Ribosomal RNA Genes of *Neurospora*: Isolation and Characterization

(gene isolation/DNA-RNA hybridization/DNA reassociation kinetics)

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ABSTRACT DNA sequences in *Neurospora crassa* that code for ribosomal RNA have been isolated in a highly purified state by DNA-RNA hybridization. About 100 separate ribosomal RNA genes exist in each nucleus. These genes are composed of repeated sequences that are present about 100 times per nucleus. The individual members of this group of DNA sequences are essentially identical.

The amount of DNA that codes for ribosomal RNA is different in different organisms (1-5). The amount of *Neurospora crassa* DNA homologous to ribosomal RNA (rRNA) is almost 1% (4). *Neurospora* contains very little (unpublished data), if any, repeated DNA, besides multiple mitochondrial and ribosomal gene sequences. Thus, DNA sequences coding for rRNA are readily isolated by techniques used for the isolation of highly purified rRNA cistrons from bacteria (1).

METHODS

Neurospora crassa strain 74A was used in these studies. Conventional methods for isolation of DNA do not work well with *Neurospora*; *Neurospora* DNA was isolated by a modification of the urea-phosphate method (6). Lyophilized mycelial powder was suspended in a solution of 8 M urea, 0.24 M phosphate buffer (made of equimolar quantities of Na_2HPO_4 and NaH_2PO_4 ; molarity refers to the phosphate; pH 6.8), 1% sodium dodecyl sulfate, and 0.1 mM ethylenediamine tetraacetic acid (EDTA). The mixture was homogenized in a closed container (filled with liquid to suppress foaming) with a Waring blender for 2 min to reduce the size of DNA pieces. The solution was passed through a hydroxyapatite (Bio-Gel HTP, BioRad Labs, Richmond, Calif.) column equilibrated with 0.24 M phosphate buffer-8 M urea at room temperature (23°); the column was washed with the same solution until the A_{260} of the effluent was zero. It was then washed with 14 mM phosphate buffer (pH 6.8) for removal of urea from the column.

DNA was removed from the column by elution with 0.4 M phosphate buffer and concentrated by centrifugation overnight at $100,000 \times g$. The pellet was suspended in a small volume of 1 mM EDTA. The resulting DNA was very pure and ready to use. 90% or more of the diphenylamine-reacting material was recovered from the *Neurospora* powder by this method. [^{32}P]DNAs (4) were checked for alkaline lability and acid solubility. [^{32}P]DNAs used in these experiments routinely had less than 1% acid-soluble radioactivity, virtually no alkali-labile radioactivity ($<0.1\%$), and 95% or better DNase lability. All DNAs were sheared before use by passing them through an orifice with a pressure drop of 50,000 lb/in.². The resulting pieces were about 300-500 nucleotides in length.

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As a final purification step, a dilute solution of sheared *Neurospora* [^{32}P]DNA (20-100 $\mu\text{g}/\text{ml}$) was heat-denatured in 0.14 M phosphate buffer-0.4% sodium dodecyl sulfate and passed immediately through hydroxyapatite equilibrated to 0.14 M phosphate buffer-0.4% sodium dodecyl sulfate at 60°. Single-stranded DNA passes through the column under these conditions. Cross-linked DNA and any [^{32}P]DNA-labeled contaminants that adsorb to hydroxyapatite were removed. Generally, about 1-2% of the total radioactivity adsorbed to the hydroxyapatite at this step. Since BioRad HT (hydroxyapatite suspended in 1 mM Na phosphate buffer, pH 6.8) works best for fractionating DNA-RNA hybrids, it was used for this and subsequent steps. *Neurospora* rRNA was isolated as described (4).

One method used here to isolate *Neurospora* rRNA cistrons is as follows: (a) Highly purified [^{32}P]DNA (190,000 cpm/ μg), sheared DNA (6 $\mu\text{g}/\text{ml}$) was mixed with unlabeled rRNA (43 $\mu\text{g}/\text{ml}$) in 0.14 M phosphate buffer, pH 6.8. (b) The mixture was denatured by heating at 100° for 2-3 min in a water

FIG. 1. Unsheared *E. coli* [^{14}C]DNA (1200 μg) was mixed with unsheared [^{32}P]DNA (8400 μg). The mixture was then sheared by passing the DNA through an orifice with a pressure drop of 50,000 lb/in.². Shearing the DNAs together ensures that the sizes of the pieces of the [^{14}C] and [^{32}P]DNAs will be the same. The mixture was then made to 0.14 M phosphate buffer, denatured, and incubated at 60°. Kinetic points were then taken. Each point was then analyzed by the hydroxyapatite technique (1). At 0 time of incubation, 1% of the [^{14}C] and 0.3% of the [^{32}P] adsorbed to hydroxyapatite. A maximum of 97% of the [^{14}C] and 98% of the [^{32}P] adsorbed after a long incubation. Open circles represent the reassociation kinetics of the *E. coli* DNA. Open triangles represent the *Neurospora* [^{32}P]DNA.

TABLE 1. Isolation of rRNA cistrons with RNase

A			B	
[³² P]DNA C ₀ t (×10 ⁻⁴)	% [³² P]DNA adsorbed in each cycle	Percent of original ³² P input adsorbed	Corresponding experiment without any rRNA added	
			[³² P]DNA C ₀ t	% ³² P input adsorbed
1. 620	1.95*	1.95	1. 0.013	1.04
2. 68% of the DNA recovered after treatment with RNase		1.238	2. 25% DNA re- covered after RNase treatment	
3. 3.4	86.5	1.071	3. 0.000125	0.014
4. 2.6	95.5	1.023	4. 0.000001	0.004
5. Base hydrolysis with NaOH				
6. 1.4	96.1	0.983		

A summary of results obtained by the modified method. Section A shows the results with rRNA present, while section B shows the control experiments without rRNA. In section A, rRNA C₀t of 0.43 was given at each step. This method is particularly useful for preparation of large amounts of the cistrons. C₀t is equal to (nucleic acid absorbance at 260 nm) times (hours of incubation divided by 2) (7).

bath, incubated at 60° for 1 hr, adjusted to 0.4% sodium dodecyl sulfate (final concentration), and passed through a hydroxyapatite column equilibrated at 60° with 0.14 M phosphate buffer-0.4% sodium dodecyl sulfate. Double-stranded DNA was removed by elution of the column with 0.3 M phosphate buffer. The DNA recovered was in the form of either DNA-DNA or DNA-RNA duplexes. At this stage about 2% of the total input DNA was adsorbed. (c) The solution of reassociated DNA was diluted to 0.14 M phosphate buffer-0.4% sodium dodecyl sulfate and passed through a hydroxyapatite column, equilibrated as above. The reassociated DNA was again eluted in 0.3 M phosphate buffer. This step removed any single-stranded DNA that had nonspecifically adsorbed to the hydroxyapatite, (d) After the second passage of the reassociated DNA through hydroxyapatite, the 0.3 M fraction was dialyzed against 10 mM phosphate buffer-0.1 mM EDTA. To this, 5 µg of RNase (bovine pancreas) per ml was added and the mixture was incubated for 1 hr at 37°, made to 0.14 M phosphate buffer-0.4% sodium dodecyl sulfate, and passed through a hydroxyapatite column, equilibrated as above. Control experiments had shown that DNA-RNA hybrids treated in this manner did not adsorb to hydroxyapatite, while the treatment did not affect DNA-DNA duplexes, which adsorbed. (e) The [³²P]DNA fraction that was applied to the hydroxyapatite column and that contained the DNA formerly involved in DNA-RNA hybrids was treated with self-digested Pronase (10 µg/ml, 37°, 1 hr); the protein was extracted with phenol and chloroform (1:1, v/v). The [³²P]DNA was concentrated with hydroxyapatite (1). (f) The concentrated [³²P]DNA was reacted with unlabeled rRNA (43 µg/ml), the mixture was applied to a hydroxyapatite column, and the reassociated DNA was collected as described above. This reassociated fraction was diluted to 0.14 M phosphate buffer-0.4% sodium dodecyl sulfate, denatured, reacted once more with rRNA, and passed again through a hydroxyapatite column. 95% or more of the [³²P]-DNA adsorbed to hydroxyapatite. This fraction was eluted with 0.3 M phosphate buffer, treated with 0.5 N NaOH at 60° for 1 hr for removal of RNA, and the [³²P]DNA was concentrated with hydroxyapatite. The products of this procedure are highly purified rRNA cistrons. Table 1 shows

data obtained by this procedure. rRNA cistrons were also isolated by a method described in ref. 1 (Table 2).

RESULTS AND DISCUSSION

The fraction of total *Neurospora* DNA complementary to rRNA

Tables 1, 2, and 3 show that in the presence of rRNA the fraction of total *Neurospora* DNA recovered as being complementary to rRNA was about 0.0095. Where no rRNA was present the fraction recovered was 0.00002 of the total DNA. 'Saturation' experiments indicated that the quantity of DNA complementary to rRNA under these conditions is about 0.95%. This value agrees well with the saturation value obtained by Wood and Luck (4). The correspondence of these two 'saturation' values indicated that all or nearly all of the

TABLE 2. Isolation of *Neurospora* rRNA cistrons

Cycle	[³² P]DNA C ₀ t (×10 ⁻⁴)	Percent of radio- activity adsorbed in each cycle	Percent of original input [³² P]DNA adsorbed
A 1	770	3.64	3.64
2	4.2	32.6	1.18
3	1.2	90	1.07
4	1	97	1.04
5	0.9	95	0.98
<i>Base hydrolysis</i>			
6	0.6	99	0.97
B 1	770	2.2	2.2
2	4	6.3	0.14
3	0.15	21.3	0.038
4	0.04	51.5	0.019

Typical experimental results obtained without RNase. The technique was described (1). Section A shows the results of the isolation when rRNA was present. At each step, rRNA C₀t of 0.7 was given. Section B presents the control isolations without rRNA.

FIG. 2. ^{32}P -Labeled isolated *Neurospora* rRNA cistrons were mixed with total sheared, unlabeled *neurospora* DNA. The mixture was then made to 0.14 M Na phosphate buffer, denatured, and incubated at 60° . Samples were taken at specified times and then analyzed on hydroxyapatite. The ratio of unlabeled to ^{32}P -labeled DNA was greater than 1500 to 1. The reaction of bulk *Neurospora* DNA was followed by absorbance at 260 nm, while the ^{32}P marked the reassociation of the ribosomal cistrons. The maximum reactions for the ^{32}P and bulk DNA were 94 and 95%, respectively. The C_0t s used are those of the unlabeled bulk DNA. Open circles and Xs represent the reassociation of bulk DNA, while the closed circles represent ^{32}P ribosomal cistrons.

rRNA cistrons were recovered from the total DNA. In order to check this further, the [^{32}P]DNA that did not react with rRNA in the first isolation reaction was recovered and reacted again with rRNA and put through the complete isolation procedure. Only 0.004% of the DNA was recovered as being homologous to rRNA. This indicates that the rRNA cistrons were essentially completely removed during the first cycle of isolation.

These data (Tables 1, 2, and 3; Figs. 2 and 3) show that the isolated rRNA cistrons react almost completely with *Neurospora* rRNA and whole unlabeled DNA. They react very poorly with rRNA from *Escherichia coli* and highly purified

TABLE 3. The amount of DNA homologous to increasing amounts of rRNA

rRNA C_0t	Percent of original input DNA recovered as rRNA cistrons
A 1. 0.35	1.04
2. (a) 0.43	0.97
(b) 0.43	0.98
A 3. (a) 0.72	0.98
(b) 0.72	1.07
(c) 0.72	1.03
4. 1.2	1.03
B 0	0.02
0	0.02
0	0.005

The data in section A show the fraction of [^{32}P]DNA recovered with increasing exposure to rRNA (larger rRNA C_0t s). At rRNA C_0t s differing by fourfold, essentially the same amount of [^{32}P]DNA is recovered. Section B shows the results of a control experiment, without rRNA.

FIG. 3. Thermal stability profiles of reassociated, unlabeled bulk *N. crassa* DNA ($\times-\times-\times$) and radioactive rRNA cistrons ($\circ-\circ-\circ$). [^{32}P]rRNA cistrons were mixed with unlabeled bulk *N. crassa* DNA. The mixture was made to 0.14 M Na phosphate buffer, denatured in a boiling water bath for 2 min, and then incubated at 60° for a very high unlabeled DNA C_0t . At the end of the reaction, the mixture was passed over hydroxyapatite (60° , 0.14 M phosphate buffer), where 97% of the radioactivity and 93% of the absorbance adsorbed. The thermal stability profile was obtained as described (1).

tRNA from *Neurospora* (less than 5%). rRNA cistrons incubated alone do not form double-stranded DNA.

In order to calculate the number of separate rRNA cistrons that are present per *Neurospora* nucleus, it was necessary to know the DNA content of a *Neurospora* nucleus. When repeated DNA sequences are absent, the rate of reassociation of a DNA can be used to determine the complexity (nucleotides per haploid nucleus) of a DNA (7). Fig. 1 summarizes a reassociation kinetic experiment that gives a good estimate of the *Neurospora* DNA complexity. At equivalent concentrations, *E. coli* DNA (51% guanine plus cytosine) reassociates 8.2 times faster than *Neurospora* DNA (50–52% guanine plus cytosine). This indicates that the *Neurospora* DNA is 8.2 times more complex than *E. coli* DNA (7). An *E. coli* chromosome contains about 2.8×10^9 daltons of DNA, so *N. crassa* must contain 23×10^9 daltons of DNA ($8.2 \times 2.8 \times 10^9$) per nucleus. Since rRNA cistrons comprise 0.0095 of the total *Neurospora* DNA, this indicates that in each nucleus there is enough DNA homologous to rRNA to code for 100 separate rRNA cistrons. This number is calculated with values of 1.4×10^6 and 0.7×10^6 for the molecular weights of 28S and 18S rRNA subunits.

Similarity of the individual rRNA cistrons

The rate of DNA reassociation is proportional to the concentration of each different DNA sequence present in the reassociation mixture (7). If each of the 100 separate rRNA cistrons of *Neurospora* represent a different nucleotide sequence occurring one time per nucleus, then the concentration of each cistron that codes for rRNA is the same as the other DNA sequences in the nucleus. In this case, the rRNA cistrons would reassociate at the same rate as the bulk of *Neurospora* DNA. If, however, all of the separate rRNA cistrons were similar, the concentration of the DNA sequences that code for rRNA will be 100 times higher than most of the other DNA sequences present in the nucleus. In this case, the rRNA cistrons will reassociate 100 times faster than the

bulk of the *Neurospora* DNA. Fig. 2 shows the reassociation kinetics of isolated [³²P]rRNA cistrons mixed with unlabeled total *Neurospora* DNA. The rRNA cistrons reassociate about 100 times faster than the bulk of the DNA. This result suggests that the majority of the DNA sequences that comprise the rRNA cistrons are present 100 times per nucleus. It does not, however, rule out the presence of a low number of different ribosomal RNA sequences in each nucleus.

It is possible for two imperfectly complementary strands to interact and reassociate together to form a stable double-stranded duplex (7). In view of this, a further test of the similarity between the repeated rRNA cistrons was made. Fig. 3 shows the thermal stability profile of reassociated rRNA cistrons and bulk *Neurospora* reassociated DNA. The thermal stabilities of the two fractions are almost identical. The thermal stability of reassociated DNA indicates the accuracy of base-pair matching between the component strands of DNA (7). Native DNA is assumed to have perfect base-pair matching. Since the thermal stability of native and reassociated *Neurospora* total DNAs are virtually the same, perfect or near perfect base-pair matching should exist also between the component strands of bulk reassociated *Neurospora* DNA. The presence of mispaired bases in reassociated DNA lowers the thermal stability of the DNA about 1° per 1.5% mispaired bases (8). If the multiple rRNA cistrons were not identical to one another, but were only similar enough to interact with each other, the thermal stability of the reassociated rRNA cistrons would be lower than that expected from their guanine-plus-cytosine content. The guanine-plus-cytosine contents of bulk and ribosomal DNAs are essentially identical (4). The data of Fig. 3 indicate that the majority of the individual repeated rRNA cistron sequences are very similar to each other. It is not possible, however, to say that these related sequences are absolutely identical to one another. The best current estimate indicates that the average similarity between the majority of the repeated sequences is greater than 98%.

DISCUSSION

The method used here to isolate rRNA cistrons uses sheared DNA pieces about 300–500 nucleotides long. Isolated rRNA cistrons are, therefore, not intact. Each cistron (28S + 18S) is chopped into about 17 pieces. If we assume that the 28S subunit gene is adjacent to the 18S subunit gene, less than 10% of the DNA recovered would consist of DNA sequences adjacent to the rRNA genes. It should also be possible to obtain intact rRNA cistrons and longer DNA pieces by the techniques described.

The amount of *Neurospora* DNA homologous to rRNA (0.0095) is enough DNA for about 100 separate rRNA cistrons (Tables 1 and 2). Figs. 2 and 3 indicate that the great majority of the rRNA cistron sequences are present 100 times per nucleus and that the individual multiple sequences are virtually identical to one another. The simplest explanation of these data is that almost all of the 100 separate rRNA cistrons are essentially identical to one another. This same type of data has been seen for various organisms. Most organisms contain enough DNA homologous to their respective rRNAs to code for multiple rRNA cistrons per haploid cell. *E. coli* contains 4–5 rRNA cistrons per haploid cell (1) while *Bacillus subtilis* (R. J. Avery, unpublished data) has 6–10 and *Xenopus laevis* has about 400 (2). Thermal stability and kinetic experi-

ments (R. J. Avery, D. E. Kohne, and D. D. Brown, unpublished data) with rRNA cistrons isolated from these organisms indicated that, in each case, the kinetic redundancy of the rRNA genes essentially equaled the number of separate rRNA cistrons present per nucleus. Again, the simplest explanation for these data is that virtually all of the rRNA genes in an organism are essentially identical. With kinetic data somewhat similar to those reported here, Kohne (1), working with *E. coli* and *Proteus mirabilis*, and Birnstiel *et al.* (9), studying *B. subtilis*, *X. laevis*, and rabbit, state that the rRNAs of these organisms are each composed of a single group (or family) of similar nucleotide sequences. Again, this is the simplest interpretation of the kinetic data.

Unfortunately, it is not possible to prove, from just kinetic data alone, that only one class of rRNA sequence exists in an organism. In most organisms, the larger rRNA (28S or 23S) subunit has no detectable homology to the small rRNA subunit (18S or 16S). *E. coli* is an exception to this as it has been observed that most of the 16S subunit of *E. coli* is homologous to the 23S subunit (3). If sequence redundancy within a single cistron exists, the kinetic and thermal stability data could be interpreted in a different manner. The following situation, for example, is compatible with the *Neurospora* data that we have presented here. It is possible that two classes of rRNA cistrons of equal size exist in each *Neurospora* nucleus. If the 28S rRNA subunit is composed of a sequence present twice per subunit, and the 18S rRNA subunit consists of a different sequence present twice per 18S subunit, the data would be consistent with this model. In this case there would be two classes of rRNA cistrons (50 members in each class), and the sequences comprising the rRNA cistrons would each be present 100 times per nucleus. Other similar models could obviously be constructed that would be consistent with the data.

Nucleotide-sequence studies (10, 11) indicate that the rRNA of *E. coli* is essentially homogeneous. It is not possible to say from this alone that the multiple rRNA genes of *E. coli* are completely identical, since only a fraction of the genes may be expressed as rRNA. However, homogeneity of the *E. coli* rRNA coupled with data similar to those presented here strongly indicates that the multiple rRNA genes of *E. coli* are essentially identical to one another. If *Neurospora* rRNA is homogeneous in sequence, a similar conclusion can be made.

No evidence exists for gross heterogeneity in the rRNA of one organism. However, small but important differences may well be present between the multiple rRNA genes. More sensitive ways of looking at these sequences are needed. Further fractionation of rRNA genes will increase the chance of seeing small differences, if they exist.

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1. Kohne, D. E. (1968) *Biophys. J.* 8, 1104–1118.
2. Brown, D. D. & Dawid, I. B. (1968) *Science* 160, 272–280.
3. Avery, R. J., Midgeley, J. E. M. & Pigott, G. H. (1969) *Biochem. J.* 115, 395–403.
4. Wood, D. D. & Luck, D. J. (1969) *J. Mol. Biol.* 41, 211–224.
5. McConkey, E. N. & Hopkins, J. W. (1964) *Proc. Nat. Acad. Sci. USA*, 51, 1197–1204.
6. Britten, R. J., Pavich, M. & Smith, J. (1970) *Carnegie Inst. Wash. Yearb.* 68, 400–402.
7. Britten, R. J. & Kohne, D. E. (1968) *Science* 161, 529–540.
8. Laird, C., McConaughy, B. L. & McCarthy, B. J. (1969) *Nature* 224, 149–154.
9. Birnstiel, M., Sells, B. H. & Purdom, I. F. (1972) *J. Mol. Biol.* 63, 21–39.
10. Nomura, M. (1970) *Bacteriol. Rev.* 34, 228–277.
11. Fellner, P., Ehresmann, T. & Ebel, J. P. (1970) *Nature* 225, 26–30.