

# Disruption and overexpression of the *Schizosaccharomyces pombe* *aph1* gene and the effects on intracellular diadenosine 5',5'''-P<sup>1</sup>,P<sup>4</sup>-tetrphosphate (Ap<sub>4</sub>A), ATP and ADP concentrations

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Diadenosine oligophosphates are ubiquitous compounds that were discovered over 30 years ago. Diadenosine 5',5'''-P<sup>1</sup>,P<sup>4</sup>-tetrphosphate (Ap<sub>4</sub>A) is the most studied member of this family, and its function in yeast is unknown. To investigate possible functions, we changed the intracellular Ap<sub>4</sub>A concentration in *Schizosaccharomyces pombe* via disruption and overexpression of the *aph1* gene, which encodes an Ap<sub>4</sub>A hydrolase (Aph1). *S. pombe* Aph1 is 52% identical with a human tumour suppressor protein, Fhit, in a core region of 109 amino acids. Disruption of *aph1* resulted in an 85% decrease in Ap<sub>4</sub>A hydrolase activity and a 290-fold increase in the intracellular Ap<sub>4</sub>A concentration. The disruption and subsequent increase in intracellular Ap<sub>4</sub>A concentration had no significant effect on the growth of *S. pombe*. Overexpression of the *S. pombe* *aph1* gene, resulting in 17- and 84-fold increases in Ap<sub>4</sub>A hydrolase activity above wild-type levels, resulted in 60 and 80% decreases re-

spectively in the intracellular Ap<sub>4</sub>A concentration. This represents the first report of a decrease in the intracellular Ap<sub>4</sub>A concentration in response to overexpression of a degradative enzyme in any eukaryotic organism. We describe a new *S. pombe* expression plasmid, pPOX, which was used to achieve the largest increase in expression of *aph1*. Overexpression of *aph1* at the highest level resulted in a 46% increase in generation time in comparison with the control strain. Neither overexpression nor disruption had any effect on the intracellular ATP or ADP concentrations. This is the first report of ADP and ATP concentrations in *S. pombe*. These data also indicate that Aph1 functions *in vivo* to degrade Ap<sub>4</sub>A, and that high-level overexpression of this enzyme reduces the growth rate.

**Key words:** adenine nucleotides, diadenosine oligophosphates, fission yeast, gene manipulation.

## INTRODUCTION

The diadenosine oligophosphates are a family of compounds first discovered over 30 years ago [1]. These compounds consist of two adenosine moieties joined together through their 5' carbons by a chain of phosphate molecules to give a diadenosine 5',5'''-P<sup>1</sup>,P<sup>*n*</sup>-oligophosphate (Ap<sub>*n*</sub>A; *n* = 2–7) [2]. Ap<sub>4</sub>A, a commonly studied family member, has been found in all tissues and organisms examined for its presence, usually at levels of between 0.01 and 1 μM [3]. The discovery that the Fhit tumour suppressor protein behaves as an Ap<sub>3</sub>A hydrolase *in vitro* [4] has increased interest in the *in vivo* function(s) of diadenosine oligophosphates. The fission yeast *Schizosaccharomyces pombe* contains an Ap<sub>4</sub>A hydrolase (Aph1) [5] which is 52% identical with Fhit in a core region of 109 amino acids [6]. The present study was aimed at gaining insight into the functions of Aph1 and Ap<sub>4</sub>A in the fission yeast *S. pombe* using genetic manipulation of the *aph1* gene. Clues as to the *in vivo* function of Aph1 and Ap<sub>4</sub>A in *S. pombe* might also be useful in determining the *in vivo* function of the Fhit tumour suppressor.

The only known method of *in vivo* synthesis of Ap<sub>4</sub>A occurs as a side reaction during the charging of a tRNA molecule [7]. This reaction was verified as an *in vivo* source of Ap<sub>4</sub>A in *Escherichia coli* by overexpressing the genes encoding four different tRNA synthetases that synthesize Ap<sub>4</sub>A *in vitro* and measuring the

concomitant increases in the intracellular Ap<sub>4</sub>A concentration [7].

Three enzymes have been identified in yeasts, two in *Saccharomyces cerevisiae* and one in *S. pombe*, that degrade Ap<sub>4</sub>A. The *S. pombe* *aph1* gene product (the subject of the present study) is an orthologue of Fhit [6], but prefers Ap<sub>4</sub>A as substrate [5]. Aph1 contains a histidine triad (HIT) sequence motif and is a member of the GAFH (GalT, Ap<sub>4</sub>A phosphorylase, Fhit, Hint) protein family [8]. Ap<sub>4</sub>A phosphorylases I and II, encoded by the *APA1* and *APA2* genes from *Sacch. cerevisiae* respectively [9–11], are both members of the GAFH protein family [8] and prefer Ap<sub>4</sub>A as substrate.

Here, we report the use of molecular genetic techniques to both raise and lower the intracellular concentration of Ap<sub>4</sub>A. Specifically, we report the disruption and overexpression of the *S. pombe* *aph1* gene. Disruption of *aph1* led to a 290-fold increase in the intracellular Ap<sub>4</sub>A concentration, but no change in growth rate. Overexpression of *aph1* via a new *S. pombe* expression plasmid, leading to an 84-fold increase in Ap<sub>4</sub>A hydrolase activity, resulted in an 80% decrease in the intracellular Ap<sub>4</sub>A concentration. This strain exhibited a 46% increase in generation time. There was no significant change in the intracellular ATP and ADP concentrations in either the disruption or the overexpression strain. These experiments show for the first time that genetic manipulation of an Ap<sub>4</sub>A degradative enzyme can be

Abbreviations used: Ap<sub>*n*</sub>A, diadenosine 5',5'''-P<sup>1</sup>,P<sup>*n*</sup>-oligophosphate (*n* = 2–7); Ap<sub>4</sub>N, adenosine (5')tetraphospho(5')nucleoside.

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used to lower the intracellular  $Ap_4A$  concentration in a eukaryotic organism. These results also indicate that the high-level over-expression of the *aph1* gene may affect the rate of cell growth. This is also the first report on the intracellular concentrations of ATP and ADP in *S. pombe*.

A preliminary report of this work has been published [12].

## MATERIALS AND METHODS

### *S. pombe* strains

The *S. pombe* strain used as wild type was PR1319  $h^- ade6-210 leu1-32 his7-366 ura4-D18$ . The *aph1* disruption and over-expression strains were constructed as described below.

### Growth

Cells were grown using standard procedures [13]. Generation times were calculated from the linear portion of a plot of  $\log_2 D_{600}$  against time. In some experiments, cell density was determined by counting cells in a haemocytometer.

### Construction of the *aph1* disruption vector

pBluescript II KS<sup>(-)</sup> (Stratagene) containing the *aph1* gene on a 1.2 kb *EcoRI* fragment was used as the source of *aph1* [5]. A 441 bp fragment of the open reading frame of *aph1* was removed by treatment of this plasmid with *BstBI* and *Bsu36I*. This fragment was replaced by a 2.1 kb fragment containing the *ura4* gene from *S. pombe* that was cut out of the pUD18 plasmid [14] with *NarI* and *AseI*. The replacement was accomplished by filling the AT overhangs of the two fragments to be ligated together with the Klenow fragment of *E. coli* DNA polymerase I, resulting in both fragments having one GC overhang and one blunt end. The two fragments were ligated together with T4 DNA ligase, and the resulting plasmid was named pDis22. This plasmid contains the *ura4* gene flanked by 359 bp of the 5' region of *aph1* and 386 bp of the 3' region of *aph1*.

### Disruption of the *aph1* gene

Approx. 5  $\mu$ g of *EcoRI*-digested pDis22 was used to transform *S. pombe* PR1319 using a lithium acetate-based procedure [15]. A total of 18 transformants were screened for a decrease in  $Ap_4A$  hydrolase activity. Transformants were grown overnight in 50 ml of rich medium. The cells were then harvested, and the crude supernatants were prepared and assayed as described below.

### Southern blots

Genomic DNA was isolated as described in [16]. Southern blotting was done as described in [17]. The *aph1* cDNA probe was labelled using a Sigma random primer labelling kit and [<sup>32</sup>P]dCTP (New England Nuclear).

### Construction of pPOX

pPOX was constructed from pDblet [18] and pCDNA 3.0 (Invitrogen). Initially, an *XhoI* site in the multiple cloning site of pDblet was eliminated by cleaving with *XhoI*, blunting both ends with the Klenow fragment of *E. coli* DNA polymerase I and religating the ends. This plasmid was named pDblet2. The cytomegalovirus-promoter–MCS–bovine-growth-hormone (where MCS is multiple cloning site) poly(A) tail cassette from pCDNA 3.0 was amplified with PCR primers 5'-ataaacccggtaggttagcgcttt-3' and 5'-atgcatcgatatactctgcctgcct-3' (*SacII* and *Clal*

sites are underlined). The resulting PCR product and pDblet2 were digested with *SacII* and *Clal* and ligated together with T4 ligase to yield pPOX (Pombe OvereXpression).

### Construction of pPOX*luc*

pPOX*luc* was constructed by ligating together *HindIII/XhoI*-digested pPOX and the *HindIII/XhoI* fragment from pGEM-*luc* (Promega) that contains *luc*.

### Assay of luciferase activity

Crude supernatant fraction from *S. pombe* PR1319 transformed with pPOX*luc* was assayed in 100  $\mu$ l of 25 mM Hepes, pH 7.75, containing 4 mM  $MgCl_2$ , 25 nM ATP and 2  $\mu$ M luciferin (Sigma). Relative light output was measured in a Trolox luminometer.

### Overexpression of *aph1*

Two plasmids were used for overexpression of *aph1*. First, *aph1* was overexpressed using pYH1 [5]. This plasmid is pFL20 containing a 3.0 kb fragment of *S. pombe* genomic DNA that contains the *aph1* gene. The second plasmid used for overexpression of *aph1* was pPOX*aph1*. pPOX*aph1* was made by removing the open reading frame of *aph1* from pYH6 [5] by digestion with *EcoRI* and *XhoI* and ligating this fragment into *EcoRI/XhoI*-digested pPOX. Both plasmids were verified by DNA sequencing. *S. pombe* PR1319 was transformed with plasmids using a lithium acetate-based procedure [15].

### SDS/PAGE analysis

Crude supernatant fractions were obtained from exponential-phase cultures of *S. pombe* PR1319 transformed with pPOX*aph1* or pPOX*luc*. Crude supernatant samples were subjected to electrophoresis on 15% (w/v) polyacrylamide gels under denaturing protein conditions using a discontinuous buffer system [19]. Gels were stained in Coomassie Blue and destained in 10% (v/v) acetic acid. Molecular mass markers were from Sigma (Sigma Mark VII-L, SDS 7).

### Preparation of cellular extracts for nucleotide measurements and measurement of $Ap_4A$ hydrolase activity

Cells were grown in 250 or 500 ml of 2  $\times$  minimal medium with the appropriate supplements [13]. When the cells reached the appropriate density, three independent samples (50 or 100 ml each) from each culture were removed and harvested by rapid filtration on 1.2  $\mu$ m-pore-size Millipore filters, suspended in 5% (w/v) trichloroacetic acid containing 2 pmol of [<sup>3</sup>H] $Ap_4A$  as an internal standard, and processed for measurement of  $Ap_4A$  as described previously [20]. One 1-ml rinse of the filter with 5% (w/v) trichloroacetic acid was added to the resuspended sample before sonication, and the sonicated trichloroacetic acid extracts sat on ice for at least 45 min before centrifugation (43 000 g, 30 min) to obtain trichloroacetic acid supernatants and crude pellets. The remaining 100 or 200 ml of cell culture was harvested and used for determining the  $Ap_4A$  hydrolase activity (see below).

### Measurement of intracellular $Ap_4A$ concentration

Trichloroacetic acid supernatants were purified using chromatography on boronate-derivatized resin, treated with alkaline phosphatase and analysed quantitatively for  $Ap_4A$  by HPLC [21] with the following modifications: a Mono-Q HR 5/5 column

(Amersham Pharmacia Biotech) was used instead of a Partisil SAX column, and the resin used in the precolumn was Source 30Q resin (Amersham Pharmacia Biotech).  $\text{Ap}_4\text{A}$  was eluted with 0.2 M ammonium bicarbonate, pH 8.5. The  $[\text{^3H}]\text{Ap}_4\text{A}$  radioactivity in a portion of the eluted  $\text{Ap}_4\text{A}$  was measured by liquid scintillation counting to determine recovery for each sample. Measured  $\text{Ap}_4\text{A}$  values were corrected for recoveries, which averaged  $50 \pm 6\%$  ( $n = 41$ ).

#### Measurement of intracellular ATP and ADP concentrations

An aliquot of each trichloroacetic acid supernatant was diluted into 20 mM  $\text{NH}_4\text{HCO}_3$ , pH 8.5, and then subjected to gradient elution from 50 mM to 383 mM  $\text{NH}_4\text{HCO}_3$ , pH 8.5, over a 30 min period on a MonoQ column, to analyse ATP and ADP levels. Mononucleotides were measured quantitatively by area integration of peaks in comparison with standards. Recoveries were assumed to be 100%. We were unable to measure AMP under these conditions due to contaminants eluting with or near the AMP peak.

#### Measurement of $\text{Ap}_4\text{A}$ hydrolase activity

Cells from the same cultures used for measurement of the nucleotides were harvested by centrifugation as above, and crude supernatants were prepared as described previously [5].  $\text{Ap}_4\text{A}$  hydrolase activity was assayed using 100  $\mu\text{M}$   $[\text{^3H}]\text{Ap}_4\text{A}$  as substrate, with subsequent separation of products and residual substrate using chromatography on boronate-derivatized resin [22]. Assays were carried out using both dialysed and undialysed crude supernatant in the presence or absence of 0.2 mg/ml BSA. Neither dialysis nor BSA affected the measured activities, but the BSA was necessary to stabilize the enzyme at high dilution. Activity was expressed as nmol of AXP formed/min per mg of protein, where  $\text{AXP} = \text{AMP} + \text{ATP}$ .

#### Determination of protein mass

Crude pellets obtained by centrifugation of trichloroacetic acid extracts were washed with 95% (v/v) ethanol and dissolved in 2–3 ml of 2 M NaOH for determination of protein [23]. Protein masses in crude supernatant fractions used in assay of  $\text{Ap}_4\text{A}$  hydrolase activity also were measured as in [23].

#### Data analysis

For measurement of nucleotides, 3–12 independent samples of each strain were analysed. Values (mean  $\pm$  S.D.) are expressed in pmol or nmol of nucleotide per mg of protein, which was measured quantitatively in trichloroacetic acid pellets as described above. Statistical analyses were performed with Excel software using a two-tailed group *t*-test assuming unequal variance. Statistical significance was assumed at  $P < 0.05$ .

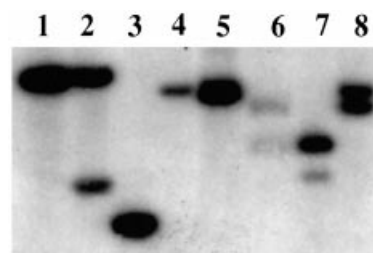
To estimate the *in vivo* concentration of nucleotides in *S. pombe*, we used the following parameters. Based on the cell density of cultures at the time of harvest and the protein mass in trichloroacetic acid pellets, we calculated a value of  $9.5 \pm 1.9$  pg of protein per cell ( $n = 12$ ). This value is in the range of values of 7–14 pg of protein per cell reported for cells grown under different conditions [24,25]. The volume of a single cell was taken as 0.14 pl, based on reported values of 0.140–0.148 pl/cell [26–28].

## RESULTS

### Disruption of the *aph1* gene and its effects on $\text{Ap}_4\text{A}$ , ATP and ADP concentrations

A total of 18 potential *aph1* disruptant transformants were tested and found to be stable *ura4* integrants. These transformants were grown overnight in rich medium, harvested, and assayed for  $\text{Ap}_4\text{A}$  hydrolase activity as previously described [5]. Several strains exhibited decreased  $\text{Ap}_4\text{A}$  hydrolase activity relative to the wild type. Five of the 18 transformants were analysed by Southern blot to verify disruption of the *aph1* gene. One of these, designated PR1319*aph1* $\Delta::ura4$ , was used in the remainder of these experiments. Figure 1 shows a Southern blot of genomic DNA from PR1319*aph1* $\Delta::ura4$ . This strain was also tested by Southern blot to verify that only a single integration of *ura4* had occurred (results not shown).

Disruption of the *aph1* gene caused a 6.6-fold decrease in  $\text{Ap}_4\text{A}$  hydrolase activity (Table 1), as well as a 290-fold increase in the intracellular  $\text{Ap}_4\text{A}$  concentration (Table 2). The residual  $\text{Ap}_4\text{A}$  degradative activity in PR1319*aph1* $\Delta::ura4$  was partially purified, and a portion of this was verified to lack detectable non-specific phosphodiesterase activity (results not shown) indicating



**Figure 1** Southern blot analysis of genomic DNA from PR1319 and PR1319*aph1* $\Delta::ura4$  probed with  $[\text{^32P}]\text{dCTP}$ -labelled *aph1* cDNA

Lanes 1 and 2, *Hind*III-cut genomic DNA from PR1319 and PR1319*aph1* $\Delta::ura4$  respectively; lanes 3 and 4, *Eco*RI-cut genomic DNA from PR1319 and PR1319*aph1* $\Delta::ura4$  respectively; lanes 5 and 6, *Stu*I-cut genomic DNA from PR1319 and PR1319*aph1* $\Delta::ura4$  respectively; lanes 7 and 8, *Bsu*36I-cut genomic DNA from PR1319 and PR1319*aph1* $\Delta::ura4$  respectively. The size (bp) of each band in each lane (calculated using a  $\lambda\text{gt}11$  *Hind*III digest as a standard curve) is given below, along with predicted sizes for each band (calculated using DNA sequence information from the *S. pombe* genome sequencing project) in parentheses: lane 1, 3600 (3063); lane 2, 3700 (3351) and 1300 (1361); lane 3, 1000 (1183); lane 4, 2700 (2738); lane 5, 2700 (2465); lane 6, 2400 (2465) and 1800 (1922); lane 7, 1800 (1994) and 1500 (1635); lane 8, 2800 (2768) and 2500 (2510).

**Table 1**  $\text{Ap}_4\text{A}$  hydrolase activity in *Schizosaccharomyces pombe*

Data were generated from exponential-phase cultures grown in minimal medium with supplements at 30 °C, as described in the Materials and methods section. AXP represents AMP + ATP. Values are means  $\pm$  S.D. ( $n = 3$ ).

Strain	$\text{Ap}_4\text{A}$ hydrolase activity (nmol of AXP/min per mg of protein)
PR1319	$1.77 \pm 0.15$
PR1319 <i>aph1</i> $\Delta::ura4$	$0.27 \pm 0.06$
PR1319 + pFL20	$1.37 \pm 0.25$
PR1319 + pFL20 <i>aph1</i>	$23.6 \pm 3.76$
PR1319 + pPOX	$1.70 \pm 0.20$
PR1319 + pPOX <i>aph1</i>	$143 \pm 22.7$

**Table 2 Intracellular Ap<sub>4</sub>A, ATP and ADP concentrations in *Schizosaccharomyces pombe***

Data were generated from exponential-phase cultures grown in minimal medium with the appropriate supplements at 30 °C, as described in the Materials and methods section. Values are means  $\pm$  S.D. for the numbers of measurements shown in parentheses. Intracellular concentrations of Ap<sub>4</sub>A, ATP and ADP were estimated using the cell parameters, as described in the Materials and methods section. Significance of differences: \* $P < 0.05$  compared with wild-type strain PR1319; † $P < 0.05$  compared with strain transformed with the corresponding control plasmid. The Ap<sub>4</sub>A concentration (pmol/mg) with PR1319 + pPOX<sub>aph1</sub> is not statistically significantly different from that with PR1319 + pFL20<sub>aph1</sub> at the  $P = 0.05$  level.

Strain	Ap <sub>4</sub> A		ATP		ADP	
	(pmol/mg of protein)	(nM)	(nmol/mg)	(mM)	(nmol/mg)	(mM)
PR1319 ( $n = 12$ )	0.84 $\pm$ 0.39	57 $\pm$ 26	13.9 $\pm$ 1.6	0.94 $\pm$ 0.11	3.2 $\pm$ 0.5	0.22 $\pm$ 0.03
PR1319 <sub>aph1</sub> $\Delta$ :: <i>ura4</i> ( $n = 6$ )	245 $\pm$ 98*	16614 $\pm$ 6645	14.3 $\pm$ 2.0	0.97 $\pm$ 0.14	3.5 $\pm$ 0.5	0.24 $\pm$ 0.03
PR1319 + pFL20 ( $n = 6$ )	0.68 $\pm$ 0.08	46 $\pm$ 5	15.0 $\pm$ 1.3	1.02 $\pm$ 0.09	3.3 $\pm$ 0.6	0.22 $\pm$ 0.04
PR1319 + pFL20 <sub>aph1</sub> ( $n = 5$ )	0.28 $\pm$ 0.18†	19 $\pm$ 12	15.7 $\pm$ 3.0	1.06 $\pm$ 0.20	3.4 $\pm$ 0.6	0.23 $\pm$ 0.04
PR1319 + pPOX ( $n = 3$ )	0.70 $\pm$ 0.10	47 $\pm$ 6.8	15.0 $\pm$ 1.2	1.02 $\pm$ 0.08	3.2 $\pm$ 0.5	0.22 $\pm$ 0.03
PR1319 + pPOX <sub>aph1</sub> ( $n = 3$ )	0.14 $\pm$ 0.06†	9.5 $\pm$ 4.1	13.0 $\pm$ 0.8	0.88 $\pm$ 0.06	3.8 $\pm$ 0.4	0.26 $\pm$ 0.03

**Table 3 Growth rates of *Schizosaccharomyces pombe* lacking or overexpressing *aph1***

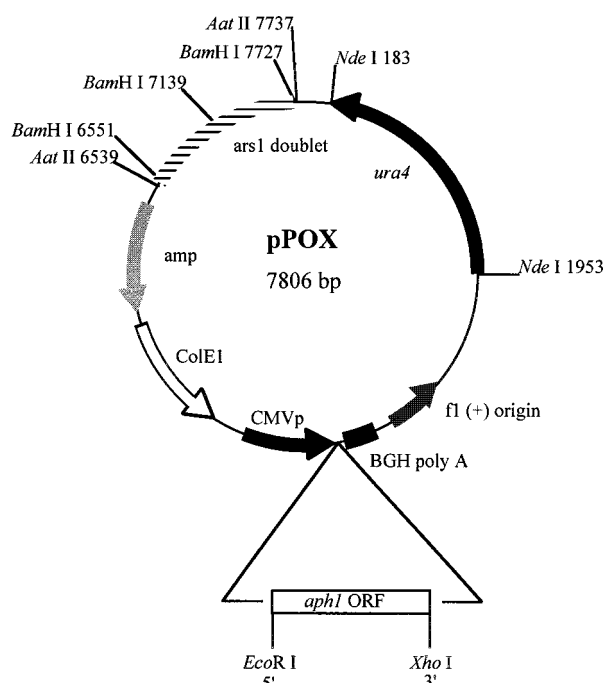
Generation times were determined from cultures at 30 °C in minimal medium with appropriate supplements, as described in the Materials and methods section. Values are means  $\pm$  S.D. ( $n = 3$ ), except where indicated († measured values in two experiments). Significance of difference compared with strain transformed with the corresponding control plasmid: \* $P < 0.05$ .

Strain	Generation time (h)
PR1319	3.3 $\pm$ 0.1
PR1319 <sub>aph1</sub> $\Delta$ :: <i>ura4</i>	3.3 $\pm$ 0.1
PR1319 + pFL20	4.1 $\pm$ 0.1
PR1319 + pFL20 <sub>aph1</sub>	3.9 $\pm$ 0.1
PR1319 + pPOX	3.7 $\pm$ 0.1
PR1319 + pPOX <sub>aph1</sub>	5.4 $\pm$ 0.4*
PR1319 + pPOX <sub>luc</sub>	3.8, 4.2†

the presence of another enzyme relatively specific for Ap<sub>4</sub>A. Disruption of the *aph1* gene had no detectable effect on the growth rate in minimal medium (Table 3) or in rich medium. The generation times in rich medium were 2.3  $\pm$  0.1 h ( $n = 3$ ) and 2.0  $\pm$  0.1 h ( $n = 3$ ) for wild-type and *aph1* disruptant strains respectively. The *aph1* $\Delta$ ::*ura4* strain mated with wild-type cells of the opposite mating type. An *aph1*<sup>+</sup>/*aph1* $\Delta$ ::*ura4* diploid sporulated, and its morphology and size at cell division were the same as that of the diploid wild-type strain. Although the intracellular concentration of Ap<sub>4</sub>A was increased by a factor of 290 in the *aph1* disruptant strain, the intracellular ATP and ADP concentrations were essentially the same as in the wild-type strain (Table 2).

### Overexpression of *aph1*

The *aph1* gene was overexpressed under the control of its own promoter on the plasmid pFL20 (pFL20<sub>aph1</sub>). *S. pombe* containing pFL20<sub>aph1</sub> exhibited a 17-fold increase in Ap<sub>4</sub>A hydrolase activity (Table 1) and a 60% decrease in intracellular Ap<sub>4</sub>A concentration (Table 2) relative to the pFL20 control strain. Overexpression of *aph1* via pFL20 had no significant effect on the intracellular concentrations of ADP or ATP (Table 2). To express *aph1* at a higher level, a new *S. pombe* expression plasmid was constructed as described in the Materials and methods section. This vector was named pPOX (Pombe Over-Expression) (Figure 2). The *aph1* gene was overexpressed under

**Figure 2 Structure of plasmid pPOX<sub>aph1</sub>**

The sizes of the fragments are designated in bp. The arrows indicate the direction of transcription of genes. The enlarged map at the bottom represents the structure of the 546 bp *aph1* open reading frame (ORF). The unique restriction enzyme sites in the multiple cloning site [located where the *aph1* ORF has been inserted between CMVp (cytomegalovirus promoter) and BGH poly A (bovine growth hormone poly(A) terminator sequence)] are: 5'-HindIII-BstXI-EcoRI-PstI-BsaBI-BstXI-NotI-XhoI-XbaI-3'.

the control of the cytomegalovirus promoter on pPOX. *S. pombe* transformed with pPOX<sub>aph1</sub> exhibited an 84-fold increase in Ap<sub>4</sub>A hydrolase activity (Table 1) and an 80% decrease in intracellular Ap<sub>4</sub>A concentration (Table 2) relative to the pPOX control strain. Overexpression of *aph1* via pPOX did not significantly affect the intracellular concentrations of ADP or ATP (Table 2), nor did it affect cell morphology. Overexpression of *aph1* via pPOX increased the generation time by about 46% relative to the pPOX control strain (Table 3).

To address the possibility that the decreased growth rate in *S. pombe* PR1319 transformed with pPOX<sub>aph1</sub> was simply related

to high-level protein expression, and not due specifically to *aph1*, we overexpressed a second gene, *luc*, via pPOX. Crude supernatant from *S. pombe* PR1319 transformed with pPOX*luc* was verified to contain luciferase activity, but no change in growth rate was detected in this strain (Table 3). In addition, the amount of endogenous Aph1 in *S. pombe* is very low [29], such that the increased expression of Aph1 via pPOX was still undetectable in a crude supernatant as a specific band on a Coomassie Blue-stained SDS/polyacrylamide gel (results not shown).

## DISCUSSION

This is the first report of the measurement of intracellular Ap<sub>4</sub>A, ATP and ADP concentrations in *S. pombe*. The measured value of 0.84 pmol of Ap<sub>4</sub>A/mg of protein and the estimated concentration of 57 nM Ap<sub>4</sub>A are generally lower than the corresponding values from *Sacch. cerevisiae*. Previously reported values in *Sacch. cerevisiae* are 3.6 pmol of Ap<sub>4</sub>A/mg of protein [11,21] and 0.58 pmol of Ap<sub>4</sub>A/mg of protein [20]. These values can be estimated as concentrations of 310 nM and 50 nM Ap<sub>4</sub>A respectively, using cell parameters for *Sacch. cerevisiae* [30]. Concentrations of 550 nM Ap<sub>4</sub>A [31], 400 nM adenosine (5')tetraphospho(5')nucleoside (Ap<sub>4</sub>N) [32] and 80 nM Ap<sub>4</sub>N [33] have also been reported. The basal concentration of Ap<sub>4</sub>A in *S. pombe* is similar to the basal concentration determined in other organisms and cells, including *Physarum*, *Tetrahymena* and CHO cells [3].

The concentrations of 0.94 mM ATP and 0.22 mM ADP in *S. pombe* are also lower than corresponding values in *Sacch. cerevisiae*, which range from 1.3 to 5.2 mM ATP [31,34–40] and from 0.30 to 0.66 mM ADP [35,37–40]. (Some values reported in the cited references were converted to mM concentrations using cell parameters for *Sacch. cerevisiae* [30].) In both fission and budding yeasts, the concentration of Ap<sub>4</sub>A is about 0.0001 times that of ATP.

Disruption of *aph1* in *S. pombe* resulted in a much larger increase in intracellular Ap<sub>4</sub>A concentration than occurred upon disruption of two genes encoding Ap<sub>4</sub>A degradative enzymes in *Sacch. cerevisiae*. Upon disruption of *APA1*, which encodes Ap<sub>4</sub>A phosphorylase I [9], the concentration of intracellular Ap<sub>4</sub>A in *Sacch. cerevisiae* increases by a factor of 3–5 [10,20], while disruption of *APA2* causes no detectable change in the intracellular Ap<sub>4</sub>A concentration [10]. However, disruption of both phosphorylase genes leads to a 38-fold increase in intracellular Ap<sub>4</sub>A concentration [10]. The 290-fold increase in intracellular Ap<sub>4</sub>A concentration upon disruption of *aph1* occurred in spite of the residual Ap<sub>4</sub>A degradative activity. This result suggests that either the degradative enzymes or Ap<sub>4</sub>A, or both, may be compartmentalized in *S. pombe*. Although disruption of the genes encoding Ap<sub>4</sub>A degradative enzymes was successful in increasing intracellular Ap<sub>4</sub>A in both yeasts, neither exhibited a detectable change in phenotype. *S. pombe* with *aph1* disrupted showed no change in growth rate in minimal and rich media, and appeared to mate and sporulate normally. *Sacch. cerevisiae* strains with either *APA1* or *APA2*, or both *APA1* and *APA2*, disrupted show no change in growth rate in minimal medium and are capable of sporulating and mating normally [10].

In addition to the manipulations performed on *APA1* and *APA2*, the *APH1* gene from *Sacch. cerevisiae*, which encodes an Ap<sub>3</sub>A hydrolase, has been disrupted alone and in an *apa1*Δ::*HIS3* *apa2*Δ::*URA3* background [32]. Disruption of *APH1* alone leads to a 31-fold increase in the intracellular Ap<sub>3</sub>N concentration and no change in growth rate. The disruption of

all three Ap<sub>n</sub>A-degrading enzymes leads to a 750-fold increase in intracellular Ap<sub>4</sub>N, to a concentration of approx. 300 μM [32]. The *apa1*Δ::*HIS3* *apa2*Δ::*URA3* *aph1*Δ::*TRP1* strain exhibits a 50% increase in generation time relative to wild type. The authors attributed this increase to the greatly increased intracellular concentration of Ap<sub>4</sub>N [32]. In contrast, no effect on growth rate was observed when the Ap<sub>4</sub>A concentration was increased to approx. 16 μM in the *S. pombe* *aph1*Δ::*ura4* strain (Tables 2 and 3).

The absence of detectable phenotypic changes accompanying the large increase in intracellular Ap<sub>4</sub>A concentration observed in the *S. pombe* *aph1*Δ::*ura4* strain has several implications. First, comparison of loss-of-function data between Fhit and Aph1 suggests that the two enzymes do not have the same function *in vivo*. Murphy et al. [41] measured the concentrations of Ap<sub>3</sub>A and Ap<sub>4</sub>A in several tumour cell lines expressing Fhit at various levels. Ap<sub>3</sub>A was undetectable in two Fhit-positive cell lines, while the levels of Ap<sub>3</sub>A were higher and variable in lines with low or undetectable levels of Fhit. The level of Ap<sub>4</sub>A was about the same in the two sets of cells. These workers concluded that Fhit regulates the level of Ap<sub>3</sub>A, and not Ap<sub>4</sub>A, *in vivo*. The decrease in the intracellular concentration of Ap<sub>4</sub>A that occurs upon overexpression of *aph1*, coupled to the increase in intracellular Ap<sub>4</sub>A concentration upon disruption of *aph1*, clearly indicate that Aph1 regulates the level of Ap<sub>4</sub>A *in vivo*. The conclusion that Aph1 degrades Ap<sub>4</sub>A *in vivo* while Fhit does not was expected, based on data on their *in vitro* substrate specificities [4,29]. In addition, loss of *aph1* appears to have no major effect on *S. pombe* cell growth, while loss of *FHIT* in mammalian cells is correlated with abnormal cell growth resulting in tumorigenesis [8]. Secondly, the lack of phenotypic change seen in the *aph1* disruptant strain also indicates that fission yeast can tolerate large increases in intracellular Ap<sub>4</sub>A concentration with no detectable effect on growth. The increase in intracellular Ap<sub>4</sub>A also does not appear sufficient to cause alterations in the size of cells at division in fission yeast, as it has been suggested to do in *E. coli* [42,43]. Nishimura [43] demonstrated that two mutants of *E. coli* with 15–100-fold increases in Ap<sub>4</sub>A exhibited reduced cell size at division without a change in growth rate. *S. pombe* PR1319*aph1*Δ::*ura4* showed no such change in cell size at division (results not shown).

The present work is the first report of the use of genetic manipulation to successfully lower the intracellular Ap<sub>4</sub>A concentration in any eukaryotic organism. Previous work attempting to achieve a similar goal in *Sacch. cerevisiae* resulted in a paradoxical increase in intracellular Ap<sub>4</sub>A concentration upon overexpression of Ap<sub>4</sub>A phosphorylase I, in spite of a maximal 90-fold increase in phosphorylase activity *in vitro* [11,20]. Mechulam et al. [44] demonstrated that overexpression of *E. coli* *apaH*, which encodes a symmetrical Ap<sub>4</sub>A hydrolase, decreased the intracellular Ap<sub>4</sub>A concentration in *E. coli* by a factor of 10.

The finding that increased expression of Aph1 in a wild-type strain of *S. pombe* decreased the rate of growth (Table 3) also has several implications. First, the decreased growth rate of *S. pombe* PR1319 transformed with pPOX*aph1* (Table 3) could be related to the decreased intracellular Ap<sub>4</sub>A concentration. However, since the intracellular Ap<sub>4</sub>A concentration in this strain is not statistically significantly different from that found in *S. pombe* PR1319 transformed with pFL20*aph1*, a strain that showed no detectable change in growth rate, a decrease in the intracellular Ap<sub>4</sub>A concentration may not be the cause of the decreased growth rate in *S. pombe* PR1319 transformed with pPOX*aph1*. Secondly, while not strictly correlated with Ap<sub>4</sub>A levels, the decreased growth rate in *S. pombe* PR1319 transformed with pPOX*aph1* does seem to be specific for overexpression of *aph1*

rather than being an effect of general high-level protein overexpression. Thirdly, comparison of overexpression data for Aph1 and Fhit reveals the following. Increased expression of Fhit in mammalian cells containing endogenous Fhit has no detectable effect on growth [45]. However, re-introduction of Fhit into *FHIT*-minus cells results in suppression of tumorigenicity of cancer cells in nude mice [45,46] and growth inhibition of cancer cells due to cell cycle arrest and increased apoptosis [45,47]. Interestingly, the decreased growth rate displayed by MKN74 gastric cancer cells overexpressing Fhit upon injection into nude mice does not appear to require  $Ap_3A$  hydrolysis. Overexpression of a mutant form of Fhit (Fhit H96N) that displays  $Ap_3A$  hydrolase activity reduced to  $1/10^6$  that of wild-type Fhit also suppresses tumorigenicity [46]. This suggests that binding of substrate and/or interaction of Fhit with another protein is more important in tumour suppression than is hydrolytic activity [46]. It has been shown that Fhit H96N binds  $Ap_3A$  with 4-fold less affinity than wild-type Fhit [48]. Overexpression of Aph1 at a high level also results in growth inhibition in *S. pombe*, but, as stated above, we have not been able to strictly correlate this growth inhibition with changes in the intracellular  $Ap_4A$  concentration. So, as is the case with Fhit, the observed growth inhibition upon overexpression of Aph1 may not involve hydrolysis of an  $Ap_nA$ . This is consistent with Aph1 and Fhit sharing a similar function *in vivo* related to cell growth that does not require  $Ap_nA$  degradation.

In conclusion, these data indicate that disruption of *aph1* and the resulting 290-fold increase in the intracellular  $Ap_4A$  concentration have no detectable effect on *S. pombe* growth under the conditions tested, and do not appear to affect mating and sporulation. On the other hand, high-level expression of Aph1 appears to slow growth rate, but the inhibition of growth is not clearly linked to changes in intracellular  $Ap_4A$  concentration. Comparison of *in vitro* data and *in vivo* overexpression and loss-of-function data for Aph1 and Fhit suggests that these two enzymes degrade different  $Ap_nAs$  in the cell, but that their mechanism of growth inhibition involving a reaction unrelated to  $Ap_nA$  degradation may yet prove similar.

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