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Detection of Thymidylate Synthase Modulators by a Novel Screening Assay

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ABSTRACT

Thymidylate synthase (TS), a key cancer chemotherapeutic target, catalyzes the conversion of deoxyuridylate to thymidylate. TS can serve as a repressor of its own synthesis by binding to its own mRNA through TS-specific binding elements (TBEs). In this report, we describe the use of a luciferase reporter plasmid containing two TBEs that can be used as a tool for the identification and initial profiling of compounds that modulate TS activity, levels, or ability to bind mRNA. To validate this model, we evaluated several groups of drugs. Thus, cells were exposed to the pyrimidine analogs 5-fluorouracil (5-FU), 5-fluorouridine (FUrd), 5-fluoro-2′-deoxyuridine (FUdR), trifluorothymidine (TFT); to the nonpyrimidine TS-inhibitors AG-331, nolatrexed (AG337), and raltitrexed (ZD1694); or to drugs with other primary sites of action (methotrexate, actinomycin D, 5-azacytidine, 8-thioguanosine). Except for 5-azacytidine and 8-thioguanosine, all compounds examined induced luciferase activity compared with untreated cells. Effects of luciferase activity inducing drugs through TS-affected translation were confirmed by examinations of TS protein and mRNA levels. Treatment of H630-C6 cells with 5-FU, FUrd, FUdR, TFT, AG331, AG337, ZD1694, and methotrexate up-regulated TS levels as determined by Western blot analysis, although TS mRNA levels remained unchanged as determined by reverse transcription-polymerase chain reaction. Our studies demonstrate a novel application of a TBE-dependent reporter plasmid that could be used for the high-throughput identification of potential chemotherapeutic agents that modulate TS RNA-binding activity, either directly or indirectly.

Thymidylate synthase [TS (EC 2.1.1.45)] catalyzes the formation of thymidylate via conversion of 2′-deoxy-UMP and 5,10-methylenetetrahydrofolate to 2′-deoxy-TMP and dihydrofolate (Friedkin and Kornberg, 1957). This catalytic reaction is the sole de novo source of 2′-deoxy-TMP in cells. For this reason, TS is considered a major target for cancer chemotherapy (Santi and Danenberg, 1984; Hardy et al., 1987). TS has also been shown to regulate translation by binding to a variety of mRNAs including its own (Chu et al., 1995, 1996), as well as p53 and c-myc (Chu et al., 1994a; Voeller et al., 1995). TS binds specifically to its own mRNA, functioning as a feedback translational inhibitor to maintain TS levels, presumably according to cellular demand for thymidylate. This binding can be alleviated by specific TS ligands, such as 2′-deoxy-UMP and 5-fluorodeoxyuridylate (FdUMP) (Chu et al., 1994b). TS binds to its cognate mRNA through two different *cis*-acting elements (Chu et al., 1991, 1993). The first binding element (TBE) is located within the 5′-untranslated region and encompasses the translation start site (Chu et al., 1993). The second binding element is located within the protein coding region within an approximately 70-nucleotide sequence corresponding to nt 480 to 550 (Chu et al., 1993; Lin et al., 2000).

Previously, Lin et al. (2000) constructed a luciferase-based reporter plasmid that contained a single TBE and luciferase driven by an early growth response gene (EGR-I) promoter and demonstrated the ability of TBE to diminish luciferase activity and the ability of TS inhibitors to induce activity. Through this translational repression mechanism, either dissociating TS from its own mRNA with TS-specific ligands such as TS inhibitors or lowering the levels of TS within cells should increase the translational activity of TBE-containing mRNAs. We reasoned that this phenomenon could be used as a tool to identify compounds that might modulate TS expression or function in ways that might not be immediately obvious or generally considered viable. For example, a compound that lowered TS by affecting its synthesis or degradation might be useful in therapy when combined with a TS inhibitor. Although inroads have been made into the mech-

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ABBREVIATIONS: TS, thymidylate synthase; TBE, thymidylate synthase-specific binding elements; nt, nucleotides; EGR-I, early growth response gene; 5-FU, 5-fluorouracil; FUdR, 5-fluoro-2′-deoxyuridine; TFT, trifluorothymidine; Act D, actinomycin D; AC, 5-azacytidine; TG, 8-thioguanosine; FUrd, 5-fluorouridine; MTX, methotrexate; bp, base pair(s); FBS, fetal bovine serum; G418, geneticin; RLU, relative light units; RT, reverse transcription; PCR, polymerase chain reaction; FdUMP, 5-fluorodeoxyuridylate; AG331, N^6[4-(*N*-morpholinosulfonyl)benzyl]-N^6-methyl-2,6-diamino-benz[*c,d*]indole glucuronate; AG337, nolatrexed; ZD1694, raltitrexed (Tomudex).

anisms controlling TS gene expression, the precise mechanisms determining TS gene expression and protein levels are not completely known. The identification of compounds that affected these regulatory mechanisms would provide for possible means to improve TS-directed therapy, and an assay amenable to high-throughput identification of these compounds would provide the means to achieve this goal.

Although it is clear that both TBEs can serve as mRNA docking sites for TS, why two TBEs are present in TS mRNA remains unclear. An obvious possibility is that the presence of multiple TBEs might provide docking sites for other proteins and might also influence translation of mRNAs containing TBEs. We therefore combined the core parts of the two TBEs (i.e., TS mRNA nt 70–111 from the upstream binding element and nt 480–550 from the second binding element) as a merged unit but separated by a randomly chosen 20-nucleotide spacer. The whole element (2TBE) was cloned into a luciferase reporter construct, and a selectable marker was added. The reporter plasmid was then stably transfected into human colon cancer H630 cells. The resultant stably transfected cell line, H630-C6, was employed to test compounds that are known either to bind TS, to affect TS indirectly, or to be unrelated to TS for their ability to modulate luciferase activity. A robust increase of luciferase activity was observed for TS-specific compounds and one compound known to affect TS indirectly. With one notable exception, TS-irrelevant compounds induced no change of luciferase activity.

Materials and Methods

Chemicals. 5-Fluorouracil (5-FU), 5-fluoro-2′-deoxyuridine (FUdR), trifluorothymidine (TFT), Actinomycin D (Act D), 5-azacytidine (AC), and 8-thioguanosine (TG) were purchased from Sigma (St. Louis, MO); 5-fluorouridine (FUrd) was obtained from Calbiochem-Behring Corp (La Jolla, CA); methotrexate (MTX) was obtained from American Cyanamid Company (Pearl River, NY); AG331, nolatrexed (AG337) and raltitrexed (ZD1694) were generous gifts from Agouron and Astra Zeneca, respectively, to Dr. John J. McGuire (Roswell Park Cancer Institute, Buffalo, NY).

Plasmid Construction. A plasmid (pG3E1-2TBE-Neo) was designed that contained a luciferase reporter gene under control of an EGR-I promoter, both TBEs, and a selectable marker suitable for use in mammalian cells (Fig. 1). The EGR-I promoter (from p644, a gift from Dr. Edward Chu, VA Cancer Center, Yale University, West Haven, CT) was added to *Bgl*II digested pGL3-basic (Promega, Madison, WI) to generate pG3E1. An oligonucleotide (150 bp) containing both TBE1 (TS mRNA untranslated region and start site) and TBE2 (protein coding region) separated by a randomly chosen 20-nucleotide segment and flanked with *Hind* III sites was synthesized on a DNA synthesizer (381A; Applied Biosystems, Foster City, CA). The sequence of this 2TBE unit is: 5′-GAT AAG CTT CCT CCG TCC CCC GCC CGC CGC GCC ATG CCT GTG GCC GGC TCG TCA GTC AGG CTA GCT ATA GCG GAC TTG GGC CCA GTT TAT GGC TTC CAG TGG AGG CAT TTT GGG GCA GAA TAC AGA GAT ATG GAA TCA GAT TAA GCT TGC-3′. The synthesized element was then amplified by PCR by using two 18-mer primers located at both terminals and was cloned into the *Hind*III sites of pG3E1. This construct is designated as pG3E1-2TBE. The identity and orientation of the insert were confirmed by sequencing. To facilitate the selection of stable

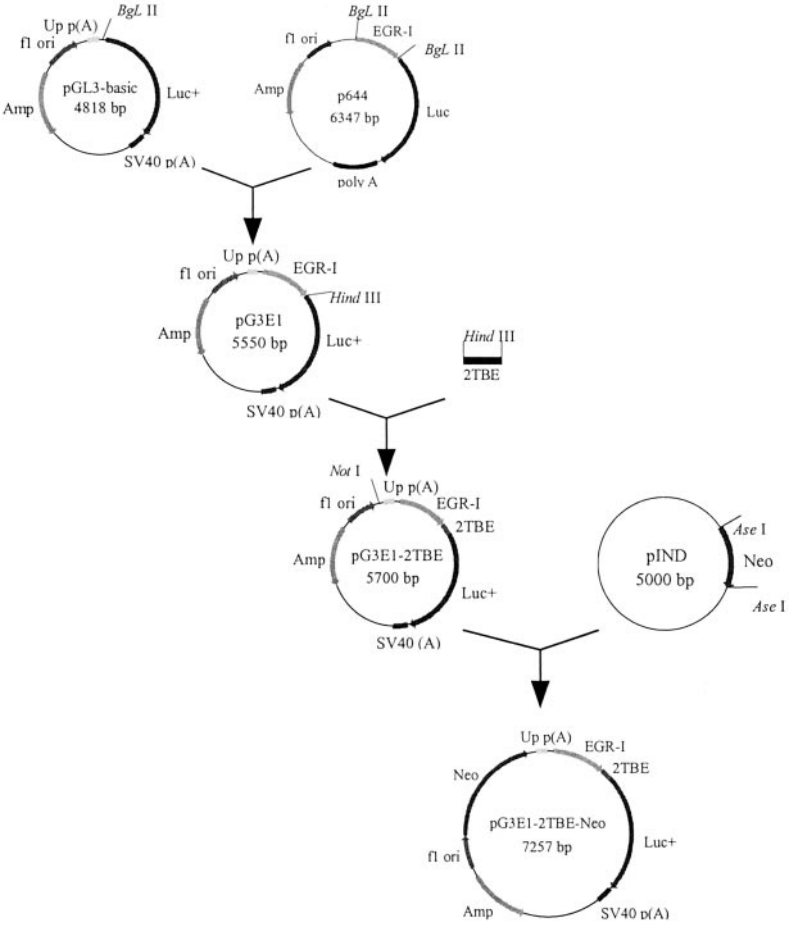

Fig. 1. Construction of pG3E1-2TBE-Neo. Key elements and restriction sites in the plasmids used for construction of pG3E1-2TBE-Neo are indicated. See *Materials and Methods* for details.

transformants, an *Ase*I fragment containing a neomycin-resistance gene (from pIND; Invitrogen, Carlsbad, CA) was blunt-ended and inserted downstream of the luciferase reporter gene by ligation to pG3E1-2TBE, which was linearized by digestion with *Not*I and blunt-ended using standard molecular biology techniques. The presence of all elements and orientation of cloned fragments was confirmed by sequencing and the final construct was termed pG3E1-2TBE-Neo (Fig. 1).

Cell Culture, Transfections, and Luciferase Assay. The human colon cancer cell line H630 (Park et al., 1987) was cultured in RPMI 1640 medium supplemented with 10% dialyzed fetal bovine serum (FBS) (Invitrogen). For transient transfection, pG3E1 or pG3E1-2TBE was electroporated into H630 cells (280 V, 600 μF, and 13 Ω, 5 μg of plasmid DNA per 2×10^6 cells) using an electroporator (ECM 600; BTX Inc., San Diego, CA). After transfection cells were cultured for 24 h before assaying for luciferase activity. For stable transfection, pG3E1-2TBE-Neo was linearized at the ampicillin-resistance gene with *Ase*I and was introduced into H630 cells as described above. Twenty-four hours after transfection, the cells were exposed to 400 μg/ml of Geneticin (G418) for 2 weeks. Individual clones were picked at that time and screened for their ability to induce luciferase in response to treatment with 5-FU. Based upon the outcome of this preliminary screen (data not shown), the clone H630-C6 was chosen for the experiments. The stably transfected H630-C6 cell line was maintained in RPMI 1640 medium plus 10% dialyzed FBS and 400 μg/ml of G418. For luciferase assays, 2×10^4 cells were seeded in each well of a 24-well plate in RPMI 1640 medium plus 10% FBS without G418 and were allowed to attach for at least 6 h, followed by drug exposure overnight (approximately 18 h). The cells were then washed with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.4 mM KH_2PO_4, pH 7.4), lysed with passive lysis buffer (Promega) and assayed for luciferase activity using the luciferase assay system (Promega) according to the manufacturer's instructions. Relative light units (RLU) were measured with a Lumat LB 9501 luminometer (EG & G Berthold, Wildbad, Germany). All experiments were done in triplicate or quadruplicate with results presented as average $\pm$ S.D.

Western Blotting. Western blotting was performed as described previously (Dolnick and Black, 1996). Briefly, H630-C6 cells were washed with phosphate-buffered saline and extracted with radioimmunoprecipitation assay buffer (Poirier et al., 1982). Total protein (10 μg) was resolved by electrophoresis in a 10% SDS-polyacrylamide gel electrophoresis and was blotted onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA). The membrane was hybridized with rabbit polyclonal antibody against TS (a gift from Dr. Edward Chu, VA Cancer Center, Yale University, West Haven, CT) diluted in SuperBlock Blocking Buffer (Pierce, Rockford, IL) plus 0.25% Tween 20 for 1 h and hybridized with horseradish peroxidase-linked anti-rabbit secondary antibody (Jackson ImmunoResearch Laboratories, Lexington, KY) for an additional 1 h. The membrane was detected with SuperSignal West Dura extended duration substrate (Pierce) and was visualized by exposing the blot to Kodak XAR-5 film (Eastman Kodak, Rochester, NY). For α-tubulin, the membrane was hybridized with mouse monoclonal anti-α-tubulin, clone B-5-1-2 (Sigma, St Louis, MO) in 5% nonfat milk dissolved in Tris-buffered saline (10 mM Tris-HCl, pH 8.0, 150 mM NaCl) plus 0.1% Tween 20, followed by horseradish peroxidase-linked anti-mouse secondary antibody IgG (JacksonImmuno Research Laboratories) in the same blocking buffer. The resulting signal was detected as described above.

RT-PCR. RNA was prepared using an RNAqueous kit (Ambion, Austin, TX). Reverse transcription was done using a Cells-to-cDNA kit (Ambion). Polymerase chain reaction (PCR) was performed as described previously (Dolnick et al., 1992). Primers used for TS mRNA [5′-TTT GGA CAG CCT GGG ATT CTC-3′ (sense) and 5′-AAA GCA CCC TAA ACA GCC ATT-3′ (antisense)] generated a 600-bp PCR product. Primers used for β-actin [5′-CAG CTC ACC ATG GAT GAT GAT A-3′ (sense) and 5′-CCA GAC GCA GGA TGG CAT-3′ (antisense)] generated a 543-bp PCR product. The PCR cycling condition used was: 95°C for 30 s, 60°C for 30 s, 72°C for 1 min, repeated for 25, 30, and 35 cycles (for TS) or 20, 25, and 30 cycles (for β-actin). Product formation was evaluated by ethidium bromide staining and was resolved by electrophoresis in 1.5% agarose gels.

Results

EGR-I Promoter and TBEs Are Constitutively Active in pGL3-Basic Plasmid. Construction of pG3E1-2TBE-Neo was accomplished in three major steps. First, we added the EGR-I promoter to pGL3-basic to generate pG3E1 (Fig. 1). To test the efficiency of the EGR-I promoter, pG3E1 DNA was transiently transfected into H630 cells by electroporation. A ~10-fold increase in RLU was observed with pG3E1 compared with mock-transfected cells (Fig. 2a). We next added the 2TBE element downstream of the EGR-I promoter to generate pG3E1-2TBE. The construct was characterized for expression of luciferase and potency of the TBEs. To accomplish this, H630 cells were transiently transfected with pG3E1 in parallel with either pG3E1-2TBE or a mock transfection. The transfected cells were assayed for luciferase activity as described. As shown in Fig. 2B the mock transfected control showed $2.08 \pm 0.19 \times 10^2$ RLU, the cells transfected with pG3E1 showed $5.19 \pm 0.75 \times 10^4$ RLU, and the cells transfected with pG3E1-2TBE showed $2.88 \pm 0.16 \times 10^4$ RLU. Thus, consistent with the anticipated inhibition of luciferase production caused by inclusion of the TBEs, there was nearly a 2-fold decrease in the luciferase activity for the cells transfected with pG3E1-2TBE compared with pG3E1 (Fig. 2b). To evaluate a role for TS binding in the suppression of luciferase activity, transfected cells were exposed to the prodrug 5-FU (10 μM). This treatment resulted in a 33 $\pm$ 13.8% increase in the RLU compared with untreated transfectants (Fig. 2c). To facilitate selection of stable transformants, in the third step we added a neomycin-resistance gene to the pG3E1-2TBE (Fig. 1) to generate a new plasmid termed pG3E1-2TBE-Neo. After selection with G418 and evaluation for changes in luciferase activity after exposure of the cells to 5-FU, the cloned subline H630/pG3E1-2TBE-Neo-L1-2-C6 (H630-C6) was chosen for further study.

Luciferase Induction by Cytotoxic Agents. To test H630-C6 as a tool to identify TS modulators three different classes of compounds (i.e., specific or predominantly specific TS inhibitors, agents that inhibit folate pathways or inhibit TS as well as having other targets, and agents that act through TS unrelated sites of action) were evaluated for their ability to induce changes in luciferase activity. For inhibitors with primary sites of action against TS, we examined 5-FU, FUrd, FUdR, TFT, ZD1694, AG331, and AG337. Treatment of the cells with the three FdUMP prodrugs resulted in increases in RLU output that reached a plateau at concentrations that reflect the metabolic proximity of the prodrugs to FdUMP (Fig. 3, a-d). These plateaus were 50 μM for 5-FU (higher concentrations not shown), 2.5 μM for FUrd, and 5 nM for FUdR. TFT, a compound that is metabolized to a tight-binding but noncovalent inhibitor of TS reached a plateau at 100 nM. The nonfolate TS inhibitors AG331 and AG337 also caused concentration-dependent increases in luciferase activity of up to 3- to 4-fold (Fig. 3, e and f). Interestingly, the TS-specific compound ZD1694 and the dihydrofolate reductase inhibitor MTX both caused increases in luciferase that were comparable despite having different ma-

jor sites of action (Fig. 3, g and h). Both of these compounds require γ-polyglutamate addition within cells to become significant TS inhibitors, although the tetrapolyglutamated ZD1694 (K_i, 1 nM) (Jackman and Calvert, 1995) is a much tighter inhibitor of TS than polyglutamated MTX (K_i, 13 μM) (Allegra et al., 1985). Surprisingly, when the cells were treated with Act D, a compound that does not target TS, a concentration-dependent increase of RLU was observed (Fig. 3i). However, as expected, no increase was observed when cells were exposed to AC, TG, or G418, the compound used for selection of cells carrying the neo gene (Fig. 3j).

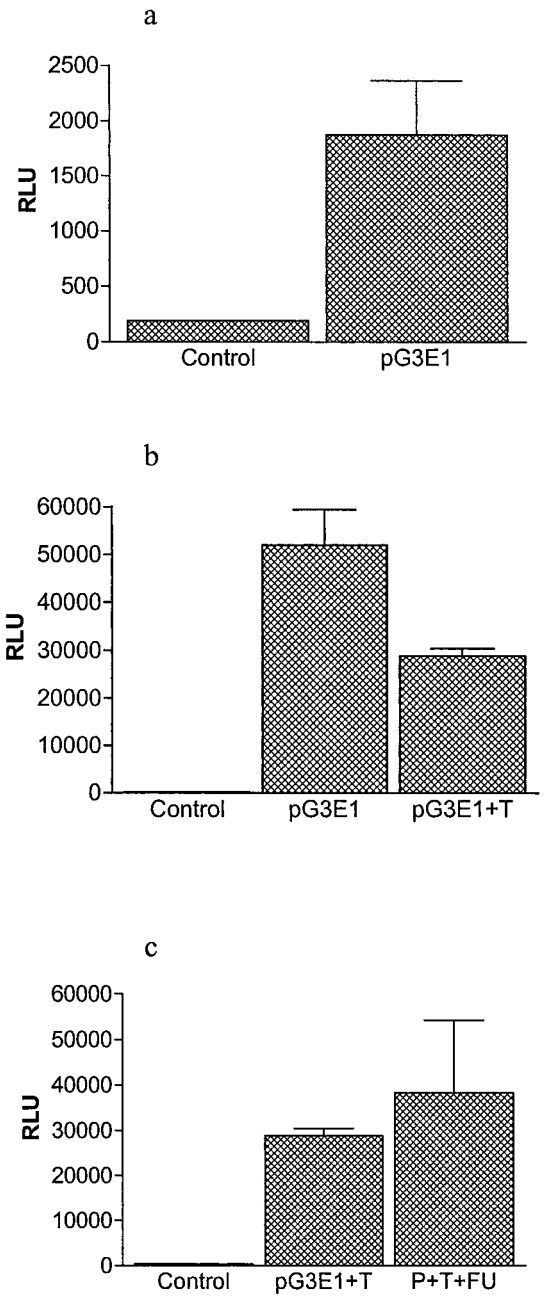

Fig. 2. Activity of the EGR-I promoter and TBEs in pGL3-basic plasmid. a, the EGR-I promoter was added upstream of the luciferase reporter gene of pGL3-basic plasmid as described under *Materials and Methods*. The resultant construct pG3E1 was transfected into H630 cells and the luciferase assay was performed 24 h later. Control, mock transfection; pG3E1, 5 μg of pG3E1 plasmid DNA per 2×10^6 cells was used for transfection; RLU, relative light units. b and c, TS-binding elements were synthesized and added into pG3E1 as described under *Materials and Methods*. The pG3E1-2TBE (5 μg) was transfected into six replicate aliquots of H630 cells (2×10^6) and plated separately. After 24 h, three of transfected samples were treated with 5-FU (10 μM) for an additional 24 h; the cells were then processed for the luciferase assay according to the manufacturer's instructions. Control, mock transfection; pG3E1, transfected with pG3E1; pG3E1+T, transfected with pG3E1-2TBE only; P+T+FU, transfected with pG3E1-2TBE and 24 h after transfection treated with 5-FU. Data are the average of triplicate experiments ± S.D.

Increased RLU Correlates with Up-Regulation of TS. An increase in RLU production in response to treatment of cells with TS inhibitors or their prodrugs is expected based upon previous demonstrations that several TS ligands can dissociate TS from the TBEs (Chu et al., 1994a, Lin et al., 2000). The induction of luciferase activity depends upon the ability to reduce the amount of endogenous TS bound to the TBEs in the recombinant luciferase transcripts. Dissociation of TS from the luciferase reporter mRNA is expected to be accompanied by the removal of TS from TBEs located in other mRNAs as well, such as endogenous TS mRNA. Therefore, an increase in TS is expected to accompany the increase in luciferase. To verify whether a translational mechanism is involved, we evaluated the levels of TS from cells treated with the compounds described above by Western blotting. The concentration for each compound was chosen based upon the results obtained in the luciferase assays (Fig. 3). Elevated levels of TS were observed in the cells treated with compounds that were found to induce production of RLU in the luciferase assay, with the exception of protein from cells treated with Act D (Fig. 4). 5-FU, FUrd, and FUdR also altered the mobility of TS. This is caused by the trapping of TS in a covalent ternary complex along with FdUMP and $N^{5,10}$-methylene tetrahydrofolate polyglutamate upon denaturation in preparation for SDS-polyacrylamide gel electrophoresis, as observed previously (Drake et al., 1993).

As expected, TG and AC, compounds that are metabolized to inhibitors of purine biosynthesis and nucleic acid precursors, did not alter TS protein levels, consistent with a lack of effect on luciferase activity, and the control compound, G418, was inactive as well (Fig. 3j). A notable exception to the observed increase in TS levels accompanying increased luciferase activity was found for Act D. Act D is known to inhibit RNA production but is not specifically associated with TS (Toku et al., 1983). Analysis of TS levels by Western blotting displays no increase of TS protein after Act D treatment (Fig. 4b).

Increases in the levels of TS protein due to relief of translational repression by binding of compounds to TS should not be accompanied by elevated TS mRNA levels, as has been demonstrated previously (Chu et al., 1994, Lin et al., 2000). TS mRNA levels were evaluated by RT-PCR, relative to β-actin. Consistent with the Western blot results, we did not detect any significant change of TS mRNA with any compound (Fig. 5).

Discussion

For decades, TS has been a major target for cancer therapy. Developing new inhibitors of TS with clinical efficacy and low side effects is likely to remain an ongoing goal of drug discovery. The development of TS inhibitors has usually proceeded from a standpoint of rational design, based upon the known structures of its substrates and, more recently, on the three-dimensional structure of TS. It occurred to us that it would be useful to be able to identify compounds that cause the down-regulation of TS levels for a variety of reasons, such

as affecting the half-life of TS, rates of gene transcription, TS pre-mRNA stability, etc. This would be expected to decrease the number of tumor cells surviving transient exposure to a TS inhibitor by helping to overcome any selective advantage conferred by transient removal of translational repression resulting from TS detachment from its mRNA. The identification of compounds that might act through targets that are not currently obvious could be accomplished through the mass screening of chemical libraries if an appropriate assay existed. An appropriate assay would have the attributes of sensitivity to compounds that affect TS by a variety of mechanisms but would also be amenable to multiwell plate formats, to facilitate screening of large numbers of compounds.

The reporter plasmid containing a TBE (Lin et al., 2000) seemed an excellent foundation from which to develop a screening model. For purposes of drug discovery and devel-

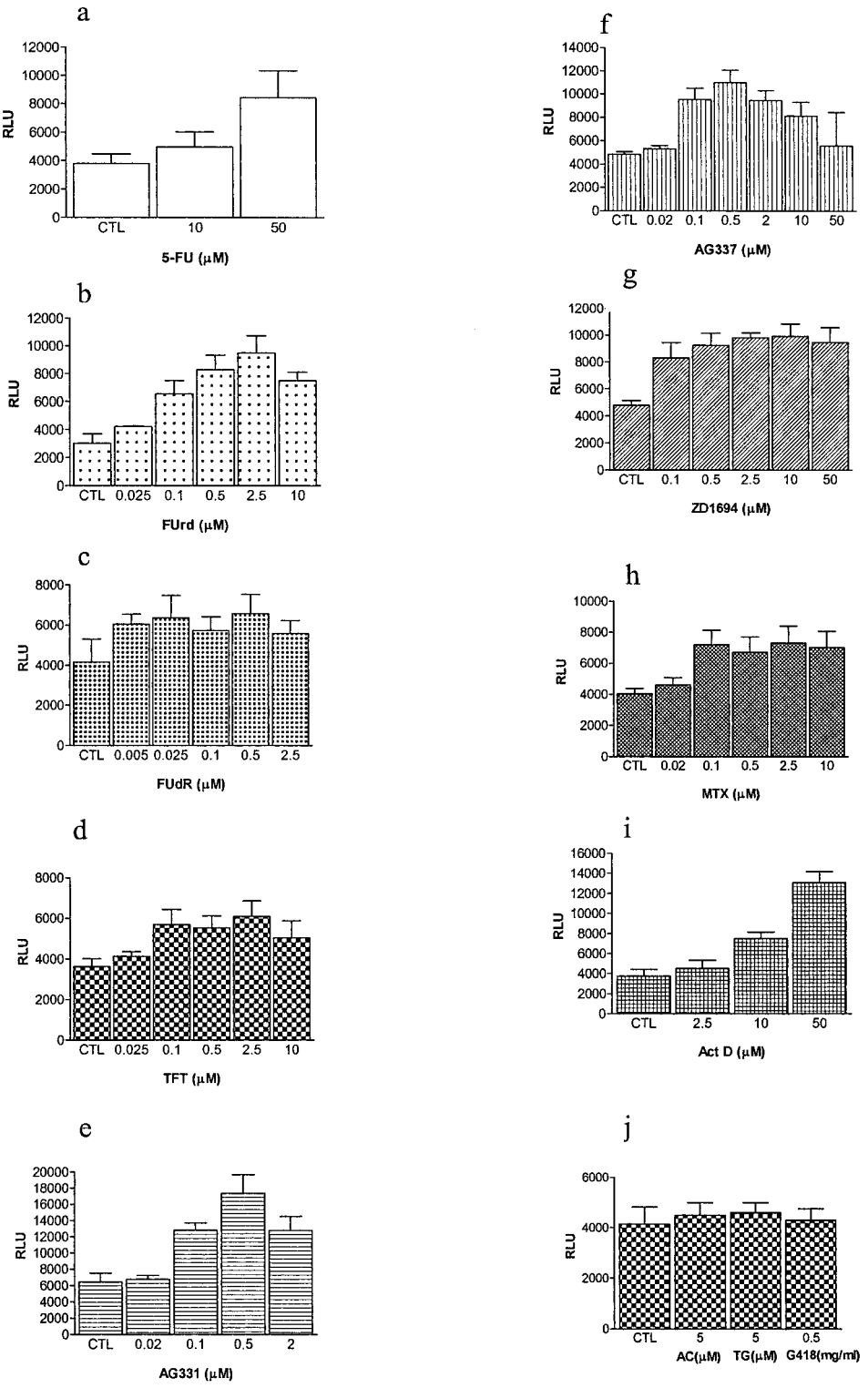

Fig. 3. RLU production of H630-C6 cells in response to TS inhibitors. H630-C6 cells were evaluated for luciferase activity in response to treatment with the indicated compounds. The cells were exposed to the compounds at the indicated concentrations for 18 h and luciferase assays were then performed according to the manufacturer's instructions. The mean values of triplicates or quadruplicates are shown along with the S.D. CTL, control.

opment, we constructed a new reporter plasmid containing both TBEs, based upon the idea that both TBEs are present in human TS mRNA and might serve multiple but as-yet-unknown purposes in vivo. Although it has been established that TS can bind to either TBE and suppress translation, we believed the possibility existed that other proteins might coordinate or modulate TS binding to one or both TBEs. If this were the case, we speculated, then the inclusion of both TBEs within the same reporter plasmid might allow us to identify translational effects on the reporter plasmid that would reflect effects on TS mRNA translation through as-yet-unidentified RNA binding proteins. The data from luciferase assays suggested that the pGL3-based construct has the desirable properties of low background luciferase expression with an S.D. below 25%, and the H630-C6 cell line responds to TS inhibitors in a dose-dependent manner (Fig. 3). The *neo* gene was introduced into pG3E1-2TBE, allowing the isolation of stable transfectants in a variety of cell lines, thus avoiding some of the limitations associated with transient transfections.

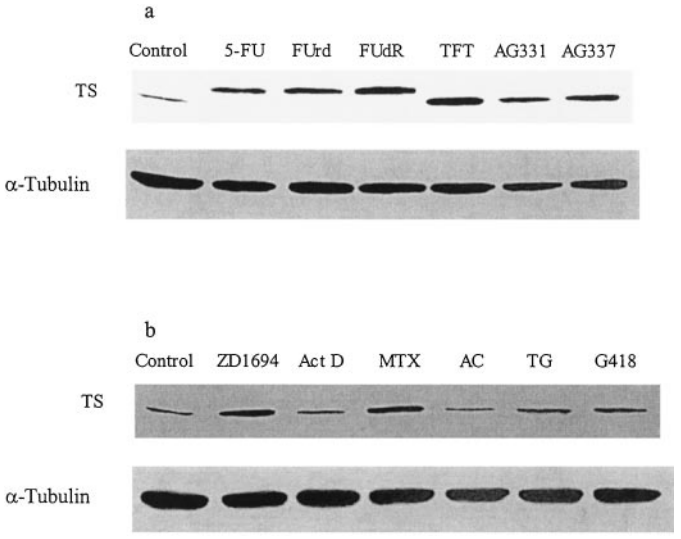

Fig. 4. Evaluation of TS expression by Western blotting. H630-C6 cells were treated with the compounds as indicated for 18 h. The cells were harvested for Western blot as described under *Materials and Methods*.

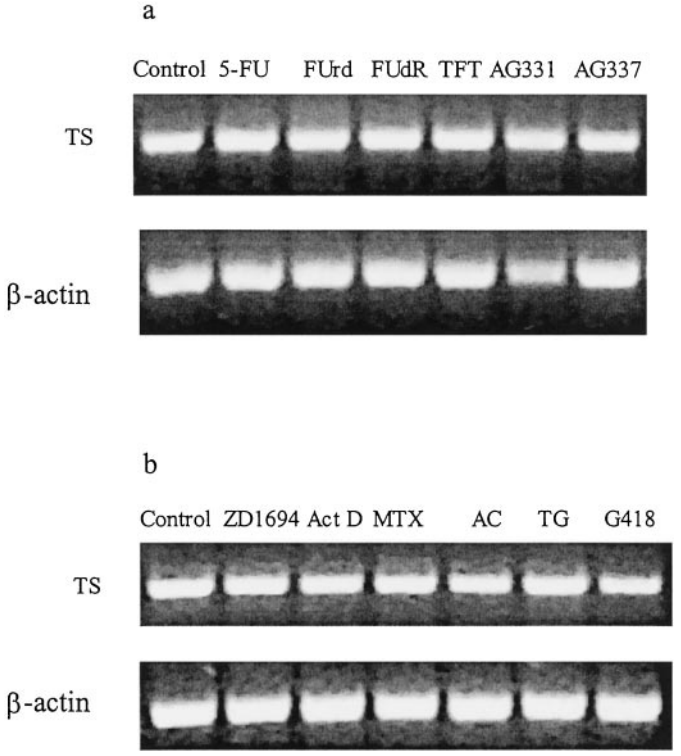

Fig. 5. Evaluation of TS mRNA expression by RT-PCR. H630-C6 cells were exposed to the indicated compounds for 18 h and total RNA was obtained for RT-PCR as described under *Materials and Methods*. Results are shown for 35 cycles of amplification. Similar results were obtained at lower cycles (data not shown).

To determine how useful the assay would be in the identification of compounds that affected TS, we took advantage of the wealth of pharmacologic information on TS inhibitors and tested major groups of compounds composed of pyrimidine TS prodrugs (5-FU, FUrd, FUdR, TFT), other direct and indirect TS inhibitors (AG331, AG337, ZD1694, and MTX), and cytotoxic compounds expected to work through pathways unrelated to TS (Act D, AC, and TG). Our expectation was that the known potent TS inhibitors would induce luciferase activity, based upon their ability to bind to TS or serve as precursors to compounds that bind TS. All of these compounds induced luciferase activity as expected. MTX, a compound with a primary site of action at dihydrofolate reductase but one that can weakly inhibit TS after polyglutamate addition or can affect TS activity by depleting cells of reduced folate, also stimulated luciferase activity. Although it was not surprising that MTX could induce luciferase activity, it was somewhat surprising that MTX was almost as effective as ZD1694 in inducing luciferase activity, with both causing a ~2-fold elevation at 100 nM (Fig. 3, g and h). This result obtained with MTX (and confirmed by a demonstration of elevated TS protein levels) suggests that the assay is likely to be useful for the identification of compounds that indirectly influence the activity or level of TS.

The ability of Act D to induce luciferase is surprising because this compound is known to be a potent inhibitor of mRNA synthesis but does not have any obvious relationship to TS (Toku et al., 1983). Treatment of H630-C6 cells with Act D (50 μM) increased luciferase up to 3.5-fold (Fig. 3i) but did not induce TS levels, indicating that its mechanism is not caused by detachment of TS from its own mRNA. Act D is a known inhibitor of RNA polymerase, and it seemed possible that there might be a differential effect on TS mRNA levels because of inhibition of synthesis that might lead to decreased TS mRNA and protein levels, which would also cause an increase in luciferase activity. A time-course experiment examining the effects of Act D on TS levels as a function of time indicated TS levels dropped by 24 h (data not shown), but not at 18 h (Fig. 4), when the extracts were prepared for Western analysis. In confirmation of this interpretation, TS mRNA levels were also not altered in cells treated with Act D (Fig. 5). Possible explanations for the increase of luciferase caused by Act D may be that Act D affects the stability of luciferase. Further studies are necessary to clarify possible mechanism(s).

Previous reports have shown that treatment of a variety of cell lines with TS inhibitors resulted in up-regulation, but not down-regulation, of TS protein (Swain et al., 1989; Chu et al., 1990; Welsh et al., 2000). One established explanation is that TS inhibitors prevent association and induce dissociation of TS to its own mRNA, releasing the mRNA for trans-

lation. Another explanation is that some TS inhibitors may increase the stability of TS by decreasing the degradation rate (Kitchens et al., 1999). Consistent with previous reports, our data showed that treatment of H630-C6 cells with 5-FU, FUrd, FUdR, TFT, AG331, AG337, ZD1694, and MTX up-regulated TS protein levels (Fig. 4). It is unlikely that MTX, a compound that is not a strong inhibitor of TS even after metabolism to its polyglutamated forms, can mediate this increase in TS by stabilization. Because all these compounds also up-regulated luciferase activity in the presence of increased TS protein, the mode of action is consistent with the detachment of TS from the TBEs on the reporter mRNA. This interpretation is further indicated by the lack of effect of any of the compounds on TS mRNA levels (Fig. 5).

The results obtained here with compounds that have fairly well defined mechanisms of action point to the potential application of this assay to identify compounds that modulate TS binding to TBEs either directly or indirectly (e.g., MTX). The assay was able to easily discriminate TS-directed from TS-independent compounds. To demonstrate the potential utility of the assay, we chose H630 cells for the current study because they have been used previously for this purpose. However, because the pG3E1-2TBE-Neo plasmid contains a selectable marker for G418 resistance, the plasmid is likely to be useful when placed within the context of other cell types with genetic alterations associated with alterations in TS behavior such as *Rb* or *rTS* (Almasan et al., 1995; Black and Dolnick, 1996). A potentially very useful application of this assay is the adaptation to a high-throughput format, where transfected cells in multiwell dishes are treated and evaluated for RLU production as a first pass to identify TS modulators. The broad concentration ranges over which the cells respond to various TS modulators with luciferase induction supports the potential utility of this assay as a means to screen large libraries of compounds for their ability to affect TS levels. These studies are currently underway.

Acknowledgments

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