Isolation of Functional cDNA Clones for Human Thymidylate Synthase*

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Thymidylate auxotrophic mutants of mouse FM3A cells due to thymidylate synthase deficiency can be transformed into prototrophs by DNA-mediated gene transfer using total human DNA (Ayusawa, D., Shimizu, K., Koyama, H., Takeishi, K., and Seno, T. (1983) J. Biol. Chem. 258, 48–53). From one such transformed cell clone, cloned recombinant λ phages containing DNA fragments were obtained recently that were concluded by circumstantial genetic evidence to have been derived from the human thymidylate synthase gene (Takeishi, K., Ayusawa, D., Kaneda, S., Shimizu, K., and Seno, T. (1984) J. Biochem. (Tokyo) 95, 1477–1483). Using a DNA segment derived from the cloned genomic DNA fragment and free of repetitive sequences as a probe, functional cDNA corresponding to thymidylate synthase mRNA could be cloned from a cDNA library of SV40 transformed human fibroblasts constructed by Okayama and Berg (Okayama, H. and Berg, P. (1983) Mol. Cell. Biol. 3, 280–289). The cloned cDNA plasmid containing an insert of approximately 1.7-kilobase transformed mouse thymidylate auxotrophic mutant cells to thymidine prototrophic cells at a frequency of 2–3 transformants/µg of DNA/10⁶ cells, a value almost comparable to the highest so far reported. The resultant transformants retained the introduced cDNA and expressed human thymidylate synthase protein sufficient for supporting normal growth of otherwise auxotrophic mouse cells.

Thymidylate synthase (EC 2.1.1.45) plays an essential role in regulating a balanced supply of the four DNA precursors for maintaining normal DNA replication. When thymidylate synthase activity is somehow blocked, various genetic alterations, such as mutation, genetic recombination, and chromosomal rearrangement, are induced, presumably by decrease in the final product of the enzyme, dTTP (1–4). The enzyme activity is usually high in rapidly proliferating cells and hardly detectable in nonproliferating cells. From these and other lines of evidence, thymidylate synthase is thought to be the target of several widely used cancer chemotherapeutic agents.

For studies on the genetic and biological function of this enzyme, we have isolated various types of thymidylate synthase mutants from mouse FM3A cells (5–8). Molecular cloning of the mouse thymidylate synthase gene seems essential for this purpose. We first tried to isolate the human thymidylate synthase gene with a view to eventually isolating the mouse gene, since in practice cloning of the human gene is easier than that of the mouse gene, for the reasons mentioned below, and once the human gene has been cloned, it should not be difficult to clone the mouse counterpart with a possible DNA sequence homologous to that of the human gene.

Recently, we introduced the human thymidylate synthase gene into thymidylate synthase-negative mouse cells by DNA-mediated gene transfer using total human DNA (9). In fact, interspecies gene transfer was found to be much easier than intraspecies transfer for locating and assessing the transferred gene and its product. Isolation of the transformants enabled us to clone human thymidylate synthase gene fragments in recombinant λ phage (10). The cloned gene fragments, however, failed to transform thymidylate synthase-negative mouse cells. We thus attempted to isolate a functional cDNA clone from the human cDNA library constructed by Okayama and Berg (11) to confirm that the above nonfunctional genomic clones were derived from the human thymidylate synthase gene. The cDNA library has been prepared so as to contain preferably a full-length cDNA sequence and to allow expression of the inserted cDNA sequences in mammalian cells (11). The SV40-pBR322 shuttle vector used in constructing the library contains a SV40 early region promoter and intron 5′ to the cDNA segment and a polyadenylation signal 3′ to the cDNA segment. These segments permit us to test for the function of the putative thymidylate synthase cDNA sequence directly in transformation assays and to identify and confirm a full-length of the genomic sequence.

This paper describes the isolation of human thymidylate synthase cDNA clones from the human cDNA library using a DNA probe, which was derived from one of the cloned thymidylate synthase gene fragments.

EXPERIMENTAL PROCEDURES

Preparation of a DNA Probe—Two DNA segments containing parts of the human thymidylate synthase gene were cloned in Charon 4A vector as recombinant phage, λHTS-1 and λHTS-2, by introducing the human thymidylate synthase gene into thymidylate synthase-negative mouse cells, preparing a genomic library from the DNA of the resulting transformant, and detecting recombinant λ phage clones containing human sequence by hybridization with a probe of the middle repetitive human Alu sequence as described previously (10) A 6.8-kb EcoRI fragment of λHTS-1 DNA was subcloned into an EcoRI site of pBR322. A 2.0-kb DNA fragment free of the repetitive sequences was cleaved out by double digestion with the restriction enzymes SalI and EcoRI from the above recombinant plasmid, separated by electrophoresis through an agarose gel, and recovered by adsorption to glass powder.

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The abbreviations used are: kb, kilobase(s); SSC, standard saline citrate; FCS, fetal calf serum; FdUMP, 5-fluoro-2'-deoxyuridine 5'-monophosphate.
as described (10). This 2.0-kb DNA, named HSR20, has been shown to hybridize to a discrete cytoplasmic poly(A) RNA of approximately 1.6 kb from the transformants expressing human thymidylate synthase, but not to any of the cytoplasmic RNA in the wild-type mouse FMI3A subclone F28-7 or the recipient thymidylate synthase-negative mutant FStlly1 (7).

Therefore, the HSR20 DNA was labeled by nick-translation with [α-32P]dCTP to a specific activity of 2 × 10^6 cpm/μg using a Nick Translation Kit (Amersham) and was used for colony hybridization to screen the Okayama-Berg cDNA library (11).

**Screening of a Human cDNA Library**—A complete human cDNA library was constructed from SV40-transformed human fibroblasts by Okayama and Berg as described (11). The plasmid DNA of the cDNA library, a generous gift from Okayama and Berg, was transferred to *Escherichia coli* HB101. A bank of approximately 3,000,000 ampicillin-resistant colonies was reproduced, and stored at −20 °C as bacterial cell suspensions in L-broth containing 50% glycerol. Aliquots of the above bacterial suspensions were grown overnight at 37 °C on L-broth agar containing 50 μg/ml of ampicillin.

The colonies were transferred to membrane filters (Schleicher & Schuell, West Germany), treated with chloramphenicol overnight at 37 °C, denatured, fixed to the filters, and then screened for the thymidylate synthase cDNA sequence by hybridization with the HSR20 DNA. The filters were hybridized to the nick-translated DNA probe in a buffer (50% formamide, 5 M NaCl, 50 mM Tris-HCl (pH 7.5), 0.1% Ficoll, 0.1% bovine serum albumin, 0.1% polyvinylpyrrolidone, 0.1% sodium dodecyl sulfate, 20 μg/ml of sonicated and denatured herring sperm DNA, and 10% sodium dextran sulfate) overnight at 42 °C as described (10). The filter sheets were washed twice with 2× SSC, 0.1% sodium dextran sulfate, and 10% sodium dextran sulfate, and then 3 times with 0.1× SSC plus 0.1% sodium dodecyl sulfate at 42 °C, dried, and autoradiographed on X-ray films with an intensifying screen at −80 °C.

The area of master agar plates showing a putative positive signal on a filter was identified. The cells in the area were aspirated with a pipette, transferred to 1 ml of L-broth, and grown overnight. An aliquot of the culture was again plated on L-broth agar in 9-cm Petri dishes, and the colonies were subjected to a second round of screening to confirm and purify the putative cDNA colonies as described above. After three to four rounds of purification steps, the candidate clones were grown in mass culture to prepare plasmid DNA.

**Restriction Mapping of cDNA**—DNA was digested with restriction endonucleases (Takara Shuzo, Kyoto) under the conditions recommended by the suppliers. Restriction enzyme mapping of cDNA was carried out by analyzing products of single and double digestion as described (10).

**Southern Blot Hybridization**—High-molecular-weight DNA prepared as described previously (9) was digested with restriction endonucleases, and 20 μg of the DNA was then subjected to electrophoresis in a 1.0% agarose gel in a buffer (pH 8.1) consisting of 40 mM Tris-HCl, 0.1% sodium dodecyl sulfate, 0.1% Ficoll, 0.1% bovine serum albumin, 0.1% polyvinylpyrrolidone, 20 mM acetic acid, 20 mM NaCl, and 2 mM EDTA. The DNA was transferred to nitrocellulose filter and the resulting blots were hybridized with a nick-translated DNA fragment prepared from a cloned cDNA plasmid as described above. Nick-translation, hybridization, and washing conditions were described in the above section.

**Cell Lines and Culture Conditions**—A wild-type mouse FMI3A subclone, F28-7, was cultured in ES medium (Nissui Seiyaku Co., Tokyo) containing 2% FCS. Two thymidine auxotrophic mutants of the F28-7 strain, FStlly1 and FStlly21, both of which had absolutely no thymidylate synthase activity, were cultured in ES medium supplemented with 2% FCS and 20 μM thymidine. The wild-type human cell lines Raji and HeLa were grown in ES medium containing 10% FCS. All the cells were incubated at 37 °C in a CO2 incubator under 5% CO2 in air at 85% humidity.

**Transformation of Thymidylate Synthase-negative Mutant Mouse Cells**—The isolated cDNA plasmid was introduced into thymidylate synthase-negative thymidylate synthase-negative thymidylate auxotrophic mutant FStlly1 and FStlly21 cells by the calcium phosphate coprecipitation method as described in detail elsewhere (9).

Briefly, 5-ml aliquots of cell suspension at 5 × 10^6 cells/ml were incubated into 6-cm glass Petri dishes and the cells were allowed to adhere to the bottom for 1 h. Then, plasmid DNA coequilibrated with calcium phosphate was added to each dish. Carrier DNA was not used. The cells were allowed to take up DNA for 5 h, with 10% dimethyl sulfoxide present in the last 30 min. Then the medium was replaced by 20 ml of normal growth medium and the cells were cultured for 20 h to allow expression of transformed phenotypes.

The cells were collected by centrifugation, washed twice with ES medium containing 2% dialyzed FCS, and seeded in triplicate on selective agarose plates (ES medium plus 5% dialyzed FCS solidified with 0.35% agarose). In the same experiment, known numbers of cells were seeded on similar agarose plates but containing 20 μM thymidine to determine viable cells. Visible colonies were counted after incubation for 7 days, picked up, and maintained in normal growth medium.

**Electrophoretic Analysis of Thymidylate Synthase**—A ternary complex of thymidylate synthase-FdUMP-5,10-methylenetetrahydrofolate was formed by incubation for 60 min at 25 °C in a volume of 100 μl of reaction mixture consisting of 50 mM Tris-HCl (pH 7.5), 0.1% Triton X-100, 10 mM dithiothreitol, 13 μl of the 0.3 mM 5,10-methylenetetrahydrofolate solution, 50 mM NaF, 1 mM [32P]FdUMP (200 Ci/mmol), prepared as described previously (8), and 0.3-0.4 mg of a cytoplasmic extract. The samples were subjected to electrophoresis in 7.5% nondenaturing polyacrylamide gel, and the gel was dried and exposed to X-ray films.

**RESULTS**

**Isolation of Thymidylate Synthase cDNA Clones**—We have screened 8 × 10^6 plasmid clones of the Okayama-Berg cDNA library using the HSR20 DNA as a probe. In all, 7 purified clones, named pcHTS-1 to pcHTS-7, were isolated. Plasmid DNAs were prepared from the 7 clones and digested with several restriction enzymes, and the products were subjected to agarose gel electrophoresis to determine the size of their inserts. The lengths of the inserts, including linkers and a small portion of SV40 vector DNA at their 3'-end, were approximately 1.7, 1.7, 1.6, 1.6, 1.3 kb, respectively (see Fig. 1). A length of 1.7 kb is long enough to cover the full-length cDNA to human thymidylate synthase mRNA, since human thymidylate synthase mRNA has been estimated to be about 1.6 kb (10). The restriction maps of the cDNA

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FIG. 1. Structures of human thymidylate synthase cDNA clones pcHTS-1 to pcHTS-7. The solid black segment represents a cDNA insert. The hatched region and the solid open segment represent the truncated simian virus 40 vector sequences and the pH9322 β-lactamase gene, respectively. The vertical dotted line in pcHTS-3 cDNA shows a possible deletion. The parentheses and the numbers in them indicate approximate positions and sizes of deletions, respectively. ori, origin of DNA replication; poly A, polyadenylation signal; Ps, PstI; Sa, SalI; Cl, ClaI; H3, HindIII; Bg, BglII; Xh, XhoI; Ba, BamHI; RI, EcoRI; PvuII.
clones were determined to be as shown in Fig. 1.

Transformation of the Mouse Mutant Cells with the cDNA Clones—At present we have no means of identifying human thymidylate synthase cDNA clones, except by their functional expression in thymidylate synthase-negative mouse cells. We thus introduced plasmid DNAs of the cDNA clones into thymidine auxotrophic mouse cells by DNA-mediated gene transfer. At least two clones, pcHTS-1 and pcHTS-2, which both have the longest cDNA inserts, were found to have potent activity to transform thymidine auxotrophs to thymidine prototrophs. Since there was no significant difference between the transforming activities of these two cDNA clones, only the results with pcTHS-1 are shown in Fig. 2. Transformant colonies were obtained dose dependently in both thymidylate synthase-negative recipients FSthy11 and FSthy21. Under the experimental conditions employed, no revertants were detected. At a dose of 20 μg of pcHTS-1 DNA, the frequency of transformants was about 80 colonies/10^5 cells.

Expression of Human Thymidylate Synthase in the Transformants—We characterized several of the transformants obtained by introduction of pcHTS-1 DNA. We tested whether the transformants expressed human thymidylate synthase. Thymidylate synthase can be easily identified because it has the unique property of forming a covalent-bonded ternary complex, i.e. enzyme-FdUMP-5,10-methylenetetrahydrofolate ternary complex. This makes it possible to locate the position of the enzyme in the complex form in polyacrylamide gel after electrophoresis when [32P]FdUMP is used as a ligand. The mouse thymidylate synthase complex migrates faster than the human complex, and so the two enzymes can be clearly distinguished as shown in Fig. 3. Two bands are seen in most lanes in Fig. 3. Since both human and mouse thymidylate synthase are dimers of identical subunits with molecular weights of 33,000 and 36,000, respectively, and each subunit has a binding site to FdUMP (8), the upper band consists of one ternary complex and one free form of the enzyme and the lower one consists of two ternary complexes (8). The results shown in Fig. 3 demonstrated unambiguously that the pcHTS-1 DNA expressed sufficient human thymidylate synthase protein to support normal growth of thymidylate synthase-negative mouse cells.

Presence of the cDNA in the Transformants—We then examined how the transformants retained the foreign DNA introduced by gene transfer. High-molecular-weight DNA from the transformants, the recipient FSthy11, and the two control human cell lines were digested with EcoRI (A) and HindIII (B) were run in agarose gel, transferred to nitrocellulose filter, and hybridized with a 32P-labeled DNA probe. The probe used is a 0.6-kb BamHI fragment derived from the cDNA insert of plasmid pcHTS-1. Lanes: a, mouse recipient FSthy11; b-e, transformant clones 1-4; f and g, human Raji and HeLa cell line, respectively.

As shown in Fig. 4, in the transformant clones 1 and 3, the introduced DNAs were integrated in multiple copies in the cells. In the transformant clones 2 and 4, in contrast, only a few copies of the cDNA plasmid were integrated in host cell chromosomes. In spite of such a significant difference in copy number of the introduced DNA between the transformants, thymidylate synthase activity did not differ between the transformants as shown in Fig. 3.

Thus, the results shown in Figs. 3 and 4 directly demonstrated that the pcHTS-1 DNA was present and expressed human thymidylate synthase in the transformants tested.
We have recently cloned two independent human thymidylate synthase gene fragments in bacteriophage λ Charon 4A vector, each having an overlapping 5.5-kb EcoRI fragment (10). The total length covered by the two genomic fragments spans 19.3 kb. We tested the functional activities of the two thymidylate synthase gene clones in thymidylate synthase-negative mouse cells but obtained negative results. Then we transferred these two in combination into mouse cells because in mammalian cells two genomic DNA fragments having an overlapping sequence can form a recombinant product in vivo by homologous genetic recombination (12), and this recombinant DNA is often shown to be functional even if each DNA fragment alone is not functional (13). Even in this case, no transformant appeared. Therefore, the identification of λ clones that carry parts of the human thymidylate synthase gene had to be based on circumstantial genetic evidence (10).

Here, we showed directly that DNA inserts in the λ recombinant clones λHTS-1 and λHTS-2 originated from the human thymidylate synthase gene, by demonstrating that the expressible cDNA clones hybridized to several restriction fragments of a DNA insert in both the recombinant phages. Preliminary characterization of genomic clones for the human thymidylate synthase gene newly isolated from the human gene library constructed by Maniatis and his co-workers (14) showed that a DNA fragment flanking the 3' end of the inserts of λHTS-1 and λHTS-2 hybridizes to pHTS-1 cDNA. It thus became evident that the above two genomic clones λHTS-1 and λHTS-2 did not include the 3' terminal portion of the thymidylate synthase coding sequence. This may account for the lack of transforming activity of the λHTS-1 and λHTS-2 in mammalian cells when combined.

Recently, Jolly et al. (15) isolated full-length cDNA clones for human hypoxanthine phosphoribosyltransferase from the Okayama-Berg cDNA library using the 1.5-kb human genomic DNA probe that hybridizes to the 3' end of the full-length cDNA. They showed that the frequency of hybridization-positive human hypoxanthine phosphoribosyltransferase cDNA clones in the library was about $2 \times 10^{-6}$ or equivalent to 2-5 copies of the mRNA/cell. They also estimated that the frequency of full-length cDNA clones was about 5% of the total number of positive clones in the complete library. In this work, we have isolated 7 cDNA clones from about 8 $\times 10^6$ E. coli transformant colonies, a frequency of about 1 $\times 10^{-3}$, using the 2.0-kb human genomic DNA probe that has recently been found to hybridize to the 5' end of thymidylate synthase cDNA. We found that 2 of 7 cDNA clones contained full-length cDNA. We do not know at present why the frequency of hybridization-positive thymidylate synthase clones was 20-times lower than that of human hypoxanthine phosphoribosyltransferase clones. Possibly, the lower frequency of thymidylate synthase-positive cDNA clones is related to the fact that in FM3A cells, the content of thymidylate synthase/cell is much less than the contents of relevant enzymes such as thymidine kinase (16), dihydrofolate reductase (7), human hypoxanthine phosphoribosyltransferase (17), and adenine phosphoribosyltransferase (17). It is also noteworthy that the mRNA level of thymidylate synthase was almost one order of magnitude lower than that of adenine phosphoribosyltransferase (10).

The pHTS-1 cDNA was found to transform thymidine auxotrophic mouse cells to thymidine prototrophs at a frequency almost comparable to that of a plasmid carrying the hamster adenine phosphoribosyltransferase gene, which is the highest gene transfer marker we have examined so far and higher by about two orders of magnitude than that of plasmid DNA carrying the thymidine kinase gene of Herpes simplex virus in our mouse FM3A cells. Therefore, the human thymidylate synthase cDNA described here will be useful in studies on control of gene transfer and genetic potential in mammalian cells.

The isolation of functional cDNA clones as well as of genomic clones now enables us to obtain human thymidylate synthase in large amount and to study the structure and regulation of the mammalian thymidylate synthase gene at a molecular level. The various genetic mutants of the thymidylate synthase gene that we have established from the mouse FM3A cells will be particularly helpful for these purposes.

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