Max-Planck-Institut für molekulare Physiologie

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Review of Ph.D. thesis entitled: Functional analysis of proteins involved in *trans*-splicing in *Trypanosoma brucei* Submited by Ms. Silvie Trantírková form the Laboratory of Molecular Biology of Protists Institute of Parasitology, Czech Academy of Sciences.

The presented thesis analyses the role of several RNA binding proteins of Kinetoplastid *Trypanosoma brucei* in biogenesis of mRNA of nuclear encoded protein genes. The proteins under study play roles in maturation of spliced leader (SL) RNA and process of *trans*-splicing. The thesis is composed of the introduction describing the field of research, the state of knowledge as well as the aim of the research. The experimental part of the thesis is represented by a summary of ongoing projects and several manuscripts already published by the author or submitted to publication.

The main subject of the thesis deals with one of the central processes of eukaryotic biology namely maturation of protein coding mRNA. In the higher eukaryotes RNA splicing of intron containing genes is believed to represent the central mechanism that generates several hundred thousand of proteins out of merely thirty thousand genes. The core of RNA splicing machinery composed of large RNA:protein complex(es) shows significant evolutionary conservation which makes the studies preformed on low eukaryotes even more interesting and relevant for understanding mRNA processing in higher mammals.

The work presented uses the tools of genetic manipulation of *Trypanosoma* to analyze the activity of selected proteins in vivo. Studies presented in first 3 publiaiotns follow a similar analysis scheme – selection of the protein, construction of transgenic *Trypanosoma brucei* strains for

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siRNA mediated knock down of the chosen protein, analysis of the resulting phenotype and effects of the knockdown on the RNA processing. Thus the experiments follow the currently most commonly used scheme of the gene functional analysis. The experiments described in the thesis are preformed and documented very well. The appropriate controls are performed and the eventual pitfalls are discussed. The fact that most of the research is published in the international peer reviewed journals further confirms that the work is being performed according to the international standards and therefore contains original intellectual contribution. The presented results are

significant for understanding of the RNA processing mechanisms of in *Trypanosoma* which in addition of a purely academic value could potential contribute to the development of anti-kinetoplastid therapy. Yet, I find it a bit difficult to single out a finding in the presented thesis which taken alone has the most scientific value attached to it. The work presented is one of many contributions to understanding of the enormously complicated machinery that phrases genetic information in eukaryotic organisms general and in *Trypanosomatids* in particular. This study along with many others assigns putative or definite roles to individual components of the machinery and thus brings us one step nearer to a still very remote comprehensive picture of RNA splicing. The presented thesis is most definitely would be awarded the fist class degree at any European university. Among the thesis I reviewed or supervised so far I would rant this thesis as top 10%.

There are several minor comments and questions that I would like make or address as a part of this review:

Comment: Preface and introduction contain a number of protein names and other abbreviations which original meaning often remains unclear or undisclosed. For instance preface refers to

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MT420 and MT 370 proteins and the respective projects. Yet to a reader not closely related to the field it remains unclear what MT short for and what does the number indicate. Although I am aware that the protein names are often contain little or no useful information it is helpful to explain the origin of their names when they are first presented to the reader.

Questions:

1)In the experiments aimed at the analysis of La protein knockdowns a strong variation in siRNA efficiency was observed among constructs used. This was attributed to the influence of the integration loci on the ability of the construct to produce double stranded RNA. How many independent clones of each transformation were analyzed and what is the evidence that the observed variation is indeed due to the positional effect and not, for instance, variation in the level of T7 polymerase mediated transcription system in some clones or other factors?

2) Along the same lines depletion of SmD1 by siRNA resulted in cytoplasmic accumulation of splice leader RNA. However the reported number of cells in which this phenomenon was observed does not exceed 25%. How does author explain the fact that only ¼ of all cells responded to siRNA induction?

3) In the manuscript describing the use of *Leishmania tarentolae expression* system for production of isotopically labeled proteins the author uses α -amanitine to suppress the expression of the endogenous proteins thus hoping to increase the signal to noise ratio in labeling experiments. The author argues that since the gene coding for the recombinant protein is transcribed by T7 RNA

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polymerases its transcription should be unaffected. However, the splice leader RNA is transcribed by RNA polymerase-II and hence its inhibition should block processing of T7 transcripts. Please comment of this consideration

Despite these minor issues I consider these thesis to be a clearly sufficient for awarding the Ph.D. and strongly advise the thesis committee to do so.

Yours sincerely

Alexandrov

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Report on thesis submission of Silvie Foldynová-Trantírková.

Dear Julius,

Thank you for the opportunity to consider Ms Foldynová-Trantírková's thesis work. I include my comments below. These should be clear, but if you require more information please contact me.

I have included a few questions, indicated by "Q" that the committee may wish to use in their examination of Ms Foldynová-Trantírková. I expect most of this material will be covered by the other examiners, but these are offered in case they are helpful.

Best regards

Mark Field

Summary

A good introduction, including very clear diagrams, which are especially commended.
Contributions to two addition papers, plus a first author one. Demonstrates teamwork as well as the ability to carry out the lead role.
Certainly an acceptable contribution to the literature and knowledge, and also a sufficient amount of work for a PhD. Work is sound, and well executed and written up. No issue at all with recommendation of award of PhD.

Aims

Q1 - What is the aim of the work? The 3 listed projects are more specific goals. Suggest ask the candidate to state, very briefly, the overall aim, and why is there interest in the problem.

Introduction

Excepting comments re the English grammar (below) and some typos that really ought to be corrected, this is a very good exposition of the field indeed. I would acknowledge that I am not expert in this area, but this is clearly a very well researched and constructed review of the literature, which includes some 'classical' studies as well as more recent work. It is well written, easy to follow and authorative. Excellent.

"At the level of RNA processing (*trans*-splicing and polyadenylation in the nucleus, editing, cleavage and polyadenylation in the mitochondrion), RNA stability, nuclear export, translational efficiency and protein degradation (Lukeš et al, 2005, 2006; Ulluet al., 1996)."

What does this sentence mean? No verb!

Similarly, the 1st line in section 3: "SL RNA dependent *trans*-splicing is the best-characterized type of this reaction ....." What reaction are we discussing?

Section 3.2: "Recently, the 3'ends of *T. brucei* small RNA was mapped." What RNA does this refer to? All in class or just specific examples?

Q1 - Discuss why the trans-splicing reaction is present, and what its function may be? Why is this so prevalent in some systems?

Q2 - can candidate discuss the differing models for SL maturation as obtained by her coworkers and Ullu and Tschudi? Is there a methodological or interpretive difference that explains this?

Q3 - Why is La essential in trypanosomes but not in yeast? Can this be explained from available data?

Publication 1

This is a nice, brief but complete analysis. The only question here is obvious - what was the candidate's precise contribution to this work?

Publication 2

Again, the analysis is clear.

Q1 - What does the candidate mean by lethal? The evidence presented here for theRNAi knockdown of La is that it is required for growth, but apparently the cells can persist in a nonreplicative form for a considerable time. Is this lethality, as no death appears to have been demonstrated here?

Q2 - How might you confirm the presence of the intron in the tRNA-Tyr in the knockdown?

Publication 3

Again, what is the candidate's contribution here? As she is third author, I assume this was a comparatively minor role. So, the couple of questions I have may not be appropriate if the candidate was not involved directly in these aspects of the project.
Q1 - The enzymology is rather brief here, and there is no indication of purity of the recombinant protein. Can additional data be described?
Q2 - There is clearly cytosolic fluorescence in one of the cells in Figure 6 - as I assume this data are being used as an argument for a difference in mechanism with the Ullu lab, this is not very convincing. Discuss.

Publication 4

Not considered as the candidate indicates this work was pursued after completion of the thesis work.

Specific questions

1. Does the thesis contain original intellectual contribution? Yes
2. Is the capability of the student to perform experiments, interpret them and present apparent from the thesis? Yes
3. Are the obtained results original? Which are the most important? Yes. The implication that La is essential in trypanosomes is likely the most original contribution.
4. Would this thesis be sufficient for awarding the PhD degree at your

university/institute? Yes

5. How would this thesis score among the theses you know of or you have reviewed so far? Would it belong among the top 10%, top 25%, medium
or worst 25%? Top 25%.

Silvie Trantirkova: Functional analysis of proteins involved in *trans*-splicing in *Trypanosoma brucei*

Presented PhD thesis consists of an introductory part, two already published papers and two manuscripts sent for a review to scientific journals.

General comments:
The introductory part is well arranged and readable but written in rather simple English and containing considerable number of mistakes and typing errors thus pointing to the rush in thesis writing. A reader would also appreciate a list of used abbreviations as well as some short introduction or a commentary to the every manuscript included. One of them has been accepted for publication three years ago and at least in this case it would have been helpful if it had been preceded by the commentary reflecting current knowledge. The introductory part does not fulfill such requirements because most of the cited literature is older than two years. If I counted or better to say estimated this well, there are only 15 citations from 2005 and 2006 years among more than 220 references presented.

Specific comments:
Two of the manuscripts presented had been reviewed in the reputable scientific journals in a course of their acceptance. Thus I would limit my comments to the manuscripts being currently submitted for the review only.

Zamudio et al.: "The methyltransferase for cap1 2'-*O*-ribose on *Trypanosoma brucei* spliced leader and U1 snRNA."

The manuscript presents an excellent piece of work describing a characterization of the cap1 ribose methyltransferase. Some of the experiments are almost reaching the edge of current possibilities in biochemical characterization of the cap structures *in vivo*. It is a pity that authors did not performed any experiments showing translational capability of the TbMTr1 knockout strains e.g. by comparison of polysome formation in the wild type and knockout strains. Primer extension experiments described at Figure 4 and 5 show that there is a pool of SL RNAs with the different cap structure both in the knockout and the wild type strains. However, the polyadenylated mRNA fraction contains only the cap 4 or the modified cap 4 structures in the wt and KO strains respectively. What is a rough molar ratio of SL RNA to matured poly(A) RNA in the cell? Can the SL RNA containing other than the cap 4 structure have some direct biological function? Nematode homologues of the eIF4E translation initiation factor differ in cap binding affinity, requirement for viability and have possible different roles in development. Dhalia *et al* reported recently that more the eIF4E homologs exist in *Leishmania major* and probably also in *T. brucei*. The same authors also observed different expression of some eIF4E isoforms in procyclic and bloodstream forms of *T.brucei*. Taking into the account these results I wonder about the possibility if other than the cap 4 structures can be used more frequently or even preferentially for matured mRNA formation in some condition.

Trantirkova et al.: "Cost-effective approach for amino-acid-type selective isotope labeling of protein expressed in *Leishmania tarentolae*"

The manuscript describes a method for production and labeling of proteins in a novel *Leishmania* based system. Publishing of such methodical reports is very important for the scientific community but at the same time it often becomes a thankless and never cited act. In

my opinion it is quite surprising that three of the proposed co-authors has published a paper on the very similar topic very recently. Could Silvie explain their role in this research? I would expect that new methods should be published when they are very well established in the inventor laboratory. In such a case it is curious, I would say, that the team of co-authors did not published this simple recommendation - consisting just of a substitution of the synthetic medium for a complex one - together with the previous paper this year. Yield or a cost of the protein production are mentioned directly or indirectly many times within the manuscript. Can Silvie summarize and compare briefly the average yields and production costs for let say a small, cytoplasmic and globular protein, like the suggested EGFP is, for the major expression systems? (e.g. *E.coli, Pichia* or *Kluyveromyces*, baculoviruses, chinese hamster ovary cells and *Leishmania*). I would prefer if you can provide, besides the normalized relative expression rates (Fig. 1., panel B), also the graph or table depicting the absolute amounts of the produced protein e.g. in micrograms per 10^7 cells or per ml of the culture. I agree with the authors that Fig. 2 evidences the proposed method of metabolic labeling to be less efficient and sensitive than the already published one. However, I would not agree that the method is suitable enough for a verification of conformational folding homogeneity for crystallization experiments. The NMR experiment presented in the manuscript does not reveal the signal from Val 5 residue and also three other minor signals presented by *Nicolae et al,* 2006, which can indicate the valine residues present in an alternative conformation of the protein.

Silvie Trantirkova presents a very good work containing important data of general interest. Both the already published manuscripts appeared in the respectable scientific journals and are cited by others, thus evidencing the importance of the obtained results and described ideas. Regardless some criticism of the introductory part of the Silvie's thesis I was really delighted to read it and recommending it without any hesitation to be accepted as her PhD dissertation.

December 10, 2006 Martin Pospíšek