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PROSPECT

Roles for Cytoplasmic Polyadenylation in Cell Cycle Regulation

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Abstract Polyadenylation of eukaryotic mRNAs in the nucleus promotes their translation following export to the cytoplasm and is an important determinant of mRNA stability. An additional level of control of gene expression is provided by cytoplasmic polyadenylation, which activates translation of a number of mRNAs important in orchestrating cell cycle events in oocytes. Recent studies indicate that cytoplasmic polyadenylation may be a mechanism of translational activation that is more widespread in eukaryotic cells. Here we discuss the roles of a recently identified family of nucleotidyl transferases (encoded by the *cid1* gene family) in cell cycle regulation. To date, this family has been characterised mainly in yeasts, but it is conserved throughout the eukaryotes. Biochemical studies have indicated that a subset of members of this family function as cytoplasmic poly(A) polymerases targeting specific mRNAs for translation. This form of translational control appears to be particularly important for cell cycle regulation following inhibition of DNA synthesis. J. Cell. Biochem. 87: 258–265, 2002. © 2002 Wiley-Liss, Inc.

Key words: *cid1*; *TRF4*; checkpoint control; nucleotidyl transferase; poly(A) polymerase

Sustainable cell division requires that in each cell cycle the genome is accurately duplicated, that the chromosomes are properly segregated, and that these processes are coordinated with cellular growth. To prevent aberrant cell division these processes must be tightly regulated and their interdependence ensured. Checkpoints are mechanisms that regulate progression through the cell cycle, ensuring that each event is complete, takes place only once per cycle, and that each event occurs in the correct sequence [Hartwell and Weinert, 1989]. The DNA damage and replication checkpoint pathways consist of a series of proteins functioning as sensors of DNA damage or replication block and signal transducers that ultimately converge on the effectors of cell cycle arrest. Arresting the cell cycle provides an opportunity for the repair of DNA lesions. Many of the checkpoint components are not essential for the normal cell cycle but are brought into play specifically when DNA is damaged or replication blocked. Studies in the yeasts *Schizosaccharomyces pombe* and *Saccharomyces cerevisiae* have defined the genetic requirements of these pathways, and our biochemical understanding of the interactions involved is increasing (Fig. 1). Briefly, DNA damage or replication blockage is detected though the 'checkpoint *rad*' proteins (Rad3, Rad26, Rad17, Rad1, Rad9, and Hus1, in the *S. pombe* nomenclature). Phosphorylation activates one of two downstream kinases, Chk1 or Cds1, via their respective adaptor proteins, Crb2 or Mrc1. In *S. pombe*, Cds1 is activated only in response to replication block or DNA damage occurring in S phase. Ultimately, inhibitory phosphorylation of Cdc2 prevents entry into mitosis (Fig. 1) [Norbury and Hickson, 2001].

Another critical level of cell cycle control is the regulation of gene expression. In concert with transcription, capping, splicing, and polyadenylation are coordinated to produce mature mRNAs prior to nuclear export. Most mRNAs are polyadenylated in the nucleus in a two step process whereby the pre-RNA is cleaved at the polyadenylation site prior to poly(A) addition. Polyadenylation is generally associated with increased stability of the mRNA and increased

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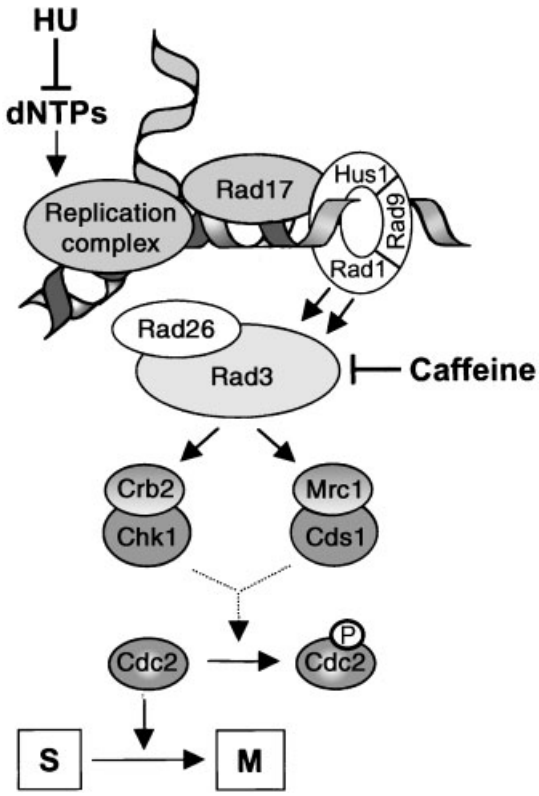

Fig. 1. DNA damage and replication checkpoints in *S. pombe*. The checkpoint *rad* proteins (Rad3, Rad26, Rad17, Rad1, Rad9, and Hus1) respond to DNA damage or replication block and transduce signals to the downstream kinases Chk1 or Cds1 via their respective adaptor proteins (Crb2 and Mrc1). Ultimately, phosphorylation of Cdc2 prevents entry into mitosis. Hydroxyurea inhibits ribonucleotide reductase resulting in the depletion of dNTP pools. Caffeine inhibits Rad3 causing checkpoint failure.

efficiency of translation [Sonenberg et al., 2000]. Studies in yeast indicate that the poly(A) tail stimulates translation through its interaction with poly(A) binding protein (PAB). In turn, PAB binds the translation initiation factor eIF4G, enabling recruitment of the 40S ribosomal subunit. eIF4G also binds eIF4E which in turn binds the mRNA cap circularising the mRNA [Wells et al., 1998].

Here we focus on how the regulation of translation, via changes in the polyadenylation state of specific mRNAs in the cytoplasm, could be responsible for some of the rapid adaptive responses of which normal cells are capable.

Cytoplasmic Polyadenylation in Early Development

Most studies of cytoplasmic polyadenylation to date have focused on early animal development. Immature *Xenopus* oocytes arrest at prophase of the first meiotic division. The formation of a mature oocyte capable of fertilisation requires re-entry into the meiotic cell cycle, a process stimulated by progesterone [Bayaa et al., 2000]. During maturation transcription ceases, while translation is controlled by the activation and repression of maternal mRNAs. Dormant mRNAs are recruited to polysomes and translationally activated following an increase in poly(A) tail length. This allows a rapid increase in the synthesis of specific proteins in the absence of transcription.

In *Xenopus*, two sequences in the 3′UTRs of mRNAs have been identified that are essential for cytoplasmic polyadenylation during meiotic maturation. The first sequence, known as the cytoplasmic polyadenylation element (CPE), is an AU-rich motif with a consensus sequence UUUUUUAU. The second is the nuclear polyadenylation motif AAUAAA [McGrew and Richter, 1990]. In addition to a poly(A) polymerase (PAP), at least two protein factors are required for cytoplasmic polyadenylation. Firstly, the CPE is bound by cytoplasmic polyadenylation element binding protein (CPEB), a zinc finger, and RNA-recognition motif containing protein, which is conserved among metazoans [Mendez and Richter, 2001]. Secondly, the cleavage/polyadenylation specificity factor (CPSF) complex that is essential for nuclear polyadenylation has also been implicated in cytoplasmic polyadenylation. A cytoplasmic form of CPSF has been identified and it is possible that it specifically interacts with CPE-containing or CPEB-bound mRNAs [Bilger et al., 1994; Dickson et al., 1999]. It has been hypothesised that CPSFs are required for all polyadenylation reactions [Dickson et al., 2001]. Cytoplasmic polyadenylation is initiated by the progesterone-stimulated phosphorylation of CPEB at serine 174 by Eg2, a member of the Aurora family of protein kinases [Andresson and Ruderman, 1998]. This phosphorylation increases the affinity of CPEB for CPSF and stabilises the association of CPSF with the AAUAAA motif (Fig. 2) [Mendez et al., 2000].

A third protein, Maskin, binds CPEB and mediates translational repression. In addition to binding CPEB, Maskin can bind eIF4E through a domain homologous to those found in other eIF4E binding proteins, including eIF4G [Stebbins-Boaz et al., 1999]. The binding of Maskin to eIF4E prevents the binding of

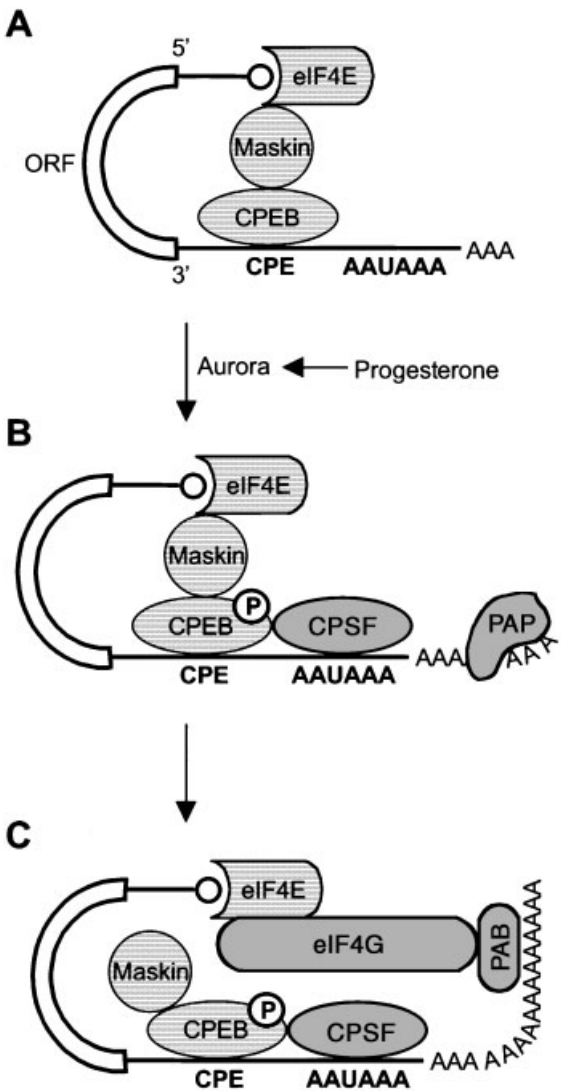

Fig. 2. Cytoplasmic polyadenylation and translational control in early *Xenopus* development. **A**: Maskin binds both CPEB and eIF4E, inhibiting assembly of the translation initiation complex. **B**: Phosphorylation of CPEB increases its affinity for CPSF and causes tighter association between CPSF and the AAUAAA motif. CPSF recruits PAP and cytoplasmic polyadenylation is initiated. **C**: PAB binds the newly formed poly(A) tail and also binds eIF4G. Once bound to PAB, eIF4G displaces Maskin from eIF4E and the translation initiation complex is assembled.

eIF4G and hence inhibits assembly of the translation initiation complex. Translation is enabled when Maskin is displaced from eIF4E by eIF4G. This requires polyadenylation because eIF4G is only able to displace Maskin when it is also bound to PAB (Fig. 2) [Groisman et al., 2000]. The binding of eIF4E to eIF4G allows recruitment of the 40S ribosomal subunit and subsequent translation of CPE containing mRNAs.

Known targets for cytoplasmic polyadenylation during *Xenopus* oocyte maturation include mRNAs encoding c-Mos, Wee1, and cyclin B [Charlesworth et al., 2002]. Cyclin B binds Cdc2 to form maturation promoting factor (MPF). The protein kinase activity of MPF drives entry into M phase, while inactivation of MPF, via the anaphase promoting complex (APC) mediated destruction of cyclin B, allows exit from M phase. In the case of cyclin B, cytoplasmic polyadenylation provides a mechanism of activating translation in specific regions of the cell. CPEB and Maskin colocalise with cyclin B1 mRNA and protein on animal pole spindles and centrosomes. A mutant CPEB protein that fails to localise on the mitotic apparatus does not affect overall cyclin B1 protein levels. However, it does disrupt the localisation of cyclin B1 mRNA and inhibits cell division [Groisman et al., 2000]. This suggests that, in these comparatively large cells, the targeting of cyclin B translation to spindles and centrosomes is necessary for the regulation of cell division.

Early translation of the c-Mos proto-oncogene product (a MAP kinase kinase kinase) is essential for progesterone-induced maturation. In contrast to cyclin B, the translation of c-Mos can be induced by the MAP kinase signalling pathway. In a study designed to investigate this mechanism, a new regulatory element (the polyadenylation response element; PRE) in the 3′ UTR of the c-*mos* mRNA was identified. During oocyte maturation, the initial translational activation of c-*mos* is mediated by the PRE and occurs in a CPE- and CPEB-independent manner. However, PRE mediated activation of c-*mos* is not sufficient to attain the c-Mos levels necessary for completion of meiotic maturation. Consequently, the CPE- and CPEB-dependent regulation of translation is required for c-Mos accumulation [Charlesworth et al., 2002].

Cytoplasmic Polyadenylation in Somatic Cells

The machinery required for cytoplasmic polyadenylation in oocytes can also be found in somatic cells, though examples of its occurrence are limited. In the adult mouse, CPEB is only expressed at high levels in the ovaries and testes, though moderate expression has been detected in the brain. In rat brain, visual experience induces polyadenylation and translation of mRNA encoding the α-subunit of

calcium/calmodulin-dependent protein kinase II (α-CaMKII) in the visual cortex. The 3′ UTR of the α-CaMKII mRNA contains two CPEs that bind CPEB and are necessary for polyadenylation-induced translation in injected *Xenopus* oocytes [Wu et al., 1998]. In the brain, this increase in translation is dependent on the *N*-methyl-D-aspartate (NMDA) receptor [Wells et al., 2001], which transduces a signal that activates Aurora kinase phosphorylation of CPEB [Huang et al., 2002]. These results provide a molecular mechanism for activity-dependent synaptic plasticity that requires both the NMDA receptor and translation of α-CaMKII mRNA. The NMDA receptor is often required for the induction of long lasting changes in synaptic strength. These changes are required for the acquisition of long-term memory and require new protein synthesis, at least some of which results from cytoplasmic polyadenylation mediated-translation.

CPE dependent polyadenylation has also been demonstrated in cytoplasmic extracts from the human MCF7 breast cancer cell line. MCF7 cells synchronised in M phase polyadenylated a CPE-containing 3′UTR fragment of cyclin B mRNA but not a similar fragment lacking the CPE. In the same experiment cytoplasmic extracts from MCF7 cells synchronised in S phase did not support polyadenylation [Groisman et al., 2002]. This indicates that the factors required for cytoplasmic polyadenylation are present in mammalian somatic cells and suggests that cytoplasmic polyadenylation might be a general mechanism of regulation in the eukaryotic cell cycle.

Some Unanswered Questions

While a lot is now known about cytoplasmic polyadenylation and its role in activating translation in early development and neural signalling there are still outstanding questions. Firstly, the PAP(s) involved in cytoplasmic polyadenylation have yet to be identified. The well-characterised PAP that catalyses nuclear polyadenylation is unlikely to be responsible. Nuclear PAP contains two bipartite nuclear localisation signals and its activity is cell cycle regulated such that multiple phosphorylation, by MPF, causes its inactivation during mitosis [Colgan et al., 1998]. This inactivation of nuclear PAP coincides with the *activation* of cytoplasmic polyadenylation in oocytes as they enter M phase [Groisman et al., 2002]. However, most cells contain multiple isoforms of PAP, and a *Xenopus* PAP has been identified as a possible candidate for the cytoplasmic activity. It lacks both a consensus NLS and the major MPF phosphorylation sites and appears to be a cytoplasmic enzyme [Gebauer and Richter, 1995].

Secondly, beyond the CPE and PRE sequence elements, little is known about how cells target specific messages for cytoplasmic polyadenylation-induced translation, though one or more of the protein factors involved could be responsible for determining specificity. In *Caenorhabditis elegans* there are four identifiable homologues of CPEB and two of these (FOG-1 and CPB-1) have distinct functions in the differentiation of germ cells into primary spermatocytes [Luitjens et al., 2000]. Identification of the mRNA targets of this family of proteins could provide insight into how they control message-specific polyadenylation.

Until recently, cytoplasmic polyadenylation had only been described in metazoans. However, the identification of a novel family of nucleotidyl transferases (encoded by the *cid1* gene family) in *S. pombe* has revealed cytoplasmic polyadenylation in yeast. This mechanism of cytoplasmic polyadenylation must be distinct from that previously characterised in metazoans, since neither the polyadenylation motif AAUAAA nor CPEB is conserved in yeast.

The *CID1* Gene Family

cid1 (for caffeine induced death suppressor) is the prototypic member of the *cid1* gene family in *S. pombe*. We identified *cid1* through its ability, when overexpressed, to confer resistance specifically to the combination of hydroxyurea (which blocks replication by inhibiting ribonucleotide reductase (RNR)) and caffeine (which overrides the replication checkpoint) [Wang et al., 1999]. Overexpression of Cid1 also partially suppresses the sensitivity of checkpoint *rad* mutants to hydroxyurea, while deletion of *cid1* confers sensitivity to the combination of hydroxyurea and caffeine. Cid1 is not essential under normal conditions but is specifically required to inhibit mitosis and promote cell survival when DNA polymerase δ or ϵ is inhibited [Wang et al., 2000a].

In addition, we identified five other members of the *cid1* gene family (*cid11*, *cid12*, *cid13*, *cid14*, *cid16*) based on sequence homology

searches. *cid13* has been identified independently based on its ability, when overexpressed, to rescue the hydroxyurea sensitivity of checkpoint *rad* mutants [Read et al., in press; Saitoh et al., 2002]. The *cid1* gene family is evolutionarily conserved. However, while there are multiple members of this family in *C. elegans*, *Drosophila*, mouse, and human cells, there are only two in *S. cerevisiae*, *TRF4*, and *TRF5*. Hence, in *S. pombe* and higher eukaryotes *cid* function is likely to be required in more diverse cellular processes than in *S. cerevisiae*.

TRF4 was initially identified through a screen for mutations that are synthetically lethal when combined with mutations in the gene encoding DNA topoisomerase 1 (*TOP1*). Top1 acts as a swivel in DNA replication to prevent the formation of positive supercoils ahead of the replication complex. The *TOP1* gene is not essential under normal circumstances, however a *top1 trf4-ts* double mutant is defective in the mitotic events of chromosome condensation, spindle elongation, and nuclear segregation [Castano et al., 1996a]. A second member of the *TRF4* family, *TRF5*, was also described. Overexpression of *TRF5* rescues the inviability of the *top1 trf4-ts* mutant strain. Trf4/5 function is indispensable because, while neither gene is essential singly, the *trf4 trf5* double mutant is inviable. Aberrant nuclear division was observed in a *trf4-ts trf5* double mutant, shifted to the restrictive temperature, indicating a role for Trf4/5 function in mitosis [Castano et al., 1996b]. Trf4 interacts both genetically and physically (by co-immunoprecipitation) with Smc1 (structural maintenance of chromosomes) a subunit of the cohesin complex that holds sister chromatids together prior to anaphase [Castano et al., 1996a].

Sequence analysis indicated that all of the *cid* and *TRF* genes belong to the DNA polymerase β superfamily [Wang et al., 2000a,b], which is characterised by the conserved sequence motif: hG[GS]x(9,13)Dh[DE]h (where x is any amino acid and h is a hydrophobic residue; Fig. 3) [Aravind and Koonin, 1999]. This family includes a diverse range of nucleotidyl transferases including: error prone DNA polymerases β and μ, terminal deoxynucleotidyl transferase, kanamycin nucleotidyl transferase, and PAP. Since nucleotidyl transferase activity has been demonstrated for many of the members of the Polβ superfamily, sequence alignment and structure prediction should be

Fig. 3. Cid1 is representative of a novel family of nucleotidyl transferases. Alignment of the amino acid sequences of Cid1, Cid13, Pla1 (*S. pombe* poly(A) polymerase), Trf4, and rat DNA polymerase β in the region of the catalytic aspartate triad (D residues highlighted in black, along with other conserved sequence motifs; conservative substitutions are highlighted in grey).

reliable indicators of function when looking at new members of this superfamily [Aravind and Koonin, 1999].

Biochemical analysis showed that purified recombinant Cid1 catalyses polyadenylation in vitro [Read et al., in press]. In support of this, Cid13-Myc partially purified from *S. pombe* has been shown to add AMP residues to a poly(A) RNA substrate [Saitoh et al., 2002]. Cid1-GFP and Cid13-Myc are cytoplasmic proteins indicating that their function is likely to be distinct from that of nuclear PAP. When incubated with whole cell *S. pombe* RNAs, Cid1 appeared to target a subset for preferential polyadenylation [Read et al., in press]. Unlike *cid1Δ*, *cid13Δ* cells are sensitive to hydroxyurea alone and steady-state dNTP levels are reduced in *cid13Δ* cells. Hence, Cid13 may have a role in the constitutive regulation of RNR. Further to this, the induction of one of the two forms of *suc22* mRNA (encoding a subunit of RNR) was slightly delayed in *cid13Δ* cells after exposure to hydroxyurea [Saitoh et al., 2002]. Other members of the Cid1 gene family presumably have different substrate specificities. The function of Cid1-related proteins appears to be conserved in more complex eukaryotes. In *C. elegans*, one such protein has recently been characterised as a cytoplasmic PAP [Wang et al., in press].

There is currently disagreement regarding the biochemical activity of Trf4. Recombinant Trf4 purified from bacteria had DNA polymerase activity in vitro. Additionally, in an elutriation-synchronised culture, cells lacking *TRF* function (*trf4-ts trf5* strain at the restrictive temperature) showed a marked delay in the G1/S transition and loss of viability occurred

when genome replication was incomplete. These data implicated Trf4/5 DNA polymerase function in DNA replication [Wang et al., 2000b]. Accordingly, it is possible that cells with mutations in both *TOP1* and *TRF4* die as a result of lack of DNA topology correction (Top1) combined with the loss of a replication factor (Trf4). There is currently no clear indication of how Trf4 function might be involved mechanistically in either DNA replication or sister chromatid cohesion. However, Carson et al. proposed a 'polymerase switch' mechanism whereby Trf4 is localised to sites of cohesion. According to this model, when a replication fork encounters a cohesion site Trf4/5 would substitute for the replicative DNA polymerase α, δ, or ε [Carson and Christman, 2001]. While cells lacking Trf4/5 fail to complete S phase, they also showed delayed budding after synchronization in early G1 [Wang et al., 2000b]. This suggests that the observed S phase defects may be secondary to a more general defect in cellular metabolism that delays progression through G1.

Recently, immunoprecipitates of Trf4-HA from *S. cerevisiae* were reported to have PAP, but not DNA polymerase, activity [Saitoh et al., 2002]. If Trf4 were to function as a PAP in vivo, defects in replication associated with loss of *TRF* function might be explained by the failure of the cells to upregulate translation of proteins with roles in cell cycle progression. The possibility that Trf4/5 represent a class of PAPs would be consistent with characterisation of *S. pombe* Cid1 and Cid13. However, while Cid1-GFP and Cid13-Myc are cytoplasmic, Trf4-GFP is a nuclear protein [Walowsky et al., 1999]. This presents the possibility that cytoplasmic polyadenylation is a mechanism of translational regulation conserved in *S. pombe* and higher eukaryotes but absent from *S. cerevisiae*. Although their biochemical activity remains controversial at the time of writing, Trf4 and Trf5 clearly have biological functions that are distinct from those of Cid1 and Cid13.

Deadenylation and Checkpoint Control

An interesting possibility is that *cid* function acts in opposition to mRNA deadenylation to provide a delicately balanced mechanism of translational control. The degradation of mRNA is an important step in the regulation of eukaryotic gene expression [Sonenberg et al., 2000]. Deadenylation, which usually results from 3′ to 5′ exonucleolytic degradation of the poly(A) tail, is the first step in targeting mRNA for degradation. Following deadenylation, decapping results in a substrate that is rapidly degraded by 5′ to 3′ exonuclease activity. Individual mRNAs have very different rates of deadenylation resulting in a variety of mRNA decay rates (see, for example, Grosset et al., 2000).

In *S. cerevisiae*, mRNA deadenylation is usually initiated by a cytoplasmic mRNA deadenylase containing Ccr4 and Caf1. The poly(A) nuclease complex (PAN), consisting of Pan2 and Pan3, also controls this regulation of poly(A) length distribution [Tucker et al., 2001]. A recent publication provides evidence for the specific regulation of *RAD5* mRNA levels by Dun1 and the Pan2/Pan3 complex [Hammet et al., 2002]. Rad5 is involved in error-free post-replicative repair and Dun1 is a protein kinase with similarity to *S. pombe* Cds1. *dun1Δ* strains have defects in cell cycle arrest at the G2-M checkpoint, reduced induction of repair genes in response to DNA damage/replication block, and increased rates of spontaneous chromosomal rearrangements [Myung et al., 2001]. A yeast two-hybrid assay detected an interaction between the FHA domain of Dun1 and Pan3. Subsequently, the *dun1Δ pan2Δ* and *dun1Δ pan3Δ* strains were found to be hypersensitive to hydroxyurea, indicating that *DUN1* and *PAN2/3* collaborate to promote cell survival following inhibition of DNA replication. Gene expression profiling indicated only a single major change in mRNA levels in the *dun1Δ pan2Δ* double mutant; upregulation of *RAD5* by greater than 60-fold. Finally, it was shown that overexpression of Rad5 was sufficient to hypersensitize *dun1Δ* strains to replicational stress. This supports the hypothesis that elevation of *RAD5* expression, due to defective deadenylation, causes the hydroxyurea-induced lethality in the *dun1Δ pan2Δ* strain [Hammet et al., 2002].

Concluding Remarks

The further characterisation of cytoplasmic polyadenylation and the identification of novel cytoplasmic PAPs have implicated this mechanism of translational control in the regulation of increasingly diverse cellular processes. The use of cytoplasmic polyadenylation to target genes for translation could be advantageous in cellular responses to stress, DNA damage,

replication block, and normal cell cycle events such as S phase. One of the main advantages of translational control is that, as a regulatory process, it enables rapid changes in gene expression without requiring transcription or mRNA transport. Additionally, cytoplasmic polyadenylation should be unaffected by aberrant DNA structures that elicit checkpoint responses. Hence, coordination of cell cycle events, stress response pathways, and DNA repair mechanisms could be attained when DNA integrity is compromised. The opposing process of deadenylation would allow this system to be finely tuned and perturbations to these processes could dramatically affect cellular events.

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