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A novel essential fission yeast gene *pad1*$^+$ positively regulates *pap1*$^+$-dependent transcription and is implicated in the maintenance of chromosome structure

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SUMMARY

Fission yeast *pap1*$^+$ gene encodes an AP-1-like transcription factor, whose overexpression can confer resistance to staurosporine, a protein kinase inhibitor. We have previously identified a target gene (*p25*) for *pap1*$^+$, and shown that, *crm1*$^+$, which is required for maintenance of higher order chromosome structure, negatively regulates pap1-dependent transcription. In this study, we have characterized a novel gene, *pad1*$^+$, which was isolated as a multicopy plasmid capable of confering staurosporine-resistance. We showed that high copy *pad1*$^+$ induces transcriptional activation of the *p25* gene and that the induction by *pad1*$^+$ is dependent on the *pap1*$^+$ gene. Furthermore, a *cis*-element analysis of the 5′-region of the *p25* gene showed that two elements (an AP-1 site and a 14 bp palindrome sequence) where pap1 binds in vitro is essential for the induction by *pad1*$^+$. These results indicate that pad1 can positively regulate pap1-dependent transcription. Through an electromobility shift assay we showed that overexpression of *pad1*$^+$ is not capable of enhancing the DNA-binding activity of pap1 directly. The *pad1*$^+$ gene encodes a 35 kDa protein that has significant identity (68%) to *Caenorhabditis elegans* F37A4.5, and is also similar to mouse Mov34 and human C6.1A. Gene disruption experiments have demonstrated that *pad1*$^+$ is essential for viability. A disruption mutant of *pad1*$^+$ obtained after spore germination exhibited an elongated cell body with abberantly folded chromosomes. A mitotic plasmid loss experiment also produced similar cells having an abnormal chromosome structure. These suggest that *pad1*$^+$ may play an important role in higher order chromosome structure. Taken concurrently with our previous results, two essential genes *pad1*$^+$ and *crm1*$^+$ regulate pap1-dependent transcription; *pad1*$^+$ and *crm1*$^+$ are positive and negative regulators, respectively.

Key words: fission yeast, AP-1-like factor, chromosome structure

INTRODUCTION

Eukaryotic chromosomes are highly organized structures which are folded and packed in the nucleus. The spatial distribution and temporal regulation of chromosomes inside the nucleus is not random but is strictly controlled during the cell cycle. For example, chromosomes in the embryo of the developing fruit fly, *Drosophila melanogaster*, are highly organized. These chromosomes have a specific orientation regarding the centromere-telomere, with the centromere situated at one side of the nucleus and the telomeres at the other side (Hiraoka et al., 1990). In addition to changes in orientation during the cell cycle, chromosomes undergo physical morphogenesis. They are condensed during mitosis and form what has been termed 'higher order structures'. The chromatin in these higher ordered structures has been proposed to contain a chromatin loop which forms an ordered network termed a chromosomal scaffold (Laemmli et al., 1977). This dynamic alteration in the chromosome structure and physical location during the cell cycle is controlled by many factors. Very little, however, is currently known about the molecular mechanisms that control these events.

We have been investigating the genetic regulation of chromatin architecture by using the fission yeast *Schizosaccharomyces pombe* (reviewed by Yanagida, 1990). In fission yeast, the interphase nucleus consists of two distinct structures; one half packed by chromosomal DNAs and the other consisting of the nucleolus (McCully and Robinow, 1971; Toda et al., 1981). Upon entry into mitosis from interphase, the chromosomes become highly condensed, revealing three individual chromosomes (Toda et al., 1981, 1983; Umesono et al., 1984). It has recently been demonstrated, using the fluorescence in situ hybridization (FISH) technique, that fission yeast contains telomeres and centromeres at specific locations within the nucleus during the cell cycle (Funabiki et al., 1993).

We have isolated, by visual screening with DAPI, a series

of conditional mutants from *S. pombe* that control chromatin structure. The mutants can be categorized into four groups. The first includes structural or regulatory proteins for mitosis, such as *nda2*$^+$ and *nda3*$^+$, which encode α1- and β-tubulin, respectively (Toda et al., 1984; Hiraoka et al., 1985) and *dis2*$^+$, which encodes a type 1 protein phosphatase (Ohkura et al., 1989). A second class of chromatin regulating proteins are the S-phase regulators, *nda1*$^+$ and *nda4*$^+$, which encode proteins of the *CDC46*/*MCM2* family proteins (Miyake et al., 1993). The third class is composed of enzymes which change the DNA topology, such as *top1*$^+$ and *top2*$^+$, which encode topoisomerase I and II, respectively (Uemura et al., 1984). The fourth class is involved in the transcription machinery and includes *nuc1*$^+$, the largest subunit of RNA polymerase I (Hirano et al., 1989). Finally a mutant that does not fall into the above categories, *dcd1*$^+$/*pim1*$^+$ gene, has recently been described (Matsumoto and Beach, 1991; Sazer and Nurse, 1994). The gene that specifies *dcd1*$^+$/*pim1*$^+$ encodes a GDP/GTP exchange factor for a nuclear small G-protein spi1 and regulates a variety of nuclear events, including mitotic checkpoint, chromosome decondensation and mRNA processing/transport (Dasso, 1993).

The cold-sensitive *crm1* mutant was isolated during visual screening with DAPI, which showed altered fibrous chromatin structures at the restrictive temperature (Adachi and Yanagida, 1989). The *crm1*$^+$ gene encodes a highly conserved 115 kDa protein whose homologue exists from yeast to human. We found that, in the *crm1* mutant cell extract, a 25 kDa protein (p25) is highly overproduced (Adachi and Yanagida, 1989). We isolated a gene for the p25 protein and, curiously, found that it is a target gene for pap1 which we previously isolated and characterized as a fission yeast AP-1-like leucine zipper transcription factor (Toda et al., 1991, 1992). Our previous work (Toda et al., 1992) identified the crm1 protein as a negative regulator of pap1. We showed that deletion of the *pap1*$^+$ gene rescues cold-sensitivity of the *crm1* mutation, suggesting that a part of phenotypes of the *crm1* mutation is due to hyper-activation of pap1-dependent transcription (Toda et al., 1992).

In this study, we describe the characterization of a novel gene, *pad1*$^+$ which is one of two genes isolated as a multicopy plasmid suppressor of the staurosporine sensitive phenotype of *S. pombe*. The first gene characterized was *pap1*$^+$ and has been described elsewhere (Toda et al., 1991). Our previous analysis incorrectly assigned a MAP-kinase homologue *spk1*$^+$ as the gene responsible for conferring drug resistance. Here we demonstrate that a novel gene, *pad1*$^+$ whose chromosomal location is in close proximity to the *spk1*$^+$ gene, is responsible for the staurosporine resistance. Moreover, we have found that overexpression of *pad1*$^+$ stimulates pap1-dependent transcription in a pap1-dependent manner similar to that seen for the *crm1* mutant (Toda et al., 1992). The *pad1*$^+$ gene is essential for cell viability and encodes a 35 kDa protein with structural similarity to three previously isolated gene products from higher eukaryotes. Interestingly, *pad1* disrupted yeast cells show altered chromosome structure. The arrested cells have an elongated cell body, suggesting that *pad1*$^+$ is crucial for cell cycle progression. This report and data described elsewhere (Toda et al., 1992) identify both positive and negative regulatory genes of an AP-1-like transcription factor.

Table 1. Yeast strains used in this study

Strains	Genotypes	Derivations
HM123	*h*$^-$*leu1*	Our stock
AC1	*h*$^-$*leu1crm1-809*	Adachi and Yanagida (1989)
TP108-3C	*h*$^-$*leu1ura4pap1::ura4*$^+$	Toda et al. (1991)
TP123-6CF	*h*$^-$*leu1ura4ade6-216p25::ura4*$^+$	Ura$^-$ mutant obtained from TP123-6C
5A/1D	*h*$^-$/*h*$^+$*leu1/leu1ura4/ura4his2/+ ade6-M210/ade6-M216*	Ohkura et al. (1989)
TP42	*h*$^-$/*h*$^+$*leu1/leu1ura4/ura4his2/+ +/pad1::ura4*$^+$*ade6-M210/ ade6-M216*	This study
TPR12-1	*h*$^-$*leu1ura4ade6pad1::ura4*$^+$ containing pST23-8	This study,

MATERIALS AND METHODS

Strains and media

S. pombe strains used in this study are listed in Table 1. Complete medium, YPD (1% yeast extract, 2% polypeptone, 2% dextrose), modified synthetic EMM2 (Moreno et al., 1991), and SPA medium for sporulation (Gutz et al., 1974) have been described. Plates contained 1.6% of agar. Staurosporine (provided by Dr H. Nakano, Kyowa Hakko Co.) was used as described (Toda et al., 1991).

Genetic techniques and nomenclatures

Standard procedures for *S. pombe* genetics were followed according to Gutz et al. (1974) and Moreno et al. (1991). *S. pombe* cells were transformed using the lithium method (Ito et al., 1983). Cold-sensitive (*cs*) mutations are abbreviated by lowercase letters *cs* followed by the gene, such as *cs crm1*. Gene disruptions are denoted by lowercase letters representing the disrupted gene followed by two colons and the wild type gene marker used for disruption, such as *pad1*::*ura4*$^+$. In the text and figures, gene disruptions are abbreviated by the gene preceded by Δ, such as Δ*pad1*.

Nucleic acids preparation and manipulation

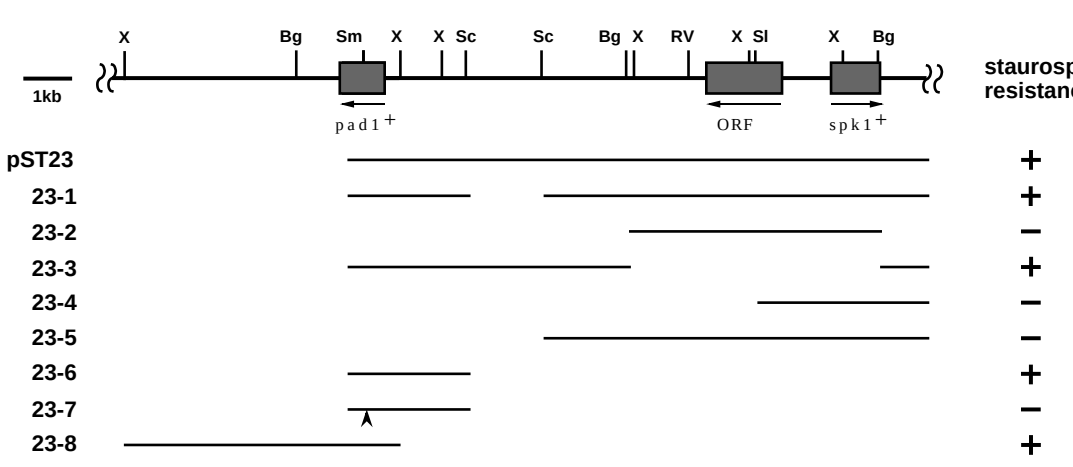
Standard molecular biology techniques were followed as described (Sambrook et al., 1989). Enzymes were used as recommended by suppliers (Takara Shuzo Co., TOYOBO Co. and New England Biolabs Co.). Total RNAs were prepared from fission yeast cells as described (Russell and Hall, 1983). RNAs were electrophoresed on a 1.2% agarose gel containing formaldehyde (Fourney et al., 1988). Restriction fragments used as DNA hybridization probes of *p25* and *pap1*$^+$ for Northern blotting have been previously described (Toda et al., 1992).

Nucleotide sequence determination

The dideoxy DNA sequencing method (Sanger et al., 1977) was used in combination with the unidirectional progressive deletion method to generate nested deletions (Henikoff, 1984) and the double-stranded plasmid DNA as template (Hattori and Sakaki, 1986). The 2.6 kb *Xba*I fragment containing the *pad1*$^+$ gene and the 3.0 kb *Eco*RI fragment containing the hypothetical ORF were isolated from pST23 (Fig. 1a) and subcloned into Bluescript (KS+, Stratagene). The 6.0 kb *Xba*I fragment containing the entire *pad1*$^+$ gene was isolated from a genomic DNA made in a cosmid vector (Mizukami et al., 1993) and subcloned into Bluescript. Nucleotide sequencing of these three fragments were determined. Only the first 1 kb sequence of the 6.0 kb *Xba*I fragment which contains the stop codon of the *pad1*$^+$ gene was determined.

Nucleotide sequence accession number

The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence data bases under accession numbers D31731 (*pad1*$^+$) and D31735 (*ORF*).



b

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ATTACGGAAGCACAGAAACATTTTGAGTCTGTTCAGGAACAAAGNTTACTTCAGAAAGCAAAGAATGAGCGATTGAAAACACATAAAGACAGNATTGATG  -601
AGTATAATAGACTACTGGAAAGTCAGTCGGAGCATTTTGACATGCCTAAAATTGGACCCGGCTAGTTTTCAAAANACGTCCTTTTTTCTCTCAATAGCTA  -501
TACTTAAATAGCAATGGATGCCTCCTCTATTCTGCATAGTTTTTCTCCATCCACCTACCATATTCTTTTATGAATTACGCACTCTAATGAAATCACTATT  -401
                                        XbaI
TTATTTTAACAATACGCATTATACCTCATTATTGTTATAGCTATTCATTTGTCTCTAGAAAAAACCTGTTTATAGCCAACTACCACGTAAACGTATACTA  -301
GCTGCCTAAGTGAGACCAAAGACATAGAATCTTGTATATTCGTTTTGTATAGTGGTTGAAACATATAGAAAGAACTTCCTTTGAAGAACAAGCACATTAT  -201
AGTGACCTTTGATTGTTTTTGTCGTCTTACTAAATATCAGTGAACAGCATTAACGTTTTGGTAACTTTATTTAAATACTTATACTGAGTTTCTCATTTAA  -101
                                                                                          NruI
TCTCCAATTACACTTTTATTTGCGGTTATTTGAGAGGAACCCCACTGATACTGATAAACTCTCATATTTCAATACATACATACGAGTCATATTTTTCGCG    -1
ATGGAATCTTTACAAAGATTGTTACAAGGTGCCCGCATGGGTACTGGTATGATGGGAGATCAGCCTCTTGTTGATAATTCCGAGTGTGTATACATTTCAT  +100
M  E  S  L  Q  R  L  L  Q  G  A  R  M  G  T  G  M  M  G  D  Q  P  L  V  D  N  S  E  C  V  Y  I  S  S    34

CTCTAGCTTTGTTAAAAATGCTTCGTCATGGGCGACATGGTACACCAATGGAAGTTATGGGTTTGATGTTAGGAGAATTCGTTGATGACTTTACAGTACG  +200
  L  A  L  L  K  M  L  R  H  G  R  H  G  T  P  M  E  V  M  G  L  M  L  G  E  F  V  D  D  F  T  V  R     67

TGTTGTTGATGTTTTTGCAATGCCTCAATCTGGTACAGGTGTCAGTGTAGAAGCAGTCGATCCAGTTTTCCAAAAAAAATATGATGGATATGCTTAAGCAA  +300
 V  V  D  V  F  A  M  P  Q  S  G  T  G  V  S  V  E  A  V  D  P  V  F  Q  K  N  M  M  D  M  L  K  Q     100
                                        SmaI
ACAGGACGGCCAGAAATGGTTGTAGGTTGGTATAATTCTCACCCGGGTTTTGGTTGTTGGCTTTCTAGTGTCGATATCAACACCCAACAATCATTTGAGC  +400
T  G  R  P  E  M  V  V  G  W  Y  N  S  H  P  G  F  G  C  W  L  S  S  V  D  I  N  T  Q  Q  S  F  E  Q    134

AATTAACACCAAGAGCGGTGGCAGTTGTTGTCGACCCTATTCAATCTGTCAAAGGAAAGGTGGTAATTGACGCCTTTCGATTGATTAATCCGTCTACCCT  +500
  L  T  P  R  A  V  A  V  V  V  D  P  I  Q  S  V  K  G  K  V  V  I  D  A  F  R  L  I  N  P  S  T  L    167

AATGATGGGTCAGGAACCCAGACAAACAACTTCCAATTTGGGTCACATCAACAAACCTAGCATTCAAGCTTTAATTCACGGTTTAGGAAGACATTACTAC  +600
 M  M  G  Q  E  P  R  Q  T  T  S  N  L  G  H  I  N  K  P  S  I  Q  A  L  I  H  G  L  G  R  H  Y  Y     200

TCCCTTCGCATCAATTACAAAAAAACTGAGCTTGAAGAGATCATGCTTTTGAATTTACACAAACAGCCTTGGGCACATGGTCTTTTACTTGAAAACTTTA  +700
S  L  R  I  N  Y  K  K  T  E  L  E  E  I  M  L  L  N  L  H  K  Q  P  W  A  H  G  L  L  L  E  N  F  N    234

ATTCTGCTGCGGAAAAAAATCATGCTTCTATTGATAAGATGAAATCCTTGTCAGAACAGTACACTGAACGTGTTCAAAATGAAGTGACTTTAAGTCCGGA  +800
  S  A  A  E  K  N  H  A  S  I  D  K  M  K  S  L  S  E  Q  Y  T  E  R  V  Q  N  E  V  T  L  S  P  E    267
                                  Sau3AI
GCAACTTCGTATTCAATACGTTGGTAAACAAGATCCCAAGAAGCATCTTGACGCTGAGGTTCAGAAATGTATAGACAACAATATTTCTTCCATGTTAGCT  +900
 Q  L  R  I  Q  Y  V  G  K  Q  D  P  K  K  H  L  D  A  E  V  Q  K  C  I  D  N  N  I  S  S  M  L  A     300

TGTATGCTTGATTCCGTTGCCTTCTAGTACATCTATTTTGGTAATACCTCTTTTCCATCATCCGTTTTTTATGAGCTACTTTCTTTCCTTACTCAAAGCA +1000
C  M  L  D  S  V  A  F  *                                                                               308

TTTATGAACTGTTTGAATAANCCTGTTGGTCGAATCTGATACTACTTTTTTACGATTTCATATTCGTGACTAGGAGTACATTTTTTTTAAAACTTTTTAC +1100
ATGATCGTGGAGTATGATTGATGATATTTTGGTAAGCTCTTGAGATTGCGAGCGTTTGCTTTGATGGATTCTTATATATTTTTTTTTCATAGATCCTTAAG +1200
ATATACCTGCACACCAGTCTTTCAAGCTTCTTCCCAATAAGAAAAAATTGTTTCAATTATTGAATTTGTAAACATCTTCTACCAACATGTTAAAAATTTT +1300
GTTTCCTTGATTGTTTGTAATGTACCTGTAAGGATCGTCGATCAGCGTCAAACCATTGAAGTTGAATACATAAAGGTATAACTGAGGCTACTTAATTTAT +1400
TATATGACATTTTAGCGTGATATGATTTTTTGATGTAGCATGATATTGTACTTGTCAAGTATNCCTATCTATCTTTCAACCTTTGCCAACCACTAACGTA +1500
TGTTGGTTTACGGAATCAACAAACAATTGTTTATTAAGAAGTTTAACAAGGTTTCTTTTTTATTTTCAACGTTTAAGACTTGCTCCAATATTTAACTGGT +1600
TTTTTGAATTCATAATGAATAGATCTGCTGCTTTATCAATCTTAAAGCGCCAAAGTTCTACGGCAGCTTCTTCTTCACTCAAGAGAACTCCTTTATATGA +1700
TCTGCATTTAAAAGAAGGAGCTACCATAGTCCCCTTTGCTGGTTTTTCAATGCCAGTACAGTATAAAGTCAAACCATTAGTGCCAGTCATAAGTGGACAC +1800
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Fig. 1. Subcloning, nucleotide sequence and predicted amino acid sequences of the pad1$^+$ gene. (a) Restriction map of the genomic DNA containing the insert of pST23 and various subclones are shown. The ability to confer drug-resistance in each subclone is also indicated. pST23-7 was constructed by inserting the *Bgl*II linker in the unique *Sma*I site, which leads to the frame shift mutation in the *pad1*$^+$ coding region. Three *ORFs* are indicated by filled boxes with the direction of transcription (arrows). *pad1*$^+$ locates in the left end of pST23 where the carboxyl portion encoding 29 amino acids is missing. *spk1*$^+$ encodes a MAP-kinase homologue which is involved in mating signal transduction (Toda et al., 1991; Gotoh et al., 1993). 1767 bp *ORF* which can encode a hypothetical protein consisting of 589 amino acid residues was found upstream of *spk1*$^+$ with opposite direction of transcription relative to *spk1*$^+$. Function of this putative *ORF* is unknown (M.S. and T.T., unpublished results). (b) Nucleotide sequence of the *pad1*$^+$ gene is shown together with the predicted amino acid sequence. Two internal restriction enzyme sites (*Nru*I and *Sma*I) were used for gene disruption (Materials and Methods). The *Sau*3AI site which is the left junction of the insert in pST23 was also shown, which is followed by carboxy-terminal 29 amino acids.

Overexpression of *pap1*$^+$ and *pad1*$^+$ in *S. pombe* cells

pST22 (containing the *pap1*$^+$ gene; Toda et al., 1991) and pST23 or pST23-8 (containing the *pad1*$^+$ gene; Fig. 1a) were used for high level protein expression.

Preparation of antisera and immunochemical assays

Production of anti-pap1 antibodies was previously described (Toda et al., 1991). Anti-pad1 antibodies were prepared as follows. The 1.6 kb *Nru*I/*Bgl*II fragment containing the entire *pad1*$^+$ gene (Fig. 1a) was inserted into the *Sma*I/*Bgl*II sites of pQF101 (a derivative of Bluescript KS+, Stratagene), yielding pQF-pad1. pQF101 had been constructed by inserting the *Bgl*II linker into the *Eco*RI site of Bluescript. The 1.6 kb *Bam*HI/*Bgl*II fragment was cut from pQF-pad1 and inserted into the *Bam*HI site of pAR3038 (Rosenberg et al., 1987),

yielding pT7-pad1. Expressed fusion protein, pad1 (38 kDa) was isolated from *E. coli* and polyclonal rabbit antibodies were raised. Affinity purification from crude serum was performed as described previously (Hirano et al., 1988). SDS-polyacrylamide gel electrophoresis was performed according to Laemmli (1970) and proteins were electrically transferred onto nitrocellulose filter (Towbin et al., 1979). Protein A conjugated with peroxidase (Bio-Rad) and chemiluminescence (ECL system, Amersham) were used to detect bound antibodies.

Analysis of the upstream region of the p25 gene

Construction of a series of subclones at the 5′ upstream regions of the *p25* gene and site-directed mutagenesis have been described previously (Toda et al., 1992). The following four oligonucleotides, AP-mt1 (CCATTTCTGAGAATTCCTATT), AP-mt2 (CCATTTCTGACGGATCCTATT), PLD-mt1 (ACCAAGAATTCGTAAACTACC) and PLD-mt2 (ACCAGGATCCCGTAAACTACC) were used for in vitro mutagenesis of either AP-1 or PLD and the mutated fragments were subcloned into pYS109 (Fig. 4a). Cells whose chromosomal *p25* gene had been disrupted (TP123-6CF, Table 1) were transformed with multicopy plasmids containing either *pap1*$^+$, *pad1*$^+$ or a vector plasmid (pDB248′, Beach and Nurse, 1981). Then, Leu$^+$ transformants were retransformed with various subclones of *p25* containing *ura4*$^+$ marker and Leu$^+$Ura$^+$ transformants were selected. Independent transformants were isolated from each transformation and analyzed by immunoblotting with the appropriate antibody.

DNA-binding analysis

A gel retardation assay was performed as described (Fried and Crothers, 1981; Toda et al., 1991). Total cellular proteins were prepared according to Hirano et al. (1988). The following complementary oligonucleotides were used for a gel retardation assay: p25-AP-1 (5′CCATTTCTGACTAATCCTATT3′/5′AATAGGATTAGTCAGAAATGG3′); and p25-PLD (5′GGTAGTTTACGTAATACTTGGT3′/5′CCATCAAATGCATTAGAACCA3′), where underlined sequences show the AP-1 site and palindromic sequence found in the 5′ upstream of the *p25* gene, respectively. Oligonucleotides were annealed and end-labeled with T4 DNA polynucleotide kinase as described (Toda et al., 1991). 20 μg cellular proteins were used per one mixture.

Gene disruption of *pad1*$^+$

The 2.7 kb *Xba*I/*Cla*I fragment containing the *pad1*$^+$ gene (Fig. 1a) was subcloned into Bluescript. Then, a 0.5 kb *Sma*I/*Nru*I internal fragment was deleted and the *Pst*I linker was inserted. The 1.8 kb *ura4*$^+$ *Pst*I fragment (Grimm et al., 1988) was inserted in the *Pst*I site, yielding p*pad1*::*ura4*$^+$. A 4.0 kb *Xba*I/*Cla*I fragment that contained the disrupted *pad1* gene (*pad1*::*ura4*$^+$) was used to transform the diploids (5A/1D, Table 1). The disruption was verified by Southern hybridization of the Ura$^+$ heterozygous diploids.

Phenotypic analysis of the *pad1*-deleted cells

pad1-deleted spores were analyzed as described previously (Booher and Beach 1987; Moreno et al., 1991; Ohkura and Yanagida, 1991) with the following minor modifications. Diploids that were heterozygous for the *pad1*$^+$ gene (TP42, Table 1) were grown to stationary phase in liquid synthetic EMM2 medium supplemented with leucine and sporulated overnight in sporulation medium (SPA, Gutz et al., 1974). Then unsporulated diploids were killed with treatment of 0.5% glusulase, followed by 30% ethanol. Free spores were germinated in synthetic EMM2 containing leucine, histidine and adenine for 36 hours incubation at 30°C and then the cell morphology was observed. Parental wild type diploids (5A/1D, Table 1) or heterozygous TP42 diploids transformed with a *pad1*$^+$-containing plasmid (pST23-8, Fig. 1a) were used as a control.

A mitotic plasmid loss experiment was performed with heterozygous TP42 diploids transformed with a *pad1*$^+$-containing plasmid (pST23-8, Fig. 1a). These cells were sporulated, treated with glusulase, and then spread on synthetic EMM2 plate supplemented with adenine to obtain a Leu$^+$Ura$^+$Ade$^-$ haploid segregant (TPR12-1, Table 1).

RESULTS

Isolation of the *pad1*$^+$ gene

Four plasmids (pST1, pST12, pST22 and pST23) that were capable of suppressing the staurosporine-supersensitive phenotype of the *sts3* mutant have been described previously (Toda et al., 1991). These plasmids did not contain the *sts3*$^+$ gene but were demonstrated to be multicopy suppressors capable of conferring a drug resistance phenotype in a wild type background. The three plasmids, pST1, pST12 and pST22, were shown to share a common insert which contained an AP-1-like transcription factor gene, *pap1*$^+$ that has been described elsewhere (Toda et al., 1991). By a combination of subcloning of pST23 and complementation with the *sts3* mutant, we previously proposed that MAP-kinase homologue *spk1*$^+$ (Fig. 1a) was responsible for conferring drug-resistant phenotype (Toda et al., 1991). Subsequent to these experiments we now show that the genomic sequence responsible for the drug resistance is not *spk1*$^+$, but a previously unidentified gene, which resides in the 5′-end of the insert (Fig. 1a). In fact, we have shown that the sequence containing *spk1*$^+$ alone does not confer drug-resistance to the wild type cells (Fig. 1a). The subclone pST23-6 which contains a 2.6 kb insert encompassing the drug-resistance suppressor activity, when mutagenized (digestion with *Sma*I to change the reading frame; see legend for Fig. 1a) leads to a plasmid (pST23-7) which is not capable of conferring drug-resistance (Fig. 1a).

The DNA sequence of the 2.6 kb fragment containing the putative drug-resistance gene was determined, and one continuous ORF which contained the essential *Sma*I site was found. This ORF did not contain a stop codon, indicating that a part of the suppressor gene was missing from this plasmid. We proceeded to isolate a flanking 6 kb *Xba*I fragment from a genomic library made in a cosmid vector (Fig. 1a, Mizukami et al., 1993). The nucleotide sequence of this fragment was determined and aligned with that determined for the 2.6 kb fragment. The entire ORF is capable of encoding a protein of 308 amino acids (Fig. 1b). The plasmid pST23 was lacking 29 amino acids from the carboxyl terminal end (Fig. 1b). We designated this gene *pad1*$^+$ (for *pa*p1 *d*ependent transcriptional activator *1*). This ORF appeared to contain the entire gene, as the molecular mass (p35) of the *pad1*$^+$ gene product identified by immunoblot (see below) was similar to that of the predicted value (34,548). Consistent with this finding is the fact that the truncated pad1 protein derived from pST23 was approximately 33 kDa (see below).

Similarity of the predicted pad1 protein to *C. elegans* and mammalian essential proteins

A search of the Genbank and EMBL data bases using the predicted amino acid sequence of pad1 revealed striking similarity to ORF of *C. elegans* F37A4.5 which was isolated from genomic sequence analysis of chromosome III (Wilson et al., 1994). As shown in Fig. 2, the amino terminal region of 200 amino acid residues are particularly similar; overall identity

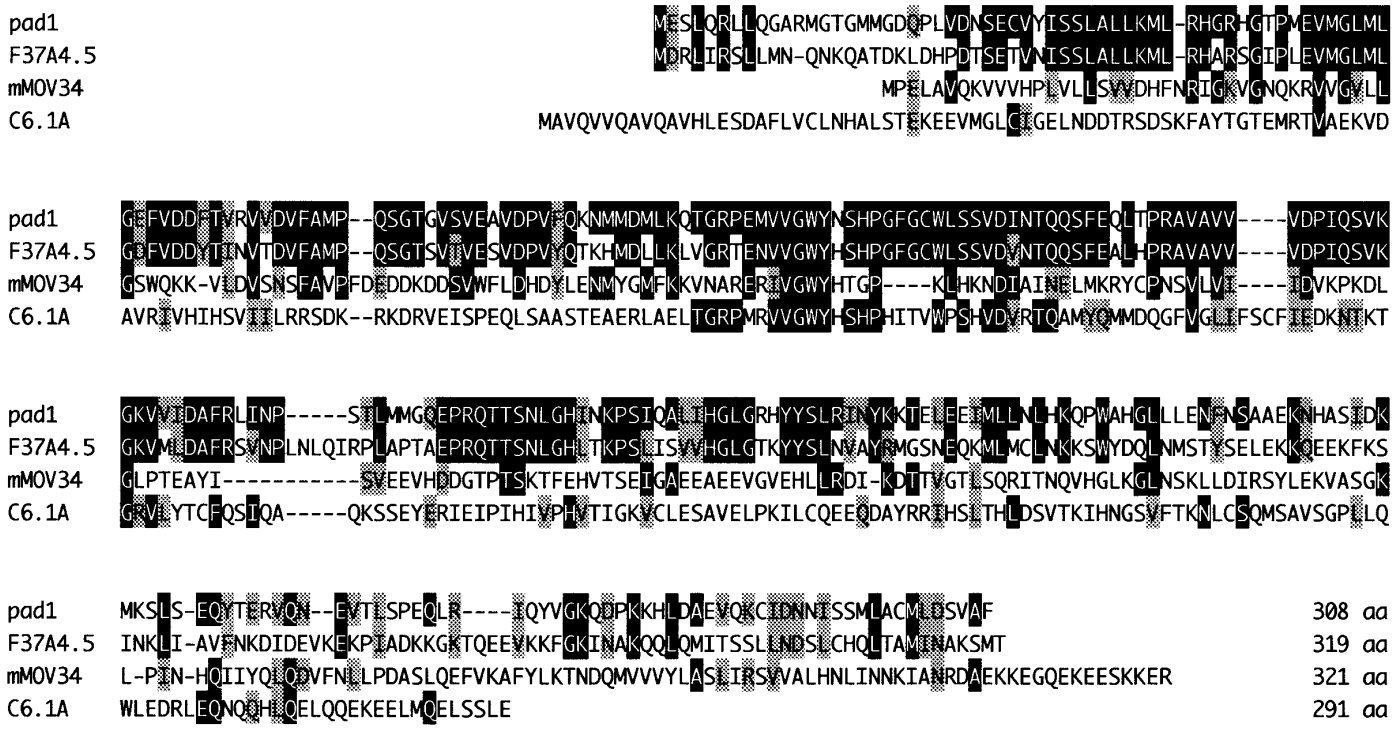

Fig. 2. Comparison of amino acid sequences among pad1, F37A4.5, Mov34 and C6.1A. Identical amino acids are emphasized by open letters with filled boxes and conservative amino acids are shown by hatched boxes. The sequence data were taken from Wilson et al. (1994, accession number: U00032), for F37A4.5; Kenwrick et al. (1992), for Mov34; and Gridley et al. (1990), for C6.1A.

reaches 68% (136 amino acids are identical). Moreover, the pad1 protein was found to be similar to that of two previously reported mammalian genes. One of these, *Mov34*, was first identified by an embryonic lethal mutation caused by integration of Molony virus into the mouse genome (Soriano et al., 1987) and subsequently isolated by Gridley et al. (1990). The other gene, termed C6.1A, was discovered as a human cDNA derived from a CpG island associated sequence of 5′ upstream of the factor VIII gene located in the X-chromosome-linked Xq28 (Kenwrick et al., 1992). Overall identity among pad1, Mov34 and C6.1A was fairly low (19% between pad1 and MOV34, 11% between pad1 and C6.1A, and 10% between MOV34 and C6.1A). However, the central region of about 30 amino acid residues (corresponding 100 to 128 of pad1; Fig. 2), shows greater than 50% amino acid identity amongst these three proteins. Unfortunately, the function of these *pad1*$^+$-related genes has not been identified, although *Mov34* clearly plays an essential role during mouse embryonic development as well as shows evolutionary conservation, being present in animal and insects (Gridley et al., 1990).

Overexpression of *pad1*$^+$ stimulates pap1-dependent transcription

A yeast cell expressing a multicopy plasmid containing the *pad1*$^+$ gene (pST23) can confer staurosporine-resistance only in the presence of *pap1*$^+$ gene; cells in which the *pap1*$^+$ gene is deleted do not show drug resistance, even in the presence of a multicopy *pad1*$^+$ (Toda et al., 1991). This suggests a functional relationship between *pap1*$^+$ and *pad1*$^+$. To determine if overexpression of the *pad1*$^+$ gene can affect the level of pap1-dependent transcripts, the levels of *p25* mRNA, whose gene is a target of pap1 (Toda et al., 1992), were examined in *pad1*$^+$ overproducing cells. As shown in Fig. 3a, the level of the *p25* mRNA greatly increased in cells containing multicopy *pad1*$^+$ plasmids (lane 2), similar to that seen in *pap1*$^+$-overproducing cells (lane 3). The level of the *pap1*$^+$ mRNA, in contrast, was not noticeably changed in *pad1*$^+$-overproducing cells (Fig. 3b, compare lanes 1 and 2). An immunoblot using anti-p25 and anti-pap1 antibodies demonstrated that, concurrent with the level of mRNAs, the amount of p25 increased. However, the level of pap1 (p75) did not change in *pad1*$^+$-overproducing cells (Fig. 3c and 3d).

In contrast to the results described above, *pad1*$^+$ multicopy plasmids, when introduced into *pap1*-deleted cells, are not able to stimulate transcription of the *p25* gene (Fig. 3a and 3c, lane 5), indicating that *pap1*$^+$ is required for transcriptional stimulation of the *p25* gene by *pad1*$^+$. These observations were consistent with our previous result that high copy *pad1*$^+$ does not confer drug resistance in *pap1*-deleted cells (Toda et al., 1991).

The *cis*-elements in the *p25* gene overlap with and are responsible for transcriptional activation by *pad1*$^+$ and *pap1*$^+$

Previous analyses on the 5′-flanking region in the *p25* gene showed that the two elements, AP-1 site (TTAGTCA, located −248 to −254, Fig. 4a) and 14 bp palindrome sequence (PLD, AGTTTACGTAATCT, located −193 to −206), are essential for transcriptional activation when *pap1*$^+$ is over-expressed or when the *crm1* gene is mutated. We demonstrated that bacterially expressed pap1 is capable of binding these two sequences

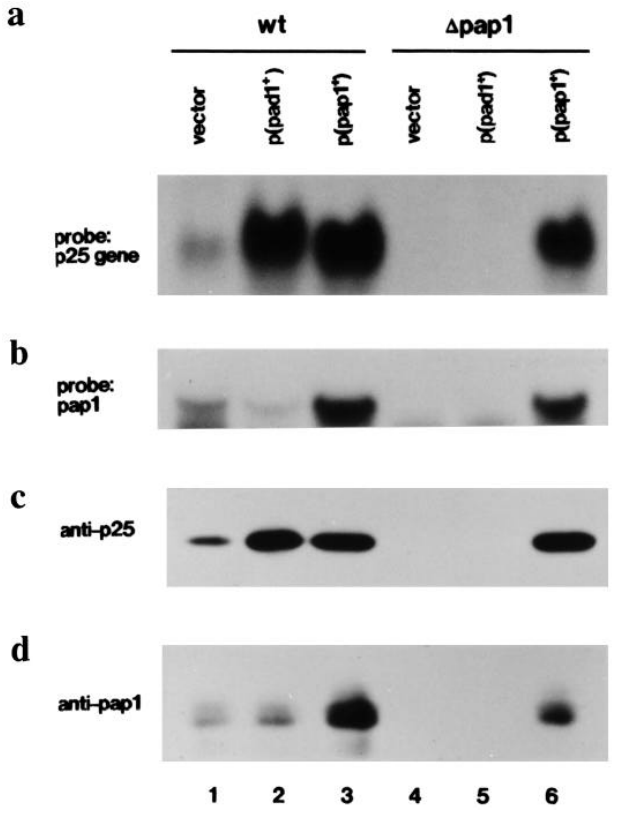

Fig. 3. Overexpression of the pad1$^+$ gene enhances the level of pap1-dependent transcription. Total RNAs (a and b) or total cell extracts (c and d) were prepared from following strains; wild type cells carrying a vector plasmid (lane 1), *pad1*$^+$-containing multicopy plasmids (lane 2) or *pap1*$^+$-containing plasmids (lane 3), or *pap1*-deleted cells (Δ*pap1*) containing a vector plasmid (lane 4), *pad1*$^+$-containing plasmids (lane 5) or *pap1*$^+$-containing plasmids (lane 6). Each filter was probed with a labeled fragment containing the *p25* gene (a), *pap1*$^+$ (b), or immunoblotted using anti-p25 antibodies (c) or anti-pap1 antibodies (d). Note that both mRNA and proteins of p25 are highly induced in *pad1*$^+$-overproducers in the wild type background (lane 2), whereas they are negligible in the *pap1*-deleted cells (lane 5).

in vitro (Toda et al., 1992). In order to determine the identity of the *cis*-element responsive to overexpression of *pad1*$^+$, a similar analysis was performed. A series of deletions in the 5′ region of the *p25* gene as well as in vitro generated mutants were constructed and used to examine the level of p25 in the presence of high copy *pad1*$^+$ (Fig. 4a). As shown in Fig. 4b, *pad1*$^+$ is capable of activating *p25* gene transcription as long as both AP-1 and PLD sequences were present (pYS108, pYS109 and pYS110 in Fig. 4b), however, it was incapable of inducing transcription if the constructs lacked the AP-1 site (pYS112 and pYS114). There is some difference between pYS108 and pYS109 in the level of the p25 protein in the absence of *pap1*$^+$ (Fig. 4B). The sequence upstream the *Cla*I site (Fig. 4A) might be important for the pap1-indepedent basal level of the expression.

Importance of AP-1 and PLD sequences was confirmed by examining site-directed mutants made in either the AP-1 or PLD sequence (Fig. 4c, Toda et al., 1992). There was no measurable induction observed when the PLD sequence was mutated (Fig. 4c). However, a mutant AP-1 sequence does contain some residual transcriptional activity. Also we saw some, although decreased, p25 protein in pYS112 which contains only PLD, but not AP-1 (Fig. 4a and 4b). These results may suggest a functional difference of the two *cis*-elements in this promoter. In any case, results obtained in this

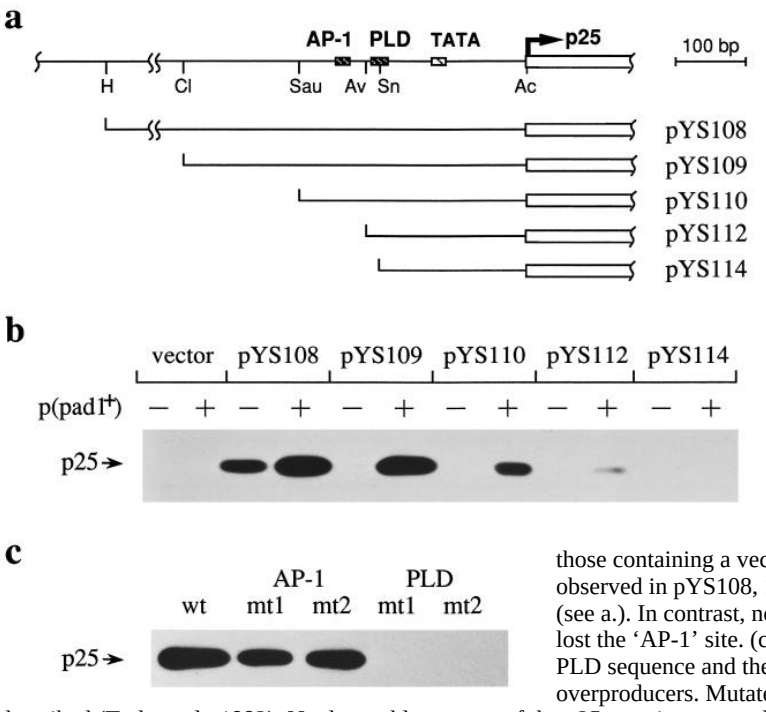

Fig. 4. Analysis of the essential *cis*-elements in the upstream region of the *p25* gene. (a) Restriction map, schematic structure of the *p25* gene and a series of subclones are indicated. Each restriction fragment that contained different 5′ segments of the *p25* gene was inserted into multicopy plasmids carrying the fission yeast *ura4*$^+$ gene (Grimm et al., 1988) as a selectable marker (see Materials and Methods). Open boxes and arrow show a *p25* open reading frame and direction of the transcription. The 'AP-1' site (AP-1) and 14 bp palindrome sequence (PLD) and putative TATA box (TATA) are schematically shown. Ac: *Acc*I, Av: *Avr*II, Cl: *Cla*I, H: *Hin*dIII, Sn: *Sna*BI, Su: *Sau*3AI. (b) Each transformant was grown and cell extracts were prepared. After electrophoresis on SDS-polyacrylamide gel, total proteins were transferred onto nitrocellulose filters. The filters were immunoblotted with anti-p25 antibodies and visualized by the peroxidase-conjugated second antibodies (Bio-Rad) and chemiluminescence (ECL system, Amersham). + represents transformants containing a multicopy plasmid carrying the *pad1*$^+$ gene and – means those containing a vector plasmid. Stimulation of the p25 expression could be observed in pYS108, 109 and 110, all of which contained the intact 'AP-1' sequence (see a.). In contrast, no stimulation was seen in pYS112 and pYS114, both of which lost the 'AP-1' site. (c) Site-directed mutation was introduced into either AP-1 site or PLD sequence and the level of the p25 protein was examined in *pad1*$^+$-overproducers. Mutated sequences of AP-1 or PLD (mt1 and mt2) were previously described (Toda et al., 1992). No detectable amount of the p25 protein was produced in either of PLD mutant, whereas reduced but detectable level of the protein was made in either of the two mutants of AP-1.

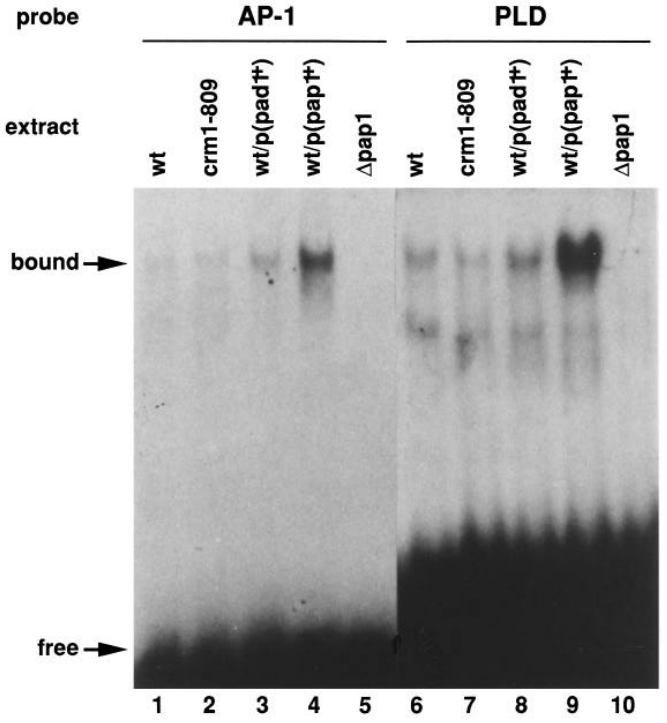

Fig. 5. DNA-binding activity of an AP-1-like factor is not augmented in cells containing the multicopy-*pad1*$^+$ plasmids. Whole cell extracts (20 μg/reaction) were mixed with end-labeled oligonucleotides (1 ng/reaction) containing the AP-1 site (lanes 1-5) or the palindrome sequence (lanes 6-10). The mixture was run on 4% native polyacrylamide gel as described before (Toda et al., 1991). Whole cell extracts were prepared from cells of wild type (HM123, Table 1, lanes 1 and 6), *crm1-809* (AC1, lanes 2 and 7), wild type cells carrying multicopy plasmids containing the *pad1*$^+$ gene (lanes 3 and 8), wild type cells carrying multicopy plasmids containing the *pap1*$^+$ gene (lanes 4 and 9) or *pap1*-deleted cells (TP108-3C, lanes 5 and 10). The position of a DNA-protein complexes (bound) and that of unbound oligonucleotides (free) were shown. The specific activity of the labeled probe containing PLD was approximately three times higher than that of AP-1, which caused apparent stronger signals produced from the PLD-containing probe. Note that DNA-binding activity was not significantly altered in cells of *cs crm1* or cells carrying multicopy *pad1*$^+$-containing plasmids, whereas the binding activity was enhanced in *pap1*$^+$-overproducing cells.

study clearly showed that overexpression of *pad1*$^+$ can induce *p25* gene expression in a pap1-dependent manner. Both the pap1 protein and the pap1-binding sequence are essential for *pad1*$^+$ to induce the *p25* transcription. We have therefore designate the gene *pad1*$^+$ (*pa*p1 *d*ependent transcriptional activator *1*).

Overexpression of *pad1*$^+$ can stimulate pap1-dependent transcription through different mechanisms than that of *pap1*$^+$

We have demonstrated that *pad1*$^+$ encodes a protein which can regulate, in a positive manner, pap1-dependent transcription. How does pad1 stimulate pap1-dependent transcription? The level of gene expression may be regulated via several different manners. For example, it is possible that the gene expression is regulated by the efficiency with which pap1 binds to DNA.

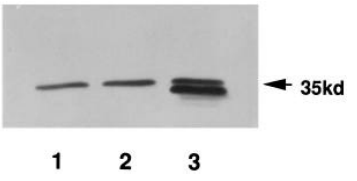

Fig. 6. Identification of the *pad1*$^+$ gene product. Cellular extracts were prepared from wild type cells containing a vector plasmid (lane 1), a multicopy plasmid containing either *pap1*$^+$ (lane 2) or truncated *pad1*$^+$ (lane 3). Note that the pad1 protein was recognized as a 35 kDa band (p35) and that the protein produced from an episomal *pad1*$^+$ gene which is missing carboxyl 29 amino acids is detected as a slightly smaller band (p33).

To address this possibility, an electrophoretic mobility gel shift assay was performed.

Total cellular proteins were prepared from wild type cells (lanes 1 and 6, Fig. 5), *crm1-809* mutant (lanes 2 and 7), *pad1*$^+$-overproducer (lanes 3 and 8), *pap1*$^+$-overproducer (lanes 4 and 9) or *pap1*-deleted cells (lanes 5 and 10) and a gel retardation assay was performed using end-labeled oligonucleotides containing either AP-1 (lanes 1 to 5) or PLD (lanes 6 to 10) (see Materials and Methods). The intensity of the slower migrating band was greatly enhanced in *pap1*$^+$-overproducing cells, whereas no significant increase in the intensity was observed in either *pad1*$^+$-overproducers or in the *crm1* mutant. Extracts that were prepared from *pap1*-deleted cells did not contain any AP-1- or PLD-binding activities (lanes 5 and 10). Thus, overproduction of *pad1*$^+$ does not increase the amount of pap1-DNA complex detectable in this bandshift assay using AP-1 or PLD substrates alone. This result was consistent with our previous data which showed the level of pap1 protein was not altered by either of overexpression of *pad1*$^+$ or a *crm1* mutation (Fig. 3 and Toda et al., 1992). These data suggest that these two proteins did not enhance the DNA-binding ability of pap1.

Identification of the *pad1*$^+$ gene product

To identify the *pad1*$^+$ gene product, polyclonal antibodies were raised against bacterially produced pad1 protein (Materials and Methods). These antibodies were affinity purified and used for immunoblotting. As shown in Fig. 6, a protein of approximately 35 kDa in molecular mass (p35) was detected in wild type cells with anti-pad1 antibodies (lane 1). Cells carrying pST23, which contained a truncated *pad1*$^+$ gene that lacks the carboxy-terminal 29 amino acids (Fig. 1a), possessed a slightly smaller form (33 kDa) of pad1, in addition to the wild type p35 protein (lane 3). Note that the protein levels of $p35^{pad1}$ was not significantly altered in cells containing a multicopy plasmid expressing the *pap1*$^+$ gene (lane 2).

Phenotypes of inviable *pad1*-deleted cells

To construct *pad1*-deleted cells, a one step gene replacement method (Rothstein, 1983) was performed to make a heterozygous diploid. The *ura4*$^+$ marker was inserted into the middle of *pad1*$^+$ coding region and used to transform the wild type yeast strain (5A/1D, Table 1, see Materials and Methods for details). Twelve tetrads were dissected from the heterozygous diploid (TP42, Table 1), and each tetrad resulted in two viable and two inviable spores. All the viable spores obtained were auxotrophic for uracil, which indicates that the *pad1*$^+$ gene is essential for cell viability. Microscopic observation of the

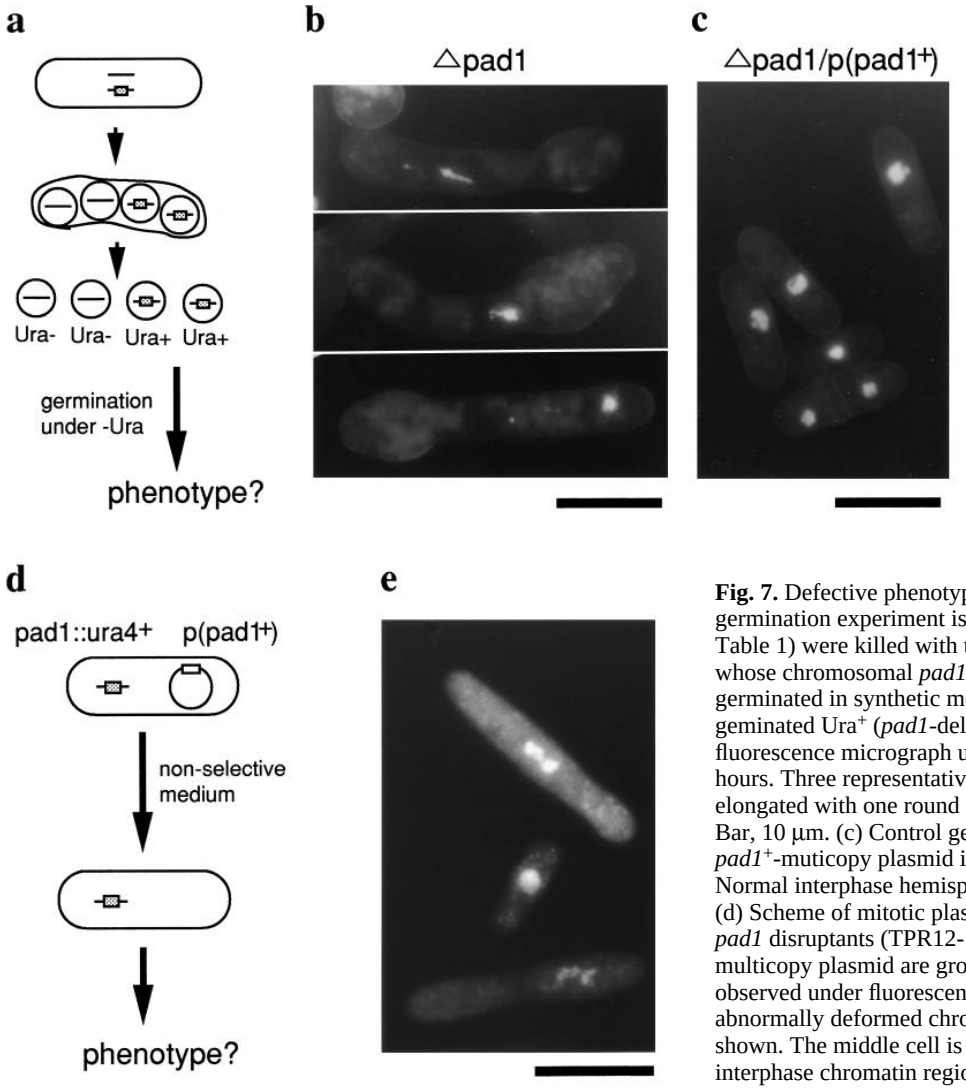

Fig. 7. Defective phenotypes of the *pad1*-deleted cells. (a) Scheme of germination experiment is shown. Unsporulated diploids (TP42, Table 1) were killed with treatment of glusulase and free Ura$^+$ spores whose chromosomal *pad1*$^+$ gene is deleted are allowed to be germinated in synthetic medium lacking uracil. (b) Morphology of geminated Ura$^+$ (*pad1*-deleted) spores were observed under fluorescence micrograph using a DNA-binding dye, DAPI after 36 hours. Three representative cells are shown. The cell body is elongated with one round end, and the chromatin region is deformed. Bar, 10 μm. (c) Control germinated cells which contain multicopy *pad1*$^+$-muticopy plasmid in the *pad1*-deleted background are shown. Normal interphase hemispherical structures are observed. Bar,10 μm. (d) Scheme of mitotic plasmid loss experiment is shown. Leu$^+$Ura$^+$ *pad1* disruptants (TPR12-1, Table 1) carrying the *pad1*$^+$ gene on a multicopy plasmid are grown under non-selective condition and observed under fluorescence micrograph. (e) Elongated cells with abnormally deformed chromatin region (upper and bottom) are shown. The middle cell is normal, showing a hemispherical interphase chromatin region. Bar, 10 μm

inviable *pad1*-deleted spores showed that most of the spores had germinated, divided once or at most a couple of times and then arrested as elongated cells.

To examine in more detail the lethal phenotype of a *pad1* gene disruption, we observed the *pad1*-deleted cells for the ability to germinate in liquid culture and by mitotic plasmid loss (Fig. 7a and d, respectively, Booher and Beach 1987; Moreno et al., 1991; Ohkura and Yanagida, 1991). The germination experiment was performed as follows. Ura$^+$ (*pad1*-deleted) spores were selectively germinated in liquid synthetic medium lacking uracil, and the cell morphology was observed after 36 hours. We observed that greater than 50% of the cells germinated showed an abnormal and elongated cell body with a deformed chromosomal DNA (Fig. 7b). Specifically, one end of the elongated cells often had a broad and round morphology, probably due to the cells arresting during the process of growth of the germ tube from a spherical spores. The nucleus was often abnormally located in the cell body; in some cases, it moved toward the growing apex (Fig. 7b, top and bottom) and in others, it remained close to the round end of the cell body (Fig. 7b, middle). Chromosomal DNAs of *pad1* disruptants were also abnormally deformed. DAPI staining of wild type cells in interphase demonstrated that the chromatin appeared as a hemispherical structure with two protrusions that consisted of ribosomal DNA and in mitosis the chromosomes condense (Toda et al., 1981; Umesono et al., 1984, see Fig. 7c). However, in the disruptants, the chromatin region looked much more compact than that in wild type and in some instances appeared ‘streaked’ (Fig. 7b). This suggests that pad1 may function to aid in establishment of the chromatin architecture during cell cycle progression.

It is also possible, by mitotic plasmid loss, to demonstrate similar results to that described above. As shown in Fig. 7e, some fraction of cells whose chromosomal *pad1*$^+$ gene was deleted, but kept viable by expression from an episomal *pad1*$^+$-containing plasmid (TPR12-1, Table 1), showed abnormal elongation. These cells often have a deformed and compact chromatin region. Moreover, the chromosomal DNA sometimes appears fibrous (upper and bottom cells in Fig. 7e). These abnormal cells, although low in frequency (less than 5%), have never been observed in the wild type culture. Taken together these observations suggest that the pad1 protein is involved in some essential aspect for integrating chromosome structure.

DISCUSSION

Identification of *pad1*$^+$ as a positive regulator of pap1-dependent transcription

In the present study, we have shown that *pad1*$^+$ is a positive regulator for pap1-dependent transcription. An electrophoretic mobility shift assay with oligonucleotides that contain either AP-1 or PLD binding site does not show an elevated level of DNA-binding activity in cell extracts prepared from *pad1*-overproduing cells. pad1 does not activate transcription by either increasing the amount of pap1 or by enhancing its DNA-binding activity. One potential mechanism is that pad1 is capable of augmenting the physical interaction between pap1 and the basic transcription machinery. However, the details of this molecular interaction are unclear and we are currently investigating the transcriptional activation by pad1.

We previously reported that a MAP-kinase homologue, *spk1*$^+$ (Fig. 1a) was responsible for conferring the drug-resistance phenotype (Toda et al., 1991). Further subcloning analysis by using the wild type strain as a host, presented in this study, leads us to conclude that in fact *pad1*$^+$, which is contiguous to *spk1*$^+$ in the plasmid pST23, confers the resistance phenotype. We attribute this discrepancy to strain differences used for subcloning. In the previous study, we used the staurosporine-supersensitive mutant strain *sts3*. As staurosporine inhibits various types of protein kinases in vitro (Tamaoki et al., 1986), in the *sts3* mutant background, overproduction of the spk1 kinase might have conferred staurosporine-resistance. On the contrary to our previous result, a subclone pST23-3, which contains the *pad1*$^+$ gene, but not *spk1*$^+$, could confer staurosporine resistance in the wild type background (Fig. 1a). We could not repeat the previous result showing that pST23-3 was incapable of conferring drug resistance in the *sts3* background. Thus, these new results obtained demonstrate that the *pad1*$^+$ gene, not *spk1*$^+$, is responsible for all the phenotypes of pST23, including drug-resistance, the transcriptional induction of the *p25* gene and a genetical interaction with *pap1*$^+$. Although it is true that the spk1 protein is induced by treatment of staurosporine as previously reported (Toda et al., 1991), *spk1*$^+$ seems to be involved only in mating signal transduction pathway (Gotoh et al., 1993).

pad1 as a regulator of chromosome structure

We have demonstrated that the *pad1*$^+$ gene is essential for spore viability and the vegetative life cycle. *pad1* deficient cells obtained either by germination from spores or by mitotic plasmid loss show altered chromosome structure with elongated cell bodies, suggesting that *pad1*$^+$ is involved in regulation of chromatin structure in cell cycle progression. By FACscan analysis with *pad1*-deleted cells, we have shown that DNA replication takes place in the absence of *pad1*$^+$ function (K. Kumada, M.S. and T.T., unpublished results). There may be two possibilities that link transcriptional regulation to chromatin structure during cell cycle progression. The first is that components or regulators of chromatin structure may affect the efficiency of transcription. For example, in fission yeast, topoisomerase I which determines chromatin organization is shown to be crucial for transcription of the ribosomal RNAs (Uemura et al., 1984). The second possibility is that transcription factors determine chromatin structure as in the case of *nuc1*$^+$, which encodes the largest subunit of RNA polymerase I (Hirano et al., 1989). At present, we are unable to define the role of pad1 to either of these two possibilities. It is also possible that pad1 may regulate gene expression other than pap1-dependent transcription. As transcription is one of the major events executed in the nucleus, it is not surprising that gene products which are neccesary for chromatin structure can directly or indirectly affect transcriptional efficiency.

The displacement of the nucleus observed in *pad1*-deleted cells also suggests a function of *pad1*$^+$ in nuclear positioning. It has been shown that nuclear localization is tightly regulated during the cell cycle. In fact, a variety of mitotic mutants defective in nuclear displacement, including *nda2* and *nda3*, which encode α1- and β-tubulin, respectively (Toda et al., 1984; Hiraoka et al., 1985) have been described. Interestingly, these mutants have a highly condensed chromosome structure, with three distinct and clearly visible chromosomes (Umesono et al., 1984). The degree of chromosome condensation in *pad1*-deleted cells is less than seen in the tubulin mutants. It is important to note that cells that have been germinated from inviable *pad1*-deleted spores elongate in the absence of nuclear division. This indicates that they are not arrested in mid mitosis, as in fission yeast cell elongation is terminated upon entry into mitosis (Mitchison, 1970). Therefore, these observations suggest that *pad1*-deficient cells are not defective in exiting from mitosis. Instead, *pad1*$^+$ can regulate some aspect of maintenance of chromatin structure although the involvement might be indirect, which then leads to abnormal chromosome structure followed by cell cycle arrest.

The transcription factor pap1 is controlled by the functional interaction between pad1 and crm1

Our previous work has identified *crm1*$^+$, a cold-sensitive mutant which showed abnormal chromosome structure at the restrictive temperature (Adachi and Yanagida, 1989), as a negative regulator for pap1-dependent transcription. Overexpression of *pad1*$^+$ exhibits a phenotype similar to the *cs crm1* mutation, including activation of pap1-dependent transcription and staurosporine resistance. Analogous to that seen in *pad1*$^+$ overexpressing cells, the *cs crm1* mutation does not enhance the DNA-binding activity of pap1. Therefore, in both cases, stimulation of *p25* transcription must occur at the transcriptional activation step. Consequently, *crm1*$^+$ and *pad1*$^+$ seem to work antagonistically in terms of regulation of pap1-dependent transcription (Fig. 8).

Both *crm1*$^+$ and *pad1*$^+$ must have functions related to chromatin structure other than the regulation of *pap1*$^+$ because *pap1*$^+$ itself is not an essential gene for viability whereas both *pad1*$^+$ and *crm1*$^+$ are essential genes. It should be emphasized that, although pad1 and crm1 function in opposition to each other, both gene products are required for the structural architecture of the chromatin regions; *a loss of function* mutation of either gene leads to abnormal chromosome structure (Fig. 8).

We performed several immunoprecipitation experiments using antisera specific for pap1, pad1 and crm1 to examine the cellular location as well as potential complex formation amongst these proteins. No physical interaction has been found (K. Kumada, M.S. and T.T., unpublished results). The crm1 protein is present in the nucleus and is especially prevalent in the nuclear periphery (Adachi and Yanagida, 1989). We were unable to determine the cellular localization of the pad1 protein

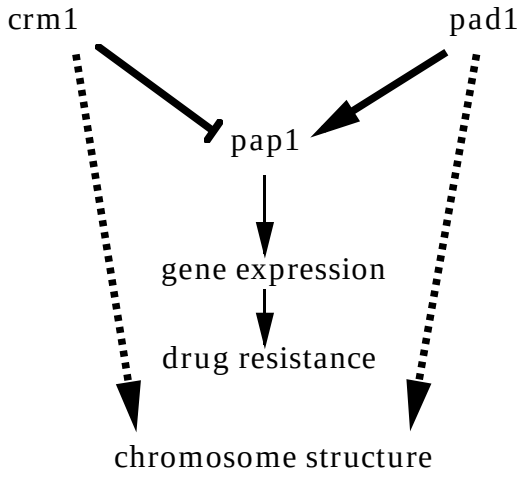

Fig. 8. Model of an interacting network in a fission yeast AP-1-like factor. A proposed model on cellular functions of a fission yeast AP-1-like factor pap1 and those of the two regulators are shown. pad1 positively regulates pap1-dependent transcription (shown by arrow) and crm1 negatively does (shown by perpendicular line on top). Both pad1 and crm1 are essential for cell viability and implicated in maintaining chromosome structure (shown by dotted lines). Either overexpression of *pad1*$^+$ or *cs crm1* mutation leads to enhancement of pap1-dependent transcription and pleiotropic drug resistant phenotype.

by immunofluorescence microscopy using anti-pad1 antibodies.

The *pad1*$^+$ gene confers resistance to several drugs

Overexpression of the *pad1*$^+$ gene confers resistance to staurosporine as well as other drugs, such as cycloheximide and caffeine (K. Kumada, M.S. and T.T., unpublished results). Moreover, we have observed this pleiotropic drug resistant phenotypes in cells containing high copy *pap1*$^+$ or *crm1* mutants (Adachi and Yanagida, 1989; K. Kumada, M.S. and T.T., unpublished results). On the other hand, cells that are deleted for *pap1*$^+$ are viable and are supersensitive to staurosporine as well as various unrelated drugs (Toda et al., 1991, 1992; K. Kumada, M.S. and T.T., unpublished results). Budding yeast *YAP1*, which encodes a structurally related AP-1-like factor (Moye-Rowley et al., 1989) has been isolated several times as plasmid suppressors that confer pleiotropic drug resistance (Hussain and Lenard, 1991; Schnell et al., 1992; Kuge and Jones, 1994). These two yeast AP-1-like factors may have a similar function in determining sensitivity to various unrelated drugs. It is possible that the drug resistant phenotype elicited by overexpression of *pap1*$^+$, *pad1*$^+$ or *cs crm1* mutation might be related to the gene regulation of oxygen detoxification enzymes (Schnell et al., 1992) or non-enzymatic antioxidants analogous to that suggested for the budding yeast *YAP1* gene (Kuge and Jones, 1994).

p25 gene and a cellular target of pap1

We and others have noticed that the budding yeast contains a homologue of the *p25* gene which encodes a protein that is 56% identical to p25 (*YCR004C*, Biteau et al., 1992; Oliver et al., 1992; Slonimski and Brouillet, 1993). Interestingly, we have found that the promoter region of *YCR004C*, like that of *p25*, contains two AP-1-like sequences (TTAGTTA and TTAGTAA). It is thus possible that *YCR004C* is also a target gene for *YAP1* in budding yeast as well.

The *p25* gene is conserved even in bacteria as the *E. coli* WrbA protein (Yang et al., 1993) is 42% identical to p25. The WrbA protein was copurified with the trp repressor (TrpR) and shown to enhance the formation and/or stability of noncovalent complexes between TrpR homorepressor and its primary operator targets (Yang et al., 1993). Unlike the case for the bacterial WrbA and TrpR, we have been unable to demonstrate any physical interaction between pap1 and p25 (Y. Adachi, M.Y. and T.T., unpublished results). It is worth pointed out that p25, YCR004C and WrbR all contain a conserved FMN-binding motif (Koonin et al., 1994). The function of p25 is not clear at this point. We know that it is non-essential for cell viability and unlike a *pap1* gene disruption, *p25* deletion has shown no obvious phenotype. Identification of the target genes for pap1 other than *p25* is crucial to understand the molecular mechanisms underlying the pleiotropic drug resistant phenotypes.

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