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Genetics

Cloning and characterization of a *Neurospora crassa* gene required for (1,3)β-glucan synthase activity and cell wall formation

(*gs-1*/osmotic 1 gene)

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ABSTRACT **The glucan synthase 1 gene (*gs-1*) is required for (1,3)β-glucan synthase activity [E.C. 2.4.1.34; UDP glucose:1,3-β-D-glucan 3-β-D-glucosyltransferase] and for cell wall formation. The *gs-1* gene was cloned by functional complementation of the cell-wall-less defect of the (1,3)β-glucan synthase-deficient mutant, TM1, by using a genomic *Neurospora crassa* cosmid library. A 2568-nucleotide *gs-1* cDNA sequence revealed a 532-amino acid open reading frame encoding a polypeptide of 59 kDa. The predicted *gs-1* gene product has no obvious signal peptide cleavage sites or transmembrane domains. A *gs-1* null mutant is defective for cell wall formation and (1,3)β-glucan synthase activity. The predicted GS-1 protein is weakly homologous to a putative *Saccharomyces cerevisiae* transcriptional regulatory protein.**

Growth and morphogenesis of fungi are the result of the synthesis and subsequent assembly of carbohydrate polymers, including (1,3)β-, (1,6)β-, and (1,4)α-linked glucans and chitin, into cell wall (for review, see ref. 1). The resulting complex cell wall provides structural rigidity and osmotic support to fungal cells (1). Unfortunately, little is known concerning the details of cell wall structure, the regulation of polymer assembly, and the subunit composition of the polymer synthases.

A common approach to the study of cell wall assembly has been the genetic dissection of polymer synthesis. This has involved several strategies: one has been to characterize mutants and genes conferring resistance to killer toxins that bind β-glucans or inhibit β-glucan synthesis. This strategy has led to the hypothesis of a complex pathway of synthesis and incorporation of (1,6)β-glucan into the cell wall in *Saccharomyces cerevisiae* (2). In addition, mutation of one killer-toxin-resistance gene affects both (1,6)β-glucan and (1,3)β-glucan syntheses (3). Two genes affecting (1,3)β-glucan synthesis were isolated using a killer toxin that inhibits (1,3)β-glucan synthesis (4–6). Another strategy used to isolate genes involved in β-glucan synthesis was to screen for mutants with altered morphology that require osmotic support for growth. Two *Aspergillus nidulans* mutants with reduced amounts of cell wall (1,3)β-glucan were isolated (7). Two groups of osmotic remedial mutants of *Schizosaccharomyces pombe* have reduced levels of (1,3)β-glucan synthase activity (8). One of these (1,3)β-glucan synthase mutants was shown to have a defective β subunit of geranylgeranyltransferase type I (9). Although there has been some success in the isolation and characterization of genes involved in cell wall β-glucan synthesis, analysis of these genes, especially those implicated in (1,3)β-linked glucan synthesis, has not resulted in a unifying model of polymer synthesis and cell wall formation.

Our approach to the study of (1,3)β-glucan synthesis has involved the purification of (1,3)β-glucan synthase activity (10) and the isolation and characterization of (1,3)β-glucan synthase mutants of *Neurospora crassa* (11). Cell-wall-less mutants were isolated by mutagenesis of a temperature-sensitive protoplast-forming osmotic 1 mutant (12) and screening for strains that did not regenerate cell wall (11). Twenty-four mutants were isolated, 22 of which were found to have significantly reduced levels of (1,3)β-glucan synthase activity, while none has reduced levels of chitin synthases (11). The (1,3)β-glucan synthase mutants were classified into three complementation groups. In this paper, we report the cloning and characterization of the glucan synthase 1 gene (*gs-1*),† which complemented one group (group III) of the cell-wall-less (1,3)β-glucan synthase defective mutants.

METHODS

Strains and Media. The *N. crassa os-1* (NM233t) *A* and *nic-1* (S1413) *a* were obtained from the Fungal Genetics Stock Center (Kansas City, KS) and crossed to obtain *os-1 nic-1 a* progeny (12). As described (12), cultures of the osmotic 1 mutant (*os-1 nic-1 a*) were grown at 37°C as a population of protoplasts; when cultures were shifted to 25°C, protoplasts regenerated hyphae. A (1,3)β-glucan synthase cell-wall-less mutant, TM1, was derived from the osmotic-1 mutant by ethylmethanesulfonate mutagenesis (11). The formation of a *gs-1* deletion strain, D7, is described in this paper.

Protoplasts were grown in Vogel's medium N (13) supplemented with 7.5% (wt/vol) sorbitol and 1.5% (wt/vol) sucrose (SS medium). Liquid cultures were incubated at 37°C with shaking at 140 rpm. For solid medium, 1.25% (wt/vol) agar was added. Strains requiring nicotinamide were grown in medium supplemented with nicotinamide at 10 μg/ml.

Escherichia coli media LB, 2× YT, and NZYM, and *E. coli* strains LE392, XL1-Blue, DH5, and DH5α are described by Sambrook *et al.* (14).

Preparation of Cosmid DNA. The Orbach/Sachs cosmid library (pMOcosX; ref. 15) was obtained from the Fungal Genetics Stock Center as 50 96-well microtiter plates (G1–G25 and X1–X25). Each well contains one *E. coli* DH5α MCR transformant frozen in LB medium supplemented with 10% (vol/vol) glycerol and ampicillin (50 μg/ml). Microtiter plates were thawed and wells were replicated onto LB solid medium containing ampicillin (50 μg/ml). After incubation of the replicated plates at 37°C overnight, cells were scraped off and suspended in 40 ml of LB medium containing ampicillin (50 μg/ml). These cultures were incubated at 37°C at 225 rpm for 2 h and cells were harvested. Cosmid DNA was isolated using a gentle alkaline lysis protocol, lithium chloride precipitation, and centrifugation in CsCl–ethidium bromide gradients (14).

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. U09275).

Sib Selection. Individual cosmids that complemented the cell-wall-less defect of TM1 were isolated using the sib selection procedure of Akins and Lambowitz (16). TM1 was transformed (see below) with 480-clone pools, 48-clone pools, 8-clone pools, and then DNA from individual wells of the Orbach/Sachs cosmid library.

Transformation. Protoplasts were transformed using a modification (17) of the Vollmer–Yanofsky procedure (18). Aliquots of the transformation mixtures were spread onto plates containing a 7-ml agar-solidified SS medium layer over a 25-ml bottom layer of agar-solidified SS medium supplemented with hygromycin (Calbiochem) at 150 units/ml or benomyl (DuPont) at 250 ng/ml.

(1,3)β-Glucan Synthase Assay. Protoplasts were grown to a density of 1–6 × 10^6 cells per ml and harvested by centrifugation (500 × *g*, 5 min, 4°C). Cell pellets were washed with ice-cold 7.5% sorbitol/1.5% sucrose/25 mM Hepes, pH 7.4, and lysed by suspension in 25 mM Hepes, pH 7.4/10 mM NaH_2PO_4/600 mM glycerol/5 mM EDTA/10 mM NaF/10 μM guanosine 5′-[γ-thio]triphosphate/1 mM dithiothreitol (10). (1,3)β-Glucan synthase activity of cell lysates was assayed with slight modifications of the procedure as described (19). The cell lysate (20 μl containing 25–300 μg of protein) was combined with a 6-μl reaction mixture containing 50 μg of α-amylase and ≈50,000 cpm of UDP[U-^{14}C] glucose (ICN). The final concentration of UDP glucose was 1.15 mM. Protein content of cell lysates was determined by the Bradford method (20) using the Bio-Rad protein dye reagent.

Product Characterization. The radioactive reaction product formed in selected (1,3)β-glucan synthase assay mixtures was incubated with bovine serum albumin (control), α-amylase, or exo-1,3-β-D-glucanase as described (21) to confirm that the product was (1,3)β-linked glucan.

Isolation of *gs-1* cDNA Clones. *gs-1* cDNA clones were isolated from a *N. crassa* λZAP I cDNA library (a gift from Matthew Sachs, Oregon Graduate Institute; ref. 22). The library was screened with a 1.8-kb genomic *gs-1* fragment, labeled with [^{32}P]dCTP (Amersham) by random prime labeling (Boehringer Mannheim) using the Benton-Davis *in situ* plaque hybridization procedure as modified by Sambrook *et al.* (14). pBluescript SK(−) derivatives containing *gs-1* cDNAs were obtained from *gs-1*-containing λZAP I clones by *in vivo* excision using the helper phage R408 (23).

Table 1. (1,3)β-Glucan synthase activities of TM1 and the osmotic 1 mutant transformed with *gs-1*-containing plasmids or a control plasmid

		(1,3)β-Glucan synthase activity	
Strain	Plasmid	Specific activity	% of *os-1 nic-1 a*
os-1 nic-1 a	pMP6	2.0 ± 0.75	100
TM1	pMP6	0.12 ± 0.11	6
TM1	pCE105	0.58 ± 0.37*	29
TM1	G23:3H	0.84 ± 0.53*	41

TM1 and the osmotic 1 mutant (*os-1 nic-1 a*) were transformed with the indicated plasmids. Liquid cultures of hygromycin-resistant transformants were grown as protoplasts at 37°C and harvested, and lysates were assayed for (1,3)β-glucan synthase activity. Specific activity is nmol of glucose incorporated into (1,3)β-linked glucan per min per mg of protein. Data are mean ± SD, based on 7–20 data points from 2–10 transformants for each value. The plasmid pMP6 contains a hygromycin-resistance gene (30) but does not contain *gs-1* DNA. The plasmid pCE105 contains a hygromycin-resistance gene and a 1.8-kb genomic *gs-1* fragment. The cosmid G23:3H contains a hygromycin-resistance gene and a *gs-1*-containing genomic fragment.
*Activities are significantly different ($P < 0.001$) from the activity of TM1 transformed with no *gs-1* DNA (pMP6).

DNA Sequencing. DNA sequencing was done by the dideoxynucleotide method (24), using Sequenase Version 2.0 (United States Biochemical), adenosine 5′-[α-[^{35}S]thio] triphosphate (Amersham), gradient polyacrylamide gels (14), and T3 and T7 primers. Sequencing templates were made by preparing nested sets of exonuclease III deletions (25) and isolating single-stranded DNA from the pBluescript SK(−) derivatives in *E. coli* XL1-Blue using the helper phage VCSM13 (14).

Restriction Fragment Length Polymorphism Mapping of *gs-1*. The *gs-1*-containing cosmid G23:3H digested with *Eco*RI was labeled with digoxigenin-dUTP by random priming (Genius kit, Boehringer Mannheim) and used to probe a Southern blot of DNA from the small set of restriction fragment length polymorphism progeny (26) digested with

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   1                                                AAGGGAATACCCCCCCTTG
  20 AATCTTCGACTCTGGTCCTTTGTCCTCTCCTGTCACCAACGACGGATTGCAAGACCAGCG
  80 CAATTCCTTCTCCCGATTTCGAGCCTCTGACCGCATACATATTCTATCTGATTGCCCAAA
 140 ATGGCTGGCTTATTCAAGGACATCTGGCATGCTATGACCAGTTATGATCGACATGCTGGC
     M  A  G  L  F  K  D  I  W  H  A  M  T  S  Y  D  R  H  A  G    20
 200 ATCGACTCTCCTTACCGAACTGGACGTCATGTCCCATTGAACCGGAACTCCGGTCTCGCG
     I  D  S  P  Y  R  T  G  R  H  V  P  L  N  R  N  S  G  L  A    40
 260 GGCGTTACTACCGCCTCCGACTCGCGTGCCGACATCAACTCGCCCTACCTTCAAGGAGAT
     G  V  T  T  A  S  D  S  R  A  D  I  N  S  P  Y  L  Q  G  D    60
 320 GGCCGTGGTTCTACCATGAGCTTCGACACGGCCTACGGTGGACGCGCCATATCTCCCATG
     G  R  G  S  T  M  S  F  D  T  A  Y  G  G  R  A  I  S  P  M    80
 380 CCGAGTCCCGCCAACGGTGGCCCCTATTCGCCCGGCCTAGTGAGCCAACGACAGTCCGTC
     P  S  P  A  N  G  G  P  Y  S  P  G  L  V  S  Q  R  Q  S  V   100
 440 CACCAGGACGCATTCGATGTACACAGCCCAACCGGCGAGATTCCCATGCAGAACTTCCAA
     H  Q  D  A  F  D  V  H  S  P  T  G  E  I  P  M  Q  N  F  Q   120
 500 AATGGCGGTCCGCCTCCCCCACCAGTCGCTAGCTCCTGGGAGAAAATTGACAGATGGGCC
     N  G  G  P  P  P  P  P  V  A  S  S  W  E  K  I  D  R  W  A   140
 560 GAGGAAAACTACCCCGAGTTGTTCGACCAACTTGGCGAGGGCTGCACCGTCAACGATCTG
     E  E  N  Y  P  E  L  F  D  Q  L  G  E  G  C  T  V  N  D  L   160
 620 AACGAACTCGAATACCAGCTTGATTGCACCCTTCCCCAAGATCTCAGGCAATCCTTGCAA
     N  E  L  E  Y  Q  L  D  C  T  L  P  Q  D  L  R  Q  S  L  Q   180
 680 ATCCACGACGGCCAGGAGCGCGGTGGTCTTCCCACTGGCATTATTTTCAGCTCCATGTTG
     I  H  D  G  Q  E  R  G  G  L  P  T  G  I  I  F  S  S  M  L   200
 740 CTCGACTGCGAAGAGATGGTTCAGGAGTGGGAGAACTGGAAGACGGTGAACCAAGAGTTT
     L  D  C  E  E  M  V  Q  E  W  E  N  W  K  T  V  N  Q  E  F   220
 800 ATGCTGGATCCCGTGCTCGTCAAGCGACAATCTCAGGCATTTGCCGCTCAGGCCTCATCA
     M  L  D  P  V  L  V  K  R  Q  S  Q  A  F  A  A  Q  A  S  S   240
 860 TCAAAAGATGCCCCTAACCGCAACCAAAACTGGCGACAAGAACTTCTCAACAAGCAGGAT
     S  K  D  A  P  N  R  N  Q  N  W  R  Q  E  L  L  N  K  Q  D   260
 920 TCCGTCCCCCCGGCCGCGATTCAGAAGGCGTATGCGCACCCTGCCTGGATTCCTCTCGTT
     S  V  P  P  A  A  I  Q  K  A  Y  A  H  P  A  W  I  P  L  V   280
 980 CGTGACTGGGGCGGCAACAACTTGGCTGTCGATTTGGCGCCTGGACCAAAGGGCCACTGG
     R  D  W  G  G  N  N  L  A  V  D  L  A  P  G  P  K  G  H  W   300
1040 GGTCAAATCATCCTCTTTGGTCGCGACTACGATACAAAGTACGTCGTGGCCCGCTCGTGG
     G  Q  I  I  L  F  G  R  D  Y  D  T  K  Y  V  V  A  R  S  W   320
1100 GCGCACTTCCTTGCCATGGTTGCCGAGGATCTCAGCAGCGGGAGGTGGTTTGTCGATGAG
     A  H  F  L  A  M  V  A  E  D  L  S  S  G  R  W  F  V  D  E   340
1160 GACACCAACGAGCTCAAGCTGCGTGAGTTTAAGGCGACCCGTGTTGAGCCGTCTTATTTC
     D  T  N  E  L  K  L  R  E  F  K  A  T  R  V  E  P  S  Y  F   360
1220 GAGATTCTGAGGTGGAGAATGGATCAGAAGTATGGTCGCACAGCCAACAAGCGCAAATCT
     E  I  L  R  W  R  M  D  Q  K  Y  G  R  T  A  N  K  R  K  S   380
1280 ATGGCGCCTTCCATGGCGTCAGCTTCCGGCATGCGCTCTCCCCCCACTCCCGGCTCTCCC
     M  A  P  S  M  A  S  A  S  G  M  R  S  P  P  T  P  G  S  P   400
1340 TACCAAAGCCCAACAGAGCACAACGAGCCTCGTGGCCGGTCGCTACACCGTCTCACTGGC
     Y  Q  S  P  T  E  H  N  E  P  R  G  R  S  L  H  R  L  T  G   420
1400 ACTTCACCCATGTCGAGTCCCATCCGACCAGGTTACGGAAAGCCAAGCCCATTGGCGCGC
     T  S  P  M  S  S  P  I  R  P  G  Y  G  K  P  S  P  L  A  R   440
1460 GTTGCTGAGGAGGCACCCCCCACAACCTCTCTCACGGCTAGCAACGCCTCCCTCGAGGCC
     V  A  E  E  A  P  P  T  T  S  L  T  A  S  N  A  S  L  E  A   460
1520 AAAGCCGCCGACAACTTGATGGAGTTGAACACCCCAAGGACAAGCGGAGAGCATAGCAAG
     K  A  A  D  N  L  M  E  L  N  T  P  R  T  S  G  E  H  S  K   480
1580 GAGGATATCAAGGTCAATGAGGATTCTCCCGCCAAGGAAAGGACAAGCGAGGATAAGGAG
     E  D  I  K  V  N  E  D  S  P  A  K  E  R  T  S  E  D  K  E   500
1640 AAGAAGCCTGAAACCGAGGCGAACGGAAAGGCGACGGAGTCAAAGGGCAAGCAAACGACA
     K  K  P  E  T  E  A  N  G  K  A  T  E  S  K  G  K  Q  T  T   520
1700 GTCGAAGACGCCGAGGACATGAAGGATATCGAGATTTAAAAGGGAACAAATCGAAGCGAC
     V  E  D  A  E  D  M  K  D  I  E  I  ***                      532
1760 CCAACGAACTCGTCAACTCATGGGTCACCATTGAGTAGCTGGCCCAGCAAACTGCTTCCT
1820 CACCATGCGCAGGGCACGGAGTCAGACAAGGTGTAATGTTTCTCCCTAATCTGATATCTT
1880 GACGCCTCCTCGAGCCAGTTGTCCTCTCTTGGCCGAATATAAGGGCCATCGCATGATGAA
1940 GGGGCAGGTCACGAATCTTTGTGTTCCAATTACTTCTTCCTCGCCACCATACGATTAGTC
2000 TCTATCGTCTAATCGGGTTCTTTCATGATGCGGGGGTTTTCCCTGCGTTGTCCTCTCGCC
2060 TGTTTGGCCAAGGAGAGGGGGGTTTTCGAGGGACAATCTCGTTGCATCGCCGAAGTTTGA
2120 ATATTGTATGTACACTAAAAGGGGATCATTTTGACTTCGACTGGTGGGTGGGACCTTGTT
2180 GTGGTCTGCCCGACGCCGCCCGCAGAAGACATTTTCAGCTTTGCACTATTGTTTACTATT
2240 ACACTGCATAGCGTCATAATAACTCTTTCTGTCTTTATTTTCTGCATCCAAGCTGTCTTG
2300 GCTTCTGGAGGGGAACTGTGGTTACTTACTTCCCCTGGTGCTGTCGTTGGCTGGTTGTCA
2360 GCCACATGGAAGGAAGTTCACGCTCGGATGCTGAGTTGGTACCGTTGTCAGTGATTTATC
2420 GAACTCGGAAGGCGCTGATTTTTTTTTTAAAGTTCGGACTGTTATTAGGGGGAGTTCGAA
2480 GAGGGTGGTCGAGGGAACAAGTGCTTTGTATTTACATGGGAAGGGTATAGACATGGATGG
2540 TAGGGAGAGAATCAACAAAGCGTAAAAAC
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FIG. 1. Nucleotide sequence and deduced amino acid sequence of a *gs-1* cDNA. The stop codon is represented by ***.

Sph I. Unlabeled pMOcosX was added to the hybridization mixture as unlabeled competitor for the labeled vector sequences.

Isolation and Southern Blot Analysis of Genomic DNA. Genomic DNA was isolated as described by Yarden and Yanofsky (27), with the following modification. After ethanol precipitation, DNA was suspended in 10 mM Tris·HCl/1 mM EDTA, pH 8.0, incubated 1–2 h at 37°C with RNase A (50 μg/ml), precipitated with isopropanol, rinsed in 70% (vol/vol) ethanol, dried, and resuspended in 10 mM Tris·HCl/1 mM EDTA, pH 8.0.

DNA digested with restriction enzymes and separated on agarose gels was transferred to Zeta-Probe GT membrane (Bio-Rad) or Hybond-N membrane (Amersham) and hybridized at high stringency with ^{32}P-labeled DNA according to the membrane manufacturers' recommendation.

Disruption of *gs-1*. The chromosomal copy of the *gs-1* gene was deleted using a one-step gene replacement (28). A selectable marker, the benomyl-resistant allele of β-tubulin (*Bml*, called *tub-2*; ref. 29) was flanked with a 1.5-kb *Eco*RI–*Hin*dIII upstream genomic fragment and a 1.9-kb *Hin*dIII–*Sal* I downstream genomic fragment (shown in Fig. 3). This gene replacement construct was digested with *Not* I and *Sal* I and transformed into protoplasts of the osmotic 1 mutant. Genomic DNA isolated from benomyl-resistant transformants was individually digested with *Eco*RI, *Hin*dIII, and *Sal* I, electrophoresed on agarose gels, and screened for the absence of *gs-1*-containing DNA and the presence of the *Bml* DNA by Southern blot analysis.

RESULTS

Cloning of the Glucan Synthase 1 Gene. A (1,3)β-glucan synthase-deficient mutant of *N. crassa*, TM1, was isolated previously by mutagenizing cells of the protoplast-forming osmotic 1 mutant (12) and screening for cultures that did not regenerate cell wall (11). TM1 was transformed with pools of DNA from the Orbach/Sachs genomic library and plated onto medium containing hygromycin. Of 17,700 hygromycin-resistant transformants visually examined for hyphal regeneration, four hyphal transformants were obtained. Hyphal transformants were only observed when TM1 was transformed with DNA pools from library plates G11–G15 and G21–G25. Single cosmids that complemented TM1 were isolated from the G21–G25 plates by sib selection. Cosmid DNA from wells G23:3H and G23:5G individually complemented the cell-wall-less defect of TM1.

To subclone the *gs-1* gene, G23:5G DNA was digested with restriction endonucleases singly and in combination. The digested cosmid DNA was cotransformed into TM1 with a plasmid containing the benomyl-resistant allele of β-tubulin (29) and resulting benomyl-resistant transformants were screened for hyphal growth. Cosmid DNA digested with *Pst* I and *Hin*dIII complemented the cell-wall-less defect of TM1 and a 4.7-kb *Pst* I fragment from G23:5G that complemented the cell-wall-less defect of TM1 was subcloned. Approximately 2.9 kb of the complementing 4.7-kb fragment contained only cosmid vector sequences. The remaining 1.8-kb fragment contained primarily genomic *N. crassa* DNA and complemented the cell-wall-less defect of TM1.

***gs-1* DNA Complemented the (1,3)β-Glucan Synthase Activity Defect of TM1.** TM1 cells transformed with a control plasmid containing a hygromycin-resistance gene (30), the 1.8-kb *gs-1* fragment, or the *gs-1*-containing cosmid G23:3H were grown and harvested, and cell lysates were assayed for (1,3)β-glucan synthase activity. As shown in Table 1, the (1,3)β-glucan synthase specific activity of TM1 transformed with the control plasmid was only 6% of the activity of the parental strain, the osmotic 1 mutant. In contrast, cells transformed with either the *gs-1*-containing cosmid G23:3H or a plasmid containing the 1.8-kb genomic *gs-1* fragment had 41 and 29%, respectively, of the level of (1,3)β-glucan synthase activity of the parental osmotic 1 mutant (Table 1). Approximately 85% of the radioactive product formed by extracts of TM1 cells transformed with the 1.8-kb *gs-1* fragment was hydrolyzed by (1,3)β-glucanase but was not hydrolyzed by α-amylase (data not shown), indicating that the product formed was (1,3)β-linked glucan.

Sequence of *gs-1*. The 1.8-kb *gs-1* fragment was labeled with [^{32}P]dCTP and used to probe a Northern blot of RNA isolated from germinating *N. crassa* macroconidia. Only a single transcript of ≈3 kb was detected (C.P.S. and M. S. Sachs, unpublished data). The labeled 1.8-kb *gs-1* fragment was also used to screen a mycelial cDNA library and a 2568-nucleotide *gs-1* cDNA was isolated and sequenced.

The *gs-1* cDNA sequence and the deduced amino acid sequence are shown in Fig. 1. The open reading frame encodes a protein of 532 amino acids (M_r, 58,939). This sequence contains no predicted signal peptide cleavage sites or transmembrane domains. Search (July 10, 1993) of the GenBank sequence data base (31) showed that the predicted protein is weakly homologous to a predicted *Saccharomyces cerevisiae* protein, Knr4 (5) or Smi1 (32) protein. As aligned in Fig. 2, the sequence identity between GS-1 and Knr4/Smi1 proteins is only 21% overall (111 of 532 identical). However, sequence identity is much higher over limited parts of the protein; e.g., 47 of 83 residues are identical (57%) from amino acids 259 to 341.

The 1.8-kb Genomic *gs-1* Fragment. As described above, the 1.8-kb genomic *gs-1* DNA fragment complemented the cell-wall-less (1,3)β-glucan synthase defect of TM1. However, restriction mapping and sequencing of the 1.8-kb *gs-1*

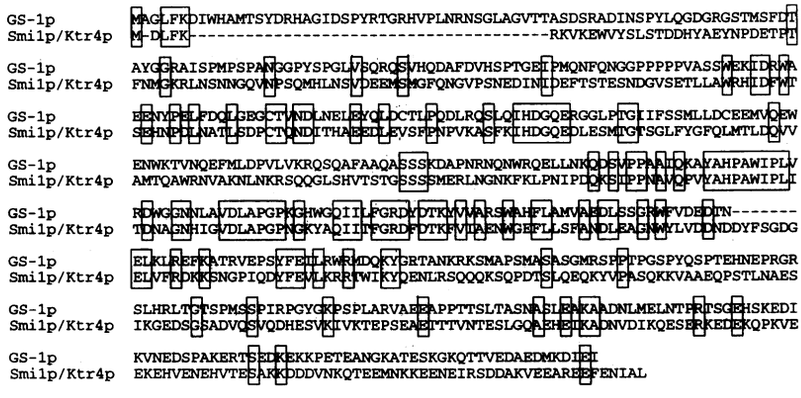

FIG. 2. Sequences and comparison of GS-1 protein (GS-1p) and Knr4/Smi1 proteins (Knr4p/Smi1p). Identical residues are boxed. Dashes represent gaps introduced for maximal alignment of the sequences.

Table 2. Segregation of restriction fragment length polymorphisms detected by G23:3H

	Genotype																			
Marker	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
G23:3H	(O)	O	O	M	O	(M)	M	M	M	M	M	M	M	M	O	M	O	M	M	M
inl	(O)	O	O	M	O	(M)	M	M	M	M	M	M	M	M	O	M	O	M	M	M

Isolates 1–20 are Fungal Genetics Stock Center strains 4411–4430. Isolate 1 is RLM33-1a; isolate 6 is Mauriceville-1cA. O, RLM33-1a parental genotype; M, Mauriceville-1cA genotype. Parentheses surround the parental markers. The *inl* data are from Metzenberg *et al.* (26).

fragment revealed that it does not include the entire open reading frame coded by the 2568-bp cDNA. This truncated genomic fragment encodes a protein ≈140 amino acids shorter (at the C terminus) than the native GS-1 protein and retains the region of high homology between Smi1 and GS-1 proteins (results not shown). Complementation of the TM1 defect by the 1.8-kb *gs-1* fragment could be due to either homologous recombination or to ectopic integration. However, the observation that the 1.8-kb DNA fragment contains 3′ vector sequence and little 5′ flanking sequence makes homologous recombination unlikely.

Chromosomal Localization of *gs-1*. Restriction fragment length polymorphism mapping (26) was used to determine the chromosomal location of *gs-1*. Polymorphisms were observed when DNA from *N. crassa* Oak Ridge and Mauriceville backgrounds was digested with a variety of enzymes and probed with the *gs-1*-containing cosmid G23:3H. When DNA from the small set of restriction fragment length polymorphism progeny (26) was digested with *Sph* I and probed with G23:3H DNA, *gs-1* segregated with the *inl* locus (Table 2), indicating that *gs-1* is located on linkage group V (26).

Deletion of *gs-1*. The chromosomal copy of the *gs-1* gene of the osmotic 1 mutant was replaced with the benomyl-resistant allele of β-tubulin, *Bml* (29), using DNA sequences that flank the *gs-1* gene (construct and flanking sequences are shown in Fig. 3). Cell lysates of a resulting *gs-1* deletion strain, D7, had <3% of the (1,3)β-glucan synthase activity of osmotic 1 mutant cells transformed with a *tub-2*-containing plasmid (0.11 ± 0.03 and 4.7 ± 0.5 nmol per min per mg of protein, respectively; mean ± SD; $n = 6$). This value was similar to the activity of the original (1,3)β-glucan synthase-deficient strain, TM1 (see Table 1). In addition, the small amount of ^{14}C-labeled material produced by D7 *in vitro* (1,3)β-glucan synthase reaction mixtures was not digested by exo-(1,3)β-glucanase (results not shown), indicating that the *gs-1* deletion strain contained no (1,3)β-glucan synthase activity.

DISCUSSION

In this manuscript, we describe the isolation of a (1,3)β-glucan synthase gene by functional complementation of a cell-wall-less (1,3)β-glucan synthase mutant. Our results indicate that the *gs-1* gene product is required for (1,3)β-glucan synthase activity and cell wall formation of *N. crassa*. In addition, our results suggest that there is only one (1,3)β-glucan synthase enzyme in *N. crassa* or that the *gs-1* gene product is required for each (1,3)β-glucan synthase activity measured under the *in vitro* conditions used.

A small region of the *gs-1*-predicted amino acid sequence has significant sequence identity to the predicted protein of a *Saccharomyces cerevisiae* gene independently isolated by two groups (5, 32). The region consists of only 83 amino acids, 16% of the predicted GS-1 protein. The yeast gene, called *KNR4* or *SMI1*, was independently isolated by two groups. The *KNR4* gene was isolated by complementation of the cell wall defects associated with a *knr4* mutation that conferred resistance to the killer toxin that inhibits (1,3)β-glucan synthesis (5). The *SMI1* gene was isolated by complementation of the growth defects associated with an *smi1* mutation isolated in a screen for suppression of the inhibition of transcription by a matrix association region (32). The results of Fishel *et al.* (32) show that the *KNR4/SMI1* gene product is localized to the nucleus, suggesting that it is a transcriptional regulatory protein. The low overall sequence identity between GS-1 and Knr4/Smi1 proteins suggests that, although they may share a common functional domain (e.g., DNA binding), they are not direct homologs. Consistent with this idea is the observation that the *gs-1* null mutant had essentially no (1,3)β-glucan synthase activity whereas the *knr4* null mutant retained 33% of the activity of the wild-type control (5).

Although we have no direct evidence yet, it is tempting to speculate that the GS-1 protein is a transcriptional regulatory protein essential for the synthesis of genes coding for subunits of the (1,3)β-glucan synthase complex. This idea is supported by the observation that the truncated GS-1 protein, coded by the 1.8-kb genomic DNA fragment, retains the area of highest homology between GS-1 protein and the putative regulatory protein Smi1 and, importantly, restores (1,3)β-glucan synthase activity and cell wall formation to TM1. Confirmation of GS-1 protein being a regulatory protein will

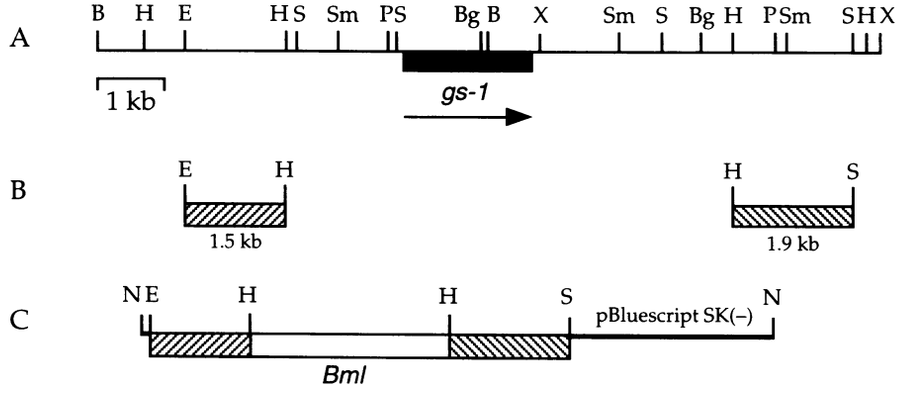

FIG. 3. (*A*) Restriction map of a *gs-1* genomic fragment. The shaded box represents the *gs-1* coding sequence and the arrow indicates the direction of transcription. B, *Bam*HI; Bg, *Bgl* II; E, *Eco*RI; H, *Hin*dIII; N, *Not* I; P, *Pst* I; S, *Sal* I; Sm, *Sma* I; X, *Xho* I. (*B*) Upstream and downstream flanking fragments used to delete the *gs-1* gene. (*C*) Gene deletion construct. The benomyl-resistant allele of β-tubulin, *Bml* (29), was flanked with a 1.5-kb *Eco*RI–*Hin*dIII upstream fragment and a 1.9-kb *Hin*dIII–*Sal* I downstream fragment in pBluescript SK(−).

involve the localization of GS-1 protein to nuclei and the demonstration of specific DNA binding.

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