Mutations of RNA polymerase II activate key genes of the nucleoside triphosphate biosynthetic pathways

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Marta Kwapisz1,2,4, Maxime Wery1,4, Daphné Després1, Yad Ghavi-Helm1, Julie Soutourina1, Pierre Thuriaux1,∗ and François Lacroute2,3
1CEA, iBiTec-S, Service de Biologie Intégrative et Génétique Moléculaire, Gif-sur-Yvette, France. 2CNRS, Centre de Génétique Moléculaire, UPR2167, Gif-sur-Yvette, France and 3Université Pierre et Marie Curie, Paris, France

The yeast URA2 gene, encoding the rate-limiting enzyme of UTP biosynthesis, is transcriptionally activated by UTP shortage. In contrast to other genes of the UTP pathway, this activation is not governed by the Ppr1 activator. Moreover, it is not due to an increased recruitment of RNA polymerase II at the URA2 promoter, but to its much more effective progression beyond the URA2 mRNA start site(s). Regulatory mutants constitutively expressing URA2 resulted from cis-acting deletions upstream of the transcription initiator region, or from amino-acid replacements altering the RNA polymerase II Switch 1 loop domain, such as rpb1-L1397S. These two mutation classes allowed RNA polymerase to progress downstream of the URA2 mRNA start site(s). rpb1-L1397S had similar effects on IMD2 (IMP dehydrogenase) and URA8 (CTP synthase), and thus specifically activated the rate-limiting steps of UTP, GTP and CTP biosynthesis. These data suggest that the Switch 1 loop of RNA polymerase II, located at the downstream end of the transcription bubble, may operate as a specific sensor of the nucleoside triphosphates available for transcription.

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Introduction
DNA transcription depends on the availability of pyrimidine (UTP and CTP) and purine (GTP and ATP) ribonucleoside triphosphate substrates, and their de novo synthesis is subjected to tight homeostatic controls. In yeast, the first two steps of UTP biosynthesis are catalysed by Ura2, a bifunctional protein endowed with carbamoyl phosphate synthetase and aspartate transcarbamoylase activities (Potier et al., 1987). These are the main rate-limiting steps of the pathway, and their allosteric control by UTP is well documented (Lacroute et al., 1965; Serre et al., 2004). Similar to other genes of the UTP biosynthetic pathway, URA2 transcription is induced in the presence of 6-azauracil, which depletes cells in UTP (Exinger and Lacroute, 1992). A previous study (Losson and Lacroute, 1981) has suggested that this does not depend on Ppr1, a transcriptional activator specific for the pyrimidine biosynthetic pathway (Loison et al., 1980; Losson and Lacroute, 1981; Flynn and Reece, 1999), as ppr1-2, a mutation unable to activate URA1 or URA3, had no effect on Ura2 activity in cell-free extracts. Moreover, we here show that a ppr1Δ null allele remains fully competent for URA2 transcription.

In this study, two classes of mutations were found to constitutively activate pURA2::HIS3 or pURA2::LacZ reporter plasmids. The first class was due to short deletions immediately upstream of the URA2 mRNA 5′-ends, indicating that this region acts as negative regulatory element of URA2 transcription. A second class altered RPBI, which encodes the largest subunit of RNA polymerase II, and specifically modified the Switch 1 loop of the active site. One of these mutations, rpb1-L1397S, was investigated in more detail. It showed a genome-wide reduced occupancy of RNA polymerase II, and specifically modified the Switch 1 loop of the active site. The Switch 1 loop of RNA polymerase II, located at the downstream end of the transcription bubble, may operate as a specific sensor of the nucleoside triphosphates available for transcription.

Results
UTP depletion activates URA2 independently of the Ppr1 activator
The URA2 open reading frame is separated by 1231 nt from the stop codon of the upstream gene TRK1 (Figure 1A). A KpnI–BamHI cassette bearing this DNA was cloned in frame with the LacZ or HIS3 open reading frames, generating the pURA2::LacZ reporter plasmids pFL80 and pFL80-H2, and the pURA2::HIS3 reporter pFL81 (Materials and methods). There was a fivefold LacZ activation in wild-type cells grown under repressing (uracil) or derepressing (6-azauracil) conditions (Figure 1B), and a similar range of activation was observed in the steady-state level of URA2 mRNAs (Figure 1C), indicating that the pURA2::LacZ reporter correctly reflects the transcriptional regulation of URA2.
Regulatory effects of RNA polymerase II

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Figure 1 Transcriptional regulation of URA2 in response to UTP shortage. (A) Schematic view of the TRK1–URA2 intergenic region. The corresponding DNA was cloned as a KpnI–BamHI cassette to generate the plFL80 and plFL80-H2 pURA2::LacZ reporter plasmids. A box denotes the 5′-UTR intron. The broken arrow corresponds to the URA2 mRNAs 5′-ends. (B) β-galactosidase activities of BMA64-URA3 (WT) and FL55 (ura8-ts ura7Δ) grown without uracil (CSM +) at 30 and 34 °C, in the presence of uracil (CSM + U), mycophenolate (CSM + MPA) and 6-azauracil (CSM + 6AU). (C) Three independent cultures of BMA64-URA3 (WT) and FL53 (ppr1Δ) were exponentially grown without (−URA) or with uracil (+URA), or with 200 mg/l of 6-azauracil (+6AU). URA1 and URA2 mRNAs were quantified by RT–PCR, and RNA polymerase II occupancy was determined by ChIP assays, using anti-CTD antibodies (8WG16). A schematic map represents the URA1 gene, with the oligonucleotide pairs used as primers. Position 1 corresponds to the start codon.

Adding 6-azauracil blocks UTP synthesis and also reduces the cellular pool of GTP (Exinger and Lacroute, 1992). However, no pURA2::LacZ activation was observed in the presence of mycophenolate, which specifically blocks IMP dehydrogenase (Shaw et al., 2001), and thus only depletes GTP. Likewise, counteracting GTP inhibition by adding guanine to the CSM +0 medium did not reduce the activating effect of 6-azauracil (data not shown). Finally, impairing the metabolic conversion of UTP to CTP in temperature-sensitive mutant (ura8-ts ura7Δ) reduced the expression of pURA2::LacZ, which presumably reflects the accumulation of UTP under these conditions and implies that URA2 does not respond to CTP shortage. These data indicated that URA2 transcription is primarily activated by a shortage in UTP, with little or no effect of GTP or CTP.

Other genes of the pyrimidine pathway, such as URA1 and URA3, are also derepressed under UTP depletion, and their activation is lost in ppr1−, a mutation that alters the Ppr1 activator but has no effect on the Ura2 activity in cell-free extracts (Losson and Lacroute, 1981). Consistent with this observation, we found here that the ppr1Δ null allele strongly reduced the activation of URA1 and URA3 in the presence of 6-azauracil, as measured by RT–PCR quantification of their mRNAs and by their RNA polymerase II occupancy in chromatin-immunoprecipitation (ChIP) assays (shown in Figure 1C for the URA1 gene). In contrast, ppr1Δ actually increased the level of URA2 mRNA produced in the presence of 6-azauracil, which may reflect a particularly effective depletion in UTP, due to its inability to activate URA1 and URA3.

Organisation of the URA2 promoter region

It was previously thought that URA2 transcription starts some 70 nt ahead of the ATG start codon (Potier et al., 1990). This is clearly inconsistent with the recent report of an untranslated intron between positions −385 and −66 relatively to the ATG (Juneau et al., 2007). As shown in Figure 2, we confirmed the existence of this intron, although with different 3′-ends corresponding to positions −63, −54 or, in one case, +12. The corresponding DNA has a 5′-end consensus (Δ584GUAUUGU−379), a canonical branch point box (Δ52 UACUAAAC−84), and a −60UAG−65 at the 3′-splice site. An intron deletion did not detectably affect the expression of pURA2::LacZ (bottom line of Figure 2A).

Using RNA ligase mediated-RACE assays, we obtained and sequenced 13 cDNAs that, by construction, extended to position +188 of the URA2 open reading frame and thus corresponded to the 5′-end domains of full-length mRNAs (Figure 2B). These 5′-ends defined six positions (−588A, −579T, −576A, −571A, −565A and −563A) clustered within an A-rich domain, matching to the initiator consensus (Δ186N(C/T)]A(A/T)NN(Arich)9 deduced from a recent survey of yeast mRNAs (Zhang and Dietrich, 2005). A parallel study (Thiebaut et al., 2008) has identified a second initiator region (positions −686/−657, denoted by a grey arrow in Figure 2A) upstream of the one shown here to be used for full-length URA2 mRNAs. This upstream initiator produces short unstable non-coding RNAs with the same transcriptional orientation as URA2. It is evidently not used to produce full-length URA2 transcripts, as the predicted 5′-ends were not found in the corresponding cDNAs (Figure 2B). Five TCTT (UCUU) boxes, present immediately upstream of the
URA2, an initiator, form a cluster of binding sites recognised by Nab3, which belongs to the transcriptional terminator system operating on non-coding intergenic RNAs (Arigo et al., 2006; Thiebaut et al., 2006).

The DNA upstream of the URA2 initiator prevents the progression of RNA polymerase II

Partial deletions of the TRK1-URA2 intergenic region were introduced in pURA2::LacZ reporters (pFL80 and pFL80-H2) and tested for β-galactosidase activity under repressing (uracil) and derepressing (6-azauracil) conditions. Deleting the first 297 nt had no effect on pURA2::LacZ, but a BsrGI/SalI deletion lacking positions -934/-580 almost entirely blocked pURA2::LacZ expression (Figure 2A). The corresponding DNA is therefore critical for URA2 transcription. It contains a TATAAA box (-776/-771) shown by Thiebaut et al. (2008) to be needed for the synthesis of the URA2 mRNA and of its short-lived upstream transcripts.

The BsrGI/SalI DNA was submitted to error-prone amplification and recombined into the pURA2::HIS3 reporter (pFL83), producing plasmids that were selected for HIS3 overexpression in the presence of uracil. No single-base mutation was isolated by this approach, but we obtained three very similar deletions up2Δ (-647/-577), up6Δ (-634/-579) or up10Δ (-650/-579) upstream of the Sall site (Figure 3A). These deletions overexpressed LacZ when subcloned into pURA2::LacZ reporters (pFL80-H2 and pFL80), and a chromosomal up2Δ mutant (FL51) constitutively expressed the URA2 mRNA under-repressing conditions (Figure 3B).

Moreover, up2Δ only acted in cis, as the expression levels in the chromosome were restored by the addition of uracil (ura1Δ, up2Δ grew as well as the ura1Δ, +ura control).

Figure 2  Organisation of the URA2 promoter region. (A) Deletion mutagenesis of promoter region. LacZ expression in BMA64-URA3 (WT) transformed with pFL80 (pURA2::LacZ), pFL80-BD (BsrGI deletion), pFL80-BSΔ (BsrGI-SalI deletion) and pFL80-INTA (no intron). Strains were exponentially grown without (-URA) or with uracil (+URA), or with 200 mg/l of 6-azauracil (+6AU). β-galactosidase was expressed in arbitrary units (Miller, 1972), where 1.0 is the level measured in wild-type cells grown in CSM. Broken arrows correspond to the transcriptional initiator regions, shown in black for full-length URA2 mRNA and in grey for short upstream untranslated RNAs (Thiebaut et al., 2008). A canonical TATAAA box and a cluster of TCTT (UCUU) motifs (stars) are also indicated (see Supplementary data). (B) Identification of the URA2 S'end region and of an URA2 intron. Thirteen cDNAs primed from an oligonucleotide corresponding to positions 167/188 of the URA2 start site (Figure 2A). The corresponding DNA is therefore critical for URA2 transcription. It contains a TATAAA box (5′-gtcgac-3′). Nine cDNAs were extracted and amplified from wild-type cells (BMA64-URA3) grown without uracil (−URA), or with 200 mg/l of 6-azauracil (6AU), and four others were prepared from FL-M9 (rpb1-L1397S) grown without uracil (−URA). This also confirmed the existence of an intron between positions −385 and −66 (Juneau et al., 2007), but with 3′-borders corresponding to positions −63, −54 or, in one case, +12.

Figure 3  The DNA upstream of the transcription start impairs URA2 expression. (A) Schematic organisation of the upΔ mutations. Thick black line corresponds to the deleted DNA up2Δ, up6Δ and up10Δ. Stars indicate TCTT (UCUU) boxes. Broken arrows correspond to the transcriptional initiator regions as shown in Figure 2A. (B) Effect of up2Δ on pURA2::LacZ expression and URA2 mRNA steady-state level. BMA64-URA3 (WT) was transformed with pFL80 or pFL80-up2Δ. β-galactosidase was tested as shown in Figure 2A. URA2 mRNA is expressed in arbitrary units (Miller, 1972), where 1.0 corresponds to wild-type cells grown in the absence of uracil (−URA). RT-PCR assays are based on three independent cultures of BMA64-URA3 (WT) and FL51 (up2Δ), grown with (+URA) or without uracil (−URA), or containing 200 mg/l of 6-azauracil (+6AU).
The ChIP assays of Figure 4A show that the region upstream of the URA2 start site is occupied by TFIIIB and TFIIH, two components of the RNA polymerase II pre-initiation complex. The corresponding RNA polymerase II signal was recognised by anti-CTD antibodies, and also by an anti-Rpb3::HA tag (see Figure 7D below), but not by antibodies raised against Ser2-phosphorylated CTD (Figure 4B). The CTD-Ser2\(^{\alpha}\) signal was only detected downstream of the URA2 initiator region. As shown in Figure 4C, this also coincided with a drop in RNA polymerase II occupancy, observed in wild-type or ppr1\(\Delta\) cells grown under repressing conditions.

URA2 activation in wild-type or ppr1\(\Delta\) cells exposed to 6-azauracil did not increase the RNA polymerase II signal detected upstream of the initiator region, but correlated with a full occupancy of the URA2 open reading frame by RNA polymerase II (Figure 4C). Likewise, the up2\(\Delta\) mutation produced a strong RNA polymerase II signal downstream of the initiator, even when grown under repressive conditions (Figure 4C). Thus, URA2 activation is not due to an increased recruitment of RNA polymerase II (as in the case of Ppr1-dependent genes such as URA1; see Figure 1C) but results from an extended RNA polymerase II occupancy downstream of the URA2 initiator region. Moreover, a relatively short DNA region, lost in up2\(\Delta\) and located immediately upstream of the URA2 initiator, impairs URA2 transcription by preventing RNA polymerase II from progressing towards the URA2 open reading frame.

**Mutants of the RNA polymerase II Switch 1 loop activate URA2 expression**

To search for trans-acting regulator(s) of URA2, mutations constitutively expressing the chromosomal pURA2::HIS3 reporter of strain FL52 were selected after UV mutagenesis. We obtained four mutants (FL-M9, M10, M13 and M23) that were resistant to 3-aminotriazol on uracil-supplemented medium and also overexpressed the pURA2::LacZ reporter. They grew slowly at 30\(^{\circ}\)C, failed to grow at 16\(^{\circ}\)C and 37\(^{\circ}\)C and were somewhat sensitive to 6-azauracil (shown in Figure 5A for the FL-M9 strain). Meiotic tetrad analysis showed that these phenotypes co-segregated in a monogenic and recessive way, and complementation tests established that the corresponding mutations belonged to one and the same gene.

We then transformed FL-M9 with a yeast genomic library and obtained one plasmid restoring growth at 37\(^{\circ}\)C. The corresponding insert harboured RPB1, which encodes the largest subunit of RNA polymerase II, and three surrounding genes. Further subcloning showed that growth at 37\(^{\circ}\)C correlated with an intact RPB1 gene, and complementation tests with the temperature-sensitive allele rpb1-1 (Scafe et al., 1990) firmly established that all four mutants were due to rpb1 mutations. In vivo gap repair (Rothstein, 1991) indicated that these mutations belonged to the Swu1-PshAI segment of RPB1. Finally, DNA sequencing revealed single amino-acid replacements corresponding to rpb1-L1397S (M9), rpb1-S1401P (M10) and rpb1-F1402L (M13 and M23), which were also present in the chromosomal DNA of the original mutant strains.

The above-mentioned data led to the rather unexpected conclusion that URA2 activation occurs by trans-acting mutations altering RNA polymerase II itself, at the level of its Switch 1 loop fold. RT–PCR and LacZ assays confirmed that

**Figure 4** Distribution of TFIIIB, TFIIH and RNA polymerase II at the URA2 locus. (A) TFIIH (Rad3-TAP) and TFIIIB (Sua7-TAP) were detected by ChIP assays as described in Materials and methods. Strains D712-10C (WT RAD3::TAP) and D714-5D (WT SUA7::TAP) were grown on SD + aa. The schematic organisation of the corresponding DNA is presented below. Broken arrows represent the URA2 mRNA start sites and the upstream initiator, as in Figure 2A.

(B) RNA polymerase II was immunoprecipitated with Dynabeads anti-mouse (Dynal Biotech), using anti-CTD (8WG16), anti-Ser2\(^{\alpha}\) and anti-Ser3\(^{\alpha}\) antibodies (Covance). Strain GR44-11C (WT) was grown on SD + aa. A schematic map indicates the oligonucleotides used as primers. (C) RNA polymerase II occupancy in wild-type, ppr1\(\Delta\) and up2\(\Delta\). Strains GR44-11C (WT), FL53 (ppr1\(\Delta\)) and FL51 (up2\(\Delta\)) were grown at 30\(^{\circ}\)C on SD + aa with 2 g/l of uracil (+ URA) or exposed to 200 mg/l of 6-azauracil (+ 6AU). ChIP signals were detected with anti-CTD antibodies (8WG16). A schematic map represents the URA2 gene, with the oligonucleotides used as primers.

promoter reporter (pURA2::LacZ) was not activated in the up2\(\Delta\) host (data not shown).
rpbl-L1397S highly expressed URA2 (Figure 5B and C). Moreover, an rpbl-L1397S up2Δ double-mutant had the same constitutive expression as up2Δ alone, and a pURA2::LacZ reporter bearing the up2Δ allele (plasmid pFL80-up2Δ) was expressed at the same level when tested in wild-type or in an rpbl-L1397S host strain (Figure 5B and C). In other words, rpbl-L1397S and up2Δ have epistatic effects on URA2 expression, thus strongly suggesting that they are defective in the same mechanism downregulating URA2 in response to uracil.

The six amino-acid segment occupied by L1397, S1401 and F1402 corresponds to Rpbl-α47β helix, one of the two α helices forming the Switch 1 loop domain of the RNA polymerase II active site (Gnatt et al., 2001). Moreover, the rpbl-G1388V allele, altering the Rpbl-α47β helix (Berroteran et al., 1994), also resulted in a high constitutive expression of URA2 (data not shown). This high clustering was not anticipated in a UV mutagenesis, as the latter evidently affects the whole yeast genome. Our genetic screen was presumably not saturating, and we cannot exclude that URA2 activation may, for some reason, be a general property of partly defective RNA polymerase II mutants. We thus tested the pURA2::LacZ reporter (pFL80) in 10 other slow-growing mutations partly impairing Rpbl (rpbl-G1437D, rpbl-E1351K and rpbl-H1367D) or Rpbl2 (rpbl2-R857K, rpbl2-E836A, rpbl2-D978A, rpbl2-P1018S and rpbl2-G1142D) or lacking the non-essential subunits Rpbl4 (rpbl4Δ) or Rpbl9 (rpbl9Δ) (Woychik and Young, 1989; Scale et al., 1990; Woychik et al., 1991). Except for a modest derepressing effect of rpbl2-E836A and rpbl2-R857K, their β-galactosidase activity was equal to or lower than the wild-type control (data not shown), thus strongly suggesting that URA2 activation, at least to a large extent, is primarily due to changes in the Switch 1 loop.

The Switch1 loop is highly conserved in all eukaryotic and archaeal RNA polymerases (Figure 6A). Together with the α25 Bridge helix, α36 Trigger helix and the α46/47 Loop of Rpb1, it forms an identical fold in the RNA polymerase of Sulfolobus solfataricus (Hirata et al., 2008) and in yeast RNA polymerase II (Gnatt et al., 2001). This fold wraps the downstream end of the transcription bubble and holds the DNA template strand by the invariant R1386-E1403 Switch 1 dipole (Figure 6B and C). As discussed elsewhere (Zaros et al., 2007), this fold is stabilised by the C-end of the Rpb5 subunit, itself strongly conserved from archaea to eukaryotes. Bacterial RNA polymerases, on the other hand, have no Rpb5 and their Switch 1 loop is considerably extended but nevertheless adopts a very similar spatial orientation (Vassiliev et al., 2007).

**rpbl-L1397S enhances RNA polymerase II occupancy downstream of the URA2 initiator**

Consistent with the partial growth defects of rpbl-L1397S, a twofold reduction in RNA polymerase II occupancy was seen for rpbl-L1397S in single-gene ChiP assays at ADH1 or PYK1 (Figure 7A). This extended to the whole genome, with an average occupancy level of 62% (R² = 0.80) relatively to wild
As expected, RNA polymerase II occupancy was increased on the URA2 open reading frame. Moreover, this increased occupancy was only seen downstream of the URA2 initiator region (Figure 7C). Similar results were obtained in ChIP assays done in RPB3 H3HA rpb1-L1397S cells, showing that this effect is independent of the precipitating antigen (Figure 7D). In contrast, two RNA polymerase II alleles (rpb2-P1018S and rpb9) with partial growth defects similar to those of rpb1-L1397S significantly reduced RNA polymerase II signal, upstream and downstream of the initiator region. Taken together, these data clearly show that rpb1-L1397S activates URA2 by extending RNA polymerase II occupancy downstream of the initiator region, thus recapitulating the effect seen above for up2Δ or for wild-type and ppr1Δ cells exposed to 6-azauracil.

rpb1-L1397S extends RNA polymerase II downstream of IMD2, IMD3 and URA8

We have seen above that rpb1-L1397S generally reduced RNA polymerase II occupancy, except for URA2. This was also consistent with genome-wide transcriptome assays, showing a moderate but significant overexpression of URA2 in rpb1-L1397S, compared with an isogenic wild-type control (Figure 8A). Furthermore, these transcriptome and genome-
wide RNA polymerase II occupancy assays identified two other genes, IMD2 (IMP dehydrogenase) and URA8 (CTP synthase), which were both overexpressed and over-occupied by RNA polymerase II in \textit{rpb1-L1397S}. A few additional genes were also overexpressed in the \textit{rpb1-L1397S} transcriptome, but with no higher RNA polymerase II occupancy of their open reading frames (data not shown). Conversely, \textit{rpb1-L1397S} enhanced RNA polymerase II at \textit{ADE12} (adenylosuccinate synthase), but had no detectable effect on its mRNA level.

\textit{Saccharomyces cerevisiae} has two CTP synthases (URA7 and URA8) and three active IMP dehydrogenase genes (IMD2, IMD3 and IMD4), with IMD1 as a pseudo-gene inactivated by a frame-shift mutation in most laboratory strains (Ozier-Kalogeropoulos et al. 1994; Nadkarni et al., 1995; Hyle et al., 2003). URA7 and URA8 are sufficiently different to be distinguished by individual RT-PCR assays, which clearly showed that \textit{rpb1-L1397S} activated only \textit{URA8} (Figure 8B). RT-PCR assays also confirmed the overexpression of the IMP dehydrogenase mRNA in \textit{rpb1-L1397S} but did not clearly distinguish between IMD2, IMD3 and IMD4, due to their very similar nucleotide sequences. However, our genome-wide ChIP assays readily discriminated between these three genes. This revealed that \textit{rpb1-L1397S} strongly activated IMD2, with some effect on IMD3 (Figure 8C) and no effect at all on IMD4 (not shown). We considered the possibility that the activation of CTP synthase and IMP dehydrogenase genes may be an indirect effect of \textit{URA2} overexpression, reflecting an increased cellular pool of UTP. However, the activation of \textit{URA2} in \textit{up2A} was not accompanied by a parallel increase in \textit{URA8} or IMP dehydrogenase mRNA, thus strongly arguing for a direct activating effect of \textit{rpb1-L1397S} (Figure 8B).
Previous studies have shown that IMD2 is controlled by an initiation switch between short transcripts, synthesised from an upstream initiator, and full-length mRNAs starting from a downstream initiator (Escobar-Henriques et al., 2003; Steinmetz et al., 2006; Kopcewicz et al., 2007; Jenks et al., 2003). RNA polymerase II is confined to the upstream initiator when wild-type cells are grown under repressing conditions, but extended to the entire URA8 open reading frame in rpb1-L1397S (Figure 8C). A similar effect may apply to ADE12, encoding the first step of the ATP biosynthetic pathway, where rpb1-L1397S moderately increased RNA polymerase II occupancy downstream of the promoter region (Figure 8D).

Discussion

Previous studies have shown that URA1 or URA3 are specifically activated by Ppr1, a Zn activator of the Gal4-type which responds to increased concentration of the orotate and/or dihydro-orotate precursors of UTP (Loison et al., 1980; Losson and Lacroute, 1981; Flynn and Reece, 1999).
This leads to an increased RNA polymerase II occupancy at the URA1 and URA3 promoters, lost in the ppr1Δ-null allele, indicating a ‘classical’ mode of gene-specific activation on the basis of a more effective recruitment of RNA polymerase II by its pre-initiation complex. URA2, which encodes the main rate-limiting enzyme of UTP biosynthesis, is activated when UTP is depleted by 6-azauracil addition. However, the ppr1Δ-null allele is fully competent for URA2 transcription. Moreover, and in contrast to URA1 or URA3, URA2 activation does not change the amount of RNA polymerase II residing at or upstream of the URA2 initiator region, but operates instead by extending RNA polymerase II occupancy to the URA2 open reading frame.

Three cis-acting mutations constitutively expressing URA2 were selected in this study. They corresponded to very similar deletions (up2Δ, up6Δ and up10A) removing a 55–70 nt DNA region upstream of the URA2 initiator. The up2Δ mutation was studied in more detail. It extended RNA polymerase II to the entire URA2 open reading frame, with no change in the RNA polymerase II signal upstream of the URA2 initiator, thus recapitulating the pattern seen in UTP-depleted wild-type or ppr1Δ cells. Further studies (Thiebaut et al., 2008) have shown that the corresponding DNA is transcribed from an initiator region located some 100 nt upstream of the main URA2 mRNA 5′-ends identified here, producing unstable RNAs with the same transcriptional orientation as URA2, which were no longer detected in up2Δ. Taken together, these data suggest that URA2 may be regulated by an attenuation mechanism related to the one recently described for IMD2 (Escobar-Henriques et al., 2003; Davis and Ares, 2006; Steinmetz et al., 2006; Kopcewicz et al., 2007; Jenks et al., 2008).

A genome-wide selection for trans-acting mutations constitutively expressing URA2 yielded three tightly clustered rpbl mutations (rpbl-L1397S, rpbl-S1401P and rpbl-F1402L), corresponding to the Rpb1α-47b helix of the Switch 1 loop. rpbl-G1388V, which was initially selected for its ability to alter transcription initiation in favour of downstream sites (Berroteran et al., 1994) and belongs to the Rpb1α-47a helix, also activates URA2 (see also Thiebaut et al., 2008). In contrast, other partly defective RNA polymerase II mutations failed to derepress URA2, indicating that URA2 activation is by no means a general property of RNA polymerase II mutants. Similar to up2Δ, rpbl-L1397S activates URA2 by extending RNA polymerase II occupancy to the entire open reading frame, with no change in the signal upstream of the URA2 initiator, thereby reproducing the effect seen in up2Δ and in UTP-depleted wild-type cells. Moreover, up2Δ and rpbl-L1397S had epistatic effects on URA2 expression, indicating that rpbl-L1397S is defective in the attenuation mechanism lost in up2Δ itself. Finally, rpbl-L1397S and wild-type URA2 mRNAs had similar 5′-ends, as determined by 5′-RACE assays, and therefore use the same transcription initiation.

The above-mentioned data indicate that changes in the Switch 1 loop specifically activate URA2, despite their adverse effects on growth and their reduced genome-wide RNA polymerase II occupancy. Moreover, we were unable to identify mutants encoding a specific regulator of URA2, although it remained possible that such mutants are lethal because they perturb some essential cellular process. The Switch 1 loop forms an invariant R1386(x47a)-E1403(x47b) dipole, holding the DNA template strand at positions +2/+3 downstream of the catalytic Mg2+/Ca2+ (Gnatt et al., 2001). This domain is highly conserved among eukaryotic and archaeal RNA polymerases (Cramer et al., 2001; Hirata et al., 2008) and a related structure exists in the bacterial enzyme, where it also binds DNA at positions +2/+3 downstream of the catalytic Mg2+ (Vassylyev et al., 2007; Zaros et al., 2007).

The relation of this domain to the entry route of NTPs in RNA polymerase II is unclear (Landick, 2005). One possibility is that NTPs reach the catalytic site of RNA polymerase II by its funnel-shaped pore (Cramer et al., 2001; Kettenberger et al., 2003; Westover et al., 2004; Landick, 2005), but it has also been suggested that NTPs enter through the large DNA channel, transiently binding the DNA template at nucleotides +2, +3 and possibly +4 (Gong et al., 2005), which implies that NTPs pass by the Switch 1 loop before reaching the catalytic Mg2+. Hence, the Switch 1 loop may act as an NTP-sensing module, enabling RNA polymerase II to adopt a processive mode of elongation when enough NTP is available. This would optimise the efficiency of transcription, which may account for the cold- and heat-sensitive defects of rpb1-L1397S, rpb1-S1401P and rpb1-F1402L, and their increased sensitivity to 6-azauracil and mycophenolate.

Strikingly, rpbl-L1397S also extended RNA polymerase II occupancy downstream of the IMD2/IMD3 (IMP dehydrogenase) and URA8 (CTP synthase) promoters, with some evidence for a similar effect on ADE12 (adenylosuccinate synthase). This makes biological sense, as the corresponding genes encode rate-limiting enzymes in the de novo synthesis of GTP, CTP and ATP. This suggests the rather non-canonical view that nucleoside triphosphate shortage or RNA polymerase II Switch 1 loop mutations (assumed here to mimic that shortage) specifically activate the expression of genes that are themselves critical for the de novo synthesis of NTPs. In other words, the transcriptional response to NTP shortage would not rely on dedicated activators or repressors (which have been vainly searched for in the case of URA2 and IMD2) but would be mediated by an NTP-sensing mechanism built in the structure of RNA polymerase II itself.

In IMD2, there is good evidence that RNA polymerase II molecules are recruited on a common pre-initiation complex, followed by a start site switch leading to the alternative production of upstream transcripts ended by Nabh/Nrd1-dependent termination or full-length mRNAs transcribed from a downstream initiator region. Start site selection is presumably dictated by the nucleotide composition of the surrounding DNA, with upstream transcription starting at Gs, whereas IMD2 transcription starts in a C-poor DNA region and is therefore favoured by low GTP pools (Escobar-Henriques et al., 2003; Davis and Ares, 2006; Steinmetz et al., 2006; Kopcewicz et al., 2007; Jenks et al., 2008). The URA2 start choice, however, depends on a different mechanism, as upstream initiation does not occur preferentially at T’s and is not sensitive to the abundance of uracil (Thiebaut et al., 2008). Conversely, we found that 3 out of 10 URA2 cDNAs produced under activating conditions started with a T, which argues against the simple idea of a selection driven by the nature of the starting nucleotide. Nevertheless, the upstream initiator is followed by an ~60 nt Trich domain, continued by 40 nt that are conspicuously poor in T’s and harbour the URA2 mRNA 5′-ends. This pattern is conserved among all Saccharomyces species sequenced to data (see...
Supplementary data), and we would be surprised if it did not provide some form of UTP-sensing. Further studies are clearly needed to better understand how RNA polymerase II responds to NTP shortage, and how this response is ultimately converted into a specific control of the IMD2, URA2 and URA8 genes.

**Materials and methods**

**Plasmids**

Newly constructed plasmids are listed in Supplementary data. Plasmids pFL35-II, pFL36-CII (CEN6 LEU2) and pFL39-CII (CEN6 TRP1), corresponding to pFL35, pFL36 and pFL39 (Bonneaud et al, 1991), with an extended polylinker of 90 nucleotides (Supplementary data). pFL80 and pFL82 are pURA2::LacZ pURa2 reporters derived from pFL39-CII and pFL36-CII, respectively. They harbour a Kpn–BamHI–NotI pURa2::LacZ cassette of 4392 nt. The Kpn–BamHI segment (1254 nt) is formed by the TRP1–URA1 intergenic region, preceded by GGGAGCAGCACACAGGTCTAATA (the KpnI site is italicised) and followed by ATGACTAC (BamHI). It is cloned in-frame to the initiator ATG of a BamHI–NotI LacZ cassette: pFL83 is pFL80 where the LacZ open reading frame has been replaced by a BamHI–NotI HIS3 (S. cerevisiae) cassette. As pFL80 poorly complemented trp1 mutant strains, which overexpressed the pURa2 promoter (due to some transcriptional interference between LacZ and TRP1), we also used pFL80-H2, a pURa2::LacZ reporter with a lower LacZ expression. This plasmid was obtained after random insertion of a genomic HindIII fragments in the unique HindIII site downstream of LacZ (Supplementary data). The HindIII fragment of pFL80-H2 corresponded to positions 7679–8251 of the TEL1 gene and was inserted in a transcriptional orientation opposite to the one of TRP1.

Plasmids pFL80-KB, pFL80-BS and pFL80-CS correspond to Kpn/BrGl, BrGl/Sall and Clal/Sall deletions of pFL80, respectively. pFL80-INTA was precisely deleted of the DNA comprised between nucleotides 384 to 63, where 1 corresponds to the URA2 start codon. The pFL83-up2A, -up6A and -up10A plasmids were obtained by error-prone amplification of the Kpn–BamHI cassette of pFL83, which was recombined into the original vector by co-transformation with linearised pFL83 DNA deleted of its BrGl/Sall fragment. Mutant plasmids were selected on 3-aminotriazole in the presence of uracil, amplified in STR mutants harboured the TRP1–URA1 fragment of pFL80 and pFL82 into pFL35-II. The resulting plasmid was linearised at the BamHI site of the pURA2 promoter region, to direct its integration by homologous recombination with the pURA2 region of BMA64-URA3. The corresponding transformants harboured the TRP1 cassette flanked by wild-type and up2 tandem copies of pURa2. Tryptophan auxotrophic clones spontaneously occurred by recombination between pURa2 and wild-type copies and were selected by nystatin enrichment (Snow, 1996), yielding single copies of pURa2. Among them, up2 mutants were identified by their weak resistance to 5-fluorouracil (10⁻³ M), and were further checked by PCR amplification.

**ChIP and genome-wide ChIP-chip assays**

Chromatin immunoprecipitation assays were described elsewhere (Chahi-Helm et al, 2008). All experiments were performed on three independent cultures of 100 ml, harvested at an OD₆₀₀ of 0.3–0.5. Cells were grown in SD + a with or without uracil, or UTP-depleted by adding 200 mg/l of 6-azaauracil to log-phase cells grown in SD + a and grown for three additional doubling times. RNA polymerase II was immunoprecipitated with Dynabeads antiamouse IgG (Dynal Biotech), using anti-CTD antibodies (8WG16), or anti-hemagglutinin A antibodies (12CAS) in the case of Rp3-3A tagged strains. Sua7-TAP and Rad3-TAP proteins were directly immunoprecipitated on Dynabeads anti-mouse IgG. Phosphorylated variants of the RNA polymerase CTD domain were immunoprecipitated with Dynabeads anti-mouse IgM, using anti-Ser5R (H5, Covance) and anti-Ser5S (H14, Covance) antibodies. ChIP signals were calculated by the immunoprecipitation/input signal. The value 1.0 was arbitrarily given to the reference signal provided by amplifying the GAL1 gene. Genome-wide ChIP assays, on the basis of three independent cultures in YPD or CSM medium for mycoplasmale experiments (10 mg/l medium), were described elsewhere (Harisemidy et al, 2003) and were based on DNA arrays with over 40 000 oligonucleotide probes covering 12 Mb of the yeast genome (Ghavi-Helm et al, 2008).

**RNA and transcriptome assays**

Total RNA was extracted from three independent, exponential cultures with hot phenol and reverse-transcribed using 1 μg of total RNA, Super-Script II reverse transcriptase (Invitrogen) and random hexamers as primers. Controls without reverse transcriptase showed negligible levels of DNA contamination. DNA was quantified by real-time PCR amplification (Applied Biosystems, SYBR®DS Software). Plasmids listed in Supplementary data. mRNA levels are calculated as a ratio of measured mRNA and ACT1 mRNA. The 5’-ends of URA2 mRNAs were mapped by sequencing cDNAs obtained by the RNA ligase mediated-RACE technique (using an RLM-RACE kit from Ambion), according to the protocol provided by the manufacturer. Yeast micro-arrays were probed against total RNA extracted from three independent cultures of strains BMA64-URA3 (wild-type) and FL89 (pFL1-L1397S) grown on SD + a at 30°C, to an OD₆₀₀ of 0.55. cDNA synthesis, purification, indirect labelling, microarray hybridisation, scanning and analysis were done according to standard protocols (Sourtourina et al, 2006), with two independent hybridisations for each RNA batch.

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