DIFFERENTIAL GENE EXPRESSION IN NEUROSPORA CRASSA CELL TYPES:
AMPLIFICATION OF rRNA GENES

MASTER

Progress Report

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Annual Progress Report
1979 to 1980.

U.S. Department of Energy Contract No. DOE-EY-76-S-05-4182

Project Title

DIFFERENTIAL GENE EXPRESSION IN NEUROSPORA CRASSA CELL TYPES:
AMPLIFICATION OF rRNA GENES

Abstract

The significant results obtained during 1979-1980 year of the current research program are as follows: (1) The differential rRNA gene amplification in germinated conidia of N. crassa was confirmed. N. crassa rDNAs showed differences in degrees of homology with isolated DNAs from other Neurospora species which could be due to heterogeneity in internal spacers. Studies with N. crassa rDNA clones were initiated to study their heterogeneities.

The organization of the Institutional Biohazard Committee (IBC) for Recombinant DNA research was completed and necessary certifications for the laboratory and the workers were obtained in accordance with the P₂EK₁ containment regulation of N.I.H.

Two rDNA clones in pBR322 plasmids, one containing only 17S rDNA and another containing whole rDNA (having 17S, 5.8S, 26S rDNAs + spacer regions) were obtained through the courtesy of Professor Metzenberg of the University of Wisconsin and were successfully cultured. ³²P or ³H labeled 17S rDNA probe was produced from the pBR322 clone containing this rDNA. In order to produce ³²P 26S rDNA probe, first it was required to produce a 26S rDNA subclone in pBR322. This was accomplished by splicing isolated DNAs from whole rDNA-pBR322 clone first by the restriction endonuclease Pst I followed by EcoR_I and Hind III restriction endonucleases. The 26S rDNA was ligated with the EcoR_I and Hind III restricted pBR322 plasmid DNA. This cloned plasmid DNA was then multiplied in E. coli strain LE 392 from which 26S rDNA probe was isolated and nick translated.

These known 17S and 26S N. crassa rDNA probes are being used to detect differences, if any, in restriction cleavage sites in rDNAs of different cell types and developmental mutants of N. crassa. DNAs from these N. crassa cells are restricted with EcoR_I and Hind III and cleaved fragments separated by gel electrophoresis, are transferred into nitrocellulose papers. Experiments are underway now to see if there is any changes in cleavage sites by annealing with ³²P or ³H-17S or 26S rDNA probes followed by autoradiography.

Progress Report

DIFFERENTIAL GENE EXPRESSION IN NEUROSPORA CRASSA CELL TYPES:
AMPLIFICATION OF rRNA GENESPersonnel
(During 1979-1980)

Name	Percent time devoted
1. Principal Investigator and Professor of Botany Dr. S.K. Dutta, Ph.D.	He devoted 20% time or effort during the year, and 50% time during summer.
*Dr. Dutta will devote the same amount of time or effort during the remainder of the current term.	
2. Dr. D. Mukhopadhyay, Ph.D. Biochemist	Research Associate (Part time)
3. Dr. S. Mannan, Ph.D. Electron Microscopist	Research Associate (Voluntary)
4. Yyan-Ksu Kang Electron Microscopist	Collaborator (Voluntary)
5. Dr. Norma P. Williams, Ph.D. Associate Professor of Botany	Voluntary Faculty Collaborator

Following Students were Benefited from This Project

6. Miss. Carolyn Chambers	Graduate Student
7. Mr. Asere Israel	" "
8. Mr. Inamul Sheikh	" "
9. Mr. Isiaka, O. Gbadamosi	" "
10. Mr. Rasheed, Mimiko	" "
11. Miss. Margnerite, Neita	" "
12. Mr. Mike John	" "
13. Mr. Davidson Curwen	" "
14. Miss. Kunda S. Deshpande	" "
15. Mr. Attoh Goziam	" "
16. Miss. Leslie Reynold	Undergraduate Student

I. Scope of the Investigation during the Tenth Year (1979-1980)

No change in the plan of work was made during this period. The scope of the investigations undertaken were as follows:

1. Continuation of the study: on heterogeneities of rRNA genes (rDNAs) in *Neurospora* species.
2. Localisation of rDNAs in cell fractions of *N.crassa*.
3. Development of P₂ facilities for "Recombinant-DNA" Research in accordance with the N.I.H. regulations.
4. Development of *N.crassa* rDNA clones.
5. Initiation of restriction analysis of rDNAs from developmental cell types.

1. Heterogeneity of rRNA cistrons (rDNAs) in Neurospora Species:

DNA sequences in *Escherichia coli* and *Neurospora crassa* that code for ribosomal RNA were isolated in a highly purified state by DNA:RNA hybridization. Three-tenths percent of *E.coli* genome and 1.0% of *N.crassa* genome were coded for rRNAs. The isolated cistrons were hybridized with total DNAs from various organisms.

E.coli rRNA cistrons (rDNAs) hybridized less with total DNAs of higher eukaryotes like pea plant leaves (25%) and rat liver (19%) respectively; whereas the percent hybridized with lower eukaryotes is about 35-38%. There was no difference in DNA sequences detected by hybridizing *E.coli* rRNA cistrons with homothallic or heterothallic species of *Neurospora*. When *N.crassa* rDNA sequences were hybridized with total DNAs of homothallic species, 2-3% differences in DNA sequences of *Neurospora* species were noticed. *N.crassa* rDNAs, however, hybridized less with *E.coli* DNAs (45%) and more with DNAs of higher eukaryotes, pea plant leaves (69%) and rat liver (59%). These observations suggest that despite the conservation of rRNA genes in evolution, heterogeneity of rDNAs among closely related *Neurospora* species does exist. This heterogeneity is likely to be in spacer regions and need further investigations.

These results are summarized in Tables 1 to 4 and figures 1 and 1a.

2. Localisation of rDNAs in Cell Fractions of N.crassa and Search for Palindromes (Inverted rDNA Repeats):

Contrary to the earlier believes that fungal genomes contained 10-20% repetitive DNA sequences, we have observed along with other laboratories that nuclear genomes of *Neurospora* and similar other fungi have small amount of (2-3%) repetitive DNA sequences. Most of these nuclear repetitive DNA sequences are

Table 1. Isolation of rDNA of E. coli
using S₁ nuclease.

Cycle	³ H-DNA Cot	Unlabelled rRNA Cot	% Recovery
1	0.065	0.5	0.5016
2	0.0006	0.5	0.4015
A 3	0.000012	0.5	0.3727
4	0.000012	0.5	0.3618
no RNA			
1	0.065	0	0.04
2	0.0006	0	0.01
B 3	0.000012	0	0.01

Data showing the fraction of original input ³H-E. coli DNA adsorbing to hydroxyapatite when reacted (A) with E. coli rRNA, (B) in the absence of any rRNA. The reassociation reactions were performed as described in methods.

Table 2. Summary of reactions among purified
Escherichia coli ribosomal-RNA cis-
trons (rDNA) and DNAs of other species.

Reaction	Total rRNA Cot	Total DNA Cot	Percentage ³ H-rDNA hybridized		
			No S ₁ Nuclease	With S ₁ Nuclease	°C Te50
<u>E. coli</u> ³ HrDNA (self reaction)	0.0	0.0	0.00	0.00	-
" + <u>E. coli</u> DNA	-	7 X 10 ²	99.00	94.00	88.00
" + <u>E. coli</u> rRNA	0.50	-	94.00	93.00	89.00
" + <u>N. crassa</u> DNA	-	1 X 10 ³	45.00	36.00	84.00
" + <u>N. dodgei</u> DNA	-	5 X 10 ²	47.00	35.00	84.00
" + <u>N. lineolata</u> DNA	-	7 X 10 ²	50.00	38.00	83.00
" + <u>N. africana</u> DNA	-	6 X 10 ²	41.00	33.00	83.00
" + Pea leaves DNA	-	8 X 10 ²	29.00	25.00	75.00
" + Rat liver DNA	-	5 X 10 ²	22.00	19.00	71.00

Cot. values of E. coli ³H-rDNA of several reactions were between 0.001 to 0.006.

Table 3. Isolation of rDNA of N. crassa

	Cycle	^3H DNA Cot	Unlabelled rRNA Cot	% Recovery
A	1	0.06	0.5	8.24
	2	0.006	0.5	3.83
	3	0.0005	0.5	1.43
	4	0.0005	0.5	1.23
	5	0.0005	0.5	1.20
No rRNA				
B	1	0.06	0	0.003
	2	0.006	0	0.001

Data showing the fraction of original input ^3H N. crassa DNA absorbing to hydroxyapatite when reacted (A) with N. crassa rRNA, (B) in the absence of any rRNA. The reassociation reactions were performed as described in methods.

Table 4. Summary of reactions among purified Neurospora crassa ribosomal-RNA cistrons and DNAs of other species.

Reaction	DNA Cot	% ³ H-rDNA hybridized (S ₁ nuclease)	Te50°C
<u>N. crassa</u> ³ H-rDNA + <u>N. crassa</u> DNA	5 X 10 ²	92	88.00
+ <u>N. dodgei</u> DNA	" "	90	87.00
+ <u>N. lineolata</u> DNA	" "	88	86.00
+ <u>N. africana</u> "	" "	85	84.00
+ <u>E. coli</u> "	7 X 10 ²	45	81.00
+ Pea leaves "	" "	69	84.00
+ Rat liver "	5 X 10 ²	59	76.00

Cot values of N. crassa ³H-rDNA of several reactions were between 0.001 to 0.006 which were negligible reactions.

Figure 1a: Thermal stability profiles of homoduplexes of ^3H -rDNA *N.crassa* total DNAs and heteroduplexes of *N.crassa*, ^3H -rDNA with total DNAs of other species. Pea (X), *E.coli* (□) and rat liver (Δ).

Figure 1b: Thermal stability profiles of homoduplexes of ^3H -rDNA *N.crassa* with total DNAs of *N.crassa* and heteroduplexes of *N.crassa* ^3H -rDNA with total DNAs of other homothallic species of *Neurospora*: *N.crassa* (O), *N.dodgei* (X), *N.lineolata* (Δ) and *N.africana* ($\square$).

rRNA genes. Hence it appears that bulk of the repetitive DNA sequences in Neurospora must be non-nuclear. These observations led us to investigate in more details characteristics of DNAs isolated from different cell fractions like, nuclear, postmitochondria, and whole cell homogenate.

Two approaches were taken to study these repetitive DNA sequences. One is (a) biochemical and the other is (b) electron microscopic.

(a) Biochemical Approaches involved isolation of DNAs from these cell fractions and study their characteristics by thermal denaturation studies in UV spectrophotometers. Results obtained are summarized in Table 5. It is apparent that whole cell homogenate DNAs were bimodal (25% low GC content) whereas nuclear and post-mitochondrial DNAs are both unimodal. See figures 2 and 3. Mitochondrial DNAs had only high AT fractions.

DNA:DNA hybridization studies done with ^3H -labeled (nick translated) repetitive DNAs obtained from nuclear and post-mitochondrial DNAs with unlabeled DNAs from whole cell homogenate, nuclei and post-mitochondrial fractions. Results obtained, as summarized in the Table 6, indicate that post-mitochondrial DNAs were all repetitive and has the same genome ($\frac{1}{2} \text{C}_0\text{t} = 0.05$) size like that of rDNA and hybridized 100% with nuclear DNAs suggesting the existence of rDNA like sequences in cytoplasm and probably are of palindromic nature.

(b) Search for inverted rDNA Repeats (Palindromes) by Electron Microscope. Work on visual observations on N.crassa rDNA were initiated with the volunteer collaboration of Dr. S. Mannan Ph.D., an electron microscopist, who had spent several days at the Molecular Genetics Laboratory of N.I.H. Preliminary observations do indicate that the existence of rDNA like double strand molecules of approx. 5-10 kb sizes in the post-mitochondrial DNAs. Presence of Palindromes could not be confirmed in the post-mitochondrial fraction yet.

3. Development of P₂ Facilities for "Recombinant-DNA" Research:

Principal Investigator S.K. Dutta had to spend enormous amount of time and efforts to form the Institutional Biohazard Committee (IBC) for Recombinant-DNA research. The entire University: 9 Faculty members of different Colleges, Offices of Academic and Health Vice Presidents, and three community leaders, participated in forming this IBC. Particulars of committee members were submitted to the Recombinant-DNA Committee of the Natl. Institute of Health. Necessary memorandum of understandings and agreements (MUA) were formed and then only we were allowed to initiate our proposed work with the N.crassa rDNA-pBR322 plasmid Recombinant molecule work. It took nearly 1½ years to form this IBC and to obtain our certifications for Recombinant-DNA research at this University. Fortunately we could at least start our work for this DOE project just in time for initiation of this project.

Figure 2: Optical melting curves of DNAs of wild type strain 74 A (O), Postmitochondrial DNA ($\square$), *N. crassa* wild type nuclear DNA (Δ) in 0.12 M phosphate buffer, pH 6.8, recorded by Gilford 2400 spectrophotometer at 260 nm. The *N. crassa* wild type and nuclear DNAs were isolated from young germinated conidial cells. The postmitochondrial DNA was isolated from freeze-dried mycelial cells. All of the DNAs were isolated by the hydroxyapatite method described in the text. The *N. crassa* wild type total DNA shows two fractions, characteristic of *N. crassa*. The *N. crassa* wild type nuclear DNA and the postmitochondrial DNA show one major fraction.

Figure 3: Optical melting curves of DNAs of slime mutant of *N. crassa* (FGSC 1118) (O) and nuclear DNA (Δ) in 0.12 M phosphate buffer, PH 6.8, recorded by Gilford 2400 spectrophotometer at 260 nm. The DNAs were isolated from fresh slime mutant cells by the hydroxyapatite method described in the text. The slime mutant total DNA shows two fractions and the nuclear DNA shows one major fraction.

Table-5. Characterization of DNAs from normal and mutant strains of N. crassa.

Type of cells	Minor fraction		Major fraction		Hyperchromicity
	T _m (% of total)	G+C mole %	T _m (% of total)	G+C mole %	
Conidia nuclear	None	-	97(100)	68	25
Conidia total	83(20)	33	92(80)	55	25
Slime mutant nuclear	None	-	97(11)	68	28
Slime mutant total	80(12)	26	96(88)	65	25
Post mitochondrial	None	-	97(100)	68	25

These data are summarized from optical melting curves of DNAs from different N. crassa strain by Gilford 2400 spectrophotometer at 0.12 M phosphate buffer (PB), pH 6.8; T_m (temperature at which 50% of the DNA dissociates) was calculated for each fraction. The GC content of the DNA fraction was determined according to the formula: $T_m = 69.3 + 0.41 \text{ GC of Marmur and Doty (1962)}$. Percent hyperchromicity $\frac{(\text{O.D./260 final} - \text{O.D./260 initial}) \times 100}{\text{O.D./260 final}}$ of 25 or above indicate purity of DNAs isolated, and that the DNA samples were free from any RNA contamination.

Table-6. Proportion of repeated and non-repeated DNA sequences of N. crassa cell types and cell fractions.

³ H-labeled DNA	Cell fractions	Percent unique sequence	Percent repeated sequences
<u>N. crassa</u> wild type	Total	80	20
<u>N. crassa</u> wild type	Nuclear	98	2
Slime mutant	Total	94	6
Slime mutant	Nuclear	97	3
Mycelial	Postmitochondrial	None	100

The purified labeled DNAs were dialyzed against appropriate buffer, sheared, and concentrated in a lyophilizer. Each of the DNAs was boiled for 3 to 4 minutes in a water bath & incubated for the C_{ot} of 2. $C_{ot} = \frac{O.D.}{2} \times \text{time in hour}$. After incubation, the DNAs were treated with S_1 -nuclease to get rid of the unhybridized single strand sequences, and then incubated at 37 C for one hour. After this, the samples were passed through the hydroxyapatite columns equilibrated with 0.001 M PB. The columns were washed to the background with 0.035 M PB at 60 C and the repetitive and unique DNA sequences were separated using 0.14 M PB to elute the unique DNA sequences and 0.48 M PB to elute the repetitive DNA sequences.

Copy of the MUA was submitted to the grantor of this Project, the Department of Energy. A new modern laboratory located at the Howard University Cancer Center has been allotted to Dr. S.K. Dutta for conducting Recombinant-DNA work for this research. This restricted laboratory expected to be occupied during this year, 1980.

4. Development of N.crassa rDNA Clones in pBR322 E.coli Plasmids.

Fortunately, by the time we were making these clones, it became known that Professor Metzenberg, of the University of Wisconsin had made some N.crassa rDNA clones for conducting different kinds of experiments. An active collaboration was then established with Professor Metzenberg who provided us with two valuable clones to speed up our work. Dr. Metzenberg sent us two stab cultures carrying Neurospora rDNA-plasmids. One is pMF2, as reported in the paper, Free, Rice, and Metzenberg, J. Bacteriol. 137: 1219 (1979). It has the entire coding region for the 17S, 5.8S and 26S rRNAs. The other is pKD001, and contains the coding region for 17S, but not 5.8S or 26S. It is a fragment terminated on the left (see map) by the EcoRI site left of 17S (Fig. 2 of the paper) and terminated on the right by the Bam HI site between 17S and 5.8S.

Culturing procedures were as suggested by Dr. Metzenberg. Permanent cultures of these were made in Luria broth with glucose (about 0.2%) and kept them with 10 µg/ml cycloheximide to guard against yeasts and molds, and also with suitable antibiotic at low concentration to guard against loss of the plasmids. In the case of pMF2, it was 2 µg/ml of tetracycline. In the case of pKD001, it was 20 µg/ml of ampicillin. Permanent cultures were made by putting 1 ml of overnight culture into 0.5 ml of sterile 100% glycerol, mixed, left at room temperature for 1 hour, then kept at -70°. It will last at least a year at -20°.

The 17S probe (i.e. radio-isotope labeled DNA sequences of 17S rDNA only) has already been made. We are now in the process of making 26S clone from pMF2 clone and expect to prepare 26S probe soon. These probes will be the basis for our future work.

5. Initiation of Restriction Analysis of rDNAs from N.crassa Developmental Cell Types:

If there is an intervening sequence in rDNAs or if there has been any change in DNA sequences anywhere in the 17S and 26S rDNAs then it was expected that cleavage sites will be different in different rDNAs. On this basis the fragments of restricted whole DNAs including rDNAs, were separated in agarose gels. Then those separated fragments were transferred onto nitrocellulose blotting papers. These unlabeled DNA fragments immobilized in the papers are in the process of hybridizing with radio-isotope labeled 17S and 26S probes. Any difference in autoradiographic bands will indicate probable changes in the rDNAs in N.crassa cell types.

Publications During 1979 - 1980

A. Peer-Reviewed Papers Published or in Press:

1. Dutta, S.K., D.K. Mukhopadhyay, and Jit Bhattacharyya. RNase Sensitive DNA Polymerase Activity in N.crassa Cell Fractions and Mutants. Biochemical Genetics (In press) 1980. (Preprint and a copy of the letter of acceptance is enclosed).
2. Mukhopadhyay, D.K., R. Mimiko and S.K. Dutta. DNA Homologies of Ribosomal RNA Genes of N.crassa. Neurospora Newsletter Vol. 28 (In press) 1980 (Preprint enclosed).
3. Dutta, S.K., I. Gbadamosi, Carolyn Chambers and D.K. Mukhopadhyay. Comparison of Nuclear DNA with whole Cell DNA Isolated from N.crassa. Neurospora Newsletter Vol 28 (In press) 1980. (Preprint enclosed).

B. International Recognition:

Principal Investigator, Dr. S.K. Dutta has been invited to lead a section on "Nuclear Control" in the Intl. Symposium on morphogenesis in Fungal Spore Morphogenesis to be held at Switzerland during August 1980. Two of our papers will be published along with other papers in a book by Academic Press, as stated below.

1. Dutta, S.K. Nuclear Controls in Fungal Spores Morphogenesis.
2. Dutta, S.K. and Lafayette Frederick. Gene Level Controls in Fungal Spore Morphogenesis.

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