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The *ste4*$^+$ gene, essential for sexual differentiation of *Schizosaccharomyces pombe*, encodes a protein with a leucine zipper motif

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ABSTRACT

***ste4*$^-$ mutants of *Schizosaccharomyces pombe* are unable to undergo both mating and meiosis. We have cloned the *ste4*$^+$ gene and its cDNA. The gene encodes a 264 amino acid protein with a typical leucine zipper motif homologous with the jun family. However, unlike the jun family, this protein does not have a typical basic region that precedes the leucine zipper. The transcription of this gene absolutely depends on the *ste11*$^+$ gene and increases several fold upon nitrogen starvation, a general signal for sexual differentiation. Whereas *ste4*$^+$ is essential for mating and meiosis, its overexpression inhibits these processes.**

INTRODUCTION

The sexual differentiation of the fission yeast *Schizosaccharomyces pombe* is regulated by a complex network of genes (1). Among the genes involved are four mating type genes (2), along with mating type specific and non-specific sterility genes (*map1*$^+$, *map2*$^+$, *mam1*$^+$, *mam2*$^+$ (3), *ste1*$^+$ through *ste13*$^+$ (4–8), *ral1*$^+$ through *ral4*$^+$ (9), *gpa1*$^+$ (10) and *spk1*$^+$ (11)). These genes are required for sexual differentiation and their inactivation makes cells sterile. Another class of genes negatively controls sexual differentiation. For example, the *pat1*$^+$ gene (12–14) encodes a protein kinase and its inactivation unconditionally induces meiosis and sporulation.

Among the ste genes, *ste5*$^+$ is involved in signal transduction in response to mating pheromone (15) and its activity is controlled by *ste6*$^+$ (16). The other ste genes appear to form complex networks controlling both mating and meiosis. One such gene is *ste4*$^+$. Despite some studies, its nature remains unknown (14,17). In the course of studies directed toward understanding the molecular mechanisms regulating the differentiation of eukaryotes, we isolated and characterized the *ste4*$^+$ gene. The *ste4*$^+$ gene encodes a protein with a typical leucine zipper motif. In this communication, we report the isolation and characterization of the *ste4*$^+$ gene and discuss its possible function.

MATERIALS AND METHODS

Yeast strains

The yeast strains used in this study are listed in Table 1. JM57, JM66, JM104, JM70, JM75, JM83, C708-5B, C766-5C, C761-5B, C756-1A, and C114-2D were obtained from C. Shimoda. Other strains, NUL11, NUS31, and KLU1 were constructed by crossing or fusing appropriate strains. DS43 and FS443 are described in the Results and Discussion section.

Libraries and vectors

The libraries used in this study were prepared by H. Nojima. The *S. pombe* genomic library was constructed by inserting the *Sau*3AI-partially-digested DNA of wild type strain L972 h^- into the *Bam*HI-digested pBluescript II KS+ vector (Stratagene). The *S. pombe* cDNA expression library was constructed with mRNA from logarithmically growing L972 cells and with the pcD2 vector (18). The transducing vector pAL7 (19), which promotes high efficiency transduction of cDNA libraries into *S. pombe*, is composed of *ars*, *stb* and *LEU2*. The pcMVL vector (20) consists of *ars*, *LEU2* and the human cytomegalovirus promotor, which is extremely active in *S. pombe* (21).

Screening for genes that complement a *ste4*$^-$ mutant

The libraries were introduced into the S. *pombe* strain C708–5B by co-transfection with pAL7 linearized with *Pst*I as described previously (19). Transfected cells were spread at a density of $1-5\times10^4$ Leu$^+$ colonies per plate and incubated on conjugation and sporulation-inducing plates. Colonies were pooled from each plate, and spores formed were selected by ethanol treatment and identified by iodine staining as described previously (22).

Other methods

Genetic and biochemical analyses and cell fusions of *S. pombe* were performed as described previously (23, 8). Southern and Northern hybridizations and dideoxynucleotide sequencing using the M13 vectors were carried out as described (24).

RESULTS AND DISCUSSION

Isolation and structures of genomic DNAs and cDNAs which complement *ste4*⁻

A leucine auxotrophic *ste4*⁻ mutant strain C708-5B was transformed with a genomic DNA library or a cDNA library together with the pAL7 vector as described previously (19). Three genomic DNA clones and 4 cDNA clones that effectively rescued the *ste4*⁻ mutation were recovered from 5.7×10^4 and 2.6×10^6 Leu⁺ transformants respectively. All the clones restored the ability of cells to conjugate as well as to sporulate. They shared common restriction sites except that one *Xba*I site was absent in #1 and #5 cDNAs (Fig. 1). The 3523 bp *Sal*I-*Xba*I fragment of the genomic DNA, the entire #7 cDNA and the region from the 5′ end to the internal *Pst*I site of #1, #5 and #6 cDNAs were sequenced. All the cDNA sequences determined were identical to the corresponding genomic sequences but #1 and #5 cDNAs were copies of spliced and #6 and #7 cDNAs were copies of unspliced mRNA. The nucleotide sequence of the genomic DNA is shown in Fig. 2. The *ste4*⁺ gene contains an open reading frame encoding a 264 amino acid protein with a calculated molecular weight of 31,000 daltons ($p31^{ste4}$). The protein has no remarkable similarity to any known ste gene product of fission or budding yeast. However, it contains 7 leucine heptad repeats, a typical leucine zipper motif, but the basic region that precedes the leucine zipper and is found in some transcriptional activators such as the jun family (25) is absent.

To assess the functional significance of the leucine zipper motif, we constructed a mutant with a deletion in this region by digestion with *Pst*I and *Spe*I followed by rejoining (Fig. 2). The downstream coding sequence remained translationally inframe. When this mutant gene was introduced into C708-5B, about 7% of the colonies (14% as complementation frequency) were stained with iodine vapor but very slightly, and only less than 1% of cells in the iodine-stained colonies had undergone sporulation, indicating that this mutant gene has a greatly diminished but detectable *ste4*⁺ activity (Table 2). This suggests that the leucine zipper is important but non-essential for function. Other mutants, i. e., deletions of the regions that precede or follow the leucine zipper, and frame-shift mutations at *Spe*I and *Sph*I sites completely inactivated the *ste4*⁺ activity (Table 2).

The first nucleotide of #7 cDNA is adenine at −313 and #1, #5 and #6 cDNAs start at −11. TATA-like sequences lie 25−30 nucleotides upstream of both cDNA start sites, suggesting the presence of two transcriptional start sites. The promotor activity may lie between the two start sites. #7 cDNA inserted into the pcMVL vector in either orientation efficiently complemented a *ste4*⁻ mutation, whereas #6 cDNA complemented only when inserted in the correct orientation (data not shown). This region contains the sequence TTCTTTGTTA, which quite resembles the proposed consensus sequence TTCTTTGTTY for the *ste11*⁺ responsive element (26). The *ste11*⁺ product is a transcriptional regulator of several genes inducible by nitrogen starvation (26). Indeed the transcription of *ste4*⁺ was induced by nitrogen starvation and the expression of *ste4*⁺ was dependent on *ste11*⁺ (Fig. 4; see below). Upstream of the polyadenylation site, a repeated sequence of twelve nucleotides CTAATATTCTAT is present in place of the common signal AATAAA (Fig. 2).

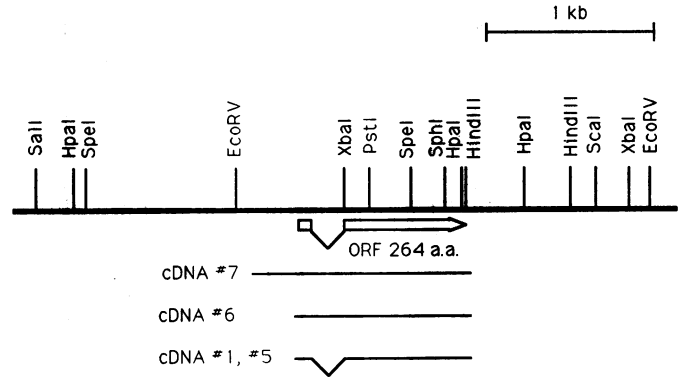

Figure 1. Restriction map of the *ste4*⁺ gene. Three genomic clones had larger inserts but only the region corresponding to the cDNA clones is presented. The open reading frame and the cDNA clones are positioned below the map according to the nucleotide sequence.

Table 1. *S.pombe* strains used in this study

Strain	Relevant genotype
L975	h^+
L972	h^-
L968	h^{90}
JM57	h^{90} *ste2 leu1*
JM66	h^{90} *ste3 leu1*
JM104	h^{90} *ste4 ade6-M216*
JM70	h^{90} *ste5 leu1*
JM75	h^{90} *ste6 leu1*
JM83	h^{90} *ste7 leu1*
C708-5B	h^{90} *ste4 leu1*
C766-5C	h^{90} *ste1-1 leu1 ade6-M216*
C761-5B	h^{90} *ste9-B36 his1-102 ade6-M216 leu1*
C756-1A	h^{90} *ste11-K1 leu1*
C114-2D	h^{90} *mam2-A84 ade6-M216 leu1*
NUL11	h^{90} *ura4-D18 leu1-32*
NUS31	h^{90} *ste3 ura4-294*
KLU1	h^+/h^- *ade6-M216/ade6-M210 leu1-32/leu1-32* ura4-D18/*ura4-D18*
DS43	h^{90} *ste4::ura4*⁺ *ura4-D18 leu1-32*
FS443	h^{90}/h^{90} *ste4/ste4::ura4*⁺ *ura4*⁺*/ura4-D18 leu1*⁺*/leu1-32 ade6*⁺*/ade6-M216*

Table 2. Complementation abilities of mutant *ste4* plasmids.

Plasmids[a]	Complementation(%)[b]
genomic DNA *Pst*I-*Spe*I deletion[c]	14[d]
genomic DNA *Sph*I-*Hind*III deletion[c]	<0.1
genomic DNA *Sph*I frame shift	0.3[e]
genomic DNA *Spe*I-*Sph*I deletion[c]	<0.4
pcD2-#7 cDNA *Spe*I frame shift	0.1[e]
pcMVL-#7 cDNA *Xba*I deletion[f]	<0.1

[a] Plasmids were introduced into C708-5B by co-transfection with *Pst*I-digested pAL7, except for the pcMVL-#7 cDNA with *Xba*I deletion. Fifty−70% of Leu⁺ colonies received the *ste4* plasmid.
[b] The % complementation was calculated by dividing the % of iodine-positive colonies generated by mutant plasmids with the % of iodine-positive colonies generated by the parental wild type plasmid.
[c] In these deletion mutants the downstream coding regions are translationally inframe with the upstream coding regions.
[d] The colonies were very slightly stained. Among the stained colonies, less than 1% of cells formed spores.
[e] Few colonies were heavily stained. These colonies were judged as revertants probably produced by recombination between the host *ste4*−1 sequence and the plasmid leading to formation of a wild type *ste4*⁺ gene.
[f] The sequence 5′ to *Xba*I was deleted from #7 cDNA.

The spliced cDNAs (#1 and #5) were less active than the unspliced cDNAs (#6 and #7)(data not shown). This is due to inhibition of mating by excess $p31^{ste4}$ expressed from the spliced cDNAs rather than the requirement of an intron for efficient expression (see below).

Disruption of *ste4*$^+$

To confirm that the gene we isolated was *ste4*$^+$, we constructed *ste4*$^-$ strains by gene disruption. The *Sal*I-*Sca*I genomic DNA fragment with insertion of the *ura4*$^+$ gene (27) into the *Pst*I site (Fig. 3) was used to transform a homothallic *S. pombe* strain NUL11 (h^{90} *ura4-D18 leu1-32*). Three independent stable Ura$^+$ transformants were obtained. All three were sterile. Southern analysis of one of the transformants, DS43, showed that the *ste4*$^+$ gene was disrupted by the *ura4*$^+$ gene (Fig. 3). While wild type DNA yielded, upon *Eco*RV digestion, a 2.5 kb single band that hybridized to the *ste4*$^+$ probe, DS43 DNA yielded two bands with sizes of 2.2 kb and 2.0 kb, as expected. To further confirm the disruption of the *ste4*$^+$ gene, DS43 was fused with JM104 (h^{90} *ste4 ade6-M216*). Fusants were selected by their ability to grow without supplementation with leucine and adenine. They did not sporulate on nitrogen-poor SSA plates. To prove that the sporulation-deficient fusants were indeed *ste4*$^-$/*ste4*$^-$ diploids, one of the fusants (FS443) was transformed with #7

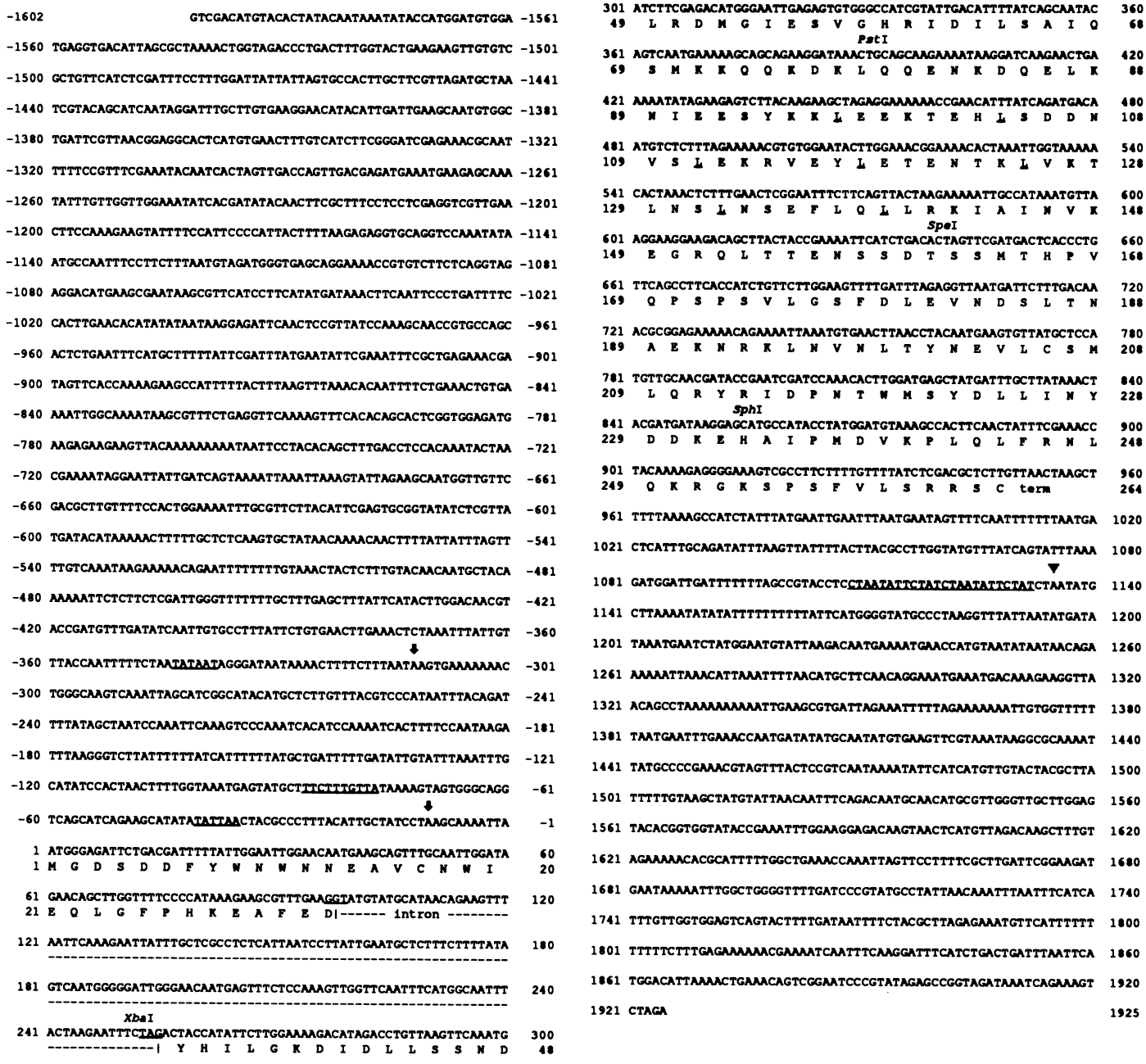

Figure 2. Nucleotide sequence of the *ste4*$^+$ gene. The 3523 nucleotides from the *Sal*I to the downstream *Xba*I sites are presented. The first nucleotides of the cDNAs are indicated by arrows. The polyadenylation site is indicated by a inverted triangle. The deduced amino acid sequence is shown below the nucleotide sequence. A TATA-like sequence, a putative *ste11*$^+$ responsive sequence, splicing junctions, a direct repeat followed by the polyadenylation site, and leucines that appeared every 7 residues are underlined. The restriction sites used for the construction of mutant genes are indicated above the sequence.

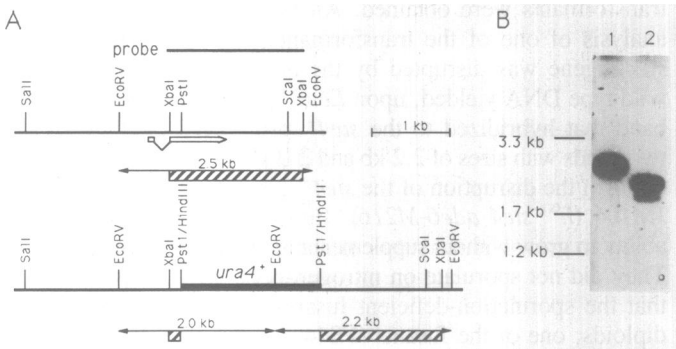

Figure 3. Disruption of the *ste4*$^+$ gene. (A) The restriction maps of wild type (above) and disrupted (below) *ste4*$^+$ genes. The *ura4*$^+$ cassette (shown by a thick line) was inserted into the *Pst*I site after blunt ending. The 1.7 kb *Xba*I fragment used as the probe is shown by a bar above the map. The fragments generated by *Eco*RV digestion are indicated by bidirectional arrows. The regions that hybridized with the probe are indicated with hatched boxes. (B) Autoradiography of Southern hybridization: lane 1, *Eco*RV digestion of DNA of the parental strain NUL11; lane 2, *Eco*RV digestion of DNA of a disruptant strain DS43. The positions of molecular weight markers are indicated on the left side.

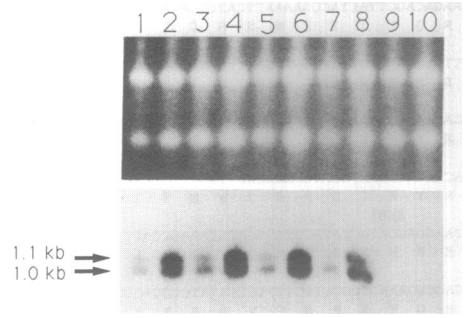

Figure 4. Northern blot analysis of *ste4*$^+$ mRNA. Five μg of total RNA was applied on each lane. Upper, ethidium bromide stained; lower, hybridized with the 0.6 kb *Pst*I-*Hpa*I fragment of *ste4*$^+$ cDNA. Lane 1, growing L975 (h^+); lane 2, nitrogen starved L975; lane 3, growing L972 (h^-); lane 4, nitrogen staved L972. Lane 5, growing L968 (h^{90}); lane 6, nitrogen starved L968; lane 7, growing KLU1 (diploid); lane 8, nitrogen starved KLU1; Lane 9, growing C756-1A (*ste11*$^-$); lane 10, nitrogen-starved C756−1A. The conditions for nitrogen starvation were as described by Egel and Egel-Mitani (31). The bands indicated by arrows are the 1.0 kb and 1.1 kb *ste4*$^+$ mRNAs.

cDNA. The transformants efficiently formed azygotic asci on SSA plates. The sterile phenotype of the disruptant was recessive because fusants between DS43 and NUS31 (*h*^{90} *ste3 ura4-294*) sporulated without conjugation. Based on these results, we concluded that the gene we isolated was indeed the *ste4*$^+$ gene.

Regulation of *ste4*$^+$ expression

Expression of the *ste4*$^+$ gene was investigated by Northern blot analysis (Fig. 4). Two mRNA bands (1.1 kb and 1.0 kb), which markedly increased upon nitrogen starvation, were detected in h^+, h^- and h^{90} haploid cells and in h^+/h^- diploid cells, but not in h^{90} *ste11*$^-$ cells. From this result we conclude that the *ste11*$^+$ gene is required for *ste4*$^+$ expression. The two transcripts correspond to spliced and unspliced mRNAs rather than mRNAs transcribed from the two putative start sites. Two additional bands (1.4 kb and 1.3 kb) were detected after long-exposure and were

Table 3. Inhibition of sporulation by overexpression of *ste4*$^+$ cDNA.

cDNA[a]	sporulation (% ± σn)[b]	
h^{90} [c]	h^+/h^- [d]	
no insert	87 ± 5 (n=10)	71 ± 17 (n=10)
ste4$^+$ #5	16 ± 4 (n=10)	15 ± 5 (n=10)
ste4$^+$ #7 *Xba*I deletion	23 ± 5 (n=4)	14 ± 5 (n=4)

[a] The cDNA was inserted into the pcMVL vector.
[b] Independent colonies were randomly picked up to count the number of sporulated or single cells. More than 200 cells were counted for each sample.
[c] The strain used was C708-5B (genotype, h^{90} *leu1*).
[d] The strain used as the host was KLU1 (genotype, h^+/h^- *ade6-M216/ade6-M210 leu1-32/leu1-32 ura4-D18/ura4-D18*).

Figure 5. Comparison of the leucine zipper region with the jun family. The amino acid sequences of regions with significant homology are aligned. Identical residues are shaded. Aligned are mouse c-jun (amino acid residues number 261 to 325) (32) and mouse jun-B (amino acid residues number 271 to 335) (33). The first 22 residues of c-jun and jun-B correspond to the basic regions and are underlined.

unchanged upon nitrogen starvation (data not shown). These bands are likely to be mRNAs that initiated at the upstream site.

Role of *ste4*$^+$ in sexual differentiation

Normally, *S. pombe* cells grow as haploid even if h^+ and h^- cells are mixed. Only when cells are starved, they mate and subsequently enter meiosis. Thus, mating and meiosis seem to be co-regulated in *S. pombe*. Accordingly, most sterile mutants are also defective in meiosis. Co-regulation is in part carried out through mating pheromone signals, and a mating pheromone signal is required for the initiation of meiosis of diploid cells as well (28). Disruptants of the *gpa1*$^+$ gene encoding the receptor-coupled Gα protein are defective in both mating and meiosis (10). Analogous to this, *ste4*$^+$ might possibly be a mediator of the signal for the mating pheromones, however, this seems unlikely (discussed below).

To obtain a clue to the action point of *ste4*$^+$ in the regulatory cascades, the *ste4*$^+$ gene was overexpressed in *ste1*$^-$, *ste2*$^-$, *ste3*$^-$, *ste5*$^-$, *ste6*$^-$, *ste7*$^-$, *ste9*$^-$, *ste11*$^-$ and *mam2*$^-$ mutants. None of these mutants were rescued by either the genomic DNA or the cDNAs. Rather, overexpression of *ste4*$^+$ cDNA in wild type cells markedly inhibited conjugation and sporulation (Table 2). A similar level of inhibition was also observed with a truncated *ste4*$^+$ gene (Table 3), which had no detectable complementation activity (Table 2). An activated form of *ste5*$^+$/*ras1*$^+$, ras1^{Val-17} is known to inhibit mating because it makes cells hypersensitive to pheromones, and consequently, cells over-react to the pheromones and become too activated to mate (29). Such cells are easily recognizable as highly elongated cells. Unlike ras, overexpression of *ste4*$^+$ cDNA did not cause the elongation of cells, suggesting that the inhibition caused by *ste4*$^+$ overexpression is not due to an over-reaction to pheromones. These data tend to suggest that the *ste4*$^+$ gene is not directly involved in the signal transduction for mating pheromones. Furthermore, the *ste4*$^+$ gene does not appear to be a key mediator of the signals for nitrogen starvation either. Despite the

induction of *ste4*$^+$ expression by nitrogen starvation, overexpression of *ste4*$^+$ did not bypass the requirement of nitrogen starvation for conjugation and meiosis.

One remarkable structural feature of p31^{ste4} is the presence of a leucine zipper motif. This motif region has significant homology with the jun trans-activator family (Fig. 5), but p31^{ste4} lacks a preceding basic region. Perhaps because of this limited similarity, c-*jun*, *jun-B* and *jun-D* cDNAs could not rescue a *ste4*$^-$ mutation (data not shown). Nevertheless, the presence of a leucine zipper motif indicates that p31^{ste4} may form dimers with itself or some other protein. Since the induction of *matPi*$^+$ or *mam2*$^+$ transcription by nitrogen starvation is reduced in *ste4*$^-$ mutants (C. Shimoda, personal communication), p31^{ste4} might function by forming a heterodimer with another protein just like jun and fos (30). In this context it is noteworthy that overexpression of the *ste4*$^+$ gene is inhibitory to mating and meiosis. This inhibitory action might be caused by competition for the targets by inactive p31^{ste4} homodimers formed by overproduction. The result that overexpression of the truncated *ste4*$^+$ gene, which had the leucine zipper motif but no detectable complementational activity (Table 2), similarly inhibited mating and meiosis (Table 3) is also consistent with this hypothesis.

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