Pneumocystis Encodes a Functional S-Adenosylmethionine Synthetase Gene

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Received 18 September 2007/Accepted 23 November 2007

S-Adenosylmethionine (AdoMet) synthetase (EC 2.5.1.6) is the enzyme that catalyzes the synthesis of AdoMet, a molecule important for all cellular organisms. We have cloned and characterized an AdoMet synthetase gene (sam1) from Pneumocystis spp. This gene was transcribed primarily as an ~1.3-kb mRNA which encodes a protein containing 381 amino acids in P. carinii or P. murina and 382 amino acids in P. jirovecii. sam1 was also transcribed as part of an apparent polycistronic transcript of ~5.6 kb, together with a putative chromatin remodeling protein homologous to Saccharomyces cerevisiae, CHD1. Recombinant Sam1, when expressed in Escherichia coli, showed functional enzyme activity. Immunoprecipitation and confocal immunofluorescence analysis using an antipeptide antibody showed that this enzyme is expressed in P. murina. Thus, Pneumocystis, like other organisms, can synthesize its own AdoMet and may not depend on its host for the supply of this important molecule.

MATERIALS AND METHODS

RNA and DNA extraction. Total RNA was extracted, using RNAzol B (Tel-Test Inc., Friendswood, TX) from, P. carinii- or Pneumocystis murina-infected lungs and from P. carinii or P. murina organisms partially purified from the infected lungs of rats or mice by Ficoll-Hypaque density gradient centrifugation, as described previously (16). Genomic DNA was isolated using a QIAamp DNA Mini Kit (Qiagen, Valencia, CA). For P. jirovecii, genomic DNA was extracted from autopsy lung samples.

PCR and DNA sequencing. PCR and sequencing were performed as described previously (17). PCR was performed using High Fidelity PCR master mix (Roche Diagnostics Corp., Indianapolis, IN) and genomic DNA or cDNA from P. carinii or P. murina organisms or from P. jirovecii-infected lung samples as templates. The sequences of the primers used for the amplifications are listed in Table 1. In certain experiments, AccuPrime Pfu (Invitrogen, Carlsbad, CA) or HotStar Taq (Qiagen) was used.

Partial genomic sequences of sam1 from both P. murina and P. jirovecii were obtained by sequencing the PCR products generated by the amplification of genomic DNA, using primers designed from the P. carinii sam1 gene sequence obtained from the Pneumocystis genome project database (7).

For reverse transcription (RT)-PCR, first-strand cDNA was synthesized from total RNA preparations obtained from partially purified P. carinii organisms or from P. murina-infected lung samples using AP primer and Superscript II reverse transcriptase (Invitrogen). PCR was performed utilizing primers designed from the known sam1 gene sequence. For 3′ rapid amplification of cDNA ends (3′ RACE), primer UAP (3′ RACE kit), and the sam1 gene-specific primers were used. RNA isolated from P. carinii organisms or from P. murina-infected lung samples was subjected to RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE), using a First Choice RLM-RACE kit (Ambion Inc., Austin, TX) according to the manufacturer’s protocol. The first- and second-round PCR were performed using outer and inner adapter primers along with the sam1 gene-specific primers.

Reverse PCR was done as described previously (20). Briefly, genomic DNA extracted from lung samples infected with P. carinii, P. murina, or P. jirovecii was digested with restriction enzyme HindIII or MboI (New England Biolabs, Beverly, MA). The digested product was ligated using T4 DNA ligase (New England Biolabs, Beverly, MA) and subjected to nested PCR.

In some cases, PCR products were subcloned into TOPO TA cloning PCR 2.1 vector (Invitrogen). The clones were verified by sequencing PCR products generated using M13 forward and reverse primers.

Southern and Northern blotting analyses. Southern and Northern blotting analyses were performed as described previously (17). Southern blotting analysis was performed using genomic DNA from P. carinii-infected lung samples digested with different restriction enzymes. The blots were hybridized with a digoxigenin (DIG)-labeled PCR product spanning nucleotides 552 to 2020 of the P. carinii sam1 genomic sequence (DIG-High Prime; Roche) or a DIG-dUTP–

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§ Published ahead of print on 7 December 2007.
| Oligonucleotide | Sequence (5’–3’) | Description of corresponding or complementary amino acid range and organism |
|----------------|-----------------|------------------------------------------------------------------|
| BH26sams       | TGCTATTTTGGATGATGTTA | Corresponds to 718–739 of the *P. carinii* sam1 gene |
| BH21sams       | TTCACAGGATTCAGATGGA | Complementary to 1811–1830 of the *P. carinii* sam1 gene |
| BH19sams       | TGTTGAACATATGGGAACAG | Corresponds to 1782–1802 of the *P. carinii* sam1 gene |
| BH29sams       | AGATCACAATTTAGAGTTGCT | Corresponds to 745–766 of the *P. carinii* sam1 gene |
| BH34sams       | GATTGGTCTTCATAGCAAGA | Complementary to 974–995 of the *P. carinii* sam1 gene |
| GK632sams      | GGATGTTGGTCTCGTTT | Corresponds to 1206–1225 of the *P. murina* sam1 gene |
| GK629sams      | GATCTCTTTTTTTACGTAC | Complementary to 728–749 of the *P. carinii* sam1 gene |
| GK628sams      | CATGCACTCAAATATGACAT | Corresponds to 716–735 of the *P. carinii* sam1 gene |
| GK1sams        | ATTTTAGGGGAAAGAACGC | Corresponds to 378–397 of the *P. murina* sam1 gene |
| GK12sams       | CTAACATTCTGATTGGTTC | Corresponds to 1949–1954 of the *P. carinii* sam1 gene |
| GK9sams        | TGTGGAACATTGGAAAGCT | Corresponds to 1985–2010 of the *P. carinii* sam1 gene |
| GK13sams       | ATGTCAGGTTATTATTATCTT | Corresponds to 552–574 of the *P. carinii* sam1 gene |
| GK14sams       | AAACATACATTITTTAGTGTTC | Corresponds to 1874–1901 of the *P. murina* sam1 gene |
| GK11sams       | CTAACATTTGATTGGTTC | Corresponds to 1904–1919 of the *P. carinii* sam1 gene |
| GK4sams        | ATTCIAGATTGGTTTGTG | Corresponds to 753–772 of the *P. carinii* sam1 gene |
| GK54sams       | GTTITTAAACAGTGGTGTG | Corresponds to 19–39 of the *P. carinii* sam1 gene |
| GK53sams       | TACGTTGTTTACATTAGCTACAC | Complementary to 6–57 of the *P. carinii* sam1 gene |
| GK7sams        | GTGCAGATGTGIGGT | Corresponds to 1496–1512 of the *P. murina* sam1 gene |
| GK20sams       | AGCTTGAAGTTTAAATTCC | Corresponds to 660–680 of the *P. jiroveci* sam1 gene |
| GK24sams       | AGTGGCTTCTTGTTATTAAAGT | Corresponds to 1547–1569 of the *P. jiroveci* sam1 gene |
| GK25sams       | GGTGTATTGTTAAGAACCTGT | Corresponds to 1655–1676 of the *P. jiroveci* sam1 gene |
| GK29sams       | CTAACAAACAAATCTTCAGAG | Corresponds to 1279–1299 of the *P. jiroveci* sam1 gene |
| GK26sams       | ATGGGACCCTTCCAACAGAT | Corresponds to 423–441 of the *P. jiroveci* sam1 gene |
| GK27sams       | CTAAGATGGCTTGATATCTG | Corresponds to 501–524 of the *P. jiroveci* sam1 gene |
| GK30sams       | TGATCAACAAATAATCTTTC | Corresponds to 1371–1393 of the *P. jiroveci* sam1 gene |

**Immunofluorescence and confocal microscopic analysis.** Immunofluorescent staining was performed by Histoserv, Inc. (Germantown, MD). *P. murina*-infected or uninfected lung sections were sectioned with affinity purified anti-Sam1 antibody and anti-Pneumocystis monoclonal antibody 4D7 (1, 23). Antibody 4D7 recognizes a Pneumocystis-specific antigen that, based on immunofluorescence, appears to be present on both cysts and trophozoites. Alexa Fluor 488 goat anti-rabbit immunoglobulin G (IgG) was used as the secondary antibody for the detection of Sam1, while biotin-conjugated anti-mouse IgG and streptavidin-conjugated Alexa Fluor 594 were used for staining Pneumocystis organisms. Nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI). In certain experiments, Sam1 antibody was preincubated with the purified recombinant *P. murina* Sam1 (insoluble form), cells were centrifuged, and the supernatant was used for immunohistochemistry.

Confocal microscopy images were collected with a Leica SP5 confocal microscope (Leica Microsystems, Exton, PA) using an ×63 oil immersion objective with a numerical aperture of 1.4, and zoom 4. Fluorochromes were excited by using an argon laser (Enterprise model 651; Coherent, Inc.) at 364 nm for DAPI, an argon laser at 488 nm for Alexa Fluor 488, and an orange helium-neon laser at 594 nm for Alexa Fluor 594. To avoid possible cross-talk, the wavelengths were collected separately and were merged later. Images were processed using Leica LAS-AF software (version 1.7.0, build 1240).

**Immunoprecipitation.** Partially purified *P. murina* organisms were resuspended in 20 mM HEPES (pH 7.5), 150 mM NaCl, 1% sodium dodecyl sulfate (SDS) and a cocktail of protease inhibitors containing EDTA. The extracts were boiled for 10 min and then centrifuged for 15 min at 13,000 rpm, and the supernatant was adjusted to a final concentration of 0.12% SDS, 1% Triton X-100, 20 mM HEPES, and 150 mM NaCl. The samples were incubated overnight at 4°C with the affinity purified anti-Sam1 antibody. Simultaneously, *P. murina* extracts were treated with preimmune serum to be used as a negative control. The samples were incubated with protein A-Sepharose beads for 2 h at 4°C. The beads were washed twice with Tris-buffered saline containing 0.1% Tween 20, followed by a final wash with Tris-buffered saline and then boiled in SDS sample denaturing buffer before they were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and Western blotting analysis.
Characterization of AdoMet synthetase genes of *Pneumocystis*. Following the identification of a partial genomic sequence (~1,600 bp) of AdoMet synthetase in the *Pneumocystis* genome project database, we undertook to determine whether a full-length functional enzyme was carried by the organism. We obtained a partial cDNA sequence of AdoMet synthetase from *P. carinii* by sequencing an RT-PCR product generated by using RNA preparations from partially purified *P. carinii* organisms and primers designed from the known genomic sequence. Partial cDNA and genomic sequences from *P. murina* and *P. jirovecii* were determined by sequencing RT-PCR or PCR products obtained using RNA or genomic DNA from *P. murina* or *P. jirovecii*-infected lung samples and primers designed from conserved areas of the *P. carinii* AdoMet synthetase sequence. Inverse PCR was utilized to obtain upstream and downstream genomic sequences. 3’ and 5’ RACE were employed to obtain the complete cDNA sequence of *P. carinii* (1,232 bp) or *P. murina* (1,309-bp) AdoMet synthetase (GenBank accession numbers EF377365 and EF377360, respectively). For *P. jirovecii*, the cDNA sequence (GenBank accession number EF377362) for the coding region was deduced by comparing the genomic sequence with the cDNA sequences of *P. carinii* and *P. murina* AdoMet synthetase. The AT content of all three genes was 70 to 72%, consistent with an origin from *Pneumocystis* rather than from a mammalian host. The accuracy of the genomic and cDNA sequences was confirmed for *P. carinii* and *P. murina* by PCR amplification and sequencing of the entire coding region, using both genomic and cDNA as a template. Figure 1 shows *P. carinii* AdoMet synthetase genomic sequence together with the deduced amino acid sequences. The figure also contains a partial sequence of the adjacent gene located immediately upstream of AdoMet synthetase, which has homology to the chromatin remodeling protein CHD1 of *Saccharomyces cerevisiae* (see below). A comparison of genomic and cDNA sequences of AdoMet synthetase identified seven introns. The GenBank accession numbers of *P. carinii*, *P. murina*, and *P. jirovecii* AdoMet synthetase genomic sequences are EF377364, EF377361, and EF377363, respectively.

Most organisms have at least two forms of AdoMet synthetase (15). In *Saccharomyces cerevisiae*, two isoenzymes encoded by the two genes *sam1* and *sam2* have been reported (35, 36), but in *Schizosaccharomyces pombe*, only *sam1* has been identified (11). We report the *Pneumocystis* AdoMet synthetase gene as *sam1*, since it has high homology to the *sam1* gene of *S. pombe*.

Deduced amino acid sequences of *Pneumocystis* *Sam1*. The cDNA sequence of *P. carinii sam1* or *P. murina sam1* contains an open reading frame encoding a protein containing 381 amino acids, while *P. jirovecii* *Sam1* contains 382 amino acids. Figure 2 shows alignment of the deduced amino acid sequences of AdoMet synthetase from *Pneumocystis*, yeast, *E. coli*, rats, mice, and humans (11–13, 21, 30, 36). The two AdoMet synthetase signature motifs GAGDQGIMFGY and GGGAFSGKD are 100% conserved among these species (11). ATP binding sites are also highly conserved (15, 28, 34). The *Sam1* protein sequence is highly conserved among *Pneumocystis*: *P. carinii* *Sam1* showed 94% identity to that of *P. murina*, and both showed 83% identity to that of *P. jirovecii*. *Pneumocystis* *Sam1* (all three species) showed 75% identity to *S. pombe* *Sam1* and 71% to that of *S. cerevisiae*. Identity to human, mouse, and rat sequences (GenBank accession numbers NM_000429, NM_133653, and NM_012860, respectively) ranged from 64% to 67%, and the *E. coli* sequence (GenBank accession number NP_289514) showed 53% to 55% identity.

Northern and Southern blotting analyses. To see whether the *sam1* gene was being transcribed, Northern blotting analysis was performed using RNA extracted from partially purified *P. carinii* organisms or from *P. murina*-infected mouse lung (Fig. 3A). A DIG-labeled PCR product corresponding to nucleotides 552 to 2020 of the *P. carinii sam1* genomic sequence was used as the probe for hybridization. In both *P. carinii* (Fig. 3, lane 1) and *P. murina* (Fig. 3, lane 2) preparations, an ~1.3-kb hybridization signal was observed, which is consistent with the size expected for the *sam1* gene transcript. However, a second, less intense band of ~5.6 kb was observed consistently in the RNA preparations from both organisms.

The 5.6-kb transcript could be derived from a gene which contained sequences with homology to the probe. To examine this, a Southern blotting analysis was performed using restriction endonuclease-digested genomic DNA from *P. carinii*-infected rat lung (Fig. 3B); the blot was hybridized with the same probe. Genomic DNA digested with AseI (Fig. 3B, lane 1) showed a single band, while that digested with XbaI (Fig. 3B, lane 2) showed two bands. This is due to the presence of one XbaI site in the probe-spanning region. Thus, the Southern blot shows that *P. carinii sam1* appears to be a single-copy gene, and no second gene with homology to the probe that could account for the higher band on the Northern blot was identified.

We also excluded the possibility that the higher transcript was related to the host *sam1* gene: the same probe did not show any reactivity with RNA from a normal (uninfected) mouse lung, while an oligonucleotide specific for mouse *sam1* gave a band of the appropriate size (~3.5 kb; data not shown).

An alternative explanation is that the 5.6-kb band represented a polycistronic RNA. Adjacent genes are rarely cotranscribed as a polycistronic RNA in eukaryotes (2). The open reading frame of the *sam1* gene is downstream of another gene that shows homology to the chromatin remodeling protein, CHD1, of *S. cerevisiae* (GenBank accession no. U18917; Fig. 1). These two genes are separated by an intergenic region of 238 bp (Fig. 1). RT-PCR performed using an upstream oligonucleotide designed from the coding region of the *chd1* homologue gene along with a downstream oligonucleotide designed from the *sam1* cDNA sequence amplified an ~2,000-bp product that contained part of the putative *chd1* cDNA sequence as well as *sam1* cDNA, confirming that these two genes are transcribed as a single RNA. The elimination of reverse transcriptase and pretreatment of the RNA with RNase-free DNase confirmed that it was RNA, not DNA, that was being amplified (data not shown). A comparison of genomic and cDNA sequences identified one intron in the partial sequence of the putative *chd1* gene and two introns in the intergenic region (Fig. 1).

To confirm that the 5.6-kb band in the Northern blotting
FIG. 1. Nucleotide and deduced amino acid sequences of *P. carinii* sam1 and partial sequences of the putative chd1 gene. Initiation and termination codons are shown in bold and are underlined. The transcription start site is indicated by an arrow, the introns are shown in lowercase letters, and the XbaI site is marked by a solid line above the sequence.
analysis was derived from this cotranscribed message, the same blot was reprobed with an oligonucleotide corresponding to the coding region of the putative \textit{chd1} gene. The probe hybridized to the 5.6-kb band but not to the 1.3-kb band (Fig. 3A, lane 3). In \textit{S. cerevisiae}, the size of \textit{chd1} cDNA is $\sim$4.4 kb. This suggests that the 5.6-kb band represents a bicistronic RNA containing the \textit{chd1} and \textit{sam1} transcripts. We also reprobed the Southern blots with the \textit{chd1} gene-specific oligonucleotide.

FIG. 2. Alignment of the deduced \textit{Pneumocystis} Sam1 amino acid sequences with those of other organisms. Sam1 sequences from \textit{P. carinii}, \textit{P. murina}, \textit{P. jirovecii}, \textit{S. pombe}, and \textit{S. cerevisiae}, MetK sequence from \textit{E. coli}, and MAT1 sequences from mice (\textit{Mus musculus}), rats (\textit{Rattus norvegicus}), and humans (\textit{Homo sapiens}) were aligned using Clustal W. Identical amino acid residues are boxed. AdoMet synthetase signature motifs are underlined. ATP binding motifs are boxed in bold.
Expression of the *P. carinii* Sam1 protein in *E. coli*. The coding region from *P. carinii* sam1 cDNA (1,145 bp) was amplified and cloned into the pET 28 expression vector and expressed as a His tag fusion protein in bacteria. SDS-PAGE analysis of whole-bacterium extract expressing recombinant protein showed a prominent band of ~45-kDa protein band when stained with Coomassie blue (Fig. 4A, lane 1), which is the size expected for Sam1, but this band was not seen when bacteria transfected with a control vector with no insert were analyzed (Fig. 4A, lane 2). The expressed protein showed immunoreactivity to His tag antibody when analyzed by Western blotting (Fig. 4B, lane 1), but no immunoreactivity was seen with the control preparation (Fig. 4B, lane 2).

Most of the expressed protein was in insoluble bacterial inclusions (data not shown). To obtain soluble, potentially functional protein, expressed recombinant protein was denatured and allowed to refold as described for AdoMet synthetase in *Leishmania donovani* (29). Control samples were processed in parallel. SDS-PAGE followed by Coomassie blue staining (Fig. 4C) and Western blotting (Fig. 4D) demonstrated solubilization of the recombinant protein.

**AdoMet synthetase assay.** The refolded protein was analyzed for AdoMet synthetase activity using 14C-labeled methionine as described previously (4). Figure 5 shows the AdoMet synthetase activity of *P. carinii* refolded recombinant protein, demonstrating increased production of AdoMet over time. The control preparation (vector alone) showed no enzyme activity. When ATP was omitted from the reaction mixture, no product was detected (data not shown). The activity was also dependent on enzyme concentration (data not shown).

**Immunological analysis of Sam1 in Pneumocystis.** Given that the sam1 mRNA was expressed by *Pneumocystis* and that the mRNA encoded a functional enzyme, we were interested in demonstrating the expression of Sam1 in *Pneumocystis* by immunological analysis. For that experiment, a polyclonal antibody was generated against a synthetic peptide corresponding to amino acid residues 199 to 219 of *P. murina* Sam1. The antibody recognized the expressed recombinant protein (Fig. 4E, lane 1), and immunoreactivity was effectively blocked when the antibody was preincubated with excess refolded recombinant *P. murina* Sam1 (Fig. 4E, lane 2). To concentrate native Sam1, partially purified *P. murina* extracts were subjected to immunoprecipitation, using this antibody; preimmune serum was used as a negative control. Western blotting analysis of immunoprecipitated samples identified an ~45-kDa band (Fig. 6, lane 1) when the antipeptide antibody was used that was not seen with preimmune serum (Fig. 6, lane 2). Preincubation of the antibody with recombinant protein led to a loss of reactivity (Fig. 6, lane 3).

To examine the expression of Sam1 protein in *P. murina*-infected tissue, lung sections were costained with anti-Sam1 and *Pneumocystis* (4D7) antibodies and subjected to confocal microscopic analysis (Fig. 7). The immunoreactivity toward Sam1 antibody colocalized with the staining of *Pneumocystis* using 4D7.
antibody, indicating the expression of Sam1 protein in Pneumocystis (Fig. 7A). It was noted that structures consistent with Pneumocystis cysts did not stain with the anti-Sam1 antibody, suggesting that Sam1 expression may be decreased or absent in cysts. When immunofluorescence analysis was done using anti-Sam1 antibody that was preabsorbed with recombinant P. murina Sam1, the immunoreactivity was lost (Fig. 7B).

DISCUSSION

In the current study we have identified genes encoding AdoMet synthetase (sam1) in Pneumocystis and have demonstrated that sam1 mRNA is transcribed, that recombinant P. carinii recombinant protein Sam1. (A) SDS-PAGE analysis of the bacterial cells expressing recombinant protein showed a band of the expected size (~45 kDa, indicated by the arrows), when stained with Coomassie blue (lane 1). When vector with no insert was analyzed, no band of that size was observed (lane 2). (B) Bacterial cells expressing the Sam1 protein showed immunoreactivity with the expected size band when probed in a Western blot with His tag antibody (lane 1), while there was no immunoreactivity when vector alone was analyzed (lane 2). (C) Refolded protein showed a band of ~45 kDa when stained with Coomassie blue (lane 1), while no band of that size was seen when vector alone was analyzed (lane 2). (D) A 45-kDa band showed immunoreactivity when Western blotting analysis shown in panel C was performed using His tag antibody (lane 1), but no immunoreactivity was observed when vector alone (negative control) was analyzed (lane 2). (E) Immunoreactivity to a 45-kDa band (lane 1) was lost when the anti-Sam1 antibody was preincubated with the antigen (recombinant protein) (lane 2). Molecular size markers (kDa) are shown to the right of panels B and D.

FIG. 4. Expression of P. carinii recombinant protein Sam1. (A) SDS-PAGE analysis of the bacterial cells expressing recombinant protein showed a band of the expected size (~45 kDa, indicated by the arrows), when stained with Coomassie blue (lane 1). When vector with no insert was analyzed, no band of that size was observed (lane 2). (B) Bacterial cells expressing the Sam1 protein showed immunoreactivity with the expected size band when probed in a Western blot with His tag antibody (lane 1), while there was no immunoreactivity when vector alone was analyzed (lane 2). (C) Refolded protein showed a band of ~45 kDa when stained with Coomassie blue (lane 1), while no band of that size was seen when vector alone was analyzed (lane 2). (D) A 45-kDa band showed immunoreactivity when Western blotting analysis shown in panel C was performed using His tag antibody (lane 1), but no immunoreactivity was observed when vector alone (negative control) was analyzed (lane 2). (E) Immunoreactivity to a 45-kDa band (lane 1) was lost when the anti-Sam1 antibody was preincubated with the antigen (recombinant protein) (lane 2). Molecular size markers (kDa) are shown to the right of panels B and D.

FIG. 5. AdoMet synthetase activity. AdoMet synthetase activity of recombinant, refolded P. carinii protein was measured at different time intervals. The figure shows the activity measured in two different experiments and performed in duplicate. Values are means ± standard deviations (error bars) (n = 4). The enzyme activity is expressed as nmol of AdoMet formed/mg protein. The protein preparation obtained by using the vector alone showed no activity.

FIG. 6. Western blotting analysis of immunoprecipitated samples from P. murina. Partially purified P. murina extracts were subjected to immunoprecipitation, using anti-Sam1 antibody (lane 1) or preimmune serum as a negative control (lane 2), followed by Western blotting analysis, using the anti-Sam1 antibody. The former (lane 1) showed a reactive band of 45 kDa (arrow), the size expected for Sam1, while the latter (lane 2) showed no immunoreactivity. The bands corresponding to IgG are marked. When anti-Sam1 antibody that was preincubated with recombinant Sam1 was used for the Western blot, immunoreactivity to the 45-kDa band was blocked (lane 3), demonstrating that the immunoreactivity is specific for Sam1.
Sam1 is enzymatically active, and that immunoreactive Sam1 can be identified in *Pneumocystis* preparations. These data strongly support the conclusion that *Pneumocystis* can synthesize AdoMet de novo and suggest that the organism does not need to rely on exogenous, host-derived AdoMet for survival.

At least two isozymes of AdoMet synthetase encoded by different but closely related genes are present in many eu-karyotes. The sequences are highly conserved among species (15). With *Pneumocystis*, we were able to identify only one gene, *sam1*, for encoding AdoMet synthetase, similar to what has been reported for *S. pombe* (11). *Pneumocystis* AdoMet synthetase showed high homology to that of yeast and less so to those of *E. coli* and mammalian species (11–13, 21, 30, 35, 36). AdoMet synthetase signature motifs and ATP binding sites are highly conserved in all these sequences (28, 34). While the recombinant protein demonstrated AdoMet synthetase activity, the specific activity under nonoptimized conditions, ~0.64 nmol/min/mg protein, was less than that seen with recombinant rat (1.5 to 30 nmol/min/mg protein, depending on the conditions for refolding) or *Leishmania* enzyme (~80 nmol/min/mg protein), though it was similar to that seen with crude extracts obtained from *S. pombe* (~0.05 to 0.55 nmol/min/mg protein, depending on the phase of growth) (11, 18, 29).

It is of interest that Northern blotting analysis identified two transcripts, a major band (~1.3 kb) that corresponded to the expected size of the *sam1* mRNA, and a minor band (~5.6 kb) that is consistent with a bicistronic mRNA containing *sam1* and a putative *chd1* homologue. The presence of polycistronic RNA is very rare in eukaryotes, and the genes derived from the polycistronic RNA are usually functionally related (3). Co-transcription of these genes was seen for both rat and mouse *Pneumocystis*, suggesting that this organization dates to the ancestor of at least these two species. Insufficient RNA was available to examine transcription in *P. jirovecii*.

A probe corresponding to the *chd1* gene recognized only the larger size band; thus, *chd1* appears to be invariably transcribed with *sam1*, while the majority of *sam1* transcripts are monocistronic. It is unknown whether the translation of both proteins occurs from the cotranscribed message and what effect the excision of introns from the intergenic region may have on the synthesis of Sam1. Bicistronic RNA in which *dmc1* and *rad 24* are cotranscribed has been reported in *S. pombe* (10). The functional significance of bicistronic RNA in fungi is currently unknown.

Our findings do not support the conclusion of Merali and Clarkson (24) that *Pneumocystis* does not possess a functional AdoMet synthetase gene. This group reported that *Pneumocystis* cannot synthesize AdoMet and that this pathogen has to depend on its hosts for the supply of this important molecule (26). AdoMet is an important molecule, and all organisms, with the exception of the xD strain of *Amoeba proteus*, are known to synthesize this molecule. It has been reported that
the xD strain of *Amoeba proteus*, which originated following the spontaneous infection of the D strain of *Amoeba proteus* by X-bacteria, must depend on its symbiont X-bacteria for its supply of AdoMet (5). Merali and Clarkson (24) noted the existence of a possible *Pneumocystis*-specific AdoMet synthetase gene in the genome project but proposed that ATP binding sites of *Pneumocystis* AdoMet synthetase might be mutated, resulting in a nonfunctional enzyme; however, our study demonstrates that this is not the case. *Pneumocystis* Sam1 retains all the consensus ATP binding sites (28, 34).

By immunochemical analysis, we were able to show the expression of the Sam1 protein in *P. murina*. The affinity purified antibody raised against a peptide that corresponds to amino acid residues 199 to 219 of purified antibody raised against a peptide that corresponds with the expected size of Sam1. Preincubation of anti-Sam1 mutated, resulting in a nonfunctional enzyme. However, our binding sites of existence of a possible interaction of the antibody with the antigen (recombinant monoclonal antibody (1, 23). While there was some nonspecific activity seen with the anti-Sam1 antibody, preabsorption of the antibody with the antigen (recombinant *P. murina* Sam1) blocked the immunoreactivity, supporting its specificity for Sam1.

In summary, we have characterized the sam1 gene from *Pneumocystis*, which is transcribed into an ~1.3-kb mRNA. The recombinant protein expressed in *E. coli* showed functional enzyme activity. Our study clearly shows that *Pneumocystis* has a sam1 gene that can encode a functional AdoMet synthetase. We were able to detect the expression of Sam1 protein in *P. murina* by immunoprecipitation and confocal immunofluorescence analyses. Thus, *Pneumocystis*, like other organisms, could synthesize its own AdoMet and does not need to depend on its hosts for the supply of this important molecule, as reported by Merali and Clarkson (24).

ACKNOWLEDGMENTS

We thank Rene Costello and Howard Mostowski for their assistance with the animal studies. This research was supported by the Intramural Research Program of the NIH Clinical Center and the National Institute of Allergy and Infectious Diseases.

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