MOLECULAR AND CELLULAR BIOLOGY, Feb. 1996, p. 704–711 Vol. 16, No. 2
0270-7306/96/$04.00+0

Schizosaccharomyces pombe pcr1$^+$ Encodes a CREB/ATF Protein Involved in Regulation of Gene Expression for Sexual Development

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Received 24 August 1995/Returned for modification 10 October 1995/Accepted 10 November 1995

The *Schizosaccharomyces pombe pcr1* gene encodes a bZIP protein that apparently belongs to the cyclic AMP response element (CRE)-binding protein/activating transcription factor family. The deduced *pcr1* gene product consists of 171 amino acid residues and is most similar to the mammalian CRE-BP1. A glutathione *S*-transferase-Pcr1 fusion protein produced in *Escherichia coli* was able to bind specifically to the CRE motif in vitro. Analysis with anti-Pcr1 serum suggested that Pcr1 is included in the major CRE-binding factors present in the *S. pombe* cell extract. Disruption of the *pcr1* gene was not lethal, but the disruptant showed cold-sensitive growth on rich medium. The disruptant was also inefficient in mating and sporulation, though it was not completely sterile. Expression of the *ste11* gene, which encodes a key transcription factor for sexual development, was greatly reduced in the disruptant, and overexpression of *ste11*$^+$ suppressed the deficiency of the *pcr1* disruptant in sexual development. It has been shown that expression of *ste11* is negatively regulated by cyclic AMP-dependent protein kinase (PKA) and that the loss of PKA activity results in ectopic sexual development. Disruption of *pcr1* blocked ectopic sexual development. Furthermore, disruption of *pcr1* reduced expression of *fbp1*, a glucose-repressible gene negatively regulated by PKA. These results suggest that Pcr1 is a putative transcriptional regulator whose activity may be controlled by PKA. Alternatively, its activity may be independent of PKA, and full induction of *ste11* and *fbp1* expression requires the function of Pcr1 in addition to elimination of the repression by PKA.

The cyclic AMP (cAMP) response element (CRE; TGACG TCA) plays an important role in cAMP-mediated gene regulation in cells of higher eukaryotes (12, 13, 34, 56). Essentially the same element, named the activating transcription factor (ATF) site, was characterized originally in viral oncogenes and was shown to be present in a wide range of promoters of cellular genes (26). Several proteinaceous factors that recognize the CRE/ATF site have been identified and characterized (13, 27, 57). These factors have a leucine zipper motif (bZIP) and form homodimers and heterodimers. The CRE-binding protein (CREB)/ATF is structurally and functionally related to the AP-1 family of eukaryotic transcription factors, which includes Jun and Fos oncoproteins, *Saccharomyces cerevisiae* GCN4 and YAP1, and *Schizosaccharomyces pombe* Pap1 (2, 5, 7, 35, 47, 50). The AP-1 site, which is also called the tetradecanoyl phorbol acetate (TPA) response element (TRE; TGAGTCA), differs from the CRE/ATF site by the deletion of the central C residue. AP-1 proteins generally recognize CRE/ATF sites efficiently, but CREB/ATF proteins bind to AP-1 sites inefficiently (12, 43). Each AP-1 or CREB/ATF protein exhibits distinct dimerization properties, and various combinations of the members within and among families are thought to generate a large number of distinct transcriptional regulatory factors (13).

In the yeast *S. cerevisiae*, CRE-binding activity was detected in cell extracts, and this activity was demonstrated to be regulated by cAMP-dependent protein kinase (PKA), suggesting conservation of the regulatory mechanism of the CREB/ATF transcription factor between *S. cerevisiae* and mammals (21). Two *S. cerevisiae* genes, *ACR1*/*SKO1* and *HAC1*, were subsequently cloned and shown to encode CREB/ATF proteins. *ACR1*/*SKO1* appears to encode a transcriptional repressor (36, 52), and *HAC1* has been proposed to be a meiotic gene (37), although critical identification of their functions in vivo awaits further investigation.

In the fission yeast *S. pombe*, the presence of CREB/ATF activity in the cell extract has been demonstrated by a gel shift assay with a mammalian CRE (21). However, little is known about the identity, function, and regulation of the putative fission yeast CREB/ATF. The cAMP cascade has been shown to play a central role in the regulation of sexual development in the fission yeast (6, 29, 30, 33, 54). A decrease in the intracellular cAMP level in response to nutritional starvation and the resultant reduction in PKA activity are essential for transcriptional activation of the *ste11* gene, the product of which is a key transcription factor for a number of genes required for sexual development (48). These observations suggest that *S. pombe* may provide a good model system for the study of transcriptional regulation by cAMP signaling.

In this paper we report the cloning and characterization of a novel gene, *pcr1*, which apparently encodes a member of the CREB family of DNA-binding proteins in *S. pombe*. We demonstrate that *pcr1*$^+$ functions in an early stage of sexual development, in association with the cAMP cascade. Furthermore, it is suggested that the function of this gene is required for proper cell growth.

MATERIALS AND METHODS

Strains, media, and genetic methods. Table 1 summarizes the *S. pombe* strains used in this study. The complete media YPD and YE and minimal medium SD were used for routine culture of *S. pombe* strains (43a). The synthetic media SSA (8) and SPA (11) were used for induction of mating and sporulation. A liquid minimal medium, MM, and its nitrogen-free derivative, MM-N, were used for nitrogen starvation experiments (55). General genetic methods have been de-

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

Strain	Genotype
JY333	h^- *ade6-M216 leu1*
JY450	h^{90} *ade6-M216 leu1*
JY879	h^{90} *ade6-M210 leu1 ura4-D18*
JZ633	h^{90} *ade6-M216 leu1 ura4-D18 pka1::ura4*$^+$
JX26	h^{90} *ade6-M210 leu1 ura4-D18 pcr1::ura4*$^+$
JX359	h^{90} *ade6-M216 leu1 ura4-D18 pcr1::ura4*$^+$ *pka1::ura4*$^+$

scribed previously (11). Transformation (38) and gene disruption (41) in *S. pombe* were performed as described previously.

Mating and sporulation assay. Mating and sporulation frequencies were calculated according to the equations previously described (23), by counting unmated or unsporulated cells, zygotes, asci, and free spores in a sample.

Plasmids and DNA sequencing. A high-copy-number vector, pDB248′ (1), and an expression vector, pREP1 (32), were used. pREP1 carries the *nmt1* promoter, which is repressed in the presence of thiamine. The original *pcr1*$^+$ clone, pDB (pcr1), was obtained from a pDB248′-based *S. pombe* genomic library previously described (30). To overexpress *pcr1*$^+$ in fission yeast cells, nucleotide residues 3 to 8 of the *pcr1* gene (GACTGC) were replaced with a *Sal*I site (GTCGAC) by site-directed mutagenesis and a resultant 1.0-kb *Sal*I-*Bgl*II fragment was cloned into pREP1. The plasmid thus obtained was named pR(pcr1). To overexpress a truncated form of *pcr1*, a 0.6-kb *Bam*HI-*Hin*dIII fragment was excised from pGEX(pcr1T) (see below) and cloned into pREP1 and then the *Bam*HI site was filled in. The resultant plasmid was named pR(pcr1T).

The pDB(pcr1) insert was recloned into the vector pBluescript (Stratagene), which is suitable for generation of single-stranded DNA for sequencing and site-directed mutagenesis (24). Subclones for sequencing were produced by progressive deletion of the insert with exonuclease III and S1 nuclease (14). DNA sequencing was performed by the dideoxy chain termination method (42).

Two plasmids derived from pGEX-KG (10) were used to produce glutathione *S*-transferase (GST)-Pcr1 fusion proteins in bacteria. pGEX(pcr1) was designed to generate a nearly complete Pcr1 protein (amino acid residues 4 to 171) fused to GST. To prepare it, a new *Sac*I site (GAGCTC) was created at nucleotide residues 4 to 9 of the *pcr1* gene (Fig. 1b) by site-directed mutagenesis. To prepare pGEX(pcr1T), a 0.6-kb *Eco*RI fragment carrying part of the *pcr1* open reading frame (ORF) was cloned into pGEX-KG. The resulting plasmid expressed truncated Pcr1 (amino acid residues 16 to 171) fused to GST.

pDB(ste11) is a pDB248′-based plasmid carrying the *ste11* ORF under the control of a cryptic read-through promoter on the vector. The level of transcription from this promoter was not greatly affected by the culture conditions (data not shown).

Gel retardation assay. Two different probes were prepared for this assay. The CRE probe was as follows:

AGCTCTTGTGA**TGACGTCA**CAGTTGCC
GAACACT**ACTGCAGT**GTCAACGGTCGA

The TRE probe was as follows:

AGCTCTTGTGA**TGAGTCA**CAGTTGCC
GAACACT**ACTCAGT**GTCAACGGTCGA

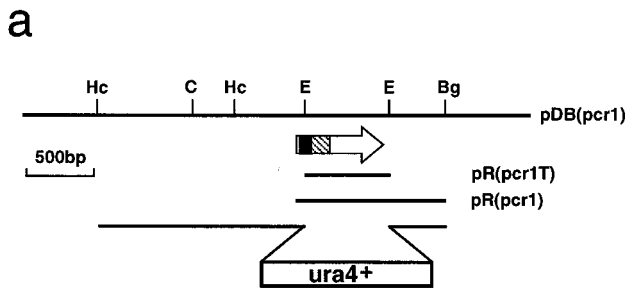
The central CRE and TRE sequences are in boldface. The CRE probe corresponds to the X box of the murine class II major histocompatibility complex Aα gene, and we designed the TRE probe by deleting the central GC base pair from the CRE probe. We adopted this CRE sequence because CRE-BP1, which is the most similar to Pcr1 among known proteins, recognizes it (20). These oligonucleotides were purified by polyacrylamide gel electrophoresis before binding reactions were performed.

GST, GST-Pcr1, and GST-Pcr1T proteins produced in *Escherichia coli* were prepared and purified as described previously (45). *S. pombe* cell extracts were prepared as described previously (4). Anti-Pcr1 serum was obtained by immunizing a rabbit with a GST fusion protein purified from *E. coli* harboring pGEX (pcr1). The binding conditions were described previously (46). When a purified GST fusion protein (0.3 pmol) was employed, the reaction was carried out in a total volume of 10 μl. Approximately 0.1 pmol of either probe, labeled with ^{32}P, was added. When an *S. pombe* cell extract (containing either 20 or 4 μg of protein) was used, the reaction was carried out in a final volume of 30 μl, with 0.15 pmol of either probe. DNA-protein complexes were resolved on a 5% polyacrylamide gel (20:1 cross-linking) in 0.5× Tris-borate-EDTA buffer, and the gel was dried on Whatman 3MM paper and autoradiographed.

Northern (RNA) blot analysis. Northern blot analysis was performed as described previously (55). DNA fragments used as probes to detect transcripts of the respective genes are as follows: a 0.5-kb *Eco*RI fragment for *pcr1* (this study), a 1.3-kb *Pvu*II fragment for *ste11* (48), a PCR-amplified ORF for *fbp1* (40), and a 0.4-kb *Eco*RI fragment for *cam1* (49).

Gene disruption of *pcr1*. A 0.5-kb *Eco*RI fragment, which covers more than 90% of the *pcr1* ORF, was replaced by a *ura4*$^+$ cassette (9) (Fig. 1a). Cells of a wild-type homothallic haploid strain, JY879 (*ura4*$^-$), were transformed with the *pcr1*::*ura4*$^+$ DNA fragment shown at the bottom of Fig. 1a and were spread on SSA plates without uracil. Ura$^+$ transformants were obtained at an expected frequency, and about half of them turned out to be sterile. Southern blot analysis showed that the *pcr1* gene was properly disrupted in Ura$^+$ sterile cells- (data not shown). To confirm that disruption of *pcr1* has no lethal effect and that the sterility and the disruption are tightly linked, we crossed the *pcr1*-disrupted cells we obtained with homothallic *ura4*$^-$ cells and performed tetrad dissection of the progeny. We obtained four viable spores essentially in every ascus, and their uracil marker segregated 2+:2−. Ura$^+$ segregants were all sterile, whereas Ura$^-$ segregants were all fertile. These observations exclude the possibility that disruption of *pcr1* is basically lethal and that the disruptants we obtained carry a cryptic mutation that suppresses the lethality. Furthermore, they strongly suggest that the sterile phenotype results from disruption of *pcr1*.

Nucleotide sequence accession number. The nucleotide sequence data in Fig. 1b will appear in the GenBank/EMBL/DDBJ nucleotide sequence database under accession number D63667.



b

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GTCAACGTGTGCGCTGTACATTTGACGTGTGTCGTGAGATTACAAAAAATTTTAGGGGTC -358
GGTTTACTTACGAATTAATTCCCCCACGTGTGTGTTTTTAATCTTAGAAGACTAGCGCTT -298
CTATAGAATTGACGTTTCTTGACGTCTTGTCATTCTTCTGTATTCCTTTAGAAACTGGCT -238
ATCTCCGTCTAATTTGCTTCCTCCTGATTTCGCGCGTGTGTCGTGCTAATTCTTTACTGC -178
GTGTTGTATTCACCAAGTAGGAGTTTCCTTTAAATTAAAATATATCCTTTAGGGTATTTC -118
TTTTTTCGTTCTTACTTTTTTTTTTCATTTCAATTTCCCATCTCCCCCTTTGTTTCCTTTG  -58
TTATATTTTTGTTTGTGATCATCTGATTCCCCCTTTTCTATACATTGATTATTTAAGATG    3
                                                          M     1
ACTGCCAAAAAAAAAGAAGTTGATGATGAAAAGCGTCGCCGAATTCTGGAGCGCAATCGC   63
T  A  K  K  K  E  V  D  D  E  K  R  R  R  I  L  E  R  N  R     21
ATTGCCGCTTCTAAATTTCGCCAGAAAAAGAAAGAGTGGATTAAAGAATTAGAGCAAACT  123
I  A  A  S  K  F  R  Q  K  K  K  E  W  I  K  E  L  E  Q  T     41
GCCAATGCTGCCTTTGAGCAATCCAAGCGTCTTCAGTTATTGCTGTCTCAGTTGCAACAG  183
A  N  A  A  F  E  Q  S  K  R  L  Q  L  L  L  S  Q  L  Q  Q     61
GAGGCTTTTCGGCTGAAAAGCCAATTACTTGCCCATCAGGGTTGTCAGTGTAGTGTAAAG  243
E  A  F  R  L  K  S  Q  L  L  A  H  Q  G  C  Q  C  S  V  K     81
ATTCGTTCTGTCCTAACAGACTTTCAAACTGCTCACAATGCTCTCCACTCTCAACATATG  303
I  R  S  V  L  T  D  F  Q  T  A  H  N  A  L  H  S  Q  H  M    101
GCTTACCGGCCGGTACAACCCCCTCCTGGTGATAACATGCTTGAATCTGTGGTTTCCGTT  363
A  Y  R  P  V  Q  P  P  P  G  D  N  M  L  E  S  V  V  S  V    121
AGCCCTACTCAAATGCATCCATCATTGCAGGGTCTCCCTCCAAATCAACACCCTCAAATG  423
S  P  T  Q  M  H  P  S  L  Q  G  L  P  P  N  Q  H  P  Q  M    141
CCCCCTTCTTCTCAGCAGCCTAATTCCGATGATGTCCAGCAACATATGTTCTCAGCAGCC  483
P  P  S  S  Q  Q  P  N  S  D  D  V  Q  Q  H  M  F  S  A  A    161
GGGCTTCCTAGATCCCTTGGTGGGCCCATCTGAAAAAGAACATTTGTTTTCATTACCCCC  543
G  L  P  R  S  L  G  G  P  I  *                               171
CTTTACAATAAAGCAATTGATTTTAATGTGATGTTGCATGTAGATTTATGGCTCCTATTT  603
TAGCCTAATGTTCTCTCCTAGATGACAGTCGGGCGTTTTTAAGTGTTGAATTACCGATGC  663
ATATGGAATTCTTTTCAAATTTCTGTCCTTATTATTTTTTTGCACAGACGTTTTCTTTGT  723
TATGACCTTTATCTATTATTATTTCGAATTTCCTCGCCGGGTTTTCAAAATATTTGTCTT  783
TATGAAATGTGAAAAGAAAATAACTTTACGATGCTTATAAACTTCTTTGAAGTATGACTT  843
TGCGCTTGCTACATCTCCACTTACTTCTTCGATGTATTTGATTTGATTTCTTGAACTATT  903
GCCAGATTGCCTATTCTCGTCAGTTACCTAAAACATAAAAAATGTTCTTCTTTCTAAATG  963
TACAGATTTATAATTTCATTTGGCAAACTGAAAAAAAGATCT                   1023
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FIG. 1. Restriction map of the *pcr1* locus and the nucleotide and deduced amino acid sequences of the *pcr1* gene. (a) Relevant restriction sites on the insert of the original *pcr1*$^+$ clone, pDB(pcr1). The extent and direction of the *pcr1* ORF, which encodes 171 amino acids, are shown by the arrow. The solid and hatched regions in the arrow indicate, respectively, basic and zipper domains. A derivative clone, pR(pcr1T), carries an *Eco*RI fragment under the control of the *nmt1* promoter and can express a truncated Pcr1 protein (residues 16 to 171), whereas pR(pcr1) can express a nearly full-length Pcr1 protein (residues 4 to 171) from the same promoter. The DNA fragment used to disrupt chromosomal *pcr1* is shown schematically at the bottom of the panel. Restriction sites shown are *Bgl*II (Bg), *Cla*I (C), *Eco*RI (E), and *Hin*cII (Hc). (b) DNA sequence of a 1.4-kb *Hin*cII-*Bgl*II fragment and deduced amino acid sequence of the *pcr1* gene product. Numbering starts with the putative first methionine codon for both nucleotide and amino acid residues.

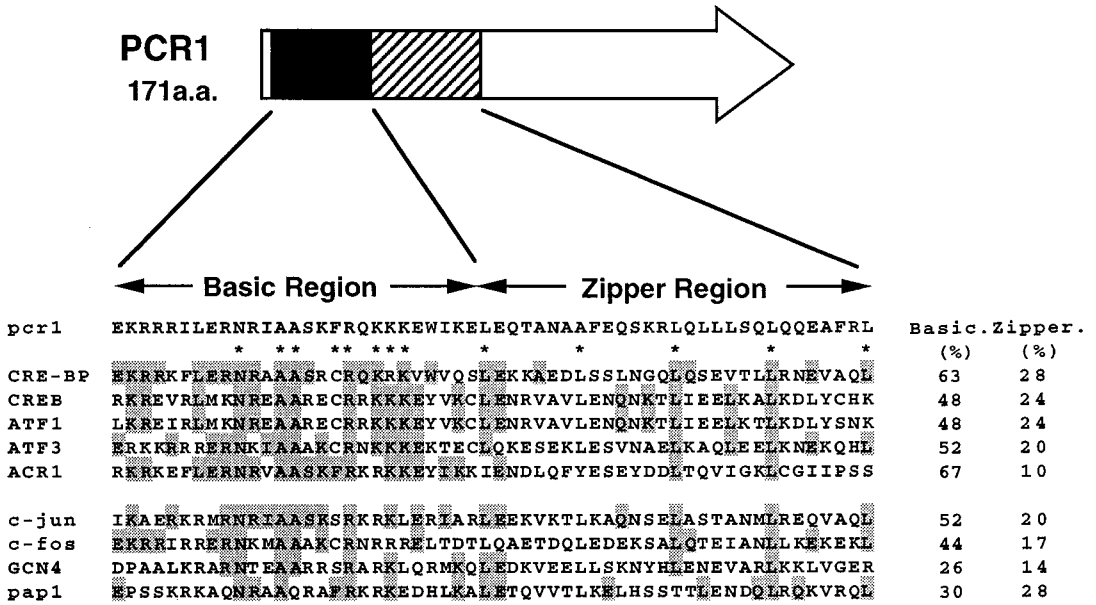

FIG. 2. Homology between Pcr1 and members of the bZIP family of transcription factors. The amino acid (a.a.) sequence of the bZIP domain of Pcr1 (residues 11 to 66) is compared with those of the following nine bZIP proteins: CRE-BP1 (20, 31), fission yeast CREB (18), ATF1, ATF3 (13), ACR1 (52), c-Jun (2), c-Fos (51), GCN4 (16), and Pap1 (50). The first five belong to the family of CREB/ATF proteins, whereas the last four are TRE-binding proteins. Amino acid residues identical to those of Pcr1 are shaded. Asterisks indicate highly conserved residues among bZIP proteins. The numbers given at the end of each sequence are the percentages of identity to Pcr1 within the basic and zipper regions.

RESULTS

Cloning and sequence determination of the *pcr1* gene. We isolated the original clone carrying the *pcr1* gene from an *S. pombe* genomic library as a weak, high-copy-number suppressor of the *spo5* mutation. Mutants defective in *spo5* grow normally but are unable to complete meiosis II properly (17). Despite this isolation procedure, subsequent analysis, as described below, indicated that the major function of $pcr1^+$ is not directly pertinent to meiosis II and suggested that the observed weak suppression of $spo5^-$ by overexpression of $pcr1^+$ might be indirect and fortuitous. The original clone, named pDB (pcr1), contained an insert 3.5 kb in length. Subcloning analysis of pDB(pcr1) suggested that the region essential for the suppression is carried on a 1.4-kb *Hin*cII-*Bgl*II fragment (Fig. 1a). Sequence analysis of this fragment revealed an ORF that potentially encodes 171 amino acids (Fig. 1b). We named this gene *pcr1*, after *S. pombe* CREB (see below).

The *pcr1* gene product carries a bZIP domain. The FASTA homology search algorithm (28) was used to look for proteins that have similarity to the predicted *pcr1* gene product. Pcr1 was found to carry a bZIP domain (25) in the amino-terminal region and to be most similar to mammalian CRE-BP1 (31) (Fig. 2). The putative basic region of Pcr1 contains the invariant asparagine and arginine residues, a pair of highly conserved alanine residues, and several other conserved residues, most of which are positively charged (39). The basic region may be wider than the area specified in Fig. 2, because Pcr1 has three contiguous lysine residues 5 amino acids upstream of the area. The leucine zipper dimerization motif is also conserved in Pcr1, although alanine rather than leucine is in the second position of the zipper. Among bZIP proteins, Pcr1 is more similar to CREB/ATF proteins than to TRE-binding proteins, especially within the basic region (Fig. 2). Pcr1 is not highly similar to Pap1, an *S. pombe* bZIP protein that has been shown to bind to the TRE/AP-1 site (50).

Pcr1 binds specifically to the CRE site. The above-described results suggested that Pcr1 might be able to bind to the CRE/ATF site. We produced two types of GST-Pcr1 fusion proteins in a bacterial system and examined their DNA-binding abilities by a gel shift assay. Two different labeled oligonucleotides, one carrying the CRE sequence and one carrying the TRE sequence, were used as the probes. The results are shown in Fig. 3. The fusion protein carrying nearly full-length Pcr1 (GST-Pcr1) could form a complex with the CRE probe but not with the TRE probe (Fig. 3, lanes 2 and 6). The other fusion protein, GST-Pcr1T, in which Pcr1 lacked the N-terminal half of the basic region, had a greatly reduced ability to bind to the CRE probe (Fig. 3, lane 3). These results are consistent with

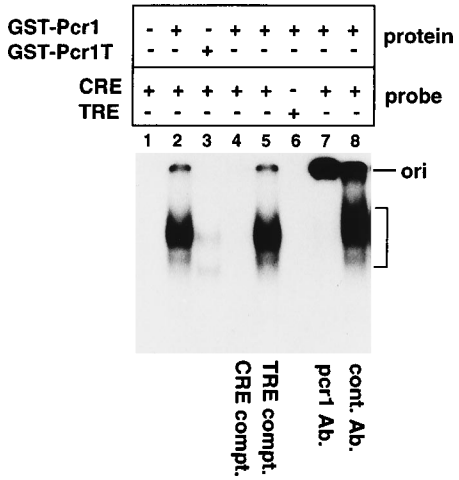

FIG. 3. Gel shift assay of the DNA-binding activity of Pcr1 protein. The GST-Pcr1 and GST-Pcr1T fusion proteins were mixed with either the CRE or the TRE probe labeled with ^{32}P. GST was used instead of the fusion proteins in lane 1. Cold CRE and TRE oligonucleotides were added to lanes 4 and 5, respectively, as competitors (compt.), in a 500-fold molar excess to the probe. Anti-Pcr1 serum (Ab.) was added to lane 7, and preimmune serum, as a control (cont.), was added to lane 8. ori, origin.

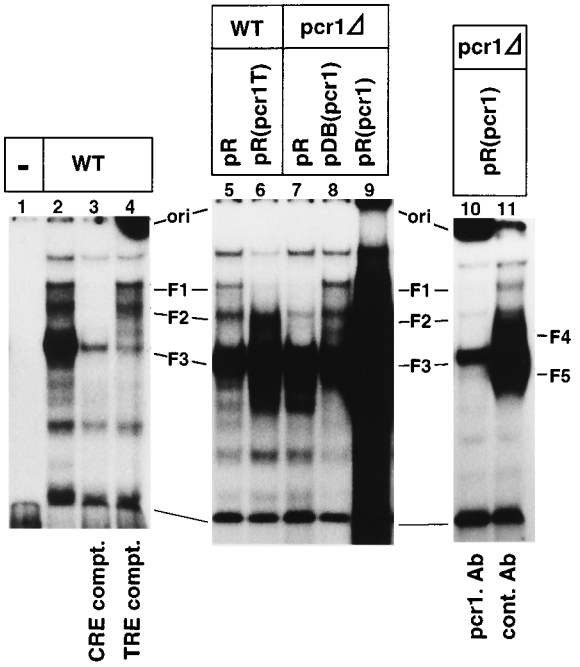

FIG. 4. CRE-binding activities in the *S. pombe* cell extract. Gel shift assays were performed with the labeled CRE probe, and total cell extracts were prepared from the following strains: a wild-type (WT) strain, JY450 (lanes 2 to 4); JY450 carrying the vector pREP1 (lane 5); JY450 carrying pR(pcr1T) (lane 6); a *pcr1*Δ strain, JX26, carrying pREP1 (lane 7); JX26 carrying pDB(pcr1) (lane 8); and JX26 carrying pR(pcr1) (lanes 9 to 11). Each lane received approximately 20 μg of protein, except for lanes 10 and 11, which received only 4 μg of protein. Lane 1 is a control with no cell extract. The extract used in lanes 2 to 4 was prepared from cells grown in YPD, whereas the extracts used in lanes 5 to 11 were prepared from respective cells grown in SD. Lanes 3 and 4, respectively, contain a 100-fold molar excess of either CRE or TRE oligonucleotide as a competitor (compt.). Anti-Pcr1 serum (Ab.) and preimmune (control [cont.]) serum were added to lanes 10 and 11, respectively. Although lane 9 is highly radioactive and is not resolved clearly, a lighter exposure of it gives essentially the same pattern as that of lane 11 (not shown). ori, origin.

the previous finding that the basic region of bZIP proteins directly contacts DNA and is essential for binding (52). The addition of anti-Pcr1 serum to the reaction mixture resulted in clustering of the protein-DNA complex at the origin of the gel, while control serum had little effect, proving that the GST-Pcr1 fusion protein indeed participates in complex formation (Fig. 3, lanes 7 and 8). Binding of GST-Pcr1 to the labeled CRE probe was inhibited by competition with an excessive amount of cold CRE oligonucleotides but not by TRE oligonucleotides (Fig. 3, lanes 4 and 5). These results indicate that Pcr1 has the ability to bind specifically to the CRE sequence.

CRE-binding complexes in the *S. pombe* extract. We examined CRE-binding activities in a cell extract prepared from a wild-type *S. pombe* strain by a gel shift assay with the probes described above. Apparently, several factors could form complexes with the labeled CRE probe (Fig. 4, lane 2). Two of these complexes, which we named F1 and F2, disappeared when cold CRE oligonucleotides were added as a competitor, but cold TRE oligonucleotides had no effect (Fig. 4, lanes 3 and 4). Thus, F1 and F2 appear to represent CRE-specific binding factors in *S. pombe* and are likely to correspond to the two major CREB/ATF complexes previously identified in the *S. pombe* nuclear extract (21). The most intensive band, F3, was efficiently inhibited by competition with both CRE and TRE oligonucleotides (Fig. 4, lanes 3 and 4). Furthermore, the F3 complex was also detected as the major retarded band when the labeled TRE probe, which had a size similar to that of the CRE probe, was used (data not shown). These observations imply that this complex may represent either the *S. pombe* AP-1-like factor reported previously (22) or a less specific DNA-binding activity that recognizes common features of the two probes. The F3 band appeared only weakly if a CRE probe carrying the same central sequence but different flanking sequences was used (22a).

Presence of Pcr1 in CRE complexes. To identify CRE-binding factors that contain Pcr1 protein, we performed a series of gel shift assays with cell extracts of various strains, including a strain with a disruption in *pcr1* (*pcr1*Δ; see below). The F1 band was undetectable in a *pcr1*Δ strain carrying the vector plasmid but was clearly seen in the same strain transformed with pDB(pcr1) (Fig. 4, lanes 7 and 8). Furthermore, if the *pcr1*Δ strain was transformed with pR(pcr1), a plasmid that produces a large amount of Pcr1 protein, the F1 band was enhanced and two additional bands were seen (Fig. 4, lanes 9 and 11). The new bands, named F4 and F5, may represent the homodimer of Pcr1 and/or another artifactual complex(es) containing Pcr1, because no significant degradation of Pcr1 was observed under these experimental conditions as judged by Western blot (immunoblot) analysis (data not shown). These observations indicate that the formation of the F1 complex depends on the presence of Pcr1. The addition of anti-Pcr1 serum to the cell extract of the pR(pcr1) transformant resulted in a supershift of the F1 complex to the origin of the gel (Fig. 4, lane 10), confirming that the F1 complex represents a CRE-binding factor that contains Pcr1 as a component. In the same lane, F4 and F5 were also supershifted, confirming the above-mentioned assumption concerning their identity. The F2 band did not disappear completely but was always weaker in the cell extract of the *pcr1*Δ strain (Fig. 4, lane 7), which may suggest that both a factor containing Pcr1 and a factor not containing it contribute to the formation of this band.

Phenotypes of the strain carrying disrupted *pcr1*. We constructed a homothallic haploid strain carrying a disrupted *pcr1* allele (*pcr1*Δ) as described in Materials and Methods. During the construction procedure, it was noted that the strain carrying disrupted *pcr1* became sterile. Cells of the *pcr1*Δ strain were poorly stained with iodine vapor on sporulation medium, suggesting that they were defective in mating and/or sporulation (Fig. 5a). Quantitative analysis showed that the mating frequency of the *pcr1*Δ strain was about 10% whereas that of the wild type was about 70% (Fig. 5b). This mating deficiency was completely suppressed by each of the two $pcr1^+$-expressing plasmids (Fig. 5).

We then examined the sporulation efficiency of a diploid *pcr1*Δ derivative. The sporulation efficiency of *pcr1*Δ cells on SSA plates was about one-fourth of that of the wild type (data not shown). Thus, we conclude that the function of $pcr1^+$ is required for efficient mating and sporulation.

As described above, a deletion in the basic domain of Pcr1 resulted in the loss of its DNA-binding ability. We overproduced the truncated Pcr1 protein (Pcr1T), not conjugated with GST in this case, in wild-type *S. pombe* cells. The mating frequency was reduced by about 50% in these cells (Fig. 5), suggesting that the *pcr1T* allele behaves in a dominant-negative manner. As shown in Fig. 4, lane 6, formation of the F1 complex was scarce in these cells. However, overproduction of Pcr1T did not appear to affect the residual mating ability of the *pcr1*Δ strain (Fig. 5). These results may suggest that Pcr1 performs its function by assuming the form of a homodimer and/or a heterodimer(s) and that the truncated Pcr1 protein restrains the wild-type Pcr1 protein from forming a functional

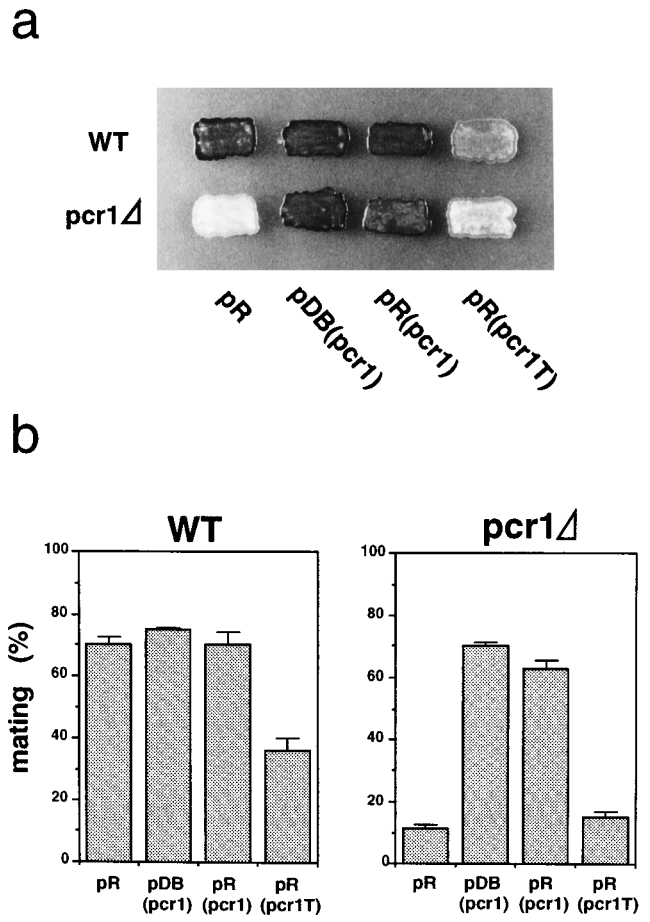

FIG. 5. Mating deficiency of the *pcr1* disruptant. (a) Wild-type (WT) strain JY450 and *pcr1* disruptant JX26, carrying a plasmid as indicated, were streaked on sporulation medium SSA and incubated at 30°C for 3 days. They were then exposed to iodine vapor, which stains sporulated cells dark brown. (b) The mating frequency of each strain shown in panel a was scored under the microscope. The averages of the scores obtained in two independent experiments are shown. The error bar for each sample represents the deviation of the two scores from the mean.

DNA-binding complex(es) by secluding the partner molecule(s) through dimerization at the leucine zipper structure.

Pcr1 may function downstream of the cAMP cascade in the regulation of sexual development. The *S. pombe ste11* gene encodes a high-mobility-group (HMG) box transcription factor that regulates expression of genes necessary for mating and sporulation. Transcription of *ste11* itself is induced in response to nutritional starvation and a concomitant decrease of the intracellular cAMP level (48). We observed that expression of *ste11* is considerably reduced in the *pcr1*Δ strain (Fig. 6). Furthermore, ectopic expression of *ste11*$^+$ could efficiently suppress the mating and sporulation defect of the *pcr1*Δ strain (Fig. 7a). These results suggest that a reduction of *ste11* expression is a major reason for the sterility of the *pcr1*Δ strain.

The cAMP cascade plays a critical role in the induction of *ste11* expression (33, 48). We therefore examined whether the function of *pcr1*$^+$ is related to the cAMP pathway. We first measured the level of intracellular cAMP of the *pcr1*Δ strain. It was not significantly different from that of a wild-type strain (data not shown). Then we examined the possibility that *pcr1*$^+$ functions downstream of the cAMP cascade. Disruption of the *pka1* gene, which encodes the catalytic subunit of PKA, has been shown to cause ectopic sexual development without nutritional limitation (30). To examine genetic epistasis between *pcr1* and *pka1*, we constructed a *pcr1 pka1* double disruptant, JX359. As shown in Fig. 7b, JX359 was sterile on sporulation medium, indicating that *pka1*Δ cannot overcome the sterility of the *pcr1*Δ strain. Furthermore, JX359 did not display any ectopic sexual development in rich medium, indicating that *pcr1*Δ can block the derepressed sexual development caused by disruption of *pka1* (Fig. 7c and d). These results suggest that *pcr1* is epistatic to *pka1* in the regulation of sexual development.

Expression of relevant genes was examined in the *pcr1*Δ, the *pka1*Δ, and the double mutants (Fig. 6). Transcription of *ste11* was constitutively derepressed in *pka1*Δ cells. Disruption of *pcr1* reduced this constitutive expression of *ste11*, which is consistent with the sterile phenotype of the double mutant. Expression of *pcr1* in wild-type cells was enhanced by nitrogen starvation, although its basal level of expression in the presence of nitrogen was considerably high (Fig. 6). However, the pattern of *pcr1* expression in cells in which Pka1 is either defective (Fig. 6) or hyperactive (data not shown) suggests that Pka1 does not regulate *pcr1* at the transcriptional level.

We examined the effects of the loss of *pcr1* function on the expression of *fbp1*, which encodes fructose-1,6-bisphosphatase,

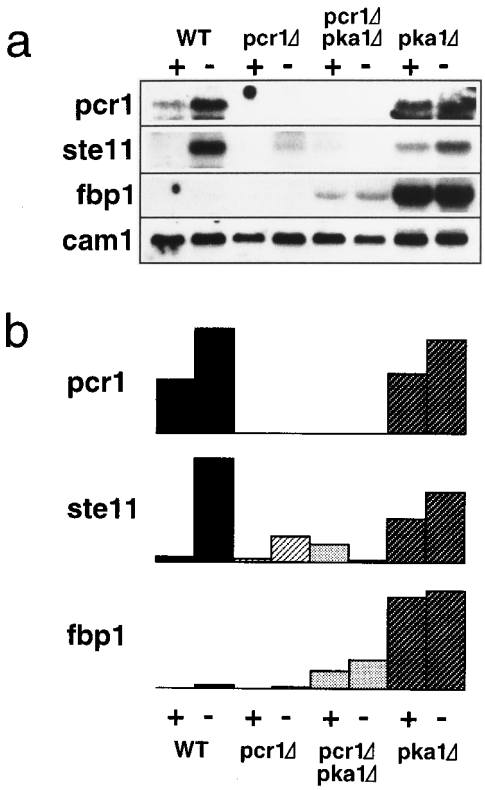

FIG. 6. Northern blot analysis of gene expression in the *pcr1*Δ or the *pka1*Δ background. (a) Total RNA was extracted from cells of either the wild-type (WT), the *pcr1*Δ, the *pka1*Δ, or the *pcr1*Δ *pka1*Δ strain. Samples were prepared from cells either growing exponentially (+) or subjected to nitrogen starvation for 6 h (−). RNA (10 μg) was loaded in each lane after denaturation by formamide. The levels of *pcr1*, *ste11*, and *fbp1* transcription were detected as described in Materials and Methods. Expression of *cam1*, the gene coding for calmodulin, was measured for each sample as an internal control not affected greatly by nutritional conditions. The size of each transcript is as follows: *pcr1*, 1.7 kb; *ste11*, 3.6 kb; *fbp1*, 1.9 kb; and *cam1*, 1.2 kb. (b) The magnitudes of *pcr1*, *ste11*, and *fbp1* transcription relative to *cam1* transcription are displayed (height in arbitrary units). The radioactivity in each transcript was quantitated with a Bioimage analyzer (BAS 2000; Fujix).

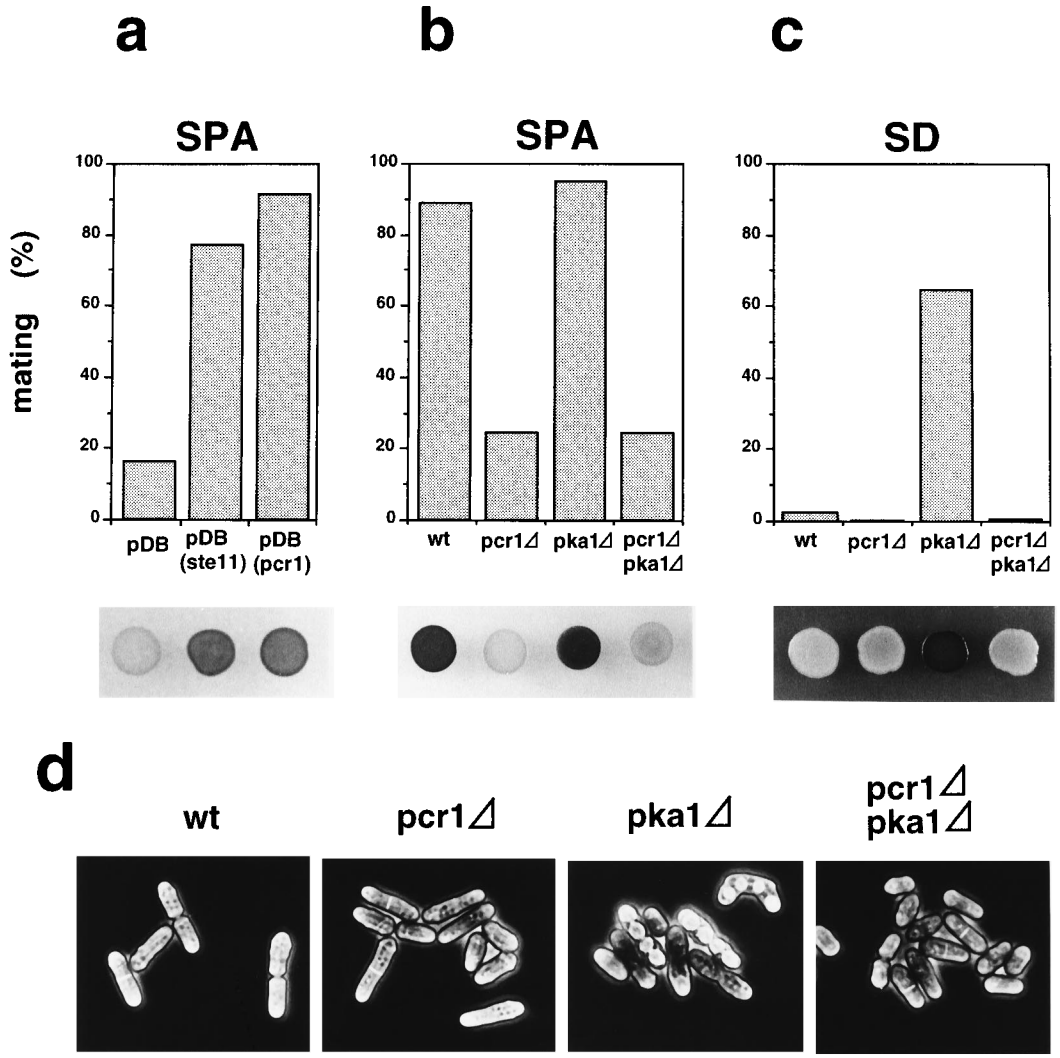

FIG. 7. Suppression of *pcr1*Δ by overexpression of *ste11*$^+$ and epistasis between *pcr1* and *pka1*. (a) Cells of JX26 (h^{90} *pcr1*Δ) were transformed by either vector pDB248′, pDB(ste11), or pDB(pcr1). They were spotted on nitrogen-free SPA, and after a 2-day incubation at 30°C, the ability of each to mate was examined qualitatively by iodine staining (bottom) and quantitatively by microscopic observation (top). (b) The mating proficiencies of homothallic strains JY450 (wild type [wt]), JX26 (*pcr1*Δ), JZ633(*pka1*Δ), and JX359(*pcr1*Δ *pka1*Δ) were examined as for panel a. (c) the same strains examined for panel b were cultured on nitrogen-rich SD plates for 3 days, and their mating efficiencies were determined. (d) Phase-contrast micrographs of the strains growing exponentially in SD liquid medium.

a key enzyme for gluconeogenesis (40). Expression of this gene is repressed in the presence of glucose and is negatively regulated by PKA (19). As shown in Fig. 6, derepressed expression of *fbp1* in *pka1*Δ cells was inhibited considerably by disruption of *pcr1*. Thus, Pcr1 is involved in the control of *ste11* expression and *fbp1* expression, which are both negatively regulated by Pka1 but are mutually independent.

Phenotypes of the *pcr1*Δ strain in vegetative cell growth. In addition to the above-mentioned deficiency in sexual development, the *pcr1*Δ strain showed growth retardation on either YPD or YE at 25°C. This kind of cold sensitivity was seen only on complete medium and was not observed on medium such as SD or SSA (Fig. 8a). Plasmids that could express *pcr1*$^+$ rescued the cold-sensitive growth of the *pcr1*Δ strain (Fig. 8b), confirming that this phenotype was indeed caused by the loss of *pcr1*$^+$ function. The reason that the *pcr1*Δ strain shows cold sensitivity on complete medium is unclear.

Cells defective in *pcr1* were slightly (~20%) larger than the wild type. The cAMP cascade of the fission yeast appears to be involved in the control of cell size, in addition to the control of sexual development. Cells defective in *pka1* grow more slowly than the wild type and are smaller, even when they grow exponentially. Because Pcr1 appeared to function downstream of Pka1 in the regulation of sexual development, we examined whether disruption of *pcr1* could suppress the reduction in the cell size of the *pka1*Δ strain. The *pka1*Δ *pcr1*Δ strain was larger than the *pka1*Δ strain but not as large as the *pcr1*Δ strain (Fig. 7d), suggesting that Pcr1 is unlikely to totally mediate the function of Pka1 in the regulation of cell size.

DISCUSSION

In this study we cloned and characterized a novel *S. pombe* gene, *pcr1*, which encodes a CREB/ATF protein. Two CRE-specific binding factors have previously been detected in an *S. pombe* cell extract (22); they appear to correspond to the F1 and F2 complexes in this study (Fig. 4). Our analysis showed that one of them (F1) uniformly contains Pcr1 as a component, whereas the other (F2) appeared to be a mixture of a complex that contains Pcr1 and a complex that does not (Fig. 4). These results leave open the possibility that *S. pombe* has additional

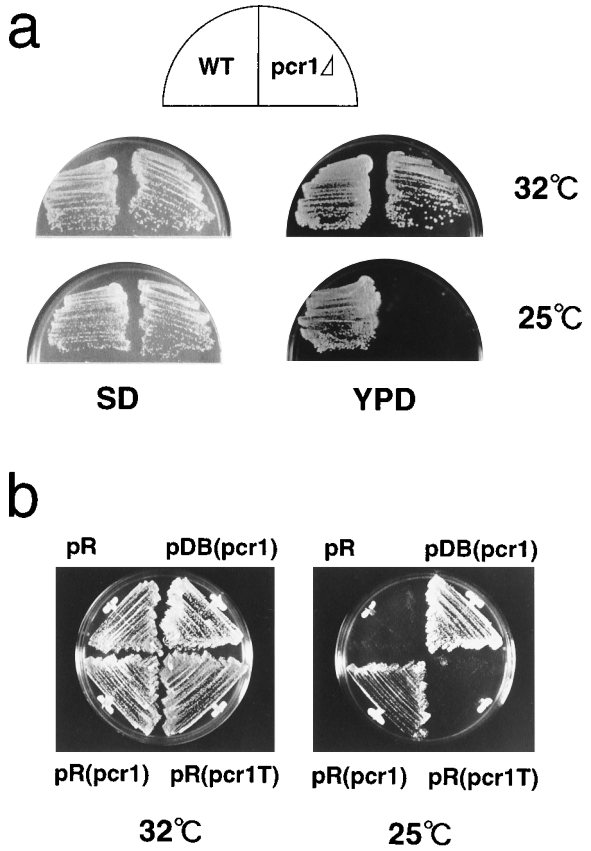

FIG. 8. The *pcr1* disruptant shows cold-sensitive growth on complete medium. (a) Wild-type strain JY450 and *pcr1* disruptant JX26 were streaked on SD or YPD plates and incubated either at 25°C for 4 days or at 32°C for 3 days. (b) Cells of JX26 transformed with each plasmid indicated were streaked on YPD plates and incubated as for panel a.

CREB/ATF proteins, which may function either as partners of Pcr1 or independently. Indeed, we have recently identified a second *S. pombe* bZIP protein, encoded by the *gad7* gene, which appears to form a heterodimer with Pcr1 (22a). It yet remains possible that a third CREB is present and plays a role in *S. pombe*. Thus, like mammalian cells (57), *S. pombe* may utilize several bZIP proteins of the CREB/ATF family to generate a large number of transcriptional regulatory systems.

Cells defective in *pcr1* were inefficient in conjugation. Induction of expression of *ste11*, which encodes an HMG box transcription factor required for sexual development, was greatly reduced in these cells. Genetically, *pcr1*Δ was epistatic to *pka1*Δ, suggesting that Pcr1 may function downstream of PKA. However, hyperactivation of PKA apparently causes more severe sterility than *pcr1*Δ (6, 33a). This suggests that Pcr1 mediates only part of the inhibitory function of PKA in sexual development if they are in the same pathway. Because *pcr1*Δ cells could mate on nitrogen-free medium if *ste11*$^+$ was overexpressed, it appears that the major function of Pcr1 in the sexual development of *S. pombe* is to promote transcription of *ste11*. Summarizing these observations, we speculate that active PKA may block induction of *ste11* expression, probably partially, through inhibition of the activity of Pcr1. The involvement of Pcr1 in the regulation of *fbp1* expression, which is also repressed by active PKA, may support this view. However, the possibilities that Pcr1 is independent of PKA and that full induction of *ste11* and *fbp1* expression calls for the function of Pcr1 in addition to the removal of repression by PKA cannot be excluded. It remains unknown, and will certainly be interesting to see, whether Pcr1 directly regulates expression of *ste11* and *fbp1* as a transcription factor.

How does PKA regulate the activity of Pcr1? Transcription of *pcr1* is not regulated by PKA (Fig. 6). Furthermore, overexpression of *pcr1*$^+$ does not induce ectopic sexual development, whereas overexpression of *ste11*$^+$ does (48). This suggests that the activity of Pcr1 is likely to be regulated posttranscriptionally. The activities of mammalian bZIP transcription factors such as Jun or CREB are known to be regulated by critical phosphorylation (3, 15, 44, 56). It is also suggested that *S. cerevisiae* CRE factor and *S. pombe* AP-1 factor are phosphorylated in vivo (21, 22). Thus, it is conceivable that the activity of a Pcr1 complex is regulated by phosphorylation, and we can speculate that Pka1, the PKA of *S. pombe* (30), is involved in this phosphorylation. However, Pcr1 itself carries no typical consensus sequence for phosphorylation by PKA and hence is unlikely to be a direct target of PKA. We found that Gad7, the putative partner of Pcr1, carries a site for phosphorylation by PKA (22a). We can thus assume that the activity of the Pcr1-Gad7 complex is modified by phosphorylation of Gad7. Further analysis of these proteins in terms of their phosphorylation status will critically answer the original question.

It has been suggested that a mitogen-activated protein (MAP) kinase cascade, which includes the *wis1* gene product as the MAP kinase kinase, functions as an integrator in the recognition of nutritional conditions (53). Further analysis suggests that *wis1* can affect the induction of *mei2* expression (45a), which is directly regulated by the transcription factor Ste11 (43). Therefore, it may also be possible that this MAP kinase cascade regulates the activity of Pcr1. However, again Pcr1 has no typical site of phosphorylation by MAP kinase, and Gad7 appears to be the target if the MAP kinase indeed controls these proteins.

It was noted that, in addition to the function required for sexual development, Pcr1 has a function crucial for cell growth in rich medium at low temperatures. This suggests that a transcriptional regulator containing Pcr1 will control gene expression essential for cell growth in accordance with the environmental nutrition. Cells defective in *ste11* do not show growth deficiency, and overexpression of *ste11*$^+$ cannot suppress the cold sensitivity of *pcr1*Δ cells. It is also unlikely that *fbp1* is related to the cold sensitivity. Hence, Pcr1 appears to activate a class of genes other than *fbp1* and *ste11* in cell growth control.

This study has demonstrated the involvement of a CREB/ATF protein, Pcr1, in the nutritional signal transduction pathway of *S. pombe*. Although more extensive analysis is required to determine the precise function and regulation of the protein, this report will certainly provide an important basis for further studies of bZIP transcription factors in the fission yeast.

ACKNOWLEDGMENTS

We thank N. Jones for helpful comments on our study.

This study was supported by grants-in-aid for scientific research from the Ministry of Education, Science, Sports and Culture of Japan.

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