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Identification of *vib-1*, a Locus Involved in Vegetative Incompatibility Mediated by *het-c* in *Neurospora crassa*

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ABSTRACT

A non-self-recognition system called vegetative incompatibility is ubiquitous in filamentous fungi and is genetically regulated by *het* loci. Different fungal individuals are unable to form viable heterokaryons if they differ in allelic specificity at a *het* locus. To identify components of vegetative incompatibility mediated by allelic differences at the *het-c* locus of *Neurospora crassa*, we isolated mutants that suppressed phenotypic aspects of *het-c* vegetative incompatibility. Three deletion mutants were identified; the deletions overlapped each other in an ORF named *vib-1* (*v*egetative *i*ncompatibility *b*locked). Mutations in *vib-1* fully relieved growth inhibition and repression of conidiation conferred by *het-c* vegetative incompatibility and significantly reduced hyphal compartmentation and death rates. The *vib-1* mutants displayed a profuse conidiation pattern, suggesting that VIB-1 is a regulator of conidiation. VIB-1 shares a region of similarity to PHOG, a possible phosphate nonrepressible acid phosphatase in *Aspergillus nidulans*. Native gel analysis of wild-type strains and *vib-1* mutants indicated that *vib-1* is not the structural gene for nonrepressible acid phosphatase, but rather may regulate nonrepressible acid phosphatase activity.

FILAMENTOUS fungi grow by hyphal tip extension and branching. Within the interior of a colony, hyphae undergo fusion to form a network that makes up the fungal individual (Buller 1933; Glass *et al.* 2000; Hickey *et al.* 2002). Filamentous fungi can also undergo hyphal fusion between isolates, resulting in the formation of a heterokaryon, in which genetically different nuclei coexist in a common cytoplasm. The viability of such heterokaryons, however, is genetically controlled by a number of loci, termed *het* (for *het*erokaryon) or *vic* (*v*egetative *i*n*c*ompatibility; Glass and Kuldau 1992; Leslie 1993). In heterokaryotic cells, a genetic difference in allelic specificity at any *het* locus between two fungal strains causes a phenomenon called vegetative incompatibility (Glass *et al.* 2000; Saupe 2000). In many fungal species, a macroscopic barrage forms when two incompatible individuals meet (Esser and Blaich 1994), which is caused by abnormal or lethal hyphal fusions in the area of contact. Vegetative incompatibility also can be triggered in forced heterokaryons, partial diploids, or transformants that contain *het* alleles of alternative specificity (Mylyk 1975; Perkins 1975; Saupe and Glass 1997; Wu and Glass 2001). In pseudohomothallic fungal species, self-incompatible progeny can be obtained from crosses between two incompatible parents (Saupe 2000; Saenz *et al.* 2001). Phenotypic aspects of vegetative incompatibility include hyphal compartmentation and death (HCD), lack of conidiation (in species that produce conidia), and growth inhibition (Garnjobst and Wilson 1956; Boucherie and Bernet 1978; Jacobson *et al.* 1998; Wu and Glass 2001).

Vegetative incompatibility has been studied extensively in two ascomycete species, *Neurospora crassa* and *Podospora anserina* (for reviews, see Glass *et al.* 2000; Saupe 2000). A total of 9 *het* loci in *P. anserina* and 11 *het* loci in *N. crassa* have been described. Genes at 4 *het* loci in *N. crassa* and 3 *het* loci in *P. anserina* have been cloned. The predicted protein products of these *het* loci are diverse. *N. crassa het-c* and *het-6* and *P. anserina het-s* and *het-e* are not essential for cellular processes other than vegetative incompatibility. They encode a plasma membrane protein, a putative protein, a prion analog, a protein with a GTP-binding site, and a C-terminal WD repeat domain, respectively (Turcq *et al.* 1990; Saupe *et al.* 1995, 1996; Smith *et al.* 2000). The other three *het* loci encode proteins involved in biological processes in addition to vegetative incompatibility. The *N. crassa* mating-type genes, *mat A-1* and *mat a-1*, regulate mating and vegetative incompatibility and encode putative transcriptional factors with predicted DNA-binding domains (Glass *et al.* 1990; Staben and Yanofsky 1990). The *un-24* locus encodes a polypeptide with high identity to the large subunit of type I ribonucleotide reductase (Smith *et al.* 2000a,b), which catalyzes the synthesis of deoxyribonucleotides that are required for DNA replication and repair (Jordan and Reichard 1998). *P. anserina het-c* encodes a putative glycolipid transfer protein required for ascospore maturation (Saupe *et al.* 1994).

Mutations that relieve vegetative incompatibility have also been identified in these two species. Mutations at

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the *tol* locus suppress mating-type vegetative incompatibility in *N. crassa* (Newmeyer 1970; Vellani *et al.* 1994), and *tol* mutations do not affect vegetative incompatibility mediated by other *het* loci (Leslie and Yamashiro 1997). *tol* encodes a putative protein (Shiu and Glass 1999) that shares three conserved amino acid regions with HET-6 in *N. crassa* and HET-E in *P. anserina* (Smith *et al.* 2000). In *P. anserina*, *mod* (for *mod*ifier) mutants that suppress some phenotypic aspects of vegetative incompatibility have been described (Saupe 2000). Mutations in *mod-A* relieve growth inhibition caused by interactions between nonallelic *het* loci, but cannot fully relieve HCD (Belcour and Bernet 1969). Complete suppression of vegetative incompatibility in a *mod-A* mutant requires a second mutation at the *mod-B* locus. *mod-A* has been cloned and encodes a novel protein (Barreau *et al.* 1998). Mutations at another *mod* locus, *mod-C*, suppress nonallelic vegetative incompatibility between *het-R* and *het-V*, but not other nonallelic incompatibility interactions. *mod* mutants also show morphological or developmental defects. Extragenic mutations (*mod-D* through *mod-G*) that suppress these morphological or developmental defects have been identified. Two of these genes, *mod-D* and *mod-E-1*, have been characterized at the molecular level. They encode an α-subunit of trimeric G protein and HSP90, respectively (Loubradou *et al.* 1997, 1999).

The *het-c* locus in *N. crassa* has been used as a model system to understand molecular mechanisms of nonself-recognition (Saupe *et al.* 1996; Saupe and Glass 1997; Wu and Glass 2001) and to assess selection mechanisms for polymorphisms at *het* loci (Wu *et al.* 1998; Muirhead *et al.* 2002). The *het-c* locus encodes three allelic specificities, termed $het\text{-}c^{OR}$, $het\text{-}c^{PA}$, and $het\text{-}c^{GR}$ (nomenclature is based on *het-c* allelic specificity of laboratory strains). The polypeptides encoded by the three *het-c* allelic specificities are similar except for a variable domain of ∼34–48 amino acids. This polymorphic region is necessary and sufficient to confer *het-c* allelic specificity (Saupe and Glass 1997; Wu and Glass 2001). HET-C is a plasma membrane protein; non-self-recognition is correlated with the formation of a HET-C heterocomplex composed of HET-C polypeptides of alternative specificity (S. Sarkar, G. Iyer and N. L. Glass, unpublished data).

In an effort to identify components of vegetative incompatibility in addition to *het-c*, we identified a number of mutants that suppressed *het-c* vegetative incompatibility. Previously, we reported on the isolation of a mutant (*ahc*) identified from a strain that had "escaped" from *het-c* vegetative incompatibility (Xiang *et al.* 2002). "Escape" is a process whereby strains that contain *het* alleles of alternative specificity (and display slow, aconidial growth) recover to wild-type-like growth (faster growth and conidiation). The escape process has been associated with deletions and point mutations in genes that relieve vegetative incompatibility, such as within the *het* locus itself, or in suppressor loci (Newmeyer 1970; DeLange and Griffiths 1975; Smith *et al.* 1996; Coustou *et al.* 1999). The *ahc* mutant carries a large deletion (∼26 kbp) covering a number of predicted open reading frames (ORFs), including a locus, *ham-2*, which is required for hyphal fusion (Xiang *et al.* 2002). The introduction of *ham-2* into the *ahc* mutant complemented morphological defects, such as the lack of aerial hyphae formation and hyphal fusion, but did not complement *het-c* vegetative incompatibility, indicating that a different ORF in the deletion region of the *ahc* mutant was required for this process. In this study, we isolated two additional mutants, *vc1* and *vc2* (for *v*egetative incompatibility and *c*onidiation), from other escape strains that suppressed *het-c*-mediated vegetative incompatibility. The mutations in *vc1* and *vc2* were also deletions in the same chromosomal region as the *ahc* deletion; the three deletions overlapped in a region covering an ORF. Mutations in this ORF (named *vib-1*, for *v*egetative *i*ncompatibility *b*locked) restored growth and conidiation in *het-c* incompatible heterokaryons, but only partially suppressed HCD.

MATERIALS AND METHODS

***N. crassa* strains and heterokaryon tests:** The strains used in this study are listed in Table 1. Strains were cultured on Vogel's medium (Vogel 1964) with supplements as required. Strains were cultured at 25° in 30- or 50-cm-long race tubes to measure growth rates. Crosses were performed on Westergaard's medium (Westergaard and Mitchell 1947). Most strains constructed in this study carry auxotrophic markers. To improve the fertility of these strains, 5–10% of recommended amounts of corresponding supplements for vegetative growth was added to the crossing medium or, alternatively, the helper strain FGSC 4564 (Perkins 1984) was used to form heterokaryons, which were subsequently used as female strains in crosses. Heterokaryon tests were performed by co-inoculating 1–2 μl each from conidial suspensions (∼10^5 conidia/μl) of two different auxotrophic strains onto plates or race tubes containing Vogel's medium (Vogel 1964).

Nucleic acid isolation, Southern hybridization, PCR, and DNA sequence analysis: Genomic DNA was isolated as described (Lee and Taylor 1990). Southern hybridization was performed as described (Sambrook *et al.* 1989). Primers used to amplify an internal fragment (∼800 bp) of *het-c* from the escape transformants were 5′-GGAGACATGGCGATATCG-3′ and 5′-CTCACCCAACACGGAGTG-3′. The $het\text{-}c^{OR}$ and $het\text{-}c^{PA}$ PCR products were distinguishable by *Apa*I digestion. The $het\text{-}c^{OR}$ PCR fragment has an *Apa*I site, while an *Apa*I site is absent in the $het\text{-}c^{PA}$ PCR fragment. Primers used to amplify mutated regions from $vib\text{-}1^{rip}$ mutants were 5′-AATCCGGTG CAGATGAATACTG-3′ at position 54–75 bp downstream of the start codon in the *vib-1* ORF and 5′-ATCTGCTTCGCA GACGTGAACGT-3′ at position 1249–1272 bp downstream of the start codon in the *vib-1* ORF. DNA sequence determinations were performed using the ABI automated DNA sequencing procedure at DNA Sequencing Facility, Berkeley, California (http://idrive.berkeley.edu/dnaseq/web).

Gene cloning: DNA sequences of cosmid H57:G1 (Xiang *et al.* 2002) were from the Munich Information Centre for Protein Sequences (MIPS; http://www.mips.biochem.mpg.de/proj/neurospora/). DNA fragments carrying the *vib-1*

ORF were subcloned from cosmid H57:G1 into plasmid pCB1004, which confers hygromycin resistance (Carroll *et al.* 1994). Spheroplast isolation and transformation were performed as described (Schweizer *et al.* 1981).

Hyphal compartmentation and death assay: Sterile pieces of cellophane (Fisher Scientific) were spread onto the surface of solid medium. Heterokaryons were forced by co-inoculating conidia of two strains ($\sim 10^5$ conidia from each strain) onto the cellophane. At different time points after inoculation, the cellophane containing hyphae was peeled off from the surface of the medium and stained with 1% Evan's Blue (Gaff and Okong'o-Ogola 1971; Jacobson *et al.* 1998). Stained hyphae were examined under bright field using a Zeiss Axioskop II microscope.

Native PAGE analysis of phosphatases: All strains were cultured in liquid Vogel's medium (phosphate rich; Vogel 1964) with corresponding supplements at 30° for 2 days. Cultures were subsequently transferred to either high-phosphate or low-phosphate conditions with constant shaking (100 rpm) at 30° overnight. For high-phosphate conditions, the liquid medium was replaced with fresh phosphate-rich Vogel's medium (Vogel 1964). For low-phosphate conditions, the hyphae were washed thoroughly with distilled water and subsequently transferred to phosphate-depleted Vogel's medium, plus 0.05 mm KH_2PO_4. Harvesting of mycelia, protein extraction, and 8% polyacrylamide gel electrophoresis were performed as described (Hochberg and Sargent 1973). Phosphatase activity was examined as described (Dorn 1965) with minor modifications: the gel was flooded with 0.6 m acetate buffer (pH 4.8) containing 0.05% sodium α-naphthyl acid phosphate (Sigma, St. Louis) plus 0.5% fast garnet G. B. C salt (Aldrich Chemical, Milwaukee) for 1 hr at room temperature. During staining, sodium α-naphthyl acid phosphate is converted into α-naphthol and phosphate by phosphatases. α-Naphthol forms an insoluble dark-brown compound with fast garnet G. B. C salt, which is deposited where phosphatases are located in the gel. The reaction was terminated by washing the gel thoroughly with distilled water.

RESULTS

Identifying suppressors of *het-c* vegetative incompatibility: Our strategy to identify additional components of vegetative incompatibility was to isolate mutants that suppressed the phenotypic aspects of *het-c* vegetative incompatibility, namely growth inhibition, repression of conidiation, and HCD. Incompatible partial diploids, heterokaryons, or transformants that contain *het* alleles of alternative specificity commonly escape from vegetative incompatibility after being maintained in culture for ~2 weeks. The escape process is associated with a sudden increase in conidiation and growth rate of the cultures. Escape has been associated with mutations either in one of the *het* alleles or at a locus required to mediate vegetative incompatibility (Newmeyer 1970; Delange and Griffiths 1975; Vellani *et al.* 1994; Smith *et al.* 1996). In our study, the *het-c^OR^* allele in plasmid pCB1004 (which confers hygromycin resistance; Carroll *et al.* 1994) was transformed into C9-2 (*het-c^PA^ thr-2 a*; Table 1). Approximately 90 hygromycin-resistant incompatible transformants were transferred to slants. Sixty escape transformants were analyzed. Heterokaryon tests and PCR analysis of these escape transformants showed that 24 isolates contained only one *het-c* allele, either $het\text{-}c^{OR}$ or $het\text{-}c^{PA}$. The other 36 escape transformants maintained both $het\text{-}c^{OR}$ and $het\text{-}c^{PA}$ alleles (data not shown). The coexistence of both *het-c* alleles in these escape transformants suggested that extragenic mutations had occurred that suppressed the phenotypic aspects of *het-c* vegetative incompatibility.

To identify the genetic basis of the escape phenotype in transformants retaining both $het\text{-}c^{OR}$ and $het\text{-}c^{PA}$, 14 escape transformants were crossed with RLM 57-30 ($het\text{-}c^{OR}$ *pyr-4 A*; Table 1). All of the escape transformants were fertile as males except for b-11-1, which showed greatly reduced fertility (Xiang *et al.* 2002). We selected for *pyr-4* progeny (and thus $het\text{-}c^{OR}$, to which *pyr-4* is closely linked). Heterokaryons were forced between *pyr-4 a* progeny from each cross and the parental escape transformant [genotype: $het\text{-}c^{PA}$ (pCB1004::$het\text{-}c^{OR}$) *thr-2 a*]. If a suppressor mutation was not linked to *het-c*, the ratio of *pyr-4* progeny forming compatible *vs.* incompatible heterokaryons with the parental escape transformant should be ~1:1. In 11 crosses, all *pyr-4* progeny (~20 progeny from each cross) were incompatible with their parental escape transformants. These mutants presumably contained mutations linked to *het-c* on LGII (which was selected against in this cross) that suppressed vegetative incompatibility or vegetative incompatibility was suppressed by epigenetic mechanisms in the original escape transformants and was not inherited by the progeny. The twelfth cross (b-11-1 × RLM 57-30) led to the identification of the *ahc* mutant (Xiang *et al.* 2002). In the remaining two crosses (b-19-5 × RLM 57-30) and (c3-1 × RLM 57-30), half of the *pyr-4 a* progeny formed compatible heterokaryons with their parental transformants, b-19-5 or c3-1, respectively. These results suggested that b-19-5 and c3-1 carried mutations unlinked to *het-c* that suppressed *het-c* vegetative incompatibility.

Phenotypic characterization of the suppressors: The *pyr-4* progeny from the above crosses that formed compatible heterokaryons with b-19-5 or c3-1 showed a similar phenotype of profuse conidiation (Figure 1A; 9-39-10 is descended from b-19-5 and 24-24-9 is descended from c3-1). Approximately one-half of the *thr-2* progeny (from both b-19-5 and c3-1 crosses with RLM 57-30) also showed a profuse conidiation phenotype, suggesting that the mutation that resulted in the profuse conidiation phenotype in these progeny was unlinked to *pyr-4* or *thr-2* (left arm of LGII, linked to *het-c*).

To determine whether the profuse conidiation phenotype segregated with suppression of vegetative incompatibility, a *thr-2* $het\text{-}c^{PA}$ progeny from b-19-5 × RLM 57-30 showing the profuse conidiation pattern was crossed with RLM 57-30; 74 progeny were analyzed. Thirty-five progeny from the cross showed the profuse conidiation pattern, while the rest of progeny were wild type in phenotype. Progeny carrying the *thr-2* (and thus $het\text{-}c^{PA}$) or the *pyr-4* (and thus $het\text{-}c^{OR}$) marker were recovered. The *pyr-4* $het\text{-}c^{OR}$ progeny with the profuse conidiation

TABLE 1

***N. crassa* strains**

Strain	Genotype	Origin/cross
C9-2	*het-c*^{PA} *thr-2 a*	Saupe *et al.* (1996)
RLM 57-30	*cyh-1; pyr-4 A*	Gift from R. L. Metzenberg
I-1-83	*ad-3A his-3 A*	Gift from A. J. F. Griffiths
I-1-51	*ad-3A nic-2 a*	Gift from A. J. F. Griffiths
RLM 57-26	*pyr-4 arg-5; inl; pan-2 a*	R. L. Metzenberg
C9-15	*het-c*^{PA} *thr-2 A*	Saupe *et al.* (1996)
FGSC 4317	*fl A*	FGSC
FGSC 4347	*fl a*	FGSC
FGSC 4564	*ad-3B cyh-1 a*^{ml}	FGSC
Xa-2	*het-c*^{PA} *arg-5; pan-2 A*	Xiang *et al.* (2002)
Xa-3	*het-c*^{PA} *arg-5; pan-2 a*	Xiang *et al.* (2002)
b-19-5	*het-c*^{PA} *thr-2*; (*het-c*^{OR}); *vcl* a	Escape transformant
C3-1	*het-c*^{PA} *thr-2*; (*het-c*^{OR}); *vc2* a	Escape transformant
8-88	*pyr-4; ahc A*	Xiang *et al.* (2002)
9-1-37	*het-c*^{OR} *pyr-4 a*	Xiang *et al.* (2002)
9-1-5	*het-c*^{OR} *pyr-4 A*	Xiang *et al.* (2002)
9-39-7	*het-c*^{PA} *thr-2; vc1 a*	(b-19-5 × RLM57-30) × RLM57-30
9-39-10	*het-c*^{OR} *pyr-4; vc1 a*	(b-19-5 × RLM57-30) × RLM57-30
X80-32	*het-c*^{OR} *pyr-4; vib-1(1) A*	*RIP* progeny from C9-2 (*vib-1*) × 9-1-5
X80-33	*het-c*^{OR}*; vib-1(2)*	*RIP* progeny from C9-2 (*vib-1*) × 9-1-5
X80-49	*het-c*^{PA} *thr-2; vib-1(1) A*	*RIP* progeny from C9-2 (*vib-1*) × 9-1-5
X39-12	*het-c*^{OR}*; lys-1 ahc a*	Xiang *et al.* (2002)
X43-12	*het-c*^{OR}*; lys-1 vc1 a*	FGSC 4070 × 9-39-32
9-39-32	*het-c*^{OR} *pyr-4; vc1 A*	(b-19-5 × RLM57-30) × RLM57-30
24-24-9	*het-c*^{OR} *pyr-4; vc2; pan-2 a*	(c3-1 × RLM57-30) × Xa-3

phenotype were forced in heterokaryons with *thr-2 het-c*^{PA} progeny with the profuse conidiation phenotype (same mating-type pairing). The conidiation pattern of the resulting heterokaryons and their growth rates were indistinguishable from the mutants by themselves (Figure 2, A and B, heterokaryon (9-39-10 + 9-39-7); Figure 1, A and B). Analysis of crosses with c3-1 yielded similar results. We named the mutants carrying these mutations as *vc1* (mutants derived from escape transformant b-19-5) and *vc2* (for mutants derived from escape transformant c3-1).

In race tubes under normal laboratory conditions, the *vc1* and *vc2* mutants formed dense patches of conidia along the length of 50-cm race tubes that later filled in to form a continuous conidial mat. By contrast, a wild-type strain forms dense conidial patches only at the two ends of the race tube; conidiation in the middle of the race tube is suppressed by a high concentration of CO_2 (Sargent *et al.* 1972). The *vc1* and *vc2* mutants have a slightly slower growth rate, 4–5 cm/day as compared to 6–7 cm/day for a wild-type strain (Figure 1B).

To determine whether the *vc1* and *vc2* mutations were recessive or dominant, heterokaryons were forced between the *vc1* and *vc2* mutants and wild-type strains with *het-c* alleles of the same or alternative *het-c* allelic specificity. Heterokaryons between a wild-type strain (FGSC 4564; Table 1) and the *vc1* or *vc2* mutants (9-39-10 or 24-24-9) of identical *het-c* specificity displayed wild-type growth rates and a normal conidiation pattern, indicating that the morphological phenotype of the *vc1* and *vc2* mutants was recessive (data not shown). Heterokaryons between *het-c*^{OR}*; vc1* or *het-c*^{OR}*; vc2* mutants with a wild-type *het-c*^{PA} strain (Xa-2; Table 1) displayed typical *het-c* vegetative incompatibility during the first 3 days. However, after 4 days an increase in growth rate was observed in the heterokaryons and conidiation began in the middle of the plate (Figure 2C). By contrast, an increase in growth rate and conidiation was not observed in wild-type *het-c* incompatible heterokaryons. These results indicate that the suppression of *het-c* vegetative incompatibility by the *vc1* and *vc2* mutations was not completely recessive.

Complementation between suppressor mutants: The *ahc*, *vc1*, and *vc2* mutants all show suppression of *het-c* vegetative incompatibility and have a similar phenotype, although the *ahc* mutant has additional morphological defects. The *ahc* mutant is female sterile, shows ascus-dominant developmental defects, and is severely restricted in its capacity to undergo hyphal fusion (Xiang *et al.* 2002). Heterokaryon tests were performed to determine if the three mutants can complement each other's morphological defects. A heterokaryon between an *ahc* mutant (X39-12) and *vc1* (9-39-10) or *vc2* (24-24-9) mutants (using a modified heterokaryon test; Xiang *et al.* 2002) of identical *het-c* specificity showed the morphology of a *vc1* or *vc2* mutant. Although the slow mycelial

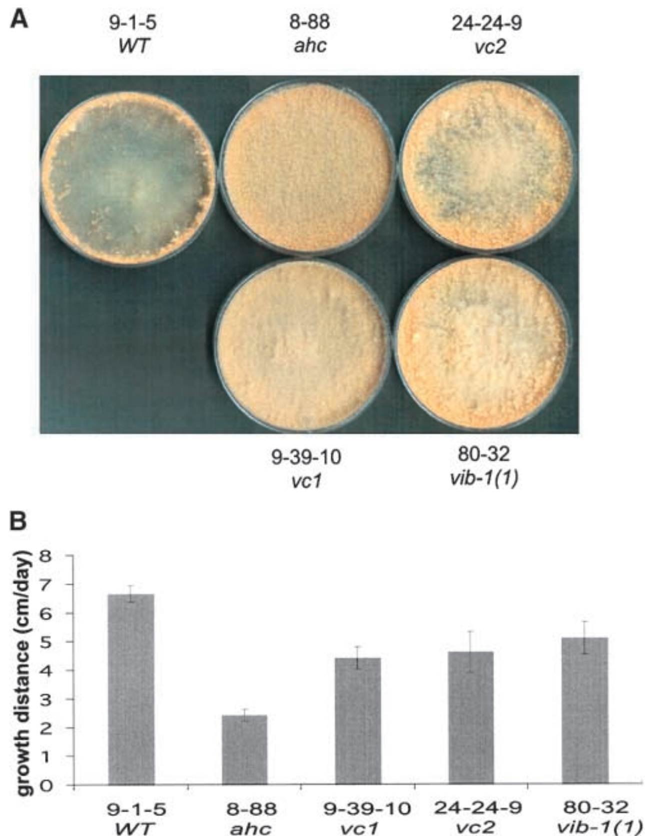


FIGURE 1.—Morphological phenotype of the *ahc*, *vc*, and *vib-1* mutants as compared to a wild-type strain. (A) The phenotype of the 8-88 (*ahc*), 9-39-10 (*vc1*), 24-24-9 (*vc2*), and 80-32 (*vib-1(1)*) mutant strains as compared to wild type when grown on petri plates. Conidiation in a wild-type strain occurs around the perimeter of the plate, while the *ahc*, *vc*, and *vib-1* mutants produce profuse conidia across the plates. (B) The growth rates of *ahc*, *vc*, and *vib-1(1)* mutants as compared to a wild-type strain.

growth of the *ahc* mutant was complemented in a heterokaryon with the *vc1* or *vc2* mutant, the profuse conidiation pattern was not. A heterokaryon between *vc1* mutant (X43-12) and *vc2* mutant (24-24-9) displayed a similar growth rate and conidiation pattern of *vc1* or *vc2* mutants themselves. The above results suggest that the mutations resulting in the conidiation defect in *ahc*, *vc1*, and *vc2* mutants were allelic. We previously mapped the mutation in the *ahc* mutant to chromosome V between *lys-2* and *ilv-2* (XIANG *et al.* 2002).

All three suppressor mutants carry deletions that overlap in an ORF: The *ahc* mutant carries a deletion covering at least eight predicted ORFs, including *ham-2*, a locus involved in hyphal fusion (XIANG *et al.* 2002). Southern hybridization was performed to determine whether *vc1* and *vc2* mutants also carried deletions in this region. Probes were generated from cosmid H57:G1, which was previously shown to span most of the deletion in the *ahc* mutant (XIANG *et al.* 2002). Figure 3 shows that the 6280-bp *Hin*dIII fragment carrying *ham-2* was present in both *vc1* and *vc2* mutants. However, a 5278-bp *Hin*dIII fragment is completely absent from the *vc1* mutant. Most of this fragment is also absent from *vc2*. In the *ahc* mutant, both *Hin*dIII fragments are missing.

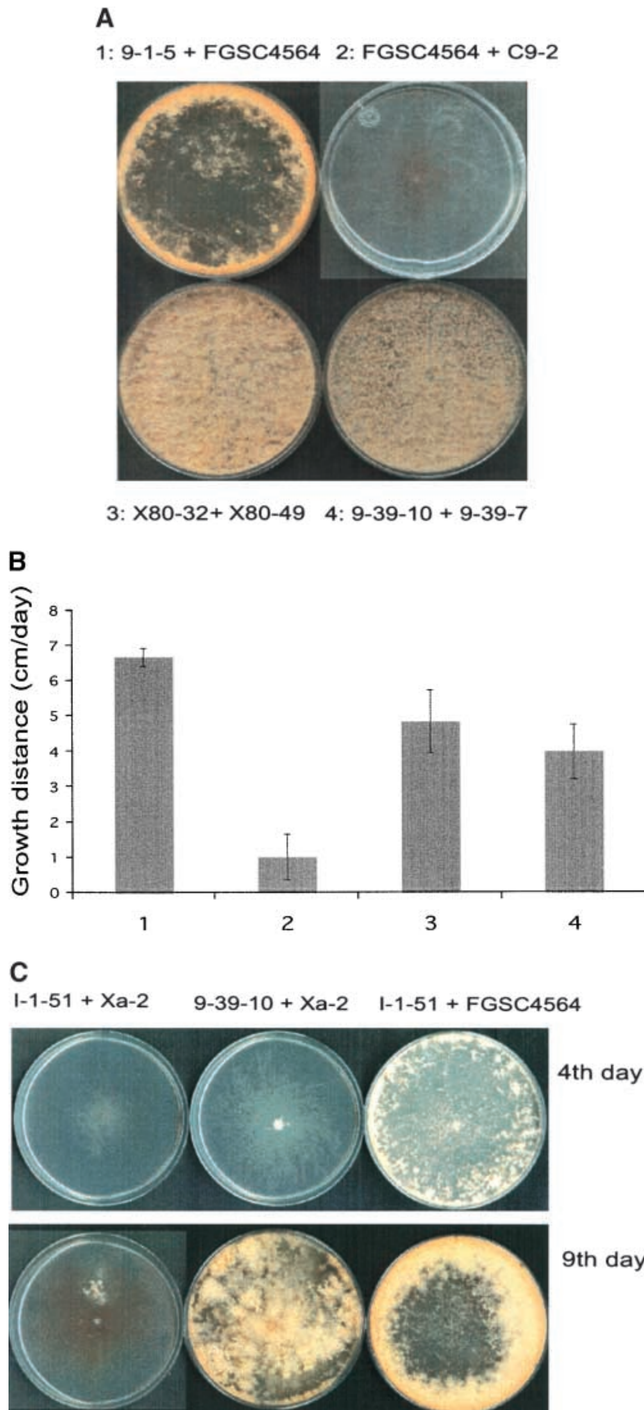
The three deletions in *ahc*, *vc1*, and *vc2* mutants overlap each other in a region (the 5278-bp *Hin*dIII fragment in Figure 3) that spans a predicted ORF. The ORF starts from position 61,496 bp (start codon) and ends at position 59,292 bp (stop codon) in contig 9a36 (http://www.mips.biochem.mpg.de/proj/neurospora/). An ~4-kbp *Sac*I-*Hin*dIII DNA fragment covering the ORF, SAH4-8 (Figure 4), was transformed into two *vc1* mutants, 9-39-7 (*het-c^{PA} thr-2; vc1 a*) and 9-39-10 (*het-c^{OR} pyr-4; vc1 a*; Table 1). The introduction of SAH4-8 into 9-39-7 and 9-39-10 did not fully complement the profuse conidiation phenotype of the *vc1* mutants. To determine whether the introduction of SAH4-8 restored vegetative incompatibility, heterokaryons were forced between 9-39-7 (SAH4-8) and 9-39-10 (SAH4-8) transformants. These heterokaryons displayed *het-c* vegetative incompatibility. We name the ORF required to restore *het-c* vegetative incompatibility in the *vc1* mutant, *vib-1*.

The predicted *vib-1* ORF has two introns and three exons and encodes a predicted polypeptide of 670 amino acids (Figure 4). A consensus sequence for translation initiation sites in Neurospora, CAGTATGGCA (EDELMANN and STABEN 1994), is present around the predicted start codon. A CAAT box is located 54 bp upstream from the start codon and a polyadenylation signal, AATAAA, is 669 bp downstream from the predicted stop codon. The 3′ untranslated region (3′ UTR) is apparently important for *vib-1* function. The introduction of deletion constructs bearing 302 bp (*Hin*dIII site) or 413 bp (*Sac*I) of the 3′ UTR (Figure 4) into the *vc1* mutant does not restore the capacity for vegetative incompatibility.

***vib-1^{rip}* mutants:** Since the deletions in the *ahc*, *vc1*, and *vc2* mutants could possibly cover additional ORFs besides *vib-1*, it was necessary to generate *vib-1* mutants. Repeat-induced point (RIP) mutation is a naturally mutagenic mechanism in *N. crassa* (SELKER 1997) that acts on duplicated sequences in the genome, such as those introduced by transformation. An ~1-kbp *Kpn*I-*Xho*I DNA fragment from the 5′ end of the *vib-1* ORF (Figure 4) was transformed into C9-2 (Table 1). A C9-2 (*Kpn*I-*Xho*I) transformant was crossed with a wild-type strain 9-1-5 (Table 1). Out of 60 progeny, 3 progeny showing a profuse conidiation pattern were recovered. The conidiation pattern of these progeny was similar to *vc1* and *vc2* mutants in plates, race tubes, and slants and their growth rates were also very similar to *vc1* and *vc2* mutants (Figure 1).

The ~1-kbp region between *Kpn*I and *Xho*I in the *vib-1* ORF of all three *RIP* mutants, X80-32, X80-49, and X80-33, was amplified by PCR and cloned. DNA sequencing of the *vib-1* fragment in the X80-32 and X80-49 mutants revealed 6 GC-to-AT transitions, which

are typical for sequences that have undergone RIP (Selker 1997). The mutations occurred in the same sites in the two mutants, suggesting that they came from the same mutagenic event (Figure 4). Three GC-to-AT transition mutations changed Met (55) to Ile, Val (227) to Met, and Trp (260) to a stop codon. The other 3 GC-to-AT transition mutations occurred in the middle of the first intron (after the stop codon). The X80-33 strain contained 26 GC-to-AT transition mutations, which caused 15 amino acid changes and three stop codons. The first stop codon is at Glu (250). Seven amino acid alterations—His (141) to Tyr, Cys (178) to Tyr, Cys (191) to Tyr, Met (200) to Ile, Ala (218) to Val, Val (227) to Ile, and Asp (249) to Asn—occurred in the *vib-1* ORF before the first stop codon at amino acid (aa) 250 (Figure 4). The *vib-1* alleles were named *vib-1(1)* (X80-32 and X80-49) and *vib-1(2)* (X80-33).



The ability of the *vib-1* mutants to suppress *het-c*-mediated vegetative incompatibility was examined by forcing heterokaryons between X80-32 [*het-c^{OR} pyr-4; vib-1(1) A*] and X80-49 [*het-c^{PA} thr-2; vib-1(1) A*]. The (X80-32 + X80-49) heterokaryons displayed a phenotype that was similar to X80-32 or X80-49 mutants by themselves (Figure 1; Figure 2, A and B). Thus, mutations in *vib-1* fully relieve growth inhibition and conidiation repression mediated by *het-c* vegetative incompatibility.

The *vc1* and *vc2* mutations were not completely recessive in heterokaryons with wild-type strains of alternative *het-c* specificity. Similar to the heterokaryon between a *vc1* or *vc2* mutant and a wild-type strain of alternative *het-c* specificity (Figure 2C), heterokaryons between a wild-type strain and a *vib-1(1)* mutant of alternative *het-c* specificity (X80-32 + Xa-3; Table 1) showed more conidiation and less growth inhibition after 3 days than did wild-type *het-c* incompatible heterokaryons. Thus, the *vib-1(1)* mutant phenotype was indistinguishable from the *vc1* and *vc2* deletion mutants.

***vib-1* mutations alter the pattern of HCD mediated by *het-c*:** In a *het-c* incompatible heterokaryon, partial diploid or transformant, ~20–30% of the hyphal compartments have plugged septa and are dead (Jacobson *et al.* 1998; Wu and Glass 2001). The percentage of dead hyphal compartments is fairly uniform across the colony and has not been associated with any obvious developmental or morphological feature (such as hyphal fusion junctions). To assess whether HCD was also suppressed in the *vib-1* mutant, we forced a heterokar-

Figure 2.—*vib-1* mutants relieve repression of conidiation and growth inhibition associated with *het-c* vegetative incompatibility. (A) A compatible heterokaryon (9-1-5 + FGSC 4564) and a *het-c* incompatible heterokaryon (FGSC 4564 + C9-2). A heterokaryon between a *het-c^{OR}; vib-1(1)* (X80-32) and a *het-c^{PA}; vib-1(1)* (X80-49) strain shows profuse conidiation. Similarly, a *vc1* heterokaryon [*het-c^{OR}; vc1* (9-39-10) + *het-c^{PA}; vc1* (9-39-7)] also shows profuse conidiation. (B) The *vib-1* and *vc1* mutations suppress growth rate inhibition mediated by allelic specificity differences at *het-c* and have growth rates similar to the mutants themselves (Figure 1B). The numbers below the columns indicate the heterokaryons with the same numbers shown in A. Standard error bars are shown. (C) The semirecessive nature of *vc1* suppression of *het-c* vegetative incompatibility. The wild-type *het-c* incompatible heterokaryon is (I-1-51 + Xa-2). The heterokaryon developed conidia in a small section of the plate on the ninth day, which was possibly due to mutational processes resulting in escape. A compatible heterokaryon (I-1-51 + FGSC 4564) is also shown. The middle panel shows a heterokaryon between a *het-c^{OR}; vc1* strain and a *het-c^{PA}* strain (9-39-10 + Xa-2). Conidiation was observed on the fourth day and spread across the plate by the ninth day.

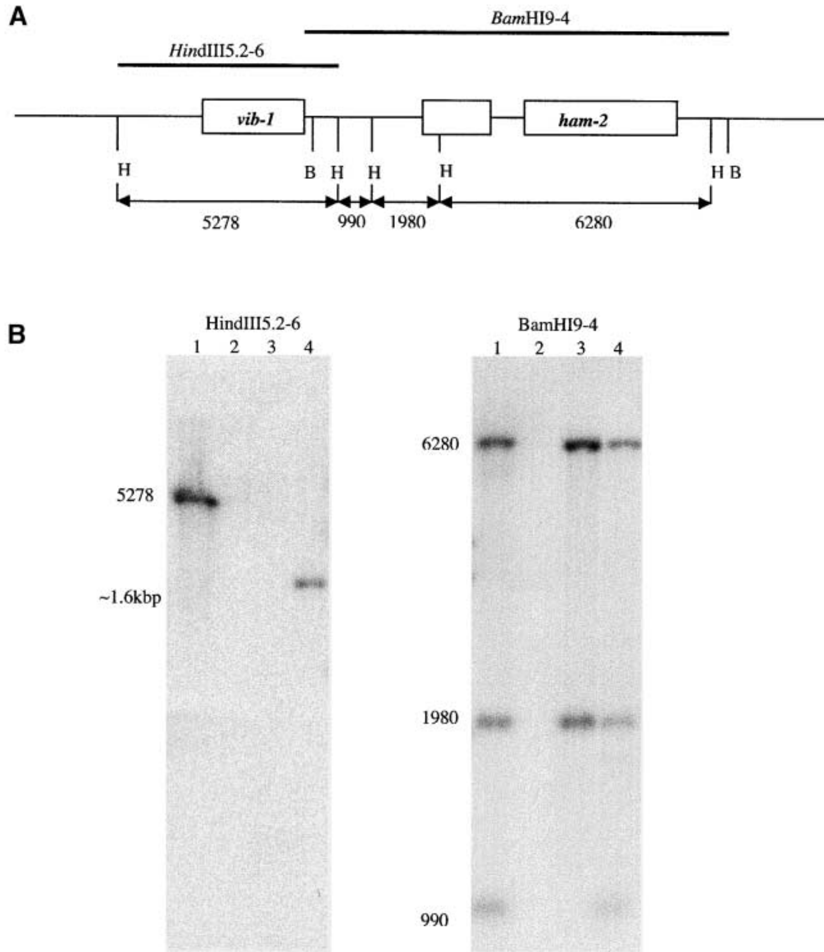


FIGURE 3.—Southern blots showing the *vib-1* region in the *ahc*, *vc1*, and *vc2* deletion mutants. (A) Restriction enzyme sites in the region covering *ham-2* and *vib-1*. The sizes of *Hin*dIII DNA fragments are indicated. H, *Hin*dIII; B, *Bam*HI. (B) Southern blots of genomic DNA probed with either *Hin*dIII5.2-6 or *Bam*HI9-4. Genomic DNA was digested by *Hin*dIII. Lane 1, C9-2 (*WT*); lane 2, 8-88 (*ahc*); lane 3, 9-39-32 (*vc1*); lane 4, 24-24-9 (*vc2*).

yon between X80-32 [*pyr-4 het-c*^{OR}*; vib-1(1) a*] and X80-49 [*thr-2 het-c*^{PA}*; vib-1(1) a*] (Table 1) and stained the hyphae with the vital dye, Evan's Blue (GAFF and OKONG'O-OGOLA 1971). Figure 5, B–D, shows the HCD pattern from a 2-day-old mycelium of the (X80-32 + X80-49) heterokaryon as compared to a wild-type *het-c* incompatible heterokaryon (Xa-3 + FGSC 4564, Figure 5A). HCD was not observed in the growth front of the mycelium of the (X80-32 + X80-49) heterokaryon (Figure 5B) in contrast to a wild-type *het-c* incompatible heterokaryon in which ~20% dead hyphal compartments were observed across the colony (Figure 5A). However, from the growth front toward the inoculation point, HCD rates in the (X80-32 + X80-49) heterokaryon increased to 5–10% (Figure 5, C and D). The above results indicate that *vib-1(1)* mutation does not fully suppress HCD and that HCD in the *vib-1(1)* heterokaryons is dependent upon the age of the mycelium. HCD was also examined in *vc1* heterokaryons with alternative *het-c* alleles (9-39-10 + 9-39-7; Table 1). The rate and pattern of HCD in the *vc1* heterokaryons (which contained a deletion covering *vib-1*) was identical to that of the *vib-1(1)* (X80-32 + X80-49) heterokaryons (data not shown).

VIB-1 has a predicted nuclear localization sequence and shows similarity to PHOG from *Aspergillus nidulans*: Database searches revealed that an internal region of VIB-1 (from 157 to 415 aa) was similar to a putative phosphate-nonrepressible acid phosphatase (*An* PHOG) from *A. nidulans* (MACRAE *et al.* 1993; 41% identity). This region also has a high similarity to a predicted PHOG in *Penicillium chrysogenum* (*Pc* PHOG; MARX *et al.* 1995) and to a hypothetical protein, NCU0429.1, in *N. crassa* (Figure 6). NCU0429.1 is a predicted polypeptide of 719 amino acids (http://www-genome.wi.mit.edu/annotation/fungi/neurospora/). VIB-1, *An* PHOG, and *Pc* PHOG did not show significant similarity to any other predicted proteins in public databases, including known phosphatases. A short region of similarity was identified between VIB-1, *An* PHOG, and *Pc* PHOG and a transcription factor from *Saccharomyces cerevisiae,* Ntd80p (23% identity over 143 aa, E value 0.003). Ntd80p is required for linking meiosis and sporulation (XU *et al.* 1995; CHU and HERSKOWITZ 1998). The N terminus (~100 amino acids) and the C terminus (~200 amino acids) of VIB-1 do not show high similarity to any other known or hypothetical proteins. Computational analysis (http://psort.ims.u-tokyo.ac.jp) showed that VIB-1 has

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   1 GAGCTCGATCTCTTTCCCTCTTTTCTGTGGGCTAAATGGGCCGTTAGCTGTTGATATCATACCCCATCTTGACTAGCAAGGGTCGCGGCCCTGCTTGCCA
 101 GATCGTCTCGTTTTCCCTCAACCTCCTTCCCTGTACGGTTGGATCGTTTCTGCAAGTGCGAGAGTGAGAAACAATCCCCTACCATCCCACCCTCGTGAAA
 201 GCCGCTAACTTCGACTTGGTCTTGTTCCGTACCCCCTGTAGCTCCCGGGACAGTGCCGAAGTTAACCCTCCTCTCACAACCGTCCTCTCGACCATTGGTG
 301 GCACTATTGGCTTGATACAAGTACATACATATGTACACCACCGCGCTCTTCTACGACTACACTCTTTCGTTATCCGATCCTCTTGTCGTTCCTTCTTATT
 401 CCTAAATCAACCAAGACGGCGTCTCTTTCGATCTCTTCTCCAAACATCTTCTTTGGTGCTCTATTCTTTCGCCCCACTACGCACGAGGAGGAGTAAAGAC
 501 TACTAGACAAAACTATCCTCCAACCTAAGCGACTCGAAGGGTTTTTTTCTGTGAAGGAAACCTTCGGGCAATCTTTGGAGGAGTGACAGCTGCCCATGTC
 601 AGCGACAGCAACATACACGGTCAGTATGGCAGAGTTGAGAGCTGAGACTCAGCACGGAGGCATCTGGCCCAACTACGGGAATCCGGTGCAGATGAATACT
                                       M A E L R A E T Q H G G I W P N Y G N P V Q M N T 25
                                                                                              A
 701 GGTCGGTACAACACTCAAGAGTCTTCGGTACCAGTAGGATCGGCGGCTTCTTCACATCTTGTACGCCCTCGCAGTCGTCAGCACACGATGGACTATCACA
      G R Y N T Q E S S V P V G S A A S S H L V R P R S R Q H T M D Y H 58
           t
 801 ACGCACCATACCATCATGGACGGCCTGCCCAGGAGGATGGCGATGGCTACGAGAGATATCCACACCCTTCGTTGATGAACATCCCCAGCATCACCACCGG
      N A P Y H H G R P A Q E D G D G Y E R Y P H P S L M N I P S I T T G 92
     t
 901 CATGAAGCGCAGCTACTCCCAGGTCGACCAAACTCCCTATACGGAAATGGTCCAAGATCTCCGCGACGACTACAAGCCGGCCATGAATCACGACCAGAAG
      M K R S Y S Q V D Q T P Y T E M V Q D L R D D Y K P A M N H D Q K 125
                                                  t
1001 CTACTCTCATTCAAGAAGGTTGGCGACAAACATACCATCGTCGACCACAAAGGGCGGATTCACGAAATCGAAATCGAAGCTCAACTTCACGGCATGTTCT
      L L S F K K V G D K H T I V D H K G R I H E I E I E A Q L H G M F 158
                                                              a                        t             a
1101 TTCTGTCGGAGTTTCCCTCGCCTGGCGACGGCAATGTCTTGAACGCCGAGTTGACTTGCTACCGCCGCAACCTGTTTCAAATCAGCGGCAATATCTGCTT
      F L S E F P S P G D G N V L N A E L T C Y R R N L F Q I S G N I C F 192
                                                                                  t
1201 TCCTCAAATACCGTTGTCTGTCATGCTAGAAACGGGCGAGACCAGCCAGATCAAGAACATGGAAGTTACTATATCGGCAATCGAATCTGTCGACGGCCAC
      P Q I P L S V M L E T G E T S Q I K N M E V T I S A I E S V D G H 225
        A
        a a           t                                                   a  t            t
1301 CCTGTGCGGCCTAATCGTCATTCCCTGGAAGACGCCCCCACCCAACTCACCAGAAGTCAACCAAGCTCCCGATCAAGAACCTCCATCACTTCCTCTGATTC
      P V R L I V I P W K T P P P N S P E V N Q A P D Q E P P S L P L I 258
        A                  t                                                           a       t
1401 CCTGGTCCGAAGAGGAGGAAGACAATGGCGGAGATCACTACGCTATCTATCCAATTGGGTGGCGTCGACTTCAATTTAGGATGTAAGCATCACTTCAGAA
      P W S E E E E D N G G D H Y A I Y P I G W R R L Q F R I 286
                               A           A  A
                    t          a  t           a                         t       a             t     t
1501 CCACCACTCACTCCACATTTCGTTATGTTCAATAAGCTGATGCGTTCCTATCTAGCGCAACGGCCAACAACGGTAGACGGAAGGAATTACAGCAACACTT
                                                                    A T A N N G R R K E L Q Q H F 301
                        t
1601 CGTCCTACACTTGAAGCTCCATGGAACCCTAGCGAATGGTACGAAACTTGTGCTTTCGGAGCTTACCACTGCGCCGATTGTTGTCCGAGGAAGAAGCCCG
      V L H L K L H G T L A N G T K L V L S E L T T A P I V V R G R S P 334
                                             t
1701 CGGAATTTCCAAGCGAGAAAGGAGATCCCGCTGTTGGGATCTAGCGCTGGTTCAAGAGGACAGACACTCGTGGAAACCGGTCACTCAATTGTTGCGCAAG
      R N F Q A R K E I P L L G S S A G S R G Q T L V E T G H S I V A Q 367
1801 CTGTCGCGCTTAACAAGCCTCCCTATGATTCGAGACCCCGTGTCTCGAGCATGGATTTGCCCAGAACAGCGTTCACGTTCACGTCTGCGAAGCAGATGCC
      A V A L N K P P Y D S R P R V S S M D L P R T A F T F T S A K Q M P 401
1901 CCAGAGTCCTATGCAAATGCGTTCGAAGTAAGTCAACAAAATTTCAAGATACGGAGATGTGGCATATTCAAGGCAGTGATGCTAGCTTCACTAGGGTCCT
      Q S P M Q M R S N 410
2001 GACTTGTTAGAGGAAAACGATACGTGATGCTAACATAGGTCAGCAGTTCTTACCCCACAAGTTGGAACCCATCTAGTCAGGTGTCGATGCCACACAACCC
                                                       S Y P T S W N P S S Q V S M P H N P 428
2101 AGGATCGACTTCTTACCCGACAACATCGATGGCCGGCCCCGAGCCCTATCCTAAAATGCCCCTTTCTGGAGCGCCCAGCTACACGGCCGAGCCCCAAGAG
      G S T S Y P T T S M A G P E P Y P K M P L S G A P S Y T A E P Q E 461
2201 ATGCCTATCCAGCAGACCTCCATGCCTTCTATGCAACTCTCTATGGTAGCCCAAGACCAACAACCGTCGGCCCCCATCCGAACACAATACGCAACTTACG
      M P I Q Q T S M P S M Q L S M V A Q D Q Q P S A P I R T Q Y A T Y 494
2301 CATCGGCACCTCCGCCGCATCTTTCGTTGCCGAGCACAGCGGACAGCTCGCTTAACGTGCCGCGCTATGTGGACAGCAATCCCCGTCCTTCAAAGAGCCC
      A S A P P P H L S L P S T A D S S L N V P R Y V D S N P R P S K S P 528
2401 TCGCCACGGTAGCCATGGCTCACTCACGAACGAGACGGCCTCGGGCGAGTATCGCTATGGCCCCCCTTCGTACCTTGGCAACAGCTCAAGTGATATAAGT
      R H G S H G S L T N E T A S G E Y R Y G P P S Y L G N S S S D I S 561
2501 CCCCAATCGCAACATCATCCGCCTACGTCAGGTGCAGGAGCAGGCGGGGCGTCGTCGGGCGCTTACGGAACACCATCTCAAGAAGGAGGTGCCTCTGCCC
      P Q S Q H H P P T S G A G A G G A S S G A Y G T P S Q E G G A S A 594
2601 CAGCGTCTGCGCCTACGAGTGCGGCTCCGCCGCGTGATTACTTCCCCCCATCTCAGTCCTGGACCAGCACTGCCGGTGAGGGGCAGACTTCATCGTACAC
      P A S A P T S A A P P R D Y F P P S Q S W T S T A G E G Q T S S Y T 628
2701 GAACGGAGGTGACAGGTCCTATTCCTTTCCTACAGGTGTGAAAACGGAGCCGCACTCGCAGCCTTCGCACTCGGGTGCACCAGTGCCAGGCGTGTATGGC
      N G G D R S Y S F P T G V K T E P H S Q P S H S G A P V P G V Y G 661
2801 AACAACCACTATGCATGGAACGCCACGTAAAAGGAGGAGAACGAAGTTTGGATCCACGACTGAAATACCAGGCTCCAGTTGCTTTTAGGCTGATGATAAC
      N N H Y A W N A T * 670
2901 GAGGAGATGAAGGCGTCTTAAATGGTAATTGTTGCTTGAAAATCAAGCCTGAAAAAGGGGGGTGCGGTCAAACAATGGTGGATATTGGTGTCTCAATCCC
3001 ATTCATGATGGGCTAATCGACGACACTATGGATGGTTTGCAGCTCGGCATTTTTACATGCCGGTGACCGGCCTGGAAACAGCGCATTACCTGTTTGGCGG
3101 GGTGCGCCTGACGTCAAAAGAGAAGTCGAGGAAGCTTGTGGATTAGAAAAGAAGTCGGCATGTGTTCTGGATGGCGGCGGCAGTTTGATTTTTTGTTGAT
3201 CCAAAATACCTTTCTCTTTGCGGTTCTATGTTTCTCAACCAAGAGCTCTGTTTTTCTATATCCGTACATACACATCTACGTCCATGTACAAATTTCATGT
3301 GCTTCTGTTGCGGGCGGTGCTTTGGCTTCACCACTGCATGATATACAATCTGGAGGGATTTCATCTGTCATTGTCTTCGGTTCTTCTTGCGCATTTTCAA
3401 CGTCCAATCGATACCACTCCAAATACCACAATGAGTAATACCTCCCGAGATGATCTGCCTGTGTTTGCATGATCAATATGGAAGATATATGCGCGTAATA
3501 AACCCAAAAATACACTGACGGCGCCTTTCCTGAGTGCTATAAGCCCCCCTTTATCTGCGTTTTCATGCACCAACTGACTGATTCCCACCCAGCCTTGGCC
3601 AGCGTAGGTAATCACAGTCACACTTTGCGGAGTGGGCACCCCGTTCAAGCTCATCATATCACGCTCCCCCGGCACTCGCTCATGTTGTGTCTCTCCATCC
3701 TCATAACGTGTCCCTCCCCCCCCTTGCCCACAGCCATAATGGTTGCTTAACCGAGCTTCACGGTAACAACCAAATCCCTGGGTGGAAATGGAGGTCAAAG
3801 GCAAATAAAGTGCTGAAGGGTATAGGTGTACGAGCTCGGTGAGAAGTTCGGCGACGAAAACCAGAAAACATGAGACATGAACTCGTGTGACTAGAGTGCT
3901 ATATCTCATTCAACCATTTCCTTTGCCGTCTTGTCACAACTCTCCTTAGCATTTACTCGACCCTCTTCATCTGGAAAGTACATGAAACCACAATGGTCCA
4001 AAAATTTCAACCCAACCCGACCAGCTTCTCGTATTTCCCCATTTCTCTTCGCAGACTTGACAAGTGAGATATGTAGTTAACATTGCATCTAACGTCCCAC
4101 AGCTTTGCAAGACAGACAAAGAAGCTT
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FIGURE 4.—DNA sequence of the 4-kbp *Sac*I-*Hin*dIII fragment, the predicted amino acid sequence of the *vib-1* ORF, and the GC-to-AT transitions in the *vib-1(1)* and *vib-1(2)* mutants. The CAAT box, consensus sequence around the start codon CAGTATGGCA, and the polyadenylation site AAT AAA are underlined. The NLS sequence is underlined and in boldface type. The *Kpn*I and *Xho*I sites used for mutational analysis and the *Hin*dIII and *Sac*I sites in the 3′ UTR region used for deletion constructs are in boldface type. The six GC-to-AT transitions in *vib-1(1)* (X80-32 and X80-49) are marked by a boldface "A" (the mutant nucleotide) above "G" (the *WT* nucleotide). The 26 GC-to-AT transition mutations in *vib-1(2)* (X80-33) are marked by boldface "a" or "t" (the mutant nucleotide) above "G" or "C" (the *WT* nucleotide; accession number for *vib-1*: BK000540).

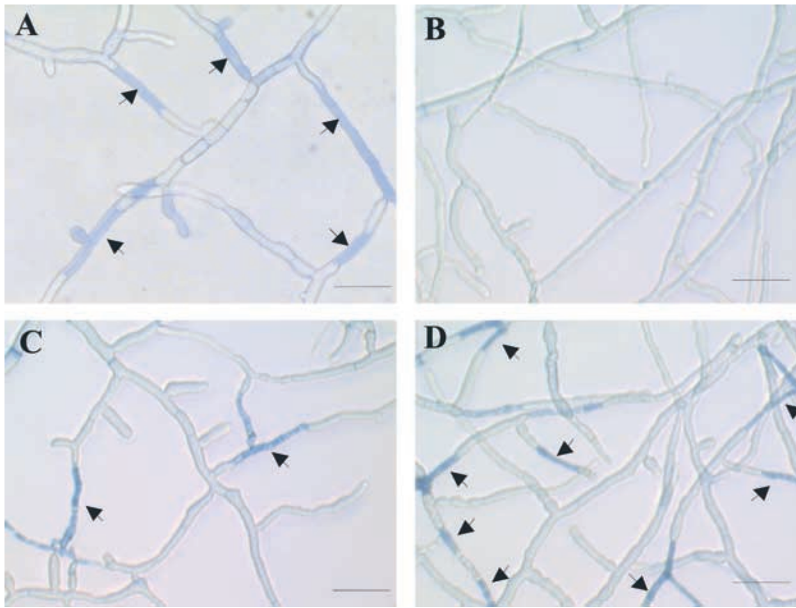

Figure 5.—The HCD pattern in heterokaryon [X80-32 (*pyr-4 het-c*^{OR}*; vib-1(1) A*) + X80-49 (*thr-2 het-c*^{PA}*; vib-1(1) A*)] is altered compared to a *het-c* incompatible heterokaryon. (A) Typical HCD in a 2-day-old *het-c* incompatible heterokaryon (Xa-3 + FGSC 4564), as observed by staining with the vital dye Evan's blue (Gaff and Okong'o-Ogola 1971). Arrows indicate dead hyphal compartments. Approximately 20% of the hyphal compartments are dead and were distributed randomly across the colony, consistent with previous observations (Jacobson *et al.* 1998; Wu and Glass 2001). (B–D) HCD in a *vib-1(1)* heterokaryon (X80-32 + X80-49; Table 1). The 2-day-old *vib-1(1)* heterokaryon growth distance is ~7 cm. (B) A region 0.5 cm away from the growth front; no HCD was observed. (C) A region ~3 cm away from the growth front. (D) A region ~6 cm away from the growth front; ~5–10% HCD was observed. Bars, 20 μm.

a predicted bipartite nuclear localization sequence (NLS), RRLQFRIATANNGRRKE, from amino acid position 280 to 297. This type of NLS consists of two basic domains separated by ~10 intervening amino acids (Robbins *et al.* 1991). The two basic domains in VIB-1 NLS are RR and RRK. This putative NLS was also conserved in the PHOG proteins (Figure 6).

***vib-1* mutants show reduced nonrepressible acid phosphatase activity:** To determine whether *vib-1* is a structural gene for nonrepressible acid phosphatase in *N. crassa*, native PAGE analysis was used to detect phosphatase activity in the *vib-1* mutants. The staining method employed in this study can detect the activities of both alkaline and acid phosphatases (Dorn 1965). As shown in Figure 7, under low-phosphate conditions, four phosphatases were observed from the two parental wild-type strains (C9-2 and 9-1-5, lanes 1 and 2) and *vib-1(1)* (X80-32) and *vc1* mutants (9-39-10, lanes 3 and 4). Under high-phosphate conditions, only nonrepressible phosphatases were detectable (Figure 7). The activity of the nonrepressible acid phosphatase (B in Figure 7) is lower in *vib-1(1)* and *vc1* mutants compared to that in wild-type strains under both low- and high-phosphate conditions. There were no obvious differences in A, C, and D in Figure 7 phosphatases between the wild-type strains and the *vib-1(1)* and *vc1* mutants, except that 9-1-5 had lower nonrepressible alkaline phosphatase activity under high-phosphate conditions. These data indicate that *vib-1* does not encode the structural gene for phosphate nonrepressible acid phosphatase, but may instead encode a positive regulator of nonrepressible acid phosphatase activity.

DISCUSSION

In this study and previous work (Xiang *et al.* 2002), we identified mutants that suppressed vegetative incompatibility from transformants that escaped from vegetative incompatibility mediated by the *het-c* locus. By contrast to partial diploids or heterokaryons that escaped from vegetative incompatibility mediated by mating type or *het-6* (Delange and Griffiths 1975; Smith *et al.* 1996), the majority of *het-c* escape transformants (60%) maintained both *het-c* alleles. The compatibility of these escape transformants presumably was caused by mutations in genes required to mediate vegetative incompatibility or by epigenetic mechanisms. The mutations in these strains could be genetically characterized because they were fertile, in contrast to the near-sterility of escaped partial diploids used to identify mutations in *tol* and *het-6* (Vellani *et al.* 1994; Smith *et al.* 1996). Crosses between 14 escape transformants and a wild-type strain revealed three suppressor mutations, *ahc*, *vc1*, and *vc2*. The compatibility of the other 11 escape transformants might have been caused by mutations closely linked to the *het-c* locus or by epigenetic mechanisms that silenced *het-c* during vegetative growth but which were not transmitted to progeny (Cogoni and Macino 1999).

The three suppressor mutants all carried independent deletions in the same region of chromosome V, between *lys-2* and *ilv-2*. The *ahc* deletion is ~26 kbp and the deletions in *vc1* and *vc2* are ~19 and ~8 kbp, respectively (our unpublished data). It is unclear how these deletions occurred, but the removal of *vib-1*, a locus responsible for mediating *het-c* vegetative incompatibility, is probably a major factor involved in their appearance. Hyphae containing a nucleus with a deletion or mutation in *vib-1* would have a selective advantage for growth and conidiation in an otherwise *het-c* incompatible colony.

Since the *ahc*, *vc1*, and *vc2* deletions possibly covered genes in addition to *vib-1*, we generated *vib-1* mutants. The phenotype of the *vib-1* mutants was identical to that of the *vc1* and *vc2* mutants. The distinguishable

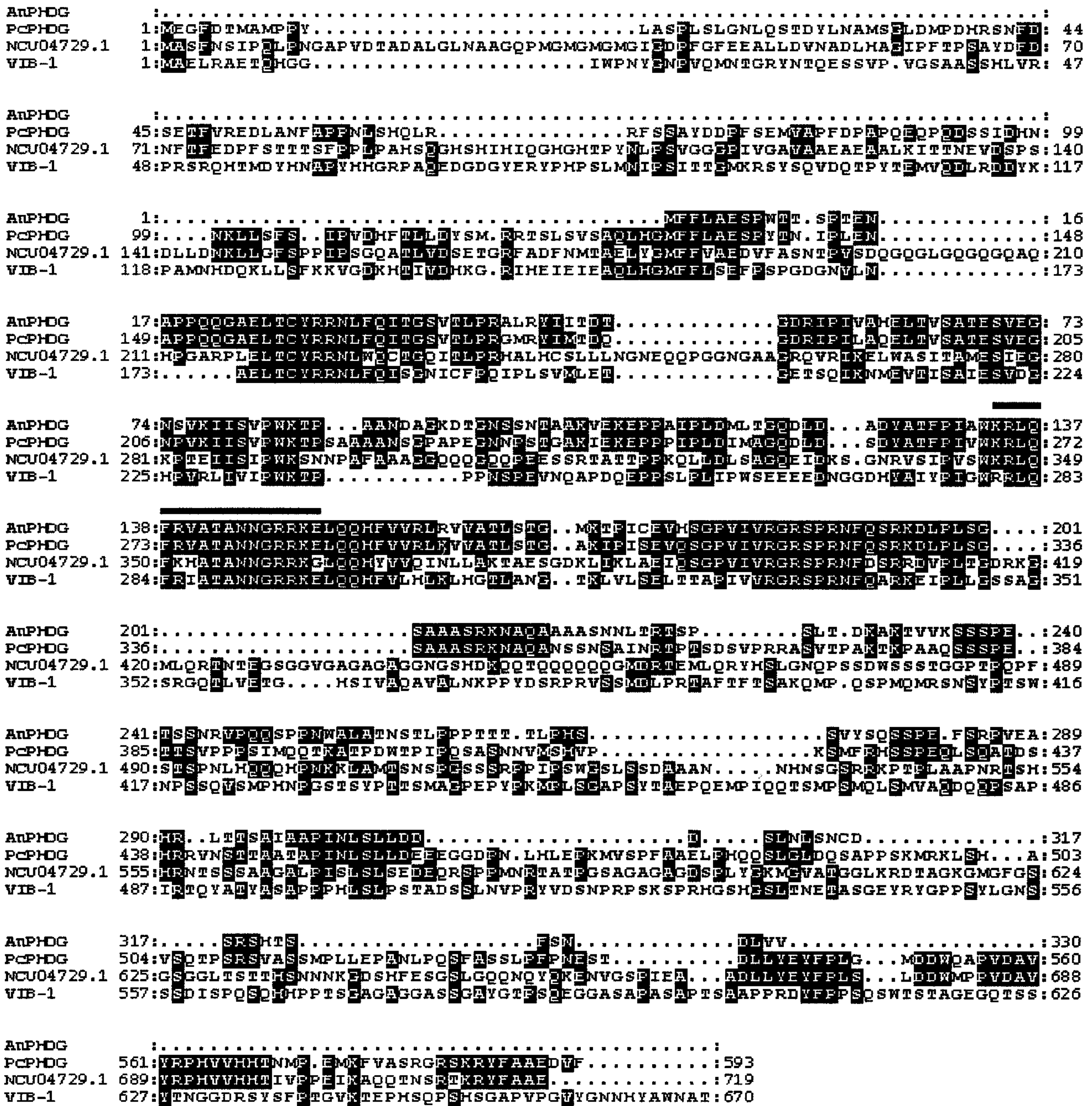

Figure 6.—Alignment of VIB-1 with *An* PHOG, *Pc* PHOG, and NCU04729.1. *An* PHOG is a 330-aa predicted polypeptide from *A. nidulans.* NCU04729.1 is a predicted 719-aa-long polypeptide in *N. crassa* (http://www-genome.wi.mit.edu/annotation/fungi/neurospora/) and *Pc* PHOG is a predicted 593-amino-acid polypeptide from *P. chrysogenum.* The shaded boxes represent identical amino acids. The predicted NLS is demarcated by a thick line above the conserved sequence.

character of the *vc* and *vib-1* mutants is the profuse conidiation phenotype, suggesting that *vib-1* negatively regulates conidiation. A characteristic phenotypic consequence of vegetative incompatibility in *N. crassa* is the suppression of conidiation (Mylyk 1975; Perkins 1975). These data suggest that VIB-1-mediated suppression of conidiation is activated or maintained during *het-c* vegetative incompatibility.

The profuse conidiation pattern of the *vib-1* mutants is similar to *cpd-1* and *cpd-2* (Hasunuma and Shinohara 1985, 1986), *cr-1* (see Perkins *et al.* 2001), and *gna-3* (Kays *et al.* 2000) mutants, all of which have reduced cAMP levels. We speculated that cAMP signaling might be involved in *het-c* vegetative incompatibility. The profuse conidiation of a *cr-1* mutant (defective in adenylyl cyclase), however, was fully suppressed in *cr-1 het-c^{OR}/het-c^{PA}* partial diploids (our unpublished data). These data suggest that *het-c* vegetative incompatibility is independent of cAMP signaling. This result is consistent with data showing that the cAMP pathway is not involved in vegetative incompatibility in *P. anserina* (Loubradou *et al.* 1999).

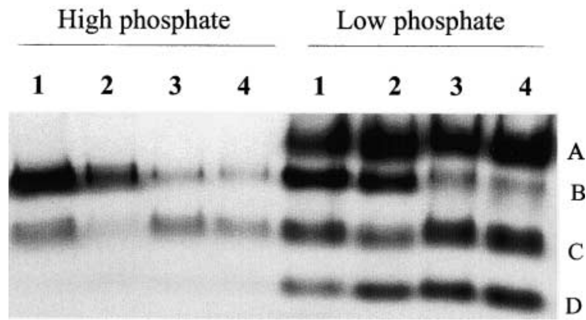

FIGURE 7.—Native gel of phosphatase activity in *N. crassa*. Fifty micrograms of total protein was loaded per lane. Cultures were grown under low-phosphate or high-phosphate conditions, as described in MATERIALS AND METHODS, prior to protein extraction. The strains used were: lane 1, C9-2 (*WT*); lane 2, 9-1-5 (*WT*); lane 3, X80-32 [*vib-1(1)*]; and lane 4, 9-39-10 (*vc1*). The predicted phosphatases are A, repressible alkaline phosphatase; B, nonrepressible acid phosphatase; C, nonrepressible alkaline phosphatase; and D, repressible acid phosphatase (HOCHBERG and SARGENT 1973).

In addition to the suppression of conidiation, vegetative incompatibility also results in growth inhibition and HCD. Mutations in *vib-1* fully relieve *het-c*-mediated growth inhibition in heterokaryon tests. They cannot, however, fully relieve HCD caused by *het-c* vegetative incompatibility. In *vib-1* or *vc1* heterokaryons with alternative *het-c* alleles, HCD was age dependent and occurred mainly in older hyphae. This pattern is in contrast with the HCD pattern in a wild-type *het-c* incompatible colony in which ~20% HCD takes place in both young and old mycelia (JACOBSON *et al.* 1998; WU and GLASS 2001). The HCD pattern caused by *vib-1* mutations is similar to that caused by *mod-A* mutations in *P. anserina* (BERNET *et al.* 1973). It is possible that the remaining level of HCD observed in *vib-1* heterokaryons with alternative *het-c* alleles is caused by NCU0429.1, a protein sharing high similarity with VIB-1. We are currently mutating NCU0429.1 to see whether a full relief of HCD in (*het-c^{PA}; vib-1* + *het-c^{OR}; vib-1*) heterokaryons will occur.

VIB-1 is a predicted polypeptide of 670 amino acids. It has been annotated to be related to acid phosphatases (Swissprot accession no. Q05534; MIPS: http://www.mips.biochem.mpg.de/proj/neurospora). The internal 240-amino-acid region of VIB-1 has a high similarity to *An* PHOG and *Pc* PHOG, possible phosphate nonrepressible acid phosphatases (nrAPase) in *A. nidulans* and *P. chrysogenum*, respectively, but is not similar to any other known phosphatases. The introduction of *An phoG* into *A. nidulans* enhanced acid phosphatase activity in a *pacG* mutant under high-phosphate conditions (MACRAE *et al.* 1993). The *pacG* mutant reduced activity of nonrepressible acid phosphatase (CADDICK and ARST 1986). *An* PHOG was thus suggested to be either a nonrepressible acid phosphatase or a regulator of acid phosphatase activity. The *Pc phoG* gene was cloned by hybridization to *An phoG* (MARX *et al.* 1995); neither an *An phoG* nor a *Pc phoG* mutant has been described in the literature. Our native gel analysis showed that *vib-1* mutants possess all four phosphatases described in *N. crassa* (KUO and BLUMENTHAL 1961a,b; NYC *et al.* 1966; JACOBS *et al.* 1971), including nonrepressible acid phosphatase. Thus, *vib-1* is not a structural gene for a nonrepressible acid phosphatase. However, VIB-1 may be a positive regulator of nrAPase because the activity of nrAPase was significantly reduced in *vib-1(1)* and *vc1* mutants. VIB-1 has a predicted NLS and could be a nuclear protein and thus may regulate multiple cellular processes at a transcriptional level. Interestingly, VIB-1 also displays limited similarity to Ntd80p in *S. cerevisiae*, a transcriptional factor involved in gametogenesis (XU *et al.* 1995; CHU and HERSKOWITZ 1998). However, the domain of Ntd80p required for DNA binding to transcriptional targets has not been well defined (E. R. JOLLY, and I. HERSKOWITZ, personal communication).

The relationship between HET-C and VIB-1 is unclear. *het-c* null mutants have no phenotype, with the exception that they are fully compatible in heterokaryons with strains with alternative *het-c* specificity (SAUPE *et al.* 1996). Non-self-recognition during *het-c* vegetative incompatibility has been associated with the formation of a heterocomplex composed of *het-c* proteins of alternative specificity (S. SARKAR, G. IYER and N. L. GLASS, unpublished data). It is possible that VIB-1 is required for formation of a HET-C heterocomplex and thus that the *vib-1* mutant suppresses *het-c* vegetative incompatibility because the HET-C heterocomplex fails to form. Alternatively, the HET-C heterocomplex may form in the *vib-1* mutant, but vegetative incompatibility is not triggered. Experiments are currently underway to determine the effect of the *vib-1* mutation on HET-C heterocomplex formation.

We conclude that *vib-1* is a gene involved in multiple cellular processes. In addition to vegetative incompatibility, it is also implicated in the regulation of conidiation and nrAPase. What is the relationship between *het-c* vegetative incompatibility and nrAPase? The first possibility is that VIB-1 regulates multiple cellular processes, such as nrAPase production, conidiation, and vegetative incompatibility, and thus there is no causal connection between vegetative incompatibility and nrAPase. The second possibility is that VIB-1 regulates nrAPase, which is required to mediate vegetative incompatibility. In *N. crassa*, it has been speculated that nrAPase participates in metabolic control systems rather than in phosphate uptake (KUO and HERSKOWITZ 1961b). Thus, nrAPase may be involved in the regulation of certain cellular processes in *N. crassa*, including vegetative incompatibility. We are currently conducting experiments to distinguish these two possibilities.

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