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Organization of Structural and Regulatory Genes That Mediate Tetracycline Resistance in Transposon Tn*10*

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The location of Tn*10* genes encoding tetracycline resistance and its regulation was determined by analyzing the properties of recombinant plasmids carrying partial *Hpa*I digestion products of λ::Tn*10* transducing phage deoxyribonucleic acid. Within a 2,700-base pair region are encoded tetracycline resistance, the structural gene (*tet*) for a tetracycline-inducible polypeptide, and the regulatory elements for the induction of both the resistance phenotype and the polypeptide. Fusion of different sequences to an *Hpa*I site in the *tet* gene alters the molecular weight and stability of the polypeptide as well as the tetracycline resistance phenotype of strains producing fusion polypeptides. These results indicate the orientation of the *tet* gene and support the conclusion that the *tet* polypeptide is required for tetracycline resistance. A *Hinc*II cleavage site immediately upstream from the *tet* gene is protected by ribonucleic acid polymerase, but only in the absence of ribonucleotide triphosphates. The possibility that *tet* transcription is initiated at this site is discussed.

Tetracycline resistance specified by the R-factor R100 (also called R222 and NR1) is carried by a 9,300-base pair-long transposon termed Tn*10* (16, 30). The mechanism of resistance to tetracycline involves an inhibition of uptake, rather than degradation or modification, of the drug (12, 20). Uptake of tetracycline by sensitive *Escherichia coli* cells involves at least two independent transport systems, one active and one passive (26); both systems are depressed in tetracycline-resistant cells (20). In addition to uptake inhibition, resistant cells are reported to possess an internal (intracellular) mechanism for inhibition of tetracycline activity (20). Thus, a combination of decreased transport and decreased sensitivity of intracellular components seems to be responsible for the resistance mediated by R100. Further, these observations suggest that several gene products may be involved in tetracycline resistance.

The expression of tetracycline resistance is regulated in that pretreatment of resistant cells with subinhibitory levels of the drug increases the level of resistance (12). Levy and McMurry (19) and Levy et al. (21) have reported the R-factor-directed synthesis of a tetracycline-inducible membrane-associated polypeptide ("TET" protein) and suggested that this protein is responsible for tetracycline resistance. That the regulation of tetracycline resistance is mediated, at least in part, by a repressor has been suggested by Yang et al. (35), who reported the partial purification from R222-containing cells of an inhibitor of TET protein synthesis in vitro. This inhibition was reversible by the addition of tetracycline.

In this communication we described experiments which examine the ability of recombinant plasmids carrying Tn*10* deletions affecting tetracycline resistance to synthesize polypeptides in *E. coli* minicells. These studies demonstrate that the TET protein is indeed encoded by Tn*10* and is necessary for tetracycline resistance. The gene encoding the TET protein (*tet*) was precisely located within Tn*10*, and its direction of transcription was determined. In addition, the probable location of the *tet* promoter region is inferred from RNA polymerase binding and protection experiments.

MATERIALS AND METHODS

Phage and bacterial strains. The λ::Tn*10* phage used here is λ::Tn*10*(1), described previously (14). Strain C600 r_K^- m_K^- (from J. Davies) is *thr leu lac* $B1^-$ and is defective in the K-12 restriction and modification system. χ984 (from R. Curtiss) is a minicell-producing strain of *E. coli* that is *met ilv his ade pdx*. DNA from λ*kan*3 (6) and R-factors JR225 and NR79 were kindly provided by J. Davies. Plasmid R100 (also known as NR1 or R222) was obtained from R. Rownd and was introduced into C600 r_K^- m_K^- and χ984 by conjugation.

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DNA preparation and restriction enzyme digestion. Phage DNA was prepared for restriction enzyme treatment by phenol extraction as described by Barnes et al. (4). Plasmid DNA was prepared by the method of Humphreys et al. (11). Enzymatic digestion of DNA was performed in 0.05 ml of 60 mM $MgSO_4$–7 mM $MgSO_4$–10 mM Tris-hydrochloride (pH 7.9)–10 mM β-mercaptoethanol.

Enzymes. *Hpa*I, *Hin*cII, *Sal*I, *Ava*I, *Bgl*I, *Bam*HI, *Xba*I, *Pst*I, and *Kpn*I restriction enzymes and T4 DNA ligase were obtained from New England Bio-Labs. *Eco*RI was a gift from J. Gardner and S. Hardies.

Agarose and polyacrylamide gel electrophoretic analysis of DNA. Horizontal agarose gel electrophoresis was carried out essentially as described by Shinnick et al. (31). DNA analysis by polyacrylamide slab gel electrophoresis was performed essentially as described by Maniatis et al. (24), except that the gels were made in 5% glycerol. DNA fragments were stained for photography under UV light by immersing the gels in a solution of 10 μg of ethidium bromide per ml for 10 min, followed by rinsing and destaining in water for 20 min.

Construction of recombinant plasmids carrying Tn*10* *Hpa*I fragments. Five micrograms of λ::Tn*10* DNA was incompletely digested with *Hpa*I, and the enzyme was inactivated by heating to 70°C for 10 min. One microgram of pVH51 DNA was digested to completion with *Hin*cII, which was also inactivated by heating. The reactions were monitored by agarose gel electrophoresis. Samples of 0.45 μg of *Hpa*I-cleaved λ::Tn*10* DNA and 0.1 μg of *Hin*cII-cleaved pVH51 DNA in ligase buffer (20 mM Tris [pH 7.5], 10 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM Na_2EDTA, and 0.05 mM ATP) were incubated in the presence of 0.4 μl of T4 DNA ligase (92 units/ml) in a 15-μl volume at 15°C overnight (29). The resulting mixture was used to transform strain C600 r_K^- m_K^- by the method of Mandel and Higa (22). Transformed cells were allowed to outgrow for 90 min in nutrient broth and were then spread on nutrient plates containing 20 μg of tetracycline per ml. Forty-eight clones so obtained were screened for plasmid size by a modification of the procedure of Barnes (3). Twenty-five clones containing plasmids of various sizes were characterized as described in the Results section. These strains were handled under P1-EK1 containment conditions as prescribed by the National Institutes of Health.

Measurement of tetracycline resistance levels. Tetracycline resistance conferred by plasmids in C600 r_K^- m_K^- was measured by a modification of the procedure of Tait et al. (32). Overnight cultures were inoculated into fresh nutrient broth with and without 1 μg of tetracycline per ml, then incubated for 45 min at 37°C and diluted. Approximately 100 to 300 cells were plated on nutrient plates containing 0, 25, 50, 75, 100, 125, 150, 175, or 200 μg of tetracycline per ml (for experiment 1; experiment 3 also used 225- and 250-μg/ml plates; see Table 1) or 0, 5, 10, 20, 30, 40, 50, 65, 80, 100, or 120 μg of tetracycline per ml (experiment 2, Table 1). The number of surviving colonies was scored after 20 h of incubation at 37°C, and the concentration of tetracycline that gave 50% efficiency of plating (the EOP_{50}) was determined graphically. Since the age of agar media containing tetracycline was found to significantly affect EOP_{50} values, strains that were to be compared were tested at the same time on the same batch of plates.

Purification and labeling of minicells. Minicells were purified essentially as described by Roozen et al. (28), except that growth of the cells was in low-phosphate medium (18) so that minicells could be labeled with $^{32}PO_4^{3-}$ (F. Schmidt, R. Jorgensen, and J. Davies, unpublished data) as well as with [^{35}S]methionine. Minicells obtained from a 400-ml culture were washed and suspended in 1 ml of 1:4 Met Assay Medium (Difco) in M9 salts with 0.5% glucose, 2 μg of pyridoxine per ml, and 40 μg of adenine per ml. Minicells were then divided into two aliquots, one of which was induced with 1 or 5 μg of tetracycline per ml. After a 30-min induction period, both samples were labeled with 20 to 50 μCi of [^{35}S]methionine (specific activity, 600 to 800 Ci/mmol, Amersham/Searle) for 30 min, pelleted, and frozen. For electrophoresis, pellets were suspended in 50 μl of sample buffer (17) and heated at 90°C for 2 to 5 min. Samples of 20 μl were loaded on 15% polyacrylamide–sodium dodecyl sulfate (SDS) slab gels (17) and electrophoresed at 35 mA for 5 to 6 h on a constant current power supply. Gels were dried by heating under a vacuum at 70°C and were autoradiographed at room temperature using Kodak X-Omat R film. Molecular weight markers used here were [^{35}S]methionine-labeled proteins from purified bacteriophage λ particles which were prepared for electrophoresis by mixing with unlabeled cells and heating to 90°C in sample buffer.

RNA polymerase "protection experiment." Four micrograms of *E. coli* RNA polymerase (4 mg/ml; a gift from S. Rothstein and L. Maquat) was incubated with 1 μg of pRT61 DNA at 37°C for 30 min in buffer PBBX + 0.1 M KCl (13) to allow formation of stable polymerase-DNA complexes. Six units of *Hin*cII was then added, and incubation was continued for 60 min. Two controls were treated identically except (i) one received no RNA polymerase and (ii) one was made 400 μM in the four ribonucleoside triphosphates immediately prior to addition of *Hin*cII. After *Hin*cII treatment, each mixture was phenol and ether extracted and subjected to electrophoresis on 8% polyacrylamide gels as described above.

RESULTS

Construction and characterization of deletions of Tn*10* defining the tetracycline resistance genes. In an earlier communication (14) we described the determination of a restriction enzyme cleavage map for Tn*10* (Fig. 1) as a first step toward studying gene organization in Tn*10*. As illustrated by this map, Tn*10* contains four *Hpa*I cleavage sites, which divide this element into five segments. Here we report the construction of a set of deletions in Tn*10*. These deletions were generated by molecular cloning of λ::Tn*10* incomplete *Hpa*I cleavage products into a plasmid vector with selection for tetracycline resistance (Materials and Methods). The plasmid cloning vehicle chosen for this experiment was the ColE1 derivative pVH51, a small

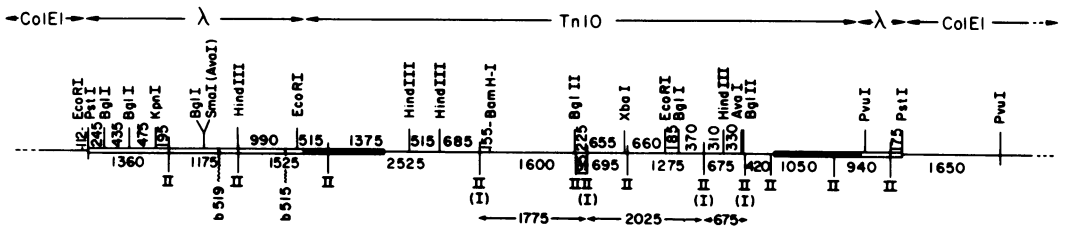

FIG. 1. *Restriction enzyme cleavage map of Tn10. Numbers refer to distances in base pairs. II refers to the HincII cleavage sites. (I) refers to those HincII cleavage sites that are also HpaI cleavage sites.*

(4-kilobase), amplifiable, multicopy plasmid with a single *Hin*cII cleavage site (10). *Hin*cII (GTPy↓PuAC) recognizes both *Hpa*I (GTT↓AAC) and *Sal*I (G↓TCGAC) cleavage sites. The plasmid, pVH51, is not cut by either *Hpa*I or *Sal*I. Therefore, the sequence of the *Hin*cII site on the plasmid is GTC↓AAC, and ligation of a *Hpa*I fragment at this site reconstitutes two *Hin*cII sites but only one *Hpa*I site.

To choose among plasmids for further study, three criteria were used: (i) that all *Hpa*I fragments cloned in each plasmid carry Tn*10* DNA (i.e., no λ *Hpa*I fragments are found in the plasmid unless that fragment also carries Tn*10* DNA); (ii) that in each recombinant plasmid *Hin*cII cleavage sites have been reconstituted at the junctions of Tn*10* and pVH51 DNA (i.e., no exonucleolytic degradation has occurred prior to ligation); and (iii) that each plasmid results from an independent ligation event.

Of 25 tetracycline-resistant clones examined, 8 met these criteria, established by *Hin*cII and *Hpa*I restriction analysis of their plasmid DNAs (*Hpa*I restriction patterns are shown in Fig. 2). The regions of Tn*10* carried in these eight recombinant plasmids are illustrated in Fig. 3. The smallest plasmid, pRT29, carries only *Hpa*I fragment 2025, indicating that this fragment alone is sufficient for generating the tetracycline resistance phenotype.

With the exception of pRT29, each plasmid carries two or more *Hpa*I fragments. The simplest way in which these plasmids could have been formed is by ligation of a single incomplete *Hpa*I cleavage product to the *Hin*cII-cleaved pVH51 molecule. An alternate route is by ligation of two *Hpa*I cleavage products (completely or incompletely digested by *Hpa*I) to the vector. The latter reaction would result in joining two fragments which do not adjoin in Tn*10*, or which do adjoin in the original sequence but in such a manner that the orientation of the fragments is reversed as compared with the original orientation (i.e., the "wrong" ends of the molecules are joined), or both. pRT4 is an obvious example of the former possibility (*Hpa*I fragments 2025 and 3000 are joined in this plasmid). Establishment

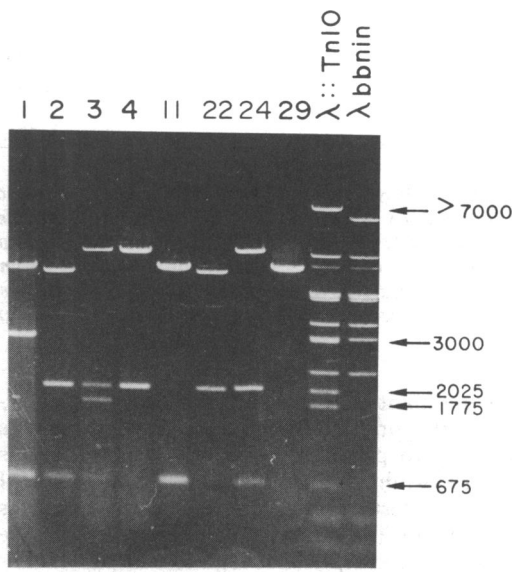

FIG. 2. *HpaI cleavage patterns of pRT plasmids and the indicated phages. 1 refers to pRT1, etc. Tn10-specific HpaI fragments are indicated by the arrows, and each fragment's size is given in base pairs.*

of the map order and relative orientations of neighboring fragments in each of these seven plasmids was accomplished by double digestion experiments (data not shown) with restriction enzymes having only one cleavage site in the cloned region (e.g., *Bam*HI, *Xba*I, *Eco*RI, *Bgl*I, *Ava*I, and *Pst*I; see map at the top of Fig. 3 for the locations of these sites). Six of these plasmids (all except pRT4) carry the cloned *Hpa*I fragments in the same map order and relative orientation as is found in Tn*10*. This suggests that these plasmids arose by cloning of a single incomplete *Hpa*I cleavage product. In pRT4, *Hpa*I fragments 2025 and 3000 are ligated with one of these fragments in the opposite orientation as found in Tn*10*, i.e., *Hpa*I fragment 3000 has reversed its orientation such that its *Pst*I cleavage site lies within 600 base pairs of *Hpa*I fragment 2025.

To complete the physical mapping of the eight

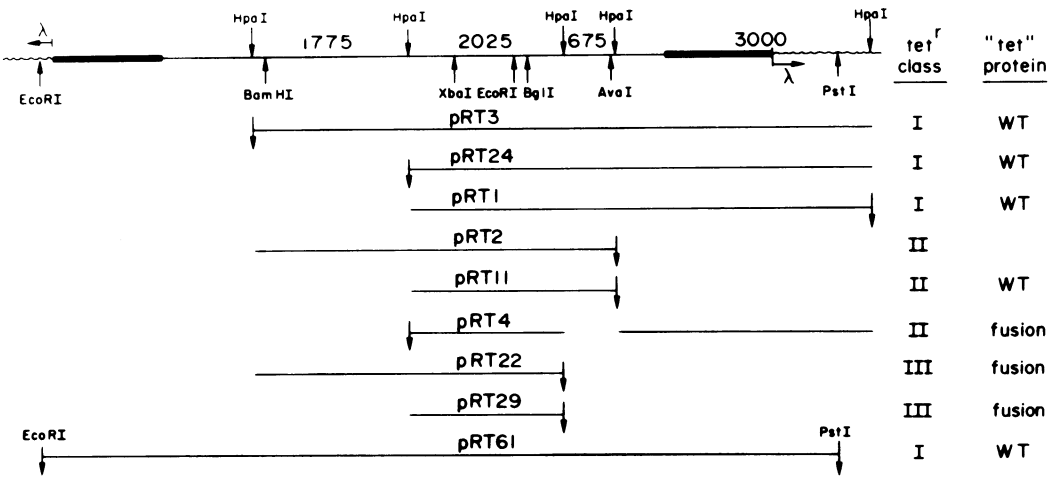

FIG. 3. *Physical maps of recombinant plasmids carrying portions of Tn10. At the top is a map of the Tn10 portion of a λ::Tn10 phage indicating appropriate cleavage sites. The lines below this map indicate the cloned portions of λ::Tn10 present in the indicated plasmid. pRT61, at the bottom, is an EcoRI-generated deletion derivation of a PstI Tn10 fragment clone from λ::Tn10. The unlabeled arrows at the end of the cloned HpaI fragments indicate the reconstituted HpaI cleavage sites. tet^r (tetracycline resistance) classes are explained in the Results section. wt refers to wild-type-size TET protein; fusion refers to the altered TET proteins (see the text).*

recombinant plasmids, it is necessary to determine the orientation of the cloned fragments relative to pVH51 sequences. For pRT29 this was determined using the results of a *Kpn*I-*Hpa*I double digestion experiment. The results of this experiment (data not shown) indicated that the reconstituted *Hpa*I cleavage site in pRT29 lies on that side of the cloned fragment nearest the *Kpn*I cleavage site (see Fig. 4 for a map of pRT29). Thus the nucleotide sequence of the pVH51 *Hinc*II site, reading from the *Eco*RI site to the *Kpn*I site, must be GTC↓AAC. This information, together with the comparisons of *Hinc*II and *Hpa*I restriction patterns described earlier, allows us to orient the insertions of the other seven plasmids. In Fig. 3, that end of an inserted segment of DNA at which the reconstituted *Hpa*I cleavage site lies is indicated by an unlabeled arrow. Interesting examples are pRT1 and pRT24, in which the same cloned fragments lie in opposite orientations relative to pVH51 sequences.

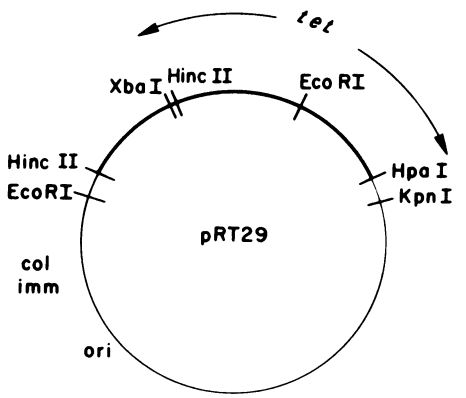

FIG. 4. *Physical and genetic map of pRT29. The heavy line indicates the 2,025-base pair HpaI fragment of Tn10; the rest of the plasmid is pVH51. tet refers to the gene encoding TET protein, col imm refers to the colicin immunity gene, and ori refers to the ColE1 origin of replication.*

Effect of deletions in Tn*10* on tetracycline resistance. Table 1 presents a comparison of tetracycline resistance levels conferred on *E. coli* C600 cells by the R-factor R100, a λ::Tn*10* prophage, and multicopy recombinant plasmids carrying various portions of Tn*10*. Resistance levels are reported here as the tetracycline concentration at which the efficiency of plating for a given strain is 50% (EOP_{50}) and were measured both before and after treatment with subinhibitory levels of tetracycline. Since the age of agar media containing tetracycline was found to significantly affect EOP_{50} values, strains that were to be compared were tested in the same experiment on the same batch of plates. Basal (or uninduced) EOP_{50} values were found to be more variable than induced EOP_{50} values (compare experiments 1, 2, and 3 in Table 1), such that basal values are not reproducible within experimental error limits. (Measurement of the basal tetracycline resistance phenotype of a strain, by plating cells on agar containing tetracycline, pre-

TABLE 1. *Tetracycline resistance phenotypes*

Expt. no.	Strain	EOP_{50} (μg/ml)		*tet*^{r} class
		Uninduced	Induced	
1	pRT61	31 ± 10	90 ± 10	I
	pRT3	38 ± 10	105 ± 10	I
	pRT24	35 ± 10	102 ± 10	I
	pRT1	38 ± 10	90 ± 10	I
	pRT11	69 ± 10	190 ± 10	II
	pRT4	77 ± 10	>200	II
	R100	80 ± 10	178 ± 10	Wild type
	λ::Tn*10*	90 ± 10	165 ± 10	Wild type
2	pRT3	64 ± 10	113 ± 10	I
	pRT22	26 ± 3	39 ± 3	III
	pRT29	26 ± 3	37 ± 3	III
	C600 r_K^- m_K^-	<2	<2	Sensitive
3	pRT3	85 ± 10	120 ± 10	I
	pRT2	135 ± 20	180 ± 10	II
	pRT11	190 ± 15	200 ± 15	II
	pRT4	150 ± 10	180 ± 10	II

sumably leads to some induction of resistance; this induction might be expected to be variable, depending on the precise experimental conditions.)

Three classes of tetracycline resistance phenotypes can be distinguished among the recombinant plasmids in Table 1 on the basis of the induced EOP_{50} levels. Class I plasmids given EOP_{50} values of 90 to 105 μg/ml for induced cells (experiment 1). This class includes plasmids pRT3, pRT24, and pRT1, as well as pRT61, a recombinant plasmid which carries the entire Tn*10* transposon (the construction of pRT61 has been described elsewhere [14]). Inspection of the physical maps of class I plasmids indicates that sequences in Tn*10* to the "left" of *Hpa*I fragment 2025 (see Fig. 3) do not contribute to tetracycline resistance. Also, from the fact that the insertions in pRT1 and pRT24 are identical but in opposite orientations relative to pVH51, we conclude that the orientation of this insertion has no effect on the expression of tetracycline resistance in these plasmids. Plasmids pRT2, pRT4, and pRT11 comprise class II; these plasmids confer resistance to much higher levels of tetracycline than do class I plasmids (experiments 1 and 3, Table 1). Class II plasmids pRT2 and pRT11 differ from class I plasmids in that they do not carry *Hpa*I fragment 3000; suggestions for a role played by fragment 3000 in tetracycline resistance will be discussed later. Class II plasmid pRT4 differs from class I plasmid pRT24 in that it is missing *Hpa*I fragment 675 and in that the orientation of *Hpa*I fragment 3000 is reversed. Explanation of the pRT4 resistance phenotype is complex and will be discussed below. Class III includes plasmids pRT22 and pRT29, which specify resistance only to low concentrations of tetracycline (experiment 2). These plasmids differ from class I plasmids in that they are missing both *Hpa*I fragment 3000 and *Hpa*I fragment 675, suggesting that sequences in fragment 675 play some role in resistance.

Tetracycline resistance levels in Table 1 are reported both before and after a 30-min induction period in the presence of 1 μg of tetracycline per ml. Resistance in all the recombinant plasmid strains described here is inducible by this treatment, indicating that the regulatory elements for the induction process lie within the 2,025-base pair *Hpa*I fragment of Tn*10*. It should be noted that the ratio of induced to uninduced EOP_{50} values may not be an exact reflection of the true induction ratio of those functions responsible for resistance, since measurement of the tetracycline resistance phenotype itself presumably leads to some induction.

Cells containing the R-factor R100 have much higher EOP_{50} values than pRT61 (a multicopy plasmid carrying the entire Tn*10* transposon) or the other class I plasmids. This observation can be explained in two ways: (i) R100 encodes functions not found in Tn*10* but which contribute to tetracycline resistance, or (ii) tetracycline resistance is less well expressed when Tn*10* is at high copy number. To distinguish these possibilities, the EOP_{50} values for cells containing a lysogenic λ::Tn*10* phage were determined (Table 1), with the result that a λ::Tn*10* lysogen is, within error, resistant to the same levels of tetracycline as a cell containing R100. This result favors the hypothesis that the low tetracycline resistance levels found in class I plasmids result from the high copy number of these plasmids. Class II plasmids have a resistance phenotype similar to R100; this observation suggests that a Tn*10*-encoded function that causes lower resistance levels at high copy number maps in *Hpa*I fragment 3000, a fragment that is missing class I plasmids. This hypothesis will be considered further in the Discussion.

Effect of deletions in Tn*10* on the synthesis of a tetracycline-inducible polypeptide. Many tetracycline-resistant R-factor strains encode a tetracycline-inducible polypeptide (referred to as TET protein) (19, 21). We have examined polypeptide synthesis in *E. coli* minicells containing recombinant plasmids to determine whether the TET protein encoded by R100 is encoded within Tn*10*, as well as to examine the effects of the various Tn*10* deletions carried by these recombinant plasmids on the synthesis of this protein. In this experiment, purified minicells were labeled with [^{35}S]methionine, and, after lysis of the minicells by heating in SDS, labeled proteins were analyzed by SDS-polyacrylamide gel electrophoresis and autoradiog-

raphy (see Materials and Methods). Minicells containing R100 or pRT3 synthesize a 36,000-dalton (36K) tetracycline-inducible polypeptide (see Fig. 5). In other experiments (data not shown), we have found that recombinant plasmids pRT24, pRT1, pRT11, and pRT61 also direct the synthesis of the same inducible polypeptide. Thus, the R100 TET protein of Levy and co-workers (19, 21) is indeed encoded by Tn*10*. (It should be noted here that the TET protein encoded by these recombinant plasmids has not always been observed in each SDS-polyacrylamide gel analysis. It may be that the inability to detect the TET protein is due to the failure to solubilize it from its site in the minicell membrane.)

Plasmids pRT22 and pRT29 direct the synthesis of a larger (37K) tetracycline-inducible polypeptide than does R100; this 37K polypeptide is probably unstable, being accompanied in the gel by many other lower-molecular-weight polypeptides whose synthesis is also induced by tetracycline (Fig. 5). pRT4, on the other hand, directs the synthesis of a stable 34K polypeptide. From the known physical structures of these plasmids (Fig. 3), it is possible to explain the occurrence of these different-sized polypeptides by concluding that a *Hpa*I site lies within the structural gene encoding the wild-type 36K polypeptide. This gene (*tet*) must then lie mostly within *Hpa*I fragment 2025 and cross one of the *Hpa*I sites defining this fragment. pRT22 and pRT29, which encode inducible polypeptides of the same size, both possess the same fusion of

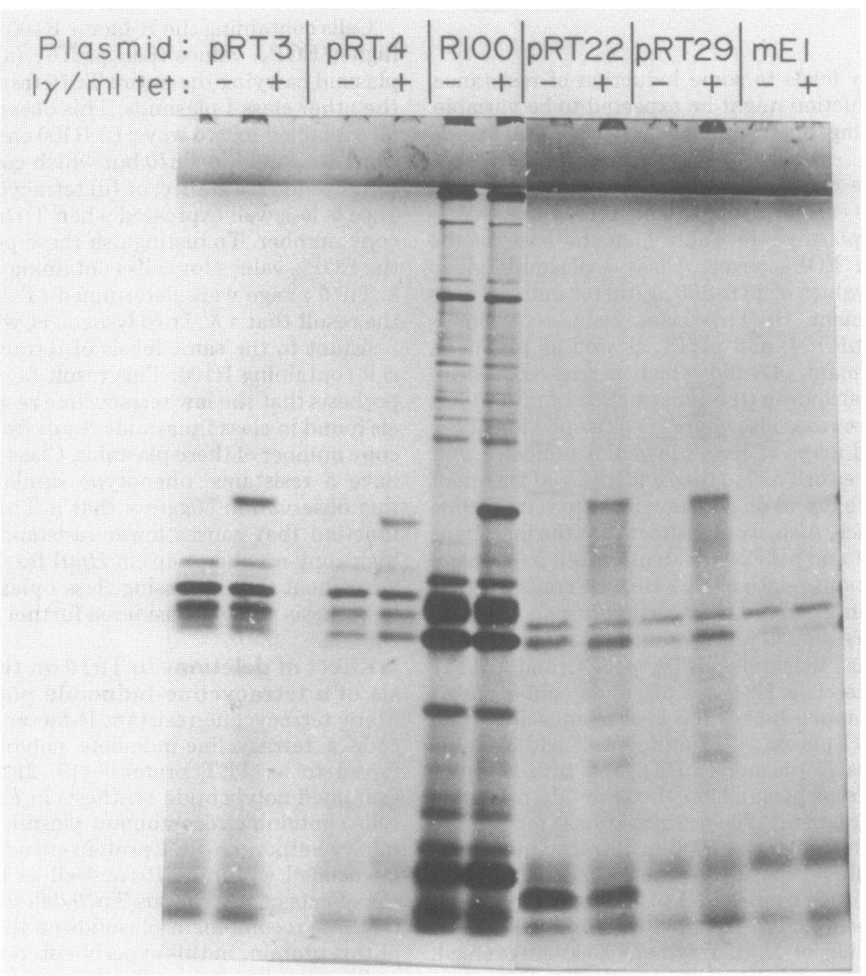

FIG. 5. *Autoradiogram of 15% polyacrylamide–SDS gel displaying plasmid-encoded polypeptides labeled with [^{35}S]methionine in minicells. Exposure time was 155 h. − and + indicate whether or not minicells were induced with 1 μg of tetracycline per ml at the time of labeling.*

fragment 2025 to pVH51 sequences, indicating that the *Hpa*I site in the *tet* gene is the one that lies between *Hpa*I fragments 2025 and 675 (see Fig. 3). In pRT4, the end of fragment 2025 defined by this site is joined to λ DNA sequences in fragment 3000 (Fig. 3), and so pRT4 encodes a different fusion polypeptide than do pRT22 and pRT29. These plasmids also encode regulatory functions for the induction of both resistance and the *tet* gene product; thus it would seem that the promoter region and the amino-terminus of the *tet* gene must be intact in plasmids pRT4, pRT22, and pRT29. If this is the case, the *Hpa*I site in the *tet* gene lies in the carboxy-terminus of the TET protein, and we can conclude that the direction of *tet* gene transcription is left to right, as the Tn*10* map is drawn in Fig. 3.

Yang et al. (35) have reported the synthesis of a 15K tetracycline-inducible polypeptide in R100-containing minicells. We have also observed this polypeptide in R100-containing minicells, but not in minicells containing any of the recombinant plasmids described here. The possibility thus exists that this protein is encoded by regions of R100 outside Tn*10*. We also observed several other tetracycline-inducible polypeptides, synthesized by both R100 and pRT series recombinant plasmid-containing minicells. These appear in the 15K-25K region of the gel but are present in relatively small amounts. These polypeptides may represent fragments of the 36K protein derived from mild proteolysis or premature translation termination.

RNA polymerase protection of an *Hin*cII cleavage site in Tn*10*: possible location of the *tet* gene promoter. We have concluded above that the start point of transcription of the *tet* gene lies in *Hpa*I fragment 2025 because a recombinant plasmid (pRT29) carrying only this fragment shows essentially normal regulation of tetracycline resistance and *tet* gene product synthesis. RNA polymerase protection of *Hin*cII cleavage sites has been observed in several *E. coli* promoters, e.g., the *trp* operon promoter (5, 13) and the bacteriophage λ P_L and P_R promoters (1, 2, 23, 25, 34). As shown by the "protection experiment" presented in Fig. 6, prior incubation of Tn*10* DNA with *E. coli* RNA polymerase inhibits *Hin*cII cleavage of the *Hin*cII site within *Hpa*I fragment 2025, i.e., *Hin*cII/*Hpa*I fragment 2025 is incompletely digested to fragments 1275 and 695. Of the nine *Hin*cII sites within Tn*10*, only this site is protected by RNA polymerase. Addition of the four ribonucleoside triphosphates to the RNA polymerase binding mixture prior to *Hin*cII treatment allows complete cleavage at this *Hin*cII site, indicating that, in vitro at least, this RNA polymerase binding site can also act as a site for transcription initiation. Although the direction of transcription from this site is not yet known, the obvious suggestion is that this site corresponds to the *tet* promoter.

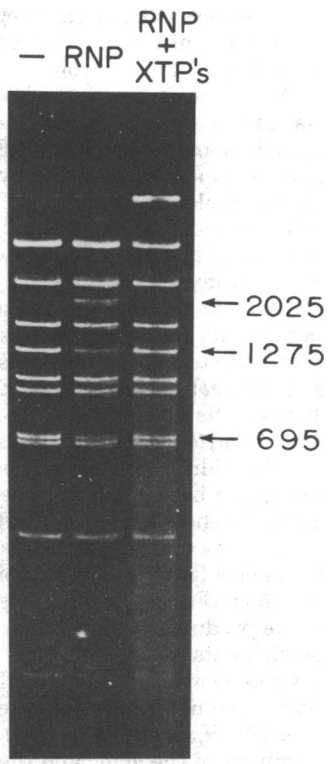

FIG. 6. *RNA polymerase "protection experiment." Columns are labeled as follows: −, no RNA polymerase added before HincII; RNP, RNA polymerase bound prior to HincII treatment; RNP + XTP's, RNA polymerase and ribonucleoside triphosphate added prior to HincII. 695 and 1275 are HincII fragments from Tn10. 2025 is the fragment resulting from RNA polymerase protection of the HincII cleavage site between fragments 1275 and 695.*

DISCUSSION

Tn*10*, which originates from the R-factor R100, carries all the structural genes necessary to confer the same level of tetracycline resistance on *E. coli* cells as R100. This was demonstrated by the observation (Table 1) that a lysogen of a λ::Tn*10* transducing phage is resistant to the same levels of tetracycline as is an R100-containing cell. Further, tetracycline resistance in both λ::Tn*10*- and R100-containing cells is inducible by subinhibitory concentrations of tetracycline, indicating that regulatory elements required for the induction process are also encoded by Tn*10*.

Levy and co-workers (19, 21) have reported the R-factor-directed synthesis of a tetracycline-inducible polypeptide (TET protein) in *E. coli* minicells. Minicell analysis of a series of recombinant plasmids carrying various portions of Tn*10* (Fig. 5) demonstrates that the TET protein is, as might be expected, encoded by Tn*10*, as are its regulatory elements.

The recombinant plasmids described in this paper provide a set of Tn*10* deletions with which it is possible to study the role played by different regions of Tn*10* in tetracycline resistance and the regulation of its expression. By inspection of the physical structures of these plasmids, the structural gene (*tet*) encoding the TET protein can be shown to lie within the 2,700-base pair-long region of Tn*10* carried by plasmid pRT11 (see Fig. 3). In addition, an *Hpa*I cleavage site within this region lies in the *tet* gene; this was demonstrated by the observation that fusion of different sequences to this site results in alterations in the molecular weight and stability of the *tet* gene product (Fig. 5). In all cases these altered *tet* gene products are inducible. Thus, the regulatory elements are not affected in the fusion strains and lie wholly within *Hpa*I fragment 2025, as do the promoter and amino-terminus of the *tet* gene. The *Hpa*I site in *tet* then lies in the carboxy-terminus of the gene, and the direction of *tet* transcription is from left to right as the Tn*10* physical map is drawn in Fig. 1 and 3.

The conclusion that the gene for tetracycline resistance resides largely within *Hpa*I fragment 2025 and more precisely within *Hinc*II fragment 1275 is consistent with structural studies of the Tn*10* transposon carried out on plasmid R6-5 by Sharp et al. (30). R6-5 is a *tet*^{s} plasmid in which an insertion is located within Tn*10* approximately 1,300 base pairs from one inverted repeat. Tetracycline-resistant derivatives of R6-5 arise by loss of this insertion. This insertion must either be in *Hin*dIII-*Hinc*II fragment 685 on the "left side" of the transposon, which our data show not to be required for tetracycline resistance, or in *Hinc*II fragment 1275 (see restriction map in Fig. 1).

In Fig. 7 we present schematically a simple model for the location and organization of the *tet* gene and its regulatory elements. The location of the *tet* gene and direction of its transcription are shown as stated above. The location of the promoter is inferred from the RNA polymerase "protection experiment" (Fig. 6). This experiment shows that an *Hinc*II cleavage site in *Hpa*I fragment 2025 lies in a region of in vitro transcription initiation. Recent experiments (L. Wray, R. Jorgensen, and W. Reznikoff, unpublished data) have shown that removal of *Hinc*II fragment 695 and fusion of other *Hinc*II fragments to *Hinc*II fragment 1275 affects the level of tetracycline resistance but not the size of the *tet* gene product. This result strongly suggests that the *Hinc*II cleavage site in *Hpa*I fragment 2025 lies in the *tet* gene promoter, with the result that fusions at this site lead to altered promoter activity.

Mapping the *tet* promoter at this site is also consistent with the location of the start point of *tet* translation, which can be approximately determined from the size of the *tet* gene product. The carboxy-terminus of this protein has been shown here to lie in *Hpa*I fragment 675, probably very near (within 100 base pairs of) the *Hpa*I site, which divides fragments 2025 and 675 (assuming that only a small portion of the carboxy-terminus of this protein can be replaced with a new amino acid sequence while maintaining the protein's resistance function). The apparent molecular weight of the *tet* gene product is 36,000 (36K) in Tris–glycine-buffered SDS–polyacrylamide gels (this paper and ref. 34) and 50,000 (50K) in a phosphate-buffered SDS–polyacrylamide gel (19). If the mass of one amino acid is 120 daltons, a 36K protein contains 300 amino acids and requires a 900-base pair-long coding sequence, while a 50K protein contains 420 amino acids and requires a 1,260-base pair-long coding sequence. With 100 base pairs as an estimate of the length of *tet* extending into *Hpa*I fragment 675, it follows that the amino terminus of the *tet* gene lies roughly 800 to 1,160 base pairs to the left of *Hpa*I fragment 675 within

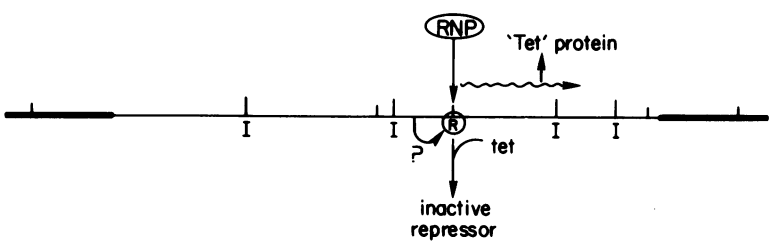

FIG. 7. *Model for organization of tet gene and its regulatory elements. RNP is RNA polymerase, R is repressor. I indicates HpaI cleavage sites in Tn10. Unlabeled vertical bars are HincII cleavage sites in Tn10.*

*Hpa*I fragment 2025. Although this is a wide range on the scale of the size of promoters, it is certainly consistent with a promoter location at the *Hinc*II site 1,275 base pairs left of *Hpa*I fragment 675. Also consistent is the finding that *Hinc*II fragments 695 and 1275 display no RNA polymerase binding activity (15; R. Jorgensen, Ph.D. Thesis, University of Wisconsin, Madison, 1978); thus the "protected" *Hinc*II site is the only known in vitro candidate for the *tet* promoter in *Hpa*I fragment 2025.

Franklin and Cook (8) and Yang et al. (35) have postulated that tetracycline resistance is negatively regulated. Our results, regardless of the mode of regulation, map the regulatory elements that control tetracycline resistance and TET protein synthesis in *Hpa*I fragment 2025. The model in Fig. 7 suggests a more precise location (in *Hinc*II fragment 695) for the coding region for the "repressor" of Yang et al. (35). This is consistent with the observation by Reeve (27) that a *tet*^{s} insertion mutation of R6 (plasmid R6-5) carries an active TET-repressor gene, although more complicated models such as the existence of overlapping *tet* and repressor genes are not ruled out.

Levy et al. (21) have suggested that the TET protein is responsible for tetracycline resistance, based on observations that the amount of this protein synthesized in minicells seems to be correlated with the level of tetracycline resistance conferred by the particular plasmid encoding it and that tetracycline-sensitive mutants of R222 do not synthesize TET protein. Our observations concerning the effect of Tn*10* deletions on tetracycline resistance and on the molecular weight and stability of the TET protein are entirely consistent with this view. We have isolated other fusions at the *Hpa*I site in the *tet* gene through attempts to use pRT29 as an *Hpa*I cloning vehicle for bacteriophage T7 *Hpa*I fragments (while maintaining selection for tetracycline resistance) and for various *Hpa*I fragments with certain selectable characters (e.g., aminoglycoside resistance) (N. Panayotatos, L. Wray, R. Jorgensen, and W. Reznikoff, unpublished data). These fusions also have altered tetracycline resistance levels, supporting a conclusion that the TET protein is both necessary and sufficient for tetracycline resistance.

Taylor et al. (33) have described a copy number mutant of NR1 called ROR12 with a markedly lower level of tetracycline resistance and suggest that this phenotype is a direct result of the higher copy number of ROR12. Recombinant plasmid pRT61, a derivative of the multicopy plasmid ColE1 containing the entire Tn*10* transposon, is resistant to only about one-half the level of tetracycline as is a single-copy λ::Tn*10* lysogen or R100 (Table 1). pRT61 appears to be resistant to higher levels of tetracycline than has been reported for ROR12; however, these two plasmids have not been tested in the same host strain. Taylor et al. (33) have further suggested that the multicopy effect on resistance in ROR12 is due to overproduction of the *tet* gene repressor by multicopy plasmids. Another possibility is that some Tn*10*-encoded function interacts directly with the *tet* gene product. Evidence for the existence of such a Tn*10*-encoded function is provided by the observed differences between class I and class II plasmids, such as pRT1 and pRT11. Although both of these plasmids encode a wild-type *tet* gene product, pRT1 is markedly less resistant to tetracycline than is pRT11. The only difference between these plasmids is that *Hpa*I fragment 3000 is not present in pRT11. This suggests that fragment 3000 encodes a function which contributes to the lowered resistance in multicopy plasmids.

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