Molecular Dissection of Human Thymidylate Synthase Gene in Relation to its Cell Cycle-Dependent Expression

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要　旨

チミジル酸合成酵素（thymidylate synthase，略称ＴＳ）は、DNA合成に必要な前駆体dTMPを*de novo*で合成する唯一の酵素で、細胞の増殖に必須な酵素の一つである。この酵素はDNA合成を律速していること、また、活性を阻害または欠損すると種々の生物学的、遺伝学的異常が引き起こされることなどが報告されている。

全長約16kbにおよぶヒトＴＳ遺伝子は当研究室の金田らによって単離され約1.6kbのcDNAとともに全塩基配列が決定されている。ヒトＴＳ遺伝子は７つのエクソンと６つのイントロンより構成されている。他の増殖関連遺伝子と同様に、プロモーター領域は、GC含量が高くCAAT box、TATA boxが見あたらず、転写開始点も複数存在する。

当研究室の鮎沢（現東大応微研）らによって報告されているように、ヒト正常２倍体繊維芽細胞内でのＴＳの発現は、細胞を低血清培地中で休止期に同調するとＴＳmRNA量、酵素活性ともに非常に低い値を示すが、次に血清を加えて増殖期に誘導した場合、DNA合成開始に先だって両者とも急激に増加し休止期の２０倍近くまで達する。しかし、単離核を用いた*in vitro*実験によると休止期と増殖期で転写活性はほとんど変化せず、このようなＴＳmRNA量、ＴＳ酵素活性の上昇は転写後の過程で調節されていることが強く示唆されている。

私は、以上のようなヒトＴＳ遺伝子の細胞周期の進行に伴った発現制

御の機構を解明することを最終目標として、まず遺伝子上のそのような発現制御に関わる領域を同定することを試みた。そのために、ヒトＴＳ遺伝子から各種ミニ遺伝子を作製し（図参照）、細胞周期の同調が容易なラット3Y1TS欠損株(3Y1TS-6)が既に得られていたので、これにミニ遺伝子を安定に導入し、得られた形質転換体でのヒトＴＳ遺伝子の発現を調べた。なお、各ミニ遺伝子について３株以上の形質転換体クローンを用い、クローンによる大きな違いがないことを確認している。

まず、上記形質転換細胞を低血清培地中で休止期に同調し（G0期）、高血清培地に移すことにより増殖刺激を与えた。DNA合成が最大になる21時間後（S期）およびG0期細胞の各々から全RNAを調製しノーザンブロット解析によりＴＳmRNA量を調べた。

オカヤマ-バーグ法によるcDNA発現ベクターに組み込んだヒトＴＳcDNA、pcHTS-1（SV40由来のプロモーターとポリＡ付加シグナルを含む3'非翻訳領域をもつ）では、G0期からＳ期へのＴＳmRNA量の変動はみられず、G0期、S期ともに高い値を示した。このことから翻訳領域には細胞周期に依存した発現を制御する配列はないと考えられる。

また、ヒトＴＳ遺伝子からイントロン１以外のイントロン２から６を除いたもの、すなわち、約4kbの5'上流域、翻訳領域、イントロン１と約1.7kbの3'下流域からなるミニ遺伝子pmHTS-1では正常遺伝子と変らない発現パターンを示した。しかし、pmHTS-1からイントロン１を除いたミニ遺伝子pmHTS-0では、G0期からＳ期へ進行するとＴＳmRNA量は増加

するものの、S期においては、pmHTS-0のほうがpmHTS-1よりＴＳmRNA量が少なかった。さらに興味深いことに、G0期において、ＴＳmRNA量はpmHTS-1ではほとんど検出されない程度であるが、pmHTS-0でははっきり検出できた。

さて、pmHTS-0でも弱いながら細胞周期に依存した発現がみられたので、これを制御している領域は、5'上流域または3'下流域であると考えられる。これを確かめるためにpcHTS-1とpmHTS-0から各々半分をつなぎ合わせたキメラミニ遺伝子を作製し3Y1TS-6細胞に導入した。キメラミニ遺伝子とは、5'上流域がpmHTS-0由来で3'下流域がpcHTS-1由来のpmcHTS-0と、それと逆の構造のpcmHTS-0である。得られた結果は、pmcHTS-0はpmHTS-0と、また、pcmHTS-0はpcHTS-1と同様であった。これにより、pmHTS-0において細胞周期に依存した発現を制御している領域は、5'上流域であることが判明した。

それでは、イントロン１にはpmHTS-0の5'上流域の役割を強化する領域が存在すると考えられるが、単独で働き得るのかが疑問となった。そこで、細胞周期に依存しない発現パターンを示すpcHTS-1にイントロン１をエクソン内の本来の位置に挿入したpcHTS-1-i1を作製し、形質転換細胞内での発現を調べた。その結果は、pmHTS-0で得られたものと同様であった。このことはイントロン１単独でも制御能力を持つが、しかし充分な制御をなし得ないことを示している。

以上のことから、G0期からＳ期に進行するときのヒトＴＳmRNA量の

正常な調節には、TS遺伝子の5'上流域とイントロン１の両者が同時に必要であることが判明した。

次に、翻訳レベルでも発現制御が行われているかどうか、すなわちTSの酵素活性はmRNA量と対応しているかどうかを、各ミニ遺伝子をもった形質転換細胞で調べた。その結果、G0期からＳ期への変動パターンはmRNAと同様であった。

前述の通り、ヒト正常細胞内におけるＴＳの発現制御は、転写後の段階で行われていることが示唆されているが、ミニ遺伝子ではどうであろうか。各形質転換細胞より核を単離し、nuclear run-on解析を行い転写活性の変化を調べた。このときプローブとしてＴＳcDNAのエクソン１とエクソン２から６の２つの断片に分けて用いた。その結果、G0期とS期においてエクソン２から６のプローブによって得られるシグナル強度に差はなかった。このことから、どのミニ遺伝子においてもG0期、S期に関わらず一定速度で転写されていること、また、イントロン１内で転写が阻害されていないことが明かになった。さらに、actinomycinDを用いた追跡実験から、G0期とS期においてＴＳmRNAの半減期は同様であることも確認した。これらのことは、ＴＳ遺伝子の5'上流域とイントロン１によるmRNA量の調節は、一次転写産物から成熟mRNAに加工される間に行われることを示唆している。特に、pmHTS-1による形質転換細胞内ではG0期においてＴＳmRNAがほとんどないことから、G0期には5'上流域とイントロン１を介した成熟mRNAの生成を抑えるメカニズムが存在すると考

えられる。

以上の結果をまとめると、各種のヒトＴＳミニ遺伝子をラット3Y1TS欠損株に安定に導入したとき、ヒトＴＳ遺伝子の発現は、5'上流域·第一イントロンのいずれか一方のみでも存在すれば細胞周期依存性を示し、さらに両者が共存すれば正常に発現制御されることが明らかになった。また、この調節は、ヒト正常細胞で得られた結果と同様に、転写後におけるmRNA前駆体のプロセッシング段階で行われていることが示唆された。

両領域の協同作業がどのような仕組みで行われるのかを明かにすることが、次の研究課題であるが、このことは増殖関連遺伝子の発現調節におけるイントロンの積極的な役割を証明する先駆的研究となるであろう。

ABSTRACT

I have determined the regulatory regions responsible for the cell cycle-dependent expression of the human thymidylate synthase (TS) gene, using a variety of chimeric minigenes constructed from segments of the human TS gene and the cDNA clone. Each construct was introduced stably into a TS-negative mutant of rat fibroblast 3Y1 cells. By serum-restricted synchronization of the cloned transformant cells, I found that the genomic 5'-flanking region and intron 1 without other introns were sufficient for the normal extent and pattern of the S-phase specific expression at the levels of both mRNA and enzymatic activity. A TS cDNA clone driven by an SV40-based expression vector showed constitutive expression. A chimeric cDNA clone in which intron 1 was inserted in the normal location, or in which the viral 5'-promoter region was replaced by the genomic 5'-flanking sequence showed S-phase dependent expression, but in both cases to an insufficient degree compared with that obtained with the above minigene. Results obtained by nuclear run-on assay showed that this regulation was largely due to posttranscriptional events that are consistent with our previous results for the *bona fide* TS gene in normal human diploid fibroblasts (Ayusawa, D., et al. J. Mol. Biol.190: 559-567, 1986).

INTRODUCTION

Thymidylate synthase (TS,EC 2.1.1.45) catalyzes the only pathway for the *de novo* synthesis of dTMP (Fig.1), and plays a key role in balancing the four nucleotide precursors for DNA replication. Impairment of this enzyme causes various biological and genetic abnormalities, such as thymineless death (6), expression of heritable fragile sites (32), and genetic recombination (8,31,64), possibly through induction of a particular type of DNA double-strand breaks (6)

Kaneda and Ayusawa et al. have isolated genomic DNA clones covering the entire human TS gene and its cDNA clone, and determined their complete nucleotide sequences (7,39,71). The general organization and its exon positions are similar to those of the mouse gene (19). The amino acid sequences of the human and mouse proteins are highly homologous (89%). However, the 5' untranslated region of the human TS mRNA contains an unusual structure consisting of triple tandem repeats of a 28bp sequence and an inverted repeat which can form three alternative stable

stem-loop structures (72). The 5' upstream regions of the two genes are G+C-rich and lack canonical transcriptional signals, such as a TATA box, and CAAT box, as it is the case in some other late-reponse genes mentioned below.

Cells proliferate through cell cycle that is classified into G_1 (gap 1), S (DNA synthesis), G_2 and M (mitosis) phase. Cells not proliferating and/or differentiated are known to be off the cell cycle, in the narrow sense, from the G_1 phase and in the resting (G_0) phase that is genetically identified from the G_1 phase (33). Signals to cause transition of the resting cells to proliferation exercises a temporal program of gene expression (2,21, and reviewed in ref.58). Genes under the program can be classified into three groups. The first group is genes expressed only or preferentially in the resting cells such as fibronectin gene (29). The second and third groups are so-called early- and late-response genes, respectively. The expression (production of mRNA) of early-response genes does not require protein synthesis and is frequently super-induced by cycloheximide. Late response genes generally require prior protein synthesis, as cycloheximide prevent their

expression.

The well-characterized early-response genes are c-*fos* and c-*myc*. Within 15 min of serum addition, the serum response element (SRE) in upstream of the c-*fos* gene mediates transcriptional activation which results in a transient increase in the level of c-*fos* mRNA (26,44,45,65,70,75). Sequences in the 3' end of c-*fos* mRNA contribute to its short half-life, which enables the steady-state of mRNA level to reflect the transcriptional activity of the gene (75). The induction of c-*fos* is closely followed by a transient increase of the expression of c-*myc*. Both transcriptional and posttranscriptional mechanisms contribute to the induction of c-*myc* mRNA (11,18,26). There is no SRE in the c-*myc* promoter. However, c-*myc* promoter can confer an appropriate serum response upon heterologous gene and this capacity may involve the E2F-binding sites in this promoter (53). The consensus sequences of the SRE and the E2F-binding site are found in 3'-half of the intron 1 and 5'-untranslated region of human TS gene, however, their functions are unknown.

Many genes encoding enzymes and proteins involved in nucleotide metabolism or DNA replication are the late-response genes, which may well possibly be under *trans*-acting gene products in the cascade of signal transduction *via* early response genes. Histone genes are in the class of them. Several *cis*-elements have been implicated in mediating S-phase-specific expression of histone genes (reviewed in ref.63). They are in the promoter region and the 3' end of the genes. There are major two differences between histone gene and the other late-response genes involved in DNA synthesis as given in the next paragraph. First, the promoters of the histone genes have a TATA box. Second, inhibition of DNA synthesis in the S phase causes rapid degradation of histone mRNAs, to which the stem-loop structure in 3' end of the mRNA is required.

TS gene is also one of the late-response genes. The TS activity and mRNA content are associated with cell proliferation. They are almost null in non-cycling cells, but increase sharply at the G_1-S border to reach about 20-fold in S phase during serum-induced transition from the resting (G_0) phase to the S phase in human diploid

fibroblasts (Fig.2 and ref.9). However, nuclear run-on analysis revealed that the transcriptional rate of the TS gene increased at most only two times in the above cell-cycle transition. This suggests that the human TS mRNA level is regulated by posttranscriptional events (9).

Similar cell-cycle dependent increases of mRNA and enzyme activity have been observed in some other late-response genes involved in DNA synthesis, such as dihydrofolate reductase (DHFR) (30,50,77), thymidine kinase (TK) (17,50,69), DNA polymerase α (76) and proliferating cell nuclear antigen (PCNA) (1,12,35). The several genes, including the mouse TS gene, have been reported to be regulated both at transcriptional and posttranscriptional levels (17,28,30,36,40,47,49,59,62,68). The regulatory regions of those genes have been reported to be in the promoter (TK, DHFR, DNA pol.α) (25,41,42,48,59,61,74), transcribed sequence including cDNA (TK) (47,68,74) and intron(s) (PCNA, mouse TS) (46,57). All enzymes mentioned above were reported to form the multienzyme complex particularly in the S phase (56,60). This suggests that the apparent cell cycle-

dependent expression of these genes must be same in spite of differences in gene regulation. The promoters of these genes can give serum inducibility upon its cDNA and/or heterologous genes, though not to a full extent (22,46,57,59,74). These findings suggested that the posttranscriptional events are indispensable for the cell cycle-dependent expression. Recently it was reported that the CCAAT element and its flanking sequence in human TK promoter were important for S-phase-specific regulation (41,42,46,61) and that cell cycle-specific nuclear protein(s) interacted with TK promoter(23,43). The CCAAT element is not found in human TS promoter region. The detailed research on the other regulatry regions has not been reported.

TS gene is expressed with the onset of DNA synthesis, which is the terminal representation of a signal transduction for proliferation. The aim of my study is to determine the regulatory regions in the human TS gene responsible for this growth-dependent expression.

Ayusawa et al. have also established the TS-negative cell mutant (TS-6) from the rat 3Y1 cells (38), which has a

spontaneous reversion frequency as low as 10^{-6} and can easily be synchronized at G_0 phase by serum starvation and stimulated to proliferate by serum addition. With the advantageous use of the human TS gene of known structure and the stable TS-negative mutant cells, human TS minigenes were constructed from the genomic gene as well as from the cDNA clone. The minigene introduced stably into the mutant cells to be prototrophic transformant cells. The transformants would be analyzed in terms of the expression of the integrated minigene during the cell-cycle transition from the resting to the DNA synthetic phase.

In the present paper I showed that either the 5'-flanking sequence or the intron 1 can invest the constitutive expressing minigene with the S-phase dependent expression but both are required for full expression equivalent to that of the *bona fide* gene. I also showed that these expressions are regulated at posttranscriptional level.

MATERIALS AND METHODS

Enzymes and chemicals.

The sources of materials used in this work were as follows: [α-^{32}P]dCTP (3000Ci/mmol,1Ci=37GBq), [α-^{32}P]UTP (400Ci/mmol) and GeneScreen membrane sheets from Du Pont-New England Nuclear, random primer DNA labeling kit and RNase-free DNase I from Takara Shuzo (Kyoto), [5-^{3}H]dUMP (18Ci/mmol) and aqueous counting scintillant ACS II from Amersham, ES medium from Nissui Seiyaku (Tokyo) and fetal calf serum (FCS) from Biocell Laboratory (Carson,CA). Phenol was saturated with Tris-HCl (pH 8.0) before use.

Cell lines and culture conditions.

TS-negative rat 3Y1 fibroblastic cell mutants (thymidine auxotrophs) were isolated previously (39) by selecting cell clones growing in the presence of methotrexate (10μM), sodium *l-5-N*-methyltetrahydrofolate (1μM) and thymidine (20μM). One clone, TS-6, was cultured in ES medium containing 10% FCS and 20μM thymidine in 5%

CO_2/95% humidity at 37°C. Its prototrophic transformants were cultured in thymidine-free medium. For experiments, cells were cultured to subconfluency and made quiescent by treatment with 0.5% FCS for 4 days. The medium was changed on the second day. The quiescent cells were stimulated to proliferate in ES medium with 20% FCS. After 21 hr, the rate of [^{3}H]thymidine incorporation into the acid insoluble fraction reached a maximum in TS-6 cells and their transformants (data not shown). Therefore, I used cells at this time as S-phase cells.

Construction of minigenes.

A recombinant plasmid clone for the functional human TS cDNA clone (pcHTS-1) and the minigene (pmHTS-1) containing only intron 1 were described by Ayusawa et al. (7) and Kaneda et al. (39), respectively. pmHTS-0 was made by substituting a 0.7kb *Sph*I-*Asu*II fragment of the TS coding region of pcHTS-1 for a 2.4kb *Sph*I-*Asu*II fragment containing intron 1 of pmHTS-1. pcHTS-1-i1 was made by substituting a 2.4kb *Sph*I-*Asu*II fragment

containing intron 1 of pmHTS-1 for a 0.7kb *Sph*I-*Asu*II fragment of the TS coding region of pcHTS-1. pmcHTS-0 is a chimeric minigene consisting of the 5'-half (4kb, *Xho*I-*Apa*I fragment) of pmHTS-0 and the 3'-half (3.8kb, *Apa*I-*Sal*I fragment) of pcHTS-1 and pcmHTS-0 has the reverse structure. The 5'-flanking region of pmcHTS-0 is shorter than that (3.8kb) of pmHTS-0 due to deletion of a 0.4kb *Eco*RV-*Xho*I fragment. The structures of these minigenes are shown in Fig.3A.

Transformation of rat 3Y1 TS-6 cells.

3Y1 TS-6 cells are not dead but quiescent in thymidine-free medium. For efficient selection, I transfected a plasmid clone, pSV2neo with the minigene into the TS-6 cells by the calcium phosphate coprecipitation method described elsewhere (5). Transfected cells were cultured without selection for a day and then cultured in ES medium containing 10% dialyzed FCS and 100μg per ml Geneticin (GIBCO) for 3 days. Then thymidine prototrophic transformants were isolated after growing the cells in ES medium containing 10% dialyzed FCS for 7

days.

Northern blot analysis.

The quiescent cells and growth-stimulated cells were lysed on dishes in 2 ml of lysis solution [8M guanidine-HCl, 20mM sodium ethylene diamine tetraacetate (EDTA), 1mM dithiothreitol and 10mM sodium acetate, pH 5.2] per 10 cm-diameter dish. The lysates were collected into centrifuge tubes with the aid of policeman. Then the lysates were sonicated for 30 to 60 seconds to shorten the DNA strand, mixed with 0.6 vol. of ethanol and stored at -20 °C for 3 hr. In this condition DNA was not precipitated. The RNAs were collected by centrifugation at 15,000 $\times g$ for 20 min, dissolved in the above lysis solution and precipitated with ethanol as above. The RNA pellets were rinsed with 80% (v/v) ethanol, dried and dissolved in 1mM EDTA (pH 8.0) containing 0.1% (w/v) sodium dodecyl sulfate (SDS) at 65°C. RNAs were extracted by mixing with an equal volume of phenol/chloroform/isoamylalcohol (25:24:1, by vol.), precipitated by addition of 2 vol. of ethanol after addition

of NaCl to a final concentration of 0.1M and stored at -80°C for more than 2 hr. Total RNAs collected by centrifugation at 17,000 $\times g$ for 20 min, washed with 80% ethanol, dried and dissolved in 1mM EDTA. Total RNAs were quantitated by measuring absorbance at 260 nm. The RNA samples were separated by electrophoresis in 1.2% agarose containing formaldehyde, as described previously (9). The RNAs were blotted onto a GeneScreen membrane in a buffer of 0.1M Na_2HPO_4/NaH_2PO_4 (pH 6.5) in a VacuGene Vacuum Blotting system (Pharmacia, Sweden) after partial alkaline hydrolysis of the RNA as described in the supplier's manual. Then RNA on the membrane was crosslinked by UV-treatment and hybridized to a labeled probe prepared with a random primer labeling kit with [α-^{32}P]dCTP. Prehybridization was omitted and hybridization was performed in hybridization buffer [50% formamide, 5×SSC (SSC is 0.15M NaCl and 0.015M sodium citrate), 0.02% polyvinyl-pyrrolidone, 0.02% bovine serum albumin, 0.02% Ficoll, 1% SDS, 100 μg sonicated and denatured salmon sperm DNA per ml] for more than 16 hr at 42°C. Then the membrane sheet was washed twice with

2×SSC at room temperature, twice with 2×SSC containing 1.0% SDS for 30 min at 65°C and twice with 0.1×SSC for 30 min at room temperature. Then, membranes were autoradiographed on an X-ray film backed with an intensifying screen at -80°C or exposed to an imaging plate and analyzed with FUJIX BAS 2000 (Fuji Photo Film, Tokyo). To rehybridize with a different probe, the probe was stripped by boiling the membrane for 30 min in 0.1×SSC containing 1% SDS. This condition was confirmed to give no signal on the membrane by autoradiography.

Assay of TS activity.

TS activity was assayed radiochemically by measuring tritium released from [5-^{3}H]dUMP by essentially the same procedure as described previously (4) . The crude extracts were prepared as follows. Cells on 2-3 of 10cm-diameter dishes were washed twice with ice-cold Ca^{2+} and Mg^{2+}-free phosphate-buffered saline [PBS(-)] and once with ice-cold Tris-sucrose [10mM Tris-HCl, 0.25M Sucrose,

20mM dithiothreitol], scraped off in 0.2-0.3 ml of ice-cold Tris-sucrose with a policeman and transferred into polyallomer microcentrifuge tube. Then the cells were disrupted by sonication on ice, followed by centrifugation at 100,000 $\times g$ for 1 hr at 4°C. The obtained supernatant was used as crude enzyme preparation. Protein contents of the supernatant was measured with BIO-RAD protein assay kit and stored at -80°C until used for assay of TS activity. The assay solution was prepared by mixing as follows: 90 μl of a solution [235mM Tris-HCl, pH 7.5, 25.8μM sodium tetrahydrofolate, 1.29%(v/v) formaldehyde, 75mM β-mercaptoethanol; stored at -125°C], 90 μl of 1M Tris-HCl (pH 7.0), 45 μl 1M sodium fluoride, 18 μl of 2.8mM dUMP, 21.6 μl of [5-^{3}H]dUMP (1mCi/ml,18Ci/mmol) and 530 μl of 1.25% bovine serum albumin. Reaction mixture consisting of 50 μl of the crude extract and 30 μl of the assay solution in a 2 ml-microcentrifuge tube was incubated at 37°C. The reaction was stopped after 60 min by the addition of 1ml of 2%(w/v) charcoal in 10mM HCl, followed by immediate mixing for 30 min. The charcoal was precipitated by centrifugation in the swinging bucket

rotor. 500 µl of the obtained supernatant was mixed with 5 ml of scintillation cocktail (ACS II) and the radioactivity was measured in a liquid scintillation counter.

Nuclear run-on assay.

The labeled nascent transcripts in the isolated nuclei were obtained essentially as described previously (9). Briefly, cells washed with ice-cold PBS(-) were scraped off from 2-3 dishes of 10 cm-diameter one, collected in PBS(-) by centrifugation, washed with ice-cold reticulocyte swelling buffer [10mM Tris-HCl (pH 7.4), 10mM NaCl, 3mM $MgCl_2$]. Cells were lysed by resuspending in the reticulocyte swelling buffer containing 0.5% Nonidet P-40 to be lysed. Nuclei obtained were washed once in this lysis buffer and resuspended by gently pipetting in 100 µl of an ice-cold solution of 40%(v/v) glycerol, 50mM Tris-HCl (pH8.3), 5mM $MgCl_2$, 0.1mM EDTA and stored at -80°C.

Reaction mixtures (200 µl) consisted of 100 µl of above nuclei suspension, 30% glycerol, 2.5mM $MgCl_2$, 30mM KCl, 2.5mM dithiothreitol, 0.25mM each of GTP and CTP,

0.5mM ATP, and 200μCi of [α-^{32}P]UTP. The mixture was incubated for 20 min at 26°C and then reactions were terminated by adding DNase I at 50μg/ml and the mixtures were further incubated for 5 min at 26°C. EDTA and SDS were added to a final concentration of 20mM and 1%, respectively and mixtures were incubated at 42°C with 40μg of proteinase K for 30 min. Then 100 μl of water and 100μg of yeast tRNA were added and the mixtures were extracted twice with phenol/chloroform/isoamylalcohol (25:24:1, by vol.) and twice with chloroform/isoamylalcohol (24:1, v/v) and precipitated with 2 vol. of ethanol. The precipitate was resuspended in 200 μl of 10mM Tris-HCl (pH 8.0), 15mM $MgCl_2$, 1mM EDTA and incubated for 60min with 10μg of DNase I at 26°C. EDTA, SDS and proteinase K were added as above, incubated for 60min and extracted once with phenol/chloroform/isoamylalcohol and chloroform/isoamylalcohol. The aqueous phase was mixed with 1ml of ice-cold 10%(w/v) trichloroacetic acid (TCA) containing 30mM sodium pyrophosphate and chilled on ice for 15min. The precipitate was collected by

centrifugation, washed with 5% TCA containing 10mM sodium pyrophosphate, dissolved in 200 µl of 0.1M sodium acetate (pH 7.0) and precipitated with ethanol.

The precipitate was dissolved in 50 µl of 10mM Tris-HCl (pH 7.5), 5mM EDTA, 1% SDS and the samples were used to quantitate specific gene transcription by filter hybridization. The following double stranded DNA probes for detection of nascent transcripts were denatured and immobilized by UV-treatment on GeneScreen: 0.12µg or 0.2µg of *Bgl*I-*Eco*RI fragment (BE in Fig. 3B, 178bp) and 0.54µg or 0.9µg of *Eco*RI-*Hpa*I fragment (EH in Fig. 3B, 935bp), respectively, of human TS cDNA and 0.6µg or 1.0µg of the cDNA fragment (1.3kb) of human glyceraldehyde-3-phosphate dehydrogenase (73) and 0.6µg or 1.0µg of λ phage DNA. The filters were prehybridized in the hybridization solution at 42°C for more than 3 hr and hybridized to the labeled RNA (1×10^6 cts/min per sample) at 42°C for 48 hr in 1 ml of the hybridization solution. The hybridization solution and washing conditions were the same as those for Northern blot analysis. Then, the membranes were washed and exposed

to an imaging plate and the hybridizing signals were analyzed with FUJIX BAS 2000 (Fuji Photo Film, Tokyo).

RESULTS

Construction of minigenes.

Previously, Ayusawa et al. isolated a functional human TS cDNA clone, pcHTS-1 which was under the control of SV40 early promoter and its 3'-flanking region (7). They also isolated recombinant phage clones covering the human TS gene (72). By the use of these clones, they constructed a minigene pmHTS-1 which consisted of 3.8kb-long 5'-flanking and 1.7kb-long 3'-flanking region of human TS gene, coding region and intron 1 in the normal location in the coding region. This minigene functions normally with respect to cell cycle-dependent regulation when stably introduced into the rat 3Y1 TS-6 cells. In contrast, the level of TS mRNA driven by pcHTS-1 was constant throughout the cell cycle (39).

To determine the regulatory regions in the human TS gene, responsible for its cell cycle-dependent expression, I constructed several kinds of chimeric minigenes by combination of fragments derived from a cDNA clone, pcHTS-1 and from pmHTS-1, as shown in Fig.3A.

pmHTS-0 is identical with pmHTS-1 except that it does not have intron 1 and pcHTS-1-i1 is identical to pcHTS-1 except it has intron 1 in the normal location in the coding region. pmcHTS-0 is a chimeric minigene consisting of the 5'-half of pmHTS-0 and the 3'-half of pcHTS-1, and pcmHTS-0 is *vice versa.*

Cell cycle-dependent expression of minigenes

I transformed rat 3Y1TS-6 cells stably with each of the chimeric TS minigenes. In each case at least three independent transformed lines were isolated and analyzed. The exponentially growing cells were made quiescent in low serum medium ("the resting cells"). Then the resting cells were growth-stimulated ("the S-phase cells") in medium with 20% FCS for 21 hours, when the rate of [^{3}H]thymidine incorporation into the acid insoluble fraction reached a maximum. The human TS mRNA levels in the resting and the S-phase cells were determined by Northern blot analysis.

Since three or more independent transformants with each minigene gave similar expression pattern, I showed

the results with one clone of each type. However, the variations of TS mRNA levels among transformed lines with pmHTS-0 or pcHTS-1-i1 tended to be greater than those in clones transformed with pmHTS-1 or pcHTS-1, the reason of which is unknown. The mRNA level of pcHTS-1 in resting cells was seem to be about twice of that in growing cells (Fig.4A, upper panel, lanes 7 and 8). Since the total RNA content per cell doubles during traverse from the resting state to the S phase, It is considered that the production of TS mRNA by pcHTS-1 per cell is constitutive. On the other hand, the level of TS mRNA expressed by pmHTS-1 was growth-dependent since S-phase cells gave a stronger band of mature mRNA (faster migrating band) than resting cells (Fig.4A, upper panel, lanes 5 and 6). Although pmHTS-0 also gave the S-phase dependent expression, the TS mRNA level of pmHTS-0 was higher in the resting cells and lower in the S-phase cells than that of pmHTS-1 (Fig.4A, upper panel, lanes 3 and 4). TS gene dosage of transformants with pmHTS-0 was not so many as that with pmHTS-1 (data not shown). This difference should be ascribed to the absence

of intron 1 in the pmHTS-0. The higher molecular weight bands seen in lanes 3 to 6 in the upper panel of Fig. 4A were characterized by rehybridizing the same filter to the probe from intron 1 or the 3'-flanking region distal to the poly(A) attachment site (see Fig.3B). The probe for intron 1 (I-1) hybridized to only the higher molecular weight bands derived from pmHTS-1 (Fig. 4A, middle panel), while the probe for the 3'-flanking region (3'FR) hybridized to the bands from both pmHTS-1 and pmHTS-0. Thus these higher molecular weight RNAs were transcribed from minigenes, but not processed properly (See discussion.). The levels of these higher molecular weight RNAs were not affected significantly during chase for eight hours in the presence of actinomycin D (10 μg/ml), while those of mature mRNAs degraded to about half of their original levels during the same treatment (Fig 5 A and B). Fig.5A also showed the half-life of TS mRNA in the resting cells is about 6 hours, as similar to that in the S-phase cells.

As the expression of TS in the pcHTS-1-transformant cells was constitutive, the cell cycle-dependent expression

observed in the case of pmHTS-0 should be due to its 5'- and/or 3'- flanking genomic sequence in this minigene. To clarify which of the two regions is necessary for the cell cycle-dependent expression, chimeric minigenes pmcHTS-0 and pcmHTS-0 were prepared. The former having the genomic 5'-flanking sequence and the viral 3'-flanking sequence, and the latter *vice versa* (Fig.3A). Fig.4B showed that the mRNA level of pmcHTS-0 increased in growing cells (lanes 1 and 2), whereas the mRNA level of pcmHTS-0 did not (lanes 3 and 4). This indicates that the 5'-flanking sequence, but not the 3'-flanking sequence, is responsible for growth-dependent increase of TS mRNA in the intronless minigene pmHTS-0.

Function of intron 1.

The relative increase of mRNA in the transition from Go to S phase was greater in transformed cell with pmHTS-1 than with pmHTS-0. Thus intron 1 plays a regulatory role in controlling the level of TS mRNA in the cell-cycle. To confirm this role of intron 1, I inserted intron 1 into pcHTS-1 (TS cDNA driven by an SV40

promoter) to see if its constitutive nature of expression would be converted to an inducible one. This construct was designated as pcHTS-1-i1 (Fig.3A). To my surprise, pcHTS-1-i1 acquired cell cycle-dependent expression to some extent as shown in Fig.4C. This ought to be the first report that the intron by itself contributes to cell cycle-dependent expression. The mRNA levels in both the resting and growing cells were lower than those with pcHTS-1. Some of the extra minor bands seen in the case of pcHTS-1-i1 may be products of the abnormal splicing between a splicing donor in the 5'-untranslated region of the SV40 sequence and the splicing acceptor at the end of intron 1 of the TS gene.

Thus, the human TS mRNA level seems to be regulated cooperatively by the 5'-flanking region and intron 1 of the gene. It should be emphasized, however, that either one of these two regions alone can exert cell cycle-dependent expression.

Human TS enzyme activities in transformant cells.

The TS activities in the transformants were examined

to find whether there are regulations at the translational or post-translational level. Fig.6 showed that the level of TS activity was essentially paralleled with that of the TS mRNA in each case, suggesting that TS gene expression was mainly regulated before translation steps. However, among the transformants we noticed, the mRNA level in cells with pcHTS-1 was the highest (Fig.4A, upper panel), but their TS activity was not the highest. These results could be due to particular 5'-untranslated sequences of the human TS mRNA (see Discussion).

Transcription of TS minigenes in isolated nuclei of the transformant cells.

It was previously shown by the nuclear run-on assay that the transcription rate of the TS gene was not altered between the resting and growth-stimulated normal human diploid fibroblasts (9). Therefore, I examined the transcription rates of the minigenes in rat 3Y1 transformant cells with the same assay system. To detect both the full and immature length transcripts, I used two probes, BE and EH fragments which cover exon 1, and

exons 2 to 7, respectively (Fig.3B). I also used λ DNA and human glyceraldehyde-3-phosphate dehydrogenase cDNA (73) as probes for non-specific hybridization and internal control, respectively. As shown in Fig.7, the pattern of hybridization signals did not alter among pmHTS-0, pmHTS-1 and pcHTS-1. The intensities of the hybridization signal to EH fragment were essentially the same in the resting and the S-phase cells. Since the EH fragment represents the sequence downstream of intron 1, transcription seems to have proceeded normally beyond intron 1 during the resting phase as in the S-phase cells in all cases. The hybridization signal to the BE fragment was unfortunately below the level of detection, probably due to its shorter sequence and the fewer of U bases to be labeled in this region. These data indicated that cell cycle-dependent expression of the human TS minigene in rat 3Y1 TS negative cells is regulated posttranscriptionally, consistent with previous observations in normal human diploid cells (9).

DISCUSSION

Cell-cycle regulation of human TS minigene in rat 3Y1TS-6 cells.

Previously, Ayusawa et al. reported that the 20-fold increase in the TS mRNA level after growth stimulation of human diploid fibroblasts was mainly due to posttranscriptional events (9). Posttranscriptional regulation of TS has also been shown in mouse cells (36). Among the various human TS minigenes expressed in rat 3Y1TS-6 cells, pcHTS-1 was expressed constitutively during cell-cycle progression, in which TS cDNA was driven by SV40 promoter and 3'-flanking sequence. This shows that the coding region of human TS gene is not concerned with its cell-cycle dependent expression. However, pmHTS-1 showed a similar pattern and extent of expression to the *bona fide* gene in human fibroblastic cells. The expressions of the intronless minigene pmHTS-0, a chimeric minigene pmcHTS-0 and an intron-inserted cDNA pcHTS-1-i1 were also growth-dependent, though lesser so than that of pmHTS-1. The result obtained with

pcHTS-1-i1 ought to be the first evidence that the intron by itself contributes to cell cycle-dependent expression. Hence, I concluded that both the 5'-flanking region and intron 1 are necessary for normal expression of the TS gene. My conclusion is consistent with the report of Li et al. (46) that not only the promoter region but also some specific introns are required for a normal level of expression of the mouse TS gene. Unfortunately they did not measure the transcriptinal activity of mouse TS minigenes in each phase of cell cycle. Because, they exprimented with wild type mouse cells cotransfected with the minigene, in which endogenous TS gene was actively expressed, therefore the transcripts from the minigene could not be distinguished from that from the endogenous gene in the nuclear run-on assay. The results obtained with pmcHTS-0 suggested that the 3' region is not important for regulating the mRNA level or the cell cycle-dependent expressions. In this connection, it should be noted that mouse TS mRNA lacks the 3'-untranslated region (37).

A similar phenomenon has been reported for the

PCNA gene. Ottavio et al. (57) reported that intron 4 is necessary for proper regulation of the PCNA mRNA level. Removal of intron 4 resulted in an abnormally high level of human PCNA mRNA in serum-deprived mouse 3T3 cells. Chang et al. (13) showed that there was very little difference between nuclear run-on transcription of PCNA minigenes in the Go phase and S phase, regardless of whether the minigenes contained intron 4. However, they did not discuss whether the intron 4 by itself is a functional element for the cell-cycle dependent expression of PCNA gene or not.

Are the human TS minigenes regulated at posttranscriptional level as well as the *bona fide* gene ? The rate of increase of mature TS mRNA during cell-cycle progression was similar to that of TS activity in each minigene (Fig.4 and Fig.7) This suggests that TS minigenes are essentially regulated before translational step. In the case of c-*myc* gene (10,54,78) and adenosine deaminase gene (14,15), transcription could be terminated in or near intron. The block of transcriptional elongation in c-*myc* gene was required a sequence in the promoter

(51,67). To examine whether the similar mechanism is involved in the expression of TS, I used two probes in nuclear run-on assay, a BE fragment corresponding to exon 1 and an EH fragment covering exons 2 to 7. My results shown in Fig.7 revealed the two evidences as follows; the first is that transcriptional arrest did not occur in or near intron 1 of pmHTS-1 in resting cells and the second is that the minigenes were transcribed in resting cells as well as in S-phase cells. These indicate that the cell-cycle dependency of the minigenes expression is controlled by events after transcription. As shown in Fig.5, the mature TS mRNA in the resting cells did not degrade more rapidly than in the S-phase cells. Thus, the cell cycle-dependent expression of human TS minigenes seem to be regulated at RNA processing step even in rat cells.

Role of intron 1.

I think that intron 1 has an interesting role in determining the level of human TS mRNA and that the function of intron 1 is different between in the resting

phase and in the S phase as discussed below.

What is the role of intron 1 in the resting phase ? One possible way is that the intron 1 sequence in the TS pre-mRNA is responsible for the rapid degradation occurring specifically in resting cells. The facts that the TS mRNA levels in resting cells with TS minigenes with an intronless construct, pmHTS-0 and pcHTS-1, were higher than those with pmHTS-1 and pcHTS-1-i1 (Fig.4A lanes 1 and 3, Fig4C lanes 1 and 3, respectively) favor this hypothesis. Two other possibilities are that intron 1 acts in resting cells as a transcriptional silencer and that transcriptional elongation is arrested or retarded in the intron 1 region. However, neither of these possibilities would apply in our study on the TS gene, because the resting cell transcribed from exon 2-7 as well as S-phase cells as described above.

In the S phase, the TS mRNA level expressed by pmHTS-1 was higher than that expressed by pmHTS-0 (Fig.4A lanes 4 and 6). Polyadenylation has been suggested to be stimulated *in vitro* by the presence of the nearest upstream intron (55). Therefore, one possibility is

that in the S phase pre-mRNA having intron 1 could be polyadenylated more efficiently than that without the intron and consequently be protected from degradation before maturation. In the case of the TK minigene, the presence of the first two introns increased the amount of human TK mRNA in the S phase in Syrian hamster fibroblasts, although the introns had no effect on the cell cycle-dependent level of expression of TK mRNA (48). In the case of chicken TK gene, introns are not required for efficient production of mRNA in mouse L cells (27).

Kaneda et al. showed interesting results about the function of intron 1 of human TS gene (Kaneda et al. submitted). Transformation efficiency of mouse FM3A TS negative mutant by pmHTS-0 was less than 1/10 of that by pmHTS-1. The effect of intron 1 on transformation frequency was further located at particular sequences within the intron 1. However, introduction of the intron 1 into pcHTS-1 (resulted in pcHTS-1-i1) did not stimulate its transformation frequency. The effects of the intron 1 on transformation frequency were also observed in 3Y1TS-6 cells (data not shown). The amounts of TS mRNA

produced by the minigene in S-phase cells may relate with its transformation efficiency. Kaneda et al. also showed that intron 1 positioned in upstream of promoter stimulated expression of a TS promoter-chloramphenicol acetyltansferase (CAT) gene construct but not that of an SV40 promoter-CAT gene construct. This suggested that a promoter-specific enhancer is present in the intron 1. And Deng et al. reported that particular introns within mouse TS minigene stimulated TS expression in transient transfection assays (20). In my results (Fig.7), however, intron 1 did not enhance transcriptional activity either in resting phase or in S phase. Only in transient state of minigenes before being integrated into chromosome, intron 1 could enhance transcription by some mechanism, such as inhibition of nucleosome formation.

Are they truly TS pre-mRNAs ?

The higher molecular weight bands detected in the Northern blots of transformants with pmHTS-0 and pmHTS-1 (Fig. 4A) were shown to have sequences which should not appear in the mature mRNA, suggesting that

they are primary transcripts of the minigenes. Higher molecular weight bands were detected at similar levels in cells in the G_0 phase as in the S phase. This fact is consistent with the results of nuclear run-on assay that transcription is active in the G_0 phase (Fig.7). However, it is unlikely that the bands in Fig.4A do represent active precursors to mature mRNA. Because, in a chase experiment in the presence of actinomycin D (10μg/ml) to repress transcription, the higher molecular weight bands did not degrade significantly, while mature sized mRNA bands did (Fig.5A and B). In northern blot analysis of mouse TK gene, TK pre-mRNAs could not be detected in resting cells and the most of them degraded rapidly within 1 hr in the presence of actinomycin D in S-phase cells though the mature TK mRNA did not (28). And little or no TK pre-mRNA could be detected in quiescent human diploid fibroblasts by a method using the polymerase chain reaction (49). It should also be noted that the higher molecular weight bands detected in the case of the two minigenes pmHTS-0 and pmHTS-1 migrated similarly although the transcripts of pmHTS-1 with the 1.6 kb-long

intron 1 should be longer than those of the intronless pmHTS-0 (Fig.4A). In the present study I did not characterize these higher molecular weight RNAs.

Translational regulation of TS.

Fig.6 shows that the change of TS activity during cell-cycle progression was essentially paralleled with that of the TS mRNA level in results with each minigene, suggesting that TS gene expression during cell-cycle progression was mainly regulated before translation steps. In TK gene, cell-cycle dependent translational and/or posttranslational regulation was reported. When human TK mRNA was expressed by a heterologous promoter in mouse cells (74) and rat cells (34), its enzyme activity was much higher in the S phase than in the resting phase, although its mRNA level was not. These are a contrast to my results with pcHTS-1, in which TS activity paralleled the TS mRNA level (Fig. 4A, upper panel; Fig. 6),

However, in each phase of cell cycle, the ratios of TS mRNA level to TS activity varied among minigenes. The fact that those of pcHTS-1 and -1-i1 was greater than those

of pmHTS-0 and -1 (Fig 4A, C and Fig.6) indicates that the chimeric TS mRNAs flanked with viral 5'- and 3'-sequences are translated with less efficiency than the native TS mRNA. Two reports (16,38) would give an answer to this phenomenon. TS mRNA transcribed from pcHTS-1 and probably pcHTS-1-i1 had additional SV40 sequences and deleted the 5' untranslated sequence inclusive of the inverted repeats of native TS mRNA (39,71,72). The native TS mRNA has an inverted repeat and triple tandem repeats in the 5' untranslated region. The directly repeated sequences in the TS mRNA transcribed from pcHTS-1 had inhibitory effect on translation of the mRNA (38). And interestingly, the addition of human TS protein to *in vitro* translation system with a rabbit reticulocyte lysate inhibited translation of TS mRNA transcribed *in vitro* from pcHTS-1 (deleting the inverted repeat) probably due to specific binding of TS protein to TS mRNA (16). Nobody showed the function of the all repeats including the inverted repeat in the native TS mRNA. The inverted one may have function to antagonize such inhibitory effect of the directed repeats on

translation.

In actively cycling cells.

I have discussed the regulation of human TS and other late-response genes to bring about 20-fold increase of their mRNA levels during transition from the resting phase to the S phase. In continuously cycling cells, however, only two- to threefold differences in the mRNA levels of these genes in the G_1 and S-G_2 phases were detected (24,52,66,76). These facts suggest that much mRNAs of these gene in the S/G_2-phase cells progressing to the G_1 phase are carried over into the G_1 phase while little mRNA is in the resting phase and in the followed G_1 phase. Therefore, the regulations of these genes in cycling cells could be different from those during progression from the resting to the proliferating phase.

A Milestone.

As reported here, human TS minigene pmHTS-1 was expressed in a cell cycle-dependent manner in rat cells in the same way as in human cells. This suggests that

common mechanisms for regulating the TS gene exist in different species. This human TS minigene/rat TS-negative cell system is simple and should be useful for elucidating cis- and trans-elements participating in posttranscriptional regulation of the TS gene. Studies on the molecular mechanisms of the posttranscriptional control of TS gene will present an interesting knowledge of a novel gene regulation and a better understanding of cell-cycle regulation of growth-dependent genes. Search for genes affecting TS gene expression would give an insight into signal transduction pathway associating cell-cycle progression.

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FIGURES

AND

FIGURE LEGENDS

Fig.1. dNTP metabolism.

Abbreviations: 1,CTP synthetase; 2, NDP kinase; 3, dCMP kinase; 4, dCMP deaminase; DHFR, dihydrofolate reductase; DNA pol, DNA polymerases; PCNA, proliferating cell nuclear antigen; RR, ribonucleotide reductase; TK, thymidine kinase; TS, thymidylate synthase

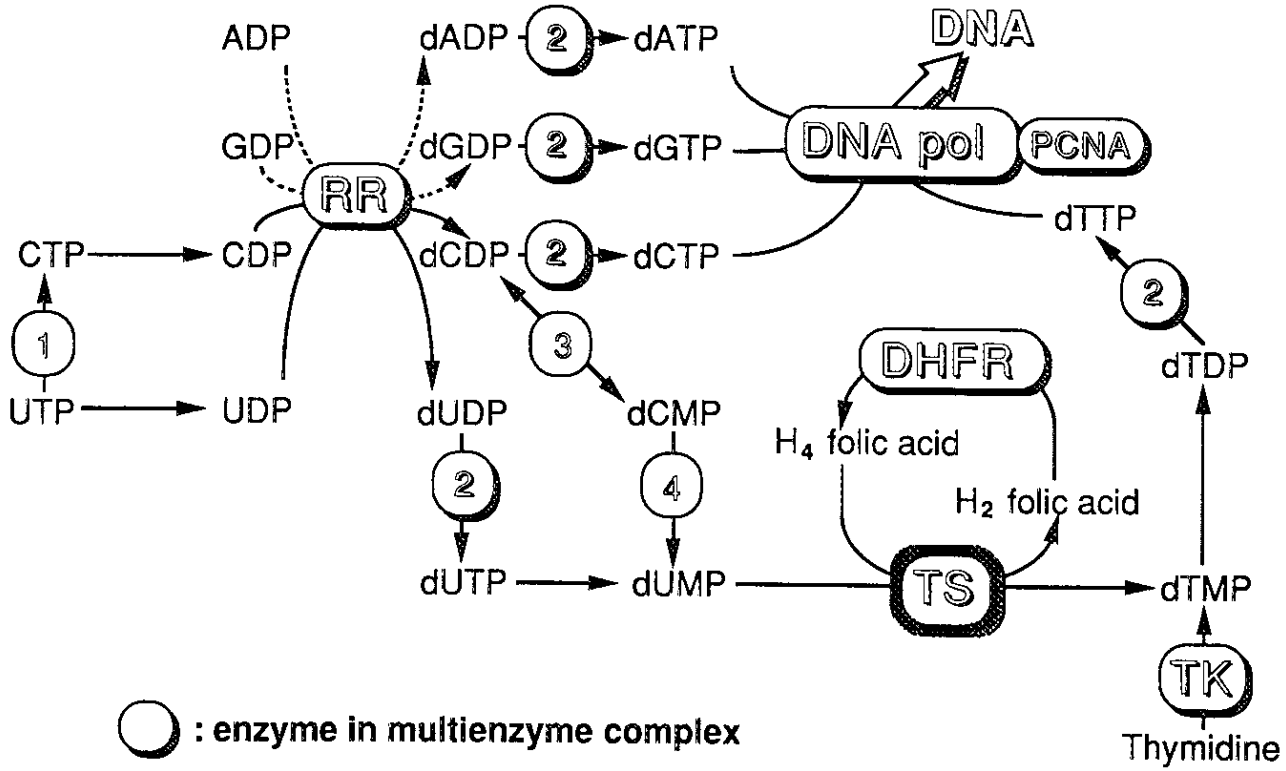

ADP
dADP
2
dATP
DNA
GDP
dGDP
2
dGTP
DNA pol
PCNA
RR
dTTP
CTP
CDP
dCDP
2
dCTP
2
1
3
DHFR
dTDP
UTP
UDP
dUDP
dCMP
H4 folic acid
2
4
H2 folic acid
dUTP
dUMP
TS
dTMP
TK
Thymidine
: enzyme in multienzyme complex

Fig.2. TS gene expression in normal human diploid fibroblasts.

Symbols: —●—, TS mRNA content of total RNA; —■—, TS activity; ---▫---, [^{3}H] thymidine incorporation; —□—, TS mRNA content of poly(A)$^+$ RNA; —▲—, nuclear run-on transcripts hybridizing with excess TS cDNA

Data taken from "**Ayusawa, D., K. Shimizu, H. Koyama, S. Kaneda, K. Takeishi, and T. Seno.** 1986. Cell-cycle-directed regulation of thymidylate synthase messenger RNA in human diploid fibroblasts stimulated to proliferate. J. Mol. Biol. **190**:559-567."

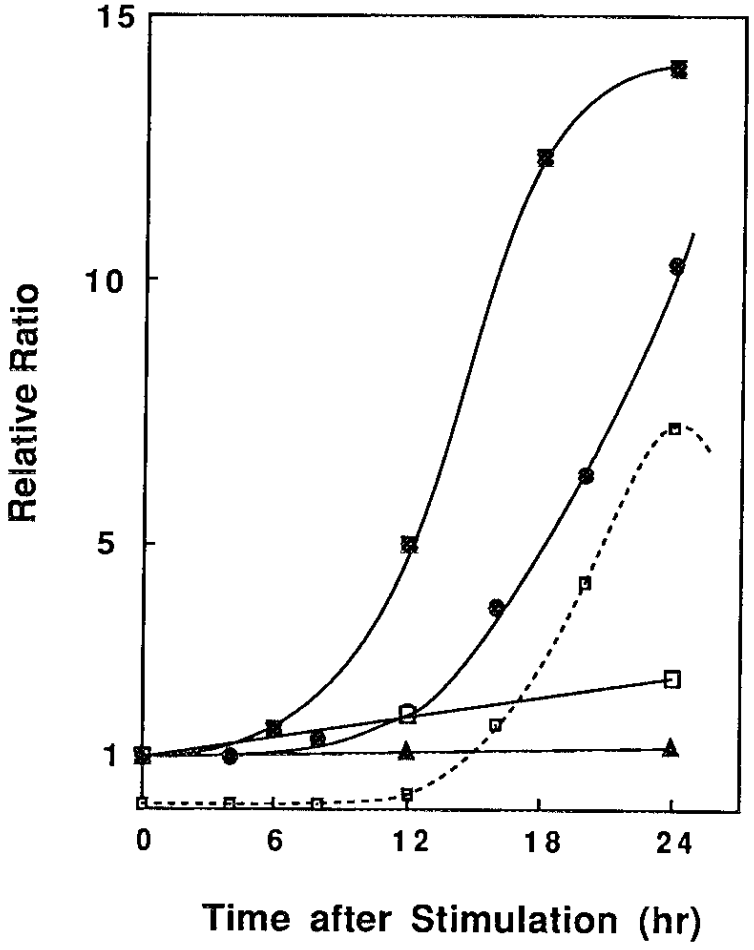

15
10
5
1
Relative Ratio
0
6
12
18
24
Time after Stimulation (hr)

Fig.3. (A) Structures of human TS gene and minigenes.

(B) The probes used for Northern blot analysis and nuclear run-on assays.

Symbols: ▓▓▓, TS coding region; □, untranslated region derived from TS cDNA; ▨, untranslated region derived from SV40; ▒▒▒, intron 1(1.7kb); ▬, 5'- or 3'-flanking region of TS gene; ≋≋≋, 5'- or 3'-flanking region derived from SV40; ——, pBR322 DNA. The human TS gene and all introns except intron 1 are not drawn to scale. BE is the portion of exon 1 and EH is the portion from exon 2 to 7 of cDNA fragment BH. Abbreviations: Ap, *Apa*I; As, *Asu*II; B, *Bgl*I; E, *Eco*RI; H, *Hin*dIII; Hp, *Hpa*I; RV, *Eco*RV; S, *Sal*I; Sp, *Sph*I; X, *Xho*I; (*Bcl*I/*Pvu*II), (S/X) and (X/S), restriction enzyme site lost by ligation.

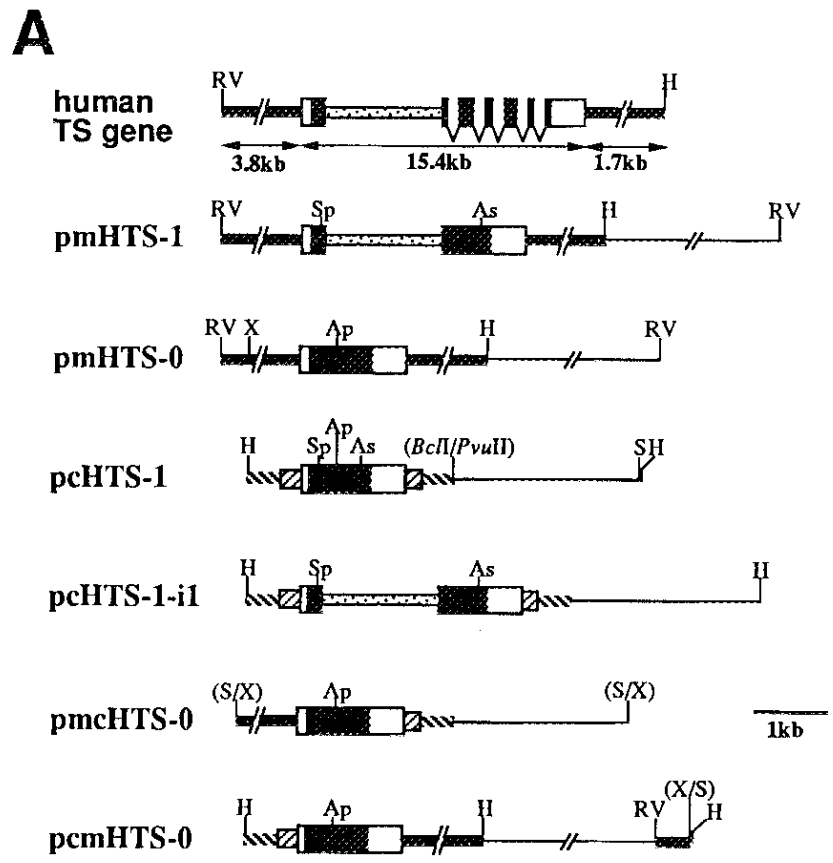

A
human
TS gene
RV
H
3.8kb
15.4kb
1.7kb
pmHTS-1
RV
Sp
As
H
RV
pmHTS-0
RV X
Ap
H
RV
pcHTS-1
H
Sp
Ap
As
(BclI/PvuII)
SH
pcHTS-1-i1
H
Sp
As
H
pmcHTS-0
(S/X)
Ap
(S/X)
1kb
pcmHTS-0
H
Ap
H
RV
(X/S)
H
B
B E
Hp
1kb
BH
BE
EH
intron 1
I-1
3'FR

Fig.4. Northern blots of TS mRNAs derived from the minigenes.

Experiments were performed as described in Materials and Methods. Total RNAs were prepared from resting (lanes 1,3,5 and 7) and S-phase cells (lanes 2,4,6 and 8) and loaded on each lane of 1.2% formaldehyde/agarose gel (20μg in A and B, 10μg in C). After electrophoresis, RNAs were partially hydrolyzed by alkali and transferred to a nylon membrane. The probes are shown in Fig.1B. Positions of ribosomal RNAs are indicated by ◀. (A), Total RNAs from TS-6 cells (lanes 1 and 2), its transformant with pmHTS-0 (lanes 3 and 4), pmHTS-1 (lanes 5 and 6), and pcHTS-1 (lanes 7 and 8) were hybridized to the BH fragment (upper panel). The probe was then stripped off, and the filter was rehybridized to fragment I-1 (middle panel) or fragment 3'FR (lower panel).

A

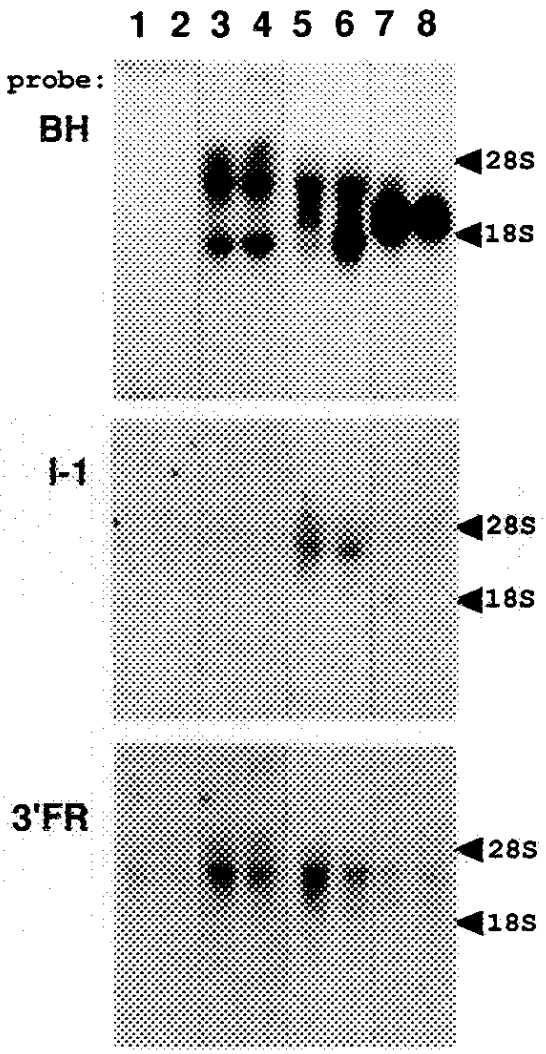
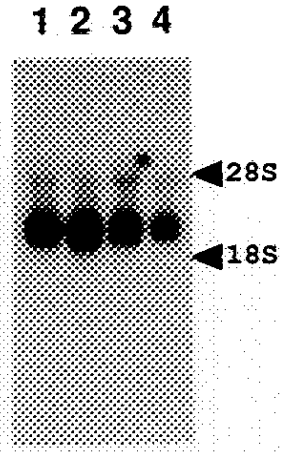


Fig.4. Northern blots of TS mRNAs derived from the minigenes. (continued)

(B), Total RNAs from transformants with pmcHTS-0 (lanes 1 and 2), and pcmHTS-0 (lanes 3 and 4) were hybridized to fragment BH. (C), Total RNAs from transformant with pcHTS-1 (lanes 1 and 2), and pcHTS-i1 (lanes 3 and 4) were hybridized to fragment BH.

B



C

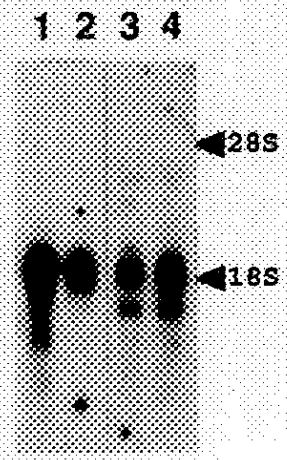


Fig.5. Northern blots of TS mRNAs in the presence of actinomycin D

The resting cells (lanes 1-8) and S-phase cells (lanes 9-16) were treated with actinomycin D (10μg/ml) for 0 min (lanes 1 and 9), 15 min (lanes 2 and 10), 30 min (lanes 3 and 11), 45 min (lanes 4 and 12), 1 hr (lanes 5 and 13), 2 hr (lanes 6 and 14), 4 hr (lanes 7 and 15) and 8 hr (lanes 8 and 16). Total RNA (20μg) at each time points was analyzed as described in Fig. 4. The membranes hybridizing with the labeled BH fragment were analyzed with FUJIX BAS 2000 (Fuji Photo Film, Tokyo). The results were printed out by Pictrography (Fuji photo film). **(A)**, Total RNAs from transformants with pmHTS-0. **(B)**, Total RNAs from transformants with pmHTS-1. **(C)** and **(D)**, photographs of gels stained with ethidium bromide before transfer of RNAs to membranes in (A) and (B), respectively. Data in this figure were obtained with the same clones as for Fig.4.

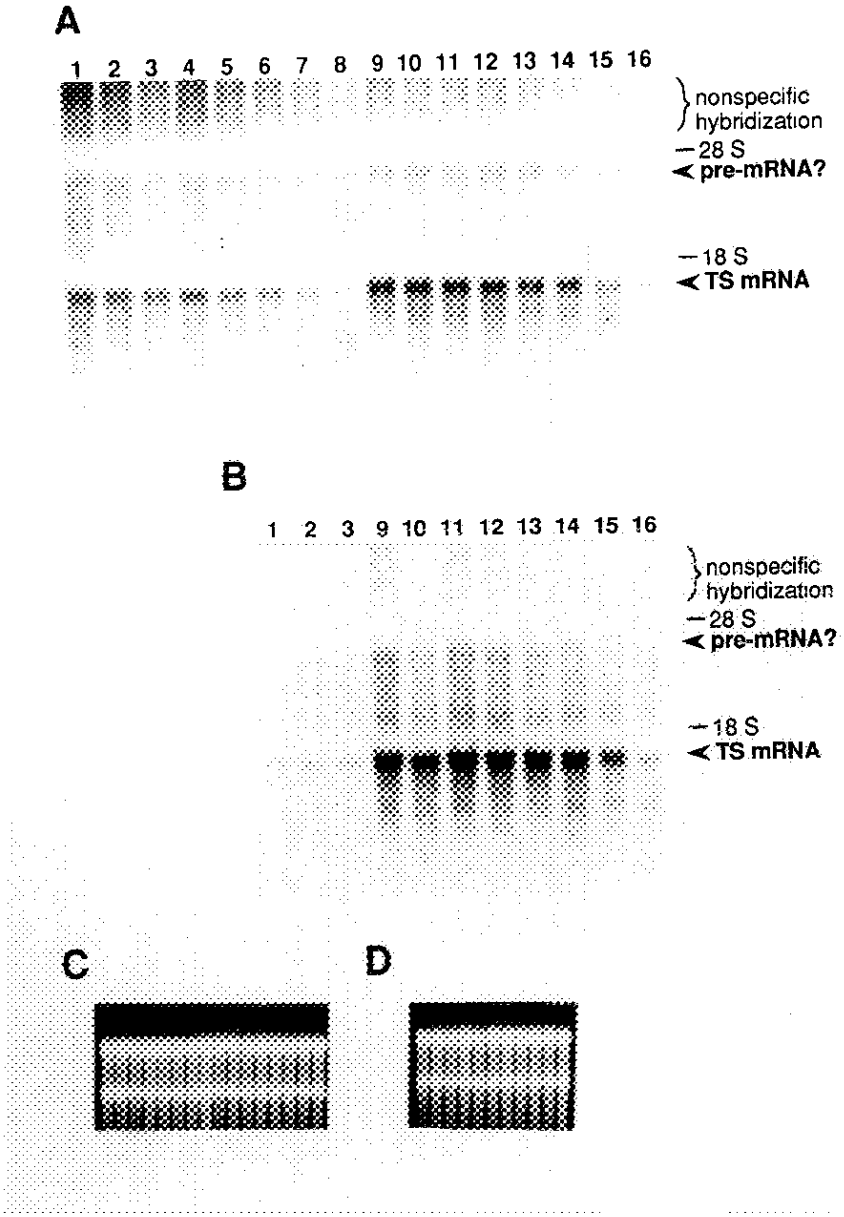

A
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16
nonspecific hybridization
28 S
pre-mRNA?
18 S
TS mRNA
B
1 2 3 9 10 11 12 13 14 15 16
nonspecific hybridization
28 S
pre-mRNA?
18 S
TS mRNA
C
D

Fig.6. TS activities expressed by the minigenes.

TS-6 (column 1) and its transformants with pmHTS-0, pmHTS-1, pcHTS-1,pcHTS-i1, pmcHTS-0, and pcmHTS-0 (column 2-7,respectively) in the resting phase (■) and S phase(▨) were sonicated and centrifuged at 100 × k*g* for one hour. The supernatants were used for assay of TS activities. Assays were performed in duplicate as shown in Materials and Methods. Data in this figure were obtained with the same clones as for Fig.4.

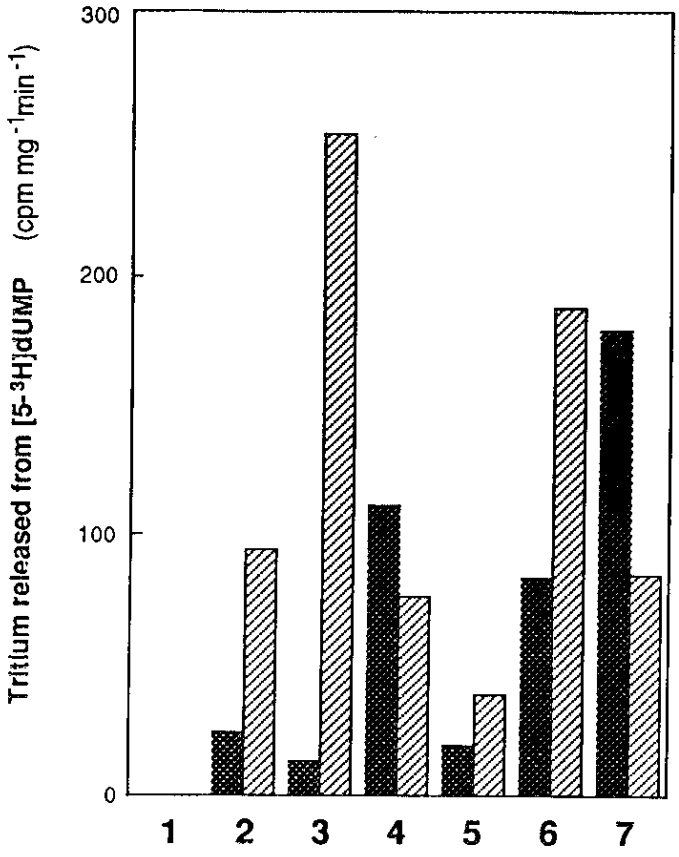
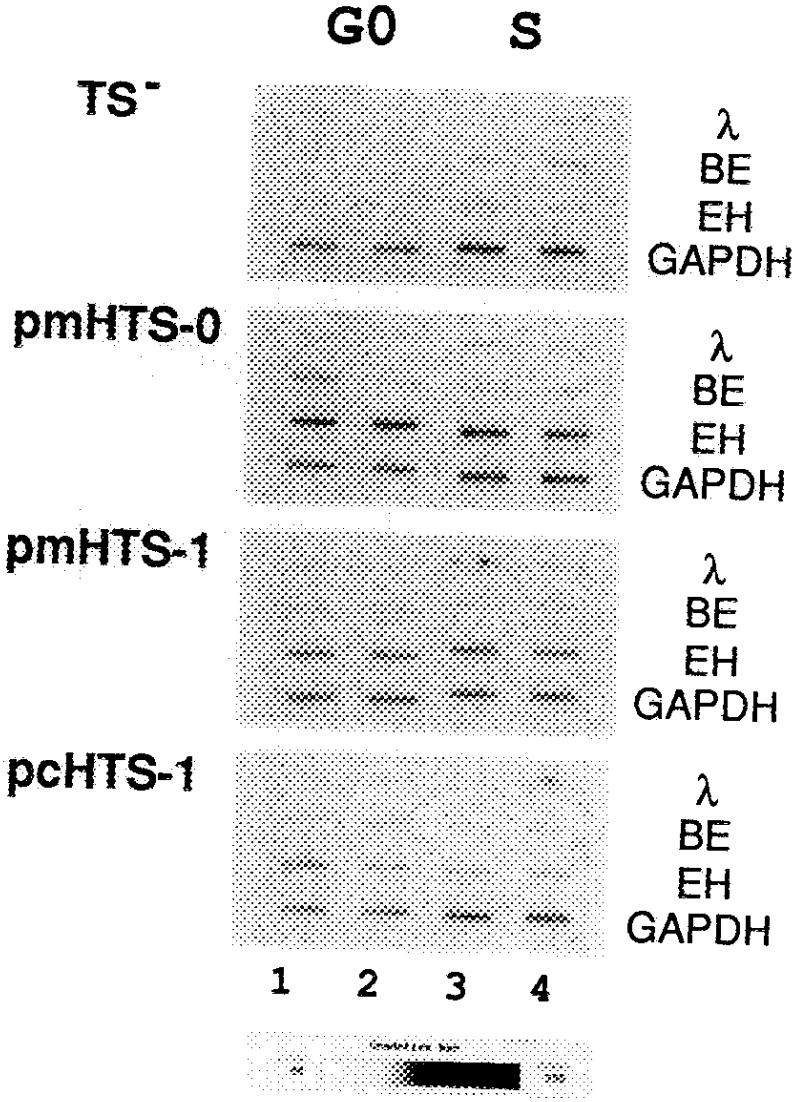

Tritium released from [5-³H]dUMP (cpm mg⁻¹min⁻¹)
300
200
100
0
1
2
3
4
5
6
7

Fig.7. Transcription of minigenes in vitro in isolated nuclei.

Nuclei were isolated from resting (G0) and S-phase cells (S) transformed with the indicated minigenes, and nuclear transcriptions were assayed as described previously (9). Data in this figure were obtained with the same clones as for Fig.4. ^{32}P-labeled transcripts were hybridized to a filter to which denatured DNA probe had been immobilized: BE and EH, the two probes shown in Fig. 3B; GAPDH, cDNA fragment (1.3kb) of glyceraldehyde-3-phosphate dehydrogenase. The nuclear transcripts hybridized to membranes were analyzed with FUJIX BAS 2000. The results were printed out by Pictrography. The gradation bar indicates that radioactivities of under level 44 were adjusted to zero for subtraction of the background image and a new shade gradation between level 44 and 252 was made.


G0
S
TS-
λ
BE
EH
GAPDH
pmHTS-0
λ
BE
EH
GAPDH
pmHTS-1
λ
BE
EH
GAPDH
pcHTS-1
λ
BE
EH
GAPDH
1
2
3
4