Expression of rTS Correlates with Altered Growth Regulation of Thymidylate Synthase

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Abstract

The recently discovered rTS gene is convergent with and overlaps the thymidylate synthase gene in the antisense orientation. K562 B1A and KB 1BT are two methotrexate-resistant cell lines that have amplified genes for dihydrofolate reductase. K562 B1A cells have increased levels of rTS mRNA and protein compared with their parental K562 cells, whereas KB 1BT cells show unaltered rTS expression compared with their parental (KB) cells. Altered levels and loss of growth-regulation of in situ thymidylate synthase activity were found in methotrexate-resistant K562 B1A cells but not in K562, KB, and KB 1BT cells. These data point to a link between rTS and the regulation of TS activity.

Introduction

TS\(^{3}\) (EC 2.1.1.45) uses 5,10-methylenetetrahydrofolates as a cofactor in the catalytic conversion of dUMP to dTMP and represents the only de novo source for intracellular thymidine nucleotides. This enzyme plays a critical role in DNA replication since it is involved not only in the production of dTTP but also in the balancing of nucleotide precursor levels during DNA synthesis (1). In keeping with its central role in cell growth, the activity of TS is under tight control in the cell and is regulated at multiple levels. Although the TS gene is known to have E2F binding sites, regulation of TS is mainly by posttranscriptional mechanisms (reviewed in Ref. 2). These mechanisms of control include posttranscriptional down-regulation of mRNA levels in non-growing cells, translational regulation by a negative feedback mechanism where the enzyme binds to its own mRNA, and posttranslational modulation of enzyme activity in a cell cycle-dependent manner. Disruption of TS activity has major effects on DNA replication that has led to the enzyme becoming a major target for cancer chemotherapy (3); therefore, a better understanding of mechanisms underlying the regulation of this enzyme is potentially of great importance in developing cancer therapies. This laboratory previously described a gene, designated rTS (4), which is convergent and overlapping with the gene for TS (Fig. 1; Ref. 5). Transcription of rTS initiates outside the TS gene but extends into it by 938 nucleotides, covering the reverse strand of TS exon 7 and part of intron VI. The juxtaposition of rTS and TS genes and aberrant expression of rTS in an MTX-resistant cell line (4) suggests a functional relationship between these two genes. Although it was previously determined that rTS produced cytoplasmic, polyadenylated RNA, the ability of rTS RNA to serve as an mRNA was not reported. Evaluation of the sequence of rTS\(^{4}\) subsequent to correction revealed that it contains two ORFs (Fig. 1A), ORF1 and ORF2, which potentially encode Mr 10,000 and Mr 42,000 proteins, respectively. The studies presented here demonstrate that rTS encodes protein in cells and that this protein is elevated in at least one cell line (i.e., K562 B1A), which is MTX-resistant. The data also show that an MTX-resistant cell line that overexpresses rTS has lost the ability to regulate TS activity with cell growth.

Materials and Methods

Cell Culture. K562 (human chronic myelogenous leukemia; ATCC CLL 243) and KB (human oral epidermoid carcinoma cells; ATCC CLL 17) cells were cultured as described previously (4). The DHFR gene-amplified, MTX-resistant K562 B1A (4) and KB 1BT (6) sublines were cultured similarly, except for the presence of 100 and 50 \(\mu\)M MTX, respectively. Doubling times of log-phase cultures were 23 h for K562 and K562 B1A cells and 26 h for KB and KB 1BT cells. All cells were grown in tissue culture flasks or 6-well plates (for in situ TS activity determinations), except for K562 cells used in polysome experiments which were grown in 1.0-liter spinner flasks. Medium in spinner flasks contained 8 mM HEPES and 4 mM 3-[N-morpholino]propanesulfonic acid, pH 7.4. For in situ TS activity and nucleotide pool determinations, cells were plated at various densities and assayed 3 days later. At this time, cultures plated at the highest densities had reached saturation. All cell culture media and sera were obtained from GIBCO/BRL (Grand Island, NY).

Probes. \(^{32}\)P-labeled RNA was produced by \textit{in vitro} transcription using the Gemini Riboprobe II kit (Promega Corp., Madison, WI) as recommended by the manufacturer using \(\alpha\)-\(^{32}\)P-CTP (\(-10^{4}\) Bq/mmol; New England Nuclear, Boston, MA). Full-length antisense rTS \(^{32}\)P-labeled RNA was transcribed with T7 RNA polymerase from XhoI cut full-length rTS plasmid (4). Antisense 5’-rTS \(^{32}\)P-labeled RNA was generated using T7 clone 6, which contains an insert corresponding to the first 381 nucleotides of rTS (4). The plasmid was linearized with Aha II and transcribed with T7 RNA polymerase to generate a 436-nucleotide \(^{32}\)P-labeled RNA complementary to nucleotides 13–381 of TS. Generation of antisense \(\beta\)-actin \(^{32}\)P-labeled RNA used cloned p\(\beta\)act, a PCR-generated insert corresponding to nucleotides 317–860 of human \(\beta\)-actin cDNA cloned into pCR II (Invitrogen Corp., San Diego, CA). p\(\beta\)act was linearized with Ava II and transcribed with SP6 RNA polymerase to produce a 243-nucleotide RNA complementary to nucleotides 618–860 of human \(\beta\)-actin. This antisense \(\beta\)-actin \(^{32}\)P-labeled RNA was radiolabeled to 100-fold lower specific radioactivity than prescribed in the Gemini Riboprobe II protocol.

\textit{In Vitro Translation}. rTS mRNA for \textit{in vitro} translations was produced by T3 RNA polymerase transcription of XhoI cut full-length rTS plasmid using the Megascript transcription system (Ambion Inc., Austin, TX). Translation was performed in the presence of a 4:1 ratio of m\(^{7}\)GpppG-GTP to produce capped mRNA. \textit{In vitro} translations were performed using the Retic Lysate IVT Kit (Ambion, Inc.). Reactions and analysis by 12\% SDS-PAGE were performed as prescribed by the manufacturers.

Polysome Analysis. Log-phase K562 cells were treated with cycloheximide (100 \(\mu\)g/ml for 10 min) and harvested by centrifugation (1000 rpm for 5 min). Cells were washed with PBS containing cycloheximide and lysed in four packed cell volumes of 10 mM Tris-HCl (pH 7.4), 10 mM KCl, 1.5 mM MgCl\(_2\), 1% (w/v) Triton X-100, 1% (w/v) sodium deoxycholate, and 10 units/ml RNase Block I (Stratagene). Nuclei were removed by centrifugation

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\(^{5}\) The abbreviations used are: TS, thymidylate synthase; MTX, methotrexate; ORF, open reading frame; HPLC, high-performance liquid chromatography; DHFR, dihydrofolate reductase.

\(^{6}\) EMBL accession number X60789

\(^{7}\) L. Hanchett, L. M. Maley, and B. J. Dolnick, unpublished results.
Fig. 1. Alignment of rTS cDNA with the TS primary transcript. The alignment of the 1.8-kb rTS RNA with the TS primary transcript is based on reported and updated sequences (4, 5, 11). *, approximate positions of introns identified previously in the rTS gene. The hatched and open boxes in the TS RNA show the position of intron VI and exon 7, respectively. Numbers represent nucleotide distances either from the start of the mature rTS RNA or over the spans represented by double arrows. ORF1 and ORF2 show the position of the two ORFs in the rTS sequence; the lengths of the predicted proteins are indicated.

(12,000 x g for 10 min), and polysomes were centrifuged for 16 h at 160,000 x g through a step gradient of 1.2 M (1.7 ml) and 2.0 M (1.7 ml) sucrose in 50 mM Tris-HCl (pH 7.4), 25 mM KCl, and 5 mM MgCl2. Pellets were stored at -80°C prior to suspension in 50 mM Tris-HCl (pH 7.4), 250 mM KCl, and 2.5 mM MgCl2. Samples were divided into two aliquots, and one was adjusted to 30 mM EDTA. Polysomes in aliquots were separated on 11 ml 10–40% sucrose gradients (in the same buffer) at 160,000 x g for 75 min. Fractions (-1 ml) were collected from the top of the gradient and extracted with phenol/chloroform:isoamyl alcohol (25:24:1) and chloroform:isoamyl alcohol (24:1). The RNA content of each fraction was estimated from the absorbance at 260 nm, and RNA was precipitated with 2-propanol. Amounts of RNA equivalent to equal volumes of each fraction were analyzed by S1 nuclease protection assay.

S1 Nuclease Protection Assay. The RNA content of each sample was adjusted to 20 µg with Escherichia coli tRNA, and nuclease protection assays were performed as described (7). Hybridization was at 55°C in 5-µl volumes containing 105 dpm of antisense 5'-rTS 32P-labeled RNA and 2 x 10^5 dpm of antisense β-actin 32P-labeled RNA. These probes generate protected fragments of 368 and 243 nucleotides, respectively. Digestion was at 35°C. Protected fragments were identified by 3.5% acrylamide/7 M urea gel electrophoresis and autoradiography. The levels of the protected fragments of β-actin and rTS RNA in each sample were quantitated by densitometric analysis using the ImageQuant system (Molecular Dynamics, Sunnyvale, CA). Autoradiograms, obtained after multiple exposures of duration, were analyzed to ensure that exposures were in the linear range of the film. The lower specific activity of the β-actin probe enabled quantitation of rTS and β-actin RNA from the same autoradiograms.

Western Blot Analysis. Western blot analysis was performed and visualized using ECL (Amersham Corp.) as described (8) using rabbit polyclonal antibodies to TS or rTS. The anti-TS antibody was a gift from Dr. Edward Chu (Bethesda Naval Research Hospital, Bethesda, MD), and the anti-rTS antibody was raised against bacterially expressed rTS ORF2 protein (8).

Northern Blot Analysis. Total cellular RNA was prepared by guanidinium isothiocyanate extraction and CsCl gradient centrifugation as described (4). RNA, denatured in the presence of ethidium bromide, was separated by electrophoresis on 1.2% agarose/formaldehyde gels and transferred to Hybond-N nylon membrane (Amersham Corp.) as described previously (4). Blots were prehybridized overnight at 68°C in 5% (w/v) blocking agent (Boehringer Mannheim, Indianapolis, IN), 50% deionized formamide, 5 x SSC (1 x SSC = 150 mM NaCl and 15 mM sodium citrate, pH 7.0), 0.2% (w/v) SDS, 0.1% (w/v) N-lauroyl sarcosine, and 20 µg/ml poly rC (Sigma Chemical Co., St. Louis, MO). Hybridization was conducted under the same conditions at a probe concentration of 2 x 10^6 dpm/ml. Blots were washed twice in 2 x SSC-0.2% (w/v) SDS and twice in 0.1 x SSC-0.2% (w/v) SDS at 68°C prior to autoradiography at -80°C. RNA species were quantitated by densitometry as described above.

In Situ TS Activity. In situ TS activities were determined using [5-3H]dCyd (5 x 10^3 Bq/mmol, 5 µM; Moravek Biochemicals, Inc., Brea, CA) as described (9). Incubations were performed at 37°C for 30 min and stopped by the addition of a 15% (w/v) charcoal suspension in 4% (v/v) perchloric acid. TS activity was determined as the difference between the 3H2O released (quantified by scintillation counting) in incubations performed in the presence and absence of cells. In situ TS activity assays using MTX-resistant cells were performed in the absence of MTX.

HPLC Analysis of Nucleotide Pools. Cells were extracted with 0.7 M HClO4 (1.0 ml/10^6 cells) for 5 min at 4°C and cleared by centrifugation (18,000 x g for 10 min) at 4°C. Supernatants were neutralized with K2CO3 (160 µl/10^6 cells), and KClO4 was removed by centrifugation as above. Separation of dCMP and dUMP from nucleosides, dinucleotides, and trinucleotides in 250 µl of extract was performed on a Whatman Partisil 10 SAX-25 column using the following protocol: 0–5 min, isocratic 100% buffer A (10 mM ammonium acetate, pH 5.0); 5–25 min, linear gradient from 100% buffer A to 20% buffer A, 80% buffer B (3.5 mM ammonium acetate, pH 5.0); 25–30 min, linear gradient from 20% buffer A, 80% buffer B to 100% buffer B; 30–35 min, isocratic 100% buffer B; and 35–40 min, linear gradient from 100% buffer B to 100% buffer A. Prior to each injection, the column was regenerated for 30 min with 100% buffer A. The elution times for nucleosides and nucleotides were determined using authentic standards. Fractions containing dCMP and dUMP were collected, lyophilized, and suspended in 250 µl of H2O. Samples were adjusted to 50 mM Tris-HCl (pH 9.0), 1 mM MgCl2, 1 mM ZnCl2, and 0.1 mM spermidine by the addition of 10 x buffer, and dCMP and dUMP were digested to dCyd and dUrd with calf alkaline phosphatase (Promega Corp.) at 2 units/100 µl. After 1 h at 37°C, the enzyme was inactivated.

All HPLC procedures were performed at a flow rate of 1 ml/min. dCyd and dUrd in digests were identified from their elution times and from the ratio of their absorbances at 260 and 280 nm. Levels of dCyd and dUrd, determined by comparison of their absorbance with that of known levels of standards, were used to calculate the levels of dCMP and dUMP in the original extracts.
Protein Alignments. Protein alignments were performed using the AALIGN program (DNAStar, Madison, WI) with a gap penalty of 4 and deletion penalty of 12.

Results

The Second ORF of rTS Encodes a Protein in vivo. Previous data (1) and the presence of large ORFs in rTS cDNA suggested that rTS RNA may encode protein (Fig. 1). Consistent with this data, translation of in vitro-transcribed rTS RNA in rabbit reticulocyte lysates produced a protein with an apparent size of \( M_r \sim 42,000 \) (Fig. 2A, arrow), which corresponds to the translation of rTS ORF2. The additional smaller proteins detected may be due to internal initiation (a common artifact with cell-free translation systems (11)) or to degradation of the \( M_r \sim 42,000 \) protein. No protein corresponding to ORF1 was detected, indicating that it may not be translated. This is consistent with the poor recognition of initiation codons close to the 5'-cap (11). rTS RNA was found associated with polysomes in vivo. Polysome analysis revealed that rTS RNA co-sedimented with total polysomal RNA (\( A_{260} \) nm) and \( \beta\)-actin mRNA on sucrose density gradients (Fig. 2B). EDTA treatment, which dissociates ribosomal subunits (12), caused a shift in the sedimentation of polysomal RNA (\( A_{260} \) nm), rTS RNA, and \( \beta\)-actin RNA to the top of the gradient (Fig. 2B), indicating that the co-sedimentation of rTS RNA is due to a specific association with polysomes. This shift in sedimentation was not due to RNA degradation since protected fragments were the same size in both control and EDTA-containing gradient fractions. A small proportion of the rTS RNA in the polysomal fraction was not affected by EDTA treatment. This was also seen with \( \beta\)-actin RNA and may represent mRNA sequestered in EDTA-insensitive ribonuclear protein. However, the identical behavior of the RNA for rTS and \( \beta\)-actin (a highly expressed protein) argues that cytoplasmic rTS RNA acts as a functional mRNA in vivo.

The above data indicated that rTS RNA is translated in vivo and that the protein produced corresponds to the second ORF in rTS. To further investigate the expression of this protein, an antibody was raised against bacterially expressed rTS protein (8). By Western blot analysis (Fig. 3A, upper panel), this antibody detected an rTS immunoreactive protein of \( M_r \sim 48,000 \) in extracts from KB, KB 1BT, K562, and K562 B1A cells (Fig. 3A, upper panel). Relative to its parental cell line, K562 B1A cells overexpressed the rTS protein, although the precise degree of expression cannot be ascertained since rTS protein is only barely detectable in K562 cells (Fig. 3A). In contrast, there was little (<25%) difference in the expression of rTS protein between KB and KB 1BT cells (Fig. 3A). To determine how expression of this protein correlated with expression of rTS RNA, Northern blot analysis was performed. Consistent with previous findings (4), multiple forms of rTS RNA were detected in the cell lines examined (Fig. 3A, middle panel). Of these, the ~18S is cytoplasmic, and the larger species are nuclear (4). Consistent with Western blot data, K562 B1A cells overexpressed all species of rTS RNA by...

Fig. 2. Translation of rTS RNA. A, in vitro translation of rTS RNA. In vitro transcribed, capped rTS RNA was translated in rabbit reticulocyte lysates in the presence of \( [\text{35S}]\text{methionine} \). Translation products were analyzed by 12% SDS-PAGE and autoradiography. Lane 1, control translation (lysate with no added RNA); Lane 2, translation of 20 \( \mu \)g/ml capped rTS RNA. Arrow, the migration of the \( M_r \sim 42,000 \) protein translated from rTS RNA. Arrowheads, the migration and size (\( M_r \times 10^3 \)) of molecular weight standards. B, analysis of rTS RNA associated with polysomes. i, a polysome preparation from log-phase K562 cell spinner cultures (5 x 10^6 cells/ml) was subjected to sucrose density gradient centrifugation; fractions were collected, and their total RNA content was determined. The levels of rTS and \( \beta\)-actin RNAs were determined by S\(_5\) nuclease protection assay and densitometric analysis of the resulting autoradiogram. Upper panel, a section of this autoradiogram with the positions of the protected fragments for rTS and \( \beta\)-actin RNA indicated. The graph shows the proportion of the total RNA (\( \bullet \)), rTS RNA (\( \square \)), and \( \beta\)-actin RNA (\( \bigcirc \)) in the individual fractions. ii, the same analysis was performed, except that sucrose density gradient centrifugation was performed in the presence of 30 \( \mu \)M EDTA.
expression of rTS RNA and protein, whereas another MTX-resistant cell line (KB 1BT) had unaltered rTS RNA expression (Fig. 3A; Ref. 4). It has been reported that TS activity in cell extracts shows no growth regulation, although TS activity measured in situ is growth regulated (13). Since decreased TS activity has been associated with MTX resistance (14) and because of the potential link of rTS and TS, in situ TS activity was measured in K562, K562 B1A, KB, and KB 1BT cells as cultures became growth saturated. Growth regulation of in situ TS activity was apparent in K562, KB, and KB 1BT cells, all of which showed a 2–3-fold decrease of in situ TS activity as cells stopped growing due to culture saturation (Fig. 3B). However, in K562 B1A cells, which overexpress rTS, there was no growth regulation of in situ TS activity. The level of activity in log-phase K562 B1A cells was 3-fold lower than seen in log-phase K562 cells but the same as that seen in nongrowing K562 cells. These differences cannot be attributed to different growth kinetics of K562 and K562 B1A cells since they both have the same doubling time of 23 h. Therefore, the rTS-overexpressing cells displayed a stationary phase level of in situ TS activity at all phases of cell growth.

Fig. 3. rTS and TS expression and in situ TS activity in MTX-resistant cell lines. A, expression of rTS and TS in MTX-resistant and parental cell lines. Cell lines analyzed are indicated at the top of the figure. Upper panel, a Western blot of rTS protein present in extracts of the indicated log-phase cells. Cells were extracted with SDS-sample buffer, and 200 μg protein were resolved on 10% acrylamide-SDS gels, transferred to polyvinylidene difluoride membrane, which was probed using anti-rTS antibody. Arrowhead, the size predicted for the translation of ORF2. The slower migration of which showed a 2–3-fold decrease of in situ TS activity as cells stopped growing due to culture saturation (Fig. 3B). However, in K562 B1A cells, which overexpress rTS, there was no growth regulation of in situ TS activity. The level of activity in log-phase K562 B1A cells was 3-fold lower than seen in log-phase K562 cells but the same as that seen in nongrowing K562 cells. These differences cannot be attributed to different growth kinetics of K562 and K562 B1A cells since they both have the same doubling time of 23 h. Therefore, the rTS-overexpressing cells displayed a stationary phase level of in situ TS activity at all phases of cell growth.

The observed changes in TS activity cannot be attributed to factors that alter relevant nucleotide pool sizes. The levels of dCMP were essentially the same in the MTX-resistant cells and their respective parental cells (Table 1; see “Discussion”), with growth cessation in saturated cultures leading to the same slight decrease in dCMP pools in the MTX-resistant and parental cell lines (Table 1). Similarly, changes in TS activity do not appear to be the direct consequence of changes in TS protein levels in the various cell lines. Despite the differences in their TS activity, K562 and K562 B1A cells have very similar TS proteins levels (Fig. 3A, bottom panel). Additionally, KB showed a somewhat lower expression of TS protein than KB 1BT cells but had very similar levels of in situ TS activity and rTS expression.

Discussion

rTS Encodes a Protein. The cloned rTS cDNA contains two overlapping ORFs (Fig. 1A): ORF1, which spans nucleotides 18–302 and corresponds to a M, 10,000; protein; and ORF2, which spans nucleotides 67–1152 and corresponds to a M, 42,000. An antibody against a protein corresponding to rTS ORF2 recognized protein in cell extracts that migrated on SDS-PAGE gels as a M, 48,000 protein. Although this apparent size is larger than that predicted from the sequence of rTS cDNA,4 several lines of evidence indicate that this protein arises from translation of rTS RNA. rTS RNA was found to be specifically associated with polysomes on sucrose density gradients (Fig. 2B), indicating that cytoplasmic rTS RNA acts as a functional mRNA in vivo. In vitro translation of rTS RNA in rabbit reticulocyte lysates yielded a protein of M, 42,000 (Fig. 2A, arrow), the size predicted for the translation of ORF2. The slower-than-predicted migration of the rTS protein detected by Western analysis may be the result of posttranslational modification of rTS. Since multiple spliced forms of rTS RNA have been detected (4), this protein may represent the translation product of an alternately spliced rTS mRNA. This latter notion is supported by preliminary data that has identified an alternately spliced form of rTS RNA that contains an additional protein coding exon (EMBL Accession No. X89602).6 Nevertheless, the association of rTS RNA with polysomes, the in vitro translation of rTS RNA, and the detection of protein immunoreactive with an antibody raised against rTS indicate that a protein corresponding to, or closely related to, that predicted for translation of ORF2 is

2–3-fold compared to the parental K562 cells (Fig. 3A), whereas KB 1BT and KB cells had very similar levels of rTS RNA. Therefore, the expression of the M, ~48,000 protein correlated with relative rTS gene expression.

Correlation of rTS Expression and in Situ TS Activity. As discussed above, MTX-resistant K562 B1A cells displayed elevated
expressed in mammalian cells. Current efforts are directed at more closely characterizing this protein.

ORF2 showed homology to mandelate racemase of Pseudomonas putida (15). Alignment of ORF2 with mandelate racemase indicated 14% identity of amino acids. Moreover, there is an internal stretch of 150 amino acids (residues 155–306) where 29% of the amino acids are identical (data not shown); these include the catalytic and metal-binding residues of mandelate racemase. In keeping with this, a recent report (15) has suggested that rTS is a member of a superfamily of genes related to mandelate racemase. The data presented here provide the first evidence for the expression of a member of this gene family in mammalian cells.

Reactions catalyzed by members of the mandelate racemase family involve abstraction of the α carbon of a carboxylate group (15). The conservation of both the metal-binding and catalytic residues in mandelate racemase indicates that rTS may catalyze a chemically similar reaction. Unfortunately, this gives little indication of the precise function of rTS because the characterized members of the mandelate racemase superfamily use diverse substrates and have distinct cellular roles (15). However, its evolutionary conservation argues that rTS performs an important role in the cell, and the data discussed below argue that this is related to TS function.

**Correlation of rTS Expression with in Situ TS Activity.** The close proximity of the rTS and TS genes and the overexpression of rTS in an MTX-resistant cell line (Ref. 4; Figs. 1A and 3A) suggested a link between rTS and TS function. This possibility was explored by comparing the levels and growth regulation of in situ TS activity with rTS expression in two MTX-resistant (K562 B1A and KB 1BT cells) and their parental cell lines (K562 and KB cells). The previous study revealed overexpression of rTS RNA in the MTX-resistant K562 B1A subline (4). In the present study, this finding was confirmed and extended because overexpression of rTS in K562 B1A cells was seen at both the RNA and protein levels. Unlike the previous study (4), however, the data shown here indicate that the pattern of rTS RNA in the K562 and K562 B1A cells is similar, and a novel RNA species at 0.6 kb was not detected. Repeated experiments using both RNA and double-stranded DNA to probe RNA from log-phase and saturated cultures yielded the same results. Thus, although the nature of the 0.6-kb rTS RNA band remains unresolved, rTS mRNA and protein are clearly overexpressed in MTX-resistant K562 B1A cells. This overexpression was accompanied by alterations in in situ TS activity in log-phase K562 B1A cells. K562 B1A cells showed a lower level of TS activity in log-phase cells and failed to show the growth-related down-regulation of TS activity apparent in K562, KB, or KB 1BT cells (Fig. 3B). The decreased activity in log-phase K562 cells, relative to their parental K562 cells, is consistent with a report by others of reduced TS activity in the MTX-resistant K562 cells from which the B1A cells were derived (4, 16). These alterations cannot be attributed to altered TS gene expression since K562 B1A cells had unaltered levels of TS protein. The MTX-resistant 1BT subline of KB cells, which is also amplified for the DHFR gene (6), showed no significant alteration in the pattern or levels of rTS RNA or protein compared to its parental KB cell line. The levels of in situ TS activity and its regulation were also very similar in KB and KB 1BT cells.

This finding indicates that the different in situ TS activities seen in the K562 and K562 B1A cells correlate with the changes in rTS expression and are not due to an MTX-resistant phenotype associated with DHFR amplification per se.

The in situ TS assay used here measures the TS-dependent conversion of dCyd to thymidylate since activation of this precursor does not require thymidine kinase, a cell-cycle and growth-dependent enzyme (9). However, due to dilution of exogenously supplied radiolabeled precursor by intracellular nucleotide pools, the in situ TS activities observed are dependent on the sizes of the intracellular dCMP and dUMP pools, in addition to the actual TS activity. To determine if this could be a significant factor affecting the results, dCMP and dUMP pools were assayed (Table 1). Although a significant difference was seen in the pools between the K562 and KB cells, the dCMP pools of the MTX-resistant K562 B1A and KB 1BT cells were the same as those seen in their respective parental cells from both log-phase and saturated cultures, indicating that the observed alterations in TS activity and growth regulation were not due to altered intracellular levels of dCMP. Culture saturation led to a slight decrease in dCMP pools in the cell lines studied, which would tend to enhance the observed TS activities in nongrowing cells. Similar to the low levels of dUMP observed in human CCRF-CEM cells (9), the levels of dUMP in the cells studied here were lower than the detection limit of the assay (<0.7 pmol/10⁶ cells). However, the specific activity of the dUMP pools should be dependent on those of the dCMP pools since: (a) the low level of dUMP pools indicate that they are turned over at least four times during the course of the assay; (b) the major source of dUMP in mammalian cells (17); and (c) the majority of dCyd is metabolized to thymidine nucleotides in both nongrowing and growing cells. Thus, any changes in dUMP pools should have only a minimal effect on the observed in situ TS activities and could not account for the differences observed. Since the observed differences in TS activities cannot be attributed to alterations in the pools of either dCMP or dUMP, the data reflect the level of TS activity in the cell.

Table 1  dCMP and dUMP pool sizes in various cell lines

<table>
<thead>
<tr>
<th>Pool size (pmol/10⁶ cells)</th>
<th>K562 log</th>
<th>K562 sat.</th>
<th>K562 B1A log</th>
<th>K562 B1A sat.</th>
<th>KB log</th>
<th>KB sat.</th>
<th>KB 1BT log</th>
<th>KB 1BT sat.</th>
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<tr>
<td>dCMP</td>
<td>3.8 ± 0.2</td>
<td>3.1 ± 0.1</td>
<td>3.7 ± 0.4</td>
<td>3.0 ± 0.1</td>
<td>9.9 ± 0.8</td>
<td>7.2 ± 1.8</td>
<td>8.9 ± 0.3</td>
<td>6.1 ± 2.9</td>
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<tr>
<td>dUMP</td>
<td>&lt;0.7</td>
<td>&lt;0.7</td>
<td>&lt;0.7</td>
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TS is dependent on 5,10-methylenetetrahydrofolates; however, limiting folate pools are unlikely to account for the changes in in situ TS activity seen here since cells were grown in folate-containing medium, conditions under which folate pools in K562 cells are greater than 4-fold higher than limiting levels (19). Folate pools in K562 B1A cells should be similar to those in K562 cells since: (a) uptake of folates should be unaltered in K562 B1A cells since the MTX-resistant cell line from which they were derived had reduced TS activity but unaltered folate/MTX uptake (16); (b) retention of folates should be the same for K562 and K562 B1A cells since their folyopolyglutamate synthase activities are very similar (843 ± 74 and 678 ± 56 units/mg protein, respectively; Ref. 20); and (c) DHFR activity should not be limiting in K562 B1A cells because the enzyme is amplified in these cells and assays were performed in the absence of methotrexate.


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Therefore, although alterations in folate pool sizes cannot be excluded, these are unlikely to contribute to the altered in situ TS activity in K562 B1A cells or to the correlation between rTS expression and altered in situ TS activity.

By demonstrating that overexpression of rTS protein correlates with altered levels and loss of growth regulation of TS activity, the present study indicates that rTS may play a role in the regulation of TS within cells. Decreased TS activity can contribute to MTX resistance (14); therefore, down-regulation of in situ TS activity, possibly linked to altered expression of rTS, may contribute to the overall MTX resistance of K562 B1A cells. Evidence for a functional interaction between rTS and TS has been found in transfection studies (8). KB cells transfected with an rTS expression vector were more resistant to 5-fluorodeoxyuridine but were more sensitive to trifluorothymidine. A similar correlation between rTS expression and sensitivity to TS interactive drugs is seen in nontransfected cells since K562 B1A cells are ~30-fold more resistant to 5-fluorodeoxyuridine than K562 cells (IC_{50} = 7.1 ± 0.2 and 0.24 ± 0.05 μM, respectively, over a 120-h continuous exposure). The altered sensitivity of rTS-overexpressing cells to 5-fluorodeoxyuridine (a substrate analogue) and trifluorothymidine (a product analogue) indicates that rTS may affect the interaction of TS with its substrate and product. It has been demonstrated previously that TS enzyme levels are not growth regulated, and it was proposed that growth regulation of in situ TS activity is due to product inhibition (13). Therefore, an rTS-induced altered interaction of TS with substrates and products could account for both the loss of growth regulation and the lower levels of in situ TS activity in the rTS-overexpressing K562 B1A cells. How rTS may modulate the interaction of TS with its substrates and/or products is not known. A possible mechanism by which rTS may affect TS comes from preliminary data indicating that these proteins are physically associated. This suggests that rTS may affect the participation of TS in a multienzyme complex. The role of this association in modulation of TS function is currently being investigated.

The overexpression of rTS in an MTX-resistant cell line and the effects of rTS overexpression indicate that it may be an important factor in drug resistance. This is further indicated by preliminary data demonstrating that a recently characterized ZD1694 and 5-fluorodeoxyuridine resistant cell line also overexpresses rTS RNA. Collectively, these data suggest that modulation of rTS expression may represent a useful tool in TS targeting therapies.

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