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A Novel Single Nucleotide Polymorphism within the 5′ Tandem Repeat Polymorphism of the *Thymidylate Synthase* Gene Abolishes USF-1 Binding and Alters Transcriptional Activity

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ABSTRACT

Thymidylate synthase (*TS*) gene expression is modulated by a polymorphism in the 5′ regulatory region of the gene. The polymorphism consists mainly of either two repeats (*2R*) or three repeats (*3R*) of a 28-bp sequence, yielding greater TS gene expression and protein levels with a *3R* genotype. Two USF family E-box consensus elements are found within the tandem repeats of the *3R* genotype, and one is found within the *2R* genotype. These elements bind USF proteins *in vitro* by electrophoretic mobility shift analysis and *in vivo* by chromatin immunoprecipitation assay. We show that the additional USF consensus element within the *3R* construct confers greater transcriptional activity relative to the *2R* construct. Mutagenesis of the USF sites shows that the transcriptional regulation of TS is dependent, in part, on USF proteins binding within the tandem repeats. In addition, we identified a novel G→C single nucleotide polymorphism in the second repeat of *3R* alleles within the USF consensus element that alters the ability of USF proteins to bind and thus alters the transcriptional activation of TS gene constructs bearing this genotype. Through RFLP analysis, we determined the respective frequencies of the C allele (*3RC*) among all *3R* alleles in non-Hispanic whites, Hispanic whites, African Americans, and Singapore Chinese to be 56%, 47%, 28%, and 37%, respectively. Based on our findings, this novel single nucleotide polymorphism should be considered when the 5′ tandem repeat polymorphism is being used as a predictor of clinical outcome to TS inhibitors.

INTRODUCTION

TS[2] is a key enzyme in the nucleotide biosynthetic pathway that methylates dUMP to produce dTMP. The TS reaction is the sole *de novo* source of thymidylate in the cell and is essential for DNA replication (1–3). The critical role of TS in nucleotide metabolism has made it an important target for a variety of chemotherapeutic agents including 5-FU, capecitabine (Xeloda), and raltitrexed (Tomudex) (4, 5). Inhibition of TS by these agents causes cytotoxicity by dTTP pool depletion, leading to thymineless death (6) and, in some instances, chronic uracil misincorporation into DNA (7, 8), which leads to strand breaks initiated by uracil-DNA-glycosylase. Limited efficacy of TS inhibitors in the treatment of human cancers has been a common phenomenon. Resistance to fluoropyrimidines arises through a variety of mechanisms, including elevated TS protein expression resulting from increases in *TS* transcription (9) and translation (10, 11).

A polymorphism within the 5′-untranslated region of the *TS* gene, consisting of tandem repeats of 28 bp, has been implicated in modulating TS mRNA expression (10, 12) and TS mRNA translational efficiency (13). Although there have been reports of four, five, and nine repeats within certain African and Asian populations (14–16), the vast majority of individual human *TS* alleles harbor either a double repeat (*2R*) or a triple repeat (*3R*) for this polymorphism, creating genotypes of *2R/2R*, *2R/3R*, and *3R/3R*. Individuals that are homozygous for the *3R* were found to have elevated intratumoral TS mRNA (17) and protein levels compared with *2R* homozygotes (18). In addition, the 5′ tandem repeat polymorphism of the *TS* gene has been identified as a predictor of clinical outcome to 5-FU-based chemotherapy in both adjuvant and metastatic settings (17, 19–21). Furthermore, the tandem repeats have been shown to predict plasma folate and homocysteine levels (22), risk of colorectal adenomas (23), and risk and outcome of acute lymphoblastic leukemia (24, 25).

The molecular mechanisms by which the tandem repeat polymorphism enhances transcription have not yet been elucidated. Furthermore, differences in the nucleotide sequences of the repeats have not been considered up to this point as playing a functional role in transcription and posttranscriptional events. In this study, we sought to identify the regulatory factor(s) responsible for binding within the polymorphic region and enhancing TS mRNA expression.

Here, we identify USF-1 and USF-2 as factors that bind within the tandem repeat polymorphism of the *TS* 5′ regulatory region. We show that USF-1 enhances transcription of TS reporter gene constructs in a luciferase assay system. We also identify a novel SNP within the tandem repeats that determines the binding and transactivating ability of USF complexes, and we demonstrate that it is a common polymorphism in non-Hispanic whites (whites), Hispanic whites (Hispanics), African Americans, and Singapore Chinese. Our data suggest that the impact of a *3R* genotype on TS transcriptional activation may ultimately be related to the presence or absence of the USF binding sites and the G→C SNP in the second repeat of *3R* alleles.

MATERIALS AND METHODS

Recombinant Protein Expression and Phosphorylation. cDNA encoding USF-1 (26) was amplified from 34Lu human lung fibroblast cDNA (upper primer 5′-CGGGATCCATGAAGGGGCAGCAGAAAACAG-3′ and lower primer 5′-GCTCTAGATTAGTTGCTGTCATTCTTGATGACGA-3′, add *Bam*HI and *Xba*I restriction sites, respectively), and the PCR was carried out under the following conditions using Accuzyme DNA polymerase (Bioline; Denville Scientific): 30 cycles of 30 s at 94°C; 30 s at 59.3°C; and 45 s at 72°C. The product was digested and cloned in-frame into the pProEX-HTb vector (Invitrogen), which adds a 6-histidine tag to the NH_2 terminus of the expressed protein. The plasmid was transformed into the DH5α strain of *Escherichia coli* (Invitrogen), and protein expression was induced by adding isopropyl-1-thio-β-D-galactopyranoside to a final concentration of 0.6 mM to the culture. After induction, the cells were centrifuged at 10,000 × *g* for 10 min and resuspended in 4 volumes of lysis buffer [20 mM Tris-HCl (pH 8.5 at 4°C), 100 mM KCl, 5 mM 2-mercaptoethanol, and 1 mM phenylmethylsulfonyl fluoride]. Cells were lysed in a French press, and cell debris was removed by centrifugation.

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[2] The abbreviations used are: TS, thymidylate synthase; SNP, single nucleotide polymorphism; CHIP, chromatin immunoprecipitation; EMSA, electrophoretic mobility shift analysis; 5-FU, 5-fluorouracil; Ni-NTA, nickel-nitrilotriacetic acid; poly(dI·dC), poly-(deoxyinosinic-deoxycytidylic acid); TBE, Tris boric acid-ethylenediamine tetra acetic acid; RFLP, restriction fragment length polymorphism.

Supernatant was run on a Ni-NTA resin column following the pProEX-HT Prokaryotic Expression System protocol (Invitrogen) to isolate recombinant 6-histidine-tagged USF-1. To activate the DNA binding ability of USF-1, the recombinant protein was phosphorylated *in vitro* using cdc2/p34. cdc2/p34 was isolated by immunoprecipitation using mouse monoclonal antibodies (sc-54; Santa Cruz Biotechnology). An *in vitro* phosphorylation reaction was carried out by adding 6 μl of 5× cdc2 kinase buffer [1 M Tris-HCl (pH 7.5), 1 M $MgCl_2$, and 1 M DTT], 1 μl of 1 mM ATP/1 mM $MgCl_2$ (1 mM [γ-^{32}P]ATP/1 mM $MgCl_2$ for visualization of phosphorylation), 200 ng of recombinant USF-1, and 8 μl of H_2O to 15 μl of the protein A-Sepharose beads bound with cdc2/p34. The reaction was carried out for 20 min at 30°C and then separated by 12.5% SDS-PAGE. The gel was dried and placed in a cartridge with Kodak Biomax Maximum Sensitivity film for visualization of [γ-^{32}P]ATP incorporation. USF-2 cDNA (27) was amplified from 34Lu cDNA (upper primer 5′-CCGGAATTCCATGCCATGGACATGCTGGACCC-3′ and lower primer 5′-GCTCTAGACATGTGTCCCTCTCTGTGCTAAGG -3′, add *Eco*RI and *Xba*I restriction sites, respectively), and PCR was carried out under the following conditions using Accuzyme DNA polymerase (Bioline; Denville Scientific): 30 cycles of 30 s at 94°C; 30 s at 62°C; and 45 s at 72°C. The USF-1 and USF-2 cDNAs were cloned into the pCI-neo plasmid vector (Promega) for expression in transient transfection experiments.

EMSA. Synthetic double-stranded oligonucleotides (Integrated DNA Technologies) corresponding to a 3RG or 3RC 28-bp tandem repeat sequence from the *TS* 5′ regulatory region were labeled with [γ-^{32}P]ATP (Amersham Pharmacia Biotech) according to the Gel-Shift Assay Kit protocol (Promega). For each gel shift reaction, 10,000 cpm of labeled probe were incubated with ~30 ng of recombinant USF-1 for 20 min at room temperature in a 20-μl reaction mixture containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 0.5 mM DTT, 0.5 mM EDTA, 4% glycerol, 1 mM $MgCl_2$, and 0.1 μg of poly(dI·dC) DNA. Where indicated, unlabeled competitor oligonucleotides were incubated for 10 min at room temperature with USF-1 before the addition of labeled probe. Samples were loaded onto a nondenaturing 4% acrylamide gel and electrophoresed in 0.5× TBE buffer at 350 V at 4°C. The gels were dried and visualized by autoradiography using Kodak BioMax Maximum Resolution Film. Sequences of the oligonucleotides were as follows: TS-3RG, 5′-CCGCGCCACTT***g***GCCTGCCTCCGTCCCG-3′; and TS-3RC, 5′-CCGCGC-CACTT***c***GCCTGCCTCCGTCCCG-3′; poly(dI·dC) (Sigma) was used as a nonspecific competitor. The reverse compliment of each oligonucleotide was synthesized and annealed with its counterpart to create duplex DNA oligonucleotides. Two hundred and fifty μl of each oligonucleotide (1.75 pmol/μl) in a 500-μl reaction volume were annealed at 95°C for 5 min and allowed to cool slowly to room temperature for 2–3 h.

CHIP Assay. CHIP from 293 cells was carried out using the CHIP assay kit (Upstate Biotechnology) according to the manufacturer's protocol. Briefly, 1×10^6 cells were plated in 10-cm dishes and incubated overnight at 37°C. The cross-linking of protein to DNA was carried out by adding 37% formaldehyde to the growth medium at a final concentration of 1% for 10 min at 37°C. Cells were washed in ice-cold PBS containing protease inhibitors (Protease inhibitor mixture set III; Calbiochem) and scraped into conical screw-cap tubes. Cells were centrifuged and resuspended in SDS lysis buffer and then sonicated three times for 10 s at full power on ice, using a Branson 450 sonifier, to shear DNA to 200-1000-bp fragments. Samples were centrifuged, and 200 μl of sonicated cell supernatant were diluted into 1800 μl of CHIP dilution buffer for each protein of interest. Salmon sperm DNA bound to protein A-agarose was added and spun down to remove nonspecific background. The rabbit polyclonal immunoprecipitating antibodies [USF-1, sc-229x; USF-2, sc-861x (Santa Cruz Biotechnology)] were added to each tube and incubated overnight at 4°C with rotation. Salmon sperm DNA/protein A-agarose was added for 1 h at 4°C and pelleted to isolate the antibody/protein/histone/DNA complexes. The protein-DNA complexes were washed and eluted, and the cross-linking was reversed by heating samples at 65°C for 4 h. DNA was recovered by phenol/chloroform extraction and ethanol precipitation. To isolate the region of DNA containing the tandem repeats, PCR primers were designed at +15 and +195 relative to transcription start. The upper primer sequence was 5′-CGAGCAGGAAGAGGCGGAG-3′, and the lower primer sequence was 5′-TCCGAGCCGGCCACAGGCAT-3′. Thirty cycles of PCR were carried out for 30 s at 94°C, 30 s at 64.8°C, and 45 s at 72°C. The PCR reactions were precipitated and run on a 1.5% agarose gel.

Construction of Reporter Plasmids. The *TS* promoter, located in the genomic sequence upstream of the 5′ exon of the gene, was identified and isolated previously (28). Primers were designed at –313 and +195 relative to transcription start, and the PCR reaction yielded a 508-bp product for the *3R* genotype and a 480-bp product for the *2R* genotype. To isolate *TS-3RC* DNA, PCR amplification was performed from a random population of human genomic DNA, and products were sequenced directly (Davis Sequencing). Fragments were cloned into the promoter-less pGL3-Basic luciferase reporter gene vector (Promega) at *Sst*I and *Xho*I sites just upstream of luciferase gene translation start. Site-directed mutagenesis was carried out according to the manufacturer's protocol (Promega) to alter the USF-1 E-box consensus elements within the first 28-bp tandem repeat of both the 2R and 3R constructs. The mutagenic oligonucleotide primer sequence was 5′-GTCCTGCCAC-CGCGC***gt***CTTGGCCTGCC-3′ (Integrated DNA Technologies) and yielded the 2RmutUSF and 3RmutUSF reporter constructs. All plasmid DNA was isolated and purified using Qiagen (Valencia, CA) mini- and midi-prep kits.

Cell Culture and Transient Transfections. Human embryonic kidney 293-S cells (American Type Culture Collection) were plated in 6-well dishes at a density of 5×10^5 cells/well and incubated overnight in 2.5 ml of DMEM supplemented with 5% (v/v) fetal bovine serum, 100 units/ml penicillin, 100 μg/ml streptomycin, 10 mM pyruvate, and 2 mM L-glutamine. The next day, growth media were aspirated from the cells and replaced with 2.5 ml of serum-free Opti-MEM (Invitrogen). A total of 5 μg of plasmid DNA [1 μg of pCMV-β-galactosidase (Invitrogen) for standardization of transfection efficiencies, 1 μg of USF-1/pCI-neo or pCI-neo, and 3 μg of reporter construct] was diluted into 250 μl of Opti-MEM. A solution containing 250 μl of Opti-MEM and 15 μl of LipofectAMINE 2000 reagent (Invitrogen) was incubated for 5 min at room temperature and mixed with the DNA-containing solution from the previous step. After a 20-min incubation at room temperature, the DNA-LipofectAMINE solution was added drop-wise to the 293 cells in a circular fashion, and cells were incubated for 4 h at 37°C. The solution was aspirated and replaced with 3 ml of growth medium, and cells were incubated overnight at 37°C to allow gene expression.

Luciferase Assays. Luciferase activity was determined using a luciferase assay system (Promega), following the manufacturer's protocol. Briefly, cells were scraped into lysis reagent, transferred to microfuge tubes, and centrifuged for 30 s at 12,000 × *g*. Luciferase activity was measured using a manual luminometer (Turner Design; TD 20/20) by mixing 100 μl of luciferase assay reagent with 20 μl of 1:10-diluted cell lysate and reading three times at 10-s intervals for each sample. Transfection efficiencies were obtained using a β-galactosidase assay (Promega) of cell lysates by reading the absorbance at 420 nm. Relative luciferase activity was quantified by standardizing luciferase activity to a transfection efficiency factor.

Genotyping by RFLP Analysis. Genomic DNA was isolated from 200 μl of whole blood using the QiaAmp kit (Qiagen). The upper primer sequence was 5′-GTGGCTCCTGCGTTTCCCCC-3′, and the lower primer sequence was 5′-CCAAGCTTGGCTCCGAGCCGGCCACAGGCATGGCGCGG-3′, as described previously (12). Briefly, a 25-μl reaction mixture containing 1.25 mM $MgCl_2$, 2 mM deoxynucleotide triphosphates, 2.5 μl of DMSO, and 40 pmol of each primer was used, and 35 cycles of PCR were carried out. Each cycle consisted of 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C. Fifteen μl of the PCR reaction were digested with the *Hae*III restriction enzyme in a 20-μl reaction volume. The digested and undigested PCR products from each patient were loaded into adjacent lanes on a 3% sea plaque-agarose (BioWhittaker Molecular Applications) gel containing ethidium bromide (0.5 mg/ml) and electrophoresed in 0.5× TBE. Genotyping was performed twice for all samples by independent investigators (M. V. M. and J. S.).

Allelic Frequency Analysis. *TS* genotype measurements were performed on 99 white, 98 Hispanic, 59 African American, and 80 Singapore Chinese subjects. The 99 white subjects represented a random sample of the 691 white controls from a recently completed population-based case-control study of bladder cancer in Los Angeles County, California (29). The 59 African-American (34 bladder cancer cases plus 25 controls) and 98 Hispanic (50 bladder cancer cases plus 48 controls) subjects also were participants in the Los Angeles Bladder Cancer Study (29). Among African-American or Hispanic subjects, there was no statistically significant difference in genotypic distributions between bladder cancer cases and controls. Therefore, frequencies were reported for all subjects combined within each race. The 80 Singapore Chinese subjects were a random sample of the 63,000 participants of the

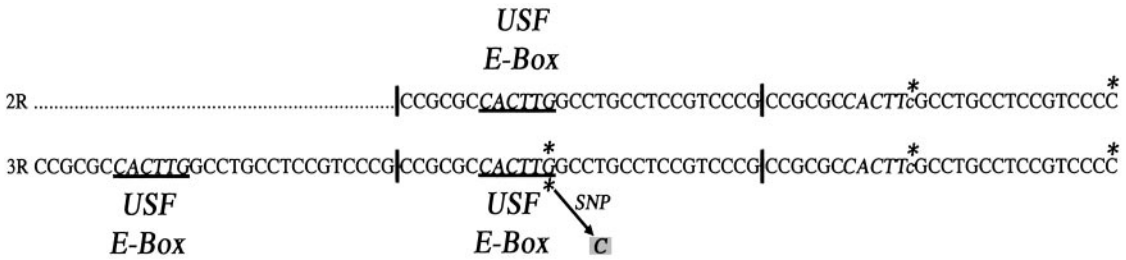

Fig. 1. The structure of a tandem repeat polymorphism within the 5′-untranslated region of the human *TS* gene. An enhancer polymorphism in the 5′-untranslated region of the *TS* gene consists of either two or three 28-bp repeats. A putative E-box binding site for upstream stimulatory factor (USF-1/USF-2) has been identified and is *underlined* and in *bold* within each repeat. Repeats one and two of *3R* and repeat one of *2R* contain USF consensus elements (*underlined*), whereas the last repeat in either construct contains an imperfect or variant consensus sequence due to a G→C base change (*asterisks*) that disrupts the putative E-box. The last nucleotide of the final repeat in *2R* and *3R* also bears a G→C base change.

Singapore Chinese Health Study, an ongoing prospective cohort study focusing on diet and cancer development (30).

RESULTS

The 28-bp Tandem Repeats in the 5′ Regulatory Region of the Human *TS* Gene Are Not Identical in Their Nucleotide Sequences. The published sequence of the human *TS* gene and its 5′ upstream regions (28) shows that there are two single base changes in the last 28-bp repeat of both the *2R* and *3R* genotypes, and recent evidence has shown that these sequence differences exist in the last repeats of the *4R* and *5R* alleles as well (16). The consequences of these base changes on *TS* gene expression, as well as the frequency of these base changes, have not been examined. Thus, we sought to verify the presence of the two sequence differences within the repeats and to look for other base changes and potential polymorphisms. By direct sequencing of 14 human genomic DNA samples, we verified the presence of the two base changes in the last repeats of *2R* and *3R*, and we identified a potential novel SNP within the second repeat of *3R* (Fig. 1, *asterisks*). To determine whether these base changes exerted a functional role on gene expression, we first sought to identify regulatory factors that bound within the 28-bp *TS* tandem repeats. Both the 28-bp sequence lacking the base changes and the 28-bp sequence bearing the base changes were scanned for putative transcription factor binding sites using the TRANSFAC database (31). A USF E-box consensus element (CACTTG) was found within the first repeat of the *2R* genotype and within the first two repeats of the *3R* genotype, but not in the last repeat of either genotype (Fig. 1). The C at the 12th nucleotide of the last repeat of *2R* and *3R* lies within the USF consensus element at a critical nucleotide for USF binding (32). The potential G→C SNP at the 12th nucleotide in the second repeat of *3R* changed the USF consensus element in a similar fashion (Fig. 1, *shaded nucleotide*). These observations suggested that USF regulatory factors might bind to sequences within the *TS* tandem repeats.

Phospho-USF-1 Binds to Consensus Elements within the *TS* Tandem Repeats but not to Repeats Containing the G→C Base Change at the 12th Nucleotide. To determine the sequence-specific binding of USF proteins to the *TS* tandem repeats *in vitro*, a 28-bp sequence bearing the putative USF consensus E-box element was used as a probe in EMSAs. Because USF-1 shows increased affinity for its DNA consensus element when it is phosphorylated, we tested the ability of the phosphorylated and unphosphorylated forms of USF-1 to bind to the putative consensus element within the 28-bp repeat sequence. Recombinant USF-1 was expressed in *E. coli* with a 6-histidine tag and purified on a Ni-NTA column (Fig. 2*A*, *left panel*,

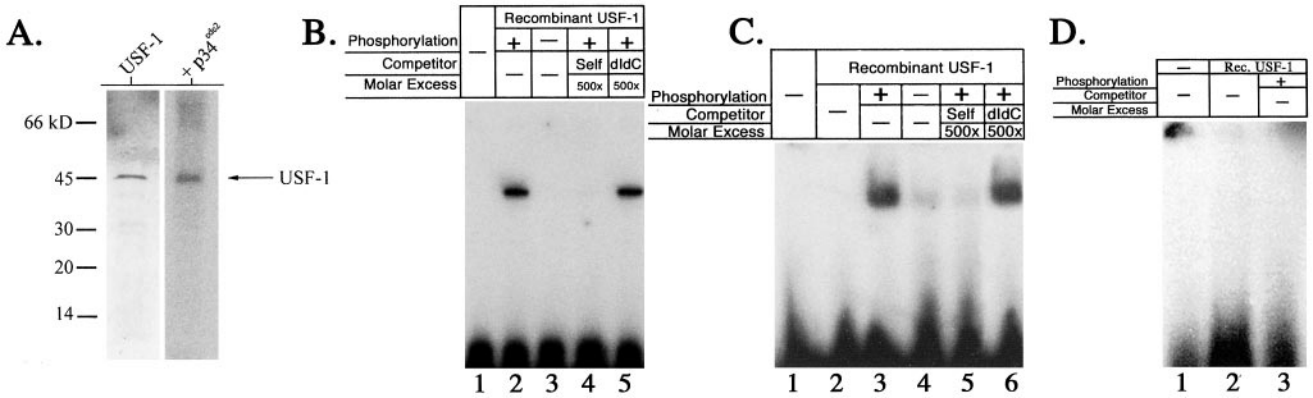

Fig. 2. Phospho-USF-1 binds to the *TS* tandem repeats *in vitro*. *A*, phosphorylation of recombinant USF-1 by cdc2/p34 *in vitro*. Recombinant USF-1 was overexpressed in *E. coli* and purified by Ni-NTA affinity chromatography (*left panel*; Coomassie Blue-stained gel). cdc2/p34 was immunoprecipitated from HeLa S3 cells and used in a phosphorylation reaction with 200 ng of recombinant USF-1. [^{32}P]ATP was used in the control reaction for confirmation of USF-1 phosphorylation after exposure of film (*right panel*; autoradiograph). *B*, phosphorylated recombinant USF-1 binds to its consensus sequence by EMSA. Gel mobility shift analyses were performed using recombinant USF-1with a ^{32}P-labeled USF-1-specific consensus probe containing an intact E-box site. *Lane 1*, free probe. *Lane 2*, 30 ng of recombinant phospho-USF-1 were incubated with probe in the absence of unlabeled competitor oligonucleotides. *Lane 3*, 30 ng of recombinant unphosphorylated USF-1 were incubated with probe in the absence of unlabeled specific competitor oligonucleotides. *Lanes 4* and *5*, phospho-USF-1 was preincubated with a 500 molar excess of unlabeled USF-1-specific competitor oligonucleotide and a 500 molar excess of nonspecific poly(dI·dC) competitor, respectively. *C*, phospho-USF-1 binds to the TS tandem repeats bearing an E-box site. Gel mobility shift analyses were performed using recombinant USF-1with a ^{32}P-labeled 28-bp probe corresponding to one tandem repeat containing an intact E-box site. *Lane 1*, free probe. *Lane 2*, 30 ng of recombinant USF-1 were incubated with probe in the absence of unlabeled competitor oligonucleotides. *Lane 3*, 30 ng of phospho-USF-1 were incubated with probe in the absence of unlabeled competitor oligonucleotides. *Lanes 4–6*, phospho-USF-1 was preincubated with a 500 molar excess of unlabeled probe, USF-1-specific competitor, and nonspecific poly(dI·dC) competitor oligonucleotides, respectively. *D*, USF-1 does not bind to the variant TS tandem repeats with a G→C base change at the 12th nucleotide. Gel mobility shift analyses were performed using recombinant USF-1 with a ^{32}P-labeled 28-bp probe corresponding to one tandem repeat containing a G→C base change at the 12th nucleotide. *Lane 1*, free probe. *Lane 2*, 30 ng of recombinant USF-1 were incubated with probe. *Lane 3*, 30 ng of phospho-USF-1 were incubated with probe.

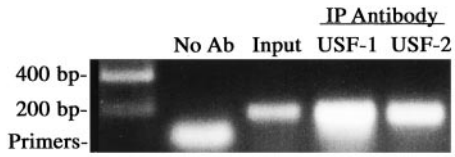

Fig. 3. USF-1 and USF-2 bind to the *TS* tandem repeats *in vivo*. The CHIP assay was performed as described in "Materials and Methods" using genomic DNA from 1×10^6 293-S cells and using antibodies for USF-1 and USF-2. Input DNA was a 20-μl aliquot of DNA taken before addition of antibodies, and the "no antibody" control was performed alongside USF-1 and USF-2 immunoprecipitations without the addition of antibody. PCR reactions were carried out at 64.8°C, ethanol precipitated, and electrophoresed on a 1.5% agarose gel.

Coomassie Blue-stained gel). cdc2/p34 was immunoprecipitated from HeLa S3 cells and used in an *in vitro* kinase reaction to phosphorylate USF-1 (Fig. 2*A*, *right panel*, autoradiograph). When both forms of USF-1 were used in an EMSA assay using a perfect USF-1 consensus element probe as a positive control, only the phosphorylated form was able to bind (Fig. 2*B*, *Lanes 2* and *3*). To determine the ability of the phosphorylated form of USF-1 to bind to its consensus element within the *TS* repeat, an EMSA assay was carried out using the 28-bp sequence as a probe. Incubation of the phosphorylated form of USF-1 with the probe caused a shift on the gel that was abolished by the addition of unlabeled specific competitor oligonucleotides (Fig. 2*C*, *Lanes 3* and *5*). These data suggest that only the phosphorylated form of USF-1 can bind its consensus element within the tandem repeats.

Because the potential G→C SNP at the 12th nucleotide of the 28-bp repeats lies within the USF binding site, we tested the ability of the recombinant USF-1 protein to bind the variant consensus element by EMSA. Neither the unphosphorylated nor phosphorylated form of USF-1 showed any affinity to this variant sequence (Fig. 2*D*). These data suggest that the potential SNP within the tandem repeats abolishes USF binding by disrupting the USF consensus E-box element.

USF-1 and USF-2 Bind to the *TS* Tandem Repeats *in Vivo*. The results of our *in vitro* assays show sequence-specific binding of USF-1 to the tandem repeats of the *TS* gene at E-box consensus sites. To determine whether USF-1 and possibly USF-2 were bound to these elements *in vivo*, we performed a CHIP assay using live 293-S (human embryonic kidney) cells. After formaldehyde cross-linking of proteins to DNA and shearing of genomic DNA by sonication, immunoprecipitations using USF-1 and USF-2 antibodies, along with a control reaction lacking antibody, were performed. After the pull-downs, PCR amplification was performed to determine whether the *TS* 5′ regulatory region containing the tandem repeats (+15 to +195 relative to the transcription start site) was bound by USF-1 or USF-2. The 180-bp fragment was amplified from the immunoprecipitations using USF-1 and USF-2 polyclonal antibodies but was not present in the control reaction lacking antibody (Fig. 3). These results show the presence of USF-1 and USF-2 on the chromatin at the *TS* locus, which includes the tandem repeats and E-box elements. This particular region of DNA contains no other putative E-box elements other than those located within the tandem repeats. The presence of USF-1 and USF-2 at the *TS* 5′ regulatory region suggests that these proteins bind to the E-box elements located within the tandem repeats. These data led us to examine the potential role of these proteins in activating transcription of *TS* 5′ regulatory region reporter constructs.

USF-1 Transactivates a TS Promoter Construct through Binding of Tandem Repeats Containing E-box Elements. To examine the ability of USF-1 and USF-2 to enhance transcription through binding within the tandem repeats, the region of −313 to +195 of the *TS* gene was cloned into the promoter-less pGL3-Basic reporter vector upstream of the luciferase reporter gene. Both 2R and 3R constructs were individually cloned into the vector, and 2RmutUSF and 3RmutUSF were created by altering the indicated USF consensus elements through site-directed mutagenesis (Fig. 4*A*). These constructs were cotransfected into 293 cells along with either a USF-1-expressing vector, a USF-2-expressing vector, or empty vector. Results from these experiments show that there was an increase in relative luciferase activity from both the 2R and 3R constructs in the presence of USF-1 (Fig. 4*B*). This 2–3-fold increase in transcriptional activity is consistent with previous reports of activation by USF-1 (33). The 3R construct had greater luciferase activity than the 2R construct in the absence and presence of exogenous USF-1 protein expression, and this difference between 2R and 3R transcriptional activity is consistent with previous reports in a similar luciferase system (12). Although these differences may appear small, subtle differences in TS gene expression have been shown to be significant in predicting response to 5-FU *in vivo* (34). Both 2RmutUSF and 3RmutUSF showed dramatically decreased transcriptional activity below endogenous levels of transcription compared with their wild-type counterparts, indicating that these USF sites, one in the 2R and two in the 3R, are critical to TS promoter activation. Consequently, these sites may be responsible for greater transcriptional activity from the 3R overall.

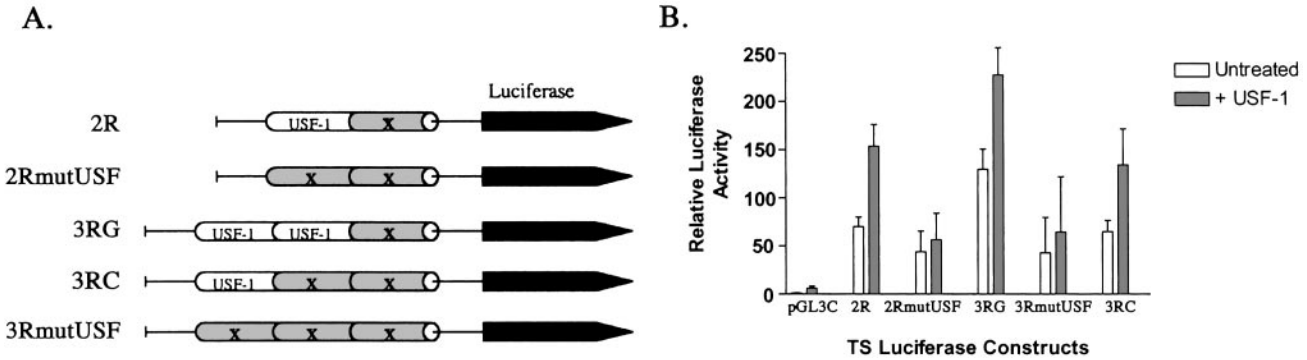

Fig. 4. The structure of the TS luciferase reporter constructs. *A*, the 5′ promoter region of the human *TS* gene from –313 to +195 (relative to the TS transcription start), including the 5′-untranslated region, was cloned into the TATA-less pGL3-Basic luciferase reporter vector just upstream of the luciferase translation start site. Site-directed mutagenesis was carried out to disrupt the E-box elements indicated in the 2RmutUSF and 3RmutUSF constructs. The 3RC construct lacks one E-box element in the second repeat due to a G→C SNP polymorphism. All E-box elements are labeled *USF*, and all C alleles (G→C) or mutant elements are labeled with an *X*. *B*, activation of the TS gene promoter by USF-1. 293 cells were transfected with 3 μg of luciferase reporter construct containing either no promoter (pGL3-Basic), the TS 5′ region containing two tandem repeats (2R), the TS 5′ region containing three tandem repeats (3R), the TS 5′ region containing the 3R with a G→C SNP at the 12th nucleotide of the third repeat (3RC), or the TS 5′ region containing two or three tandem repeats with mutated E-box sites (2RmutUSF and 3RmutUSF). Cells were cotransfected with 1 μg of empty pCI-NEO vector, 1 μg of vector containing USF-1 cDNA, 1 μg of vector containing USF-2 cDNA, or 0.5 μg of USF-1- and USF-2-containing vectors, respectively. Cells were also cotransfected with 1 μg of the pCMV-β-galactosidase vector for standardization of transfection efficiencies. 24 h after transfection, cells were harvested, lysed, and assayed for β-galactosidase activity and luciferase activity.

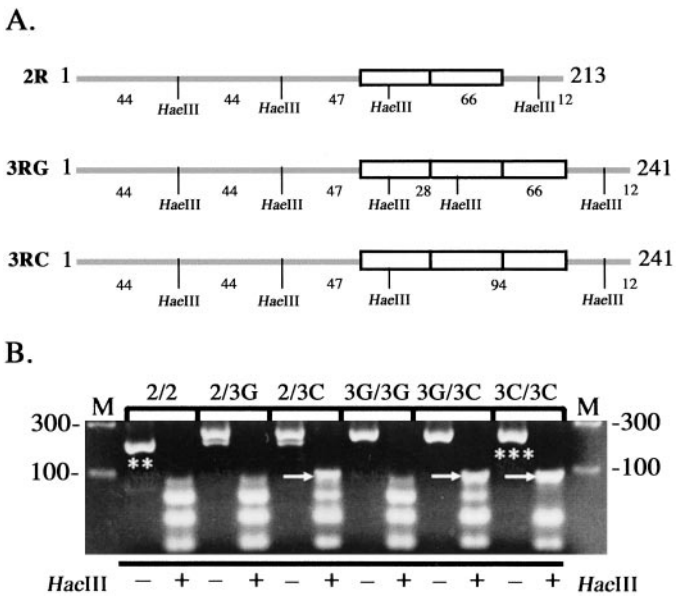

Fig. 5. *Hae*III restriction map of the *TS* tandem repeat fragments produced in the RFLP analysis. *A*, this map shows the *Hae*III restriction endonuclease sites within the fragments produced by PCR for the RFLP analysis. The size of the DNA fragments produced after digestion with *Hae*III is shown. A restriction site is removed in *3RC*, producing a larger, 94-bp fragment that allows for screening of the G→C SNP. *B*, RFLP analysis used for screening of the tandem repeats as well as the G→C SNP. Fragments of 241 (***) and 213 bp (**) were amplified from patient genomic DNA, corresponding to *3R* and *2R* genotypes, respectively. Half of the PCR products were digested with the *Hae*III restriction enzyme, and half were left undigested. Samples were run on a 3% sea plaque-agarose gel in 0.5× TBE. The *arrows* point to the additional 94-bp fragment that is absent in wild-type samples but present in samples positive for the G→C polymorphism. Genotypes are listed above corresponding lanes showing repeat polymorphism (2 or 3) and G→C SNP (G or C).

Because the single G→C base change at the 12th nucleotide of the 28-bp repeats can abolish the ability of USF proteins to bind to this site by EMSA, we wanted to determine whether this base change would alter the ability of USF-1 to transactivate the 3RC (C at position 12 of second repeat) TS promoter construct. The 3RC reporter construct (Fig. 4*A*) had decreased transcriptional activity compared with the 3RG in the absence and presence of exogenous USF-1. In addition, 3RC had a similar ability to transactivate the luciferase reporter gene as the 2R construct (Fig. 4*B*). These data suggest that the ability of the tandem repeats to enhance transcription increases only as the number of USF consensus elements increase and not necessarily as tandem repeats increase. Hence, the potential SNP within the second repeat of 3R is a determinant of the ability of the 3R construct to act as an enhancer of transcription, relative to the 2R construct. Overall, USF-2 activation led to a modest increase in relative luciferase activity alone, and USF-1 and USF-2 cotransfections showed no significant increase in luciferase activity compared with USF-1 alone (data not shown). These data suggest that USF-1 homodimers and possibly USF-1/USF-2 heterodimers contribute to the activation of the TS promoter constructs in this system.

Characterization of a Novel SNP by RFLP Analysis. To determine the frequency of the potential SNP in a large population, we developed a RFLP analysis (Fig. 5*A*). PCR was carried out using genomic DNA samples yielding PCR fragments of 213 bp for *2R* alleles, 241 bp for *3R* alleles, and both fragments for *2R/3R* heterozygotes (Fig. 5*B*, undigested samples). The G→C base change in *3RC* removes a *Hae*III restriction endonuclease site and changes the banding pattern of the digested PCR fragment on a 3% sea plaque-agarose gel. The digested banding patterns are shown (Fig. 5*B*, *Hae*III-digested lanes). Digested and undigested PCR products from each patient were run in adjacent lanes to determine the repeat polymorphism genotypes and the G→C SNP genotypes of each allele. Running undigested product next to digested product was necessary because there are similar banding patterns for *2R/2R*, *2R/3RG*, and *3RG/3RG* as well as for *2R/3RC* and *3RG/3RC* when they are digested with the enzyme. In most samples, a nonspecific DNA product was observed at ~100 bp in length in the undigested samples. This nonspecific DNA resulted in the presence of an ~60-bp band in the *Hae*III-digested samples that did not interfere with interpretation of the genotype. Nevertheless, a single PCR reaction followed by digestion of half the sample with *Hae*III yielded patient genotypes for the tandem repeat polymorphism and the SNP within the tandem repeats.

Table 1 shows the genotypic and allelic frequency distributions of the *TS* tandem repeats and SNP in whites, Hispanics, African Americans, and Singapore Chinese. The frequencies of *2R* and *3R* alleles in white, African-American, and Chinese populations are consistent with previous reports (14–16). The *3RC* allele frequency varied by 2.2-fold across the four races and was lowest in African Americans (15%) and highest in whites (33%). Hispanics (26%) and Chinese (30%) exhibited rates that were modestly lower than that of whites. When only *3R* alleles were counted, the frequencies of the *3RC* allele in whites, Hispanics, African Americans, and Singapore Chinese were 56%, 47%, 28%, and 37%, respectively. Our data suggest that the G→C base change at the 12th nucleotide of the second repeat of *3R* alleles is a relatively common polymorphism among all major racial/ethnic groups.

DISCUSSION

In this study, we characterized the role of USF-1 in transcriptional activation from the tandem repeat polymorphism of the human *TS* gene, and we describe how an additional 28-bp repeat can enhance transcriptional activity. We also identified a common G→C SNP within the *3R* allele that can abolish its increased transcriptional activity relative to the *2R*, and we show that sequence variations within the tandem repeats have functional significance.

It has been postulated that the number of tandem repeats in the *TS* gene may be a determinant of the levels of TS expression (12). However, a novel SNP within the tandem repeats alters the enhancer function of an extra repeat. A single G→C base transition found at the 12th nucleotide of the second repeat in the *3R* genotype changes a critical residue in the USF E-box consensus element (32). By EMSA

Table 1 *Distribution of the 5′ TS tandem repeat polymorphism and the novel G→C SNP polymorphism in the second repeat of the 3R allele among non-Hispanic white, Hispanic white, African-American, and Singapore Chinese individuals*

		Genotype (%)							Allele frequency (%)			
Ethnic group	n^a	*2/2*	*2/3G*	*2/3C*	*3G/3G*	*3G/3C*	*3C/3C*	Other	*2*	*3G*	*3C*	Other
White	99	19	13	31	11	16	9		41	26	33	
Hispanic	98	17	26	25	10	13	7	2	42	30	26	2
African American	59	29	25	13	19	12	2		48	37	15	
Chinese	80	2	19	15	32	18	14		19	51	30	

[a] *n*, total number of individuals in sample population.

assay, we showed that this base change abolishes the ability of USF complexes to bind within the repeat and effectively eliminates the E-box site. A 3R construct bearing this variation, 3RC, was isolated from patient genomic DNA and used in luciferase reporter assays to analyze the effects of this polymorphism on transcription. The 3RC construct displayed a similar transcriptional activity as a 2R construct. These results suggest that the addition of a 28-bp repeat alone is not sufficient for enhanced transcriptional activity of the *TS* gene but that a USF E-box element is required within the extra repeat to enhance transcription.

We have revised the previous PCR-based method for determining tandem repeat polymorphism genotype (12) into a RFLP technique that includes a screen for the G→C SNP. The high frequency of the novel SNP (*3RC*) among all *3R* alleles in the four given populations identifies this G→C substitution as a common event (Table 1).

The tandem repeat polymorphism of the *TS* 5′ regulatory region has been shown to be associated with TS expression levels, and evaluation of its predictive value in various clinical trials identified a treatment benefit to 5-FU-based chemotherapy for individuals possessing a *2R* genotype (13, 14, 17–21). However, a sizeable fraction of patients with *3R/3R* genotypes demonstrated low TS expression (17) or showed some short-term benefit to 5-FU-based chemotherapy (21). Based on our results, it seems that the novel SNP may alter the ability of the repeats to function as enhancers of transcription and may be useful in explaining discrepancies in predicting response to 5-FU treatment using the tandem repeat polymorphism alone as a marker. Recent evidence shows a role for the tandem repeats in increasing the translational efficiency of TS transcripts but suggests that a transcriptional component is absent (13). These studies were performed using real-time PCR on patient samples and included screening for the tandem repeat polymorphism but did not include screening for the SNP that we describe. Thus, we demonstrate that a transcriptional component within the tandem repeats exists and show evidence that this component is altered by differences in the nucleotide sequence of the repeats. A future study correlating intratumoral TS mRNA levels with the tandem repeats and the G→C SNP awaits future investigation. The SNP is easily screened for with the addition of a simple restriction digestion and may generate useful information for clinicians to tailor individual chemotherapy with respect to both tumor response and host toxicity.

Considering the importance of the TS reaction in folate metabolism, this novel polymorphism may have influence in the modulation of various folate-dependent pathways. In addition to thymidylate biosynthesis, other pathways such as purine synthesis, methionine regeneration, and other one-carbon donor reactions such as those involved in DNA methylation (23) could all be influenced by this polymorphism. Taken together, our data suggest that this novel SNP in the 5′ regulatory region of the *TS* gene should be considered in prospective studies in which the tandem repeat polymorphism is being used as a predictive marker.

The functional regulation of USF proteins adds further complexity to the TS inhibition pathway. The USF transcription factors have been traditionally described as ubiquitous regulatory factors, but recent evidence has shown that the DNA binding activity of these proteins is differentially regulated from multiple signal transduction pathways through phosphorylation by kinases including p38 (35), protein kinase A, protein kinase C (36), and cdc2/p34 (37). Of particular interest is the phosphorylation of USF-1 by the stress-responsive p38 kinase. It has been postulated that this activation may provide a link between stress stimuli and the subsequent changes in gene expression that occur as a result of treatment with stress-inducing agents (35), possibly including chemotherapeutic agents. The USF family of proteins can also be misregulated in some forms of cancer (38) and are overexpressed during periods of malnutrition, particularly protein-free diets (39). Thus, it can be hypothesized that overexpression or fraudulent activation of USF proteins through phosphorylation could cause increased activation of genes targeted by USF-1/USF-2 complexes, thereby implicating the USF proteins as mediators of TS overexpression *in vivo*. Taken together, these events may represent factors that could alter the prediction of patient TS mRNA levels and, ultimately, response to 5-FU.

We provide evidence for a direct role of USF proteins in the regulation of *TS* gene expression and suggest that the inhibition of USF-1, specifically its regulation through phosphorylation, could be considered as a modulating therapy for TS-directed anticancer drugs. Based on these combined observations, the role of USF proteins in carcinogenesis and clinical response is intriguing and warrants further investigation at the molecular level as well as in the clinic.

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A Novel Single Nucleotide Polymorphism within the 5′ Tandem Repeat Polymorphism of the *Thymidylate Synthase* Gene Abolishes USF-1 Binding and Alters Transcriptional Activity

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