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# Studies on Resistance Transfer Factor Deoxyribonucleic Acid in *Escherichia coli*

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A variant of the derepressed R factor, R1, which does not contain any of the drug resistance markers, and represents, in large part, the resistance transfer factor (RTF) was studied in *Escherichia coli*. RTF deoxyribonucleic acid (DNA) was specifically labeled in a female cell after conjugation. Physical characterization of the molecule showed that RTF possessed an average molecular weight of $50 \times 10^6$ daltons and a buoyant density of 1.709 g/cm$^3$. By comparison to R1, we calculate that the region of DNA carrying the drug resistance genes is therefore about 20% of the R1 molecule and has a buoyant density of approximately 1.716 g/cm$^3$. These results support the hypothesis that the single species of R-factor DNA observed in *E. coli* represents a composite of the 1.709 and 1.716 g/cm$^3$ replicons seen in *Proteus*.

In a previous communication (10), we described a method for the specific radiolabeling and physical characterization of the derepressed R factor R1 in *Escherichia coli*. Only a single molecular species of R-factor deoxyribonucleic acid (DNA) was observed with a molecular weight of $65 \times 10^6$ daltons and a buoyant density of 1.711 g/cm$^3$ (10). In *Proteus mirabilis*, R1 has been shown to be composed of independently replicating components of buoyant density 1.709, 1.711, and 1.716 g/cm$^3$ with molecular weights of $55 \times 10^6$, $65 \times 10^6$, and $12 \times 10^6$ daltons, respectively (3; D. Haapala, Ph.D. Dissertation, Georgetown University, Washington, D.C., 1969; Cohen, *personal communication*). Furthermore, the transfer function of the R factor, RTF, is apparently associated with the 1.709 g/cm$^3$ replicon, whereas the drug resistance determinants reside on the 1.716 g/cm$^3$ species (S. Cohen, Bacteriol. Proc., p. 49, 1969; D. Haapala, Ph.D. Dissertation, 1969). We suggested that the single molecule of R1 observed in *E. coli* represents a composite structure resulting from a recombinational assemblage of a 1.709 and 1.716 g/cm$^3$ replicon (10). In this paper, we present evidence which infers that the drug resistance determinants associated with this single molecular species do, in fact, reside on a guanine plus cytosine (GC) rich stretch of DNA of approximately $12 \times 10^6$ daltons and that the buoyant density of the remainder of the molecule is, indeed, 1.709 g/cm$^3$.

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## MATERIALS AND METHODS

The bacterial strains and media used were previously described, together with centrifugation, DNA isolation, and mating techniques (10). The procedure for determining molecular size of an extrachromosomal element utilizing the techniques of alkaline sucrose sedimentation and X-ray target size was also previously described (6, 10). Assays of the male-specific phage, MS2, were performed by using Nutrient Agar (Difco) as a base agar overlayed with soft agar containing 0.75% Nutrient Agar supplemented with $10^{-3}$ M $CaCl_2$.

A segregant of the derepressed R factor, R1 (streptomycin sulfate, SM; sulfadiazine, SU; chloramphenicol, CM; ampicillin, AM; kanamycin, KM), lacking all drug resistance determinants was kindly provided by Elinor Meynell. The strain, although now sensitive to SU, SM, CM, AM, and KM, was still sensitive to the male-specific phage, MS2. Sex factor function, or RTF, was therefore still present. Furthermore, the strain could not be induced by mutagenesis to revert to transmissible drug resistance. RTF was transferred to DF110, the 5-bromouracil-resistant ($BU^r$) strain used as the host for the R1 factor. Since direct selection was not available, the recipient colonies were screened for male phage sensitivity to detect successful transfer. Of the initial six clones tested, all proved to be sensitive to MS2. The loss of drug resistance determinants had not effected the derepression of RTF function since transfer occurred at high frequency.

RTF DNA was specifically radiolabeled in a manner similar to that described for the R1 parent (10). The system consists of a male cell which contains a genetic block (resistance to BU) which prevents the incorporation of exogenous thymine and a female cell which cannot replicate its own DNA as a result of its inability to repair lesions produced by a heavy dose of ultraviolet (UV) light (7). When these cells are mated in the presence of radioactive thymine, the R factor, or any other transmissible element, is transferred with normal efficiency to the killed female and can replicate and be transcribed in the irradiated female. The specificity of the DNA labeled in this manner was demonstrated by DNA-DNA hybridization in agar gels (7, 10).

## RESULTS

The RTF-containing male donor (DF110-RTF) was mated with the UV-irradiated female (AB2500) and specifically radiolabeled RTF DNA was obtained as previously described (10). Figure 1 shows the sedimentation analysis of $^3$H-RTF-DNA after sucrose gradient centrifugation in comparison with the sedimentation of the $^3$H-R1 parent DNA. RTF preparations showed both a nicked circular form and the more rapidly sedimenting covalent circular species that had been observed with R1 (10).

The presence of covalently closed circular molecules in the preparations of $^3$H-RTF-DNA was verified, however, by centrifugation in alkaline sucrose gradients. If DNA is sedimented in alkali at a *p*H level above that necessary for denaturation, covalent circles will sediment three to four times faster than a single-strand filament with a molecular weight one-half that of the circle. Moreover, this species is confirmed to be a covalently closed molecule rather than a high-molecular-weight aggregate if a single-strand break in the molecule results in a discontinuous transition to a species which sediments three to four times more slowly. Figure 2 shows the sedimentation of $^3$H-RTF-DNA in alkaline sucrose after 20 min of centrifugation. A rapidly sedimenting molecule can clearly be seen. Single strand breaks can be introduced in a DNA molecule by X irradiation (5), and the results of various X-ray doses on the rapidly sedimenting species is shown in Fig. 3. The amount of fast-sedimenting material decreased as a result of the irradiation, and this decrease was discontinuous since DNA trailing behind the fast peak was not observed (Fig. 3).

It is apparent from Fig. 1 that the sedimentation of RTF is slower than the complete R factor. Clearly RTF is physically smaller than the parent R1, and the loss of drug resistance determinants resulted from a deletion rather than a mutational event. $S_{20,w}$ values of 64 and 44*S*

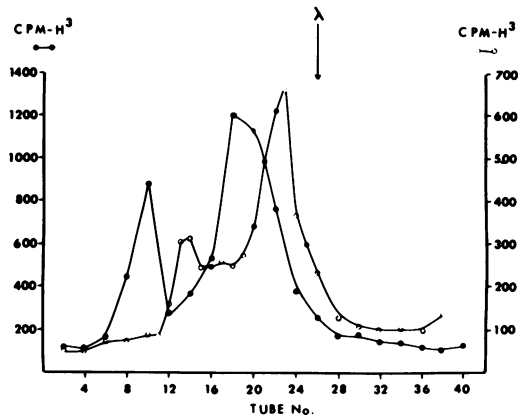


FIG. 1. *Sucrose gradient sedimentation of $^3$H-thymine RTF DNA. DNA was extracted 60 min after DF-110 RTF and AB2500 F$^-$ were mixed at 37 C in glucose-Casamino Acid medium containing 1 μg of $^3$H-thymine per ml. A 0.1-ml sample of the lysate was layered over a 5 to 20% sucrose gradient made up in 0.5 M NaCl; 0.01 M potassium phosphate (pH 6.8). Centrifugation was carried out for 2 hr at 35,000 rev/min (100,000 × g) in the SW39 rotor of a Spinco preparative ultracentrifuge at a chamber temperature of 4 C. $^{14}$C-thymine-labeled λ bacteriophage DNA (13,000 counts per min per μg) was added as a sedimentation reference (34S). Five-drop fractions were collected through a hole punctured in the bottom of the tube. The fractions were precipitated with trichloroacetic acid, collected on membrane filters, and counted. The sedimentation of $^3$H-thymine-labeled R1 drd DNA is shown (●) for comparison since it was the plasmid from which RTF was derived. The R1 drd DNA was prepared the same day, treated identically, and centrifuged in a separate tube at the same time as the $^3$H-thymine-labeled RTF DNA. The gradients had an identical number of fractions and the λ reference sedimented identically in each.*

were obtained for the closed and open forms of the RTF molecule, respectively, as compared to 75*S* and 50*S* for R1. The theoretical *S* value of the linear RTF molecule could be calculated since it has been shown that circular DNA with single-strand scissions sediments 1.14 times as rapidly as the linear form (12). The *S* value (39*S*) calculated in this manner corresponds to a molecular weight of 50 × 10$^6$ daltons (11) as compared to a molecular weight of the parental R1 molecule, also determined by sedimentation in neutral sucrose gradients, of 65 × 10$^6$ daltons (10). Thus, the loss of drug resistance determinants from R1 had apparently resulted in a deletion of R factor DNA of approximately 15 × 10$^6$ daltons or 23% of the molecule.

The molecular size of RTF DNA was also determined by using the techniques of alkaline sucrose sedimentation and X-ray target size as

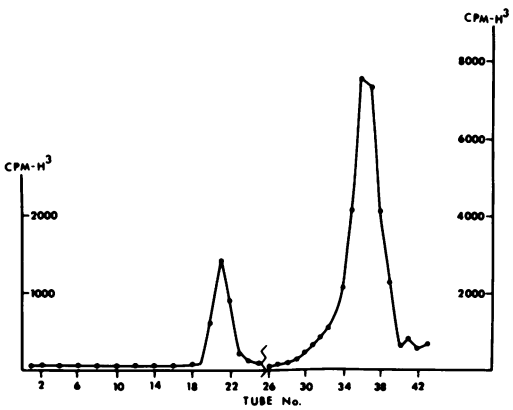


FIG. 2. *Alkaline sucrose gradient sedimentation of ³H-thymine-labeled RTF DNA. A 0.1-ml sample of DNA extracted from a 60-min mating was layered over a 5 to 20% sucrose gradient made up in 0.3 N NaOH, 0.01 M ethylenediaminetetraacetate (pH 12.5). Centrifugation was carried out at 38,000 rev/min (115,000 × g) in a SW39 rotor of a Spinco preparative ultracentrifuge at a chamber temperature of 4 C. Five-drop fractions were collected through a hole punctured in the bottom of the tube, and the fractions were assayed for trichloroacetic acid-precipitable radioactivity. Note the change of scale.*

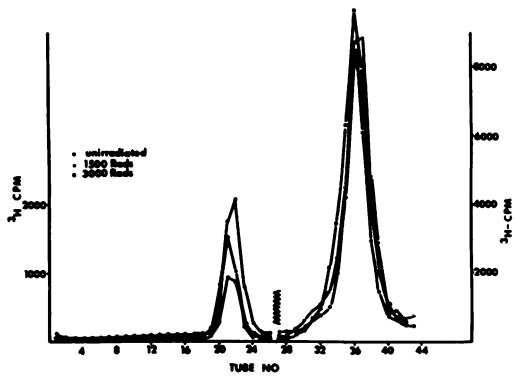


FIG. 3. *Effect of X irradiation on the sedimentation of ³H-thymine-labeled RTF DNA. Portions of ³H-thymine-labeled RTF DNA were placed in cellulose nitrate tubes 35 cm from an X-ray source calibrated to give 256 rad/min at the level of the sample. A 0.1-ml sample of unirradiated (●), 1,500 rad (○), and 3,000 rad (□) DNA was layered over an alkaline 5 to 20% sucrose gradient and treated as described in the legend to Fig. 2.*

described by Freifelder (6). We previously used this procedure to determine the molecular weight of R1 DNA and obtained a size of 65 × $10^6$ daltons (10). The decay of covalently closed RTF DNA was determined from sedimentation in alkaline sucrose gradients (Fig. 3). The $D_{37}$ dose (the dose at which 37% of the initial covalent circles remained intact) was used to calculate the relative size of RTF DNA by the following relationship: molecular weight = ($D_{37F\text{-}lac}$/$D_{37RTF}$) × (100 × $10^6$) (reference 10). A molecular weight of RTF DNA of 52 × $10^6$ daltons was obtained from a $D_{37}$ ratio of 0.52, indicating a deletion of 13 × $10^6$ daltons or 20% of the R1 parent. These data are consistent with the results obtained from sucrose gradient analysis, indicating that the loss of drug determinants from R1 is concomitant with a deletion of a large segment of the molecule.

The ³H-RTF-DNA was then centrifuged to equilibrium in a CsCl density gradient to determine whether the deletion had effected the buoyant density of the molecule. The results in Fig. 4 show that RTF bands with a buoyant density equivalent to that of lambda DNA or 1.709 g/cm³. The loss of drug determinants, therefore, had not only decreased the size of the molecule but had also resulted in a decrease in the buoyant density of the molecule from 1.711 g/cm³ to 1.709 g/cm³. The density of the deleted portion of the R-factor molecule could be calculated by

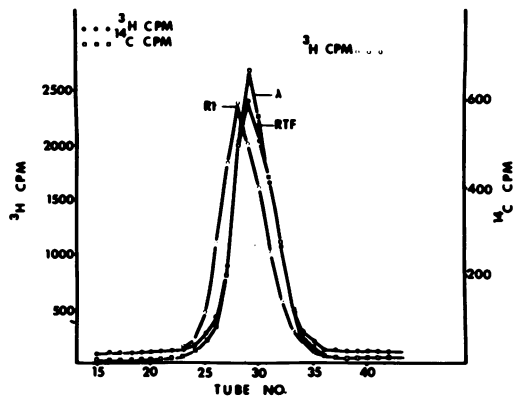


FIG. 4. *Equilibrium centrifugation of ³H-thymine-labeled RTF DNA in CsCl. ³H-thymine-labeled RTF DNA from a 60-min mating mixture was added to a CsCl solution and adjusted to a final density of 1.710 g/cm³ (refractive index, 1.4010). ¹⁴C-thymine-labeled λ bacteriophage DNA was added as a density marker (1.709 g/cm³). The mixture was centrifuged for 60 hr at 35,000 rev/min (80,000 × g) in a type 40 rotor of a Spinco preparative ultracentrifuge at a chamber temperature of 4 C. Seven-drop fractions were collected through a hole punctured in the bottom of the tube, and each fraction was assayed for trichloroacetic acid-precipitable radioactivity. The banding of ³H-thymine-labeled drd R1 DNA is shown for comparison since it was the plasmid from which RTF was derived. The R1 drd DNA was prepared the same day, treated identically, and centrifuged in a separate tube at the same time. The gradients had an identical number of fractions, and the λ reference DNA banded identically in each gradient.*

a simple equation relating the whole to the sum of its parts: 1.711 = 1.709 (size RTF/size R1) + density deletion (size deletion/size R1). When the equation was solved it became apparent that the composition of the DNA deleted from R1, which represents (at least in part) the drug resistance determinants (Am, Cm, Su, Sm, Km) was associated with the loss of material with a high buoyant density (1.716 to 1.720 g/cm³ in different experiments). Moreover, these data suggest that the independent replicon with an average density of 1.716 g/cm³ observed in *Proteus* does indeed exist as an integral part of a single replicon in *E. coli*.

## DISCUSSION

There have been some differing views with regard to the molecular nature of the drug resistance determinants carried by R factors. The initial observations on the DNA of R factors from this laboratory indicated that most drug resistance genes (Cm, Sm, Km, Su) resided on a stretch of DNA with a buoyant density of 1.716 g/cm³ with the notable exception of tetracycline which was identified on a DNA molecule with an average buoyant density of 1.709 g/cm³ (2). Rownd, Nakaya, and Nakamura (9) concluded, however, that all the drug resistance genes and the loci responsible for infectivity reside on a 1.712 g/cm³ component. The results which we have presented tend to confirm the view that most drug resistance determinants associated with an R factor reside on DNA with a high GC content. Furthermore, we conclude that the independent 1.709 g/cm³ replicon evident in *Proteus* does represent the resistance transfer factor, whereas most drug resistance determinants are associated with the 1.716 g/cm³ replicon.

It is clear that the ability of the 1.709 and 1.716 g/cm³ replicons to exist autonomously and independently in *Proteus* indicates the presence of separate and independent determinants controlling the replication of each. Our results in *E. coli* show, however, that the 1.716 g/cm³ replicon can also behave as a functional part of the 1.709 g/cm³ replicon. We believe that this situation is formally analogous to the insertion of phage lambda into the *E. coli* chromosome. We would predict then that the single composite molecule in *E. coli* possesses more than one replication system although one would presumably be repressed. The multiple representation of replication genes could account for the inability to isolate replication defective R factors, as has been suggested by Novick (8). It should be noted that the proposed repression of replication of the 1.716 g/cm³ replicon does not affect the constitutive expression of the drug resistance determinants. This suggests that the drug determinants have their own controlling genes.

The control mechanism which we have described is clearly operative and stable in *E. coli*. However, in *Proteus*, this control appears to be ineffective, permitting the release of the 1.716 g/cm³ replicon and the independent replication of each of the components. This is not a unique situation with regard to regulatory behavior of "foreign genes" in *Proteus*. Falkow et al. (4) transferred the F-merogenote F-*lac*, from *E. coli* to *Proteus*. *Proteus* F-*lac* strains synthesized β-galactosidase which was indistinguishable from that produced in *E. coli*. The *Proteus* F-*lac* strains, however, showed constitutive enzyme synthesis and were not derepressed by inducers known to function in *E. coli*. Hence, it appears that the regulatory functions of some *E. coli* genes are impaired when they are transferred to *Proteus*.

The isolation of the 1.709 g/cm³ RTF replicon directly in *E. coli* could be interpreted to mean that the composite molecule is also capable of dissociation in *E. coli*. It is conceivable that a small degree of dissociation would have been below the level of detection of the techniques we used. This is particularly cogent in view of the recent observations of Cohen and Miller (1), in which electron microscopic examination of R1 DNA in *E. coli* revealed that an amount of DNA totaling less than 1 per cent of the total R-factor preparation was represented by a 5.5 μm replicon. The only other species of R-factor DNA detected, however, was the 65 × 10⁶ dalton composite molecule (1).

### ACKNOWLEDGMENT

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