

Rapid GAL Gene Switch of *Saccharomyces cerevisiae* Depends on Nuclear Gal3, Not Nucleocytoplasmic Trafficking of Gal3 and Gal80

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ABSTRACT The yeast transcriptional activator Gal4 localizes to UAS_{GAL} sites even in the absence of galactose but cannot activate transcription due to an association with the Gal80 protein. By 4 min after galactose addition, Gal4-activated gene transcription ensues. It is well established that this rapid induction arises through a galactose-triggered association between the Gal80 and Gal3 proteins that decreases the association of Gal80 and Gal4. How this happens mechanistically remains unclear. Strikingly different hypotheses prevail concerning the possible roles of nucleocytoplasmic distribution and trafficking of Gal3 and Gal80 and where in the cell the initial Gal3–Gal80 association occurs. Here we tested two conflicting hypotheses by evaluating the subcellular distribution and dynamics of Gal3 and Gal80 with reference to induction kinetics. We determined that the rates of nucleocytoplasmic trafficking for both Gal80 and Gal3 are slow relative to the rate of induction. We find that depletion of the nuclear pool of Gal3 slows the induction kinetics. Thus, nuclear Gal3 is critical for rapid induction. Fluorescence-recovery-after-photobleaching experiments provided data suggesting that the Gal80–Gal4 complex exhibits kinetic stability in the absence of galactose. Finally, we detect Gal3 at the UAS_{GAL} only if Gal80 is covalently linked to the DNA-binding domain. Taken altogether, these new findings lead us to propose that a transient interaction of Gal3 with Gal4-associated Gal80 could explain the rapid response of this system. This notion could also explain earlier observations.

GENETIC regulation of transcriptional initiation occurs through environmentally responsive molecular switching systems that facilitate cellular differentiation (Reece 2000; Forsberg and Ljungdahl 2001). One such system in eukaryotes is the *Saccharomyces cerevisiae* GAL gene switch that regulates galactose-responsive transcription through the interplay of the Gal4, Gal80, and Gal3 proteins (Carlson 1987; Johnston 1987; Lohr *et al.* 1995; Traven *et al.* 2006). Gal4 is a DNA-binding transcription activator that has a single DNA sequence-specific binding domain (Pan and Coleman 1989; Reece and Ptashne 1993; Schjerling and Holmberg 1996) and a strong transcription activation domain (ADII; aa 768–881) (Giniger and Ptashne 1987; Johnston *et al.* 1987; Melcher and Johnston 1995; Ansari *et al.* 1998). Gal4 is found associated with the UAS_{GAL} DNA site(s) of the GAL genes in both absence and presence of galactose, but in the absence of galactose it is unable to activate tran-

scription due to masking of its ADII by Gal80 (Torchia *et al.* 1984; Lue *et al.* 1987; Ma and Ptashne 1987; Chasman and Kornberg 1990; Leuther and Johnston 1992; Mizutani and Tanaka 2003). Galactose facilitates association of the Gal3 and the Gal80 proteins, an event that brings about dissociation of Gal80 from Gal4 (Peng and Hopper 2002; Jiang *et al.* 2009) and subsequent Gal4-mediated recruitment of RNA polymerase to UAS_{GAL}-associated promoters (Torchia and Danzinger 1980; Johnston *et al.* 1987; Lue *et al.* 1987; Chasman and Kornberg 1990; Yun *et al.* 1991; Leuther and Johnston 1992; Parthun and Jaehning 1992; Melcher and Johnston 1995; Suzuki-Fujimoto *et al.* 1996; Wu *et al.* 1996; Blank *et al.* 1997; Yano and Fukasawa 1997; Koh *et al.* 1998; Platt and Reece 1998; Vollenbroich *et al.* 1999; Bhaumik and Green 2001; Jeong *et al.* 2001; Larschan and Winston 2001; Melcher and Xu 2001; Carrozza *et al.* 2002; Timson *et al.* 2002; Bryant and Ptashne 2003; Menezes *et al.* 2003).

A hallmark of the GAL gene switch is its rapid induction response. A *de novo* increase in GAL1 and GAL10 mRNAs is observed within 4–6 min of galactose addition to cells cultured at 30° (Yarger *et al.* 1984). This rapid induction of

transcription stems in part from the fact that a round of protein synthesis subsequent to galactose addition is not required for target gene activation (Perlman and Hopper 1979). The rapid response correlates well with the documented rapid recruitment of RNA Pol II to the *GAL1* promoter that begins ~2–4 min after galactose addition (Bryant and Ptashne 2003). Because Gal4-mediated recruitment of SAGA and Mediator complexes precedes recruitment of Pol II (Bryant and Ptashne 2003), it is apparent that galactose-activated Gal3 begins to relieve Gal80 inhibition of Gal4AD by 1–3 min following galactose addition. Accordingly, the mechanisms by which the galactose-activated Gal3 brings about Gal80 dissociation from Gal4 must be fast acting.

The inherent mechanisms and molecular species involved in the process by which Gal3–Gal80 interaction overcomes Gal80 inhibition of Gal4 remain unclear and subject to conflicting hypotheses. In this work, we addressed two unresolved questions: Where in the cell does Gal3 initially bind to Gal80 to trigger induction—the cytoplasm, the nucleus, or both? And, does nucleocytoplasmic trafficking of Gal3 and/or Gal80 play a role in the switch? There are two published hypotheses concerning the first question. One suggests that Gal3 interacts with Gal80 exclusively in the cytoplasm and sequesters it away from nuclear Gal4 (Peng and Hopper 2002). A strikingly different hypothesis specifies that cytoplasmic Gal3 binds galactose and then moves into the nucleus to bind to Gal80 (Wightman *et al.* 2008). These two hypotheses specify not only different subcellular compartments in which the initial interaction of Gal3 and Gal80 occurs, but also different nucleocytoplasmic trafficking dynamics for these proteins.

Deciphering the mechanisms that overcome Gal80's inhibition of Gal4 in live cells requires imaging the Gal3, Gal80, and Gal4 molecules, their associations, and their subcellular distributions and dynamics prior to and during the first few minutes of galactose addition. However, imaging these molecules expressed from their native promoters under such conditions has been hampered, as their levels in uninduced cells are very low (Wightman *et al.* 2008). Here we have employed spinning disc confocal microscopy (SDCM) to alleviate this problem and facilitate critical tests of competing hypotheses specifying GAL gene switch mechanisms. SDCM requires less excitation light to achieve an equivalent quality of signal compared to conventional confocal microscopy and thus provides a high quality of spatial and temporal resolution with relatively low photobleaching. These features make SDCM optimally suited for fluorescence recovery after photobleaching (FRAP) analyses and for tracking of small foci in live cells (Graf *et al.* 2005).

Using SDCM-based FRAP, we tested nucleocytoplasmic exchange rates of Gal80 and Gal3 molecules and dynamics of protein–protein interactions at the uninduced *GAL1* promoter. Our FRAP experiments showed that neither Gal80 nor Gal3 rapidly shuttle between nucleus and cytoplasm. These results challenge central mechanistic elements of two current hypotheses concerning the GAL gene switch,

one of which stems from this lab. Moreover, we discovered that a marked reduction of Gal3 levels in the nucleus diminishes the rate of induction from *GAL1* promoters. These results strongly suggest that the nuclear concentration of Gal3 is a critical feature of the GAL gene switch regardless of whether Gal3 interacts with Gal4-associated Gal80 or free Gal80. We also show that, in the absence of the galactose-induced Gal3–Gal80 interaction, the Gal80–Gal4 complex at the promoter-associated *UAS_{GAL}* is kinetically stable relative to the induction rate. The results are discussed in light of the older models of the GAL gene switch and lead us to propose a new model that highlights the importance of nuclear Gal3.

Materials and Methods

Strains

Genotypes of all the strains used in this study are presented in Table 1. *MATa* and *MATα* strains used in this study were derived from *S. cerevisiae* ScTEB652 (*MATa ade1 ile leu2-3,112 ura3-52 trp1-HIII his3-Δ1 MEL1*) and FY630 (*MATα his4-917δ lys2-173R2 leu2Δ1 ura3-52 trp1Δ63*), respectively (Arndt *et al.* 1995; Blank *et al.* 1997). Details of strain construction will be provided upon request. The array strains were constructed by consecutive insertion of pFJ58×64 and pFJ57×8 (Jiang *et al.* 2009) into Sc652 (*MATa* strains) and FY650 (*MATα* strains). The *GAL3*, *GAL80*, and *GAL4* genes were tagged in the genome with the green fluorescent protein (GFP) ORF using insertion cassettes obtained from pFJ120N, pFJ109N, and pFJ110N, respectively.

Plasmids

The plasmids used in this study are listed in Table 2. The NAT selection marker in plasmids that were used as a source of genomic insertion cassettes was isolated from Toda10 (a gift from Jian-Qiu Wu). pFJ04 was constructed by insertion of *SmaI/XhoI* fragments of pGP15 (Peng and Hopper 2000) and pSA80N1 (S. Alam and J. E. Hopper, unpublished results) into pRS416 (New England Biolabs). pFJ109N was constructed by simultaneous insertion of the NAT marker and a 2GFP cassette obtained from pKT209 (Sheff and Thron 2004) into pFJ04 by PCR-based and other molecular methods. pFJ110N was constructed by insertion of the NAT marker and the 2GFP cassette into the *GAL4*-containing plasmid, pLM34 (L. Mylin and J. E. Hopper, unpublished results). pFJ120N was constructed by insertion of the NAT cassette into pGP17 (Peng and Hopper 2000). Plasmids pOE180 [nuclear export sequence (NES)-Gal3] and pOE182 [mutant NES (mNES)-Gal3] were constructed as follows. To introduce *Rna1* NES and mNES (L₃₂₆, I₃₂₈) into Gal3, the region of *RNA1*-encoding residues (AA 316–357) was isolated from pGEM-T *Rna1* (AA 316–357) and pGEM-T *Rna1* (AA 316–357) (Feng *et al.* 1999) by PCR with primers OE167 (5'-CTCGTCGACGTCGAAAAGGGAAATTTACCTGA-3') and OE168 (5'-CTCGTCGACGTCCTCTTCAAAAATCGTCAACCT-3'). The resulting PCR products were inserted into the *AatII* site of pAKS15 to yield pOE180 (NES-Ga3) and

Table 1 Strains used in this study

Strain	Genotype	Source
RLY2800	MAT α his3 Δ 1 leu2 Δ 0 met15 Δ 0 ura3 Δ 0 Fus3-GFP	Slaughter <i>et al.</i> (2007)
M5739	MAT α ura3-52 leu2-3, 112 ade2-101 kar1-1	Vallen <i>et al.</i> (1992)
Sc723	MAT α ade1 ile leu2-3, 112 ura3-52 trp1-HIII his3-1 P _{GAL1} -HIS3	Blank <i>et al.</i> (1997) M5730
Sc724	MAT α ade1 ile leu2-3, 112 ura3-52 trp1-HIII his3-1 P _{GAL1} -HIS3 gal3 Δ	Blank <i>et al.</i> (1997)
Sc859	MAT α ade1 ile leu2-3, 112 ura3-52 trp1-HIII his3-1 GAL3-2GFP::NAT	Jiang <i>et al.</i> (2009)
Sc862	MAT α ade1 ile leu2-3, 112 ura3-52 trp1-HIII his3-1 GAL80-2GFP::NAT	This study
Sc920	MAT α ade1 ile leu2-3, 112 ura3-52 trp1-HIII his3-1 LacOX64::LEU2 (P _{GAL1} -GST) \times 8::Kan ^r GAL80-2GFP::NAT	This study
Sc930	MAT α ade1 ile leu2-3, 112 ura3-52 trp1-HIII his3-1 LacOX64::LEU2 (P _{GAL1} -GST) \times 8::Kan ^r GAL4-2GFP::CaURA3	This study
Sc932	MAT α ade1 ile leu2-3, 112 ura3-52 trp1-HIII his3-1 LacOX64::LEU2 (P _{GAL1} -GST) \times 8::Kan ^r GAL80-GFP::NAT gal3 Δ ::CaURA3 Gal80-2GFP::NAT	This study
Sc963	MAT α his4-9178 lys2-173R2 leu2 Δ 1 ura3-52 trp1 Δ 63 LacOX64::LEU2 (P _{GAL1} -GST) \times 8::Kan ^r Gal80-2GFP::NAT	This study
Sc965	MAT α his4-9178 lys2-173R2 leu2 Δ 1 ura3-52 trp1 Δ 63 LacOX64::LEU2 (P _{GAL1} -GST) \times 8::Kan ^r Gal80-2GFP::NAT	This study
Ds300 (Sc920 \times Sc963)	MAT α /ADE1/ade1 ile/ILE his3- Δ 1/HIS3 HIS4/his4-9178 LYS2/lys2-173R2 leu2-3, 112/leu2 Δ 1, ura3-52/ura3-52 trp1-HIII/trp1 Δ 63 LacOX64::LEU2 (P _{GAL1} -GST) \times 8::Kan ^r /LacOX64::LEU2 (P _{GAL1} -GST) \times 8::Kan ^r Gal80-2GFP::NAT/Gal80-2GFP::NAT	This study
Ds301 (Sc930 \times Sc965)	MAT α /ADE1/ade1 ile/ILE his3- Δ 1/HIS3 HIS4/his4-9178 LYS2/lys2-173R2 leu2-3, 112/leu2 Δ 1, ura3-52/ura3-52 trp1-HIII/trp1 Δ 63 LacOX64::LEU2 (P _{GAL1} -GST) \times 8::Kan ^r /LacOX64::LEU2 (P _{GAL1} -GST) \times 8::Kan ^r Gal4-2GFP::NAT/Gal4-2GFP::NAT	This study

pOE182. A single GFP ORF was inserted at the 3' end of Gal3 in pOE180 and pOE182 to make pOE183 and pOE185, respectively. The P_{GAL1}-2GFP reporter plasmid, pOE33, was constructed by PCR-based and other molecular methods in pRS416 (New England Biolabs). Construction of pFJ35 was described previously (Jiang *et al.* 2009). All PCRs for plasmid construction purposes were carried out with high-fidelity *Pfu* DNA polymerase (Stratagene). The oligonucleotides were purchased from Integrated DNA Technologies.

Microscopy

All microscopy experiments were performed with a Nikon TE-2000U spinning disc confocal microscope that was equipped with a \times 100/1.4 N.A. objective lens (Nikon, Melville, NY); 488-, 514-, and 568-nm argon ion lasers; and a charge-coupled device camera (ORCA-AG, Hamamatsu, Bridgewater, NJ). Live yeast cells were concentrated to appropriate cell density by centrifugation and immobilized on Superfrost slides (Fisher Scientific) coated with 2% gelatin mix. The experiments in which we monitored the effect of different media on a single cell were carried out using Y04C Cellasik Microfluidics plates (Cellasik) mounted onto the microscope stage.

The FRAP experiments testing the nucleocytoplasmic mobility of Gal3-GFP and Gal80-2GFP molecules were carried out with Sc859 and Sc862 cells, respectively. The cells were grown to mid-log phase in glycerol–lactic acid media. To produce sufficient levels of Gal80-2GFP signal, the Sc862 cells were induced with 2% galactose for 4 hr and then shifted back to glycerol–lactic acid media for 4 hr to establish nonexpressing conditions for GAL genes. It was previously demonstrated that Gal4–Gal80 interaction is reconstituted 2 hr after the cells were shifted from galactose to non-inducing conditions (Jiang *et al.* 2009). The nuclear or cytoplasmic regions of these cells were then bleached for 100 ms at 20% laser power to ablate the GFP signal in the target area. The cells were then monitored for signal recovery for indicated time periods. All fluorescence quantifications of the acquired images were carried out with the software ImageJ (Wayne Rasband, National Institutes of Health).

The photobleaching experiments that tested the stability of Gal80–Gal4 and Gal4–DNA interactions were carried out with diploid cells (Ds300 and Ds301, Table 1). A small sub-nuclear region overlapping one of the GFP spots and excluding the other spot was briefly bleached at 30% laser power for 100 ms.

GFP reporter assay

Sc724 cells expressing 2GFP from the native GAL1 promoter (P_{GAL1}) on pOE33 and wild-type Gal3 or NES-Gal3 or mNES-Gal3 from pAKS15, pOE180, or pOE182, respectively, were grown in 25 ml of glycerol–lactic acid media. Galactose was added (2% final) to induce synthesis of reporter GFP molecules. At indicated time points after induction, 3 ml of cells at OD₆₀₀ = 0.5 was taken from the culture, and

Table 2 Plasmids used in this study

Plasmid	Genotype	Source
pAKS15	CEN ARS1 TRP1 P _{GAL3} -GAL3	Sil <i>et al.</i> (1999)
pOE33	CEN ARS1 URA3 P _{GAL1} -2GFP	This study
pOE180	CEN ARS1 TRP1 P _{GAL3} -NES-GAL3	This study
pOE182	CEN ARS1 TRP1 P _{GAL3} -mNES-GAL3	This study
pOE183	CEN ARS1 TRP1 P _{GAL3} -NES-GAL3-GFP	This study
pOE185	CEN ARS1 TRP1 P _{GAL3} -mNES-GAL3-GFP	This study
pGP15Δ	CEN ARS1 URA3 P _{GAL80} -GAL80	Peng and Hopper (2000)
pGP17	CEN ARS1 TRP1 P _{GAL3} -GAL3-GFP	Peng and Hopper (2000)
pGP56GFP	CEN ARS1 TRP1 P _{GAL3} -myf-GAL3-GFP	Peng and Hopper (2002)
PGP57GFP	CEN ARS1 TRP1 P _{GAL3} -mom-GAL3-GFP	Peng and Hopper (2002)
pFJ35	CEN ARS1 TRP1 P _{ADH2} -Htb2-mCherry	Jiang <i>et al.</i> (2009)
pFJ109N	CEN ARS1 TRP1 P _{GAL80} -GAL80-2GFP::NAT	This study
pFJ110N	CEN ARS1 TRP1 P _{GAL4} -GAL4-2GFP::NAT	This study
pFJ120N	CEN ARS1 TRP1 P _{GAL3} -GAL3-GFP::NAT	This study
pME9	CEN ARS1 HIS3 P _{ADH2} -Lacl-mCherry	This study
pPS1739	CEN ARS1 URA3 P _{HOG1} -Hog1-GFP	Ferrigno <i>et al.</i> (1998)

cell pellets were collected. Protein extracts were prepared using the sodium hydroxide method, as described elsewhere (Kushnirov 2000). Proteins were separated with sodium dodecyl sulfate–10% polyacrylamide gel electrophoresis, and GFP was detected by Western blotting using anti-GFP (Invitrogen).

Results

Two previous studies failed to detect Gal3 in the nucleus of uninduced cells (Peng and Hopper 2000; Wightman *et al.* 2008). The apparent lack of Gal3 in the nucleus of uninduced cells in conjunction with other data led to two strikingly different hypotheses concerning how Gal3 works. One hypothesis posits that Gal3 works primarily, if not exclusively, in the cytoplasm to bind to Gal80, which at the time was thought to shuttle between cytoplasm and nucleus (Peng and Hopper 2002). Accordingly, it was proposed that cytoplasmic Gal3–Gal80 interaction would sequester Gal80 in the cytoplasm to free Gal4 from Gal80's inhibitory effect. In another study, Gal3 could not be detected anywhere in the uninduced cells (Wightman *et al.* 2008). Those authors hypothesized that galactose-bound Gal3 protein moves from the cytoplasm into the nucleus (Wightman *et al.* 2008). These hypotheses are called into question by recent observations. First, making Gal3 predominantly nuclear by fusing it to a strong nuclear localization sequence (NLS) does not alter the kinetics of induction (Jiang *et al.* 2009). Second, Gal3 expressed from its native gene is detectable within both the nucleus and the cytoplasm of live yeast cells grown in the absence of galactose (uninduced cells) under similar conditions as in the previous works mentioned above (Jiang *et al.* 2009). Considering these new observations, we used SDCM to evaluate the subcellular dynamics of Gal3 and Gal80 and to determine whether nuclear Gal3 plays a role in induction.

Cytoplasmic:nuclear ratio of Gal3 does not change in response to galactose

In a recent study, Gal3 was not detectable in uninduced cells but was readily detected in both cytoplasm and nucleus 45 min following galactose addition (Wightman *et al.* 2008). Accordingly, those authors proposed that, upon binding galactose, Gal3 translocates from the cytoplasm to the nucleus where it binds to Gal80. Here we used SDCM and a highly sensitive camera capable of detecting uninduced levels of wild-type Gal3-GFP in living cells to evaluate whether Gal3 moves from cytoplasm to nucleus in the absence or presence of galactose. In one series of experiments, we imaged Gal3-GFP nucleocytoplasmic distribution in cycloheximide-treated Sc859 cells before and after galactose induction. Htb2-mCherry expressed from pFJ35 served as a nuclear marker. In glycerol–lactic acid media, the Gal3-GFP was uniformly distributed between the two compartments, and this did not notably change throughout the 45 min following the addition of galactose (Figure 1A). In contrast, our imaging setup readily detected rapid subcellular redistribution of Hog1-GFP, which rapidly concentrated in the nucleus within 10 min after NaCl was added to the media (final: 500 mM) (Figure 1B) as was previously demonstrated (Ferrigno *et al.* 1998).

In another series of experiments, we utilized FRAP to evaluate whether Gal3 rapidly enters the nucleus. The nucleus of Sc859 cells as marked by Htb2-mCherry was photo-bleached briefly to eliminate the nuclear Gal3-GFP signal. Limited recovery of the GFP fluorescence in the nucleus was detectable beginning only after 450 sec, and the compartments did not fully re-equilibrate by 10 min (Figure 2A). This result was obtained for both cells in glycerol–lactic acid and galactose media. As a positive control, we performed FRAP with cells expressing Fus3-GFP, which is a known nucleocytoplasmic shuttling protein (Blackwell *et al.* 2003). The GFP signal in the bleached nuclei of these cells

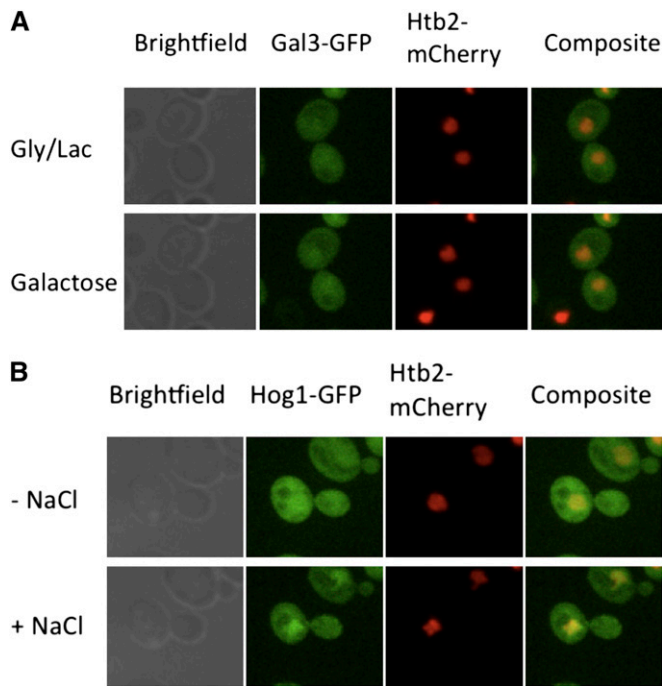


Figure 1 Gal3 distribution does not change in the course of early induction. (A) Sc859 (Gal3-2GFP) cells expressing Htb2-mCherry from pFJ35 were grown to mid-log phase in glycerol-lactic acid media and were placed into the Cellasik Onyx microfluidics system. Prior to imaging, cells were treated with cycloheximide for 15 min to prevent galactose-induced increases in Gal3-GFP. Images were acquired before and 45 min after galactose induction. (B) Sc723 cells expressing Hog1-GFP from pPS1739 and Htb2-mCherry from pFJ35 were grown to mid-log phase and were placed into the Cellasik Onyx microfluidics system. Images were acquired before and 10 min after osmotic stress with 500 mM NaCl.

exhibited apparently full re-equilibration within 15 sec (Figure 2C). These results provide direct evidence that Gal3 does not exhibit rapid nuclear import and challenge the hypothesis that Gal3 moves from the cytoplasm into the nucleus in response to galactose.

Diminishing the nuclear pool of Gal3 impairs the kinetics of induction

In earlier work from this lab, we were unable to detect Gal3 in the nucleus (Peng and Hopper 2000). Subsequently, it was found that fusing Gal3 to peptides for targeting to outer mitochondrial membranes (mom-Gal3) or vesicular membranes (myr-Gal3) outside the nucleus did not diminish maximal levels of induction (Peng and Hopper 2002). Taken together, those results led to the hypothesis that Gal3 acts in the cytoplasm and need not enter the nucleus to promote Gal4 activation (Peng and Hopper 2002). That hypothesis was called into question by the recent detection of Gal3 in the nucleus of uninduced cells (Jiang *et al.* 2009). However, the possible relevance of the nuclear Gal3 to the regulatory mechanism has not been addressed to date.

Here, we directly addressed whether depletion of the nuclear Gal3 affects induction. For this test, we fused Gal3-GFP to the strong NES consisting of residues 316–357 of

Rna1 (Feng *et al.* 1999). The NES-Gal3-GFP was observed only in the cytoplasmic region (Figure 3A). This striking exclusion from the nucleus was, as expected, attenuated in control experiments with the L326A, L328A double-mutant version of the NES (mNES) (Figure 3B) previously shown to impair NES activity (Feng *et al.* 1999). We then assessed the galactose-induction activity of NES-Gal3 compared to wild-type Gal3 and mNES-Gal3 using a galactose-inducible P_{GAL1} -2GFP reporter. Cells with NES-Gal3 showed impaired induction kinetics when compared to cells with wild-type Gal3. Moreover, the effect of the NES tag was alleviated when the leucine residues were replaced by alanines (Figure 4). These results indicate that a critical level of nuclear Gal3 is required for the normally very rapid GAL gene switch response to galactose.

The finding that some critical level of Gal3 within the nucleus is required for rapid induction challenges the previous hypothesis that Gal3 need not enter the nucleus to support induction (Peng and Hopper 2002). That hypothesis stemmed in part from results of studies using myr- and mom-tagged versions of Gal3 that highly localized Gal3 to vesicular membranes and mitochondrial outer membrane, respectively (Peng and Hopper 2002). Such tagged versions of Gal3 were shown to be similar to wild-type Gal3 in supporting maximal levels of induction as determined at 6 hr following galactose addition.

Although the myr-Gal3-GFP and mom-Gal3-GFP signals were seen in those previous experiments to be highly localized to the appropriate membranes outside the nucleus (Peng and Hopper 2002), the imaging sensitivity at the time did not permit detection of residual nuclear pools of these molecules. With our current greatly improved imaging capabilities afforded by SDCM and a more sensitive camera, we sought to determine the effectiveness of the myr, mom, NES, and mNES tags in depleting the nuclear pool of Gal3. We found that all of these tagged forms of Gal3-GFP, when compared to Gal3-GFP, show reductions of the nuclear Gal3-GFP pool (Figure 3). To better assess the relative effectiveness of the various tags in reducing nuclear levels of Gal3, we developed a novel sensitive assay for nuclear Gal3-GFP. This assay utilizes yeast containing a previously developed chromosomal UAS_{GAL} array (Jiang *et al.* 2009) consisting of 32 Gal4 UAS_{GAL} -binding sites associated with eight tandem GAL1 promoter-GST genes. The Gal3-GFP expressed in such cells formed a fluorescent spot at the site of the chromosomal UAS_{GAL} array within the nucleus only in galactose-induced cells that also expressed Gal80 fused to the Gal4 DNA-binding domain (DBD-Gal80) (Figure 5, A and B). When mNES-Gal3-GFP, myr-Gal3-GFP, and mom-Gal3-GFP were expressed, each of these formed spots at the array (Figure 5, C, D, and F). In contrast, NES-Gal3-GFP did not (Figure 5E). These results indicate that the myr, mom, and mNES tags are not as effective as NES in depleting the nuclear pool of Gal3 and thus provide a plausible explanation for the erroneous earlier proposal (Peng and Hopper 2002) that normal induction does not require Gal3 in the nucleus.

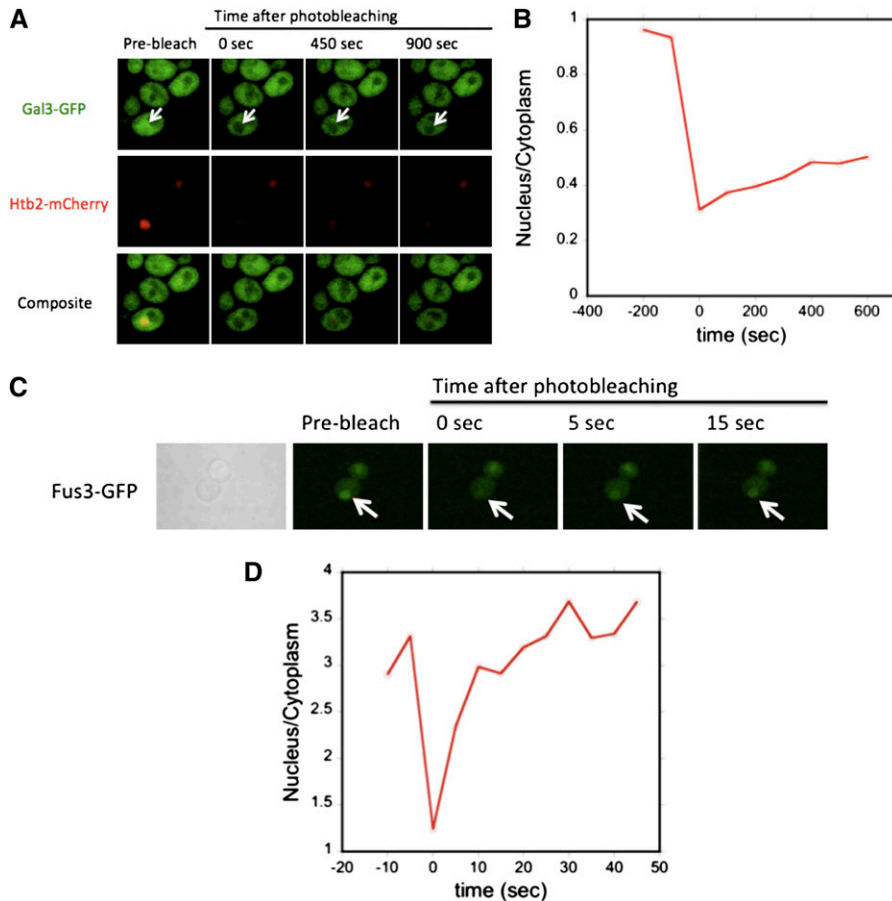


Figure 2 Gal3 does not move into the nucleus rapidly. (A and B) Yeast strain Sc859 nuclei, defined by the nuclear marker Htb2-mCherry from pFJ35, were bleached briefly. The recovery of GFP signal from Gal3-GFP was monitored up to 10 min, at which point only 19% re-equilibration was observed. (C and D) RLY2800 (Fus3-GFP) cells were grown to mid-log phase in glycerol-lactic media. Distribution of Fus3-GFP was imaged, and the indicated nucleus was bleached. The nuclear:cytoplasmic ratio of the molecules completely re-equilibrated <20 sec after bleaching.

Exchange between nuclear and cytoplasmic pools of Gal80 is slow

Earlier studies showed that either one of two distinct regions of Gal80, amino acids 1–321 and amino acids 341–423, were able to localize β -galactosidase to the yeast nucleus in long-term grown cultures (Nogi and Fukasawa 1989). Those results suggested that Gal80 has two independent NLSs. More recent studies showed movement of NLS^{SV40}-Gal80-GFP molecules from one nucleus to the other within the binucleate heterokaryon zygote formed in a *kar1-1* cross, suggesting the presence of an NES (Peng and Hopper 2000). These previous data led us to hypothesize that Gal80 shuttles between nucleus and cytoplasm. Thus, we surmised that galactose-activated Gal3, which was not detected in the nucleus at the time, sequestered Gal80 in the cytoplasm (Peng and Hopper 2002). The rapid shuttling of Gal80 molecules is one central feature of such a model; however, no evaluation of the kinetics of Gal80 nucleocytoplasmic trafficking has been reported.

Here we used FRAP to directly assess the subcellular dynamics of Gal80 in Sc862 cells. We bleached Gal80-2GFP in the cytoplasm and observed only ~15% recovery 10 min after bleaching (Figure 6B). When we bleached Gal80-2GFP in the nucleus, we observed only ~8% recovery 10 min after bleaching (Figure 6A). These results were in stark contrast to our FRAP results with Fus3-GFP, a known shuttling pro-

tein, that showed apparently full recovery 15 sec after nuclear bleaching (Figure 2C). We also used FRAP to test the kinetics of NLS-Gal80-GFP exchange between the two nuclei in a heterokaryon resulting from a *kar1* cross performed identically to that in the work of Peng and Hopper (2000). In the present work, and as previously reported (Peng and Hopper 2000), the NLS-Gal80-GFP appeared within both nuclei of the heterokaryons, even though it was expressed in only one of the two mating haploids and its expression was terminated immediately prior to the *kar1-1* cross (Figure 7). However, the FRAP analyses clearly showed that the NLS-Gal80-GFP in the unbleached nucleus did not re-equilibrate with the molecules within the bleached nucleus for up to 300 sec post bleach (Figure 7). Taken together, our FRAP results with the NLS-Gal80-GFP in heterokaryon zygotes and Gal80-2GFP in vegetatively grown haploid cells reveal that Gal80 does not shuttle rapidly between the nucleus and cytoplasm.

Assessment of the dynamics of the Gal80-Gal4-UAS_{GAL} and Gal4-UAS_{GAL} complexes

Because Gal3 is in the nucleus prior to galactose addition (Figure 1) (Jiang *et al.* 2009) and the nuclear pool of Gal3 is important for rapid induction kinetics (Figure 4), we wondered whether Gal3 might work by contacting Gal4-associated Gal80, as posited earlier in a nondissociation model (Platt

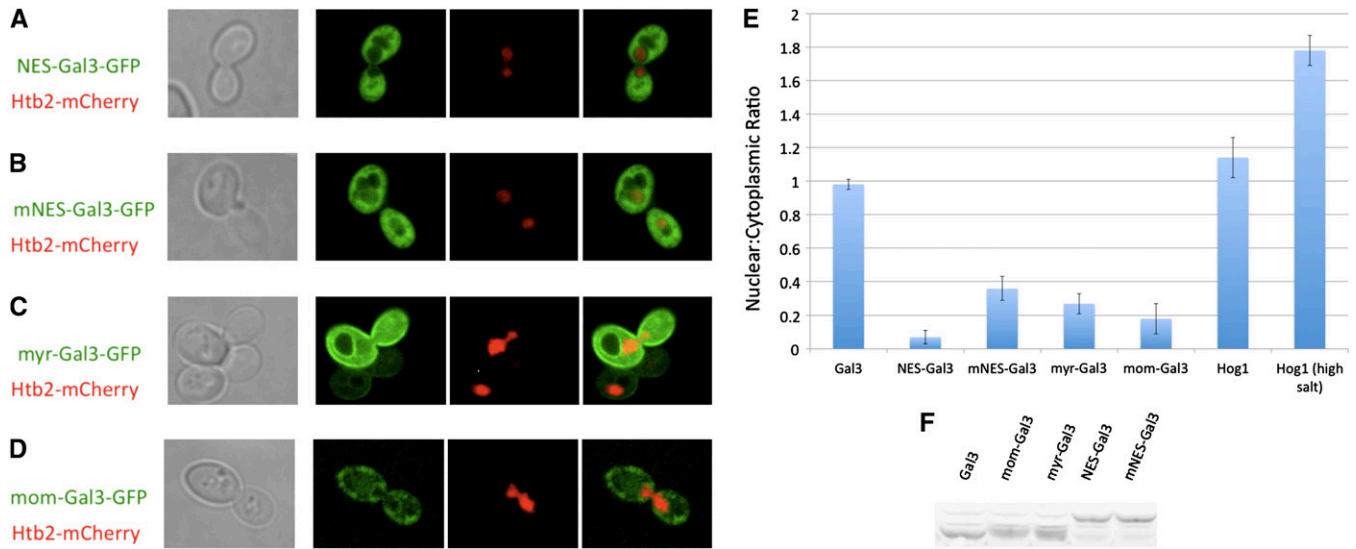


Figure 3 Effect of different tags on Gal3 distribution. Sc724 (*gal3Δ*) cells expressing (A) NES-Gal3-GFP from pOE183, (B) mNES-Gal3-GFP from pOE185, (C) myr-Gal3-GFP from pGP56GFP, or (D) mom-Gal3-GFP from pGP57GFP. All cells were grown to mid-log phase in glycerol–lactic media. (E) The ratio of the nuclear:cytoplasmic signal exhibited by the indicated molecule tagged with GFP was determined by measuring signal density. Ten cells were evaluated for each molecular species, and the results were averaged. The cells were induced for 4 hr in 2% galactose to enhance the GFP signal and were shifted back to gly/lac for 4 hr, at which time image acquisition began. The nuclear regions were marked by Htb2-mCherry that was expressed from pFJ35. (F) Total cellular levels of the various Gal3 molecules in the whole-cell extracts are demonstrated by the Western blot.

and Reece 1998). Although it is now clear that Gal80 does dissociate from Gal4, a galactose-triggered interaction of nuclear Gal3 with Gal4-associated Gal80 could be the event that triggers such dissociation. In such a case, rapid induction would be expected to be independent of the kinetic stability of the Gal80–Gal4 complex. If, on the other hand, Gal3 binds exclusively to free Gal80, we would expect that high kinetic stability (slow off-rate) for the Gal80–Gal4 complex at the UAS_{GAL} site would be rate limiting for induction. Accordingly, we evaluated the kinetic stability of Gal80–Gal4 complexes at UAS_{GAL} sites using a novel genetic assay consisting of diploid cells that have two of the above-mentioned UAS_{GAL} chromosomal arrays (Jiang *et al.* 2009). Expression of Gal80-2GFP or Gal4-2GFP in these cells allowed us to visualize Gal4–Gal80 complexes as two separated nuclear spots, one for each array (Figure 8). We bleached one of the spots and monitored the fluorescence recovery in the bleached array as well as loss of fluorescence in the unbleached array. The signal in the targeted spots of Gal4–Gal80-2GFP did not recover >10% within 10 min after bleaching. The lack of re-equilibration between the two spots within the same nucleus signifies very little dissociation of either the Gal4–Gal80-2GFP complex from the DNA or the Gal80-2GFP from the Gal4–DNA complex within 10 min (Figure 8C). In contrast, our experiments with Gal4-2GFP:Gal80 showed that the Gal4-2GFP molecules re-equilibrated between the two arrays within 5 min after photobleaching when the cells were in galactose media (Figure 8A), but not when they were in glycerol–lactic acid media (Figure 8B). These results indicate that the Gal4–Gal80 complex is kinetically stable.

Discussion

It is well established and widely agreed that the GAL gene switch functions through a galactose-triggered binding of Gal3 to Gal80 that relieves Gal80 inhibition of Gal4. How this occurs mechanistically is controversial (Platt and Reece 1998; Peng and Hopper 2002; Wightman *et al.* 2008; Jiang *et al.* 2009). Lack of agreement centers primarily on three issues: whether the molecular events that initiate induction involve the movement of Gal80 or Gal3 molecules between cytoplasm and nucleus; where in the cell the initial, induction-triggering interaction between Gal3 and Gal80 occurs; and whether Gal3 binds to Gal4-associated Gal80, to exclusively free Gal80, or to both forms of Gal80. To address these issues, we evaluated the kinetics of Gal80 and Gal3 movements between cytoplasm and nucleus and the dynamics of the Gal80–Gal4 complex.

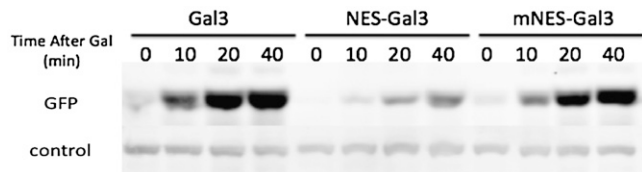


Figure 4 Effect of depleting nuclear Gal3 on induction kinetics. Sc724 (*gal3Δ*) cells were transformed with pOE33 (P_{GAL1}-2GFP) and pAKS15 (Gal3^{WT}), pOE180 (NES-Gal3), or pOE182 (mNES-Gal3). Cultures were grown to mid-log phase in glycerol–lactic acid media and induced for indicated times. The GFP produced at each time point was determined by a Western blot. A nonspecific band from the same blot served as a loading control.

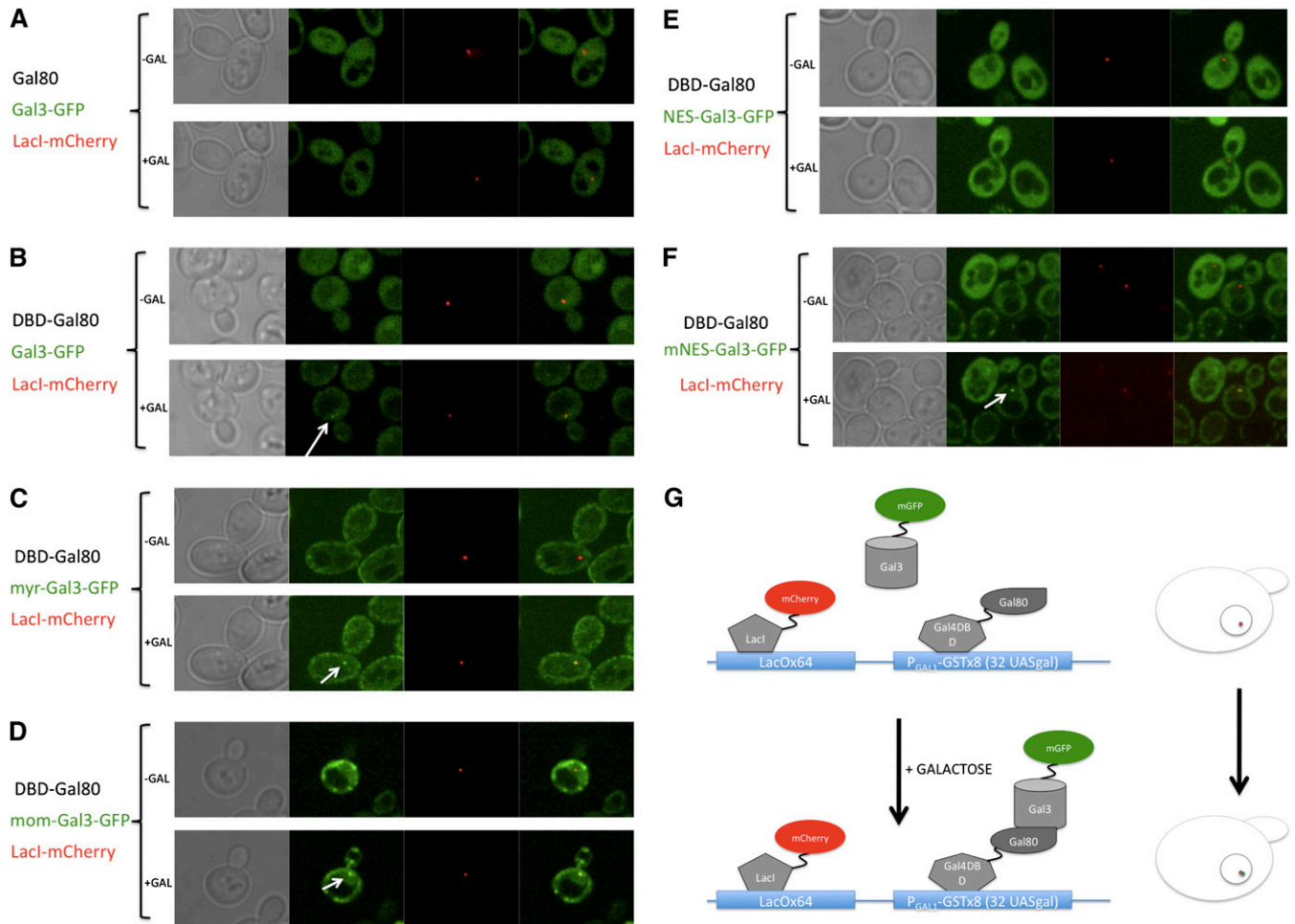


Figure 5 Effect of mis-localization tags on the nuclear Gal3 pool. In Sc932 (*gal3Δ*, [P_{GAL1}-GSTx8]:[LacOx64]:LEU2) cells, (A) Gal80 or (B–F) DBD-Gal80 was expressed from pOE142 or pOE165, respectively. The indicated Gal3 species were expressed from (A and B) pGP17, (C) pGP56GFP, (D) pGP65, (E) pOE183, or (F) pOE185. (G) Schematic presentation of the array system. The cells were grown in glycerol–lactic to mid-log phase and placed into chambers of Cellasik Microfluidics plates. Synthetic drop-out media with 3% glycerol and 2% lactic acid as carbon source were fed through the chambers for 20 min. Images of cells were obtained before and at 1 hr after galactose addition. The same cells were monitored for Gal3-GFP spots, which colocalized with LacI-mCherry expressed from pME9.

Gal80-2GFP movement between subcellular compartments is relatively slow

Our FRAP analyses involving Gal80-GFP in haploid cells and in heterokaryons revealed very slow kinetics for nucleocytoplasmic transport of Gal80-GFP molecules. This was in striking contrast to our results with Fus3-GFP, a protein known to exhibit rapid nucleocytoplasmic shuttling (Blackwell *et al.* 2003). These results, considered with respect to the rapid response of the GAL gene switch, challenge the hypothesis that nucleocytoplasmic shuttling of Gal80 is a central feature of the GAL gene switch mechanism (Peng and Hopper 2002). That hypothesis stemmed in large part from two studies. One previous study identified two different fragments of Gal80, designated NLS I and NLS II, which independently localized the *Escherichia coli* β-galactosidase protein to the yeast nucleus (Nogi and Fukasawa 1989). However, in that study, the subcellular localization was de-

termined following several generations of growth. Consequently, those results could be explained by slow entry of the Gal80–β-galactosidase fusion protein into the nucleus. The other previous study determined that NLS^{SV40}-Gal80-GFP moved from one nucleus (donor) to the other nucleus in a yeast heterokaryon, leading to the conclusion that Gal80 has an NES (Peng and Hopper 2000). In that work and in identical experiments carried out here, the time lag between the formation of the heterokaryon and image acquisition of the NLS^{SV40}-Gal80-GFP was 1–2 hr. Consequently, the results of the heterokaryon experiments can be explained by slow movement of NLS^{SV40}-Gal80-GFP from the one nucleus to the cytoplasm, followed by rapid entry into the other nucleus, due to the strong SV40 NLS tag on Gal80. Therefore, we conclude that Gal80 does not exhibit a rate of nucleocytoplasmic exchange typical of shuttling proteins.

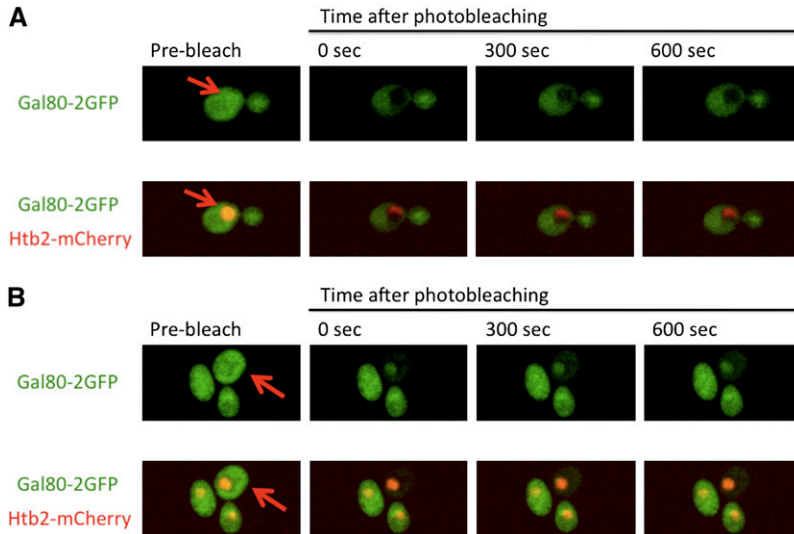


Figure 6 Subcellular distribution and dynamics of Gal80. Sc862 (Gal80-2GFP) cells expressing Htb2-mCherry from pFJ35 were grown to mid-log phase in glycerol-lactic acid media. The cells were induced with galactose for 4–6 hr to enhance the GFP signal and then shifted back to non-inducing media for 4 hr. The (A) nuclear or (B) cytoplasmic region was briefly bleached.

Gal3-GFP does not move rapidly into the nucleus

If Gal3 enters the nucleus upon binding galactose, we expected to see evidence of such redistribution by a decrease in cytoplasmic fluorescence and a corresponding increase in nuclear fluorescence in cells expressing Gal3-GFP. Our results show that the subcellular distribution observed for preexisting Gal3 (in non-induced cells) does not change rapidly in response to galactose. This conclusion is corroborated by the results of our FRAP experiments in which nuclear Gal3-GFP was bleached, and only a small fractional recovery was detected 10 min after bleaching. Thus, it is very unlikely that galactose causes entry of Gal3 into the nucleus to account for the rapid Gal4-mediated gene activation (induction).

Nuclear-localized Gal3 is required for rapid induction

The fact that Gal3 is in the nucleus prior to galactose addition and does not show rapid movement from cytoplasm to the nucleus in response to galactose prompted us to test the significance of nuclear Gal3. We find that strongly reducing the nuclear level of Gal3 by fusing it to an exogenous NES impairs the kinetics of induction. Thus, some level of Gal3 is required in the nucleus for the normal rapid induction kinetics. This conclusion is at odds with the conclusion from previous work that Gal3 entry to the nucleus is not required for maximal levels of induction (Peng and Hopper 2002). Peng

and Hopper's work showed that the levels of MEL1 gene-encoded α -galactosidase enzyme activity produced by cells at 6 hr after galactose addition was similar for cells expressing wild-type Gal3, myr-, or mom-tagged Gal3 variants (Peng and Hopper 2002). To explain the contradictory conclusions, we reasoned that the myr and mom tags might not be as effective as the NES tag in excluding Gal3 from the nucleus and that the magnitude of induction might not be as sensitive as the kinetics of induction to reduced nuclear levels of Gal3. The effectiveness of misplacing Gal3 with myr and mom tags was determined using conventional wide-field imaging (Peng and Hopper 2002). In addition, in this study when we used confocal imaging of non-array cells to compare the myr, mom, and NES tags, we found that they similarly depleted nuclear levels of Gal3-GFP. However, when we used a more sensitive photon-concentrating assay employing cells with the 32 Gal4-binding site (UAS_{GAL}) array and expressing DBD-Gal80, we detected a difference in the effects of the tags. The myr- and mom-Gal3-GFP but not NES-Gal3-GFP formed spots at the array, indicating that the myr and mom tags are not as effective as the NES tag in reducing the nuclear concentration of Gal3. Thus, we surmise that, in Peng and Hopper (2002), small pools of myr- or mom-tagged Gal3 in the nucleus were not detectable but were sufficient to support the maximal levels of induction observed at 6 hr, leading to the view at the time that Gal3 need not act in the nucleus.

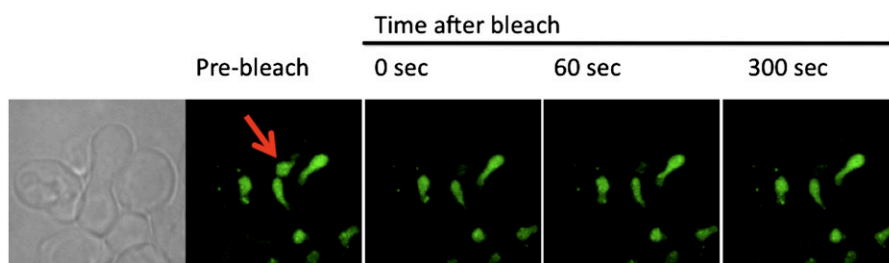


Figure 7 NLS-Gal80-GFP does not shuttle rapidly in heterokaryons. NLS-Gal80-GFP was expressed in wild-type MAT α cells from a GAL1/CYC1 promoter. The expression was repressed with 2% glucose for 2 hr, after which the cells were mated with MAT α kar1-1 cells. Binucleate heterokaryons appeared within 2 hr. The GFP signal was found in both nuclei of the heterokaryons. One of the two nuclei was bleached briefly, and the heterokaryon nuclei were monitored for 5 min.

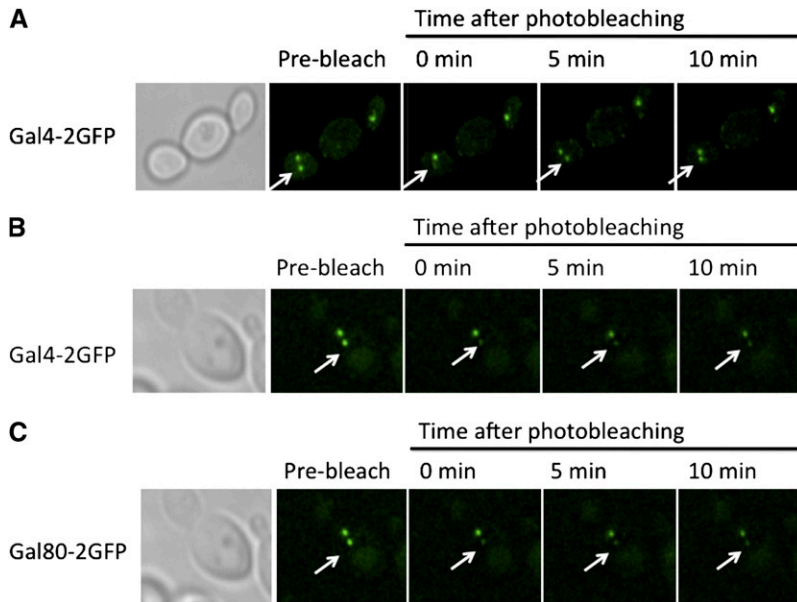


Figure 8 Dynamics of Gal4–DNA and Gal80–Gal4 interactions. (A and B) Sc930 (*MAT α* Gal4-2GFP [*P_{GAL1}-GSTx8*]:[LacOx64]:LEU2) and (C) Sc920 (*MAT α* Gal80-2GFP [*P_{GAL1}-GSTx8*]:[LacOx64]:LEU2) strains were mated with Sc965 (*MAT α* Gal4-2GFP [*P_{GAL1}-GSTx8*]:[LacOx64]:LEU2) and Sc963 (*MAT α* Gal80-2GFP [*P_{GAL1}-GSTx8*]:[LacOx64]:LEU2), respectively. The resulting diploid cells containing two arrays were grown to mid-log phase in (A) galactose or (B and C) glycerol–lactic acid media. One of the GFP spots in the cells was briefly bleached, and the cells were monitored for 10 min. (A) The Gal4-2GFP signal in the bleached array of the cells grown with galactose recovered completely within 5 min, while no detectable re-equilibration was observed for (B) Gal4-2GFP and (C) Gal80-2GFP spots in cells grown in glycerol–lactic acid media.

That even a small fraction of myr- or mom-tagged Gal3 in the nucleus is likely sufficient to support high levels of GAL gene expression is suggested also by our comparison of NES and mNES versions of Gal3-GFP. We found that mNES, like NES, diminished nuclear Gal3 in non-array cells (Figure 3B), but mNES-Gal3 supported induction kinetics very similarly to wild-type Gal3 (Figure 4). As expected, our sensitive array system showed mNES-Gal3-GFP spots at the Gal4-binding sites occupied by DBD-Gal80 (Figure 5F). Taken together, these results indicate that some minimal nuclear level of Gal3 is required to support the rapid induction kinetics that is characteristic of the GAL gene switch.

Stable Gal3–Gal80–Gal4 complex is not detectable in galactose-induced cells

Our finding that a reduction in the nuclear pool of Gal3 slows the induction kinetics together with our evidence against rapid movements of Gal80 and Gal3 between nucleus and cytoplasm implies that the nuclear pool of galactose-activated Gal3 might be critical for initiating rapid relief of Gal80 inhibition. Accordingly, nuclear Gal3 might act through binding to only free Gal80 or to both free Gal80 and Gal80 that is in complex with Gal4. Good evidence exists for Gal3 binding to free Gal80 *in vivo*, as Gal3-YFP–Gal80-CFP complexes have been detected in both cytoplasm and nucleus 25 min after galactose addition (Wightman *et al.* 2008). Also, Gal3 is able to bind to Gal80 *in vitro* in the absence of Gal4 (Yano and Fukasawa 1997). On the other hand, direct evidence for *in vivo* association of Gal3 with Gal4-associated Gal80 is lacking. In the course of these investigations, we attempted to detect such a complex. We first confirmed that we could detect Gal3-GFP as a fluorescent spot at the array in galactose-exposed cells expressing DBD-Gal80 (Figure 5B). However, when such cells expressed Gal80 instead of DBD-Gal80, a Gal3-GFP spot did not appear at the array in response to galactose (Figure 5A).

This result is inconsistent with a previously proposed model specifying nondissociation of Gal80 from Gal4 in response to galactose (Leuther and Johnston 1992; Platt and Reece 1998), but is consistent with the observed galactose- and Gal3-dependent dissociation of Gal80 from Gal4 in live cells (Jiang *et al.* 2009). While our results are negative, they are not inconsistent with a mechanism wherein Gal3 interacts with Gal4-associated Gal80 to cause immediate dissociation of Gal80 (presumably as a Gal3–Gal80 complex) from Gal4.

Gal80 complexed with Gal4 on DNA does not exchange rapidly with free Gal80

We lack any direct evidence that would distinguish between transient Gal3 binding and Gal4-associated Gal80 and Gal3 binding to only free Gal80. However, if the GAL gene switch works by Gal3 binding to only free Gal80, we would expect to observe rapid exchange between Gal4-associated Gal80 and free Gal80. Otherwise, a kinetically stable Gal80–Gal4 complex would be rate limiting for induction. To our knowledge, the rate of exchange, or dynamics, between Gal4-associated Gal80 and free Gal80 has not been determined. To evaluate the kinetic stability of the Gal80–Gal4 complex on the DNA, we carried out FRAP experiments with diploid array cells. We monitored re-equilibration between bleached and unbleached Gal80-2GFP spots. Re-equilibration is possible only if either the Gal4–Gal80-2GFP complex dissociates from a UAS_{GAL} or the Gal80-2GFP molecules dissociate from the Gal4–UAS_{GAL} complex at the array. Our results show that a bleached Gal80-2GFP spot did not re-equilibrate substantially with the unbleached spot over 10 min (Figure 8C). This lack of evident re-equilibration challenges a mechanism wherein Gal3 binds to only free Gal80.

On the other hand, Gal4 molecules exchanged between the two arrays within 5 min, when the cells were in galactose, but not in glycerol–lactic acid media. According to a previously proposed Gal4 cycle model, it was suggested that

Gal4 molecules became more dynamically associated with the DNA-binding sites after galactose induction (Muratani *et al.* 2005). However, other more recent work counters this idea by showing that Gal4 became more stable on the GAL promoters of induced cells (Ferdous *et al.* 2007). Our results from the FRAP experiments using diploid array strains are consistent with Gal4 becoming less stable on the DNA upon induction.

Transient association of Gal3 with Gal4-associated Gal80: a plausible mechanistic feature of the GAL gene switch

In light of the very rapid induction in this system, the relatively slow exchange of Gal80 between its Gal4-bound and free states observed here and the importance of nuclear Gal3 suggests a new plausible model. Transient interaction of nuclear Gal3 with Gal4-associated Gal80 might be what initiates the rapid galactose-mediated dissociation of Gal80 from Gal4. This notion is seemingly at odds with the previously reported detection of a Gal3–Gal80:Gal4 ternary complex by DNA-electrophoretic mobility shift analyses (EMSA) in polyacrylamide gels (Platt and Reece 1998). However, the form of Gal3 used in those EMSA experiments was not the wild-type Gal3 protein but rather a misense mutant Gal3 protein identified as not requiring galactose to bind to Gal80 (Blank *et al.* 1997). Moreover, it has been shown that exchange rates for macromolecular complexes can be 100-fold slower in gels than in free solution (Fried and Crothers 1981; Fried and Bromberg 1997; Vossen and Fried 1997). Thus, off-rates can be substantially underestimated by EMSA (Yang *et al.* 2002). Accordingly, we suggest the possibility that the association of Gal3 with Gal80–Gal4:DNA complexes observed previously might have been artificially stabilized by the gel environment and possibly also by the use of the Gal3 mutant protein.

The possibility of transient interaction of Gal3 with Gal4-associated Gal80 as a mechanistic feature of the GAL gene switch has two important testable implications. First, Gal3 interaction with Gal80 must occur on a surface separate from that to which Gal4 binds. Although the binding surface of Gal80 for Gal4 has been determined partially (Kumar *et al.* 2008), its surface for binding to Gal3 has not. Second, Gal3 transient binding to Gal80 must alter some property of Gal80 that is essential to its high-affinity binding with Gal4. One property of Gal80 that has been implicated in its association with Gal4 is its capacity to dimerize (Pilauri *et al.* 2005; Kumar *et al.* 2008), but no direct evidence exists for whether Gal3 interaction with Gal80 affects Gal80 self-association. These issues highlight the fact that much remains to be learned concerning the mechanisms involved in this seemingly simple gene switch.

Acknowledgments

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