

Transient responses and adaptation to steady state in a eukaryotic gene regulation system

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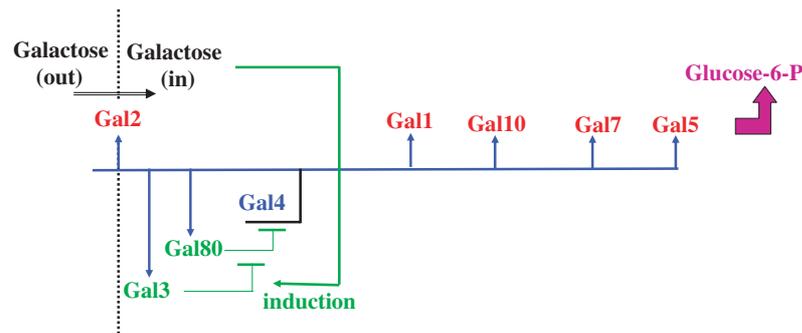
Abstract

Understanding the structure and functionality of eukaryotic gene regulation systems is of fundamental importance in many areas of biology. While most recent studies focus on static or short-term properties, measuring the long-term dynamics of these networks under controlled conditions is necessary for their complete characterization. We demonstrate adaptive dynamics in a well-known system of metabolic regulation, the *GAL* system in the yeast *S. cerevisiae*. This is a classic model for a eukaryotic genetic switch, induced by galactose and repressed by glucose. We followed the expression of a reporter *gfp* under a *GAL* promoter at single-cell resolution in large population of yeast cells. Experiments were conducted for long time scales, several generations, while controlling the environment in continuous culture. This combination enabled us, for the first time, to distinguish between transient responses and steady state. We find that both galactose induction and glucose repression are only transient responses. Over several generations, the system converges to a single robust steady state, independent of external conditions. Thus, at steady state the *GAL* network loses its hallmark functionality as a sensitive carbon source rheostat. This result suggests that, while short-term dynamics are determined by specific modular responses, over long time scales inter-modular interactions take over and shape a robust steady state response of the regulatory system.

1. Introduction

Genetic regulatory networks, their structure and functionality, are the focus of current research in several areas of biology. These networks control the level of gene expression in response to intracellular and external signals. Specific stimulus-response relationships, such as switch-like behaviour and graded induction, are important for determining cell fate (Davidson 2001, Gerhart and Kirschner 1997, Wilkins 2002). The response of a genetic regulatory system is usually characterized by a well-defined function of a small number of inputs. However, recent large-scale analyses have revealed that genetic regulatory networks are highly complex and multifunctional (Brem *et al* 2002, Ideker *et al* 2001, Lee *et al* 2002, Pilpel *et al* 2001). These properties suggest that

adaptive dynamics, context and history dependence should play important roles in the response of genetic regulatory systems, as they do in many other physiological systems. Thus, uncovering the adaptive behaviour of genetic regulation systems over time must complement the efforts to map their network wiring. An important example for adaptation was recently provided in a study of the genome-wide stress response in yeast (Gasch *et al* 2000). This work showed that following a specific response to an environmental change, the cells adapt their gene expression programme to a new steady state not very different from the one before the environmental shift; the global changes in transcript abundance immediately following the environmental change were largely transient. Specific cell responses to non-stressful signals are also likely to be composed of a transient part, followed by adaptation to



Scheme 1. Outline of the *GAL* network in *S. cerevisiae*. This network consists of a set of structural genes responsible for galactose metabolism (GAL1, GAL10, GAL7, GAL5), a permease responsible for galactose transport (GAL2), and regulatory proteins (GAL4, GAL80, GAL3). GAL4 is the transcription factor, inhibited by GAL80; galactose activates GAL3 which in turn alleviates this inhibition. All genes except for GAL4 are regulated by a similar mechanism.

more robust steady states. The aim of the present paper is to explore adaptive dynamics in a well-studied regulation system under non-stressful conditions.

The typical time scale for gene expression processes is of the same order as that of the cell cycle, and therefore adaptation effects are expected to be manifested over several generations. This necessarily calls for the investigation of these effects at the level of a cell population. On the other hand, the time scale for genetic variability to become significant in a large population is well separated from these physiological time scales. In order to study physiological adaptation, the system of choice is therefore a large isogenic population studied over several generations. Maintaining large populations in steady growth conditions requires either a serial-dilution batch (Lenski and Travisano 1994) or a chemostat (continuous culture) (Novick and Szilard 1950, Paquin and Adams 1983). These techniques are widely used to study evolutionary processes, but are less commonly used in the context of physiological processes. However, in a propagating population, even over a small number of generations, dynamic factors such as protein inheritance and specific history are important and therefore similar controlled growth techniques are required. Among these, only the chemostat enables growth in true steady state, and should therefore be used to distinguish transient behaviour from steady state of the population.

A cell population is best characterized by the distribution of a property, rather than its mean value, since cell individuality is important even in a clonal population (Spudich and Koshland 1976). Long-term measurements of gene expression distributions in an isogenic population of cells grown in a fixed environment and having a well-defined history have not been carried out till now. We have constructed an experimental system that allows measurements of single cell gene expression in clonal populations. Large populations of yeast ($\sim 10^9$ – 10^{10} cells) were grown in a serially diluted batch or in a home-made chemostat for many generations under various controlled homogeneous minimal media. The number of generations in all experiments reported here is less than required for genetically-based adaptive shifts (<50 generations) (Ferea *et al* 1999, Paquin and Adams 1983).

Thus, the populations are isogenic and adaptations are solely physiological.

We concentrate on the *GAL* system of the yeast *Saccharomyces cerevisiae*, which is one of the best characterized transcriptionally regulated networks in eukaryotes (Jayadeva and Murthy 2001, Johnston and Carlson 1992, Lohr *et al* 1995). It is responsible for galactose metabolism and becomes an essential pathway in the presence of galactose as a sole carbon source. Scheme 1 shows an outline of the *GAL* network. Experiments till now have shown that this network exhibits a strong level of induced transcription in galactose, modulated by its concentration, and strong repression by glucose (Biggar and Crabtree 2001, Carlson 1999, Johnston and Carlson 1992, Johnston *et al* 1994, Li *et al* 2000). How these specific responses at the single cell level are reflected in an adaptive population is not known. We followed single-cell transcription regulation by inserting the gene for green fluorescence protein (*gfp*) downstream the promoter of *GAL10*, one of the structural genes of the *GAL* network, either on $2 \mu\text{m}$ plasmids or into the chromosome. Previous studies have demonstrated that $2 \mu\text{m}$ plasmid reporter systems accurately reflect the regulation patterns of native *GAL* genes (Yocum *et al* 1984). Also, it has been shown that *gfp* expressed from *GAL* promoters exhibits a fluorescence level closely correlated with the activity of the corresponding *GAL* genes (Biggar and Crabtree 2001, Li *et al* 2000, Niedenthal *et al* 1996, Tatchell and Robinson 2002). In addition to measuring fluorescence, we measured population averages of mRNA transcription levels of *gfp* and various *GAL* network genes using real-time PCR.

The main result of these experiments is that the *GAL* network behaves as a sensitive switch only transiently. In the presence of ample carbon source the population adapts, over several generations, to a single robust steady state. This state is characterized by a broad expression distribution that is largely independent of external conditions. Thus, in the adapted state the *GAL* network loses its hallmark functionality as a sensitive carbon source rheostat. This result can be understood in light of the significant interactions between the *GAL* network and other modules in the cellular regulatory network (Ideker *et al* 2001) and the ability of the *GAL* system to respond to multiple inputs (Griggs and Johnston 1993, Rohde *et al* 2000).

2. Materials and methods

2.1. Construction of yeast strains

Unless otherwise specified all experiments were carried out with the haploid yeast strain YPH499 (*a*, *ura3-52 lys-801 ade2-101 trp1-Δ63 his3-Δ200 leu2-Δ1*) which was transformed with the 2 μm plasmid vector (pESC-LEU, Stratagene) cloned with GFPS65T (from plasmid pFA6a-GFP(S65T)-TRP1 (Helm *et al* 1995, Longtine *et al* 1998), gift from Y Kassir, Technion) downstream the *GAL10* promoter. The ability of *gfp* to faithfully report the state of the network was verified by following the turn-on of the GAL network after switching from raffinose to galactose in the chemostat (data not shown). The experiments on a single *gfp* copy integrated into the genome were done by using the plasmid PRS405 (Sikorski and Hieter 1989) (gift from Y Kassir, Technion) cloned with the *gfp* downstream *GAL10* promoter and the same terminator used in the plasmid above, and integrated in a single copy into the *LEU2* promoter region in the genome. The mean plasmid copy-number in the propagating population was measured by real-time PCR. Total DNA was purified from cells harvested from the chemostat (Dneasy Tissue kit, Qiagen). The number of *gfp* DNA-sequences was compared with that of *GAL4* and found to be 16 ± 2 . As a control, the strain with the integrated *gfp* was measured with the same protocol and primers leading to a ratio of one between the *gfp* and *GAL4*.

The experiment described in figure 3(b) used a different strain DBY9497 (Mata, *leu2-3,112*; a gift from D Botstein and M Dunham) which was transformed with the *gfp* downstream *GAL10* promoter on the 2 μm plasmid vector as above.

2.2. Cell growth and microscopy

Throughout this work, a home-made 100 ml chemostat was used under continuous mixing and filtered air supply and stabilized temperature of 30 °C. All chemostats were inoculated with cells from a single colony on an agar plate, grown to exponential phase in a medium similar to the chemostat medium. Dilution rates were varied between experiments in the range of 0.06–0.2 h⁻¹. In all experiments we used minimal media without *LEU*, varying the concentration of the different components depending on the choice of the limiting nutrient: for amino-acid limited medium and the serial-dilutions batch experiment: (concentrations in g l⁻¹) 1.7 yeast nitrogen base without amino acids and ammonium sulfate, 5 ammonium sulfate (1/2 in nitrogen-limited), 1.4 amino acids dropout powder (Sigma), 0.02 L-histidine, 0.04 L-tryptophan, 0.02 uracil, 20 galactose (5 in galactose-limited) or 20 glucose. The experiment on the strain DBY9497 was done in a minimal phosphate-limited medium with no amino acids: salts (concentrations in g l⁻¹): 0.1 calcium chloride, 0.1 sodium chloride, 0.5 magnesium sulfate, 5 ammonium sulfate, 0.1 potassium chloride, 0.02 potassium phosphate monobasic; metals and vitamins (concentrations in μg l⁻¹): 500 boric acid, 40 copper sulfate, 100 potassium iodide, 200 ferric chloride, 400 manganese sulfate, 200 sodium molybdate, 400 zinc sulfate, 1 biotin, 200 calcium pantothenate, 1 folic acid, 1000 inositol,

200 niacin, 100 p-aminobenzoic acid, 200 pyridoxine, 100 riboflavin, 200 thiamine; 20 g l⁻¹ galactose or glucose.

Samples were harvested from the chemostat occasionally for fluorescence and cell density measurements (optical density at 600 nm). The identity of the limiting nutrient in the chemostat was verified by changing its concentration and observing a corresponding cell density change. Galactose or glucose concentration in the chemostat was measured using the lactose/D-galactose or D-glucose kit, respectively (Scil Diagnostics, Martinsried, Germany). Fluorescence measurements were performed using an inverted microscope (Zeiss Axiovert 135) with 100× oil-immersed objective (filter set O-5717, Omega) and 100 W mercury lamp. The sample was scanned and at each area both a bright-field image and a fluorescence image were captured (digital cooled CCD, microMax, Princeton Instruments).

2.3. Image analysis

Bright field images were used to identify cell boundaries using ImagePro software (Media-Cyberentics). The cell boundaries were superimposed on the corresponding fluorescence images and further analysis was performed by homemade software written in Matlab. In order to compensate for background variations in intensity, the signal for each cell was calculated as the local contrast: $(I_{\text{cell}} - I_{\text{background}})/I_{\text{background}}$, where $I_{\text{background}}$ was computed as an average over the close vicinity of the cell. This quantity is dimensionless and invariant under stretching of the image grey scale. All data presented in figures are scaled by the *same* constant factor (typical number of pixels per cell area) such that the contrast scale is of order unity. Histograms were constructed from several hundred to several thousand cells, and normalized for unit area. Thus the histograms represent the probability density function, allowing a comparison among histograms computed over different bin sizes. Histogram stability was checked in each case by dividing the data into two and comparing the resulting histograms from the two halves. Note that the number of data points required for a stable histogram strongly depends on the nature of the distribution: broader distributions, with a larger standard deviation to mean ratio, require a larger number of points. The fraction of cells in each bin is the probability density multiplied by the bin size.

Spatial variance was estimated by summing the square of fluorescence signal at all pixels inside the cell volume. Although this does not correspond to a true variance of a normalized distribution, we have found that this measure gives a good indication of the inhomogeneity, and distinguishes reliably between transient and steady state populations.

2.4. mRNA transcription level using real-time PCR

Cells were harvested from the chemostat, frozen in liquid nitrogen and kept at -70 °C. Total RNA was purified by a commercial kit (RNeasy, Qiagen). cDNA was prepared using a commercial kit (TAQMAN—Reverse Transcription Kit, Applied Biosystems) and oligo-dT. Real-time PCR measurements were performed with AB 7700 using a set of designed primers (primer express, AB). The ORFs of *ADHI*,

ACT1 and *GAL4* were prepared by PCR using the *S. cerevisiae* S288C genome template and a set of commercial primers (Research Genetics). Purified PCR products were measured (Lambda Bio 40 spectrophotometer, Perkin Elmer) and later used as calibration scales. The different primers worked with uniform efficiency and led to the same quantitative results as compared by using calibrated genomic DNA. Sybr-Green PCR master mix (AB) was used and specificity was verified by melting temperatures. In all experiments a non-template control for each of the primer pairs resulted in at least two orders of magnitude lower signal than the ones measured for the relevant gene. All measurements were normalized by the *ACT1* transcription level and the results are reported in these units. The measurement of *ADHI* serves as a control and was found at the expected level of ~ 2.5 times *ACT1* level (*ADHI* ~ 51 copies/cell, *ACT1* ~ 21 copies/cell (Young Lab Home Page)) and stable over time. Each experiment was performed at least twice and the results reported are the average over these repetitions.

3. Results and discussion

We started by studying glucose repression of the *GAL* system upon transforming a population of yeast cells from pure galactose to pure glucose as a sole carbon source. In order to maintain the cell population in an approximately constant environment of rich non-depleted medium we used the method of serial dilutions. Cells were first grown in pure galactose to an optical density of 0.5 and then diluted into a rich glucose medium. Dilution was repeated every time the population grew to a density corresponding to $OD = 0.5$. The activity of the *GAL* network was measured by following the fluorescence signal of *gfp* expressed under the *GAL10* promoter using fluorescence microscopy (see section 2). Figure 1(a) shows the kinetics of the mean fluorescence signal from such a population. As expected, it shows strong repression following the medium change from galactose to glucose. The ‘fold repression’ is similar to other published fluorescence measurements (Li *et al* 2000). We note that the fold repression cannot be accurately inferred from such measurements, since the *gfp* fluorescence signal is bounded from below by autofluorescence. We have measured autofluorescence in a separate experiment and found it to be approximately the same as the value measured here at the maximally repressed level. The time scale of maximal glucose repression is about 15 h, similar to time scales reported in the literature (Johnston *et al* 1994). The exact fold change in gene expression and its characteristic time scale depend on precise growth conditions and culture history, as illustrated below (see figure 4).

Extending the experiments to longer time scales while maintaining the population in pure rich glucose revealed that repression is only a transient response, as seen in figure 1(a). It was followed by de-repression, monitored as an increase in the fluorescence signal. The *gfp* expression was eventually stabilized at an intermediate level, between the highly induced level in pure galactose ($t = 0$) and the most repressed one in pure glucose ($t = 17$ h). The characteristic time

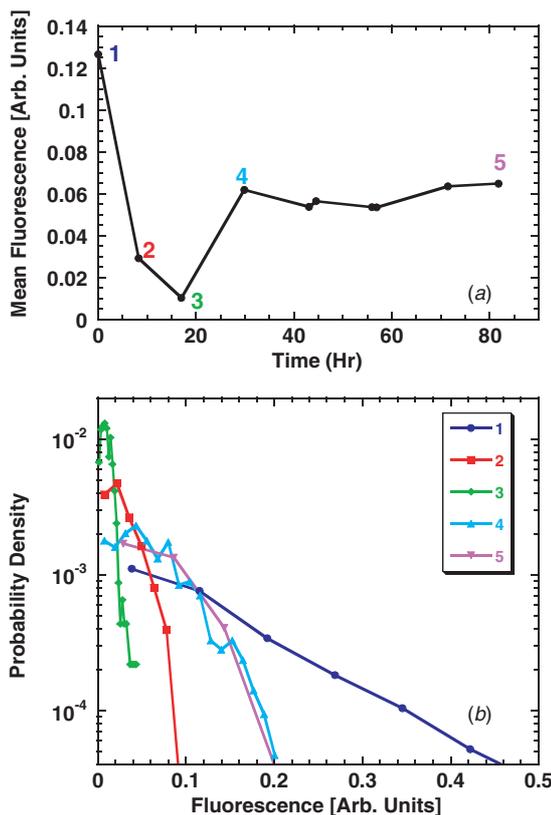


Figure 1. Glucose repression and de-repression in a serial-dilution batch experiment. (a) Kinetics of mean fluorescence signal in a population of yeast cells grown in minimal medium batch culture (see section 2) and serially diluted into a fresh medium upon reaching an optical density (OD) of 0.5. The fluorescence signal is from *gfp* downstream the *GAL10* promoter on a $2 \mu\text{m}$ plasmid. The sample was diluted from a medium containing 2% pure galactose to 2% pure glucose as sole carbon sources at time $t = 0$. The rest of the dilutions are into 2% pure glucose medium. (b) The population fluorescence distributions for the same experiment as in (a), see methods section for details. The numbers correspond to the time points marked in panel (a). Note the convergence of the distributions at the adapted state. Cell numbers and bin sizes in histograms: (1) 2123, 0.077; (2) 819, 0.014; (3) 419, 0.0022; (4) 353, 0.012; (5) 3026, 0.057.

scale for de-repression is >20 h, which is longer than the duration of classic experiments on glucose repression (Johnston *et al* 1994). Figure 1(b) shows the distribution of *gfp* signal in the population, computed as a histogram of several thousand single-cell measurements from the same fluorescence microscopy images as in figure 1(a) (see section 2 for details). Note the convergence of distribution curves at the adapted state in pure glucose. Since a distribution contains more information than the average value, the convergence of the expression distributions in pure glucose points to a high degree of stable expression in the population during the adapted state. It is worth emphasizing that the cells were not under stress at any stage; all measured phenotypic characteristics such as cell size, remained constant during the experiment. We also note that the culture in the steady state could be induced back to original fluorescence level by diluting it into galactose.

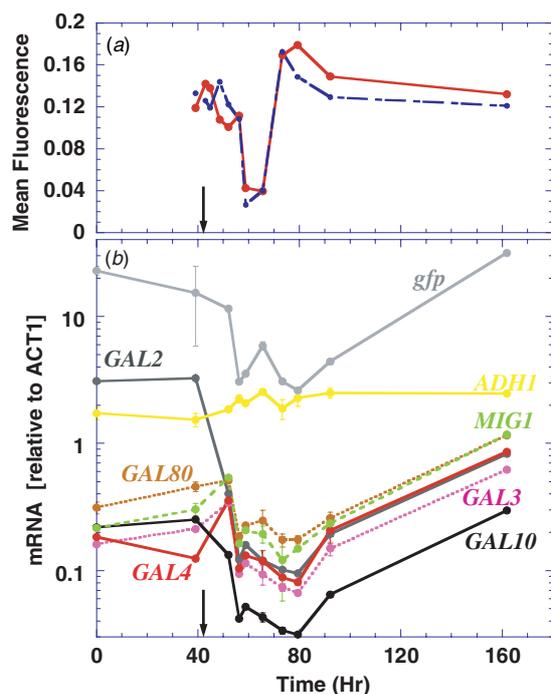


Figure 2. Glucose repression and de-repression in continuous culture (chemostat). (a) Mean (red) and standard deviation (blue) of fluorescence signal as a function of time for the same yeast strain as in figure 1 but now grown in continuous culture. The arrow marks the time of switching from pure 2% galactose to pure 2% glucose as sole carbon sources. The chemostat is amino-acid limited with a dilution rate 0.16 h^{-1} and OD 0.2 in galactose and 1.2 in glucose (see section 2). Similar results were obtained for nitrogen-limited chemostats (data not shown). (b) Real-time PCR measurements of mRNA transcription level (relative to *ACT1*) for various genes of the *GAL* network, *gfp*, *ADH1* and *MIG1* at different time points before and after a medium-switch from galactose to glucose (arrow). Cells are harvested from the same chemostat experiment as in (a). Note that both the mean fluorescence and mRNA adapt back to the same level as in galactose, though they do not follow exactly the same kinetics during adaptation.

The serial dilution method in the experiment of figure 1 does not provide steady growth condition, since dilution of a small subpopulation into a rich medium presents a considerable perturbation. Growing cells in a chemostat, however, allows steady growth in which the external environment and the mean population parameters (growth rate, cell density, etc) are all constant in time (Novick and Szilard 1950, Paquin and Adams 1983). To understand the meaning of de-repression in pure glucose and to characterize the adapted steady state we turn, for the rest of this paper, to chemostat experiments. Figure 2(a) shows the mean fluorescence signal as a function of time for the same strain as in figure 1, but now grown in an amino-acid-limited chemostat. The medium was switched from pure 2% galactose to pure 2% glucose at the point marked by the arrow and the rest of the experiment is in this fixed glucose environment. The cell density at steady state in the chemostat is different in glucose and galactose under the same limiting nutrients since glucose metabolism is more efficient. The fluorescence signal exhibits repression and de-repression kinetics similar to the serial dilution experiment

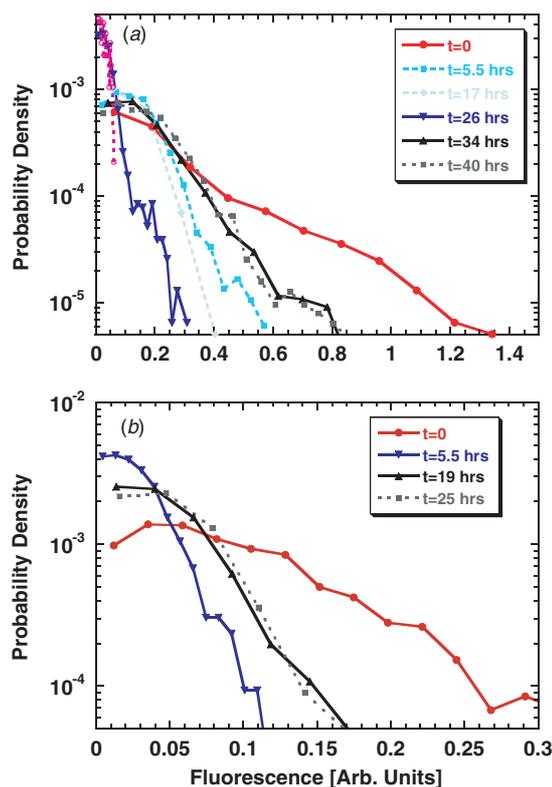


Figure 3. Population expression distributions during adaptation. (a) Population fluorescence distributions for the same chemostat experiment as in figure 2. The medium was switched from 2% pure galactose to 2% pure glucose as sole carbon sources at $t = 0$. The pink dotted line is the autofluorescence signal for cells containing no *gfp*. Cell numbers and bin sizes: 2169, 0.13; 2906, 0.05; 2392, 0.11; 1862, 0.02; 2945, 0.08; 2589, 0.05. (b) Population fluorescence distributions for the strain DBY9497 in phosphate-limited chemostat (dilution rate 0.2 h^{-1} , OD 0.3 in galactose and OD 1 in glucose) lacking amino acids in the medium (see section 2). The medium was switched from 2% pure galactose to 2% pure glucose as sole carbon sources at $t = 0$. Cell numbers and bin sizes: 1017, 0.023; 1014, 0.009; 792, 0.026; 728, 0.031. In both experiments, before switching from galactose to glucose, the cells were grown in pure galactose for long enough so the chemostat was in steady state.

of figure 1: the fluorescence signal was repressed to a level of approximately the autofluorescence, over a time scale of about 20 h. It adapted back to a level similar to the initial one over about 40 h. As discussed above, the precise values of these parameters depend on many details of the experiment, and were slightly different for chemostat and batch experiments. However, the basic phenomenon of de-repression appeared in both methods of culture growth. Moreover, the expression distributions shown in figure 3(a) are robust and largely independent of the chemostat conditions (dilution rates and limiting nutrient). For example, the results are essentially the same for a nitrogen-limited chemostat (data not shown) or a phosphate-limited one (see figure 3(b)). Thus, the chemostat itself was not involved in the mechanism of de-repression and played no significant role in the adaptation kinetics.

To further characterize the dynamics of transient glucose repression and subsequent de-repression, the population-mean mRNA transcription levels of different genes in the *GAL*

Table 1. Comparison of mRNA transcription levels in strains with *gfp* in plasmids and integrated. mRNA transcription levels (relative to Act1) were measured by real-time PCR as in figure 2. The cells were grown in a steady-state nitrogen-limited chemostat with pure 2% galactose (dilution rate 0.12, OD 1; see section 2). Errors are derived from two repeated measurements.

Gene	<i>GAL2</i>	<i>GAL3</i>	<i>GAL4</i>	<i>GAL10</i>	<i>GAL80</i>	<i>gfp</i>	<i>ADHI</i>
Integrated	5.8 ± 0.19	0.17 ± 0.004	0.22 ± 0.02	0.43 ± 0.05	0.42 ± 0.03	3.43 ± 0.13	2.45 ± 0.17
Plasmids	5.8 ± 0.15	0.15 ± 0.002	0.11 ± 0.003	0.69 ± 0.02	0.41 ± 0.006	20.8 ± 0.29	2.40 ± 0.13

network were measured directly as a function of time using real-time PCR (figure 2(b), see section 2). *GAL2* and *GAL10* are structural genes whose products participate in galactose metabolism; their dynamics show strong repression and de-repression. This behaviour is closely followed by *gfp* transcription. Importantly, the mean fluorescence signal (figure 2(a)) grossly follows the mRNA transcription pattern of the *GAL* network genes, ruling out the possibility that the observed adaptation is an artefact related to *gfp*. The regulatory genes (*GAL4*, *GAL3* and *GAL80*) show a behaviour similar to that of the structural genes during glucose repression and de-repression. Note that the ratios between the regulatory genes are preserved over time throughout the dynamics of repression and de-repression. The control measurement of *ADHI* is nearly constant during the experiment, while *MIG1*, a glucose repression mediator (Johnston and Carlson 1992, Johnston *et al* 1994), follows the same kinetics as the *GAL* genes. This may indicate that *MIG1* and the *GAL* network genes are co-regulated. In this experiment, *gfp* was introduced on vector plasmids. Populations with integrated *gfp* (single copy) exhibited similar behaviour (data not shown). Comparison of the real-time PCR measurements of a strain with plasmids and one with an integrated *gfp* shows that the transcription levels of all *GAL* network genes before and during adaptation are similar in both strains under the same chemostat conditions. Table 1 displays the steady state transcription levels of the two strains in galactose, showing the similarity in expression of all genes except for *gfp*. This shows that neither the load exerted by plasmid copy-number nor by *gfp* expression plays a role in the observed phenomenon.

To rule out the possibility that the observed de-repression is due to glucose depletion we measured the concentration of glucose in the chemostat. In the adapted state after de-repression the concentration of glucose was over 1%. In a separate experiment, we observed transient glucose repression followed by de-repression at external glucose concentration of ½% (data not shown). This shows that the observed adaptation was not due to a deficit of glucose (Brown *et al* 1998, Ferea *et al* 1999), but due to the fundamental difference in transcription regulation between transient response and steady state.

Single cell fluorescence measurements enable a complete characterization of the population expression by a distribution function. Combining steady-state growth in the chemostat with single-cell measurements, allowed us to explore the nature of expression in the population at steady state. Figure 3(a) depicts population distributions of the fluorescence signal constructed from measurements of several thousand cells from the same experiment as in figure 2. At the time point $t = 0$ the medium was switched from 2% pure galactose

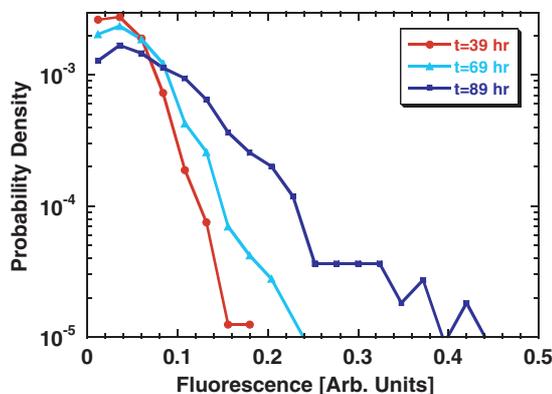


Figure 4. De-repression in pure glucose. The same experiment as in figure 3(a), in a minimal medium, amino-acid limited chemostat (dilution rate 0.2 h⁻¹, OD 2), with cells that have not been exposed to galactose and are grown continuously in pure 2% glucose as a sole carbon source. Adaptation dynamics nevertheless occur under this condition. Note the much slower kinetics of adaptation compared to that observed in figure 3(a) where the initial conditions were steady-state growth in pure galactose. Cell numbers: 3939 2933; 1993. Bin size 0.024 for all histograms.

to 2% pure glucose as a sole carbon source. Comparison of figures 3(a) and 1(b) shows that the serial dilution experiment and the chemostat experiment led to qualitatively similar expression distributions. The exact ratio between the highly induced state and the repressed state depends on the history of the population and the exact metabolism, and cannot be directly compared between the two experiments. Note also that since the chemostat is sampled at sparse time points, the lowest curve shown ($t = 26$ h) is not necessarily the deepest repression possible.

The generality of the de-repression phenomenon in rich pure glucose was further supported by repeating the same experiment with a different yeast strain (DBY9497) grown in a phosphate-limited chemostat (see section 2); figure 3(b) shows repression and de-repression and convergence of the steady-state distribution similar to figure 3(a). The total *gfp* expression levels are smaller in the phosphate-limited medium due to the weaker metabolism, hence the quantitative differences between the distributions of figures 3(b) and (a).

Cells grown in pure galactose and then transformed into pure glucose may retain memory of their galactose phase. Figure 4 shows population expression distributions for a similar chemostat experiment as in figure 3(a), for cells that were grown continuously in 2% pure glucose medium without having been previously exposed to galactose. Here, also, the repressed state was unstable and de-repression took over, but

with a characteristic time scale much longer than the one shown in figure 2 or 3(a). This demonstrates that the cells indeed have a memory of their history, even on a scale of many generations; previous exposure to galactose accelerated de-repression and adaptation.

It is of interest also to look at the dynamics and stability in pure galactose. The classic picture is that in the presence of rich galactose, the *GAL* system genes are highly induced (Johnston and Carlson 1992). We have found that high induction by galactose is also a transient response. Figure 5(a) shows that in pure 2% galactose the population of cells grown in a chemostat exhibited one out of two possible distributions. In particular, the distributions I–IV were measured from the same culture at different time points. The highly induced distribution (lines I–II) is thus a meta-stable state; it decays spontaneously to a lower one. This spontaneous transition occurred randomly in time with no apparent change in any of the chemostat parameters or the external environment. It occurred repeatedly in our experiments under different chemostat conditions, with nitrogen or amino-acid limitation, and within a range of dilution rates, 0.06–0.2 h⁻¹. While the onset of transition occurred at a random time, the transition itself once started lasted only a small number of generations (<5, see figure 5(b)). Therefore sampling populations at random time points revealed one of the two distributions shown in figure 5(a), but intermediate distributions were encountered only rarely and were not stable. The robustness of the two distributions is evident by the other curves shown in figure 5(a) (V–VI), which were measured from two other separate chemostat experiments. We emphasize that only the lower distribution is a true steady state of the system; a spontaneous transition backwards from the lower to the higher distribution has never been observed in our experiments. It is here that the chemostat technique becomes crucial: following the culture for long time scales enables the identification of its steady state.

Note that the minimal fraction of cells that must modify their expression level to account for the difference between the two distributions in figure 5(a) is ~0.2–0.3. This implies that the *GAL* network functionality changed significantly between two well-defined distributions in spite of galactose saturation and independent of its exact concentration. In the experiments discussed here, galactose was not the limiting nutrient (at least 1% inside the chemostat, as routinely measured during the experiments, see section 2). Changing medium from 2% to ½% galactose so that galactose became the limiting nutrient in the chemostat, induced a backward transition from the lower distribution to the higher one (figure 5(b)). The low concentration of galactose forced a higher mean expression level of the *GAL* system and consequently a reverse transition to the high distribution. The reversibility and rapidity of the spontaneous transition (measured here to be less than 25 h, ~5 generations) rule out genetic mutations as the mechanism of transition.

We can now fully characterize the steady-state expression distribution of the *GAL* system under conditions of saturated carbon source. Figure 6 summarizes the possible expression distributions and transitions observed in our experiments.

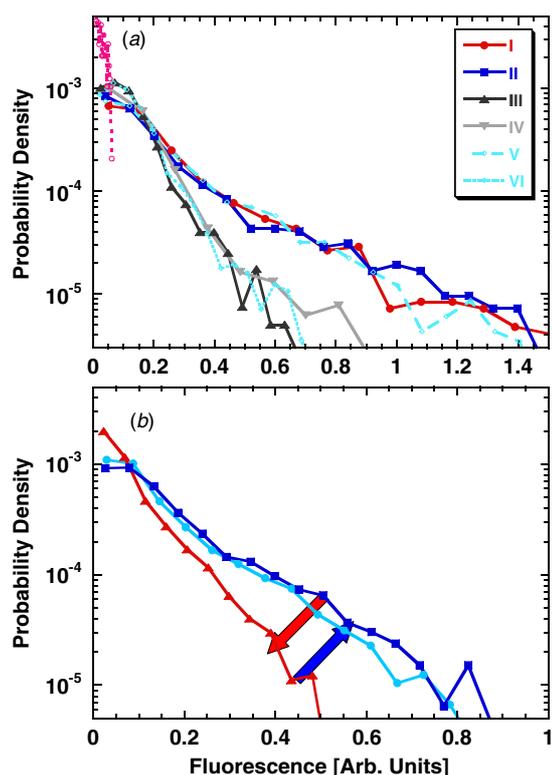


Figure 5. Spontaneous transition in galactose-rich medium and the reverse transition under galactose-limited conditions. (a) Population fluorescence distribution for the same yeast strain as in figure 1 grown in amino-acid limited chemostats in a fixed medium containing pure 2% galactose as a sole carbon source. Curves I–IV are measured for samples from the same chemostat (dilution rate 0.1 h⁻¹, OD 1) at different time points, after the chemostat has reached a steady state. The time points are (in hours): I-0, II-62, III-343 and IV-439. The transition between II and III is spontaneous and occurs at a random time without any apparent change in chemostat parameters. This transition is reproducible and occurred routinely in many of our chemostat experiments. The transition kinetics is very rapid once it starts and lasts only a small number of generations (<5). Once the transition has occurred, the population distribution is stabilized at the lower level and a spontaneous transition backwards to the upper level has never been observed in our experiments. Curves V (dilution rate 0.12 h⁻¹, OD 0.8) and VI (dilution rate 0.06 h⁻¹, OD 1.7) are measured from cells harvested from two separate chemostat experiments, demonstrating the stability of the two discrete distributions. Cell numbers and bin sizes: (I) 1613, 0.1; (II) 1075, 0.08; (III) 1720, 0.05; (IV) 2387, 0.11; (V) 2892, 0.08; (VI) 2547, 0.04. (b) The same phenomenon as in (a) for cells grown in a nitrogen-limited chemostat (dilution rate 0.2 h⁻¹, OD 0.3). Fluorescence distribution showing a spontaneous transition in a constant galactose-rich (2%) medium (light-blue to red curves—25 h difference). A reverse transition is found after switching the same chemostat to a galactose-limited (½%) medium (blue curve—53 h after switching media, OD 0.17). The rapidity of the spontaneous transition and its reversibility exclude genetic mutations as the underlying mechanism. Cell numbers and bin sizes: (light blue) 3644, 0.06; (red) 3946, 0.05; (blue) 1746, 0.05.

Remarkably, both in pure rich galactose medium and in pure rich glucose medium the *GAL* system exhibited the same steady-state expression distribution shown by the solid red and blue lines. The fact that the distributions coincide over more

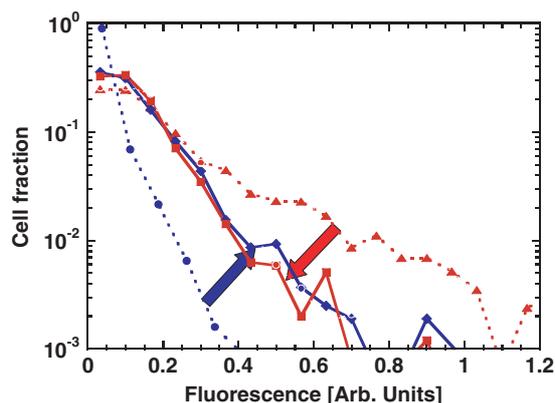


Figure 6. Summary of adaptive transitions to a robust steady state. Population fluorescence distributions as shown in figures 3(a) and 5(a). Cells grown in a chemostat with pure 2% galactose as a sole carbon source exhibit a spontaneous transition (red arrow) from a high expression induced state (red dotted line) into a lower expression steady state (red line). Cells grown in rich galactose medium and then switched to a pure 2% glucose medium exhibit a transient repression (blue dotted curve) and then adapt (blue arrow) to the same steady state (blue line) as in rich galactose medium. The dotted red line is the highly-induced state in

than two decades provides strong evidence for the similarity of the two expression states in the population, beyond an equality of means. The dotted red line is the highly-induced state in

pure rich galactose (same as curves I, II and V in figure 5(a)). This is a meta-stable state that decays eventually to the red solid line of lower mean (same as curves III, IV and VI in figure 5(a)). This decay is illustrated by the red arrow. The dotted blue line, on the other hand, is the repressed state in pure glucose (same as curve $t = 26$ h in figure 3(a)). De-repression, illustrated by the blue arrow, leads to the adapted state—the blue solid line (same as curves $t = 34, 40$ h in figure 3(a)), that is exactly the same as in pure galactose.

The fundamental difference between transient responses to stimuli and steady state is not surprising, since it is a property of many physiological systems. Specifically for yeast, this was shown to be the case for stress response (Gasch *et al* 2000). Measurements of other phenotypic characters in the population simultaneously with *gfp* expression levels enabled us to distinguish between transient and steady-state populations. During transient responses the fluorescence signal within each cell volume became spatially inhomogeneous. Figure 7 shows several characteristics of this inhomogeneity at different time points during the repression and adaptation transition in glucose. Typical cell images (top insets) show the transition from a uniform distribution of fluorescence signal (figure 7(a)) through a strong inhomogeneity (figures 7(b), (c)) and back to a uniform distribution at steady state (figure 7(d)). The spatial variance is a quantitative measure of *gfp*

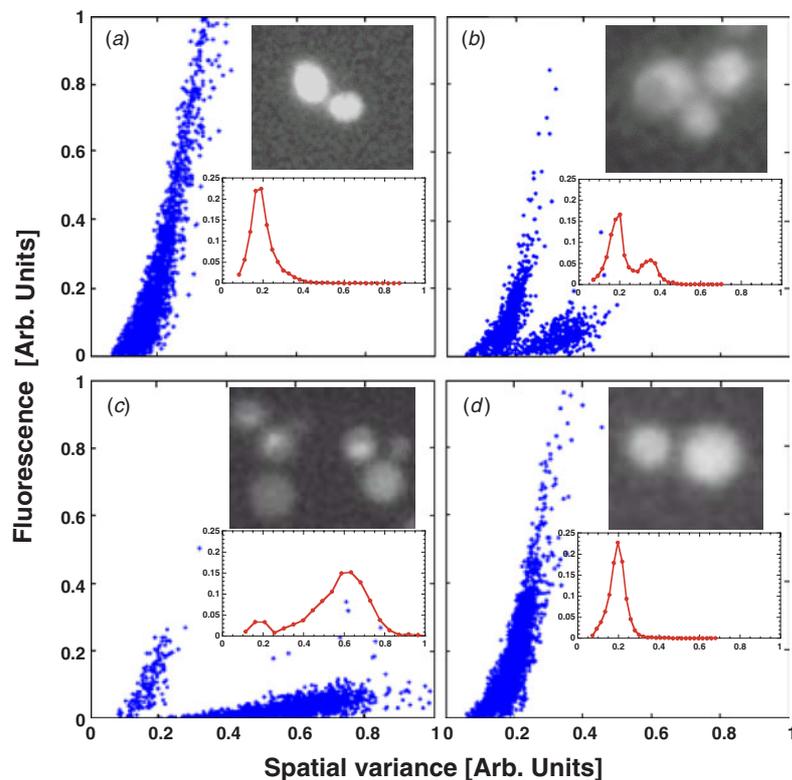


Figure 7. Fluorescence inhomogeneity distinguishes between transient and steady states. Scatter plot of the fluorescence signal versus spatial variance, for the same experiment as in figure 3(a), before (a), during (b-13 h, c-26 h) and after (d-40 h) the transient glucose repression (hours from switching media). Insets: upper—fluorescence images of typical cells; lower—distributions of spatial variance in the population.

inhomogeneity over the cell volume, and was computed for each cell in the population from the high-resolution microscopy images (see section 2). The main panels show scatter plots of this spatial variance versus total fluorescence in the cells. Lower insets show the population distribution of the spatial variance. Note the coexistence of two subpopulations at the transition phase. The increase in spatial variance is a clear signature of many transient responses observed in our experiments and is not unique to the glucose transient. Although the underlying mechanism is not yet understood, spatial variance distribution appears to be a sensitive phenomenological indicator of the state of the population that distinguishes in general between transient and steady states.

4. Conclusion and outlook

The *GAL* system in yeast is known as a model for metabolic regulation, switching between an induced state in the presence of galactose and a repressed state in the presence of glucose. In this work, we have extended the scope of previous experiments characterizing this system by opening a larger time window in a controlled environment, and by single-cell measurements in a large population. These extensions shed new light on the *GAL* system's adaptive dynamics.

A combination of a long-term growth in a chemostat under steady environment and single-cell measurements enabled us to distinguish specific transient response of the *GAL* system from its steady state. The steady state is a robust single state independent of the external carbon source. It can be reached by two different transient metabolic routes: (A) in rich galactose, the highly induced state is unstable and decays. This spontaneous transition involves a considerable fraction of the population and occurs over a small number of generations. Understanding the mechanism underlying this transition remains an important open issue: if the intracellular biochemical network is bistable, then a mixture of cellular behaviours is expected, which would be reflected by a wide range of distributions at the population level (Biggar and Crabtree 2001). Our experiments, however, result in one out of two discrete distributions with essentially no intermediates. (B) In rich glucose, the repressed state cannot be maintained and de-repression leads to exactly the same steady state as in galactose. The kinetics of de-repression has a typical time scale of several tens of hours, and seems to be a continuous transition. The details of these kinetics depend on the history of the culture: exposure to galactose prior to glucose accelerates the transition.

The existence of a single steady-state expression distribution under saturated carbon source, insensitive to the carbon source type, is a significant result. It shows that the *GAL* system at steady state loses its hallmark functionality as a sensitive switch. The simple picture of a two-state system—on in galactose and off in glucose—is only a transient one. The fact that multiple signals regulate the *GAL* transcription in yeast (Griggs and Johnston 1993, Rohde *et al* 2000) enables, in principle, an identical response under different environments.

The marked difference between steady-state behaviour and specific transient responses emphasizes the importance of physiological adaptive processes in gene regulation. There are several indications in the recent literature, which point to the fact that genetic regulatory systems exhibit a high degree of multifunctionality. In addition to responses that are specific to each input, there are also common stereotypical responses. For example, an environmental change that is suboptimal to yeast cell growth leads to a stereotypical stress response, common to many types of stresses and involving a remarkable fraction of the genome plus features that are specific to each stress (Causton *et al* 2001, Gasch *et al* 2000, Gasch and Werner-Washburne 2002). Other pieces of evidence imply that there are significant inter-modular interactions in the regulatory system. For example, it was shown specifically for the *GAL* system that gene deletions lead to the emergence of genome-wide changes in expression involving >900 genes (Ideker *et al* 2001). It is possible that at steady state there is a general genomic expression programme that results from the integration of the various input signals to the cells. This is a direct outcome of a highly interconnected regulatory system. Individual regulatory modules then respond transiently to specific inputs but the dynamics lead at steady state to a converged response when the inter-modular interactions take over. The principles governing this convergence at steady state may involve energy conservation, optimized metabolic fluxes and others. Like other physiological responses, there exists homeostasis in gene regulation; transients are then deviations from the homeostatic set-point.

A natural extension of the work presented here is to measure other regulatory modules by the same combination of chemostat and single-cell techniques to identify the steady-state expression distributions of the cell population. It is then possible to use DNA microarray to study the genome-wide expression pattern of the cells during adaptation and at steady state to gain insight into the inter-modular regulation mechanisms.

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