

## Transition of R Factor *NR1* in *Proteus mirabilis*: Molecular Structure and Replication of *NR1* Deoxyribonucleic Acid

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The structure of R factor *NR1* DNA in *Proteus mirabilis* has been studied by using the techniques of CsCl density gradient centrifugation, sedimentation in neutral and alkaline sucrose gradients, and electron microscopy. It has been shown that the nontransitioned form of *NR1* DNA isolated from *P. mirabilis* cultured in drug-free medium is a 37- $\mu$ m circular deoxyribonucleic acid (DNA) with a density of 1.712 g/ml in a neutral CsCl gradient. This circular molecule is a composite structure consisting of a 29- $\mu$ m resistance transfer factor containing the tetracycline-resistance genes (RTF-TC) and an 8- $\mu$ m r-determinants component conferring resistance to chloramphenicol (CM), streptomycin/spectinomycin, and the sulfonamides. There are one to two copies of *NR1* per chromosome equivalent of DNA in exponential-phase cells cultured in Penassay broth. After growth of Pm15/*NR1* in medium containing 100  $\mu$ g of CM per ml, the density of the *NR1* DNA increased from 1.712 g/ml to approximately 1.718 g/ml and the proportion of *NR1* DNA relative to the chromosome is amplified about 10-fold. The changes in R factor DNA structure which accompany this phenomenon (termed the transition) have been studied. DNA density profiles of the transitioned *NR1* DNA consist of a 1.718-g/ml band which is skewed toward the less dense side. The transitioned *NR1* DNA consists of molecules containing the RTF-TC element attached to multiple copies of r-determinants DNA (poly-r-determinant R factors) and multimeric and monomeric autonomous r-determinants structures. Poly-r-determinant R factors have a density intermediate between the basic composite structure (1.712 g/ml) and r-determinants DNA (1.718 g/ml). These species presumably account for the skewing of the 1.718-g/ml DNA band toward the less dense side. When transitioned cells are subsequently cultured in drug-free medium, poly-r-determinant R factors and autonomous poly-r-determinants undergo dissociation to form smaller structures containing fewer copies of r-determinants. This process continues until, after prolonged growth in drug-free medium, the *NR1* DNA returns to the nontransitioned state which consists of an RTF-TC and a single copy of r-determinants.

Drug resistance factors (R factors) are extra-chromosomal genetic elements in bacteria which confer multiple drug resistance to host cells (1, 5, 10, 28, 29). Genetic (28, 29) and physical (1, 3, 4, 5, 7, 16, 21, 22, 24, 25) analyses of R factors have shown that these plasmids consist of two distinguishable components: the resistance transfer factor (RTF) component which is involved in the infectious transfer of the R factor and the r-determinants component which harbors most of the drug resistance genes. In the case of the R factor *NR1*, the RTF carries the tetracycline (TC) resistance genes (18, 26) (referred to as an RTF-TC), whereas the r-determinants carries the genes for resistance to

chloramphenicol (CM), streptomycin/spectinomycin, and the sulfonamides.

Several generalizations can be made about the properties of R factor deoxyribonucleic acid (DNA) when these plasmids are harbored by *Escherichia coli*, *Salmonella typhimurium*, or *Serratia marcescens*. The R factor DNA usually forms a single band in either neutral or alkaline CsCl density gradients (1, 3, 4, 21). A single band is also observed when the covalently closed circular form of the R factor DNA is examined in ethidium bromide-CsCl density gradients (1, 21). When the size of the plasmid DNA has been examined, all of the R factor DNA in these hosts has usually been found to

consist of molecules of the same molecular weight. In *E. coli* the RTF and r-determinants components are united to form a composite R factor structure. There are approximately one to two copies of the R factors *NR1*, R6, and R1 per chromosome equivalent of DNA in *E. coli* or *S. marcescens* (1, 3, 4, 21).

In studies on the molecular structure of a number of different R factors in several different strains of *Proteus mirabilis*, on the other hand, multiple satellite DNA bands have been observed in a CsCl density gradient (3, 4, 6, 7, 16, 21, 22, 24, 25). The density of the R factor DNA as well as the proportion of the multiple satellite bands relative to the host chromosomal DNA can vary widely, depending on the conditions under which the host cells are cultured (21, 22, 24, 25).

The explanation for the origin of the multiple R factor DNA satellite bands in *P. mirabilis* has been a subject of considerable controversy. Four groups of investigators have suggested that the multiple R factor DNA bands are due to the dissociation of the composite R factor into the RTF and r-determinants components (1, 3, 4, 7, 16, 19). This interpretation was based primarily on the contour lengths of circular R factor DNA molecules measured from electron micrographs. The observed contour lengths were consistent with those expected for the RTF, the r-determinants, and composite R factor molecules. These observations, together with the observation of multiple satellite bands in a CsCl density gradient, suggested dissociation of the composite R factor in *P. mirabilis*. However, in none of these investigations was compelling evidence presented that the circular molecules identified by electron microscopy were representative of the major fraction of the R factor DNA present in the host cells. It should also be noted that several of the drugs to which the R factor confers resistance were always included in the culture medium in all of these experiments.

Our laboratory has also suggested that dissociation of R factors into the RTF and r-determinants components is possible in *P. mirabilis*. Moreover, we have proposed that multiple copies of r-determinants can be incorporated in tandem sequences into R factors in this host to form poly-r-determinant R factors. Autonomous poly-r-determinants consisting of multiple copies of r-determinants DNA also can be formed. We have suggested that these poly-r-determinant structures are formed when *P. mirabilis* is cultured in medium containing drugs to which r-determinants confer resistance. In this way the number of copies of r-determinants per cell,

and hence the level of drug resistance of the host cells, is increased. These changes in the structure of the R factor DNA (referred to as the transition) can account for the presence of the multiple R factor DNA bands which are observed when *P. mirabilis* is cultured in medium containing appropriate drugs (21, 22, 24, 25). According to the transition model, monomeric units of the autonomous RTF and r-determinants components need not be present in any significant amount during growth of R<sup>+</sup> *P. mirabilis* in medium containing drugs.

In this communication we describe a number of interrelated experiments on the structure of R factor *NR1* DNA in *P. mirabilis* when the host cells have been cultured under several different conditions. The R factor DNA was characterized using CsCl density gradient centrifugation, sucrose gradient sedimentation, and electron microscopy. These experiments have shown that when *P. mirabilis* is cultured in drug-free medium essentially all of the *NR1* DNA is in the form of the composite structure which undergoes no detectable dissociation into the RTF-TC and r-determinants components. This will be referred to as the nontransitioned state of *NR1* DNA. However, after the host cells have been cultured in medium containing CM, there is an increase in the density and the proportion of the plasmid DNA relative to the host chromosomal DNA. Moreover, the *NR1* DNA has a much higher molecular weight than nontransitioned *NR1* DNA and is also quite heterogeneous in size (referred to as transitioned *NR1* DNA). These changes are consistent with the formation of poly-r-determinant R factors and autonomous poly-r-determinants as a consequence of growth of the *P. mirabilis* cells in medium containing CM. The transition can be reversed by culturing transitioned cells in drug-free medium for many generations. This gradual return to the nontransitioned state is termed the "back transition." Our experiments suggest that r-determinants are dissociated from poly-r-determinant R factors and autonomous poly-r-determinants during the back transition and are subsequently diluted from the cells. After several hundred generations of growth in drug-free medium, the *NR1* DNA returns to the nontransitioned composite R factor structure which consists of an RTF-TC and a single copy of r-determinants.

#### MATERIALS AND METHODS

**Bacterial strains and R factors.** Both *P. mirabilis* strains used in this study were derived from *P. mirabilis* Pm1 which has been described previously

(20). Pm15/*NR1* is lactose and galactose negative and requires nicotinic acid, tryptophan, leucine, and thymine for growth and is TC resistant, owing to a chromosomal TC resistance gene (11). The R factor *NR1* confers resistance of TC, CM, streptomycin/spectinomycin, and sulfonamides (15, 23). Two segregants of *NR1* which had deleted the r-determinants component and thus confer only TC resistance (RTF-TC) were also used in this study. A TC-sensitive host strain ( $\phi$ S-3) which has been described previously (15) was used in these experiments. A thymine-requiring mutant of  $\phi$ S-3/RTF-TC was isolated by the aminopterin method (2).

**Media and culture conditions.** To maintain reproducible starting cultures of nontransitioned Pm15/*NR1*, 2 volumes of a nontransitioned Penassay broth culture were mixed with 1 volume of 50% glycerol, and aliquots were distributed to a series of vials which were frozen at  $-70^{\circ}\text{C}$ . These vials were thawed and used to inoculate staining cultures. Cells were cultured at  $37^{\circ}\text{C}$  with vigorous aeration in either Penassay broth (Difco) or minimal M9 medium containing 0.03% Casamino Acids (Difco) and other supplements essentially as described previously (13, 20). Penassay broth which contains approximately  $2\ \mu\text{g}$  of thymine per ml was usually supplemented with an additional  $3\ \mu\text{g}$  of thymine per ml; minimal medium contained  $5\ \mu\text{g}$  of thymine per ml. When [ $^{14}\text{C}$ ] and [ $^3\text{H}$ ]thymine (New England Nuclear) were used to label DNA, typical specific activities were approximately 0.1 and  $5\ \mu\text{Ci}/\text{ml}$  of thymine for [ $^{14}\text{C}$ ] and [ $^3\text{H}$ ]thymine, respectively.

**DNA isolation and CsCl density gradient centrifugation.** About  $10^{10}$  cells were harvested, washed two times with cold saline-ethylenediamine-tetraacetic acid (0.1 M disodium ethylenediaminetetraacetic acid, 0.1 M NaCl; adjusted to pH 8.0 with NaOH) and resuspended in saline-ethylenediaminetetraacetic acid. DNA was isolated by the procedure described previously (20) and redissolved in  $0.1\times$  SSC (0.015 M NaCl, 0.0015 M sodium citrate, pH 7.0). The DNA solution was heated to  $65^{\circ}\text{C}$  for 30 min. The DNA was analyzed by analytical CsCl density gradient centrifugation, as described previously (13, 20). For preparative CsCl density gradient centrifugation, the cell suspension ( $10^{10}$  cells/ml) was mixed with Pronase (CalBiochem) which had been heated at  $80^{\circ}\text{C}$  for 10 min; the final concentration was  $2\ \text{mg}/\text{ml}$ . The cells were lysed with 0.5% sodium dodecyl sulfate and then heated to  $65^{\circ}\text{C}$  for 30 min. It was cooled, mixed with 4 ml of saturated CsCl in  $0.1\times$  SSC and centrifuged for 65 h at 35,000 rpm in a Ti50 rotor (Beckman) at  $23^{\circ}\text{C}$ . The rotor was coasted (unbraked) to a stop. Five-drop fractions were collected slowly through a 22-gauge needle which had been used to pierce the bottom of the centrifuge tube. By measuring the absorbancies (260 nm) of the fractions containing the chromosomal DNA (assuming an absorbance of 1.0 corresponds to  $50\ \mu\text{g}$  of DNA per ml), it was found that typical gradients contained about  $150\ \mu\text{g}$  of DNA.

**Counting of radioisotopes.** Gradients containing

radioactive DNA were fractionated and approximately  $5\text{-}\mu\text{l}$  aliquots of each fraction were placed on Whatman no. 42 filter paper squares. These were then washed three times with 7.5% trichloroacetic acid and then three times with 95% ethanol, and dried in an oven at  $160^{\circ}\text{C}$ . The filter paper squares were added to scintillation vials containing 5 ml of toluene and 2,5-diphenyloxazole at a concentration of 5 g/liter and counted using a Nuclear Chicago model 6848 scintillation counter. Counting accuracy was typically  $\pm 3$  to 5%.

**Sedimentation of DNA in sucrose gradients.** Fractions from CsCl preparative gradients containing *NR1* DNA were stored in the concentrated CsCl (approximately 1.70 g/ml) until used for sedimentation analysis. The DNA was then dialyzed against  $10\times$  SSC using dialysis tubing which had been boiled in a solution of 5% sodium bicarbonate and then in a solution of 0.01 M ethylenediaminetetraacetic acid. The tubing was finally boiled in  $0.1\times$  SSC just before use. Less than  $1\ \mu\text{g}$  of DNA in a volume of  $50\ \mu\text{l}$  was applied to a 5-ml 5 to 20% linear sucrose gradient. Neutral sucrose gradients contained  $0.1\times$  SSC and alkaline sucrose gradients contained 0.3 M NaOH. Linear phage  $\lambda$  DNA labeled with [ $^{14}\text{C}$ ]thymine was used as a sedimentation standard. The gradients were centrifuged at 35,000 rpm in an SW50 rotor at  $20^{\circ}\text{C}$ . Fractions were either collected and analyzed as described previously for the CsCl preparative gradients' or five-drop fractions were collected directly onto Whatman no. 42 filter paper squares lying on a strip of aluminum foil. The filter paper squares were dried in an oven at  $160^{\circ}\text{C}$  for 3 to 5 min and counted as described above. After the fractionation of sucrose gradients, the lower part of all centrifuge tubes were cut out, dried, and counted. These pelleted counts were always included in the counts of fraction number one of the gradient profiles.

**Electron microscopy.** Freshly dialyzed DNA samples ( $1$  to  $4\ \mu\text{g}/\text{ml}$ ) in  $0.1\times$  SSC were mixed with formaldehyde buffer, formamide, and cytochrome *c* and spread on an aqueous hypophase as described by Inman and Schnös (12). The DNA-cytochrome *c* films were picked up on carbon films on mica disks and rotary shadowed with platinum. The shadowed films were floated onto distilled water, picked up on 200-mesh copper grids, and examined in a Philips model 300 electron microscope (EM) at about  $\times 7,000$  magnification. For calibration of magnification, a 54,800-line-per-inch cross-lined standard grid (Pelco) was used. In addition, the contour lengths of linear phage  $\lambda$  DNA molecules which were spread in an identical manner were measured. The film negatives were projected onto a paper screen and contour lengths of the DNA molecules were traced and measured with a Keuffel and Esser map measurer. Final graphical magnification was about 100,000 times.

## RESULTS

**Molecular weight and structure of non-transitioned *NR1* DNA in *P. mirabilis*.** To examine the structure of nontransitioned *NR1*

DNA, single colonies of Pm15/*NR1* were picked from drug-free MacConkey agar plates and grown to stationary phase in Penassay broth or minimal medium. The DNA prepared from these nontransitioned cultures was examined in an analytical ultracentrifuge and showed a single 1.712-g/ml *NR1* satellite DNA band in a neutral CsCl density (Fig. 1A). This single *NR1* DNA band of density 1.712 g/ml was reproducibly observed in both exponential and stationary

phase Penassay broth or minimal medium cultures. A consistent difference has been observed in the percentage of *NR1* DNA relative to the chromosomal DNA depending on the growth phase of the cells (13). There is much as a fourfold increase in the percentage of copies of nontransitioned *NR1* DNA after entry of an exponential phase culture into stationary phase (data not shown).

The structure of nontransitioned *NR1* DNA in *P. mirabilis* was examined by first labeling the DNA with [<sup>3</sup>H]thymine and then fractionating the DNA in a preparative CsCl density gradient. A typical fractionation profile is shown in Fig. 1B. The fractions containing *NR1* DNA were pooled, dialyzed to remove CsCl, and then studied by sedimentation in a sucrose gradient and by EM. In the majority of sedimentation experiments with *NR1* DNA in neutral (pH 7.0) sucrose gradients, a single 48.5S band was observed (Fig. 1C) using <sup>14</sup>C-labeled linear phage λ (34.4S) as a sedimentation standard (8).

EM preparations of purified nontransitioned *NR1* DNA showed only one class of nicked circular molecules having a contour length of 37 μm (Fig. 2B). The mean contour length of 40 molecules was 37.0 μm (σ = 0.4 μm) (Table 1). The same results were obtained for *NR1* DNA from exponential or stationary phase cells which were cultured either in Penassay broth or minimal medium. Phage λ DNA which was spread under identical conditions measured 17.5 μm relative to a calibration grid. Since many studies have shown EM contour lengths of DNA to vary with the spreading conditions, the λ DNA standard was itself a calibration standard; i.e., the *NR1* DNA measured 2.1 λ units. Some shorter heterogeneous linear molecules representing about 25% of the *NR1* DNA were observed in some regions of the EM grids; other areas of the grids showed nearly 100% intact

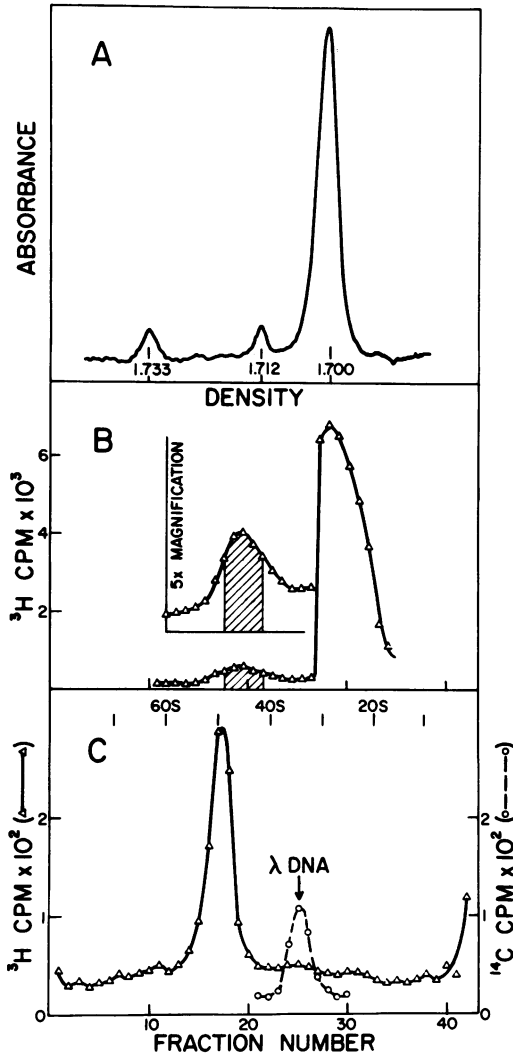


FIG. 1. Characterization of nontransitioned R factor *NR1* DNA in *P. mirabilis*. DNA was isolated from a stationary phase culture of nontransitioned Pm15/*NR1* in Penassay broth containing [<sup>3</sup>H]thymine. (A) Microdensitometer tracing showing the distribution of DNA at equilibrium in a neutral (pH 8.0) CsCl density gradient at 44,000 rpm. The ordinate repre-

sents the DNA concentration as a function of distance from the axis of rotation. *Micrococcus lysodeikiticus* DNA (1.733 g/ml) was included in the gradient as a reference density. (B) Fractionation profile of the DNA in a CsCl preparative gradient. The *NR1* DNA in the fractions which are included in the cross-hatched area was pooled and dialyzed for analysis by sucrose gradient sedimentation. (C) Sedimentation profile of fractionated *NR1* DNA in a 5 to 20% linear neutral sucrose gradient at 35,000 rpm for 2.5 h at 20 C. The linear form of <sup>14</sup>C-labeled λ DNA (34.4S) was co-sedimented with the *NR1* DNA as a sedimentation coefficient standard. The linear S scale shown at the top of profile (C) was calculated from the distance that the λ DNA sedimented under the conditions of the experiment.

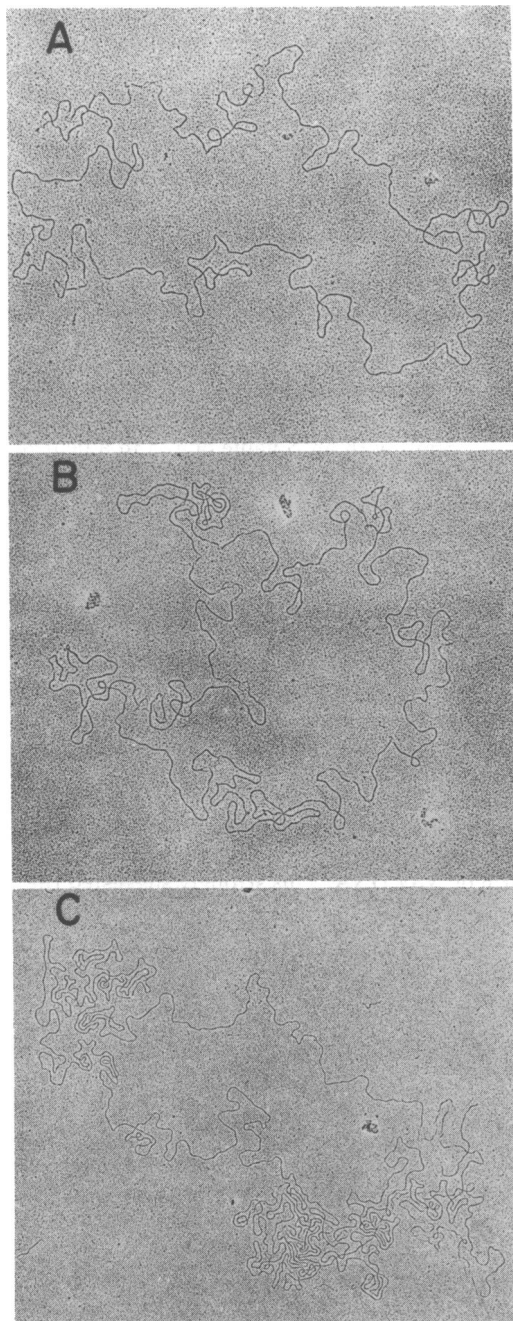


FIG. 2. EM of RTF-TC, composite R factor, and poly-r-determinant R factor NR1 DNA. R factor DNA molecules from the experiments described in the text were prepared for EM and examined in the electron microscope, and their contour lengths were measured as described. (A) RTF-TC DNA, contour length 28.7  $\mu\text{m}$ ; (B) composite R factor DNA, contour length 37  $\mu\text{m}$ ; (C) poly-r-determinant R factor DNA, contour length 70  $\mu\text{m}$ . A few small denatured

TABLE 1. Contour lengths of circular R factor NR1 DNA molecules in nontransitioned and transitioned cultures of *Proteus mirabilis*<sup>a</sup>

R factor	Buoyant density (g/ml)	Contour lengths <sup>b</sup>
RTF-TC DNA	1.710	28.7 $\pm$ 0.2 (18)
Nontransitioned composite NR1 DNA	1.712	37.0 $\pm$ 0.4 (40)
NR1 DNA during back transition	$\sim$ 1.713	45.9 $\pm$ 0.4 (6)
	$\sim$ 1.715	53.8 $\pm$ 0.4 (8)
	$\sim$ 1.715	62.2 $\pm$ 0.5 (9)
Transitioned NR1 DNA	1.718	8.2 $\pm$ 0.05 (7)
	1.718	16.3 $\pm$ 0.1 (7)
	1.718	24.4 (1)
	1.718	33.9 (7)
	1.718	42.0 (1)

<sup>a</sup> Using CsCl preparative density gradient centrifugation, R factor DNA was purified from cultures of *P. mirabilis* harboring either the RTF-TC component, nontransitioned composite NR1 DNA, or transitioned NR1 DNA. R factor DNA from the specified density regions of the preparative CsCl gradients was examined in an electron microscope and the contour lengths of the indicated number of circular molecules were measured.

<sup>b</sup> Parentheses indicate number of molecules.

37- $\mu\text{m}$  circular DNA. These observations, together with the single 48.5S species seen in sucrose gradients, suggests that the linear DNA molecules are artifacts due to fragmentation of the DNA during the spreading procedure.

If we reduce the 48.5S value of the nicked circular DNA by 10% to correspond to the S value for linear DNA of the same molecular weight (27), we can use the Freifelder equation (8)  $[S_1/S_2 = (M_1/M_2)^{0.38}]$  to determine the molecular weight of the linear form of the NR1 DNA. Using linear phage  $\lambda$  DNA as a sedimentation and molecular weight ( $M$ ) standard ( $30 \times 10^6$  daltons; 34.4S), the molecular weight calculated for the NR1 DNA is  $53 \times 10^6$ . However, EM determinations relative to phage  $\lambda$  indicate a value of  $63 \times 10^6$  daltons ( $2.1 \times 30 \times 10^6$ ). All of our S values from sucrose gradient sedimentation have been 5 to 10% lower than

regions appear in the RTF-TC component of these molecules since the DNA was spread for EM at pH 10.7 in our studies on partial denaturation mapping of R factor DNA (18). At this pH the r-determinants DNA remains relatively undenatured because of its higher guanine plus cytosine content. In the molecules shown in (C) the poly-r-determinant region is to the left and the RTF-TC region is at the lower right.

the values expected from EM determinations of molecular weight. This discrepancy has been discussed elsewhere (14).

Of the several hundred circular molecules which have been viewed by EM, some rare 37- to 37- $\mu\text{m}$  catenated dimers and 74- $\mu\text{m}$  concatenated dimers have been observed. The sucrose gradient profiles lack significant radioactive counts in the 66S region where such molecules would sediment. This places a rough upper estimate on the fraction of molecules in these categories of about 2%.

This laboratory has previously observed that a maximum of 30% of nontransitioned *NR1* DNA can be isolated as covalently closed circular (CCC) DNA from *P. mirabilis* when the cells are cultured in Penassay broth (C. Morris and R. Rownd, submitted for publication). In the experiments described here, some degree of nicking of the *NR1* DNA must have occurred during fractionation in a CsCl preparative gradient and in the steps preceding sucrose gradient sedimentation of the DNA. In the majority of our experiments using Penassay broth cells, most of the *NR1* DNA sedimented as a single 48.5S band (Fig. 1C) in a neutral sucrose gradient and very little *NR1* DNA was observed which had an S value corresponding to the CCC form (ca. 70S) under the conditions of our experiments.

*Pm15/NR1* cells cultured in M9 minimal medium yielded a higher proportion of CCC *NR1* DNA in agreement with our previous

observations (C. Morris and R. Rownd, submitted for publication). When fractionated *NR1* DNA isolated from cells cultured in minimal medium was examined in a neutral sucrose gradient (Fig. 3A), in addition to the 48.5S peak, a 70S peak was observed which corresponds to the CCC form of *NR1* DNA. Since the proportion of CCC *NR1* DNA in *P. mirabilis* has been examined in detail in other experiments in this laboratory (C. Morris and R. Rownd, submitted for publication), we have not examined this point in the experiments described in this communication.

In some experiments using nontransitioned *Pm15/NR1* cells which were cultured in either Penassay broth or minimal medium, *NR1* DNA (1.712 g/ml) with an S value of 42S in a neutral sucrose gradient was observed in addition to the nicked circular (48.5S) form of the R factor DNA (Fig. 3B). The 42S DNA was most often observed in experiments where the DNA was purified by precipitation prior to fractionation or after longer periods of storage of *NR1* DNA which, when first examined, showed only the 48.5S peak. The DNA used in the sedimentation experiment shown in Fig. 3B is from the same DNA sample used in the experiment shown in Fig. 3A, except that the DNA had been stored at 4 C for 2 months. Isolation and fractionation of the *NR1* DNA by a procedure which does not involve precipitation of the DNA, however, usually yielded only the 48.5S form of *NR1* DNA. This strongly suggested that

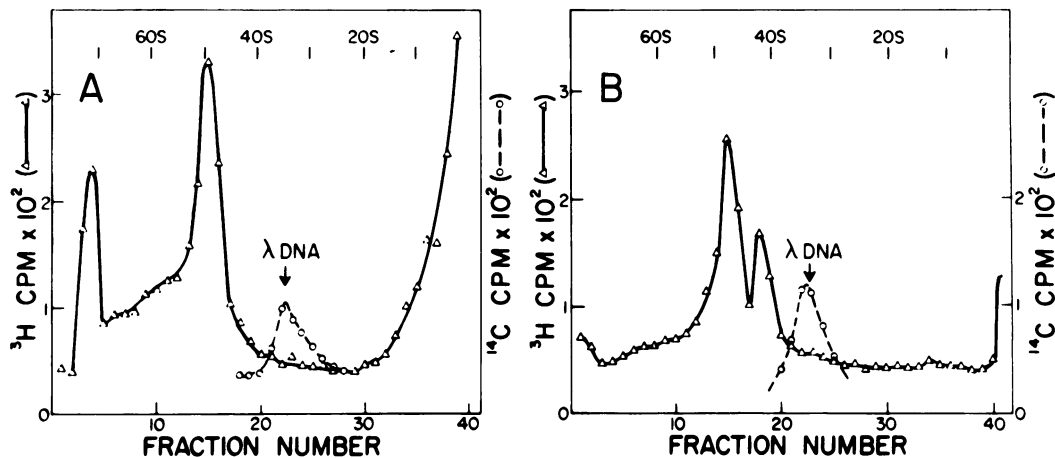


FIG. 3. Sucrose gradient sedimentation profiles of nontransitioned R factor *NR1* DNA. DNA was prepared from a stationary phase culture of nontransitioned *Pm15/NR1* which was cultured in M9 minimal medium containing [<sup>3</sup>H]thymine. (A) Sedimentation profile of fractionated *NR1* DNA in a neutral sucrose gradient under the same conditions described in the legend to Fig. 1. (B) Sedimentation profile of the fractionated *NR1* DNA in a neutral sucrose gradient after the DNA had been stored for 2 months at 4 C. Both sucrose gradients included <sup>14</sup>C-labeled λ DNA as a sedimentation standard.

the 42S DNA is linear DNA derived from the 37- $\mu$ m circular form due to a double-strand break, as will be discussed in further detail in the following section.

**Molecular weight and structure of the RTF-TC component.** The most common genetic segregant of NR1 has TC resistance and transferability but has lost the antibiotic resistances which reside on the r-determinants component (30). If, in fact, the r-determinants component is deleted, physical studies should confirm this. Two such genetic segregants of NR1 were examined. One was a spontaneous segregant, isolated by H. Hashimoto; the other was derived from an interrupted mating experi-

ment by D. Taylor in this laboratory. A TC-sensitive *P. mirabilis* host,  $\phi$ S-3, was used in these experiments.

A thymine auxotroph of  $\phi$ S-3/RTF-TC was cultured in Penassay broth. Analytical CsCl density gradient centrifugation of the DNA (Fig. 4A) showed that both RTF-TC segregants had a density of 1.710 g/ml. [ $^3$ H]thymine-labeled RTF-TC DNA was fractionated in a CsCl preparative gradient (Fig. 4C). When RTF-TC DNA was sedimented in a neutral sucrose gradient, a single 44.5S band was observed (Fig. 4D). The same DNA samples were examined by EM. Both RTF-TCs were circular molecules measuring 28.7  $\mu$ m ( $\sigma = 0.25$

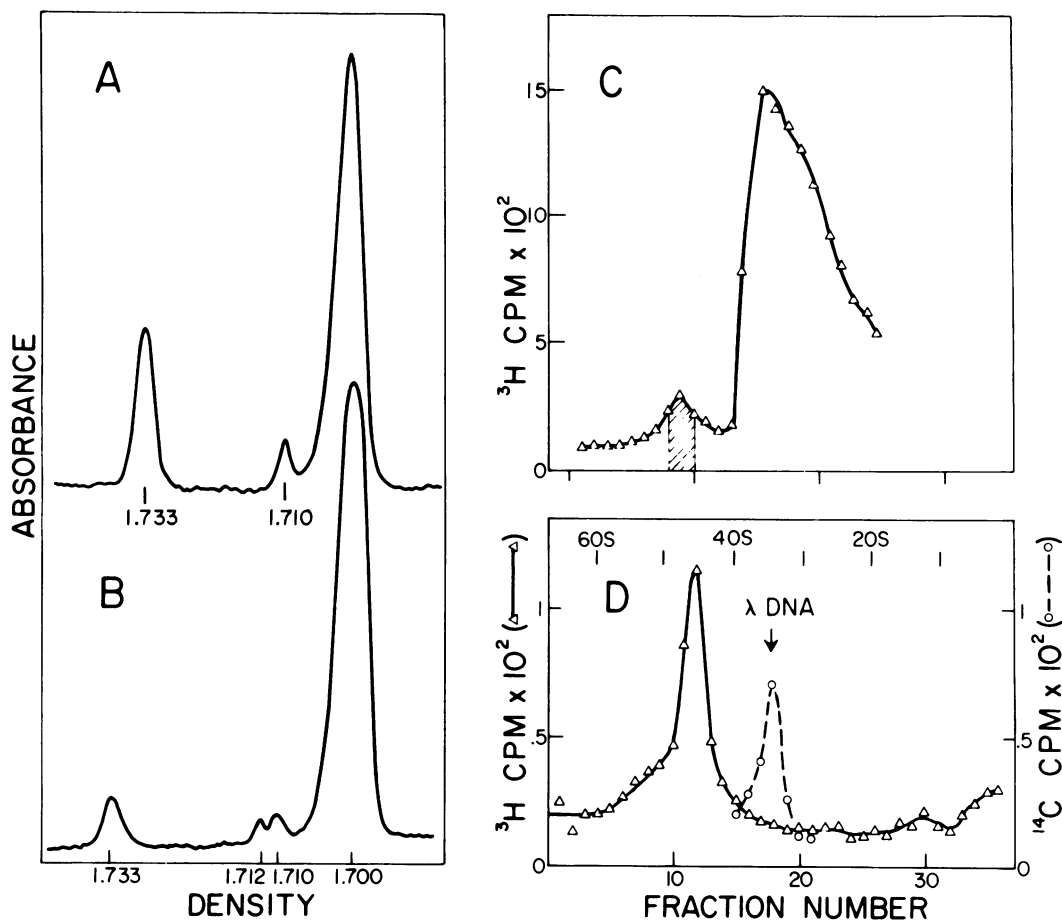


FIG. 4. Characterization of RTF-TC DNA in *P. mirabilis*. DNA was isolated from a stationary phase culture of  $\phi$ S-3/RTF-TC in Penassay broth containing [ $^3$ H]thymine. (A) Density profile of the DNA in an analytical CsCl density gradient. *Micrococcus lysodeikticus* DNA (1.733 g/ml) was included in the gradient as a reference density. (B) Density profile of the DNA isolated from the mixture of  $\phi$ S-3/RTF-TC and Pm15/NR1 cells in an analytical CsCl density gradient. (C) Fractionation profile of  $\phi$ S-3/RTF-TC DNA in a preparative CsCl density gradient. The RTF-TC DNA in the fractions contained in the cross-hatched area was pooled for sedimentation analysis. (D) Sedimentation profile of the fractionated RTF-TC DNA in a neutral sucrose gradient under the conditions described in the legend to Fig. 1.

$\mu\text{m}$ ; based on 18 molecules measured) (Fig. 2A and Table 1). Some 80 to 90% of the DNA on the EM grids was circular. The single 44.5S band observed in the neutral sucrose gradient suggests that the RTF-TC DNA consists of a homogeneous class of molecules. The EM contour length of the RTF-TC DNA corresponds to a molecular weight of  $49 \times 10^6$ .

From side by side contour length measurements of RTF-TC and *NR1* DNA molecules prepared for EM under identical conditions, we found that the RTF-TC was 22.4% shorter, a difference of 8.3  $\mu\text{m}$  ( $\sigma = 0.6 \mu\text{m}$ ). This difference in length of approximately 8.3  $\mu\text{m}$  between the two RTF-TC segregants and the nontransitioned composite R factor corresponds well with the length of the r-determinants component of density 1.718 g/ml described in the following sections.

It should be noted that in some experiments carried out in this laboratory up to 100% of the RTF-TC DNA in  $\phi\text{S-3}$  has been isolated as CCC DNA (C. Morris, and R. Rownd, submitted for publication). The high percentage of nicked circular DNA must again be due to nicking of the DNA during purification in our experiments.

There are at least four reasons for ruling out the possibility that the 42S DNA species observed in some experiments with nontransitioned *NR1* DNA (Fig. 3B) could correspond to RTF-TC nicked circular molecules which result from the dissociation of the composite R factor DNA. First, it is possible to distinguish between RTF-TC DNA and composite R factor *NR1* DNA on the basis of their density differences, if both are present in an analytical CsCl gradient. When approximately equal numbers of  $\phi\text{S-3}$ /RTF-TC cells were mixed with Pm15/*NR1* cells and the DNA was isolated and analyzed in an analytical CsCl gradient, two distinct bands of density 1.710 and 1.712 g/ml were observed which correspond to the RTF-TC DNA and *NR1* DNA, respectively (Fig. 4B). The clear resolution of these two bands would suggest that, even if RTF-TC DNA was present as a minor component in nontransitioned cells, it would appear as a shoulder on the low density side of the *NR1* DNA band. This, however, has not been observed. Second, in an experiment in which the *NR1* DNA in the 42S region of a neutral sucrose gradient was dialyzed against  $0.1 \times \text{SSC}$ , concentrated, and examined by EM, most of the DNA was 37- $\mu\text{m}$  linear molecules. Some 37- $\mu\text{m}$  circular molecules were also observed which were probably due to spill-over from the 48.5S band. No 29- $\mu\text{m}$  circular RTF-

TC DNA molecules were observed. Third, the RTF-TC nicked circular DNA has consistently an *S* value of 43.9 ( $\sigma = 0.4$ ; eight experiments) in neutral sucrose gradients which is definitely different than the 41.9S value ( $\sigma = 0.4$ ; 10 experiments) of the band under discussion in which both 48.5S and 42S R factor DNA bands were present. (The linear form of RTF-TC DNA would have an *S* value of 39 to 40S and therefore is not a possibility.) Fourth, if dissociation of the composite R factor was significant, we would have detected a 1.718-g/ml r-determinants DNA band in CsCl gradients in addition to the 1.712-g/ml DNA as well as bands corresponding to the nicked circular or CCC forms of r-determinants DNA in neutral sucrose gradients. These bands, however, have not been observed.

**Molecular weight and structure of transitioned *NR1* DNA.** When nontransitioned Pm15/*NR1* is cultured in medium containing CM, there is an increase in the density of the R factor DNA and in the ratio of plasmid to chromosome DNA (21, 22, 24, 25). This is referred to as a transition. Usually the original 1.712-g/ml band is no longer observed in the *NR1* DNA density profile and the 1.718-g/ml band is markedly skewed toward the less dense side. In some experiments a separate distinct intermediate density band (1.715 to 1.716 g/ml) has also been observed in the DNA density profile. This laboratory has previously suggested that this transitioned *NR1* DNA consists of multiple, tandem copies of r-determinants which are attached to the RTF-TC component. This would explain the increase in the density and the proportion of the *NR1* DNA relative to the host chromosomal DNA (21, 22, 24, 25). We therefore examined the structure of transitioned *NR1* DNA. Some differences in the size distribution of transitioned *NR1* DNA have been observed in different experiments so several independent experiments will be described.

In the first experiment a nontransitioned stationary phase culture of Pm15/*NR1* was diluted  $10^6$ -fold into Penassay broth containing 100  $\mu\text{g}$  of CM per ml. After the cells had reached an optical density at 650 nm ( $\text{OD}_{650}$ ) of 0.4, they were then diluted  $10^5$ -fold into similar medium containing [ $^3\text{H}$ ]thymine. When this culture reached an OD of 0.4, a sample of the cells was taken. A second sample was taken after this culture had been in stationary phase for about 5 h (OD approximately 2.5).

The analytical CsCl density gradient profile of the exponential phase transitioned DNA sample showed both an intermediate density



NR1 band and a 1.718-g/ml NR1 DNA band (Fig. 5A). The proportion of total plasmid DNA to chromosome DNA was 0.15 in this experiment. In the density profile of the transitioned

DNA from the stationary phase cells (Fig. 5B), the two NR1 DNA bands of density 1.716 and 1.718 g/ml were clearly resolved. The ratio of total NR1 DNA to chromosome DNA was 0.27,

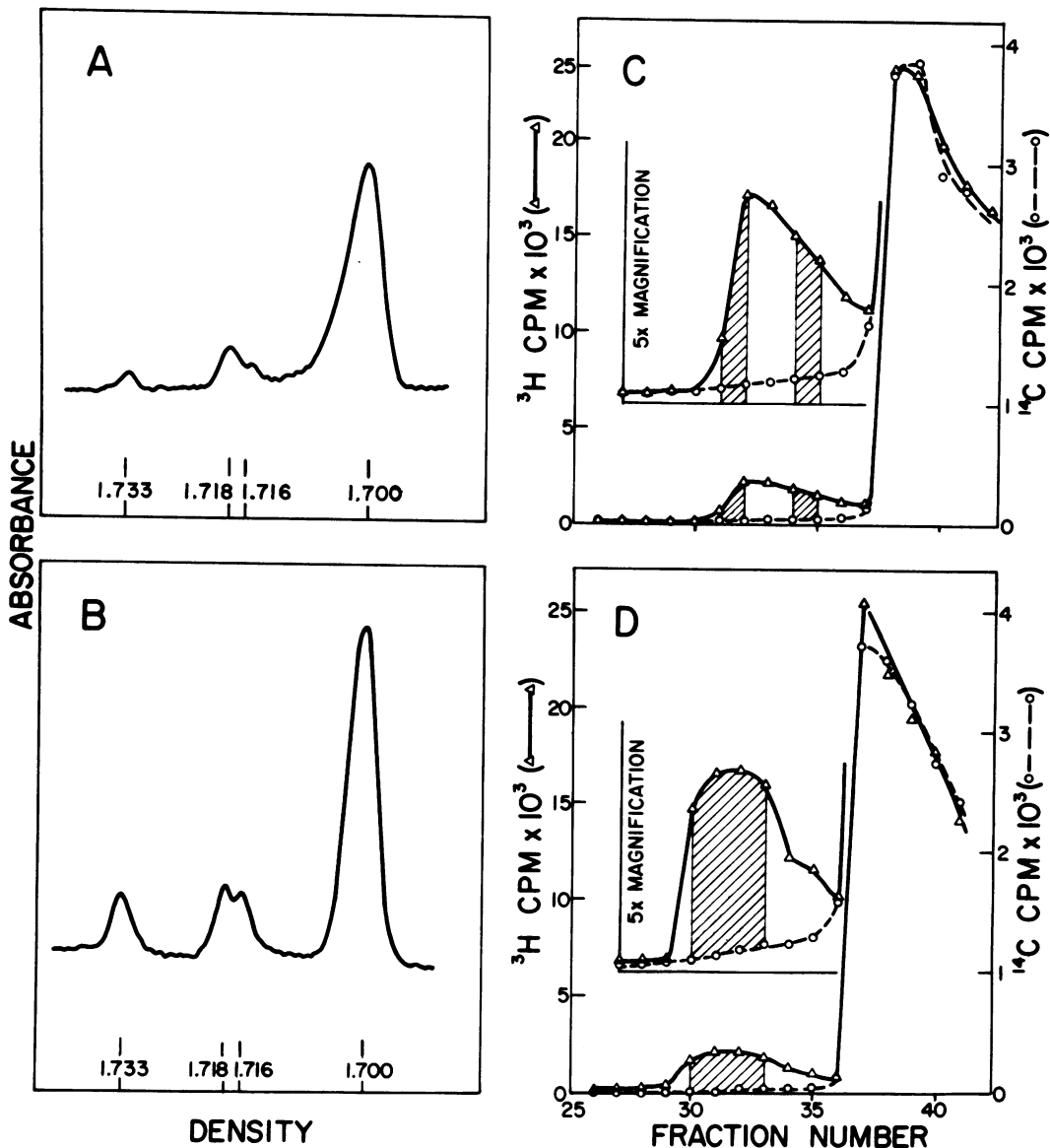


FIG. 5. Analytical and preparative CsCl density gradient profiles of transitioned Pm15/NR1 DNA from exponential and stationary phase cells. The cells were cultured in Penassay broth containing 100 µg of CM per ml and [<sup>3</sup>H]thymine as described in the text. (A) Density profile of the DNA isolated from exponential cells in an analytical CsCl density gradient. (B) Density profile of the DNA from the same culture of cells after 5 h in stationary phase in an analytical CsCl density gradient. (C) Fractionation profile of the DNA from exponential cells in a preparative CsCl density gradient. (D) Fractionation profile of the DNA prepared from stationary phase cells in a preparative CsCl density gradient. In the fractionation profiles shown (C and D), <sup>14</sup>C-labeled *P. mirabilis* chromosomal DNA from R<sup>-</sup> cells was included in the gradient to monitor chromosomal contamination of the NR1 DNA fractions. The fractions which were pooled for sedimentation analysis are indicated by cross-hatching.

about twice the ratio observed for the exponential phase cells.

These same DNA samples were fractionated in a preparative CsCl gradient (Fig. 5C and D). To monitor the degree of contamination of the *NR1* DNA by the chromosome DNA, [ $^{14}\text{C}$ ]thymine-labeled  $\text{R}^-$  cells were mixed with the  $\text{R}^+$  cells prior to DNA isolation and fractionation. When the  $^{14}\text{C}/^3\text{H}$  ratios of the *NR1* DNA fractions and the chromosome DNA fractions were compared, it was found that there was less than 5% contamination of the *NR1* DNA by the chromosomal DNA. The two peaks observed in the analytical density profiles of the *NR1* DNA were not resolved in the preparative gradient fractionation profiles (Fig. 5).

The *NR1* DNA fractions from the preparative CsCl gradients were pooled as indicated (Fig. 5C and D), dialyzed, and sedimented in neutral and alkaline sucrose gradients. The neutral sedimentation profiles of the DNA isolated from both exponential and stationary phase cells showed considerable heterogeneity in the *S* values of the DNA (Fig. 6A, B, and C). Most of the DNA has an *S* value in the range 55 to 60S. The *NR1* DNA from the exponential phase cells had a minor component (5 to 10% of the DNA) which had an *S* value of 28 to 30 (Fig. 6A and B); this component was not observed in the transitioned *NR1* DNA from the stationary phase cells (Fig. 6C). The alkaline sucrose sedimentation profiles of the same DNA samples (Fig. 6C, E, and F) indicate the presence of little, if any, CCC transitioned *NR1* DNA. CCC DNA would sediment about 2.5 times faster in an alkaline sucrose gradient than in a neutral sucrose gradient and would have pelleted under the conditions of our experiments. This would have been detected in counting the bottom of the gradient tube, as was done in our experiments. Therefore, the 55 to 60S DNA is either linear or open circular in form and has a high molecular weight. The observed increase in heterogeneity and the reduction in *S* value of the DNA in the alkaline sucrose gradients suggest that multiple nicks were present in the DNA. The approximate molecular weight of the transitioned *NR1* DNAs was calculated from the neutral sucrose gradient data using the Freifelder equation:  $S_2/S_1 = (M_2/M_1)^{0.38}$ . Using the 34.4S, linear form of  $\lambda$  DNA ( $30 \times 10^6$  daltons) as a standard, the 60S *NR1* DNA would have a molecular weight of about  $150 \times 10^6$ .

EM preparations of this transitioned *NR1* DNA confirmed that the bulk of the material was high-molecular-weight, linear DNA. Linear

molecules having contour lengths of 75 to 100  $\mu\text{m}$  were measured. It was considered pointless to determine a contour length distribution since such large molecules are very susceptible to breakage during sample preparation for EM and also run off the EM grid squares. The estimate of molecular weights from sucrose gradient sedimentation data, however, avoided breakage problems and possible bias in the selection of molecules for contour length measurement. Sucrose gradient data are therefore considered to be a more reliable representation of the distribution of sizes of transitioned *R* factor DNA. A number of 8- $\mu\text{m}$  open circular molecules consistent with the length expected for the autonomous *r*-determinants monomer were observed in the EM. The minor 28S band observed in neutral sucrose gradients (Fig. 6A and B) is consistent with the *S* value expected for the nicked circular form of monomeric *r*-determinants DNA.

*P. mirabilis* chromosomal DNA from the preparative CsCl gradient (Fig. 5D) was also sedimented in a neutral sucrose gradient to determine the molecular weight of chromosomal DNA isolated and fractionated under our experimental conditions. The sedimentation profile of the chromosomal DNA (Fig. 7) is similar to those obtained with transitioned *NR1* DNA (Fig. 6A, B, and C). Since the 60S chromosomal DNA almost certainly represents breakage products of the *P. mirabilis* chromosome, it is also possible that the bulk of 1.718-g/ml *NR1* DNA could also be fragments of larger, perhaps circular, *in vivo* structures.

Another transition experiment was carried out under similar conditions except that the cells were diluted  $10^6$ -fold into Penassay broth containing 100  $\mu\text{g}$  of CM per ml and grown to stationary phase two successive times. The analytical and preparative CsCl gradient profiles of DNA from the stationary phase cells are shown in Fig. 8A and B. The analytical profile (A) showed a single 1.718-g/ml *NR1* DNA band which was skewed toward the less dense side. The preparative profile (B) was very similar.  $^{14}\text{C}$ -labeled  $\text{R}^-$  chromosomal DNA was included in the preparative CsCl gradient to show that there was negligible contamination of the transitioned *NR1* DNA by the chromosomal DNA. The *NR1* DNA in the indicated fractions of the CsCl preparative gradient (Fig. 8B) was sedimented in neutral and alkaline sucrose gradients (Fig. 8C and D). The 60S value observed for the DNA in the neutral sucrose gradient, taken with the alkaline sucrose gradient data, show that the double-stranded DNA had a high

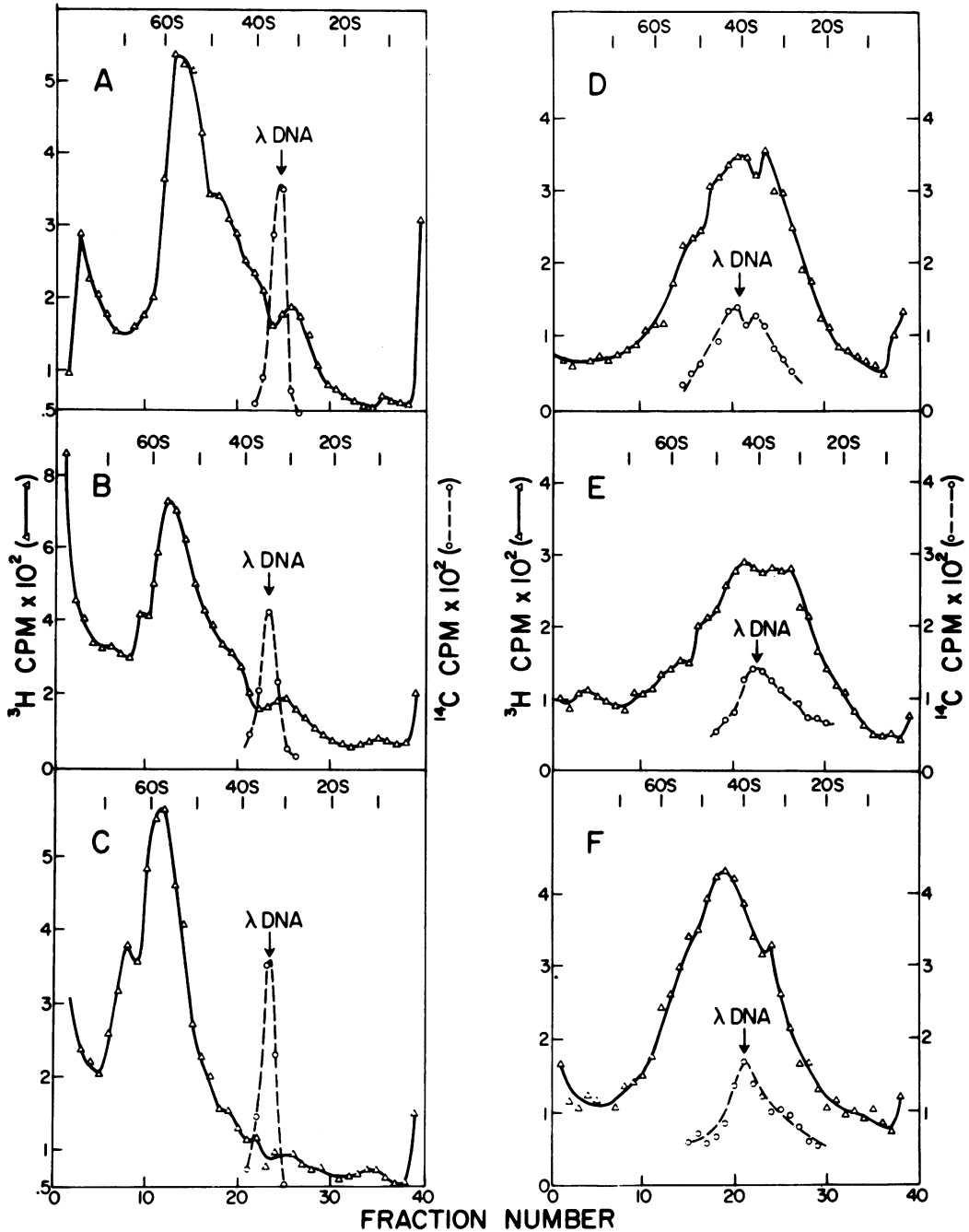


FIG. 6. Neutral and alkaline sucrose gradient sedimentation profiles of transitioned NR1 DNA. The  $^3\text{H}$ -labeled NR1 DNA from the pooled fractions indicated by cross-hatching in Fig. 5 (C and D) was sedimented through both neutral and alkaline sucrose gradients. (A and D) Sedimentation profiles of the NR1 DNA in the more dense pooled fractions in Fig. 5C in neutral and alkaline sucrose gradients, respectively. (B and E) Sedimentation profiles of the NR1 DNA in the less dense pooled fractions in Fig. 5C in neutral and alkaline sucrose gradients, respectively. (C and F) Sedimentation profiles of the NR1 DNA in the pooled fractions in Fig. 5D in neutral and alkaline sucrose gradients, respectively.  $^{14}\text{C}$ -labeled  $\lambda$  DNA was included as a sedimentation standard in all of the sucrose gradients.

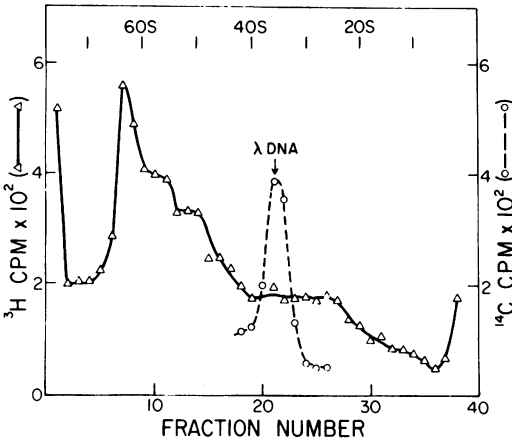


FIG. 7. Sedimentation of *P. mirabilis* chromosomal DNA in a neutral sucrose gradient. Fractions containing [ $^3\text{H}$ ]thymine-labeled chromosomal DNA in the preparative density gradient profile shown in Fig. 5D were pooled and sedimented through a neutral sucrose gradient. [ $^{14}\text{C}$ ]labeled  $\lambda$  DNA was included as a sedimentation standard in the sucrose gradient.

molecular weight and probably contained multiple single-strand nicks.

The presence of a significant 28S *NR1* DNA band in the neutral sucrose gradient in this experiment differed from the results of the first experiment (Fig. 5 and 6) and suggested the presence of an increased proportion of r-determinant monomers in the transitioned *NR1* DNA. The 28S sucrose gradient peak is about 25% of the size of the major 60S *NR1* DNA band. (This figure would actually have been about 15% if all of the fractions containing *NR1* DNA had been pooled [Fig. 8B] and analyzed since r-determinant DNA which is unattached to the RTF-TC component would not have been present in the lower density fractions [45 to 49, Fig. 8D] which were not included in the pooled *NR1* DNA.) This figure reflects a weight-average percentage. Since the average molecular weight of the DNA in the 60S band is on the order of  $100$  to  $150 \times 10^6$ , whereas this smaller species is only about  $15 \times 10^6$  daltons, the number-average percentage of the smaller species would be about seven to ten times greater than its weight percentage. Thus, we would expect a ratio of monomers to multimers of approximately 1.5:1. EM confirmed that approximately half the molecules observed were 8- $\mu\text{m}$  circular DNA molecules.

In addition to these 8- $\mu\text{m}$  circles many other circular molecules were observed by EM and the lengths of some of these molecules are included in Table 1. The data strongly indicate that these molecules are multimers of 8.3- $\mu\text{m}$

r-determinants. Figure 9 shows examples of circular poly-r-determinant molecules which were observed in this experiment. Large linear DNA was also observed in the EM, but the contour lengths of these molecules were not measured since the sedimentation data were considered to be a more reliable description of the size distribution of these molecules and also because of the problem of fragmentation of large DNA molecules during sample preparation for EM. This experiment illustrates that in at least some transition experiments autonomous circular r-determinant monomers may be present along with much larger linear *NR1* DNA molecules. It is also interesting to note in Table 1 that trimeric and pentameric r-determinants were relatively rare in occurrence. A subsequent transition experiment also suggested this. In this experiment a circular molecule having a contour length of 70  $\mu\text{m}$  was also observed, suggesting that this molecule consisted of an RTF-TC plus five r-determinants (Fig. 2C).

**Molecular weight of *NR1* DNA during the early stages of a transition.** In the majority of the experiments carried out in this laboratory, transitioned R factor DNA has been found to have a molecular weight similar to *P. mirabilis* chromosomal DNA which has been isolated under the same conditions (ca.  $150 \times 10^6$ ). In these experiments the cells were usually cultured for about 40 generations in Penassay broth containing 100  $\mu\text{g}$  of CM per ml. In some preliminary experiments in which the cells were cultured for fewer generations in medium containing drugs, a substantial fraction of the *NR1* DNA has had a lower molecular weight, although larger *NR1* DNA was present as well.

In the experiments shown in Fig. 10 and 11 a nontransitioned culture of Pm15/*NR1* was diluted  $10^4$ -fold into Penassay broth containing 100  $\mu\text{g}$  of CM per ml to begin the transition. After this culture reached stationary phase, it was diluted  $10^3$ -fold into Penassay broth containing 150  $\mu\text{g}$  of CM per ml. In this medium the cellular DNA was uniformly labeled with [ $^{14}\text{C}$ ]thymine (0.2  $\mu\text{Ci}/\text{ml}$ ) for six generations. Then to identify the *NR1* DNA species which had recently replicated, [ $^3\text{H}$ ]thymine (10  $\mu\text{Ci}/\text{ml}$ ) was added, and the cells were labeled for only 0.25 generation. After this period, a sample of the cells was removed and added to an equal volume of ice cold saline (0.85%) containing 0.02 M sodium azide. These cells were washed and the DNA was isolated as previously described. The remainder of the culture was grown to stationary phase and DNA was isolated by the standard procedure. Both the exponential

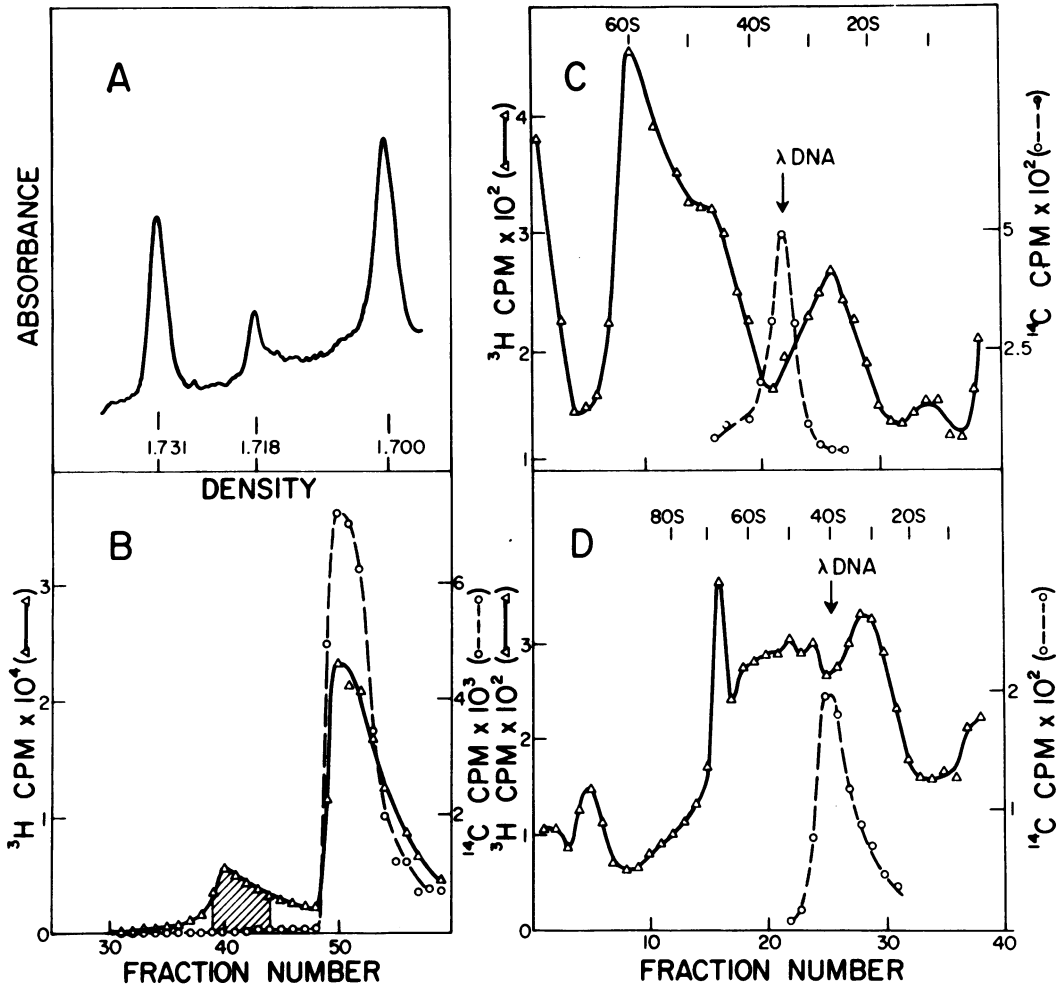
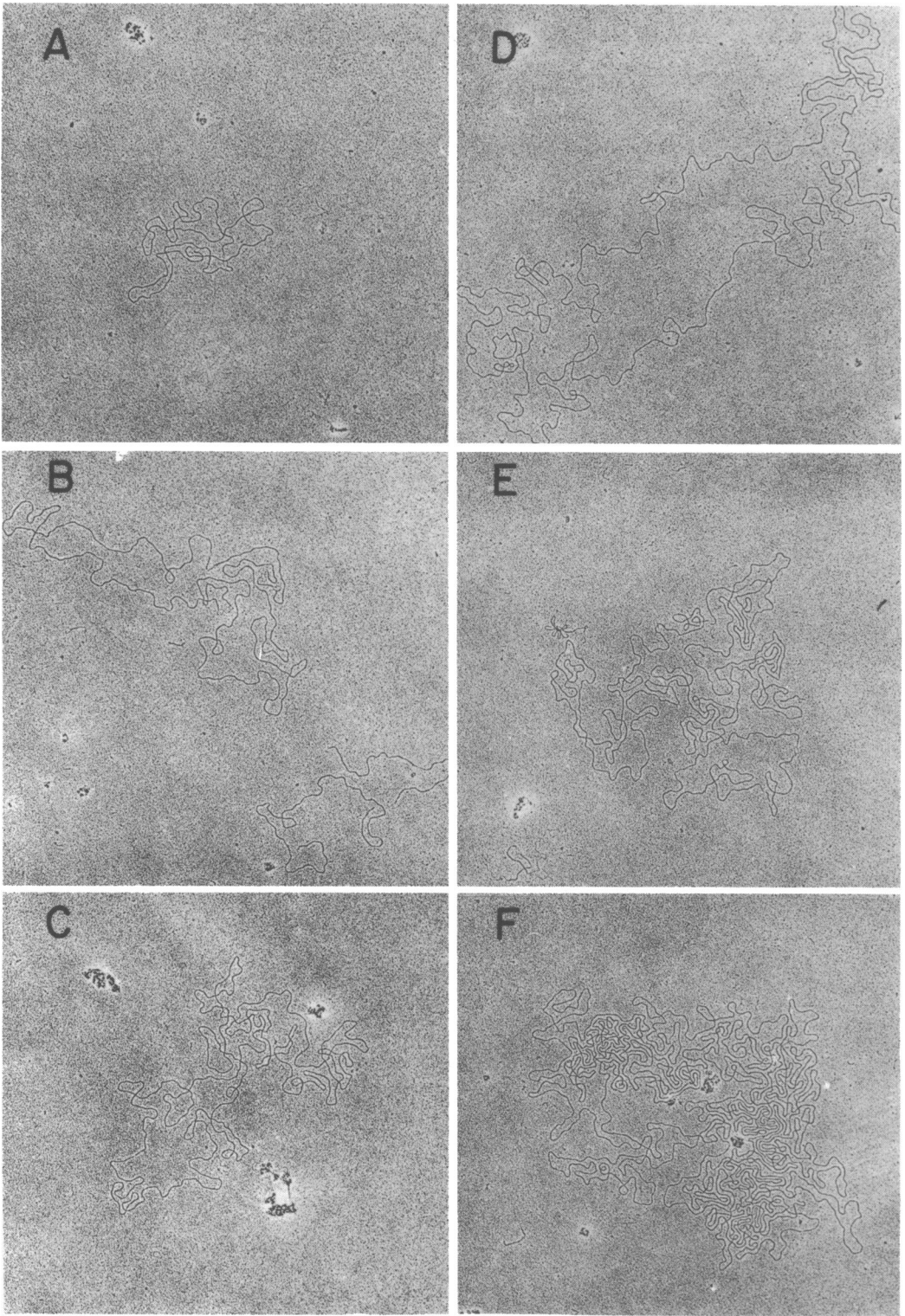


FIG. 8. Characterization of transitioned NR1 DNA having an appreciable 28S monomeric r-determinants DNA component.  $^3\text{H}$ -labeled DNA was isolated from stationary phase transitioned cells as described in the second transition experiment in the text. (A) Density profile of DNA in an analytical CsCl gradient. (B) Fractionation profile of DNA in a preparative CsCl density gradient.  $^{14}\text{C}$ -labeled *P. mirabilis* chromosomal DNA from R<sup>-</sup> cells was included in the gradient to monitor chromosomal contamination of the NR1 DNA. The NR1 DNA in the fractions indicated by cross-hatching was analyzed in neutral and alkaline sucrose gradients. (C) Sedimentation profile of purified NR1 DNA in a neutral sucrose gradient. (D) Sedimentation profile of purified NR1 DNA in an alkaline sucrose gradient.  $^{14}\text{C}$ -labeled  $\lambda$  DNA was included as a sedimentation standard in the sucrose gradients.

and the stationary DNA samples had a 1.718-g/ml band and an intermediate density NR1 DNA band in an analytical CsCl gradient (Fig. 10). Both DNA samples were fractionated in a preparative CsCl gradient (Fig. 11A and B) and the indicated fractions were pooled and sedimented in neutral sucrose gradients. The ratio of  $^{14}\text{C}/^3\text{H}$  in the stationary phase DNA sample was very low (approximately 1:50) since by this time the DNA had been labeled with [ $^3\text{H}$ ]thymine for about five generations. We were thus able to add  $^{14}\text{C}$ -labeled phage  $\lambda$

DNA to this DNA sample for calibrating the sucrose gradients. A prominent 28S peak corresponding to the r-determinants monomer was observed in the sucrose gradient profiles of both the exponential and the stationary phase NR1 DNA samples. The DNA in both the exponential (Fig. 11C) and stationary (Fig. 11D) NR1 DNA samples had a very heterogeneous distribution of S values, corresponding to molecular weights ranging from the 28S monomeric r-determinants DNA ( $14 \times 10^6$ ) to values greater than  $150 \times 10^6$ . It is interesting to note



**FIG. 9.** *Electron micrographs of monomeric and multimeric r-determinants DNA. In these experiments the DNA was spread for electron microscopy at pH 11.1 and a few partial denaturations are visible in the r-determinants DNA. The molecules shown represent multimers of the 8.3- $\mu\text{m}$  monomeric structure. (A) Monomer; (B) dimer; (C) trimer; (D) tetramer; (E) tetramer; (F) decamer. These poly-r-determinant molecules and the 70- $\mu\text{m}$  poly-r-determinants R factor molecule shown in Fig. 2C were obtained in the second transition experiment described in the text.*

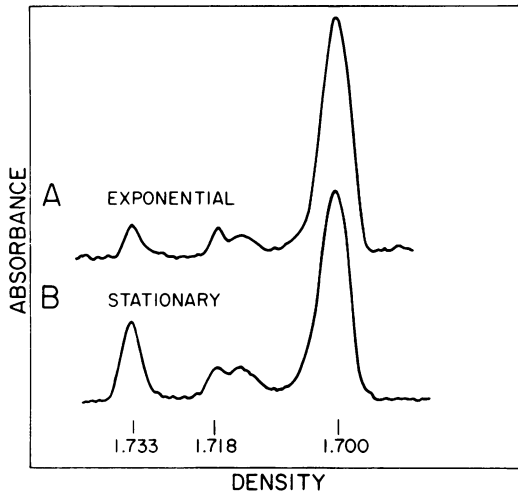


FIG. 10. Analytical CsCl density gradient profiles of transitioned *NR1* DNA isolated from exponential and stationary phase cells during the early stages of a transition. The details of this experiment are described in the text. (A) DNA from exponential phase cells. (B) DNA from stationary phase cells. *Micrococcus lysodeikticus* DNA was included in the gradients as a reference density.

that the percentage of the 28S monomeric r-determinants DNA is lower in the stationary phase DNA sample than in the exponential phase DNA sample. This is consistent with the transition experiment shown in Fig. 5 and 6 where the minor 28S peak was no longer detectable by the time the culture had reached stationary phase.

The distribution of  $^{14}\text{C}$ -labeled DNA (uniform label) and  $^3\text{H}$ -labeled DNA (pulse label) was essentially the same in the sucrose gradient profile of the *NR1* DNA from the exponential phase cells (Fig. 11C). This suggests that there was no preferential replication of either the monomeric r-determinants DNA or the more heterogeneous, higher-molecular-weight DNA within the time scale and the sensitivity of this experiment.

Although more detailed data are not presently available, these experiments are consistent with the view that lower-molecular-weight and more heterogeneous R factor structures are present in more substantial proportions when  $R^+$  *P. mirabilis* is cultured for fewer generations in medium containing CM; i.e., during the earlier stages of a transition.

**Molecular weight and structure of *NR1* DNA during the back transition.** Growth of a transitioned culture in drug-free medium results in a decrease in the proportion of 1.718-g/ml *NR1* DNA and an increase in the proportion of *NR1* DNA having a density inter-

mediate between 1.712- and 1.718-g/ml DNA (21, 22, 24, 25). This has been termed the back transition. We have suggested that the intermediate density *NR1* DNA molecules are R factors consisting of an RTF-TC plus several r-determinants (poly-r-determinant R factors). Such molecules should be smaller in size than the large, heterogeneous *NR1* DNA found in the 1.718-g/ml band of transitioned cultures since they would have fewer copies of r-determinants. Therefore, breakage of the DNA should be less of a problem during isolation and handling.

A stationary phase transitioned culture, whose DNA was characterized by analytical neutral CsCl gradient centrifugation (Fig. 12A, top profile) was diluted  $10^2$ -fold into drug-free Penassay broth containing  $[^3\text{H}]$ thymine. The medium was supplemented with  $10\ \mu\text{g}$  of thymine per ml since it previously was observed by S. Mickel in our laboratory that higher concentrations of thymine in the growth medium accelerated the back transition. (Penassay broth [Difco] contains approximately  $3\ \mu\text{g}$  of thymine per ml.) The cells were harvested at an  $\text{OD}_{660}$  of 0.80. The DNA was analyzed in the analytical ultracentrifuge (Fig. 12A, bottom profile) and fractionated in a preparative CsCl gradient (Fig. 12B). Comparing the initial transitioned DNA profile to the back transition profile, we can see an appreciable decrease in the amount of 1.718-g/ml DNA relative to intermediate density *NR1* DNA. Fractions containing intermediate and higher density *NR1* DNA from the preparative CsCl gradient were separately pooled as indicated in Fig. 12B and sedimented in neutral sucrose gradients.

The major fraction of the intermediate density *NR1* DNA had an *S* value of 56S in a neutral gradient (Fig. 12C). Examination of this DNA in the EM revealed that about 75% of the DNA was circular molecules. The 17 circular molecules which were photographed and analyzed comprised two sizes classes:  $53.8\ \mu\text{m}$  ( $\sigma = 0.4\ \mu\text{m}$ ) and  $62.2\ \mu\text{m}$  ( $\sigma = 0.5\ \mu\text{m}$ ). These data are included in Table 1. The contour lengths of these two classes are consistent with a 29- $\mu\text{m}$  RTF-TC plus three and plus four r-determinants, respectively. Since approximately one-half of the DNA of these molecules would have a density of 1.710 g/ml (RTF-TC DNA) and the other one-half a density of 1.718 g/ml (r-determinants DNA), the composite structures would have a density intermediate between these two values. The molecular weight of this intermediate density *NR1* DNA can also be calculated from its sedimentation coefficient. Since the *S* value of linear DNA is 10% lower than that of nicked

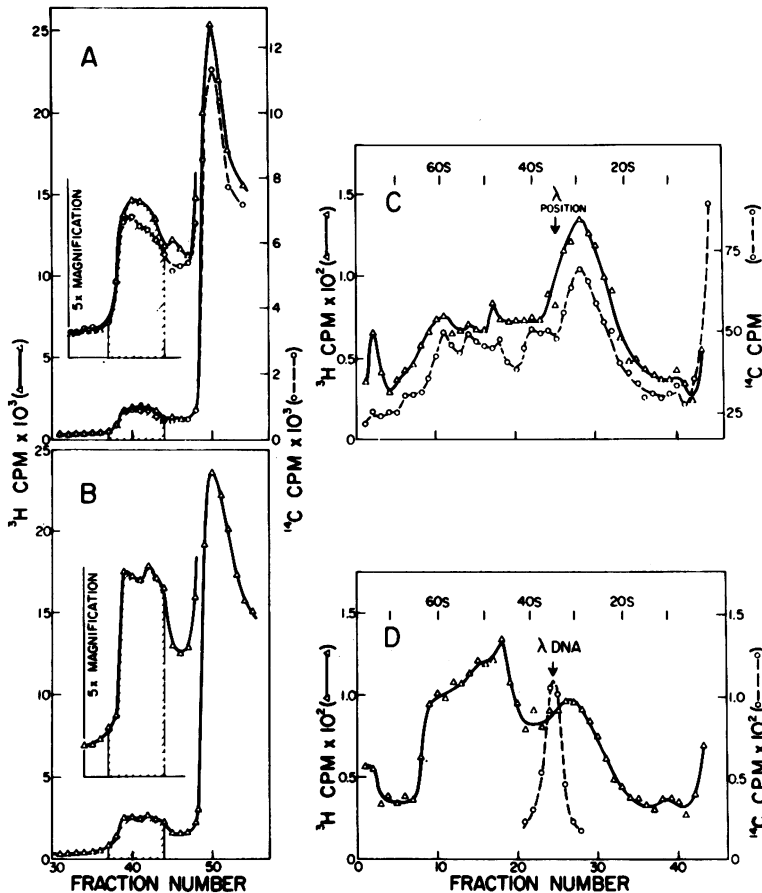


FIG. 11. Fractionation profiles and sedimentation profiles of transitioned NR1 DNA isolated from exponential and stationary phase cells during the early stages of a transition. DNA was isolated from exponential and stationary phase cells during the early stages of a transition as described in the text. The analytical density profiles of these DNA samples are shown in Fig. 10. (A) Fractionation profile of DNA from exponential phase cells in a preparative CsCl gradient. (B) Fractionation profile of DNA from stationary phase cells in a preparative CsCl density gradient. (C) Sedimentation profile of NR1 DNA in the fractions indicated by cross-hatching in (A) in a neutral sucrose gradient. (D) Sedimentation profile of NR1 DNA in the fractions indicated by cross-hatching in (B) in a neutral sucrose gradient.  $^{14}\text{C}$ -labeled  $\lambda$  DNA was co-sedimented in the experiments shown in (D).  $^{14}\text{C}$ -labeled  $\lambda$  DNA was not co-sedimented in the experiments shown in (C) because of the  $^{14}\text{C}$  label contained in the uniformly labeled NR1 DNA; the peak fraction of the  $\lambda$  DNA in the parallel gradient shown in (D) is denoted in the sedimentation profile shown in (C).

circular DNA (27), the linear form of the intermediate density NR1 would be about 50S. This corresponds to a molecular weight of about  $110 \times 10^6$ , using the S value and molecular weight of  $\lambda$  DNA as the standard in the Freifelder equation. This agrees well with the average of the DNA contour lengths observed in the EM. A number of 8- $\mu\text{m}$  circular molecules were also observed in the EM which presumably resulted from the overlap of the 1.718-g/ml DNA in the preparative CsCl gradient. The rest of the intermediate density DNA was linear in struc-

ture and of high molecular weight. Two linear molecules which were measured had a contour length of 62  $\mu\text{m}$  and probably originated from circles which had been broken during preparation of the DNA for EM.

When NR1 from the higher density region of the preparative CsCl gradient was sedimented in a neutral sucrose gradient, about 60 to 70% of the DNA had an S value of 28 to 35S (Fig. 12D). EM confirmed that most of the DNA was monomeric 8- $\mu\text{m}$  r-determinant DNA. The preponderance of lower-molecular-weight NR1



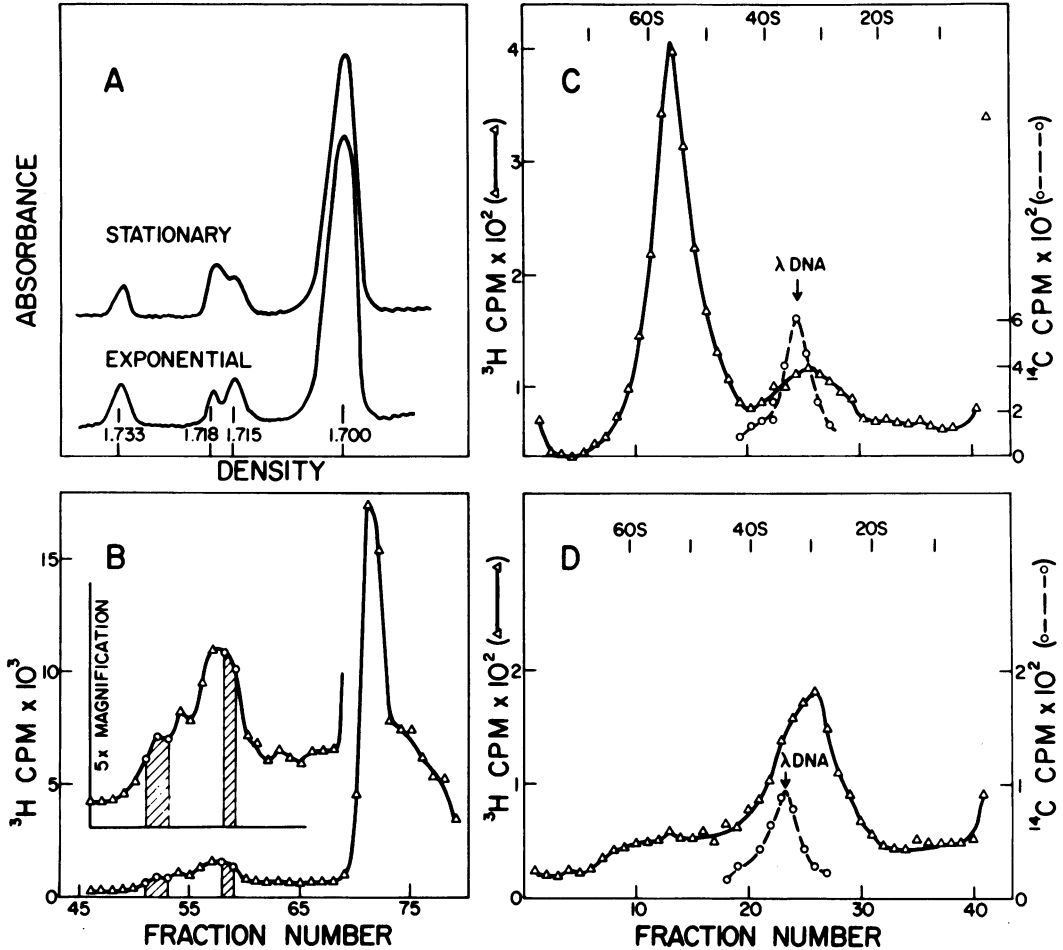


FIG. 12. Characterization of R factor NR1 DNA during a back transition. A stationary phase transitioned culture of Pm15/NR1 was diluted 100-fold into drug-free Penassay broth containing [ $^3\text{H}$ ]thymine. DNA was isolated from the original stationary phase culture and from the  $^3\text{H}$ -labeled culture after it had reached late exponential phase of growth. (A) Density profiles of DNA isolated from stationary and exponential phase cultures in an analytical CsCl density gradient. (B) Fractionation profile of DNA isolated from exponential phase culture in a preparative CsCl density gradient. (C) Sedimentation profile in a neutral sucrose gradient of the NR1 DNA in the less dense fractions indicated by cross-hatching in (B). (D) Sedimentation profile in a neutral sucrose gradient of the NR1 DNA in the more dense fractions indicated by cross-hatching in (B).  $^{14}\text{C}$ -labeled  $\lambda$  DNA was co-sedimented in both sucrose gradient experiments as a sedimentation standard.

DNA in this experiment is not necessarily a typical result since other studies have indicated that the 1.718-g/ml DNA can be considerably more heterogeneous and of much higher molecular weight during the back transition (R. Rownd and N. Goto, manuscript in preparation).

In an extended back-transition experiment in which the cells were cultured for several hundred generations in drug-free Penassay broth, the density of the plasmid DNA returned to a value which was only slightly greater than

nontransitioned NR1 DNA (data not shown). When this 1.712- to 1.713-g/ml NR1 DNA was fractionated and examined by EM, both 37- and 46- $\mu\text{m}$  NR1 circular molecules were observed (Table 1), corresponding to an RTF-TC plus one and two r-determinants, respectively. From the data presented in this table, it can be seen that the density and the contour lengths of poly-r-determinant R factors increase in the manner expected for the consecutive addition of 8.3- $\mu\text{m}$  r-determinants to R factor molecules. At the limiting density of 1.718 g/ml, most of the

circular molecules correspond to autonomous poly-r-determinants. Presumably poly-r-determinant R factors having this density would be so high in molecular weight that they would be fragmented during isolation and handling.

### DISCUSSION

This paper presents our analysis of the structure of the R factor *NR1* in nontransitioned and transitioned cultures of *P. mirabilis*. These experiments demonstrate that in the nontransitioned state *NR1* DNA is a circular molecule consisting of an RTF-TC plus a single copy of the r-determinants component. This composite structure has a density of 1.712 g/ml, and a contour length of 37  $\mu\text{m}$  ( $63 \times 10^6$  daltons). Our analysis of the two RTF-TC genetic segregants of *NR1* suggests that this element is a circular molecule with a contour length of 29  $\mu\text{m}$  ( $49 \times 10^6$  daltons). The fact that two independent RTF-TC segregants had a density of 1.710 g/ml and the same contour lengths suggests they may have originated by dissociation or "excision" of the r-determinants via specific recombination sites in the R factor. After *P. mirabilis* is cultured in medium containing CM, the original 1.712-g/ml *NR1* DNA is no longer observed and 1.718-g/ml DNA and intermediate density *NR1* DNA appear in the *NR1* DNA density profile. The contour length of the 8.3- $\mu\text{m}$  ( $14 \times 10^6$  daltons) r-determinants circular monomer observed in transitioned cultures is in good agreement with the difference in contour lengths of the composite *NR1* and the RTF-TC component. Our results on the densities, contour lengths, and molecular weights of the RTF-TC and r-determinants components and of the composite R factor are in general agreement with the results obtained in several other laboratories (1, 3, 4, 7, 16). However, our results and their interpretation with regard to the molecular state of composite R factors in *P. mirabilis* are quite different in several fundamental respects from the interpretation published by these other groups.

In our original transition model (21, 22, 25) it was assumed that dissociation of the composite R factor into the RTF-TC and r-determinants components was extensive even when the *P. mirabilis* host cells were cultured in drug-free medium. This assumption seemed logical since dissociation would provide a source of autonomous r-determinants which could then replicate separately from the RTF-TC to produce the extra copies of r-determinants required for the formation of poly-r-determinant structures. Our characterization of the nontransitioned form of

*NR1* DNA, however, has consistently shown that the 1.712-g/ml *NR1* DNA corresponds to the composite R factor structure. This indicates that there is only a very small extent of dissociation of *NR1* into the RTF-TC and r-determinants when *P. mirabilis* is cultured in drug-free medium. This has been found to be true for both exponential and stationary phase cultures.

It should be noted that extensive dissociation of the composite R factor is not an essential feature of the transition model. What is important is that R factor dissociation can occur in *P. mirabilis* to provide an autonomous source of r-determinants for amplification of this component during the transition. It is of course also possible that growth of nontransitioned cells in medium containing drugs may lead to an increase in the extent of R factor dissociation.

We have previously shown that there is a small fraction of the cells of a nontransitioned population which exist in a partially transitioned state even during growth in drug-free medium (9). These cells have a higher level of drug resistance and outgrow the nontransitioned cells in the population during growth in medium containing appropriate drugs. This results in the change observed in the *NR1* DNA density profile during the transition. The amplified r-determinants DNA from these partially transitioned cells would not have been detected in our physical characterization of nontransitioned *NR1* DNA, since these cells represent only a very small fraction of the nontransitioned cell population.

The high molecular weight of the *NR1* DNA isolated from transitioned cultures is consistent with the increase in size of the DNA expected to accompany the formation of poly-r-determinant R factors and autonomous poly-r-determinants. The major fraction of transitioned DNA is linear in structure, heterogeneous in size, and of molecular weight similar to *P. mirabilis* chromosomal DNA which had been isolated under the same conditions (ca.  $150 \times 10^6$ ). Since the linear chromosomal DNA results from the fragmentation of the bacterial chromosome, these findings suggest that the large, linear 1.718-g/ml *NR1* DNA molecules are probably breakage products of larger circular R factor DNA molecules. The large size of transitioned *NR1* DNA is not unexpected since on the order of 10 to 15 copies of r-determinants would have to be attached to an RTF-TC to increase its density to values approaching 1.716 to 1.718 g/ml, as observed during the transition. The resulting poly-r-determinant R factors would have in situ molecular weights in the range 200 to

$400 \times 10^6$  and would be subject to shear breakage during isolation and fractionation of the DNA by CsCl preparative density gradient centrifugation, just as the *P. mirabilis* chromosomal DNA.

The contour lengths of the circular *NR1* DNA molecules having a density intermediate between 1.712 and 1.718 g/ml fit the size distribution expected for molecules consisting of a single copy of an RTF-TC and multiple copies of r-determinants. Moreover, the densities of these molecules are correlated with their contour lengths in the expected way. *NR1* DNA molecules present in the 1.713-g/ml density region of a CsCl preparative gradient have contour lengths of 45.9  $\mu\text{m}$ , corresponding to an RTF-TC plus two r-determinants. In the 1.715- to 1.716-g/ml region, the molecules have contour lengths of 53.8 and 62.2, corresponding to an RTF-TC harboring either three or four copies of r-determinants, respectively. Thus, these experiments clearly show that multiple copies of r-determinants can be incorporated into R factors in *P. mirabilis* during the transition, which results in an increase in the density of the *NR1* DNA. Circular molecules consisting of multiple sequences of r-determinants (autonomous poly-r-determinants) are also observed in the 1.718-g/ml region of the transitioned *NR1* DNA density profile. Transitioned *NR1* DNA evidently consists of a heterogeneous collection of poly-r-determinant structures which may be attached to an RTF-TC or exist in the autonomous state.

Using denaturation mapping of R factor DNA, it has been possible to identify both circular and linear transitioned *NR1* DNA molecules which consist of an RTF-TC plus an integral number of tandem r-determinants which are joined in a head-to-tail fashion. Poly-r-determinants are located in the RTF-TC at the same integration site as the single copy of the r-determinants component in the nontransitioned, composite R factor DNA of density 1.712 g/ml (18).

Since the molecular weight of nontransitioned *NR1* DNA is  $63 \times 10^6$  and that of the *P. mirabilis* chromosome is about  $2.5 \times 10^9$ , it follows that the ratio of *NR1* DNA to chromosome DNA would be about 0.025 if there is one copy of the R factor per chromosomal equivalent of DNA. This is the ratio which is observed at rapid growth rates in Penassay broth. Since Penassay broth cultures of *P. mirabilis* contain three to four chromosomal DNA equivalents per cell (R. Rownd and R. Stickgold, submitted for publication), it follows that there must be three

to four copies of *NR1* per cell under these conditions. Although there is a decrease in the chromosome DNA content per cell at slower growth rates to one to two chromosomal DNA equivalents per cell (R. Rownd and R. Stickgold, submitted for publication) there is an increase in the *NR1* DNA to chromosome DNA ratio to 0.05 to 0.07. Thus under these conditions there are also about three copies of nontransitioned *NR1* per cell. These values are only approximate since the relationship between the actual number of *NR1* copies per cell and the ratio of *NR1* DNA to chromosome DNA will depend upon the time during the bacterial division cycle at which *NR1* replication occurs.

In transitioned cells it is more difficult to estimate the number of copies of the various R factor species per cell because of the heterogeneous nature of the R factor DNA. Basically two different types of *NR1* DNA density profiles have been observed in transition experiments performed in this laboratory. In the first (which is the more common type), a single *NR1* DNA band which is markedly skewed toward the less dense side is present. In the second, an intermediate density *NR1* DNA band (typically 1.715 to 1.716 g/ml) is present in addition to the 1.718-g/ml *NR1* DNA band. In both cases, the 1.712-g/ml nontransitioned *NR1* DNA band is no longer observed in the density profile, indicating that essentially all of the RTF-TC component is associated with multiple copies of r-determinants. The intermediate density *NR1* DNA band observed in the second type of transition experiment must correspond to poly-r-determinant R factors. The 1.718-g/ml *NR1* DNA observed in both types of transition experiments does not necessarily correspond to a unique class of R factor DNA molecules. This DNA could represent autonomous monomeric r-determinants, autonomous poly-r-determinants, poly-r-determinant R factors with a large number of copies of r-determinants, or poly-r-determinant DNA resulting from the fragmentation of poly-r-determinant R factor DNA during isolation and handling.

Although there is no detailed evidence at present which indicates the distribution of r-determinants DNA among these various molecular structures, there is reason to believe that most of the r-determinants DNA in the cells is included in poly-r-determinant R factors. The argument supporting this statement is as follows. In an exponential phase nontransitioned culture the ratio of 1.712-g/ml *NR1* DNA to chromosome DNA is about 0.03. To increase the density of the 1.712-g/ml nontransitioned DNA

to values in the range 1.716 to 1.718 g/ml due to the recombination of multiple copies of r-determinants, it would be necessary to recombine about four times the mass of r-determinants DNA with the original 1.712-g/ml *NR1* DNA. Assuming that the number of copies of the RTF-TC component per cell remains constant regardless of the number of copies of r-determinants attached to it, the *NR1* DNA to chromosome DNA would then increase about fourfold to a value of 0.12 in the transitioned state when the density of all of the original 1.712-g/ml *NR1* DNA approaches 1.718 g/ml during the transition. This is a typical ratio of transitioned *NR1* DNA to chromosomal DNA in exponential phase (13, 21, 25). These considerations suggest that a major fraction of the r-determinants DNA of transitioned cells is associated with poly-r-determinant R factors.

Autonomous monomeric and poly-r-determinant DNA has also been observed in most of our transition experiments. In the majority of our experiments monomeric r-determinants DNA (28S in a neutral sucrose gradient) represented less than a few percent of the total R factor DNA of transitioned cultures. This corresponds to less than one copy of monomeric r-determinants DNA per chromosomal equivalent of DNA. However, in a few experiments a larger fraction of monomeric r-determinants DNA was observed. For example, in the experiment shown in Fig. 8, about 15% of transitioned *NR1* DNA had an *S* value of 28S. Since the ratio of total *NR1* DNA to chromosome DNA was 0.27 in this experiment, the monomer r-determinants DNA accounted for about 5% of the total chromosome DNA, corresponding to about 10 copies of monomer r-determinants DNA per chromosomal equivalent of DNA. In the experiment shown in Fig. 10 and 11 where the cells were grown for a smaller number of generations in medium containing CM an ever larger fraction of the *NR1* DNA was in the monomeric r-determinants form. However, after this culture had entered stationary phase, the fraction of monomeric r-determinants DNA had decreased considerably. An analogous situation also occurred in the transition experiment shown in Fig. 5 and 6 where the minor 28S band observed in exponential phase (Fig. 6A and B) was no longer observed in the *NR1* DNA sedimentation profile after the culture had entered stationary phase (Fig. 6C). In this transition experiment, however, the major fraction of the *NR1* DNA from both the exponential and stationary phase cultures had a molecular weight greater than  $100 \times 10^6$ , as is typical of transition

experiments in which the cells are cultured in medium containing appropriate drugs for a prolonged period.

Although there is indication that the relative amounts of monomeric and multimeric r-determinant structures in transitioned cultures can be variable depending on the growth phase and the drug concentration in the growth medium, the variables influencing the degree of association of r-determinants are not understood at the present time. It is important to note that the distribution of monomeric and poly-r-determinant structures in transitioned cultures cannot be considered to be a steady-state situation. Continued growth of the culture in medium containing drugs should favor the formation of larger poly-r-determinant structures. On the other hand, growth of the culture in drug-free medium (or after inactivation of the drug) should result in the dissociation of poly-r-determinant structures and the dilution of r-determinants DNA from the cells; i.e., the back transition.

Despite the fact that r-determinants are dissociated from poly-r-determinant R factors during the back transition, dissociation does not go to completion since the final R factor structure after prolonged growth in drug-free medium appears to be the basic composite R factor consisting of an RTF-TC and one r-determinants. Just as in the case of nontransitioned *NR1* DNA, the basic composite structure does not dissociate further during the back transition. It is quite possible that r-determinants:r-determinants recombination sites and/or functions differ from those of the r-determinants:RTF-TC couple. Put more generally, the greater homology between two or more r-determinants than the homology between an r-determinants and an RTF-TC may explain why an R factor with only one r-determinants is less susceptible to r-determinants dissociation than a poly-r-determinant R factor.

The experiments described here have shown that the behavior of R factors in *P. mirabilis* can be quite different than that reported by several other laboratories (1, 3, 4, 7, 16, 19). These groups have suggested that extensive R factor dissociation normally occurs in *P. mirabilis* and that the multiple R factor bands observed in this host correspond to the RTF-TC and the r-determinants components and the basic composite R factor. Two of these groups have reported that neither intermediate density R factor DNA nor poly-r-determinants structures are present in their experiments (1, 19). We have recently obtained the *P. mirabilis* strains and R factors

(R3W, R6, R1) used by these other laboratories and carried out a detailed characterization of the R factor DNA in both the nontransitioned and transitioned states. Our experiments have revealed no basic differences in the behavior of R factors in these other strains of *P. mirabilis* in comparison with our own results with *NR1* in the strain used in our laboratory (R. Rownd and N. Goto, manuscript in preparation). During growth of the *P. mirabilis* host strains in drug-free medium, there was no detectable dissociation of the R factor DNA into the RTF-TC and r-determinants components. As a consequence of growth in medium containing CM, poly-r-determinant R factors and autonomous poly-r-determinants were formed. For reasons which will be discussed when the full accounts of this work are published, we think that the description of the behavior of R factors in *P. mirabilis* published by these other laboratories is based on the characterization of only a small fraction of the total R factor DNA by EM. The experiments described in this communication emphasize the necessity of a thorough characterization of the molecular structure of R factor DNA when there is considerable heterogeneity in the population of plasmid DNA molecules. It is especially important to analyze the total population of plasmid DNA by methods such as sucrose gradient sedimentation to insure that the more limited number of molecules viewed by electron microscopy are representative of the total plasmid population.

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