JOURNAL OF BACTERIOLOGY, June 1974, p. 855-866
Vol. 118, No. 3

Round of Replication Mutant of a Drug Resistance Factor

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Received for publication 13 November 1973

A derivative of the R factor *NR1* (called R12) has been isolated which undergoes an increased number of rounds of replication each division cycle in *Proteus mirabilis*, *Escherichia coli*, and *Salmonella typhimurium*. The alteration resulting in the increased number of copies (round of replication mutation) is associated with the transfer factor component of the R factor. R12 has the same drug resistance pattern as *NR1*, is the same size as shown by sedimentation in a sucrose gradient and electron microscopy (63×10^6 daltons), and has the same partial denaturation map. The level of the R factor gene product chloramphenicol acetyltransferase has been examined in *P. mirabilis* and was found to be consistent with gene dosage effects. The plasmid to chromosomal deoxyribonucleic acid ratio of *NR1* increases several fold after entry into stationary phase, whereas this ratio for R12 remains approximately constant. Individual copies of R12 are selected at random for replication from a multicopy plasmid pool. A smaller percentage of R12 copies replicate during amino acid starvation than has previously been found for *NR1* in similar experiments.

A comparison of the data on the replication of a number of bacterial plasmids indicates that the mechanisms controlling the replication of various extrachromosomal elements can be quite different (2-4, 10, 14, 22). Although enough similarities do exist to be able to fit various plasmids into loose classification groups (4, 22), the differences observed make it seem very unlikely that they will all share the same deoxyribonucleic acid (DNA) replication control mechanisms. At present, it is not known what controls the number of rounds of replication of any plasmid during the bacterial division cycle. To gain an understanding of these controls, it would be desirable to isolate and to characterize mutants of genes which are involved in the control of plasmid DNA replication. At present, very few plasmid DNA replication control mutants are available.

In our laboratory we have been studying the control of extrachromosomal DNA replication in bacteria, particularly of the drug resistance factor (R factor) *NR1* (14, 24-26; C. Morris and R. Rownd, manuscript in preparation). *NR1*, as well as a number of other R factors, has previously been shown to be composed of two distinguishable units (4, 5, 26, 36): a resistance transfer factor (RTF) and a unit referred to as an r-determinant. The RTF mediates the infectious transfer of multiple drug resistance among bacteria and also carries the gene specifying tetracycline (TC) resistance. The r-determinant carries the other drug resistance genes which direct the synthesis of enzymes which specifically inactivate the antibiotics to which the R factor confers resistance.

When *Proteus mirabilis* harboring *NR1* is cultured in drug-free medium, the DNA of this composite R factor appears as a satellite band of density 1.712 g/ml to the chromosomal DNA (1.700 g/ml) in a neutral CsCl gradient (24, 26, 29, 30; H. Hashimoto and R. Rownd, manuscript in preparation). As described previously (26, 29), the proportion of the *NR1* DNA band is about 2.5 to 3% of the chromosomal DNA in exponential phase and increases to 6 to 8% after entry into stationary phase. If cells harboring *NR1* are cultured for many generations in medium containing any of the drugs to which *NR1* confers resistance (except TC), a much larger satellite band of density 1.718 g/ml is observed and the 1.712 g/ml band no longer appears in the *NR1* DNA density profile. This shift in density, termed the "transition," results from the selective amplification of r-determinants (1.718 g/ml) to form polygenic R factors consisting of an RTF component and multiple tandem copies

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of r-determinants (14, 26, 29, 30; D. Perlman, H. Kasamatsu, and R. Rownd, manuscript in preparation).

In this communication we describe the isolation of a mutant of *NR1* that has an increased number of rounds of replication (ROR) per cell division cycle. This ROR mutant, called R12, most noticeably differs from *NR1* in exponential cultures of *P. mirabilis* where there are about four times as many R factor copies per chromosomal DNA equivalent as observed for *NR1*. A preliminary account of this work has been presented previously (C. Morris, H. Hashimoto, S. Mickel, C. Hershberger, and R. Rownd. 1972. Abstr. Annu. Meet. Amer. Soc. Microbiol. p. 62).

MATERIALS AND METHODS

Bacterial strains. The strains of *P. mirabilis*, Pm15 (11) and ϕS-3 (20), which were used were derived from strain Pm1 (25) and have been described previously. The *Escherichia coli* K-12 strains used were W677, described previously (28), and CR34,-Thy$^-$ obtained from W. Dove. CR34,Thy$^-$ requires threonine, leucine, thiamine, and thymine for growth. *Salmonella typhimurium* LT-2 was obtained from H. Hashimoto.

R factors. The R factor *NR1* confers resistance to chloramphenicol (CM), streptomycin/spectinomycin (SM/SP), sulphonamide (SA), and TC and has been described previously (28). The isolation and characterization of the R factor R12 is the subject of this paper. The RTF-TC of R12 was isolated as a naturally occurring R factor segregant in a *S. typhimurium* (LT-2) host by H. Hashimoto.

Isolation of R12. The R factor *NR1* harbored in *P. mirabilis* Pm15 was transferred to *E. coli* W677 by bacterial mating as described previously (28). R$^+$ W677 was selected on W677 minimal agar plates containing 50 μg of SM per ml. A clone was isolated and purified by repeated streaking on agar plates containing drug. The R factor was then transferred from W677 to Pm15 by the same bacterial mating procedure except that R$^+$ Pm15 was selected on Pm15 minimal agar plates containing 50 μg of SM per ml. A colony was selected and purified by repeated streaking on agar plates containing drug. The R factor harbored in the Pm15 host was found to have the same drug resistance pattern as *NR1* (CM, SM, SA, TC) but showed a higher percentage plasmid to chromosomal DNA than *NR1* in a CsCl density gradient.

Drugs. CM and streptomycin sulfate (SM) were purchased from Parke Davis and Co., Detroit, Mich., and Merck and Co., Inc., Rahway, N.J., respectively. TC hydrochloride, sulfadiazine, and sulfathiazole were generously provided by the American Cyanimide Co., Pearl River, N.Y.

Media. Penassay broth (Difco), M9 (1), and M9 buffer (M9 lacking NH_4Cl) were used. The M9 and M9 buffers used for growth of Pm15/R12 were supplemented to yield a medium of the following composition: 10^{-3} M $MgSO_4$; 0.2% glucose; thymine, 2.5 μg/ml; nicotinic acid, 10 μg/ml; leucine, 20 μg/ml; tryptophan, 20 μg/ml; vitamin-free Casamino Acids (Difco), 0.03% in M9 and 0.01% in M9 buffer. A 300-μg amount of either $^{14}NH_4Cl$ per ml or $^{15}NH_4Cl$ per ml was added to the M9 buffer. Appropriately supplemented M9 agar plates were used to select for R$^+$ recipient strains in mating experiments between *E. coli* and *P. mirabilis* (20).

Measurement of CM acetyltransferase specific activity. The enzyme activity was determined by the procedure described by T. Franklin and R. Rownd (8).

Isolation of DNA and CsCl density gradient centrifugation. Isolation of DNA and CsCl density gradient centrifugation were carried out as described previously (14, 25) except that the pH of the saline-EDTA buffer (0.15 M NaCl and 0.1 M disodium ethylenediaminetetraacetic acid) that was used to resuspend the cells for lysis was 10.2 instead of 8. *Micrococcus lysodeikticus* DNA (1.731 g/ml) was added as a reference DNA. In alkaline CsCl gradient experiments, the density of *P. mirabilis* chromosomal DNA was used as a reference in the calculation of the densities of the other bands.

In the preparative ethidium bromide-CsCl density gradient (23) experiment, the DNA (500 μg) was centrifuged in 8 ml of CsCl solution (final density 1.61 g/ml) containing: 0.005 M Na_2EDTA, pH 8.0; 0.8 mg of ethidium bromide per ml (gift of the Boots Pure Drug Company, Ltd., Nottingham, England); and 6.3 g of CsCl (Harshaw, optical grade). The solution was centrifuged in polyallomer tubes at 35,000 rpm in a Spinco Ti 50 rotor for 60 h at 20 C. The rotor was then allowed to coast to a stop (unbraked) and 0.08- to 0.10-ml fractions were collected from the bottom of the tube. In the neutral preparative CsCl gradients, the DNA (200 μg) in 5 ml of CsCl solution (1.710 g/ml) containing 0.01 M Na_2EDTA, pH 8.0, was centrifuged and fractionated as described above except that 0.04- to 0.05-ml fractions were collected.

Sucrose gradient centrifugation. Sucrose gradients (5 to 20% wt/wt) containing either SSC buffer (0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) or 0.01 M tris(hydroxymethyl)aminomethane (Tris) and 0.001 M Na_2EDTA, pH 10.0, were prepared by using a Buchler Polystaltic pump and mixing chamber. Samples containing 0.4 μg or less DNA were layered on the gradient and centrifuged at 20 C in an SW 50L rotor at 35,000 rpm for either 75 min or 2 h as described in the text. The rotor was coasted (unbraked) to a stop and 0.1-ml fractions were collected from the bottom of the tube onto 1.25-inch squares of Whatman no. 40 filter paper. After drying, these squares were washed in cold 7.5% trichloroacetic acid, ethanol, and then ether, and counted in a Beckman or a Nuclear Chicago liquid scintillation counter. Toluene containing 5 mg of 2,5-diphenyloxazole per ml served as the standard fluor.

Sedimentation coefficients were calculated from the distances sedimented relative to [^{14}C]thymine-labeled λ DNA which was included with each sample as a reference. The sedimentation coefficient of λ DNA is taken as 34.4*S* (9).

Amino acid starvation experiment. A supplemented M9 medium lacking tryptophan was divided equally into four sterile flasks (referred to as flasks A,

B, C, and D). After one-fourth of the medium had been transferred to flask A, [^{3}H]thymine (5 μCi/ml) was added to the remaining three-fourths of medium which was then transferred to flasks B, C, and D. To flasks A and C were added 20 μg of tryptophan per ml and [^{14}C]thymine (0.1 μCi/ml). Flasks A and C were inoculated with Pm15/R12 and incubated at 37 C. When the cultures (A and C) reached mid-exponential phase they were filtered, washed, and resuspended into flasks B and D, respectively. A portion from the resuspended cultures in flask D was taken immediately as a zero time sample. After 3.5 h of incubation at 37 C the cultures from B and D were harvested by centrifugation and the DNA was isolated. The DNA was centrifuged in a preparative neutral CsCl density gradient, fractionated, and counted.

To determine the amount of chromosomal DNA synthesis during amino acid starvation, the zero time and 3.5-h DNA profiles from culture D were examined. By normalizing the chromosomal ^{14}C peaks for both profiles, the percentage of increase in [^{3}H]DNA after amino acid starvation could be calculated. This percentage of increase was determined to be 43%.

Electron microscopy. The purified DNA samples were prepared for electron microscope examination by the procedure described by Inman and Schnös (13). When the DNA contour length was to be determined, the buffer solution was titrated to pH 8.0. When the DNA was to be partially alkaline denatured, the buffer solution was titrated to pH 10.69. A calibration grid (Polaron accessories for microscopy, 54,800 LPI) was used to determine the micron length of the DNA molecules examined.

RESULTS

Isolation and characterization of R12. During a series of mating experiments in which the R factor *NR1* harbored in *P. mirabilis* (Pm15) was transferred to *E. coli* (W677) and then back to Pm15, an R factor, called R12, which had a number of different properties than *NR1* was isolated (Materials and Methods). In *P. mirabilis*, *NR1* DNA appeared as a satellite band of density 1.712 g/ml which was 2.5 to 3% of the chromosomal DNA (1.700 g/ml) in exponential phase cultures (Fig. 1A); the percentage of *NR1* DNA increased to about 7% of the chromosomal DNA in stationary phase (data not shown) (24, 26, 29, 30). Unlike *NR1*, the percentage of R12 DNA (1.712 g/ml) was 10% of the chromosomal DNA in both exponential (Fig. 1C) and stationary phase cultures (data not shown). When *P. mirabilis* cells harboring *NR1* are cultured in Penassay broth containing appropriate concentrations of either CM or SM, there is an increase in the density of *NR1* DNA to a 1.718 g/ml form which represents a much larger percentage of the chromosomal DNA (Fig. 1B) (26, 27, 29, 30). Although R12 confers resistance to the same antibiotics as *NR1*, R12 DNA does not undergo

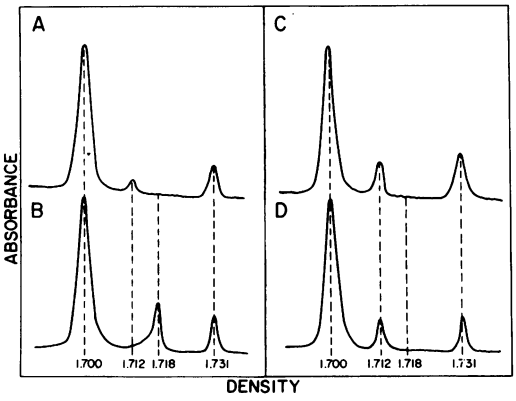

FIG. 1. *Density profiles of DNA prepared from P. mirabilis harboring the R factors NR1 and R12. Microdensitometer tracings of ultraviolet absorption photographs at equilibrium in a neutral (pH 8.0) CsCl density gradient at 44,000 rpm. DNA was prepared from exponential Pm15/NR1 (A) or Pm15/R12 (C) cells cultured in Penassay broth. DNA was prepared from stationary phase Pm15/NR1 (B) or Pm15/R12 (D) cells cultured in Penassay broth containing 100 μg of CM per ml for approximately 25 generations. M. lysodeikticus DNA (1.731 g/ml) was added as a reference DNA.*

such a transition when *P. mirabilis* host cells are cultured in Penassay broth containing CM (Fig. 1D). However, an increase in the density and the amount of R12 DNA is obtained when *P. mirabilis* is cultured in Penassay broth containing 50 to 100 μg of SM per ml. The details of these experiments are presented in the accompanying communication (19).

Size and number of copies of R12. The increased size of the R12 satellite band DNA could be caused by either an increased number of copies per chromosomal DNA equivalent or an increased size of the R factor DNA. To distinguish between these two possibilities, R12 DNA was purified and analyzed by electron microscopy and sedimentation in a sucrose gradient. Pm15/R12 was cultured in medium containing [^{3}H]thymine to label the DNA. After isolation of the DNA, covalently closed circular (CCC) R12 DNA was fractionated by using an EtBr-CsCl gradient (23) (Fig. 2). Since the sedimentation coefficient (S) of CCC DNA increases with decreasing ionic strength while the S value of open circular and linear DNA remains nearly constant (15), the purified R12 DNA was sedimented in a low salt (0.01 M Tris and 0.001 M EDTA, pH 10) 5 to 20% sucrose gradient (Fig. 3A) and a 5 to 20% sucrose gradient containing SSC (0.15 M NaCl plus 0.015 M Na citrate, pH 7) (Fig. 3B). In each gradient there are two major bands in addition to the 34.4 S λ DNA (9) which was added as a

reference: a 48*S* band corresponding to open circular DNA which is present in both gradients, and a more rapidly sedimenting species at 79*S* in the low salt sucrose gradient and 70*S* in the SSC sucrose gradient. This behavior suggests that the latter bands correspond to CCC DNA. The remainder of the purified R factor DNA was stored at 4 C for 2 weeks. A sample of the stored DNA was then sedimented in a 5 to 20% sucrose gradient (SSC) (Fig. 3C). It can clearly be seen that the 70*S* species has decreased in proportion during storage and the 48*S* band has increased. This result also supports the assignment of the 70*S* peak as a single CCC DNA species that has degraded to a 48*S* open circular DNA form during storage. The *S* value of the linear form of R12 DNA was calculated to be 43*S* by reducing the *S* value of the open circular form by 11%. This corresponds to a molecular weight (MW) of 53×10^6 for R12 DNA when compared to linear λ DNA (34.4*S*; 30×10^6 MW) using the relationship between these two parameters presented by Freifelder (9).

Purified R12 DNA was examined in an electron microscope following the procedure outlined by Inman and Schnös (13). The average contour length found for 40 open circular molecules was 37 ± 0.7 μm. Since the contour length of DNA depends upon the ionic strength and

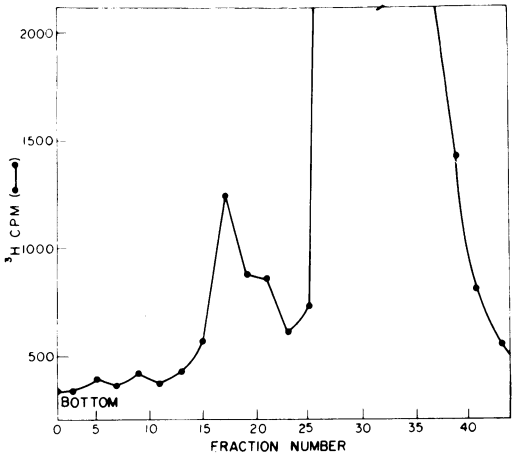

FIG. 2. *Ethidium bromide-CsCl density gradient centrifugation of DNA prepared from a culture of P. mirabilis harboring R12. The [^{3}H]thymine-labeled DNA was isolated and prepared for centrifugation as described in Materials and Methods. The DNA (500 μg) in 8 ml of ethidium bromide-CsCl solution was centrifuged to equilibrium in a Spinco Ti 50 rotor (20 C) at 35,000 rpm for 60 h. Fractions 15 to 21 were pooled for subsequent analysis on sucrose gradients.*

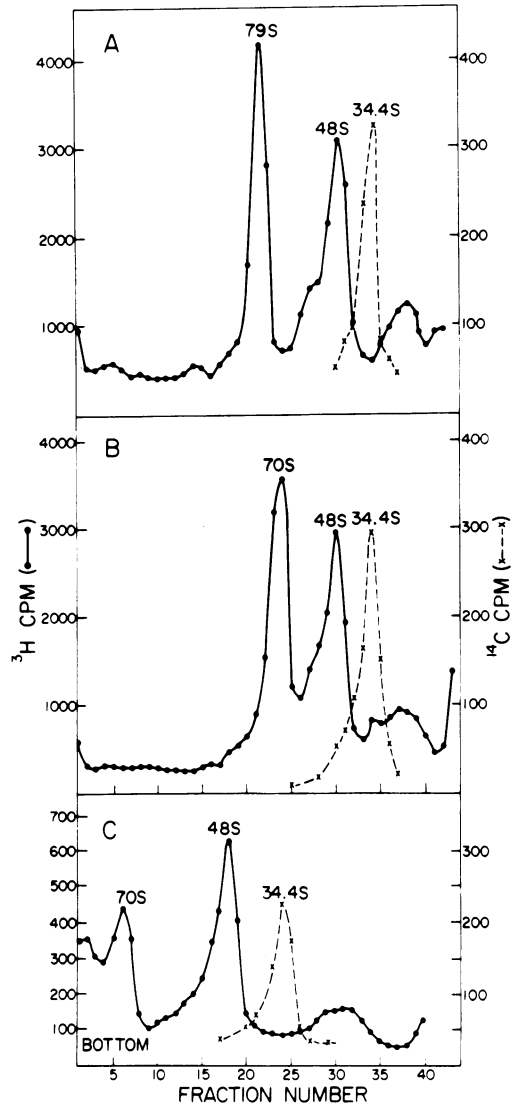

FIG. 3. *Sucrose gradient analysis of R12 DNA. The pooled fractions from the satellite peak in Fig. 2 were dialyzed to remove ethidium bromide from the CCC DNA. Portions of this DNA were sedimented through 5 to 20% sucrose gradients containing different salt concentrations in an SW50 L rotor at 35,000 rpm for 75 min at 20 C. (A) Sucrose gradient containing 0.01 M Tris and 0.001 M Na_3EDTA, pH 10. (B) Sucrose gradient containing SSC (0.15 M NaCl and 0.015 M sodium citrate, pH 7.0). (C) The DNA sample which had been kept at 4 C for 2 weeks was sedimented through a 5 to 20% sucrose gradient containing SSC in an SW50 L rotor at 35,000 rpm for 2 h at 20 C. Sedimentation was from right to left. Sedimentation coefficients were calculated relative to [^{14}C]thymine-labeled λ DNA (34.4S; ref. 9) co-sedimented with each gradient.*

composition of the reagents used in preparing the DNA for electron microscopy (12, 17, 18), the length of λ DNA (30×10^6 MW) was also determined. Using our conditions for sample preparation, λ DNA had a contour length 17.5 μm. R12 DNA should have a molecular weight of 63×10^6 ([37/17.5] $\times$ 30 $\times$ 10^6).

The MW of R12 DNA obtained by electron microscopy is higher than that estimated by sedimentation in a sucrose gradient by using the Freifelder (9) equation. The observed *S* value for R12 DNA appears to be about 10% too small. This discrepancy has been seen repeatedly in our laboratory and has also been reported by other laboratories. For example, Clewell and Helinski (2) have reported an *S* value of 45*S* (45×10^6 MW using the Freifelder equation) for the open circular form of the colicin factor Col I_6-P9, whereas the MW obtained from electron microscopy was 61×10^6 (2). Until the cause of this discrepancy is resolved, our MW assignments will be based primarily on electron microscopy. Sedimentation in a sucrose gradient will be used to determine approximate DNA sizes and forms. Analysis of a DNA sample in a sucrose gradient has the advantage of examining the entire molecular population and is therefore very useful for identifying the number of molecular species present.

Since *NR1* DNA also sediments as a 48*S* open circular species and has a contour length of 37 μm (29; D. Perlman et al., manuscript in preparation), the difference in band size between the *NR1* and R12 is not a result of a difference in the MW of the R factor DNA. Rather there must be three to four times as many copies of R12 as *NR1* per chromosomal DNA equivalent in exponential *P. mirabilis* cells. This trait is stable and inherited. R12 can be transferred into different strains of *P. mirabilis* with the R factor band size always remaining 10% of the host chromosomal DNA. This alteration must result from a mutation on the R factor itself.

Replication of R12 in P. mirabilis during exponential and stationary growth phases. The replication of R12 in *P. mirabilis* was examined by monitoring the percentage of the R factor DNA satellite band relative to the host chromosomal DNA band in DNA samples prepared from exponential and stationary phase Penassay broth cultures. No significant increase in the percentage of R12 DNA relative to the chromosomal DNA was observed after entry into stationary phase. An accumulation of data from many such experiments has consistently yielded values between 9 and 12% for both exponential (10.1 ± 0.9%) and stationary (11.3 ± 1.4%) cultures.

This is in sharp contrast with studies on "transitioned" *NR1* DNA (1.718 g/ml) (14) and on the less detailed but similar studies conducted on *NR1* in the non-transitioned (1.712 g/ml) form (R. Rownd and H. Kasamatsu, unpublished data). Here the percentage of *NR1* DNA relative to the *P. mirabilis* chromosomal DNA consistently increased 2.4-fold after entry into stationary phase when the cells were cultured in Penassay broth containing 20 μg of thymine per ml. Since the chromosomal DNA content of *P. mirabilis* cultures also increases about twofold after entry into stationary phase in Penassay broth (H. Kasamatsu and R. Rownd, unpublished data), the increased percentage of R factor DNA must correspond to approximately a fivefold increase in the number of *NR1* copies. For R12, the constancy of the percentage of R factor DNA in both exponential and stationary phases must mean that there is a doubling of the number of R factor copies after entry into stationary phase.

When Pm15/R12 is cultured in minimal M9 medium containing the required growth supplements for this auxotrophic strain, there is again no significant change in the percentage of R12 DNA for cultures in exponential (11.5 ± 0.5%) or stationary phase (12.5 ± 0.5%). Since the chromosomal DNA content of members of the *Enterobacteriaceae* increases as a function of the growth rate of the cells (6, 33, 34), there must be a mechanism which coordinates the amount of R12 and chromosomal DNA replication in such a way that the percentage of R12 DNA remains the same irrespective of the growth rate.

Gene dosage effects in P. mirabilis harboring R12. Having established that there is an increased number of copies of R12 as compared to *NR1* in exponentially growing cells, it was of interest to examine if the extra copies produced a proportionate increase in R factor gene products due to gene dosage effects. A number of experiments were conducted in which the proportion of R factor DNA and the specific activity of the enzyme that inactivates CM, chloramphenicol acetyltransferase (31, 32), were monitored for the R factors R12, *NR1* in the nontransitioned form (1.712 g/ml), and *NR1* in the transitioned form (1.718 g/ml). Gene dosage effects have already been demonstrated for *NR1* in the 1.718 g/ml form by comparing the specific activity of CM acetyltransferase of exponential cultures having different size R factor DNA bands (26, 30). The results of several experi-

ments are summarized in Table 1. The specific activity of CM acetyltransferase in an exponential culture of Pm15/R12 is threefold higher than in an exponential culture of Pm15/*NR1* (1.712 g/ml). This increase is in reasonable agreement with the three- to fourfold increase in the number of R factor copies. In this study the transitioned form of *NR1* (1.718 g/ml) was included as a control to relate the already published *NR1* CM acetyltransferase studies (26, 30) with our present work. In that previous study of *NR1* (1.718 g/ml) the enzyme specific activity was found to increase about 4.5-fold after entry into stationary phase in Penassay broth containing 20 μg of thymine per ml. The enzyme specific activity of the *P. mirabilis* cells harboring *NR1* (1.718 g/ml) in the present study increased fourfold after entry into stationary phase, which corresponds to the 4.5-fold increase in the amount of R factor DNA/cell (26, 30). The approximate twofold increase in the enzyme specific activity of the Pm15/R12 culture after entry into stationary phase is consistent with our previous conclusion that there is a doubling of the amount of R12 DNA per cell in stationary phase. When cells harboring these R factors were cultured in M9 minimal medium, there was a threefold increase in the exponential phase enzyme specific activities for all three factors relative to their values in Penassay broth. It is interesting to note, however, there was no significant difference in the percentage of R factor DNA in M9 medium compared to the values observed in Penassay broth (Table 1). Thus, *P. mirabilis* cells harboring R12 have higher specific activities of CM acetyltransferase than cells harboring *NR1*, as would be expected from gene dosage effects.

ROR mutation located on RTF-TC. Since R12 represented an R factor replication mutant, we wished to determine whether this alteration resided on the r-determinant or the RTF-TC component of the R factor. This was accomplished by transferring the R factor R12 to *S. typhimurium* and selecting naturally occurring segregants that had segregated the r-determinant component but retained transferability and TC resistance. An R12 RTF-TC segregant of R12 was transferred back to *P. mirabilis* φS-3 which is a TC-sensitive mutant of strain Pml (20). The satellite band of the R12 RTF-TC had a density of 1.710 g/ml and was 8% of the chromosomal DNA in both exponential and stationary phase cultures. The contour length of 20 open circular R12 RTF-TC molecules was 30 ± 1 μm which corresponds to a MW of 50×10^6. An RTF-TC isolated from the R factor *NR1* has the same contour length (D. Perlman et al., manuscript in preparation); however, its percentage in exponential phase cultures is only 2% of the chromosomal DNA. Thus, the increased band size of the R12 RTF-TC must result from an increased number of copies. The fact that the R12 RTF-TC band size is only three-fourths of that of the composite R factor is consistent with the reduction in MW expected from the loss of the r-determinant which accounts for about one-fourth of the R factor DNA. Thus, whatever change is involved in the increased number of copies of R12 appears to be associated with the RTF-TC component of the R factor and does not depend upon the presence of the r-determinant.

^{14}N-^{15}N density shift experiments. Copies of *NR1* are selected at random for replication from a multicopy plasmid pool without discrimina-

TABLE 1. *Comparison of the specific activities of CM acetyltransferase in P. mirabilis cells harboring the R factor R12 or NR1*[a]

No. of exp.	Strain	R factor density (g/ml)	Media	Exponential phase		Stationary phase		Ratio of CM acetyltransferase (stationary)/CM acetyltransferase (exponential)
				CM acetyltransferase sp act (units per mg of cell protein)	% R factor DNA/chromosomal DNA	CM acetyltransferase sp act (units per mg of cell protein)	% R factor DNA/chromosomal DNA	
6	Pm15/R12	1.712	PAB + thy	3.6 ± 0.4	10.1 ± 0.9	8.4 ± 1.8	11.5 ± 1.5	2.3
3	Pm15/*NR1*	1.712	PAB + thy	1.3 ± 0.2	2.2 ± 0.6	4.0 ± 1.0	5.4 ± 1.0	3.1
2	Pm15/*NR1*	1.718	PAB + thy	7.5 ± 0.7	7.5 ± 0.5	30.7 ± 2.5	20.8 ± 2.4	4.1
2	Pm15/R12	1.712	M9	10.8 ± 0.8	11.5 ± 0.5			
2	Pm15/*NR1*	1.712	M9	4.0 ± 0.4	3.0 ± 0.5			
2	Pm15/*NR1*	1.718	M9	25.5 ± 2.0	7.0 ± 0.5			

[a] Cells were cultured in either Penassay broth (PAB) supplemented with 20 μg of thymine per ml or M9 medium (Materials and Methods). DNA was isolated from samples of the cells and examined in an analytical CsCl density gradient (pH 8.0); the specific activity of CM acetyltransferase in samples of the cells was determined as described by T. Franklin and R. Rownd (8).

tion as to whether any particular copy has been duplicated one or more times during the cell division cycle (14, 25). To examine how copies of R12 are picked for replication, an exponential culture of Pm15/R12 was transferred from ^{14}N-labeled minimal medium to ^{15}N-labeled minimal medium. At exactly one doubling time (70 min) after the medium transfer, the ^{15}N culture was immediately chilled, 0.001 M sodium azide was added, and the cells were harvested by centrifugation. The DNA was isolated for examination in a CsCl gradient.

To be able to resolve the R12 bands clearly and not have the ^{14}N:^{15}N hybrid chromosome (1.705 g/ml) overlap and interfere with the resolution of the ^{14}N:^{14}N light plasmid (1.712 g/ml), it was necessary to use an alkaline CsCl density gradient and examine R factor CCC DNA (34). At pH values greater than 12.4, CCC DNA is 20 mg/ml more dense than single stranded linear or circular DNA of the same base composition (35). The R12 CCC fraction cannot be argued to be unrepresentative of the plasmid DNA because 90 to 100% of R12 DNA is in the CCC form when isolated as described in Materials and Methods (C. Morris and R. Rownd, manuscript in preparation).

In Fig. 4A is shown the density profile in an alkaline CsCl gradient of Pm15/R12 DNA isolated after one doubling time in ^{15}N-labeled medium. Due to a difference in base composition between the complementary strands of both chromosomal and R12 DNA, two bands are formed for each type of DNA (11). The single-stranded chromosomal light (1.760 and 1.764 g/ml) and heavy (1.770 and 1.774 g/ml) as well as the plasmid light (1.772 and 1.776 g/ml) and heavy (1.782 and 1.786 g/ml) complementary strands cannot be clearly resolved due to the overlap of adjacent bands. The light (1.795 g/ml), hybrid (1.800 g/ml), and heavy (1.805 g/ml) CCC R12 DNA bands can be resolved reasonably well. An enlarged tracing of this portion of the density profile is shown directly above the corresponding bands. Better resolution can be achieved by lowering the pH of the alkaline CsCl gradient from 12.4 to 11.7. The CCC R12 DNA decreases in density over 70 mg/ml as a result of lowering the pH, whereas the single-stranded chromosomal and R12 DNA remains at the density characteristic of the fully titrated DNA (Fig. 4B). The greater resolution of the R12 CCC DNA from the chromosomal DNA bands allows a more accurate calculation of their proportions. As shown in Table 2, essentially all of the *P. mirabilis* chromosomal DNA is hybrid after one generation in ^{15}N-labeled medium. On the other hand, light (1.724 g/ml), hybrid (1.729 g/ml), and heavy (1.734 g/ml) CCC R12 DNA are all observed (Fig. 4B). The ratio of light to hybrid to heavy is 1:2.7:1, which is in good agreement with the theoretical

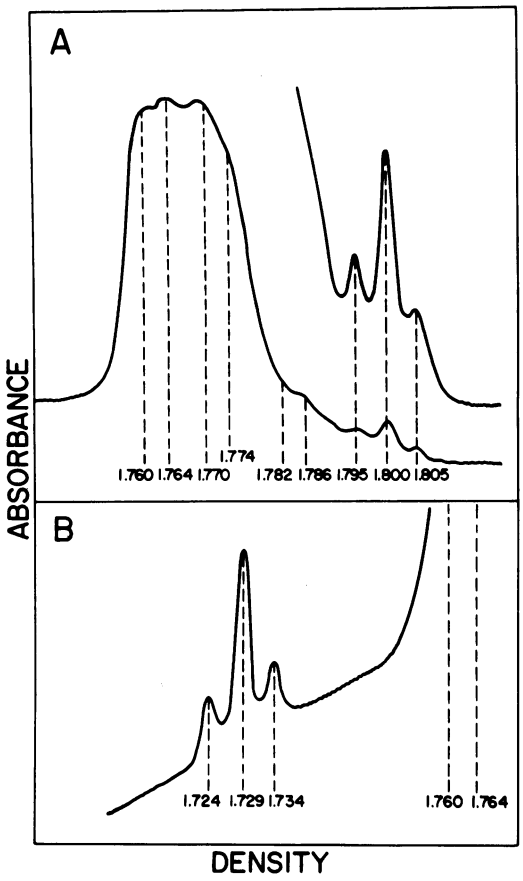

FIG. 4. *Replication of DNA in Pm15/R12 after transfer to ^{15}N-labeled medium for one generation of growth. Shown are microdensitometer tracings of the distribution of DNA in alkaline CsCl gradients at pH 12.5 (A) or after back titration from pH 12.5 to pH 11.7 (B) prior to centrifugation. The DNA was isolated from samples of the cells which were harvested 70 min after transfer to ^{15}N-labeled medium as described in Materials and Methods. Portions of the DNA were then placed in CsCl solutions containing 0.04 M K_3PO_4 (pH 13) to give a final solution pH of 12.5 and a density of 1.750 g/ml. The DNA shown in profile B was then titrated back to pH 11.7 with 1 M KH_2PO_4 (pH 5.5). The densities of the single-stranded light and heavy P. mirabilis chromosomal DNA bands and the light, hybrid, and heavy CCC R12 DNA bands are described in the text. Directly above the CCC bands in density profile (A) is a magnified profile of this region of the density gradient. In density profile (B) the chromosomal DNA bands are not shown. The proportion of the light, hybrid, and heavy CCC R12 DNA (Table 2) was calculated from density profile (B).*

TABLE 2. *Proportion of light, hybrid, and heavy chromosomal DNA and CCC R12 DNA after transfer to ^{15}N-labeled medium*[a]

Time in ^{15}N-labeled medium (min)	Generations in ^{15}N-labeled medium	Chromosomal DNA			CCC R12 DNA			Theoretical distribution of R12 DNA for random replication		
		Light	Hybrid	Heavy	Light	Hybrid	Heavy	Light	Hybrid	Heavy
70	1.0		1.00		0.21	0.57	0.22	0.233	0.534	0.233

[a] The proportion of light, hybrid, and heavy *P. mirabilis* chromosomal DNA was calculated from microdensitometer tracings of photographs of the DNA distribution in a neutral (pH 8.0) CsCl gradient. The proportion of light, hybrid, and heavy CCC R12 DNA was calculated from the alkaline (pH 11.7) CsCl density gradient profile shown in Fig. 4B. The theoretical distribution expected for random selection of copies of R12 for replication was calculated (25) on the assumption that there were eight copies of R12 in the plasmid pool at the time of transfer to ^{15}N-labeled medium. As described previously (14, 25), the distribution of light, hybrid, and heavy plasmid DNA expected from the random model of replication varies only modestly as a function of the pool size.

values of 1:2.3:1 expected for random selection of R factors for replication from a multicopy pool of eight R factors (25).

Comparison of R12 and NR1 replication after amino acid starvation. When an exponential culture of an auxotrophic strain of *P. mirabilis* harboring *NR1* is starved for a required amino acid for 3 to 4 h, about 25% of the *NR1* DNA copies replicate during the period in which protein synthesis is inhibited (24, 26; D. Perlman and R. Rownd, unpublished data). A preliminary experiment revealed that the amount of R12 DNA replication during amino acid starvation was considerably smaller than that found for *NR1*. To increase the sensitivity of the analysis, an exponential culture of Pm15/R12 labeled with [^{14}C]thymine was filtered, washed, and resuspended into medium lacking tryptophan and containing [^{3}H]thymine. The amount of R12 DNA replication during tryptophan starvation could then be calculated by comparing the ratio of ^{3}H-labeled R12 DNA to ^{3}H-labeled chromosomal DNA if the amount of chromosomal DNA synthesized during the amino acid starvation could be accurately determined. In a parallel culture grown under exactly the same growth conditions except that [^{3}H]thymine was present at the same specific activity before and during tryptophan starvation, the percentage of increase in chromosomal DNA after starvation was found to be 43%. By normalizing the ^{3}H-labeled chromosomal DNA which was synthesized during amino acid starvation to 43% of the ^{14}C-labeled chromosomal peak representing the amount of DNA present in the exponential culture just prior to starvation, the amount of R12 DNA replication during amino acid starvation could be determined (Fig. 5). Within the sensitivity of this technique, it is estimated that 10 to 15% of the R12 DNA is replicated during amino acid starvation.

Examination of R12 in E. coli and S. typhimurium. Because the densities of *E. coli* DNA (1.710 g/ml) and *S. typhimurium* DNA (1.710 g/ml) are similar to that of R12 DNA (1.712 g/ml), the plasmid DNA cannot be resolved as a satellite band in a neutral CsCl density gradient. CCC R12 DNA, however, can be distinguished from the host chromosomal DNA in an alkaline (pH 12.5) CsCl gradient. Figure 6 shows an alkaline CsCl density profile of DNA prepared from *E. coli* harboring R12. Since CCC DNA is converted to the open circular form with first order kinetics in alkaline CsCl solutions (C. Morris and R. Rownd, manuscript in preparation), it is necessary to take a series of ultraviolet absorption photographs at different times after equilibrium is reached and to extrapolate the percentage CCC R12 DNA observed to zero time of centrifugation to find the percentage of CCC DNA present. In both *E. coli* and *S. typhimurium* this percentage is 10% of the chromosomal DNA which is in agreement with the value obtained in *P. mirabilis*. The percentage of CCC R12 DNA in *E. coli* and *S. typhimurium* is, of course, only a minimal estimate since open circular and linear R12 DNA, if present, would not have been resolved from the chromosomal DNA. Since the *NR1* CCC DNA band is only 4% of the chromosomal DNA in exponential phase cultures of *E. coli* and *S. typhimurium*, there appears to be a greater number of copies of R12 than *NR1* in these hosts as well.

Comparison of the DNA partial denaturation maps of R12 and NR1. Another means of comparing *NR1* and R12 is by their DNA partial denaturation maps. In these experiments, the procedures described by Inman and Schnös (18) were used. Conditions of sample preparation for electron microscopy were determined so that enough small denatured regions were introduced into R12 DNA to orient the circular

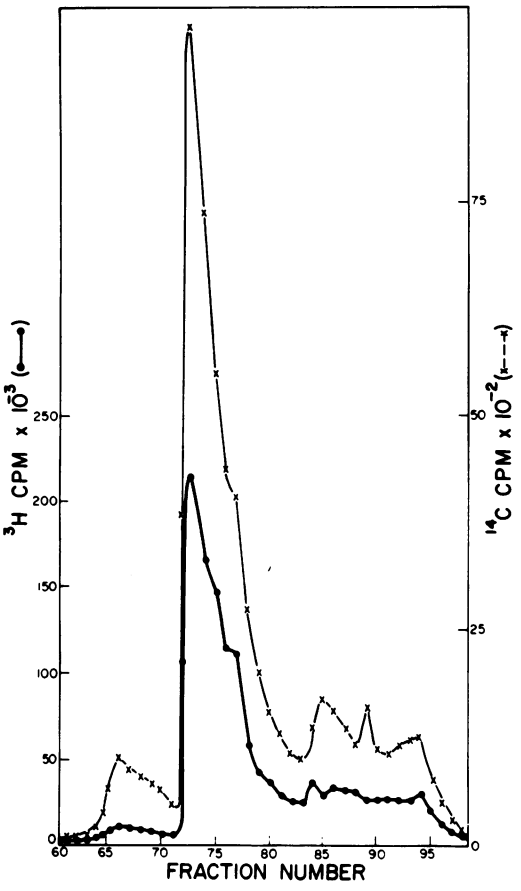

FIG. 5. *Fractionation by preparative CsCl density gradient centrifugation of DNA prepared from Pm15/R12 after amino acid starvation. An exponential culture of Pm15/R12 growing in supplemented M9 medium containing [^{14}C]thymine as described in Materials and Methods was filtered, washed, and suspended into supplemented M9 medium containing [^{3}H]thymine but lacking tryptophan. After 3.5 h of tryptophan starvation, the cells were harvested by centrifugation and the DNA was isolated. The DNA (400 μg) was placed in a neutral (pH 8.0) CsCl solution and centrifuged to equilibrium in a Spinco Ti 50 rotor (20 C) at 35,000 rpm for 60 h. The [^{3}H]thymine DNA profile representing DNA synthesis after amino acid starvation was normalized to 43% of the [^{14}C]thymine chromosomal DNA. The 43% figure represents the amount of host chromosomal DNA synthesis which occurred during amino acid starvation in a parallel culture.*

molecules uniquely. In our experiments the pH range of 10.69 to 10.73 provided the best results. Comparison of the partial denaturation maps of R12 and *NR1* DNA (Fig. 7) reveals that each denatured region found on *NR1* (D. Perlman et al., manuscript in preparation) is also present at the same corresponding map location on R12. No detectable deletion, translocation, or rearrangement of any of the R12 DNA was observed.

DISCUSSION

Little is known about the mechanism of control of plasmid DNA replication. In this communication we have described the properties of a replication control mutant derived from the R factor *NR1*. Because R12 replicates an increased number of times each bacterial division cycle, it is called a "round of replication" mutant. There are four copies of R12 per chromosomal DNA equivalent compared to one to

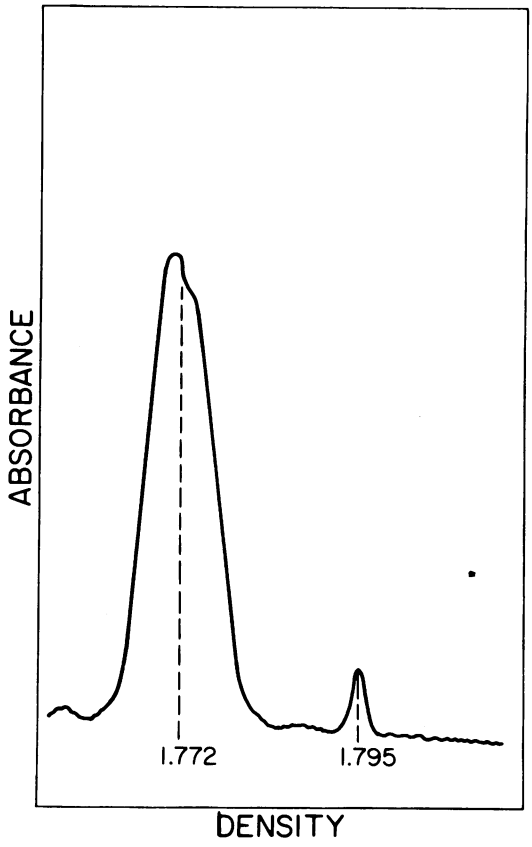

FIG. 6. *Density profile of DNA prepared from E. coli harboring R12 in an alkaline CsCl gradient. Shown is a microdensitometer tracing of the distribution of CR34/R12 DNA in an alkaline (pH 12.5) CsCl gradient. This profile was one of several taken at various time intervals to monitor the first order decay of CCC DNA. The percent CCC R12 DNA (1.795 g/ml) to chromosomal DNA for each profile was determined. Extrapolation to zero time of centrifugation of a first order plot of the logarithm of the percent CCC R12 DNA versus time gives the percent CCC DNA present in the DNA sample (C. Morris and R. Rownd, manuscript in preparation).*

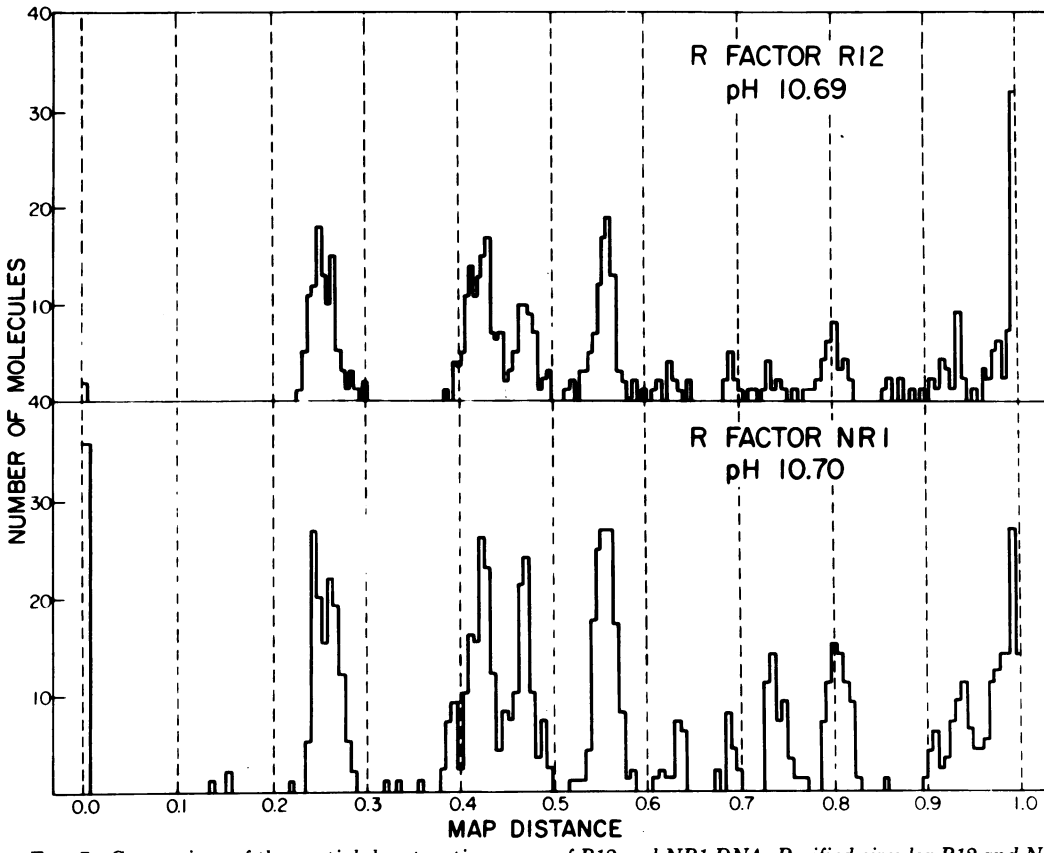

FIG. 7. *Comparison of the partial denaturation maps of R12 and NR1 DNA. Purified circular R12 and NR1 DNA was prepared for electron microscopy using the procedure described by Inman and Schnös (13) for alkaline partial denaturation. The R factor DNA was placed in pH 10.69 buffer solution for 10 min at room temperature just prior to spreading. The denaturation map of NR1 DNA was constructed by Perlman et al. (manuscript in preparation).*

two copies of nontransitioned *NR1* (1.712 g/ml) in exponential cultures of *P. mirabilis*. The ROR mutation is a property of the R factor since the increased number of copies of R12 is maintained after transfer to a new host. The gene(s) controlling this altered replication character resides on the RTF-TC component of the R factor. Both *NR1* and R12 have the same drug resistance pattern. However, cells harboring R12 have a higher level of resistance to CM and SM (19) and a higher specific activity of CM acetyltransferase (Table 1), as would be expected from gene dosage effects. R12 DNA has the same size and density as *NR1* DNA (1.712 g/ml). No genetic data are yet available to suggest whether this naturally occurring ROR mutant is due to a point mutation or a deletion. By using the electron microscopic procedures outlined by Inman and Schnös (13), we found no detectable differences in the contour length or the partial denaturation maps of R12 DNA and *NR1* DNA.

It is very interesting to note that in addition to the increase in the number of copies per cell, R12 differs from *NR1* in a number of other properties, many of which are concerned with replication of the plasmid DNA. While there is a 2.4-fold increase in the percentage of *NR1* DNA after entry into stationary phase (14, 24), the increase in R12 DNA parallels the increase in host chromosomal DNA in stationary phase so that there is essentially no change in the percentage of R12 DNA. When cells harboring R12 are cultured at different growth rates, the percentage of R12 DNA relative to the chromosomal DNA also remains approximately constant. The amount of *P. mirabilis* chromosomal DNA per cell increases as a function of the growth rate (34; R. Rownd and R. Stickgold, manuscript in preparation). The number of R12 copies per cell must also increase in such a way that the percentage of R12 DNA remains the same. About 25 to 30% of the copies of *NR1* undergo replication when protein synthesis is

inhibited by amino acid starvation (26; R. Rownd, Fed. Proc. 30:1313, 1971). In similar experiments only 10 to 15% of the R12 copies undergo replication. R12 DNA does not undergo a transition in density from its 1.712 g/ml form to a 1.718 g/ml form when *P. mirabilis* cells are cultured in very high concentrations of CM (up to 800 μg/ml). However, R12 DNA does undergo a transition when cultured in medium containing SM (19). Almost 100% of R12 DNA in the 1.712 g/ml density form can be isolated as CCC DNA, whereas a maximum of 35% of nontransitioned *NR1* DNA (1.712 g/ml) can be isolated in the supercoiled form (C. Morris and R. Rownd, manuscript in preparation). All of these changes appeared simultaneously which suggests that the R12 phenotype resulted from a single pleiotropic event and not a series of individual mutational events. These findings suggest that there is a complex mechanism controlling R12 replication such that an alteration in one aspect of the control mechanism may have a simultaneous effect on a number of replication characteristics of the plasmid.

Our observations that only a small amount of *NR1* or R12 replication occurs during amino acid starvation indicates that one or more proteins are required for the initiation of *NR1* and R12 replication. This residual replication can reasonably be explained by the existence of a small pool of initiator protein for *NR1* and R12 DNA replication in the cells. The percentage of copies of *NR1* which replicate during amino acid starvation (25 to 30%) is about three times the percentage of copies of R12 which replicate during inhibition of protein synthesis (10 to 15%). However, there may not be much difference in the absolute number of copies of *NR1* and R12 which replicate during amino acid starvation since there are about three times as many copies of R12 per cell than there are of *NR1*. Thus the steady-state initiator protein pools for *NR1* and R12 replication may be of comparable sizes in exponential cells. Since R12 was derived from *NR1*, it seems quite likely that they utilize the same initiator protein(s). If this hypothesis is correct, then the size of the R factor initiator protein pool does not appear to determine the number of rounds of R factor replication during the bacterial division cycle since there are about three times as many copies of R12 per cell as of *NR1*.

Nordström, Ingram, and Lundbäck (21) have independently isolated ROR mutants of the R factor R1. Although their characterization of these mutants was not as extensive as our studies with R12, there is a general agreement between their results and our own with one notable exception. They concluded that the ROR mutation resided on the r-determinant component of the R factor. This conclusion was based on experiments in which *P. mirabilis* cells harboring either the wild-type R factor or the ROR mutant were cultured in medium containing 100 μg of CM per ml. The 1.718 g/ml DNA band corresponding to the r-determinant component of the R factor was found to be larger in the culture with the ROR mutant than in the culture with the wild-type R factor. They suggested that the r-determinant might be governing replication under conditions where both r-determinant and RTF are present as a combined replicon. We do not feel that this interpretation is justified. Culturing *P. mirabilis* cells harboring an R factor in medium containing appropriate drugs for many generations can certainly result in an enlarged plasmid DNA band which has the same modal density as the r-determinant DNA (compare Fig. 1A and B). However, the R factor DNA density profiles of these cultures are highly variable both in size and in the extent of the density shift (19, 26, 27). As described in the accompanying communication (19), when *P. mirabilis* harboring R12 is cultured in medium containing SM, there is an increase in both the proportion and the density of the R12 DNA to a limiting value of 1.718 g/ml, which is the density of the r-determinant DNA. In some experiments the R12 DNA band was larger than the chromosomal DNA band. These changes are due to the formation of polygenic R factors consisting of an RTF and multiple tandem copies of r-determinants and polygenic r-determinants consisting of repeated sequences of r-determinants. They are not a consequence of the ROR mutation which resides on the RTF component of R12. A much better way to determine whether a ROR mutation resides on the RTF or the r-determinant component of an R factor would be to isolate RTF segregants of the ROR mutant and the wild-type R factor. The number of copies of each RTF can then be compared as we have done for R12.

Our preliminary results from genetic complementation experiments between a TC-sensitive mutant of *NR1* and the RTF-TC component of R12 have shown that there is no decrease in the number of copies of the RTF-TC of R12 and there is no increase in the number of copies of *NR1* when both R factors are present simultaneously in an *E. coli* host (C. F. Morris and R. Rownd, unpublished data).

ACKNOWLEDGMENTS

This work was supported (in part) by Public Health Service research grant GM 14398 and by Research Career Development Award GM 19206 (to R.R.), both from the National Institute of General Medical Sciences. C. F. Morris

and S. Mickel were predoctoral trainees of a Public Health Service Research Training grant.

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