Molecular Biology of the Cell
Vol. 11, 3205–3217, September 2000

A Zinc-Finger Protein, Rst2p, Regulates Transcription of the Fission Yeast *ste11*$^+$ Gene, Which Encodes a Pivotal Transcription Factor for Sexual Development

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Submitted May 1, 2000; Revised June 20, 2000; Accepted July 11, 2000
Monitoring Editor: Chris Kaiser

Schizosaccharomyces pombe ste11 encodes a high-mobility group family transcriptional activator that is pivotal in sexual development. Transcription of *ste11* is induced by starvation of nutrients via a decrease of the cAMP-dependent protein kinase (PKA) activity. Here we report the identification of a novel transcription factor, Rst2p, that directly regulates *ste11* expression. Cells in which the *rst2* gene was disrupted expressed *ste11* poorly and were sterile, and this sterility could be suppressed by artificial expression of *ste11*. Disruption of *rst2* suppressed hypermating and hypersporulation in the PKA-null mutant, whereas overexpression of *rst2* induced sexual development in the PKA-activated mutant. Cloning analysis indicated that Rst2p was a Cys_2His_2 zinc-finger protein carrying 567 amino acid residues. Rst2p could bind specifically to a stress response element–like *cis* element located in the *ste11* promoter region, which was important for *ste11* expression. Meanwhile, transcription of *ste11* was reduced significantly by a defective mutation in itself. An artificial supply of functional Ste11p circumvented this reduction. A complete Ste11p-binding motif (TR box) found in the promoter region was necessary for the full expression of *ste11*, suggesting that Ste11p is involved in the activation of *ste11*. We conclude that transcription of *ste11* is under autoregulation in addition to control through the PKA–Rst2p pathway.

INTRODUCTION

Cells of the fission yeast *Schizosaccharomyces pombe* initiate sexual development under starvation of nutrients, especially that of nitrogen (Egel, 1973; Egel and Egel-Mitani, 1974). Starvation reduces the level of intracellular cAMP, which in turn results in the inactivation of cAMP-dependent protein kinase (PKA) (Yamamoto, 1996). Genes encoding the catalytic and regulatory subunits of *S. pombe* PKA have been identified, *pka1* for the catalytic subunit (Maeda *et al.*, 1994) and *cgs1* for the regulatory subunit (DeVoti *et al.*, 1991). Physiological and mutational analyses established that a high level of PKA activity blocks *S. pombe* cells from initiating sexual development, whereas a low level promotes sexual development irrespective of nutritional conditions (reviewed by Yamamoto, 1996).

Inactivation of PKA triggers expression of the *ste11* gene, which encodes a transcription factor required to activate transcription of a number of genes involved in the progression of sexual development (Sugimoto *et al.*, 1991; Yamamoto, 1996). Expression of *ste11* is not inducible in cells defective in *cgs1*, i.e., with a high PKA activity (H.K. and M.Y., unpublished results). Ste11p is a DNA-binding protein that belongs to the high-mobility group (HMG) family. It binds to a nucleotide motif, TTCTTTGTTY, that is termed the TR box (Sugimoto *et al.*, 1991). TR boxes have been found in the promoter regions of a number of genes regulated by Ste11p, including *mat1-P*, *mat1-M*, *mei2* (Sugimoto *et al.*, 1991), *esc1* (Benton *et al.*, 1993), *ste6* (Hughes *et al.*, 1994), and *fus1* (Petersen *et al.*, 1995).

Subsequent studies revealed that expression of *ste11* is regulated by a stress-responsive MAPK, Phh1/Spc1/Sty1p (Kato *et al.*, 1996; Shiozaki and Russell, 1996), in addition to PKA. This MAPK is regulated by the Wis1p MAPK kinase (Millar *et al.*, 1995; Shiozaki and Russell, 1995) and has been shown to phosphorylate a CRE-binding protein encoded by the *atf1/gad7* gene. Loss of function of *wis1*, *phh1/spc1/sty1*, or *atf1/gad7* greatly reduces the level of *ste11* transcription (Shiozaki and Russell, 1995; Takeda *et al.*, 1995; Kanoh *et al.*, 1996). Atf1/Gad7p apparently forms a complex with another CRE-binding protein, Pcr1p, which is also required to

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

Strain	Genotype
JX231	*h*^{90} *ade6-M216 leu1 rst2::ura4*$^{+}$ *ura4-D18*
JX232	*h*^{90} *ade6-M210 leu1 rst2::ura4*$^{+}$ *ura4-D18*
JX233	*h*$^{-}$ *ade6-M216 leu1 rst2::ura4*$^{+}$ *ura4-D18*
JX239	*h*^{90} *ade6-M216 leu1 pka1::ura4*$^{+}$ *rst2::ura4*$^{+}$ *ura4-D18*
JX250	*h*$^{+}$*/h*$^{-}$ *ade6-M210/ade6-M216 leu1/leu1 rst2::ura4*$^{+}$ */rst2::ura4*$^{+}$ *ura4-D18/ura4-D18*
JY333	*h*$^{-}$ *ade6-M216 leu1*
JY362	*h*$^{+}$*/h*$^{-}$ *ade6-M210/ade6-M216 leu1/leu1*
JY450	*h*^{90} *ade6-M216 leu1*
JY476	*h*^{90} *ade6-M210 leu1*
JY858	*h*^{90} *ade6-M216 leu1 ste11-029*
JZ396	*h*^{90} *ade6-M216 leu1 ste11::ura4*$^{+}$ *ura4-D18*
JZ633	*h*^{90} *ade6-M216 leu1 pka1::ura4*$^{+}$ *ura4-D18*
JZ858	*h*^{90} *ade6-M216 leu1 cgs1::ura4*$^{+}$ *ura4-D18*

activate *ste11* transcription (Kanoh *et al.*, 1996; Watanabe and Yamamoto, 1996). Thus, a heterodimeric transcription factor is likely to play a role in the regulation of *ste11* expression, although it is not known if its involvement is direct or indirect.

Elucidation of regulatory elements that may directly activate transcription of *ste11* is undoubtedly important to understand how fission yeast cells commit themselves to the initiation of sexual differentiation. Hence, we set out to search for new factors that might affect *ste11* expression. We also analyzed the promoter region of *ste11* precisely. In this report, we show that expression of *ste11* is directly regulated by two transcription factors. One is a novel zinc-finger protein, Rst2p, which binds to a stress response element (STRE)-like *cis* element located in the upstream regulatory region of *ste11* by means of its two Cys_2His_2 zinc-finger motifs. The other is the *ste11* gene product itself.

MATERIALS AND METHODS

Yeast Strains, Media, and Genetic Methods

S. pombe strains used in this study are listed in Table 1. Cells were routinely grown in complete medium or minimal medium at 30°C (Sherman *et al.*, 1986). Either malt extract agar medium (Gutz *et al.*, 1974) or synthetic sporulation medium (Egel and Egel-Mitani, 1974) was used for the induction of mating and sporulation. Liquid minimal medium (PM) and its nitrogen-free version (PM-N) (Beach *et al.*, 1985; Watanabe *et al.*, 1988) were used in nitrogen-starvation experiments. General genetic methods were described previously (Gutz *et al.*, 1974). Transformation of *S. pombe* was done as described (Okazaki *et al.*, 1990).

Plasmids

Two *S. pombe–Escherichia coli* shuttle vectors, pDB248′ (Beach *et al.*, 1982) and pREP1 (Maundrell, 1990), were used. pDB-ste11^{+} was pDB248′-based and carried the entire *ste11* ORF under the control of the cryptic read-through promoter on the vector (Watanabe and Yamamoto, 1996). pREP-ste11^{+} was constructed by connecting a 2.5-kilobase (kb) *Nde*I–*Bgl*II fragment, which contained the complete *ste11* ORF (Sugimoto *et al.*, 1991), to the *nmt1* promoter on pREP1. pDM+, which carried a 1.4-kb *Sph*I–*Bam*HI fragment corresponding to the 5′ regulatory region of *ste11* and part of the *mei2* gene as a reporter, was derived from pDB(mei2)3 (Shimoda *et al.*, 1987). Modification of pDM+ was performed by site-directed mutagenesis (Kunkel, 1985). Oligonucleotides used for construction of modified plasmids were as follows (altered nucleotides are underlined): pDM1, 5′-AAAATCAAAAAAAGAAATTC-3′; pDM2, 5′-AAGTCAAAAAAAAGAAAAGA-3′; pDM3, 5′-CAAAATGTGTATGATCAGAAGGGAC-3′; and pDM4, 5′-GAGTTAAGGATCAGTGGAGAAAG-3′. pDM12 carried both mutations introduced in pDM1 and pDM2, and pDM34 carried both mutations introduced in pDM3 and pDM4.

Northern Blotting

S. pombe cells either growing logarithmically or starved for nitrogen were prepared as described above. Total RNA was extracted from them, and RNA blot analysis was performed according to Watanabe *et al.* (1988). A 1.3-kb *Pvu*II–*Pvu*II DNA fragment was used as the probe to detect *ste11* mRNA (this study), and a 3.3-kb *Pvu*II–*Hin*dIII fragment was used to detect *mei2* mRNA (Watanabe *et al.*, 1988). The intensity of each band on the blot was quantified with the use of a built-in program of the image-analysis software Adobe Photoshop (Adobe Systems, Mountain View, CA). The relative intensity of transcription was then calculated with the amount of rRNA, which was similarly quantified, as the loading control.

Isolation of rst2

An *S. pombe* genomic library constructed in the vector pREP1 (Maundrell, 1990) was introduced into a haploid *cgs1*-disruption strain JZ858 (*h*^{90} *cgs1::ura4*$^{+}$). Transformants were plated on sporulation medium and incubated at 30°C for 4 d. Colonies formed were exposed to iodine vapor to stain cells that could conjugate and sporulate. After confirming the dependence of their fertility on plasmids, 11 independent plasmids were recovered from the positive colonies. These plasmids could be classified into four groups by Southern blot analysis (our unpublished results). After elimination of known genes, including *ste11*, a plasmid named pRD2-27, which apparently carried a new gene, was chosen for further analysis.

Nucleotide Sequence Determination

The 1.7-kb *Sph*I–*Eco*RV fragment carrying the *ste11* promoter region and the 3.8-kb *Sac*I–*Sph*I fragment carrying the *rst2* gene were subcloned into pUC119 (Takara, Kusatsu, Japan). The DNA sequence was determined with the use of the dideoxy chain-termination method (Sanger *et al.*, 1977). Subclones for sequencing were produced by unidirectional deletion (Henikoff, 1984). The nucleotide sequences shown in this paper have been determined in both strands.

Disruption of the rst2 Gene

The 1.2-kb *Kpn*I–*Nde*I fragment within the *rst2* ORF was removed and replaced by the 1.8-kb *ura4*$^{+}$ cassette (Grimm *et al.*, 1988). A *Cla*I–*Eco*RI fragment containing this disruption construct was introduced into JY879 (*h*^{90} *ade6-M210 leu1 ura4-D18*). Successful disruption of *rst2* was confirmed by Southern blot analysis (our unpublished results). To exclude the possibility that the *rst2*-disruption strain had acquired any additional mutation, we crossed it with both homothallic and heterothallic *ura4-D18* strains and performed tetrad analyses. In every case, we obtained four viable progeny, which segregated in two Ura^{+}:two Ura^{-}, indicating that disruption of *rst2* is not lethal.

Mating and Sporulation Assay

Mating and sporulation frequencies were calculated according to the procedure described previously (Kunitomo *et al.*, 1995). Each value in Table 2 is an average of the results obtained from at least two independent colonies.

Primer Extension Analysis

Total RNA was prepared from a wild-type strain, JY450, and primer extension analysis for the *ste11* transcript was performed as described (Watanabe *et al.*, 1988). The oligonucleotide used as the primer was 5′-AACGAGGCAAAAGCTCT-3′, which corresponds to nucleotides +178 to +162 on the *ste11* antisense strand.

Assay of β-Galactosidase Activity

pSL3 carried a *ste11–lacZ* translational fusion composed of a 5.6-kb *Sma*I–*Pvu*II fragment that covered nucleotides −3400 to +2230 of *ste11* and the *lacZ* gene derived from pMC1871 (Clontech, Palo Alto, CA). The vector was a modified version of pREP1 lacking the *nmt1* promoter. Deletion derivatives of pSL3 were constructed by inserting the following fragments in place of the *Sma*I–*Pvu*II fragment in the chimeric plasmid: pSL6, −833 to +2230; pSL7, −366 to +2230; pSL8, −194 to +2230; pSL9, −113 to +2230; pSL10, −159 to +2230; and pSL11, +7 to +2230. pSL6(ΔEV) and pSL6(ΔNd) were derivatives of pSL6 that lacked +7 to +1771 and −227 to +1771, respectively. pSLN(ΔEV) carried −229 to +2230, excluding +7 to +1771. A heterothallic haploid strain, JY333, was transformed with these plasmids. Transformants were grown in PM to 1×10^7 cells/ml. A portion was sampled (log-phase cells), and the remainder was shifted to PM-N and grown for another 4 h (nitrogen-starved cells). After harvesting of cells by centrifugation, the β-galactosidase activity was determined as described (Guarente, 1983). The data presented in Table 3 are averages of at least two independent measurements.

Gel Mobility Shift Assay

To assess the DNA-binding ability of Rst2p, two kinds of wild-type probes (WTa and WTb) and four kinds of mutant forms (Ma, Mb1, Mb2, and Mb3) were prepared. WTa: 5′-GTCCCTTCCCCTCATACACATTTTG-3′ annealed with 3′-CAGGGAAGGGGAGTATGTGTAAAAC-5′, a blunt-end dsDNA fragment corresponding to −202 to −178 of the *ste11* promoter region. WTb: 5′-TTGTCCCTTCCCCTCATACACATTT-3′ annealed with 3′-GGGAAGGGGAGTATGTGTAAAACCG-5′, a dsDNA fragment corresponding to −200 to −180 with four additional nucleotides protruding from each 5′ end. Ma: a derivative of WTa carrying TGA (as of the sense strand) instead of CCC at −194 to −192. Mb1: a derivative of WTb carrying A instead of C at −195. Mb2: a derivative of WTb carrying TGA instead of CCC, similar to Ma. Mb3: a derivative of WTb carrying G instead of C at −190. These oligonucleotide probes were labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP.

Two plasmids derived from pET19b (Novagen, Madison, WI) were used to produce histidine-tagged Rst2p derivatives in bacteria. The initiation codon of the *rst2* gene (AGTATG) was replaced by the *Nde*I target site (CATATG). A 0.6-kb *Nde*I–*Sph*I fragment was used to construct pET-Rst2ZF, which could produce a tagged protein carrying the N-terminal 183 amino acid residues of Rst2p with the two zinc-finger motifs. Similarly, a 1.7-kb *Hin*cII–*Hin*cII fragment was cloned into the pET19b to generate pET-Rst2ΔZF, which could produce a tagged protein carrying the C terminus of Rst2 with no zinc-finger motifs (amino acids 113–567).

For analysis of Ste11p–TR box interaction, a histidine-tagged HMG domain of Ste11p (amino acids 1–239) was expressed from pETste11HMG, which was constructed by inserting a 0.7-kb *Nde*I–*Hin*cII fragment within the *ste11* gene (Sugimoto *et al.*, 1991) into pET19b.

Recombinant proteins were prepared and purified as instructed by the vector supplier (Novagen), and the buffer was exchanged with buffer A (Thukral *et al.*, 1989, 1991) through ultrafiltration. We used binding conditions described for the *Saccharomyces cerevisiae ADR1* gene product (Eisen *et al.*, 1988; Thukral *et al.*, 1989), with minor modifications. Each reaction was carried out in a total volume of 10 μl, with 1 μg of recombinant protein, 0.05 pmol of ^{32}P-labeled probe, and 1 μg of poly[d(I-C)]–poly[d(I-C)] (Pharmacia, Piscataway, NJ). In some cases, 0.01 μg of recombinant protein was used with a supplement of 300 μg/μl BSA. Protein–DNA complexes were separated in a prerun polyacrylamide gel (3.5 or 5%) containing glycerol in Tris–glycine buffer. The gel was dried on Whatman (Clifton, NJ) 3MM paper and autoradiographed.

DNase I Footprinting

DNase I footprinting was done essentially as described (Sawadogo and Roeder, 1985). We cloned a 1.3-kb *Sph*I–*Bam*HI fragment of the *ste11* promoter region (−834 to +575) into pUC119. The coding strand was labeled with [γ-^{32}P]ATP at the *Nde*I site (−228) with the use of T4 polynucleotide kinase, the DNA preparation was cut with *Hin*dIII, and a 0.8-kb fragment was recovered by electrophoresis. The noncoding strand was labeled at the *Eco*RV site (+6) and cut with *Eco*RI to obtain a 0.8-kb fragment. About 0.2 μg of each end-labeled probe was allowed to bind with 0.3 and 1.5 μg of recombinant Rst2ZF, or 0.2 and 1.0 μg of recombinant Ste11HMG protein, in 70 μl of buffer A containing 4 mg of poly[d(I-C)]–poly[d(I-C)] for 10 min on ice. DNase I was added to the final concentration of 0.5 μg/ml and incubated for 2 min at room temperature. Reaction was stopped by adding 25 μl of saturated ammonium acetate followed by 325 μl of ethanol for ethanol precipitation. Reaction products were loaded on a 7% sequencing gel together with the probes subjected to Maxam-Gilbert sequencing reactions. After separation, the gel was autoradiographed.

RESULTS

Identification of the rst2 Gene Encoding a Zinc-Finger Protein

To obtain possible new factors involved in the regulation of sexual development, we isolated high-copy-number suppressors of the sterility of the *cgs1*-deficient mutant, which retained a high PKA activity, as described in MATERIALS AND METHODS. A suppressor plasmid, named pRD2-27, could recover both mating and sporulation in the *cgs1* mutant (Table 2), thereby restoring transcription of *ste11* (our unpublished results; see below).

The nucleotide sequence of a 3.8-kb *Sac*I–*Sph*I genomic fragment carried by pRD2-27, which has been deposited in DDBJ/EMBL/GenBank under the accession number AB025941, contained an uninterrupted ORF of 567 amino acids (Figure 1B). The direction of transcription of this ORF was opposite that of the cryptic promoter on the vector, suggesting that the cloned fragment carried the authentic promoter for the ORF. Subcloning, as summarized in Figure 1A, confirmed that this ORF was responsible for the suppression of Δ*cgs1*. We hereafter call this suppressor gene *rst2* (recovery of *ste11* expression). The C-terminal 176 amino acid residues of the deduced *rst2* gene product (Rst2p) were apparently dispensable for the suppression (Figure 1A, 2.6-kb *Sac*I–*Nde*I fragment).

Features of Rst2p were investigated by the FASTA homology search algorithm (Lipman and Pearson, 1985). Rst2p carried two zinc-finger motifs of the Cys_2His_2 class at its N terminus (Figure 1B). They were most similar to the pair of zinc fingers carried by the *Saccharomyces cerevisiae ADR1* gene product, which is a key transcription factor involved in glucose repression (Shuster *et al.*, 1986; Eisen *et al.*, 1988) (Figure 1C). In addition, Rst2p carried five consecutive arginine residues at positions 134–138, which might be a nuclear localization signal, followed by three possible phosphorylation sites by PKA (Figure 1B; see DISCUSSION).

Table 2. Mating and sporulation frequency of *S. pombe* strains and transformants

Strain	Relevant genotype	Plasmid carried	Mating (*sporulation) frequency (%)
JY476	h^{90}		61.9
JZ858	h^{90} *cgs1*Δ	pREP1	<0.1
JZ858	h^{90} *cgs1*Δ	pDB-cgs1$^+$	79.4
JZ858	h^{90} *cgs1*Δ	pRD2-27 (*rst2*$^+$)	53.8
JZ858	h^{90} *cgs1*Δ	pREP-ste11$^+$	10.5
JX232	h^{90} *rst2*Δ		0.1
JX232	h^{90} *rst2*Δ	pDB248′	<0.1
JX232	h^{90} *rst2*Δ	pDB-rst2$^+$	83.6
JX232	h^{90} *rst2*Δ	pDB-ste11$^+$	29.7
JY362	h^+/h^-		87.5*
JX250	h^+/h^- *rst2*Δ		<0.1*

Northern blot analysis of *rst2* mRNA in various *S. pombe* strains indicated that the gene was transcribed only weakly into a single mRNA species of 3.0 kb in length. The level of *rst2* expression was not significantly affected by nutritional conditions, nor was it affected by mutations in *cgs1*, *pka1*, *ste11*, or *phh1/spc1/sty1* (our unpublished results).

Phenotypes of the rst2-Disruptant

The *rst2* gene was disrupted as detailed in MATERIALS AND METHODS (Figure 1A). Disruption of *rst2* was not lethal. Δ*rst2* cells appeared normal in shape, and they grew at the same rate as wild-type cells on any conventional medium examined (our unpublished results). However, a haploid Δ*rst2* strain (JX232) turned out to be impaired in conjugation, and a diploid Δ*rst2* strain (JX250) was unable to sporulate (Table 2). Transcription of *ste11* was greatly reduced in the *rst2*-disruptant (Figure 2). The sterility of JX232 could be rescued by artificial expression of *ste11* (Table 2). These results indicate that Rst2p plays an essential role in the activation of *ste11* transcription and that loss of *ste11* expression is the major reason that *rst2*-deficient cells become sterile.

Cells defective in *rst2* did not lose their viability under nutrition-depleted conditions, unlike Δ*cgs1* cells (DeVoti *et al.*, 1991) (our unpublished results). They displayed shortened cell morphology in the stationary phase (Figure 3B), resembling the *ste11* mutant rather than the *cgs1* mutant, the latter of which maintained elongated cell morphology under starvation (DeVoti *et al.*, 1991). These observations suggest that the sterility of the *rst2* disruptant is unlikely to be due to increased PKA activity. This was confirmed by analysis of a Δ*pka1* Δ*rst2* double mutant. If disruption of *rst2* induces sterility through hyperactivation of PKA, the double mutant should behave like the Δ*pka1* strain and hence be derepressed for sexual development. The results obtained were the opposite. The Δ*pka1* Δ*rst2* strain JX239 was sterile (Figure 3D), suggesting that Rst2p would function downstream of PKA in a cascade.

Table 3. Deletion analysis of *ste11* promoter activity

Construct	5′ end	Internal deletion	UASst	TR1	β-Galactosidase (10^3 U)
pSL3	−3400		+	+	9.0
pSL6	−833		+	+	30.6
pSL6(ΔEV)	−833	+7 to +1771	+	+	11.6
pSL6(ΔNd)	−833	−227 to +1771	−	−	0.1
pSL7	−366		+	+	8.7
pSLN(ΔEV)	−229	+7 to +1771	+	+	8.7
pSL8	−194		−	+	0.2
pSL10	−159		−	+	0.1
pSL9	−113		−	−	0.2
pSL11	+7		−	−	0.1

Transcription Start Site of the ste11 Gene

We previously reported the nucleotide sequence of the *ste11* locus over 3.6 kb, including a 1.6-kb upstream noncoding region (Sugimoto *et al.*, 1991). Because subsequent analyses indicated that this sequence was unlikely to cover the authentic transcription start site, we isolated a 1.7-kb *Sph*I–*Eco*RV genomic fragment that carried another upstream region (Figure 4A). The nucleotide sequence of the proximal 0.3 kb of this fragment was the same as we reported previously (Sugimoto *et al.*, 1991), whereas the sequence of the remaining 1.4 kb was totally new. We found two complete TR boxes, which we call TR1 and TR2 hereafter, in this new sequence. They were located at −155 to −146 and +357 to +366, respectively, relative to the major transcription start site (Figure 4A; see below). The new sequence has been deposited in DDBJ/EMBL/GenBank under the accession number AB025942.

To clarify the transcription start site of *ste11*, we carried out primer extension analysis as detailed in MATERIALS AND METHODS. The majority of *ste11* mRNA was found to start from either of the two adjacent adenine residues located 2183 and 2182 nucleotides upstream of the translation initiation site (Figure 4B). Because the latter residue was used more frequently as the start site, we assigned it to position +1 (Figure 4A). A cluster of nucleotides A and T, which might contain a TATA element, was found at −72 to −55.

Upstream Sequences Required for the Expression of ste11

To identify sequences required for *ste11* expression, we performed deletion analysis of a chimeric gene carrying the upstream region of *ste11*. The parental plasmid pSL3 carried a *ste11–lacZ* fusion gene in which the *lacZ* ORF was connected to a 3.0-kb DNA fragment that covered the promoter region of *ste11* down to the initiation codon. The product of this fusion gene was functional as β-galactosidase (Table 3). Various deletion derivatives of pSL3 were introduced into a host strain, and each transformant was examined for the expression of β-galactosidase activity under nitrogen-depleted conditions. Although we may be able to postulate a number of scattered sequences that can partially increase or decrease the level of *ste11* expression from the results summarized in Table 3, we assume that an unequivocal inference from the data will be the presence of a sequence(s) essential for *ste11* expression between nucleotides −229 and −194. Any derivative carrying nucleotides −229 to +1 could exhibit β-galactosidase activity at a comparable level to pSL3. In contrast, pSL8, in which the nucleotides preceding −194

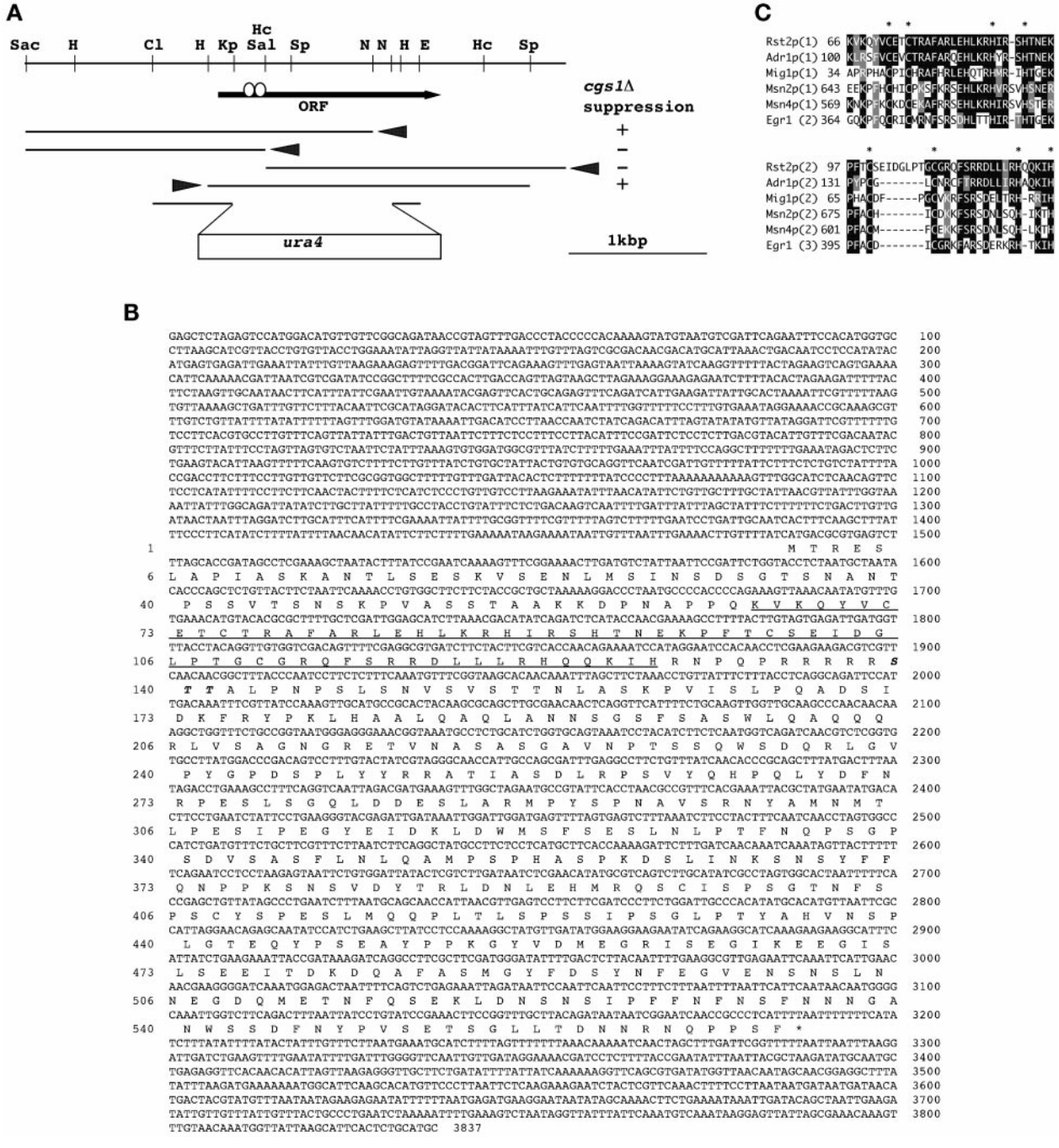

Figure 1. The structure of the *rst2* gene. (A) A restriction map of the *rst2* locus and functional analysis of the subclones. The arrow indicates the position and direction of the *rst2* ORF. Open circles on the arrow represent the two Cys_2His_2 zinc-finger motifs. Arrowheads indicate the orientation of transcription from a cryptic promoter on the vector. Each subclone was examined for the ability to promote mating and sporulation in JZ858 (*cgs1*Δ). The construct used to disrupt the *rst2* gene is shown at the bottom. Restriction sites are abbreviated as follows: Cl, *Cla*I; E, *Eco*RI; H, *Hin*dIII; Hc, *Hin*cII; Kp, *Kpn*I; N, *Nde*I; Sac, *Sac*I; Sal, *Sal*I; and Sp, *Sph*I. (B) The nucleotide sequence of a 3.8-kb *Sac*I–*Sph*I fragment carrying *rst2* and the amino acid sequence of the deduced gene product. The two zinc-finger motifs are underlined. Amino acid residues that may be phosphorylated by PKA are italicized. (C) Comparison of zinc-finger motifs among Rst2p and its close homologues. The two zinc-finger motifs of Rst2p (residues 66–128) are aligned with *S. cerevisiae* Adr1p (residues 100–155; Hartshorne *et al.*, 1986), *S. cerevisiae* Mig1p (residues 34–90; Nehlin and Ronne, 1990), *S. cerevisiae* Msn2p (residues 643–698), *S. cerevisiae* Msn4p (residues 569–624; Estruch and Carlson, 1993), and the human *EGR1* gene product (residues 364–419; Sukhatme *et al.*, 1988). Amino acid residues identical to those of Rst2p are shown in white against black. Conserved amino acids are shown in white against gray. The number of fingers assigned for each individual protein is indicated in parentheses. Asterisks indicate cysteine and histidine residues conserved in the zinc-finger motif.

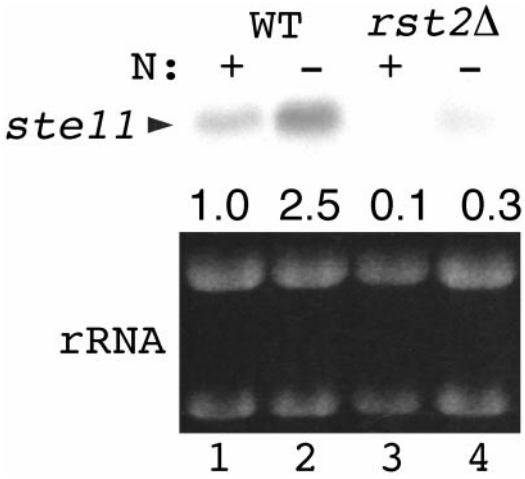

Figure 2. Necessity of *rst2* for the transcription of *ste11*. Expression of *ste11* was examined in the *rst2*$^+$ (JY333) and *rst2*Δ (JX233) strains by Northern blot analysis. Total RNA was prepared from wild-type cells (lanes 1 and 2) and *rst2*Δ cells (lanes 3 and 4), either growing vegetatively (N+; lanes 1 and 3) or being starved for nitrogen (N−; lanes 2 and 4). rRNA stained with ethidium bromide is shown in the lower panel as loading controls. The relative intensity of *ste11* transcription in each lane was calculated as described in MATERIALS AND METHODS and is presented under the top panel.

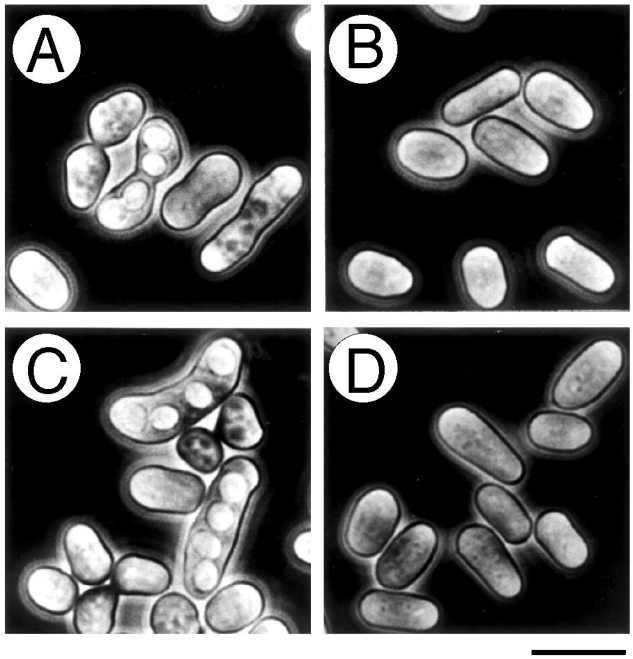

Figure 3. Suppression of the hypersporulation phenotype of the *pka1*Δ strain by disruption of *rst2*. The homothallic wild-type strain JY450 (A), the *rst2*Δ strain JX231 (B), the *pka1*Δ strain JZ633 (C), and the *pka1*Δ *rst2*Δ strain JX239 (D) were grown on malt extract agar medium for 2 d at 30°C, and cells were photographed under the phase-contrast microscope. Bar, 10 μm.

were deleted, exhibited only 2% of the β-galactosidase activity compared with the parent.

Rst2p Binds to the Promoter Region of ste11 In Vitro

We speculated that Rst2p might directly regulate *ste11* transcription. To determine whether Rst2p could bind to the promoter region of *ste11*, we carried out DNase I footprint analysis. A histidine-tagged polypeptide corresponding to the N-terminal 183 amino acid residues of Rst2p, which contained the two zinc-finger motifs, was mixed with *ste11* DNA, and nucleotides protected from nuclease digestion were examined as detailed in MATERIALS AND METHODS. When the sequence between −228 and +6 was investigated, nucleotides −198 to −183 on the coding strand and −185 to −195 on the noncoding strand were found to be protected (Figure 5). Together with the observations described in the previous section, these results suggest that Rst2p and the protected region are likely to regulate *ste11* expression in cooperation, as a *trans* and a *cis* element, respectively. Hereafter, we call the sequence from −198 to −183 UASst (upstream activating sequence for *ste11*).

Zinc-Finger–dependent Binding of Rst2p to UASst

The DNA-binding specificity of Rst2p was characterized with the use of double-stranded oligonucleotide probes corresponding to nucleotides −202 to −178 of *ste11*, which covered UASst. Gel mobility shift assay was done as detailed in MATERIALS AND METHODS. The wild-type probe (WTa) could bind to the recombinant Rst2p protein (Figure 6A, lanes 3–5), whereas no binding was observed when the central three nucleotides (CCC; −194 to −192) were substituted by TGA (probe Ma; lane 12). The addition of the unlabeled wild-type oligonucleotide interfered with the binding of the labeled probe in a quantitative manner (lanes 8 and 9), whereas the unlabeled mutant oligonucleotide was not effective (lanes 10 and 11). When a cation-chelating reagent, 1,10-phenanthroline, was added to the mix at the final concentration of 10 mM, Rst2p lost its DNA-binding ability (lane 6). The addition of zinc ions in excess could rescue it (lane 7). Rst2p without the zinc-finger motifs did not form a complex with DNA (lane 2). These results indicate that Rst2p binds to the target sequence through the zinc-finger motifs.

The Core Sequence of UASst Resembles S. cerevisiae STRE

We noticed the apparent similarity between the Rst2p-binding sequence and the STRE identified in *S. cerevisiae* (Marchler *et al.*, 1993). The core sequence of STRE is 5′-CCCCT-3′, which is recognized by zinc-finger proteins Msn2p and Msn4p (Martínez-Pastor *et al.*, 1996; Schmitt and McEntee, 1996). By homology modeling, the first three CG pairs of STRE are supposed to interact with one of the zinc fingers carried on each Msn protein, and the remaining two pairs and the following sixth pair are supposed to interact with the other zinc finger (Martínez-Pastor *et al.*, 1996). Thus, if the similarity between STRE and UASst is significant, the sequence CCCCTC (−195 to

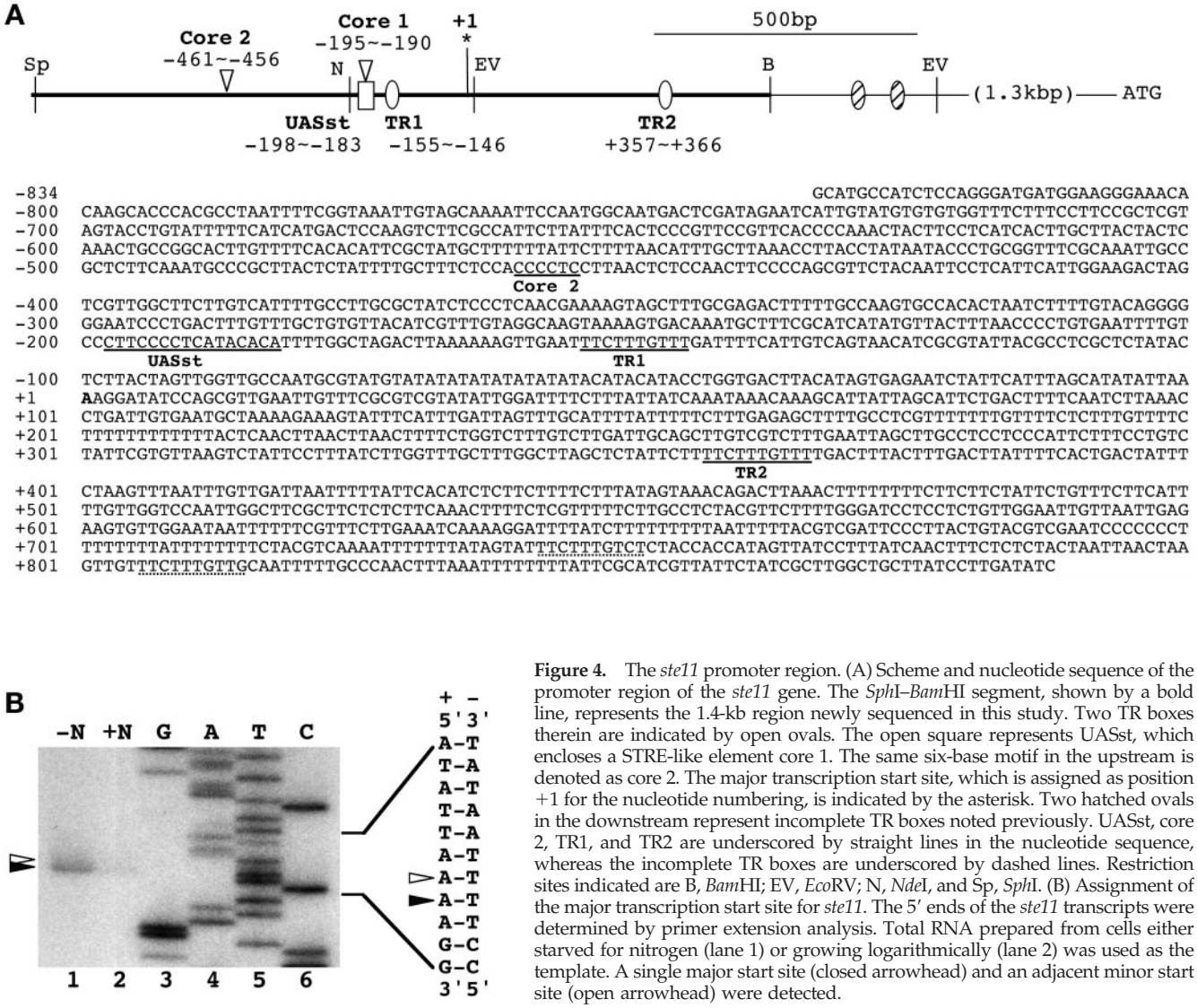

Figure 4. The *ste11* promoter region. (A) Scheme and nucleotide sequence of the promoter region of the *ste11* gene. The *Sph*I–*Bam*HI segment, shown by a bold line, represents the 1.4-kb region newly sequenced in this study. Two TR boxes therein are indicated by open ovals. The open square represents UASst, which encloses a STRE-like element core 1. The same six-base motif in the upstream is denoted as core 2. The major transcription start site, which is assigned as position +1 for the nucleotide numbering, is indicated by the asterisk. Two hatched ovals in the downstream represent incomplete TR boxes noted previously. UASst, core 2, TR1, and TR2 are underscored by straight lines in the nucleotide sequence, whereas the incomplete TR boxes are underscored by dashed lines. Restriction sites indicated are B, *Bam*HI; EV, *Eco*RV; N, *Nde*I, and Sp, *Sph*I. (B) Assignment of the major transcription start site for *ste11*. The 5′ ends of the *ste11* transcripts were determined by primer extension analysis. Total RNA prepared from cells either starved for nitrogen (lane 1) or growing logarithmically (lane 2) was used as the template. A single major start site (closed arrowhead) and an adjacent minor start site (open arrowhead) were detected.

−190) in UASst is likely to be unchangeable. This was examined by gel mobility shift assay with the use of modified probes. The wild-type probe used here (WTb) consisted of a double-stranded oligonucleotide corresponding to nucleotides −200 to −180, with four additional nucleotides protruding from each 5′ end (Figure 6B). We prepared three variants of it. One of them, called Mb1, carried A instead of the first C (−195) in the core sequence. Mb2 carried TGA instead of the central CCC (−194 to −192), similar to Ma used above. Mb3 carried G instead of the last C of the six nucleotides (−190). Gel mobility shift assay with these mutant probes demonstrated that none of them could bind to Rst2p (Figure 6B, lanes 5–7), suggesting strongly that the CCCCTC sequence is a pivotal element of UASst recognized by the zinc-finger motifs of Rst2p.

Mutation in UASst Decreases ste11 Expression

The necessity of UASst for transcription of *ste11* was examined by Northern blot analysis with the use of a *ste11–mei2* fusion gene. This fusion gene was constructed by connecting a 1.4-kb *Sph*I–*Bam*HI fragment, which carried the entire regulatory region of *ste11*, to the C-terminal half of the *mei2* ORF, which could be conveniently detected in Northern analysis. We constructed a mutant plasmid in which the core sequence of UASst (CCCCTC; core 1) was altered to CTGATC. In addition, it came to our notice that the *ste11* gene carried another core sequence (core 2) in the farther upstream region (−461 to −456), and we also made a mutant plasmid carrying an identical mutation in this sequence. Cells transformed with either the parental plasmid or one of the mutant plasmids were tested for expression of the fusion gene in the presence and absence of nitrogen. As shown in Figure 7A, the mutation in core 1 reduced transcription of the gene significantly (lanes 1 and 2 versus lanes 3 and 4). In contrast, the mutation in core 2 rather increased transcription (lanes 5 and 6; see DISCUSSION). The double mutant gave results consistent with these observations (lanes 7 and

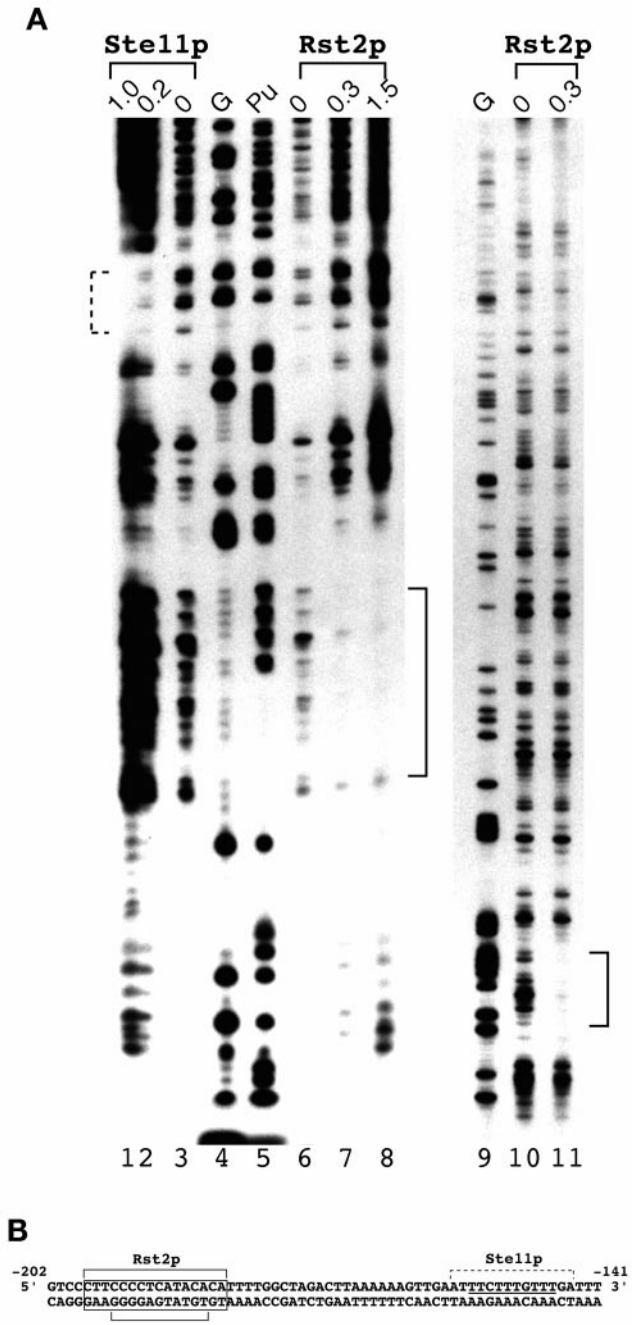

Figure 5. DNase I footprint analysis of the *ste11* promoter region. (A) Left panel, The coding strand of the *ste11* promoter region, labeled and processed as described in MATERIALS AND METHODS, was incubated with either 1.0 μg (lane 1) or 0.2 μg (lane 2) of recombinant Ste11p and either 0.3 μg (lane 7) or 1.5 μg (lane 8) of recombinant Rst2p. Each sample was subjected to DNase I digestion. Lanes 3 and 6 represent control analysis with no protein addition. Lanes 4 and 5 represent the G and the purine ladders, respectively. Right panel, Similar analysis was done with the noncoding strand. DNA was labeled and incubated with either no (lane 10) or 0.3 μg (lane 11) of recombinant Rst2p before DNase I digestion. Lane 9 represents the G ladder. Nucleotides protected from DNase I digestion are indicated by parentheses. (B) A summary of the protected sequences.

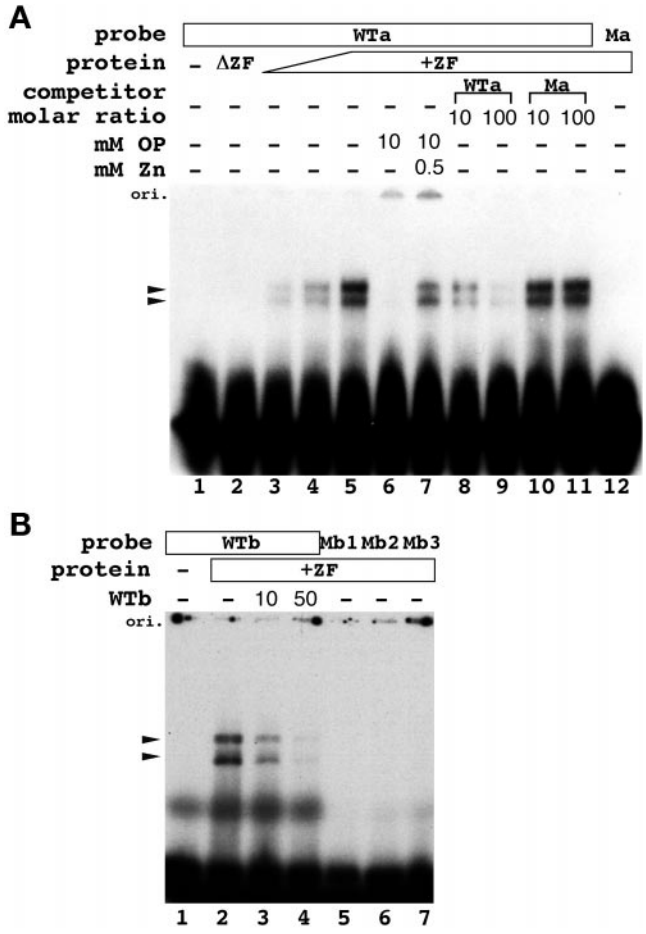

Figure 6. Specific binding of Rst2p to UASst in vitro. Recombinant Rst2p, either carrying the two zinc-finger motifs or lacking them, was incubated with labeled DNA probes, and formation of a protein–DNA complex was examined by gel mobility shift assay. (A) The protein with the zinc-finger motifs was mixed with a wild-type probe (WTa) in gradually increasing amounts (lane 3, 0.01 μg; lane 4, 0.1 μg; and lane 5, 1 μg of protein). Lane 6 is the same as lane 5 except for the addition of 10 mM 1,10-phenanthroline. Lane 7 is the same as lane 6 but it further accepted 0.5 mM zinc sulfate. Lanes 8–11 are the same as lane 5 except that cold competitor oligonucleotides were added to them in the molar ratios, as indicated in the panel. Lane 1 represents a mock experiment with no protein addition. Binding of the wild-type probe to the protein with no zinc-finger motifs (1 μg of protein) was examined in lane 2, and binding of a mutant probe (Ma) to the intact protein was examined in lane 12. (B) Mutational dissection of the binding sequence. A wild-type probe (WTb) was incubated with 0.01 μg of the protein carrying the zinc-finger motifs, together with (lanes 3 and 4) or without (lane 2) cold competitors. Mock reaction with no protein addition (lane 1) and binding of mutant probes (lane 5, Mb1; lane 6, Mb2; and lane 7, Mb3) to the protein were also examined.

8). Reduction of gene expression was also observed when we removed core 1 completely by deleting nucleotides −225 to −185 (our unpublished results). However, the reduction brought by the loss of core 1 function never reached zero (see DISCUSSION).

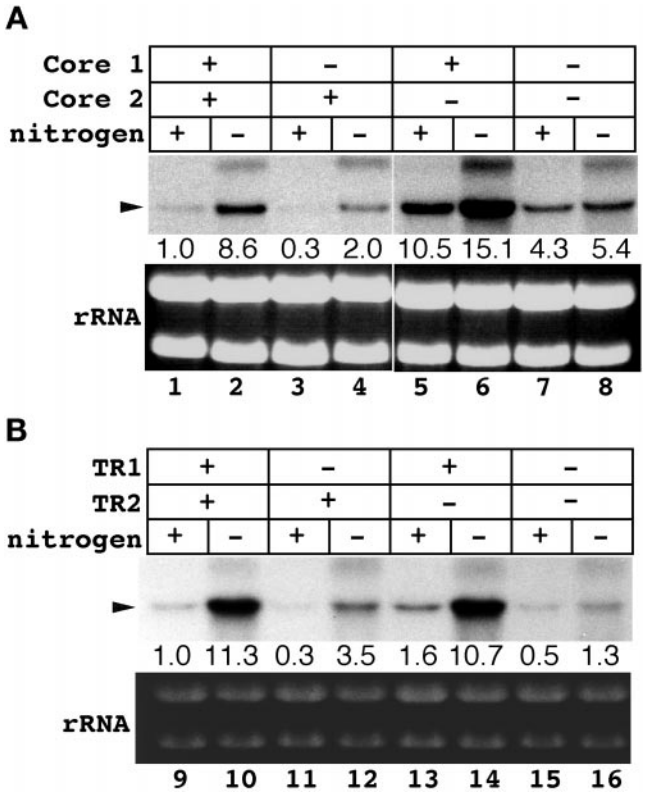

Figure 7. Core 1 and TR1 are essential *cis* elements for the promoter function of *ste11*. Transcription of the *ste11–mei2* fusion gene driven by variously modified *ste11* promoters was examined by Northern blot analysis. Cells harboring each reporter construct on a plasmid were harvested either during vegetative growth (+nitrogen) or after being starved of nitrogen (−nitrogen). RNA was prepared from each sample and analyzed by the *mei2* probe. (A) Lanes 1 and 2, expression from the wild-type promoter (pDM+); lanes 3 and 4, mutant in core 1 (pDM3); lanes 5 and 6, mutant in core 2 (pDM4); and lanes 7 and 8, mutant in both core 1 and core 2 (pDM34). (B) Lanes 9 and 10, expression from the wild-type promoter (pDM+); lanes 11 and 12, mutant in TR1 (pDM1); lanes 13 and 14, mutant in TR2 (pDM2); and lanes 15 and 16, mutant in both TR1 and TR2 (pDM12). The major transcript of the reporter gene is indicated by the arrowhead. rRNA stained with ethidium bromide is shown in the lower panels as loading controls. The relative intensity of transcription is presented under the top panels.

TR1 Is Required for the Full Expression of ste11

TR1 and TR2, the two TR box motifs newly found in this study, perfectly matched the consensus sequence we proposed previously (Sugimoto *et al.*, 1991). In contrast, the two imperfect TR box motifs in the noncoding region of *ste11*, which we noticed before (Sugimoto *et al.*, 1991), now turned out to be rather far downstream from the transcription start site. As shown in Table 3, deletion of nucleotides +7 to +1771, which covered the two imperfect TR motifs, did not appear to affect the transcription of *ste11* significantly, indicating that these motifs contribute little to *ste11* expression. Because this deletion included TR2, the contribution of TR2 also appeared to be negligible.

To examine the roles of TR1 and TR2 in the regulation of *ste11* expression, we carried out mutational analysis with the use of the *ste11–mei2* fusion gene (Figure 7B). When the conserved G in TR1 was replaced by T, both the basal and induced levels of transcription decreased considerably (lanes 11 and 12), indicating that TR1 is an important element for *ste11* expression. In contrast, the same mutation in TR2 caused no significant effect (lanes 13 and 14), reinforcing the previous inference. However, if combined with the TR1 mutation, the TR2 mutation appeared to decrease the level of transcription further (lanes 15 and 16), leaving the possibility that TR2 is potentially functional and may play a role under certain conditions.

Autoregulation of ste11 by Its Own Gene Product

The involvement of TR1 in the transcriptional activation of *ste11* suggested that this gene was under an autoregulatory mechanism, stimulating its transcription by its own product. Consistently, we found that *ste11* transcripts were much less abundant in cells carrying a point mutation in *ste11* (*ste11-029*) compared with wild-type cells (Figure 8, lane 6 versus lane 2). The amount of transcript from the *ste11-029* allele was increased when functional Ste11p was supplied from a plasmid-borne *ste11* gene whose transcripts were truncated and hence distinguishable from those of the chromosomal allele (lane 8). These results indicate that full activation of *ste11* transcription requires the presence of intact Ste11p, decreasing the possibility that the mutant form of *ste11* transcripts is more susceptible to degradation. We examined a few more *ste11*-defective mutants and obtained essentially the same results (our unpublished results). Furthermore, DNase I footprint analysis confirmed that the HMG domain of Ste11p could protect TR1 (Figure 5). Therefore, we conclude that the transcription of *ste11* is positively regulated by Ste11p, mainly through its binding to the upstream *cis* element TR1.

DISCUSSION

Two Transcription Factors Regulating ste11

This study has demonstrated that *ste11* is regulated directly by two transcription factors, namely Rst2p and its own gene product Ste11p. This and previous observations establish that expression of *ste11* is controlled in at least three ways, i.e., by the cAMP cascade, by the stress-responsive MAPK cascade, and by autoregulation. Ste11p is a key transcription factor for a number of genes required for mating and meiosis, and the level of *ste11* expression appears to be a measure of the ability to execute sexual development. Thus, we assume the following as a feasible scenario. Fission yeast cells recognize a variety of environmental parameters, including nutrients and stresses. Integrating these parameters, they set expression of *ste11* at an appropriate level through the function of regulators, including Rst2p. Thereby, the positive feedback loop contributes to amplify the magnitude of *ste11* expression and probably also to create a sharp transition in the level of accumulated *ste11* mRNA. Once the level exceeds a threshold, the cells become committed to sexual development.

Our analysis has indicated that transcription of *ste11* is decreased if a cell lacks function of either Rst2p or Ste11p. It

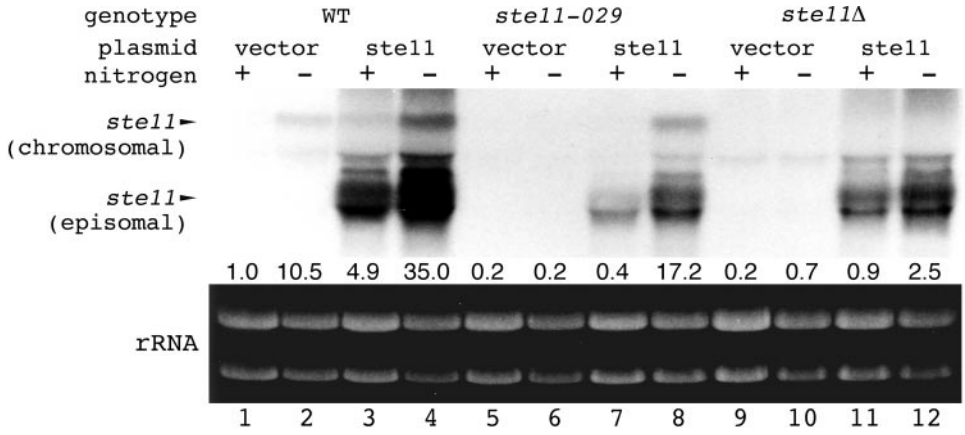

Figure 8. Activation of *ste11* transcription by Ste11p. The multicopy vector pDB248′ (lanes 1, 2, 5, 6, 9, and 10) and the *ste11*-expressing plasmid pDB-ste11$^+$ (lanes 3, 4, 7, 8, 11, and 12) were introduced into the wild-type strain JY450 (lanes 1–4), the *ste11* point mutant JY858 (lanes 5–8), or the *ste11*Δ strain JZ396 (lanes 9–12). Total RNA was prepared from each strain either growing logarithmically (odd-numbered lanes) or starved for nitrogen (even-numbered lanes) and analyzed by Northern blotting. Transcripts from the chromosomal *ste11* allele (wild type or *ste11-029*) and truncated transcripts from the episomal allele on pDB-ste11$^+$ are indicated by arrowheads. rRNA stained with ethidium bromide is shown in the lower panel as loading controls. The relative intensity of transcription is presented under the top panels.

has also been shown that their respective binding sequences, namely UASst and TR1, are necessary for the full activation of *ste11* transcription. Because the core sequence of UASst, namely core 1, and TR1 are separated by only 34 nucleotides, it is possible that Rst2p and Ste11p may cooperate synergistically in activating transcription. Indeed, Ste11p, which is a member of the HMG protein family, has been shown to cooperate with another HMG protein Mat1-Mcp to activate transcription of M cell–specific genes, in which Mat1-Mcp is thought to assist Ste11p to bind to an imperfect TR box (Kjærulff *et al.*, 1997). In the case of *ste11*, however, TR1 is a perfect TR box, and Ste11p alone can bind to this motif effectively, at least in vitro. Thus, if Ste11p and Rst2p interact with each other, the mode of interaction is likely to be different from that observed between Ste11p and Mat1-Mcp.

How PKA Controls Rst2

The Rst2p-binding sequence has turned out to be similar to *S. cerevisiae* STRE, a *cis*-acting element involved in the response to multiple stresses. Two zinc-finger proteins of the Cys_2His_2 type, encoded by the *MSN2* and *MSN4* genes, target STRE (Martínez-Pastor *et al.*, 1996; Schmitt and McEntee, 1996). The stress response in *S. cerevisiae* is regulated positively by the HOG1 MAPK cascade and negatively by the PKA cascade (Varela *et al.*, 1995; Görner *et al.*, 1998). Msn2p and Msn4p accept signals from both of these cascades and change their localization from cytoplasm to nucleus when activated (Görner *et al.*, 1998). Although phosphorylation of Msn2p/Msn4p by PKA has not been demonstrated yet, nuclear localization of these proteins has been shown to be correlated inversely with cellular PKA activity (Görner *et al.*, 1998). The lethality caused by the loss of PKA activity in *S. cerevisiae* can be suppressed by the loss of Msn2p and Msn4p, giving rise to a suggestion that Msn2p/Msn4p-dependent gene expression may account for the pleiotropic effects caused by PKA (Smith *et al.*, 1998).

Our analysis has indicated that Rst2p is likely to function downstream of the PKA cascade in *S. pombe*. The most straightforward speculation is that Rst2p is a substrate of PKA and is negatively regulated by phosphorylation. Although we assume that this possibility is very high, unequivocal biochemical evidence for it remains to be obtained. It will be especially interesting to determine to what extent Rst2p behaves as a counterpart of Msn2p/Msn4p, including whether it translocates to nucleus like the *S. cerevisiae* proteins, because Rst2p apparently lacks the sequence thought to determine the nuclear localization of Msn2p/Msn4p (Görner *et al.*, 1998).

Similarity between UASst and a cis Element in S. cerevisiae IME1

The sequence of UASst is particularly similar to a STRE sequence found in the 5′ upstream region of the *S. cerevisiae IME1* gene, termed IREu (Sagee *et al.*, 1998). Although the consensus motif for STRE is CCCCT, UASst and IREu share 10 consecutive nucleotides encompassing the consensus (CCTTCCCCTC). *IME1* encodes a key transcriptional activator for meiosis-specific genes in *S. cerevisiae* that does not belong to any specific family of transcription factors (Smith *et al.*, 1990; Mandel *et al.*, 1994). Thus, Ime1p is not a structural homologue of Ste11p, and unlike Ste11p, it is not required for mating. However, because *S. cerevisiae* cells mate in the presence of rich nutrition and require starvation of nutrients only for meiosis, Ime1p is the major transcription factor of *S. cerevisiae* that regulates gene expression to promote sexual development under starved conditions. Together, *S. pombe* and *S. cerevisiae* appear to use similar *cis* and *trans* transcriptional elements to activate the gene encoding the pivotal transcription factor that promotes sexual development in response to nutritional starvation. This is noteworthy because the two yeast species are distantly related in phylogeny, and so far no homologous regulatory proteins have been found to function in their early meiotic steps.

Rst2p Target Sites

It was rather surprising that a mutation (three-base substitution) in core 2 did not reduce the promoter activity of *ste11*. We have shown that Rst2p can bind to the core 2

region but not to the mutant form in vitro (T.H. and M.Y., unpublished results). Because the core 2 region is not particularly homologous to UASst except for the central six bases and hence is less similar to IREu, it may be that binding of Rst2p to core 2 affects *ste11* expression rather negatively. Alternatively, our assay system that used plasmids may not precisely reproduce physiological regulations. At any rate, it appears likely that UASst is not the single target of Rst2p, because the three-base substitution in core 1 and deletion of UASst both decreased the level of *ste11* mRNA only to one-fourth (Figure 7A), whereas deletion of *rst2* decreased it to one-eighth (Figure 2). Because the regulation of *ste11* expression involves various factors, as discussed above and below, it is possible that *ste11* may use cryptic or provisional Rst2p-binding sites depending on conditions. Furthermore, the results shown in Figure 2 indicate that Rst2p is essential for the full activation of *ste11* in the absence of a nitrogen source and, in addition, that induction of *ste11* expression by nitrogen starvation still occurs without Rst2p. This finding suggests two alternative possibilities. One is that Rst2p mediates the starvation signal but *S. pombe* has another protein that partially fulfills the function of Rst2p. The other is that, although Rst2p delimits the maximal level of *ste11* expression according to the level of intracellular cAMP, a factor other than Rst2p is responsible for the induction of *ste11* expression in response to nitrogen starvation. The latter view is consistent with some previous observations (Kunitomo *et al.*, 1995; Okazaki *et al.*, 1998). Obviously, more extensive characterization of Rst2p and related factors is needed to illuminate the regulation of *ste11* expression.

Does Rst2p Mediate a Stress Signal?

S. cerevisiae Msn2p and Msn4p respond to a number of stresses, including osmotic and oxidative stress, heat shock, low pH, and nutrient starvation. In *S. pombe*, the Phh1/Spc1/Sty1 MAPK cascade has been shown to affect the expression of *ste11* via the function of Atf1/Gad7p transcription factor, which resembles mammalian CRE-binding protein and binds to the CRE sequence (Kanoh *et al.*, 1996; Shiozaki and Russell, 1996). Thus, another important question is whether the stress-responsive MAPK cascade modulates Rst2p activity to regulate *ste11* expression. We scanned the *ste11* promoter region, including the sequence newly identified in this study, but found no probable CRE motif. This suggests that Atf1/Gad7p is likely to regulate the transcription of *ste11* indirectly. Our preliminary analysis indicated that Atf1/Gad7p does not significantly affect the level of *rst2* expression (T.H. and M.Y., unpublished results), suggesting that Atf1/Gad7p regulates *ste11* expression either independently of Rst2p or by modifying the activity of Rst2p at the protein level. The relationship between these two transcription factors remains an interesting question.

Physiological Importance of ste11 Autoregulation

Fission yeast cells recognize environmental conditions and make a decision whether they should continue to grow, stay in rest, or initiate sexual development. Although how they recognize the abundance of nutrients is largely unknown, the availability of nutrients, especially glucose and nitrogen, appears to affect the level of intracellular cAMP through the function of a Gα protein encoded by *gpa2* (Isshiki *et al.*, 1992). A reduction in the intracellular cAMP level leads to the initiation of sexual development. Under natural conditions, however, *S. pombe* cells may have difficulty deciding whether they should enter sexual development if they meet a fluctuation of environmental nutrition or other critical factors. The positive feedback loop of *ste11* revealed in this study will help reinforce the decision and make the cell fate irreversible, once cells decide to commit themselves to sexual development. Thus, even under compromising conditions partially favorable for sexual development, some cells will be able to undergo sexual development and complete it, whereas others will stay securely in the mitotic cell cycle.

ACKNOWLEDGMENTS

We thank Yoshinori Watanabe for helpful discussion and Asako Sugimoto for construction of some plasmids. This work was supported by Grants-in-Aid for Scientific Research on Priority Areas (A) and for Specially Promoted Research from the Ministry of Education, Science, Sports and Culture of Japan and by the Mitsubishi Foundation. A partial cDNA sequence of *rst2* has been independently deposited by S. Yoshikawa *et al.* in the DDBJ/EMBL/GenBank database under the accession number D89221.

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