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Fission Yeast pap1-Dependent Transcription Is Negatively Regulated by an Essential Nuclear Protein, crm1

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The fission yeast *pap1*$^+$ gene encodes an AP-1-like transcription factor that contains a leucine zipper motif. We identified a target gene of pap1, the p25 gene. The 5′ upstream region of the p25 gene contains an AP-1 site, and by DNase I footprint analysis, we showed that the pap1 protein binds to the AP-1 site as well as to a 14-bp palindrome sequence. p25 is overproduced when the *pap1*$^+$ gene is overexpressed, whereas p25 is not produced at all in the *pap1* deletion mutant. p25 was previously found to be overproduced in strains carrying cold-sensitive *crm1* mutations whose gene product is essential for viability and is thought to play an important role in maintenance of a proper chromosomal architecture. Deletion and site-directed mutagenesis of sequences upstream of the p25 gene demonstrated that the AP-1 site as well as the palindrome sequence are crucial for transcriptional activation either by *pap1* overproduction or by the cold-sensitive *crm1* mutation; *pap1*$^+$ is apparently negatively regulated by *crm1*$^+$. Moreover, we found that cold-sensitive *crm1* mutations are suppressed by the deletion of *pap1*$^+$, further indicating a close relationship between *crm1*$^+$ and *pap1*$^+$. The crm1 protein is highly conserved; the budding yeast homolog, *CRM1*, which complements the fission yeast cold-sensitive *crm1* mutation, was isolated and found to also be essential for viability. These results suggest the functional importance of chromosome structure on the regulation of gene expression through the pap1 transcription factor.

The nucleus is the final destination to which numerous external and internal cellular signals are transmitted; the cell responds to a particular signal by altering the expression of one gene or a set of targeted genes. Many eukaryotic genes contain multiple *cis*-acting elements that are recognized by various types of sequence-specific *trans*-acting factors (reviewed in references 29 and 30). Dissection of molecular mechanisms involved in the regulation of these transcription factors is thus crucial for understanding how transcriptional control results from signal transduction. In addition to these *trans*-acting factors, it has been suggested that the chromatin structure of the *cis*-acting sequences can also play an important role in determining the pattern of gene expression. Transcriptional activation and repression of certain genes correlates with the proper positioning of nucleosomes, and higher-order chromatin structure is known to affect gene expression (4, 11). For example, in the budding yeast *Saccharomyces cerevisiae*, repression of the silent mating-type loci is thought to be the result of altered chromatin structure (35). Genetic evidence has indicated that the nucleosomal component histone H4 is responsible for the repression of these regions (24, 41). It has also been proposed that the nuclear scaffold has an important role in transcriptional control (14). In the fly, specific DNA sequences that associate with the nuclear scaffold are often close to promoters and colocalize with upstream regulatory regions (13).

The leucine repeat motif (leucine zipper) present in many eukaryotic transcription factors contains periodic leucine residues at every seventh position, preceded by a region rich in basic amino acids (27). In mammals, products of the proto-oncogenes c-*jun* and c-*fos*, which constitute an AP-1 transcription factor complex, are also shown to be transcriptionally and posttranslationally regulated (1, 7, 9, 10, 40, 48). These posttranslational modifications of an AP-1 factor include phosphorylation-dephosphorylation mediated through the ras protein, protein kinase C, and MAP kinase and by redox regulation. However, studies on the regulation of AP-1 factors have failed to convincingly demonstrate a causal relationship between an AP-1 factor and these potential regulatory factors. The experimental systems so far used have not been amenable to detailed genetic manipulation. Although binding of the jun-fos peptides to the AP-1 site in vitro has been shown to cause DNA bending (25), an in vivo functional relationship between DNA bending and chromatin structure has not yet been established.

We previously cloned the *pap1*$^+$ gene from the fission yeast *Schizosaccharomyces pombe* as a multicopy plasmid which conferred resistance to staurosporine, a drug which is a potent protein kinase inhibitor (33, 38, 49, 51). Nucleotide sequencing of the gene revealed that *pap1*$^+$ encodes a transcription factor which contains a leucine zipper motif, and biochemical analysis has shown that the pap1 protein behaves identically to the fission yeast AP-1-like factors that Jones et al. (23) had previously reported (52). Our work has focused on regulation of this fission yeast AP-1-like factor, pap1. In addition to multicopy genes that confer staurosporine resistance, we had previously isolated a cold-sensitive (cs) and staurosporine-resistant mutant, *crm1* (*crm* stands for chromosome region maintenance) (3). The *crm1* mutants were originally isolated by visually screening 4,6-diamidino-2-phenylindole (DAPI)-stained cells among a collection of cs mutants to identify gene products which are essential for maintaining the proper higher-order chromosome structure (3). The aberrant chromosome structure of the *crm1* mutants at the restrictive temperature was so conspicuous that all

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TABLE 1. Yeast strains used

Strain	Genotype	Source or reference
HM123	*h⁻ leu1*	Our stock
JY6	*h⁺ leu1 his2*	Our stock
AC1	*h⁻ leu1 crm1-809*	3
AT1	*h⁺ leu1 ura4 crm1-809*	Segregant between AC1 and JY6
TP108-3C	*h⁻ leu1 ura4 pap1::ura4⁺*	52
TP113-6A	*h⁻ leu1 ura4 pap1::ura4⁺crm1-809*	Segregant between AT1 and TP108-3C
TP113-6B	*h⁺ leu1 ura4 crm1-809*	Segregant between AT1 and TP108-3C
TP113-6C	*h⁻ leu1 ura4 pap1::ura4⁺*	Segregant between AT1 and TP108-3C
TP113-6D	*h⁺ leu1 ura4*	Segregant between AT1 and TP108-3C
5A/1D	*h⁻/h⁺ leu1/leu1 ura4/ura4 his2/+ ade6-M210/ade6-M216*	37
AY1	*h⁻/h⁺ leu1/leu1 ura4/ura4 his2/+crm1::LEU2/+ade6-M210/ade6-M216*	This study
TP21	*h⁻/h⁺ leu1/leu1 ura4/ura4 his2/+ pap1::ura4⁺ ade6-M210/ade6-M216*	50
TP26	*h⁻/h⁺ leu1/leu1 ura4/ura4 his2/+ pap1::ura4⁺ crm1::LEU2/+ ade6-M210/ade6-M216*	This study
TP123-6C	*h⁻ leu1 ura4 ade6-M216p25::ura4⁺*	2
TP123-6CF	*h⁻ leu1 ura4 ade6-M216p25::ura4*	Ura⁻ mutant obtained from TP123-6C
TP160-1B	*h⁻ leu1 ura4 ade6-M216p25::ura4 crm1-809*	This study
OK372-10B	*MATa ade3 leu2 rad2*	Obtained from B. Ono
MY324	*MATa leu2 ura3 his4 rad2*	This study
HY34	*MATa leu2 ura3 his4 rad2 CRM1-URA3*	This study

three strains visually selected out of 900 cs mutants were found to carry mutations in the *crm1* locus. In addition, even at the permissive temperature, they show pleiotropic phenotypes, such as Ca^{2+} hypersensitivity, staurosporine resistance, and accumulation of a protein with a molecular mass of 25 kDa (designated p25).

In this report, we show that the p25 gene is a cellular target of the pap1 transcription factor and describe regulation of p25 gene expression through negative control of pap1-dependent transcription. Our results support current models of the functional importance of chromosome structure on the regulation of gene expression.

MATERIALS AND METHODS

Strains, media, and chemicals. *S. pombe* strains used in this study are listed in Table 1. Rich YPD (1% yeast extract, 2% polypeptone, 2% dextrose), minimal SD (0.67% yeast nitrogen base without amino acids, 1% dextrose), modified EMM2 (31), and SPA medium for sporulation (16) were used. Plates contained 1.6% agar. Staurosporine (provided by H. Nakano, Kyowa Hakko Co.) was used as described previously (52).

Genetic techniques and nomenclature. Standard procedures for *S. pombe* genetic manipulation as described by Gutz et al. (16) and Moreno et al. (31) were followed. The lithium method (22) was used for yeast transformation. Cold-sensitive mutations are referred to simply as "cs." Gene disruptions are indicated by lowercase letters representing the gene followed by two colons and the wild-type gene marker used for disruption. In the text, gene disruptions are abbreviated by the gene name followed by a superscript minus sign, e.g., *pap1⁻*.

Search for the AP-1 site in known fission yeast genes. A homology search of the EMBL and SWISS data bases was carried out to find the AP-1 site [T(T/G)AGTCA] in the 5′- or 3′-flanking regions of the fission yeast genes sequenced. Our personal sequence data base containing nucleotide sequences of unpublished fission yeast genes was also searched. The complete AP-1 site was found in 12 genes containing the p25 and *scy1⁺* genes. The *scy1⁺* gene was isolated as a multicopy plasmid that confers staurosporine resistance (46).

Nucleic acid preparation and manipulation. Standard molecular biology techniques were followed as described elsewhere (43). Enzymes were used as recommended by the suppliers (Takara Shuzo Co., TOYOBO Co., and New England Biolabs, Inc.). Total RNAs were prepared from fission yeast cells after disruption of cells with glass beads as described previously (31). RNAs were run in a 1.2% agarose gel containing formaldehyde (12).

Preparation of antisera and immunochemical assays. Preparation of rabbit anti-pap1 antisera and affinity purification of the antisera were carried out as described by Hirano et al. (21) by using *pap1⁺*-fusion proteins expressed in *Escherichia coli* (52). Anti-p25 antisera were prepared by using p25 proteins purified from *crm1-809* mutant whole cell extracts, followed by ammonium sulfate extraction, gel filtration chromatography, and electroelution on sodium dodecyl sulfate (SDS)-polyacrylamide gels (2). SDS-polyacrylamide gel electrophoresis (PAGE) was performed as described previously (26), and proteins were electrically transferred onto a nitrocellulose filter (54). Immunoblots were stained by using peroxidase-conjugated goat anti-rabbit immunoglobulin G (Bio-Rad Co.) and the ECL (enhanced chemiluminescence) nonradioactive detection kit (Amersham Co.). Immunoprecipitation was carried out as described previously (52).

Cloning of the p25 gene. Partial amino acid sequence was determined after digestion of purified p25 proteins with peptidase and fractionation of the resulting fragments by reverse-phase high-pressure liquid chromatography. Oligonucleotide p2520 [5′GT(T/C)TC(G/A/T/C)GG(G/A)AA(T/C)TG(G/A)TA(G/A/T)AT3′], which corresponds to the determined amino acid sequence (Thr-Glu-Pro-Phe-Gln-Tyr-Ile), was used to isolate a genomic fragment containing the p25 gene from a fission yeast library (21).

DNase I footprint analysis using bacterially expressed *pap1⁺* proteins. Construction and expression of the truncated *pap1⁺* protein (pap7K) will be described elsewhere (47). A 557-bp *ScaI-AccI* fragment containing the promoter region of the p25 gene was labeled by filling the 3′ end of the *AccI* site with Klenow fragment and [α-^{32}P]dCTP, and the labeled single-stranded fragment was gel purified. The labeled DNA was mixed with various amounts of the pap7K protein in 22 mM Tris-Cl (pH 7.5)–110 mM KCl–5.5 mM $MgCl_2$–0.6 mM dithiothreitol and incubated at room temperature for 20 min.

After partially digested with DNase I, mixtures were run on a 5% sequencing gel.

Overexpression of the *pap1*$^+$ gene in *S. pombe* cells. pST22 (containing the *pap1*$^+$ gene [52]) was used for overexpressing *pap1*$^+$.

Suppression test of the *crm1* mutation by the *pap1* gene disruption. Strains *crm1-809* (AT1; Table 1) and *pap1*$^-$ (TP108-3C) were mated, and tetrad analysis was carried out. Among 12 tetrads dissected, 2 showed a 2:2 segregation pattern for cs$^+$:cs$^-$, 7 showed a 3:1 pattern, and 3 showed 4:0 pattern. Backcrossing between one of *pap1*$^-$ (Ura$^+$) segregants (TP113-6A; Table 1) and a wild-type mater strain (TP108-3D) gave segregants showing a cs$^-$ Ura$^-$ staurosporine resistance phenotype, demonstrating that the original segregant, TP113-6A, contains double mutations of *crm1-809* and *pap1*::*ura4*$^+$. In contrast, backcrossing another *pap1*$^-$ segregant in the same tetrad (TP113-6C; Table 1) gave only cs$^+$ segregants, indicating that strain TP113-6C contains *crm1*$^+$.

For construction of a complete null allele of the *crm1*$^+$ gene, a 4.7-kb *Hin*dIII fragment of *crm1*::*LEU2* (3) was used for transformation (42) of wild-type diploids (5A/1D; Table 1) or *pap1*::*ura4*$^+$/+ heterozygous diploids (TP21). After selection of stable Leu$^+$ diploid transformants (designated AY1 and TP26, respectively), tetrads were dissected in each of heterozygous diploids. Among eight tetrads analyzed in either AY1 or TP26, two viable and two nonviable spores were obtained, and viable colonies were always Leu$^-$ (i.e., *crm1*$^+$). Uracil prototrophy (*pap1*$^-$) segregated randomly in two viable Leu$^-$ (*crm1*$^+$) cells, indicating that *crm1*$^+$ is an essential gene and that *pap1*$^-$ deletion could not rescue the lethality of the *crm1*$^-$ disruption. Random spore analysis of TP26 also did not produce any Ura$^+$ Leu$^+$ haploid segregants among approximately 10^4 viable spores. Microscopic observation of dissected spores after 6 days of incubation at 33°C showed that inviable cells were arrested in round ungerminated spores, demonstrating that the *crm1*$^+$ gene is required for spore germination.

Analysis of the upstream region of the p25 gene. Serial subclones containing different 5′ upstream regions of the p25 gene (see Fig. 6B) were constructed as follows. A 3.4-kb *Hin*dIII fragment of fission yeast centromeric repeat dg, derived from chromosome II, which has autonomously replicating sequence activity (34), was inserted in the *Kpn*I site of pYC6 in which the 1.8-kb *ura4*$^+$-containing fragment (15) was inserted into the *Nae*I site of Bluescript (Stratagene); the resulting construct was designated pYS107. From pYS107, the following subclones were made. pYS108 was made by inserting 4.3-kb *Hin*dIII-*Xba*I fragment containing 2.9 kb of the upstream region of the p25 gene. pYS109 was constructed by inserting a 1.9-kb *Cla*I-*Xba*I fragment containing 484 bp of upstream sequence from the initiator methionine of the p25 gene. pYS110 was constructed by inserting a 1.4-kb *Sau*3AI fragment containing 321 bp of upstream sequence. pYS112 was made by inserting a 3.6-kb *Avr*II-*Hin*dIII fragment containing 225 bp of upstream sequence. pYS114 was made by inserting a 1.3-kb *Sna*BI-*Xba*I fragment containing 202 bp of upstream sequence. pYS108, pYS109, and pYS110 contain the AP-1 site, whereas pYS112 and pYS114 do not. The following oligonucleotides were used for in vitro site-directed mutagenesis of the AP-1 site or the 14-bp palindrome (PLD) sequence: AP-mt1 (CCATTTC TGAGAATTCCTATT), AP-mt2 (CCATTTCTGACGGATC CTATT), PLD-mt1 (ACCAAGAATTCGTAAACTACC), and PLD-mt2 (ACCAGGATCCCGTAAACTACC). pYS117, which contains the 1.0-kb *Cla*I-*Eco*RI fragment in Bluescript (Stratagene), was used as a template for mutagenesis. Each *Cla*I-*Eco*RI mutated fragment was subcloned into pYS109. The chromosomal p25 gene was disrupted by inserting the *ura4*$^+$ marker, and then Ura$^-$ revertants were selected on 5-fluoro-orotic acid-containing plates (2, 15) (TP123-6CF; Table 1). TP123-6CF cells were transformed with multicopy plasmids containing either *pap1*$^+$ (pST22) or vector plasmid pDB248′ (5, 6). Then, Leu$^+$ transformants were retransformed with the various *ura4*$^+$-containing subclones described above, and Leu$^+$ Ura$^+$ transformants were selected. cs *crm1* cells (TP160-1B; Table 1) were also transformed with various subclones, and Ura$^+$ transformants were selected. Multiple transformants were picked from each transformation and analyzed by immunoblotting.

Cloning, nucleotide sequencing, mapping, and disruption of the budding yeast *CRM1* gene. A 2.6-kb *Hin*dIII fragment containing the fission yeast *crm1*$^+$ gene (3) was used as a probe to clone a budding yeast homolog. An *S. cerevisiae* genomic library constructed in pDB248′ (37) was screened under low-stringency conditions (50). Sixteen positive clones were obtained, and restriction enzyme mapping showed that they could be classified into three different but overlapped sets of clones. The 6.0-kb *Eco*RI fragment was found to complement the cold sensitivity of *crm1-809*. The 6.0-kb fragment was inserted into Bluescript (Stratagene), and the nucleotide sequence was determined by the dideoxy-chain termination method (44), using double-stranded plasmid DNAs as templates (19). A unidirectional stepwise deletion method (20) was used to prepare each template plasmid, and a Sequenase kit (U.S. Biochemical Corp.) was used for sequencing reactions. Nucleotide sequences of both strands in the coding region of the *CRM1* gene were determined.

The 2.0-kb *Cla*I fragment was inserted into pYC5, which had been made by inserting a *Not*I linker in the *Bam*HI site of YIp5 (8); the resulting construct was designated p*CRM1-URA3*. This plasmid was linearized with *Bgl*II and transformed into *ura3* strain MY324 (Table 1). One stable Ura$^+$ transformant, designated HY34, was selected. Integration of the fragment into the *CRM1* locus via homologous recombination was confirmed by Southern hybridization (data not shown). HY34 was used for genetic mapping of the *CRM1* locus and also for physical mapping by using pulsed- field gel electrophoresis and Southern hybridization.

To disrupt the *CRM1* gene, a 1.8-kb *Hin*dIII fragment that contains only the coding sequence of the *CRM1* gene was cloned in pYC5. The resulting construct, designated pRK51, was linearized by cutting with *Xba*I and transformed into diploids (SP1/DC124 [53]). Since the 1.8-kb *Hin*dIII fragment in pRK51 contains only the coding sequence of the *CRM1* gene, integration of the linearized plasmid into the *CRM1* locus was expected to disrupt the gene (36). Stable Ura$^+$ transformants were picked, and correct integration was confirmed by Southern hybridization (data not shown). Tetrad analysis of the diploids produced two viable and two inviable spores in 16 tetrads dissected, and viable colonies were always Ura$^-$, indicating that the *CRM1* gene is essential. Microscopic observation of inviable cells indicated that they were arrested in ungerminated spores.

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 D13038 (*S. pombe* p25 gene) and D13039 (*S. cerevisiae CRM1* gene).

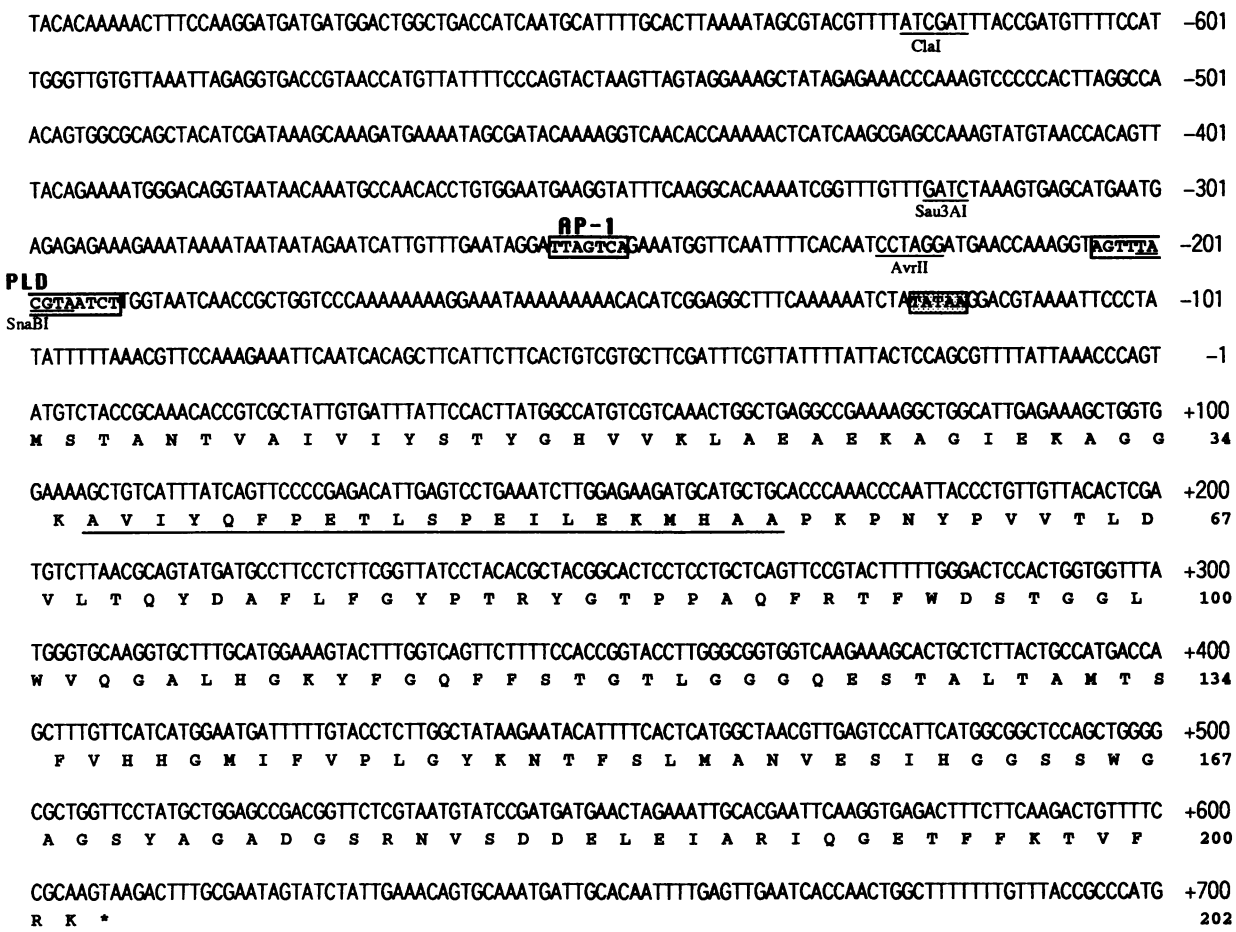

FIG. 1. Nucleotide sequence of the p25 gene. The AP-1 consensus sequence (TTAGTCA) and the PLD sequence (AGTTTACGTAATCT) are boxed. The putative TATA box (TATAA) is indicated by a hatched box. The last three bases are the putative initiator methionine (ATG). Restriction sites used for subcloning are also shown. The sequenced peptide of the purified protein is underlined.

RESULTS

Identification of a putative *S. pombe* AP-1 site. We attempted to identify a gene(s) under the control of *pap1*$^+$ by searching a data base of *S. pombe* nucleotide sequences (Materials and Methods) for AP-1 recognition sequences [T(T/G)AGTCA], which we found in the 5'-flanking regions of several genes. We then examined whether the transcript levels of those genes were elevated upon the introduction of multicopy plasmids carrying the *pap1*$^+$ gene into *S. pombe* cells. We found that only one gene among those tested, the p25 gene (3) of unknown function, showed an increased level of transcript upon overexpression of *pap1*$^+$. p25 is present in large quantity when the essential gene *crm1*$^+$ is defective. The cs *crm1* mutations cause, in addition to increased levels of p25 at the permissive temperature, aberrations in higher-order chromosome structure at the restrictive temperature. Immunofluorescence microscopy indicated that the crm1 protein resides in the entire nucleus but may be enriched in the nuclear periphery. The molecular function of the crm1 protein is unknown except for the finding that it is essential for maintaining proper chromosome structure and for cell viability. The gene for p25 was isolated by hybridization using oligonucleotide probes based on the amino acid sequence of purified p25, and its nucleotide sequence was determined (Fig. 1). The predicted p25 protein consists of 202 amino acids with no significant homology to any known protein.

High dosage of the *pap1*$^+$ gene causes overproduction of p25. As shown in Fig. 1, the p25 gene contains an AP-1 recognition consensus sequence (TTAGTCA) located 251 bp upstream from the putative initiation codon. We addressed the question of whether p25 transcription is regulated by pap1. The level of p25 mRNA was estimated under different *pap1*$^+$ gene dosage conditions in Northern (RNA) blots probed with the p25 gene. As shown in Fig. 2, we found that expression of the p25 gene is under the control of *pap1*$^+$. Total RNA was prepared from wild-type cells (lane 1), a *pap1* deletion mutant (lane 2), cs *crm1-809* mutant cells (lane 3), wild-type cells carrying the multicopy vector (lane 4), and wild-type cells carrying a plasmid with the *pap1*$^+$ gene (lane 5). The levels of p25 and *pap1*$^+$ transcripts are shown in Fig. 2A and B, respectively. The amount of p25 mRNA is negligible in the *pap1* deletion mutant and greatly elevated with a high dosage of *pap1*$^+$. This result is consistent with the hypothesis that p25 transcription is positively regulated by *pap1*$^+$. The level of p25 mRNA is also increased in cs *crm1* cells (Fig. 2A, lane 3), without increasing the amount of *pap1*$^+$ mRNA (Fig. 2B, lane 3). In comparison, the result of Northern hybridization probed with another gene (*scy1*$^+$ [46]) which contains an AP-1 consensus sequence (TTAG

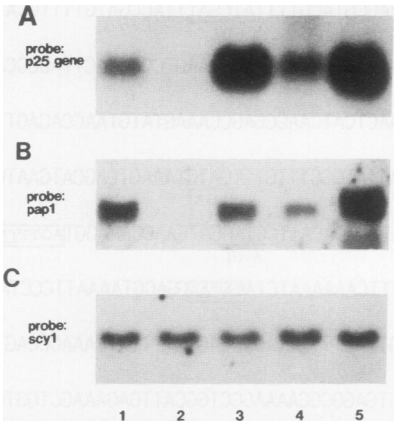

FIG. 2. Regulation of the level of the p25 transcript by *pap1*$^+$ and *crm1*$^+$. Total RNA (10 μg per lane) was run on a formaldehyde-containing 1.2% agarose gel, transferred onto a membrane filter, and hybridized with ^{32}P-labeled DNA containing either the p25 gene (A), *pap1*$^+$ (B), or *scy1*$^+$ (C). Sources of total RNA were as follows: lane 1, wild-type (HM123) cells; lane 2, *pap1*$^-$-deleted (TP108-3C) cells; lane 3, cs *crm1* (AC1) cells; lane 4, wild-type cells carrying a vector plasmid; and lane 5, wild-type cells carrying a *pap1*$^+$ plasmid. A 2.5-kb *Eco*RI fragment (the p25 gene) (A), a 2.0-kb *Hin*dIII fragment (the *pap1*$^+$ gene [52]) (B), and a 3.0-kb *Bgl*II fragment (the *scy1*$^+$ gene [46]) (C) were used as the probes. Approximate sizes of the transcripts are 1.0 kb for the p25 gene, 2.7 kb for *pap1*$^+$, and 3.1 kb for *scy1*$^+$.

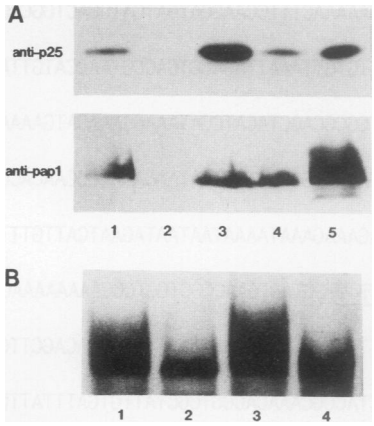

FIG. 3. Immunoblotting of various mutant cells with anti-p25 antibodies. (A) Cell extracts were prepared from wild-type (lane 1), *pap1* disruptant (lane 2), and cs *crm1* (lane 3) cells and from wild-type cells carrying a vector plasmid (lane 4) or a *pap1*$^+$ multicopy plasmid (lane 5). The blotted filter was incubated with 1/200-diluted anti-p25 antisera or affinity-purified anti-pap1 antibodies, as indicated, and stained with peroxidase-conjugated secondary antibody, using the ECL detection kit (Amersham). The lowermost band of pap1 corresponds to 75 kDa. (B) The pap1 protein was immunoprecipitated with protein A-Sepharose-conjugated anti-pap1 antibody. Extracts were prepared from wild-type cells carrying a vector plasmid (lanes 1 and 2) or a *pap1*$^+$ plasmid (lanes 3 and 4) as described previously (52). Then, immunoprecipitates were incubated with (lanes 2 and 4) or without (lanes 1 and 3) 0.1 U of potato acid phosphatase (Boehringer Mannheim) for 60 min at 37°C. Reaction mixtures were electrophoresed on an SDS–7.5% polyacrylamide gel, and the pap1 proteins were detected by a horseradish peroxidase kit (Wako Co.).

TCA) in its 5′ upstream sequences (283 bp from ATG) is shown in Fig. 2C. The transcript levels for *scy1*$^+$ are approximately the same with different levels of the p25 transcript, suggesting that the AP-1 consensus sequence itself is not sufficient for the regulation.

We raised antisera against p25 and pap1 proteins by using bacterially made proteins as antigens (Materials and Methods) and carried out immunoblotting in order to estimate the corresponding amounts of p25 and pap1 proteins. As shown in Fig. 3A, the levels of p25 and pap1 (around 75-kDa) proteins are similar to the levels of p25 and *pap1*$^+$ mRNAs shown in the Fig. 2A and B. The pap1 and p25 proteins are missing in *pap1* deletion mutant cells (Fig. 3A, lane 2). The amount of pap1 protein is not increased in cs *crm1* cells, but that of p25 is highly enhanced (lane 3). Both pap1 and p25 proteins are overproduced in wild-type cells carrying a multicopy *pap1*$^+$ plasmid (lane 5). From these results, we conclude that the *pap1*$^+$ gene is required for the production of p25 and that the amount of p25 is increased either by overexpression of *pap1*$^+$ or by mutation of the *crm1*$^+$ gene.

The pap1 protein band is broad (Fig. 3B); the pap1 protein immunoprecipitated by anti-pap1 antibodies showed a dispersed thick band when wild-type cells carry a vector (lane 1) or a plasmid with the *pap1*$^+$ gene (lane 3). This result may be due to posttranslational modification, possibly by phosphorylation (39). Consistently, when the immunoprecipitates were treated with potato acid phosphatase (lanes 2 and 4), the pap1 protein band became much narrower.

Footprinting of the AP-1 site in the p25 gene by bacterially made pap1 protein. To determine whether binding takes place between the pap1 protein and TTAGTCA sequence found in the 5′-flanking region of the p25 gene, we performed DNase I footprinting. To this end, a 7-kDa truncated pap1 polypeptide (designated pap7K) containing only the basic region and adjacent leucine repeats was expressed in *E. coli* and then purified (47). As shown in Fig. 4, an increasing amount of the pap7K protein protected a region of sequence that exactly matched the TTAGTCA sequence in the 5′ upstream region of the p25 gene (lanes 3 to 10; indicated as AP-1 in Fig. 4).

The pap7K protein protects not only the AP-1 site but also another region, marked as PLD in Fig. 4. This region corresponds to a 14-bp palindrome sequence (Fig. 1) located 52 bp downstream from the AP-1 sequence. The degrees of protection by pap7K are similar for AP-1 and PLD. These results suggest that the pap1 protein directly activates transcription of the p25 gene and that the activation may require not only the AP-1 but also the PLD sequence.

Deletion of the *pap1*$^+$ gene rescues the cs *crm1* mutant. We addressed the question of how the overexpression of p25 in cs *crm1* is related to *pap1*$^+$ gene function. To investigate a possible genetic interaction between *pap1*$^+$ and *crm1*$^+$, a cross was made between the *pap1* gene disruptant and the cs *crm1* mutant. A prediction was that the cs phenotype of *crm1-809* might be due to the deregulated activation of the pap1 transcription factor. If this were the case, the *pap1* deletion mutant might rescue cs *crm1-809*. Indeed, we found cs$^+$ segregants of the double mutant *pap1*::*ura4*$^+$ *crm1-809* by tetrad analysis (Table 2). Genotypic and phenotypic analyses showed that segregants C in tetrad 1 and segregants C and D in tetrad 2 were double mutants. However, they could form colonies at 22°C, although their colony sizes were smaller than those of a single *pap1* deletion mutant (Fig. 5A, plate c). Fluorescence microscopy of the double-mutant cells incubated at 20°C showed a normal-looking nuclear chromatin structure by DAPI staining (Fig. 5B, plate c),

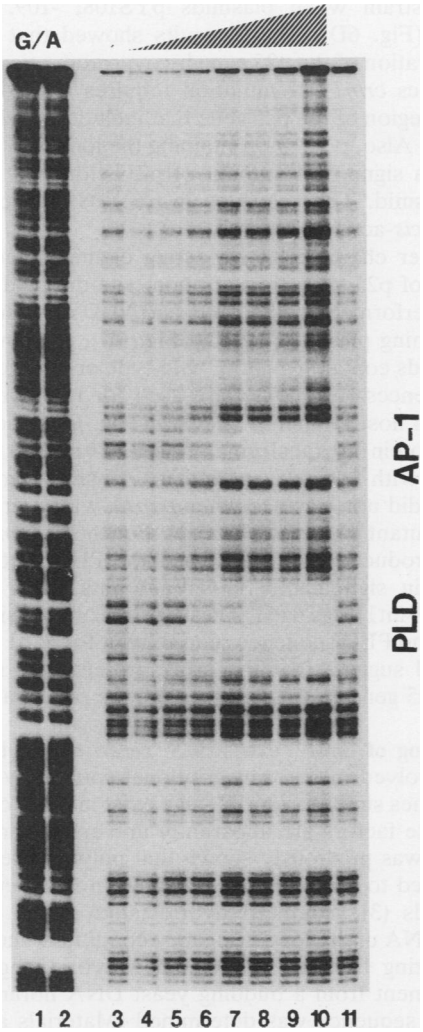

FIG. 4. DNase I protection assay of the promoter region of the p25 gene. A 557-bp *Sca*I-*Acc*I fragment containing the promoter region of the p25 gene was end labeled with Klenow fragment. The single-stranded fragment was gel purified and incubated with various amounts of truncated pap1 protein (pap7K) purified from *E. coli* (47). The amounts of the pap7K protein added were 0 (lanes 3 and 11), 0.24 (lane 4), 0.475 (lane 5), 0.95 (lane 6), 1.9 (lane 7), 3.8 (lane 8), 7.6 (lane 9), and 15.2 (lane 10) μg. A G/A ladder is shown in lanes 1 and 2.

similar to that of the wild-type cells and *pap1* deletion mutant (plate a). In single *crm1-809* mutant cells at 20°C, the nuclear chromatin region was much deformed, revealing filamentous structures (plate b). Figure 5C shows results of immunoblotting with anti-p25 antibodies for three sets of segregants derived from different tetrads. It is clear that the overexpression of p25 was found only in cs *crm1* and that the double mutant *pap1*$^-$ *crm1* had no p25.

It was previously shown (3) that the *crm1* mutation causes pleiotropic phenotypes. We examined whether the *pap1* deletion suppressed other phenotypes of *crm1-809* such as staurosporine resistance. As shown in Table 2 and Fig. 5A, plate d, this staurosporine resistance of *crm1-809* is lost when the *pap1*$^+$ gene is deleted. Thus, four characteristic features of the cs *crm1* mutants, namely, the accumulation of p25, staurosporine resistance at the permissive temperature,

TABLE 2. Tetrad analysis of a cs *crm1* and *pap1*-disruptant genetic cross[a]

Strain or segregants	Phenotype			Genotype	
	cs	STS	p25	*crm1*	*pap1*
Parents					
crm1-809	cs	r	op	*809*	+
pap1$^-$	+	s	−	+	−
Tetrad 1					
A	+	s	−	+	−
B	cs	r	op	*809*	+
C	+	s	−	*809*	−
D	+	s	+	+	+
Tetrad 2					
A	+	s	+	+	+
B	+	s	+	+	+
C	+	s	−	*809*	−
D	+	s	−	*809*	−

[a] Two strains, *crm1-809* (AT1; Table 1) and *pap1*$^-$ (TP108-3C), were mated, and tetrad analysis was performed. Two types of tetrad segregants obtained are shown. In tetrad 1, the phenotype of cold sensitivity (cs), staurosporine (STS) resistance, and accumulation of p25 segregated 3:1. In tetrad 2, the phenotypes segregated 4:0. The cs phenotype was examined by streaking each strain on rich YPD plates and incubating the plates for 6 days at 22°C. Drug resistance (r) or sensitivity (s) was determined by streaking each segregant on YPD plates containing 1.0 μg of staurosporine per ml and incubating the plates for 4 days at 33°C. The amount of p25 protein was examined by immunoblotting with anti-p25 antibodies. op, overproduction of p25; + and −, normal and undetectable amounts, respectively, of p25.

cold-sensitive growth and disorganization of chromosome structures at the restrictive temperature, were lost in the *pap1*$^-$ *crm1* double mutant. Most phenotypic defects of *crm1* mutants seem to be suppressed by the *pap1* deletion. We found that the *pap1* deletion also suppressed phenotypes of p25 accumulation and staurosporine resistance of another *crm1* mutant allele (46), *crm1-119* (3), although in this case cold sensitivity of *crm1-119* is not suppressed. Failure of the cs suppression by the *pap1* deletion might be due to the severity of the effect of this allele, since *crm1-119* cells grow much more slowly even at the permissive temperature (46). Indeed, the *pap1* deletion cannot rescue a complete disruption of the *crm1*$^+$ gene, as shown by tetrad analysis of a cross between *pap1* deletion and *crm1* deletion mutants. We could not obtain any viable doubly disrupted haploid segregants (Materials and Methods).

***cis*-acting elements required for activation of p25 transcription by *pap1*$^+$ and cs *crm1*.** The results described above showed that cs *crm1* causes overproduction of p25 which appears to be mediated by the pap1 protein. To more precisely examine the relationship between pap1 and crm1 with respect to the regulation of p25 transcription, we dissected the *cis*-acting element of the p25 upstream region (Fig. 6A and B).

We constructed a series of plasmids carrying the truncated 5′ upstream and coding regions of the p25 gene and the *S. pombe* marker *ura4*$^+$ (Fig. 6B). The insert in pYS110 contains the AP-1 site, whereas the 100-bp-shorter insert in pYS112 does not. We then introduced these plasmids into a host strain whose p25 gene had been deleted (Δ*p25 leu1 ura4*) or that contained the cs *crm1* mutation in addition to the p25 gene deletion (Δ*p25 leu1 ura4 crm1-809*; Fig. 6A). The p25 gene is nonessential for viability, and the haploid null strain produced normal colonies. Therefore, the amount of p25 detected should be solely from the p25 gene on the plasmid. To examine the effect of the high-dosage *pap1*$^+$ genes on the level of p25, we further introduced a multicopy

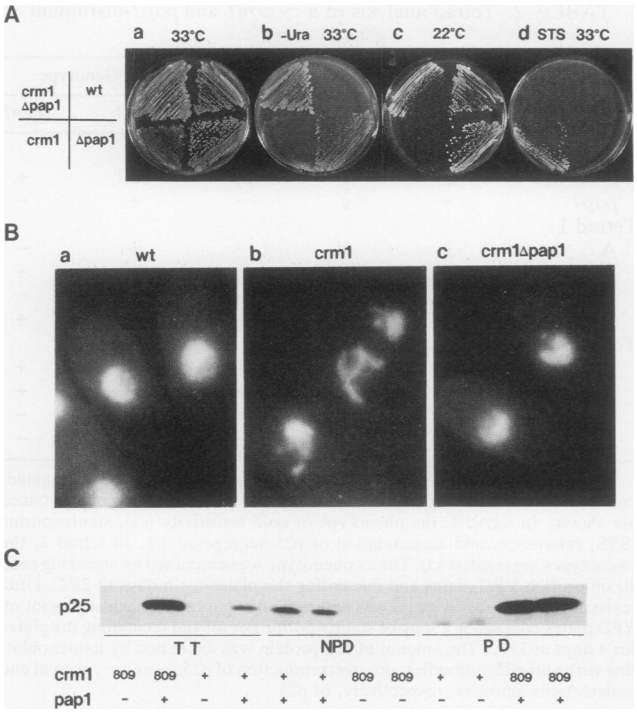

FIG. 5. Rescue of the *crm1* mutation by deletion of the *pap1*$^+$ gene. (A) A set of tetrad segregants derived from the cross between cs *crm1* and *pap1*$^-$ (AT1 and TP108-3C, respectively; Table 1) was streaked on rich YPD plates (plates a and c), a synthetic EMM2 plate lacking uracil to examine the *pap1* mutant which is disrupted with the *ura4*$^+$ gene (plate b), or a YPD plate containing 1.0 μg of staurosporine (STS) per ml (plate d). Plates were incubated at 33°C for 4 days (plates a, b, and d) or at 22°C for 6 days (plate c). Cold sensitivity segregated 3:1 (plate c), and staurosporine resistance segregated 1:3 (plate d). (B) Three types of strains (wild type [HM123; a], cs *crm1-809* [AC1; b], and *pap1*$^-$ *crm1-809* [TP113-6A; c] [see Table 1] were grown exponentially at 33°C, transferred to 20°C, and then incubated for 12 h. Cells were fixed by glutaraldehyde and stained with DAPI. The bar represents 10 μm. (C) Cell extracts were prepared from three sets of the tetrad segregants, which represent tetratype (TT), nonparental ditype (NPD), and parental ditype (PD), respectively. Total proteins were electrophoresed in an SDS–15% polyacrylamide gel and transferred onto a nitrocellulose filter. p25 protein was identified by immunoblotting with anti-p25 antibodies. Note that the p25 protein was overproduced only in the cs *crm1-809* segregants and was absent in *pap1*$^-$ *crm1* double mutants. Faint bands, smaller than p25, seen in *pap*$^-$-deleted segregants of the parental ditype are due to nonspecific binding of the serum; in a longer exposure, the same band appeared in all lanes.

plasmid carrying the *pap1*$^+$ gene and the *S. cerevisiae LEU2* marker into the p25-disrupted *leu1 ura4* strain. Leu$^+$ Ura$^+$ transformants obtained should carry these two plasmids. The amount of p25 in different transformants was estimated by immunoblotting (Fig. 6C and D).

Transcriptional activation by the multicopy *pap1*$^+$ plasmid occurred only when the p25 plasmid (pYS108, -109, or -110) contains the AP-1 sequence of the p25 gene (Fig. 6C; the plus and the minus signs indicate the presence and absence, respectively, of the *pap1*$^+$-carrying plasmid in transformants). In contrast, production of the p25 protein is barely detectable in pYS112 and pYS114, which lack the AP-1 sequence. Similarly, p25 was overproduced in the cs *crm1-809* strain when plasmids pYS108, -109, and -110 coexisted (Fig. 6D). These results showed that transcriptional activation of the p25 gene by overproduction of *pap1*$^+$ or by the cs *crm1-809* mutation requires a 100-bp-long 5′ upstream region of the p25 gene that includes AP-1 and PLD sequences. Also, pYS108 containing the longest 5′ sequence produced a significant amount of p25 without a multicopy *pap1*$^+$ plasmid. The sequence in pYS108 may contain an additional *cis*-acting element.

To further characterize *cis*-acting elements required for activation of p25 gene transcription, site-directed mutagenesis was performed on the AP-1 and PLD sequences in the p25-containing plasmid pYS109 (Fig. 6B), as shown in Fig. 7A. Plasmids containing either wild-type or mutated AP-1 or PLD sequences (Fig. 7A) were used for transformation of p25-deleted host strains as shown in Fig. 6A. The levels of the p25 protein in transformants were examined by immunoblotting with anti-p25 antibodies. As shown in Fig. 7B, p25 levels did not increase when *pap1*$^+$ was overexpressed with the mutant p25 gene plasmids. For the cs *crm1* mutant, p25 was produced not at all when the PLD mutants were used and in significantly lower amounts when the AP-1 mutant (AP-mt1) was used. These results are consistent with the AP-1 and PLD sequence requirement for pap1 activation of p25 and suggest that the crm1 protein may negatively regulate p25 gene expression through the pap1 transcription factor.

A homolog of *crm1*$^+$ exists in *S. cerevisiae*. If the *crm1*$^+$ gene is involved in the transcriptional control by AP-1-like factors, genes similar to *crm1*$^+$ may exist in other organisms, as AP-1-like factors are apparently universally present (28, 32, 52). It was previously shown that polypeptides antigenically related to the crm1 protein exist in *S. cerevisiae* and human cells (3). Southern hybridization of *S. cerevisiae* genomic DNA under low-stringency conditions showed a set of hybridizing bands. We isolated a hybridizing genomic DNA fragment from a budding yeast DNA library, and its nucleotide sequence was determined (Materials and Methods; the entire nucleotide sequence has been submitted to EMBL/GenBank). One open reading frame encoding 1,084 amino acid residues, which was highly similar to that of the fission yeast *crm1*$^+$ (55% identity; Fig. 8), was found and designated *CRM1*. We were informed by T. Beppu and his associates that our previous nucleotide sequence data for the fission yeast *crm1*$^+$ contained sequencing errors in seven positions; consequently, the corrected sequence is compared here with the budding yeast *CRM1* sequence.

The budding yeast *CRM1* gene is located on chromosome VII, judging from the pulsed-field gel electrophoretic pattern after probing with the *CRM1* gene (Materials and Methods). Further analysis, including examination of the integration of the *CRM1* gene with a newly engineered *Not*I site, showed that *CRM1* is located 150 kb distal from the right end of chromosome VII. This finding was confirmed by tetrad dissection of the integrant with the nearby markers; the genetic distance was 5.7 centimorgans from *ADE3* and 26 centimorgans from *RAD2* (Materials and Methods). No mutants have been mapped in this region; thus, *CRM1* is a novel gene in *S. cerevisiae*. *CRM1* was disrupted by homologous recombination and integration and shown to be an essential gene like the fission yeast *crm1*$^+$ gene. *CRM1* is perhaps a homolog of the fission yeast *crm1*$^+$ gene, as the cs phenotype of *crm1-809* was rescued by introduction of *CRM1* into the cs mutant.

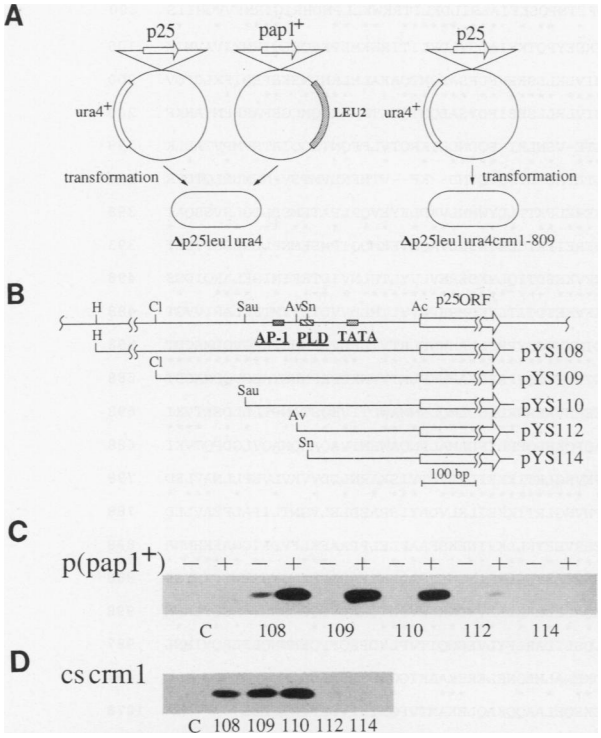

FIG. 6. Dissection of the upstream region of the p25 gene. (A) Method used to identify the DNA sequences required for activation of p25 gene expression. Two kinds of host cells (TP123-6CF [left] or cs *crm1* mutant [TP160-1B; right]) whose chromosomal p25 gene was deleted were transformed with a series of truncated subclones (carrying *ura4*$^+$ as the selectable marker) (B). The Ura$^+$ *crm1*$^+$ cells (left) were also transformed with the *pap1*$^+$ multicopy plasmid (carrying the budding yeast *LEU2* gene as the selectable marker for the fission yeast *leu1* mutation). (B) The 5′-flanking region of the p25 gene and a series of truncated subclones that contain various 5′ upstream regions of the p25 gene. Shown are the AP-1 site, the PLD sequence, and a putative TATA box. ORF, open reading frame; Ac, *Acc*I; Av, *Avr*II; Cl, *Cla*I; H, *Hin*dIII; Sn, *Sna*BI; Sau, *Sau*3AI. (C and D) Immunoblots. Leu$^+$ Ura$^+$ transformants (for *pap1*$^+$ overproducers, − indicates transformants with a vector plasmid and + indicates those with a *pap1*$^+$ multicopy plasmid; see the left side of panel A) (C) or Ura$^+$ cs *crm1* transformants (right side of panel A) (D) were grown, and cell extracts were prepared. After SDS-PAGE (26), total proteins were transferred onto nitrocellulose filters (54). The filters were immunoblotted with the anti-p25 antibodies and stained by peroxidase-conjugated secondary antibodies (Amersham). Lanes C represent transformants of a *ura4*$^+$-containing vector plasmid without the p25 gene (no endogenous p25 was observed).

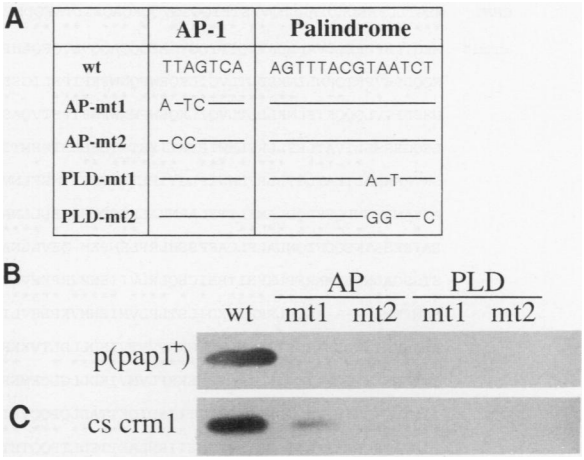

FIG. 7. Essential *cis*-acting elements for p25 gene expression revealed by site-directed mutagenesis. (A) Site-directed mutations in both AP-1 and palindrome sequences are shown together with the wild-type sequences. Only mutated nucleotides are indicated. All of the mutants were introduced into plasmid pYS109 (see Fig. 6B). (B and C) Each mutated plasmid was used for transformation of wild-type cells carrying a multicopy *pap1*$^+$ plasmid [p(pap1$^+$; or B] or cs *crm1* mutant cells (C), and then the level of p25 was examined by immunoblotting with anti-p25 antibodies.

DISCUSSION

p25 is a target for *pap1*$^+$. In this study, we showed that two gene products, the transcription factor pap1 and the essential nuclear protein crm1, are closely involved in activation and repression of the transcription of the p25 gene. We demonstrated that the p25 gene, whose product was previously shown to be overproduced in cs *crm1* mutants, was a cellular target for *pap1*$^+$.

The following results from this study support the notion that the p25 gene is a target of the AP-1-like protein pap1. First, the 5′ upstream region of the p25 gene contains an AP-1 consensus, TTAGTCA. Second, bacterially made pap1 protein binds to this AP-1 sequence in addition to a nearby palindrome, PLD. Third, overproduction of the pap1 protein causes a large increase of p25 mRNA and protein. Fourth, deletion of the *pap1*$^+$ gene abolishes expression of the p25 gene. Experiments using a bacterially made pap1 protein indicated that the protein directly binds not only to the AP-1 sequence but also to the PLD sequence (AGTTTACG TAATCT). Although the PLD sequence shows little sequence similarity to the AP-1 sequence, it may be more related to a recognition sequence of ATF/CREB (TGACGT CA [18]); the core of PLD (TTACGTAA) differs at only two positions from the ATF/CREB site (underlined). It is known that some AP-1 proteins can also efficiently binds to the ATF/CREB site (17, 18, 45). The pap1 protein may have recognition properties similar to those of AP-1 proteins. However, it should be noted that both AP-1 and PLD are essential for the overexpressed pap1 protein to activate transcription of the p25 gene (Fig. 7). Thus, the two sites might somehow synergistically interact for transcriptional activation by pap1.

The cellular function of p25 remains to be determined; disruption of the p25 gene failed to show any phenotype, including cold or temperature sensitivity, staurosporine resistance, and suppression of the *crm1* mutation. A multicopy p25 plasmid introduced into fission yeast cells did not confer staurosporine resistance. The amount of overexpressed p25 was very high, recognizable as an intense band by Coomassie brilliant blue staining of whole cell extracts subjected to SDS-PAGE (3). It exists as a dimer (2). It should be noted that the p25 gene is unlikely to be the sole target of pap1; Coomassie brilliant blue staining of cell extracts from wild-type and *crm1* mutants has shown, in addition to the p25 protein, several other proteins that were overproduced in *crm1* mutant cells (3). Like the p25 gene, genes encoding these proteins might also be targets for pap1. It seems likely that the crm1 protein negatively regulates the transcription of multiple genes.

Transcriptional control and higher-order chromosome structure. A surprising result of this study was that the

FIG. 8. Amino acid sequence comparison between fission yeast *crm1*$^+$ and budding yeast *CRM1* proteins. Identical amino acid residues between fission yeast crm1 and budding yeast CRM1 are marked by asterisks. Amino acid sequences between residues 20 and 31 of crm1 are corrected from our previous report (3) according to information from T. Beppu, M. Yoshida, and K. Nishi (University of Tokyo). Positions 119 and 484 are also corrected from proline to leucine and from valine to isoleucine, respectively.

phenotype of the cs *crm1* mutant was suppressed by deletion of the *pap1*$^+$ gene. We have shown further evidence that the *pap1*$^+$ and *crm1*$^+$ gene functions are closely interrelated. The p25 transcript was greatly increased in the cs *crm1* mutant only when the *pap1*$^+$ gene was present. The *cis*-acting sequences required for transcriptional activation of the p25 gene were basically identical for the *pap1*$^+$ overproducer and the cs *crm1* mutant, defined by AP-1 and PLD sequences. Since the level of pap1 protein was unchanged in cs *crm1* mutant cells, the crm1 protein function is unlikely to regulate the amount of pap1 protein through transcription or protein stability. The crm1 protein appears to negatively regulate the pap1 protein by direct or indirect modification or a configurational change, although we have not found a quantitative difference in phosphorylation of the pap1 protein between wild-type and cs *crm1* cells. The loss of *crm1*$^+$ appears to cause the deregulated activation of pap1-dependent transcription. The mechanism by which staurosporine resistance is conferred by a high-dosage *pap1*$^+$ gene or cs *crm1* mutation is not understood. Preliminary results, however, have shown that the resistance may not be specific to staurosporine because both products seem to be involved in multidrug resistance phenomena (46).

The molecular function of the crm1 protein (115 kDa) is unknown. It is essential for viability, highly conserved, and localized within the nucleus and at its periphery (3). It may be an enzyme that directly modifies the pap1 protein or indirectly down regulates pap1. Alternatively, it could be a chromatin protein controlling transcription in general; pap1 could be one of the transcription factors that interacts with crm1. Cells overproducing pap1 do not show the phenotypes exhibited by cs *crm1*, indicating that high pap1 activity may not be sufficient for the phenotypic consequences of cs *crm1*. The essential function of the crm1 protein cannot be substituted by *pap1* deletion; *pap1* disruption failed to rescue the complete deletion of the *crm1*$^+$ gene. A gross alteration in chromosomal organization was observed in cs *crm1* cells at the restrictive temperature, suggesting that the crm1 protein may play an important role in the maintenance of higher-order chromatin structure. Disruption of either fission yeast *crm1*$^+$ or budding yeast *CRM1* resulted in inviable spores which could not germinate. Budding yeast cells carrying a *CRM1* multicopy plasmid did not sporulate, suggesting that overexpressed *CRM1* apparently inhibited sporulation. Hence, the crm1 protein seems to be involved in diverse aspects of cellular growth, such as maintenance of chromosome organization, transcriptional control through an AP-1-like factor(s), sporulation, and germination. The gross alteration of chromatin structure and the chromosomal disorganization in cs *crm1* mutants may be the cause or possibly the result of an uncoordinated activation of transcription factors such as pap1.

crm1 is conserved throughout evolution. We cloned a budding yeast homolog, *CRM1*, and showed that it can be substituted for the fission yeast *crm1*$^+$ gene. Immunoblotting with affinity-purified anti-crm1 antibodies clearly showed the existence of antigenically related peptides of similar size (around 110 kDa) in human cells (3). An intriguing possibility is that the crm1 protein function as a negative regulator of an AP-1 factor is also conserved in mammals. A variety of extracellular stimuli and gene products are known to activate AP-1 factors; these include mitogens, the tumor promoter tetradecanoyl phorbol acetate, reducing agents, oncogenic ras protein, and MAP/ERK kinases (1, 9, 40, 48). The human

crm1 homolog might be involved in regulation of an AP-1 factor by negatively interacting with such activators. An understanding of crm1 function at the molecular level will be a central aim of future studies.

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