An Extragenic Suppressor of the Mitosis-Defective *bimD6* Mutation of *Aspergillus nidulans* Codes for a Chromosome Scaffold Protein

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Manuscript received June 7, 1995
Accepted for publication November 16, 1995

ABSTRACT

We previously identified a gene, *bimD,* that functions in chromosome segregation and contains sequences suggesting that it may be a DNA-binding protein. Two conditionally lethal mutations in *bimD* arrest with aberrant mitotic spindles at restrictive temperature. These spindles have one-third the normal number of microtubules, and the chromosomes never attach to the remaining microtubules. For this reason, we hypothesized that BIMD functioned in chromosome segregation, possibly as a component of the kinetochore. To identify other components that function with *bimD,* we conducted a screen for extragenic suppressors of the *bimD5* and *bimD6* mutations. We have isolated seven cold-sensitive extragenic *su*ppressors of *bimD6* heat sensitivity that represent three or possibly four separate *sud* genes. We have cloned one of the suppressor genes by complementation of the cold-sensitive phenotype of the *sudA3* mutation. SUDA belongs to the DA-box protein family. DA-box proteins have been shown to function in chromosome structure and segregation. Thus *bimD* and the *sud* genes cooperatively function in chromosome segregation in *Aspergillus nidulans.*

CONSERVATION of cell cycle molecules has made genetic and biochemical studies of simple organisms particularly attractive. Genetic approaches in the filamentous fungus *Aspergillus nidulans* have fueled progress toward unraveling complexities of nuclear division (DOONAN 1992). A collection of heat-sensitive (Hs^-) mutations in *A. nidulans* that cause an increase in mitotic index at restrictive temperature were designated *bim* for *b*locked *i*n *m*itosis (MORRIS 1976). Conditional mutations that disrupt transit through specific cell cycle stage(s) have provided the opportunity to identify, clone and characterize genes coding for structural and regulatory components of the cell cycle machinery. We have reported cloning and sequencing of the *bimD* gene (DENISON *et al.* 1992). *bimD* encodes a protein that shares sequence motifs with known DNA-binding transcription factors. *bimD6* mutant germlings at restrictive temperature block in anaphase and eventually undergo nuclear fragmentation (DENISON *et al.* 1992). Since the defective mitotic phenotype of *bimD6* mutants, as viewed by electron microscopy, includes the failure of chromosomes to attach to spindle microtubules, the BIMD protein may function in kinetochore assembly and competency (OAKLEY 1981). Additionally, *bimD* mutants are sensitive to DNA-damaging agents (DENISON *et al.* 1992; DENISON and MAY 1994). Therefore, another possible BIMD function is to regulate mitotic progression in response to DNA integrity. This observation and the fact that BIMD overexpression results in arrest of the nuclear division cycle led us to investigate whether BIMD may function as a checkpoint in cell cycle progression in response to DNA damage. *bimD6* mutant strains respond like wild type to DNA damage with delay of the nuclear division cycle, but upon resumption of the cell cycle, they enter a catastrophic mitosis (DENISON and MAY 1994). These experiments prove that BIMD does not perform a checkpoint function.

We describe here the isolation and characterization of extragenic supressors, *sud* (*su*ppressor of *bimD6*) genes, for the *bimD6* Hs^- phenotype. At least three, and possibly four, *sud* genes were identified during the screen. In addition to their ability to suppress *bimD6*, *sud* genes are themselves required for successful nuclear division in *A. nidulans.* Complexities in genetic interactions between *sud* alleles were uncovered that may indicate that the SUD proteins function together in a protein complex. For example, the *sudA3* and *sudC4* mutations exhibit unlinked noncomplementation. Our data suggest that the *sudA, sudB, sudC* and *sud7* gene products participate in the process of nuclear division and cooperate with *bimD* during mitosis.

We also report the cloning of the *sudA* gene and show that *sudA* encodes a member of a newly emerging family of proteins, the DA-box proteins, involved in chromosome condensation and segregation at mitosis (PETERSON 1994). The similarity of the SUDA sequence and members of the DA-box protein family suggests that restoration of the integrity of the *bimD6* kinetochore microtubule system by *sudA* alleles is accomplished through modification of chromosome structure.

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TABLE 1

Strains used in this study

Strain name	Genotype	Source
A122	*riboA1 yA2; nicB8*	FGSC
A612	*AcrA1; riboB2 chaA1*	FGSC
A618	*SulA1 adE20; AcrA1; ActA1 galA1; pyroA4 nicA2; sB3; oliC2 malA1; fwA2*	FGSC
A773	*pyrG89; wA3; pyroA4*	FGSC
4.2.18	*pabaA1; bimD5; sC12; chaA1*	This study
D6.9	*riboA1; bimD6 pyroA4; sC12*	DENISON *et al.* (1992)
D6.1	*pyrG89 yA2; wA3; sC12*	DENISON *et al.* (1992)
5-8	*sudA6 riboA1; bimD6 pyroA4; sC12*	This study
5-8(4-9)	*sudA6 pyrG89; pyroA4; sC12*	This study
5-8(2-5)	*sudA6 pyrG89; wA3; pyroA4; sC12*	This study
3-10	*sudA5 riboA1; bimD6 pyroA4; sC12*	This study
3-10(1-6)	*sudA5 pyrG89; bimD6 pyroA4*	This study
3-10(4-20)	*sudA5 riboA1; wA3; bimD6 pyroA4*	This study
8-16	*riboA1; bimD6 pyroA4 sud7; sC12*	This study
8-16(3-6)	*pyrG89; bimD6 pyroA4 sud7*	This study
8-16(1-3)	*riboA1; wA3; bimD6 pyroA4 sud7*	This study
2-16	*riboA1; sudC4; bimD6 pyroA4; sC12*	This study
2-16(2-7)	*pyrG89; sudC4; pyroA4*	This study
2-16(1-4)	*riboA1; wA3 sudC4; pyroA4; sC12*	This study
2-4	*sudA3 riboA1; bimD6 pyroA4; sC12*	This study
2-4(3-10)	*sudA3 pyrG89; wA3; pyroA4*	This study
2-4(1-5)	*sudA3 pyrG89; bimD6 pyroA4*	This study
2-4(2-11)	*sudA3 riboA1; wA3; bimD6 pyroA4*	This study
B3	*sudA1 riboA1; bimD6 pyroA4; sC12*	This study
B3(3-18)	*sudA1 pyrG89; pyroA4*	This study
B3(1-20)	*sudA1 riboA1; wA3; pyroA4*	This study
C25	*riboA1; bimD6 pyroA4 sudB2; sC12*	This study
C25(1-6)	*riboA1; wA3; bimD6 pyroA4 sudB2*	This study
Diploids		
GR5/A618	*SulA1/SulA pyrG89/pyrG89 adE/adE20; wA/wA3 AcrA/AcrA1; galA/galA1 ActA/ActA1; pyroA4/pyroA4; nicA/nicA2; sB/sB3; oliC/oliC2 malA/malA1; fwA/fwA2*	This study
2nsudA3-1	*SulA1/SulA sudA3/sudA3 pyrG89/pyrG89 adE/adE20; wA/wA3 AcrA/AcrAl; galA/galA1 ActA/ActA1; pyroA4/pyroA4; nicA/nicA2; sB/sB3; oliC/oliC2 malA/malA1; fwA/fwA2*	

MATERIALS AND METHODS

Media and strains: Media for *A. nidulans* growth and mating were as previously described (COVE 1977; KÄFER 1977). Strains carrying the *pyrG89* mutation were grown on 2% malt extract, 0.2% peptone, 1% dextrose, trace elements, 5 mM uridine and 10 mM uracil and 2% agar. For microscopic studies all strains were grown in 0.5% yeast extract, 20 mM dextrose, trace elements, 5 mM uridine, 10 mM uracil and 8.8 μg/ml riboflavin liquid medium. The *A. nidulans* strains used in this study are listed in Table 1 and carried markers previously described (CLUTTERBUCK 1974). Bacterial media and techniques were essentially as described (SAMBROOK *et al.* 1989).

Mutagenesis and identification of cold-sensitive extragenic suppressors of *bimD*: Conidia from strain D6.9 at a density of 10^8/mL in 0.1 M potassium phosphate buffer pH 7.0 were mutagenized by exposure to 1 μg/mL 4-nitroquinoline 1-oxide (4-NQO) (Sigma) at 37° for 30 min. Mutagen was inactivated by addition of an equal volume of 5% sodium thiosulfate. Mutagenized conidia were washed twice in minimal medium and resuspended in 0.1 M potassium phosphate buffer pH 7.0. Three separate experiments were performed. Aliquots were plated and incubated at 32° for 3 days to determine survival rate. The remaining mutagenized spores were then plated in aliquots to give a density of ~50 colonies per plate and incubated at 42° for 3 days. Revertants were colony purified and retested for growth at 42°. Revertant colonies (1505) were replica plated and tested for cold sensitivity (Cs^-) at 25° for 5–7 days. The spontaneous reversion frequency of *bimD6* Hs^- was 8.5×10^{-8}. Two percent of *bimD6* conidia remained viable following 4-NQO treatment. The frequency of induced mutation to an Hs^+ phenotype was 1.9×10^{-5}.

Genetic manipulations: Sexual crosses were performed using standard genetic techniques (PONTECORVO *et al.* 1953). Linkage group assignments were by traditional methods of parasexual genetic analysis (PONTECORVO *et al.* 1953; KÄFER 1977) using diploids made between strain A618 that carries genetic markers on each of the eight *A. nidulans* linkage groups and the following suppressor strains: 5-8(2-5), 3-10, 8-16(3-6), 2-16(1-4), 2-4(3-10), B3(3-18), C25. Diploids were spotted on complete medium containing 2 μg/mL benomyl (DuPont) and incubated at 37° for 1–2 days. Under these conditions random chromosome loss occurs. Mycelial growth was torn from the small colonies that formed on the benomyl plates and rescued onto complete medium. Haploid sectors of growth formed after 2 days of growth in the absence of benomyl. These were picked onto grids, replica plated and incubated at 25° to score Cs^-. Replicas were also made onto appropriate selective media at 37°. Mutations were assigned

to linkage group by virtue of linkage with markers whose chromosomal location is known.

MMS sensitivity assays: MMS survival studies were conducted as previously described (DENISON *et al.* 1992).

Microscopy: Conidia were inoculated into petri dishes containing liquid medium and sterile coverslips. Strains were incubated at their respective permissive temperatures during which time germlings adhered to the glass coverslips. Temperature shifts were performed by placing coverslips into medium preincubated at appropriate temperature. Microscopic techniques for glutaraldehyde fixation and 4,6-diamidino-2-phenylindole (DAPI) staining was performed as described (MAY *et al.* 1992).

Chromosome loss assays: Diploid strains GR5/A618 and 2nsudA3-1 (Table 1) were constructed as described below for this study. Homozygotes at *pyrG89* and *sudA* or *sudA3* resulting from mitotic crossover between the centromere of linkage group I and the *pyrG89* locus to produce GR5/A618 and 2nsudA3-1, respectively, were identified by recovering 5-fluoro-orotic acid (5-FOA)-resistant colonies after plating on rich media containing 5-FOA (PCR, Inc.) at 37° (MAY *et al.* 1989). Colonies that were Cs^- were maintained on minimal medium supplemented with 5 mM uridine, 10 mM uracil and 1 μg/ml pyrodoxine. Colored sectors produced at permissive conditions either alone or after shift from restrictive conditions were easily identified as white (*wA3/wA3*), fawn (*fwA2/fwA2*), or diploid green (*wA/wA3; fwA/fwA2*). Colored sectors were isolated, purified, analyzed for their nutritional requirements, and classified as mitotic crossovers, nondisjunctional diploids or haploids as previously described (MORPUGO *et al.* 1979). Genotypes of purified colored sectors from the *sudA3* diploid 2nA3-1 were determined to discriminate between different events that led to homozygosity for the conidial color mutations. The process of haploidization can occur by a variety of mechanisms distinct from nondisjunction. Such loss of a complete set of chromosomes, including *II* and *VIII*, would produce, in addition to white and fawn sectors, haploid green (blue-green) sectors that are easily distinguished from the bright diploid-green parental color. This type of sector was not recovered among the colored sectors produced by the *sudA3* mutation at restrictive temperature.

Cloning and nucleotide sequencing of *sudA*: Transformation of *A. nidulans* was performed as described (DENISON *et al.* 1992; MAY *et al.* 1992). The wild-type *sudA* gene was cloned from chromosome *I*-specific *A. nidulans* genomic cosmid libraries (BRODY *et al.* 1991) by complementation of Cs^- *sudA3*. Pools of 40 cosmids were cotransformed with pPyrG into strain 2-4(3-10), and transformants were incubated at 20° for 5–7days. Genomic DNA from 14 Cs^+/*pyrG*+ transformants was digested with *Bam*HI and subjected to Southern hybridization with ^{32}P-labeled pLORIST2 (GIBSON *et al.* 1987). To rescue cosmid inserts, genomic DNA from four single integrants was treated with *in vitro* λ packaging extracts and used to transduce *Escherichia coli* LE392 to kanamycin resistance (YELTON *et al.* 1985). Three of the four samples yielded kanamycin-resistant colonies from which cosmids of 40–50 kb were purified. Cosmids rescued from each transformant were identical. The three independently rescued cosmids displayed related but distinguishable electrophoretic patterns following *Eco*RI digestion and they cross hybridized with each other. All three could transform strain 2-4(3-10) to Cs^+. An ~3.2-kb *Pst*I/*Sal*I fragment retained complementing activity and recognized an ~4 kb message on Northern blots of *A. nidulans* poly-A^+ RNA. UniZAP and λgt10 *A. nidulans* cDNA libraries were screened with restriction fragments of *sudA* genomic clones to identify full-length and overlapping cDNA clones (OSMANI *et al.* 1988; MAY *et al.* 1992). Sequencing of *sudA* cDNAs and genomic clones on both strands was performed on dsDNA using the T7 DNA polymerase system (Sequenase, U.S. Biochemical Corp., Cleveland). Ambiguous sequences were resolved using 20-bp primers adjacent to problem areas. Sequences were assembled and analyzed with the aid of the MBCR computer facility at Baylor College of Medicine and the GCG Package of programs. DNA gel electrophoresis and genomic Southern analysis were performed as described (MAY *et al.* 1987).

RESULTS

Genetic characterization of *sud* mutants: Of 3000 ultraviolet light-induced revertants of *bimD5* heat-sensitive (Hs^-) lethality in strain 4.2.18, all *bimD5* extragenic suppressor mutations were Cs^+. In contrast, eight of 1505 4-nitroquinoline 1-oxide (4-NQO)-induced Hs^+ revertants of the *bimD6* allele, in strain D6.9, were extragenic to *bimD6* and additionally Cs^- (Figure 1). The genes in which these suppressor mutations lie were designated *sud* genes (*su*ppressors of *bimD6*) and the eight revertant strains as *sud1–8* in order of isolation. Once allelism was established, each gene was given an alphabetical letter designation (*sudA1, sudA3, sudA5, sudA6; sudB2; sudC4* and *sud7*). Strain *sud8* was subsequently lost and will not be further described.

Some genetic characteristics of *sud* mutants are summarized in Table 2. Mutants were placed into complementation groups to estimate the number of *sud* loci. Genetic complementation of the *sud* cold-sensitive phenotype was analyzed by measuring the ability of appropriate heterozygous *sud* diploid strains to grow at 25°. These tests placed mutations carried by the seven *sud* strains into six complementation groups. Of all diploids generated, only *sud3/4* diploids remained Cs^- for growth at 25°.

Subsequent genetic mapping experiments led to the assignment of the *sud1, sud3, sud5* and *sud6* mutations to linkage group I. Using mitotic recombination in diploids heterozygous for *suds 1, 3, 5,* and *6,* we found that all four mapped to the left arm of linkage group I distal to *fpaB*. As will be discussed later, all four of these *sud* mutants are complemented by the same clone and are thus allelic. We therefore designated them as alleles of the *sudA* gene. *sud2* and *sud7* map to linkage group IV, but we are unable to establish linkage to known genetic markers and so these two suppressors were designated *sudB2* and *sud7*. The *sudB2* and *sud7* mutations are potentially allelic, but we have not clearly demonstrated this by showing linkage between the two mutations. The remaining suppressor, *sud4,* mapped to linkage group II and was designated *sudC4*. These linkage assignments indicate that at least three different *sud* genes have been identified.

***sudA1* and *sudA3* are dominant suppressors of *bimD6*:** The dominant or recessive nature of the two easily scored *sud* phenotypes, cold sensitivity and suppression of *bimD6* heat sensitivity, was determined. Diploids homozygous for the *bimD6* allele and heterozygous at each *sud* locus were constructed. *A. nidulans* diploids

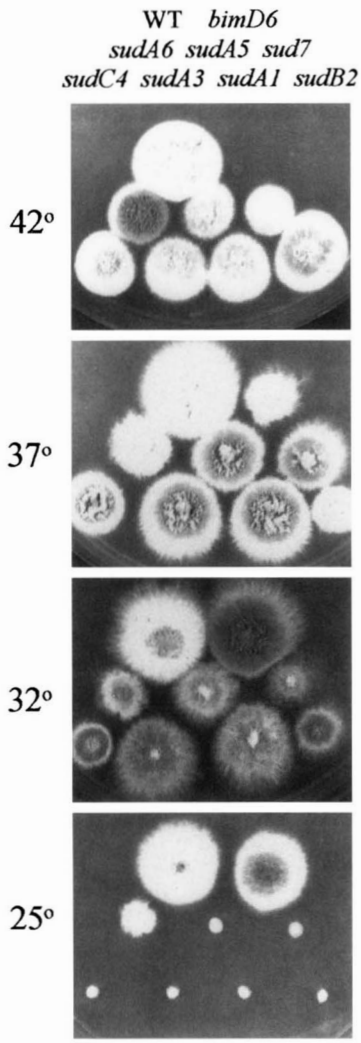


FIGURE 1.—Cold sensitivity of *sud* mutant strains. Wild-type (WT) *A. nidulans* strain GR5, *bimD6* mutant strain D6.9 and *sud/bimD6* double mutant strains 5-8, 3-10, 8-16, 2-16, 2-4, B3 and C25, which carry *sud* mutations *sudA6, sudA5, sud7, sudC4, sudA3, sudA1* and *sudB2,* respectively, were inoculated on complete medium in the pattern indicated by the key at the top of the figure. Plates were incubated at either 42° or 37° for 2 days, at 32° for 4 days or at 25° for 6 days. Wild-type (WT) *A. nidulans* grows well at all four temperatures. The conditional *bimD6* allele is lethal at 42°. The *sud* mutants revert the *bimD6* heat-sensitive growth phenotype and display cold sensitivity at 25°.

were generated by nutritional selection between each of seven *sud* strains and *bimD6* strain D6.1 (Table 1). Diploids were tested for growth at 25° and 42°. The Cs^- phenotype of all *sud* mutations is recessive as all diploids grew at 25°. Diploids heterozygous for either *sudA1* or *sudA3* suppressed the *bimD6* Hs^- phenotype at 42°. Thus, *sudA1* and *sudA3* act as dominant suppressors of *bimD6* heat sensitivity.

Some *sud* alleles do not suppress *bimD6* DNA damage sensitivity: Extragenic suppression need not correct all mutant phenotypes. *bimD6* mutant strains are Hs^- for growth at 42° and display a mitotic defect upon shift to this restrictive temperature. Increased sensitivity to

TABLE 2

Characteristics of *bimD6* extragenic suppressors

Mutation	Linkage group	Cs^- phenotype[a]	Suppression of *bimD6* Hs^-[b]
sudA1	I	Recessive	Dominant
sudA3	I	Recessive	Dominant
sudA5	I	Recessive	?
sudA6	I	Recessive	Recessive
sudB2HIV	Recessive	Recessive	
sudC4[c]	II	Recessive	Recessive
sud7[d]	IV	Recessive	Recessive

[a] Dominance of the Cs^- phenotype of the *sud* mutations was determined in heterozygous diploids.

[b] Dominance of suppression of the Hs^- phenotype of the *bimD6* mutation was determined in diploids homozygous for the bimD6 mutation and heterozygous for each of the *sud* mutations.

[c] Exhibit unlinked noncomplementation.

[d] Cs^- phenotype suppressed by extra copies of *sudA*.

DNA damaging agents, including MMS and ultraviolet light, is also conferred by the *bimD6* mutation at permissive temperature (DENISON *et al.* 1992). This MMS sensitivity (MMSS) is displayed by a loss of conidial viability relative to untreated controls. Survival curves were constructed for a wild-type strain, a *bimD6* strain and each of the *bimD6* and suppressor double mutant strains. Wild-type conidia remained >50% viable after treatment in 0.1% MMS, while the *bimD6* strain remained <10% viable following the same treatment (Figure 2). The suppressor strains fell into two groups relative to their MMSS phenotype. The first group was as sensitive as the original *bimD6* mutant strain and included the suppressors *sudA1, sudA3, sudA5* and *sud7.* The second group showed partial suppression of the MMSS phenotype and comprised the suppressors *sudA6, sudB2* and *sudC4.*

***sud* strains are defective in nuclear division:** Cold sensitivity of *sud* alleles allowed analysis of *sud* genes in a wild-type background, providing insight into the normal role of *sud* gene products in *A. nidulans.* A quantitative approach to describe the terminal phenotype at restrictive temperature of the *sud* mutants was to monitor the chromosomal mitotic index (CMI) up to 8 hr at 25° after temperature shift from 37°. Scorable mitosis in the wild-type strain A122 and *sud7* strains revealed a CMI of 4–5% up to 8 hr (Figure 3A), at which point a subset of *sud7* germlings acquired nuclear characteristics that could not be definitively attributed to any nuclear division cycle stage. In contrast, after 4 hr at restrictive temperature, the CMI of *sudA6* strains was elevated to 14.5%, *sudA3* strains to 25% and *sudA1* strains to 28%. CMI elevation was also seen in *sudA5* strains, reaching a maximum, 17%, after only 2 hr after the temperature shift. Typical chromosome staining indicative of cell cycle stage could be discerned only in a subpopulation of *sudA5* germlings viewed at the 4-hr

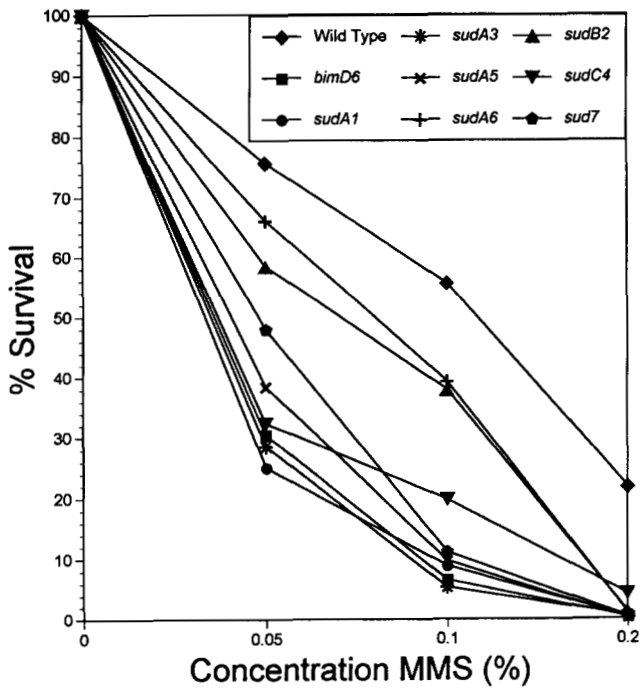


FIGURE 2.—Effect of *sud* mutations on *bimD6* sensitivity to MMS. Conidia from wild-type strain A122, *bimD6* mutant strain D6.9 and *sud-/bimD6* double mutant (strains 5-8, 3-10, 8-16, 2%hyphen;16, 2-4, B3 and C25) were treated and plated as described in MATERIALS AND METHODS to determine the percentage viability. Values represent averages from three independent experiments for each strain.

time point and at the 6-hr time point for *sudA1, sudA3* and *sudA6*. Nuclei frequently appeared abnormal, accumulating large nuclear masses and/or a "fragmented" staining pattern (Figure 3B, d–j). The tendency of *sudA1, sudA3, sudA5* and *sudA6* to stall in mitosis at restrictive temperature and the disorderly nuclear structure derived from mutant *sud* function is reminiscent of that caused by the *bimD6* mutation after shift to its restrictive temperature (Figure 3B, c). At no point following temperature shift did strains carrying the *sudB2* and *sudC4* mutations display normal mitotic configurations, since malformed nuclei prevailed at the first (2-hr) time point. Therefore, CMIs for *sudC4* and *sudB2* strains could not determined. One possible explanation for this is that *sudC* and *sudB* function not solely at mitosis but more generally in maintaining interphase nuclear structure.

Because the *sud* genes have been identified as suppressors of a mutation that disrupts mitosis in *A. nidulans*, it was important to observe the terminal nuclear morphologies of *sud* strains at their restrictive temperature. Wild-type strain A122 conidia germinated at 37° for 5.5 hr and then shifted to 25° produced nuclei displaying typical interphase and mitotic configurations. Interphase is easily identified by the presence of nonstaining, round nucleolar regions and a DAPI-fluorescent crescent of diffuse chromatin (Figure 3B, a). A typical mitosis is characterized by the disappearance of the nucleolus and the condensation of chromatin into a discrete brightly staining chromosome mass (Figure 3B, b). *A. nidulans* germlings harboring *sud* mutations with the *bimD6* mutation undergo normal rounds of interphase and mitosis at 37°. After a shift to 25° for 8 hr, the approximate length of one cell cycle, nuclear morphology of all *sud* strains is strikingly altered. Nuclei are not seen as delimited organelles but rather are stretched about the hyphal interior as disorganized masses of chromatin. *sudA6* nuclei appear to have broken into several smaller DNA-containing pieces (Figure 3B, d) as does the original *bimD6* mutant (Figure 3B, c). The abnormal chromatin structures of *sudA5, sud7, sudC4* and *sudB2* exhibit characteristics of both mitosis and interphase (Figure 3B, e, f, g and j, respectively). Strains carrying the *sudA1* or *sudA3* alleles produce two diffuse chromatin masses connected by a thin chromatin string (Figure 3B, h and i). At 8 hr and at subsequent 4-hr intervals over 24 hr, there is no visible evidence of cycles of chromosome condensation and decondensation. These same abnormal nuclear morphologies are also observed in *sud* strains with a wild-type *bimD* background (not shown).

***sudA3* mutants "lose chromosomes" at high frequency due to nondisjunction of sister chromatids:** *sudA* mutant alleles conditionally disrupt nuclear division in *A. nidulans* germlings (Figure 3). Specifically, the *sudA1* and *sudA3* alleles produce extended nuclear regions in which a string of DNA is seen to connect two masses of diffuse chromatin. Such abnormal nuclear morphologies indicate that some level of nuclear separation, and thus proper chromosome transmission, is defective in *sudA* mutants. We have devised a method to monitor chromosome transmission in *sudA* mutant diploid strains. The first phase of this test detects general abnormal segregation of chromosomes and the second phase discriminates between mechanisms such as "simple" loss, mitotic crossing over, and mitotic nondisjunction. This assay is based on genetic diploid tester analysis used to study environmental agents that cause chromosome abnormalities (MORPUGO *et al.* 1979; KÄFER 1984; CREBELLI *et al.* 1990). Diploids homozygous for *sudA1, sudA3* or *sudA6* were made that allow monitoring of chromosome inheritance. Two strains were generated independently for each mutant allele. *sudA5* was not analyzed due to an apparent dependence on *bimD6*. A potential dependence of the *sudA5* mutant phenotype on the *bimD6* mutation was revealed when all the Cs^- progeny tested from a cross to a wild-type strain were found to still have the *bimD6* mutation. We interpret this to mean that the *sudA5* mutation is either lethal in the presence of the wild-type *bimD* gene or phenotypically silent.

A. nidulans diploid strain 2nsudA3-1 is homozygous at *sudA3* and therefore cold sensitive. The genotype of this *sudA3* diploid was designed to allow obvious visual monitoring of chromosome loss events in a growing

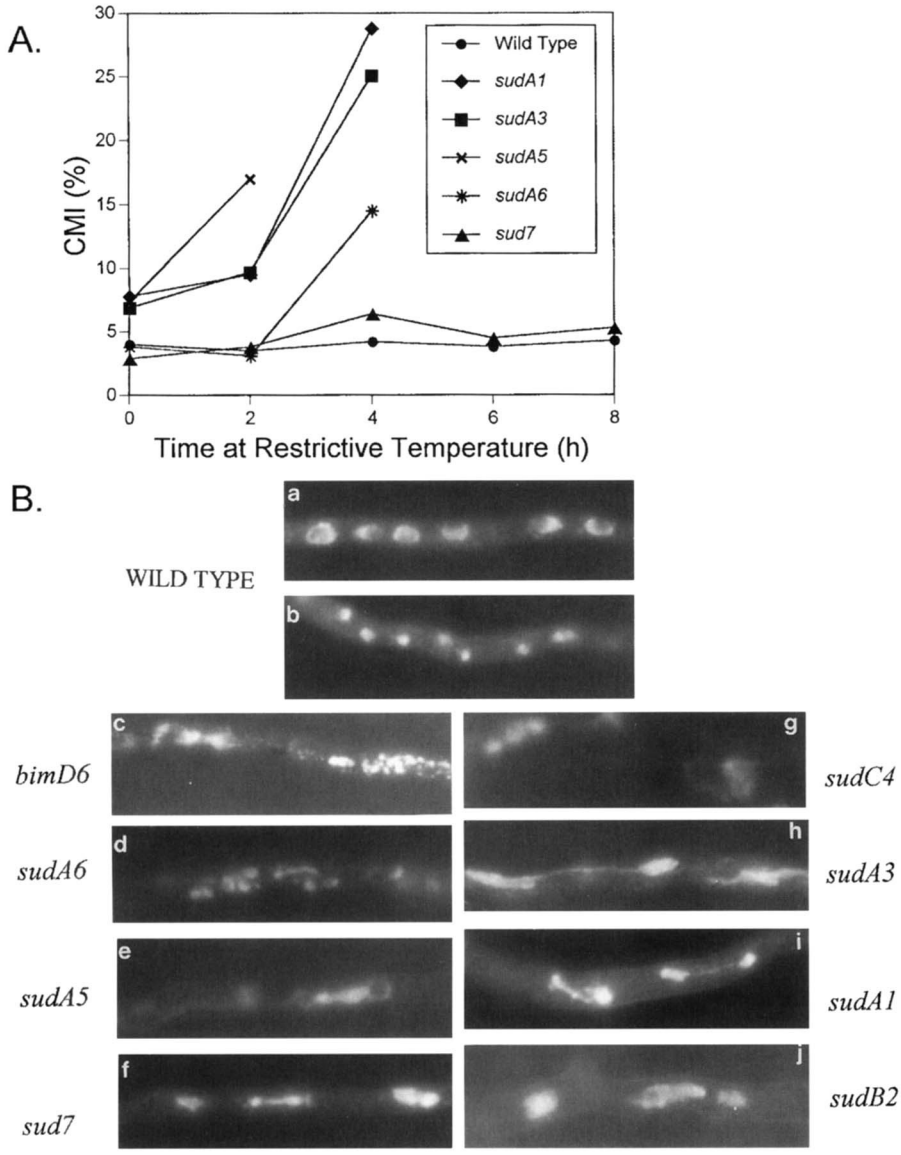


FIGURE 3.—(A) Conidia of wild-type strain A122 and *sud* mutant strains B3(*sudA1, bimD6*), 2-4(*sudA3, bimD6*), 3-10(*sudA5, bimD6*), 5-8(*sudA6, bimD6*), C25(*sudB2, bimD6*), 2-16(*sudC4, bimD6*) and 8-16(*sud7, bimD6*) were germinated at 37° for 5.5 hr and then transferred to 25° at time 0. At 2-hr intervals, 200 germlings were examined for determination of the chromosomal mitotic index (CMI). At time points later than those plotted on the graph, *sud* mutants do not remain blocked in a recognizably mitotic state but rather become abnormal. (B) Nuclear morphology of *sud* mutant strains after shift to restrictive temperature. DAPI-stained *A. nidulans* germlings are shown. A typical wild-type germling with multiple interphase nuclei is shown in a. Condensed mitotic chromatin of a wild-type cell in mitosis is shown in b. A *bimD6* mutant strain after 4 hr at restrictive temperature (c) displays fragmented and/or abnormally extended chromatin masses. d–j are micrographs of each of the *sud* mutants after 8 hr at 25°. All of the mutants display abnormal nuclear morphology that is similar to that seen in *bimD6* mutant strains.

colony (Figure 4A). One linkage group II homologue is marked such that if a nucleus fails to inherit it, a white-spore colored sector is produced due to loss of the wild-type *wA* allele, which is resistant to acroflavin. Likewise, loss of linkage group VIII results in fawn-colored sectors. Colonies of this *sudA3* diploid were grown at 37° following incubation at the restrictive temperature of 20° for 24 or 48 hr, approximately two and four cell cycles, respectively. Abnormal diploid segregation of the *wA3* and *fwA2* markers was seen as white and fawn-colored sectors radiating from the central point of inoculum (Figure 4B, c and d). Loss of these traceable linkage groups was not seen in the control diploid strains between parents GR5 and A618 (Figure 4B, a). The spontaneous incidence of crossovers and nondisjunction in *A. nidulans* are rare, $\sim 1 \times 10^{-4}$ and 1×10^{-3} per generation, respectively (MORPUGO 1963; MORPUGO *et al.* 1979). *sudA3* is likely to be a hypomorphic allele as 2nsudA3-1 kept at 37° on rich medium and not shifted to restrictive temperature also sectored, but to a lesser extent than after temperature shift (Figure 4B, b). Like the control diploid, the *sudA/sudA3* heterzygous diploid used to construct 2nsudA3-1 did not frequently form colored sectors. Thus, unlike the ability of the *sudA3* mutation to act as a dominant suppressor of the *bimD6* heat-sensitive phenotype, it does not act in a dominant manner to affect chromosome segregation.

Genotypes of purified colored sectors from the *sudA3* diploid 2nA3-1 were determined to discriminate between different events that induce mitotic malsegregation. The process of haploidization can occur by a variety of mechanisms distinct from nondisjunction. Such loss of a complete set of chromosomes, including *II* and *VIII*, would produce, in addition to white and fawn sectors, haploid green (dark blue-green) sectors that are easily distinguished from the bright diploid-green parental color. This type of sector was not recovered

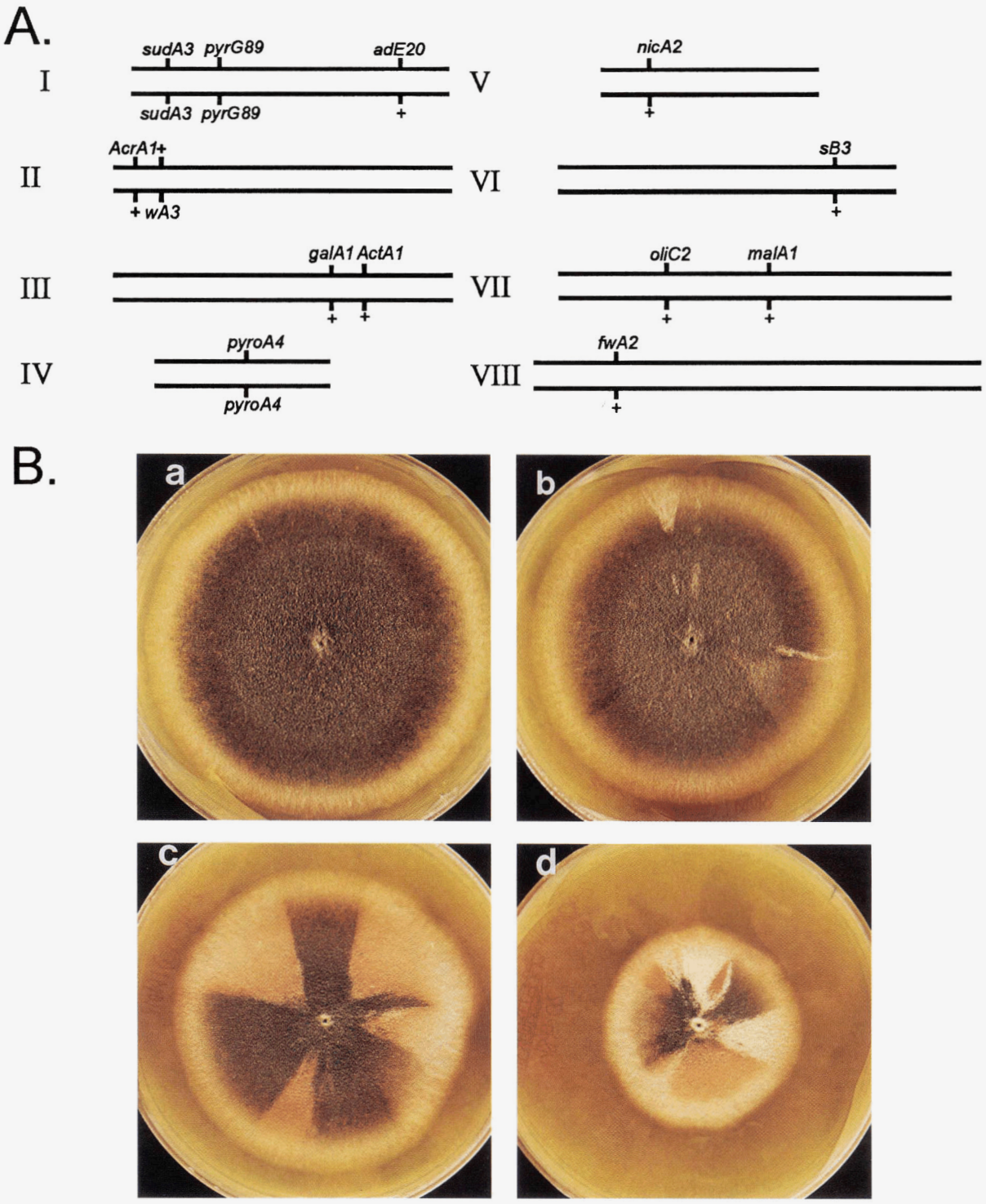


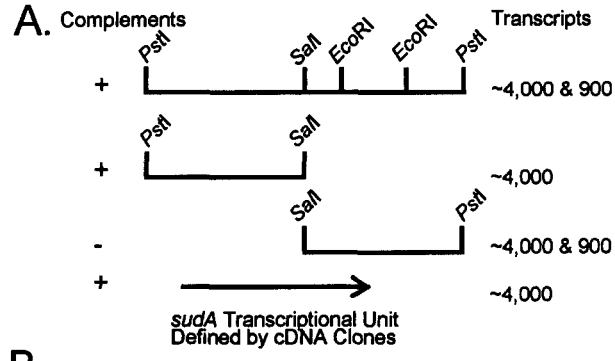
FIGURE 4.—(A) Genotype of the *sudA3* homozygous diploid strain used to assess chromosome stability. The genetic markers on each homologue of the eight linkage groups are shown. (B) Assay for mitotic stability of chromosome segregation. Instability of segregation caused by *sudA3*. Diploid conidia were inoculated at the center of petri dishes containing complete solid medium. (a) Wild-type parental diploid strain GR5/A618 grown at 37° for 3 days is stable. (b) *sudA3* mutant diploid 2nsudA3-1 grown at permissive temperature of 37° for 3 days exhibits a small number of chromosome loss events, seen as small sectors of white or fawn color. 2nsudA3-1 was incubated at the restrictive temperature of 25° for 24 hr (c) or 48 hr (d), and allowed to recover at 37° for 3 days. Elevated chromosome instability is illustrated by the cold-sensitive mitotic sectoring phenotype of the *sudA3* mutation.

among the colored sectors produced by the *sudA3* mutation at restrictive temperature. The diploid *sudA3* tester strain requires uridine/uracil (homozygous for *pyrG89*) and pyridoxine (homozygous for *pyroA4*) and is resistant to acroflavin (due to partial dominance of the *AcrA1* mutation). This diploid is heterozygous for

the genetic markers *adE20; AcrA1; galA1 ActA1; nicA2; sB3; malA1* and *oliC2* so that seven of the eight linkage groups are marked (Figure 4A). A diploid chromosome number, in which the entire chromosome *II* (white sectors) or chromosome *VIII* (fawn sectors) had become homozygous, with no additional rearrangements of genetic loci on any chromosome, was revealed in analysis of nutritional requirements of the recovered sectors. The genotype of 17 colored sectors was determined. Thirteen of the colored sectors were prototrophic. Since the probability of acquiring the wild-type allele for each of the seven genetic markers on the different chromosomes is one out of 128, the prototrophic sectors are likely diploid. The remainder were haploid as they expressed one or more auxotrophic phenotypes. Thus, neither mitotic crossing over (or deletions) or simple chromosome loss leading to haploidization caused instability of chromosome transmission. Instead, production of *sudA3* nondisjunctional diploids has identified failed sister chromatid separation. This demonstrates that *sudA* functions in maintaining the fidelity of chromosome segregation at mitosis.

The *sudA* gene encodes a chromosome scaffold protein: The *sudA* gene was cloned by complementation of the Cs^- phenotype of the *sudA3* mutation. Cosmid pools from two linkage group I-specific *A. nidulans* genomic libraries were cotransformed with the *pyrG* selective marker into strain *sudA3*(3-10) (BRODY *et al.* 1991). Complementing cosmid DNA was recovered from genomes of site-specific single integrants by *in vitro* λ packaging (YELTON *et al.* 1985). A ~6.6 kb *PstI* fragment was found to contain the Cs^--complementing activity. Subclones of this activity were screened for *sudA3*-complementing activity and for the number and size of bands recognized by hybridization to Northern transfers of polyadenylated *A. nidulans* RNA (Figure 5A). These data indicated a transcriptional unit of ~4 kb as the complementing activity. Five overlapping cDNA clones were isolated for the *sudA* transcriptional unit. The longest cDNA clone was able to rescue the cold sensitivity of *sudA3* when cotransformed with the *pyrG* gene. That recombination of this promoterless cDNA results in rescue of Cs^-, presumably by gene conversion, identifies the cloned sequence as containing information wild type to *sudA3*. Further evidence that we had cloned *sudA* was obtained by showing linkage of a transforming plasmid containing the ~6.6-kb *PstI* fragment to the Cs^- *sudA3* mutation in a single copy transformant crossed to an unrelated wild-type strain. The complementing *PstI/SalI* fragment (Figure 5A) not only complements the *sudA3* mutation but also *sudA1, sudA5* and *sudA6*. That only part of the *sudA* transcriptional unit is represented on this genomic fragment indicates that these tightly linked Cs^- loci are alleles of one gene and confirms our genetic mapping.

The nucleotide sequence of both strands of five overlapping *sudA* cDNA clones and derived amino acid sequence was determined (Figure 5B). The longest cDNA clone was 3981 bp in length and so is considered to be full or nearly full length. A single 3633-bp open reading frame (ORF) initiated at a methionine residue at nucleotide position 163 was identified. The predicted 1211 amino acid protein of ~139 kD molecular mass identifies proteins of the DA-box family in searches of the sequence data bases. The DA-box proteins named for a conserved sequence motif found in their carboxyl-terminal domain consisting of aspartate (D) and alanine (A) residues. In addition to *sudA*, 11 other DA-box proteins have been identified in diverse species such as: purple nonsulfur bacteria (FALK and WALKER 1988), mycoplasma as 115p (NOTARNICOLA *et al.* 1991), budding yeast SMC1 and SMC2 (STRUNNIKOV *et al.* 1993), fission yeast as *cut3* and *cut14* (SAKA *et al.* 1994), *C. elegans* as *dpy-27* (CHUANG *et al.* 1994), chicken as ScII (SAITOH *et al.* 1994), and *Xenopus laevis* as XCAP-C and XCAP-E (HIRANO and MITCHISON 1994). A third gene encoding a DA-box protein has been recently



B.

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   1: myvkqiiiqgfksykdqtviepfspkhnvivgrngsgksnffaairfvlsdaythlgree
  61: rqallhegsgsavmsayveiifdnsderfptgkpelvlrrtiglkkdeytldrknatknd
 121: vmnllesagfsrsnpyyivpqgrvtaltnmkdserlnllkevagtqvyearraeslkimh
 181: etnskrekidelldfinerlaeleeekdelrnfqekdkerrcleytiysreqqeiasfld
 241: sleeqrqtgvedtdinfiqgekemaqvdaeiaeckqqieflkvdkaqlederreaskala
 301: qvelqakslsdnqaaaqeskarhdeslkavqsaieerqtelkelvprfisakdaedaara
 361: klteaetarqrlyakqgrnsrfknkserdkwlqaeiknnnasissvqsvlsqtqedindi
 421: endiallepeterlrqidgrgdtiqsveqqvqaakderdrlmdqrkyvdwprtscatlti
 481: hrelwreeakldsilinasnevdraernlsqmmdhntsrgiaavrrikrqhnlegvygtl
 541: aelfevndryrtavevtagqslfhyvvdtddtatkvleilqhekagrvtfmplnrlrtkp
 601: lnmpkasdtipmieklqydrayekafqhvfgktiicpnlqvasqyarshgvnattpegdr
 661: sdkrgaltggfhdsrqsrldavknlakwrdeyetkksrgseirkeleeldqlitravgel
 721: qkleqqrhqvqnssgplrqelrskrdllqkqndnldakrralrniegnlaalkdqvdafe
 781: aelsspfhkaltdeeearleslnsnvqeyrreyqelsgkrseletrksvlevelrenlnp
 841: rldqllaqdadiadedqqgniketqreqkrltkvldklaqrlaqvdesmeqansrvtelt
 901: qrnaesrreleelaksiekhqrrmeksmqkkaaltkqaaecaanirdlgvlpdeaftkyk
 961: ntdsntvvkklhkvnealkkyahvnkkafeqynnftkqretltsrreeldasqksiddli
1021: svldhrkdeaiertfkqvsrefatifeklvpagrgrliiqrktdrtqraeddlesedeea
1081: khsvenyvgvgisvsfnskhddqqriqqdlsggqkslcalalvfaiqacdpapfylfdeid
1141: anldaqyrtavaqmlktisdstngqficttfrpemlhvaekcygvsfrqkastidvvsre
1201: ealkfveeqks
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FIGURE 5.—(A) *sudA* genomic clone analysis and isolation of *sudA* cDNAs. The ability of restriction fragments subcloned, from the ~6.6 kb *sudA3* complementing fragment, to complement Cs^- (+/−) is indicated at the left, and the size of messenger RNA species in nucleotides each subclone recognized by hybridization is indicated at the right. An unidentified transcript unit of 900 nucleotides is present downstream of the *sudA* gene. The location and direction of the *sudA* transcriptional unit is indicated below the genomic map. (B) Predicted amino acid sequences derived from the *sudA* cDNAs. The largest *sudA* cDNA (3981 bp) encodes a single ORF with a predicted polypeptide of 1211 amino acids. The highly conserved sequences found in other DA-box proteins and the putative Walker A and Walker B sites are enclosed in boxes.

identified in *Saccharomyces cerevisiae* (W. JONES and S. ELLEDGE, personal communication). SUDA shares the highest overall amino acid sequence similarity of 49% with SMC2.

Extra copies of *sudA* suppresses the *sud7* Cs$^-$ phenotype: The complex genetic interactions that we encountered among *sud* genes led us to test the possibility that extra copies of *sudA* might act to suppress the Cs$^-$ phenotype of the other suppressor mutations. Another reason to examine this possibility was the fact that the *Scizosaccharomyces pombe cut3-477* mutation was partially suppressed by a multicopy plasmid containing the *cut14* gene. Similarly, at least one other gene in high copy was able to suppress the temperature sensitivity of the *smc1-2* mutation. Transformation of one or more "extra" genomic copies of *sudA* did not affect *sudC4* strains but complemented the cold sensitivity conferred by the *sud7* mutation. The *sud7* suppressor gene maps to linkage group IV and thus represents a genetically separate activity. *sud7* activity may be redundant to that of the SUDA protein or may cooperate with SUDA to carry out a common function.

DISCUSSION

We have isolated seven Cs$^-$ extragenic suppressors, *sud* genes, of the Hs$^-$ *bimD6* mutation. These suppressors formed six complementation groups, but genetic mapping and molecular cloning indicates that we have identified three and possibly four extragenic suppressor genes. The complementation tests were misleading because of the unusual and complex genetic interactions displayed by the suppressor genes. The four *sudA* alleles we isolated displayed linked complementation and the *sudA3* and *sudC4* mutations exhibited unlinked noncomplementation. The nuclear morphology displayed by the extragenic suppressors at restrictive temperature was reminiscent of that seen for the *bimD6* mutation. We have cloned *sudA* by complementation of the Cs$^-$ phenotype of the *sudA3* mutation and have shown *sudA* to code for a DA-box protein.

The DA-box family of proteins was founded by Smc1p (STRUNNIKOV *et al.* 1993). Through genetic and biochemical analyses, DA-box proteins have been shown to contribute directly to the composition of chromosomes, to facilitate higher order mitotic chromosome organization and ensure the fidelity of chromosome segregation. We have shown with a color sectoring assay that *sudA* functions in chromosome segregation. Further insight into the role that *sudA* plays in chromosome maintenance and *bimD6* suppression may be gathered from clues provided by *sudA* relatives. Members of the DA-box protein family function as known chromosomal scaffolding proteins like the ScII protein of chickens (SAITOH *et al.* 1994), while others, like the *S. cerevisiae SMC1* (instability of minichromosomes) gene, functions in chromosome segregation. *SMC1* was the first extensively characterized member of the DA-box family. The *smc1-1* allele was identified in a genetic screen for mutants exhibiting an elevated rate of minichromosome nondisjunction (STRUNNIKOV *et al.* 1993). A second budding yeast DA-box protein was identified in the *SMC2* gene as a partial ORF in a chromosome *VI* clone in attempts to locate and characterize ARS elements. Similarly, in *S. pombe* the *cut3* and *cut14* genes have been shown to function in chromosome condensation and segregation (SAKA *et al.* 1994). In addition to the ScII chromosomal scaffold protein, intimate associations of two *X. laevis* DA-box proteins, XCAP-C and XCAP-E, with chromosomal architecture have been identified (HIRANO and MITCHISON 1994). Finally, the *C. elegans* "dumpy" genes (*dpy*) are required by hermaphrodite individuals (*XX*) for proper dosage compensation (CHUANG *et al.* 1994). Hermaphroditic worms reduce transcription of both *X* chromosomes to achieve a level of *X*-linked gene expression produced by males (*XO*) (HSU and MEYER 1993). It has been suggested that DPY-27 regulates dosage compensation through association with the *X* chromosome. The DPY-27 protein is expressed in both sexes but is specifically localized to the *X* chromosome in only wild-type *XX* animals. DPY-27 may affect a global reduction in gene expression through general modification of chromatin structure specifically of the *X* chromosome.

The eukaryotic DA-box proteins fall into two size classes, large and small. The small family includes SUDA, Smc2p, cut14, XCAP-E and ScII and the large family is composed of cut3, XCAP-C, dpy-27 and Smc1p. The degree of amino acid similarity between SUDA and any other member of the small family ranges from 47.5 to 49%, suggesting that they are all equally divergent from SUDA. The only pair that shows a high degree of similarity is between ScII and XCAP-E with 81% similarity and 68% identity, suggesting that these two may be functionally homologous proteins.

That *sudA* encodes a DA-box protein and was identified as a suppressor of the *bimD6* mutation is consistent with BIMD functioning in chromosome segregation during mitosis. The mitotic defective phenotype of *bimD6* mutant strains at restrictive temperature is characterized by a failure of chromosomes to attach to the spindle microtubules and a reduction in the total number of spindle microtubules. Restoration of BIMD function in *bimD6* mutant strains by the suppressors must then restore the ability of the chromosomes to establish a linkage with the spindle microtubules. One model that can explain this restoration of function is if *bimD* and the *sud* genes function in the assembly and maintenance of the mitotic chromosome. That other DA-box proteins function in chromosome assembly and maintenance of chromosome structure, most notably XCAP-C, XCAP-E and ScII, is consistent with the idea that *sudA* may function in this capacity in *A. nidulans*. Alternatively, the *bimD* and *sud* genes may code for proteins

that have specific functions at the centromere in assembling the kinetochore.

The genetic analysis of the suppressors provides additional insight into the complex interactions of the *sud* genes. The classical inference of the complementation test is that two mutations that fail to complement in *trans* must both block the same function and are therefore assumed to lie within one gene. Exceptional behavior in genetic complementation tests may then expose underlying structure and function relationships between products of different genes whose distinct proteins perform redundant or highly similar activities and/or associate in a complex (BENDER and PRINGLE 1991; DRUBIN 1991; SWANSON and WINSTON 1992). Screens for unlinked noncomplementers have been used to isolate genes whose protein products participate in the same structure or pathway (reviewed in DUTCHER and LUX 1989; FULLER *et al.* 1989; REGAN and FULLER 1990; JAMES and LEFEBVRE 1992; WELCH *et al.* 1993; ERICKSON and JOHNSTON 1993). Allele specificity of noncomplementation may allow an understanding of the actual physical means in which the wild-type polypeptide does not function normally in the presence of two different mutant proteins. Specific noncomplementing alleles may out-compete wild-type proteins for binding to each other so that the relative amount of active complexes falls below a critical threshold. On the other hand the combination of both mutant proteins may produce a complex that dominantly inhibits or "poisons" the process to be complemented (STEARNS and BOTSTEIN 1988). The observed unlinked noncomplementation between *sudC4* and *sudA3* defines a possible physical relationship between the products of the *sudA, sudC* and *bimD* genes in a complex and/or association of SUDA and SUDC. This proposed SUDA/SUDC protein component would function in the same pathway/process as *bimD* either up- or downstream of the BIMD execution point.

At permissive temperature *bimD5* and *bimD6* mutant strains are sensitive to agents that damage DNA. We have previously shown that the sensitivity to DNA-damaging agents was not a failure in DNA damage checkpoints but rather a failure to successfully complete mitosis following reentry into the cell cycle (DENISON and MAY 1994). The *bimD5* and *bimD6* DNA damage sensitive phenotype may result from a general failure to organize chromatin that is structurally competent to support DNA repair processes.

Chromosomes support a variety of activities like DNA replication, repair and transcription. Proteins must act on chromatin to ensure that chromosomes are properly condensed and made ready for segregation at mitosis. We have shown that *A. nidulans* chromosomes need BIMD and SUDA to become competent for segregation at mitosis. For this reason, it is likely that *bimD* and the remaining extragenic suppressor genes code for evolutionarily conserved proteins as well. The identification of the remaining genes and the products they encode will help us to unravel the molecular functions of these proteins in chromosome structure.

This work was supported by a grant from the National Institutes of Health to G.S.M.

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Communicating editor: R. H. DAVIS