Alternate Splicing of the rTS Gene Product and Its Overexpression in a 5-Fluorouracil-resistant Cell Line

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Abstract

rTS is a gene with convergent and overlapping transcription relative to thymidylate synthase (TS). Screening of a cDNA library has identified a second rTS RNA (rTSβ) which is related to a previously described rTS RNA (rTSα). rTSβ differs from rTSα by the insertion of an extra 116 nucleotides after base 128 of rTS and an altered 3'-noncoding region such that rTSβ RNA is not complementary to TS mRNA. rTSα and rTSβ RNAs have open reading frames predicted to encode proteins of 361 and 416 amino acids, respectively. Antibody to rTSβ protein detects two proteins of the predicted molecular weight in the H630 colon tumor cell line. rTSβ is overexpressed 40–70-fold in a 5-fluorouracil-resistant H630 subline compared with 7–8-fold for TS and rTSα.

Introduction

In 1993, our laboratory reported the existence of the rTS gene (1). The rTS gene was identified from the isolation of a cDNA clone which had a region complementary to TS5 mRNA (1). The predicted protein encoded by the large ORF of rTS RNA (M, 41,028, now referred to as rTSα) was found to have homology to a superfamily of proteins which includes mandela racemase and muconate-lactonizing enzyme from Pseudomonas putida. These enzymes recognize diverse substrates but have the common ability to extract protons from the α carbons of carboxylic acids (2, 3). rTS was found to be overexpressed at both the RNA and protein levels in a human leukemic cell line (K562 B1A) selected for resistance to MTX (1, 3). The colocalization of the rTS gene with the TS gene and its overexpression in a cell line selected for resistance to MTX suggested the possibility that rTS function may be related to the enzymes TS and dihydrofolate reductase. We also reported that rTS-overexpressing K562 B1A cell line has lost its ability to down-regulate TS activity with cell growth, further suggesting that rTS expression is related to TS activity (3).

In this communication, we report the isolation and characterization of a second mRNA (rTSβ). rTSβ mRNA is predicted to encode a protein larger than rTSα due to the presence of an extra exon retained in rTSβ mRNA as a result of alternate splicing. Immunoblot data indicate that rTS proteins of the predicted molecular weight for both rTSα and rTSβ are expressed in a human colon tumor cell line. Furthermore, the rTSβ protein is preferentially overexpressed in a human colon tumor cell line selected for resistance to FU compared with TS and rTSα.

Materials and Methods

Oligonucleotides and DNA Sequencing. Automated DNA sequencing was performed by either the DNA sequencing facility at the State University of New York at Buffalo (Hochstetter Hall) or the Roswell Park Cancer Institute Biopolymer Facility.

Cloning of rTSβ cDNA. A ZAP cDNA library was generated from cytoplasmic RNA, isolated from KB cells, as described previously (1). Plaque-forming units (106) were screened for cDNA inserts homologous to the RACE-generated 5' rTS cDNA clone 6 described previously (1). A cDNA clone (i.e., rTS33) in the pBlueScript II SK– vector was identified with homology to 5' rTS cDNA clone 6 and sequenced. Sequencing revealed the identity to the cDNA clone previously identified as rTS cDNA (renamed rTSα) over most of its length, corresponding to rTSα positions 86–128 and 129–1296 (see Fig. 1). The 5' end of rTS33 was found to correspond to position 86 of rTS. The rTS33 was also found to diverge from the rTSα 3' of position 1296 of rTSα and to have an extra 116 nucleotides inserted in the position corresponding to 128 of rTSα. Since rTS33 did not have a large ORF with an initiation codon, it was concluded that the potential initiation codons previously identified in rTSα were absent as a result of premature termination during the reverse transcription step used to generate the cDNA library. This conclusion was based on the high degree of identity of rTS33 and rTSα and previous primer extension studies that had identified the likely 5' ends of rTS RNAs (1).

The region of cDNA corresponding to positions 1–85 of rTSα was added to the 5' end of rTS33 to generate rTSβ. This was accomplished by generating overlapping DNA fragments from the 5' ends of both rTSα and rTS33 cDNAs using PCR, combining these DNA products, and performing a second round of PCR to generate a new DNA fragment corresponding to the predicted 5' end of rTSβ. The first DNA fragment (using rTSα as a template) generated by PCR is defined by the region bordered by the T3 promoter upstream of the rTSα insert to position 127 of rTSα (primers: 425, sense, 5'-ATTAAACCTTCACTAAAAGGAAACAA-3' and 424, antisense, 5'-ACTCTAGTGGCTTTTCCAGAGT-3'). The second DNA fragment was generated using plasmid DNA containing rTSα as a template and primers defined by positions 105–127 of rTSα (423, sense, 5'-ACTCTGGAAAGCGACTGAAGT-3') and 361–381 of rTSα (337, antisense, 5'-GTATCTTTGTCGCAAGTATT-3'). The two PCR products were gel purified, combined, and amplified with primers 425 and 337 to generate a unique PCR product which includes the region defined by nucleotides 1–381 of rTSα but with the unique 116 nucleotide insertion found in rTSβ (see Fig. 1). An Xbal/EcoRI restriction fragment, corresponding to the 5' region, was subcloned into pBlueScript II SK–/rTS33 to generate pBlueScript II SK–/rTSβ. Sequencing revealed the newly generated rTSβ to have a silent T for C substitution at position 104 (see "Results") of the predicted large ORF. The nucleotide sequence rTSβ has been entered in the European Molecular Biology Laboratory nucleotide sequence data base as accession no. X89602.

Cell Culture. H630 colon carcinoma cells and the FU-resistant variant cell line H630-1 (4) were obtained from Dr. Edward Chu (Bethesda Naval Research Hospital). Both cell lines were maintained in RPMI 1640 supplemented with 5% fetal bovine serum at 37°C in a 95% air/5% CO2 humidified atmosphere. H630-1 cells were maintained in the presence of 1 μM FU, but were passaged once in the absence of drug prior to extraction.

Immunoblotting. Cells were grown to approximately 50% confluence, the culture medium was aspirated, the cells were washed twice with PBS (0.21 g/liter KH2PO4, 9 g/liter NaCl, and 0.726 g/liter Na2HPO4·7H2O), and then extracted by boiling for 10 min in Laemmli sample buffer (5) supplemented to contain 5% SDS, centrifuged at 14,000 × g for 10 min, and stored at −20°C.
The protein contents of samples to be analyzed using gel electrophoresis were quantitated using a BCA kit (Pierce, Rockford IL) after acid precipitation. Samples to be analyzed using immunoblotting were denatured in Laemmli sample buffer at 85°C for 5 min, and the indicated amounts of protein were resolved with SDS-10% polyacrylamide using a minigel format (Bio-Rad, Hercules, CA). Immunoblotting was performed with proteins transferred to polyvinylidene difluoride membranes (Immobilon-P; Millipore Corp.) using BLOTTO/PBS-Tween 20 as described (6). Primary antibodies were diluted 1:2000 and the secondary horseradish peroxidase-conjugated antibody [goat anti-rabbit IgG (Fab')2; Jackson ImmunoResearch] 1:5000. ECL reagent (Amersham) was used to locate the secondary antibody and Renaissance X-ray film (DuPont) used for fluorography. For probing of filters with different antisera, membranes were stripped essentially as described (7). Polyclonal antisera to rTS was prepared in rabbits against a fusion protein comprised of malseose-binding protein and rTSβ. The serum was preabsorbed to fixed bacteria (XLI-Blue-MRF') as described (8), then to malseose-binding protein coupled to Sepharose prior to use in immunoblotting. Polyclonal antisera to human TS was a gift from Dr. E. Chu.

Results

rTSα, rTSβ RNAs, and Proteins. Sequencing of rTSβ indicated identity with rTSα in the region of rTSβ 5' of rTSα nucleotide 128 and 3' of rTSα nucleotide 129 to rTSα nucleotide 129. This relationship is depicted in Fig. 1. rTSβ RNA contains an extra 116 nucleotides inserted at the position equivalent to the rTSα nucleotide 128. Unlike other RACE cDNA products previously isolated and having sequence elements characteristic of introns (1), the 116-nucleotide insert found in rTSβ (Fig. 2) lacks a polypyrimidine tract and consensus splice donor and acceptor sites. This insertion modifies the predicted large ORFs (ORF1 and ORF2, Fig. 1) of rTSα, so as to place the first initiation codon (ATG) at position 18 in frame with an ORF predicted to code for a 416-amino acid protein versus 361 amino acids for rTSα. Unlike the initiation codon predicted to start the large ORF of rTSα at position 67 (Fig. 1), the initiation codon located at position 18 in rTSβ is in a context of bases shown to effect efficient translational initiation in eukaryotes (9). rTSβ mRNA encodes a protein with a predicted Mr of 46,898 with a predicted Mr of 41,028 for rTSα. The proteins predicted to be encoded by the large ORFs of rTSα and rTSβ are identical for the 341 C-terminal amino acids of each protein.

The divergence of the 3' noncoding regions of rTSβ and rTSα RNAs appears to be the result of transcriptional termination and cleavage/polyadenylation in a previously unidentified intron to generate rTSβ (Fig. 1). rTSβ has a consensus cleavage/polyadenylation site (i.e., AUUAAA; Ref. 10) located 139 nucleotides downstream from where rTSβ and rTSα diverge (nucleotides 1296 and 1412 of rTSβ and rTSβ, respectively). The nucleotide sequences downstream of position 1296 of rTSα are complementary to the published 3' end of the TS gene (Refs. 1 and 3; Fig. 1). This suggests that rTSβ RNA contains nucleotides absent from rTSα transcripts as a result of splicing and that the rTS gene has at least seven introns and eight exons. Previously it was estimated that the rTS gene had at least six introns (1). As a result of the differential utilization and processing of the 3' end of the rTS gene, rTSβ RNA, unlike rTSα, is not complementary to either the primary transcript or mRNA for TS. The two mRNAs differ in length by approximately 350 nucleotides but are not distinguishable by Northern blot analysis (1, 3, 11). Thus, rTS, previously identified because it produced antisense RNA to TS, also encodes the RNA species which are not complementary to TS.

Detection of rTS Proteins of the Predicted Sizes for rTSα and rTSβ. Previously, we reported that two human cell lines (KB and K562) express an rTS protein of Mr ~48,000, larger than that predicted for rTSα. Immunoblotting of cell extracts prepared from H630...
proteins which differ in their N-terminal amino acids, but have 341 identical amino acids. Although rTSα mRNA is longer than rTSβ mRNA (Fig. 1), the alternative utilization of the AUG initiation codon at position 18 for rTSβ predicts for the translation of a much larger ORF for rTSβ than either ORF (ORF1 and ORF2; Fig. 1B) found in rTSα mRNA. In H630 cells, rTSβ is expressed at levels much higher than rTSα (Fig. 3). Higher expression of rTSβ protein compared to rTSα is to be expected based on the context of the nucleotides surrounding the initiation codons for each predicted protein (Refs. 1 and 9; Fig. 1A), and possibly as a result of the difference in the levels of rTSα and rTSβ mRNAs. Identification of rTSβ as a Mr~48,000 protein detected by immunoblotting explains the discrepancy previously observed in the size of the rTS proteins found in K562 B1A and KB cells (3) and that predicted based on the structure of rTSα mRNA.

A potential role for rTS proteins, particularly rTSβ in cellular resistance to TS inhibitors, is suggested by a relatively large increase in the level of rTSβ protein in the FU-resistant H630–1 cell line that also overproduces TS and rTSα proteins compared with H630 cells. H630–1 cells have coamplified the genes and overexpress mRNAs for TS, rTS, and the c-yes oncogene, which are located adjacent to one another on chromosome 18 (15). The 7–8-fold overexpression of rTSα and TS proteins in H630–1 cells is comparable to their levels of gene amplification and overproduction of mRNAs (12, 14). However, the significantly greater expression of rTSβ in the FU-resistant cell line suggests that, in addition to gene amplification, expression of rTSβ protein in these cells is enhanced by transcriptional or posttranscriptional mechanisms. Therefore, overexpression of rTSβ is likely an adaptive response to the cytotoxicity of FU. Overexpression of an rTS protein with the predicted molecular weight of rTSβ was previously reported in a K562 cell line selected for resistance to MTX, which is also 30-fold cross-resistant to fluorodeoxyuridine, but which does not have altered levels of TS (2). Based on the data presented here, we now conclude that rTSβ is probably the protein overexpressed in both the FU-resistant H630–1 cells and in the MTX-resistant K562 B1A cells. Thus, rTSβ levels are elevated, relative to TS, in at least two cell lines resistant to fluorinated pyrimidines.

Elevated expression of TS has been demonstrated to occur as a result of continued passage of cultured cells in media containing TS inhibitors such as ZD 1694 and 5-fluorodeoxyuridine (16–18). The increased levels of rTSβ in H630–1 cells demonstrate that the elevation of TS can be accompanied by larger changes in the expression of other proteins. Preliminary data indicate that rTSβ is also elevated in cultured cells selected for resistance to the TS inhibitors ZD 1694 and fluorodeoxyuridine and that rTSβ levels are induced in H630 and HCT8-DP2 cells in response to TS inhibitors. Therefore, increased expression of rTSβ may be a previously unknown but common phenomenon associated with resistance to TS inhibitors. Both rTSα and rTSβ are members of the mandelate racemase enzyme superfamily (2, 3, 15). This family of proteins catalyzes diverse enzymatic reactions with similar underlying chemistry (2). Thus, at this time, we are not able to predict the precise function of rTS. Preliminary immunoprecipitation data suggest that rTS proteins interact with both TS and dihydrofolate reductase, suggesting the function of each may be modified by direct interactions with rTS proteins (11). Preliminary data in several asynchronously growing cell lines also indicate that rTSβ levels increase as cells progress from log to stationary phase growth, whereas TS levels decrease. Thus, although the precise function of rTS has yet to be elucidated, it seems reasonable to expect

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**Discussion**

Although a number of genes are now known to produce antisense RNAs, the number of genes found in higher eukaryotes that produce proteins encoded by both strands of DNA is exceedingly small (13, 14). We now report that the rTS gene, an antisense gene for the chemotherapeutically important TS, produces two functional mRNAs that produce two closely related proteins (rTSα and rTSβ) through an alternate splicing pathway. Previous studies indicated that rTSα RNA is a functional mRNA, and data presented here indicate rTSα and rTSβ mRNAs code for proteins of molecular weight predicted from the sequences of their ORFs (Figs. 1 and 3). The two mRNAs encode...
that expression of rTS may be a second factor (in addition to TS levels) in affecting tumor response to TS inhibitors.

References


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