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Supplemental Data

Transcription Elongation by RNA Polymerase I Is Linked to Efficient rRNA Processing and Ribosome Assembly

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Supplemental Experimental Procedures

Mutant Isolation

RPA135 was mutagenized by amplification with Taq DNA polymerase using standard conditions for PCR. The resulting DNA fragment was co-transformed into NOY975 with plasmid pNOY742 from which most of the WT *RPA135* coding region was excised by digestion with *Bsp*EI and *Nru*I (resulting in 165 bp of overlap between 5' end of the fragment and the plasmid encoded gene and 298 bp of overlap at the 3' end). Recombination between the plasmid and fragment *in vivo* resulted in galactose independent growth due to restored expression of A135 variants. Colonies that grew on glucose (SD –Ura) were then screened for growth on SD –Ura +250 μg/ml 6AU. Colonies that grew substantially slower in the presence of the drug than those containing a WT A135 control plasmid were picked, plasmid was prepared, and retransformed into NOY975 to confirm the defect. The mutation was identified by sequencing both strands of the *RPA135* gene using standard methods.

Analysis of Steady-State Amounts of Total and Individual RNA Species Separated by Gel Electrophoresis

Since both the WT (NOY388) and *rpa135* (NOY2172) strains require uracil for growth, all cellular RNA can be uniformly labeled by growing cells in SD-Ura supplemented with [^{14}C]uracil. Two cultures, WT and *rpa135*, which were growing in SD-Ura containing uracil (5 µg/ml) exponentially were diluted with the SD-Ura medium supplemented with [^{14}C]uracil (5 µg/ml; 1 µCi/ml). Dilution was sufficiently high so that after overnight growth at 30˚C cell density (A_{600}) reached 0.05 to 0.1 at which time measurement of ^{14}C-labeled RNA was begun. Thus, WT and mutant cells were maintained ~11 and ~7 generations in exponentially growing states, respectively, i.e., cells were uniformly labeled with [^{14}C]uracil. Both cell density (A_{600}) and the amounts of total RNA in the two cultures were measured at every hour until cell density reached A_{600} = ~0.4. Total RNA was measured by taking triplicate samples (50 µl) and counting the amounts of ^{14}C in RNA precipitated with ice-cold 10% TCA. The ratio of [^{14}C]RNA to A_{600} remained constant as expected and the average values were used to compare "total RNA/A_{600}" between the *rpa135* mutant and WT cells in exponentially growing state. The doubling times were 80 min for WT and 145 min for the mutant.

In the above experiments, cells were collected by centifugation at the end of total RNA measurements (A_{600} = 0.3 to 0.4), and total RNA was isolated by a standard method as described previously (Elder et al., 1983; Nogi et al., 1991). To analyze 25S, 18S rRNA separately from the small RNA fraction (5.8S rRNA, 5S RNA and tRNA), RNA samples containing equal amounts of ^{14}C-radioactivity were subjected to electrophoresis on formaldehyde-agarose gels followed by transfer of RNA to GeneScreen Plus nylon

membrane according to manufacturer's recommendations (NEN Research, Boston, MA) (see Fig. 3B, lanes 1 and 2). Radioactive RNA bands were detected by autoradiography, and the amount of radioactivity in bands corresponding to 25S rRNA, 18S rRNA and the mixture of 5.8S, 5S and tRNA were measured by a scintillation counter after cutting out pertinent membrane regions. Since the separation of 5.8S, 5S and tRNA is not complete by this formaldehyde-agarose gel system, the same RNA preparations were also analyzed by electrophoresis on 2% polyacrylamide / 0.5% agarose composite gels as described previously (Peacock and Dingman, 1968; Nogi et al., 1991). The gels were dried on filter paper and positions of radioactive 5.8S, 5S and tRNA (as well as larger rRNAs) were identified by autoradiography (see Fig. 3B, lanes 3 and 4). Bands corresponding to these individual RNAs were cut and the amounts of ^{14}C-radioactivity were measured in 0.1 N NaOH which ensures elution of RNA from the filter paper (our unpublished experiments). The ratios of the amount of separated rRNA species, e.g. for 18S rRNA ("18S"), per cell mass (A_{600}) in the *rpa135* mutant cells to those in the WT cells given in Table 3B were calculated from the observed ratio (*rpa135* / WT = 0.82) obtained from the gel analysis in the following way: $(18S / A_{600})_{rpa135} / (18S / A_{600})_{WT} = [(18S / \text{total RNA}) \times (\text{total RNA} / A_{600})]_{rpa135} / [(18S / \text{total RNA}) \times (\text{total RNA} / A_{600})]_{WT} = [(18S / \text{total RNA})_{rpa135} / (18S / \text{total RNA})_{WT}] \times [(\text{total RNA} / A_{600})_{rpa135} / (\text{total RNA} / A_{600})_{WT}] = 0.82 \times 0.64 = 0.52$.

"Pol I RNA" in Fig. 3B is the sum of the amount of 25S, 18S and 5.8S rRNAs. Since cells were maintained in a steady state, accumulation rate of individual RNA species can be calculated from the steady state amounts of individual RNA species and growth rate. For example, the ratio of the accumulation rate of Pol I RNA for the mutant

to that for WT given in Fig. 3B was calculated as follows: [Accumulation rate of Pol I RNA]$_{rpa135}$ / [Accumulation rate of Pol I RNA]$_{WT}$ = [(Pol I RNA/A_{600}) x Growth rate]$_{rpa135}$ / [(Pol I RNA/A_{600}) x Growth rate]$_{WT}$ = [(Pol I RNA/A_{600})$_{rpa135}$ / (Pol I RNA/A_{600})$_{WT}$] x [(Growth rate)$_{rpa135}$ / (Growth rate)$_{WT}$] = 0.46 x 0.55 = 0.25.

The values given in Table 3B were averages of values obtained from two independent [^{14}C]uracil labeling experiments as described above. All the values obtained agreed within the range of 7%. We note that all the values are normalized to cell density (A_{600}) as shown in Table 3B. We measured number of cells per cell density and found that one A_{600} unit of cells corresponds to 3.6 ± 0.5 x 10^7 for both the *rpa135* mutant and WT cells (in seven independent measurements). Therefore, the parameters given in Table 3 as per A_{600} can be considered to be approximately the same as those per cell.

Elongation Assay for Pol I

The template for this analysis was prepared by PCR amplification of the promoter region (-247 to +763 relative to transcription start site) from a variant of pNOY373 (Wai et al., 2000) where the 6 CTP residues between +1 and +56 were mutated to GTP (now called pNOY746). The resulting PCR product was extracted from an agarose gel, purified and used at a final concentration of 2 μg/ml.

Purified Pol I was pre-incubated with recombinant Rrn3, and then preinitiation complexes were formed *in vitro* with purified UAF, CF, and TBP. Conditions were identical to those used for standard multi-round transcription (Keener et al., 1998) except for the different template. Instead of a final volume of 20 μl per reaction, large 440 μL reactions were prepared for WT and the *rpa135 (D784G)* mutant Pol I preparations. After

incubation for 5 minutes at room temperature (20° C) a mixture of 3 nucleotides (200 μM final concentration of ATP and UTP, and 30 μM final concentration of GTP) was added, including 220 μCi [α^{32}P]GTP. This allowed transcription initiation and elongation to +56, where the first CTP would be encountered. After 5 minutes, heparin (50 μg/ml) was added to prevent reinitiation of transcription and to disrupt remaining preinitiation complexes (data not shown). At time zero, CTP was added to 200 μM final concentration and 20 μl samples were collected and added to 50 μl 1:1 phenol:chloroform to stop transcription at multiple time points for 20 minutes.

RNA samples were processed as described (Keener et al., 1998) and the 763 nt runoff transcript was analyzed and quantified by polyacrylamide gel electrophoresis and phosphorimaging (BioRad Personal FX, Hercules, CA).

Oligonucleotides Used in This Study

Oligonucleotides used as primers in RT-PCR and Northern analyses are listed in Table S1.

Supplemental References

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Table S1. Oligonucleotides Used in This Study

Name	Direction	Position*	Location	Sequence (5’ – 3’)
a	reverse	+151	5’ ETS	ACACGCTGTATAGAGACTAGGCA
b	reverse	+702	A_0 - A_1	TAACTATCTTAAAAGAAGAAGCAACAAGC
c	reverse	+2675	D - A_2	GCACAGAAATCTCTCACCGT
d	reverse	+2845	A_2 - A_3	TCCAGTTACGAAAATTCTTG
e	reverse	+3235	C_2 - C_1	GCCTAGACGCTCTCTTCTTA
P+674	forward	+674	A_0 - A_1	TGCCTAGTCTCTATACAGCGTGT
oligo $(dT)_{16}$-adapter	reverse -			GACTCGAGTCGACATCGAT-$(T)_{16}$
adapter	reverse -			GACTCGAGTCGACATCGAT
MLO42	reverse		U14	AGACATCCTAGGAAGGTCTCTAAAGAAGAG
MLO44	reverse		U3	ACTTGTCAGACTGCCATTTGTACCCACCCA

* relative to the 5’ end of the 35S pre-rRNA