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Review of the Ewa Leśniewska PhD thesis titled "Novel layers of tRNA transcription regulation – from assembly of polymerase RNA III complex to its degradation"

The PhD thesis I have reviewed describes original experiments performed by Ewa Leśniewska in the Department of Genetics at the Institute of Biochemistry and Biophysics Polish Academy of Sciences in Warsaw, Poland. In this very interesting dissertation Ewa Leśniewska presents the results of her original investigations of different mechanisms that are responsible for regulation of RNA polymerase III activity in yeast. Professor Magdalena Boguta serves as a director of the Ewa Leśniewska PhD thesis. The research conducted by Ewa in the frame of her PhD studies has a very high quality, and the results are interesting and stimulating for the broad scientific community. I confirmed that the subject of Ewa Leśniewska's PhD studies, the results discussed in her PhD thesis, as well as the way of their presentation meets all official requirements of a PhD dissertation.

Pol III, one of three main eukaryotic polymerases, synthesizes tRNA, 5S rRNA and

other small non-coding RNAs. A characteristic feature of the genes transcribed by Pol III is the presence of internal promoter elements within the region encoded a mature RNA molecule and a relatively simple transcription termination sequence which consists of a short stretch of Ts at the end of the Pol III transcribed genes. As all eukaryotic DNA-dependent RNA polymerases, Pol III is a big enzyme composed of 17 subunits, some of them are shared with two other polymerases, Pol I and Pol II. As a multi-subunit complex, Pol III requires precise assembly which takes place mostly in the cytoplasm. The polymerase complex is then transported from the cytoplasm to the nucleus in assistance of special adaptors and so called assembly factors which dissociate from the enzyme in the nucleus and are exported back to the cytoplasm. Transcription of tRNA genes has to be controlled at different cell growth conditions. This regulation, however, can take part at various levels of gene expression. To understand such complex regulation Ewa decided to uncover and characterize novel mechanisms that mediate activity of the yeast Pol III. Her research had four very well defined and clear goals. First, she decided to take a closer look at Pol III transcription in a wide-genome scale. To this end, Ewa applied the CRAC method, in which a tagged form of the largest Pol III subunit, C160, was used to localize Pol III along all nascent transcripts synthetized by this enzyme. Second, she asked a question about a potential RNA binding property of Rbs1, a protein involved in assembly of Pol III. Third, Ewa wanted to study an effect of nucleotide depletion on Pol III transcription. The fourth main goal of the studies performed by Ewa Leśniewska was molecular investigations of C160 Pol III subunit stability, ubiquitination and its degradation.

To globally characterize Pol III transcription in yeast the UV-crosslinking and analysis of cDNA method (CRAC) was used. The Pol III largest catalytic subunit in fusion with the tripartite tag HTP (His6/TEV protease site/protein A) was used to pull down Pol III nascent transcripts. This allowed to monitor the distribution of Pol III on the genes transcribed by the tagged enzyme. Inspection of individual tRNA transcription units revealed unexpected

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uneven Pol III density across transcripts: with a high density of reads at the 5' end of the transcription unit and a weaker peak before the 3' end of the tRNA sequence. This Pol III distribution was independent of growth conditions (glucose vs. glycerol) and the presence or absence of the yeast Pol III repressor Maf1. Based on the results, Ewa postulated that TFIIIC bound to the A and B internal promoter elements can interact with and impede transcribing Pol III, in consequence leading to transient polymerase pausing. During the PhD thesis defense I would like to ask Ewa how TFIIIC, which is a transcription factor, can inhibit Pol III activity. Is any model of such dual role of TFIIIC proposed? In addition, the CRAC data also showed transcription read-through events on many tRNA genes, typically extending 50-200 nt beyond the expected terminator. The level of such read-throughs decreased under permissive conditions, but was unaffected in the *Δmaf1* mutant. *In silico* analyses performed by the author failed to identify any correlations between the predicted structural elements in nascent transcripts and the efficiency of read-through events. Beyond tRNA genes, distribution of Pol III was also analyzed across other genes transcribed by Pol III: *5S, RPR1, SCR1, SNR6, SNR52* and *RNA170*. In many cases the profile of Pol III was similar to those described for tDNA transcripts, but some examples of different distribution of Pol III was also observed. This can be easily explain by different structures of the promoters recognized by Pol III. I found particular interesting the results showing in stress conditions the increased level of transcription of some genes normally transcribed by Pol II. During the defense I would like to know Ewa's opinion what is a potential mechanism of this unusual switch. In the dissertation Ewa Leśniewska analyzed also a function of RNA170 and showed that the *ΔRNA170* strain grows slower on YPD at low temperature. This phenotype allows to perform a suppressor screen to find functions of the RNA170 gene. Has anyone from the Magda Boguta laboratory carried out this experiment?

The second part of the Ewa Leśniewska dissertation described her investigation on the Rbs1 protein which is involved in assembly of the RNA III polymerase complex. Ewa

noticed that RBS1 contains the R3H domain, a potential single-stranded nucleic acids binding fragment. This observation has suggested that Rbs1 can be involved, in addition to its function in Pol III assembly, in other processes. Ewa used the CRAC method to verify the hypothesis that Rbs1 binds RNA. The experiment confirmed that Rbs1 is indeed an RNA binding protein. The data collected after sequencing of RNAs crosslinked to Rbs1 revealed that Rbs1 preferentially binds to transcripts originated from intergenic regions as well as to mRNAs. In general, the identified targets of Rbs1 are rather heterogeneous. Ewa was able to show that Rbs1 binds poly(A) tracts in mRNAs. Interestingly, one of Rbs1 target RNA encodes the ABC 10β protein which is shared by all three RNA polymerases and is involved in Pol III assembly. This observation suggests a potential regulatory role of Rbs1 in coordination of all polymerases assembly. Has it been tested if the level of ABC 10β is changed in *rbs1* mutants?

Next, Ewa asked a question about the effect of nucleotide depletion on the Pol III machinery . She used 6-AU and mycophenolic acid (MPA) to lower the level of nucleotides in the cell. These experiments showed that after treatment of yeast cells with both transcription inhibitors the accumulation of selected tRNA gene transcripts significantly decreased. However, some adaptation to the drugs used in this study were observed in two or three hours after the beginning of treatment. Interestingly, this adaptation was faster in the *Δmaf1* mutant than in wt yeast cells. Moreover, it has been demonstrated that the renewal of Pol III transcription after two hours of MPA treatment is most likely due to the induction of expression of the *IMD2* gene, which leads to the recovery of GTP synthesis. Is this effect observed also when cells were treated with 6-AU? Ewa wanted also to correlate the level of Pol III transcription with occupancy of Pol III and its transcription factors, TFIIIB and TFIIIC across the Pol III genes. To this end, she performed interesting ChIP experiments showing that in general the level of Pol III on its genes was almost unchanged after treatment of yeast cells with MPA. Ewa concluded that most likely upon nucleotide depletion Pol III is stalled on transcribed genes until better growth conditions will allow to

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re-start efficient RNA synthesis. Interesting results came, however, when Ewa checked the level of expression of Pol III subunits upon nucleotide depletion. In such stress conditions the levels of Pol III subunits decreased. The most dramatic effect was seen on the level of the biggest Pol III subunit, C160, which was lower roughly 5-times. In addition, subcellular localization of C160 and Maf1 was monitored upon treatment of yeast with MPA. Ewa concluded from this experiment that MPA had no significant effect on the cellular distribution of Maf1, but changes in the nuclear accumulation of C160 was observed. I found these experiments difficult to interpret since no measurement scores were provided.

The observation that under stress conditions the level of the C160 Pol III subunit decreased encouraged Ewa to start a new direction in her PhD studies: She decided to analyze stability and degradation of the C160 Pol III subunit. Using cycloheximide (CHX) Ewa blocked translation and monitored by western blot the levels of three subunits of Pol III and Maf1. The results showed that, in contrast to Maf1, the level of C160, C82 and C53 decreased in time. Noteworthy, the effect on C160 was the most evident. Next, Ewa used rapamycin that inhibits genome-wide Pol III transcription, and showed that this treatment also down-regulated C160. A similar effect was observed after treatment with MPA and 6-AU. The decrease in the C160 protein level under conditions that cause Pol III repression suggested that expression of the largest subunit of Pol III C160 might be controlled by the ubiquitin proteasome system. Ewa expressed the tagged version of C160 and at the same time overexpressed ubiquitin. She used a mutant strain defective in maturation of 20S proteasome to increase chances of C160-ubiquitin conjugates detection. C160 was immunoprecipitated and analyzed by western blot. Slower migrated bands were detected and their levels increased when cells were grown in glycerol-containing medium, suggesting multi ubiquitylation of C160 in these conditions. Then, the experiment was performed in which a specific proteasome inhibitor MG132 was used. When MG132 was applied the degradation of C160 observed in glycerol was blocked. Moreover, Ewa has checked whether blocking the proteasome (to this end she used the proteasome maturation mutant *Δump1*) regulates Pol III activity. The results show clearly that the proteasome degradation of C160 is not coupled with inhibition of Pol III transcription. Does it mean that the degradation of Pol III

subunits is an effect of low activity of Pol III? It was also verified if the Rsp5 ubiquitin ligase is involved in degradation of C160. To answer this question the analysis of degradation of two subunits of Pol III and the transcription inhibitor Maf1 in yeast wt cells and two *rsp5* mutants were performed. Because no difference was seen after transferring the yeast cells from glucose- to glycerol-containing medium, the final conclusion was drawn that Rsp5 is not responsible for the observed degradation of Pol III after switching from a fermentable to non-fermentable source of carbon. Ewa tried also to identify in C160 sites of ubiquitination. Despite some technical difficulties, she proposed three lysines (K1432, K1242, K1249) which might be ubiquitinated in the cell. In addition, it has been demonstrated that two subunits of Pol III, C82 and C53 are phosphorylated.

I am really impressed by the results described and discussed in the Ewa Leśniewska thesis. I am convinced that she presents in her PhD dissertation novel and original data which have allowed to propose new, biologically important mechanisms involved in the regulation of RNA polymerase III activity. I have no doubt that Ewa Leśniewska has performed an excellent research, and the results obtained by her are reliable and important. An article based on the data presented in her thesis was already published in *Genome Research*. Ewa Leśniewska is also the first author of a review article that appeared last year in *Open Biology*.

The results presented and discussed in the thesis I reviewed, provide a significant contribution towards understanding how RNA polymerase III is regulated in yeast, and Ewa Leśniewska proved to be able not only to design and carry out experiments but also critically interpret the results of the experiments performed. Moreover, Ewa has demonstrated that she knows well all scientific literature concerning RNA polymerase III, its activity and regulation. She was also able to confront her own data with the observations obtained by other researchers.

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I recommend the Scientific Board of Institute of Biochemistry and Biophysics to accept the thesis and proceed with all necessary procedural steps to confer Ewa Leśniewska a PhD degree. In addition, because of the high quality results presented in the thesis, I also recommend to award this work with a special prize.

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