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A Gene Encoding a Protein with Seven Zinc Finger Domains Acts on the Sexual Differentiation Pathways of *Schizosaccharomyces pombe*

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Byr3 was selected as a multicopy suppressor of the sporulation defects of diploid *Schizosaccharomyces pombe* cells that lack *ras1*. Like cells mutant at *byr1* and *byr2*, two genes that encode putative protein kinases and that in multiple copies are also suppressors of the sporulation defects of *ras1* null diploid cells, cells mutant at *byr3* are viable but defective in conjugation. Nucleic acid sequence indicates *byr3* has the capacity to encode a protein with seven zinc finger binding domains, similar in structure to the cellular nucleic acid binding protein (CNBP), a human protein that was identified on the basis of its ability to bind DNA. Expression of CNBP in yeast can partially suppress conjugation defects of cells lacking *byr3*.

INTRODUCTION

RAS proteins are small guanine nucleotide binding proteins that have been structurally conserved throughout the evolution of eukaryotes, and homologs have been found in two highly divergent species of yeast, *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Fukui and Kaziro, 1985). RAS function is best understood in *S. cerevisiae*, where RAS1 and RAS2 regulate adenylyl cyclase in a guanine nucleotide triphosphate (GTP) dependent manner (Broek *et al.*, 1985; Field *et al.*, 1987). Paradoxically, regulation of adenylyl cyclase does not appear to be the function of RAS proteins in vertebrates (Beckner *et al.*, 1985; Birchmeier *et al.*, 1985) or even in the yeast *S. pombe* (Fukui *et al.*, 1986; Nadin-Davis *et al.*, 1986b). We have begun to study the function of RAS in *S. pombe* in the hope of uncovering a common underlying mechanism of its action.

S. pombe contains a single *ras* homolog, *ras1* (Fukui and Kaziro, 1985; Nadin-Davis *et al.*, 1986b). The biochemical function of ras1 is not known. *ras1* null haploid strains fail to conjugate, and *ras1* null diploid strains fail to sporulate (Fukui *et al.*, 1986). *ras1* null strains are more spherical in shape than the rod-shaped wild-type *S. pombe* strains (Nadin-Davis and Nasim, 1988). Cells expressing the activated mutant $ras1^{val17}$ gene are partially defective in conjugation (Fukui *et al.*, 1986; Nadin-Davis *et al.*, 1986a). When exposed to mating pheromone, they become pear shaped and develop an elongated conjugation tube, but mate poorly. Otherwise, $ras1^{val17}$ cells are normal. These observations indicate that, in *S. pombe*, *ras1* is not an essential gene, but its product participates in the processes of sexual differentiation and the determination of cell shape.

S. pombe is predominantly a haploid organism with two mating types, designated h^+ and h^- (Egel, 1989). Homothallic (h^{90}) haploid strains switch mating type and hence cultures of h^{90} cells can self-mate. When starved, and in the presence of mating pheromone, haploid cells will conjugate and, with high probability, undergo meiosis and sporulation without intervening vegetative growth. Several *ste* (sterile) genes have been defined (Michael and Gutz, 1987). Cells mutant in these genes are frequently defective both in conjugation and sporulation, indicating that the regulation of these distinct processes have many shared components. At least one *ste* gene, *ras1* (or equivalently, *ste5*), comes in two alleles: one causing defective conjugation and one causing defective conjugation and sporulation. This indicates that the requirement for an intact signaling pathway is more stringent for conjugation than for sporulation.

Table 1. Genotype of *S. pombe* strains

Sp870[a]	h^{90} *leu1.32 ade6.210 ura4-D18*
SpBU	h^{90} *leu1.32 ade6.210 ura4-D18 byr1::ura4*
SpBUD	h^{90}/h^{90} *leu1.32/leu1.32 ade6.210/ade6.210 ura4-D18/ura4-D18 byr1::ura4/byr1::ura4*
SpB2U	h^{90} *leu1.32 ade6.210 ura4-D18 byr2::URA4*
SpB2UD	h^{90}/h^{90} *leu1.32/leu1.32 ade6.210/ade6.210 ura4-D18/ura4-D18 byr2::ura4/byr2::ura4*
SpB3U	h^{90} *leu1.32 ade6.210 ura4-D18 byr3::ura4*
SpR2A	h^{90} *leu1.32 ade6.216 ura4::RAS2*^{val17}
SpRU	h^{90} *leu1.32 ade6.210 ura4-D18 ras1::ura4*
SpRUD	h^{90}/h^{90} *leu1.32/leu1.32 ade6.210/ade6.210 ura4-D18/ura4-D18 ras1::ura4/ras1::ura4*
Sp255[a]	h^{-S} *ade6.210*
Sp256[a]	h^{-S} *ade6.216*
Sp258[a]	h^{+N} *ade6.216*
SpRN1	h^{90} *leu1.32 ade6.210 ura4-D18 ras1-DBglII-NheI*

See MATERIALS AND METHODS for more details.

[a] The strains are generous gifts from David Beach.

Several nonessential genes are candidates for encoding components of the ras1 signaling pathway. ste6 encodes a protein that is homologous to the CDC25 protein of *S. cerevisiae* (Hughes *et al.*, 1990). Hence, ste6, like CDC25, is thought to catalyze guanine nucleotide exchange on ras1. *ste6* is required only for conjugation. *sar1* encodes a protein homologous to mammalian GAP, the human *NF1* product and the yeast IRA proteins (Wang *et al.*, 1991a). These proteins accelerate GTP cleavage by RAS proteins (Trahey *et al.*, 1987; Ballester *et al.*, 1990; Tanaka *et al.*, 1990a). Cells lacking *sar1* have a phenotype virtually indistinguishable from cells with activated *ras1*^{val17}. *byr1* and *byr2* were each found as multicopy suppressors of the sporulation defect of *ras1* null diploid cells, and both are predicted to encode serine/threonine kinases (Nadin-Davis and Nasim, 1988; Wang *et al.*, 1991b). Overexpression of the *byr1* and *byr2* genes only poorly suppress the conjugation defects of *ras1*$^-$ haploid cells (see RESULTS). Cells lacking the *byr1* and *byr2* genes are absolutely defective in sporulation and conjugation. *byr1* is equivalent to *ste1*, and *byr2* is probably equivalent to *ste8*.

In the present report we describe the identification and cloning of *byr3*, another gene that is a multicopy suppressor of the sporulation defects of *ras1* null diploids. *byr3* can encode a protein with seven zinc finger binding motifs that are most homologous to the human cellular nucleic acid binding protein (CNBP) (Rajavashisth *et al.*, 1989). *byr3* is not an essential gene, but its product appears required for efficient conjugation.

MATERIALS AND METHODS

Yeast Strains and Microbial Methods

The genotypes of all *S. pombe* strains used in this paper are listed in Table 1. *Escherichia coli* strain DH5α (Hanahan, 1983) was used for plasmid preparation and transformation. Yeast strains were grown in either rich medium (YEA, dextrose/yeast extract/adenine) or synthetic medium with appropriate auxotrophic supplements (PMA) (Nadin-Davis *et al.*, 1986b). The iodine vapor staining was performed as previously described (Nadin-Davis *et al.*, 1986b). *S. pombe* strains were transformed by the lithium acetate procedure (Ito *et al.*, 1983). Diploidization of haploid strains was accomplished by treating haploid cells with the lithium acetate procedure (Ito *et al.*, 1983) and then screening for diploid cells with phloxin B (Gutz *et al.*, 1974).

Nucleic Acid Manipulation and Analysis of byr3

A *S. pombe* genomic library comprised of Sau3A partial digested DNA fragments cloned into pWH5, a high copy shuttle vector expressing the *S. cerevisiae LEU2* gene, was obtained from David Beach. The average size of the insert DNA was 10 kbp. The pWH5 vector contained the bacterial marker for ampicillin resistance for selection in *E. coli* (Wright *et al.*, 1986). A plasmid containing the 12-kbp genomic DNA from the *byr3* locus (pWH5BYR3) was isolated from the extrachromosomal DNA of the transformed strain of SpRUD as described in the text by method of Nasmyth and Reed, 1980. This DNA was transformed into *E. coli* (Holmes and Quigley, 1981), and plasmid DNA was obtained from individual *E. coli* (Katz *et al.*, 1973). Nucleotide sequencing was performed by the dideoxynucleotide chain-termination method with oligo-nucleotide primers (Sanger *et al.*, 1977; Biggin *et al.*, 1983). Serial complementary oligomers for each strand of the gene were synthesized progressively by ABI 380A & 380B DNA synthesizers to serve as primers for complete double stranded sequencing.

Plasmid Constructions

The *S. pombe* plasmids used are listed in Table 2. pWH5 is a shuttle vector for *E. coli*, *S. cerevisiae* and *S. pombe* (Wright *et al.*, 1986). Plasmid

Table 2. Plasmids used in transforming *S. pombe*

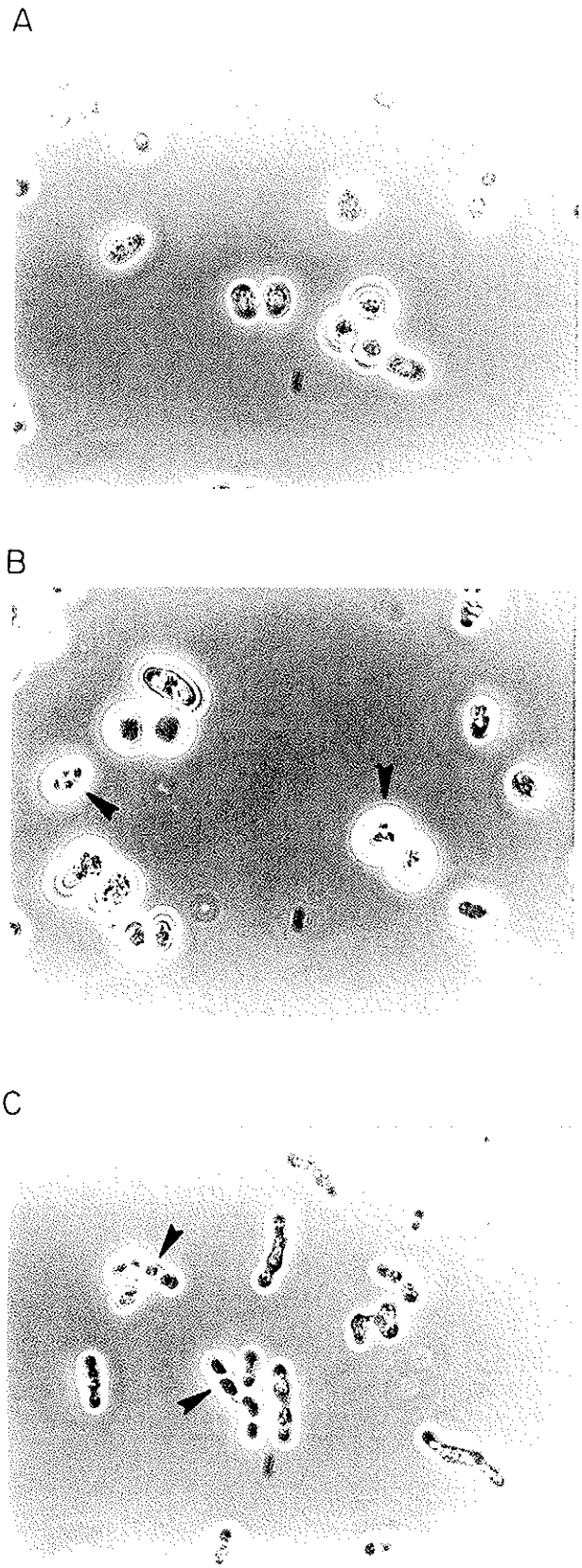
pAL	Derived from pUC118, with ARS[a] and *LEU2*[b] marker
pIRT5	Derived from pUC118, with ARS and *ura4*[c] marker
pART1	Derived from pUC118, with ARS, *LEU2* marker and *adh*[d] promoter
pAAUN	Derived from pUC118, with ARS, *ura4*, *adh* promoter and NotI site in polylinker
pAALN	Same as pAAUN except *ura4* is replaced with *LEU2*
pAAUNL	Derived from pAAUN, encoding peptide epitope from the HA antigen of influenza virus
pAAUNLBYR3	*S. pombe byr3* gene in pAAUNL
pAALNLBYR3	*S. pombe byr3* gene in pAALNL
pWH5	A shuttle vector for *E. coli*, *S. cerevisiae*, and *S. pombe* containing the *LEU2* marker
pALR	*S. pombe ras1* in pAL vector
pALRV	*S. pombe ras1*^{val17} in pAL vector
pARTBYR1	*S. pombe byr1* gene in pART1 vector
pWH5BYR2	*S. pombe byr2* gene in pWH5 vector
pWHBYR3	*S. pombe byr3* in pWH5
pALBYR3	*S. pombe byr3* in pAL
pARTCNBP	Human *CNBP* in pART1.
pRAS::ura4	*S. pombe ras1* disrupted with *S. pombe ura4* gene
pALBYR3::ura4	*S. pombe byr3* disrupted with *ura4*

[a] *S. pombe* autonomously replicating sequences.

[b] *S. cerevisiae LEU2* marker.

[c] *S. pombe ura4* marker.

[d] *S. pombe* alcohol dehydrogenase promoter.



pIRT5 is a vector containing a 1.2-kbp *S. pombe* ARS fragment (Losson and Lacroute, 1983) at the *Eco*RI site and a 1.8-kbp *S. pombe ura4* fragment at the *Hin*dIII site of pUC118. The plasmid pAL is a pUC118 based vector containing the 2.2-kbp *Hin*dIII fragment of the *S. cerevisiae LEU2* gene cloned into the *Hin*dIII site, and a 1.2-kbp *S. pombe* ARS fragment cloned at the *Eco*RI site. The plasmid pART1 has, in addition, a 0.7-kbp *S. pombe adh* promoter fragment inserted between the *Sph* I and *Pst* I sites. Plasmid pAAUN was derived from plasmid pART1 by first replacing the *S. cerevisiae LEU2* gene with a 1.8-kbp *Hin*dIII *ura4* fragment from *S. pombe* and then adding *Not* I linkers at the *Sma*II site. pAALN is the same as pAAUN except the *ura4* gene was replaced by *S. cerevisiae LEU2* gene. Plasmid pAALNL and pAAUNL was derived from pAAUN and pAALN by inserting an oligonucleotide encoding an influenza hemagglutinin antigen epitope peptide (Field *et al.*, 1988) downstream of the *adh* promoter. This oligonucleotide contained a *Sal* I site for cloning coding sequences downstream and in frame with the peptide epitope.

Plasmid pARTBYR1 contains the *S. pombe byr1* gene, which was obtained from *S. pombe* genomic DNA by the polymerase chain reaction (PCR) (Wang *et al.*, 1991b), cloned into the *Sma* I site of pART1. Plasmid pALR was constructed by inserting a 1.4-kbp *Bam*HI-*Bgl*II *S. pombe* wild-type *ras1* gene fragment (Nadin-Davis *et al.*, 1986b) into the *Sma* I site of pAL. Plasmid pALRV is the same as pALR except that the activated mutant $ras1^{val17}$ gene was inserted (Nadin-Davis *et al.*, 1986a). Plasmid pWH5BYR2 contains the *S. pombe* genomic *byr2* gene in the pWH5 vector (Wang *et al.*, 1991b). pWHBYR3 (Figure 2) has a 12-kbp *S. pombe* genomic DNA sequence containing *byr3* gene in the pWH5 vector. pALBYR3 was constructed by subcloning a 6.0-kbp *Bam*HI fragment containing the *byr3* gene from pWHBYR3 into the *Sma* I site of the pAL vector. Plasmid pAALNLBYR3 and pAAUNLBYR3 were constructed by inserting the coding sequences of *byr3* into the *Sal* I site of pAALNL and pAAUNL. These coding sequences were obtained with PCR and the following primers:

5′-GTAAAGGAGTGTCGACGATGGAGTCT-3′

5′-AAGTGTGCGAGTCGACAAGGACTGAAG-3′,

with plasmid pALBYR3 as template. The primers contain internal *Sal* I restriction enzyme cleavage sites to facilitate cloning into pAAUNL. As the first step in the construction of expression plasmid pARTCNBP, an *Eco*RI fragment corresponding to full-length human CNBP cDNA was subcloned into the *Eco*RI site of Bluescript (Stratagene, La Jolla, CA). A 1.4-kbp long fragment containing the complete coding region of human CNBP was then excised with *Sma* I and *Dra* I and inserted into the *Sma* I site of pART1. The resulting plasmid pARTCNBP contains the inserted human sequences under the control of the strong *S. pombe adh* promoter.

Plasmid pRAS, obtained from David Beach, was derived by cloning a 2.5-kbp *S. pombe* genomic DNA fragment containing the *ras1* gene into the *Bam*HI site of pUC118 (Nadin-Davis *et al.*, 1986b). pRAS::ura4 contains the *ras1* gene inactivated by replacing a part of the gene with the *S. pombe ura4* gene. The plasmid pRAS was digested with *Nhe* I and *Bgl*II, blunt-ended, and a 1.8-kbp blunt-ended fragment of the *ura4* gene was inserted. The construction, pALBYR3::ura4, was made by replacing the *Bal* I fragment containing part of the *byr3* gene in pALBYR3 with a 1.8 kbp of the *S. pombe ura4* gene (Figure 2).

A plasmid, pIH-byr3, which expresses byr3 fusion protein was constructed as follows. The polymerase chain reaction was used to

Figure 1. Sporulation induced by the *S. pombe byr3* gene in *S. pombe ras1* null diploid strain SpRUD. The strain SpRUD was transformed with (A), pAL, a *S. pombe* expression vector, (B), pWH5BYR3, a plasmid containing the *S. pombe byr3* gene, or (C), pALR, a plasmid containing *S. pombe ras1* gene. The arrow heads indicate asci, abundant in B and C. Only asexual asci are observed in B because pWHBYR3 suppresses the sporulation defects of the *ras1* null diploid cells but not the conjugation defects of *ras1* null haploid cells.

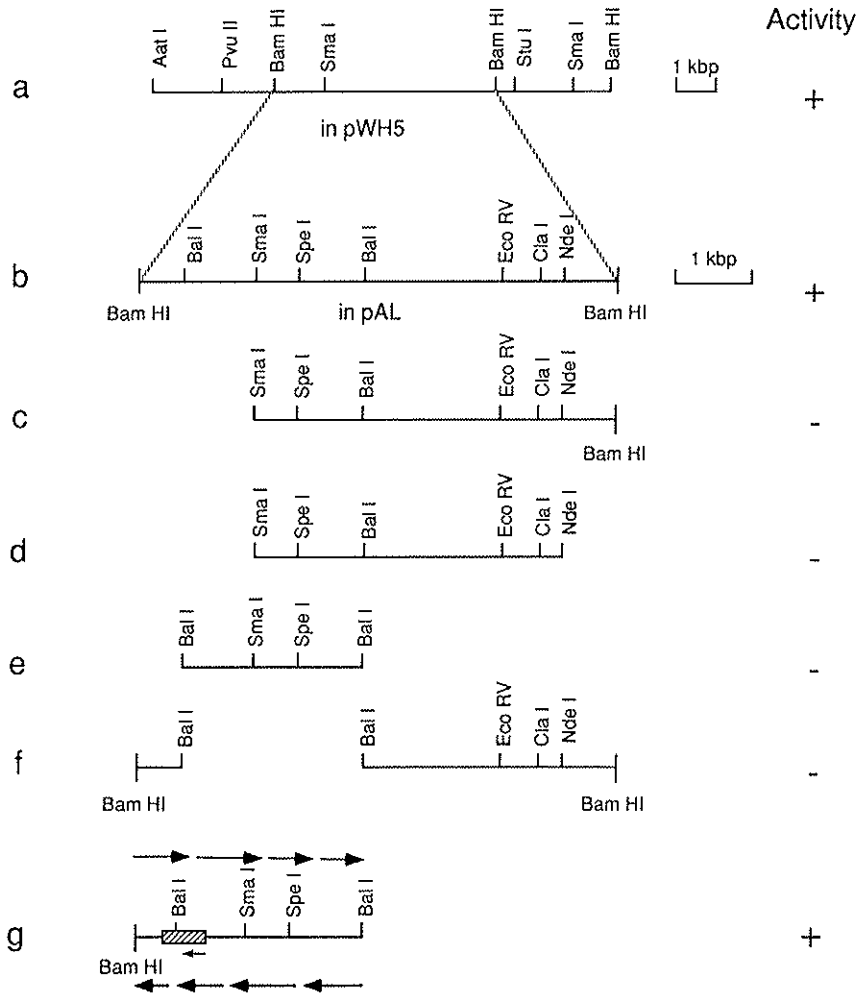


Figure 2. A restriction endonuclease map of the *S. pombe byr3* gene and its deletion/activity analysis. The restriction map of the *S. pombe* genomic locus cloned in pWH5 (pWH5BYR3) is shown in a, and the region cloned in pAL (pALBYR3) is shown in b. The inserts of various plasmids derived by deleting regions of *byr3* locus from pALBYR3 are shown in c through g. The ability of these plasmids to confer sporulation competence to ras1 null diploid cells is indicated on the right. The region of the *byr3* locus indicated in g was sequenced on both strands, as indicated by the arrows, the small arrow indicates the gene translation direction. Part of this sequence is presented in the next Figure. The hatched region in g indicates coding sequences.

generate *Sal* I restriction sites flanking the open reading frame of byr3 with the use of the following primers:

byr3#1 5′-GTAAAGGAGTGTCGACGATGGAGTCT-3′

byr3#2 5′-AAGTGTGCGAGTCGACAAGGACGAAG-3′.

The putative initiating codon is indicated in bold type and the *Sal* I sites are underlined. The PCR product was digested with *Sal* I and cloned into an identical site in vector pGEM4 (Promega, Madison, WI) to generate plasmid pGEM-byr3. The vector pMAL-cRI is designed to express fusion proteins containing the maltose-binding protein (MBP) at the amino terminal, facilitating rapid and simple purification by amylose affinity chromatography (New England Biolabs, Beverly, MA). Byr3 was cloned downstream of the MBP by the following strategy: The vector pMAL-cRI was digested with *Hin*dIII and the 5′ overhang partially filled in by Klenow polymerase in the presence of dATP, dCTP, and dGTP. The *byr3* fragment was excised from pGEM-byr3 by *Sal* I digestion and the 5′ overhang partially filled in by Klenow polymerase in the presence of dATP, dCTP, and dGTP. The resulting compatible ends were ligated to generate plasmid pMAL-byr3 encoding an in-frame fusion of MBP with byr3. Vector pIH-902 is identical to pMAL-cRI except that it encodes a spacer of 10 asparagines separating the MBP from the inserted protein. Byr3 was introduced into this vector by exchanging an EcoRV/XbaI fragment of pMAL-byr3, with an EcoRV/XbaI fragment of pIH-902 to create plasmid pIH-byr3. Plasmid pIH-byr3 was transformed into bacterial host BL21(DE3) for protein expression. The fusion protein expressed from pIH-byr3 bound more strongly to amylose resin than the protein expressed from pMAL-cRI and was used for all further studies.

Gene Disruptions in S. pombe

The *ras1* gene was disrupted in Sp870 to generate the haploid strain SpRU (h^{90} *leu1.32 ade6.210 ura4-D18 ras1::ura4*). The *ura4*-disrupted *ras1* gene fragment was released from the pRAS::*ura4* plasmid by digestion with *Bam*HI, purified by agarose gel electrophoresis, and then transformed into the Sp870 wild-type strain. Individual *ura4*$^+$ transformants were grown, and Southern blots confirmed that the proper disruption of the single-copy endogenous *ras1* gene was obtained. The *ras1* null diploid strain, SpRUD, was created from SpRU haploid cells by treatment with the lithium acetate procedure (Ito *et al.*, 1983). The SpRN21 strain was created by deleting the *Nhe* I-*Bgl*II fragment from the *ras1* gene in the h^{90} haploid wild-type strain Sp870. The *Nhe* I-*Bgl*II fragment of the *ras1* gene includes the C-terminal 20 codons and ~300 bps of 3′ flanking sequences. The strain SpRN1 is sterile because the *ras1* gene has been inactivated. We named the *ras1* allele of this strain *ras1-DBgl*II-*Nhe* I.

The *byr3* gene was disrupted in Sp870 to obtain the haploid strain SpB3U (h^{90} *leu1.32 ade6.210 ura4-D18 byr3::ura4*). A disrupted *byr3* gene fragment was separated from pALBYR3::ura4 by *Bam*HI diges-

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-45                    TAA TTT TCT TTA ATA ATT TTT TTT AAT TGT AAA GGA GTT GTT AAG

  1  ATG GAG TCT GAA TCT GTT CCC ACC GTT CCT CAA ACC ACT CGT CCC GGT CCT CGA TGC TAT
  1  MET Glu Ser Glu Ser Val Pro Thr Val Pro Gln Thr Thr Arg Pro Gly Pro Arg Cys Tyr

 61  AAC TGT GGT GAA AAC GGT CAT CAA GCT CGT GAG TGC ACC AAG GGT TCA ATC TGC TAC AAT
 21  Asn Cys Gly Glu Asn Gly His Gln Ala Arg Glu Cys Thr Lys Gly Ser Ile Cys Tyr Asn

121  TGC AAT CAG ACC GGT CAC AAG GCT AGC GAA TGC ACT GAG CCT CAA CAG GAA AAA ACT TGC
 41  Cys Asn Gln Thr Gly His Lys Ala Ser Glu Cys Thr Glu Pro Gln Gln Glu Lys Thr Cys

181  TAT GCT TGT GGT ACC GCC GGA CAT CTC GTT CGT GAT TGC CCC AGC AGC CCT AAC CCC CGT
 61  Tyr Ala Cys Gly Thr Ala Gly His Leu Val Arg Asp Cys Pro Ser Ser Pro Asn Pro Arg

241  CAA GGT GCG GAA TGT TAC AAG TGT GGT CGT GTT GGT CAC ATT GCT AGA GAC TGT CGT ACA
 81  Gln Gly Ala Glu Cys Tyr Lys Cys Gly Arg Val Gly His Ile Ala Arg Asp Cys Arg Thr

301  AAT GGT CAA CAA AGT GGC GGA CGA TTT GGT GGT CAT CGC TCC AAC ATG AAT TGC TAT GCT
101  Asn Gly Gln Gln Ser Gly Gly Arg Phe Gly Gly His Arg Ser Asn MET Asn Cys Tyr Ala

361  TGT GGC TCT TAT GGC CAT CAA GCC CGT GAT TGC ACT ATG GGC GTG AAA TGC TAC TCT TGT
121  Cys Gly Ser Tyr Gly His Gln Ala Arg Asp Cys Thr MET Gly Val Lys Cys Tyr Ser Cys

421  GGT AAG ATT GGA CAC CGC AGC TTT GAA TGT CAA CAA GCT TCA GAT GGT CAA CTT TGT TAC
141  Gly Lys Ile Gly His Arg Ser Phe Glu Cys Gln Gln Ala Ser Asp Gly Gln Leu Cys Tyr

481  AAG TGT AAT CAA CCA GGC CAC ATC GCC GTC AAT TGC ACC TCT CCT GTA ATT GAG GCA TAA
161  Lys Cys Asn Gln Pro Gly His Ile Ala Val Asn Cys Thr Ser Pro Val Ile Glu Ala  .

541  GTG CTT GCT ATT TAT AAC CCT TTG TCG TGG TAT GAA GAT ATT TTT TCT TTT TCT TGT CCG

601  ATT TAT TCA AAT TTG GCA AGT GGT CAT ATT CTT ACA AAT CCC TTG AGC GAT
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Figure 3. The sequence of the *S. pombe byr3* coding region and the predicted 179 amino acid product. The in-frame stop codons located either directly upstream or downstream of the open-reading frame are indicated in bold. The seven metal-binding motifs are underlined, and the Cys and His residues of this motif are indicated in bold. The numbers in the left margin represent nucleotide and amino acid coordinates.

tion, purified by agarose gel electrophoresis and transformed into the Sp870 wild-type strain. Transformed strains in which the *byr3* gene had been disrupted properly were identified by Southern blots.

Induction and Expression of MBP-byr3 Fusion Protein

A 500-ml culture of *E. coli* cells containing plasmid pIH-byr3 was grown at 37°C in LB medium (1% bacto-tryptone; 0.5% yeast extract; 1% NaCl) containing 0.2% glucose and 100 μg/ml of ampicillin to an OD_{600} of 0.4. The P_{tac} promoter was derepressed by adjusting the media to 0.3 mM isopropyl-β-D-thiogalactopyranoside (IPTG) followed by a 3-h induction. Cells were pelleted, resuspended, and frozen in 25 ml of lysis buffer (10 mM phosphate buffer, pH 7.0; 30 mM NaCl; 0.25% Tween-20; 10 mM β-mercaptoethanol [βME]; 1 mM phenylmethylsulfonyl fluoride [PMSF]; 10 μM $ZnSO_4$). Zinc was included in all buffers on the assumption that it would stabilize the seven zinc finger motifs of byr3. All work, unless otherwise indicated, was carried out at 4°C. The cells were lysed by thawing and sonicating. Cellular debris was removed by centrifugation at 10 000 rpm for 30 min in a Sorvall HB-4 rotor. The fusion protein was purified from the supernatant with a one-step batch purification procedure: 2.5 ml of amylose resin, previously equilibrated in column buffer (10 mM phosphate buffer, pH 7.0; 0.5 M NaCl; 10 mM βME; 10 μM $ZnSO_4$) was added to the supernatant and incubated on ice for 30 min. The resin was pelleted by low speed centrifugation (500 rpm in an IEC DPR-6000 centrifuge) and washed twice with 10 ml column buffer containing 0.25% Tween-20 and 10 mM βME, twice with 10 ml column buffer containing 10 mM βME, twice with 10 ml DNA-binding buffer (50 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES], pH 7.5; 50 mM KCl; 5 mM $MgCl_2$; 10 mM βME; 10 μM $ZnSO_4$; 20% glycerol), once in 10 ml DNA-binding buffer containing 50% glycerol, and finally resuspended in the same buffer to twice the resin volume and stored at −20°C.

Binding of ^{32}P-DNA to MBP-byr3

S. pombe DNA, sonicated to an average size of 200–300 bps, was labeled by ^{32}P-dCTP with random priming to a specific activity of ~10^8 cpm/μg. Conditions used for DNA binding and washing were previously described by Kinzler and Vogelstein (1989). MBP-byr3/amylose resin suspension (20 μl) was washed once in 1 ml of DNA binding-buffer. The pellet was resuspended in 20 μl of the same buffer containing ~80 000 cpm of ^{32}P-labeled DNA in the presence, or absence, of cold competitor double-stranded DNA (either poly dI-dC [Pharmacia, Piscataway, NJ] or sonicated salmon sperm DNA). The

17	PRCYNCGENGHQARECT	byr3
4	NECFKCGRSGHWARECP	CNBP
36	SICYNCNQTGHLASECT	byr3
52	DICYRCGESGHLAKDCD	CNBP
58	KTCYACGTAGHLVRDCP	byr3
72	DACYNCGRGGHIAKDCK	CNBP
83	AECYKCGRVGHIARDCR	byr3
96	QCCYNCGKPGHLARDCD	CNBP
116	MNCYACGSYGHQARDCT	byr3
117	QKCYSCGEFGHIQKDCT	CNBP
135	VKCYSCGKIGHRSFECQ	byr3
135	VKCYRCGETGHVAINCS	CNBP
157	QLCYKCNQPGHIAVNCT	byr3
156	VNCYRCGESGHLARECT	CNBP

Consensus	
byr3	XXCYXCGXXGHXAXXCX
CNBP	XXCYXCGXXGHXAXXCX
NBPs	XXCXXCGXXGHXXXXCX

Figure 4. Alignment of the zinc finger repeats of the *S. pombe* byr3 and of the human CNBP protein sequences (Rajavashisth *et al.*, 1989). The amino acid positions of the repeats are indicated on the left. The consensus sequences (bottom) show the characteristic features of a typical repeat unit and is matched with the consensus sequence of zinc finger repeats in retroviral NBPs (Covey, 1986). Considerable conservation is also observed between the invariant residues of the motifs.

mixture was incubated for 30 min at 4°C in a rotating shaker. The resin was pelleted at 3000 rpm for 30 s in an Eppendorf microfuge, the supernatant removed, and the pellet washed 4× with wash buffer (50 mM HEPES, pH 7.5; 150 mM KCl; 5 mM $MgCl_2$; 10 μM $ZnSO_4$; 1% Triton X-100; 0.05% sodium dodecyl sulfate [SDS]). The ^{32}P-labeled DNA adhering to the washed pellet was quantitated by Cerenkov counting. SDS-polyacrylamide gel electrophoresis (PAGE) analysis confirmed that MBP-byr3 or MBP remained stably bound to amylose resin under these DNA binding and washing conditions.

Mating Assays

The *ras1* null h^{90} strain, SpRN1, was transformed with multicopy plasmids expressing the *ras1*, *byr1*, *byr2*, or *byr3* genes to test their ability to overcome the mating defective phenotype of SpRN1. Cultures of transformed SpRN1 were mated with Sp256, a h^- strain, or with Sp258, a h^+ strain. The different mating type cells were cultured separately for 2 d in PMA (minimum medium with adenine), then 5 × 10^7 cells from each mating type cells were mixed and cultured for another 4 h in the same medium. The mixture was spread on PMA plates for 24 h and then replicated onto PM plates (same as PMA except without adenine) for 5 d to select diploid cells by adenine prototrophy. The diploid colonies were verified by another replica plating onto PM plates and further confirmed by microscopic examination.

Immunoprecipitation of ^{35}S-met Labeled Fusion byr3 Protein

The *ras1* null h^{90} strain (SpRU), the *byr1* null h^{90} strain (SpBU), the *byr2* null h^{90} strain (SpB2U), and the wild-type strain (Sp870) were transformed with pAALNLBYR3 to express a byr3 protein, "pbyr3," that is fused with an influenza hemagglutinin antigen epitope peptide and to test the possibility that the level of expression of byr3 protein or its apparent mobility on SDS-PAGE was related to the expression of the *ras1*, *byr1*, or *byr2* genes. Cells were grown in PMA medium to a concentration of 5 × 10^7/ml at 30°C. 5 × 10^8 cells were used per immunoprecipitate. The cells were labeled in PMA medium with 0.2 mCi ^{35}S-met for 4 h at 30°C and washed with IP (immunoprecipitation) buffer #1 (50 mM NaF, 5 mM EDTA, 1 mM dithiothreitol [DTT], and 0.1 mM phenylmethylsulfonyl fluoride [PMSF], 0.1 mM aprotinin, 0.1 mM leupeptin, and 0.1 mM pepstatin in PBS) at 4°C. The cells were broken by glass beads in 0.3 ml IP buffer #1 at 4°C, then 1 ml ice cold IP buffer #2 (0.1% Triton X-100, 0.1% deoxycholate in IP buffer #1) was added. The supernatant of the cell lysate was precleared with 40 μl of 1:1 protein A-agarose suspension (Boehringer Mannheim Biochemicals, Indianapolis, IN) for 30 min on a rotator at 4°C, and the supernatant was collected by spinning at 12 000 × *g* in a microcentrifuge. The first antibody, mouse monoclonal antibody to influenza hemagglutinin antigen epitope peptide (Field *et al.*, 1988), was added to the supernatant and incubated for 1 h on a rotator at 4°C. The reaction sat on wet ice for another hour, and the supernatant was collected by spinning (as mentioned above) to remove debris. Protein A-agarose suspension (40 μl) was added to the supernatant and rotated for 20 min at 4°C. The precipitate was collected by spinning (as mentioned above). The protein A-agarose pellets were washed three times with ice cold IP buffer #1. For electrophoresis, 35 μl of sample buffer was added to the pellets and heated to 100°C for 2 min. The samples were loaded on a 10–20% acrylamide gradient gel and run at 25 μA.

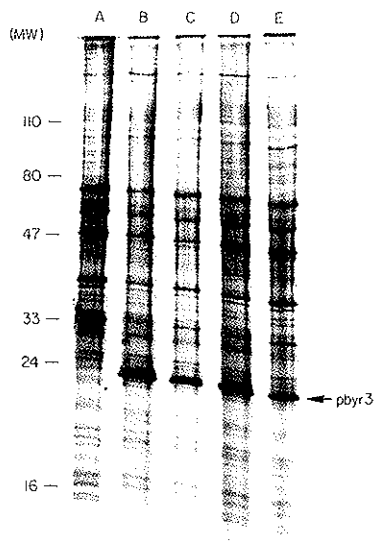


Figure 5. Immunoprecipitation of byr3 fusion protein. Cells with various genetic backgrounds were labeled with ^{35}S-methionine. Extracts were prepared from: (lane A), Sp870, a wild-type strain, transformed with pAL, a *S. pombe* expression vector; (lane B), Sp870, transformed with pAALNLBYR3; (lane C), SpBU, a *byr1* null strain, transformed with pAALNLBYR3; (lane D), SpB2U, a *byr2* null strain, transformed with pAALNLBYR3; (lane E), SpRU, a *ras1* null strain, transformed with pAALNLBYR3. The proteins were run on a 10–20% acrylamide gradient gel. The arrows point to the expressed byr3 fusion protein ("pbyr3"). The molecular weight standards in daltons (Bio-Rad Laboratories, Richmond, CA) are marked on the left.

Table 3. Binding of ^{32}P-labeled DNA to MBP-byr3 fusion protein

	poly dI-dC		Salmon sperm DNA			
Competitor	0 μg	10 μg	0 μg	10 μg	20 μg	40 μg
Protein						
MBP	786	303	nd	nd	nd	nd
MBP-byr3	22 169	20 900	18 310	2 764	1 271	696

8×10^4 cpm of ^{32}P-labeled, sonicated *S. pombe* DNA was incubated with either maltose-binding protein (MBP), as a negative control, or the byr3 fusion protein (MBP-byr3). Both MBP and MBP-byr3 were expressed in *E. coli* and were used complexed with amylose resin. Values are the cpm of labeled DNA adhering to the amylose resin/MBP complex. Either poly dI-dC or salmon sperm DNA was used as a competitor during incubation in the amounts indicated. See MATERIALS AND METHODS for more details. nd, not done.

Immunoprecipitation of ^{32}P-Labeled Fusion byr3 Protein

The wild-type strain, Sp870, was transformed with pAL or pAALNLBYR3, respectively. The *byr1* null strain, the *byr2* null strain, and the *ras1* null strain were transformed with pAALNLBYR3, respectively. All the transformed strains mentioned above were grown in the minimum medium, PMA, to a concentration of 5×10^7 cells/ml. Sixty milliliters of each cell culture were spun down at 2000 rpm and were washed with nitrogen and phosphate-free PMA medium once, and then resuspended in 10 ml of nitrogen and phosphate-free PMA medium with 0.2 mCi of phosphate-^{32}P (9000 Ci/mmol, NEN Du Pont, Wilmington, DE). The cells were harvested, after growing 6 h, by spinning at 2000 rpm. The treatment of the cells and the following steps for examining the phosphorylated *byr3* gene product were the same as those used for immunoprecipitation of ^{35}S-labeled fusion byr3 protein except the IP buffer #1 contains 2 mM of sodium orthovanadate.

RESULTS

Isolating byr3

The *byr1* and *byr2* genes, each capable of bypassing the requirement for *ras1* during sporulation, are believed to act downstream of *ras1*. (Nadin-Davis *et al.*, 1990; Wang *et al.*, 1991b). We sought to discover additional genes of *S. pombe* that, when contained on a multicopy plasmid, could induce sporulation in a *ras1* null diploid cell. We designed our screen to exclude genes that can also suppress the loss of *byr1*. The diploid SpRUD (h^{90}/h^{90} *leu1.32/leu1.32 ade6.210/ade6.210 ura4-D18/ura4D-18 ras1::ura4/ras1::ura4*) fails to form spores, and hence colonies of SpRUD do not stain black when exposed to iodine vapor. SpRUD was transformed with an *S. pombe* genomic library made from the *LEU2* vector pWH5. Leu$^+$ transformants (5×10^4) were selected and stained with iodine vapor to detect colonies capable of forming spores. Seven of eleven positively staining transformants showed a plasmid-dependent phenotype, and plasmids were rescued from these into *E. coli*. These plasmids were next tested for the ability to induce conjugation in the *byr1* null haploid strain SpBU (see Table 1). Plasmids that could not induce conjugation in SpBU were further characterized. These plasmids fell into three classes. One class contained *ras1;* a second class contained *mei3;* and a third class defined a new genetic

Table 4. Conjugation efficiency of a *byr3* null strain

SpB3U	Sp870
85/701	485/679
55/670	394/605
67/601	323/665
62/678	403/655
51/630	460/650
9.8[a]	80.2[a]

Five independent clones of SpB3U, a *byr3* null strain, and five of the wild-type strain, Sp870, from which it derived, were starved on PMA plates for 4 d. The number of conjugated cells to the total number of cells counted, as determined microscopically, is presented for each clone.

[a] Average, as a percentage of total, is as indicated.

Table 5. Conjugation efficiency of a *byr3* null strain transformed with the human CNBP gene

Plasmid	pAL	pARTCNBP	pALBYR3
	67/584	202/645	282/589
	72/607	129/602	327/603
	82/608	151/583	302/639
	71/649	146/614	329/621
	80/599	131/610	176/566
	12.2[a]	24.9[a]	46.5[a]

The *byr3* null strain, SpBU3, was transformed with the indicated plasmids, and five independent Leu$^+$ transformants were picked from each transformation. The number of conjugating cells/total number of cells counted is presented after each transformant was cultivated for 4 d on PMA plates. Plasmids were pAL, a control vector; pARTCNBP, expressing the human *CNBP* gene from an adh1 promoter; and pALBYR3, a vector expressing *byr3* from an adh1 promoter.

[a] Average number of conjugated cells expressed as a percentage.

Table 6. Mating suppression assay of *byr* genes in the *ras1* null strain SpRN1

Gene on multicopy plasmid	Mating partner	Number of diploid colonies	Mating partner	Number of diploid colonies
none	Sp256	0	Sp258	0
byr1	Sp256	74	Sp258	51
byr2	Sp256	10	Sp258	6
byr3	Sp256	0	Sp258	0
ras1	Sp256	$\sim 2 \times 10^3$	Sp258	$\sim 2 \times 10^3$

The strain SpRN1 was transformed with either the control plasmid pAL (none), pARTBYR1 (for *byr1*), pWH5BYR2 (for *byr2*), pALBYR3 (for *byr3*), or pALR (for *ras1*), and tested for mating with Sp256 (h^-) or Sp258 (h^+) by cocultivation and selection for adenine prototrophs. The number of verified diploid colonies is presented. See MATERIALS AND METHODS and text for more details.

locus we call *byr3* (see Figure 1). The previously identified gene, *byr2*, was not found in this genomic screen, and hence the screen was not exhaustive. *mei3* is known to encode a product that down regulates the *ran1/pat1* gene product (McLeod *et al.*, 1987) and thereby induces sporulation. *mei3* induces sporulation even in the haploid SpBU strain, and therefore acts downstream of *byr1*. We do not discuss this gene further here.

Nucleic Acid Sequence and Predicted Product of byr3

The limits of *byr3* were determined by analysis of subclones (see Figure 2). The smallest restriction endonuclease fragment, capable of encoding a suppressor of the sporulation defects of *ras1* null diploid cells, was sequenced. Nucleotide sequence of this fragment revealed the long open-reading frame shown in Figure 3. The open-reading frame of 179 codons is initiated by an ATG codon, which is preceded by a stop codon 42 nucleotides upstream in the same frame, and an in-frame stop codon 537 nucleotides downstream from this ATG. The predicted amino acid sequence of the byr3 product is also shown in Figure 3. Searches of GenBank and EMBL data banks, with the FASTDB program, revealed that two regions of the *byr3* product, amino acids 30–90 and 100–150, were 43 and 38% identical to the gag polyprotein of Caprine Arthritis Encephalitis Virus CG-1 (CEAVCG-1) (Clements, unpublished data) and human immunodeficiency virus type 2 ST-1 (HIV2ST-1) (Kumar *et al.*, 1990), respectively. These regions were located in nucleic acid binding protein (NBP) region in the gag polyproteins. The NBP region has the metal binding motif: $CX_2CX_4HX_4C$, where C is cysteine, H is histidine, and X is any amino acid (Berg, 1986). This motif was first discovered in low-molecular-weight proteins, encoded by retroviruses, which bind to single stranded DNA or RNA (Covey, 1986). The same metal-binding motif appears seven times in a cellular nucleic acid binding protein (CNBP) (Rajavashisth *et al.*, 1989). CNBP binds a sterol regulatory element (SRE) in both a sequence-specific and a single-strand-specific manner (Rajavashisth *et al.*, 1989). In the byr3 protein, there are also seven such metal-binding domains. The homologous regions between the byr3, CNBP, and NBP proteins are located in the metal-binding motif (Figure 4).

To confirm that the predicted open reading frame belonged to *byr3* and was sufficient to encode a functional protein, we constructed plasmids pAAUNLBYR3 and pAALNLBYR3 that joined this sequence in frame to a sequence encoding a peptide epitope from the HA antigen of the influenza virus, driven from the strong *S. pombe adh* promoter (see MATERIALS AND METHODS and Table 2). Both plasmids were capable of inducing sporulation in *ras1* null diploid cells. Cells containing these vectors produce a protein of the correct molecular size that immunoprecipitates with antibodies to the peptide epitope (see Figure 5). A protein of the same molecular weight is observed on Western blotting.

DNA Binding Assay of pBYR3 Fusion Protein

Human CNBP is reported to be a nucleic acid binding protein. To test if byr3 protein has the same property, we engineered an *E. coli* system expressing byr3 as a fusion to a maltose binding protein (MBP). MBP-byr3 and MBP were expressed from plasmids pIH-byr3, and pIH-902, respectively, and were used as a complex with amylose resin for these studies. MBP-byr3 protein was

Table 7. Effects of expression of various genes on the conjugation of a *byr3* null strain

Gene	None	*ras1*	*byr1*	*byr2*	*byr3*
Transformants					
#1	40/602	163/523	49/600	184/556	171/517
#2	28/437	216/537	4/618	298/640	176/563
#3	73/523	303/572	61/550	221/551	178/592
#4	61/589	227/580	11/588	221/551	159/554
#5	52/589	249/611	26/558	219/597	138/538
	9.3[a]	41.0[a]	5.2[a]	39.5[a]	29.7[a]

Vectors expressing various genes were used to transform the *byr3* null strain SpB3U: pAL (none) is a *S. pombe* expression vector; pALR carries the *S. pombe ras1* gene in the pAL vector; pALBYR1 carries the *S. pombe byr1* gene in the pAL vector; pALBYR2 carries the *S. pombe byr2* gene in the pAL vector; pAALNLBYR3 carries the *S. pombe byr3* gene in the pAALNL vector, expressed as a fusion protein with an influenza hemagglutinin antigen epitope, downstream of the *adh1* promoter. Independent transformants were picked and maintained on PMA plates for 3 d. The ratio of the conjugated cells to the total cells counted is presented.

[a] Average is expressed as a percentage.

stably maintained as a complex with the amylose resin. Analysis by SDS-PAGE indicates that the protein bound to the resin corresponded to the predicted molecular weight of 66 000 Da for the MBP-byr3 fusion protein. Approximately 10 μg of either MBP, expressed from plasmid pIH-902, or 15 μg of the MBP-byr3 fusion protein, expressed from pIH-byr3 was bound to 50 μl of the resin suspension. This was determined by comparison of Coumassie-Blue staining with known concentrations of bovine serum albumin after separation by SDS-PAGE. *S. pombe* DNA was sonicated to an average size of 200–300 bps, labeled with ^{32}P-dCTP by random priming, and incubated with MBP or MBP-byr3. Approximately 80 000 cpm of ^{32}P-labeled DNA was used in each reaction in the presence or absence of double stranded competitor DNA. The amount of radiolabeled material associated with the proteins was then determined. The results (Table 3) indicate that the byr3 fusion protein is capable of binding to the *S. pombe* double stranded DNA.

Phenotype of byr3 Null Cells

The cloned *byr3* gene carried on the plasmid pAL was disrupted with the *ura4* gene as described in MATERIALS AND METHODS, thus creating plasmid pALBYR3::ura4 in which the promoter and first 125 codons (out of 179) of *byr3* are deleted. The haploid *ura4*$^-$ strain Sp870 was then transformed with the *Bam*HI restriction endonuclease fragment containing the disrupted gene flanking the *ura4* marker. Many Ura4$^+$ colonies were obtained and blotted with the *byr3* gene as probe. Most of the Ura4$^+$ colonies contained *ura4* insertions at the *byr3* locus, suggesting that *byr3* disrupted cells are viable. This was subsequently confirmed by tetrad analysis of diploids disrupted at a single *byr3* locus. The *byr3* null strain SpB3U was chosen for further studies.

The *byr3* null strain SpB3U has a normal morphology. It is homothallic and hence is capable, in principle, of self-mating and sporulating when starved. The strain has an apparently normal growth rate, but colonies of this strain stain weakly with iodine vapor. Microscopic examination of cells from such colonies revealed that the level of conjugation, as determined by the observation of zygotic asci, was considerably lower than that observed in wild-type h^{90} colonies (see also Table 4). To determine if the *byr3* null strain was defective in sporulation, diploidized colonies were picked as described in MATERIALS AND METHODS. Colonies of the diploidized strains stain as dark black on exposure to iodine vapor as do wild-type diploid cells, and microscopic examination revealed numerous asci. Haploid progeny of such diploids display the same deficiency in conjugation as the starting *byr3* null strain SpB3U. Thus cells with *byr3* disruptions are partially defective in conjugation but appear wild type with respect to sporulation and in other respects appear normal.

Human CNBP Restores Conjugation of the byr3 Null Strain

The *S. pombe* byr3 and the human CNBP protein both contain seven repeats of the consensus sequence, $CX_2CX_4HX_4C$, which is a "zinc finger" motif found in a number of nucleic acid binding protein. The sizes of these two proteins are similar; the *S. pombe* byr3 is predicted to have 179 residues, and the human CNBP 180. The similarity between byr3 and CNBP suggests that a similar biological function may be found. To test this possibility, a plasmid, pARTCNBP, was constructed to express the human CNBP protein in yeast. This plasmid was transformed into the *byr3* null strain, SpB3U (see Table 5), and transformants were examined for the ability to conjugate. The results suggest that expression of the human CNBP is capable of restoring conjugation of the SpB3U strain towards wild-type levels, although not as efficiently as expression of byr3 itself from a similar plasmid. Expression of the human *CNBP* gene in *ras1* null diploid yeast failed to restore sporulation.

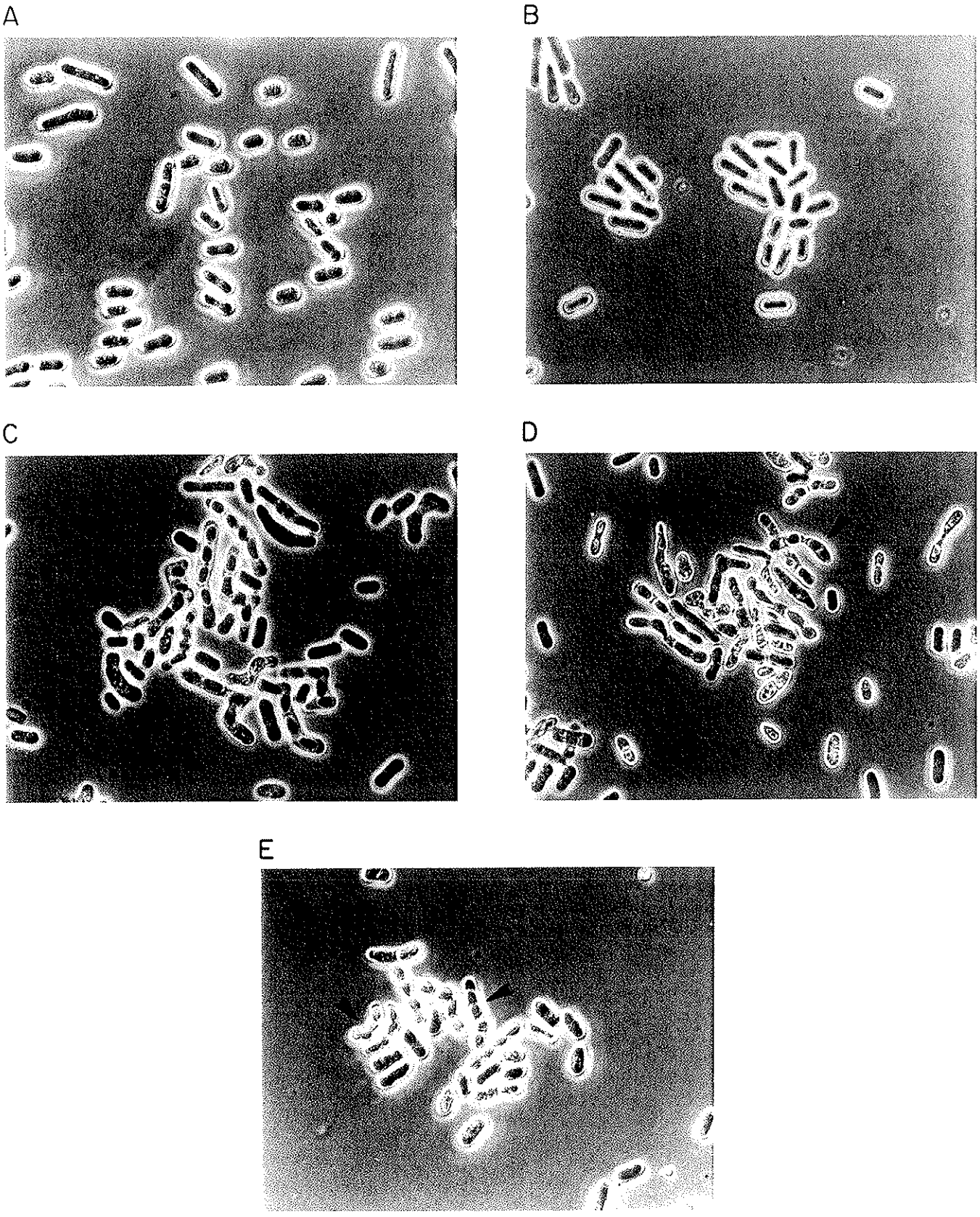
Suppression of Other Phenotypes of ras1 Null Cells by byr Plasmids

S. pombe strains lacking *ras1* are also defective in conjugation and have a round cell shape. Multicopy plasmids expressing *byr1* and *byr2* do not restore cell shape to *ras1*$^-$ strains (Nadin-Davis and Nasim, 1988; Wang *et al.*, 1991b). Multicopy plasmids expressing *byr3* likewise fail to restore cell shape to these strains.

It has been reported by others, and by us, that multicopy plasmids expressing either *byr1* (Nadin-Davis and Nasim, 1988) or *byr2* (Wang *et al.*, 1991b) could not suppress the conjugation defects of *ras1* null cells. These conclusions were based upon microscopic examination of *h*^{90} *ras1* null cells grown in dense patches on solid medium. We have reexamined this question with the use of a more sensitive genetic selection for conjugation with a mating-competent partner. *ras1* null, *h*^{90} cells, transformed with various test plasmids, were incubated with either *h*$^+$ or *h*$^-$ *ras1* wild-type strains under nutrient conditions promoting conjugation (see MATERIALS AND METHODS). The *ras1* null, *h*^{90} cells contained the *ade6.210* marker, whereas the *ras1* wild-type cells contained the *ade6.216* marker. After cocultivation and replica plating, adenine prototrophic cells were selected, and colonies containing sporulating cells were counted. From these experiments it was evident that multicopy plasmids expressing either *byr1* or *byr2* were capable of restoring low levels of conjugation to *ras1* null cells (see Table 6). In contrast, pALBYR3, which expresses the *byr3* gene, failed to induce measurable levels of conjugation.

Further Genetic Interactions of byr3

Multicopy plasmids expressing *ras1*, *ras1*^{val17}, *byr1*, and *byr2* were all tested for the ability to alter the phenotype


A
B
C
D
E

of the *byr3* null strain SpB3U. Plasmids expressing *ras1* and *byr2* effectively restored normal levels of conjugation (see Table 7 and Figure 6). In contrast, the plasmid pARTBYR1, expressing *byr1*, was not capable of restoring this activity even though this plasmid was capable of restoring sporulation to *ras1* and to *byr2* null diploid cells, restoring conjugation and sporulation to *byr1* null cells (Wang *et al.*, 1991b), and was more effective than the *byr2* plasmid, pWH5BYR2, at restoring conjugation to *ras1* null cells (see Table 6). Expression of $ras1^{val17}$ made the *byr3* null strain more sterile and increased its agglutinability. Moreover, expression of $ras1^{val17}$ induced conjugation tubes and a pear shape morphology in the *byr3* null strain, as it does in wild-type homothallic strains. Hence byr3 function is not required for the phenotypes associated with activated $ras1^{val17}$.

Next we explored the ability of multicopy plasmids expressing *byr3* to restore phenotypic defects in *S. pombe* strains carrying mutations in other loci. We used the following host strains: SpBUD, a diploid strain that carries a complete disruption of *byr1* at both alleles and is absolutely defective in sporulation; SpB2UD, a diploid strain that is completely defective at both alleles of *byr2* and is absolutely defective in sporulation; and SpR2A, a haploid strain that expresses the dominant interfering $RAS2^{ala22}$ gene of the yeast *S. cerevisiae*, and is thus partially sterile (Powers *et al.*, 1989; Wang *et al.*, 1991b). The multicopy plasmid pALBYR3 was not able to restore sporulation to SpBUD, or to SpB2UD, but was able to restore conjugal competence to the $RAS2^{ala22}$ strain (see Figure 7).

Mutation in ras1, byr1, or byr2 do not Affect the Physical Properties of the byr3 Product

In view of the apparent involvement between *byr3* and the sexual differentiation pathway, and that at least two genes required for this pathway probably encode protein kinases, we sought to determine if the covalent state of byr3 protein is affected by mutations in genes involved in sexual differentiation. To this end, we expressed an epitope fusion of byr3 from an *adh1* promoter in strains that were either otherwise wild type, *ras1* null, *byr1* null, or *byr2* null, and labeled cells with either ^{32}P phosphate or with ^{35}S-methionine. Cell extracts were immunoprecipitated with monoclonal antibody specific to the epitope, and immunoprecipitates resolved by SDS-PAGE. The results of ^{32}P labeling suggest that byr3 is a phosphoprotein but that the amount of label is not dependent on genetic background. Moreover, the results of ^{35}S labeling, shown in Figure 5, indicate that the mobility of the byr3 protein is not affected either. These experiments fail to find evidence that the byr3 protein is a substrate for protein kinases that function on the sexual differentiation pathway.

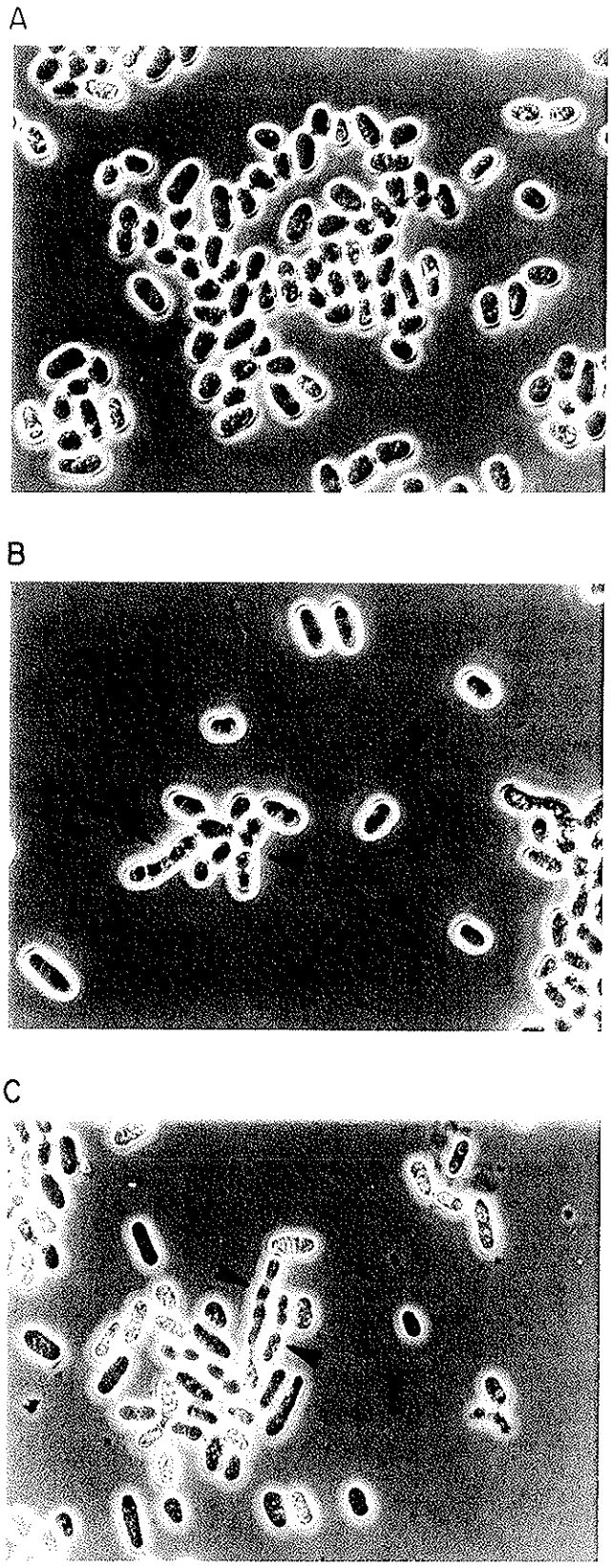
DISCUSSION

The function of RAS in the yeast *S. cerevisiae* is at least partly understood: one effector is adenylyl cyclase (Toda *et al.*, 1985). The function of ras1 in *S. pombe* is still unclear, but adenylyl cyclase is not its effector (Fukui *et al.*, 1986). In an attempt to define the ras1 dependent signal transduction pathways in *S. pombe*, we have sought to identify genes that can suppress *ras1* null phenotypes when carried on multicopy plasmids. In this manner, both *byr1* and *byr2* were previously identified (Nadin-Davis and Nasim, 1988; Wang *et al.*, 1991b). In this report we have described a third gene, *byr3*.

The significance of these identifications extends beyond the narrow objective of defining the effectors of RAS and encompasses the broader question of how signal transduction pathways are composed and woven together. We know that ras1 participates in the pathways of sexual differentiation, culminating in both conjugation and sporulation. Many components in addition to ras1 are shared in the consumation of these two events. The two genes, *byr1* and *byr2*, each expected to encode protein kinases, and also to participate in both conjugation and sporulation. Like *byr1* and *byr2*, the overexpression of *byr3* can influence sporulation and conjugation. Overexpression of *byr3* restores sporulation in *ras1* null diploids and restores conjugation in *S. pombe* strains that express the interfering *S. cerevisiae* $RAS2^{ala22}$ gene. Overexpression of *byr3* does not have as profound an effect on these events in *ras1* null cells as does overexpression of either *byr1* or *byr2*, because the latter can restore measurable mating activity to *ras1* null haploid cells, whereas overexpression of *byr3* cannot.

Disruption of *byr3* leads to diminished conjugal activity in haploid cells, without a measurable effect on sporulation, whereas disruption of *byr1* and *byr2* leads to defects in both conjugation and sporulation. This result can be readily explained, because disruption of *byr3* leads to only a partial debilitation of the signal transduction pathways of sexual function: disruption of *byr1* or *byr2* leads to a complete inhibition of mating and sporulation. Several lines of evidence indicate that efficient conjugation is more sensitive to perturbation of this signaling pathway than is sporulation: cells with

Figure 6. Effects of multicopy expression of *byr1*, *byr2*, and *ras1* on the phenotype of the *byr3* null strain SpB3U. The strain SpB3U was transformed with (A) pAL, a *S. pombe* expression vector, (B) pARTBYR1, a plasmid containing the *S. pombe byr1* gene, (C) pWH5BYR2, a plasmid containing the *S. pombe byr2* gene, (D) pALR, a plasmid containing the *S. pombe ras1* gene, and (E) pALBYR3, a plasmid containing the *S. pombe byr3* gene. Cells were maintained for 3 d in PMA before photographing. The arrow heads indicate asci resulting from conjugation, evident in C, D, and E, but lacking in A and B.



interfering $RAS2^{ala22}$ are defective in conjugation, but not in sporulation (Wang *et al.*, 1991b); cells with activated $ras1^{val17}$ are defective in conjugation but not sporulation (Fukui, *et al.*, 1986; Nadin-Davis *et al.*, 1986a); cells lacking *ste6*, which probably encodes a ras1 nucleotide exchange catalyst, are defective in conjugation but not in sporulation (Hughes *et al.*, 1990); overexpression of either *byr1* or *byr2* readily suppresses the sporulation defects of *ras1* null diploid cells, but only very weakly suppress the conjugation defects of *ras1* null haploid cells (Wang *et al.*, 1991b and this report); overexpression of *byr1* suppresses the sporulation defects of *byr2* null diploid cells but not the conjugation defects of *byr2* null haploid cells (Wang *et al.*, 1991b).

We do not understand why byr3 does not appear to be essential in the sexual differentiation pathway. One possibility is that byr3 does not carry out an essential function but merely improves conjugal efficiency. Alternately, byr3 may carry out an essential function, but that a byr3 homolog exists in *S. pombe* with redundant function. Low stringency hybridization experiments with the *byr3* gene as probe have been performed but have not yielded an unambiguous answer (Xu, unpublished data). Given the structural and functional homology between byr3 and the human protein CNBP, (see below), it is quite feasible that functional homologs of byr3 exist in *S. pombe* that are sufficiently diverged to escape detection by conventional hybridization techniques. Many examples can be found of proteins with homologous function that have only a bare amount of conserved primary structure.

The discovery of each new gene operating in the sexual differentiation pathway presents an opportunity to order it with respect to other genes in the pathway. Although this ordering cannot be made without certain assumptions, and these assumptions are often unstated and unproven, we believe the tentative model created thereby is useful. First, each of *byr1*, *byr2*, and *byr3* can suppress the sporulation defect of *ras1* null diploid cells. These results suggest that each acts downstream of *ras1*. One assumption required for this conclusion is that *ras1* disruption creates an impenetrable blockade of the signal transduction cascade at its point of action. Second, disruption of *byr1*, *byr2*, or *byr3* does not produce the morphological abnormality of *ras1* disruption, namely abnormally round cells. Thus ras1 must control more than the ensemble of the known byr proteins. The assumption required for this conclusion is that there are

Figure 7. Effects of expressing *byr3* on the phenotype of SpR2A. SpR2A is a haploid strain that expresses the dominant interfering $RAS2^{ala22}$ gene of the yeast *S. cerevisiae*, and is thus partially sterile. The strain was transformed with (A), pAL, a *S. pombe* expression vector, (B), pALBYR3, a plasmid containing the *S. pombe byr3* gene, (C), pALR, a plasmid containing the *S. pombe ras1* gene. The arrow heads in B and C indicate asci that have resulted from haploid conjugation.

no other genes that duplicate the function of the *byr* genes. Although we can have considerable confidence in this assumption for the *byr1* and *byr2* genes, which, as already discussed, create absolute blocks in conjugation and sporulation, we can have little confidence in this assumption for *byr3*. Third, multicopy *ras1* and multicopy *byr2* can each restore conjugation efficiency to cells lacking *byr3*, but multicopy *byr1* fails to do this. Because multicopy *byr1* is superior to multicopy *byr2* in suppressing the conjugation defects of *ras1* null haploid cells (Table 6), we infer from this that byr2 and ras1 each have some function that is not shared by byr1. This conclusion is consistent with our previous analysis that placed *byr2* upstream of *byr1* (Wang *et al.*, 1991b).

Another way to order protein/protein interactions is by biochemical analysis. We therefore sought evidence that byr3 might be a "substrate" of one of the components of the ras1/byr dependent signal transduction cascade. byr3 is a phosphoprotein, but neither its degree of phosphorylation nor its apparent mobility in SDS polyacrylamide gels appears to be a function of the presence or absence of *ras1*, *byr1*, or *byr2*.

The predicted primary sequence of the byr3 product is quite unusual in that it contains seven repeats of the motif $CX_2CX_4HX_4C$. A similar zinc finger motif is found in a number of nucleic acid binding proteins, and matches a motif found in the gag polyproteins of retroviruses (Berg, 1986). A mammalian protein, CNBP, has been described that, like byr3, contains precisely seven units of this repeat (Rajavashisth *et al.*, 1989). The homology between CNBP and byr3 repeats is the closest we have found in the sequence data banks. The similarity in the overall structure of byr3 and CNBP suggests that a similarity in biochemical function will be found. To test this hypothesis, we expressed CNBP in *S. pombe*. Expression of CNBP increased the conjugal efficiency of cells lacking byr3, consistent with the idea that both proteins have common cellular functions. Expression of CNBP failed, however, to induce sporulation in *ras1* null diploids cells suggesting that it does not function equivalently to byr3 in *S. pombe*.

CNBP was found by its ability to bind to the DNA motif GTGCGGTG that has been found near sterol responsive genes. Like CNBP, byr3 binds to DNA. byr3 was expressed as a fusion to maltose-binding protein and purified as a complex to amylose resin. The MBP-byr3 fusion protein stably associates with the amylose resin under the DNA binding and washing conditions used. This stable association permits for a rapid separation, by low-speed centrifugation, of unbound from bound ^{32}P-labeled DNA. We have found that the fusion protein can bind ^{32}P-labeled DNA and that this binding can be competed by unlabeled DNA.

On the basis of on its DNA binding characteristics, Rajavashisth *et al.*, 1989, hypothesized that CNBP is involved in the repression of transcriptional activity of sterol responsive genes. This hypothesis has received no direct experimental support, at this time. Another hypothesis is that byr3 and CNBP bind RNA. Although several proteins with the zinc finger binding motif $CX_2CX_{12-14}HX_2H$ are transcriptional activators, the motif $CX_2CX_4HX_4C$ is associated with retroviral nucleocapsid proteins (Berg, 1986), and the similar metal-binding motif $CX_2CX_6HX_2H$ is found in *E. coli* alanyl-tRNA synthetase (Miller *et al.*, 1991). The discovery of the conservation of proteins with seven units of this zinc finger binding motif, and, in particular their discovery in yeast, may lead to understanding of the way these molecules participate in cellular events.

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