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Genetic System for Analyzing *Escherichia coli* Thymidylate Synthase

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Random in vitro mutagenesis of the *thyA* gene is being used to delineate its regulatory elements as well as the functional domains of its product, thymidylate synthase (EC 2.1.1.45). Streamlined procedures have been developed for the isolation and characterization of the mutants. Positive selection for synthase-deficient *thyA Escherichia coli* permitted the isolation of 400 mutants, which are being categorized by phenotypic and genetic criteria. An in situ 5-fluorodeoxyuridylate binding assay was devised to rapidly probe the substrate binding domain, whereas facile mapping procedures, based on pBR322- or M13-borne *thyA* deletion derivatives, were developed to localize mutations. The sequence changes of one amber mutation and another mutation that abolishes catalysis while maintaining substrate binding activity are presented. The orientation of the *thyA* gene on the *E. coli* chromosome was established.

The metabolic importance of thymidylate synthase (EC 2.1.1.45) stems from its role in providing the replication machinery with dTMP. This vital precursor is specific to DNA synthesis, making the enzyme an attractive cellular focus for regulation and an important target in chemotherapy (reviewed in reference 14). These considerations have resulted in extensive kinetic (24) and structural studies (reviewed in references 3 and 20), which provide insights into the catalytic process of thymidylate synthase. The enzyme, a dimer composed of identical subunits of about 30,000 daltons, is responsible for catalyzing the reductive methylation of dUMP to yield dTMP. Methylene tetrahydrofolate (CH_2FH_4) acts as both methyl donor and reductant in this process. The antitumor drug 5-fluorouracil inactivates the enzyme after conversion to 5-fluorodeoxyuridylate (FdUMP), a substrate analog, which interacts with the synthase and CH_2FH_4 to form a covalent ternary complex. The study of this complex, believed to be analogous to a transition state intermediate in the enzymatic reaction, has provided a working knowledge of enzyme mechanism (24).

Given this body of biochemical information available for the enzyme and our interest in the regulation of its synthesis, the synthase provides an attractive model system for further study by genetic analysis. A random mutagenesis approach to highlight functional regions and residues seems particularly useful for this enzyme, since X-ray crystallographic data are not yet available. The *thyA* gene of *Escherichia coli* and its thymidylate synthase product were chosen for the development of such a genetic system. This choice seemed expedient specifically because positive selection techniques exist for synthase-deficient, thymine-requiring (*thyA*) mutants in the presence of the antifolates such as aminopterin or trimethoprim and thymine (23). The availability of the cloned *thyA* gene, of thymidylate synthase-overproducing strains (4), and of the sequence of both gene and gene product (3) greatly facilitate such a study.

As a first step toward establishing structure-function correlates, we have adopted a random mutagenesis strategy and developed simple phenotypic screening procedures based on growth properties and FdUMP-binding potential of mutant clones. Rapid deletion mapping strategies facilitated genetic analysis of a variety of *thyA* mutants. Several interesting genetic features of the *thy* locus itself emerged in the course of these studies.

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MATERIALS AND METHODS

Bacterial strains. The Thy$^-$ mutant strains used include the *recA* strain Rue10, which is a *thyA* derivative of HB101 (28), the *thyA*(Ts) strain N4316 (9), and the *thyA* alleles *Δ2*, *3*(Ts), *7*, *Δ64*, *83*, and *108* of strain Hfr3.OSO *thi lac* (1). The strains used for propagation of M13 derivatives were JM103 (22) or JM103 *recA*. We constructed this *recA* derivative by P1 transduction of the *Δ(srl-recA)306*::Tn*10* region from strain JC10289 into JM103 (12).

Phage and plasmid constructs. The intact *thyA* gene is contained within a 1.16-kilobase fragment that was cloned with linkers into the *Hin*dIII site of pBR322 (6) to yield plasmid pBTAH (4) or into the *Hin*dIII site of pACYC184 (8) or M13mp9 (22) to yield plasmid pATAH or phage M13TAH, respectively (this study). The pBTAHΔN1 through pBTAHΔN8 deletion constructs were derived from pBTAH, which was linearized at the 5′ end of the *thyA* gene and subsequently treated for increasing times with BAL 31 nuclease. The plasmids were then recircularized after insertion of *Bam*HI linkers (3). The truncated ΔN-*thyA* fragments, which carry a constant 3′ *Hin*dIII end and progressively deleted 5′ termini abutting the *Bam*HI linkers, were transferred into the *Hin*dIII through *Bam*HI interval of M13mp9 to yield M13TAHΔN1 through M13TAHΔN8. By analogy, the BAL 31 deletion derivatives extending from the 3′ terminus of *thyA* were used directly to construct the series M13TAHΔC3 through M13TAHΔC7 (3). These constructs have fixed 5′ *Hin*dIII ends and progressively truncated 3′ termini (Fig. 1).

Growth media. Tryptone broth (TB) (1.0% Bacto-tryptone [Difco Laboratories] and 0.5% NaCl) and TB supplemented with 50 μg of thymine per ml or 0.5% yeast extract or both was used for cell growth or M13 phage propagation as indicated. The composition of the minimal medium (Davis) has been previously reported (5). Solid medium was prepared by adding 1.5% Bacto-agar (Difco). Ampicillin (Amp) (100 μg/ml) or chloramphenicol (Cam) (25 μg/ml) was added when selecting for pBR322 derivatives or for pACYC184 derivatives, respectively. Selection for *thyA* colonies was

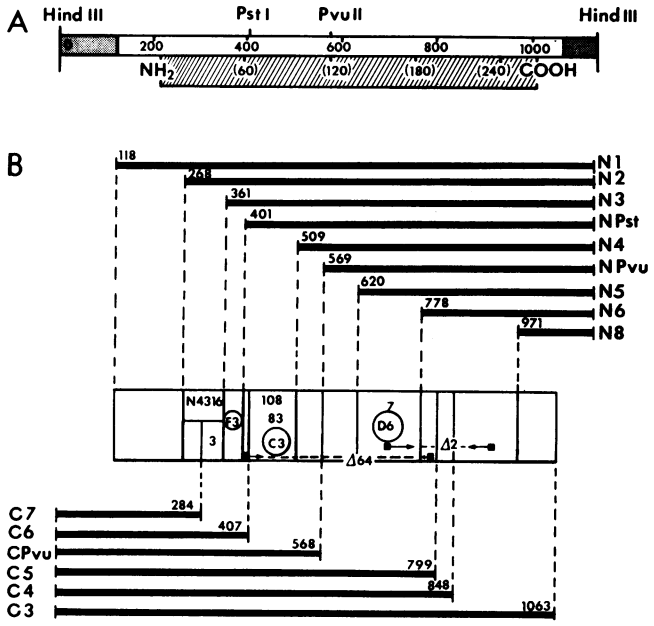

FIG. 1. Map of the *thyA* gene. (A) The 1.16-kilobase fragment encoding the *thyA* gene. Nucleotides are numbered within the double line, whereas amino acid residue numbers are indicated within parenthesis below, between the NH_2 and COOH ends of the synthase structural gene (hatched). Areas of the fragment dispensable to *thyA* function are shaded. (B) Deletion map of the *thyA* gene. The ΔN deletion series extending progressively from the 5′ end of the *thyA* gene appears above the map, whereas the ΔC series extending from the 3′ end is shown below. The deletions ΔN-*Pst*, ΔN-*Pvu*, and ΔC-*Pvu* represent subclones of the gene spanning the length between the indicated restriction enzyme site and the relevant *Hin*dIII end. The constructs ΔN1 through ΔN6 and ΔN8 as well as ΔC3 through ΔC7 were derived with BAL 31 nuclease (3) (see the text). The thick line represents the DNA present in each deletion derivative with the nucleotide number at the deletion endpoint shown above each line. The *thyA* mutations mapped to the resulting deletion intervals are as follows: derivatives of pATAH generated in this laboratory, C3, D6, and F3 (encircled); derivatives of Hfr3.OSO, *Δ2*, *3*, *7*, *Δ64*, *83*, and *108*(1); and the *thyA*(Ts) allele in strain N4316 (9). The mutations *Δ2* and *Δ64* are chromosomal *thyA* deletions (32) that are located between the respective arrows on the map. N4316 is M13 resistant and was therefore mapped only by the transformation method with the ΔN series deletions. All other mutations were localized by the M13 infection method as well as by transformation.

achieved on minimal agar medium supplemented with 50 μg of thymine per ml and 20 μg of trimethoprim per ml.

Recombinant DNA procedures. Plasmid DNA preparation, restriction, ligation, and transformation procedures have been previously documented (5). The sequence change in point mutants was determined, after subcloning the mutated DNA fragment into M13 vectors, by the dideoxy chain termination method of Sanger et al. (29) as previously reported (3). To minimize the rescue of wild-type sequences from the bacterial chromosome, M13-*thyA* mutant recombinants were usually propagated on JM103 *recA*.

Mutant isolation. Mutants were isolated from a purified preparation of plasmid pATAH (Cam^r, $ThyA^+$) after hydroxylamine mutagenesis (34). Briefly, plasmid (30 μg/ml) was incubated in the presence of 1 M NH_2OH–2 mM EDTA (pH 6.0) at 75°C. Samples were removed at intervals of up to 30 min into 3 volumes of chilled TB containing 5 mM EDTA and dialyzed in the cold for 1 to 2 h against TB plus EDTA by using dialysis disks (VSWP; Millipore Corp.). Dialysis was continued overnight against 10 mM Tris (pH 8)–1 mM EDTA. After competent *thyA recA* cells (Rue10) were transformed, they were plated on TB containing thymine and chloramphenicol and incubated overnight at 38°C to measure total number of survivors and on thymine-trimethoprim medium plus chloramphenicol for 48 h at 38°C to select for cells that had been transformed with plasmids carrying a defective *thyA* gene. At 20 to 50% survival, Thy^- mutant clones arose at a frequency of 10^{-2} to 10^{-3} (10 to 20 min of NH_2OH treatment). The Thy^- transformants were purified by two more passages on thymine-trimethoprim medium plus chloramphenicol (38°C) and stored on TB plates supplemented with thymine and chloramphenicol at 4°C in arrays of six-by-eight corresponding to the microtiter dish template. Mutants were grown in TB plus thymine and chloramphenicol in microtiter dishes before printing out under the specific screening conditions.

In situ FdUMP binding assay. Arrays of mutant clones, grown overnight at 30°C on TB supplemented with thymine and chloramphenicol, were lifted onto dry 9-cm Whatman no. 42 filter paper disks by carefully placing a disk onto the agar surface and peeling it off with growth patches adhering to its underside. Cells were lysed by wetting the filter in 2 ml of a solution of lysozyme (10 mg/ml) in 10 mM EDTA (pH 6) in a petri dish. After the excess liquid was gently aspirated off, the filters were saturated for 2 min in 10 mM potassium phosphate (pH 7.5), rinsed in 10 mM Tris (pH 7.5), damp-dried on a Buchner funnel, and frozen at −20°C (7). All rinse steps were carried out without agitation and with barely enough liquid to saturate the filter (about 2 ml) to minimize loss of cell contents from the lysed spots. After thawing, filters were dried to fix the cell lysates to their surface. They were then rehydrated in 0.8 ml of Tris-$MgCl_2$ (10 mM, pH 7.5) at 0°C, 0.2 ml of FdUMP-binding solution (0.6 μM [6-^{3}H]FdUMP [18 Ci/mmol], 0.6 mM CH_2FH_4, 100 mM mercaptoethanol, 10 mM ascorbate, 100 mM potassium phosphate, pH 7.1) was added, and the filters were subsequently incubated at 0°C for 10 min. Filters were then flooded with 10% trichloracetic acid at 0°C and rinsed in a Buchner funnel with chilled 5% trichloracetic acid, which washed away the unbound label and fixed the FdUMP-enzyme-CH_2FH_4 ternary complex. After drying, the filters were impregnated with fluor by spraying with En^{3}Hance (New England Nuclear Corp.) and autoradiographed. Films were exposed for 4 to 14 days at −80°C.

Genetic mapping. The deletion mapping system was based on the two overlapping deletion libraries extending from either the 5′ end of the *thyA* gene (the ΔN series) or from the 3′ end of the 1.16-kilobase *thyA* fragment (the ΔC series) (3) (Fig. 1). Mapping was performed in a $recA^+$ background with either transformation by plasmid borne deletions or infection with M13mp9 recombinants containing the truncated *thyA* constructs. Chromosomal *thyA* mutations from other laboratories were mapped directly in the strains in which they were isolated (1, 9), whereas point mutations in pATAH were mapped after transfer of the plasmid to strain Hfr3.OSO *ΔthyA64* (1). This strain not only carries a substantial deletion of the *thyA* gene, but also (being an Hfr) is M13 sensitive.

(i) Mapping by M13 infection. An overnight culture of the Hfr strain containing the *thyA* mutation to be mapped was grown up in TB containing yeast extract and thymine. The media were supplemented with chloramphenicol if the strain harbored a pATAH mutant. This culture was then streaked across a plate containing the same agar-based medium and

allowed to dry. Recombinant M13 phage lysates (about 10^{11} phage per ml), cleared of contaminating host bacteria by centrifugation (30 min, 13,000 rpm), were then systematically cross-streaked at right angles to the *thyA* bacteria, and the plate was incubated at 37°C overnight. The cross-streaks were subsequently replica plated onto minimal medium lacking thymine. Thy^+ growth at the intersection of phage and bacterial streaks resulted from recombination events between the *thyA* deletion derivative in the phage and the mutated *thyA* gene harbored by the host cell.

(ii) Mapping by transformation. Strains carrying *thyA* point mutations, either on the chromosome or on pATAH (Cam^r), were made competent by the procedure of Dagert and Ehrlich (13) and transformed systematically to Amp^r by each member of the pBR322-borne *thyA* ΔN deletion series. (The ΔC deletion fragments have not yet been transferred into pBR322). Transformant colonies were patched onto minimal medium containing ampicillin, but lacking thymine. Thy^+ growth within the patch places the point mutation to the right of a particular ΔN deletion endpoint. Preliminary map positions assigned on the basis of patch tests were confirmed where necessary by growing up relevant transformants and screening these cultures more extensively for the occurrence of Thy^+ recombinants.

RESULTS

Mutant isolation and characterization. Mutations in the cloned *thyA* gene were isolated after hydroxylamine treatment of plasmid pATAH, which comprises the well-characterized 1.16-kilobase *thyA* fragment (3, 4) cloned into the *Hin*dIII site of vector pACYC184 (8). This vector choice was based on its compatibility with pBR322, a property which has been exploited in one of the deletion mapping procedures.

The mutant isolation strategy was based on the marked growth advantage that thymidylate synthase-defective Thy^- mutants have over their Thy^+ counterparts on media containing trimethoprim and thymine (23). Thus Rue10 (*recA thyA*) host cells were transformed with mutagenized pATAH, and cells with mutations in the cloned gene were isolated by selecting transformants able to grow at 38°C in the presence of chloramphenicol, trimethoprim, and thymine. Purified mutant clones were screened for their growth properties on different selective media and under the various conditions of temperature indicated in Fig. 2. Mutants were broadly classified into the following three groups: I, absolute defective, unable to grow in the absence of thymine; II, temperature sensitive, with a temperature-dependent ability

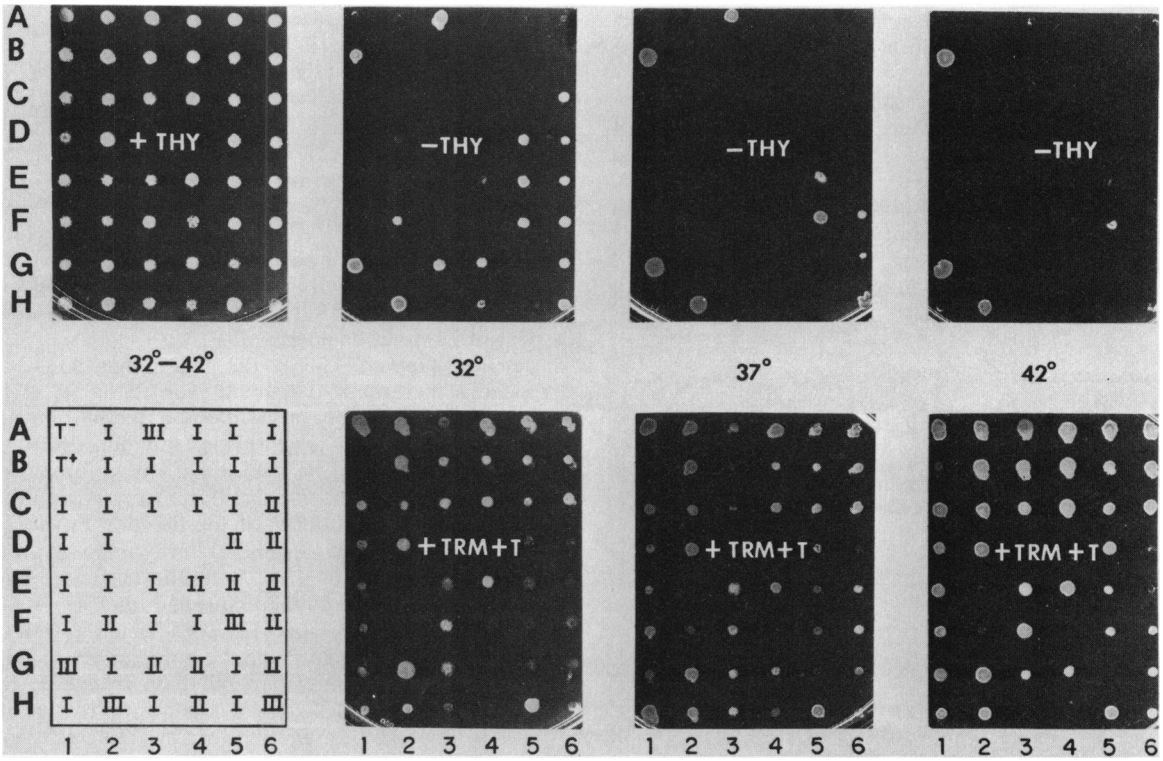

FIG. 2. Plating characteristics of Thy^- mutants. Purified mutant clones, selected at 38°C on thymine-trimethoprim medium plus chloramphenicol, were grown up in wells of a microtiter dish containing TB plus yeast extract, thymine, and chloramphenicol and printed onto selective media. Minimal agar-chloramphenicol plates lacking thymine (−THY), or supplemented with trimethoprim and thymine (+TRM+T) and thymine- and chloramphenicol-containing TB plates (+THY) were incubated for 16 to 24 h at the indicated temperature of 32, 37, or 42°C. The +THY plates yielded similar results at all three temperatures, and only the 32°C plate is shown. Similar growth patterns were also obtained with minimal medium containing a thymine supplement. The mutant classification scheme, which assigns mutants to one of three groups, is shown to the bottom left of the figure: I, absolute defective; II, temperature sensitive; III, partially defective. The *thyA* control (T^-) in well A1 was Rue10(pACYC184), whereas the *thyA*$^+$ control (T^+) in well B1 was Rue10(pATAH). All mutants are derivatives of Rue10(pATAH).

TABLE 1. Enzyme activity of selected mutants and map position of selected mutations

Plasmid[a]	Class[b]	Growth on Thy$^-$[c]			Synthase Activity[d]		FdUMP[e] binding	Map[f] segment
		30°C	37°C	42°C	30°C	37°C		
pACYC184	Thy$^-$	−	−	−	0.1	0.5	−	NA
pATAH	Thy$^+$	+	+	+	100	100	+++	NA
C3	I	−	−	−	0.1	0.9	−	407–509
F3	I*	−	−	−	2.0	2.0	++	361–401
D6	II	+	−	−	5.0	0.8	+	620–778
G1	III	+	+	+	12.0	14.0	+	ND

[a] All plasmids were in *thyA recA* strain Rue10. pATAH is the Thy$^+$ parent from which mutants C3, F3, D6, and G1 were isolated. Mutant numbering corresponds to microtiter dish coordinates.

[b] As per Fig. 2 and 3. I*, Class I, which has retained FdUMP-binding ability.

[c] As per Fig. 2. +, Growth; −, no growth on minimal media lacking thymine.

[d] Sonic extracts prepared from cells grown at 30°C were assayed for thymidylate synthase activity at the indicated temperature by measuring tritium release from the substrate [5−^{3}H]dUMP (26). The specific activity of the Thy$^+$ parent pATAH (2 mU/mg of protein at 30°C and 1.5 mU/mg of protein at 37°C) was assigned a value of 100, and the other activities are expressed relative to this.

[e] As per Fig. 3. +++, Very strong FdUMP binding; ++, strong binding; +, moderate binding; −, no detectable binding. The FdUMP binding for F3 is indicated as ++, since in shorter exposures than that depicted in Fig. 3 the labeling appears to be somewhat less intense than that of the parent clone.

[f] As per Fig. 1. NA, Not applicable; ND, not determined.

to grow in the absence of thymine; and III, partial defective, able to grow in the absence of thymine as well as in the presence of trimethoprim and thymine. Although this classification is somewhat arbitrary and overlap does exist between the three mutant groups, the scheme is operationally useful. We have further classified 400 mutants into 42 subgroups based on both phenotypic (e.g., degree of temperature sensitivity, enzyme activity, and patency of the substrate-binding domain) and genetic criteria (e.g., reversion frequency and map position), indicating the diverse nature of the mutant collection.

To reconcile plating characteristics with thymidylate synthase activity, enzyme assays were performed on 10 each of class I and II mutants and on 5 class III mutants. All had varied degrees of reduced activity. From the typical examples shown in Table 1 it appears that mutants with ≤2.0% of the synthase activity of the parent Thy$^+$ plasmid are unable to grow in the absence of exogenous thymine (C3, F3, and D6 at elevated temperature), whereas mutants with ≥5% of parental activity can grow on media lacking thymine (G1 and D6 at low temperature).

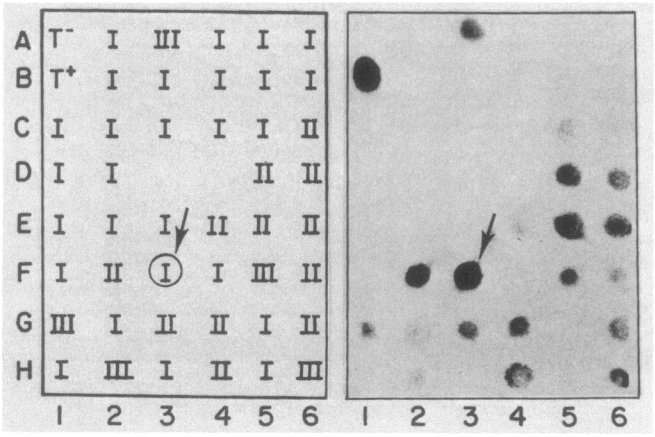

FIG. 3. In situ FdUMP-binding assay. The same array of mutants depicted in Fig. 2 was subjected to ternary complex formation with CH_2 FH_4 and [^{3}H]FdUMP as described in the text. The fluorogram to the right of the figure (2-week exposure, −80°C) demonstrates residual binding activity of the mutants relative to the *thyA*$^+$ control (B1). Mutant F3 (arrow) is unusual in that it was classified as absolute defective, yet has retained substantial FdUMP-binding affinity.

In situ FdUMP-binding assay. To investigate the properties of the mutant enzymes on a large scale, we developed an in situ FdUMP-binding assay based on ternary complex formation between thymidylate synthase, the substrate analog [6-^{3}H]FdUMP, and CH_2FH_4. The ability of Thy$^+$ colonies, lysed on a filter, to bind FdUMP is demonstrated in Fig. 3. FdUMP binding seems to be correlated in most cases with plating characteristics (Fig. 2) and enzyme activity (Table 1). Thus, whereas type I mutants generally did not bind ^{3}H-FdUMP, type II and III mutants, which retain at least some ability to grow in the absence of thymine, bound the ligand to various degrees. The notable exception was mutant F3, which was unable to grow at all in the absence of thymine (Fig. 2) and had only 2% of parental enzyme activity (Table 1), yet bound FdUMP almost as avidly as the Thy$^+$ wild type. It would appear that F3 enzyme has maintained its FdUMP-binding capacity and must therefore have suffered a lesion outside of the substrate-binding site in some other aspect of its functional apparatus.

Deletion mapping. The deletion mapping system, designed to localize mutations to specific gene segments, was based on the two nested sets of deletions extending inward from opposite ends of the gene (3) and dividing the functional portion of the *thyA* gene (945 nucleotides) into 13 deletion intervals (Fig. 1). The precise extent of these intervals is known since the sequences of the deletion endpoints were previously determined (3) (Fig. 1). Two mapping procedures have been developed: in the first, the truncated fragments were phage borne, and mapping relied upon rescue of wild-type sequences from an M13-*thyA* deletion after phage infection of the strain carrying the point mutation (Fig. 4); in the second, the strategy depended on rescue of the *thy*$^+$ sequences from a pBR32-*thyA* deletion derivative that had been introduced by transformation into the strain harboring the point mutation (Fig. 5). Chromosomal *thyA* mutant alleles *Δ2*, *3*, *7*, *Δ64*, *83*, and *108* (1) and that in strain N4316 (9) have, on the basis of this bidirectional deletion mapping system, been assigned the positions shown in Fig. 1.

To use our mapping systems to localize plasmid-borne *thyA* mutations we sought to generate a complete deletion of the chromosomal *thyA* gene so as to confine Thy$^+$ rescue to recombination events between the cloned point mutation and cloned deletion. These attempts, like those previously reported by Chung and Greenberg (11), have, however, been

unsuccessful (see below). The deletion Δ*64* (1, 32) has, instead, been useful in providing an appropriate chromosomal background for mapping point mutations in the cloned *thyA* gene. Despite the fact that the background level of Thy^+ recombinants is sometimes somewhat elevated, especially when working with mutations at the extremities of the map, we have been able to assign unambiguous map positions to many *thyA* mutations generated in pATAH. Map locations of C3, D6, and F3 are shown (Fig. 1), with the positions of C3 and F3 having been verified by DNA sequencing experiments.

Sequence changes in two *thyA* mutants. The sequence change in C3, an absolute defective class I mutant, is a G→A transition in codon number 98 changing UGG (Trp) to UAG (amber). Since this null phenotype was selected in the *thyA* host Rue10, which carries an efficient *supE* Gln-inserting amber suppressor allele (15), the effective change, leading to the abolition of thymidylate synthase activity and FdUMP binding (Table 1), is Trp-98→Gln-98. The F3 mutation is another G→A mutation, converting Cys-50 (UGC) to Tyr-50 (UAC). Both of these sequence changes were the only mutations detected within 150 nucleotides to either side of the relevant single-base substitutions in C3 and F3.

DISCUSSION

The straightforward mutant isolation procedure described, coupled with streamlined phenotypic screening methods (Fig. 2 and 3) and rapid genetic mapping of the *thyA* gene (Fig. 1, 4, and 5), makes its thymidylate synthase product amenable to the techniques of in vitro genetics. Whereas class I mutations, which destroy catalytic activity, will be useful in defining residues that are vital to the catalytic process, class II and class III mutations, which alter rather than abolish enzyme activity, should eventually allow kinetic measurements to identify more subtle roles and interplays of the synthase domains that contribute to the catalytic process.

The in situ FdUMP-binding assay that we have developed facilitates the screening of large numbers of mutants for their ability to form a ternary complex with this substrate analog, CH_2FH_4, and thymidylate synthase. In the majority of cases the ability of mutant clones to grow in the absence of thymine as well as their synthase activity assayed in crude extracts correlates with their FdUMP-binding capacity (Fig. 2 and 3, Table 1). One interesting exception is mutant F3, which is unable to grow in the absence of thymine and which produces inactive enzyme, yet retains the ability to bind this ligand. We are presently investigating the possibility of using this assay to distinguish between temperature-sensitive synthesis of the enzyme and temperature-dependent ligand binding by employing differential mutant growth and assay temperatures. This would corroborate genetic mapping data, used presently as the basis for distinguishing between mutations in the structural gene and those in the transcriptional and translational control regions 5′ to the coding sequence.

The deletion mapping procedures, based on tester deletions carried in phage M13 or in plasmid pBR322, localize point mutations in the *thyA* gene to one of 13 deletion intervals. It should be emphasized that these deletions were generated as part of the DNA sequence strategy for the wild-type *thyA* gene, and that no additional recombinant construction for mapping purposes was necessary. Whereas the M13-Δ*thyA* cross-streak test is more rapid, results are sometimes less clear cut than with the pBR322-Δ*thyA* patch test. This latter test also has the advantage that it is not confined to M13-sensitive F^+, F′, or Hfr strains, being feasible in any strain that is transformable by pBR322. These mapping techniques serve to guide DNA sequence experiments de-

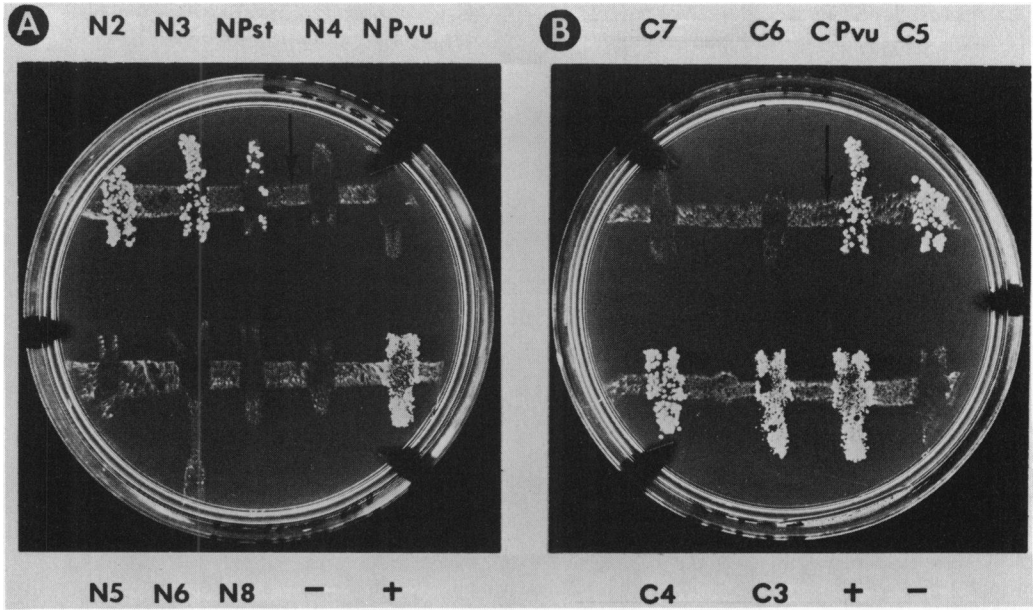

FIG. 4. Deletion mapping by M13 infection. M13mp9 recombinant phages that had been cross-streaked against Hfr3.OSO *thyA83* cells on rich medium were subsequently replica plated onto minimal agar plates lacking thymine. Thy^+ recombinants are seen as heavy bacterial growth or distinct isolated colonies in the phage cross-streak. (A) Amino-terminal (N) deletion series; (B) carboxy-terminal (C) deletions. The M13TAHΔN or M13TAHΔC phage designations are indicated at the appropriate cross-streak in A or B, respectively. The negative control (−) was M13mp9, and the positive Thy^+ control (+) was M13TAH. Arrows indicate the recombinational cutoff point, i.e., the deletion interval to which the *thyA83* mutation maps. The deletion endpoints are shown in Fig. 1.

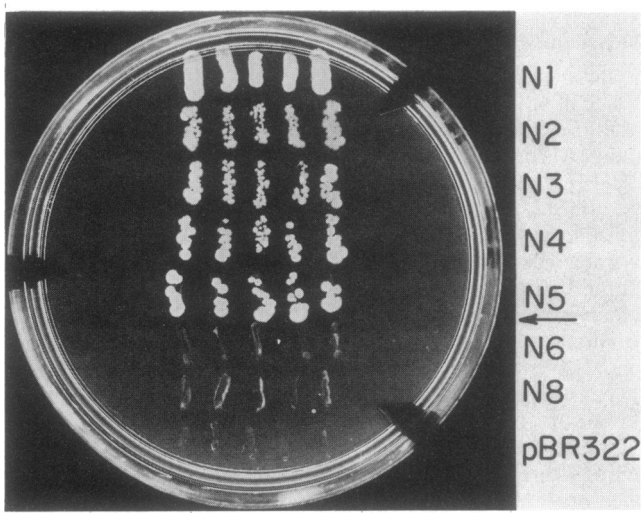

FIG. 5. Deletion mapping by transformation. The patch test shown represents transformants of strain Hfr3.OSO Δ*thyA2* with the constructions pBTAHΔN1 through pBTAHΔN8, designated N1 through N8, respectively. pBR322 transformants of this strain provided the negative control. Single colony transformants were selected on TB plus yeast extract, thymine, and ampicillin and patched onto minimal agar plates lacking thymine. The arrow indicates the deletion interval to which the 5′ end of the Δ*thyA2* mutation maps.

signed to define the precise mutational change. Further, the bidirectional confinement of a mutation to a particular deletion interval minimizes the possibility of second-site mutations existing outside of the interval, thereby obviating sequence analysis of the entire gene.

The sequence determination of two of the cloned *thyA* point mutations confirms their map assignments. Although the mapping and sequence alterations of a large number of mutations will be reported elsewhere, it is worthwhile to note several features of the two sequence changes for mutations C3 and F3 reported here. The mutation in C3 converting Trp-98 to an amber codon is not suppressed by insertion of Gln by the *supE* suppressor allele in host strain Rue10. This is not surprising given that tryptophan is a hydrophobic, aromatic amino acid, whereas glutamine is a hydrophilic residue. A number of other efficient amber suppressors are available, including *supD*, *supF*, *supP*, and *supU*, inserting serine, tyrosine, leucine, and tryptophan, respectively (15), and it will be worthwhile to determine the phenotypic consequences of these amino acid substitutions. The stringency of the amino acid requirement in position 98 and those features (e.g., charge, hydrophobicity) that are compatible with enzyme activity may thereby be ascertained. F3, on the other hand, has suffered a cysteine→tyrosine change at residue 50. This amino acid, which is distant in the primary sequence from Cys-146, the FdUMP-binding residue in the substrate-binding domain (3), borders a 10-residue stretch (50 to 60) that is similar to that implicated in the binding of folate derivatives in the *Lactobacillus casei* synthase (21). A possibility, therefore, is that whereas the cofactor-binding domain has been altered, leading to loss of the catalytic process, the substrate-binding specificity is little changed, and therefore the ability to complex with FdUMP is retained. Whether FdUMP can, in fact, bind to this mutant enzyme in the absence of CH_2FH_4 to form a stable binary complex is a possibility that remains to be tested. Interestingly, a clustering of mutations has been noted in two regions of the structural gene, namely, in the vicinity of the F3 mutation and near the substrate-binding cysteine residue (Fig. 1; unpublished data). These observations further implicate these two domains of the synthase as of functional importance.

The order of chromosomal *thyA* alleles Δ*2*, *3*(Ts), *7*, Δ*64*, *83*, and *108*, which we determined with these methods (Fig. 1), is in partial agreement with the original map assignments of Alikhanian et al. (1) and resolves those discrepancies between recombination frequency and the order of these markers later noted by Sukhodolets et al. (33; personal communication). Spurious ordering of alleles inherent in classical three-factor crosses is clearly obviated by this deletion mapping strategy.

The *thyA* allele in strain N4316, a temperature-sensitive *thyA* mutant that was noted by Cheung and Herrington (9) to

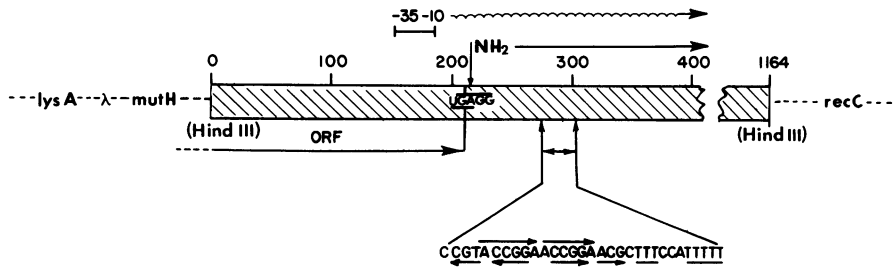

FIG. 6. Chromosomal orientation and structure of the 5′ region of the *thyA* gene. The double line (hatched), reflecting the extent of DNA of known sequence in the *thyA* region (3), corresponds to the *thyA* fragment shown in Fig. 1. The areas flanking the artificial *Hin*dIII linker sites (4) represent the neighboring chromosomal regions. The relative order of the *lysA* and *mutH* genes on the 5′ side of the *thyA* gene and the *recC* locus at the 3′ end is shown. Although the precise distances between these loci are not yet known, they cluster within <1 map unit (i.e., <40 kilobases) (2). Neither RecC nor MutH activity is encoded within 3 kilobases to either side of *thyA* (unpublished data; P. Modrich, personal communication). The orientation of the *thyA* gene has been inferred by its coding characteristics (3, 4) coupled with its relationship to the *recC* gene in the constructs of Hickson and co-workers (17, 18). The location of a λ prophage between *lysA* and *mutH* in an atypical lysogen isolated by Shimada et al. (30) is shown (11, 19). Nucleotide numbering is above the *thyA* map. The synthase-coding sequence starts at NH_2 (solid arrow), whereas the *thyA* transcript (wavy arrow) originates at a yet undetermined point beyond the *thyA* promoter (−35, −10) (3). The *thyA* ribosome-binding sequence GAGG (overscored) overlapping the UGA termination codon (underscored) of the potential coding sequence (ORF) (solid arrow below map) is shown within the map. The sequence of a putative regulatory region bearing resemblance to a transcriptional terminator is shown (↔), with arrows above the sequence marking direct repeats and below the sequence demarcating inverted repeats. The poly-T tracts are underscored.

have an associated multiple suppressor phenotype, maps in the same leftward region of the gene as allele *3*, which is also a *thyA*(Ts) mutation. This is a further example of the clustering of chromosomal temperature-sensitive mutations in *thyA* previously reported by Alikhanian et al. (1), who found that 62 of their 150 *thyA* mutations were temperature sensitive and mapped to one end of the gene. Interestingly, we have found no such position effect among temperature-sensitive mutations isolated in the cloned gene (unpublished data), suggesting a relationship between the clustering of temperature-sensitive mutations and some property of the chromosomal *thyA* locus (see below).

Although we have been able to force small internal deletions generated in vitro into the chromosome by using strategies developed by Greener and Hill (16), large deletions spanning the entire gene have not been transferable from a plasmid construct into the *thyA* locus. It is important to note that, whereas Chung and Greenberg (11) were able to cure abnormal λ lysogens with the prophage inserted between *thyA* and *lysA* (30) (Fig. 6) and to obtain equivalent numbers of λ *thy* and λ *lys* transducing particles, they obtained roughly 200 Lys^-, but no Thy^-, mutants among the cured survivors. Their experiments suggested that the desired deletions appeared to cause loss of an essential diffusible function bordering *thyA*. On the other hand, the *mutH* locus, between the λ prophage and *thyA* gene (Fig. 6), has been successfully deleted by using this strategy (19). These observations prompted us to reexamine the sequence of the 5′ portion of the *thyA* gene, which we have been able to orient relative to neighboring chromosomal loci as described in Fig. 6. We found an open reading frame which extends for 200 nucleotides from the 5′ end of the *thyA* fragment and which presumably begins in an area of unknown sequence 5′ to this region. This potential coding sequence ends beyond the *thyA* promoter (nucleotides 153 through 184) with a UGA termination codon that overlaps the presumptive ribosome-binding sequence of *thyA* (Fig. 6). This stop codon is followed 60 nucleotides downstream within the *thyA* structural gene by a structure containing a GC-rich inverted repeat region and two strings of Ts. This region, spanning nucleotides 275 through 304, has some properties of a transcriptional terminator (27) and also contains two immediately adjacent 6-base-pair direct repeats. Some questions stimulated by these observations include the following. (i) Is there a neighboring gene with its distal 3′ end overlapping the 5′ portion of the *thyA* gene? (ii) Does this putative gene code for the vital function without which the cell cannot survive, precluding the isolation of complete *thyA* deletions? (iii) Does this overlap account for the preponderance of temperature-sensitive mutations in this region of the *thyA* gene (1) (Fig. 1)? (iv) Is the suppressor phenotype associated with the *thyA*(Ts) mutation in N4316 (9) the result of concomitant mutation of this overlapping function? Although these speculations are providing a basis for further experiments, no answers to these questions are yet available. Nevertheless, an interesting precedent for the unusual configuration of procaryotic thymidylate synthase genes has been provided by the *td* gene of bacteriophage T4: not only is there a 4-nucleotide overlap between the *td* initiation codon and the termination codon of the neighboring dihydrofolate reductase gene (10, 25), but also an intervening sequence has been discovered recently in the synthase-coding region of the *td* gene (10).

A phenotypic-genetic analysis of the 400 *thyA* mutants with the techniques developed here should clarify the regulatory sequences of the gene as well as functional domains of the synthase. The correlation of map position and sequence alteration with a specific defect will serve to guide directed mutagenesis strategies, for which several efficient methods are available (31), to further probe the catalytic process and regulatory mechanisms that govern the expression of this metabolically important enzyme.

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