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Protein Specificity and Sexual Morphogenesis in *Schizophyllum commune*

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Soluble proteins of a number of strains of the basidiomycete *Schizophyllum commune* were separated on polyacrylamide gel by disc electrophoresis. The presence or absence, R_F values, and relative intensity of protein bands, observed on gel columns and densitometric tracings, were used as criteria for analysis and comparison. Eight mycelial types were studied, namely, normal homokaryon, dikaryon, homokaryons carrying primary mutation(s) at one or both of the $A\beta$ and $B\beta$ loci, and three mutant *B* homokaryons, each carrying a different type of modifier mutation. Similarities and dissimilarities in protein spectra among these mycelial types, observed with crude extracts, "total protein precipitates," and protein fractions, were consistent with predictions based on an earlier genetic model for the action of the two incompatibility factors known to control "sexual morphogenesis" in this fungus.

Mating competence and sexual morphogenesis in the wood-rotting basidiomycete *Schizophyllum commune* are controlled by two complex incompatibility factors, each consisting of two linked loci, $A\alpha$, $A\beta$ and $B\alpha$, $B\beta$ (3, 10). Among a very large number of alternate *A* and *B* factors in the natural population of this fungus (7), four basic combinations of incompatibility factors are possible in matings between homokaryotic mycelia: (i) compatible, different *A* factors and different *B* factors, yields the sexually fertile dikaryon; (ii) hemicompatible-*a*, like *A* and different *B* factors, yields the infertile common-*A* heterokaryon; (iii) hemicompatible-*b*, different *A* factors and like *B*, yields the infertile common-*B* heterokaryon; and (iv) noncompatible, like *A* and like *B*, yields the infertile common-*AB* heterokaryon.

In a mating between two compatible homokaryotic mycelia, contiguous cells of the two mycelia fuse and establish binucleate fusion cells. After this, a sequence of events takes place that converts the two homokaryotic mycelia into a uniformly dikaryotized mycelium. This morphogenetic sequence consists of six distinct stages, namely, nuclear migration, nuclear pairing, conjugate division, hook-cell formation, hook-cell septation, and hook-cell fusion. All of these events take place only when both the *A* and the *B* factors of the two mates are different ($A \neq B \neq$). None of these events takes place when both the *A* and the *B* factors of the two mates are in common ($A = B =$). When the *A* factor of the two mates is in common ($A = B \neq$), only nuclear migration takes place. When the *B* factor of the two mates is in common ($A \neq B =$), there is no extensive nuclear migration, but nuclear pairing, conjugate division, hook-cell formation, and hook-cell septation take place (3). A uniformly dikaryotized mycelium eventually forms fruiting bodies and basidia. After karyogamy and meiosis, the basidia produce basidiospores of four distinct genotypes. These spores germinate and give rise to homokaryotic mycelia, thereby completing the sexual cycle.

Mutations at the $A\beta$ and $B\beta$ loci in homokaryons (2, 4) negate both the specificity and the normal discriminatory function of the respective factors. These mutations in homokaryons also lead to heterokaryon mimicry. Thus, a homokaryon carrying a mutation at the $A\beta$ locus (*Amut Bx*) mimics the common-*B* heterokaryon, a homokaryon carrying a mutation at the $B\beta$ locus (*Ax Bmut*) mimics the common-*A* heterokaryon, and a homokaryon carrying mutations at both the $A\beta$ and the $B\beta$ loci (*Amut Bmut*) mimics the dikaryon. In addition, numerous mutations of several distinct types of "modifier" genes, scattered throughout the genome, effect drastic alterations in many characters of the heterokaryons. These mutations have no expression in homokaryons; they are expressed only in heterokaryotic mycelial types (or their mimics) in which morphogenetic processes under the control of the incompatibility factors have been "released" (8, 9).

Of a number of possible models for the action of the incompatibility factors that have been

examined in the light of known genetic structures and the physiological functions of the factors, a preferred model, based on derepression, casts the two loci of each incompatibility factor in the role of dual regulator genes (3). An important, though preliminary, finding was central to the formulation of this model: the essential identity of the protein spectra of two isogenic homokaryons and the marked differences between these protein spectra and that of the dikaryon formed by the mating of the two homokaryons (5). The model postulates (i) specific inhibitors of dikaryosis as the direct or indirect products of the two loci of each of the incompatibility factors in homokaryons and (ii) the suppression or inactivation of the inhibitor(s) as the result of the interaction of different factors of the same series. Although mycelia of the different types would be expected to have most protein species in common, on the basis of these postulates, certain specific predictions on the similarities and dissimilarities in protein spectra of the various mycelial types of this fungus have been made (3).

(i) The protein spectra of normal homokaryons are not correlated with specific incompatibility factors; all normal homokaryons should yield similar protein spectra.

(ii) The protein spectra of normal dikaryons will be different from those of normal homokaryons.

(iii) The protein spectrum of an *Amut Bmut* homokaryon will be essentially identical to that of a normal dikaryon, because proteins characteristic of the dikaryon will be present when the morphogenetic sequences controlled by both the *A* and *B* factors are expressed, regardless of the specific cause of their derepression.

(iv) The protein spectrum of the *Amut Bx* homokaryon and that of the *Ax Bmut* homokaryon will have certain, but not all, features of the spectrum of the dikaryon; this should permit the differentiation of the proteins specifically involved in the sequences controlled by the *A* and *B* factors.

(v) Mutations of "modifier" genes should affect those proteins peculiar to the dikaryon or other mycelial types in which the morphogenetic processes are expressed.

The present investigation was designed to assess the validity of these predictions. This paper presents the results obtained from electrophoretic analyses of soluble proteins of a number of mycelial types of *S. commune* and a discussion of the significance of these results in the light of our current concept of the mode of action of the incompatibility factors in controlling sexual morphogenesis in this fungus.

MATERIALS AND METHODS

Culture work. Nine isogenic strains (backcrossed for 10 or more generations with a single homokaryotic strain) were used in this study: two compatible normal homokaryons (*A41 B41* and *A43 B43*), one normal dikaryon (*A41 B41* + *A43 B43*), one homokaryon carrying a primary mutation at the *Bβ* locus (*A43 Bmut*; 2), one homokaryon carrying a primary mutation at the *Aβ* locus (*Amut B41*; 4), one homokaryon carrying primary mutations at both the *Aβ* and *Bβ* loci (*Amut Bmut*; 4), and three *Ax Bmut* homokaryons that also carry different types of modifier mutations, namely, *A41 Bmut M2* (type II), *A43 Bmut M11* (type IV), and *A43 Bmut M14* (type V; 9).

Stock cultures were maintained on peptone-glucose-yeast extract-agar medium (11). Seven-day-old mycelia, grown on this medium in standard plastic petri dishes at 25 C, were macerated for 3 min with cold, sterile distilled water in a Waring Blendor. The macerated material was used to inoculate 100-ml batches of a minimal liquid medium (10 g of dextrose, 1 g of DL-asparagine, 1 g of K_2HPO_4, 0.46 g of KH_2PO_4, 0.5 g of $MgSO_4 \cdot 7H_2O$, 0.12 mg of thiamine hydrochloride, 1 liter of distilled water with added trace elements) in 500-ml Erlenmeyer flasks. These liquid cultures were incubated for 4 days at 22 C on a reciprocating shaker. The mycelia were then macerated, and the macerated material was used to inoculate 6-liter batches of the minimal liquid medium in 10-liter carboys. Liquid cultures in carboys were incubated at 25 C with aeration for 48 hr. Mycelia were collected by filtration and were immediately frozen. Proteins were extracted from the harvested mycelia within 1 week.

Preparation of protein extracts. Frozen mycelia were homogenized at freezing temperature by passage through a pressure cell (6) at 18,000 psi. The homogenates were extracted for 60 min at 0 C with appropriate buffers (see below). The mixtures were centrifuged for 30 min at 0 C at 37,000 × *g*. The supernatant fluids (crude extracts) either were used directly or were treated in various ways for different experimental purposes.

(i) Sodium phosphate (0.1 M, *p*H 8.6) extracts were used for electrophoretic analysis without further treatment.

(ii) Tris(hydroxymethyl)aminomethane (Tris)-hydrochloride (0.2 M, *p*H 9.1) extracts were treated with 0.1 volume of 2% protamine sulfate at 0 C. The clear supernatant fluids collected after centrifugation were used for electrophoretic analysis.

(iii) Sodium phosphate (0.1 M, *p*H 7.0) extracts were treated as described in (ii).

(iv) Sodium phosphate (0.1 M)-0.01 M ethylenediaminetetraacetic acid (EDTA; *p*H 7.0) extracts were treated with 0.1 volume of 2% protamine sulfate at 0 C and centrifuged. The clear supernatant fluids either were used directly for electrophoretic analysis or were subjected to one of the following treatments. For precipitation of "total protein," the supernatant fluids were brought to 90% saturation with solid ammonium sulfate. Protein precipitates collected by centrifugation were dissolved in cold distilled water and dialyzed for 24 hr at 4 C against three changes of

large volumes of dilute buffer (0.02 M sodium phosphate-0.002 M EDTA) at *p*H 7.0. Dialyzed samples were freeze-dried and kept in a desiccator at −18 C. For protein fractionation, soluble proteins in the partially purified extracts were fractionated by precipitation with stepwise increases in concentration of ammonium sulfate. The number of fractions was determined on the basis of the quantity of precipitates formed and hence the ease with which electrophoretic analyses could be made. The following "cuts" were made: 0 to 50%, 50 to 55%, 55 to 60%, 60 to 65%, 65 to 70%, 70 to 75%, 75 to 80%, and 80 to 90% saturation. Protein precipitates collected by centrifugation were dialyzed and dried as described above.

Dry extracts were dissolved in cold buffer immediately before use. A quantitative colorimetric biuret method (1) was routinely used for determination of proteins.

Analytical procedures. Disc electrophoresis on polyacrylamide gel columns was used as the major tool of separation according to the methods and formulations provided by Canal Industrial Corp. with the model 12 apparatus. The 7.5% "standard gel" at a running *p*H of approximately 9.4 was used for the separation of medium-sized proteins. Five per cent (large-pore) and 15% (small-pore) "standard gels" at the same running *p*H were used for the separation of large and small proteins, respectively. The length and diameter of the glass tubes used for preparation of gel columns and separation were as follows: 10 by 0.8 cm, 14 by 0.8 cm, and 20 by 0.9 cm. Protein samples were either incorporated into a sample gel or mixed with an equal volume of a sample buffer (containing 40% glycerol, a few drops of 2-mercaptoethanol, and a trace amount of tracking dye, namely, bromophenol blue) and placed between two layers of spacer gel. Electrophoresis was performed at room temperature with a constant current of 4 ma per tube. After electrophoresis, gels were removed from glass tubes and stained for 60 min in 0.5% Naphthol Blue Black in a mixture of methanol-acetic acid-water (5:1:5, v/v). Destaining was done either by soaking or by electrophoresis with 7.5% acetic acid.

Patterns and densities of protein bands on destained gel columns were recorded by means of a recording densitometer (Photovolt Densicord 542), and the R_F values of protein peaks were calculated. The presence or absence, R_F values, and relative intensity of protein bands were used as criteria for analysis and comparison.

RESULTS

Typical banding patterns of "total proteins" of eight types of mycelia of *S. commune* are shown in Fig. 1. These proteins were precipitated with ammonium sulfate from *p*H 7.0 sodium phosphate-EDTA extracts and were separated on 20-cm, 7.5% standard gel columns. The protein spectra of the two normal homokaryons were practically identical, and the banding pattern of the *Ax Bmut M11* homokaryon closely resembled that of the normal homokaryon from which it is

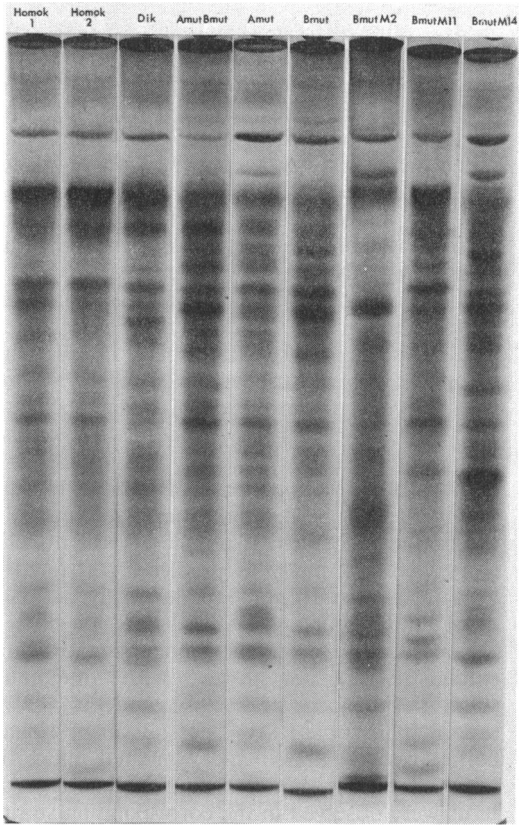

FIG. 1. *Total protein precipitates of 0.1 M sodium phosphate-EDTA (pH 7.0) extracts separated on 20-cm, 7.5% standard gel columns.*

morphologically indistinguishable. Whereas the protein spectra of these homokaryons were noticeably different from those of the other mycelial types, the banding patterns of the dikaryon and the *Amut Bmut* homokaryon were quite similar. The details of such similarities and dissimilarities in protein spectra among the various mycelial types represented here are better recognized in a comparison of the protein peaks recorded by densitometry. Many of the differences in banding pattern observed in the other mycelial types, as compared to the normal homokaryons, may be the result of "R_F shift," i.e., changes in electrophoretic mobility, of the same proteins. In many instances, the appearance of a protein peak was accompanied by the disappearance of another having a very close R_F value in one or more of the other mycelial types as compared to the normal homokaryons. A slight modification of the structure or properties, or both, of a protein, as a result of the expression of one or both of the morphogenetic sequences, may well lead to a change in its electrophoretic mobility and hence

its R_F value. The designation of "new" and "missing" proteins accordingly becomes somewhat arbitrary. Because the R_F values of the same protein bands in duplicate gel columns of normal homokaryons consistently differed by less than 0.01, whereas distinct bands differing by R_F values of 0.01 were routinely seen in the same duplicate sets, only protein bands having R_F values that differed by 0.01 or more in the several mycelial types were considered as distinct.

The results of an analysis of these details are summarized in Fig. 2. Clearly, the special features of the protein spectra displayed by the other mycelial types in comparison with the normal homokaryons, the presence of certain proteins, the absence of certain other proteins, and quantitative differences in yet other proteins, fell in a number of distinct patterns (Table 1). In sum, the similarities and dissimilarities in the spectra of the proteins obtained from the several mycelial types were essentially as predicted, as stated in the introduction, from the model for the action of the *A* and *B* factors.

For the purpose of comparison, "whole extracts" (i.e., not treated with ammonium sulfate) of the same strains, prepared with *p*H 7.0 sodium phosphate-EDTA or sodium phosphate buffer, were separated and examined under the same conditions, and comparable banding patterns were observed in the corresponding strains. Although, with the whole extracts, a larger number of bands were observed in each strain, the presence of similarities and dissimilarities as predicted for the various mycelial types was confirmed.

When the whole extracts of the two normal homokaryons, the dikaryon, the *Amut Bmut* homokaryon, the *Amut Bx* homokaryon, and the *Ax Bmut* homokaryon, prepared with *p*H 8.6 sodium phosphate buffer or *p*H 9.1 Tris-hydrochloride buffer, were separated and examined under the same conditions, the banding patterns

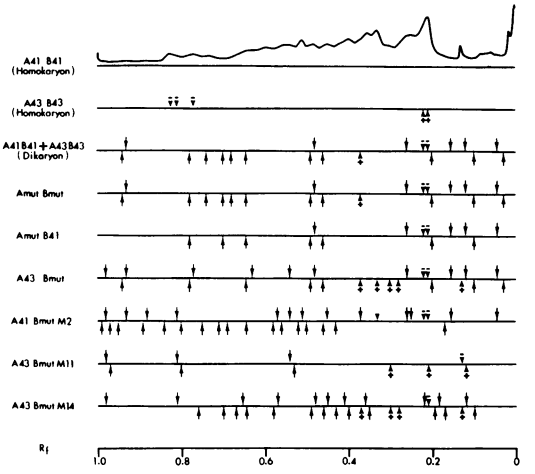

FIG. 2. *Analysis of densitometric tracings of the same gel columns shown in Fig. 1. Symbols: ↓, protein peak present in normal homokaryons but absent from other mycelial types; ↑, protein peak observed in other mycelial types but not in normal homokaryons; ⇕, protein peak increased in intensity as compared to the corresponding peak of the normal homokaryon A41 B41; ⇳, protein peak decreased in intensity as compared to the corresponding peak of the normal homokaryon A41 B41.*

TABLE 1. *Differences in protein spectra observed among five mycelial types*

Pattern no.	Patterns of differences in protein spectra among mycelial types[a]					No. of protein peaks showing the same pattern in				
	Homokaryon	Dikaryon	*Amut Bmut*	*Amut Bx*	*Ax Bmut*	Total protein	Protein fractions[b]			
							65–70%	70–75%	75–80%	80–90%
1	−	+	+	+	+	6	9	10	1	0
2	−	+	+	+	−	1	3	1	3	0
3	−	+	+	−	+	2	0	1	1	1
4	−	+	+	−	−	2	0	2	2	1
5	−	−	−	−	+	0	3	2	4	5
6	+	−	−	−	−	5	11	7	2	1
7	+	−	−	−	+	0	5	4	4	0
8	+	−	−	+	−	1	0	1	1	3
9	+	−	−	+	+	0	0	3	1	1
10	+	+	+	+	−	4	2	3	3	4
11	−	+	−	−	−	0	0	0	0	1
12	−	−	+	+	−	0	0	0	1	1
13	+	−	+	+	+	0	1	0	0	0

[a] Protein peak present (+) or absent (−).
[b] Protein fractions obtained at various levels of saturation.

were somewhat different from those given by the *p*H 7.0 extracts. Although the precise banding patterns were different, the same predicted relationships among the extracts of the several mycelial types were found as in the previous case.

To expand the protein spectra and obtain a better resolution of the banding patterns, protein precipitates of the same six strains, obtained from *p*H 7.0 sodium phosphate-EDTA extracts by fractionation with ammonium sulfate, were further separated by electrophoresis on 14-cm, 7.5% standard gel columns under the same experimental conditions. On examination of the protein spectra of these preparations, the following features were observed.

(i) The first fraction, namely, 0 to 50% saturation, of all six strains studied gave practically identical patterns for the several mycelial types.

(ii) Protein fractions obtained at 50 to 55% and 55 to 60% saturation showed essentially only quantitative differences among mycelial types.

(iii) The remaining fractions each showed a number of qualitative differences, as defined by their electrophoretic mobility, in addition to quantitative differences between the normal homokaryons and the other mycelial types.

(iv) The banding patterns of the two normal homokaryons were essentially identical in each of the fractions.

(v) The protein peaks peculiar to or absent from the dikaryon, the *Amut Bmut* homokaryon, the *Amut Bx* homokaryon, and the *Ax Bmut* homokaryon, in comparison with those of the two normal homokaryons, were generally better resolved (Table 1).

(vi) Protein peaks present in all mycelial types, but quantitatively different from one mycelial type to another, stood out more clearly than in the case of whole extracts.

(vii) Most of the differences in protein spectra displayed by the various fractions of the other mycelial types, in comparison with the corresponding fractions of the normal homokaryons, fell in 10 distinct patterns (patterns 1 to 10 in Table 1). Other patterns (patterns 11 to 13) were only found in one or two fractions and hence were uncertain.

Figures 3 to 6 summarize the results of analyses of the protein spectra of the last four fractions, namely, 65 to 70%, 70 to 75%, 75 to 80%, and 80 to 90% saturation.

When "total protein precipitates" of *p*H 7.0 sodium phosphate-EDTA extracts of five representative mycelial types were separated on 15% (small-pore) standard gel, no appreciable differences in banding pattern were detected. Furthermore, only minor quantitative differences were detected among mycelial types when these protein preparations were separated on 5% (large-pore) standard gel columns.

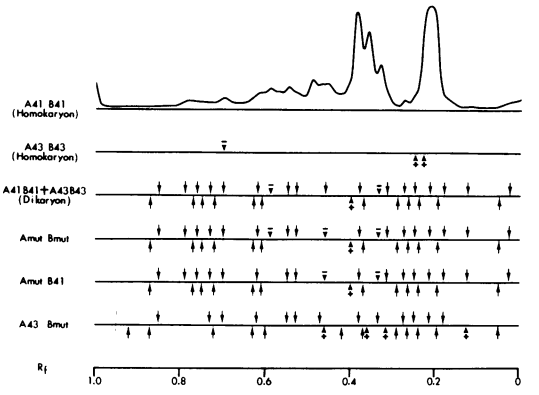

FIG. 3. *Analysis of densitometric tracings of proteins precipitated at 65 to 70% saturation with ammonium sulfate from pH 7.0 phosphate-EDTA extracts and separated on 14-cm, 7.5% standard gel columns. Symbols as for Fig. 2.*

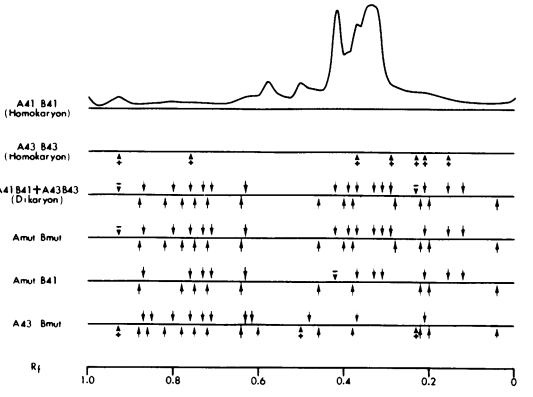

FIG. 4. *Analysis of densitometric tracings of proteins precipitated at 70 to 75% saturation with ammonium sulfate from pH 7.0 phosphate-EDTA extracts and separated on 14-cm, 7.5% standard gel columns. Symbols as for Fig. 2.*

DISCUSSION

The primary objective of the present work has been to recognize specific biochemical manifestations of the activities of the incompatibility factors of *S. commune* as reflected in the protein spectra of the several types of mycelia. For a study of this type, an ideal approach would be one that would permit the extraction, separation, and analysis of all proteins. The extreme diversity of the chemical and physical properties of proteins and the lability of some of these complex molecules, however, make such an ideal method impossible. Of the methods tested, those finally adopted were found to cause the least difficulty in

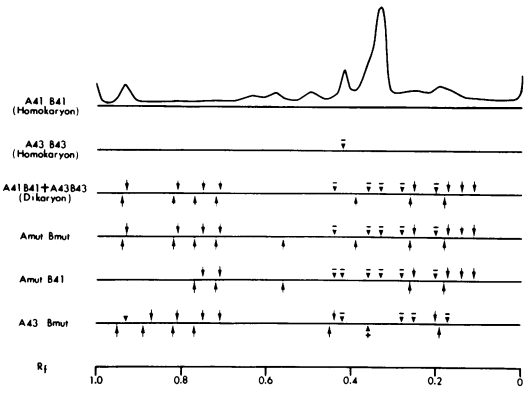

FIG. 5. *Analysis of densitometric tracings of proteins precipitated at 75 to 80% saturation with ammonium sulfate from pH 7.0 phosphate-EDTA extracts and separated on 14-cm, 7.5% standard gel columns. Symbols as for Fig. 2.*

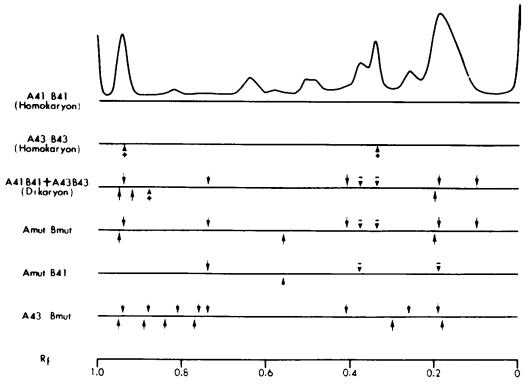

FIG. 6. *Analysis of densitometric tracings of proteins precipitated at 80 to 90% saturation with ammonium sulfate from pH 7.0 phosphate-EDTA extracts and separated on 14-cm, 7.5% standard gel columns. Symbols as for Fig. 2.*

studying "total" proteins under the experimental conditions described.

Disc electrophoresis, so extensively used by various workers in recent years, is recognized as a very sensitive method for the separation of proteins. We have found, with a given method of preparation and a given set of experimental conditions, the reproducibility of banding patterns to be quite good. In the present study, in which large numbers of proteins were involved, techniques and skills of using 20-cm glass tubes for electrophoresis had to be developed to increase the power of resolution. Nevertheless, for a variety of reasons, we feel that minor quantitative differences in banding patterns must be interpreted with extreme caution.

The results obtained from analyses of the total protein precipitates and whole extracts of the various strains studied here certainly provide strong support for the predictions with regard to similarities and dissimilarities in protein spectra among the several mycelial types. The persistence of the differences in protein spectra displayed by the other mycelial types, in comparison with the normal homokaryons, signals the central role of many proteins in the morphogenetic progression.

The special features of banding patterns observed in the various fractions of the dikaryon and the homokaryons carrying primary mutations at the incompatibility loci, as compared to the banding patterns of the corresponding fractions of the normal homokaryons, further support the results obtained with total protein precipitates and whole extracts. It is thus felt that the qualitative and quantitative differences observed between normal homokaryons and other mycelial types can be interpreted in terms of the derepression model (Table 2).

The presence or absence of a protein in a particular type of mycelium should signal the activity or the inactivity of a component process of the morphogenetic progression. Extended to the full range of mycelial types determined by the incompatibility factors, the pattern of presence or absence of a given protein in the various mycelia should relate the relevant component process to the overall morphogenetic progression. Ten such different patterns were observed in the protein spectra compared above (patterns 1 to 10 in Table 1).

The complexity of the banding patterns observed in each of the protein fractions and the technical limitations encountered, however, only serve to emphasize a difficulty recognized from the very beginning of the present study. Demonstration of differences in protein spectra, even when such differences are positively correlated with a specific incompatibility factor on the one hand and with a particular component of the morphogenetic sequence on the other hand, provides no information about what precise roles the proteins, or for that matter the incompatibility factors, are performing in the sequence.

In further studies and with more sensitive methods, it may be possible, in some cases, to isolate individual proteins and to identify their specific activities as relevant to particular stages in the morphogenetic progression. Alternatively, it may be feasible to search for the activities of certain enzymes responsible for a number of postulated biochemical events as likely components of the morphogenetic progression. Limited success (to be reported elsewhere) has been achieved along these lines in the alteration

TABLE 2. *Interpretations for differences in protein spectra observed among five mycelial types*

Pattern no.	Differences					Interpretation
	Homokaryon	Dikaryon	*Am Bm*	*Am Bx*	*Ax Bm*	
1	−	+	+	+	+	Derepressed when either *A*- or *B*-sequence is in operation
2	−	+	+	+	−	Derepressed when *A*-sequence is turned on whether *B*-sequence is on or off
3	−	+	+	−	+	Derepressed when *B*-sequence is turned on whether *A*-sequence is on or off
4	−	+	+	−	−	Derepressed only when *A*- and *B*-sequences are simultaneously in operation
5	−	−	−	−	+	Derepressed when *B*-sequence is turned on while *A*-sequence is off
6	+	−	−	−	−	Repressed when either *A*- or *B*-sequence is in operation
7	+	−	−	−	+	Repressed when *A*-sequence is turned on whether *B*-sequence is on or off
8	+	−	−	+	−	Repressed when *B*-sequence is turned on whether *A*-sequence is on or off
9	+	−	−	+	+	Repressed only when *A*- and *B*-sequences are simultaneously in operation
10	+	+	+	+	−	Repressed when *B*-sequence is turned on while *A*-sequence is off
11[a]	−	+	−	−	−	Protein(s) unique to a uniformly dikaryotized mycelium (possibly related to constitutive dikaryosis)
12[a]	−	−	+	+	−	Protein(s) common to *Amut Bmut* and *Amut Bx* homokaryons only (possibly related to certain common morphological features in these two mycelial types)
13[a]	+	−	+	+	+	Repressed only in a uniformly dikaryotized mycelium (possibly related to dikaryosis)

[a] There are (i) minor phenotypic differences between dikaryon and *Amut Bmut* homokaryon and (ii) rather substantial phenotypic similarities between *Amut Bmut* and *Amut Bx* homokaryons (9).

of isozymes of identified proteins, correlated with different mycelial types as representing different expressions of incompatibility and sexual morphogenesis.

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