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Genetics

Translocation of a plasmid DNA sequence which mediates ampicillin resistance: Molecular nature and specificity of insertion

(R factor/antibiotic resistance/inverted repeat sequence/insertion sequence)

FRED HEFFRON, CRAIG RUBENS, AND STANLEY FALKOW

Department of Microbiology, School of Medicine, University of Washington, Seattle, Wash. 98195

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ABSTRACT **A series of recombinant plasmids was generated in *Escherichia coli* in which the TEM β-lactamase translocon (TnA) was inserted into the small plasmid RSF1010. RSF1010 is a 5.5 × 10^6 dalton nonconjugative plasmid which confers resistance to streptomycin and sulfonamide. The recombinant plasmids can be classified into three clearly defined phenotypic groups. Group I is ampicillin-, streptomycin- and sulfonamide-resistant. Group II is ampicillin- and sulfonamide-resistant but has lost streptomycin resistance. Group III is ampicillin-resistant but is sensitive to sulfonamide and shows a simultaneous 30-fold reduction in the minimal inhibitory concentration of streptomycin. It was possible to map the site of insertion of TnA within RSF1010 by electron microscope studies of DNA heteroduplexes formed between RSF1010 and recombinant plasmids. Insertions of TnA occur at, at least, 12 distinct sites in a region corresponding to one-third of the RSF1010 DNA molecule. Those insertions giving rise to particular phenotypes are clustered. Insertions of TnA-like insertion sequences (IS) appear to give rise to strongly polar mutations.**

Plasmid-mediated resistance to ampicillin (Ap) is most commonly associated with the TEM β-lactamase (1). Recent work by Hedges and Jacob (2) and from our laboratory (3) has shown that the TEM β-lactamase gene is located on a 3 × 10^6 dalton sequence of plasmid DNA, called TnA, which can be translocated from one replicon to another. In addition, an examination of a large variety of naturally occurring, largely nonhomologous, R plasmids representing many incompatibility groups showed that those plasmids which specified the TEM β-lactamase possessed the TnA sequence. These data suggested that the wide distribution of TEM-mediated Ap resistance among R plasmids was associated with the dissemination of TnA by a translocation event rather than conventional reciprocal recombination mechanisms (3).

In its ability to translocate from one replicon to another, TnA resembles the insertion sequences (IS) of *Escherichia coli* (4). In this paper we demonstrate that TnA resembles IS sequences in another way, namely its insertion results in polar mutations. Plasmids which have acquired TnA show a single insertion of 3.2 ± 0.3 × 10^6 daltons of DNA which is bounded by inverted complementary sequences. Moreover, the insertion of TnA into the small recipient plasmid RSF1010 can occur at a large number of discrete sites.

MATERIALS AND METHODS

Bacterial Strains and Plasmids. Strain 1485-1 is a thymine-requiring, nalidixic-acid-resistant derivative of *Escherichia coli* K-12 described previously (3). *E. coli* C600 was employed as the recipient strain for plasmid DNA transformation (3). R1*drd* is a 63 × 10^6 dalton conjugative plasmid which confers resistance to Ap, streptomycin (Sm), sulfonamide (Su), chloramphenicol, and kanamycin (5). R64-1 is a 75 × 10^6 dalton conjugative plasmid containing the TnA translocation sequence and, in addition to Ap resistance, it confers Sm and tetracycline resistance (2). The R plasmid RSF1010 is a nonconjugative 5.5 × 10^6 dalton plasmid which confers resistance to Sm and Su (6). R684 is a 6.3 × 10^6 dalton nonconjugative plasmid which is related to RSF1010 (7).

Bacterial Conjugation and Determination of Minimum Inhibitory Concentration (MIC). The conditions used for growing cultures on solid and liquid medium and for bacterial mating have been described previously (8). The concentrations of antibiotics used in selection medium were ampicillin, 100 μg/ml; streptomycin, 15 μg/ml; and sulfadiazine 2000, μg/ml (the latter in Mueller-Hinton medium supplemented with 5% lysed horse blood; ref. 9). The MIC for antibiotics was determined by the method of Steers (10).

Preparation of Plasmid DNA. The labeling and isolation of covalently closed circular plasmid DNA, sucrose gradient, and CsCl dye buoyant density centrifugation have been described previously (11, 3). Purified covalently closed DNA was stored in TNE buffer (100 mM Tris·HCl, 50 mM NaCl, and 1 mM EDTA; pH = 7.5).

Cleavage of Plasmid DNA with *Eco*RI. Plasmid DNA (0.1–0.5 μg) in TNE buffer with 15 mM $MgCl_2$ was cleaved with the *Eco*RI restriction endonuclease (the generous gift of H. W. Boyer) in a total volume of 20 μl as described by Greene *et al.* (12). *Eco*RI-digested plasmid DNA was electrophoresed in 0.8% agarose, stained with ethidium bromide, and photographed under long wave ultraviolet light (12). *Eco*RI-generated fragments of lambda DNA were included in each gel as a molecular weight standard.

Analysis of DNA Heteroduplexes by Electron Microscopy. DNA heteroduplex analysis of *Eco*RI-cleaved plasmid DNA was carried out as described by Lai and Nathans (13) using a JEOL model 100B electron microscope.

Isolation of Recombinant Plasmids Containing the TnA Insertion. Bacterial isolates of 1485-1 containing either coexisting R64-1 and RSF1010 or R1*drd* and RSF1010 were grown in 1 liter of brain heart infusion broth to allow translocation to take place. Purified plasmid DNA for transformation was prepared by Triton X-100 lysis (14) and CsCl dye buoyant density centrifugation. To this DNA was added approximately 2 μg of ^3H-labeled plasmid DNA (specific activity 1 × 10^5 cpm/μg) from the same isolate as a marker. About 20 μg of plasmid DNA in 0.5 ml of TNE buffer was layered on the top of a 12 ml 5–20% neutral sucrose gradient and centrifuged in a Beckman SW41 rotor for 2 hr at 15°, 39,000 rpm (185,000 × *g*). Fractions (0.4 ml) were collected and 10 μl of each fraction was spotted on filters and counted as described previously (11). The fractions were dialyzed

Abbreviations: Ap, ampicillin; Sm, streptomycin; Su, sulfonamide; IS, insertion sequence; CCC, covalently closed circular; kb, kilobase.

Table 1. Phenotypic properties of RSF1010 recombinants

Plasmid	Minimal inhibitory concentration of antibiotics		
	Ap (mg/ml)	Sm (μg/ml)	Su (mg/ml)
No plasmid	0.0001–0.001	0.5	0.1
RSF1010	0.0001–0.001	60	>10
Group I, Ap Sm Su resistant			
Ap130	>5	20	>10
Ap136	>5	60	>10
Ap230	5	60	>10
Ap234	5	60	>10
Group II, Ap Su resistant			
Ap110	>5	0.5	>10
Ap111	>5	0.5	>10
Ap210	5	0.5	>10
Ap211	5	0.5	>10
Group III, Ap resistant			
Ap101	>5	2	0.01
Ap102	>5	2	0.01
Ap103	>5	2	0.01
Ap201	5	2	0.01

against TEN buffer (20 mM Tris·HCl, 20 mM NaCl, 1 mM EDTA, pH 8) and used to transform $CaCl_2$-treated *E. coli* C600 as described by Cohen *et al.* (15).

RESULTS

Isolation of Recombinant Plasmids. Plasmid DNA from independent isolates of *E. coli* strain 1485-1 containing either coexisting RSF1010 and R1*drd* or RSF1010 and R64-1 was sedimented through 5–20% neutral sucrose in order to separate the larger molecules of R1*drd* or R64-1 from the much smaller RSF1010 molecules. Individual fractions from the sucrose gradient were employed as a source of DNA to transform $CaCl_2$-treated C600 cells and ampicillin-resistant cells were selected. When artificial mixtures of R64-1 (or R1*drd*) and RSF1010 were prepared and sedimented through a sucrose gradient, ampicillin-resistant transformants were found only in fractions of the gradient which corresponded to the location of covalently closed circular (CCC) molecules of the larger plasmid species. However, when plasmid DNA isolated from cells containing RSF1010 and R64-1 (or R1*drd*) was sedimented through sucrose and employed for the transformation, ampicillin-resistant transformants were found in two distinct regions of the gradient. One region (I) corresponded to the peak of the larger CCC molecular species; the DNA in the other peak (II) yielded Ap transformants at a frequency at least 4 orders of magnitude lower and sedimented slightly faster (about 30S) than that of CCC RSF1010 molecules (26S). One hundred and twenty region II Ap transformants have been examined. The transformants fall into three distinct phenotypic groups. Group I transformants (93) were Ap Sm Su resistant, group II transformants (10) were Ap Su resistant, while group III transformants (17) were resistant to Ap and showed a low level of Sm resistance. Table 1 summarizes the phenotype and MIC levels for 12 representative transformant plasmids.

The Molecular Nature of RSF1010 Recombinants. The DNA from each of 21 representative transformant plasmids was isolated and the molecular mass of the plasmids was obtained by sedimentation in 5–20% neutral sucrose gradients and by the measurement of their contour length in the electron microscope relative to RSF1010. All of the recombinant plasmids, regardless of phenotype, were $8.7 \pm 0.4 \times 10^6$ daltons in size. RSF1010 has a single site susceptible to cleavage by the *Eco*RI restriction endonuclease (16). When the recombinant plasmids were treated with *Eco*RI and analyzed by agarose gel electrophoresis, it was found that they likewise had but a single *Eco*RI site (Fig. 1). This finding permitted us to examine directly the homology between the recombinant plasmid molecules and RSF1010.

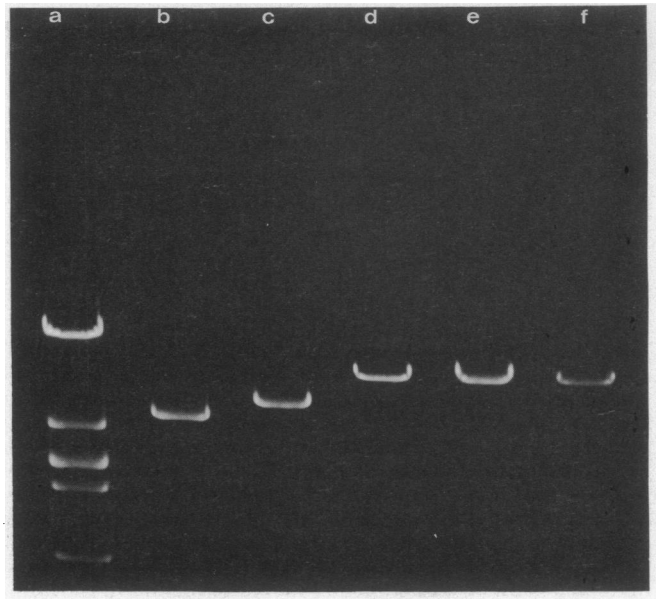

FIG. 1. Agarose gel electrophoresis of *Eco*RI-cleaved RSF1010 and recombinant plasmid DNA. Purified plasmid DNA was cleaved with *Eco*RI as described in *Materials and Methods* and the fragments were subjected to agarose gel electrophoresis (12). The gel was stained with ethidium bromide and the fluorescing DNA bands were photographed under long wavelength ultraviolet light. (a) λ cI857 DNA, (b) RSF1010 DNA, (c) R684 DNA, (d) recombinant plasmid Ap131 (Group I phenotype) DNA, (e) recombinant Ap211 (Group II phenotype) DNA, (f) recombinant Ap120 (Group III phenotype) DNA.

Each of the recombinant plasmids was individually mixed with RSF1010, treated with the *Eco*RI endonuclease and, following denaturation and renaturation, viewed in the electron microscope. Examination of heteroduplex molecules indicated that each of the 21 recombinant plasmids was formed by a single insertion of about 3×10^6 daltons of DNA into RSF1010. A typical heteroduplex is shown in Fig. 2a. This insertion was shown to be identical to the TnA DNA sequence by heteroduplexing the recombinant plasmids with RSF1030 (data not shown), a plasmid previously employed to define the TnA sequence (3). The site of insertion of TnA within RSF1010 could be determined by measurement of the distance from one end of the *Eco*RI-cleaved molecule to the site of the insertion loop in heteroduplex molecules. While it was readily apparent that TnA had inserted into different sites within RSF1010, it was necessary to distinguish the "right hand end" from the "left hand end" of the *Eco*RI-generated recombinant DNA molecules. For this purpose, the related but larger plasmid R684 was employed (shown in the agarose gel, Fig. 1). Fig. 2b shows the single insertion loop found when R684 is heteroduplexed with RSF1010, while Fig. 2c shows a heteroduplex between R684 and a recombinant plasmid in which two noninteracting in-

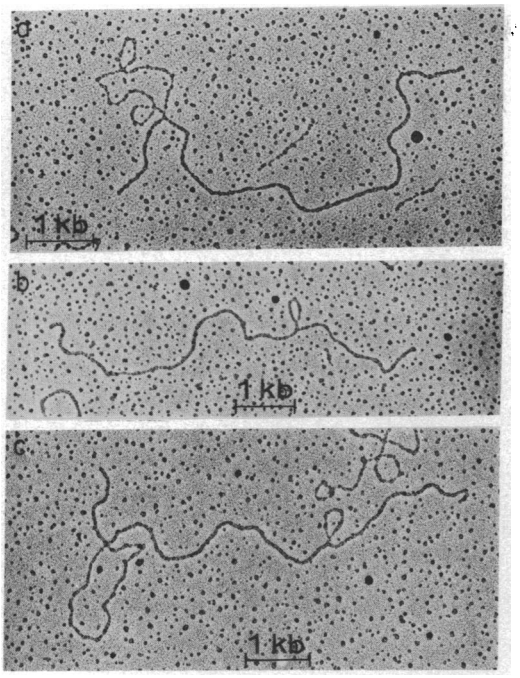

FIG. 2. The demonstration of the insertion of TnA into the RSF1010 DNA. Top, *Eco*RI-cleaved RSF1010 DNA and the DNA of the recombinant plasmid Ap230 were heteroduplexed and spread for electron microscopy by a formamide technique (26). The insertion of DNA representing TnA is seen as a single loop of single-stranded DNA. Middle, heteroduplex of RSF1010/R684 DNA showing the single small insertion loop of R684 which serves to distinguish the left and right end of the *Eco*RI-cleaved linear molecule. The *Eco*RI-generated end closest to the R684 insertion marker is arbitrarily designated as the right-hand end of the molecule. Bottom, heteroduplex of R684 and the recombinant plasmid Ap230 illustrating two non-interacting insertion loops corresponding to TnA and the R684 marker insertion. kb = kilobase, 1000 base pairs.

sertion loops, one corresponding to TnA and the other to the "marker" insertion from R684, are apparent. The *Eco*RI-generated end of the molecule closest to the R684 insertion loop was arbitrarily designated the right-hand end.

Fig. 3 shows several examples of heteroduplex molecules prepared between R684 and the DNA of independently isolated recombinant plasmids. Fig. 3a and b shows insertions which have occurred 3% and 9%, respectively, from the left hand end of the molecule. Both of these recombinant plasmids were of the Group II phenotype (Ap Su resistant). The heteroduplexes illustrated in Fig. 3c, d, and e show insertions of TnA at 15, 21, and 26% from the left hand end, respectively. These recombinant plasmids all belonged to the Group 1 phenotype, Ap Sm Su resistant. The final example, Fig. 3f, shows a recombinant plasmid in which TnA has inserted some 5% from the right hand end of the molecule. This recombinant plasmid is a representative of the Group III phenotype (Ap and low Sm resistance).

The examination of single-stranded molecules of recombinant DNA that have been reannealed only a short period of time has shown that the TnA insertion is bounded by inverted repeated sequences (inverted repeat; refs. 17–19). A single-stranded molecule in which this inverted repeated sequence has self-annealed is shown in Fig. 3e. The length of this inverted repeated sequence is estimated to be 140 ± 39 base pairs. However, due to the difficulty of measuring such a short sequence, we feel that this estimate represents the maximum length of the inverted repeat.

The Distribution of TnA Insertions into RSF1010. A map of RSF1010 showing the sites of TnA insertion from the heteroduplex analysis of the 21 recombinant plasmids is shown in Fig. 4. In all cases, 25 to 50 separate heteroduplex molecules were measured, which permitted the assignment of the site of insertion to within 1% of the RSF1010 molecule (about 83 base pairs). The insertion of TnA has occurred in at least 12 distinct sites within a contiguous 2×10^6 dalton sequence of RSF1010 DNA. Statistical analysis of the observed distribution of TnA either by the χ^2 method or by a "goodness of fit" test (20) indicates the distribution with the 2×10^6 dalton sequence is nonrandom ($P = 0.90$ to 0.95). Nevertheless, it appears that the underlying specificity of insertion is not high. A striking feature of this map is that TnA insertions into a contiguous stretch of DNA comprising 60% of the RSF1010 genome have never been observed. We assume that much of this region of RSF1010 encodes for essential plasmid functions; however, the observed distribution may also reflect the A+T-rich regions found near the *Eco*RI ends of the molecule (16). Insertions of TnA into the left hand end of the RSF1010 molecule from 3 to 9% all lead to a loss of streptomycin resistance and we presume, though we have no direct evidence, that this loss is a result of the insertions' having occurred within the structural gene for streptomycin phosphotransferase. Similarly, TnA insertions which inactivate sulfonamide resistance have all been observed within a small region of the plasmid. The insertion of TnA into what we presume to be the structural gene for sulfonamide resistance is strongly polar, however, since there is a sharp reduction in the level of streptomycin resistance (Table 1).

A large number of R factors code for both streptomycin and sulfonamide resistance. The observed loss of resistance phenotypes upon the insertion of TnA suggests that they may be frequently closely linked genes which are transcribed together.

DISCUSSION

RSF1010 recombinant plasmids which acquired ampicillin resistance from two different R factors show an identical insertion (based on heteroduplexes with RSF1030) of $3.2 \pm 0.3 \times 10^6$ daltons of DNA which is bounded by short inverted complementary sequences in all 21 recombinant plasmids. This 3×10^6 daltons sequence, TnA, resembles the IS sequences found in bacterial, viral, and plasmid DNA (4, 21) since it can be translocated and its insertion leads to a loss of gene function. However, TnA differs from the IS sequences in at least two respects. Whereas the IS sequences are of relatively small size (800 to 1400 base pairs; ref 4) that are recognized by their mutational effects, TnA is larger (4500 base pairs) and possesses a readily identifiable marker, ampicillin resistance. R-plasmid-mediated tetracycline and kanamycin resistance are other members of this new class of translocatable elements (22, 23). Of course, translocatable sequences would not be expected to be limited to only antibiotic resistance genes. However, antibiotic resistance is an easily observed phenomenon and it is not surprising that resistance genes can become widely disseminated because of their distinct advantage to host cells. Translocation events in which antibiotic resistance genes are moved from plasmid to plasmid clearly play an important role in plasmid evolution (3), and it is tempting to speculate that translocation of genes may also be important in eukaryotic organisms.

Many of the translocatable antibiotic resistance determinants are bounded by inverted repeated sequences and, at least in the case of tetracycline resistance, the inverted repeat has been identified as IS3 (24). One may imagine that genetic elements such as the tetracycline resistant determi-

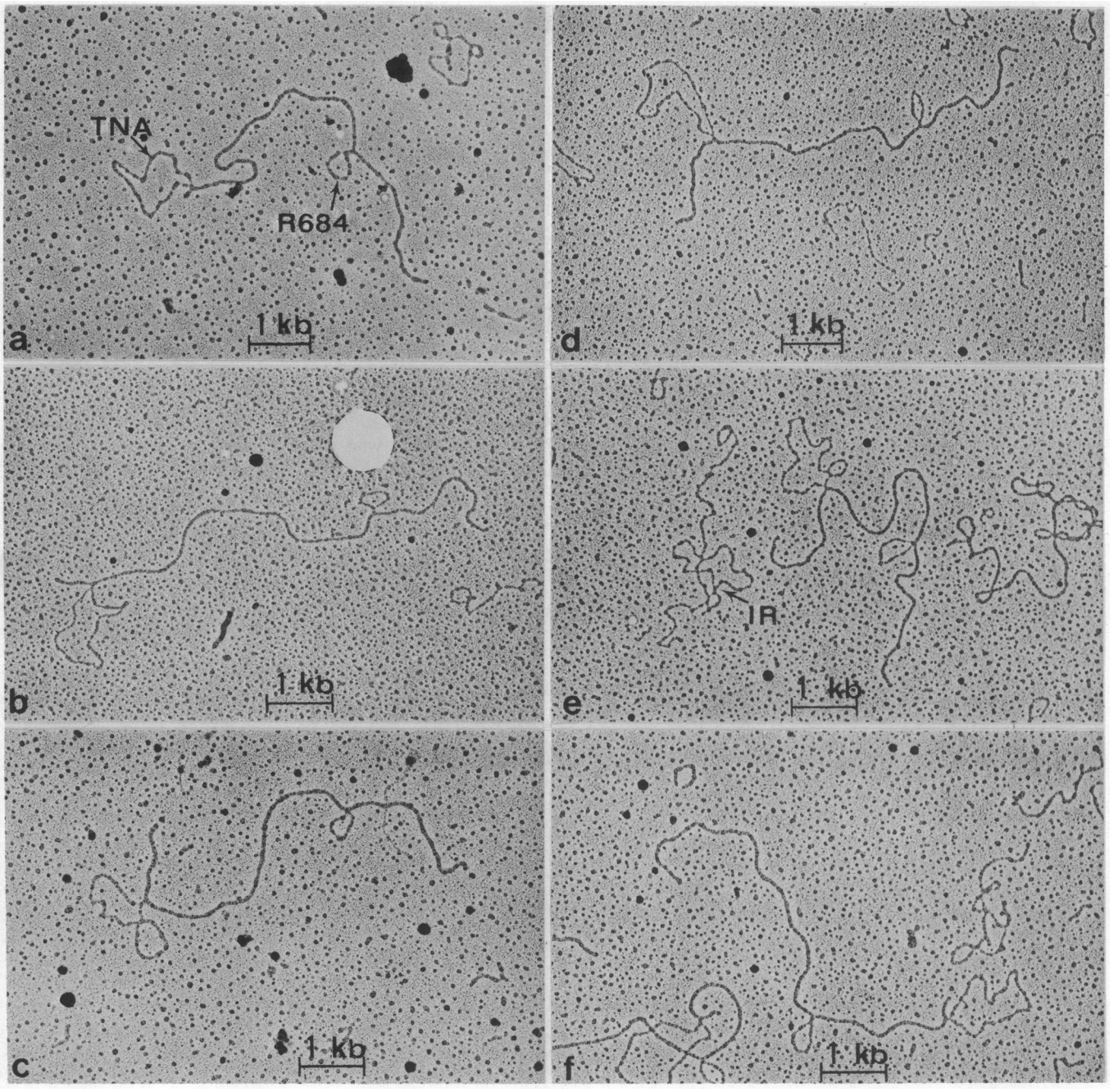

FIG. 3. The insertion of TnA at different sites on RSF1010. (a) Heteroduplex of R684 recombinant Ap210 (Su Ap) DNA showing TnA insertion 3.4% (0.28 kb) from the left hand end of the *Eco*RI-cleaved molecule. (b) Heteroduplex of R684/recombinant Ap211 (Su Ap) DNA showing the TnA insertion at 8.5% (0.70 kb) from the left hand end of the molecule. (c) Heteroduplex of R684/recombinant Ap130 (Su Sm Ap) DNA showing the TnA insertion at 15% (1.24 kb) from the left hand end of the molecule. (d) Heteroduplex of R684/recombinant Ap233 (Su Sm Ap) DNA showing the TnA insertion 21% (1.74 kb) from the left hand end of the molecule. (e) Heteroduplex of R684/recombinant Ap135 (Su Sm Ap) showing the TnA insertion 25.8% (2.14 kb) from the left hand end of the molecule. A single-stranded molecule of Ap135 DNA is also shown in this photograph in which the inverted, repeated sequences (IR) have reannealed to form a short duplex region (arrow). (f) Heteroduplex of R684/recombinant Ap201 (Ap) showing insertion of TnA 95.2% (7.9 kb) from the left hand end of the molecule.

nant were formed by insertion of IS sequences at either side of a drug resistance gene. Subsequent recombinational events, together with antibiotic selection, has led to the dissemination of these translocatable genetic elements. One reason these IS sequences are inverted and not direct repeats may be that traditional host-mediated recombination between inverted and repeated sequences reverses but does not excise the intervening sequence (18). Direct repeats of IS sequence might be selected against because recombination between directly repeated sequences would excise a drug resistance gene, proving lethal for the host bacteria in the presence of antibiotics. While the tetracycline resistance determinant is bounded by IS3, the much shorter inverted repeat found in TnA corresponds to no known IS sequence.

The mechanisms of TnA translocation and of IS sequence translocation is obscure. Our observation that TnA is bounded by inverted complementary sequences in each of the recombinant plasmids would indicate that these sequences play an essential role in translocation. The short inverted complementary sequences which are found on either end of

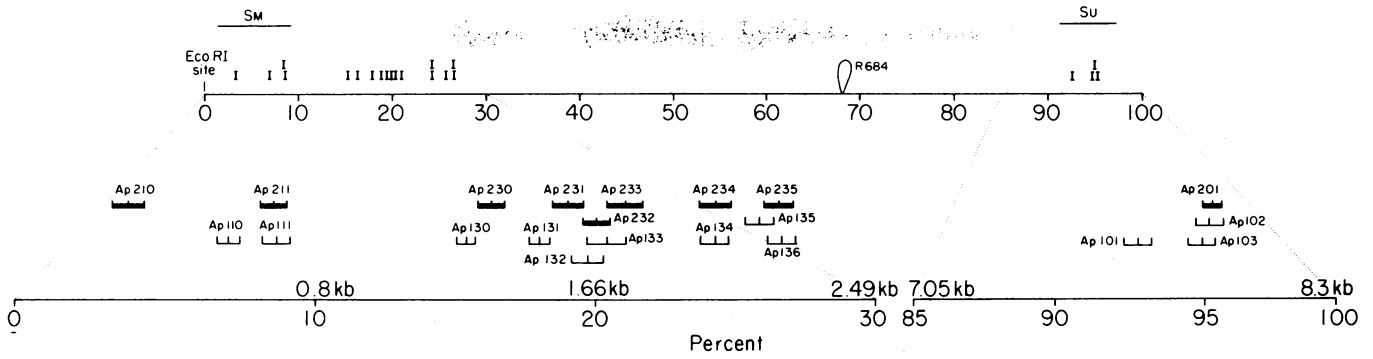

FIG. 4. A map of the sites of TnA insertion into RSF1010. The observed sites of insertion of TnA in the upper line representation of the entire RSF1010 genome are each shown as an 'I'. All recombinant plasmids are named as derivatives of RSF1010. For example, RSF1010 Ap210 represents an ampicillin-resistant derivative of RSF1010. The Ap100 and Ap200 series are derivitives of RSF1010 in which TnA translocated from R64-1 and R1, respectively, and inserted into what presumably is the gene encoding for sulfa resistance. The 110 and 210 series are insertions from R64-1 and R1, respectively, in which TnA has inserted in the gene which presumably specifies streptomycin phosphotransferase. The 130 and 230 series are insertions from R64-1 and R1 in which TnA has not affected either of the two drug resistance genes nor any other phenotype of this plasmid. Insertions of TnA from R64-1 are denoted by lines on the expanded map of RSF1010; the mark in the center of each line represents the mean of the measurements for the site of insertion. The solid bars represent insertions of TnA from R1.

TnA might in fact be specific recognition sequences for enzymes used in excision and insertion. We have shown that insertion of TnA can take place at a minimum of 12 distinct sites within one third of the RSF1010 genome. The distribution of insertion sites within a 2×10^6 dalton contiguous portion of RSF1010 is nonrandom, suggesting that insertion of TnA is site-specific. Judged by the large number of insertion sites in RSF1010 this sequence must be short and quite common. Kopecko and Cohen have reported a site-specific *recA*-independent event which they interpreted as the insertion of an entire plasmid genome at a unique site of another plasmid and excision of a DNA segment (containing the Ap gene) originally present at the point of this insertion (25). It is clear, however, that the events they have described and the translocation phenomenon which we have reported in this paper are essentially similar. We have also found that translocation of TnA, like the translocation of IS sequences (4), is independent of the *E. coli recA* gene (Rubens, Heffron, and Falkow, unpublished observations). This and other results dealing with the specificity of excision and insertion will be published elsewhere. The ease with which translocation of TnA can be selected, its low specificity of insertion, and ready visualization in the electron microscope in DNA heteroduplexes make the technique outlined in this paper one that may be usefully applied to the genetic mapping of plasmids.

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