

Polypeptides Expressed in *Escherichia coli* K-12 Minicells by Transposition Elements Tn1 and Tn3

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Escherichia coli K-12 minicells were employed to examine polypeptides encoded by plasmids carrying wild-type and mutant Tn1 or Tn3 transposition elements. Tn1- and Tn3-containing minicells express high levels of four transposon-specified polypeptides. Three, of molecular weights 30,000, 28,000, and 25,000, are related immunologically to β -lactamase, the enzyme responsible for ampicillin hydrolysis. A fourth polypeptide of molecular weight 19,000 is encoded by the Tn1 or Tn3 region which spans the *Bam*HI cleavage site. Mutant transposons which no longer produce this polypeptide transpose at higher than wild-type frequencies to give aberrant transposition products (Gill et al., *J. Bacteriol.* **136**: 742-756, 1978; Heffron et al., *Proc. Natl. Acad. Sci. U.S.A.* **72**:3632-3627, 1975). No expression could be detected from a region of the transposons extending from the inverted repeat sequence distal to the β -lactamase gene to more than half the distance into the Tn1 or Tn3 sequence.

A number of plasmid-mediated genetic determinants, including some antibiotic and heavy-metal resistance genes, reside on discrete DNA sequences capable of genetic transposition in bacteria (3, 4, 12, 14, 19). Recent reports have provided information about the structure and transposition properties of these DNA sequences, now known as transposable genetic elements or transposons (7, 16, 18), but little insight has been gained into the mechanisms controlling and executing the transposition events themselves. Two transposons, Tn1 and Tn3 (known generally as TnA), possess about 85% base sequence homology (24) and mediate ampicillin resistance (14, 16). Both are 4.8 kilobases in size and flanked by small, inverted, repeated sequences which appear to be about 140 base pairs in length in an electron microscope (16). Normally, transposition of Tn1 or Tn3 into a recipient plasmid replicon in the same cell occurs at a frequency between 10^{-3} and 10^{-5} per cell, depending on growth conditions (20).

Heffron et al. isolated a series of deletion mutants of the pMB8::Tn3 plasmid RSF1050 which exhibited greatly impaired frequencies of transposition of the Tn3 element (15). Complementation analysis with these deletion mutants and an ampicillin-sensitive (Ap^r) Tn1 derivative

located on RSF103 (24) indicated the deletion mutants could be divided into three types. Type I mutants were complemented in *trans* by RSF103 functions and transposed at a frequency about 25% that of wild-type Tn3. These were deletions that covered internal regions of Tn3 extending from the left-hand inverted repeat sequence to just over 50% of the way into the sequence (see Fig. 1). Type II deletions were also complemented and were unique in that they transposed at a frequency higher than that of wild-type Tn3. The transposed sequence included the pMB8 portion of the RSF1050 genome (11). All type II deletions extended over unique regions of the Tn3 sequence adjacent to the *Bam*HI site. Type III mutants, which had all lost the inverted repetition distal to the β -lactamase gene, had irreversibly lost the ability to transpose.

Heffron et al. postulated that Tn3 encodes gene products essential for transposition. We previously reported that *Escherichia coli* minicells containing the Tn1 sequence produced large amounts of three polypeptides, at least one of which is β -lactamase, the enzyme responsible for ampicillin resistance (9, 10). In this work, we used isolated minicells containing representative Tn1 and Tn3 deletion plasmids to examine polypeptide expression from defined regions of the Tn1 and Tn3 sequences. Our results suggest that β -lactamase may exist in the cell as three poly-

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peptides. Further, we identified a polypeptide, specified by Tn1 and Tn3 and distinct from β -lactamase, which is encoded in a region of Tn3 close to the β -lactamase gene. Type II deletion plasmids and a Tn3 plasmid with a lesion at the BamHI site failed to express detectable levels of this polypeptide in minicells.

MATERIALS AND METHODS

Bacterial strains and plasmids. All experiments were performed with derivatives of the minicell-producing *E. coli* K-12 substrain DS410 (9). Plasmids were introduced into DS410 by transformation (7), using purified plasmid DNA. A spectinomycin-resistant, streptomycin-sensitive (Sp^r Sm^s) derivative of strain DS410, strain DS411, constructed by P1 transduction from DS446 (9), was used to facilitate selection of certain plasmids. Plasmid pREG118 was constructed by using T4 DNA polymerase to fill in the single-stranded ends of linear RSF1050 DNA generated by BamHI restriction endonuclease, followed by rejoining the flush ends using T4 ligase. Other plasmids are listed in Table 1 or in Fig. 1. Gene symbols are as described by Bachmann et al. (2). Plasmid nomenclature is according to Novick et al. (23). All strains were maintained on nutrient agar (Difco Laboratories) plates.

Isolation and labeling of minicells. Minicells were isolated as previously described (13). The minicells were suspended in 1 ml of labeling medium (9) and incubated at 37°C for 15 min with aeration. [³⁵S]methionine (5 μ Ci; >1,000 Ci/mmol; Amersham Corp.) was added for protein labeling, and incubation continued for 30 min. The minicells were then chilled, pelleted, and suspended in 100 μ l of sample buffer (0.0625 M Tris [pH 6.8], 3% sodium dodecyl sulfate, 5% mercaptoethanol, 0.1% bromophenol blue, 10%

glycerol), heated to 100°C for 2 min, and frozen at -20°C until needed.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. Polyacrylamide gels were run with the discontinuous buffer system of Laemmli (21) and the apparatus described by Studier (25). Gels consisted of 4.5% stacking gels

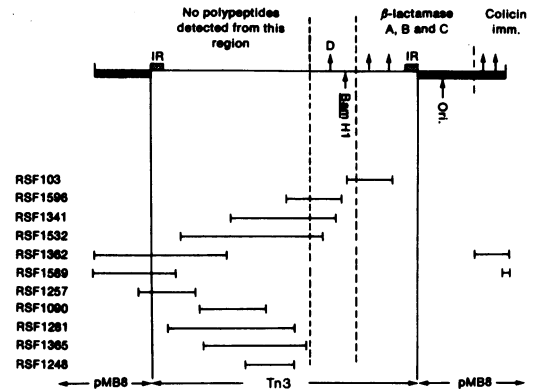


FIG. 1. Position of deletions in RSF1050 Tn3 transposition-deficient deletion mutants. RSF1050 is arbitrarily represented as a linear form cleaved at the single EcoRI restriction endonuclease cleavage site. pMB8 is represented as the thicker line and the Tn3 sequences are represented as the thinner line between the two inverted repeat sequences (IR). Ori, Origin of replication; colicin imm., colicin immunity; \pm , position and extent of deletion in the respective mutants (listed on the left; see Table 1 for nomenclature). The solid vertical lines delineate the ends of Tn3, and the broken lines separate the map into functional regions. The deletion in RSF103 is in the Tn1 element which resides on RSF1010.

TABLE 1. Sources and derivations of plasmids used^a

Plasmid	Other names	Derivation	Phenotype	Source
RSF1050		pMB8::Tn3	Ap ^r Iel ⁺	S. Falkow (15)
RSF1362	Δ 362	RSF1050 deletion	Ap ^r Iel ⁻	S. Falkow (15)
RSF1589	Δ 589	RSF1050 deletion	Ap ^r Iel ⁺	S. Falkow (15)
RSF1257	Δ 257	RSF1050 deletion	Ap ^r Iel ⁺	S. Falkow (15)
RSF1090	Δ 90	RSF1050 deletion	Ap ^r Iel ⁺	S. Falkow (15)
RSF1281	Δ 281	RSF1050 deletion	Ap ^r Iel ⁺	S. Falkow (15)
RSF1365	Δ 365	RSF1050 deletion	Ap ^r Iel ⁺	S. Falkow (15)
RSF1248	Δ 248	RSF1050 deletion	Ap ^r Iel ⁺	S. Falkow (15)
RSF1596	Δ 596	RSF1050 deletion	Ap ^r Iel ⁺	S. Falkow (15)
RSF1341	Δ 341	RSF1050 deletion	Ap ^r Iel ⁺	S. Falkow (15)
RSF1532	Δ 532	RSF1050 deletion	Ap ^r Iel ⁺	S. Falkow (15)
RSF1010		Naturally occurring	Sm ^r Su ^r	S. Falkow (16)
RSF1010::Tn3			Ap ^r	S. Falkow (24)
RSF103	Δ Ap	RSF1010::Tn1	Sm ^r	S. Falkow (24)
pREG100	RSF1010::Tn3 (Ap ^r)	Possible point mutant in β -lactamase gene of Tn3 carried on RSF1010	Sm ^r Su ^r Ap ^s	R. Gill (11)
pSC101			Tc ^r	R. Gill (19)
pREG126	pSC101::Tn3		Tc ^r Ap ^r	R. Gill (unpublished)
pREG118		See Materials and Methods	Ap ^r Iel ⁺	R. Gill (unpublished)

^a Abbreviations: Tc, tetracycline; Sm, streptomycin; Su, sulfonamide; s, sensitive; r, resistant.

and either 12.5% or 18% separating gels (7.5 cm long). Minicell samples (10 to 20 μ l) were subjected to electrophoresis at 20 mA under constant current until the bromophenol blue dye front reached the end of the gel. Proteins were precipitated with 100% (wt/vol) trichloroacetic acid and stained with Coomassie brilliant blue R250 (Sigma Chemical Co.). Destaining was in 7.5% (wt/vol) trichloroacetic acid. Destained gels were dried and autoradiographed as previously described (26). Molecular weight standards were phosphorylase B (100,000), catalase (58,500), alcohol dehydrogenase (41,000), DNase (31,000) chymotrypsinogen (25,000), and RNase A (13,600).

Precipitation of antibody-antigen complexes with *Staphylococcus aureus* protein A. Protein which reacts with specific antibody was identified by a modification of the Kessler procedure (17), which is based on the observation that *Staphylococcus aureus* (Cowan strain 1) contains protein A in its cell wall and therefore binds strongly to the Fc portion of immunoglobulin G antibodies. Minicells containing either RSF1010::Tn3 or RSF1050 were isolated from 600 ml of culture and labeled as described above. They were then suspended in 1 ml of phosphate buffer (pH 7.5) containing 0.15 M NaCl, 1 mM EDTA, and Triton X-100 and broken by sonic oscillation (six 30-s periods with chilling). The unbroken minicells and minicell debris were removed by centrifugation at $40,000 \times g$ for 30 min. The supernatant was split into two equal volumes. (The pellet was suspended in 100 μ l of sample buffer, boiled for 2 min, and frozen at -20°C until required.) Rabbit antibody raised against purified TEM β -lactamase (20 μ l) (a generous gift of R. B. Sykes, Squibb Institute) was added to one-half of the supernatant. Thirty minutes later, 200 μ l of fixed *S. aureus* cells, prepared as described by Kessler (17), was added to both halves of the supernatants. After a further 45 min, the *Staphylococcus* cells were removed by sedimenting twice at $17,000 \times g$ for 10 min each through 2 ml of 1 M sucrose. They were then suspended in 200 μ l of sample buffer, boiled for 2 min, and frozen at -20°C until required.

RESULTS

Polypeptides expressed in plasmid-free minicells. Plasmid-free minicells possess the capacity to synthesize various levels of a number of polypeptides. The polypeptides are believed to be translated from stable mRNA molecules which are chromosomally encoded and segregate into minicells during cell division (22). The three major polypeptides have molecular weights of 37,000, 34,000, and 22,000. The levels of synthesis of these polypeptides were dependent on the age of the cultures from which the minicells are isolated. Figure 2, track F shows the polypeptides produced in plasmid-free minicells isolated from 12-h-old cultures when cells were just entering stationary phase. The chromosomally encoded polypeptides can be clearly seen. Track C shows the polypeptides produced by minicells isolated after a further 5 h of culture growth.

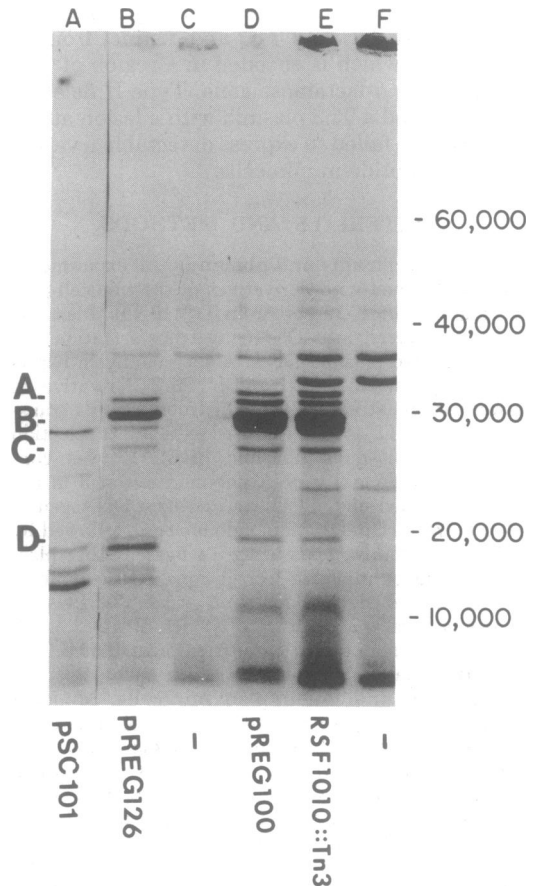


FIG. 2. Autoradiogram of extracts from [^{35}S]methionine-labeled minicells. Minicell samples shown in tracks A through C were isolated from cultures grown 5 h longer than the cultures from which minicells in tracks E and F were isolated. Electrophoresis was on 12.5% gels, and the autoradiogram was exposed for 5 days. Tracks C and F show plasmid-free DS410 and DS411, respectively. pREG100 is a RSF1010::Tn3 derivative which is Ap^r due to a putative point mutation in the β -lactamase gene. pREG126 is a pSC101::Tn3 plasmid. Molecular weights are indicated on the right. Putative Tn1/Tn3 polypeptides A, B, C, and D are indicated on the left.

The levels of synthesis of these chromosomal polypeptides were reduced. To fully interpret the autoradiographs presented in this communication, the reader should be aware of the positions of the chromosome-encoded polypeptides.

Polypeptides expressed from the Tn1 and Tn3 elements in minicells. Heffron et al. isolated transposition-deficient Tn3 deletion mutants from plasmid RSF1050. RSF1050 contains a complete Tn3 sequence inserted into plasmid

pMB8, a derivative of plasmid ColE1 which encodes colicin E1 immunity but not colicin E1 production (5, 8). Figure 3, track H, shows the pattern of ^{35}S -labeled polypeptides produced by minicells harboring the parental RSF1050 plasmid. Four polypeptides, synthesized in relatively large amounts, were designated A (30,000 daltons), B (28,000 daltons), C (25,000 daltons), and D (19,000 daltons). Polypeptides A, B, and C comigrated with three Tn1-specified polypeptides we have previously described (9, 10). Polypeptide B comigrated with a polypeptide which possessed β -lactamase activity (9). Evidence has accumulated indicating that polypeptides A, B, and C are forms of the β -lactamase gene product. These three polypeptides have formerly been identified in minicells containing a variety of plasmids carrying all or part of the Tn1 or Tn3 transposons: ColE1::Tn1, ColE2::Tn1 (9), CloDF13::Tn1 (10), pSC101::Tn3 (Fig. 2, track B), RSF1010::Tn3 (Fig. 2, track E), and pBR322 (5) (not shown). However, they were not detected when the β -lactamase gene was inactivated as, for example, with the RSF103 Ap^r deletion mutant (Fig. 3, track B). The RSF1010 polypeptide that runs in a similar position to

polypeptide B is probably the phosphotransferase that specifies streptomycin resistance.

We decided to use immunological tests to determine whether polypeptides A, B, and C were all related to β -lactamase. Kessler developed a method which employs *S. aureus* protein A to purify antigen-antibody complexes (17). We modified this procedure (see Materials and Methods) to determine which polypeptides, expressed in minicells containing RSF1050 or RSF1010::Tn3, would cross-react with purified anti-TEM β -lactamase antibody. Figure 4, track D, shows the polypeptides expressed in the RSF1050-containing minicells used in this experiment. A higher-than-normal level of whole-cell contamination was allowed so that we could observe whether any chromosome-encoded polypeptides were precipitated by the antibody. Tracks B and C show the labeled polypeptides which are precipitated by the *Staphylococcus* cells from the RSF1050 (pMB8::Tn3) and RSF1010::Tn3 supernatants, respectively. Polypeptides A and B were clearly precipitated. Polypeptide C could be seen more clearly after a longer exposure of the autoradiograph. None of the other polypeptides was precipitated to a

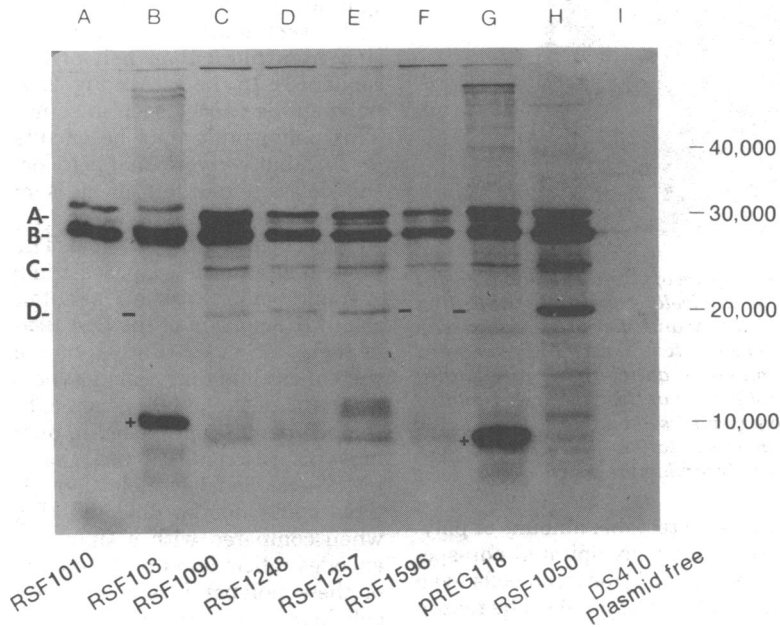


FIG. 3. Autoradiogram of extracts from [^{35}S]methionine-labeled minicells containing RSF1050, RSF1010, and a selection of Tn3 and Tn1 deletion mutants. Electrophoresis was on a sodium dodecyl sulfate-polyacrylamide gel, with 4.5% stacking and 18% separating gel acrylamide concentrations. The autoradiogram was exposed for 7 days. Molecular weights are indicated on the right, and polypeptides A through D are indicated on the left. -, Absence of band; +, appearance of new band. Track I shows labeled polypeptides from plasmid-free minicells. The two RSF1010 polypeptides of molecular weights 28,500 and 31,000 comigrated on the gels in positions similar to those of polypeptides A and B but could be distinguished especially after short exposure of the autoradiogram.

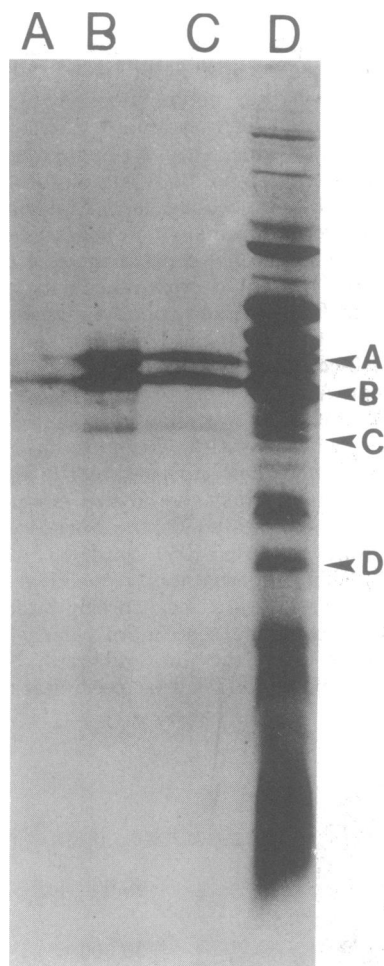


FIG. 4. Autoradiogram of [^{35}S]methionine-labeled polypeptides from minicells containing RSF1010::Tn3 and RSF1050 before and after precipitation with *Staphylococcus* cells (see text). Track A, Precipitated RSF1050 polypeptides - antibody; B, precipitated RSF1050 polypeptides - antibody; C, precipitated RSF1010::Tn3 polypeptides - antibody; D, RSF1050 minicells used in precipitation. 4.5% stacking and 12.5% separating polyacrylamide gel.

significant degree. A detectable amount of polypeptide B was sometimes precipitated non-specifically by the fixed *Staphylococcus* cells even in the absence of antibody (track A). The reason for this is unclear, although the amount of precipitation was not increased when antibody raised against an unrelated antigen (LT enterotoxin) was present.

Polypeptide D is the only other Tn1 and Tn3 gene product we can reproducibly detect in minicells. It was detected in minicells containing RSF1050 (Fig. 3, track H), pSC101::Tn3 (Fig. 2,

track B), and RSF1010::Tn3 (Fig. 2, track E). We used the transposition-deficient Tn3 deletion mutants listed in Fig. 1 to examine expression in minicells from defined regions in Tn1 and Tn3. All the Tn3 mutants expressed near-normal levels of polypeptides A, B, and C (Fig. 2, tracks C to F). As they all encode for β -lactamase, this is in agreement with the above-mentioned observations. Type I and type III mutants synthesized polypeptide D, but none could be detected in minicells containing any of the type II mutants (Fig. 3, track F). The deletions in the type II mutants extend into a unique region of Tn3 adjacent to the *Bam*HI site. Minicells harboring either of two other plasmids with lesions at or near the *Bam*HI site also failed to produce detectable amounts of polypeptide D. These plasmids, pREG118 and RSF103 (Fig. 3, tracks G and B), expressed large amounts of two unique low-molecular-weight polypeptides. The origin of these polypeptides is unknown, but it is possible they are truncated forms of polypeptide D. If this is so, the high levels of the truncated polypeptide would indicate that D is under autogenous control and represses its own synthesis. Taken together, the data indicate that polypeptide D can only be encoded in a cistron spanning the *Bam*HI site.

We were unable to detect polypeptide expression from more than half of the Tn3 or Tn1 sequences. In the gels in Fig. 2, a 29,000-dalton polypeptide can be seen in some of the tracks. This polypeptide may be encoded somewhere on Tn3, but we were unable to locate it by using the deletion mutants as it is made in small amounts and is often obscured by polypeptides A and B. Much of Tn3 and Tn1 must be expressed at a low level, at least in minicells.

A number of the deletion mutants extend into the pMB8 portion of the RSF1050 genome. One of these, RSF1362 (Fig. 1), no longer expresses the colicin immunity phenotype which is characteristic of the pMB8 genome. Examination of the polypeptides produced in minicells containing this plasmid showed that two putative pMB8-encoded low-molecular-weight polypeptides could not be detected (Fig. 5, track B) when compared with a similar deletion which encodes colicin immunity (track A). One or both of these polypeptides could play a role in the colicin immunity function.

DISCUSSION

Minicells proved to be a useful tool for analyzing the expression of genes present in Tn1 and Tn3. Large amounts of four polypeptides were detected in minicells harboring a variety of plasmids carrying the Tn1 and Tn3 sequences.

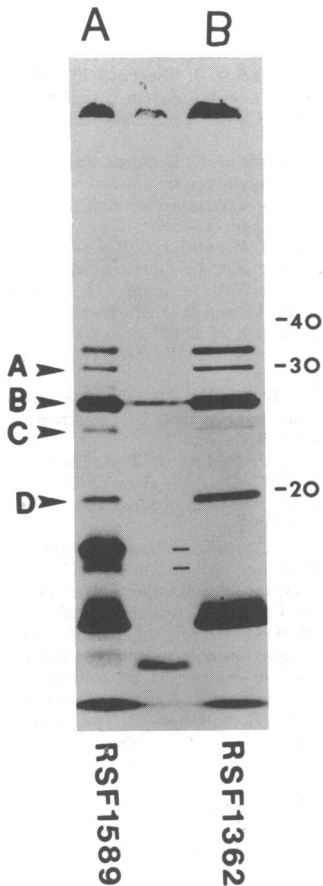


FIG. 5. Autoradiogram of [^{35}S]methionine-labeled minicells. Electrophoresis was on 12.5% gels, and exposure of the autoradiography was for 28 days. Short dashes indicate missing polypeptides. Molecular weights ($\times 10^3$) are indicated on the right. The track between A and B is a ^{14}C -labeled preparation of RSF1362 minicells.

Three of the polypeptides, A (30,000 daltons), B (28,000 daltons), and C (25,000 daltons), are derived from β -lactamase. Polypeptide B was previously shown to possess β -lactamase activities. RSF103, a plasmid that contains a deletion within Tn1 which extends into the β -lactamase gene (Fig. 1), failed to express any of these three polypeptides in minicells (Fig. 3, track B). Interestingly, pBR322, a plasmid which contains the β -lactamase gene but has little else of the Tn3 sequence (5), still expressed these polypeptides in minicells (data not shown). Taken together, the results suggest the polypeptides are three forms of β -lactamase. The immunological data obtained by using anti-TEM β -lactamase antibody support this view. Two recent reports describing the sequencing of the β -lactamase gene

of Tn3 (1, 26) and the partial sequencing of the amino-terminal end of the TEM β -lactamase polypeptide indicate that β -lactamase is translated in a precursor form containing 23 additional amino acids at the amino-terminal end. The additional amino acids, many of which are hydrophobic, are believed to play a role in transportation of the β -lactamase across the cell membrane. Polypeptide A is likely to be the precursor form, polypeptide B is likely to be the active form, and polypeptide C is likely to be a breakdown form of β -lactamase. Tn3-specified β -lactamase synthesized *in vitro* has the same molecular weight as does polypeptide A (M. Achtman, personal communication).

Polypeptide D is a Tn1- and Tn3-specified gene product which is distinct from β -lactamase. It is expressed in minicells when Tn3 or Tn1 is inserted in a variety of plasmids. However, minicells containing mutant Tn1 or Tn3 elements with lesions at or adjacent to the *Bam*HI site fail to synthesize detectable amounts of the polypeptide. These mutants include all type II Tn3 deletion mutants, pREG118, and RSF103. All of these mutant transposition elements, including the Tn1 element on RSF103 (A. Arthur and D. Sherratt, unpublished data), transposed at a frequency higher than of the wild type either independently or during complementation tests. Thus, there is a correlation between the high frequency of transposition and the inability to express detectable levels of polypeptides D in minicells. Heffron et al. originally employed plasmid RSF103 in complementation tests with the transposition-deficient Tn3 deletion mutants. The Tn1 sequence on RSF103, however, contains a substantial deletion that extends out of the β -lactamase gene (Fig. 1). It is not surprising then that no polypeptide D could be detected in RSF103 harboring minicells. Gill et al. (11) modified the complementation analysis developed by Heffron et al. (15) by replacing RSF103 with an RFS1010::Tn3(Ap^r) plasmid, pREG100, which probably has a point mutation in the β -lactamase gene. Under these conditions type II Tn3 mutants transposed at the same frequency as that of the type I mutants, but the colicin immunity gene was still transposed to the recipient replicon. pREG100 expresses normal levels of polypeptide D (Fig. 2, track D). This is further evidence that polypeptide D could play a direct or indirect role in regulating the transposition frequency of Tn1 and Tn3, as well as being necessary for a normal transposition product. Recently, Heffron et al. (F. Heffron, M. So, and M. McCarthy, *in press*) used an *in vitro* mutagenesis method to introduce defined mutations into the region close to the *Bam*HI site.

They found mutations that resulted in a high frequency of transposition in a region large enough to encode for a polypeptide of molecular weight 25,000. Polypeptide D seems to be encoded in this region.

Minicells enable us to detect polypeptide expression from one end of the Tn3 and Tn1 sequence. We were unable to detect any expression from the region distal to the β -lactamase gene. Heffron et al. showed that this region is essential for transposition and encodes diffusible gene products involved in the transposition mechanism. The fact that we were unable to detect expression suggests that this region may only be expressed at a very low level, perhaps only during the transposition event itself. The region contains enough DNA to encode for over 100,000 daltons of protein from nonoverlapping genes. Obviously, a new approach is required to characterize the polypeptides encoded within this region.

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