2001).

The DExD/H-box proteins are known to participate in essentially all aspects of cellular RNA metabolism, such as pre-mRNA splicing, ribosomal biogenesis, mRNA export, RNA decay, and translation (de la Cruz et al., 1999). In these processes, RNA-RNA, RNA-protein, and protein-protein interactions transiently form and dissociate in a specific temporal and spatial order (Tanner and Linder, 2001). As a result, enzymes are expected to be required for the regulation of the processes. The DExD/H-box proteins have been proposed to contribute to the abilities of unwinding folded RNA, and remodeling RNA-protein and protein-protein interactions.
1.4.1 RNA helicases

Because a number of the DExD/H-box proteins are known to couple ATP hydrolysis to unwind short RNA duplexes *in vitro*, consequently DExD/H-box proteins are often referred to as “RNA unwindases” (or RNA helicases) (Lorsch and Herschlag, 1998a; Lorsch and Herschlag, 1998b; Schmid and Linder, 1992). Extensive studies have been done in eukaryotic initiation factor 4A (eIF4A), a translation initiation factor, as a model system for DExD/H-box proteins. eIF4A is thought to unwind the secondary structures in the 5′-untranslated region of mRNA and then facilitate binding of the 40S ribosomal subunit to eukaryotic mRNA. Mutations in the ATP binding motif, the ATPase activity motif, and the RNA unwinding motif of eIF4A have been known to abolish its function. Moreover, the results also suggested that there is a series of changes in conformation and substrate affinity throughout the ATP hydrolysis reaction cycle (Lorsch and Herschlag, 1998a; Lorsch and Herschlag, 1998b). As such RNA helicase may act as ATP-driven conformational switch that produce movements or structural rearrangements of attached protein domain or associated proteins. Yet, the movements could then be used to remodel RNA structures or RNA-protein interaction.
Two most widely discussed models have been proposed to explain the RNA helicase activity (Figure 1.5) (Tanner and Linder, 2001). The first model is “active rolling model”. It requires at least a dimer to be functional. The dimers are in two different conformational states. One has a higher affinity for dsRNA, and the other has a higher affinity for single-strand RNA (ssRNA). The two different conformational states differ by the binding to ATP and the hydrolysis of ATP. In this manner, the dimer acts “hand-over-hand” to move along the RNA duplex. The second model is “inchworm model”. The inchworm will work with monomers and oligomers. The binding and hydrolysis of ATP is associated with monomer’s conformational change that moves the head and the tail closer or further apart relative to each other.

1.4.2 Ribonucleoproteinases

Even though DExD/H-box proteins are traditionally called RNA helicases, recent works from our lab and other labs showed that Prp28p, an essential splicing factor, could be eliminated by alteration of components of U1 small ribonucleoprotein complexes resulting in less binding ability to the 5’ splicing site of pre-mRNA (Chen et al., 2001; Staley and Guthrie, 1999). This
suggests that Prp28p may function \textit{in vivo} as a ribonucleoproteinase (RNPase) to counteract a specific RNA-binding protein for remodeling ribonucleoprotein (RNP) complexes. Moreover, the DExH protein NPH-II from the vaccinia virus was shown to displace the protein U1A from RNA in an ATP-dependent manner \textit{in vitro} (Jankowsky et al., 2000). It was found that NPH-II increases the rate of U1A dissociation from the duplex RNA substrate. This indicates that DExD/H proteins can effectively catalyze protein displacement from RNA deplexes and thereby may participate in the structural reorganization of RNP complex assemblies.

More recently, it was demonstrated that NPH-II and Ded1p could function in single-stranded RNA substrates to remodel RNP complexes (Fairman et al., 2004). These results suggest that the essential functions of DExD/H proteins are not confined to RNA duplexes, but can be exerted on a wide range of ribonucleoprotein substrates.

\section*{1.5 Ded1p, a DExD/H box protein, is a translation initiation factor}

The DED1 protein (Ded1p) is a member of the evolutionarily conserved DExD/H-box protein family in the yeast \textit{Saccharomyces cerevisiae}. DED1 was first identified in a genetic screen, in which a \textit{ded1} mutant allele was
found to suppress the splicing defect caused by a mutation in PRP8, which encodes an essential splicing factor (Beggs et al., 1995; Jamieson et al., 1991). Thus, it was speculated that Ded1p might be involved in pre-mRNA splicing. However, there was no direct evidence to support this hypothesis. In addition, it was reported that overexpression of wild-type DED1 could suppress the growth phenotype of an RNA polymerase III (Pol III) mutant. This suggests that Ded1p may also influence Pol III transcription, although it may not normally participate in this process (Thuillier et al., 1995).

On the other hand, our lab was the first to find that Ded1p is a translation initiation factor (Chuang et al., 1997). Dr. Ray-Yuan Chuang, a former graduate student in the lab, found that cells harboring ded1 cold-sensitive mutant alleles stopped synthesizing proteins after being shifted to 16°C. The ded1 mutant alleles were also found to be synthetic lethal with tif1, which encodes an altered translation initiation factor. Most convincingly, depletion of Ded1p abolishes translation in vitro and the translation ability in Ded1p-depleted cell extract can be restored by adding back recombinant GST-Ded1p. Altogether, these data strongly suggest that Ded1p is directly involved in translation. In further examinations, Dr. Chuang found that inactive forms of 80S ribosomes were accumulated in cells with ded1 cold-sensitive
mutant alleles after shifting to 16°C. This suggests that Ded1p could be involved in the very early stages of translation initiation, before the binding of 40S ribosomes to mRNAs.

Although Ded1p’s ATPase activity and unwinding activity of RNA duplexes have recently been analyzed in vitro, its in vivo target has not been defined (Iost et al., 1999). Therefore, identification of Ded1p’s association proteins is crucial to understand Ded1p’s mechanistic role in translation initiation. On the basis of the earlier work done by Dr. Chuang, I set out to further characterize Ded1p’s role in translation. Unexpectedly, I found that Ded1p is recruited by the L-A virus for its own replication advantage, as will be described in Chapter 3.
**Figure 1.1.** Replication cycles of the L-A virus. Both (+)- and (-)-strand synthesis take place in the virus particle. The newly synthesized (+)-strand RNA, which is extruded from the particle to serve as template for making (-)-strand RNA and as substrate for translation to produce Gag and Gag-Pol proteins, which in turn package the L-A and the M1 (+)-strand RNAs. Gag protein (filled circles), Gag-Pol fusion protein (filled ovals) are shown.
Figure 1.1.
**Figure 1.2.** BMV genome. The open boxes represent the coding regions. The single black lines represent the noncoding regions. Viral proteins translated from BMV genome are listed as 1a, 2a, 3a, and coat.
Figure 1.3. Conserved sequence motifs of DExD/H-box proteins. (A) Positions of conserved motifs are shown as black boxes. (B) The conserved amino acid sequences of DEAD-box proteins are shown. Amino acids conserved at least 80% of the time are shown as upper case letters while those conserved 50%—79% of the time are in lower case letters. Adapted from Tanner and Linder, 2001.
<table>
<thead>
<tr>
<th>Motif</th>
<th>Known or Suggested Function</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>P-loop; Walker A NTP-binding domain motif; binds phosphates of NTP</td>
</tr>
<tr>
<td>Ia</td>
<td>Binds substrate through sugar-phosphate backbone</td>
</tr>
<tr>
<td>Ib</td>
<td>Substrate binding; not as highly conserved and may not always be present</td>
</tr>
<tr>
<td>II</td>
<td>Walker B NTP-binding domain motif; binds $\beta$ and $\gamma$ phosphate through Mg$^{2+}$; coordinates hydrolysis of NTP with water molecule</td>
</tr>
<tr>
<td>III</td>
<td>Binds $\gamma$ phosphate; link NTP hydrolysis with unwinding activity</td>
</tr>
<tr>
<td>IV</td>
<td>Substrate binding; known as IVa in SF1 DNA helicases</td>
</tr>
<tr>
<td>V</td>
<td>Binds substrate through sugar-phosphate backbone; may interact with NTP</td>
</tr>
<tr>
<td>VI</td>
<td>Binds $\gamma$ phosphate; converts NTP binding/hydrolysis with domain 1 and domain 2 movement</td>
</tr>
</tbody>
</table>

**Figure 1.4.** Summary of known or suggested functions of DExD/H-box-protein motifs. Adapted from Tanner and Linder, 2001
Figure 1.5. Models of helicase activity. Adapted from Tanner and Linder, 2001
CHAPTER 2

MATERIALS & METHODS

2.1 Yeast strains

All yeast strains used in this work contain endogenous L-A virus in an isogenic background. They are listed in Table 2.1.

2.2 Plasmids

All plasmids used in this study are listed in Table 2.2.

2.3 Affinity purification and characterization of Ded1p-PA-associated proteins

Yeast cells collected from 1 liter of culture grown to 1 OD$_{600}$ unit was spheroplasted using 7 mg of zymolyase 100T (Seikagaku), in buffer B (100 mM Tris-HCl [pH 7.6], 1 M sorbitol, 20 mM 2-mercaptoethanol) at 30°C for 30
min. Then spheroplast was resuspended in 15 ml of lysis buffer (150 mM KCl, 20 mM Tris-HCl [pH 7.5], 5 mM MgCl₂, 0.1 % Triton X-100), and kept on ice for 5 min. Cell debris was removed by centrifugation at 16,000 r.p.m. for 20 min in an SS-34 rotor (Sorvall). An aliquot of clarified cell extract containing 20 mg of total proteins was mixed with 100 µl of IgG Sepharose 6 Fast Flow (Pharmacia) in a closed Poly-Prep Chromatography column (BioRad) and incubated at 4°C for 2.5 hour (hr) on a nutator. Following flow through, the column was extensively washed 10 times, each with 5 ml of lysis buffer, and the bound materials were eluted in steps by a buffer (20 mM Tris-HCl [pH 7.5], 0.05% Triton X-100) containing increased concentrations of MgCl₂ (50 mM, 100 mM, 200 mM, 500 mM, 1 M, 2 M, and 4.5 M) as described (Siniossoglou et al., 1996). For RNA analysis, IgG-bound materials were extracted by phenol/chloroform (pH 5.2), ethanol precipitated, and analyzed in a 1% agarose gel. To assess the RNase sensitivity of the viral dsRNA in the affinity-purified L-A particles, the bound materials were treated with 2.5 µg/ml of pancreatic RNase (Sigma) at 4°C for 60 min. For mass spectrometry analysis, the Ded1p-PA co-precipitation assay was scaled up in order to collect enough
materials. The prominent proteins were cut out from SDS-PAGE and were analyzed in “Mass Spectrometry and Microsequencing Facilities in Beckman Research Institute”, City of Hope, CA.

2.4 Purification of GST-Ded1p and GST from bacteria

XL-1 blue (Stratagene) in 100 ml overnight culture was transferred into 1 liter of 2X YT (1% Bacto yeast extract, 0.5% NaCl, 1.6% Bacto-tryptone) with 250 µg/ml Ampicillin and grown for 4 hr at 37°C. IPTG was added to a final concentration 0.1 mM and the culture continued growing for 3 hr. Cells were then collected and washed with ice-cold phosphate-buffered saline (PBS). Cell pellets were frozen in liquid nitrogen and thawed three times. A 25-ml lysis buffer (50 ml Tris-HCl [pH 8.0], 5 mM EDTA, 1% Triton X-100) with lysozyme (4 mg/ml, Sigma) was added and incubated on ice for 20 min. After sonication, the extract was clarified by centrifugation of 3,0000 g for 30min at 4°C. The supernatant was mixed with 3 ml of Glutathione (GSH)/agarose beads at 4°C with gentle agitation for 12 hr. The beads were washed extensively with a wash buffer (50 mM Tris-HCl [pH 7.5], 500 mM NaCl, 1% NP-40, 0.5% Na-deoxycholate, 0.1% SDS) and then equilibrated with buffer
TB (25 mM Tris-HCl [pH 8.0], 120 mM NaCl) for elution by 20 mM GSH (6 mg/ml).

2.5 Purification of L-A viral particles by CsCl gradients

L-A virus particles were purified according to the standard protocol by Esteban and Wickner (Esteban et al., 1989) and advice by Dr. R. Wickner (NIH). Cells were harvested from 2 liters of culture grown to mid-log phase (for positive-strand-RNA-containing virus particles) or saturation (for dsRNA-containing virus particles) as needed (Fujimura and Wickner, 1987). After washing once with water, cells were suspended in 1 volume of buffer A (100 mM Tris-HCl [pH 7.6], 1 M sorbitol, 20 mM 2-mercaptoethanol, and 2 mg of zymolyase 100T [Seikagaku] per ml) and incubated at 37°C for 1 hr. The resultant spheroplasts were resuspended in 15 ml of buffer B (50 mM Tris-HCl [pH 7.6], 5 mM Na-EDTA, 150 mM NaCl and 1 mM DTT) and lysed by a single passage through a French press (14,000 lb/in2). Cell debris was removed by low-speed centrifugation at 10,000 r.p.m. for 20 min in a SS-34 rotor (Sorvall). Subsequently, L-A viral particles were collected by high-speed centrifugation at 32,000 r.p.m. in a 70Ti rotor (Beckman) for 1 hr. The re-suspended pellet in 20 ml of buffer B was then homogenized by 10 strokes in a glass dounce homogenizer (Kontes) and clarified by low-speed centrifugation at 10,000
r.p.m. for 20 min in a SS-34 rotor. The density of the solution was adjusted to 1.35 g/ml by addition CsCl and virus particles were banded by centrifugation at 38,000 r.p.m. for 20 hr at 4°C in a 70Ti rotor (Beckman). The gradient was fractionated from top to bottom, 1 ml/per fraction. Thirty µl of each fraction was used for extraction of L-A dsRNA to determine which fractions contained dsRNA virus particles. The positive-strand-RNA-containing virus particles were collected in the sixth fraction with the density lighter than that of dsRNA-containing virus particles. Fractions containing virus particles were dialyzed against buffer B containing 20% (vol/vol) glycerol for 4 hr and stored at –80°C. Purified virus particles were used within two weeks for an in vitro replication assay.

2.6 In vitro binding assay

[^35S]-labeled Gag was synthesized in a 50-µl reaction using a coupled transcription/translation rabbit reticulocyte system as instructed by the supplier (Promega). For the binding assay, 15 µl of the translation product was used for binding to 20 µl of the glutathione beads pre-coated with either GST-Ded1p or GST in a binding buffer (150 mM KCl, 20 mM Tris-HCl [pH 7.5], 5 mM MgCl₂, 0.1 % Triton X-100) for 2 hr at 4°C. After extensive washes with the
binding buffer, beads were boiled in SDS-PAGE loading dye. Proteins in the supernatant were separated by 8% SDS-PAGE and the gel was dried for autoradiography. To test the binding of L-A particles to GST-Ded1p, 15 µl of CsCl-purified L-A particles was incubated in a binding buffer (150 mM KCl, 20 mM Tris-HCl [pH 7.5], 5 mM MgCl₂, 0.1 % Triton X-100) at 4°C for 2 hr. After extensive washing with the binding buffer (1 ml/each time, 10 times), the binding efficiency was assessed by the recovered L-A dsRNA or the Gag protein.

2.7 In vitro virus replication assay

L-A virus particles purified from CsCl gradient centrifugation was incubated in a 20-µl reaction (50 mM Tris-HCl [pH 7.6], 5 mM MgCl₂, 0.1 mM Na-EDTA, 20 mM NaCl, 5 mM KCl, 10 mM 2-mercaptoethanol, 0.5 mM each of ATP, CTP And GTP, 20 µM [α-³²P]-UTP) with or without 500 ng GST-Ded1p at 30°C for 1 hr (Fujimura and Wickner, 1988b). The newly synthesized [³²P]-labeled RNA were extracted with phenol/chloroform (pH 5.2) and precipitated with ethanol. The products were analyzed in 1% agarose gel and autoradiographed.
2.8 Electron microscopy

CsCl- and affinity-purified L-A virus particles were negatively stained by 2% uranyl acetate and then imaged on a Philips CM12 transmission electron microscope (Fujimura et al., 1992). This procedure was done in the Ohio State University Campus Microscopy and Imaging Facility (CMIF).

2.9 Cytoduction and killer activity assay

To introduce the M₁ virus into Ded1p-PA containing strain, YTC598 was mixed with YTC212 for mating at 30°C for 7 hr on YPD plates (Conde and Fink, 1976; Fujimura and Wickner, 1987). The mixed cells are then re-suspended into 1 ml of water and plated onto YPD plates. After incubation at 30°C for 2 days, cells were replica-plated to different plates to check their genotypes. Cells with both YTC212 genotype and killer activity were selected. For the killer assay, yeast cells containing the M₁ virus were grown on YPD plates at 30°C for 2 days. Cells were then replica-plated into killer assay plates (0.04% Adenine sulfate, 1% yeast extract, 2% peptone, 2% dextrose, and 2% agar 100mM Na-Citrate [pH 4.7] and 0.003% methylene blue), which contain M₁-minus yeast cells, and incubated at 22°C for 4 days. Yeast cells
with the $M_1$ virus can secret the toxin protein, which kills $M_1$-minus yeast cells and forms a clear zone around the $M_1$ containing cells.
<table>
<thead>
<tr>
<th>Strains:</th>
<th>Genotype</th>
</tr>
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<tr>
<td>YTC127</td>
<td>\textit{MATa} ded1::TRP1 ura3-52 lys2-801 ade2-101 trp1-Δ1 his3Δ200 leu2-Δ1 pDED1009 [L-A]</td>
</tr>
<tr>
<td>YTC212</td>
<td>\textit{MATa} ura3-52 lys2-801 ade2-101 trp1-Δ1 his3Δ200 leu2-Δ1 ded1::TRP1 pDED1033 [L-A]</td>
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<tr>
<td>YTC433</td>
<td>\textit{MATa} dbp5::HIS3 ura3-52 lys2-801 ade2-101 trp1-Δ1 his3Δ200 leu2-Δ1 pCA5032 [L-A]</td>
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<tr>
<td>YTC420</td>
<td>\textit{MATa} dbp3::HIS3 ura3-52 lys2-801 ade2-101 trp1-Δ1 his3Δ200 leu2Δ1 pCA3053 [L-A]</td>
</tr>
<tr>
<td>YTC598</td>
<td>\textit{MATa} kar1-1his4 [L-A] [M₄]</td>
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</table>

Table 2.1
### Table 2.2

<table>
<thead>
<tr>
<th>Plasmids:</th>
<th>Description</th>
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<tbody>
<tr>
<td>pGAG1001</td>
<td>a L-A cDNA clone containing the entire length of GAG in T-vector (Promega) is used for the synthesis of L-A positive-strand cDNA and for the preparation of random primer labeling in Northern analysis</td>
</tr>
<tr>
<td>pGAG1004</td>
<td>a L-A cDNA clone containing the entire length of GAG in T-vector (Promega) is used for the <em>in vitro</em> translation and for the synthesis of L-A negative-strand cDNA</td>
</tr>
<tr>
<td>p596</td>
<td>a M1 cDNA clone is a gift from Reed Wickner for the preparation of random primer labeling in Northern analysis</td>
</tr>
<tr>
<td>pDED1009</td>
<td>A 2.9 kb <em>XhoI-SalI</em> fragment containing the entire length of the DED1 gene isolated from pUC-Sc2605 (gift from K. Struhl) was cloned into pRS315 with <em>XhoI+SalI</em></td>
</tr>
</tbody>
</table>

continued
Table 2.2 continued

**pDED1033**  A 3.4 kb DNA fragment with the *XhoI/PstI* site containing DED1-Protein A from pDED1032 was cloned into pRS315 with *XhoI* and *PstI*. This fusion gene is controlled under its own promoter and terminator sequences for expression in yeast.

**pDED1036**  A 2.7 kb *Ndel/HindIII* DNA fragment containing the *DED1* gene was removed from pDED1002. The *Ndel* 5' overhang was blunted by Klenow enzyme. The fragment was then cloned into the *SmaI* site of pGEX-2T vector (Pharmacia).

**pCA5032**  An 0.65 kb protein A fragment from pCA3052 was cloned into the *AatII* site of pCA5031 for the expression of Dbp5p-protein A fusion protein under its own promoter in yeast.

**pCA3052**  An 0.65 kb protein A fragment of PCR product with *AatII* ends was ligated into the *AatII* site of pCA3006 for the expression of Dbp3p-protein A fusion protein under its own promoter in yeast.
CHAPTER 3

RESULTS

3.1 The Gag protein Interacts specifically with Ded1p

Our lab has previously shown that Ded1p is an essential translation factor (Chuang et al., 1997). To elucidate Ded1p's role in translation, we sought to identify its interacting proteins by using a yeast strain in which the chromosomal $DED1$ gene has been deleted and complemented by a plasmid-born recombinant $DED1$-Protein A gene, in which the Protein A moiety (PA) was fused in-frame to the C-terminus of Ded1p. The growth rate of this strain is indistinguishable from that of the wild-type strain under a variety of conditions, suggesting that the recombinant Ded1p-PA protein is functional.
(Chuang et al., 1997). Yeast extracts were made from this strain and then incubated with IgG-Sepharose beads. The recombinant Ded1p-PA is expected to bind to IgG via its PA moiety. After extensive washes, Ded1p-PA and its associated proteins were eluted from IgG beads by 0.5 M acetic acid and analyzed by SDS-PAGE (Grandi et al., 1993).

One prominent ~80-kDa protein was found to co-elute with the 94-kDa Ded1p-PA (Figure 3.1, lane 2). Internal peptide sequencing of this 80-kDa protein yielded two peptide sequences, FAYRHALT and VYGDTHGLTK, matching perfectly with the amino-acid sequences 240—247 and 527—536, respectively, of the L-A virus Gag protein. The identity of the 80-kDa protein was confirmed by its specific reaction with the anti-Gag antibody (data not shown). The observed Gag-Ded1p interaction was specific because, using the same experimental protocol, Gag protein could not be recovered from the wild-type yeast extract (Figure 3.1, lane 1), which contained untagged Ded1p, nor from control extracts containing either Dbp3p-PA (lane 3) or Dbp5p-PA fusion proteins (lane 4). The Dbp5p-PA extract was a good control, because Dbp5p is also a cytoplasmic DExD/H-box protein of similar abundance to Ded1p. Experiments using extracts containing a N-terminally tagged PA-Ded1p also allowed Gag recovery at the same level (data not shown), arguing
that the binding of Ded1p to Gag does not simply result from fusion of the PA moiety to the C-terminus of Ded1p.

Because Ded1p is an RNA-binding protein (Iost et al., 1999), we wondered whether the observed Ded1p-Gag interaction was RNA-dependent. This was not the case, as extensive treatment of the IgG-Sepharose-bound material by RNase did not affect Gag recovery (Chuang, 1997b). To show that Ded1p directly interacts with Gag, I synthesized [35S]-labeled Gag by \textit{in vitro} transcription and translation and used it for binding to glutathione beads pre-coated with either GST-Ded1p fusion (Figure 3.2, lane 1) or GST alone (lane 2). Binding of Gag to GST-Ded1p was at least 10-fold more efficient than to GST.

I then assessed the binding affinity of Ded1p-PA to Gag by step-eluting the Gag protein using elution buffer containing increasingly concentrated MgCl$_2$ (Sinosossoglou et al., 1996). The Gag protein was eluted in a peak around 0.5 M MgCl$_2$, suggesting high affinity between Gag and Ded1p (Figure 3.4). The same strong interaction was also observed between the purified GST-Ded1p and the [35S]-labeled Gag \textit{in vitro} (data not shown). Thus, Ded1p interacts specifically and directly with Gag with high affinity.
3.2 Specific interaction of Ded1p with the L-A Virus Particle

Careful inspection of co-precipitation results (Figure 3.1) revealed that a 180-kDa protein of lesser abundance also co-eluted with Ded1p-PA, prompting us to speculate that it could be the Gag-Pol fusion protein (Fujimura and Wickner, 1988a), which is present in the L-A virus at only one to two copies per particle (Dinman et al., 1991; Fujimura and Wickner, 1988a). This prediction was validated by Western analysis using an antibody recognizing only the Pol moiety (data not shown) (Fujimura and Wickner, 1988a). These results thus suggest that Ded1p may interact with the L-A virus particle. To test this hypothesis, I asked whether L-A dsRNA could also be co-purified with Ded1p-PA. Indeed, extraction of the IgG-bound material from the Ded1p-PA extract (Figure 3.3, lane 1; top panel) yielded an RNA species with similar gel mobility to that of the cellular L-A dsRNA (lanes 3 and 4). This RNA was not detected in a control experiment using Dbp5p-PA extract (lane 2), despite its presence in both Ded1p-PA and Dbp5p-PA extracts (figure 3.4, lanes 3 and 4).

To prove that the co-purified RNA species is indeed the L-A dsRNA, I synthesized $^{32}$P-labeled positive-strand and negative-strand probes from an L-A cDNA clone. Both probes hybridized to the RNA species (Figure 3.3, lane
and to the L-A dsRNA present in the total cellular RNA (lanes 3 and 4, middle and bottom panels), but not to any corresponding RNA species in the co-purified fraction from the Dbp5p-PA-extract (lane 2). Thus, the recovered RNA species is the L-A dsRNA. Because the L-A Gag protein is also used to encapsidate the satellite M₁ dsRNA (Wickner, 1996a), I checked whether Ded1p also binds to the M₁ virus particle. Similar experiments using extracts prepared from a yeast strain containing Ded1p-PA and M₁ virus particles (see Materials and Methods) showed that M₁ dsRNA, as monitored by Northern blotting, was also efficiently recovered (Figure 3.5).

If Ded1p interacts with the L-A virus particles, the L-A dsRNA should be eluted from the IgG beads in a profile identical to that of the Gag protein. This indeed was the case, in that both the L-A dsRNA and the Gag protein were co-eluted in a peak around 0.5 M MgCl₂ (Figure 3.4). To further show that L-A virus particles interact with Ded1p, the IgG-bound material was eluted and subjected to electron microscopy imaging. A population of particles measuring ~40 nm in diameter was observed (Figure 3.7B). These particles were identical in shape and dimension as the L-A virus particles (Fujimura et al.,
1992) purified from a wild-type yeast strain using a conventional CsCl purification protocol (Figure 3.7A).

To demonstrate that Ded1p is capable of interacting with the purified L-A virus *in vitro*, I used the CsCl-purified L-A virions for binding to either GST-Ded1p- or GST-coated glutathione beads. GST-Ded1p allowed recovery of >50% of the input L-A virions, judging from the amount of L-A dsRNA recovered (Figure 3.6A, lane 1). In contrast, little, if any, L-A dsRNA was recovered from GST-coated beads (lane 2). Western analysis using anti-Gag antibody yielded the same result (Figure 3.6B). This higher yield of recovery, in comparison to the recovery of the *in vitro* synthesized Gag by GST-Ded1p (Figure 3.2), raised a possibility that the conformation of the L-A virion is critical for its effective binding to Ded1p.

### 3.3 Affinity-purified L-A virions are transcriptionally active

It is possible, although unlikely, that Ded1p-PA interacts only with either damaged or defectively assembled L-A particles, which could be functionally inactive. To address this issue, I first assessed the RNase susceptibility of the L-A dsRNA molecules in the affinity-purified material. Incubation of the IgG-bound material in the presence of an internal control, i.e. a [\(^{32}\)P]-labeled L-A
transcript, with pancreatic RNase (Fujimura et al., 1986) resulted in complete
destruction of the added L-A transcript, yet still permitted quantitative recovery
of the L-A dsRNA (Figure 3.8). Since the RNase-treatment was shown to
readily degrade naked L-A dsRNA (Fujimura et al., 1986) these results
suggest that the affinity-purified L-A particles were sufficiently intact to shield
the encapsidated L-A dsRNA from the RNase attack.

Since CsCl-purified L-A virions are known to synthesize both positive-
and negative-strand RNAs \textit{in vitro} (Fujimura et al., 1986), I next examined the
affinity-purified L-A virions for such activities. Affinity-purified L-A virions were
bound to the Ded1p-PA-conjugated IgG beads and incubated under the
standard \textit{in vitro} transcription condition. The production of several major RNA
species was readily detected by denaturing agarose gel electrophoresis
(Figure 3.9A, lane 1). The 4.6-kb species, because of its apparent size, most
likely corresponds to a full-length L-A transcript, whereas the shorter species
may represent either truncated or degraded products of \textit{in vitro} transcription.
As expected, the control experiment using Dbp5p-PA extract yielded no
detectable RNA synthesis (Figure 3.9A, lane 2). To determine the
strandedness of the synthesized products, we gel-purified the $^{32}$P-labeled
4.6-kb species and used it as a probe for Southern analysis. This probe
hybridized to two single-stranded DNA clones harboring either the positive or the negative strand of the L-A dsRNA (Figure 3.9B, lanes 3 and 4) and to a double-stranded L-A cDNA clone (lane 2), but not to an empty vector (lane 1). These data suggest that Ded1p interacts with at least two functional forms of the L-A virion: one that contains the L-A dsRNA, which can synthesize the positive-strand transcript, and the other that contains the positive strand RNA, which can produce the negative-strand transcript.

3.4 Ded1p promotes L-A negative-strand RNA synthesis

Although a number of chromosomal MAK genes are essential for viral dsRNA, especially M₁, replication, their gene products are not known to stably associate with the virus particles (Wickner, 1996a). The fact that Ded1p interacts specifically and strongly with the L-A particle thus raises a possibility that it may participate in the L-A life cycle. Since earlier data suggest that a host factor(s) in a 0—50% ammonium sulfate fraction of a crude extract is required for negative-strand RNA synthesis in vitro (Fujimura and Wickner, 1988b), we wondered whether Ded1p could be a part of this activity. To test this hypothesis, I purified a fraction of the L-A particles that contain predominantly, if not exclusively, the positive-strand RNA from mid-log-phase
yeast cultures (Fujimura et al., 1986). When incubated with GST alone, these positive-strand RNA particles produced little negative-strand RNA (Figure 3.10, lane 1). In sharp contrast, addition of GST-Ded1p greatly stimulated the negative-strand synthesis to at least 10-fold (lane 2). Time-course studies further revealed that Ded1p significantly accelerated the rate of negative strand synthesis (Figure 3.12). Notably, this stimulation is specific, as Prp28p (Chen et al., 2001), a DExD/H-box splicing factor, and a mutant Ded1p with altered NTP binding pocket (DEAD-to-DAAD) known to be devoid of ATPase and RNA-winding activities (Iost et al., 1999) both failed to appreciably promote the negative-strand RNA synthesis (Figure 3.11). I confirmed that the Ded1p-dependent product is indeed the negative-strand RNA by strand-dependent Southern analysis (data not shown). Finally, to test whether Ded1p can also promote positive-strand RNA synthesis, L-A dsRNA-containing virus particles purified from stationary-phase yeast cultures (Fujimura et al., 1986) were used in the same reaction. Neither GST nor GST-Ded1p was found to stimulate the production of the positive-strand RNA (Figure 3.10, lanes 4 and 5). Taken all together, we conclude that Ded1p is likely to be a host factor recruited by the L-A virus to promote its negative-strand synthesis.
Figure 3.1. Ded1p interacts specifically with Gag. Extracts prepared from yeast strains containing Ded1p (lane 1), Ded1p-PA (lane 2), Dbp3p-PA (lane 3), or Dbp5p-PA (lane 4) were incubated with IgG-Sepharose. After washes, IgG-bound proteins were eluted with acetic acid and analyzed by SDS-PAGE. Molecular size markers are indicated (kDa). This experiment was done by Dr. Ray-Yuan Chuang.
Figure 3.2. *In vitro* binding of Ded1p with Gag. *In vitro* synthesized [*35S*]-labeled Gag was incubated with glutathione beads pre-coated with either GST-Ded1p (lane 1) or GST alone (lane 2). Materials bound were analyzed by SDS-PAGE followed by autoradiography. Lanes 3—7: 4% (lane 3), 1% (lane 4), 0.5% (lane 5), 0.25% (lane 6), and 0.125% (lane 7) of the input [*35S*]-labeled Gag.
Figure 3.3. L-A dsRNA co-precipitates with Ded1p-PA. Extracts made from yeast strains harboring Ded1p-PA (lane 1) or Dbp5p-PA (lane 2) were incubated with IgG-Sepharose. The bound RNAs were extracted with phenol/chloroform, precipitated (Ppt.) by ethanol, separated by agarose gel electrophoresis, and visualized by ethidium bromide (EtBr) staining (top panel; lanes 1 and 2). Total RNAs (Total) prior to immunoprecipitation were extracted and used as positive controls (lanes 3 and 4). The identity of the L-A dsRNA was verified by Northern blotting using either $[^{32}P]$-labeled positive-strand probe (middle panel) or negative-strand probe (bottom panel).
Figure 3.3.
Figure 3.4. The L-A dsRNA co-elutes with Gag. Extracts from Ded1p-PA-containing strain was incubated with IgG-Sepharose. The IgG-bound proteins were then sequentially eluted with increased concentrations (0.05 to 4.5 M) of MgCl$_2$ as shown (bottom panel). RNAs were extracted from MgCl$_2$-eluted fractions, separated by agarose gel electrophoresis, and then visualized by EtBr staining (top panel).
Figure 3.5. The $M_1$ dsRNA co-precipitates with Ded1p-PA. Extracts made from yeast strains harboring Ded1p-PA and $M_1$ viruses were incubated with IgG-Sepharose. The bound RNAs were extracted with phenol/chloroform, precipitated by ethanol, separated by agarose gel electrophoresis, and visualized by ethidium bromide staining (lanes 1). The identity of the $M_1$ dsRNA was verified by Northern blotting using $M_1$ cDNA probe (lane 2).
Figure 3.6. Direct interaction of Ded1p to L-A particles. (A) L-A dsRNA co-precipitates with GST-Ded1p. CsCl-purified L-A particles were incubated with glutathione beads pre-coated with GST-Ded1p (lane 1) or GST (lane 2). Lane 3 represents 33% of the input L-A particles. The bound RNAs were extracted with phenol/chloroform, precipitated by ethanol, separated by agarose gel electrophoresis, and detected by Northern blotting. (B) Gag co-precipitates with GST-Ded1p. The glutathione-bound proteins were analyzed on SDS-PAGE and probed with anti-Gag antibody in Western blotting.
Figure 3.6.

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Figure 3.6.
Figure 3.7. Electron microscopy imaging of purified L-A particles. (A) L-A particles were purified from a wild-type strain by conventional CsCl-gradient method or (B) from a Ded1p-PA-containing strain by affinity purification, in which L-A particles were eluted in one step by 2 M MgCl₂. Virus particles were then imaged by transmission electron microscopy. Bar, 100 nm.
Figure 3.8. The co-precipitated L-A dsRNA is resistant to RNase treatment. Ded1p-PA-bound IgG beads were incubated with pancreatic RNAase in the presence of a $^{32}\text{P}$-labeled L-A transcript as described in Materials and Methods. After incubation, RNAs were recovered and run on an agarose gel, which was stained with ethidium bromide (top panel) and autoradiographed (bottom panel).
Figure 3.9. Affinity-purified L-A particles are active in synthesizing L-A RNA. (A) *In vitro* synthesis of the L-A transcript. L-A particles were bound to IgG beads precoated with Ded1p-PA (lane 1) or Dbp5p-PA (lane 2) for *in vitro* RNA polymerase activity assay. The $^{32}$P-labeled RNA products were separated on a denaturing agarose gel and autoradiographed. RNA size markers (kb) are indicated to the left. (B) Production of both (+)- and (-)-RNA by affinity-purified virions. The virus-synthesized RNA from lane 1 of (A) was used as a probe to hybridize to vector DNA alone (lane 1), vector DNA containing L-A cDNA (lane 2), single-stranded DNA containing the (+)-strand L-A cDNA (lane 3), single-stranded DNA containing the (-)-strand L-A cDNA (lane 4). Top panel: ethidium bromide (EtBr) staining; bottom panel: autoradiogram of the Southern blotting result.
Figure 3.9.
**Figure 3.10.** Ded1p accelerates the rate of *in vitro* negative-strand RNA synthesis. Ded1p promotes negative-strand RNA synthesis but not positive-strand RNA synthesis. Positive-strand-RNA-containing (Log phase) or dsRNA-containing (Stationary phase) L-A particles were incubated in the presence of either GST (lanes 1 and 4) or GST-Ded1p (lanes 2 and 5). Lane 3, dsRNA-virus alone. After incubation, RNAs were extracted and analyzed by denaturing agarose gel electrophoresis (lanes 1 and 2) and autoradiographed.
Figure 3.11. Ded1p specifically promotes the in vitro negative-strand RNA synthesis. The dsRNA-containing (stationary phase) L-A particles were incubated in the presence of His$_6$-tagged Ded1p (lanes 2), His$_6$-tagged Ded1p with alteration in DEAD motifs (DEAD→DAAD) (lanes 3), or His$_6$-tagged Prp28p (lane 4). Lane 1, dsRNA-virus alone. After incubation, RNAs were extracted and analyzed by native agarose gel electrophoresis and autoradiographed.
Figure 3.12. Time-course study of Ded1p-promoted negative-strand RNA synthesis. Aliquots of reaction in Fig 3.10 using the positive-strand-RNA-containing L-A particles (Log phase) were withdrawn at various time points and analyzed in native agarose gel electrophoresis.
CHAPTER 4

DISCUSSION

To successfully multiply in the cell, RNA viruses, such as the positive-strand RNA and the dsRNA viruses, must overcome a series of challenges. One of the immediate tasks is to compensate for their lack of mRNA 5’ cap and/or 3’ poly(A) tail, which in theory would severely handicap viral mRNAs in terms of their stability and translatability. RNA viruses are known to evolve a variety of strategies to circumvent this problem. For example, poliovirus uses its virus-encoded proteinase 2A to cleave the translation initiation factor 4G (eIF4G) and poly(A)-binding protein, thereby inhibiting the host cell mRNA translation (Gradi et al., 1998; Joachims et al., 1999; Kerekatte et al., 1999). Yet, poliovirus RNA translation proceeds in a cap-independent fashion by
recruiting ribosomes via its internal ribosome entry site (IRES) in the absence of intact eIF4G. The L-A virus, on the other hand, uses its Gag protein to covalently bind to and cleave the cellular mRNA 5’ cap, thereby yielding cap-less decoys for shielding the also cap-less L-A transcript from RNase attack (Masison et al., 1995). Once the virus-encoded RNA-dependent RNA polymerase (RdRp) and other viral proteins are produced, viruses, owing to their gene-poor nature, face another layer of challenge in effectively organizing an RNA replication complex by recruiting host factors for producing the negative-strand RNA from the positive-strand template. Classic examples of this borrowing act include the bacteriophage Qß, which incorporates EF-Ts and EF-Tu and the ribosomal protein S1 into its replicase and vesicular stomatitis virus, whose RdRp binds strongly to EF-1 (homologous to the bacterial EF-Tu), which in turn binds EF-1β and 1 (both homologous to EF-Ts) (Blumenthal and Carmichael, 1979; Das et al., 1998). Remarkably, these and other examples, including those of the BMV (Diez et al., 2000; Noueiry and Ahlquist, 2003; Noueiry et al., 2000; Quadt et al., 1993) and the tobacco mosaic virus (TMV) (Osman and Buck, 1997; Taylor and Carr, 2000), appear to indicate that components of the host translation machinery are favored
targets for viral recruitment. In this work, I provide a novel example that appears to be consistent with this general proposal.

4.1 Biological significance of Ded1p and Gag interaction

The earlier work from our lab showed that Ded1p, a highly abundant cytoplasmic DExD/H-box protein, is a novel translation initiation factor (Chuang et al., 1997). However, its mechanistic role in translation remains to be defined. To examine Ded1p’s role further, we sought to identify its interacting proteins. We unexpectedly discovered that Ded1p, an evolutionarily conserved DExD/H-box translation factor, binds specifically and tightly to the yeast L-A virus particle and, in doing so, promotes the L-A negative-strand RNA synthesis in vitro. Although work is still in progress to demonstrate that Ded1p is required for L-A replication in vivo, several lines of evidence have already implicated Ded1p in some aspects of the viral role. First and foremost, a recessive ded1 allele (ded1-18), isolated from a genetic screen aiming to identify host factors required for BMV replication, represses BMV replication in yeast by selectively inhibiting BMV RNA2 translation without impacting on general cellular translation or translation of BMV RNA1 (Noueiry et al., 2000). Thus, Ded1p must play a role in promoting BMV
RNA2 translation, at least in yeast. Since Ded1p is highly conserved among eukaryotes, its involvement in BMV’s replication in plants is almost certainly expected. Second, DBX (DDX3 or CAP-Rf), Ded1p’s human ortholog (54% sequence identity), was found by three independent studies (Mamiya and Worman, 1999; Owsianka and Patel, 1999; You et al., 1999) to interact directly and strongly with the HCV core protein, reminiscent of Ded1p’s interaction with the Gag protein. While the physiological significance of this interaction remains to be elucidated, the apparent evolutionary relatedness among positive-strand RNA viruses and dsRNA viruses argues for the case. For example, an evolutionary link between dsRNA viruses and flaviviruses, including HCV, has been proposed on the basis of the striking similarity in the catalytic side-chain positions, overall molecular architecture and topology, and other shared biochemical properties of their respective RdRp’s (Butcher et al., 2001). In addition, recent studies on the assembly of the BMV replication complex by Schwartz et al. (Schwartz et al., 2002) clearly demonstrated that positive-strand RNA viruses, dsRNAs viruses, and retroviruses share fundamental similarities in replication and, therefore, may have common evolutionary origins. The fact that Ded1p is implicated in all classes of viruses that replicate through mRNA intermediates raises the tantalizing possibility
that it may be widely favored among these RNA viruses and therefore may represent a viable target for antiviral therapeutic intervention.

4.2 Possible Ded1p’s binding site on the L-A virus particle

At the moment, we do not know how many molecules of Ded1p interact with each L-A virus particle. Calculation done in this lab indicates that, for many actively growing laboratory yeast strains, there are \( \sim 5.3 \times 10^4 \) and \( \sim 1.2 \times 10^5 \) molecules of Ded1p and Gag per cell, respectively. In addition, it has been reported that overexpression of Gag driven by inducible \( \text{GAL1} \) promoter could lead to its constitution of 0.5% of total protein (R. Wickner, personal communication). Thus, Gag can in practice be nearly 100-fold more abundant than that of Ded1p. Assuming that each Gag molecule is capable of binding to Ded1p in a 1:1 stochiometric ratio, then there will be essentially no free Ded1p available in the cell. Under such a situation, if this interaction abolishes Ded1p’s activity in translation, the host translation would be brought to a halt. Yet, there is no evidence that the L-A virus imposes any selective disadvantage on its host. In contrast, the presence of the toxin-producing M₁ virus may even be beneficial to its host in the wild for eliminating competitor cells harboring no M₁ virus.
The fact that Ded1p can interact with the whole L-A particle (Figure 3.7) makes it conceptually difficult to imagine that all 120 Gag proteins ($1.2 \times 10^5$ per cell) on a single L-A virion interact with Ded1p in a 1:1 ratio. Furthermore, it has been reported that Gag alone is sufficient to form empty virus particle (Fujimura et al. 1992) leaving little, if any, free Gag in the cell (R. Wickner, personal communication). Taken together, an alternative hypothesis would seem more plausible. That is to assume that each of the 1,000 L-A virions in the cell can only bind to perhaps one molecule of Ded1p. If so, a vast majority of Ded1p (~98%) would still remain unbound, consistent with the lack of deleterious effect imposed by the presence of Gag. Given that, a priori, all Gag are presumed structurally equivalent on a single virion, what, then, determines which Gag is to bind to Ded1p on the virus particle? One alternative, but attractive, untested hypothesis is that the binding of Ded1p to virion is mediated preferentially through the “unique” Gag-Pol fusion protein, which is responsible for the positive-strand RNA synthesis. As a result, Ded1p would be “tethered” to the emerging transcript for its immediate employment.
4.3 Working models for Ded1p’s role in L-A negative-strand RNA synthesis

Many RNA viruses encode DExD/H-box or helicase-like proteins, such as NS3 (HCV), 2C (poliovirus), and 1a (BMV), in their genomes. These helicase-like proteins are thought to participate in viral replication via their putative RNA helicase activities because some can unwind short RNA duplexes \textit{in vitro}. Yet, their precise roles in the viral life cycles remain largely a mystery. For example, NS3 turns out to be a highly processive helicase on DNA, but a poor helicase on RNA, thus prompting a speculation on its role in influencing host DNA (Pang et al., 2002). Perhaps this offers a partial explanation as to why viruses may need to recruit another DExD/H-box protein, such as Ded1p, for their replication. What, then, could possibly be Ded1p’s role in RNA virus replication? Previous work by Noueiry \textit{et al.} (Noueiry \textit{et al.}, 2000) on BMV replication provides some interesting clues. They showed that the dependency of BMV RNA2 translation on Ded1p is correlated with the presence a 31-nucleotide region found only in RNA2. Thus, a logical prediction would be that Ded1p exerts its function on this region by resolving a putative inhibitory RNA structure. This model fits well with the conventional view that DExD/H-box proteins bind directly to and
unwind specific RNA duplexes and that eIF4A, the prototypical DExD/H-box translation factor, is responsible for unwinding stable RNA structures in the 5’ untranslated region to promote mRNA translation (Tanner and Linder, 2001). In this light, a plausible model for Ded1p’s role in L-A replication would be that Ded1p modulates the positive-strand RNA structure to facilitate the negative-strand RNA synthesis. Implicit in this model is the notion that Ded1p must be able to reach the positive-strand L-A RNA molecule encapsidated in the virus particle. Structural studies of the L-A virus (Caston et al., 1997; Naitow et al., 2002) revealed that the capsid wall of the particle is perforated by openings of 18 Å in diameter at the icosahedral five-fold axes. These openings are thought to function as molecular sieves to allow the exit of transcript and the exchanges of metabolites, while retaining dsRNA and excluding degradative enzymes. Although we have yet to completely rule out the possibility that a fraction of purified L-A particles were damaged, thereby allowing Ded1p to access the L-A RNAs, the fact that the affinity- and CsCl-purified particles are resistant to RNase treatment (Figure 3.8) and that the CsCl-purified particles, which were used for structural analysis by other groups, bind to GST-Ded1p (Figure 3.6) argues that Ded1p most likely binds to the virus particle externally.
If so, it would be difficult to envisage how Ded1p directly touches the L-A RNAs.

An alternative model would be to evoke a recently emerged “RNPase” hypothesis (Schwer, 2001; Will and Luhrmann, 2001) built on several lines of experimental evidence. First, biochemical studies of eIF4A suggest that the DExD/H-box proteins may perform functions distinct from RNA unwinding, which include mediating large-scale RNA structural rearrangements, disrupting protein-RNA or protein-protein interactions, and functioning as fidelity sensors in RNA-RNA interactions and rearrangements (Lorsch and Herschlag, 1998a; Lorsch and Herschlag, 1998b). Second, it was demonstrated that the essential requirement of two DExD/H-box splicing factors, Prp28p and Sub2p, can be eliminated by specific mutations that, by genetic definition, define their corresponding *in vivo* protein targets (Chen et al., 2001; Kistler and Guthrie, 2001). Third, the DExD/H-box protein NPH-II from vaccinia virus can displace the protein U1A from RNA in an active adenosine triphosphate-dependent fashion (Jankowsky et al., 2001). On the basis of these studies, it was proposed that DExH/D proteins may act as “RNPases” to reorganize, or “remodel”, the structures of ribonucleoprotein assemblies. Thus, it is tempting
to speculate that Ded1p’s recruitment by the positive-strand RNA-containing L-A particles may result in conformational changes of the viral particles, which in turn influence the L-A Gag-Pol activity to favor the negative-strand synthesis. Alternatively, Ded1p may specifically modify Gag-Pol alone to yield the same outcome. To differentiate these possibilities, works are in progress to map the contact points and to determine the stoichiometric ratio between Ded1p and the L-A particle.

4.4 Prospectus

All together, this study shows that Ded1p binds to the L-A particles and accelerates its negative-strand RNA synthesis in vitro. However, it remains formally possible that Ded1p may also have a role in promoting L-A translation, because it is a general translation factor (Chuang et al., 1997; de la Cruz et al., 1997). Unpublished biochemical analysis done by Dr. Ray-Yuan Chuang, a former graduate student in the lab, suggests that Ded1p plays a critical role prior to the formation of 43S translation initiation complex, because depletion of Ded1p results in a dramatic buildup of mRNPs (Chuang, 1997b). Critically, Ded1p appears to function in a cap- and poly(A)-tail-independent manner (Chuang, 1997b). This latter observation is of particular interest and
may provide an underlying rationale for the specific recruitment of Ded1p, among other equally abundant cytoplasmic DExD/H-box proteins, by L-A, because L-A transcript possesses neither 5’ cap nor 3’ poly(A) tail. Future insights into Ded1p’s mechanistic role in translation initiation may shed new lights on its viral role.

It is critical to note that, despite my work has shown that Ded1p is capable of accelerating L-A negative-strand RNA synthesis \textit{in vitro}, it remains to be established that is also the case \textit{in vivo}. To firmly show that Ded1p is a host factor that promotes L-A replication \textit{in vivo}, one needs to isolate specific \textit{ded1} mutant alleles that affect only L-A replication without impacting on Ded1p’s essential role in cellular translation. Finally, this thesis work has paved the road for future studies on the function of human DBX in the HCV life cycle. If indeed the binding of DBX to the HCV core protein is important for HCV replication, screening of small compounds aiming to interfere with this interaction may lead to development of promising therapeutics to treat HCV infection in the future.
5.1 Introduction

The gene expression pathway in the eukaryotic cells spans two separate compartments, the nucleus and the cytoplasm. Upon its synthesis in the nucleus, precursor messenger RNA (pre-mRNA) must undergo a series of covalent modifications to produce mature mRNAs. These nuclear modification processes include 5’ capping, the removal of introns or splicing, and 3’ end processing and polyadenylation. Mature mRNAs are then exported into the cytoplasm for protein synthesis or translation. Conventionally and conceptually, all these modification processes, such as splicing, export, and translation, have been considered and treated as functionally discrete steps leading to the final production of protein. However, a growing body of evidence emerged in recent years has strongly argued otherwise. In this apparent paradigm shift, the machineries involved in the gene expression
pathway are thought to be both kinetically and functionally coupled, although this coupling appears not be obligatory because most of the steps in the gene expression pathway can be individually studied *in vitro*. Accordingly, it is believed that the observed coupling of these processes probably exists to offer proofreading and to streamline the gene expression *in vivo*. Consistent with this recent paradigm shift, in this Chapter, I will provide preliminary evidence that Ded1p, a known cytoplasmic translation factor, may also have a nuclear role in influencing the splicing events in the nucleus.

### 5.1.1 Pre-mRNA splicing

Conserved sequence elements within the primary RNA transcript provide the signals required for precise intron removal (Staley and Guthrie, 1998). These sequences include 5’ and 3’ splice sites (SS), a branch point sequence containing a strictly conserved adenosine residue, and a stretch of pyrimidines that is located between the branch point and the 3’ splice site. The 5’ SS sequence signal in yeast is almost always GUAGU while in humans only the first two positions (i.e., GU) are very highly conserved. Similarly, the branch point sequence found in yeast introns is almost always UACUAAC, but the sequence is very degenerate in mammalian introns.
In both yeast and mammals, the 3' SS signal is rather short, consisting of a pyrimidine (U or C) followed by AG. The differences in signal sequence conservation likely reflect the relative complexity of the systems.

Splicing occurs by a two-step trans-esterification mechanism (Madhani and Guthrie, 1994; Staley and Guthrie, 1998). In the first step, the phosphodiester bond at the 5' SS is cleaved by a nucleophilic attack by the 2' hydroxyl group of the conserved adenosine (A) located at the intron branch point. This generates a 2'-5' phosphodiester bond between the branch site and the 5' end of the intron as well as a free 3' hydroxyl group on the 5' exon. The cleavage also results in two RNA molecules, including a free exon 1 and a “lariat” intron-exon 2. In the second step, the free 3' hydroxyl on the end of the 5' exon attacks the phosphodiester bond at the 3' splice site, resulting in the joining of the exons and release of the lariat intron.

5.1.1.1 Spliceosome assembly

Splicing is accomplished in a complex cellular machine called the spliceosome, which orchestrates the removal of introns from pre-mRNAs (Staley and Guthrie, 1998). Since the discovery that a large complex was responsible for splicing, the list of spliceosome components has grown to
include five small nuclear RNAs (snRNAs) and over 75 proteins. Each of the five snRNAs (U1, U2, U4, U5 and U6) is found in a complex with a number of proteins to form small nuclear ribonucleoprotein particles or snRNPs. Non-snRNP proteins are also required for splicing and are likely to interact with other splicing components only transiently (Staley and Guthrie, 1998).

Studies in both the yeast and mammalian systems revealed a cycle of spliceosome assembly and disassembly on a pre-mRNA (Figure 5.1). The first step that commits a pre-mRNA to the splicing pathway is recognition of the 5'SS by the U1 snRNP, initiating the commitment complex (CC) in yeast and the early (E) complex in mammals. The initial formation of the commitment complex 1 (CC1) (Seraphin and Rosbash, 1989; Seraphin and Rosbash, 1991) is involved in primarily the recognition of 5' SS, which is then followed by binding of the branchsite binding protein (BBP) (or the yeast Msl5p) and U2AF65 (or the yeast Mud2p) respectively to the branch site and the polypyrimidine tract within the intron to form the CC2 complex (Abovich and Rosbash, 1997; Zamore and Green, 1989). After CC2 formation, Mud2p and Msl5p are replaced by the binding of the U2 snRNP to the branch-site region, forming complex B in yeast (Abovich and Rosbash, 1997; Rain and Legrain, 1997). Addition of the U2 snRNP is the first energy (ATP)-dependent
step in the splicing pathway. Following U2 snRNP binding, the U4/U6.U5 tri-

snRNP particle then joins complex B to form complex A2-1 in yeast or B1 in 
mammals to form a complete pre-spliceosome (Staley and Guthrie, 1998). 
Finally, before splicing reaction can take place, a major remodeling process 
involved in dissociating the U4 snRNP must occur to form the final active 
spliceosome consisting of the remaining U2, U5, and U6 snRNPs.

5.1.1.2 Roles of DExD/H box proteins in pre-mRNA splicing

According to the prevailing model, pre-mRNA splicing in yeast requires 
at least eight DExD/H box proteins: Prp5p, Brr2p, Prp28p, Sub2p (yUAP56), 
Prp2p, Prp16p, Prp22p and Prp43p (Staley and Guthrie, 1998). Although their 
specific targets and mechanisms of function remain unclear, the stages of 
spliceosome maturation requiring each factor have been delineated and 
putative RNA/RNA targets have been identified.

The first RNA/RNA duplex formed during commitment of a pre-mRNA 
to splicing is the base pairing between the 5’ end of the U1 snRNA and the 5’ 
SS (Seraphin and Rosbash, 1989). Before the first catalytic step, this 
interaction is disrupted and replaced by a mutually exclusive interaction 
between the U6 snRNA and the 5’ SS (Staley and Guthrie, 1998). This
rearrangement requires not only the unwinding of the U1 snRNA/5’ SS duplex but also the disruption of the extensive base pairing between the U4 and U6 snRNAs. Prp28p is required for the switch of U1 for U6 at the 5’ SS and is thought to disrupt the early interaction between the U1 snRNP and the 5’ SS (Chen et al., 2001). Prp28p may also disrupt the interaction between the U1-C protein and the pre-mRNA, supporting the model of DExD/H box proteins as RNPases. The function of Prp28p at the 5’ SS appears to be coupled to the function of Brr2p, another DExD/H protein thought to disrupt the U4/U6 RNA duplex (Raghunathan and Guthrie, 1998). This rearrangement also releases the U4 snRNP, releasing it from the spliceosome at approximately the same time that the U1 snRNP is released.

The second step during spliceosome assembly is binding of the U2 snRNP to the branch site, an association that is the first energy-requiring step during splicing. Two DExD/H box proteins, Prp5p and Sub2p (mammalian UAP56), are required for U2 snRNP binding to the branchpoint (Kistler and Guthrie, 2001; Ruby et al., 1993). In a manner similar to Prp28p counteracting U1-C, Sub2p has been proposed to displace Mud2p, a protein that initially binds to the branch point and must be displaced to allow binding of the U2 snRNP.
snRNP (Kistler and Guthrie, 2001). Prp5p is thought to modulate an ATP-dependent conformational change in the U2 snRNA, possibly exposing the branch point recognition sequence within the U2 snRNA to facilitate binding to the branch point (Wells and Ares, 1994).

Following binding of U2 and the ensuing rearrangements that release U1 and U4 and allow pairing between U6 and U2, two additional DExD/H box proteins catalyze rearrangements that lead to spliceosome activation. The first is Prp2p, a factor that interacts transiently with the spliceosome through Spp2p and is required to function before the first catalytic step (Kim and Lin, 1996). The second is Prp16p, a protein that also interacts with the spliceosome only transiently (Schwer and Guthrie, 1991). Prp16p is required after the release of the U4 snRNP for the second transesterification reaction and affects a rearrangement at the 3’ SS (Schwer and Guthrie, 1992). After both transesterification reactions have taken place, several factors are required for the disassembly of the spliceosome and recycling of factors for subsequent rounds of splicing. The DExD/H box protein Prp22p is involved in disassembly of the spliceosome, aiding in the release of the mature messenger RNA molecule (Company et al., 1991). The final DExD/H box factor assigned a role in the splicing cycle is Prp43p, a protein required for
release of the lariat intron from the spliceosome (Martin et al., 2002). After its release, the lariat intron is recognized by the debranching enzyme that recognizes the 2'-5' bond and cleaves it, allowing rapid degradation of the now linear intron. Finally, A mutation in *DED1* was found to suppress the temperature-sensitivity of the *prp8-1* mutation, thus implicating a putative role of Ded1p in splicing. It is noted that among the spliceosomal proteins, Prp8p is the largest and most conserved component that is most likely contribute to the formation of the spliceosomal catalytic center together with other snRNAs.

### 5.1.1.3 Penta-snRNP

The concept of step-wise spliceosome assembly has been supported by numerous studies in yeast and mammalian systems (Parker et al., 1987; Seraphin and Rosbash, 1989; Staley and Guthrie, 1998). However, many observations are difficult to reconcile within this assembly model. For example, distinct spliceosomal intermediates were studied under salt concentrations that are incompatible with the physiological condition (Stevens et al., 2002). In addition, it has been reported that U4/U6.U5 tri-snRNP can be crosslinked to the 5’ splice site without the base pairing between pre-mRNA and U1 or U2 snRNPs (Maroney et al., 2000).
Interestingly, in this regard, Stevens et al. has identified and purified a functional splicing complex, termed penta-snRNP, containing all the snRNPs present in the active spliceosome (Stevens et al., 2002). They found that the U4/U6.U5 tri-snRNP could be purified using a buffer with 250 mM KCl. However, when the monovalent salt was lowered to 150 mM, a U2.U4/U6.U5 tetra-snRNP was instead isolated. Remarkably, at 50 mM salt concentration, a functional U1.U2.U4/U6.U5 penta-snRNP was purified. It was thus hypothesized that, in vivo, splicing may proceed by directly binding of a preassembled spliceosome (i.e. the penta-snRNP) to the pre-mRNA substrate and that the stepwise assembly process may reflect stepwise stabilization of interactions, not stepwise recruitment of components.

5.1.2 Coupling of the gene expression pathway

The now prevailing functional coupling model for the gene expression pathway was built on numerous experimental findings, which I will briefly summarize as follows. First, components functioning in one process were often co-purified or co-localized with components involved in other processes. For example, the 5’-end capping and 3’-end formation machineries are physically associated with the C-terminal domain (CTD) of the largest subunit
of RNA polymerase II (Cho et al., 1998; Zhao et al., 1999). Additionally, partially spliced transcripts still attaching to transcribing RNA polymerase II were found to co-localize with splicing factors, strongly suggesting splicing in higher eukaryotes occurs co-transcriptionally (Misteli and Spector, 1999; Osheim et al., 1985). Second, certain factors required in one process proved to be functionally indispensable for another process both in vivo and in vitro. Such a functional linkage has been reported among transcription, pre-mRNA splicing, and mRNA export. A prominent example is Sub2p, a DExD/H-box protein originally shown to be required for splicing (Kistler and Guthrie, 2001). Later studies unexpected found that Sub2p is also required for mRNA export and that both Sub2p and Yra1p, a mRNA export factor, are associated with the THO complex, which is involved in transcription elongation (Strasser et al., 2002). Moreover, the THO complex also genetically interacts with YRA1 and SUB2, and null mutations in any of the THO complex subunits are defective in mRNA export (Strasser et al., 2002). As a result, the THO complex together with Yra1p and Sub2p are now called the TREX complex. A third example is the link between transcription and RNA turnover. It was found that Spt5p and Spt6p, transcription elongation factors, co-purified with the exosome, whose function is to degrade RNAs (Andrulis et al., 2002). Therefore, it was
proposed that the exosome was recruited to the transcription machinery at an early stage for the degradation of improperly processed pre-mRNAs. Taken together, growing evidence suggests that the coupling of processes may be common in vivo.

5.1.3 Nuclear translation?

The concept that translation occurs exclusively in the cytoplasm faced few serious challenges over the years. Yet, there are observations that make this “textbook” statement slightly unsettled. For example, some translation factors, such as eIF4E, are also present in the nucleus and ribosomal proteins and translation factors could be co-immunoprecipitated and co-localized with spliceosome and transcription machineries (Dostie et al., 2000; Lund and Dahlberg, 1998). Furthermore, studies of nonsense-mediated mRNA decay (NMD) pathway have raised the possibility of a translation-like mechanism in the nucleus (Maquat and Carmichael, 2001). NMD is a cellular mechanism for eliminating mRNAs with premature stop codon. Because the recognition of nonsense codon ought to occur at the translation level, NMD is naturally expected to take place in the cytoplasm (Maquat and Carmichael, 2001). However, studies have indicated that NMD may have occurred while mRNA is
still in association with the nucleus. A prominent example is that nonsense
mutations can affect pre-mRNA splicing efficiency in vivo (Carter et al., 1996;
Cheng and Maquat, 1993; Dietz et al., 1993). Since it is conceptually difficult
to envision translation, a cytoplasmic event, can impact on an early step in
RNA processing, it has been proposed that a translation pathway may exist in
the nucleus (Dahlberg et al., 2003). While this subject remains highly
controversial, recently data supporting this contention were reported, in that
protein synthesis was found to occur in the mammalian nuclei (Iborra et al.,
2001) and ribosome components, including ribosomal RNAs, are associated
with active transcription sites in Drosophila salivary gland chromosomes
(Brogna et al., 2002).

Ded1p is a translation initiation factor shown to predominately localize
in the cytoplasm in Saccharomyces cerevisiae (Chuang et al., 1997).
Interestingly, several lines of evidence suggest that Ded1p may have a role in
the nucleus. First, spp81-1, a ded1 mutant allele can suppress the splicing
defect caused by prp8-1, which encodes an altered form of Prp8p, an
essential splicing factor (Jamieson et al., 1991). Second, both DDX3 and An3,
orthologues of Ded1p in human and in Xenopus laevis respectively, shuttle
between the cytoplasm and the nucleus (Askjaer et al., 1999; Owsianka and
Patel, 1999). In line with finding, the yeast Ded1p also contains a N-terminal nucleus export signal (NES) conserved within DDX3 and An3 (Figure 5.2) (Askjaer et al., 1999). Third, the Ded1p homolog in *Chironomus Tentans* was reported to load onto nascent transcripts and accompanies them to enter polysomes in the cytoplasm (Daneholt, 2001). Fourth, Ded1p has been reported to associate with purified splicing machineries in yeast and in human (Stevens et al., 2002; Zhou et al., 2002). In this Chapter, I will report some preliminary data consistent with the idea that a translation factor such as Ded1p may have a nuclear role, in particular in the splicing pathway.
Figure 5.1. The spliceosome formation cycle. The spliceosome is assembled in a highly ordered and stepwise manner by adding snRNPs onto pre-mRNA. After undergoing a series of rearrangements, the catalytic reactions occur and components are then released. snRNPs are recycled for subsequent assembly. Each complex is named according to the yeast terminology. The mammalian complex is noted in parentheses.
Figure 5.1.
Figure 5.2. A conserved NES sequence in Ded1p homolog family. The conserved N-terminal NES sequence in Ded1p homolog family is revealed by alignment of the N termini of *Xenopus* AN3, human DBX, mouse PL10, *S. cerevisiae* Ded1p and Dbp1p. The leucine-rich NES consensus sequence is shown at the bottom. The * sign denotes amino acids M, V, I, L, F, or W.
5.2 Materials and methods

5.2.1 Yeast strains

All yeast strains used in this work are in an isogenic background. They are listed in Table 5.1.

5.2.2 plasmids and oligomers

All plasmids used in this study are listed in Table 5.2. Oligomers are listed in Table 5.3.

5.2.3 Glycerol gradient centrifugation

Cell extract was prepared as in affinity purification method in chapter 2.3. An aliquot of cell extract containing 250 µg of total proteins was loaded on the top of 10—25% glycerol gradient containing 10 ml of buffer C (100 mM KCl, 20 mM Tris-HCl [pH 7.5], 5 mM MgCl₂) and then subjected to centrifugation at 36,000 r.p.m. at 4°C for 20 hr in a SW41 rotor (Beckman). The gradient was fractionated in 250-µl aliquots from bottom to top. Proteins in even-number fractions were precipitated by 10% trichloroacetic acid and then analyzed in SDS-PAGE.
5.2.4 *In vitro* binding assay

Cell extracts made from strains containing Imd2p-TAP, Imd3p-TAP, or Imd4p-TAP individually were prepared as in affinity purification method in chapter 2.3. An aliquot of clarified cell extract containing 10 mg of total proteins was then added to 20 µl of glutathione beads pre-coated with either GST-Ded1p or GST in a binding buffer (150 mM KCl, 20 mM Tris-HCl [pH 7.5], 5 mM MgCl₂, 0.1 % Triton X-100). After incubation at 4°C for 2 hr on a nutator, the beads were extensively washed 10 times, each with 5 ml of the binding buffer. Beads were boiled in SDS-PAGE loading dye and analyzed by Western blot.

5.2.5 Preparation of splicing extract

Cells were collected at late log phase (OD600 ~2-3) by centrifugation in a cold GS3 rotor at 4,500 r.p.m. at 4°C for 15 min. Cells were then resuspended in 200 ml of cold buffer AGK (10 mM HEPES [pH 7.9], 1.5 mM MgCl₂, 200 mM KCl, 10% glycerol, 0.5 mM DTT). After centrifugation, cell pellets were resuspended in 20 ml of AGK buffer and transferred to 50-ml Falcon tubes. Cells were collected again by centrifugation in a GS3 rotor at
3,000 r.p.m. at 4°C for 15 min) and then 0.4 volumes of AGK buffer with protease inhibitors (Roche) was used to resuspend cells. The suspension was frozen by liquid nitrogen immediately and stored at −80°C. Frozen cells were then taken out from −80°C into pre-cooled mortar and broken by a lot of brute force of pounding and grinding until cells become a very fine powder. After grinding, the powder was collected and thawed in a room temperature water bath with gentle swirling. Once thawed, cell extract was spun in a SS34 rotor at 1,700 r.p.m. at 4°C for 30 min. The clarified cell extract was immediately removed into a 60 Ti ultracentrifuge tube and spun in a Ti 70.1 rotor (Beckman) at 38,000 r.p.m. at 4°C for one hour. After centrifugation, two thirds of the clear pale yellow liquid in the middle of the tube was transferred into dialysis tubing for dialysis against 4 liters of buffer D (20 mM HEPES [pH 7.9], 2 mM EDTA, 50 mM KCl, 20% glycerol, 0.5 mM DTT) for 3 hr. The dialyzed splicing extract was spun to remove sediments and stored at −80°C for use.

5.2.6 In vitro transcription

A plasmid with an intron-containing actin DNA was linearized with EcoRI at its 3’ end. The transcription reaction was set up with 1.5 µg of
linearized DNA, 1X T7 Transcription Buffer (NEB), 10 mM DTT, 10 mM NTP, 15 Units of RNasin (Promega), 3 µl of α-[³²P]-UTP (3000Ci/mmol), and 1.5 µl of T7 RNA Polymerase (Gibco). After 2 hr incubation at 37°C, 15 µl of formamide dye was added and the reacting tube was heated at 65°C for 3 min to denature newly synthesized transcripts. The transcripts were then loaded into a 5% polyacrylamide gel with 8M urea and run in 1X TBE at 500 V until xylene cyanol was 3-4 cm from the bottom. The sliced gel containing transcripts was transferred into 2 eppendorfs with 500 µl elution buffer (500 mM NaOAc [pH 5.0], 1 mM EDTA [pH 8.0]) and stored at 4°C overnight. The next day, the eppendorfs were inverted into a poly-prep chromatography column (BioRad) in a 50 ml falcon tube and spun to remove the gel debris. The spun liquid was extracted by phenol/chloroform (pH 5.2) and precipitated by ethanol. The [³²P]-transcripts were then resuspended in water and standardized to 60,000 cpm/µl for an in vitro splicing assay or to 200,000 cpm/µl for analysis of spliceosome formation on native gel electrophoresis.

5.2.7 In vitro splicing assay

The splicing reaction was set up with 4 µl of splicing extract in a 10-µl reaction (60 mM KPO₄ [pH 7.0], 3% PEG 8000, 2.5 mM MgCl₂, 2 mM ATP, 1
mM spermidine, [$^{32}$P]-labeled pre-mRNA transcript [60,000 cpm]) at 25°C for 20 min or 16°C for one hour. Subsequently, a 200-µl splicing extraction buffer (50 mM Na-Acetate, 1 mM EDTA [pH 8.0], 0.1% SDS) and 200 µl of phenol/chloroform (pH 4.8) were added for extraction of RNAs. RNAs were precipitated by ethanol and dissolved in 3 µl of ddH₂O and 7 µl of sequencing gel loading dye. After incubation at 65°C for 2 min and then on ice for 2 min, an aliquot of 5-µl suspension was loaded on 8% polyacrylamide gel with 8 M urea and run in 1X TBE at 500V until xylene cyanol to approximately 1-2 cm from the bottom. The gel was transferred onto 3 MM paper for autoradiography.

5.2.8 Affinity purification of snRNAs

A 20-µl splicing extract was incubated with 5 µl of IgG Sepharose 6 Fast Flow (Pharmacia) in an eppendorf and gently nutated for one hour at 4°C. The beads were then washed in buffer D (20 mM HEPES [pH 7.9], 2 mM EDTA, 50 mM KCl, 20% glycerol, 0.5 mM DTT) with 0.05% NP-40 and the bound material was extracted with phenol/chloroform (pH 5.2) and ethanol precipitated. The extracted RNA was analyzed on 6% polyacrylamide gel with 8 M urea and then transferred onto Nylon membrane for Northern analysis to
detect snRNAs. Templates of snRNAs for random-primer labeling were synthesized from Polymerase Chain Reaction (PCR) and primers used for PCR are listed in Table 5.3

5.2.9 Affinity purification of active spliceosome

A 50-µl splicing reaction with exogenous [³²P]-labeled pre-mRNA transcripts was assembled and incubated at 25°C for 15 min. The reaction was transferred into an eppendorf with 5 µl of IgG Sepharose 6 Fast Flow (Pharmacia) and gently nutated at 4°C for one hour. The beads were washed 5 times, each with 1 ml of buffer D (20 mM HEPES [pH 7.9], 2 mM EDTA, 50 mM KCl, 20% glycerol, 0.5 mM DTT) with 0.05% NP-40 and the bound material was extracted with phenol/chloroform (pH 5.2) and ethanol precipitation. The extracted transcripts were analyzed on 6% polyacrylamide gel with 8 M urea for autoradiography.

5.2.10 Commitment Complex and Spliceosome in Native Gel electrophoresis

Spliceosome were assembled in a 5-µl reaction containing 2 µl of splicing extract, 2 µl of splicing salts (150 mM K-phosphate [pH 7.0], 6.25 mM
MgCl₂, 7.5% of PEG 6000), 2 mM ATP, [³²P]-labeled pre-mRNA (100,000 cpm). After incubation at 25°C for 20 min or 16°C for one hour, 2.5 µg of yeast transfer-RNA (Sigma) in R buffer (2 mM magnesium acetate, 20 mM EDTA, 50 mM HEPES [pH 7.5]) was added to dissociate nonspecific binding proteins from spliceosome for 10 min at 4°C. Then the loading buffer (50% glycerol, 2.5X TBE, 0.1% bromphenol blue) was added and spliceosome were analyzed in a native gel (3% acrylamide [50:1], 0.5% agarose, 0.5 X TBE, 4.15% glycerol) with 0.5X TBE at 70V for 20 hr at 4°C. Commitment complexes were analyzed in the same native gel, except the reaction was assembled in a 5-µl reaction containing 2 µl of splicing extract, 2 µl of splicing salts (150 mM K-phosphate [pH 7.0], 6.25 mM MgCl₂, 7.5% PEG 6000), [³²P]-labeled pre-mRNA (100,000 cpm), and 125 ng RB60.

5.2.12 Microscopy

To image the Ded1p-GFP, YTC555 and TYC954 were grown in synthetic medium lacking tryptophan and adenine to early-log phase (OD₆₀₀ = 0.4). Cells were harvested, stained with DAPI (1.5 µg/ml) at room temperature for 10 min, washed three times with 1 ml sterile distilled water, and then spotted onto glass slides for imaging using an FITC filter. For treatment with
Leptomycin B, 0.1 µg/ml of Leptomycin B (Sigma) was added into YTC825, which contains a *crm1* mutant allele sensitive to Leptomycin B, for 60 min at room temperature before DAPI staining.
**Table 5.1**

**Strains:**

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<th>Strain</th>
<th>Genotype</th>
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<tbody>
<tr>
<td>YTC127</td>
<td>MATa</td>
<td>ded1::TRP1 ura3-52 lys2-801 ade2-101 trp1-Δ1 his3Δ200 leu2-Δ1 pDED1009</td>
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<tr>
<td>YTC142</td>
<td>MATa</td>
<td>ded1::TRP1 ura3-52 lys2-801 ade2-101 trp1-Δ1 his3Δ200 leu2-Δ1 pDED1018</td>
</tr>
<tr>
<td>YTC143</td>
<td>MATa</td>
<td>ded1::TRP1 ura3-52 lys2-801 ade2-101 trp1-Δ1 his3Δ200 leu2-Δ1 pDED1019</td>
</tr>
<tr>
<td>YTC212</td>
<td>MATa</td>
<td>ura3-52 lys2-801 ade2-101 trp1-Δ1 his3Δ200 leu2-Δ1 ded1::TRP1 pDED1033</td>
</tr>
<tr>
<td>YTC825</td>
<td>MATa</td>
<td>crm1::KAN leu2 his3 trp1 ura3 pDC-CRM1T539C (LEU2/CEN) pDED1117</td>
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</tbody>
</table>

Continued
Table 5.1 continued

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<th>Strain</th>
<th>Genotype</th>
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<tr>
<td>YTC555</td>
<td>MATa ded1::TRP1 ura3-52 lys2-801 ade2-101 trp1-Δ1 his3Δ200 leu2-Δ1 pDED1058</td>
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<tr>
<td>YTC954</td>
<td>MATa ded1::TRP1 ura3-52 lys2-801 ade2-101 trp1-Δ1 his3Δ200 leu2-Δ1 pDED1060</td>
</tr>
</tbody>
</table>
Table 5.2

**Plasmids:**

- pDED1018 Derived from pDED1009 by hydroxylamine mutagenesis, carrying *ded1-120* mutant allele (mutations = G108D and G494D).
- pDED1019 Derived from pDED1009 by hydroxylamine mutagenesis, carrying *ded1-199* mutant allele (mutation = G368D).
- pDED1008 A 2.9 kb *XhoI-SalI* fragment containing the entire length of the *DED1* gene isolated from pUC-Sc2605 (gift from K. Struhl) was cloned into pRS316/*XhoI*+
- pDED1009 A 2.9 kb *XhoI-SalI* fragment containing the entire length of the *DED1* gene isolated from pUC-Sc2605 (gift from K. Struhl) was cloned into pRS315/*XhoI*+

Continued
Table 5.2 continued

pDED1117  \( \text{ded1}\Delta\text{NES-GFP/pASZ11 ded1}\Delta\text{NES-GFP} \) allele in a \( Smal-Xhol \)
fragment isolated from pDED1058 was recloned into \( Smal-SalI \)
sites of pASZ11 (\( \text{ADE2/CEN} \)); (NES deletion of ded1 with GFP)
pDED1033  A 3.4 kb DNA fragment (\( Xhol-PstI \)) containing DED1/ProtA
fusion gene from pDED1032 was cloned into pRS315 (\( Xhol-PstI \))
pDED1058  The pDED1057 was cut by \( \text{AatII} \) to remove ProA and replaced
by GFP. The orientation of this GFP clone was checked by \( PvuII \)
(NES deletion of ded1 with GFP)
pDED1060  The pDED1033 was cut by \( \text{AatII} \) to remove ProA and replaced
by GFP. The orientation of this GFP clone was checked by \( pvuII \)
(wild type DED1 with GFP)
Table 5.3

**Oligomers:**

U1-1: GAATGGAAACGTCAGCAAACAC for PCR of U1 snRNA DNA
U1-2: GACGTTAAGCATTCTCATTTGAAC for PCR of U1 snRNA DNA
U2-2: CTTCCTCTTGCAGCCACCAG for PCR of U2 snRNA DNA
U2-3: ATAGTAGGGTTGGGAGATATTATCCGAG for PCR of U2 snRNA DNA
U4-4: CGCATATCAGTGAGGATTCGTCCGAG for PCR of U4 snRNA DNA
U4-5: CCCTACATAGTCTTGAAG for PCR of U4 snRNA DNA
U5-1: ACACCCGGATGGTTCTGGTA for PCR of U5 snRNA DNA
U5-2: TTACAGATCAATGGCGGGAGGGAGTC for PCR of U5 snRNA DNA
RB60: CAGATACTACACTTTG for commitment complex formation
5.3 Results

5.3.1 Ded1p is present in a 400-kDa complex

We have previously shown that Ded1p is a novel translation initiation factor (Chuang et al., 1997). However, Ded1p’s mechanistic role in translation initiation remains to be defined. To examine Ded1p’s role further, I sought to identify its interacting proteins. In Chapter 3, I describe the unexpectedly finding that Ded1p binds to the yeast L-A virus particles and promotes L-A’s negative-strand RNA synthesis in vitro (Chong et al., 2004). Because the L-A Gag protein is one of the most abundant proteins in the cell, I decided to pursue the question in a different way using yeast strains cured of the L-A virus. L-A virus can be cured from its host strains by repeatedly growing the yeast strains at 39°C (Weinstein et al., 1993). Such L-A-minus yeast strains were used in studies described below.

To begin to examine whether Ded1p binds to other cellular proteins, I prepared yeast extracts from the L-A-minus strain and subjected them to 10—25% glycerol gradient centrifugation analysis. The majority of Ded1p remained on the top of the gradient, indicating that Ded1p (a 68-kDa protein) primarily exists in the free form (Figure 5.3). However, a minor fraction of
Ded1p was found to sediment to a position measured ~400 kDa, suggesting that these Ded1p molecules may be in association with other cellular proteins. The finding that a fraction of Ded1p exists in a large complex thus formed the experimental basis for me to pursue cellular proteins that may interact with Ded1p.

5.3.2 Ded1p is associated with Imd2p, Imd3, and Imd4p

To that end, I used a Ded1p-protein A (PA) strain cured of L-A virus and employed the same experimental approach described in Figure 3.1, Chapter 3. A 55-kDa protein was found to reproducibly co-precipitate with Ded1p-PA (Figure 5.4, lanes 5 and 8). Control experiments using a wild-type \textit{DED1} extract containing no protein-A-tagged Ded1p (lanes 1, 4, 7, 10, and 13) and a Dbp5p-PA extract (lanes 3, 6, 9, 12, and 15) failed to detect the same protein, suggesting that this protein’s association with Ded1p-PA is specific. Stepwise-elution analysis revealed that the association between this 55-kDa protein and Ded1p-PA is of a reasonable affinity, the majority of which could be disrupted by >100 mM MgCl\textsubscript{2} (lanes 2, 5, 8, 11, and 14). To further substantiate this observation, I tested this interaction \textit{in vitro}. Recombinant GST-Ded1p overexpressed in \textit{E. coli} was first purified on the glutathione beads, which was...
then used to incubate with the Ded1p-PA extract. If the 55-kDa protein indeed binds to Ded1p, one would expect that it will also bind to the GST-Ded1p, although the yield would be less in this case due the competition between the endogenous Ded1p-PA and GST-Ded1p. Indeed, a GST-Ded1p-bound protein (Figure 5.5, lanes 3, 7, 11, and 15) was found to co-migrate with the previously identified 55-kDa protein (lanes 4, 8, 12, and 16). Control experiments using the purified GST alone (lanes 1, 5, 9, and 13) and GST-Prp28p (lanes 2, 6, 10, and 14) failed to recover the same protein, again suggesting that this protein’s association with GST-Ded1p is specific. Prp28p is chosen as a control here, because it is also a DExD/H-box protein, which is required in splicing. To examine whether the association of the 55-KDa protein with Ded1p is mediated by RNA, RNase was added to the reaction during incubation (10 µg /ml RNase A, at 30°C for 60 min) and the recovery of the 55-kDa protein was then examined. Interestingly, the 55-kDa protein could no longer be recovered after this treatment, suggesting its association with Ded1p is likely depend on RNA (Figure 5.6, lane 3). Since Ded1p is known to hydrolyze ATP, I also examined the binding behavior of 55-kDa protein in the presence of ATP. Incubation of yeast extract with GST-Ded1p-bound
glutathione beads in the presence of 2 mM ATP appeared to abolish this interaction, resulting in an inability to recover the 55-kDa protein (Figure 5.7).

To pursue the identity of the 55-kDa protein, the purification procedure was scaled up, and the band corresponding to the 55-kDa protein was excised from gel, in-gel digested by trypsin, and the resulting peptides were subjected to mass spectrometry analysis. This analysis matched the masses of five separate peptides to five of the tryptic peptides by prediction derived from inosine 5’-monophosphate dehydrogenase (IMPDH), a rate-limiting enzyme for the de novo synthesis of guanine nucleotide (GTP) (Figure 5.8) (Escobar-Henriques and Daignan-Fornier, 2001; Weber et al., 1992). Yeast contains four IMPDH-like genes (IMD1 [YAR073W], IMD2 [PUR5; YHR216W], IMD3 [YLR432W], and IMD4 [YML056C]) that display 83—96% amino acid identity between themselves when aligned pairwise (Figure 5.9) (Hyle et al., 2003). IMD1 is transcriptionally silent and considered as a pseudogene. Because the identified peptides are within the highly conserved regions among these proteins, it is not possible to distinguish which one(s) is associated with Ded1p.

To confirm the presumed interaction between Ded1p and IMPDHs and to sort out which IMPDHs interact with Ded1p, I asked whether any of the
IMPDHs directly interacts with Ded1p in vitro. Cell extracts were prepared from yeast strains individually expressing C-terminally TAP-tagged Imd2p, Imd3p, and Imd4p (Imd2p-TAP, Imd3p-TAP, and Imd4p-TAP, respectively) and were incubated with glutathione beads pre-coated with either GST-Ded1p or GST. All three IMPDHs could be recovered (Figure 5.10, lanes 6—8), and their yields were significantly above the background (lanes 5 and 9—12). Interestingly, the recovery of Imd3p-TAP and Imd4p-TAP were at least five times more than that of the Imd2p, suggesting Imd3p and Imd4p are functionally different from Imd2p, a proposition consistent with the fact that over-expression of either Imd3p or Imd4p failed to rescue the loss of Imd2p (Hyle et al., 2003).

5.3.3 Ded1p is associated with active spliceosome

Although the biological underpinning of Ded1p’s association with Imd2p, Imd3p, and Imd4p remains to be elucidated, several lines of evidence suggest that this interaction may be related to the splicing process. First, mass spectrometry analysis of penta-snRNP, a functional splicing machinery recently identified in yeast, has documented the presence of both Ded1p and IMPDHs (Stevens et al., 2002). Second, a similar analysis showed that DDX3,
the human Ded1p ortholog is also present in the human spliceosome (Zhou et al., 2002). Third, it has long been documented that spp81-1, a ded1 mutant allele can suppress the splicing defect of prp8-1, which encodes an altered form of Prp8p, an essential splicing factor (Jamieson et al., 1991). Fourth, both DDX3 and An3, a Ded1p ortholog in *Xenopus laevis*, shuttle between cytoplasm and nucleus. Interestingly, the yeast Ded1p also harbors a N-terminal nuclear export signal (NES) conserved among DDX3, An3, and PL10, a mouse ortholog of Ded1p (Askjaer et al., 1999), suggesting that Ded1p may also shuttle. Fifth, the Ded1p homolog in *Chironomus tentans* is loaded co-transcriptionally onto nascent transcripts and accompanies them to polysomes in the cytoplasm (Daneholt, 2001). Taken together, it is tempting to speculate that the yeast Ded1p has a nuclear role in relation to pre-mRNA splicing.

To test this hypothesis, I first examined if Ded1p is associated with penta-snRNP under the established conditions for purifying penta-snRNP. Splicing extracts made from a Ded1p-PA strain was incubated with IgG Sepharose to capture Ded1p-PA and its associated components. After extensive washes, RNAs were extracted from the bound materials and analyzed by Northern blotting using probes hybridizing to the U1, U2, U4, U5, and U6 snRNA. Indeed, the amount of U1, U2, U4, U5, and U6 snRNAs
recovered by Ded1p-PA (Figure 5.11, lanes 5 and 8) is reproducibly 5—10X higher than the background obtained from the control experiments (lane 4, 6, 7, and 9)), suggesting Ded1p is specifically associated with penta-snRNP.

To further test the association of Ded1p with active spliceosome, I prepared splicing extracts from Ded1p-PA and other control strains for performing in vitro splicing assays. Splicing reactions were done using [32P]-labeled pre-mRNA. If Ded1p is associated with active spliceosome, precipitation of Ded1p-PA is expected to bring down splicing intermediates (exon 1, lariat, and lariat-exon 2) (Figure 5.12 and 5.13). This indeed was the case for experiments using Ded1p-PA splicing extract (Figure 5.12 and 5.13, lanes 5 and 8), but not for other control extracts (lanes 4, 6, 7, and 9). The co-precipitation behavior of Ded1p-PA with RNAs remained significantly higher than background in the presence of 150 mM KCl (lanes 8). Notably, spliced mRNA could also be efficiently recovered, raising the possibility that Ded1p continues to associate with mRNP produced from the splicing event (Figure 5.13). This intriguing observation is reminiscent of the report that Chironomus tentans Ded1p is co-transcriptionally loaded onto nascent transcripts and continue to stay on mRNP after splicing and then enter polysome (Daneholt, 2001).
5.3.4 Inactivation of Ded1p impacts on the formation of splicing complexes

The association of Ded1p with penta-snRNP and spliceosome argues that Ded1p may have role in influencing nuclear pre-mRNA splicing. To probe this possibility, I asked whether inactivation of Ded1p allows detection of splicing defect \textit{in vitro}. However, preliminary Ded1p-depletion analysis did not yield detectable splicing defect \textit{in vitro} (data not shown), a scenario that has also been documented for several proteins shown to be involved in splicing (Fortes et al., 1999; Perriman and Ares, 2000). To circumvent this difficulty, I turned to the analysis of splicing complex formation in the absence of functional Ded1p.

Standard splicing reactions were assembled using extracts prepared from both wild-type and \textit{ded1-120} cells. After incubation, the reactions were run on native gel to separate splicing complexes. To specifically examine the commitment complex formation, an oligonucleotide promoting the RNaseH-dependent destruction of the endogenous U2 snRNA was added into the reaction to block the formation of spliceosome. For reactions using the wild-type extract, CC2 is the predominant complex formed in reactions done at both 25°C and 16°C (Figure 5.14, lanes 1 and 3), which then progressed to the
formation of complete spliceosome (lanes 5 and 7). In contrast, in \textit{ded1-120} reactions, little CC2 was formed and the RNA transcript accumulates substantially more in CC1 than in CC2 (lanes 2 and 4) and in some fast-migrating complexes (marked by *). This suggests that the conversion of CC1 to CC2 is either slowed down or the commitment complex formed is unstable, or both. These data are in line with previous observations that the \textit{ded1-120} mutant grows substantially slower than the isogenic wild-type strain at 25°C, and at 16°C, \textit{ded1-20} completely stops growing (Chuang et al., 1997).

Intriguingly, when allowed to progress to spliceosome formation, the \textit{ded1-120} reactions yielded a “spliceosome-like” complex migrating faster than the wild-type spliceosome and a fraction of the transcript remains in the fast-migrating complexes (lanes 6 and 8). Again, these data suggest that, although a “spliceosome-like” complex could be formed in \textit{ded1-120} extract, this complex is likely to adopt an altered conformation. Alternatively, this complex could represent a break-down form of the wild-type spliceosome.
Figure 5.3. Ded1p was found to sediment into a 400-kDa complex. Cell extract was laid on top of 10–25% glycerol and subjected to centrifugation. Fractions were collected from top to bottom. The even-numbered fractions were analyzed by SDS-PAGE and then Western blotting using anti-Ded1p antibody. Molecular size markers are indicated (kDa).
Figure 5.4. Imd2p, Imd3p, and Imd4p interact with Ded1p. Extracts prepared from yeast strains containing Ded1p (lane 1, 4, 7, 10, and 13), Ded1p-PA (lane 2, 5, 8, 11, and 14) or Dbp5p-PA (lane 3, 6, 9, 12 and 15) were incubated with IgG-Sepharose. After washes, IgG-bound proteins were eluted with 50 mM (lanes 1—3), 100 mM (lanes 4—6), 200 mM (lanes 7—9), 500 mM (lanes 10—12), and 1 M (lanes 13—15) MgCl₂-elution buffer. Proteins were precipitated and analyzed by SDS-PAGE. Molecular size markers are indicated (kDa). The * sign indicates Imd2p, Imd3p, and Imd4p.
Figure 5.4.
Figure 5.5. Ded1p specifically binds to Imd2p, Imd3p, and Imd4p in vitro. Cell extract made from the Ded1p-PA strain was incubated with recombinant GST (lane 1, 5, 9, and 13), GST-Prp28p (lane 2, 6, 10, and 14), GST-Ded1p (lane 3, 7, 11, and 15) or IgG-sepharose (lane 4, 8, 12, and 16). After washes, bound materials were eluted with 50 mM (lanes 1—4), 100 mM (lanes 5—8), 200 mM (lane 9—12), and 500 mM (lane 13—16) MgCl₂-elution buffers. Proteins were precipitated and analyzed by SDS-PAGE. Molecular size markers are indicated (kDa). The * sign indicates Imd2p, Imd3p, and Imd4p.
Figure 5.5.
Figure 5.6. The binding of Ded1p to Imd2p, Imd3p, and Imd4p is RNA dependent. Cell extract made from the Ded1p-PA strain was incubated with (lane 3) or without RNase (lane 1, and 2) at 30°C (lane 2, and 3) or at 4°C (lane 1) for 60 min and then transferred into eppendorfs with IgG-Sepharose. After washes, bound materials were eluted using elution buffers containing different concentrations of MgCl₂. This figure only shows the fraction eluted by 100 mM MgCl₂ elution buffer. Molecular size markers are indicated (kDa).
Figure 5.6.
Figure 5.7. The association of Ded1p to Imd2p, Imd3p, and Imd4p is sensitive to ATP treatment. Cell extract made from the Ded1p-PA strain was incubated with IgG sepharose with 0 mM ATP (lane 1), 2 mM ATP (lane 2) or with 5 mM glucose (lane 3). After washes, bound materials were eluted with 1000 mM MgCl₂ elution buffer. Molecular size markers are indicated (kDa).
Figure 5.7.
Figure 5.8. Schematic representation of purine nucleotides *de novo* and salvage pathways. Solid lines represent the plasma membrane. The following abbreviations are used: *Ade*, adenine; *ext*, extracellular medium; *Gua*, guanine; *Hyp*, hypoxanthine; *int*, intracellular compartment; *PRPP*, 5-phosphoribosyl-1-pyrophosphate. Gene names are italicized and encode the following enzymatic activities: *AAH1*, adenine deaminase; *APT1*, adenine phosphoribosyltransferase; *FCY2*, purine cytosine permease; *GUA1*, guanosine-5'-monophosphate synthetase; *GUK1*, guanosine-5'-monophosphate kinase; *HPT1*, hypoxanthine-guanine phosphoribosyltransferase; *IMDs*, inosine-5'-monophosphate dehydrogenases; *RNR*, ribonucleotide reductase; *YNK1*, nucleoside-5'-diphosphate kinase. Adopted from Escobar-Henriques et al. 2001
Figure 5.8.
Figure 5.9. Sequence alignment of Imd2p, Imd3p, and Imd4p. Protein sequences were aligned using PILEUP program in the GCG package and adjusted by importing into the BOXSHADE server (http://www.ch.embnet.org/software/BOX_form.html) for graphic presentation. Black boxes: identical amino acid. Gray boxes: similar amino acid. The open boxes indicate peptides analyzed in mass spectrometry.
Figure 5.9.
**Figure 5.10.** *In vitro* binding of Imd2p, Imd3p, and Imd4p to GST-Ded1p. Cell extracts made from wild-type (lane 1, 5, and 9), Imd2p-TAP (lane 2, 6, and 10), Imd3p-TAP (lane 3, 8, and 11), and Imd4p-TAP (lane 4, 8, and 12) strains were incubated with glutathione beads precoated with recombinant GST-Ded1p (lanes 5—8) or GST (lanes 9—12). Lanes 1—4 represent 4.5% of cell extract inputs.
Figure 5.11. Ded1p is associated with penta-snRNP. Splicing extracts made from Ded1p (lane 1, 4, and 7), Ded1p-PA (lane 2, 5, and 8), and PA alone (lane 3, 6, and 9) were incubated with IgG sepharose. The IgG-bound materials were eluted with 50 mM KCl (lanes 4—6) or 150 mM KCl (lanes 7—9) elution buffer and extracted by phenol/chloroform (pH 5.2). The identities of RNAs were determined by Northern blotting by probing with U1, U2, U4, U5, and U6 snRNA. Lanes 1—3 represent 10% of input RNAs.
Figure 5.11.
Figure 5.12. Ded1p binds to active spliceosome. Splicing reactions were set up with [32P]-labeled actin pre-mRNA and splicing extracts made from Ded1p (lane 1, 4, and 7), Ded1p-PA (lane 2, 5, and 8), and PA alone (lane 3, 6, and 9). After incubation at 25°C for 20 min, reactions were transferred into eppendorfs with IgG sepharose for binding. The IgG-bound materials were eluted with 50 mM KCl (lanes 4—6) or 150 mM KCl (lanes 7—9) elution buffer and extracted by phenol/chloroform (pH 5.2). RNAs were analyzed in 6% polyacrylamide gel with 8 M urea for autoradiography.
Figure 5.12.
**Figure 5.13.** Ded1p interacts with active spliceosomes. Splicing reactions were set up as in Figure 5.3.10, except using $[^{32}P]$-labeled RP51A pre-mRNA. Splicing extracts were made from strains containing Ded1p (lane 1, 4, and 7), Ded1p-PA (lane 2, 5, and 8), and PA alone (lane 3, 6, and 9). The IgG-bound materials were eluted by 50 mM KCl (lanes 4—6) or 150 mM KCl (lanes 7—9) elution buffer and extracted by phenol/chloroform (pH 5.2). RNAs were analyzed in 6% polyacrylamide gel with 8 M urea for autoradiography.
Figure 5.13.
Figure 5.14. Inactivation of Ded1p affects spliceosome formation. The formation of commitment complex (lanes 1—4) and spliceosome (lane 5—8) are set up by using splicing extracts made from strains with wild-type DED1 (lane 1, 3, 5, and 7) or ded1-120 (lane 2, 4, 6, and 8). SP: spliceosome. CC1: commitment complex 1. CC2: commitment complex 2. The fast-migrating complex is marked by *.
Figure 5.14.
5.4 Discussion

5.4.1 Functional coupling in the gene expression pathway

One of the defining features of eukaryotic cells is the nucleus, which separates the activities of replication and transcription from that of translation. The existence of the nucleus affords a higher level of regulation for cellular processes but also presents a difficult task of communication between the nucleus and the cytoplasm. For example, RNA molecules are synthesized in the nucleus via a series of complicated processes and most of them are needed to be exported to the cytoplasm, where they participate in protein synthesis. Traditionally each of the processes along the gene expression pathway was characterized in a separate manner, in that the components of each process were identified and their potential roles elucidated. Yet researchers working on one process rarely need to worry about the other processes.

It was not until recently has this “linear” and “discreet” manner of thinking begun to receive some serious challenges. A growing body of data has since revealed the intricate inter-dependence among the nuclear processing steps (Reed, 2003). For example, it has been well-characterized
that, at least in metazoans, the experience of the splicing event greatly stimulates the export of a given transcript (Luo and Reed, 1999). This is achieved by the loading onto the spliced transcript of the EJC complex, which helps to recruit the export machinery (Gatfield et al., 2001; Le Hir et al., 2000; Reed, 2003). Thus, the history or itinerary of an RNA is apparently linked to its ultimate functionality. In addition, it is now not uncommon to observe the sharing of components among the various nuclear information-processing machineries. For example, as described earlier in Introduction, Sub2p is involved in transcriptional elongation, splicing, and export (Reed, 2003; Strasser et al., 2002).

Yet, hardly has a component been shown to participate in both the nuclear and cytoplasmic processes of the gene expression pathway (Dostie et al., 2000; Kamath et al., 2001), despite that evidence suggests a linkage of the NMD, a potential nuclear event, to the cytoplasmic translation process (Cheng and Maquat, 1993) (see Introduction). In this Chapter, I provide a preliminary example illustrating that Ded1p, a previously characterized translation initiation factor predominantly localized in the cytoplasm, has a role in impacting on splicing in the nucleus. This finding appears to forecast a much closer
functional coupling between the two compartments for the gene expression pathway.

5.4.2 Interaction of Ded1p with IMPDH

Seeking to deepen our understanding of Ded1p’s cellular roles, I searched for proteins that interact with Ded1p. Interestingly, Ded1p was found to associate with Imd2p, Imd3p, and Imd4p, the yeast orthologs of the human inosine 5'-monophosphate dehydrogenase (IMPDH), an enzyme that catalyzes the rate-limiting step in the de novo synthesis of GTP (Escobar-Henriques and Daignan-Fornier, 2001). This finding immediately suggests a role for Ded1p in relationship to the known enzymatic activity of IMPDH. Yeast cells with individual deletion of IMD2, IMD3, or IMD4 are viable. However, simultaneous deletions of all three genes is lethal but can be rescued by supplementing with 0.5 mM guanine to the deletion strain, consistent with the notion that Imd2p, Imd3p, and Imd4p are required for maintaining the critical GTP level in the cell. Nonetheless, supplementing 0.5 mM guanine to the cold-sensitive ded1-120 strain failed to rescue its growth defect at 16°C (data not shown), raising the possibility that Ded1p may not be critical solely for the de novo synthesis of GTP and that it may have an
uncharacterized aspect of functional association with IMPDH. In this regard and in light of Ded1p’s potential link to splicing (see below), it is tempting to speculate that this GTP aspect of functional association may be related to Snu114p, a conserved U5-snRNP protein homologous to the ribosomal GTPase EF-2 (Bartels et al., 2003; Fabrizio et al., 1997). Snu114p, a proven GTPase is involved in dissociation of U4 snRNA from U6 snRNA during splicing (Bartels et al., 2003). Interestingly, it is noted that a ded1 mutant allele was reported to rescue the splicing defect caused by the prp8-1 allele, which encodes an altered form of Prp8p, an U5 snRNP protein present in a subcomplex that also includes Snu114p (Dix et al., 1998).

5.4.3 Requirement of Ded1p for correct function of the splicing apparatus

The preliminary data described herein strongly suggest that Ded1p is required for the correct function of the splicing apparatus in the nucleus. First, the co-immunoprecipitation experiments validate earlier reports (Stevens et al., 2002) that a fraction of Ded1p is associated with the penta-snRNP (Figure 5.3.9). Second, co-immuprecipitation of the in vitro splicing reactions showed that this interaction is likely to occur on the functional splicing apparatus
Third, inactivation of Ded1p \textit{in vitro} causes aberrant formation and/or instability of both commitment complexes and spliceosome, as shown by the native gel analysis (Figure 5.3.12). These arguments are further strengthened by examining the genome-wide splicing defects, using splicing-specific microarrays (Clark et al., 2002), of the \textit{ded1-120} mutant. Remarkably, upon inactivation of Ded1p by the \textit{ded1-120} mutation at 16°C, there is a dramatic buildup of intron-containing species corresponding to almost exclusively the ribosomal-protein (rp) gene transcripts (T. Burkin, R. Nagel, Y. Mandel-Gutfreund, L. Shiue, T. Clark, J.-L. Chong, T.-H. Chang, G. Hartzog, and M. Ares, Jr., submitted). Control experiments using mutations that inactivate eIF4A (a translation initiation factor), Dbp5p (a mRNA export factor), and Dhh1p (a factor involved in mRNA turnover), as well as using cycloheximide, to shut down translation and other key steps of the mRNA metabolism pathway did not result in any significant genome-wide splicing phenotype, arguing strongly against the idea that the unique \textit{ded1-120} splicing phenotype was due to an indirect effect of inactivation of translation (T.-H. Chang, unpublished). Finally, extensive computational analysis by conventional hierarchical clustering and multi-class support vector machine (SVM)

If Ded1p plays a direct chemical role in the splicing process, one would anticipate that depletion or inactivation of Ded1p in the splicing extracts should lead to a scoreable splicing defect in vitro. However, preliminary analysis showed that splicing still occurred at 16°C in splicing extracts made from ded1-120 cold-sensitive strain (data not shown). Additionally, depletion of >97% of Ded1p-PA in Ded1p-PA-containing splicing extract also did not block the in vitro splicing activity (data not shown). Admittedly, such a lack of distinct splicing phenotype in vitro may simply reflect a possibility that appropriate reaction conditions remain to be established. However, one of the plausible explanations would be that Ded1p is not a rate-limiting factor in the biochemical reaction of the splicing process per se, because similar observations have been documented for several known splicing factors. For example, Cus2p is not essential for splicing, but helps to enforce the ATP dependence of U2 snRNP recruitment and spliceosome assembly (Perriman and Ares, 2000); Msl5p, which is required for the formation of commitment complex 2, is not essential for in vitro splicing (Rutz and Seraphin, 2000);
inactivation of Luc7p, an U1-snRNP protein essential for cell viability, resulted in no detectable splicing phenotype \textit{in vitro} \cite{Fortes1999}. Invariably, the ultimate detection of the splicing phenotypes in all these cases depends on stressing the \textit{in vitro} system by using RNA transcripts with compromised splice sites. The fact that the microarrays experiments indicate that the splicing of the rp pre-mRNA are particularly sensitive to the loss of Ded1p appears to be consistent with this notion. Future experiments aiming to reveal the \textit{in vitro} splicing defect, already hinted by the aberrant formation of the splicing complexes in splicing reactions using the \textit{ded1-120} extracts, by employing various transcript variants and adjusting for reaction conditions may help to resolve this issue.

\textbf{5.4.4 Potential shuttling behavior of Ded1p}

The fact that Ded1p is predominantly localized in the cytoplasm \cite{Chuang1997} is consistent with its essential role in translation. However, its potential role in the correct formation of splicing apparatus \textit{in vitro} would suggest that it is also present in the nucleus. Interestingly, Ded1p harbors a standard N-terminal nuclear export signal (NES) conserved among DDX3, An3, and PL10, a mouse ortholog of Ded1p, which have all been
demonstrated to shuttle between the nucleus and the cytoplasm (Askjaer et al., 1999). Consequently, one would expect that Ded1p may also be a shuttle protein, whose export from the nucleus is mediated by its intrinsic NES, which works through the Crm1p-dependent pathway. If so, deletion of NES in Ded1p will result in blocking the export of Ded1p. To test this idea, I constructed strains containing NES-deleted Ded1p tagged by the green fluorescence protein (NES\textsuperscript{Δ}-Ded1p-GFP). Unexpectedly, these yeast strains are viable, although their growth rates are slower than that of the corresponding wild-type Ded1p-GFP strain at 16°C (data not shown). Furthermore, NES\textsuperscript{Δ}-Ded1p-GFP is localized again predominately in the cytoplasm, rather than in the anticipated nucleus. One way to reconcile these observations is that Ded1p may harbor other uncharacterized NESs, allowing the presumed NES\textsuperscript{Δ}-Ded1p-GFP to be exported via the Crm1p-dependent pathway. Surprisingly, attempts to use Lepomycin B to shut down the Crm1p-dependent pathway did not result in the accumulation of NES\textsuperscript{Δ}-Ded1p-GFP in the nucleus. (data not shown) (see Chapter 5.2). Taken together, one plausible explanation would be that Ded1p’s import rate might be substantially slower than its export rate and than the rate of the cell division cycle, so that the nuclear accumulation of NES\textsuperscript{Δ}-Ded1p-GFP is perpetually below the detection limit during each cell
division cycle. Future experiments exploring the heterokaryon strategy used to monitor the shuttling behavior of Npl3p may allow one to resolve this issue (Lee et al., 1996).

5.4.5 Prospectus

Although this study suggests that Ded1p has a role in pre-mRNA splicing, several critical issues main to be resolved. First, the identity of the spliceosome-like complex needs to be clarified, as it may represent a so-far uncharacterized functional spliceosome intermediate or simply a breakdown product. To this ends, one needs to determine that whether the formation of the spliceosome-like complex is dependent on functional splice sites and on splicing conditions. Second, it is not known if the association of Ded1p with mature spliced mRNA is splicing-dependent. A further examination may help to determine if Ded1p is similar to the *Chironomus tentans* Ded1p homolog, which remains to associate with the spliced RNAs and accompany with them to cytoplasm for translation. Third, it is of interest to know why introns of ribosomal protein genes are particularly sensitive to inactivation of Ded1p. This may allow one to understand Ded1p’s mechanistic role in pre-mRNA splicing. Fourth, although the possession of a highly conserved NES strongly
implicates a nuclear presence for Ded1p, this remains to be shown. In conclusion, the data described in this Chapter represent an exciting starting point to chart a potentially unknown territory, i.e. the coordination of the nuclear and the cytoplasmic machineries involved in the gene expression pathway. With luck, continued studies of the molecular basis of Ded1p’s involvement in splicing may prove to unite the two worlds divided by the seemingly alienating nuclear boundary.
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