# The *ste4*<sup>+</sup> gene, essential for sexual differentiation of *Schizosaccharomyces pombe*, encodes a protein with a leucine zipper motif

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# ABSTRACT

ste4<sup>-</sup> mutants of Schizosaccharomyces pombe are unable to undergo both mating and meiosis. We have cloned the ste4<sup>+</sup> 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<sup>+</sup> gene and increases several fold upon nitrogen starvation, a general signal for sexual differentiation. Whereas ste4<sup>+</sup> 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 Sau3AI-partially-digested DNA of wild type strain L972  $h^-$  into the BamHI-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 *PstI* as described previously (19). Transfected cells were spread at a density of  $1-5\times10^4$  Leu<sup>+</sup> 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<sup>-</sup> 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<sup>-</sup> mutation were recovered from  $5.7 \times 10^4$  and  $2.6 \times 10^6$ Leu<sup>+</sup> 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 XbaI site was absent in #1 and #5 cDNAs (Fig. 1). The 3523 bp SalI-Xbal fragment of the genomic DNA, the entire #7 cDNA and the region from the 5' end to the internal PstI 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<sup>ste4</sup>). 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 PstI and SpeI 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

Table 1. S. pombe strains used in this study

Strain	Relevant genotype
L975	$h^+$
L972	h <sup>-</sup>
L968	h <sup>90</sup>
JM57	h <sup>90</sup> ste2 leul
JM66	h <sup>90</sup> ste3 leu1
JM104	h <sup>90</sup> ste4 ade6-M216
JM70	h <sup>90</sup> ste5 leul
JM75	h <sup>90</sup> ste6 leu1
JM83	h <sup>90</sup> ste7 leu1
C708-5B	h <sup>90</sup> ste4 leu]
C766-5C	h <sup>90</sup> stel-1 leu1 ade6-M216
C761-5B	h <sup>90</sup> ste9-B36 his1-102 ade6-M216 leu1
C756-1A	h <sup>90</sup> stell-Kl leul
C114-2D	h <sup>90</sup> mam2-A84 ade6-M216 leu1
NUL11	h <sup>90</sup> ura4-D18 leu1-32
NUS31	h <sup>90</sup> ste3 ura4-294
KLU1	h <sup>+</sup> /h <sup>-</sup> ade6-M216/ade6-M210 leu1-32/leu1-32
	ura4-D18/ <i>ura4-D18</i>
DS43	h <sup>90</sup> ste4::ura4 <sup>+</sup> ura4-D18 leu1-32
FS443	h <sup>90</sup> /h <sup>90</sup> ste4/ste4::ura4 <sup>+</sup> ura4 <sup>+</sup> /ura4-D18 leu1 <sup>+</sup> /leu1-32 ade6 <sup>+</sup> /ade6-M216

zipper, and frame-shift mutations at SpeI and SphI sites completely inactivated the ste4<sup>+</sup> 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<sup>-</sup> 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 TTC-TTTGTTY for the stell+ responsive element (26). The stell+ 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 stell<sup>+</sup> (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<sup>+</sup> 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 2. Complementation abilities of mutant ste4 plasmids.

Plasmids <sup>a</sup>	Complementation(%) <sup>b</sup>					
genomic DNA PstI-SpeI deletion <sup>c</sup>	14 <sup>d</sup>					
genomic DNA SphI-HindIII deletion <sup>c</sup>	<0.1					
genomic DNA SphI frame shift	0.3 <sup>e</sup>					
genomic DNA SpeI-SphI deletion <sup>c</sup>	<0.4					
pcD2-#7 cDNA Spel frame shift	0.1 <sup>e</sup>					
pcMVL-#7 cDNA XbaI deletion <sup>f</sup>	<0.1					

\* Plasmids were introduced into C708-5B by co-transfection with PstI-digested pAL7, except for the pcMVL-#7 cDNA with XbaI deletion. Fifty-70% of colonies received the ste4 plasmid.

<sup>b</sup> 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.

In these deletion mutants the downstream coding regions are translationally inframe with the upstream coding regions.

<sup>d</sup> 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<sup>+</sup> gene. <sup>†</sup> The sequence 5' to XbaI 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 p31ste4 expressed from the spliced cDNAs rather than the requirement of an intron for efficient expression (see below).

### Disruption of ste4<sup>+</sup>

To confirm that the gene we isolated was  $ste4^+$ , we constructed ste4<sup>-</sup> strains by gene disruption. The SalI-ScaI genomic DNA fragment with insertion of the ura4<sup>+</sup> gene (27) into the PstI site (Fig. 3) was used to transform a homothallic S. pombe strain NUL11 (h<sup>90</sup> ura4-D18 leu1-32). Three independent stable Ura<sup>+</sup>

-1602	GTCGACATGTACACTATACAATAAATATACCATGGATGTGGA	-1561
-1560	TGAGGTGACATTAGCGCTAAAACTGGTAGACCCTGACTTTGGTACTGAAGAAGTTGTGTC	-1501
-1500	<b>GCTGTTCATCTCGATTTCCTTTGGATTATTATTAGTGCCACTTGCTTCGTTAGATGCTAA</b>	-1441
-1440	TCGTACAGCATCAATAGGATTTGCTTGTGAAGGAACATACAT	-1381
-1380	TGATTCGTTAACGGAGGCACTCATGTGAACTTTGTCATCTTCGGGATCGAGAAACGCAAT	-1321
-1320	TTTTCCGTTTCGAAATACAATCACTAGTTGACCAGTTGACGAGATGAAATGAAGAGCAAA	-1261
-1260	TATTTGTTGGTTGGAAATATCACGATATACAACTTCGCTTTCCTCCTCGAGGTCGTTGAA	-1201
-1200	CTTCCAAAGAAGTATTTTCCATTCCCCATTACTTTTAAGAGAGGTGCAGGTCCAAATATA	-1141
-1140	ATGCCAATTTCCTTCTTTAATGTAGATGGGTGAGCAGGAAAACCGTGTCTTCTCAGGTAG	-1081
-1080	AGGACATGAAGCGAATAAGCGTTCATCCTTCATATGATAAACTTCAATTCCCTGATTTTC	-1021
-1020	CACTTGAACACATATATAATAAGGAGATTCAACTCCGTTATCCAAAGCAACCGTGCCAGC	-961
-960	actetgaattteatgettttattegatttatgaatattegaaatttegetgagaaacga	-901
-900	TAGTTCACCAAAAGAAGCCATTTTTACTTTAAGTTTAAACACAATTTTCTGAAACTGTGA	-841
-840	ANATTGGCAAAATAAGCGTTTCTGAGGTTCAAAAGTTTCACACAGCACTCGGTGGAGATG	-781
-780	алдадалдалдттасалалалалататтсстасасадстттдасстссасалатастал	-721
-720	CGAAAATAGGAATTATTGATCAGTAAAATTAAATTAAAGTATTAGAAGCAATGGTTGTTC	-661
-660	GACGCTTGTTTTCCACTGGAAAATTTGCGTTCTTACATTCGAGTGCGGTATATCTCGTTA	-601
-600	TGATACATAAAAAACTTTTTGCTCTCAAGTGCTATAACAAAACAACTTTTATTATTTAGTT	-541
-540	TTGTCAAATAAGAAAAACAGAATTTTTTTTGTAAACTACTCTTTGTACAACAATGCTACA	-481
-480	AAAAATTCTCTTCTCGATTGGGTTTTTTTGCTTTGAGCTTTATTCATACTTGGACAACGT	-421
-420	ACCGATGTTTGATATCAATTGTGCCTTTATTCTGTGAACTTGAAACTCTAAATTTATTGT	-360
-360	ттассаатттттстаа <u>татаат</u> аgggataaтаалаастттстттаатааgggaaaлааас	-301
-300	TGGGCAAGTCAAATTAGCATCGGCATACATGCTCTTGTTTACGTCCCATAATTTACAGAT	-241
-240	TTTATAGCTAATCCAAATTCAAAGTCCCAAATCACATCCAAAATCACTTTTCCAATAAGA	-181
-180	TTTAAGGGTCTTATTTTTATCATTTTTTATGCTGATTTTTGATATTGTATTTAAATTTG	-121
-120	CATATCCACTAACTTTTGGTAAATGAGTATGCT <u>TTCTTTGTTA</u> TAAAAGTAGTGGGCAGG	-61
-60	TCAGCATCAGAAGCATATA <u>TATTAA</u> CTACGCCCTTTACATTGCTATCCTAAGCAAAATTA	-1
1 1	ATGGGAGATTCTGACGATTTTATTGGAATGGAACAATGGAGGGGTTGGAATTGGAA M g d s d d f y w n w n n e a v c n w i	60 20
61	GAACAGCTTGGTTTTCCCCCATAAAGAAGCGTTTGAA <u>GGT</u> ATGTATGCATAACAGAAGTTT	120
21	E Q L G F P H K E A F E D  intron	
121		180
181	GTCAATGGGGGATTGGGAACAATGAGTTTCTCCAAAGTTGGTTCAATTTCATGGCAATTT	240
241	XDAI ACTAAGAATTTC <u>TAG</u> ACTACCATATTCTTGGAAAAGACATAGACCTGTTAAGTTCAAATG 	300 48

## Nucleic Acids Research, Vol. 19, No. 25 7045

transformants were obtained. All three were sterile. Southern analysis of one of the transformants, DS43, showed that the ste4<sup>+</sup> gene was disrupted by the  $ura4^+$  gene (Fig. 3). While wild type DNA yielded, upon EcoRV digestion, a 2.5 kb single band that hybridized to the ste4<sup>+</sup> 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<sup>+</sup> gene, DS43 was fused with JM104 (h<sup>90</sup> 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

301	AT	CTI	rcg	NGA	CAT	GGGJ	AT	rg <b>a</b>	GAG	TGT	GGGG	CA	rcg'	TAT	TGA	AT	TT	ATC.	AGC	NAT/	\C 3	60
49	1	L	R	D	H	G	I	E	5	v	G <b>Ps</b> (	H	R	I	D	I	L	5	A	I	Q	61
361 69	AG	TC/ 5	NTA M	GAN K	K K	Q Q	Q Q	SAA K	GGA:	TAA K	L	Q Q	Q Q	NGA) E	N N	raac K	GA' D	ICA Q	nga E	L	ia 4 K	20
421		•••	CAT/	AGAJ	AGA	STC	гта		-	GCT	NGAC	GN		MC	CGA		TT	NTC.	AGA	rg <b>a</b>	:A 4	
89	1	N	I	Ľ	E	8	Y	ĸ	ĸ	T	E	E	ĸ	T	E	H	T	5	D	D	N 1	.08
481	AT	GTC	TC	TTT/	<b>IGA</b>	~~	CG	GTG	GN	ATA	CTTO	GN	VAC (	GGA		CAC	raa/	ATT	GGT		VA 5	40
109		v	S	T	B	ĸ	R	v	E	Y	T	E	T	E	N	T	ĸ	T	v	K	т 1	.28
541	CM	CT/	~~~	CTC	CTT(	GAA	CTC	GN	\TT:	TCT'	IC AC	STT/	\CT/	NAG	~~~	UAT1	rgc	CAT.	***	FGT	TA 6	00
129	1	6	N	5	T	N	5	E	F	L	Q	T	L	R Spe	K PI	I	x	I	N	v	K 1	.48
601	AG	GAJ	GG	VYC	CA	CT:	TAC!	CACO	:GN	***	TTC	TC	(GA	CAC	rag:	TCO	SAT	GAC	TCA	CCC	rg 6	60
149	1	5	G	R	Q	L	T	T	E	N	S	S	D	т	5	5	M	Т	н	P	V 1	.61
661	TT	CAC	CC.	ITC/	VCCI	ATCI	IGT1	CT	rGG/	AAG!	TTT	GA	TT7	NGA	GGT	'AA'	GA'	TTC	TTT	SACI	<b>A</b> 7	20
169	(	2	P	5	P	S	v	L	G	S	F	D	L	E	v	N	D	S	L	T	N 1	. 8 8
721	AC	GCG	GM	GAN	~~~	CAG!	ŵ	\TT/	NAA!	IGTO	GAAC	:TT/	vice	CTA	CAA1	GA	\GT(	GTT.	ATG	CTC	X 7	80
169		•	E	ĸ	N	R	ĸ	L	N	v	N	L	т	Y	N	E	v	L	с	5	M 2	:04
209	TG:	гтс 1.	с <b>л</b>	ACG/	ATA(	CG/	VATC 7	GA1	1001	MAAG	CACI	TG	AT(	GAG	CTA:	GA	TTC	χT	TAT	~~~~	T 8	40
841	AC	-	¥ 'CA'	-	-	Sphi		• <b>•</b> • • •	F		-	GTI		3					I TCC		· · ·	
229	1	5	D	K	E	H	A		P	M	נאסי	V	K	P	L II	0	L T	F	100		τ. 9	4.
901	TA	2.8.8		IAGO	GGJ		TCG	-	TC		-	• • • • • •	TC	-	-	• • •	-	-	NCT:		 -	60
249	ç	2	ĸ	R	G	ĸ	s	P	s	F	v	L	s	R	R	5	C	te	CM 11		2	64
961	TT	CT?		GCC	ATC	TAT	TTP	TG	ATT	[GA]	\TTI	-	GA	TAC	TT	TC	AT	TTT'	TTT/	ATO	A 10	20
1021	сто	: 1	TTC	CAG	AT.	\TT7	'AAG	TT <b>/</b>	TTI	TAC	TTA	CGC	CT	rGG1	TATO	TTI	TAT	CAG	[AT]		A 10	
1081	GAT	GG	ATI	GAI	TTI	TTT	AGC	CGI	ACC	стс	TA	TAT	TC	TAT		TAT	TC	TAT	▼ CTA	TAT	G 11	40
1141	CTI			TAT.	TAT	TTT	TTT	TTT	TAT	ICA1	GGG	GTA	TGO	cci		GTI	'TA1	TA	ATA:	GAT	A 12	00
1201	TN	NAT	GAJ	TCT	ATC	GAJ	TGT	ATT	'AAG	AC	ATG		ATC	AAC	CAT	GTA	AT,	TA	NT A	CAG	A 12	60
1261	~~	~~	TT		:AT1		TTT	TAR	CAT	GCI	тся	лсл	GCI	~~	GAA	ATG	AC.		-	GTI	A 13	20
1321	AC4	GC	CT/		~~		TTG	AAG	CG1	GAI	TAG		TTI	TT/	GAA		<b>A</b> A1	TG	rGG7	TTI	T 13	80
1381	TA	TG	771	TTG		CCA	ATG	ата	TAT	GC/	АТА	TGT	GN	GTI	CGI	***	TA	GGG	GCJ		T 14	40
1441	TAT	GC	ccc	GAA	ACG	TAG	TTT	ACT	cca	TC	ATA	<b>, , , ,</b>	TAT	TC	TCA	TGT	TGI	ACI	race	CTI	A 15	00
1501	TTI	TT	GTA	AGC	TAT	GTA	TTA	АСА	ATI	TC	GAC	AAT	GC1	ACP	TGC	GTT	GGG	TTO	CTI	GGA	G 15	60
1561	TAC	AC	GGI	GGT	ATA	CCG		TTT	GGA	AGG	AGA	CAA	GT/	ACI	CAT	GTT	AGA	CN	\GC1	TTG	T 16	20
1621	AGA		YYC	ACG	CAT	TTT	TGG	CTG		CCA	AAT	TAG	TTC	CTI	TTC	GCT	TGA	TTC	GGJ	AGA	T 16	80
1681	GAN	TA	ллл 	ATT	TGG	CTG	GGG	TTT	TGA	TCC	CGT	ATG	cci	ATT	AAC	***	TTI	'AA'	TTC	ATC	A 17	40
1.001	TTT	GT	TGG	TGG	AGT		TAC	TTT	TGA		TTT	TCT	ACG	ст1 	AGA	GAA	ATG	TTC	ATI	TTT	T 18	00
1861	TGG	11 	с ГТ атт	IGA	.unn 2017	~~~	ACG	~^^	ATC		TTC	AAG	GAI	TTC	ATC	TGA	CTG	AT1	TAA	TTC	A 18	60 20
1921	CTA	GA		~~~	I	ann		910	- <b></b>	m10		1 VI	nul	w.C	Joel	nuA		ar r c	.nGA	AAG	1 19	20 25

Figure 2. Nucleotide sequence of the ste4<sup>+</sup> gene. The 3523 nucleotides from the Sall to the downstream Xbal 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 stell<sup>+</sup> 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.

1 1 1



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 PstI site after blunt ending. The 1.7 kb XbaI fragment used as the probe is shown by a bar above the map. The fragments generated by EcoRV 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, EcoRV digestion of DNA of the parental strain NUL11; lane 2, EcoRV 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<sup>+</sup> mRNA. Five  $\mu g$  of total RNA was applied on each lane. Upper, ethidium bromide stained; lower, hybridized with the 0.6 kb *PstI-HpaI* fragment of  $ste4^+$  cDNA. Lane 1, growing L975  $(h^+)$ ; lane 2, nitrogen starved L975; lane 3, growing L972  $(h^{-})$ ; lane 4, nitrogen starved 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 (stel1<sup>-</sup>); 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<sup>+</sup> 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<sup>+</sup> expression

Expression of the ste4<sup>+</sup> 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  $h^{90}$  stell<sup>-</sup> cells. From this result we conclude that the stell<sup>+</sup> 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<sup>+</sup> cDNA.

cDNA <sup>a</sup>	sporulation $(\% \pm \sigma n)^b$						
h <sup>90 c</sup>	$h^+/h^-$ d						
no insert ste4 <sup>+</sup> #5 ste4 <sup>+</sup> #7 XbaI deletion	$\begin{array}{r} 87 \pm 5 \ (n=10) \\ 16 \pm 4 \ (n=10) \\ 23 \pm 5 \ (n=4) \end{array}$	$71 \pm 17 (n=10) 15 \pm 5 (n=10) 14 \pm 5 (n=4)$					

<sup>a</sup> The cDNA was inserted into the pcMVL vector.

<sup>b</sup> Independent colonies were randomly picked up to count the number of sporulated or single cells. More than 200 cells were counted for each sample. <sup>c</sup> The strain used was C708-5B (genotype,  $h^{90}$  leul). <sup>d</sup> The strain used as the host was KLU1 (genotype,  $h^{+}/h^{-}$ 

ade6-M216/ade6-M210 leu1-32/leu1-32 ura4-D18/ura4-D18).

Ste4	89	9 NIEESYKKLEEKTEHLSDDNVSEKREY ET ETKVKLESINSEFLELRIAI	NER TT 1	55
c-jun	261	KRMRNRIAASKCRKRKLERIAR EK KTEKAQ SE AS A M REQVA KO VMN	HINSICHML 32	27
jun-B	271	KRLENRLAATKCRKRKLERIAR CON KTLKA AG SSAAGL REQVAL KQ VMT	HISNICHIL 3:	37

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<sup>+</sup> 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  $gpal^+$  gene encoding the receptorcoupled G $\alpha$  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<sup>+</sup> gene was overexpressed in ste1<sup>-</sup>, ste2<sup>-</sup>, ste3<sup>-</sup>, ste5<sup>-</sup>, ste6<sup>-</sup>, ste7<sup>-</sup>, ste9<sup>-</sup>, ste11<sup>-</sup> and mam2<sup>-</sup> 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<sup>+</sup> gene (Table 3), which had no detectable complementation activity (Table 2). An activated form of ste5+/ras $\tilde{l}^+$ , ras1<sup>Val-17</sup> 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<sup>+</sup> 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 p31ste4 is the presence of a leucine zipper motif. This motif region has significant homology with the jun trans-activator family (Fig. 5), but p31ste4 lacks a preceding basic region. Perhaps because of this limited similarity, c-jun, jun-B and jun-D cDNAs could not rescue a ste4mutation (data not shown). Nevertheless, the presence of a leucine zipper motif indicates that p31ste4 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), p31ste4 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<sup>+</sup> gene is inhibitory to mating and meiosis. This inhibitory action might be caused by competition for the targets by inactive p31<sup>ste4</sup> 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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