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Poznań, 28 February 2025

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Review of the thesis

The role of ISRE and GAS composite-containing genes in long-term IFN-I and IFN-II responsiveness

PhD Student: **Sanaz Hassani**

This thesis was prepared in the Human Molecular Genetics research group led by Professor Hans Bluyssen at the Institute of Molecular Biology and Biotechnology, Poznań University, under his supervision. Among its various research topics, the group is particularly distinguished by its studies on interferon I and II-induced antiviral responses mediated by Signal Transducer and Activator of Transcription (STAT) and Interferon Regulatory Factors (IRFs). Elucidating this complex process is not only scientifically compelling but also critically important for paving the way toward future therapies targeting viral infections.

One of the significant challenges in this field is deciphering the diverse interactions between STAT/IRF transcription factors (TFs) and the promoters of the response genes (ISGs) to which they bind, an interplay that is induced upon interferon (IFN) stimulation. In this context, two groups of ISGs were previously identified: one containing the ISRE motif and the other containing the GAS sequence motif, with each group recruiting distinct members of the STAT/IRF TF families.

Sanaz's thesis specifically focussed on a third group of ISGs, distinct from the aforementioned groups, that comprises both ISRE and GAS motifs in their promotor/5'UTR regions, and is therefore referred to as ISRE+GAS composite ISGs. Interestingly, this group exhibits heterogeneity in several aspects: first, the number of ISRE and GAS motifs; second, the order in

which these motifs appear (i.e., whether ISRE precedes GAS or vice versa); and third, the distances between the two types of motifs.

The Introduction section is very well written, providing a solid background for the study and including informative figures that clearly illustrate the various mechanisms of IFN stimulation described.

The Results section begins with a description of ChIP-seq and RNA-seq data, which enabled the initial selection of 30 composite genes, all of which were sensitive to both IFNα and IFNγ stimulation in a WT human hepatocellular carcinoma cell line (Huh 7.5) model. What was the rationale for choosing this particular cell line as the model of study? Based on the presented GO analysis, these genes were confirmed to be involved in processes induced by viral infection. The 30 composite genes were then categorized into five groups, each representing a different arrangement of ISRE and GAS motifs (e.g., ISRE/GAS or GAS/ISRE order) and varying distances between motifs – from overlapping to up to 200 bases apart. Based on this initial classification, the hypothesis was proposed that these composite genes, depending on their specific ISRE and GAS motif arrangements, recruit distinct groups of STAT/IRF TFs upon IFNα or IFNγ stimulation and, as a result, exhibit group-specific expression levels. My main question is: was there a potential scenario(s) envisaged?

However, as analysed in the WT Huh 7.5 cell line, the temporal expression patterns and levels of the 30 selected ISGs did not segregate according to the previous defined criteria. Moreover, despite differences in the arrangement of ISRE and GAS motifs, these composite genes displayed similar binding profiles of the canonical TF-complexes previously known to bind ISRE or GAS motifs. In other words, the STAT/IRF TF complexes did not assemble at the promoters based on the specific motif arrangements representing each of the five groups of composite genes.

Subsequent analysis confirmed that both ISRE and GAS motifs are active in the WT cell line. Similarly, the composite genes demonstrated diverse expression patterns in response to both IFNα and IFNγ indicating that their binding characteristics merge features of both GAS-only and ISRE-only genes. Notably, when the expression of these composite genes was examined in a series of KO cell lines, each lacking a different canonical STAT/IRF TF, there was evidence of a potential switch between ISRE and GAS binding, dependant on the specific canonical TF missing in each KO cell line. The switch mechanism between ISRE and GAS was further confirmed in both WT and KO cell lines by combining qPCR results of 13 genes, selected from the initial set of 30, following IFNα and IFNγ activation. Importantly, this time-course experiment allowed the prediction of the composition of the canonical TFs bound to these 13 ISGs. Overall, these experiments further confirmed that there is no correlation between the order or distance of ISRE/GAS sites and the binding or expression profiles of the composite genes.

The importance of ISRE/GAS sites for ISG stimulation by IFNs was studied in greater detail using 6 selected ISGs from the previous group of 8, each representing different ISRE and GAS motif arrangements. These motifs were mutated by applying site-directed mutagenesis. This approach enabled a comparison of wild-type promoter activity with that of promoters containing mutated ISRE and GAS motifs through luciferase assays that evaluated the response of individual ISGs to both IFNα and IFNγ. These elegant series of experiments were conducted in both WT and KO cell lines, allowing the prediction of the transcriptional components bound to each composite gene promotor. Similarly to previous series of experiments, the data revealed that the ISRE motif exerts a more potent effect than the GAS motif.

Interestingly, in the case of the NMI gene, removal of one GAS motif enhanced the response to both IFNs, suggesting that this particular GAS motif may exert an inhibitory effect on gene activation and add a potential additional layer of regulation to the response to IFN stimulation.

Considering that the discussion section is highly complex and includes lengthy descriptive paragraphs, I would appreciate a few final summary points highlighting the most important findings during the defence presentation.

Collectively, this multi-level study demonstrates that composite genes can undergo transcriptional activation in response to IFNα and IFNγ through variable mechanisms based on the interaction of distinct combinations of STAT/IRF TFs. However, the observed differences in gene expression and the predicted binding of specific STAT/IRF factors to the promoters of these composite genes did not correlate with the expected patterns on ISRE or GAS distribution expected from this study, including the spacing between these motifs, upon IFN stimulation.

Moreover, given that some of the composite genes studied in this dissertation exhibit long distances between ISRE and GAS motifs, it is likely that the aforementioned complexes interact with those motifs separately and do not form a single functional complex. I wonder whether, in the case of very closely located ISRE and GAS motifs, the interaction of STATs/IRFs would result in the formation of one functional complex, as is the case, for example, with PARP14?

Minor comments

1. It is confusing to refer to blue as purple in the figure descriptions in the Introduction section.
2. Regarding the location of ISRE and GAS motifs in composite genes, it is preferable to use “order” rather than “orientation”, as the latter implies positioning on different strands of the double helix.
3. What was the reasoning behind placing chapters 4.4.1.-4.4.5, which describe the strategy and methodology of construct generation, in the Results section rather than in the Methods section?

Final conclusion

The Ph.D. dissertation by Sanaz Hassani is a highly valuable scientific contribution, particularly given the complex methodologies employed. It provides new insights into the molecular mechanisms underlying the variability of ISG responses to IFN stimulation during viral infection. Without a doubt, the dissertation fulfils the expected scholarly standards. Therefore, in accordance with the provisions specified in Art. 187, ust. 1-2, and Art. 190, ust. 3 Ustawy z dn. 20.07.2018 r. Prawo o Szkolnictwie wyższym i nauce (Dz. U. 2024 poz. 1571), I kindly request that the Scientific Council of the Faculty of Biology at A. Mickiewicz University proceed with the next steps in Sanaz Hassani's Ph.D. process.