JOURNAL OF BACTERIOLOGY, Jan. 1977, p. 482-491

Vol. 129, No. 1
Printed in U.S.A.

Isolation, Mapping, and Examination of Effects of TnA Insertions in ColE1 Plasmids

JOSEPH INSELBURG

Department of Microbiology, Dartmouth Medical School, Hanover, New Hampshire 03755

Received for publication 23 July 1976

Twelve TnA insertions of ColE1 deoxyribonucleic acid have been isolated and mapped by electron microscopic studies of heteroduplex molecules. Insertions only blocking the production of active colicin clustered in one region of the map, whereas insertions only blocking the expression of deoxyribonucleic acid nicking activity associated with the plasmid "relaxation complex" clustered in another region of the map. The location of one insertion that blocks both the expression of colicin immunity and the production of active colicin suggests that the expression of both characteristics are coordinately controlled.

Plasmid-mediated drug resistance to ampicillin has been shown to be associated with a translocatable sequence of deoxyribonucleic acid (DNA; TnA) which is approximately 3.2×10^6 daltons in size (9, 11). So et al. (25) have recently isolated colicin E1 plasmid DNAs (ColE1) carrying the TnA insertion. The location of those insertions in ColE1 DNA and their effect on colicin production was demonstrated by them (25). In this paper the results of the study of 12 independently isolated TnA insertions of ColE1 DNA are reported. Map locations of the insertions were established by heteroduplex mapping. The effects of the various insertions on the expression of (i) colicin production, (ii) colicin immunity, and (iii) the "relaxation" of ColE1 DNA was also examined. The implications of the locations of the various insertions on alterations in the expression of these characteristics is discussed.

MATERIALS AND METHODS

Bacterial strains. All strains are derivatives of *Escherichia coli* K-12: C600 (*thr⁻ leu⁻ thy⁻*); B1; and C600(ColE1), a colicinogenic derivative. J5(R1*drd-19*), obtained from S. Falkow, carries the 6.3×10^7-dalton R1-*drd-19* plasmid, which codes for resistance to ampicillin (Ap), chloramphenicol (Cm), streptomycin, sulfonamide, and kanamycin. C600-(ColE1, R1*drd-19*) was constructed by transforming C600(ColE1) with R1*drd-19* DNA. Plasmid pMB9, constructed in the laboratory of H. Boyer, was obtained from D. Botstein. The pMB9 plasmid contains approximately 40% of the ColE1 plasmid (unpublished data and personal communication of H. Boyer) to which has been fused a fragment carrying the tetracycline resistance gene originally carried by the plasmid pSC101 (6).

Media. Cells were grown in M9 medium. M9 medium contains Na_2HPO_4 (6.0 g), KH_2PO_4 (3.0 g), NaCl (0.5 g), NH_4Cl (1.0 g), $MgSO_4 \cdot 7H_2O$ (0.25 g), glucose or glucerol (0.2%), and vitamin B_1 (1 μg/ml) in a final volume of 1 liter. Amino acids and thymine were added as needed.

Isolation of ColE1-Ap^r plasmids. The method used was identical to that described by So et al. and Hefron et al. (10, 11, 25). C600 (ColE1) was transformed with purified R1*drd-19* plasmid according to the procedure of Cohen et al. (7), and a ColE1-Ap^r Cm^r clone was isolated (in this cell the 3.2×10^6-dalton TnA fragment will translocate to some ColE1 molecules). A mixture of [^{3}H]thymidine-labeled and cold plasmid DNA from a culture derived from one colony was purified as previously described (10, 25) and layered on top of a 16-ml, 5 to 20% neutral sucrose gradient and centrifuged in a Beckman SW27 rotor for 8 h at 15°C at 27,000 rpm (95,000 × *g*). Fractions (0.4 ml) were collected. The location of the ^{3}H-labeled ColE1 DNA band was determined, and the appropriate gradient fractions containing DNA sedimenting faster than ColE1 DNA (ColE1-TnA DNA being 7.4×10^6 daltons) were pooled and dialyzed against TEN buffer [20 mM tris(hydroxymethyl)aminomethane(Tris), 20 mM NaCl, and 1 mM disodium ethylenediaminetetraacetic acid (pH 8.0)] and used to transform $CaCl_2$-treated C600 cells to Ap^r (7) (see Fig. 1). These transformants were further studied.

Tests of the transformants for production of active colicin and colicin immunity were performed as previously described (14).

Agarose gel electrophoresis of DNA isolated from transformants was done in 1.0% agarose prepared in 0.04 M Tris (pH 7.8), 5 mM sodium acetate, and 1 mM disodium ethylenediaminetetraacetic acid. The pH adjustment is with glacial acetic acid (17).

Tests of the presence of "relaxation complexes" of ColE1-Ap^r. C600 cells carrying ColE1-Ap^r plasmids were grown to 4×10^8 cells/ml in M9-glycerol medium with 5 μg of thymine per ml and [^{3}H]thymidine (5 μCi/μg of thymine). The cultures were lysed using the "cleared lysis procedure" (4)

with Triton X-100. The radioactive plasmid DNA was then divided into two parts, either untreated or sequentially treated at 37°C for 10 min with predigested Pronase (250 μg/ml) and 1% sodium dodecyl sulfate (SDS). The DNA was layered onto a 15 to 50% sucrose gradient in TES buffer (0.05 M Tris [pH 8.0], 0.05 M NaCl, and 0.005 M ethylenediaminetetraacetic acid) and centrifuged in a Spinco SW50.1 rotor for 3 h at 15°C at 45,000 rpm (189,000 × g). ^{32}P-labeled ColE1-Ap^r DNA was used as a sedimentation marker to define the position of covalently closed circular (CCC) and open circular (OC) DNA. The convertibility of CCC ColE1-Ap^r DNA to OC DNA by the Pronase-SDS treatment was taken as the criterion for the existence of a functional relaxation complex (3, 4).

Heteroduplex mapping of TnA insertions. The preparation of DNA, its denaturation, renaturation in formamide, and final preparation for electronmicroscopic measurements are exactly as previously described (13). The relative location of TnA insertions was determined by cleaving all DNAs with *Eco*RI restriction endonuclease (16) as previously described and preparing linear heteroduplex molecules containing one strand of ColE1-Ap^r DNA no. 5-42, in which the TnA insertion was shown to be about 88% of the total distance from one end of the *Eco*RI-cleaved DNA molecule (Table 1) and one strand of another ColE1-Ap^r DNA. The location of TnA insertions relative to the 5-42 TnA insertion were therefore obtained. Heteroduplexes between a ColE1-Ap^r DNA and ColE1 DNA were also formed to verify the location of an insertion when heteroduplexing conditions led to the interaction between the TnA sequences in the heteroduplex molecules. The absolute location of the TnA insertions was determined by locating the site of one of the TnA insertion in ColE1-Ap^r DNA (no. 6-30) that was 26% of the total ColE1 distance from the *Eco*RI-sensitive site in a heteroduplex with the plasmid pMB9 which contains less than half of the ColE1 DNA molecule (12, 18; H. Boyer, personal communication). As that half of the ColE1 contains the unique replication origin of the plasmid (18), a relationship between all TnA insertions to a specific end of an *Eco*RI-cleaved molecule could be defined.

RESULTS

Isolation of ColE1-Ap^r plasmids. C600 *thy*$^-$ cells were transformed with extrachromosomal DNA isolated from cells carrying both ColE1 and R1*drd-19* DNA as described in Materials and Methods (Fig. 1). Seventy Ap^r transformants were selected purified and tested for colicin production and colicin immunity (14). All colicin-producing (Col$^+$) transformants were colicin immune (Imm$^+$). Seven transformants that did not produce active colicin (Col$^-$) were Imm$^+$, and two transformants that were Col$^-$ failed to produce active colicin and were also nonimmune (Imm$^-$). The test for colicin immunity was a plus-minus test, and no attempt was

TABLE 1. *Location of TnA insertions in ColE1-Ap^r plasmids as determined by electron microscopic measurements of heteroduplex DNAs*[a]

DNA in heteroduplex	No. of molecules measured	Distance of a loop from left end (%)	Distance of a loop from right end (%)
5-42 + E1	30	88.1	11.9 ± 2.0
6-30 + 5-42	33	25.8 ± 2.4	11.2 ± 1.8
6-30 + E1	32	26.4 ± 3.9	
6-30 + PMB9	14	26.3 ± 7.2	
6-32 + 5-42	23	29.3 ± 3.1	11.7 ± 1.8
5-36 + 5-42	12	29.4 ± 3.7	14.1 ± 2.4
7-12 + 5-42	16	31.4 ± 3.6	12.2 ± 3.2
2-14 + 5-42	32	40.0 ± 3.9	12.7 ± 2.1
0-1 + 5-42	26	44.2 ± 4.2	11.9 ± 2.0
0-1 + E1	32	45.2 ± 3.8	
3-12 + 5-42	15	53.7 ± 5.1	13.6 ± 3.7
3-12 + E1	38	54.0 ± 5.7	
3-1 + 5-42	17	72.4 ± 6.6	13.1 ± 2.9
3-1 + E1	31	73.6 ± 6.7	
7-6 + 5-42	20	79.6 ± 4.0	12.1 ± 3.2
2-35 + 5-42	18	87.1 ± 5.4	12.9 ± 2.9
2-35 + E1	27	86.6 ± 2.7	
6-12 + 5-42	29	100 ± 5.6%	

[a] The left end of the *Eco*RI-cleaved ColE1 DNA molecule is arbitrarily designated to be that end nearest the ColE1 replication origin. All distances are given as percentages of the total length of the linear ColE1 DNA in the heteroduplex. The distance of insertion 5-42 from the left end when calculated from all molecules in which it is found is 87.5 ± 2.5%. The measurements in the column "distance of a loop from left end" is the distance of the insertion loop that is not the 5-42 loop from the left end with the exception of the first entry. The measurement in the column "distance of a loop from right end" represents the distance of the 5-42 insertion from the right end. The difference between the percentages in both columns represents the distance between the two insertions in the same heteroduplex.

made to distinguish degrees of immunity. All Col⁻ Imm⁻ cells and 40 Col⁺ Imm⁺ cells were grown to 5 × 10^8 cells/ml in M9-glucose medium, incubated in the presence of 180 μg of Cm per ml overnight (4), lysed by the cleared lysis procedure, phenol extracted, and screened for

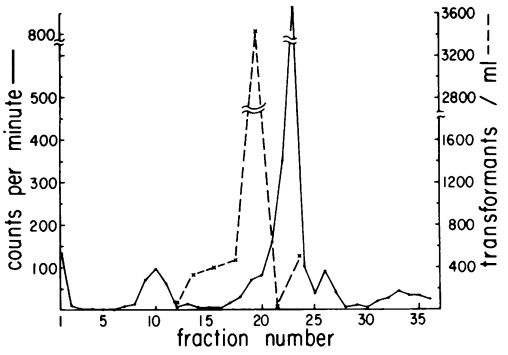

FIG. 1. *Sucrose density gradient of a mixture of ^{3}H-labeled and cold DNA from C600 (ColE1, R1drd-19). The DNA was centrifuged in an SW27 rotor for 8 h at 15°C at 27,000 rpm (95,000 × g). Fractions were pooled, dialyzed, and used to transform C600 cells to Ap^r. Symbols: ^{3}H-labeled DNA (·——·), AP^r transformants (×---×). The principal peak of ^{3}H-labeled DNA (fraction no. 23) represents CCC ColE1 DNA. The transformations were carried out as described in Materials and Methods.*

the presence of extrachromosonal DNA by examining the lysates by electrophoresis of DNA in 1% agarose gels containing 0.5 μg of ethidium bromide per ml. The mobility of the extrachromosonal DNA from 12 transformants which were further studied are shown in Fig. 2. Eleven of the plasmid DNAs were the same size, and one was slightly smaller than the others. All were larger than ColE1 DNA (Fig. 2). The size of the 11 DNAs of the same size was determined by electron microscopic measurements to be 3.90 ± 0.20 μm in length. This confirmed the reported size of the TnA insertion fragment, usually being 3.2 × 10^6 daltons (9, 25), as the size of ColE1 DNA which is 4.2 × 10^6 daltons and measures 2.17 ± 0.15 μm under the same conditions.

Mapping of the TnA insertions in ColE1-Ap^r plasmids. As *Eco*RI restriction endonuclease cleaves both ColE1 and ColE1-Ap^r DNA at the same single site, the mapping of the TnA insertions was initiated by preparing heteroduplexes between *Eco*RI-cleaved ColE1 and various ColE1-Ap^r DNAs. The heteroduplex of ColE1-Ap^r DNA no. 5-42 and ColE1 DNA contained an insertion loop at approximately 88% of the distance from one end of the *Eco*RI-cleaved DNA. (Fig. 3L). All other ColE1-Ap^r DNAs were subsequently heteroduplexed with the 5-42 DNA to provide an internal

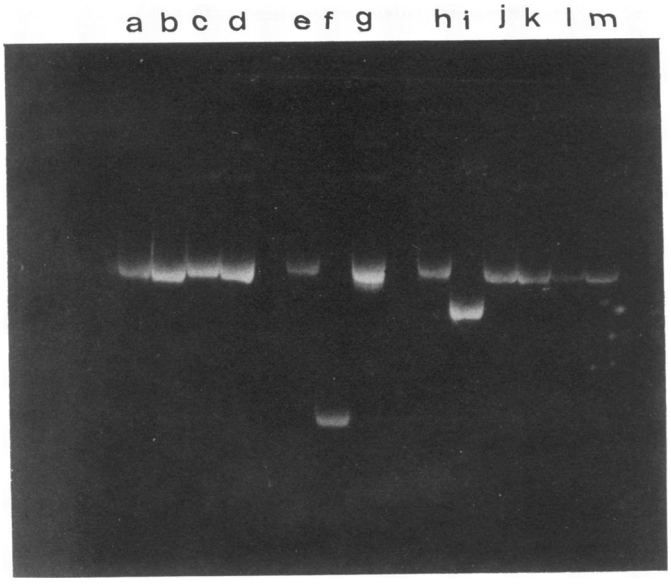

FIG. 2. *Electrophoresis of extrachromosomal DNA obtained from cleared lysates of C600 (ColE1-Ap^r) transformants. A 25-μl portion of phenol-extracted cleared lysates of ColE1-Ap^r-transformed cells were electrophoresed in 1% agarose gels containing 0.5 μg of ethidium bromide per ml. The DNAs from left to right are (a) 7-12, (b) 7-6, (c) 6-32, (d) 6-30, (e) 3-12, (f) ColE1 CCC DNA, (g) 5-42, (h) 6-12, (i) 5-36, (j) 3-1, (k) 2-35, (l) 2-14, and (m) 0-1. A small amount of ColE1 DNA was added to each sample to insure the parallel migration of each sample. That band is just visible at the ColE1 CCC DNA position.*

marker within the heteroduplexes to determine the relative positions of the other TnA insertions. Electron micrographs of various heteroduplexes are shown in Fig. 3. In several instances the heteroduplexes formed between 5-42 DNA and other ColE1-Ap^r DNAs exhibited interaction between the TnA elements on both strands (Fig. 3-G, I, M). In such instances the location of the TnA insertions could be determined from the lengths of the linear double-stranded DNA joining the double-stranded TnA loop. These results were confirmed by preparing a heteroduplex between the particular ColE1-Ap^r and ColE1 DNA (See Fig. 3F and H). Figure 3F represents a heteroduplex between one strand of 3-12 DNA and three strands of ColE1 DNA, giving a cruciate structure with four ends and a loop at the intersection of the cross. If one arbitrarily placed the 5-42 TnA insertion near the right end of an *Eco*RI-cleaved ColE1 molecule, then the locations of the various insertion relative to it are shown in Table 1.

The absolute location of insertions is based on the observation that the ColE1 replication initiation site is approximately 20% of the total molecular distance from one end of an *Eco*RI-cleaved ColE1 molecule (16, 21, 27). (We will arbitrarily call that near end, the left end.) We have shown that the mini ColE1 DNA in plasmid pML21, which contains about 50% of the ColE1 plasmid, contains the replication origin and so would carry the left half of the ColE1 DNA (18). The plasmid pMB9, which replicates as ColE1, was constructed from the mini ColE1 plasmid and so also contains the left half of the ColE1 DNA molecule. It has one *Eco*RI-sensitive site which is the same as that in ColE1 DNA. The *Eco*RI-cleaved ColE1-Ap^r plasmids 5-42 and 6-30 which have TnA insertions at 88 and 26%, respectively, of the total distance from the same end of the molecule (Table 1) were heteroduplexed with *Eco*RI-treated pMB9 DNA to determine which insertion resides within the ColE1 segment carried by pMB9. No heteroduplexes with an insertion loop was noted between 5-42 and pMB9 DNAs, whereas heteroduplexes with an insertion loop were noted between 6-30 and pMB9 DNA (Fig. 4; Table 1). This result provides an absolute orientation for the insertion map shown in Fig. 5. Two additional points of interest are determinable from the heteroduplex structures. The heteroduplex between 5-42 and 5-36 DNA suggests that the portion of the 5-36 DNA that is deleted resides in the TnA insertion as the linear duplex DNA is equal to that of ColE1 DNA. The heteroduplex structure generated between 5-42 and 2-35 DNAs suggests that they are inserted in opposite orientations.

The ability of plasmids carrying TnA insertions to produce active colicin and express immunity to colicin was tested as previously described (14). The distribution of Col$^+$ and Col$^-$ plasmids are shown in Fig. 5. The result suggests that genetic information affecting the expression of active colicin resides in the right quarter of the map. A ColE1 Col$^-$ insertion mutant previously isolated in this laboratory (15) was found to be located very near the *Eco*RI-sensitive site of ColE1. A heteroduplex between the mutant and ColE1 DNA is shown in Fig. 6. That result further substantiates the observation that that region of the ColE1 map is involved in colicin expression. Although these results are similar to those of So et al. (25) who found that that insertion in the same region caused a Col$^-$ phenotype, they also found that insertions located between 40 and 65% of the total distance from the left end of the map shown in Fig. 5 also were Col$^-$. We found three insertions located between 40 and 53% of the distance from the left end of the ColE1 map that give a Col$^+$ phenotype. As one possible explanation of this discrepancy was that our strains might be carrying a Col$^+$ plasmid as well as the Col$^-$ plasmid with the observed insertion, DNA was isolated and used to transform C600 cells to Ap^r. Each of 20 of the new Ap^r transformants prepared from 2-14, 0-1, and 3-12 DNAs were tested and found to be Col$^+$. Three of each were tested, and all had aquired a plasmid identical in size to the original plasmid. This result appears to rule out this trival explanation for the observed difference. Further explanations of the discrepancy between these results and those of So et al. (25) will be discussed later.

A Col$^-$ Imm$^-$ plasmid, 6-32, with an insertion located at 29 ± 3% from the left end of the map, was found. The implication of the finding of this insertion of ColE1 DNA on gene organization will be discussed later.

It has been shown that under appropriate growth conditions a significant number of ColE1 DNA molecules carry a protein complex, a relaxation complex, which will nick (relax) the CCC ColE1 DNA at a single specific site in one strand when the complex is exposed to a variety of protein denaturing or inactivating treatments (3–5). As this site is near (26), though not necessarily at the origin of replication initiation, it has been postulated that this nicking activity is involved in some aspect of replication (4). If any of the proteins that are associated with the plasmid relaxation complex and are necessary for its nicking activity are

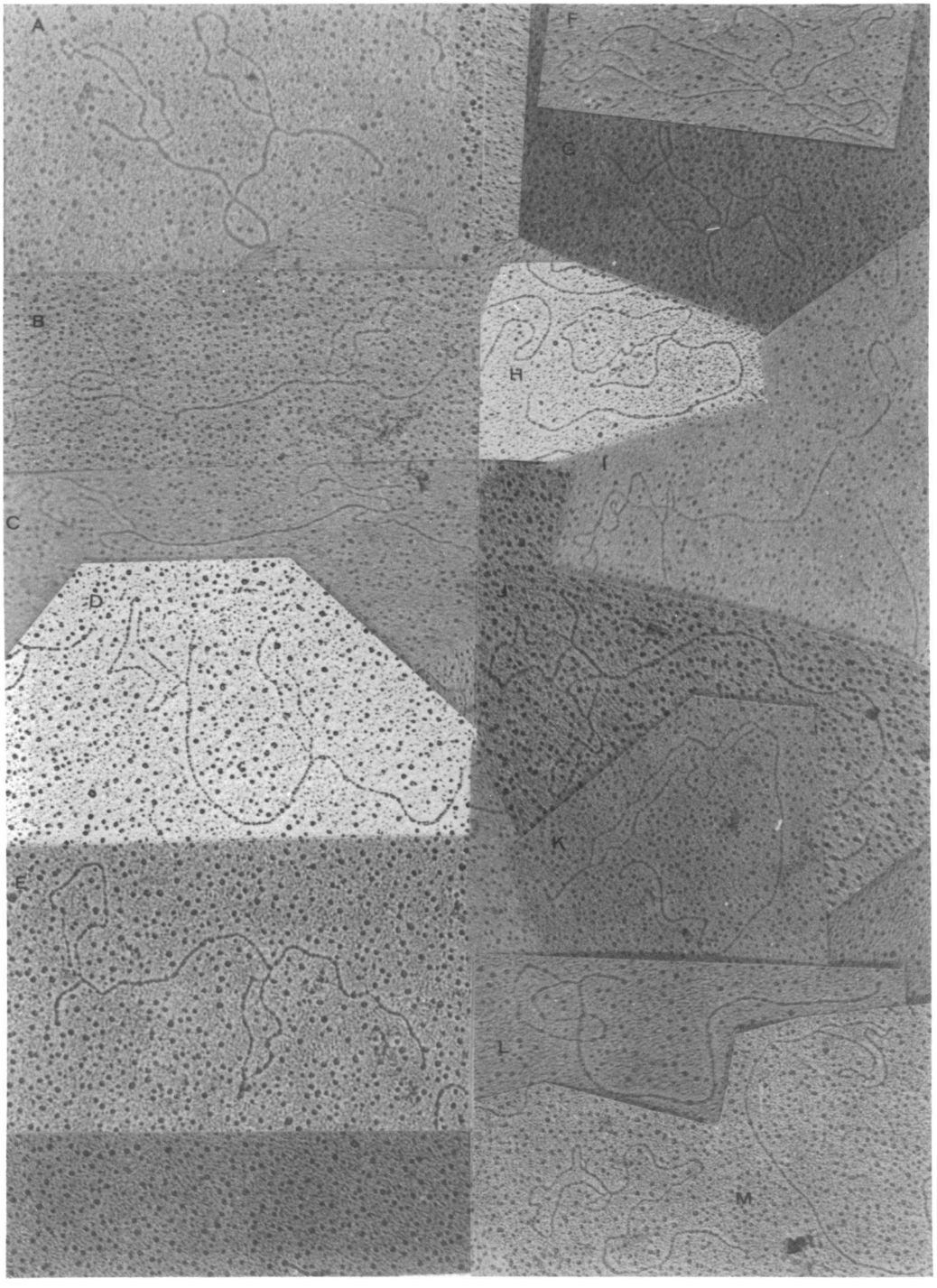

FIG. 3. *Electron micrographs of some heteroduplexes prepared between two ColE1-Ap^r plasmid DNAs or between ColE1-Ap^r and ColE1 DNAs. The heteroduplexes prepared or described in Materials and Methods were (A) 5-42 and 6-30; (B) 5-42 and 6-32; (C) 5-42 and 5-36; (D) 5-42 and 2-14; (E) 5-42 and 0-1; (F) Col E1 and 3-12; (G) 5-42 and 3-12; (H) Col E1 and 3-1; (I) 5-42 and 3-1; (J) 5-42 and 7-6; (K) 5-42 and 2-35; (L) Col E1 and 5-42; and (M) 5-42 and 6-12. In samples (G), (I), and (M) the TnA insertions interacted, forming*

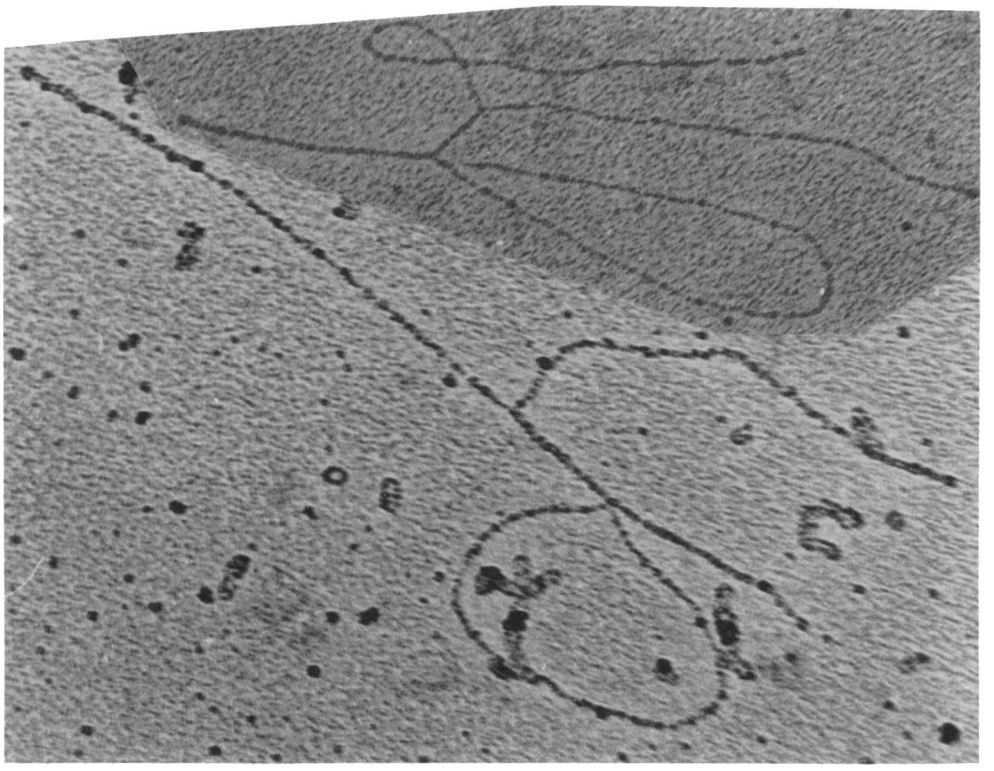

FIG. 4. *Heteroduplex between 6-30 and pMB9 DNA. The double-stranded region of the heteroduplex represents the left side of the ColE1 plasmid DNA. The rest of the ColE1 DNA in the 6-30 molecule is the longer single strand. The TnA insertion loop is 26.3 ± 7.2% of the total distance from the left end of the ColE1 molecule which comprises the 6-30 DNA. (See Table 1.)*

coded for by ColE1, the presence of some or all of the TnA insertions could affect their production by either disrupting a structural gene sequence or inhibiting normal transcription of the gene(s). In either case one might find plasmids prepared under conditions where a large proportion should be relaxable that are not converted from CCC to OC forms when they are exposed to pronase or SDS, which normally causes relaxation of the protein-complexed ColE1 DNA molecules. All the ColE1-Ap^r plasmids mapped in this paper were examined to see if they retain the relaxation complex nicking activity. The plasmid-containing cells were labeled for three generations in M9-glycerol medium containing [^{3}H]thymidine (5 mCi/μg of thymidine). Growth in glycerol as opposed to glucose medium increases the content of relaxation-complexed DNA (5). The DNA was isolated by the cleared lysis procedure using Triton X-100 as the detergent, and the plasmid DNA in the lysate was examined by sucrose density-gradient centrifugation to determine if nicking was induced by sequential 10-min treatments at 37°C with 100 μg of Pronase per ml and 1% SDS. Figure 7 shows sucrose density-gradient analyses of three treated ColE1-Ap^r DNAs that showed little or no nicking

double-stranded loops. The location of the insertion was determined by the lengths of the linear double-stranded DNA joining the forks and was confirmed by measuring heteroduplexes formed with ColE1 DNA [(F) and (H)]. In (F) a single 3-12 DNA strand has interacted in an excess of ColE1 DNA so the heteroduplex interaction involves three ColE1 and one 3-12 single strands. Note one loop attached to the "X"-shaped structure at the crossing point in which the arms on the same side of the cross are the same length, and the sum of one long and one short arm is equal to ColE1 DNA. In (H) the single TnA loop is that of 3-1 DNA which interacted with the 5-42 TnA loop in the heteroduplex shown in (I). In the heteroduplex molecules in (M) the molecular structure obtained involves an insertion much nearer the EcoRI site than 5-42. The absence of a second tail suggests that it is very close to the EcoRI site as does the measurement of the linear portion of the heteroduplex which is equal to the ColE1 DNA length.

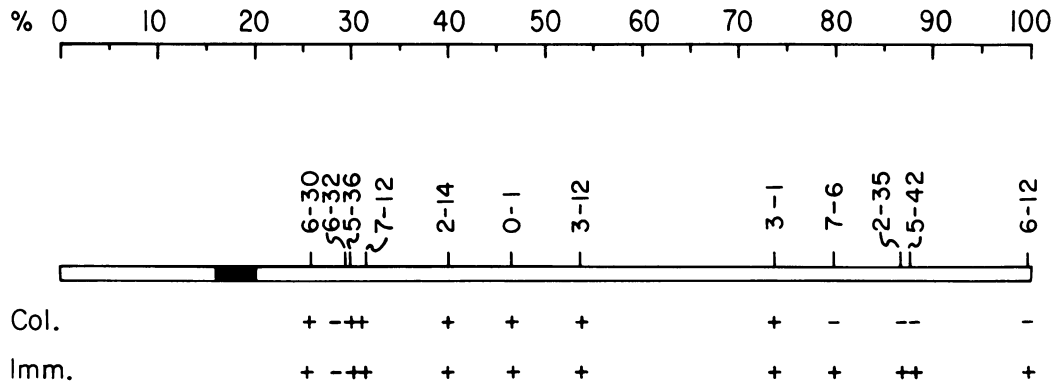

FIG. 5. *Summary map of the location of various TnA insertions in ColE1 DNA and the effect of the insertions on colicin production and immunity. This data represents material from Table 1 and the results of tests of colicin production and immunity described in the text.*

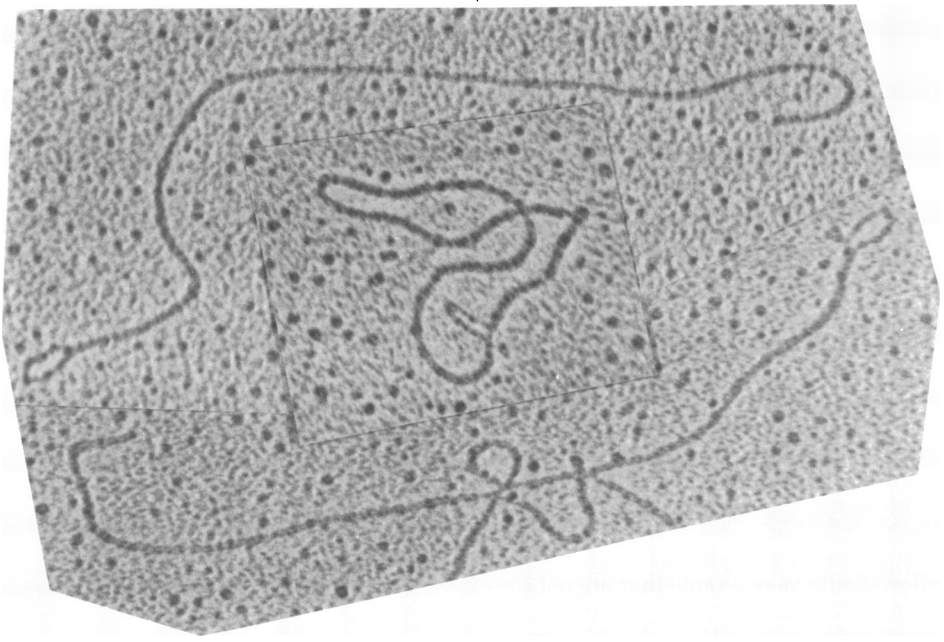

FIG. 6. *Electron micrographs of heteroduplexes between the Col⁻ plasmid ColE1 4A carrying a small insertion and ColE1 DNA. Note the small loop at one end of the heteroduplex. The insertion size was reported to be about 10% of the length of ColE1 DNA (15).*

(Fig. 7A and B) and a degree of nicking comparable to that of ColE1 DNA (Fig. 7C) after isolation under identical conditions. The ^{32}P-labeled sedimentation marker DNA was 6-30 ColE1-Ap^r DNA labeled during extended replication in Cm, a condition in which like ColE1 DNA nonrelaxable CCC DNA accumulated in the cell. The nonrelaxed, untreated DNAs from the cleared lysates sedimented, as previously reported (4), as a single band with a sedimentation coefficient that is greater than CCC DNA. In the case of ColE1-Ap^r DNA it is about 40S (data not shown). The percentage of OC DNA found after treatment is shown in Table 2. The results indicate that insertions clustering between 30 and 50% of the total molecular distance from the left end of the map (Fig. 5) have a markedly reduced sensitivity to being nicked, suggesting that the expression of a ColE1 product that is necessary for relaxation is affected by the insertions in that region.

In studying the plasmids carrying the insertions described here, no unusual instability as expressed in segregation was noted. The yields

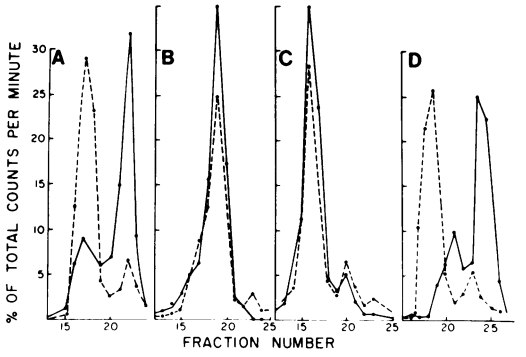

FIG. 7. *Sucrose density gradients of Pronase-SDS-treated ColE1-Ap^r DNA. Cells were grown in M9-glycerol medium in the presence of [^{3}H]thymidine and lysed by the cleared lysis method, and the cleared lysates were either treated with Pronase and SDS or untreated as described in Materials and Methods. DNA samples (0.2 ml) were layered onto sucrose density gradient (15 to 50% sucrose made in TES buffer) and centrifuged for 3 h at 15°C at 189,000 × g in a Spinco SW50.1 rotor. ^{32}P-labeled ColE1-Ap^r no. 6-30 DNA was the sedimentation marker and is primarily in the CCC form. The treated DNAs shown are (A) 6-30, (B) 0-1, (C) 3-12, and (D) ColE1. Untreated DNAs from the cleared lysates of ColE1-Ap^r carrying cells were centrifuged under identical conditions and sedimented as a single peak (4) that had a sedimentation coefficient of about 40S as compared with one of about 27S for the CCC form after Pronase-SDS treatment (data not shown). In (D) the difference in sedimentation properties of ^{32}P-labeled 6-30 DNA and treated ColE1 DNA is shown. Symbols: ^{32}P-labeled DNA (●---●); ^{3}H-labeled DNA (●——●). A 10,000-cpm portion of ^{3}H-labeled DNA was applied to each gradient.*

of DNA isolatable from identical Cm-treated cultures carrying different plasmids have been very similar, when lysis efficiency was considered. This was determined by the yields of [^{3}H]thymidine-labeled plasmid DNA isolatable from those cultures and by examining the fluorescence of DNA samples obtained from those same cultures that were examined by agarose gel electrophoresis. All plasmids continued to replicate in the presence of 180 μg of Cm per ml. Although more precise kinetic measurements must be made to note differences in plasmid replication in the absence of Cm, it appears that an absence of nicking activity normally associated with relaxation-complexed DNA probably does not greatly reduce the ability of the plasmids to replicate.

DISCUSSION

TnA insertions of ColE1 DNA have been isolated and mapped by electron microscopic studies of heteroduplex molecules. Various insertions were shown to affect the production of active colicin (7-6, 2-35, 5-42, and 6-12), of both active colicin and colicin immunity (6-32), and of the relaxability of molecules in the presence of Pronase and SDS (5-36, 7-12, 2-14, 0-1, and 3-12).

TABLE 2. *Percent conversion of CCC ColE1-Ap^r DNA to OC forms by Pronase-SDS treatment*[a]

ColE1-Ap^r plasmid	% Conversion of CCC to OC form by SDS-Pronase treatment
6-30	71
6-32	34
5-36	4
7-12	11
2-14	<1
0-1	<1
3-12	11
3-1	42
7-6	59
2-35	68
5-42	44
6-12	56
ColE1	60

[a] The percent conversion of ^{3}H-labeled ColE1-Ap^r DNA was obtained from cleared lysates of cells grown in M-9-glycerol medium to OC forms by Pronase-SDS treatment. All untreated ColE1-Ap^r DNAs banded as a single peak with a sedimentation coefficient of approximately 40S in identical sucrose gradients, whereas untreated ColE1 DNA banded as a single peak with a sedimentation coefficient of about 34S (results not shown) (4) (see Fig. 7). The ordering of plasmids from top to bottom is the same as the left to right sequence shown on the insertion map shown in Fig. 5.

The Col$^-$ Imm$^+$ plasmids reported here have insertions clustering in the right 20% of the insertion map (Fig. 5). Insertions having similar effects were reported by So et al. (25) to cluster in that region and also in a second region between 40 and 65% of the total distance from the left end of the map. Insertions 2-14, 0-1, and 3-12 which fall in that second region described here are Col$^+$. Although it may be argued that insertions 2-14 and 0-1, because of the variation in measurements ±4%, may be located just beyond the edge of a cistron affecting active colicin production, the Col$^+$ activity of the plasmid carrying insertion 3-12 can not be explained in that way. S. Falkow (personal communication) indicated that an explanation of the discrepency may be that colicin production in the particular bacterial strain carrying the plasmids they studied may be low, thus giving a negative result. Another potential explanation, however, could involve a possible effect of the orientation of the TnA insertion sequences on cistrons located between 45 and

65% on the map affecting colicin production. It has been shown that insertions of IS sequences can have polar effects (1, 8, 20, 23), and other evidence suggests that depending on the orientation of an IS II sequence the insertion may play a role in the expression of a termination or promoter action on adjacent cistrons (22). Although such an affect has not been reported for TnA sequences, such an effect remains a possibility.

The insertion, 6-32, which was found to be located 29 ± 3% of the total length from the left end of the map, caused a simultaneous loss of active colicin production and colicin immunity, whereas many insertions located to the immediate right and only one insertion located to the immediate left of it had no effect on these plasmid functions. Although it would not be unreasonable to interpret this finding to indicate that the cistrons for immunity and colicin activity are transcribed together, such an interpretation would be better supported by demonstrating through finer mapping that the 6-32 insertion really lies just to the left of the 6-30 insertion (a point that measurement variation does not rule out) or by demonstrating that the TnA insertions such as the IS II insertions can have polar effects in some and not other circumstances (22). It should be noted here that the coordinate expression of a colicin structural gene and immunity gene is suggested from results of studies of colicin E3 and its immunity substance (19, 24). In vitro studies of their interaction indicates that immunity substance in stoichiometric amounts with respect to colicin blocks the colicin activity. The coordinate expression of both cistrons would protect a cell from the effects of a low-level expression of the plasmid genes. An operator proximal location for the immunity cistron in such an operon would seem to be functionally beneficial.

The insertions 5-24 and 2-35, which both cause a Col^- phenotype, are inserted in opposite orientations at adjacent locations on the map. If the orientation of the TnA insertion could influence the expression of a polar effect, then the absence of an effect of either of these insertions on the expression of colicin immunity might be considered to indicate that they are both located distal to an immunity cistron in an immunity-colicin operon that is being transcribed from right to left through the Col^- region.

Ultimately, these speculations regarding the effects of insertions on ColE1 gene expression, and in particular insertion 6-32, will have to be substantiated by studies of ColE1 gene products and a finer mapping of the 6-30 and 6-32 insertion sites with respect to each other.

The finding that a cluster of insertions between the 30 and 50% region on the map (Fig. 5) affects the relaxability of ColE1 DNA suggests that some or all of the proteins associated with the plasmid relaxation complex are coded for by the plasmid.

Insofar as little or no relaxation of CCC DNA was inducible in plasmids with the insertions clustered between the 30 and 50% locations on the map (Fig. 5) and insofar as those plasmids replicate well enough to be maintained, it would appear that any role which relaxation nicking activity may have in replication is not essential for the maintenance of the plasmid. In vitro replication of ColE1 DNA that does not carry a relaxation complex was found to be as efficient as replication of DNA carrying the complex (unpublished data). If the relaxation complex does have a role in ColE1 replication, it may be at a moderating or control level.

ACKNOWLEDGMENTS

I thank S. Falkow for sending me bacterial strains and preprints of work done in his laboratory on the isolation and characterization of TnA mutants and D. Botstein and N. Kleckner for the pMB9 plasmid. I also thank P. Ware for invaluable technical assistance in the course of this work.

This work was supported by grants 4 ROI-AI-08937-08 and 5KO4-AI 28818-03 from the National Institute of Allergy and Infectious Diseases.

LITERATURE CITED

1. **Adhya, S., M. Gottesman, and B. deCrombrugghe.** 1974. Release of polarity in *Escherichia coli* by gene N of phage λ: termination and antitermination of transcription. Proc. Natl. Acad. Sci. U.S.A. 711:2534–2538.
2. **Clewell, D. B.** 1972. Nature of Col E_1 plasmid replication in *Escherichia coli* in the presence of chloramphenicol. J. Bacteriol. 110:667–673.
3. **Clewell, D. B., and D. R. Helinski.** 1969. Supercoiled circular DNA-protein complex in *Escherichia coli*: purification and induced conversion to an open circular DNA form. Proc. Natl. Acad. Sci. U.S.A. 62:1159–1166.
4. **Clewell, D. B., and D. R. Helinski.** 1970. Properties of a supercoiled deoxyribonucleic acid-protein relaxation complex and strand specificity of the relaxation event. Biochemistry 9:4428–4440.
5. **Clewell, D. B., and D. R. Helinski.** 1972. Effect of growth conditions on the formation of the relaxation complex of supercoiled ColE1 deoxyribonucleic acid and protein in *Escherichia coli*. J. Bacteriol. 110:1135–1146.
6. **Cohen, S. N., A. C. Y. Chang, H. B. Boyer, and R. B. Helling.** 1973. Construction of biologically functional plasmids *in vitro*. Proc. Natl. Acad. Sci. U.S.A. 70:3240–3244.
7. **Cohen, S. N., A. C. Y. Chang, and Y. Hsu.** 1972. Nonchromosomal antibiotic resistance in bacteria: genetic transformation of *Escherichia coli* by R-factor DNA. Proc. Natl. Acad. Sci. U.S.A. 69:2110–2114.
8. **Fiandt, M., W. Szybalski, and M. H. Malamy.** 1972. Polar mutations in lac, gal and phage λ consist of a few DNA sequences inserted with either orientation. Mol. Gen. Genet. 119:223–231.
9. **Hedges, R. W., and A. E. Jacob.** 1974. Transposition of

ampicillin resistance from RP4 to other replicons. Mol. Gen. Genet. 132:31-40.
10. **Hefron, F., C. Rubens, and S. Falkow.** 1975. The translocation of a plasmid DNA sequence which mediated ampicillin resistance: molecular nature and specificity of insertion. Proc. Natl. Acad. Sci. U.S.A. 72:3623-3627.
11. **Hefron, F., R. Sublett, R. W. Hedges, A. E. Jacob, and S. Falkow.** 1975. Origin of the TEM beta-lactamase gene found on plasmids. J. Bacteriol. 122:250-256.
12. **Hershfield, V., H. B. Boyer, L. Chow, and D. R. Helinski.** 1976. Characterization of a mini-ColE1 plasmid. J. Bacteriol. 126:447-453.
13. **Inselburg, J.** 1973. Colicin factor DNA: a single nonhomologous region in Col E2-E3 heteroduplex molecules. Nature (London) New Biol. 241:234-237.
14. **Inselburg, J.** 1974. Incompatibility exhibited by colicin plasmids E1, E2, and E3 in *Escherichia coli*. J. Bacteriol. 119:478-483.
15. **Inselburg, J.** 1974. Isolation and characterization of mutants of colicin plasmids E1 and E2 after Mu bacteriophage infection. J. Bacteriol. 119:469-477.
16. **Inselburg, J.** 1974. Replication of colicin E1 plasmid DNA in minicells from a unique replication initiation site. Proc. Natl. Acad. Sci. U.S.A. 71:2256-2269.
17. **Inselburg, J., and V. Johns.** 1975. Mapping of colicin E2 and colicin E3 plasmid dioxyribonucleic acid *Eco*R1-sensitive sites. J. Bacteriol. 121:381-389.
18. **Inselburg, J., and A. Oka.** 1975. Discontinuous replication of colistin E1 plasmid deoxyribonucleic acid. J. Bacteriol. 123:739-742.
19. **Jakes, K., N. D. Zinder, and T. Boon.** 1974. Purification and properties of colicin E3 immunity protein. J. Biol. Chem. 249:438-444.
20. **Jordon, E., H. Saedler, and P. Starlinger.** 1968. 0° and strong-polar mutations in the gal operon are insertions. Mol. Gen. Genet. 102:353-364.
21. **Lovett, M. A., L. Katz, and D. R. Helinski.** 1974. Unidirectional replication of plasmid Col E1 DNA. Nature (London) 251:337-340.
22. **Saedler, H. H., J. Reif, S. Hu, and N. Davidson.** 1974. A genetic element for turn-off and turn-on of gene activity in *E. coli*. Mol. Gen. Genet. 132:265-289.
23. **Shapiro, J. A.** 1969. Mutations caused by the insertion of genetic material into the galactose operon of *Escherichia coli*. J. Mol. Biol. 40:93-105.
24. **Sidikaro, J., and M. Nomura.** 1974. E3 immunity substance: a protein from E3-colicinogenic cells that accounts for their immunity to colicin E3. J. Biol. Chem. 249:445-453.
25. **So, M., R. Gill, and S. Falkow.** 1975. The generation of a Col E1-Ap^r cloning vehicle which allows detection of inserted DNA. Mol. Gen. Genet. 142:239-249.
26. **Sugino, Y., J. Tomizawa, and T. Kakefuda.** 1975. Location of non-DNA components of closed circular colicin E1 plasmid DNA. Nature (London) 253:652-654.
27. **Tomizawa, J., Y. Sakakibara, and T. Kakefuda.** 1974. Replication of colicin E1 plasmid DNA in cell extracts. Origin and direction of replication. Proc. Natl. Acad. Sci. U.S.A. 71:2260-2264.