Characterization of the Structure and Expression of Mouse *Itpa* Gene and its Related Sequences in the Mouse Genome

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Abstract

In the mouse genome, we found one processed *Itpa* gene-like sequence and two processed *Itpa* pseudogenes as well as the *Itpa* gene itself with introns, located on chromosome 2F3, which was isolated by a retro-recombination method. We also identified three types (A, B, C) of *Itpa* transcripts in mouse tissues. The processed *Itpa* gene-like sequence located on chromosome 2E1 has a complete open reading frame for exactly the same polypeptide as ITPA encoded by the type A transcript, with a polyadenylation signal. However, no transcribed sequence derived from the *Itpa* gene-like sequence was detectable in any of the mouse tissues examined, thus naming the sequence as *Itpa* processed pseudogene α. The type A *Itpa* mRNA, which was expressed in all mouse tissues examined, only encodes mouse ITPA polypeptide consisting of 198 amino acid residues with a capacity to hydrolyze dITP into dIMP. *Itpa* mRNA was detected in all tissues examined, and its expression is especially high in the testis, brain, and thymus. ITPA protein was mostly detected in the cytoplasm, to a lesser extent in the nuclei of neurons in the brain, and also those of hepatocytes, epithelial cells lining the bile duct, and endothelial cells lining the portal vein in the liver.

Key words: ITPase; ITP; XTP; pseudogene; retro-recombination; oxidative deamination

1. Introduction

The accumulation of modified or damaged bases in genomic DNA is a major cause of altered genetic information that results in mutagenesis or even programmed cell death.1 It has been established that such damaged bases in genomic DNA arise from two independent pathways: one is a consequence of the direct modification of the normal bases in the DNA and the other is incorporation of modified nucleotides generated in resident nucleotide pools.2,3,4

The incorporation of dUTP into DNA which arises from the deamination of dCTP or as a byproduct of UTP biosynthesis is a major cause for shortening the Okazaki fragment during nascent strand synthesis that occurs during base excision repair by uracil DNA glycosylase.4 dUTP is thus detrimental to organisms and it is eliminated by a specific nucleotidase, dUTPase, from the resident nucleotide pools.5–7 8-Oxo-2′-deoxyguanosine triphosphate (8-oxo-dGTP) is one of the major causes of spontaneous mutagenesis, because 8-oxo-dGTP is formed by the spontaneous oxidation of dGTP in the nucleotide pool and it is incorporated into the nascent strand opposite both adenine and cytosine in the template strand during DNA replication.8 We have demonstrated that 8-oxo-dGTP is specifically hydrolyzed by MutT family proteins from prokaryotes to humans, and, as a result, organisms maintained a low spontaneous mutation rate.9–12

Genome projects revealed the existence of many MutT-like proteins with different substrate specificities,13–17 and a structure-based approach recently identified another novel enzyme, namely inosine triphosphate pyrophosphatase (ITPase), which hydrolyzes deaminated purine nucleoside triphosphates such as ITP, dITP, and XTP.18–20 In *Escherichia coli*, a mutant of the *rdgB* gene coding ITPase protein is viable but it shows synthetic lethality with *recA* or *recBC* mutation.21 Recently, the lethality of *rdgB recA* or *rdgB recBC* double mutants has been shown to be suppressed by the inactivation of endonuclease V (EndoV) which initiates the excision of...
deoxyinosine (dI) or deoxyxanthosine (dX) incorporated into DNA.\textsuperscript{22} It is likely that an ITPase deficiency results in the accumulation of its substrate nucleotides, dITP or dXTP in the nucleotide pools, thus causing an increased accumulation of dI or dX into DNA, and further excision repair initiated by EndoV leads to chromosomal fragmentation in \textit{recA} or \textit{recBC} mutants. Following this study,\textsuperscript{22} the missense mutants of the \textit{dut} gene encoding dUTPase, were reported to exhibit synthetic lethality with \textit{recA} or \textit{recBC} mutations, and synthetic lethality was suppressed by inactivation of the \textit{ung} gene encoding uracil DNA glycosylase, thus confirming that the accumulation of abnormal nucleotides in the nucleotide pools increased chromosomal fragmentation as a consequence of the excision repair of such abnormal bases incorporated into DNA.\textsuperscript{7}

The first reported deficiency in the ITPase in a human was characterized by an elevated ITP level in erythrocytes as an inherited abnormality.\textsuperscript{23} However, such individuals do not exhibit any abnormal phenotype even with accumulation of ITP in erythrocytes. A cDNA for human ITPase was isolated and its gene \textit{ITPA} was identified,\textsuperscript{19} and the structure of the \textit{hITPA} gene and nucleotide alterations responsible for the ITPase deficiency were reported.\textsuperscript{24,25}

In contrast to \textit{E. coli}, the biological importance of ITPase protein and the pathological consequences of its deficiency in humans or mammals have not yet been elucidated. To explore the biological significance of ITPase in mammals, disruption of the mouse \textit{Itpa} gene and characterization of such mutant mice is considered to be one of the best experimental approaches. During isolation of the \textit{Itpa} gene from the mouse genome, we found one processed \textit{Itpa} gene-like sequence and two processed \textit{Itpa} pseudogenes as well as the \textit{Itpa} gene itself with introns, which was isolated as well as a retro-recombination method in the present study.

2. Materials and Methods

2.1. RT-PCR

Total RNAs from \textit{CCE} mouse embryonic stem (ES) cells derived from 129SvEv mouse,\textsuperscript{26} serum-starved and serum-stimulated BALB/c3T3 cells,\textsuperscript{27,28} and C57BL/6J adult mouse tissue specimens were prepared using ISOGEN (Nippon Gene) according to the manufacturer’s instructions. Total RNAs from BALB/c mouse tissues were purchased from Clontech. Total RNA (10 µg) was treated with 20 units of RNase-free deoxyribonuclease I (DNase I) (Amersham Biosciences) at 37°C for 15 min in 100 µl of reaction buffer containing 40 mM Tris-HCl (pH 7.5) and 6 mM MgCl\textsubscript{2}. The treated RNA was purified by extraction with Phenol/CHCl\textsubscript{3} (1:1) followed by ethanol precipitation. The purified RNA (2 µg) was used for synthesis of the first strand cDNA using a First-strand cDNA synthesis Kit (Amersham Biosciences) according to the manufacturer’s instructions. PCR was performed in 20-µl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl\textsubscript{2}, 0.3 µl of the first-strand cDNA, 0.4 U of recombinant Taq DNA polymerase (Takara), 4 µM of each primer (Table 1), and 200 µM of each deoxynucleoside triphosphate. The initial denaturation was performed at 95°C for 1 min and the amplification was performed by 27, 32, 40 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 20 sec, and extension at 72°C for 60 sec, followed by a final extension at 72°C for 5 min. PCR products were subjected to agarose gel electrophoresis, and the band intensity on the gel stained with ethidium bromide was measured using the LAS1000-plus Luminescent Image Analysis System (FUJI FILM).

Table 1. The synthetic oligonucleotides used in this study. These were obtained from Greiner Japan and Hokkaido System Science.

| Oligomer   | Sequence                  |
|------------|---------------------------|
| mltpa_EX1f | GACGGGAACGGCAAGAAGGCTG   |
| mltpa_EX2r | CAAAGTCATGGAAATTTATCTCCG |
| mltpa_EX2f | TCAATGCATGGGAAGGCTCAAG   |
| mltpa_EX3r | TGGAAAACATCCTCGGTTTCTCC  |
| mltpa_EX3f | CCACTACGAAAGTGGGACAGGC   |
| mltpa_EX4r | AGGTATCCTTCACGAGACAGG    |
| mltpa_EX4f | TTATACGACCTTGAGGAGCTC    |
| mltpa_EX5f | AGGGTTCTACGGTGGCTACAG    |
| mltpa_EX5r | AAATGGTTCCTACGAAGACTGA   |
| mltpa_EX6f | GTGCTATGCGCTTTTGTCTCT    |
| mltpa_EX6r | CAACGTGGCTCTGTGCGCAG     |
| mltpa_EX7f | CCGCCTGTCCTGCGGATCAG     |
| mltpa_EX7r | TCTCTGCTTTCAGCGCTGTTG    |
| mltpa_EX8f | CACCACGCGGATGCGCGCTTTC   |
| mltpa_EX8r | AGTTATAGTATAAATAATATACAGA |
| Psa-46    | GGAGTTTCACTGCGGTGCTGAG   |
| Psa-46    | GGAGTTTCACTGCGGTGCTGAG   |
| Psa-46    | GGAGTTTCACTGCGGTGCTGAG   |
| Psa-46    | GGAGTTTCACTGCGGTGCTGAG   |
| Psa-46    | GGAGTTTCACTGCGGTGCTGAG   |
| Psa-46    | GGAGTTTCACTGCGGTGCTGAG   |

\*Italic letters indicate a sequence added to create restriction sites for HindIII and Nco I.

Biosciences) according to the manufacturer’s instructions. PCR was performed in 20-µl reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl\textsubscript{2}, 0.3 µl of the first-strand cDNA, 0.4 U of recombinant Taq DNA polymerase (Takara), 4 µM of each primer (Table 1), and 200 µM of each deoxynucleoside triphosphate. The initial denaturation was performed at 95°C for 1 min and the amplification was performed by 27, 32, 40 cycles of denaturation at 95°C for 30 sec, annealing at 55°C for 20 sec, and extension at 72°C for 60 sec, followed by a final extension at 72°C for 5 min. PCR products were subjected to agarose gel electrophoresis, and the band intensity on the gel stained with ethidium bromide was measured using the LAS1000-plus Luminescent Image Analysis System (FUJI FILM).

2.2. Genomic PCR

Genomic DNA (25 µg) from CCE cells was treated with RNase A (20 units, Sigma), which was boiled for 15 min to inactivate contaminated DNase, in 150 µl of a reaction mixture containing 5 mM Tris-HCl (pH 7.6) and
0.5 mM EDTA at 37°C for 30 min to eliminate the residual RNA completely. DNA was purified by extraction with Phenol/CHCl₃ followed by ethanol precipitation. A PCR reaction was performed with 100 ng of the purified genomic DNA with appropriate primers (Table 1), as described in the RT-PCR section. The PCR products were subcloned into pT7 Blue T vector (Novagen) plasmid and their nucleotide sequences were confirmed by sequencing the insert from the both strands using ABI Sequencing Kits and a model 3100 automated DNA sequencer (ABI), according to the manufacturer’s instructions.

2.3. Isolation of genomic clones for Itpa gene

A PCR fragment carrying intron 2 or 3 of Itpa amplified from CCE genomic DNA was subcloned into the multi-cloning site of the pANγ plasmid, and each plasmid was introduced into E. coli MC1061[P3] cells. Each cell was infected with λ phage genomic library constructed in ATK phage with 129 SvJ mouse genomic DNA. Four out of 6 clones isolated with a probe plasmid carrying intron 2, and 8 out of 14 clones isolated with a probe plasmid carrying intron 3, using the retro-recombination methods, contained the appropriate probe sequence and identical adjacent sequences for each other, respectively.

2.4. Plasmid construction

Mouse Itpa cDNA was amplified from the mouse testis library (ML 4007AB, Clontech) and from total RNA prepared from CCE cells using 5-3 mIT and 3-3 mIT primers (Table 1). Human ITPA cDNA was amplified from a human leukocyte library (HL 4021AB, Clontech) using 5-3 hIT and 3-2 hIT primers (Table 1). PCR products were subcloned into pT7 Blue T Vector. The entire coding region of type A Itpa cDNA (615 bp) was amplified with 5-1 mIT and 3-1 mIT primers, in which a NcoI site at the initiation site and an EcoRV site after the termination codon was introduced, respectively. The PCR product digested with NcoI and EcoRV was subcloned into a Neo I/BamHI site of the pET8c and pET32a plasmids (Novagen), in which a BamHI site was converted to a blunt end by filling in with Klenow fragment. Obtained plasmids were designated pET8c:mITPA and pET32a:mITPA, respectively. A Neo I/BamHI fragment of hITPA cDNA was also subcloned into a Neo I/BamHI site of pET32a or pET8c plasmid yielding pET32a:hITPA and pET8c:hITPA, respectively.

2.5. Immunodetection of ITPA

Rabbit antiserum against the fusion protein TrxA-hITPA were prepared as described previously. The antiserum was able to detect both TrxA-hITPA and mITPA proteins with almost the same efficiency, and thus was designated as anti-ITPA. Western blotting analyses were performed as previously described, using anti-ITPA serum (1/500 dilution). Immunohistochemistry with anti-ITPA were performed as follows. The mice were deeply anesthetized with 5% pentobarbital, and perfused transcardially with 10 ml of heparinized saline (0.9%) followed by 30 ml of phosphate buffer (0.1 M) containing 4% paraformaldehyde. Tissue specimens were fixed in 4% paraformaldehyde at 4°C for 12 to 24 hr and embedded in paraffin. The sections (4 μm) were deparaffinized, pretreated in 3% hydrogen peroxide in methanol, and subjected to immunohistochemistry with anti-ITPA serum (1/500 dilution). The sections were processed by Vectastain ABC KITs (Vector Laboratories) with an anti-rabbit biotinylated secondary antibody, and the peroxidase reaction product was detected using 3,3′-diaminobenzidine-tetrahydrochloride (Sigma). The slides were subjected to counterstaining by hematoxylin. Digital images were acquired using Axioskop2 plus equipped with an AxioCam (Carl Zeiss).

3. Results and Discussion

3.1. Isolation of Mouse cDNA and Genomic Sequences Homologous to Human ITPA cDNA sequence

We searched the DNA sequence databases to retrieve the mouse cDNA or genome sequences homologous to human ITPA cDNA sequence (accession no. AF219116), using the BLASTN program (http://www.ncbi.nlm.nih.gov/BLAST/), and found many mouse expressed sequence tags (ESTs) and several mouse genomic sequences. Among the many EST sequences retrieved, the longest cDNA sequence (accession no. AK008279) which encodes a polypeptide highly homologous to the amino acid sequence of human ITPA protein (hITPA) was selected, and it was used for the BLASTN search with the Ensembl Genome Data Resources for mouse (http://www.ensembl.org/Mus_musculus/). The alignment revealed that there are several different mouse chromosomes carrying highly homologous but not identical sequences to that of AK008279 itself, thus suggesting that there are multiple genes for mouse ITPA protein (mITPA) or related proteins.

To isolate cDNA or genomic sequences which encode mITPA, we amplified the sequences from genomic DNA and cDNA prepared from a mouse ES cell line CCE cells which was established from a 129 SvEv mouse, using two different primer sets for the AK008279 EST sequence (Fig. 1A, Table 1). From the RNase A-treated genomic DNA, a major single fragment approximately 1100 bp in length was amplified with primer set I (5-1 mIT and 3-3 mIT), but not with primer set II (5-3 mIT and 3-3 mIT; Fig. 1B, lanes 2, 7). In primer set I, the 5’-primer hybridizes a region including the initiation codon while the 5′ primer in the latter set hybridizes its 5′-untranslated region (UTR). In contrast, two major fragments of approximately 1100 and 830 bp in length from primer set I,
or 1140 and 860 bp in length from primer set II were amplified from the cDNA prepared from the DNase I-treated RNA, respectively (Fig. 1B, lanes 3, 8).

A sequence analysis of subcloned PCR products (102 independent clones) obtained by primer set II, revealed three different types of cDNA sequences (types A, B, and C) to be amplified from the RNA prepared from the ES cells (Fig. 2A). In the RT-PCR products, the ratio of each PCR product. 

Figure 1. Amplification of the mouse Itpa cDNA-related sequences from genomic DNA and total RNA prepared from CCE ES cells. A. Primer sets for mouse Itpa cDNA. Two primer sets (I and II) were designed based on a mouse Itpa cDNA (accession no. AK008279). Primer set I with primers 5-1 mIT and 3-3 mIT, in which the 5′-primer hybridizes into the initiation codon with 9-base extra sequence (see Table 1), was expected to amplify the 1111-bp fragment, while primer set II with primers 5-3 mIT and 3-3 mIT, in which the 5′-primer hybridizes into 5′-UTR sequence, was expected to amplify the 1143-bp fragment from Itpa cDNA. The coding regions for ITPA and poly(A) sequences are shown with an open box and a wavy line, respectively. B. Agarose gel electrophoresis of the PCR products. To avoid cross-contamination of genomic DNA or RNA into total RNA or genomic DNA prepared for PCR templates, genomic DNA was treated with DNase-free RNase A, while total RNA preparation was pre-treated with RNase-free DNase I before cDNA synthesis. PCR was performed with a template and primer set shown under each lane. Lanes 1, 6, size markers. The arrowheads indicate the major PCR products in each reaction: closed, 1108-bp genomic PCR product; gray, 1111- or 1143-bp RT-PCR product; open, 832- or 864-bp RT-PCR product.

or 1140 and 860 bp in length from primer set II were amplified from the cDNA prepared from the DNase I-treated RNA, respectively (Fig. 1B, lanes 3, 8).

A sequence analysis of subcloned PCR products (102 independent clones) obtained by primer set II, revealed three different types of cDNA sequences (types A, B, and C) to be amplified from the RNA prepared from the ES cells (Fig. 2A). In the RT-PCR products, the ratio of each cDNA identified was type A : B : C = 1 : 0.3 : 0.05. The longest type A cDNA isolated was 1143 bp length with an open reading frame (ORF) of 597 nucleotides, whose sequence was identical to AK008279 and was predicted to encode a polypeptide of 198 amino acid residues with a molecular weight of 21,883 Da. The amino acid sequence was 89.9% identical to that of human ITPA (194 aa).

Type B and C cDNAs were 864 and 720 bp length, respectively, and had the same 5′- and 3′-UTR sequences as did the type A cDNA. Both of the cDNAs had the same reading frame as that of the type A, but they were shorter than that of type A, and were 318 and 168 nucleotides in length, respectively. Both type B and C cDNA shared the first ATG codon and the stop codon (TAG) with type A cDNA, and were predicted to encode polypeptides with 105 or 55 amino acid residues missing central parts (94–186 aa or 44–186 aa) of the full-length mITPA protein, respectively (Fig. 2A). These results were confirmed by a sequence analysis of RT-PCR products (100 clones) obtained by primer set I, whose length was 1111, 832, or 688 bp, respectively (data not shown).

A sequence analysis of 50 clones of DNA fragments amplified from genomic DNA with primer set I revealed a single 1108-bp fragment whose sequence was 99% identical to that of the type A Itpa cDNA (Fig. 2B). The more 5′- and 3′-regions of the genomic sequence were amplified using a new primer set based on the mouse DNA sequence from clone RP23-16015 on chromosome 2 (accession no. AL672251) which contained exactly the same sequence shown in Fig. 2B (Table 1). The genomic sequence (1135 bp) retained the same ORF of 597 nucleotides which encodes the same polypeptide as does the type A cDNA, with a canonical polyadenylation signal (AATAAA) in the same position as in the type A cDNA. There are three silent nucleotide substitutions in the ORF of the genomic sequence in comparison to that in type A cDNA (Fig. 2B). In addition, one of the five repeats of the CAA triplet found in the 3′-UTR of the type A Itpa cDNA sequence was missing in the genomic sequence. These results strongly suggest that there are indeed multiple genomic sequences and transcripts which are capable of encoding mITPA protein or its variants.

3.2. Transcripts for mouse Itpa expressed in adult mouse tissue

Since the genomic sequence amplified with the primer set I has no intron-like sequence with a complete ORF for mITPA protein, it is likely that this genomic sequence is a processed type gene for the mITPA protein. Although we did not obtain such a sequence in the RT-PCR products from the CCE ES cells with primer set I, it is possible that the genomic sequence is transcribed in other types of cells or tissues. To clarify this point, we amplified cDNA prepared from various adult mouse tissues using primer set I, and the RT-PCR products were digested with two different restriction enzymes, ApaLI and Hae II. The former digests the processed gene-like sequence but not the three types of cDNA, while the latter digests only the
Figure 2. Mouse Itpa cDNA-related sequences. A. Alignment of the coding sequences of three types of mouse Itpa cDNA and amino acid sequences of their translation products (accession no. AB100501). The number of nucleotides and amino acid residues are shown on the right.

B. Alignment of DNA sequences for type A Itpa cDNA and the genomic PCR product containing Itpa cDNA-like sequence. Type A Itpa cDNA was amplified from CCE RNA with a primer set of 5'-mIT and 3'-4 mIT, and the genomic PCR product was obtained from CCE genomic DNA with the primer set of Pα-45 and Pα-46 (Table 1) designed based on a mouse genome sequence (accession no. AL672251) which contained a sequence identical to that of the genomic PCR product shown in Fig. 1B (lane 2). Identical nucleotides between the two sequences are shaded and the initiation and stop codons and the polyadenylation signal are boxed. The recognition sequences for Hae II in the Itpa cDNA and Apa I in the genomic PCR product are underlined.
three types of cDNA for mITPA (Fig. 2B, 3A). A single 1108-bp fragment was amplified from CCE genomic DNA pre-treated with DNase-free RNase A, while two or three bands corresponding to 1111-, 832-, or 688-bp fragments were detected in the RT-PCR products from all the tissues examined as well as from the CCE cells (Fig. 3B).

ApaLI digestion of the 1108-bp genomic PCR product yielded 814- and 294-bp fragments as predicted from its sequence (Fig. 3C, lane 10), while no such digested band was detected in the RT-PCR products from all adult mouse tissues examined as well as from the CCE ES cells (Fig. 3C, lanes 2–9). On the other hand, HaeII digestion produced 609- and 502-bp fragments from all RT-PCR products (Fig. 3D, lanes 2–9), and the genomic PCR product remained undigested (Fig. 3D, lane 10). From the cDNA prepared from the lung, cerebrum and cerebellum as well as from CCE cells, three fragments — 1111-bp, 832-bp, and, to a much lesser extent, 688-bp fragments — were amplified, and the latter two fragments were also digested by HaeII but not by ApaLI. These results were confirmed by simultaneous digestion with the two enzymes (Fig. 3E).

As a result, we concluded that the 1108-bp genomic sequence highly homologous to the mouse Itpa cDNA sequence is not transcribed in any of the adult mouse tissues examined so far, and thus we designated the genomic sequence as Itpa processed pseudogene α (accession no. AB100502). Furthermore, we confirmed that the three types of Itpa transcripts identified in CCE ES cells were also expressed in adult mouse tissue.

3.3. Type A Itpa mRNA encodes a functional deoxyinosine triphosphate pyrophosphatase

In order to confirm that a polypeptide encoded by the type A Itpa transcript possesses the capability of hydrolyzing nucleotides such as ITP or dITP, the type A cDNA was placed under the control of T7 promoter in pET8c or pET32a. In E. coli cells harboring pET8c:mITPA or pET32a:mITPA plasmids, a single 22-kDa or 34-kDa polypeptide was expressed after IPTG induction, respectively (Fig. 4A, lanes 5, 11). The former corresponds to the native mITPA protein with the expected molecular weight of 21,883 Da, and the latter is a fusion protein with thioredoxin (Trx-mITPA) whose molecular weight is predicted to be 33,558 Da. Crude extracts prepared from E. coli cells harboring vector itself or pET8c:mITPA, pET32a:mITPA were incubated with dITP or dATP, and the reaction products were separated on a DEAE column attached to an HPLC system.12 Exports from E. coli cells harboring vector itself had no detectable activity to hydrolyze dITP (Fig. 4B, top panel; Fig. 4C, open circles and squares), while the extracts from E. coli cells expressing mITPA efficiently converted dITP to dIMP (Fig. 4B, bottom panel; Fig. 4C,
Extracts containing Trx-mITPA also hydrolyzed dITP to dIMP (Fig. 4C, closed squares). dATP was barely hydrolyzed by extracts prepared from *E. coli* cells with or without mITPA (Fig. 4C, triangles), thus demonstrating that mITPA encoded by the type A *Itpa* mRNA specifically hydrolyzes dITP into dIMP.

### 3.4. Isolation and characterization of mouse *Itpa* gene

A detailed analysis of mouse genomic sequences revealed that there are several pseudogene-like sequences highly homologous to mouse *Itpa* cDNA in the mouse genome (Table 2). In order to isolate the functional mouse *Itpa* gene, we initially intended to isolate unique intronic sequences for mouse *Itpa* gene. Based on the genomic structure of the human *ITPA* gene consisting of 8 exons and 7 introns, each exon of the mouse *Itpa* gene was predicted based on the type A mouse *Itpa* cDNA sequence, and each intron sequence, except for introns 1 and 6, was amplified from genomic DNA prepared from CCE ES cells by two primers hybridized to the adjacent exons (Table 1).

With the genomic fragment containing the intron 2 or 3 as a probe, we applied the retro-recombination method, in order to isolate the genomic sequences encompassing *Itpa* gene from a ATK phage genomic library derived from

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**Figure 4.** Type A *Itpa* mRNA encodes functional deoxyinosine triphosphate pyrophosphatase. **A.** The expression of recombinant mITPA proteins in *E. coli* cells. Isopropyl β-D-thiogalactoside (IPTG, 1 mM) was added to the exponentially growing cultures of *E. coli* BL21 cells carrying pET8c (lanes 1, 2), pET8c:mITPA (lanes 4, 5) or pET32a (lanes 8, 9, pET32a:mITPA (lanes 10, 11), and each culture was further incubated at 37°C for 3 hr. An aliquot was harvested before (lanes 1, 4, 8, 10) and after the addition of IPTG (lanes 2, 5, 9, 11). Whole cell extracts prepared from each cell were subjected to 15% (Lanes 1–6) or 12.5% SDS-PAGE (lanes 7–12), and the gels were stained with Coomassie brilliant blue. Lanes 3, 6, 7, 12, molecular weight markers. The closed arrowhead indicates a band corresponding to the 22-kDa mITPA protein, an arrow indicates the 34-kDa Trx-mITPA fusion protein, and the open arrowhead indicates the Trx itself (20 kDa).

**B.** Hydrolysis of dITP by the recombinant mITPA. Whole cell extracts (50 ng of protein) were incubated with 1 mM dITP for the time noted, and the products were separated on a TSK-Gel DEAE-2SW column. The amount of nucleotides was determined by UV absorbance at 249 nm. Closed circles, dITP reacted with extracts prepared from cell carrying pET8c:mITPA; open circles, dITP reacted with extracts prepared from cells carrying pET8c; closed squares, dITP reacted with extracts prepared from cells carrying pET32a:mITPA; open squares, dITP reacted with extracts prepared from cells carrying pET32a; closed triangles, dATP reacted with extracts prepared from cells carrying pET8c:mITPA; open triangles, dATP reacted with extracts prepared from cell carrying pET8c.
Table 2. Restriction fragments containing a homologous sequence with mouse type A Itpa cDNA predicted from the mouse genome database.

| Nomenclature   | Chromosome | DDBJ/EMBL/GenBank accession no. | EcoRI   | HindIII | XbaI   |
|----------------|------------|---------------------------------|---------|---------|--------|
| Itpa gene      | 2P3        | AL772162 AB101662               | 3.17, 7.4, 9.95 | 0.84, 0.91, 2.35, 21 | 4.7, 10.4 |
| Itpa pseudogene α | 2B1       | AL672251 AB100502               | 5.9, 12.7 | 4.8    | 4.7, 7.4 |
| Itpa pseudogene β | 9B4       | AC091531                        | 11.8, >2.6 | 9.8    | 12.5   |
| Itpa pseudogene γ | XA3.2     | AL672147                        | 8.8     | 4      | 4.4    |

129 SvJ mouse. Fourteen clones were isolated and classified into three groups as shown in Fig. 5A. The sequences of PCR products and λTK phage clones together with the mouse DNA sequence from clone RP23-175J8 on chromosome 2 (accession no. AL772162) revealed that mouse Itpa gene consists of 8 exons and 7 introns as expected from the genomic structure of the human ITPA gene, and it spans about 13.8 kb of the region on the F3 band of chromosome 2 (Table 2, Fig. 5A,B). The alignments of type B and C Itpa cDNA sequences with the genomic sequence revealed that the two transcripts are generated by an unusual joining between exon 5 and exon 8 or between exon 3 and exon 8, respectively (Fig. 5A).

Type A mRNA, a major transcript from Itpa gene, is generated by splicing at canonical splice sites with GT-AG dinucleotides at the splice junctions. However, type B and C transcripts are most likely generated by splicing at non-canonical splice sites with GA-AG or AA-AG dinucleotides at each unusual splice junction, respectively (Fig. 5A). In both cases, the same 3'-acceptor site in the exon 8 was used. The 5'-donor sequence (GAGTAC) in exon 3 was also found at the 5'-end of alternatively spliced exon 8, while the sequence (CTACAG) at the 3'-end of alternatively spliced exon 5 was also present at the 3'-acceptor site in exon 8, thus suggesting that these repeated sequences may be involved in such unusual splicing.

To confirm the genomic structure for the mouse Itpa gene, genomic DNA prepared from ES cells was digested with restriction enzymes EcoRI, HindIII or XbaI, and subjected to Southern blot hybridization with a DNA fragment containing the entire coding region from type A cDNA (731 bp) as a probe (Fig. 6). In addition to the bands expected from the genomic sequence for Itpa (Table 2; Fig. 6, arrows), three extra bands for each digested sample were identified which likely represent the processed pseudogenes α, β, and γ, respectively (Fig. 6, arrowheads). Among them, the Itpa pseudogene α located on chromosome 2E1 has a complete ORF for exactly the same polypeptide as ITPA encoded by the type A transcript, with a polyadenylation signal (Fig. 2B).

However, we could not detect any transcribed sequence derived from the Itpa pseudogene α in CCE ES cells or from any mouse tissues examined, as shown in Fig. 3, thus concluding that the mouse has only one functional Itpa gene in its genome.

The existence of such process pseudogenes in the mice were confirmed by Southern blot analysis of 129, C57BL/6J and BALB/c mouse strains, and essentially the same results were obtained from all strains examined as well as for CCE ES cells (data not shown). Although there was one more pseudogene-like sequence on chromosome 6 in the mouse genome database, we could not identify any band corresponding to the sequence in our Southern blots (Fig. 6). This may be because the sequence has much less homology with the Itpa cDNA probe used.

3.5. Expression of mouse Itpa gene in mouse tissues and its association with cell proliferation

Expression of Itpa gene in adult mouse tissues was examined by a Northern blot analysis (Fig. 7A, top panel). A band corresponding to 1.35 kb in length was detected in all examined tissues, however, its levels significantly varied from tissue to tissue. Measurements of the radioactivities of bands on the blot revealed the highest expression to be seen in the testis and brain, followed by the thymus (Fig. 7A, lanes 2, 13, 14), and most other tissues except for smooth muscle, the salivary gland and stomach, expressed about a 50% of the level of Itpa mRNA detected in the brain or testis (Fig. 7A, bottom panel).

We then monitored the expression of Itpa in serum-starved BALB/c 3T3 cells with or without serum stimulation (Fig. 7B). A quantitative analysis of the Northern blots revealed the level of Itpa mRNA in quiescent cells to be low, however, this level increased twofold within 15 hr after serum stimulation and then gradually decreased after entering the S phase, 18 hr after serum stimulation, and then returned to the basal level after 24 hr or later (Fig. 7C).

The genomic sequence of the Itpa gene revealed a...
Figure 5. Genomic organization and alternative splicing of the mouse *Itpa* gene. *A*. A schematic diagram of the structure of the *Itpa* gene and its transcripts. The alignment of the DNA fragments derived from mouse genomic libraries is shown in the upper part. The approximate insertion size of each clone is also shown in parentheses. In the middle part, the structure of the gene, together with the appropriate restriction enzyme sites (E: EcoRI, H: HindIII, X: XbaI), is shown. The boxes represent the exons for the *Itpa* gene and the shaded regions represent the protein-coding region. In the bottom part, three types of alternatively spliced *Itpa* mRNAs are shown. The sequence derived from each exon is shown as a box with a number of bases consisting of each exon, and the dashed lines indicate the normal splicing sites, while solid lines indicate the unusual splicing sites found in type B and type C transcripts. A number of bases for the protein-coding region derived from exon 1 or exon 8 are shown in parenthesis. *B*. The nucleotide sequences of intron/exon boundaries of *Itpa* gene. The nucleotide sequences of the exons and parts of the introns determined by a comparison of the sequence of type A *Itpa* cDNA with the mouse genomic sequence (Accession nos. AL772162, AB101662), are shown in bold uppercase and lowercase, respectively, and the flanking sequences in plain lowercase. The genomic sequences shown were confirmed by sequencing of phage clones from a λTK phage genomic DNA library derived from 129SvJ mouse or genomic PCR products. The start of exon 1 was based on the most 5′-extended EST clone for *Itpa* mRNA (Accession no. BB6647208). The initiation codon ATG, the termination codon TGA and a putative polyadenylation signal are underlined. The arrows indicate the unusual 5′-splicing sites in the exon 3 and 5, and the arrowhead indicates the unusual 3′-splicing site in the exon 8, found in type B and type C transcripts. The dotted lines indicate the repeated sequences at the unusual splicing junctions.
Figure 6. Southern blot analyses of genomic DNA for mouse Itpa-related sequences. Genomic DNA (10 µg) prepared from CCE ES cells was digested with EcoRI (lane 2), HindIII (lane 4) or XbaI (lane 6), and was subjected to Southern blot analyses with 32P-labeled fragment containing the entire coding region from the type A Itpa cDNA (731 bp). Southern hybridization was done as previously described,27 and the membrane was washed twice in 2× SSC/0.1% SDS, and then once in 0.2× SSC/0.1% SDS. The arrows indicate bands corresponding to the expected fragments from Itpa gene, closed arrowheads indicate bands corresponding to the expected fragments from pseudogene α, the gray arrowheads with solid line indicates the bands corresponding to the expected fragments from pseudogene β, while the gray arrowheads without solid line indicate band(s) whose size could not be predicted precisely because of incompleteness of the database. The open arrowheads indicate bands corresponding to the expected fragments from pseudogene γ (Table 2).

TATA-less promoter with a sequence (CTACTTC) which exactly matched the consensus sequence for the initiator (PyPyANT/APyPy), just upstream of the 5′ end of the longest Itpa cDNA (Fig. 5B),34 indicating that the Itpa gene is likely to be one of the housekeeping genes which are ubiquitously expressed. A consensus-like sequence for ELK1 or ETS-1 proteins (ACMGGAAGTNC, ACMGGAWRTT), which are known to be activated during serum stimulation,35,36 and one for GATA transcription factors (WGATAR)37 were found in the Itpa promoter region (Fig. 5B). Those factors may be responsible for the inducible expression of Itpa in some tissues.

3.6. Expression and distribution of mouse ITPA protein in mouse tissue

In extract prepared from mouse brain, only a single band corresponding to a polypeptide with a molecular

Figure 7. Expression of mouse Itpa gene in mouse tissue and its association with cell proliferation. A. The expression of Itpa mRNA in various types of adult mouse tissue. Total RNAs (20 µg each) extracted from various types of mouse tissue were electrophoresed, transferred onto a HybondTM-N+ nylon membrane, and subsequently probed with 32P-labeled fragment containing the entire coding region from the type A Itpa cDNA (731 bp) (top panel) and the 18S rRNA probe49 (middle panel), as previously described.50 The arrowhead indicates Itpa mRNA. In the bottom panel, the relative amounts of Itpa mRNA to 18S rRNA were calculated based on the radioactivity. The ratio of the relative amount of each transcript to that in the testis is shown. B. The expression of Itpa mRNA in quiescent and serum-stimulated BALB/c3T3 cells. The total RNA isolated was subjected to Northern blot analyses to determine the expression amount of Itpa mRNA and 18S rRNA. C. The relative amounts of Itpa mRNA to 18S rRNA were calculated based on radioactivity. The ratio of the relative amount of each transcript to that in the quiescent cells is shown (closed circle). The percentages of the cells in the S phase of the cell cycle were determined by flow cytometry as previously described28 and then were plotted with a dotted line.
Figure 8. The expression of mouse ITPA protein in mouse tissues. A. Western blotting analysis. Brain extracts (20 µg protein) were separated on 12.5% SDS-PAGE and subjected to Western blotting as previously described, with preimmune serum (lane 1) or anti-ITPA serum (lane 2). The arrowhead indicates the 22-kDa polypeptide. B. Immunohistochemistry. Brain (panels a–d) and liver (panels e, f) sections embedded in paraffin blocks were subjected to immunohistochemical analyses with the anti-ITPA serum (panels b, d, f) and preimmune serum (panels a, c, e) as controls. The nuclei were counterstained with hematoxylin (blue).

weight of 22 kDa was reacted with the anti-ITPA serum but not with preimmune serum (Fig. 8A), thus indicating that the anti-ITPA serum specifically reacts with mouse ITPA in the extract. We could not detect smaller polypeptides in either cellular or tissue extracts reacted with the anti-ITPA serum; therefore, it remains to be established as to whether type B or C Itpa transcripts produce any polypeptide in vivo.

Next, brain and liver sections embedded in paraffin blocks were subjected to immunohistochemistry using the anti-ITPA and preimmune sera as controls (Fig. 8B). In the brain, the anti-ITPA serum exhibited apparent immunoreactivity throughout the brain section in comparison to the preimmune serum (data not shown). In the brain section, most of the hippocampal neurons in CA1 to CA3 and DG exhibited relatively strong ITPA immunoreactivity, especially in the soma of CA3 pyramidal cells and mossy fibers (Fig. 8Ba, b). In the cerebellum, the cell bodies of Purkinje cells and mossy fibers in the cerebellar white matter exhibited a strong ITPA immunoreactivity, while granule cell bodies exhibited a relatively weak immunoreactivity (Fig. 8c, d). In the liver, hepatocytes exhibited an evenly distributed ITPA immunoreactivity mainly in the cytoplasm, and a significantly strong immunoreactivity was seen in the cytoplasm and nuclei of the epithelial cells lined bile ducts, and to a lesser extent those of the endothelial cells lined the portal vein (Fig. 8Be, f).

The expression profile of Itpa suggests that the ITPA function may be required for proliferative tissues as well as postmitotic neurons. In neurons, ITPA protein was mostly detected in the cytoplasm, to a lesser extent in the nucleus and also in nerve fibers. A particularly high level of expression was apparent in hippocampal CA3 pyramidal cells and Purkinje cells in the cerebellum. Those neurons were postmitotic, thus suggesting that ITPA may function to sanitize the nucleotide pools for RNA synthesis or DNA synthesis in the mitochondria. However, the PSORT II program (http://psort.nibb.ac.jp/) to predict the protein localization sites in cells predicted that human and mouse ITPA proteins are likely to mostly localize in the cytoplasm and some in the nucleus but not in the mitochondria, and thus ITPA may not function in mitochondria.

On the other hand, the localization of ITPA in nerve fibers may indicate that ITP/dITP or XTP/dXTP, substrate nucleotides for ITPA, may be toxic for the neural function when such abnormal nucleotides accumulated in the nerve fibers. It is well known that nitric oxide (NO), a neurotransmitter, promotes the deamination of various molecules such as nucleic acids and proteins, thus increasing the intracellular concentration of ITP/dITP or XTP/dXTP which can be generated by the deamination of ATP/dATP or GTP/dGTP. It has recently been shown that ITP or XTP disturbs the small G protein function through competition with GTP, thus suggesting that the hydrolysis of ITP/XTP by ITPA is critical for maintaining such signal transduction through small G proteins, especially in neurons. As a result, ITPA may protect the neurons from damage caused by these abnormal nucleotides.

In the liver, a much higher level of ITPA was detected in the epithelial cell-lined bile duct and also in the endothelial cell-lined portal vein than in hepatocytes. In the endothelial cells, again NO plays an important role in regulating vasoconstriction, thus a high level of ITPA may be required. In the bile duct, a high level of ITPA was detected in the nuclei of epithelial cells, suggesting that those cells have greater exposure to deamination by chemicals excreted through the bile duct as well as...
In conclusion, among various genomic sequences highly homologous to human ITPA coding sequences in the mouse, the mouse ortholog (mItpa) for hITPA was successfully isolated by the retro-recombination method, while a processed-Itpa-like sequence was found in any of the examined mouse tissue specimens, we thus conclude that the mouse has only one functional Itpa gene, which is highly expressed in the testis, brain and thymus.

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