MOLECULAR AND CELLULAR BIOLOGY, Feb. 1999, p. 1582–1594 Vol. 19, No. 2
0270-7306/99/$04.00+0

Thymidylate Synthase Protein and p53 mRNA Form an In Vivo Ribonucleoprotein Complex

EDWARD CHU,[1]* SITKI M. COPUR,[2] JINGFANG JU,[1] TIAN-MEN CHEN,[1] SAMIR KHLEIF,[2] DONNA M. VOELLER,[2] NOBUYUKI MIZUNUMA,[2] MAHENDRA PATEL,[2] GLADYS F. MALEY,[3] FRANK MALEY,[3] AND CARMEN J. ALLEGRA[2]

Department of Medicine and Pharmacology, Yale Cancer Center and VA CT Cancer Center, Yale University School of Medicine, New Haven, Connecticut 06520[1]; Medicine Branch, Division of Clinical Sciences, National Cancer Institute, Bethesda, Maryland 20889-5105[2]; and Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, New York 12201[3]

Received 15 June 1998/Returned for modification 18 August 1998/Accepted 22 October 1998

A thymidylate synthase (TS)-ribonucleoprotein (RNP) complex composed of TS protein and the mRNA of the tumor suppressor gene p53 was isolated from cultured human colon cancer cells. RNA gel shift assays confirmed a specific interaction between TS protein and the protein-coding region of p53 mRNA, and in vitro translation studies demonstrated that this interaction resulted in the specific repression of p53 mRNA translation. To demonstrate the potential biological role of the TS protein-p53 mRNA interaction, Western immunoblot analysis revealed nearly undetectable levels of p53 protein in TS-overexpressing human colon cancer H630-R10 and rat hepatoma H35(F/F) cell lines compared to the levels in their respective parent H630 and H35 cell lines. Polysome analysis revealed that the p53 mRNA was associated with higher-molecular-weight polysomes in H35 cells compared to H35(F/F) cells. While the level of p53 mRNA expression was identical in parent and TS-overexpressing cell lines, the level of p53 RNA bound to TS in the form of RNP complexes was significantly higher in TS-overexpressing cells. The effect of TS on p53 expression was also investigated with human colon cancer RKO cells by use of a tetracycline-inducible system. Treatment of RKO cells with a tetracycline derivative, doxycycline, resulted in 15-fold-induced expression of TS protein and nearly complete suppression of p53 protein expression. However, p53 mRNA levels were identical in transfected RKO cells in the absence and presence of doxycycline. Taken together, these findings suggest that TS regulates the expression of p53 at the translational level. This study identifies a novel pathway for regulating p53 gene expression and expands current understanding of the potential role of TS as a regulator of cellular gene expression.

Thymidylate synthase (TS) catalyzes the reductive methylation of dUMP by 5,10-methylenetetrahydrofolate to generate thymidylate and dihydrofolate. This enzymatic reaction provides for the sole intracellular de novo source of thymidylate, an essential precursor for DNA biosynthesis. As a result, TS remains a critical target enzyme in cancer chemotherapy (25, 60a).

In addition to its role in enzyme catalysis, there is evidence that TS also functions as an RNA binding protein (5–7). Studies from this laboratory have demonstrated that the translation of human TS mRNA is regulated by its own protein product via a negative autoregulatory mechanism (5). The repression of TS mRNA translation by TS is mediated by specific binding of the protein to at least two distinct *cis*-acting sequences on its own mRNA (6). The first site corresponds to a 188-nucleotide (nt) sequence that includes the translational start site, while the second site is contained within a 100-nt sequence in the protein-coding region. However, in the presence of the nucleotide substrate dUMP or 5-fluoro-2′-deoxyuridine-5′-monophosphate (FdUMP), TS is unable to directly interact with its own mRNA, thus allowing for the synthesis of new protein to proceed (5, 7). Several in vitro studies have shown that short-term exposure of human colon and breast cancer cells to TS-inhibitory compounds, such as 5-fluorouracil (5-FU) or the antifolate analog ZD1694, is associated with an increased level of TS protein expression but no corresponding change in the levels of TS mRNA (8, 9, 35). These findings are consistent with earlier observations that both the RNA binding and the translational inhibition activities of TS are impaired in the presence of either nucleotide and/or folate substrates. Thus, the ability to regulate the expression of TS at the translational level in the setting of acute cytotoxic stress suggests that this regulatory event has biological relevance. Moreover, this process may represent an important mechanism by which normal cellular synthetic function can be controlled and a mechanism for the rapid development of cellular resistance in response to exposure to nucleotide inhibitors of TS, such as 5-FU, and antifolate inhibitors of TS, such as ZD1694, LY231514, and AG331.

An immunoprecipitation–reverse transcription (RT)-PCR technique was recently developed to isolate from an intact cultured human colon cancer cell line a TS-ribonucleoprotein (RNP) complex made up of TS protein and TS mRNA (10). In addition to complexing with its own mRNA, TS formed an RNP complex with the mRNA of the c-*myc* transcription factor. Subsequent studies with RNA electrophoretic gel mobility shift assays (EMSAs) confirmed that the interaction between TS and c-*myc* mRNA was specific and identified the C-terminal coding region as being an important *cis*-acting regulatory element (11). Furthermore, in vitro translation experiments demonstrated that TS protein specifically repressed the translation of c-*myc* mRNA (11). Recent work has shown that TS is

* Corresponding author. Mailing address: VA CT Healthcare System, Cancer Center-111D, 950 Campbell Ave., West Haven, CT 06516. Phone: (203) 937-3421. Fax: (203) 937-3803.

capable of forming RNP complexes in vivo with several cellular RNA species in addition to those of TS and the *myc* family of transcription factors (13). Included in this list is an mRNA sequence corresponding to the p53 tumor suppressor gene, and preliminary binding studies have suggested that this sequence binds with a high affinity to human TS protein (K_d, 1.5 nM) (13). Taken together, these studies suggest that TS may play a role in the regulated expression of various cellular genes.

The p53 tumor suppressor gene encodes a nuclear phosphoprotein which plays an essential role in the regulation of cell cycle progression, DNA synthesis, and apoptosis (1, 26, 38, 46, 47, 66, 67). There is an extensive body of literature highlighting the role of deletion and missense mutations of this gene in the eventual development of a number of human malignancies (24, 30). The expression of p53 has been associated with activation of the G_1 checkpoint leading to growth arrest of cells. It has been proposed that the p53-mediated G_1 checkpoint allows sufficient time for cells to repair DNA damage before DNA synthesis and replication can proceed. Various studies have shown that the levels of p53 protein are acutely increased in both normal and malignant cells following exposure to DNA-damaging agents (35, 48, 50, 71). This induced expression of p53 protein appears to be regulated in part by both translational and posttranslational events. As further support for the role of translational regulation in the control of p53 expression, there is now growing evidence that the biosynthesis of p53 is controlled by a translational autoregulatory feedback mechanism whereby the p53 protein binds to its own mRNA, resulting in translational repression (18, 21, 54).

Given that the biosynthesis of p53 is regulated in part at the translational level and given recent studies showing that TS can form an RNP complex with the p53 mRNA in vivo (13), we investigated whether TS might be able to regulate the expression of p53 through a translationally mediated mechanism. In the present study, we used both a cell-free RNA EMSA and an immunoprecipitation–RT-PCR method to show that TS, an enzyme critical for de novo pyrimidine and DNA biosynthesis, binds specifically in vitro and in vivo to p53 mRNA. Our preliminary studies suggested that the binding of TS to p53 mRNA occurs within a sequence that includes the protein-coding region. Further evidence demonstrating that the binding of TS is associated with the translational repression of p53 mRNA translation both in vitro and in intact biological systems is presented. These studies may have important implications with regard to identifying a novel molecular pathway for regulating p53 expression.

MATERIALS AND METHODS

Cell culture. The characteristics of the human colon cancer cell line H630, its TS-overexpressing subline H630-R10, and the revertant subline H630-R10rev (34, 56) as well as the rat hepatoma cell line H35 and its TS-overexpressing subline H35(F/F) (14, 58) have been previously described. The human colon cancer RKO cell line was a gift from Peter Danenberg (University of Southern California, Los Angeles). All cell lines were grown in 75-cm^2 plastic tissue culture flasks (Falcon Labware, Oxnard, Calif.) with growth medium consisting of RPMI 1640 medium containing 10% dialyzed fetal bovine serum and 2 mM glutamine. Dialyzed fetal bovine serum and all other components were obtained from GIBCO, Grand Island, N.Y.

The doubling times for the human colon cancer H630, H630-R10, and H630-R10rev cell lines were 25, 27, and 26 h, respectively. The doubling times for the rat hepatoma H35 and H35(F/F) cell lines were 24 and 28 h, respectively.

Measurement of DNA synthesis. Human colon cancer and rat hepatoma cells in the logarithmic phase of growth were incubated with 1 μCi of [*methyl*-^{3}H]thymidine (specific activity, 60 Ci/mmol) per ml for 30 min. Cells were then processed as previously described (9). No significant differences were observed in the level of tritiated thymidine incorporation into DNA among human colon cancer H630, H630-R10, and H630-R10rev cells, nor were any differences observed between rat hepatoma H35 and H35(F/F) cells.

Whole-cell extraction and immunoprecipitation of RNP complexes. Whole-cell extracts were prepared from 10^8 cells as previously described (10, 45, 62) and used as the source of antigen. Immunoprecipitation of RNP complexes was performed as described by Steitz (62). In brief, whole-cell extracts were first cleared of nonspecific binding material by incubation with Immunoprecipitin (300 μl; GIBCO BRL, Gaithersburg, Md.) for 30 min on ice and then centrifuged to remove Immunoprecipitin. The cleared extracts were incubated for 30 min with the appropriate antibody; Immunoprecipitin (300 μl), yeast carrier RNA (35 μg; U.S. Biochemicals, Cleveland, Ohio), and Prime RNase Inhibitor (30 U; 5 Prime-3 Prime, Boulder, Colo.) were added for an additional 30 min of incubation. The Immunoprecipitin-immune complex precipitates were centrifuged at 14,000 × *g* for 3 min and then washed four times with 350 μl of NET-2 buffer (50 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.05% [vol/vol] Nonidet P-40). After the addition of 300 μl of NET-2 buffer, the immunoprecipitate pellets were subjected to phenol-chloroform extraction. The RNA fraction of the RNP complex was isolated by ethanol precipitation and then used in the RT reaction.

RT-PCR analysis. The entire immunoprecipitated RNA sample was subjected to RT with a first-strand cDNA synthesis protocol (Stratagene, San Diego, Calif.) as previously described (10). The RT products were stored at −20°C until further use.

All DNA oligonucleotide primers were synthesized on an Applied Biosystems model 391 DNA synthesizer. Their sequences are as follows, and the regions of p53 cDNA to which they correspond are shown in parentheses: p53-1 (1 to 24), 5′-ACCGTCTAGAGCCACCGTCCAGGGAGC-3′; p53-2 (400 to 376), 5′-ACCGGCTGGTGCAGGGGCCGCCGGTGT-3′; p53-3 (531 to 548), 5′-TTGGATCATGTTTTGCCAACTGGCC-3′; p53-4 (1020 to 1005), 5′-TTGAATTCAGGCTCCCCTTTCTTGCG-3′; p53-5 (1321 to 1346), 5′-ACCGGATCCCTCCACCTTCTTGTTCCCCACTGACAG-3′; and p53-6 (1560 to 1537), 5′-ACCGAATTCCCCTCACAGTAAAAACCTTAAAAT-3′.

The single-stranded cDNA obtained from the RT reaction was then used as a template for PCR amplification. The reaction conditions were those outlined by a protocol from Perkin-Elmer Cetus, Emeryville, Calif., and the total volume of the reaction was 100 μl. Mineral oil (40 μl) was placed on top of the aqueous solution to prevent evaporation. Reactions were cycled in a Perkin-Elmer Cetus thermal cycler, and samples were incubated at 96°C for 1 min, 62°C for 1 min, and 72°C for 1 min for 35 cycles. At the end of cycle 35, the samples were incubated for an additional 10 min at 72°C and then cooled to 4°C. The amplified DNA products were resolved on a 1.5% nondenaturing agarose gel, which was stained with ethidium bromide.

Preparation of plasmid constructs and in vitro mRNA transcription. A 1,587-nt wild-type human p53 cDNA was a gift from Frederic Kaye (Medicine Branch, National Cancer Institute), and its cRNA transcript was synthesized with SP6 RNA polymerase following linearization of pSP65/p53 cDNA with *Hin*dIII (63). Two mutant p53 cDNAs, pTp53-154 and pTp53-247, were also gifts from Frederic Kaye. Human chromogranin A cDNA was a gift from Lee Helman (Pediatric Branch, National Cancer Institute), and its corresponding mRNA was synthesized as previously described (27). Human dihydrofolate reductase (DHFR) mRNA was synthesized with SP6 RNA polymerase as previously described (12). The p53/1-400 DNA construct was prepared by PCR amplification under standard conditions with 100 pmol each of primers p53-1 and p53-2 and 0.5 pmol of full-length p53 cDNA. The p53/531-1020 DNA construct was prepared by PCR amplification with 100 pmol each of primers p53-3 and p53-4 and 0.5 pmol of full-length p53 cDNA. The p53/1321-1560 DNA construct was prepared by PCR amplification with 100 pmol each of primers p53-5 and p53-6 and 0.5 pmol of full-length p53 cDNA. Amplified DNAs were cloned into the *Srf*I site of pCR-Script SK(+) (Stratagene), and the cRNA was synthesized with T7 RNA polymerase after linearization with *Sst*I. Mutant pTp53-154 cRNA with an A-to-T point mutation at nt 875 was synthesized with T7 RNA polymerase following linearization with *Nhe*I, while the pTp53-247 cRNA transcript with a G-to-T point mutation at nt 596 was synthesized with SP6 RNA polymerase following linearization with *Hin*dIII (3).

In vitro transcription reactions were performed as previously described (5, 42). Labeled RNA transcripts were made by inclusion of [α^{32}P]CTP at 200 Ci/mmol. All RNA transcripts were evaluated on a 1% agarose–formaldehyde gel to verify their integrity and size. The concentration of unlabeled RNA was determined by UV spectrophotometry. The concentration of radioactively labeled RNA was determined from the specific activity of ^{32}P incorporation.

Construction of a tetracycline-responsive human TS plasmid, pUHD10-3HTS. Tetracycline-responsive plasmid pUHD10-3 (23) was a gift from Bruce Dolnick (Roswell Park Memorial Cancer Institute, Buffalo, N.Y.), and it was digested with the restriction enzymes *Xba*I and *Sac*II. The full-length TS cDNA (nts 1 to 1524) was isolated from plasmid pcEHTS (5) by PCR amplification with 100 pmol each of primers TS-1 and TS-2 and 0.5 pmol of plasmid pcEHTS DNA. The sequences of the primers were as follows: TS-1, 5′-CAGTCCGCGGACCACTTGGCCTGCCTCCGTCC-3′; and TS-2, 5′-CAGTTCTAGAGCAGAACACTTCTTTATTATAGC-3′.

Reactions were cycled in a Perkin-Elmer Cetus thermal cycler, and samples were incubated at 96°C for 1 min, 60°C for 1 min, and 72°C for 2.5 min for 35 cycles. At the end of cycle 35, the samples were incubated for an additional 7 min at 72°C and then cooled to 4°C. The amplified DNA products were resolved on a 1.5% agarose gel, gel purified with a gel purification kit from Qiagen, Santa Clarita, Calif., and digested with the restriction enzymes *Xba*I and *Sac*II. Amplified, digested DNAs were cloned into the *Xba*I and *Sac*II sites of pUHD10-3 to yield plasmid pUHD10-3HTS.

Stable transfection of pUHD10-3HTS into RKO cells. Human colon cancer RKO cells were initially transfected with plasmid pUHD172-1neo (23), a gift from Bruce Dolnick, by use of Lipofectin in accordance with the manufacturer's protocol (GIBCO BRL). The cells were grown in RPMI 1640 medium containing 10% dialyzed fetal bovine serum and 600 μg of Geneticin (G418; GIBCO BRL) per ml. After 3 weeks of growth, colonies were selected by a cloning cylinder method and subsequently expanded into cell lines. Plasmids pTK-Hyg (Clontech, Palo Alto, Calif.) and pUHC10-3HTS were cotransfected into RKO cells already expressing plasmid pUHD172-1neo by the protocol described above. Cells were grown in RPMI 1640 medium containing 10% dialyzed fetal bovine serum, 600 μg of G418 per ml, and 200 μg of hygromycin per ml. Approximately 40 colonies were selected and subsequently expanded into cell lines. Each clone was treated with 1 μg of doxycyline, a tetracycline derivative, per ml for 24 h, and the level of induced TS expression was analyzed by Western immunoblot analysis. The clone with the highest level of induced TS expression was chosen for further study, and this stable transfected cell line was designated RKO-HTStet.

Purification of proteins. Human recombinant TS protein was purified by previously published methods (5, 16, 59). Following purification, TS was stored in a buffer containing 25 mM Tris-HCl (pH 7.4), 25 mM 2-mercaptoethanol, and 10% glycerol at −20°C. Human recombinant deoxycytidylate deaminase was purified as previously described (68).

RNA-protein binding assay. RNA EMSAs were performed as previously described (5, 6, 39, 44). In brief, radiolabeled RNAs (2.2 fmol, 100,000 cpm) were incubated with human recombinant TS protein (3 pmol) in a reaction mixture containing 10 mM HEPES (pH 7.4), 40 mM KCl, 3 mM $MgCl_2$, 0.1 U of Prime RNase Inhibitor per μl, 250 mM 2-mercaptoethanol, and 5% glycerol for 15 min at room temperature. RNase T_1 (12 U; 5 Prime-3 Prime) was then added for 10 min, followed by heparin sulfate (5 mg/ml; Sigma Chemical Co.) for an additional 10 min at room temperature. A 4% polyacrylamide gel (acrylamide/methylenebisacrylamide ratio, 60:1) was preelectrophoresed for 30 min at 150 V, and the entire reaction mixture (total volume, 30 μl) was then resolved on the gel for approximately 40 min at 500 V. The gel was transferred to Whatman filter paper, dried, and visualized by autoradiography.

Competition experiments were performed with human recombinant TS protein (3 pmol) and ^{32}P-radiolabeled TS mRNA (2.2 fmol, 100,000 cpm). These conditions were based on control experiments done with a fixed amount of radiolabeled RNA probe and increasing TS protein concentrations to determine the linearity of binding. Unlabeled competitor RNAs were mixed with labeled probes prior to the addition of TS protein. The relative binding affinity of unlabeled RNAs was determined in terms of the concentration of unlabeled RNA at which specific binding of radiolabeled full-length TS RNA was inhibited by 50%. Each RNA competition experiment was performed three to five times. Quantitation by densitometry was done with a ScanJet Plus scanner (Hewlett-Packard) and analyzed with NIH Image 1.59 software (Wayne Rasband, National Institute of Mental Health, Bethesda, Md.).

In vitro translation. Translation reactions (final volume, 30 μl) were performed with a rabbit reticulocyte lysate in vitro translation system (New England Nuclear Corp., Boston, Mass.) as previously described (5). The translation products were analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) (12.5% acrylamide) by the method of Laemmli (41), and the gels were processed as previously described. After the gels were dried for 2 h, the translation products were visualized by autoradiography. Quantitation of signal intensities was performed by densitometry with a ScanJet Plus scanner and analyzed as described above.

Western immunoblot analysis. Human colon cancer cells and rat hepatoma cells were harvested and extracted as previously described (9). Protein concentrations were determined with a protein assay (Bio-Rad Laboratories, Richmond, Calif.), and equivalent amounts of protein (250 μg) from each sample were resolved on SDS-polyacrylamide gels (10% acrylamide) by the method of Laemmli (41). The gels were electroblotted onto nitrocellulose membranes (Schleicher & Schuell, Inc., Keene, N.H.). Primary antibody staining was performed by incubating filter membranes with either anti-TS monoclonal (1/100 dilution) (34), anti-p53 monoclonal (1/500 dilution; p53 Ab-2; Oncogene Science), anti–beta-actin monoclonal (1/500 dilution; Oncogene Science), anti–alpha-tubulin monoclonal (1/500 dilution; Oncogene Science), or anti-DHFR polyclonal (1:1,000 dilution) antibody overnight at 4°C. After three 5-min washes with blocking solution (5% nonfat milk, 10 mM Tris [pH 7.4], 0.01% thimerosal), filter membranes were incubated with horseradish peroxidase-conjugated secondary antibody (1/500 dilution; Bio-Rad) for an additional 2 h at room temperature. Filters were incubated for three 5-min washes with blocking solution and then for four 10-min washes with 0.1% Tween–phosphate-buffered saline (PBS). They were then processed by the enhanced chemiluminescence method (Amersham Life Science, Amersham, England), and protein bands were visualized by autoradiography. Quantitation of signal intensities was performed by densitometry with a ScanJet Plus scanner and analyzed with NIH Image 1.59 software.

Isolation of total RNA and RNA blot hybridization analysis. Human colon cancer cells and rat hepatoma cells were harvested from 75-cm^2 plastic tissue culture flasks and washed three times with ice-cold PBS. Total RNA was isolated by the method of Chomczynski and Sacchi (4). After extraction, 20 μg of total cellular RNA per sample was denatured, resolved on a 1% agarose–formaldehyde gel, and transferred to a Nytran filter membrane (Schleicher & Schuell). Filter membranes were hybridized to a human p53 cDNA insert probe ^{32}P radiolabeled by the method of Feinberg and Vogelstein (19). Filters were washed as previously described (9), and autoradiography was performed with Kodak XAR-5 film exposed for 12 to 24 h at −70°C.

Polysome analysis. Polysome analysis was performed as previously described (57, 64). In brief, 1.5×10^7 rat hepatoma cells were treated with cycloheximide (CHX; 100 μg/ml) for 10 min and harvested by gentle scraping. The cells were pelleted, washed with ice-cold PBS containing 100 μg of CHX per ml, and then lysed by the addition of 0.5 ml of polysome lysis buffer, containing 0.3 M KCl, 5 mM $MgCl_2$, 10 mM HEPES (pH 7.4), 0.5% Nonidet P-40, 100 μg of CHX per ml, 5 mM dithiothreitol, 250 μg of heparin per ml, 0.1 U of RNasin per ml, and 10 U of Prime RNase Inhibitor per ml. The lysate was passed through a 27-gauge needle and centrifuged at $12,000 \times g$ for 5 min to remove nuclei and cell debris. Aliquots of 20 A_{260} units were loaded on 11 ml of 5 to 47% sucrose gradients prepared in a buffer containing 20 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 0.5 M KCl, 50 mM NaCl, 1 mM dithiothreitol, 100 μg of CHX per ml, 100 μM phenylmethylsulfonyl fluoride, 0.15 μM aprotonin, 0.1 μM pepstatin A, 1 μM leupeptin, 10 mM NaF, and 50 mM beta-glycerophosphate and were subjected to centrifugation at 39,000 rpm for 3 h in a Beckman SW41 rotor at 4°C. Gradients were fractionated with an ISCO VA-5 absorbance monitor set at 260 nm, and 0.6-ml fractions were collected. RNA was prepared from each fraction by phenol-chloroform extraction and ethanol precipitation and was then applied to a nitrocellulose membrane with a Schleicher & Schuell Minifold apparatus. Hybridization to a ^{32}P-radiolabeled p53 cDNA probe was performed as described above.

Cell cycle analysis. Samples were prepared for flow cytometry as previously described (20). In brief, cells (5×10^6/ml) were washed in ice-cold PBS and resuspended in a 0.1% (wt/vol) sodium citrate–0.1% (vol/vol) Triton X-100 hypotonic solution. Cells were then treated with RNase A (1 μg/ml; 5 Prime-3 Prime) and propidium iodide (50 μg/ml; Sigma) overnight at 4°C. In experiments examining the effect of drug treatment on cell cycle distribution, rat hepatoma H35 and H35(F/F) cells were incubated with 10 and 100 nM fluorodeoxyuridine (FUdR), respectively, for 8 h. The concentrations selected for these studies were based on growth inhibition studies and corresponded to the concentration of drug that inhibited by 50% (IC_{50}) the growth of rat hepatoma cells. Cells were then harvested as described above. Cell cycle analysis was performed with a Becton-Dickinson fluorescence-activated FACStar^{PLUS} cell analyzer and the SOBR model analysis program provided by the manufacturer.

RESULTS

Previous studies from this laboratory have shown that TS, as an RNA binding protein, interacts in intact human colon cancer cells with its own mRNA, the mRNA of the c-*myc* transcription factor, and the mRNA of the p53 tumor suppressor gene (10, 11, 13). Given that the p53 gene encodes a protein product involved in cell cycle regulation and cellular growth and proliferation (38, 47), we investigated in further detail the interaction between TS and p53 mRNA. Both partially pure (Fig. 1A, lane 2) and homogeneously pure (Fig. 1A, lane 3) human recombinant TSs interacted with a radiolabeled p53 RNA probe to form an RNA-protein complex. Further studies showed that native TS purified from human cervical cancer HeLa cells also specifically interacted with a radiolabeled p53 mRNA probe (data not shown). Thus, both recombinant and native sources of TS possess RNA binding activity. The results obtained with homogeneously pure protein suggested that TS alone is sufficient for RNA binding and that it does not require the presence of other cofactors or proteins. We also incubated radiolabeled p53 mRNA with various concentrations of human recombinant TS protein (0.3 to 30 pmol). Scatchard analysis (61) of the experimental data revealed that TS bound the 1,587-nt p53 mRNA with an equilibrium K_d of 1.6 ± 0.3 nM. When TS protein was initially denatured by treatment with either proteinase K (Fig. 1A, lane 4) or heating to 95°C (Fig. 1A, lane 5), no RNP complex was formed. The inability of denatured TS to interact with p53 mRNA indicated that the native form of TS is important for RNA binding. Furthermore, no complex was formed when the radiolabeled p53 mRNA probe was incubated with unrelated proteins, such as human deoxycytidylate deaminase (Fig. 1A, lane 6), a protein similar to TS in its essential role in one-carbon metabolism and DNA biosynthesis, or with bovine serum albumin (data not shown).

Previous studies revealed that the binding of TS protein to

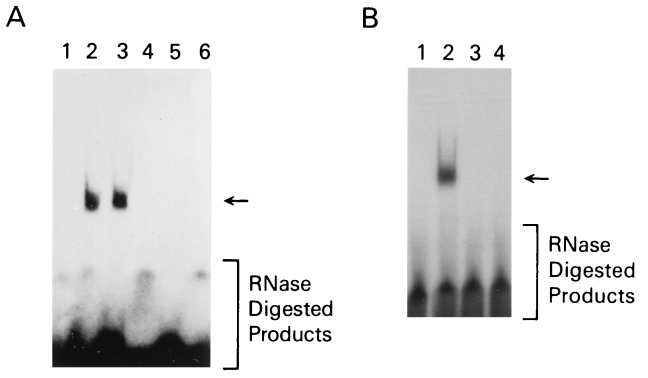

FIG. 1. (A) Specific interaction between human TS and p53 RNA. RNA EMSAs were performed with a 1,587-nt radiolabeled p53 RNA probe (2.2 fmol, 100,000 cpm) incubated in the absence (lane 1) or presence of partially purified human recombinant TS (specific activity, 0.01 U/mg; 3 pmol; lane 2) and pure human recombinant TS (specific activity, 0.7 U/mg; 3 pmol; lane 3). Pure human recombinant TS (3 pmol) was preincubated with proteinase K (1 μg/μl) for 15 min (lane 4) or heat denatured at 95°C for 15 min (lane 5) and then included in a reaction mixture with a radiolabeled p53 RNA probe. Labeled p53 RNA was incubated with pure human recombinant deoxycytidylate deaminase (specific activity, 1,000 U/mg; 3 pmol; lane 6). (B) Effect of nucleotides on RNA binding of TS protein. Radiolabeled p53 RNA (2.2 fmol; 100,000 cpm) was incubated in the absence (lane 1) or presence (lanes 2 to 4) of human recombinant TS (3 pmol). TS protein was preincubated with either dUMP (30 μM; lane 3) or the 5-FU metabolite FdUMP (30 μM; lane 4) for 15 min, followed by the addition of radiolabeled p53 RNA probe. Reactions were performed at room temperature for 15 min. Then, RNase T_1 (12 U) was added for 10 min, followed by heparin (5 mg/ml) for an additional 10 min. Samples were electrophoresed in a nondenaturing 4% acrylamide gel. The specific complex is indicated by the arrows.

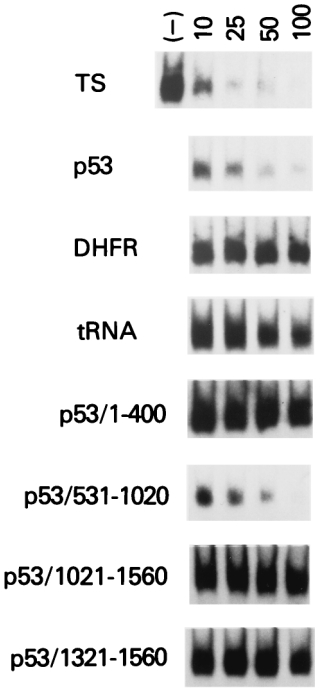

FIG. 2. Competition experiment to determine the relative binding of various p53 RNA sequences. Radiolabeled p53 RNA (2.2 fmol; 100,000 cpm) was incubated with TS (3 pmol) alone (−) or in the presence of a 0- to 100-fold molar excess of either the 1,524-nt TS RNA, the 1,587-nt p53 RNA, or the 700-nt DHFR RNA, yeast tRNA, p53/1-400 RNA, p53/531-1020 RNA, p53/1021-1560 RNA, or p53/1321-1560 RNA. Each RNA sequence was synthesized in vitro as described in Materials and Methods.

its own mRNA was repressed when TS was treated with either of its nucleotide ligands, dUMP and FdUMP (7). Similar experiments were performed to determine whether ligand binding could alter the interaction between TS protein and p53 mRNA. Incubation of TS with either the physiologic substrate dUMP (30 μM) (Fig. 1B, lane 3) or the 5-FU nucleotide FdUMP (30 μM) (Fig. 1B, lane 4) completely inhibited RNA binding. In contrast, incubation of TS with either of the parent substrate compounds, 2′-deoxyuridine and 5-FU, or with nonsubstrate nucleotide analogs, such as dAMP (30 μM) and dGMP (30 μM), did not alter RNA binding (data not shown).

Competition studies were performed with RNA EMSAs to confirm the specificity of the p53 mRNA-TS protein interaction. Complex formation was inhibited upon the addition of a 10- to 100-fold molar excess of either unlabeled full-length TS mRNA or 1,587-nt p53 mRNA (Fig. 2). The relative binding affinities of TS for TS mRNA and for p53 mRNA were nearly identical (1.0 ± 0.5 and 1.5 ± 0.4 nM, respectively). Recent studies identified a specific interaction between the mRNA of the c-*myc* transcription factor and TS protein (11). With a radiolabeled 1,587-nt p53 mRNA as a probe, competition analysis revealed that the addition of unlabeled c-*myc* mRNA inhibited complex formation between the p53 mRNA probe and TS protein in a dose-dependent fashion and that c-*myc* mRNA bound TS protein with a relative affinity of 2 nM (data not shown). In contrast, the addition of unrelated RNAs, such as human DHFR mRNA and yeast tRNA, did not block complex formation (Fig. 2).

To begin to localize the binding sites on p53 mRNA, four p53 mRNA sequences were synthesized in vitro and then tested as unlabeled RNA competitors. A 489-nt sequence corresponding to nts 531 to 1020 within the protein-coding region bound TS with a relative affinity similar to that of full-length p53 mRNA (2.0 ± 0.5 nM) (Fig. 2). In contrast, three different p53 mRNA sequences, a 400-nt sequence corresponding to nts 1 to 400, a 539-nt sequence corresponding to nts 1021 to 1560, and a 249-nt sequence corresponding to nts 1321 to 1560, were each unable to compete for binding to TS protein (Fig. 2).

In addition to studying wild-type p53 mRNA sequences, we also synthesized mutant p53 mRNAs in vitro to determine whether they could interact with TS. Moreover, the particular mutant p53 mRNAs selected for study were chosen because they represent "hot-spot" mutations (24, 30). Two different mutant p53 mRNAs, the first with a point mutation at nt 596 (2.3 ± 0.3 nM) and the second with a point mutation at nt 875 (2.5 ± 0.4 nM), both bound TS with relative affinities nearly identical to that of wild-type p53 mRNA (1.5 ± 0.4 nM).

To begin to investigate the potential functional role of the interaction between TS and p53 mRNA, a series of in vitro translation experiments was performed. In the absence of exogenous mRNA, translation of the rabbit reticulocyte lysate gave rise to a protein product resolving at a molecular mass of approximately 50 kDa (Fig. 3A, lane 1). When p53 mRNA was included in the rabbit reticulocyte lysate, two major protein products resolving at approximately 53 and 45 kDa were observed (Fig. 3A, lane 2). To confirm that these protein products were p53 associated, the gel was transferred to a filter membrane and subjected to Western immunoblot analysis with an anti-p53 monoclonal antibody. Positive staining was observed for both the 53- and the 45-kDa proteins (data not shown). Since the specific anti-p53 antibody used in these studies recognizes an N-terminal epitope on p53, the 45-kDa protein most likely represents a truncated form of p53 at the C-terminal region. The addition of exogenous TS protein (1 to 4 pmol) to a rabbit reticulocyte lysate containing p53 mRNA

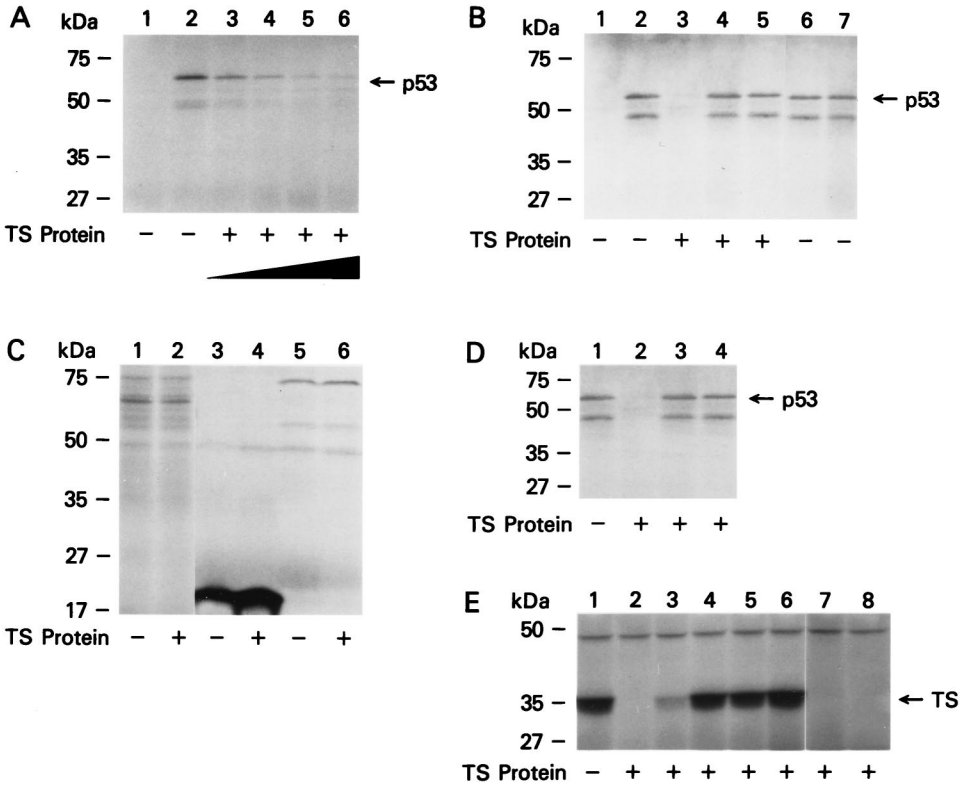

FIG. 3. (A) Dose-dependent inhibition of p53 mRNA translation in vitro by TS. Translation reaction mixtures containing rabbit reticulocyte lysate were incubated with either no exogenous RNA (lane 1) or p53 mRNA (0.2 pmol; lanes 2 to 6). Various amounts of TS protein—1 (lane 3), 2 (lane 4), 3 (lane 5), and 4 (lane 6) pmol—were added to the reaction mixture as indicated. (B) Specificity of inhibition of p53 mRNA translation in vitro by TS. Translation reaction mixtures were incubated with either no RNA (lane 1) or p53 mRNA (0.2 pmol; lanes 2 to 7), and TS (3 pmol; lanes 3 to 5) was included in the reaction mixture as indicated. TS was treated with either proteinase K (1 μg/μl) (lane 4) for 15 min or heat denaturation at 95°C (lane 5) for 15 min and then added to p53 mRNA-containing rabbit lysate reaction mixtures. p53 mRNA was incubated with bovine serum albumin (3 pmol; lane 6) or recombinant deoxycytidylate deaminase (3 pmol; lane 7). (C) Translation reaction mixtures were incubated with 0.2 pmol of yeast mRNA (lanes 1 and 2), 0.2 pmol of human DHFR mRNA (lanes 3 and 4), and 0.2 pmol of human chromogranin A mRNA (lanes 5 and 6). TS (3 pmol) was included in the rabbit reticulocyte lysate reaction mixtures as indicated. (D) p53 mRNA (0.2 pmol) was incubated in the absence (lane 1) or presence (lanes 2 to 4) of human TS protein (3 pmol) as indicated. The nucleotides dUMP (30 μM; lane 3) and FdUMP (30 μM; lane 4) were included in the reaction mixtures. (E) TS mRNA (0.2 pmol) was incubated alone (lane 1) or in the presence of TS protein (3 pmol; lanes 2 to 8). Various exogenous RNAs were also included in the rabbit reticulocyte lysate mixtures: p53/531-1020 RNA at 2 pmol (lane 3) and 4 pmol (lane 4), 1,587-nt p53 mRNA at 2 pmol (lane 5) and 4 pmol (lane 6), p53/1-400 RNA (4 pmol; lane 7), and p53/1321-1560 RNA (4 pmol; lane 8). All translation reaction mixtures were incubated at 37°C for 60 min, and protein products were analyzed by SDS-PAGE and autoradiography. The TS and p53 protein products are indicated by the arrows.

resulted in dose-dependent repression of the synthesis of both the 53- and the 45-kDa proteins (Fig. 3A, lanes 3 to 6). Careful inspection of the protein gels did not reveal the presence of truncated p53 protein products.

The specificity of the effect of TS on p53 mRNA translation was further supported by control experiments in which the addition of either bovine serum albumin (Fig. 3B, lane 6) or recombinant deoxycytidylate deaminase (Fig. 3B, lane 7) did not alter the translation of p53 mRNA from control levels (Fig. 3B, lane 2). Moreover, compared to intact, native TS (Fig. 3B, lane 3), TS treated with either proteinase K (Fig. 3B, lane 4) or heat denaturation (Fig. 3B, lane 5) was unable to inhibit translation. Finally, the addition of exogenous TS did not alter the translational efficiencies of unrelated mRNA transcripts, such as yeast (Fig. 3C, lanes 1 and 2), human DHFR (Fig. 3C, lanes 3 and 4), and human chromogranin A (Fig. 3C, lanes 5 and 6). Further experiments were also performed to document that exogenous TS protein repressed, in a dose-dependent manner, the translation of a mutant p53 mRNA with a point mutation at nt 596 and that of a mutant p53 mRNA with a point mutation at nt 875 (data not shown).

The effect of nucleotide binding on the ability of human TS protein to repress p53 mRNA translation was also examined. When p53 mRNA was incubated in the presence of exogenous TS, significant repression of translation was observed (Fig. 3D, lane 2). However, the addition of either of the nucleotide ligands dUMP and FdUMP to reaction mixtures containing both TS protein and p53 mRNA completely prevented the translation-repressing effects of TS (Fig. 3D, lanes 3 and 4).

As demonstrated by RNA competition experiments in Fig. 2, the mRNAs of both TS and p53 bound with nearly equal affinities to TS. Given this observation, we next examined whether the presence of excess p53 mRNA might abrogate the translation-inhibiting effects of TS protein on TS mRNA translation. When either a 10- or a 20-fold molar excess of p53/531-1020 RNA (Fig. 3E, lanes 3 and 4) or the 1,587-nt p53 RNA (Fig. 3E, lanes 5 and 6) was included in the rabbit reticulocyte lysate reaction mixtures containing TS mRNA and human TS protein, the translation of TS mRNA was restored back to control levels (Fig. 3E, lane 1). In contrast, the presence of excess p53/1-400 RNA (Fig. 3E, lane 7) or p53/1321-1560 RNA (Fig. 3E, lane 8) was unable to abrogate the translational repression by human TS. The inability of these two p53 RNA sequences to compete with TS mRNA for binding to TS protein provides further support for the specific interaction be-

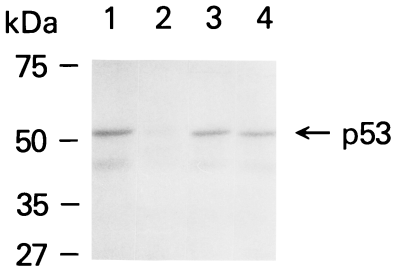

FIG. 4. Specific effect of TS protein on p53 mRNA translation. p53 mRNA (1,587 nts) was incubated in rabbit reticulocyte lysates in the absence (lane 1) or presence (lane 2) of human TS protein (3 pmol). At the end of the reaction, RNA was extracted from each reaction sample and incubated in a fresh rabbit reticulocyte lysate (lanes 3 and 4) at 37°C for an additional 60 min. Protein products were analyzed by SDS-PAGE and autoradiography. The p53 protein product is indicated by the arrow.

tween TS and the 489-nt sequence contained between nts 531 and 1020 of p53 mRNA.

While these studies suggested that the specific regulatory effect of TS occurred via direct repression of p53 mRNA translation, the possibility of TS activating the degradation of p53 mRNA could not be excluded. In addition, the presence of even low levels of RNases contaminating the preparations of TS might have contributed to the rapid degradation of p53 mRNA. To rule out these two possibilities, p53 mRNA was incubated with rabbit reticulocyte lysates in the absence (Fig. 4, lane 1) and presence (Fig. 4, lane 2) of exogenous human TS as described above. At the end of the 1-h incubation, total RNAs were isolated from lysates and then retranslated with fresh rabbit reticulocyte lysates for an additional 1 h. The presence of exogenous TS protein in the first reaction did not alter the relative amount of p53 that was subsequently translated in vitro in the second reaction (Fig. 4, lane 4) compared to that in the control reaction (Fig. 4, lane 3). These results indicated that the repressive effect of TS protein was, in fact, specific for p53 mRNA translation.

In previous studies, we had developed an immunoprecipitation–RT-PCR method to identify a TS-RNP complex composed of TS and its own mRNA in cultured human colon cancer cells (H630-R10) (10). In the present study, the same method was used to isolate a TS-RNP complex containing TS and p53 mRNA in the H630-R10 cell line. The RNP complexes were immunoprecipitated with TS-specific antibody, and the nucleic acid fraction specifically bound to TS was isolated and then subjected to RT-PCR amplification to yield a 489-nt product corresponding to nts 531 to 1020. Immunoprecipitation of H630-R10 cell extracts with either a polyclonal antibody (Fig. 5A, lane 3) or a monoclonal antibody (Fig. 5A, lane 4) to human TS followed by RT-PCR amplification gave rise to the predicted 489-nt DNA product. This band resolved at the same position as a DNA product obtained from a PCR with the same set of primers and the 1,587-nt p53 cDNA as a DNA template (Fig. 5A, lane 1). The gel was then transferred to a filter membrane, and Southern blot hybridization analysis with a ^{32}P-radiolabeled p53 cDNA probe confirmed that the DNA product was, in fact, specific for p53 (Fig. 5B, lanes 1, 3, and 4).

When the immunoprecipitated nucleic acid fraction was treated with RNase A before the RT-PCR amplification step, no amplified DNA product was observed (Fig. 5A, lane 5). In contrast, when the isolated nucleic acid fraction was treated with DNase before the RT-PCR step, the DNA product could still be PCR amplified (data not shown). No DNA product was observed when immunoprecipitated nucleic acids were directly

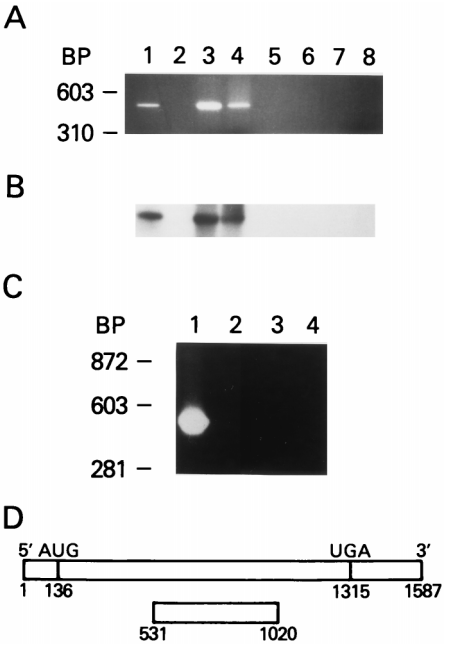

FIG. 5. RT-PCR analysis of p53 RNA immunoprecipitated from human colon cancer cells (H630-R10). (A) Ethidium bromide stain. (B) Southern hybridization. The 1,587-nt p53 cDNA was used as a DNA template in a PCR amplification with p53-specific primers p53-3 and p53-4 (lane 1). Whole-cell extracts were immunoprecipitated with either anti-TS polyclonal antibody (lane 3), anti-TS monoclonal antibody (lane 4), no antibody (lane 6), preimmune antisera (lane 7), or anti–alpha-tubulin monoclonal antibody (lane 8) as described in Materials and Methods. The isolated nucleic acid fraction was reverse transcribed and PCR amplified with p53-specific primers. The immunoprecipitated nucleic acid in lane 5 was treated with RNase A prior to RT. A control PCR was performed with p53-specific primers and no DNA template (lane 2). BP, base pairs. (C) Analysis of immunoprecipitated RNAs from human colon cancer cells. RNAs isolated by immunoprecipitation of H630-R10 cell extracts with anti-TS polyclonal antibody were RT-PCR amplified with either p53-specific primers to yield DNA products corresponding to nts 531 to 1020 (lane 1) or *max*-specific (lane 2), DHFR-specific (lane 3), or GAPDH-specific (lane 4) primer sets. Amplified DNA products were resolved on a 1% nondenaturing agarose gel and analyzed by staining with ethidium bromide. (D) p53 RNA sequence. The p53 RNA sequence predicted to be RT-PCR amplified is shown in relation to its position on the full-length p53 mRNA. The translational start (AUG) and stop (UGA) sequences are identified.

PCR amplified in the absence of the RT step or when whole-cell supernatants were initially deproteinized by phenol-chloroform extraction prior to immunoprecipitation (data not shown). To confirm the specificity of antibody recognition, either no antibody (Fig. 5A, lane 6) or unrelated antibodies, such as preimmune antisera (Fig. 5A, lane 7) or anti–alpha-tubulin antibody (Fig. 5A, lane 8), were used in the immunoprecipitation reaction. Under each of these conditions, no amplification of the 489-nt DNA product was observed. Taken together, the results of these control experiments demonstrated that the nucleic acid of origin for the 489-nt amplified DNA product was specific for p53 RNA.

To determine whether TS-specific antibody was capable of immunoprecipitating RNAs other than p53 RNA (Fig. 5C, lane 1), we used the same RT-PCR method but included primer sets specific for unrelated genes, such as *max* (Fig. 5C, lane 2), the DHFR gene (Fig. 5C, lane 3), and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene (Fig. 5C, lane 4). None of these cellular RNAs formed an RNA complex with TS protein. Control experiments were performed to show that each of these primer sets was able to PCR amplify the respective genes from total cellular RNA isolated from the

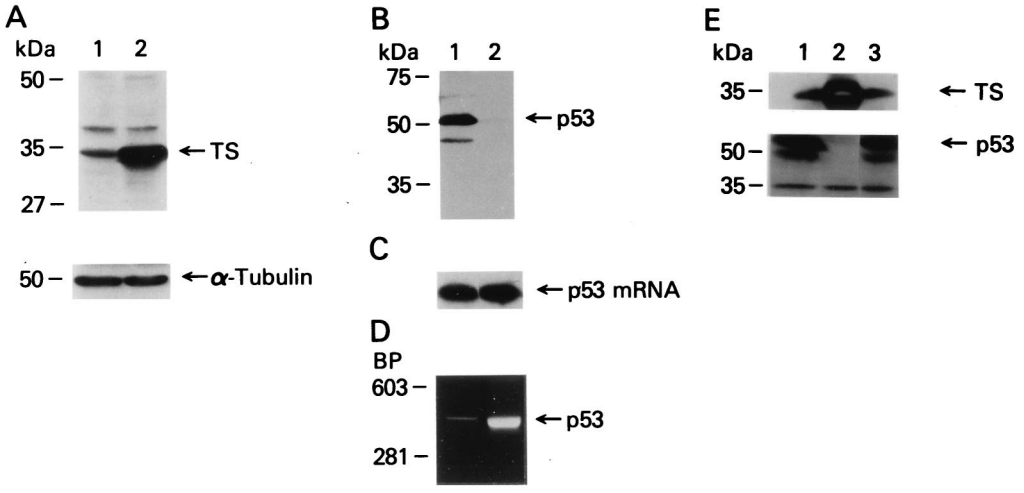

FIG. 6. (A) Western immunoblot analysis of TS in H630 cells. Cytosolic extracts from human colon cancer H630 (lane 1) and H630-R10 (lane 2) cells were prepared as described in Materials and Methods. Equal amounts of protein (250 μg) were loaded onto each lane. TS protein was detected by immunoblot analysis with an anti-TS monoclonal antibody (1/100 dilution) (top panel). Filter membranes were stripped and reprobed with an anti–alpha-tubulin monoclonal antibody (1/500 dilution) to control for the loading and integrity of the protein (bottom panel). (B) Western immunoblot analysis of p53 in parent H630 (lane 1) and resistant H630-R10 (lane 2) cells. Filter membranes used in panel A were stripped and reprobed with an anti-p53 monoclonal antibody (1/500 dilution). (C) Northern blot analysis of p53 mRNA in parent H630 (lane 1) and resistant H630-R10 (lane 2) cells. Total cellular RNA (20 μg) was resolved on a 1% agarose–formaldehyde gel, transferred to a Nytran filter membrane, and hybridized with a ^{32}P-radiolabeled p53 cDNA insert. (D) Analysis of p53 RNA immunoprecipitated from parent H630 (lane 1) and resistant H630-R10 (lane 2) cells. p53 RNA in the form of RNP complexes was immunoprecipitated with an anti-TS polyclonal antibody and RT-PCR amplified with p53-specific primers as described in Materials and Methods. BP, base pairs. (E) Western immunoblot analysis of TS in human colon cancer H630 (lane 1), H630-R10 (lane 2), and H630-10rev (lane 3) cells. Equal amounts of protein (250 μg) were loaded onto each lane, and TS protein was detected as described for panel A (top panel). Filter membranes were stripped and reprobed with an anti-p53 monoclonal antibody (1/500 dilution) (bottom panel). Quantitation of signal intensities was performed by densitometric scanning (ScanJet Plus scanner).

human colon cancer H630-R10 cell line (data not shown). The inability to detect other genes, such as the *max*, DHFR, and GAPDH genes, in the immunoprecipitated samples lends further support for the specificity of the p53 RNA-TS protein in vivo RNP complex.

We next attempted to quantitate the fraction of total cellular p53 mRNA bound to TS in the H630-R10 cell line. Approximately 12% of the total p53 mRNA within the cells was bound to TS in the form of an RNP complex (data not shown). However, this value may not be completely representative of the true fraction of p53 mRNA in the complex form. The immunoprecipitation–RT-PCR method used for these studies is associated with several technical limitations, including the potential for contaminating degradative RNase activity released during the isolation of RNP complexes from cells and the relative inefficiencies of the various steps associated with this process, including immunoprecipitation, RT, and/or PCR amplification. Moreover, other factors, such as the intracellular levels of the physiologic nucleotide substrate dUMP and reduced 5,10-methylenetetrahydrofolate, the redox state within the cell, and the intracellular localization of the TS protein, may play key roles in determining the final ability of TS to bind to its cellular target RNAs. For all these reasons, the levels of TS-RNP complexes at a given point in time may vary considerably within the cell.

As a first step toward investigating the potential biological significance of the interaction between TS and p53 mRNA, we measured the level of p53 protein in cells overexpressing TS. Using Western immunoblot analysis, we compared the level of expression of TS in the parent H630 human colon cancer cell line and its 5-FU-resistant H630-R10 subline. H630-R10 cells (Fig. 6A, lane 2) demonstrated a nearly 30-fold-increased level of expression of TS compared to parent H630 cells (Fig. 6A, lane 1). To control for protein loading, this blot was stripped and then stained for alpha-tubulin, which revealed no difference in the expression of this "housekeeping" protein between these two cell lines (Fig. 6A, lanes 1 and 2). However, a significant difference was observed in the level of p53 expression between H630 and H630-R10 cells. Parent H630 cells (Fig. 6B, lane 1) stained quite strongly for p53, in contrast to H630-R10 cells (Fig. 6B, lane 2), which expressed a nearly undetectable level of p53.

The H630-R10 cell line was established by maintaining parent H630 cells under continued selective pressure with 10 μM 5-FU (10). When these 5-FU-resistant cells were grown in the absence of the selective pressure of the drug for more than 4 years (H630-R10rev), the sensitivity to the fluoropyrimidine was restored to the same level as that observed in parent H630 cells (data not shown). Western immunoblot analysis revealed that the level of TS protein in the revertant H630-R10rev cells (Fig. 6E, lane 3) was significantly decreased relative to that in H630-R10 cells but was virtually identical to that in parent H630 cells (Fig. 6E, lane 1). As was shown in Fig. 6B, H630-R10 cells overexpressing TS were found to express a virtually undetectable level of p53 (Fig. 6E, lane 2). However, the level of p53 in the revertant H630-R10rev cells (Fig. 6E, lane 3) was identical to that in parent H630 cells (Fig. 6E, lane 1).

As determined by RNA hybridization analysis, the levels of p53 mRNA were identical in both H630 and H630-R10 cells (Fig. 6C, lanes 1 and 2). RNAs from each cell line were intact and equally loaded, as determined by ethidium bromide staining of the gel and by hybridization analysis of the filter membrane with a radiolabeled human beta-actin probe (data not shown). In addition, the cDNA sequence corresponding to the protein-coding region of p53 was isolated from each cell line and subcloned into a pGEM-4Z in vitro transcription vector. p53 cRNA transcripts were synthesized in vitro and then subjected to in vitro translation with the rabbit reticulocyte lysate system. The levels of in vitro-translatable p53 were identical for H630 and H630-R10 cells, suggesting that no gross muta-

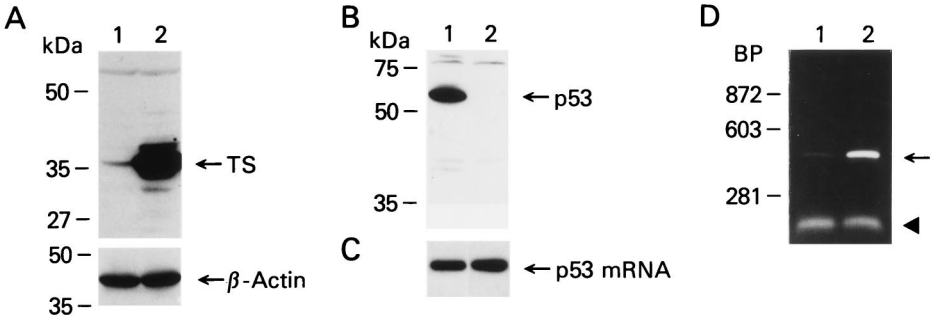

FIG. 7. (A and B) Western immunoblot analysis of TS in rat hepatoma H35 (lane 1) and H35(F/F) (lane 2) cells. Equal amounts of protein (250 μg) were loaded onto each lane. Filter membranes were stripped and reprobed with an anti–beta-actin monoclonal antibody (1/500 dilution) to control for the loading and integrity of the protein (A) or were reprobed with an anti-p53 monoclonal antibody (1/500 dilution) (B). (C) Northern blot analysis of p53 mRNA in parent H35 (lane 1) and resistant H35(F/F) (lane 2) cells. Total cellular RNA (20 μg) from each cell line was resolved on a 1% agarose–formaldehyde gel, transferred to a Nytran filter membrane, and hybridized with a ^{32}P-radiolabeled p53 cDNA insert. (D) Analysis of p53 RNA immunoprecipitated from parent H35 (lane 1) and resistant H35(F/F) (lane 2) cells. p53 RNA in the form of RNP complexes was immunoprecipitated by an anti-TS polyclonal antibody and RT-PCR amplified with p53-specific primers as described in Materials and Methods. The p53 DNA product is indicated by the arrow, and the primers are indicated by the arrowhead. BP, base pairs.

tion in the protein-coding region of the p53 mRNA sequence was present in the resistant H630-R10 cells (data not shown). An immunoprecipitation–RT-PCR analysis was then performed to quantitate the level of p53 RNA bound to TS in cellular RNP complexes. The amount of complexed p53 RNA in H630-R10 cells (Fig. 6D, lane 2) was significantly higher (approximately 40-fold) than that in parent H630 cells (Fig. 6D, lane 1). This result is consistent with the Western blot analysis, which revealed a 30-fold increase in TS expression in the resistant H630-R10 cells compared to the parent H630 cells.

A similar set of experiments was performed with the rat hepatoma H35 cell line and its FUdR-resistant, TS-overexpressing H35(F/F) subline to determine whether a similar relationship existed between the expression of TS and the expression of p53. The H35(F/F) subline was established by maintaining the parent H35 cells under continued selective pressure with 100 nM FUdR (14, 58). Previous studies had revealed a 15-fold amplification of the TS gene in H35(F/F) cells relative to H35 cells (14). As shown in Fig. 7A, H35(F/F) cells expressed a 40-fold-higher level of TS protein than parent H35 cells. However, in sharp contrast to parent H35 cells (Fig. 7B, lane 1), which stained strongly for p53, H35(F/F) cells did not express a detectable level of p53 (Fig. 7B, lane 2). RNA hybridization analysis demonstrated that the levels of p53 mRNA were identical in these two cell lines (Fig. 7C). In addition, no mutations were detected upon sequencing analysis of the p53 cDNA isolated from each cell line (data not shown). An immunoprecipitation–RT-PCR analysis was performed to measure the level of p53 RNA bound to TS in an RNP complex. The level of complexed p53 RNA in H35(F/F) cells (Fig. 7D, lane 2) was markedly higher (approximately 45-fold) than that in parent H35 cells (Fig. 7D, lane 1).

As further in vivo evidence for the effect of TS on p53 expression, a human colon cancer RKO cell line (RKO-HTStet) was transfected with human TS cDNA under the control of a tetracycline-responsive plasmid. The RKO cell line was selected for these transfection studies because it has been shown to express wild-type p53 and relatively low basal levels of TS. Preliminary studies with the RKO-HTStet cell line revealed that treatment with 1 μg of doxycycline per ml for 24 h resulted in maximal induction of TS expression (data not shown). Incubation of RKO-HTStet cells with 1 μg of doxycycline per ml for 24 h gave rise to a 15-fold-induced expression of TS, while the level of p53 was nearly undetectable compared to that in untreated cells (Fig. 8A, lanes 1 and 2). As important controls, the expression of unrelated proteins, such as alpha-tubulin and DHFR, was examined with cells treated under the same conditions, and the levels of these two proteins were identical in the absence or presence of doxycycline. In addition, treatment of parent RKO cells with doxycycline did not change the expression of TS and p53 from their baseline levels (data not shown). RNA hybridization analysis revealed the levels of p53 mRNA in untreated cells (Fig. 8B, lane 1) and doxycycline-treated cells (Fig. 8B, lane 2) to be identical. The cellular RNAs isolated from each cell line were intact and equally loaded, as determined by ethidium bromide staining of the gel (Fig. 8B) and by subsequent hybridization analysis of the filter membrane with a radiolabeled human beta-actin probe (data not shown).

To provide further evidence for the role of translational control in the regulation of p53 expression, polysome analyses were performed with the rat hepatoma H35 model system. The association of p53 mRNA with polysomes in parent H35 cells and in TS-overexpressing H35(F/F) cells was analyzed by sucrose gradient fractionation. As shown in Fig. 9, p53 mRNA isolated from parent H35 cells was associated with larger-sized polysomes than was that isolated from TS-overexpressing H35(F/F) cells. Approximately 60% of the p53 mRNA in H35 cells was isolated in fractions 8 to 12, containing higher-molecular-weight polysomes, while nearly 80% of the p53 mRNA in H35(F/F) cells was found in the monosome fractions. As an internal control, the distribution of beta-actin mRNA in these two cell lines was compared and found to be identical (data not shown).

Given the well-documented role of p53 in controlling the G_1 cell cycle checkpoint, we next determined whether the decreased level of p53 expression in TS-overexpressing cells might result in alterations in this critical cellular function. For these experiments, rat hepatoma H35 and H35(F/F) cell lines were used as the representative model system. Flow cytometric analysis did not reveal significant differences in the normal cell cycle distribution between these two cell lines during exponential growth. The ability of each of these cell lines to arrest in G_1 following exposure to a cytotoxic drug was determined by incubating exponentially growing cells in toxic concentrations of FUdR for 8 h. As shown in Fig. 10, the G_1-S block was determined by comparing the ratio of the percentage of cells in the G_1 phase to that in the S phase in both untreated and treated cells. Parent H35 cells, expressing wild-type p53, exhibited

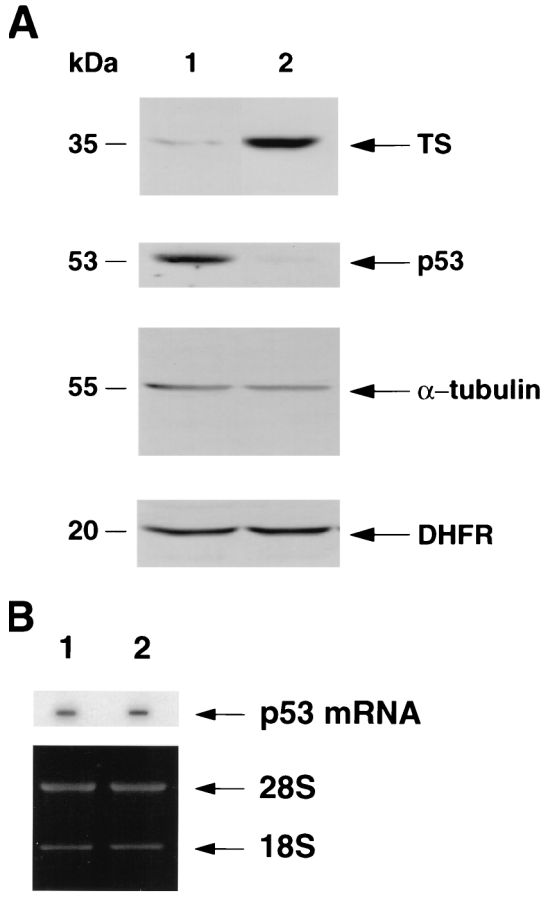

FIG. 8. (A) Western immunoblot analysis of TS and p53 in human colon cancer RKO cells. RKO cells were incubated for 24 h in the absence (lane 1) or presence (lane 2) of 1 μg of doxycycline per ml. Total cellular protein (250 μg) was extracted and used in a Western immunoblot analysis as described in Materials and Methods. Filter membranes were stripped and reprobed with either an anti–alpha-tubulin monoclonal antibody (1/5,000 dilution) or an anti-DHFR polyclonal antibody (1/1,000 dilution) to control for the loading and integrity of the protein. (B) RNA hybridization analysis of p53 mRNA in human colon cancer RKO cells. RKO cells were incubated for 24 h in the absence (lane 1) or presence (lane 2) of 1 μg of doxycycline per ml. Total cellular RNA was extracted, and RNA hybridization analysis was performed as described in Materials and Methods.

pronounced G_1-S cell cycle arrest, as evidenced by a significant increase (threefold) in the G_1/S ratio following exposure to FUdR. In marked contrast, H35(F/F) cells overexpressing TS and expressing undetectable levels of p53 were unable to arrest in G_1 and actually showed a slight increase in the G_1/S ratio following drug treatment. To provide further evidence that the p53-dependent checkpoint activity was abolished by excess TS, we investigated the effect of gamma irradiation, a different DNA-damaging agent, in these same two rat hepatoma cell lines. There was a significant increase (twofold) in the G_1/S ratio in parent H35 cells 24 h after treatment with 8 Gy of gamma irradiation. In contrast, H35(F/F) cells did not arrest in the G_1 phase following exposure to the same dose of gamma irradiation, as there was no change in the G_1/S ratio. In addition to the effect on the G_1 checkpoint, we observed an even more pronounced effect on the G_2 checkpoint. Treatment of H35 cells with 8 Gy of gamma irradiation resulted in a significant increase (sevenfold) in the G_2/S ratio, while no such change from baseline was seen in TS-overexpressing H35(F/F) cells (data not shown).

DISCUSSION

In this study, we have characterized a specific interaction between TS protein and the mRNA of the p53 tumor suppressor gene. In initial RNA binding studies, we identified a 489-nt sequence within the protein-coding region of the p53 mRNA as being a *cis*-acting element required for protein recognition. The affinity of TS for this RNA sequence is on the same order of magnitude as that observed for a longer, more complete, 1,587-nt p53 mRNA sequence. Of note, DNA sequences corresponding to either the 1,587-nt p53 cDNA or the 489-nt p53 sequence were unable to compete for binding to TS protein, suggesting that sequence alone is insufficient for protein recognition by TS. This observation is in keeping with those for other well-described RNA binding proteins that appear to rely upon sequence within the context of a specific secondary structure or secondary structure alone for RNA binding. The specificity of this interaction is supported further by the inability of three distinct p53 RNA sequences, p53/1-400, p53/1021-1560, and p53/1321-1560, to interact with TS.

Earlier work had shown that wild-type human p53 mRNA is an approximately 2.8-kb species with a heterogeneous 5′ untranslated region (UTR) of 161 to 246 nts and a 3′ UTR of approximately 1,200 nts (42, 51, 52, 69, 70). While the p53 mRNA sequence investigated in the present study is only 1,587 nts long, our in vitro binding studies identified a 489-nt sequence within the protein-coding region as being one binding site for TS. In addition to the in vitro RNA binding experiments, an immunoprecipitation–RT-PCR method demonstrated that TS forms an in vivo RNP complex with p53 RNA in human colon cancer cells. The sequence of p53 RNA isolated by this technique was identical to that determined by the RNA binding studies. While it is conceivable that other sequences in the 5′ UTR RNA and/or the 3′ UTR RNA interact with TS, the 489-nt RNA sequence contained within the protein-coding region of p53 mRNA appears to be sufficient for high-affinity binding.

Our studies with the rabbit reticulocyte lysate in vitro translation system showed that the binding of TS results in the specific inhibition of p53 biosynthesis. Furthermore, the suppressive effect of TS on p53 expression is mediated by direct inhibition of p53 mRNA translation and not by enhanced degradation of p53 mRNA. Many of the initial studies characterizing the essential *cis*-acting elements required for protein recognition and translational initiation focused on sequences contained within the 5′ UTR (37, 40, 53). One issue raised by the present study relates to how the binding of TS to a sequence in the protein-coding region of p53 mRNA is able to repress translational initiation. The absence of truncated p53 protein products would suggest that the p53 mRNA-TS protein interaction either interferes with binding of the translational initiation factors near or at the cap site or inhibits the process of ribosomal scanning. For such events to occur, however, some type of interactive cross talk between the 5′ end of the mRNA and the protein-coding region must take place. Similar findings were observed with regard to the translational repression of c-*myc* mRNA mediated by the direct binding of TS to the C-terminal region of the c-*myc* transcript (11). While the precise molecular mechanism by which this process takes place remains to be elucidated, there is now growing evidence to suggest that sequences in both the protein-coding region and in the 3′ UTR may play active roles in the process of translational initiation (22, 32, 43, 55). Sophisticated molecular modeling studies will be required, however, to more precisely characterize the molecular events underlying this complex process.

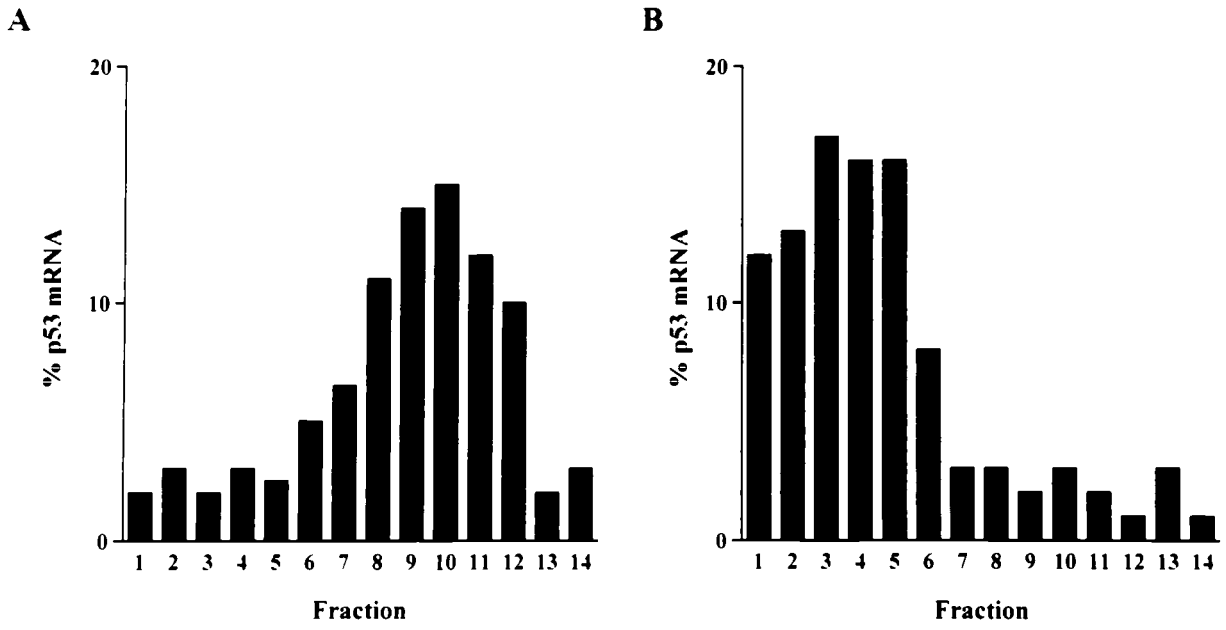

FIG. 9. Polysome analysis of p53 mRNA in rat hepatoma H35 (A) and H35(F/F) (B) cells. Cell extracts containing polysomes were prepared as described in Materials and Methods. Sample fractions were collected, and the level of p53 mRNA in each fraction was determined by dot blot hybridization analysis with a ^{32}P-radiolabeled human p53 cDNA probe. Fractions 1 to 5 correspond to free ribosomes and monosomes, and fractions 6 to 14 represent polysomes.

To begin to address the biological role for the interaction between TS and p53 mRNA, we examined the level of p53 expression in cells overexpressing TS. For these studies, two different experimental systems were used: a paired set of parent and resistant TS-overexpressing human colon cancer and rat hepatoma cell lines. In each system, a striking inverse relationship was observed between the levels of expression of TS and p53. In resistant human colon cancer H630-R10 and rat hepatoma H35(F/F) cell lines, which each overexpressed TS relative to their respective parent cell lines (30- to 40-fold-higher levels), no detectable levels of p53 were observed (Fig. 6 and 7). As further support for the potential role of TS in controlling p53 expression, revertant H630-R10rev cells were established by maintaining H630-R10 cells in the absence of the selective pressure of the fluoropyrimidine 5-FU. The level of TS in these revertant cells had decreased to a level nearly identical to that observed in parent H630 cells (Fig. 6E). In contrast to the results for H630-R10 cells, in which there was undetectable staining for p53, the expression of p53 in H630-R10rev cells was restored to a level nearly identical to that observed in parent H630 cells (Fig. 6E). There was no evidence of gross alterations in the p53 gene in either human colon cancer or rat hepatoma cell lines, as evidenced by identical levels of p53 mRNA expression and by identical levels of in vitro-translatable p53 mRNA isolated from each parent and TS-overexpressing cell line. However, the level of p53 RNA

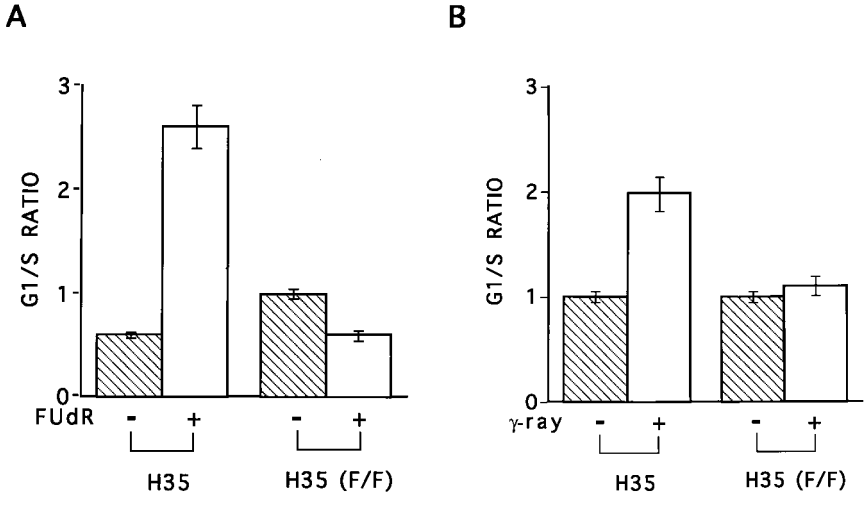

FIG. 10. Measurement of G_1-S arrest following exposure to FUdR (A) and gamma irradiation (B) in rat hepatoma H35 and H35(F/F) cells. G_1/S ratios for rat hepatoma cells were determined in the absence and presence of FUdR as indicated. H35 cells were treated for 8 h with 10 nM FUdR, while H35(F/F) cells were treated with 100 nM FUdR. Cells were exposed to 8 Gy of gamma irradiation and harvested after 24 h. Cell cycle distribution was quantitated by flow cytometry as described in Materials and Methods.

complexed to TS in each of the TS-overexpressing cell lines was significantly increased compared to that in the parent cell lines.

Additional experiments showed that neither human recombinant TS protein nor whole-cell extracts from either human colon cancer H630-R10 cells or rat hepatoma H35(F/F) cells promoted the degradation of p53 protein, suggesting that alterations in protein stability are unable to account for the decreased levels of p53 protein in TS-overexpressing cells (data not shown). In the paired human H630 and rat H35 cell lines, the overexpression of TS resulted from chronic exposure of the parent cells to either 5-FU or FUdR. One potential confounding feature in the TS-overexpressing cell lines relates to whether the effect of TS on p53 expression is mediated through a direct interaction or whether drug treatment may affect the expression of some other gene that is the true regulator of p53 gene expression. For this reason, the effect of overexpression of TS on p53 expression was investigated with a tetracycline-inducible expression system. Upon exposure of transfected RKO cells to doxycycline, the induced expression of TS protein significantly inhibited the expression of p53 protein but did not alter the levels of p53 mRNA. Finally, comparison of the polysome profiles of rat hepatoma H35 cells and TS-overexpressing H35(F/F) cells, which contained identical levels of p53 mRNA, revealed that a significantly higher proportion of the p53 mRNA was associated with higher-molecular-weight polysomes in parent H35 cells than in TS-overexpressing H35(F/F) cells. Taken together, these experimental findings suggest that the synthesis of p53 in cells overexpressing TS is regulated at the translational level and mediated by direct binding of TS to p53 mRNA.

Previous studies demonstrated that the expression and function of p53 can be controlled by several different pathways (24, 26, 30, 38, 47, 67). The most commonly reported change observed in p53 in human malignancies is a point mutation within the coding sequence of the p53 gene that gives rise to an altered protein product. In addition, deletions, structural rearrangements, and genomic insertions have also been described. The function of p53 may be inactivated by the formation of protein complexes either with viral proteins, such as simian virus 40 large T antigen, adenovirus E1B protein, or papillomavirus E6 protein, or with cellular proteins, such as the Mdm-2 transcription factor. In addition, it has been shown that the biosynthesis of wild-type p53 can be controlled by both transcriptional (17, 31) and translational (18, 21, 54) regulatory processes. While the precise mechanism by which transcriptional autoregulation is mediated remains to be elucidated, this effect appears to be cell type specific and to involve the binding of p53 to other transcription factors. The inhibition of p53 biosynthesis at the translational level requires wild-type p53 and presumably arises through a negative autoregulatory feedback loop process (21, 54). While it has been shown that mouse p53 protein can bind directly to its corresponding mouse p53 mRNA, we were unable to document a similar interaction between human recombinant p53 protein and its own p53 mRNA (data not shown). Of note, the binding site on the mouse p53 mRNA was previously localized to a 216-nt sequence within the 5′ UTR. As the 5′ UTR of the human p53 mRNA used in our RNA binding experiments was only 136 nts long, it is conceivable that this shorter sequence did not contain the required elements for binding by the p53 protein. Nevertheless, in the present study, we identified a novel mechanism for regulating the expression of p53 whereby p53 biosynthesis is regulated through the binding of TS protein to the p53 mRNA, with resultant translational repression.

TS and p53 are proteins involved in cellular growth and DNA biosynthesis. Despite the undetectable expression of p53 protein, TS-overexpressing cells do not display any significant changes relative to their parent cells in either cellular growth rate and/or level of DNA biosynthesis, as determined by cell doubling time and thymidine incorporation. Wild-type p53 plays a pivotal role in inducing G_1 cell cycle arrest and/or initiating a pathway of programed cell death following exposure to DNA-damaging antineoplastic agents or ionizing radiation (reviewed in references 38 and 47). There is now recent evidence to suggest that a functional p53 pathway is also critical for the G_2 checkpoint (reviewed in references 38 and 47). Our studies demonstrated that drug-resistant, TS-overexpressing rat hepatoma H35(F/F) cells that express wild-type p53 mRNA but that do not express wild-type p53 protein fail to arrest in G_1 following exposure to DNA-damaging agents, such as the fluoropyrimidine FUdR and gamma irradiation. This defect in the ability to activate the G_1 checkpoint may not allow for the program of cell death to be triggered and thereby may render these cells more resistant to the cytotoxic effects of a given drug. Moreover, it is interesting to note that in response to either a fluoropyrimidine or gamma irradiation, activation of the G_2 checkpoint was significantly impaired in cells expressing high levels of TS in this study. These studies suggest that TS may control p53-dependent G_1 and G_2 checkpoints.

Recent studies by Linke et al. (48) showed that ribonucleotide depletion in the absence of detectable DNA damage in response to treatment with the antimetabolites *N*-phosphonoacetyl-L-aspartic acid and pyrazofurin results in G_1 cell cycle arrest. This work suggests that p53 is able to function as a critical sensor that is activated upon depletion of ribonucleotides, products, and/or processes dependent upon ribonucleotides (48). Moreover, this same group observed that inhibitors of deoxynucleoside triphosphate biosynthesis, such as methotrexate and the fluoropyrimidine 5-FU, resulted in p53-independent accumulation of cells in the early S phase, with no significant G_1 arrest. These results would appear to be in direct contrast to those reported here, since we observed effective G_1 arrest following fluoropyrimidine exposure in H35 cells expressing functional p53. Experiments are currently in progress to more carefully elucidate the complex relationship among TS, p53, and cell cycle function in this experimental system and to confirm that the G_1 and G_2 checkpoints are, in fact, p53 dependent. In addition, it will be important to document the critical downstream events that mediate the checkpoint functions. Our preliminary evidence suggests that treatment of parent H35 cells with FUdR results in a significant increase (five- to sixfold) in the expression of both p53 and p21 proteins, a finding consistent with the transcriptional activation of p21 by p53. In contrast, the drug-mediated induction of p53 and p21 is significantly blunted in TS-overexpressing H35(F/F) cells. Thus, the ability of TS to abrogate the usual induced expression of p53 and p21 in response to cytotoxic stress offers a potential mechanism for alteration of these critical cell-cycle-associated events. Further studies are ongoing in our laboratory to identify the other key downstream events resulting from the translational regulation of p53 by TS.

Our work demonstrates that TS specifically interacts with its own mRNA as well as with the mRNAs of c-*myc* and p53. There is a precedent for regulatory processes being mediated by a single RNA binding protein. Several groups have shown that the binding of the iron-responsive factor to its target iron-responsive element located in the UTRs of three different mRNAs, ferritin (29), transferrin receptor (2), and erythroid-5-aminolevulinate synthase (53), regulates the expression of each of these iron-associated proteins. Moreover, recent investigations with an in vitro RNA selection approach revealed

that the iron-responsive factor can bind with a high affinity to a number of RNAs with sequences quite different from that of its own wild-type target iron-responsive element. Secondary structure analysis showed that these variant sequences had the potential to form alternative RNA secondary structures (28). Although our own studies have yet to identify a consensus nucleotide sequence contained within the mRNAs of TS, c-*myc*, and p53, one potential drawback to such a "consensus" approach is that the majority of the RNA binding proteins characterized to date appear to recognize a combination of structure and sequence (65). We have observed that the binding of TS to its own 5′ upstream target RNA sequence is dependent upon the presence of a stable stem-loop structure (6). Given that the 5′ upstream binding site for TS appears to be characteristic of other RNA *cis*-acting elements, identification of a consensus RNA binding site by simple sequence analysis may be inadequate. Moreover, except for the 5′ upstream binding site whose sequence has been localized to a 36-nt element, the target sequences on the mRNAs of both c-*myc* and p53 remain rather large (165 and 489 nts, respectively). Additional studies are required to more narrowly define the binding sites on each of these mRNAs. Once sequences on the order of 25 to 30 nts are defined, a combination of mutational, deletional, and secondary structure analyses may then be performed to help identify a consensus TS-responsive sequence(s) and/or secondary structure required for protein recognition and for the regulated expression of the respective genes.

In conclusion, our studies have identified a novel molecular mechanism by which the expression of p53 is regulated in vitro and in vivo. These findings suggest that the intracellular level of TS may represent a critical determinant of p53 expression. Further studies are necessary to more completely characterize the molecular basis for the TS protein-p53 mRNA interaction. One issue that may shed further light is the intracellular location of TS and TS-RNP complexes. Using an immunogold electron microscopy method, Samsonoff et al. (60) recently observed that TS was mainly associated with the nucleolar region of parent H35 cells. In contrast, the protein was located primarily in the cytoplasm of TS-overexpressing H35(F/F) cells. Based on the experimental findings reported here, it is interesting to speculate whether the biologically active TS protein-p53 mRNA complex is localized within the nucleolus of the cell. Studies are now under way in our laboratory to address this point.

It will also be important to further define the biological role for this specific RNA-protein interaction. Our initial studies suggested that this interaction may determine the cell cycle response of cells following exposure to DNA-damaging agents. Previous work showed that malignant tumors express higher levels of TS than the corresponding normal tissue from which they are derived (34). It is conceivable that the ability of TS to suppress p53 expression may represent an additional mechanism for malignant cells to ensure their transformed phenotype. Studies are now in progress to characterize the tumorigenic potential of cell lines expressing different levels of TS and wild-type p53. Finally, it has been suggested that the loss of p53 function would result in the development of resistance to chemotherapy, presumably due to the inability to induce G_1 arrest and/or the process of apoptosis (49). Our preliminary studies suggest that TS-overexpressing H35(F/F) cells may indeed be resistant to a number of different anticancer agents. Thus, this work expands our current concept of the potential role of TS as a regulatory RNA binding protein and offers new insight into the molecular regulatory mechanisms controlling p53 expression.

ACKNOWLEDGMENTS

We thank Kristen Carey for technical assistance; Bruce Chabner, Leslie Parsels, and John Schmitz for helpful discussions and critical reading of the manuscript; and Janet Edds and Edna McCarthy for editorial preparation of the manuscript. We also thank Alan Hinnebusch and Matthew Melton for advice and input regarding the polysome experiments.

This work was supported in part by grants from the National Science Foundation (MCB93-16321 to G.F.M.), the National Cancer Institute (CA44355 to F.M. and CA16359 and CA75712 to E.C.), and the Department of Veteran Affairs (VA Merit Review to E.C.).

REFERENCES

1. **Caelles, C., A. Heimberg, and M. Karin.** 1994. p53-dependent apoptosis in the absence of transcriptional activation of p53-target genes. Nature **370:** 220–223.
2. **Casey, J. L., M. W. Hentze, D. M. Koeller, S. W. Caughman, T. A. Rouault, R. D. Klausner, and J. B. Harford.** 1988. Iron-responsive elements: regulatory RNA sequences that control mRNA levels and translation. Science **240:**924–928.
3. **Chiba, I., T. Takahashi, M. M. Nau, D. D'Amico, D. T. Curiel, T. Mitsudomi, D. L. Buchhagen, D. Carbone, S. Piantadosi, H. Koga, P. T. Reissman, D. J. Slamon, E. C. Holmes, and J. D. Minna.** 1990. Mutations in the p53 gene are frequent in primary, resected non-small cell lung cancer. Oncogene **5:**1603–1610.
4. **Chomcyznski, P., and N. Sacchi.** 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate phenol-chloroform extraction. Anal. Biochem. **162:**156–159.
5. **Chu, E., D. M. Koeller, J. L. Casey, J. C. Drake, B. A. Chabner, P. C. Elwood, S. Zinn, and C. J. Allegra.** 1991. Autoregulation of human thymidylate synthase messenger RNA translation by thymidylate synthase. Proc. Natl. Acad. Sci. USA **88:**8977–8981.
6. **Chu, E., D. M. Voeller, D. M. Koeller, J. C. Drake, C. H. Takimoto, F. Maley, G. F. Maley, and C. J. Allegra.** 1993. Identification of an RNA binding site for human thymidylate synthase. Proc. Natl. Acad. Sci. USA **90:**517–521.
7. **Chu, E., D. M. Voeller, P. F. Morrison, K. L. Jones, T. Takechi, G. F. Maley, F. Maley, and C. J. Allegra.** 1994. The effect of reducing reagents on binding of thymidylate synthase protein to thymidylate synthase messenger RNA. J. Biol. Chem. **269:**20289–20293.
8. **Chu, E., S. Zinn, D. Boarman, and C. J. Allegra.** 1990. The interaction of gamma interferon and 5-fluorouracil in the H630 human colon carcinoma cell line. Cancer Res. **50:**5834–5840.
9. **Chu, E., D. M. Koeller, P. C. Johnston, S. Zinn, and C. J. Allegra.** 1993. Regulation of thymidylate synthase in human colon cancer cells treated with 5-fluorouracil and interferon-gamma. Mol. Pharmacol. **43:**527–533.
10. **Chu, E., D. M. Voeller, K. L. Jones, T. Takechi, G. F. Maley, F. Maley, S. Segal, and C. J. Allegra.** 1994. Identification of a thymidylate synthase ribonucleoprotein complex in human colon cancer cells. Mol. Cell. Biol. **14:**207–213.
11. **Chu, E., T. Takechi, K. L. Jones, D. M. Voeller, S. M. Copur, G. F. Maley, S. Segal, and C. J. Allegra.** 1995. Thymidylate synthase protein binds to c-*myc* RNA in human colon cancer cells and in vitro. Mol. Cell. Biol. **15:**179–185.
12. **Chu, E., C. H. Takimoto, D. Voeller, J. L. Grem, and C. J. Allegra.** 1993. Specific binding of human dihydrofolate reductase protein to dihydrofolate reductase messenger RNA in vitro. Biochemistry **32:**4756–4760.
13. **Chu, E., T. Cogliati, S. M. Copur, A. Borre, D. M. Voeller, C. J. Allegra, and S. Segal.** 1996. Identification of in vivo target RNA sequences bound by thymidylate synthase. Nucleic Acids Res. **24:**3222–3228.
14. **Ciesla, J., K. X. B. Weiner, R. S. Weiner, J. T. Reston, G. F. Maley, and F. Maley.** 1995. Isolation and expression of rat thymidylate synthase cDNA. Phylogenetic comparison with human and mouse thymidylate synthases. Biochim. Biophys. Acta **1261:**233–242.
15. **Dandekar, T., R. Stripecke, N. K. Gray, B. Goossen, A. Constable, H. E. Johansson, and M. W. Hentze.** 1991. Identification of a novel iron-responsive element in murine and human erythroid 8-aminolevulinic acid synthase mRNA. EMBO J. **10:**1903–1909.
16. **Davisson, V. J., W. Sirawaraporn, and D. V. Santi.** 1989. Expression of human thymidylate synthase in *Escherichia coli*. J. Biol. Chem. **264:**9145–9148.
17. **Deffie, A., H. Wu, V. Reinke, and G. Lozano.** 1993. The tumor suppressor p53 regulates its own transcription. Mol. Cell. Biol. **13:**3415–3423.
18. **Ewen, M. E., and S. J. Miller.** 1996. p53 and translational control. Biochim. Biophys. Acta **1242:**181–184.
19. **Feinberg, A. P., and B. Vogelstein.** 1984. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. **137:**266–267.
20. **Fried, J., A. G. Perez, and V. D. Clarkson.** 1978. Rapid hypotonic method for flow cytofluorometry of monolayer cell culture. J. Histochem. Cytochem. **26:**921–926.

21. **Fu, L., M. D. Minden, and S. Benchimol.** 1996. Translational regulation of human p53 gene expression. EMBO J. **15:**4392–4401.
22. **Gallie, D. R.** 1991. The cap and poly (A) tail function synergistically to regulate mRNA translational efficiency. Genes Dev. **5:**2108–2116.
23. **Gossen, M., S. Freundlieb, G. Bender, G. Muller, W. Hillen, and H. Bujard.** 1995. Transcriptional activation by tetracyclines in mammalian cells. Science **268:**1766–1769.
24. **Greenblatt, M. S., W. P. Bennett, M. Hollstein, and C. C. Harris.** 1994. Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. Cancer Res. **54:**4855–4878.
25. **Hardy, L. W., J. S. Finer-Moore, W. R. Montfort, M. O. Jones, D. V. Santi, and R. M. Stroud.** 1987. Atomic structure of thymidylate synthase: target for rational drug design. Science **235:**448–455.
26. **Harris, C. C., and M. Hollstein.** 1993. Clinical implications of the p53 tumor-suppressor gene. N. Engl. J. Med. **329:**1318–1327.
27. **Helman, L. J., T. G. Ahn, M. A. Levine, A. Allison, P. S. Cohen, M. J. Cooper, D. V. Cohn, and M. A. Israel.** 1988. Molecular cloning and primary structure of human chromogranin A (secretory protein 1) cDNA. J. Biol. Chem. **263:**11559–11563.
28. **Henderson, B. R., E. Menotti, C. Bonnard, and L. C. Kuhn.** 1994. Optimal sequence and structure of iron-responsive elements. J. Biol. Chem. **269:** 17481–17489.
29. **Hentze, M. W., S. W. Caughman, T. A. Rouault, J. G. Barriocanal, A. Dancis, J. B. Harford, and R. D. Klausner.** 1987. Identification of the iron-responsive element for the translational regulation of human ferritin mRNA. Science **238:**1570–1573.
30. **Hollstein, M., D. Sidransky, B. Vogelstein, and C. C. Harris.** 1991. p53 mutations in human cancers. Science **253:**49–53.
31. **Hudson, J. M., R. Frade, and M. Bar-Eli.** 1995. Wild-type p53 regulates its own transcription in a cell-type specific manner. DNA Cell Biol. **14:**759–766.
32. **Jackson, R. J., and N. Standard.** 1990. Do the poly(A) tail and 3′ untranslated region control mRNA translation? Cell **62:**15–24.
33. **Jackson, R. J.** 1993. Cytoplasmic regulation of mRNA function: the importance of the 3′ untranslated region. Cell **74:**9–14.
34. **Johnston, P. G., C. M. Liang, S. Henry, B. A. Chabner, and C. J. Allegra.** 1991. Production and characterization of monoclonal antibodies that localize human thymidylate synthase in the cytoplasm of human cells and tissue. Cancer Res. **51:**6668–6676.
35. **Kastan, M. B., O. Onyekwere, D. Sidransky, B. Vogelstein, and R. W. Craig.** 1991. Participation of p53 protein in the cellular response to DNA damage. Cancer Res. **51:**6304–6311.
36. **Keyomarsi, K., J. Samet, S. Gyongyi, G. Molnar, and A. B. Pardee.** 1993. The thymidylate synthase inhibitor, ICI D1694, overcomes translational detainment of the enzyme. J. Biol. Chem. **268:**15142–15149.
37. **Klausner, R. D., T. A. Rouault, and J. B. Harford.** 1993. Regulating the fate of mRNA: the control of cellular iron metabolism. Cell **72:**19–28.
38. **Ko, L. J., and C. Prives.** 1996. p53: puzzle and paradigm. Genes Dev. **10:**1054–1072.
39. **Konarska, M. M., and P. A. Sharp.** 1986. Electrophoretic separation of complexes involved in the splicing of precursors to mRNAs. Cell **476:**845–855.
40. **Kozak, M.** 1991. Structural features in eukaryotic mRNAs that modulate the initiation of translation. J. Biol. Chem. **266:**19867–19870.
41. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
42. **Lamb, P., and L. Crawford.** 1986. Characterization of the human p53 gene. Mol. Cell. Biol. **6:**1379–1385.
43. **Leathers, V., R. Tanguay, M. Kobayashi, and D. R. Gallie.** 1993. A phylogenetically conserved sequence within viral 3′ untranslated RNA pseudoknots regulates translation. Mol. Cell. Biol. **13:**5331–5347.
44. **Leibold, E. A., and H. N. Munro.** 1988. Cytoplasmic protein binds in vitro to a highly conserved sequence in the 5′ untranslated region of ferritin heavy- and light-subunit mRNAs. Proc. Natl. Acad. Sci. USA **85:**2171–2175.
45. **Lerner, M. R., and J. A. Steitz.** 1979. Antibodies to small nuclear RNAs complexed with proteins are produced by patients with systemic lupus erythematosus. Proc. Natl. Acad. Sci. USA **76:**5495–5499.
46. **Levine, A. J., J. Momand, and C. A. Finlay.** 1991. The p53 tumour suppressor gene. Nature **351:**453–456.
47. **Levine, A. J.** 1997. p53, the cellular gatekeeper for growth and division. Cell **88:**323–331.
48. **Linke, S. P., K. C. Clarkin, A. DiLeonardo, A. Tsou, and G. M. Wahl.** 1996. A reversible, p53-dependent G0/G1 cell cycle arrest induced by ribonucleotide depletion in the absence of detectable DNA damage. Genes Dev. **10:**934–947.
49. **Lowe, S. W., H. E. Ruley, T. Jacks, and D. E. Houseman.** 1993. p53-dependent apoptosis modulates the cytotoxicity of anticancer agents. Cell **74:**957–968.
50. **Maltzman, W., and L. Czyzyk.** 1984. UV irradiation stimulates levels of p53 cellular tumor antigen in nontransformed mouse cells. Mol. Cell. Biol. **4:** 1689–1694.
51. **Matlashewski, G., P. Lamb, J. Pim, J. Peacock, L. Crawford, and S. Benchimol.** 1984. Isolation and characterisation of a human p53 cDNA clone: expression of the human p53 gene. EMBO J. **3:**3257–3262.
52. **Matlashewski, G. J., S. Tuck, D. Pim, P. Lamb, J. Schneider, and L. V. Crawford.** 1987. Primary structure polymorphism at amino acid residue 72 of human p53. Mol. Cell. Biol. **7:**961–963.
53. **Melefors, O., and M. W. Hentze.** 1993. Translational regulation by mRNA/protein interactions in eukaryotic cells: ferritin and beyond. Bioessays **15:** 85–90.
54. **Mosner, J., T. Mummenbrauer, C. Bauer, G. Sczakiel, F. Grosse, and W. Deppert.** 1995. Negative feedback regulation of wild-type p53 biosynthesis. EMBO J. **14:**4442–4449.
55. **Ostareck-Lederer, A. D., D. H. Ostareck, N. Standard, and B. J. Thiele.** 1994. Translation of 15-lipooxygenase mRNA is inhibited by a protein that binds to a repeated sequence in the 3′ untranslated region. EMBO J. **13:** 1476–1481.
56. **Park, J. G., H. K. Oie, P. H. Sugarbaker, J. G. Henslee, T. R. Chen, B. E. Johnson, and A. Gazdar.** 1987. Characteristics of cell lines established from human colorectal carcinoma. Cancer Res. **47:**6710–6718.
57. **Ramirez, M., R. C. Wek, and A. G. Hinnebusch.** 1991. Ribosome association of GCN2 protein kinase, a translational activator of the *GCN4* gene of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **11:**3027–3036.
58. **Rhee, M. S., M. Balinska, M. Bunni, D. G. Priest, G. F. Maley, and F. Maley.** 1990. Role of substrate depletion in the inhibition of thymidylate biosynthesis by the dihydrofolate reductase inhibitor trimetrexate in cultured hepatoma cells. Cancer Res. **50:**3979–3984.
59. **Rode, W., B. L. Dolnick, and J. R. Bertino.** 1980. Isolation of a homogeneous preparation of human thymidylate synthetase from HeLa cells. Biochem. Pharmacol. **29:**723–726.
60. **Samsonoff, W. A., J. Reston, M. McKee, B. O'Connor, J. Galivan, G. Maley, and F. Maley.** 1997. Intracellular location of thymidylate synthase and its state of phosphorylation. J. Biol. Chem. **272:**13281–13285.

60a. **Santi, D. V., and P. V. Danenberg.** 1984. Folates in pyrimidine nucleotide biosynthesis, p. 345–398. *In* R. L. Blakely and S. J. Benkovic (ed.), Folates and pteridines, vol. 1. John Wiley & Sons, Inc., New York, N.Y.

61. **Scatchard, G.** 1949. The attraction of proteins for small molecules and ions. Ann. N. Y. Acad. Sci. **51:**660–692.
62. **Steitz, J. A.** 1989. Immunoprecipitation of ribonucleoproteins using autoantibodies. Methods Enzymol. **180:**468–481.
63. **Takahashi, T., M. M. Nau, I. Chiba, M. J. Birrer, R. K. Rosenberg, M. Vinocour, M. Levitt, H. Pass, A. F. Gazdar, and J. D. Minna.** 1989. p53: a frequent target for genetic abnormalities in lung cancer. Science **246:**491–494.
64. **Theodorakis, N. G., and R. I. Morimoto.** 1987. Posttranscriptional regulation of *hsp70* expression in human cells: effects of heat shock, inhibition of protein synthesis, and adenovirus infection on translation and mRNA stability. Mol. Cell. Biol. **7:**4357–4368.
65. **Uhlenbeck, O. C., H.-N. Wu, and J. R. Sampson.** 1987. Recognition of RNA by proteins, p. 285–294. *In* M. Inouye and B. S. Dudock (ed.), Molecular biology of RNA. Academic Press, Inc., New York, N.Y.
66. **Ullrich, S. J., C. W. Anderson, W. E. Mercer, and E. J. Appela.** 1992. The p53 tumor suppressor protein, a modulator of cell proliferation. J. Biol. Chem. **267:**15259–15262.
67. **Vogelstein, B., and K. W. Kinzler.** 1992. p53 function and dysfunction. Cell **70:**523–526.
68. **Weiner, K. X. B., R. S. Weiner, F. Maley, and G. F. Maley.** 1993. Primary structure of human deoxycytidylate deaminase and overexpression of its functional protein in *Escherichia coli*. J. Biol. Chem. **168:**12983–12989.
69. **Wolf, D., and V. Rotter.** 1985. Major deletions in the gene encoding the p53 tumor antigen cause lack of p53 expression in HL-60 cells. Proc. Natl. Acad. Sci. USA **82:**790–794.
70. **Wolf, D. I., Z. Laver-Rudich, and V. Rotter.** 1985. In vitro expression of human p53 cDNA clones and characterization of the cloned human p53 gene. Mol. Cell. Biol. **5:**1887–1893.
71. **Zhan, Q., F. Carrier, and A. J. Fornace, Jr.** 1993. Induction of cellular p53 activity by DNA-damaging agents and growth arrest. Mol. Cell. Biol. **13:** 4242–4250.