Proc. Natl. Acad. Sci. USA
Vol. 75, No. 8, pp. 3673–3677, August 1978
Biochemistry

Low molecular weight RNA species encoded by a multiple drug resistance plasmid

(RNA purification/two-dimensional chromatography/minicells/Southern hybridization)

MICHEL DE WILDE*†, JULIAN E. DAVIES*‡, AND FRANCIS J. SCHMIDT§

Departments of * Biochemistry and § Bacteriology, University of Wisconsin, Madison, Wisconsin 53706

Communicated by Lawrence Bogorad, May 19, 1978

ABSTRACT **Multiple drug resistance plasmid NR1 is shown to code for at least 10 low molecular weight RNAs. These species, ranging in size from 60 to 120 nucleotides, have been purified from minicells by two-dimensional gel electrophoresis and characterized by RNase T1 fingerprinting. Hybridization of purified RNAs to restriction endonuclease digests of NR1 DNA indicates that most are derived from the resistance transfer factor region of the plasmid genome. One RNA was found to be coded by the transposable tetracycline resistance element Tn10, and several are associated with DNA fragments that contain origins of replication.**

The number of functions known or presumed to be coded by multiple drug resistance plasmids is very small compared to the size and coding capacity of the plasmid DNA. Also, our understanding of the mechanisms by which plasmid functions are expressed and regulated is limited. Many plasmid functions may be required only under certain circumstances (such as a given environment or a given host), which makes their detection difficult. In this respect they may be similar to the low molecular weight RNAs, in particular tRNAs, that are synthesized by some bacteriophages. The biological role(s) of these molecules is(are) unclear; they are essential for growth under certain conditions and may have evolved to meet rather specialized requirements of bacteriophage propagation (1). In this study, we have investigated the possibility that drug resistance plasmids may also specify small RNA molecules. This was first indicated by Roozen *et al.* (2), who showed that a substantial portion of the RNA synthesized in plasmid-containing minicells was of 4S size.

We have studied the drug resistance plasmid NR1 (also called R100 or R222), which has been well characterized (3–5), to examine if specific low molecular weight RNA molecules are made and to ask if such RNA molecules are associated with known plasmid functions.

MATERIALS AND METHODS

Strains. *Escherichia coli* K12 strains used were W677 (6) and the minicell-producing strain χ984 (7). Plasmid NR1 (also called R100 or R222) (F_{II}, Cm^R, Str^R Hg^R, Tet^R, Fus^R) was introduced by conjugation. pRS5 and pRS30 are hybrid plasmids containing pSC101 and parts of the F genome (8); they were obtained from A. J. Clark.

Minicell Preparation and Labeling. Minicells were prepared as described (2) except that cells were grown in low phosphate medium (9) and sucrose gradients were run in phosphate-free medium. Purified minicells were resuspended in low phosphate medium at an optical density (550 nm) of 1 and incubated at 37° for 90 min before addition of 2 mg ml^{-1} of ampicillin to lyse any whole cells in the minicell preparation (7). Ninety minutes later, between 0.2 and 1 mCi ml^{-1} of carrier-free [^{32}P]orthophosphate was added; labeling was for 30 min. Cells were labeled in the same medium with 1 mCi ml^{-1} for 15 min.

RNA Purification. At the end of the labeling period the cell or minicell suspension was treated with 0.5 vol of phenol saturated with 10 mM Tris·HCl, pH 7.5. RNA was precipitated from the aqueous phase by addition of 0.04 vol of 5 M potassium acetate, pH 5.0, and 3 vol of cold absolute ethanol. The precipitate was redissolved in 10 mM Tris·HCl, pH 7.5/10 mM NaCl/10 mM $MgCl_2$ and treated with 10 mg ml^{-1} pancreatic DNase (RNase-free) at 37° C for 30 min. The solution was applied to a 2-ml column of DEAE-cellulose and washed with 5 vol of 10 mM Tris·HCl, pH 7.5/250 mM NaCl. The RNA was eluted with 1.5 ml of 10 mM Tris·HCl, pH 7.5/1.0 M NaCl/6.5 M urea and precipitated with ethanol. After centrifugation the redissolved RNA was applied to a polyacrylamide gel; one-dimensional gel electrophoresis was on 10% slab gels (10). Two-dimensional electrophoresis was as described (11) except that urea (7 M in the first dimension and 4 M in the second) was included in the gel (N. Pace, personal communication). After autoradiography, bands or spots were cut from the gel, crushed in a syringe, and extracted with 10 mM Tris·HCl, pH 7.5/500 mM NaCl/20 mM $MgCl_2$. The gel debris was removed by centrifugation and the supernatant was diluted twice with water and applied to a DEAE-cellulose column. After elution, the RNA was precipitated twice with ethanol before use for hybridization or two-dimensional chromatography.

Nucleotide Sequence Analysis. Two-dimensional chromatography of purified RNA species was by the Sanger method (12). RNA species were characterized by RNase T1 or RNase A fingerprinting. Oligonucleotides were analyzed by further digestion with the complementary RNase (i.e., RNase T1 products were digested with RNase A) as described (12). Base analyses of either whole RNA species or individual T1 oligonucleotides were carried out by chromatographing RNase T2 digests on cellulose thin-layer plates (13).

DNA Preparation. Covalently closed plasmid DNA was prepared as described (14) and was purified by two successive bandings in CsCl/ethidium bromide. DNA from phage λbb::Tn10 was a gift from R. Jorgensen.

Endonuclease Digestions and Agarose Gel Electrophoresis. *Eco*RI, *Sal* I, and *Hpa* I were purchased from New England Biolabs (Beverly, MA) and used as recommended by the supplier. Digestion with a mixture of *Eco*RI and *Sal* I was in *Eco*RI salts. Electrophoresis in 0.8% agarose was performed in a horizontal slab gel apparatus with 60 × 3 × 1 mm slots (15).

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Abbreviation: RTF, resistance transfer region.

† Present address: Laboratorium voor Histologie en Genetica, Rijksuniversiteit Gent, Ledeganckstraat, 35, B-900 GENT, Belgium.
‡ To whom reprint request should be addressed.

Hybridization. The technique of Southern (16) was used for the transfer of DNA fragments, separated by gel electrophoresis, to nitrocellulose sheets. Three- to 4-mm-wide strips were cut from the DNA-containing sheets and soaked in 0.60 M NaCl/0.060 M Na citrate (pH 7.0) containing 50% formamide. Hybridizations were at 43° for 48 hr in 1 ml of the same solution, and the nitrocellulose strips were processed as described (16), including an RNase treatment. Hybridization was detected by autoradiography on Kodak RP Royal XOmat film.

RESULTS

Evidence for Synthesis of Low Molecular Weight RNAs Coded by Plasmid DNA. Our first approach was to determine whether a fraction of the 4S RNA isolated from a strain containing plasmid NR1 would hybridize to the purified plasmid DNA. RNA was prepared by phenol extraction from cells labeled with ^{32}P for 15 min and the low molecular weight fraction was purified by electrophoresis on 10% acrylamide slab gels by the procedure used for preparation of tRNA from bacteriophage T4-infected cells (10). Total cellular 4S RNA was then hybridized to DNA fragments generated by restriction endonuclease digestion of the plasmid DNA. DNA fragments were separated by agarose gel electrophoresis and transferred from the gel to nitrocellulose sheets as described (16). In addition to its sensitivity, this method allows one to locate DNA sequences complementary to the RNA species on the physical map of the plasmid.

Fig. 1 shows results of hybridization of 4S [^{32}P]RNA prepared from R^+ and R^- bacteria to both chromosomal and plasmid DNA digested with *Eco*RI. As expected, total 4S RNA from both strains hybridized extensively to *E. coli* DNA. However, only the 4S RNA obtained from R^+ cells contained components that hybridized specifically to plasmid DNA. This result indicates that the species involved are not synthesized by the host; furthermore, they appear to be specific products since they hybridize to some but not all restriction fragments of the plasmid DNA.

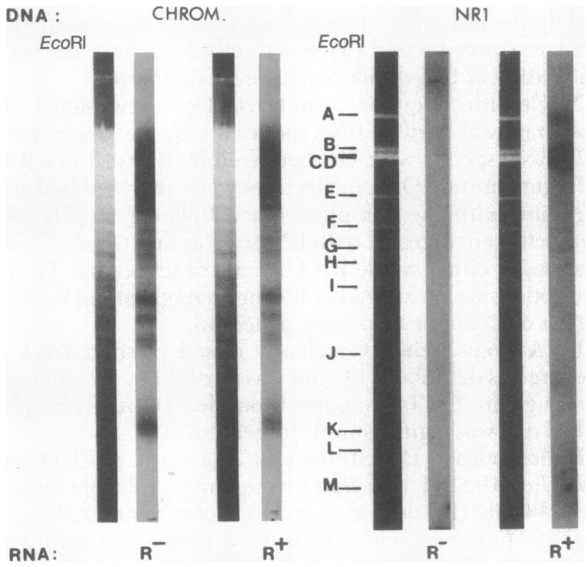

FIG. 1. Hybridization of total 4S RNA extracted from R^+ and R^- cells to chromosomal and plasmid DNA. 4S RNA, extracted from a 10% slab gel, was hybridized to the products of *Eco*RI digestion transferred to nitrocellulose after separation on 0.8% agarose slab gels. The nomenclature of *Eco*RI fragments of plasmid NR1 DNA is that of Tanaka *et al.* (5). Photographs of the original stained gel and of the autoradiograms, adjusted to the same size photographically, are presented side by side.

The *Eco*RI cleavage map of plasmid NR1 has been determined (5). The DNA fragments derived from the R determinant portion of the plasmid, e.g., *Eco*RI fragments G–M, do not hybridize with (i.e., do not code for) low molecular weight RNA, even though the most active transcription occurs in the R determinant portion of resistance plasmids (D. Vapnek, personal communication) and high molecular weight RNA, presumably mRNA, does hybridize to fragments G–M (data not shown). The RNA species we observe hybridize only to DNA fragments contained in the resistance transfer region (RTF) of the composite plasmid (see Fig. 5).

Purification and Stability of Low Molecular Weight RNAs. The plasmid RNA species could not be easily observed in gels from whole cells because of the large background of host tRNAs. Therefore, we obtained [^{32}P]RNA of exclusively plasmid origin by isolating minicells and adding [^{32}P]phosphate to this preparation (2). Minicells are products of aberrant cell division and do not contain chromosomal DNA (7); however, plasmid DNA is segregated into minicells, and plasmid-coded RNA species can be obtained free of contamination by *E. coli* tRNAs. Fig. 2*a* shows that minicells from an R^- strain did not synthesize any 4S RNA species; however, minicells containing plasmid NR1 (Fig. 2*b*) synthesized at least 10 small RNA species which were separated by gel electrophoresis.

The most prominent RNA species (continuous circles, Fig. 2*d*) were chosen for further study. The stability of these molecules was investigated by examination of the RNAs remaining after 15–30 min of incubation of the labeled minicell suspension with rifampicin, which blocks RNA synthesis. As shown in Fig.

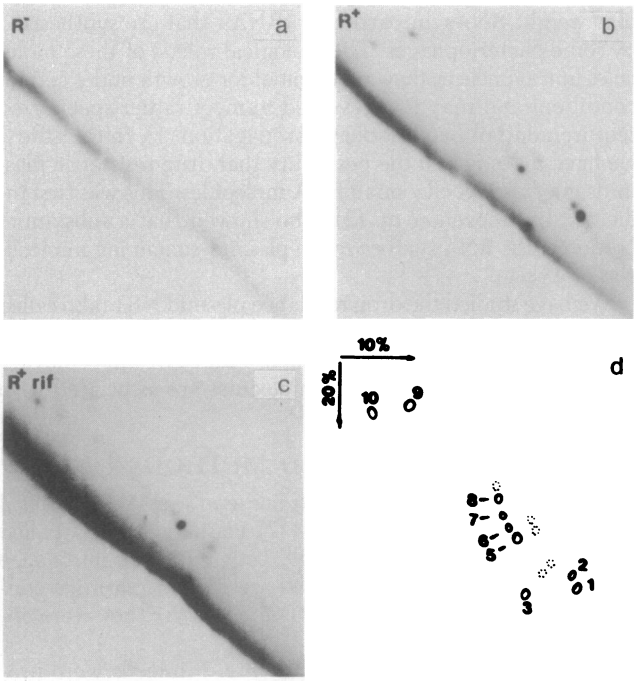

FIG. 2. Two-dimensional gel electrophoresis of the low molecular weight RNAs labeled in minicells. RNA was extracted from ^{32}P-labeled minicells and applied to a polyacrylamide gel. The first dimension in 10% polyacrylamide/7 M urea is from left to right; the second, in 20% polyacrylamide/4 M urea is from top to bottom. RNA species were visualized by autoradiography. (*a*) Plasmid-free minicells; (*b*) minicells containing NR1 plasmid; (*c*) same as *a* but at the end of the labeling period, the minicell suspension was further incubated with rifampicin (100 $\mu g\ ml^{-1}$) for 30 min; (*d*) diagrammatic representation of the autoradiogram. The spots denoted by a full circle were chosen for further study.

2*c*, components 1, 2, 8, and 9 are metabolically unstable and disappear after rifampicin treatment; by contrast, species 3, 5, 7, and 10 are stable and must have half-lives much longer than 30 min.

Two-Dimensional Chromatography. RNase T1 fingerprints (two-dimensional chromatograms) of several species were prepared in order to obtain information on their relatedness and purity. (RNase T1 cleaves RNA sequences next to the nucleotide Gp and generates about 20 products upon complete digestion of an average 80-nucleotide molecule.) RNA species 1, 2, and 8 have closely related fingerprints (Fig. 3). The oligonucleotides generated by RNase T1 digestion of species 1 and 2 were eluted and further digested with RNase A (which cleaves after pyrimidine residues) and with RNase T2 (which cleaves RNA chains to mononucleotides). These analyses show that the two RNA species are smaller than tRNAs (60–65 nucleotides long) and are identical except for a single oligonucleotide which is present in species 2 only. Both have an identical 5′-oligonucleotide with the sequence pU-U-A-A-Gp, which indicates that they are not primary transcripts (since they do not contain a 5′-triphosphate) and that species 2 is most probably longer than species 1 at the 3′ end.

The possible sequence relationship of RNA species 1 and 2 to the larger species 8 was investigated by comparing the products obtained upon further digestion of the RNase T1 products by RNase A and RNase T2. The RNase T1 products of RNA species 1 and 2 were also found in RNA species 8. Species 8 contained several additional RNase T1 products. In the absence of complete sequence determinations of these molecules, we cannot conclude that species 8 is a precursor to the other RNAs; however, they do share similar sequences which may be a consequence of a metabolic relationship between these RNAs. Hybridization analysis indicates that these RNAs are coded by the same portion of the plasmid genome.

The stable species 5 migrates at a position where host tRNA would be found; RNase T1 digestion (Fig. 3*e*) shows that this component is about 75 nucleotides long and that its 3′-terminal oligonucleotide is C-C-A. No modified bases could be detected upon digestion with RNase T2; it is thus unlikely that species 5 is a tRNA. Since the lack of modification could be due to a low level (or absence) of the appropriate modifying enzymes in minicells, we tested whether a plasmid that contains the genes for two *E. coli* tRNAs (17) produced modified tRNA in minicells. These [^{32}P]tRNAs ($tRNA_{1B}^{Ala}$ and $tRNA_{1}^{Ile}$) were purified by two-dimensional gel electrophoresis. Analysis of the products of RNase T2 digestion revealed that at least some tRNA-modifying enzymes are present in minicells (data not shown). Thus, the lack of modification of species 5 must be due to a property of the RNA molecule itself. Species 6 and 7 exhibit fingerprints very similar to that of species 5, but were not further investigated due to their low recovery and impurity. Component 3 migrates rapidly in the second dimension of the gel; its fingerprint shows that it has no relationship to the other RNA species (Fig. 3*d*).

In addition to the 4S RNA species, two 5S RNAs were observed in the two-dimensional gel. The fingerprint of one of these, component 9, is shown in Fig. 3*f*. This RNA is not related to any of the other RNAs in Fig. 3, and in addition, has no similarity to *E. coli* 5S ribosomal RNA.

Hybridization. Radioactively labeled RNAs were hybridized

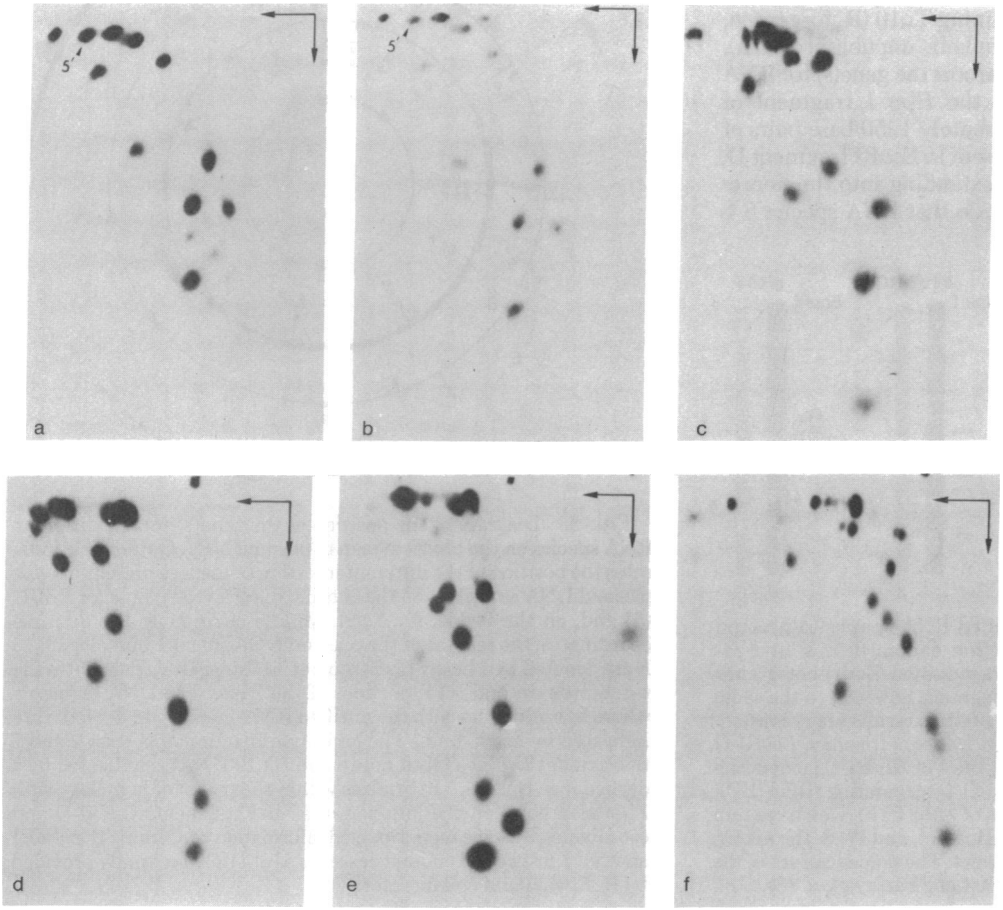

FIG. 3. RNase T1 fingerprint analysis of plasmid NR1-coded low molecular weight RNAs. RNAs were digested with RNase T1 and the products were separated by electrophoresis at pH 3.5 on cellulose acetate in the first dimension (from right to left) and on DEAE-paper in 7% formic acid in the second dimension (from top to bottom). (*a*) RNA species 1; the 5′ oligonucleotide, pU-U-A-A-Gp, is indicated. (*b*) RNA species 2; this RNA also has the 5′ terminus pU-U-A-A-Gp. (*c*) RNA species 8, which is related to species 1 and 2. This fingerprint was run longer in the second dimension than those in *a* and *b*. (*d*) Stable RNA species 3 which is coded by the tetracycline resistance element Tn10. (*e*) Stable RNA species 5, which is coded by the transfer genes. The 3′ C-C-A sequence can be seen as the most slowly moving spot in the first dimension. (*f*) 5S RNA species 9.

to fragments of NR1 DNA produced by *Eco*RI and *Sal* I (to determine the regions of the plasmid DNA that code for the individual RNA species). Hybridization to *Eco*RI fragments indicated that different RNAs were derived from different regions of the RTF. Since fragments C and D are not well separated by electrophoresis (Fig. 1), RNA hybridizing to either of these two fragments could not be located by hybridization. This difficulty was circumvented by hybridizing RNA to fragments obtained by digestion with a mixture of *Eco*RI and *Sal* I; *Eco*RI fragment C is cleaved by *Sal* I, while fragment D is not (see Fig. 5). The two DNA fragments were separated by electrophoresis, permitting the RNA to be effectively localized.

In spite of the relatedness of the sequences of RNA species 1 and 2, their behavior on hybridization was different. Both species hybridized to the small *Sal* I fragment E, which is contained in *Eco*RI fragment B (Fig. 4). However, whereas component 1 hybridizes weakly to *Eco*RI fragment D, component 2 hybridizes more strongly to this fragment. Since components 1 and 2 are related in sequence (Fig. 3), this behavior is puzzling. We do not know whether the apparent discrepancy between the sequence and hybridization data is due to hybridization of a single species to two different segments of the plasmid DNA or to a minor contaminant (in greater amount) in species 2. The latter possibility seems to be more likely since the closely related component 8 hybridizes to *Sal* I fragment E only.

Components 3 and 5 hybridize to *Eco*RI fragment D, and RNA species 9 hybridizes to the contiguous fragment *Eco*RI-A (Fig. 4). Each of these plasmid DNA fragments contains part of the transposable tetracycline resistance determinant Tn10, which is flanked by IS3 insertion sequences. We have investigated the possibility that these RNAs are coded by Tn10 by hybridizing isolated RNA to DNA derived from λbb::Tn10, a derivative of bacteriophage λ containing Tn10 (R. Jorgensen, D. E. Berg, B. Allet, and W. S. Reznikoff, unpublished data). The data in Fig. 4 show that Tn10 harbors the gene(s) for RNA species 3. Species 3 hybridizes to the *Hpa* I fragment of λbb::Tn10, which contains approximately 1250 base pairs of λ DNA, part of the IS3 sequence present in *Eco*RI fragment D, and about 500 base pairs of DNA extending into the nonrepeated portion of Tn10. We conclude that RNA species 3 is coded by the last since it does not hybridize to λ DNA; neither does it hybridize to the other IS3 sequence present in the largest *Hpa* I fragment of λbb::Tn10 and in *Eco*RI fragment A of plasmid NR1. RNA species 5 and 9 did not hybridize to λbb::Tn10 DNA and must be coded by regions adjacent to the Tn10 element; these results are summarized in Fig. 5.

Plasmid NR1 shares considerable homology with the bacterial sex factor F (4). This homology is contributed mainly by the transfer genes and extends from coordinate 36 to 3 on the NR1 map, excluding Tn10 (Fig. 5). Previous studies (4, 8) indicate that approximately 6.6 kilobases of *Eco*RI fragment D is homologous to a region of F; this region is thought to contain the origin of transfer replication, the gene *finP*, and possibly part of the *tra* operon. Since RNA species 5 is coded by NR1 fragment *Eco*RI-D, we have asked if it also hybridizes to the F plasmid, using the recombinant plasmid pRS5 (8), which contains the homologous segment of F DNA. Fig. 4 shows that RNA species 5 does indeed hybridize to the *Eco*RI fragment f6 of pRS5. Comparison of the *Eco*RI restriction maps of NR1 and F and their heteroduplex analysis locate the gene for species 5 probably between coordinate 58 and the R1 site between C and D (Fig. 5).

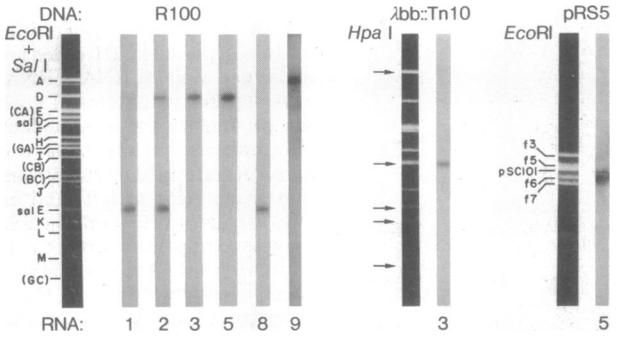

FIG. 4. Hybridization of the purified RNA species to plasmid and phage DNA. The DNAs, restriction endonucleases, and radioactively labeled RNA species used are indicated. Both photographs of the stained gels and of the autoradiograms, adjusted to the same size photographically, are shown. Only positive results are presented. NR1 (R100) DNA is cut five times by *Sal* I; once in fragment *Eco*RI-G [generating fragments (GC) and (GA)], once in *Eco*RI-C [generating (CB) and (CA)], and three times in *Eco*RI-B [generating (BB), (BC), sal E and sal D]. The *Hpa* I fragments of λbb::Tn10, which contain Tn10 DNA (R. Jorgensen, D. E. Berg, B. Allet, and W. S. Reznikoff, unpublished data), are indicated by arrows. The nomenclature of the *Eco*RI fragments present in pRS5 is that of Skurray *et al.* (8).

DISCUSSION

We have shown that plasmid NR1 codes for at least 10 RNAs which are approximately 60–120 nucleotides long. These RNAs include both stable (RNAs 3, 5, 6, and 7) and unstable (RNAs 1, 2, 8, and 9) species; they have been characterized by fin-

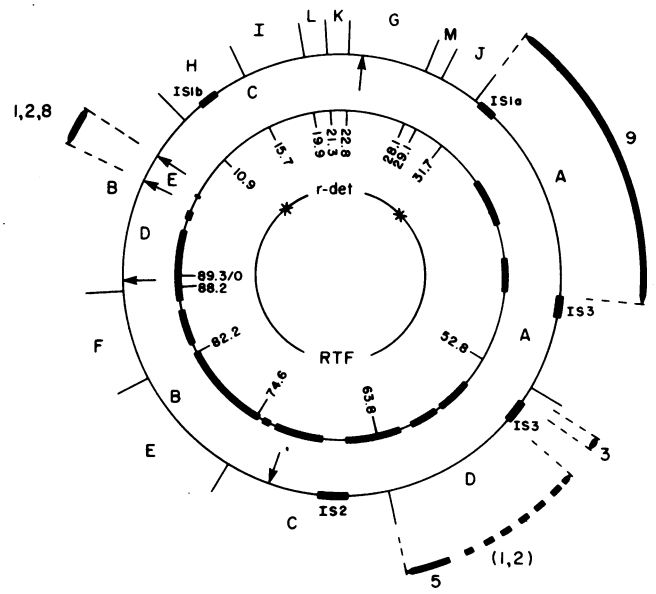

FIG. 5. Diagram of the position of the gene(s) for the different RNA species on the physical map of plasmid NR1. Outer circle indicates the position of the different insertion sequences present in NR1 plasmid DNA as well as, on the outside, the cleavage points for *Eco*RI (5) and, on the inside, for *Sal* I. Middle circle gives the kilobase coordinates; the regions of the plasmid DNA that are homologous to F are denoted by a heavy line (from ref. 5). Inner circle delineates the r-determinant and RTF portions of the DNA. The DNA segments where hybridization with the purified RNA species was detected are indicated by heavy lines. The joint hybridization of species 1 and 2 to fragment *Eco*RI-D (but not to λbb::Tn10 DNA) is indicated by a dashed line. Species 5 hybridizes within the region of homology with F located between the junction of *Eco*RI fragments C and D and coordinate 58 on the map. Detailed information concerning the *Eco*RI and *Sal* I restriction endonuclease maps of NR1 was kindly provided by R. Rownd and collaborators (18).

gerprint analysis and by hybridization to different portions of the plasmid genome. We have examined other resistance plasmids and have found that they code for their own specific collection of small RNA molecules unrelated to those of NR1 (unpublished observations).

The hybridization experiments show that these RNAs are derived from the RTF portion of NR1 and from the tetracycline resistance determinant Tn10. The physiological functions of these RNAs are not known, but their hybridization to plasmid DNA offers a possibility of correlating genetic function and RNA production. Fig. 5 summarizes results of localization of the plasmid RNAs on the plasmid genome.

One can speculate on functions that may be served by some or all of these RNA molecules. (*i*) They may be components of the translation apparatus that ensure the synthesis of certain plasmid functions under specialized circumstances. In this connection, they would serve the same purpose as that hypothesized for bacteriophage tRNA species (1). Since resistance plasmids can replicate and be expressed in various host bacterial species, they may synthesize components of the translation machinery necessary for plasmid maintenance in some hosts, under some conditions. RNA species 5 is one RNA that may serve such a function; it is similar to a tRNA in size, it is stable, and it contains a 3′ C-C-A sequence. However, species 5 does not contain modified bases and may not be a functional tRNA. The possibility that component 5 and 5S RNA molecules 9 and 10 may be required for translation under certain circumstances cannot be excluded; they may be derived from an organism unrelated to *E. coli* and not have sequence relationships with RNA molecules of similar size coded by *E. coli*. In addition, they may not be substrates for the RNA-modifying enzymes of *E. coli*.

(*ii*) Several bacterial operons are regulated by leader RNAs, which are involved in transcription termination (19). Some plasmid RNA species could serve this function; species 3, for example, is coded by the tetracycline resistance determinant Tn10, and species 5 appears to be associated with both plasmid and F transfer function. Any of these RNAs could be leader RNAs; obviously, species 3 may be involved in the expression of a function of Tn10 other than tetracycline resistance.

(*iii*) DNA replication, at least in some instances, is initiated from an RNA primer (20–22). It is possible that RNA species 1, 2, and 8 serve such a function in the replication of NR1. Warren *et al.* (23) have located an origin of plasmid DNA replication on the small *Sal* I fragment E, where the genes for RNA species 1, 2, and 8 are located (Fig. 5); it is tempting to speculate that these unstable RNAs serve as origin RNAs. In addition, we note that RNA species 5 hybridizes to a portion of NR1 DNA that is thought to contain the origin of plasmid DNA transfer, and it might be the primer for this purpose.

(*iv*) Bacterial cell division requires lipid and cell wall synthesis in addition to DNA replication. Transfer RNAs have been implicated in the biosynthesis of aminoacyl lipid (24) as well as of cell wall peptidoglycan (25). In the latter case, specialized tRNA molecules, which lack some of the base modifications of other tRNAs, are used for this purpose (26). RNA species 5 may be a similar molecule; its apparent lack of modification could be explained by its involvement in cell wall rather than protein synthesis. In view of the composite nature of R-plasmids, it is conceivable that they may have evolved with the capacity to synthesize some membrane functions that are necessary for their own replication.

Notwithstanding our failure to substantiate any of these speculations, it is apparent that resistance plasmids code for a variety of specific RNA molecules that may play important roles in their maintenance and expression.

We thank W. H. McClain for his interest and support and G. Högenauer, R. Jorgensen, D. Vapnek, C. Barton, R. Warren, and R. Rownd for communicating data before publication. We thank A. J. Clark, M. Nomura, and R. Jorgensen for strains. M.D.W. was a recipient of a long-term fellowship from the European Molecular Biology Organization. F.J.S. was a postdoctoral fellow of the National Institutes of Health (GM05450). This work was supported by funds from the University of Wisconsin Graduate School and by National Institutes of Health Grants AI10257 to W. H. McClain and AI10076 to J.E.D.

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