Curr Genet (2000) 37: 200–208

ORIGINAL PAPER

N. B. Averbeck · O. N. Jensen · M. Mann
H. Schägger · H. D. Osiewacz

Identification and characterization of PaMTH1, a putative *O*-methyltransferase accumulating during senescence of *Podospora anserina* cultures

Received: 17 September / 1 December 1999

Abstract A differential protein display screen resulted in the identification of a 27-kDa protein which strongly accumulates during the senescence of *Podospora anserina* cultures grown under standard conditions. After partial determination of the amino-acid sequence by mass-spectrometry analysis of trypsin-generated fragments, pairs of degenerated primers were deduced and used to amplify parts of the sequence coding for the protein. These PCR products were utilized to select specific cDNA and genomic clones from DNA libraries of *P. anserina*. A subsequent DNA-sequence analysis revealed that the 27-kDa protein is encoded by a discontinuous gene, *PaMth1*, capable of coding for 240 amino acids. The first three amino-terminal residues appear to be removed post-translationally. The deduced amino-acid sequence shows significant homology to S-adenosylmethionine (SAM)-dependent methyltransferases. We hypothesize that the 27-kDa protein, PaMTH1, is involved in age-related methylation reactions protecting aging cultures against increasing oxidative stress.

Key words *Podospora anserina* · Age dependent · Copper · Methyltransferase

Communicated by K. Esser

N. B. Averbeck · H. D. Osiewacz (✉)
Molekulare Entwicklungsbiologie und Biotechnologie,
Johann Wolfgang Goethe-Universität,
Botanisches Institut, Marie-Curie-Strasse 9,
60439 Frankfurt am Main, Germany
e-mail: Osiewacz@em.uni-frankfurt.de;
Fax: +49-69-798 29363

O. N. Jensen · M. Mann
Department of Molecular Biology,
Odense University, Campusvej 55,
DK-5230 Odense M, Denmark

H. Schägger
Zentrum für Biologische Chemie,
Universitätsklinikum Frankfurt,
D-60590 Frankfurt am Main, Germany

Introduction

The enzymatic methylation of a diverse range of compounds, such as proteins, nucleic acids, polysaccharides, lipids and small molecules, is a ubiquitous process occurring in both prokaryotic as well as eukaryotic organisms (Ibrahim 1997; Ibrahim et al. 1998). Methylation is involved in various biosynthetic pathways (e.g., phenylpropanoid metabolism of plants), in the inactivation of active molecules such as the neurotransmitters adrenaline and noradrenaline (Axelrod and Tomchick 1958; Rivett et al. 1982), and in other regulatory processes. Among these, the methylation of DNA plays an important role. In plants, some filamentous fungi, and probably in mammals, DNA methylation is related to the inactivation of duplicated sequences and appears to represent a defense mechanism against the "unwanted" spread of "selfish" DNA species like transposable elements (Singer and Selker 1990; Meyer 1996; Selker 1999). In addition, DNA methylation is involved in the control of gene expression during differentiation and development (for review see Russo et al. 1996). Finally, there is some evidence that methylation of DNA and other substrates also plays a role during the aging of biological systems (Barber and Clarke 1983; Galletti et al. 1983; Wilson and Jones 1983; Fairweather et al. 1987).

The ascomycete *Podospora anserina* is a filamentous fungus employed as a model system in experimental aging research. In contrast to many other fungi, the life-span of *P. anserina* cultures is limited (Rizet 1953) and is controlled by environmental factors as well as genetic traits (for detailed reviews see Esser 1985; Osiewacz 1997; Osiewacz and Kimpel 1999). During the course of investigations to elucidate the molecular mechanisms involved in life-span control in *P. anserina* we performed a differential protein display of cultures of different age. Here we report the identification and characterization of a protein, named PaMTH1, which accumulates during the aging of *P. anserina* cultures. This protein displays

strong homology to the S-adenosylmethionine (SAM)-dependent methyltransferases (Kagan and Clarke 1994), a group of methyltransferases catalyzing the transfer of methyl groups from SAM to various types of substrates. We suggest that PaMTH1 is a component of the molecular machinery protecting aging cultures of *P. anserina* against oxidative stress.

Materials and methods

Strains and plasmids

P. anserina wild-type strain s and the long-lived nuclear mutant grisea were used in this study. For details of strain origin see Esser (1974) and Prillinger and Esser (1977). In order to investigate strains of defined age, juvenile cultures were generated by crossing strains and selecting mononuclear ascospores. For both strains, the wild-type and the long-lived mutant, two independent cultures were selected and analyzed. These isolates were named s1 and s2 for wild-type s, and g1 and g2 for the long-lived mutant grisea, respectively. The mating-type of isolates s1, s2, and g1 is minus. The mating-type of g2 is plus. The life-span of these isolates corresponds well to those of the corresponding strains grown under the conditions reported in Osiewacz and Nuber (1996). Strains of a specific age were isolated and used for protein extraction. Juvenile cultures were grown for 6 days after the germination of a single ascospore. Middle-aged cultures were defined as cultures grown for a period corresponding to half of the time the cultures grew until reaching the senescent stage. Senescent cultures are characterized by a growth arrest (Table 1).

The plasmids pBCMth1 and pADMth1 were constructed by cloning the degenerated B/C-primer- and A/D-primer-PCR product into pUC18 (for the construction of the degenerated primer-PCR products see section "PCR and degenerated primers"). Subcloning of a 3.6-kb *Bam*HI and 3.8-kb *Xho*I fragment derived from a genomic cosmid clone of *P. anserina* hybridizing to the degenerated A/D primer resulted in the construction of pMth1-1 and pMth1-2. The cloning vector was pBSSK$^+$. The insert sequences of pMth1-1 and pMth1-2 are overlapping. Hybrid plasmid pMth1-1 contains the 5′ part of *PaMth1*; pMth1-2 the 3′ part. Two cDNA clones were selected which either contain the complete reading frame of *PaMth1* (pcMth1-1) or only the 3′ part of it (pcMth1-2).

Media and culture conditions

Mycelia of germinated spores were grown under standard growth conditions (Esser 1974) with or without the addition of 250 μmol/l of $CuSO_4$ to the medium. For protein isolation, mycelia were first cultured for 2 days on a piece of sterile cellophane covering the surface of an agar plate. The medium was either BMM (Esser 1974) without additional copper salts, or BMM containing 250 μmol/l of $CuSO_4$. Subsequently, the mycelium was transferred into liquid CM (complete medium) with or without additional copper (250 μM of $CuSO_4$). CM was prepared according to Hermanns and Osiewacz (1992) with the following modifications: instead of 1 mg/l of $CuSO_4$, 1 mg/l of Zn SO_4 was used and Mn $SO_4 \times 7\ H_2O$ was replaced by 1 mg/l of $MnCl_2 \times 2\ H_2O$. Cultures were grown for 2 days in CM on a rotary shaker (125 rpm) at 27 °C in permanent light.

DNA isolation

The DNA isolation of *P. anserina* was performed as described in Lecellier and Silar (1994). Plasmid and cosmid DNA was isolated using a commercial plasmid-isolation kit (Qiagen).

cDNA Synthesis

cDNA was synthesized from total *P. anserina* RNA using the *Superscript Preamplification System for First Strand cDNA Synthesis* (Gibco BRL).

PCR with degenerated primers

Degenerated primer sequences were deduced from the oligopeptide sequences generated by nanoelectrospray tandem mass spectrometry: WML/IFTA and PFNEETA. For the construction of the DNA sequences corresponding to these oligopeptides we had to take into account that this method of protein sequencing cannot distinguish between isoleucine and leucine. In addition, since the relative orientation of the oligopeptides in the protein was unknown, both possibilities were considered for the sequences of primer A, primer B, primer C and primer D, as well as their orientation, see Table 2 and Fig. 2a, respectively. The PCR-amplifications were carried out with 100 ng of cDNA from *P. anserina* or 70 ng of genomic *P. anserina* DNA as a template and 12 pmol/μl of each degenerated primer. The reactions contained 20 μM of dNTPs, 1.5 mM $MgCl_2$ and 0.05 U of *Taq* polymerase (Gibco BRL). The PCR-amplification was performed under the following conditions: denaturation for 1 min at 94 °C and 35 amplification cycles (94 °C for 1 min, 50 °C for 1 min, 72 °C for 2 min) followed by 9 min at 72 °C. The annealing temperature of 50 °C was defined by the method of Wu et al. (1991).

Table 1 Age (in days, d) of the examined *P. anserina* wild-type s and mutant grisea isolates cultivated on standard solid medium BMM without and with copper addition (250 μM $CuSO_4$), respectively

Isolate	s1	s1 + $CuSO_4$	s2	s2 + $CuSO_4$	g1	g1 + $CuSO_4$	g2	g2 + $CuSO_4$
Juvenile	6 d	6 d	6 d	6 d	6 d	6 d	6 d	6 d
Middle-aged	17 d	25 d	24 d	19 d	36 d	19 d	32 d	31 d
Senescent	35 d	51 d	47 d	37 d	71 d	37 d	63 d	61 d

Table 2 Peptide sequences derived from nanoelectrospray tandem mass-spectroscopy of trypsin-generated fragments of the 27-kDa protein and the deduced degenerated primers of two polypeptides

Oligopeptide	Sequence	Deduced, degenerated primers
1	L/IQGSWML/IFTAR	A: TGGATGHTNTTYACNGCN B: NGCNGTRAANADCATCCA
2	L/IPFNEETADR	C: CNTTYAAYGARGARACNGCNGAY D: RTCNGCNGTYTCYTCRTTRAANGG
3	L/IL/IEGPAENTL/IK	
4	FSAGL/IMEDPR	
5	AEL/IVTL/IEYSPK	

Gene bank screening

For gene-bank screening we used a genomic cosmid library (Osiewacz 1994) and a pUC18 cDNA library of *P. anserina* wild-type s. Both gene banks were constructed using RNA isolated from strains grown under standard growth conditions without the addition of copper. The screening of these libraries was performed using the cloned Digoxigenin (DIG)-labelled PCR products. The primer B/C-PCR-product sequence was DIG-labelled by the Feinberg-Vogelstein technique. The primer A/D-PCR-product was DIG-labelled using oligonucleotides A and D as PCR primers. Labelling, hybridization, and detection of fragments was carried out as described by van Miltenberg et al. (1997).

DNA sequencing

Both strands of the cDNA clone and the genomic gene were sequenced. DNA sequencing was performed using the *T7 polymerase sequencing kit* from Pharmacia according to the dideoxy chain-termination technique (Sanger et al. 1979).

Protein extraction and protein sequencing

The extraction of proteins from *P. anserina* was performed according to Brakhage et al. (1992). The extracts were stored at −80 °C. Protein extraction from polyacrylamide gels (PAA), trypsin digestion and peptide sequencing by nanoelectrospray tandem mass spectrometry was performed as described in Shevchenko et al. (1996). Protein extraction for Edman degradation was performed according to Schägger (1994). For Edman degradation a protein sequencer (473 , Applied Biosystems) was used.

Protein gel-electrophoresis/differential protein-display analysis

Approximately 15 µg of *P. anserina* protein were separated in 12% denaturing PAA gels (Laemmli 1970). The proteins were stained with Coomassie brilliant blue G250 (Sambrook et al. 1989).

Homology search

The homology search was performed using the FASTA algorithm (Pearson and Lipman 1988) and the protein entries in the SWISSPROT data base. For multiple sequence alignment the CLUSTAL W algorithm was used the De Cypher II Similarity Search System by Time Ligic, Inc.; (Thompson et al. 1994).

Accession number

The genomic and the cDNA sequence of *PaMth1* will appear in the EMBL database under the accession number AJ245505.

Results

A differential protein-display analysis revealed a 27-kDa protein accumulating during the senescence of *P. anserina* cultures

Earlier investigations revealed that life-span extension in the mutant grisea of *P. anserina* is due to a defect in copper-uptake, as the result of a loss-of-function mutation in a gene coding for the copper-dependent transcription factor GRISEA (Marbach et al. 1994; Osiewacz and Nuber 1996; Borghouts et al. 1997; Borghouts and Osiewacz 1998). As a consequence, cellular copper levels are low, most likely resulting in a reduced generation of reactive oxygen species (ROS) and a reduction of molecular damage. Since GRISEA is a transcription factor, it is possible that the expression of multiple genes is affected in the mutant. This may be visible on protein gels. Consequently, we compared the protein profiles of the wild-type and the mutant obtained after separation of proteins on SDS-PAGE. In these initial experiments, a protein of about 27-kDa was identified in the wild-type strain that was missing – or present only in very low amounts – in the mutant, suggesting that the gene encoding this protein may be a target gene of the transcription factor GRISEA. However, in subsequent experiments this conclusion could not be verified since the protein was also found in cultures of the mutant grisea. We therefore performed a more detailed analysis using cultures of different age for both strains.

In a first series of experiments, cultures were grown in standard medium. In both strains, a protein of 27-kDa was found to accumulate strongly in cultures of older age (Fig. 1 a, b, panels 2, 4, 8, 10, 14, 16). While the protein was either completely absent or only present in very low amounts in juvenile and middle-aged strains of the wild-type, it was found in high abundance in senescent cultures of the two isolates s1 (mating-type minus) and s2 (mating-type plus) grown for 35 days and 47 days, respectively. The rather strong difference in the life-span of these two isolates is partly due to the fact that they differ in the mating-type which is known to affect life-span. In the two grisea isolates, which were both mating-type minus, high amounts of the same 27-kDa protein were found in cultures grown for about the same time (36 days and 32 days) as wild-type isolate s1. However, since grisea is a mutant with an increased life-span this stage corresponds to the middle of the life-span (middle-aged cultures) and not to the senescent stage. These data indicate that, under standard growth conditions, the accumulation of the 27-kDa protein is independent of the relative life-stage of the culture – juvenile, middle-aged, and senescent – but rather depends on the absolute time of cultivation.

Since grisea is a copper-uptake mutant, we asked the question whether or not a difference in the amount of the 27-kDa protein also occurs in middle-aged and senescent strains grown in medium containing additional copper. In these experiments, the two grisea isolates were permanently grown on medium supplemented with 250 µmol/l of $CuSO_4$ (permanent copper stress). Under these conditions, the life-span, the growth rate, and the color of the mycelium of the mutant revert to wild-type. However, the addition of copper did not lead to the same age-dependent protein pattern as in wild-type strains grown in standard medium without copper-supplementation (Fig. 1b, panels 6, 7, 12, 13, 18, 19). Interestingly, in copper-supplemented medium the 27-kDa protein does not accumulate significantly in cultures of any age.

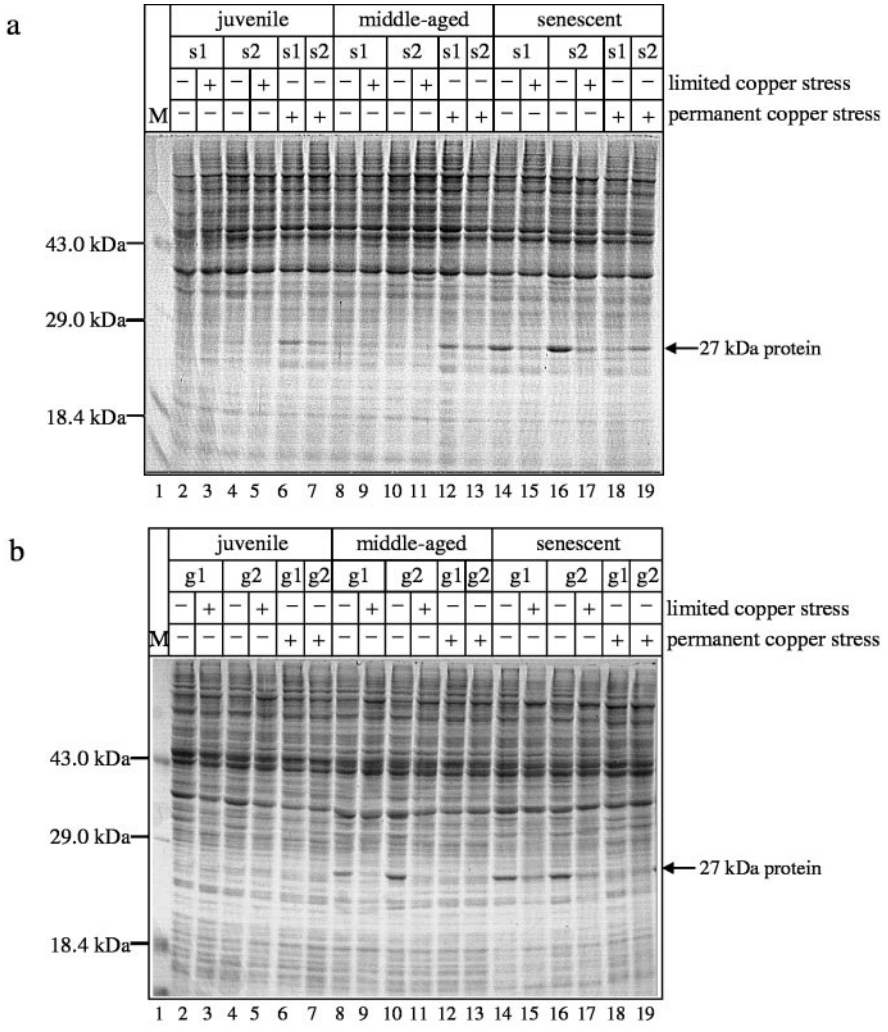

Fig. 1 SDS-polyacrylamide gel electrophoresis of protein extracts from *P. anserina* (**a**) wild-type strain s (isolates s1 and s2) and (**b**) long-lived mutant grisea (isolates g1 and g2). Approximately 15 μg of protein from each strain were separated on a 12% PAA gel. Staining was performed with Coomassie brilliant blue. Total protein was isolated from strains of different age: juvenile, middle-aged, senescent. Cultures were grown either under standard growth conditions (no copper stress), under limited copper stress (growth for 4 days on medium containing 250 μmol/l of $CuSO_4$) or under permanent copper stress (permanently grown on 250 μmol/l of $CuSO_4$-supplemented medium). The position of a 27-kDa protein accumulating during aging of the wild-type and long-lived mutant grisea is indicated by an *arrow*. *M* molecular-weight marker

In order to determine whether high copper concentrations also affect the age-related accumulation of the 27-kDa protein in the wild-type, we grew the two wild-type isolates under permanent copper stress. As may be seen from Fig. 1a (panels 6, 7, 12, 13, 18 and 19) no significant difference in the amount of the 27-kDa protein occurs in cultures of different age under these stress conditions. However, in contrast to the mutant grisea, it appears that the amount of the protein grown under permanent copper stress is slightly increased when compared to the cultures grown in medium without additional copper. These differences may be explained by the fact that, although identical copper concentrations were used in the growth medium, the final cellular copper concentrations are not identical in the two strains due to differences in the copper-uptake machinery.

Finally, we asked whether the copper concentration in the solid medium on which the cultures were grown until they reached the appropriate relative life-stage (juvenile, middle-aged, senescent) influences the age-related accumulation of the 27-kDa protein. To address this question, the wild-type and the mutant were first cultivated on solid medium not supplemented with additional copper. After the cultures reached a specific age, mycelium was transferred to medium containing 250 μmol/l $CuSO_4$ and inoculated for 4 days (limited copper stress). As may be seen from Fig. 1 (a, b, panels 3, 5, 9, 11, 15, 17) only a very slight increase if at all, in the 27-kDa protein level was observed. Interestingly, when compared to senescent strains of the wild-type and to middle-aged and senescent strains of the mutant grown in standard medium, a strong decrease in the amount of the 27-kDa protein was observed.

Taken together, the results clearly demonstrate that the 27-kDa protein identified in this study accumulates in both the wild-type and the long-lived mutant grisea. This accumulation is time-dependent but not dependent on the relative life-stage. In addition, it is affected by copper in the growth medium. Moreover, the results clearly demonstrate that the gene coding for the 27-kDa protein is not a target gene of the transcription factor GRISEA, since it is also expressed in the long-lived mutant which does not contain this regulatory factor.

Cloning and characterization of *PaMth1*, the gene encoding the 27-kDa protein of *P. anserina*

In order to elucidate the nature of the 27-kDa protein, we set out to clone the gene coding for this protein via reverse genetics. First, partial protein sequences were generated by nanoelectrospray tandem mass-spectrometry of the trypsin-digested 27-kDa protein isolated from PAA gels (Shevchenko et al. 1996). Five oligopeptide sequences were obtained. From two of these sequences degenerated oligonucleotide primers were designed (Table 2). Since the relative position of the polypeptide fragments determined by mass-spectrometry was not clear, we had to design two pairs of primers, namely primers A and B for WML/IFTA and primers C and D for PFNEETA (Table 2 and Fig. 2a). In addition, we had to take into account that the mass-spectral determination of the protein sequence cannot distinguish between leucine and isoleucine.

In subsequent PCR experiments, both pairs of primers (primer A and C, primer B and D) led to a strong amplification of one PCR product each (Fig. 2b). These amplification products were not obtained when only one oligonucleotide was used in control PCR experiments (Fig. 2b).

Subsequently, the two amplification products of 120 bp and 600 bp in size were used as probes to screen a cDNA library of the *P. anserina* wild-type strain. In these experiments, only the 120-bp probe gave rise to the selection of two positive clones. No positives were obtained in screens using the 600-bp probe. The insert sequences of the two selected cDNA clones, termed pcMth1-1 and pcMth1-2, were determined and analyzed. The insert of pcMth1-1 was found to code for all five polypeptides sequenced by nanoelectrospray tandem mass-spectrometry (Fig. 3), as well as for 14 peptides detected by matrix-assisted laser desorption/ionization mass-spectrometry (data not shown). The insert of pcMth1-2 was partial and contained only the sequence encoding four of the five known polypeptides (Fig. 3).

Using the same probe that led to the selection of the positive cDNA clones, we screened a genomic cosmid library of the wild-type strain s of *P. anserina*. Subsequently, two overlapping DNA fragments were isolated and subcloned from the insert of a positive cosmid clone which strongly hybridized to the selected cDNA. Both strands of these genomic inserts were sequenced and analyzed. The results of this analysis and of the sequence analysis of the cDNA clones are summarized in Fig. 3. An open reading frame of 723 bp was identified capable of coding for a protein of 240 amino acids. This reading frame is interrupted by two short introns of 57 bp and 50 bp, respectively. The translational start was verified by sequencing the amino-terminus of the 27-kDa protein by Edman degradation, resulting in the amino-acid sequence: SILPFNEETADRVSAYXEKNXHGI. This sequence is in good agreement with the sequence deduced from the nucleotide sequence and with the data obtained from mass-spectrometry (Fig. 3). However, the first three amino acids are missing, suggesting that the protein is post-translationally processed at the amino-terminus. The molecular weight of 27 kDa estimated from SDS PAGE corresponds very well to the molecular weight of the mature protein of 237 amino acids.

In order to elucidate the function of the 27-kDa protein, we compared the deduced amino acid sequence

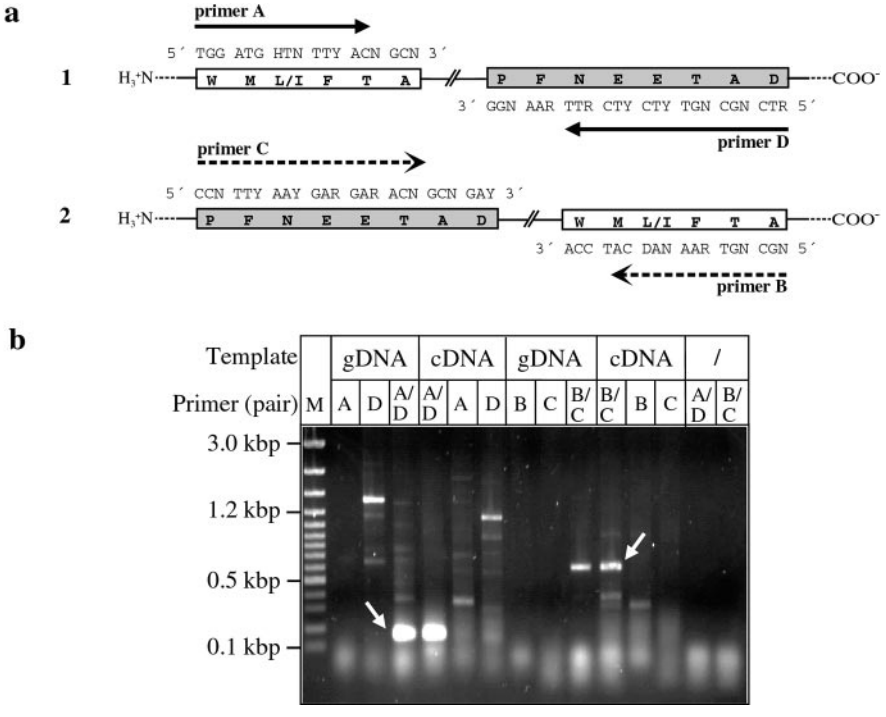

Fig. 2a, b PCR-amplification of DNA fragments using degenerated primers deduced from two polypeptides determined by mass-spectrometric analysis of fragments of the 27-kDa protein of *P. anserina*. **a** Relative orientation (*1, 2*) of the two polypeptides in the 27-kDa protein. The sequences of the oligopeptides and the deduced primers are indicated. L/I indicates an amino acid that either is a leucine or an isoleucine. **b** PCR products obtained from PCR-amplification of genomic (*gDNA*) or cDNA from *P. anserina*. The PCR products were separated on a 1.3% agarose gel and stained with ethidium bromide. *M* DNA ladder

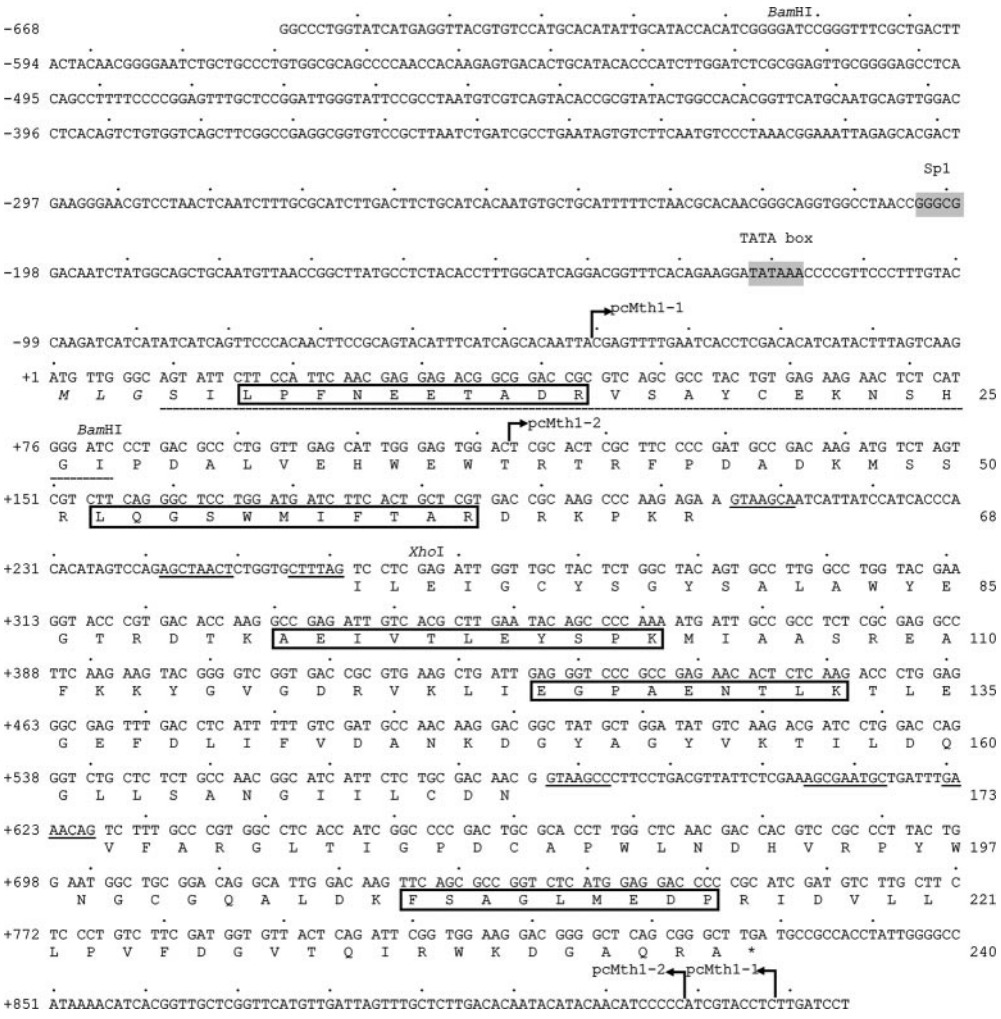

Fig. 3 Nucleotide sequence of *PaMth1* and the deduced amino acid sequence encoded by this gene. The *asterisks* mark a stop codon. *Boxed regions* in the amino-acid sequence correspond to the five sequence fragments identified by mass-spectrometry. Amino acids written in *italics* are processed from the mature protein. The amino acid sequence determined by Edman degradation is indicated by a *dashed line*. Fungal consensus sequences at the intron-exon boundaries and the internal lariat formation consensus sequences are *underlined* in the intron sequences (Ballance 1986). Upstream of the open reading frame a putative TATA box and a putative SP1-binding site are indicated by *gray boxes*. Sequences of the cDNA clones pcMth1-1 and pcMth1-2 (except for the intron sequences) are delimited by *arrows*

with the entries in the SWISSPROT database. This analysis revealed that the *P. anserina* sequence displays significant homology to various methyltransferases. The highest degree of homology was found to a caffeoyl-CoA *O*-methyltransferase (CCoAMT) of the higher plant *Vitis vinifera* (Busam et al. 1997) and to the catechol *O*-methyltransferase (COMT) of *Rattus norvegicus* (Tenhunen and Ulmanen 1993; Tenhunen et al. 1993). These results strongly suggest that the 27-kDa protein of *P. anserina* also encodes an *O*-methyltransferase (Fig. 4). Accordingly, the *P. anserina* protein was named PaMTH1 and the corresponding gene *PaMth1*. A Southern-blot analysis revealed that this gene is present as a single-copy in the genome of *P. anserina* (data not shown).

Discussion

Here we report the identification and characterization of a novel protein of *P. anserina*, a putative SAM-dependent *O*-methyltransferase. The amino acid sequence of this protein contains six sequence stretches (Fig. 4, 1–6) with a high degree of identity to the corresponding stretches in CCoAMT from *V. vinifera* (Busam et al. 1997) and of COMT from *R. norvegicus* (Tenhunen and Ulmanen 1993; Tenhunen et al. 1993). The six stretches contain all three sequence motifs (I, II, III) which previously were suggested to be crucial for the methylation function of SAM-dependent *O*-methyltransferases (Ingrosso et al. 1989; Kagan and Clarke 1994). The highest degree of identity is found to motif I, a motif conserved in all methyltransferases described until now (Haydock et al. 1991; Kagan and Clarke 1994). In addition, PaMTH1 displays significant homology to motifs B, C, F and H, four out of eight motifs of functional significance in plant CCoAMTs (Joshi and Chiang 1998). Finally, PaMTH1 contains several of the amino acids which, by X-ray diffraction analysis, were shown to be relevant for the binding of different compounds by COMT (Vidgren et al. 1994). These are four out of eight SAM-binding amino acids (Fig. 4 a), all three amino acids involved in Mg^{2+}-binding (m), and two out of four substrate-binding amino acids (s). Taken together, these data strongly suggest that PaMTH1 is a SAM-dependent *O*-methyltransferase which probably catalyzes the methylation of a hydroxyl group of catechol derivatives. However, at this point we do not know the

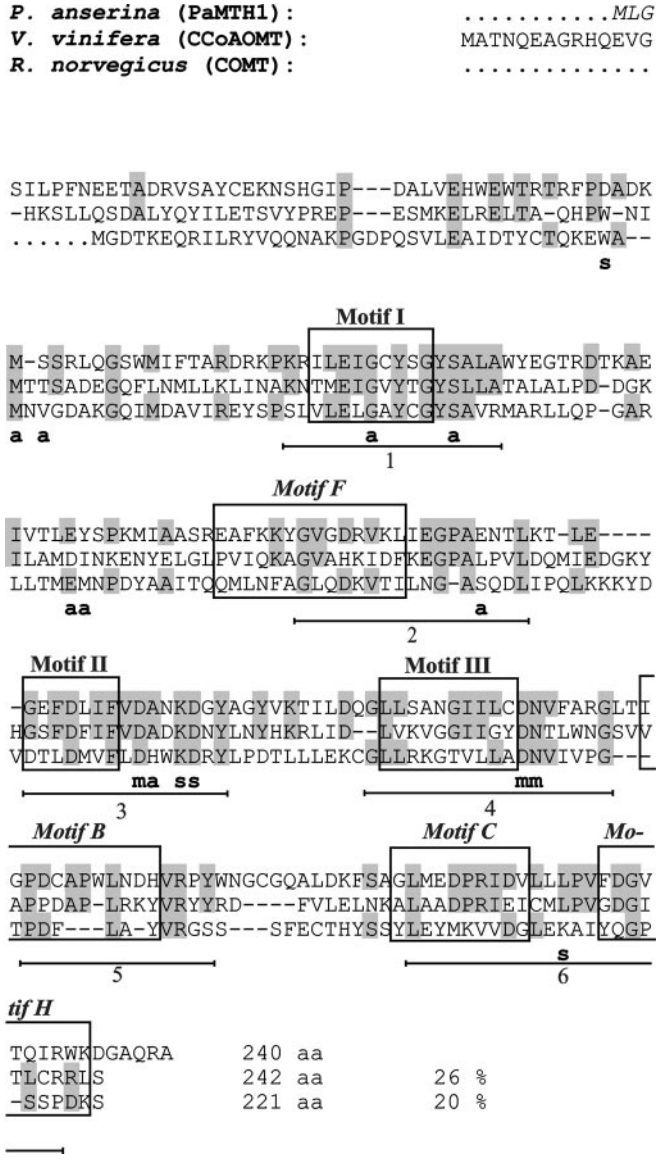

Fig. 4 Alignment of the amino acid sequences of PaMTH1 from *P. anserina* and methyltransferases from *V. vinifera* (caffeoyl-CoA-3-*O*-methyltransferase, CCoAMT) (Busam et al. 1995) and *R. norvegicus* (catechol *O*-methyltransferase, COMT) (Tenhunen and Ulmanen 1993; Tenhunen et al. 1993). The analysis was performed using the multiple alignment program CLUSTAL W (Thompson et al. 1994). Amino acids which are identical to amino acids of PaMTH1 are marked by *gray boxes*. Six sequence stretches strongly conserved in the compared proteins are indicated (1–6). The motifs I, II, and III (Kagan and Clarke 1994) and the motifs B, C, F, and H (Joshi and Chiang 1998) are indicated by *boxes*. At the end of each protein sequence the amino acid (*aa*) number and percentage of identity in comparison to PaMTH1 are indicated. Amino acids of COMT involved in SAM binding (*a*), binding of the substrate (*s*), and binding of Mg^{2+} (m) are also indicated

definitive substrate(s). In *P. anserina* neither a phenylpropane metabolism, in which caffeoyl-CoA is a reactive intermediate, is known, nor are compounds like adrenaline or noradrenaline. However, the catechol derivative L-DOPA (3,4-dihydroxyphenylalanine), from which these neurotransmitters and hormones are derived, is an intermediate in the synthesis of melanin, a pigment produced by *P. anserina* cultures.

Although the sequence conservation of PaMTH1 is not as high as that of CCoAMT and COMT, two other types of methyltransferases are noteworthy for mention at this point. One group of enzymes are the cytosine-DNA methyltransferases which are involved in DNA methylation. Previously it was shown that DNA methylation is reduced during the aging of mammalian cell cultures (Wilson and Jones 1983). In addition and most significantly, the experimental reduction of DNA methylation by 5-azacytidine shortens the life-span of cultured mammalian cells (Fairweather et al. 1987). The reduction of DNA methylation may cause uncontrolled activation of genes resulting in aging and death.

A second group of age-relevant methyltransferases are the SAM-depending L-isoaspartyl (D-aspartyl)-*O*-methyltransferases (Kim et al. 1997; Barber and Clarke 1983; Galletti et al. 1983; Visick et al. 1998). These enzymes are involved in protein repair proceeding via the methylation of D-aspartyl and L-isoaspartyl residues (Johnson et al. 1987; McFadden and Clarke 1987; Brennan et al 1994; Kim et al. 1997). Significantly, this type of methylation has been reported to increase during the aging of mammalian cells (Barber and Clarke 1983; Galletti et al. 1983).

From the available data it is tempting to speculate about the function of PaMTH1 in *P. anserina*. We hypothesize that this protein is involved in the defense of reactive oxygen species (ROS) which leads to damage of the different cellular components and consequently to dysfunction and death. PaMTH1 may catalyze the methylation reactions of hydroxyl groups of phenolic compounds. Specifically, these reactions may lead to the modification of reactive hydroxyl groups which, if not modified, may be converted to a free radical and participate in radical-chain reactions leading to the formation of increased levels of ROS. We suggest that *PaMth1* is constitutively expressed in both, the wild-type and the long-lived mutant grisea. As demonstrated in this study in both strains under standard growth conditions, protein levels accumulate after approximately the same absolute growth period. In the wild-type this corresponds to the senescent stage, in the mutant to the middle-aged phase. Since ROS generation is reduced in the mutant grisea, less substrate molecules of PaMTH1 become damaged in the mutant than in the wild-type prior to the protective methylation catalyzed by PaMTH1. Consequently, within the same growth period, cultures of the mutant accumulate lower levels of damaged molecules than the wild-type, resulting in an increased life-span.

Clearly, at this time there is uncertainty and many unanswered questions about the definitive significance of PaMTH1 identified in this study. However, it will be of interest for future investigations to experimentally address the hypothesis introduced above. The analysis of the age-dependent expression of *PaMth1* at the RNA level and the construction of *PaMth1* knock-out strains and of strains over-expressing *PaMth1*, followed by the analysis

of the corresponding transgenic strains, will certainly help to answer some of the relevant questions. Moreover, apart from providing specific clues about the role of PaMTH1 in the aging of *P. anserina* this type of experiment can be expected to provide additional data about the significance of methyltransferase for aging in general.

Acknowledgements We thank A. Werner for excellent technical assistance. This work was supported by the Deutsche Forschungsgemeinschaft (Bonn Bad-Godesberg, Germany) by a grant to H.D.O.

References

Axelrod J, Tomchick R (1958) Enzymatic *O*-methylation of epinephrine and other catechols. J Biol Chem 233: 702–705

Barber J, Clarke S (1983) Membrane protein carboxyl methylation increases with human erythrocyte age. Evidence for an increase in the number of methylatable sites. J Biol Chem 258: 1189–1196

Ballance DJ (1986) Sequences important for gene expression in filamentous fungi. Yeast 2: 229–236

Borghouts C, Osiewacz HD (1998) GRISEA, a copper-modulated transcription factor from *Podospora anserina* involved in senescence and morphogenesis, is an ortholog of MAC1 in *Saccharomyces cerevisiae*. Mol Gen Genet 260: 492–502

Borghouts C, Kimpel E, Osiewacz HD (1997) Mitochondrial DNA rearrangements of *Podospora anserina* are under the control of the nuclear gene *grisea*. Proc Natl Acad Sci USA 94: 10768–10773

Brakhage A, Browne P, Turner G (1992) Regulation of *Aspergillus nidulans* penicillin biosynthesis and penicillin biosynthesis genes *acvA* and *ipnA* by glucose. J Bacteriol 174: 3789–3799

Brennan T, Anderson J, Jai Z, Waygood E, Clarke S (1994) Repair of spontaneously deamidated Hpr phosphocarrier protein catalyzed by the L-isoaspartate-(D-aspartate) *O*-methyltransferase. J Biol Chem 269: 24586–24595

Busam G, Junghans KT, Kneusel RE, Kassemeyer H-H, Matern U (1997) Characterization and expression of caffeoyl-coenzyme A 3-*O*-methyltransferase proposed for the induced resistance response of *Vitis vinifera* L. Plant Physiol 115: 1039–1048

Esser K (1974) *Podospora anserina*. In: King RC (ed) Handbook of genetics, vol I. Plenum Press, New York, pp 531–551

Esser K (1985) Genetic control of aging. The mobile intron model. In: Bergener M, Ermini M, Stähelin HB (eds) The 1984 Sandoz Lectures in Gerontology, Thresholds in Aging. Academic Press, London, pp 3–20

Fairweather DS, Fox M, Margison GP (1987) The in vitro lifespan of MRC-5 cells is shortened by 5-azacytidine-induced demethylation. Exp Cell Res 168: 153–159

Galletti P, Ingrosso D, Nappi A, Gragnaniello V, Iolascon A, Pinto L (1983) Increased methyl esterification of membrane proteins in aged red-blood cells. Eur J Biochem 135: 25–31

Haydock S, Dowson JA, Dhillon N, Roberts G, Cortes J, Leadlay PF (1991) Cloning and sequence analysis of genes involved in erythromycin biosynthesis in *Saccharopolyspora erythraea*: sequence similarities between EryG and a family of S-adenosylmethionine-dependent methyltransferases. Mol Gen Genet 230: 120–128

Hermanns J, Osiewacz HD (1992) The linear mitochondrial plasmid pAL2-1 of a long-lived *Podospora anserina* mutant is an invertron encoding a DNA and RNA polymerase. Curr Genet 22: 491–500

Ibrahim RK (1997) Plant *O*-methyltransferase signatures. Trends Plant Sci 2: 249–250

Ibrahim RK, Bruneau A, Bantignies B (1998) Plant *O*-methyltransferases: molecular analysis, common signature and classification. Plant Mol Biol 36: 1–10

Ingrosso D, Fowler AV, Bleibaum J, Clarke S (1989) Sequence of the D-aspartyl/L-isoaspartyl protein methyltransferase from human erythrocytes. J Biol Chem 264: 20131–20139

Johnson B, Langmack E, Aswad D (1987) Partial repair of deamidation-damaged calmodulin by protein carboxyl methyltransferase. J Biol Chem 262: 12283–1287

Joshi CP, Chiang VL (1998) Conserved sequence motifs in plant *S*-adenosyl-L-methionine-dependent methyltransferases. Plant Mol Biol 37: 663–674

Kagan RM, Clarke S (1994) Widespread occurrence of three sequence motifs in diverse S-adenosylmethionine-dependent methyltransferases suggests a common structure for these enzymes. Arch Biochem Biophys 310: 417–427

Kim E, Lowenson JD, MacLaren DC, Clarke S (1997) Deficiency of a protein-repair enzyme results in the accumulation of altered proteins, retardation of growth, and fatal seizures in mice. Proc Natl Acad Sci USA 94: 6132–6137

Laemmli U (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685

Lecellier G, Silar P (1994) Rapid methods for nucleic acid extraction from Petri-dish grown mycelia. Curr Genet 25: 122–123

Marbach K, Fernandez-Larrea J, Stahl U (1994) Reversion of a long-living, undifferentiated mutant of *Podospora anserina* by copper. Curr Genet 26: 184–186

McFadden PN, Clarke S (1987) Conversion of isoaspartyl peptides to normal peptides: implications for the cellular repair of damaged proteins. Proc Natl Acad Sci USA 84: 2595–2599

Meyer P (1996) Repeat-induced gene silencing: common mechanisms in plants and fungi. Biol Chem Hoppe-Seyler 377: 87–95

Miltenburg R van, Rüger B, Grünewald-Janho S, Leons M, Schröder C (eds) (1995) The DIG system user's guide for filter hybridization. Boehringer Mannheim GmbH, Mannheim, Germany

Osiewacz HD (1994) A versatile shuttle cosmid vector for the efficient construction of genomic libraries and for the cloning of fungal genes. Curr Genet 26: 87–90

Osiewacz HD (1997) Genetic regulation of aging. J Mol Med 75: 715–727

Osiewacz HD, Kimpel E (1999) Mitochondrial-nuclear interactions and lifespan control in fungi. Exp Gerontol 34: 901–909

Osiewacz HD, Nuber U (1996) GRISEA, a putative copper-activated transcription factor from *Podospora anserina* involved in differentiation and senescence. Mol Gen Genet 252: 115–124

Pearson WR, Lipman DJ (1988) Improved tools for biological sequence comparison. Proc Natl Acad Sci USA 85: 2444–2448

Prillinger H, Esser K (1977) The phenoloxidases of the ascomycete *Podospora anserina*. Mol Gen Genet 156: 333–345

Rivett AJ, Eddy BJ, Roth JA (1982) Contribution of sulfate conjugation, deamination, and *O*-methylation to metabolism of dopamine and norepinephrine in human brain. J Neurochem 39: 1009–1016

Rizet G (1953) Sur l' impossibilité d' obtenir la multiplication végetative in interompue et illimité de l'ascomycete *Podospora anserina*. Comp Rend de l'Acad Sci Paris 237: 838–855

Russo VEA, Martienssen RA, Riggs AD (1996) Epigenetic mechanisms of gene regulation. Cold Spring Harbor Laboratory Cold Spring Harbor, New York

Sambrook J, Fritsch E, Maniatis T (1989) Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York

Sanger F, Nicklen S, Coulson A (1979) DNA sequencing with chain-termination inhibitors. Proc Natl Acad Sci USA 74: 5463–5468

Schägger H (1994) Denaturing electrophoretic techniques. In: von Jagow G, Schägger H (eds) A practical guide to membrane protein purification. Academic Press, London, pp 59–79

Selker EU (1999) Gene silencing: repeats that count. Cell 97: 157–160

Shevchenko A, Jensen ON, Podtelejnikov AV, Sagliocco F, Wilm M, Vorm O, Mortensen P, Shevchenko A, Boucherie H, Jensen M (1996) Linking genome and proteome by mass spectrometry:

large-scale indentification of yeast proteins from two-dimensional gels. Proc Natl Acad Sci USA 93: 14440–14445

Singer MJ, Selker EU (1990) Genetic and epigenetic inactivation of repetitive sequences in *Neurospora crassa*: RIP, DNA methylation, and quelling. Curr Top Microbiol Immunol 197: 165–177

Tenhunen J, Ulmanen I (1993) Production of rat soluble and membrane-bound catechol *O*-methyltransferase forms from bifunctional mRNAs. Biochem J 296: 595–600

Tenhunen J, Salminen M, Jalanko A, Ukkonen S, Ulmanen I (1993) Structure of the rat catechol-*O*-methyltransferase gene: separate promoters are used to produce mRNAs for soluble and membrane-bound forms of the enzyme. DNA Cell Biol 12: 253–263

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22: 4673–4680

Vidgren J, Svensson LA, Liljas A (1994) Crystal structure of catechol *O*-methyltransferase. Nature 368: 354–358

Visick JE, Cai H, Clarke S (1998) The L-isoaspartyl protein-repair methyltransferase enhances survival of aging *Escherichia coli* subjected to secondary environmental stresses. J Bacteriol 180: 2623–2629

WilsonVL, Jones PA (1983) DNA methylation decreases in aging but not in immortal cells. Science 220: 1055–1057

Wu DY, Ugozzoli L, Pal BK, Qiang J, Wallace RB (1991) The effect of temperature and oligonucleotide primer length on the specificity and efficiency of amplification by the polymerase chain reaction. DNA Cell Biol 10: 233–238