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Alternative Polyadenylation in Yeast: 3´-UTR Elements and Processing Factors Acting at a Distance

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1. Introduction

One of the key steps necessary to obtain a messenger RNA (mRNA) is 3´-end RNA processing. The two specific 3´-end processing reactions for genes transcribed by RNAP-II (RNA polymerase II) are pre-mRNA cleavage followed by the addition to the cleaved 3´-end of a polyadenine "tail" (polyadenylation) catalyzed by the poly-A polymerase. For some genes this 3´-end processing gets more complex due to the fact that a single gene can be transcribed in two or more mRNAs differing in their 3´-UTR length due to the presence of two or more cleavage and polyadenylation points. This multiple (or alternative) 3´-end processing is also referred to as alternative polyadenylation (APA). In this chapter we will analyze some aspects of alternative RNA processing in eukaryotes using yeast as model. Hence, we will focus on the involvement of RNA processing machinery factors and elements in the UTRs (3´-UnTranslated Regions) necessary for this processing.

1.1 Elements for 3´-end RNA processing

In the yeast *Saccharomyces cerevisiae*, this RNA processing is catalyzed by multi-subunit complexes (Fig. 1): CFIA (Cleavage Factor IA) consists of Rna14, Rna15, Clp1 and Pcf11; CFIB (Cleavage Factor IB) consists of Hrp1/Nab4 factor; Holo-CPF (holo-Cleavage and Polyadenylation Factor) consists of an APT (Associated with Pta1) subcomplex which includes Pti1, Swd2, Syc1, Ref2, two protein phosphatases, Glc7 and Ssu72, and the Pta1 factor, which appears to be a scaffold that bridges holo-CPF sub-complexes (Nedea et al., 2003) but is also involved in different interactions that modulate processing (Ghazy et al., 2009). Regarding the CPF subcomplex it consists of Cft1/Yhh1, Brr5/Ysh1 and Cft2/Ydh1, the last one is able to interact with factors involved in cleavage and also polyadenylation as Pfs2 (Kyburtz et al., 2003).

The RNA 3´-end processing machineries are relatively conserved in eukaryotes, with moderate homologies between yeasts and human processing factors (reviewed by Keller & Minvielle-Sebastia, 1997; Zhao et al., 1999; Kyburz et al., 2003).

The UTR sequences necessary for 3´-end processing (*cis* elements) are less conserved. In the mammalian system 3´-end processing is driven mainly by three elements at the 3´-UTR: the sequence AAUAAA separated 10-30 positions to the cleavage site itself and followed by a

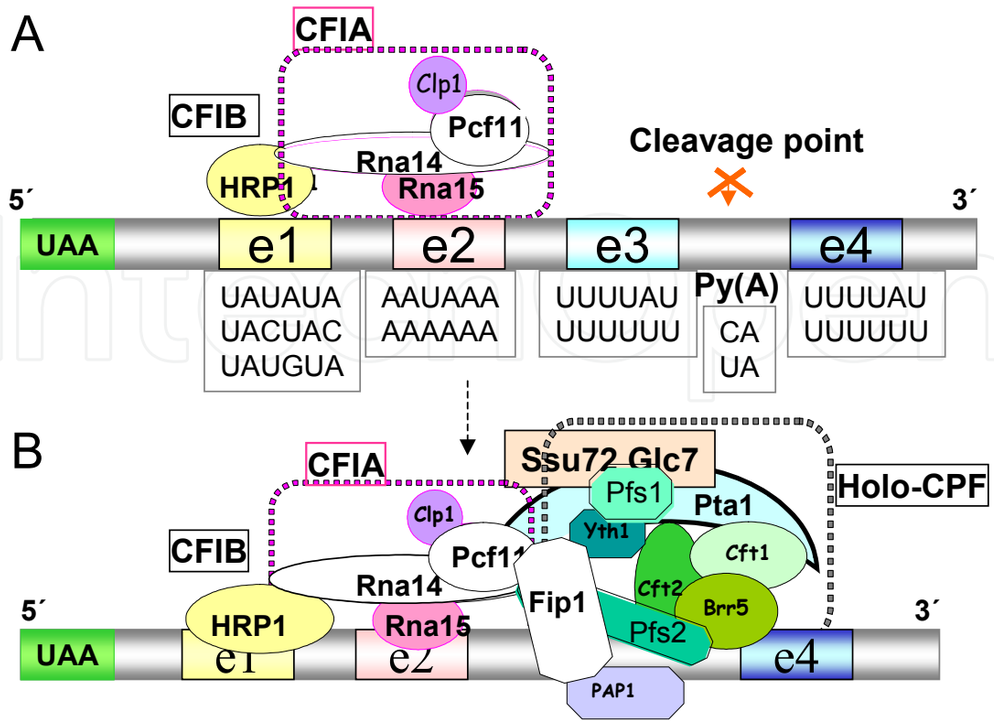

Fig. 1. Model of yeast RNA processing machinery including components of the different sub-complexes. A The UTR elements (e1 to e4) necessary for processing and their interaction with Hrp1 and Rna15 are also indicated. B A further step positioning the 3´-end machinery components at the UTR, indicating the complexes.

Downstream Sequence Element (DSE) located 30 nucleotides downstream of the cleavage site (Bilger et al., 1994).

A recent review (Danckwardt et al., 2008) summarizes variations in this pattern as the Upstream Sequence Elements (USEs). In yeast there is a more complex combination, global analyses of yeast UTRs showed (Van Helden et al., 2000; Graber et al., 1999) five elements as necessary to position the 3´-end machinery, **e1** (positioning, A-rich element), **e2** (efficiency; UA-rich element), **e3** (5´U-rich), the cleavage (Py(A)) site and **e4** (downstream, U-rich element) (reviewed by Graber et al., 2003) (Fig. 1A).

1.2 Genes with alternative polyadenylation

The yeast genes with regulated alternative polyadenylation have in common the presence of a canonical RNA processing element and a second (and in some instances more positions) non-canonical that does not include all the elements. The common feature for the less conserved is the presence of multiple AT-rich regions (Sparks & Dieckerman, 1998).

The biological sense of using regulatory alternatives of RNA processing with complex 3´-UTR is to have an alternative way to modulate gene expression and is described in many eukaryotes. In the yeast *Saccharomyces cerevisiae* APA is associated with differential regulation of gene expression. As examples, the *CBP1* gene is processed in two mRNAs (2.2 and 1.2kb) by alternative 3´-end processing, the predominance being regulated by the carbon source (Sparks et al., 1997). The *SUA7* transcript predominance changes with the

growth phase (Hoppes et al., 2000), after a heat shock or when copper concentrations cause stress to cells (Kim Guisbert et al., 2007).

Alternative polyadenylation is a widespread mechanism in eukaryotes. Expressed Sequence Tag analyses estimated that 40-50% of human genes undergo alternative polyadenylation (Tian et al., 2005; Beaudoning et al., 2000), an important regulatory mechanism with implications in different types of illnesses (Edwalds- Gilbert et al, 1997; Hall-Pogar et al., 2005; Dumont et al., 2004; Caballero et al., 2004). More recently (Shepard et al., 2011) developed a method, PAS-Seq (Poly(A) Site Sequencing), to analyze polyadenylation at the transcriptome level showing a dynamic regulation of APA during stem cells differentiation in mammals. In plants APA is also a widespread mechanism; in *Arabidopsis thaliana* the flowering time control pathway depends on the alternative polyadenylation of the FCA pre-mRNA (Simpson et al., 2003). FCA is an RNA-binding protein and required for the alternative polyadenylation of its own pre-mRNA. The FY and FCA factors control these processing positions. Additionally PCFS4 (homolog to yeast Pcf11) also regulates FCA alternative processing (Xing et al., 2008).

The yeast genes with alternative polyadenylation may be classified following several criteria:

i. Regarding the UTR length and the possibility of APA regulation, there are genes processed with regulatory alternatives. In these the alternative processing positions allow to differentiate transcripts (ie, *HIS3* with 13 sites (Mahandevan et al., 1997), *SUA7* with two transcripts (1.2 and 1.4) (Hoopes et al., 2000) or the two *KlCYC1* (1.14 kb and 1.5 kb) (Freire-Picos et al., 2001). The genes with apparently non-regulatory alternatives are where the alternative sites are separated by as few as 10-11 nucleotides in the *ACT1* transcripts (Gallwith et al., 1981) or the seven variants of *YPT1* 3´-end transcripts (Heidmann et al., 1992).
ii. Regarding if the transcript alternatives include truncated coding regions as the *RNA14* or non-truncated ones as *SUA7* (Sparks & Dieckerman 1998).

While analyzing alternative polyadenylation in rice; Shen and coworkers (Shen et al., 2008) defined "microheterogeneity" in processing as the phenomenon of finding alternative poly(A) sites located within 30 nucleotides of another in the same gene due to the probably slack nature of the polyadenylation machinery. We consider that the APA for yeast genes where the alternative sites are separated by as few as 10-11 nucleotides, as the *ACT1,* are examples of microheterogeneity.

1.3 RNA processing factors involved in APA

Some components of the yeast RNA processing machinery have been involved in alternative processing selection. Different groups have described that mutations in the RNA processing factors affect poly(A) choice (Table 1). The involvement of Pcf11 (Licatalosi et al., 2002), Yhh1 (Dichtl et al., 2002), Ydh1 (Kyburz et al 2003) and Rat1 (Wong et al 2003) was studied in the gene *ACT1*, belonging to the non-regulated group and included in Table 1 as examples of microheterogeneity (M). The effects on *RNA14* processing, with truncated alternatives (T), were shown in Ssu72 (He et al., 2003) and Npl3 (Wong et al., 2007) mutants. However, until more recently with Hrp1 (Kim Guisbert et al., 2007) or with Pta1 and Pcf11 (Seoane et al., 2009a), little was known about the factors specifically involved in alternative 3'-end selection in yeast genes with regulated (and non-truncated) 3'-end alternatives, or their possible interaction with specific elements at the 3'-UTR.

Factor /mutation	Processed gene / Type	Reference
ssu72-3	*RNA14* /T	He et al., 2003
Npl3	*RNA14* /T	Wong et al., 2007
Ydh1/Cft2p	*ACT1* /M	Kyburtz et al., 2003
Rat1	*ACT1* /M	Luo et al., 2006
pcf11-2 & *mpe1-1*	*ACT1* /M	Vo et al., 2001
Pcf11	*ACT1* /M	Licatalosi et al., 2002
Yhh1	*ACT1* /M	Ditchll et al., 2002b
Hrp1	*SUA7*/**C**	Kim Guisbert et al., 2007
Pta1& Pcf11	*KlCYC1*/**C** *CBP1*/T	Seoane et al., 2009
fip1-1 & *rna14-2*	*KlCYC1*/**C**	Lamas-Maceiras et al., this chapter

Table 1. Yeast factors involved in alternative RNA processing. Type of APA: truncated (T) or non-truncated (C= complete) and microheterogeneity (M).

The proximal *KlCYC1* 3´-UTR contains the AU-rich element important for both proximal and distal processing (at the two wild type positions) showing long distance APA regulation, in which Pta1 and Pcf11 are also involved. Clearly, there must be more factors connecting the ***cis*-**elements with the RNA processing machinery. Kim Guisbert and coworkers (Kim Guisbert et al., 2007) previously showed that Hrp1 (an RNA-binding factor and the only component of yeast CFIB) is involved in *SUA7* alternative polyadenylation. Pta1 establishes a physical interaction with Pcf11 (GST pull-down assays) (Ghazy et al., 2009). In the same work the Pta1 does not interact directly with Hrp1. Gross and Moore (2001a) previously had proposed that the Hrp1 binds directly to the e1 element and by means of its interaction with Rna14 directs Rna15 towards the e2 element (Fig. 1). Rna14 also binds Pcf11. All these interactions point out the interest to check if Rna14 is also involved in APA. We also analyzed the effect of Fip1 since this factor has an inhibitory effect on the Pap1 (polyA polymerase) activity (Zhelkovvsky et al., 1998). The *fip1-1* protein extract is defective in the poly(A) addition of *CYC1* substrate; ***in vitro***, *pta1-1* mutants have a 3´-end processing defect very similar to *fip1-1* defects (Zhao et al., 1999).

1.4 APA studies on *KlCYC1*: *cis* elements and factors involved

The long 3´-UTR (1.2 kb) of the *KlCYC1* gene from *Kluyveromyces lactis* is processed at two alternative positions (698 or 1092). The two *KlCYC1* transcripts change their predominance along the growth phase, but not with the carbon source (Freire-Picos et al., 2001). We have shown that when the UTR is split after the first processing point, thereby separating the proximal or distal sequences, they may act as independent processing elements in both *S. cerevisiae* and *K. lactis* yeast species (Seoane et al., 2005). The *KlCYC1* APA is also conserved when expressed in *S. cerevisiae*; the separation of the alternative processing positions (395 nucleotides) is a reason to use it as model to study APA in *S. cerevisiae*.

The role of several alleles of Pta1 and Pcf11 in alternative processing was studied using Pta1 and Pcf11 mutants where the predominance of *KlCYC1* and *CBP1* transcript isoforms was changed (Seoane et al., 2009). In the same work we showed that the *KlCYC1* APA is dependent on the AU-rich element located at the proximal 3´-UTR at positions 670 to 699. Mutations of this element change the transcript predominance and this effect is compensated when the mutated UTR is expressed in *pta1-1*, *pcf11-2* mutant strains. Therefore, the preference for the two alternative processing positions (proximal and distal)

is directed through this AU-rich element located at the proximal UTR, and therefore acting at a distance in combination with Pta1 and Pcf11 (Seoane et al., 2009). More information is needed to better understand the mechanism. In *S. cerevisiae* Hrp1 binds to the e1, UTR element, and does not interact with Pta1, therefore, more factors need to be identified to complete the relation of factors involved in APA.

The APA determinants are the intrinsic strength of sequence elements, the concentration or activity of polyadenylation factors and/or tissue or stage-specific regulatory factors (Barabino & Keller, 1999). The involvement of RNA structure in rRNA processing has been well characterized (Rauhé and Planta, 1995). Histone mRNAs 3´-end processing is also dependent on a secondary structure, a stem-loop, positioned upstream of the cleavage site (Marzluff, 2005; Gilmartin 2005). The possible combination of RNA secondary structures with *cis* elements and/or polyadenylation factors constitutes a possible determinant for processing that is not clear in yeast genes with APA.

Taking into account the relevance of the proximal AU-rich element in the *KlCYC1* APA in the present work we analyzed two aspects of alternative RNA processing in yeast: at first an analysis of the changes caused by mutations at the *KlCYC1* proximal AU-rich element (that change transcript predominance) related to changes in the predicted RNA secondary structure and stability. Secondly, we show the involvement and effect of not previously characterized yeast RNA processing factors (Rna14 and Fip1) in *KlCYC1* APA.

2. Methodology

2.1 Strains and growth conditions

Strains FY23 (*MATa ura3-52 trp163 leu2-1*) were described previously (Madison & Winston 1997). Strains XH6 (*MAT*a *ura3-52 leu2*Δ*1 trp1*Δ*63 his3*Δ*200 pta1*::*TRP1* [*YCpLEU2-PTA1*]) and XH15 (*MAT*a *ura3-52 leu2*Δ*1 trp1*Δ*63 his3*Δ*200 pta1*::*TRP1* [*YCpLEU2-pta1-3*]) are an isogenic pair that have been disrupted at the chromosomal *pta1* locus (*pta1*::*TRP1*) (He et al., 2003) and differ from each other only by the plasmid-borne *pta1* allele. *pcf11-2*, *pcf11-9* (Amrani et al., 1997). BMA64 (*MATa ura3 leu2 ade2 his3*) ***rna14-1*** (*MAT*α *ura3-1 trp1-1 ade2-1 leu2-3-112 his3-11,15 rna14-1*); *rna14-2* (*MATa ura3 leu2 trp1 ade2 his3 rna14-2*) (Minvielle-Sebastia et al., 1991) *fip1-1* (*MATa leu2-3 112 ura3-52 trp1 his4 fip1::LEU2/pIA23 (CEN4 TRP1 fip1-1*) (Preker et al., 1997).

Yeasts cultures, manipulations and transformations were done as previously (Seoane et al., 2009).

2.2 Northern analysis

Total RNA extractions and Northern experiments were carried out according to (Zitomer & Hall, 1976). Hybridization signals were quantitated using the Image-Quant program (Molecular Dynamics) and normalized with respect to U3 RNA. The U3 probe, corresponding to *S. cerevisiae* small nuclear RNA (snRNA) R17A, was obtained by PCR amplification with primers U3F: 5´-CGACGTACTTCAGTATGTAA-3´ and U3R: 5´-ATTTGTACCCACCCATAGAG-3'. *ACT1* and *CYC1* probes were prepared by digestion of cloned versions with specific restriction enzymes.

Normalization of mRNA signals: a double normalization was performed for better comparison among the different mutants. First, all transcripts were normalized with respect to U3. Secondly, all signals were normalized with respect to the wild type band (for single

transcripts) or with respect to the upper band in the wild type strain (for multiple processing factors).

2.3 mRNA-stability experiments

Cultures of the *rpb1-1* thermosensitive RNA polymerase II mutant strain (Nonet et al., 1987) transformed with different versions of *KlCYC1* 3´-UTR were grown in CM-Ura at 30ºC until they reached an OD_{600} of 0.6. Cultures were shifted to 37ºC to induce a rapid shutdown of mRNA synthesis, and 10 mL samples were taken, at the times described in Figure 4.

2.4 Site-directed mutagenesis

Specific mutations (M3a) on *KlCYC1* 3´-UTR were introduced using the Stratagene QuickChange™ site-directed mutagenesis kit, using specific oligos:
ossMUT1C-1: 5´- CTATGTACTACTATTCCAAGAATACCAAATTTTATGATTATTTCT TTCTTTTTTAACAACTCTACTACTTCTATTATCAAC-3´.
ossMUT1C-2: 5´-GTTGATAATAGAAGTAGTAGAGTTGTTAAAAAAGAAAGAAATAA TCATAAAATTTGGTATTCTTGGAATAGTAGTACATAG-3´.
Plasmid pCT2 (Freire-Picos et al., 2001) was used as *KlCYC1* template for the site-directed mutagenesis experiments, and as a source of wild type 3´-UTR in expression experiments.

2.5 Computer programs

For secondary structure analysis the program used was RNA-Draw (Matzura et al., 1996) (http://iubio.bio.indiana.edu/soft/molbio/ibmpc/rnadraw-readme.html)
The *S. cerevisiae* mRNA 3'-processing site predictor was used to search for the yeast RNA processing determinants at 3´-UTRs http://harlequin.jax.org/polyA/ (Graber et al., 2002).

3. Results and discussion

3.1 Analysis of 3´-end processing elements in a secondary structure context

The previous analyses showed that the *KlCYC1* 3´-UTR contains multiple putative processing elements but most of them do not match canonical consensus (Freire-Picos et al., 2001). In Figure 2A the RNA processing prediction output for *KlCYC1* 3´-UTR (*S. cerevisiae* mRNA 3'-processing site predictor) is shown. The graph is combined with the experimental data of real polyadenylation positions (P1 and P2) (Freire-Picos et al., 2001). The *KlCYC1* sequences at the first processing position (P1) are identified by the program showing the highest probability peak, matching the five elements (detailed in Fig. 2B). The location of the determinant elements for processing in the proximal region contrast with the lower probability peaks shown with the sequence near the second polyadenylation position (P2) (Fig. 2A).
The *KlCYC1* complete 3´-UTR secondary structure prediction was calculated (using RNA Draw program) for the wild type sequence (Fig. 2C). As shown in the complete view, the whole UTR forms a complex squiggle structure. There is a central region (CR) where there is a confluence for: the end of the coding region, the end of the distal 3'-UTR (both encircled) and the first polyadenylation point determined experimentally. Part of this region is magnified to show the end-triplet and polyadenylation position. Interestingly, the CR includes the five elements for the proximal processing position. The distal processing point

(P2) is located almost 400 bases downstream in a region structurally different from the proximal one (Fig. 2C). The AU-rich element, previously shown as APA determinant element, partially overlaps with the e2 element and therefore is also located at the CR.

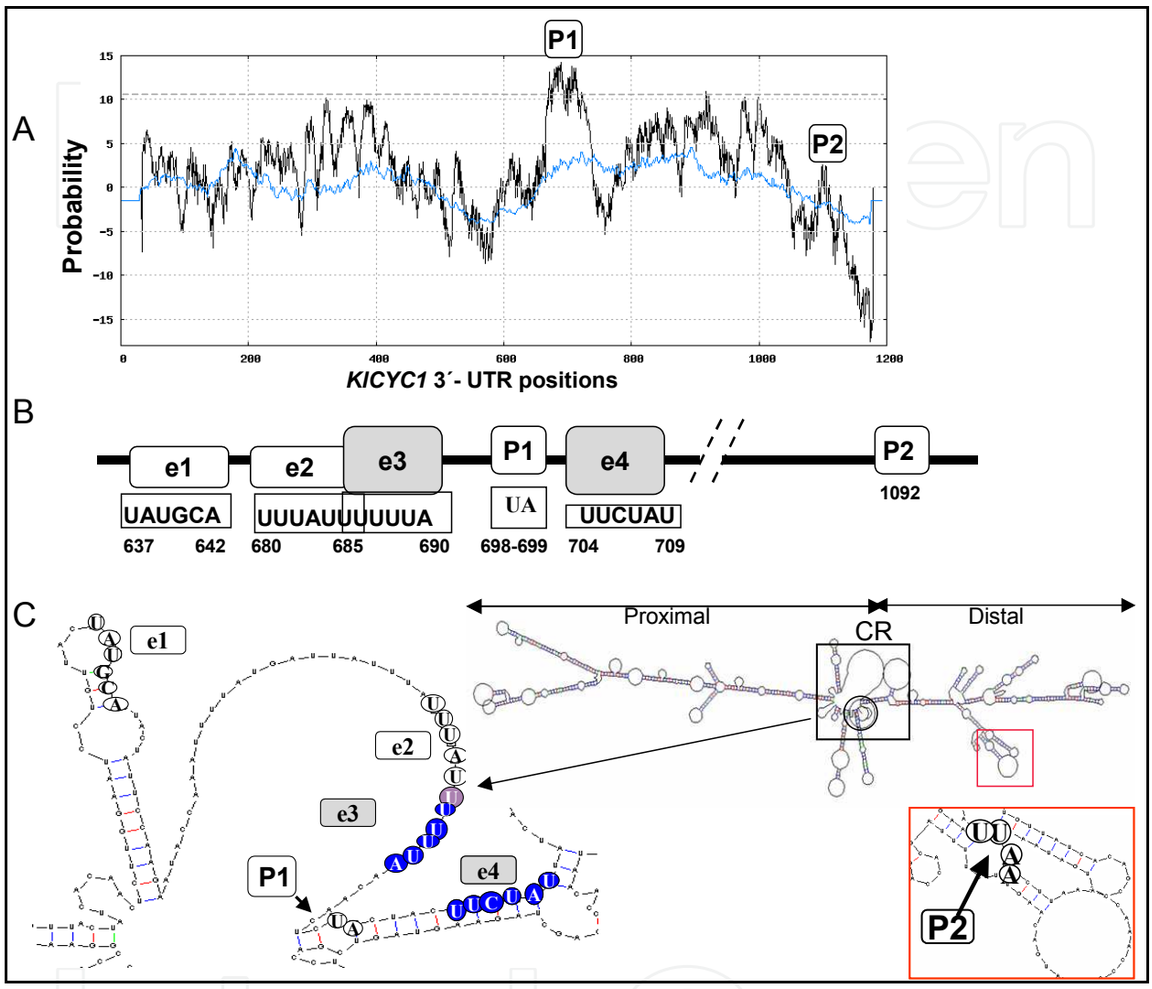

Fig. 2. The *KlCYC1* 3´-UTR. A Processing prediction output using the *S. cerevisiae* site predictor. B Detail of sequences matching the elements for the first (and canonical) processing point (P1). C Processing elements located in the context of the 3´-UTR secondary structure.

In summary, the sequences surrounding the proximal processing point (P1) correspond to canonical RNA processing sequences that are located in a central region of confluence of secondary structures and *cis* elements including the proximal AU-rich element. The sequences surrounding the distal processing point (P2) do not match the canonical consensus and do not share similarity with the secondary structure prediction surrounding P1.

The differences found in both, the *cis*-elements and the putative secondary structures, at the alternative processing points can be an important feature for a differential regulation of *KlCYC1* APA.

3.2 The APA changes caused by AU-rich mutations are more related to Pta1 than to the secondary structure

The involvement of the proximal AU-rich element (wild type and M1 or M2 mutated forms) and the *pta1-1* mutation in switching short or long distance RNA processing has been recently shown (Seoane et al., 2009). To analyze the possible implications of changes in the secondary structure derived of mutations at the AU-rich element, we analyzed the secondary structure prediction for each UTR variant (Fig. 3). In this analysis we also included a new mutant M3b where the substitution was A for C at the same positions as in M1 (Fig. 3A). To complete this analysis, the effects on *KlCYC1* transcript predominance were studied by expressing the gene in either a wild type or a *pta1-1* mutant strain.

As shown in Figure 3C, the punctual mutations in M1 clearly abolish several secondary structures at the CR encompassing the first polyadenylation site. The M2 mutant (3D), despite it having a 5 bp deletion, shows no relevant change in the structure, just a shorter duplex region indicated by an asterisk (Fig. 3D). The new mutant M3a does not change the structure prediction with respect to the wild type (Fig. 3E).

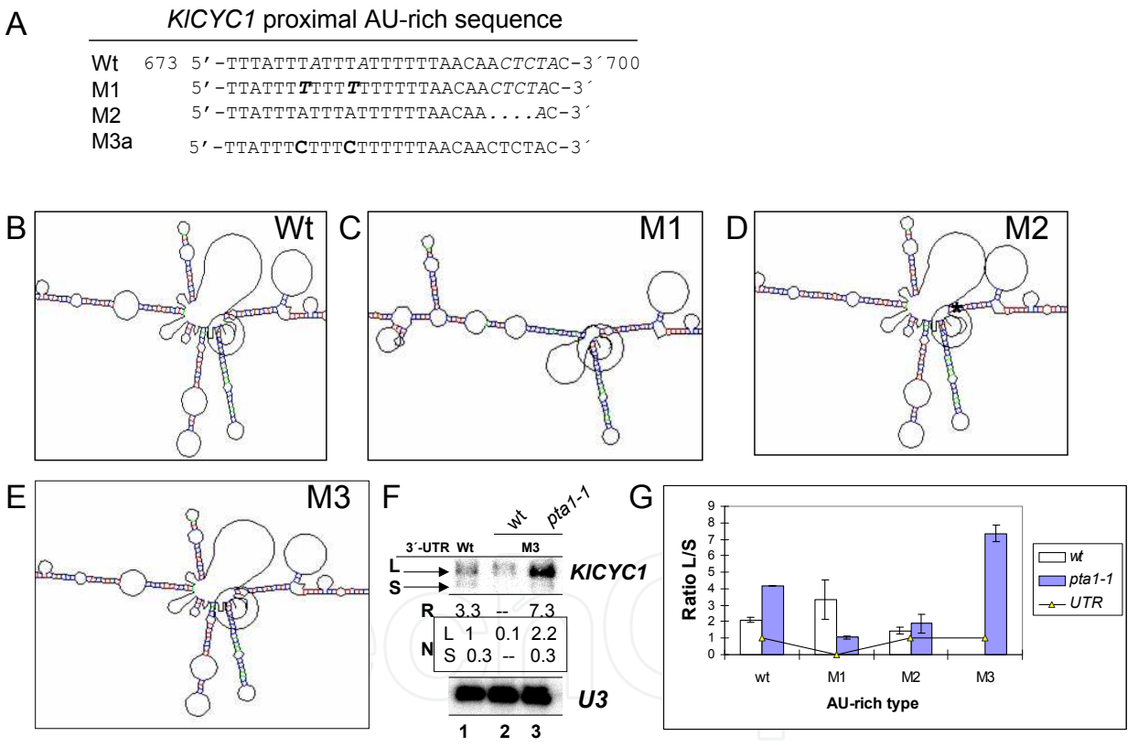

Fig. 3. Study of the predicted secondary structure changes (caused by mutations at the proximal AU-rich element) in relation to *KlCYC1* transcripts ratio. A Sequence of M1 and M2 and M3a mutations. B to E Secondary structures calculated with RNA Draw. F Expression of M3a mutation in either wild type (lane1) or *pta1-1 S. cerevisiae* strains (lanes 2 and 3) compares with the expression of a wild type 3´-UTR in a wildtype strain for Pta1 (XH6). G *KlCYC1* L/S ratios in the different UTR mutations expresed in either a wild type or a *pta1-1* mutant strain. The information of the UTR secondary structure in each mutant was included in the same graph giving to the secondary structure a value of 1 (equal or very similar to wild type) or 0 (different structure).

The M3a mutation causes a strong reduction in the two *KlCYC1* transcripts when expressed in the wild type strain (compare lanes 1 and 2) while there is a strong increase in the long transcript signal in the *pta1-1* mutant strain (lanes 2 and 3). The increase is also two fold respect to the normal expression with a wild type UTR (compare lanes 1 and 3). Therefore we found a clear enhancement in the usage of the distal processing point in the *pta1-1* mutant strain expressing the M3a mutation. This last result can be considered as a gain in the processing efficiency at the distal position only when Pta1 is mutated.
The changes in *KlCYC1* transcripts ratio (L/S) analyzed in strains and 3´-UTR mutants are shown in Figure 3G. To be able to include the secondary structure in the graph we used a binary code where the presence of the wild type UTR was considered as 1 and its absence as 0. By comparing the secondary structure variations and the changes in ratio shown in Figure 3G, we conclude that the changes in the L/S ratios are more dependent on the sequence mutations and the *pta1-1* mutant alelle than on the possible secondary structure alterations.
The *pta1-1* mutation was characterized as a non-sense mutation (*pta1-1 ocre* fragment) in the *PTA1* coding region, however, despite this mutation, a small amount of Pta1 protein is able to maintain cell viability (Zhao et al., 1999). The changes in *KlCYC1* transcript predominance are due to the low Pta1 levels present in the *pta1-1* mutant, a result that was confirmed by using the conditional degradation in Pta1-*td* degron strain (Seoane et al., 2009).
Therefore, the results with the new mutant M3a show that the *KlCYC1* APA is dependent on the AU-rich element and the Pta1 levels. The parallel secondary structure analysis with the AU-rich mutants does not seem to have an effect on *KlCYC1* APA.

3.3 Effect of the *KlCYC1* AU-rich element mutants on transcript stability

Recent stability analysis of *KlCYC1* transcripts revealed the presence of different mRNA turnover mechanisms able to operate on *KlCYC1* mRNAs under different physiological conditions (Seoane-Rosende et al., 2009). The AU-rich elements that match with the consensus UUAUUUAUU are also known as ARE (AU-Rich Elements) acting as signals to determine mRNA stability, not only in higher eukaryotes but also in yeasts (Vasudevan & Peltz., 2001). It is reasonable to consider that the AU-rich element mutated in M1 can be governing *KlCYC1* transcript stability. To determine if the AU-rich sequence present at positions 673 to 686 was also involved in *KlCYC1* transcript stability, the wild type *KlCYC1* plasmid pCT2 (Freire-Picos et al., 2001) or the site-directed mutagenesis derivatives M1 and M2 were expressed in the *rpb1-1* thermosensitive mutant strain, Figure 4 shows the effect of the mutations in *KlCYC1* transcript predominance.

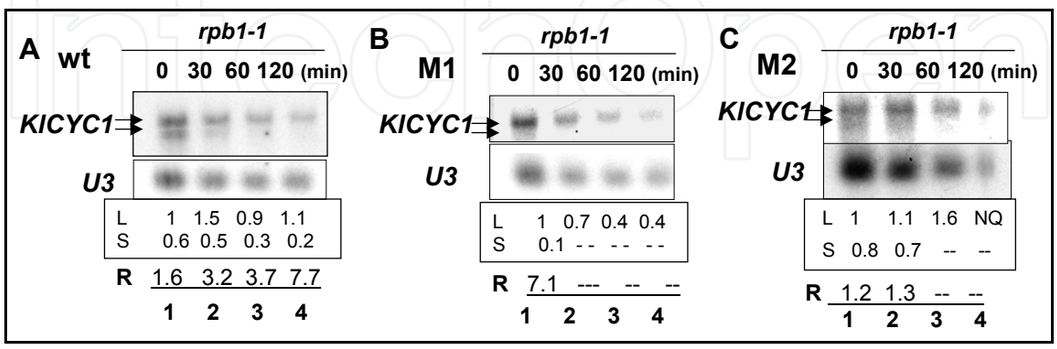

Fig. 4. Involvement of the proximal AU-rich element in transcript stability: expression in an *rpb1-1* thermosensitive mutant. A: Expression of wild type *KlCYC1* 3´-UTR (pCT2), B: Expression of M1 C: Expression of M2. Normalized values were taken with respect to U3. R: L/S Ratio. NQ: not quantitated.

At permissive temperature (30ºC) the wild type *KlCYC1* 3´-UTR expressed in *rpb1-1* has a ratio upper/lower of 1.2 (Figure 4A, lane 1). This ratio increases two-fold when the temperature is changed to 37ºC for 30 min and keeps increasing up to 7.7 after 1 hour (Figure 4A, lanes 1 to 4). This increase is due to a progressive decrease of the shorter transcript, while the longer transcript is almost invariant. Thus, both *KlCYC1* transcripts have different stabilities once transcription is abolished. The distal part of the 3´-UTR region, present only in the long transcript, is important for this stability.

Analyzing expression in the M1 mutant, the signal of the shorter transcript is very low at permissive temperature, due to effects in processing, and after 30 minutes at 37ºC is not possible to detect a signal (Figure 4B). It is important to note that in this mutant, the longer transcript reduces to the half after one hour. Therefore the AU-rich element mutated in M1 is important for the stability of the long transcript since the A to C changes cause a faster degradation of mRNAs. Hence, this AU-rich sequence is not equivalent to an ARE motif since its deletion should have caused the opposite effect.

When the same experiment is performed in M2 (where the AU-rich sequence is not mutated), the longer transcript remains stable at least up to one hour (Figure 4C), which reinforces the idea that the AU-rich element at position 674 to 686 is acting at a distance, affecting the stability of the long 3´-UTR.

3.4 The involvement of other yeast RNA processing factors in APA

Although the interplay between Pta1, Pcf11 and the AU-rich region is clear, the connection between the *cis* and *trans* factors driving APA is unknown. In the S*UA7* system the Hrp1 factor binds to an e1-like element, however, Hrp1 does not interact with Pta1 (Ghazy et al., 2009), and therefore, it is necessary to search for new factors. To identify new RNA processing factors involved in APA, and following the same procedures done previously, we expressed *KlCYC1* in yeast mutants for the RNA processing factors Rna14 and Fip1 belonging to the CFIA and PF1 complexes.

The analysis of *KlCYC1* transcript predominance is shown in Fig. 5 . The *S. cerevisiae CYC1* is included as a gene with a well characterized single processing (Guo & Sherman 1996). The *pcf11-2* and *pcf11-9* alleles, previously characterized as involved in *KlCYC1* and *CBP1* APA (Seoane et al., 2009), were included as a positive controls.

In the *rna14-2* mutant the *KlCYC1* transcripts show a 0.8 L/S ratio (Fig. 5, compare lanes 6 and 7). This ratio change is similar to that obtained by expressing the gene in *pcf11* mutants (compare lane 6 with lanes 3 and 4). Although in this mutant there is a clear reduction in mRNA levels, the results on *CYC1* and *ACT1* also show a lower expression therefore that´s a general processing effect. The *KlCYC1* transcripts are undetectable in the *rna14-1* mutant (lane 5), and from this result we cannot make conclusions on APA, nevertheless, this drastic effect does not affect the other genes tested, suggesting a more relevant role of Rna14 in *KlCYC1* 3´-end processing. Rouillard and Coworkers (2000) characterized two types of *rna14* mutants the poly(A) negatives as *rna14-1* causing defects in 3´-end maturation and those with no apparent effect in polyadenylation. The drastic result with *KlCYC1,* but not with the other analyzed genes, points towards gene specific effects in this mutant.

With respect to the *fip1-1* mutant, this mutation causes a preference of usage of the proximal processing position with respect to the wild type (Fig. 5, compare lanes 2 and 1, respectively). Regarding Fip1 the normalized transcript values with respect to U3 indicate

that there is a change in processing preference rather than in upper transcript degradation, especially when comparing the result of the wild type 3´-UTR in a wild type strain.

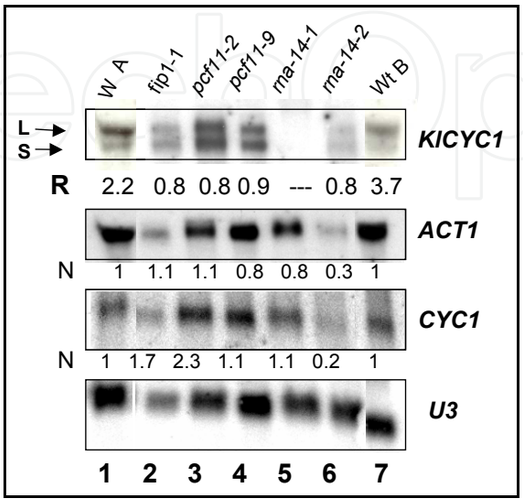

Fig. 5. Effect of RNA processing factors on *KlCYC1* APA. R Long/Short (L/S) ratio, to compare transcript predominance. N Represents the double normalized signals. Two wild type strains were included in the blot: WtA WtB corresponding to XH6 and BMA64 strains, respectively.

Transcription is coupled to RNA-processing (Proufoot, 2004) and is dependent on the carboxy-terminal repeat domain (CTD) of the Rpb1 subunit of RNAP II as reviewed in (Bentley, 2005). The coupling is mediated, in part, by Ssu72, which is a CTD phosphatase with specificity for the serine-5-P of the CTD (Hausmann et al., 2005; Krisnamurthy et al., 2004) and Pcf11, which bridges the CTD to the nascent transcript (Zhang et al., 2005). Pcf11 contains an N-terminus CID (CTD Interaction Domain) able to interact with serine-2-phosphorylated heptapeptide repeats; the *pcf11-9* mutation affects CTD binding, however, mutations in *pcf11-2* (located downstream of the CID) do not (Sadowski et al., 2003). Our results on *KlCYC1* expressed in the two mutants show similar L/S ratios (Fig. 1A), suggesting that the alternative processing effect of *pcf11-2* and *pcf11-9* does not depend on Pcf11-CTD interactions.

Future studies combining APA processing studies and *S. cerevisiae* mutants for specific CTD serine 2 kinases and phosphatases will help to clarify the regulatory role.

The data presented, combined with the information of protein-protein interactions led us to propose a model for APA in the *KlCYC1* system (Fig. 6). The factors involved in APA are shown in white. This model explains the possibility that the relationship of both pta1 and pcf11 with the *KlCYC1* AU-rich element is mediated by Rna14. This possibility is consistent with the result in Figure 4 showing the *rna14-2* effect on *KlCYC1* APA. The role of Rna14 driving Rna15 towards the e2 element (Gross & Moore 2001) makes Rna15 an excellent

candidate in binding this proximal AU-rich element (which partially overlaps with the e2 canonical sequence). The specific APA changes in the *pta1-1* mutant can be explained for a more regulatory role of these factors and its specific interactions with phosphatases (as Ssu72 or Glc7) that regulate its activity (Ghazy et al., 2009). This modulation must be in response to changes in cellular conditions as the growth phase, or stress conditions. The involvement of Fip1, as a factor conecting the RNA processing machinery with the polyA polymerase (Pap1), in APA is a new data that, following the model in Figure 6, connects the RNA binding factors as Hrp1 and Rna14, and the HoloCPF, where Pta1 is the regulator, with the actual cleavage and polyadenylation site.

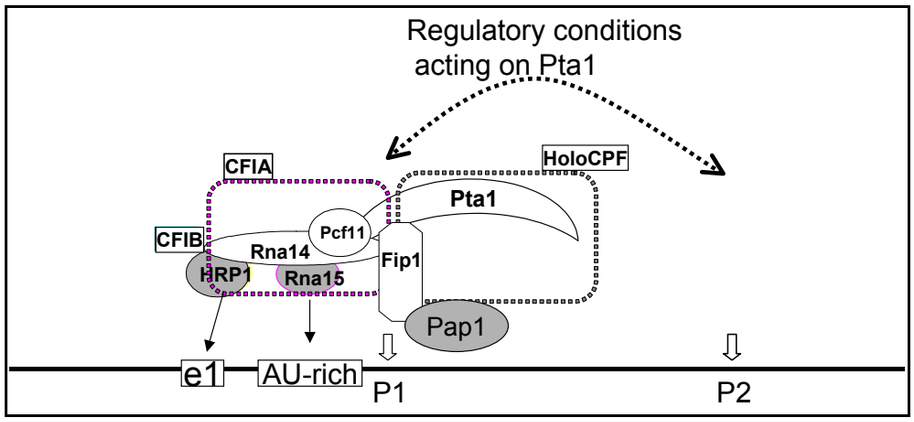

Fig. 6. Model for the interplay between factors of the RNA processing machinery involved in APA and the *KlCYC1* proximal AU-rich element. Those with effect on *KlCYC1* APA are in white. The model includes Hrp1 as factor involved in *SUA7* APA (in grey), and Rna15 as the factor connecting e2 element with the factors characterized for *KlCYC1.* The curved arrow indicates the role of Pta1 in the preferential processing point usage. Small white arrows indicate the two processing points.

4. Conclusions

The complexity of the *KlCYC1* is shown by the multiple elements present in its long 3´-UTR. The secondary structure prediction for the long *KlCYC1* 3´-UTR indicates a complex squiggle structure with a central region where there is a confluence of the 3´- UTR ends and also contains the first processing point, which corresponds with the canonical yeast RNA processing elements. The alternative processing position (P2) is in a suboptimal region and in a separate region of the secondary structure. The parallel analysis of *KlCYC1* processing in different AU-rich mutations and their corresponding secondary structure predictions do not indicate that the structures may play a relevant role in *KlCYC1* APA.

The mutation M3a combined with *pta1-1* mutation causes a strong increase in distal RNA processing, as an example of gain of function in usage of distal processing point. This is clearer when comparing with the transcripts ratio in a wild type context (for both UTR and

strain). This result indicates both the importance of Pta1 in selecting proximal position and the fact that low Pta1 levels (RNA processing machineries with subestequiometric changes due to low Pta1 levels) cause an increase in processing efficiency at the distal point only when the proximal AU-rich element is mutated. In the future our goal is to analyze if conditions that regulate Pta1 *in vivo* causing changes in protein levels, phosphorylation status or interactions under different conditions are associated with APA.

KlCYC1 transcripts stability analyses indicate that the changes caused in the AU-rich element affects, the long transcript stability since its mutation causes a faster degradation of the long mRNA. Hence, this AU-rich sequence is not equivalent to an ARE motif since its deletion should have caused the opposite effect.

The analysis of *fip1-1* and *rna14-2* mutations shows an effect in alternative processing with a predominance pattern similar to that in Pcf11 mutants. This information together with the Rna14 ability to connect RNA binding factors (such as Rna15) with other components of the processing machinery is important to understand the effects of 3´-UTR mutations combined with processing factors.

The results obtained allowed us to propose the connection between the factors involved in alternative processing and the proximal AU rich element driving long and short distance 3´-end processing as shown in Figure 6.

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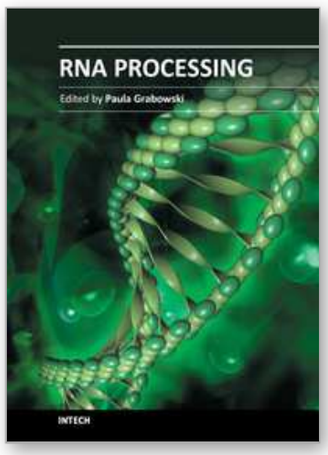

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RNA functions broadly as informational molecule, genome, enzyme and machinery for RNA processing. While these functions reflect ancient activities, they also remain vital components of contemporary biochemical pathways. In eukaryotic cells RNA processing impacts the biogenesis of RNA molecules of essentially every shape and function. The collection of articles in this volume describes the current state of understanding of the broad array of RNA processing events in animal and plant cells, key unanswered questions, and cutting edge approaches available to address these questions. Some questions discussed in this volume include, how viruses subvert the RNA processing machinery of the host cell, how the coordination of co-transcriptional RNA processing is regulated at the level of chromatin, the status of RNA processing in plant organelles, and how micro RNA machinery is biosynthesized and regulated.

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