JOURNAL OF BACTERIOLOGY, Dec. 1977, p. 931-949

Vol. 132, No. 3
Printed in U.S.A.

Characterization of Hybrid Plasmids Carrying Individual Ribosomal Ribonucleic Acid Transcription Units of *Escherichia coli*†

MARIAN ELLWOOD KENERLEY, EDWARD A. MORGAN, LEONARD POST, LASSE LINDAHL,†† AND MASAYASU NOMURA*

Institute for Enzyme Research, Departments of Genetics and Biochemistry, University of Wisconsin, Madison, Wisconsin 53706

Received for publication 9 June 1977

We have screened the strains with ColE1 hybrid plasmids constructed by Clarke and Carbon (Cell **9**:91-99, 1976) for the presence of ribosomal ribonucleic acid (rRNA) genes on the plasmids and identified 16 strains whose plasmids carry rRNA genes. The structures of these 16 plasmids were compared by heteroduplex analysis, and the plasmids were classified into six groups on the basis of their chromosomal origins. Homology with known transducing-phage deoxyribonucleic acids and genetic mapping have assigned locations on the *Escherichia coli* chromosome to three of the six groups. These are *rrnB* near *rif* at 88 min, *rrnC* near *ilvE* at 83 min, and *rrnD* near *aroE* at 71 min. A fourth group is probably *rrnA* at 85 min (T. Ikemura and M. Nomura, Cell, **11**:779-793, 1977). We conclude that the minimum number of rRNA transcription units per haploid chromosomes is seven, that is, the six groups identified in this work plus a known operon (*rrnE* near *metA* at 89 min) that we failed to find among the hybrid plasmids. This heteroduplex analysis also suggests that there are only two kinds of rRNA operons with respect to their spacer region; three of the six rRNA operon groups studied here have one kind, whereas the remaining three have the other kind.

Ribosomal ribonucleic acid (rRNA) genes in *Escherichia coli* are organized in transcription units (called "rRNA operons" in this paper) containing 16S, 23S, and 55 RNA genes and a "spacer" transfer RNA (tRNA) gene(s) (13, 16, 35, 39). Deoxyribonucleic acid (DNA)-RNA hybridization data and analysis of minor oligonucleotides in enzyme digests of rRNA's indicate the presence of six to seven copies of the rRNA transcription unit per genome (for reviews, see 9, 41). Several of these transcription units have been mapped by using trasducing phages and an F′ episome, and all are located in single copies at discrete sites (12, 24, 25, 30, 40, 49; see also Fig. 7).

There are several unsolved questions related to the presence of multiple copies of rRNA operons in *E. coli*. For example, we do not know whether all the rRNA operons function in all growth conditions, whether all the rRNA operons are regulated in the same way, or whether and how rRNA's produced from one rRNA operon are chemically and functionally different from those produced from other rRNA operons. To study these and other problems, we have initiated an attempt to identify and isolate all the rRNA operons in *E. coli*.

Using the polydeoxyadenylate · polydeoxythymidylate "connector" method (34), Clarke and Carbon constructed a population of hybrid plasmids, each consisting of colicin E1 (ColE1) DNA and a fragment of *E. coli* chromosomal DNA produced by mechanical shearing (4, 5). From the average size of sheared fragments, they suggested that the hybrid plasmid collection, which comprises about 2,000 independently constructed plasmids, is likely to include plasmids representative of the entire *E. coli* genome. We screened these 2,000 ColE1 hybrid plasmids for the presence of rRNA genes ("ribosomal DNA" or "rDNA") and identified 16 plasmids that carry rDNA. In this paper, we describe characterization of these plasmids, using genetic and biochemical methods as well as heteroduplex analysis. Based on the results obtained, we have classified these plasmids into six groups with respect to their chromosomal locations.

MATERIALS AND METHODS

Bacterial and transducing-phage strains. *E. coli* K12-30, originally obtained from P. Fredericq,

† Paper no. 2136 from the Laboratory of Genetics, University of Wisconsin.

†† Present address: Department of Molecular Biology, University of Aarhus, DK-8000, Aarhus, Denmark.

was used to prepare ColE1 DNA, and *E. coli* strain NO79 (E1 colicinogenic derivative of *E. coli* K-12 strain W4573) was used to prepare ColE1.λcI857S7*dilv*5 (called λ*ilv*5; 8, 25) was obtained from P. Jørgensen and N. P. Fiil. λcI857S7*rif*^{d}18 (called λ*rif*^{d}18; 27) and φ80*rif*^{s} (28) and their preparation have been described (35, 48). λ*metA*20 was previously described (49). Phages were prepared by standard methods (36). The bank of 2,000 hybrid ColE1 plasmids was made by transformation of JA200 (F^{+} Δ*trpE5 recA thr leu thi*) with sheared *E. coli* DNA fragments connected to ColE1 DNA (4). The strains carrying these plasmids were obtained from L. Clarke and J. Carbon. Recipients used in genetic crosses are described in Tables 4 and 5.

Screening for plasmids with rRNA genes. To screen the bank plasmid strains for the presence of rRNA genes on the plasmids, 2-ml cultures were grown overnight in Luria broth (1% tryptone–0.5% yeast extract–0.5% NaCl) and five cultures were pooled. Cells were centrifuged and lysed by using a lysozyme-ethylenediaminetetraacetate-sodium dodecyl sulfate method (44). After centrifugation at 10,000 rpm for 30 min, the supernatant was taken and applied directly to 0.9% agarose gels, and electrophoresis was carried out for 15 h at 1 V/cm according to Shinnick et al. (45). Gels were then stained with ethidium bromide and photographed to record the locations of plasmid DNA bands. The gels with plasmid DNAs were mixed with ^{32}P-labeled 16S and 23S rRNA (ca. 3×10^{6} to 6×10^{6} cpm/gel), and hybridization was carried out in situ according to the procedure of Shinnick et al. (45), except that whole gels were hybridized to RNA in beakers instead of in dialysis bags. When a plasmid band visible in both the agarose gel and the autoradiogram was found, each of the five strains pooled to make the sample was grown separately, and the hybridization was repeated to determine the origin of the plasmid. Approximately 20 strains were found to bear plasmids that hybridized rRNA by this method. Plasmid DNA of these strains was then purified and hybridized to purified ^{3}H-labeled 16S or 23S rRNA on nitrocellulose filters (see Table 2), and the 16 plasmids that showed significant hybridization were selected for further study.

Preparation of plasmid DNA. Strains carrying hybrid plasmids were grown at 37°C in Luria broth and treated with chloramphenicol (150 μg/ml). After 8 to 12 h, cells were harvested and plasmid DNA was isolated by using a method adapted from that of Guerry et al. (17). Details are described in a previous paper (46). Two cycles of the CsCl-ethidium bromide equilibrium centrifugation were used for purification. After removal of the dye, the DNA solutions were dialyzed against 0.1× SSC (SSC = 0.015 M NaCl plus 0.0015 M sodium citrate, pH 7.9) and stored frozen at −80°C.

Radioactive 16S and 23S rRNA. ^{3}H-labeled 16S and 23S rRNA used for filter hybridization was prepared according to Morgan and Kaplan (37). The ^{32}P-labeled 16S and 23S rRNA used for in situ hybridization was prepared from *E. coli* K-12 strain NO1310 (a uracil-requiring mutant of PR13; PR13 is an *argG*$^{+}$ *pnp* derivative of strain LRN2 [cf. ref. 42] and was obtained from W. Gilbert) in a manner similar to that described for 5S RNA (35). RNA was extracted from 70S ribosomes by a phenol method and subjected to centrifugation on a sucrose gradient. Fractions containing 16S and 23S rRNA were pooled, and RNA was recovered by alcohol precipitation.

Electron microscopic analysis of heteroduplexes. Solutions containing purified plasmid DNA (20 μg, usually in 0.1 to 0.5 ml of 0.1× SSC) were lyophilized to dryness and dissolved in 100 μl of buffer containing 36 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 8.0), 33 mM NaCl, 3 mM ethylenediaminetetraacetate, and 13 mM K_2HPO_4. A 50-μl amount of ribonuclease A [deoxyribonuclease-free, 3 mg/ml in 9 mM tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.5; preheated] was added, and the solution was incubated at 37°C for 30 min to nick supercoiled circles (2). Predigested Pronase [50 μl of 5 mg/ml in 50 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 8.0)–50 mM NaCl–5 mM ethylenediaminetetraacetate] was added, and the mixture was incubated for an additional 10 min at 37°C. This method was adapted from that described by Blair et al. (2). DNA heteroduplexes were prepared for electron microscopy by the method of Davis et al. (10). φX174 DNA was used as a length standard for single-stranded regions (5.25 kilobases [kb]; 44), and PM2 DNA was used as a length standard for double-stranded regions (9.5 kb; 15, 44). Measurements in each case were based on at least 15 independent heteroduplex molecules and, in most of the cases, had a standard deviation of 2 to 10% of the length of the interval measured.

Other methods. ColE1 was prepared according to Schwartz and Helinski (43) through the ammonium sulfate precipitation step. In vitro protein synthesis and detection of radioactive ribosomal proteins by reconstitution followed by immunodiffusion were done according to Lindahl et al. (33). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis analysis of radioactive proteins was done using the system of Laemmli (29), as described by Lindahl et al. (31).

RESULTS

Identification of hybrid plasmids carrying rRNA genes. We examined the strains constructed by Clarke and Carbon (4, 5) for the presence of rRNA genes on the plasmids. For initial screening, we hybridized radioactive rRNA to plasmid DNA in situ after gel electrophoresis of the DNA, as described in Materials and Methods. Plasmid DNA was then isolated from the strains, which were positively scored, and radioactive rRNA was hybridized to purified plasmid DNA on nitrocellulose filters. We found that 16 strains have plasmids that contain DNA complementary to rRNA and, therefore, at least part of an rRNA operon. Plasmids isolated from these 16 strains were further analyzed with a variety of techniques. The results are summarized in Table 1.

DNA-RNA hybridization with 16S and 23S rRNA. To determine whether the isolated plasmid DNAs carry the 16S rRNA gene, the 23S rRNA gene, or both, ^{3}H-labeled purified

TABLE 1. *Hybrid plasmids that carry rRNA genes*

Group	Plasmid designation (pLC-)	DNA-RNA hybridization[a]		Heteroduplex analysis[b]			Length of plasmid (kb)			Heteroduplex homology to DNA from:	Genetic mapping (markers detected)	Probable identification
		16S	23S	16S	Spacer	23S	Total	"Non-ColE1"	rDNA			
I	7-21	+	+	+	+	+	15.7	9.3	5.2			(unknown)
	43-34	−	+	(0.1)	+	+	15.9	9.5	3.6			
	28-21	−	+	(0.3)	+	+	23.6	17.2	3.7			
II	22-11	+	+	+	+	+	20.0	13.6	5.2		*aroE*	*rrnD* (71 min)
	12-24	+	+	+	+	+	21.0	14.6	5.2		*aroE*	
	16-6	+	+	+	+	+	20.1	13.7	5.2		*aroE*	
	16-1[c]	+	+	+	+	+	20.1	13.7	5.2		*aroE*	
III	19-3	+	+	+	+	+	16.7	10.3	5.2			(*rrnA*)[d] (85 min)
	5-31	−	+	−	(?)	+	14.6	8.2	3.2			
	16-11[c]	−	+	−	(?)	+	14.6	8.2	3.2			
IV	34-34	+	+	+	+	+	19.2	12.8	5.0	λ*rif*[d]18		*rrnB* (88 min)
V	23-30	+	+	+	+	+	22.7	16.3	5.2			(unknown)
VI	21-9	−	+	−	(+)	+	13.1	6.7	3.5	λ*ilv*5		*rrnC* (83 min)
	22-36	+	+	+	+	+	27.2	20.8	5.2	λ*ilv*5	*ilvE*	
Unclassified[e]	27-40	−	+	−	(?)	+	9.6	3.2	2.9			
	24-26	+	−	(?)	(?)	(?)	7.3	ca. 1.0	0.9			

[a] The presence of rDNA regions is indicated by +; − indicates no evidence for the presence obtained.

[b] The symbols +, −, and (?) mean present, absent, and no conclusion obtained, respectively. pLC21-9 probably has a spacer region (see legend to Fig. 4). Presence of small portions of 16S rRNA gene on pLC43-34 and pLC28-21 was demonstrated by heteroduplex analyses. Their sizes in kilobases are shown in parentheses.

[c] pLC16-1 and pLC16-11 are very similar or identical to pLC16-6 and pLC5-31, respectively (see text).

[d] Tentative identification (see text).

[e] pLC27-40 has only a 23S rRNA gene (2.9 kb) and a small amount of adjacent non-rDNA (0.3 kb) in addition to ColE1 DNA. It was difficult to examine the possible homology of its nonribosomal bacterial DNA with DNA in other hybrid plasmids. For the same reason, pLC24-26 could not be classified by heteroduplex analyses.

16S or 23S rRNA was hybridized to each of the plasmids. The results of one such experiment are shown in Table 2. Several plasmid DNAs (pLC43-34, pLC28-21, pLC5-31, pLC16-11, pLC21-9, and pLC27-40) have very little ability to hybridize 16S rRNA but have the ability to hybridize 23S rRNA. These plasmids appear to carry the 23S rRNA gene but none or only a small portion of the 16S rRNA gene. In contrast, one plasmid (pLC24-26) showed an ability to hybridize 16S rRNA but no significant hybridization to 23S rRNA. Other plasmid DNAs hybridized to both 16S and 23S rRNA's, indicating that they carry both rRNA genes and thus at least a major portion of a complete rRNA operon.

Analysis of heteroduplexes between plasmid DNAs and rDNA-bearing transducing-phage DNAs. Electron microscope analysis of heteroduplexes formed between plasmid DNAs and reference transducing-phage DNAs, which carry rDNA and whose structures are known, was used to determine the extent of rDNA carried by the plasmids, the "spacer" identity of the plasmids, and the presence of possible homology outside rRNA operons between plasmid DNAs and phage DNAs. The transducing-phage DNAs we have used are λ*rif*[d]18 (27), λ*ilv*5 (25), and λ*metA*20 (49). λ*rif*[d]18 carries an rRNA operon near *rif* (*rrnB* at 88

TABLE 2. *Hybridization of radioactive 16S and 23S rRNA to hybrid plasmid DNA*[a]

Plasmid DNA	rRNA hybridized (cpm)		
	16S	23S	16S/23S
pLC 7-21	8,577	15,000	0.57
pLC43-34	166	17,313	0.01
pLC28-21	571	7,634	0.07
pLC22-11	10,780	20,640	0.52
pLC12-24	11,845	18,591	0.62
pLC16-6	5,701	11,190	0.51
pLC16-1	3,835	6,497	0.59
pLC19-3	3,312	5,775	0.57
pLC5-31	469	14,900	0.03
pLC16-11	253	7,284	0.03
pLC34-34	5,570	9,834	0.57
pLC23-30	5,351	8,167	0.65
pLC21-9	0	22,377	0.00
pLC22-36	2,316	3,858	0.60
pLC24-26	21,905	0	∞
pLC27-40	0	11,391	0.00

[a] Plasmid DNA was prepared as described in Materials and Methods and affixed to nitrocellulose membrane filters, and hybridization assays were performed under the conditions of DNA excess as described before (11). Each filter had 3.5 μg of DNA. Two hybrid plasmid DNAs that are known not to have rDNA and ColE1 DNA were used as controls. Averages of values obtained with these three DNAs (630 cpm for 16S and 1,020 cpm for 23S rRNA) were used to correct experimental values. The corrected values considered to be significant are underlined.

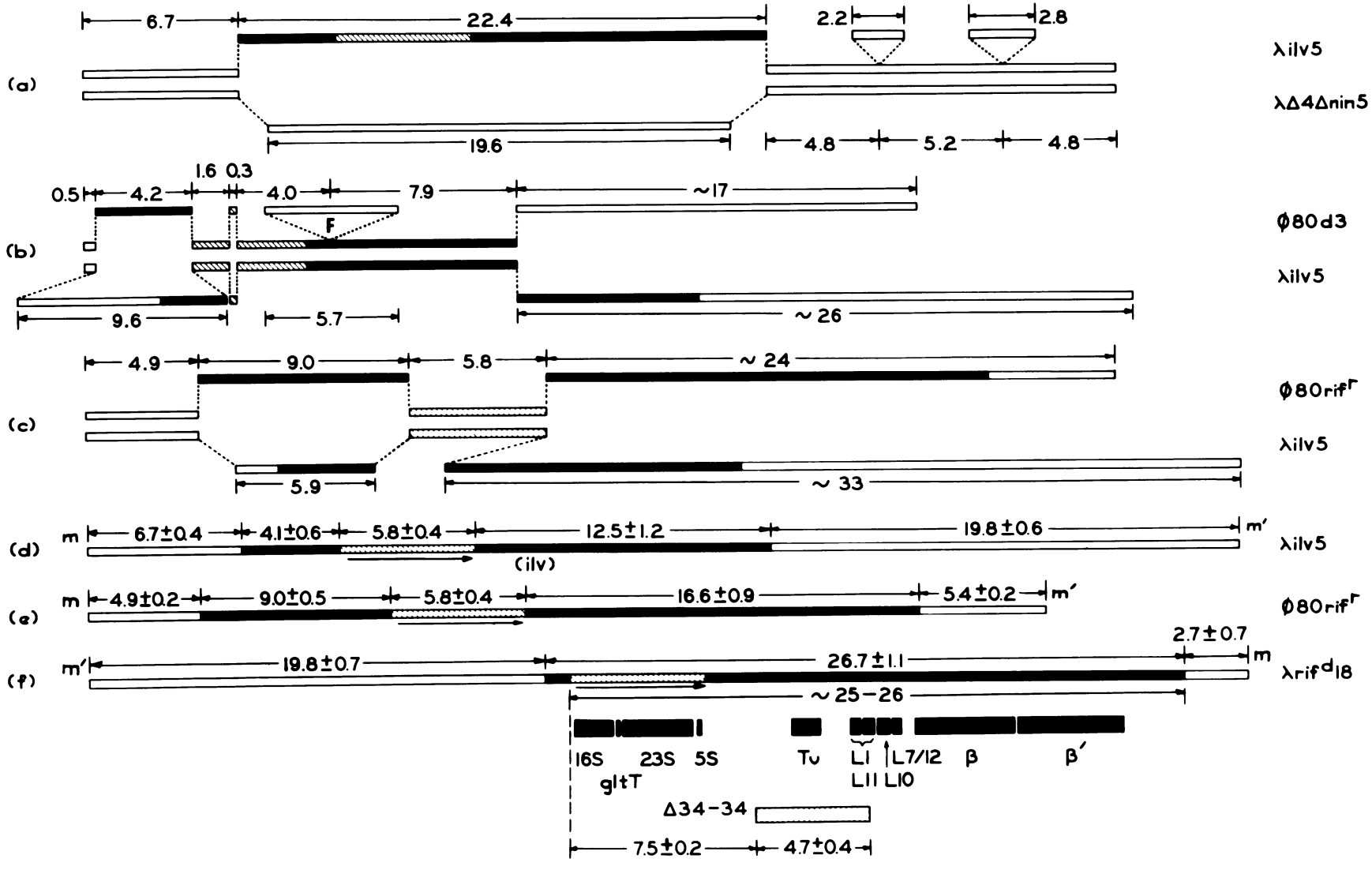
min; 30). λ*ilv*5 carries an rRNA operon and *ilv* genes and has been studied by *Eco*RI restriction endonuclease (8). Locations of rRNA operons as well as other bacterial DNA on the λ*ilv*5 DNA molecule and the λ*rif*^{d}18 molecule, as determined by heteroduplex analysis, are shown in Fig. 1d and f, respectively. The structure of λ*ilv*5 (Fig. 1d) was derived from the results of heteroduplex analysis shown in Fig. 1a, b, and c.

Davidson and co-workers first discovered that the two different rRNA operons (called *rrnA* and "*rrnB*"; cf. Fig. 8, legend) on the F'14 plasmid have a nonhomology region ("spacer region") between the 16S rRNA and the 23S rRNA genes (12). Subsequent experiments demonstrated the presence of tRNA genes in spacer regions; the rRNA operon carried by λ*rif*^{d}18 transducing phage has a gene for $tRNA_2^{Glu}$ in the spacer region, whereas φ80*rif*^{r} transducing phage carries a $tRNA_1^{Ile}$ gene in the spacer region (35). It was found that a considerable fraction of heteroduplex molecules formed between λ*rif*^{d}18 and φ80*rif*^{r} shows a nonhomology bubble ("spacer bubble") at a place corresponding to the spacer region (35). Similarly, heteroduplexes formed between λ*ilv*5 and λ*rif*^{d}18 showed a spacer bubble (data not shown), whereas those between λ*ilv*5 and φ80*rif*^{r} failed to show any such bubble (cf. Fig. 1c). Like φ80*rif*^{r}, λ*ilv*5 carries a gene for $tRNA_1^{Ile}$ but not for $tRNA_2^{Glu}$ (36a). The presence of a spacer bubble in a heteroduplex indicates that the two rRNA operons have different spacer regions and helps to orient 16S and 23S genes on plasmid DNA molecules. Failure to detect a spacer bubble in heteroduplex analysis stongly suggests that the two rRNA operons being tested have the same spacer tRNA genes.

Heteroduplex molecules formed between each of the 16 isolated plasmid DNAs and λ*rif*^{d}18 or λ*ilv*5 DNA were examined. As expected, we found DNA homology between the transducing phage DNA and plasmid DNA due to the presence of rRNA genes on these molecules. Some of the structures of these heteroduplex molecules are shown in Fig. 2a and b and 3. Eight plasmid DNAs showed a spacer bubble when heteroduplexes were made with λ*rif*^{d}18 but not when heteroduplexes were made with λ*ilv*5. Therefore, these eight plasmids carry a spacer different from that carried by λ*rif*^{d}18. Examples are pLC7-21, pLC22-11, and pLC19-3 (Fig. 3a–c). Three plasmid DNAs (pLC34-34, pLC23-30, and pLC22-36) showed a spacer bubble when heteroduplexes were made with λ*ilv*5 but not when heteroduplexes were made with λ*rif*^{d}18 (Fig. 3d, e, and h). Thus, these three plasmids carry a spacer different from that carried by λ*ilv*5. Five remaining plasmids showed no spacer bubbles with either λ*rif*^{d}18 or λ*ilv*5; these plasmids were found to possess only one of the 16S or 23S rRNA genes intact, and detection of spacer regions by heteroduplex analysis would have been difficult, even if they were present (cf. Table 1).

Analysis of heteroduplexes between plasmid DNAs and transducing-phage DNAs gave information on the extent of the rRNA operons carried by plasmids that is roughly consistent with DNA-RNA hybridization data (Table 2). The double-stranded homology region (plus the length of the spacer bubble if present) was about 5.2 kb long, which is approximately equal to the length of a complete rRNA operon as reported by Davidson and co-workers (12). Therefore, these plasmids probably carry a single complete set of rRNA genes. In other cases, the length of the homology region was significantly smaller than 5.2 kb. For example, pLC21-9 showed only about 3.5 kb of homology in heteroduplexes with λ*rif*^{d}18 (Fig. 3g). Other heteroduplex analysis and DNA-RNA hybridization experiments showed that pLC21-9 has only the 23S rRNA

FIG. 1. *Schematic representation of the structure of heteroduplexes (a–c) and the structures of the chromosomes of λilv5 (d), φ80rif^{r} (e), and λrif^{d}18 (f). The regions corresponding to bacterial DNA are either filled (nonribosomal DNA) or hatched (rRNA operon). F factor DNA in the φ80d3 genome is shown as an open bar, indicated as F. (For the structure of φ80d3, see 40). Arrows under the rRNA operons show their orientations (from 16S to 23S rRNA genes). Distances are given in kilobases (1 kb is 1,000 base pairs). For some pertinent distances, the standard deviations are included. The structures of λrif^{d}18 and φ80rif^{r} are based on heteroduplex analyses published previously (48). The structure of λilv5 is based on the results of heteroduplex analyses given in (a), (b), and (c). It should be noted that the orientation of the λrif^{d}18 genome given in the figure is opposte that of λilv5 and φ80rif^{r} with respect to phage genes (m and m' designate the "left" and "right" cohesive ends of phage molecules). The approximate sizes and locations of bacterial genes carried by λrif^{d}18 are given below the λrif^{d}18 chromosome, as is the location of DNA deleted in pLC34-34 ("△ 34-34"). The location of the L1 and L11 genes was previously determined using several deletions of λrif^{d}18 (Yamamoto and Nomura, unpublished data; see also 32). The length of an rRNA operon estimated from the length of the homology region between φ80rif^{r} and λilv5 is 5.8 ± 0.4 kb. This length appears to be significantly different from that estimated from the length (5.2 ± 0.3 kb) of the homology region between different plasmid DNAs (cf. Fig. 4). The reason for the apparent discrepancy is not known. The value 5.8 ± 0.4 kb is used in the structure of the three phages shown in (d), (e), and (f).*


6.7
22.4
2.2
2.8
λilv5
(a)
λΔ4Δnin5
19.6
4.8
5.2
4.8
1.6 0.3
0.5
4.2
4.0
7.9
~17
F
ϕ80d3
(b)
λilv5
9.6
5.7
~26
4.9
9.0
5.8
~24
ϕ80rif^r
(c)
λilv5
5.9
~33
m
6.7±0.4
4.1±0.6
5.8±0.4
12.5±1.2
19.8±0.6
m′
(d)
λilv5
(ilv)
m
4.9±0.2
9.0±0.5
5.8±0.4
16.6±0.9
5.4±0.2
m′
(e)
ϕ80rif^r
2.7±0.7
m′
19.8±0.7
26.7±1.1
m
(f)
λrif^{d}18
~25-26
16S
23S
5S
Tυ
L1
L7/12
β
β′
gltT
L11
L10
Δ34-34
7.5±0.2
4.7±0.4

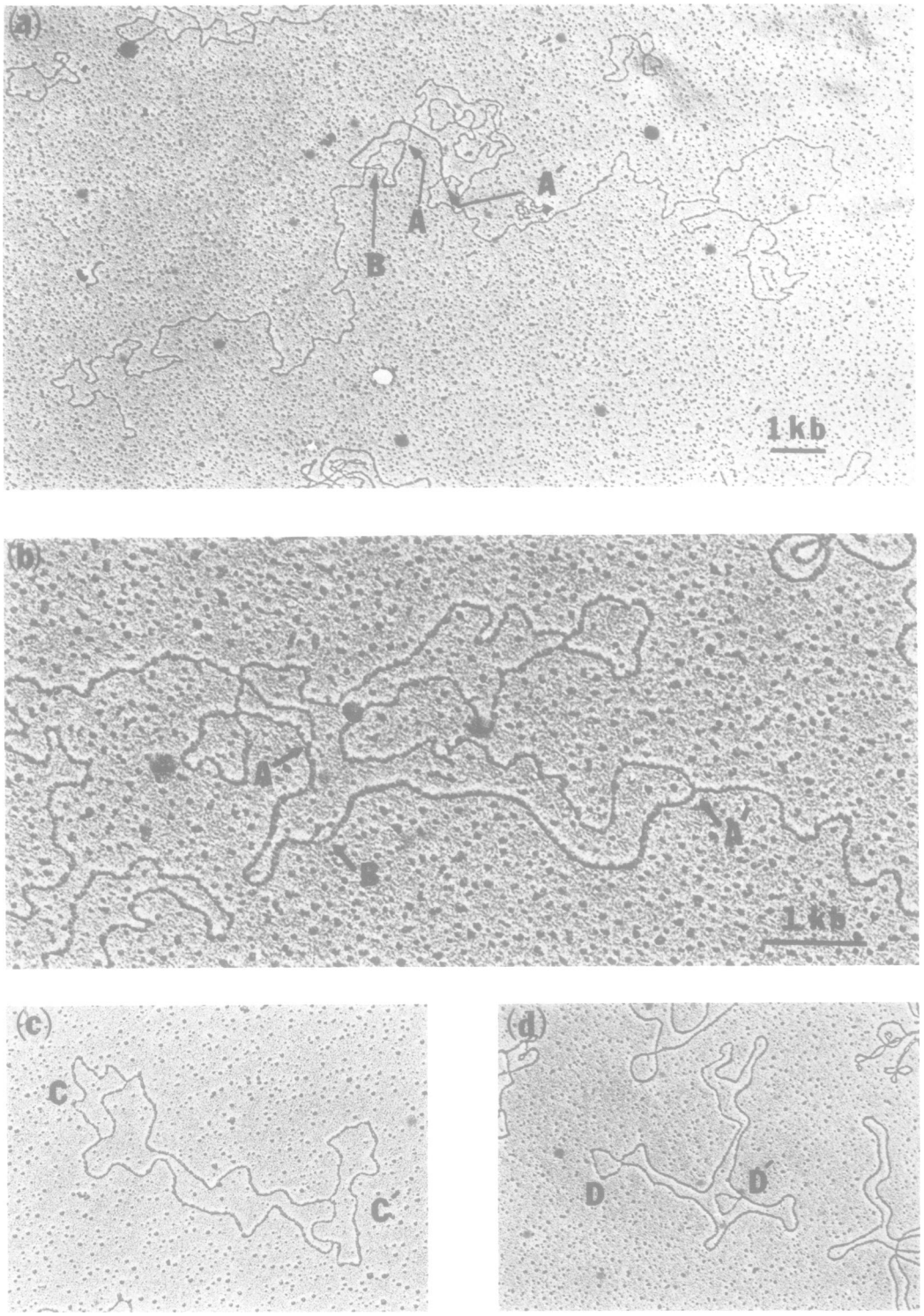


FIG. 2. *Electron micrographs of DNA heteroduplexes between pLC22-11 and λrif^{d}18 (a and b), pLC22-11 and pLC19-3 (c), and pLC22-11 and pLC16-6 (d). A and A′ are the left and right ends of the duplex region created by the homology of rRNA operons (see Fig. 3b for schematic representation). B indicates a nonhomology "spacer bubble" in the spacer region. C and C′ are nonhomology loops formed by bacterial DNAs surrounding the rRNA operon [see Fig. 4f for schematic representation of the heteroduplex shown in (c)]. D and D′ are deletion loops formed by bacterial DNAs, which are present only in one or the other of the two plasmids [see Fig. 5e for schematic representation of the heteroduplex shown in (d)]. (a), (c), and (d) are in the same magnification; (b) shows a heteroduplex similar to that given in (a) in a higher magnification.*

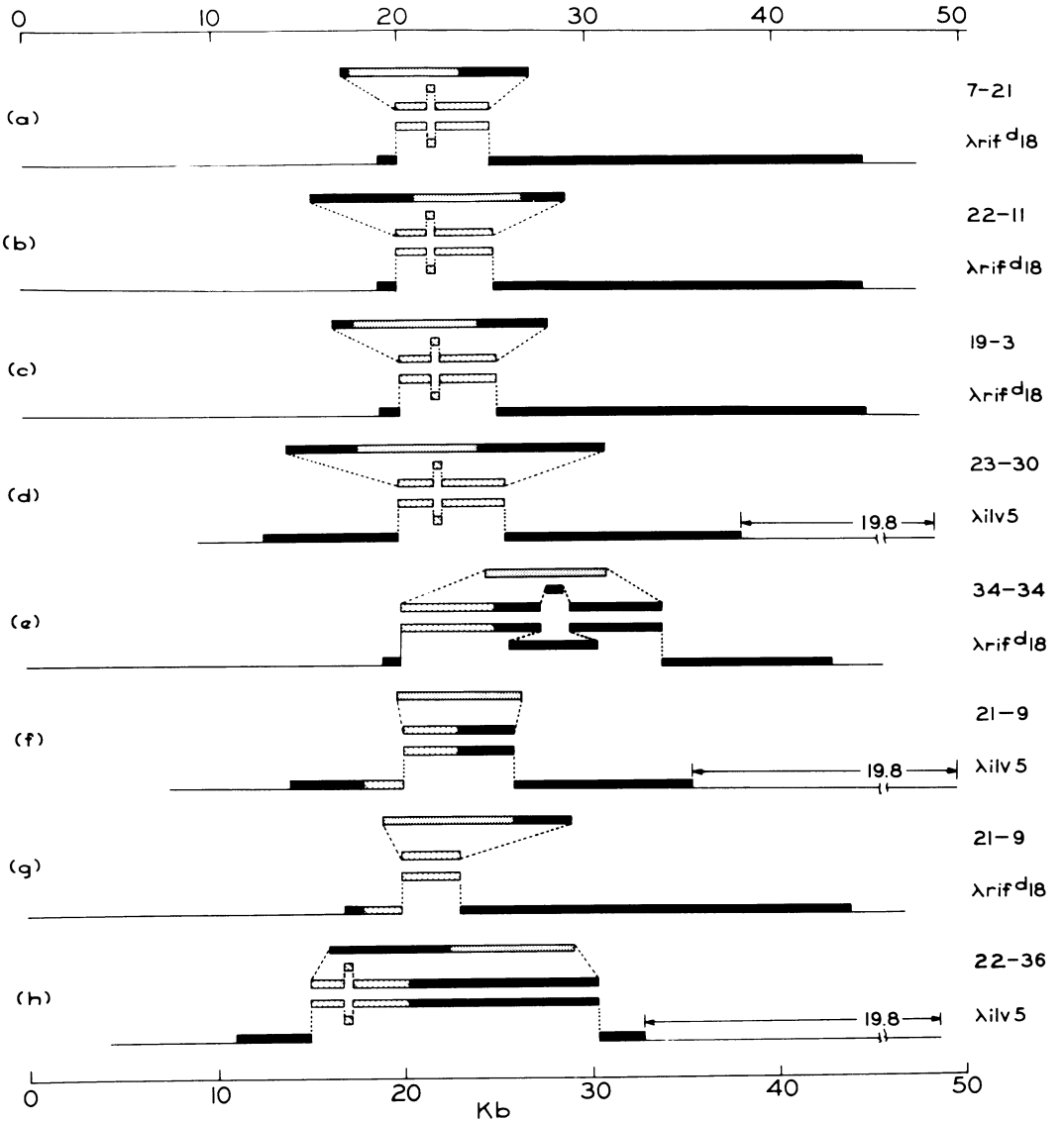
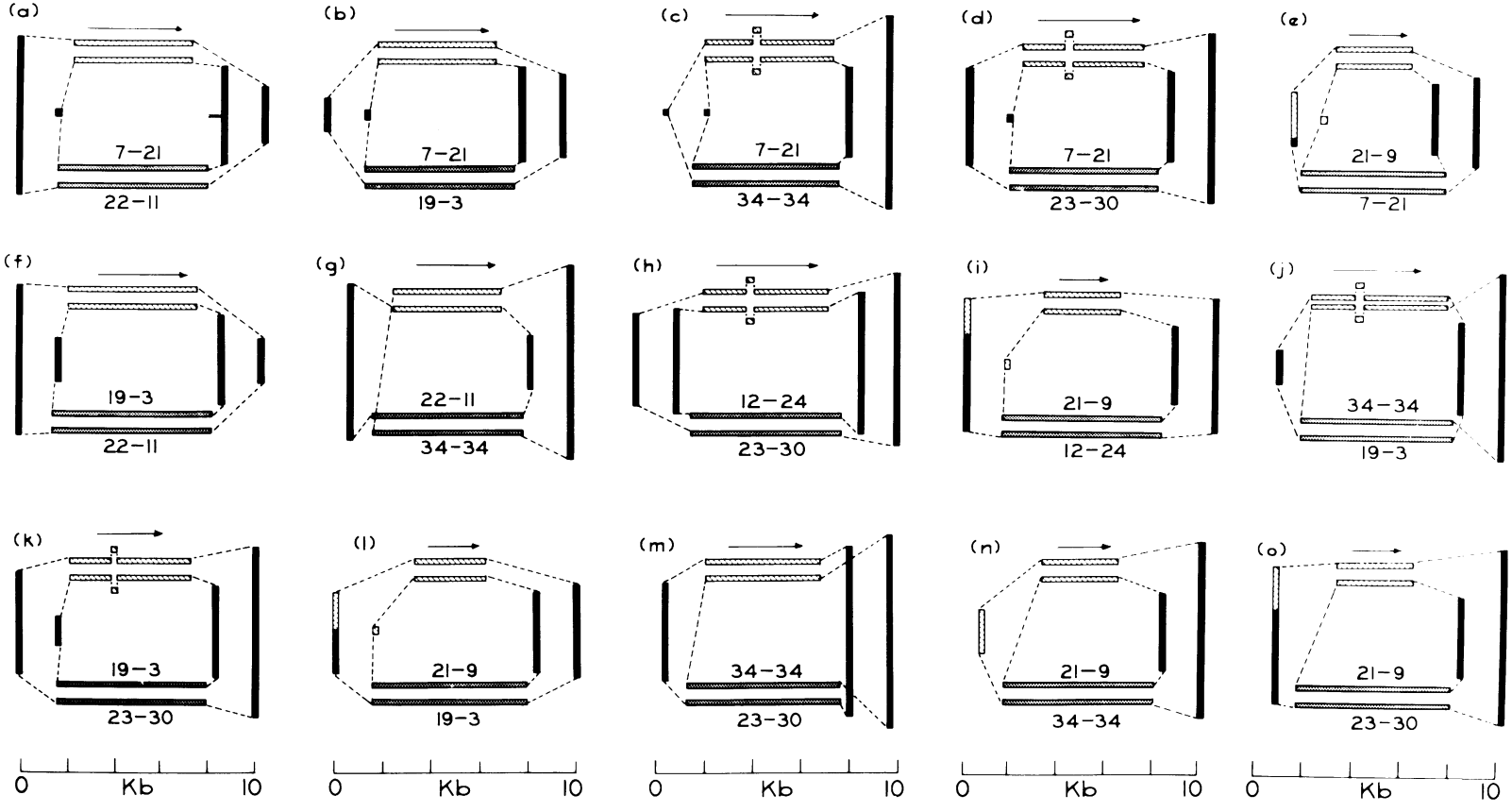


FIG. 3. *Schematic representation of the structure of heteroduplexes formed between reference transducing-phage DNAs (λrif^{d}18 or λilv5) and some representative plasmid DNAs. Thin solid lines represent λ phage DNA; filled bars represent nonribosomal bacterial DNA; hatched bars represent rDNA containing rRNA genes; and stippled bars represent ColE1 DNA. Locations of rDNA and ColE1 DNA have been inferred from information on the structures of phage DNAs and plasid DNAs obtained from various heteroduplex analyses (Fig. 1, 4, 5, this figure, and other data not shown).*

gene (and a spacer region) and little, if any, of the 16S rRNA gene (cf. Fig. 4, legend; 36a).

In three cases, homology outside rRNA operons was detected between plasmid DNA and transducing-phage DNA. The plasmid pLC34-34 showed two double-stranded regions, 7.5 and 4.5 kb long, in heteroduplexes with λ*rif*^{d}18 (Fig. 3e). Although this structure is complex, the observed homology indicates that the chromosomal DNA in this plasmid has originated from the *rif* region (88 min) on the *E. coli* chromosome, and therefore we conclude that the rRNA operon carried by pLC34-34 is probably derived from the *rrnB* locus. No other plasmid DNA showed homology to λ*rif*^{d}18 DNA in the region outside the rRNA operon.

Two plasmids, pLC21-9 and pLC22-36, showed homologies to λ*ilv*5 DNA outside the rRNA operon (Fig. 3f and h). pLC22-36 has bacterial DNA (about 9.4 kb in length), which is probably


(a)
7–21
22–11
(b)
7–21
19–3
(c)
7–21
34–34
(d)
7–21
23–30
(e)
21–9
7–21
(f)
19–3
22–11
(g)
22–11
34–34
(h)
12–24
23–30
(i)
21–9
12–24
(j)
34–34
19–3
(k)
19–3
23–30
(l)
21–9
19–3
(m)
34–34
23–30
(n)
21–9
34–34
(o)
21–9
23–30
0 Kb 10
0 Kb 10
0 Kb 10
0 Kb 10
0 Kb 10

homologous to the DNA at the *ilv* region (83 min on the *E. coli* genetic map). Analysis of heteroduplexes formed between pLC21-9 and λ*ilv*5 (Fig. 3f) and λ*rif*^{d}18 (Fig. 3g) indicates that pLC21-9 carries about 3 kb of bacterial DNA, which also probably comes from the *ilv* region. Technical problems have prevented analysis of heteroduplexes between these two plasmids, but we conclude that pLC22-36 and pLC21-9 are derived from the *ilv* region and contain the *rrnC* genes. No other plasmids showed homology to λ*ilv*5 DNA in the region outside the rRNA operon.

Heteroduplexes between λ*metA*20 DNA and the following plasmids were analyzed: pLC7-21, pLC12-24, pLC19-3, pLC23-30, and pLC21-9 (data not shown). In no case was DNA homology outside rRNA operons detected.

Analysis of heteroduplexes formed between two plasmid DNAs. Analysis of heteroduplexes between two plasmid DNAs can ascertain homology outside the rRNA operon and thus determine whether two rRNA operons arise from the same or different chromosomal locations. Lack of homology between the DNA sequences adjacent to rRNA operons would indicate that the plasmids were derived from different regions of the chromosome. Conversely, presence of homology between the two plasmid DNAs adjacent to the rRNA operons would show that they originated from the same region of the chromosome.

Heteroduplex analyses were done on plasmid DNAs in various combinations. Structures of some pertinent heteroduplex molecules are given in Fig. 4 and 5. For example, the heteroduplex formed between pLC22-11 and pLC19-3 (Fig. 2c and 4f) indicates that the rRNA operon carried by pLC22-11 has adjacent bacterial DNA segments (about 6 kb on one side and about 2 kb on the other side), which are different from the bacterial DNA segments (about 2 and 4 kb in length, respectively) surrounding the rRNA operon on pLC19-3 plasmid. (Assignment of single-stranded DNA regions to one of the two plasmids comes from comparison of structures of heteroduplex molecules involving pLC22-11 [Fig. 4a, f, and g] and those involving pLC19-3 [Fig. 4b, f, j and k].) We conclude that the chromosomal location of the rRNA operon on pLC22-11 is different from that of the rRNA operon on pLC19-3. In contrast, the structure of the heteroduplexes formed between pLC22-11 and pLC16-6 (Fig. 2d and 5e) reveals two double-stranded regions of DNA homology; one is 6.4 kb long, which is about the same length as ColE1 DNA (about 6.4 ± 0.4 kb), and the other is about 11 kb long, much longer than the homology expected from a complete rRNA operon (about 5.2 kb; see Fig. 1 and 4, legends). We conclude that the bacterial DNA adjacent to the rRNA operon on both pLC22-11 and pLC16-1 originates from the same chromosomal location and therefore classify both in the same group (group II, Table 1).

From the results of these analyses, we have classified the plasmids into six groups (Table 1). Two plasmids (pLC27-40 and pLC24-26) had non-ColE1 DNAs, which contain segments of an rRNA operon with little or no adjacent DNA, and therefore could not be classified.

The evidence supporting the classification given in Table 1 is summarized as follows (cf. Table 3). Heteroduplex structures shown in Fig. 5 classify pLC7-21, pLC43-34, and pLC28-21 in the same group (group I); pLC22-11, pLC12-24, pLC16-6, and pLC16-1 in another group (group II); and pLC19-3 and pLC5-31 in the third group (group III). Heteroduplex structures shown in Fig. 4 then define the presence of six different groups. Here, one representative plasmid was taken from each group (pLC7-21 for group I; pLC22-11 or pLC12-24 for group II; pLC19-3 for group III; pLC34-34 for group IV; pLC23-30 for group V; and pLC21-9 for group VI) and heteroduplexed with a member of each other group. As mentioned above, both pLC22-11 and pLC12-24 belong to group II, and heteroduplex struc-

FIG. 4. *Schematic representation of the structure of heteroduplexes formed between two plasmid DNAs representing two different groups. Concerning the three different kinds of bars, see legend to Fig. 3. The length of an rRNA operon defined by the homology surrounded by two substitution loops in the heteroduplex molecules in this figure is 5.2 ± 0.3 kb. It is not clear whether pLC34-34 has a complete rRNA operon including the presumptive promoter adjacent to the 16S rRNA gene. In heteroduplex molecules with three other plasmids (g, j, and m), the structures suggest that no or very little nonribosomal DNA exists outside 16S rRNA on pLC34-34. However, heteroduplex molecules made with pLC7-21 (c) suggest the presence of a very small amount of "nonribosomal" DNA outside the rDNA homology region. The plasmid pLC21-9 has a 23S rRNA gene and a spacer region but no or very little 16S rRNA gene (see text; 36a). The spacer region carried by pLC21-9 is probably very similar to those carried by pLC34-34 and pLC23-30, but not to those carried by pLC7-21, pLC12-24, and pLC19-3. This may explain the absence of substitution loops due to the spacer homology in the heteroduplexes with the former two plasmids (n and o), and their presence in those with the latter three plasmids (e, i, and l). Analysis of spacer tRNA genes carried by these plasmids confirmed this inference (36a).*

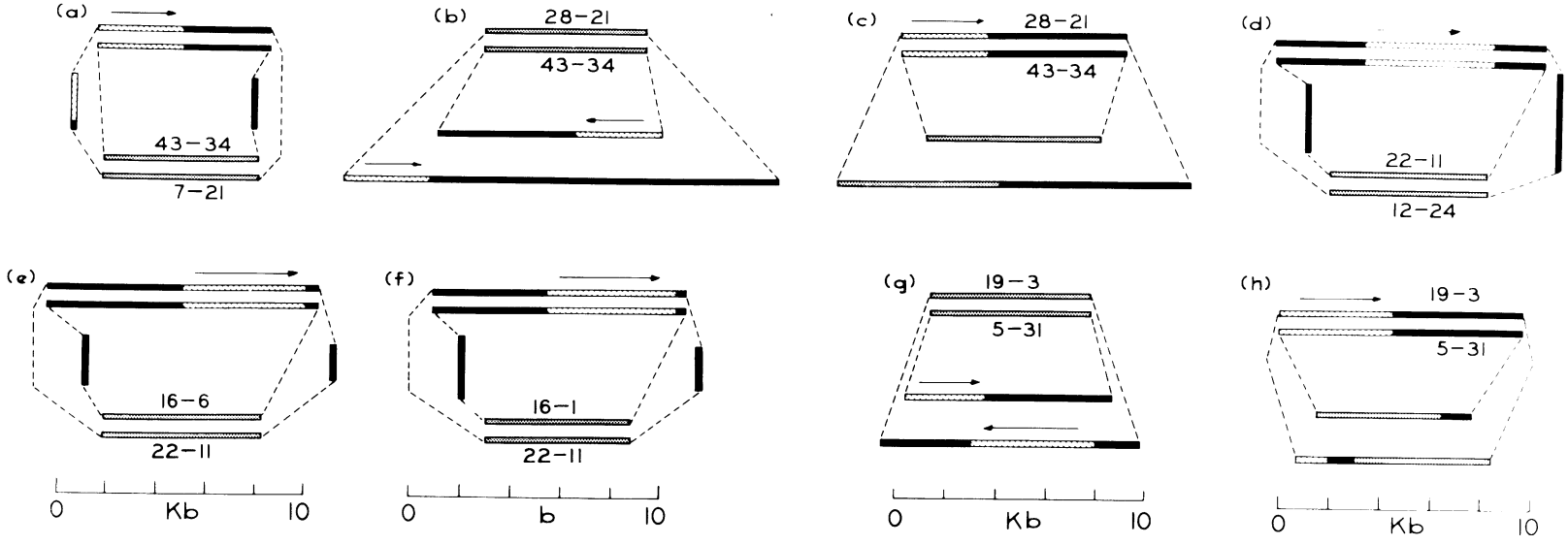


FIG. 5. *Schematic representation of the structure of heteroduplexes formed between two plasmid DNAs that are classified in the same group. Concerning the three different kinds of bars, see legend to Fig. 3. As described in the text, two different structures were observed in heteroduplexes formed between pLC28-21 and pLC43-34 [(b) and (c)], and those formed between pLC19-3 and pLC5-31 [(g) and (h)].*

TABLE 3. *Classification of rRNA operons on hybrid plasmids using outside DNA homology detected by heteroduplex analysis*[a]

Group	Plasmid designation (pLC-)	Presence of outside nonhomology in heteroduplexes with pLC:						
		7-21	22-11	12-24	19-3	34-34	23-30	21-9
I	7-21		+		+	+	+	+
	43-34	−						
	28-21	−						
II	22-11	+		−	+	+		
	12-24		−				+	+
	16-6		−					
	16-1		−					
III	19-3	+	+			+	+	+
	5-31				−			
	16-11				−		+	+
IV	34-34	+	+		+			
V	23-30	+		+	+	+		+
VI	21-9	+		+	+	+	+	
	22-36							

[a] +, Presence of nonhomology of DNA outside the rRNA operon ("outside DNA") detected; −, presence of homology of outside DNA detected. Results shown in Fig. 4 and 5 (and some other analyses) are summarized that are essential for the classification of hybrid plasmids into six groups. Heteroduplex analyses were done in many other combinations. All the results were consistent with the above classifications.

tures shown in Fig. 4 include both, to represent group II. From these results, we conclude that there are at least six rRNA operons with different chromosomal origins in the plasmid collection.

It should be noted that heteroduplexes formed between a member of group I, II, or III and one of group IV or V (or VI, see legend to Fig. 4) always showed a spacer bubble, whereas other combinations did not show any spacer bubble (Fig. 4 and 5). This observation will be discussed later in connection with spacer tRNA genes found in these plasmids.

The orientation of ColE1 DNA with respect to bacterial DNA on pLC28-21 is opposite to that on pLC43-34. This conclusion has been deduced from the presence of two different kinds of structures seen among the heteroduplexes formed between pLC28-21 and pLC43-34 (Fig. 5b and c), each of which has only one double-stranded region of homology. Similarly, the orientation of ColE1 DNA with respect to bacterial DNA on pLC5-31 is opposite to that on pLC19-3. All the plasmid DNAs except pLC28-21 and pLC5-31 (and pLC16-11, see below) had the orientation of ColE1, which is the same as that on pLC43-34 or pLC19-3. It is not clear why we did not observe about equal numbers of hybrid plasmids for each type of the two orientations.

We also note that the structures of pLC16-1 and pLC16-6 are very similar or identical (Fig. 5e and f). Heteroduplex analysis between pLC16-1 and pLC16-6 DNAs failed to detect any difference between the two DNA molecules. Similarly, the structure of pLC16-11 was found to be very similar or identical to the structure of pLC5-31.

Figure 6 summarizes the structures of the plasmids deduced from heteroduplex analyses described above.

In vitro protein synthesis directed by plasmid DNAs. We have previously shown that genes for four r-proteins (L1, L7/L12, L10, and L11) and EF-Tu (23, 30, 32, 47) are present very close to the rRNA operon at the *rif* region (cf. Fig. 1f). We explored the possibility that various plasmid DNAs carrying rRNA operons derived from other chromosomal locations might also have some r-protein genes. In addition, we compared protein products synthesized with various template DNAs to see whether or not plasmid DNAs classified in the same group (based on DNA homology surrounding rRNA operons) direct the synthesis of some common proteins.

Proteins were synthesized in vitro in the presence of [^{35}S]methionine, using each of 15 plasmid DNAs listed in Table 1 as templates. (Plasmid pLC24-26 was not studied, because it has very little chromosomal DNA besides the 16S rRNA gene.) Ribosome reconstitution techniques were used to see whether there were any radioactive r-proteins produced in vitro that could incorporate into ribosomal particles (26, 30). Using this method, r-proteins were detected in the samples made with pLC34-34 as template, but not with other plasmid DNAs.

As mentioned above, pLC34-34 has DNA sequence homology to λ*rif*^{d}18 DNA outside the rRNA operon. λ*rif*^{d}18 DNA carries genes for ribosomal proteins L1 (*rplA*), L7/L12 (*rplL*), L10 (*rplK*), and L11 (*rplJ*) and directs the synthesis of these four proteins in vitro (30, 32).

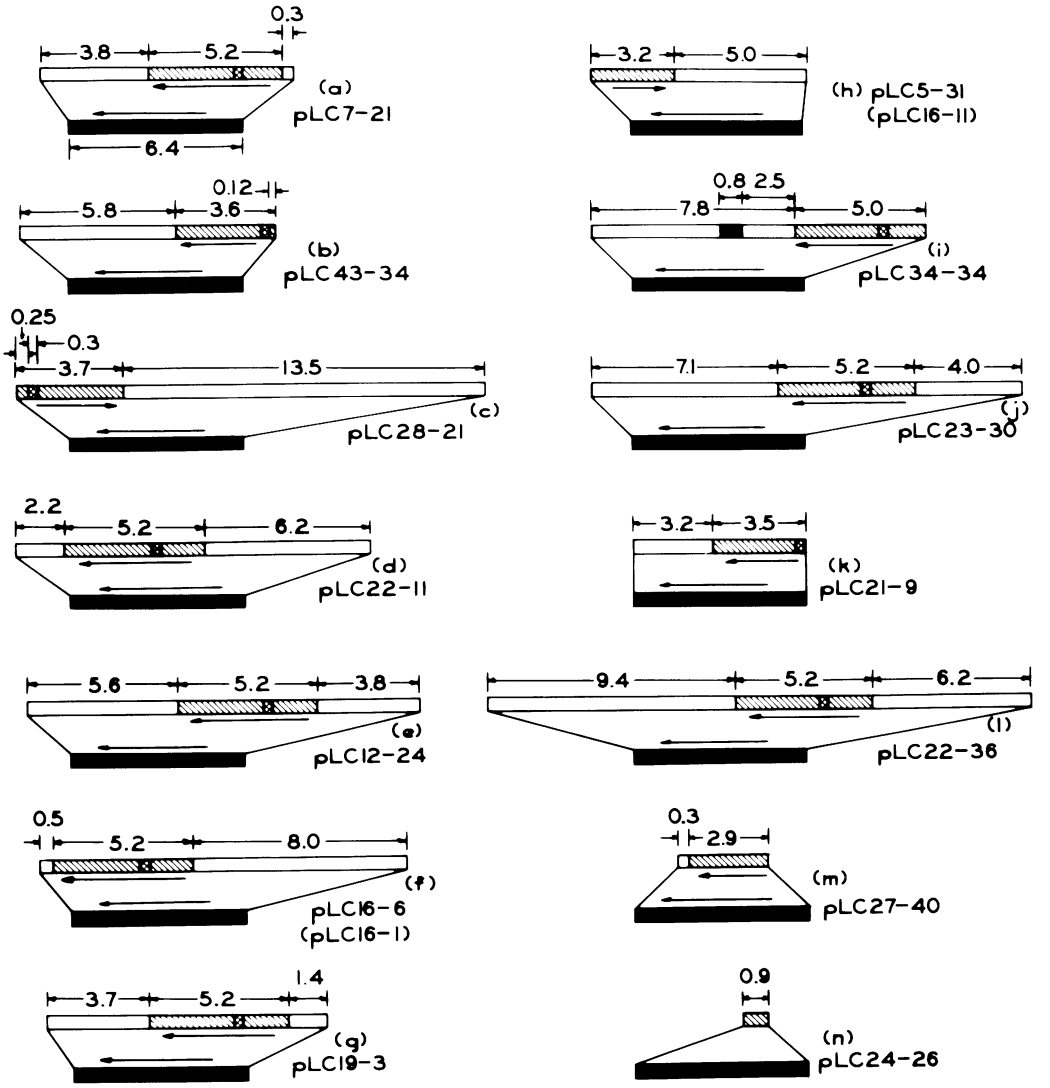


FIG. 6. *Structures of the hybrid plasmids with rRNA genes. Structures of the plasmids deduced from heteroduplex analyses are summarized. Circular molecules are shown with two horizontal bars, one representing ColE1 DNA (stippled bars) and the other representing bacterial DNA (open and hatched bars). The hatched regions represent rRNA genes, and the cross-hatched regions are spacer regions. Distances are given in kilobases. Arrows show the orientations of rRNA operons (from 16S to 23S rRNA genes) and the orientation of ColE1 DNA. The latter was arbitrarily determined in the present work, but the direction of the arrow is the same as the direction of transcription of the ColE1 gene. This gene is probably separated into two parts by the insertion of bacterial DNA. Its promoter and a proximal portion are at the left end of the stippled bar in this figure and the remaining distal portion is at the right end of the stippled bar (J. Collins, personal communication).*

Therefore, radioactive proteins synthesized in vitro with pLC34-34 DNA as template were analyzed for these proteins by an immunodiffusion method, using specific antisera against these proteins, followed by autoradiography. Reference samples obtained with λ*rif*[d]18 DNA as template showed positive results with all four antisera. With pLC34-34 samples, radioactive L7/L12 and L10 were detected, but L1 and L11 were not detected (data not shown). Apparent absence of L1 and L11 genes on the pLC34-34 plasmid is consistent with the deletion in pLC34-34 plasmid detected by heteroduplex analysis, long) to the right of the rRNA operon. These

which includes the region approximately corresponding to the locations of the L1 and L11 genes (see Fig. 3e and 1f and Fig. 1 legend). The presence of L10 and L7/L12 genes on pLC34-34 supports the conclusion obtained by heteroduplex analysis that the chromosomal location of the rRNA operon carried by this plasmid is *rrnB* near *rif*. [The location of the L10 gene relative to the other r-protein genes in this region has not been published. The present results confirm the earlier deletion mapping results (M. Yamamoto and M. Nomura, unpublished data) and show that the order of the genes is *rrnB*, *tufB*, (*rplA*, *rplK*), (*rplJ*, *rplL*), *rpoB*, *rpoC* in clockwise direction on the chromosome.]

Radioactive proteins synthesized in vitro using various plasmid DNAs were also analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Figure 7 shows autoradiograms of the dried sodium dodecyl sulfate-polyacrylamide gels. In addition to radioactive protein bands corresponding to those observed with control ColE1 DNA, several other radioactive protein bands are present. These latter protein bands probably reflect the presence of non-ColE1 bacterial DNA outside rRNA operons. It can be seen that plasmids in each of the groups I, II, and III share some unique protein bands with the other plasmids in the same group. The results shown in Fig. 7 are consistent with the classification of hybrid plasmids based on the heteroduplex analysis.

Among the protein products synthesized with pLC34-34 plasmid DNA as template, we detected two strongly radioactive proteins with the same mobility in the sodium dodecyl sulfate-polyacrylamide gel elecrophoresis as reference L10 and L7/L12 (Fig. 7b). The results support the conclusion obtained from experiments using the immunodiffusion method described above.

Mapping of rRNA operons by genetic crosses. Previous studies established chromosomal locations of five rRNA operons: *rrnA* at 85 min between *rha* and *metE* (12); *rrnB* at 88 min between *bfe* and *rif* (30); *rrnC* at 83 min near *ilv* (8); *rrnD* at 71 min near *aroE* (24); and *rrnE* at 89 min between *purD* and *metA* (49) (Fig. 8). (Davidson and co-workers identified an rRNA operon in addition to *rrnA* on F'14 [12, 40]. It is not clear whether this additional operon corresponds to *rrnB* or *rrnC* [see Fig. 8, legend].) In addition, Jarry and Rosset (22) mapped 5S RNA genes at four chromosomal regions. The first two (*cqsA* and *cqsB*) probably correspond to *rrnA* and *rrnC*. The third (*cqsC*) is between *malA* and *aroB*, and the fourth (*cqsD*) is between *aroE* and *argR*, which may correspond to *rrnD*. As described above, heteroduplex analyses indicate that the rRNA operon carried by the group IV plasmid corresponds to *rrnB* and that carried by group VI plasmids probably corresponds to *rrnC*.

Genetic crosses were made to search for chromosomal locations of the rRNA operons carried by other hybrid plasmids. Since the strains carrying the hybrid plasmids were derived from JA200, which is F^+, the plasmids carrying the rRNA operons can be transferred to F^- strains (5). We used recipient strains carrying genetic markers that are close to the previously studied rRNA gene locations mentioned above. The following genetic markers were examined in preliminary experiments: *metA*, *argA*, *purD*, *argH*, *rha*, *glnA*, *trkA*, *malA*, *bioH*, *aroB*, and *aroE*. The strains carrying the hybrid plasmids listed in Table 1 were cross-streaked with recipient strains on suitable selective media. We found that the strains carrying plasmids pLC22-11, pLC12-24, pLC16-6, and pLC16-1 are able to transfer the $aroE^+$ marker to *aroE* recipient cells. No other cross-streaking tests gave positive results.

The presence of the *aroE* gene on the group II plasmids was confirmed by crosses in liquid cultures. All of the recipient cells which became immune to ColE1 proved to be $aroE^+$ (Table 4). Conversely, all the $aroE^+$ "recombinants" acquired ColE1 immunity. As a control, a strain carrying the plasmid pLC22-36, which belonged to group VI, was tested. This strain did not donate $aroE^+$ to the recipient, even though it transferred ilv^+ to an *ilv* recipient (see below). We conclude that the rRNA operon carried by the group II plasmids is located close to *aroE* at 71.5 min on the *E. coli* chromosome. The presence of an rRNA operon near *aroE* was independently demonstrated by isolation of several λ transducing phages carrying both *aroE* and an rRNA operon, called *rrnD* (24; Yamamoto and Nomura, unpublished data). Analysis of the structure of these transducing phages located *rrnD* counterclockwise from *aroE*. Since there are no rRNA genes between *aroE* and *str*, that is, clockwise from *aroE* (31), and since the group II plasmids do not carry any r-protein genes in the *str-spc* region (see above), we conclude that the rRNA operon carried by the group II plasmids is *rrnD*.

As mentioned above, heteroduplex analysis has indicated that both pLC22-36 and pLC21-9 carry bacterial DNA, which originated from the *ilv* region at 83 min. Genetic experiments shown in Table 5 demonstrated that the pLC22-36 plasmid carries the *ilvE* gene. pLC21-9 has only a short segment (about 3.2 kb long) of bacterial DNA to the right of the rRNA operon (in Fig. 3; see also λ*ilv*5 structure in Fig. 1d), whereas pLC22-36 has a longer segment (about 9.4 kb

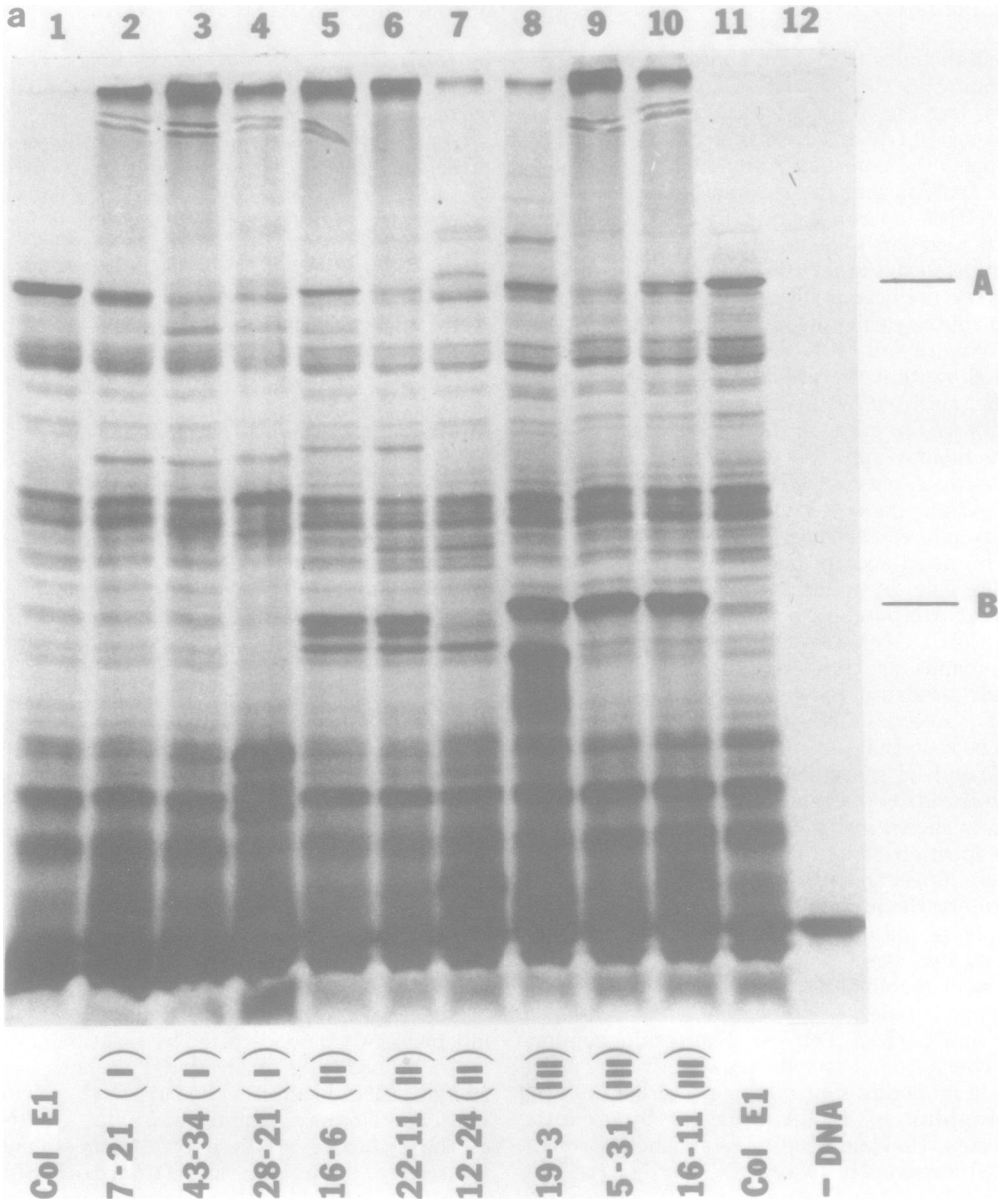
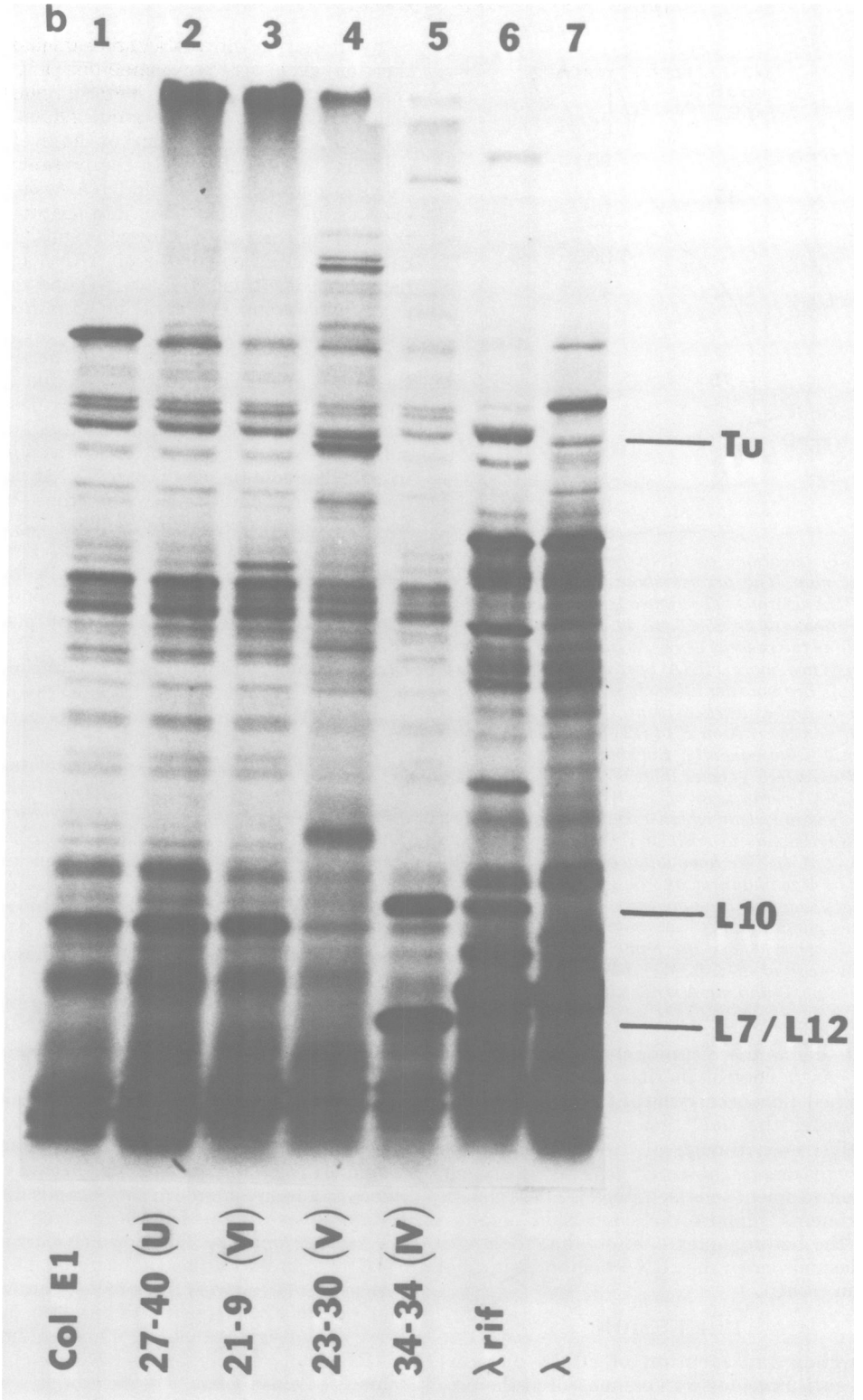


FIG. 7. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteins synthesized in vitro using various hybrid plasmid DNAs as templates. Proteins were synthesized in the presence of [^{35}S]methionine (60 μCi/40-μl reaction mixture) in the DNA-dependent in vitro system using various hybrid plasmid DNAs, ColE1 DNA, or λrif^{d}18 DNA as template, as indicated in the figure. Electrophoresis (from the top to the bottom) was performed on a 12% acrylamide gel slab. A 2-μl reaction mixture was loaded in each slot. Several reference proteins were also subjected to the electrophoresis along with the samples. The gels were stained and dried. Autoradiograms of the dried gels are shown. Positions of EF-Tu, L10, and L7/L12 are indicated. It can be seen that the synthesis of EF-Tu with pLC34-34 DNA as template was strongly reduced (or absent) compared to that with λrif^{d}18 DNA as template [(b), gels 5 and 6]. A weak radioactive protein band with the mobility of EF-Tu is probably not EF-Tu, but rather a protein coded for by ColE1 DNA (see gel 1). As described in the text, plasmid DNAs that are classified in the same group produced some common unique protein(s). For example, all the group III plasmid DNAs directed the synthesis of a protein indicated as B. ColE1 DNA directed the synthesis of ColE1, whose position is indicated as A. Proteins with similar mobilities were also produced with all other hybrid plasmid DNAs as template. At least some of them are related to ColE1 protein (W. Lotz and M. Nomura, unpublished data). Template DNA concentrations were between 30 and 40 μg/ml, except that pLC34-34 DNA was 20 μg/ml.*


b
1
2
3
4
5
6
7
Tu
L10
L7/L12
Col E1
27-40 (U)
21-9 (VI)
23-30 (V)
34-34 (IV)
λ rif
λ

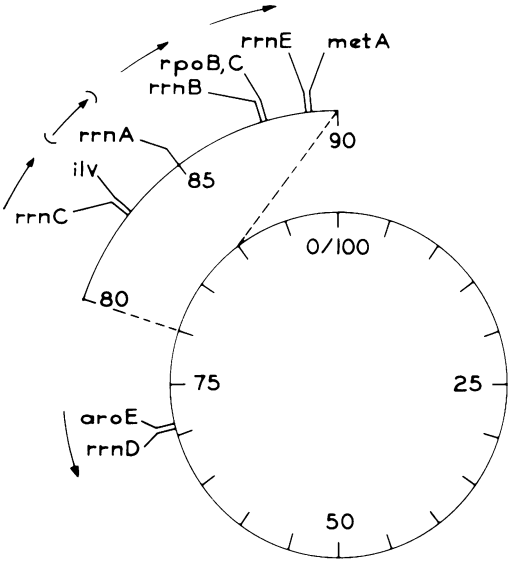


FIG. 8. *Locations of rRNA genes on the E. coli genetic map. The arrows show the orientations of the rRNA operons. The figure is based on previous publications (12, 24, 25, 30, 32, 49; Hill et al., in press) as well as the present work. As discussed in the text, at least two more rRNA operons exist, but their locations are not identified. It should be noted that Davidson and co-workers identified an rRNA operon in addition to rrnA on F'14 (12, 40), and they called it "rrnB." Subsequently, Bachmann et al. (1) published a revised genetic map of E. coli and called the rRNA operon near rif rrnB, and that near ilv rrnC. It is not clear whether Davidson's "rrnB" on F'14 corresponds to rrnB or rrnC named by Bachmann et al. (1). We have followed the nomenclature given by Bachmann et al. (1). Hill and co-workers (in press) suggested the presence of an rRNA operon between purD and metA and named it "rrnD." However, Jørgensen gave the name rrnD to the rRNA operon near aroE (24). We call the rRNA operon between purD and metA rrnE (49).*

results define the mapping position of the *ilvE* gene. As a control in these experiments, we examined a strain carrying pLC16-1 for its ability to transfer the *ilvE*$^+$ gene. The results were negative, even though pLC16-1 did show the ability to transfer the *aroE*$^+$ gene on the plasmid to *aroE* recipient cells (see above). These genetic experiments support the conclusion obtained from the heteroduplex analysis that the group VI plasmids carry the rRNA operon near *ilv* at 83 min (*rrnC*).

DISCUSSION

Number and location of rRNA operons in *E. coli*. Previous workers measured the proportion of total *E. coli* DNA that is saturated by hybridization with rRNA and estimated that there are about six to seven operons per haploid chromosome of *E. coli* (see 43 for a review). As mentioned above, previous studies have established chromosomal locations of the five rRNA operons shown in Fig. 8. In the present study, we have identified 16 plasmid DNAs with rRNA operons and classified them into six groups on the basis of their chromosomal origins. The locations of three out of the six rRNA operons have been identified; they correspond to *rrnB* at 88 min (group IV), *rrnC* at 83 min (group VI), and *rrnD* at 71 min (group II).

In addition, there is evidence to suggest that the rRNA operon on group III plasmids corresponds to *rrnA* at 85 min. As will be described in a separate paper, overproduction of not only spacer tRNA's, but also 5S RNA, was observed in cells that contain many copies of hybrid plasmids carrying complete rRNA operons (19a). It was found that the 5S RNA (called "5S RNA-III") overproduced by cells harboring group III plasmids contains a unique "minor" oligonucleotide, UCUCCUCAUG, among T_1 ribonuclease digests. Strains harboring the hybrid plasmids classified in other groups overproduced 5S RNA, which did not contain this minor oligonucleotide. Jarry and Rosset (21, 22) have mapped a gene (*cqsA*) responsible for the production of 5S RNA with this minor oligonucleotide between *metE* and *rha*. This mapping position corresponds to the location of *rrnA* studied by Deonier et al. (12). If, as suggested by Jarry and Rosset (21, 22), there is only a single genetic locus responsible for the production of this unique 5S RNA, our group III rRNA operon must correspond to *cqsA* and, hence, to *rrnA* at 85 min on the chromosome. We tentatively conclude that the group III rRNA operon corresponds to *rrnA*.

The locations of the two rRNA operons carried by the plasmids in groups I and V are not known. An additional rRNA operon, whose location is known to be between *purD* and *metA* at 89 min, is called *rrnE* (49; C. W. Hill, R. H. Grafstrom, and B. S. Hillman, *DNA Insertion Elements, Plasmids and Episomes*, in press). It is unlikely that either of the two plasmid groups whose origins are unknown corresponds to *rrnE*. Heteroduplexes formed between these plasmid DNAs and λ*metA*20 DNA, which carries the *rnnE* operon and adjacent bacterial DNA on both sides (49), showed no homology other than that of the rRNA operon. Thus, the present study demonstrates that the minimum number of rRNA operons per haploid *E. coli* chromosome is seven, that is, the six groups given in Table 1 plus *rrnE*.

Spacer regions and spacer tRNA genes. The present heteroduplex analysis has demonstrated that among the six groups of rRNA operons, there are only two different kinds of spacer regions that can be distinguished by the heteroduplex technique. In groups I, II, and III, rRNA operons have the same spacer region, whereas groups IV, V, and VI have another, different kind of spacer region. Biochemical analysis to be published elsewhere (36a) demonstrated that the rRNA operons in groups I, II, and III carry two tRNA genes in their spacer regions, namely, genes for $tRNA_{1B}^{Ala}$ and $rRNA_1^{Ile}$, and the remaining rRNA operons (groups IV, V, VI, and *rrnE*) carry the gene for $tRNA_2^{Glu}$ in their spacer regions. Thus, the classification of spacers based on the presence or absence of nonhomology spacer bubbles in heteroduplex analysis is entirely consistent with that based on the biochemical identification of spacer tRNA genes.

Expression of ribosomal protein and rRNA genes. Our previous studies indicate that the arrangement of genes on the bacterial DNA carried by λ*rif*^{d}18 is probably the same as that on the *E. coli* chromosome, with the possible exception of the spacer region in the rRNA operon (32, 35). The heteroduplex formed between the hybrid plasmid pLC34-34 and λ*rif*^{d}18 (Fig. 3e) indicates the presence of a deletion (about 4.7 kb in length) and an insertion (about 0.8 kb in length) in the bacterial DNA carried by pLC34-34. The deletion has eliminated genes including *tufB* (a gene for EF-Tu) and perhaps a part of the r-protein gene cluster. The results of the in vitro protein synthesis experiments are consistent with the absence of genes for r-proteins L1 and L11 and the *tufB* gene (see Fig. 7, legend). However, the genes for L10 and L7/L12 are still present. Although the location of promoter(s) for these r-protein genes has not been exactly determined, our preliminary experiments suggested that the genes for these proteins (genes for L1, L11, L10, and possibly L7/L12) are cotranscribed from one promoter, which is to the left of these genes in Fig. 1 (Yamamoto and Nomura, unpublished data). If so, this promoter must have also been deleted. The size of the insertion in this plasmid suggests that the inserted DNA might be IS1, which is known to prevent transcription of distal genes in many transcription units and is frequently accompanied by deletions of genetic material at the site of integration (46a). We speculate that the presence of at least some r-protein genes or the *tufB* gene in many copies in an active state may be harmful to cells. Such considerations may explain why only pLC34-34, which carries

TABLE 4. *Detection of the aroE$^+$ gene on hybrid plasmids by genetic crosses*[a]

Donor	Plasmid group	Expt 1			Expt 2		
		Selection	No. tested	E1-immune cells that were *aroE*$^+$ (%)	Selection	No. tested	*aroE*$^+$ that were E1 immune (%)
JA200(pLC22-11)	II	E1 immunity	32	100	*aroE*$^+$	34	100
JA200(pLC12-24)	II	E1 immunity	33	100	*aroE*$^+$	22	100
JA200(pLC16-1)	II	E1 immunity	36	100	*aroE*$^+$	50	100
JA200(pLC16-6)	II	E1 immunity	38	100	*aroE*$^+$	23	100
JA200(pLC22-36)	VI	E1 immunity	18	0			

[a] Recipient: NO1352 (F$^-$ *aroE recA ery^r spc^r str^r fus^r*). Both donor and recipient strains were grown overnight, diluted 50-fold, and incubated for an additional 3 to 4 h in LB media. Donor and recipient cells were mixed about 1:1 in cell numbers and held at 37°C for 1 h. A 0.1-ml amount of a 100-fold dilution of this mixture was plated on selective agar media. Colonies were purified and tested for unselected markers. Colicin and BF23 resistances were determined by cross-streaking on rich medium; auxotrophy was determined by streaking on minimal agar. Counterselection of donors was done by omitting leucine, tryptophan, and threonine required for the growth of donors. NO1352 was constructed from AB2834 (*aroE malA*), which was obtained from J. Davies.

TABLE 5. *Detection of the ilvE$^+$ gene on a hybrid plasmid by genetic crosses*[a]

Donor	Plasmid group	Selection	No. tested	E1-immune cells that were *ilvE*$^+$ (%)
JA200(pLC22-36)	VI	E1 immunity	25	100
JA200(pLC21-9)	VI	E1 immunity	18	0
JA200(pLC16-1)	II	E1 immunity	34	0

[a] Recipient: 2530E (F$^-$ *ilvE metB/H argG his xyl lac gal tsx rha recA*). Experiments were done similarly to those in Table 4. Strain 2530E is described in reference 38.

the *rrnB* operon, has an aberrant structure with a deletion and an insertion.

In contrast to the structure seen in pLC34-34, heteroduplex analysis did not show any sign of deletion of promoter(s) for rRNA operons. As described in a separate paper (19a), we have in fact observed an increased rate of transcription of rRNA genes, especially under conditions where the number of plasmid copies was selectively increased. It appears that the large increase in the number of intact rRNA gene copies and active transcription of these genes are not harmful to *E. coli* cells.

Heterogeneity of rRNA transcription units. Heterogeneity of rRNA sequence was first detected in 5S RNA (3, 20) and subsequently in larger rRNA's (14). However, the sequence heterogeneity in larger rRNA's has been difficult to analyze because of the complexity of oligonucleotides in enzyme digests used for sequencing the RNAs. Even in the case of 5S RNA molecules where sequence heterogeneity was established definitively at several sites, separation of individual "pure" 5S RNA molecules was very difficult and comparison of complete sequences of each molecular species has not been possible. Since we now have six different rRNA operons separate on plasmid DNAs, comparison of sequences of rRNA's derived from each of these operons would be easier. We have already mentioned our studies on the 5S RNA sequences derived from each of the rRNA operons in connection with mapping of these operons (19a). In addition, analyses of plasmid DNAs with *Eco*RI restriction endonuclease, as well as other restriction endonucleases, have demonstrated sequence differences among rRNA's encoded by different isolated rRNA operons (our unpublished data). The functional significance of the heterogeneity of rRNA's is not known.

In addition to the structural differences among RNA transcripts coming from different rRNA operons, there might be differences related to the regulation of transcription among different rRNA operons. At the moment, we do not know whether the expression of each of the multiple rRNA operons is regulated in the same way. Availability of the hybrid plasmids, each carrying a unique rRNA operon, may help in studying these and other problems related to the multiplicity of rRNA operons in bacterial cells.

ACKNOWLEDGMENTS

We thank L. Clarke and J. Carbon for making their plasmid bank strains available to us to screen plasmids with rRNA genes, and S. R. Jaskunas, A. E. Arfsten, and G. D. Strycharz for their assistance in various aspects of the project.

This work was supported by the College of Agriculture and Life Sciences, University of Wisconsin, and by grants from the Public Health Service, National Institute of General Medical Sciences (GM-20427 administered by M.N.), and the National Science Foundation (GB-31086 administered by M.N.).

LITERATURE CITED

1. **Bachmann, B. J., K. B. Low, and A. L. Taylor.** 1976. Recalibrated linkage map of *Escherichia coli* K-12. Bacteriol. Rev. **40**:116-167.
2. **Blair, D. G., D. J. Sherratt, D. B. Clewell, and D. R. Helinski.** 1972. Isolation of supercoiled colicinogenic factor E_1 DNA sensitive to ribonuclease and alkali. Proc. Natl. Acad. Sci. U.S.A. **69**:2518-2522.
3. **Brownlee, G. G., F. Sanger, and B. G. Barrell.** 1968. The sequence of 5S ribosomal ribonucleic acid. J. Mol. Biol. **34**:379-412.
4. **Clarke, L., and J. Carbon.** 1975. Biochemical construction and selection of hybrid plasmids containing specific segments of the *Escherichia coli* genome. Proc. Natl. Acad. Sci. U.S.A. **72**:4361-4365.
5. **Clarke, L. and J. Carbon.** 1976. A colony bank containing synthetic ColE1 hybrid plasmids representative of the entire E. coli genome. Cell **9**:91-99.
6. **Clewell, D. B.** 1972. Nature of ColE1 plasmid replication in *Escherichia coli* in the presence of chloramphenicol. J. Bacteriol. **110**:667-676.
7. **Clewell, D. B., and D. R. Helinski.** 1972. Effect of growth conditions on the formation of the relaxation complex of supercoiled ColE1 deoxyribonucleic acid and protein in *Escherichia coli*. J. Bacteriol. **110**: 1135-1146.
8. **Collins, J., N. P. Fiil, P. Jørgensen, and J. D. Friesen.** 1976. Gene cloning of *Escherichia coli* chromosomal genes important in the regulation of ribosomal RNA synthesis, p. 356-367. *In* N. O. Kjeldgaard and O. Maaløe (ed.), Alfred Benzon symposium IX: control of ribosome synthesis. Academic Press Inc., New York.
9. **Davies, J., and M. Nomura.** 1972. The genetics of bacterial ribosomes. Annu. Rev. Genet. **6**:203-234.
10. **Davis, R. W., M. Simon, and N. Davidson.** 1971. Electron microscope heteroduplex methods for mapping regions of base sequence homology in nucleic acids. Methods Enzymol. **210**:413-428.
11. **Dennis, P. P., and M. Nomura.** 1975. Regulation of the expression of ribosomal protein genes in *Escherichia coli*. J. Mol. Biol. **97**:61-76.
12. **Deonier, R. C., E. Ohtsubo, H. J. Lee, and N. Davidson.** 1974. Electron microscope heteroduplex studies of sequence relations among plasmids of *Escherichia coli*. VII. Mapping the ribosomal RNA genes of plasmid F14. J. Mol. Biol. **89**:619-629.
13. **Dunn, J. J., and F. W. Studier.** 1973. T7 early RNAs and *Escherichia coli* ribosomal RNAs are cut from large precursor RNAs *in vivo* by ribonuclease III. Proc. Natl. Acad. Sci. U.S.A. **70**:3296-3300.
14. **Fellner, P.** 1974. Structure of the 16S and 23S ribosomal RNAs, p. 169-191. *In* M. Nomura, A. Tissières, and P. Lengyel (ed.), Ribosomes. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
15. **Fiandt, M., W. Szybalski, and F. Imamoto.** 1974. Physical mapping of the *trp* endpoint in the N-t_{L1} segment of phage λ*trp*E-A. Virology **61**:312-314.
16. **Ginsburg, D., and J. A. Steitz.** 1975. The 30S ribosomal precursor RNA from *Escherichia coli*. J. Biol. Chem. **250**:5647-5654.
17. **Guerry, P., D. J. LeBlanc, and S. Falkow.** 1973. General method for the isolation of plasmid deoxyribonucleic acid. J. Bacteriol. **116**:1064-1066.
18. **Helinski, D. R.** 1973. Chemistry of colicinogenic factors, p. 15-39. *In* L. P. Hager (ed.), Chemistry and functions of colicin. Academic Press Inc., New York.
19. **Herschfield, V., H. W. Boyer, C. Yanofsky, M. A. Lovett, and D. R. Helinski.** 1974. Plasmid ColE1 as

a molecular vehicle for cloning and amplification of DNA. Proc. Natl. Acad. Sci. U.S.A. **71**:3455-3459.

19a. **Ikemura, T., and M. Nomura.** 1977. Expression of spacer tRNA genes in ribosomal RNA transcription units carried by hybrid ColE1 plasmids in E. coli. Cell **11**:779-793.

20. **Jarry, B., and R. Rosset.** 1971. Heterogeneity of 5S RNA in *Escherichia coli*. Mol. Gen. Genet. **113**:43-50.

21. **Jarry, B., and R. Rosset.** 1973. Localization of some 5S RNA cistrons on *Escherichia coli* chromosome. Mol. Gen. Genet. **121**:151-162.

22. **Jarry, B., and R. Rosset.** 1973. Further mapping of 5S RNA cistrons in *Escherichia coli*. Mol. Gen. Genet. **126**:29-35.

23. **Jaskunas, S. R., L. Lindahl, M. Nomura, and R. R. Burgess.** 1975. Identification of two copies of the gene for the elongation factor EF-Tu in *E. coli*. Nature (London) **257**:458-462.

24. **Jørgensen, P.** 1976. A ribosomal RNA gene of *Escherichia coli* (*rrnD*) on λd*aroE* specialized transducing phages. Mol. Gen. Genet. **146**:303-307.

25. **Jørgensen, P., and N. P. Fiil.** 1976. Ribosomal RNA synthesis *in vitro*, p. 370-382. *In* N. O. Kjeldgaard and O. Maaløe (ed.), Alfred Benzon symposium IX: control of ribosome synthesis. Academic Press Inc., New York.

26. **Kaltschmidt, E., L. Kahan, and M. Nomura.** 1974. *In vitro* synthesis of ribosomal proteins directed by *Escherichia coli* DNA. Proc. Natl. Acad. Sci. U.S.A. **71**:446-450.

27. **Kirschbaum, J. B., and E. B. Konrad.** 1973. Isolation of a specialized lambda transducing bacteriophage carrying the beta subunit gene for *Escherichia coli* ribonucleic acid polymerase. J. Bacteriol. **116**:517-526.

28. **Konrad, B., J. Kirschbaum, and S. Austin.** 1973. Isolation and characterization of φ80 transducing bacteriophage for a ribonucleic acid polymerase gene. J. Bacteriol. **116**:511-516.

29. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227**:680-685.

30. **Lindahl, L., S. R. Jaskunas, P. P. Dennis, and M. Nomura.** 1975. Cluster of genes in *Escherichia coli* for ribosomal proteins, ribosomal RNA, and RNA polymerase subunits. Proc. Natl. Acad. Sci. U.S.A. **72**:2743-2747.

31. **Lindahl, L., L. Post, and M. Nomura.** 1976. DNA-dependent in vitro synthesis of ribosomal proteins, protein elongation factors, and RNA polymerase subunit α: inhibition by ppGpp. Cell **9**:439-448.

32. **Lindahl, L., M. Yamamoto, M. Nomura, J. B. Kirschbaum, B. Allet, and J.-D. Rochaix.** 1977. Mapping of a cluster of genes for components of the transcriptional and translational machineries of *Escherichia coli*. J. Mol. Biol. **109**:23-47.

33. **Lindahl, L., J. Zengel, and M. Nomura.** 1976. Organization of ribosomal protein genes in *Escherichia coli*. II. Mapping of ribosomal protein genes by *in vitro* synthesis of ribosomal proteins using DNA fragments of a transducing phage as templates. J. Mol. Biol. **106**:837-855.

34. **Lobban, P. E., and A. D. Kaiser.** 1973. Enzymatic end-to-end joining of DNA molecules. J. Mol. Biol. **78**:453-471.

35. **Lund, E., J. E. Dahlberg, L. Lindahl, S. R. Jaskunas, P. P. Dennis, and M. Nomura.** 1976. Transfer RNA genes between 16S and 23S rRNA genes in rRNA transcription units of E. coli. Cell **7**:165-177.

36. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

36a. **Morgan, E. A., T. Ikemura, and M. Nomura.** 1977. Identification of spacer tRNA genes in individual ribosomal RNA transcription units of *Escherichia coli*. Proc. Natl. Acad. Sci. U.S.A. **74**:2710-2714.

37. **Morgan, E. A., and S. Kaplan.** 1976. Transcription of *Escherichia coli* ribosomal DNA in *Proteus mirabilis*. Mol. Gen. Genet. **147**:179-188.

38. **Morgan, E. A., and S. Kaplan.** 1977. Expression and stability of *Escherichia coli* F-prime factors in *Proteus mirabilis*. Mol. Gen. Genet. **151**:41-47.

39. **Nikolaev, N., L. Silengo, and D. Schlessinger.** 1973. A role for ribonuclease III in processing of ribosomal ribonucleic acid and messenger ribonucleic acid precursors in *Escherichia coli*. J. Biol. Chem. **248**:7967-7969.

40. **Ohtsubo, E., L. Soll, R. C. Deonier, H. J. Lee, and N. Davidson.** 1974. Electron microscope heteroduplex studies of sequence relations among plasmids of *Escherichia coli*. VIII. The structure of bacteriophage φ80d$_3$*ilv*$^+$*su*$^+$7, including the mapping of the ribosomal RNA genes. J. Mol. Biol. **89**:631-646.

41. **Pace, N. R.** 1973. Structure and synthesis of the ribosomal ribonucleic acid of prokaryotes. Bacteriol. Rev. **37**:562-603.

42. **Reiner, A. M.** 1969. Genetic locus for ribonuclease I in *Escherichia coli*. J. Bacteriol. **97**:1522-1523.

43. **Schwartz, S. A., and D. R. Helinski.** 1971. Purification and characterization of Colicin E_1. J. Biol. Chem. **246**:6318-6327.

44. **Sharp, P. A., M.-T. Hsu, E. Ohtsubo, and N. Davidson.** 1972. Electron microscope heteroduplex studies of sequence relations among plasmids of *Escherichia coli*. I. Structure of F-prime factors. J. Mol. Biol. **71**:471-497.

45. **Shinnick, T. M., E. Lund, O. Smithies, and F. R. Blattner.** 1975. Hybridization of labeled RNA to DNA in agarose gels. Nucleic Acids Res. **2**:1911-1929.

46. **Sidikaro, J., and M. Nomura.** 1975. *In vitro* synthesis of the E3 immunity protein directed by Col E3 plasmid deoxyribonucleic acid. J. Biol. Chem. **250**:1123-1131.

46a. **Starlinger, P., and H. Saedler.** 1976. Is-elements in microorganisms. Curr. Top. Microbiol. **75**:111-152.

47. **Watson, R. J., J. Parker, N. P. Fiil, J. G. Flaks, and J. D. Friesen.** 1975. New chromosomal location for structural genes of ribosomal proteins. Proc. Natl. Acad. Sci. U.S.A. **72**:2765-2769.

48. **Yamamoto, M., L. Lindahl, and M. Nomura.** 1976. Synthesis of ribosomal RNA in E. coli: analysis using deletion mutants of a λ transducing phage carrying ribosomal RNA genes. Cell **7**:179-190.

49. **Yamamoto, M., and M. Nomura.** 1976. Isolation of λ transducing phages carrying rRNA genes at the *metA-purD* region of the *Escherichia coli* chromosome. FEBS Lett. **72**:256-261.