At least two nuclear gene products are specifically required for translation of a single yeast mitochondrial mRNA

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Mitochondrial translation of the *oxi2* mRNA, encoding yeast cytochrome *c* oxidase subunit III (coxIII), has previously been shown to specifically require the mitochondrially located protein product of the nuclear gene *PET494*. We show here that this specific translational activation involves at least one other newly identified gene termed *PET54*. Mutations in *PET54* cause an absence of the coxIII protein despite the presence of normal levels of its mRNA. *pet494* mutations are known to be suppressible by mitochondrial gene rearrangements that replace the normal 5'-untranslated leader of the *oxi2* mRNA with the leaders of other mitochondrial mRNAs. In this study we show that *pet54*, *pet494* double mutants are suppressed by the same mitochondrial gene rearrangements, showing that the *PET54* product is specifically required, in addition to the *PET494* protein, for translation of the *oxi2* mRNA. Since, as we show here, *PET54* is not an activator of *PET494* gene expression, our results suggest that the products of both of these genes may act together to stimulate coxIII translation. **Key words: mitochondria/*oxi2* gene/translational control/yeast**

Introduction

The expression of yeast mitochondrial genes is accomplished by a genetic system composed largely of proteins encoded by nuclear genes (reviewed in Dujon, 1981). While many of these proteins are general components of the mitochondrial genetic system, others function only in the expression of particular mitochondrial genes. In all of the cases investigated to date, nuclear genes required for the expression of specific mitochondrial genes have been found to act at post-transcriptional steps, either in processing of particular transcripts or in translation of specific mRNAs (reviewed by Fox, 1986).

We are studying the role of nuclear gene products in the expression of subunit III of cytochrome *c* oxidase (coxIII), encoded by the mitochondrial gene termed *oxi2* (Thalenfeld and Tzagoloff, 1980). Mutations in the nuclear gene *PET494* result in a specific deficiency of coxIII, even though normal levels of the *oxi2* mRNA are present (Cabral and Schatz, 1978; Mueller *et al.*, 1984). Mitochondrial second-site mutations which restore coxIII synthesis in a *pet494* mutant background have been isolated and found to consist of fusions between the 5'-flanking sequences of the *oxi2* gene and those of other mitochondrial genes. Novel *oxi2* mRNAs carrying the 5'-untranslated leaders of other mitochondrial transcripts are copied from these rearranged genes and are translated to yield wild-type coxIII (Costanzo and Fox, 1986). The fact that 5' leader substitutions on the *oxi2* mRNA can suppress the *pet494* mutant phenotype shows that in wild-type cells *PET494* protein is required for coxIII translation, and further suggests that its site of action is in the 5' leader of the *oxi2*

mRNA. Since the *PET494* protein is known to be localized to mitochondria (Costanzo and Fox, 1986), it is probably directly involved in activating coxIII translation.

To investigate the complexity of the specific activation of coxIII translation, we have searched for new nuclear mutations that specifically block this process. We report here on mutations in a newly identified gene, termed *PET54*, that result in a phenotype identical to that of *pet494* mutants. *pet54*, *pet494* double mutants are suppressed by the mitochondrial gene rearrangements isolated as *pet494* suppressors, showing that *PET54* is also required for coxIII translation in wild-type cells. We have excluded the possibility that *pet54* mutations prevent coxIII translation by blocking expression of *PET494*. Thus these data suggest that the *PET54* and *PET494* proteins may act in concert, perhaps interacting with each other in a complex, to specifically activate coxIII translation.

Results

Isolation of pet54 mutations and characterization of the pet54 mutant phenotype

To search for new nuclear genes involved specifically in the expression of the mitochondrial gene product coxIII, we first generated a collection of nuclear *pet* mutants from the *Pet*⁺ strain LH114AC (*MAT α* , *ade2*, *ino1-13*, *ino4-8*, [*rho*⁺]). This strain carries mutations in two genes for inositol biosynthesis, allowing efficient enrichment of non-respiring mutants by the inositol starvation procedure (Henry *et al.*, 1975). Following mutagenesis by u.v. irradiation, the cells were starved for inositol in the presence of non-fermentable carbon sources. Survivors were recovered by replica plating to glucose-containing medium and checked for their ability to grow on non-fermentable medium. One hundred and fifty four nuclear *pet* mutants were identified by testing the ability of the non-respiring survivors to form respiring diploids when mated to a *Pet*⁺, *rho*⁰ tester strain. Among these strains one new *pet494* mutant, ECS124, was identified by its inability to complement a *pet494* deletion strain (Costanzo *et al.*, 1986).

Since mutants lacking coxIII are known to lack cytochrome *c* oxidase activity (Ebner *et al.*, 1973), we assayed this enzymatic activity in each mutant using a convenient assay recently developed by McEwen and Poyton (McEwen *et al.*, 1985). Eighty four strains were deficient in cytochrome oxidase. To identify those strains specifically lacking coxIII, mitochondrial translation products were examined by radioactively labeling cultures of each mutant strain in the presence of cycloheximide and analyzing the labeled products by SDS-gel electrophoresis and autoradiography. In addition to the *pet494* mutant mentioned above, four other strains specifically failed to accumulate coxIII. The phenotype of one of these strains, ECS54, at the level of mitochondrial translation products is shown in Figure 1. Since ECS54 complemented a *pet494* deletion mutant, its nuclear lesion, termed *pet54-1*, defines a new gene that is specifically required for accumulation of coxIII.

Fig. 1. The *coxIII* protein is specifically absent from the mitochondrial translation products of a *pet54* mutant strain. Mitochondrial gene products of various strains were radioactively labeled by growth in the presence of [³⁵S]methionine and cycloheximide. Crude mitochondria were subjected to electrophoresis on an SDS-containing 10–15% polyacrylamide gradient gel and the gel was dried and autoradiographed. **Lane A**, wild-type (*Pet*⁺) strain LH114AC; **lane B**, *pet54* mutant ECS54; **lane C**, respiring revertant ECS54R1, carrying an ochre suppressor (see text); **lane D**, TF148, a *pet54*, *pet494* double mutant carrying the mitochondrial suppressor *MSU494-2* (see text). The arrow indicates the position of *coxIII*. *CoxIII* is absent in the *pet54* mutant (**lane B**) but is restored when the *pet54-1* mutation is suppressed by either nuclear (**lane C**) or mitochondrial (**lane D**) suppressors.

To determine whether *PET54* is required for transcription of the mitochondrial gene coding *coxIII*, total cellular RNA was isolated from the wild-type strain LH114AC and the mutant ECS54. Following electrophoresis and blotting to nitrocellulose the RNA was hybridized to ³²P-labeled DNA from the mitochondrial gene *oxi2*, which codes for *coxIII* (Thalenfeld and Tzagoloff, 1980). Autoradiography revealed that the *pet54* mutant strain contained levels of the *oxi2* mRNA comparable with those in the wild-type strain (Figure 2). Thus the nuclear *pet54* mutant must be blocked in a post-transcriptional step leading to the accumulation of the mitochondrially coded protein *coxIII*. Its phenotype is indistinguishable from that of *pet494* mutants (Figure 2; Mueller *et al.*, 1984), which are known to be blocked in translation of the *oxi2* mRNA (Costanzo and Fox, 1986).

Genetic analysis and molecular cloning of *PET54*

PET54 is genetically unlinked to *PET494*. A diploid heterozygous at both genes was sporulated and the phenotype of haploid progeny from 26 asci was examined. Three quarters of the progeny (74/98) were *Pet*[−] as expected for unlinked *pet* mutations. In a cross to a *Pet*⁺ strain carrying the centromere-linked marker *trp1*, the *pet54-1* mutation of ECS54 segregated 2:2 and did not exhibit centromere linkage. The three remaining mutants that specifically lacked *coxIII*, ECS70, ECS78 and ECS101, failed to complement when mated to a *pet54-1* segregant of the latter

Fig. 2. The *oxi2* mRNA is present at wild-type levels in both *pet54* and *pet494* mutants. Total RNA was isolated from various strains and 25 µg per lane were subjected to electrophoresis on a formaldehyde–agarose gel and transferred to a nitrocellulose filter. The filter was incubated with radioactively labelled DNA probes and autoradiographed. **Lane A**, RNA from the wild-type (*Pet*⁺) strain LH114AC; **lane B**, RNA from the *pet54* mutant ECS54; **lane C**, RNA from the *pet494* mutant ECS124. **Top**, the filter was hybridized with the plasmid pZHS3, which carries a 1.17-kb fragment internal to the region copied into *oxi2* mRNA (Mueller *et al.*, 1984). The position of the 3.6-kb *oxi2* transcript is indicated. **Bottom**, the same filter was re-hybridized with mtDNA then isolated from the *rho*[−] strain ROS-31, which retains an ~1.6-kb fragment carrying the mitochondrial 15S rRNA gene (Fox and Staempfli, 1982). The position of the 15S rRNA is shown. In this experiment and in several others not shown, the amount of *oxi2* mRNA was quantitated by densitometry and normalized to the amount of 15S rRNA present in the same lane. The normalized amount of *oxi2* mRNA in *pet54* and *pet494* mutant strains differed by <25% from the amount found in wild-type cells.

cross. Thus, this study yielded four alleles of the newly identified gene *PET54*.

The *pet54-1* mutation is ochre suppressible: a spontaneous revertant, ECS54R1, contained a suppressor unlinked to *pet54* which also suppressed the known ochre alleles *arg4-19* and *lys-1* (Fogel and Mortimer, 1970). Thus *PET54* most probably encodes a protein product. We have cloned *PET54* by selecting a plasmid that complements the *pet54-1* mutation from a yeast genomic bank in the shuttle vector YEp13 (Nasmyth and Tatchell, 1980). The complementing DNA fragment was shown to be tightly linked genetically to *PET54* by tetrad analysis of integrative transformants crossed to wild-type (three recombinants among 444 spores). The smallest fragment retaining *PET54* activity was a 1-kbp piece generated by digestion with *Cla*I and *Hind*III.

pet54-1 is suppressed by replacements of the 5' leader of the mitochondrial *oxi2* mRNA

In a previous study, mitochondrial suppressors of a nuclear *pet494* deletion mutant, termed *MSU494*, were selected and characterized. The requirement for the *PET494* protein in the translation of the *oxi2* mRNA was suppressed in strains that contained rear-

Fig. 3. A respiring *pet54*, *pet494* double mutant strain carries both *rho*⁺ and *MSU494-2* suppressor *rho*⁻ genomes. Total DNA from (A) strain MCC3[*MSU494-2*] (*pet494*, [*rho*⁺, *MSU494-2*]), (B) from strain TF133 (*pet54*, *pet494*, [*rho*⁺]) or (C) from TF148, a respiring *pet54*, *pet494* segregant of a cross between MCC3[*MSU494-2*] and TF133, was digested with *Mbo*I, subjected to electrophoresis on an agarose gel, transferred to nitrocellulose and hybridized with plasmid DNA radioactively labeled by nick-translation. The probe was plasmid pZHS1, which carries a 2.9-kb *Mbo*I fragment containing the 5' end and 5'-flanking sequences of the *oxi2* gene (Mueller *et al.*, 1984). The probe hybridized to the wild-type 2.9-kb fragment from the *rho*⁺ genome in each strain and, in addition to the 750-bp fragment from the *MSU494-2* genome (Costanzo and Fox, 1986) in strain MCC3[*MSU494-2*] (A) and in the respiring *pet54*, *pet494* double mutant TF148 (C).

rangements of *oxi2* sequences, carried on *rho*⁻ mtDNAs, in addition to wild-type (*rho*⁺) mtDNA (Costanzo and Fox, 1986). In several cases, the *MSU494* rearrangements resulted in the fusion of the *oxi2* structural gene to the 5'-untranslated leader coding regions of mitochondrial genes (*cob*, *aap1* and *oli1*) whose expression is independent of *PET494*. The novel transcripts copied from these fusion genes, consisting of the 5' leaders of the other mitochondrial mRNAs attached to the *oxi2* structural gene transcript, were translated in the *pet494* mutant to yield wild-type coxIII. These findings demonstrated that the PET494 protein is required for synthesis of coxIII, and suggested that its site of action is in the 5' leader of the *oxi2* mRNA.

Since the *pet54* mutant ECS54 was phenotypically similar to *pet494* mutants, we wished to determine whether *PET54* was also required to activate translation of the *oxi2* mRNA. To do this we asked whether a previously characterized mitochondrial suppressor of *pet494*, termed *MSU494-2*, would allow a *pet54* mutant to respire. *MSU494-2* mtDNA is transcribed to yield an mRNA with the 5' leader of the *aap1* transcript fused to the *oxi2* coding sequence (Costanzo and Fox, 1986). The double *pet* mutant strain TF133 (*MAT α* , *ade2*, *pet494-41*, *pet54-1*, [*rho*⁺]) was crossed to the *pet494* 'mitochondrial revertant' strain MCC3[*MSU494-2*] (*MAT α* , *ura3-52*, *pet494-41*, [*rho*⁺, *MSU494-2*]) (Costanzo and Fox, 1986). Prototrophic respiring diploids were selected and sporulated. All the haploid progeny of this cross contained the *pet494-41* mutation while half the spores in each tetrad were *pet54-1*. Due to the known instability of the heteroplasmic combination of *rho*⁺ and *MSU494-2* mtDNAs during cell division (Costanzo and Fox, 1986) we expected that most haploid segregants would be unable to respire since they would contain one or the other but not both mtDNAs.

Fig. 4. The PET494 protein is present in *pet54* mutant cells carrying the plasmid pMC210. Crude mitochondria were prepared from strain TF130 (*MAT α* , *pet54-1*, *ura3-52*, *leu2-3*, *leu2-112*, *ade2*, *his4-519*) carrying either the vector pAAH5 (Ammerer, 1983) or the *PET494* expression plasmid pMC210 (Costanzo and Fox, 1986), and 100 μ g per lane were loaded onto an SDS-polyacrylamide gel. The gel was electrophoretically blotted to a nitrocellulose filter and the filter was reacted with anti-PET494 antiserum and [¹²⁵I]protein A and autoradiographed. Lane A, TF130 carrying pAAH5; lane B, TF130 carrying pMC210. The arrow indicates the position of the PET494 protein. PET494 is undetectable at wild-type levels in this experiment. The *pet54* mutant cells of lane B failed to respire despite the presence of excess PET494 protein.

However, one of 14 tetrads dissected yielded four respiring haploids. Since the *pet54-1* mutation should have segregated 2:2 in the cross, this result immediately suggested that *MSU494-2* was suppressing *pet54*, *pet494* double mutants.

To confirm that *MSU494-2* suppressed both *pet494* and *pet54* mutations, the nuclear and mitochondrial genotypes of the four respiring haploid segregants were directly determined. *rho*⁰ derivatives of each were isolated and used for complementation testing with *pet54* and *pet494* testers. As expected, *pet54-1* had segregated 2:2 in this tetrad, while all four segregants were *pet494-41*. To confirm the mitochondrial genotype of these segregants, Southern analysis was used to demonstrate that they contained both wild-type and *MSU494-2* copies of the *oxi2* structural gene (Figure 3). Finally, analysis of mitochondrial translation products by radiolabeling in the presence of cycloheximide confirmed that the *pet54-1*, *pet494-41*, [*rho*⁺, *MSU494-2*] segregants synthesized coxIII (shown for strain TF148 in Figure 1). Similar results were obtained with another mitochondrial suppressor of *pet494*, *MSU494-4* (not shown). These results clearly demonstrated that the *oxi2* gene rearrangements that suppress the translation defect of *pet494* mutants also bypass the block in coxIII accumulation caused by the *pet54* mutation. Since the data of Figure 2 show that *PET54* activity is not required for synthesis of the *oxi2* mRNA, it must, like *PET494*, be required for translation.

PET54 activity is not required for *PET494* expression

Since the PET494 protein is specifically located in mitochondria (Costanzo and Fox, 1986), it presumably acts directly on *oxi2* translation and does not affect expression of *PET54*. However, since the subcellular location of the *PET54* protein is currently unknown, one plausible explanation for the phenotype of *pet54* mutants would be that the wild-type *PET54* gene product is a

positive regulator of *PET494* gene activity. We tested this hypothesis by measuring the levels of β -galactosidase activity expressed from a *pet494::lacZ* fusion gene in strains carrying a mutant or wild-type copy of *PET54*. The fusion gene, which joined the 5'-flanking sequences and first 119 codons of *PET494* to the 8th codon of the *Escherichia coli lacZ* gene (Costanzo and Fox, 1986), replaced the wild-type copy of *PET494* on chromosome XIV (Mueller and Fox, 1984; D.Marykwas and T.Fox, unpublished data). The specific activities of β -galactosidase in *pet54, pet494::lacZ* cells (strain TF152) and in *PET54, pet494::lacZ* cells (strain TF153) were 0.21 and 0.18 units/mg protein, respectively, showing that *PET54* is not required to activate *PET494* expression.

As a further demonstration that the *pet54* phenotype was not due to a deficiency of PET494 protein, we asked whether overproduction of the PET494 protein in *pet54* mutant cells would suppress the *pet54* mutant phenotype. A *pet54* mutant strain was transformed to Leu⁺ by the plasmid pMC210 (Costanzo and Fox, 1986), which directs high level expression of wild-type PET494 in yeast under the control of the *ADC1* promoter (Ammerer, 1983). The transformants were unable to grow on non-fermentable carbon sources, even though immunologically detectable levels of PET494 protein were present in the cells (Figure 4). Therefore, since *pet494::lacZ* expression was not affected by a *pet54* mutation and since the presence of abundant amounts of PET494 protein did not allow a *pet54* mutant to respire, we conclude that the wild-type *PET54* gene is not required for *PET494* expression.

Discussion

Translation of the yeast mitochondrial *oxi2* mRNA to yield the coxIII protein was previously shown to require the product of the nuclear *PET494* gene (Mueller *et al.*, 1984; Costanzo and Fox, 1986). The results presented in this report reveal that the mechanism of coxIII expression is even more complex than was previously thought. We show that mutations in yet another nuclear gene, termed *PET54*, also specifically block this process. Thus the products of at least two nuclear genes, *PET54* and *PET494*, are both necessary for translation of one mitochondrial mRNA.

The *pet54* mutant phenotype demonstrates that *PET54* is necessary for a post-transcriptional step in coxIII expression: the *oxi2* mRNA is present at levels comparable with those in wild-type cells, yet the coxIII protein fails to accumulate. This phenotype could be caused either by a block in translation of the *oxi2* mRNA, if *PET54* normally activates translation, or by rapid degradation of the coxIII polypeptide, if *PET54* normally stabilizes coxIII during assembly into cytochrome *c* oxidase. As shown here, coxIII does accumulate in *pet54, pet494* double mutants carrying chimeric mitochondrial genes that encode novel *oxi2* mRNAs bearing the 5'-untranslated leaders of other mitochondrial transcripts. [These mitochondrial mutations were isolated as suppressors of a *pet494* mutation (Costanzo and Fox, 1986)]. The *oxi2* coding sequences in these chimeric suppressor genes are not altered; only the 5'-untranslated leaders are different from the wild-type *oxi2* mRNA leader. Thus, this result establishes that *PET54* activity is required in wild-type cells for translation of the *oxi2* mRNA rather than to stabilize the coxIII protein, since the wild-type coxIII produced from the mitochondrial suppressor genes is stable and functional in a *pet54, pet494* mutant background.

It is not yet unknown whether the PET54 protein acts directly on the *oxi2* mRNA or whether it acts indirectly. If PET54 acts indirectly, its function could conceivably be to activate expres-

sion of the *PET494* gene, whose product is known to be a mitochondrial protein (Costanzo and Fox, 1986) and therefore is likely to be directly involved in coxIII translation. However, we have demonstrated that expression of a *pet494::lacZ* fusion gene is not affected by a *pet54* mutation and that overproduction of the PET494 protein in *pet54* mutant cells does not suppress the *pet54* mutant phenotype. Thus *PET54* is not required for *PET494* expression. Instead, it appears more likely that *PET54* encodes a protein that functions together with the PET494 protein to activate translation of the *oxi2* mRNA in mitochondria. Clearly, such a multi-subunit complex might also contain products of as yet unidentified genes. While it must be stressed that the mechanism by which the *PET494* and *PET54* products stimulate translation of the *oxi2* mRNA remains to be determined, a plausible working hypothesis is that they are subunits of a complex that functions as an *oxi2* mRNA-specific translation initiation factor. It is worth noting that at least two eucaryotic translation initiation factors are multi-subunit complexes (Maitra *et al.*, 1982). Unfortunately, there is virtually no biochemical information on translation initiation in yeast mitochondria.

As discussed above, chimeric mitochondrial genes encoding novel *oxi2* mRNAs whose 5'-untranslated leaders are replaced with those from other mitochondrial transcripts suppress *pet54, pet494* double mutants. This suggests that PET54 and PET494 normally promote coxIII translation by acting at a site encoded in the 5'-leader of the *oxi2* mRNA. Similarly, translation of the cytochrome *b* apoprotein from the *cob* mRNA in yeast mitochondria requires specific activation, mediated by the product of the *CBS1* gene (formerly MK2; Rödel *et al.*, 1985), at a site in the 5' leader. A *chs1* mutation is suppressed by a mitochondrial gene rearrangement that fused the 5' leader of another mitochondrial transcript to the *cob* mRNA (Rödel *et al.*, 1985). Furthermore, *CBS1* activity is required for translation of coxIII from a novel *oxi2* mRNA bearing the *cob* 5'-untranslated leader (Rödel and Fox, 1986). Thus specific translational activation at sites in the 5'-untranslated leaders of mRNAs may be a general feature of yeast mitochondrial gene expression. 5' Leaders have been implicated as sites for positive control of translation in several other systems, including mRNAs coding heat shock proteins of *Drosophila* (McGarry and Lindquist, 1985; Klemenz *et al.*, 1985; Hultmark *et al.*, 1986), late mRNAs of adenovirus (Logan and Shenk, 1984) and mRNAs of HTLV-III/LAV (Rosen *et al.*, 1986; Sodroski *et al.*, 1986). In the case of the translationally regulated yeast gene *GCN4*, a detailed analysis of regulatory sites in the 5'-untranslated leader has been carried out (Mueller and Hinnebusch, 1986).

While it is perhaps surprising that the translation of a single yeast mitochondrial mRNA is specifically dependent on the function of at least two nuclear genes, this degree of complexity may not be unique to coxIII expression. In addition to the *CBS1* gene, described above, the product of the *CBP6* gene may also be required for translation of the mitochondrial *cob* mRNA, although the possibility that the *cbp6* mutant phenotype results from instability of the cytochrome *b* peptide has not been eliminated (Dieckmann and Tzagoloff, 1985). Two nuclear genes, *PET111* (formerly *PETE11*; Cabral and Schatz, 1978; Poutre, 1986) and *PET112* (E.Seaver and T.Fox, unpublished data) are necessary for the expression of subunit II of cytochrome *c* oxidase (coxII), encoded by the mitochondrial *oxi1* gene. Mutations in these genes block *oxi1* expression post-transcriptionally, probably at the level of translation.

It is as yet unknown whether these complex pathways of expression are employed to modulate synthesis of mitochondrial

gene products in response to environmental conditions. However, there is evidence that the synthesis of coxIII is modulated at the translational level (Falcone *et al.*, 1983; Zennaro *et al.*, 1985), leading to the speculation that *PET54* and *PET494* might be involved in this process. With respect to the control of organellar genes in general, it is particularly interesting to note that light regulation of some chloroplast genes has recently been shown to occur largely at the translational level in several species (Miller *et al.*, 1983; Berry *et al.*, 1985; Fromm *et al.*, 1985).

Materials and methods

Genetic methods

Most genetic techniques were performed as described by Sherman *et al.* (1974). Yeast transformations were carried out with intact cells treated with lithium acetate (Ito *et al.*, 1983). *Rho*⁰ strains were made by prolonged growth in 25 µg/ml ethidium bromide (Goldring *et al.*, 1970).

Radioactive labeling and isolation of mitochondrial proteins and immunological methods

Mitochondrial translation products were labeled by incubation of yeast cells in medium containing [³⁵S]methionine and cycloheximide, and crude mitochondria were isolated and electrophoresed on SDS-polyacrylamide gels, as previously described (Douglas and Butow, 1976; Mueller *et al.*, 1984). Gels were dried and autoradiographed.

Unlabeled crude mitochondria were isolated as described by Daum *et al.* (1982) except that spheroplasts were lysed by stirring with 0.1 mm glass beads. Protein concentrations were determined by the methods of Bradford (1976) or of Lowry *et al.* (1951). Mitochondrial proteins were subjected to electrophoresis on SDS-10% polyacrylamide gels. Gels were electrophoretically blotted to nitrocellulose and reacted with antiserum as described by Towbin *et al.* (1979). Rabbit antiserum raised against an ompF-PET494-β-galactosidase fusion protein (Costanzo and Fox, 1986) was incubated with blots at 1:1000 dilution and immune reactions were visualized by incubation with [¹²⁵I]Protein A (New England Nuclear, Boston, MA) followed by autoradiography.

RNA and DNA gel-blot analysis

Total yeast RNA was isolated by the method of Sprague *et al.* (1983). RNA was subjected to electrophoresis on 1% agarose-formaldehyde gels, blotted to nitrocellulose and hybridized with radioactively labeled probes as described (Mueller *et al.*, 1984). Total yeast DNA was isolated from small (5 ml) cultures by the method of Sherman *et al.* (1974), subjected to electrophoresis on agarose gels, blotted to nitrocellulose, and hybridized with radioactively labeled probes as described (Mueller *et al.*, 1984). DNA was radioactively labeled with [α-³²P]-dATP by nick-translation (Rigby *et al.*, 1977).

β-Galactosidase assays

Strains TF152 and TF153 (see text) were grown to mid-logarithmic phase in YPGal medium (1% yeast extract, 2% peptone, 2% galactose). Cells were harvested, washed and converted to spheroplasts in 1.35 M sorbitol, 20 mM KPO₄, pH 7.4, containing 5 mg zymolyase 20 000 per g (wet weight) of cells. Spheroplasts were collected by centrifugation, washed and lysed by stirring with 0.1 mm glass beads. A minimal volume of 0.1 M Tris pH 8.0, 20% glycerol, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride was added to the lysate and it was centrifuged at 3500 r.p.m. in a Sorvall SS-34 rotor. The supernatant was assayed for protein concentration as described by Lowry *et al.* (1951) and for β-galactosidase activity as described by Miller (1972) except that 1% Triton was added to the assays. One unit of β-galactosidase activity is the amount that hydrolyzes 1 nmol of o-nitrophenyl β-D-galactoside in 1 min at 28°C.

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References

- Ammerer, G. (1983) *Methods Enzymol.*, **101**, 192–201.
- Berry, J.O., Nikolau, B.J., Carr, J.P. and Klessig, D.F. (1985) *Mol. Cell. Biol.*, **5**, 2238–2246.
- Bradford, M.M. (1976) *Anal. Biochem.*, **72**, 248–254.
- Cabral, F. and Schatz, G. (1978) *J. Biol. Chem.*, **253**, 4396–4401.
- Costanzo, M.C. and Fox, T.D. (1986) *Mol. Cell. Biol.*, **6**, 3694–3703.

- Costanzo, M.C., Mueller, P.P., Strick, C.A. and Fox, T.D. (1986) *Mol. Gen. Genet.*, **202**, 294–301.
- Daum, G., Boehni, P. and Schatz, G. (1982) *J. Biol. Chem.*, **257**, 13028–13033.
- Dieckmann, C.L. and Tzagoloff, A. (1985) *J. Biol. Chem.*, **260**, 1514–1520.
- Douglas, M. and Butow, R.A. (1976) *Proc. Natl. Acad. Sci. USA*, **73**, 1083–1086.
- Dujon, B. (1981) In Strathern, J.N., Jones, E.W. and Broach, J.R. (eds), *The Molecular Biology of the Yeast Saccharomyces: Life Cycle and Inheritance*. Cold Spring Harbor Laboratory Press, NY, pp. 505–635.
- Ebner, E., Mason, T.L. and Schatz, G. (1973) *J. Biol. Chem.*, **248**, 5369–5378.
- Falcone, C., Agostinelli, M. and Frontali, L. (1983) *J. Bacteriol.*, **153**, 1125–1132.
- Fogel, S. and Mortimer, R.K. (1970) *Mol. Gen. Genet.*, **109**, 177–185.
- Fox, T.D. (1986) *Trends Genet.*, **2**, 97–100.
- Fox, T.D. and Staempfli, S. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 1583–1587.
- Fromm, H., Devic, M., Fluhr, R. and Edelman, M. (1985) *EMBO J.*, **4**, 291–295.
- Goldring, E.S., Grossman, L.I., Krupnick, D., Cryer, D.R. and Marmur, J. (1970) *J. Mol. Biol.*, **52**, 323–335.
- Henry, S.A., Donahue, T.F. and Culbertson, M.R. (1975) *Mol. Gen. Genet.*, **143**, 5–11.
- Hultmark, D., Klemenz, R. and Gehring, W. (1986) *Cell*, **44**, 429–438.
- Ito, H., Fukuda, Y., Murata, K. and Kimura, A. (1983) *J. Bacteriol.*, **153**, 163–168.
- Klemenz, R., Hultmark, D. and Gehring, W. (1985) *EMBO J.*, **4**, 2053–2060.
- Logan, J. and Shenk, T. (1984) *Proc. Natl. Acad. Sci. USA*, **81**, 3655–3659.
- Lowry, O., Rosebrough, N., Farr, A. and Randall, R. (1951) *J. Biol. Chem.*, **193**, 265–275.
- Maitra, U., Stringer, E.A. and Chaudhuri, A. (1982) *Annu. Rev. Biochem.*, **51**, 869–900.
- McEwen, J.E., Cameron, V.L. and Poyton, R.O. (1985) *J. Bacteriol.*, **161**, 831–835.
- McGarry, T.J. and Lindquist, S. (1985) *Cell*, **42**, 903–911.
- Miller, J.H. (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory Press, NY.
- Miller, M.E., Jurgenson, J.E., Reardon, E.M. and Price, C.A. (1983) *J. Biol. Chem.*, **258**, 14478–14484.
- Mueller, P.P. and Fox, T.D. (1984) *Mol. Gen. Genet.*, **195**, 275–280.
- Mueller, P.P. and Hinnebusch, A.G. (1986) *Cell*, **45**, 201–207.
- Mueller, P.P., Reif, M.K., Zonghou, S., Sengstang, C., Mason, T.L. and Fox, T.D. (1984) *J. Mol. Biol.*, **175**, 431–452.
- Nasmyth, K.A. and Tatchell, K. (1980) *Cell*, **19**, 753–764.
- Poutre, C.G. (1986) Ph.D. Thesis, Cornell University.
- Rigby, P.W.J., Dieckmann, M., Rhodes, C. and Berg, P. (1977) *J. Mol. Biol.*, **113**, 237–251.
- Rödel, G. and Fox, T.D. (1986) *Mol. Gen. Genet.*, in press.
- Rödel, G., Körte, A. and Kaudewitz, F. (1985) *Curr. Genet.*, **9**, 641–648.
- Rosen, C.A., Sodroski, J.G., Goh, W.C., Dayton, A.I., Lippke, J. and Haseltine, W.A. (1986) *Nature*, **319**, 555–559.
- Sherman, F., Fink, G.R. and Lawrence, C.W. (1974) *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory Press, NY.
- Sprague, G.F., Jr., Jensen, R. and Herskowitz, I. (1983) *Cell*, **32**, 409–415.
- Sodroski, J., Goh, W.C., Rosen, C., Dayton, A., Terwilliger, E. and Haseltine, W. (1986) *Nature*, **321**, 412–417.
- Thalenfeld, B.E. and Tzagoloff, A. (1980) *J. Biol. Chem.*, **255**, 6173–6180.
- Towbin, H., Staehlin, T. and Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA*, **76**, 4350–4354.
- Zennaro, E., Grimaldi, L., Baldacci, G. and Frontali, L. (1985) *Eur. J. Biochem.*, **147**, 191–196.

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