Cloning of the *Schizosaccharomyces pombe* gene encoding diadenosine 5′,5‴-P¹,P⁴-tetraphosphate (Ap_4A) asymmetrical hydrolase: sequence similarity with the histidine triad (HIT) protein family

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Diadenosine 5′,5‴-P¹,P⁴-tetraphosphate (Ap_4A) asymmetric hydrolase (EC 3.6.1.17) is a specific catabolic enzyme of Ap_4A found in *Schizosaccharomyces pombe*. We have previously described the partial purification of Ap_4A hydrolase from *S. pombe* [Robinson, de la Peña and Barnes (1993) Biochim. Biophys. Acta **1161**, 139–148]. We determined the sequence of the N-terminal 20 amino acids of Ap_4A hydrolase and designed two degenerate PCR primers based on the sequence. The 60 bp DNA fragment obtained by PCR, which is specific to Ap_4A hydrolase, was used to isolate the Ap_4A hydrolase gene, *aph1*, from *S. pombe* by screening a genomic DNA library in a multicopy plasmid. Ap_4A hydrolase activity from the crude supernatant of a positive *S. pombe* transformant was about 25-fold higher than the control. There was no detectable stimulation of enzymic activity by phosphate. The *aph1* gene from *S. pombe* contains three introns. The intron boundaries were confirmed by sequencing the cDNA of the *aph1* gene from a *S. pombe* cDNA library. The deduced open reading frame of the *aph1* gene codes for 182 amino acids. Two regions of significant local similarity were identified between the Ap_4A hydrolase and the histidine triad (HIT) protein family [Séraphin (1992) DNA Sequence **3**, 177–179]. HIT proteins are present in prokaryotes, yeast, plants and mammals. Their functions are unknown, except that the bovine protein inhibits protein kinase C *in vitro*. All four histidine residues which are conserved among the HIT proteins, including the HxHxH putative Zn^{2+}-binding motif, are conserved in the Ap_4A hydrolase. In addition, there are two regions of similarity between the Ap_4A phosphorylases I and II from *Saccharomyces cerevisiae* and Ap_4A hydrolase from *S. pombe*. These regions overlap with the HIT protein similarity regions. The *aph1* gene from *S. pombe* is the first asymmetrical Ap_4A hydrolase gene to be cloned and sequenced.

INTRODUCTION

Diadenosine 5′,5‴-P¹,P⁴-tetraphosphate (Ap_4A) is present in a variety of prokaryotic and eukaryotic organisms, with a basal concentration of 10 nM–1 μM, except in secretory granules in platelets and chromaffin cells, where it is present at > 100 μM [1,2]. Ap_4A may play important physiological roles in organisms both as an intracellular and extracellular regulatory molecule [3–5]. As an intracellular regulatory molecule, Ap_4A has been proposed to regulate the cell's ability to adapt to metabolic stress, such as heat, oxidation, nutritional state and DNA damage. However, the specific role of Ap_4A is still unknown. As an extracellular regulatory molecule, Ap_4A inhibits platelet aggregation, and may function as an neurotransmitter in chromaffin cells and brain synaptosomes. Related diadenosine polyphosphates may play a role in the regulation of blood pressure [6].

Although Ap_4A is synthesized in different organisms by the same kinds of aminoacyl-tRNA synthetases [7], it is degraded by a variety of specific enzymes (reviewed in ref. [8]). The first type of enzyme, from prokaryotes such as *Escherichia coli* and the slime mould *Physarum polycephalum*, is an Ap_4A symmetrical hydrolase which yields ADP. The second type of enzyme, from the fission yeast *Schizosaccharomyces pombe* and higher eukaryotes, including humans, is an asymmetrical Ap_4A hydrolase, which forms AMP and ATP. The third type of enzyme, from *Saccharomyces cerevisiae*, *Kluyveromyces lactis*, and *Euglena gracilis*, is an Ap_4A phosphorylase, which forms ADP and ATP. All cells possess only a single type of Ap_4A catabolic enzyme, except the green alga *Scenedesmus obliquus* which contains both Ap_4A asymmetrical hydrolase and Ap_4A phosphorylase [9].

The availability of genes encoding Ap_4A-catabolic enzymes will be helpful in determining the specific function(s) of Ap_4A, catalytic mechanisms of Ap_4A-catabolic enzymes, and the evolution of Ap_4A-catabolic enzymes. One Ap_4A symmetrical hydrolase gene, *apaH* from *E. coli* [10,11], the Ap_4A phosphorylase I gene from *S. cerevisiae*, *APA1*, and the Ap_4A phosphorylase II genes, *APA2*, from *S. cerevisiae* and *K. lactis* [12–15], have been cloned and sequenced. The two *S. cerevisiae* Ap_4A phosphorylases are 60% identical [13], and the Ap_4A phosphorylases II of *S. cerevisiae* and *K. lactis* are 51% identical [15]. There is no sequence similarity between the *E. coli* Ap_4A hydrolase and the Ap_4A phosphorylases [12–14]. Here we report the cloning and sequencing of the gene encoding Ap_4A asymmetrical hydrolase from *S. pombe*. The *aph1* gene is the first gene cloned for an asymmetrical Ap_4A hydrolase and the first intron-containing gene encoding an Ap_4A-catabolic enzyme. We also

Abbreviations used: Ap_4A, diadenosine 5′,5‴-P¹,P⁴-tetraphosphate; Ap_3A, diadenosine 5′,5‴-P¹,P³-triphosphate; *aph1*, Ap_4A hydrolase gene from *Schizosaccharomyces pombe*; *APA1*, Ap_4A phosphorylase I gene from *Saccharomyces cerevisiae*; *APA2*, Ap_4A phosphorylase II gene from *S. cerevisiae* or *Kluyveromyces lactis*; aph1p, APA1p or APA2p, the proteins coded by each of these genes; HIT, histidine triad; PKCI-1, protein kinase C inhibitor 1; SSPE, 0.18 M NaCl, 10 mM sodium phosphate, 1 mM EDTA, pH 7.7; CAI, codon adaptation index; uORF, upstream open reading frame.

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The nucleotide sequence reported in this paper has been submitted to the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession number U32615.

show here that the deduced amino acid sequence from the *aph1* gene is related to the histidine triad (HIT) proteins [16] and has local similarities to Ap_4A phosphorylase I and II from *S. cerevisiae*.

EXPERIMENTAL

Strains and growth conditions

E. coli strain DH5αF′ was used as a host for routine genetic manipulations. *S. pombe* 972*h*⁻, used for isolation of *S. pombe* genomic DNA, was grown in medium containing 1% yeast extract, 2% bactopeptone and 3% glucose. *S. pombe* strain *h*⁻ *leu1-32 ura4 his3* was used as a host for transformation of the cloned *aph1* gene and assay of Ap_4A hydrolase activity. *S. pombe* transformed with plasmids was grown in YNB minimal glucose medium (0.67% bacto–yeast nitrogen base without amino acids, 2% glucose and 50 mg/l each supplements to satisfy auxotrophic requirements). *S. cerevisiae* strain PGY135 [*MATa ura3-52 lys2-801 ade2-101 trp1 HIS3 leu2-1 apa1-Δ1::HIS3 apa2::LEU2*] was used as a host strain for transformation of plasmids bearing the *aph1* cDNAs. *S. cerevisiae* strains without plasmids were cultured in YPDA (1% yeast extract, 2% peptone, 2% glucose and 30 mg/l adenine). *S. cerevisiae* strains transformed with plasmids were cultured in liquid YNB minimal glucose medium or YNB minimal sucrose medium (2% sucrose instead of 2% glucose) or grown on 2% agar plates containing YNB minimal glucose medium.

Determination of the N-terminal sequence of Ap_4A hydrolase

The major Ap_4A hydrolase fraction isolated from a Mono Q HPLC column was subjected to SDS/PAGE under conditions that allowed renaturation. Both the partial purification of Ap_4A hydrolase and the electrophoretic conditions for recovery of activity have been described [17]. After electrophoresis, the proteins were electrophoretically blotted on to Immobilon P membrane and the membrane was stained with Coomassie Blue [18,19]. The protein band corresponding to Ap_4A hydrolase was cut out and subjected to protein sequence determination on an Applied Biosystems 477A sequencing system, as described by Matsudaira [19].

Preparation of DNA probe for colony hybridization

A DNA probe based on the N-terminal sequence of aph1p was synthesized using PCR. PCR was conducted using an Ericomp thermal cycler. The reaction was performed with 1 unit of *Taq* DNA polymerase (BRL), 230 pmol of each degenerate primer [CCIAA(A/G)CA(A/G)(C/T)TT(C/T)GC and TTIGTIC-(G/T)(A/G)TA(A/G)AAIAC(C/T)TG corresponding to the potential coding sequences for observed amino acids 1–7 and 14–20 respectively], 0.2 μg of *S. pombe* genomic DNA and 0.2 mM dNTPs in a total volume of 50 μl. The *S. pombe* genomic DNA was isolated as described previously using NovoZym 234 (Sigma) to prepare spheroplasts [20]. The PCR was first cycled three times for 30 s at 94 °C, 30 s at 42 °C, 30 s at 72 °C, and then shifted to 32 cycles of 30 s at 94 °C, 30 s at 50 °C and 30 s at 72 °C. The 60 bp DNA fragment amplified by PCR was cloned directly into the pCR-II vector (Invitrogen) and transformed into *E. coli* INvαF′. Plasmid DNAs from the positive transformants were treated with restriction endonucleases and the reaction mixtures were analysed by electrophoresis on 4% Nusieve 4:1 agarose gels (FMC) and 3% Metaphor agarose gels (FMC). The plasmid DNA from positive transformants was sequenced using the dideoxynucleotide termination method as described below.

Colony hybridization

The probe for hybridization was generated by *Eco*RI digestion of pCR-II plasmid bearing the 60 bp insert. The 80 bp fragment was ^{32}P-labelled with [α-^{32}P]dCTP (3000 Ci/mmol; Dupont/NEN) by the random primer method [21]. A genomic DNA library of *S. pombe* in the shuttle vector pFL20 [22,23] was transformed into *E. coli* strain DH5αF′. The colonies were transferred on to Hybond-N (Amersham). The hybridization was carried out for 18 h at 55 °C in a solution comprised of 5 × SSPE, 5 × Denhardt's, 0.5% SDS and 20 μg/ml sonicated salmon sperm DNA. After hybridization, the filters were washed three times in 2 × SSPE/0.1% SDS at room temperature for 10 min and once in 1 × SSPE/0.1% SDS at 55 °C for 15 min. The positives identified in the first screen were rescreened and purified twice such that all colonies were positive.

Transformation of yeast

The plasmids were transformed into *S. cerevisiae* and *S. pombe* using lithium protocols [24,25].

DNA sequencing of the *aph1* gene and cDNA

The genomic *aph1* gene was sequenced from both strands by the double-stranded dideoxynucleotide sequencing method described by Toneguzzo et al. [26], using Sequenase Version 2.0 (United States Biochemical Corp.). Eight primers complementary to pBluescript sequences or to internal *aph1* sequences were used in the DNA sequencing. The cDNA of the *aph1* gene was sequenced using the same method. Two additional primers based on the plasmid pPGY1 sequences were used in the cDNA sequencing.

Cloning the *aph1* cDNA from *S. pombe*

The *aph1* cDNA was obtained by PCR amplification from a cDNA library of *S. pombe* [27]. The two PCR primers,

5′-TCGAATTCCAAAATGCCAAAACAGCTATAT-3′

(*Eco*RI site is underlined) and

5′-CTTCTCGAGTTTATTCCTTCTCTTGCTCTT-3′

(*Xho*I site is underlined), overlap the start codon and stop codon respectively of the *aph1* gene. PCR was carried out in 100 μl containing 50 pmol each of the two primers, 0.1 mM dNTPs, 10 ng of the cDNA library of *S. pombe* and 2.5 units of *Taq* DNA polymerase. The reaction schedule was 94 °C for 30 s, 56 °C for 45 s and 72 °C for 1 min for 34 cycles. The amplified cDNA was digested with *Eco*RI and *Xho*I, purified on a low-melting-point agarose gel and ligated into the yeast expression vector pPGY1 (constructed in this laboratory) between the *S. cerevisiae GAL1* promoter [28] and the *UTR1* transcription terminator [29]. This plasmid is pYH6.

Expression of the *aph1* cDNA from *S. pombe* in *S. cerevisiae*

The *S. cerevisiae* strain PGY135 bearing pYH6 was first grown in YNB minimal glucose medium to an A_{600} of 1.0. The cells were then diluted to an A_{600} of 0.1 in YNB minimal sucrose medium. When the density of cells reached an A_{600} of 0.5, galactose was added to a final concentration of 1% and the cells were harvested by centrifugation 5.5 h later and broken with glass beads to obtain crude supernatants.

Southern-blot hybridization

S. pombe genomic DNA (3 μg) was digested with *Eco*RI, *Hin*dIII or *Bam*HI overnight. The DNA fragments were separated on a

0.8% agarose gel at 3 V/cm for 9 h. After depurination of the DNA, the gel was denatured, neutralized and blotted on to Hybond-N. A 571 bp *Eco*RI–*Xho*I fragment from the cDNA of the *aph1* gene was labelled with [α-^{32}P]dCTP (3000 Ci/mmol) by the random primer method [21] and was used as a probe for hybridization. Hybridization was performed under both high- and moderate-stringency conditions. For the high-stringency conditions, it was carried out at 65 °C in a solution containing 5 × SSPE, 5 × Denhardt's, 0.5% SDS and 20 μg/ml sonicated salmon sperm DNA for 18 h. After hybridization, the filter was washed twice in 2 × SSPE/0.1% SDS at room temperature for 10 min, once in 1 × SSPE/0.1% SDS at 65 °C and once in 0.1 × SSPE/0.1% SDS at 65 °C. For the moderate-stringency conditions, the hybridization was carried out at 60 °C, and the filter was washed twice in 2 × SSPE/0.1% SDS at room temperature and once in 1 × SSPE/0.1% SDS at 60 °C.

Other recombinant DNA techniques

Routine cloning procedures were performed according to standard protocols [30].

Preparation of crude supernatants from *S. pombe*

S. pombe transformants were cultured in 400 ml of minimal medium in a 2-litre flask at 30 °C with agitation at 200 rev./min. The cells were harvested in late exponential phase by centrifugation at 27000 *g* for 10 min at 4 °C. The pellet was washed with 50 mM Hepes/NaOH, pH 7.5, containing 10% glycerol, frozen and stored at −80 °C. The cells were resuspended in 50 mM Hepes/NaOH, pH 7.5, containing 10% glycerol, 0.5 mM PMSF, 0.5 μg/ml pepstatin and 0.5 μg/ml leupeptin at a ratio of 2:1 (v/w) (buffer/wet weight of pellet) and broken by vortexing with glass beads [1:1 (w/w) glass beads/wet weight of pellet]. Homogenates were centrifuged at 27000 *g* for 20 min at 4 °C. The crude supernatants were collected and the protein concentrations were determined by the method of Lowry et al. [31]. A portion of each crude supernatant was dialysed at 4 °C overnight against 50 mM Hepes/NaOH, pH 7.5, containing 10% glycerol and 0.5 mM PMSF.

Assay of Ap_4A hydrolase activity

Ap_4A hydrolase activity was assayed by hydrolysis of [^{3}H]Ap_4A as previously described [17]. The reaction products, [^{3}H]ATP and [^{3}H]AMP, were separated from residual [^{3}H]Ap_4A by column chromatography on a boronate-derivatized resin [32]. The activity was expressed as nmol of AMP + ATP formed/min per mg of protein.

Analysis and alignment of sequences

The codon adaptation index (CAI) was calculated as described by Sharp and Li [33] using the reported relative synonymous codon usage (RSCU) values for high-expression genes of *S. pombe* [34]. Codons with RCSU of 0 were substituted with the lowest non-zero value in the table, 0.045. The NCBI BLAST network server was used to search for related protein sequences in the NCBI non-redundant protein database [35]. Searches were also carried out on the Blocks database of ungapped homology blocks [36]. The alignment shown in Figure 4 was carried out using the multiple sequence alignment program MACAW and the procedures described by Schuler et al. [37], but similar alignments were produced by Clustal V [38]. The Blosum 62 scoring matrix was used [39].

RESULTS

Isolation of the *aph1* gene

The strategy for cloning the *aph1* gene was to use a probe based on the N-terminal sequence of Ap_4A hydrolase to screen an *S. pombe* genomic DNA library. The N-terminal sequence of the Ap_4A hydrolase was determined to be:

P-K-Q-L-Y-F-S-K-F-P-V-G-r-Q-V-F-Y-R-T-K.

No methionine residue was detected at the N-terminus. Degenerate oligonucleotides corresponding to the potential coding sequence for observed amino acids 1–7 and 14–20 were used as primers for PCR amplification of the *S. pombe* genomic DNA (see the Experimental section).

The 60 bp PCR product was cloned into the pCR-II vector. The resulting plasmid was called pCR-II-I. Sequence analysis of the 60 bp fragment revealed that the amplified sequence was consistent with that predicted from the N-terminal peptide sequence of Ap_4A hydrolase, except that residue 13 (numbering from the proline) was found to be serine instead of arginine. There was an uncertainty at this position in the peptide sequence. A probe specific for the *aph1* gene was generated by *Eco*RI digestion of pCR-II-I, which yielded an 80 bp fragment. This fragment was used as a probe instead of degenerate oligonucleotides to minimize false positive clones [40]. An *S. pombe* genomic DNA library carried on the multicopy shuttle vector pFL20 [22,23] was screened by hybridization. Seven positives were obtained in a screen of 60000 colonies. Restriction mapping analysis indicated that there were three different inserts among the seven positives. They all had a common 1.2 kb *Eco*RI fragment.

To confirm further that the DNA inserts in positive transformants contained the *aph1* gene, the plasmids were transformed into *S. pombe* and crude supernatants were prepared from transformants. Ap_4A hydrolase activity from all of the positives was 10- to 25-fold higher than that from *S. pombe* transformed with pFL20. In order to detect any Ap_4A phosphorylase activity of the cloned gene, all crude supernatants were dialysed and assayed for Ap_4A phosphorylase activity. P_i had no significant effect on the enzyme activity. A plasmid with a 2.5 kb insert (pYH1) was selected for sequence analysis.

Nucleotide sequence of the *aph1* gene

Because all positives contained a 1.2 kb *Eco*RI fragment, we subcloned this fragment from pYH1 into pBluescript II KS(−) and sequenced the fragment. The sequencing strategy is shown in Figure 1. Amino acids 2–20 deduced from the DNA sequence (Figure 2) exactly matched the sequence determined from peptide sequencing, except as noted above. The DNA sequence also revealed that the coding sequence of the *aph1* gene is disrupted by three introns.

The 5′ flanking DNA sequence of the *aph1* gene was extended beyond the *Eco*RI site by sequencing the original plasmid pYH1. Analysis of the 5′ sequence revealed a putative TATA element, TATTATAT, at positions −120 to −113, numbering from the start codon. The sequence in the putative 5′ promoter region is AT-rich. An upstream open reading frame (uORF) is present, following the putative TATA element. The 28-codon uORF has two in-frame ATG codons separated by 21 nucleotides, and a stop codon 6 nucleotides away from the start codon of the *aph1* gene (Figure 2).

S. pombe genes often possess either the classical AATAAA polyadenylation sequence or close derivatives near the 3′ end,

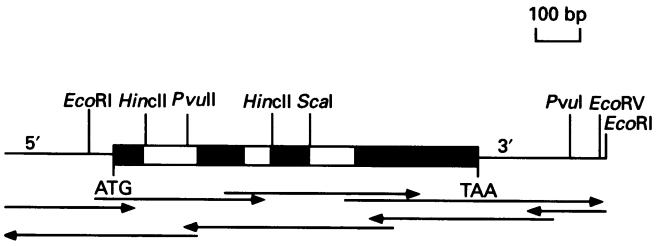

Figure 1 Restriction map of the 1.2 kb *Eco*RI fragment and the DNA-sequencing strategy

The filled boxes represent the exons of the *aph1* gene from *S. pombe*. The open boxes represent the introns. The arrows indicate the direction and extent of the sequences obtained by the dideoxynucleotide-termination reaction. The bar represents the flanking DNA sequences. The start, ATG, and stop, TAA, codons are indicated. The relevant restriction enzyme sites within this fragment are indicated also.

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-265                                              GAAACTTGTGTGCATACGAATAATA
-240  AAATTCGATAATATTGGAATTTTTAGTCCGCTTTATCTGTTCCATGATACTGTTACTTAC
-180  ATATATGCAAGACGCTATTTTCTCATAGTCTGTTTGTTTTTTAAGTATATCAATCTTTCT
-120  TATTATATTCCATAGACACTTTCGCACATGACTCTCCAGGGACTCCGCGATATGGGTTGT
                                  M  T  L  Q  G  L  R  D  M  G  C
 -60  GAGCATCGTGAAGCTGAATTCAACCAACAACTTAGATTCTTACAATATTCGTAAGCCAGA
       E  H  R  E  A  E  F  N  Q  Q  L  R  F  L  Q  Y  S  *
   1  ATGCCAAAACAGCTATATTTCTCCAAGTTTCCTGTTGGAAGTCAAGTTTTTTATCGTACT
       M  P  K  Q  L  Y  F  S  K  F  P  V  G  S  Q  V  F  Y  R  T
  61  AAGGTAAGTTAACGGTCTCATGTGTGTAGATATTGGTGTTTGCAAACTTTTGTTTGTCAT
       K
 121  TCTTATTTATTCTATAACGGCAGACAGTTTGTGATTTTTCTTTGGTTGAGGTCAGCTGCT
 181  AACGATTTTAGTTATCTGCCGCGTTTGTAAACCTGAAACCAATTTTACCAGGTCATGTTT
                     L  S  A  A  F  V  N  L  K  P  I  L  P  G  H  V
 241  TGGTAATTCCGCAACGGGCGGTCCCTAGATTGAAAGATTTGACACCTTCAGAGGTAGGAT
       L  V  I  P  Q  R  A  V  P  R  L  K  D  L  T  P  S  E
 301  TCTTATGCTATTCGAAAAAATAATGGAATCTGCATACGCTAACTAATGAAAACTTAGTTG
                                                                 L
 361  ACGGATTTGTTTACTTCTGTTCGCAAAGTGCAACAGGTAATCGAAAAGGTGTTTTCGGCA
       T  D  L  F  T  S  V  R  K  V  Q  Q  V  I  E  K  V  F  S  A
 421  TCTGCATCAAACATTGGTATTCAAGTAAGTACTTTGATAGTCAAGGAATAAATAAAAAAA
       S  A  S  N  I  G  I  Q
 481  CATATTCCTTTTCACATTCAAAATAAAAAATCGTTTTAATTTAGAAGCTGACATTTTGCT
 541  TTTAACTCAATAGGATGGTGTAGACGCTGGTCAAACAGTTCCTCATGTACATGTTCACAT
                     D  G  V  D  A  G  Q  T  V  P  H  V  H  V  H  I
 601  TATCCCTCGTAAAAAGGCAGATTTTTCAGAAAACGATCTAGTCTACAGTGAGTTGGAAAA
       I  P  R  K  K  A  D  F  S  E  N  D  L  V  Y  S  E  L  E  K
 661  AAACGAAGGAAATCTTGCTTCCCTTTATCTTACGGGAAATGAGCGGTATGCAGGAGATGA
       N  E  G  N  L  A  S  L  Y  L  T  G  N  E  R  Y  A  G  D  E
 721  GAGACCGCCAACCAGTATGAGGCAAGCTATTCCTAAGGACGAGGATCGTAAGCCAAGAAC
       R  P  P  T  S  M  R  Q  A  I  P  K  D  E  D  R  K  P  R  T
 781  ACTTGAGGAAATGGAAAAGGAAGCTCAGTGGTTGAAAGGGTACTTTTCCGAAGAGCAAGA
       L  E  E  M  E  K  E  A  Q  W  L  K  G  Y  F  S  E  E  Q  E
 841  GAAGGAATAAAAAGTTGAAGTACCTCAATACCACAGGGGTAGTGTTTACGTATGAATTAA
       K  E  *
 901  GCTAAATATTATATGACCCTTTTTTTTTATTTCACCCAAGGTTACAAGAAAAATTTCCTT
 961  TTTTCTCTCTACCCTGCTTACATTGCATCTGTCTGCTGAGCTTTAGCAACACAACGTAAC
1021  CATACATATTGTGATGAACCCTTCTACAATTCGATCGAATTAGCTTCAGTTCCCTATTTT
1081  GATTTTGCTCTCTTTCTTTCATCCTTTCCTCATAACCCTACTAGATATCCATCTTTTTGA
1141  ATTC
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Figure 2 Nucleotide sequence of the *aph1* gene from *S. pombe* and the predicted amino acid sequence

The nucleotide positions are numbered at the left. The putative TATA element is underlined. The uORF is underlined with dots. The stop codons of the uORF and the *aph1* ORF are indicated by asterisks. The intron sequences are underlined. The intron sequences of the 5′-site, putative branch site and 3′-site are double underlined. The deduced amino acid sequence is indicated by one-letter code. The amino acids of the histidine triad are underlined. The AATAAA-like sequences are indicated in bold. The 3′-AT-rich sequence is underlined. Palindromic sequences in the 3′-flanking region are double underlined.

followed by an extremely AT-rich sequence [41]. An AATAAA was not identified in the 3′ untranslated region of *aph1*. However, two AATAAA-like sequences, AATTAA and AATATTA, were present, followed by the AT-rich sequence, TTTTTTTTTATTT.

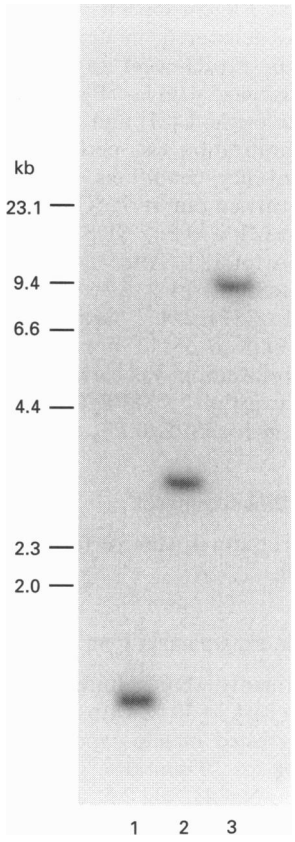

Figure 3 Autoradiograph of a Southern blot of genomic DNA from *S. pombe* 972h⁻

S. pombe genomic DNA was cleaved with restriction nucleases *Eco*RI (lane 1), *Hin*dIII (lane 2) and *Bam*HI (lane 3). The Southern blot was hybridized with α-^{32}P-labelled cDNA of the *aph1* gene of *S. pombe* under high-stringency conditions. The sizes of the standards are shown on the left.

In addition, two palindromic sequences were identified (Figure 2). Recently the sequence segments of the *S. pombe ura4* gene required for mRNA 3′-end formation have been defined [42]. The sequences required did not include an AATAAA-like sequence or an AT-rich sequence. The 3′-flanking sequences of the *aph1* gene do not contain any regions with notable similarity to the *ura4* 3′-end-forming elements.

Cloning and expression of a cDNA of the *aph1* gene from *S. pombe*

To confirm the intron boundaries, we cloned a cDNA of the *aph1* gene by PCR amplification from an *S. pombe* cDNA library [27]. The expected DNA fragment was cloned into the *S. cerevisiae* expression vector pPGY1 and sequenced. The three introns deduced from the *aph1* gene sequence were confirmed by the sequence of the cDNA. The intron sequences of the 5′- and 3′-splice sites and the putative branch sites are shown in Figure 2. After removal of introns, the deduced amino acid sequence of Ap_4A hydrolase has 182 amino acids, with a molecular mass of 20642 Da. A CAI [33] of 0.325 was calculated, which is compatible with a moderate level of expression.

The *aph1* cDNA was expressed in *S. cerevisiae* strain PGY135, in which both the Ap_4A phosphorylase genes are disrupted. The

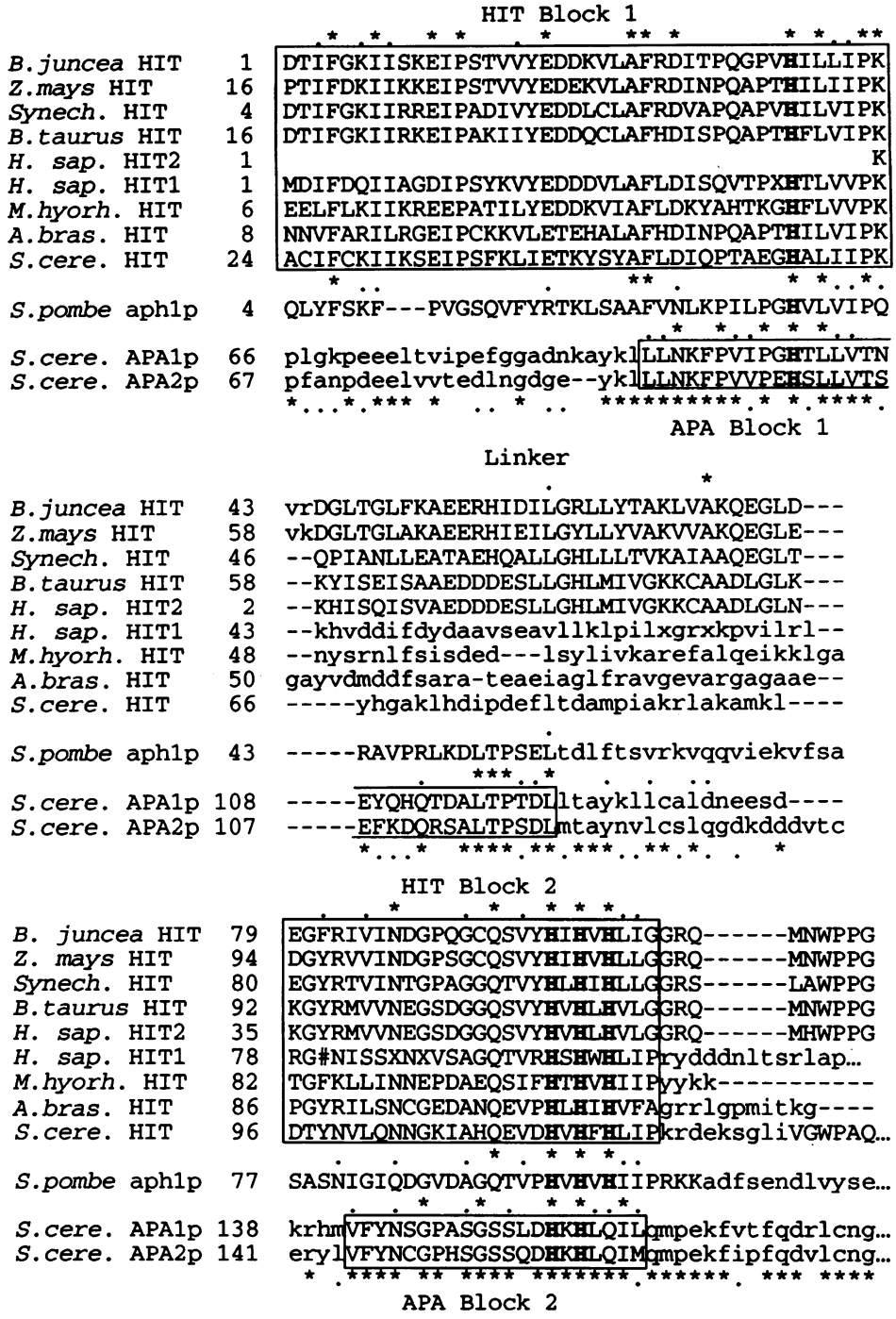

Figure 4 Multiple alignment of the amino acid sequence of the Ap_4A hydrolase from *S. pombe* with the HIT protein family and *S. cerevisiae* Ap_4A phosphorylases I and II

The Ap_4A hydrolase sequence was aligned separately with the HIT proteins and with the Ap_4A phosphorylases using MACAW. The two alignments were reconciled for the Figure by introducing the five residue gap in the hydrolase and the phosphorylases at the beginning of the linker region. The main blocks of similarity between the HIT proteins or the Ap_4A phosphorylases and the Ap_4A hydrolase are indicated by boxes and capital letters. Regions of similarity among subsets of the HIT proteins are indicated by capital letters only. The four conserved histidines (three in the Ap_4A phosphorylases) are indicated in bold. Residues that are identical or similar among all the HIT proteins or between APA1p and APA2p are indicated above (HIT proteins) or below (APAp) them by asterisks and dots respectively. Residues identical or similar between aph1p and the HIT proteins or APA1p and APA2p are marked next to the aph1p sequence in the same fashion. The two human sequences were added after the alignment process, and the scoring of conserved residues does not include them, since these single-pass sequences may contain a number of errors. The # marks a stop codon in the human HIT1 sequence and the site used to change reading frames. The lack of similarity in the linker region makes it impossible to tell where the sequence error is that results in the reading frame change. Numbers show the leftmost amino acid residue positions in the aligned amino acid sequences. Sequences that extend beyond the region shown are indicated by an ellipsis. The species and GenBank/EMBL accession numbers are: *B. juncea* HIT, *Brassica juncea* (Chinese cabbage), U09406; *Z. mays* HIT, *Zea mays* (maize), Z29643 [51]; *Synech.* HIT, *Synechococcus* sp. (cyanobacterium), M34833 [52]; *B. taurus* HIT, *Bos taurus* (bovine), U09405 [48]; *H. sap.* HIT2, *Homo sapiens*, T57609 and F00830; *H. sap.* HIT1, Z15890; *M. hyorh.* HIT, *M. hyorhinis* (mycoplasma), M37339 [54]; *A. bras.* HIT, *Azospirillum brasilense* (bacterium), X61207 [53]; *S. cere.* HIT, *S. cerevisiae* (yeast), X56956 [55]; *S. pombe* aph1p, Ap_4A hydrolase from *S. pombe* (yeast), U32615; *S. cere.* APA1p, Ap_4A phosphorylase I from *S. cerevisiae* (yeast), M35204 [12,14]; *S. cere.* APA2p, Ap_4A phosphorylase II from *S. cerevisiae* (yeast), M34354 [13].

Table 1 Statistics on the amino acid sequences compared with the Ap_4A hydrolase

The size of each complete protein is shown along with the Poisson probability (P) calculated by BLAST for the scores against the Ap_4A hydrolase and the percentage of identical residues and identical plus conservative substitutions over the segments including the homology blocks and the linking region. The P values for the human sequences are calculated on the basis of the size of the expressed sequence tag database, and are thus not comparable with the other P values. Values in parentheses are percentage similarity. Abbreviations are as defined in Figure 4.

Protein	Size (amino acids)	P	Identity (%)
B. juncea HIT	113	0.99	24.5 (53.6)
Z. mays HIT	128	0.059	22.7 (50.9)
Synech. HIT	114	6.3×10^{-5}	23.1 (51.9)
B. taurus HIT	126	3.1×10^{-3}	24.1 (53.7)
H. sapiens HIT2	> 70	1	19.2 (49.3)
H. sapiens HIT1	> 115	4.6×10^{-7}	22.0 (43.2)
M. hyorh. HIT	111	2.9×10^{-5}	27.8 (52.8)
A. bras. HIT	122	10^{-3}	24.5 (53.6)
S. cere. HIT	158	10^{-6}	28.6 (58.1)
S. cere. APA1	321	1	19.7 (57.9)
S. cere. APA2	325	1	21.1 (57.9)
S. pombe aph1	182	–	–

plasmid pPGY1 has an inducible *S. cerevisiae GAL1* promoter and an *S. cerevisiae UTR1* terminator. Expression was induced with galactose, and crude supernatants of transformants were prepared. The Ap_4A hydrolase specific activity in PGY135 bearing the plasmid pYH6 was 18 nmol/min per mg, whereas that in the control PGY135 bearing the plasmid pPGY1 was 0.9 nmol/min per mg. The latter activity arises from hydrolysis of Ap_4A by an Ap_3A hydrolase present in *S. cerevisiae* [43]. The level of expression observed did not inhibit growth, but it is a low level of activity, comparable with the wild-type Ap_4A phosphorylase activity. The reason for the low level of expression is not yet known.

Analysis by Southern blotting and hybridization

Organization of the *aph1* gene from *S. pombe* and detection of related genes were studied by Southern-blot hybridization of the genomic DNA of *S. pombe* with the cDNA of the *aph1* gene. When *S. pombe* genomic DNA was digested with *Eco*RI and hybridized under different stringency conditions, a single band corresponding to a 1.2 kb *Eco*RI fragment was detected (Figure 3). Thus *aph1* is present in *S. pombe* chromosomes as a single copy. Similar results were obtained with *Bam*HI and *Hin*dIII digests. Southern-blot hybridization under moderate-stringency conditions did not detect any other genes closely related to *aph1* in *S. pombe*.

Amino acid sequence comparisons

Based on searches of the NCBI non-redundant protein database, the expressed sequence tag database and the Blocks database, we located two blocks of local similarity between the Ap_4A hydrolase and the HIT protein family [16] (Figure 4). The HIT family was originally defined on the basis of sequences of proteins from three prokaryotes (*Mycoplasma hyorhinis*, *Synechococcus* sp. and *Azospirillum brasilense*) and *S. cerevisiae* with similarity to a bovine protein kinase C inhibitor (PKCI-1) [16]. The function of these proteins *in vivo* is not known. All are small proteins containing a histidine triad (HxHxH, where x is hydrophobic), which in PKCI-1 has been shown to bind Zn^{2+}. The sequence around the histidine triad has been used to define a signature for the HIT proteins (Prosite PDOC00694) [44]. All of the HIT proteins were among the sequences scoring the highest in an NCBI BLAST search with the Ap_4A hydrolase. The matches of the original five HIT proteins to the Ap_4A hydrolase all had Poisson probabilities of less than 0.005 reported by BLAST (Table 1). Two plant sequences (*Zea mays* and *Brassica juncea*) gave insignificant *P* scores, but were clearly related to the other HIT proteins. Several partial cDNA sequences from the expressed sequence tag database had translated blocks that were related to the HIT proteins and to the Ap_4A hydrolase. Two of these (human HIT1 and human HIT2) are shown in Figure 4. The two HIT protein blocks were the highest scoring blocks against the Ap_4A hydrolase in the Blocks database. All four histidines conserved in the HIT proteins are present in the Ap_4A hydrolase. Alignments of the two similar blocks and the linking region of Ap_4A hydrolase with nine HIT proteins and statistics on the alignment are shown in Figure 4 and Table 1. The alignments of the two HIT similarity blocks are insensitive to the parameters or alignment method used and are statistically highly significant by the method used in MACAW. In the linker region, a subset of the HIT proteins show significant similarity to each other, as indicated in Figure 4, but the Ap_4A hydrolase has weak similarity only to the budding yeast HIT protein.

In addition to the similarity to the HIT proteins, a more limited similarity to the *S. cerevisiae* Ap_4A phosphorylases was detected, as shown also in Figure 4. The two blocks of the Ap_4A hydrolase similar to the Ap_4A phosphorylases overlap or are contained within the HIT blocks, and the length of the linker region is approximately conserved in all the sequences. The third histidine of the triad is replaced in the Ap_4A phosphorylases by glutamine. The degree of similarity observed between the Ap_4A hydrolase and the Ap_4A phosphorylases is not statistically significant given the size of the database searched [35], but may represent structural similarity nonetheless, since both bind Ap_4A and catalyse nucleoside monophosphoryl transfer. Other than the few residues that are conserved between all the sequences shown in Figure 4, there is no significant similarity between the Ap_4A phosphorylases and the HIT proteins.

DISCUSSION

The *aph1* gene from *S. pombe* is the first asymmetrical Ap_4A hydrolase gene that has been cloned and sequenced. The deduced size of the hydrolase (182 amino acids, 20642 Da) is in reasonable agreement with a value of 22 kDa for the subunit molecular mass based on SDS/PAGE [17]. Ap_4A asymmetrical hydrolases from other organisms have molecular masses of 17–26 kDa [8].

Like about 40% of *S. pombe* genes, the *aph1* gene contains introns. The three introns found in the *aph1* gene have features that are generally typical of *S. pombe* introns [45,46], although the 5′-splice-site sequence of the second intron is unusual in containing a rare G at the +4 position and an A at position +6. As expected, these mismatches with the U1 snRNA-complementary consensus sequence (5′-CAG GTAAGT-3′) are compensated by matches in nucleotides upstream of the splice site (5′-GAG -3′) [46].

The uORF identified in the *aph1* gene is probably not translated. If the identified TATA site is utilized, the transcription start site is probably downstream of the first AUG and may be downstream of the second, based on a typical distance of 35–45 nucleotides between the two elements [22]. *S. pombe* is similar to

other eukaryotes in the sequence context around utilized start codons, including the favoured A at position −3 [46]. In the few *S. pombe* mRNAs known to contain untranslated uORFs, there are no As at position −3 [46]. Unlike the Ap_4A hydrolase reading frame, neither of the upstream AUGs has an A at position −3. This, combined with their probable proximity to the 5′-end of the mRNA, suggests that, if transcribed, they are not utilized as start codons.

The Ap_4A hydrolase from *S. pombe* has weak but significant similarity to the HIT protein family. The HIT proteins are a group of hypothetical proteins related to bovine brain PKCI-1 [16,47]. The brain protein inhibits protein kinase C with an IC_{50} of 2.2 μM, whereas other kinases are unaffected [48], although its physiological importance as an inhibitor has been questioned [49]. It has also been shown to bind Zn^{2+} at the HIT [50]. A maize cDNA with sequence similarity to PKCI-1 was obtained in a screen with an antibody to a protein component of the U1 ribonucleoprotein [51]. It has been expressed in *E. coli*, and the protein binds Zn^{2+} and has antigenic cross-reactivity with PKCI-1, but little protein kinase C inhibitor activity [47]. cDNAs from Chinese cabbage (*Brassica juncea*) and rice (*Oryza sativa*) [47] that are similar to the maize cDNA are also known, but nothing is known of their function. The HIT gene in *Synechococcus*, a cyanobacterium, is upstream of one of the three *psbA* genes (*psbA*II), which code for the D1 thylakoid protein of photosystem II [52]. The three *psbA* genes, coding for two forms of the D1 protein, are expressed on three light-regulated monocistronic transcripts. The HIT-coding region is expressed on a low-level constitutive transcript which includes the *psbA*II-coding region downstream. A mutant that does not make the constitutive transcript has a slow growth rate, but expression and light regulation of the other *psbA* transcripts is unaffected. HIT-coding regions have also been identified in the histidine-biosynthetic operon of the nitrogen-fixing bacterium *Azospirillum brasilense* [53], in an operon of *Mycoplasma hyorhinis* containing genes for components of periplasmic binding-protein-dependent transport systems [54] and in *S. cerevisiae* [55]. A human partial cDNA sequence was reported to be similar to the N-terminal part of the HIT proteins [47]. We found that the similarity of this protein extends to the HIT region if it is assumed that a sequence error caused a change of reading frame (human HIT1 in Figure 4). In addition, we found homologies to HIT proteins and the Ap_4A hydrolase in other partial cDNA sequences from human [human HIT2 (Figure 4) (Z15890) and (T78708)], *Arabidopsis thaliana* (T44544), *Plasmodium falciparum* (T18067) and *S. cerevisiae* (T37045).

The HIT proteins may represent a group of structurally similar proteins that have several different functions. They appear to fall into at least two groups. One group, represented by the top five sequences in Figure 4, has pairwise identities among themselves of 54–92%. They all retain similarity to each other in the linker region, and all contain a distinctive C-terminal region ending in WPPG. It is striking that the *Synechococcus* HIT protein is more similar to the mammalian and plant proteins in this group than it is to the prokaryotic non-WPPG HIT proteins [16]. The remaining HIT proteins have pairwise identities with each other and the WPPG HIT proteins that range from 25 to 35%, and there is little similarity in their linker regions or C-termini. These observations suggest that the WPPG HIT proteins have a common function and are under different functional constraints from the other HIT proteins. The other HIT proteins may have multiple functions or one function that allows greater structural variation. The two human HIT proteins in Figure 4 and a third one not shown (T78708) indicate that a single organism may need HIT proteins from both classes.

The Ap_4A hydrolase is the most distantly related of all the HIT proteins, with slightly lower pairwise scores (Table 1), and it fails to conserve several residues that are completely conserved in the other HIT proteins (Figure 4). It will be interesting to determine whether any of the other HIT proteins have nucleotide-hydrolytic activity. Pearson et al. [48] suggested that bovine PKCI-1 may be a multimer or a very asymmetric monomer, based on an apparent mass assessed by gel filtration that is more than double the calculated subunit molecular mass. The Ap_4A hydrolase behaves similarly [17], suggesting that a general structural similarity may have been conserved. The Ap_4A hydrolase is not known to bind Zn^{2+}; it requires Mg^{2+}, Co^{2+} or, optimally, Mn^{2+} for activity [17]. Zn^{2+} does not support activity. We cannot rule out the possibility that tightly bound Zn^{2+} may purify with the enzyme. On the basis of hydrogen-exchange NMR studies of PKCI-1, it was suggested that the three histidines are buried in the interior of the protein. If this is true, the HIT may be a core structural element of the protein.

The similarity of the Ap_4A hydrolase to the Ap_4A phosphorylases is weaker than that to the HIT proteins, but may represent homology nonetheless. A number of examples are known of enzymes catalysing similar reactions in which only limited regions show recognizable similarity [56]. The limited regions of similarity between all three classes of protein in Figure 4 suggest that the conserved histidines, especially the HIT, may be involved in catalysis. We previously proposed residues 63–69 as a possible glycine-containing nucleotide-binding loop in Ap_4A phosphorylase I [14]. However, those residues are not well conserved in the Ap_4A phosphorylases II (Figure 4 [15]). We note here that the two glycines 5 and 9 residues upstream of the histidine pair or triad are conserved between the Ap_4A hydrolase and the Ap_4A phosphorylases. This segment was predicted to be β-turn and random coil in PKCI-1 [48], which conserves the glycines as well, suggesting this segment as a possible phosphate-binding loop [57].

Note added in proof (received 19 October 1995)

While the present paper was in press, a paper on a human gene encoding Ap_4A hydrolase was published [58]. The authors report the cloning and sequencing of a gene encoding an asymmetrical Ap_4A hydrolase for the first time.

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