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Yap, a Novel Family of Eight bZIP Proteins in *Saccharomyces cerevisiae* with Distinct Biological Functions

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Saccharomyces cerevisiae **contains eight members of a novel and fungus-specific family of bZIP proteins that is defined by four atypical residues on the DNA-binding surface. Two of these proteins, Yap1 and Yap2, are transcriptional activators involved in pleiotropic drug resistance. Although initially described as AP-1 factors, at least four Yap proteins bind most efficiently to TTACTAA, a sequence that differs at position ±2 from the optimal AP-1 site (TGACTCA); further, a Yap-like derivative of the AP-1 factor Gcn4 (A239Q S242F) binds efficiently to the Yap recognition sequence. Molecular modeling suggests that the Yap-specific residues make novel contacts and cause physical constraints at the ±2 position that may account for the distinct DNA-binding specificities of Yap and AP-1 proteins. To various extents, Yap1, Yap2, Yap3, and Yap5 activate transcription from a promoter containing a Yap recognition site. Yap-dependent transcription is abolished in strains containing high levels of protein kinase A; in contrast, Gcn4 transcriptional activity is stimulated by protein kinase A. Interestingly, Yap1 transcriptional activity is stimulated by hydrogen peroxide, whereas Yap2 activity is stimulated by aminotriazole and cadmium. In addition, unlike other *yap* mutations tested, *yap4* (*cin5*) mutations affect chromosome stability, and they suppress the cold-sensitive phenotype of *yap1* mutant strains. Thus, members of the Yap family carry out overlapping but distinct biological functions.**

Eukaryotic organisms from yeast to humans contain AP-1 transcription factors that stimulate the expression of specific classes of genes in response to a wide variety of extracellular stimuli (2, 27, 50). By definition, AP-1 proteins contain a conserved bZIP DNA-binding domain (36) consisting of a leucine zipper that mediates dimerization (45) and an adjacent basic region that specifically interacts with DNA sequences termed AP-1 sites (10). Mammalian cells contain a large number of AP-1 proteins, and distinct family members can often interact to form heterodimeric complexes with AP-1 sites. However, analysis of transcriptional activation, oncogenic potential, and loss-of-function phenotypes in mice clearly indicates that individual family members play different biological roles.

In the yeast *Saccharomyces cerevisiae*, the best-characterized AP-1 factor is Gcn4, which coordinately activates the transcription of at least 40 genes in response to amino acid starvation and other environmental circumstances (11, 20, 22). Biochemical and crystallographic analysis has defined the optimal AP-1 site (TGACTCA), the structure of the Gcn4-DNA complex at atomic resolution, and many of the protein-DNA contacts that mediate specific binding to AP-1 sites (10, 19, 29, 31, 42). In functional terms, Gcn4 is remarkably similar to the Jun and Fos oncoproteins. Gcn4, Jun, and Fos have the same DNA-binding specificity (3, 8, 54), and they are functionally interchangeable for transcriptional activation from AP-1 sites in yeast and mammalian cells (43, 44, 55). Moreover, Gcn4 resembles mammalian AP-1 factors in that it mediates a protective response against UV involving the Ras signal transduction pathway and a translational control mechanism that leads to increased AP-1 transcriptional activity (11).

Two other *S. cerevisiae* bZIP proteins, termed Yap1 and Yap2, have been described as AP-1 factors (5, 17, 39). However, in comparison to conventional AP-1 factors, we have noticed that Yap1 and Yap2 differ at two of the five highly conserved residues that directly contact bases in the AP-1 site (see Results). Overexpression of Yap1 or Yap2 leads to increased resistance to a variety of drugs and metals (5, 16, 18, 21, 51, 65), with Yap1 typically having stronger effects. Both Yap1 and Yap2 can stimulate transcription from an artificial promoter containing a simian virus 40 (SV40) sequence (TG ACTAA) that differs from the optimal AP-1 site by a single base pair.

Yap1 has also been described as being involved in the oxidative stress response (32, 52). Loss of Yap1 function results in decreased resistance to hydrogen peroxide. Yap1 directly affects the transcription of at least four genes involved in the oxidation status of the cell: *GSH1*, which encodes γ-glutamylcysteine synthetase (66); *TRX2*, which encodes thioredoxin (32); *YCF1*, which encodes an ATP-binding transporter (64); and *GLR1*, which encodes glutathione reductase (15). The Yap1 recognition sites in the *GSH1* promoter resemble the SV40 site (TGACTAA), whereas those in the *TRX2*, *YCF1*, and *GLR1* promoters differ at two positions from the conventional AP-1 site (TTACTAA). In addition, Yap1 is important for stress-induced and protein kinase A (PKA)-inhibited transcription of *TPS2*, which encodes trehalose phosphate phosphatase, but these effects do not appear to involve direct Yap1 binding to the *TPS2* promoter (14).

The functional relationship between Yap1 and Yap2 is poorly understood. One possibility is that these proteins are functionally equivalent except that Yap1 is a stronger activator. Alternatively, as is the case for mammalian AP-1 factors, Yap1 and Yap2 may have overlapping, but distinguishable, biological functions. In addition, the drug sensitivity of *yap1* and *yap2* deletion strains does not directly correspond with the drug resistance observed upon Yap1 or Yap2 overexpression, suggesting the possibility of other Yap-like proteins that functionally overlap with Yap1 and Yap2.

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In this study, we identify an extended Yap family of transcription factors and demonstrate that they bind DNA with a specificity distinct from that of conventional AP-1 factors. In contrast to that of Gcn4, transcriptional activation by several Yap proteins is inhibited by PKA. However, Yap1 and Yap2 are not functionally equivalent, because they respond to different environmental stimuli, and Yap4 has distinct genetic and phenotypic properties. These observations indicate that individual members of the Yap family can have distinct biological functions.

MATERIALS AND METHODS

Computer analysis. A search for *S. cerevisiae* bZIP proteins was performed on the complete genome sequence by using a pattern-matching program written by Stewart Scherer. The degenerate query sequence (RQK, N, TRK, X, ASY, ASQV, X, X, CSFY, R, X, RK, RK, LAEVRK, where X indicates no amino acid preference) was derived from a list of 30 bZIP proteins from a variety of organisms and corresponds to the basic region (28). All yeast protein sequences that exactly or closely matched the query sequence contained leucine zippers in the position required for a functional bZIP protein (49). Because more-imprecise matches to the query sequence were clearly incompatible with a functional bZIP domain, it is very likely that the 14 identified proteins represent the complete set of yeast bZIP proteins. To make comparisons among the Yap proteins, BLAST searches were performed with individual Yap basic regions (17 residues corresponding to Gcn4 residues 231 to 247) or full bZIP domains in comparison with the translated sequence of the *S. cerevisiae* genome. To compare Yap proteins to other bZIP domains from other organisms, Yap1, Yap4, and Yap5 basic regions were individually used as query sequences for BLAST searches of GenBank sequences.

DNAs. For expression of Yap proteins in vitro, the following DNA fragments were cloned downstream of the SP6 promoter in pYX314 (47): Yap1, *Dra*I-*Hin*cII fragment encoding the N-terminal 155 residues; Yap2, *Bsr*I fragment encoding the N-terminal 182 residues; Yap3, *Bam*HI-*Ssp*I fragment (*Bam*HI is an artificial site) encoding residues 104 to 167; Yap4, *Bam*HI-*Ssp*I fragment (*Bam*HI site is artificial) encoding the C-terminal 112 residues; and Yap5, *Bam*HI-*Xmn*I (*Bam*HI site is artificial) fragment encoding the full-length protein. Molecules expressing LexA-Yap fusions were generated by cloning full-length Yap coding sequences into the YCp91 expression vector (between *Sma*I-*Kpn*I or *Sma*I-*Sac*I sites) in frame with the cassette harboring the LexA coding sequence (residues 1 to 202), SV40 nuclear localization signal, and HA-1 epitope (61). Plasmids overexpressing Yap1, Yap2, Yap3, and Yap5 contain the following chromosomal fragments cloned into the YEplac series of vectors (12): Yap1, *Sph*I-*Xba*I; Yap2, *Pst*I-*Bam*HI; Yap3, *Xho*I-*Bst*UI; and Yap5, *Eco*RV-*Xmn*I. To overexpress Yap4, the full-length coding and termination sequence was placed downstream of the *DED1* promoter in vector based on pRS314. Gcn4 and the A239Q S242F derivative (which was generated by PCR mutagenesis) were expressed from the *DED1* promoter as described previously (48). *HIS3* promoter constructs were based on a derivative of *his3-Δ101,189* (23) in which the *Xho*I site immediately upstream of the *Eco*RI site was replaced by a *Bam*HI site; oligonucleotides containing the different binding sites were cloned between the *Bam*HI and *Eco*RI sites. The following deletion genes for the various proteins were used: Δ*gcn4*, YIp56-Sc3674 (11); Δ*yap1*, which lacks a *Hin*cII fragment that includes the ATG initiation codon and bZIP domain; Δ*yap2*, which lacks an internal *Cla*I-*Eco*RI fragment that includes the initiation codon and bZIP domain; Δ*yap4*::*TRP1*, which lacks an internal *Bgl*II-*Msc*I fragment of the open reading frame that is replaced by *TRP1* and contains one stop codon upstream and in frame with the remaining portion of the open reading frame which contains the bZIP domain; and Δ*yap5*, which lacks an *Hpa*I-*Msc*I fragment with the initiation codon and bZIP domain.

DNA-binding assays. ^{35}S-labeled proteins were synthesized by transcription and translation in vitro using SP6 RNA polymerase and rabbit reticulocyte lysates (Promega) as described previously (56). Translated products were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and quantified by PhosphorImager analysis. The proteins used for the DNA-binding assays were not further purified from the in vitro translation reaction mixture. Oligonucleotide probes (65 bp) containing the various target sites were generated by PCR amplification, purification on a nondenaturing polyacrylamide gel, 5′ end labeling with ^{32}P, and separation on a Sephadex G-50 spin column. The binding reaction mixtures (20 μl total) contained 1 μl of in vitro translation product, 50 fmol of the radiolabeled probe, 500 ng of poly(dI · dC), 50 mM KCl, 3 mM $MgCl_2$, 12.5% glycerol, 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, and 100 mg of gelatin per ml. The reaction mixtures were incubated for 30 min at 25°C, and protein-DNA complexes were analyzed on a native polyacrylamide gel run with 0.5× Tris-borate-EDTA (TBE) buffer as described previously (56).

Yeast strains and phenotypic analysis. Yeast strains were generated by gene replacement of FT4 (61) with *his3*, *yap*, and *gcn4* alleles as described above. *bcy1* deletion strains were generated by one-step gene replacement and used immediately after construction to prevent accumulation of suppressor mutations (30). To determine growth phenotypes, strains grown to late log phase in appropriate media were harvested by centrifugation and resuspended in a solution containing 10 mM Tris-HCl (pH 8) and 5 mM EDTA. Cells were spotted onto yeast-peptone-dextrose (YPD) medium or on glucose minimal medium with appropriate supplements that either contained histidine or aminotriazole (AT) (0.1 or 10 mM) or lacked histidine. For analysis of LexA-Yap fusions and *his3* RNA levels, strains were grown in glucose minimal medium with appropriate supplements in the presence or absence of histidine. To analyze the response to AT, cells were grown in medium containing histidine and shifted for 4 h to a medium with 10 mM AT and lacking histidine. For other stress agents, cells were grown to early log phase in glucose medium containing histidine and treated with 1 mM hydrogen peroxide for 1 h or 400 μM $CdCl_2$ for 4 h.

For analysis of LexA-Yap fusions, derivatives of strain FT4 carrying the plasmids expressing LexA-Yap proteins and the LexA-dependent *lacZ* reporters JK103 (26) and Lop*HIS3*TATA (7) were grown in glucose minimal medium in the absence or presence of 10 mM AT, 400 μM $CdCl_2$, or 1 mM hydrogen peroxide as described above. Cells were harvested in early log phase, permeabilized with chloroform, and assayed for β-galactosidase activity. Values were normalized to A_{600} and are the averages for at least six independent transformants; they are accurate to ±20%.

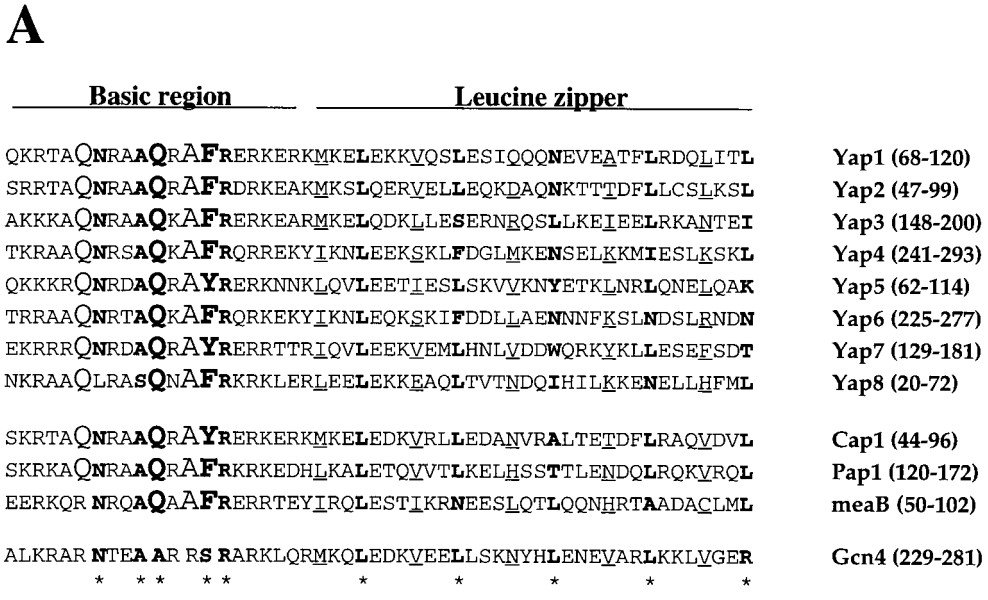
Western blotting. Cells were resuspended in 20 mM HEPES (pH 7.9)–150 mM NaCl–10 mM $MgCl_2$–0.3 M ammonium sulfate–10% (wt/vol) glycerol–2 mM phenylmethylsulfonyl fluoride–20 μg of aprotinin per ml–1.5 μg of benzamidine per ml–10 μg of leupeptin per ml–1 μg of pepstatin per ml. Whole-cell extracts were prepared by lysing the cells with glass beads and subjecting them to microcentrifugation at 4°C for 15 min; they were stored at −70°C. Protein from each extract was quantitated by Bradford assay and subjected to electrophoresis on a 10% denaturing polyacrylamide gel. To measure Yap1 levels, Western blots were performed by using 100 μg of electrophoretically separated yeast protein, Yap1 antibody (obtained from W. Scott Moye-Rowley and used at a 1:4,000 dilution), anti-mouse immunoglobulin G linked to horseradish peroxidase (1:200 dilution; Amersham), and enhanced chemiluminescent detection. To measure intracellular levels of the LexA-Yap fusion proteins, Western blotting was performed with 20 μg of electrophoretically separated yeast protein, LexA antibody (obtained from Roger Brent and used at a 1:2,000 dilution), and anti-rabbit immunoglobulin G conjugated to alkaline phosphatase (1:2,500 dilution; Promega).

Transcriptional analysis. For analysis of Yap-dependent transcription, RNAs (20 μg as quantitated by A_{260}) were hybridized to completion with a 10- to 100-fold excess of ^{32}P-labeled *HIS3* and *DED1* oligonucleotide probes and treated with S1 nuclease as described previously (24). *HIS3* mRNA levels were quantitated with respect to the *DED1* internal control by PhosphorImage analysis and expressed as molecules per cell as defined previously (24). The error for individual determinations by this method is ±25% (24). The specific activity of the *DED1* probe relative to that of *HIS3* is reduced by 50%. To analyze the effect of PKA on *YAP* transcription, RNAs (20 μg as quantitated by A_{260}) from wild-type and *bcy1* strains containing multicopy *YAP* plasmids were electrophoretically separated on agarose gels, blotted onto a nylon membrane, and hybridized with *YAP*, *TUB2*, *DED1*, and *TRP1* probes generated by random-primed synthesis.

RESULTS

A family of eight Yap proteins in *S. cerevisiae*. The most conserved feature of bZIP domains is the basic region that directly interacts with DNA. Within the basic region, the five residues (corresponding to Asn235, Ala238, Ala239, Ser242, and Arg243 in Gcn4) that make base-specific contacts in the Gcn4 and Fos/Jun cocrystal structures (10, 13, 31) are most highly conserved. To identify *S. cerevisiae* bZIP proteins, we searched the complete genome with a degenerate motif based on the sequences of a large number of basic regions in bZIP proteins from various organisms. This search revealed 14 bZIP proteins, which include Gcn4, Yap1, Yap2, Met28 (35), and the ATF/CREB proteins Acr1/Sko1 (40, 63) and Hac1 (41). These 14 proteins are likely to represent the complete set of *S. cerevisiae* bZIP proteins.

Yap1 and Yap2 are unusual among bZIP proteins in that they contain a glutamine at the position corresponding to Ala239 and a phenylalanine at the position corresponding to Ser242 (Fig. 1) (see Discussion). Interestingly, six of the newly identified *S. cerevisiae* bZIP proteins contain these characteristic glutamine and phenylalanine (or tyrosine in two cases) residues. These six proteins further resemble Yap1 and Yap2 in that they all contain a glutamine at position 234 and an alanine at position 241; such residues are rarely, if ever, observed in other bZIP proteins. None of these proteins contain



B

	Yap2	Yap3	Yap4	Yap5	Yap6	Yap7	Yap8
Yap1	12.3	8.1	7.4	7.5	7.1	5.4	5.1
Yap2		7.7	6.0	4.9	6.4	5.3	2.4
Yap3			5.0	3.7	6.0	3.6	3.3
Yap4				4.4	17.8	6.0	3.0
Yap5					3.0	11.8	2.5
Yap6						3.6	4.4
Yap7							0

FIG. 1. Comparison of the Yap bZIP domains. (A) Sequences of the eight *S. cerevisiae* Yap bZIP domains compared with corresponding regions from Gcn4, a classical AP-1 factor, and Yap-like proteins from *S. pombe* (Pap1) and *A. nidulans* (meaB), and *C. albicans* (Cap1). In the basic region, residues that directly interact with base pairs (boldface) and Yap-specific residues (large font) are indicated. In the leucine zipper, the conserved leucines (or other residues) at position d of the coiled coil (boldface) and hydrophobic (typically) residues at position a of the coiled coil (underlined) are indicated. (B) Pairwise sequence comparison of the Yap bZIP domains, with entries indicated by LOD score as determined by BLAST searches.

a basic residue at position 247 that is characteristic of the ATF/CREB family of bZIP proteins and is important for their half-site spacing specificity (28). Thus, *S. cerevisiae* contains a novel family of eight bZIP proteins that is defined by idiosyncratic residues in the basic region; for this reason, the previously undescribed proteins are designated Yap3, Yap4 (previously identified as Cin5), Yap5, Yap6, Yap7, and Yap8 (Table 1). As determined by SAGE (62), the *YAP* genes are poorly expressed (≤0.5 mRNA molecule/cell).

More-detailed comparison (Fig. 1B) of the bZIP domains indicates that the Yap proteins can be subdivided into related subfamilies: Yap1 and Yap2, Yap4 and Yap6, and Yap5 and Yap7. Yap3 shows comparable similarity to other Yap proteins, whereas Yap8 is least closely related. In addition, Yap8 contains a leucine at the position of the invariant asparagine in the basic region of bZIP domains; such a substitution abolishes binding of Gcn4 homodimers to DNA (48). The Yap leucine zippers are compatible with coiled-coil structure, but they are unrelated in sequence, with the exceptions of Yap4 and Yap6. Outside the bZIP regions, the Yap proteins are unrelated, with the exceptions of Yap1 and Yap2, which have two additional regions of similarity. One of these conserved regions has recently been implicated in regulating nuclear localization of Yap1 in response to oxidative stress (33).

Yap proteins have a DNA-binding specificity distinct from those of conventional AP-1 factors. The optimal sequence for Gcn4 binding, TGACTCA, contains two TGA half-sites and a

TABLE 1. Description of *YAP* genes

Gene	Chromosome	Length of ORF (aa)[a]	Predicted molecular mass (kDa)	Other name(s)	Yeast ORF designation
YAP1	XIII	650	72.5	*PDR4*; *PAR1*; *SNQ3*	YML007w
YAP2	IV	409	46	*CAD1*	YDR423c
YAP3	VIII	330	38		YHL009c
YAP4	XV	295	33	*CIN5*	YOR028c
YAP5	IX	245	28.3		YIR018w
YAP6	IV	383	43.6		YDR259c
YAP7	XV	245	27.4		YOL028c
YAP8	XVI	294	33.2		YPR199c

[a] ORF, open reading frame; aa, amino acids.

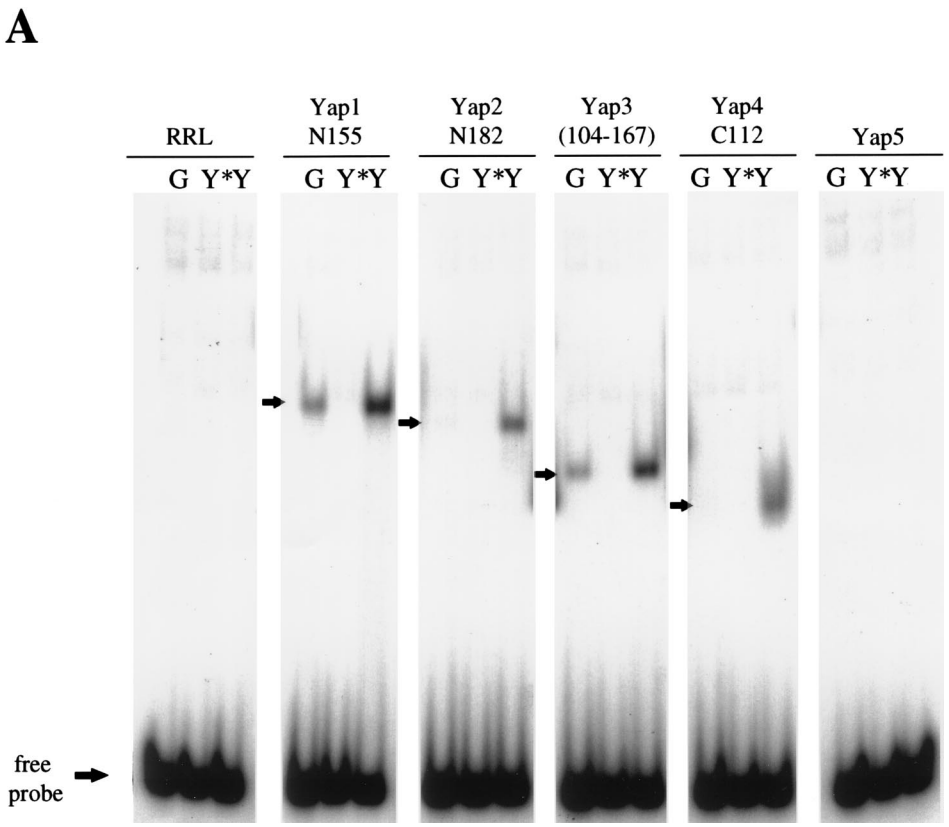


FIG. 2. Specific DNA-binding activities of the Yap proteins and Gcn4. (A) Yap derivatives and a control translation reaction mixture in the absence of RNA (RRL) were incubated with the following ^{32}P-labeled sequences: TGACTCA (G, Gcn4 optimal binding site), TAACTTA (Y*, Yap mutated binding site), and TTACTAA (Y, Yap binding site). Specific Yap-DNA complexes are indicated (arrows). Weak complexes present in all lanes result from binding of proteins in the rabbit reticulocyte lysate to the respective DNA probes. Equimolar amounts of proteins (determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis) were used except for the Yap4 derivative, which was used at a fivefold-lower concentration. (B) Gcn4 and the A239Q S242F derivative were analyzed as described above; the protein concentrations were equivalent to those of the Yap proteins. Specific Gcn4-DNA complex is indicated (arrow). wt, wild type.

central C · G base pair (19, 42, 53). Because Yap1 was initially defined by its ability to bind an SV40 sequence (TGACTAA) that contains a TGA and a TTA half-site, the optimal half-site (and thus the optimal binding site) was unclear. To clarify the preferred half-site for Yap1 and to define the DNA-binding specificities of the other Yap proteins, we examined in vitro-synthesized proteins for their ability to bind an optimal AP-1 site and a related sequence (TTACTAA) that contains two TTA half-sites and coincides with sequences in the *TRX2*, *YCF1*, and *GLR1* promoters.

As shown in Fig. 2A, Yap1, Yap2, Yap3, and Yap4 preferentially interact with TTACTAA, which we now term the Yap site. This preference for the Yap site clearly distinguishes the Yap proteins from Gcn4, which strongly prefers the optimal AP-1 site (Fig. 2B). The sequence preferences of the Yap proteins tested are not identical; Yap1 and Yap3 bind with modest affinity to the optimal AP-1 site, whereas Yap2 and Yap4 bind poorly (weak bands are detectable upon longer exposure of the autoradiograph). DNA-binding activity was not detected for Yap5 either as a homodimer (Fig. 2A) or as a heterodimer (obtained by cotranslation) with Yap1, Yap2, Yap3, or Yap4 (data not shown). In addition, Yap het-

TABLE 2. Transcriptional regulation by LexA-Yap proteins

Control or LexA hybrid	Activity with the indicated *lacZ* reporter[a]					
	JK103				Lop*HIS3*TATA	
	With no addition	With AT	With Cd^{2+}	With H_2O_2	Wild type	Δ*bcy1*
Vector alone	0.5	0.5	0.5	0.6	55	5.5
LexA alone	0.4	0.5	0.5	0.6	57	4.4
Yap1	427	406	700	449	478	139
Yap2	2	1.4	10.9	1.3	85	16
Yap3	1,808	1,700	NT	NT	830	212
Yap4	1	1	NT	NT	82	24
Yap5	9.3	9	NT	NT	213	58

[a] β-Galactosidase activities (averages for six independent transformants) of derivatives of strain FT4 carrying the indicated promoters and proteins that were grown in glucose medium in the presence of the indicated stress agents. Values are normalized to the A_{600} of cells at the time of collection and are accurate to ±20%. β-Galactosidase levels in *bcy1* strains are generally reduced by a factor of 3 to 6 due to poor growth. NT, nontested.

erodimers that bound to the Yap site were not observed for any pairwise combination involving Yap1, Yap2, Yap3, and Yap4 (data not shown).

Yap-specific residues contribute to Yap-like DNA-binding specificity. The distinct DNA-binding specificities of Yap and AP-1 proteins are most easily explained by the differences in amino acids that directly contact DNA. To test this hypothesis, we analyzed the DNA-binding properties of a Gcn4 derivative (A239Q S242F) in which the relevant residues were replaced by those diagnostic of a Yap basic region (Fig. 2B). In comparison to Gcn4, the mutant derivative exhibits increased binding to the Yap site, indicating that the Yap-specific residues in the basic region contribute to Yap-like DNA-binding specificity. However, these residues are not sufficient for conversion of Gcn4 into a derivative with true Yap-like specificity because the A239Q S242F derivative recognizes the optimal AP-1 site with wild-type affinity. Other residues are also necessary for conversion of Gcn4 into a derivative that preferentially binds the Yap site; perhaps these include those corresponding to Gln234 and Ala241, which are specific to the Yap family. These observations are consistent with the views that adaptability at the protein-DNA interface is an important aspect of sequence recognition by bZIP proteins and that the five residues that directly contact base pairs are critical, but not sufficient, for DNA-binding specificity (10, 29). In any event, the results indicate that the Yap-specific residues in the basic regions are important for the distinct DNA-binding properties of the Yap proteins and conventional AP-1 factors.

Inherent activation potential of the Yap proteins. Using LexA fusion proteins and appropriate reporter constructs, we tested the ability of several Yap proteins to activate transcription independently of their DNA-binding properties (Table 2). LexA-Yap1 and LexA-Yap3 strongly activate transcription from two different reporters and LexA-Yap5 activates transcription to a modest degree, whereas LexA-Yap2 and LexA-Yap4 are essentially inactive (although perhaps slightly more active than the LexA and vector controls). Interestingly, transcriptional activation by Yap2 is increased fivefold upon treatment with 400 μM $CdCl_2$ (see below). Western blotting (Fig. 3) indicates that the LexA fusion proteins are comparably expressed except for LexA-Yap3, which is present at higher levels. Thus, the differences in transcriptional activity by the LexA-Yap fusions are due primarily to differences in the functional quality of the activation domains.

Yap proteins preferentially activate transcription through the Yap site. Yap1 and Yap2 can activate transcription from heterologous promoters containing the SV40 sequence TGA CTAA (39, 65), and Yap1 can activate transcription from TT ACTAA (32), the preferred Yap site. To address the functional activities and DNA-binding specificities of the various Yap proteins in vivo, we overexpressed the Yap proteins in four strains that differ only in the regulatory sequences that are upstream of the *his3* TATA element and mRNA coding sequence (diagrammed in Fig. 4); these sequences include the optimal AP-1 site, the Yap site, the SV40 sequence, and a mutated Yap site. The resulting strains were analyzed for *his3* expression by growth in the presence of AT, a competitive inhibitor of the *HIS3* gene product (Fig. 4), and by measurement of *his3* RNA levels under standard growth conditions (Fig. 5A and B).

In accord with the DNA-binding studies, Yap1 and Yap2 activate transcription from the Yap site but not from the optimal AP-1 site. Cells expressing Yap1 show about fourfold-higher levels of *his3* transcripts than cells expressing Yap2. Yap1 and Yap2 also activate transcription from the SV40 sequence (as evidenced by the growth assay) but with much lower efficiency than that from the Yap site. Yap3 displays the same promoter specificity in vivo, but it is much less efficient than Yap1 and Yap2 (*his3* transcripts are detected upon overexposure of the autoradiogram in Fig. 5B). Yap4 and Yap5 fail

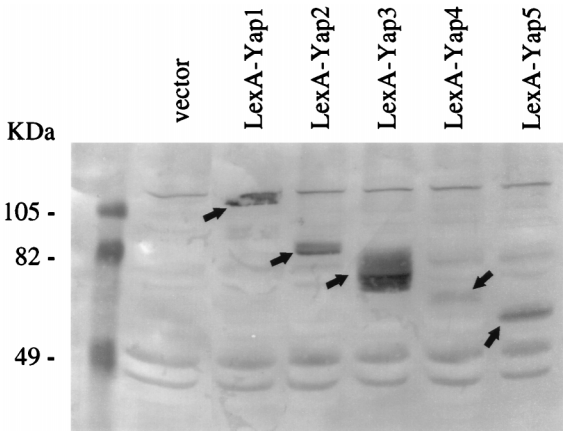


FIG. 3. Western analysis of the LexA-Yap hybrid proteins. Protein (20 μg) from strains overexpressing the indicated LexA-Yap protein was analyzed by electrophoresis on a 10% denaturing polyacrylamide gel followed by Western blotting using LexA antibody. The positions of the LexA-Yap hybrid proteins are indicated (arrows); in all cases, the apparent molecular mass does not precisely correspond to that predicted from the amino acid sequence. Bands observed in all strains (including the parental strain lacking LexA proteins) are due to nonspecific reaction of the LexA antibody with yeast proteins.

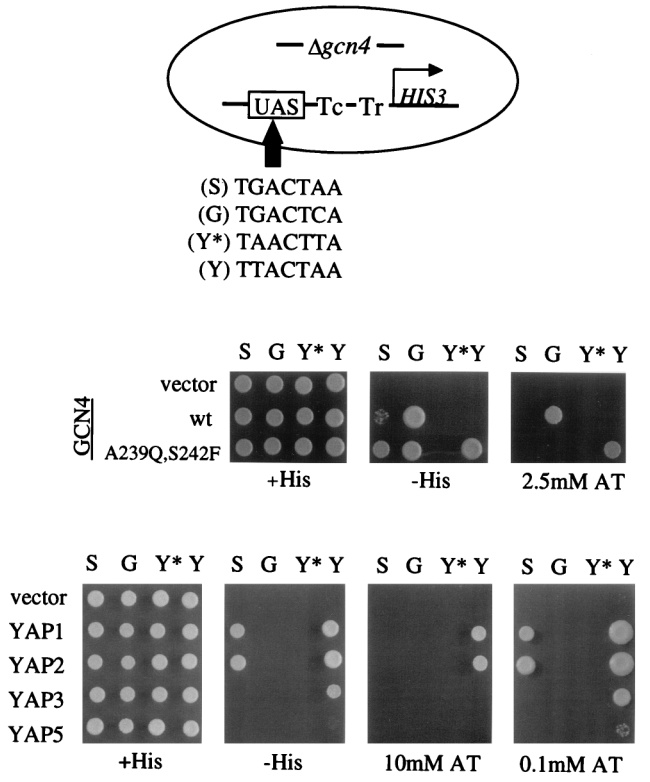


FIG. 4. Phenotypic assay for Yap-dependent activation. Plasmids expressing the indicated Yap proteins and Gcn4 derivatives were introduced into *gcn4* deletion strains containing *his3* alleles with the indicated upstream activated sequence (UAS) (S, SV40 sequence; G, optimal Gcn4/AP-1 site; Y*, mutated Yap site; Y, preferred Yap site) upstream of the *his3* T_C and T_R TATA elements and structural gene. The resulting strains were analyzed in glucose minimal medium containing histidine (+His) or the indicated concentrations of AT or lacking histidine (−His). wt, wild type.

to stimulate transcription under normal growth conditions, although Yap5-expressing cells show weak growth in medium containing low concentrations of AT (see below).

In contrast to the Yap proteins and in accord with previous results (29, 53), Gcn4 behaves like a conventional AP-1 factor in vivo. It strongly activates transcription from an optimal AP-1 site binding site, weakly activates transcription from the SV40 sequence, and does not detectably stimulate transcription from the Yap site. Although Gcn4 can detectably bind the Yap site in vitro (Fig. 2B), this interaction is not sufficiently strong to mediate transcriptional activation in vivo. Interestingly, the A239Q S242F Gcn4 derivative preferentially activates transcription from the Yap site. Although this protein binds with comparable affinities to the optimal AP-1 and Yap sites in vitro, subtle quantitative differences might be magnified in vivo. In any event, this observation provides independent evidence for the importance of the Yap-specific residues in distinguishing the Yap proteins from conventional AP-1 factors.

Structural basis for Yap-specific DNA-binding specificity. Molecular modeling was performed by substituting Yap-specific amino acids and base pairs into the crystal structure of the Gcn4–AP-1 complex (10). Gln239 can be positioned so that it makes novel hydrogen bonds to the T at position ±2 and the A at ±1 (top-strand residues) while retaining the hydrophobic contact with the T at ±1 (bottom-strand residue) observed in the Gcn4–AP-1 complex. In contrast, the canonical AP-1 residue, Ala239, is smaller and is unable to make hydrogen bonds to the ±2 base pair. Unlike the smaller Ser/Cys242, Phe242 can be positioned to make a novel hydrophobic contact to the thymine methyl at ±2 while relieving crowding at residue ±3. Moreover, an interaction between Phe242 and a G at ±2 would be disfavored by crowding and by the close juxtaposition of hydrophobic and polar atoms. Thus, Gln239 and Phe242 are likely to contribute directly to recognition of the ±2 T · A base pair in the Yap site and discrimination against the ±2 G · C pair in the AP-1 site.

The other Yap-specific residues, Gln234 and Ala241, are smaller than their AP-1 counterparts (typically lysine and arginine), but their structural roles are less clear. Gln234 is likely to contact a different phosphate than the corresponding AP-1 residues, whereas the Ala241 substitution results in the loss of a phosphate contact. However, these phosphate contacts are located at positions ±4 and ±5 and hence are unlikely to directly affect DNA-binding specificity at position ±2. We speculate that the relatively small size of Gln234 and Ala241 might contribute to Yap specificity by permitting an adjustment of the basic region that alleviates the crowding at the DNA interface caused by Gln239 and Phe242. Gln234 has the additional property that it alleviates crowding without loss of a phosphate contact.

Yap proteins respond differentially to environmental stress. The experiments described above indicate that Yap proteins can activate transcription from the preferred Yap site to various degrees, but they don't address potential functional differences among the Yap proteins. Because the growth phenotypes on AT (Fig. 4) are not strictly correlated with *his3* transcription under normal growth conditions (Fig. 5B), we first analyzed *his3* transcription in strains grown in the presence of 10 mM AT, conditions of histidine starvation. Interestingly, when assayed on the promoter containing the optimal Yap site, Yap2- and Yap3-dependent transcription under these conditions is three- to fourfold higher than the level observed in standard medium (compare Fig. 5B and C). In addition, transcription dependent on Yap5 was detected (when the autoradiograph is overexposed [data not shown]); this probably accounts for the ability of Yap5-overexpressing strains to grow in medium containing 0.1 mM AT but not in medium lacking AT (Fig. 4). In contrast, Yap1-dependent activation was minimally affected by the presence of AT in the medium.

We next examined the effect of other stress agents in wild-type and *yap* deletion strains. In a wild-type strain containing normal levels of the Yap proteins, *his3* transcription is stimulated by hydrogen peroxide but not by AT or cadmium (Fig. 6A). This transcriptional induction is due to Yap1, because it is abolished in a *yap1* deletion strain. However, activation by LexA-Yap1 is not increased by hydrogen peroxide (Table 2), and cells overexpressing Yap1 do not show significantly increased *his3* transcription in the presence of hydrogen peroxide (Fig. 6B). These results and the observation that hydrogen peroxide can affect Yap1 DNA-binding activity (32) suggest that Yap1-dependent transcription is limited at the level of promoter occupancy and that overexpression of Yap1 results in saturation of the Yap target sequence in the *his3* promoter, even under normal growth conditions. Nevertheless, under physiological conditions, Yap1-dependent transcription is strongly stimulated by hydrogen peroxide but not by AT or cadmium.

The stress-responsive properties of Yap2 were analyzed in strains lacking Yap1 and overexpressing Yap2 (Fig. 6B). In contrast to the case for Yap1, Yap2-dependent transcription is strongly induced by AT (4.8-fold) and cadmium (7.3-fold) but is minimally affected by hydrogen peroxide. Regulation of

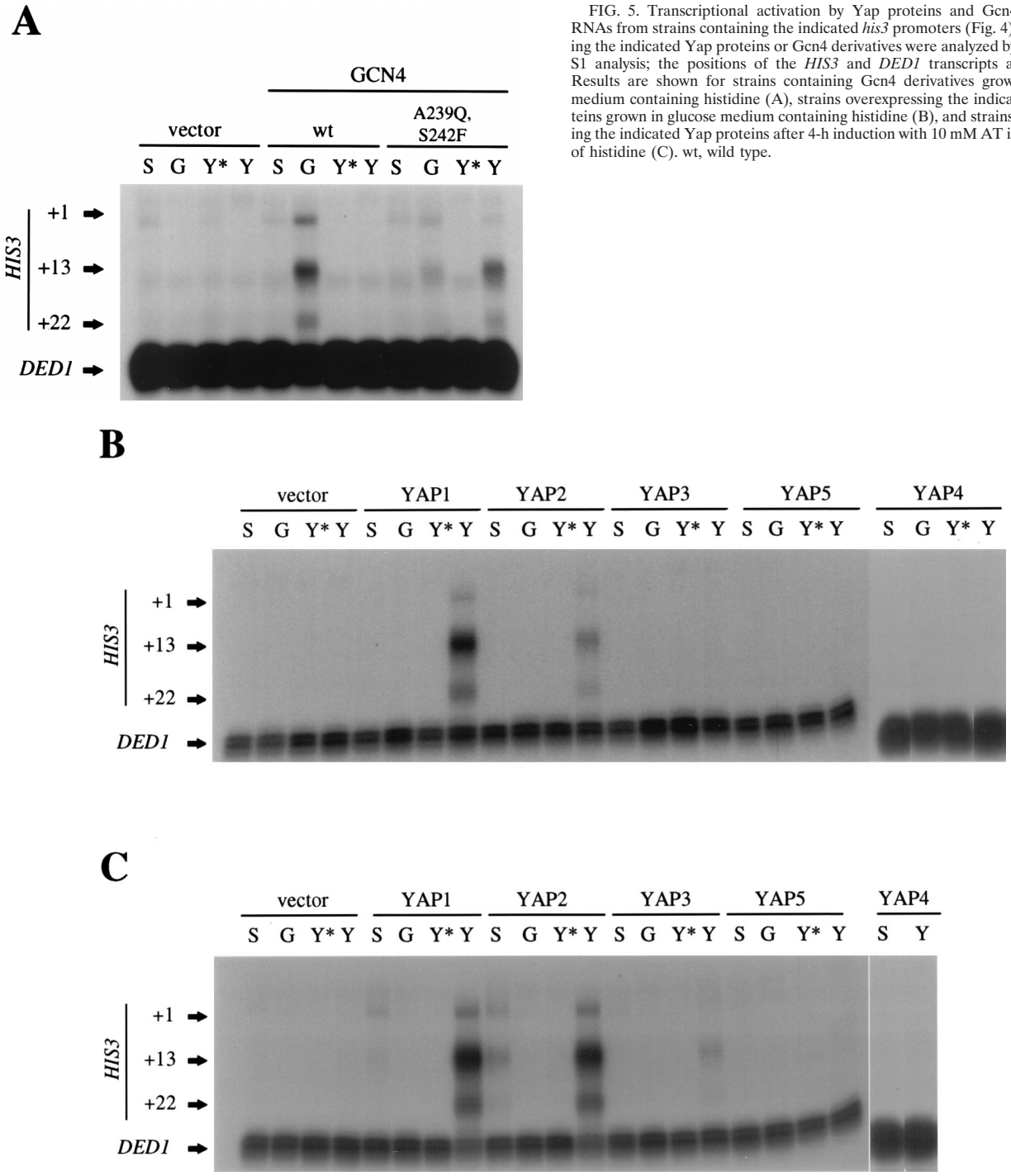


FIG. 5. Transcriptional activation by Yap proteins and Gcn4 derivatives. RNAs from strains containing the indicated *his3* promoters (Fig. 4) and expressing the indicated Yap proteins or Gcn4 derivatives were analyzed by quantitative S1 analysis; the positions of the *HIS3* and *DED1* transcripts are indicated. Results are shown for strains containing Gcn4 derivatives grown in glucose medium containing histidine (A), strains overexpressing the indicated Yap proteins grown in glucose medium containing histidine (B), and strains overexpressing the indicated Yap proteins after 4-h induction with 10 mM AT in the absence of histidine (C). wt, wild type.

Yap2-dependent transcription occurs at different levels with cadmium and AT. Activation by LexA-Yap2 is induced by cadmium (Table 2), suggesting that regulation occurs, at least in part, at the level of the activation domain. In contrast, AT does not affect activation by LexA-Yap2 (Table 2) or *YAP2* mRNA levels (data not shown). These results suggest that AT regulates Yap2-dependent transcription at the level of promoter occupancy.

Similar analysis of Yap3 indicates that it responds to AT but not to hydrogen peroxide or cadmium (Fig. 6B). As is the case for Yap2, AT does not affect activation by LexA-Yap3 and LexA-Yap5 (Table 2) or Yap3 and Yap5 mRNA levels (data not shown). Although Yap4 (Cin5) appears to be involved in chromosome stability (22a), transcriptional stimulation of the *his3* reporter was not observed under any conditions, including treatment with nocadozole and colchicine (data not shown).

Yap-dependent activation is inhibited by high levels of PKA. Although PKA and Yap1 have been implicated in the inhibition of stress-inducible transcription from a DNA sequence element termed the STRE (14, 37), the relationship between

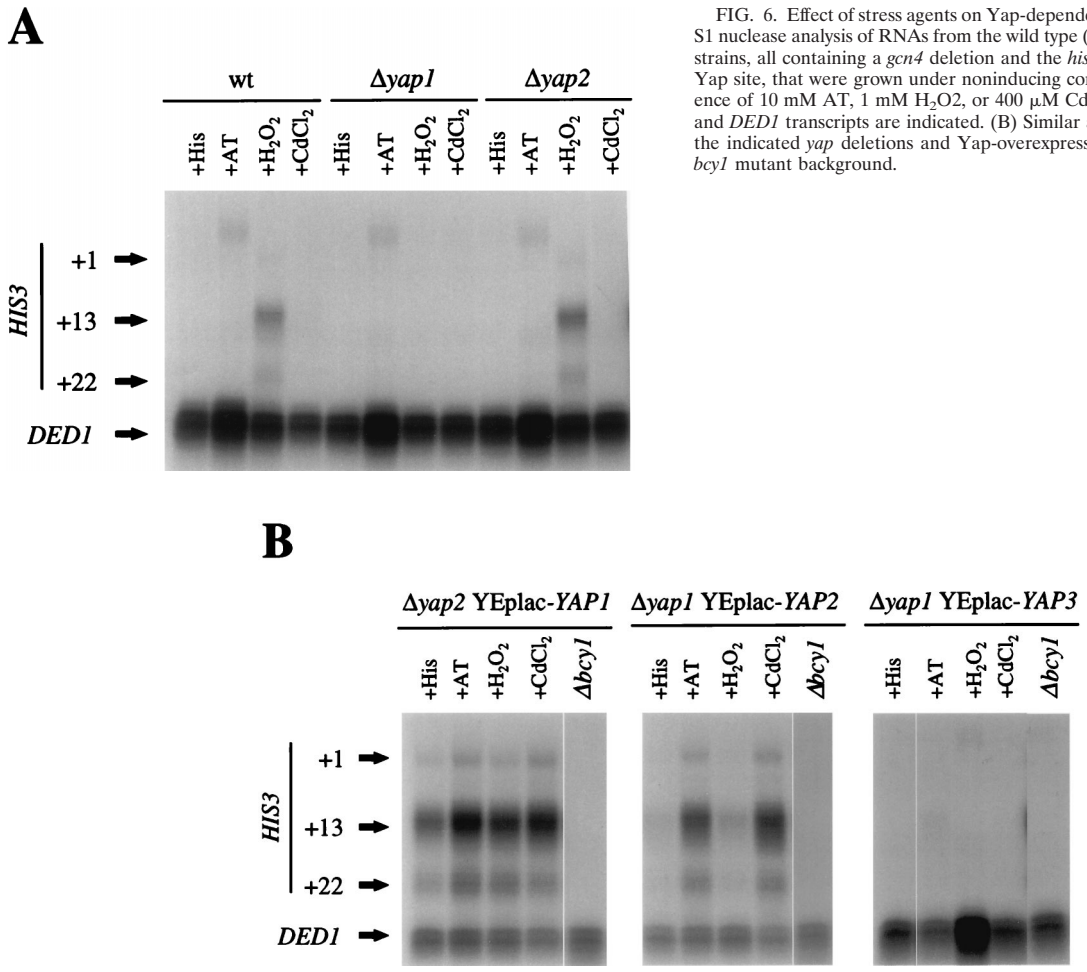


FIG. 6. Effect of stress agents on Yap-dependent activation. (A) Quantitative S1 nuclease analysis of RNAs from the wild type (wt) and *yap1* and *yap2* deletion strains, all containing a *gcn4* deletion and the *his3* promoter with the preferred Yap site, that were grown under noninducing conditions (+His) or in the presence of 10 mM AT, 1 mM H_2O2, or 400 μM $CdCl_2$. The positions of the *HIS3* and *DED1* transcripts are indicated. (B) Similar analysis with strains containing the indicated *yap* deletions and Yap-overexpressing plasmids in a wild-type or *bcy1* mutant background.

PKA and Yap-dependent activation is unclear. We therefore examined Yap-dependent transcription in *bcy1* strains that contain high, unregulated levels of PKA (Fig. 6B). Under all conditions tested, *his3* transcription dependent on Yap1, Yap2, and Yap3 is virtually abolished in *bcy1* strains. Although Yap1, Yap2, and protein kinase C are involved in a novel heat shock response independent of the classical heat shock element (25, 38), Yap-dependent transcription activity is not affected when cells are treated with staurosporine, a potent inhibitor of this kinase (data not shown). The inhibition of Yap-dependent transcription by PKA is in marked contrast to the ability of PKA to stimulate Gcn4-dependent activation (11).

Additional experiments suggest that PKA inhibits Yap1-dependent transcription by at least two mechanisms. First, Yap1 protein and RNA levels are reduced two- to threefold in *bcy1* strains (Fig. 7A and B). It is likely that this modest effect only partially accounts for the severe reduction in Yap1-dependent activation observed in *bcy1* strains. Second, high levels of PKA inhibit activation by LexA-Yap1 from a promoter dependent on a Yap site (Fig. 7C) but not from a promoter dependent on a LexA operator (Table 2); this promoter specificity is not easily explained simply by decreased protein levels in *bcy1* strains. Instead, this observation suggests that PKA inhibits Yap1-dependent transcriptional activity via an effect on occupancy of Yap sites, not on inherent activation function.

In the case of Yap2, *bcy1* strains display a two- to threefold reduction in *YAP2* RNA (and presumably protein) levels; we suspect that this reduction contributes to, but does not fully account for, the severe reduction in Yap2-dependent activation. PKA causes a more dramatic reduction in *YAP3* RNA levels which might be sufficient for the observed loss of Yap3-dependent activation. As observed for LexA-Yap1, activation by LexA-Yap2 and LexA-Yap3 through LexA operators is not significantly affected by high PKA levels (Table 2), suggesting that PKA does not affect the function of the activation domains.

Distinct phenotypes of *vap* deletion strains. Yap1, Yap2, Yap4, and Yap5 are not essential; all single- and multiple-deletion strains are viable, able to grow at high temperature (37°C), and able to grow in medium lacking inositol or containing galactose, sucrose, raffinose, or glycerol as the sole carbon source. As described previously (52), *yap1* deletion strains are sensitive to hydrogen peroxide; in contrast, a *yap2 yap4 yap5* deletion strain retains wild-type sensitivity. In addition, loss of Yap1, but not the other Yap proteins, results in a cold-sensitive phenotype (Fig. 8). Interestingly, this cold sensitivity is suppressed by a *yap4* deletion but not by a *yap2* or *yap5* deletion. However, the *yap4* deletion is unable to suppress the sensitivity of *yap1* deletion strains to hydrogen peroxide. In accord with the previous identification of *yap4* as *cin5*, a mutation that increases chromosome instability, strains lacking Yap4 (but not the other Yap proteins tested) show a modest sensitivity to nocadozole (18% survival after 4-h exposure to 20

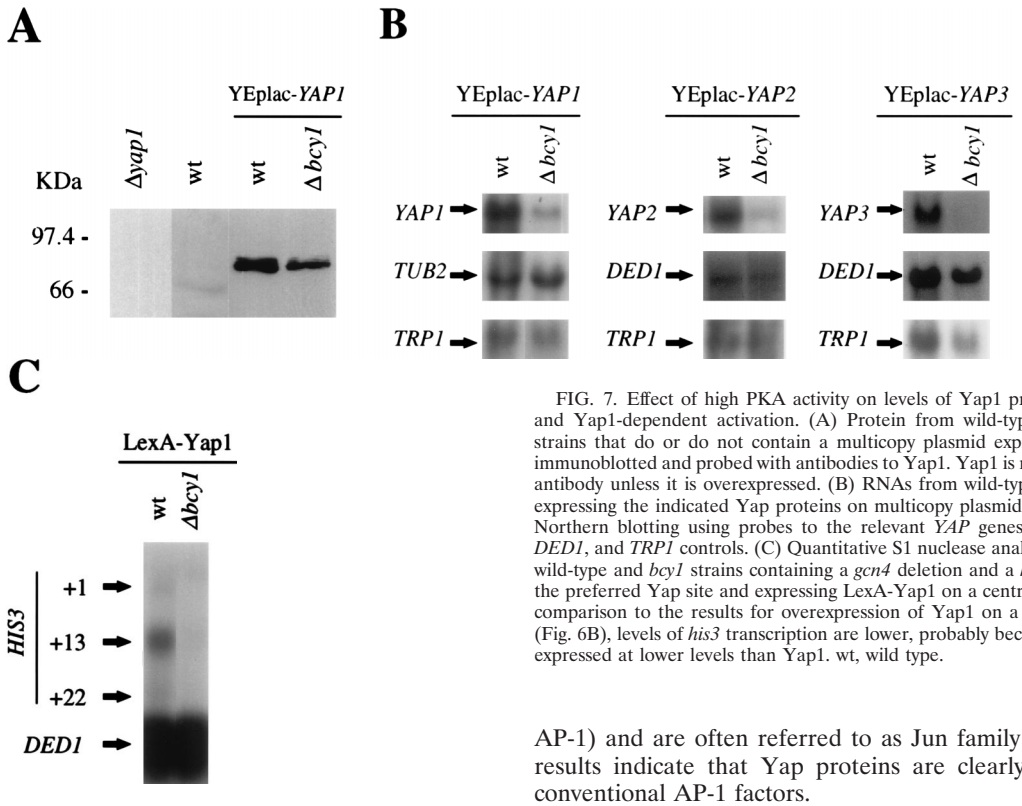


FIG. 7. Effect of high PKA activity on levels of Yap1 protein, Yap RNAs, and Yap1-dependent activation. (A) Protein from wild-type, *yap1*, and *bcy1* strains that do or do not contain a multicopy plasmid expressing Yap1 were immunoblotted and probed with antibodies to Yap1. Yap1 is not detected by this antibody unless it is overexpressed. (B) RNAs from wild-type and *bcy1* strains expressing the indicated Yap proteins on multicopy plasmids were analyzed by Northern blotting using probes to the relevant *YAP* genes as well as *TUB2*, *DED1*, and *TRP1* controls. (C) Quantitative S1 nuclease analysis of RNAs from wild-type and *bcy1* strains containing a *gcn4* deletion and a *his3* promoter with the preferred Yap site and expressing LexA-Yap1 on a centromeric plasmid. In comparison to the results for overexpression of Yap1 on a multicopy plasmid (Fig. 6B), levels of *his3* transcription are lower, probably because LexA-Yap1 is expressed at lower levels than Yap1. wt, wild type.

mg of nocadozole per ml compared to 40% survival of wild-type cells).

DISCUSSION

Yap proteins are members of a fungus-specific class of bZIP proteins. Detailed sequence analysis indicates that the Yap family represents a distinct and novel subgroup of bZIP proteins. The Yap proteins contain four diagnostic residues in the basic region (Q234, Q239, A241, and F/Y242) that are rarely or never observed in other bZIP proteins. Moreover, individual BLAST searches using the Yap1, Yap4, or Yap5 basic region uncover all the Yap family members in *S. cerevisiae* but do not identify any of the numerous bZIP proteins in plants and animals. Aside from the Yap proteins, the only other proteins identified in these BLAST searches are Pap1, a *Schizosaccharomyces pombe* protein that mediates resistance to a variety of drugs (34, 58, 60); meaB, a protein from *Aspergillus nidulans* that is involved in nitrogen repression (46); and Cap1, a *Candida albicans* protein that mediates resistance to fluconazole (1). These considerations strongly suggest that the Yap proteins represent a subfamily of bZIP proteins that is restricted to fungi.

In addition to their diagnostic sequence characteristics, the Yap proteins display a novel DNA-binding specificity in vitro (Fig. 2A) and in vivo (Fig. 4 and 5B and C). The preferred recognition site for at least four Yap proteins (TTACTAA) is different from the optimal AP-1 site (TGACTCA) at position ±2, and it diverges even more from the preferred recognition sequences for other bZIP proteins. The properties of the A239Q S242F derivative of Gcn4 (Fig. 2B and 5A) indicate that two of the Yap-specific residues in the basic regions contribute significantly to the novel DNA-binding specificity of the Yap proteins. Thus, although Yap proteins were originally named as yeast AP-1 factors (and Pap1 was named as *pombe* AP-1) and are often referred to as Jun family members, our results indicate that Yap proteins are clearly distinct from conventional AP-1 factors.

Molecular modeling provides a plausible hypothesis for the DNA-binding specificity of Yap proteins. Two Yap-specific residues, Gln239 and Phe242, are likely to contribute directly to recognition of the ±2 T · A base pair in the Yap site and discrimination against the ±2 G · C pair in the AP-1 site. In both cases, the Yap-specific residues are larger than their AP-1 counterparts (Ala239 and Ser/Cys242), so they can make novel contacts to the ±2 thymine (top strand). A guanine is probably disfavored at position ±2 because the bulky purine ring crowds the larger Yap-specific residues. The structural roles of Gln234 and Ala242 are less clear, but they may contribute to Yap specificity by permitting an adjustment of the basic region that alleviates the crowding at the DNA interface caused by Gln239 and Phe242.

Yap proteins have common properties but are functionally distinct. In many respects, the Yap proteins are functionally redundant in vivo. Overexpression of Yap1 and Yap2 confers increased resistance to a common group of stress agents, with Yap1 generally having stronger effects than Yap2. At least four Yap proteins preferentially activate transcription through the Yap site, and in the three cases tested, transcription is strongly inhibited by PKA. The magnitude of activation through the Yap site varies considerably; Yap1 activates strongly, Yap2 activates moderately, Yap3 activates weakly, and Yap5-dependent activation is barely detectable. The observed differences in transcriptional output mediated by the various Yap proteins could reflect quantitative differences in one of more of the following: intracellular level, nuclear localization, and inherent DNA-binding and/or transcriptional activity.

Despite their common properties, several observations in this study indicate that the Yap proteins are functionally distinct. First, Yap2 and Yap4 severely discriminate against the optimal AP-1 site, whereas Yap1 and Yap3 show only a modest discrimination (Fig. 2A). This suggests that some yeast promoters might be differentially affected by individual Yap pro-

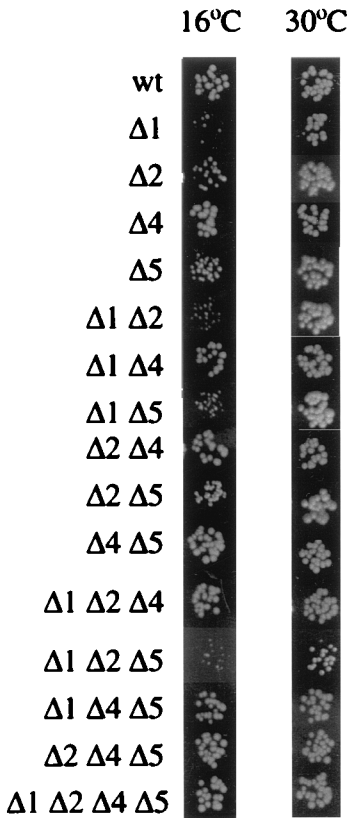


FIG. 8. Cold sensitivity of *yap1* cells is suppressed by a *yap4* mutation. Equal numbers of cells of the indicated strains were spotted onto YPD plates and incubated at 16 and 30°C.

teins. Second, the Yap proteins differ in their response to stress agents. Yap1 transcriptional activity is strongly induced by hydrogen peroxide but is relatively unaffected by AT and cadmium (Fig. 5 and 6). Conversely, Yap2 responds strongly to cadmium and AT but not to hydrogen peroxide. The cadmium response is mediated at least in part through the Yap2 activation function, because cadmium increases the function of LexA-Yap2. Yap3 and Yap5 also respond to AT, and like Yap2, this response does not seem to occur at the level of the activation domain. Third, Yap4 is phenotypically distinct from the other Yap proteins tested. Unlike other *yap* mutations, *yap4* mutations increase chromosome instability (evidence by the previous isolation as *cin5* and by sensitivity to nocadozole), and they suppress the cold sensitivity caused by a *yap1* deletion.

Yap transcriptional activity is inhibited by PKA. PKA, the ultimate target of the Ras pathway in yeast (6, 59), has diverse effects on gene expression. PKA stimulates Rap1 transcriptional activity in response to growth signals, an effect that is mediated at the level of the activation domain (30). In addition, the Ras-PKA pathway stimulates Gcn4 transcriptional activity in response to UV light and nutritional stress by increasing both the translation and the activity of Gcn4 (11). In contrast, PKA inhibits transcription of *ADR1*, *ADH2*, *CTT1*, *SSA3*, *TPS2*, and *UBI4* (4, 9, 14, 37, 57), and it also reduces the transcriptional activity of Adr1 (9).

Our results indicate that PKA inhibits Yap1-dependent transcription both by reducing Yap1 protein levels and by inhibiting Yap1 function. As the reduction in Yap1 protein levels is quantitatively modest (two- to threefold), we suspect that inhibition of Yap1 activity contributes more to the drastic loss of Yap1-dependent transcription in *bcy1* strains. Moreover, the observation that PKA inactivates LexA-Yap1 function on promoters containing Yap, but not LexA, sites suggests that Yap1 transcriptional activity is probably inhibited at the level of promoter occupancy. As is the case for Yap1, PKA probably affects Yap2-dependent transcription both by reducing protein levels and by inhibiting promoter occupancy, although direct evidence for the latter mechanism is lacking; for Yap3, the reduction in protein levels might be sufficient to account for the loss of Yap3-dependent activation. Our results for Yap1 (and perhaps Yap2 and Yap3) are similar to those observed for Adr1, for which PKA reduces both the amount and the activity of the protein (9).

Physiological roles of the Yap proteins. Given that *S. cerevisiae* proteins are typically encoded by single genes or by a small family of related genes, it is striking that the Yap family of bZIP proteins has eight members. It seems unlikely that the Yap proteins are evolutionary vestiges or are derived from pseudogenes; the proteins are of typical size for yeast, and all the features of a functional bZIP domain are present (with the exception that Yap8 has a leucine at the position of the invariant asparagine). Yap1 is a phenotypically important member of the family, because it is the strongest activator when assayed on a simple promoter containing an optimal Yap site. Moreover, *yap1* mutant strains have the most pronounced phenotypes (sensitivity to hydrogen peroxide and low temperature), indicating that the other Yap proteins cannot efficiently substitute for these Yap1 functions. By these criteria, Yap2 is quantitatively less important than Yap1, although under certain conditions (cadmium or AT) Yap1 and Yap2 are comparable in the ability to activate transcription through the Yap site. Yap4 has a distinct role in chromosome stability, but it is transcriptionally inert in the assays we have employed. Yap4 might be a repressor, or it might activate transcription only in combination with other proteins. In this regard, Yap interactions with other proteins might alter DNA-binding specificity and/or transcriptional potential involving other DNA sequences (e.g., STRE [14]). It seems likely that the Yap proteins will differentially respond to a variety of environmental stimuli that have not yet been examined and that such responses might contribute to the viability of the organism over evolutionary time.

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