Eco RI or Bam, respectively. The linear plasmid DNA was mixed with double-stranded HbF cDNA and the mixture was treated with S1 nuclease, followed by T4 ligase and *Escherichia coli* DNA polymerase I. The ligated DNA's were used to transform *E. coli* strain HB101. We then looked for colonies containing hybrid DNA molecules by selecting for antibiotic resistance (pMB9) or, as in the case of pBR313, using the cycloserine selection procedure developed by Bolivar *et al.* (*11*), followed by in situ hybridization. Table 1 shows the results of such a transformation. No transformants were obtained when *E. coli* cells were transformed with closed circular pBR313 DNA. Neither were transformants obtained when cells were incubated with plasmid DNA converted to linear form by enzymatic cleavage with Bam and digested with S1 nuclease. However, the mixture of plasmid and HbF cDNA which had been treated with T4 ligase gave rise to 60 transformants.

Hemoglobin mRNA from patients with sickle cell anemia was used to synthesize Hb cDNA. This cDNA was then used as a template for the production of ^{32}P-labeled sickle cell cRNA for use as a hybridization probe. Transformed colonies were lysed, fixed onto nitrocellulose filters (*12*), and hybridized with the ^{32}P-labeled cRNA. After hybridization, filters were audioradiographed. Repeated hybridizations showed that several colonies hybridized to the ^{32}P-labeled Hb cRNA (Fig. 3A).

Plasmid DNA was isolated from two colonies that hybridized to [^{32}P]cRNA. These plasmid DNA's were electrophoresed on a 0.7 percent agarose gel with pBR313 DNA (Fig. 3B). On this gel, plasmid DNA's from transformed colonies showed slower mobilities. This indicates plasmids of larger molecular weight, as one would expect after insertion of Hb cDNA into the plasmid.

With the issuance of the National Institutes of Health guidelines, further characterization of these colonies and plasmid DNA's was postponed until the certification of a EK2 host vector system and the official certification of a P3 facility. However, this research was conducted in a limited-access laboratory with negative pressure. All microbiology was done in that laboratory, in a special biocontainment hood meeting the specifications of the NIH guidelines.

During the period when this research was conducted, the laboratory was monitored twice weekly for containment of the host bacteria. At no time was any evidence found for escape of the bacteria into the laboratory environment. Fecal samples were taken throughout the study and found to be free of resistance markers used in this study.

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Cloned Ribosomal RNA Genes from Chloroplasts of *Euglena gracilis*

Abstract. *Fragments of* Euglena *chloroplast DNA generated by endonuclease R·Eco RI were separated by agarose-gel electrophoresis into 24 distinct bands. At least five fragments contain sequences complementary to chloroplast ribosomal RNA. Most of the Eco RI fragments have been cloned in a plasmid of* Escherichia coli. *Three of the cloned fragments were shown to contain chloroplast ribosomal RNA sequences by DNA-RNA hybridization.*

Chloroplasts from the unicellular alga *Euglena gracilis* contain multiple copies of a circular, double-stranded DNA molecule (Chl-DNA) whose molecular weight is about 92×10^6 (*1*). These chloroplasts also contain ribosomes with sedimentation values similar to those of prokaryotic ribosomes (*2, 3*), and hybridization of chloroplast ribosomal RNA (Chl-rRNA) to Chl-DNA shows that Chl-DNA contains about two to three rRNA cistrons per chloroplast genome (*3, 4*). The existence of DNA and a protein-synthesizing apparatus in chloroplasts poses some of the most intriguing problems of modern cell biology. How did these organelles arise, and what are their genetic capabilities? How does the nuclear genome interact with the cytoplasmic genome to regulate the biogenesis of the organelle and its energy-producing reactions? Recombinant DNA methodology provides an approach to answer these questions, for it enables us to dissect the chloroplast genome and to determine the genes contained on specific segments.

As a first step toward understanding the organization of chloroplast genes and their functions, we have cloned most of the chloroplast DNA as discrete fragments inserted in a set of RSF2124 plasmids of *Escherichia coli*. These fragments were generated by treatment of Chl-DNA with the restriction endonuclease Eco RI (*5*) and were identified by agarose-gel electrophoresis, which separates DNA molecules according to size (*6*). The purpose of this report is to show that molecular cloning permits unambiguous identification of unique Chl-DNA fragments containing Chl-rRNA genes.

Electrophoresis of Eco RI–digested Chl-DNA on agarose gels yields 24 distinct bands (Fig. 1a). We were able to separate several bands of similar mobility which had not been resolved (*4, 7*). In particular, bands D and E can be separated, and therefore were given individual letter designations. However, bands I, M, and W each contain two DNA fragments so nearly identical in size that we were unable to separate them. These bands are designated I_1, I_2, and so forth. The staining intensity of these bands indicates that each contains more than one

Table 1. Hybridization of plasmid DNa with chloroplast rRNA. Preparation of ^{125}I-labeled rRNA and its hybridization to DNA on filters was performed as described (*13*). Hybridization values are the averages of duplicate filters and are corrected for adsorption of rRNA to blank filters. Duplicate filters in the hybridization vials were placed between blank filters. A zero value here indicates that the amount of radioactivity on the test filters fell within the range of that bound to the blank filters. Other values represent 1.3 to 9.3 times as much radioactivity bound to test filters as to blank filters. Experiments were repeated with different preparations of ^{125}I-labeled rRNA, and specific activities ranged from 9.6×10^6 to 1.6×10^7 count/min per microgram. Band N DNA has not been cloned, and therefore it is not represented.

Plasmid	Chloroplast DNA band	DNA per filter (μg)	[^{125}I]rRNA hybridized per filter (ng)	Plasmid	Chloroplast DNA band	DNA per filter (μg)	[^{125}I]rRNA hybridized per filter (ng)
pML10	J	0.41	0	pML22	I + O	0.32	1.58
		0.82	0	pML15	L + O	0.72	1.8
		1.9	0	pML14	O + S + (V or W_1 or W_2)	0.40	0.75
pML19	K	0.30	0.82	pML18	O + (T or U) + X	0.29	1.26
		0.60	3.85	pML35	P	1.0	0
pML31	K	0.27	1.03	pML17	P + R + W(W_1 or W_2)	0.45	1.23
		0.54	1.28			1.35	1.84
pML21	L	0.33	0			5.7	4.79
		0.66	0	pML20	S + (T or U)	0.16	0
		1.1	0			0.31	0
pML23	M	2.3	0	pML26	T or U	0.63	0
pML25	O	0.19	1.35	pML28	W(W_1 or W_2)	0.18	0
pML12	O	0.52	3.5			0.35	0
				pML34	W(W_1 or W_2) + X	1.23	0

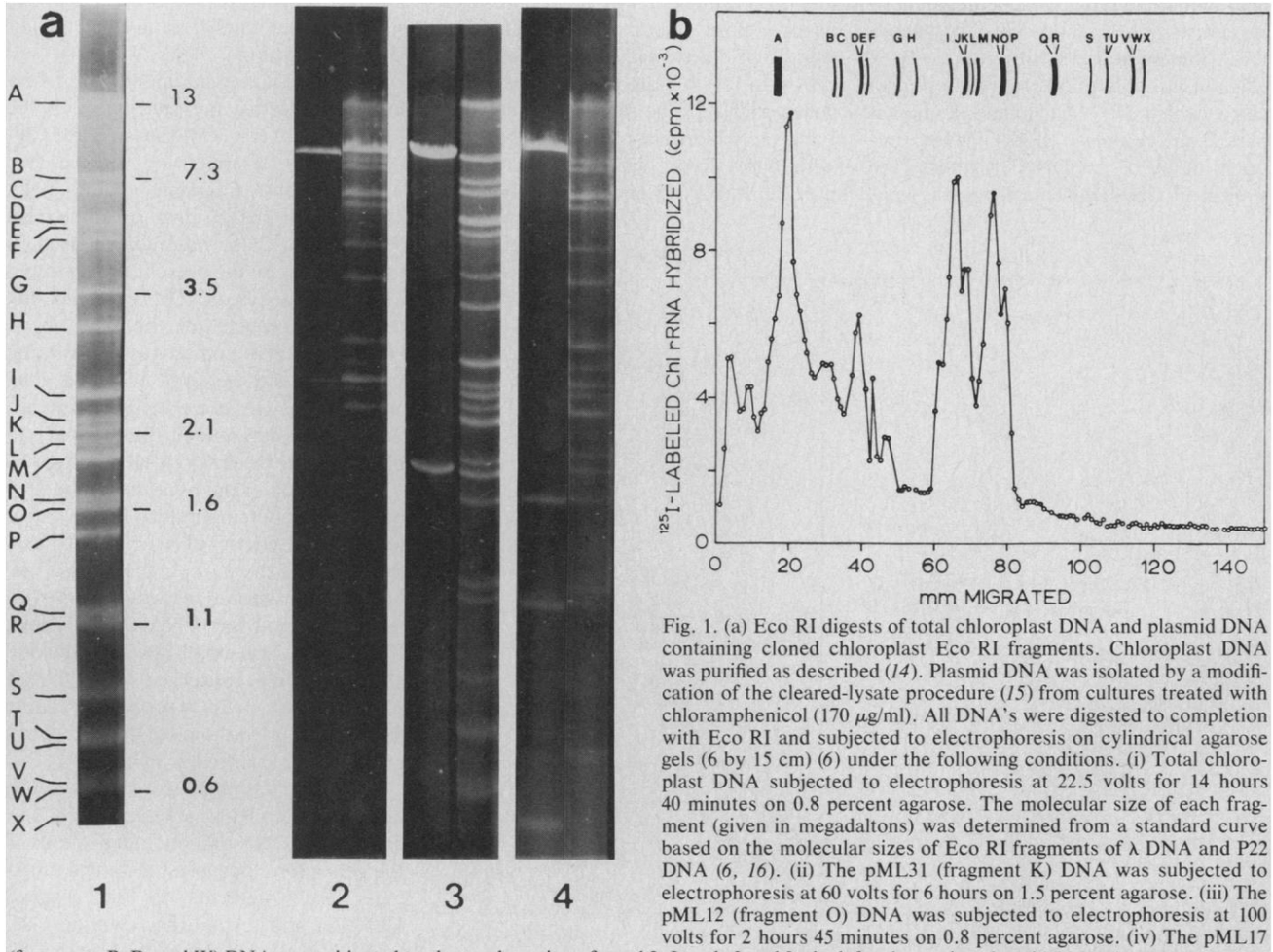

Fig. 1. (a) Eco RI digests of total chloroplast DNA and plasmid DNA containing cloned chloroplast Eco RI fragments. Chloroplast DNA was purified as described (*14*). Plasmid DNA was isolated by a modification of the cleared-lysate procedure (*15*) from cultures treated with chloramphenicol (170 μg/ml). All DNA's were digested to completion with Eco RI and subjected to electrophoresis on cylindrical agarose gels (6 by 15 cm) (*6*) under the following conditions. (i) Total chloroplast DNA subjected to electrophoresis at 22.5 volts for 14 hours 40 minutes on 0.8 percent agarose. The molecular size of each fragment (given in megadaltons) was determined from a standard curve based on the molecular sizes of Eco RI fragments of λ DNA and P22 DNA (*6*, *16*). (ii) The pML31 (fragment K) DNA was subjected to electrophoresis at 60 volts for 6 hours on 1.5 percent agarose. (iii) The pML12 (fragment O) DNA was subjected to electrophoresis at 100 volts for 2 hours 45 minutes on 0.8 percent agarose. (iv) The pML17 (fragments P, R, and W) DNA was subjected to electrophoresis as for gel 2. In gels 2 and 3, the left gel contains plasmid DNA alone and the right gel contains plasmid plus chloroplast DNA. In gel 4, the left gel contains only plasmid DNA and the right gel, only chloroplast DNA. (b) Hybridization of chloroplast ^{125}I-labeled rRNA with Eco RI fragments of chloroplast DNA. Eco RI–digested chloroplast DNA (5 μg) was subjected to electrophoresis on an 0.8 percent agarose slab gel (*6*). DNA fragments from the agarose gels were transferred to a Millipore membrane filter strip (0.45 μm HAWP) by the procedure of Southern (*8*). The Millipore strip containing the DNA fragments was hybridized in 3 ml of solution containing 10 μg of ^{125}I-labeled Chl-rRNA in 2 × SSC plus 0.1 percent sodium dodecyl sulfate (SSC is 0.15*M* sodium chloride and 0.015*M* sodium citrate, *p*H 7.0). After hybridization and ribonuclease treatment, strips were cut into 1-mm pieces and counted in a scintillation counter. Abbreviation: *cpm*, counts per minute.

fragment. Densitometry measurements confirmed this (not shown). In addition, band A probably contains two fragments. Stutz *et al.* were able to resolve two bands of DNA in the molecular weight range of 13×10^6 to 15×10^6 (*3*). On this basis, the sum of the molecular weights of the identified Eco RI fragments accounts for 97 percent of the molecular weight of the intact Chl-DNA.

The DNA fragments were transferred from a slab gel to a membrane filter strip by the procedure of Southern (*8*) and hybridized with ^{125}I-labeled Chl-rRNA. The strips were cut into 1-mm sections, and the radioactivity associated with each section was determined. Ribosomal RNA hybridizes with the DNA of bands A; with D, E, or F; and with several bands in the set J through P (Fig. 1b). By autoradiography of the hybridized filter strips, the radioactive hybrids can be located more precisely in specific DNA bands (*4, 8*). Using this procedure, Stutz *et al.* concluded that bands equivalent to A, D or E, K or L, and O or P contained DNA that hybridizes with rRNA (*4*). It was not possible to determine more precisely which DNA fragments contained partial or complete rRNA genes, because many of the DNA fragments are similar in size (and hence poorly separated on gels). Therefore, we tested DNA from plasmids containing unique fragments of Chl-DNA. In order to demonstrate the utility and sensitivity of the method, we concentrated on the DNA fragments in bands J through P.

Eco RI–generated Chl-DNA fragments were randomly inserted into the single Eco RI site of plasmid RSF2124 (*9*), a derivative of Col E1, which contains a gene conferring resistance to ampicillin. The *E. coli* K12 strain M94 (*thr leu thi hsd*) was transformed with the ligated DNA preparation (*10*). Ampicillin-resistant transformants that contained added DNA were identified by their failure to produce colicin E1 (*11*). The specific fragment (or fragments) of Chl-DNA present in a particular plasmid was determined by electrophoresis of the Eco RI–digested plasmid DNA (for example, see Fig. 1a).

Purified DNA from recombinant plasmids containing Eco RI–generated fragments J through P was immobilized on nitrocellulose filters. The bound DNA was hybridized with ^{125}I-labeled Chl-rRNA to test for the presence of rRNA genes (rDNA). The results are shown in Table 1. Chloroplast rRNA hybridized with DNA from plasmids pML19 and pML31, which contain band K DNA, but did not hybridize with DNA from plasmids pML10, pML21, or pML23, which contain Eco RI fragments J, L, or M(M_1 or M_2), respectively. The rRNA also hybridized with DNA from every plasmid that contains fragment O. Plasmid pML17 DNA contains three fragments of Chl-DNA: P, R, and W. The rRNA bound to pML17 DNA, but not to pML35 DNA which contains fragment P alone. Therefore, rDNA must be present in fragment R or W(W_1 or W_2) DNA. Neither of these fragments appeared to hybridize when Eco RI–digested whole Chl-DNA was hybridized with Chl-rRNA (Fig. 1b). This may be due to the decreased efficiency of hybridization of DNA fragments of molecular weight less than 10^6 (*12*), which are not retained well by the nitrocellulose filter. The hybridization observed with the cloned pML17 DNA was not an artifact resulting from the use of smaller molecular weight DNA segments, because plasmids pML26, pML28, pML20, and pML34 also containing smaller molecular weight DNA did not bind rRNA (Table 1).

Two types of experiments were performed to verify the specificity of the hybrids between Chl-rRNA and Chl-DNA. First, competition experiments with each DNA fragment binding rRNA showed that the binding of label from ^{125}I-labeled rRNA was reduced from 50 to 86 percent by the presence of a tenfold excess of unlabeled rRNA, indicating that both compete for the same DNA binding site. (In some cases, saturating levels of rRNA were not achieved, thus competition was not always complete.) Second, we determined the thermal stability of the three DNA-rRNA hybrids (Fig. 2). Each of the hybrids melted (T_m) over a narrow temperature range, a feature characteristic of true RNA-DNA hybrids, and the T_m's, in 0.1 × SSC (saline, sodium citrate), are consistent with those expected for rRNA-DNA hybrids (*3, 13, 14*). The small but reproducible differences in T_m observed with different plasmid DNA hybrids probably reflects differences in the nucleotide sequences of rDNA in a particular hybrid.

In these experiments, we have identified three Eco RI fragments of *Euglena* chloroplast DNA that contain portions of the genes for chloroplast rRNA, by linking these fragments to the plasmid RSF2124 and testing the resulting plasmid DNA's for hybridization with Chl-rRNA. Chloroplast rRNA contains two major molecular species of 1.1×10^6 and 0.55×10^6 daltons (*3*). In our experiments, total rRNA containing both species was used, and, consequently, hybridization to a given fragment may re-

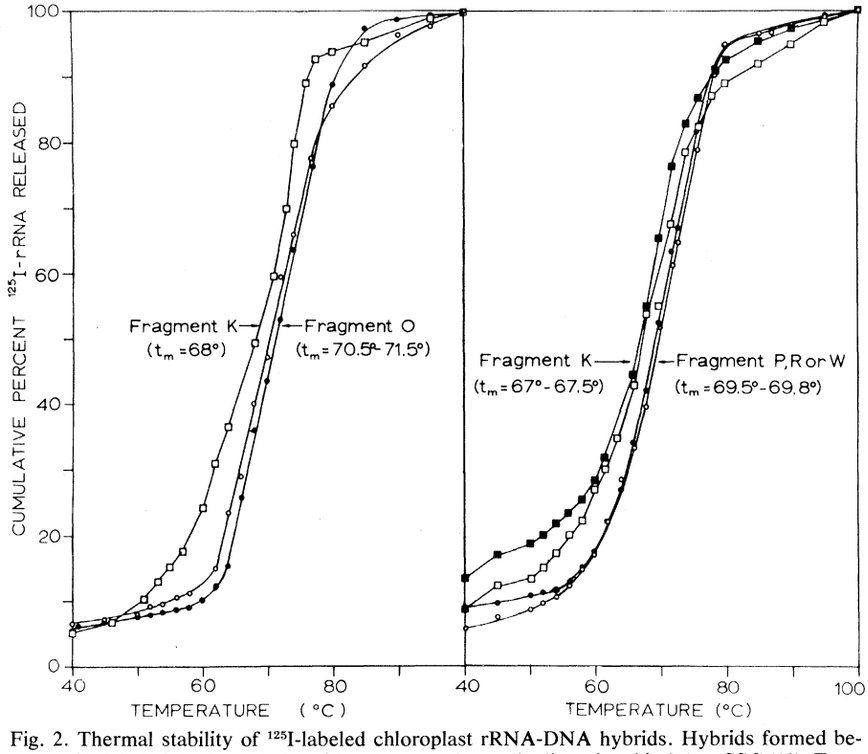

Fig. 2. Thermal stability of ^{125}I-labeled chloroplast rRNA-DNA hybrids. Hybrids formed between ^{125}I-labeled rRNA and plasmid DNA were thermally dissociated in 0.1 × SSC (*13*). Fragment K came from pML19; fragment O, from pML12; and fragments P, R, and W, from pML17. The calculated T_m (in 0.1 × SSC) for a DNA-DNA hybrid with a G + C content of 52 percent is 75.2°C (*17*). The observed T_m's for the rRNA-DNA hybrid for fragment O are 4° to 5°C lower, in good agreement with the previously reported decreased stability of the corresponding RNA-DNA hybrids (*18*).

flect the presence of cistrons for either the 23*S* or the 16*S* rRNA, or both. We also found in hybridization studies with an Eco RI digest of total Chl-DNA that at least two other fragments (A and D or E) of Chl-DNA contain rRNA sequences. These fragments can now be used to construct a physical map of the chloroplast genome and to locate precisely the position and orientation of the rRNA cistrons. Furthermore, other genes, such as those for chloroplast transfer RNA cistrons, can be identified by similar hybridization experiments.

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Cloning of Yeast Transfer RNA Genes in *Escherichia coli*

Abstract. *Four thousand* Escherichia coli *clones containing yeast DNA inserted into the plasmid pBR313 have been isolated. Of these, 175 clones were identified as carrying yeast transfer RNA genes. The initial analysis of the inserted transfer RNA genes via the colony hybridization technique with individual radioactive transfer RNA species is reported. The data indicate that yeast transfer RNA genes are not highly clustered, although some clustering exists. In addition, it was observed that the reiteration number of different transfer RNA genes may vary extensively.*

In order to study the principles of gene organization and expression in yeast we have undertaken a project to isolate and characterize yeast transfer RNA (tRNA) genes. There are several reasons for choosing yeast tRNA genes as a system for studying gene organization and expression. The gene products have been well characterized; most yeast tRNA's have been purified, and many sequenced (*1*). As in *Escherichia coli*, nonsense suppressors have been isolated (*2*), and it has been demonstrated that these suppressor genes are the structural genes of tRNA (*3*, *4*). Thus, there is extensive information available on the genes and gene products for this class of molecules. Recombinant DNA technology now supplies us with a method for isolating the tRNA genes in pure form. We report here our initial efforts to collect and catalog the yeast tRNA genes.

The first step was to obtain an adequate number of *E. coli* clones carrying random fragments of yeast DNA (the "shotgun experiment"). These clones were then screened by hybridization to identify those containing yeast tRNA genes. This

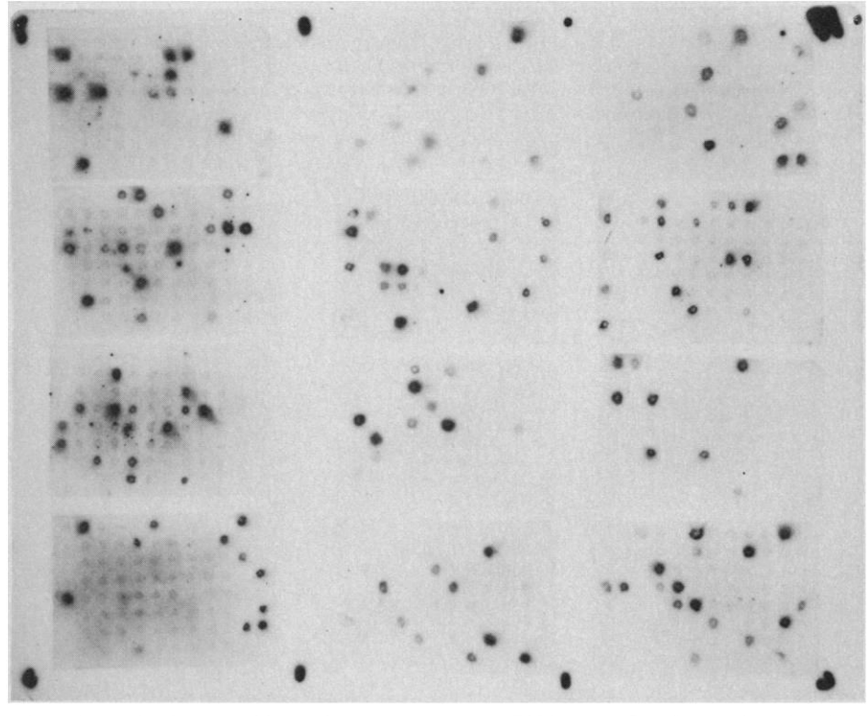

Fig. 1. Screening for recombinant clones carrying yeast tRNA genes. One thousand one hundred and forty clones carrying endo R Hind III restricted fragments of the yeast genome inserted into pBR313 were transferred to 12 filters (95 clones per filter) and assayed for yeast tRNA genes by hybridization with in vivo ^{32}P-labeled 4*S* RNA. Labeling and extraction of stable RNA's were performed according to Rubin (*12*). The RNA was subsequently separated on a 10 percent polyacrylamide gel in 4*M* urea. The positions of 4*S*, 5*S*, and 5.8*S* RNA were revealed by autoradiography. Appropriate bands were cut out, and the RNA's were eluted in 0.3*M* NaCl and purified by DEAE-cellulose column chromatography. We have modified the colony hybridization procedure developed by Grunstein and Hogness (*11*). The candidate clones were replica-plated in an 8 by 12 cm matrix onto 15-cm (diameter) petri plates (95 clones per dish). After an overnight growth period, a sheet of Whatman 540 filter paper was pressed onto the plate and removed (most of the bacteria adhere to the filter). The filter was then treated with 0.5*M* NaOH, neutralized, dried, and used in the hybridization test. We have routinely used 10^5 to 10^6 count/min of purified labeled RNA per filter. However, when total 4*S* RNA was used the hybridization solution contained 10^6 to 10^7 count/min per filter. Hybridization was for 12 to 18 hours at 37°C in a solution of 50 percent formamide, 0.6*M* NaCl, 0.06*M* sodium citrate, *p*H 7.0, and 0.5 percent sodium dodecyl sulfate. The filters were then washed, treated with ribonuclease (optional), and autoradiographed.