A RIBOSOMAL RNA GENE CLONE ISOLATED FROM *NEUROSPORA CRASSA*, WILD STRAIN 74A DIFFERS SIGNIFICANTLY FROM A SIMILAR CLONE ISOLATED FROM ITS ABNORMAL DEVELOPMENTAL SLIME MUTANT

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ABSTRACT

We have constructed a clone, pCC103, of rDNA of *Neurospora crassa* wild strain 74A which contains 960 bp more sequences than the previously isolated rDNA clone, pMF2, of the slime mutant of *N. crassa* developed by a similar cloning strategy[1]. pCC103 contains genes coding for 17S, 5.8S, and 26S rRNA with external and internal transcribed spacers (ITS-1 and ITS-2). The clone was isolated by inserting one Pst I fragment of rDNA from *N. crassa* wild strain 74A into plasmid pBR322 in the ampicillin gene at the Pst I site. The restriction endonuclease analysis and hybridization experiments confirmed our results, and the physical map of pCC103 was constructed for enzymes Bam HI, Bgl II, Eco RI, Hinc II, Hind III, Pst I, Sma I, Sst II, Xba I, Xho I, etc. We believe that this clone should help in understanding the regulation of transcription and processing of rRNA genes in *N. crassa*.

INTRODUCTION

RIBOSOMAL RNA genes play a significant role in the proper functioning of an organism. The isolation, organization, expression, cloning, and sequencing of rRNA genes from fungal species have been well documented by us[2-9]. Earlier, we cloned the external spacer region of the *Neurospora crassa* wild strain 74A rDNA in pBR325 to study the initiation codons for 17S and termination codons of 26S gene[7], and now we have isolated a new clone, named as pCC103, which contains genes coding for 17S, 5.8S and 26S rRNA with external and internal transcribed spacers. This clone not only contains a larger rDNA insert (6.860 kbp) than pMF2 (5.900 kbp) but it also shows a different restriction endonuclease pattern for a number of restriction endonucleases. In this paper, we have described the procedure for constructing this new clone and have given its physical map. This information is expected to help in studying regulation of rRNA gene transcription, rRNA processing and sequencing of transcription promoter, enhancer (if any) and termination sequences, as well as rRNA processing sites, which are part of our ongoing studies.

MATERIALS AND METHODS

Chemicals and Enzymes

All restriction endonucleases, DNA polymerase I, DNase I, T4 ligase, salmon sperm DNA, λ DNA, calf thymus DNA, pBR322 DNA, and bacterial alkaline phosphatase were obtained from Bethesda Research Laboratory, USA, and the guidelines of the manufacturer were strictly followed. Tris (hydroxymethyl)-aminomethane, agarose, acrylamide, bis acrylamide, bovine serum albumin, isopropyl-β-D-thiogalactopyranoside (IPTG), 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), polyvinyl pyrrolidone, ficol, ethidium bromide, boric acid, and all antibiotics (ampicillin, tetracycline, and chloramphenicol) were purchased from Sigma Chemicals, USA. Other chemicals were of Analytical grade. Nitrocellulose filter sheets (BA

85) were from Schleicher and Schuell, West Germany, and α^{32}P-labelled deoxynucleoside triphosphates were obtained from New England Nuclear, USA.

Cultures

Neurospora crassa strain 74A was obtained from the Fungal Genetics Stock Center (no. 587), Humboldt, CA. The slime mutant rDNA clone pMF2 was obtained from Dr. R. Metzenberg, University of Wisconsin, Madison, USA. Conidia were grown on Vogel's minimal medium[10] plus 1.5% sucrose and 1.5% agar at 30°C for 5 days in the dark and then 2–5 days in light. Conidia were germinated by growing in Vogel's minimal medium[10] plus 1.5% sucrose for 4–6 hours at room temperature with aeration. *Escherichia coli* LE392 was grown in Luria broth at 37°C, and clones pMF2 and pCC103 were also grown in Luria broth. Tetra-cycline was included in the growth medium at a final concentration of 10 μg/ml to avoid any contamination in the clones.

Isolation of Plasmid DNA

For the isolation of plasmid DNA, the method described by Maniatis *et al*[11] was followed.

Isolation of Neurospora crassa Wild Type DNA

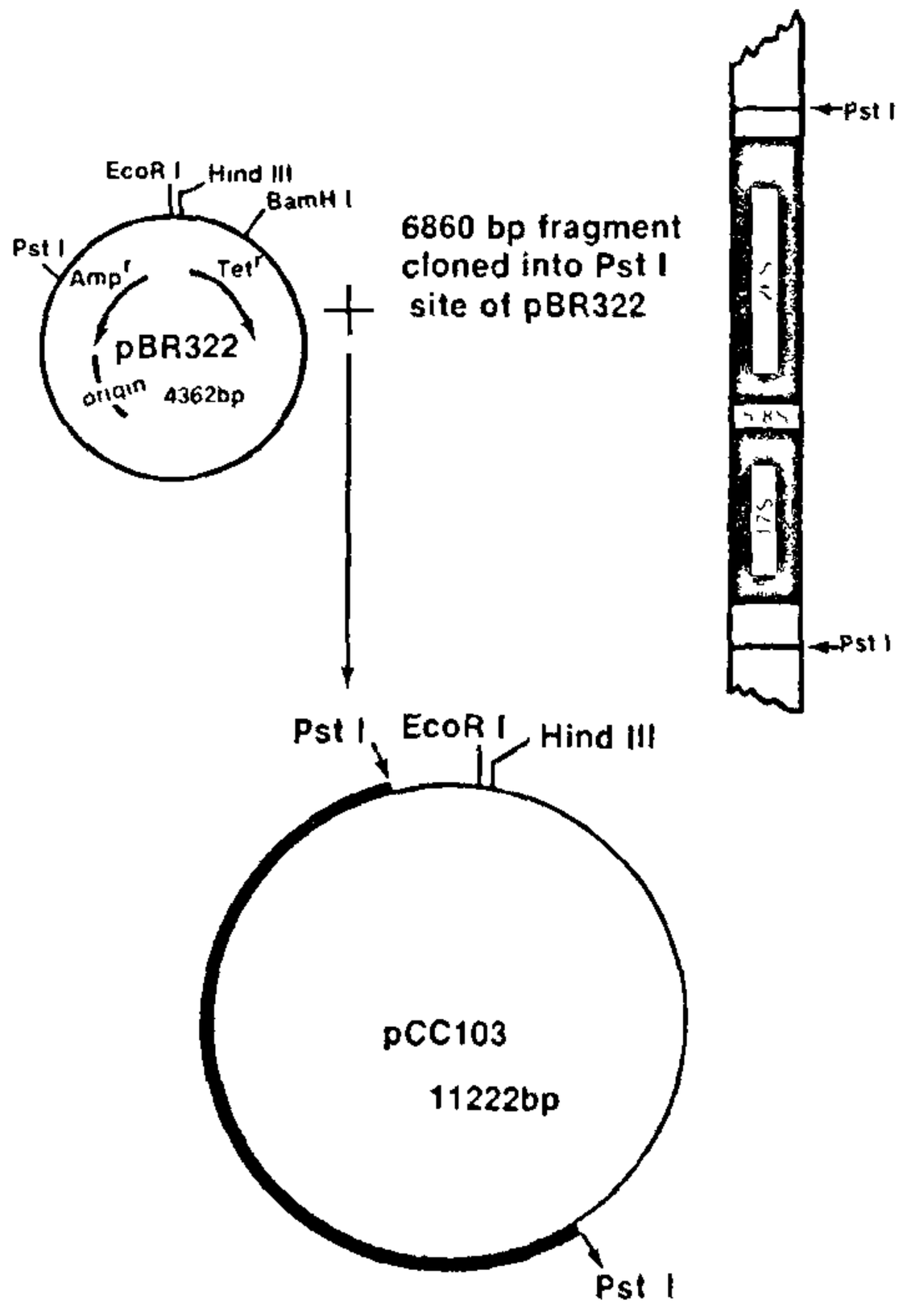
Neurospora DNA was isolated according to a modification of the ureaphosphate method which has been described earlier[12].

Digestion of Neurospora and Clone DNAs with Restriction Enzymes and Electroelution of Fragments

For digestion of DNA with different restriction endonucleases, the manufacturer's instructions were followed. Restriction endonuclease cleavage products were analyzed by electrophoresis through vertical agarose slab gels (16 cm × 22 cm × 3 mm) with a trisborate buffer system described by Maniatis *et al*[11]. DNA samples were mixed with glycerol (final concentration of glycerol being 10%) and bromophenol blue (0.005%) and then layered under buffer into the slot of the gel. Just before layering, samples were heated for 10 minutes at 65°C to disrupt hydrogen bonding between short cohesive ends. Electrophoresis was performed at 75 volts at room temperature. For determining the molecular weight of small DNA fragments, 5% polyacrylamide gels were run. The electroelution of digested fragments was done according to the methods described by Smith[13].

Cloning of rDNA of Neurospora crassa into pBR322 and Southern Hybridization

The strategy of cloning has been depicted in figure 1. The rDNA clone pCC103 was identified using ^{32}P-labelled nick-translated pMF2 as a probe. Nick-translation was done as described by Rigby *et al*[14]. DNA from agarose gels was transferred to nitrocellulose filters by following



Cloning Strategy of pCC103

Figure 1. Cloning strategy for pCC103.

Southern's technique[15]. The hybridization of Southern blots with ^{32}P-labelled nick-translated probe was done mainly by following the procedure as described by Maniatis *et al*[11] with few modifications. For washing of filters, special attention was paid to avoid the nonspecific adsorption of probe DNA to the denatured DNA loaded on filters. After removing the filters from bag, they were washed with (i) 4 × Denhardt's solution, 0.5 % SDS, 3 × SSC two times at 60°C for 30 minutes each; (ii) 2 × SSC, 0.5 % SDS at 60°C, 3 times for 15 minutes each; and (iii) 2 × SSC, 0.1 % SDS at room temperature, 3 times for 15 min each. At no stage was a filter allowed to dry after removing it from the bag.

After the filter was air-dried, it was exposed to Kodak X-Omat X-ray film using Dupont Cronex intensifying screen at −70°C for 12 hours.

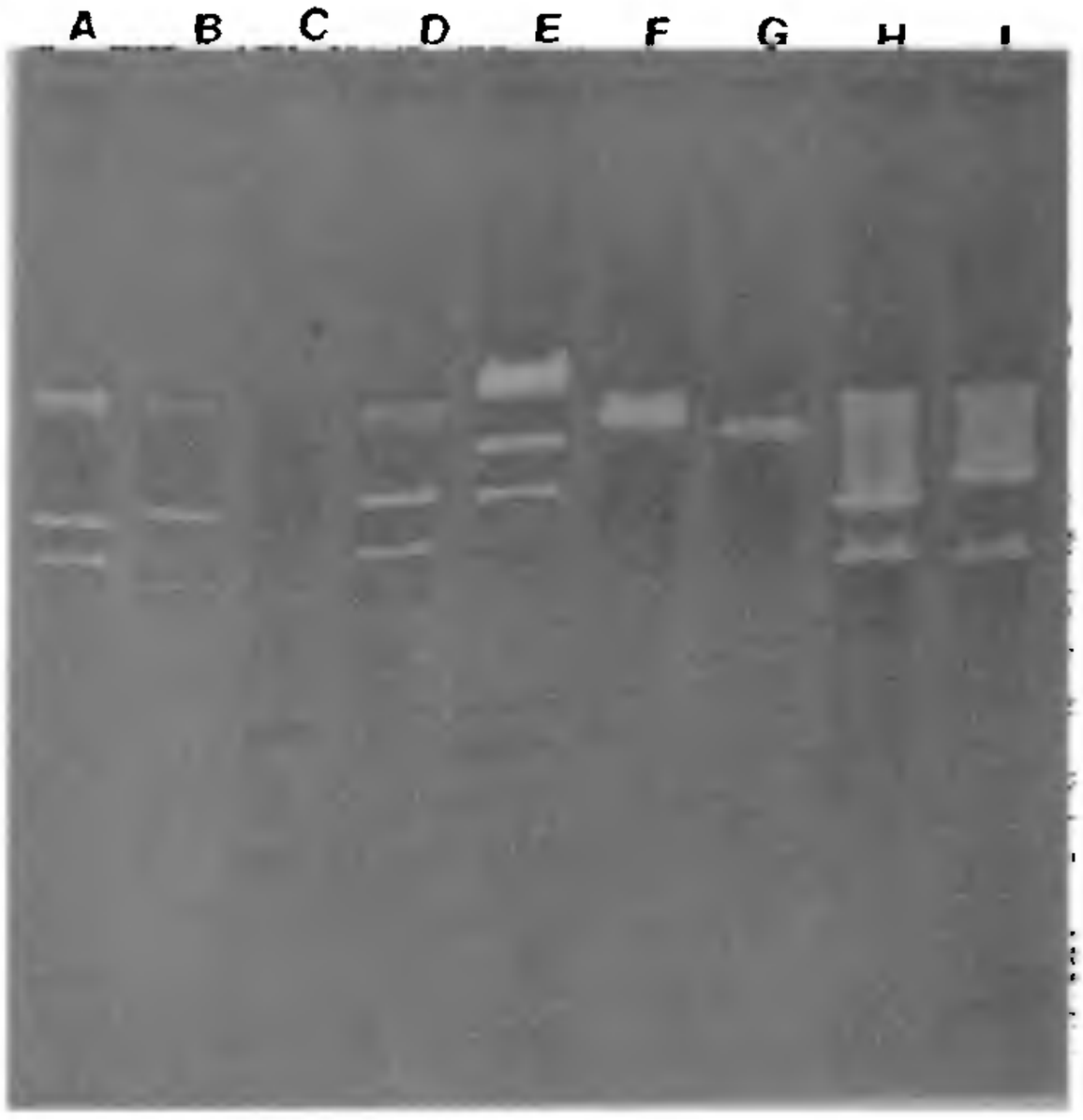


Figure 2. Analysis of restriction products of pCC103 and pMF2 DNA cleaved with various enzymes. Electrophoresis was carried out on a 0.8 % agarose gel. Track A: pCC103 digested with Hind III; Track B: pMF2 digested with Hind III; Track C: pMF2 digested with Eco RI; Track D: pCC103 digested with Eco RI; Track E: λ DNA digested with Hind III; Track F: pCC103 digested with Bgl II; Track G: pMF2 digested with Bgl II; Track H: pMF2 digested with Pst I; Track I: pCC103 digested with Pst I.

RESULTS

The strategy of cloning is shown in figure 1. The Pst I fragment containing rDNA was isolated from the total *N. crassa* wild type strain 74A DNA digested with Pst I using pMF2 as a probe and was inserted at the Pst I site of pBR322. The ligated product was introduced into *E. coli* strain LE392, and clone PCC103, which is sensitive to ampicillin and resistant to tetracycline, was isolated and characterized.

The insert in pCC103 is 6.860 kbp compared to 5.900 kbp of pMF2 (figure 2). As indicated in the figure, the linear band of pBR322 (the vector DNA) moves to the same distance in pCC103 and pMF2 after digesting both the clones with Pst I. One extra band of 0.800 kbp is seen in Hind III digested pCC103 (figure 3a) and, moreover, the movement of other two Hind III fragments of pCC103 is different than pMF2 because of the size differences.

The gels containing the digested fragments of pCC103 and pMF2 DNAs with different enzymes were Southern-blotted and hybridized with ^{32}P-labelled probe (i) pMF2 and (ii) pCC103. The autoradiograph of one of those gels has been shown in figure 3b. The intensity of bands is the same whether we use ^{32}P-pMF2 or ^{32}P-pCC103 DNA as a probe, indicating that the insert in pCC103 is from rDNA. Moreover, nonspecific adsorption of probe DNA to λ DNA is not seen.

The physical map of pCC103 has been shown in figure 4 for enzymes Pst I, Hind III, Eco RI, Bam HI, Sst II, Xho I, Xba I, Bgl II, Sma I, Hinc II, etc. From the results of double digestions (table 1), it was concluded that the extra site of Hind III is present just outside the 3′ end of 26S gene while one site for Bam HI is present towards 5′ end to last Sma I site in the 26S gene. Most of the sites of different enzymes are conserved in the 17S, 5.8S, or 26S region, and it seems reasonable that the extra size of rDNA in pCC103 mostly belongs to the external spacer or internal transcribed spacer regions (ITS-1 and ITS-2).

The clone contains the initiation and termination codons of rRNA genes, although their

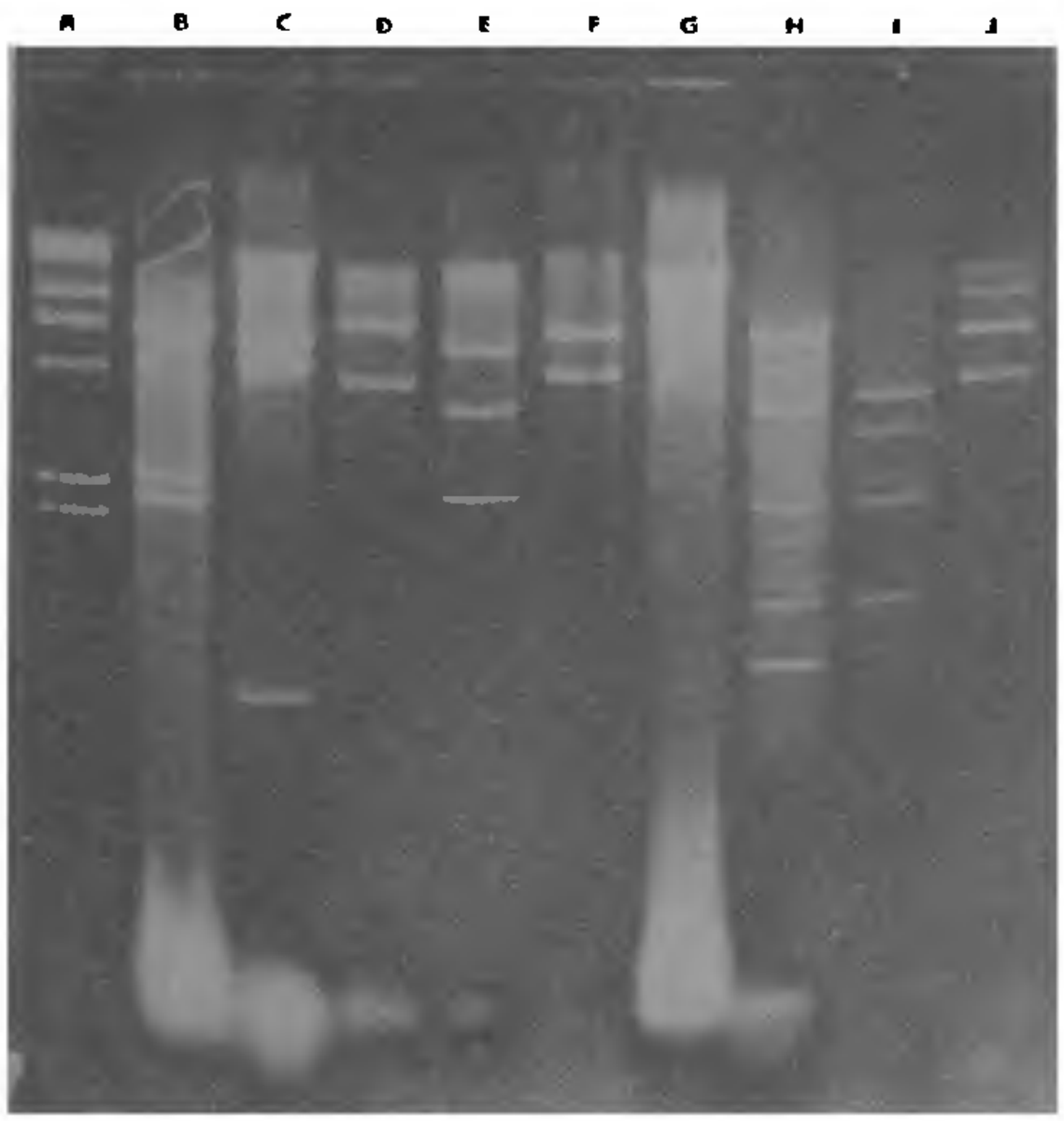


Figure 3a. Analysis of restriction products of pCC103 and pMF2 DNA cleaved with various enzymes. Electrophoresis was carried out on a 0.8% agarose gel. Track A: λ DNA digested with Hind III; Track B: pCC103 digested with Bgl I; Track C: pCC103 digested with Hind III; Track D: pCC103 digested with Pst I; Track E: pCC103 digested with Bam HI; Track F: pMF2 digested with Pst I; Track G: pCC103 digested with Bgl II; Track H: pCC103 digested with Eco RI; Track I: pMF2 digested with Eco RI; Track J: pMF2 digested with Hind III.

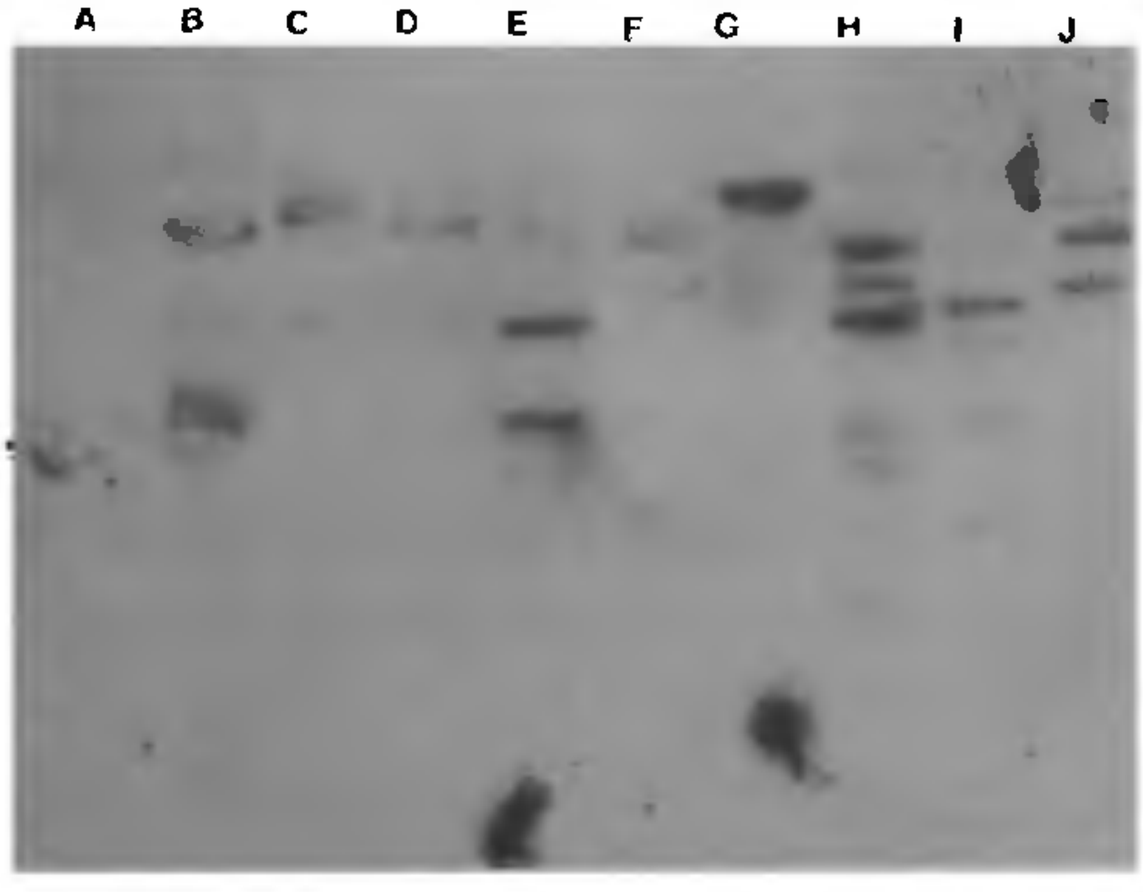


Figure 3b. Autoradiogram of the gel shown in figure 3a using ^{32}P-labeled pMF2 DNA.

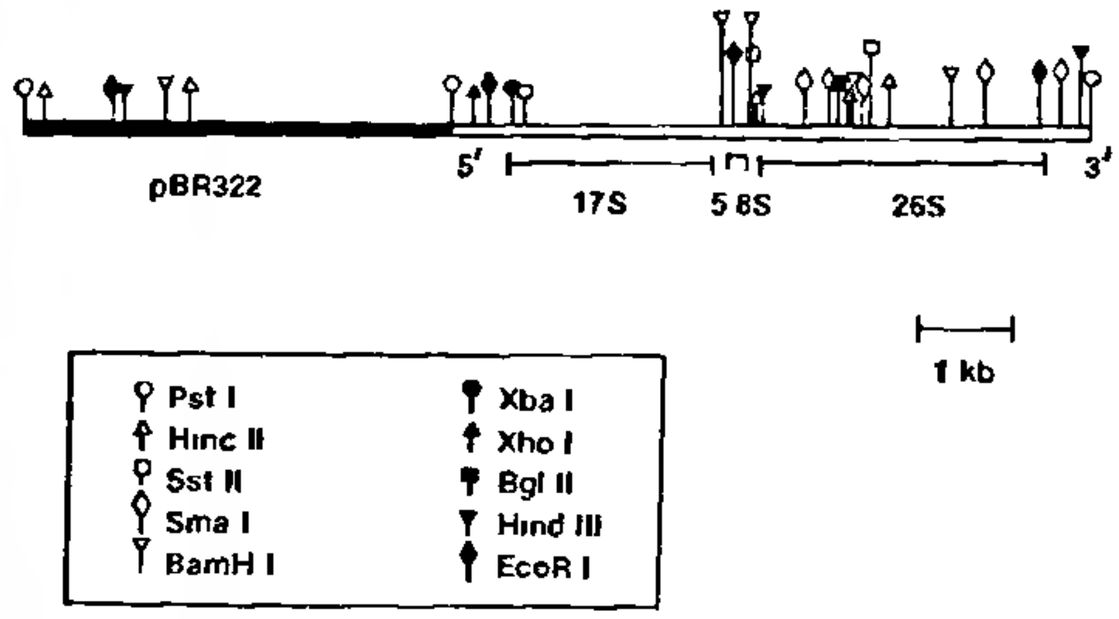


Figure 4. Restriction map of pCC103.

precise location is yet to be found. We have sequenced the ITS-1, 5.8S, and ITS-2 regions[8], and the sequencing of the termination and initiation regions of rRNA genes as well as the rRNA processing sites is underway.

The orientation of the insert is the same in pCC103 andpMF2 as indicated by the digestion patterns of the DNAs from these clones. Few enzymes like Bgl II, Xho I, and Xba I cut pCC103 only at one site.

DISCUSSION

Ribosomal RNA genes (rDNA) of *N. crassa* contain DNA sequences which code for 17S, 5.8S, and 26S rRNAs, in addition to internal and external spacers. Free *et al*[1] isolated a rDNA clone, pMF2, from the slime mutant of *N. crassa*, using Pst I enzyme and pBR322 as vector; this clone has been extensively used so far as a probe for identifying the rDNA in various species. Since pMF2 contains rDNA from slime mutant of *N. crassa*, it seems likely that it carries some additions or deletions. Chaudhuri *et al*[16] reported several years ago that the total DNA isolated from the slime mutant of *N. crassa* differed significantly (more than 2% non-homology) when compared with the wild strain based on thermal denaturation and DNA-DNA hybridization procedures. Some of the DNA non-homology could be attributed to non-homology of rDNAs between *N. crassa* and its slime mutant.

This rDNA clone pCC103 isolated from wild type *N. crassa* strain 74A not only contains a

Table 1: *Fragments generated with restriction enzymes on the DNA of clone pCC103 and pMF2.*

Enzymes	pCC103	pMF2	Enzymes	pCC103	pMF2	Enzymes	pCC103	pMF2
Single Digestion			*Double Digestions*					
Hind III	5 680	6 200	Pst I + Sst II	4 320		Pst I + Hind III	5 650	3 500
	4 700	4 100		3 050			4 450	3 400
	0 800			1 700	ND		0 750	2 400
				1 642			0 230	0 780
Pst I	6 860	5 900		0 600			0 070	
	4 362	4 362						
			Pst I + Xho I	6 850	5 900	Eco RI + Xho I	6 000	
Sst II	6 500	6 700		4 300	4 360		2 030	
	3 050	1 900		0 050	0 050		1 520	ND
	1 700	1 700					1 340	
			Hinc II + Bgl II	3.340			0.300	
Hinc II	3 350	5 200		3.255				
	3 250	1 700		2 250	ND	Eco RI + Bgl II	6 000	3 700
	2.250	1 560		1 250			2 305	2 200
	1 400	1 100		1 800			2 150	1 650
	0 330	0 540		0.300			0 405	1.500
Bam HI	5 110	5 200	Pst I + Bgl II	6 855			0 300	1.300
	3 780	4 700		3 150	ND	Eco RI + Hind III		
	2 000	0 300		1 205			4 500	3.750
	0.300						4 100	2.750
Sma I	7.900	7 500	Bam HI + Xho I	3 800			1 350	2 100
	1 650	0 950		3 505			0.650	1 300
	0 780	0 860		2 050	ND		0 300	0 400
	0 630	0 760		1.550			0.300	0 400
	0.260	0 260	Hind III + Xho I	0 300	4 020	Eco RI + Bam HI	0.250	0 030
Xho I	11 200	10 300		4 650	3.600		0.050	
Xba I	11 220	10 290		0 050	2 650		3 655	3.200
Bgl II	11.220	10 300		2.655			2.455	2.900
			Bam HI + Hind III	0 800	5 200		2 150	1.900
				ND	4 200		1.250	1 300
					0 346		0.800	0 375
					0.300		0 550	0.200
					0.272		0 300	0 100

ND means not done; sizes are in kbp

larger size of rDNA than pMF2 but also shows some interesting restriction endonuclease patterns with a number of restriction enzymes (table 1, figure 3). We believe that clone pCC103 should be a better choice to use as a probe for identifying rDNA sequences for studying the regulation of transcription; and for processing; clone pCC103 should also be very useful as it contains all the processing sites and transcription initiation and termination signals.

At this juncture it is not possible to correlate that the extra base pairs present in pCC103 compared to pMF2 are because clone pMF2 contains an insert from an abnormal developmental mutant strain slime of *N. crassa*. The available data from our laboratory from homothelic species *e.g. N. dodgei*, *N. terricola* and *N. africana* and also from a temperature sensitive mutant of *N. crassa*[17,18] suggest that rDNA is 8.7 kbp in all the cases except in slime mutant.

ACKNOWLEDGEMENTS

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ANNOUNCEMENTS

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