Conditional Thymidine Auxotrophic Mutants of Mouse FM3A Cells Due to Thermosensitive Thymidylate Synthase and Their Prototrophic Revertants*

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A number of temperature-sensitive conditional thymidine auxotrophs were isolated from mutagenized mouse FM3A cells. Upon temperature shift from 33.5 °C to 39.5 °C, the mutant cells rapidly lost thymidylate synthase activity with concomitant decrease in intracellular dTTP and changes in other dNTP pools. Thymidylate synthase obtained from these mutants was inactivated in a manner following first order kinetics by heat treatment, which did not affect the parental enzyme. When bound covalently to 5-fluoro-2'-deoxyuridine 5'-[14C]monophosphate and 5,10-methylenetetrahydrofolate, the enzyme of one mutant migrated slower than the parental enzyme on non-denaturing polyacrylamide gel electrophoresis, whereas it did not do so on sodium dodecyl sulfate gel electrophoresis. Spontaneous prototrophic revertants were isolated from most of the mutant lines. In some revertants, the enzyme regained the heat resistance of the wild type completely or partially. In other revertants, the enzyme was overproduced, but its heat-sensitive nature was unaltered. The results demonstrate unequivocally that the conditional thymidine auxotrophy in the mutants was caused by thermosensitive thymidylate synthase due, at least in one particular line, to a missense mutation in its structural gene.

Thymidylate synthase (EC 2.1.1.45) is a key enzyme in DNA synthesis because it catalyzes the only de novo pathway for supply of dTTP. It is closely coupled with the proliferative potency of cells and its gene expression is cell-cycle dependent. Because of these characters, the enzyme has been a direct and indirect target of cancer chemotherapeutic agents. The enzyme can be easily purified to homogeneity by one-step affinity chromatography and easily identified biochemically by its specific binding to FdUMP*. Therefore, establishment of a genetic system of the thymidylate synthase gene, which is probably located on autosomes (2), should be useful in the study of the problems in somatic cell genetics, such as mechanisms of recessive mutation, especially autosomal mutation.

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The abbreviations used are: FdUMP, 5-fluoro-2'-deoxyuridine 5'-monophosphate; FdUrd, 5-fluoro-2'-deoxyuridine; ts, temperature-sensitive.

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**Experimental Procedures**

**Materials**—Powdered ES medium, designed by one of us (H. K.), was purchased from Nissui Seiyaku Co., Tokyo. ES medium was made on the basis of Eagle's minimum essential medium with some modifications and will be described in detail elsewhere. It contains all essential and nonessential amino acids, 0.15 μM vitamin B12, and 1 mM pyruvate, but is devoid of purines and pyrimidines. Fetal calf serum was purchased from GIBCO, Grand Island, NY. Methotrexate, d,l-5-N-methyltetrahydrofolate, and FdUMP were obtained from Sigma Chemical Co., MO. The other chemicals used were of reagent grade and were obtained from Wako Pure Chemicals Co., Osaka, Japan. Plastic tissue culture dishes were products of NUNCROM, Denmark. [14C]FdUMP (18 Ci/mmol) was purchased from Moravek Biochemicals, CA. [33P]FdUMP was prepared by enzymatic phosphorylation of FdUrD as follows. Reaction mixture (0.1 ml) contained 1 μM [7-32P]ATP (200 Ci/mmol, Radiochemical Centre, Amersham), 50 mM Tris-HCl (pH 7.5), 1 mM FdUrD, 3 mM MgCl2, 0.7 mM MnCl2, 5 mM NaF, and 50 μl of thymidine kinase sample extracted from Escherichia coli as described previously (7) except that column chromatography steps were omitted. The mixture was incubated for

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2 H. Koyama and H. Kodama, manuscript in preparation.
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60 min at 37 °C and boiled for 1 min. The ["\(^3\)P"]dUMP formed was monitored by polyethyleneimine cellulose thin layer chromatography with authentic dUMP as a standard.

**Cell Culture—**Cells were cultured at 33.5 °C or 39.5 °C in suspension in plastic dishes containing ES medium supplemented with 2% fetal calf serum, which had been dialyzed against ES medium. When indicated, 10 μM thymidine was added to the medium as a nutritional repressor. The semisolid agar medium (2% agar in a CAH/He mouse (4) were mutagenized with 0.5 gg/ml of N-methyl-N"-nitro-N-nitrosooguanidine (Sigma Chemical Co., MO) for 3 h at 33.5 °C (a condition under which the surviving fraction was reduced to 0.5) and then cultured for 2 days at 33.5 °C in thymidine-free medium to allow expression of mutated genes. The cells then seeded on selection plates, which contained agar medium supplemented with 10 μM thymidine, 1 μM d-5-N-methylthymidylate, and 30 mM methotrexate, and incubated for a week at 39.5 °C. Colonies grown on the selective plates were scored and picked up. Clones that were capable of growing at 33.5 °C but not at 39.5 °C in thymidine-free medium were reared on agar medium and then plated for characterization. The method used for selecting thymidylate synthase-deficient mutants on the basis of methotrexate resistance (8) was described in detail elsewhere (2).

**Assay of Thymidylate Synthase—**A sample of about 10 cell harvested from an exponentially growing culture was washed once with phosphate-buffered saline and resuspended in 1 ml of 50 mM Tris-HCl (pH 7.5) containing 10 mM dithiothreitol, 0.1% Triton X-100. The samples were disrupted by sonication and then the supernatant obtained by centrifugation at 12,000 x g for 10 min was used as a crude enzyme preparation.

Thymidylate synthase was assayed by the method described previously (9) with minor modifications. A 50-μl aliquot of the enzyme preparation was mixed with 30 μl of substrate mixture consisting of 0.15 mM Tris-HCl (pH 7.5), 0.8% bovine serum albumin, 0.1 mM thymidylate (both were from Sigma Chemical Co., MO), 0.5% formamide, 50 mM NaF, 5 mM dithiothreitol, 0.06 mM dUMP and 1-2 μl of [\(^3\)H]FdUMP (1 μCi/ml, specific activity, 10 Ci/mmol, Radichemical Centre, Amsbaugh). After incubation for 60 min at 33.5 °C, the reaction was terminated by adding 0.5 ml of 2% charcoal in 0.01 M HCl. The mixture was vigorously mixed, stood for 15 min, and centrifuged for 5 min at 3000 rpm. Then 0.4 μl of the supernatant was mixed with 4 ml of aqueous counting scintillant (ACS 11, American). After electrophoretic runs, the gels were soaked in 7% acetic acid and stained with Coomassie blue. The stained bands were excised from the gel, treated with 1 N HCl for 30 min, and subjected to polyacrylamide gel electrophoresis in two systems. One of the gels contained 0.1% Triton X-100, 10 mM dithiothreitol, 13 mM potassium phosphate (pH 7.5), 0.1% Triton X-100, 10 mM dithiothreitol, 13 μl of the 0.5 mM N5,N10-methylenetetrahydrofolate solution, 50 mM NaF, 10 mM [\(^3\)P]FdUMP (200 Ci/mmol) or 200–500 mM [\(^3\)H]FdUMP (18 Ci/mmol), and 0.3–0.4 mg of crude cell extract. The reaction mixture was incubated for 60 min at 33.5 °C and then dialyzed against 10 mM Tris-HCl (pH 7.5) containing 0.1% Triton X-100. The samples were subjected to polyacrylamide gel electrophoresis in two systems. One was similar to that described previously (10) except that separation gels (10%) contained 0.1% Triton X-100 and spacer gels were omitted. For the other system, the samples were denatured in 1% sodium dodecyl sulfate by heating for 2 min at 100 °C and applied to sodium dodecyl sulfate separation gels (10%) as described previously (11). After electrophoretic runs, the gels were soaked in 7% acetic acid and exposed to Kodak XR-1 x-ray films backed with DuPont Cronex (P-1) X-ray film (Dupont de Nemours, Wayne, N.J.). The activities of dTTP pools were extracted with ice-cold 30% methanol from cell pellets prepared rapidly from exponentially growing cultures as described previously (12). The extracts were processed and analyzed enzymatically for the content of dNTPs using E. coli DNA polymerase I (Boehringer Mannheim, Germany) and alternating co-polymers poly(dA-dT)-(P-L Biochemicals Inc., CA and poly(dI-dC) (Miles Laboratories Inc., IN) as templates by the methods described previously (13, 14).

**Cell Hybridization—**The mutant cells were fused with each other and with protoporphyrin FS5oual cells carrying a recessive marker of temperature-sensitive conditional growth and a dominant marker of ouabain resistance by means of polyethylene glycol as described previously (4, 12). Hybrids arising from the crosses between thy" mutants and FS5oual were selected on agar medium containing 2 mM ouabain (Sigma Chemical Co., MO) and 10 μM thymidine by incubation at 39.5 °C and then tested for their thymidine requirement. Hybrids from the crosses between thy" mutants were subjected to selection on thymidine-free agar medium at 39.5 °C. Hybrid clones were analyzed for their karyotypes to ensure that the cells were hybrids as described previously (4, 12) and maintained in medium containing thymidine.

**RESULTS**

**Isolation of Temperature-sensitive Conditional Thymidine Auxotrophs—**Thymidine auxotrophs were first isolated at 39.5 °C (nonpermissive temperature) by single step selection as described under "Experimental Procedures" and then cells that could grow in thymidine-free medium at 33.5 °C (permissive temperature) were cloned and defined as thy" mutants. More than 10 thy" mutants, all of independent origin, have been obtained thus in several experiments. Thymidine auxotrophs including thy" mutants were isolated at a frequency of 5 x 10^{-5} when cells were mutagenized with N-methyl-N"-nitro-N-nitrosooguanidine. The average frequency of distinct thy" mutants was about 7 x 10^{-7}. Thus, about one-seventeenth of the thy" auxotrophs showed the temperature-sensitive phenotype. When the mutation treatment was omitted, no thymidine auxotrophs were obtained at a frequency of less than 10^{-6}. In contrast, spontaneous temperature-insensitive prototrophic revertants of thy" mutants could be isolated at a frequency of about 10^{-4} as shown later. In this study, four mutants thy"1, thy"2, thy"3, and thy"4 were extensively characterized. These mutants, which were named simply in their order of isolation, are listed in Table I.

**Growth of Thy" Mutants—**Fig. 1 shows the growth of the thy" mutants under various conditions. When the cells were shifted from 33.5 °C to 39.5 °C in the absence of thymidine, thy"1, thy"2, and thy"4 cells immediately stopped growing and began to lyse within 1 day, whereas thy"3 cells showed a slight increase in cell number and then started to lyse by day

<table>
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<th>Table I</th>
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<td>Activity and content of thymidylate synthase in the parent and its thy&quot; mutants</td>
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<td>FSthy&quot;3</td>
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Cells harvested from an exponentially growing culture in ES medium at 33.5 °C were disrupted and the supernatants obtained by centrifugation at 12,000 x g were used as enzyme preparations. For details, see "Experimental Procedures." Activities are shown as averages for three to four independent experiments. Enzyme contents were determined as follows: the enzyme-[\(^3\)H]FdUMP 5,10-methylenetetrahydrofolate complex was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis as described under "Experimental Procedures." The gel segments containing the complex were cut out after extensive washing in 7% acetic acid and then counted in toluene-based scintillation mixture containing 10% tissue solubilizer (NCS, Amsbaugh).
2. In the presence of thymidine, the four mutants grew quite normally at both 33.5°C and 39.5°C, suggesting that only a biosynthetic step of thymidylate is blocked in the mutants.

Decrease in Thymidylate Synthase Activity in Thy" Mutant Cells upon Shift Up to 39.5°C—Although the thy" mutants can grow normally at the nonpermissive temperature when thymidine is supplied exogenously, the thymidylate synthase activity in these mutant cells decreased rapidly in response to shift-up of temperature, as shown in Fig. 2 (see Table I for net enzyme activity before shift-up). By 12 h after the temperature shift, the enzyme level in the thy" mutants reached a minimum constant level, amounting to less than 5% of that of the wild type. The presence of 10 μM thymidine in the culture had no significant effect on the enzyme level in the FM3A cell lines used here (data not shown) and this enzyme has been shown to be stable in mouse fibroblasts with a half-life of 20–25 h (15). Thus, the rapid loss of the enzyme activity in the thy" mutants suggests that the enzyme itself is temperature-sensitive and also that its loss is responsible for the immediate cessation of growth of the mutants in response to the temperature shift-up.

Thymidylate Synthase Level in Thy" Mutants—Table I shows that the thymidylate synthase activities in cell extracts of mutants thy"1, thy"2, thy"3, and thy"4 were reduced to about 30%, 30%, 70%, and 20%, respectively, of that of the wild type even though the mutant cells were grown at the permissive temperature.

Next, we determined the net amount of the enzyme. The enzyme can be titrated directly with labeledFdUMP owing to the unique fact that FdUMP binds covalently to the active site of the enzyme at the sixth position and simultaneously to 5,10-methylenetetrahydrofolate at the fifth position of the pyrimidine ring, thus, forming a stable ternary complex that is resistant to acid and to sodium dodecyl sulfate treatment (16). The enzyme content of cells measured by this affinity ligand was reduced to almost the same level as that measured by enzyme activity (Table I). Thus, the decrease in enzyme activity in thy" mutants was due to decrease in the active form of enzyme molecules, not merely to change in the catalytic activity.

The decrease in the level of active enzyme in the mutant cells could be affected by two factors, the stability of the enzyme in vivo and number of functional gene copies. The enzyme from thy" mutants was in fact unstable in vitro owing to a structural alteration, as described later. In Table I, it is noted that the mutant thy"3 has an enzyme content roughly 2 times as much as those of the other three mutants. When this instability of the enzyme was assumed for all four mutants and the value in mutant thy"3 was normalized to that in the wild type, the results could be interpreted to mean that two copies of the thymidylate synthase gene were functioning in mutant thy"3 (homozygous), but only one copy of the gene was active in the other three mutants (hemizygous) (see a later part of "Discussion").

Changes in Intracellular Pool Sizes of dNTPs in Thy" Mutants after Temperature Shift-up—The rapid loss of intracellular thymidylate synthase activity in response to the temperature shift-up in the mutants should affect the biosynthesis of thymidylate and thereby of other deoxyribonucleotides. As expected, when the cells were transferred to 39.5°C from 33.5°C in thymidine-free medium, the intracellular pool of dTTP in all four mutants decreased progressively to a minimum within 6 h, whereas that of the parent decreased only slightly, as shown in Fig. 3. These results provide direct evidence for the temperature-sensitive auxotrophic phenotype in our thy" mutants.

The pool size of dCTP at the permissive temperature was significantly higher in the mutants thy"1, thy"2, and thy"4 than that in the parent and the mutant thy"3. Thus, it was inversely correlated with the thymidylate synthase activity, which is lower in the three former mutants than in mutant thy"3 and the parent. There are many reports (see Ref. 17) that in various cell lines the lowering of the dTTP, the higher is the dCTP, and this phenomenon is thought to be partly due to the effect of dTTP as a negative allosteric effector of CDP reduction by ribonucleotide reductase (EC 1.17.4.1). After the temperature shift-up, however, the dCTP pool was markedly decreased, like the dTTP pool, in all of the mutant lines, but not in the parent. These results are not consistent with the above rule. It must be noted in this connection, however, that the dATP pool was increased in the mutants thy"1, thy"2, and thy"4. Since dATP is known to inhibit reduction of all
Heat Stability of Thymidylate Synthase from Thy mutants—The heat sensitivity of thy mutants was tested in the presence of dUMP, a substrate of the enzyme, using a crude cell extract as enzyme source. The wild type enzyme was not affected by pretreatment at 42 °C for more than 2 h. In contrast, the enzymes from the four mutants lost more than 50% of their activities within 1 h under the same conditions (Fig. 4). The heat inactivation in all four thy mutants followed first order kinetics, indicating that the heat-sensitive enzymes are homogeneous. Similar results to those with cell extracts were obtained with purified enzyme preparations obtained by column chromatography on methotrexate-bound Sepharose 4B as described previously (21). When a mixture of the wild type and mutant enzyme was treated in the same way, the remaining activity was consistent with the theoretical value calculated from the inactivation curves as the sum of those of the two enzymes, thereby excluding the possibility of an inhibitor in the mutant cells.

Next, heat inactivation was examined at various temperatures (Fig. 5). The curves for the wild type and the four thy mutants all seemed to be monophasic. The temperatures at which the enzyme activities of the wild type and mutants thy1, thy2, thy3, and thy4 were reduced by 50% in 30 min were 46 °C, 42 °C, 43 °C, 41 °C, and 41 °C, respectively. Although the differences were small, the results were quite

Fig. 3. Changes in the intracellular pool size of dNTPs in the parent (○), mutants thy1 (△), thy2 (●), thy3 (▲), and thy4 (■) after shift-up of culture temperature. Cells grown to a cell density of about 5 × 10^6 cells/ml in thymidine-free medium at 33.5 °C were shifted up to 42 °C. At intervals after the temperature shift, cells were harvested and then deoxyribonucleotides were extracted for determination as described under “Experimental Procedures.” Two independent experiments gave similar results to those presented above.

Fig. 4. Effect of time of preincubation at 42 °C on the activity of thy mutants. The curves for the wild type and the four thy mutants seemed to be monophasic. The temperatures at which the enzyme activities of the wild type and mutants thy1, thy2, thy3, and thy4 were reduced by 50% in 30 min were 46 °C, 42 °C, 43 °C, 41 °C, and 41 °C, respectively. Although the differences were small, the results were quite
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The complex gave can be specifically marked with labeled FdUMP. We, thus, from the parent a single band on sodium dodecyl sulfate-polyacrylamide gel (lower panel). The ternary complexes were formed and subjected to electrophoresis as described under “Experimental Procedures.” The samples applied to the gels were from the parent (wt), mutants thy”1 (ts-1), thy”2 (ts-2), thy”3 (ts-3), and thy”4 (ts-4), and mixtures of those of the parent and each of the thy” mutants. For other abbreviations, see text.

Electrophoretic Analysis of Thymidylate Synthase—The enzyme from FM3A cells consists of two identical subunits and has a molecular weight of 37,000.4 Similar results have been reported for human leukemic cells (MW = 44,000) (1) and for mouse L1210 cells (MW = 38,500) (22).

As described earlier, thymidylate synthase forms a covalently bound ternary complex with FdUMP and 5,10-methylenetetrahydrofolate. One enzyme molecule has been shown to bind two FdUMP's sequentially; that is, each subunit binds 1 FdUMP molecule (23). Thus, the enzyme in the cell extract can be specifically marked with [3H]FdUMP on polyacrylamide gel electrophoresis to detect any possible difference in mobility of the mutant enzyme. The complex gave a single band on sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the single stained band coincided with the radioactivity on the autoradiogram obtained using enzyme purified to near homogeneity as a standard.4

Fig. 6 (upper panel) shows that the enzyme complex of the mutant thy”3 migrated slower than those of the parent and the other mutants (indicated by arrows). Each sample gave two bands. The complex with two FdUMP's (denoted as FdUMP-E) had higher mobility than the complex with one FdUMP (denoted as FdUMP-E). The slower band tended to decrease with concomitant increase in the faster band when a higher ratio of FdUMP to enzyme was used and also when freshly prepared enzyme or highly purified enzyme was used. The two bands were not detected in extracts from thymidylate synthase-deficient mutants (data not shown).

On sodium dodecyl sulfate gel electrophoresis, one major band corresponding to a dissociated form of the dimer enzyme (denoted as FdUMP-E) migrated the same distance in each case, including that of mutant thy”3 (Fig. 6, lower panel). These results indicate that the heat-sensitive enzyme in the mutant thy”3 has an altered net charge but not a different polypeptide chain length, thus providing evidence for a missense mutation in the thymidylate synthase structural gene. The results in Fig. 6 also indicate that thymidylate synthase in the ts mutants, at least in mutant thy”3, is composed of one species.

Genetic Analysis of Thy” Mutants—The results of cell-cell hybridization in Table II show that ts conditional thymidine auxotrophy is a recessive trait. Each of the ts mutants was crossed to the prototroph FSsoual as described under “Experimental Procedures.” The frequency of appearance of hybrid colonies was above 1 × 10⁻¹ at each cross when selected at 39.5 °C on agar plates containing 2 mM ouabain and 10 μM thymidine. Under our conditions, hybrid colonies usually appeared at a frequency of 5 × 10⁻¹ to 5 × 10⁻² for complemented crosses. When the four ts mutants were crossed with each other, colonies selected at 39.5 °C on thymidine-free agar plates were not obtained within a frequency of 10⁻² in any crosses. Moreover, the ts mutants did not complement some constitutive thymidylate synthase-deficient mutants obtained previously (data not shown).

These results indicate that the thy” mutants so far tested fall into a single complementation group, which presumably contains mutants having defects in a structural gene of thymidylate synthase.

Table II

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+ = Hybrid colonies appeared at a frequency of over 10⁻¹. | |
- = No hybrid colony appeared within a frequency of 10⁻².

Table III

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<thead>
<tr>
<th>Thymidylate synthase activity and doubling time of the revertants of mutants thy”3 and thy”4</th>
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Revertants of Thy⁻ Mutants—Spontaneous temperature-insensitive prototrophic revertants could be isolated from mutants thy⁻², thy⁻³, and thy⁻⁴. When cells from several populations starting from small inocula of mutants thy⁻², thy⁻³, and thy⁻⁴ were plated on thymidine-free agar medium at 39.5 °C, colonies appeared with an average frequency of 1 × 10⁻⁴, 8 × 10⁻⁴, and 5 × 10⁻⁴, respectively. No revertant colonies could be obtained within a frequency of 1 × 10⁻⁵ for mutant thy⁻¹. The characteristics of two revertants from each of the mutants thy⁻³ and thy⁻⁴ are presented.

As shown in Table III, all revertants grown at 39.5 °C in thymidine-free medium had regained thymidylate synthase activity, but not at the level of wild-type cells. However, these enzyme levels are sufficient to allow almost normal growth, because we have evidence that about one-tenth of the wild type enzyme level can support growth of mutants although it results in somewhat prolonged doubling times (see Ref. 5). In a revertant of the mutant thy⁻³, designated as thy⁻³-R₁, and a revertant of the mutant thy⁻⁴, thy⁻⁴-R₁, the enzyme levels as measured by both activity and affinity label with FdUMP in cell cultures at 33.5 °C were significantly elevated although their heat-sensitive nature was unaltered, as shown in Figs. 7 and 8. The reverted phenotype can be explained by this overproduction of the enzyme, which represents one type of suppression mechanism of the ts conditional phenotype in mammalian cells.

In revertant thy⁻⁴-R₂, thymidylate synthase from cells grown at 33.5 °C and at 39.5 °C both showed heat resistance comparable to that of the wild type (Fig. 7). Judging from the inactivation curves of the enzyme, the enzyme in the revertant was also homogeneous.

In revertant thy⁻³-R₂, thymidylate synthase from cells grown at 33.5 °C was slightly but reproducibly more resistant to heat than that of its parent thy⁻³, but was far less resistant than that of the wild type (Fig. 8). When enzyme extracted from revertant cells grown at 39.5 °C was examined, increase in heat resistance toward the wild type level was more evident (Fig. 8). Moreover, the curve of inactivation kinetics seemed to be biphasic, suggesting that two molecular species of thymidylate synthase are present in this particular revertant line (for genetic evaluation of the results, see "Discussion") and that the heat-resistant enzyme fraction is responsible for the revertant phenotype. If this is so, the partial shift toward the wild type level of the enzyme from thy⁻³-R₂ may be explained by supposing that only the enzyme formed from two reverted subunits is heat-resistant, while enzyme from two heat-sensitive subunits or a hybrid combination of the two subunits is heat-sensitive. Thymidylate synthase of all four revertant

DISCUSSION

Mutant Enzyme and ts Conditional Phenotype—Of 17 thy⁻ mutants isolated in the present study and in previous work (5), we have extensively characterized four clones. Biochemical analyses unequivocally showed that thymidylate synthase in cell extracts and partially purified preparations of these four clones was thermostable. Furthermore, the different mobility on gel electrophoresis of the enzyme from a mutant thy⁻³ provided a convincing evidence for structural alteration of the enzyme. On shift-up to the nonpermissive temperature, all of the ts mutants tested underwent rapid loss of intracellular thymidylate synthase activity with concomitant decrease in the dTTP pool. Thus, the results account well for the ts conditional thymidine auxotrophic phenotype. It should also be noted that these mutant enzymes are homogeneous judging from the profile of heat inactivation kinetics and results of electrophoretic analysis.

Ts conditional thymidine auxotrophy in these mutants behaved recessively in cell-cell hybrids. None of the four ts mutants described here complemented each other, indicating that these mutants have lesions in the same gene. Many thymidylate synthase-negative mutants have been assigned to this complementation group. Thus, the enzyme-negative mutants are likely to have defects in the thymidylate synthase gene.

Spontaneous Revertants of Thy⁻ Mutants—Revertants of mutants thy⁻³ and thy⁻⁴ were classified into at least two classes: one in which the enzyme is overproduced but has unaltered heat sensitivity, probably as a result of gene multiplication or mutation in a regulatory region, if any, in the thymidylate synthase gene, and the other in which the heat-sensitive enzyme has regained heat stability by a second mutation introduced in the same structural gene. The two classes of revertant phenotype described here should be included in the few examples so far reported of the reversion mechanisms in mammalian nonsense or missense mutants (25–27). A different type of suppression than the above two also seems to be involved in the other revertants because about one-half of them showed neither of the two phenotypes. In fact, a variety of reversion or suppression mechanisms are known in bacterial ts mutants. We conclude from these genetic and biochemical lines of evidence that temperature-sensitive

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growth in these mutants is brought by heat-sensitive thymidylate synthase, very probably as a consequence of a missense mutation in the thymidylate synthase gene.

Since thymidylate synthase is typical housekeeping enzyme, genetic and biochemical studies on the structure of this gene and its regulatory mechanism will be of great interest. Studies along these lines on isolation of the thy gene by the DNA-mediated gene transfer technique and structural analysis of thymidylate synthase from thy" mutants are underway.

Possible Mechanisms of Autosomal Recessive Mutation

The thy gene is probably located on autosomes because 6-thioguanine-resistant (hypoxanthine phosphoribosyltransferase-deficient, hprt) segregants from thy"×thy" hprt" hybrids appeared with a frequency of about 10^-3 regardless of whether or not thymidine was present in the selection plates (2).

Recessive thymidylate synthase-negative mutation was induced in N-methyl-N'-nitro-N-nitrosoguanidine-treated cells with a frequency of 10^-10^-7, whereas spontaneous mutants were not obtained when at least 1 x 10^9 cells were plated. Thy" mutants could be obtained with a frequency of 10^-7^-10^-8 from mutagenized cells. In contrast, spontaneous prototrophic revertants could be obtained with a frequency of about 10^-6 and with a frequency of about 10^-5 from mutagenized cells of thy" and thy" mutants. Taking into account the normal and higher reversion frequency than the extremely low frequency of the spontaneous forward mutation, it seems probable that both copies (possibly alleles) of the thymidylate synthase gene are active in FM3A cells rather than that only one gene copy is active (hemizygous or functionally hemizygous) as in some autosomal genetic markers in Chinese hamster ovary cells (28-30). The following results support this possibility. In FM3A cells, a variety of dominant mutations, e.g. resistance to ouabain, aphidicolin, tunicamycin, and arabinosylcytosine, and a recessive 6-thioguanine resistance mutation occur spontaneously with a frequency of about 10^-4 (4, 12, 31, 32). As examples of autosomal recessive mutations, mutants deficient in thymidine kinase, adenine kinase, and adenine phosphoribosyltransferase could be obtained at frequencies of more than 10^-6 by the mutagen treatment, but they appeared spontaneously at a frequency of less than 10^-8 (8).

In connection with these findings, it is relevant to discuss here the mechanisms involved in thy" mutation. Three possible mechanisms for autosomal recessive mutations are shown in Fig. 9. (i) A ts missense mutation and a second mutation that inactivates enzyme function occurred simultaneously in each of two copies of the gene (double mutations); (ii) a ts missense mutation on one copy of the gene can be manifested by an accompanying deletion or modification of a segment covering the other allelic gene on the complementary autosome, giving rise to a hemizygous state; (iii) a ts missense mutation on one copy of the gene is followed by a yet unknown recombinational event or gene conversion, e.g. somatic recombination like a mitotic intra-diplo chromatin interchange or somatic segregation (33, 34), thereby leading to a homozygous state for the mutated gene. These alternatives could overlap in the overall reactions induced by a multipotent mutagen such as N-methyl-N'-nitro-N-nitrosoguanidine, and, hence, a high frequency of autosomal recessive mutations could be obtained in FM3A cells. It is worth mentioning that in thy" mutants and tk" mutants, more than 90% of the cells had 40 chromosomes as in the wild type FM3A subclone F28-7 (4, 8). In addition, the chromosome banding analysis demonstrated no significant difference between thy" and the parental F28-7 cells. Thus, in mechanism (ii), a deleted segment, if any, would be very small.

In this connection, revertants of mutants thy"3 and thy"4 provided useful information. In revertant thy"4-R2, the thermostable enzyme regained heat stability and its inactivation curve indicated that the reverted enzyme was homogeneous, as was the mutant enzyme in the parental mutant (Fig. 7), suggesting that only one copy of the gene was active in mutant thy"4 possibly as a result of mechanism (ii) or (i). In contrast, in revertant thy"3-R2, the enzyme seemed to consist of two molecular species, a temperature-sensitive and a temperature-insensitive one (Fig. 8). These results suggest that mechanism (iii) was responsible for mutant thy"3 and also that the revertant resulted from a mutation in only one copy of the homozygous genes in the mutant since the reverted enzyme seems to have resulted from its parental enzyme judging from its electrophoretic behavior (Fig. 6). However, the occurrence of mechanism (i) or involvement of a missense suppressor tRNA in phenotypic reversion cannot entirely be excluded in the case of revertant thy"3-R2. The fact that the thymidylate synthase levels in mutants thy"3 and thy"4 are, respectively, the same as and one-half that of the wild type (Table I) favors mechanism (iii) for the genetic origin of mutant thy"3 and (i) or (ii) for that of mutant thy"4 according to the gene dosage effects. Isolation of more adequate revertants of mutant thy"3 or isolation of a dominant mutant with structurally altered thymidylate synthase is necessary for determining the more exact number of functional copies of the thy" gene in FM3A cells. Evidence that both alleles in autosomes are active has been reported (28, 35). This has also been shown more recently in, for example, a ribonucleotide reductase mutant of mouse S49 cells. It has also been suggested in the case of the regulatory subunits of CAMP-dependent protein kinase from mutant cells of mouse S49 cells (36) and a β-tubulin mutant of Chinese hamster ovary cells (37).

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Temperature-sensitive Thymidylate Synthase Mutants