Introduction

The primary cause of PD is still unknown, but oxidative stress and mitochondrial respiratory failure with a resultant energy crisis have been implicated as two major contributors to nigral neuronal death. An increase of superoxide dismutase (1), the accumulation of iron (2-5), the loss of gultathione (2, 6), and an increase of lipid peroxidation (3, 7) are reported to be evidence of oxidative stress, while complex I deficiency (8-10) or loss of α-ketoglutarate dehydrogenase complex (11) are evidence of mitochondrial failure.

Reactive oxygen species (ROS), such as superoxide anions and hydroxyl radicals, are constantly produced during normal cellular metabolism. However, defense mechanisms exist to limit the levels of ROS and the damage inflicted on cellular components such as proteins, lipids, and mitochondrial or nuclear DNAs. It has been proposed that the imbalance between production and destruction of ROS is skewed in pathological conditions such as in PD. Nigral dopaminergic neurons are particularly exposed to a high risk of oxidative stress since the metabolism of dopamine can leads to formation of H_2O_2, which can give rise to highly toxic hydroxyl radical in the presence of ferrous iron (12, 13). As nigral neurons degenerate, the remaining neurons compensate with increased dopamine production, which also increases the oxidative deamination of monoamines and the formation of dopamine (14).

Excessive ROS cause damage to intracellular macromolecules, including proteins, lipids, and DNA. In the oxidized damage of DNA, an oxidized form of guanine, 8-oxo-deoxyguanine (8-oxo-dG; 8-oxo-2, 8-dihydroguanine), is the major mutagenic molecule formed by ROS, since it can pair with adenine or cytosine, with almost equal efficiency during DNA

replication (15). In *Escherichia coli*, two DNA glycosylase encoded by the *mutM* and *mutY* genes act to repair 8-oxo-dG. The enzyme mutM protein removes 8-oxo-dG paired with cytosine (16) while mutY protein removes adenine paired with 8-oxo-dG in DNA (17). Oxidized form of guanine is also formed in the intracellular nucleotide pool and is eliminated by the *mutT* gene product. MutT protein (8-oxo-dGTPase) hydrolyzed 8-oxo-dGTP to 8-oxo-dGMP, thereby preventing misincorporation of 8-oxo-dGMP into DNA (18). In *mutT*-deficient strain, the rate of spontaneous occurrence of A:T to C:G transversion is increased hundreds- to thousands-fold compared to wild type *E. coli* (19, 20). In contrast the spontaneous mutation rate in *mutM* or *mutY*-deficient strain is only 10-50 times higher than in wild-type *E. coli*(19, 20), suggesting that MutT is the most important enzyme involved in preventing mutagenesis. Mammalian cells contain enzymes similar to MutM, MutY, and MutT of *E. coli* . 8-oxo-dGTPase has been purified from Jurkat cells, a human T cell leukemia cell line (21). The cDNA was isolated and the genomic structure was identified (22, 23). Human 8-oxo-dGTPase showed a considerable degree of amino acid sequence homology with the *E. coli* MutT protein (23). Therefore, this enzyme is considered to be the human counterpart of MutT protein, and its gene has been named human *mutT* homologue 1 (*hMTH1*) (22).

In eukaryotic cells, the dNTP pool for nuclear DNA replication is mainly present in the cytosol (24), while one of the dNTPs involved in mitochondrial DNA replication appears to be present in the mitochondrial matrix. In fact, *hMTH1* is localized in both the mitochondrial matrix and cytosol (25). In recent studies, 8-oxo-dG was found to be increased in the substantia nigra of PD patients compared to age-matched controls (26), indicating that oxyradical-mediated DNA damage to the nigrostriatum was

greater in the patients than in controls. The level of 8-oxo-dG may be determined by a dynamic equilibrium between its generation and the excision repair of DNA. Accordingly, impairment of *hMTH1* may be one of the causes for neuronal damage in PD. In the present study, we used immunohistochemical and immunoblotting techniques to examine the association between *hMTH1* and PD. In addition, we determined the *in situ* distribution of 8-oxo-dG using specific antibodies to reveal the relationship between the distribution of 8-oxo-dG and *hMTH1* expression.

Methods

Subjects

The subjects consisted of six patients with PD, three patients with multiple system atrophy (MSA), and four controls. The age, sex, clinical features and pathological features of the subjects are shown in Table 1. The histologic diagnosis was confirmed by the Department of Pathology of Juntendo University School of Medicine in all cases. One of the three MSA patients had striatonigral degeneration plus olivopontocerebellar atrophy (SND), while the remaining two patients had olivopontocerebellar atrophy (OPCA) alone. None of the control subjects had nigral or striatal lesions. The postmortem delay between death and fixation of the brains in formalin was 3.5-11.5 hours for the controls, 3.5-11.5 hours for the PD patients, and 5.0-13.5 hours for the MSA patients. All the PD and MSA patients were treated with levodopa and a peripheral dopa decarboxylase inhibitor until death, except for the terminal period in some cases. The maintenance dose of levodopa shortly before death was about 540 ± 89 mg/day (mean ± SD) in the PD patients and 380 ± 68 mg/day in the MSA patients. The duration of levodopa therapy was shorter in MSA (2-11 years) than in PD patients (6-12 years).

Preparation of anti-hMTH1 antibody and monoclonal antibody N45.1 specific for 8-oxo-dG

A peptide corresponding to Gln^{140} to Val^{156} having an additional cystein residue at the amino-terminus for conjugation with a carrier protein was synthesized and purified by HPLC equipped with a reversed phase C-18 column. The polypeptide was conjugated with Maleimid activated Keyhole limpet hemocyanin (Pierce, Rockford, IL, USA) according to the manufacturer's instruction. The conjugated peptide (200 μg) in 500 μl of phosphate buffered saline was emulsified with the same volume of Freund complete adjuvant and injected into a Japanese white rabbit. Hundred μg of the conjugated peptide emulsified with Freund incomplete adjuvant was injected every 2 weeks after the first immunization. The antibody was designated anti-M910; characterization of anti-M910 has been confirmed by immunoblotting (K. M.,unpublished data).

A monoclonal antibody specific for 8-oxo-dG, N45.1, was obtained from Nippon Yushi Corporation (Tokyo, Japan). The specificity of N45.1 was confirmed in previous studies (27).

Immunohistochemistry for hMTH1 and 8-oxo-dG

Immunohistochemical studies were performed on paraffin-embedded midbrain sections obtained at the superior colliculus level. The 5-um thick transverse sections were deparaffinized with xylene and immersed in 70%, 90%, and 100% ethanol for dehydration. To inhibit endogenous peroxidase, the sections were incubated with 0.3% H_2O_2 in methanol for 30 min. After rinsing in 0.01 M phosphatebuffered saline (PBS), the sections were incubated with normal goat serum (Dako, Kyoto, Japan; diluted to 1:10) to inhibit nonspecific binding. After removing the serum, the slides were incubated for for overnight at 4C° with polyclonal anti-M910 (diluted 1:500)

and with monoclonal N45.1 specific for 8-oxo-dG (5 μg / ml). After rinsing with 0.01M PBS, sections were incubated with biotin-labeled goat anti-rabbit IgG antiserum (Dako, Kyoto, Japan; diluted 1:200) for 60 min and then with avidin-biotin complex (Vectastain ABC kit, Vector Laboratories; 1:100) for 60 min. After rinsing, sections were finally incubated with 0.02% 3,3-diaminobenzidine and 0.03% H_2O_2 in deionized water for 20-25 min. Control sections were incubated with normal rabbit serum as well.

Tissue fractionation samples for immunoblot analysis of hMTH1

Blocks of tissue containing appropriate brain regions were brought from -70 C° to -20 C°, and tissue samples (150mg to 300mg) were punched out with a hollow bore instrument. The regions sampled included the SN. Approximately 200mg of tissue was homogenized for 20-30 seconds in 500μl of 0.32 M sucrose, 3 mM Tris-HCl (pH 7.4), 15 μg/ml leupeptin, 5 ug/ml (p-aminophenyl) methanesulfonyl fluoride hydrochloride (APMSF), and 50 ng/ml pepstatin using ultrasonic homogenizer. The following procedures were performed at 4 C°. The homogenate was centrifuged at 600 x g for 10 min, and the supernatant (post-nuclear supernatant) was centrifuged again at 600 x g for 10 min to minimize contamination of the post-nuclear supernatant with nuclei and intact cells. The 600 g pellet was used as the nuclear fraction. The post nuclear supernatant was centrifuged at 7,000 x g for 10 min. The pellet (crude mitochondrial fraction) was used for further purification of mitochondria. The 7,000 x g supernatant was centrifuged at 320,000 x g for 1h and resulting supernatant was used as the cytosolic fraction.. The 320,000 x g pellet was served as the microsomal fraction. The crude mitochondrial fraction was layered on discontinuous sucrose gradient made by successive layering 4.5 ml of 1.5 M and 1.0 M

sucrose from the bottom and then centrifuged at 80.000 x g for 1 h. MR51 cells which overexpress *hMTH1* was used as positive control (25).

Immunoblotting of hMTH1

Proteins were separated by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (PAGE) (28), and immunoblotting analysis was performed as described elsewhere (29, 30). Protein concentrations were determined with a protein assay kit (Bio-Rad, Hercules, CA). Briefly, a blot was blocked in Tris-buffered saline (10 mM Tris-HCl, pH 7.6, and 150 mM NaCl) containing 0.05% Tween 20 and 5% bovine serum albumin at 52 C° for 1 hr, and was incubated with anti-M910 antibody (dilution 1: 3000) in the blocking solution at 4 C° overnight, followed by washing with Tris-buffered saline containing 0.05% Tween 20. This was followed by reaction with the biotin-labeled goat anti-rabbit IgG antiserum (Dako; diluted 1:200) and peroxidase-labeled streptoavidin. Then the reaction was visualized using a chemiluminescence reagent (Dupon, New England Nuclear, Boston, MA). The intensity analyzed by the Masterscan™ Interpretive Densitometer (Scanalytics).

Results

Immunohistochemistry for hMTH1

All the six PD patients showed a moderate to severe degree of neuronal loss in the SN. The remaining nigral neurons showed various degrees of immunostaining for *hMTH1* (Fig. 1B). The overall impression was that PD patients showed a higher proportion of positively stained neurons and more intense immunostaining compared to the controls. No Lewy bodies were immunostained for *hMTH1*. The positive neurons showed homogeneous staining in the cytoplasm; both degenerated and intact neurons were stained

(Fig. 1B). In patient 2, for whom nigral neuronal loss was most severe among the patients studied, there appeared to be a correlation between the amount of neuromelanin within the cell body and the intensity of immunostaining. Neurons with much neuromelanin occupying the cytoplasm were stained more strongly. In addition, neurons located the lateral part of the pars compacta of the SN showed intensely immunostaining for *hMTH1*. Most of the oculomotor neurons and the neuropil showed weak immunostaining. However, none of the glial cells were stained. Other neurons in the midbrain, such as those in the red nucleus, superior colliculus, and midbrain raphe, were not stained either. In addition, neurons in frontal lobe were not stained for *hMTH1* (data not shown). More than 90% of melanized neurons in the SN did not show positive immunostaining for *hMTH1* in the controls (Fig 1A). As seen in Figure 1A, most of the positively stained neurons only showed weak staining. Weak immunostaininig of oculomotor neurons for *hMTH1* was also observed in four controls. Oculomotor neurons and the surrounding neuropil were stained with a variable intensity ranging from negative to weak. In the younger controls (controls 1 and 2), most of the oculomotor neurons and surrounding neuropil were not stained, while scattered oculomotor neurons were stained weakly in the elderly controls (controls 3 and 4). There was severe loss of melanized neurons in the SN of patients with SND, but the loss was mild in the patients with OPCA. Nigral neuronal loss was severe and the remaining neurons were shurunken. Melanized neurons were not immunostained as those of PD patients (Fig. 1C). In MSA patients, most of the oculomotor neurons showed weak staining and the intensity was similar to that in PD patients.

Immunohistochemistry for 8-oxo-dG

In the PD patients, the cytoplasm of neurons in the SN showed intense and punctuate staining (Fig. 1E), while the nuclei were not stained. In contrast, in the MSA patients (Fig. 1F) and the controls (Fig. 1D), neither cytoplasmic nor nuclear staining was observed. Accumulation of 8-oxo-dG in neurons was not related to the extent of degenerative changes. Lewy bodies were not immunostained. Other neurons in the midbrain, such as those in the red nucleus, superior colliculus, and midbrain raphe were not stained.

Immunoblotting analysis of hMTH1

An aliquot (100 μg) of total homogenate tissue sample from each subject was analyzed by immunoblotting using anti-M910 (Fig. 2A). Lane C represents the total homogenate of Hela MR51 cells, which show about 10-to 300-fold increased production of *hMTH1* protein. A single band corresponding to the 18-kDa polypeptide was only detected in the SN of PD patients, and no band was found in controls or MSA patients. There was no clear association between the duration of disease and the amount of *hMTH1*. In addition, the 18-kDa band was not detected any part of the total homogenate samples in all subjects except for the SN sample of PD patients (data not shown).

Each subcellular fraction of the SN samples from PD patients was analyzed by immunoblotting using the anti-M910 (Fig. 2B). A single band corresponding to the 18-kDa polypeptide was detected in the mitochondria and in the cytosol . The intensity of the signal / mg of protein in each fraction, mitochondria / cytosol was 0.56.

Discussion

In the present study, we demonstrated that *hMTH1* was markedly increased in the SN of PD patients, but not in the frontal lobe using

immunohistochemical and immunoblotting analyses. We also found that 8-oxo-dG was marked increased in the cytoplasm of SN neurons of PD patients, suggesting that the oxidative modification of mitochondrial DNA was markedly increased. Evidence of nuclear DNA modification could not be detected in any of the subjects studied. There was no immunostaining for both *hMTH1* and 8-oxo-dG in MSA patients and controls. These findings have some important implications: 1) The increase of *hMTH1* in PD was restricted to the SN and so probably influences some active process selective to that region. 2) The absence of an increase of *hMTH1* and 8-oxo-dG in MSA patients indicates that these changes were not the result of neuronal degeneration of the SN.

The cytoplasmic location of 8-oxo-dG in PD nigral neurons would indicate that mitochondrial DNA is preferentially affected by oxidative damage. Complex I deficiency is specific for the SN in PD, and is not present in other neurodegenarative disorders. Mitochondrial respiratory dysfunction increases the generation of ROS; indeed, increased production of ROS *in vivo* is a known consequence of inhibition of complex I by the MPTP derivate MPP^+ (31). Therefore, it is likely that oxidative stress occurs within the mitochondria of nigral neurons in PD. We have previously shown that a high level of *hMTH1* presents in the mitochondrial matrix[25]. Mitochondria synthesize their own dNTPs (32) and form dNTP pool that is independent from the other pools in the cytosol and nucleus (24, 33). The mitochondrial dNTP pool is probably exposed to a stronger oxidative stress than those of the cytosol and nucleus due to presence of the respiratory chain, generates a large amount of ROS. Several studies have indicated that mitochondrial was much more susceptibile to oxidative stress than nuclear DNA (34-36), and that it showed a 16-fold increase in oxidazed

bases as compared with nuclear DNA (36). We demonstrated that *hMTH1* was increased in the mitochondria of the SN tissue samples from PD patients compared to MR51 cells which overexpress *hMTH1*. The ratio of *hMTH1* in the mitochondria / cytosol of PD SN sample showed a 4-fold increases compared to MR51 cells.

The increase of *hMTH1* in the SN of PD brains may be a reactive response to elevated levels of 8-oxo-dG within the mitochondria. Okamoto et al. have detected elevated the expression of *hMTH1* mRNA in human renal carcinoma (37). In addition, Kennedy et al. confirmed that expression of *hMTH1* was inversely proportional to the cellular level of 8-oxo-dG in lung cancer (38). The level of oxidative stress in renal carcinoma increases with the clinical stage of the disease, while lung cancer cells apparently up-regulate expression of *hMTH1* in response to increased oxidative stress (38) and thereby keep 8-oxo-dG levels relatively constant throughout the course of the disease. In addition, the *hMTH1* promoter contains many AP-1 and NFκB recognition sequences (39). It has been reported that exposure of a variety of cells to oxidants induces increases of AP-1 and NFκB DNA-binding activity (40, 41). Therefore, activation of AP-1 transcription factors may be potentially important signaling pathways for cellular responses to oxidative stress. Thus, our results indicates that the synthesis of *hMTH1* increases to remove 8-oxo-dGTP from the mitochondrial matrix by hydrolyzing it to 8-oxo-dGMP after 8-oxo-dG was accumulated due to oxidative stress. 8-oxo-dGTPase specifically hydrolyzes 8-oxo-dGTP to the monophosphate and the 8-oxo-dGMP thus formed cannot be rephosphorylated. By the action of nucleotidase, 8-oxo-dGMP is then further degraded to 8-oxo-dG (35).

Recenty, it was reported that 8-oxo-dG was increased in the nigro-striatum of PD brains (26). There are at least two different metabolic pathways for the presence of 8-oxo-dG in DNA: (1) direct hydroxylation of dG that causes a G:C to T:A transversion upon replicatin (15, 42), and (2) hydroxylation of dGTP that induces A:T to C:G and possibly G:C to T:A transversions when miscorporated into DNA upon replication (18). Thus, our results indicate that part of the 8-oxo-dG in mitochondrial DNA may be derived from 8-oxo-dGTP, which is located mainly in mitochondrial matrix. Furthermore, even in postmitotic cells, mitochondrial DNA is replicated independently from nuclear DNA. Therefore, 8-oxo-dGTP must be the origin of 8-oxo-dG that enters DNA strands during replication.

In summary, we showed that synthesis of *hMTH1* and oxidative modification of mitochondrial DNA were markedly increased in PD patients compared to controls and MSA patients. In addition, our findings exclude the possibility that L-DOPA therapy imposes oxidative stress on the mitochondrial and nuclear DNA.

Accumulation of mutations in mitochondrial DNA has been correlated with the decline of oxidative phosphorylation with aging and with impairment of the respiratory chain in degenerative diseases (43). Our studies suggest that mitochondrial oxidative damage may be important in the pathogenetic mechanism of nigral neuronal death in PD. Further research to develop drugs that can prevent oxidative stress within nigral neurons, particularly within the mitochondria, appears to be very important, and such drugs may turn out to be a neuroprotective treatment for PD.

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Legends for figures

Figure 1

Immunohistochemistry for 8-oxo-dGTPase (**A, B, C**) and 8-oxo-dG (**D, E, F**) in control, PD patient, and MSA patient midbrain sections (5 μm). (**A, D**) Control midbrain (Case 1). (**B, E**) PD patient midbrain (Case 1). (**C, F**) MSA patient midbrain (Case 1). The cytoplasm of the SN neurons in the PD patient shows homogeneously staining for both 8-oxo-dGTPase (**B**) and 8-oxo-dG (**E**) antibodies, but control (**A, D**) and MSA patient (**C, F**) show no staining. Arrows indicate the cytoplasm of neuron and arrowheads show melanin. (Bar = 50 μm.)

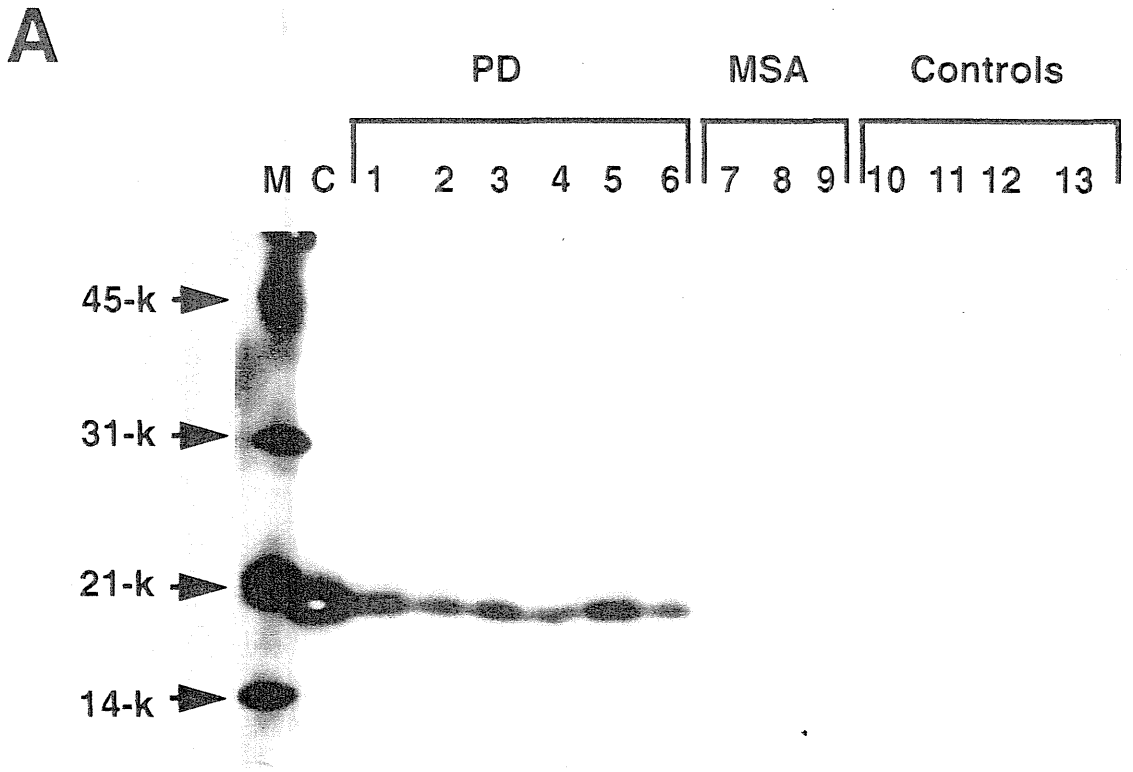
Figure 2

A, Immunoblotting of 8-oxo-dGTPase in punchedout substantia nigra tissue samples (100 μg) from PD patients (Lanes **1-6**), controls (Lanes **7-10**), and MSA patients (Lanes **11-13**). (**C**) total homogenized sample of MR51, which produced about 10 - to 300 - fold increase of *hMTH1* protein. 18-kDa band was detected in all six PD patient samples (Lanes **1-6**), but not in all four controls (Lanes **7-10**) and all three MSA patients (Lanes **11-13**). (**M**) Molecular weight marker (Bio-Rad). **B,** *hMTH1* in each fraction was analyzed. Applied protein contents were 20 μg.

Table 1 Patients with PD, MSA and Control Subjects

	Age (year)	Sex	Duration of disease	L-Dopa therapy	Postmortem time (hours)
Parkinson's disease					
1 PD	76	F	8	6	3.5
2 PD	66	F	10	9	11.5
3 PD	75	M	10	9	4.0
4 PD	63	F	8	7	5.5
5 PD	81	F	8	7	2.5
6 PD	51	M	13	12	4.5
Control subjects					
1 Ovarian cancer	38	F		none	4.0
2 Gastric cancer	47	F		none	4.0
3 Brain infarction	82	M		none	3.5
4 Brain infarction	92	M		none	11.5
MSA					
1 OPCA	58	F	12	11	13.5
2 OPCA	63	M	8	6	6.0
3 SND	69	F	7	2	5.0

PD: Parkinson's disease, MSA: Multiple system atrophy with striato-nigral degeneration and olivopontocerebellar atrophy. SND: Multiple system atrophy with striatonigral degeneration without olivopontocerebellar atrophy, OPCA: Multiple system atrophy with olivo-ponto-cerebellar atrophy without striatonigral degeneration.


A
PD
MSA
Controls
M C 1 2 3 4 5 6 7 8 9 10 11 12 13
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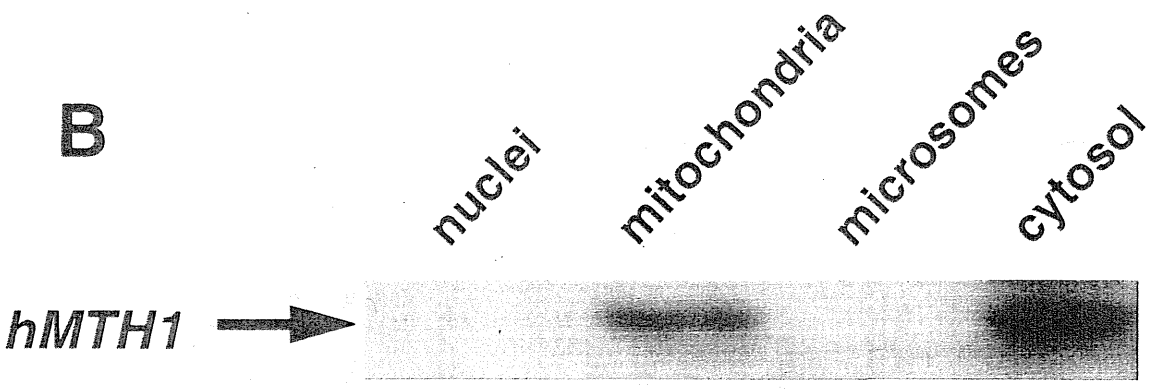
B
nuclei
mitochondria
microsomes
cytosol
hMTH1