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Dual regulation of the expression of the polyubiquitin gene by cyclic AMP and heat shock in yeast

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The expression of the polyubiquitin gene in yeast, *UBI4*, is repressed by cAMP-dependent protein phosphorylation. Since cycloheximide does not inhibit the induction of the *UBI4* transcription, no *de novo* protein synthesis is required in this process. The expression of the *UBI4* gene is also induced by mild heat shock by a mechanism other than depletion of cAMP. From the genetic analysis of the *UBI4* gene, we propose that the *UBI4* gene is one of the genes which are part of the cAMP-effector pathway and required for G_0/G_1 arrest in *Saccharomyces cerevisiae*.

Key words: cAMP/G_0/G_1 phase/heat shock/polyubiquitin/yeast

Introduction

Ubiquitin is a 76-residue protein whose sequence is well conserved; ubiquitin of humans is identical to that of insects and differs at only three positions from that of yeast (Goldstein *et al.*, 1975; Gavilanes *et al.*, 1982; Özkaynuk *et al.*, 1984; Wilkinson *et al.*, 1986). This protein is usually found either free or covalently coupled via its carboxyl terminus to ϵ-amino groups of lysine residues in a wide variety of intracellular protein species (Atidia and Kulka, 1982). It was found that ubiquitin is identical to the ATP-dependent proteolysis factor I of rabbit reticulocytes (Wilkinson *et al.*, 1980). Hershko and Ciechanover (1982) proposed that ubiquitin serves as a signal for an attack by proteinases specific for ubiquitin–protein conjugates. Expression of the ubiquitin gene is also interesting as ubiquitin is one of the heat shock proteins in chicken cells (Bond and Schlesinger, 1985). Recently, genetic analysis of the yeast polyubiquitin gene (*UBI4*), which encodes five ubiquitin repeats in a spacerless head-to-tail arrangement, revealed that the *UBI4* gene is essential for resistance to high temperatures, starvation and other stresses, and that the expression of the *UBI4* gene is specifically induced by heat shock and in the stationary phase of growth (Finley *et al.*, 1987). It was reported that the synthesis of several heat shock proteins was induced by starvation (Iida and Yahara, 1984b).

Recent study of cell cycle control in yeast suggests that nutrient starvation results in a low level of intracellular cAMP (Matsumoto *et al.*, 1985a). Cyclic AMP controls the cell cycle of yeast via activation of cAMP-dependent protein kinase. In the absence of cAMP, cells enter a resting state (G_0/G_1) equivalent to the G_0 state of higher eukaryotes (Iida and Yahara, 1984a), while the presence of cAMP activates cAMP-dependent protein kinase thereby allowing the cells to enter the mitotic cycle (Matsumoto *et al.*, 1985a). A *cyr1-2* mutant which has a heat-labile adenylate cyclase cannot enter the mitotic cycle because of the failure to activate cAMP-dependent protein kinase at a non-permissive temperature. In contrast, a *bcy1* mutant which is defective in the gene encoding the regulatory subunit of cAMP-dependent protein kinase cannot be arrested at G_0/G_1 phase because of the constitutive activation of the catalytic subunit of the cAMP-dependent protein kinase (Matsumoto *et al.*, 1982). More recently, it was reported that several heat shock proteins were constitutively synthesized in the *cyr1-2* mutant (Shin *et al.*, 1987).

In this report we show that the expression of the polyubiquitin gene in *Saccharomyces cerevisiae* is regulated not only by heat shock but also by cAMP. Interestingly, these divergent signals controlled the expression of the *UBI4* gene by different mechanisms. We also report that the induction of the *UBI4* gene by cAMP depletion occurs in the absence of protein synthesis. Analysis of a gene disruption mutant of the *UBI4* gene revealed that ubiquitin is part of the cAMP-effector pathway.

Results

Cyclic AMP represses the expression of the polyubiquitin gene

To examine the effect of cAMP on the expression of the *UBI4* gene, the amount of *UBI4* transcript was analyzed in cells of yeast R28-3 (*cyr1-1/cyr1-1*) grown in the presence or absence of cAMP. When cAMP was depleted from the medium, R28-3 cell growth ceased gradually and stopped after 5.5 h. Microscopic observation of the culture showed that most cells were arrested at an unbudded state, possibly at the G_0/G_1 phase (date not shown). Exponentially growing R28-3 cells at 28°C in YPD medium containing 0.5 mM cAMP were collected by centrifugation, washed once with YPD and re-inoculated into YPD containing various combinations of cAMP and cycloheximide. After incubation for 5.5 h at 28°C, cells were harvested and total RNA was extracted. Northern hybridization experiments using a fragment containing the entire *UBI4* gene as a probe clearly indicate that the 1.2-kb polyubiquitin mRNA was induced by depletion of cAMP (Figure 1A, lane 1). Surprisingly, the induction was seen in the presence of cycloheximide (lane 4). Another experiment in which cycloheximide was added 15 min before the cAMP depletion showed the same results (data not shown). In the presence of cAMP, the cells continued growing and the *UBI4* mRNA was not produced (lane 2). The hybridization band seen in lane 3 indicates that cycloheximide itself cannot induce the *UBI4* mRNA, although a very faint band appeared. Cycloheximide seemed to enhance the derepression of the *UBI4* transcription; the level of the 1.2-kb RNA is higher in lane 4 than in lane 1.

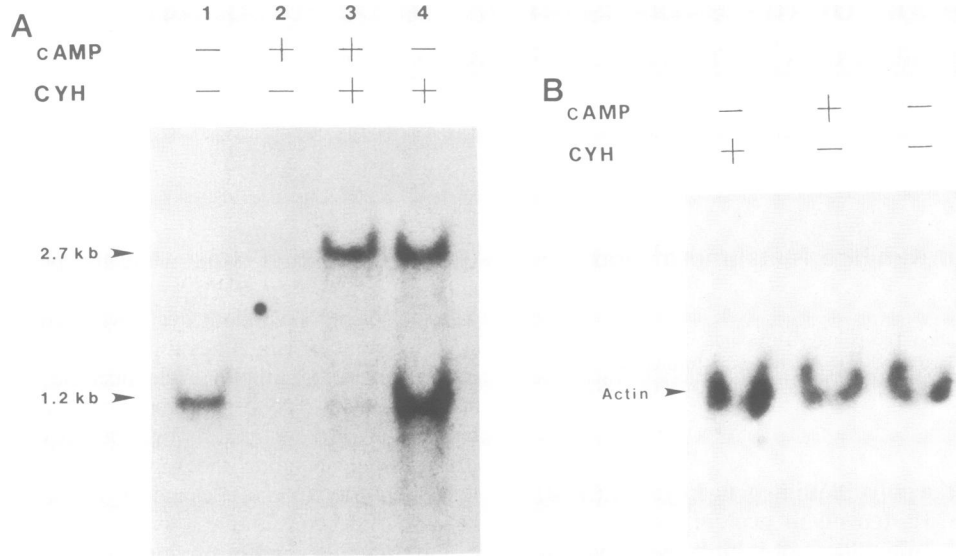

Fig. 1. Regulation of expression of the *UBI4* gene by cAMP. **A.** Washed cells of R28-3 grown in YPD with 0.5 mM cAMP were shaken for 5.5 h at 28°C in YPD with no addition (**lane 1**), 0.5 mM cAMP (**lane 2**), 0.5 mM cAMP and 50 μg/ml cycloheximide (**lane 3**) or 50 μg/ml cycloheximide (**lane 4**). 15 μg of total RNA extracted from each culture was electrophoresed in an agarose gel. Northern hybridization was carried out using the 3.4-kb *Bam*HI−*Eco*RV fragment containing the entire *UBI4* gene as a probe. 1.2-kb RNA corresponds to the ubiquitin mRNA. **B.** The level of the actin mRNA is not affected by either cAMP or cycloheximide. Northern hybridization was carried out using the same amount of RNA used in lanes 1, 2 and 4 of Figure 1A. The blotted filter was probed with the 1.6-kb *Bam*HI−*Hin*dIII fragment of the yeast actin gene.

Unexpectedly a similar extent of hybridization was seen at the size of 2.7 kb in lanes 3 and 4, possibly due to the presence of cycloheximide. 2.7-kb RNA is more abundant in strain R27-7C than in R28-3 since it could be detected in the absence of cycloheximide (Figure 2, lanes 8 and 9). The difference in relative abundance is probably due to the different genetic backgrounds of these two strains. The fact that the 2.7-kb hybridization band was not seen in the *UBI4* gene disruption mutant (Figure 2, lane 7) indicates that the 2.7-kb RNA does contain the polyubiquitin sequence. When a sequence far upstream of the *UBI4* gene was used as a probe, only the 2.7-kb RNA was detected. Moreover, analysis using a single strand specific probe indicates that the 2.7-kb RNA is transcribed in the same orientation as that of the 1.2-kb RNA (data not shown). From these results we conclude that the 2.7-kb RNA is a readthrough product transcribed from an upstream region of the *UBI4* gene. We did not analyze this species of RNA further because various lines of evidence indicated that the 1.2-kb RNA is the true polyubiquitin mRNA. Thus when yeast cells harboring *UBI4-lacZ* plasmids were subjected to heat treatment at 39°C, β-galactosidase activity increased (data not shown). The 1.2-kb RNA was induced by heat shock, while the 2.7-kb RNA was not (Figure 2, lanes 8 and 9). A plasmid (pJU10) having a chimeric gene in which the promoter of the *GAP-DH* (glyceraldehyde-3-phosphate dehydrogenase) gene was fused to the entire polyubiquitin open reading frame was constructed (see Materials and methods). The pJU10 plasmid, when introduced into yeast cells, resulted in a very high amount of the 1.2-kb RNA (Figure 5, lane 1). Moreover, the *GAP-DH-UBI4* gene complemented the sporulation deficiency of the *ubi4::LEU2* mutant (see Table V).

The results presented above clearly indicate that the expression of the 1.2-kb polyubiquitin mRNA is induced by the absence of cAMP, where *de novo* protein synthesis is not required. We also examined the expression of the yeast

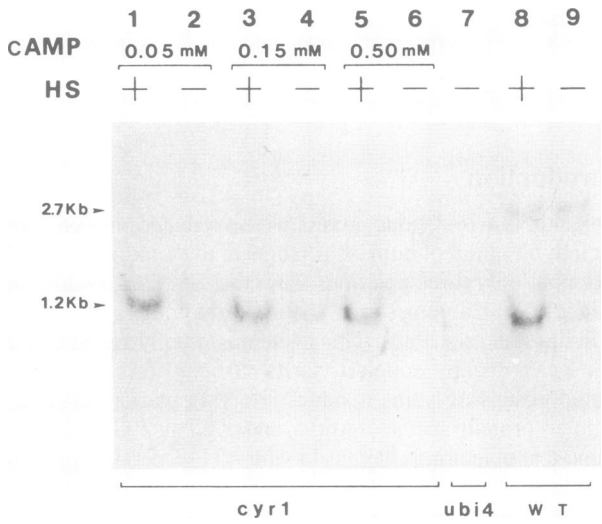

Fig. 2. Heat shock response of the *UBI4* gene in the *cyr1-1* mutant. Northern hybridization experiment was carried out using 15 μg each of total RNA extracted from cells of the *cyr1-1* mutant, *ubi4::LEU2* mutant and wild-type strain. Exponentially growing cells at 28°C in YPD medium of the strains R28-3 (*cyr1-1/cyr1-1*; lanes 1−6), R27-7C-UD (*ubi4::LEU2*; **lane 7**), and R27-7C-D (wild type; **lanes 8** and **9**) were shaken at 28°C for 2 h in YPD medium (**lanes 2, 4, 6, 7** and **9**) or heated at 39°C for 2 h in YPD medium (**lanes 1, 3, 5** and 8). R28-3 was cultured in YPD medium containing various amounts of cAMP (0.05 mM, **lanes 1** and **2**; 0.15 mM, **lanes 3** and **4**; 0.50 mM, **lanes 5** and **6**), while R27-7C-UD and R27-7C-D were cultured in YPD medium without cAMP. OD_{660} nm of each culture was ~1.0 at the time of harvest. The 3.4-kb *Bam*HI−*Eco*RV fragment was used as a DNA probe.

actin gene (Gallwitz and Seidel, 1980) by Northern analysis. However, neither cAMP nor cycloheximide affected the mRNA level of this gene (Figure 1B).

Because, in yeast, cAMP exerts its action via a cAMP-dependent protein kinase (Matsumoto *et al.*, 1985a), it is possible that expression of *UBI4* gene is regulated by cAMP-

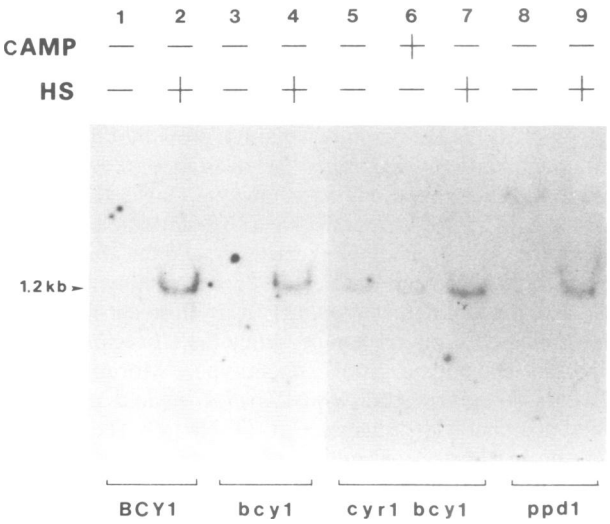

Fig. 3. The *UBI4* gene is induced by heat shock in the *bcy1* mutant and the *ppd1* mutant. Northern hybridization experiment was carried out as described in Figure 2 except that the synthesized probe A (see Materials and methods) was used. Strains used were RA1-13D (a parent of RA1-13D-R; **lanes 1** and **2**), RA1-13D-R (*bcy1::URA3*; **lanes 3** and **4**), AM9-10A (*cyr1-1 bcy1-1*; **lanes 5, 6** and **7**) and R59-1C (*ppd1*; **lanes 8** and **9**). Cells grown exponentially at 28°C were shaken at 28°C (**lanes 1, 3, 5, 6** and **8**) or heated at 39°C (**lanes 2, 4, 7** and **9**) for 2 h. OD_{660} nm of each culture was ~1.0 at the time of harvest. AM9-10A was cultured in the absence (**lane 5**) or presence (**lane 6**) of 0.5 mM cAMP.

dependent protein phosphorylation. If this is the case, the *UBI4* gene should not be induced by the depletion of cAMP in the *cyr1 bcy1* double mutant whose cAMP-dependent protein kinase is constitutively active. Northern blotting experiments (Figure 3, lanes 5 and 6) clearly support our idea. The level of the *UBI4* mRNA was not affected by the cAMP concentration in *cyr1 bcy1* mutant. Thus we conclude that the expression of the ubiquitin gene is regulated by the cAMP-dependent protein kinase in yeast.

Cyclic AMP is not involved in the heat shock response of the UBI4 gene

In chicken and yeast cells, polyubiquitin is one of the heat shock proteins (Bond and Schlesinger, 1985; Finley and Varshavsky, 1985). These observations led us to examine whether or not induction of the ubiquitin gene by temperature shift-up is mediated through the cAMP cascade. Cells of the *cyr1-1* mutant (R28-3) or wild type were cultivated in duplicate at 28°C in YPD supplemented with or without various concentrations of cAMP. The incubation time of R28-3 in YPD with low concentrations of cAMP (0.05 mM or 0.15 mM) was ~16 h. When the OD_{660} of each culture reached ~0.5, one of the cultures was heated at 39°C for 2 h whereas the other was kept at 28°C. Total RNA was extracted from each culture and the amount of *UBI4* mRNA was estimated by Northern hybridization (Figure 2). In the RNA from R28-3 grown at 28°C, we observed the effect of cAMP concentration on the amount of *UBI4* mRNA; the *UBI4* mRNA was slightly induced by lowering the cAMP concentration in the cells without heat shock. The *UBI4* gene was clearly induced by the temperature shift-up in the *cyr1-1* mutant even in the presence of sufficient cAMP. This result suggests that heat treatment induces the expression of the *UBI4* gene by some signal other than cAMP.

This was further confirmed by the experiment shown in Figure 3. The heat shock response of the *UBI4* gene was examined in a *bcy1* mutant and a *ppd1* mutant which are deficient in the regulatory subunit of cAMP-dependent protein kinase and in the phosphoprotein phosphatase respectively (Matsumoto *et al.*, 1985b). Northern hybridization data shown in Figure 3 clearly indicate that the *UBI4* gene is actively transcribed in both the *bcy1* mutant and the *ppd1* mutant after heat treatment. These results exclude the involvement of the cAMP-dependent protein phosphorylation in the induction of the *UBI4* gene by heat shock.

Phenotypes of disruption mutant of the UBI4 gene

From our study of the induction of the *UBI4* gene, it can be presumed that ubiquitin is an essential factor for the G_0/G_1 arrest caused by cAMP depletion. This idea is consistent with the fact that the *ubi4* mutants are sensitive to starvation and are defective in sporulation (Finley *et al.*, 1987). To know whether the *UBI4* gene is essential for G_0/G_1 arrest morphologically in yeast, we also constructed a mutant in which the *UBI4* gene is disrupted (R27-7C-UD; Table I, see Materials and methods).

R27-7C-UD and its parental strain (R27-7C-D) were starved in medium from which nitrogen and sulfate was omitted (−NS medium) and the population of unbudded cells was determined. As shown in Table II, the *ubi4::LEU2* strain cannot be arrested at the G_0/G_1 phase in this starvation medium. Probably because of failure to be arrested at the G_0/G_1 phase, cell viability of R27-7C-UD decreased rapidly in the −NS medium, and R27-7C-UD showed no sporulation capability (Table II, Finley *et al.*, 1987). Microscopic observation of *ubi4::LEU2/ubi4::LEU2* cells in the sporulation medium stained with DNA-specific fluorescent dye, DAPI (4′, 6′-diamidino-2-phenylindole), revealed that most cells have only a single nucleus, although we did not determine whether or not premeiotic DNA synthesis had occurred (data not shown). From these observations, we conclude that the *UBI4* gene is essential for the G_0/G_1 phase.

The fact that the ubiquitin gene is activated by heat treatment also indicates that ubiquitin may be involved in heat tolerance in yeast. Exponentially growing cells of R27-7C-D or R27-7C-UD were heat-shocked at 52°C with or without prior incubation at 37°C, and surival fractions were determined at various time points. Data shown in Figure 4 support our hypothesis. That is, R27-7C-UD cells grown at 28°C are more sensitive by two orders of magnitude to exposure to 52°C than R27-7C-D cells grown at 28°C. However, prior incubation at 37°C for 2 h conferred thermal tolerance on R27-7C-UD cells as well as R27-7C-D cells. From these results we conclude that ubiquitin is functional in rendering yeast cells thermally tolerant, although it is not involved in the acquisition of heat tolerance by a mild heat shock.

Genetic analysis of the ubi4::LEU2 mutant

The results presented above indicate that ubiquitin is required for cells to enter the G_0/G_1 phase. In this respect, ubiquitin functions downstream of the cAMP cascade, because the expression of the *UBI4* gene was enhanced by a low level of cAMP. However, it is equally possible that ubiquitin is involved in the control of cAMP-dependent protein phosphorylation because the phenotypes of the *ubi4::LEU2* mutant resemble those of the *bcy1*, *RAS2*^{val19} (Toda *et al.*,

1985) and *ppd1* mutants. These hypotheses can be genetically tested by using the *cyr1-2* mutant which has a heat-labile adenylate cyclase (Matsumoto *et al.*, 1983). If the *UBI4* gene is essential for G_0/G_1 arrest, the *ubi4::LEU2 cyr1-2* double mutant will show the phenotypes of both the *ubi4::LEU2* mutant and the *cyr1-2* mutant. If the *UBI4* gene is required for the control of cAMP-dependent protein phosphorylation, the double mutant will show either phenotype; if, like the *bcy1* mutant, the *ubi4::LEU2* strain is defective in a pathway downstream of adenylate cyclase in the cAMP cascade, the *ubi4::LEU2* mutation will suppress the growth deficiency of the *cyr1-2* mutant at a non-permissive temperature. On the other hand, if the phenotypes of the *ubi4::LEU2* mutant are caused by a deficiency upstream of adenylate cyclase in the cAMP cascade, like the $RAS2^{val19}$ mutation, the *cyr1-2* mutation will suppress the phenotypes of the *ubi4::LEU2* strain at 25°C because the adenylate cyclase activity in the *cyr1-2* mutant is 5−10% of that in the wild-type strains even at a low temperature (Matsumoto *et al.*, 1983).

C16-3B was crossed with T50-3A (see Table III) and the resultant diploid was sporulated and dissected. The temperature-sensitive phenotype for growth segregated $2^+:2^-$ in a manner showing non-linkage to the Leu^+ phenotype (Table III). Thus, the *ubi4::LEU2* mutation does not suppress the temperature-sensitive growth of the *cyr1-2* mutation. Next, we examined the resistance of each spore culture to NS-starvation. As shown in Table III, all the *ubi4::LEU2* spores were sensitive to NS-starvation irrespective of the *cyr1-2* mutation. We also crossed the *ubi4::LEU2 cyr1-2* double mutants with each segregant shown in Table III. All the *ubi4::LEU2/ubi4::*LEU2 diploids showed a sporulation-deficient phenotype, while the *UBI4/ubi4::LEU2* diploids showed a Spo^+ phenotype. Moreover, the *ubi4::LEU2/ubi4::LEU2 cyr1-2/cyr1-2* diploid could not arrest at an unbudded state at 37°C (Table IV). These results indicate that the *cyr1-2* mutation cannot suppress the starvation sensitivity, sporulation deficiency and G_0/G_1 arrest deficiency of the *ubi4::LEU2* mutation. Thus we conclude that the *ubi4::LEU2* mutation and *cyr1-2* mutation cannot suppress each other. These observations indicate that the function of the *UBI4* gene is independent of the activity of

Table I. List of strains used in this study

Strain	Genotype	Reference
R28-3	*MATa/MATα cyr1-1/cyr1-1*	
RA1-13D	*MATa ura3 leu2 trp1 his3*	Yamano *et al.*, 1987
RA1-13D-R	*MATa ura3 leu2 trp1 his3 bcy1::URA3*	Yamano *et al.*, 1987
R27-7C	*MATα ura3 leu2 trp1*	
R27-7C-U	*MATα ura3 leu2 trp1 ubi4::LEU2*	
R27-7C-D	*MATa/MATα ura3/ura3 leu2/leu2 trp1/trp1*	
R27-7C-UD	*MATa/MATα ura3/ura3 leu2/leu2 trp1/trp1 ubi4::LEU2/ubi4::LEU2*	
R59-1C	*MATa ura3 lys2 thr4 ppd1-1*	
AM9-10A	*MATα cyr1-1 bcy1-1*	Matsumoto *et al.*, 1982
T50-3A	*MATα ura3 leu2 trp1 his3 cyr1-2*	Kataoka *et al.*, 1985
R145-6A	*MATa ura3 leu2 lys2 thr4? cyr1-2*	
C16-3B	*MATa ura3 leu2 his3 trp1 ade8 ubi4::LEU2*	
C17-3A[a]	*MATa ura3 leu2 ubi4::LEU2 cyr1-2*	
C17-4B[a]	*MATα ura3 leu2 lys2 ubi4::LEU2 cyr1-2*	
KMY82-1C[b]	*MATa ura3 leu2 his3 trp1 ade8 ubi4::LEU2 cyr1-2*	
KMY82-3A[b]	*MATa ura3 leu2 his3 trp1 cyr1-2*	
W935-1B	*MATα ura3 trp1 bcy1-1*	Yamano *et al.*, 1987

[a]C17-3A and C17-4B are haploid progeny obtained from a cross between R27-7C-U and R145-6A.
[b]KMY82-1C and KMY82-3A are haploid progeny obtained from a cross between C16-3B and T50-3A.

Table II. The *ubi4::LEU2* cells cannot enter the G_0/G_1 phase

Strain	Incubation time (h)	−NS medium		Sporulation efficiency (%)
		Unbudded cells (%)	Viability (%)	
R27-7C-D	0	36	100	–
	48	91	290	18
R27-7C-UD	0	33	100	–
	48	49	8	<0.1

Exponentially growing cells in YPD medium of each strain were inoculated in −NS medium or sporulation medium at 28°C. For each determination see Materials and methods.

the cAMP-dependent protein kinase and is specifically required for the G_0/G_1 arrest in yeast.

Over-expression of the UBI4 gene does not suppress the bcy1 mutation

We demonstrated that the mRNA of the polyubiquitin gene cannot be induced in the *cyr1-2 bcy1* mutant by depletion of cAMP (Figure 3). This phenomenon led us to assume that the phenotype of the *bcy1* mutant is caused by the failure of induction of the polyubiquitin gene. To test this hypothesis, pJU10 was introduced into the *bcy1* mutant. A Northern blotting experiment of the transformant revealed that the *UBI4* gene mRNA was produced at a level higher than that induced by heat shock or the depletion of cAMP (Figure 5). However, the high expression of the *UBI4* gene could not suppress the sporulation deficiency of the *bcy1* mutant (Table V). This observation suggests that the failure of induction of the polyubiquitin gene is not the only cause of the Spo^- phenotype of the *bcy1* mutant.

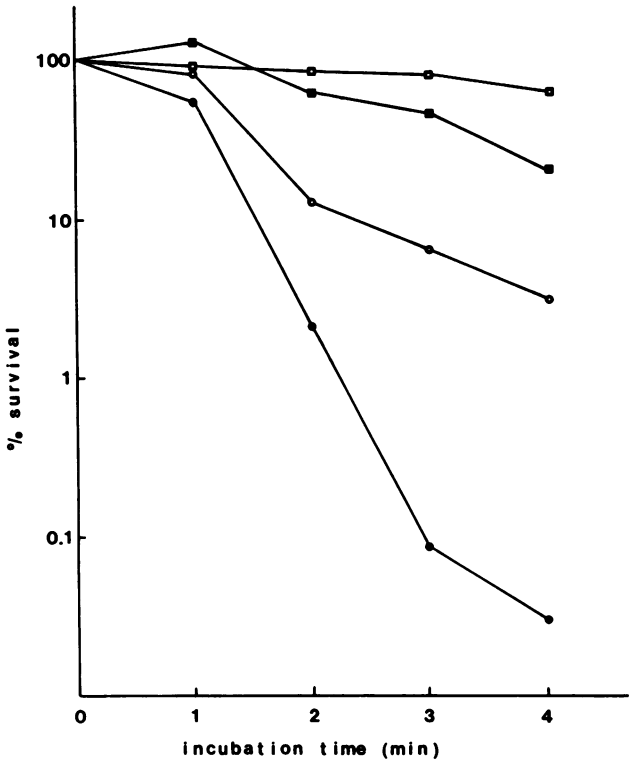

Fig. 4. Heat shock sensitivity of the disruption mutant of the polyubiquitin gene. Exponentially growing cells of R27-7C-D (○, □) or R27-7C-UD (●, ■) at 28°C were preincubated at 28°C (○, ●) or 37°C (□, ■) for 2 h respectively. Cells were then incubated at 52°C and survival fractions were determined at each time point.

Discussion

We have shown that the expression of the polyubiquitin gene is regulated by cAMP in *S.cerevisiae*. In yeast, cAMP regulates the cell cycle via cAMP-dependent protein kinase, and expression of the polyubiquitin gene is most probably modulated by cAMP-dependent protein phosphorylation. In contrast to the genes whose expression needs the presence of cAMP (Montminy *et al.*, 1986; Comb *et al.*, 1986), the *UBI4* gene in the *cyr1-1* mutant was induced by depletion of cAMP. No *de novo* protein synthesis was required in this process. This result implies that the cAMP-dependent protein kinase directly regulates the positive or negative transcriptional factor(s) by phosphorylation. In this context, results reported by Celenza and Carlson (1986) are of interest. They showed that the *SNF1* gene, which is required for the release from carbon catabolite repression in yeast, encodes a protein kinase. Mediation by a protein kinase may be one of the general mechanisms of gene regulation in yeast.

Because cAMP depletion causes G_0/G_1 arrest in yeast cells, we presume that the *UBI4* gene is specifically required for the G_0/G_1 arrest. Phenotypes of the *ubi4::LEU2* mutant and the *ubi4::LEU2 cyr1-2* double mutant support this idea. That is, ubiquitin is essential for sporulation, resistance to starvation (Finley *et al.*, 1987) and morphological G_0/G_1 arrest. However, Finley *et al.* (1987) reported that the *ubi4* mutants could accumulate glycogen and acquire thermal tolerance in stationary-phase cells. These observations indicate that the *ubi4* mutants are not defective in all of the processes of the G_0/G_1 phase. Thus we presume that another gene(s) specifically required for the G_0/G_1 arrest (G_0-specific genes) should be present in yeast. This specula-

Table III. Genetic analysis of the *ubi4::LEU2* mutation

Segregant[a]	Genotype		Leu phenotype	Growth at 35°C	Sporulation[b] Phenotype	Resistance to NS-starvation[c]
1A	*UBI4*	*CYR1*	−	+	+	+
1B	*UBI4*	*cyr1-2*	−	−	+	+
1C	*ubi4::LEU2*	*cyr1-2*	+	−	−	−
1D	*ubi4::LEU2*	*CYR1*	+	+	−	−
3A	*UBI4*	*cyr1-2*	−	−	+	+
3B	*ubi4::LEU2*	*CYR1*	+	+	−	−
3C	*ubi4::LEU2*	*CYR1*	+	+	−	−
3D	*UBI4*	*cyr1-2*	−	−	+	+
4A	*UBI4*	*CYR1*	−	+	+	+
4B	*UBI4*	*CYR1*	−	+	+	+
4C	*ubi4::LEU2*	*cyr1-2*	+	−	−	−
4D	*ubi4::LEU2*	*cyr1-2*	+	−	−	−

[a]Three tetrads obtained from a cross, KMY82, between C16-3B (*ubi4::LEU2 CYR1*) and T50-3A (*UBI4 cyr1-2*) were examined.
[b]Each segregant was crossed with C17-3A (*MATa ubi4::LEU2 cyr1-2*) or C17-4B (*MATα ubi4:: LEU2 cyr1-2*) and the sporulation phenotype at 25°C of the resultant diploids was tested.
[c]Resistance to NS-starvation was scored at 25°C.

Table IV. The *ubi4::LEU2 cyr1-2* cells cannot arrest at the G_0/G_1 phase at high temperature

Strain[a]	Unbudded cells at 37°C[b] (%)		Sporulation efficiency (%)
	0 h incubation	6 h incubation	
CK-1	32	48	<0.1
CK-2	44	80	21

[a]CK-1 or CK-2 was obtained by a cross between C17-4B (*ubi4::LEU2 cyr1-2*) and KMY82-1C (*ubi4::LEU2 cyr1-2*) or C17-4B and KMY82-3A (*UBI4 cyr1-2*).
[b]Exponential-phase cells of CK-1 or CK-2 at 25°C were shifted to 37°C. After incubation for the indicated times, cells were sampled and the percent unbudded cells was determined under the microscope.
[c]Sporulation efficiency of each strain was determined after incubation in sporulation medium at 25°C for 3 days.

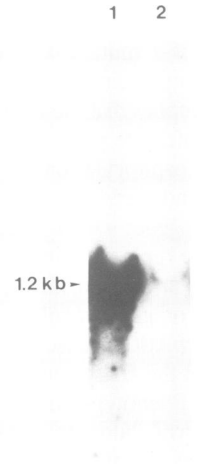

Fig. 5. Over-expression of the *UBI4* gene in the *bcy1* mutant. RA1-13D-R (*bcy1::URA3*) was transformed with pJU10 (**lane 1**) or pYS6 (**lane 2**). Northern hybridization was carried out using 15 μg of total RNA extracted from the cultures of each transformant grown under selective conditions. Plasmid pYS6 is a reference which has no expression unit. The blotted filter was hybridized with probe A.

Table V. Over-expression of the *UBI4* gene cannot suppress the *bcy1* mutation

Strain[a]	Plasmid[b]	Sporulation efficiency (%)
RW-1	pJU10	<0.1
RW-2	pSY2-1	14
R27-7C-UD	pJU10	15
R27-7C-UD	pYS3	<0.1

[a]Transformants of RA1-13D-R (*bcy1::URA3*) with pJU10 or pSYS2-1 were mated with W935-1B (*bcy1-1*) obtaining RW-1 or RW-2.
[b]Plasmids, pJU10 or pSYS2-1, contain the *GAP-UBI4* gene (see Materials and methods) or the *BCY1* gene (Yamano *et al.*, 1987) respectively. Plasmid pYS3 which contains the *PHO5* gene (Arima *et al.*, 1983) in place of the *UBI4* gene under the *GAP-DH* promoter was used as a reference.

tion is suppprted by the fact that the over-expression of the *UBI4* gene could not suppress the *bcy1* mutation (Table V). From these results, we propose that cAMP controls the G_0/G_1 phase in yeast by regulating the synthesis of G_0-specific proteins including polyubiquitin (Figure 6).

Induction of transcription of the *UBI4* gene by heat shock

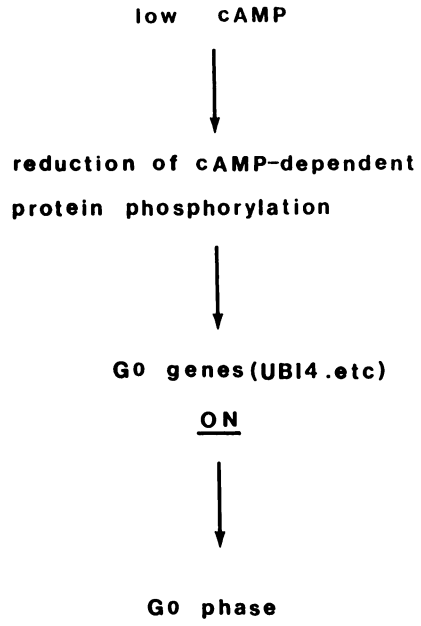

Fig. 6. Proposed model for transcriptional regulation exerted by the cAMP cascade. Genes which are required for G_0/G_1 arrest and are repressed by cAMP-dependent protein phosphorylation were designated as G_0-specific genes. *UBI4* gene is a member of the G_0-specific genes but not the only gene required for G_0/G_1 arrest (see text).

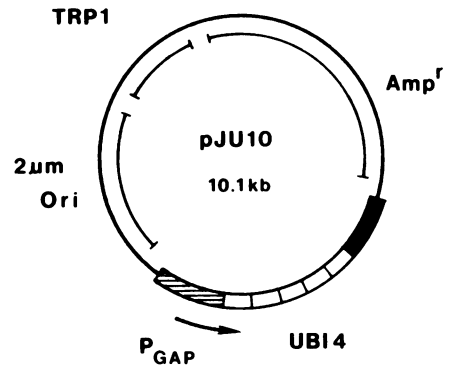

Fig. 7. Structure of the plasmid, pJU10, used for high expression of the *UBI4* gene. Plasmid pJU10 is a 2 μm DNA-based plasmid which has the *TRP1* gene and the Amp^r gene for selection in yeast and *E.coli* respectively. Shaded box; *GAP-DH* promoter. Open boxes; *UBI4* coding region. Black box; *UBI4* 3'-region.

was independent of the cAMP cascade (Figures 2 and 3). Recently it was reported that several heat shock proteins were not synthesized in the *bcy1* mutant even at a high temperature (Shin *et al.*, 1987). This discrepancy can be reconciled by assuming that the *bcy1* gene regulates the synthesis of the heat shock proteins at the translational level. It was reported that the synthesis of several heat shock proteins are regulated not only at the transcriptional level but also at the translational level in *Drosophila* (McGarry and Lindquist, 1985; Hultmark *et al.*, 1986). For further study of the regulation of the *UBI4* gene, it is important to determine whether an upstream activation sequence (UAS) responding to the cAMP level is the same as that responding to heat shock or not. Such studies are now under way.

Induction of the *UBI4* gene by heat shock suggests that the *UBI4* gene is functional in thermal tolerance. Data shown in Figure 4 support this hypothesis. However, it was reported

that *ubi4* mutants were sensitive at 38.5°C rather than 52°C (Finley *et al.*, 1987). We have no explanation for this discrepancy at present.

Gene disruption of the *UBI4* gene revealed that ubiquitin is functional in both heat shock stress and starvation stress. In mammalian systems, it is proposed that ubiquitin may be a signal for attack by proteinases specific for ubiquitin−protein conjugates (Hershko and Ciechanover, 1982). Thus the function of the ubiquitin in yeast is presumably proteolytic elimination of the toxic proteins generated by these stresses. Because starvation stress may be caused by cAMP depletion, these stresses appear to elicit the activation of the *UBI4* gene by different mechanisms. This may indicate that these stresses are not the same in their biochemical nature and, in this respect, ubiquitin appears to recognize more than one target protein *in vivo*.

Materials and methods

Plasmids

The DNA sequence of the yeast ubiquitin (*UBI4*) gene was reported by Özkaynak *et al.* (1984). We cloned the *UBI4* gene into pBR322; the clone was detected by the colony hybridization method using the synthetic oligonucleotide probe A, 5′-ATGCAAATTTTCGTCAAAACTCTAACA-GGGAAGACTATAA−3′. We confirmed that the cloned DNA fragment contained the *UBI4* gene by DNA sequencing of the cloned fragment. A plasmid, pJU2, was constructed by replacing the 2.3-kb *Bam*HI−*Hin*cII fragment containing the entire *UBI4* coding region, a 0.7-kb upstream sequence and a 0.4-kb downstream sequence, with a 2.0-kb *Hpa*I−*Bam*HI fragment containing the *LEU2* gene.

For the high expression of the *UBI4* gene, we connected the *UBI4* coding region to the promoter of the *GAP-DH* gene (Holland and Holland, 1979) by the *Eco*RI site created immediately before the initiation codons of two genes by *in vitro* mutagenesis (Zoller and Smith, 1983). Introduction of a plasmid (pJU10, Figure 7) harboring the *GAP-DH-UBI4* fusion gene into R27-7C-UD restored the sporulation deficiency (Table V). Predicted nucleotide sequence changes of the mutated region were confirmed by DNA sequencing (Sanger *et al.*, 1977).

Strains and media

Strains used are listed in Table I. Gene disruption mutants were constructed by a one-step gene disruption method (Rothstein, 1983). RA1-13D-R was obtained by inserting a 1.2-kb *URA3* fragment into the *BCY1* locus. C16-3B and R27-7C-U were constructed by replacing the *UBI4* gene with the disrupted gene of pJU2. Diploid strains, R27-7C-D and R27-7C-UD were obtained by transformation with the plasmid carrying the homothallic gene, *HO* (Jensen *et al.*, 1983). *Escherichia coli* strain DH1 (Hanahan, 1983) was used for construction and amplification of plasmids. YPD medium contains 10 g of yeast extract (Oriental), 20 g of polypeptone (Daigo Eiyo) and 20 g of glucose per liter. −NS medium is the synthetic medium (Burkholder, 1943) in which $(NH_4)_2SO_4$ and asparagine were omitted and $MgSO_4$ was replaced with $MgCl_2$. Appropriate nutrients were supplemented in −NS medium. Agar plates were prepared by adding 20 g of agar per liter. Standard genetic techniques were carried out as described (Nogi *et al.*, 1977). Yeast transformation was by the lithium acetate method (Ito *et al.*, 1983).

Determination of heat sensitivity

Cells growing exponentially in YPD at 28°C were preincubated at 28°C or 37°C for 2 h. Each tube was then transferred to 52°C and small samples were taken at the indicated time and chilled in an ice bath. After appropriate dilution, cells were plated on a YPD plate. Three days later, colonies were counted and percentage survival was determined.

Determination of sporulation efficiency, sensitivity to starvation and degree of cell cycle arrest

Cells to be tested were freshly grown on YPD plates, transferred to sporulation media and incubated at various temperatures for 3 days. The population of sporulated cells was determined under the miroscope. At least 150 cells were counted.

Sensitivity to NS starvation was determined as follows. For the scoring on plate medium, cells to be tested were grown on YPD plates replica-plated on −NS plates and incubated at 25°C for 1 week. Then, the starved cells were replica-plated on YPD plates. After 2 days incubation at 25°C, cell viability was scored. For examination in liquid medium, exponentially growing cells in YPD medium were washed twice and inoculated into liquid −NS medium. After incubation for the indicated times, cells were plated on YPD plates after appropriate dilution. Three days later, colonies were counted and the percentage viability was determined.

For the determination of cell cycle arrest by nutrient starvation, starved cells in liquid −NS medium, as described above, were examined microscopically. Small samples were taken at time 0 and after 2 days incubation, and the population of unbudded cells was determined. For each determination at least 180 cells were counted.

Northern hybridization

Total RNA was extracted as described from a culture whose OD_{660} nm reached ~1.0 (Jensen *et al.*, 1983). Fifteen micrograms of total RNA was electrophoresed in a 1.2% agarose−7% formaldehyde gel. We checked the amount of each RNA electrophoresed by briefly staining the gel with ethidium bromide. After blotting, a filter was hybridized at 42°C in a buffer containing 50% formamide, 900 mM NaCl, 50 mM Na-phosphate (pH 8.3), 5 mM EDTA, 0.3% SDS, 170 μg/ml calf thymus DNA and 2.5 × Denhardt's solution. After hybridization, the filter was washed and autoradiographed as described (Toh-e and Shimauchi, 1986). DNA probes used for hybridization were either the 3.4-kb *Bam*HI−*Eco*RV fragment containing the entire *UBI4* gene or the synthetic probe A used for cloning the ubiquitin gene. The 3.4-kb *Bam*HI−*Eco*RV fragment and the oligonucleotide A were labeled with [α-^{32}P]dCTP by the multiprime method (Feinberg and Vogelstein, 1983) or with [γ-^{32}P]ATP by T4-polynucleotide kinase. The actin mRNA was detected by using the 1.6 kb *Bam*HI−*Hin*dIII fragment of the yeast actin gene as a probe (Gallwitz and Seidel, 1980). Probe A was used for hybridization and washing at 37°C and 45°C respectively. Essentially the same results were obtained using either of these two probes.

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References

Arima,K., Oshima,T., Kubota,I., Nakamura,N., Mizunaga,T. and Toh-e,A. (1983) *Nucleic Acids Res.*, **11**, 1657−1672.
Atidia,J. and Kulka,R.G. (1982) *FEBS Lett.*, **142**, 72−76.
Bond,U. and Schlesinger,M.J. (1985) *Mol. Cell. Biol.*, **5**, 949−956.
Burkholder,P.R. (1943) *Am. J. Bot.*, **30**, 206−211.
Celenza,J.L. and Carlson,M. (1986) *Science*, **233**, 1175−1180.
Comb,M., Birnberg,N.C., Seasholtz,A., Herbert,E. and Goodman,H.M. (1986) *Nature*, **323**, 353−354.
Feinberg,A.P. and Vogelstein,B. (1983) *Anal. Biochem.*, **132**, 6−13.
Finley,D. and Varshavsky,A. (1985) *Trends Biochem. Sci.*, **10**, 343−347.
Finley,D., Özkaynak,E. and Varshavsky,A. (1987) *Cell*, **48**, 1035−1046.
Gallwitz,D. and Seidel,R. (1980) *Nucleic Acids Res.*, **8**, 1043−1059.
Gavilanes,J.G., deBuitrago,G.G., Perez-Castells,R. and Rodriguez,R. (1982) *J. Biol. Chem.*, **257**, 10267−10270.
Goldstein,G., Scheid,M., Hammerling,U., Boyse,E.A., Schlesinger,D.H. and Niall,H.D. (1975) *Proc. Natl. Acad. Sci. USA*, **73**, 11−15.
Hanahan,D. (1983) *J. Mol. Biol.*, **166**, 557−580.
Hershko,A. and Ciechanover,A. (1982) *Annu. Rev. Biochem.*, **51**, 335−364.
Holland,J.P. and Holland,M.J. (1979) *J. Biol. Chem.*, **254**, 9839−9845.
Hultmark,D., Klemenz,R. and Gehring,W.J. (1986) *Cell*, **44**, 429−438.
Iida,H. and Yahara,I. (1984a) *J. Cell Biol.*, **98**, 1185−1193.
Iida,H. and Yahara,I. (1984b) *J. Cell Biol.*, **99**, 199−207.
Ito,H., Fukuda,Y., Murata,K. and Kimura,A. (1983) *J. Bacteriol.*, **153**, 163−168.
Jensen,R., Sprague,G.F.,Jr and Herskowitz,I. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 3035−3039.
Kataoka,T., Broek,D. and Wigler,M. (1985) *Cell*, **43**, 493−505.
Matsumoto,K., Uno,I., Oshima,Y. and Ishikawa,T. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 2355−2359.
Matsumoto,K., Uno,I. and Ishikawa,T. (1983) *Cell*, **32**, 417−423.
Matsumoto,K., Uno,I. and Ishikawa,T. (1985a) *Yeast*, **1**, 15−24.
Matsumoto,K., Uno,I., Kato,K. and Ishikawa,T. (1985b) *Yeast*, **1**, 25−38.

McGarry,T.J. and Lindquist,S. (1985) *Cell,* **42**, 903–911.
Montminy,M.R., Sevarino,K.A., Wagner,J.A., Mandel,G. and Goodman,R.H. (1986) *Proc. Natl. Acad. Sci. USA,* **83**, 6682–6686.
Nogi,T., Matsumoto,K., Toh-e,A. and Oshima,T. (1977) *Mol. Gen. Genet.,* **152**, 137–144.
Özkaynak,E., Finely,D. and Varshavsky,A. (1984) *Nature,* **312**, 663–666.
Rothstein,R.J. (1983) *Methods Enzymol.,* **101**, 202–211.
Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA,* **74**, 5463–5467.
Shin,D.-Y., Matsumoto,K., Iida,H., Uno,I. and Ishikawa,T. (1987) *Mol. Cell. Biol.,* **7**, 244–250.
Toda,T., Uno,I., Ishikawa,T., Powers,S., Kataoka,T., Broek,D., Cameron,S., Broach,J., Matsumoto,K. and Wigler,M. (1985) *Cell,* **40**, 27–36.
Toh-e,A. and Shimauchi,T. (1986) *Yeast,* **2**, 129–139.
Wilkinson,K.D., Urban,M.K. and Haas,A.L. (1980) *J. Biol. Chem.,* **255**, 7529–7532.
Wilkinson,K.D., Cox,M.J., O'Connor,L.B. and Shapira,R. (1986) *Biochemistry,* **25**, 4999–5004.
Yamano,S., Tanaka,K., Matsumoto,K. and Toh-e,A. (1987) *Mol. Gen. Genet.,* **210,** 413–418.
Zoller,M.J. and Smith,M. (1983) *Methods Enzymol.,* **100**, 468–500.

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