Mol Gen Genet (1994) 244:456–464

ORIGINAL PAPER

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The *ste13*$^+$ gene encoding a putative RNA helicase is essential for nitrogen starvation-induced G1 arrest and initiation of sexual development in the fission yeast *Schizosaccharomyces pombe*

Received: 25 November 1993 / Accepted: 10 February 1994

Abstract When the fission yeast *Schizosaccharomyces pombe* is starved for nitrogen, the cells are arrested in the G1 phase, enter the G0 phase and initiate sexual development. The *ste13* mutant, however, fails to undergo a G1 arrest when starved for nitrogen and since this mutant phenotype is not suppressed by a mutation in adenylyl cyclase (*cyr1*), it would appear that *ste13*$^+$ either acts independently of the decrease in the cellular cAMP level induced by starvation for nitrogen, or functions downstream of this controlling event. We have used functional complementation to clone the *ste13*$^+$ gene from an *S. pombe* genomic library and show that its disruption is not lethal, indicating that, while the gene is required for sexual development, it is not essential for cell growth. Nucleotide sequencing predicts that *ste13*$^+$ should encode a protein of 485 amino acids in which the consensus motifs of ATP-dependent RNA helicases of the DEAD box family are completely conserved. Point mutations introduced into these consensus motifs abolished the *ste13*$^+$ functions. The predicted Ste13 protein is 72% identical to the *Drosophila melanogaster* Me31B protein over a stretch of 391 amino acids. ME31B is a developmentally regulated gene that is expressed preferentially in the female germline and may be required for oogenesis. Expression of ME31B cDNA in *S. pombe* suppresses the *ste13* mutation. These two evolutionarily conserved genes encoding putative RNA helicases may play a pivotal role in sexual development.

Key words Mating · G1 arrest · RNA helicase · DEAD box protein · *Schizosaccharomyces pombe*

Introduction

The mechanism by which a eukaryotic cell exits the resting G0 phase and re-enters the mitotic cell cycle in response to a variety of stimuli has been well investigated. In contrast, the change from a growth phase to a resting phase is poorly understood, even though this transition is a prerequisite for cell differentiation.

The fission yeast *Schizosaccharomyces pombe* provides a suitable system for studying this transition, since nitrogen starvation of mitotic cells causes a G1 arrest and entry into a resting G0 phase (Costello et al. 1986; Kitamura et al. 1990). *S. pombe* exists in two mating types, h^+ (Plus) and h^- (Minus) (Gutz et al. 1974; Egel 1989), and when haploid cells of opposite type are shifted to nitrogen-free medium they conjugate to form a diploid zygote, which then undergoes meiosis and sporulation (Egel 1971, 1989). Genetic analysis has defined 13 sterility (*ste*) genes (Thuriaux et al. 1980; Girgsdies 1982; Michael and Gutz 1987; Kitamura et al. 1990; Leupold and Sipiczki 1991), most of which are required for meiosis (Girgsdies 1982; Kitamura et al. 1990). The *ste12* and *ste13* mutations seem to affect the transition from growth to a resting phase (Kitamura et al. 1990). Molecular analysis of *ste12*$^+$ and *ste13*$^+$ may therefore provide clues as to the mechanism of this transition.

In this study we report the structure and function of *ste13*$^+$, which appears to encode an ATP-dependent RNA helicase of the DEAD box family. Furthermore, we demonstrate that Me31B, a germline-specific *Drosophila* member of the DEAD box family, is structurally and functionally homologous to Ste13.

Communicated by K. Isono

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Materials and methods

Yeast strains and media

Yeast strains used in this study are listed in Table 1. Culture media used were YEA (yeast extract agar) for growth and MEA (malt extract agar) for mating and sporulation (Moreno et al. 1991). Defined minimal media, PM an SSA, and their nitrogen-free versions were also used (Castello et al. 1986; Gutz et al. 1974). Mating and sporulation were induced in liquid medium, SSL-N (Egel and Egel-Mitani 1974).

Flow cytometry

Flow cytometric analysis for cellular DNA content was performed according to the method of Costello et al. (1986). Cells were fixed in 70% ethanol, treated with RNase A and then stained with propidium iodide. Flow cytometry was carried out on a Becton Dickinson FACScan.

DNA sequencing

The 3.0 kb *Bam*Hl-*Pvu*ll fragment containing *ste13*$^+$ was recloned into the pUC119 and pBluescript vectors. A series of nested deletions was produced by exonuclease III and S1 nuclease (Henikoff 1984). Nucleotide sequencing was performed by dideoxy-chain termination (Sanger et al. 1977; Yanisch-Perron et al. 1985). Homology search was carried out for proteins deposited in the SwissProt and NBRF databases by Genetyx-CD software (Software Development, Tokyo).

Gene disruption

Two disrupted alleles of *ste13* were produced by the one-step gene disruption method according to Rothstein (1983). The 0.3 kb *Eco*RV fragment was removed from the coding region, and replaced by a *Saccharomyces cerevisiae LEU2* cassette (*ste13*::*LEU2*). A diploid strain, C509, was transformed with a *Hin*dIII fragment of this disrupted *ste13* allele, stable Leu$^+$ transformants were sporulated and the tetrads were dissected and analyzed. A set of segregants in one ascus were subjected to Southern blotting for confirmation. To obtain a second disruptant, the 1.7 kb *Hin*dIII fragment was replaced by an *S. pombe ura4*$^+$ cassette (*ste13*::*ura4*$^+$). A diploid strain, C525, was transformed with a *Bam*HI-*Pvu*II fragment. Stable Ura$^+$ transformants were analyzed as mentioned above.

Southern and Northern analysis

Genomic DNA was prepared from a wild-type strain of *S. pombe* by the method of Hereford et al. (1979). Restriction fragments were electrophoresed on a 0.8% agarose gel and transferred onto nylon membranes (Biodyne A, Pall). For Northern analysis, RNA was prepared from *S. pombe* cells by the method of Jensen et al. (1983). Poly(A)$^+$RNA was enriched by oligo (dT) cellulose chromatography and fractionated on a 1% agarose gel as previously reported (Kitamura and Shimoda 1991). A. 1.7 kb *Hin*dIII fragment containing *ste13*$^+$ was labeled with [α-^{32}P]dCTP and used as a hybridization probe. Hybridization was performed in 50% formamide at 42° C (Thomas 1980).

Site-directed mutagenesis

Oligonucleotide-directed mutagenesis of the DEAD and HRIGR regions was performed according to Sambrook et al. (1989). Oligonucleotides used were 5′-TTGTTATGGATCCAGCCGATAAAT-3′ for DPAD and 5′-CACGGCATCGGTAGATCTGG-3′ for HGIGR.

cDNA analysis

The 5′ region of the *ste13*$^+$ cDNA was amplified by the polymerase chain reaction (PCR) using DNA from an *S. pombe* cDNA library constructed in the pcD2 expression vector (Okazaki et al. 1991) The PCR product was cloned in pBluescript and then sequenced.

Plasmid construction

Two *S. pombe-Escherichia coli* shuttle vectors, pDB248′ and pAU-SK, were used (Beach and Nurse 1981; K. Tanaka, personal communication). The selectable markers in *S. pombe* are *LEU2*

Table 1 *Schizosaccharomyces pombe* strains. C509, CD16-1, C525 and HM316 are diploid strains and other strains are haploid

Strain	Genotype	Source
L968	h^{90}	U. Leupold
L972	h$^-$	U. Leupold
JZ298	h$^+$ *cyr1*::*ura4*$^+$ *ade6-M216 leu1 ura4-D18*	T. Maeda
FM103-5C	h$^+$ *ste13*::*ura4*$^+$ *ade6-M216 leu1 ura4-D18*	This study
HM513-4C	h$^+$ *ste13*::*LEU2 cyr1*::*ura4*$^+$ *ade6-M216 leu1 ura4*	This study
HM315-1A	h^{90} *ste13*::*ura4*$^+$ *leu1 ura4-D18 ade6-M210*	This study
HM315-2C	h^{90} *ste13*::*ura4*$^+$ *leu1 ura4-D18 ade6-M216*	This study
C522-4B	h$^-$ *ste13*::*LEU2 ura4 his2 leu1*	This study
C458-4C	h$^-$ *ste13-N50 leu1 ade6 ura1 his2*	K. Kitamura
C451-1A	h^{90} *ste13-N50 leu1*	K. Kitamura
C509	h$^+$ *ura4-D18 leu1 ade6-M216* +	C. Schimoda
	h$^-$ *ura4-D18 leu1* + *his2*	
C525	h^{90} *ura4-D18 leu1 ade6-M210*	This study
	h^{90} *ura4-D18 leu1 ade6-M216*	
CD16-1	h$^+$ *ade6-M210 cyh1* +	C. Shimoda
	h$^-$ *ade6-M216* + *lys5-391*	
HM316	h^{90} *ste13*::*ura4*$^+$ *leu1 ura4-D18 ade6-M216*	This study
	h^{90} *ste13*::*ura4*$^+$ *leu1 ura4-D18 ade6-M210*	

(PDB248') and *URA3* (pAU-SK). pREP1 and pREP2 are *S. pombe* expression vectors with a thiamine-repressible *nmt* promoter (Tommasino and Maundrell 1991). The cDNA (1.5 kb) containing the entire ME31B open reading frame (ORF) and the 1.7 kb *Hin*dIII fragment containing *ste13*$^+$ were inserted into *Sal*I/*Bam*HI sites of the pREP1 or pREP2 plasmids downstream of the *S. pombe nmt* promoter.

Results

Ste13$^+$ is required for G1 arrest

The *ste13* mutant can neither enter the G0 phase of the cell cycle nor initiate sexual reproduction (Kitamura et al. 1990). To examine whether such mutants are arrested in G1 following nitrogen starvation, flow cytometry was used to measure the DNA content of wild-type cells and cells harboring an ochre nonsense mutation (*ste13-N50*) (Fig. 1). Cells were cultured to mid-log phase (5×10^6 cells/ml) in PM minimal medium, transferred to nitrogen-free PM medium (PM-N) and cultured at 26° C for a further 48 h. *S. pombe* has a relatively long G2 phase and so the majority of logarithmically growing cells have a DNA content of 2C. Following transfer to PM-N, about 60% of the wild-type cells shifted to a 1C peak but no discrete peak of 1C DNA content was detected in the *ste13* culture. This indicates that *ste13* is essential for the G1 arrest of *S. pombe* cells induced by nitrogen starvation.

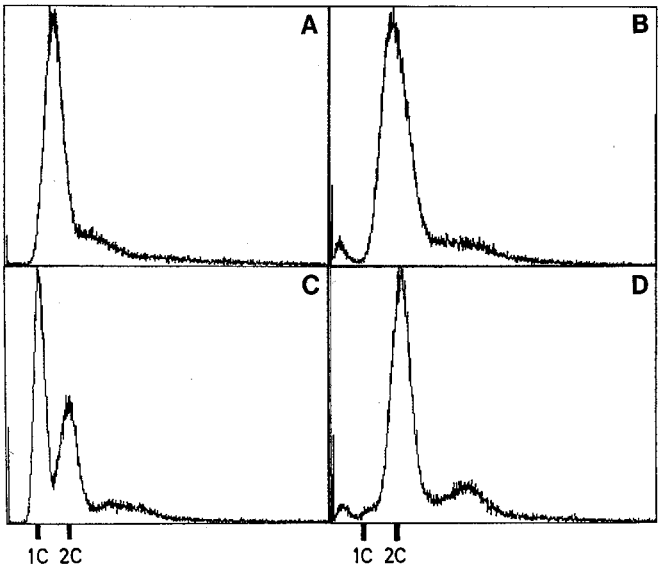

Fig. 1A–D Flow-cytometric analysis of the distribution of DNA content per cell. **A** and **B** log phase cells in minimal medium (PM); **C** and **D** stationary phase cells cultured in the absence of a nitrogen source (PM-N). **A** and **C** L972 (h$^-$ wild type); **B** and **D** C458-4C (h$^-$ *ste13-N50*) The 1C and 2C DNA contents represent those of a single haploid nucleus in G1 and G2 phase, respectively

Cloning of the *ste13*$^+$ gene

A genomic library constructed in the multicopy plasmid pDB248' was introduced into C451-1A (h^{90} *ste13-N50 leu1*) and plasmids able to produce Ste$^+$ transformants were rescued in the *E. coli* strain HB101. Subcloning localized the complementing activity to a 3.0 kb *Bam*HI-*Pvu*II fragment (pTN4) (Fig. 2), which was shown to contain the *ste13*$^+$ gene by chromosomal integration mapping (data not shown). Southern blot analysis of genomic DNA from a wild-type strain showed that *ste13*$^+$ is a single-copy gene (Fig. 3). Northern blot analysis showed that *ste13*$^+$ is constitutively transcribed as a 2.1 kb mRNA, although transcription may be slightly enhanced in nitrogen-free medium (Fig. 4).

Nucleotide sequencing of the *ste13*$^+$gene

The nucleotide sequence of the 3.0 kb *Bam*HI-*Pvu*II fragment (pTN4, see Fig. 2) containing *ste13*$^+$ was determined and indicated the possibility of two introns in the amino-terminal region of the gene. To investigate this, PCR was used to isolate the corresponding region

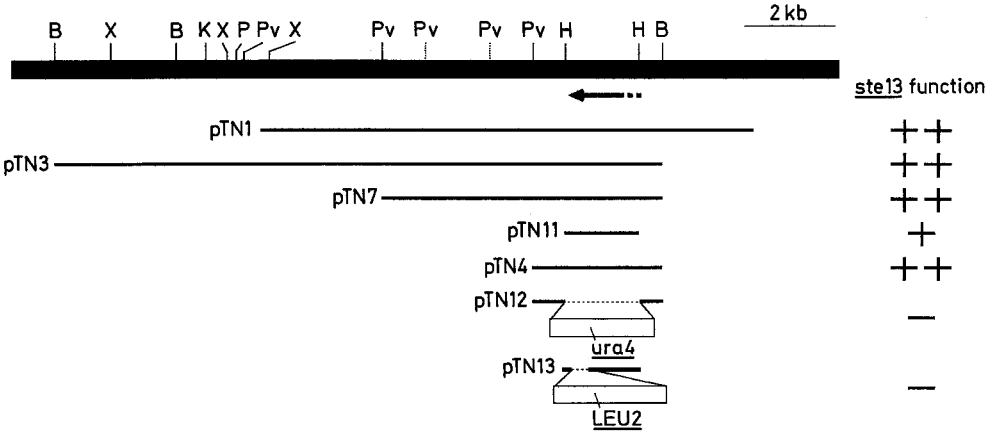

Fig. 2 Restriction map of the cloned *Schizosaccharomyces pombe* genomic DNA and subcloning and gene disruption of *ste13*$^+$. (Restriction enzymes: *B Bam*HI; *P Pst*I; *X Xba*I; *Pv Pvu*II; *H Hin*dIII; *K Kpn*I.) A *leftward arrow* indicates the *ste13*$^+$ open reading frame (ORF). Complementation of the *ste13-N50* ochre nonsense mutation: ++, completely complements; +, partially complements; –, does not complement

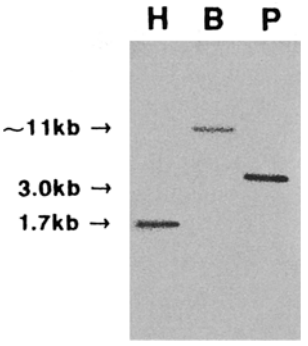

Fig. 3 Southern analysis of genomic DNA. Genomic DNA prepared from a wild-type strain, L968, was digested with *Hin*dIII (*H*), *Bam*HI (*B*) and *Pvu*II (*P*). Hybridization was performed under stringent conditions for 24 h. The 1.7 kb *Hin*dIII fragment containing *ste13*$^+$ was used as a probe

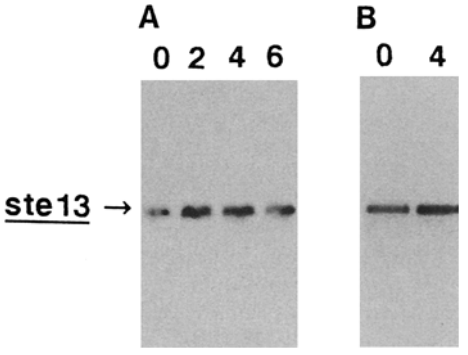

Fig. 4A, B Northern analysis of *ste13*mRNA. Poly(A)$^+$ RNA was prepared from a heterothallic h$^-$ haploid strain, L972 (**A**), and a heterothallic h$^+$/h$^-$ diploid strain, CD16-1 **B**. Mid-log phase cultures in minimal medium were inoculated into nitrogen-free sporulation medium, SSL-N, and incubated with shaking at 26° C. Hours after the shift to sporulation medium are shown over each lane. The size of the *ste13*$^+$mRNA was estimated to be 2.1 kb using 18S and 25S rRNA as size markers

from an *S. pombe* cDNA library, which was then cloned and sequenced. Comparison of the genomic and cDNA sequences confirms that the *ste13*$^+$ gene contains two introns of 46 and 51 nucleotides. The nucleotide sequence is shown in Fig. 5 and defines a single ORF with the potential coding capacity for a protein of 52 kDa, composed of 485 amino acids.

Phenotype of the *ste13* null mutation

The mutant *ste13-N50* exhibits normal growth behavior in rich medium (Kitamura et al. 1990) and nucleotide sequence analysis of this mutant allele revealed a TAA ochre nonsense mutation in the CAA codon encoding glutamine at position 130 (see Fig. 5). Such a mutation would generate a truncated protein that would be unlikely to retain full activity and so the apparently normal growth phenotype of the mutant would indicate that Ste13 is not essential for cell proliferation. Since *ste13*$^+$ is a single-copy gene (Fig. 3), we were able to test this suggestion directly by constructing *ste13*$^+$ null mutants. Two different disrupted constructs of *ste13*$^+$ (see Fig. 2) were introduced into the chromosomal *ste13*$^+$ locus and, while both strains grew

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-511                                                    GGATCCATCTTGGGAAGATGAACTTTCAT
-482 GCTCTCTTTTCAAACCATTCATGACCTCAAATTAAAGGTTCTAAATCGTAGGTCGCAAGTTTTTTAAACAGT
-410 CGAGTGTAAAGTTCAAGTTCGAGCTCCAAAGGTGAAGAACTTCCGAAGATATAGAAAATCGCATAAAATTAA
-338 TATAATACTACCTTTTAGAAAATTTTGAATTAATTTCAATAATTATTAATGTATTTTCGATCCACATATTTT
-266 CAAGTATTGAGTAACCTTGCTAAACCACTAAACCTATCCAACACCAACAACGGCTTTTTTTCGAAATCACCC
-194 ACAAAGGGGTTTGTTTCAGGCTAAACACGACGATTCTTGTTTATTTTGTAGAAAATAATACGTTCCATTTTG
-122 AACCTTTGTTACATATATTTACGACTAGCAAATTCCTTAAGAATTAAAAGGAAACTAGAAACCATTTACAAA
 -50 ATTATTTTAAAGTTACCCAGTCTTTCATTATTGATTAAGATTAAAACAACATGGCTGAAAGCTTGATTCAAA
   1                                                              M  A  E  S  L  I  Q  K
  23 AGTTAGAAAACGCTAATTTGAATGACCGAGAATCCTTTAAAGGACAAATGAAGGCTCAGCCTGTAGACATGC
   9  L  E  N  A  N  L  N  D  R  E  S  F  K  G  Q  M  K  A  Q  P  V  D  M  R
  95 GGCCGAAAACAGAGgtgagtgactaagtaattagaattggcgttgattaacaattaccagGATGTTACGAAA
  33  P  K  T  E  ---------- intron I ----------D  V  T  K
 167 ACCAGAGGAACAGAGTTCGAAGATTATTATTTGAAACGgtaagatggtttctttcttatgaaatttgtatat
  41 T  R  G  T  E  F  E  D  Y  Y  L  K  R---------- intron II ------
 239 tgacacatggtttacagCGAACTTTTGATGGGCATCTTTGAGGCGGGCTTCGAGCGTCCATCTCCAATTCAA
  54 ----------E  L  L  M  G  I  F  E  A  G  F  E  R  P  S  P  I  Q
 311 GAAGAATCTATCCCTATTGCTCTTAGCGGCCGTGATATTTTAGCTAGAGCTAAAAACGGTACTGGTAAAACA
  72 E  E  S  I  P  I  A  L  S  G  R  D  I  L  A  R  A  K  N  G  T  G  K  T
 383 GCCGCCTTTGTAATTCCCTCCCTTGAAAAAGTTGATACTAAGAAGAGTAAAATTCAAACATTAATTCTCGTC
  96 A  A  F  V  I  P  S  L  E  K  V  D  T  K  K  S  K  I  Q  T  L  I  L  V
 455 CCCACTCGTGAACTTGCTCTTCAGACATCTCAAGTCTGTAAGACTTTAGGAAAACATATGAATGTCAAAGTT
 120 P  T  R  E  L  A  L  Q  T  S  Q  V  C  K  T  L  G  K  H  M  N  V  K  V
 527 ATGGTCACAACTGGTGGTACAACTTTAAGAGATGATATTATCCGTTTAAACGATACTGTCCATATTGTGGTT
 144 M  V  T  T  G  G  T  T  L  R  D  D  I  I  R  L  N  D  T  V  H  I  V  V
 599 GGTACTCCTGGTCGTGTCCTTGACCTTGCTGGTAAAGGCGTTGCTGATTTCAGTGAATGTACTACTTTTGTT
 168 G  T  P  G  R  V  L  D  L  A  G  K  G  V  A  D  F  S  E  C  T  T  F  V
 671 ATGGATGAAGCCGATAAATTATTAAGTCCGGAATTTACTCCCATCATTGAACAACTTCTTTCCTATTTTCCT
 192 M  D  E  A  D  K  L  L  S  P  E  F  T  P  I  I  E  Q  L  L  S  Y  F  P
 743 AAAAATCGACAAATCTCTTTGTATAGTGCTACCTTTCCGTTAATTGTTAAAAACTTTATGGATAAACATCTT
 216 K  N  R  Q  I  S  L  Y  S  A  T  F  P  L  I  V  K  N  F  M  D  K  H  L
 815 AATAAACCTTATGAAATTAACTTGATGGACGAGCTTACCCTTCGTGGTGTTACTCAATATTATGCTTTCGTT
 240 N  K  P  Y  E  I  N  L  M  D  E  L  T  L  R  G  V  T  Q  Y  Y  A  F  V
 887 GACGAGAGCCAGAAGGTGCATTGTCTTAACACCTTGTTTTCAAAACTTCAGATCAACCAGTCGATCATATTC
 264 D  E  S  Q  K  V  H  C  L  N  T  L  F  S  K  L  Q  I  N  Q  S  I  I  F
 959 TGTAACTCCACTAACCGTGTCGAGTTACTTGCCAAAAAAATTACTGAACTTGGATATTCATGCTTTTATTCT
 288 C  N  S  T  N  R  V  E  L  L  A  K  K  I  T  E  L  G  Y  S  C  F  Y  S
1031 CATGCTAAAATGTTACAGTCTCATAGAAACCGTGTTTTTCATAATTTTAGAAATGGCGTTTGCCGCAATTTG
 312 H  A  K  M  L  Q  S  H  R  N  R  V  F  H  N  F  R  N  G  V  C  R  N  L
1103 GTTTGTTCTGATCTGTTGACTCGTGGTATTGATATCCAAGCTGTAAATGTTGTCATCAATTTTGATTTTCCG
 336 V  C  S  D  L  L  T  R  G  I  D  I  Q  A  V  N  V  V  I  N  F  D  F  P
1175 AAGAACGCCGAAACTTATTTGCACCGCATCGGTCGTTCTGGTCGATTTGGTCATCGTGGTTTGGCTATTAGT
 360 K  N  A  E  T  Y  L  H  R  I  G  R  S  G  R  F  G  H  R  G  L  A  I  S
1247 TTCATCTCTTGGGCTGACAGATTTAACCTTTATCGAATTGAAAATGAACTTGGCACTGAAATTCAACCAATT
 384 F  I  S  W  A  D  R  F  N  L  Y  R  I  E  N  E  L  G  T  E  I  Q  P  I
1319 CCCCCTAGTATTGATCCCTCCCTGTATGTTTTTCCTAATGGTGATTATCAAATCCCTCGACCCTTGACTGCA
 408 P  P  S  I  D  P  S  L  Y  V  F  P  N  G  D  Y  Q  I  P  R  P  L  T  A
1391 TCTGCCGATCAAGTATTGGCTGCTCAACAAGCCAAAGGACAGGAGGGATATCATAATCGTCCGAACAATAAT
 432 S  A  D  Q  V  L  A  A  Q  Q  A  K  G  Q  E  G  Y  H  N  R  P  N  N  N
1463 CGTGGTGGTCATCCTCGTGGAGGTGGAAATCGTGGTGGTTATCGTCAGTCCAATCGACAACCCAGATACCGT
 456 R  G  G  H  P  R  G  G  G  N  R  G  G  Y  R  Q  S  N  R  Q  P  R  Y  R
1535 GGTCAACAAAAAGCAGACTAATGTATTAAATATGTCGGTAAAGCCTCGTTGATAGTTTAGAGTCCTGGACTT
 480 G  Q  Q  K  A  D
1607 TTCATCATTTTTTTTTTGAAAGTGAATTATGTGGTAATTGATTCTCACACAATTTGTTTATACTGGACAAGG
1679 ACTGATTTACTATTTCCAAGCTT
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Fig. 5 Nucleotide sequence of the 2212 bp *Bam*HI-*Hin*dIII fragment and the amino acid sequence of the putative *ste13*$^+$ ORF. Amino acids are displayed in the one-letter notation. *Lowercase letters* represent the intron sequence. Exact exon/intron junctions were determined from the nucleotide sequence of the *ste13*$^+$ cDNA. The nucleotide sequence has been deposited in the GSDB/DDBJ/EMBL/NCBI databases under the accession number D29795

normally in rich medium, they failed to enter G0 phase and to undergo mating (Fig. 7A). A diploid strain, HM316, homozygous for the *ste13* null allele was unable to initiate meiosis (data not shown). It would thus appear that *ste13*$^+$ is not required for growth but is essential for the appropriate response to nitrogen starvation.

Ste13 acts downstream or independently of a cyclic AMP pathway

Nitrogen starvation of *S. pombe* causes a reduction in the cytoplasmic concentration of cyclic adenosine-3′,5′-monophosphate (cAMP), and a mutation in the *cyr1*$^+$ gene, which encodes adenylyl cyclase, induces both

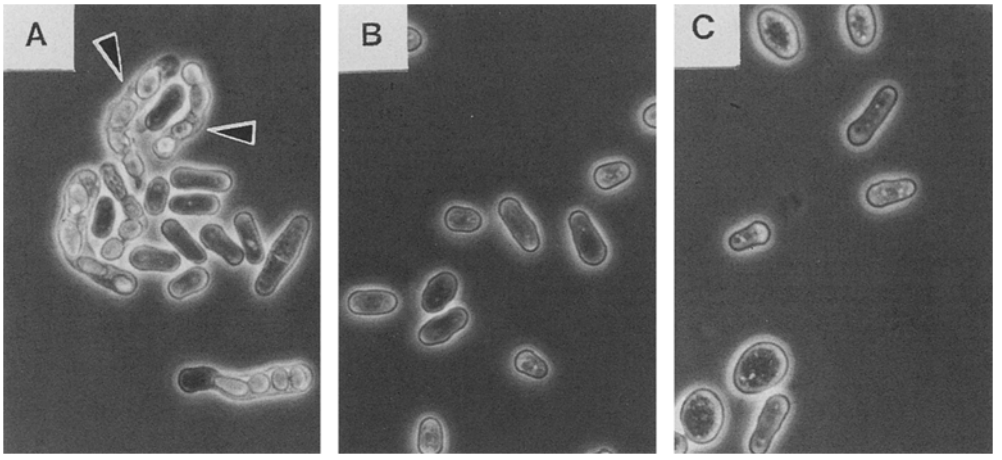

Fig. 6A–C Phenotype of a *cyr1 ste13* double mutant. **A** JZ298 (h^+ *cyr1::ura4*$^+$); **B** FM103-5C (h^+ *ste13::ura4*$^+$); **C** HM513-4C (h^+ *cry1::ura4*$^+$ *ste13::LEU2*). Each mutant strain was mixed with L972 (h^-) and incubated on MEA sporulation medium for 2 days at 28° C. *Arrowheads* indicate some typical zygotic asci

mating and meiosis in growth medium (Maeda et al. 1990). Since mutations in the *cgs1*$^+$ and *cgs2*$^+$ (*pde1*$^+$) genes, which encode a regulatory subunit of cAMP-dependent protein kinase and cAMP phosphodiesterase, respectively, exhibit phenotypes which resemble that of the *ste13* mutant (DeVoti et al. 1991), it is possible that *ste13*$^+$ is also involved in lowering the cellular cAMP concentration. However, reducing the cAMP level using a *cyr1* mutation failed to suppress an *ste13* mutation (Fig. 6), indicating that the function of *ste13*$^+$ in arresting cell growth in response to nitrogen starvation is either down-stream of cAMP or is independent of the cAMP pathway.

Ste13$^+$ may encode an RNA helicase

Comparison of the putative Ste13 protein with known proteins demonstrates a significant homology with the ATP-dependent RNA helicases known as the "DEAD box family" proteins (Fig. 8) (Linder et al. 1989). Each of the eight consensus motifs normally found in members of this family, including the ATPase A and B motifs and the RNA-unwinding motif, are conserved in the Ste13 protein. In addition to these DEAD box consensus motifs, we found three repeats of "RGG", which was supposed to be an RNA-binding motif (Kiledjian and Dreyfuss 1992), in the carboxy terminal region of Ste13. ATPase and RNA helicase activities have been demonstrated in some of the DEAD box proteins (Iggo and Lane 1989; Rozen et al. 1990; Pause and Sonnenberg 1992) and we sought to investigate these functions in Ste13 by introducing appropriate point mutations into the *ste13*$^+$ gene using oilgonucleotide-directd in vitro mutagenesis. The regions subjected to mutation were the DEAD motif, which is believed to couple the ATPase and RNA helicase activities, and the HRIGR motif which interacts with the RNA (Linder et al. 1989): we converted DEAD to DPAD and HRIGR to HGIGR. The mutant alleles were introduced into an *ste13* disruptant strain using

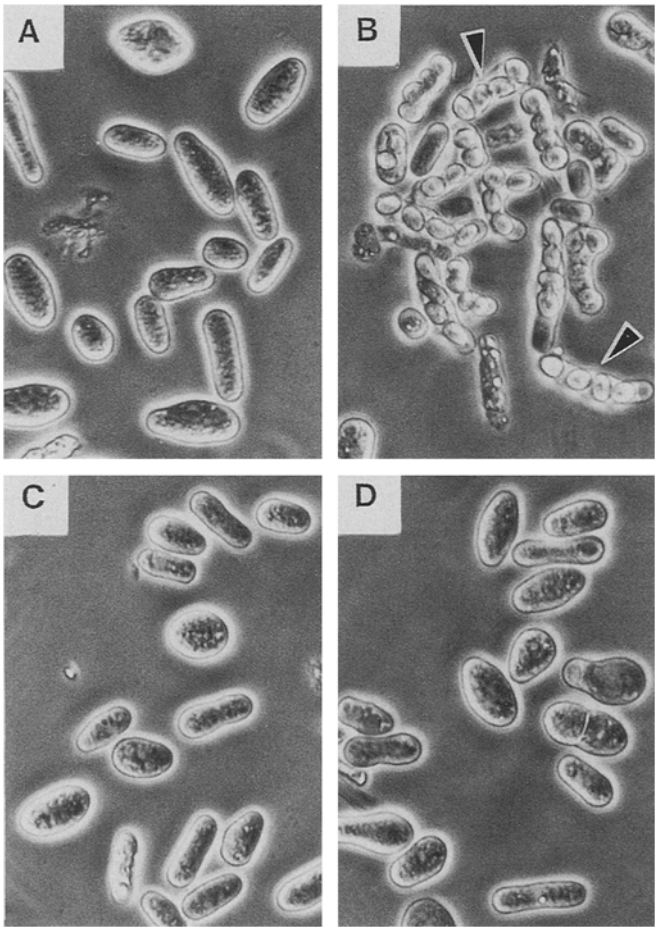

Fig. 7A–D Phenotypes of in vitro generated *ste13* mutants. A host strain HM315-1A (h^{90} *ste13::ura4*$^+$ *ura4-D18 leu1 ade6-M216*) was transformed by plasmids carrying *ste13*. **A** pAL-SK, a vector plasmid having no insertion. **B** pTN8, a 2.3 kb *Bam*HI-*Hin*dIII fragment containing *ste13*$^+$. **C** pAL (*ste13-EP*), the same fragment with a missense mutation (glutamic acid to proline at 194). **D** pAL (*ste13-RG*), the same fragment with a missense mutation (arginine to glycine at 368). Cells were cultured on SSA sporulation medium at 28° C for 2 days. *Arrowheads* indicate some typical asci

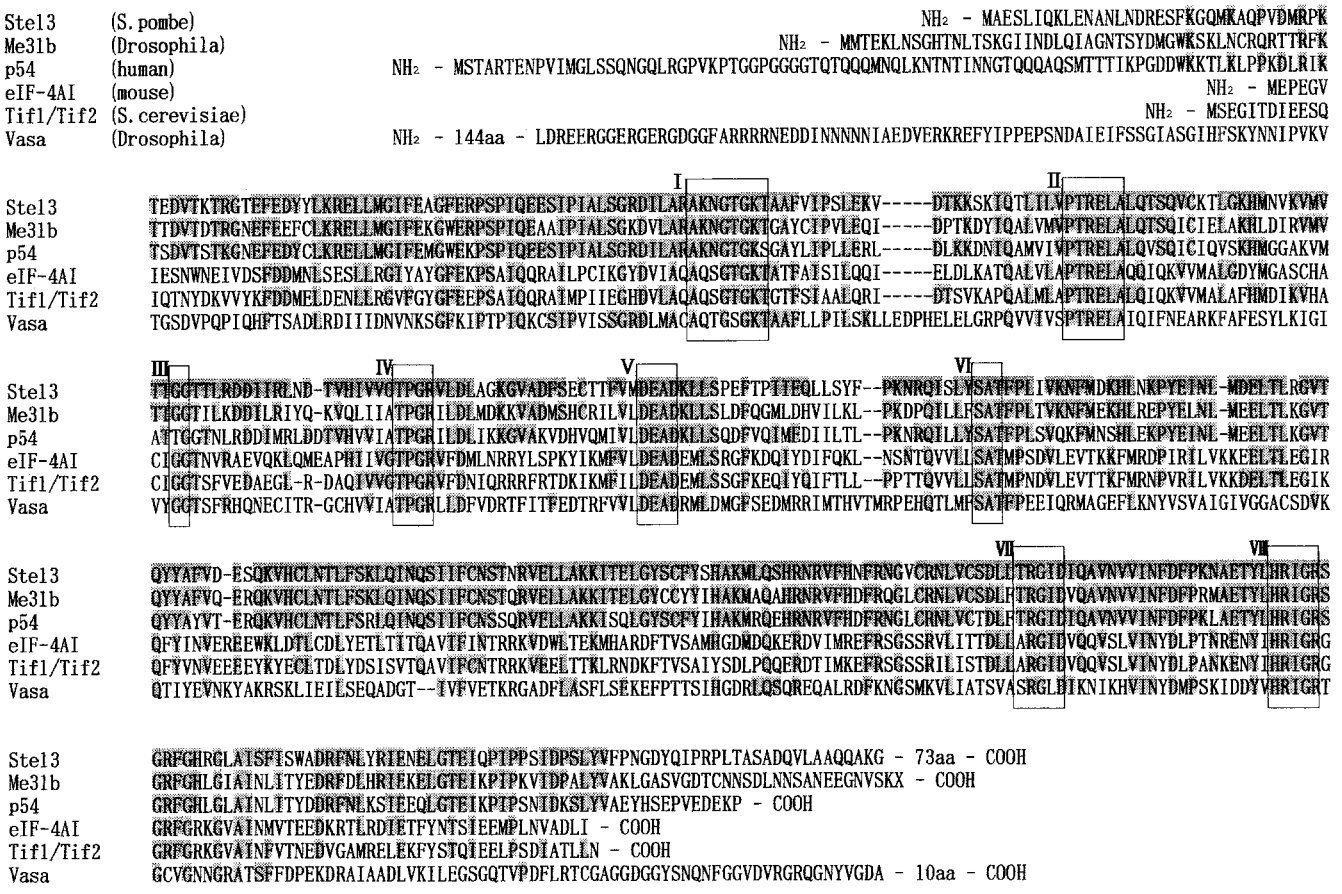

Fig. 8 Amino acid alignment of Ste13 with several DEAD proteins. DEAD proteins: Ste13 fission yeast *S. pombe* (this study); Me31B *Drosophila melanogaster* (De Valoir et al. 1991); p54 human (Lu and Yunis 1992); eIF-4AI mouse (Nielsen et al. 1985); Tif1/Tif2 budding yeast *Saccharomyces cerevisiae* (Linder and Slonimski 1989); *Vasa D. melanogaster* (Lasko and Ashburner 1988). Identical amino acids are *shaded*. Eight conserved DEAD box motifs are *boxed*. Consensus boxes I and V represent ATPase A and B motifs, respectively.

the shuttle vector pAU-SK. Neither mutant could complement the *ste13* disruptant and this strongly suggests that the Ste13 protein functions as an ATP-dependent RNA helicase (Fig. 7).

Suppression of the *ste13* mutation by ME31B

The DEAD box family of proteins perform a wide variety of functions, which include the initiation of protein synthesis and mRNA splicing (Schmid and Linder 1992). Several members, including Vasa (Lasko and Ashburner 1988; Hay et al. 1988) and Me31B of *Drosophila* (De Valoir et al. 1991) and PL10 of mouse (Leroy et al. 1989), are involved in germline cell differentiation. Since Ste13 plays a role in sexual development in *S. pombe*, it would seem to be closely related to this particular subgroup of the family. Indeed, the alignment of amino acid sequences reveals that Ste13 is very similar to the *Drosophila melanogaster* developmental protein Me31B (Fig. 8). Ste13 is 72% identical to Me31B over a stretch of 391 amino acids. The primary structure of two recently identified DEAD proteins, p54 from human (Lu and Yunis 1992) and Dhh1 of *S. cerevisiae* (Strahl-Bolsinger and Tanner 1993), are also similar to Ste13/Me31B, though their cellular functions have not been elucidated.

Given the similarities in structure and function, we wondered whether the ME31B gene product from *Drosophila* could replace the *ste13*$^+$ gene of *S. pombe*. Thus, the ME31B cDNA was inserted downstream of the thiamine-repressible *nmt* promoter in the pREP plasmids and introduced into an *ste13* mutant. ME31B was able to restore mating and sporulation abilities and reduced the lethality caused by nitrogen starvation (Fig. 9). It is reasonable to conclude that *Drosophila* ME31B is not only a structural homolog of the *S. pombe ste13*$^+$ gene but is also a functional homolog. Similar experiments revealed that the Vasa protein of *Drosophila*, another member of the DEAD family (Lasko and Ashburner 1988), which is less similar to Ste13/Me31B, failed to complement the *ste13* mutation (data not shown) and demonstrates that conservation of the DEAD box consensus motifs is not sufficient to suppress the *ste13* mutation.

Discussion

Results presented in this paper suggest that the *S. pombe* sterility gene, *ste13*$^+$, encodes an ATP-dependent RNA

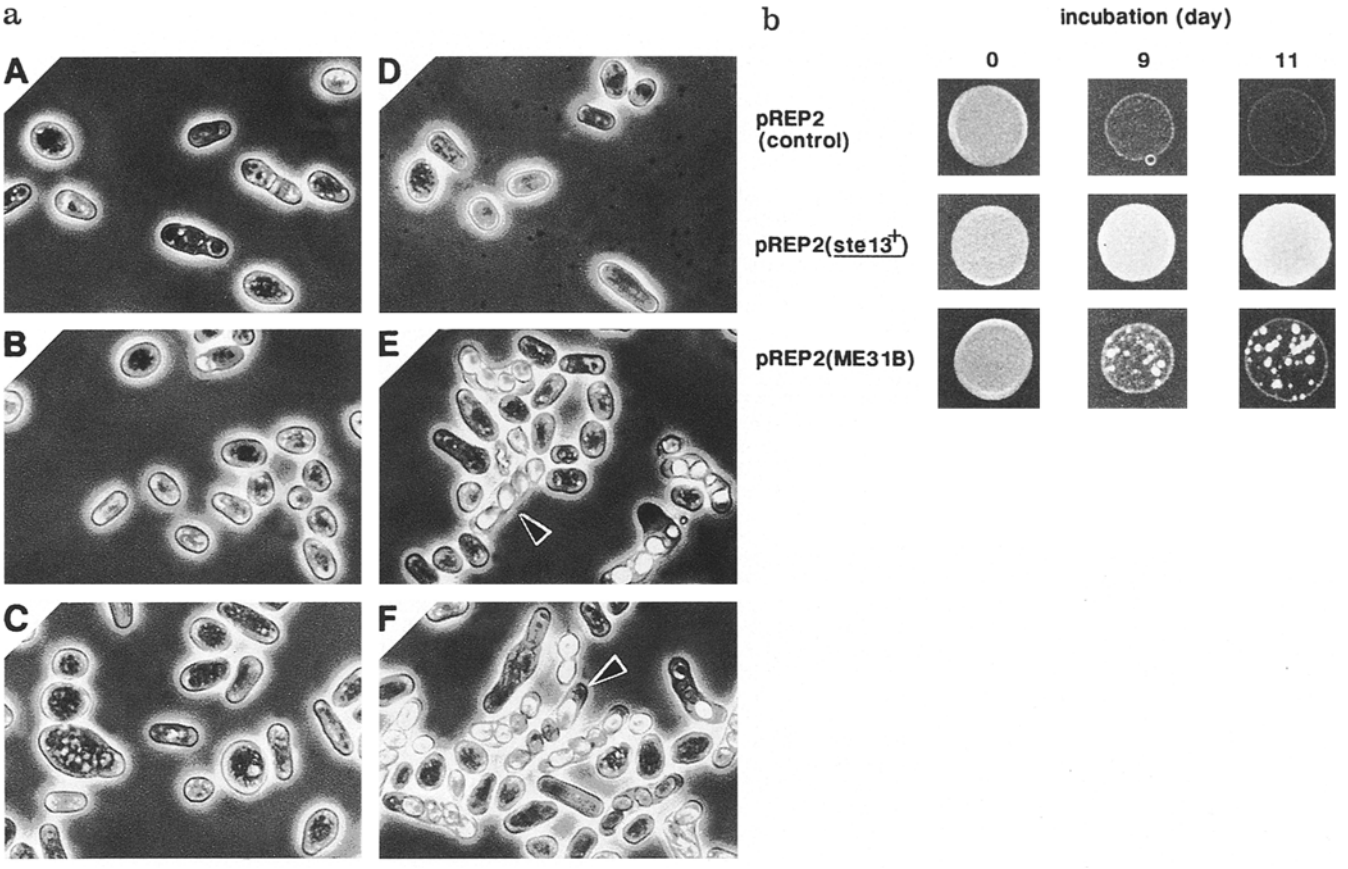

Fig. 9a, b Suppression of the *ste13* mutation by expression of the *Drosophila* ME31B cDNA in *S. pombe*. **a** Complementation of mating and sporulation ability. A *ste13* nonsense mutant strain, C451-1A, was transformed with pREP1 (*A* and *D*), pREP1 (*ste13*$^+$) (*B* and *E*) or pREP1 (ME31B) (*C* and *F*). Complementation of the mating and sporulation ability was tested by incubating the transformed strains for 3 days at 26° C on SSA sporulation plates with or without thiamine. *A–C* the *nmt* promoter was repressed by adding thiamine at a concentration of 2 μM; *D-F* the promoter was switched on by depletion of thiamine. *Arrowheads* represent some typical zygotic asci. **b** Complementation of survival in starvation medium. The *S. pombe* strain C522-4B was transformed by pREP2 (ME31B) or pREP2 (*ste13*$^+$). Each transformant was cultured in nitrogen-free PM-N liquid medium for 0, 9 and 11 days at 26° C with moderate shaking. A portion of the culture was spotted onto agar plates of YEA rich medium and photographed after 2 days incubation at 30° C

helicase of the DEAD box family of proteins. Although we have no direct biochemical evidence for it, the suggestion is based on both sequence comparisons and, more importantly, on mutational analysis. Members of this family contain several conserved motifs including the DEAD motif, a specialized version of the ATPase B motif (Linder et al. 1989), and the HRIGR motif, which is essential for RNA unwinding (Pause and Sonenberg 1992). Mutational analysis has demonstrated that these two completely conserved motifs are essential for the activity of proteins in this family; neither EEAD nor DQAD can replace DEAD (Pause and Sonenberg 1992) and the first arginine residue of the HRIGR motif of the yeast eIF-4A (Tif1/2) could not be replaced with either E, G, I, S or T (Schmid and Linder 1991). Here we have used site-directed mutagenesis of *ste13* to change DEAD to DPAD and HRIGR to HGIGR and show that neither mutant could suppress an *ste13* deletion mutant. Our results strongly suggest that Ste13 functions as an ATP-dependent RNA helicase.

Sequence comparison with a variety of DEAD box proteins indicates that Ste13 is similar to initiation factors such as eIF-4A of mouse (Schmid and Linder 1992) but, since the *ste13*$^+$ gene is not essential for growth, it is unlikely to be an initiation factor itself. One possibility is that Ste13 provides an RNA helicase activity, which enhances translational efficiency by unwinding secondary structures of specific mRNA species that are produced or active under certain conditions or during cell differentiation. This possibility could be further investigated by performing binding assays using bacterially produced Ste13 and in vitro transcripts from a variety of sterility and meiotic genes of *S. pombe*. A similar role can be proposed for the ME31B of *D. melanogaster*, which appears homologous in terms of structure and function.

Depletion of nitrogen from the culture medium causes an apparent G1 block, though the G1 phase is extremely short in the normal *S. pombe* mitotic cell cycle. Ste13 does not seem to be required for the G1/S transition of the normal cell cycle in rich medium, since a *ste13* disruptant is visible, but is required for a G1 arrest in response to nitrogen starvation. It remains

possible that, under conditions of nitrogen starvation, Ste13 acts as a negative regulator of a G1-specific cell cycle gene such as *puc1*$^+$, which encodes a putative G1 cyclin (Forsburg and Nurse 1991). One suggestion is that Ste13 acts in concert with other proteins and we have now identified two further *ste* genes, *ste12* and *ste14* (Kitamura et al. 1990; Y. Uno and C. Shimoda, unpublished) whose defective mutants show a similar phenotype to *ste13* mutants in nitrogen-free medium. It is important to analyze further these genes in an attempt to understand better how the exit from the mitotic cell cycle is regulated.

The *ste13*$^+$ mRNA contains two short introns in the 5′ terminal region and since both contain in-frame stop codons correct splicing is indispensable for expression of the functional Ste13 protein. Interestingly, the splice donor site of the first intron (GTGAG) deviates from the consensus for *S. pombe* (GTANG) and demonstrates that GTG can be used as a splice donor signal in *S. pombe*. It remains to be examined whether these introns are efficiently spliced under all culture conditions, since meiosis dependent splicing has been demonstrated in both *S. cerevisiae* (Engebrecht et al. 1991) and *S. pombe* (Kishida et al. 1994).

Acknowledgements We thank Dr. K. Beckingham of Rice University for the gift of the ME31B cDNA clone, Dr. K. Maundrell of Glaxo for the pREP plasmids, Dr. O. Niwa of Kyoto University for FACScan analysis, Dr. P. Linder of Biozentrum Basel for amino acid alignment of Ste13 with other DEAD proteins, Dr. M. Yamamoto of the University of Tokyo for *cyr1* disruptant strain, Dr. H. Okayama and Dr. H. Nojima of Osaka University for an *S. pombe* cDNA library and Dr. J. Davey of University of Birmingham for critically reading the manuscript. This work was supported by Grants-in-Aid for Scientific Research by the Ministry of Education, Science and Culture of Japan. H.M. is supported by JSPS Fellowships for Japanese Junior Scientists.

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