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The *Saccharomyces cerevisiae* gene *CDC40/PRP17* controls cell cycle progression through splicing of the *ANC1* gene

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ABSTRACT

The timing of events in the cell cycle is of crucial importance, as any error can lead to cell death or cancerous growth. This accurate timing is accomplished through the activation of specific *CDC* genes. Mutations in the *CDC40/PRP17* gene cause cell cycle arrest at the G_2/M stage. It was previously found that the *CDC40* gene encodes a pre-mRNA splicing factor, which participates in the second step of the splicing reaction. In this paper we dissect the mechanism by which pre-mRNA splicing affects cell cycle progression. We identify *ANC1* as the target of *CDC40* regulation. Deletion of the *ANC1* intron relieves the cell cycle arrest and temperature sensitivity of *cdc40* mutants. Furthermore, we identify, through point mutation analysis, specific residues in the *ANC1* intron that are important for its splicing dependency on Cdc40p. Our results demonstrate a novel mechanism of cell cycle regulation that relies on the differential splicing of a subset of introns by specific splicing factors.

INTRODUCTION

The orderly progression through the mitotic cycle in eukaryotic cells is ensured by the highly regulated transition from one phase to the next. The accuracy of this process is critical for the high fidelity transmission of genetic information. Accuracy is achieved by the timely activation/inactivation of specific cell division cycle (*CDC*) genes, and is genetically controlled by surveillance mechanisms termed checkpoints (reviewed in 1).

In this work we have investigated the role of a specific *CDC* gene, *CDC40*, in connecting cell cycle progression and pre-mRNA splicing. *CDC40* was first identified through the temperature-sensitive mutation *cdc40–1*. This mutation affects both the mitotic and meiotic cycles (2). At the restrictive temperature, *cdc40* strains arrest as large-budded cells with one undivided nucleus containing a duplicated content of DNA, a phenotype characteristic of G_2/M arrest (2,3). Besides the role played in the transition from G_2 to M, Cdc40p is required for efficient entry into the S phase following release from α-factor-induced G_1 arrest (3–5). In addition, *cdc40* cells are also slightly sensitive at the restrictive temperature to UV and ionizing irradiation, and show sensitivity to DNA damaging agents such as methyl methane sulfonate (MMS) (6,7). Independently, genetic screens identified *PRP17* as a gene involved in the second step of the splicing reaction; mutations in this gene accumulate small amounts of splicing intermediates (8). Sequence comparison revealed that *CDC40* and *PRP17* are the same gene. Thus the *PRP17/CDC40* gene is involved in two seemingly unrelated processes: pre-mRNA splicing and progression through G_1/S and G_2/M phases of the cell cycle. For simplicity, henceforward we will refer to the gene as *CDC40.*

Eukaryotic genes are often interrupted by intervening sequences (introns) that must be cleaved out precisely during gene expression. Although introns need to be removed with single-nucleotide precision to avoid introducing catastrophic errors of frame, they contain only very few conserved sequences. Only three short regions are conserved among all yeast introns: the 5′ splice site (GUAUGAGU), the branchpoint (UACUAACA) and the 3′ splice site (PyAG, usually preceded by a pyrimidine-rich tract). These short sequences are highly important for the efficient splicing of pre-mRNA. For example, it has been shown that mutations in the first two nucleotides (GU) of the 5′ splice site or in the AG dinucleotide at the 3′ splice site of *Saccharomyces cerevisiae* introns block the first or second step of splicing, respectively (9,10).

Pre-mRNA splicing occurs by two transesterification reactions. In the first catalytic step, the 2′ hydroxyl of the conserved intronic adenosine attacks the phosphate at the 5′ splice site, producing a free 5′ exon and a branched species termed the lariat intermediate. In the second catalytic step, the 3′ hydroxyl of the 5′ exon attacks the phosphate at the 3′ splice site, yielding ligated mRNA and a lariat intron. Pre-mRNA splicing requires a large number of *trans*-acting factors that constitute the spliceosome (11). The splicing reaction is highly dynamic and requires a large number of protein–protein, protein–RNA and RNA–RNA interactions. The spliceosome includes several small nuclear ribonucleoprotein particles (snRNPs) composed of small nuclear RNA molecules (U1, U2, U4, U5 and U6 snRNA) associated with a large number of specific proteins. In addition, a large number of non-snRNP proteins are either transiently associated with, or constitute an integral part of, the spliceosome (11). Briefly, the 5′ splice site

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is initially recognized through a base-pairing interaction with U1 snRNA (12). The branchpoint sequence is first recognized by the branchpoint binding protein (BBP) in a sequence-specific fashion. This interaction is later replaced by base pairing with U2 snRNA. In the next step, the U4/U5/U6 triple snRNP binds. This binding heralds a large number of RNA:RNA rearrangements. U1 is released from the pre-mRNA, the stable interaction between U4 and U6 is disrupted and U4 is released. This release frees the U6 snRNA, which is now available for base pairing with U2 snRNA (12). Recent findings suggest that the RNA rearrangements are then followed by the recruitment of an additional protein complex (named NTC or CSC) which interacts and modifies the U5 snRNP (13). Having executed these RNA rearrangements, the spliceosome is competent to carry out the two steps of splicing. Although much progress has been made in understanding how the 5′ splice site and branchpoint are recognized, the mechanism that recognizes 3′ splice sites remains ill defined. Following the first transesterification reaction, another rearrangement occurs which aligns the two splicing sites and positions them for the second transesterification reaction (12). Recent findings suggest that all of the above rearrangements may occur within the context of a pre-existing higher-order particle [reviewed in (11)].

Cdc40p plays a role in the second step of the splicing reaction along with Prp8p, Prp16p, Prp18p, Prp22p and Slu7p (8,14). Previously, we implemented a screen to identify mutants that are lethal in the absence of Cdc40p. This screen identified several genes, including three novel genes *SYF1–3*, which were shown to play a role in both cell cycle progression and splicing (15,16). When the Syf proteins were used as baits in two-hybrid screens, we uncovered a complex network of interactions which suggested the existence of a protein complex (CSC) that plays a role in both splicing and cell cycle progression (15,16). Biochemical analysis in both *S.cerevisiae* and *Schizosaccharomyces pombe* has confirmed the existence of this complex (17,18; O. Dahan and M. Kupiec, unpublished results).

In addition to *CDC40* and the *SYF*s, a handful of genes in both *S.cerevisiae* and *S.pombe* have also been identified in genetic screens for splicing factors and independently in screens for cell cycle regulators. These include *PRP3* (19), *PRP8* (20), *PRP22* (21) and *CEF1* (22) in *S.cerevisiae*, and *prp2* (23), *prp5* and *prp6* (24), *prp8* (25) and *prp12* (26) in *S.pombe*. All of the above strongly indicate that pre-mRNA splicing and cell cycle control are linked *in vivo*. Despite this wealth of evidence, the exact molecular mechanism underlying the connection between cell cycle progression and splicing is still unclear.

In this paper we examine the role played by *CDC40* in splicing and cell cycle regulation. By screening for cDNAs that can overcome the splicing defect in a *cdc40* strain we show that the cell cycle arrest phenotype observed in this mutant is due to the inefficient splicing of the *ANC1* pre-mRNA. We identify, through point mutation analysis, specific residues in the *ANC1* intron that are important for its splicing dependency on Cdc40p. Our results suggest that cell cycle progression can be regulated at the level of splicing and that Cdc40p is important for the efficient splicing of a subset of introns with suboptimal splicing sequences.

MATERIALS AND METHODS

Media and growth conditions

Yeast cells were grown at various temperatures (in accordance with the required experimental conditions) in either YEPD (1% yeast extract, 2% bactopeptone, 2% dextrose) or SD media (0.67% yeast nitrogen base and 2% dextrose, supplemented with appropriate nutrients). In order to induce expression from the *GAL* promoter, cells were grown on YEPGal (1% yeast extract, 2% bacto peptone, 2% galactose) or SGal (0.67% yeast nitrogen base and 2% galactose, supplemented with appropriate nutrients) media. Bacto Agar (1.8%) was added for solid media. Selective media lacking one nutrient are designated SD-nutrient (e.g. SD-Ura). Ura$^-$ colonies were selected on SD complete medium with uracil (50 mg/l) and 5-fluoroorotic acid (5-FOA) (0.8 g/l). $CuSO_4$ (Merck) was diluted into solid or liquid medium at the concentrations indicated from a stock 1 M solution.

Yeast strains

YR4: *MAT***a** ade2-1 his3-RS21 leu2Δ1 lys2-801 trp1Δ1 ura3-52 can1-100. SB37: MAT**a** cdc40*::*URA3 ade2-1 his3-RS21 leu2Δ1 lys2-801 trp1Δ1 ura3-52 can1-100. MK89: MAT**a** leu2-3,115 trp1-1 ade2-1 ura3-NcoΔ his3-11 can1-100. IM2: MAT**a** cdc40::LEU2 leu2-3,115 trp1-1 ade2-1 ura3-NcoΔ his3-11 can1-100. IM2–M5: MAT**a** cdc40::LEU2 TRP1-P_{GAL10}-TUB1Δi leu2-3,115 trp1-1 ade2-1^{oc} ura3-Nco his3-11 can1-100. YOD6: *MAT***a** *cdc40::LEU2 TRP1-* P_{GAL10}-*TUB1Δi ANC1ΔI leu2-3,115 trp1-1 ade2-1^{oc} ura3-NcoΔ his3-11 can1-100.* W303: *MAT***a** *leu2-3,112 trp1Δ1 can1-100 ade2-1 ura3-1 his3-11,15.* YOD1-A: *MAT***a** *anc1::HIS3 leu2-3,112 trp1Δ1 can1-100 ade2-1 ura3-1 his3Δ300.* YCL51: *MAT***a** *ura3 leu2 his3 trp1 lys2 cup1::ura3.* SB90: *MAT*α *cdcd40::leu2::HIS3 ura3 leu2 his3 trp1 lys2 cup1::ura3.*

Plasmids

Saccharomyces cerevisiae cDNA library: yeast cDNA expression library, regulated by the *GAL1* promoter cloned into pRS316 vector (*URA3-CEN*) (kindly provided by Professor D. Kornizer, Technion, Israel). YIplac211: integrative *URA3* marked plasmid. pJU83: *CUP1-ACT1* reporter plasmid (*CEN-LEU2*) (27).

The following plasmids contain an *ANC1-CUP1* reporter, and were constructed as explained below. pSBY87: *ANC1-CUP1* reporter plasmid, based on pJU83 (*CEN-LEU2*). BOD3: ACT-ACT, contains the *ACT1* branchpoint and the *ACT1* 3′ splice site. BOD6a: ANC-ACT, contains the *ANC1* branchpoint and the *ACT1* 3′ splice site. BOD7: ANC-ANC, contains the *ANC1* branchpoint and the *ANC1* 3′ splice site. BOD8: ACT-ANC, contains the *ACT1* branchpoint and the *ANC1* 3′ splice site. BOD9: ANC T71A-ACT, is BOD6a with a T71A mutation. BOD10: ACT-ANC A97T, is BOD8 with an A97T mutation.

Construction of the *CUP1* reporting plasmids

Plasmid pSBY87 was constructed as follows: pJU83 (28) was digested with BamHI followed by partial digestion with Asp718, causing the release of ACT1 intron. A PCR fragment containing the *ANC1* intron was produced using the following primers: upper primer: 5′-CGCGGATCCATGGTAGCTGT-

ATGT-3′; lower primer: 5′-CGGGGTACCTTTTACTGTC-TGTCTGTCTGTTG-3′. After digestion of the PCR fragment with both BamHI and Asp718, it was ligated to the linear plasmid to create pSBY87.

The plasmids containing the *ACT1-ANC1* chimeric introns were constructed as follows. First, pJU83 was digested with both XhoI and PacI causing the release of the branchpoint and 3′ splice site of the *ACT1* intron. PCR fragments containing different combinations of *ACT1* and *ANC1* branchpoint and 3′ splice site were obtained using the following as templates: pJU83 (in the case of BOD3 and BOD6a), pSBY87 (in the case of BOD7 and BOD8), BOD6a (in the case of BOD9) or BOD8 (in the case of BOD10). The sequence of primers used to construct each of the PCR fragments is available upon request.

All PCR fragments were digested with XhoI and PacI and ligated to the purified linear pJU83 plasmid, yielding the desired clones.

Cell synchronization

Exponentially growing cells were arrested in G_1 by incubation in the presence of 4 μM α-factor (Sigma-Aldrich, Israel) for 3 h at 25°C. In order to release cells from G_1 arrest, they were washed three times and resuspended in fresh YEPD.

Screening for temperature-sensitivity suppressors

IM2-M5 (*cdc40*Δ) cells were transformed with a cDNA expression library regulated by the *GAL1* promoter on the *URA3-CEN* vector pRS316. The transformation mixture was plated on SGal-Ura plates at 25°C. After 12 h, cells were shifted to the restrictive temperature (33°C) for 3–4 days until the appearance of colonies. Colonies were then streaked on YEPGal plates and replicated on YEPGal and 5-FOA plates at 25 and 33°C. Colonies that were able to grow on YEPGal plates at 25 and 33°C and on 5-FOA plates at 25 but not at 33°C were further analyzed. Plasmids were extracted, re-checked for their ability to support growth at the restrictive conditions and then their cDNA insert was sequenced.

Creation of a genomic *ANC1*Δ*i* allele

A PCR fragment containing 785 bp upstream of the *ANC1* open reading frame (ORF) and the first 30 bp of the *ANC1* cDNA was amplified using the OR1 and OR3 oligonucleotides and the pMW13 plasmid as a template. A second PCR fragment containing the entire *ANC1* cDNA was amplified using the OR2 and OR4 oligonucleotides. Both PCR fragments were then used as templates in a third PCR using the OR1 and OR2 oligonucleotides, yielding a 1.6 kb fragment containing 785 bp upstream of the *ANC1* ORF fused to the entire *ANC1* cDNA. This fragment was cloned into the integrative *URA3* marked plasmid YIplac211 and transformed into IM2-M5 cells. 5-FOA-resistant colonies (pop-outs) were checked by PCR in order to identify cells in which the genomic *ANC1* was replaced by its cDNA. Primers used are listed below (restriction sites are underlined). OR1: 5′ CGCGGATCCGCTATTACAAGAAGCTTTG 3′. OR2: 5′ CCGGAATTCGCGGTTTTGGAATAAAGTTTAT 3′. OR3: 5′ ACGGATGGTTCTTTTTACTGTAGCTACCATGATTA-GTTATCT 3′. OR4: 5′ AGATAACTAATCATGGTAGCT-ACAGTAAAAAGAACCATCCGT 3′.

RT–PCR analysis

RNA was extracted from 5×10^7 cells using the RNeasy kit (QIAGEN Inc.) according to the manufacturer's instructions. Prior to the RT–PCR step, genomic DNA was degraded by RQ1 RNase-free DNase (Promega Inc.). Complete removal of contaminating DNA was verified by negative control PCRs with a specific set of primers. One microgram of total RNA was used as a template for cDNA synthesized using the Expand™ Reverse Transcriptase Kit (Boehringer Mannheim) and 500 ng of oligo $(dT)_{15}$ (Boehringer Mannheim) as a primer. A quarter of each cDNA preparation was used as a template in a PCR using specific primers spanning the intron of either *ANC1* or *RPL25*.

Copper growth assays

Copper-containing plates were made by diluting $CuSO_4$ into SD agar lacking the relevant nutrients at the concentrations indicated from a stock solution of 1 M. Copper-resistant growth was determined by plating assays. Logarithmic cultures of the strains of interest containing the relevant plasmids were grown to the same cell number (10^7 cells/ml). Consecutive decimal dilutions were carried out, and drop-out assays were performed on plates with different concentrations of copper. Growth was assayed after 3 days of incubation at either 25 or 30°C.

Mutagenesis of *ANC1* 3′ splice site

Two complementary oligonucleotides, each containing the *ANC1* 3′ splice site, were synthesized (named DEG5 and DEG3). Random nucleotides were incorporated at five different sites in DEG5 and DEG3 (corresponding to positions 94, 95, 97, 99 and 101 in the *ANC1* intron) as follows: DEG5, 5′ CTATTCTCATCATTANNTNANCNA-CAGACGTTAAAG 3′; DEG3, 5′ CTTTAACGTCTGTNG-NTNANNTAATGATGAGAATAG 3′.

Each degenerate oligonucleotide was then used to generate a PCR fragment using BOD8 as a template in combination with oligonucleotides CUP5 or CUP3 which are complementary to upstream and downstream sequences from BOD8: CUP5, 5′ CTTTTAGATTTTTCACGC 3′; CUP3, 5′ GACT-ATTCGTTTCATTTC 3′.

The two PCR fragments were then used as templates in a new PCR using the CUP5 and CUP3 primers, yielding PCR fragments containing random sequences at the desired positions.

Screening for mutated clones that confer copper resistance

The mutated PCR fragments were co-transformed into SB90 cells along with a linear BOD8 plasmid (digested with *Xho*I and *Pac*I). The cells were then plated on SD-Leu plates containing 0.2 mM copper and incubated at 25°C for 2–3 days until the appearance of colonies. This copper concentration was chosen because it was just above the maximal copper concentration at which BOD8 allowed growth. After rechecking the ability of the colonies to grow at high copper concentrations, the colonies were streaked on YEPD plates to allow plasmid loss and then replica plated on SD plates with or without 0.2 mM copper. Colonies that were unable to grow on plates containing copper without the plasmid were further

analyzed. Plasmids were extracted, rechecked for their ability to support growth at high copper concentration and subjected to sequence analysis.

RESULTS

The cell cycle arrest phenotype of *cdc40*Δ cells can be suppressed by *ANC1* cDNA

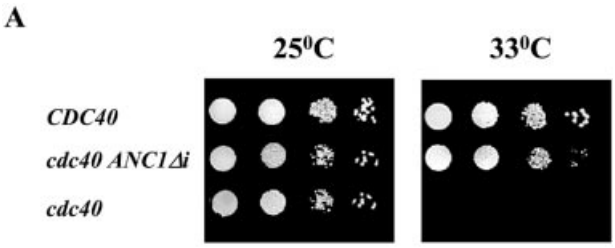
Since splicing constitutes a key cellular mechanism, genes encoding splicing factors are usually essential. Many temperature-sensitive splicing mutants have been isolated; most of them arrest at the restrictive temperature without any defined cell cycle morphology (16). However, as has been shown previously, *cdc40* mutant cells are temperature sensitive, arresting at the restrictive temperature with a large bud and one undivided nucleus (2). FACS analysis and β-tubulin staining showed that *cdc40* mutant cells held at the restrictive temperature contain 2C DNA content and a short spindle indicating that cells are arrested at the G_2/M phase of the cell cycle (2,3). We reasoned that inefficient processing of a small number of intron-containing genes particularly dependent on Cdc40p for their pre-mRNA splicing might cause the cell cycle specific arrest of *cdc40* mutant cells. If this were the case, we predicted that introduction of the relevant target gene(s) in the form of cDNA should be sufficient to bypass the need for Cdc40p and should allow growth at the restrictive temperature.

To test this hypothesis, we conducted a screen to isolate cDNAs able to suppress the temperature-sensitive cell cycle arrest of *cdc40*Δ cells. A *cdc40*Δ strain was transformed with a yeast cDNA expression library and screened for cDNAs that support cell growth at 33°C, a restrictive temperature for *cdc40* cells. About 100 000 colonies were screened; in addition to the *CDC40* gene itself, we isolated five independent clones, all carrying the *ANC1* cDNA.

In order to ensure that the suppression by *ANC1* cDNA results from the ability to bypass the *cdc40*Δ splicing defect, and not simply from over-expression of *ANC1,* we constructed a *cdc40*Δ strain in which the chromosomal *ANC1* gene was replaced by its cDNA under the control of its endogenous promoter (designated *ANC1*Δ*i*). As shown in Figure 1A, the *ANC1*Δ*i* allele was able to support growth of *cdc40*Δ cells at the restrictive temperature of 33°C. However, no growth was observed at higher temperatures. Microscopic examination of cultures held at 37°C revealed that in contrast with *cdc40*Δ mutants, *cdc40*Δ *anc1*Δ*i* cells do not exhibit a cell cycle specific arrest (Fig. 1B). The fact that *ANC1* cDNA was unable to support full vigorous growth of *cdc40*Δ cells at 37°C (data not shown), suggests that other genes, in addition to *ANC1*, are dependent on Cdc40 for splicing. These genes, however, are not required for a specific cell cycle stage.

ANC1 pre-mRNA splicing is not cell cycle regulated

ANC1 was first isolated in a screen for mutants that fail to complement a temperature-sensitive allele of *ACT1* in the heterozygous state. *anc1* mutant cells grow slowly at 30°C and are unable to grow at 37°C (29). In addition, *anc1* mutants exhibit defects in actin cytoskeleton organization and several morphological aberrations which correlate with cytoskeletal imperfections such as unusual cell morphology, sensitivity to high osmolarity and low mating (29). However, Anc1p resides exclusively in the nucleus, which is devoid of actin, suggesting that Anc1p is not involved in the actin cytockeleton function *per se*. Subsequently, it was shown that Anc1p is a component of several transcription complexes, including the Mediator (30), the TFIID and TFIIF basal transcription complexes (31), the SWI/SNF chromatin remodeling complex (32) and the NuA3 histone aceytelation complex (33).



B

	○	♂	∞
cdc40	12%	4%	84%
cdc40 ANC1Δi	40%	33%	27%

Figure 1. *ANC1* cDNA suppresses the temperature-sensitive phenotype of a *cdc40*Δ strain. (**A**) A wild-type strain (MK89), a *cdc40*Δ strain containing *ANC1* cDNA under its endogenous promoter (*ANC1*Δ*i*, YOD6) and a *cdc40*Δ strain (IM2) were diluted, spotted on YEPD plates and assayed for viability at either permissive (25°C) or restrictive (33°C) temperatures. (**B**) IM2 (*cdc40*Δ) and YOD6 (*ANC1*Δ*i*) were synchronized at G_1 with α-factor and transferred to 37°C. After 6 h incubation cells were analyzed by microscopic observation and classified as being in the G_1 (unbudded), S (small-budded cells) or G_2/M (large-budded cells) cell cycle stage. More than 300 cells were classified in this manner.

It should be noted that Anc1 is not a 'sticky' protein that is co-purified artifactually with all yeast complexes. It has been identified only in a selective minority of yeast complexes that affect transcription/chromatin, and always in stoichiometric quantities. Anc1p is remarkable in that it is an abundant protein present in several regulatory complexes, and yet, unlike the other members of these complexes, it is not essential for cell viability. The role of *ANC1* remains unclear; it may play a role in regulating transcriptional regulators or it may act as a mediator between several complexes, linking different processes in the cell.

Since the expression of *ANC1* cDNA was able to suppress the *cdc40*Δ cell cycle arrest phenotype, we assumed that Anc1p is required for cell cycle progression through the G_2/M transition at high temperatures. A search in the current databases of DNA microarray experiments shows that the steady state RNA level of *ANC1* is very low and its transcript levels remain constant and are not cell cycle regulated. Therefore *ANC1* constitutes a good candidate for a gene whose expression is regulated by splicing. In an attempt to find out whether the splicing pattern of *ANC1* pre-mRNA varies at different cell cycle stages, we measured its expression by RT–PCR. Wild-type cells were synchronized at the G_1 stage of the cell cycle with α-factor and then released into fresh YEPD medium at 30°C. At different time points, samples were taken,

A

Time (hours)	0	0.5	1	1.5	2	2.5	3
% G_1	**96**	64	1	7	1	21	23
% S	1	35	**97**	27	18	58	52
% G_2/M	3	1	2	66	**81**	21	25

B

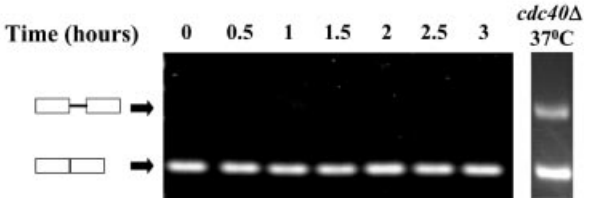


Figure 2. *ANC1* pre-mRNA splicing is not cell cycle regulated. Wild-type cells (strain W303) were synchronized in G_1 with α-factor and then released into fresh YEPD media at 30°C. At timely intervals aliquots were collected and samples were analyzed by microscope for cell cycle distribution (**A**), or were subject to RT–PCR analysis using *ANC1* specific primers (**B**). Unbudded cells were classified as being in G_1, cells with small buds (less than half the size of the mother cell) were considered to be in S phase and cells with large buds were classified as being in G_2/M. As a control an RT–PCR was carried out with a *cdc40*Δ strain incubated for 2 h at 37°C.

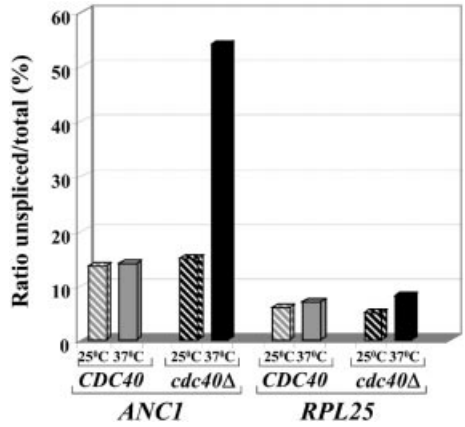


Figure 3. Cdc40 protein is required for efficient splicing of the *ANC1* intron. *cdc40*Δ (IM2) and wild-type (MK89) strains were grown exponentially at 25°C and then transferred to either permissive (25°C) or restrictive (37°C) temperatures for 3 h. RNA was extracted and analyzed by RT–PCR using *ANC1* or *RPL25* specific oligonucleotides spanning the relevant introns. The level of products for spliced and unspliced transcripts of *ANC1* and *RPL25* was quantified after gel electrophoresis. For each strain and temperature, the ratio of unspliced to total is shown. Similar results were obtained after 1.5 and 6 h incubation at 37°C.

RNA was extracted and RT–PCR analysis using primers spanning the *ANC1* intron was performed. In addition, the cell cycle distribution of the cell population was determined. As shown in Figure 2, *ANC1* pre-mRNA splicing in wild-type cells remains efficient and constant through all cell cycle stages and does not seem to be regulated during cell cycle progression.

Cdc40p is required for the efficient splicing of *ANC1* pre-mRNA

The ability of *ANC1* cDNA to suppress the cell cycle arrest of *cdc40*Δ cells in restrictive conditions supports the notion that Cdc40p is required for the efficient splicing of *ANC1* pre-mRNA. To test this possibility, we carried out RT–PCR analysis. Total RNA from *cdc40*Δ and wild-type strains was isolated before and after the cells were shifted to the non-permissive temperature. RNA was subjected to RT–PCR analysis using primers spanning either the *ANC1* intron or, as a control, the intron-containing ribosomal gene *RPL25*. As shown in Figure 3, when cells deleted for *CDC40* were incubated at the non-permissive temperature, an accumulation of unspliced *ANC1* was observed. Such an accumulation was not seen in cells incubated at the permissive temperature, or in wild-type cells at either temperature. No pre-mRNA accumulation was seen using primers specific to the ribosomal gene *RPL25* (Fig. 3) or to six additional intron-containing genes (data not shown). These results indicate that the splicing of *ANC1* pre-mRNA is highly dependent on the Cdc40 protein, and that the requirement for Cdc40p as a splicing factor is restricted to a subset of intron-containing genes.

Additionally, in order to monitor pre-mRNA splicing *in vivo* and further examine the *ANC1* splicing dependency on Cdc40p, we have developed a reporting assay based on the *ACT1-CUP1* system (28). *CUP1* is a non-essential gene that encodes a 61 amino acid metallothionein protein. Cup1p protects cells from copper toxicity by chelating the metal in a dosage-dependent manner, therefore allowing cells to grow in the presence of otherwise lethal concentrations of copper. *CUP1* thus provides a sensitive assay for studying splicing (28).

In the splicing reporting system, the *CUP1* gene was cloned downstream to an intron-containing fragment of the *ACT1* gene. The *ACT1* promoter was replaced by the strong constitutive GPD promoter (28). In order to use this reporter system for the analysis of *ANC1* splicing, we replaced the *ACT1* intron by the intervening sequence of *ANC1*. The reporter-containing plasmids (carrying either the *ACT1* or the *ANC1* intron) were introduced into either *cup1*Δ *cdc40*Δ or *cup1*Δ *CDC40* strains. The transformants were then spotted on copper-containing plates. Since the ability to grow in the presence of different copper concentrations depends on the amount of correctly spliced *CUP1* pre-mRNA, one can estimate the splicing efficiency of the *ANC1* intron by assaying the level of copper resistance. The results are summarized in Figure 4. *cup1*Δ *CDC40* cells were able to grow at high copper concentrations (0.5 mM) in the presence of either *ACT1* or *ANC1* introns. Note that splicing of the *ANC1* intron at 25°C is slightly less efficient in these cells than splicing of the *ACT1* intron. Similarly, *cup1*Δ *cdc40*Δ cells containing the *ACT1* intron were able to grow at high copper concentrations (0.6 mM). However, when the same cells were transformed with the *ANC1*-intron-containing plasmid, they exhibited a marked decrease of copper tolerance (≤0.15 mM copper). These results prove that the splicing efficiency of the *ANC1* intron (but not that of *ACT1*) is highly reduced in the absence of Cdc40p.

Taken together, the RT–PCR and *ACT1-CUP1* reporting system results show that the splicing of *ANC1* is extremely dependent on Cdc40p.

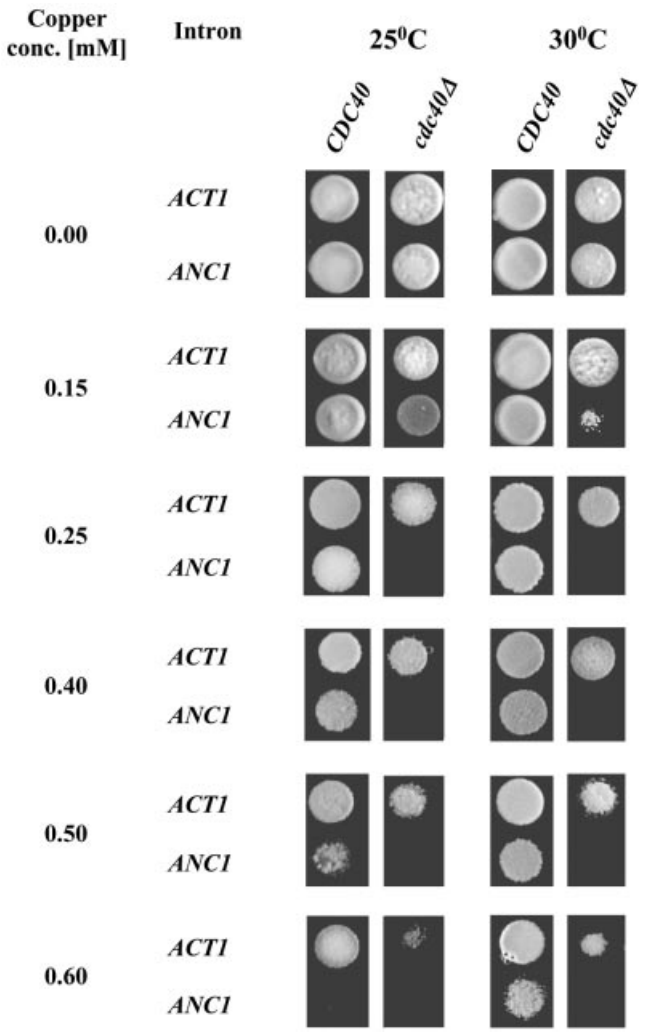


Figure 4. Cdc40 protein is required for efficient splicing of the *ANC1* intron. *cup1*Δ *cdc40*Δ (SB90) and *cup1*Δ *CDC40* (YCL51) strains were transformed with plasmids bearing *ACT1-CUP1* or *ANC1-CUP1* reporter gene fusions. The transformants were diluted, spotted on minimal selective plates and tested for the ability to grow in the presence of increasing copper concentrations.

Both the branchpoint and the 3′ splice site of *ANC1* pre-mRNA cause splicing dependency on the Cdc40 protein

The results presented so far support the possibility that splicing factors such as Cdc40p are differentially required for the efficient processing of a subset of intron-containing transcripts. This dependency could be due to unique intron features. For example, it has been shown that the requirement for Slu7p during the second step of splicing increases with the distance between the branchpoint and the 3′ splice site. Slu7p is necessary only for efficient splicing of introns in which the distance between the 3′ splice site and the branchpoint is >7 nt (34). Since Cdc40p is required for the splicing of only a subset of genes (including *ANC1*), we reasoned that Cdc40p target genes might contain some unique features that make their splicing highly dependent on Cdc40p.

In order to identify sites in the *ANC1* intron which are responsible for its splicing dependency on Cdc40p, we compared the *ANC1* intron splice sites (5′ splice site, branchpoint and 3′ splice site) with the yeast consensus sites (35). This comparison reveals that while the *ANC1* 5′ splice site is identical to the consensus sequence, unusual bases in conserved positions are found at both the branchpoint and the 3′ splice site. *ANC1* splicing dependency on Cdc40p could be due to the unusual *ANC1* branchpoint, the 3′ splice site or both. In order to examine this hypothesis and localize the feature in the *ANC1* intron that confers its splicing dependency on Cdc40p, we used the reporting systems based on *ANC1* and *ACT1* introns described. These two introns are differently affected by the absence of Cdc40p (Fig. 4). By shuffling the branchpoint and the 3′ splice site of the *ANC1* and *ACT1* introns, we created a series of chimeric introns fused to the *CUP1* reporting gene (Fig. 5A). All the constructs analyzed shared the same (*ACT1*) intron sequences upstream of the branchpoint. Plasmids carrying these fusions were introduced into *cup1*Δ *CDC40* and *cup1*Δ *cdc40*Δ cells and examined for their ability to allow growth at different copper concentrations. As can be seen in Figure 5B, *cdc40*Δ cells transformed with a clone containing the *ACT1* branchpoint and 3′ splice site (ACT-ACT) were able to grow at high copper concentrations (>0.5 mM). In contrast, all the other chimeric clones, which contain either the *ANC1* branchpoint (ANC-ACT), the *ANC1* 3′ splice site (ACT-ANC) or both (ANC-ANC), were defective in their splicing ability and allowed *cup1*Δ *cdc40*Δ cells to grow only in the presence of low copper concentrations (≤0.1 mM). However, these clones were spliced normally in the presence of Cdc40p, and supported growth of *CDC40* cells at high copper concentrations (Fig. 5B). The fact that both ANC-ACT (*ANC1* branchpoint and *ACT1* 3′ splice site) and ACT-ANC (*ACT1* branchpoint and *ANC1* 3′ splice site) clones were barely spliced in the absence of Cdc40p indicates that both the branchpoint and the 3′ splice site of *ANC1* pre-mRNA participate in the dependency on *CDC40* for efficient splicing.

Characterization of the *ANC1* intron

In order to identify the specific residues in each site (branchpoint and 3′ splice site) that make *ANC1* splicing highly dependent on Cdc40p, we carried out site-directed mutagenesis experiments.

The *ANC1* branchpoint differs from the consensus at three positions (62, 63 and 71). Position 71 is the most conserved among yeast introns. A mutation was introduced replacing the unusual thymidine at position 71 by the conserved adenine in the ANC-ACT clone (Fig. 6A). The mutated T71A clone was transformed into *cup1*Δ *CDC40* and *cup1*Δ *cdc40*Δ cells and its ability to support growth at different copper concentrations was examined. As shown in Figure 6B, whereas *cup1*Δ *cdc40*Δ cells containing wild-type *ANC1* branchpoint (ANC-ACT clone) grew only at a low copper concentration (0.1 mM), the same strain containing the mutant clone (ANC T71A-ACT) was extremely resistant to copper and grew at high copper concentrations, similar to cells containing the *ACT1* branchpoint (ACT-ACT). These results demonstrate that a single nucleotide is responsible for the *ANC1* branchpoint reliance on Cdc40p for splicing, and mutating this nucleotide is sufficient to alleviate this dependency.

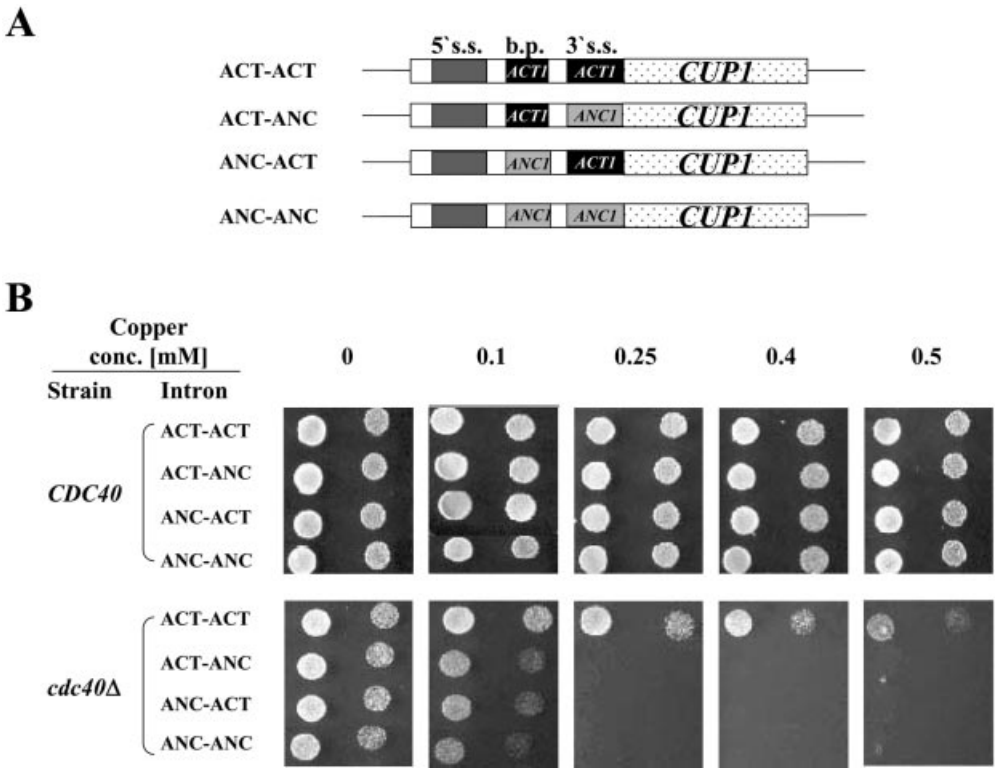


Figure 5. Both the branchpoint and the 3′ splice site of the *ANC1* intron contribute to the dependency on Cdc40p for splicing. (**A**) A series of chimeric plasmids containing different combinations of *ANC1* and *ACT1* splice sites fused to the *CUP1* reporting gene were constructed. (**B**) *cdc40*Δ *cup1*Δ (SB90) and *cup1*Δ *CDC40* (YCL51) strains were transformed with each of the chimeric clones. Serial dilutions of each transformant were spotted on minimal selective media at 30°C and tested for the ability to grow in the presence of different copper concentrations.

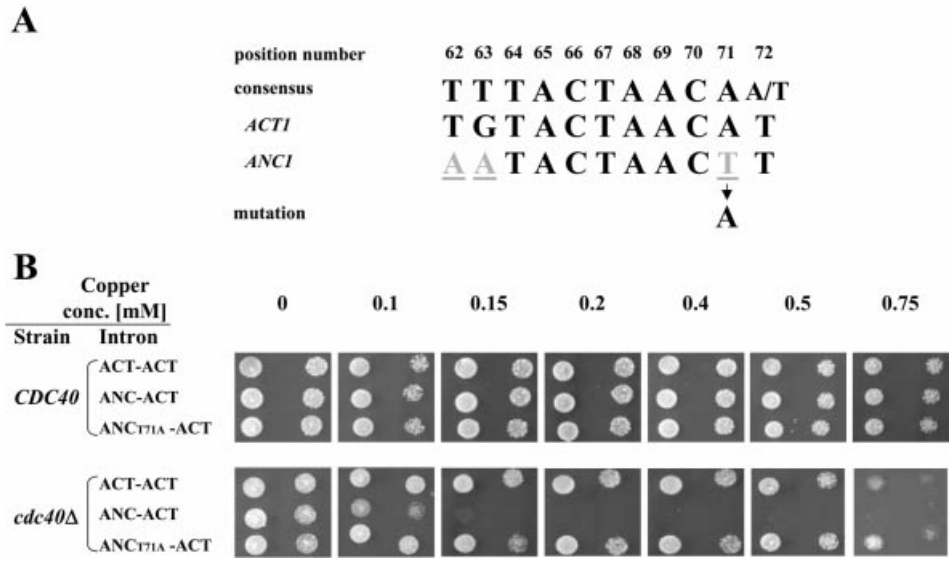


Figure 6. Site-directed mutagenesis of the *ANC1* intron branchpoint. (**A**) The branchpoint sequence of both *ACT1* and *ANC1* introns compared with the consensus sequence. The position number of each residue of the *ANC1* branchpoint (numbering starting from the first nucleotide of the *ANC1* intron) is indicated. Non-canonical nucleotides are underlined. (**B**) *cup1*Δ *cdc40*Δ (SB90) and *cup1*Δ *CDC40* (YCL51) strains were transformed with wild-type and mutated chimeric clones. The transformants were diluted and tested at 30°C for the ability to grow in the presence of different copper concentrations.

The *ANC1* 3′ splice site contains several nucleotides that differ from the consensus (Fig. 7A). The ninth nucleotide from 3′ end of the intron (position 97 of the *ANC1* intron) is occupied by a thymidine in 86% of all the yeast introns (35). However, in the *ANC1* intron there is an adenine at this position. To test the possible role of this nucleotide, we

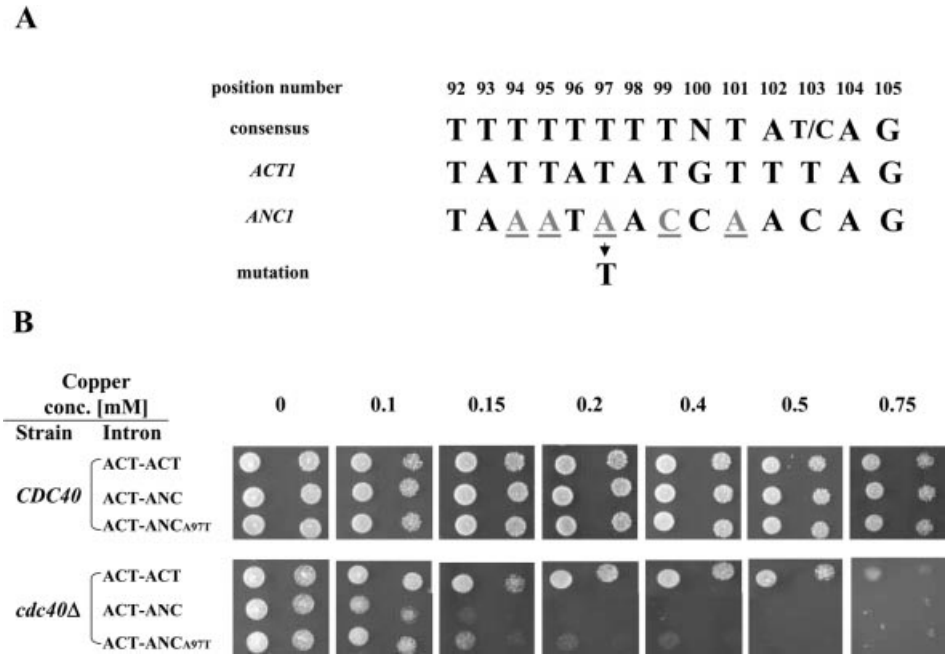


Figure 7. Site-directed mutagenesis of the *ANC1* intron 3′ splice site. (**A**) The sequences of the 3′ splice site of both *ACT1* and *ANC1* introns are compared with each other and with the consensus sequence. The position number of each residue of the *ANC1* 3′ splice site is indicated. Non-canonical nucleotides are underlined. (**B**) *cup1*Δ *cdc40*Δ (SB90) and *cup1*Δ *CDC40* (YCL51) strains were transformed with wild-type and mutated chimeric clones. The transformants were diluted and tested at 30°C for the ability to grow in the presence of different copper concentrations.

introduced the A97T transversion in the ACT-ANC clone. The mutated clone was transformed into *cup1*Δ *CDC40* and *cup1*Δ *cdc40*Δ cells and its ability to support growth at different copper concentrations was examined. Unlike the results obtained by mutating the *ANC1* branchpoint, the single-nucleotide exchange only partially increased *ANC1* splicing efficiency (as indicated by the ability of *cup1*Δ *cdc40*Δ carrying ACT-ANC A97T clone to grow in the presence of 0.4 mM copper) (Fig. 7B). However, the splicing efficiency of cells containing the mutant clone was lower than that of the ACT-ACT clone, indicating that this nucleotide is not solely responsible for the dependency of the splicing of the *ANC1* 3′ splice site on the Cdc40 protein.

In an attempt to identify other residues in the *ANC1* 3′ splice site that are necessary for its efficient splicing in the absence of Cdc40p, we adopted a random mutagenesis strategy. Five non-canonical positions at the *ANC1* 3′ splice site (positions 94, 95, 97, 99 and 101) in the ACT-ANC clone were mutagenized using degenerate primers. A thymidine (T) is present at all of these positions in the consensus sequence, as well as in the efficient *ACT1* 3′ splice site, whereas the *ANC1* intron carries either adenine (A) or cytosine (C). The mutated clones were then transformed into *cup1*Δ *cdc40*Δ cells and screened for their ability to support growth in the presence of restrictive copper concentrations. Clones that supported growth were extracted and further checked in both *cup1*Δ *CDC40* and *cup1*Δ *cdc40*Δ cells, and the nature of mutations was determined by sequencing. The results are summarized in Table 1. Unlike the results obtained for the branchpoint, our mutation analysis of the *ANC1* 3′ splice site failed to identify a single position crucial for the *ANC1* splicing dependency on Cdc40p. However, several points could be inferred from our experiments. The nucleotides at positions 94 and 101 do not seem to play an important role, since all four nucleotides were found at similar frequencies at these positions. At position 99, there seems to be a bias against the cytosine present at the *ANC1* intron, suggesting that C at this position lowers splicing efficiency. Positions 95 and 97 clearly have the strongest impact on splicing efficiency. Out of 15 copper-resistant clones, 8 and 11 carried thymidines at positions 95 and 97, respectively. Thus our results indicate that thymidines are important and cannot be replaced by the other pyrimidine, cytosine, which seems to be under-represented in the mutant clones (especially at positions 95 and 97). A genome-wide search revealed a total of 12 introns carrying adenine residues at positions 95 and 97. We are presently investigating whether these introns are also controlled by a differential requirement of Cdc40p activity.

In summary, our analysis was able to identify specific residues in both the branchpoint and the 3′ splice site that are important for *ANC1* splicing dependency on Cdc40p.

DISCUSSION

Cell cycle progression and pre-mRNA splicing are two apparently independent processes in eukaryotic cells. Despite this, a growing body of evidence has recently accumulated indicating that these processes may be connected. However, understanding the molecular basis underlying this connection has remained a challenge. Our results provide a molecular explanation for this connection.

Mutations in most splicing genes do not cause a cell cycle arrest. Furthermore, in some cases, different alleles of splicing genes behave differently. For example, not all splicing-

Table 1. Sequence of mutagenized clones conferring resistance to high levels of copper

Clone	Position 94	Position 95	Position 97	Position 99	Position 101
ACT-ACT	T	T	T	T	T
ACT-ANC	A	A	A	C	A
P47	T	**T**	**T**	T	C
P22	T	**T**	**T**	Δ	Δ
P53	A	**T**	**T**	G	T
P83	A	**T**	**T**	G	T
P89	A	**T**	**T**	G	T
P105	T	**T**	**T**	T	C
P65	C	A	**T**	T	G
P49	A	A	**T**	G	C
P81	T	A	**T**	T	T
P35	T	G	**T**	C	A
P1	A	A	**T**	C	A
P75	C	T	A	G	A
P31	A	**T**	G	A	C
P4	G	G	A	T	T
P28	C	G	G	T	G
T	5	**8**	**11**	6	5
A	6	4	2	1	3
G	1	3	2	5	2
C	3	**0**	**0**	2	4

defective alleles of *PRP8* cause a cell cycle stage specific arrest (20). One possible explanation for the association between pre-mRNA splicing and cell cycle progression may be the need for specific splicing factors for the efficient splicing of subsets of intron-containing genes ('target' genes). According to this model, a small number of genes that are essential for the different transitions through the phases of the cell cycle contain introns with unique features. These introns are differentially dependent on specific splicing factors. Failure to accurately splice these genes prevents the synthesis of the specific protein(s) and leads to cell cycle arrest. By transforming *cdc40*Δ cells with a cDNA library we have isolated the *ANC1* cDNA as a suppressor of cell cycle arrest of *cdc40*Δ cells. By deleting the intron in the genomic copy of *ANC1*, we were able to show that this suppression is truly due to bypass of the need for *ANC1* pre-mRNA splicing, rather than to *ANC1* over-expression.

Anc1p was found to be associated with several protein complexes in the cell (30–33). The precise role of Anc1p in all these complexes is presently unclear. A common feature of these complexes, however, is that they are all involved in transcription and chromatin remodeling, and thus the expression of Anc1p is likely to be important for the proper transcription of many genes, including cell cycle specific genes. Two human homologs of *ANC1* were identified. These genes, ANL and AF9, were shown to be the target of reciprocal translocations that are common in myeloid leukemias. New evidence suggests that the resulting fusion proteins affect transcription partly by interacting with a repressor of transcription, BcoO. The negative effect on transcription deregulates cell cycle progression and thus increases susceptibility to leukemia (36).

The steady state level of *ANC1* RNA is very low; its transcription is not cell cycle regulated and remains constant in most experimental conditions. We have shown that elimination of the *ANC1* intron is sufficient to allow suppression of the *cdc40*Δ cell cycle arrest phenotype. Using both RT–PCR analysis and the *CUP1* reporting system, we have proved that the efficient splicing of *ANC1* pre-mRNA requires Cdc40p. Thus regulation of the *ANC1* gene occurs not at the level of transcription, but at the level of splicing. The dependence of *ANC1* on Cdc40p is not universal, since other intron-containing genes are less dependent on Cdc40p for splicing. Using microarray experiments specially designed to detect splice junctions and introns, the Ares group has recently examined the genome-wide response to the loss of individual splicing factors. They showed that intron-containing genes depend on different splicing factors to different extents. These findings are in accordance with our target gene hypothesis. Furthermore, in the same study, *ANC1* splicing was shown to be extremely dependent on Cdc40/Prp17p (as well as on Prp18p) (37) in agreement with our RT–PCR and *CUP1* reporting analysis.

The *ANC1* intron sequence differs from most other yeast introns at several positions. By creating chimeric introns bearing different combinations of the *ACT1* and *ANC1* branchpoint and 3′ splice sites, we have shown that both the branchpoint and the 3′ splice site of *ANC1* are essential for its splicing dependency on Cdc40p. Each of the two splice sites seems to contain unique features that generate dependency on Cdc40p. The degree of nucleotide conservation at each position of the intron splice sites is highly correlated with its importance in the splicing reaction. For instance, mutations in the first and fifth positions of the 5′ splice site (invariant Gs for all naturally occurring yeast introns) adversely affect both the efficiency of splicing and the specificity of the first catalytic step, whereas mutations at other positions have less severe effects (10). Similarly, branchpoint mutations at positions not found to vary naturally are the most detrimental to splicing efficiency, in contrast with the weak effect of mutations in less

conserved residues (38). The existence of natural introns bearing non-standard signals indicates that these residues may possess some regulatory role. Comparison of the *ANC1* splice site with the consensus sequence (35) revealed the existence of several unorthodox residues at highly conserved positions in both the branchpoint and the 3′ splice site (Figs 6 and 7). We have shown that in the case of the *ANC1* branchpoint, replacing the unusual nucleotide (T) at position 71 with the most conserved nucleotide (A) completely abolished the splicing dependency of the ANC-ACT clone on Cdc40p. Similarly, replacement of the adenine at position 97 of the *ANC1* intron with the consensus (T) residue increased splicing efficiency in the absence of *CDC40*. These results indicate that positions 71 and 97 are important for regulating the *ANC1* pre-mRNA splicing involving Cdc40p. Sequence comparison of the *ANC1* intron with its orthologs from other related yeast species (*Saccharomyces paradoxus*, *Saccharomyces mikatae* and *Saccharomyces bayanus*) (39) revealed that both unorthodox nucleotides at position 71 in the *ANC1* branchpoint and position 97 in the *ANC1* 3′ splice site are conserved in all the yeast species examined. This strengthens the notion that these residues may play an important role in controlling *ANC1* splicing.

Position 71 in *ANC1* is located near the highly conserved branchpoint residues that are engaged in base pairing with U2 snRNPs during the splicing reaction (40). We propose that Cdc40p is important for stabilizing the interaction between U2 snRNA and branchpoint sequences containing residues different from the consensus (as in the *ANC1* intron). In the absence of Cdc40p this interaction is disrupted, especially at higher temperatures, resulting in a reduction in splicing efficiency. This hypothesis is supported by the ability of a compensatory mutation in U2 snRNA (T33A) that restores the stable base paring at position 71 of the *ANC1* intron to support cell growth in the absence of Cdc40p (data not shown) presumably by allowing efficient splicing of the *ANC1* branchpoint even in the absence of Cdc40p.

As for the 3′ splice site, although there is no indication for base pairing between the 3′ splice site and any known snRNA, the 3′ splice site region has been shown to interact with several splicing proteins such as Prp8, Prp16, Prp22 and Slu7 (41,42). Recently, McPheeters and Muhlenkamp (43) monitored, by cross-linking analysis, the RNA–protein interactions involving the 3′ splice site during yeast pre-mRNA splicing. According to their results, extensive associations of the U2-snRNP-associated splicing factor Hsh155p with the branchpoint and 3′ splice site region are established upon pre-spliceosome formation. Following the first catalytic step of splicing, Prp8p, and later Prp16p, interacts with the 3′ end of the intron, leading to binding of Prp22p (43). Our results indicate that Cdc40p cooperates with these factors, directly or indirectly interacting with the 3′ splice site.

Previously, extensive genetic interactions between *CDC40* and *PRP8* were described. In the absence of Cdc40p, mutations in *PRP8* can either suppress the temperature-sensitive phenotype or confer synthetic lethality (44). In addition, a strong correlation between the ability of *prp8* alleles to suppress the temperature-sensitive phenotype of *cdc40*Δ cells and their ability to recognize mutant 3′ splice sites was observed (44). Similar interactions were also found between *prp8* alleles and mutants of the CSC (data not shown). Since we have shown that Cdc40p plays a role in governing the splicing of the *ANC1* intron through its branchpoint and 3′ splice site, one can propose the following model. Prp8p plays a central role in the second splicing reaction, presumably by helping in the recognition of the 3′ splice site. However, the efficient splicing of some introns (presumably those with non-canonical splice sites) requires the assistance of Cdc40p (or other factors such as the CSC). Cdc40p, in addition to its role in stabilizing the base pairing between the branchpoint of some introns and the U2 snRNA, may help in positioning Prp8p (and probably other proteins as well) at the correct site on the pre-mRNA. Alternatively, Cdc40p may help stabilize interactions within the spliceosome. In the absence of the Cdc40p, at the restrictive temperature, the interactions become unstable and splicing of introns with suboptimal sequences such as *ANC1* is disrupted, causing cell cycle arrest.

The relationship between Cdc40p and the CSC is still not clear. The CSC was originally discovered in a screen for mutants that depend on Cdc40p for survival (15). This dependency hints at an overlapping role for CSC and Cdc40p. Subsequently, it was found that mutations in components of the CSC, such as *cef*1 or *isy1*Δ *syf*2Δ, also cause arrest at the G_2/M phase of the cell cycle. The arrest is due to inefficient splicing of the *TUB1* and *TUB3* genes encoding α-tubulin (45,46). We have also shown that this cell cycle arrest is mediated by the spindle checkpoint pathway (46). However, introduction of *TUB1* or *TUB3* cDNA does not suppress the temperature-sensitive phenotype of *cdc40*Δ cells (5, data not shown). These results are supported by a DNA microarray analysis of *cdc40*Δ cells in which no increase in *TUB1* or *TUB3* unspliced signals were observed (37). Thus, although the CSC and Cdc40p seem to have somewhat overlapping functions, their specificity may differ. Different subgroups of introns in essential genes may require Cdc40p, the CSC or both, explaining both the synthetic lethality and the specificity observed.

Budding yeast cells have a relatively small number of intron-containing genes. Although several essential yeast genes have intervening sequences, there is a clear evolutionary trend of discarding introns, perhaps by a cDNA-directed homologous recombination mechanism as proposed by Fink (47). It is conceivable that those introns that remained may serve in the regulation of cellular processes. A similar mechanism, for example, is used for the regulation of meiosis. A number of genes that are essential for meiotic functions are transcribed in both vegetative and meiotic cells. Their expression, however, is meiosis specific because they are spliced only in meiotic cells. The meiosis-specific splicing depends on *MER1*, a meiosis-specific U1-associated splicing factor that is required for efficient splicing of introns containing non-canonical 5′ splice sites (48). Mutation in the 5′ splice site of one target gene (*MER2*) that recreates the consensus bypasses the Mer1p requirement for splicing (49). Thus the meiosis-specific splicing factors, together with the unique 5′ splice site, ensure efficient splicing of introns only in the right circumstances.

Similarly, we have shown that Cdc40p serves as an auxiliary splicing factor that is dispensable for most yeast introns but has become essential for splicing of the *ANC1* gene. We have provided a mechanism for this dependence which relies on the presence of non-canonical sequences at the

branchpoint and 3′ splice site. The evolutionary conservation of Cdc40p (50), as well as that of the non-canonical residues in the *ANC1* gene, suggests that the mechanism of splicing-dependent cell cycle regulation uncovered here may be conserved in other organisms. Current work is aimed at analyzing the dependence on Cdc40p of additional introns with non-canonical sequences.

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