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S. Chabane · F. Képès

Expression of the yeast *BFR2* gene is regulated at the transcriptional level and through degradation of its product

Abstract The essential *Saccharomyces cerevisiae* gene *BFR2* has been isolated as a high-copy suppressor of the growth defects induced by Brefeldin A, a drug that disrupts the Golgi apparatus and its protein influx. Furthermore, *BFR2* has been found to display genetic interactions with four mutations affecting protein transport to the Golgi apparatus. Here we show that the level of *BFR2* mRNA rapidly increased over fivefold in response to cold shock, and over threefold following nutrient replenishment by dilution of cells from exhausted to fresh minimal medium. During subsequent growth, the transcript level returned to its basal values, except for a transient drop toward the end of the exponential phase. The early burst of transcription was not caused by toxic compounds in the fresh medium, or by synchrony among cells that had simultaneously entered their first cell cycle. The *BFR2* gene product (Bfr2p) was synthesized following the early burst of mRNA, and was no longer produced when the mRNA was back to basal level. Bfr2p was finally degraded after growth became limited, and reached undetectable levels in exhausted medium. Under steady-state conditions of lengthened exponential phase, the intracellular level of Bfr2p remained constant. This peculiar pattern of gene expression suggests that Bfr2p is essential for mass growth or cell proliferation, whereas it is either toxic or not required during nutrient-limited growth.

Key words *Saccharomyces cerevisiae* · Transcriptional regulation · Protein degradation · Lag phase · Growth cycle

S. Chabane · F. Képès (✉)
Service de Biochimie et de Génétique Moléculaire,
DBCM/DSV, Bât. 142, CEA/Saclay,
F-91191 Gif cedex, France
Tel.: +33-1-69-08-34-60; Fax: +33-1-69-08-47-12
E-mail: kepes@jonas.saclay.cea.fr

Introduction

Cell division and growth of the yeast *Saccharomyces cerevisiae* are controlled by the level of available nutrients (Hartwell 1974). When the growth medium becomes depleted, cells undergo a regulated arrest of proliferation, ultimately leading to stationary phase. Addition of nutrients to arrested cells induces them to begin growth and division, following a lag phase of varying length. Cells then grow exponentially until one nutrient becomes limiting, leading to a slowdown in accumulation of biomass. If the carbon source is glucose, cells will grow primarily by fermentation during the exponential phase. The diauxic shift occurs when glucose concentration becomes low and cells adapt to respiratory metabolism. The post-diauxic phase is characterized by very slow growth. This residual growth stops when cells reach stationary phase (reviewed in Werner-Washburne et al. 1993).

Upon resupply of nutrients, resumption of growth and reentry into the cell cycle constitute active processes with unique requirements, as revealed by mutational analysis (Drebot et al. 1987). The *gcs1* mutant is cold sensitive for the resumption of cell proliferation from stationary phase. At the restrictive temperature of 15° C, mutant cells respond normally to nutrient replenishment and quit stationary phase to resume mass growth. However, *gcs1* mutant cells are blocked after the exit from stationary phase, never reach the start point of the first mitotic cell cycle and fail to divide (Drebot et al. 1987). The *GCS1* gene product is similar in sequence to a mammalian protein that stimulates the GTPase activity of ARF1 (ADP-ribosylation factor), a small G-protein involved in intracellular protein transport. Indeed, the *GCS1* product is, at least in vitro, a GTPase-activating protein for the yeast ARF proteins, and the *GCS1* and *ARF* genes show genetic interactions (Poon et al. 1996). In contrast to *gcs1* mutants, *arf* mutants were not described as blocked for the resumption of cell proliferation. *GCS1* function is required for the efficient secretion

of invertase (Poon et al. 1996) and for endocytosis of the dye FM 4–64 (Wang et al. 1996).

The fungal metabolite Brefeldin A (BFA) disrupts the Golgi apparatus and its incoming flux, in animal cells (reviewed in Klausner et al. 1992) and in yeast cells (Graham et al. 1993; Jackson and Képès 1994; Rambourg et al. 1995). BFA is thought to exert its effects by inhibiting guanine nucleotide exchange on ARF (Donaldson et al. 1992; Helms and Rothman 1992). The *BFR2* gene, also known as YDR299W, has been isolated as a high-copy suppressor of the growth defects induced by BFA in a sensitive strain of *S. cerevisiae* (Chabane et al. 1998). In addition, *BFR2* shows genetic interactions with five secretory mutations, four of which affect protein traffic from the endoplasmic reticulum to the Golgi apparatus. The essential *BFR2* gene was predicted to encode a very hydrophilic product (Bfr2p) containing two short regions with potential coiled-coils, i.e., regions that are generally responsible for protein-protein interactions (Chabane et al. 1998). During subsequent analysis, it appeared that Bfr2p was easy to detect from cultures grown to early exponential phase and undetectable from cultures taken just beyond the end of exponential phase. In the present study, we analyze the peculiar transcriptional regulation of the *BFR2* gene with respect to the cell growth cycle and to environmental stress.

Materials and methods

Strains, growth conditions and materials

Yeast strains used in this study are listed in Table 1. The *Escherichia coli* strain DH5α was grown at 37°C in LB medium (2.5% Luria broth base). Where appropriate, ampicillin was added at 100 µg/ml. Yeast strains were usually grown at 30°C in YPD (2% Bacto peptone, 1% Bacto yeast extract and 2% glucose) or in minimal medium (0.67% Yeast Nitrogen Base without amino acids and 2% glucose). Cell density was monitored in liquid culture by measuring optical density at 600 nm (OD_{600}) using a DU68 spectrophotometer (Beckman Instruments France, Gagny). DNA content was evaluated with a fluorescence-activated cell sorter (FACS, BioRad, Ivry/Seine, France). Culture media were obtained from Difco Laboratories (OSI, Paris, France). Ampicillin, cycloheximide, glyoxal, phenylmethyl sulfonyl fluoride (PMSF), sodium azide and Tween 20 were purchased from Sigma (Saint Quentin Fallavier, France). Dimethyl sulfoxide was purchased from Merck

(Nogent/Marne, France). Glucose concentration was quantified using a kit [diagnostic kits Glucose (HK) 10, Sigma].

Vectors, plasmids and manipulation of nucleic acids

pSC10 and pMET25::BFR2-HA are *E. coli-S. cerevisiae* shuttle vectors. pSC10 was constructed from the YCplac33 vector (Gietz and Sugino 1988) by inserting the *BFR2* gene with a short sequence encoding the hemagglutinin (HA) epitope from influenza virus (nine amino acids: Tyr-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala) fused on the 5′ side of its open reading frame. pMET25::BFR2-HA is the pMET25 plasmid (Mumberg et al. 1994) containing the *BFR2* gene amplified by the polymerase chain reaction (PCR) in fusion with the hemagglutinin-encoding sequence. DNA manipulations were performed as previously described (Sambrook et al. 1989). Yeast DNA transformation was performed by electroporation (Meilhoc et al. 1990) or by lithium acetate treatment (Ito et al. 1983).

RNA preparation and Northern blot analysis

Approximately 6×10^7 cells were harvested by centrifugation, washed once in ice-cold water and resuspended in 0.1 ml of LETS 1% (0.01 M TRIS, pH 7, 0.02 M LiEDTA, 0.1 M LiCl, 1% SDS). After addition of 30 µl PDI (phenol:dichloromethane:isoamylalcohol 25:24:1) and 0.1 ml of glass beads (0.5 mm diameter), the cells were lysed by agitation with a vortex for 2 min on ice. Then 0.4 ml of LETS (0.01 M TRIS, pH 7, 0.02 M LiEDTA, 0.1 M LiCl, 0.2% SDS) was added and after vortexing, 0.5 ml of phenol was added. After vortexing for 1 min, the lysate was incubated for 3 min at 65°C. This step was repeated three times. After centrifugation in a microfuge for 5 min at 10 000 rpm, the upper phase was extracted once with 0.5 ml of PDI. After an identical centrifugation, the upper phase was again extracted with 0.5 ml of DI and precipitated with 0.3 M LiCl and 2 vol. of ethanol overnight at –20°C. RNA was recovered by centrifugation in a microfuge for 20 min at 10 000 rpm and dissolved in 30–50 µl of sterile water. The RNA concentration was determined by measuring the absorbance at 260 nm.

For Northern blot analysis, 20 µg of RNA denatured with glyoxal and dimethyl sulfoxide, was subjected to electrophoresis on a 1% agarose gel and then transferred overnight (Sambrook et al. 1989) to a positively charged nylon membrane (Boehringer Mannheim, Meylan, France). The transferred RNA was crosslinked to the membranes by UV irradiation (UV stratalinker 1 800, Stratagene, Basle, Switzerland). Hybridization and detection were carried out with digoxygenin (DIG)-labeled RNA probes at 50°C according to the instructions accompanying the DIG Chemiluminescent Detection Kit (Boehringer Mannheim). Two DNA fragments were used as probes: *BFR2* and *ACT1*. They were amplified by PCR using the Goldstar polymerase enzyme (Eurogentec, Seraing, Belgium) and the dNTP-DIG mixture (Boehringer Mannheim). Northern blots were exposed to Biomax MR film (Kodak, sold by Amersham, Les Ulis, France) for 5–120 min to obtain

Table 1 Strains of *Saccharomyces cerevisiae* used in this study

Strain	Genotype	Source
W303	*MATα ura3-52, leu2-3,-112, his3-11,-15, trp1-1*, ade2-1, *CanR*	F. Lacroute
Δ*bfr2*/pSC10	As W303 but Δ*bfr2*, complemented by a single-copy plasmid (*URA3*⁺) bearing the *BFR2* gene tagged with the hemagglutinin epitope	This study
Δ*bfr2*/pSC10	As W303 but Δ*bfr2* and *MATa* complemented by a single-copy plasmid (*URA3*⁺) bearing the *BFR2* gene tagged with the hemagglutinin epitope	This study
Δ*bfr2*/pMET25::BFR2-HA	As W303 but Δ*bfr2* complemented by a single-copy plasmid (*TRP1*⁺) bearing the *BFR2* gene, tagged with the hemagglutinin epitope, downstream of the *MET25* promoter	This study

optimal signal intensities for quantification. The films were scanned and band intensities were quantified with NIH Image software (NIH, Bethesda, Md., USA).

Western blot analysis

10 ml of cells at an OD_{600} of 0.2 were collected. The cells were washed with 10 mM sodium azide and resuspended in 160 µl of buffer (100 mM TRIS-Cl, pH 6.8, 4 mM EDTA, 4% SDS, 1 mM PMSF). After 50 s vortexing with a mini-beadbeater (Biospec products, Bartlesville, Okla., USA) in the presence of 0.1 ml of glass beads, the samples were heated for 5 min at 95° C. Protein was measured by the Bradford method according to the manufacturer's description (BioRad, Ivry-sur-Seine, France). Then, 32 µl of 100% glycerol, 6 µl of β-mercaptoethanol and 2 µl of 2.5% bromophenol blue were added to each sample. The samples were heated for 5 min at 95° C and equal amounts of protein were loaded on an 8% SDS-polyacrylamide gel. After migration, proteins were transferred to nitrocellulose filters (Amersham). Equal loading per lane was confirmed by brief staining with Ponceau S. Protein detection was performed with the ECL kit (Amersham). Preincubation, antibody incubation and washes were conducted in TBST buffer (100 mM TRIS, 150 mM NaCl, 0.05% Tween-20) containing 5% nonfat dry milk. Antisera were used at a 1/500 dilution for HA and a 1/2000 dilution for hexokinases (I and II). Western blots were exposed to Biomax MR film (Kodak) for 0.5–20 min to obtain optimal signal intensities and were quantified as described above.

Results

Expression of the *BFR2* gene is induced during lag phase

Expression of the *BFR2* gene was monitored during the growth cycle of Δbfr2/pSC10 cells, which express from a single-copy plasmid a hemagglutinin-tagged version of Bfr2p (Bfr2p-HA) complementing a null chromosomal allele. An overnight culture of *Δbfr2*/pSC10 cells was diluted from an OD_{600} of 2 to an OD_{600} of 0.04 in the same minimal selective medium with 2% glucose, and allowed to grow in batch culture at 30° C (Fig. 1). After about 130 min of lag phase, mass growth resumed and was exponential until about 560 min postdilution; it slowed down thereafter (Fig. 1A, D). Glucose concentration in the medium was still around 1.5% at the growth breakpoint (Fig. 1D), indicating that the initial slowdown was not caused by carbon source limitation (diauxic shock) but by limitation of another nutrient. The period just following the growth breakpoint will be referred to as the prestationary phase. At 1440 min postdilution, however, glucose concentration was low and, thus, cells were in early stationary stage. Since the culture had reached the maximal OD_{600} for this type of medium, this stage will be referred to as early stationary phase. At various growth phases, RNA and protein were extracted from a fixed amount of cells and analyzed. *BFR2* mRNA was detected by Northern blotting (Fig. 1B). Since no specific mRNA could be taken as a reliable standard over various growth phases, the rRNA species were used as a control of comparable gel loading on an identical gel stained with ethidium bromide
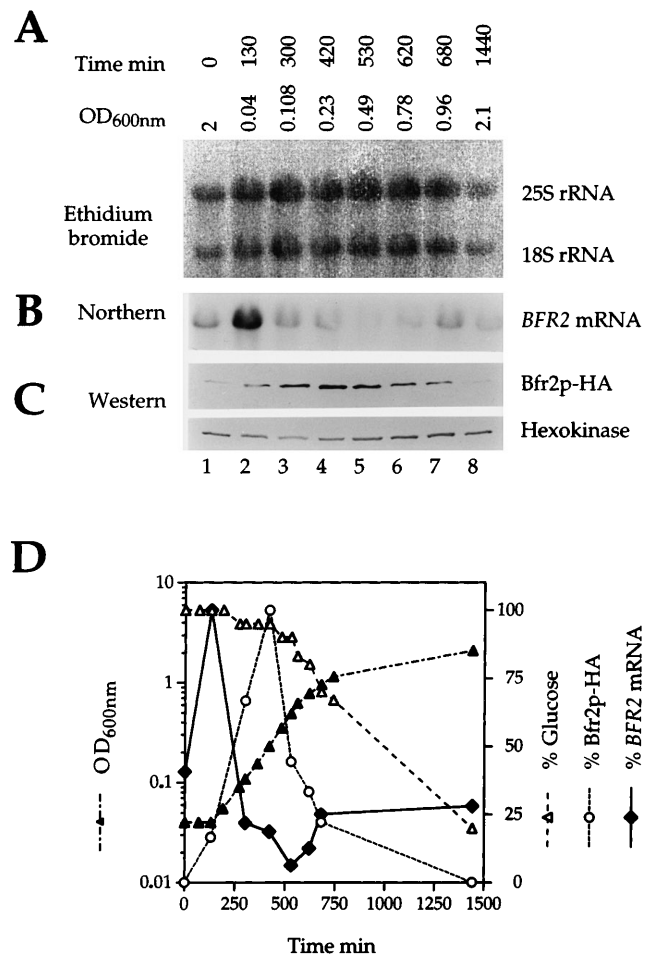
Fig. 1A–D Expression of the *BFR2* gene as a function of growth phase. An overnight culture of *Δbfr2* /pSC10 cells was diluted 50-fold into minimal medium at time zero. Growth at 30° C was subsequently monitored by measuring the OD at 600 nm. At various stages of growth, total RNA and total protein extracts were prepared and quantified. **A** Equal amounts of RNA were fractionated on a 1% agarose gel and stained with ethidium bromide. The positions of the 25S and 18S rRNA species are indicated on the right side. **B** The gel was Northern blotted and probed for *BFR2* mRNA. **C** Equal amounts of protein were fractionated on an 8% polyacrylamide gel. Bfr2p-HA and hexokinases were detected by Western blotting and enhanced chemiluminescence with appropriate antibodies. **D** The results shown in **A–C**, and the glucose concentration in the growth medium, were quantified and plotted. The ratios of Bfr2p-HA over hexokinases, and of *BFR2* mRNA over rRNAs, were scaled to 100% at their peak values. Note the log scale for OD and the linear scale for the other parameters

(Fig. 1A). Bfr2p-HA was detected by Western blotting (Fig. 1C). As an internal standard, the constitutively expressed and stable cytoplasmic enzyme hexokinase was detected on the same blot (Fig. 1C). The band intensities were quantified and plotted after correction by the appropriate control values (Fig. 1D). As can be seen from the blots of both Fig. 1B and C, and from the corrected data of Fig. 1D, the level of *BFR2* mRNA was particularly high around the end of the lag phase (lane 2). Bfr2p-HA was undetectable in early stationary phase (lanes 1, 8), although the transcript was present at about 25% of its

maximal level. It began to be detectable at 130 min, when the mRNA peaked (lane 2). The level of Bfr2p-HA was highest during exponential phase (lanes 3–5), when the level of transcript was back to ≤25%. The level of Bfr2p-HA decreased as the level of mRNA was particularly low (lane 6), and dropped further when the transcript had increased to about 25% again (lane 7). It reached undetectable levels during early stationary phase (lane 8), indicating that Bfr2p-HA had been degraded.

To resolve the lag phase better, the experiment shown in Fig. 1 was repeated but samples were collected at earlier time points (Fig. 2A). Toxic compounds (heavy metal ions) are present in trace amounts in the fresh dilution medium and might potentially be responsible for induction of the *BFR2* gene. Upon inoculation, yeast cells rapidly detoxify their medium by internalizing these ions into harmless complexes. Fresh medium was thus preconditioned by allowing limited yeast cell growth, followed by sterilization through a filter. In parallel with the experiment reported in Fig. 2A, dilution of the early stationary phase culture was carried out in such preconditioned medium. The lag phase and the early burst in the level of *BFR2* mRNA were not affected by preconditioning of the medium. The first time point, even as early as 20 min postshift (Fig. 2B), consistently corresponded to the peak of transcript levels (Figs. 1D, 2A, B). Bfr2p-HA was also detectable as early as 20 min postshift (Fig. 2B), and might have appeared earlier in the preconditioned medium than in the fresh medium (Fig. 2A, B). In a reverse test, cadmium sulfate was added to an exponential-phase culture to induce oxidative stress (Fig. 1C). This stress was probably much stronger than that induced by traces of heavy ions in fresh medium, as judged by the growth slowdown. However, expression of the *BFR2* gene was not induced. If anything, cadmium addition caused a further decrease in the transcript levels. The levels of Bfr2p-HA decreased with the usual pattern, given the OD_{600} of the culture.

It remained possible that the early burst of *BFR2* transcription was caused by synchrony among cells resuming growth simultaneously. During the lag phase, it appeared that cells were partially synchronized with respect to their DNA content, evaluated with a FACS. In contrast, cell shapes were not homogeneous, as judged by microscopic inspection (not shown). In any case, this possibility was tested by blocking the cell cycle with a mating pheromone (α-factor) at the time of culture dilution. The experiment described in Fig. 1 was repeated with cells of the opposite mating type (*MATa*), with or without 2.5 μM α-factor in the fresh dilution medium. Although cells were efficiently blocked by α-factor for at least 360 min according to FACS analysis, the levels of *BFR2* transcript were highest at the 20 and 60 min time points, as in the control (not shown).

Expression of the *BFR2* gene is induced by cold shock

Wild-type cells were grown to exponential phase on rich medium, and shifted from 30° to 10°C. Northern blot
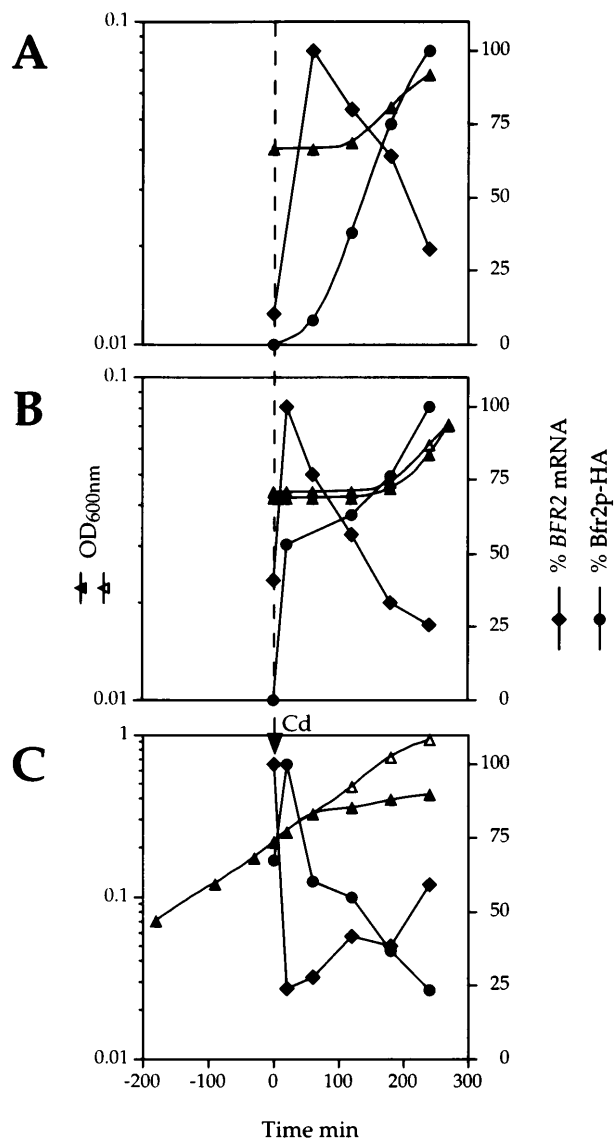

Fig. 2A–C Expression of the *BFR2* gene and oxidative stress. Details as in Fig. 1. **A** Lag phase following dilution of the culture in prewarmed minimal medium at time zero. **B** Lag phase following dilution of the culture in preconditioned minimal medium. Preconditioned medium was obtained by growing yeast cells from an OD_{600} of 0.11 to 0.18 and filtering them out. *Open* and *closed triangles* correspond to growth in minimal medium and in preconditioned minimal medium, respectively. **C** 10 μM cadmium sulfate was added at time zero to an exponentially growing culture in minimal medium. *Open* and *closed triangles* correspond to growth without, or with cadmium sulfate, respectively

analysis showed that this cold shock resulted in a strong increase in the level of the *BFR2* transcript, by a factor of 3.5 in 20 min and by a factor of 5.6 in 180 min. In contrast, heat shock had very minor effects (not shown). Finally, the level of the *BFR2* transcript in exponentially growing cultures was independent of the carbon source added to rich medium (2% glucose, galactose, raffinose or glycerol) and of the richness of the medium (minimal or rich, with 2% glucose; not shown).

The Bfr2 protein is present at steady-state levels during the exponential phase of growth, and is degraded during early stationary phase

An overnight culture was diluted as before (Fig. 1). Cells were subsequently grown to OD_{600} of 0.2, i.e., when Bfr2p was at a maximal level (Fig. 1D). At that point, the culture was inoculated in a batch-fed chemostat and maintained in exponential phase at almost constant OD_{600} for 300 min by dilution every 30 min. The OD_{600}, corrected for chemostat dilutions, increased exponentially during the entire experiment. Protein was extracted every 30 min from the chemostat and analyzed by Western blotting. Under such conditions of lengthened exponential phase, the ratio of Bfr2p-HA over hexokinase (or total protein) remained strictly constant (not shown).

Figure 1 had shown that Bfr2p-HA was degraded, starting at about the growth breakpoint. To monitor protein degradation directly, cultures in exponential phase (OD_{600} of 0.22) or at the growth breakpoint (OD_{600} of 0.86) were subjected to the addition of 0.2 mg/ml cycloheximide, an inhibitor of protein synthesis. Western blotting showed that the level of Bfr2p-HA remained constant over 300 min in both cultures (not shown), indicating the absence of degradation. To resolve the apparent contradiction between the results obtained around the growth breakpoint in the presence or absence of cycloheximide, protein degradation was evaluated with a conditional expression mutant. The *BFR2* open reading frame was amplified with the addition of a hemagglutinin tag, and cloned in a single-copy vector, downstream of the *MET25* promoter. This promoter is active in the absence of methionine and repressed in the presence of 1 mM methionine (Mumberg et al. 1994). A *Δbfr2/BFR2* heterozygous diploid was transformed with the resulting plasmid, pMET25::BFR2-HA, and then sporulated to generate a *Δbfr2* haploid. Two cultures of a *Δbfr2* haploid strain complemented by pMET25::BFR2-HA were grown to early stationary phase in minimal medium supplemented or not with methionine. The culture deprived of methionine was then diluted to OD_{600} of 0.03 in minimal medium, with or without methionine. Protein extracts were prepared from these cultures at various time intervals during the growth cycle. Bfr2p-HA and hexokinase were detected by Western blotting (Fig. 3). Both arrested cultures were devoid of Bfr2p-HA (lanes 1, 2). Under repression with methionine, Bfr2p-HA became detectable but remained at low levels (lanes 4, 6 and 8). In the absence of methionine, Bfr2p-HA clearly accumulated during exponential phase (lanes 3, 5), reached a high level at 520 min (lane 5), and strongly decreased at the growth breakpoint (lane 7). In the presence of methionine, this mutant had little Bfr2p-HA, yet it had no obvious abnormal phenotype, suggesting that a very low level of Bfr2p was sufficient to support growth. Indeed, the level of Bfr2p was detectable (close to the limit of detection) by pulse-chase/immunoprecipitation experiments only when overproduced (not shown), suggesting that the physiological levels of Bfr2p are rather low.
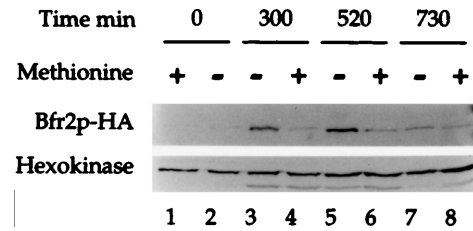
Fig. 3 Stability of Bfr2p-HA expressed from an inducible, heterologous promoter. An early stationary phase culture without methionine of the *Δbfr2*/pMET25::BFR2-HA conditional expression mutant (lane 2) was diluted to an OD_{600} of 0.03 in the same minimal medium supplemented (lanes 4, 6, 8) or not (lanes 3, 5, 7) with 1 mM methionine. Lane 1 corresponds to an early stationary phase culture with 1 mM methionine. At the time intervals indicated, total protein extracts were prepared and quantified. Equal protein amounts were fractionated on a gel, blotted, and probed for Bfr2p-HA and hexokinases

Discussion

The cellular concentration of Bfr2 protein appears to be regulated at two levels. Although the present data do not exclude translational control, transcriptional control and protein turnover suffice to account for the results. Upon dilution of cells from early stationary phase into fresh medium, the levels of *BFR2* mRNA increase three- to tenfold within the initial 20 min in the absence of net mass growth, and decrease soon afterwards. Thus, the differential rate of *BFR2* transcription must be very high, relative to an average gene. Culture dilution provides yeast cells with nutrients and traces of toxic compounds. Either of these two modifications could cause the observed burst of transcription. However, strong oxidative stress does not induce *BFR2* expression, and clearing of toxic compounds from the dilution medium does not modify the transcriptional response. Because cells from overnight cultures in exhausted medium are in a G1-arrested stage (Hartwell et al. 1974), resumption of growth allows the expression of this latent synchrony, and cells are transiently homogeneous with respect to their DNA content. If *BFR2* were preferentially expressed at a particular stage of the cell cycle, a peak in the level of mRNA would be observed during the first cell cycle following dilution, and not observed thereafter owing to loss of synchrony among the cells. However, preventing the progression of cells through their first cell cycle by treating them with pheromone does not alter the transcriptional response. Hence nutrient provision must be the cause of the burst of *BFR2* transcription during the early lag phase of growth, accounting for high levels of mRNA during the whole lag phase.

Replenishing nutrients results in an early change in cellular metabolism and, following a lag phase, in an increase in biomass (Hartwell 1974). Resumption of growth cannot constitute the signal for elevated transcription, since resumption occurs when transcription is already decreasing. Thus, metabolic modifications might be the most direct signal for elevated transcription. In

accord with this line of thought, the *BFR2* promoter contains one RPG-like box (two mismatches compared with the degenerate consensus RMAYCCRYNCAYY) at position –57 from the translational start codon. Such boxes represent the recognition sites for DNA-binding factors, including RAP1 (Vignais et al. 1990; Moehle and Hinnebusch 1991). RPG boxes have been shown to mediate a three- to fourfold increase in expression of genes encoding ribosomal proteins and glycolytic enzymes, upon addition of glucose to a culture growing on a nonfermentable carbon source (Herruer et al. 1987; Butler and McConnell 1988). By analogy, the RPG-like box found in the *BFR2* promoter might play a role in the initial strong burst of transcription upon dilution, which involves replenishment of glucose and other nutrients. During exponential growth, however, our data show that the levels of *BFR2* transcript are not affected by the fermentability of the carbon source.

Bfr2p rises to detectable levels at 20–60 min postshift, i.e., at the peak of *BFR2* transcript levels, shortly after the initial increase in concentration of *BFR2* transcript. This result is compatible with immediate translation of the new transcript as soon as it is available, taking into account the fact that the translation machinery must regain activity, following an arrested stage. The peak level of Bfr2p (standardized by total protein) is reached around 420 min, i.e., when the transcript is back to its basal level. From 420 min on and during mid- and late exponential phase, levels of Bfr2p are high but tend to decrease before the end of the exponential phase. However, under conditions of lengthened exponential phase at constant cell concentration, levels of Bfr2p remain constant, which means that Bfr2p is synthesized at the same rate as total protein (or at a higher rate if Bfr2p is continuously degraded). Finally, during early stationary phase, Bfr2p is degraded. Two models could account for these results. In the first model, Bfr2p degradation would be triggered by nutrient depletion and prevented by nutrient replenishment. Bfr2p would be synthesized at a high rate as a consequence of the early accumulation of mRNA; during exponential phase, Bfr2p would be synthesized at a lower rate, owing to a low level of transcript. At the growth breakpoint, the limited resumption of production of mRNA would weakly boost Bfr2p synthesis just when protein degradation resumes, resulting in a net loss of the protein. This first model is entirely consistent with the data. In the second model, a constant degradation rate would bring the protein to undetectable levels when the transcript concentration was basal; only when the transcript was abundant would synthesis overcome degradation. However, transcript was at its lowest level when protein concentration was still increasing between 300 and 420 min postdilution. This second model can therefore be rejected, unless a third degree of control is postulated, at the translational level. For instance, translation would be up-regulated during early exponential phase.

Whatever the interpretation, the observation of a full growth cycle indicates that Bfr2p degradation does occur, at least around early stationary phase. Using a heterologous promoter to observe Bfr2p degradation without the complications of a highly regulated transcriptional pattern, it appears that levels of Bfr2p decrease as early as around the growth breakpoint. Altogether, the conclusion is that Bfr2p is actively degraded from the prestationary to the early stationary phase of growth. It is thus surprising that Bfr2p is stabilized when protein synthesis is prevented by the presence of cycloheximide in a culture taken at the growth breakpoint. One reason might be that new protein synthesis is required to effect degradation of Bfr2p.

The concentration of Bfr2p is not regulated solely by growth phase. A cold shock rapidly and strongly induces transcription of *BFR2*, although Bfr2p is not a member of any of the families of temperature shock proteins that have already been identified (Kondo and Inouye 1991). The peculiar pattern of regulation of *BFR2* transcription has not been described so far for any other yeast gene (see review by Werner-Washburne et al. 1993), not to mention the additional level of control provided by protein degradation. The pattern of expression of *BFR2* is, however, reminiscent of a cellular process that also depends on the growth phase: interconversion between steryl esters and free sterols. The concentration of free sterols, the usable form, is low in stationary phase, and high toward the end of the lag phase and during exponential growth phase (Taylor and Parks 1978; Lewis et al. 1987). The *BFR2* gene has been selected as a high-copy suppressor of the lethality induced by BFA (Chabane et al. 1998). This selection was carried out in a sensitive *erg6* mutant, defective in the enzyme S-adenosyl-methionine: Δ24-sterol-C-methyltransferase, which is required for ergosterol methylation (Gaber et al. 1989). This mutant is sensitive to a number of drugs, including BFA, probably because of enhanced permeability of the plasma membrane (Winsor et al. 1987; Nitiss and Wang 1988; Gaber et al. 1989; Jackson and Képès 1994). One interesting possibility is that the *BFR2* gene is not directly involved in BFA resistance, but regulates the concentration of free sterols. Membranes are poor in sterols in an *erg6* mutant. Up-regulating the concentration of free sterols should increase the proportion of sterols in membranes, thus decreasing the permeability of the plasma membrane to BFA. This effect would have been achieved by increasing the copy number of the *BFR2* gene in the above genetic selection. Similarly, the concentration of free sterols would be high during the lag and exponential phases of growth as a consequence of the elevated levels of Bfr2p.

References

Butler G, McConnell DJ (1988) Identification of an upstream activation site in the pyruvate decarboxylase structural gene (*PDC1*) of *Saccharomyces cerevisiae*. Curr Genet 14:405–412

Chabane S, Gachet E, Képès F (1998) Overexpression of the yeast *BFR2* gene partially suppresses the growth defects induced by Brefeldin A and by four ER-to-Golgi mutations. Curr Genet, 33:21–28

Donaldson JG, Finazzi D, Klausner RD (1992) Brefeldin A inhibits Golgi membrane catalysed exchange of guanine nucleotide onto ARF protein. Nature 360:350–352

Drebot MA, Johnston GC, Singer RA (1987) A yeast mutant conditionally defective only for reentry into the mitotic cell cycle from stationary phase. Proc Natl Acad Sci USA 83:7948–7952

Gaber RF, Copple DM, Kennedy BK, Vidal M, Bard M (1989) The yeast gene *ERG6* is required for normal membrane function but is not essential for biosynthesis of the cell-cycle-sparking sterol. Mol Cell Biol 9:3447–3456

Gietz RD, Sugino A (1988) New yeast-Escherichia coli shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. Gene 74:527–534

Graham TR, Scott PA, Emr SD (1993) Brefeldin A reversibly blocks early but not late protein transport steps in the yeast secretory pathway. EMBO J 12:869–877

Hartwell LH (1974) *Saccharomyces cerevisiae* cell cycle. Bacteriol Rev 38:164–198

Hartwell LH, Culotti J, Pringle JR, Reid BJ (1974) Genetic control of the cell division cycle in yeast. Science 183:46–51

Helms JB, Rothman JE (1992) Inhibition by brefeldin A of a Golgi membrane enzyme that catalyzes exchange of guanine nucleotide bound to ARF. Nature 350:352–354

Herruer MH, Mager WH, Woudt LP, Nieuwint RTM, Wassenaar GM, Groeneveld P, Planta RJ (1987) Transcriptional control of yeast ribosomal protein synthesis during carbon-source upshift. Nucleic Acids Res 15:10133–10144

Ito H, Fukuda Y, Murata K, Kimura A (1983) Transformation of intact yeast cells treated with alkali cations. J Bacteriol 153:163–168

Jackson CL, Képès F (1994) *BFR1*, a multicopy suppressor of brefeldin A induced lethality, is implicated in secretion and nuclear segregation in *Saccharomyces cerevisiae*. Genetics 137:423–437

Klausner RD, Donaldson JG, Lippincott-Schwartz J (1992) Brefeldin A: insights into the control of membrane traffic and organelle structure. J Cell Biol 116:1071–1080

Kondo K, Inouye M (1991) *TIP1*, a cold shock-inducible gene of *Saccharomyces cerevisiae*. J Biol Chem 166:17537–17544

Lewis TA, Rodriguez RJ, Parks LW (1987) Relationship between intracellular sterol content and sterol esterification and hydrolysis in *Saccharomyces cerevisiae*. Biochim Biophys Acta 921:205–212

Meilhoc E, Masson JM, Teissié J (1990) High efficiency transformation of intact cells by electric field pulses. Bio/Technology 8:223–227

Moehle CM, Hinnebusch AG (1991) Association of RAP1 binding sites with stringent control of ribosomal protein gene transcription in Saccharomyces cerevisiae. Mol Cell Biol 11:2723–2735

Mumberg D, Müller R, Funk M (1994) Regulatable promoters of *Saccharomyces cerevisiae*: comparison of transcriptional activity and their use for heterologous expression. Nucleic Acids Res 22:5767–5768

Nitiss J, Wang JC (1988) DNA topoisomerase-targeting antitumor drugs can be studied in yeast. Proc Natl Acad Sci USA 85:7501–7505

Poon PP, Wang X, Rotman M, Cukierman E, Cassel D, Singer RA, Johnston GC (1996) *Saccharomyces cerevisiae Gsc1* is an ADP-ribosylation factor GTPase-activating protein. Proc Natl Acad Sci USA 93:10074–10077

Rambourg A, Clermont Y, Jackson CL, Képès F (1995) Effects of brefeldin A on the three-dimensional structure of the Golgi apparatus in a sensitive strain of *Saccharomyces cerevisiae*. Anat Rec 241:1–9

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY

Taylor FR, Parks LW (1978) Metabolic interconversion of free sterols and steryl esters in *Saccharomyces cerevisiae*. J Bacteriol 136:531–537

Vignais M-L, Huet J, Bühler J-M, Sentenac A (1990) Contacts between the factor TUF and RPG sequences. J Biol Chem 265:14669–14674

Wang X, Hoekstra MF, DeMaggio AJ, Dhillon N, Vancura A, Kuret J, Johnston GC, Singer RA (1996) Prenylated isoforms of yeast casein kinase I, including the novel Yck3p, suppress the *gcs1* blockage of cell proliferation from stationary phase. Mol Cell Biol 16:5375–5385

Werner-Washburne M, Braun E, Johnston GC, Singer R (1993) Stationary phase in the yeast *Saccharomyces cerevisiae*. Microbiol Rev 57:383–401

Winsor B, Potter AA, Karst F, Nestmann ER, Lacroute F (1987) Characterization of the mutation *ise1* that alters permeability of *Saccharomyces cerevisiae*. Environ Mutagen 9:114–120