

# Isolation and characterization of *S.cerevisiae* mutants defective in somatostatin expression: cloning and functional role of a yeast gene encoding an aspartyl protease in precursor processing at monobasic cleavage sites

Yves Bourbonnais<sup>1,3</sup>, Josée Ash<sup>1</sup>,  
Mireille Daigle<sup>1</sup> and David Y. Thomas<sup>1,2</sup>

<sup>1</sup>Eukaryotic Genetics Group, National Research Council of Canada, Biotechnology Research Institute, 6100 avenue Royalmount, Montréal, Québec, H4P 2R2 and <sup>2</sup>Department of Biology, McGill University, Montréal, Québec, H3A 1B1, Canada

<sup>3</sup>Corresponding author

Communicated by Hugh R.B. Pelham

**The peptide somatostatin exists as two different molecular species. In addition to the most common form, somatostatin-14, there is also a fourteen amino acid N-terminally extended form of the tetradecapeptide, somatostatin-28. Both peptides are synthesized as larger precursors containing paired basic and monobasic amino acids at their processing sites, which upon cleavage generate either somatostatin-14 or -28, respectively. In some species of fish two distinct, but homologous, precursors (prosomatostatin-I and -II) give rise to somatostatin-14 and -28, respectively. Whereas anglerfish prosomatostatin-II was previously shown to release exclusively somatostatin-28, the yeast *Saccharomyces cerevisiae* proteolytically matures the homologous prosomatostatin-I precursor to somatostatin-28 and -14 as well as to a lysine-extended form of somatostatin-14. The Kex2 endoprotease appears to be essential for the formation of lysine somatostatin-14 and is involved either directly or indirectly in the release of mature somatostatin-14. The isolation of yeast mutants defective in somatostatin-28 expression (*sex* mutant) allowed the cloning of a non-essential gene, which encodes an aspartyl protease, whose disruption severely affects the cleavage of mature somatostatin-28 from both somatostatin precursors. We conclude that two distinct endoproteases, which demonstrate some cross specificity *in vivo*, are involved in the proteolytic maturation of prosomatostatin at mono- and dibasic processing sites in yeast.**

**Key words:** heterologous gene expression/prohormone processing/somatostatin/yeast aspartyl protease/yeast mutants

## Introduction

Most small peptide hormones and neuropeptides are synthesized as larger polypeptides that undergo a series of post-translational modifications to generate the bioactive molecule (Douglass *et al.*, 1984). A common feature of many precursors is that pairs of basic amino acids flank the active peptide sequences and constitute the endoproteolytic cleavage site. However, there are several examples of prohormones in which the bioactive peptide is cleaved at monobasic processing sites rather than at paired basic residues (Schwartz, 1986; Devi, 1991). This cleavage usually occurs at single arginine residues. Somatostatin-28 is an N-

terminally 14-amino acid extended form of SRIF-14 (somatotropin releasing inhibitory factor). Both peptides display widespread effects inhibiting the secretion of numerous hormones including growth hormone, glucagon and insulin. Although there is some overlap in activity of SRIF-14 and -28, each also has distinctive biological effects. In mammals these bioactive peptides are synthesized from a common precursor and released by endoproteolytic activation; a single arginine residue (Arg-15) constitutes the cleavage site for the release of SRIF-28, whereas proteolytic cleavage at an internal dibasic sequence (Arg-2 Lys-1) generates SRIF-14. Differential processing of these cleavage sites has been shown to be tissue-specific (Patel *et al.*, 1981; Noe and Spiess, 1983). However, in several species of fish, SRIF-14 and -28 are encoded by distinct but related precursors, preproSRIF-I and -II, which are expressed in different subpopulations of pancreatic islet cells (McDonald *et al.*, 1987). Despite possessing the same potential cleavage sites (i.e. Arg-5 and Arg-2 Lys-1), proSRIF-I is cleaved at the Arg-Lys pair to yield SRIF-14, whereas SRIF-28 is the unique end product of maturation of proSRIF-II.

In an attempt to understand the molecular basis for differential processing of anglerfish prosomatostatins, we initiated studies on the biosynthesis of these precursors in the yeast *Saccharomyces cerevisiae*. The choice of yeast as a model was predicated on the observation that at the time yeast was the unique eukaryote in which a prohormone-converting enzyme had been unambiguously identified (Julius *et al.*, 1984). Furthermore, recent studies indicated that the yeast *KEX2* gene product, which is a member of the subtilisin family of serine proteinases, is both functionally and structurally related to its mammalian counterparts (Thomas *et al.*, 1988; Seidah *et al.*, 1990; Smeekens and Steiner, 1990; Smeekens *et al.*, 1991). Our previous study revealed that the native somatostatin precursors were not faithfully transported in the yeast secretory pathway unless their signal peptides were replaced by the leader sequence of the yeast  $\alpha$ -factor precursor (Bourbonnais *et al.*, 1991). Expression of such an  $\alpha$ -factor-proSRIF-II chimera promoted efficient transport and led to the secretion of mature SRIF-28, thus demonstrating accurate proteolytic cleavage at the monobasic processing site (Arg-15). Most importantly, we provided genetic evidence that the convertase involved in the maturation of proSRIF-II at Arg-15 is distinct from the paired basic-specific endoprotease encoded by the *KEX2* gene (Julius *et al.*, 1984). In the present study we have investigated the biosynthesis of a similar chimeric protein with proSRIF-I and developed a screening procedure for the isolation of yeast mutants defective in somatostatin-28 expression (*sex* mutants). Genetic complementation of the *sex1-1* mutant strain led to the cloning of a gene, *YAP3*, recently isolated by another group based upon its ability to partially suppress the phenotype of a *kex2* null mutant (Egel-Mitani *et al.*, 1990). We show here that this gene, which encodes an aspartyl protease, is required for the release of SRIF-28 from

both SRIF precursors by cleavage after a single arginine residue (Arg-15). Consistent with its role in pro  $\alpha$ -factor maturation in the absence of a functional *KEX2* gene (Egel-Mitani *et al.*, 1990), our data show that the endoproteases encoded by *KEX2* and *YAP3* demonstrate some cross specificity *in vivo*.

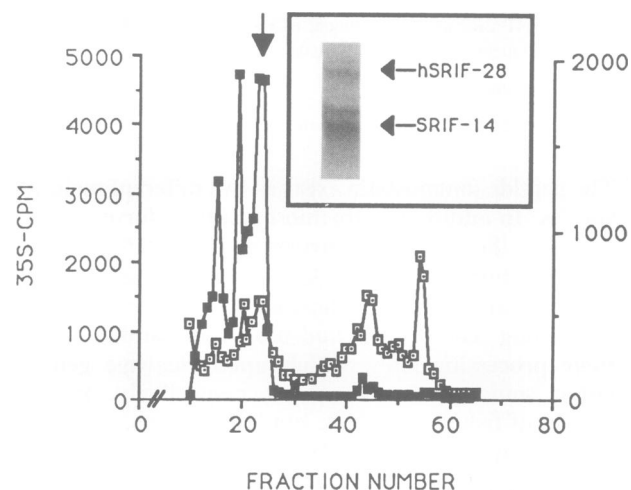
## Results

### *Mature SRIF-14, mature SRIF-28 and Lys-SRIF-14 are the end products of maturation of the proSRIF-I chimera in wild type yeast*

The same strategy was used for the construction of the proSRIF-II (Bourbonnais *et al.*, 1991) and the proSRIF-I chimeric genes. The plasmid carrying the proSRIF-I fusion gene was called pS1D and gene expression is under the control of the  $\alpha$ -factor promoter. The hybrid protein is composed of the first 49 amino terminal residues of prepro  $\alpha$ -factor fused in frame to full length proSRIF-I (preproSRIF-I 26–121) (Figure 1). The peptide SRIF-14 is located at the carboxyl-terminal end of the precursor and is flanked by a pair of basic amino acid residues (Arg-2 Lys-1). A single arginine residue, whose cleavage generates SRIF-28, is located 15 residues upstream from SRIF-14 (Arg-15). The hybrid precursor possess only two cysteine residues, which both reside in the SRIF-14 moiety at positions 3 and 14 respectively. Consequently, [<sup>35</sup>S]cysteine was used in all metabolic labelling experiments. The labelled proteins from the cell lysates and the media were then immunoprecipitated with an anti-SRIF-14 polyclonal antibody (RSSI B23; see Materials and methods). Analysis of the SRIF-14 immunoreactive material secreted from wild type yeast transformed with pS1D (strain 20B-12) by high performance liquid chromatography (HPLC) revealed the presence of major SRIF-14-like species eluting from the C<sub>18</sub> column at fractions 15, 19 and 23, respectively (Figure 2). The labelled immunoreactive material from fraction 23 co-eluted with synthetic SRIF-14 used as a standard. The three major secreted peptides could also be resolved on a high resolution SDS-Tricine gel (Figure 2, inset). A calibration experiment performed with the HPLC-purified peptides indicated that the 3, 2.2 and 1.8 kDa species correspond to the immunoreactive material eluting at fraction 15, 19 and 23, respectively (not shown). The 3 kDa peptide co-migrated with synthetic human SRIF-28, whereas the 1.8 kDa species co-migrated with synthetic SRIF-14. The identity of these peptides was confirmed by partial amino acid sequencing of the HPLC-purified radiolabelled peptides (data not shown). As expected from the amino acid sequence of mature SRIF-14, the radioactivity of the 1.8 kDa peptide (fraction 23 from HPLC) came out at cycles 3 and 14. Amino acid sequence determination of the 2.2 kDa species (fraction 19 from HPLC) revealed the presence of cysteine residues at positions 4 and 15 respectively, therefore identifying this peptide as a lysine-extended form of SRIF-14 (Lys-SRIF-14). For the material eluting at fraction 15, it was not possible to get a sequence. This was most probably due to the relatively low amount of radioactive material available for radiosequencing and the remote locations (at positions 17 and 28) of the cysteine residues in SRIF-28. In addition to these secreted peptides, HPLC analysis of the intracellular material that had been previously treated with endoglycosidase H identified two other SRIF species eluting at fractions



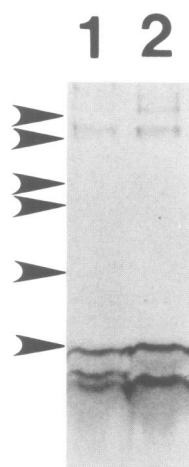
**Fig. 1.** Schematic representation of the chimeric protein encoded by plasmid pS1D. The fusion protein encoded by plasmid pS1D is 145 amino acids long and is composed of the first 49 N-terminal residues of prepro  $\alpha$ -factor (■), including the first N-linked glycosylation site (○), fused in frame to anglerfish proSRIF-I. The SRIF-I precursor is composed of an 82 amino acid proregion (▨) containing both a mono- and a dibasic processing site (○). The basic residues of the cleavage sites are indicated by their single letter code. The mature SRIF-14 peptide (■) is located at the carboxyl terminal end of the precursor.



**Fig. 2.** Biosynthesis of the  $\alpha$ -factor–proSRIF-I fusion protein in wild type yeast. Strain 20B-12 transformed with plasmid pS1D was labelled with [<sup>35</sup>S]cysteine and both the culture medium (■—■) and the intracellular labelled material (□—□) were submitted to immunoprecipitation with anti-SRIF-14 antibodies (see Materials and methods). The SRIF-14 immunoreactive material was then loaded onto a C<sub>18</sub> column, eluted with a gradient of acetonitrile then 1 min fractions were collected and the radioactivity determined by scintillation counting as described previously by Bourbonnais *et al.* (1988). The arrow indicates the position of elution of synthetic SRIF-14. Note that the ordinates for the secreted and intracellular material are on the left- and right-side of the figure respectively. Inset, analysis of the secreted SRIF-14 immunoreactive material from 20B-12 transformed with plasmid pS1D on high-resolution SDS–Tricine gel (see Materials and methods for details). Note that only the lower portion of the corresponding autoradiogram is shown. hSRIF-28 and SRIF-14 indicate the positions of migration of human synthetic SRIF-28 and synthetic SRIF-14, respectively.

44 and 54, respectively. Based on their elution position and their molecular masses, as estimated by SDS–Tricine gels (17 and 19 kDa), they correspond to the pro and prepro forms of the hybrid protein.

Quantification of the radioactivity recovered in the five SRIF species that were found intracellularly indicates that the processed forms accounted for >40% of the total SRIF-immunoreactive material. Overall (cell lysate plus medium) ~80% of the radioactivity was recovered in the mature peptides and of this, 86% was found in the culture medium at the end of the labelling period (30 min). The precursor forms were not secreted in significant amounts (<5%). These observations strongly argue that the chimeric protein was transported efficiently in the secretory pathway and that processing of the precursor did not take place in the culture medium. Maturation of the hybrid precursor to SRIF-14 and



**Fig. 3.** High-resolution SDS-Tricine gel of the secreted SRIF-14 immunoreactive material from wild type and *kex2* mutants transformed with pS1D. Wild type (strain 20B-12, lane 1) and *kex2* yeast (strain DS20, lane 2) transformed with plasmid pS1D were labelled with [<sup>35</sup>S]cysteine and the culture media were immunoprecipitated with anti-SRIF-14 antibodies as described earlier (Figure 2). The labelled proteins were then resolved in a high resolution SDS-Tricine gel. Arrowheads on the left indicate the position of migration of the prestained low molecular weight standards (Gibco/BRL): ovalbumin (43.0 kDa); carbonic anhydrase (29.0 kDa);  $\beta$ -lactoglobulin (18.4 kDa); lysozyme (14.3 kDa); bovine trypsin inhibitor (6.2 kDa) and: insulin  $\beta$ - and  $\alpha$ -chains (3.0 kDa).

Lys-SRIF-14, which together accounted for approximately two-thirds of the processed peptides, is, however, in sharp contrast to processing of the proSRIF-II chimera. The latter was found to be matured exclusively to SRIF-28 and there was no evidence of further processing at the Arg-2 Lys-1 cleavage site (Bourbonnais *et al.*, 1991).

#### **Proteolytic processing of the proSRIF-I chimera to Lys-SRIF-14 requires a functional KEX2 gene**

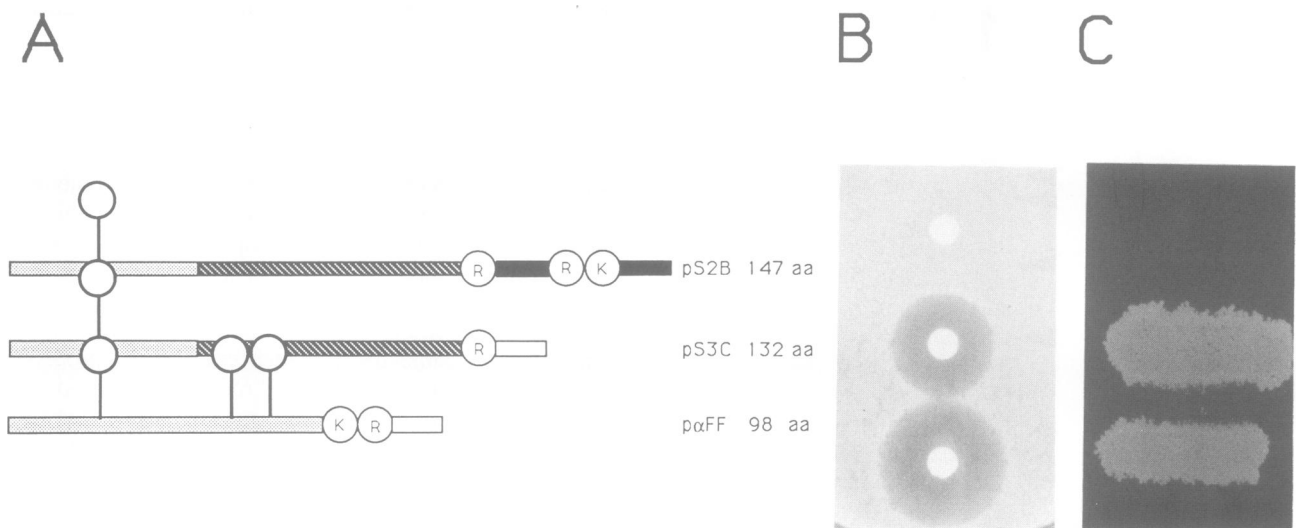
The release of SRIF-28 from the proSRIF-II chimera through cleavage at a monobasic processing site (Arg-15) was previously shown to occur in the absence of a functional *KEX2* gene (Bourbonnais *et al.*, 1991). This is consistent with the known *in vitro* specificity of the Kex2 endoprotease for paired basic amino acids (Julius *et al.*, 1984). To test whether any of the SRIF-14-like peptides secreted from yeast carrying pS1D were released through the action of Kex2, a *kex2* mutant yeast (strain DS20) was transformed with plasmid pS1D. A comparison of the labelled SRIF-immunoreactive material secreted from wild type and *kex2* yeast strains on SDS-Tricine gels (Figure 3, compare lanes 1 and 2) showed that the release of both SRIF-14 and SRIF-28 was not affected by the absence of a functional Kex2 endoprotease. However, the peptide Lys-SRIF-14 was completely absent from the culture medium of the *kex2* transformant. The same results were obtained when pS1D was expressed in a strain carrying a disrupted *KEX2* gene (strain M213-10C) (not shown). This indicates that there are no differences between the *kex2-1* and the *kex2* null alleles for the release of SRIF-14 and SRIF-28. The most likely explanation of the results is therefore that the Kex2 endoprotease is involved in the cleavage of the proSRIF-I chimera to Lys-SRIF-14. It also extends our previous observations with the proSRIF-II hybrid (Bourbonnais *et al.*, 1991) and demonstrates that the cleavage at the single

arginine residue (Arg-15), which releases SRIF-28 from both SRIF precursors, is effected by an endoprotease distinct from Kex2.

#### **Isolation and partial characterization of somatostatin-28 expression mutants**

Problems inherent in the purification of non-abundant proteinases prompted us to clone the gene encoding this novel activity through direct genetic complementation of a mutant yeast that is unable to recognize and/or cleave after the monobasic site present in proSRIFs. To facilitate the screening procedure involved in the isolation of such a mutant the plasmid pS2B, which encodes the proSRIF-II fusion protein (Figure 4A), was modified by site-directed mutagenesis in order to replace the SRIF-28 moiety by a single copy of the  $\alpha$ -factor peptide. The resulting gene was named pS3C and encodes a fusion protein of 132 amino acids (see Figure 4A for its structure). Expression of the gene is under the control of the  $\alpha$ -factor promoter. Plasmid pS3C was introduced into  $\alpha$  cells that carry a disrupted copy of the *MF $\alpha$ 1* and *MF $\alpha$ 2* genes (strain DS7) so that secretion of bioactive  $\alpha$ -factor from that strain requires proper maturation of the hybrid precursor at the monobasic site. The rationale was to use assays based on the secretion of a bioactive  $\alpha$ -factor (i.e. 'halo' and mating assays, Julius *et al.*, 1984). To distinguish between mutants that specifically affect maturation of the hybrid precursor and those that perturb the transcriptional regulation of the  $\alpha$ -factor promoter, or any mutant whose primary defect does not directly involve the monobasic-specific endoproteolytic activity, two other plasmids were constructed. Plasmid pGS3C is a derivative of pS3C where the chimeric gene is under the control of the *GAL1* promoter and p $\alpha$ FF is a truncated version of the *MF $\alpha$ 1* gene and encodes a polypeptide of 98 amino acids that has retained a single copy of the mature  $\alpha$ -factor peptide immediately preceded by the Lys Arg dibasic processing site. We reasoned that a mutant strain which is still capable of secreting bioactive  $\alpha$ -factor when carrying p $\alpha$ FF, but not pS3C, would be a potential candidate for a defect in the gene encoding the monobasic-specific endoprotease.

Control 'halo' and mating assays showed that only the DS7 transformants expressing pS3C and p $\alpha$ FF, but not those expressing pS2B, could elicit a zone of inhibition ('halo') on a lawn of supersensitive  $\alpha$  cells (Figure 4B) and were competent for mating (Figure 4C). Strain DS7, carrying pS3C, was next subjected to UV irradiation (see Materials and methods). From two independent experiments a total of >9000 colonies that survived the UV exposure were screened first for their mating competence with *MATa* cells. From these mutated colonies, 39 were found to be mating defective (Table I). Halo assays performed with these mutants identified a class of mutant (16 colonies, class I) whose mating defect was not associated with a reduced production and/or secretion of bioactive  $\alpha$ -factor (Table I). From the remaining 23 mutants, where a correlation between mating and secretion of biologically active  $\alpha$ -factor was observed, one could further distinguish two classes. The class II mutants (15 colonies) were unable to produce a zone of inhibition even on a lawn of the supersensitive strain M200-6C whereas the size of the 'halo' produced by the class III mutants (eight colonies) was significantly reduced. Expression of the plasmid pGS3C in representative members



**Fig. 4.** Strategy used for the isolation of somatostatin expression mutants. (A) Schematic representation of plasmids pS2B, pS3C and p $\alpha$ FF: (▨)  $\alpha$ -factor preproregion; (P) N-linked glycosylation site; (▨) full length proSRIF-II; (○) cleavage site; (■) mature SRIF-28; and (□) mature  $\alpha$ -factor. Note that the basic residues of the cleavage sites are indicated by their single letter code. (B) Halo assay (see Materials and methods) performed with the indicated DS7 transformants on a lawn of the indicator *MATa* strain M200-6C (*sex1 sst2*). (C) Prototroph selection of diploid yeast resulting from a cross of the indicated DS7 transformants with the *MATa hisI* tester strain.

of both classes showed that in contrast to class III mutants, mutants pertaining to class II could elicit a wild type 'halo' when the hybrid gene was expressed under the control of the *GAL1* promoter. This strongly suggests that the class II mutants are only defective in expression of the hybrid gene when under the control of the  $\alpha$ -factor promoter. To test whether the class III mutants had retained the ability to secrete bioactive  $\alpha$ -factor when it was flanked by its normal processing site (Lys Arg) in the precursor, plasmid p $\alpha$ FF was introduced in three members of class III. One transformant (B51) was found to secrete very low levels of the pheromone regardless of the nature of the cleavage site (Lys Arg in p $\alpha$ FF; single Arg in pS3C), suggesting that the mutation had disrupted a cellular function not directly involved in the maturation at the monobasic processing site. Two strains (B34 and B22), however, secreted wild type or near wild type levels of bioactive  $\alpha$ -factor when carrying p $\alpha$ FF as estimated by the size of the 'halo' on a lawn of supersensitive *a* cells (strain M200-6C). We therefore conclude that strains B34 and B22 carry mutations affecting either directly (the gene encoding the endoprotease itself) or indirectly (e.g. any gene involved in the proper localization or function of the endoprotease) the putative monobasic endoprotease activity. Without further characterization, strain B34 (*sex1-1*) was used as a recipient cell for the cloning of the putative monobasic-specific endoprotease gene.

#### Cloning and characterization of a multicopy suppressor of the *sex1-1* mutation

A yeast genomic library constructed in the multicopy plasmid YEP24 was used to transform strain B34 carrying pS3C. From a total of >10 000 Ura<sup>+</sup> Trp<sup>+</sup> transformants, we isolated seven clones in which the mating defect as well as the ability to produce a 'halo' on a lawn of sensitive *a* cells (M200-6C) had been corrected (not shown). Given the phenotype of the parental strain (*MF $\alpha$ 1::LEU2 MF $\alpha$ 2::LEU2*) and since the *sex1-1* mutation did not significantly affect proteolytic processing of the native  $\alpha$ -factor precursor, we anticipated cloning also functional

**Table I.** Isolation of mutants defective in somatostatin expression (*sex* mutants)

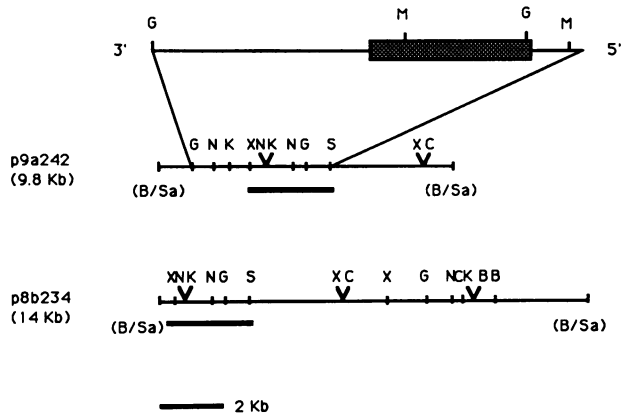
Phenotypic class	No. of mutants	Mating	Production of $\alpha$ -factor <sup>a</sup> with		
			pS3C	pGS3C	p $\alpha$ FF
Wild type	NA	+	+++	+++	+++
Class I	16	-	+++	ND	ND
Class II	15	-	-	+++ (4) <sup>b</sup>	ND
Class III	8	-	+	+ (2) + (1)	+++ (2) + (1)

<sup>a</sup>Production of mature  $\alpha$ -factor as estimated by a 'halo' assay on a lawn of supersensitive strain (M200-6C; Table III).

<sup>b</sup>Number of mutants from that phenotypic class that were transformed with the indicated plasmid and assayed for the production of a halo on M200-6C.

Absence of mating or of a halo is indicated (-). The size of the halo produced by the wild type strain is indicated by (+++); a reduction of 25–50% in the diameter of the halo is indicated by (++) ; a reduction of 50–75% in the diameter of the halo is indicated by (+). NA, not applicable; ND, not determined.

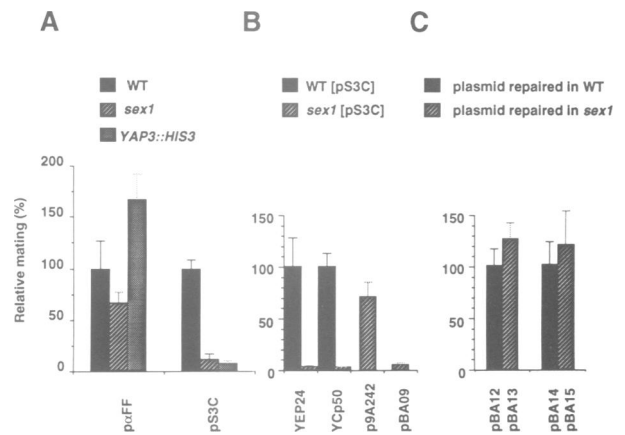
copies of the *MF $\alpha$*  genes. From the initial 7 positive clones, 5 were shown to mate efficiently and secrete wild type levels of  $\alpha$ -factor even in the absence of plasmid pS3C. Hybridization of the plasmid DNA carried by these clones with a radiolabelled probe derived from p $\alpha$ FF confirmed that a functional copy of the *MF $\alpha$*  genes had been introduced into these strains (data not shown). In the other positive clones, expression of both plasmids was required for the restoration of the wild type pheromone production and mating competence. The YEP24-based plasmids carried by these strains were rescued by transformation into *Escherichia coli* and designated p9A242 and p8B234. Partial restriction maps of p9A242 (9.8 kb insert) and p8B234 (14 kb insert) suggested that they share a common *SalI*-*XbaI* DNA fragment of ~2.7 kb (Figure 5). This was confirmed by hybridizing the plasmids with a probe derived from the *SalI*-*XbaI* fragment of p9A242 (not shown). Upon subcloning this common fragment into pTZ18R and the generation of a more precise restriction map, it became



**Fig. 5.** Partial restriction map of plasmids p9A242 and p8B234. A partial restriction map of the inserts carried by plasmids p9A242 and p8B234 was established with the following restriction enzymes: *Bam*HI (B); *Bg*III (G); *Kpn*I (K); *Mlu*I (M); *Nco*I (N); *Sac*I (C); *Sal*I (S); and *Xba*I (X). The solid bar below the restriction maps indicates the position of the common *Xba*I–*Sal*I fragment in these two inserts. The enlarged portion of the restriction map shows the location and orientation of the coding region of the cloned gene (■).

obvious the gene we had cloned by genetic complementation of a *sex1* mutant was remarkably similar to another yeast gene recently identified by Egel-Mitani *et al.* (1990) and called *YAP3*. The *YAP3* gene (Yeast Aspartyl Protease 3) was cloned because of its ability to partially suppress the *kex2* mutation when present on a multicopy plasmid. However, its normal function in the cell had not been investigated. Partial DNA sequencing confirmed the two genes are identical (data not shown).

Quantitative mating assays are quite sensitive and provide a good estimate of the relative amount of secreted  $\alpha$ -factor. Quantitative mating assays performed with strain YBAD1 carrying a disrupted copy of the cloned gene indicated that the null mutant was slightly more competent for mating (167%) than either the wild type (set at 100%) or the *sex1-1* mutant (67%) strain when expressing plasmid p $\alpha$ FF (Figure 6A). In contrast, the mating efficiency of the null mutant expressing plasmid pS3C was greater than 10-fold lower (7.3%) than the wild type strain and very similar to that obtained for the strain carrying the *sex1-1* mutation (12.4%). However, the cloned gene was shown to restore near wild type mating competence to strain B34 when present on a multicopy plasmid (YEP24), but was unable to do so when expressed from a centromere-based plasmid (YCp50) (Figure 6B). This provided an indication that the gene we had cloned is not allelic to *SEX1*, but rather acts as a multicopy suppressor of the *sex1-1* mutation. Because the phenotype of the *sex1-1* mutation could only be observed in  $\alpha$  cells, complementation tests with a strain carrying a disrupted copy of the *YAP3* gene could not be performed. To circumvent this problem we decided to clone the *YAP3* locus from the *sex1-1* mutant using the ‘gap repair’ technique (Orr-Weaver *et al.*, 1981) and test its ability to correct the mating deficiency of strain YBAD1 ( $\Delta$ *YAP3*) expressing pS3C. Centromere-based plasmids (YCp50) containing either a *Bg*III or an *Mlu*I deletion, which together covered the whole coding region of the *YAP3* gene (see Figure 5), were used to transform either the parental or the original *sex1-1* mutant yeast to allow repair by homologous recombination. The resulting plasmids were then rescued by transformation into *E. coli* and introduced into strain YBAD1 ( $\Delta$ *YAP3*) (Figure

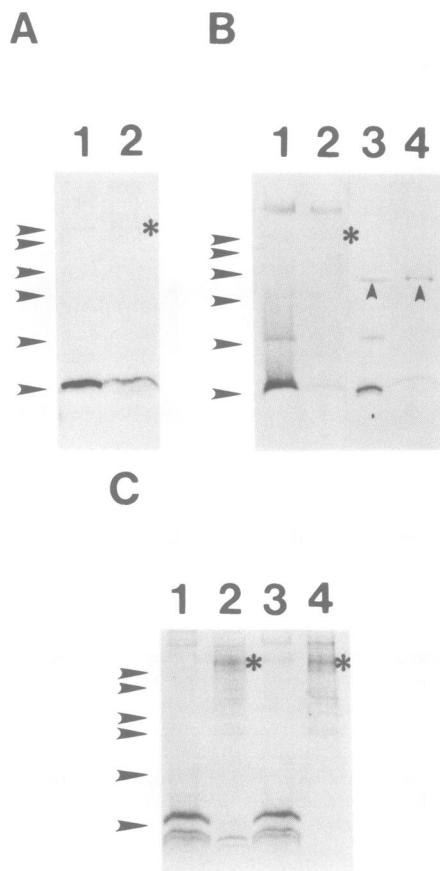


**Fig. 6.** The gene carried by plasmids p9A242 and p8B234 acts as a multicopy suppressor of the *sex1-1* mutation. Quantitative mating assays (see Materials and methods) were performed with the indicated *MAT $\alpha$*  transformants and the *MAT $\alpha$  his1* tester strain. The efficiency of mating is expressed in percentage of diploid cells formed by the indicated transformant relative to mating efficiency of the parental strain DS7 (set to 100%). All assays were performed in duplicate and the average mating efficiency is reported with the standard deviation. In (A) the averages were obtained from three independent determinations. (A) Wild type (WT; strain DS7), *sex1-1* (*sex1*; strain B34) and a strain carrying a disrupted copy of the cloned gene (*YAP3::HIS3*, strain YBAD1) were transformed with the indicated plasmids and challenged to mate. (B) Wild type (WT; strain DS7) and *sex1-1* (*sex1*; strain B34) yeast co-transformed with pS3C and the indicated multicopy or centromere-based plasmids carrying the cloned gene (p9A242 and pBA09, respectively) or not (YEP24 and YCp50, respectively) were challenged to mate. (C) Yeast carrying a disrupted copy of the cloned gene (*YAP3::HIS3*; strain YBAD1) were co-transformed with pS3C and the indicated gap-repaired plasmids. Plasmids pBA12-13 and pBA14-15 (see Materials and methods) contain a *Bg*III and a *Mlu*I deletion in the *YAP3* gene respectively. They were then assayed for their relative mating efficiency.

6C). As expected for a gene which is not allelic to *SEX1*, the mating efficiency was the same regardless of the host (i.e. wild type or *sex1-1* mutant) in which the plasmids containing deletions were repaired. We therefore conclude the *YAP3* gene acts as a multicopy suppressor of the *sex1-1* mutation.

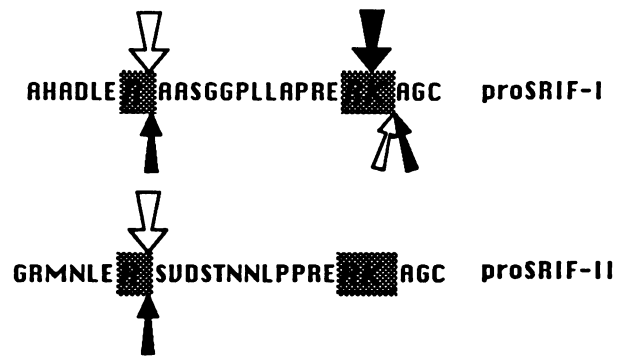
**Functional role of the *YAP3* gene in prosomatostatin processing**

Deletion of the *YAP3* gene, which encodes an aspartyl protease, had a profound effect on the mating competence of the strain expressing pS3C. Its role in proSRIF proteolytic maturation was further investigated by metabolic labelling of an appropriate yeast strain that had been transformed with either pS2B or pS1D, followed by specific immunoprecipitation of the secreted SRIF species. Identical amounts of radiolabelled material secreted from wild type and mutant yeast were then loaded on SDS–Tricine gels. Strain YBAD1 ( $\Delta$ *YAP3*) transformed with pS2B was found to secrete considerably less mature SRIF-28 (<24% as estimated by densitometric analysis) than the wild type strain (Figure 7A, compare lanes 1 and 2). This supports the hypothesis that the *YAP3* gene is required for proteolytic processing at monobasic cleavage sites. In its absence, however, clearly alternative endoproteolytic activities could generate some mature peptide. Although the *K<sub>m</sub>* for the reaction was considerably higher (>10-fold) with single arginine residues than with Lys Arg- or Arg Arg-containing substrates, previous studies on the specificity of the Kex2 endoprotease



**Fig. 7.** Biosynthesis of the prepro  $\alpha$ -factor–proSRIF-I and -II chimeras in wild type, single ( $\Delta YAP3$ ) and double ( $\Delta YAP3 \Delta KEX2$ ) mutant yeast. Yeast were labelled with [ $^{35}$ S]cysteine and the radioactive material was specifically immunoprecipitated with anti-SRIF-28 (A and B) or anti-SRIF-14 (C) antisera and resolved on a high resolution SDS–Tricine gel as described in Figure 3. (A) Secreted immunoreactive material from wild type (strain DS7; lane 1) and  $\Delta YAP3$  (strain YBAD1; lane 2) yeast transformed with plasmid pS2B. (B) Secreted immunoreactive material from wild type (strain DS7; lanes 1 and 3) and  $\Delta YAP3 \Delta KEX2$  (strain YBAD2; lanes 2 and 4) yeast transformed with plasmid pS2B. Samples in lanes 3 and 4 were incubated with endoglycosidase H prior to electrophoresis. (C) Secreted immunoreactive material from wild type (strains DS7 and W303-1b; lanes 1 and 3, respectively),  $\Delta YAP3$  (strain YBAD1; lane 2) and  $\Delta YAP3 \Delta KEX2$  (strain YBAD2; lane 4) yeast transformed with plasmid pS1D. Note that an identical amount of radioactivity was loaded in each pair of lanes. Asterisks indicate the migration position of the major glycosylated precursor form of the chimeras. Upward pointing arrowheads indicate the position of the deglycosylated proSRIF-II fusion protein (16 kDa). Arrowheads on the left indicate the position of the prestained protein standards as described in Figure 3.

indicated that this enzyme recognizes and cleaves, at least *in vitro*, after single arginine residue (Brenner and Fuller, 1992). To test the idea that Kex2 could, in the absence of YAP3, excise mature SRIF-28 from the hybrid precursor the double mutant strain YBAD2 ( $\Delta YAP3 \Delta KEX2$ ) was constructed. Expression of plasmid pS2B in this strain resulted in a further decrease (<10% of the wild type production of SRIF-28) in proSRIF-II conversion to SRIF-28. It also totally prevented the appearance of a 6 kDa peptide, which was consistently observed in the culture medium of wild type and  $\Delta YAP3$  yeast. Although its nature remains to be confirmed, its mobility on SDS–Tricine gels



**Fig. 8.** Model for the proteolytic processing of the anglerfish somatostatin precursors in the yeast *S.cerevisiae*. The amino acid sequence of proSRIF-I and -II, comprising the two processing sites (grey boxes), is represented using the single letter code. Filled and open arrows represent the KEX2- and YAP3-dependent cleavage, respectively. Larger arrows emphasize the prevalence of the indicated gene in the proteolytic cleavage.

is that expected for preproSRIF-II 63–125, a previously described SRIF-28 processing intermediate whose release requires cleavage at a Lys Arg doublet (Noe *et al.*, 1986). Any decrease in the production of SRIF-28 should yield a concomitant increase in the precursor forms. Accumulation of the secreted, hyperglycosylated proSRIF-II hybrid precursor could not easily be detected on SDS–Tricine gels. That the absence of both Kex2 and Yap3 leads to the secretion of unprocessed precursor forms was best demonstrated by first subjecting the immunoreactive material to endoglycosidase H treatment (Figure 7B, lanes 3 and 4). The deglycosylated precursor (16 kDa band) could be detected from the culture media of both the wild type and the double mutant yeast. However, its relative abundance with respect to mature SRIF-28 indicated that it was a minor species secreted by the wild type strain but the predominant secreted peptide of the  $\Delta YAP3 \Delta KEX2$  mutant.

As shown above, expression of proSRIF-I in yeast leads to the secretion of three major peptides (SRIF-28, Lys-SRIF-14 and SRIF-14) and genetic evidence suggested Kex2 is directly involved in the release of Lys-SRIF-14 through cleavage in the middle of a pair of basic residues (Arg Lys). Consistent with this finding, expression of pS1D in the absence of a functional YAP3 gene did not affect conversion of the precursor to Lys-SRIF-14 (Figure 7C, lanes 1 and 2). However, it nearly completely prevented accumulation of SRIF-28 and significantly reduced the amount of secreted SRIF-14. This was accompanied by the appearance of a diffuse band whose molecular mass (45 kDa) is consistent with hyperglycosylation, which contributes 30–50 kDa per chain (Julius *et al.*, 1984) of the proSRIF-I hybrid precursor (17 kDa). That mature SRIF-14 was secreted from a *kex2* (Figure 3, lane 2) as well as from a  $\Delta YAP3$  mutant yeast (Figure 7C, lane 2) suggested that both gene products are involved in the release of this peptide. Expression of pS1D in the double mutant strain YBAD2 confirmed this hypothesis (Figure 7C, lanes 3 and 4). In this mutant yeast the proteolytic maturation of this fusion protein was completely abolished and only intact hyperglycosylated precursor forms (45 kDa peptide) could be recovered from the culture medium.

## Discussion

Our main objective is to define the molecular basis for the differential processing of the anglerfish SRIF-I and -II precursors using the yeast *S.cerevisiae* as a model system. As a first step toward that goal we recently expressed proSRIF-II in yeast, as a fusion protein, and identified a novel endoproteolytic activity capable of generating *in vivo* mature SRIF-28 (Bourbonnais *et al.*, 1991). In the present study we wanted to investigate the biosynthesis of the analogous anglerfish somatostatin precursor (SRIF-I) and characterize the gene encoding the novel yeast endoprotease. The recent cloning of the mammalian dibasic processing enzymes PC2, PC1/PC3 and PC4 (Seidah *et al.*, 1990; Smeekens and Steiner, 1990; Smeekens *et al.*, 1991; Nakayama *et al.*, 1992) was based upon sequence similarity with the yeast Kex2 enzyme. A key finding in the cloning of these mammalian Kex2 homologues was the observation that the Kex2 endoprotease, which is required for the maturation of the yeast  $\alpha$ -factor (Julius *et al.*, 1984), could faithfully recognize and cleave analogous dibasic processing sites (i.e. Lys Arg and Arg Arg) in heterologous precursors (Thomas *et al.*, 1988; Germain *et al.*, 1990; Zollinger *et al.*, 1990). The cloning of the mammalian monobasic processing enzyme might therefore benefit from the isolation of the gene for the cognate enzyme from yeast.

We have shown that expression of anglerfish proSRIF-I in yeast leads to the secretion of mature SRIF-28, mature SRIF-14 and an extended form of SRIF-14 (Lys-SRIF-14). Accumulation of SRIF-28 in the culture medium indicates processing in yeast at the internal Arg-2 Lys-1 basic pair does not proceed to completion. This is in contrast to synthesis of the prohormone in the pancreatic islets, which exclusively yield mature SRIF-14. In higher eukaryotes, however, the secretion of peptide hormones and neuropeptides is regulated, whereas yeast rapidly and constitutively secretes the proteins destined for the extracellular medium. The very limited contact time between the prohormone convertase and its substrate in yeast may therefore account for the apparent discrepancy observed between yeast and the pancreatic islets. Expression of proSRIF-I in a *kex2* yeast mutant extended our previous observation (Bourbonnais *et al.*, 1991) and provided genetic evidence that both SRIF precursors are processed to mature SRIF-28 by a Kex2-independent cleavage. Genetic complementation of a yeast mutant (*sex1-1*), which specifically affects cleavage at the monobasic processing site (Arg-15), resulted in the cloning of a gene encoding an aspartyl protease. This gene, *YAP3*, which was also recently cloned by another group (Egel-Mitani *et al.*, 1990) based on its ability to partially suppress the phenotype of a *kex2* null mutant, was shown to act as a multicopy suppressor of the *sex1-1* mutation. It is unclear at this point what the primary defect of the *sex1* mutant is. Because of the null phenotype of the *YAP3* gene (see below) it is, however, unlikely that Sex1 is directly involved in the proteolytic maturation of proSRIFs. Most probably, it is involved in either the function (i.e. proteolytic activation of a zymogen) or the intracellular localization of the *YAP3* gene product. Hence, although our screening procedure was primarily designed to facilitate cloning of the gene encoding the putative monobasic-specific endoprotease, further characterization of our class III mutants and cloning

of the corresponding *SEX* genes will undoubtedly provide new insights on the yeast cellular machinery involved in precursor processing. This work is currently in progress. Similarly, the rapid and sensitive biological assay used in the isolation of the *sex* mutants, combined with 'cassette'-directed mutagenesis, may provide a powerful tool for investigating the role of the amino acid residues upstream from the processing sites in the differential maturation of the anglerfish SRIF precursors. This is of particular interest since despite possessing the same mono- and dibasic cleavage sites they appear to be differentially processed in an identical cellular environment (see below).

Expression of proSRIF-I and -II fusion proteins in single and double mutant yeast enabled us to propose a model for the role of the *KEX2* and *YAP3* genes in the proteolytic maturation of somatostatin precursors by yeast (Figure 8). In both hybrid precursors efficient cleavage at the single arginine residue (Arg-15) requires a functional *YAP3* gene. Although its absence was more dramatic for the production of SRIF-28 from proSRIF-I, clearly other enzymatic activities could, at least partially, process the SRIF precursors to generate this peptide. Consistent with previous *in vitro* and *in vivo* studies on the substrate specificity of the Kex2 endoprotease (Brenner and Fuller, 1992; Zhu *et al.*, 1992), our results implicate the *KEX2* gene in this alternative, less efficient processing. As reported for the pancreatic islets (Noe and Spiess, 1983), proteolytic maturation of proSRIF-II in yeast is restricted to that single arginine residue (this study; Bourbonnais *et al.*, 1991). In contrast, proSRIF-I is further processed, presumably at the Arg-2 Lys-1 site, to generate Lys-SRIF-14 and mature SRIF-14. Since the basic pair is present in both precursors this supports our hypothesis (Bourbonnais *et al.*, 1991) that lack of proSRIF-II cleavage at Arg-2 Lys-1 results from a specific conformation rendering that site inaccessible to endoproteolytic processing. The dibasic-specific Kex2 endoprotease is essential for the release of Lys-SRIF-14, suggesting that the endoproteolysis occurs in between the basic doublet. In favor of this idea, previous studies on the specificity of the purified Kex2 endoprotease with peptidyl-MCA substrates revealed that the P<sub>1</sub> binding site of the enzyme was highly specific for Arg and also that with Arg at the P<sub>1</sub> position, substitution at P<sub>2</sub> had little effect on the overall catalytic rate (Brenner and Fuller, 1992). However, additional experiments are required to confirm this hypothesis (see below). The Kex2 enzyme is also involved either directly (i.e. cleavage after the Arg Lys doublet) or indirectly (i.e. a limiting aminopeptidase B-like activity would remove the extra lysine residue in the Lys-SRIF-14 processing intermediate) in the release of mature SRIF-14. In support to this hypothesis, an aminopeptidase B-like enzyme purified from rat brain cortex was recently proposed to be required for the conversion of processing intermediates to mature SRIF-14 (Gluschkof *et al.*, 1987; Gomez *et al.*, 1988). Finally our data showed that the *YAP3* gene, which is predominantly involved in processing at monobasic cleavage site (Arg-15), also contributes to some extent in the release of mature SRIF-14. This is consistent with a previous study on the proteolytic maturation of an overexpressed pro  $\alpha$ -factor-insulin chimera in the absence of a functional *KEX2* gene (Egel-Mitani *et al.*, 1990). Under these conditions up to 20% proteolytic processing, ascribed to the action of Yap3,

occurred at the pro  $\alpha$ -factor Lys Arg cleavage site. Hence, proteolytic maturation of anglerfish SRIF precursors in yeast implicates two endoproteases with distinct specificities for the mono- and dibasic processing sites. The present model of proSRIF processing by yeast is based largely on genetic arguments. The actual sites of endoproteolytic cleavage need therefore to be confirmed by *in vitro* incubation of the SRIF precursors with the purified endoproteases. For instance, our data cannot exclude the possibility that the Lys-SRIF-14 peptide is generated by Kex2 through endoproteolytic cleavage C-terminal to Arg-4 (i.e. after the Pro-5 Arg-4 putative monobasic site), instead of after Arg-2, followed by exoproteolytic cleavage by an aminopeptidase. This would be supported by our present finding implicating Kex2 in the release of some mature SRIF-28, as well as from a recent report on the Kex2-dependent processing of the yeast K<sub>1</sub> killer toxin at the Pro43 Arg44 site (Zhu *et al.*, 1992).

Although the precise site of action of the YAP3 gene product has yet to be determined, preliminary studies on the localization of the Yap3-dependent cleavage of proSRIFs support its role in precursor processing. Namely, the accumulation of significant amount of the various SRIF peptides in cell extracts of pS1D- (this study) and pS2B-transformants (Bourbonnais *et al.*, 1991) and the virtual absence of precursor forms in the culture media strongly

argued that the proteolytic maturation occurs intracellularly. The presence of the mature SRIF peptides in cell extracts prepared from spheroplasts of *kex2*-transformed yeast confirmed the Yap3-dependent processing occurs in the secretory pathway and not in the periplasmic space (not shown). In addition, our previous observation that the processing of proSRIF-II is completely blocked when expressed in a *sec18* mutant grown at the restrictive temperature, further indicated that the proteolytic maturation is a post-ER event (Bourbonnais *et al.*, 1991). These results are thus in good keeping with the  $\alpha$ -factor maturation by the Kex2 endoprotease which, most probably, takes place in a late-Golgi compartment (Julius *et al.*, 1984; Redding *et al.*, 1991). Consistent with the physiological relevance of Kex2 and Yap3 in proSRIF maturation, two groups have documented the action of distinct somatostatin convertases in the production of SRIF-28 and SRIF-14, respectively, in higher eukaryotes (Gluschankof *et al.*, 1987; Mackin and Noe, 1987; Beinfeld *et al.*, 1989). More recently Mackin *et al.* (1991a, 1991b) purified to apparent homogeneity two candidate SRIF convertases from anglerfish secretory granules capable of generating the SRIF-28 and -14 peptides respectively, *in vitro* from their physiological precursor (i.e. proSRIF-II and -I). Partial N-terminal sequencing of the purified proteins revealed the candidate SRIF-14 con-

**Table II.** Plasmids used in this work

Name	Description	Reference
pS2B	A 2 $\mu$ m based vector derived from p82-5 (Green <i>et al.</i> , 1986) expressing the prepro $\alpha$ -factor-proSRIF-II chimeric gene under the control of the $\alpha$ -factor promoter	Bourbonnais <i>et al.</i> (1991)
pS1D	Same as pS2B, but expressing the prepro $\alpha$ -factor-proSRIF-I chimeric gene	This study
pS3C	Same as pS2B, but expressing the prepro $\alpha$ -factor-proSRIF-II-mature $\alpha$ -factor chimeric gene	This study
pGS3C	A centromere-based vector derived from pJK6 (kindly provided by J.Kurjan) where the prepro $\alpha$ -factor-proSRIF-II-mature $\alpha$ -factor chimeric gene is under the control of the <i>GAL1</i> promoter	This study
p $\alpha$ FF	Same as pS2B, but expressing the truncated version of prepro $\alpha$ -factor in which a single copy of the mature peptide has been retained	This study
YEP24G-B	A yeast genomic library constructed in plasmid YEP24	Rose and Broach (1991)
pBA09	Plasmid YCp50 in which the 5.8 kb <i>Xba</i> I (filled-in) fragment of p9A242 has been cloned in the <i>Sa</i> II (filled-in) site	This study
pBA12	Derived from YCp50 carrying the 6.0 kb <i>Sa</i> II of p9A242, this plasmid contained a <i>Bg</i> II deletion (3.8 kb) that removed all but the first seven codons of the Yap3 protease and ~2.1 kb of 3' flanking nucleotides that had been repaired <i>in vivo</i> by homologous recombination in the parental strain DS7	This study
pBA13	Same as pBA12, except repair of the plasmid was done in the mutant strain B34	This study
pBA14	Derived from pBA09, this plasmid contained a <i>Mlu</i> I deletion (1.8 kb) that removed 0.4 kb of 5' flanking sequence and the first 459 codons (out of 569) of the YAP3 gene, that had been repaired <i>in vivo</i> by homologous recombination in the parental strain DS7	This study
pBA15	Same as pBA14, except repair of the plasmid was done in the mutant strain B34	This study

**Table III.** Yeast strains

Name	Genotype	Source of reference
20-B12	<i>MAT<math>\alpha</math> pep4-3 trp1</i>	Green <i>et al.</i> (1986)
DS20	<i>MAT<math>\alpha</math> kex2-1 trp1 ura1</i>	Bourbonnais <i>et al.</i> (1991)
DS7	<i>MAT<math>\alpha</math> MF<math>\alpha</math>1/<math>\alpha</math>2::LEU2 ade2 his3 leu2 trp1 ura3</i>	J.Kurjan
M200-6C	<i>MAT<math>\alpha</math> sst1 sst2 ade1 ilv3 ura3</i>	Whiteway <i>et al.</i> (1988)
B34	<i>MAT<math>\alpha</math> sex1-1 MF<math>\alpha</math>1/<math>\alpha</math>2::LEU2 ade2 his3 leu2 trp1 ura3</i>	This study
YBAD1	<i>MAT<math>\alpha</math> YAP3::HIS3 MF<math>\alpha</math>1/<math>\alpha</math>2::LEU2 ade2 his3 leu2 trp1 ura3</i>	This study
M213-10C	<i>MAT<math>\alpha</math> KEX2::HIS3 his3 leu2 trp1 ura3</i>	Dignard <i>et al.</i> (1991)
W303-1b	<i>MAT<math>\alpha</math> ade2 his3 leu2 trp1 ura3</i>	S.Henry
YBAD2	<i>MAT<math>\alpha</math> YAP3::LEU2 KEX2::HIS3 his3 leu2 trp1 ura3</i>	This study



vertase was homologous to human and mouse PC2, a recently identified member of the Kex2-like mammalian homologues (Seidah *et al.*, 1990; Smeekens and Steiner, 1990). Both the inhibition profile and the partial N-terminal sequencing of the protein indicated the putative SRIF-28 convertase belongs to the aspartyl protease family. It is therefore tempting to speculate that the *YAP3* gene is the yeast functional homologue of this endoprotease. However, it is also possible that while functionally related to higher cell convertase involved in cleavage at monobasic sites, the Yap3 endoprotease is not closely structurally related. This would be analogous to the carboxypeptidase B-like activity required in processing the transient intermediates generated by endoproteolysis after the pair of basic residues. Hence, while both carboxypeptidases are thought to fulfil the same function in the cell, the sequence of the yeast *KEX1* gene product (Dmochowska *et al.*, 1987) does not resemble that of mammalian carboxypeptidase E (Fricker, 1988). Future experiments addressing the physiological function of these endoproteases, and information on the complete amino acid sequence of the anglerfish aspartyl protease, are thus needed to clarify the exact relationship between the two enzymes. As a first step toward that goal we are currently investigating the nature of the Yap3 substrate(s) in yeast.

## Materials and methods

### Plasmids and strains

Plasmids used in this study and a brief description of the genes they carry are listed in Table II. All DNA modifications and subcloning were performed according to standard procedures. Site-directed mutagenesis was performed on single-stranded DNA isolated from *dut<sup>-</sup> ung<sup>-</sup>* bacteria using the single primer annealing technique according to the procedure developed by Kunkel (1985). Restriction endonucleases were from New England Biolabs and T4 DNA polymerase was from Pharmacia LKB Biotechnology Inc. Yeast strains used in this study are listed in Table III. Yeast transformation was carried out using the lithium acetate procedure described by Ito *et al.* (1983).

### Yeast mutagenesis

Strain DS7, which expresses plasmid pS3C, was grown overnight and plated on selective medium. The dishes were then immediately exposed to short-wave UV light for 35 s, wrapped in aluminum foil and placed at 22°C for 3 days to allow formation of colonies. Control plates indicated this treatment resulted in 90% killing. Colonies that survived the UV exposure were then screened for production of bioactive  $\alpha$ -factor as described below.

### Halo and quantitative mating assays

Halo assays were performed with the strain M200-6C, which carries mutant alleles of the *SST1* and *SST2* genes and thus is supersensitive to  $\alpha$ -factor. Following an overnight incubation at 30°C in YPD medium, 5  $\mu$ l of M200-6C were mixed with 10 ml of YPD medium containing 0.7% agar, which had been prewarmed at 42°C, and overlaid onto YPD plates. Colonies to be tested were then spotted directly onto the solidified top agar and the plates were incubated overnight at 30°C. For the mating assay, haploid yeast were first grown overnight in YPD medium (*MATa* tester strain) or the appropriate SC medium (*MAT $\alpha$*  strains). The next day  $\alpha$  cells were diluted 1/10 in SC medium and the incubation was resumed for an additional 2 h at 30°C. The *MAT $\alpha$*  strains were then mixed with the tester strain (*MATa*) in the following ratio: 100  $\mu$ l of the diluted *MAT $\alpha$*  cells, 50  $\mu$ l of the *MATa* cells (undiluted overnight culture) and 100  $\mu$ l of YPD medium. The mixture was then gently filtered over sterile nitrocellulose filters (Sartorius), the filters were put, colonies side up, over YPD plates and these were incubated at 30°C for 4–6 h. Following incubation the cells were gently washed off the filters with 300  $\mu$ l of selective medium and appropriate dilutions were plated onto SC plates allowing diploid cells to grow by prototroph selection. Parallel nitrocellulose filters containing only YPD medium and the  $\alpha$  cells were plated onto YPD plates following appropriate dilutions. The percentage of mating was determined according to the following equation: (No. of colonies on SC plate/No. colonies on YPD plate)  $\times$  100. Alternatively, a semi-quantitative mating assay was performed by directly replicating the nitrocellulose filters onto SC plates.

### Gene disruption

To disrupt the yeast *YAP3* locus the *YAP3 SphI–XbaI* fragment was first subcloned into plasmid pTZ18R. Most of the *YAP3* coding region was then deleted (544/569 and 467/569 codons, respectively) and replaced by the *HIS3* gene (1.8 kb *BamHI* fragment from plasmid pJ215; Jones and Prakash, 1990) or the *LEU2* gene (2.0 kb *BamHI–HindIII* fragment from pJ283; Jones and Prakash, 1990). One step disruptions (Rothstein, 1983) with the *HIS3* marker were obtained through the use of the *SphI–BamHI* fragment for transformation of yeast strains. The disruption with the *LEU2* marker was obtained using the *HindIII–BamHI* fragment. Each disruption was confirmed by Southern analysis.

### Metabolic labelling of yeast, immunoprecipitation and protein analysis

Exponentially growing yeast were labelled with 250  $\mu$ Ci of [<sup>35</sup>S]cysteine (Amersham) during a 30 min incubation at 30°C as described previously by Bourbonnais *et al.* (1988, 1991). Cell extracts and media prepared as described by Bourbonnais *et al.* (1988, 1991) were then subjected to immunoprecipitation with anti-SRIF-28 (R33 B5) and anti-SRIF-14 (RSSI B23) antibodies as indicated. The antisera were generous gifts of Drs Shields and Danoff (Albert Einstein College of Medicine, Bronx, NY). Production and specificity of the antibodies have been described previously by Bourbonnais *et al.* (1991) and Danoff and Shields (1991). Anti-SRIF-28 specifically recognizes SRIF-28 and its precursor form, but does not cross-react with either SRIF-14 or proSRIF-I. Anti-SRIF-14 efficiently immunoprecipitates SRIF-14 and its precursor; it also recognizes SRIF-28 and proSRIF-II, albeit with much lower affinity. Radiolabelled proteins were then analyzed by high resolution Tricine–SDS gels or reversed-phase HPLC using a C<sub>18</sub> column (Vydac) as described previously by Bourbonnais *et al.* (1991). Samples were eluted from the column with 0.1% trifluoroacetic acid containing acetonitrile at a flow rate of 1.5 ml/min according to the following gradient: 5% acetonitrile for the first 5 min; 5–20% in 1 min; 20–35% in 30 min; 35–50% in 15 min; 50–80% in 1 min; and isocratically at 80% for 13 min.

### Partial amino acid sequencing

Peptides to be sequenced were first purified by HPLC and submitted directly to Edman degradation on a gas phase sequenator (Applied Biosystem model 470A) as described earlier by Bourbonnais *et al.* (1991).

### Endoglycosidase H treatment

Treatment of the immunoprecipitates with endoglycosidase H was carried out as described previously by Bourbonnais *et al.* (1991).

## Acknowledgements

We are grateful to Susan Henry, Janet Kurjan and Malcolm Whiteway who kindly provided yeast strains, Mark Rose for the generous gift of the yeast genomic library and Dennis Shields and Ann Danoff who supplied us with the SRIF antibodies; France Dumas for the amino acid sequencing of the labelled peptides and Caroline Kunze for her precious technical assistance in the early part of this work; Malcolm Whiteway and Thierry Vernet for critical reading of the manuscript. This is NRCC publication no. 33655.

## References

- Beinfeld, M.C., Bourdais, J., Kuks, P., Morel, A. and Cohen, P. (1989) *J. Biol. Chem.*, **264**, 4460–4465.
- Bourbonnais, Y., Bolin, D. and Shields, D. (1988) *J. Biol. Chem.*, **263**, 15342–15347.
- Bourbonnais, Y., Danoff, A., Thomas, D.Y. and Shields, D. (1991) *J. Biol. Chem.*, **266**, 13203–13209.
- Brenner, C. and Fuller, R.S. (1992) *Proc. Natl. Acad. Sci. USA*, **89**, 922–926.
- Danoff, A. and Shields, D. (1991) *J. Biol. Chem.*, **266**, 10004–10010.
- Devi, L. (1991) *FEBS Lett.*, **280**, 189–194.
- Dignard, D., Whiteway, M., Germain, D., Tessier, D. and Thomas, D.Y. (1991) *Mol. Gen. Genet.*, **227**, 127–136.
- Dmochowska, A., Dignard, D., Henning, D., Thomas, D.Y. and Bussey, H. (1987) *Cell*, **50**, 573–584.
- Dougllass, J., Civelli, O. and Herbert, E. (1984) *Annu. Rev. Biochem.*, **263**, 665–715.
- Egel-Mitani, M., Flygenring, H.P. and Hansen, M.T. (1990) *Yeast*, **6**, 127–137.
- Fricker, L.D. (1988) *Annu. Rev. Physiol.*, **50**, 309–321.

- Germain, D., Zollinger, L., Racine, C., Gossard, F., Dignard, D., Thomas, D.Y., Crine, P. and Boileau, G. (1990) *Mol. Endocrinol.*, **4**, 1572–1579.
- Gluschankof, P., Gomez, S., Morel, A. and Cohen, P. (1987) *J. Biol. Chem.*, **262**, 9615–9620.
- Gomez, S., Gluschankof, P., Lepage, A. and Cohen, P. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 5468–5472.
- Green, R., Schaber, M.D., Shields, D. and Kramer, R. (1986) *J. Biol. Chem.*, **261**, 7558–7565.
- Ito, H., Fukada, Y., Murata, K. and Kimura, A. (1983) *J. Bacteriol.*, **153**, 163–168.
- Jones, J.S. and Prakash, L. (1990) *Yeast*, **6**, 363–366.
- Julius, D., Brake, A., Blair, L., Kunisawa, R. and Thorner, J. (1984) *Cell*, **37**, 1075–1089.
- Kunkel, T.A. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 488–492.
- Mackin, R.B. and Noe, B.D. (1987) *J. Biol. Chem.*, **262**, 6453–6456.
- Mackin, R.B., Noe, B.D. and Spiess, J. (1991a) *Endocrinology*, **129**, 1951–1957.
- Mackin, R.B., Noe, B.D. and Spiess, J. (1991b) *Endocrinology*, **129**, 2263–2265.
- McDonald, J.K., Greiner, F., Bauer, G.E., Elde, R.P. and Noe, B.D. (1987) *J. Histochem. Cytochem.*, **35**, 155–162.
- Nakayama, K., Kim, W.-S., Torii, S., Hosaka, M., Nakagawa, T., Ikemizu, J., Baba, T. and Murakami, K. (1992) *J. Biol. Chem.*, **267**, 5897–5900.
- Noe, B.D. and Spiess, J. (1983) *J. Biol. Chem.*, **258**, 1121–1128.
- Noe, B.D., Andrews, P.C., Dixon, J.E. and Spiess, J. (1986) *J. Cell Biol.*, **103**, 1205–1211.
- Orr-Weaver, T.C., Szostak, J. and Rothstein, R.J. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 6354–6358.
- Patel, Y.C., Wheatley, T. and Ning, C. (1981) *Endocrinology*, **109**, 1943–1949.
- Redding, K., Holcomb, C. and Fuller, R.S. (1991) *J. Cell Biol.*, **113**, 527–538.
- Rose, M.D. and Broach, J.R. (1991) *Methods Enzymol.*, **194**, 195–230.
- Rothstein, R.J. (1983) *Methods Enzymol.*, **101**, 202–211.
- Schwartz, T.W. (1986) *FEBS Lett.*, **200**, 1–10.
- Seidah, N.G., Gaspar, L., Mion, P., Marcinkiewicz, M., Mbikay, M. and Chrétien, M. (1990) *DNA Cell Biol.*, **9**, 415–424.
- Smeekens, S.P. and Steiner, D.F. (1990) *J. Biol. Chem.*, **265**, 2997–3000.
- Smeekens, S.P., Avruch, A.S., LaMendola, J., Chan, S.J. and Steiner, D.F. (1991) *Proc. Natl. Acad. Sci. USA*, **88**, 340–344.
- Thomas, G., Thorne, B.A., Thomas, L., Allen, R.G., Hraby, D.E., Fuller, R. and Thorner, J. (1988) *Science*, **241**, 226–230.
- Whiteway, M., Hougan, L. and Thomas, D.Y. (1988) *Mol. Gen. Genet.*, **214**, 85–88.
- Zhu, Y.-S., Zhang, X.-Y., Cartwright, C.P. and Tipper, D.J. (1992) *Mol. Microbiol.*, **6**, 511–520.
- Zollinger, L., Racine, C., Crine, P., Boileau, G., Thomas, D.Y. and Gossard, F. (1990) *Biochem. Cell. Biol.*, **68**, 635–640.

Received on July 7, 1992; revised on September 30, 1992