Schizosaccharomyces pombe ste11⁺ encodes a transcription factor with an HMG motif that is a critical regulator of sexual development

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Schizosaccharomyces pombe ste11 encodes a member of the family of HMG-box proteins. Its transcript is induced in response to nitrogen starvation and a concomitant decrease of the intracellular cAMP level. Expression of *ste11* is essential for induction of sexual development, and its ectopic expression stimulates uncontrolled mating and sporulation. Ste11 protein regulates positively transcription of the following genes required for sexual development: the mating type genes, *matP* and *matM*, and the *mei2* gene, which is essential for commitment to meiosis. Ste11 protein synthesized in vitro binds specifically to a DNA fragment carrying a 10-base motif TTCTTTGTTY that is an essential *cis*-acting element for the induction of *mei2* and is commonly seen in the upstream regions of the genes inducible by nitrogen starvation. These observations strongly suggest that Ste11 serves as a key transcription factor for sexual development.

[Key Words: *Schizosaccharomyces pombe*; nitrogen starvation; sexual differentiation; transcription factor; high-mobility group protein box; DNA-binding activity]

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Nutritional starvation, particularly that for nitrogen, induces sexual development in *Schizosaccharomyces pombe* (Egel 1973; Egel and Egel-Mitani 1974). Haploid cells mate and diploid cells initiate meiosis under nitrogen starvation, and ascospores are subsequently formed. Many genes have to be regulated in an organized manner during these differentiation processes. This study focuses on how nitrogen starvation induces concerted gene expression that commits *S. pombe* cells to sexual development.

Genes that are activated transcriptionally in response to nitrogen starvation have been reported. They include the four transcription units of the mating-type genes (*matPc*, *matPi*, *matMc*, and *matMi*) (Kelly et al. 1988), and the *mei2* gene (Shimoda et al. 1987), which encodes an essential factor for the initiation of meiosis (Bresch et al. 1968). Function of these genes is required in the initial stages of sexual development. Three lines of evidence indicate that cAMP is involved in the control of the expression of these genes. (1) Addition of cAMP to the medium inhibits induction of these genes (Watanabe et al. 1988); (2) *S. pombe* cells that lack the gene for adenylyl cyclase and do not have a measurable amount of cAMP are highly derepressed for sexual development

(Maeda et al. 1990); and (3) nitrogen starvation lowers the level of intracellular cAMP by ~50% in *S. pombe* (Fukui et al. 1986; Maeda et al. 1990; N. Mochizuki and M. Yamamoto, in prep.). Thus, it appears that nitrogen starvation results in a decrease in the intracellular cAMP level which, in turn, functions as a signal for the induction of nitrogen starvation-responsive genes in *S. pombe*.

We demonstrate in this discussion that the *ste11* gene is also inducible by nitrogen starvation and repressible by cAMP and, furthermore, that the function of *ste11* is essential for the induction of all the other known nitrogen-responsive genes. These findings indicate a pivotal role for *ste11* in the regulation of sexual development in response to nitrogen starvation. Analysis of the *ste11* gene product strongly suggests that this protein binds to DNA and functions as a transcription factor.

Results

ste11 mutation

Originally we identified the *ste11* mutation as a suppressor of the uncontrolled meiosis driven by the *pat1* (also called *ran1*) mutation. *S. pombe* strains carrying the temperature-sensitive *pat1-110* or *pat1-114* allele initiate meiosis and hence halt growth at the restrictive temperature (Iino and Yamamoto 1985a; Nurse 1985). More than 150 spontaneous revertants able to grow at 37°C

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were isolated from parental *pat1* mutants. All of these revertants had acquired suppressor mutations; 90% of the mutations mapped in *mei2*, whose deficiency had been shown to be a potent suppressor of *pat1* (Beach et al. 1985; Iino and Yamamoto 1985a,b). The remainder defined a new gene, *ste11*, which we referred to as *steX* in a previous publication (Watanabe et al. 1988), and is now known to be allelic both to the *ste11* gene described recently (Kitamura et al. 1990) and to *aff1* isolated independently as a *pat1* suppressor (Sipiczki 1988). The *ste11* mutations separated from the *pat1* background conferred complete sterility and meiotic deficiency. Although there are other *S. pombe* sterility genes that are essential for both mating and meiosis (Girgsdies 1982), *ste11* is distinct from them in that none of the others can arrest the uncontrolled meiosis driven by *pat1*.

Cloning of the *ste11* gene

Cloning of the *ste11* gene was achieved by complementation of the *ste11-29* allele. An *S. pombe* genomic library was prepared in the vector YIp468, which was expected either to integrate into *S. pombe* chromosomes or replicate autonomously but poorly. *Spo*⁺ transformants of JY736 (*h*⁹⁰*ste11-29*) were isolated and plasmids were recovered from them. Among plasmids recovered successfully in *Escherichia coli*, only three had unarranged sequences, two of which (named pSX1) were identical and able to retransform JY736 to *Spo*⁺. The insert in pSX1 was a 9.5-kb *Bgl*II fragment. This fragment was recloned in pUC118, and the resultant plasmid was named pSX1-U1.

Structure of the *ste11* gene

Deletion analysis of pSX1 suggested that *ste11* is carried on a 3.7-kb *Bam*HI–*Eco*RI fragment (Fig. 1). Sequence analysis of this region revealed an open reading frame (ORF) that potentially encodes 468 amino acids (Fig. 2). To confirm that this ORF corresponds to *ste11*, gene disruption was performed. A 2.0-kb *Bam*HI–*Pst*I fragment, which covers the amino-terminal one-fourth of the ORF, was excised from pSX1-U1 and replaced by a *ura4*⁺ cassette (Fig. 1). The wild-type allele in a homothallic hap-

loid strain JY878 was substituted with this construct. Southern analysis of two sterile *Ura*⁺ transformants indicated that the disrupted allele had integrated by homologous recombination (data not shown). These transformants were fused with an original *ste11* isolate JY736 by protoplast fusion. All diploid fusion products were *Spo*⁺. Because both the original and the disrupted sterile mutations are recessive, these results strongly suggest that the cloned gene is indeed *ste11*.

The *ste11* product carries a conserved motif found in the nuclear high-mobility group proteins

Homology of the deduced *ste11* gene product to known proteins was pursued with the aid of Dr. Mark Goebel (Indiana University School of Medicine, Indianapolis). The Ste11 protein was found to have the highest similarity to the product of *S. pombe* *matMc*, which is a transcription unit in the M-mating type locus (Kelly et al. 1988). The *matMc* gene product has been shown to have homology with *SRY* (Sinclair et al. 1990), a human gene that is likely to be the testis-determining factor (TDF) (Berta et al. 1990; Jäger et al. 1990; Koopman et al. 1990). Ste11 and this putative TDF also exhibited considerable homology (Fig. 3). The homologous region in these cases matches the HMG box (Jantzen et al. 1990; Kolodrubetz 1990), which corresponds to the conserved domains in nuclear high-mobility group protein HMG1 (Tsuda et al. 1988; Wen et al. 1989) and has been demonstrated to be a DNA-binding domain in some of the family members (Wright and Dixon 1988; Jantzen et al. 1990). These suggest that Ste11 also could be a DNA-binding protein.

ste11 is inducible by nitrogen starvation and repressible by cAMP

The effect of nitrogen starvation on expression of *ste11* was examined. Expression of this gene was higher under nitrogen starvation (Fig. 4, lanes 1,2), irrespective of the mating type of the cell (data not shown). However, a low level of *ste11* expression was consistently observed when cells were growing in rich medium at the maximum growth rate, which contrasts to much lower expression of *mei2* under the same conditions (data not

Figure 1. A restriction map of the *ste11* locus and complementation analysis of subclones. The insert in the original clone pSX1 is illustrated at the top. The extent and direction of the *ste11* ORF, which encodes 468 amino acids, is shown by a solid arrow. Open arrows indicate the orientation of readthrough transcription from a cryptic promoter on the vector pDB248'. The fragment inserted in pSX-D1 and pSX-

D2 apparently carries the authentic *ste11* promoter, whereas that in pSX1-D3 and pSX1-D4 does not. Complementation was assayed by mating and sporulation proficiency of the recipient *ste11*[−] cells. The construct used in disruption of *ste11* is shown schematically at bottom. The presumed promoter region and amino-terminal one-fourth of the *ste11* ORF was replaced by a *ura4*⁺ cassette (Grimm et al. 1988). Restriction sites shown are *Bam*HI (B), *Bgl*II (Bg), *Cla*I (C), *Eco*RI (E), *Hind*III (H), *Mlu*I (M), *Pst*I (P), *Pvu*II (Pv), *Sph*I (S), and *Xho*I (X).

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-1612 GGATCCTCCTCTGTTGGAATTTGTTAATGAGAAGTGTGGAATAATTTTTCTGTTTCTGAAATCAAAGGATTTATCTTTTTTTTAAATTTTACGTC -1513
-1512 GATTCCTCTACTGTACGTGGAATCCGCCCTTTTTTTTATTTTTTTCTACGTCAAATTTTTTTATATATTTCTTTGTCTCTACACCCTAGTTATCCT -1413
-1412 TTATCAACTTTCTCTCTACTAATTAAGTGTGTTCTTTGTTGCAATTTTTGCCCACTTTAAATTTTTTTTATTCGCATCGTTATTTCTATCGCTGG -1313
-1312 CTGCTTATCCTTGATATCTGATCTATTAGATTTCATTCTCTCAATTTTAAATATCCTTTTTTAACGTGTTCTTTTGTGATTCCATTAGTACACAC -1213
-1212 CATTGAGAATTAAGTTGCAATCCACATCGGCTTTGTTGGCTCATGTTTATAACATTATTTCTCCCTGTCTATGTCGATTATTCAGTATTCATTGTTA -1113
-1112 TTCTCTCACTTTATTTGATACCCAAAGTTTCACCATCTTACAGAGCTCAACCATCTTCAGTTATCGGTGTCCTCTGAAATTTCTCCCTGTTACGCTTT -1013
-1012 TTATTTGACTCACTTTCCGTTATTTAGTCTTCGTTCTGTTCTGTTTCTGTTAACTATTCGCTCTTTCTGTTTCTAGTTTTCCTCCGTTTATA -913
-912 TAGAGAAAAAAGCCTTAGAGGCTCGTGAATTTGTTCTCGTTTCAAATCTCCTCAACTTAAATTAGGCTTATAAGAAATCACTTTTAAAGCTTCAA -813
-812 TTTTTTCTTGTGGCTTCTATTATATATCACTTTCACCTCTCTCCACTTGAGCCTCTACTAATTTGCTCTTTCAAGTTATCTCTGATTCAATCGCT -713
-712 TTACGAAAAATATCCTTCATCATTCCACTCGATCTAGTAACCTTTTCACTCTCACTTTTCATCTTTTTTTCTACGCTCTTTACATTTTCAAATCGG -613
-612 CTTTGAGTGTGCTTCTGTTTTTTCTCTTCTGAGCTGAAATCCATTAGTTTCATTATTTATTTCACTTACTTTTCTGTTTAGGAAAAACCTGGC -513
-512 ATCAAAATATACCTTTGTCACCTTTTTCTTTTTTTAGTCCAGTCAGTCTCTATTCAATTAAGCTTGAACCTGATTCTTTGGCGAACAAGCTGTTTAATA -413
-412 TCTAGATTGACGGAAATTTTATTTTCATTATTTGTTGATACATACAAGGAATTTTACTTTTACTATATTCATCTGTTACTTCACTGTGAATAATTTGT -313
-312 TCATTTTTCATTCTGTTAGTTTGTAGATTCTTTCTATCTTAATACCATTTTCTTTTTTAAATAGAAAAATCCTTCACACAGTTTATTATCGGTG -213
-212 TTTATTTCTTACTGTTTCTGATCTCATCCCTCTCCCTCGGTTTGCTGTTGCTGCTTCTTTCTACTTCTGTTCTTCTGACTGCGAACTTCATTATG -113
-112 TTTGCTTTGTTCTTGAATCATCTTCTAATTAATCTAAGAACCTTCATTGCTGTTTATTTTCTCCCTTACTTTTACTACATTTTATATTACT -13
-12 CTTCTCTACACAAATGTCTGCTTCTTTAACAGCGAGCAAAGGACCAAAAGTCTGTTAAGGACCTTTAAACAGTTTATGCTTTATCGTCGAGATC 88
      M S A S L T A E Q K D Q K S S V K R P L N S F M L Y R R D R
89 GCCAAGCTGAAATCTCTAATCAACCAAGATATCTACGATGATTTGGTCAGCTGTGGAGAAATGAAAGTCCCAAGTCAAGAAGTATTTCTGA 188
      Q A E I P T S N H Q S I S R I I G Q L W R N E S A Q V K K F Y S D
189 TTTATCCGCCCTCGAAAGGCGAAGACATATGCTGAAATCCCGAATACAAGTACACCCCTAAAAAGCGGTCTACTGTTCTGAGACCCCATAAAAAGGTA 288
      L S A L E R Q K H M L E N P E Y K Y T P K K R S T V R R P H K K V
289 TCACCTTCTTCGGATCTTTTGTGGCTCGGATTACGTTGTACTGCAAGAAATGCAACATCTTCTAAGACTCTAAAAAGACCCGCAAGCGGAAAGCCTG 388
      P S S D S F V A S D Y V L V L A Q S S K T L K Q T E P K F Y P V
389 TTAATGAGGAGAACTTTAGCAGCTTTATAGTCTCCGCTCTTTTATATCCAAAGTCAGGTAATCGAATCTTATTGAAACAGTGAATTTGCTTTGCT 488
      N E E T L A A L L A P A L S Y P K S G K S N L I E T S E L S C L
489 TTCGTTCTCCCAATGATTCGAAGTCATACAAATCTCTCTTATCTTCAACGATCAAGTTTCTACAATATTTCTACATTTGGATAAATCCGAGCAAGCT 588
      S S S P M I P S L S F T D Q V S T T I S T L D K E Y Q A
589 CCTAGTTCTTTGGGCATCTATTATAGATCAACCGAGTAGCGGTAGTCCAAATCGGGCGAACAATCTGTGTTTAGCTAATAAGCCCGAATAGTACCGA 688
      P S S L G I Y Y R S P S S G S P I G R T K S V C L A N K A R I V P K
689 AAAGATCAATGTCTGATGGCTGTGTTGACAAATCTTATCAATGTCAAAACACCGAGCTTGGAGCGAAGTTCGCTCAAAATTCAGCAATGTCTC 788
      R S M S S D G C V D K S Y Q M S K T P S L E A N L P Q N S N C S
789 TGCTCGAAGAGTTCAAAATTTGATTCTAAAGGAACCGTAGTGAGCAAGTAACTCTGATAGTCCAGAGCTGTGAGCGGATAAAGTGTGTTCTCATTGT 888
      A R R V P K F D S K G T V S E Q S N S D S P E L S A D K V L S H C
889 AGTCCTATGATGCTGACCTCACTCCCTCTTGCCTCAATGCCAGTATATCACTAAAACCCCGAATACCGGTGATCATTATGTTTGTATGAGGCTG 988
      S P I D A R P S T P S C P N A S I S P K T P N T G D H Y G F D G A E
989 AGTACCTAGGAACCTCTCTCAGTGTAGGAGCACTACTGCTATTATATACGGCAAGAGACCGAAGCTTTTATCTACGCTTATTTGCCATCTTCTTACCC 1088
      Y L G T P L S V G S T T A Y L Y G Q E T E L L S T P Y C H T S Y P
1089 AGCAATGAGTCGATTGAACAGCAGTCTCTGGCTATACATGGCTCTCTCTGCTAGTGTACCAATTTCTGGACATCTGAAATAATACCTGGAGAAGCGAC 1188
      A M S R L N S S S G Y T T C V S S S T V T N S G H T E N T W R E Q A
1189 GAACAAAGTAAAGCTTTGTTGACATTAATAGTTTCTCAAGTTTCTTCAATGGCAATATGAGTTCGCGCTCATTCTCAAGATTTGGATGATT 1288
      E Q S K G F V D I N S F S Q S L F S N G N Y E F A A H S Q E L D D L
1289 TATTTTCAAAATACGATTTTACATCAACTGATCTATTGCTTCTCTCAAGATGCTAATCTATTGGACCTTCTCTTTTAGAACCTTGGTTGCC 1388
      F S Q I T D F T S T D P I A S S L K D A N S L G P S L L E P L P
1389 CAATCTAATTTATTTTGAATAATTTCTATGCAACATGGCTTTGTTTCAACAAACGATCATTTTAAACGCTCACAAGCATGCAGCTGATTGTT 1488
      N S N L F
1489 CATGTCATGCTTAAAAATCTATATTTTTTAAATTTTCATATCTTTGTTATGCTAGATGATGCCTTCCATAATTTATTTCACTTTTAACTCTTT 1588
      AATTTATCCCTTTTATGTTTATATAGGAATATGTATATCTAGTTTCCAAAGAGCATTGGGCAACATTGAATAGTTGCGCACTAATTTCTTTT 1688
      TTTTAAATTTGAACAATGCTTACGTTATGAATGATTCTTACATCAATTTCTCTGTCATCTAATTTGGGCCGCTTATTACATATATATTGATGTA 1788
      AAAATATATATATATATCGCAACGTAATTTCTAATTTTCTCTTCCATTACTTTCTGTAAGAAATGTAAGTATATATATGTTCAATTTCTTTACGTAT 1888
      CAAGCAATCATCAATGAGTATGTTATCACCAATTTCACTAGATATACTATCGATGAAGGTTTACTTGGCACCCACACCTACTGGGGTAAATGTAAGAT 1988
      AACTCAACCAATAAATGATTAAGAATAAAGGTAATTAATTAACCTGACAAGAACTTATAGAATTC 2056

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Figure 2. DNA sequence and deduced amino acid sequence of the *ste11* gene. The DNA sequence of the 3.67-kb *Bam*HI–*Eco*RI fragment, carried on pSX1-D1 and pSX1-D2 (Fig. 1), is shown. The deduced amino acid sequence is shown below the DNA sequence in the single-letter code, starting with the first methionine codon. These sequence data have been submitted to the EMBL/GenBank data libraries.

shown). Thus, the regulation of *ste11* expression by nutritional conditions appears to be less rigorous.

Because induction of *mei2* in response to nitrogen starvation has been shown to be blocked by cAMP (Watanabe et al. 1988), the effect of cAMP on *ste11* expression was examined. The addition of 10 mM cAMP, together with 5 mM caffeine, a phosphodiesterase inhibitor, severely suppressed expression of *ste11* both in the presence and absence of a nitrogen source (Fig. 4, lanes 3,4). Conversely, *ste11* was highly expressed in cells defective in *cyr1*, which have no detectable level of adenylyl cyclase and cAMP (Maeda et al. 1990), even in the presence of a nitrogen source (Fig. 4, lanes 5,6). These results es-

tablish that *ste11* expression is responsive to changes in the level of cAMP.

ste11 is essential for expression of genes required for sexual development and cells expressing ste11 are sexually derepressed

Induction of *mei2* by nitrogen starvation did not occur in a *ste11* mutant (Watanabe et al. 1988; also see Fig. 5). Similarly, the four transcripts of the mating-type genes, which are normally inducible by nitrogen starvation (Kelly et al. 1988), were not induced in *ste11*[−] cells (Fig. 5). Apparently, *ste11* regulates these genes positively.

A

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steII      E Q K D Q K S S V K R P L N S F M L Y R R D R A - - - E I P T S N H Q S I S R I I G L W R N E S A Q V K K Y Y S D L S A L E R O K H M L E N P E Y K Y T P K K R S T V R R
matMc      K D T T S T E R T E R P P N A F I L Y R K E K H A T L K S N P S I N N S Q V S K L V G E M W R N E S K E V R M R Y F K M S E F Y K A Q H O K M Y P G Y K Y Q P - R R N K V K R
TDF        S K G N V Q D R V K R P M N A F I V W S R D O R R K M A L E N P R M R M S E I S K O L G Y Q W K M L T E A E K W F F F Q E A Q K L Q A M H R E K Y E N Y K Y R P R R K A K M L P
HMG1       K K F K D E N A P K R P S A F F L F C S E Y R P K T K G S P G L S I G D V A K K L G E M W N N T A A D K H P Y E K K A K L K E K Y E K D I A A Y R A K G - K P D A A K K
NHP6A      R K K K D E N A P K R A L S A Y M F F A N E N R D I V R S E N P D I T F G Q V G K K L G E K W R A L T P E E K Q E Y E A K A Q A D K R Y E S E K E L Y N A T L A
hUBF       K L K K H P D F F K K P L T P Y F R F F M E K R A Y A K L H P E M S N L D L T K I L S K K Y K E L P E K K M K Y I Q D F O R E K O E F E R N L A R F R E D H P D L I Q N A K
consensus  . . . . . K R P . . . F . . F . . E . . . . . P . . . . . V . K . L G . . W K . . . . . K . . Y . . . . . K . . . . . Y . . . . .

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B **C**

	matMc	TDF	HMG1	NHP6A	hUBF
steII	57	42	38	36	33
matMc		48	44	36	41
TDF			41	42	38
HMG1				63	44
NHP6A					41

Figure 3. (A) Comparison of amino acid sequences of the HMG boxes in the *ste11* gene product and its homologs. The amino acid sequence of Ste11 (residues 8–91) is aligned with the following homologs: product of the *S. pombe* mating-type gene *matMc*, amino acid residues 95–181 (Kelly et al. 1988); putative human testis-determining factor (TDF), amino acid residues 71–158 (Sinclair et al. 1990); porcine nuclear high-mobility group protein HMG1 (middle segment), amino acid residues 86–172 (Tsuda et al. 1988); *S. cerevisiae* nonhistone chromosomal protein NHP6A, amino acid residues 12–92 (Kolodrubetz and Burgum 1990); human nucleolar transcription factor (hUBF) (upstream binding factor), amino acid residues 104–191 (Jantzen et al. 1990). Amino acids identical or conserved between Ste11 and at least two of the homologs are shown in white against black, whereas those identical or conserved in three or more of the homologs but not in Ste11 are shown in white against gray. Amino acid residues that suggest a close relationship among Ste11, MatMc, and TDF are indicated by asterisks (*). Conserved amino acid substitutions are grouped as follows: K,R; E,D; Q,N; S,T; F,Y,W,H; and L,I,V,A. (B) Schematic illustration of the structure of *ste11* homologs. Assigned HMG boxes are shaded. The total number of amino acid residues in each protein is shown. (C) The degree of similarity (%) between *ste11* homologs in the HMG box region. Calculations were performed using the DISTANCES program of the UWGCG Sequence Analysis Software Package (Devereux et al. 1984).

To examine the consequences of unregulated expression of *ste11* in *S. pombe* cells, three plasmids, based on the vector pDB248' (Beach et al. 1982), were constructed that express *ste11* under the control of different promoters. pSX1-D4 carries a 3.2-kb *HindIII*–*BglII* fragment, which covers the entire *ste11* ORF but lacks its authentic promoter. The *ste11* ORF on this plasmid, however, could be transcribed in *S. pombe* cells from a cryptic promoter on the vector. pSX1-V2 carries the same fragment connected to the SV40 promoter. pSX1-A1 carries the same fragment connected to the *S. pombe adh* promoter. A homothallic *ste11*-disrupted strain, JZ396, was transformed with these three plasmids. Cells that became Leu⁺ as a result of the *S. cerevisiae* *LEU2* marker on the vectors were selected. Apparently homogeneous transformants could be obtained with pSX1-D4 and pSX1-V2, whereas pSX1-A1 gave <10% as many transformants as the other two plasmids and these transformants showed heterogeneous aberrant phenotypes in growth and sexual development. This is probably because the *S. pombe adh* promoter is too strong; therefore, overproduced Ste11 protein causes deleterious effects on cell growth (see below). The pSX1-D4 and pSX1-

V2 transformants could grow; but unlike wild-type cells, they readily produced zygotes and asci on nitrogen-rich SD medium (Fig. 6). Cells (10.1%) of the pSX1-V2 transformant were found to have undergone mating on SD. These observations strongly suggest that artificial expression of *ste11* can induce sexual development irrespective of the nutritional conditions, as was seen in *cyr1*[–] mutants (Maeda et al. 1990). A large portion of these transformants continued to proliferate, although the truncated gene on pSX1-V2 was efficiently transcribed (see Fig. 5). This may mean that the truncated mRNA, lacking most of the 5'-untranslated region, is unable to direct production of a sufficient amount of Ste11 protein. Alternatively, Ste11 protein may not be fully active in the presence of a nitrogen source (see Discussion). As expected from its phenotype, the mating-type genes and *mei2* were expressed in the presence of nitrogen in the pSX1-V2 transformant (see Fig. 5).

Ste11 protein synthesized in vitro binds to DNA

The results shown above suggest that the Ste11 protein could be a transcription factor that positively regulates

Figure 4. Effect of nitrogen starvation and cAMP on the expression of *ste11*. Total RNA was extracted from cells either rapidly growing or starved for nitrogen for 4 hr, and analyzed by Northern blotting. The probe used was the *Hind*III–*Bgl*II fragment covering the entire *ste11* ORF, whereas a shorter fragment was used to detect *ste11* mRNA in other experiments. (Lane 1) JY333 (*cyr1*⁺) in the presence of nitrogen; (lane 2) JY333 in the absence of nitrogen; (lane 3) the same as lane 1 but 10 mM cAMP and 5 mM caffeine were added; (lane 4) the same as lane 2 but cAMP and caffeine were added; (lane 5) JZ300 (*cyr1*[−]) in the presence of nitrogen; (lane 6) JZ300 in the absence of nitrogen. Equal loadings of RNA in these lanes were confirmed by ethidium bromide staining of rRNAs (not shown).

at least *matP*, *matM*, and *mei2*. Inspection of these genes revealed that they carry nearly identical 10-base motifs (TTCTTTGTTY) in their 5'-upstream regions (Fig. 7). Hereafter, we call them TR (T-rich) boxes. Five TR boxes are oriented in the same direction (regular orientation) in *mei2*. Two TR boxes were found in both *matP* and *matM*, and they locate in the central region of the two diverging transcription units in each case (Kelly et al. 1988). In the *matP* locus, they are regularly oriented toward *matPi* and hence in reverse order toward *matPc*, whereas they are regularly oriented toward *matMc* in the *matM* locus. Gel-shift experiments using oligonucleotides with this motif as a probe showed that *S. pombe* extracts exhibit a Ste11-dependent activity that binds specifically to the probes (data not shown). To demonstrate that Ste11 protein itself binds to DNA, we performed reverse gel-shift experiments with Ste11 protein produced in wheat germ cell extract (Fig. 8). The results showed that the Ste11 protein could bind to double-stranded probes with TR motifs (Fig. 8C, lanes 3,5) but not to a mutated TR box in which the invariant G was replaced by T (Fig. 8C, lane 4) or to single-stranded probes (data not shown). Furthermore, a truncated Ste11 protein that lacks the HMG domain lost the ability to bind to the probe (Fig. 8C, lane 7), suggesting that this domain is necessary for DNA binding.

The TR box is an essential cis-acting element for gene induction under nitrogen starvation

We wanted to determine whether the TR box is required in vivo for gene induction in response to nitrogen starvation. A plasmid carrying part of the *mei2* gene was conveniently used for this purpose. This plasmid, pDB-(*mei2*)Δ12, carries two of the four transcription initiation

sites of *mei2* identified previously (Watanabe et al. 1988), two of the five TR boxes found in their vicinity (c and d in Fig. 7), and most of the *mei2* ORF (Fig. 9A). A *mei2*-disrupted strain, JY776, was transformed with pDB-(*mei2*)Δ12 and its derivatives and analyzed by Northern blotting. As shown in Figure 9B, transcription of *mei2* from pDB(*mei2*)Δ12 was considerably inducible by nitrogen starvation (lanes 1,2). However, this induction was reduced when the conserved G in TR box d was altered to T [pDB(*mei2*)Δ12M1] (Fig. 9B, lanes 3,4). Induction of the shorter transcript appeared to be inhibited more severely. If the same mutation was also introduced into the remaining TR box c, the inducibility was almost completely abolished [pDB(*mei2*)Δ12M2] (Fig. 9B, lanes 5,6). These observations establish the requirement of the TR box as a controlling cis-acting element for induction of the *mei2* gene. Together with the fact that Ste11 protein binds to a TR box in vitro (Fig. 8), this result strongly suggests that induction of nitrogen starvation-responsive genes is due to binding of the Ste11 transcription factor to TR boxes upstream of these genes.

Figure 5. Summary of Northern blot analysis of genes required for sexual development. Total RNA was prepared from *ste11*⁺ and *ste11*[−] cells, either growing exponentially or starved for nitrogen. RNA from cells expressing *ste11* in the presence of nitrogen was also analyzed. Genes analyzed are listed at left; the probes to detect each transcript are described in Materials and methods. (Lanes 1,2) JY450 (*ste11*⁺); (lanes 3,4) JZ396 (*ste11*[−]) carrying the vector pDB248; (lane 5) JZ396 carrying pSX1-V2. Cells were either grown in the presence of nitrogen (lanes 1,3,5), or starved for nitrogen for 4 hr (lanes 2,4). The *ste11*-transcript in lane 5 (2.4 kb), expressed from the truncated gene on pSX1-V2, is shorter than the authentic ones seen in the other lanes (3.6 kb). The *matMc* and *matMi* transcripts, which are both 0.6 kb in length (Kelly et al. 1988), were measured collectively in this analysis. The sizes of other transcripts are as follows: *mei2*, 4.2 kb (Watanabe et al. 1988); *matPc*, 0.75 kb (Kelly et al. 1988); *matPi*, 0.56 kb (Kelly et al. 1988). (Bottom) The ethidium bromide-stained patterns of rRNA, which verifies approximately equal loadings of total RNA in the five lanes.

Figure 6. Unconditional mating and sporulation displayed by *ste11*-expressing cells. Cells were cultured on SD medium, which does not allow wild-type cells (*ste11*⁺) to mate and sporulate ($<1 \times 10^{-4}$). (A) JZ396 (*ste11*⁻) carrying the vector pDB248'. (B) JZ396 carrying pSX1-V2 and expressing *ste11* constitutively. White arrowheads point to asci and a possible zygote. Larger cells seen here but not in A are likely to represent diploids.

Discussion

S. pombe cells initiate sexual development in response to nitrogen starvation (Egel 1973; Egel and Egel-Mitani 1974). We have proposed that depletion of nitrogen results in a reduction in the level of intracellular cAMP which, in turn, serves as a signal for the subsequent gene expression. The activity of adenylyl cyclase, which catalyzes production of cAMP, appears to be regulated at the enzyme level in *S. pombe* cells (Maeda et al. 1990), but the mechanism that connects nitrogen depletion to reduction in this activity remains unknown. This study, however, has shed light on the subsequent steps of sexual development. It appears that *ste11* is initially induced in response to reduction in the intracellular cAMP level and that other genes required for the commitment to sexual development are subsequently activated by the Ste11 protein. Because constitutive expression of *ste11* can induce sexual development under conditions otherwise favorable for vegetative growth, expression of *ste11* appears to be the principal target of the regulation by cAMP during sexual development. Consistent with this idea, we recently found that expression of *S. pombe ste6*, which encodes a putative activator of the Ras1 protein (Hughes et al. 1990), is also regulated by Ste11 (D. Hughes and M. Yamamoto, unpubl.). Because *ras1* function is essential for mating (Fukui et al. 1986; Nadin-Davis et al. 1986), this observation reinforces the view that Ste11 plays a pivotal role in sexual development in *S. pombe*.

The deduced Ste11 protein has an HMG box, which has been shown to bind to DNA in some cases (Wright and Dixon 1988; Jantzen et al. 1990), although it may not direct DNA sequence specificity by itself (Bell et al. 1990). The results of the reverse gel-shift assay (Fig. 8) indicate that the Ste11 protein has intrinsic sequence specificity for DNA binding. However, we note that apparently only a small fraction of Ste11 protein migrated

in association with DNA in the reverse gel-shift assay, and it remains an open question whether Ste11 can perform its function *in vivo* by itself or whether it collaborates with other elements.

Among the HMG-box family of proteins, the function of Ste11, MatMc, and TDF may be related most closely, as they share conserved amino acid residues in the carboxy-terminal region of the HMG box (Fig. 3A, asterisks), although it is not yet clear whether the HMG-box proteins can be classified into subfamilies. Another interesting feature is that Ste11 regulates *matMc*, which encodes an HMG-box protein. Thus, *S. pombe* uses HMG-box proteins twice, in a cascading manner, to regulate sexual development.

Expression of the *ste11* gene is induced when the level of intracellular cAMP is reduced (see Fig. 4). How does the cAMP pathway control *ste11* expression? One possibility is that the Ste11 protein is a transcriptional activator of the *ste11* gene itself, which is inactive while phosphorylated by protein kinase A. This hypothesis assumes that nitrogen starvation lowers the activity of protein kinase A and leads to accumulation of dephosphorylated Ste11 protein, which results in transcriptional activation of *ste11* in an autoregulatory fashion. Consistent with this idea, we found a tentative TR box in the 5'-upstream region of *ste11* (Fig. 7). It remains an interesting question whether such an autoregulatory mechanism operates as a key switch for *S. pombe* sexual development.

A motif similar to the TR box, YYYATTGTTCTC, has been proposed as a consensus operator sequence of *Saccharomyces cerevisiae* hypoxic regulons and is probably recognized by the *ROX1* gene product (Lowry et al. 1990). Interestingly, Rox1 protein also has an HMG box (C. Lowry and S. Zitomer, pers. comm.). Although Rox1 regulates gene expression negatively, whereas Ste11 does

<i>mei2</i>	a	CGATTTCCTTTGTTCTAT
	b	AAGTTTCCTTTGTTTACA
	c	GAGATTTCCTTTGTTTACTT
	d	TAACCTTCCTTTGTTCTCTA
	e	TCTTTTCCTTTGTTTGT
<i>matP</i>		CTAATGCTTTGTTCCCTC
		CTCTTTCCTTTGTTCTCTA
<i>matM</i>		TTGTTGCTTTGTTTGTGG
		GGGTTTCCTTTGTTCTGTA
<i>ste11</i>		TTGTTTCCTTTGTTGCAAT
Consensus		TTCTTTGTTY

Figure 7. Conserved elements, named TR boxes, of the genes induced at the initial stage of sexual development. These sequences are found in the 5'-upstream region of each ORF. The conserved G in box d of *mei2* is essential for binding to Ste11 protein (see text). The sequences shown here correspond to the following nucleotides defined in the original publication. *mei2*: a, -1896 to -1879; b, -1874 to -1857; c, -1702 to -1685; d, -1522 to -1505; e, -917 to -900 (Watanabe et al. 1988). *matP*: 528-545 and 543-560 (Kelly et al. 1988). *matM*: 298-315 and 314-331 (Kelly et al. 1988). *ste11*: -1381 to -1364 (this study).

Figure 8. Reverse gel-shift assay of the DNA-binding activity of Ste11 protein. (A) Nucleotide sequences of three oligonucleotide probes used. ME1 carries TR box d in the *mei2* upstream region (see Figs. 7 and 9). ME2 has the same sequence as ME1 except that the invariant G-C pair in the TR box is substituted by T-A. MP1 corresponds to the upstream region of *matP* and carries two TR boxes. (B) Ste11 protein produced in wheat germ extract (for details see Materials and methods). (Lane 1) No RNA was added to the wheat germ extract; (lane 2) RNA transcribed from pET[ste11]-2 was added; (lane 3) RNA transcribed from pET[ste11]-1 was added. A full-length Ste11 protein (M_r 51,000) and a truncated Ste11 protein that lacks the HMG domain (M_r 43,000) are apparently produced in lanes 2 and 3, respectively. Samples were run in a 10% SDS-polyacrylamide gel and fluorographed. Lines at left represent markers for a molecular mass: from top to bottom, 92, 66, 42, 34, and 26 kD. (C) Mobility-shift assay of radioactive Ste11 bands. Each lane received the following combination of protein and probe DNA. (Lane 1) No Ste11, probe ME1; (lane 2) full-length Ste11, no probe; (lane 3) full-length Ste11, ME1; (lane 4) full-length Ste11, ME2; (lane 5) full-length Ste11, MP1; (lane 6) truncated Ste11, no probe; (lane 7) truncated Ste11, ME1. The majority of radioactive Ste11 protein was stacked at the top of the gel, and new shifted bands were seen only in lanes 3 and 5.

so positively, the two regulatory systems, both of which respond to changes in environmental conditions, may have conserved *cis*- and *trans*-acting elements.

Failure of *ste11* mutants to transcribe *matPi* may result from a defect in synthesis of mating factors. The *matPi* gene is highly induced by nitrogen starvation in homothallic h^{90} cells, which contain both P and M cells, but not in heterothallic h^- cells (Nielsen and Egel 1990). Thus, nitrogen starvation alone is not sufficient for induction of *matPi* transcription; the M-mating factor is essential for its expression. Because *ste11* mutants do not secrete mating factors (Y. Iino and M. Yamamoto, unpubl.), the absence of *matPi* mRNA in homothallic

ste11 cells may simply reflect the lack of gene expression necessary to produce or secrete mating factors. This explanation may also be the case for *matMi*, although there is no direct evidence on this point. The *matPc*, *matMc*, and *mei2* genes are inducible in heterothallic cells by nitrogen starvation and, hence, are likely to be regulated directly by *ste11*. Some other genes, like *ste6*, appear to

Figure 9. Changes in inducibility of *mei2* expression by mutations in TR boxes. (A) The structure of the 5'-upstream region of *mei2* is illustrated at top. (Δ) Five TR boxes shown in Fig. 7. Arrows indicate four transcription initiation sites identified previously (Watanabe et al. 1988). Some restriction sites are also indicated. The oligonucleotide ME1 used for the in vitro-binding assay (Fig. 8) and three plasmids used in the following analysis are shown schematically below. pDB[*mei2*] $\Delta 12$ has intact TR boxes c and d. pDB[*mei2*] $\Delta 12M1$ has intact box c, but box d has a G $\rightarrow$ T change. The two TR boxes in pDB[*mei2*] $\Delta 12M2$ are both mutated. Only intact TR boxes are shown as Δ . (B) JY776 transformed with either of the above three plasmids was analyzed by Northern blotting for expression of the plasmid-borne *mei2* gene in the presence or absence of a nitrogen source. Experimental conditions were the same as those in Fig. 5. (Lane 1) pDB[*mei2*] $\Delta 12$ transformant growing exponentially; (lane 2) the same transformant starved for nitrogen; (lane 3) pDB[*mei2*] $\Delta 12M1$ transformant growing exponentially; (lane 4) the same transformant starved for nitrogen; (lane 5) pDB[*mei2*] $\Delta 12M2$ transformant growing exponentially; (lane 6) the same transformant starved for nitrogen. Equal loadings of RNA in these lanes were confirmed by ethidium bromide staining of rRNAs (not shown).

be regulated both by nutritional conditions and by mating factors (D. Hughes, N. Yabana, M. Yamamoto, unpubl.). Investigation of the interaction between the nutrient-sensing and the mating factor response pathways should help us to understand further how different signals are integrated to drive cell differentiation.

Materials and methods

Strains, media, and genetic methods

Table 1 summarizes *S. pombe* strains used in this study. Complete medium YPD and minimal medium SD (Sherman et al. 1986) were used for routine culture of *S. pombe* strains. Malt extract agar medium (MEA) (Gutz et al. 1974), which contains a limited amount of nitrogen, was used to induce mating and sporulation after permitting some cell growth. Minimal medium PM and its nitrogen-free derivative PM-N (Beach et al. 1985; Watanabe et al. 1988) were used for nitrogen-starvation experiments. General genetic methods were as described by Gutz et al. (1974). Transformation (Beach et al. 1982; Okazaki et al. 1990) and gene disruption (Rothstein 1983; Fukui et al. 1986) were done as described.

Plasmids

Ylp468, in which the *ste11* gene was cloned originally, is a chimeric plasmid that consists of YIp33 developed for *S. cerevisiae* (Botstein et al. 1979) and a 10.4-kb *HindIII* fragment that carries one repeating unit of the *S. pombe* rRNA gene (Schaak et al. 1982). Because centromere-bearing plasmids, like *S. cerevisiae* YCp plasmids (Hsiao and Carbon 1981), are not available in *S. pombe*, Ylp468 was developed as a possible low-copy vector, although its copy number has not been measured accurately. In other experiments, pDB248' (Beach et al. 1982) was used as the vector to transform *S. pombe*. Three plasmids based on pDB248' were used to express *ste11* constitutively. pSX1-D4 has the *ste11* ORF inserted at the *HindIII* site of pDB248' and uses a cryptic promoter on the vector to express *ste11*. pSX1-V2 has the same structure as pSX-D4 except that a 342-bp *HindIII*–*PvuII* fragment carrying the SV40 early promoter is inserted upstream of the *ste11* ORF. This promoter has been shown to function in *S. pombe* (Käuffer et al. 1985). pSX1-A1 has a 0.7-kb *SphI*–*EcoRI* fragment carrying *S. pombe* *adh* promoter (Russell and Hall 1983) inserted at the same site. pDB(me12)Δ12, used in the experiments shown in Figure 9, was a derivative of the *mei2* clone pDB(me12)3 (Shimoda et al. 1987). Mutagenesis of pDB(me12)Δ12 to pDB(me12)Δ12M1 or pDB(me12)Δ12M2 was performed by using oligonucleotides as described previously (Kunkel 1985).

Table 1. *S. pombe* strains used

Strain	Genotype		
JY333	<i>h</i> [−]	<i>ade6-M216 leu1</i>	
JY450	<i>h</i> ⁹⁰	<i>ade6-M216 leu1</i>	
JY685	<i>h</i> ⁹⁰	<i>ade6-M216 pat1-110 ste11-29</i>	
JY736	<i>h</i> ⁹⁰	<i>ade6-M210 leu1 ste11-29</i>	
JY776	<i>h</i> ⁺	<i>ade6-M210 leu1 ura4-D18 mei2::ura4⁺</i>	
	<i>h</i> [−]	<i>ade6-M216 leu1 ura4-D18 mei2::ura4⁺</i>	
JY878	<i>h</i> ⁹⁰	<i>ade6-M216 leu1 ura4-D18</i>	
JY990	<i>h</i> ⁹⁰	<i>ade6-M216 pat1-110</i>	
JZ300	<i>h</i> [−]	<i>ade6-M216 leu1 ura4-D18 cyr1::ura4⁺</i>	
JZ396	<i>h</i> ⁹⁰	<i>ade6-M216 leu1 ura4-D18 ste11::ura4⁺</i>	

Nucleotide sequence determination

The DNA sequence of the 3.7-kb *BamHI*–*EcoRI* fragment was determined with the dideoxy chain-termination method (Sanger et al. 1977). Subclones for sequencing were produced by progressive deletion with exonuclease III and S1 nuclease (Henikoff 1984). The region shown in Figure 2 has been sequenced in both directions at least once.

Northern blotting analysis

S. pombe cells growing in PM were sampled at a cell density of 5×10^6 /ml, and the remaining cells were starved for nitrogen in PM-N for 4 hr. Total RNA was prepared from these cells, and RNA-blotting analysis was performed essentially according to Thomas (1980) with modifications as described previously (Watanabe et al. 1988). DNA fragments used as the probe to detect each transcript are as follows: *ste11*, 1.3-kb *PvuII*–*PvuII* (this study); *mei2*, 3.3-kb *PvuII*–*HindIII* (Watanabe et al. 1988); *matPc* and *matPi*, 0.9-kb *HinfI*–*MluII* (Kelly et al. 1988); *matMc* and *matMi*, 0.37-kb *BclI*–*EcoRI* (Kelly et al. 1988).

In vitro production of Ste11 protein

Two plasmids were prepared based on the vector pET-3a, which carries a bacteriophage T7 promoter (Rosenberg et al. 1987). pET[ste11]-1 carries a 2.3-kb *NdeI*–*BglII* fragment that covers amino acid residues 73–468 of Ste11 protein. pET[ste11]-2 was designed to express the complete Ste11 protein. To do so, the *NdeI* site at histidine 72–methionine 73 of the cloned *ste11* gene was mutagenized to CACATG, and a new *NdeI* site was created at the initiation codon (Kunkel 1985). The new 2.5-kb *NdeI*–*BglII* fragment was cloned in pET-3a. These two plasmids were linearized with *MluI* and the *ste11* constructs were transcribed in vitro by using T7 RNA polymerase (Tabor and Richardson 1985). After phenol–butanol extraction, RNA was translated in wheat germ extract (Promega) containing 60 mM potassium acetate and L-[³⁵S]methionine (Amersham) in 25 μl of reaction mixture. Incubation was at 25°C for 60 min.

Reverse gel-shift assay of protein–DNA binding

The DNA-binding activity of Ste11 was assayed essentially according to Hope and Struhl (1985). Each assay sample contained in a total volume of 10 μl, 3 μl of Ste11 solution (made in vitro), labeled with ³⁵S, 2 μg of poly[d(I-C)]–poly[d(I-C)] (Pharmacia), 0.5 μg of pUC118 DNA, 6 mM MgCl₂, 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 10% glycerol, and 0.1 μg of probe DNA to be examined. The assay mixture was incubated at 25°C for 30 min and analyzed by nondenaturing 5% polyacrylamide gel electrophoresis. The gel was fixed and fluorographed.

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