We have introduced the T4 thymidylate synthetase gene, resident in a 2.7-kilobase EcoRI restriction fragment, into an amplification plasmid, pKC30. By regulating expression of this gene from the phage λp promoter within pKC30 in a thyA host containing a temperature-sensitive λ repressor, the T4 synthetase could be amplified about 200-fold over that after T4 infection. At this stage, a 20-fold purification was required to obtain homogeneous enzyme, mainly by an affinity column procedure. The purified plasmid-amplified T4 synthetase appeared to be identical with the temperature-sensitive 2thetase expressed in E. coli and other hosts. The T4 synthetase purified from phage-infected Escherichia coli was indistinguishable from the synthetase produced in vivo from pKC30.(14). Moreover, it appears that co-regulation of these enzymes as well as others involved in deoxyribonucleotide synthesis occurs post-translationally by formation of an active multienzyme complex (16). The regulation of these enzymes at several levels may account for discrepancies in their apparent order of expression in vivo and in vitro (17). Further interest in T4 thymidylate synthetase is generated by the fact that the enzyme is a structural component of the phage base-plate (18, 19).

To increase our understanding of the regulation, mode of action, effect of mutation, and functional domains of thymidylate synthetases from various sources, we have amplified this enzyme by using DNA-cloning techniques. This paper describes the linkage of the T4 td gene, which has previously been isolated (14), to the strong regulatable promoter p(20). Phage λ in expression plasmid pKC30 (20). Purification and properties of the amplified gene product are also presented along with a preliminary comparison of the phage and host enzymes.

**EXPERIMENTAL PROCEDURES**

*Strains, Vectors, and Recombinant Plasmids*

The bacterial and bacteriophage strains used in this study are listed in Table I. The recombinant plasmids which were constructed by using the plasmid vectors pBR322 (21) and the expression plasmid pKC30 (20) are described in Table I and Fig. 1. pKC30 comprises the p(21)-containing HindIII-BamHI fragment of phage λ cloned into the HindIII-BamHI interval of pBR322.

*Media*

Tryptone broth (1.0% Bacto-tryptone and 0.5% NaCl) supplemented with 0.5% yeast extract and 50 μg of thymine/ml (TRYET)
TABLE I

| Derivatives of E. coli | Strain | Relevant features | Ref. |
|----------------------|--------|------------------|------|
| E. coli K12          | Rue10  | thyA derivative of HB101 Rue10 + wild type λ prophage | 21, 22 |
|                      | Rue10(λ+ts)a | Rue10 + thermosensitive λ prophage with cI857 repressor | MB"
| Phage λ              | λNM816 | λimm21c1ts EcoRI vector | 23 |
|                      | X74td1 | 2.7-kb EcoRI T4 td fragment in λNM816 | 14 |
|                      | λthyA (NM589) | 7.8-kb HindIII E. coli thyA fragment in λ HindIII vector | 13 |
| pBR322               | pBTd1  | 2.7-kb EcoRI T4 td fragment in EcoRI site of pBR322 | MB*
|                      | pKC30  | λP1-containing expression plasmid | 20 |
|                      | pKTd2  | 2.7-kb EcoRI T4 td fragment in HpaI site of pKC30 | MB*

* This work.

† Rue10(λ+ts) transformed with pKTd2 was used initially for overproduction of T4 thymidylate synthetase. We later switched to an MB147(λcI857del) transformant since the defective prophage in this strain allowed for longer induction times and yielded higher enzyme levels. Here, prophage is thermosensitive (cI857), deleted for host-killing function (bio10), and replication-defective (Pam3).

‡ Lysogen constructed by D. L. Wulff, State University of New York, Albany; thyA derivative, this work.

was used to grow cultures for crude extract preparation and phase purification. Ampicillin (50 μg/ml) was added for culturing plasmid-containing strains. Minimal medium contained 7 g of K2HPO4, 2 g of KH2PO4, 0.5 g of sodium citrate, 0.1 g of MgSO4, and 1.0 g of (NH4)2SO4/liter and was supplemented with 0.1% Norit A-treated casamino acids and 0.2% glucose. Solid medium was prepared by adding 1.5% Bacto-agar.

Enzymes and Radiochemicals

Restriction enzymes were purchased from Bethesda Research Laboratories or New England Biolabs and T4 DNA ligase was purchased from New England Biolabs; all were used according to the manufacturers' instructions. E. coli DNA polymerase I was from Bethesda Research Laboratories. [5-3H]dUTP (10-15 Ci/mmol) was from Amersham Corp.; [2-3H]dUDP (50 mCi/mmol) and [5-3H]dUMP (15 Ci/mmol) were from Moravek Biochemicals. [α-32P]dATP (3000 Ci/mmol) was from New England Nuclear.

Methods

Preparation of Phage and Plasmid DNAs and DNA Fragment Purification

Phage λ was prepared and purified and the DNA extracted in 50% formamide, as described by Davis et al. (24). Plasmid DNA to be used as a vector or for fragment isolations was purified by banding in CsCl-ethidium bromide gradients (25). Rapid plasmid preparations were made by the procedure of Holmes and Quigley (26). Fragment purification was achieved by electrophoresis onto strips of DEAE-cellulose (Schleicher & Schuell, NA-45, 0.45 μm) and subsequent elution by adaptations of the procedure of Dretzen et al. (27).

Construction of Recombinant Plasmids

pBTd—EcoRI-treated λX74td1 (500 ng) was ligated to EcoRI-cleaved pBR322 (200 ng) by incubation with 30 units of T4 DNA ligase in ligase buffer at 15 °C for 18 h. The ligation mixture was used to transform a CaCl2-treated Rue10 (Thy-) culture (28). While no Thy+ colonies were obtained when transformants were selected directly on minimal medium, about 10% of ampicillin-resistant transformants produced zones of weak but discernible growth when replica plated onto minimal medium plates. Several of these transformants were purified and used for preparation of plasmid DNAs by rapid isolation procedures. Restriction analyses and Southern blotting experiments (Fig. 6) confirmed the presence of the 2.7-kb EcoRI fragment of T4 cloned in either orientation into the EcoRI site of pBR322. These constructions are designated pBTd. B, cloning the T4 td fragment into expression plasmid pKC36. The 2.7-kb EcoRI restriction fragment was excised from a pBTd derivative, purified, and made blunt-ended by treatment with E. coli DNA polymerase I in the presence of the four deoxynucleotide triphosphates. This blunt-ended td fragment was ligated into the HpaI sites of pKC30, 321 nucleotides downstream from the λP1 promoter. Recombinants carrying the T4 td insert in the correct transcriptional orientation relative to p, are designated pKTd.

Methods

Hybridization and Autoradiography

Restriction endonuclease-digested DNAs were separated on an 0.8% agarose gel and transferred to a nitrocellulose filter (Schleicher & Schuell) as described by Thomas (30). Nick-translated 32P-labeled

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Assays of Enzyme Activity

Assay of Roberts (33) was used with [5-3H]dUMP as substrate. One assay of Wahba and Friedkin (32). In some cases, the tritium release indicated.

Enzyme Purification

The phage synthetases were purified to homogeneity from crude extracts by a modification of the quinazoline affinity column procedure of Rode et al. (34).

Ternary Complex Formation

To form this complex, enzyme, FdUMP, and 5,10-CH2H4PteGlu were incubated for 10 min at 37 °C in a solution containing 100 mM potassium phosphate (pH 7.1), 10 mM ascorbate, and 100 mM β-mercaptopetanol. 5,10-CH2H4PteGlu was present at 0.6 mM and [3H]FdUMP at 0.6 µM or [2,4-C]FdUMP at 25 µM. When it was important to ensure conversion of all of the enzyme present in a sample to the ternary complex (as in Fig. 2), the sample was incubated for an additional 10 min in the presence of unlabeled FdUMP (250 µM). After incubation, the samples were either lyophilized or added directly to electrophoresis loading buffer.

Gel Electrophoresis

All samples were heated at 95 °C for 3 min in loading buffer containing 1% SDS and 1% β-mercaptoethanol before being applied to the gels. Separations were performed on either a 26% acrylamide low cross-linked gel (bisacrylamide/acrylamide ratio of 3:00) or on a 12.5% acrylamide high cross-linked gel (ratio 8:300). The gel composition, electrophoresis conditions, and differences in separating properties of the two gel types have been described (35). T4 thymidylate synthetase is more clearly resolved from neighboring bands after Coomasie blue staining in the low cross-linked gels.

Carboxymethylation and Sequence Determination

Enzyme (5–6 mg) was reduced in 6 mM guanidine-HCl, 1 mM Tris-HCl (pH 8.0), and 0.1 mM 2-mercaptoethanol for 4 h at room temperature under a nitrogen atmosphere. S-Carboxymethylation was accomplished with iodoacetate (36). After 20 min, the reaction was terminated by adding 2-mercaptoethanol to a final concentration of 1%. The carboxymethylated protein was then dialyzed at 4 °C against four changes of 5% acetic acid and lyophilized. The carboxymethylated proteins (50 nmol) were sequenced by using 0.1 M Quotrol as buffer with a Beckman Model 890 automatic sequencer. The released thiazolinone derivative were converted to the latter were characterized by thin layer chromatography on silica gel (37) and quantitated by high performance liquid chromatography in a modified Waters Associates Model 440 system equipped with a Model 660 solvent programmer. The ClxpBondapak columns (Waters) were developed with a gradient formed from 0.01 M sodium acetate (pH 5.0) and 38% acetonitrile in 0.01 M sodium acetate (pH 5.0) (38).

Preparation of Antibody to Thymidylate Synthetase

Antibody to T2-induced thymidylate synthetase was prepared in rabbits (39), and the globulin fraction was partially purified by ammonium sulfate fractionation as described previously (40). The globulin fraction was dialyzed against a 5 mM potassium phosphate (pH 7.1), 0.65% NaCl solution and stored at −10 °C. Antibody to the protein antigen was determined by immunodiffusion on a microscope slide (41). Precipitin lines were visible after several hours.

Amino Acid Analysis

Carboxymethylated protein samples were hydrolyzed in sealed evacuated tubes containing 1.0 ml of constant boiling HCl for 24 h at 110 °C. Single column amino acid analysis was performed on a Model 119CL Beckman analyzer in triplicate.

RESULTS

Expression of the T4 td Gene in Plasmid Vectors

Subcloning the T4 td Gene—Phage λT4td1, which contains the td gene of T4, was selected by its ability to complement thymidylate synthetase-negative (thyA) mutants of E. coli (14). The 2.7-kb EcoRI fragment of T4, on which the td gene resides, was carried in this λ phage in the orientation shown in Fig. 1. This arrangement permits gene expression from the distant ρ1 promoter of phage λ during a lytic infection. Lyso- gens of λT4td1 made in a thyA host have been reported to be

| Host strain | Infecting phage plaque | Resident plaque | Time after infection or induction | Specific Activity | Amplification factor |
|-------------|-----------------------|----------------|---------------------------------|------------------|---------------------|
| T4          | 40                     | 0              | 10 min                          | 1                | 1                   |
| T4           | 40                     | 0              | 15 min                          | 2                | 2                   |
| T4           | 40                     | 0              | 30 min                          | 3                | 3                   |
| T4           | 40                     | 0              | 60 min                          | 4                | 4                   |
| T4           | 40                     | 0              | 90 min                          | 5                | 5                   |

Degree of enhancement of thymidylate synthetase activity

Enzyme activity was measured as described under "Experimental Procedures" in crude cell-free extracts. Trimethoprim (0.3 mM) was added to the T4-infected extract to inhibit dihydrofolate reductase (line 2). All cells were grown in TYEBET medium to midlog phase. Phage infections and temperature inductions were carried out for the times indicated, and the cultures were chilled to 2 °C in an ice-ethanol bath before harvesting.

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Thy', suggesting that the T4 insert includes a sequence that can function as a promoter for the td gene since the main promoters are repressed in the lysogenic state (14). To obtain this 2.7-kb td fragment from χT4td1 for further study, it was subcloned into the EcoRI site of pBR322. ThyA cells transformed by these constructions, designated pTtd, could not be selected on Thy⁻ medium and contained only trace amounts of thymidylate synthetase, with the insert on either orientation (Table II, line 4). This observation implies that if there is a promoter-like sequence within the insert, it functions very inefficiently. The presence of the insert was verified by restriction analysis (data not shown) and Southern hybridization (see below).

To amplify expression of the td gene, the 2.7-kb td fragment was placed immediately proximal to the χp promoter contained within expression plasmid pK230 (20). E. coli DNA polymerase I was used to make the 5'-EcoRI extensions double-stranded. Subsequently, this blunt-ended fragment was ligated into the HpaI site of pK230, 321 base pairs from the χp transcriptional start site. Because we were concerned about potentially detrimental effects of T4 thymidylate synthetase overproduction in E. coli and aware of the fact that dTMP excess can be both mutagenic (42) and lethal (43) in yeast, transformants were selected in a thy⁻ lysogen, Rue10(λc'). The λ repressor protein (cI) in these cells prevents transcription from χp, and thereby permits the cloning of lethal functions which would be expressed if cI were not present (20). Ampicillin-resistant transformants which appeared on replica plates to be weakly Thy⁻ were screened by size and restriction analysis. All eight of the transformants checked contained the 2.7-kb fragment in the correct transcriptional orientation relative to χp. This bias may be accounted for by low level transcription originating from χp in the presence of λ repressor (Table II, line 5), leading to a weak Thy⁻ phenotype, an effect which influenced our choice of recombinants in favor of those with the td gene oriented appropriately relative to χp. Low level transcription from χp may also account for the fact that χT4td1 lysogens are Thy⁺ (14).

To induce high level expression from χp, pKTdt2 was transferred into thy⁻ lysogens harboring prophages with a temperature-sensitive repressor gene (cIt). These cells were maintained at 32 °C and were shifted to 42 °C when expression of the td gene was desired. The mutant repressor was inactivated at 42 °C, enabling transcription to proceed from χp.

**High Level Production of T4 Phage Thymidylate Synthetase**—Lysogens containing the multicopy pKTdt2 plasmid (Table II, lines 6 and 7) produced about 4-5-fold more enzyme before temperature shift than was obtained after T4 phage infection (line 2). This was roughly the same degree of amplification (4.5-fold) as was effected by infection with χT4td1, where the td gene is under remote control of χp in a lytic infection (line 3). However, 30 min after the temperature was raised to 42 °C, the synthetase was elevated 115-fold in Rue10(AcIe)/pKTdt2 (line 6) and 155-fold in the defective lysogen MB147(AcItsddef)/pKTdt2 (line 7). Since cell lysis occurs beyond 30 min in the former lysogen, later time points were feasible only with the strain harboring a defective prophage. In this experiment, a 240-fold amplification was achieved in 60 min (line 7), corresponding to 480 milliunits/min in the former lysogen, later time points were feasible only with the strain harboring a defective prophage. In this experiment, a 240-fold amplification was achieved in 60 min (line 7), corresponding to 480 milliunits/min in the form of purified T2 phage thymidylate synthetase.

**Identification of T4 thymidylate synthetase subunit in crude extracts**—The temperature-sensitive lysogen MB147(AcItsddef)/pKTdt2 was grown at 32 °C to midlog phase in TBYET medium containing 50 µg of ampicillin/ml. One-half of the culture was shifted to 42 °C for 1 h. Crude cell-free extracts were prepared from each half of the culture, and aliquots containing 25 µg of protein were either taken directly for electrophoresis or pretreated with [2-¹⁴C]FdUMP and 5,10-CH₂H₄PteGlu for ternary complex formation (see "Experimental Procedures"). Purified T2 thymidylate synthetase was used as a marker in this experiment, either in native form or as the [2-¹⁴C]FdUMP-labelled ternary complex. The Coomassie-stained SDS-gel electrophoresis and sedimentation equilibrium measurements (39). It should be noted, however, that values in the range of 25,000 daltons...
In each sample was varied such that 0.02-0.1 milliunit of enzyme was applied to each lane except where otherwise indicated. Lane 1, crude extract of Rue10 (thyA-) (no assayable activity); lane 2, purified T2 thymidylate synthetase; lane 3, purified T4 thymidylate synthetase; lane 4, Rue10(sclts)/pKTd2, crude extract of culture infected for 10 min at 37 °C; lane 5, crude extract of Rue10 infected for 10 min with T4 at 37 °C; lane 6, crude extract of Rue10 infected for 30 min at 37 °C with XT4tdl; lane 7, crude extract of Rue10 infected for 30 min at 37 °C with XthyA; lane 8, crude extract of E. coli C600 thyA; lanes 9 and 10, ternary complexes corresponding in size to the synthetases of B. subtilis (strain 168). The molecular weight scale (in kilodaltons) to the left of the figure was derived from a series of protein standards run on the same gel and stained with Coomassie blue.

(44) to 29,000 daltons (14, 45) have been obtained for the T4 phage enzyme. Discrepancies in molecular weight estimates from polyacrylamide gels are common (35) and our molecular weight assignments must be considered tentative. Nevertheless, it is clear from Fig. 3 that the T2 synthetase ternary complex (lane 2), that of the T4 phage enzyme from extracts of heat-treated pKTd2-containing lysogens (lane 4), and that formed by T4 synthetase purified from this source (lane 3) appear identical in size. Furthermore, these are indistinguishable in size from the derivatized subunit encoded by wild type T4 phage (lane 5) and by the XT4tdl-transducing phage (lane 6). This method clearly distinguishes the T-even enzymes from those of E. coli (lanes 7 and 8), Bacillus subtilis (lanes 9 and 10), and H-35 hepatoma cells (data not shown).

Comparative Analysis of T-Even and E. coli Thymidylate Synthetases

The plasmid-amplified T4 td gene product was purified to homogeneity by affinity chromatography (Fig. 4), as were the enzymes produced after T2 and T4 infection. These products were then sequenced from their NH₂-terminal ends, and for at least 20 cycles, the amino acid sequences of the T2-induced, T4-induced, and T4-cloned enzymes appeared to be identical: Met-Lys-Gln-Tyr-Gln-Leu-Ile-Lys-Asp-Ile-Phe-Glu-Asn-Gly-Tyr-Glu-Thr-Asp-Asp.

This information, coupled with the molecular weight data in Figs. 3 and 4, suggests that T2- and T4-induced synthetases are very closely related, if not identical. The immunological similarity of these proteins is also evident from the fact that the T2-induced (Fig. 5A, wells 1 and 4) and T4-cloned synthetases (wells 2 and 5) both cross-reacted with antibody to the former enzyme. No cross-reaction was obtained with the E. coli synthetase. Similarly, antibody to the latter reacted only with the E. coli synthetase (Fig. 5B, wells 3 and 6) and not with the phage enzymes.

These differences between the T4 and E. coli enzymes are corroborated by apparent differences in their coding sequences. The Southern hybridization experiment, in which 32P-labeled pBTd was used as probe (Fig. 6), showed the expected homology between the probe and the 2.7-kb EcoRI fragment in both XT4tdl (lane b) and pBTd (lane c). In the latter case, the 4.4-kb band corresponds to the vector portion of pBTd. No hybridization was evident, however, either with λ vector alone (lane a) or with a 7.8-kb E. coli thyA fragment cloned into λ (13) (lane d). This absence of apparent homology between the T4 and E. coli thymidylate synthetase fragments under these hybridization conditions is somewhat surprising in view of certain homologies in amino acid sequence between the phage and host enzymes.
this band to its corresponding FdUMP-5,10-CH₂H₄PteGluta-
ternary complex.

Comparison of the amplified T4 enzyme with the previously purified T2 synthetase (39) revealed them to be identical in subunit molecular weight (32,000), amino end group analysis through 20 amino acids, and immunochemical cross-reactivity (Fig. 5). These data and the apparent sequence homology in the td region of the T2 and T4 genetic maps (47) suggest that the T2 and T4 synthetases may be identical.

In contrast to the similarities between the T-even phage synthetases, striking differences have been found between the phage and host enzymes. We have shown here that differences exist in the electrophoretic mobility (Fig. 3) and immunological specificity (Fig. 5) of the synthetases. These findings were corroborated by DNA hybridization experiments in which no homology was apparent between the td gene of the T4 phage and the thyA gene of E. coli K12 (Fig. 6). These differences are somewhat surprising in view of the similarities in amino acid content and peptide patterns previously reported by us for the T2 and E. coli B synthetases (48). On the other hand, their differential response to inhibition by the folylpolyglutamate (9) implicates distinctive differences in their folate-binding regions. A comparative sequence analysis of the T-even phage and E. coli synthetases and the genes encoding them is in progress. These results should not only help to explain the molecular basis for the differential behavior of these proteins, but may also give us some insights into the evolutionary relationships between duplicated phage and host functions.

Acknowledgments—We wish to thank Norreen Murray and Marty Rosenberg who generously provided us with recombinant phage, vectors, and sound advice. We also thank Gary Wilson and Dan Wulff for bacterial strains.

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DISCUSSION

To obtain adequate amounts of T-even phage thymidylate synthetases for comparison with each other, as well as with the E. coli enzyme and with those from other sources, it was found advantageous to exploit the expression plasmid pK30 described by Shimatake and Rosenberg (20). By positioning a 2.7-kb fragment containing the td gene of T4 phage down- stream from the phage λ p₈ promoter residing within this plasmid (Fig. 1), the expression of the td gene was amplified more than 200-fold above that produced by phage infection (Table II). This was achieved in lysogenic cells containing a thermosensitive repressor gene (cl), which could be inacti- vated by raising the temperature of the cultures to 42 °C. Transcription could then proceed from p₈ into the adjacent td gene to an extent that the gene product represented as much as 5-7% of total cellular protein in crude extracts prepared from such cultures. Similar results have been obtained by using pK30 to amplify the expression of the cII gene of phage λ (20) and the utrA gene of E. coli (46). The use of thyA cells lacking host synthetase aided in the selection of plasmids expressing the td gene and in the ensuing enzyme purification since extracts were devoid of the E. coli synthetase. Purification of the amplified T4 synthetase was greatly simplified with an affinity column procedure (34).

The amplified T4 td gene product is easily visible in gel patterns of crude cell extracts prepared after inactivation of the temperature-sensitive cl repressor (Fig. 2, lane 4). The identity of the amplified band was verified either directly on the Coomassie-stained gel or on autoradiograms by converting
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J. Biol. Chem. 1983, 258:2045-2051.

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