Role in translation of a triple tandemly repeated sequence in the 5'-untranslated region of human thymidylate synthase mRNA

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ABSTRACT

A triple tandem repeat (TTR) consisting of 90 nucleotides exists immediately upstream of the ATG initiator codon in human thymidylate synthase (TS) cDNA (pcHTS-1). To investigate the role of the TTR in the expression of the TS cDNA, we used pcHTS-1 to construct mutant cDNA clones in which part of the TTR was deleted or an additional element was inserted. The mutant cDNA plasmid was introduced into murine TS-negative mutant cells and the relative translation efficiencies of the mutant cDNAs were determined by measuring the transient expression of TS activity and the amount of TS mRNA transcribed. The translation efficiency in transient expression of the mutants was increased by deletions covering all the first two repeated elements, and the part of the third closest to the ATG initiator codon, but was not affected by deletions of only parts of the first two repeated elements at the 5' end. The translation efficiency was also not affected by insertion of an additional repeated element into the TTR. These results suggest that the first two repeated elements at the 5' end both have inhibitory effects on translation of the TS mRNA, probably due to the unique structural feature of this element.

INTRODUCTION

Thymidylate synthase (TS, EC 2.1.1.45) catalyzes the reductive methylation of deoxyuridylate to thymidylate and is essential for the survival of proliferating cells. The expression of TS activity (1-3) has been shown to be cell-cycle regulated like those of other enzymes involved in DNA synthesis such as dihydrofolate reductase (4-7), DNA polymerases (8), thymidine kinase (5,9-11), and ribonucleotide reductase (12-14). However, little is known about the structure of the mammalian TS genes or the molecular mechanism regulating its expression.

By use of DNA-mediated gene transfer techniques and TS-negative mutants isolated from murine FM3A cells (15), we recently cloned genomic DNA fragments that encode human TS (16) and isolated functional human TS cDNA clones (17). With a DNA fragment from the cDNA clone as a probe, we investigated expression of the TS gene during the cell cycle in normal

human fibroblasts, and from the results we suggested that expression of the gene is mainly controlled by post-transcriptional events (18). Similar results were reported in the case of the murine TS gene (19). We also sequenced the entire 1,600 nucleotide-long cDNA insert of the functional human TS cDNA clone, pcHTS-1, and found that the cDNA insert contains a unique structure of triple tandem repeats (TTR) in the 5'-untranslated region immediately upstream of the ATG initiator codon (20). Two of the three repeated elements have exactly the same sequence of 28 nucleotides and the third element is identical, except for a six-base insertion and a single substitution. In the present study, we constructed mutant TS cDNA clones to investigate the role of the TTR sequence in the translation of TS mRNA, and measured the transient expression of the mutant clones in transfected cells.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

The thymidylate synthase-negative mutant of murine FM3A cells, FSt_{hy}21 was described previously (15). FSt_{hy}21 was cultured at 37°C in ES medium (Nissui Seiyaku Co., Tokyo, ref. 21) supplemented with 2% fetal calf serum (FCS) and 20 µM thymidine (dThd). FCS was obtained from Reheis Chemical Co., Arizona.

Assay of Transient Expression of TS Activity

TS cDNA plasmids were introduced into TS-negative mutant FSt_{hy}21 cells by the calcium phosphate co-precipitation method described in detail elsewhere (22). The transient expression of TS activity in these cells by the cDNA plasmid was assayed by measuring incorporation of [³H]deoxyuridine (dUrd) via dTMP into the trichloroacetic acid (TCA)-insoluble fraction.

Briefly, exponentially growing FSt_{hy}21 cells were suspended at 5×10^5 cells/ml in ES medium, supplemented with 0.5% FCS and 20 µM dThd. Two ml of the cell suspension were transferred to glass dishes of diameter 2 cm, and the cells were allowed to adhere to the bottom of the dish for 1 hr. Plasmid DNA, co-precipitated with calcium phosphate, was then added to each dish. The cells were allowed to take up DNA for 5 hr. Then the medium was replaced by 2 ml of normal growth medium (ES medium containing 2% FCS and 20 µM dThd) and the cells were cultured for 16 hr. The cells were then collected by centrifugation, washed twice with ES medium containing 2% FCS and 1 µM dThd, and cultured for 6 hr, unless otherwise specified, in the

same medium (2 ml) supplemented with 10 μ Ci of [6- 3 H]dUrd (New England Nuclear, 21.4 Ci/mmol).

In a time-course experiment (Fig. 1), cells that had taken up DNA were cultured for appropriate times, and then collected, washed, cultured in ES medium containing 2% FCS and 1 μ M dThd for 1 hr, and finally incubated with 10 μ Ci of [3 H]dUrd for 1 hr.

In all cases, 3 H-labelled cells were collected in a centrifuge tube and treated with 5% TCA, and the radioactivity in the TCA-insoluble fraction was measured.

Construction of Mutant TS cDNA Clones

Mutant clones with a 5'-deletion or insertion in the TTR sequence of TS cDNA were constructed from a functional human TS cDNA clone, pcHTS-1 (17, 20), by the three procedures described below (see also Figure 2). The cDNA clone, pcHTS-1, is a TS cDNA clone isolated from the cDNA library constructed by Okayama and Berg (23). The plasmid vector (pcD) in pcHTS-1 contains simian virus 40 (SV40) early region promoter, SV40-derived intron sequences and the SV40 polyadenylation signal, which permit expression of the cloned cDNA segment in mammalian cells (23). *Escherichia coli* HB101 (a gift of Dr. Fumio Imamoto) was used as host to obtain transformants that carried plasmids. The restriction endonucleases and Bal 31 exonuclease used were purchased from New England Biolabs. T4 DNA ligase and the large fragment of DNA polymerase I (Klenow fragment) were obtained from Boehringer-Mannheim. DNA was digested with restriction endonucleases under the conditions recommended by the supplier. Preparation of plasmid DNA and isolation of DNA fragments from agarose gels were carried out as described previously (16).

Plasmid DNA of pcHTS-1 was digested with PstI to yield a 3.3 kilobase pair (kb) DNA fragment, a 1.3 kb DNA fragment and a 200 base pair (bp) DNA fragment. The 3.3 kb PstI fragment, which contained most of the TS cDNA insert, an SV40 polyadenylation signal sequence and part of the β -lactamase gene, was used to construct mutant TS cDNA clones as described in procedures I and II. The 1.3 kb PstI fragment, containing SV40 enhancer, SV40 early region promoter and introns in addition to part of the β -lactamase gene, was further digested with SalI to produce a 750 bp PstI-SalI fragment and a 580 bp SalI-PstI fragment. The 750 bp PstI-SalI fragment containing part of the β -lactamase gene was used as a portion of a vector DNA for construction of mutant cDNA clones in procedures I and II. The 580 bp SalI-PstI fragment, containing the SV40 regulatory sequences

upstream of TTR, was used as a portion of a vector DNA in procedure III. The 200 bp PstI fragment, containing TTR, was used to prepare deleted or inserted TTR as described in procedures II and III.

Procedure I: The pcHTS-1 DNA was digested with BamHI. The 490 bp BamHI fragment isolated, containing TTR, was treated with Bal 31 exonuclease under conditions where approximately 100 to 200 bp of DNA were cleaved from both ends of the fragment. After phenol extraction, the digests were further treated with PstI. Then Bal 31-digested PstI fragments of appropriate size (100-200 bp) were ligated with a 580 bp SalI-PstI fragment, of which the PstI site was blunted by treatment with the Klenow fragment. The resultant DNA fragments of 650-800 bp were further ligated with the 750 bp PstI-SalI fragment and 3.3 kb PstI fragment mentioned above, as shown in Figure 2. *E. coli* HB101 was transformed by the resultant plasmid DNA. Ampicillin-resistant clones were selected and subjected to analysis by restriction endonucleases for isolation of the relevant clones.

Procedure II: The pcHTS-1 DNA was digested with PstI. A 200 bp PstI fragment, containing TTR, was isolated and partially digested with MnI. The digests, containing 95, 123 and 151 bp fragments, were ligated with the same DNA fragments, i. e., a PstI-SalI fragment of 750 bp and a 3.3 kb PstI fragment, as used in procedure I (Fig. 2), and processed in the same manner as described in procedure I.

Procedure III: The MnI partial digests of the PstI fragment, isolated in procedure II, were religated and then digested with both PstI and BglI. The resultant PstI-BglI fragment of 70-200 bp was ligated with a SalI-PstI fragment of 580 bp and a BglI-EcoRI fragment of 180 bp and the desired product, the SalI-EcoRI fragment of 800-1000 bp shown in Figure 2, was isolated. This DNA fragment was further ligated with a 4 kb EcoRI-SalI fragment (Fig. 2.) to yield circular plasmid DNA. After this step, the same protocol was followed as for procedure I.

DNA Sequence Analysis

The sequences of the 5'-untranslated regions of mutant TS cDNAs were determined by the dideoxynucleotide chain termination method (24). The plasmid DNA was used directly, without subcloning into M13 phage, in accordance with the protocol recommended by the supplier of the M13 Sequencing Kit (Takara Shuzo, Kyoto). Plasmid DNA was used as a template, after denaturation with alkali. A synthetic "19-mer" (5'-AGCTCCGAGCCGCCACAG-3'), which is complementary to the sequence between

positions 5 and 23 (see Figure 3 for numbering of nucleotides) in the coding region, was used as a primer. [α - ^{32}P]dCTP(400 Ci/mmol, 1 Ci=37GBq), used for labelling, was obtained from Amersham.

RESULTS

Transient Expression of TS cDNA Clones in Murine TS-negative Mutant Cells

When cDNA clones, isolated from the Okayama and Berg cDNA library (23), are introduced into mammalian cells, they can be transcribed and translated transiently without integration into chromosomal DNA. We have utilized a transient expression system in murine TS-negative cells for quantitative measurement of the expression of parental and mutant TS cDNA. The strategy of the method is as follows: When dUrd is incorporated into cells, it is converted to deoxyuridylate by phosphorylation. The subsequent reductive methylation to thymidylate is catalyzed by TS, and the

Figure 1. Transient expression of pcHTS-1 DNA, introduced into murine TS-negative FStHy21 cells. The assay was carried out as described in Materials and Methods. (a) Dose dependency. Murine FStHy21 cells were treated for 5 hr with pcHTS-1 DNA co-precipitated with calcium phosphate, at the doses indicated, and then cultured first for 16 hr in normal growth medium with 20 μM dThd, and then for 6 hr in normal growth medium with [^3H]dUrd and 1 μM dThd instead of 20 μM dThd. (b) Time-course. Murine FStHy21 cells treated with pcHTS-1 DNA (5 μg DNA/dish) were cultured in normal growth medium for the indicated periods. The medium was then changed to ES medium containing 2% FCS and 1 μM dThd and the cells were cultured for 1 hr without and 1 hr with [^3H]dUrd. In both (a) and (b), the radioactivity of [^3H]dUrd incorporated into the acid-insoluble fraction was determined. In (a), the radioactivity incorporated without added plasmid DNA was 5,700 cpm per 5×10^5 cells. The values obtained after correction for this count are plotted. In (b), values corrected for the radioactivity at zero time (450 cpm) are plotted.

thymidylate formed is incorporated into DNA. Thus if [6-³H]dUrd is used as substrate, the amount of radioactivity incorporated into the TCA-insoluble fraction of cells that have been transfected with the TS cDNAs must reflect the amount of transiently expressed TS activity. Although dUrd can be incorporated into RNA via uridylate by enzymes other than TS, the amount incorporated has been shown to be less than 0.5% of that incorporated into DNA, as shown by a comparison of the radioactivity incorporated into the TCA-insoluble fractions of murine wild type FM3A cells and TS-negative cells incubated with labelled dUrd (data not shown).

The incorporation of [6-³H]dUrd into DNA of murine TS-negative cells, transfected with pcHTS-1, increased with increase in the dose of added plasmid DNA (Fig. 1a), and reflected the expression of TS activity, although the incorporation was only two- to five-fold that of [6-³H]dUrd incorporated into DNA in untransfected cells. A study of the time-course

Figure 2. Strategy for construction of human TS cDNA mutant clones with deletions or an insertion in TTR. Open and solid bars represent tandemly repeated sequences and coding sequences of human TS cDNA, respectively. Thin lines indicate vector DNA sequences. PstI sites in parentheses indicate a blunt end formed by treatment with the Klenow fragment. Abbreviations: B, BamHI; P, PstI; M, MnII; Bg, BglII; S, SalI; R, EcoRI; Bal 31, Bal 31 exonuclease; ligase, T4 DNA ligase; Klenow, E. coli DNA polymerase I large fragment; Amp, β -lactamase.

of transient expression of TS showed that the incorporation of [6-³H]dUrd reached a maximum 12 hr after treatment with the DNA-calcium phosphate co-precipitate and then decreased gradually (Fig. 1b). This decrease was due to the fact that the cDNA plasmid was introduced into TS-negative cells, but not integrated into their chromosomal DNA under the conditions used, and so was diluted at every cell division. The profile of Figure 1b reflects the doubling time (12 hr) of FSty21. In this study, we routinely assayed the transient expression of TS 16 hr after treatment with the DNA-calcium phosphate co-precipitate.

Construction and Structure of TTR Mutants

We constructed TTR mutants from the TS cDNA clone pcHTS-1 by the three procedures shown in Figure 2. Deletion mutants were obtained by

Figure 3. Structure of 5'-untranslated region of parental and mutant human TS cDNA clones and their relative translation efficiencies. The sequence of the 5'-untranslated region of the parental human TS cDNA clone pcHTS-1 is shown at the top. The relative translation efficiencies of TS mRNAs transcribed from mutant TS cDNA clones were calculated by normalizing the individual TS activity transiently expressed from the cDNA clones to the amount of transcribed TS mRNA, which was estimated by Northern blot hybridization, and are listed as a percentage of that of pcHTS-1. The values for each cDNA clone indicate the average and standard deviation of values obtained in four separate experiments. Open and solid bars represent tandemly repeated elements and coding sequences of human TS cDNA, respectively. Hatched bars indicate the sequence of a 5'-untranslated region beyond the TTR sequence. Thin lines indicate vector DNA sequences. Broken lines indicate deleted sequences. A six-base insertion and a single base substitution in the third repeated element are indicated by a wavy underline and a closed triangle, respectively. Open triangles indicate MnlI sites. The inverted repeated sequences in each repeated element are boxed. The first nucleotide of the ATG initiator codon is position 1. The nucleotides on the 5' side of position 1 are indicated by negative numbers.

procedures I and II, and insertion mutants by procedure III, as described in Materials and Methods. In procedure I, deletion in the TTR sequence was achieved by limited digestion with Bal 31 exonuclease of a BamHI fragment that contained TTR. Clones pcHTS-8 through pcHTS-11 were obtained by this procedure. The structures of TTR in these clones were determined by the dideoxy chain termination method, as described in Materials and Methods, and their repeated sequences were found to include nucleotides from positions -84, -63, -47 and -30 from the ATG initiator codon, respectively, as shown in Figure 3. In procedure II, a deleted TTR was prepared by partial digestion of a PstI fragment that contained the TTR with MnI. Plasmids pcHTS-12 and pcHTS-13 were isolated by this method and the structures of their deleted TTRs were determined by DNA sequencing. As shown in Figure 3, these plasmids contain the repeated sequence as far as nucleotides -41 and -13, which are the MnI sites in the second and third repeated elements, respectively. The 5' insertion mutant, pcHTS-14, was constructed by procedure III. In this procedure, a TTR structure with an insert was obtained by ligation of the MnI partial digests obtained in procedure II. Sequence determination confirmed that the inserted sequence created an additional repeated element that had the same sequence as the first and second ones (Fig. 3). The 5' deletion mutant clones mentioned above can be classified as follows. Clones pcHTS-8 and pcHTS-9 have 5' deletions only in the first repeated element of pcHTS-1; pcHTS-10 and pcHTS-12 lack the entire first repeated element and a part of the second repeated element; clones pcHTS-11 and pcHTS-13 lack all the repeated elements except for a part of the third element, as shown in Figure 3.

Transient Expression of Mutant TS cDNA Clones

The total DNA sequences of the parent and mutant TS cDNA clones described above are identical, except in the TTR region. Thus, all the clones have the same SV40-derived DNA sequences involved in transcriptional regulation. As was expected, there were no big differences in the relative transcription efficiencies of these clones in the transient expression assay system described in Materials and Methods, judging from the amount of TS mRNA estimated by Northern blot hybridization (data not shown). In the assay used, however, the incorporation of [³H]dUrd into DNA varied significantly from experiment to experiment, even when the same DNA preparation was used. This variation may have been due to variation in the amount of DNA incorporated into cells in each experiment. Therefore, to compare the accurate translation efficiencies of the TS mRNAs transcribed

from parent and mutant TS cDNA clones in transfected cells, we measured the amount of human TS mRNA expressed in transfected cells by Northern blot hybridization (data not shown) and normalized the transiently expressed TS activity to the amount of TS mRNA. The values thus obtained for the relative translation efficiencies of the TS cDNA clones with deleted or inserted TTR are summarized in Figure 3. The mutant clones pcHTS-8, pcHTS-9, pcHTS-10, pcHTS-12 and pcHTS-14 expressed comparable TS activity to that of the parental clone, pcHTS-1. On the other hand, the translation efficiencies in the transient expression of pcHTS-11 and pcHTS-13, which have no intact repeated elements, were two to three times that of the parental clone. From these data we conclude that the translation efficiency increases only when the deletion extends into the third repeated element.

DISCUSSION

We established an assay system for measuring transient expression of human TS cDNA introduced into murine, TS-negative, mutant cells. The assay system is so sensitive that as little as one percent of the TS activity observed in wild type cells can be detected. Using this system, we examined the effect of deletions in the repeated elements in the 5'-untranslated region of functional human TS cDNA, pcHTS-1 (17,20), on the expression of TS activity. Deletions of the first and second repeated elements had no effect on the translation efficiency of TS mRNA. When the deletion extended into the third repeated element, however, the translation efficiencies of mutant TS mRNAs increased to 2 to 3 times that of TS mRNA from parental pcHTS-1. To determine the influence of the length of the 5'-untranslated region on the translation efficiency, we constructed a plasmid, pcHTS-14, containing an additional repeated element (identical to the first and second elements) as well as the original TTR. TS mRNA from this plasmid showed essentially the same translation efficiency as that of TS mRNA from pcHTS-1 in our assay. As described above, no differences were found between the translation efficiency of the parental pcHTS-1 and those of mutant TS cDNA clones in which the first, entire, repeated element and most of the second repeated element were deleted. From these data, it seems very unlikely that the translation efficiency of the TS mRNA is affected by a change in length of the 5'-untranslated region. This conclusion is consistent with the suggestion of Kozak (25) that the translatability of a given mRNA is not perturbed when the 5'-noncoding

Table 1. Stabilities of the stem-loop structures that can be formed between the repeated elements

Sequences involved	ΔG^* (kcal)
First and second repeated elements	-9.0
First and third repeated elements	-8.1
Second and third repeated elements	-8.8
Heptanucleotide CGCCGCG making a stem	-14.5

* ΔG is the summation of free energy differences (at 25°C) between the base-paired state and the unpaired state calculated by the method of Salser (26)

region is altered by deletion, insertion, or recombination. Therefore, the repeated elements upstream from the third repeated element may be assumed to exert an inhibitory effect on the efficiency of translation of TS mRNA, due to their unique structural features.

The TTR structure is not an artifact generated during cloning of the cDNA, because the same structure has been shown to be present in the chromosomal TS gene (Takeishi et al., unpublished data). As we suggested in a previous paper (20) it is possible that any two of the three elements of the TTR form a stem-loop structure by association of the inverted repeated sequence CGCCGCG, which is present in each element. The calculated stabilities of the hypothetical stem-loop structures are listed in Table 1. All the possible secondary structures have significant stability ($\Delta G = -8.1$ to -9.0 kcal), comparable to the stability of secondary structures predicted from sequences within the 5'-untranslated region of rat and human insulin mRNAs (27), and to those of the stable association between the 3'-end of 18s rRNA and the secondary structure, predicted from sequences within the 5'-untranslated regions, of several mRNAs (28). The inhibitory effect of the first and second elements in the TTR sequence, described above, may result from formation of a stem-loop structure within the TTR sequence. This suggestion is similar in nature to the observation of Pelletier and Sonenberg (29) that insertion of a small hairpin loop structure into the 5'-noncoding region of the mRNA for thymidine kinase from herpes simplex virus type 1 reduces the translational efficiency.

The repeated elements in the TTR sequence did not have a strong inhibitory effect on the translation efficiency of TS mRNA, perhaps because a stem-loop structure formed within a TTR sequence is relatively unstable. The stability might be increased or diminished by association of the stem-

loop structure with cellular protein factors in vivo. In this connection an interesting possibility is that the TS cDNA clone, pcHTS-1, in which the expression of TS is in a partially suppressed state, as shown in this study, might require some protein factor(s) for expression of maximal TS activity. The level of the pool of dTTP in cells depends on the level of TS activity and is involved in regulating the balanced supply of the four DNA precursors required for normal DNA replication. The TTR sequence play an important role in this delicate regulation.

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