Additional Organizational Features of the Murine Folylpolyglutamate Synthetase Gene

TWO REMOTELY SITUATED EXONS ENCODING AN ALTERNATE 5' END AND PROXIMAL OPEN READING FRAME UNDER THE CONTROL OF A SECOND PROMOTER

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The enzyme folylpolyglutamate synthetase (FPGS)1 in mammalian cells has both physiological and pharmacological significance. FPGS mediates the anabolism of folates and their analogs to γ-polyglutamate peptides (1–5). Polyglutamated forms of folate coenzymes are conserved and are more efficient as cofactors for folate-dependent biosynthetic reactions (1–5). Polyglutamylation of folate analogs, on the other hand, renders these agents more cytotoxic (2–8). However, a differential in anabolism of these analogs among normal tissues and tumors often favors (8, 9) their accumulation as polyglutamates in tumor cells and thus contributes to their therapeutic selectivity.

Earlier studies in several laboratories (10–12) suggest that the expression of FPGS activity in various tissues is under stringent regulation. The basis for this regulation and details as to its mechanism are important to our understanding of cellular folate homeostasis (1–6) and also to the metabolic disposition of folate analogs (2–8) during therapy of tumors in animal models and patients. The recent cloning by Shane and co-workers (13) of human FPGS cDNA now makes it possible to address these issues at the level of gene expression. Other recent studies by Moran and co-workers (14) have provided information pertaining to the most 5′ sequence of the human FPGS gene which included the sequence of a putative promoter-like region. Preliminary functional studies reported recently (15) provided evidence of promoter activity for this region. The earlier (14) studies also suggested alternate transcription start sites as a basis for the regulation of synthesis of both mitochondrial and cytosolic forms of human FPGS with the former requiring the inclusion of a NH2-terminal leader peptide encoded by nucleotide sequence at the 5′ end of exon 1. This same group (16) and Shane and co-workers (17) provided detailed information on the structural organization of the human FPGS gene in the form of 15 exons spanning approximately 11 kb of DNA sequence. Shane and co-workers (17) also provided evidence for the existence of alternate splice variants of exon 1. Recent studies of our own (18) characterized the murine FPGS gene, a promoter-like region with somewhat different characteristics than the human promoter and revealed an interesting complexity with regard to exon 1. This was expressed in the form of three splice variants that differed in their content of sequence homologous to human exon 1. As only some variants incorporated a nucleotide sequence encoding both a mitochondrial leader peptide as well as cytosolic FPGS, the results of these studies raised the possibility that the regulation of synthesis of mitochondrial and cytosolic forms of FPGS could occur as a result of alternate splicing.

Nucleotide sequence analysis of independently isolated clones from a mouse liver cdNA library identified two additional splice variants of folylpolyglutamate synthetase (FPGS) mRNA with novel sequence at the 5′ end. These variants incorporate two new alternatives (exons A1a and A1b) of exon 1 in the murine FPGS gene which are also spliced to exon 2. Exon A1a encodes most of the 5′-untranslated region. Exon A1b encodes a downstream segment of the 5′-untranslated region, two ATG start codons, and a unique mitochondrial leader peptide as well as 15 additional amino acids of cytosolic FPGS not encoded by all previously identified (Roy, K., Mitsugi, K., and Sirotnak, F. M. (1996) J. Biol. Chem., 271, 23820–23827) splice variants. It was also found by direct sequencing of genomic fragments that although exon A1b to exon 1 are found approximately 9.5 kilobases upstream from exons B1a, B1b, and B1c. Exons A1a and A1b are separated from each other by a 124-nucleotide intron. Sequencing of the region 5′ to exon A1a revealed a nucleotide sequence that was promoter-like and different from the downstream promoter region in the content of putative cis-acting elements. Primer extension analysis identified a number of potential transcription start sites within the more 3′ end of this region. FPGS RNA transcripts incorporating exons A1a and A1b were detected in both normal mouse tissues, particularly, liver and kidney, and also to a varying extent in tumors; FPGS RNA transcripts incorporating exons B1a, B1b, and B1c were detected mainly in tumors. Thus, transcription of the FPGS gene in this tissue-specific manner appears to reflect the different usage of alternates to exon 1 under the control of different promoters. An unusual splice variant identified infrequently in a mouse liver cDNA library was 2.67 kilobases in size and incorporated exons A1a and A1b and a segment of the downstream promoter region along with exons B1c and B1b and exons 2–15.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank/EBI Data Bank with accession number(s) U35557, U54783, and U59817.

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1 The abbreviations used are: FPGS, folylpolyglutamate synthetase; kb, kilobase(s); PCR, polymerase chain reaction.
We now report other results that identify two additional splice variants found in the form of cDNA in a murine liver cDNA library. These variants incorporate distinct alternatives to exons designated as exons 1a, 1b, and 1c of the murine FPGS gene in our previous publication (18) and encode a distinctly different 5' end and proximal open reading frame within the mRNA transcript. Hybridization screening of DNA restriction fragments showed that these new alternatives to exon 1 exist in the genome >10 kb upstream from the previously designated exons 1a, 1b, and 1c, and their transcription along with exons 2–15 also incorporated in these variants, appears to be under the control of a different promoter. We have putatively identified such a promoter in the sequence immediately 5' of these new alternates to exon 1. We also report on data that show that transcription of these various splice variants that incorporate alternatives to exon 1 occurs in a tissue-specific manner reflecting the alternate usage of different promoters.

**EXPERIMENTAL PROCEDURES**

**Selection of Murine FPGS cDNA Variants**—Full-length cDNA clones were selected by hybridization (19) screening of a murine liver cDNA library prepared from L1210 cells (18) or murine adult liver and by quantitative reverse transcriptase PCR. Additional details are provided under “Experimental Procedures.”

**Isolation of Genomic Clones by Hybridization Screening**—A BALB/c mouse liver genomic library in EMBL3 SP6/T7 (Clontech; Palo Alto, CA) was screened with an exon-specific radioactive murine FPGS cDNA as a template. Isolation of poly(A)⁺ RNA was prepared as discussed earlier (21) and utilized in the synthesis of cDNA by a standard procedure (22).

**FIG. 1.** Organization of the murine FPGS gene showing a restriction map, exon/intron junctions, and different splice variant formation. The composition of the different splice variants is shown to scale in panel A. The three genomic clones shown in panel B had inserts of 17 (clone 3), 12 (clone 5), and 18 kb (clone 10), respectively. The lengths of the exons and introns are shown to scale. The details of the methodology employed are provided under “Experimental Procedures.”

**TABLE I**

Relative content of splice variants found in L1210 cell and murine liver cDNA libraries

The relative frequencies of these variants were estimated from their representation in a cDNA library prepared from L1210 cells (18) or murine adult liver and by quantitative reverse transcriptase PCR. Additional details are provided under “Experimental Procedures.”

| Variant | L1210 cells | Murine liver |
|---------|-------------|--------------|
| I       | 5           | <5           |
| II      | 50          | <5           |
| III     | 20          | <5           |
| IV      | 20          | 80           |
| V       | <5          | 5            |

We now report other results that identify two additional splice variants found in the form of cDNA in a murine liver cDNA library. These variants incorporate distinct alternatives to exons designated as exons 1a, 1b, and 1c of the murine FPGS gene in our previous publication (18) and encode a distinctly different 5' end and proximal open reading frame within the mRNA transcript. Hybridization screening of DNA restriction fragments showed that these new alternatives to exon 1 exist in the genome >10 kb upstream from the previously designated exons 1a, 1b, and 1c, and their transcription along with exons 2–15 also incorporated in these variants, appears to be under the control of a different promoter. We have putatively identified such a promoter in the sequence immediately 5' of these new alternates to exon 1. We also report on data that show that transcription of these various splice variants that incorporate alternatives to exon 1 occurs in a tissue-specific manner reflecting the alternate usage of different promoters.
submitted to GenBank (GenBank/EMBL Accession U59517). After screening of a large number of plaques and purification several positive clones were obtained. The DNA insert from these clones was purified and characterized by restriction mapping and Southern hybridization (23). One of these nonidentical clones designated clone 10 was selected by the above procedure for further analysis because of the large size of its insert (18 kb). Restriction fragments of these clones generated with SacI or EcoRI were selected for further study on the basis of hybridization with the region-specific probes. The fragments of interest after extraction were subcloned into Bluescript SK (Stratagene) for sequencing.

Sequencing of DNA Fragments and Intron Size Determination—Double-stranded DNA was sequenced in both directions according to the dideoxymethod of Sanger et al. (24) using Sequenase version 2.0 (U. S. Biochemical Corp.). Oligonucleotide primers based on the mouse FPGS cDNA were used initially. Additional oligonucleotide primers were prepared on the basis of the sequence data generated when necessary for extending the sequencing. Exon/intron junctions were determined by direct sequencing across these junctions using primers based upon the mouse FPGS cDNA sequences. Intron sizes were determined by sequencing through the region in question.

Enzymatic Primer Extension—Ten pmol of an antisense oligonucleotide primer corresponding to nucleotide sequence in the 3' end of exon A1b (5'-GGAGGCAGTCTAGCTTCAG-3') of the murine FPGS gene was endlabeled (Promega, Madison, WI) at the 5' terminus using T4 polynucleotide kinase and [γ-32P]ATP. The labeled primer was hybridized to 5 μg of poly(A)+ RNA isolated from mouse liver cells which was first treated with methyl merccuric hydroxide and the primer extension reaction carried out as well with a standard kit as specified (Promega). An mRNA preparation derived from a 1.2-kb kanamycin-resistant plasmid cDNA with an antisense primer was used as a control. Electrophoresis on an 8% polyacrylamide gel was carried out by a standard protocol (25).

Northern Hybridization Analysis—Using a standard procedure, sam-
samples of poly(A) RNA were blotted (26, 27) and analyzed by radioautography using exon-specific or nonspecific cDNA probes. The exon A1-specific probe incorporating all of the nucleotide sequence in exons A1a and A1b (see below) was prepared by PCR using variant IV murine liver cDNA as template. The exon B1-specific probe incorporating all of the nucleotide sequence in exons B1a, B1b, and B1c and intron B1c was prepared by PCR using a mouse liver genomic clone as template (clone 3, Ref. 18). A nonspecific probe incorporating the complete nucleotide sequence of the most upstream 5' region of the murine FPGS gene. The DNA sequence is numbered from the ATG start codon for the mitochondrial leader sequence in variant IV. The boundaries for exon A1a and A1b are designated (a) in the figure. Major putative transcriptional start sites identified by primer extension are indicated by vertical arrows in boldface, and other putative transcriptional start sites are designated by vertical arrows. Potential cis-regulatory elements are designated by the appropriate symbols.
sequence in exon 14 was prepared by PCR using murine liver cDNA as template (variant IV). The poly(A)\(^+\) RNA content of each blot was normalized with a mouse 36B4 probe (acidic ribosomal phosphoprotein PO, Ref. 28). Labeling of each probe was by random priming (Random Primers DNA Labeling Kit, Boehringer Mannheim) using \(\alpha\)-\[^{35}\mathrm{S}\]dCTP and a 10–20-ng insert.

**Quantitative PCR**—The relative amount of cell FPGS mRNA transcript representing different splice variants in L1210 cells and murine liver was determined by reverse transcriptase PCR. Prior to CDNA synthesis (see above), poly(A)\(^+\) RNA was digested with RNase-free DNase I, treated with phenol chloroform and precipitated with ethanol followed by washing twice with 70% ethanol before dissolving in \(\mathrm{H}_2\mathrm{O}\). All poly(A)\(^+\) RNA was treated with methyl mercuric hydroxide to reduce secondary structure. Five \(\mu\)l of the prepared cDNA was utilized in a PCR using four different exon 1-specific sense primers and an exon 5 antisense primer. Ten pmol of an 18-mer oligonucleotide encompassing the ATG start site were used for each PCR. 5\(^\prime\)-GAGCCGGGAGCATGGAGT-3\(^\prime\) was used as the A1b-specific sense primer, and 5\(^\prime\)-TAA-GACTATGTCGCTGGC-3\(^\prime\) was used as the B1a-specific sense primer. As a control, the cDNA reaction mixture without reverse transcriptase was used to initiate a PCR to make sure that there is no genomic contamination (data not shown). As an internal standard, sense (5\(^\prime\)-CCTGTTGCTGCTGCTT-3\(^\prime\)) and antisense (5\(^\prime\)-TCTATTGCCATGGACTG-3\(^\prime\)) primers of FPGS representing exons 9 and 14, respectively, were used in a parallel reaction. The experimental reaction was run for 25 cycles using a standard procedure (28), and the relative amount of product generated with each set of primers was determined by 1.5% agarose gel electrophoresis and staining with ethidium bromide (29).

**Materials**—All radioactive isotopes used for the above studies were obtained from DuPont NEN. Specific activities for \(\alpha\)-\[^{32}\mathrm{P}\]dCTP, [\(\alpha\)-\[^{32}\mathrm{P}\]dATP, and \(\gamma\)-\[^{32}\mathrm{P}\]ATP were 3,000, 1,000, and 3,000 Ci/mmol, respectively. DNA restriction enzymes were purchased from Boehringer Mannheim. AmpliTaq DNA polymerase was obtained from Perkin-Elmer. Nitrocellulose was purchased from Schleicher & Schuell, and oligonucleotide primers were synthesized by Genelink. Solutions for polyacrylamide sequencing gels were obtained from National Diagnostics.

**RESULTS AND DISCUSSION**

**Novel Splice Variants of the Murine FPGS Gene**—Screening of an L1210 cell cDNA library with a murine FPGS cDNA probe (19) and DNA sequencing originally identified (18) two different classes of clones exhibiting complexity with regard to exon 1 of the murine FPGS gene. The least common cDNA clone incorporates (Fig. 1A and Ref. 18) all of the nucleotide sequence (now designated exons B1a and B1b in variant I) homologous to human exon 1 (16) spliced to exons 2–15 and encodes both a mitochondrial leader peptide and the cytosolic form of FPGS. The most common variant (variant II) incorporates (Fig. 1 and Ref. 18) only a portion (exon B1b spliced to exons 2–15) of the nucleotide sequence homologous to the human exon 1 and encodes only the cytosolic form of FPGS. A third variant was identified (Fig. 1 and Ref. 18) initially in a liver cDNA library and subsequently in an L1210 cell cDNA library and incorporates an alternate to exon B1a (exon B1c) which is spliced to exon B1b plus exons 2–15. The 5\(^\prime\) nucleotide sequences of these variants aligned with respect to the sequences in exon 2 are given in Fig. 2 where it can be seen that variants I and III incorporate different 5\(^\prime\) ends and a different nucleotide sequence within the proximal segment of the open reading frame. Further screening of the mouse liver cDNA library was carried out using the same cDNA probe. In this library, cDNA representatives of variants I, II, and III were found to be relatively rare (Table I) compared with their relative frequency in the L1210 cell cDNA library. Instead, the most common variant found (variant IV) incorporates (Fig. 1A and 2) a novel sequence in the form of alternates (exons A1a and A1b) to exons B1a and B1b or B1c and B1b spliced to exons 2–15. These clones were derived from mature transcripts with an appropriate poly(A)\(^+\) tail and properly positioned polyadenylation signal sequences at the 3\(^\prime\) end. The length and sequence of the 5\(^\prime\)-untranslated region of variant IV, the position of the two ATG start codons, and the encoded mitochondrial leader peptide (MKGTRSLPMVWVFQY) were distinctly different from those encoded by either exons B1a and B1b or exons B1c and B1b. The nucleotide sequence and the encoded amino acid sequence of all of these splice variants are compared in Fig. 2. These data show that the exons included in these splice variants differ considerably in their composition. Variants I, III, and IV all have in-frame upstream and downstream ATG start codons with properly positioned Kozak (29) consensus sequences. Variant II incorporates only the downstream ATG. In addition to the untranslated region incorporated in exon B1a, exons B1a and B1b in variants I encode different segments of a mitochondrial leader peptide. Along with a shorter untranslated region incorporated in exon B1c, exons B1c and B1b in variant III incorporate different segments of another mitochondrial leader peptide larger in size than that encoded by variant I. By contrast, exon A1a in variant IV only incorporates a untranslated region much longer than that found in variants I and III, whereas exon A1b encodes a different mitochondrial leader peptide in its entirety as well as 15 additional amino acids of cytosolic protein not encoded in variant I, II, or III. The results of quantitative reverse transcriptase PCR obtained earlier (18) and in the current studies (data not shown) using poly(A)\(^+\) RNA from L1210 cells and mouse liver were consistent with the relative frequency of these variants observed in
the L1210 cell and mouse liver cDNA libraries summarized in Table I.

During further screening, another splice variant (variant V) was eventually identified (Fig. 1) in the liver cDNA library which was also rare and extremely unusual. This cDNA was larger (2.67 kb in length) than the other variant cDNAs and incorporated not only exons A1a and A1b but also a portion (identified as exon B1d) of the putative promoter region described previously (18) along with exon B1c and B1b and exons 2–15.

Identification of a Second Promoter-like Region in the Murine FPGS Gene—Sequencing of DNA 5' of exon A1a identified a 2-kb region with stretches of the sequence that included a number of putative binding sites for various cis-acting factors known to affect transcription. The characteristics of this region (Fig. 3) are quite different from that of the promoter-like region 5' of exon B1a (18). In contrast to this downstream region, there are no putative SP-1 binding sites. However, in addition to a GATA/TATA box, there are putative sites for 15–20 different transcription factors not found in the downstream promoter-like region, notably MyoD, Myc, Myb, and p53.

Enzymatic primer extension analysis was carried out using a primer matching the 3' end of exon A1b and methyl mercuric hydroxide-treated poly(A)+ RNA from murine liver. The data suggest (Fig. 4) multiple transcription start sites distributed within this region spanning approximately 200 nucleotides (Fig. 3) upstream from the location of the primer. Start sites were found at +52, +55, +62, and +64 nucleotides. Major start sites were found at +53 and especially at −184. The usage of only the latter would result in the formation of a transcript encoding the putative mitochondrial leader peptide. The results of primer extension analysis pertaining to the downstream promoter-like region using exons B1b- and B1c-specific primers have already been reported (18).

Tissue-specific Expression of FPGS in the Form of Alternate RNA Transcripts—Using antisense probes specific for the A1 and B1 alternates of exon 1, a series of Northern blots was performed with poly(A)+ RNA from a variety of normal and neoplastic murine tissues. Poly(A)+ RNA from these same tissues was also blotted with a nonspecific probe incorporating the nucleotide sequence from a downstream exon. All of these blots were normalized as well by blotting with a 36B4 cDNA probe. The data in Fig. 5 show that FPGS mRNA transcripts in the range of 2.3 kb could be detected in all of the tissues examined using the exon nonspecific probe. In contrast, the detection of FPGS mRNA transcripts with the A1- and B1-specific probes was highly tissue-specific. With the nonspecific probe there was considerable variability in the relative amount of 2.3-kb FPGS mRNA detected depending upon the tissue. Among normal tissues examined, FPGS mRNA content was highest in kidney and liver and lowest in spleen, lung, and small intestine. Using the same probe, FPGS mRNA was readily detectable in all of the tumors except the hepatoma. This also included the Ehrlich tumor, which had less mRNA in the sample employed than was assumed (see also the blot with the 36B4 probe). With the exon A1-specific probe, relative levels of FPGS mRNA detected among these different tissues were similar to that obtained with the nonspecific probe. However, with the B1-specific probe, the level of FPGS mRNA detected was highest in the tumors with the exception of the mouse hepatoma. The level of mRNA detected by this probe in this tumor was relatively low by comparison and extremely low in kidney and small intestine and virtually undetected in spleen, lung, and liver. The low intensity overall of the blot obtained with this probe most likely reflects the relatively high GC content of this probe compared with the other probes used in this study. In addition to the tumors identified in Fig. 5, poly(A)+ RNA from several other murine tumors was also probed with these two cDNA probes with similar results (data not given). These included B16 melanoma, T241 fibrosarcoma, P388 lymphoma, EO771 mammary carcinoma, and taper liver tumor.

These studies provide further evidence for substantial heterogeneity at the 5' end among murine FPGS RNA transcripts. Our earlier (18) studies documented 5' end heterogeneity in the form of a splice variant (variants I and III) incorporating alter-
among the different tumors examined.

5’ end heterogeneity associated with differential promoter usage has been documented or suggested to occur in the case of a variety of mammalian genes. These include rodent genes for γ-glutamyl transferase (30), acyl-CoA synthetase (31), and human genes for aminopentidase N (32), carbonic anhydrase I (33), and phosphofructokinase (34). Despite this prece

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