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Synthesis of an R plasmid protein associated with tetracycline resistance is negatively regulated

(plasmid/*in vitro* protein synthesis/*Escherichia coli* minicells)

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ABSTRACT **Synthesis of proteins encoded by the R222 plasmid was observed in a DNA-directed cell-free system and the products were compared to those plasmid proteins synthesized in *Escherichia coli* minicells. A greater number of plasmid-specified proteins was detected in the *in vitro* system than in the minicell, suggesting the presence of control factors for plasmid gene expression in the minicell. Synthesis of a newly detected plasmid protein (*TET* protein) is induced by tetracycline in minicells containing tetracycline-resistant plasmids, including R222, and this induced synthesis correlates with induced host resistance to the drug. This *TET* protein was synthesized *in vitro* from R222 DNA in the absence of tetracycline, indicating that no positive regulatory role for tetracycline is required for the protein's synthesis. *TET* protein synthesis was inhibited *in vitro* when cell-free extracts prepared from cells containing the R222 plasmid were used. This inhibition was reversed by adding tetracycline or a tetracycline analog to the system. A protein factor that selectively inhibits *TET* protein synthesis *in vitro* was partially purified from R222-containing cells. This protein, presumed to be plasmid encoded, appears to function as a negative regulator that is antagonized by tetracycline.**

Plasmids are extrachromosomal genetic elements capable of autonomous replication in bacteria (1–3). R plasmids (R factors) confer multiple drug resistance to their bacterial host. The increase in R plasmids in our environment, following the widespread use of antibiotics, has provoked both clinical and epidemiologic concerns that have prompted studies of the mechanisms of antibiotic resistance and of plasmid metabolism. Most plasmid-conferred drug resistances in *Escherichia coli* are constitutively expressed (2, 3). R plasmid-mediated tetracycline (Tc) resistance, however, is inducible (4, 5, 6, 7). The R222 plasmid contains genes responsible for resistance to Tc, chloramphenicol, streptomycin, and sulfonamides (1). Whereas initially resistant to low levels of Tc, R222-containing cells become refractory to high levels of Tc after exposure to subinhibitory levels of Tc (6–8).

The study of particular R plasmid gene products has been aided by the use of the minicell system (9–14). Minicells, tiny DNA-less cells formed by abnormal cell division (15), receive R plasmids segregating into them from the parent cell. In the minicell, the R plasmids find cell components necessary for the synthesis of R plasmid-specified proteins. With the use of this system, certain R plasmid gene products have been identified, including a membrane protein (*TET* protein) associated with induced Tc resistance (8). Only R plasmids expressing Tc resistance code for this protein (7). Since Tc resistance correlates with decreased permeability of the cell to the drug (4, 5), the presence of this protein in the cell membrane suggests that it functions by impeding entry of the drug into resistant cells.

The minicell system has limitations when one is attempting to characterize certain regulatory events. To investigate the regulatory system for Tc resistance we have focussed on the synthesis of *TET* protein in an *in vitro* coupled transcription–translation system (16). Identification of this protein and other plasmid proteins has been performed by coincident gel electrophoresis of proteins synthesized *in vitro* and in minicells. Utilizing the two systems, we have identified the synthesis of *TET* protein *in vitro* and have been able to study the regulation of its synthesis.

Abbreviations: *TET*, plasmid protein synthesized in response to tetracycline; Tc, tetracycline; 514, *E. coli* strain 514; 514/R222, *E. coli* strain 514 containing the R222 plasmid.

MATERIALS AND METHODS

Bacterial Strains. *E. coli* minicell strain D1-7 (9), which contains the R222 plasmid, was used for studies of protein synthesis in intact cells. *E. coli* strain 514 and its R222-containing derivative, 514/R222 (D1-83), were used for cell-free syntheses.

Media. Minicell strains were grown in enriched media (11), and R plasmid proteins were labeled with [^{35}S]methionine in methionine assay medium as reported (11). Growth and collection of cells for S-30 preparation followed previous descriptions (16).

Chemicals. Tetracycline was purchased from Calbiochem. The Tc analog 5α-11α-dehydro-7-chlortetracycline was a gift from M. Inoue and S. Mitsuhashi.

Minicell Analyses. Minicell purification followed previously reported methods (10, 17). R222 plasmid DNA was obtained from minicells collected from 30 liters of cultured strain D1-7 (11). Preparation of ^{35}S-labeled minicell protein for gel analysis was as described (11, 12). Gradient gel analysis of the radioactively labeled proteins was as described below.

***In Vitro* R222 DNA-Directed Protein Synthesis.** Procedures used for preparation of S-30 and conditions for *in vitro* synthesis have been described elsewhere (16) except for the following modifications: Synthesis was carried out in a final volume of 100 μl to which 30 μCi of [^{3}H]leucine (New England Nuclear, 1 mCi/ml) was added. Forty to sixty μg/ml of DNA was used to program synthesis. After 90 min at 37°, synthesis was terminated by the addition of 100 μg/ml of DNase (Worthington). DNase treatment was advantageous for subsequent gel analysis. A 5 μl sample containing about 0.8 μCi of ^{3}H-labeled protein was used in each gel analysis. Tc, 5α-11α-dehydro-7-chlortetracycline, 3′:5′-cAMP, and ppGpp when tested were added at concentrations of 2 μg, 2.5 μg, 0.5 μmol, and 0.1 μmol/ml, respectively.

Procedure for Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis and Photofluorography. The samples

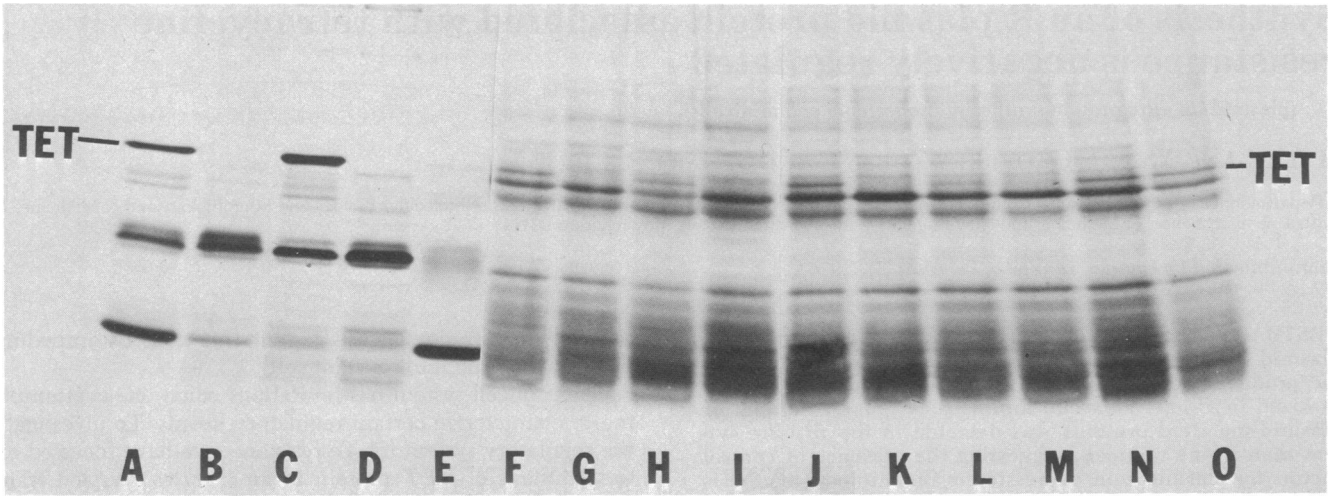

FIG. 1. Fluorogram of sodium dodecyl sulfate–acrylamide gel pattern obtained by electrophoresis of *in vivo* ^{35}S-labeled protein from R222-containing minicells (A–E) and ^{3}H-labeled protein from R222 DNA-directed cell-free synthesis (F–O). *TET* indicates the position of *TET* protein, which has a molecular weight of about 3.6×10^4 as determined from a standard calibration curve using several protein markers of known molecular weight. Migration is from top to bottom. A—protein from Tc-induced minicells. B—same as A without Tc. C—membrane fraction of A. D—membrane fraction of B. E—supernatant fraction of A. F and O—proteins from R222 DNA-directed cell-free system using S-30 from strain 514. G—same as F except strain 514/R222 was used for S-30 preparation. H—same as G except 2 μg/ml of Tc was added prior to synthesis. I—same as G except 10 μg/ml of streptomycin was present during synthesis. J—same as F. K—same as F except 0.3 mg/ml of 514/R222 protein from a DEAE-cellulose column was added prior to synthesis. L—same as F except that 0.24 mg/ml of 514/R222 protein from the run through fraction of a DNA-cellulose column was added prior to synthesis. M—same as F except that 0.014 mg/ml of 514/R222 protein from the 0 to 0.3 M NaCl fraction of a DNA-cellulose column was added prior to synthesis. N—same as F except that 0.004 mg/ml of 514/R222 protein from the 0.3 to 1.0 M NaCl fraction of a DNA-cellulose column was added prior to synthesis. See *Materials and Methods* and *Results and Discussion* for further explanation.

synthesized *in vitro* were washed briefly with 90% ice-cold acetone and loaded onto a polyacrylamide slab. Electrophoresis was done by the method of Studier (18). The gel consisted of a 5% acrylamide stacking gel on top of a 10–20% linear gradient of acrylamide. Electrophoresis was performed at room temperature at 150 V for 1.7 hr. The gel was immediately processed and photofluorographed according to the method of Bonner and Laskey (19) except that 0.04% (dimethyl POPOP) 1,4-bis[2(4-methyl-5-phenyloxazolyl)]benzene was added to enhance activation. For *in vivo* and *in vitro* samples film was exposed for 15 days and 3 days, respectively. The resulting fluorographs on Kodak X-O-mat RP film were scanned on a Gilford 240-spectrophotometer.

Partial Purification of Protein Fractions Containing Inhibitor of *TET* Protein Synthesis. Inhibitor-containing fractions were prepared from strain 514/R222 by chromatography on DEAE-cellulose and double-stranded calf thymus DNA-cellulose as described (20).

RESULTS AND DISCUSSION

Induction of Increased Tetracycline Resistance and Synthesis of *TET* Protein by Exposure of R222-Containing Cells to Low Levels of Tetracycline. The antibiotic tetracycline (Tc) inhibits the growth of a broad range of bacteria by blocking translation at the level of the binding of aminoacyl-tRNA to the ribosome (21). Many R plasmids, including R222, confer resistance to Tc. This inducible resistance has been correlated with the induced synthesis of a membrane protein called the *TET* protein (8). The effect of Tc on induced Tc resistance in strain 514/R222 was examined prior to its use for S-30 in the *in vitro* system. As in other R222-containing bacteria, resistance to Tc was enhanced after growth of the host cell at subinhibitory concentrations of Tc. Although cell growth was originally inhibited by 100 μg of Tc, preincubation of cells for 30 min with as little as 1 μg of Tc induced resistance to Tc at 100 μg/ml.

The effect of Tc on R222-programmed protein synthesis *in vivo* is advantageously studied in minicells containing R222 DNA but little, if any, *E. coli* chromosomal DNA. Proteins synthesized in R222 DNA-containing minicells in the absence and presence of Tc are compared in Fig. 1. The presence of Tc causes a large increase in the synthesis of the (*TET*) protein with an estimated molecular weight of 3.6×10^4, whereas molecular weight estimation of this *TET* protein on gel using phosphate buffer system is 5×10^4 (8) (compare A and B in Fig. 1). That this protein is, in fact, the *TET* protein is further demonstrated by its association with the membrane fraction (compare C and E in Fig. 1). Inspection of frames A, B, C, and E shows that tetracycline appears to induce the synthesis of at least one other protein in the low molecular weight region (estimated molecular weight about 15×10^3). This protein has not been further studied in the cell-free system because of the high backgrounds in this region of the fluorogram.

Synthesis of the *TET* Protein in an R222 DNA-Directed Cell-Free System for Coupled Synthesis of RNA and Protein. With the evidence that Tc induces *TET* protein synthesis, we set out to define the mechanism for this action by using an R222 DNA-directed system for RNA and protein synthesis. This type of system has been useful in studying the regulation of a number of genes (20) because it offers the possibility to add or withdraw the factors involved in the regulation and thereby to clarify their function. R222 DNA was used in an S-30 extract of strain 514 with the essential salts and substrates for RNA and protein synthesis (see *Mate-*

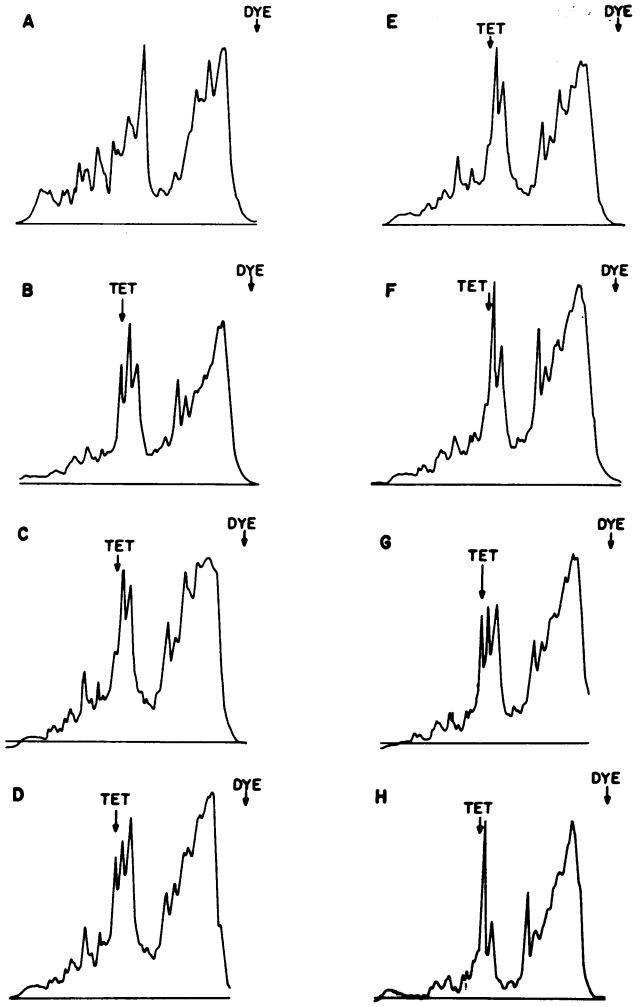

FIG. 2. Densitometry tracings of fluorograms obtained from sodium dodecyl sulfate–acrylamide gel electrophoresis of ^{3}H-labeled protein from DNA-directed cell-free synthesis. Whereas the data presented in Figs. 1 and 2 are comparable, quantitative measurements are facilitated by densitometry. Migration in gel is from left to right with the dye front indicated. The position of *TET* protein is also indicated. R222 DNA was used to direct synthesis except in A where *E. coli* chromosomal DNA was used. A—S-30 from strain 514. B—S-30 from strain 514. C—S-30 from strain 514/R222. D—same as C except that 2 μg/ml of Tc was added prior to synthesis. E—same as C except that 10 μg/ml of streptomycin was added prior to synthesis. F—same as B except that 0.3 mg/ml of 514/R222 protein from a DEAE-cellulose column was added prior to synthesis. G—same as F except that 2 μg/ml of Tc was added prior to synthesis. H—same as B except 0.014 mg/ml of 514/R222 protein from a 0 to 0.3 M NaCl fraction of a DNA-cellulose column was added prior to synthesis. See *Materials and Methods* and *Results and Discussion* for further explanation.

rials and Methods), and more than 30 distinct [^{3}H]leucine-labeled protein bands were characterized as products of cell-free synthesis by sodium dodecyl sulfate–polyacrylamide gel electrophoresis and fluorography (F–O in Fig. 1). No labeled proteins are visible if DNA is left out of the cell-free system, and a very different profile of proteins is visible when *E. coli* DNA is used in place of R222 DNA (A in Fig. 2). When the proteins from the cell-free system were compared with those synthesized in minicells, certain bands of similarity and dissimilarity were found (compare A–E with F–O in Fig. 1). More proteins were detectable in the *in vitro* system. The relatively small number of detectable proteins synthesized in either system and the differences in the amounts of each protein indicated that not all regions of the R222 DNA were being equally expressed. Longer exposure or more intense labeling revealed some additional protein products. To determine if any alteration in this *in vitro* system would occur with S-30 from an R plasmid-infected cell, R222 DNA was studied in S-30 from strain 514/R222. One major protein was greatly diminished when the latter S-30 system was used (compare F and G in Fig. 1 and B and C in Fig. 2); this protein migrated with the same mobility in the gel as *TET* protein.

Factors Affecting Expression of the *TET* Gene *In Vitro*. The gene for the *TET* protein is expressed in relatively large amounts in an S-30 extract from strain 514 in the absence of Tc. (F in Fig. 1 and B in Fig. 2.) The level of expression is not appreciably altered by cAMP, cGMP, or ppGpp (not shown). Rifampicin, at a level of 2 μg/ml, completely eliminated *TET* synthesis. The fact that *TET* protein is made in an S-30 extract in the absence of Tc suggests that no special regulatory process involving Tc is required for turning on the *TET* gene in R222 DNA.

If the S-30 extract used in the *in vitro* synthesis came from the R222-containing strain (514/R222), only trace amounts of the *TET* protein were synthesized (G in Fig. 1 and C in Fig. 2). Other proteins were less affected. This inhibition indicated that an inhibitor was present in the S-30 extract prepared from 514/R222 that was not present in 514 S-30 extracts. It seems likely that R222 DNA contains a gene for this inhibitor although no such gene has yet been identified from genetic studies. Studies were undertaken to antagonize this "repression" with Tc. A level of Tc was sought that would affect the hypothesized repressor without producing gross inhibitory effects on protein synthesis. As a test system for these studies, β-galactosidase synthesis was studied in a λ *plac5* DNA-directed system (22) at various levels of Tc. At 2 μg/ml of Tc no inhibition of β-galactosidase synthesis was seen when an S-30 from strain 514/R222 was used, whereas with S-30 from strain 514, 20% inhibition was observed. As would be expected, higher levels of Tc (5 μg/ml) inhibited β-galactosidase in either S-30 system. Although it has been noted that R222-conferred Tc resistance is associated with decreased permeability of the drug in the cell, the reduced sensitivity to Tc in the soluble 514/R222 S-30, where little if any membrane protein would be expected, suggests that Tc resistance may not result exclusively from a permeability effect. Possibly this is related to the low-molecular-weight cytoplasmic protein induced in R222-containing minicells commented on above.

In the 514/R222 S-30 system with R222 DNA, 2 μg/ml of Tc produced a large increase in *TET* protein synthesis (compare G and H in Fig. 1 and C and D in Fig. 2). This is also true for S-30 made from such cells grown in the presence of Tc. The inhibitor of *TET* protein synthesis in such extracts was antagonized by Tc. The Tc analog 5α-11α-dehydro-7-chlortetracycline, which does not inhibit protein synthesis, showed the same effect, whereas streptomycin, another *Streptomyces*-derived antibiotic, showed no such effect (I in Fig. 1 or E in Fig. 2; note: since strains used in this study were streptomycin resistant, streptomycin produced no nonspecific inhibitory effects on translation). This reversal *in vitro* by Tc established a strong parallel between the effects of Tc in whole cells, minicells, and the cell-free system. Although 2 μg/ml of Tc inhibited gross protein synthesis to some extent when a 514 S-30 was used, no selective effect on *TET* protein synthesis was seen.

Partial Purification of the Inhibitor of *TET* Protein Synthesis. Further studies focussed on using the cell-free system as an assay tool for purification of the inhibitor. For this purpose R222 DNA and S-30 from strain 514 were used and the effects of adding fractionated extracts from strain 514/R222 were determined. A nucleic-acid-free DEAE-cellulose eluate (see *Materials and Methods*) of strain 514/R222 protein contained the inhibitor (K in Fig. 1 and F in Fig. 2) whose activity *in vitro* was reversed by Tc (compare F and G in Fig. 2). Comparable extracts of strain 514 showed no inhibitory activity. The DEAE-cellulose eluate was further fractionated on a DNA-cellulose column (see *Materials and Methods*). Although some inhibitor activity was present in the run-through fraction (L in Fig. 1) the bulk of the activity was eluted in the 0–0.3 M NaCl fraction (M in Fig. 1 and H in Fig. 2); this latter fraction was estimated to be greater than 50-fold enriched over the DEAE step, since it accounted for only about 1% of the soluble protein in the cell. Both the DEAE-cellulose fraction and the 0–0.3 M NaCl DNA-cellulose fraction gave maximum inhibition at about one-fifth of the levels used in Figs. 1 and 2.

It seems highly likely that the inhibitor of *TET* protein synthesis is a negative control protein operating at the level of transcription or translation. Schematically the control system could function very much like the negative control systems seen for various bacterial operons like the *lac* operon (22), the inhibitor functioning as repressor and Tc as inducer. Further purification work is necessary to establish the exact nature of this inhibitor and its mode of action in regulating *TET* protein synthesis.

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