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Genetics

Molecular alteration in a *Neurospora crassa* morphological mutant and its phenocopy

(glycan/cell wall/sorbose)

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ABSTRACT **A procedure using ion exchange chromatography has been developed to detect alterations in a polysaccharide produced by *Neurospora crassa*. The polysaccharide, isolated from medium that has supported the growth of a culture, is highly responsive to the 3-methyl-2-benzothiazolinone hydrazone assay, indicating a high hexosamine content. The substance elaborated by wild-type *N. crassa* can be fractionated into two components that appear by rechromatography to be closely related. When isolated from mutants of the *peak (pk)* locus, the corresponding polysaccharide cannot be resolved into two components. Instead, a single component is consistently found. This variant chromatographic pattern cosegregates with morphological effects of the *pk* allele after crosses with the wild type. The polysaccharide isolated from a wild-type culture that has been induced by sorbose to phenocopy the hyphal characteristics of *pk* mutants elutes from the ion exchange column in a manner similar to the corresponding polysaccharide from the *pk* mutants.**

Neurospora crassa may undergo two distinct developmental phases. One phase involves the asexual production of conidia that germinate to produce a mycelial mat producing more conidia. The second, the sexual developmental phase, consists of protoperithecia production, fertilization of the protoperithecia, and then maturation of the perithecia, or fruiting bodies. Perithecia maturation includes differentiation of the perithecial wall and internal development of asci containing ascospores. By use of appropriate mutants, loci that regulate development of one phase only (1, 2) as well as loci regulating both phases (3) have been identified. A number of loci of the second type regulate morphology of both asci and vegetative hyphae. Mutations at these various loci offer the potential for dissecting ascus development and providing insight into the relationship of the zygote to the developing ascus. Full utilization of these mutants to study development requires some knowledge of their molecular function. No consistent molecular alteration that can be directly related to alterations in morphology has been previously detected for mutations at any of these loci. The careful examination of a molecular component of *Neurospora* now reveals a consistent molecular alteration for members of one set of allelic mutants.

Reissig and Glasgow (4) reported the isolation of a growth-regulating substance both from purified hyphal wall of *Neurospora* and from medium that had supported a culture. They found no difference between the materials from the two sources. The substance was found to be composed primarily of 2-amino-2-deoxygalactose, with several neutral sugars and amino acids as minor components. A portion of the 2-amino-2-deoxygalactose residues was found to be *N*-acetylated. Originally referred to as MP (for mucopolysaccharide), the designation was later changed to galactosaminoglycan (5).

Mycelial morphology in *Neurospora* is determined in part by the frequency and location of branching and by growth rate. The galactosaminoglycan isolated by Reissig and Glasgow was shown to be capable of restricting growth rate. Because of the reported growth-regulating properties of this substance and the conclusion of Mahadevan and Tatum (6) that the most consistent molecular alteration of purified hyphal wall from morphological mutants of *Neurospora* occurred in a fraction containing all of the 2-amino-2-deoxygalactose, we decided to develop a procedure by which the isolation product of Reissig and Glasgow could be more critically examined. The substance studied by us has a high hexosamine content and probably is the same substance as Reissig and Glasgow's galactosaminoglycan. Because component analysis has not yet been completed to the point of our being sure that 2-amino-2-deoxygalactose is the primary component of the substance we have examined, we refer to it here simply as hexosaminoglycan (HAG).

We have developed an ion exchange chromatography procedure by which HAG from wild-type cultures can be fractionated into several components and have examined also the corresponding material elaborated by mutants at the *pk* (*peak*) locus. Mutation at the *pk* locus results in dichotomous branching of vegetative hyphae, restricted colonial growth habit, and nonlinear arrangement of ascospores within abnormally shaped asci (7). These properties contrast with the loose, spreading mycelium with few dichotomous branches of wild-type cultures which, when appropriately crossed, produce cylindrically shaped asci with a linear arrangement of ascospores. Both zygotic recessive and dominant alleles have been isolated and characterized for the *pk* locus (3).

MATERIALS AND METHODS

Strains. The standard laboratory St. Lawrence 74*A* and 77*a* were the wild-type strains utilized in this study. The zygotic dominant alleles *Pk1*, *Pk3*, and *Pk4* and the zygotic recessive alleles *pk2* and *pk5* of the *pk* locus were all isolated in this laboratory from the St. Lawrence genetic background and, after isolation, were backcrossed repeatedly to the same wild-type strains.

Culture. Maintenance of stocks and preparation of cultures for HAG isolation were performed by using Vogel's minimal medium (8) with 2% sucrose. The minimal medium was sup-

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Abbreviations: HAG, hexosaminoglycan; MBTH, 3-methyl-2-benzothiazolinone hydrazone; TES, 2-{[tris(hydroxymethyl)methyl]amino} ethanesulfonic acid.

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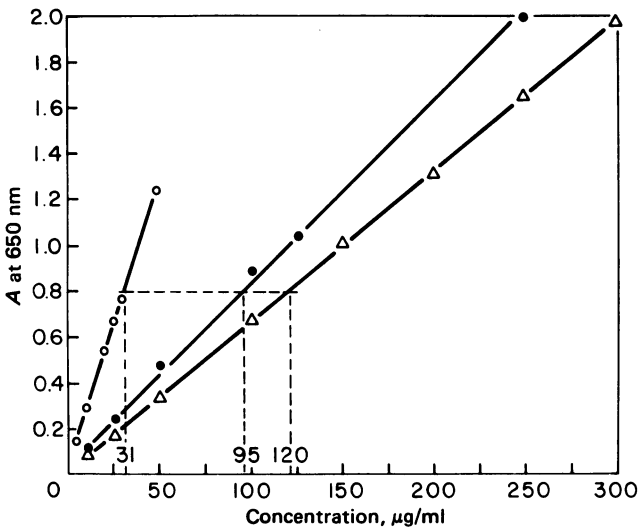

FIG. 1. Comparison of HAG concentration to MBTH assay response. HAG response is also compared to the response of 2-amino-2-deoxyD-galactose HCl. The dashed line intersecting the three response lines is at an arbitrarily chosen position to demonstrate the relative concentration of each compound required to produce a given MBTH assay response. O, (2-Amino-2-deoxy-D-galactose HCl; ●, HAG from *Pk1*; △, HAG from wild type.

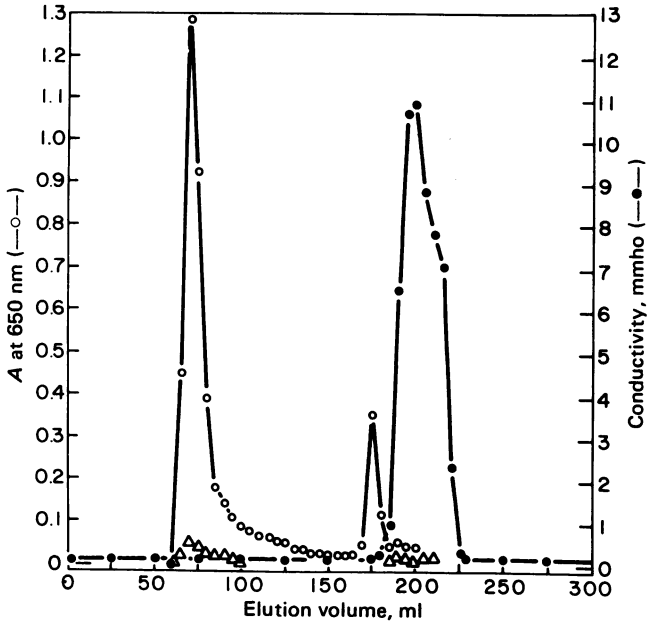

FIG. 2. Elution diagram of wild-type HAG eluted from Sephadex G-50 (fine). △, Folin phenol assay-positive material.

plemented with 2% agar when solid medium was required. Crosses were made on the crossing medium of Westergaard and Mitchell (9) with 2% agar.

All stocks were maintained on solid minimal medium at 25°. For preparation of HAG, a culture technique described by Brody (10) and modified by Novak (11) was used, as follows. Approximately 6 ml of solid minimal medium in a 16 × 150-mm culture tube was inoculated with the appropriate strain and incubated for 5 days at 25°. A heavy inoculum was then transferred to 30 ml of liquid medium in a 125-ml Erlenmeyer flask and incubated on a rotary shaker for 48 hr at 25°. The medium was decanted from the culture and replaced with 50 ml of fresh liquid medium. The culture was then sheared in a semimicro blender cup for 15 sec at high speed on a two-speed Waring Blendor. The contents of the blender cup were then used to inoculate 1 liter of liquid medium in a 2.8-liter Fernbach flask. The culture was incubated on a rotary shaker for 48 hr at 25°. A sample of the mycelium from the Fernbach flask, prior to harvest, was transferred to solid medium in a culture tube and grown for 72 hr in order to test for contamination.

For phenocopy experiments, Vogel's minimal medium was supplemented with 0.5% sucrose and 2% sorbose. The sorbose-supplemented medium was used in place of the standard 2% sucrose Vogel's minimal medium in Fernbach flasks in the above procedure.

HAG Preparation. The procedure of Reissig and Glasgow (4) was modified as follows for the preparation of HAG. The medium and mycelium from Fernbach flask cultures were separated by filtration through Whatman no. 1 filter paper. Two volumes of cold 95% ethyl alcohol was added to the medium, the precipitate was collected by centrifugation (3000 × *g*, 10 min), and the supernatant was discarded. The precipitate was dissolved in 1 M NaCl (40 ml/liter of medium precipitated) and the solution was extracted several times with an equal volume of chloroform/isoamyl alcohol, 24:1 (vol/vol), to remove proteins.

When testing the linearity of the 3-methyl-2-benzothiazolinone hydrazone (MBTH) assay to HAG concentration, the chloroform-washed sample was precipitated with 1.3 volumes of isopropyl alcohol. The precipitate was collected by centrifugation (3000 × *g*, 10 min) and redissolved in H_2O. The solution was lyophilized and weighed prior to being dissolved in H_2O or 5 mM 2-n{[tris(hydroxymethyl)methyl]amino}ethanesulfonic acid (TES), pH 8.0.

Fractionation. An aliquot of the HAG sample was applied to a column of Sephadex G-50 (fine) (100-cm bed height in a 1.6-cm diameter column) equilibrated with 5 mM TES, pH 8.0, in order to desalt the sample and equilibrate it to the TES buffer for ion exchange fractionation. The first 150 ml of effluent from the Sephadex column was washed directly onto a column (20-cm bed height in a 1.6-cm column) of CM-cellulose equilibrated to the same buffer. The CM-cellulose was eluted with

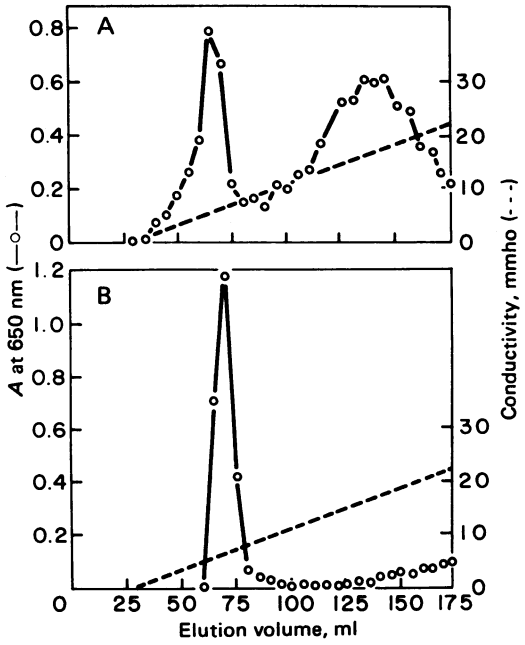

FIG. 3. Elution diagrams of HAG from wild-type (*A*) and mutant *Pk1* of the *pk* locus (*B*). HAG was eluted from cm-cellulose by the use of a NaCl gradient.

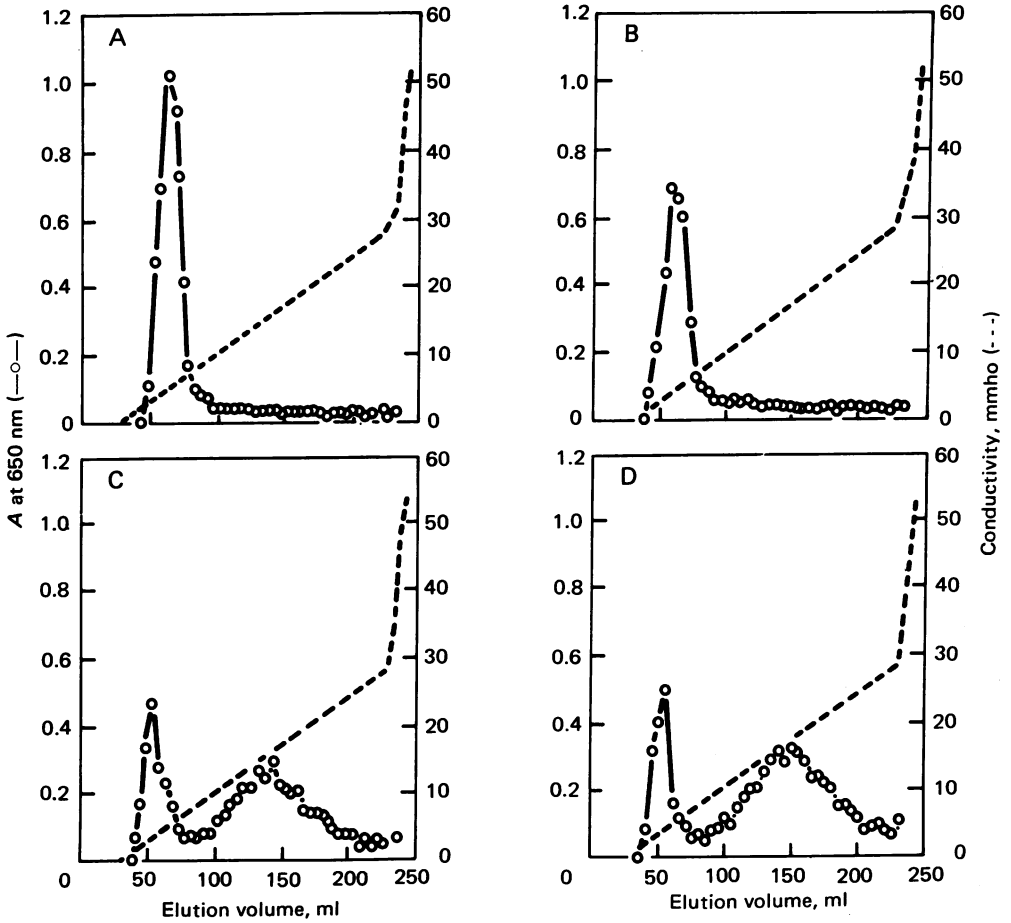

FIG. 4. CM-Cellulose elution diagrams of HAG prepared from the four meiotic products of a tetra-type ascus from a *Pk1* × wild-type cross. (*A*) Spore pair having colonial morphology and *A* mating type. (*B*) Spore pair having colonial morphology and *a* mating type. (*C*) Spore pair having wild-type morphology and *a* mating type. (*D*) Spore pair having wild-type morphology and *A* mating type

a 200 ml 0–0.5 M NaCl linear gradient and flushed with 50 ml of 1 M NaCl in buffer. Fractions of 5 ml each were collected throughout the gradient and column flushing. All fractions were assayed for hexosamine content, and the conductivity of every fifth fraction was measured to determine the position and the linearity of the gradient.

Assays. Hexosamine was assayed by the MBTH procedure of Tsuji *et al.* (12, 13). Protein was assayed by the method of Lowry *et al.* (14). NaCl concentration was assayed by measuring the conductivity of appropriate fractions with a Radiometer type CDM 2e conductivity meter.

RESULTS

The MBTH assay showed a linear response to increasing concentration of HAG (Fig. 1) over a concentration range of 5 to at least 250 μg of HAG/ml (Fig. 1). Only 3 times as much *Pk1* HAG was required to produce a response equivalent to that to a given amount of free hexosamine hydrochloride, indicating that 33% of *Pk1* HAG is *N*-unsubstituted hexosamine. Four times as much HAG from wild type was required to produce the same response, indicating that only 25% of wild-type HAG is *N*-unsubstituted hexosamine. The response of HAG in the MBTH assay was lower in TES buffer but remained linear.

The majority of the MBTH-positive material in the samples prepared as described eluted in the void volume from the Sephadex G-50 column (Fig. 2). Although two peaks of MBTH-positive material were eluted from the column, only the first component was washed onto the CM-cellulose column for fractionation. Very little protein or peptide material was present in the samples and it appeared to elute as at least two components. The first protein component coeluted with the majority of the MBTH-positive material. The second protein component eluted with the NaCl front and, because it appeared after the first 150 ml of effluent, it was not carried to the CM-cellulose column. The Sephadex G-50 elution behavior of HAG from wild-type and *pk* mutants was similar.

NaCl gradient elution from the CM-cellulose column of HAG from wild type resulted in fractionation of the sample into two components (Fig. 3*A*). Washing the CM-cellulose column with several column volumes of buffer after sample application removed none of the MBTH-positive material. Estimates of the amount of sample applied to the column and the material recovered under the two peaks indicated 80–95% recovery. To test any possible relationship between the material in the low-salt-eluted component and that in the high-salt-eluted component, the material under each peak was pooled, dialyzed, and rechromatographed on the CM-cellulose column. Each peak was refractionated into two components in a pattern similar to that generated by the original sample from which each was derived. The peaks of the rechromatographed samples occurred in positions similar to the peak positions of the original sample. The size of each peak, relative to the other, was also similar to that found in the original sample, although some variability in relative peak sizes was found for various samples. However, preparation of HAG from wild-type cultures always demonstrated two distinct peaks of MBTH-positive material.

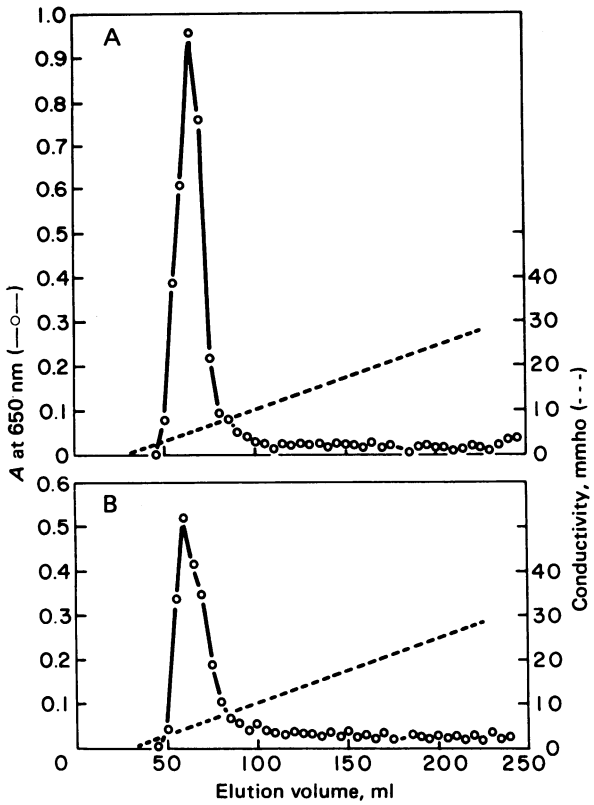

FIG. 5. CM-Cellulose elution behavior of HAG from sorbose-grown wild type (*A*) and sorbose-grown *Pk1* (*B*).

When HAG from *Pk1* was eluted from CM-celluose, only a single peak of MBTH-positive material was recovered (Fig. 3*B*). The amount of MBTH-positive material found in the single peak was equivalent to the total amount applied to the column. This single peak also was found when mutants *Pk3*, *Pk4*, *pk2*, and *pk5* were tested in the same manner. The single peak eluted in a position similar to that of the low-salt peak from wild type. Rechromatography of the pooled and dialyzed material from the single peak resulted in the appearance of only the single peak in the same position as the original.

Pk1 and wild type were crossed in order to test whether the HAG elution behavior cosegregated with morphology. Ascospores were dissected from isolated nonlinear asci. The ascospores were germinated and each was tested for mating type and classified for morphology (colonial versus wild type). A tetra-type tetrad was selected for extraction and fractionation of the HAG elaborated by each. The results, shown in Fig. 4, indicated cosegregation of the altered HAG elution behavior and altered morphology (i.e., presence of the *Pk1* allele). Mating type had no influence on the ion exchange elution characteristics of HAG from either wild type or mutant.

Phenocopies of mutants characterized by dichotomous branching and restricted growth habit can be produced in wild type by growing it in the presence of sorbose (15). The similarity in gross morphology at the vegetative hyphal level between *Pk1* and sorbose-grown wild type led us to test the elution behavior of HAG from sorbose-grown wild type. Fig. 5 shows that sorbose-grown *Pk1* had no alteration in its HAG elution characteristics whereas sorbose-grown wild-type demonstrates an HAG elution pattern similar to that of *Pk1*. The typical wild-type HAG elution pattern (Fig. 3*A*) was restored when a sample of the sorbose-grown wild type was returned to non-sorbose-containing medium and prepared in the typical manner. The loose, spreading appearance of the culture also was restored.

DISCUSSION

The results presented indicate that HAG preparations from wild-type cultures contain primarily a substance having high hexosamine content. The substance can be fractionated into several components by ion exchange chromatography on CM-cellulose. The components appear to be closely related and the results obtained by rechromatography seem to indicate a reversible equilibrium between the components. It is not evident whether the reversible equilibrium involves molecular interaction between several of the polysaccharide molecules (multimer or network formation) or the reversible binding of some other component in the system (ion binding or the binding of some other small molecule).

The elution behavior of HAG from *pk* mutants suggests that it is not undergoing the interconversion found for HAG from wild type. This elution behavior is stable to rechromatography. The findings strongly suggest that the observed difference in elution behavior is based on a molecular difference between *pk* HAG and wild-type HAG. The precise nature of that molecular difference is not known at this time. Identification and quantitation of the molecular components of HAG are required in order to understand the nature of the change in HAG effected by mutation at the *pk* locus.

That the molecular differences between wild-type and mutant HAGs are due to mutation at the *pk* locus itself is quite clear. The segregation data (Fig. 4) from the *Pk1* × wild-type cross are necessarily limited but the identity of elution diagrams for cultures representing five independent mutations at the *pk* locus provides strong evidence that these mutations account for the molecular alterations observed.

The relationship between hyphal morphology and HAG is evidenced further by the results of the phenocopy experiments. Although the mechanism by which sorbose is able to cause morphological change is not known, morphology and HAG ion exchange elution behavior can be simultaneously altered in a particular way, either by mutation or by sorbose.

The difference in response to the MBTH assay demonstrated by HAG from *Pk1* and from wild type suggests a particular kind of molecular alteration. The MBTH assay for the determination of hexosamines requires that the amino sugar have an unsubstituted amino group in the 2-position because the first step in the procedure is a nitrous acid deamination of the amino sugar (12). The greater response of the *Pk1* HAG would seem to indicate either less *N*-substitution of the hexosamine residues present or a greater total number of hexosamine residues. Because HAG is probably the same as Reissig and Glasgow's galactosaminoglycan (4), the *N*-substitution should be acetylation.

The results do not specify whether the observed alteration in HAG is the cause of the altered morphology. It is not yet clear whether the alteration in HAG is a concomitant result of a change in morphology or whether HAG is directly determined by the molecular function of the *pk* locus. Testing the HAG elution behavior of mutants at other loci which regulate hyphal and ascus morphology may help to resolve this question and may also provide additional information on the molecular basis of morphological determination.

Most previous studies that have attempted to correlate changes in *N. crassa* morphology with chemical changes in the cell wall have dealt with the analysis of chemical degradation products of the cell wall (6, 16, 17). The present study demonstrates that additional information can be obtained by the analysis of undegraded macromolecular components. As suggested by Kirkwood (18), the determination of morphology may

involve the matrix substances within which major cell wall structural components are embedded rather than involving the structural components themselves. These matrix substances may be present in relatively minor proportions [i.e., galactosamine represents only 1–2% of the total cell wall of *Neurospora* (6)] and a molecular alteration of one of these minor components could easily go undetected if only degradation analysis of whole wall or wall fractions is performed. Such a change would be identified most easily by assaying for a change in some chemical or physical parameter of the macromolecular structure, as presented here.

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