

## Identification of a Second Tetracycline-Inducible Polypeptide Encoded by Tn10

THOMAS J. ZUPANCIC, STEVEN R. KING, KAY L. POGUE-GEILE, AND S. RICHARD JASKUNAS\*

*Department of Chemistry and the Program in Molecular, Cellular and Developmental Biology, Indiana University, Bloomington, Indiana 47405*

Three Tn10 polypeptides were detected by analyzing the proteins synthesized in ultraviolet light-irradiated *Escherichia coli* cells after infection with  $\lambda$ ::Tn10. One of these polypeptides was the previously identified 36,000-dalton TET polypeptide. The other two had approximate sizes of 25,000 and 13,000 daltons. The syntheses of both the TET polypeptide and the 25,000-dalton polypeptide were inducible by tetracycline in  $\lambda$ -immune hosts. Similarly, the synthesis of the TET polypeptide was inducible in nonimmune hosts. However, the synthesis of the 25,000-dalton polypeptide was constitutive in nonimmune hosts. An amber mutation in a gene required for tetracycline resistance on  $\lambda$ ::Tn10 was isolated that eliminated the synthesis of the TET polypeptide in *sup*<sup>+</sup> hosts but not the synthesis of the 25,000-dalton or the 13,000-dalton polypeptides. The expression of tetracycline resistance from wild-type Tn10 was found to be anomalous in *E. coli* strains carrying the amber suppressors *supD*, *supE*, and *supF*. In general, strains containing these nonsense suppressors were less resistant to tetracycline.

Tn10 is a transposable sequence that encodes resistance to tetracycline on several bacterial resistance factors including R100 (19, 30). It contains 9,300 base pairs including 1,400 base pair inverted repeats at the ends (19).

The mechanism of tetracycline resistance (Tet<sup>r</sup>) appears to involve an inhibition in the uptake of tetracycline (1, 5, 6). In addition, there may be an intracellular system that contributes to the resistance (24, 33). The expression of Tet<sup>r</sup> in several bacterial species is inducible by low levels of tetracycline (6, 11, 23, 32). For the Tet<sup>r</sup> encoded by R100 and Tn10, the induction is coincident with an increase in the synthesis of a 36,000-dalton polypeptide that has been called the TET polypeptide (16, 23, 31, 33). This polypeptide has been found in the inner membrane of *Escherichia coli* (23, 31, 33), suggesting that it is responsible for the decreased uptake of tetracycline. The gene for the TET polypeptide has been mapped on Tn10 by Jorgensen et al. (16). The synthesis of the TET polypeptide appears to be controlled by a repressor system encoded by Tn10 that can be inactivated by tetracycline (16, 33).

### MATERIALS AND METHODS

**Phage and bacterial strains.** All bacterial strains were derivatives of *E. coli* K-12. Strain 159 (from M. Pearson and C. Epp) is F<sup>-</sup>*uurA rpsL sup*<sup>+</sup>. Strains 159 *supD*, 159 *supE*, and 159 *supF* (from M. Pearson and C. Epp) are isogenic with strain 159. CSH25 is F<sup>-</sup>*supF*.

$\lambda$ ::Tn10(1) and  $\lambda$ ::Tn10(2), which were gifts from D. Berg, had the genotype  $\lambda$  b515 b519 *xis6 c1857 nin5*

*s7*::Tn10 ( $\lambda$ ::Tn10) (15). The parent phage shall be called  $\lambda$  b b *nin*. The Tn10 in these phages was inserted in different sites in the b2 region of  $\lambda$  b b *nin*, but with the same orientation.  $\lambda$ <sup>+</sup> is wild-type lambda phage.  $\lambda$  *rif*<sup>+</sup>18 carries bacterial DNA from the *rif* region of the *E. coli* chromosome (18).

The  $\lambda$ ::Tn10(2)*tet575* amber mutant was isolated after hydroxylamine mutagenesis of  $\lambda$ ::Tn10(2) as follows (10). Approximately 10<sup>12</sup>  $\lambda$ ::Tn10(2) phages were incubated for 20 h at 37°C in 5 ml of 0.4 M hydroxylamine-Cl, 0.05 M NaPO<sub>4</sub>, 0.5 mM EDTA, and 2 mM MgSO<sub>4</sub> (pH 6.0), and titrated with CSH25 on H-plates with H top agar (25). The fraction of hydroxylamine-treated  $\lambda$ ::Tn10(2) that was capable of plaque formation compared to a sample incubated in the absence of hydroxylamine was 5 × 10<sup>-4</sup>. Individual plaques were spotted on a lawn containing a 1:1 mixture of CSH25 (*supF* Str<sup>r</sup>) and 159 (*sup*<sup>+</sup> Str<sup>r</sup>) in H top agar on an H plate (25) and incubated overnight at 30°C. This master plate was replicated onto LB plus 25  $\mu$ g of tetracycline per ml (LB-tet) plates (25) and LB plus 25  $\mu$ g of tetracycline per ml plus 200  $\mu$ g of streptomycin per ml (LB-tet-str) plates, which were incubated overnight at 30°C.  $\lambda$ ::Tn10(2) lysogens of both strains grew on LB-tet plates, but only lysogens of 159 could grow on LB-tet-str plates. Thus, we looked for lysogens that grew on LB-tet but not LB-tet-str, which would be *supF* suppressible Tet<sup>r</sup> transductants of CSH25. From 1,400 plaques we found one  $\lambda$ ::Tn10(2) phage with an amber mutation in a *tet* gene,  $\lambda$ ::Tn10(2)*tet575*. The mutant phage was recovered from the CSH25 lysogen that grew on the LB-tet replica of the master.

**Infection of UV light-irradiated *E. coli*.** Infection of UV light-irradiated strain 159 and various derivatives was performed as described (13, 14), except that the bacteria were irradiated for 4 min rather than 12 min. Briefly, the procedure was as follows. Bacteria

were grown to a cell density of  $2 \times 10^8$  cells/ml at 30°C in AB medium plus 0.4% maltose plus 1 µg of thiamin per ml (3), concentrated fivefold in the same medium, irradiated, infected with λ at a multiplicity of 5 to 10 in the presence of 0.02 M MgSO<sub>4</sub> (0.2 ml of bacteria per sample), incubated with shaking for 15 min at 30°C, and diluted fivefold with prewarmed medium. For the tetracycline-induced samples with Tet<sup>r</sup> bacteria, the bacteria were grown and infected in the presence of 10 µg of tetracycline per ml. For the tetracycline-induced samples with Tet<sup>s</sup> bacteria, 1 µg of tetracycline per ml was added 15 min after infection. Polypeptides were labeled with [<sup>3</sup>H]leucine between 20 and 65 min after infection (20 µCi/ml). The labeled cells were collected by centrifugation, washed once with 1 ml of 0.05 M Tris-Cl (pH 8), and solubilized by boiling for 3 min in 50 µl of sodium dodecyl sulfate (SDS) sample buffer (22).

**Polyacrylamide gel electrophoresis of labeled polypeptides.** Electrophoresis of labeled polypeptides on one-dimensional SDS-15% polyacrylamide gels was performed on slabs (27 mm by 13 mm by 1.5 mm) at 200 V for 16 h at 20°C as described (22). About one-third of the sample was applied per lane. Initially we had considerable difficulty detecting the 36,000-dalton TET polypeptide encoded by Tn10 (16, 23, 31, 33). It aggregated readily and remained at the top of the gel or electrophoresed as a smear. However, this was less of a problem if the labeled cells were boiled in SDS sample buffer and electrophoresed immediately without freezing.

Two-dimensional NEPHGE were performed essentially as described by O'Farrell et al. (27). The washed cell pellet ( $2 \times 10^8$  cells) was frozen and thawed in 10 µl of 0.1-mg/ml lysozyme (0.1 M Tris-chloride, pH 8), incubated on ice for 15 min with 1 µl of 2-mg/ml RNase A plus 2-mg/ml of DNase I, and boiled for 2 min after the addition of 1 µl of 10% SDS and 2 µl of β-mercaptoethanol. A 10-mg amount of urea and 25 µl of SDS lysis buffer (9.5 M urea, 4% Nonidet P-40, 2% ampholines [pH 3 to 10, Bio-Rad], 5% β-mercaptoethanol) were added, and the entire sample was applied to a cylindrical 4% polyacrylamide gel (100-mm by 3.0-mm inner diameter) in 9 M urea-2% Nonidet P-40-2% ampholines (pH 3 to 10). The sample was overlaid and electrophoresed for 3 h at 400 V as described (27). The gels were equilibrated and run in the second dimension on a 12 to 20% linear gradient polyacrylamide-SDS gel as described by O'Farrell (26).

**In vivo rates of protein synthesis.** These experiments were performed essentially as described by Franklin and Cook (7). The culture was grown exponentially at 32°C to  $2 \times 10^8$  cells/ml, diluted 1 → 40 with LB (plus 0.5 µg of tetracycline per ml for induced samples), and shaken at 32°C for 60 min. Aliquots of 0.2 ml were transferred to test tubes containing 2 µCi of [<sup>3</sup>H]leucine and tetracycline and shaken at 32°C for 60 min. The protein was precipitated with trichloroacetic acid, collected on glass fiber filters, and counted in a scintillation counter. The relative rate of protein synthesis was calculated by subtracting the background (counts per minute retained on the filter for a sample without bacteria) and normalizing to the rate of protein synthesis for the sample without added tetracycline. These latter samples incorporated about

5,000 cpm. All samples were done in duplicate and averaged.

**Efficiency of plating.** Fresh overnight cultures of bacteria in LB ( $\pm 25$  µg of tetracycline per ml) were diluted and plated on LB and LB-tet plates containing 10, 25, 50, 100, and 150 µg of tetracycline per ml. Results were obtained from plates that contained at least 100 colonies after overnight incubation at 30°C.

## RESULTS

**Identification of three polypeptides synthesized under control of Tn10.** The polypeptides encoded by Tn10 were investigated by analyzing the polypeptides synthesized in UV light-irradiated *E. coli* cells after infection with λ *b b nin*::Tn10. Two λ *b b nin*::Tn10 phages were used, λ::Tn10(1) and λ::Tn10(2), in which Tn10 was inserted at different sites in the *b2* region of the λ genome (15). Several different bacterial hosts were used. Some were λ lysogens and immune to λ infection and others were nonimmune. In the immune cells, the expression of Tn10 genes presumably would come from Tn10 promoters. However, in nonimmune cells the Tn10 genes conceivably could be expressed from phage promoters. In addition, some cells contained an endogenous Tn10 so that the effect of tetracycline on the expression of the Tn10 genes could be examined. Polypeptides synthesized after phage infection were labeled with [<sup>3</sup>H]leucine and analyzed by polyacrylamide gel electrophoresis on one-dimensional SDS gels (22) or two-dimensional NEPHGE gels (27) followed by autoradiography.

Polypeptides synthesized after infection of 159 [λ::Tn10(1)] and analyzed on SDS gels are shown in Fig. 1 (lanes 1 to 9). After infection of this immune host with λ *b b nin*, two λ polypeptides, labeled b and d, could be detected (compare lanes 1 and 2), which were the products of the *rex* and *cI* genes, respectively (29). Two additional bands, labeled P36 and P25, could be observed after infection with λ::Tn10(1) or λ::Tn10(2) in the presence (lanes 7 and 8) but not in the absence (lanes 3 and 4) of tetracycline. Thus, the synthesis of P36 and P25 could be induced by adding tetracycline. The sizes of P36 and P25 were estimated to be 36,000 and 25,000 daltons, respectively, by comparison of their electrophoretic mobility with that of the known polypeptides synthesized after infection with λrif<sup>r</sup>18 (lane 14) (12).

Similar experiments were done with nonimmune host cells. The polypeptides synthesized after infection of *E. coli* 159 are shown in Fig. 1 (lanes 10 to 13). One band that was present after infection with λ::Tn10(1) and λ::Tn10(2) but not after infection with λ *b b nin* had the same mobility as P25. This band was one of the most

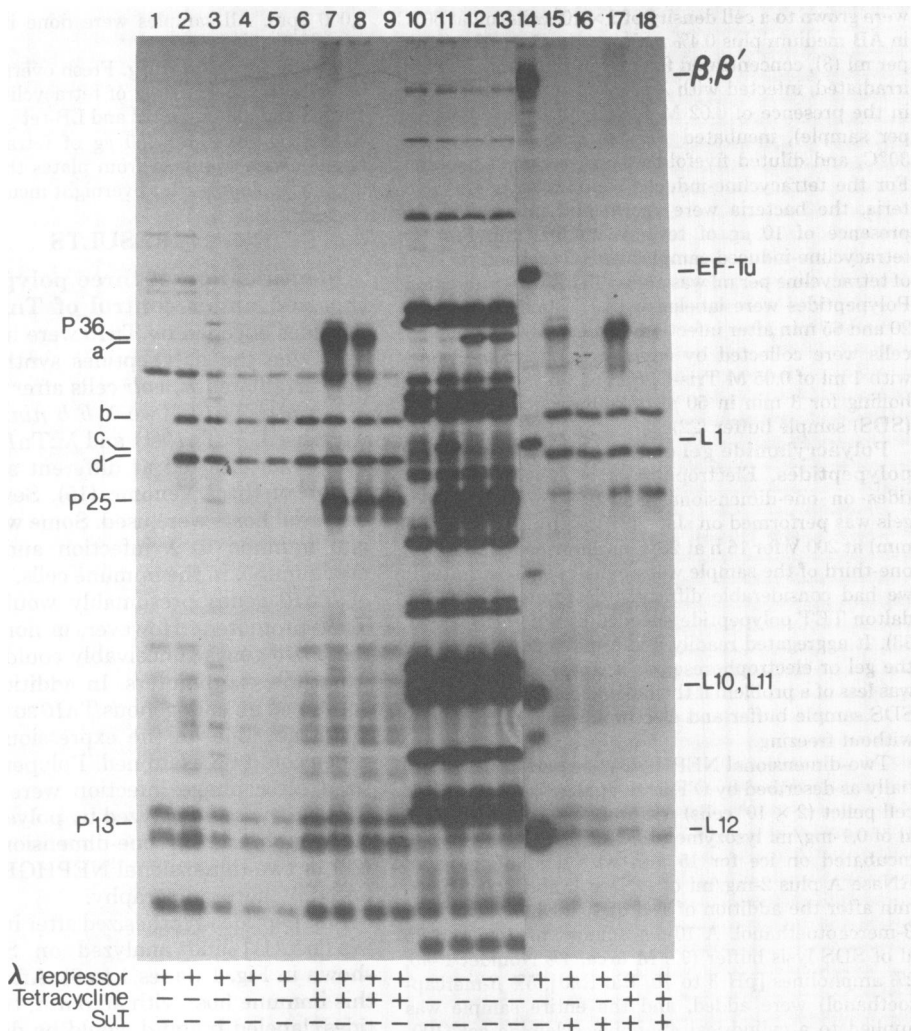


FIG. 1. Autoradiogram of 15% polyacrylamide-SDS gel of polypeptides synthesized after  $\lambda$ ::Tn10 infection of UV-irradiated *E. coli*. Bacterial hosts were strains 159 [ $\lambda$ ::Tn10(1)] (lanes 1 to 9, 14), 159 (lanes 10 to 13), and 159 supD [ $\lambda$ ::Tn10(1)] (lanes 15 to 18). Infecting phages were: no infection (lane 1),  $\lambda$  b b nin (lanes 2, 6, and 10),  $\lambda$ ::Tn10(1) (lanes 3, 7, and 11),  $\lambda$ ::Tn10(2) (lanes 4, 8, 12, 15, and 17),  $\lambda$ ::Tn10(2)tet575 (lanes 5, 9, 13, 16, and 18), and  $\lambda$  rif<sup>d</sup>18 (lane 14). Conditions for infection are indicated at the bottom. Symbol: +, infection was carried out in the presence of the  $\lambda$  repressor (immune cells), 10  $\mu$ g of tetracycline per ml, or supD (SuI). Positions of various polypeptides are indicated on the left (see text). Names on right identify polypeptides synthesized from  $\lambda$  rif<sup>d</sup>18 in lane 14 that were used as molecular weight standards (14).

intensely labeled bands when the polypeptides were labeled with [<sup>3</sup>H]leucine. However, it was only weakly labeled when [<sup>35</sup>S]methionine was used (not shown). There also appeared to be a Tn10-specific band labeled P13 that had a size of about 13,000 daltons. Surprisingly, there was no evidence for the synthesis of P36. There was a band in the  $\lambda$ ::Tn10(2) sample, labeled a, that had a slightly greater mobility than P36. However, this band was probably a truncated  $\lambda$  polypeptide since it was not in the  $\lambda$ ::Tn10(1) sample.

Similarly, there was a band in the  $\lambda$ ::Tn10(1) sample, labeled c, that also appeared to be a truncated  $\lambda$  polypeptide because it was not in either the  $\lambda$  b b nin or  $\lambda$ ::Tn10(2) samples. When these samples were analyzed on lower percent polyacrylamide-SDS gels, a 40,000-dalton polypeptide in the  $\lambda$  b b nin sample was missing in the  $\lambda$ ::Tn10(1) and  $\lambda$ ::Tn10(2) samples (not shown). This polypeptide was not resolved from the other 40,000-dalton  $\lambda$  polypeptide on the gel in Fig. 1. Polypeptides a and c presumably were

truncated derivatives of the missing band rather than Tn10-encoded polypeptides. Tn10 was inserted in slightly different positions in  $\lambda::$ Tn10(1) and  $\lambda::$ Tn10(2), which probably accounts for the two different-sized truncated  $\lambda$  polypeptides.

Since the synthesis of P36 was inducible in the immune cells, we investigated the possibility that its synthesis was also inducible in nonimmune cells. For these experiments the host cell was 159::Tn10, in which the Tn10 was inserted at an unknown location on the bacterial genome. The polypeptides synthesized after  $\lambda::$ Tn10(1) infection of 159::Tn10 in the presence and absence of tetracycline are shown in Fig. 2. The synthesis of P36 was clearly inducible (compare lanes 3 and 4). By contrast, the synthesis of P25 was constitutive (lane 3).

The mobility on two-dimensional NEPHGE gels of the Tn10-specific polypeptide synthesized in immune and nonimmune cells is compared in Fig. 3. The polypeptides synthesized after infection of 159 with  $\lambda$  b b nin and  $\lambda::$ Tn10(2) are shown in gels A and C, respectively. Polypeptides synthesized after infection of 159 with  $\lambda::$ Tn10(1) were also analyzed on NEPHGE gels (not shown). Only two spots could be found that were present in both the  $\lambda::$ Tn10(1) and  $\lambda$  b b nin samples but not the  $\lambda$  b b nin sample, which are labeled P25 and P13 on gel C.

Analysis of the polypeptides synthesized after infection of *E. coli* 159 [ $\lambda::$ Tn10(1)] are shown in gels B, D, and F. Polypeptides b and d were the products of the *rex* and *cI* genes since they were present in the  $\lambda$  b b nin sample (gel B) and the  $\lambda::$ Tn10(1) samples (gels D and F) (29). Infection with  $\lambda::$ Tn10(1) resulted in the synthesis of two new polypeptides when the infection was done in the presence of tetracycline (gel F) but not in its absence (gel D). One, labeled P36, remained at the origin and the other, labeled P25, was below d. The second dimension of the NEPHGE gel system was essentially the same as the one-dimensional SDS gel system used in Fig. 1 and 2. Thus, the spot below d was the P25 polypeptide in Fig. 1 and 2 because it had the same mobility in the second dimension, relative to b and d, and its synthesis was tetracycline-inducible. The spot at the origin appeared to be P36, since no major spot appeared on the gel in the expected position above b that could correspond to P36. This observation was consistent with our observation that P36 aggregated readily.

To determine whether the Tn10-specific P25 synthesized in immune and nonimmune cells were the same, we analyzed a mixture of the samples used for gels C and F on gel E. P25 from both samples clearly had the same mobility,

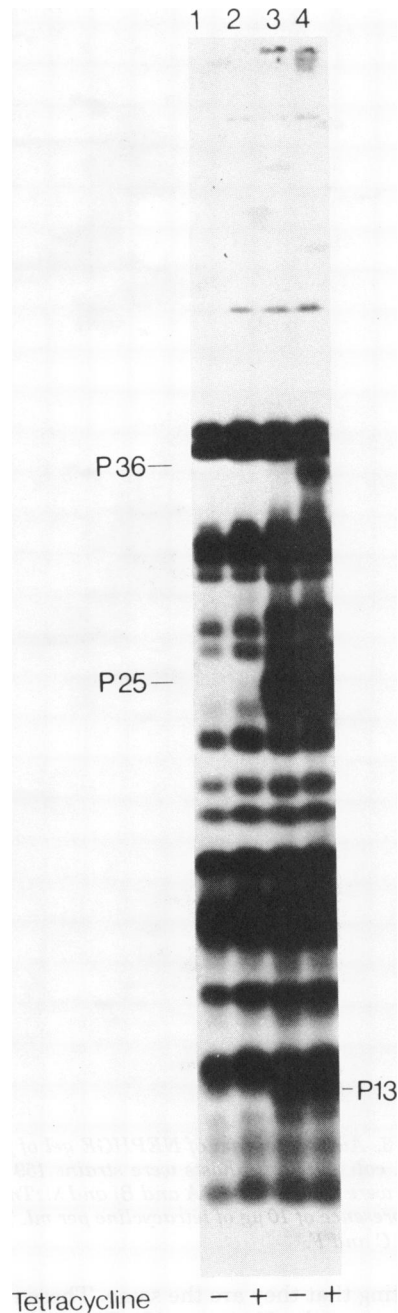


FIG. 2. Autoradiogram of 15% polyacrylamide-SDS gel of polypeptides synthesized after  $\lambda::$ Tn10 infection of UV-irradiated *E. coli*. Bacterial host was 159::Tn10. Infecting phages were  $\lambda$  b b nin (lanes 1 and 2) and  $\lambda::$ Tn10(1) (lanes 3 and 4). Infections were carried out in the presence and absence of tetracycline as indicated at the bottom. Note that no  $\lambda$  polypeptides or truncated polypeptides occurred at the position of P36 after infection of 159 with  $\lambda::$ Tn10(1) in the absence of tetracycline (Fig. 1, lane 11).

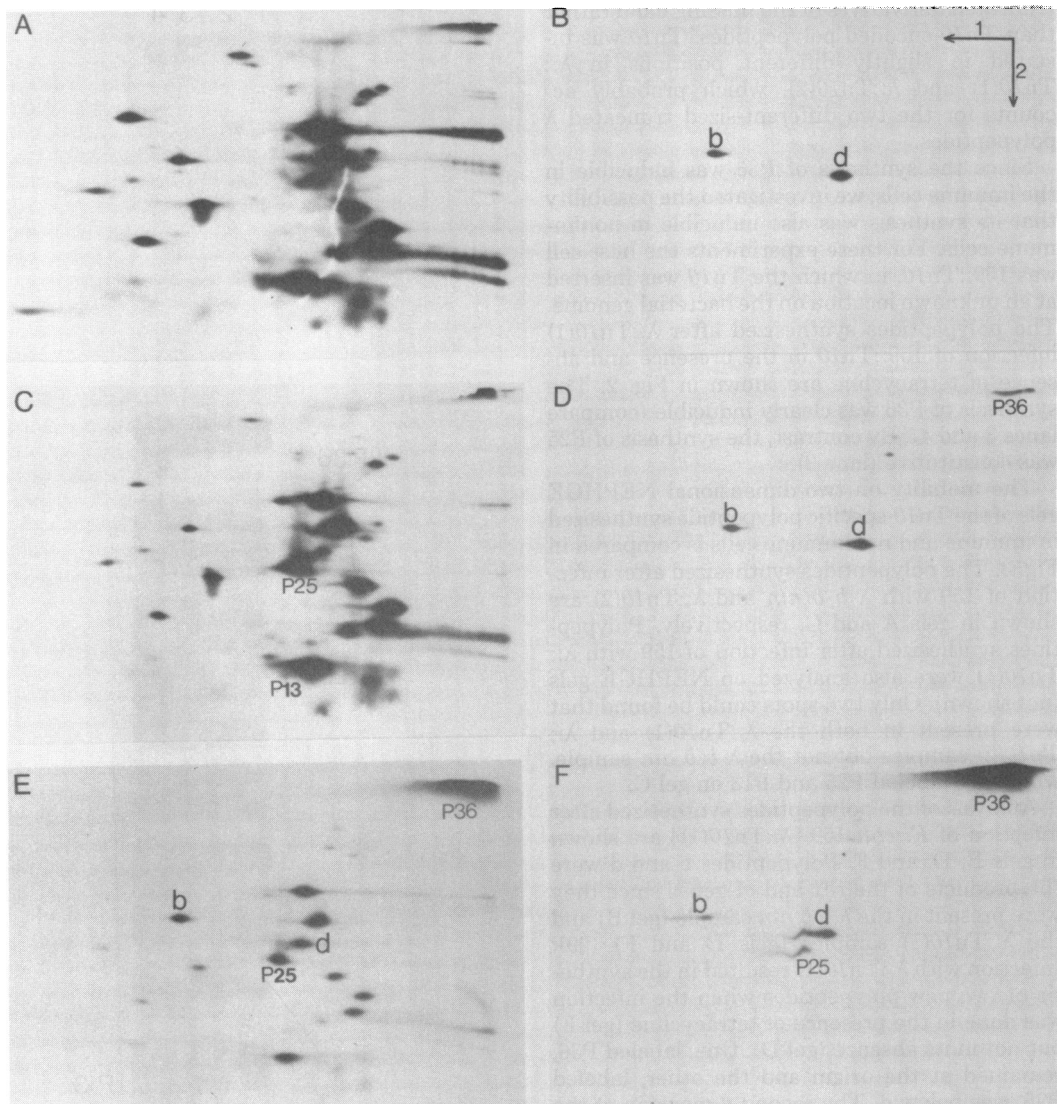


FIG. 3. Autoradiogram of NEPHGE gel of polypeptides synthesized after  $\lambda::Tn10$  infection of UV-irradiated *E. coli*. Bacterial hosts were strains 159 (gels A and C) and 159 [ $\lambda::Tn10(1)$ ] (gels B, D, F). Infecting phages were  $\lambda$  b b nin (gels A and B) and  $\lambda::Tn10(2)$  (gels B, D, F). Only the infection for gel F was carried out in the presence of  $10 \mu\text{g}$  of tetracycline per ml. The sample for gel E was a 1:4 (vol:vol) mixture of the samples on gels C and F.

indicating that they are the same. These results suggest that the Tn10-specific P25 bands that were seen in Fig. 1 and 2 after infection of immune and nonimmune hosts contained the same polypeptide, and both bands contained only one molecular species.

Similarly, the spot labeled P13 on gel C appeared to be the same as P13 in Fig. 1 and 2 because both could be labeled with [ $^3\text{H}$ ]leucine but not [ $^{35}\text{S}$ ]methionine (not shown). In addition, this spot had the size expected for P13 and

was only observed after infection of nonimmune cells, but not after infection of immune cells (gels D and F).

Thus, we have only been able to detect the synthesis of three Tn10-specific polypeptides after infection of nonimmune cells, two of which are also synthesized after infection of immune cells. The polypeptides synthesized after  $\lambda::Tn10$  infection were also analyzed on standard two-dimensional O'Farrell gels (26) (not shown). No additional Tn10-specific spots were detected. It

is possible, of course, that some of the Tn10 polypeptides coelectrophoresed with  $\lambda$  polypeptides synthesized in nonimmune hosts.

**Effect of a *tet* amber mutation on the synthesis of Tn10 polypeptides.** To aid in the identification of polypeptides involved in tetracycline resistance, we isolated an amber mutation, called *tet-575*, in a gene on  $\lambda$ :Tn10(2) that was required for Tet<sup>r</sup>. The efficiency of transduction of 159 (*sup*<sup>+</sup>) to Tet<sup>r</sup> compared to *supD*, *supE*, or *supF* derivatives of 159 was 10<sup>-6</sup>. The mutation was not in a gene required for lysogeny because stable lysogens of 159 could be isolated that were Tet<sup>s</sup>. Revertants of  $\lambda$ :Tn10(2)*tet575* were isolated that could transduce a *sup*<sup>+</sup> strain to Tet<sup>r</sup> at normal frequency, indicating that the mutation was probably a point mutation. Thus, the *tet-575* mutation appeared to be a conventional amber mutation.

Analyses of the polypeptides synthesized after infection with  $\lambda$ :Tn10(2)*tet575* are shown in Fig. 1 (lanes 5, 9, 13, 16, 18). The results indicated that only the synthesis of P36 was affected. Similar results were obtained when the samples were analyzed on NEPHGE gels (not shown). No evidence of P36 could be detected after infection of 159, even in the presence of tetracycline (compare lanes 8 and 9 of Fig. 1). However, a small amount of P36 appeared to be synthesized after infection of a *supD* derivative of *E. coli* 159 in the presence of tetracycline (lane 18) but not in its absence (lane 16). This result was consistent with the genetic analysis that indicated the mutation was suppressible by *supD*. However, no evidence for an amber fragment was detected.

Neither the amount nor the inducibility of the synthesis of P25 in immune cells was affected by the mutation (Fig. 1, lanes 5 and 9). Similarly, the synthesis of P25, as well as P13, in nonimmune cells was the same for  $\lambda$ :Tn10(2) and  $\lambda$ :Tn10(2)*tet575* (compare lanes 12 and 13 of Fig. 1).

The repressor that controls the expression of Tet<sup>r</sup> is encoded by Tn10 (16). Thus, we investigated whether the *tet-575* mutation had inactivated the repressor gene. If the repressor gene was inactivated, we would expect that the synthesis of P36 and P25 in immune hosts would be constitutive. The results in Fig. 1 did not answer this question because the immune cells contained a wild-type Tn10 that could supply the *tet* repressor. Thus, we analyzed the polypeptides synthesized after  $\lambda$ :Tn10(2) and  $\lambda$ :Tn10(2)*tet575* infection of 159 ( $\lambda$ <sup>+</sup>) and 159 [ $\lambda$ :Tn10(2)*tet575*]. Results for the infection of the latter host are shown in Fig. 4. The synthesis of P36 from  $\lambda$ :Tn10(2) and P25 from  $\lambda$ :Tn10(2) and  $\lambda$ :Tn10(2)*tet575* was stimulated by the ad-

dition of tetracycline (compare lanes 2 and 5, and lanes 3 and 6). Similar results were obtained when the host was 159 ( $\lambda$ <sup>+</sup>) (not shown). In other words, it was not necessary to use a host that carried Tn10 to observe the induction of the synthesis of these two Tn10 polypeptides. Presumably, the infecting phage could supply the repressor. However, the inducibility of the synthesis of P25 after infection of a Tn10*tet575* lysogen with  $\lambda$ :Tn10(2)*tet575* indicated that the *tet-575* mutation had not inactivated the repressor gene that controls the synthesis of P25. We quantitated the stimulation in the synthesis of P25 by densitometry analysis of the autoradiogram in Fig. 4. The intensity of P25 was normalized to the intensity of the  $\lambda$  b and d bands. The results indicated there was approximately a fivefold stimulation in the synthesis of P25 after infection with either  $\lambda$ :Tn10(2) or  $\lambda$ :Tn10(2)*tet575*.

**Expression of Tet<sup>r</sup> in the presence of amber suppressors.** The effect of the *tet-575* mutation on the expression of Tet<sup>r</sup> in stable lysogens was examined as described by Franklin and Cook (7). The rate of protein synthesis in exponentially growing cultures was measured by the incorporation of [<sup>3</sup>H]leucine in the presence of 10, 25, 50, and 100  $\mu$ g of tetracycline per ml. The effect of tetracycline induction was tested by preincubating the culture with 0.5  $\mu$ g of tetracycline per ml before addition of [<sup>3</sup>H]leucine and the remaining tetracycline. The results are given in Table 1. The resistance of protein synthesis to tetracycline in lysogens of  $\lambda$ :Tn10(1) and  $\lambda$ :Tn10(2) was tetracycline-inducible (compare the -Tet and +Tet columns for strain 159 in Table 1). The strain 159 *sup*<sup>+</sup> lysogen of  $\lambda$ :Tn10(2)*tet575* was more sensitive to tetracycline as expected. However, the induced culture was more resistant to tetracycline than a strain that did not contain any Tn10. This suggested that the tetracycline resistance system of Tn10 was not entirely inactivated by the *tet-575* mutation. Furthermore, since this intermediate level of tetracycline resistance was inducible, the mutation did not appear to have inactivated the repressor, which was consistent with the results of the experiment in Fig. 4.

When these experiments were done with *supD*, *supE*, and *supF* derivatives of the lysogens, anomalous results were obtained for the  $\lambda$ :Tn10(1) and  $\lambda$ :Tn10(2) strains. The results indicated that each of the strains carrying a suppressor allele was significantly more sensitive to tetracycline than the 159 strains without suppressors. Similar results were obtained for the nonlysogens 159:Tn10 and 159 *supD*:Tn10. This phenomenon was more pronounced for the *supD* and *supF* strains than the *supE* strains,



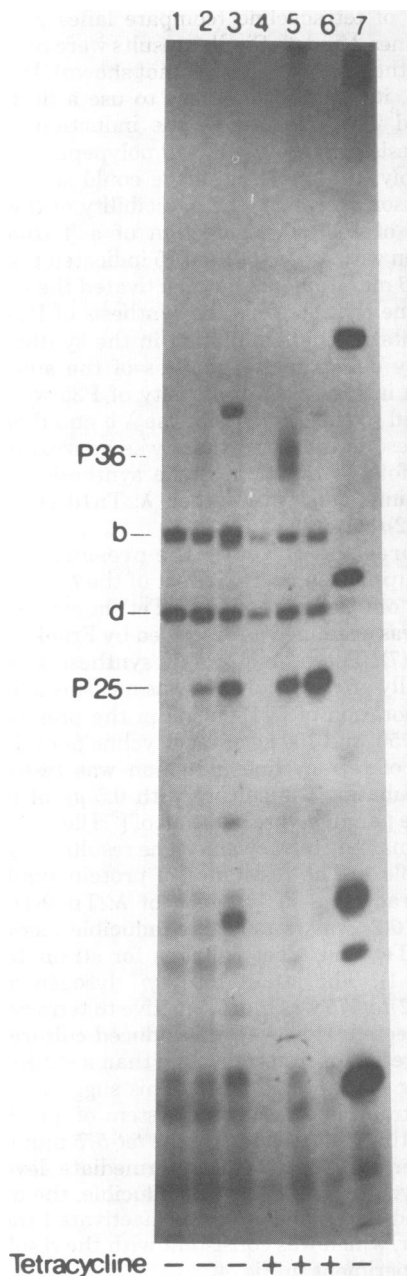


FIG. 4. Autoradiogram of 15% polyacrylamide-SDS gel of polypeptides synthesized after  $\lambda$ :Tn10 infection of UV-irradiated *E. coli*. Bacterial host was strain 159 [ $\lambda$ :Tn10(2)*tet*575]. Infecting phages were  $\lambda$  b b nin (lanes 1, 4),  $\lambda$ :Tn10(2) (lanes 2, 5),  $\lambda$ :Tn10(2)*tet*575 (lanes 3, 6), and  $\lambda$  rif<sup>d</sup>18 (lane 7). Samples in lanes 1, 2, 3, and 7 were from uninduced hosts (-tetracycline). Samples in lanes 4 to 6 were from hosts that were induced by the addition of 1  $\mu$ g of tetracycline per ml 15 min after infection. See text for identification of polypeptides indicated on left, and

which parallels the efficiency of suppression by these suppressors (8, 17). It was also more pronounced for the uninduced cultures, although the resistance was still inducible. The  $\lambda$ :Tn10(2)*tet*575 lysogens of the *supD* and *supE* strains showed about the same level of resistance as the  $\lambda$ :Tn10(2) lysogens, and the *supF* lysogen of the mutant was less resistant.

The physiological experiments in Table 1 indicated that the presence of amber suppressors decreased the level of tetracycline resistance. Further evidence of this phenomenon has come from measurements on the efficiency of plating of *sup*<sup>+</sup>, *supD*, and *supE* lysogens of  $\lambda$ :Tn10(1). When the strains were grown in the absence of tetracycline, the efficiency of plating the *supD* strain on 100 and 150  $\mu$ g of tetracycline per ml was ca.  $10^{-3}$  of that for the *sup*<sup>+</sup> strain. The efficiency of plating the *supE* strain was reduced ca.  $10^{-1}$ . When the strains were grown in the presence of 25  $\mu$ g of tetracycline per ml, the efficiency of plating was approximately 1.0 for each of the strains and drug concentrations tested.

#### DISCUSSION

Three polypeptides were detected with sizes of 36,000, 25,000, and 13,000 daltons that appeared to be encoded by Tn10. The 36,000-dalton polypeptide was clearly the TET polypeptide that has been associated with Tet<sup>r</sup> (11, 16, 23, 33). Its synthesis was inducible with tetracycline, and its size was the same as previously reported (16, 31, 33). The isolation of an amber mutation that eliminated the synthesis of the TET polypeptide and also the expression of Tet<sup>r</sup> provides further proof that this polypeptide is required for Tet<sup>r</sup>. The mutation did not decrease the synthesis of P13 and P25, although the synthesis of P25 was also inducible. Thus, the amber mutation may be in the gene for the TET polypeptide. Although the TET polypeptide is required for Tet<sup>r</sup>, it is not certain that it is the only polypeptide required for Tet<sup>r</sup>. Other gene products of Tn10, such as P25, may be required.

Yang et al. also detected a 15,000-dalton polypeptide encoded by R100 that was *tet* inducible (33). Since the *tet* genes of R100 are from Tn10 (30), we would expect that this 15,000-dalton polypeptide would be encoded by Tn10. However, Jorgensen and Reznikoff suggested that this polypeptide may be encoded by non-Tn10 sequences on R100 because they did not find any evidence of its synthesis from several clones

Fig. 1 for identification of polypeptides synthesized from  $\lambda$  rif<sup>d</sup>18 (lane 7), which were used as molecular weight markers.

TABLE 1. Relative rates of protein synthesis in  $\lambda$ ::Tn10 lysogens<sup>a</sup>

Prophage	Tet ( $\mu\text{g/ml}$ )	Relative rate of protein synthesis (%)							
		159		159 <i>supD</i>		159 <i>supE</i>		159 <i>SupF</i>	
		-Tet	+Tet	-Tet	+Tet	-Tet	+Tet	-Tet	+Tet
$\lambda$ ::Tn10(1)	0	100	100	100	100	100	100	100	100
	10	90	95	16	83	35	98	22	58
	25	57	88	5	40	11	88	16	46
	50	27	87	5	23	7	70	15	16
	100	8	64	9	7	4	28	9	10
$\lambda$ ::Tn10(2)	0	100	100	100	100	100	100	100	100
	10	88	100	31	78	41	97	26	83
	25	28	95	16	58	11	93	18	58
	50	6	83	4	37	5	52	8	32
	100	7	39	4	12	2	18	2	12
$\lambda$ ::Tn10(2) <i>tet575</i>	0	100	100	100	100	100	100	100	100
	10	12	45	10	70	21	84	11	40
	25	5	17	12	21	14	41	6	40
	50	8	10	6	17	8	15	4	46
	100	6	5	6	13	17	11	2	18
Nonlysogen::Tn10	0	100	100	100	100				
	10	80	92	19	85				
	25	20	80	9	41				
	50	8	50	7	9				
	100	5	25	3	7				
Nonlysogen (-Tn10)	0	100							
	10	8							
	25	1							
	50	0							
	100	2							

<sup>a</sup> Concentration of tetracycline during the labeling is indicated on the left. Induced cultures, +Tet, were grown in the presence of 0.5  $\mu\text{g}$  of tetracycline per ml for 60 min before labeling. Thus, the "0" tetracycline samples for these cultures actually contained 0.5  $\mu\text{g}$  of tetracycline per ml. The rate of protein synthesis for each strain is expressed as the percent of the rate obtained for the "0" tetracycline culture. Each measurement is an average of duplicate samples. The error in each value was approximately  $\pm 5\%$ . The nonlysogen ::Tn10 *sup*<sup>+</sup> and *supD* strains contained Tn10 inserts at unknown sites on the bacterial chromosome. Results for the induced culture of the nonlysogen (-Tn10) are not given because the preincubation in 0.5  $\mu\text{g}$  of tetracycline per ml significantly inhibited protein synthesis.

of Tn10 (16). They did observe some tetracycline-inducible Tn10 polypeptides with sizes in the range of 15,000 to 25,000 daltons. However, since they were present in relatively small amounts, they suggested these other tetracycline-inducible polypeptides were fragments of the 36,000-dalton polypeptide.

The inducible 25,000-dalton polypeptide we detected did not appear to be a derivative of P36. The best evidence for this conclusion was that the synthesis of P25 did not always parallel the synthesis of P36. The synthesis of both was inducible in  $\lambda$ -immune cells. However, in non-immune cells the synthesis of P25 was constitutive and the synthesis of P36 was still inducible. Also, the *tet-575* mutation appeared to completely eliminate the synthesis of P36 in a *sup*<sup>+</sup> strain, but did not decrease the synthesis of P25.

Finally, P25 did not appear to be a minor product since it labeled as intensely as P36 in immune cells and more intensely in nonimmune cells when the labeling was done with [<sup>3</sup>H]leucine. One possible explanation for the observation of Jorgensen and Reznikoff (16) is that they labeled with [<sup>35</sup>S]methionine, which does not label P25 very intensely (not shown).

The gene products of the *tet* genes on pSC101 have also been investigated (31). The relationship between these *tet* genes and those on Tn10 is not known. However, there are several similarities. For example, pSC101 encoded three tetracycline-inducible polypeptides that were about the same size as the three Tn10-specific polypeptides we detected. Additional experiments are required before we can compare the results for pSC101 and Tn10.



What could be the role of P25? Since its synthesis was tetracycline inducible, it could be involved in the expression of Tet<sup>r</sup>. However, the conjugative ability of some R-factors that encode Tet<sup>r</sup> is also tetracycline inducible (28). Thus, a polypeptide whose synthesis is tetracycline inducible could be involved in other processes than the expression of Tet<sup>r</sup>. If it is involved in the expression of Tet<sup>r</sup>, then it could be a component of the system that confers Tet<sup>r</sup>, or it could be involved in the regulation of the synthesis of the components.

The regulatory system that controls the expression of Tet<sup>r</sup> is encoded by R-factors rather than the bacterial chromosome (7). Since the expression of Tet<sup>r</sup> from Tn10 was inducible, it was clear that Tn10 encodes the regulatory system, presumably a repressor. Jorgensen and Reznikoff found that all of their plasmids that encoded the TET polypeptide and conferred Tet<sup>r</sup> also encoded the repressor (16). They suggested the repressor was encoded by a 695-base pair region adjacent to the gene for the TET polypeptide, which would be large enough to code for P25. The observation that the synthesis of P25 was tet inducible would appear to be inconsistent with the hypothesis that it is the repressor. However, it is conceivable that the synthesis of the tet repressor is also regulated by tetracycline. Another possibility is that the TET polypeptide (P36) is the repressor. However, we found that the tet-575 mutation, which eliminated the synthesis of P36, did not inactivate the regulatory system that controls the synthesis of P25. Thus, the TET polypeptide is probably not the repressor.

Whatever the role of P25, it is clear that λ::Tn10(1)- or λ::Tn10(2)-infected nonimmune cells would be a good source of this polypeptide since large amounts of it were synthesized in these cells. One possible explanation for the constitutive synthesis of P25 and the inducible synthesis of P36 in these cells is that the two polypeptides are encoded on opposite strands of DNA. In nonimmune cells, transcription of the gene for P25 could have come from phage promoters whereas transcription of the gene for P36 probably came from the normal Tn10 promoter. Other explanations are possible.

The effect of amber suppressors on the expression of Tet<sup>r</sup> was anomalous. In general, these suppressors seemed to decrease the resistance of uninduced cultures more than induced cultures. This observation was most clear from the measurements on the efficiency of plating λ::Tn10(1) lysogens. If the synthesis of the repressor was regulated by an attenuator mechanism (2), then it is conceivable that the frequency of transcription termination at an attenuator could be influ-

enced by translation termination in a leader. If that was the case, then rho mutants might have the same effect as amber suppressors. Thus, we examined the effect of tetracycline on the rate of protein synthesis as described in Table 1 for the rho mutants isolated by Korn and Yanofsky (20, 21), and the rho15 mutant of Das et al. (4) (not shown). None of them exhibited the anomalous expression of Tet<sup>r</sup> that occurred with strain 159 supD. These observations suggested amber suppressors have their effect on the expression of Tet<sup>r</sup> directly at the translational level, presumably due to the presence of extra amino acids at the C-terminus of some polypeptides.

Tn10 could code for 300,000 daltons of protein, even if there are no overlapping genes and only one strand of the DNA is sense. We have detected polypeptides with an aggregate size of about 85,000 daltons. Thus, Tn10 could code for several more polypeptides. However, it would have been difficult to detect these polypeptides with the approach used here if only small amounts were synthesized. The transposase polypeptide encoded by Tn3, which had a size of about 120,000 daltons, could only be detected on gels when the repressor that controls its synthesis was inactivated (9). Thus, Tn10 could code for such a polypeptide that we have not been able to detect.

#### ACKNOWLEDGMENTS

We thank Doug Berg for λ::Tn10(1) and λ::Tn10(2), B. Reznikoff for communicating the results of his experiments with Tn10 before publication, Chris Epp and Mark Pearson for the set of *E. coli* 159 strains, and Janice Hartman for preparation of the manuscript. This work was supported by a grant from the National Science Foundation (PCM7714983), a Public Health Service grant from the National Institutes of Health (GM2434702), and a Public Health Service Career Development Fellowship from the National Institutes of Health (GM0040102) to S.R.J.

#### LITERATURE CITED

1. Arima, K., and K. Izaki. 1963. Accumulation of oxytetracycline relevant to its bactericidal action in the cells of *Escherichia coli*. *Nature* (London) **200**:192-193.
2. Bertrand, K., L. Korn, F. Lee, T. Platt, C. L. Squires, C. Squires, and C. Yanofsky. 1975. New features of the regulation of the tryptophan operon. *Science* **189**: 22-26.
3. Clark, D. J., and O. Maale. 1967. DNA replication and the division cycle in *Escherichia coli*. *J. Mol. Biol.* **23**: 99-112.
4. Das, A., D. Court, and S. Adhya. 1976. Isolation and characterization of conditional lethal mutants of *Escherichia coli* defective in transcription termination factor rho. *Proc. Natl. Acad. Sci. U.S.A.* **73**:1959-1963.
5. De Zeeuw, J. R. 1968. Accumulation of tetracyclines by *Escherichia coli*. *J. Bacteriol.* **95**:498-506.
6. Franklin, T. J. 1967. Resistance of *Escherichia coli* to tetracyclines. Changes in permeability to tetracyclines in *Escherichia coli* bearing transferable resistance factors. *Biochem. J.* **105**:371-378.
7. Franklin, T. J., and J. M. Cook. 1971. R-factor with a mutation in the tetracycline resistance marker. *Nature*

- (London) **229**:273-274.
8. Garen, A. 1968. Sense and nonsense in the genetic code. *Science* **160**:149-159.
  9. Gill, R. E., F. Heffron, and S. Falkow. 1979. Identification of the protein encoded by the transposable element Tn3 which is required for its transposition. *Nature* (London) **282**:797-801.
  10. Hong, J.-S., and B. N. Ames. 1971. Localized mutagenesis of any specific small region of the bacterial chromosome. *Proc. Natl. Acad. Sci. U.S.A.* **68**:3158-3162.
  11. Izaki, K., K. Kiuchi, and K. Arima. 1966. Specificity and mechanism of tetracycline resistance in a multiple-drug-resistant strain of *Escherichia coli*. *J. Bacteriol.* **91**:628-633.
  12. Jaskunas, S. R., R. R. Burgess, L. Lindahl, and M. Nomura. 1976. Two clusters of genes for RNA polymerase and ribosome components in *Escherichia coli*, p. 539-552. In R. Losick and M. Chamberlin (ed.), RNA polymerase. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
  13. Jaskunas, S. R., L. Lindahl, and M. Nomura. 1975. Identification of two copies of the gene for the elongation factor EF-Tu in *E. coli*. *Nature* (London) **257**:458-462.
  14. Jaskunas, S. R., L. Lindahl, and M. Nomura. 1975. Specialized transducing phages from ribosomal protein genes of *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* **72**:6-10.
  15. Jorgensen, R. A., D. E. Berg, B. Allet, and W. S. Reznikoff. 1979. Restriction enzyme cleavage map of Tn10, a transposon which encodes tetracycline resistance. *J. Bacteriol.* **137**:681-685.
  16. Jorgensen, R. A., and W. S. Reznikoff. 1979. Organization of structural and regulatory genes that mediate tetracycline resistance in transposon Tn10. *J. Bacteriol.* **138**:705-714.
  17. Kaplan, S., A. O. W. Stretton, and S. Brenner. 1965. Amber suppressors: efficiency of chain propagation and suppressor specific amino acids. *J. Mol. Biol.* **14**:528-533.
  18. Kirschbaum, J., and E. B. Konrad. 1973. Isolation of a specialized lambda transducing phage carrying the beta subunit gene for *Escherichia coli* ribonucleic acid polymerase. *J. Bacteriol.* **116**:517-526.
  19. Kleckner, N., R. Chan, B.-K. Tye, and D. Botstein. 1975. Mutagenesis by insertion of a drug-resistance element carrying an inverted repetition. *J. Mol. Biol.* **97**:561-575.
  20. Korn, L. J., and C. Yanofsky. 1976. Polarity suppressors defective in transcription termination at the attenuator of the tryptophan operon of *Escherichia coli* have altered rho factor. *J. Mol. Biol.* **106**:231-241.
  21. Korn, L. J., and C. Yanofsky. 1976. Polarity suppressors increase expression of the wild-type tryptophan operon of *Escherichia coli*. *J. Mol. Biol.* **103**:395-409.
  22. Laemmli, U. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* (London) **227**:680-685.
  23. Levy, S. B., and L. McMurry. 1974. Detection of an inducible membrane protein associated with R-factor-mediated tetracycline resistance. *Biochem. Biophys. Res. Commun.* **56**:1060-1068.
  24. Levy, S. B., and L. McMurry. 1978. Plasmid-determined tetracycline resistance involves new transport systems for tetracycline. *Nature* (London) **276**:90-92.
  25. Miller, J. H. 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
  26. O'Farrell, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. *J. Biol. Chem.* **250**:4007-4021.
  27. O'Farrell, P. Z., H. M. Goodman, and P. H. O'Farrell. 1977. High resolution two-dimensional electrophoresis of basic as well as acidic proteins. *Cell* **12**:1133-1142.
  28. Privitera, G., M. Sebald, and F. Fayolle. 1979. Common regulatory mechanism of expression and conjugative ability of a tetracycline resistance plasmid in *Bacteriodes fragilis*. *Nature* (London) **278**:657-659.
  29. Ptashne, M. 1967. Isolation of the lambda phage repressor. *Proc. Natl. Acad. Sci. U.S.A.* **57**:306-312.
  30. Sharp, P., S. Cohen, and N. Davidson. 1973. Electron microscope heteroduplex studies of sequence relations among plasmids of *Escherichia coli* II. Structure of drug resistance (R) factors and F. factors. *J. Mol. Biol.* **75**:235-255.
  31. Tait, R. C., and H. W. Boyer. 1978. On the nature of tetracycline resistance controlled by the plasmid pSC101. *Cell* **13**:73-81.
  32. Unowsky, J., and M. Rachmeler. 1966. Mechanisms of antibiotic resistance determined by resistance-transfer factors. *J. Bacteriol.* **92**:358-365.
  33. Yang, H.-L., G. Zubay, and S. Levy. 1976. Synthesis of an R plasmid protein associated. *Proc. Natl. Acad. Sci. U.S.A.* **73**:1509-1512.