Identification of Functional Elements in the Promoter Region of the Human Gene for Thymidylate Synthase and Nuclear Factors That Regulate the Expression of the Gene*

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To identify the essential motifs of the promoter of the human gene for thymidylate synthase (TS), we constructed a set of deletion mutants from the 5′-terminal region of the human TS gene. From the results of assays of the expression of chloramphenicol acetyltransferase (CAT), we identified two functional elements with positive effects on the promoter activity: a CACCC box (CCA-CACC) and an Sp1-binding motif (GAGGCGGA) that was homologous to the Sp1-binding site in the mouse TS gene. In addition, negative regulatory sequences were identified between the two positive elements and in the region upstream of the CACCC box. The results of gel mobility shift analyses suggested that Sp1 binds to the Sp1-binding motif of the human TS gene promoter and that multiple nuclear factors that are related to Sp1 bind to the CACCC box. Furthermore, the binding of Sp1 to mutated Sp1-binding motifs in the promoter region of the human TS gene was correlated with the promoter activity, as measured by the CAT assay. Therefore, the Sp1 motif seems to be a major contributor to the basic promoter activity of the human TS gene, although multiple positive and negative regulatory elements are involved in the regulated expression of this gene.

Thymidylate synthase (TS; N5,N10-methylenetetrahydrofolate:UMP C-methyltransferase; EC 2.1.1.45) catalyzes the conversion of deoxyuridylate to thymidylate, and the enzyme is known as the key enzyme in nucleotide metabolism. Tight regulation of TS activity is essential for the normal replication of DNA, and impairment of this enzyme causes various biological and genetic abnormalities, such as thymine-less death (1), chromosome breakage and exchange (2), the expression of heritable fragile sites (3), and genetic recombination (4–6). The regulation of the expression of the TS gene depends on the proliferative state of cells (7–10). For example, the activity of the enzyme and the level of mRNA for human TS (hTS) are very low in quiescent human fibroblast cells, but both increase dramatically when the cells are stimulated by serum to enter the G1/S phase (11).

To study the regulated expression of the hTS gene, we cloned and characterized the hTS gene (12–15). The hTS gene is 18 kilobase pairs in length and consists of seven exons. Regions essential for the regulation that is dependent on the stage of the cell cycle have been identified both in the first intron (15, 16) and in the 5′-flanking region (16) of the hTS gene. Although the expression of the hTS gene is mainly regulated at the post-transcriptional level (11), these findings suggest the involvement of the promoter region in the cell cycle-dependent regulation of the gene. However, functional motifs in the promoter region and mechanism for regulation of the promoter activity of the hTS gene remain to be elucidated. The 5′-flanking region of the hTS gene has neither a TATA box nor a CAAT box (13). The mouse TS gene also has neither a TATA box nor a CAAT box in its promoter region and an Sp1-binding site in the promoter region has been reported to be essential for the promoter activity (17). The Sp1-binding site in the mouse TS gene exhibits 90% homology with the corresponding region of the hTS gene, suggesting that the human and mouse genes for TS might share common motifs that are essential for promoter activity. However, the regulatory elements identified in the region upstream from the Sp1-binding motif of the mouse TS gene (17) are not conserved in the hTS gene. Furthermore, the region upstream of the cap site of the hTS gene exhibits only 36% homology over 100 bp with the corresponding region of the mouse TS gene. These data suggest that most of the regulatory elements are not conserved between the human and mouse genes for TS in the upstream region, implying the possibility that some elements might contribute specifically to the regulation of the promoter of the hTS gene. In previous studies, we showed that the essential promoter sequence is in the region from −242 to −148 (nucleotide positions in the hTS gene in this report are numbered from the first nucleotide of the codon for initiation of the translation of the hTS gene) and that, in addition to sequences upstream of the cap site, the tandemly repeated sequences downstream from the cap site are necessary for the sufficient expression of the hTS gene (18, 19). In this study, we examined sequences that were essential for promoter activity using a transient expression system and we demonstrated that a CACCC box (CCACACCC) and the region that includes the sequence homologous to the Sp1-binding site of the mouse TS gene are necessary for efficient promoter activity. We also identified two negative regulatory sequences in the promoter region of the hTS gene and characterized the nuclear factors that interact with the positive elements in the promoter region of the hTS gene.

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The abbreviations used are: TS, thymidylate synthase; hTS, human thymidylate synthase; PCR, polymerase chain reaction; CAT, chloramphenicol acetyltransferase; EKLF, erythroid Krüppel-like factor; NRS, negative regulatory sequence; GRE, glucocorticoid response element; CREB, cAMP response element-binding protein; TFIID, transcription factor IID; NF1/CTF, nuclear factor-I/CAAT-box binding transcription factor; OCT, octamer motif.
Experimental Procedures

Reagents—Enzymes were purchased from Takara Shuzo (Kyoto, Japan) and Toyobo (Osaka, Japan). ES medium (20) and fetal calf serum for cell culture were products of Nissui Seiyaku (Tokyo, Japan) and HyClone Laboratories (Logan, UT), respectively. DNA polymerase from *Thermus aquaticus* (AmpliTaq) was obtained from Perkin-Elmer (Foster City, CA), agarose and electrophoresis from Nippon Gene (Toyama, Japan), [α-32P]dCTP (3,000 Ci/mmol) and n-threo-(1,2,3)-chloramphenicol (40–60 mCi/mmol) from ICN Biomedicals Inc. (Costa Mesa, CA). A mouse monoclonal antibody against Sp1 was purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). A nonspecific mouse IgG was purchased from Bio-Rad.

Plasmids—The 587-bp SacI-BglII fragment, including the cap sites and the site of initiation of translation of the hTS gene, was prepared from pHR668, a subclone of the hTS gene (12), and inserted into the HindIII site of the pGCMAT vector (18) via a 10-bp HindIII linker, such that the initiation codon of the hTS gene was in frame with a CAT gene on the pGCMAT vector. From the resultant plasmid (pScBg6CAT), we prepared a set of deletion plasmids using exonuclease III. Each plasmid was sequenced to determine the deletion site. A cassette mutation or a point mutation was introduced into the promoter region of the hTS gene by PCR (21). To introduce a BgIII linker sequence at a specific site in the promoter region of the hTS gene, we synthesized oligonucleotide primers (LS2a, etc. in Table I) that contained a BgIII site at the 5′-end and used them with a 5′-primer and a 3′-primer (Primer 1 and Primer 2, respectively, in Fig. 2) for amplification of the promoter region of the hTS gene. A HindIII site was added to the 5′-end of Primer 2. The sequences of the primers used for PCR are listed in Table I. For the PCR, pScBg6CAT was used as template. The amplified fragment included the region from –283 to +22 and had a HindIII site at its 3′-end. The products of PCR were cloned into pUC19, and the sequences of DNA fragments with cassette or point mutations were used. These fragments were labeled with [α-32P]dCTP using the Klenow fragment of DNA polymerase I and used for the assay as probes. Competitor DNA fragments of each containing a cassette or a point mutation were used as competitors. The gel mobility shift assays were performed using nuclear extract, as indicated in legends to the figures, for 5 min prior to the addition of the labeled probe. To identify nuclear factors that bind to DNA probes, 0.25 or 0.5 μg of mouse monoclonal antibody against Sp1 was added to the reaction mixture containing 2.5 μg of protein of nuclear extracts, prior to the addition of the labeled probe.

Results

Multiple Positive and Negative Regulatory Sequences Are Present in the Promoter Region of the hTS Gene—In a previous study, the essential region of the promoter of the hTS gene was identified as the region from –242 to –148 (18). To identify the essential motifs in this promoter region, we constructed a set of deletion mutants that covered the region from –441 to +28 of the hTS gene. The structures of the deletion mutants were indicated on the left in Fig. 1. Among the fragments shown in Fig. 1, the fragments including the region upstream of position –270 had no significant promoter activity. Promoter activity was detected with the fragments that included the regions from –229 to –223 and from –213 to –147. These results suggest that the negative regulatory element is in the region from –342 to –269. Although deletion mutant plasmids d223 and d212 included the region from –187 to –147, these two plasmids had lower promoter activity than d201. This observation suggests that the region from –212 to –201 decreased the promoter activity of the region from –187 to –147. Thus, the results shown in Fig. 1 suggest that the promoter region of the hTS gene contains two negative regulatory sequences and two positive elements that influence the promoter activity.

A CACCC Box and an Sp1-binding Motif Are Positive Elements Required for the Promoter Activity of the hTS Gene—We found several DNA motifs in the two positively acting regions that we had identified from the results of the CAT assay with the deletion mutants. The upstream positively acting region, namely the region from –229 to –223, overlapped with the CACCC box (Ref. 24; CCACACCC from –228 to –221). The downstream positively acting region, from –187 to –147, contained a sequence homologous to the Sp1-binding site (AAGAGGCGGA, from –152 to –143 of the hTS gene) of the mouse TS gene (17).

### Table I

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<th>Nucleotide positions of each oligonucleotide in the human TS gene are shown in parentheses.</th>
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<td>Primer 1</td>
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This region contains the consensus sequence of an Sp1-binding site (27). To examine the function of these DNA motifs, we introduced cassette mutations and a point mutation into the promoter region of the hTS gene. The structures of these mutants are shown in Fig. 2 and the results of CAT assays with these constructs are shown in Fig. 3. The M-CA mutation in Fig. 2 had three substitutions of nucleotides in the CACC box in the promoter of the hTS gene. The promoter activity of the mutant plasmid (M-CA) was 65% of the activity of the native promoter of the hTS gene (“No mutation” in Fig. 3). The fragment with the mutation indicated by M-Sp had five substitutions of nucleotides in the Sp1-binding motif of the promoter of the hTS gene and had no significant promoter activity in the CAT assay (M-Sp in Fig. 3). A single nucleotide substitution in the Sp1-binding motif reduced the promoter activity to half of that of the parental fragment (see d229 and d229/146A in Fig. 3). These results suggest that, in the hTS gene, the CACC box and the sequence homologous to the Sp1-binding site of the mouse TS gene function as positive elements for the promoter activity and that the Sp1-binding site is essential for the expression of the gene. Furthermore, we introduced a cassette mutation into the negative regulatory region that we had identified in the CAT assays of the deletion mutants. The M-NRS mutant had four substitutions in the negative regulatory sequence that was located between two positive elements in the promoter region. Its promoter activity was 2.5-fold higher than that of the native promoter of the hTS gene. This result suggests that this region represses the promoter activity of the hTS gene.

Sp1 Binds to the CACC Box and the Sp1-binding Motif of the hTS Gene Promoter—To identify nuclear factors that regulate the promoter activity of the hTS gene, we examined the specific binding of nuclear factors to the functional motifs in the promoter of this gene. To examine the individual nuclear factors that bind to the CACC box or the Sp1-binding motif in the promoter, we used a PvuII-XbaI fragment and an XbaI-BssHII fragment (Fig. 2) from the promoter region of the hTS gene as probes in the gel mobility shift assay. Fig. 4 shows the formation of nucleoprotein complexes that contained the XbaI-BssHII fragment. The mutated fragments used in the CAT assays were also used for the preparation of the probes. When the fragment with the mutation indicated by M-Sp (Fig. 2) was used as the probe, the rate of formation of the major nucleoprotein complex was markedly reduced (compare the band a in lanes 1 and 4 in Fig. 4A). B in Fig. 4 indicates that the point mutation in the Sp1-binding motif also inhibited the formation of the major complex with the XbaI-BssHII fragment. The results obtained in the competition assays with DNA fragments that contain known binding motifs for specific transcription factors are shown in C in Fig. 4, and the results indicate competition for the formation of the major complex upon the addition of a DNA fragment with the binding motif for Sp1 (lanes 9 and 10 in Fig. 4C). These results suggest that the major complex involved the Sp1-binding motif in the XbaI-BssHII fragment of the hTS gene promoter together with Sp1. Taking into consideration the fact that the M-Sp mutant had no significant promoter activity in the CAT assay, as compared with other mutants or the parental fragment of the promoter (Fig. 3), and that the point mutation in the Sp1-binding motif reduced the promoter activity of the fragment in the CAT assay, we concluded that the formation of the complex that involved the Sp1-binding motif was correlated with the promoter activity of the mutants that had been measured by the CAT assay.

When the PvuII-XbaI fragment (Fig. 2) was used as a probe, several shifted bands were observed in the gel mobility shift
assay (lane 1 in Fig. 5A). Among these bands, two bands (bands b, d, and e in Fig. 3) were not detected when the fragment with the mutation in the CACCC box (M-CA) was used as the probe. To examine the sequence specificity of the nuclear factors that bind to the fragment, competitor DNA fragments that contain known binding motifs for specific transcription factors were added to the binding reaction mixture. The results of the competition assay showed that intensities of almost all the bands, including the bands whose intensities diminished when the fragment with the mutation indicated by M-CA was used as the probe, were reduced upon the addition of the fragment with the Sp1-binding motif (bands b, d, and e in lanes 9 and 10 in Fig. 5B). The remaining band was subject to competition by the DNA fragment with the AP2-binding motif (band c in lanes 5 and 6 in Fig. 5B). These results suggest that multiple nuclear factors that recognize the Sp1 binding motif bind to the PvuII-XbaI fragment of the promoter of the hTS gene, and at least two of them interact with the CACCC box in the fragment, while a nuclear factor that binds to the consensus sequence for binding of AP2 also binds to the PvuII-XbaI fragment. In fact, computer search did reveal that an AP2 consensus motif (YC-SCCMNSSS; Ref. 25) was present in the region from −206 to −197 in the XbaI-BssHII fragment (see Fig. 2). Thus, the AP2-binding motif is a candidate for the binding site of an AP2-like factor.

Then, we examined the binding of Sp1 to the XbaI-BssHII fragment of the hTS gene using monoclonal antibody against Sp1. Fig. 6 shows the effect of the antibody on the formation of nucleoprotein complexes when the Sp1 monoclonal antibody was added to the binding mixture of the gel mobility shift assay. The result indicated that the major retarded band disappeared upon the addition of the antibody (band a in lane 6 in Fig. 6). The band that disappeared corresponds to the nucleoprotein complex formed with the Sp1-binding motif of the hTS gene (band a in Fig. 4). These results suggested that the nucleoprotein complex formed with the Sp1-binding motif in the XbaI-BssHII fragment includes Sp1.

**DISCUSSION**

**Essential Motifs in the Promoter of the hTS Gene**—The promoter region of the hTS gene does not contain any DNA motifs that are typical of eukaryotic promoters, such as a TATA box, a CAAT box, or a typical GC box. Therefore, we attempted to identify the motifs that are essential for the promoter activity. In studies of the mouse TS gene, multiple nuclear factors, including Sp1 and Ets-like factors, were reported to bind to the promoter region, and the Ets/Sp1-binding motifs were shown to play an important role in the expression of the mouse TS gene (17, 26). In a previous study, we identified the region that is
Fig. 4. Identification of the nuclear factors that bind to the Sp1-binding motif in the promoter region of the human TS gene. A, gel mobility shift assay using an XbaI-BssHII fragment from the native and mutated promoter region of the hTS gene (see Fig. 2) as a probe. The following probes were used: a DNA fragment without mutations (lane 1), a DNA fragment with the mutation indicated by M-Cap (lane 2), M-CG (lane 3), and M-Sp (lane 4). An open triangle indicates nucleoprotein complexes formed with the probe. A closed triangle indicates bands of free probe. B, effect of a point mutation in the Sp1-binding motif of the hTS gene on the formation of nucleoprotein complex. The XbaI-BssHII fragment without mutation (lane 1) or with a point mutation in the Sp1-binding motif of the hTS gene (lane 2) was used as a probe. The DNA fragment with the point mutation was prepared from plasmid d229(146A). An open triangle indicates nucleoprotein complexes formed with the probe. A closed triangle indicates bands of free probe. C, competitive gel mobility shift assay for identification of nuclear factors that bind to the promoter region of the hTS gene. An end-labeled XbaI-BssHII fragment (0.5 ng) from the native promoter region of the hTS gene was incubated with a nuclear extract of HeLa cells in the presence of 12.5 ng (lanes 5, 7, 9, 11, 13, 15, 17, and 21) or 25 ng (lanes 4, 6, 8, 10, 12, 14, 16, 18, 20, and 22) of a competitor DNA fragment that included the consensus sequence for binding of the specific transcription factor indicated below. Lane 1, no extract; lane 2, no competitor; lanes 3 and 4, AP1; lanes 5 and 6, AP2; lanes 7 and 8, AP3; lanes 9 and 10, Sp1; lanes 11 and 12, NF-κB; lanes 13 and 14, NF-κB; lanes 15 and 16, TFIIID; lanes 17 and 18, GRE; lanes 19 and 20, CREB; lanes 21 and 22, OCT. An open triangle indicates nucleoprotein complexes formed with the probe. A closed triangle indicates bands of free probe.

Fig. 5. Identification of nuclear factors that bind to a CACCC box in the promoter region of the human TS gene. A, gel mobility shift assay using a Puu1Ⅰ-Xba1 fragment from the native and mutated promoter region of the hTS gene (see Fig. 2) as a probe. The following probes were used: a DNA fragment without mutation (lane 1), a DNA fragment with the mutation indicated by M-CA (lane 2), and by M-NRS (lane 3). Open triangles indicate nucleoprotein complexes formed with the probe. A closed triangle indicates bands of free probe. B, competitive gel mobility shift assay for identification of nuclear factors that bind to the promoter region of the hTS gene. An end-labeled Puu1Ⅰ-Xba1 fragment (0.5 ng) from the native promoter region of the TS gene was incubated with nuclear extracts of HeLa cells in the presence of 12.5 ng (lanes 3, 5, 7, 9, 11, 13, 15, 17, and 19) or 25 ng (lanes 4, 6, 8, 10, 12, 14, 16, 18, and 20) of a competitor DNA fragment that included the consensus sequence for binding of a specific transcription factor, as indicated below. Lane 1, no extract; lane 2, no competitor; lanes 3 and 4, AP1; lanes 5 and 6, AP2; lanes 7 and 8, AP3; lanes 9 and 10, Sp1; lanes 11 and 12, NF-κB; lanes 13 and 14, NF-κB; lanes 15 and 16, TFIIID; lanes 17 and 18, GRE; lanes 19 and 20, CREB. Open triangles indicate nucleoprotein complexes formed with the probe. A closed triangle indicates bands of free probe.

Fig. 6. Identification of a nuclear factor using a monoclonal antibody against Sp1. Gel mobility analyses were performed using the XbaI-BssHII fragment (lanes 1–6) of the hTS promoter as probes. The probe was incubated without nuclear extracts (lane 1) or with nuclear extracts of HeLa cells (lanes 2–6). For identification of nuclear factors that bind to the probe, 0.25 μg (lane 5) or 0.5 μg (lane 6) of a mouse monoclonal antibody against Sp1 was added to the binding mixture. In negative control experiments, 0.25 μg (lane 3) or 0.5 μg (lane 4) of nonspecific IgG was added to the binding mixture. Open triangles indicate nucleoprotein complexes formed with the probe. Closed triangles indicate bands of free probe.

The major cap sites of the mouse TS gene have been identified at positions downstream from the Sp1-binding site (30). By contrast, the major cap site of the hTS gene was located at position −179 from the results of a primer extension experiment (14). This position is located upstream of the Sp1-binding motif. In a previous study, Takeishi et al. (13) suggested the presence of cap sites downstream from the Sp1-binding site.
from the results of S1 nuclease mapping. However, the results could not be confirmed by primer extension experiments, because the unique, tandemly repeated structure of the hTS gene interfered with the identification of the cap sites around the Sp1-binding site (14). The finding that the common Sp1-binding motif is essential for the promoter activity of the human and mouse genes for TS suggests that the major cap site of the hTS gene is also present in the region downstream of the Sp1-binding motif.

The CACCC box in the hTS gene is located at 71 bp upstream of the Sp1-binding motif, and it appears to have positive effect on the promoter activity of the hTS gene. This motif is not conserved in the corresponding region of the mouse and rat genes for TS. However, in the case of the mouse and rat genes, a CACCC box is found about 160 bp upstream of the Sp1-binding motif. The function or biological significance of the CACCC box in the mouse and rat genes is unknown. However, it is possible that the motif might play a role in the regulation of expression of the mouse or rat gene. The CACCC motif is found in the promoter region of the gene for β-globin, in the enhancer region of the early gene of SV40, and in many other genes (31). Multiple transcription factors, including Sp1 and EKLF (erythroid Kru¨ppel-like factor), have been reported to bind to the CACCC box (31, 32). This observation suggests that the motif can function as either a promoter element or an enhancer element. In the case of the hTS gene, inactivation of the Sp1-binding motif downstream of the CACCC box reduced the promoter activity to the background level, whereas three substitutions of nucleotides in the CACCC box reduced the promoter activity to 65% of that of the native hTS gene promoter. Therefore, the CACCC box seems not to be a promoter element but, rather, to be a regulatory element essential for the appropriate promoter activity of the hTS gene.

**Nuclear Factors That Bind to the Promoter Region of the hTS Gene**—We also examined the specific interaction of nuclear factors with the functional DNA motifs found in the promoter region of the hTS gene. The results of gel mobility shift analysis revealed that Sp1 bound to the Sp1-binding motif of the hTS gene (Figs. 4 and 6). Sp1 has been reported to bind also to the corresponding region of the mouse TS gene, and the binding is important for the expression of the gene (17). In the case of the mouse TS gene, Johnson and his colleagues (33, 34) reported that an E2F-binding motif and Ets-binding motifs (GGAAG) play a role in the growth-regulated expression of the gene. These functional motifs in the mouse TS gene are not conserved in the corresponding regions of the human TS gene, whereas an Ets-binding motif, GGAAG, adjacent to the Sp1-binding motif is conserved in the human TS gene. When the DNA fragment with an Sp1-binding motif was used as a competitor, the band due to formation of the nucleoprotein complex that included the Sp1-binding site was completely lost (Fig. 4C). Furthermore, the formation of the complex that included the Sp1-binding site was inhibited by the addition of the monoclonal antibody against Sp1 (Fig. 6). This result suggests that, at least in HeLa cells, the binding of Sp1 is essential for the formation of the complex at the Sp1-binding site.

The results of gel mobility shift assays suggest that nuclear factors bind to the CACCC box (bands b and c in Fig. 5A). The binding of the factors was affected by the introduction of mutations into the CACCC box (Fig. 5A) and subject to competition by the DNA fragment with the Sp1-binding motif (Fig. 5B). Based on these results, we suggest that the major factor that bound to the CACCC box of the hTS gene is Sp1 or an Sp1-related factor. This finding is consistent with the previous finding that Sp1 binds weakly to the CACCC box (31). The CACCC box of the gene for β-globin is reported also to be a target of EKLF, and the binding of EKLF is known to regulate tissue-specific expression of the gene (32). It will be of interest to examine whether the CACCC box of the hTS gene is a target for tissue-specific factors, including EKLF, because some growth-related genes, such as the gene for DNA polymerase β (35), have been reported to be expressed in a tissue-specific manner.

Two negative regulatory sequences were identified by CAT assays with the deletion mutants of the promoter of the hTS gene (Fig. 1), and the function of one of these sequences was confirmed by CAT assays with the cassette mutant of the promoter (M-NRS in Fig. 3). Negative regulation of the expression of the TS gene should be important, because it is necessary that the level of thymidylate in cells should remain constant throughout the cell cycle and the overexpression of the TS gene is toxic to cells, as is a defect in the expression of the gene. Comparison of the sequences of these two negative regulatory regions revealed the presence of a consensus sequence, TTCCCA (Fig. 7). The TTCCCA sequence is included in the consensus motif of the E2F-binding sequence (36), which is important in
the regulation of transcription that is dependent on the stage of the cell cycle. Many genes expressed in the G1-S phase, such as genes for dihydrofolate reductase and thymidine kinase and the mouse TS gene, have E2F-binding motifs in the promoter region (36). The hTS gene also has a potential E2F-binding site in the mouse TS gene, have E2F-binding motifs in the promoter region of the gene.

In conclusion, we have identified the basic functional motifs in the promoter region of the hTS gene. Among these motifs, the Sp1-binding motif was identified as a core promoter motif or a related motif functions as a negative motif in the regulation of the hTS gene.

REFERENCES

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