Evidence for Interaction of *Schizophyllum commune* Y Mating-Type Proteins *in Vivo*

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ABSTRACT

The Aα mating-type locus of *Schizophyllum commune* regulates sexual development and contains the code for two proteins, Y and Z, which are thought to form a complex and function as a transcription factor. Import of these proteins into the nucleus may be an essential step in Aα-regulated sexual development. The Y proteins contain a bipartite basic sequence, which is an excellent candidate for a nuclear localization sequence (NLS), while Z proteins contain no such sequence. Here we describe experiments in which deletions were made in the putative NLS sequence of Y4. We show that this putative NLS is essential to the function of the Y protein and capable of mislocalizing green fluorescent protein (GFP) to the nucleus in *Saccharomyces cerevisiae*. Further, we describe genetic experiments that demonstrate the first Y-Y protein interactions *in vivo*. These results are consistent with our previously postulated hypothesis that the Y-Z complex is likely to be of a higher order than dimer.

IN the basidiomycete fungus *Schizophyllum commune*, fusion of homokaryotic mycelia with different mating types leads to sexual development. This development consists of two developmental pathways, A and B, that together constitute formation of the fertile dikaryon (Kniep 1920; Papazian 1951; Raper 1966). The A-pathway may be activated by either of two functionally redundant genetic switches termed the Aα and Aβ loci. The B-pathway is controlled by another redundant pair of genetic switches, the Bα and Bβ loci. There are 9 Aα mating types and 32 Aβ mating types in the worldwide population of *S. commune* (Raper *et al.* 1960; Stamberg and Koltin 1973). Previous work has shown that the Aα locus contains two divergently transcribed genes, *Y* and *Z*, which encode mating-type proteins (Stankis *et al.* 1992). Each Aα locus encodes a unique *Y* and a unique *Z* (*e.g.*, the Aα4 locus contains the *Y4* and *Z4* genes) except Aα1, which has a *Y1* gene but no *Z* gene. Activation of the A-pathway by Aα occurs through the interaction of Y and Z proteins from different mating types (*non-self* pairs, *e.g.*, *Y4-Z5*). Y proteins contain an essential homeodomain, and Z proteins contain a nonessential homeodomain-like motif (Luo *et al.* 1994). Y and Z proteins contain other motifs associated with transcription factors (Stankis *et al.* 1992) and *self-nonself* recognition determinants termed specificity regions (Wu *et al.* 1996; Yue *et al.* 1997). *In vitro* protein affinity assays have shown that protein interactions occur between both *non-self* (*e.g.*, *Y4-Z5*, *Y4-Y5*, and *Z5-Z4*) and *self* (*e.g.*, *Y4-Z4*, *Y4-Y4*, and *Z5-Z5*) combinations of Y and Z proteins (Asada *et al.* 1997).

Current dogma suggests that the Y and Z proteins function as transcription factors; however, there is no direct evidence to support this. If the dogma is correct, Y and Z must be transported from the site of translation in the cytoplasm into the nucleus. Evidence for nuclear localization would be informative to our studies of Aα-regulated development. Several types of amino acid motifs are thought to mediate transport of proteins into the nucleus (Garcia-Bustos *et al.* 1991; Boulikas 1994; Nigg 1997). The bipartite nuclear localization sequence (NLS) has been shown to consist of two predominantly basic sequences separated by a predominantly nonbasic spacer of three to eight amino acids; this class of NLS sequences is found in many transcription factors including v-Jun, c-Fos, CREB, GCN4, and NF-E2 (Boulikas 1994). Visual inspection of the Aα protein sequences shows a bipartite basic sequence at approximately the same location within each AαY protein; however, no NLS-like sequence has been found within Z. This suggests that transport of Z into the nucleus may represent a regulation point in the A-pathway signal transduction cascade. Regulation of the nuclear entry of signaling proteins has been the subject of considerable study. Masking and unmasking of NLSs is one proposed mechanism for such regulation (Nigg 1990). Another proposed mechanism is piggybacked transport such as that thought to occur between TFIIE-α and TFIIE-β (Bouli-

Published posthumously, this article is dedicated to Alexander McMahon Kende, a promising young researcher who died prematurely.

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kas 1994). In the basidiomycete *Coprinus cinereus*, evidence shows that complex formation between two mating-type proteins (one lacking an identifiable NLS) affects import of the two into nuclei in an onion skin heterologous test system (Spit *et al.* 1998).

An important physical characteristic of Y and Z regulation of the A developmental pathway is the *in vivo* state of the Y and Z proteins. Development requires Y and Z proteins of different mating types within a common cytoplasm. It may seem intuitive to this system of combinatorial control that these two proteins participate together in a protein complex. Despite the fact that protein affinity assays demonstrate the formation of Y-Z protein complexes *in vitro* (as well as other combinations of Y and Z proteins; Asada *et al.* 1997), there is no direct evidence for such complexes *in vivo*. Eukaryotic gene expression is mediated in many instances by complexes containing combinations of proteins, *i.e.*, by combinatorial control. Combinatorial control allows individual proteins to participate in multiple regulatory activities, thus allowing the complex regulatory networks found in eukaryotes (Wolberger 1999). Among proteins of the leucine zipper family of transcription factors, homo- and heterodimerization of proteins (*e.g.*, Fos and Jun) provide a means by which complex cascades of events can be regulated by a modest set of proteins (Karin and Hunter 1995). Similar combinatorial regulation is seen in the regulation of yeast mating-type events by complexes formed by the α2 protein homeodomain with MATa1 and MCM1. DNA-bound regulatory proteins have also been found associated in higher-order complexes; *e.g.*, mammalian PBX and MEIS proteins form trimeric complexes with HOXD4, HOXD9, and HOXD10 proteins (Shanmugam *et al.* 1999), and in zebrafish, *pbx4*, *meis3*, and *hox1b* form trimeric complexes (Vlachakis *et al.* 2000).

Here we describe experiments that demonstrate the presence of a bipartite NLS in Y proteins. Cytological experiments show that this sequence from *S. commune* is capable of mislocalizing green fluorescent protein (GFP) to the nucleus in *Saccharomyces cerevisiae*. Genetic analysis shows the NLS sequence to play an essential role in sexual development; the implication is that the essential NLS functions to direct Y protein to the nucleus in Schizophyllum. Further, the genetic experiments demonstrate that Y proteins form complexes with one another *in vivo*; however, whether this interaction is present along with Z protein in the complex that activates A-regulated development is yet to be determined.

MATERIALS AND METHODS

Strains: Homokaryotic strains of *S. commune* used in this study are listed in Table 1. *Escherichia coli* strain XL1-Blue (Stratagene, La Jolla, CA) was used for gene cloning. *Saccharomyces cerevisiae* strain RAK21 (Akada *et al.* 1996) was the gift of J. Kurjan.

TABLE 1

***S. commune* homokaryotic strains and genotypes used in this study**

Strains	Mating type Aα	Aβ	Bα	Bβ	Auxotrophic markers
18-20	Δ	1	2	2	*ade2*[b]
18-24	Δ	1	3	2	*ade2*[b]
21-29	Δ, *Y4*[a]	1	2	2	
21-30	Δ, *Y4*[a]	1	2	2	
21-31	Δ, $Y4_{NLS1/2\Delta}$[a]	1	2	2	
21-32	Δ, $Y4_{NLS1/2\Delta}$[a]	1	2	2	
21-63	Δ, *Z6*[a]	1	3	2	
21-64	Δ, *Z6*[a]	1	3	2	
21-65	Δ, *Z6*[a]	1	3	2	
21-66	Δ, *Z6*[a]	1	3	2	
21-71	Δ, *Z5*[a]	1	3	2	
21-72	Δ, *Z5*[a]	1	3	2	
21-73	Δ, *Z5*[a]	1	3	2	
21-74	Δ, *Z5*[a]	1	3	2	
21-75	Δ, $Y4_{\Delta NLS}$[a]	1	2	2	
21-76	Δ, $Y4_{\Delta NLS}$[a]	1	2	2	
21-77	Δ, $Y4_{\Delta NLS}$[a]	1	2	2	
21-78	Δ, $Y4_{\Delta NLS}$[a]	1	2	2	
21-79	Δ, $Y4_{\Delta NLS}$[a]	1	2	2	
21-85	Δ, $Y4_{\Delta NLS1/2}$[a]	1	2	2	
21-86	Δ, $Y4_{\Delta NLS1/2}$[a]	1	2	2	
21-87	Δ, $Y4_{\Delta NLS1/2}$[a]	1	2	2	
21-88	Δ, $Y4_{\Delta NLS1/2}$[a]	1	2	2	
21-89	Δ, $Y4_{\Delta NLS1/2}$[a]	1	2	2	
19-2	1	1	1	1	
T29	2	1	3	2	*ura1, trp1*[b]
T22	3	1	2	2	*ura1, trp1*[b]
T33	3	1	4	7′	*ura1, trp1*[b]
T2	4	1	3	2	*ura1, trp1*[b]
T34	5	1	1	1	*ura1, trp1*[b]
T30	6	1	3	2	*trp1*[b]
T35	7	1	4	7′	*ura1, trp1*[b]
T31	8	1	3	2	*ura1, trp1*[b]
T32	9	1	3	2	*ura1, trp1*[b]

[a] Genes inserted by transformation.

[b] *ade2* mutant for a gene of unidentified function in the adenine biosynthetic pathway (Froeliger *et al.* 1987); *ura1* mutant for orotidine-5′-phosphate decarboxylase (DiRusso *et al.* 1983); and *trp1* mutant for indole-3-glycerol phosphate synthetase (Muñoz-Rivas *et al.* 1986).

Construction of plasmids deleted for part or all of the *Y4* putative NLS: Full-length cDNA of the wild-type *Y4* gene (Yue *et al.* 1997) was cloned into plasmid pALTER (Promega, Madison, WI) and used as a substrate for mutagenesis. Deletions were made in the putative NLS sequence of the *Y4* gene (nucleotides 7196–7279; nucleotide positions specified in this article correspond to the genomic sequence positions of GenBank accession no. M80822) by site-directed mutagenesis using an Altered Sites II mutagenesis kit (Promega). Primers (Life Technologies) synthesized to make the NLS deletions were as follows: in $pY4_{\Delta 1/2NLS}$, 5′ CAGAGGAAGGCGCTGGCCTC GAGGTCGCAA 3′ deletes nucleotides 7196–7238; in $pY4_{NLS1/2\Delta}$, 5′ ATCGCCCGGGCCGAGGAGGAGAAGCAGGCGAA 3′ de-

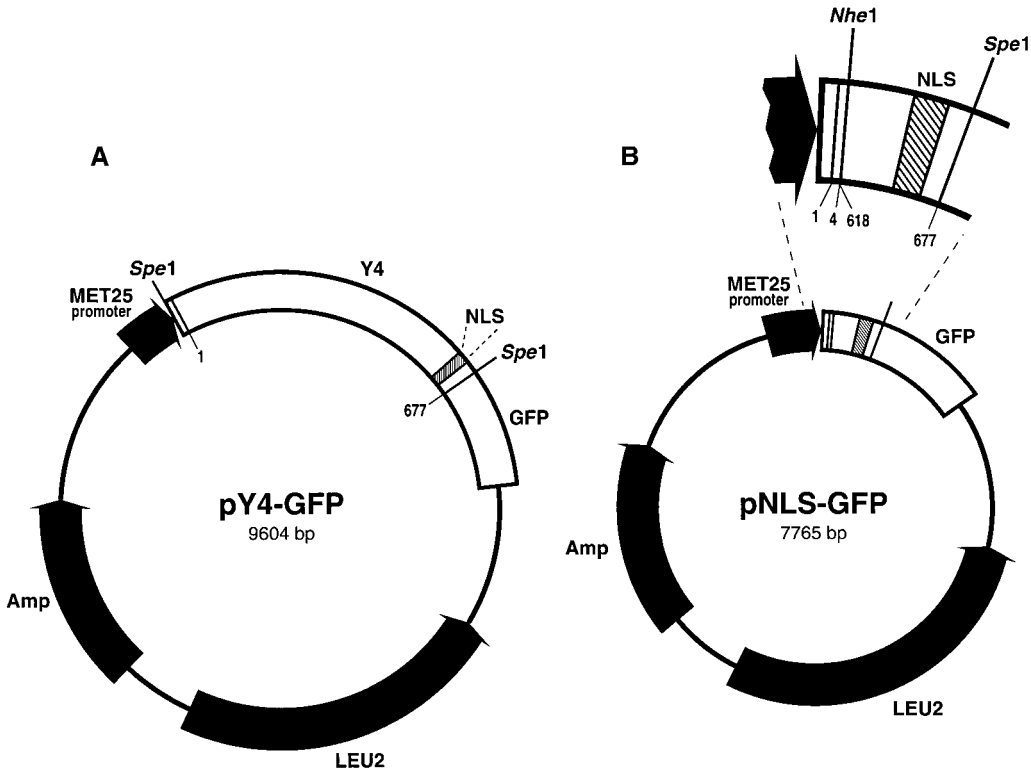

FIGURE 1.—Construction of GFP-containing plasmids. (A) Plasmids pY4-GFP and pY4$_{\Delta NLS}$-GFP differ only by the presence or absence of the NLS code (only pY4-GFP is shown). They were constructed using pTef1Y4 and pTef1Y4$_{\Delta NLS}$. Plasmid pTef1Y4 was created for experiments not described in this article. Plasmid pTef1Y4 contains the *S. commune TEF1* (translation elongation factor 1) promoter fused to the 5′ end of Y4 cDNA in pALTER; pTef1Y4$_{\Delta NLS}$ is identical to pTef1Y4 except that the entire Y4 NLS was deleted by the methods (see MATERIALS AND METHODS) used to delete the NLS in the construction of pY4$_{\Delta NLS}$. Two *Spe*I sites were created in pTef1Y4 and pTef1Y4$_{\Delta NLS}$ using a Quickchange mutagenesis kit (Stratagene). Complementary pairs of primers were used as follows: 5′ CGAGATCTTGTGCGAACTAGTTTACTGACA 3′ and its complement bind in the TEF1 promoter 36 bp 5′ of the *Y4* start ATG, and 5′ TCCGTTGCCTCTACTAGTTCGCTGCCCGCGCGA 3′ and its complement bind within *Y4* at Aα4 nucleotide 7359. The resulting 2.2-kb *Spe*I fragment, which encodes amino acids 1–677 of Y4 and 36 bp of the TEF1 promoter, was recovered from pTef1Y4 and ligated to the *Spe*I site located at the 5′ end of the GFP gene and immediately downstream of the yeast MET25 promoter in the yeast 2μ plasmid p415 MET25 to yield plasmid pY4-GFP. The *Spe*I fragment from pTef1Y4$_{\Delta NLS}$ was ligated similarly to obtain the fusion to GFP in pY4$_{\Delta NLS}$-GFP. (B) Plasmid pNLS-GFP was produced as follows. A Quickchange mutagenesis kit (Stratagene) was used to introduce two *Nhe*I sites in pY4-GFP at Aα4 nucleotides 5036 and 7172 using the following complementary primer pairs: 5′ ATGGCAGAGCTGCTAGCCTGCCTGCAGAGC 3′ and its complement bound at nucleotide 5025 and 5′ ATGCAT TGGCTAGCTTCAAATCGGCTCGGGCC 3′ and its complement bound at nucleotide 7164. The resulting *Nhe*I fragment containing the code for amino acids 5–617 of the Y4 protein was excised from the modified pY4-GFP. Ligation of the remainder of pY4-GFP leaves the coding region for amino acids 1–4 fused and in frame to that for amino acids 618–677, which in turn are fused and in frame to the 5′ end of the GFP sequence.

letes nucleotides 7238–7279; and in pY4$_{\Delta NLS}$, 5′ ATCGCCCG GGCCGAGGCCTCGAGGTCGCAAC 3′ deletes nucleotides 7196–7279. The resulting plasmids, pY4$_{\Delta 1/2NLS}$, pY4$_{NLS1/2\Delta}$, and pY4$_{\Delta NLS}$, contain deletions of the first half of the NLS, second half of the NLS, and the entire NLS, respectively. In each case, the deletion was designed to conserve the reading frame of the protein. Each product was confirmed by sequencing and a 2.8-kb *Bam*HI fragment of the deleted gene was used to replace the wild-type fragment of the *Y4* gene in plasmid pY4T. Plasmid pY4T is pALTER into which the entire *Y4* and *TRP1* genes, including their promoters, have been cloned. The resulting constructs were transformed into *S. commune* protoplasts and analyzed for function by mating tests as described below.

Construction of plasmids to test mislocalization of GFP in yeast: Plasmids pY4-GFP and pY4$_{\Delta NLS}$-GFP contain gene fusions of *Y4* or mutant *Y4* to the gene encoding GFP (Figure 1A). They were created to test the ability of the *Y4* NLS to misdirect the nonnuclear GFP protein to the nucleus in yeast. Plasmid pNLS-GFP was created to test the ability of the Y4 NLS to mislocalize GFP to the yeast nucleus in a construct containing minimal surrounding Y4 sequences, namely, in Y4 amino acids 1–4 and 618–677 where the putative NLS of Y4 is encoded by amino acids 624–651. The construction of pNLS-GFP is shown in Figure 1B.

Transformation of *S. commune* and analysis of transformants: Transformation was performed by methods previously described (FROELIGER *et al.* 1987; SPECHT *et al.* 1988). Protoplasts derived from strain T33 (*trp1*) were transformed with 2.5 μg of plasmid containing wild-type or mutant *Y4* and *TRP1*. Transformants were selected for Trp$^+$ phenotype on CYM medium (SPECHT *et al.* 1988). Cotransformation of protoplasts derived from strain 18-20 and 18-24 used 2.5 μg of plasmid containing the *ADE2* gene (FROELIGER *et al.* 1987) and 10 μg of plasmid containing wild-type or mutant *Y4*. ADE2 transformants were selected on the ammonium phosphate minimal medium of EPSTEIN and MILES (1966). To determine the mating types or developmental activity of the transformants, each was paired with a series of appropriate tester strains on CYM medium for 3 days at 30° and then examined microscopically for developmental state. All recipient strains contained the Aβ1 locus, and all tester strains used contained Aβ1 and Bα and/or Bβ mating types different from the recipient strain. In each case the matings depended on the presence of functional Y4 protein to activate the A-pathway.

Pairings between strains differing in both Aα and/or Aβ

TABLE 2

Mating test results: transformants of AαΔ strain 18-20 containing various Y4 NLS deletions paired with tester strains

Tester strain	18-20 + $Y4_{NLS1/2\Delta}$		18-20 + $Y4_{\Delta NLS1/2}$					18-20 + $Y4_{\Delta NLS}$				
	21-31	21-32	21-85	21-86	21-87	21-88	21-89	21-75	21-76	21-77	21-78	21-79
19-2	F	F	F	F	F	F	F	F	F	F	F	F
T29	+	+	+	+	+	+	+	+	+	+	+	+
T33	+	+	+	+	+	+	+	+	+	+	+	+
T2	F	F	F	F	F	F	F	F	F	F	F	F
T34	+	+	+	+	+	+	+	+	+	+	+	+
T30	+	+	+	+	+	+	+	+	+	+	+	+
T35	+	+	+	+	+	+	+	+	+	+	+	+
T31	+	+	+	+	+	+	+	+	+	+	+	+
T32	+	+	+	+	+	+	+	+	+	+	+	+

F, flat morphology.

and Bα and/or Bβ activate both the A- and B-pathways and produce dikaryotic cells with plentiful aerial hyphae and lateral cellular appendages termed "clamp connections" at every septum of the hyphae. Clamp connections can be visualized with a light microscope at ×200 magnification. In contrast, matings between strains identical at Aα and/or Aβ, but different at Bα and/or Bβ, produce mycelia with a different morphology, hyphae with short, perpendicular branches, few aerial hyphae, and no clamp connections. This morphology is termed the "flat" phenotype. T33 cells transformed with wild-type *Y4* show full and normal dikaryotic development when paired with T22. A deletion that causes a loss of *Y4* function would result in the flat phenotype.

Growth and fluorescence microscopy of yeast cells: Yeast were transformed as described by Sherman *et al.* (1986). Yeast strain RAK21 was grown at 23° to mid-log phase in synthetic complete media lacking methionine and leucine. Fresh wet mounts of cells stained with 4′6-diamidino-2-phenylindole (DAPI) by methods previously described (Pringle *et al.* 1989) were viewed on a Nikon E400 fluorescent microscope using an Omega XF06 optical filter cube. An Omega XF100 optical filter cube was used to visualize cells expressing GFP. Images were obtained on a cooled CCD camera and analyzed with Adobe Photoshop 5.0.

RESULTS

Testing the Y4-NLS deletion constructs for loss of function in wild-type strains: Recipient strain T33 was transformed separately with plasmids containing the *Y4* gene with a deletion of the putative NLS ($pY4_{\Delta 1/2NLS}$, $pY4_{NLS1/2\Delta}$, and $pY4_{\Delta NLS}$). In each case 25 Trp^+ transformants were selected on CYM medium and paired with tester strain T22. Strains T33 (Aα3 Aβ1 Bα4 Bβ7′) and T22 (Aα3 Aβ1 Bα2 Bβ2) contain identical genes at the Aα and Aβ loci and differing genes at the Bα and Bβ loci. Therefore, in pairings of *Y4* transformants of T33 with T22, activation of the A-pathway depends on the presence of functional *Y4* in the T33 transformants. In pairings of transformants containing $pY4_{\Delta 1/2NLS}$, $pY4_{NLS1/2\Delta}$, or $pY4_{\Delta NLS}$ with tester strain T22, several transformants (8, 9, and 14 of each 25 Trp^+ transformants, respectively) yielded pairings with clamps. Thus each of the NLS deletion constructs in *Y4* transformants activate the A-pathway in pairings with strain T22.

Testing the Y4-NLS deletion constructs for loss of function in strains deleted for Aα: The deletion constructs were tested further as follows. Strain 18-20, a strain from which the Aα locus has been deleted by gene replacement (AαΔ Aβ1 Bα2 Bβ2; Robertson *et al.* 1996), was cotransformed separately with one of the *Y4* deletions and a plasmid containing *ADE2*. In each case 25 ADE2 transformants were selected on ammonium phosphate minimal medium and each was paired with tester strain T33 to distinguish the *Y4* transformants. These transformants are listed in Table 1 as strains deleted for Aα and transformed with *Y4* deleted for part or all of the putative NLS. These transformants were screened for *Y4* function by pairing them with a series of tester strains. Each tester strain contained one of the nine Aα mating types, Aβ1, and Bα and/or Bβ differing from recipient strain 18-20. All matings depend on functional Y4 protein to activate the A-pathway. Again, surprisingly, these mating tests showed that transformants carrying any of the mutant *Y4* genes were able to activate A-regulated development. Only strains 19-2 and T2 failed to activate development (Table 2). The lack of development in the transformants paired with the strain 19-2 is expected because strain 19-2 is an Aα1 strain; thus it lacks a *Z* gene and both *Y* and *Z* are required in the fusion cell for mating-type activity. Strain T2 is an Aα4 strain, and therefore is *self* with the *Y4* constructs. This experiment shows that *Y4* deleted part or all of its NLS functions normally in pairings with wild-type strains.

Testing the Y4 deletions in pairs of strains deleted for AαY: Our *in vitro* protein affinity studies of Y and Z show that each Y and Z protein interacts with every other Y and Z protein (Asada *et al.* 1997). Therefore we considered the possibility that protein interactions support complementation of the Y4 NLS mutants above. We deemed it essential to test the function of the dele-

TABLE 3

Mating test results: transformants of AαΔ strain 18-24 containing *Z5* or *Z6* paired with transformants of AαΔ strain 18-20 containing various Y4 NLS deletions

	AαΔ + Z strain								
	18-24 + Z5 strains				18-24 + Z6 strains				T33
AαΔ + $Y4_{NLS\Delta}$ strains	21-71	21-72	21-73	21-74	21-63	21-64	21-65	21-66	wild type
18-20 + $Y4_{NLS1/2\Delta}$									
21-31	F	F	F	F	F	F	F	F	+
21-32	F	F	F	F	F	F	F	F	+
18-20 $Y4_{\Delta NLS1/2}$									
21-85	F	F	F	F	F	F	F	F	+
21-86	F	F	F	F	F	F	F	F	+
21-87	F	F	F	F	F	F	F	F	+
21-88	F	F	F	F	F	F	F	F	+
21-89	F	F	F	F	F	F	F	F	+
18-20 + $Y4_{\Delta NLS}$									
21-75	F	F	F	F	F	F	F	F	+
21-76	F	F	F	F	F	F	F	F	+
21-77	F	F	F	F	F	F	F	F	+
21-78	F	F	F	F	F	F	F	F	+
21-79	F	F	F	F	F	F	F	F	+
18-20 + Y4									
21-29	+	+	+	+	+	+	+	+	+
21-30	+	+	+	+	+	+	+	+	+
T33	+	+	+	+	+	+	+	+	−

Positive controls using strains 21-20, 21-30, and T33 are also shown. F, flat morphology.

tion constructs in backgrounds devoid of wild-type Y proteins. Appropriate tester strains (21-71 to 21-74 or 21-63 to 21-66) containing a compatible *Z5* or *Z6* gene, respectively, but no *Y* gene (Table 1) were created by cotransformation of AαΔ strain 18-24 with *Z5* or *Z6* and *ADE2*. Twenty-five Ade^+ colonies from each transformation were selected on ammonium phosphate minimal medium and the presence of *Z5* (or *Z6*) was confirmed by pairings with strain T22. The strains shown to contain *Z5* or *Z6* were paired with strains containing either of the Y4-NLS deletion constructs (Table 3). In every pairing a flat reaction was obtained. These results differ from those obtained by pairing *Y4*-NLS deletion transformants with tester strains containing a wild-type Y gene (Table 2 or the original T33 transformants paired with tester T22). The NLS deletion constructs of *Y4* are complemented by the presence of wild-type Y protein of any mating type, but do not function in the absence of another Y protein.

Testing the function of the putative Y4 NLS in yeast sufficiency experiments: The putative NLS of Y4 was tested for function in a sufficiency experiment. A portion of Y4 containing the NLS was cloned in front of the gene coding GFP as described in MATERIALS AND METHODS. Plasmids pY4-GFP or $pY4_{\Delta NLS}$-GFP were transformed into yeast cells. Both sets of yeast transformants show fluorescence of GFP from the cytoplasm, but not the nucleus. To test the effect of the putative NLS in a smaller construct containing minimal Y4 sequence surrounding the NLS, we used a third plasmid, pNLS-GFP, with amino acids 5–617 of Y4 deleted from pY4-GFP. These amino acids lie between the N terminus and the NLS in Y4. Yeast transformants containing the pNLS-GFP construction fluoresce from the nucleus. The same transformants stained with DAPI show that the regions of fluorescence from DAPI correspond to those from GFP (Figure 2). This experiment shows that the Y4 NLS is sufficient to misdirect a nonnuclear protein to the nucleus in yeast.

DISCUSSION

It should not surprise us that the *S. commune* Y proteins, which contain a homeodomain and regulate complex development, would also possess a nuclear localization sequence. Furthermore, on the basis of other motifs common to Y or Z we infer that the Y-Z complex functions as a transcription factor and, as such, may be expected to be imported into the nucleus.

This study has shown that the bipartite sequence of

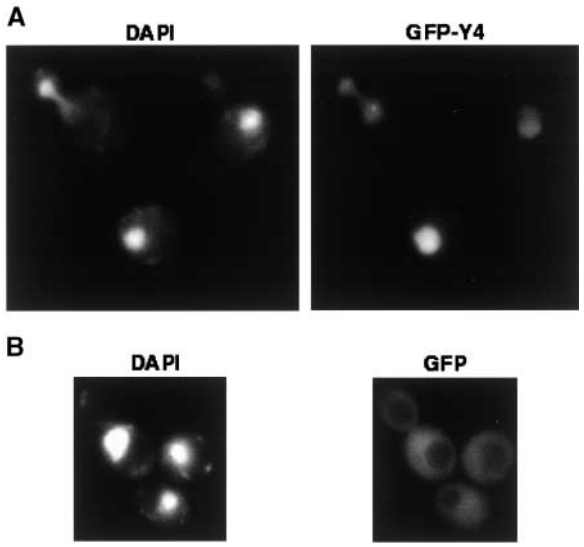

FIGURE 2.—Nuclear localization of fluorescence from pNLS-GFP in yeast. Fluorescence from both DAPI and GFP are shown. (A) Yeast cells transformed with pNLS-GFP or (B) pGFP. Note that in A fluorescence from DAPI and GFP are coincident and located in the nucleus. In B, fluorescence from DAPI (nuclear) is not coincident with that from GFP (cytoplasmic).

A

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Y1  RRARRKARKEKKRQEEKQAKKDKKERKK   635
Y3  KRAKRRARKEKKKQAEKEARKEEKRARK   647
Y4  RRARRKERKQRKALEEKQAKKDRKERQK   651
Y5  KRAKRKARKEKKLQEEKQIRKAEKKARK   642
Y6  RRARRKERKEKKRLEEKLAKRERKKAGL   649
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B

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    ++ +++ ++ +++   +  ++ +++ ++
    BRABRBARKEBKBQEEKQABBDBKBABK
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FIGURE 3.—(A) Comparison of deduced polypeptides of putative NLS sequences from Y1, Y3, Y4, Y5, and Y6. The most C-terminal amino acid residue in each NLS is specified to the right of each NLS sequence. (B) Consensus sequence of the putative NLS for the five Y proteins shown in A. +, positively charged residue; B, basic amino acid; amino acids according to the single letter code.

basic amino acids found in Y proteins is essential to the function of those proteins. Cells containing a single form of mutant Y4 protein, missing either half of the NLS or all of it, brought together via matings with cells devoid of Y protein, but containing Z5 or Z6 protein, failed to activate the A-regulated developmental pathway.

Lacking a reporter system that we can use in Schizophyllum, we turned to GFP and its expression in yeast cells. GFP is not a nuclear protein and is not normally imported into the nucleus. Our first fusions, which linked 677 amino acids of nearly full-length Y4 to GFP, failed to mislocalize to the nucleus, perhaps because of the sheer bulk and folding of the fusion protein. Nevertheless, fusion proteins in which the Y4 NLS with minimal surrounding sequence was fused to GFP were localized to the nucleus of *S. cerevisiae*. A similar observation was made by SPIT *et al.* (1998) for *C. cinereus* where the NLS from an HD1 mating-type protein was fused to the bacterial *uidA* gene and expressed in onion epidermal cells; the fusion protein was targeted to the nucleus in the onion cells.

The presence of an NLS motif in the Y proteins and the absence of such a motif in the Z proteins suggest that Y and Z form a complex in the cytoplasm that is transported into the nucleus by virtue of the Y NLS. We examined the predicted amino acid sequence for each of the five AαY genes that our lab has cloned and studied, *i.e.*, *Y1*, *Y3*, *Y4*, *Y5*, and *Y6*. The predicted amino acid sequences of each contain two predominantly basic sequences separated by a six-amino-acid "spacer" (Figure 3). These basic sequences are found at approximately the same position in each Y protein in spite of the generally poor amino acid homology found between the Y proteins. In contrast, inspection of the predicted amino acid sequence of each studied Z gene, *i.e.*, *Z3*, *Z4*, *Z5*, and *Z6*, shows no candidates for basic NLS sequences. The absence of an identifiable NLS in Z proteins is not totally unexpected given that Y and Z protein interaction is essential to activation of Aα-regulated development (STANKIS *et al.* 1992) and Y and Z have been shown to interact in protein affinity assays *in vitro* (ASADA *et al.* 1997). Furthermore, not all known transcription factors contain an identifiable NLS; it is thought likely that these nuclear proteins gain entry into the nucleus by formation of a complex linking them to a protein that contains an NLS, *e.g.*, TFIIE-α and TFIIE-β (BOULIKAS 1994). In fact, in *C. cinereus* a pair of bipartite NLS motifs have been identified in the HD1 class of mating-type proteins and, similarly to the case in *S. commune*, no NLS motif has been found in the HD2 class of proteins (TYMON *et al.* 1992).

Our *in vitro* protein affinity studies show that Y binds with both Y and Z and Z similarly binds with both Y and Z (ASADA *et al.* 1997). All of these protein interactions occur regardless of the mating types involved (*i.e.*, *self* or *non-self*). In the present genetic study the Y4 mutants that had essential NLS sequences deleted are complemented by the NLS of wild-type Y proteins when present in the matings. The simplest explanation of this result is that multiple Y proteins are involved in a protein complex by which the wild-type Y transports the mutant Y protein into the nucleus. In this context the mutant protein can participate in protein complex formation necessary to activate development by interacting with the Z protein of a different mating type. Because Y-Z interaction is the basis for activating development (SPECHT *et al.* 1992) we postulate that Z also is most likely part of the same protein complex, but we do not yet have proof for this. The proposed mechanism of complex formation is consistent with our previous data that showed the *in vitro* association of Y proteins with

self and/or non-self Y proteins, as well as self and/or non-self Z proteins (Asada *et al.* 1997). Could it be that only non-self Y and Z proteins bind together in complex formation with sufficient tenacity to allow Z protein to be transported into the nucleus, thus providing a mechanism underlying the specificity behavior of the Aα mating-type proteins?

Our experiments are the first demonstration of Y-Y protein interactions *in vivo*. We paired strains containing Y4 deleted for the NLS: (1) with strains containing a wild-type Y or (2) with strains containing no other Y. In the former, A-regulated development occurred, whereas no development occurred in the latter. Because the presence or absence of wild-type Y was the single genetic difference distinguishing the results we conclude that Y-Y protein interactions occur *in vivo* as we had demonstrated *in vitro* (Asada *et al.* 1997). This result is also consistent with our previously postulated hypothesis that the Y-Z complex in Schizophyllum is likely to be higher order than dimer (Asada *et al.* 1997).

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