A novel RING-finger-like protein Ini1 is essential for cell cycle progression in fission yeast

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Summary

We have cloned a fission yeast (Schizosaccharomyces pombe) homologue of Ini, a novel RING-finger-like protein recently identified in rat that interacts with the connexin43 (cx43) promoter and might be important for the response of the cx43 gene to estrogen. S. pombe cells deleted for ini1+ fail to form colonies and arrest with an elongated cell phenotype, indicating a cell cycle block. Cell cycle arrest is dependent on expression of Weel, but not Rad3, suggesting that it occurs independently of the DNA damage checkpoint control. Analysis of mRNA intermediates in cells depleted for Ini1 demonstrates that Ini1 is required for pre-mRNA splicing. We observe an accumulation of pre-mRNA for six of seven genes analysed, suggesting that Ini1 is required for general splicing activity. Interestingly, loss of Ini1 results in cell death that is partially suppressed by elimination of the Weel kinase. Therefore, Weel might promote cell death in the absence of Ini1.

Key words: Ini1, Cell cycle, Ring-finger, Wee1, Pre-mRNA, Fission yeast

Introduction

Many factors influence the steady state levels of cellular mRNA. These include regulation of the rate of transcription, the rate of mRNA turnover, and the efficiency of post-transcriptional processing of pre-mRNA to form mature functional mRNA. To identify protein factors that are involved in the differential regulation of connexin43 (cx43) gene expression in rat heart and uterus, a uterine cDNA expression library was screened for proteins that interact directly with the cx43 gene promoter. This screen identified a protein called Ini, which binds to a 38-nucleotide region of the cx43 promoter (Oltra et al., 2003; Oltra and Werner, 1998). This sequence had been shown previously to function as a cis-activator in the transcription of the cx43 gene (Chen et al., 1995).

Analysis of the Ini gene sequence demonstrates that it is evolutionarily conserved, sharing >70% identity with proteins from a wide variety of organisms from yeast to human. To further elucidate the potential function of the Ini protein, we have cloned a homologous sequence from fission yeast (Schizosaccharomyces pombe), called ini1+, and investigated its putative function by gene disruption experiments. Deletion of ini1+ from S. pombe was lethal, and loss of Ini1 resulted in a block to cell cycle progression. Cell cycle arrest required the activity of the Weel protein kinase, a negative regulator of mitosis, but not Rad3, a kinase required for the G2-M checkpoint control. Therefore, the DNA damage checkpoint is not involved in the cell cycle delay.

Some of the characteristics of the ini1+ gene and the phenotype of the Sin1 mutant were reminiscent of a collection of cdc/prp genes that have been shown to be required for normal pre-mRNA splicing in yeast (Burns et al., 1999; Habara et al., 2001; Lundgren et al., 1996; Ohi et al., 2002; Potashkin et al., 1998; Potashkin et al., 1989; Urushiyama et al., 1996). At least three of these genes, cdc5, cdc28 and prp12+ are required for cell viability and cell cycle progression (Habara et al., 2001; Lundgren et al., 1996; Ohi et al., 1994). Furthermore, a Saccharomyces cerevisiae homologue of ini1+ is present in purified preparations of the yeast U2/U5/U6 snRNP complex, which is required for pre-mRNA splicing (K. Gould, personal communication). These findings suggest that S. pombe ini1+ is required for pre-mRNA splicing. Consistent with this hypothesis, we show that six of seven intron-containing genes are incompletely spliced in cells depleted of Ini1. Therefore, Ini1 may be required for general splicing of pre-mRNA, at least some of which encode proteins required for normal cell cycle progression.

Materials and Methods

Yeast strains and methods

All yeast strains were derived from S. pombe 972 and 975. All growth conditions, and genetic manipulations were as previously described (Moreno et al., 1991). For flow cytometry analysis of DNA content, S. pombe cells were stained with propidium iodide and analyzed using a Becton Dickinson FACScan as previously described (Sazier and Sherwood, 1990).

Cloning and deletion of ini1+ gene

To delete the ini1+ gene, a 0.5 kb fragment, containing all four exons encoding Ini1 were replaced by a single copy of the ure4+ gene. A PCR-generated fragment containing the ure4+ gene flanked by approximately 1 kb of genomic sequence upstream and 0.8 kb downstream of the ini1 coding sequence was transformed into the diploid strain, h+/h− ure4-D18/ure4-D18 leu1-32/leu1-32 ade6-M210/ade6-M216. The primers used for PCR amplification of the genomic sequence upstream of exon 1 were: 5’-Sall-tagged- GTGTGCA TAAA TACCCA TACTTAG-3’ and 5’- HindIII-tagged- TGAATATCCTCAGGATTATTTCC-3’. The primers used for PCR amplification of the genomic sequence downstream of exon 4...
were: 5'-HindIII-tagged-GTACATCTAATGGTGAGACTTGTGTTTCC-3' and 5'-GAAAGATTTCGCAAGCATGTC-3'. Stable integrants were isolated on minimal medium lacking uracil at 32°C and the deletion of ini1+ coding sequence was confirmed by Southern blot analysis. p1K-ini3X and p1K-ini81X were constructed by first inserting a cDNA encoding amino acids 1-117 of S. pombe obtained by RT-PCR into the SalI and BamHI sites of the pRep3X or pRep81 vectors. The PstI to BamHI fragment from the resulting plasmid containing the nmtI or nmt81 promoter and ini1+ gene were then subcloned into pJK148 (Keeney and Boeke, 1994). The resulting plasmids pJK-ini3X and pJK-ini81X were linearized with NdeI and transformed into the Δini1/ini1+ diploid strain. Stable integrants were selected that grew in the absence of leucine, were sporulated and plated to isolate Δini1 cells expressing either pJK-ini3X or pJK-ini81X. For expression of human Ini, a cDNA encoding Ini protein was cloned into the SalI site of the pRep3X vector (pRep3X-hIni).

Localization of GFP-tagged Ini1
For expression of GFP-tagged Ini1, the ini1+ cDNA was linked in-frame to the carboxy-terminal end of the GFP coding sequence in the pEGFP-C1 plasmid (Clontech). A SalI-BamHI PCR-amplified fragment containing the cDNA encoding GFP-tagged Ini1 was inserted either in the pRep3X or the pRep81 vectors. The resultant constructs were transformed into Δini1 strain and following sporulation, haploid cells containing both the Δini1 and RepGFP-Ini1 were selected, stained with DAPI and visualized using a Zeiss Axiophot microscope equipped with an Endow GFP BP filter (Chroma Technology Corp.) and Openlab 3.0.2 software (improvision®).

RT-PCR and northern blot analysis
Preparation of total RNA was carried out as described previously (Schmitt et al., 1990). Total RNA (1 μg) isolated from wild-type or mutant strains was subjected to DNasel digestion with the DNA-free kit (Ambion). DNasel-treated RNA was converted to cDNA using the SuperScript™ choice system (Gibco-BRL, Gaithersburg, MD). Primers used for RT-PCR amplification included the following: 5'-GGTTTATACACACTGAGTGC-3' and 5'-GAAAGATTTCGCAAGCATGG-3' (for ini1+), 5'-GGAGATGGCGGATTGAGCGGCTG-3' and 5'-GGCAATGAGTGGCAGAAAGGCTG-3' (for cwf8 + intron 2), 5'-AAGCTGAGAAGCTCATTGTTG-3' and 5'-GCAAGCTGAGATGGCAGAAAGGCTG-3' (for cdc2+ introns 3 and 4), 5'-ATTTCGTTTACCCACCACG-3' and 5'-GGTTTATACACACTGAGTGC-3' (for tfld intron 1), 5'-ACTTCTGGCGCAGGTACCAAG-3' and 5'-GGTTTATACACACTGAGTGC-3' (for nDA3 intron 4), 5'-GGAGATGAGTGGCAGAAAGGCTG-3' and 5'-GGCAACAGATGTAATTCTAG-3' (for rpl7 intron) and 5'-GGGTAACCTGGTTGATATCCCTTG-3' and 5'-CGAATGGTTTCACTTCAACATC-3' (for ade2 intron 2). PCR products were resolved on 2-3% agarose gels and stained with ethidium bromide.

For northern blot analysis of U6 snRNA, 5 μg of total RNA from each mutant grown under permissive or restrictive conditions was mixed with a dye solution (80% formamide, 1X Tris-Borate-EDTA (TBE), 0.1% Bromophenol Blue and 0.1% xylene cyanol) and electrophoresed for 40 minutes at 300 V on an 8% polyacrylamide gel. The RNAs were electrobotted onto positively charged Qiabrane nylon membranes (Qiagen) in 1X TBE at 100 V for 40 minutes. A biotinylated oligonucleotide complementary to the exon 2 of the U6 snRNA (5'-CTCTCAATGTGCAAGTGGCTACCTTG-3') and the streptavidine-HRP system (cat. no. 20148Y Pierce) was used for detection, according to the manufacturer’s recommendations.

Results
Characterization of the ini1+ gene in yeast.
Potential homologues of the mammalian Ini gene were identified in both S. cerevisiae and S. pombe by searching their respective genome databases. The structure of the S. pombe ini1+ gene was shown in Fig. 1A, and its alignment with the human and budding yeast Ini sequences is shown in Fig. 1B. The ini1+ genes of S. cerevisiae and S. pombe encode proteins that are 55% and 74% identical with the human Ini gene, respectively. In S. pombe, ini1+ is located on chromosome I between nucleotides 3190 and 2686 of the cosmid 23H3. The four ORFs shown in Fig. 1A encode a protein of 117 amino acids, making S. pombe Ini1 seven amino acids longer than its mammalian counterpart. Using RT-PCR and DNA sequencing we confirmed the presence of an Ini1-encoding transcript containing all four predicted exons. All three predicted introns were removed (GenBank accession no. AY279206).

Ini1+ is required for cell cycle progression
To determine whether the Ini1 protein of S. pombe is essential for cell viability, we constructed a diploid strain in which one copy of the ini1+ gene was replaced with ura4+. Construction of the deletion strain (ade6-M210/ade6-M210 ura4-D18/ura4-D18 leu1-32/leu1-32 ini1+ ini1::ura4+ h+) was confirmed by Southern blot analysis (data not shown). When this strain was induced to sporulate and the germinating spores were subjected to tetrad analysis, a 2:2 segregation pattern of viable:non-viable cells was observed indicating disruption of
Ini1 is required for cell cycle progression

Fig. 2. Germination of spores disrupted for the ini1+ gene. (A) Phase contrast images of spores derived from an ini1+/Δini1 tetrad upon germination in YE medium. A 2:2 segregation pattern is observed. (B) DNA content of germinating haploid spores derived from ini1+/Δini1 diploids grown on the presence of uracil, to allow all spores to germinate (left), or in the absence of uracil, to restrict germination to only those spores deleted for ini1+ (right). The positions of 1C and 2C DNA content are indicated. Cells were collected at 2-hour intervals for 12 hours and processed for flow cytometry (FACS) analysis as previously described (Sazer, 1990).

diploid integrants were incubated in the presence or absence of thiamine. In the absence of thiamine both transformants appeared identical to wild-type cells suggesting that ini1+ expression (from either the nmt1 or nmt81 promoter) can complement the deletion strain and that overexpression of ini1+ has no deleterious effects on cell growth (Fig. 3A, Δini1, minus thiamine). Upon addition of thiamine to the medium, cell division was blocked in cells containing the nmt81-ini1+, while cells expressing nmt1-ini1+ continued to divide (data not shown). In the case of cells expressing nmt81-ini1+, significant cell elongation was observed 10–12 hours after thiamine addition (Fig. 3A, Δini1, +thiamine), and cell number no longer increased (Fig. 3B, Δini1, +th). FACS analysis suggests that cells depleted for Ini1 complete DNA replication and arrest with a G2 (2C) DNA content (Fig. 3C, Δini1, + thiamine). Please note that for the remainder of this manuscript the mutant Δini1pKrep81ini1+ will be referred to as Δini1.

As noted above, S. pombe ini1+ is highly conserved, sharing 74% identity with human Ini1 at the amino acid level. Therefore we set out to determine whether the human gene could functionally complement the Δini1 strain. For these experiments, we cloned the human Ini cDNA into the Rep3x plasmid pRep3x-hINI. Transformation of the deletion strain with this construct yielded colonies able to grow in the absence of uracil, leucine and thiamine, indicating that Ini1's essential function was not compromised by fusion to GFP. As shown in Fig. 4, GFP-Ini1 is localized predominantly in the nucleus, consistent with its proposed role in transcriptional

an essential gene. As expected the two viable colonies failed to grow in the absence of uracil. Microscopic examination demonstrated that although spores deleted for ini1+ germinated, they arrested with a 'cdc' elongated cell phenotype following one to three cell divisions (Fig. 2A). Further analysis of germinating spores demonstrated that cells lacking ini1+ fail to increase in cell number (data not shown), and arrest with a 2C DNA content (Fig. 2B), consistent with these cells being blocked in late S phase or in the G2 phase of the cell cycle. As a control, if uracil was added to the medium to allow the ini1+ura+ spores to germinate, cell number steadily increased throughout the course of the experiment.

Cells deleted for ini1+ are rescued by expression of ini1+ or the human Ini gene

Since the construct used to generate the ini1+ deletion was made by PCR, including a mixture of Pfu and Taq polymerases, it was possible that the observed phenotype was due to mutations in the sequences overlapping, or adjacent to, the ini1+ gene. To rule out this possibility, we re-established Ini1 expression in the deletion strain by introducing a plasmid expressing the wild-type ini1+ gene. If Ini1 is indeed required for normal cell progression, expression of ini1+ should rescue the observed cell cycle arrest phenotype.

For these experiments, an S. pombe ini1+ gene (encoding amino acids 1-117) was placed under the control of thiamine repressible nmt1 or nmt81 promoter in plasmid vector Rep3x or Rep81, respectively (Maundrell, 1993). Both promoters are repressed by addition of thiamine; the wild-type nmt1 promoter being more leaky than the stringently repressed nmt81 promoter (Maundrell, 1993). The nmt1 wild-type and nmt81-ini1+ constructs were then sub-cloned into the pJK148 integrating vector and transformed into the Δini1 strain as described in Materials and Methods. Haploid Δini1 spores expressing either nmt1- or nmt81-ini1+ isolated from the stable
regulation. In contrast, the nucleolus appears to lack Ini1 protein (Fig. 4, see arrows).

Cell cycle arrest in $\Delta ini1$ cells requires the activity of the Wee1 kinase, but not Rad3 kinase

The G2 to M phase transition in eukaryotic cells is controlled by the activity of the cyclin-dependent kinase, Cdc2 (Nurse, 1990). Activation of Cdc2 relies on the activity of the protein phosphatase Cdc25 that dephosphorylates a critical tyrosine residue in the ATP binding site of the enzyme (Gautier et al., 1991; Kumagai and Dunphy, 1991; Russell and Nurse, 1986; Sebastian et al., 1993; Strausfeld et al., 1991). This activity is opposed by the activity of the Wee1 kinase that phosphorylates this site (Lundgren et al., 1991; Parker et al., 1992; Parker and Piwnica-Worms, 1992; Russell and Nurse, 1987). During the cell division cycle there is a strict size control over entry into mitosis, and this is achieved in part through the activity of Wee1 (Nurse, 1975; Russell and Nurse, 1987). When Wee1 kinase activity is compromised by mutation, cells enter mitosis at a significantly reduced size. Therefore the Wee1 kinase is a general negative regulator of the G2 to M phase transition. To test whether Wee1 is required for the cell cycle arrest observed in the $\Delta ini1$ mutant we constructed the double mutant wee1-50 $\Delta ini1$ and examined its phenotype following shut off of the ini1 + gene by addition of thiamine. In the absence of thiamine the double mutant behaves like the wee1-50 mutant alone, smaller cells at the restrictive temperature of 36°C, when compared to wild-type cells grown at the same temperature (Fig. 5A a and b and Fig. 5B, wt and wee1$\Delta ini1$–th). To test whether Wee1 is required for cell elongation in the absence of Ini1 we incubated the double mutant wee1-50$\Delta ini1$ at 36°C in the presence of 15 $\mu$M thiamine. Thiamine was added at the same time as the shift to the restrictive temperature. Under these conditions, no cell elongation was observed 12 hours after shift to restrictive conditions suggesting that Wee1 is required for the cell cycle block (Fig. 5A d and Fig. 5B, wee1$\Delta ini1$+th). As a control, $\Delta ini1$ cells treated with thiamine at 36°C for 12 hours showed the expected cell elongation phenotype (Fig. 5A c and Fig. 5B, $\Delta ini1$+th). Moreover, if thiamine was added to the double mutant wee1-50$\Delta ini1$ at 25°C, when Wee1 kinase is still active, cell elongation was observed (data not shown). Microscopic analysis demonstrated that a high percentage of cells lacking both Ini1 and Wee1 (>30%) undergo a catastrophic mitosis, displaying the (cell untimely torn) ‘cut’ phenotype (Fig. 5A d, see arrows). This indicates that in the absence of both Wee1 and Ini1, the inability to arrest the cell cycle leads to a significant increase in the percentage of aberrant mitoses.

We also examined cell viability following addition of thiamine at 36°C. Cells were harvested at two-hour intervals, washed extensively to remove any traces of thiamine and plated at 25°C on minimal agar lacking thiamine. After 5 days of incubation, viable cell colonies were counted and plotted (Fig. 5C). Interestingly, cell viability of the $\Delta ini1$ strain was significantly reduced (Fig. 5C, closed squares) following even a relatively brief treatment with thiamine (2 hours). This is well before any noticeable effect on cell division is observed (Fig. 5C).
A. Significant proportion of the event. Perhaps even more surprising was the observation that a deletion of \( \text{Bentley et al., 1996; Carr and Hoekstra, 1995}. \) We observed \( \Delta \) kinase Rad3 is essential for the cell cycle arrest observed in the \( \text{O’Connell, 2000}, \) we also tested whether the checkpoint induced by the DNA damage checkpoints \( \text{Raleigh and} \) implies that the consequence of losing Ini1 is a cellular defect that leads to a Wee1-dependent cell death.

Since the Wee1 kinase plays a role in cell cycle arrest induced by the DNA damage checkpoints \( \text{(Raleigh and} \) O’Connell, 2000), we also tested whether the checkpoint kinase Rad3 is essential for the cell cycle arrest observed in the \( \Delta \text{ini1} \) mutant. Rad3 is related to the mammalian checkpoint genes ATR/ATM \( \text{(Cahill et al., 1998)}, \) and is essential for all known DNA damage-dependent checkpoints in \( \text{S. pombe} \) \( \text{(Bentley et al., 1996; Carr and Hoekstra, 1995).} \) We observed that deletion of \( \text{rad3}^+ \), which is a non-essential gene, has no effect on the observed cell cycle arrest of \( \Delta \text{ini1} \) cells (data not shown). Therefore, activation of the Rad3-dependent checkpoint does not play a role in the cell cycle arrest triggered by depletion of Ini1.

Pre-mRNA splicing defects are observed in the \( \Delta \text{ini1} \) mutant

Many of the characteristics of the \( \Delta \text{ini1} \) mutant were similar to those reported for mutants defective in pre-mRNA splicing. These include the mutants \( \text{cdc5, cdc28 and} \) \( \text{ppr12, all of which appear to arrest in G2 upon shift to the restrictive temperature (Burns et al., 1999; Habara et al., 2001; Ohi et al., 1994).} \) It has also been reported that \( \text{cdc5 mutants, in the absence of} \) Wee1, undergo mitotic catastrophe similar to what we observe following depletion of Ini1 \( \text{(Ohi et al., 1994).} \) In addition, mass spectrometric analysis of the purified U2/U5/U6 snRNP complex from yeast identified additional proteins that are likely to be required for pre-mRNA splicing \( \text{(Ohi et al., 2002).} \) Like Ini1, many of these proteins share extensive homology with human proteins, and in some cases, contain RING-finger-like motifs. Therefore, the Ini1 sequence was compared to a large number of polypeptide sequences obtained from the mass spectrometry analysis of the U2/U5/U6 snRNP purified from yeast identified additional proteins that are likely to be involved in pre-mRNA splicing. To test this possibility directly, we used RT-PCR to measure the steady state levels of pre-mRNA and mRNA in Ini1-depleted cells for intron-containing genes transcribed by RNA Pol I \( \text{(rpl7*), Pol II (cdc2^+}, \) \( \text{cwp8}^+ \), and \( \text{tfld^+}, \text{ade2}^+, \text{nda3}^+ \) and Pol III \( \text{(U6 snRNA).} \) As a control, we measured the levels of pre-mRNA that accumulate at 36°C in \( \text{ppr2-1, a mutant previously shown to be defective for pre-mRNA splicing (Potashkin et al., 1989; Urushiyama et al.,} \text{)}.} \)
As expected, significant accumulation of pre-mRNA of all seven genes is observed in the prp2-1 mutant at 36°C (Fig. 6A-G). With the exception of cdc2, we also observed an accumulation of pre-mRNA in the ∆ini1 mutant following addition of thiamine (Fig. 6A-C-G). For most genes, maximum inhibition of splicing was observed 12-14 hours following addition of thiamine suggesting that depletion of Ini1 may take several hours. Although the levels of accumulation of pre-mRNA did not reach the levels detected in the prp2-1 mutant, they were clearly above background and are similar to data published for other splicing defective mutants (Bishop et al., 2000; Burns et al., 1999; Habara et al., 2001; Shimoseki and Shimoda, 2001; Urushiyama et al., 1996). The prp2-1 mutant is unique in displaying a very strong inhibition of splicing at the restrictive temperature. Although splicing of the cdc2 transcript was effectively blocked in the prp2-1 mutant control, no detectable cdc2 pre-mRNA was observed in the ∆ini1 mutant. Interestingly, splicing of cdc2 transcripts was not affected in mutant prp12-1 (Habara et al., 2001), suggesting that Prp12, like Ini1, may not be required for splicing of cdc2 + message. However, we did notice a slight decrease in the levels of cdc2 + spliced product in the ∆ini1 mutant (+th) suggesting that depletion of Ini1 did lead to a decrease in cdc2 + mRNA. Thus, it is possible that cdc2 + pre-mRNAs may be highly unstable and rapidly degraded following inhibition of splicing. Consequently, the accumulation of cdc2 + pre-mRNA might only be observed in stronger mutants like prp2-1.

To ensure that accumulation of pre-mRNA is not just a consequence of a cell cycle block in G2, we tested whether pre-mRNA accumulates in the cdc25-22 mutant, a mutant that undergoes cell cycle arrest in G2 when incubated at the non-permissive temperature of 36°C. No accumulation of pre-mRNA was observed in the cdc25-22 mutant at 36°C, even after prolonged incubation (data not shown). We conclude that the Ini1 protein is required for efficient splicing of cellular pre-mRNAs. Some of these mRNAs are likely to encode factors required for onset of mitosis.

**Discussion**

We identified an *S. pombe* homologue of Ini, a mammalian protein that associates with the cx43 gene promoter and has been implicated in the transcriptional regulation of the cx43 gene (Oltra et al., 2003). Although the precise function of the Ini protein in mammalian cells is currently unknown, our analysis of ini1 + in *S. pombe* has revealed at least one of its potential activities. Cells depleted of Ini1 undergo a cell cycle arrest and show defects in pre-mRNA splicing. Although our study only examined a limited set of genes, the fact that an accumulation of pre-mRNA was observed for six of the seven genes tested strongly supports a role for Ini1 in general mRNA splicing. Our hypothesis that Ini1 is required for pre-mRNA splicing is further supported by the observation that a *S. cerevisiae* homologue of Ini1 has been identified in association with the purified U2/U5/U6 snRNP complex from budding yeast (#3695) (K. Gould, personal communication). Other studies have shown that, consistent with our yeast data, the human homologue of Ini, called SF3b14b, is associated with 17S U2 snRNP, which is required for assembly of the spliceosome (Will et al., 2002).

Interestingly, cells lacking Ini1 undergo a cell cycle arrest that is dependent on expression of the Wee1 kinase. Although mutations in wee1 + are frequently observed to be epistatic to mutations that block in G2, the observation that at least some of the ∆ini1 cells display a cut phenotype in the absence of
wee1+ suggests that cells lacking Ini1 are incapable of completing a successful mitosis. The loss of viability observed in the absence of Ini1 is not limited to cells that contain a mutation in Wee1, but is also observed following depletion of Ini1 from wild-type cells. In fact, loss of Wee1 function appears to suppress rather than enhance the lethality associated with depletion of Ini1. This indicates that although the loss of Wee1 in Ini1-depleted cells can lead to mitotic catastrophe in some cells, the remaining cells that fail to enter mitosis may survive. This implies that Wee1 may have a positive role in promoting cell death in the absence of Ini1.

It is still not clear why loss of Ini1 leads to a cell cycle arrest. One possibility is that Ini1 is required to accumulate some critical factors required for the G2 to M phase transition. Under these conditions Cdc2 may be maintained in an inactive state by the Wee1 kinase. In this model, Wee1 would be part of a checkpoint control, distinct from the DNA damage checkpoint, that blocks mitosis in the absence of some critical factor(s) whose expression or synthesis is dependent on Ini1. One example of such a protein might be β-tubulin (encoded by nda3+), whose activity is required for assembly of the mitotic spindle, and whose levels might be dependent on the activity of Ini1 (Fig. 6E). Although the cell cycle arrest phenotype is consistent with Ini1 having a specific function during G2, we cannot exclude the possibility that Ini1 is essential for other cellular processes that occur throughout the cell cycle and are required to maintain cell viability.

Mammalian Ini was originally identified in rat uterus as a protein that binds directly to a cis-acting regulatory region of the cx43 gene promoter, and was found to be upregulated in response to estrogen (Oltra et al., 2003). Overexpression of Ini has been shown to increase transcription from a cx43-reporter gene approximately twofold in response to estrogen (Oltra et al., 2003). This would suggest that the Ini protein might be involved in both transcriptional regulation and pre-mRNA processing (as suggested by our own experiments). One interesting possibility is that the Ini protein binds specifically to cis-acting elements and is important in coordinating active transcription with pre-mRNA splicing, in a process known as ‘cotsplicing’ or ‘transcriptional-splicing coupling’ (Goldstrohm et al., 2001; Neugebauer, 2002). Such a mechanism has been proposed for androgen receptor-mediated transcriptional regulation in human cells (Zhao et al., 2002). Also, more recently, it has been shown that the transcriptional activation domain AF-1 of the estrogen receptor can also regulate alternative splicing, suggesting that steroid hormone receptors might simultaneously control both transcription and exon usage of the product mRNA (Auboeuf et al., 2002). Interestingly, Ini was found to stimulate estrogen receptor-mediated induction of a cx43 promoter-luciferase reporter in a manner that was dependent on the presence of the AF-1 domain (Oltra et al., 2003), suggesting that Ini might influence AF-1’s activity on transcription and/or splicing. Although the loss of Ini1 clearly leads to a splicing deficiency in yeast, it is not yet known whether gene transcription is also affected. However, it is worth noting that in budding yeast a homologue of Ini has been shown to interact with components of the transcription elongation complex (Ito et al., 2001). Based on the observations in mammalian cells, it will be interesting to determine if Ini1 has any affinity for yeast gene promoters, especially those that control expression of genes required for cell cycle progression.

Finally, Ini homologues have been identified in other organisms, including Caenorhabditis elegans and zebrafish (Golling et al., 2002; Trappe et al., 2002). Depletion of Ini protein by introduction of RNAi (in C. elegans) or by insertion of mutagenesis (in zebrafish) is lethal and leads to gross developmental defects, underscoring the high degree of evolutionary conservation and functional importance of the Ini protein. In worms, expression of the Ini homologue phf-5 was restricted to the muscle tissue, and was essential for normal morphogenetic development and muscle function (Trappe et al., 2002). These observations are at least consistent with Ini functioning as a regulator of transcription and/or splicing.

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