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Regulation of Dihydrofolate Reductase Synthesis in *Escherichia coli*

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Summary. Two clones from the Clarke-Carbon *Escherichia coli* colony bank were resistant to inhibition by trimethoprim, a potent inhibitor of dihydrofolate reductase. Both clones had elevated levels of dihydrofolate reductase. Furthermore, trimethoprim resistance and elevated enzyme levels were associated with ColE1 plasmids that carried DNA from the *trkC ksgA pdxA* region of the *E. coli* chromosome. Plasmid pLC1437a was shown by two criteria to carry the structural gene for dihydrofolate reductase: 1) A partial diploid containing plasmid pLC1437a produced a kinetically-recognizable dihydrofolate reductase that was not present in the parent haploid strain. 2) Plasmid pLC1437a coded for dihydrofolate reductase in vitro. A 1,000 base pair fragment of plasmid pLC1437a containing *fol* was used as a probe to measure *fol* mRNA in a mutant strain isolated by Sheldon and Brenner (Molec. gen. Genet. 147, 91–97, 1976). The mutation in this strain, which results in constitutively-high levels of dihydrofolate reductase and in the inability of the strain to grow at 42° C, is *cis* dominant (Sheldon and Brenner, 1976). The results of kinetic hybridization and pulse-labeling experiments indicated that the regulatory mutant produced elevated levels of dihydrofolate reductase in response to an increased rate of synthesis of *fol* mRNA.

Introduction

The NADPH-dependent reduction of dihydrofolate to tetrahydrofolate, which is catalyzed by dihydrofolate reductase (DFRase, EC 1.5.1.3), is an important reaction in metabolism because tetrahydrofolate serves as a reducing agent during the conversion of deoxyuridylic acid to deoxythymidylic acid (Osborn and Huennekens, 1958; Friedkin, 1973). DFRase binds tightly to and is inhibited by analogues of folic acid such as methotrexate (Werkheiser, 1961), and a number of them are useful clinically as antibacterial, antiprotozoal, immunosuppressant, and antineoplastic agents (Bertino and Johns, 1972). Some attention has been focused on mechanisms by which cells become resistant to analogues of folic acid. Of particular interest in terms of significance to gene expression are those cases in which resistance results from an increased amount of DFRase per cell. For example, some methotrexate-resistant mutants of *Diplococcus pneumoniae* have levels of DFRase2- to 100-fold higher than the wild-type level (Sirotnak, 1970). Unexpectedly, the mutations that lead to high enzyme levels appear to map in the structural gene for DFRase (Sirotnak, 1970). Mammalian cells resistant to folate analogues have also been isolated and studied extensively (Kellems et al., 1976; Chang and Littlefield, 1976). In one case, a several hundred-fold increase in enzyme level was correlated with a corresponding increase in the number of genes coding for DFRase (Alt et al., 1978).

Analogue-resistant mutants of *Escherichia coli* have also been isolated (Breeze et al., 1975; Sheldon and Brenner, 1976) but have not been studied in as much detail as the systems mentioned above. Of several dozen mutants resistant to trimethoprim studied by Sheldon and Brenner (1976), about half produced a DFRase that was less inhibitable by trimethoprim (Ki mutants) and the other half had 2- to 30-fold elevated levels of DFRase (regulatory mutants). Those regulatory mutations that were analyzed were dominant to the wild type allele and caused increased expression of structural genes when in a *cis* but not a *trans* position. Of particular interest was their finding that strains having the *cis*-acting mutation *fol-60* (20-fold elevated levels of DFRase) were unable to

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grow at 42° C. That a single mutation resulted in both temperature-sensitive growth and altered levels of DFRase was indicated from an analysis of revertants that grew at the restrictive temperature. Most revertants had enzyme levels different from the parent *fol-60* strain, individual isolates having from less than a tenth to more than fifteen times the wild-type enzyme level (Sheldon and Brenner, 1976).

Cis-dominant effects are usually associated with regulatory regions such as promoters or operators. The observation of a *cis*-dominant mutation with an associated temperature-sensitive phenotype invites further study. A number of models can explain the pleiotropic phenotype of *fol-60*. To aid in distinguishing between some of them, we screened the Clarke-Carbon *E. coli* colony bank (Clarke and Carbon, 1976) and identified two plasmids carrying *fol*, the structural gene for DFRase. We used one of these plasmids as a probe in hybridization studies to show that *fol-60* causes an increase in the rate of synthesis of *fol* mRNA.

Materials and Methods

Bacterial Strains and Growth Conditions. The strains used in this study are described in Table 1. The Clarke-Carbon colony bank (Clarke and Carbon, 1976), containing plasmid ColE1 attached to portions of the genome of strain CS520 in host JA200, was a gift from J. Carbon. Rich media included L-broth (Miller, 1972), L-agar (Miller, 1972), and LS-agar (Sparling et al., 1973). Minimal media included M9 (Miller, 1972), SSA (Calvo et al., 1969) and these two solidified with 1.5% agar. Minimal medium was supplemented with sugars (1%), vitamins (1 μg/ml), casamino acids (1%) or individual amino acids (50 μg/ml), and pyrimidines (20 μg/ml) when required.

The following conditions were employed for growing cells: for assay of DFRase, A_{550}-0.9 (Zeiss Spectrophotometer), L-broth, 37° C; for purification of DFRase, A_{550}-1.2, L-broth, 37° C; for isolation of RNA, A_{550}-0.9, M9-casamino acids-glucose 30° C; for isolation of plasmid DNA, A_{550}-0.9, M9-casamino acids-glucose, 37° C; for preparation of competent cells, A_{550}-0.6, L-broth, 37° C.

Plasmid Preparation, Restriction Endonuclease Digestions and Transformation Procedures. Plasmid DNA was amplified and cleared lysates prepared by procedures described by Kupersztoch-Portnoy et al. (1974) except that the concentration of Triton X100 was increased to 1%. CsCl gradients were prepared as follows: 15 g of CsCl was dissolved in 16 ml of cleared lysate and after warming to 22° C, the mixture was centrifuged at 22° C for 15 min at 16,000 × *g*. Following addition of 0.7 ml of ethidium bromide (10 mg/ml, Sigma) to the supernatant, the samples were centrifuged again and the resulting supernatant was centrifuged to equilibrium as described (Kupersztoch-Portnoy et al., 1974).

For isolation of the 1,000 base-pair *PstI*/*SalI* fragment (Fig. 4), 500 μg of plasmid pLC1437a DNA was digested for 16–24 h with 50 units of each enzyme (New England Bio Labs) in a total volume of 500 μl containing 50 mM NaCl, 6 mM 2-mercaptoethanol, 6 mM $MgCl_2$, 6 mM Tris-Cl, pH 8. Following electrophoresis through a 1% agarose slab gel (20 cm × 40 cm × 0.6 cm, 50 mM Tris-acetate, pH 8.3, 0.5 μg/ml ethidium bromide, 80V, 16 h), fragments were visualized by fluorescence and extracted from the gel by the procedure of Zain and Roberts (1978).

Transformation was carried out according to Clarke and Carbon (1975), except that 0.1 M $CaCl_2$ was used throughout and incubation in the presence of DNA was for 30 min and was preceded by warming to 37° C for 30 s.

Table 1. Strains of *E. coli* K12 used in this study

Strain	Genotype	Source
RSO	Hfr *met thi*	R. Sheldon
RS16	Hfr *met thi fol-38*	R. Sheldon
RS35	Hfr *met thi fol-60*	R. Sheldon
JA200	F^+ C600 *recA*	J. Carbon
CS520	HfrC *trpA58 metB glyV su-58*	J. Carbon
TK118	F^- *kdpABC5 lacZ82 gal-33 rha-4 thi-1 trkC118*	W. Epstein
TPR201	F^- *thr leu str lac xyl mtl mal rns pnp thi ksgA*	O.A. Andresson
OSA14	F^- *thi rha lacZ gal kdpABC5 pdxA ksgA*	O.A. Andresson
MI178	Hfr *carA178 relA1 metB1*	B. Bachmann
JC411/ColE1	*arg his leu met lacY malA* λ^r *mtl str*	V. Hershfield
M94	F^- *thr-1 leu-6 thi 1 lacY1 tonA21* λ-supE44 hsr-*1*	B. Bachmann

Hybridization Kinetic Analysis. RNA was isolated from 100 ml cultures by a hot phenol method (Ikemura and Dahlberg, 1973) using the lysozyme treatment described by Cooper et al. (1974). It was dissolved in 100 mM sodium acetate containing 10 mM magnesium acetate (pH 7.7) and incubated at 37° C with 40 μg of DNase I (Sigma-electrophoretically purified). The latter had previously been dissolved in water (0.5 mg/ml) containing 0.25% diethylpyrocarbonate and incubated for 2.5 h at 37° C. The sample was extracted two times with phenol, four times with ether, brought to 0.3 M in sodium acetate (pH 5.6), precipitated with three volumes of ethanol, and dissolved in TEN buffer (10 mM Tris-Cl, pH 8, 10 mM NaCl, 1 mM EDTA) containing 0.1% sodium dodecyl sulfate (SDS) at a concentration of 10 mg/ml.

The 1,000 base pair *PstI*/*SalI* fragment from plasmid pLC1437a was labeled by nick translation (Rigby et al., 1977) to 4×10^7 cpm/μg, denatured by heating at 100° C for 5 min, and rapidly chilled to −10° C. Samples of labeled probe were incubated with different amounts of RNA in 0.2 M NaCl, 50 mM Tris-Cl, pH 7.5, 0.5 mM EDTA containing 0.2% SDS and 100 μg/ml of denatured calf thymus DNA for 5 h at 67° C. Samples were treated with an excess of S1 nuclease (20 units, purified by the method of Vogt) (1973) in 2 ml of 0.1 M sodium acetate, 0.1 M NaCl, 2 mM $ZnSO_4$, pH 4.6 for 45 min at 45° C. After addition of 50 μg of calf thymus DNA and trichloroacetic acid (TCA) to a final concentration of 5%, samples were incubated 10 min on ice, filtered under vacuum on glass fiber filters (Gelman), washed with 5%

TCA followed by 95% ethanol, dried, and counted in a toluene-based solvent in a scintillation counter.

Measurement of mRNA Decay Rate. A 25 ml culture of each strain was labeled with 20 μCi of 5.6-^{3}H uridine (40 Ci/mmole) per ml. After 3 min at 30° C, rifampin (Sigma) was added to 200 μg/ml and cold uridine to 1 mg/ml. Five ml samples were taken at the times indicated in Fig. 6 and RNA was isolated as described above. The specific activity varied from 58,000 to 40,000 cpm/μg for RNA from strain RSO and from 73,000 to 56,000 for RNA from strain RS35. RNA pellets were dissolved at a concentration of 1 mg/ml in 0.6 M NaCl, 0.06 M sodium citrate containing 0.1% SDS. Nitrocellulose filters (B6, A.H. Thomas) with bound 1,000 base pair *PstI/SalI* fragment were prepared (Bovre and Szybalski, 1971), and hybridization carried out as described by Cooper et al. (1974). Each 6 mm filter contained sufficient DNA to permit a linear increase in bound label when hybridized with up to 16 μg of RNA from strain RS35.

Cell-Free Synthesis of Dihydrofolate Reductase. In vitro synthesis was carried out following procedures established by Zubay (1973) except that the total methionine concentration was reduced to 44 μM and a 0.1 ml reaction contained 10 μCi of ^{35}S-methionine (Amersham, 500 Ci/mmole). Plasmid DNA, when included, was at a concentration of 10 μg per 0.1 ml reaction. Incubations were for 1 h at 37° C. Samples were dialyzed against distilled water, lyophilized, dissolved in 100 μl of buffer containing SDS (Laemmli, 1970) and boiled 2 min. Twenty five μl samples were applied to a 20×20×0.15 cm, 14% acrylamide-0.2% bis-acrylamide slab gel containing 0.1% SDS (Laemmli, 1970). After electrophoresis for 10 h at 110V, the gel was stained for 4 h in a solution containing 28% ethanol, 11.5% TCA. 4% sulfosalicylic acid, and 0.12% Coomassie blue, destained in 38% ethanol-15% acetic acid, dried under vacuum, and autoradiographed for 24 h.

After incubation, some Zubay reaction mixtures were brought to 1 M in NaCl and passed three times through a 0.5 ml methotrexate-aminohexyl-Sepharose 4B column equilibrated with 1 M NaCl, 0.1 M Tris-Cl, 10 mM 2-mercaptoethanol, 1 mM EDTA, pH 7.2 (buffer B) (Poe et al., 1972). The column was washed with 12 ml of the same solution and DFRase was eluted with 3 ml of buffer B containing 4 mM dihydrofolate. The latter was prepared from folic acid (Sigma) by the method of Blakely (1960). Methotrexate-aminohexyl Sepharose 4B was prepared from methotrexate (Sigma) and CNBr-activated Sepharose 4B (Sigma) by the method of Poe et al. (1972) except than an equimolar amount of diaminohexane was substituted for ethylenediamine.

Results

Trimethoprim Resistance is Conferred by Plasmids pLC1437 and pLC446. A preliminary experiment showed strain JA200, the parent strain of the Clarke-Carbon colony bank, to be unable to grow on minimal agar plates containing 2 μg of trimethoprim (Trm) per ml. Two clones from the colony bank were resistant to this level of trimethoprim: 14-37 and 04-46. The specific activities of DFRase in cell extracts prepared from these clones were 7 to 15-fold higher than in the Trm-sensitive (Trm^s) parent strain. The plasmid from strain 14-37 (pLC1437) was transferred to Trm^s strain RSO by transformation, selection being made for resistance to colicin E1. The resulting transformants were resistant to trimethoprim (Trm^r) and had elevated specific activities of DFRase. That the latter was due to elevated levels of DFRase rather than to an increased turnover number for the enzyme was demonstrated by titration with methotrexate under conditions where binding of the drug to the enzyme was stoichiometric (Fig. 1). As described by Williams et al. (1973), the binding of drug is stoichiometric when the enzyme is incubated with methotrexate and NADPH prior to the addition of dihydrofolate. The difference in enzyme levels between parent and plasmid-containing strains determined in this way was 15-fold. However, there was no difference in the turnover number (600 ± 10 molecules dihydrofolate reduced per min per methotrexate binding site) for the enzyme from the two sources.

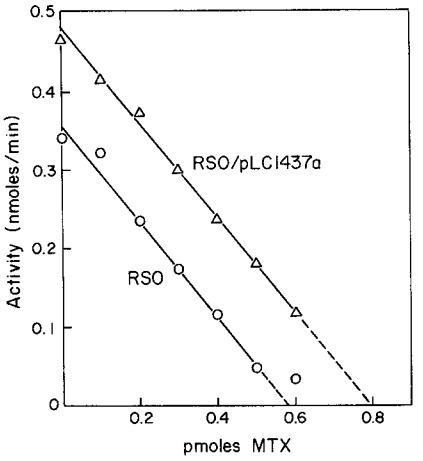

Fig. 1. Dihydrofolate reductase levels in extracts of strains RSO and RSO/pLC1437a determined by methotrexate (MTX) binding. The conditions for these experiments were as described by Sheldon and Brenner (1976). RSO, 50 μg protein; enzyme level, 0.012 pmoles MTX bound per μg protein; turnover number, 612 nmoles/min/nmole MTX bound. RSO/pLC1437a, 4.6 μg protein; enzyme level, 0.172 pmoles MTX bound per μg protein; turnover number, 604 nmoles/min/nmole MTX bound

During the course of this work, plasmid DNA was isolated from another single colony derived from position 14-37 in the colony bank. This plasmid was 3.5 megadaltons larger than the first. All of the work reported in this paper was carried out with the first isolate, designated henceforth, pLC1437a. A complete description of plasmids pLC1437 and pLC1437a as well as pLC446 will be published elsewhere.

Plasmid pLC1437a Carries the Structural Gene for Dihydrofolate Reductase. The only published data on the map position of the structural gene for DFRase relate to trimethoprim-resistant mutants of *E. coli* having elevated levels of the enzyme (Breeze et al., 1975). From these results and some unpublished ob-

Table 2. Complementation tests indicating that plasmid pLC1437a carries *trkC*, *ksgA*, and *pdxA*

Strain	Relevant genotype	Transformant selected by[a]	Phenotype[b]
M1178	*carA*		Arginine + uracil auxotroph
CV600	*carA*/pLC1437a	Trm^r	Arginine + uracil auxotroph
TK118	*trkC*		High K^+ required
CV601	*trkC*/pLC1437a	Col^r	Low K^+ required
CV601	*trkC*/pLC1437a	Trm^r	Low K^+ required
TPR201	*ksgA*		Resistant to kasugamycin
CV602	*ksgA*/pLC1437a	Trm^r	Sensitive to kasugamycin
OSA14	*pdxA*		Pyridoxine auxotroph
CV603	*pdxA*/pLC1437a	Trm^r	Pyridoxine prototroph

[a] Resistance to trimethoprim was selected on agar plates containing M9 salts, glucose, casamino acids, 2 µg trimethoprim per ml (Sigma) and any required supplements. Resistance to colicin E1 was selected on L-agar plates on which were spread 0.1 ml of a sterile crude colicin E1 preparation having a titer of 100 units/ml. Colicin E1 was prepared by the procedure of Shafferman et al. (1978) except that cells were disrupted by sonication in a solution containing 1 M NaCl and 0.1 M potassium phosphate, pH 7, and dialysis was against 0.1 M potassium phosphate, pH 7

[b] Requirement for high potassium was tested on plates described by Epstein and Kim (1971). Sensitivity to kasugamycin was tested on LS-agar containing 100, 150, 200, and 250 µg of kasugamycin per ml (Sigma). Kasugamycin sensitivity is dominant to resistance (Sparling et al., 1973). Auxotrophy was tested on appropriately supplemented SSA minimal medium

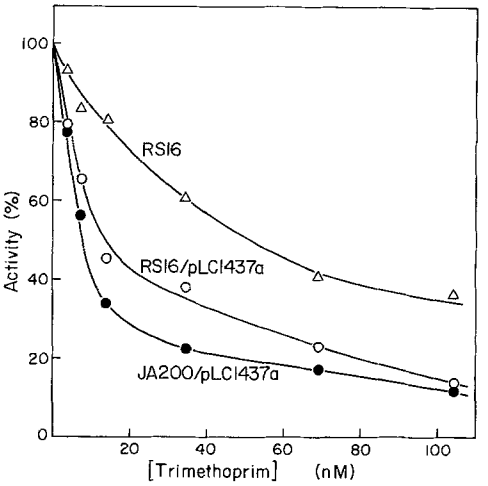

Fig. 2. Competitive inhibition of dihydrofolate reductase by trimethoprim. Activity was measured as described by Sheldon and Brenner (1976). To reduce the background of NADPH oxidase activity, the enzyme was carried through the purification steps described by Burchall and Hitchings (1965) except that only a single G75 Sephadex column was run

servations by R. Sheldon, *fol* (the structural gene for DFRase) has been assigned a position at minute 1 on the *E. coli* genetic map between *maf* and *trkC* (Bachmann et al., 1976). We tested a number of markers in that region of the chromosome (*carA*, *trkC*, *ksgA*, *pdxA*) to determine whether they were complemented by plasmid pLC1437a. The plasmid did not restore function to a *carA* strain, but did restore function to a *trkC* mutant (growth on a low potassium medium) (Epstein and Kim, 1971) and a *pdxA* mutant (growth in the absence of pyridoxine) (Table 2). Furthermore, plasmid pLC1437a conferred sensitivity to kasugamycin upon a kasugamycin-resistant strain indicating that it also carries *ksgA* (Sparling et al., 1973).

The following experiments demonstrate that the structural gene for DFRase is carried on plasmid pLC1437a. Mutant strain RS16 produces a DFRase with an increased Ki for trimethoprim (Sheldon and Brenner, 1976). Strain RS16/pLC1437a was constructed by transformation, selection being made for colicin E1 resistance. The inhibition of DFRase in extracts of this strain by trimethoprim was measured under conditions in which trimethoprim is a competitive inhibitor of the enzyme (Fig. 2). Such competitive inhibition occurs when the enzyme is incubated with both substrates prior to addition of trimethoprim (Williams et al., 1973). For comparison, inhibition curves were also obtained for strains RS16 and JA200/pLC1437a. The I_{50} (concentration of trimethoprim giving 50% inhibition) for DFRase from strain RS16/pLC1437a is close to that for DFRase from strain JA200/pLC1437a (8 versus 13 nM). This is the result expected if plasmid pLC1437a leads to the production of a large amount of DFRase having the low I_{50} characteristic of wild-type strains. If the plasmid carried a gene that increased the expression of the chromosomal *fol* gene, the expected result would have been an enzyme with an I_{50} similar to that for DFRase from strain RS16 (about 50 nM).

A second line of evidence that *fol* is on the plasmid came from the results of in vitro experiments employing the system described by Zubay (1973). Polypeptide synthesis directed by plasmid pLC1437a DNA was carried out in the presence of ^{35}S-methionine and the products were separated on SDS-polyacrylamide gels. Proteins specified by plasmid pLC1437a were clearly visible above a background synthesis caused by endogenous nucleic acids (Fig. 3, compare tracks

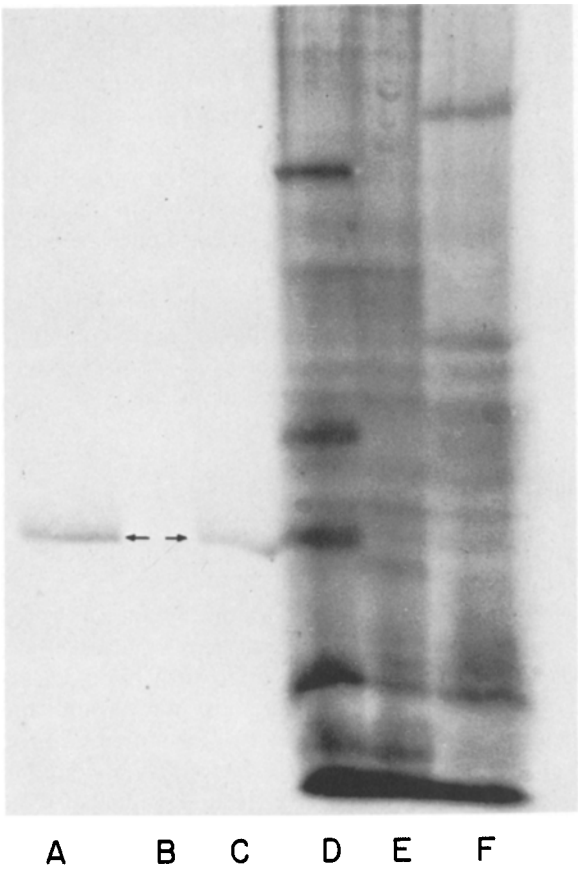

Fig. 3. In vitro synthesis of polypeptides programmed by plasmid pLC1437a. Polypeptides newly synthesized in the presence of ^{35}S-methionine were visualized by autoradiography following separation by electrophoresis on a SDS-polyacrylamide gel. Track E, no plasmid DNA. Track F, ColE1 DNA. Tracks A, C, and D, plasmid pLC1437a. Samples A and C were applied and eluted from a methotrexate-Sepharose affinity column prior to electrophoresis. DFRase purified from strain RSO/pLC1437a and run on the same gel, migrated to the position indicated by the arrows

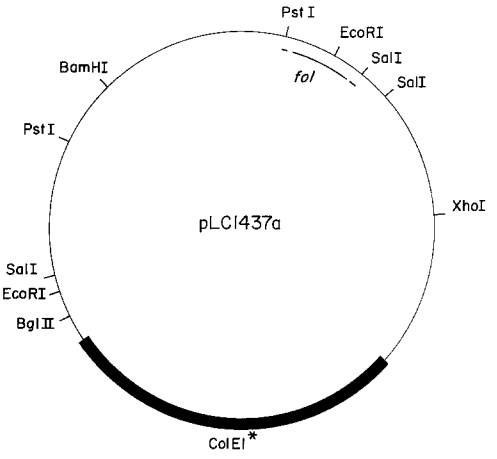

Fig. 4. Diagrammatic representation of plasmid pLC1437a. A molecular weight of 1×10^{-7} was determined by agarose gel electrophoresis following cleavage of the plasmid into two fragments by restriction endonuclease EcoRI. *fol* was located on this map by determining the trimethoprim resistance of clones containing different portions of plasmid pLC1437a. Details of the restriction endonuclease map and of the location of *fol* will be published. elsewhere. ColE1* signifies that part of the ColE1 vector is missing in plasmid pLC1437a

D and E). One prominent pLC1437a-specified protein comigrated with a sample of DFRase purified from strain RSO/pLC1437a (position noted by arrows in Fig. 3). That the newly-synthesized material was indeed DFRase was confirmed by demonstrating that it bound specifically to a methotrexate-Sepharose column (Fig. 3, tracks A and C).

These experiments demonstrate that plasmid pLC1437a carries *fol*. Since *fol* and mutations leading to high DFRase levels are both closely linked to *trkC*, a regulatory region for *fol* lies close to or within the structural gene.

Strain RS35 (fol60) has Elevated Levels of fol mRNA. Strain RS35, a trimethoprim-resistant mutant isolated and characterized by Sheldon and Brenner (1976), has elevated levels of DFRase. We determined *fol* mRNA levels in strain RS35 and its parent (RSO) by hybridization kinetics. A 1,000 base pair fragment of plasmid pLC1437a was isolated following cleavage of the plasmid with restriction endonucleases *Pst*I and *Sal*I (Fig. 4). This fragment, which contains the entire *fol* gene (Smith and Calvo, unpublished results), was labeled to a high specific activity by nick translation and hybridized to RNA isolated from strains RS35 and RSO to different R_0t values (Fig. 5). Strain RS35 contained 15 times more *fol*-specific mRNA than strain RSO. The corresponding DFRase levels in the two strains differed by a factor of 20 to 25 when grown under the same conditions.

RNA from strain RS35 protected over 40% of the probe at high R_0t values (Fig. 5). Since at high C_0t values, plasmid pLC1437a protected 80% of the probe against S1 nuclease digestion (data not shown), this indicates that one entire strand of the probe (or portions of both strands) was homologous to RNA that is derepressed in strain RS35. The minimum required to code for DFRase is about 500 base pairs, considerably less than the 1,000 base pair fragment used as a probe.

Elevated fol mRNA Levels in Strain RS35 are Due to Increased Transcription of the fol Gene. Elevated *fol* mRNA levels in strain RS35 could be a result of either increased transcription or decreased degradation of the mRNA. In order to distinguish between these two possibilities, we measured the degradation

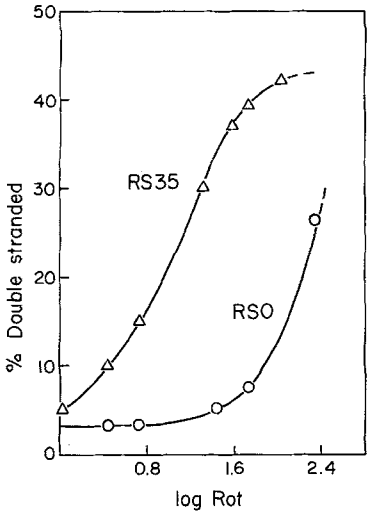

Fig. 5. Hybridization of *fol* DNA to RNA from strains RS35 and RSO. A 1,000 base pair ^{32}P-labeled *PstI/SalI* fragment (Fig. 4) served as probe and unlabeled RNA as driver. For the longest hybridization time (5 h), less than 5% of the probe was resistant to S_1 nuclease when RNA was omitted from the reaction. R_0t units are in moles·seconds·liter^{-1}

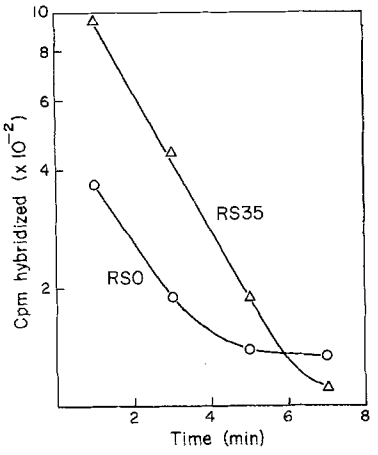

Fig. 6. Rate of *fol* mRNA degradation in strain RS0 and RS35. Zero on the abscissa is the time at which the labeling was terminated by addition of rifampin and a large excess of non-radioactive uridine. For each strain analyzed, a constant amount of RNA from each sample was hybridized to filters containing an excess of denatured *PstI/SalI* fragment (Fig. 4). Strain RSO, 50 μg RNA; strain RS35, 10 μg RNA

rate of RNA hybridizable to the 1,000 base pair *PstI/SalI* fragment bound to nitrocellulose filters. Pulse labeled (^{3}H-uridine) RNA samples were prepared from strains RSO and RS35. Labeling was terminated by the addition of rifampin and a large excess of unlabeled uridine so that further transcription initiation and incorporation of label were inhibited. There was no significant difference in the half life of *fol* mRNA from strain RSO or strain RS35 (about 2 min, Fig. 6). The reason for the apparent decrease in the rate of degradation of RNA at longer times is not clear. The rate of synthesis of *fol* mRNA (proportion of total cpm hybridized to *fol* DNA), on the other hand, differed substantially between the two strains. For the sample taken 1 min after rifampin addition, there was a greater than 12-fold difference between the two.

Taken together, the results of the hybridization kinetic experiments and pulse-label experiments indicate clearly that the mutation in RS35 (*fol-60*) results in an increased rate of synthesis of *fol* mRNA.

Discussion

A search of the Clarke-Carbon colony bank for clones resistant to trimethoprim was undertaken with the expectation that an increased number of *fol* genes (carried on a multiple copy plasmid) would lead to increased DFRase levels which, in turn, would lead to increased resistance to trimethoprim. We did not measure the number of copies of plasmid pLC1437a per cell but assuming a value of 20 found for a similar sized ColE1 *leu* plasmid (Davis and Calvo, 1977), an enzyme level 20-fold higher than normal would have been expected assuming that *fol* expression is unaffected by ColE1 genes and that enzyme level is proportional to gene frequency. The level of DFRase in strain 14-37 is somewhat lower than expected on the basis of these estimates. However, *E. coli* strains containing *fol* cloned on plasmid pBR322 do produce the expected levels of DFRase (our unpublished data).

Two lines of evidence demonstrate that *fol*, the structural gene for DFRase, is carried on plasmid pLC1437a. 1) Strains having *fol-38* on the chromosome (mutation affecting the Ki of DFRase) and plasmid pLC1437a produce a DFRase with a Ki value characteristic of the wild-type enzyme 2) A polypeptide with the molecular weight and methotrexate binding properties of DFRase is synthesized in an in vitro system programmed with plasmid pLC1437a DNA. Since plasmid pLC1437a also carries *trkC*, *ksgA* and *pdxA*, these results confirm the location of *fol* at minute 1 on the genetic map of *E. coli*.

In trying to unravel the cause for the pleiotropic phenotype of *fol-60* several questions are pertinent.

1. Where are regulatory mutations such as *fol-60* located relative to the *fol* structural gene? At the moment, we can say only that *fol-60* is within three kilobases of the structural gene. This estimate follows from a comparison of plasmids cloned from *BamHI* digests of strains RSO (*fol*$^+$) and RS35 (*fol-60*); whereas the two have identical sizes and similar re-

striction maps, the latter plasmid confers 20-fold higher levels of DFRase than does the former (our unpublished results).

2. Is *fol* part of a larger operon? In comparison with the parent, RNA from strain RS35(*fol-60*) has a relatively large proportion of sequences homologous to the 1,000 base pair *Pst/Sal* fragment of plasmid pLC1437a. Furthermore, these relatively frequent sequences protect an entire strand of the 1,000 base pair probe, or parts of both strands. Assuming that bidirectional transcription does not occur in vivo in the *fol* region, these results suggest that *fol* is part of a transcript at least twice as long as the 500 nucleotides necessary to code for DFRase. Consistent with this interpretation is our finding that the largest RNA species from strain RS35(*fol-60*) that hybridizes to the 1,000 base pair *Pst/Sal* fragment is about 1,000 nucleotides long (unpublished data). Whether this RNA species codes for one or several polypeptides is unknown.

3. Why is strain RS35(*fol-60*) unable to grow at high temperatures? *A priori,* at least two possibilities can be imagined. a) An excess of some inhibitory substance accumulates at 42° C and inhibits growth. The putative inhibitor cannot be a product of the DFRase-catalyzed reaction because other strains have equally high DFRase levels and are not temperature-sensitive (Sheldon and Brenner, 1976). b) *fol-60* results in the synthesis of a temperature-sensitive protein whose function is indispensable for growth. Since *fol* mRNA is longer than is necessary to code for DFRase, it is possible that the putative indispensable protein is coded for by part of this mRNA, and that the indispensable protein is DFRase itself. In strains carrying *fol-60*, the catalytic activity of DFRase is not temperature sensitive nor does the synthesis of the enzyme appear to be temperature sensitive (Sheldon and Brenner, 1976). On the other hand, the possibility is not excluded that DFRase has some function other than to catalyze the reduction of folate and dihydrofolate. Mutants of *E. coli* lacking DFRase activity have not been reported. The enzymatic function associated with DFRase is dispensable when *E. coli* is supplied with those nutrients requiring tetrahydrofolate for their synthesis (methionine, glycine, purines and pyrimidines). Thus, Harvey has demonstrated that *E. coli* grows, albeit at a slow rate, in appropriately supplemented media containing an amount of trimethoprim in excess of that needed to totally inhibit DFRase activity (Harvey, 1973). These results suggest that if DFRase has a second function, that function is not destroyed upon binding of the enzyme to trimethoprim. The question of whether *E. coli* is viable when totally lacking the DFRase polypeptide remains to be answered.

4. How can the pleiotropic effects of *fol-60* be explained? The *cis*-dominant properties of *fol-60* are most simply explained by assuming that the mutation is either in the structural gene itself or in a regulatory region adjacent to it. Considering the case where the mutation is in the structural gene, several possibilities come to mind. a) DFRase is formed by cleavage of a longer precursor polypeptide and the *fol-60* mutation increases the rate of cleavage. b) The *fol-60* mutation overcomes a bottleneck that exists during translation of wild-type *fol* mRNA. c) DFRase is regulated by an autogenous mechanism in which DFRase binds to *fol* DNA and prevents transcription. By this model, *fol-60* would be a mutation in the binding region which prevented the interaction of that region with DFRase. All of these models are consistent with the temperature-sensitive and *cis* dominance effects of *fol-60*. However, the first model is not easily reconciled with our finding that the high DFRase levels are correlated with high *fol* mRNA levels in *fol-60* strains. Moreover, this same finding makes the second model unlikely unless it is assumed that the translation bottleneck is early in translation and that the mRNA synthesized past the block is so unstable that an appreciable amount of label was not incorporated during the experiment in which the decay rate was measured.

The remaining discussion deals with the possibility that the mutation is in a regulatory region contiguous with the structural gene, for example, an attenuator region or a region interacting with RNA polymerase, a repressor, or an activator. Examples of *cis*-acting mutations leading to increased operon expression have been reported for operator regions (Jacob and Monod, 1961), activator regions (Englesberg et al., 1961), promoter regions (Scaife and Beckwith, 1966), and attenuator regions (Bertrand et al., 1976). Mutations leading to temperature-sensitivity could be imagined if the corresponding region were both transcribed and translated. Such would be the case if the region in question were part of two genes, a phenomenon known to exist in several phages (Sanger et al., 1977; Schwarz et al., 1978). Finally, the possibility must be considered that *fol-60* is in a separate cistron that codes for a *cis*-acting protein. The products of the *cisA* gene of phage ϕX174 (Levine and Sinsheimer, 1969) and the *Q* gene of phage lambda (Echols et al., 1976) have been postulated to be *cis*-acting proteins.

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