The EMBO Journal vol.9 no.10 pp.3225 - 3232, 1990

The adenylate cyclase/protein kinase cascade regulates entry into meiosis in *Saccharomyces cerevisiae* through the gene *IME1*

Akira Matsuura[1], Millet Treinin[2], Hiroshi Mitsuzawa[1], Yona Kassir[2], Isao Uno[1] and Giora Simchen[2]

[1]Institute of Applied Microbiology, University of Tokyo, Bunkyo-ku, Tokyo 113, Japan and [2]Department of Genetics, The Hebrew University, Jerusalem 91904, Israel

Communicated by A.Levitski

Entry into meiosis in *Saccharomyces cerevisiae* cells is regulated by starvation through the adenylate cyclase/cAMP-dependent protein kinase (AC/PK) pathway. The gene *IME1* is also involved in starvation control of meiosis. Multicopy *IME1* plasmids overcome the meiotic deficiency of *bcy1* and of *RAS*^{val19} diploids. Double mutants *ime1 cdc25* and *ime1 ras2* are sporulation deficient. These results suggest that *IME1* comes after the AC/PK cascade. Furthermore, the level of *IME1* transcripts is affected by mutations in the AC/PK genes *CDC25, CYR1* and *BCY1*. Moreover, the addition of cAMP to a *cyr1-2* diploid suppresses *IME1* transcription. The presence in a *bcy1* diploid of *IME1* multicopy plasmids does not cure the failure of *bcy1* cells to arrest as unbudded cells following starvation and to enter the G_0 state (thermotolerance, synthesis of unique G_0 proteins). This indicates that the pathway downstream of the AC/PK cascade branches to control meiosis through *IME1*, and to control entry into G_0 and cell cycle initiation, independently of *IME1*.

Key words: cAMP cascade/*IME1* gene/meiosis/*Saccharomyces cerevisiae*

Introduction

Diploid cells of the yeast *Saccharomyces cerevisiae* are able to initiate meiosis and sporulation only under starvation conditions. Diploidy and starvation are both required for meiosis. Diploidy is mediated through the mating type genes, *MATa1* and *MATα2*, and diploid cells defective in or homozygous for one of these genes are incapable of undergoing meiosis. The *rme1* mutation, when homozygous, suppresses this deficiency (Kassir and Simchen, 1976). The *RME1* gene is transcriptionally regulated by the genes *MATa1* and *MATα2* (Mitchell and Herskowitz, 1986). *RME1* has no role in the regulatory pathway by starvation, as *rme1* mutations do not permit unstarved cells to sporulate (Mitchell and Herskowitz, 1986).

Starvation seems to induce meiosis in *S.cerevisiae* through the adenylate cyclase/cAMP-dependent protein kinase (AC/PK) cascade system. Mutations in the adenylate cyclase gene, *CYR1* (*CDC35*) (Matsumoto *et al.*, 1982), and in two of its regulators *CDC25* (Broek *et al.*, 1987) and *RAS2* (Toda *et al.*, 1985), result in low levels of cellular cAMP. In these mutants, meiosis and sporulation take place in rich medium (Shilo *et al.*, 1978; Matsumoto *et al.*, 1983b; Tatchell *et al.*, 1985; Toda *et al.*, 1985; Mitsuzawa *et al.*, 1989). Mutations in the gene *BCY1* (Matsumoto *et al.*, 1983a; Toda *et al.*, 1987), which codes for the regulatory subunit of cAMP-dependent protein kinase, suppress the mutations *cyr1*, *cdc25* and *ras2* by making the protein kinase independent of cAMP. The mutation *bcy1*, when homozygous, results in diploids being meiosis and sporulation deficient (Matsumoto *et al.*, 1983a). The *RAS2*^{val19} mutation results in high levels of cellular cAMP and constitutively activates the cAMP-dependent protein kinase (Toda *et al.*, 1985); this dominant mutation also results in diploids being meiosis and sporulation deficient. This implies that high activity of the protein kinase inhibits meiosis, and that the reduced activity might be a necessary intermediate step in the regulation of meiosis by starvation (Matsumoto *et al.*, 1983b).

The gene *IME1* was originally identified by its ability, when present on a multicopy plasmid, to promote sporulation regardless of the constitution of *MAT* (Kassir *et al.*, 1988). Multicopy plasmids carrying *IME1* also enabled sporulation to occur in various media which contained nutrients (Granot *et al.*, 1989), thus also overriding the requirement for the starvation signal. Furthermore, transcription of *IME1* was shown to be induced by starvation (Kassir *et al.*, 1988). Thus, the *IME1* gene may serve as the merging point for the signals representing the two basic requirements for meiosis and sporulation, namely diploidy and starvation.

Here we present evidence that the expression of *IME1* is regulated by the AC/PK system. This is shown by Northern analysis (of *IME1* transcripts) in various mutants (*cdc25, cyr1* and *bcy1*), as well as by genetic interactions in double mutants with *ime1* or with the multicopy *IME1* plasmid. The multicopy *IME1* overrides the *bcy1* and *RAS2*^{val19} defects in meiosis. This epistatic effect is confined, however, to the meiotic defect of *bcy1*; other defects of *bcy1* homozygotes, the failure to respond to starvation by arresting at G_0 (Shin *et al.*, 1987b), becoming thermo-resistant, and synthesizing G_0 proteins, are not relieved by multicopy *IME1*.

Results

The gene IME1 functions downstream of the adenylate cyclase/protein kinase cascade

The involvement of the adenylate cyclase pathway in transmitting the starvation signal required for the initiation of yeast meiosis has been demonstrated by the behavior of diploids, homozygous for mutations in the cyclase gene *CYR1* (*CDC35*) or one of its two regulators, *CDC25* and *RAS2*. Such diploids undergo meiosis and sporulation in rich media (Shilo *et al.*, 1978; Matsumoto *et al.*, 1983b; Tatchell *et al.*, 1985; Toda *et al.*, 1985; Mitsuzawa *et al.*, 1989). Thus reduction of cyclase activity and low levels of cAMP simulate the starvation signal required for initiation of meiosis. Elevated cyclase activity and cAMP levels found

in diploids carrying the mutant allele *RAS2*^{val19} suppress sporulation even in sporulation (starvation) medium (Toda *et al.*, 1985), thus counteracting the starvation signal. Likewise, a reduction in sporulation efficiency is brought about by the presence of multicopy plasmids carrying the gene *CDC25* (G.Simchen and M.Treinin, unpublished results). The sporulation deficient phenotype is also observed in diploids homozygous for mutations in the gene *BCY1* (Matsumoto *et al.*, 1983b), which codes for the regulatory unit of the cAMP-dependent protein kinase, thus suggesting that increased phosphorylating activity, rather than an increased level of cAMP, overrides the starvation signal. This interpretation is further supported by the reduction of sporulation frequency resulting from the presence of multicopy plasmids carrying the *TPK1* gene, coding for the structural unit of the protein kinase (G.Simchen and M. Treinin, unpublished results).

The *IME1* gene also has a role in transmitting the starvation signal for the initiation of meiosis, as multicopy plasmids with *IME1* enable sporulation to occur in rich media (Granot *et al.*, 1989). In order to elucidate the interaction between *IME1* and the AC/PK cascade in transmitting the nutrition/starvation signal towards the initiation of meiosis, we constructed double mutant diploids *cdc25/cdc25 ime1/ime1* and *ras2/ras2 ime1/ime1*. Table I shows that the double mutants, strains 2175 (*cdc25/cdc25 ime1/ime1*) and 2181 (*ras2/ras2 ime1/ime1*), were sporulation deficient, whereas their isogenic counterparts 2169 (*cdc25/cdc25*) and 2178 (*ras2/ras2*) underwent sporulation on YEPA as well as SP medium. The results showed that *ime1* mutation is epistatic to the mutations in AC/PK cascade, supporting the model that *IME1* comes after the AC/PK cascade. According to this model, nutrients activate the latter, which in turn represses *IME1*. However, one should bear in mind the (unlikely) possibility that *IME1* may have separate transmissions for the diploidy signal from *MAT–RME1* (Kassir *et al.*, 1988) and for the starvation signal, and that the failure of the double mutants to sporulate may result only from the failure to transmit the diploidy signal.

A strong prediction of the model is that the multicopy *IME1* plasmid would overcome the sporulation deficiency of the *bcy1* homozygous diploids. Strains MTD2 and MTD9, which are homozygous for the mutation *bcy1*, were used for these experiments. These strains grew slowly on acetate-based media, in contrast to strains carrying null alleles of *bcy1* (for instance, *bcy1::TRP1* or *bcy1::URA3*) which cannot grow at all on presporulation media such as YEPA. Both strains were transformed with the multicopy plasmid YEpK26-7, which carries the *IME1* gene without its upstream region which is negatively regulated by the nutrients signal (Kassir *et al.*, 1988; Granot *et al.*, 1989). As a control, MTD2 and MTD9 were also transformed with the centromeric plasmid pSY2-1, which carries the *BCY1* gene, thus complementing the *bcy1* deficiency. The six strains (Table II) were grown in YEPA at 25°C to a titer of $\sim 10^7$/ml, washed once in water and transferred to sporulation medium. Sporulation was examined microscopically after 48 h. As shown in Table II, the *bcy1* homozygotes did not form any asci, whereas the same strains carrying the gene *BCY1* on a plasmid sporulated rather well. The isogenic *bcy1* homozygotes which carried the multicopy plasmid with *IME1* were also able to undergo sporulation (Figure 1) although sporulation frequency was only ~2% (Table II). These results suggest that multicopy *IME1* suppresses the sporulation defect of the *bcy1* mutation, but that the suppression is only partial. To further investigate this partial suppression, cells of strains MTD2 and MTD2-I from the sporulation medium were stained with propidium iodide, and were examined under a fluorescence microscope (Table II). In the strain carrying the multicopy *IME1* plasmid, almost half of the cells that did not form asci appeared to have initiated meiosis and progressed to the binucleated or even the tetranucleated stage (30% and 13% respectively). Another intermediate event during sporulation is meiotic

Table I. Sporulation efficiency (%) of isogenic strains in three media

Strain	Homozygous mutation		Medium		
			SP	YEPA	YEPD
2166	–		73	1	0
2169	*cdc25*^{ts}	23°C :	71	5	0
		33.5°C:		23	1
2172	*ime1*		0	0	0
2175	*cdc25*^{ts} *ime1*	23°C :	0	0	0
		33.5°C:		0	0
2178	*ras2*		75	25	3
2181	*ras2 ime1*		0	0	0

Cells grown overnight in YEPD were transferred to SP, YEPA or YEPD and incubated for 4 days. Unless otherwise indicated, cultures were incubated at 30°C. Each value is based on a count of at least 200 cells.

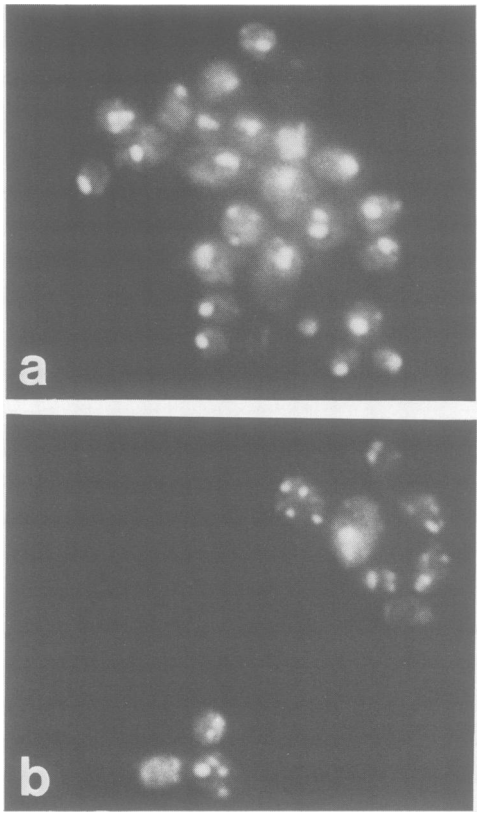

Fig. 1. Photomicrographs of *bcy1/bcy1* diploid cells and *bcy1/bcy1* diploid cells carrying YEpK26-7 stained with propidium iodide. The *bcy1/bcy1* diploid cells (MTD2) (**a**) and *bcy1/bcy1* diploid cells carrying YEpK26-7 (MTD2-I) (**b**) were incubated in sporulation medium at 25°C for 48 h. These cells were stained with propidium iodide and photographed under fluorescent microscopes.

recombination. In an experiment with the MTD9 series of strains, intragenic recombination in the *ADE2* gene was examined in cultures that were incubated in sporulation medium. At times 0 and 24 h, appropriate dilutions of cells were spread onto −ADE plates, to obtain Ade^+ prototrophs, and onto YEPD plates, to obtain an estimate of the number of viable, colony forming cells. The results (Table II) indicate that the level of recombination achieved in the *bcy1* strain with the multicopy *IME1* plasmid is ~80% of the level found in its isogenic control strain MTD9-B, where the *BCY1* gene on a plasmid complemented the *bcy1* lesion. Thus we may conclude as follows: the *bcy1* cells with multicopy *IME1* plasmid initiate meiosis and reach the recombination stage [or recombination commitment (Esposito and Esposito, 1974)] at slightly less frequency than those with the *BCY1* plasmid. About 30% of the total cell population reach the binucleate stage, 13% reach the tetranucleate stage, and 2% form asci. The spores of these asci had very low viability (Table II). The implications of the abnormal sporulation process revealed here will be discussed later.

Analogous experiments were carried out with strains carrying the $RAS2^{val19}$ mutation, which are incapable of sporulation. Introducing the multicopy *IME1* plasmid into such a strain also partly suppressed the sporulation deficiency, resulting in low frequency sporulation and several-fold increases in the frequencies of binucleated and tetranucleated cells (Table II).

Based on the experiments reported in this section, we may conclude that *IME1* comes after the AC/PK cascade in the pathway transmitting the nutrients/starvation signal to the initiation of meiosis.

Table II. Sporulation and intermediate stages of meiosis in *bcy1* and $RAS2^{val19}$ diploid strains carrying the plasmids YEpK26-7 [*IME1*] or pSY2-1 [*BCY1*]

Strain	Genotype [plasmid]	Sporulation efficiency (%)	Spore viability (%)	Binucleated cells (%)	Tetranucleated cells (%)
(a)					
MTD2	*bcy1/bcy1*	0	–	8.4	0
MTD2-I	*bcy1/bcy1* [*IME1*]	1.8	2.8	30.0	13.2
MTD2-B	*bcy1/bcy1* [*BCY1*]	49.0	93.0	NT	NT
MTD3	$RAS2^{val19}$/+	0.2	NT	3.3	0.6
MTD3-I	$RAS2^{val19}$/+ [*IME1*]	1.1	NT	12.3	3.8

Strain	Genotype [plasmid]	Incubation time in SP medium (h)	No. of Ade^+ prototrophs/10^7 viable cells
(b)			
MTD9	*bcy1/bcy1*	0	21
		24	89
MTD9-I	*bcy1/bcy1*	0	91
	[*IME1*]	24	4126
MTD9-B	*bcy1/bcy1*	0	0
	[*BCY1*]	24	5159

(a) Diploid cells were incubated in SP medium for 48 h and the number of sporulating cells (asci) were counted. These cells were stained with propidium iodide and the number of binucleated and tetranucleated cells were counted. Each sample consisted of at least 300 cells. Spore viability was determined by ascus dissection on YEPD plates. NT, not tested.
(b) Diploid cells were grown in YEPA at 25°C to a titer of 10^7/ml, washed in water and transferred to SP medium at 25°C. Ade^+ frequency was determined as described in the text.

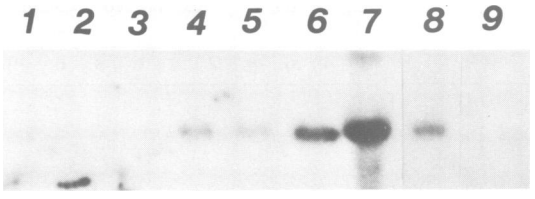

Fig. 2. Expression of *IME1* gene in various diploid cells. RNA was prepared from 2 × 10^9 cells of MTD2 (*bcy1/bcy1*) (lanes 1–3), MTD2-B (*bcy1/bcy1* pSY2-1) (lanes 4–6), MTD2-I (*bcy1/bcy1* YEpK26-7) (lane 7) and IT2 (*cyr1-2/cyr1-2*) (lanes 8 and 9). Cells were harvested during growth in YEPA (lanes 1, 4 and 7) or after a shift to SP medium for 2 h (lanes 2 and 5) and 4 h (lanes 3 and 6). IT2 cells were harvested after a shift to YEPA medium in the absence of cAMP (lane 8) or presence of 1 mM cAMP (lane 9) for 6 h. Northern blots were probed with *IME1*, then reprobed with *URA3* to confirm the presence of RNA in all lanes.

Expression of the IME1 gene is regulated by the adenylate cyclase/protein kinase cascade

To complement the genetic data in the previous section, we examined whether expression of *IME1*, at the level of transcription, is affected by mutations in the AC/PK cascade.

Northern analysis of a *bcy1/bcy1* diploid transferred to sporulation medium did not reveal expression of *IME1* (Figure 2, lanes 1–3), whereas the same strain, with the gene *BCY1* on a centromeric plasmid showed *IME1* expression (lanes 4–6). These results correlated with the sporulation behavior of the two strains (Table II). The same strain with the multicopy *IME1* plasmid showed, as expected, a high level of transcripts of the gene even in YEPA (Figure 2, lane 7) [YEpK26-7 lacks the nutrition regulatory sequences upstream of *IME1* (Granot *et al.*, 1989)].

A second mutant diploid which was tested for the expression of *IME1* was IT2 (*cyr1-2/cyr1-2*). Like other diploids homozygous for mutations in the adenylate cyclase gene *CYR1* (*CDC35*), this strain can sporulate in rich media (Shilo *et al.*, 1978; Matsumoto *et al.*, 1983b). It is also capable of taking up cAMP from the medium (Matsumoto *et al.*, 1983b), so the effect on *IME1* expression of adding cAMP may be examined. As shown in Figure 2, lane 8, there is a high level of transcripts in cells of this strain which were transferred from YEPD to YEPA without cAMP. When cAMP was added (lane 9), the *IME1* transcripts disappeared.

The third mutation whose effect on *IME1* expression was examined was *cdc25-2* (Daniel and Simchen, 1986), a temperature sensitive mutation in a regulator of adenylate cyclase. Diploid strains 2166 and 2169 are isogenic (Table V) and differ from each other only in that the latter is homozygous for *cdc25-2*. As shown in Table I, cells of strain 2169 growing in YEPA at 23°C, when shifted to the temperature of 33.5°C (which is restrictive for vegetative growth), undergo meiosis and sporulation at a high frequency. Northern analysis with *IME1* probe was performed on RNA samples of the two strains at the time of the temperature shift (0 h) and at 1, 2, 4 and 6 h thereafter (Figure 3). Very rapid induction of *IME1* transcription was observed in the *cdc25* strain, comparable with the rate of induction in normal meiosis (Kassir *et al.*, 1988). Transcripts

of *IME1* were not observed in strain 2166. In the mutant diploid 2169, a noticeable level of *IME1* transcript was already present at time 0, i.e. in cells growing in YEPA at 23°C. This correlates with the 5% asci observed under these conditions (Table I).

Thus we have shown that the two genes, *CDC25* and *BCY1* regulate the expression of *IME1*, as does the addition of cAMP to *cyr1-2/cyr1-2* cells.

Regulation of entry into G_0 by BCY1 is not affected by IME1

The main role of the AC/PK cascade in *S.cerevisiae* is to regulate the cell cycle (Matsumoto *et al.*, 1983a, 1985). Under rich nutritional conditions, a new cell cycle is initiated, whereas upon starvation, the cells arrest at G_1 and undergo a series of changes towards the G_0 stage (Shin *et al.*, 1987b). The *bcy1* homozygous diploid cells, in addition to their sporulation deficiency, cannot arrest at G_1 following nutritional starvation (Matsumoto *et al.*, 1983a), and fail to enter G_0. It was therefore of interest to examine whether these deficiencies are also relieved by the multicopy *IME1* plasmid, as is the sporulation deficiency. First, we compared the percentage of unbudded cells following transfer to starvation conditions in *bcy1* diploids with or without the multicopy *IME1* plasmid, and with the *BCY1* plasmid (the strains MTD2-I, MTD2 and MTD2-B respectively). Cells grown in YEPD or YEPA medium were transferred to starvation conditions (for nitrogen and sulfur) or to SP medium respectively, and the proportion of unbudded cells was examined after shaking at 25°C for 2 days. As shown in Table III, cells of strain MTD2 starved for nitrogen, sulfur or nutrients did not accumulate as unbudded cells,

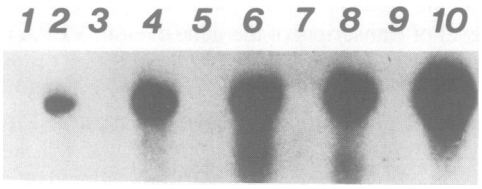

Fig. 3. Expression of *IME1* gene in *cdc25* cells. RNA was prepared from 2166 (CDC25/CDC25) (lanes 1, 3, 5, 7 and 9) and 2169 (cdc25/cdc25) (lanes 2, 4, 6, 8 and 10). Cells were harvested during growth in YEPA at 23°C (lanes 1 and 2) or after a shift to 33.5°C for 1 h (lanes 3 and 4), 2 h (lanes 5 and 6), 4 h (lanes 7 and 8) and 6 h (lanes 9 and 10). Northern blot was probed with *IME1*.

Table III. Proportion of unbudded cells of *bcy1* diploid strains carrying the plasmids YEpK26-7 [*IME1*] or pSY2-1 [*BCY1*], incubated in various conditions

Strain	Genotype [plasmid]	Unbudded cells (%)			
		Exponential phase	Nitrogen starvation	Sulfur starvation	SP medium
MTD2	*bcy1/bcy1*	51.7	27.5	49.1	25.1
MTD2-I	*bcy1/bcy1* [*IME1*]	48.1	30.3	60.6	30.0
MTD2-B	*bcy1/bcy1* [*BCY1*]	30.2	84.7	79.8	74.8

Exponentially growing cells (in YEPD) were collected and washed with water then resuspended in nitrogen-free or sulfur-free medium. After shaking at 25°C for 2 days, the number of unbudded cells was determined. Cells grown in YEPA medium were collected and transferred to sporulation medium (SP medium). After shaking at 25°C for 2 days, the number of unbudded cells was determined.

a phenotype characteristic for arrest at the G_1 phase. Likewise, the cells carrying YEpK26-7 were unable to arrest at G_1 under nutritional starvation. These data indicate that *IME1* expression cannot suppress the defect of G_1 arrest in the *bcy1* cells.

To test whether the *IME1* gene suppresses the failure of *bcy1* cells to enter into the G_0 phase, the acquisition of thermotolerance by sulfur starvation and the synthesis of G_0 proteins were examined in the MTD2 series of strains. These cells were grown on YEPD plates at 25°C for 6 h, and heat-treated in a 57°C water bath for various times. The *bcy1* cells carrying pSY2-1 were still viable following a heat treatment of >6 min, but the *bcy1* mutant cells carrying YEpK26-7 were not viable even following heat treatment for 2 min (Table IV). We also examined the acquisition of thermotolerance by incubating cells of the three strains at 37°C for 90 min on YEPD plates, and then exposing them to a temperature of 57°C for various times. The *bcy1* mutant cells carrying pSYS2-1 acquired thermotolerance to the lethal heat treatment, but the *bcy1* mutant cells carrying YEpK26-7 did not. Sulfur starvation effect was tested on cells grown on YEPD plates which were transferred to sulfur-free plates and incubated at 30°C for 3 days. These cells were then transferred to YEPD plates and immediately exposed to a temperature of 57°C for various times. Again, the *bcy1* mutant cells carrying pSY2-1 were viable after heat treatment of >20 min, whereas the *bcy1* cells carrying YEp26-7 were not viable after a treatment of 10 min. These results indicate that the *bcy1* mutant cells carrying YEpK26-7 could not enter the G_0 phase following sulfur starvation.

In order to examine whether the *IME1* gene regulates the synthesis of G_0 proteins, the MTD2 series of strains were starved for sulfur. Extracts of cells incubated in sulfur-free medium were analyzed for patterns of protein synthesis. A considerable number of proteins were stimulated or repressed after the *bcy1* mutant cells carrying pSY2-1 (Figure 4a) were incubated in sulfur-free medium for 20 h. The synthesis of the proteins designated G_0 specific was stimulated by sulfur starvation, and was repressed when the sulfur-starved cells were transferred to sulfate-containing medium, as described previously (Shin *et al.*, 1987b). However, these G_0 proteins

Table IV. Effect of heat treatment on the growth of *bcy1* diploid cells carrying the plasmids YEpK26-7 [*IME1*] or pSY2-1 [*BCY1*]

Strain	Genotype [plasmid]	Growth after heat treatment (min)										
		Exponential phase[a]				Heat-shocked[b]				Sulfur starvation[c]		
		2	4	6	8	2	4	6	8	6	10	20
MTD2	*bcy1/bcy1*	+	−	−	−	+	−	−	−	+	−	−
MTD2-I	*bcy1/bcy1* [*IME1*]	+	−	−	−	+	−	−	−	+	−	−
MTD2-B	*bcy1/bcy1* [*BCY1*]	+	+	+	−	+	+	+	+	+	+	+

[a]Cells were inoculated onto YEPD plates and incubated at 25°C for 6 h. The plates were then heat treated in a 57°C water bath for the indicated periods, and incubated at 25°C for 3 days.
[b]Cells were inoculated onto YEPD plates and incubated at 25°C for 6 h. The plates were then incubated at 37°C for 90 min and heat treated in a 57°C water bath for the indicated periods.
[c]Cells grown on YEPD plates were transferred to sulfur-free plates and incubated at 30°C for 3 days. These plates were then transferred to YEPD plates and were immediately heat treated for the indicated periods.

were not synthesized in the *bcy1* mutant cells carrying YEpK26-7 nor in the *bcy1* cells without plasmids (Figure 4b and c).

To check the effect of the *IME1* gene alone (not in a *bcy1* strain) on G_0 arrest, the acquisition of tolerance to a lethal heat treatment following heat-shock treatment or sulfur starvation, as well as the synthesis of G_0 proteins, were examined in *IME1*$^+$ and in *ime1*::*TRP1* haploid and diploid strains, as described above. The *ime1*::*TRP1* haploid and diploid cells acquired tolerance to a lethal heat treatment (data not shown) and synthesized G_0 proteins, as the wild-type cells (Figure 4d and e). These data indicate that the presence of the *IME1* gene did not affect thermotolerance or the synthesis of G_0 proteins following sulfur starvation.

Taken together, these results suggest that the *IME1* gene is probably not involved in the regulatory effects of the AC/PK cascade on the vegetative cell cycle and possibly not on entry into G_0 following starvation.

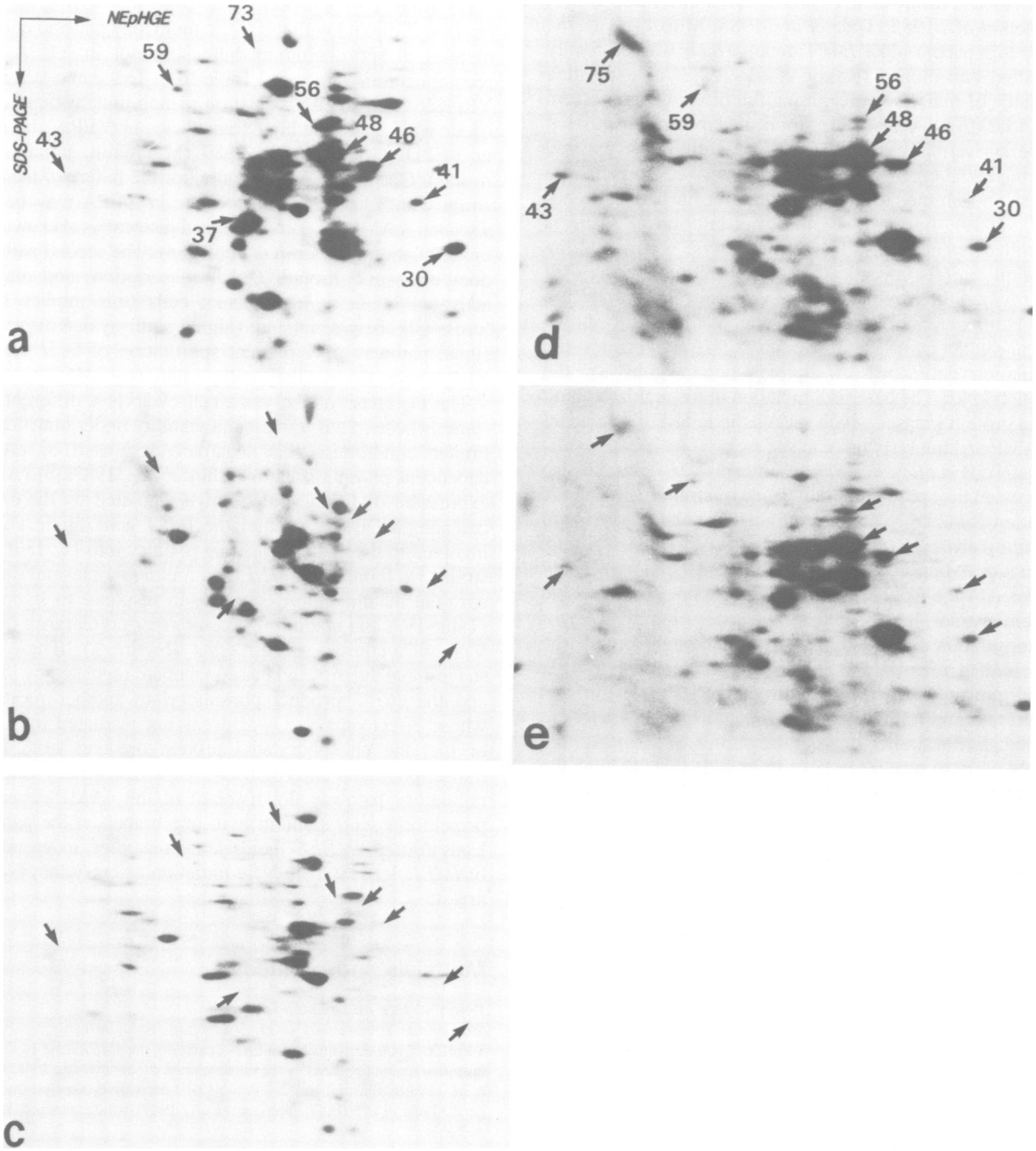

Fig. 4. Two-dimensional gel electrophoresis of L-[^{35}S]methionine-labeled proteins in sulfur-free medium. MTD2-B (*bcy1/bcy1* pSY2-1) (**a**), MTD2 (*bcy1/bcy1*) (**b**), MTD2-I (*bcy1/bcy1* YEpK26-7) (**c**), 2166-4D (*IME1*) (**d**) and 2172-2B (*ime1*::*TRP1*) (**e**) cells were incubated in sulfur-free synthetic medium for 20 h after transfer from the synthetic complete medium. These cells were pulse-labeled with L-[^{35}S]methionine, and total proteins extracted from the labeled cells were analyzed in two-dimensional gel electrophoresis. Arrows indicate the G_0 proteins synthesized in wild-type cells of the same genetic background, and numbers indicate mol. wts of the proteins (in kd). NEpHGE, non-equilibrium pH gradient electrophoresis; SDS−PAGE, SDS−polyacrylamide gel electrophoresis.

Discussion

The adenylate cyclase/cAMP-dependent protein kinase (AC/PK) pathway has been shown to mediate the regulation of the cell cycle by nutrients in the environment (Matsumoto *et al.*, 1983a, 1985). Starvation serves as a signal for diploid *MATa/MATα* cells to initiate meiosis and sporulation, and this signal is transmitted by the same AC/PK cascade, as shown by the altered sporulation behavior of strains carrying mutations or multicopy plasmids of genes in the cascade (Shilo *et al.*, 1978; Matsumoto *et al.*, 1983b; Tatchell *et al.*, 1985; Toda *et al.*, 1985; Mitsuzawa *et al.*, 1989; this paper). The *IME1* gene, which was originally identified and cloned (Kassir *et al.*, 1988) as a positive regulator of meiosis, transmitting the diploidy signal from *MAT* to *RME1*, has also been implicated in the transmission of the starvation signal to meiosis: a multicopy plasmid carrying *IME1* enabled diploid *S.cerevisiae* cells to undergo meiosis in rich media, in the presence of nutrients (Granot *et al.*, 1989). Furthermore, the transcription of *IME1* is controlled by nutrients. In this paper, we report that the mutation *ime1* is epistatic to the AC/PK mutations *cdc25* and *ras2*; double mutants *ime1 cdc25* or *ime1 ras2* are sporulation deficient, like *ime1* alone. We have also shown that a multicopy plasmid carrying *IME1* overrides the sporulation deficiency of diploids homozygous for the mutation *bcy1*. The latter is a mutation in the regulatory unit of the protein kinase, which makes the kinase independent of cAMP and not responsive to starvation (Matsumoto *et al.*, 1983a; Toda *et al.*, 1987). The multicopy *IME1* plasmid also suppressed the sporulation deficiency of strains carrying the mutation $RAS2^{val19}$, in which intracellular cAMP levels are constitutively high (Toda *et al.*, 1985). These epistatic interactions support a model in which *IME1* is negatively regulated by the AC/PK cascade. The data presented in Table III demonstrate, however, that the suppression by *IME1* of the sporulation defect is only partial; although most of the cells initiate meiosis and undergo recombination, only a proportion progress through subsequent stages of meiosis (binucleate and tetranucleate cells), and only few asci are formed. Furthermore, most of the spores of these asci are inviable. These results lead us to conclude that although the regulation of entry into meiosis by the AC/PK cascade is likely to be mostly mediated by the *IME1* gene, the cascade may affect also later stages of meiosis and sporulation, such as carbohydrate storage (Uno *et al.*, 1983), not through *IME1*.

We have shown that the regulation of *IME1* by the AC/PK cascade is transcriptional (Figures 2 and 3). Mutations in the genes *BCY1* and *CDC25* affected the level of *IME1* transcripts, as did the addition of cAMP to a *cyr1-2/cyr1-2* strain. Transcriptional regulation of *IME1* suggests the existence of at least one unknown intermediary protein, which is phosphorylated by the cAMP-dependent protein kinase. According to the simplest model, the phosphorylated form of this protein represses *IME1* transcription, for instance by binding to sequences upstream of the *IME1* coding region. The starvation signal in sporulation medium causes dephosphorylation of the intermediary protein through the reduction of cellular cAMP level, and in its dephosphorylated state, the protein no longer represses *IME1*. Multicopy *IME1* plasmids may overcome the repression by the AC/PK system (in rich medium) either by titrating the limited amount of phosphorylated repressor, or by avoiding it due to absence of the repressor binding site, for instance in plasmid YEpK26-7 (Granot *et al.*, 1989). Support for this interpretation comes also from an experiment in which multicopy plasmids carrying sequences upstream of *IME1*, but without the coding region, enabled 4% sporulation to occur in YEPD (Granot *et al.*, 1989). However, control of *IME1* transcription may be more complicated. Supposing three intermediary proteins, it is possible that the substrate of cAMP-dependent protein kinase is an activator which is inactivated in its phosphorylated form.

A further complication stems from the finding that the *bcy1/bcy1* diploid with the *IME1* multicopy plasmid entered meiosis in sporulation medium, but not in YEPA (see recombination values in Table II). The same plasmid, YEpK26-7, enabled sporulation of non-mutant diploids to occur with relatively high frequencies in YEPA and even in YEPD (Granot *et al.*, 1989; M.Treinin, unpublished results). One possible explanation for the failure of the *bcy1* strain with YEpK26-7 to sporulate in YEPA may be that a second, cAMP-independent signal pathway is also involved in sporulation (Cameron *et al.*, 1988). The second pathway does not work through *IME1* transcription, and may be activated in these *bcy1* mutant cells (this implies some 'cross-talk' between the two signal pathways). This second signal pathway may prevent sporulation of *bcy1* cells in YEPA even though they carry multicopy *IME1*.

The G_0 phase of the yeast cell cycle is a differentiated stage, whose role is to maintain viability under nutritionally limited conditions; it is negatively regulated by cAMP-dependent phosphorylation (Shin *et al.*, 1987a,b). As the expression of *IME1* is also down-regulated by the cAMP cascade, we asked whether the *IME1* gene might also be involved in the pathway leading to entry into G_0. In a *bcy1* strain, we found that *IME1* on a multicopy plasmid did not cure the inability to arrest at G_1 in response to starvation (Table IV), and inability to acquire thermotolerance (Table V) and synthesize G_0 proteins (Figure 4). These data suggest that the protein kinase(s) that are regulated by cAMP and the *BCY1* product phosphorylate another protein(s) whose effect on G_1-G_0 arrest is not mediated by *IME1*, or that the phosphorylated protein prevents both *IME1* transcription and entry into G_1-G_0 arrest. In any case, the regulatory pathway downstream of the cAMP-dependent protein kinase(s) splits in two: one branch of the pathway leads through *IME1* to meiosis, and the other, independently of *IME1*, leads to cell cycle initiation or entry into G_1-G_0 arrest.

Materials and methods

Yeast strains and plasmids

S.cerevisiae strains used in this study are listed in Table V. Plasmid YEpK26-7 is a derivative of YEp24, carrying the *IME1* gene lacking the upstream region regulated by the nutrients signal (Kassir *et al.*, 1988; Granot *et al.*, 1989). Plasmid pSY2-1 is a YCp19 derivative carrying the *BCY1* gene (Toda *et al.*, 1987; Yamano *et al.*, 1987) which was provided by A.Toh-e.

Media

Rich medium (YEPD) was composed of 1% yeast extract (Difco), 2% peptone and 2% glucose. Presporulation medium (YEPA) was prepared by adding 1% postassium acetate to YEPD instead of glucose. Sporulation medium (SP) contained 1% potassium acetate. The composition of sulfur-free synthetic medium was the same as that of the liquid synthetic medium (Hartwell, 1970) except that all sulfate salts were replaced by chlorides, and yeast extract was omitted. Nitrogen-free medium contained 2% glucose

Table V. Strains used in this study

Strain	Genotype	Origin
Haploids		
AM203-1B	*MATα his7 bcy1*	Shin *et al.* (1987b)
IU-1B	*MATa leu2 ura3 his3 trp1*	Uno *et al.* (1987)
MB1-1D	*MATα leu2 ura3 trp1 ade2-R8 bcy1*	Progeny of MT5-6Ax2166-2B
MB2-6D	*MATa leu2 ura3 trp1 ade2-101 bcy1*	Progeny of MT5-6Dx2166-2C
MT5-4C	*MATα leu2 ura3 trp1*	Progeny of R31-1BxR31-1D
MT5-6A	*MATa leu2 ura2 bcy1*	Progeny of R31-1AxR31-1D
MT5-6D	*MATα leu2 ura3 bcy1*	Progeny of R31-1BxR31-1D
MT5-9B	*MATa leu2 ura3 bcy1*	Progeny of R31-1BxR31-1D
MT5-9D	*MATα ura3 trp1 bcy1*	Progeny of R31-1BxR31-1D
T41-7D	*MATa leu2 ura3 his ade tyr1 can1 cyh^R RAS2^{val19}*	Toda *et al.* (1985)
TM26-8A	*MATα leu2 ura3 trp1 cyr1-2*	Progeny of AM26-2C (Matsumoto *et al.*, 1983b)
TM26-13C	*MATa ura3 his3 trp1 cyr1-2*	Progeny of AM26-2C (Matsumoto *et al.*, 1983b)
R31-1B	*MATa ura3 trp1 bcy1*	Progeny of AM203-1BxIU-1B
R31-1D	*MATα leu2 ura3*	Progeny of AM203-1BxIU-1B
2164	*MATα leu2-3,112 ura3-52 trp1(del) metx ade2-101 can1*	This work
2165	*MATa leu2-3,112 ura3-52 trp1(del) metx ade2-R8*	This work
2166-2B	*MATa leu2 ura3 trp1 ade2-R8*	Progeny of 2166
2166-2C	*MATα leu2 ura3 trp1 ade2-101 can1*	Progeny of 2166
2166-4D	*MATα leu2 ura3 trp1 ade2 can1*	Progeny of 2166
2172-2B	*MATα leu2 ura3 trp1 ade2 can1 ime1-1*	Progeny of 2172 with YEpK26-7
Diploids		
MTD2	MT5-9B × MT5-9D	This work
MTD2-I	MTD2 carrying YEpK26-7	This work
MTD2-B	MTD2 carrying pSY2-1	This work
MTD9	MB1-1D × MB2-6D	This work
MTD9-I	MTD9 carrying YEpK26-7	This work
MTD9-B	MTD9 carrying pSY2-1	This work
MTD3	T41-7D × MT5-4C	This work
MTD3-I	MTD3 carrying YEpK26-7	This work
IT2	TM26-8A × TM26-13C	This work
2166	2164 × 2165	This work
2169	Isogenic to 2166, with *cdc25-2* replacements	This work
2172	Isogenic to 2166, with *ime1-1* disruptions	This work
2175	Isogenic to 2166, with *cdc25-2* and *ime1-1*	This work
2178	Isogenic to 2166, with *ras2::LEU2* disruptions	This work
2181	Isogenic to 2166, with *ras2::LEU2* and *ime1-1*	This work

Strains 2169 and 2175 are homozygous for the *cdc25-2* mutation. This mutation was cloned out of strain 352-5A2 (Daniel and Simchen, 1986) by gap-repair and combined with *URA3* between the sites *Xho*I–*Pvu*II downstream of the coding region. The *Sal*I–*Pvu*II fragment containing *cdc25-2* and *URA3* was used for gene replacements into the parental haploids 2164 and 2165. Strains 2169, 2175 and 2181 are homozygous for the gene disruption *ime1-1::TRP1* (Kassir *et al.*, 1988). Strains 2178 and 2181 are homozygous for the gene disruption on *ras2::LEU2* (Tatchell *et al.*, 1984). *metx* is a methionine auxotropy mutation in an unidentified gene.

and 0.17% yeast nitrogen base without amino acids and ammonium sulfate (Difco).

Fluorescent microscopy

Samples were placed on a glass slide, and a drop of staining solution containing 10 μg/ml of propidium iodide and 500 μg/ml RNase A in N buffer (Suzuki *et al.*, 1982) was immediately added to them. The materials were then squashed gently under a coverslip. The cells were viewed with a UVFL 100× objective using a UV excitation filter (545 nm) in combination with a 610 nm suppression filter.

Northern analysis

RNA was isolated from 2 × 10^9 cells as described by Carlson and Botstein (1982). Separation of RNAs on formaldehyde agarose gel, its transfer to GeneScreen membrane and hybridization with dextran sulfate to DNA probes were done as described in the instruction manuals of GeneScreen (New England Biolabs). Northern blots were probed with *IME1*, then reprobed with the control probe *URA3*.

Heat-shock treatment

The heat-shock treatment of plates was carried out by transferring cultures to a water bath maintained at 57°C. The heat treated plates were cooled in an ice box and incubated at 30°C for 3 days.

Determination of the proportion of unbudded cells

Small fractions of cell cultures were pipetted, briefly sonicated to dissociate the cell clumps, and examined under a microscope. To determine the proportion of unbudded cells, at least 600 cells were examined.

Labeling and extraction of proteins

S.cerevisiae cells were grown with shaking in a liquid synthetic medium having low methionine content. Subsequently, a part of the cultures was transferred to a sulfur-free medium with a low methionine content. The cultures incubated in sulfur-free medium for 20 h were pulse-labeled with 10 μCi L-[^{35}S]methionine (1200 Ci/mmol, Amersham) per ml for 10 min, and chased for 3 min by the addition of nonradioactive L-methionine to 0.5 mg/ml. The radioactively labeled cells were chilled, washed twice with Tris–HCl buffer (pH 8.8) containing 2 mM $CaCl_2$, and kept frozen until protein extraction. Protein extraction from whole cells to prepare the samples for two-dimensional gel electrophoresis was performed at 4°C. Frozen *S.cerevisiae* cells were vortexed four times, 30 s each time, with 0.3 g of glass beads (0.5 mm in diameter) and 2.5 μl 100 mM phenylmethylsulfonyl fluoride. Lysates were incubated for 5 min with 200 μl 200 mM Tris–HCl (pH 8.8) containing 2 mM $CaCl_2$ and 10 μl 1 mg micrococcal nuclease (Sigma Chemical Co., St Louis, MO) per ml. The samples were mixed with 20 μl of a 2% sodium dodecyl sulfate (SDS)–10% 2-mercaptoethanol solution, and 20 μl of a 0.5 M Tris–HCl solution containing 1 mg pancreatic

DNase I (Sigma) per ml, 2 mg RNase A (Sigma) per ml and 50 mM $MgCl_2$, and incubated for 5 min. The mixture was then lyophilized and dissolved at room temperature in the lysis buffer described by O'Farrell (1975).

Two-dimensional gel electrophoresis

Two-dimensional non-equilibrium pH gradient electrophoresis/SDS–polyacrylamide gel electrophoresis was conducted essentially following the procedures of O'Farrell *et al.* (1977). About 20 μg of protein sample was electrophoresed. Electrophoresis in the first dimension was performed for 4 h at 400 V as a total of 1600 V in a glass tube (2.5 × 130 mm) containing 4% acrylamide-bisacrylamide, 2% Ampholine (LKB, Bromma, Sweden, pH range 3.5–10), 2% Nonidet P-40 (Nakarai Chemicals Ltd, Tokyo) and 9.2 M urea. SDS–polyacrylamide gel electrophoresis for the second dimension was carried out on a discontinuous SDS–polyacrylamide gel with 11% acrylamide–bisacrylamide in the separation gel and 4.75% in the stacking gel. Electrophoresis gels were stained for 1 h with a solution containing 0.025% Coomassie brilliant blue G-250 (Difco Laboratories, Detroit, MI), 25% isopropanol and 10% acetic acid. They were destained twice for 1 h each time and then overnight with a 7% acetic acid solution. To obtain autoradiograms, the gels were dried and exposed to Kodak X-Omat AR film (XAR-5). The mol. wts were estimated by co-electrophoresis with mol. wt markers (Pharmacia, Uppsala, Sweden).

Other procedures

Plasmid DNA was prepared from *Escherichia coli* cultures by the standard alkaline lysis procedure (Maniatis *et al.*, 1982). Transformation of yeast cells was carried out by the lithium acetate procedure (Ito *et al.*, 1983).

Acknowledgements

Part of this research was supported by Grant-in-Aid for Scientific Research (A) from the Ministry of Education, Science and Culture of Japan. Part of this research was supported by grant 86-00139 from the US–Israel Binational Science Foundation, Jerusalem, Israel.

References

Broek,D., Toda,T., Michaeli,T., Levin,L., Birchmeier,C., Zoller,M., Powers,S. and Wigler,M. (1987) *Cell*, **48**, 789–799.
Cameron,S., Levin,L., Zoller,M. and Wigler,M. (1988) *Cell*, **53**, 555–566.
Carlson,M. and Botstein,D. (1982) *Cell*, **28**, 145–154.
Daniel,J. and Simchen,G. (1986) *Curr. Genet.*, **10**, 643–646.
Esposito,R.E. and Esposito,M.S. (1974) *Proc. Natl. Acad. Sci. USA*, **71**, 3172–3176.
Granot,D., Margolskee,J.P. and Simchen,G. (1989) *Mol. Gen. Genet.*, **218**, 308–314.
Hartwell,L.H. (1980) *J. Bacteriol.*, **104**, 1280–1285.
Ito,H., Fukuda,Y., Murata,K. and Kimura,A. (1983) *J. Bacteriol.*, **153**, 163–168.
Kassir,Y. and Simchen,G. (1976) *Genetics*, **82**, 187–206.
Kassir,Y., Granot,D. and Simchen,G. (1988) *Cell*, **52**, 853–862.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning: a Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
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. (1983a) *Exp. Cell Res.*, **146**, 151–161.
Matsumoto,K., Uno,I. and Ishikawa,T. (1983b) *Cell*, **32**, 417–423.
Matsumoto,K., Uno,I. and Ishikawa,T. (1985) *Yeast*, **1**, 15–24.
Mitchell,A.P. and Herskowitz,I. (1986) *Nature*, **319**, 738–742.
Mitsuzawa,H., Uno,I., Oshima,T. and Ishikawa,T. (1989) *Genetics*, **123**, 739–748.
O'Farrell,P.H. (1975) *J. Biol. Chem.*, **250**, 4007–4021.
O'Farrell,P.Z., Goodman,H.M. and O'Farrell,P.H. (1977) *Cell*, **12**, 1133–1142.
Shilo,V., Simchen,G. and Shilo,B. (1978) *Exp. Cell Res.*, **112**, 241–248.
Shin,D.-Y., Matsumoto,K., Iida,H., Uno,I. and Ishikawa,T. (1987a) *Mol. Cell. Biol.*, **7**, 244–250.
Shin,D.-Y., Uno,I. and Ishikawa,T. (1987b) *Curr. Genet.*, **12**, 577–582.
Suzuki,T., Nishibayashi,S., Kuroiwa,T., Kanbe,T. and Tanaka,K. (1982) *J. Bacteriol.*, **152**, 893–896.
Tatchell,K., Chaleff,D.T., DeFeo-Jones,D. and Scolnick,E.M. (1984) *Nature*, **309**, 523–527.
Tatchell,K., Robinson,L.C. and Breitenbach,M. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 3785–3789.
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.
Toda,T., Cameron,S., Sass,P., Zoller,M., Scott,J.D., McMullen,B., Hurwitz,M., Krebs,E.G. and Wigler,M. (1987) *Mol. Cell. Biol.*, **7**, 1371–1377.
Uno,I., Matsumoto,K., Adachi,K. and Ishikawa,T. (1983) *J. Biol. Chem.*, **258**, 10867–10872.
Uno,I., Mitsuzawa,H., Tanaka,K., Oshima,T. and Ishikawa,T. (1987) *Mol. Gen. Genet.*, **210**, 187–194.
Yamano,S., Tanaka,K., Matsumoto,K. and Toh-e,A. (1987) *Mol. Gen. Genet.*, **210**, 413–418.

Received on May 30, 1990; revised on June 25, 1990