

Construction of genetic circuits to study protein decay rates

Vivien Miczán^{1*}, Mümün Gencoğlu², Attila Becskei²

¹ Faculty of Information Technology, Pázmány Péter Catholic University, H-1083, Budapest, Hungary

² Institute of Molecular Biology, University of Zurich, CH-8057, Zurich, Switzerland

*Correspondence: micvi@digitus.itk.ppke.hu

Abstract

The phenotypes of different cells are encoded by complex networks, one of the most studied one is the galactose network in yeast. Interactions between proteins and genes tune the transcription and one major issue is the response of the cells to galactose. In the first part of our experiments we wanted to analyze the decay of the Gal80 protein in single cells, because most of the publications of this topic are based on bulk measurements. That is why we made a construct of Gal80 gene with an intron U3 and a tetracycline operator. We added a plasmid with Gal1.GFP to monitor the decay and one with pcln3.tTA to control the production of the Gal80 protein. Then we transformed these into a Gal1-Gal3 double deletion strain in order to be able to avoid the effect of a feedback loop.

In the second part of our study we used a Gal2 deleted *Saccharomyces cerevisiae* strain to investigate whether cutting this particular positive feedback loop we get binary or graded response to galactose. We analyzed the response in a galactose gradient using GFP as the reporter of the transcription of the Gal genes. The results showed that there is a binary response in the wild type cells but it's a graded one in the case of the Gal2 deletion strain. This observation is essential when further protein decay measurements are conducted in the Gal network.

Introduction

Proteins are continuously degraded and replaced in the human body even in adults. We use the term 'half life' for the time taken for half of the protein in question to be replaced. The values of half-life of different proteins range from a few minutes to months.

Protein amount can change in response to external stimuli, developmental programs or disease states, eventually establishing a new equilibrium. Transcription in eukaryotes is a highly controlled process, both activators and inhibitors can affect it. One of the main parameters that control their concentration is their half life. In the first part of the project we investigated the decay of the Gal80 protein (Gal80p).

Measurement of protein decay

Until recently, changes in cell states could only be

measured in aggregate by considering all proteins or in a highly targeted fashion, whereby specific proteins or the mRNA species that lead to them are individually assayed, these are mainly bulk measurements⁽¹⁾

We wanted to measure the protein decay in an indirect way using genetic regulatory circuits. They are functional clusters of genes that impact each other's expression through inducible transcription factors and cis-regulatory elements. One of their advantages is that we can determine protein levels even in single cells so we are able to examine the behavior of different cells.

It is important because for example during the budding of yeast the protein levels of the daughter cells might differ.

Model organism

We used *Saccharomyces cerevisiae* yeast strains for the project. It is one of the most intensively studied eukaryotic model organisms in molecular and cell biology. It is a small single celled organism with a short generation time (It has a doubling time about 2 hours at 30 °C) and can be easily cultured. It can be transformed allowing for either the addition of new genes or deletion through homologous recombination. Furthermore, it can be grown as a haploid simplifying the creation of gene knockouts strains. As a eukaryote, *S.cerevisiae* shares the complex internal cell structure of plants and animals.

The galactose signaling pathway

Cellular regulation is a result of complex interaction between various mechanisms such as DNA–protein and protein–protein binding, autoregulation, compartmentalization and shuttling of regulatory proteins. One of the most studied genetic networks is the galactose signaling pathway ⁽²⁾ (Figure 1)

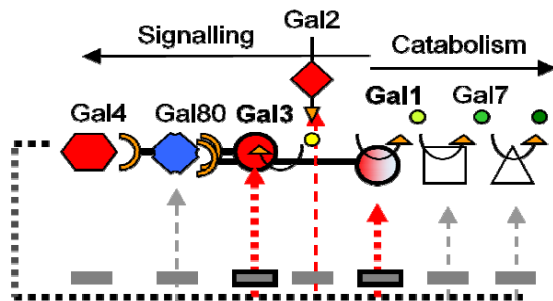


Figure 1. Schematic diagram of the *S.cerevisiae* GAL system

When galactose is added to a logarithmically growing culture of the yeast *Saccharomyces cerevisiae*, a set of genes (collectively called GAL genes) that encode galactose-metabolizing enzymes start to be transcribed within a few minutes and this way they can use galactose as a carbon source when no glucose is available. This rapid induction involves GAL2p as transporter, Gal3p, in addition to Gal4p as transcription activator and Gal80p, the antagonist of Gal4p. The structural genes GAL1, GAL7 and GAL10 are clustered but separately transcribed by individual promoters.

In the presence of inducer, galactose and adenosine triphosphate (ATP), Gal3p is activated

to form a complex with Gal80p in the cytoplasm. This leads to shuttling of Gal80p from the nucleus to the cytoplasm, thereby reducing the availability of Gal80p for binding with Gal4p in the nucleus. Thus, galactose relieves the repression through nucleocytoplasmic shuttling of Gal80p. Once the switch is turned on, the product of the gene Gal2 (permease Gal2p) mediates transport of galactose into the cells, which further activates Gal3p. In the presence of glucose (inhibitor), the synthesis of Gal4p is inhibited. In a non-inducing and non-repressing medium (such as glycerol and raffinose), Gal80p is bound to the gene–Gal4p dimer complex in the nucleus and maintains the switch in a repressed state.

GAL system consists of three feedback loops, a positive activation of galactose uptake by Gal2p, an upregulation of the inducer Gal3p to sense intracellular galactose, and a negative feedback caused by upregulation of the repressor Gal80p. ⁽³⁾

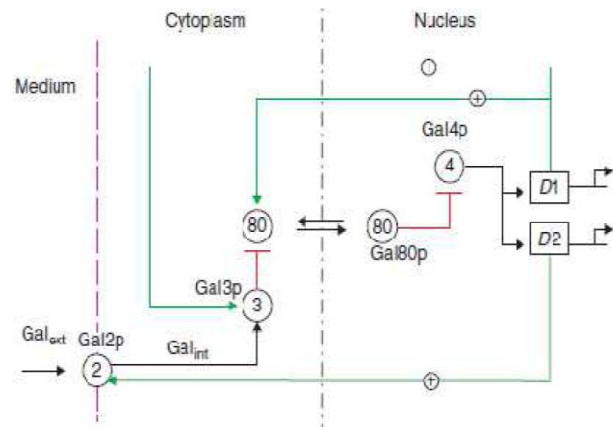


Figure 2. Schematic diagram showing feedback effects in GAL system of *Saccharomyces cerevisiae*

Gal2 and *Gal3* form positive and *Gal80* a negative feedback loop

The aim of the first part of the project was to measure the decay of the Gal80 protein using a genetic circuit, because it helps us to understand more about the GAL system.

Direct measurements concerning the half-life of a specific protein are not always satisfying, because for example from the RNA of Gal80 multiple proteins can be made. We chose an indirect measurement instead. As the Gal80p is an inhibitor of the transcription of Gal4, we made a

construct with Gal80 and 'tet'O operator in it to be able to control the production of Gal80p.

Synthetic systems

Tetracycline-Controlled Transcriptional Activation is a method of inducible expression where transcription is reversibly turned on or off in the presence of the antibiotic tetracycline or one of its derivatives (e.g. doxycycline). This system makes use of the tetracycline transactivator (tTA) protein created by fusing one TetR (tetracycline repressor) protein with another protein, VP16. The tTA protein binds on DNA at a 'tet'O operator. Once bound the 'tet'O operator will activate a promoter coupled to the 'tet'O operator, activating the transcription of Gal80p. Doxycycline binds tTA and make it incapable of binding to the DNA, preventing transactivation of Gal80.⁽⁴⁾

If we stop the Gal80 transcription like this, the amount of the protein starts to decrease. But we cannot measure it directly, that is why we put pGal1.GFP as a reporter and since Gal80p inhibits the pGal1-GFP construct, if the Gal80p starts to decrease, the amount of GFP starts to grow in parallel. We can measure the fluorescent activity using a flow cytometer.

Flow cytometry

It is a technique for counting and examining microscopic particles, such as cells and chromosomes, by suspending them in a stream of fluid and passing them by an electronic detection system. It allows simultaneous multiparametric analysis of the physical and/or chemical

Our second aim was to assess complications in the Gal network. If we would like to analyze the decay of further proteins in the system, it is necessary to know whether Gal2 induces a binary response or not. We use the term 'graded' to describe a promoter response that achieves a continuous range of activity from fully on to fully off. In contrast we use 'binary' to refer to

Methods

To measure the decay of the Gal80p we had to get rid of the Gal1 and the Gal3 genes because with them there had been feedback loops in the system as Gal1p can substitute for Gal3 when present at

characteristics of up to thousands of particles per second.

One unique feature of flow cytometry is that it measures fluorescence per cell or particle. This contrasts with spectrophotometry in which the percent absorption and transmission of specific wavelengths of light is measured for a bulk volume of sample.

Introns

In multicellular organisms most genes contain at least one intron, usually more. In contrast only 5% of *S. cerevisiae* genes are interrupted by introns (most by a single intron) and all are constitutively removed during gene expression.

In order to improve the construct an intron has been placed right after the ATG codon of Gal80 gene. The reason to put an intron in the gene is to screen a more processed mRNA. Moreover, K. Juneau et al.⁽⁵⁾ has presented data showing intron-containing genes produce more RNA and more protein than single exon genes in yeast. H.-F. Wang et al.⁽⁶⁾ has also pointed out that intron containing genes are more stable than intronless ones. These features of intron addition can possibly increase the dynamic range of the transcription and expression of Gal80 gene and Gal80p respectively. A wild type U3 intron has been inserted in Gal80 since it has been stated by Pikielny et al.⁽⁷⁾ that the splicing efficiency of transcripts containing the wild-type intron is well in excess of what is necessary to generate normal mRNA levels.

the situation when a promoter produces only two levels of activity: on and off.⁽⁸⁾

To find out the answer we used Gal2 deletion strain with a pGal1.GFP construct and we measured the response to a galactose gradient and then we compared the results with a wild type strain. This way we were able to check whether the response is binary or graded.

high concentrations.⁽¹⁰⁾ It was necessary because we wanted to analyze the decay of the Gal80 protein instead of measuring the response to galactose. That is why we chose a Δ Gal1 and a

Δ Gal3 strain with different mating types and after mating and random sporulation we eliminated the wild types using kanamycine plates because in the deletion strains there were kanamycine resistance gene. Then we chose the Δ Gal1- Δ Gal3 double deletion strains using colony PCR.

After that we made a construct with the Gal80 gene and an intron named U3 using fusion PCR and we ligated this into a plasmid with 'tet' operon in it. Then we transformed this plasmid

into the double deletion strain.

Then we transformed pGal1.GFP and pcln3.tTA containing plasmids into this strain to be able to control the production of tTA and this way the Gal80p. After the production of Gal80p is stopped by adding doxycycline to the system the protein starts to decay and becomes incapable to inhibit the transcription of the Gal1 promoter driven GFP gene, this way the amount of GFP and the fluorescent activity starts increasing which can be measured by a flow cytometer.

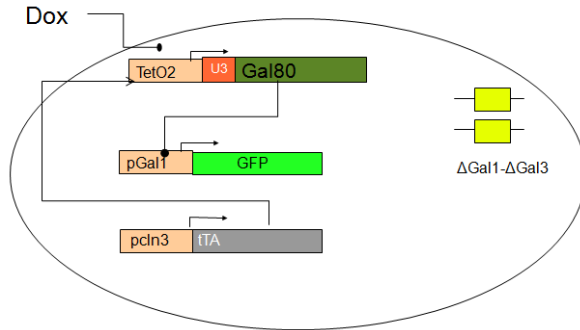


Figure 3. The construct to measure the Gal80p decay

It contains the target gene Gal80 with an intron in it. The transcription of these genes are driven by tet operon and after adding doxycycline to the system it can be stopped. As the Gal80p level starts dropping the GFP level starts increasing.

In the second part of the project we used Δ Gal2 and wild type (SY991) *S. Cerevisiae* strains and we transformed a pGal1.GFP containing plasmid in them. This way in the first case we cut off one of the positive feedback loops while in the second case we were able to observe the natural response

to galactose. After growing both strains in non-inducing non-inhibiting raffinose media overnight we put them under a galactose gradient for 4 hours. Then we measured the fluorescent activity caused by the induction of the Gal1 promoter.

Results and Discussion

In the first part of the project we succeeded in making the construct for the indirect measurement of the Gal80p protein decay but the actual analysis is taking place later. The significance of this type of measurement that the protein decay rates can be determined in single living cells in a way that we do not disturb them very much and do not change the structure of the original proteins.

In the second part of the project we found that the response of the wild type and the Gal2 deletion strains are different, moreover we can say that in the first case it is a binary response, there is a jump between two states. In the second case it is rather a graded response, there are continuous steps in the system. (Figure 4)

Using this approach, binary transcriptional

responses generate two peaks of GFP fluorescence: one of high intensity that corresponds to cells expressing GAL1-GFP, and one representing background fluorescence corresponding to cells in which GAL1-GFP is not expressed. In this case, increasing levels of expression raise the height of the high intensity peak as the number of expressing cells increased, but the positions (i.e. fluorescence intensities) of the two peaks do not change. Alternatively, graded transcriptional responses generate a single fluorescence peak that increases in intensity and therefore changes position on a FACS plot as expression levels increase. In this case, the single peak represents a homogeneous transcriptional response across an entire population of cells.⁽⁸⁾

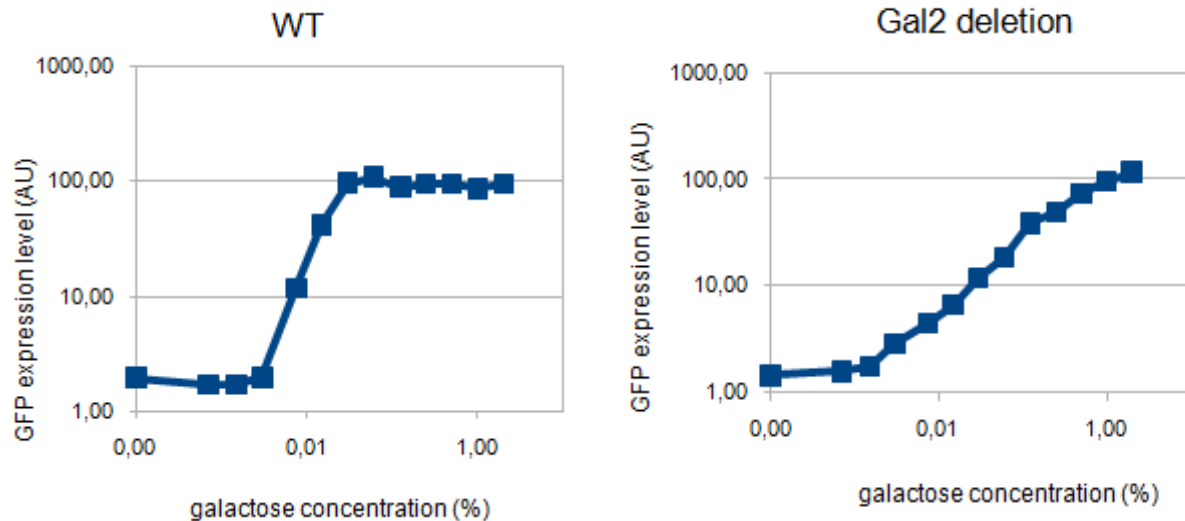


Figure 4. Response to galactose gradient in WT and Δ Gal2 strains

In the first case we can see a rather binary response, there is a gap between the ON and the OFF states, while in the second case we see a graded response to galactose gradient.

We cannot say that the Gal2 is the only gene that is responsible for the binary response but it is clear that without this positive feedback loop

we can see a graded response to galactose. This observation can help the future experiments on the decay of the proteins of the Gal system.

Acknowledgements

This study was conducted under the guidance of Mümün Gencoğlu and supervisor Prof. Dr. Attila Becskei at the Institute of Molecular Biology in Zürich, Switzerland. Special thanks is extended to the 2011 Biology Undergraduate Summer School for hosting this internship and opportunity for students.

References

- 1) Hinkson V., Elias E. The dynamic state of protein turnover: It's about time. *Trends in Cell Biology*. 21: 293-307 (2011)
- 2) Bhat PJ. Galactose Regulon of Yeast: From Genetics to System Biology. Berlin Heidelberg: Springer-Verlag; 2008, 30–31
- 3) Pannala et al. Systems biology of GAL regulon in *Saccharomyces cerevisiae*. John Wiley & Sons, Inc. Volume 2. (2010)
- 4) Bujard, Hermann; M. Gossen, Tight Control of Gene Expression in Mammalian Cells by Tetracycline Responsive Promoters. *Proc. Natl. Acad. Sci. U.S.A.* 89 (12): 5547–51. (1992)
- 5) Juneau K. Introns Regulate RNA and Protein Abundance in Yeast. *Genetics* 174: 511–518 (2006)
- 6) Wang F. Relationship between mRNA stability and intron presence. *Biochemical and Biophysical Research Communications* 354: 203–208 (2007)
- 7) Pikielny W. mRNA Splicing Efficiency in Yeast and the Contribution of Nonconserved Sequences. *Cell*. 41: 119-126 (1985)
- 8) Biggar, S. R. & Crabtree, G. R. Cell signaling can direct either binary or graded transcriptional responses. *EMBO J.* 20, 3167–3176 (2001).
- 9) Becskei, A., Seraphin, B. & Serrano, L. Positive feedback in eukaryotic gene networks: cell differentiation by graded to binary response conversion. *EMBO J.* 20, 2528–2535 (2001).
- 10) Kundu, S., Peterson, C.L. Dominant Role for Signal Transduction in the Transcriptional Memory of Yeast GAL Genes. *Molecular and Cellular Biology*, 2330-2340 (2010)