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# Oscillatory nucleocytoplasmic shuttling of the general stress response transcriptional activators Msn2 and Msn4 in *Saccharomyces cerevisiae*

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Msn2 and Msn4 are two related transcriptional activators that mediate a general response to stress in yeast *Saccharomyces cerevisiae* by eliciting the expression of specific sets of genes. In response to stress or nutritional limitation, Msn2 and Msn4 migrate from the cytoplasm to the nucleus. Using GFP-tagged constructs and high-resolution time-lapse video microscopy on single cells, we show that light emitted by the microscope also triggers this migration. Unexpectedly, the population of Msn2 or Msn4 molecules shuttles repetitively into and out of the nucleus with a periodicity of a few minutes. A large heterogeneity in the oscillatory response to stress is observed between individual cells. This periodic behavior, which can be induced by various types of stress, at intermediate stress levels, is not dependent upon protein synthesis and persists when the DNA-binding domain of Msn2 is removed. The cAMP–PKA pathway controls the sensitivity of the oscillatory nucleocytoplasmic shuttling. In the absence of PKA, Msn4 continues to oscillate while Msn2 is maintained in the nucleus. We show that a computational model based on the possibility that Msn2 and Msn4 participate in autoregulatory loops controlling their subcellular localization can account for the oscillatory behavior of the two transcription factors.

## Introduction

All living cells have developed regulatory responses to stress. In addition to stress-specific transcriptional regulators, the yeast *Saccharomyces cerevisiae* uses two related transactivators, Msn2 and Msn4, that become active under most stress conditions and in the face of nutritional changes (Martinez-Pastor et al., 1996; Boy-Marcotte et al., 1998). These proteins are located in the cytoplasm, but in the presence of stress, they accumulate in the nucleus. Msn2 and Msn4 share a conserved NLS that is negatively controlled by high cellular PKA activity (Gorner et al., 1998, 2002). They bind to DNA cis-acting stress response elements (STREs)* (Martinez-Pastor et al., 1996) that control a large set of genes (Boy-Marcotte et al., 1998; Moskvina et al., 1998; Gasch et al., 2000; Causton et al., 2001). Activation of the transcription factors is transient and depends upon the strength of the stress (Gasch et al., 2000). The cytoplasmic localization of Msn2–GFP during normal growth reflects a dynamic steady state in which nuclear export predominates over import, because a block of export by the deletion of *MSN5*, which encodes the exportin, leads to nuclear accumulation of Msn2 (Alepuz et al., 1999; Gorner et al., 2002).

Using high-resolution time-lapse video microscopy on single cells, we followed the kinetics of the nuclear translocation of an Msn2–GFP fusion protein. Here we show that yeast cells sense as a stress light emitted by the microscope, as evidenced by Msn2 migration into the nucleus. Moreover, and unexpectedly, the populations of Msn2 and Msn4 molecules display an oscillatory behavior, shuttling repeatedly between the nucleus and cytoplasm with a periodicity of a few minutes. The DNA-binding domain of Msn2 is not essential for this periodic behavior. The cAMP–PKA system interferes with the nucleocytoplasmic shuttling by controlling the sensitivity of the stress-dependent transport of Msn2 into and out of

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*Abbreviation used in this paper: STRE, stress response element.

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the nucleus. In the absence of PKA, oscillatory shuttling of Msn4 continues while Msn2 is permanently localized in the nucleus. The occurrence of repetitive nucleocytoplasmic shuttling raises the possibility that Msn2 and Msn4 participate in autoregulatory loops, producing oscillations in their subcellular localization. A computational model based on such a regulation provides a theoretical framework accounting for the main observations on stress-induced oscillatory shuttling of the transcription factors.

## Results

### Oscillatory shuttling of Msn2 molecules

It has been shown that Msn2–GFP migrates to the nucleus shortly after application of acidic and other stresses and returns to the cytoplasm when cAMP is added to the medium (Gorner et al., 1998). To measure the kinetic parameters of nucleocytoplasmic trafficking of Msn2, we followed the previously described Msn2–GFP fused protein (Gorner et al., 1998) by using ultrafast three-dimensional time-lapse video microscopy, which gives improved images resolved in space and time (Sibarita et al., 2002). In the control experiments, with untreated yeast cells being maintained at a constant temperature under the microscope, we noted the migration of Msn2 to the nucleus in some cells of the population after a few minutes. More surprisingly, we observed a global oscillatory behavior of Msn2–GFP molecules entering and exiting the nucleus with a periodicity in the range of 3–6 min. This phenomenon, first recorded in a W303 genetic background (Fig. 1; see Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200303030/DC1), was reproduced in other yeast strains, such as SP1 and derivatives of S288c, with similar temporal patterns. It was also obtained with a construction expressing the Msn2–GFP fusion at a lower level, where the GFP is fused at the $NH_2$-terminal part instead of the COOH-terminal part of Msn2. Msn4–GFP was found to oscillate under similar conditions, with a comparable period (see Fig. 5 A). Oscillations were also observed under constant illumination and are therefore not due to operation of the shutter system.

As shown in Fig. 1 (A and B), the pattern of oscillations is heterogeneous; brief and partial migrations were often observed in cells in which complete translocations also took place. Despite the differences observed in sensitivity to stress, the kinetics of Msn2 nuclear import or export appear to be relatively constant within and between cells; when

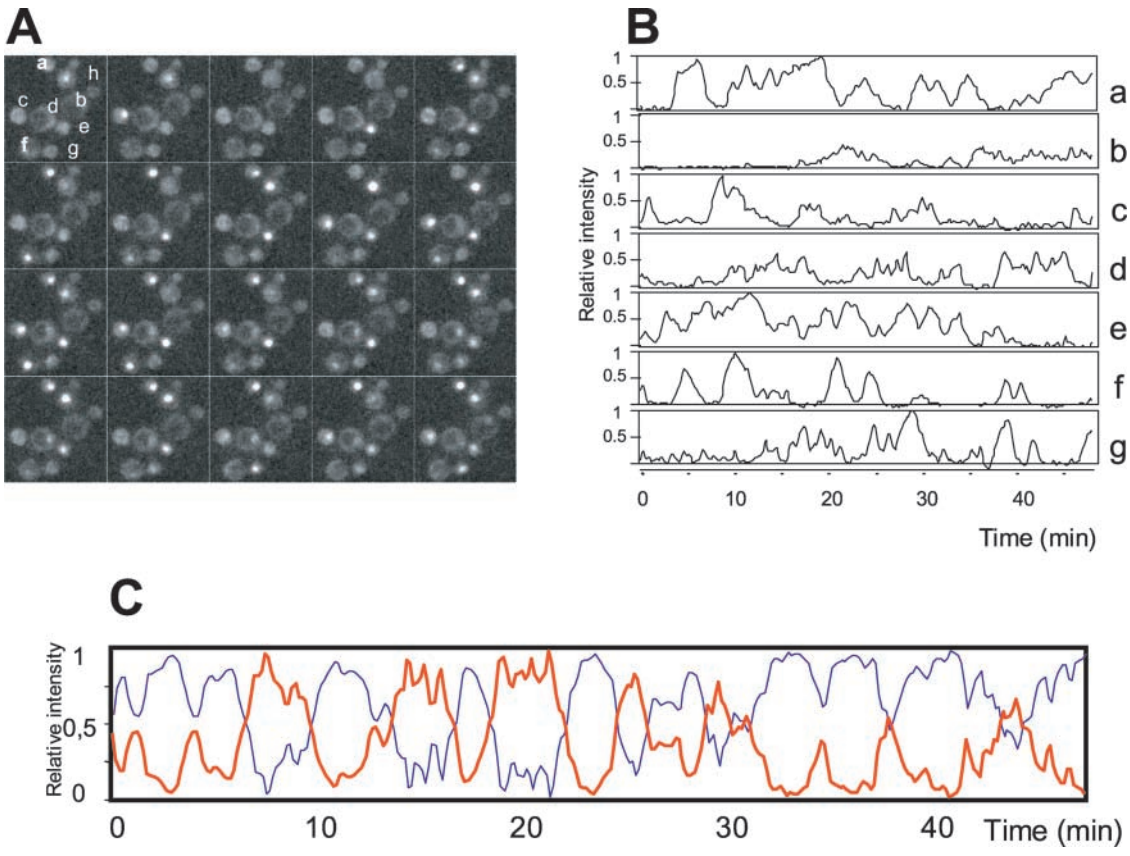


Figure 1. **Oscillatory nucleocytoplasmic shuttling of Msn2.** Video microscopic examination of W303 yeast cells containing the plasmid pAMG growing in YNB glucose at 30°C. The subcellular localization of Msn2–GFP was determined by means of time-lapse video microscopy. Fluorescent images were recorded over 48 min. (A) A sequence of pictures taken every minute during the first 20 min, displayed from left to right and top to bottom. (B) The normalized variation of fluorescence in the nucleus of the cells labeled a–g in A. (C) The kinetics of nuclear (red line) and cytoplasmic (blue line) intensity of fluorescence for cell h in A. Curves presented in B and C have been drawn from values taken every 10 s and smoothened by taking the average between two consecutive values (see Video 1, available at http://www.jcb.org/cgi/content/full/jcb.200303030/DC1).

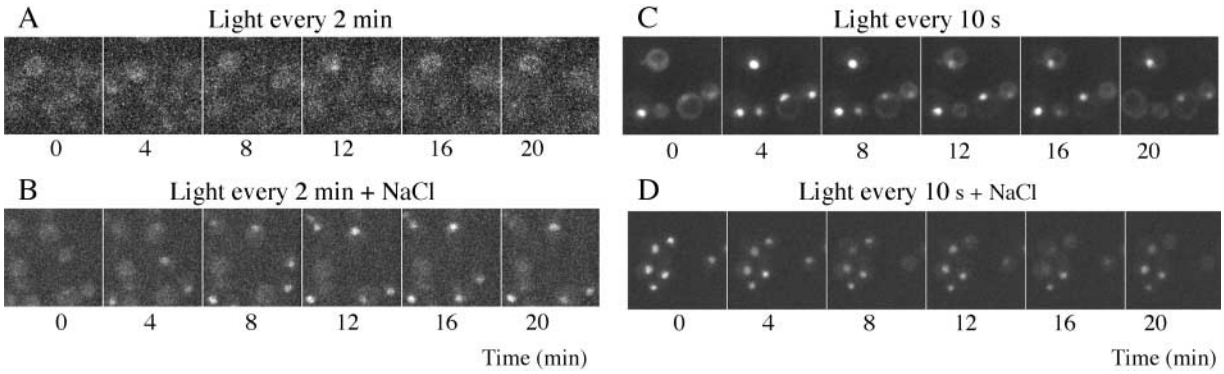


Figure 2. **Effect of light and stresses on Msn2 subcellular localization.** The same cells as in Fig. 1 were examined under various conditions of illumination as indicated above the images. (A and B) Pictures were taken every 2 min with a light intensity reduced to a minimum, giving a fourfold reduction in GFP fluorescence. No other stress was applied in A, whereas 0.5 M NaCl was added to the cell prior to examination in B. (C and D) Standard illumination was used (light applied for 1 s every 10 s) without other stress (C) and with 0.5 M NaCl (D). A subset of images taken at 4-min intervals is presented.

complete transfer from cytoplasm to nucleus occurred, as illustrated in Fig. 1 C, it was usually achieved in <2 min. Oscillatory shuttling occurred in cells at all stages of the cell cycle, even during mitosis. We recorded the cellular localization of Msn2–GFP for up to 3 h; if oscillations were sustained in some cells all over this time, Msn2–GFP stayed in the cytoplasm of most cells after 1–2 h. The progressive disappearance of oscillations is consistent with the transient nature of the transcriptional response to stress (Gasch et al., 2000; Causton et al., 2001).

### Light triggers migration of Msn2–GFP to the nucleus

To assess whether the illumination of cells under the microscope was responsible for the transfer of Msn2–GFP to the nucleus, we set the intensity of light to a minimum and took pictures every 2 min instead of 10 s. Under these conditions, as shown in Fig. 2 A, Msn2–GFP remained cytoplasmic during the 1-h period of observation, in contrast to the oscillatory shuttling observed under usual conditions with a higher light intensity (Fig. 2 C). However, some abortive episodes of shuttling, occurring in <2 min, were observed in a subset of the cells in the conditions of Fig. 2 A. We also determined the proportion of labeled nuclei in a cell population fixed with formaldehyde before examination, and found Msn2–GFP in the nucleus of <3% of several hundred cells. This is to be compared with an average occupancy of the nuclei ranging from 30 to 60% under illumination at higher light intensity during regular time-lapse recording. Thus, we may conclude that light induces nuclear localization of Msn2. The observation of a delayed cell division cycle in most experiments is another indication of a cellular stress response to illumination (Belli et al., 2001).

### Effect of other stresses on oscillatory shuttling

To know whether the oscillations induced under illumination are a general phenomenon associated with other stress responses of Msn2, we took advantage of the lack of response under minimal exposure to light and applied a variety of stresses to the cells. As illustrated in Fig. 2 B, the addition of 0.5 M NaCl induces nuclear localization as well as oscillations. Similar results were obtained with $K^+$ sorbate and hydrogen peroxide (unpublished data). We also applied various stresses on cells examined under the usual illumination conditions and found that although Msn2–GFP was present in the nucleus during longer periods of time than in the control, the oscillatory behavior persisted (Fig. 2 D). Probably due to a stronger stress effect, the addition of ethanol at a final concentration of 7.5% sent Msn2–GFP permanently to the nucleus, but lower amounts induced oscillations (unpublished data).

### Uncoupling oscillations from transcription activation

Entry into the nucleus per se is not sufficient for Msn2 activation. Indeed, as described by others and confirmed by us (unpublished data), despite nuclear localization of Msn2 in *msn5*Δ exportin mutants, transcriptional activation occurs only if a stress signal is applied to the cell. Once in the nucleus, activated Msn2 must interact with the regulatory elements of responsive genes through the transcription machinery. By removing the zinc finger domain of Msn2, we investigated whether the fixation of Msn2 to the STREs was required for oscillatory behavior. The truncated construct continues to respond to stress in regard to its nuclear localization and oscillates with a period similar to that observed for the complete protein (Fig. 3; see Video 2, available at http://www.jcb.org/cgi/content/full/jcb.200303030/DC1). To eliminate the possibility that the truncated Msn2 was driven through association with Msn2 or Msn4, we checked that the oscillatory behavior was maintained in a strain with

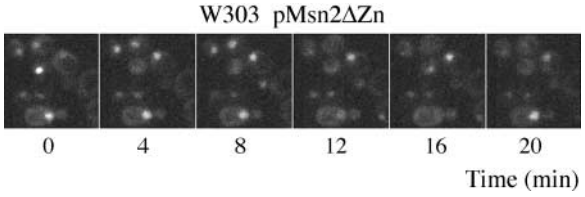


Figure 3. **Oscillatory behavior of Msn2ΔZn–GFP.** Video-microscopic images of localization are presented for a W303 cell containing pMsn2ΔZn–GFP (see Video 2, available at http://www.jcb.org/cgi/content/full/jcb.200303030/DC1).

a double deletion of these two genes (unpublished data). Therefore, the mechanism underlying the oscillations involves only the control of nuclear localization and not the STRE-dependent gene activation process.

Oscillatory shuttling also continued in the presence of defects of components of the transcriptional machinery that have been associated with the control of Msn2 activity. Thus, oscillations persisted in the absence of protein kinase Srb10, which belongs to the transcriptional complex and displays a negative effect on Msn2-dependent transcription and Msn2 phosphorylation (Chi et al., 2001). The Ccr4–Not complex, which negatively regulates Msn2-dependent transcription (Lenssen et al., 2002), is also not essential for the oscillatory mechanism because the *not1-1* mutation, known to affect the function of this complex, does not prevent oscillatory behavior. However, the average time spent in the nucleus for each of these mutants appears slightly longer than in the isogenic counterpart (unpublished data).

## Differential sensitivity of Mns2 and Mns4 oscillations to cAMP–PKA

Both the NLS and the putative nuclear export or cytoplasmic retention signal of Msn2 contain PKA-dependent phosphorylation sites whose substitutions alter nuclear import or export, respectively (Gorner et al., 1998, 2002). Thus, Msn2 nucleocytoplasmic shuttling is modulated by the activity of the cAMP–PKA pathway. In the absence of PKA, in a cell deleted for the three genes *TPK1*, *TPK2*, and *TPK3*, which encode the kinase catalytic subunits, Msn2–GFP stays permanently in the nucleus of all the cells (Fig. 4 C). To perform these experiments, the growth defect due to the lack of PKA was rescued by a deletion in the gene of kinase *YAK1* (Garrett et al., 1991); we controlled that this deletion does not significantly alter the oscillatory behavior of Msn2–GFP (Fig. 4 B). Conversely, high levels of cAMP in the phosphodiesterase mutant *rca1*/*pde2* (Wilson et al., 1993) maintain Msn2–GFP in the cytoplasm of most cells (Fig. 4 A). However, the shuttling mechanism was still operative in these cells. Oscillations indeed resume when an additional stress, such as osmotic or oxidative shock, is applied (unpublished data). Thus, the cAMP–PKA system increases the threshold level of the Msn2 response without disrupting the mechanism of oscillations. The role of PKA in the control of translocation of Msn2 was further demonstrated by the use of a mutant of *BCY1* (BC-N4), which encodes the regulatory subunit of PKA, deleted from its $NH_2$-terminal part involved in its nuclear localization (Griffioen et al., 2001). In this mutant strain, Msn2–GFP enters the nucleus upon stress but never leaves it (Fig. 4 D), which indicates a key role for nuclear PKA in the export.

In a cell defective in all three catalytic subunits of PKA, while Msn2–GFP remains accumulated in the nucleus as discussed above for low levels of cAMP (Fig. 4 C), Msn4 still responds to stress with an oscillatory behavior (Fig. 5 C; see Video 3, available at http://www.jcb.org/cgi/content/full/jcb.200303030/DC1), as in the wild type (Fig. 5 A) and in the control *yak1*Δ (Fig. 5 B). This dissimilarity in behavior reflects a differential sensitivity to stress of the two transcriptional regulators, as previously described in regard to their level of hyperphosphorylation (Garreau et al., 2000). It appears that Msn2 and Msn4 differ in their response thresh-

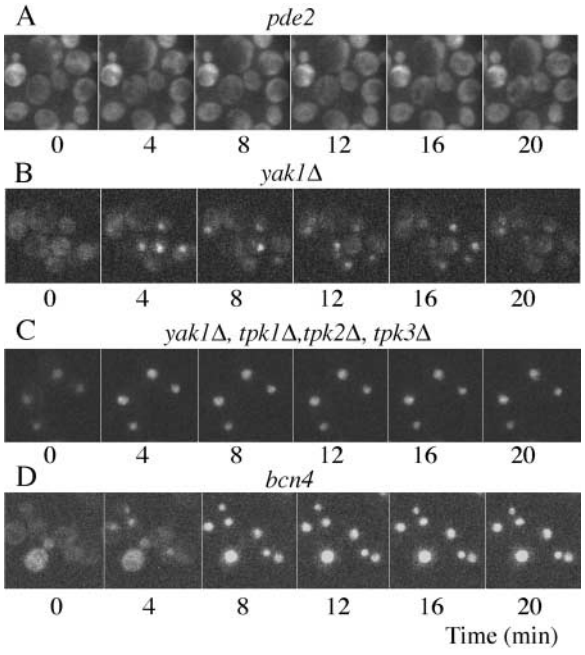


Figure 4. **Effect of mutations in the cAMP–PKA system.** Time-lapse video microscopy images of Msn2–GFP localization in various mutants of the cAMP–PKA system are presented. *pde2* stands for the OL564-1B strain with the phosphodiesterase mutation *rca1* in the *PDE2* gene (Wilson et al., 1993). *yak1Δ* corresponds to the OL607-47B strain deleted for this protein kinase. *yak1Δ, tpk1Δ, tpk2Δ, tpk3Δ* corresponds to the OL625-3 strain lacking all three PKA catalytic subunits (see Video 3, available at http://www.jcb.org/cgi/content/full/jcb.200303030/DC1). *bcn4* corresponds to the strain BC-N4 in which the *BCY1* gene coding for the regulatory subunit of PKA has been deleted from the $NH_2$-terminal part responsible for PKA nuclear localization (Griffioen et al., 2001).

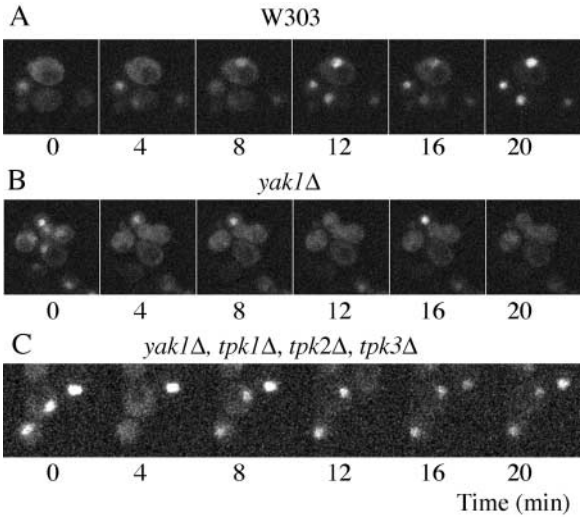


Figure 5. **Oscillatory behavior of Msn4–GFP.** Msn4–GFP behavior is shown in W303 cells (A), in *yak1Δ* cells (B), and in *yak1Δ, tpk1Δ, tpk2Δ, tpk3Δ* cells (C). Only the images taken at 4-min intervals are presented (see Video 3, available at http://www.jcb.org/cgi/content/full/jcb.200303030/DC1).

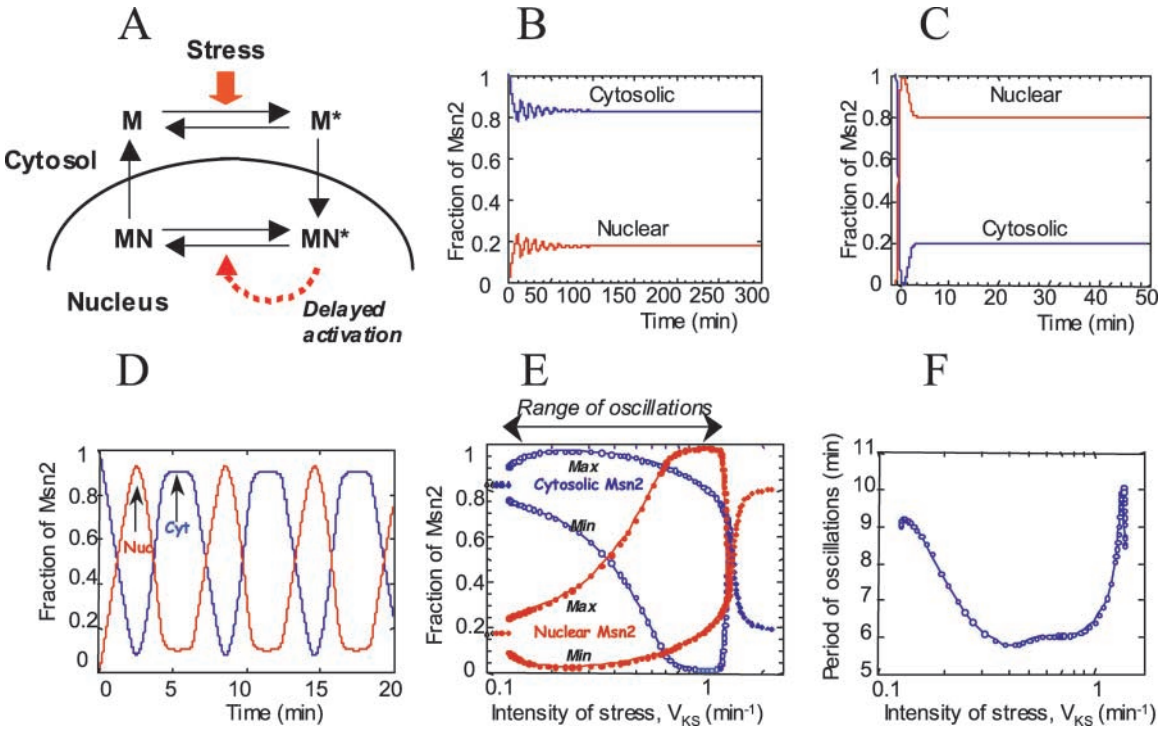


Figure 6. **Computational model for oscillatory nucleocytoplasmic shuttling of the transcription factor Msn2.** (A) A schematic model showing interconversions between the different states of Msn2: cytoplasmic, inactive (M); cytoplasmic, primed to enter the nucleus (M*); nuclear, active (MN*); nuclear, primed to leave the nucleus (MN). In the absence of stress, form M predominates. Under stress, the nuclear form MN* increases. Oscillatory nucleocytoplasmic shuttling relies on an autoregulatory loop involving Msn2 (dashed arrow marked "delayed activation"). One possible implementation of such a mechanism, capable of producing oscillatory shuttling, involves the activation, by the nuclear form of Msn2, of a bicyclic phosphorylation–dephosphorylation cascade, leading to the activation of a protein (e.g., a kinase or phosphatase) that would elicit Msn2 export from the nucleus (see Materials and methods for details and kinetic equations of the computational model; see Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200303030/DC1). (B–D) The time evolution of the subcellular distribution of Msn2 between the cytoplasm and nucleus predicted by the model. The curves illustrate different types of evolution as a function of the intensity of the stress measured by the maximum activity ($V_{KS}$, in $min^{-1}$) of the stress-activated enzyme. The transcription factor is predominantly in the cytosol (B, weak stress; $V_{KS} = 0.1$) or in the nucleus (C, strong stress; $V_{KS} = 2$), or oscillates between the cytosol and nucleus (D, intermediate stress; $V_{KS} = 0.7$). (E) Envelope of Msn2 oscillations showing the maximum and minimum values of the total fractions of Msn2 in cytoplasm and nucleus as a function of $V_{KS}$, the stress-controlled parameter. For the choice of parameter values (see Materials and methods), the range of sustained oscillations extends from $V_{KS} = 0.13$ to 1.39 $min^{-1}$. On each side of the oscillatory domain, a stable steady state is reached. (F) Variation of the period of nucleocytoplasmic shuttling as a function of $V_{KS}$. B–D show the total fractions of Msn2 in the cytosol (M + M*) and nucleus (MN + MN*). Data points in E and F represent results from numerical simulations. The curves were obtained as described in the Materials and methods.

old, thereby allowing the cell to exhibit a larger range of reaction to stress.

## A computational framework for sustained oscillations

If we try to simulate the translocations of Msn2 in a simple cyclical model (as schematized in Fig. 6 A in the absence of the dashed arrow in red), we cannot generate sustained oscillatory patterns because these require the existence of regulatory feedback. Two kinds of models could account for the oscillatory shuttling associated with the stress response: either an external biochemical oscillator (Goldbeter, 1996) drives Msn2 nucleocytoplasmic transitions, or oscillatory shuttling results from an autoregulatory loop involving Msn2. A putative external oscillator should be sensitive to stress to explain the induction of oscillatory shuttling and the increase in time spent in the nucleus as a function of stress intensity. What could be the nature of this putative external oscillator? Among known biochemical processes that oscillate with the appropriate period, glycolytic oscillations have for a long time been demonstrated in yeast, but they are induced by glucose rather than stress or illumination (Hess and Boiteux, 1971). Given that $Ca^{2+}$ oscillations rely in many cell types on $Ca^{2+}$-induced $Ca^{2+}$ release usually triggered by a rise in inositol 1,4,5-trisphosphate ($InsP_3$) (Berridge, 1997), their occurrence in yeast, which has not been reported, is unlikely, because the yeast genome analysis shows the absence of ryanodine or $InsP_3$ receptors. The possible occurrence of oscillations due to the peroxidase reaction (Olsen et al., 2001), which are generally much faster, remains to be investigated in yeast.

Nevertheless, the observation that in cells devoid of PKA activity, Msn2 remains confined to the nucleus while oscillatory nucleocytoplasmic shuttling continues for Msn4 can be explained more readily by assuming that each of these proteins directs its mechanism of subcellular localization. To produce oscillations, nuclear Msn2 (Msn4) should elicit, after a delay, the process that eventually leads to its exit from the nucleus, as schematized in Fig. 6 A by the ar-

row marked "delayed activation." This mechanism likely involves phosphorylation–dephosphorylation processes. Indeed, although PKA-dependent phosphorylation prevents nuclear localization by inactivating an NLS domain and activating nuclear export (Gorner et al., 2002), hyperphosphorylation by (an)other kinase(s) has been shown to correlate with activation and nuclear localization (Garreau et al., 2000). A computational model capable of producing oscillations, based on a simple implementation of the delayed activation mechanism, is presented in the Materials and methods section. We would like to stress that the modeling is not meant to "prove" a particular mechanism but to provide a theoretical framework, and to illustrate how a minimal mechanism based on phosphorylation–dephosphorylation can give rise to sustained oscillatory shuttling of the transcription factor. The interest of computational models is to throw light on the conditions in which sustained oscillations occur in regulated biochemical and cellular systems (Goldbeter, 2002).

The dynamic behavior predicted by the model is illustrated in Fig. 6 (B–D) for increasing values of parameter $V_{KS}$, which measures the maximum activity of the stress-activated enzyme that acts on Msn2 by catalyzing the conversion of M into M* in the scheme of Fig. 6 A. We assume that the value of parameter $V_{KS}$ increases with stress in the cytosol, but a similar model could be generated with an effect of stress within the nucleus. In the absence of stress, for a low value of $V_{KS}$ (Fig. 6 B), the system reaches a stable steady state in which Msn2 is located predominantly in the cytoplasm; this stable steady state is reached after damped oscillations. For a large stress corresponding to a high value of $V_{KS}$ (Fig. 6 C), the situation is reversed; close to 80% of Msn2 now resides in the nucleus while some 20% of the transcription factor is in the cytosol. It is at intermediate values of $V_{KS}$ (Fig. 6 D) that sustained oscillations occur, with Msn2 shuttling back and forth between the cytosol and the nucleus. In the case illustrated in Fig. 6 D, the total nuclear and cytosolic fractions of Msn2 oscillate in antiphase between ~10 and 90%, with a period of ~6 min.

The model predicts the existence of a range of $V_{KS}$ values producing Msn2 oscillations, bounded by two critical values of the stress enzyme activity (Fig. 6 E). As observed in the experiments, outside this domain, a stable steady state is found in which Msn2 is predominantly either in the cytosol, in the absence of stress, or in the nucleus, when stress is strong. The variation of the period in the oscillatory domain remains less than a factor of two (Fig. 6 F). Interestingly, when sustained oscillations begin as $V_{KS}$ passes its first, lower bifurcation value, the magnitude of Msn2 oscillations is relatively reduced (Fig. 6 E). Such behavior could account for the "aborted" oscillations, which are sometimes observed in the experiments. The oscillations predicted by the model are much more regular than those shown in Fig. 1. Fluctuations due to molecular noise could be included in the model by resorting to stochastic simulations. Such simulations yield good agreement with the predictions of deterministic models, as previously shown for circadian oscillations (Gonze et al., 2002; Goldbeter, 2002).

## Discussion

In this report, we described a novel dynamic behavior for transcriptional regulators in yeast, the stress-induced oscillatory shuttling of Msn2 and Msn4 between the cytoplasm and nucleus, with a period of several minutes. Although the nuclear translocation of a growing number of proteins in response to diverse signals is well documented, the synchronous oscillatory shuttling of transcriptional factors on a short time scale has not been reported so far. As suggested by the retention of Msn2 in the nucleus of cells lacking the exportin Msn5 (Alepuz et al., 1999; Gorner et al., 2002), the nuclear localization of a transcription factor results from a dynamic balance between import and export. Individually, transcription factors may go into and out of the nucleus, but these movements are largely uncorrelated, and when they are, they generally lack the repetitive nature associated with periodic behavior. The present results point to the occurrence of rapid, collective oscillatory movements between the cytoplasm and nucleus resulting from stress-induced synchronization of shuttling of a large number of transcription factor molecules.

The analysis of a computational model shows that, as in the case of other oscillatory phenomena such as $Ca^{2+}$ oscillations (see Box 1 figure in Goldbeter, 2002), such periodic behavior occurs only in precise conditions, in a range bounded by critical parameter values. At low stress intensities, the transcription factors remain in the cytoplasm, whereas at high stress levels, they remain in the nucleus. Only at intermediate strength of the stress do they oscillate between the cytoplasm and nucleus. These observations, accounted for by the model, explain why oscillations occur only for sufficiently high light intensity and why moderate ethanol stress triggers oscillations, whereas at higher levels, ethanol sends Msn2 permanently to the nucleus. The occurrence of aborted translocations, observed in some cells, is consistent with the existence of thresholds in the molecular mechanism underlying oscillations. Aborted transitions can also be viewed as corresponding to the low-amplitude oscillations predicted by the computational model at low stress intensity (see the envelope of the oscillations as a function of stress in Fig. 6 E).

Our results demonstrate the ability of light to trigger nuclear localization of Msn2 and Msn4. The shuttling behavior of these stress-sensitive transactivators was not induced at very low intensity but occurred readily with stronger illumination. The time spent in the nucleus by Msn2 also correlated with the intensity of light (unpublished data). The excitation of GFP fluorescence is likely to release reactive oxygen species that could then produce an oxidative stress. More generally, these observations suggest that the state of the cells containing GFP molecules can be modified by simple observation with a fluorescent microscope.

The sensitivity of the cells is controlled by the activity of the cAMP–PKA pathway. In contrast to normal cells, in which oscillations were induced by illumination under the microscope, in cells defective in phosphodiesterase Pde2, additional stress was required to trigger nuclear migration of Msn2. Thus, the cAMP–PKA system controls the sensitivity of Msn2 oscillations by affecting the kinetics of import and export of Msn2. Indeed, PKA-dependent phos-

phorylation negatively regulates the NLS of Msn2 (Gorner et al., 1998). In addition, we showed here that delocalization from the nucleus of the PKA with a truncated regulatory subunit prevents exit of Msn2 from the nucleus. As a consequence, without PKA, Msn2–GFP accumulates in the nucleus (Fig. 4) whereas Msn4–GFP still oscillates in the same strain. Thus, Msn2 and Msn4 present differences in regulation by, or sensitivity to, cAMP, as previously reported at the level of phosphorylation (Garreau et al., 2000). With a narrow window for transcription activation due to thresholds in transport to the nucleus, it might be useful for the cell to have two sensors with high and low sensitivity. Msn2 and Msn4 are involved in lower and higher stress response, respectively.

The large variability in shuttling behavior observed among cells in the same microscopic field (Fig. 1) likely reflects cellular heterogeneity in sensitivity to stress. In line with a recent study that demonstrated, by FACS® analysis, the heterogeneity of gene expression in response to stress in a yeast population (Attfield et al., 2001), the present data highlight the variability at the single-cell level of a so-called homogenous population. In spite of this heterogeneity, the time required for Msn2 and Msn4 to enter the nucleus is quite constant among cells, irrespective of the triggering conditions. Such transfer time is similar to that measured in a mammalian cell for the entry of a GFP-fused protein possessing an NLS (Ribbeck and Gorlich, 2001).

In the cases where an oscillatory expression program has been described (Darlington et al., 1998; Lev Bar-Or et al., 2000), an autoregulatory loop involving the expression of some controlling element is involved. Thus, periodic nucleocytoplasmic shuttling of the transcriptional regulators PER and TIM occurring on a circadian time scale in *Drosophila* involves autoregulation of gene expression (Sehgal et al., 1995; Young, 1998). Autoregulation of protein synthesis and degradation underlies oscillations of the tumor suppressor p53, which occur with a period of ~3 h (Lev Bar-Or et al., 2000). On a similar time scale, oscillations have recently been described for NFκB (Hoffmann et al., 2002). The phenomenon involves synthesis and degradation of an inhibitor and has been accounted for by a computational model. Yet another example of oscillatory gene expression is provided by the segmentation clock that controls somite formation in embryogenesis with a periodicity of ~90 min (Maroto and Pourquie, 2001). The latter oscillations, based on negative autoregulatory feedback on transcription (Dale et al., 2003), have recently been shown to occur with a 2-h period in serum-treated cultured cells (Hirata et al., 2002).

We have ruled out the possibility that the mechanism of Msn2 oscillations involves the control of protein synthesis because oscillations are not affected by the addition of cycloheximide (unpublished data). Moreover, the fact that oscillations occur with an Msn2 truncated from its DNA-binding domain, which is not capable of transcriptional activation, eliminates an effect directly mediated by STRE-dependent gene expression. The molecular mechanism underlying the oscillations remains to be further characterized. The fact that protein synthesis and DNA binding of Msn2 are not required suggests a control at the level of import or export of the transcription factors.

To our knowledge, the present experimental observations provide the first report of rapid oscillatory shuttling of a transcriptional activator between the cytoplasm and nucleus. Because they are not based on transcriptional regulation or de novo protein synthesis, oscillations in the subcellular localization of Msn2 inaugurate a new class of periodic processes controlling gene expression. The proposed computational model provides a unifying explanatory framework for the observations and should help to unravel the molecular mechanism of the phenomenon. It predicts the existence of one or more novel components in an autoregulatory loop that primes Msn2 and/or Msn4 for nuclear export. These components are likely to be kinases and/or phosphatases. In regard to the physiological significance of oscillations, shuttling between the cytoplasm and nucleus permits the sensing by Msn2 of the state of the cell and its adaptation in both compartments to the level of stress. Oscillations allow the generation of maximum responses during brief episodes during which the transcription factor reaches high levels that could have detrimental effects if maintained over an extended period of time. Furthermore, stress-dependent oscillations in the nuclear localization of transcription factors could play a role in fine tuning the cellular response to stress by modulating the level and the order of expression of genes after stress. Such a view is consistent with the results of Gasch et al. (2000), which showed that the number of genes activated, the level of induction, and the duration of the effect depend on the strength of the stress.

## Materials and methods

### Cell and plasmids

The yeast strains used were W303–1B, *MATα*; OL564–1B, an *rca1/pde2* mutation in a W303 *MATa* background; and OL607–47D (*ura3*, *his3*, *leu2*, *trp1*, *yak1Δ::URA3*), which is a progeny of OL550–11B (*MATa*, *ura3–52*, *his3Δ200*, *leu2Δ1*, *trp1Δ63::TRP1-RAS2$^{ile152}$*, *cdc25Δ::HIS3*) and OL595–1 (*MATα*, *ura3*, *his3*, *leu2*, *trp1*, *cyr1Δ::kanMX3*, *yak1Δ::URA3*). The triple *tpk* mutant OL625–3 (*MATa*, *ade2*, *ura3*, *his3*, *leu2*, *trp1*, *tpk1Δ::KanMX2*, *tpk2Δ::HIS3*, *tpk3Δ::TRP1*, *yak1Δ::hisG-URA3-hisG*) was obtained as a progeny of the *tpk$^{w}$* strain RS13–58A-1 (Nikawa et al., 1987), in which the *YAK1* gene was deleted by a kanMX2 cassette, and the OL596–2A strain, a derivative of W303–1B, in which the *TPK1* gene was deleted with a kanMX2 cassette. In the BC-N4 strain, the *BCY1* gene has been replaced by recombination of *bcy1ΔN::HIS3* (Zhu et al., 2000). OL610-2D (*ura3Δ0*, *his3Δ1*, *leu2Δ0*), which is a progeny of two EUROSCARF® mutants, was used as reference for the S288c background.

To delete *YAK1*, the gene was cloned in a PRS314 plasmid from −609 to 2840 relative to the ATG, the internal fragment 247–2293 was replaced by an EcoRI–Sal1 fragment of pMPY.ZAP containing the *URA3* gene, and a Pvu2–Pvu2 fragment encompassing the *yak1Δ* gene with its flanking regions was transferred to the OL583 yeast strain (*cyr1Δ*, *rca1/pde2*) (Mallet et al., 2000), resulting in OL595-1. To delete *CDC25*, a PCR fragment containing the *HIS3* gene between two *CDC25* sequences, respectively, from −60 to −26 and 4818 to 4853 relative to ATG was transferred by homologous recombination to the OL549-3 strain isogenic to FY16792 (Thierry et al., 1995) except the integration in *trp1* of the pGR113 containing the *RAS2$^{ile152}$* and the *TRP1* gene.

The plasmid pAMG (gift from C. Schüller, Institute of Biochemistry, Vienna, Austria) encodes the fusion MSN2–GFP (GFP at the COOH-terminal part) downstream of an ADH1 promoter in a centromeric LEU2 plasmid (Gorner et al., 1998). PJL42 (gift from L.J. Parrou, INSA Toulouse, Toulouse, France) is a plasmid where the SalI *MSN2* fragment is inserted at the SalI site of pGFP-N-Fus (Niedenthal et al., 1996). The plasmid pGR247 is derived from pAMG in which the *MSN2* ORF, cut out by Sal1 and Nco1, has been replaced by the *MSN4* ORF amplified from a plasmid containing it (pAL32–45) with oligos containing, respectively, a Sal1 and an Nco1 site. To construct the pAMG-ΔZ plasmid, the flanking region of the zinc

finger–coding region (amino acids 642–698) had been first amplified by PCR with overlapping oligos and then amplified together with the external oligos containing the Sal1 site at the 5′ end and the HindIII site at the 3′ end. The resulting fragment, Msn2ΔZn–GFP, replaced, after restriction cutting and ligation, the Sal1–HindIII Msn2–GFP fragment in pAMG.

### Time-lapse microscopy

Time-lapse microscopy was performed using the set-up previously described (Sibarita et al., 2002). It consists of an inverted microscope, Leica DM IRBE, mounted on an antivibration table and placed in an incubator at constant temperature. The objective was a 100× PL APO z-positioned by a piezo-electric driver. Fluorescence was recorded by a CCD detector (Roper Micromax™ RTE-782-YHS; Roper Scientific). For illumination, we used a Sutter Instrument Co. DG4 instrument with a 175-W Xenon lamp. The experimental set-up was steered by Metamorph™ 4.6 Software (Universal Imaging Corp.).

### Imaging

Every 10 s, during a 1-s period, a stack of 21 frames along the Z-axis (every 0.3 μm) was recorded. Images were deconvoluted using an algorithm developed by Sibarita et al. (2002). For each stack, a brightest point z projection was done using the Metamorph™ program to obtain the final reconstructed image. Pixel values in the nucleus and the cytoplasm, after removal of background noise, were used to monitor the distribution of Msn2–GFP over time. Normalization was based upon the lowest and the highest pixel values in the nucleus when complete transfer to the cytoplasm and into the nucleus was observed. For cells showing incomplete translocation, the values were calculated with respect to the size of the cell and the nucleus.

### Computational model for oscillatory shuttling of Msn2

One possible implementation, among others, of the delayed activation mechanism (schematized in Fig. 6 A) involves the switching on, by the nuclear form of Msn2, of a bicyclic cascade of phosphorylation–dephosphorylation reactions, leading to the activation of a protein (e.g., a kinase or phosphatase) that would elicit Msn2 export from the nucleus (see Fig. S1, available at http://www.jcb.org/cgi/content/full/jcb.200303030/DC1). Oscillations can also be obtained with a monocyclic cascade, but their amplitude is more reduced because the delay in the feedback loop is shorter. We assume that within the nucleus, activated Msn2 (MN*) promotes the conversion of an enzyme, X, from an inactive form, XI, into an active form, XA. The latter activates a regulator of Msn2, R, from RI into RA. The active form, RA, triggers the conversion of MN* into the form, MN, that leaves the nucleus. Both R and X, as well as Msn2, could be controlled by the antagonistic interplay of kinases and phosphatases. An example of a related mechanism that generates oscillations is provided by a cascade model for the periodic activation of cdc2 kinase during early mitotic cycles in amphibian embryonic cells (Goldbeter, 1991).

The model is governed by the following system of kinetic equations describing the time evolution of the fractions of M, M*, MN*, MN, XA, and RA:

$$\frac{dM}{dt} = -V_1 + V_2 + k_2 MN, \tag{1a}$$

$$\frac{dM^*}{dt} = V_1 - V_2 - k_1 M^*, \tag{1b}$$

$$\frac{dMN^*}{dt} = k_1 M^* + V_3 - V_4, \tag{1c}$$

$$\frac{dMN}{dt} = V_4 - V_3 - k_2 MN, \tag{1d}$$

$$\frac{dXA}{dt} = V_5 - V_6, \tag{1e}$$

$$\frac{dRA}{dt} = V_7 - V_8, \tag{1f}$$

with

$$V_1 = V_{KS}\left(\frac{M}{K_1 + M}\right), \tag{2a}$$

$$V_2 = V_P\left(\frac{M^*}{K_2 + M^*}\right), \tag{2b}$$

$$V_3 = V_{KSN}\left(\frac{MN}{K_3 + MN}\right), \tag{2c}$$

$$V_4 = V_{PN}\left(\frac{RA}{K_{a1} + RA}\right)\left(\frac{MN^*}{K_4 + MN^*}\right), \tag{2d}$$

$$V_5 = V_{KX}\left(\frac{MN^*}{K_{a2} + MN^*}\right)\left(\frac{1 - XA}{K_5 + 1 - XA}\right), \tag{2e}$$

$$V_6 = V_{PX}\left(\frac{XA}{K_6 + XA}\right), \tag{2f}$$

$$V_7 = V_{KR} \cdot XA\left(\frac{1 - RA}{K_7 + 1 - RA}\right), \tag{2g}$$

$$V_8 = V_{PR}\left(\frac{RA}{K_8 + RA}\right). \tag{2h}$$

The curves of Fig. 6 (B–F) were obtained by numerical integrations of equations 1a–1f using the Berkeley Madonna™ software for the following parameter values: $V_P = 0.3$, $V_{KSN} = 0.5$, $V_{PN} = 2$, $V_{KX} = 1.3$, $V_{PX} = 0.6$, $V_{KR} = 1.6$, $V_{PR} = 0.9$, $k_1 = 6.6$, $k_2 = 5$ (all these parameters are in $min^{-1}$), $K_i = 0.01$ ($i = 1, \ldots 8$), and $K_{a1} = K_{a2} = 0.2$. Initial conditions in panels B–F of Fig. 6 are $M = XI = RI = 1$, and $M^* = MN = MN^* = XA = RA = 0$. These values correspond to the situation where, before stress is applied, the transcription factor is in its cytosolic state M, while regulators X and R are in their inactive form.

### Online supplemental material

The supplemental material (Videos 1–3; Fig. S1) is available at http://www.jcb.org/cgi/content/full/jcb.200303030/DC1. The videos show nucleocytoplasmic shuttling of Msn2–GFP in W303 cells, shuttling of Msn2ΔZn–GFP in W303 cells, and a montage of Msn2–GFP and Msn4–GFP in the cell deleted for the three *TPK* genes and *YAK1*. Fig. S1 represents a scheme for the autoregulatory loop of the model.

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