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Protein Changes During the Asexual Cycle of *Neurospora crassa*

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A method for synchronizing conidiation and isolating large numbers of cells at discrete stages of conidia development is described. Using two-dimensional gel electrophoresis, we analyzed the protein profiles of mycelia, aerial hyphae, and conidia and observed that the concentration of 14 polypeptides increase and 38 decrease during the asexual cycle. Twelve polypeptides were present in extracts of aerial hyphae or conidia, but not mycelia, suggesting that they may be conidiation specific. The protein profiles of mutants defective in conidiation were also analyzed. Differences were detected in the two-dimensional profiles of protein extracts from fluffy and wild-type aerial hyphae. Polyadenylated RNA isolated from wild-type mycelia and conidiating cultures was translated in vitro in a rabbit reticulocyte lysate. Differences were detected in the polypeptide products specified by the two RNA populations, suggesting that changes in steady-state levels of polyadenylated RNAs also occur during conidiation.

During asexual development, *Neurospora crassa* undergoes a series of morphological changes resulting in the production of asexual spores or conidia. Vegetative hyphae or mycelia grow by hyphal elongation and branching to produce a filamentous mat. Each vegetative hypha is a linear sequence of multinucleate cells divided by incomplete cross-walls. Cytoplasmic flow and nuclear migration occur between cells through a central pore in the cross-walls. During growth, vegetative hyphae differentiate into aerial hyphae to begin the developmental steps leading to conidiation. What induces mycelia to grow aerially is unclear. The age of the culture and nutrient limitation may be contributing factors (12), although evidence supporting this notion is not definitive. Eight to ten percent of the aerial hyphae that form differentiate into conidiophores. In these differentiating aerial hyphae, nuclear migration occurs to the hyphal tips. Hyphal tips containing at least one nucleus swell to become spheres 4 to 6 μm in diameter and then form chains of immature conidia by apical budding. When terminal budding ceases, septa form between adjacent conidia. Nuclear division ceases, followed by the disarticulation of the proconidial chain and the formation of mature conidia (11).

Changes in enzyme activities occurring during the stages in the asexual cycle have been measured. Most of the enzyme activities that have been determined are lower in conidia than in mycelia, although the activities of several extracellular hydrolytic enzymes such as trehalase and invertase are elevated in conidia. However, these enzymes are not required for conidium formation since mutants deficient in each conidiate normally (16).

Mutants have been isolated that are defective in several steps leading to conidium formation. The mutations in these strains map to single chromosomal loci in several linkage groups. The alterations in most of these mutants are phase specific in that each affects a relatively discrete developmental process such as formation of aerial hyphae, migration of nuclei to the tips of aerial hyphae, swelling of hyphal tips, formation of proconidial chains by repeated apical budding, and septation along the length of the conidial chain (11, 19). For the most part, the primary biochemical defects in these mutants have not been characterized. Thus, the molecules that induce *N. crassa* to conidiate and the products required for the differentiation of aerial hyphae and conidia are unknown.

As described above, the asexual cycle of *N. crassa* involves a well-ordered series of events well suited for the study of gene expression during development. The work presented here describes a method for inducing conidiation and isolating large numbers of cells at discrete stages in conidium development. By examining the protein profiles of mycelia, aerial hyphae, and conidia, we have been able to estimate the proportion of cellular proteins differentially produced during the asexual cycle and to identify proteins present only in aerial hyphae or conidia. Our results suggest that synthesis of some of the proteins differentially produced in conidiating cultures is regulated at the level of RNA synthesis and provide the rationale for our current efforts to isolate and characterize genes that are induced during asexual development in *N. crassa*.

MATERIALS AND METHODS

Strains and culture conditions. The strains used in this work are indicated in Table 1. Strains were grown in Vogel minimal medium (22) containing 1.5% (wt/vol) sucrose as the carbon source. Liquid mycelial cultures of all strains except *fl* were grown by inoculating 10^6 conidia per ml of minimal medium. Conidia were counted with a hemocytometer. Hyphal fragments were used to inoculate *fl* cultures. Liquid cultures were grown at room temperature in Florence flasks for approximately 20 h until cultures reached the late log phase (dry weight, ~0.8 mg/ml). Sterile air was continuously bubbled through cultures for aeration and to disperse cells during growth. At the end of the growth period, mycelia were either harvested for protein analysis or induced to conidiate.

To induce conidiation, the cells in 200-ml samples of a liquid mycelial culture grown to the late log phase were harvested by filtering onto sterile, 9-cm Whatman no. 1 filter paper disks. The filter paper disks and mycelium were transferred to petri dishes containing a single layer of 5-mm glass beads and 10 ml of medium. In some experiments a second piece of filter paper moistened with medium was placed over the mycelium. The cultures harvested onto filter paper were placed in trays lined with moist paper towels and were incubated uncovered at room temperature in a sterile hood under fluorescent lights for various lengths of time. When wild-type mycelia were covered with a filter paper

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TABLE 1. *N crassa* strains used in this study

Strain	Allele	Phenotype	Reference(s)
74-OR23-1a		Wild type	
Fluffy (*fl a*)	P	Aconidial	9
Crisp (*cr-1 A*)	B123	Conidiates prematurely on short aerial hyphae	10
Easily wettable (*eas a*)	UCLA 191	Produces conidia lacking the rodlet layer	2, 18
Sulfur control (*scon a*)	c	Produces osmotically fragile conidia	5, 17

disk, aerial hyphae of the wild-type strain grow through the top filter paper and in 24 h produce approximately 10^9 conidia per plate. For protein analysis, aerial hyphae and conidia growing above the top filter paper were harvested and separated from the underlying mycelium by scraping the cells from the top filter paper.

Scanning electron microscopy. Mycelia grown to the late log phase were induced to conidiate. Harvested mycelia were not covered with a filter paper disk. Various times after induction samples were fixed in 2% glutaraldehyde–1% paraformaldehyde–0.1 M phosphate buffer (pH 7.2) for 12 to 24 h at 4°C. The samples were then treated for 2 h with 1% OsO_4 followed by treatment with 1% uranyl acetate for 1 h. Samples were dehydrated in increasing concentrations of acetone, dried in a Sorvall critical point dryer, and coated with gold palladium. Samples were visualized with a scanning electron microscope.

Labeling conditions. Liquid mycelial cultures were grown as described above. The cells in 100-ml samples were harvested by filtration through cheesecloth and suspended in 10 ml of medium containing 20 μCi of L-[^{35}S]methionine (New England Nuclear Corp.; 1,000 Ci/mmol) per ml. Cultures were grown for an additional 90 min at 30°C in a shaking water bath. At the end of the labeling period mycelial cultures were harvested by filtration through Whatman no. 1 filter paper and were washed with 20 ml of 1 mM cold methionine.

To label conidiating cultures, late log phase mycelia were harvested onto filter paper, transferred to petri dishes containing minimal medium plus 20 μCi [^{35}S]methionine per ml, and grown for an additional 24 h. Aerial hyphae and conidia were harvested and suspended in 1 mM methionine containing 0.1% Tween 80 (Sigma Chemical Co.). To separate conidia and aerial hyphae, the suspension was vortexed and filtered three times through four layers of cheesecloth. The conidia that pass through the cheesecloth were collected by centrifugation at 3,000 × *g* for 5 min. Aerial hyphae, which do not pass through the cheesecloth, were washed with 1 mM methionine and were transferred to a test tube for extraction of cellular proteins.

Extraction of cellular proteins. Proteins were extracted from mycelia, aerial hyphae, and conidia by a modification of the procedure of Bowman et al. (4). Approximately 1 g (wet weight) of mycelia was suspended in 10 ml of buffer A (0.59 sucrose, 5 mM EDTA, 50 mM NaH_2PO_4 [pH 6.5]). Aerial hyphae and conidia harvested from two petri dishes were suspended in 3 and 8 ml of buffer A, respectively. β-Mercaptoethanol (25 mM) and 6,000 U of glusulase (Sigma; β-glucuronidase type H-2) per ml were added to cell suspensions. The cell suspensions were incubated at 30°C for 30 min with gentle shaking. Cells were then pelleted by centrifugation for 10 min at 4,000 × *g*. Pellets were washed two times with 0.68 M sucrose and suspended in buffer B (0.33 M sucrose, 10 mM Tris [pH 7.5], 1 mM EDTA, 1 mM phenylmethanesulfonyl fluoride [Sigma], 100 μM leupeptin [Sigma]) in one-half the original volume. Cells were broken by vortexing with glass beads, and the homogenate was centrifuged at 2,500 × *g* for 10 min to pellet cell debris and unbroken cells. The pellet was suspended in a small volume of buffer B, vortexed with glass beads, and centrifuged at 2,500 × *g* for 10 min. The two supernatants were combined and stored at −80°C.

mRNA isolation. Total cellular RNA was prepared from mycelia and unfractionated conidiating cultures as described by Reinert et al. (15). Polyadenylated [poly(A)$^+$] RNA was purified by oligodeoxythymidylate-cellulose chromatography by the method of Aviv and Leder (1) as modified by Chirgwin et al. (6).

In vitro translation of poly(A)$^+$ RNA. Rabbit reticulocyte lysate was prepared as described by Pelham and Jackson (14) and was supplemented with amino acids minus L-methionine. The lysate was kindly provided by Debbie Brown. Reactions containing 8 μl of lysate, 10 μCi of [^{35}S]methionine, and 0.3 μg of poly(A)$^+$ RNA in a total volume of 11 μl were incubated at 30°C for 60 min. Reactions were stored at −80°C until they were analyzed by gel electrophoresis.

Electrophoresis of proteins. Cellular proteins and in vitro translation products labeled with [^{35}S]methionine were analyzed by two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) as described by O'Farrell et al. (13) and Jones (8). Proteins were separated in the first dimension by non-equilibrium pH gradient electrophoresis in cylindrical gels containing ampholines pH 3.5 to 10 (LKB Instruments

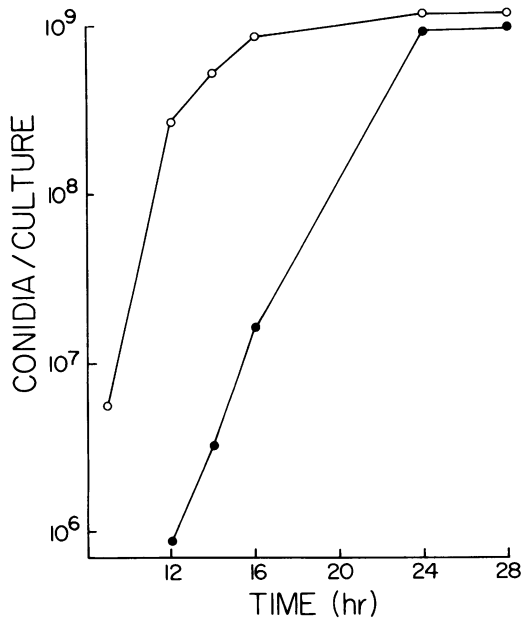


FIG. 1. Conidia production per culture versus time after induction. Samples (200 ml) of a mycelial culture grown to the late log phase were harvested by filtering onto sterile, 9-cm Whatman no. 1 filter paper disks to induce conidiation. Half of the cultures were covered with a second filter paper disk. At various times after induction, cultures were harvested, and the number of conidia produced per culture was counted with a hemocytometer. Conidium production per culture assayed before the first time points shown was lower than the resolution of the hemocytometer (10^3). Results show conidia per culture grown with (●) or without (○) a filter paper disk placed over the mycelial mat.

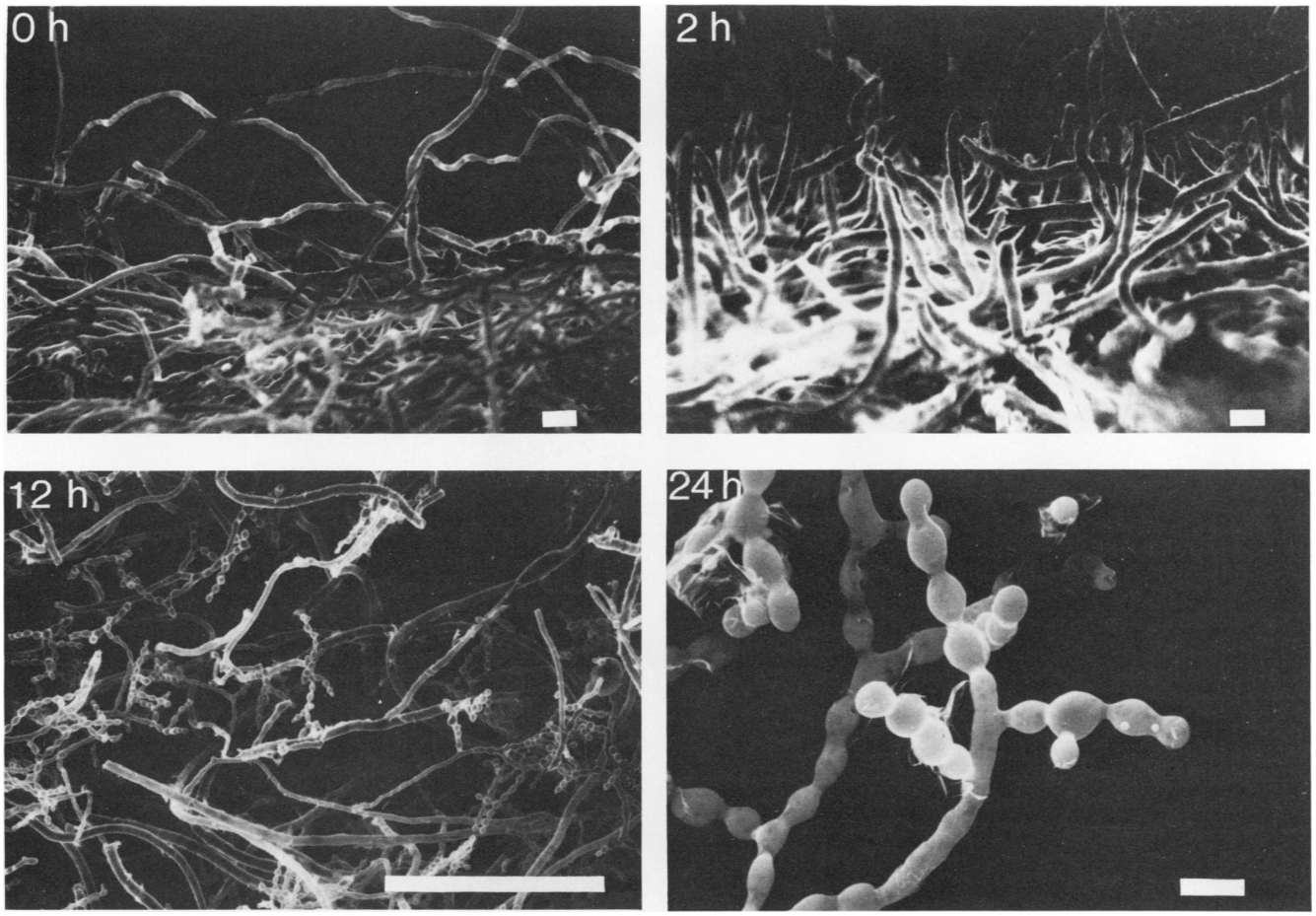


FIG. 2. Changes in cell morphology during conidiation. Mycelia grown to the late log phase were harvested onto filter paper to induce conidiation. Cultures grown for 0, 2, 12, and 24 h after induction were fixed, dried, and coated with gold palladium as described in the text. Bars: 10, 10, 100, and 10 μm at 0, 2, 12, and 24 h, respectively.

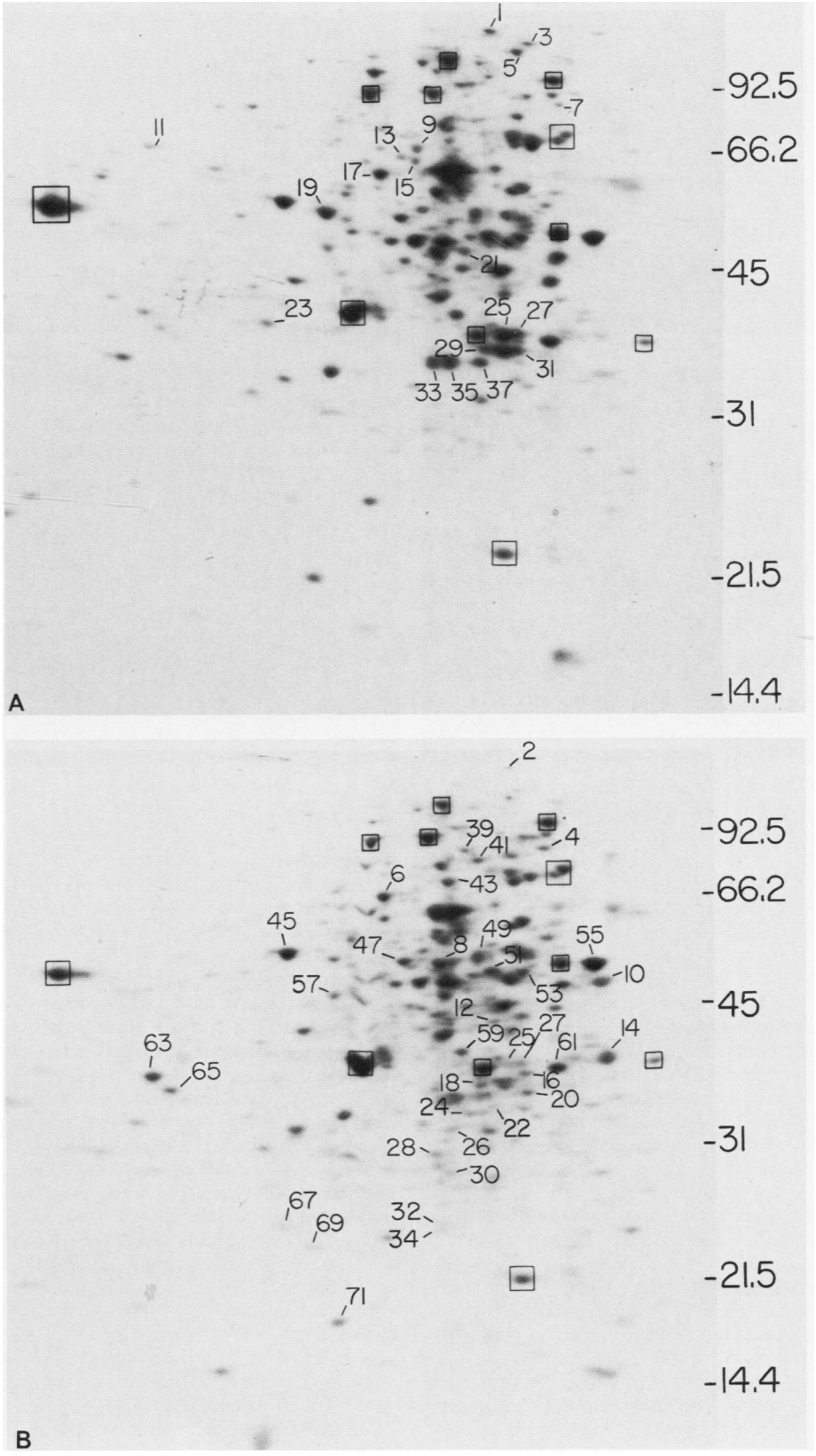
Inc.) and in the second dimension by electrophoresis in 12% sodium dodecyl sulfate–polyacrylamide slab gels. The proteins in slab gels were fixed and stained in 50% trichloroacetic acid and 0.1% Coomassie blue for 30 min and were destained in 7% acetic acid. Gels were rinsed in water for 30 min, transferred to 1 M sodium salicylate for 30 min for gel fluorography, and exposed to X-ray film (Eastman Kodak Co.; XAR-5). Exposure times were inversely proportional to the total counts per minute of ^{35}S in protein applied to each gel as judged by counts in material precipitable by trichloroacetic acid in a separate sample. A gel containing 10^6 cpm of ^{35}S-labeled protein was exposed for 24 h at −80°C.

RESULTS

Induction of conidiation. Conidiation was induced by a modification of methods of others (20, 21). In earlier studies, mycelial mats were transferred from minimal medium to phosphate buffer to induce conidiation. In the present study both vegetative and conidiating cultures were grown in minimal medium to eliminate the effect of nutrient supply on protein synthesis during various stages of the asexual cycle. Conidiating cultures were grown for various times after induction to examine the time course of conidium formation. During the initial period of growth, aerial hyphae grew through the filter paper placed over the mycelial mat and were first visible under the dissecting microscope (70× magnification) at 6 h after induction. Conidium formation was not evident at this time or at 9 h after induction. However, at 9 h a uniform layer of aerial hyphae was visible growing above the top filter paper. By 12 h a single culture produced 8.7×10^5 conidia. The number of conidia produced per culture increased until 24 h and reached a plateau at approximately 10^9 conidia per petri dish. Growth of aerial hyphae through the top filter paper delayed the onset of conidiation, but had no effect on the maximal conidial yield per culture (Fig. 1).

The morphological changes that occur during conidiation were examined by scanning electron microscopy. For these studies mycelia harvested onto filter paper to induce conidiation were not covered with a second filter paper disk so that we could visualize the initial morphological changes that occur during the differentiation of mycelia into aerial hyphae. At 2 h after induction, nascent aerial hyphae approximately 40 μm long were visible, growing perpendicular to the mycelial mat. By 12 h, aerial hyphae had elongated and branched. A majority of the hyphal tips had budded to produce chains of conidia consistent with the time course of conidium formation shown in Fig. 1. No differences in morphology were apparent between cultures grown for 12 and 24 h after induction (Fig. 2).

Analysis of protein synthesis during the asexual cycle in wild-type cultures. During growth of mycelia in liquid culture, 60% of the [^{35}S]methionine added to the medium was incorporated into trichloroacetic acid-precipitable material.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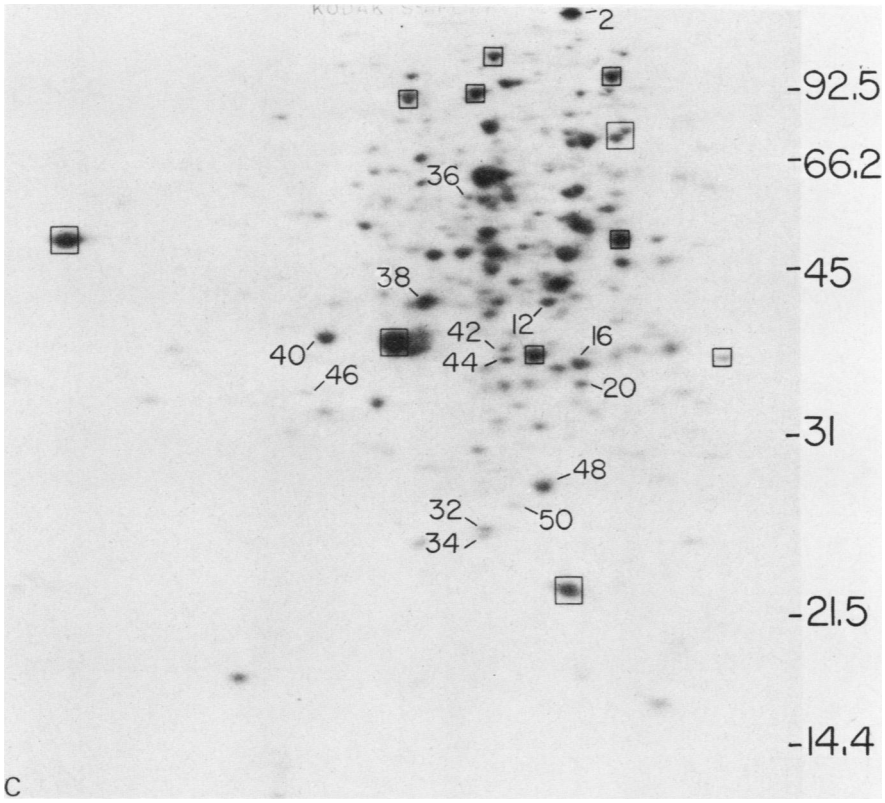

FIG. 3. Two-dimensional profiles of total labeled cellular proteins extracted from wild-type mycelia, aerial hyphae, and conidia. Cultures were grown and labeled with [^{35}S]methionine as described in the text. Aerial hyphae and conidia were harvested 24 h after inducing conidiaton. The polypeptides extracted from mycelia, aerial hyphae, and conidia were separated by non-equilibrium pH gradient electrophoresis in the first dimension in a pH gradient from pH 3.5 (right) to pH 10 (left) and by electrophoresis in 12% sodium dodecyl sulfate–acrylamide slab gels in the second dimension. The molecular weights of size standards ($\times 10^3$) are shown in the vertical axis at the right. Spots enclosed in boxes represent selected polypeptides present at comparable levels in the three cell types and serve as reference polypeptides to orient the autofluorograms with respect to each other. Odd-numbered polypeptides decrease, and even-numbered polypeptides increase in concentration in conidiating cultures. (A) Polypeptides extracted from mycelia. Numbered polypeptides are present at lower concentrations in aerial hyphae and conidia. (B) Polypeptides extracted from aerial hyphae. Even-numbered polypeptides are absent or present at lower concentrations in mycelia. Polypeptides 4 and 18 are not detectable in protein extracts of conidia. All other even-numbered polypeptides are present at comparable or higher levels in conidia. Odd-numbered polypeptides are present at lower concentrations in conidia. (C) Polypeptides extracted from conidia. Numbered polypeptides are absent or present at lower concentrations in mycelia and aerial hyphae. Autofluorograms were exposed at −80°C for 12 h to 3 days.

When mycelia were harvested onto filter paper and induced to conidiate, only 5% of the [^{35}S]methionine added to the medium was incorporated into trichloroacetic acid-precipitable material. The incorporation of label into trichloroacetic acid-precipitable material isolated from aerial hyphae and conidia was fivefold greater than the incorporation of label into trichloroacetic acid-precipitable material isolated from the mycelial mat (data not shown). Protein turnover during the 24-h labeling period could account in part for the relatively low level of incorporation of label into trichloroacetic acid-precipitable material in conidiating cultures. It is also possible that some of the methionine was desulfomethylated or otherwise metabolized.

Extracts were prepared from cultures labeled with [^{35}S]methionine, and their proten profiles were analyzed by 2-D PAGE. The profile of proteins synthesized in mycelia, aerial hyphae, and conidia is shown in Fig. 3. Major differences are apparent when the protein profiles of the three cell types are compared. Of approximately 300 polypeptides resolved by 2-D PAGE, 61 were identified that are differentially expressed in conidiating cultures; 38 of these decrease and 14 increase in concentration in aerial hyphae or conidia. Twelve polypeptides are conidiation specific in that they are present in extracts of aerial hyphae or conidia, but not mycelia. Several polypeptides are cell type specific; i.e., polypeptides 4 and 18 were detected only in extracts of aerial hyphae, as were polypeptides 36, 38, 40, and 46 in extracts of conidia. Polypeptides 4, 18, and 34 fall into more than one category (Fig. 3; Table 2).

Changes in protein synthesis or degradation or both could account for the differences observed in the protein profiles of mycelia, aerial hyphae, and conidia. Conidiating cultures were grown with label for 24 h, whereas mycelia were grown in liquid culture for 90 min after the addition of [^{35}S]methionine to the medium. Proteins that decrease in concentration during the asexual cycle could turn over more rapidly in aerial hyphae and conidia than in mycelia or may be synthesized at a lower rate during conidiation. Although a decrease in the rate of protein turnover could account for the increase in concentration of certain proteins in conidiating cultures, this is unlikely since the growth period of conidiating cultures after the addition of label was much longer than that of mycelia. A more likely explanation is that the rate of synthesis of proteins present at higher concentra-

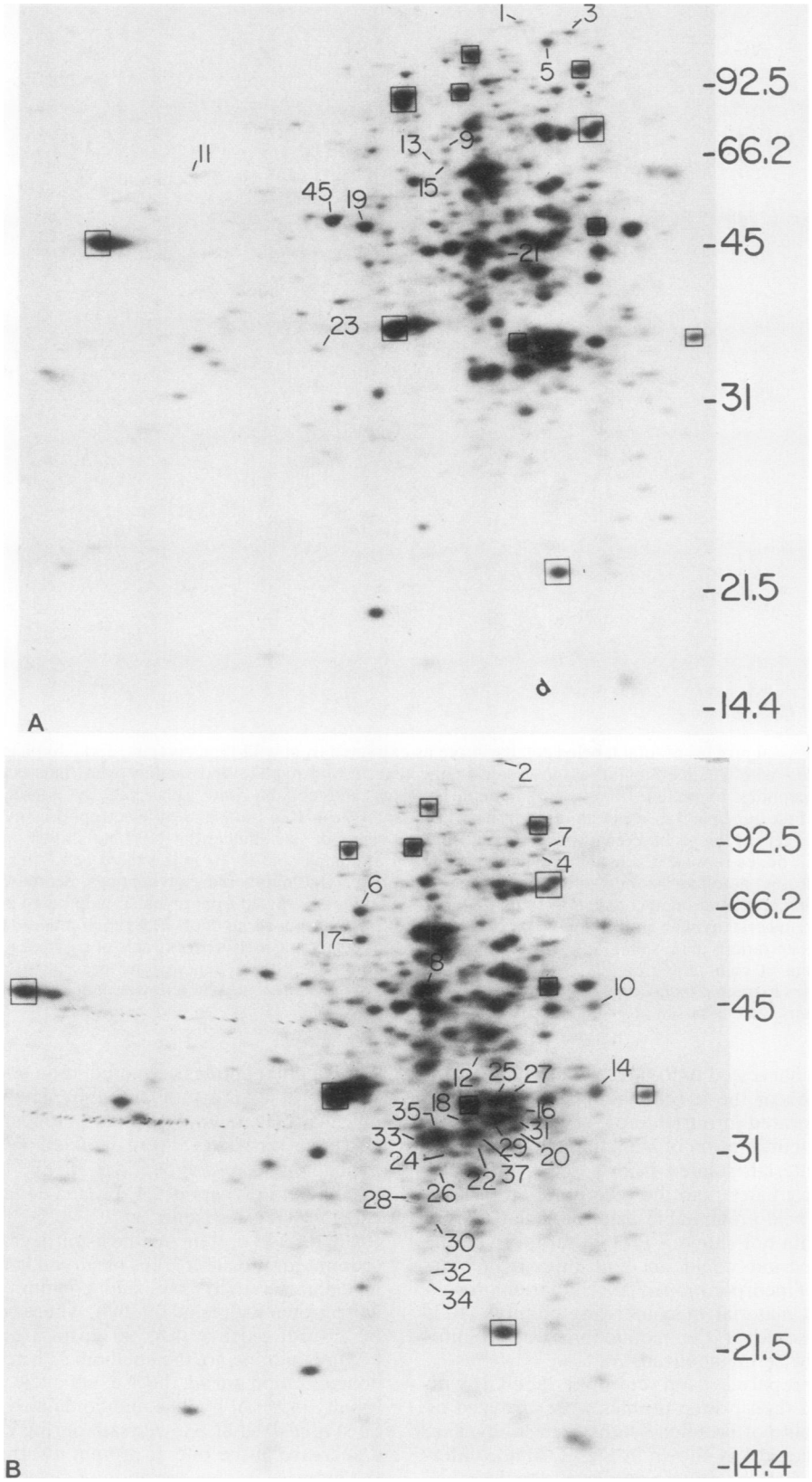

FIG. 4. Two-dimensional profiles of total cellular proteins extracted from *fl* mycelia and aerial hyphae. Cultures were grown and proteins were labeled, extracted, and analyzed on two-dimensional gels as described in the legend Fig. 3. Spots enclosed in boxes represent selected polypeptides present at comparable levels in *fl* mycelia and aerial hyphae. The molecular weights of size standards ($\times 10^3$) are shown in the vertical axis at the right. (A) Polypeptides extracted from *fl* mycelia. Numbered polypeptides are present at lower concentrations in *fl* aerial

TABLE 2. Changes in polypeptide synthesis during the asexual cycle in wild-type and *fl* strains[a]

change	Strain	Identity[b] and no. of polypeptides differentially expressed during asexual development in:			
		Aerial hyphae		Conidia	
		Spot no.	No. of spots	Spot no.	No. of spots
Concn decrease	74-OR23-1a	1–37[c]	19	4, 18, 25, 27, 39–71[c]	21
	fl a	1, 3, 5, 11, 13, 15, 19, 21, 23, 45	10		
Concn increase	74-OR23-1a	2, 6, 12, 14, 16, 20, 24, 26, 32	9	2, 12, 32, 34, 42, 44, 48, 50	8
	fl a	2, 6, 10, 12, 14, 16, 20, 24, 26, 32	10		
New proteins	74-OR23-1a	4, 8, 10, 18, 22, 28, 30, 34	8	36, 38, 40, 46	4
	fl a	4, 8, 18, 22, 28, 30, 34	7		

[a] Strains are described in Table 1.
[b] Numbers correspond to polypeptides in Fig. 3 and 4.
[c] Range includes only odd-numbered polypeptides.

tions in conidiating cultures increases during the asexual cycle.

Analysis of protein synthesis in mutants defective in asexual development. Protein synthesis during asexual development was also analyzed in the mutant strains listed in Table 1. The two-dimensional profiles of polypeptides extracted from wild-type and mutant cultures were compared to identify differences between the two that would provide information about the biochemical defects caused by the mutations. For example, *cr-1* conidiates prematurely on short aerial hyphae (10). Therefore we reasoned that proteins induced in wild-type aerial hyphae might be expressed prematurely in *cr-1* mycelia, thereby promoting conidiation before the differentiation of aerial hyphae. On the other hand, in the aconidial strain *fl*, aerial hyphae might lack polypeptides required for asexual spore formation due to the failure of induction. Unlike *cr-1* and *fl*, *eas* and *scon* produce wild-type levels of aerial hyphae and conidia. However, the phenotypes of *eas* and *scon* (Table 1) suggested that both could be defective in the synthesis of a major structural protein of the conidium. By analyzing the polypeptide profiles of these mutants, we hoped to identify polypeptides possibly required during the asexual cycle for the differentiation of aerial hyphae and conidia.

Protein extracts were prepared from wild-type and the mutant cultures and were analyzed by 2-D PAGE. With the exception of *fl*, we could detect no differences in the two-dimensional profiles of protein extracts from wild-type and mutant cultures (data not shown). However we observed major differences in the two-dimensional profiles of protein extracts from *fl* and wild-type aerial hyphae. Of the 19 polypeptides that decreased in concentration during wild-type aerial hypha formation, 10 decreased in concentration in *fl* aerial hyphae (Fig. 4A; Table 2). Nine polypeptides did not decrease in concentration to the same low level in *fl* aerial hyphae as in wild-type aerial hyphae (odd-numbered proteins, Fig. 4B). Polypeptide 45 decreased in concentration in wild-type conidia and in *fl* aerial hyphae. Contrary to what might be expected, all of the proteins that increased in concentration in wild-type aerial hyphae were present at comparable elevated levels in *fl* aerial hyphae (compare Fig. 3B with Fig. 4B; Table 2). One possible difference in the induction of protein synthesis in *fl* and wild-type cultures concerns polypeptide 10. Although it appeared as a novel labeled polypeptide in extracts of wild-type aerial hyphae and increaed in concentration in *fl* aerial hyphae, it was detected in extracts of *fl* mycelia (compare Fig. 3A with Fig. 4A; Table 2).

In vitro translation of poly(A)$^+$ RNA. The results described above suggested that changes in specific protein synthesis occur during the asexual cycle. We wanted to know in a general way whether changes in the steady-state levels of mRNAs could account for any of these changes. To address this question, poly(A)$^+$ RNA was isolated from mycelia and conidiating cultures. The RNA was translated in a rabbit reticulocyte lysate system, and the products synthesized in vitro were analyzed by 2-D PAGE. The profiles of polypeptide products specified by poly(A)$^+$ RNA isolated from mycelia and conidiating cultures indicated that there were major differences in the two RNA populations (Fig. 5). It is unlikely that RNA degradation is responsible for the differences observed in the 2-D PAGE profiles of products synthesized in vitro, since RNA isolated from mycelia and conidiating cultures directed the synthesis of proteins of comparable size, ranging in molecular weight from less than 14,000 to greater than 90,000.

There were a number of polypeptides synthesized in vitro that had no obvious counterpart in vivo. The converse was also true. The fact that translation of *N. crassa* RNA was performed in a heterologous system could account in part for these discrepancies. Proteins modified or processed in vivo may not be modified or processed identically in the heterologous system in vitro. Also, premature termination of polypeptide synthesis could occur in vitro, producing dis-

hyphae. (B) Polypeptides extracted from *fl* aerial hyphae. Even-numbered polypeptides are absent or are present at lower concentrations in *fl* mycelia. Odd-numbered polypeptides are present at comparable levels in *fl* mycelia and aerial hyphae, whereas they decrease in concentration in wild-type aerial hyphae.

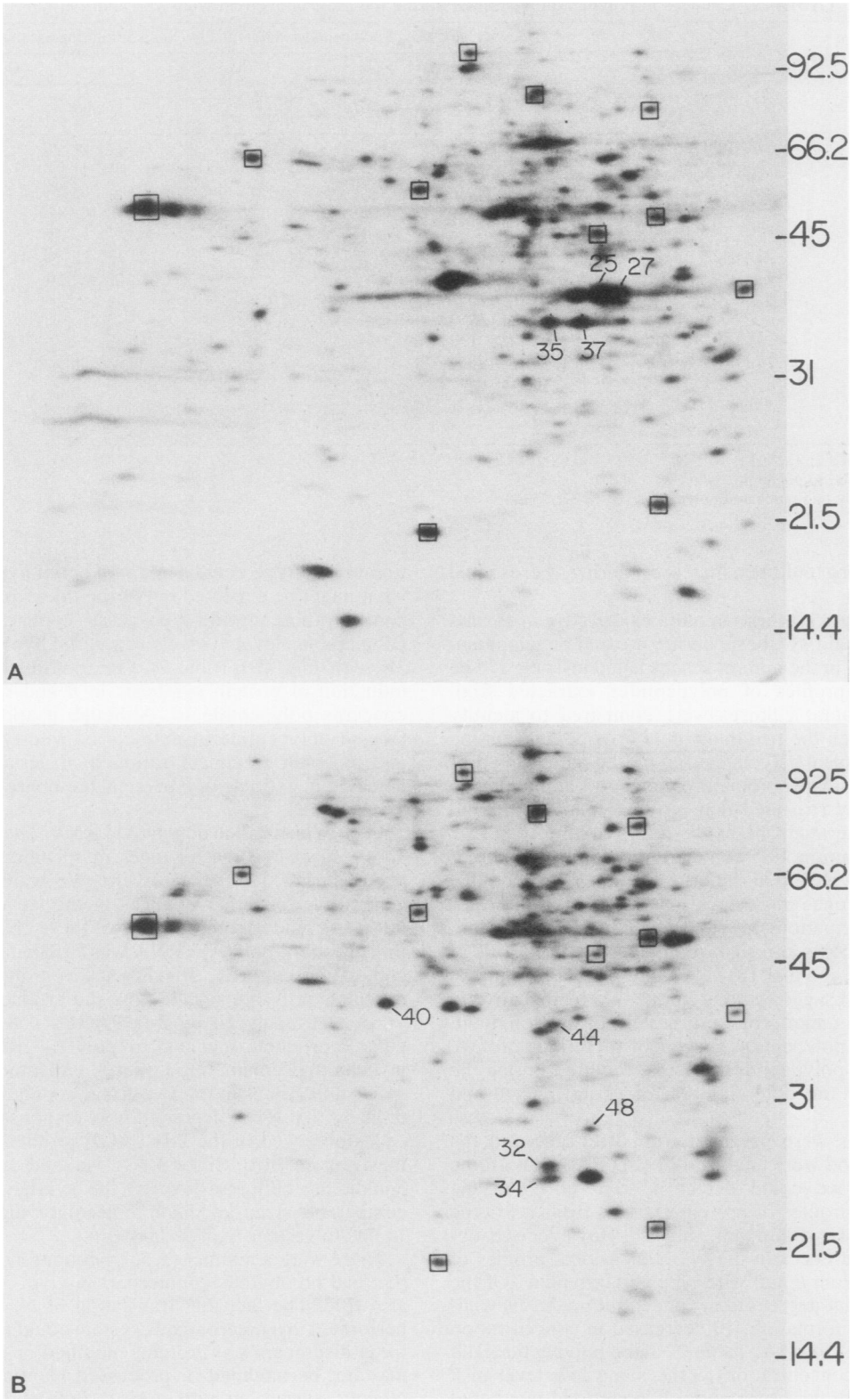

FIG. 5. Two-dimensional profiles of proteins synthesized by rabbit reticulocyte lysates programmed with poly(A)$^+$ RNA isolated from mycelia (A) and conidiating cultures (B). Cultures were grown as described in the text. Aerial hyphae and conidia were harvested 24 h after inducing conidiation. Poly(A)$^+$ RNA was prepared from total RNA (1, 6) isolated from mycelia and unfractionated conidiating cultures containing a mixture of aerial hyphae and conidia. Spots enclosed in boxes represented selected polypeptides specified by both RNA populations. These serve as reference polypeptides to orient one autofluorogram with respect to the other. The numbered polypeptides appear to comigrate with polypeptides synthesized in vivo (Fig. 3). The molecular weights of size standards ($\times 10^3$) are shown on the vertical axis at the right. The autofluorograms were exposed to −80°C for 12 to 16 h.

crete species that do not comigrate with any polypeptides synthesized in vivo.

Despite the limitations of the heterologous in vitro translation system, mRNAs that may correspond to a number of develomentally regulated proteins were detected by in vitro translation. RNA isolated from mycelia directed the synthesis of products in vitro that comigrated with mycelial polypeptides 25, 27, 35, and 37 detected by in vivo labeling (compare Fig. 3A with Fig. 5A). Also mRNA isolated from conidiating cultures specified several in vitro translation products that comigrated with polypeptides synthesized in aerial hyphae (polypeptides 32 and 34) and conidia (polypeptides 40, 44, and 48) (compare Fig. 3B and C with Fig. 5B). Whether the cell-free translation products are identical to comigrating polypeptides synthesized in vivo was not determined.

DISCUSSION

The work presented here describes a method for inducing conidiation and isolating large numbers of cells at discrete stages of conidium development. In liquid culture only vegetative growth occurs. However, conidiation is induced when mycelia are harvested onto filter paper and incubated under aerobic conditions. Within 2 h, vegetative hyphae differentiate into aerial hyphae (Fig. 2), which produce chains of conidia by 4 h. The number of conidia produced per culture increases until 12 h and then begins to plateau. By 16 h, approximately 10^9 conidia are produced per petri dish (Fig. 1). Covering mycelia with a filter paper disk to separate aerial hyphae and conidia from the underlying mycelial mat delays conidiation, but has no effect on maximum conidia production (Fig. 1). The rapid appearance of aerial hyphae and the time course of conidium formation suggest that these processes proceed in a synchronous fashion and account for our ability to detect changes in the levels of specific polypeptides during the asexual cycle.

The protein profiles of mycelia, aerial hyphae, and conidia were examined by 2-D PAGE. The levels of approximately 20% of the polypeptides resolved on two-dimensional gels are regulated during the asexual cycle. Most of the changes we could detect involve an increase or decrease in the concentration of proteins synthesized in mycelia during subsequent stages of differentiation. However, 12 proteins were identified that were only detected in cultures induced to conidiate. These proteins either could be synthesized de novo in aerial hyphae or conidia or could appear as new species on two-dimensional gels as a consequence of post-translational modification.

The methods used in this study to analyze developmental changes in protein synthesis have advantages as well as several limitations. Non-equilibrium pH gradient electrophoresis resolves proteins that have a wide range of isoelectric points and is useful for detecting changes in the levels of major cellular products. It is likely that many of these polypeptides are structural proteins or enzymes. Certainly any proteins present in small amounts would have been overlooked. Also, basic proteins such as histones and other DNA-binding proteins with isoelectric points greater than pH 9 are not resolved by the electrophoretic conditions employed. Therefore the number of changes in specific protein synthesis that occur during asexual development certainly exceeds the number that we have detected on our two-dimensional gels.

We wanted to know whether the differences observed in the polypeptide profiles of mycelia, aerial hyphae, and conidia were developmentally significant. To address this question, we compared the protein profiles of mutant and wild-type cultures. Of the mutants examined, only the aconidial mutant *fl* differed significantly from the wild type in the profile of proteins synthesized during the asexual cycle. Although the same proteins increased in concentration in wild-type and *fl* aerial hyphae, several proteins were present in extracts of *fl* aerial hyphae whose levels decreased in wild-type aerial hyphae. These findings indicate that the proteins that increase in concentration in *fl* aerial hyphae are not sufficient for asexual spore formation. Perhaps a change in the ratio of key products synthesized in aerial hyphae signals terminal differentiation. If this notion were correct, mutations that affect the induction of specific protein synthesis in aerial hyphae should also block conidiation.

The results of in vitro translation experiments suggest that changes in the steady-state levels of mRNAs account in part for differential gene expression during the asexual cycle of *N. crassa*. Major differences were detected in the profile of proteins specified by poly(A)$^+$ RNA isolated from mycelia and conidiating cultures. Several changes in the population of translatable RNA corresponded to changes in protein synthesis in vivo. However, many proteins differentially expressed in vivo did not correspond to any of the proteins synthesized in vitro. Therefore we could not estimate what proportion of the changes in protein synthesis detected on two-dimensional gels was due to changes in the steady-state synthesis of mRNA.

More changes were detected in the profiles of proteins synthesized in vitro than in those synthesized in vivo. This discrepancy can be explained in several ways. Proteins may be modified or processed differently in vitro than in vivo. In addition, translational regulation may occur during conidiation, but not in vitro. Finally, proteins that turn over rapidly in vivo may be detected more readily in vitro.

We have shown that the formation of aerial hyphae and conidia is associated with the synthesis of new gene products. How the expression of genes induced during asexual differentiation is regulated at the molecular level is the subject of our current work. Our results (3) suggest that it is feasible to clone genes induced in conidiating cultures on the basis of their differential expression during the asexual cycle. This approach has been used by others to isolate the conidiation-specific genes of *A. nidulans* (23) and, more recently, to isolate T-cell receptor genes (7). Isolation and characterization of genes induced in aerial hyphae and conidia should begin to elucidate the molecular events involved in differential gene expression during the asexual cycle of *N. crassa*.

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LITERATURE CITED

1. **Aviv, H., and P. Leder.** 1972. Purification of biologically active globin messenger RNA by chromatography of oligothymidylic acid-cellulose. Proc. Natl. Acad. Sci. U.S.A. **69**:1408–1412.
2. **Beever, R. E., and G. P. Dempsey.** 1978. Function of rodlets on

the surface of fungal spores. Nature (London) **272**:608–610.
3. **Berlin, V., and C. Yanofsky.** 1985. Isolation and characterization of genes differentially expressed during conidiation of *Neurospora crassa*. Mol. Cell. Biol. **5**:849–855.
4. **Bowman, E. J., B. J. Bowman, and C. W. Slayman.** 1980. Isolation and characterization of plasma membranes in wild type *Neurospora crassa*. J. Biol. Chem. **256**:12336–12342.
5. **Burton, E. G., and R. L. Metzenberg.** 1972. Novel mutation causing derepression of several enzymes of sulfur metabolism in *Neurospora crassa*. J. Bacteriol. **109**:140-151.
6. **Chirgwin, J. M., A. E. Przybyla, R. J. MacDonald, and W. J. Rutter.** 1974. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry **18**:5294–5299.
7. **Hedrick, S. M., D. I. Cohen, E. A. Nielsen, and M. M. Davis.** 1984. Isolation of cDNA clones encoding T cell-specific membrane associated proteins. Nature (London) **308**:149–153.
8. **Jones, P.** 1980. Analysis of radiolabeled lymphocyte proteins by one- and two-dimensional polyacrylamide gel electrophoresis. *In* B. B. Mishell and S. M. Shiigi (ed.), Selected methods in cellular immunology. W. H. Freeman & Co., San Francisco.
9. **Lindegren, C. C.** 1933. The genetics of *Neurospora* III. Pure bred stocks and crossing-over in *N. crassa*. Bull. Torrey Bot. Club **60**:133–154.
10. **Lindegren, C. C.** 1936. A six-point map of the sex chromosome of *Neurospora crassa*. J. Genet. **32**:243–256.
11. **Matsuyama, S. S., R. E. Nelson, and R. W. Siegel.** 1974. Mutations specifically blocking differentiation of macroconidia in *Neurospora crassa*. Dev. Biol. **41**:278–287.
12. **Nelson, R. E., C. P. Selitrennikoff, and R. W. Seigel.** 1975. Cell changes in *Neurospora*. p. 291–310. *In* J. Reinert and H. Holzer (ed.), Results and problems in cell differentiation. Springer-Verlag, Berlin.
13. **O'Farrell, P. Z., H. M. Goodman, and P. H. O'Farrell.** 1977. High resolution two-dimensional electrophoresis of basic as well as acidic proteins. Cell **12**:1133–1142.
14. **Pelham, H. R. B., and R. J. Jackson.** 1976. An efficient mRNA-dependent translation system from reticulocyte lysates. Eur. J. Biochem. **67**:247–256.
15. **Reinert, W. R., V. B. Patel, and N. H. Giles.** 1981. Genetic regulation of the *qa* gene cluster of *Neurospora crassa*: induction of *qa* messenger ribonucleic acid and dependency on *qa-1* function. Mol. Cell. Biol. **1**:829–835.
16. **Schmidt, J. C., and S. Brody.** 1976. Biochemical genetics of *Neurospora crassa* conidial germination. Bacteriol. Rev. **40**:1–41.
17. **Schmidt, J. C., M. Cohen, and S. Brody.** 1974. Conidial germination in *scon*^{c}. *Neurospora* Newsl. **21**:17–18.
18. **Selitrennikoff, C. P.** 1976. Easily-wettable, a new mutant. *Neurospora* Newsl. **23**:23.
19. **Selitrennikoff, C. P., R. E. Nelson, and R. W. Siegel.** 1974. Phase-specific genes for macroconidiation in *Neurospora crassa*. Genetics **78**:679–690.
20. **Siegel, R. W., S. S. Matsuyama, and J. C. Urey.** 1968. Induced macroconidia formation in *Neurospora crassa*. Experientia **24**:1179–1181.
21. **Stine, G. J., and A. M. Clark.** 1967. Synchronous production of conidiophores and conidia of *Neurospora crassa*. Can. J. Microbiol. **13**:447–453.
22. **Vogel, H. J.** 1964. Distribution of lysine pathways among fungi: evolutionary implications. Am. Naturalist **98**:435–446.
23. **Zimmermann, C. R., W. C. Orr, R. F. Leclerc, E. C. Barnard, and W. E. Timberlake.** 1980. Molecular cloning and selection of genes regulated in *Aspergillus* development. Cell **21**:709–715.