A Human Testis-specific mRNA for Phosphoribosylpyrophosphate Synthetase That Initiates from a Non-AUG Codon*

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Two highly homologous subunits for phosphoribosylpyrophosphate synthetase are encoded by human X-linked genes, PRPS1 and PRPS2 (Taira, M., Kudoh, J., Minoshima, S., Iizasa, T., Shimada, H., Shimizu, Y., Tatibana, M., and Shimizu, N. (1989b) Somat. Cell Mol. Genet. 15, 20-31). These genes are expressed in most tissues, whereas an additional unique mRNA (1.4 kilobases) is present in the testes of rats as well as mice and humans (Taira, M., Iizasa, T., Yamada, K., Shimada, H., and Tatibana, M. (1989a) Biochim. Biophys. Acta 1007, 203-208). In this paper, cDNA cloning revealed that the human testis-specific mRNA was encoded by an autosomal gene, termed PRPS3. RNA blot analysis showed that the expression of this gene began at 4 weeks of age in rats, coinciding with the reported appearance of primary spermatocytes. A cDNA clone of PRPS3 was sequenced and found to encode a predicted product of 317 amino acids which was highly homologous to those of PRPS1 and PRPS2 (94.3% and 91.2% identities, respectively). However, the PRPS3 cDNAs lacked an ATG initiator for translation at the expected position, although the deduced amino acid sequence of PRPS3 cDNA was highly homologous to those of rat PRPS1 as well as rat and human PRPS2 cDNAs (Taira et al., 1989b). In vitro mutagenesis and in vitro transcription/translation studies suggested that the ACG codon at this position could direct initiation of translation at the expected position, although the deduced amino acid sequence of PRPS3 cDNA was highly homologous to those of rat PRPS1 as well as rat and human PRPS2 cDNAs (Taira et al., 1987; Iizasa et al., 1988b). Human gene mapping showed that PRPS1 and PRPS2 genes, referred to as PRPS1 and PRPS2, respectively, are located on different regions of the X chromosome, while two additional PRPS1-related sequences were present on autosomes (Taira et al., 1989b). These results led to the idea that the testis-specific mRNA is probably transcribed from one of the two autosomal sequences, since X-chromosome inactivation during spermatogenesis has been proposed in several animals (Lifschitz and Lindsay, 1972), as discussed (Taira et al., 1989a and 1989b).

To examine this possibility, we cloned human cDNAs for the testis-specific mRNA. We report here that this mRNA is encoded by an autosomal gene, termed PRPS3. Sequence analysis revealed that the mRNA lacks an AUG translational initiator at the expected position, although the deduced amino acid sequence of PRPS3 cDNA was highly homologous to those of rat PRPS1 as well as rat and human PRPS2 cDNAs (Taira et al., 1987; Iizasa et al., 1988b). In vitro mutagenesis and in vitro transcription/translation studies suggested that the ACG codon at this position could direct initiation of translation in the mRNA.

Initiation from non-AUG codons in naturally occurring eukaryotic mRNAs has been reported only for the upstream "weak" initiator for the alternate translational initiations in the mRNAs of adeno-associated virus (Becker et al., 1985). Sendai virus (Curran and Kohakosky, 1986; Gupta and Patwardhan, 1988), c-myc proto-oncopgene (Hann et al., 1988), and basic fibroblast growth factor gene (Prats et al., 1989). In contrast, the ACG codon in the PRPS3 mRNA may be the sole initiator codon for the biologically functional product of this gene.

EXPERIMENTAL PROCEDURES

cDNA Cloning and Nucleotide Sequencing—A λgt10 cDNA library was constructed from human testis poly(A)+ RNA as described (Taira et al., 1987). About 1,350,000 independent plaques were screened using rat PRPS1 and PRPS2 cDNA probes (Taira et al., 1987) and 17 clones were obtained, three of which strongly hybridized with the PRPS1 cDNA probe. The insert from one of the three, named λPRSIII-7, was recloned into a plasmid vector pUC18 (Takara Shuzo) to construct pHPRSII-1, and the PouI "3'"-base pair fragment (probe A; see Fig. 1) from pHPRSII-7 was used for blot analysis. cDNA inserts and their restriction fragments were subcloned into M13mp18 or -19 and sequenced by the dideoxy chain termination method (Sanger et al., 1977). The nucleotide sequence(s) reported in this paper has been submitted to the GenBank/EMBL Data Bank with accession number(s) 005602.

The abbreviations used are: PP-Rib-P, 5-phosphoribosyl 1-pyrophosphate; kbp, kilobases; PRS I, PRS II, and PRS III, phosphoribosylpyrophosphate synthetase subunits I, II, and III, respectively; SDS, sodium dodecyl sulfate; tRNA, initiator methionine tRNA; tRNA, methionine tRNA.
method (Sanger et al., 1977) using deoxy-7-deazaadenosine triphosphate as a substrate instead of dGTP (Mizusawa et al., 1986). The sequence of AC at nucleotide 1 together with an ambiguous sequence at nucleotides 393-394 was confirmed by using dITP and a modified T7 DNA polymerase (Sequenase, U. S. Biochemical Corp.; Tabor and Richardson, 1987).

RNA and DNA Blot Analyses—The procedures were as described (Taira et al., 1987).

Recombinant Plasmids and Construction of Deleted or Chimeric cDNAs—The recombinant plasmids were constructed as follows: pGEM2-r1 (rH) is from insertion of the 2.0-kilobase (kb) rat PRPS1 cDNA (XPRS1; Taira et al., 1987) into the EcoRI site of pGEM2-2 (2.9 kb). pGEM2-r1 (rII) is from insertion of the 3.0-kb rat PRPS2 cDNA (XPRS2-1; Taira et al., 1987) into the EcoRI site of pGEM2-2 (2.9 kb; Promega); pGEM2-h3 (hIII) and pGEM2-h3r2 (its orientation reverse to that of hIII) are from insertion of the 1.1-kb human PRPS3 cDNA (hPRPSIII-7) into the EcoRI site of pGEM-2. Construction of the hPRPSIII-7 cDNA was as described in detail above (Fig. 5 A). pGEM2-h3R was digested with Accl to isolate a 3.5-kb fragment. This 3.5-kb fragment and fragment b were ligated and the 3'-recessed ends were filled by Klenow DNA polymerase and religated to form pGEM1-h3R (hIII). This 3.5-kb fragment was filled by Klenow DNA polymerase and then self-ligated to generate pGEM1-h3R2 (hIII2). Chimeric plasmids, hIII-r1 and hII-rIII, were constructed by using fragments c, d, b, and c, or a and e, respectively (Fig. 5A). No ATG sequence is created by ligation of these DNA fragments.

Construction of Mutant cDNAs—Site-directed mutagenesis was carried out as follows. The insert from hPRPSIII-7 cloned into the EcoRI site of M13mp18 and single-stranded DNA was prepared for use as the template. Mutant oligonucleotides used for priming DNA synthesis were antisense 19-mer, ATATCGGCGCTGTAGCCTATG (from 1 to 18) for hIIAC or ATATCAGCTCTATGTGCTATGC (from 11 to 28) for hIIATG, and antisense 22-mer, AGATTTGGATATTCGGC- -TTGGCCAACTACCA (from 20 to -15) for hIIIAACG. Mutagenesis was carried out by using a kit purchased from Amersham Corp., and the conditions were as described by the supplier. The altered sequences were confirmed by sequence analysis of the mutant M13 clones. The mutant cDNAs were cloned into the EcoRI site of pGEM-2. The orientation of cDNA was the same as that of hII, as shown in Fig. 5A.

In Vitro Transcription/Translation—In vitro transcription of linearized plasmids (5 µg), digested with EcoRI (hIIL1 and hIIL2) or BamHI (hIIL1, hIIL3, hII-rIII, and rI), was performed at 37 °C for 2 h in 25 µl of 40 mM Tris-Cl, pH 7.5, 3.6 mM MgCl2, 2 mM spermidine, 10 mM dithiothreitol, 1.2 units/µl RNasin (Promega), 0.5 mM ATP/UTP/CTP, 20 µM GTP, and 0.25 µM GTP (Boehringer, Mannheim), using 30 units of SP6 RNA polymerase (Takara Shuzo) for hII, hIIL1, hIIL3, and rI, or 100 units of T7 RNA polymerase (Bethesda Research Laboratory) for hII-rIII and hII-rII, as described (Melton et al., 1984; Pelletier and Sonenberg, 1985; Nielsen and Sharp, 1986). The reaction mixture was extracted with phenol/ chloroform, and nucleic acids were recovered by ethanol precipitation and resuspended in 20 µl of H2O. Two µl of each was electrophoresed in formaldehyde-agarose gel to estimate integrity and amount of synthesized RNA. Four µl of each was used for in vitro translation. The synthesized capped mRNA was translated in rabbit reticulocyte lysates (Cade N.90, Amersham) or in wheat germ extracts (Du Pont Nuclear) with [35S]methionine (1300 Ci/mmol, Amersham), and 1.1 µg of protein/µl Escherichia coli S-100 fraction containing aminoacyl-tRNA synthetase and methionyl-tRNA synthetase (kindly gifted from Dr. S. Yokoyama) (Muramatsu et al., 1988), in the presence or absence of 0.25 mM formyl donor. The formyl donor (N-formyltetrahydrofolate) was prepared from folic acid (calcium salt) as described (Dubnoff and Maizel, 1971). After incubation, incorporation of [35S]methionine into mRNA was measured as described (Muramatsu et al., 1988). Since E. coli methionyl-tRNA synthetase recognizes only mammalian tRNA, no [35S]methionyl-tRNA will be formed (Stanley, 1974). The mixture was passed through a spin column C 60 (Boehringer Mannheim), extracted with phenol saturated with water and then with chloroform/isooamyl alcohol (24:1). The aminoacylated tRNA was ethanol-preципitated and dissolved in 20 µl of 10 mM sodium acetate, pH 5.0, and stored at -20 °C until used. This preparation did not contain free [35S]methionine. Formylation efficiency of [35S]methionyl-tRNA was determined as described (Leder and Bursztyn, 1966) with slight modification. In vitro translation was carried out in 12.5 µl of the reaction mixture composed of 10 µl of reticulocyte lysates, 0.5 µl of the mRNA solution, and 2 µl of the formylated or nonformylated [35S]methionyl-tRNA solution (about 2 x 106 dpm); otherwise in 13 µl of the reaction mixture supplemented with 1 mM methionine. The products were separated by SDS-polycrylamide gel electrophoresis and visualized by fluorography as described above.

RESULTS
cDNA Cloning of the Testis-specific mRNA—We obtained PRPS1-related clones, hPRPSIII-7, -18, and -1, from a human testis cDNA library with use of rat PRPS1 cDNA as a probe (Fig. 1). To examine whether these clones originated from the testis-specific transcript, Northern blot analysis using RNAs from rat, mouse, and human testes was performed. As shown

![Fig. 1. Restriction map and nucleotide sequencing strategy for human PRPS5 cDNA. The cDNA clones, hPRPSIII-7, -18, and -1, are shown under the restriction map. The box represents the coding region and the thick bars indicate the noncoding region. The arrow shows the direction and extent of sequencing. The upper bar labeled Probe A corresponds to the PouII 0.74 kb restriction fragment used as a probe for the blot analyses. A16 and A13 represent numbers of adenine nucleotides from the 3' end. The open circle in hPRPSIII-18 indicates a cytosine residue replaced with an adenine in hPRPSIII-7 at nucleotide 1010. This may represent a difference in start sites for poly(A) tailing. The closed circle in hPRPSIII-1 indicates a thymine residue at nucleotide 837, which causes an amino acid substitution from Glu (GAG) to Asp (GAT). This difference seems to represent a polymorphic change between alleles.](https://www.jbc.org/content/jbc/321/16/16492/F1.large.jpg)
in Fig. 2, the rat PRPS1 cDNA probe, as a control, hybridized to PRPS1 2.3-kb and the testis-specific 1.4-kb mRNAs (panel A), whereas the human cDNA probe (Probe A, see Fig. 1) hybridized to the 1.4-kb mRNA more preferentially in each species (panel B) and little to 3.7-kb (rat and mouse) or 2.7-kb (human) PRPS2 mRNA. These results suggested that λPRSIII-7 originated from the 1.4-kb RNA and that this mRNA was transcribed from a third gene.

To investigate the relationship between the expression of this gene and testicular maturation, RNAs from the testes of 1-3-week-old rats were analyzed. As shown in Fig. 2C, the 1.4-kb transcript, barely detectable at 3 weeks of age, became clearly visible after 4 weeks, whereas the 2.3-kb band of PRPS1, which was cross-hybridized with probe A, was present throughout development. This dramatic increase in the 1.4-kb mRNA roughly correlated with the appearance of primary spermatocytes (Allsop and Watts, 1986).

Gene Mapping of PRPS3—Southern blotting with the rat PRPS1 cDNA probe detected three major bands of 20, 6.7, and 4.3 kb in BamHI-digested human DNA (Taira et al., 1989b). In Fig. 3A, under nonstringent conditions, probe A hybridized to 20-, 6.7-, and 4.3-kb fragments (lane 1), whereas, under stringent conditions, only the BamHI 20-kb fragment was detected (lane 2). In the human-mouse somatic cell hybrid TA4 containing human chromosome 7 but not X (Shimizu et al., 1980), the 20-kb band was detected in addition to three bands of mouse DNA fragments (Fig. 3B). The locus of this band was previously assigned to chromosome 7 and was tentatively named PRPS1L1 (Taira et al., 1989b). As these results are taken to mean that the testis-specific 1.4-kb mRNA was transcribed from the PRPS1L1 gene, this testis-specific gene was renamed PRPS3.

Sequence Analysis of cDNA—Fig. 4A shows the nucleotide and deduced amino acid sequences of human PRPS3 cDNA (λPRSIII-7 clone), along with the nucleotide sequences of rat PRPS1 and PRPS2 cDNAs. The nucleotide identity in the region from nucleotide 1 to 954 is 89.8% between human PRPS3 and rat PRPS1; this value is greater than the nucleotide identity between rat PRPS1 and rat PRPS2 (81.0%) and between rat PRPS2 and human PRPS3 (78.1%). The sequence similarity agreed well with the strong cross-hybridization between PRPS1 and PRPS3 genes.

The putative PRPS3 product, designated as PRS III, was predicted from the cDNA sequence to start at Pro-1 and to terminate at Leu-317 with a calculated molecular mass of 34,676 Da. This amino acid sequence is strikingly homologous to those of rat PRS I and rat PRS II (Fig. 4B); identities between the amino acid sequences are 94.3% and 91.2%, respectively. The amino-terminal sequences of rat PRS I and PRS II start with Pro-1, as determined by protein analysis (Kita et al., 1989). The presence of an upstream in-frame terminator (indicated by underline in Fig. 4A) suggests that the ATG triplet adjacent to the Pro-1 codon is the translation initiator in the PRPS1 and PRPS2 mRNAs.

However, in the human PRPS3 cDNA, the nucleotide sequence just upstream of the Pro-1 codon lacks an ATG and instead contains, unexpectedly, an ACG triplet (Fig. 4A and C). This ACG sequence was also found by nucleotide sequencing of another cDNA clone, λPRSIII-18 (starting with nucleotide −51, see Fig. 4A). The same result was obtained by using either deoxy-7-deazaguanosine (data not shown) or dITP (Fig. 4C) as a sequencing substrate for elimination of band compression. Furthermore, this sequence was confirmed by nucleotide sequencing of a genomic clone obtained from the DNA of another individual.

An in-frame termination codon exists at nucleotide −12 and no splicing acceptor site is found upstream from the Pro-1 codon, thereby suggesting that an upstream ATG, if present, could not be used for the initiation of translation. Therefore, the ACG triplet at nucleotide 1 should serve as the initiation codon for human PRPS3 mRNA. In addition, the nucleotide sequence surrounding the ACG (GCCAAGACGC, from nucleotide −6 to 3) resembled the sequence considered as a favorable context for the initiation of translation (GCCACCATGG; Kozak, 1986a).

In Vitro Transcription/Translation—In vitro transcription and cell-free translation was used to examine the possibility that the human PRPS3 mRNA is translated from a non-AUG codon. Recombinant plasmid DNA was used as template in an in vitro transcription reaction with SP6 or T7 RNA polymerase (Fig. 5A). The resulting RNA was translated in rabbit reticulocyte lysates in the presence of [35S]methionine. In control experiments, the translation products from rat PRPS1 and PRPS2 constructs, rI (Fig. 5B, 6th lane) and II (data not shown), both co-migrated with the 34-kDa subunit of purified rat PP-Rib-P synthetase in the SDS-polyacrylamide gel electrophoresis. The two subunits of this enzyme, PRS I and PRS II, were not separated by electrophoresis or by conventional chromatographic procedures (Kita et al., 1989).

In the human PRPS3 construct hIII, three major bands of 38, 28.5, and 27 kDa were detected (Fig. 5B, 1st lane). The 38-kDa band was abolished in the deletion plasmid hIIIΔ1, and all three bands were abolished in hIIIΔ2 (Fig. 5, A and B).
Testis-specific PRPS3 mRNA Initiates from ACG Codon

Fig. 4. Nucleotide and deduced amino acid sequences of human PRPS3 cDNA. A, nucleotide sequence of human PRPS3 cDNA and comparison of nucleotide sequences of human PRPS3, rat PRPS1, and rat PRPS2 cDNAs. b3, complete nucleotide sequence of human PRPS3 cDNA. Numbers above sequences (AAATCC in 5' and GG in 3', are omitted); r1, partial sequence of rat PRPS1 cDNA; r2, partial sequence of rat PRPS2 cDNA (Taira et al., 1987). Dots indicate bases identical with those of the human cDNA. Deduced 2nd and 3rd lanes). The bands were also not detected in the product of the antisense hII1 transcript (see Fig. 6, 5th lane). These results demonstrate that the synthesized RNA from hII1 was translated, and that translation for the 38-kDa product initiates from a site existing between the 5' end and the Accl site at nucleotide 157 (Fig. 5A). On the other hand, the 28.5- or 27-kDa band in hII1 or the 28- or 26.5-kDa band in r1 might be a product initiating from either of four internal ATG codons at nucleotides 202, 217, 343, and 379 (indicated by dots in Fig. 5A) which exists between the two Accl sites at nucleotides 157 and 424 in hII1.

If it is assumed that the 38-kDa band of hII1 was the putative product of PRPS3, i.e. PRS III, the question is why the product behaved as a 38-kDa species, not co-migrating with the 34-kDa subunit of the rat PP-Rib-P synthetase. The calculated molecular mass of 34,676 for PRS III was all but identical with the 34,703 for rat PRS I or 34,682 for rat PRS II.

To determine whether the coding frame of the PRPS3 cDNA predicted in Fig. 4A was actually used for the translation of the 38-kDa product from hII1, chimeric cDNAs of r1/hII1 and hII1/r1 were constructed, by using the common Accl sites (GTCTAC) at nucleotide 157 (Fig. 5A). The products of these constructs were 37 kDa for r1/hII1 and 36 kDa for hII1/r1, which were intermediate between those of hII1 (38 kDa) and r1 (34 kDa) (Fig. 5B, 4th and 5th lanes). The two lower bands of r1/hII1 and hII1/r1 appeared to be identical with those of hII1 and r1, respectively. These results show that the reading frames of the hII1 DNA fragments a, b, and c (see Fig. 5A) were aligned with the frame of r1. In addition, differences in the product size between hII1 (38 kDa) and r1 (34 kDa) were roughly correlated with the number of amino acid substitutions against hII1: 1 (r1/hII1), 17 (hII1/r1), or 18 (r1) residues, respectively, as indicated by vertical lines in Fig. 5A. Therefore, the higher molecular mass found for hII1, r1/hII1, and hII1/r1 (compared to r1) could be accounted for by some amino acid sequences of human PRS III, rather than a difference in translational start position. The 38-kDa product was also detected using the wheat germ extract translation system (Fig. 6, 2nd lane).

To determine the precise site of translation initiation in hII1, we used oligonucleotide-directed site-specific mutagenesis to construct mutant cDNAs, hII1ACC, hII1ATG, and hII1ACG: the ACG codon at nucleotide 1 in hII1 was converted to ACC (hII1ACC) or ATG (hII1ATG) or deleted (hII1ACG). Of the four threonine codons (ACU, ACC, ACA, and ACG), ACC is most frequently used in mammalian cells.

Amino acid sequence of human PRPS3 is shown above the nucleotide sequence, b3. The sequence of ACG at nucleotide 18 started from nucleotide -51, as indicated. Underlines, in-frame termination codons; bold underline, the poly(A) addition signal. The 5' nucleotide sequence in parentheses (nucleotides -112 to -82) was not found in the genomic gene and was identical with the sequence from nucleotides 113 to 193. Thus the 5' sequence of 21 bases appears to have been artificially connected during cDNA synthesis. B, comparison of predicted amino acid sequences of PP-Rib-P synthetase subunits. Human PRS III (Hum III), rat PRS I (Rat I), rat PRS II (Rat II) (Taira et al., 1987), and the E. coli enzyme (E. coli) (Hove-Jensen et al., 1986) were aligned. Dots indicate amino acid residues identical with those of human PRS III, and gaps have been introduced for alignment. Little different residues between human PRS III and rat PRS I; +, identical residues among the four; , conservative substitutions among the four, which were based on Dayhoff's mutation data (Dayhoff et al., 1978). C, nucleotide sequencing of the 5' coding region in human PRPS3 cDNA. Sequencing reaction was performed using dGTP (left panel) or dITP (right panel). Nucleotide sequence from 12 to 12 is shown on the right. ACG triplet at nucleotide 1 is indicated by closed circles.
Testis-specific PRPS3 mRNA Initiates from ACG Codon

(Aota et al., 1988). Fig. 6 shows that the 38-kDa product was not found in hIIIACC and hIIIAACG, whereas the level of hIIIATG product, the mobility of which cannot be distinguished from the 38-kDa one, was about 5 times higher than that of hIII product. Thus, these results suggested that the ACG triplet at nucleotide 1 served as translation initiation of the 38-kDa product but not as a threonine codon, and that this native ACG initiator might have a lesser degree of efficiency than the ATG mutant in initiation of translation, at least in this rabbit reticulocyte lysates.

Sequence Analysis of in Vitro Translation Products—To analyze the amino-terminal sequences of the products of 38 kDa generated from hIII and of 34 kDa from rI, radiolabeling with [3H]leucine in in vitro translation was performed. The products were subjected to the amino acid sequence analysis. As shown in Fig. 7, the peaks of radioactivity were found at residues 3 and 5 in both rI and hIII. These results were consistent with the predicted amino-terminal sequences of rI and hIII as well as that of rat PP-Ribo-P synthetase purified from the liver (Kita et al., 1989). This implies that the amino-terminal methionine was cleaved with methionine aminopeptidase from the nascent polypeptide of rI in this cell-free translation system, and further that the hIII product might initiate with methionine from the ACG codon and then the initiating methionine was removed to yield amino-terminal proline.

![Fig. 6. In vitro transcription/translation of PRPS3 mutant cDNAs constructed by site-directed mutagenesis. A, RNA, no addition of RNA sample to the translation system; hIII, human PRPS3 cDNA; rI, rat PRPS3 cDNA; hIIIACC, hIIIAACG, and hIIIATG, mutated constructs (see text); hIII Δ, deleted construct; anti-sense hIII, reverse orientation to that of hIII. The left two lanes, translated in wheat germ extracts; other lanes, translated in rabbit reticulocyte lysates. Two minor bands lower than 38 kDa appeared in hIIIACC and hIIIAACG may result from initiation at other non-AUG triplets downstream of the ACG, as suggested (Peabody, 1989).](http://www.jbc.org/content/16495/Fig6.large.png)

![Fig. 7. Amino-terminal sequence analysis of radiolabeled products of rI and hIII. The in vitro translation products labeled with [3H]leucine were subjected to automated sequential Edman degradations as described under “Experimental Procedures.” Upper panel, the 34-kDa product of rI (17,000 cpm were applied to the sequencer); lower panel, the 38-kDa product of hIII (21,000 cpm applied). The predicted amino acid sequence corresponding to residues 1 to 11 (Fig. 4B) is given at the bottom of each panel. Ordinate represents the amount of radioactivity released from each Edman degradation cycle.](http://www.jbc.org/content/16495/Fig7.large.png)

**Translation Initiation with Methionine—Formyl[35S]methionyl-tRNA was used to determine whether methionine was incorporated at the amino-terminal position corresponding to the ACG codon, since a formylmethionine residue blocks cleavage from nascent polypeptides and formylmethionyl-tRNA should not be incorporated at positions corresponding to internal AUG codons.**

The hIII products were synthesized in vitro with nonformylated [35S]methionyl-tRNA, or N-formylated [35S]methionyl-tRNA, with or without 1 mM nonradioactive methionine (Fig. 8). Although the 38-kDa product was labeled with nonformylated [35S]methionyl-tRNA (–F in left panel), the 38-
kDa band was diminished in the presence of 1 mM nonradioactive
methionine (−F/+Met in right panel), thereby indicating
that most of the radioactivity of the 38-kDa product labeled
with nonformylated [35S]methionyl-tRNA, was derived from
free [35S]methionine which might be generated by cleavage of
initiating methionine of nascent polyribosomes. In contrast,
the 38-kDa product labeled with formylated [35S]methionyl-
tRNA, (Fig. 8, +F) was not so affected by the addition of 1
mM methionine (+F/+Met in right panel) compared to that
labeled with the nonformylated one (−F/+Met). The activity
of the radioactive 38-kDa product in +F/+Met probably
represents the N-formyl[35S]methionine residue at the amino
terminus of the product. These results together with the
amino acid sequence analysis strongly suggest that the 38-
kDa product of hII could initiate with [35S]methionyl-tRNA,
at the position corresponding to the ACG codon.

**DISCUSSION**

**Tissue Differential Expression of the PP-Rib-P Synthetase**

Gene Family—PP-Rib-P serves as a coordinate regulator for
the biosynthesis of purine and pyrimidine nucleotides: it is a
limiting substrate for amidophosphoribosyltransferase (Rose-
benloom et al., 1968) and a critical activator for carbamoyl-
phosphate synthetase II, key enzymes in purine and pyrimi-
dine de novo biosynthesis, respectively (Tatibana and Shige-
sada, 1972). Therefore, modulation of PP-Rib-P synthetase
activity could regulate the synthetic rate of both nucleotides,
in vivo. This is supported by observations that superactive
PP-Rib-P synthetase leads to nucleotide overproduction in
human fibroblasts (Becker et al., 1987).

We have identified three genes for subunits of PP-Rib-P
synthetase (X-linked PRPS1 and PRPS2 and autosomal
PRPS3) and also showed the tissue-differential expression of
the three genes (Taira et al., 1988a) as well as the develop-
mental expression of PRPS3 in the testis (Fig. 2C). These
observations may imply some functional differences among
enzymatic properties of the protein products of the three
genes. In addition, the presence of the three genes may also
allow for the tissue-differential (or tissue-specific) control of
PRPS expression by linking them to different regulatory sequences.

It is also tempting to speculate that the expression of auto-
somal PRPS3 may be associated with X-chromosome inacti-
vation in the spermatogenic cells, as has been postulated for
the phosphoglycerate kinase genes: X-linked PGK-1 and au-

**Highly Conservative Structure of PP-Rib-P Synthetase—**
The predicted amino acid sequences of the three isoforms
(PRPS I, PRPS II, and PRPS III) and the E. coli enzyme (Hove-
Jensen et al., 1996) indicate a remarkable conservation of the
enzyme structure: 221 conservative amino acids (69.7%) are
common to the four, based on Dayhoff’s mutation data (Dayhoff
et al., 1978) (Fig. 4B). In addition, 148 (PRS III, 46.7%) or
151 (both PRS I and PRS II, 47.6%) identical residues exist
between the mammalian and E. coli sequences. These per-
centages are comparable with the homology seen between
some of the most highly conserved proteins, such as the β-
subunit of ATPase (69%: Runswick and Walker, 1983) and
dnaK/hsp70 products (48%; Bardwell and Craig, 1984).

**PRPS3 Derived from PRPS1—**The evolutionary distances
among the three isoforms were calculated from the
sequence proposed by Miyata and Yasunaga (1980) K values,
representing the nucleotide differences per site in synonymous
changes of amino acids, are 0.380 for PRPS3 versus PRPS1,
0.735 for PRPS1 versus PRPS2, and 0.785 for PRPS2 versus
PRPS3. These findings clearly show that the PRPS3 gene
is derived from the PRPS1 gene after divergence from the
PRPS1 and PRPS2 genes. This suggests that the ancestor
gene of PRPS3 contained an ATG initiation codon at nucleo-
tide 1, and thereafter this sequence was changed to a AC
triplet. During evolution of this gene, the coding frame from
Pro-1 to Leu-317 has been remarkably conserved, a finding
interpreted to mean that ACG codon at nucleotide 1 rather
than a thrineo codon is the initiator of translation.

**Non-AUG Initiation Codon of PRPS3 mRNA—**The in vivo
transcription/translation assay showed that the human
PRPS3 mRNA was indeed translatable and the in vitro mu-
genesis experiment suggested that the 38-kDa product was
initiated from the ACG triplet at nucleotide 1. The amino-
terminal sequence of the radiolabeled PRPS3 product was
consistent with the predicted sequence starting at Pro-1 (Fig.
7). Moreover, since the product was labeled with N-for-
myl[35S]methionyl-tRNA, the nascent peptide probably initi-
ates with methionine at the amino-terminal position corre-
sponding to the AC codon. These results are supported by
the observation that an ACG codon replacing the AUG initi-
ation codon functions as an initiator of translation, in cell-
free translation or in in vivo expression systems (Anderson
(1987) also showed that in vivo translation of a mutant
dihydrofolate reductase mRNA in which the AUG initiator
was converted to ACG triplet was initiated with methionine.

Certain mRNAs of the adeno-associated virus (Beccera
et al., 1985) and Sendai virus (Curran and Kolakofsky, 1988;
Gupta and Patwardhan, 1988) are reportedly initiated from
ACG codons. In these cases, the non-AUG codon was thought
to serve as an unfavorable context for the upstream initiator,
causing an advance to the second initiator, supporting the
“leaky scanning” mechanism of ribosomes (Kzoak, 1986b);
accordingly, two or more kinds of functional products are
produced from one mRNA species. In the case of PP-Rib-P
synthetase, however, truncated molecules of functional PP-
Rib-P synthetase have never been reported in any species
from bacteria to humans (Fox and Kelley, 1971; Switzer and
Gibson, 1978; Kita et al., 1989), although this possibility has
not been completely excluded. Moreover, the primary struc-
ture, both amino acid sequence and its number, of this enzyme
is highly conservative. Therefore, it is unlikely that the testis-
specific PRS III has an isoform truncated by alternate initi-
ation; the ACG triplet at nucleotide 1 is possibly the sole
initiator codon for the functional PRPS3 product in vivo.

**Why does PRPS3 gene lack an AUG initiator codon?** The
non-AUG initiator may play an important role in reducing
translational efficiency so that PRPS3 mRNA accidentally
expressed in somatic cells is not easily translated, thereby
strictly limiting the expression of PRS III subunit in the testis.

We searched for the 38-kDa product in the crude homogenate and partially purified sample of human testis by immunoblot analysis using polyclonal antisera against the rat PP-Rib-P synthetase or synthetic peptides corresponding to the conserved sequences among the subunits. However, a band of 38 kDa was not evident on the blot above the background of immunoreactive bands, although the 34-kDa band was clearly visible on the blot. The human testis appeared to have an undetectable level of the 38 kDa product in these experiments, whereas the PRPS3 mRNA was comparatively abundant. At this time, we have no direct evidence for the translation of the human PRPS3 mRNA in vivo. However, a possibility that PRPS3 mRNA is not translated is unlikely because (i) the mRNA is present in rats, mice, and humans (Taira et al., 1989a; Fig. 2, A and B), (ii) the expression of the mRNA is developmentally regulated (Fig. 2C), and (iii) the coding sequence has been conserved over a long period of evolution since divergence of PRPS1 and PRPS3 (at least before divergence of rats, mice, and humans), thereby suggesting that the product, PRS III, should exist in the mature testes and have an important biological function.

We are now trying to prepare PRS III-specific antisera to detect the presence of PRS III protein in the testis and also are isolating the rat PRS III cDNA to examine whether this cDNA has an ACG codon at the predicted location.

While it is difficult to verify that non-AUG codons are physiologically functional, our finding that in the highly conservative isoform genes, a non-AUG triplet is substituted for AUG initiation codons, does provide evidence for non-AUG initiation systems in eukaryotic cells.


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