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External conditions inversely change the RNA polymerase II elongation rate and density in yeast

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ABSTRACT

Elongation speed is a key parameter in RNA polymerase II (RNA pol II) activity. It affects the transcription rate, while it is conditioned by the physicochemical environment it works in at the same time. For instance, it is well-known that temperature affects the biochemical reactions rates. Therefore in free-living organisms that are able to grow at various environmental temperatures, such as the yeast *Saccharomyces cerevisiae*, evolution should have not only shaped the structural and functional properties of this key enzyme, but should have also provided mechanisms and pathways to adapt its activity to the optimal performance required. We studied the changes in RNA pol II elongation speed caused by alternations in growth temperature in yeast to find that they strictly follow the Arrhenius equation, and that they also provoke an almost inverse proportional change in RNA pol II density within the optimal growth temperature range (26–37 °C). Moreover, we discovered that yeast cells control the transcription initiation rate by changing the total amount of available RNA pol II.

1. Introduction

Living cells have evolved within the constraints imposed by physical and chemical laws. This rule acts on every chemical reaction within a cell and has limited the concentrations, structures, affinities and specific locations of all enzymes and metabolites within ranges that are compatible with life and evolution. For instance, it has been recently demonstrated that metabolic enzyme thermostability is rate-limiting at superoptimal temperatures in *Escherichia coli* [1].

Chemical reactions are governed by the thermodynamics and kinetics laws that depend on concentrations of reactants and products, and also on environmental factors, such as pH or temperature. In the specific case of nucleic acid synthesis, reactants (nucleotide triphosphates, NTPs/dNTPs) are usually well above K_m [2], and the main product of the reaction is a long polymer to which the catalytic enzyme (polymerase) is continuously bound during its synthesis (processive synthesis). The use of highly processive enzymes is necessary because a high synthesis rate must avoid the delay that a distributive mechanism for monomer incorporation would cause [3].

Synthesis of mRNAs is usually the most important step in gene regulation in eukaryotes, and RNA polymerase II (RNA pol II) is the acting enzyme in this process. RNA pol II molecules are first recruited to gene promoters to then transcribe along the coding region of the gene (see [4] for a review). RNA pol II is a complex of 12 subunits that is helped by many other ancillary proteins during initiation and elongation [4]. During elongation, RNA pol II undergoes different modifications which mark their particular stage throughout the process [4]. All eukaryotic RNA pol II have a long C-terminal tail (CTD) in their largest subunit (Rpb1) with repeated heptads (from 26 in yeast to 52 in humans); *e.g.*, Tyr-Ser-Pro-Thr-Ser-Pro-Ser. The CTD is a target for many reversible modifications, including phosphorylation, prolyl-isomerization and glycosylation, at various positions [4]. Unphosphorylated molecules form the PIC (pre-initiation complex) at gene promoters. Then Ser5 phosphorylation in the CTD is required to start early elongation. Ser2 phosphorylation starts later and progressively replaces Ser5 during elongation, thus rendering it a marker for elongating RNA pol II (see [4,3] for a review). Moreover, it should also be considered that differences in transcription depend not only on RNA pol II molecules, but also on the ancillary factors accompanying them along the gene.

Genes differ not only in the particular amino acid sequence they encode, but also in other sequence-dependent features. Genes have different lengths, G + C content, dinucleotide distribution, exon/intron

Abbreviations: 6AU, 6-azauracil; RNA pol, RNA polymerase; NTP, nucleotide triphosphate; CTD, carboxy terminal domain of the largest RNA pol II subunit; TR, transcription rate; nTR, nascent TR; GRO, Genomic run-on; O.D., optical density; ORF, open reading frame; TSS, transcription start site; Nucleotides, nt; mRNA degradation rate, DR; qPCR, quantitative PCR

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organization in general, and a chromatin structure in particular. All these features, which are intrinsic to the transcriptional apparatus or to the template that RNA pol reads, can influence elongation [3]. As explained before however, RNA pol II elongation can also be conditioned by extrinsic factors, such as temperature, NTP concentration, and pH.

The speed at which processive RNA pol II elongates has been found to be 1–2 kb/min on average in several eukaryotes [5–7]. However, it has been discussed that it varies within a broader range (1–6 kb/min, see [8]) and has been recently shown to be variable among and along human genes [9], thus raising the important question of the role that the elongation rate plays in regulating the TR. The elongation rate of a prokaryotic RNA pol has been kinetically modeled on the basis of the single one-nucleotide successive steps as a thermal ratchet molecular motor that depends on NTP concentrations and DNA sequences [10]. In prokaryotes, *in vitro* experiments have demonstrated that RNA pol is dependent on temperature [11].

The nascent transcription rate (nTR) depends on the density of RNA pol II molecules and their speed. Density directly depends on the rates of initiation events and abortive elongation that cause RNA pol II to drop off [4]. When the remaining factors are fixed, density and the elongation rate are mutually inverse parameters. Thus, if an external factor that affects the elongation rate is changed, it will be observed as a reciprocal inverse change in RNA pol density. Current techniques to determine the nTR, such as chromatin immunoprecipitation of RNA pol II molecules or run-on analysis, are based on the determination of RNA pol II densities [12]. When doing comparative analyses between samples, presumably elongation rates do not vary [13–15]. In some cases they have been assumed to change with temperature [16]. However, this assumption has not been experimentally tested and it is not known if cells react in any way to a change in the RNA pol II elongation rate when it happens.

In this study, we experimentally show that *in vivo* RNA pol II in yeast elongates at different speeds depending on external factors, such as temperature or NTP concentration. The change in RNA pol II density is mainly reciprocal to that in speed, and the TR does not alter. Nonetheless, we also observed minor changes in the TR within the optimal yeast growth temperature range (26–37 °C), which are brought about by an alteration in the initiation rate, mainly in response to changes in the total cell amount of RNA pol II.

2. Materials & methods

2.1. Yeast strains, media and growth conditions

For most experiments, we used the *Saccharomyces cerevisiae* THJ2400 strain (*MATa*, *pGAL1-YLR454W*::*URA3*, *ura3Δ*, genetic background S288C), which was grown in liquid YPD medium (1% yeast extract, 2% peptone, 2% glucose), YPGal (1% yeast extract, 2% peptone, 2% galactose) or synthetic complete media lacking uracil and containing galactose as the carbon source (SCGal 0.17% Yeast Nitrogen Base, amino acid complement lacking uracil, 0.5% ammonium sulfate, 2% galactose). To test the effect of 6-azauracil (6AU) on RNA pol II density, we utilized an *imd2Δ* strain (BY4741 *imd2*::*HphMX4*) by growing cells at 30 °C in SC-URA to O.D.$_{600}$ ~0.5. At that point, 6AU (Sigma) was added to the culture at a final concentration of 50 μg/mL and was incubated for 30 min. In all cases, cells grew for at least seven generations in the correspondent growth condition before performing the assay.

For cell growth rate determination, cells were grown in 250 mL flasks in 100 mL of liquid YPD medium at the corresponding temperatures from O.D.$_{600}$ ~0.1 to the mid-late log phase. The growth rate was calculated as the inverse of the slope of the line of best fit obtained when representing the $\log_2$ value of the measured O.D. against time.

Table 1
Primer sequences for the quantitative PCR analysis.

GAL1-YLR454W
+3/+66
GY0-A: 5′-GATGTTTCCGATTAATGTTCTACTGTACAA-3′
GY0-B: 5′-GCTCCATAAGAAAGTCACTGCAAA-3′
+308/+370
GY0.3-A: 5′-AAGATAATGGTGGGGGCTTC-3′
GY0.3-B: 5′-CCCCATTGAGCCAGTATTGT-3′
+1903/+1963
GY2-A: 5′-AGACAGAAGGAAATTTTACCAAGCG-3′
GY2-B: 5′-AATCGAAAAAATCAGGTAGTTGCTG-3′
+3991/+4058
GY4-A: 5′-GATATGCTTCAATCCGACAGAGAG-3′
GY4-B: 5′-TCAACAGTTACCGATGGTATTAAAGG-3′
+5802/+5871
GY6-A: 5′-AGCCGGACAAACAGAACAGC-3′
GY6-B: 5′-CAGGGTCTTTTTGGTGTTTTTCA-3′
+7621/+7692
GY8-A: 5′-GGCAAAGGAAAGATGAGATTGG-3′
GY8-B: 5′-GTTGGACAATCTTAAAGTCGGGA-3′
ORF-Free Chr 5
Positions 9754/9837 (intergenic) of chromosome V
CHR5-A: 5′-TGTTCCTTTAAGAGGTGATGGTGAT-3′
CHR5-B: 5′-GTGCGCAGTACTTGTGAAAACC-3′

2.2. RNA pol II elongation rate assays

The procedure to determine the RNA pol II elongation rate was adapted from Mason and Struhl [17]. Cells were grown in the specified galactose media until O.D.$_{600}$ ~0.5 at the corresponding temperature. Upon glucose addition, 50 mL of culture aliquots were harvested at the required time points. After collection, cells were treated with 1% formaldehyde for 15 min at room temperature. Then cells were resuspended in lysis buffer (50 mM HEPES-KOH pH 7.5, 140 mM NaCl, 1 mM EDTA, 0.1% sodium deoxycolate, 1 mM PMSF, 0.15% benzamidine, 1 tablet each of 50 mL of *Protease inhibitor cocktail tablets*, Roche) and were broken with glass beads by Fastprep (MP Biomedicals). The lysate was sonicated in a Bioruptor device to yield DNA fragments with an average size of 350 bp. Immunoprecipitation was performed by incubating chromatin with an antibody (8WG16, Covance) against the C-terminal domain of the RPB1 subunit of the RNA pol II bound to magnetic beads (Dynabeads Pan Mouse IgG, Invitrogen) for 90 min with rotation. The immunoprecipitated material was eluted from the beads by incubating twice at 65 °C for 10 min in elution buffer (50 mM Tris–HCl pH 8, 10 mM EDTA, 1% SDS). To revert the crosslinking, samples were incubated in elution buffer for 15 h at 65 °C by agitation and for 90 min at 37 °C with 0.05% Proteinase K (Roche). DNA was obtained by phenol–chloroform extraction. A quantitative analysis DNA was done by qPCR using the oligonucleotides listed in Table 1. Background noise and immunoprecipitation homogeneity were tracked by a reference DNA fragment contained in the non-coding region of Chromosome 5 (ORF-Free Chr 5). Relative RNA pol II occupancy was determined by dividing the immunoprecipitation efficiency of each relevant segment of the gene by the immunoprecipitation efficiency of the reference (ORF-Free Chr 5). The relative occupation values are expressed as the ratio of the RNA pol II occupation upon glucose treatment divided by occupation in galactose. Having obtained the kinetics of the RNA pol II dissociation on each gene fragment, we calculated the linear tendencies and extrapolated the line to the X-axis to obtain the time (min) at which the last wave of RNA pol II abandoned that specific segment. The RNA pol speed was calculated as the position (kb) divided by the obtained time (min) value (see Supplementary Fig. S1).

2.3. In vitro transcription

For the average intensity normalization in GRO experiments, we performed an *in vitro* transcription of the PCR fragments amplified

from the *thr* gene of *Bacillus subtilis* using T7 RNA polymerase (Ambion) and [α-^{33}P] UTP (Perkin Elmer).

2.4. Genomic run-on (GRO) assays

The GRO protocol was adapted from [13]. In all cases, cells were grown to O.D.$_{600}$ ~0.5. Radioactively-labeled RNA was hybridized to macroarrays containing probes for the 769 ORFs of the *S. cerevisiae* genome (see Suppl. Table S1) and the 32 internal controls of the *thr B. subtilis* gene (ATCC clone # 87484) at random positions, produced in the DNA chips service S.C.S.I.E. (University of Valencia). The same amount of *in vitro* transcribed *B. subtilis* clones was added to each GRO sample. Macroarrays were exposed to an Imaging plate (BAS-MP, Fujifilm) and were scanned in a Fujifilm FLA3000 Phosphorimager. The signal intensity of the spots was quantified with the Array Vision™ image analysis software. sARM values (background-corrected Artifact Removed Median density) were used for the analysis and were normalized by the average intensity value of the internal controls to avoid technical variation. The RNA pol II total density value, under all the tested conditions, was calculated as the median of the intensities of all the genes analyzed.

2.5. Western blot

Cells were grown in synthetic medium to O.D.$_{600}$ ~0.5 at their respective temperatures in biological triplicates and were counted in a Coulter-counter device. To prepare protein extracts, cultures were harvested by centrifugation and resuspended in lysis buffer (50 mM Tris–Base, 150 mM NaCl, 5 mM MgCl2, 1% NP40, pH = 7.5) containing phosphatase inhibitors (1 mM Na-orthorvanadate, 60 mM β-glycerol phosphate, 50 mM NaF) and Complete Mini Protease Inhibitor Cocktail Tablets (Roche). Cells were broken with glass beads by FastPrep. Protein concentration was determined by the Bradford Assay (Bio-Rad). Then 1 μg of total protein was redissolved on a 7% SDS-PAGE gel (7% acrylamide, 0.1% Bis-Acrylamide, 0.2% SDS, 0.75 M Tris–Base) and was transferred onto a nitrocellulose membrane (GE Healthcare) in transfer buffer (25 mM Tris–Base, 192 mM Glycine, 0.1% SDS, 20% Methanol). The total RNA polymerase II protein expression was detected using the 8WG16 antibody, while phosphorylated RNA polymerase II was detected using the *anti-RNA polymerase II CTD repeat YSPTSPS* (*phospho S2*) *antibody [H5]* (Abcam 24758). This antibody, however, has been described to recognize both Ser2 and Ser5 phosphorylations [18]. Therefore, we assume that it quantifies all forms of elongating RNA pol II. The Pgk1 protein expression was detected as an endogenous control using the anti-Pgk1 antibody (Invitrogen). The anti-mouse HRP-conjugated secondary antibody was purchased from Promega. Immunoblots were developed using the ECL Prime kit (GE Healthcare). Band intensities were analyzed with the Image Quant TL program (GE Healthcare).

3. Results

3.1. The RNA pol II elongation rate varies with the growth temperature

Enzymatic reactions increase velocity with temperature according to the Arrhenius dependence of the catalytic constant [19]. For RNA synthesis, this dependence affects the individual catalytic steps of nucleotide incorporation, but it should not necessarily be converted into a quantitative identical effect on the elongation rate. To test if this was actually the case for yeast RNA pol II, we used the assay developed by Mason and Struhl [17]. In this assay, the last elongation wave of RNA pol II after the shut-off of the *GAL1* promoter was followed by qPCR over time using different primer sets along the 8 kb-long *YLR454W* gene to determine by extrapolation the time that RNA pol molecules require to elongate from the promoter to the primer pair (see Fig. S1). Although, in principle, only one primer pair was necessary to measure the elongation rate, we used four pairs placed at 2, 4, 6 and 8 kb from the TSS. This allowed us to obtain a more robust result by averaging the RNA pol speed calculated from several independent reference points.

For the experiment that determines how temperature influences the elongation rate, we utilized two different growth conditions: rich and synthetic media. The results for three independent experiments were repetitive and similar in both growth media. As expected, Fig. 1A depicts that the RNA pol II speed increased linearly with growth temperature from 0.67 to 1.31 kb/min between 23 °C and 37 °C. This increase (about 0.5 kb/min every 10 °C) perfectly fitted the Arrhenius equation (Fig. 1B), which suggests that it is mostly the result of the effect of temperature on overcoming a fixed activation energy step.

3.2. RNA pol II density inversely correlates with RNA pol II speed

A change in the elongation rate should be concomitantly seen as a change in RNA pol II density (i.e., not altering the TR) if the rate of initiation and the drop-off index are constant. Therefore, we decided to check whether these premises are fulfilled exactly in yeast. A plot of the time zero ChIP values from the *YLR454W* experiment *vs.* temperature shows that RNA pol II density decreased with temperature (Fig. 2A). Since the particular fusion gene used can have specific effects on transcription initiation (i.e., a temperature effect on the *GAL1* promoter [20]), we decided to use the genome-wide techniques to avoid any bias in the density calculations.

To do this, we performed a genomic run-on experiment [13] to measure the total elongating RNA pol II density in the ORF-containing genes. This approach offers the advantage of avoiding having to count RNA pol

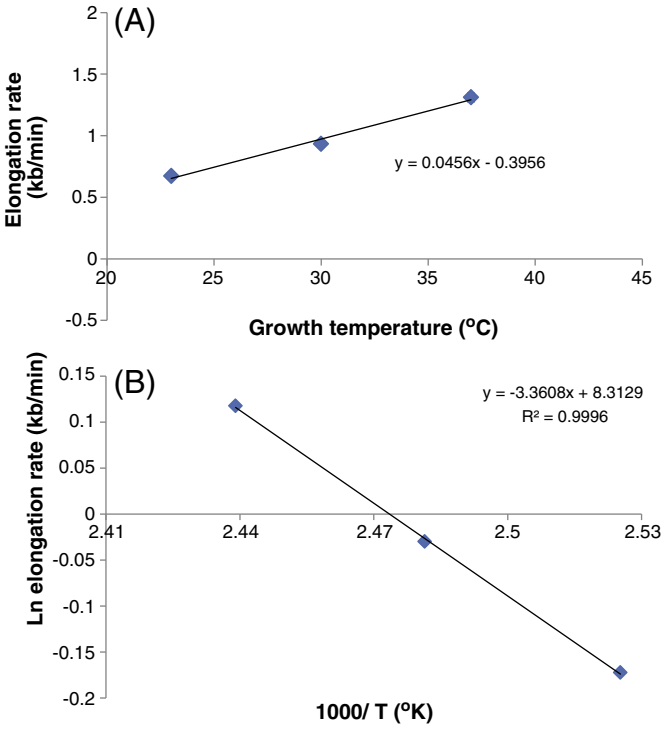

Fig. 1. Analysis of the RNA pol II elongation rate at different growth temperatures. A) RNA pol II elongation rate (V) was measured using the *GAL-YLR454W* construction [17] at three different growth temperatures. Elongation speed was calculated as distance/time, as explained in Fig. S1. Data represent the average of three different replicates of the experiment that were done in different growth media: two in YPGal and one in SCGal. The standard deviation (SD) for the replicates is provided. In each experiment, the elongation rate was obtained using four different probes at 2, 4, 6 and 8 kb (see the oligonucleotides in Table 1) and the results were averaged. B) Graphical fitting of data to the Arrhenius equation ($\ln V = \ln A - E_a/RT$, ref. [45]). After that representation, the activation energy (E_a) of 6.6 kcal/mol is obtained.

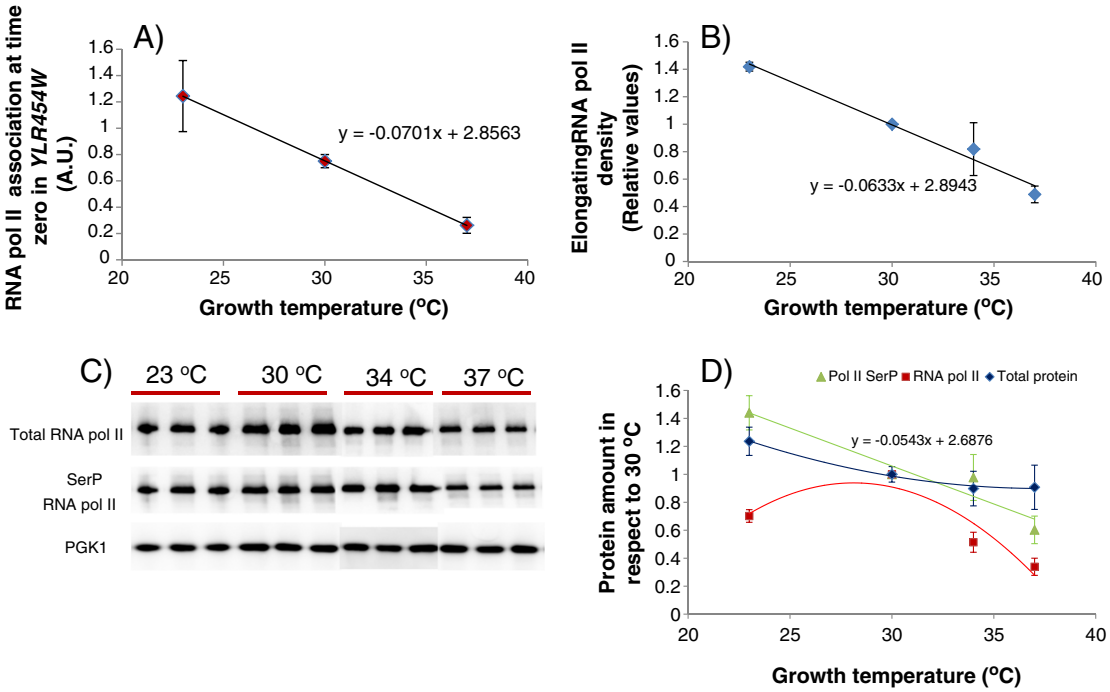

Fig. 2. Analysis of RNA pol II density and abundance according to growth temperature. A) The experimental data from the same experiments in Fig. 1 were used to obtain the relative values of the presence of RNA pol II along the *YLR454W* gene at time zero to represent a measure of RNA pol II density before transcription shut-off. For this purpose, the values of occupancy at all four positions (2, 4, 6 and 8 kb, see Fig. S1) were averaged to obtain one total RNA pol II occupation value per growth temperature. SD bars represent the deviation of the four probe positions. B) RNA pol II density was calculated by run-on incorporation using macroarrays containing 769 *S. cerevisiae* gene probes (see Table S1), as described in M & M. The average values of 2–3 experiments at each growth temperature and their SD are shown. Values are relative to that of 30 °C, which was arbitrarily taken as 1. C) Western blot analysis of the RNA pol II amounts according to growth temperature. Western blots of three independent biological replicates from each growth temperature were probed with the 8WG16 (total RNA pol II), H5 (phosphorylated Ser2 + Ser5) and anti-Pgk1 antibodies. All the lines correspond to the same Western blots, but the individual three-sample repeats were cut and pasted to better show the comparisons. D) Analysis of the total protein and of phosphorylated and total RNA pol II amounts at different temperatures. The total amount of protein was determined by the Bradford assay for the same number of cells. Quantification of phosphorylated RNA pol II (green triangles) and total RNA pol II amount (blue diamonds) from the experiments in Fig. 2C, normalized by the Pgk1 signal and by the total protein/cell (red squares). The results in the graph are displayed as relative values in respect to 30 °C, which was taken arbitrarily as 1. Three biological replicates were measured in technical triplicates. Values are expressed as mean ± SD.

I and RNA pol III contribution, which account for >70% of yeast cell transcription [21]. As run-on counts all the elongating RNA pol molecule in the regions covered by probes, and since our macroarrays contain whole-length ORF sequences for almost 800 genes of the *S. cerevisiae* genome, the sum of all their signals is a good estimator of the total RNA pol II average density in canonical genes.

Fig. 2B and Table 2 illustrate how the median run-on signal drastically dropped from 23 °C to 37 °C over the set of canonical RNA pol II genes. This result was then confirmed by evaluating the amount of elongating RNA pol II by Western blot. The quantification of phosphorylated polymerase using the H5 monoclonal antibody confirmed a severe decrease of elongating RNA pol II from 23 °C to 37 °C (Fig. 2C & D, Table 2). It is interesting to note that the drop in density did not exactly compensate the increase in speed with temperature, which caused a bell-shaped TR curve (Fig. 3). As the growth rate can impose different constraints to yeast physiology, including variations in the TR, we checked the exact growth rates for our particular *S. cerevisiae* strain at several common laboratory temperatures ranging from 18 °C to 37 °C. Fig. S2 shows that the growth rate did not significantly differ within the 26–37 °C range, whereas it was 70% slower at 23 °C than it was at 30 °C. These results are similar to those obtained by other authors for the BY4741 yeast strain [22]. In that case however, the growth rate at 37 °C was slightly better than it was at 30 °C. The behavior of the TR at temperatures under 26 °C can be influenced by major changes in growth rates, which precludes a quantitative comparison. Finally, although the observed growth rates were similar between 30 °C and 37 °C, the TR slightly lowered (by around 25%) at 37 °C (Fig. 3).

To further verify the inverse relationship of RNA pol II speed and density, we decided to investigate another cause of RNA pol II speed reduction: the effect of the [NTP] depletion caused by the 6-azauracil (6AU)

Table 2
The relative global RNA pol II density data from Fig. 2B & D are displayed and their averages are shown in the last column.

Temperature	SerP	SD	Run-on	SD	Av.
23 °C	1.46	0.12	1.42	0.03	1.44
30 °C	1.00	0.00	1.00	0.00	1.00
34 °C	0.98	0.16	0.82	0.19	0.90
37 °C	0.60	0.10	0.49	0.06	0.55

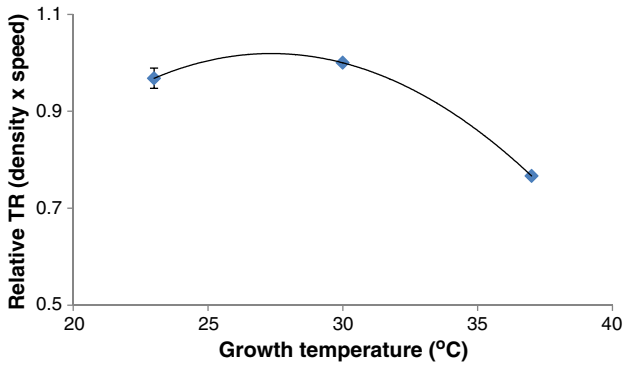

Fig. 3. The RNA pol II transcription rate according to growth temperature. The elongation rate (Fig. 1A) and density (Table 2 average) data were multiplied to obtain a relative TR at three different growth temperatures. SD bars are shown.

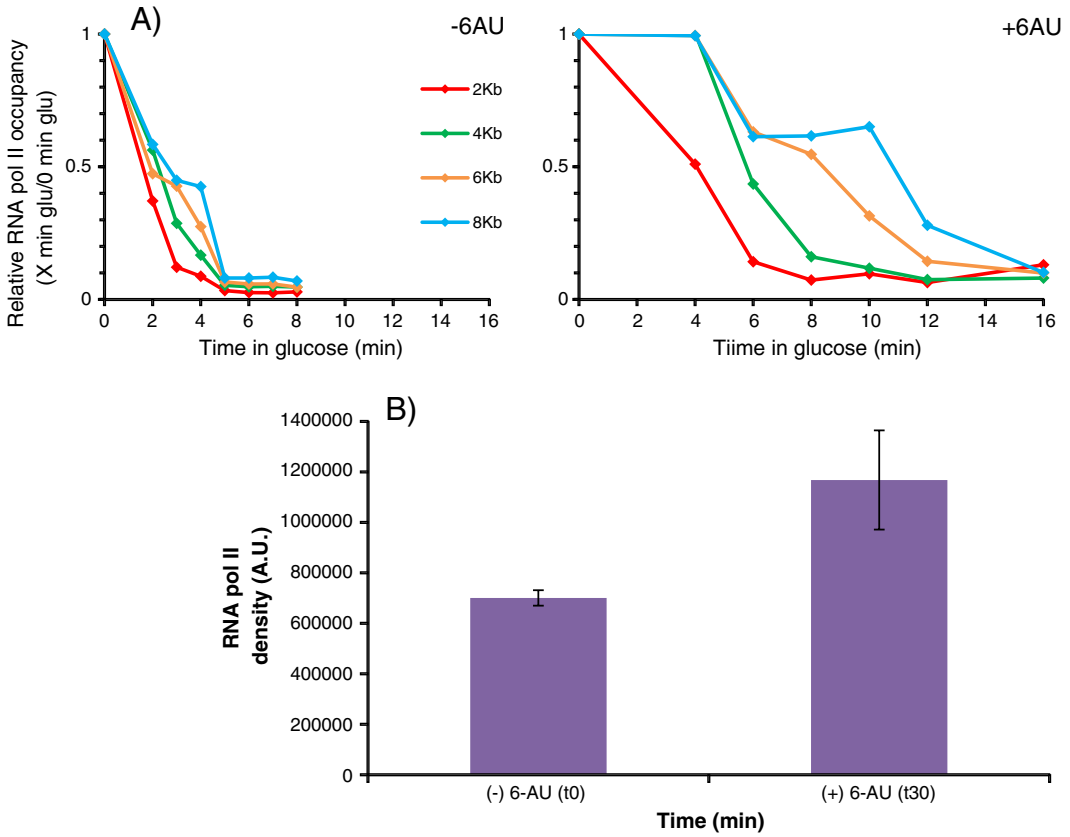

Fig. 4. The RNA pol II elongation rate and density in the presence of 6AU in wt and *imd2* cells. A) The RNA pol II elongation rate was measured using the *GAL-YLR454W* construction [17] in a wt strain before (−6AU, left panel) and after (+6AU, right panel) the addition of the nucleotide-depleting drug 6AU (50 μg/mL). Values are normalized to the time zero value for each probe. Decay curves were obtained from the cells growing in SC–URA at 30 °C after changing from galactose to glucose medium. Elongation rates (1 kb/min and 0.4 kb/min, respectively) were calculated from the decay curves as distance/time, as described in Fig. S1, and were averaged for all the probes to obtain a final speed value for each growth condition. B) Histogram showing the total RNA pol II density calculated by run-on (GRO assay) in an *imd2Δ* strain growing at 30 °C in the absence of (left), or 30 min after the addition of, 50 μg/mL 6AU (right). The SD bars from three replicates are shown.

drug. In our case, we noted that the results were similar to those previously published [17]. As seen in Fig. 4A, we found that 50 μg/mL of 6AU led to a reduction in RNA pol II speed in a wt strain after a 30-minute treatment, and this reduction was estimated to be about 60%. Next we determined by run-on the RNA pol II density in the cells in which a decrease in the NTP pool caused the elongation rate to lower (Fig. 4B). The experiment was done in an *imd2* mutant strain to avoid the partial recovery of NTP pools, which occurs in a wild-type strain due to the induction of the *IMD2* gene during nucleotide depletion [23,24]. We used the same 6AU concentration as in the experiment of Fig. 4A. This concentration allows *imd2* strain growth, be it at a lower rate than in its absence, and it provokes constant [NTP] depletion. Fig. 4B depicts how density increased by 40% after 30 min in 6AU as compared to time 0 in an *imd2* mutant. Taken together, the fact that the addition of 6AU lowers the RNA pol II elongation rate (Fig. 4A) and increases Pol II density (Fig. 4B) supports the idea that the RNA pol II elongation rate is reflected by its density. This means that, given the possible variations in RNA pol II speed, the TR cannot always be compared between two samples if it has been estimated directly from RNA pol density values.

Another factor that might influence RNA pol II density is the drop-off, which might vary with temperature and cause differences in the density observed. We checked this hypothesis by looking at the distribution of the RNA pol II molecules along the *YLR454W* gene for each growth temperature. Figs. 5 and S3 reveal that the distribution along

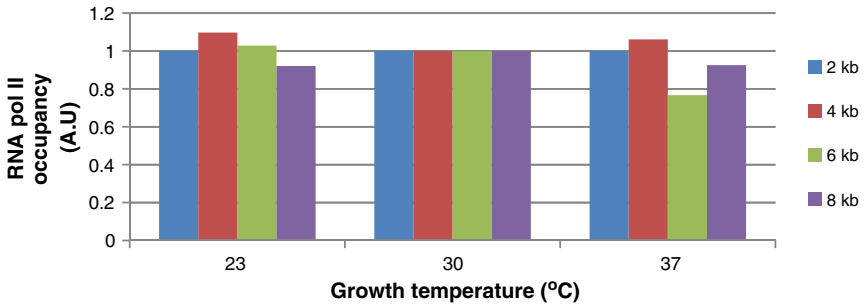

Fig. 5. Analysis of the RNA pol II distribution on the *YLR454W* gene according to growth temperature. The same experimental data as in Fig. 1A were used to obtain the RNA pol II relative distribution along the *YLR454W* gene (probes 2, 4, 6 and 8 kb; see Fig. S1) by taking the values of 30 °C as 1. Fig. S3 is a representation of these data without the normalization by the 30 °C values.

the coding region, from 2 to 8 kb probes, decreased from 5′ to 3′ for all three temperatures, which was expected given previously published results [17]. This decrease, however, did not reveal a significantly different distribution for 23 °C or 37 °C if compared to 30 °C.

As mentioned before, density directly depends on the rates of initiation events and abortive elongation that cause RNA pol II to drop-off [4]. Since we have demonstrated that drop-off is not variable in our particular case, the 25% drop in the TR observed at higher temperatures (Fig. 3) suggests that the cause is a decrease in transcription initiation events. This led us to investigate if this reduction is caused by a drop in the total amount of RNA pol II or only in the proportion of that involved in elongation.

3.3. RNA pol II amount varies with growth temperature

To further investigate the cause of the decreased RNA pol II density at high temperatures, we measured by Western blot the RNA pol II amount per cell in the cells grown at different temperatures using an antibody against total RNA pol II. Fig. 2C and D shows that the amount of total RNA pol II/cell lowered from 30 °C to 37 °C, similarly to elongating RNA pol II density. It is interesting to note that this reduction was specific for RNA pol II because total protein/cell did not reduce (Fig. 2D). Conversely at 23 °C, the amount of RNA pol II was lower than at 30 °C. As we have previously shown, both run-on and H5 antibody data, which quantifies only actively elongating polymerase, indicate that the amount of elongating RNA pol II continuously diminished with an increase in temperature (Fig. 2B–D). Therefore, yeast cells seem to adjust the total RNA pol II amounts per cell to accomplish an appropriate initiation rate on gene promoters within the range of the similar growth rates tested in these experiments (30–37 °C). At lower temperatures and growth rates (23 °C, Fig. S2), the level of RNA pol II/cell did not increase further. In this case, it seems that the proportion between elongating and total RNA pol II molecules increases.

4. Discussion

The TR is a fundamental parameter of every living cell because the synthesis of all its biomolecules relies directly or indirectly on the expression of its genetic information. The rate at which genes are transcribed is the first, and probably one of the most important, regulated steps along the flux of genetic information. RNA polymerases are, therefore, highly regulated machines in both prokaryotes and eukaryotes.

In recent years, several different techniques, mostly genome-wide, have been developed to measure the TR in both yeast and higher eukaryotes (revised in [12]). Many calculate RNA pol II density by analyzing either the DNA sequence where it is placed onto (chromatin immunoprecipitation, ChIP, i.e., [25,26]) or the nascent RNA sequence being transcribed (genomic run-on, GRO; i.e. [13,15,27]). In this kind of studies, the TR is defined, as the number of mRNA molecules synthesized by time units (molecules or moles/min). This TR, defined in molecular terms instead of kinetic terms, can be called the "nascent TR" [12].

When employing high resolution techniques for nucleic sequence analyses, such as next generation sequencing or tiling arrays, the RNA pol II distribution inside genes can be determined. This distribution depends on the elongation rate and the drop-off of these processive molecules. Drop-off has been calculated to be, on average for the whole genome, about 20% of each kb in *S. cerevisiae* wt cells [8], and to have slightly higher values (up to 10% more) for elongation mutants or during NTP depletion [8,17,28]. The transcription abortion caused by the drop-off of RNA pol is presumptively preceded by a pause. This pause has been shown to be independent of temperature within the 21–37 °C range *in vitro* in prokaryotic RNA pol [11,29]. Thus, it seems that the drop-off rate would also be constant between those temperatures. There is no study available on the possible changes in the drop-off proportion regarding the temperature *in vivo* in any eukaryotic RNA pol. Nonetheless, here we see that the RNA pol II distribution onto the *YLR454W* gene shows no major differences between 23 °C and 37 °C (Fig. 5, which suggests similar drop-off indexes).

The RNA pol II distribution along the gene is also inversely related to the elongation rate [30], just as the density of cars running on a highway is inversely proportional to their average speed [31]. The elongation rate is a feature of every single RNA pol. Moreover, it has been shown to be variable along and among genes in human cells [9]. However, it can be averaged for the whole length of the "gene highway" that it travels along with single molecule techniques (see refs. [6,7]) or, more commonly, for the whole set of RNA pol molecules that transcribe many copies of the same gene in a cell population over a given time lapse [32–34].

In many instances, RNA pol II density has been interpreted as a measure of the nTR by assuming an average constant elongation rate. In order to be rigorous, this assumption needs the experimental verification of absence of variation in the elongation rate or in the drop-off rate between the compared samples. In fact, the changes in RNA pol II speed have been calculated *in vivo* or *in vitro* in very few cases [17,35]. Most of the studies conducted in yeast have been done at 28–30 °C. In a previous work [16], we used a correction of the elongation rate in the nTR based on the Arrhenius equation during a heat-shock response at 37 °C; however, it was not experimentally verified. Here we confirm that our prediction was correct, and we show that the current protocols used to evaluate the TR are all based on the determination of RNA pol densities [12] and require an independent estimation of the existence, or not, of the external factors that induce changes in the elongation rate.

In *E. coli*, it has been shown that RNA pol elongates at a maximum speed of 80 to 90 nucleotides (nt) per s in rRNA operons [36] and at a speed of 45 nt/s for mRNA operons at 37 °C [37]. The speed of RNA pol II in eukaryotes is believed to be slower, 25 nt/s on average, but varies considerably (13–100 nt/s), which could be either a real biological property or caused by the experimental method to determine it [5]. In yeast growing at 28–30 °C, measuring the last wave of RNA pol II after blocking the transcription [17] determined an average of 18–25 nt/s (1–1.5 kb/min), which is similar to that established by single molecule live tracking techniques [6,7].

In this study, we used two external factors that should presumptively affect the elongation rate and demonstrate that RNA pol II is affected by them at exactly the magnitude predicted by the Arrhenius equation (temperature) or within expectations (NTP depletion). Published RNA pol elongation models [10,38] have predicted its kinetic behavior based on a general ratchet model for processive enzymes. Each individual step depends on thermal fluctuations and [NTP]. In fact in single molecule studies, RNA pol from *E. coli* has been reported to increase the elongation rate *in vitro* with a temperature fitting the Arrhenius equation, including pauses, between 21 °C and 37 °C [11,29]. From those results, it can be anticipated that the RNA pol II elongation rate will increase with temperature. However, this had not been demonstrated in eukaryotic cells *in vivo* to date, that is, not until this study.

A yeast cell growing under optimal conditions (i.e., 30 °C in YPD) probably performs transcription at its best. If temperature changes, but the growth rate does not alter (i.e., at 37 °C), at first sight, one might expect the TR to remain constant. This can be achieved by keeping the initiation rate constant. In this way, the average number of elongating RNA pol IIs will decrease in exactly the same proportion in which their elongation rate increases because they spend less time in the elongation phase.

To check this hypothesis, we used a genomic run-on [13] to measure the total RNA pol II density in ORF-containing genes. We discovered that reduced RNA pol II density compensated the increased elongation rate. However the drop in density was slightly higher (25%) than required at 37 °C, which means that the initiation rate lowered (Fig. 3). Since the elongation rate passively responded to kinetics rules (Fig. 1), and as no significant change in the drop-off index was noted (Fig. 5), it can be concluded that the main control of the TR response to temperature took place at the initiation level and not at the elongation level, as

expected (see the Discussion in [30,39,40]). This conclusion is based on the comparison of genomic run-on data with single-gene data for elongation rate and drop-off. Accordingly, we cannot exclude that specific subsets of genes control their TR response to temperature at post-initiation steps. In this regard, we have previously shown that some yeast regulons, like those encompassing ribosome-related and mitochondrial-related genes, can be regulated at the level of RNA pol II activity during elongation [41,42].

What is the mechanism that a eukaryotic cell uses to globally control the initiation rate? The objective to increase/decrease the number of initiating RNA pol II molecules can be accomplished by changing the total amount of RNA pol II in the cell or by acting in any (or several) successive steps from the formation of closed PIC to the productive elongation by phosphorylated RNA pol II [3,4]. In *E. coli* cells, the RNA polymerase concentration increases with a higher growth rate [31] and the percentage of transcription-engaged RNA pol molecules also rises [37]. Therefore, this prokaryote controls the TR, as far as its growth requirements are concerned, by increasing both the total RNA pol available and the fraction of it devoted to transcribe, be it with differences between the rRNA and mRNA operons [31]. In this case, the increase in the growth rate is studied at a constant temperature, but the elongation rate in *in vitro* studies increases with temperature by the same relative amounts as the general growth rates [11]. Here we demonstrate in yeast that the increase in the growth rate from 23 °C to 30 °C involved an increment in the total amount (Figs. 2C & D), but not in the *in vivo* elongating RNA pol II (Fig. 2B–D & Table 2). Furthermore from 30 °C to 37 °C, we observed that the total RNA pol II amount diminished per cell (Fig. 2D diamonds) and, probably as a result of this, the phosphorylated RNA pol II lowered (Fig. 2D squares). This reduction in elongating pol II may respond to the principle of reducing the number of costly machineries, which become faster due to the additional energy supply at higher temperatures, as previously seen with ribosomes [43,44].

5. Conclusions

We have found an *in vivo* dependence of the RNA pol II elongation rate and density on transcribed genes with the growth temperature in yeast cells. The variation of elongation rate and density with temperature is reciprocal, as expected [30]. Accordingly, RNA pol II density cannot be directly used to compare TRs without verifying constant elongation rates. Our results also imply that global variations in the TR seem to be mainly due to the control at the transcription initiation level. Within the usual growth temperature range in *S. cerevisiae*, initiation of transcription is, in turn, controlled by adjusting the total amount of RNA pol II molecules present in the cell.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.bbagrm.2013.09.008.

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