JOURNAL OF BACTERIOLOGY, July 1976, p. 619-636
Vol. 127, No. 1
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*Eco*RI Restriction Endonuclease Map of the Composite R Plasmid NR1

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Received for publication 12 January 1976

A physical map of the composite R plasmid NR1 has been constructed using specific cleavage of deoxyribonucleic acid (DNA) by the restriction endonuclease *Eco*RI. Digestion of composite NR1 DNA by *Eco*RI yields thirteen fragments. The six largest fragments (designated A to F) are from the resistance transfer factor component that harbors the tetracycline resistance genes (RTF-TC). The seven smallest fragments (designated G to M) are from the r-determinants component that harbors the chloramphenicol (CM), streptomycin-spectinomycin (SM/SP), and sulfonamide (SA) resistance genes. The largest fragment of several RTF-TC segregants of NR1 that have deleted the r-determinants component is 0.8×10^6 daltons larger than fragment A of composite NR1. Only a part of fragment H of the r-determinants component is amplified in transitioned NR1 DNA in *Proteus mirabilis,* which consists of multiple, tandem sequences of r-determinants attached to a single copy of the RTF-TC component. Both of these changes can be explained by the locations of the excision sites at the RTF-TC: r-determinants junctions that are involved in the dissociation and reassociation of the RTF-TC and r-determinants components. The thirteen fragments of composite NR1 DNA produced by *Eco*RI have been ordered using partial digestion techniques. The order of the fragments is: ┌A-D-C-E-F-B-H-I-L-K-G-M-J┐. The approximate locations of the TC, CM, SM/SP, and SA resistance genes on the *Eco*RI map were determined by analyzing several deletion mutants of NR1.

The genome of the R plasmid NR1 is a deoxyribonucleic acid (DNA) molecule that has a molecular weight of 63×10^6 (16, 18). This R plasmid confers resistance to tetracycline (TC), chloramphenicol (CM), streptomycin-spectinomycin (SM/SP), and sulfonamide (SA) to host cells (17). Like many R plasmids (2, 5), NR1 is a composite structure that is composed of two distinguishable components: a resistance transfer factor (RTF) and a unit referred to as r-determinants. The RTF mediates the infectious transfer of multiple drug resistance among bacteria; the TC resistance genes of NR1 also reside on the RTF component (referred to as an RTF-TC) (19, 24, 27). The r-determinants component of NR1 harbors the CM, SM/SP, and SA drug resistance genes.

In *Proteus mirabilis,* the RTF and r-determinants of composite R plasmids dissociate and reassociate, which results in the regulation of the number of copies of r-determinants per host cell (23, 25). When *P. mirabilis* harboring NR1 is cultured in drug-free medium, NR1 DNA forms a single satellite band with a density of 1.712 g/ml in a CsCl density gradient that represents about 7% of the *P. mirabilis* chromosomal DNA (1.700 g/ml) in stationary-phase cultures. In this "nontransitioned" state, the NR1 DNA exists in the form of the composite structure, consisting of one copy of the RTF component and one copy of the r-determinants component. After prolonged growth in medium containing CM, SM, or SP, the 1.712-g/ml NR1 DNA band is no longer detectable, and a much larger satellite band with a density of 1.718 g/ml is observed in the NR1 DNA density profile. This replacement of the 1.712-g/ml NR1 DNA band by the 1.718-g/ml NR1 DNA band during growth in medium containing appropriate drugs is called a "transition" (23, 25). Transitioned cells have an increased level of resistance to the antibiotics CM, SM/SP, and SA, but not to TC (8, 22). In the transitioned state, the NR1 DNA consists of multiple, tandem copies of r-determinants that are joined to a single copy of the RTF component (poly-r-determinant R plasmids) and molecules containing multiple copies of r-determinants (autonomous poly-r-determinants) (18, 19, 23-25).

This report describes the construction of a physical map of the specific fragments of NR1

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DNA produced by the restriction endonuclease *Eco*RI (10). The alterations in the *Eco*RI restriction fragments of several deletion mutants of NR1 and the related R plasmid NR84 (17) were also studied. The locations of the drug resistance genes on the map were determined. Such a physical map will be important in determining a number of other chemical, genetic, and biological properties of the R plasmid, such as the location of the origin and terminus of replication and the location of specific single-strand breaks.

MATERIALS AND METHODS

Bacterial strains and R plasmids. The *P. mirabilis* (Pm15 and ϕS-3) and *Escherichia coli* K-12 (CSH-2 and ML1410) strains used in this study have been previously described (8, 17, 18, 23). R plasmids and their origins are described in Table 1.

Growth of cells and radioisotope labeling of DNA. Cells were cultured overnight in Penassay broth (Difco) to stationary phase. In experiments where plasmid DNA was to be used as carrier and was not radioactively labeled, 20 μg of thymine per ml was added to the medium. No unlabeled thymine was added when isotope labeling was done. To prepare transitioned NR1 DNA for r-determinants mapping, stationary-phase cells of Pm15/NR1 were diluted 10^8-fold into Penassay broth containing 100 μg of CM per ml and grown to stationary phase twice in succession. The cells reached stationary phase at 3 days after the first dilution, but they reached stationary phase in less than 2 days after the second dilution because of their higher level of resistance to CM. Examination of the DNA from these stationary-phase transitioned cells by analytical CsCl gradient centrifugation showed that the NR1 DNA had the density characteristic of transitioned DNA (1.718 g/ml) (18, 23) and represented 18% of the chromosomal DNA.

Plasmid DNA used for studies of endonuclease restriction digestion patterns was isolated from cells grown in Penassay broth supplemented with 2 μCi of [*methyl*-^{3}H]thymine (New England Nuclear, approximately 50 mCi/mmol) per ml so that it could be identified by radioactivity measurements during purification procedures. For partial digestion mapping, DNA of much higher specific activity was prepared from cells grown in Penassay broth containing 50 μCi of [*methyl*-^{3}H]thymine per ml. Plasmid DNA labeled with [^{14}C]thymine was obtained from cells that were cultured in Penassay broth containing 1 μCi of [*methyl*-^{14}C]thymine (New England Nuclear, approximately 50 mCi/mmol) per ml.

TABLE 1. *R plasmids used*

R plasmid	Resistance marker					Reference
	TC	CM	SA	SM	AP	
NR1	+	+	+	+		17
ROR12	+	+	+	+		16
RTF-TC-C+	+					D. Taylor[a]
NR1-1	+					R. Nakaya[a]
RTF-TC of ROR12	+					16
UCR122		+	+	+		13, 27
NR1-2		+	+	+		17
NR1-22	+	+		+		R. Nakaya[a]
NR1-T22	+	+				L. Taichman[a]
NR1-24	+		+	+		R. Nakaya[a]
NR84	+	+	+	+	+	17

[a] Unpublished data.

Preparation of plasmid DNA. Stationary-phase *P. mirabilis* cells were harvested, suspended in SV (0.15 M NaCl and 0.1 M disodium ethylenediaminetetraacetate, pH 10.2) and lysed with sodium dodecyl sulfate as described previously (21). *E. coli* cells were lysed by incubating them for 30 min at 65 C in 1.2% sodium dodecyl sulfate after treatment with 500 μg of lysozyme per ml for 15 min at 37 C. The DNA isolation method has been described (21).

Purification of covalently closed circular plasmid DNA was carried out using nitrocellulose column chromatography by the method of Boezi and Armstrong (1). The covalently closed circular DNA in the column effluent was concentrated by dialysis against 25% polyethylene glycol (Carbowax, Union Carbide) in 0.1× SSC (SSC is 0.15 M NaCl-0.015 M sodium citrate, pH 7.0) and further purified by preparative centrifugation in a neutral CsCl gradient as described previously (18). Transitioned NR1 DNA was fractionated by preparative centrifugation in a neutral CsCl gradient without using the nitrocellulose step. Plasmid DNA from the CsCl gradients was dialyzed against 0.1× SSC and frozen at −20 C until use. The purity of isolated plasmid DNA was examined by *Eco*RI digestion and electrophoretic analysis on agarose-ethidium bromide gels.

***Eco*RI preparation and endonuclease digestion.** *Eco*RI was purified according to Tanaka and Weisblum (29) and stored in 50% glycerol at −20 C. The reaction mixture for endonuclease digestion contained 90 mM tris(hydroxymethyl)aminomethane-hydrochloride, pH 7.4, 10 mM $MgCl_2$, 2.5 μg of plasmid DNA, and 5 μl of *Eco*RI in a total volume of 50 μl. Under these conditions, the DNA was completely digested in less than 30 min at 37 C. Digestion reactions were terminated by the addition of 20 μl of 2× BJ (BJ is 30% sucrose [wt/vol] and 0.005% bromphenol blue in E buffer [see below] with the disodium ethylenediaminetetraacetate concentration increased to 0.04 M).

Agarose-ethidium bromide gel electrophoresis. The method of Sharp et al. (28) was used for electrophoretic separation of DNA fragments. In different experiments the agarose (Sigma, electrophoresis grade) concentration was varied between 0.5 and 1.0% (wt/vol). Electrophoresis buffer (E buffer), which contained 0.04 M tris(hydroxymethyl)aminomethane, 0.02 M sodium acetate, 0.001 M disodium ethylenediaminetetraacetate and 0.5 μg of ethidium bromide per ml, was prepared as a 10-fold concentrated solution, adjusted to pH 7.9 with glacial acetic acid before the addition of ethidium bromide, and diluted just prior to use.

(i) Analytical agarose gels. Samples of DNA (0.7 to 2.5 μg) mixed with 2× BJ were applied to agarose gels (0.6 cm in diameter, 11 or 40 cm in length), and

electrophoresis was performed at 50 V (constant voltage) for 5 to 8 h at 23 C, depending on the experiment. After adequate separation of the DNA bands was obtained, gels were removed from the glass tubes, illuminated with a short-wavelength mineral light (UVS-54, Ultraviolet Products, Inc., San Gabriel, Calif.), and photographed using type 57 film (ASA 3000) with a Polaroid MP-3 Land camera equipped with Wratten K2 (yellow) and 25 (red) filters. Direct visualization of the DNA bands without removal of gels from the glass tubes was possible by illuminating the gels with a long-wavelength ultraviolet light (Blak-Ray B-100 A, Ultraviolet Products, Inc.).

To monitor radioactivity on the gels, separated DNA bands were cut out with a razor blade, and the individual gel slices were dissolved in 0.5 ml of 2 N HCl by incubation at 65 C overnight in capped scintillation vials. A 10-ml portion of Triton X-100 scintillation mixture (500 ml of Triton X-100, 1,000 ml of toluene, 7.5 g of PPO, and 0.15 g of POPOP) was added to each vial, and the radioactivity was counted in a Nuclear Chicago liquid scintillation counter.

(ii) Preparative agarose gels. Large-scale 0.55 or 0.6% preparative agarose gels (26 mm in diameter, 50 or 70 cm in length) were used to separate partial digestion fragments of plasmid DNA for mapping studies. Electrophoresis was performed at 15 mA per gel (constant current) for 3 to 5 days to obtain adequate separation of the DNA bands. Higher current resulted in skewing of the bands. While the electrophoresis was in progress, E buffer in the reservoirs was changed each day.

Coelectrophoresis of the DNA of different R plasmids. Coelectrophoresis of *Eco*RI digests of two different R plasmids on analytical agarose gels was routinely done to determine the identity or nonidentity of restriction fragments from the two sources. The DNA species were digested separately, the reactions were terminated, and the mixtures were combined in the desired proportions and subjected to electrophoresis. Two different gels were done for each pair of R plasmids, using two different ratios of R plasmid DNA, so that the source of each restriction fragment could be unambiguously determined. Low concentrations of DNA were used on these gels so that fragments with similar but not identical mobilities could be clearly distinguished.

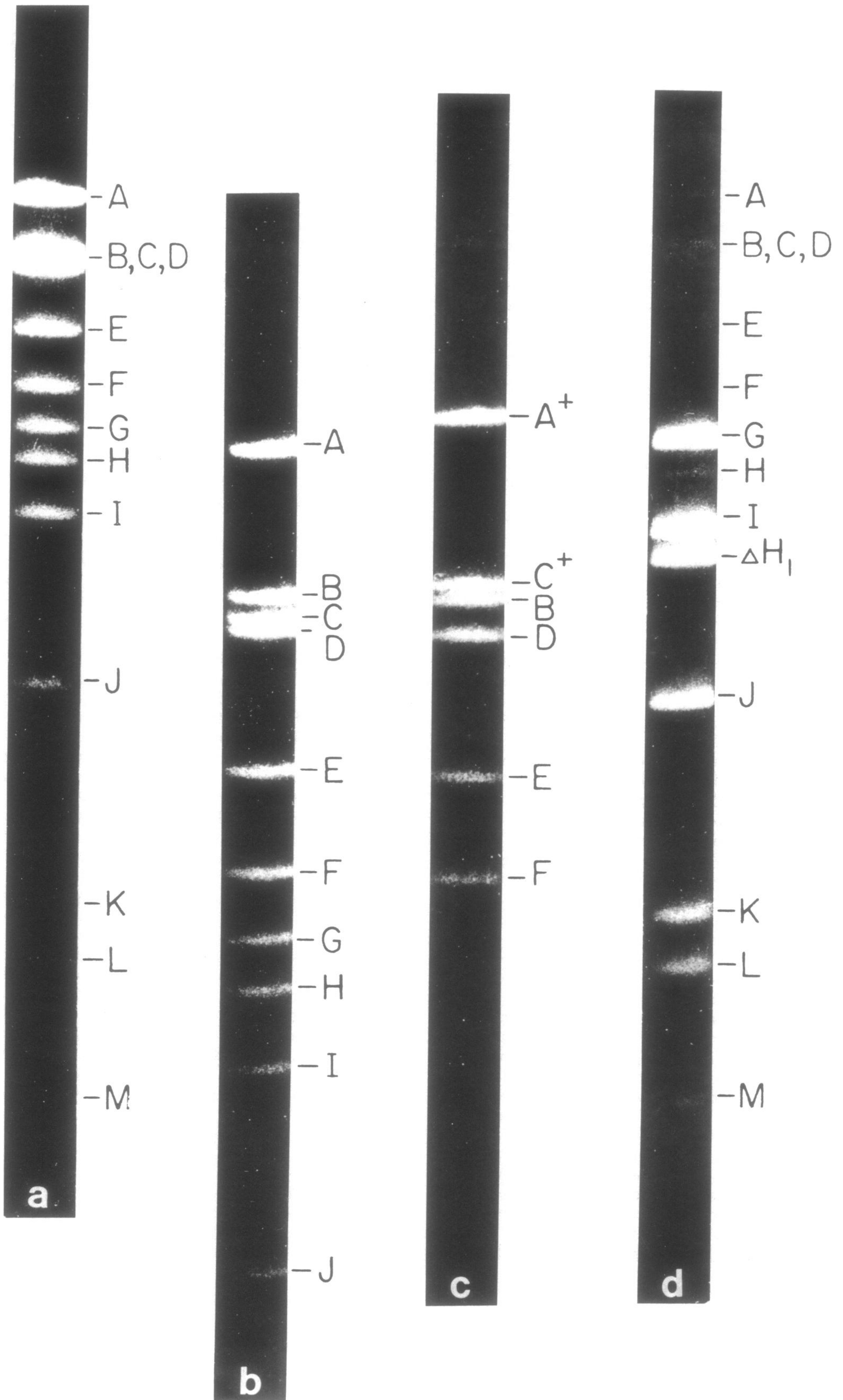
Partial digestion mapping. The mapping was done by analysis of partial digestion products and overlapping sets of fragments as described by Danna et al. (4). About 70 μg of plasmid DNA in a total volume of 1.0 ml was digested with 50 μl of *Eco*RI at 37 C for the time periods indicated in Fig. 4, mixed with 0.4 ml of 2× BJ, applied to a preparative gel, and subjected to electrophoresis as described above. After the DNA bands were adequately separated, the gel was removed from the glass tube and photographed, and the bands were sliced out with a razor blade. Each gel slice was crushed by forcing it once through a 25-gauge hypodermic needle into a Pyrex centrifuge tube, and the DNA was extracted as follows. A 2-ml portion of 0.1× SSC containing 10 μg of appropriate unlabeled carrier DNA (i.e., transitioned NR1 DNA for transitioned NR1 partial digestion, etc.) was added to each tube, the contents were mixed, frozen in dry ice-acetone, and thawed in a 37 C water bath twice, and the agarose was removed by centrifugation at 4 C and 12,000 rpm for 30 min in a Sorvall RC2-B with an SS-34 rotor. The agarose pellets were reextracted with 2 ml of 0.1× SSC, the two supernatants were combined, and the DNA solutions were dialyzed first against 25% polyethylene glycol in 0.1× SSC to concentrate them and then against 0.1× SSC. The final volume of each extract was about 500 μl.

Five milliliters (0.7 to 0.8 μg) of the appropriate [^{14}C]DNA (not previously treated with *Eco*RI), 10 μl of 10× salt solution [0.9 M tris(hydroxymethyl)-aminomethane-hydrochloride (pH 7.4)-0.1 M $MgCl_2$], and 10 μl of the *Eco*RI preparation were added to 75 μl of each DNA extract. The DNA was completely digested by a 1-h incubation at 37 C, and the final digestion fragments were separated by analytical agarose gel electrophoresis. The RTF-TC C$^+$ DNA final digestion products were separated on 0.6% gels (0.6 cm by 40 cm) to obtain sufficient resolution of the bands, whereas those of r-determinants DNA were adequately separated on 1% gels (0.6 by 11 cm). To resolve all of the fragments of composite NR1 DNA, double-layer gels (0.55% top layer, 1.0% bottom layer, 0.6 by 40 cm) were used. After separation was achieved, each band was sliced out with a razor blade, and the ^{3}H/^{14}C ratio was determined. This ratio was used as an indicator for the presence or absence of the final digestion fragments in each partial digestion DNA band.

Electron microscopy. NR1 *Eco*RI fragments E, K, L, and M were mounted for electron microscopy by the method of Inman and Schnös (15) and rotary shadowed with platinum. Purified λ DNA was included with fragment E, and P4 DNA was included with K, L and M as internal length markers. Grids were examined and photographed with a Philips 300 electron microscope. Fragment E molecules were projected on a paper screen, traced, and measured with a Keuffel and Esser map measurer. Length measurements for fragments K, L, and M were made on a digitizer (Numonics Corp.) interfaced to a programmable calculator and plotter (Hewlett-Packard). Length and molecular weight determinations were made relative to the marker DNAs. The λ DNA has a contour length of 17.06 μm (Inman, personal communication) and a molecular weight of 30×10^6 (7); P4 DNA has a contour length of 4.1 μm and a molecular weight of 7.35×10^6 (32).

RESULTS

***Eco*RI restriction fragments of NR1 DNA.** When *Eco*RI digestion products of NR1 DNA were separated by 1% agarose-ethidium bromide gel electrophoresis, 11 bands were detected (Fig. 1a). The second band from the top of the gel, however, was very broad and more intense than expected if it had been composed of a single fragment. Therefore, attempts were made to obtain better resolution of the larger



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-F
-G
-H
-I
-J
-K
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-A
-B
-C
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-A+
-C+
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c
-A
-B,C,D
-E
-F
-G
-H
-I
-ΔH1
-J
-K
-L
-M
d

(slower moving) fragments. Gel electrophoresis using lower concentration of agarose (0.5 to 0.7%) clearly showed that the second band was actually a "triplet" consisting of three separate DNA fragments (Fig. 1b). The smaller fragments ran off the gel when electrophoresis was carried out long enough to resolve the triplet into the three distinct bands. Thus, 13 fragments, designated A through M in order of decreasing size (4), were observed in the *Eco*RI digest of NR1 DNA. Electrophoresis on 1.4% agarose gels failed to demonstrate the presence of any fragments with a higher mobility than fragment M.

The molecular weights of the *Eco*RI fragments of NR1 DNA are shown in Table 2. Fragments E, K, L, and M were purified, their contour lengths were measured in the electron microscope, and their molecular weights were determined as described above. Fragment J coelectrophoresed with an *Eco*RI restriction fragment of the ribosomal DNA genes of the yeast *Saccharomyces cerevisiae* (γ DNA), which has a molecular weight of 1.79×10^6 (J. H. Cramer, unpublished data). The molecular weights of the remaining fragments were determined by coelectrophoresis with the *Eco*RI fragments of a bacteriophage λ mutant (λ_{cb2}) whose molecular weight is known (F. Blattner, personal communication). In Table 2, the *Eco*RI fragment C of λ_{cb2} has been assigned an arbitrary mobility of 100 units, and the mobilities of the other λ_{cb2} and NR1 fragments are expressed relative to it. The standard curves obtained by plotting the log molecular weight of the λ_{cb2} fragments and NR1 fragments E, J, K, L, and M versus their relative mobilities on 1.0% agarose (Fig. 2A) and 0.6% agarose (Fig. 2B) were similar to the curve obtained recently by Thomas and Davis (30) for *Eco*RI restriction fragments of λ DNA. Our data extend the range of this curve to lower molecular weight values. Using these standard curves, the molecular weights of the other NR1 *Eco*RI fragments were determined from their mobilities. Values for B, C, and D could be calculated only from the 0.6% gels, since these fragments were not sufficiently separated on 1.0% gels. The estimates for fragments F through I from the 0.6% and 1.0% gels agree quite well (Table 2).

The *Eco*RI digest of a round of replication mutant of NR1 (ROR12) that has an increased number of R plasmid copies per cell (16) has a gel pattern indistinguishable from that of NR1 DNA (data not shown). However, at a later stage of this study it was found that a basic change had occurred in the *Eco*RI fragments of an NR1 that had been harbored in another strain of *P. mirabilis* Pm15, which had been maintained in this laboratory. The *Eco*RI digest of the DNA of this variant NR1 (referred to as "NR1 C+") had the same pattern as the NR1 DNA shown in Fig. 1a and b, except that fragment C was not present and a new fragment with a lower mobility (larger molecular weight)

TABLE 2. *Relative mobility and molecular weights of EcoRI restriction fragments of NR1 DNA*

Gel	DNA source	Fragment	Relative mobility[a]	Size ($\times 10^6$ daltons)
1.0% Agarose-ethidium bromide	λcb2	A	57.9	13.5[b]
		B	87.7	4.67[b]
		C	100	3.70[b]
		D	116.0	2.76[b]
		E	135.4	2.06[b]
	NR1	A	57.9	13.5
		B–D	68.5–71.2	8.0–7.2
		E	84.3	5.00[c]
		F	95.4	4.0
		G	102.7	3.5
		H	108.8	3.2
		I	119.2	2.7
		J	153.6	1.79[c]
		K	196.9	1.10[c]
		L	208.3	1.01[c]
		M	237.7	0.75[c]
0.6% Agarose-ethidium bromide	λcb2	A	43.4	13.5[b]
		B	84.8	4.67[b]
		C	100	3.70[b]
		D	117.8	2.76[b]
		E	135.8	2.06[b]
	NR1	A	43.4	13.5
		B	59.0	8.0
		C	61.7	7.55
		D	63.3	7.2
		E	80.5	5.00[c]
		F	94.3	4.0
		G	103.4	3.5
		H	110.5	3.2
		I	120.9	2.7
		J	151.6	1.79[c]

[a] Relative mobilities are averages of five gels.

[b] These values were obtained from F. Blattner (personal communication).

[c] Molecular weights determined from DNA contour lengths in an electron microscope. Between 80 and 100 molecules of each fragment were measured.

FIG. 1. *Agarose-ethidium bromide gel electrophoresis of EcoRI restriction fragments. (a) NR1 DNA on 1.0% agarose gel; (b) NR1 DNA on 0.6% agarose gel; (c) RTF-TC C+ DNA on 0.6% agarose gel; (d) transitioned NR1 DNA on 1.0% agarose gel.*

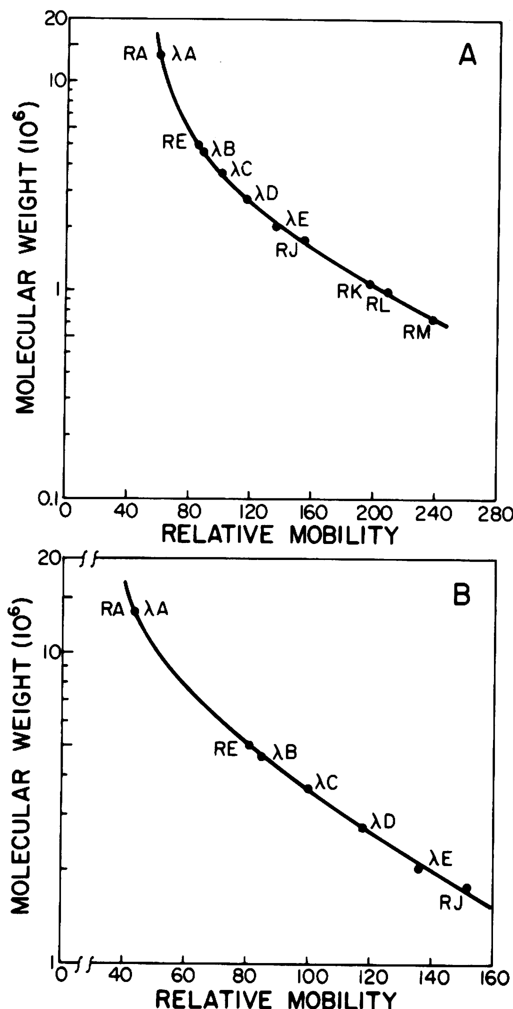

FIG. 2. *Relative electrophoretic mobility on agarose-ethidium bromide gels of EcoRI restriction fragments of NR1 DNA. The log (molecular weight) of the EcoRI fragments of NR1 DNA is plotted against their mobility. The molecular weights were estimated from the contour lengths of the fragments, which were measured by electron microscopy. Mobilities were expressed relative to the mobility of the fragment C of* λ_{cb2} *DNA, which was assigned an arbitrary value of 100. λA-λE are EcoRI restriction fragments of* λ_{cb2} *DNA. RA, RE, and RJ-RM are EcoRI restriction fragments of NR1 DNA. (A) 1.0% agarose gel; (B) 0.6% agarose gel.*

than NR1 fragment B was observed in the gel pattern. Since all the other fragments of NR1 C^+ coelectrophoresed with the corresponding fragments of NR1, it appears that the new fragment in the NR1 C^+ digest originated by an insertion in fragment C of NR1. This is the reason why we have designated the variant NR1 C^+. (In other cases where fragments of mutant R plasmids appear to have arisen by insertions in specific fragments, we routinely follow this nomenclature and label the new fragment by its original letter in NR1 with a superscript plus.) The insertion in fragment C of NR1 DNA must have occurred during the maintenance of strain Pm15 harboring NR1 as a stock culture in this laboratory. All other mutants of NR1 used in these studies contained fragment C rather than C^+, with the exception of an RTF-TC segregant (RTF-TC C^+), which probably was derived from NR1 C^+. These observations are consistent with the view that NR1 C^+ was derived from NR1 by an insertion in fragment C rather than NR1 being derived from NR1 C^+ by a deletion.

***Eco*RI restriction fragments of RTF-TC segregant DNA.** The *Eco*RI digest of an RTF-TC segregant of NR1 that had lost the r-determinants component was examined, since it was expected that it would yield fewer *Eco*RI fragments than the composite R plasmid and therefore simplify the mapping of the fragments. These experiments had been initiated before the difference between fragment C of NR1 and of NR1 C^+ was recognized. The R plasmid that was most extensively characterized (for no special reason) was an RTF-TC C^+ derived from NR1 C^+. Six *Eco*RI fragments, designated A^+, C^+, B, D, E, and F in order of increasing mobility, were found (Figure 1c). Fragment A, which was previously observed in both RN1 DNA and NR1 C^+ DNA, was not observed in RTF-TC C^+ DNA. The largest fragment of RTF-TC C^+ DNA (A^+) had a lower mobility (hence, a higher molecular weight) than fragment A of NR1 C^+ DNA and, therefore, fragment A of NR1 DNA, since the A fragments of NR1 C^+ DNA and NR1 DNA coelectrophorese. Fragment C^+ of RTF-TC C^+ DNA coelectrophoresed with fragment C^+ of NR1 C^+ DNA and displayed the expected difference in mobility with fragment C of NR1 DNA in a coelectrophoresis experiment. Figure 3 illustrates the differences in mobility of fragments A and A^+ and fragments C and C^+ in coelectrophoresis of RN1 DNA and RTF-TC C^+ DNA after *Eco*RI digestion. Fragments B, D, E, and F of RTF-TC C^+ DNA coelectrophoresed with the corresponding fragments of both NR1 C^+ and NR1 DNA.

The increase in size of the largest *Eco*RI fragment of RTF-TC DNA (A to A^+) was found not only for the RTF-TC C^+ described above but also for two other RTF-TC segregants that have unaltered C fragments. As will be explained below, the change in fragment A is a consequence of the location of one of the r-determinants excision sites in the composite R plasmid.

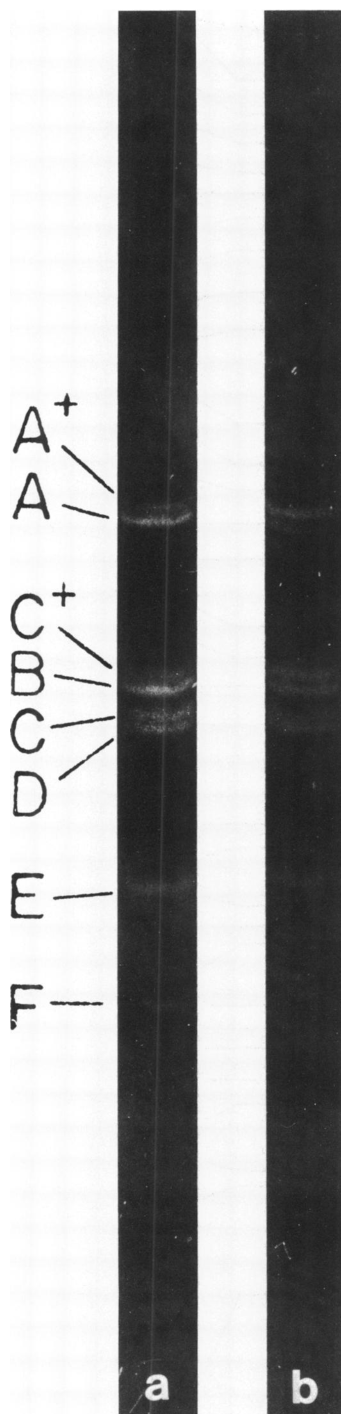

FIG. 3. *Coelectrophoresis of EcoRI-digested NR1 DNA and RTF-TC C+ DNA. (a) 0.5 μg of RTF-TC C+ DNA plus 1.0 μg of NR1 DNA on 0.6% agarose gel; (b) 0.8 μg of RTF-TC C+ DNA plus 0.4 μg of NR1 DNA on 0.6% agarose gel.*

In all of the RTF-TC segregants that were examined, only fragments A^+ through F were present; all of the fragments with higher mobilities were missing. Thus, the largest six fragments of the R plasmid DNA must be from the RTF-TC component and, as will also be shown below, fragments G through M must be from the r-determinants component.

***Eco*RI restriction fragments of transitioned NR1 DNA.** In the transitioned state the amount of r-determinants DNA is amplified, whereas that of RTF-TC DNA apparently is not (18, 23, 25). When an *Eco*RI digest of transitioned NR1 DNA was analyzed on 1% agarose-ethidium bromide gels (Fig. 1d), fragments A through F and also fragment H were present only in minor quantities; fragment G, fragments I through M, and a new fragment that had a mobility between that of I and that of J (referred to as fragment ΔH_1) were present in much larger quantities. As will be discussed later, ΔH_1 appears to have originated from fragment H as a result of the transition. This electrophoresis pattern of *Eco*RI fragments is consistent with the mode of transition previously reported (18, 23, 25), i.e., transitioned DNA consists of poly-r-determinant R plasmids plus autonomous poly-r-determinants, and therefore results in a large amplification of r-determinants fragments. The amplification in the relative amounts of fragments G, I through M, and ΔH_1 is consistent with our previous assignment of these fragments to r-determinants DNA because they were not present in RTF-TC DNA. We will explain below why fragment H is not amplified in transitioned DNA even though ΔH_1 and the other r-determinants fragments are amplified.

Partial digestion mapping. To simplify the mapping of R plasmid DNA using the partial digestion technique, the RTF-TC was first mapped using RTF-TC C^+ DNA. The r-determinants component was then mapped using transitioned NR1 DNA. The orientation between RTF-TC fragments and the r-determinants fragments was then determined using composite NR1 DNA.

Partial digestion products of RTF-TC C^+ were prepared and fragments were analyzed as described above. Due to the large molecular weight of RTF-TC DNA (49×10^6), partial digestion products were generally very large and difficult to separate even on 0.6% gels that were 50 or 70 cm long. Figure 4a shows an example of an RTF-TC C^+ partial digestion reaction on a preparative agarose gel.

Table 3 is a summary of partial digestion experiments of RTF-TC C^+ DNA. Some of the DNA bands in the partial digest appeared to be

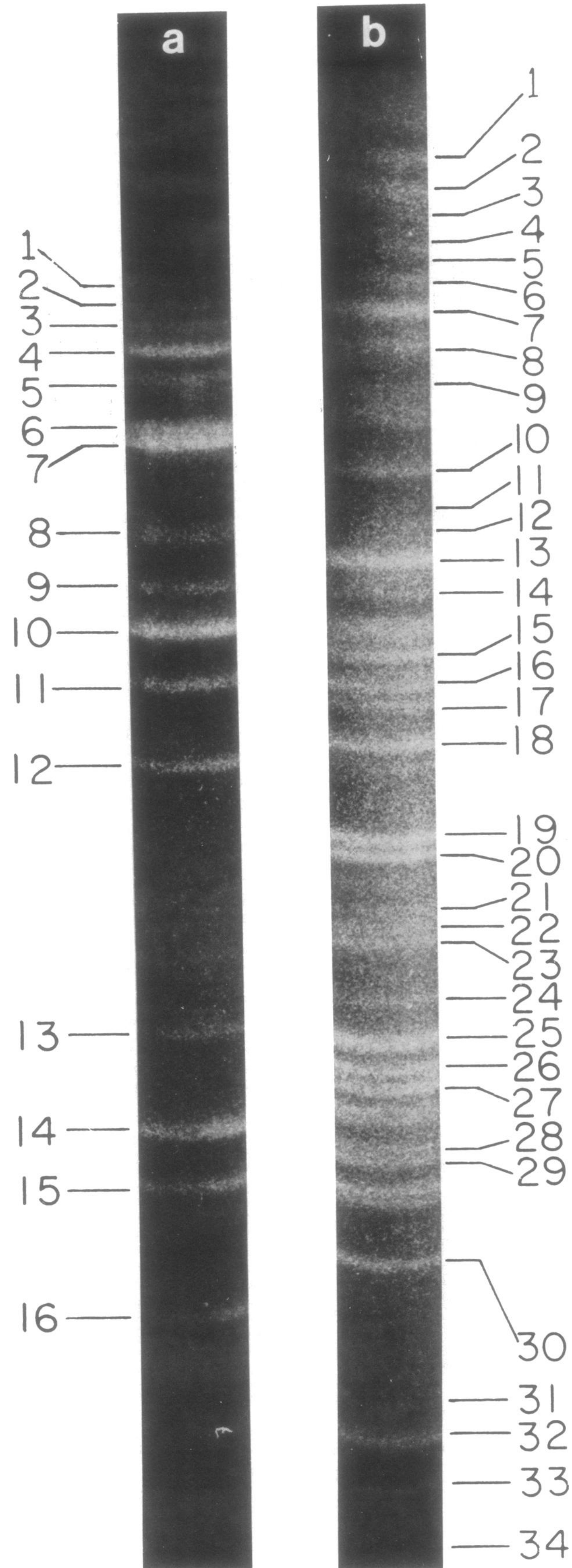
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TABLE 3. *Summary of RTF-TC-C⁺ DNA partial digestion experiments*

Partial fragment	$^3H/^{14}C$ ratios in final fragments						Final fragments present	Estimated mol wt[a] of partial fragments ($\times 10^6$)	Order of final fragments
	A^+	C^+	B	D	E	F			
Expt 1									
6[b]	0.0	0.2	0.3	0.3	4.2	4.9	E, F	9.0	EF
5	0.1	1.2	13.7	0.9	0.7	18.1	B, F	12.0	FB
4	0.5	15.5	1.6	1.4	23.6	1.8	C^+, E	13.2	C^+E
3	23.9	2.1	2.3	2.1	2.4	2.1	A^+	14.3	A^+
2	4.5	1.8	2.1	8.0	2.0	1.8	A^+, D	21.5	A^+D
1	9.2	2.4	11.4	8.6	3.5	3.0	A^+, B, D	29.5	BA^+D
Expt 2[c]									
15	0.1	0.5	7.8	0.7	0.7	0.7	B	8.0	B
14	0.1	13.9	7.0	1.1	1.6	1.4	C^+	8.2	C′
13	0.0	0.2	0.3	0.3	4.2	4.9	E, F	9.0	EF
12	0.1	1.2	13.7	0.9	0.7	18.1	B, F	12.0	FB
11	0.5	15.4	1.6	1.4	23.7	1.8	C^+, E	13.2	C^+E
10	23.9	2.1	2.3	2.0	2.4	2.1	A^+	14.3	A^+
9	2.3	6.7	1.3	7.9	0.9	1.3	C^+, D	15.4	DC^+
8	0.7	2.4	3.7	1.1	6.4	4.7	B, C^+, E, F	25.2	Mixture of C^+EF:17.25 and EFB:17.0 ?
7	4.5	1.9	2.2	8.0	2.6	1.8	A^+, D	21.5	A^+D
6	9.2	2.4	11.4	8.6	3.6	3.0	A^+, B, D	29.5	BA^+D
2	0.5	1.1	1.3	1.2	1.0	1.6	B, C^+, D, E, F	32.4	DC^+EFB
									⌐A^+DC^+EFB¬

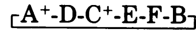
[a] Sum of molecular weights of final fragments present.

[b] Numbers assigned to the partial fragments are not equivalent in the two experiments since the digestion conditions were slightly different and the total number of partial digestion fragments was not the same.

[c] This experiment is the same as that shown in Fig. 4a.

mixtures of two or more partial products, and it was not possible to determine unequivocally which final digestion products were present. In the mapping, therefore, we used only those partial digestion products that gave distinctly higher $^3H/^{14}C$ ratios for some of the final digestion fragments and distinctly lower ratios for others. The $^3H/^{14}C$ ratios vary among the different partial digestion fragments because the relative amounts of the partial digestion fragments shown in Fig. 4 were different; as a result, different quantities (^3H counts per minute) of the partial digestion fragments were subsequently mixed with a constant amount of the ^{14}C-labeled carrier DNA.

By analysis of the partial digestion products that contained overlapping sets of fragments, the order of the final digestion fragments of RTF-TC C⁺ DNA was determined to be:



Every fragment was found to be contiguous with two other fragments, as would be expected for a circular DNA molecule (Fig. 5). As mentioned previously, fragment A⁺ of the RTF-TC corresponds to fragment A of the composite NR1. Fragment C⁺ of NR1 C⁺ or RTF-TC C⁺ corresponds to fragment C of either NR1 or the RTF-TC of NR1.

Partial digestion mapping of r-determinants DNA was carried out next using the DNA isolated from transitioned cells (Fig. 4b). The presence of small amounts of RTF-TC fragments in

FIG. 4. *Preparative agarose-ethidium bromide gel electrophoresis of partial digestion products. RTF-TC C⁺ DNA and transitioned NR1 DNA were partially digested with EcoRI for 4.5 and 10 min, respectively, and subjected to electrophoresis on 0.6% preparative agarose gels. The fragment numbers correspond to the numbers in the tables. Some of the more diffuse bands were assumed to be mixtures of more than one fragment. These bands are not numbered since they were not analyzed further. (a) RTF-TC C⁺ DNA (experiment 2 of Table 3); (b) transitioned NR1 DNA (experiment 2 of Table 4).*

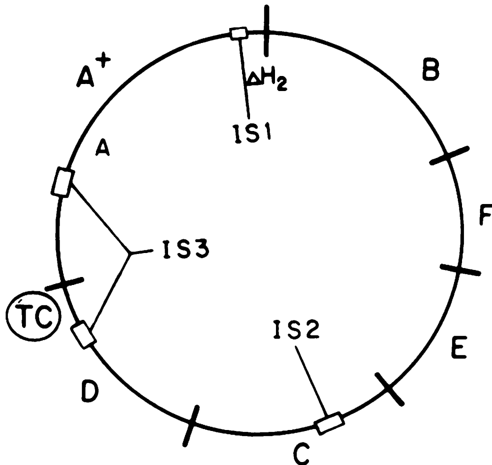


FIG. 5. *EcoRI restriction map of RTF-TC DNA. The order of the fragments was determined as described in the text. The RTF-TC DNA that was mapped contained an insertion in fragment C to form fragment C^+ (see text). Fragment A^+ is composed of fragment A and a part of the fragment H (designated ΔH_2) as described. The expected location of the insertion sequence IS1 that has been identified in the DNA of several composite R plasmids (14, 20) is indicated on fragment A^+ at the A-ΔH_2 junction. We have assumed that one copy of the IS1 is present on the RTF-TC component of NR1. The TC resistance genes are located on fragment A or D near the EcoRI site separating them as described in the text. The locations of the insertion sequences IS2 and IS3 were determined by Hu et al. (14) and Ptashne and Cohen (20).*

addition to the seven r-determinants fragments complicated the mapping by making the separation of the partial digestion products more difficult. However, the use of appropriate digestion conditions and of long (70 cm) 0.6% gels allowed adequate separation of the partial digestion products for unambiguous mapping of the fragments. The order of the final r-determinants fragments in transitioned NR1 DNA was determined to be (Table 4): - ΔH_1-I-L-K-G-M-J-ΔH_1-I-L-K-G-M-J-. For simplicity, we have presented the order of the r-determinants fragments in the sequence in which they actually exist in composite (nontransitioned) NR1 DNA (see below). However, the correct orientation of the r-determinants fragments relative to the RTF-TC fragments could not be determined in these experiments with transitioned NR1 DNA that contains multiple, tandem copies of the r-determinants component. In poly-r-determinant molecules, fragment ΔH_1 is adjacent to fragment J (Table 4) even though fragment H (from which ΔH_1 is derived) and fragment J are not contiguous in composite (nontransitioned) NR1 DNA.

We next examined which fragments of the RTF-TC and the r-determinants components were adjacent to each other in the composite NR1 molecule. To answer this question, partial digestion mapping of composite NR1 DNA was done. Owing to the large size of NR1 DNA and the number of final digestion fragments, the separation of partial digestion products was extremely difficult and only a few clearly resolved partial fragments were obtained. However, one of these was a critical partial product which produced, upon complete digestion, fragments B and H (Table 5). Thus, based on the partial digestion mapping data, two arrangements are possible in the circular composite R plasmid:

┌A-D-C-E-F-B-H-I-L-K-G-M-J┐ or ┌A-D-C-E-F-B-H-J-M-G-K-L-I┐

The A fragment would be attached to either the I or the J fragment, since the composite NR1 is a circular molecule. Studies of NR1 deletion mutants (presented below) have shown that fragment H is not next to J in the composite NR1 molecule; therefore, H must be next to I. The map of the *Eco*RI fragments of NR1 must therefore be the first order given above (Fig. 6 and 7).

***Eco*RI digestion of deletion mutant R plasmid DNA and location of drug resistance genes.** DNA was isolated from bacterial strains harboring several deletion mutants of NR1 or the related R plasmid NR84. The plasmid DNA was digested with *Eco*RI, and electrophoresis patterns of the fragments were compared with that of the NR1 fragments on 1% and 0.6% agarose-ethidium bromide gels. Coelectrophoresis of the digested DNA of two different plasmids was extensively used to detect differences in the mobilities of fragments with similar sizes.

Comparison of three different RTF-TC segregants derived from NR1 showed that all three have an enlarged A fragment (A^+) and that all are missing fragments G through M (Table 6). The RTF-TC C^+ that had been derived from NR1 C^+ has been described previously (see Fig. 5). NR1-1 is an RTF-TC that had been isolated from the original NR1 by Nakaya and Rownd using P1 transduction (17). NR1-1 had a normal C fragment but had an insertion of 0.75×10^6 daltons in fragment E to form fragment E^+. The *Eco*RI fragments of the RTF-TC derived from the round of replication mutant ROR12 were the same as the *Eco*RI fragments of NR1 or ROR12 except for the enlarged A fragment.

TABLE 4. *Summary of r-determinants (transitioned NR1) partial digestion experiments*

Partial fragment	$^3H/^{14}C$ ratios in final fragments							Final fragments present	Estimated mol wt[a] of partial fragments ($\times 10^6$)	Order of final fragments
	G	I	ΔH_1	J	K	L	M			
Expt 1										
20	0.0	0.0	0.3	68.9	0.1	0.4	0.3	J	1.79	J
19	0.0	0.2	0.8	0.0	7.6	3.8	0.0	K, L	2.11	LK
18	0.7	2.0	103.0	2.3	0.6	0.6	0.5	ΔH_1	2.4	ΔH_1
17	0.1	6.5	4.6	14.6	0.3	1.1	8.0	J, M	2.54	MJ
16	2.5	54.7	1.1	3.8	0.7	1.0	2.7	I	2.7	I
13	73.4	1.3	1.5	1.2	0.7	0.8	0.8	G	3.5	G
11	1.8	3.8	0.7	0.9	0.6	2.9	0.1	I, L	3.71	IL
10	1.6	7.4	13.6	13.0	0.5	1.1	0.5	ΔH_1, J	4.19	J ΔH_1
9	70.7	3.2	4.6	4.2	28.5	0.0	0.7	G, K	4.6	KG
4	7.0	13.6	14.4	8.3	9.7	8.4	7.5	ΔH_1, I	5.1	ΔH_1I
2	6.7	17.5	13.5	16.1	3.8	14.5	9.2	ΔH_1, I, J, L	7.9	J ΔH_1IL
Expt 2[b]										
33	13.0	1.2	1.4	1.8	0.9	1.1	1.0	G	3.5	G
31	0.7	2.5	0.5	0.4	1.0	3.3	0.4	I, L	3.71	IL
30	0.8	2.8	10.1	8.7	1.0	1.0	1.2	ΔH_1, J	4.19	J ΔH_1
29	5.2	0.8	1.0	1.2	4.7	1.2	1.7	G, K	4.6	KG
27	2.9	4.0	2.3	1.1	9.0	4.4	1.8	I, K, L	4.81	ILK
26	1.2	3.3	6.9	5.7	2.7	1.9	7.0	ΔH_1, J, M	4.94	MJ ΔH_1
25	0.6	7.7	10.0	3.7	1.0	1.5	4.4	ΔH_1, I	5.1	ΔH_1I
23	4.3	0.9	0.9	0.9	5.5	3.8	3.4	G, K, L, M	6.36	LKGM
18	1.4	7.7	7.7	8.4	1.7	2.2	2.2	ΔH_1, I, J	6.89	J ΔH_1I
15	1.9	6.6	5.6	7.9	2.3	2.2	7.3	ΔH_1, I, J, M	7.64	MJ ΔH_1I
14	5.6	4.7	1.6	2.1	5.9	6.4	1.9	G, I, K, L	8.31	ILKG
13	5.0	7.0	8.7	8.4	3.3	7.7	10.3	ΔH_1, I, J, L, M	8.65	MJ ΔH_1IL
12	1.5	3.9	4.0	4.4	4.0	4.7	3.6	ΔH_1, I, J, K, L, M	9.75	MJ ΔH_1ILK
										ΔH_1ILKGMJ (circular)

[a] Sum of molecular weights of final fragments present.

[b] This experiment is the same as that shown in Fig. 4b. See Table 3, footnote *b*.

TABLE 5. *Summary of partial digestion experiment of composite NR1 DNA*

Partial fragment	$^3H/^{14}C$ ratios in final fragments													Final fragments present	Estimated mol wt[a] of partial fragments ($\times 10^6$)	Order of final fragments
	A	B	C	D	E	F	G	H	I	J	K	L	M			
10	0.3	35.0	4.5	4.2	3.3	3.5	3.0	2.9	3.4	2.7	1.3	1.1	2.4	B	8.0	
9	0.3	3.5	2.3	2.6	11.7	10.8	3.1	0.0	3.0	3.0	2.0	2.4	2.0	E, F	9.0	EF
8	0.2	14.9	3.3	3.8	3.6	4.5	4.3	12.6	4.0	3.1	0.8	1.2	2.4	B, H	11.2	BH
7	1.3	16.8	6.5	4.8	7.3	15.9	4.7	6.3	6.4	3.0	3.8	3.4	3.8	B, F	12.0	FB
																EFBH

[a] Sum of molecular weights of final fragments present.

Several deletion mutants which had lost resistance to some antibiotics were also examined (Fig. 8 and Table 7). The R plasmids of the TC-sensitive mutants UCR122 (Fig. 8c and d) and NR1-2 had a smaller A fragment (referred to as A^-) and lacked fragment D. Denaturation mapping (19) and heteroduplex mapping (27) of UCR122 DNA have shown that this plasmid DNA has one continuous deletion that is about 10×10^6 daltons in size. A comparison of the *Eco*RI restriction map with the denaturation map or the heteroduplex map shows that the

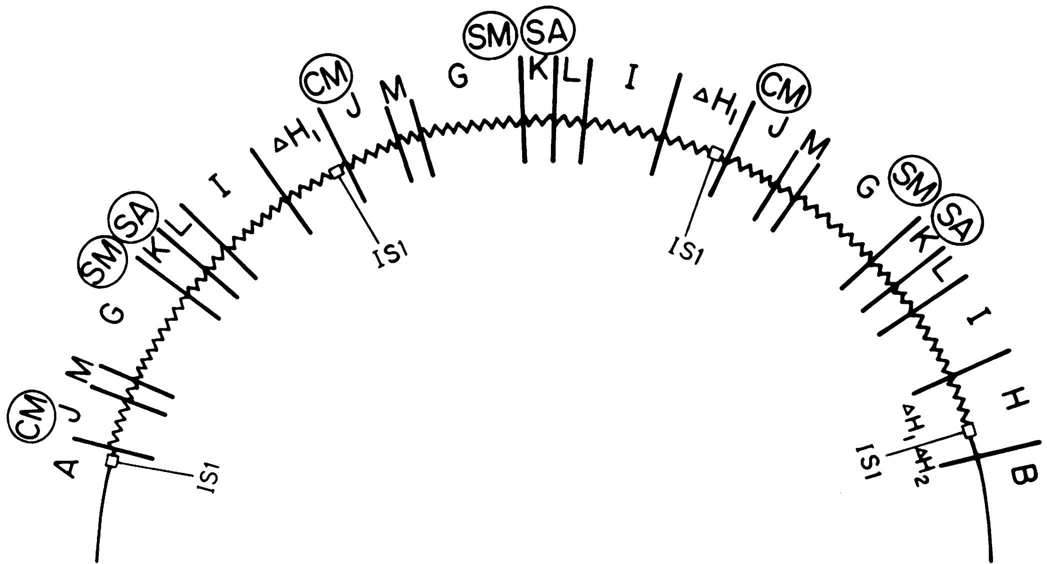


FIG. 6. *EcoRI restriction map of poly-r-determinant R plasmid DNA in transitioned NR1. A poly-r-determinant R plasmid molecule containing three tandem copies of the r-determinants component that are arranged head-to-tail at the unique r-determinants integration site on the RTF-TC component (19) is shown; for simplicity of presentation, only a part of fragments A and B of the RTF-TC component is shown. The order of the fragments was determined as described in the text. The expected locations of the IS1 insertion sequences, which were previously identified on the DNA of several composite R plasmids (14, 20), are indicated on the assumption that the copies of IS1 are amplified in the formation of the multiple, tandem copies of the r-determinants component. Owing to the internal location of the IS1 sequence on fragment H, EcoRI digestion of this poly-r-determinant R plasmid molecule will yield one copy of fragment H from the r-determinants component immediately adjacent to fragment B of the RTF-TC and two copies of fragment ΔH_1 from the other two r-determinants components. The other r-determinants fragments will be amplified in proportion to the amplification of the entire r-determinants component in the transitioned NR1 DNA. The approximate location of the drug resistance genes was determined as described in the text.*

deletion spans parts of both fragment A and fragment D and includes the restriction site between the two fragments. The sum of the molecular weights of fragments A and D is 20.7 $\times 10^6$. The deletion in UCR122 has a molecular weight of 10 $\times 10^6$. The difference is about 10.7 $\times 10^6$, which is in good agreement with the size of the A^- fragment of UCR122 (10.2 $\times 10^6$) (Table 7). Therefore, the A^- fragment of UCR122 is probably a fusion fragment that originated from the nondeleted DNA of fragments A and D. In addition to fragment A^-, a new fragment (referred to as X) with a molecular weight of 1.4 $\times 10^6$ was observed for UCR122 DNA. The origin of this fragment is unknown. Fragment A^- of NR1-2 is also probably a fusion fragment originating from the nondeleted DNA in fragments A and D after deletion of the *Eco*RI site between the two fragments. From the known location of the TC gene(s) on the heteroduplex map (27), the TC resistance gene(s) should be located very near the *Eco*RI restriction site between fragments A and D. The alignment of the *Eco*RI restriction endonuclease map of NR1 with its heteroduplex map is not sufficiently precise to allow us to determine whether the TC resistance gene is on fragment A or on fragment D.

The R plasmid NR1-22 is a deletion mutant of NR1 that does not confer resistance to SA. NR1-T22 is a mutant of NR1-22 which, in addition, has lost SM resistance. The *Eco*RI restriction patterns of the two plasmids are indistinguishable and do not have fragments G, H, I, K, and L but display a new fragment with a molecular weight of about 4.4 $\times 10^6$ (Fig. 8i and j; Table 7). This new fragment probably originated from a fusion of the remaining parts of fragments H and G, owing to a deletion that removed part of H, all of I, L and K, and part of fragment G (see Fig. 7). Since fragment H is at an RTF-TC–r-determinants junction and is adjacent to fragment B, only one of the other r-determinants fragments can be a neighbor of fragment H. As was mentioned above, according to the partial digestion map either fragment I or fragment J

could be the r-determinants neighbor of fragment H. If the deletion in NR1-22 is a single, continuous one (which is most likely the case), fragment J, which is present in the *Eco*RI digest of NR1-22 DNA, could not be next to fragment H. Hence, fragment I must be next to H, and the fragment order ⌐A-D-C-E-F-B-H-I-L-K-G-M-J⌐ is confirmed.

The R plasmid NR1-24 is a deletion mutant of NR1 that does not confer CM resistance. After *Eco*RI digestion, NR1-24 DNA was found to have lost fragment J and to have a much larger fragment A (fragment A^{++}) and a smaller fragment D (fragment D^{-}) than NR1 DNA (Fig. 8f and g). Since fragment D is in the RTF-TC component of the plasmid, the decrease in the size of fragment D is not believed to be directly related to the loss of CM resistance. The large increase in the size of fragment A to A^{++} and the loss of fragment J may be due to the loss of the restriction site between fragments A and J and the resulting fusion of these two fragments. This interpretation is consistent with the order of the *Eco*RI fragments of the composite NR1 DNA presented above.

The R plasmid NR84 confers resistance to AP in addition to TC, CM, SA and SM/SP. NR84 DNA has two altered fragments when compared to NR1 (Table 7). Fragment D of NR84 is about 30% larger than fragment D of NR1. Fragment L of NR84 (4.3×10^6 daltons) is much larger than fragment L of NR1 (1.0×10^6 daltons). This larger fragment L (L^+) of NR84 probably contains the AP resistance genes because this is the only difference between the r-determinants fragments of NR84 and those of NR1. Examination of the DNA of an RTF-TC segregant of NR84 has shown that the fragments A through F in Table 7 are from the RTF-TC component (22).

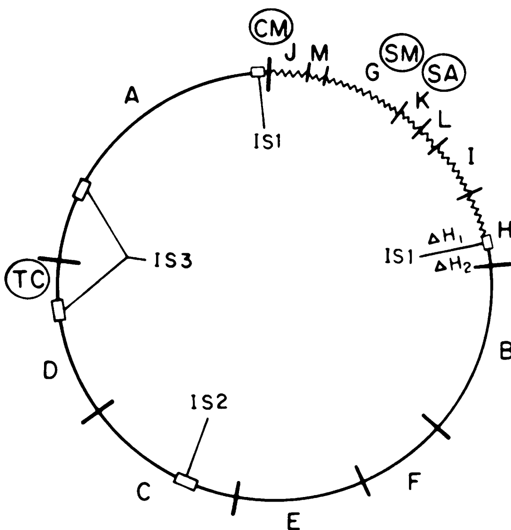


FIG. 7. *EcoRI restriction map of composite NR1 DNA. The order of the fragments was determined as described in the text. The locations of the insertion sequences IS1, IS2, and IS3 that were previously identified on the DNA of several composite R plasmids (14, 20) are shown. The approximate location of the drug resistance genes was determined as described in the text.*

DISCUSSION

When the DNA of the R plasmid NR1 is digested with *Eco*RI, 13 fragments are produced. The six largest fragments are of RTF-TC origin, and the seven smallest fragments are of r-determinants origin. It is interesting that there are more r-determinants fragments than RTF-TC fragments. If restriction sites were distributed randomly in the NR1 DNA molecule, the number of restriction sites in the r-determinants component should be smaller than the number in the RTF-TC component, since the r-determinants component (14×10^6 daltons) is much smaller than the RTF-TC component (49×10^6 daltons) and also has a lower mole fraction of adenine plus thymine residues (18, 23). The unique base sequence of the *Eco*RI restriction site is -GAATTC- (10), which is high in adenine plus thymine content. Apparently, the *Eco*RI restriction sites are not distributed randomly between the RTF-TC and the r-determinants components of the composite NR1 DNA molecule.

The relationship between the molecular weight and the mobility of *Eco*RI fragments observed in our experiments (Fig. 2a and b) is similar to that reported by Thomas and Davis (30). Our data extend the range of this curve to lower-molecular-weight values. The sum of the molecular weights of the NR1 fragments in Table 2 (59.1×10^6) is in good agreement with our recent estimates of the molecular weight of 57.5×10^6 for composite NR1 DNA molecules using electron microscopy (D. Taylor, unpublished results).

Two basic changes were consistently observed in the DNA of several RTF-TC segregants of NR1 and in transitioned NR1 DNA. In all three RTF-TC's that were examined, fragment A of the composite R plasmid was missing and a larger fragment (designated A^+) was observed. In transitioned NR1 DNA, fragment H was present, and its proportion relative to the six RTF-TC fragments (A to F) was the same as observed for the composite NR1 DNA fragments. However, a new fragment (referred to as ΔH_1) was observed in transitioned NR1

TABLE 6. *Molecular weights of EcoRI fragments of composite NR1 DNA, transitioned NR1 DNA, and the DNA of several RTF-TC segregants*

Fragment	Molecular weight ($\times 10^6$)					
	NR1	ROR12	Transitioned NR1	RTF-TC C^+	RTF-TC of ROR12	NR1-1
A	13.5	S[a]	S	14.3	14.3	14.3
B	8.0	S	S	S	S	S
C	7.5	S	S	8.25[b]	S	S
D	7.2	S	S	S	S	S
E	5.0	S	S	S	S	5.75
F	4.0	S	S	S	S	S
G	3.5	S	S[c]			
H	3.2	S	S, 2.4[c,d]			
I	2.7	S	S[c]			
J	1.79	S	S[c]			
K	1.10	S	S[c]			
L	1.01	S	S[c]			
M	0.75	S	S[c]			

[a] S indicates that the size (mobility) of the fragment is the same as the corresponding fragment in NR1 DNA.

[b] RTF-TC C^+ DNA has an insertion of 0.75×10^6 daltons in fragment C; owing to this insertion, fragment C is replaced by fragment C^+, which is larger than fragment B.

[c] The proportions of these r-determinants fragments in transitioned NR1 DNA were amplified relative to the RTF-TC fragments as described in the text.

[d] The H fragment (molecular weight, 3.2×10^6) was not amplified and was present in the same proportion as the RTF-TC fragments A-F. The ΔH_1 fragment (molecular weight, 2.4×10^6) was amplified and was present in essentially the same proportion as the r-determinants fragments G and I-M.

DNA which was amplified to the same extent as the other r-determinants fragments. These finding appear to be related and can be explained in the following way.

As shown in the map of the *Eco*RI fragments of the composite NR1 structure (Fig. 7), fragment J is located next to fragment A and fragment H is located next to fragment B. If one of the RTF-TC–r-determinants junctions (referred to as an excision site or an IS1 insertion sequence [see below]) is located within fragment A at a point very near the *Eco*RI restriction site between fragments A and J and the other excision site is located within the interior of fragment H, excision of the r-determinants component to form an RTF-TC segregant will delete the *Eco*RI site between A and J and all of the r-determinants *Eco*RI sites (see Fig. 7). As a result, in an RTF-TC segregant fragment A will be united with the part of fragment H (referred to as ΔH_2) that is distal to the excision site on H to form fragment A^+ (Fig. 7).

In transitioned NR1 DNA, the entire fragment H is present but is not amplified relative to the RTF-TC fragments because there is only one fragment H in a poly-r-determinant R plasmid molecule that is located immediately adjacent to fragment B of the RTF-TC component (Fig. 6). In the tandem copies of r-determinants in poly-r-determinant R plasmids and in autonomous poly-r-determinant molecules, fragment ΔH_1 is present and will be amplified in the same proportion as the other r-determinants fragments in the transitioned DNA. If this interpretation is correct, then fragment A^+ of RTF-TC DNA should be 0.8×10^6 larger than fragment A of the composite NR1, since this is the difference in molecular weight between fragment H and fragment ΔH_1, i.e., the size of ΔH_2. Within the accuracy of our molecular weight estimates, these relationships between the molecular weights of the various fragments appear to be correct. Fragment ΔH_1 will also contain a small part of the end of fragment A that is located between the excision site in fragment A and the *Eco*RI site between fragments A and J.

By comparing Fig. 6 and 7 it is evident that fragments A and J should have the same molecular weight in both transitioned NR1 DNA and nontransitioned (composite) NR1 DNA, since the location of the *Eco*RI site between these two fragments is not altered in transitioned DNA. As a result of the formation of tandem sequences of the r-determinants component during a transition, fragments ΔH_1 and J will be brought into juxtaposition, but this should not change the size of fragment J. Detailed coelectrophoresis experiments of mixtures of transitioned and nontransitioned NR1 DNA that had

been treated with *Eco*RI have verified these expectations.

The examination of the fragments produced by *Eco*RI digestion of the DNA of several mutant R plasmids that have lost drug resistance genes has confirmed several aspects of the *Eco*RI fragment map shown in Fig. 7. The patterns of UCR122 and NR1-2 DNA are consistent with the map in showing that fragments A and D are contiguous. The pattern of NR1-22 DNA indicates that fragments I, L, and K have been deleted, as would be expected if they are contiguous, and that fragments H and G are apparently fused to form a new fragment "X." Also, as discussed above, the increase in fragment A to form fragment A^+ in RTF-TC segregants and the appearance and amplification of fragment ΔH_1 in transitioned NR1 DNA are consistent with the locations of these fragments on the *Eco*RI map. There are, nevertheless, a few unexplained observations in these experiments. There is a fragment of molecular weight 1.4×10^6 that is observed in *Eco*RI-digested UCR122 DNA, whose origin is unknown. The largest fragment in NR1-24 DNA may be larger than the sum of the molecular weights of fragments A and J, and fragment D also appears to be smaller in NR1-24 DNA. It is possible that there was a translocation of a part of fragment D onto fragment A in NR1-24 due to a change in the location of the *Eco*RI restriction site between fragments A and D.

In several of the R plasmids that we have examined, there have been insertions of unknown origin in one of the *Eco*RI fragments. In the case of the composite NR1, there has been an insertion in fragment C to form NR1 C^+; the RTF-TC C^+ that was derived from NR1 C^+ also had the C^+ fragment. Thus, until this study, we have unknowingly maintained two different forms of composite NR1 in this laboratory, which are now designated NR1 and NR1 C^+. An RTF-TC isolated from NR1 by R. Nakaya (called NR1-1) had a normal C fragment but had an insertion in fragment E to form fragment E^+. These different insertions apparently occurred spontaneously, and there are no observable differences in the phenotypes of the plasmids owing to these insertions. Since these two insertions were detected purely by chance,

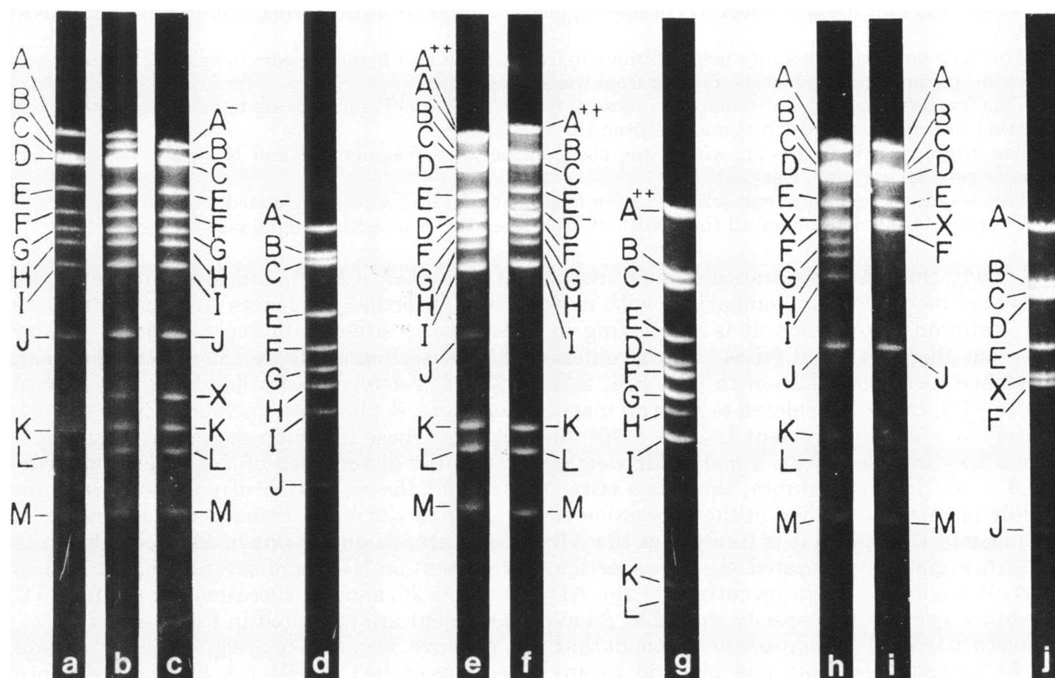


FIG. 8. *Agarose-ethidium bromide gel electrophoresis of EcoRI-digested NR1 and mutant R-plasmid DNAs. (a) NR1 DNA on 1.0% agarose gel; (b) coelectrophoresis of NR1 DNA and UCR122 DNA on 1.0% agarose gel; (c) UCR122 DNA on 1.0% agarose gel; (d) UCR122 DNA on 0.6% agarose gel; (e) coelectrophoresis of NR1 DNA and NR1-24 DNA on 1.0% agarose gel; (f) NR1-24 DNA on 1.0% agarose gel; (g) NR1-24 DNA on 0.6% agarose gel; (h) coelectrophoresis of NR1 DNA and NR1-T22 DNA on 1.0% agarose gel; (i) NR1-T22 DNA on 1.0% agarose gel; (j) NR1-T22 DNA on 0.6% agarose gel.*

TABLE 7. *Molecular weights of EcoRI fragments of several deletion mutants of NR1 and of the R plasmid NR84*

Fragment	Mol wt ($\times 10^6$)						
	NR1	NR1-2	UCR122	NR1-22	NR1-T22	NR1-24	NR84[a]
A	13.5	11.5[b]	10.2[b]	S[c]	S	16.5	S
B	8.0	S	S	S	S	S	S
C	7.5	S	S	S	S	S	S
D	7.2			S	S	4.4	9.3[d]
E	5.0	S	S	S	S	S	S
F	4.0	S	S	S	S	S	S
G	3.5	S	S			S	S
H	3.2	S	S			S	S
I	2.7	S	S			S	S
J	1.79	S	S	S	S		S
K	1.10	S	S			S	S
L	1.01	S	S			S	4.3[e]
M	0.75	S	S	S		S	S
X			1.4[f]	4.4[g]	4.3[g]		
Lacks resistance to:		TC	TC	SA	SA SM	CM	

[a] The R plasmid NR84 carries AP resistance in addition to TC, CM, SM, and SA.

[b] This fragment probably represents a fusion fragment containing the nondeleted parts of fragments A and D owing to the deletion of the *Eco*RI site between the two fragments (see text).

[c] S indicates that the size (mobility) of the fragment is the same as the corresponding fragment in NR1 DNA.

[d] This fragment has a mobility between those of fragments A and B and appears to have originated by an insertion in fragment D which is missing from the gel pattern.

[e] This fragment has a mobility between those of fragments E and F and appears to have originated by an insertion in fragment L which is missing from the gel pattern.

[f] The origin of this fragment, which was observed between fragments J and K on agarose-ethidium bromide gels, is unknown (see text).

[g] This fragment probably represents a fusion fragment containing the nondeleted parts of fragments G and H owing to the deletion of all the *Eco*RI sites between the two partial fragments (see text).

it is likely that the appearance of such insertions may be common in comparison with normal mutation frequencies. It is interesting to note that the R plasmid NR84, which confers resistance to AP in addition to CM, SM, SA, and TC (17), can be considered to have an insertion of 3.3×10^6 in fragment L (1.01×10^6) to form a new fragment with a molecular weight of 4.3×10^6. In this instance, there is a recognizable phenotypic change in the expression of the plasmid DNA, and it is likely that the AP resistance genes are located on the insertion DNA. It has been shown recently that the AP resistance genes which specify the TEM β-lactamase are located on an insertion element that can be translocated from one plasmid to another and also to the host chromosome (9, 11). This is one mechanism by which R plasmids can acquire additional drug resistance genes.

Recently, several of the insertion sequences that were previously shown to cause strong polar mutations in the galactose and lactose operons of *E. coli* (6, 12, 26) have been identified on a number of F, F′, and R plasmids (14, 20). These insertion sequences are thought to be involved in site-specific recombination. A copy of the insertion sequence IS1 is located at both RTF-TC–r-determinants junctions of several composite R plasmids, including NR1 (14, 20) (Fig. 7). These insertion sequences may play a role in the dissociation of the R plasmid structure and the formation of poly-r-determinant structures during a transition. The insertion sequences IS2 and IS3 have also been shown to be present on NR1, and several related R plasmids (14, 20) and their locations on the RTF-TC component are indicated in Fig. 5 and 7.

We have been able to assign the approximate locations of the CM, SM, SA, and TC resistance genes by analyzing the digestion patterns of several mutant R plasmids that have lost drug resistance genes (Table 7; Fig. 6–8). The CM resistance gene seems to be located near the restriction site between A and J, because the *Eco*RI restriction pattern of NR1-24 that had lost CM resistance had also lost fragment J and

had an enlarged fragment A. This is most likely due to the deletion of the *Eco*RI site between fragments A and J. The R plasmid NR1-22 that had deleted the SA resistance genes did not have fragments G, K, L, I, and H. However, it did have a new fragment with a molecular weight of 4.4×10^6. This new fragment appears to be a fusion fragment containing a part of fragment G and a part of fragment H. The SA resistance gene must reside in the deleted region of the r-determinants. Other experiments in this laboratory suggest that the SA resistance gene is on fragment K and that the SM resistance gene is on fragment G (N. Goto and R. Rownd, unpublished data). A comparison of the *Eco*RI restriction map of NR1 DNA with the heteroduplex map (27) or the denaturation map (19) of NR1 DNA has shown that the TC resistance genes are located near the *Eco*RI restriction site between fragments A and D. As mentioned previously, the AP resistance gene of the R plasmid NR84 appears to be located on fragment L.

Cohen et al. (3) have reported that there are 12 fragments that result from digestion of R plasmid R6-5 DNA with *Eco*RI. Heteroduplex analysis has shown that R6-5 and NR1 are completely homologous R plasmids except for three insertions on R6-5 with molecular weights of 0.92×10^6, 1.12×10^6, and 2.51×10^6 (27). The 0.92×10^6 insertion is an IS3 sequence that inactivates TC resistance (20), and the 2.51×10^6 insertion carries the gene for kanamycin (KM) resistance. In the experiments of Cohen et al. the band of second lowest mobility was quite diffuse and had a higher intensity than the band of lowest mobility. They suggested that this diffuse band contained two *Eco*RI fragments of R6-5 DNA that have very similar molecular weights. We have recently shown that this band contains three fragments, corresponding to fragments B, C, and D of NR1 DNA (P. Jezo and R. Rownd, unpublished data). This explains why Cohen et al. (3) observed only 12 fragments for R6 DNA instead of the 13 fragments that we have observed for the essentially homologous NR1 DNA.

Watanabe et al. (31) have concluded from the pattern of segregation of the drug resistance of R6 (the R plasmid from which R6-5 was derived [27]) in *Salmonella typhimurium* that the KM resistance gene is located between the SM and CM resistance genes. We have recently shown in coelectrophoresis experiments with *Eco*RI-digested NR1 and R6 DNA that the KM resistance gene on R6 is located on an insertion of 2.5×10^6 daltons on fragment J of NR1 DNA. Thus, the KM gene would be located between the SM and CM resistance genes on the r-determinants component. The locations of the SA, SM, KM, and CM resistance genes on the *Eco*RI map of the r-determinants component is consistent with the order of these genes previously determined by Watanabe et al. (31).

ACKNOWLEDGMENTS

We thank Bernard Weisblum for his advice, help, and encouragement provided in the preparation of the restriction endonuclease *Eco*RI and in carrying out these experiments, and Dean Taylor and David Womble for their contributions and discussions in establishing the relationship between the R plasmids NR1 and NR1 C$^+$. The assistance of Dean Taylor in the preparation of the R plasmid DNA used in some of these studies is also gratefully acknowledged.

This work was supported by Public Health Service research grant GM 14398 from the National Institute of General Medical Sciences to R. R. and National Science Foundation grant GB 41551 to R. R. and J. H. C.

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