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Differential Gene Action in *Neurospora crassa*

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Molecular hybridization experiments with pulse-labeled total ribonucleic acid (RNA) or isolated nonribosomal rapidly labeled RNA species and deoxyribonucleic acid from growth periods of 8, 16, and 24 hr of *Neurospora crassa* showed differential transcription. Hybridization competition experiments between RNA species isolated from 8, 16, and 24 hr of growth showed qualitative differences in the types of RNA synthesized during these periods.

Gene function can be assessed by the amount and type of ribonucleic acid (RNA) that is synthesized at any growth period. McCarthy and Hoyer (7) showed that there was no difference in the polynucleotide sequences of deoxyribonucleic acid (DNA) from different organs, although large differences were seen among rapidly labeled RNA isolated from these organs. The method of molecular hybridization as developed by Gillespie and Spiegelman (4) has allowed the determination of the extent of gene activity. Such a technique has also allowed the identification of tissue-specific RNA by means of hybridization competition experiments (3, 10). All such studies indicated the existence of regulated gene activity as expressed by differential gene functioning. Such a regulation has been suggested as a basic molecular event behind the processes of differentiation and morphogenesis. In the present paper we report experimental data that show the extent and differences in the gene activity during three growth periods of *Neurospora crassa*.

Even though there is lack of synchrony in the early growth phase of *Neurospora*, an 8-hr-old culture is made up of unbranched hyphae of various lengths. A 16-hr-old culture consists predominantly of branching hyphae. At 24 hr the hyphae have several branches, and the growth is in the form of collective mycelial mass. In spite of some heterogeneity in the age of the hyphae obtained from any single conidium, the three stages do show distinct morphological differences.

MATERIALS AND METHODS

N. crassa strain RL-3-8A (Rockefeller wild type) was used for all experiments. The culture was routinely kept in 2% agar slants containing 2% sucrose and minimal salt solution (12). Cultures of various growth periods (e.g. 8, 16, and 24 hr) were obtained by growing the fungus in 100 ml of 2% sucrose minimal salt solution after inoculation with a conidial suspension. The flasks were kept on a rotary shaker during the entire growth period. Cultures were grown in a medium containing a minimum of 2 μCi of ^{3}H-uridine per ml (specific activity 16 Ci/mmole) when RNA was to be isolated. Pulse labeling of the cultures was done by addition of the radioactive compounds 5 or 10 min before harvesting at the desired growth period. Amount of labeled precursor was suitably increased for the increasing growth periods. After growth for 8, 16, and 24 hr, the mycelia were filtered repeatedly through double layers of Mira cloth (Chickopee Mills, N.Y.). This repeated filtration separated ungerminated conidia from the long germ tubes. This was checked by microscope observation. The mycelial pad free from conidia was washed with distilled water and after lyophilization was used for extraction of RNA or DNA.

Extraction and chromatography of nucleic acids. DNA was isolated from lyophilized mycelia by Marmur's method (8). RNA was isolated by Kirby's method (6). Modification in these methods to suit *Neurospora* was reported earlier (1). The nucleic acid preparations were chromatographed on methylated albumin Kieselghur (MAK) columns to separate RNA species. Pooled messenger RNA (mRNA) fractions, ribosomal RNA, and transfer RNA isolated in this manner were used for DNA hybridization experiments. Isolation of species of RNA characterized as mRNA was based on some of the expected properties of such RNA. Their separation and characterization have been detailed in our earlier paper (1).

RNA-DNA hybridization on membrane filters. The hybridization experiments were carried out essentially by the method of Gillespie and Spiegelman (4). The optimal conditions for such hybridization with preparations from *Neurospora* were reported earlier (1). In hybridization competition experiments, labeled RNA, used at the saturation level obtained from Fig. 1, for a particular growth period, was hybridized to 25 μg of DNA in the presence of increasing amounts of competing unlabeled RNA from another growth period. Membrane filters containing labeled RNA hybridized to DNA were suspended in BBOT scintillator, and radioactivity was

determined in a Packard Tri-Carb liquid scintillation spectrometer with 50% and 18% efficiency, respectively, for ^{14}C and ^{3}H labels. Hybridization values are expressed as percent DNA hybridized, which was calculated as micrograms of RNA hybridized to 100 μg of DNA, and also as radioactivity in hybrids. The amount of RNA bound to DNA was calculated from the radioactivity associated with the filter after hybridization; and with the specific activity of RNA being known, micrograms of RNA associated with the DNA in the filter was determined. RNA was estimated by the orcinol method (12) and by DNA by using the diphenylamine reagent (2).

Hydroxyapatite fractionation. DNA was prepared by Marmur's method from a culture of *Neurospora* grown for 16 hr in Vogel's minimal salt solution containing 20 μCi of ^{32}P as orthophosphoric acid per ml, 2% sucrose, and half the normal phosphate concentration. The extraction was done from lyophilized mycelia, and after the DNA was obtained, it was repeatedly subjected to ribonuclease and Pronase treatments in a dialysis bag. The enzymes were removed by deproteinization with phenol, and the final DNA preparation was further purified by MAK chromatography. The final preparation of DNA was dissolved in 0.24 M phosphate buffer, *p*H 6.8. DNA samples (10 μg/ml) were then sheared by ultrasonic disintegration at 20 kc/sec for 1 min, and the buffer strength was diluted to 0.12 M with distilled water. DNA was denatured in a boiling water bath for 15 min and transferred immediately to an ice bath.

In hybridization experiments, 5 μg of ^{32}P-labeled DNA was incubated in 0.12 M phosphate buffer with 250 μg of ^{3}H-uridine-labeled RNA (pulse-labeled total RNA) for 52 hr at 70 C in a water bath. After the incubation period, the sample was immediately frozen and kept until it was analyzed on a hydroxyapatite column. The hydroxyapatite column was prepared by the method of Miyazawa and Thomas (9). The chromatography was done by the method of Kohne (6).

Chemicals. All labeled compounds were obtained from the Isotope division, Bhabha Atomic Research Centre, Bombay. Ribonuclease was a product of Sigma Chemical Co., and Pronase was purchased from Calbiochem, Switzerland.

RESULTS AND DISCUSSION

Hybridizable mRNA during various growth periods. Nonribosomal rapidly labeled RNA species from growth periods of 8, 16, and 24 hr, which were labeled with ^{3}H-uridine, were used for hybridization with DNA. Figure 1 shows the maximum hybridizable RNA present in the RNA sample isolated from the growth periods of 8, 16, and 24 hr. From these it is clear that the saturation values are different for the different growth periods. This probably shows that the numbers of active genes during the above three growth periods are different and that the number of active genes at 8 hr is higher than the other two.

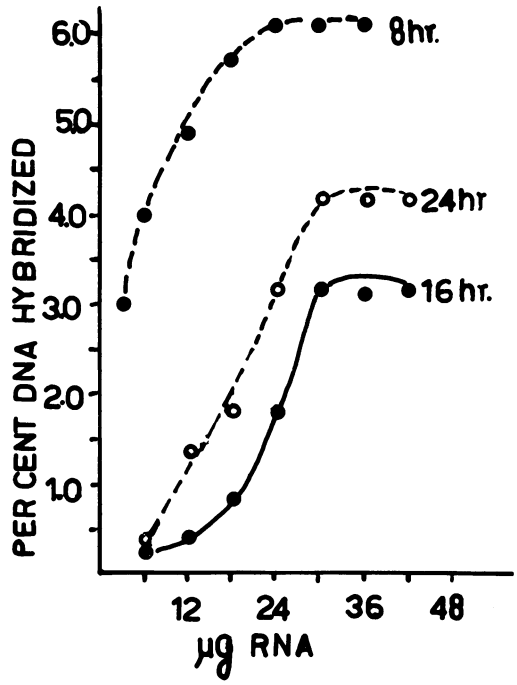

FIG. 1. *Saturation level for hybridization of mRNA from 8, 16, and 24-hr growth periods isolated by MAK column chromatography. Each point is an average of three separate experiments. Specific activity of RNA: 8 hr, 22,650 counts per min per µg; 16 hr, 10,320 counts per min per µg; 24 hr, 12,430 counts per min per µg.*

Total RNA extracted from a culture pulse-labeled with ^{3}H-uridine was also used for hybridization. The results (Table 1) indicate the total hybridizable RNA from three growth periods. The higher saturation value for RNA at 8 hr is still evident.

Another experiment was done in which DNA was first hybridized with unlabeled transfer RNA (tRNA) and ribosomal RNA (rRNA), both obtained from MAK chromatography. Duplicate samples were treated with ribonuclease to remove loosely bound RNA on the membrane filter. Later on, one set was further exposed to labeled tRNA and rRNA to show that the sites on DNA for these species are occupied by unlabeled tRNA and rRNA. The other one was allowed to hybridize with pulse-labeled total RNA preparation. This was done for samples from all three growth periods. Table 2 shows the data indicating the hybridization level with pulse-labeled total RNA after saturation of sites for tRNA and rRNA. The values for hybridization of 7.5, 3.4, and 3.9 for 8, 16, and 24 hr cultures, respectively, seen in this experiment are close to the data

TABLE 1. *Hybridization values of total pulse-labeled RNA with DNA*[a]

Total RNA from growth periods (hr)	Percent DNA hybridized		
	Expt 1	Expt 2	Expt 3
8	9.8	9.7	9.82
16	6.4	6.48	6.35
24	7.6	7.8	7.49

[a] Specific activity of the RNA: 8 hr, 850 counts per min per μg; 16 hr, 352 counts per min per μg; 24 hr, 690 counts per min per μg.

TABLE 2. *Hybridization values for pulse-labeled total RNA after saturating the DNA sites with tRNA and rRNA*

Total RNA from growth periods (hr)	Percent DNA hybridized	
	Expt 1	Expt 2
8	7.55	7.6
16	3.55	3.52
24	3.9	4.1

obtained as hybridization values for isolated mRNA species (see Fig. 1). This clearly shows that the saturation values obtained in earlier experiments with isolated nonribosomal RNA have no contribution from species of tRNA and rRNA.

Hybridizable DNA or RNA isolated from different growth periods (as determined by hydroxyapatite column). Hybridization of ^{32}P-labeled DNA was carried out with ^{3}H-uridine-labeled RNA as detailed in Materials and Methods (for hydroxyapatite chromatography), and the RNA-DNA hybrids were separated from single-stranded DNA and RNA by chromatography of the samples in hydroxyapatite columns at elevated temperature. In this system the amount of labeled DNA which remained absorbed to the column, but was eluted only by higher molarity of phosphate buffer (0.48 M), was considered as the amount hybridized to RNA. The percent hybridization could be expressed as the percent of the input DNA that remained in the form of a hybrid with RNA. The amount of input DNA and RNA was kept constant in all the experiments. From the specific activity of the DNA preparation, the amount of DNA hybridized to RNA was calculated. These data are presented in Table 3, after correction for self-reassociation. This was achieved by hybridizing ^{32}P-labeled *Neurospora* DNA to nonhomologous *Escherichia coli* RNA (unlabeled). It is clear that the hybridizable DNA was 9.9, 5.2, and 6.0% (experiment no. 1) for 8, 16, and 24 hr of growth, respectively. The values are very comparable to those obtained by the membrane technique as the amount of RNA hybridized and form an independent verification of the saturation values obtained earlier (see Table 1).

Hybridization competition (by membrane technique). To test whether similar species of mRNA are produced during three growth periods, hybridization competition was done. The amount of radioactivity in hybrids, when 8-hr RNA is used in the presence of increasing amounts of 16-hr RNA, is gradually reduced (Fig. 2a) until it levels off with 66% of the original hybridized level. This indicates that between 8 and 16 hr the RNA has homology to some extent (up to 34%) and at the same time some new species are present at 8 hr which are not seen at 16 hr. The reciprocal experiment (Fig. 2b) also showed that at 16 hr there are

TABLE 3. *Hybridizable amount of ^{32}P-DNA to RNA (hydroxyapatite chromatography)*[a]

Total RNA from growth period (hr)	Percent DNA hybridized (after correction for self-reassociation)		Comparable data from Table 1[b]
	Expt 1	Expt 2	
8	9.9	9.88	9.8
16	5.2	5.15	6.4
24	6.0	6.02	7.4

[a] Specific activity of RNA: 8 hr, 2244 counts per min per μg; 16 hr, 1,982 counts per min per μg; 24 hr, 800 counts per min per μg.

[b] Data from molecular hybridization by membrane technique.

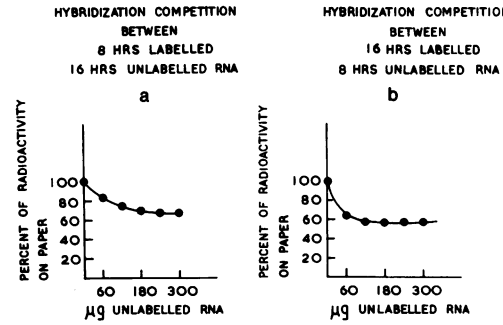

FIG. 2. *(a) Hybridization competition between mRNA (unlabeled) from 16 hr and mRNA (labeled) from 8 hr. Increasing amounts of unlabeled mRNA were added to 30 μg of ^{3}H-labeled mRNA exposed to 25 μg of DNA on the filter. (b) Similar to (a) but with 16-hr labeled mRNA and 8-hr unlabeled mRNA.*

common species (up to 42%) with 8 hr, and the rest are new species characteristic of 16 hr. Figure 3 shows data for such an experiment between 8-hr and 24-hr RNA. This shows also the presence of new species at 24 hr and common species or homology (up to 68%) in the RNA of 8 and 24 hr.

In all membrane hybridization experiments, the controls were those wherein hybridization was done by using labeled RNA in the presence of unlabeled RNA from the same culture (growth period); in this case the competition was nearly 100%.

Saturation value, expressed as percent of DNA hybridized (Fig. 1), is essentially a measure of the number of genes that are active in the particular growth period. Gene activity thus is quite high at 8 hr compared to 16 and 24 hr of growth. A very similar observation was made by DNA-RNA hybridization and hydroxyapatite chromatography for estimation of hybridized RNA. It is also clear from the competition experiments that there are new species of RNA during the various stages of growth. Thus the three growth periods showed differences in the extent and quality of active genes, indicating differential gene action in *Neurospora* during the growth period of 8 to 24 hr. It is noteworthy that in *Neurospora* there is very little, if any, redundant DNA (Dutta, *personal communication*).

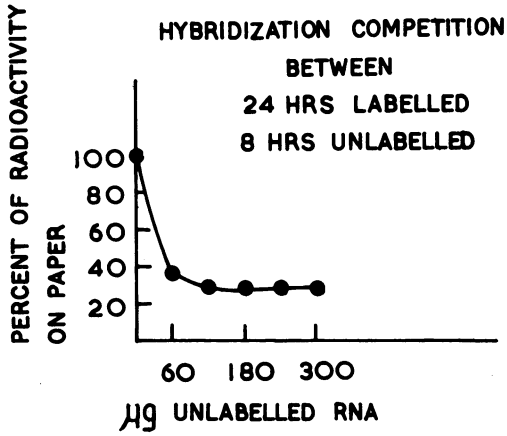

FIG. 3. *Hybridization competition between mRNA of 24 hr (labeled) with 8-hr unlabeled mRNA. Amount of DNA and labeled RNA was the same as in Fig. 2.*

It is to be noted that the estimate of gene activity shown from our experiments concerns only RNA molecules synthesized during the short period of labeling. Nevertheless, differential gene action was clearly exhibited.

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LITERATURE CITED

1. Bhagwat, A. S., and P. R. Mahadevan. 1970. Conserved mRNA from the conidia of *Neurospora crassa*. Mol. Gen. Genet. **109**:142-151.
2. Burton, R. 1956. Estimation of deoxyribose by diphenylamine. Biochem. J. **62**:315-323.
3. Davidson, E. H., M. Crippa, and A. E. Mirsky. 1968. Evidence for the appearance of novel gene products during amphibian blastulation. Proc. Nat. Acad. Sci. U.S.A. **60**:152-159.
4. Gillispie, D., and S. Spiegelman. 1965. A quantitative assay for DNA-RNA hybridization with DNA immobilized on a membrane. J. Mol. Biol. **12**:829-842.
5. Kirby, K. S. 1965. Isolation and characterization of ribosomal RNA. Biochem. J. **96**:266-269.
6. Kohne, D. E. 1968. Isolation and characterization of bacterial ribosomal RNA cistrons. Carnegie Inst. Year Book **67**:310-311.
7. McCarthy, B. J., and B. H. Hoyer. 1964. Identity of DNA and diversity of messenger RNA molecules of normal mouse tissue. Proc. Nat. Acad. Sci. U.S.A. **52**:915-922.
8. Marmur, J. 1961. Isolation of bacterial DNA. J. Mol. Biol. **3**:208-221.
9. Miyazawa, Y., and C. A. Thomas. 1965. Nucleotide composition of short segments of DNA molecules. J. Mol. Biol. **11**:223-237.
10. Paul, J., and R. S. Gilmour. 1968. Organ specific restriction of transcription in mammalian chromatin. J. Mol. Biol. **34**:305-316.
11. Schneider, W. C. 1957. Determination of nucleic acids in tissues by pentose analysis, p. 680-684. *In* S. P. Colowick and N. O. Kaplan (ed.), Methods in enzymology, vol. 3. Academic Press Inc., New York.
12. Vogel, H. J. 1956. A convenient growth medium for *Neurospora*. Microbial Genet. Bull. **13**:42-45.