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Stimulation by cadmium of myohemerythrin-like cells in the gut of the annelid *Nereis diversicolor*

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Summary

Isolated guts of *Nereis diversicolor* revealed the existence of a cadmium-binding protein, the MPII, belonging to the group of hemerythrins and myohemerythrins. The presence of MPII in the cells of the intestine was demonstrated by immunocytochemistry, using anti-MPII, a monoclonal antibody. In addition, using *in situ* hybridization and northern blotting, it was shown that MPII-cells are the site of synthesis of this molecule. Exposure of the worms to cadmium led to the cellular activation process of MPII-cells (i.e. transformation of the nucleolus, development of the endoplasmic reticulum and the Golgi apparatus), although MPII mRNA transcript levels were unchanged. Enzyme-linked immunosorbent assay (ELISA) of gut extracts revealed that MPII levels were increased after exposure to Cd, so it appears that this protein is synthesized as a response to Cd exposure without any new synthesis of mRNA. This mechanism of regulation is quite similar to that reported in the case of mammalian ferritin and may be involved in the regulation of Cd levels in this worm.

Key words: annelid, *Nereis diversicolor*, myohemerythrin, MPII, cadmium-binding protein, midgut.

Introduction

Trace metals, whether essential such as Cu and Zn or non-essential such as Cd, are taken up by aquatic invertebrates from both food and solutions. After ingestion, the metal is transported around the body, perhaps to be accumulated in particular target organs or even to be excreted (Rainbow, 1998). In most invertebrates, body concentrations of the non-essential metal Cd do not appear to be regulated (Rainbow, 1985; Amiard et al., 1987; Rainbow, 1998). Metal toxicity is often postulated to arise from reactions occurring in the cytosol, through non-specific binding of the metal to non-thionein ligands, which are physiologically important molecules (metalloenzymes or small peptides such as glutathione) and are inactivated by metal binding (Mason and Jenkins, 1995). Control of intracellular metal toxicity and detoxification of accumulated trace metals is generally achieved *via* the production of metal-binding ligands that sequester metals. These cellular ligands can be found in the particulate fraction of the cells such as mineral deposits, granules or lysosomes (George, 1990; Mason and Jenkins, 1995) and in the cytosol such as metallothioneins (Engel and Roesijadi, 1987). Metallothioneins, a family of low molecular mass cysteine-rich proteins, have been shown to occur in most zoological taxa (Amiard and Cosson, 1997). However, in polychaetous annelids this type of protein has not yet been isolated.

Nereis diversicolor (*Hediste diversicolor*, recent denomination) is a polychaete living in the mud of estuaries which, in Europe, is routinely contaminated by heavy metals. Previous work (Nejmeddine et al., 1988) showed that in animals exposed to Cd the metal was bound to two pools of proteins of high molecular mass (metalloprotein I, MPI, >67 kDa) and low molecular mass (MPII, of about 20 kDa). MPI is the extracellular hemoglobin of this annelid (Demuynck and Dhainaut-Courtois, 1993). The primary structure of MPII isolated from whole worms was different from that of a metallothionein, consisting of 119 amino acid residues with only one cysteine residue (Demuynck et al., 1993), but shares 80.8% identity with the myohemerythrin isolated by Takagi and Cox (1991) from the same species. MPII is a Cd-binding protein but is not a metallothionein. Metallothioneins are known to be induced by essential (Cu or Zn) and non-essential (Cd) metals and are thought to be important both in the detoxification of trace metals and in the metabolism of intracellular Cu and Zn, as in the scavenging of reactive oxygen species (Palmiter, 1998; Klaassen et al., 1999). However, MPII appeared to correspond to a myohemerythrin-like pigment, known to play a mainly respiratory role and with no known function of detoxification. Immunocytochemical studies showed that MPII is located in granulocytes I

(Dhainaut-Courtois et al., 1987; Porchet-Henneré et al., 1987), a specific type of coelomic cells (Dhainaut, 1984; Dhainaut and Porchet-Henneré, 1986). Electron microscopy revealed that the protein was located in the cytoplasmic granules of these cells (Dhainaut-Courtois et al., 1987). In addition, Salzet-Raveillon et al. (1993) showed, by *in situ* hybridization, that MPII mRNA is located in perineural and oblique muscles and in clusters of free cells in the coelom, thought to differentiate later into type I granulocytes, which would contain the MPII protein without the corresponding mRNA.

MPII can be also observed in other cell types, however, such as cells from intestine epithelium. In fact, in the present work, we show that intestine epithelium cells are immunoreactive with MPII mAb. Our biochemical approach has been to confirm the presence of MPII (or MPII-like protein) in the gut and to verify the Cd-binding capacity of this molecule on exposure of the worms to this metal. The cells were identified by electron microscopy, and variations in MPII levels in the gut of animals in response to acute or chronic exposure to Cd were investigated by enzyme-linked immunosorbent assay (ELISA). Finally, the gene expression was studied in the gut of non-exposed and Cd-exposed animals by *in situ* hybridization and northern blotting using a specific probe in order to see whether or not there is transcriptional regulation of the MPII gene after Cd intoxication.

Materials and methods

Animals and exposure conditions

Nereis diversicolor O. F. Müller were collected in the estuary of the river Aa near Gravelines (Northern France). Worms of mass 0.4–0.6 g were used for the experiments. The first group of animals (acute Cd-exposed worms) were exposed to 20 mg l^{-1} of cadmium added as $CdCl_2$ to seawater for 2 days at 15°C. The second group (chronically Cd-exposed worms), were subjected to progressive exposure for 6 days (1.25 mg l^{-1} for 3 days; 2.5 mg l^{-1} for 2 days and 5 mg l^{-1} for 1 day). The concentrations tested were lower than the LC_{50}, which is about 100 mg l^{-1} for 192 h of exposure (Bryan, 1976). At the end of the exposure period, animals were rinsed with natural seawater. Before manipulations, worms were anesthetized with 1% chloretone added to seawater.

Immunohistochemical procedure

The worm stubs were immersed for 12 h in Bouin–Hollande fixative and embedded in cytoparaffin. The immunohistochemical method was performed as described previously (Engelhardt et al., 1982). In brief, 8 μm sections were prepared and mounted on chrome alum/gelatin-coated slides and briefly air-dried. A monoclonal antibody raised against MPII (MPII mAb) produced previously (Porchet-Henneré et al., 1987) and used as pure culture supernatant was applied for 24 h at 4°C. After washing with Coons buffer, pH 7.2, horseradish peroxidase-conjugated anti-mouse immunoglobulin was applied at 1:40 dilution for 2 h. The peroxidase activity was revealed by allowing the sections to react with 0.01% H_2O_2:40% 4-chloro-1-naphthol (w/v) in 0.1 mol l^{-1} Tris-HCl buffer, pH 7.6.

Electron microscopy

Small pieces of gut were fixed for 3 h at 4°C with 3% glutaraldehyde, 2.5% NaCl in 0.1 mol l^{-1} phosphate buffer, pH 7.4. They were then washed for 24 h with the phosphate buffer in presence of 0.33 mol l^{-1} sucrose, post-fixed for 1 h with 1% osmium tetroxide in the buffer and embedded in Epon resin. The sections were stained with uranyl acetate and observed using a Jeol 100 CX electron microscope (Peabody, USA).

Preparation of samples for biochemical analysis

The guts were isolated by dissection under a stereo microscope. For each experimental sample, guts from 25 worms were pooled to reduce individual variation and homogenized in 4 ml of 10 mmol l^{-1} Tris-HCl buffer, pH 8.6 using a polytron. The homogenate was then centrifuged at 4°C for 1 h at 15 000 ***g***. The pellet was discarded and the supernatant used for chromatographic analysis.

Chromatography

2 ml of the supernatant were loaded onto a 16/60 Hi-Load Superdex 75 Prep grade (separation range 3–70 kDa) column (Amersham Pharmacia Biotech, Uppsala, Sweden) equilibrated with the homogenization buffer. The elution was then performed at 1 ml min^{-1} using FPLC system (Amersham Pharmacia Biotech). The absorbance at 280 nm was read continuously.

Electrophoresis

Equal amounts of proteins from peak II chromatographic fractions derived from gut supernatants of both control and acute Cd-exposed worms were analysed by SDS-polyacrylamide gradient gel electrophoresis (SDS-PAGGE) on a 5–25% acrylamide gradient slab gel (0.75 mm thick) under reducing conditions (5% v/v β-mercaptoethanol). The migration was performed at 15 mA for 4 h. Half of the gel was then stained for proteins with Coomassie Brilliant Blue R 250. The second part was processed for the immunodetection of proteins.

Western blotting

Proteins were blotted onto an Immobilon P membrane (Millipore, Billerica, USA) activated in methanol for a few seconds, washed in milli-Q water (Millipore) for 10 min then equilibrated for 10 min in 25 mmol l^{-1} Tris, pH 8.3, containing 192 mmol l^{-1} glycine and 10% methanol. The conditions used were 250 mA for 4 h.

Immunodetection of proteins

The membrane was first saturated with 1% low-fat powdered milk, 0.05% Tween 20 in Tris-buffered saline (TBS; 20 mmol l^{-1} Tris-HCl, pH 7.4, containing 150 mmol l^{-1} NaCl) for 1 h and then incubated for 12 h with the MPII mAb

used as the pure culture supernatant. The membrane was then rinsed four times with 0.05% Tween 20 in TBS (TBS-Tween) and incubated for a further 2 h with anti-mouse antibody labelled with peroxidase diluted 1/1000 in the buffer. The peroxidase activity was revealed with a solution composed of 50 ml TBS, 3 ml 0.3% 4-chloro-1-naphthol in methanol and 10 μl H_2O_2.

Metal analysis

Chromatographic fractions were analysed for their Cd content by Flame Absorbance Spectrometry using a Perkin-Elmer 2380 (Boston, USA) spectrophotometer.

Enzyme-linked immunosorbent assay (ELISA)

Chromatographic fractions derived from the gut of acute Cd-exposed worms and gut supernatant from both control and acute Cd-exposed and chronically Cd-exposed worms were analysed by ELISA using the MPII mAb. In brief, 96-well plates were coated with 100 μl of the different samples for 12 h at 4°C. The saturation was realized for 2 h at ambient temperature with 200 μl of a 2% bovine serum albumin (BSA) in 0.01 mol l^{-1} phosphate-buffered saline (PBS) at pH 7.4. After four rinses with 0.1% BSA; 0.05% Tween 20 in PBS, 100 μl of the monoclonal antibody were added to each well and incubated for 2 h at 37°C. After four rinses with the previous buffer, 100 μl of peroxidase-labelled second antibody diluted 1/10 000 in TBS-Tween were added and incubated for 2 h. Finally, peroxidase activity was revealed by the addition of 100 μl of a solution containing 0.04% (w/v) O-phenylene diamine in 0.1 mol l^{-1} sodium citrate–citric acid buffer, pH 5.5, and 0.83% (v/v) H_2O_2. The reaction was stopped after 30 min at 37°C by adding 100 μl 1 mol l^{-1} HCl. Absorbance of the plate at 490 nm was read. The results obtained were corrected for protein content. Since this protein has not yet been purified from this tissue and is not available from commercial sources, the results of the ELISA were not expressed in terms of MPII concentration but as absorption only.

Protein content determination

The protein content of supernatants and chromatographic fractions was determined using protein reagent (Biorad, Hercules, USA) by the method of Bradford (1976) using 96-well plates.

Data analysis

The results obtained by ELISA for the supernatants were compared for mean statistical differences by one-way analysis of variance (ANOVA) and Student–Newman–Keuls comparison post tests. $P<0.05$ was considered significant.

Cloning of MPII probe for northern blot and in situ hybridization

Poly(A)$^+$ RNA was extracted from stubs of *Nereis diversicolor* using the Quick Prep mRNA Purification kit (Amersham Pharmacia Biotech). 2 μg of RNA was reverse-transcribed with 1 μmol l^{-1} of oligo(dT)$^{12–18}$ primer, 0.5 mmol l^{-1} dNTPs, 10 i.u. RNase inhibitor and 4 i.u. Omniscript reverse transcriptase (Qiagen, Valencia, USA) for 1 h at 37°C. A 333-base pair (bp) fragment was amplified with 5′ CCATATAAGCAGGACGAGTC 3′ and 5′ TCCCTT-GTAGCCGAAGTCGG 3′ primers (Deloffre et al., 2003) using 2 μl of cDNA, 100 ng of each primer, 1.5 mmol l^{-1} $MgCl_2$, 0.2 mmol l^{-1} dNTPs, 1× Taq polymerase buffer and 1 i.u. of Taq DNA polymerase (Promega, Madison, USA). Amplification conditions were: 5 min at 94°C, 40 cycles consisting of 1 min at 94°C, 1 min at 55°C and 1 min at 72°C, and a final elongation step of 10 min at 72°C. The resulting 333 bp fragment was cloned in pGEM-T Easy vector (Promega) and both strands were sequenced to verify the orientation of the insert.

Northern blot analysis

RNA from guts of *Nereis diversicolor* was prepared using Tri-Reagent (Molecular Research Centre, Cincinnati, USA), and quantitated spectrophotometrically. 15 μg of RNA were denatured in formaldehyde, fractionated on a 1.2% agarose gel, and then transferred to nitrocellulose membranes (Hybond C extra, Amersham Biosciences, Uppsala, Sweden). The MPII 333 bp and the leech 18S (generous gift from Dr Christophe Lefebvre) probes were labeled with ^{32}P deoxy-CTP using the Megaprime DNA labelling system (Amersham). The membranes were washed twice in 2× SSC, 0.1% SDS at 42°C for 20 min, followed by two washes in 0.5× SSC, 0.1% SDS at 50°C. Membranes were finally exposed for several hours (18S probe) or days (MPII probe) to Hyperfilm (Amersham) at –80°C with two intensifying screens.

Preparation of probes for in situ hybridization

Sense and anti-sense digoxygenin (DIG)-labelled riboprobes were transcribed from SP6 and T7 RNA polymerase promoters, respectively, from 1 μg of linearized template following the conditions of Roche Diagnostics (DIG RNA Labelling kit, Basel, Switzerland). The labelling yield of each probe was then estimated after purification by comparison with a DIG-labelled control RNA.

Tissue preparation

Pieces (several mm in length) from worm guts were rinsed in PBS, then fixed in 4% paraformaldehyde in PBS for 1–2 h at 4°C, rinsed in PBS and dehydrated in a graded ethanol series. Pieces were then transferred into butanol and embedded in Paraplast (VWR International, West Chester, USA). Frontal sections (7 μm) were prepared and stored at 4°C until *in situ* hybridization.

In situ hybridization

After deparaffinization of sections and hydration, slides were treated with 10 μg of proteinase K (Sigma) for 30 min at 37°C, post-fixed in 4% paraformaldehyde in PBS, washed in PBS, acetylated by 0.25% acetic anhydride in 0.1 mol l^{-1} triethanolamine, and incubated for prehybridization for 2 h at

37°C in 4× SSC containing 50% formamide. Hybridization was then performed overnight at 42°C in 100 µl of hybridization solution per slide (40% formamide, 10% dextran sulfate, 1× Denhardt's solution, 4× SSC, 10 mmol l^{-1} DTT, 1 mg ml^{-1} yeast t-RNA, 1 mg ml^{-1} denaturated salmon sperm DNA containing 20 ng of denaturated DIG-labelled RNA probe).

Posthybridization and immunological detection

First washes were performed at 37°C, twice for 15 min with 2× SSC, and twice for 15 min with 1× SSC. Sections were incubated for 30 min at 37°C in 0.5 mol l^{-1} NaCl, 10 mmol l^{-1} Tris, 1 mmol l^{-1} EDTA, pH 8.0, containing 20 µg ml^{-1} RNase A. Additional washes were performed at 52°C with 2× SSC containing 50% formamide. Immunological detection was then performed according to the manufacturer's instructions (Roche Diagnostics, Basel, Switzerland).

Results

Chromatography and metal analysis

Analyses were performed on guts isolated from acute Cd-exposed worms. The chromatographic profile observed at 280 nm (Fig. lA) revealed the presence of three peaks, a feature identical to that observed on analysis of whole worms exposed to Cd (Nejmeddine et al., 1988).

The metal content of the eluted fractions (Fig. 1B) was determined by Flame Absorption Spectrometry and revealed that Cd was bound to three types of ligands: (i) a high molecular mass fraction (>70 kDa) present in peak I that would correspond to hemoglobin (Demuynck and Dhainaut-Courtois, 1993), (ii) lower molecular mass component(s) appearing as a shoulder of peak I, already seen in the case of an acute Cd stress of the worms (Demuynck and Dhainaut-Courtois, 1994), and (iii) a third fraction present in peak II. Finally, ELISA was performed on all the eluted fractions and revealed that the immunoreactivity was mainly associated with peak II (Fig. 1C). These results showed that Cd was actually bound to the animals' gut and coeluted with a MPII or a MPII-like molecule.

Electrophoresis and immunodetection of proteins

There were no significant differences between the peak II proteins from control and Cd-exposed worms, as determined by electrophoretic analysis and subsequent Coomassie Blue staining (Fig. 2, lanes 1 and 2, respectively). After western-blotting using mAb anti-MPII, a main reactive band of quite similar intensity was obtained from both controls and Cd-exposed worms (Fig. 2, lanes 4 and 5, respectively). According to the molecular mass markers used (Fig. 2, lane 3), this immunoreactive band would be slightly smaller than 14.4 kDa, which is in agreement with the previously determined molecular mass of 13.7 kDa of the MPII (Demuynck et al., 1993). An additional band was observed in the region of about 40 kDa that would correspond to the artefactual polymerized MPII molecules.

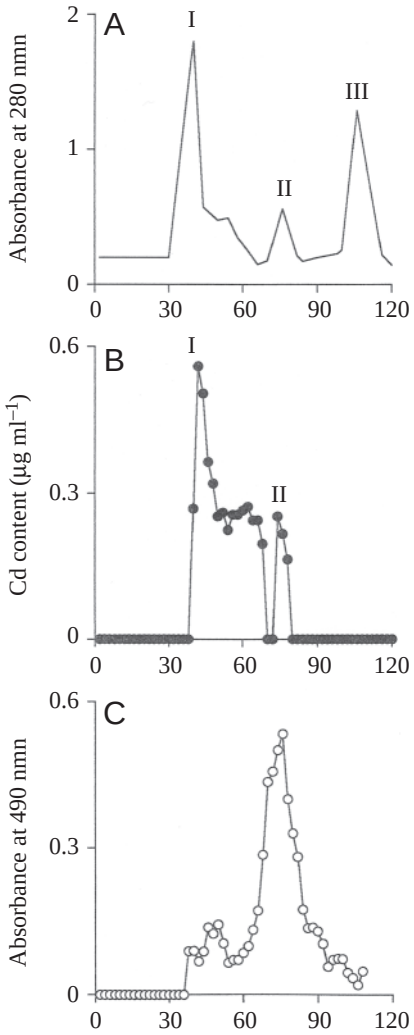

Fig. 1. Gel-permeation chromatography of acute Cd-exposed (20 mg l^{-1}, 2 days) *N. diversicolor* gut supernatant. Column: Hi-Load Superdex 75 Prep grade (Pharmacia); sample volume: 2 ml; flow rate: 1 ml min^{-1}; elution buffer: 10 mmol l^{-1} Tris-HCl, pH 8.6. (A) The absorbance profile at 280 nm shows three main protein peaks, I, II and III. (B) Analysis of the Cd content (µg ml^{-1}) of the chromatographic fractions shows that this metal is associated with components eluted in the void volume of the column (>70 kDa) likely to represent the hemoglobin (peak I in A) and components of the second main peak (peak II in A). (C) Enzyme-linked immunosorbent assay (ELISA) using the mAb anti-MPII revealed that peak II fractions contain the Cd-binding protein MPII.

Immunocytochemistry

Examination of the sections processed for immunohistochemistry revealed that the wall of the midgut or intestine of *Nereis diversicolor* is composed of a pseudo-stratified epithelium laying on a basal lamina that is frequently in contact with blood sinus. In addition to the MPII cells (MPII-C), the intestinal epithelium contains absorptive cells (enterocytes) interspersed with a few mucous cells. The immunoreactive MPII-containing cells (MPII-C) are numerous and rather regularly distributed in the midgut. They are

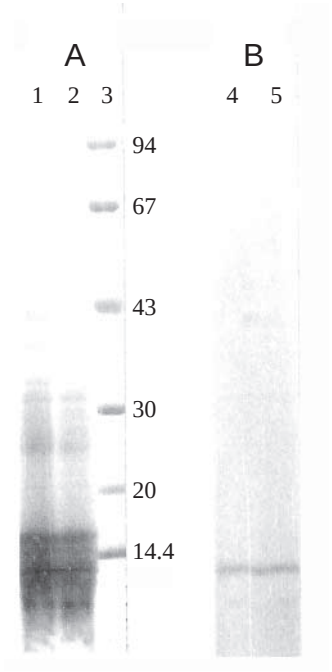

Fig. 2. SDS-polyacrylamide gradient gel electrophoresis (SDS PAGGE) (5–25% acrylamide gradient slab gel) under reducing conditions. (A) Gel stained with Coomassie Brilliant Blue R250 (lanes 1 and 2) and (B) immunodetection of proteins blotted on Immobilon-P (Millipore) membrane using mAb anti-MPII of gut supernatants from control and acute Cd-exposed worms (20 mg l^{-1}, 2 days). Lane 3, molecular mass markers (kDa): 14.4, α-lactalbumin; 20, trypsin inhibitor; 30, carbonic anhydrase; 43, ovalbumin; 67, albumin; 94, phosphorylase. Immunodetection of proteins from chromatographic fractions (see text) of gut supernatants revealed a main band of molecular mass <14.4 kDa and of similar intensity in control (lane 4) and Cd-exposed worms (lane 5).

specially abundant in the folds of the epithelium (Fig. 3A). The apex of these cells reaches the intestinal lumen. The nucleus is located in the lower part of the epithelium near the basal lamina and contains a single large nucleolus, which is very typical of this cell type (Fig. 3B). Identification of these MPII-C was checked in a sipunculid species *Sipunculus nudus*, whose hemerythocytes are immunoreactive with the MPII mAb (Demuynck et al., 1991). In contrast to *N. diversicolor*, immunohistochemistry in *S. nudus* did not reveal a positive reaction in the epithelium, but only in rounded cells quite similar to hemerythrocytes in the blood sinus of the connective tissue surrounding the intestinal epithelium (Fig. 3C). In *Nereis diversicolor*, light microscopy did not reveal any significant differences in the number of immunoreactive cells in the gut of control and Cd-exposed animals (data not shown).

In situ hybridization

We performed *in situ* hybridization to identify synthesis sites of MPII RNA in the midgut of control worms. As shown in Fig. 4A, labelling was observed in cells of the pseudo-stratified epithelium. According to their particular location (in the folds of the epithelium) and their shape, these cells seem to correspond to MPII cells. The specificity of this labelling was confirmed by the absence of labelling in the control (Fig. 4B).

Electron microscopy

MPII-C were characterized by electron microscopy. Detection of this cell type was facilitated by their characteristic nucleolus. The cytoplasm of these cells contains numerous mitochondria localized around the nucleus and also near the apex of the cell. The cisternae of rough endoplasmic reticulum are poorly extended and specially distributed near the nucleus (Fig. 5A). In the same region, the Golgi apparatus shows flat saccules containing a moderate dense material (not shown). Granules of dense electron material are distributed in the cytoplasm. They have an irregular size and their material shows a homogenous structure (see Fig. 5B). In the nucleus, chromatine elements are scarce. The central core of the nucleolus contains moderately dense material of fibrillar structure (Fig. 5A). It is surrounded by granular material

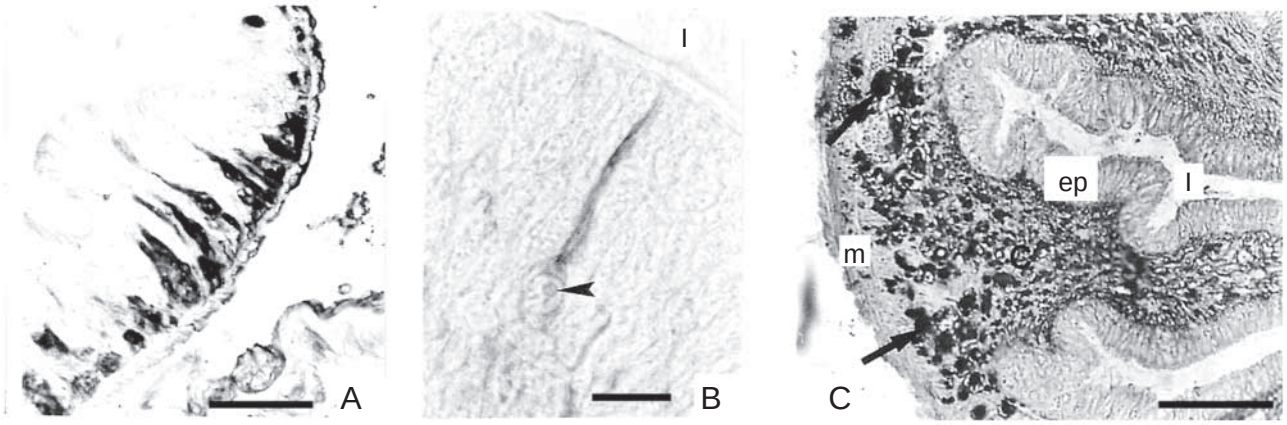

Fig. 3. Immunocytochemical characteristics of MPII-containing cells (MPII-C). (A) Anti-MPII immunoreactive cells (MPII-C) in the intestine of a control worm. Scale bar, 50 μm. (B) Immunoreactive MPII-C showing the characteristic nucleolus (arrow). l, gut lumen. Scale bar, 10 μm. (C) Immunoreactive cells (arrow) in the intestine of the sipunculid *Sipunculus nudus*. l, gut lumen; ep, epithelium; m, muscles. Scale bar, 50 μm.

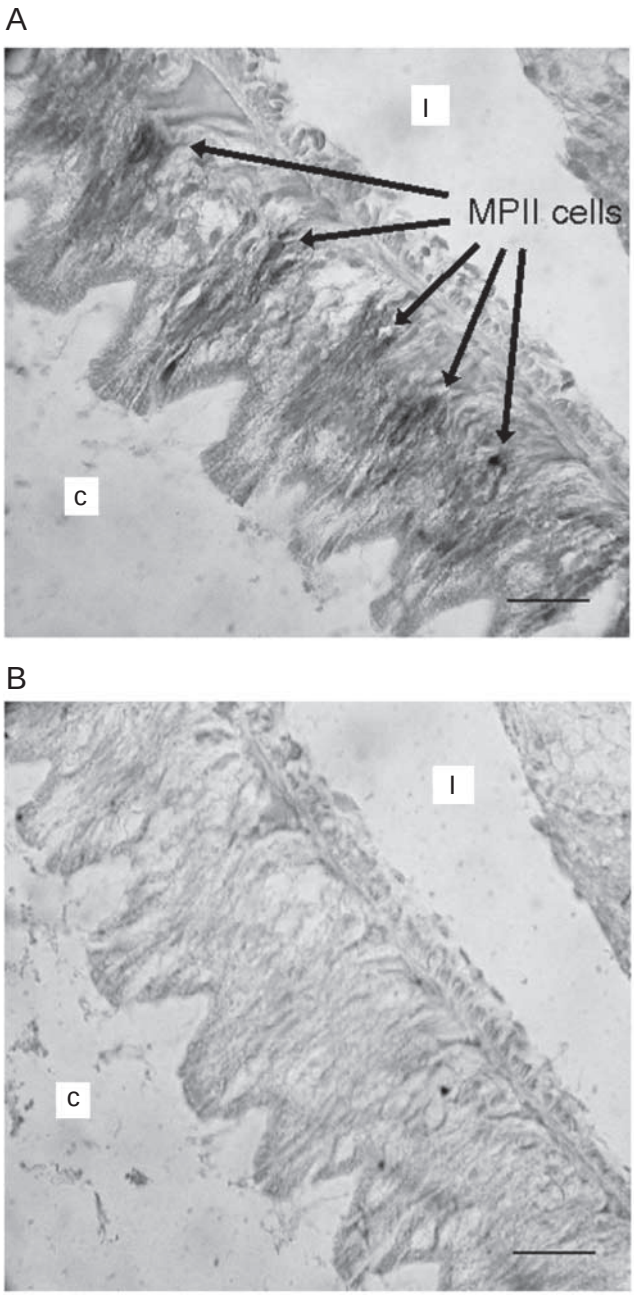

Fig. 4. *In situ* hybridization of MPII mRNA in intestine of control worms. (A) Labelling obtained using a digoxygenin anti-sense riboprobe. Note the labelling is specific to the MPII cells. l, lumen; c, coelom. (B) Using the sense riboprobe, no labelling was detected. Scale bar, 10 μm.

comprising 15–20 nm granules. Dense fibrillogranular material, probably associated with chromatin, is distributed at the periphery of the nucleolus. By contrast with control worms, several ultrastructural modifications were observed in the MPII-C of worms exposed to Cd, of which the most typical one occurred in acute Cd-exposed worms; similar but more discrete modifications were recorded in chronically Cd-exposed worms. These modifications concern the appearance, in the cytoplasm, of long ribbons of endoplasmic reticulum (ER) surrounding the nucleus. In addition, the Golgi complex shows dilated saccules containing dense material related to the development of secretion granules (Fig. 5C). In the nucleolus, granular material becomes more extensive and appears to be either integrated with the fibrillar component (Fig. 5B) or an important network around the central core (Fig. 5D).

Estimation of MPII content by ELISA

The MPII (or an MPII-like) content in gut extracts from control and Cd-exposed worms was estimated by ELISA, where a significantly higher reaction, and so a higher content, was obtained from gut extracts of both chronically (Fig. 6B) and acute (Fig. 6C) Cd-exposed worms compared to controls (Fig. 6A).

Northern blot

Semi-quantitative analysis of MPII expression was undertaken by northern blotting using RNA from guts of both control and acute or chronically Cd-exposed worms. The results (Fig. 7) are the means of two independent experiments and were obtained using Quantity One software (Bio-Rad) after normalization relative to 18S RNA expression level. The data (Fig. 7B) show that the MPII mRNA expression level in Cd-exposed worms remained almost unchanged from control levels. Thus, acute or chronic exposure to cadmium did not induce expression of MPII mRNA.

Discussion

The biochemical results of this study confirm that the gut of intoxicated worms was able to bind Cd. Binding appeared at least on three types of ligands. One of these was eluted in the void volume of the column used (molecular mass >70 kDa) and could correspond to the hemoglobin. In fact, previous results showed that hemoglobin was one of the main ligands for Cd in this species (Demuynck and Dhainaut-Courtois, 1993). In addition, numerous blood vessels are located in the wall of the gut and their presence in gut extracts is likely. A second Cd-binding protein was immunoreactive with MPII mAb and could be considered as the MPII or an isoform of this protein. The binding of metal could occur near the brush border of intestinal cells. Indeed, radioautography performed after injection of ^{109}Cd in the coelom showed an intense labelling located at the apical area of the intestine cells (Septier et al., 1991). The gut of Nereids has been well studied in two species, *Nereis virens* (Michel and Devilliez, 1979; Punin and Lukyanova, 1984; Saunier-Michel, 1992) and *Perinereis cultrifera* (Dakhama and Dhainaut, 1985). Although the gut structure of *Nereis diversicolor* has not been described, it appears to be closely similar to that of other Nereidae. In fact, the epithelium of the intestine part is composed of numerous typical absorptive cells and of fewer secretory cells, although the nature of the secretion is unknown (Punin and Lukyanova, 1984). However, MPII-containing cells, as described in the present study, have not previously been reported in the gut of Nereidae. Morphologically, these cells are distinguishable

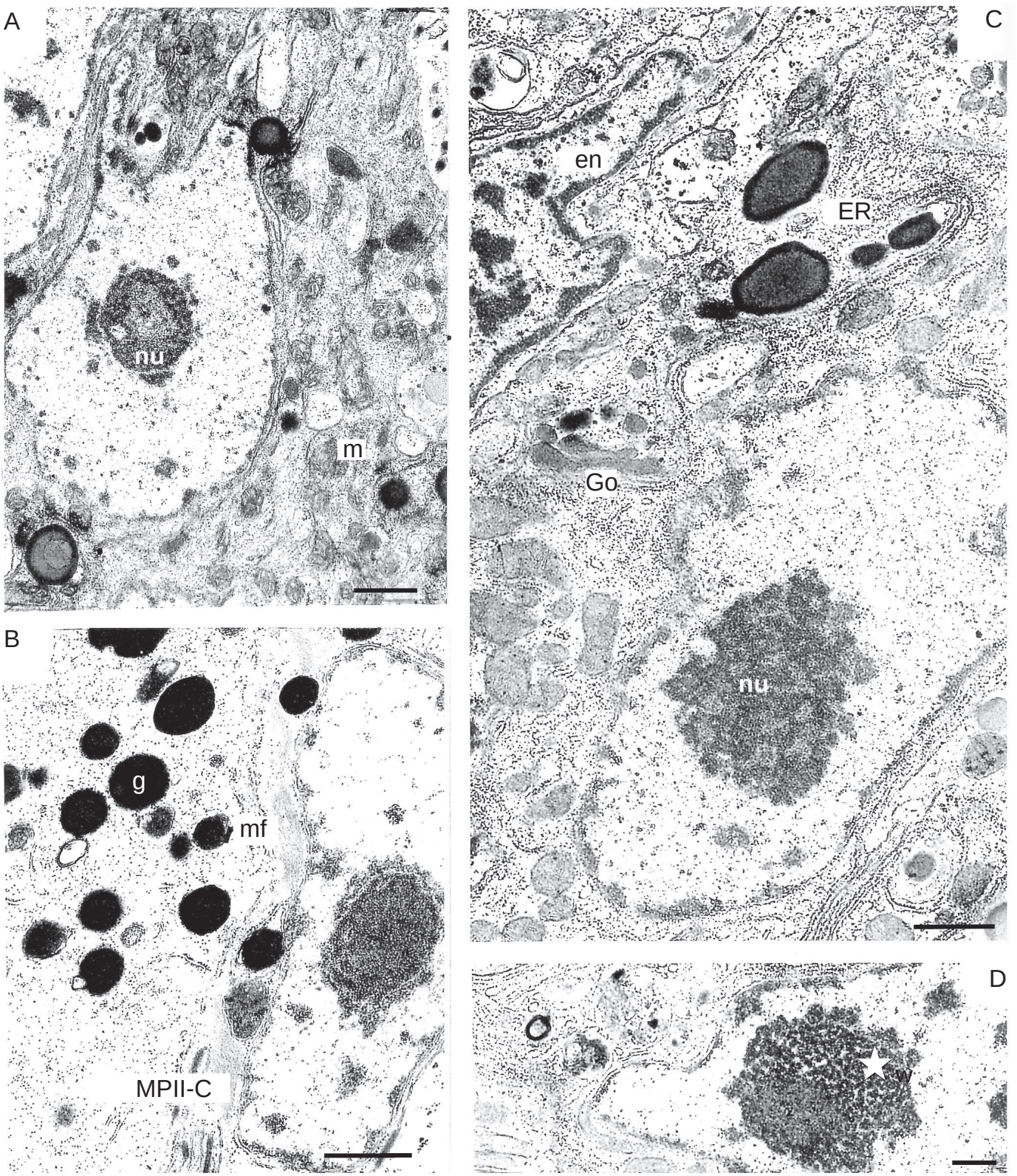

Fig. 5. Ultrastructural characteristics of MPII-containing cells (MPII-C). (A) Control worm intestine. Numerous mitochondria (m) are dispersed in the cytoplasm. ER and Golgi apparatus are poorly developed. nu, nucleolus. (B) MPII-C of the intestine from a chronically Cd-exposed worm, showing characteristic granules (g). mf, micro-filaments. (C) MPII-C from an acute Cd-exposed worm. Note the development of endoplasmic reticulum (ER) and Golgi apparatus (Go). Nucleolus (nu) shows intrication of fibrillar and granular material. En, enterocyte nucleus. (D) Proliferation of granular nucleolar material (asterisk) in MPII-C from a chronically Cd-exposed worm. Scale bar, 1 μm.

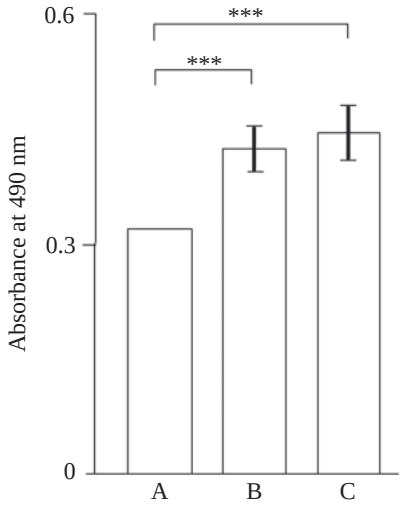

Fig. 6. Results of the ELISA on midgut extracts using the mAb anti-MPII. (A) Control worms, (B) chronically Cd-exposed worms, (C) acute Cd-exposed worms. Values are means ± S.E.M. (N=3) ***Significant difference (P<0.001).

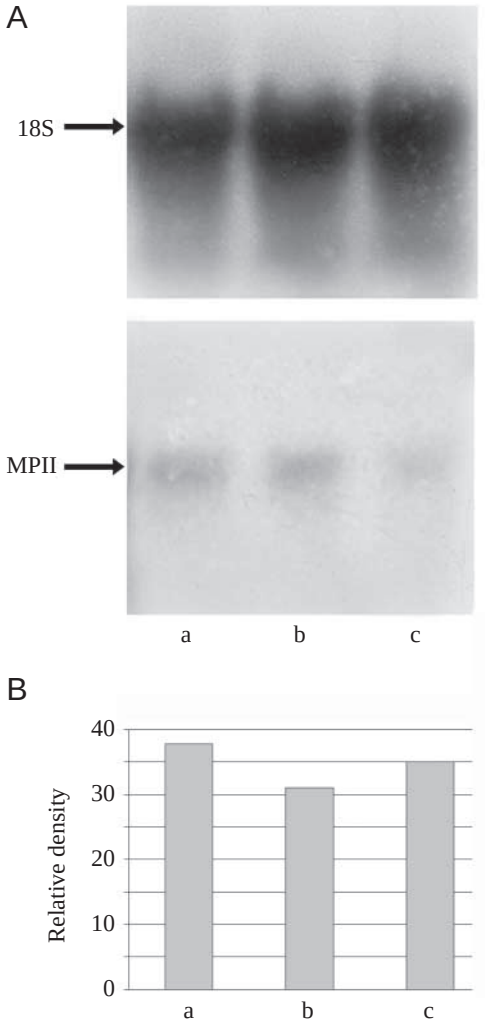

Fig. 7. Semi-quantitative northern blot analysis: MPII mRNA levels were unchanged regardless of intoxication in control (A), acute (B) or chronically (C) intoxicated worms. (A) Northern blot of 18S and MPII RNA (see Materials and methods). (B) Density quantification of MPII RNA content after analysis using Quantity One software (Bio-Rad), relative to 18S expression level.

from typical serous cells by possessing a large nucleolus and dense cytoplasmic granules with homogenous material. Indeed, according to Michel and Devilliez (1979), zymogen granules of serous cells are characterized by spiral internal condensations.

In comparison with control animals, no abnormal structures such as necrosis or degenerative patterns were observed in the gut epithelium of individuals exposed to Cd. However, cytological observations seemed to indicate the appearance of a real cellular activation process, viewed by the structural modification of the nucleolus. The nucleolus is the site of rRNA synthesis processing and ribosome assembly (reviewed in Hadjiolov, 1985). It is generally agreed that the granular component of nucleoli represents a region where ribosomes are assembled and from which they pass out of the nucleolus (reviewed in Goessens, 1984). In the cytoplasmic region of MPII-C, the increased importance of rough endoplasmic reticulum and the secretory activity of the Golgi apparatus suggested that the effect of Cd on these cells resulted in a synthesis of proteins. The MPII or MPII-like content of the gut was shown by ELISA to be increased in the case of Cd exposure, so these ultrastructural modifications could reflect an increased synthesis of MPII. In addition, the detection of MPII mRNA transcripts, realized by *in situ* hybridization, in the cells of the intestine of control worms indicates clearly that this organ is implicated in the synthesis of this protein, which would play a role in the normal metabolism of this worm.

A possible role for MPII could be involvement in the binding of an essential metal Fe during hemoglobin metabolism. In fact, metallothionein of the digestive gland of the winkle was thought to be associated with regulation of the essential metal Cu during metabolism of hemocyanin (Bebianno et al., 1992; Bebianno and Langston, 1995; Langston and Zhou, 1987). However, comparison of the relative abundance of MPII mRNA transcripts in the gut of non-exposed and Cd-exposed worms realized by northern blotting did not reveal any changes in the expression levels of MPII mRNA following exposure to Cd. These findings seem to indicate that MPII is apparently not transcriptionally regulated when cadmium levels are high (compared to control worms), but there is nevertheless synthesis of the protein in acute and chronically intoxicated worms.

In contrast with metallothionein mRNA, which has been proposed as a biomarker in the field of environmental toxicology (Chan, 1995; Tom et al., 1999), MPII mRNA does not seem to be a good candidate. This kind of regulation at the protein level appears similar to that reported for mammalian ferritin, an iron-storage protein. In this case, there is translational regulation of ferritin synthesis in response to iron but not transcriptional regulation. When cellular iron content

is low, an iron-regulatory protein (IRP) is able to bind to the 5′ untranslated region of the ferritin mRNA and thus repress the translation. When cellular iron content is high, in the absence of new transcription, ferritin is thus synthesized (Harrison and Arosio, 1996).

The higher levels of MPII in Cd-exposed worms could also be explained by a longer half-life of the molecule when bound to Cd. In fact, Bebianno and Langston (1998) reported that the half-life of metallothionein induced by exposure to Cd in the digestive gland of the winkle is very long and probably linked to the formation of a very stable complex of Cd and metallothionein. In addition, it was reported that the turnover rates of metallothionein induced by exposure to Cd (a non-essential metal) are slow by comparison with those induced by copper or zinc that are essential metals.

An alternative hypothesis from these results is that protein synthesis of products reactive to the mAb used in this study does occur, but without an increase in MPII mRNA expression, as the specific probe used in the molecular biology experiments revealed that there was not any relevant change in MPII mRNA levels after stimulation with Cd. We can speculate that the mAb used here could recognize several isoforms of MPII, since at least two isoforms have actually been identified in *N. diversicolor*, whereas the probe used for northern blot and *in situ* hybridization is specific for only one kind of MPII RNA, as shown by the controls. It is thus possible that we detected the modulation of one isoform at the protein level. In fact, Lemoine and Laulier (2003), studying the metallothionein mRNA level in the mussel *Mytilus edulis* following metal exposure, found different induction patterns of mRNA for two different metallothioneins.

There are several hypotheses concerning the role of this MPII. MPII isolated from whole worms by Demuynck et al. (1993) showed 80.8% identity with *N. diversicolor* myohemerythrin (Takagi and Cox, 1991), so MPII could be considered as an isoform of this myohemerythrin. Thus, the present molecule (MPII or MPII-like) detected in the intestine, can be considered to be similar. However, the physiological function of a myohemerythrin-like molecule in digestive cells is quite puzzling. The literature reveals two related respiratory pigment candidates: hemerythrins and myohemerythrins.

Hemerythrins are multisubunit, non-heme-iron, O_2-carrying proteins distributed among species of four groups of invertebrates, especially sipunculids (Klippenstein, 1972; Loehr et al., 1978), but also in brachiopods, priapulids (Terwilliger, 1998; Kurtz, 1992) and one polychaete annelid, *Magelona* (Benham, 1897). They are localized in free blood cells called hemerythrocytes, where they act as high-affinity dioxygen carrier molecules (Mangum, 1976).

Myohemerythrins are monomeric proteins resembling hemerythrin subunits in structure. They were first described in sipunculids as a storage form for dioxygen in the muscles of the trunk, and then reported in various annelids, including the polychaete annelid *Nereis diversicolor* (Takagi and Cox, 1991; Demuynck et al., 1991, 1993), the oligochaete species *Allolobophora caliginosa* (Nejmeddine et al., 1992) and two species of achaete worms, *Theromyzon tessulatum* (Coutte et al., 2001) and *Hirudo medicinalis* (Wang et al., 2002). Depending on the animal group, the localization of this molecule differs. Thus, in the leech *T. tessulatum*, the molecule was purified from yolk granules and coelomic fluid (Baert et al., 1992) but the site of synthesis was not reported by the authors. By contrast, in the species *H. medicinalis* (Wang et al., 2002), myohemerythrin was seen to be expressed in serotonergic neurons (Retzius cells). For oligochaetes, as described here for *N. diversicolor*, the molecule was recorded in the gut of the earthworm (Nejmeddine et al., 1992) and it was also able to bind Cd. In *Nereis diversicolor* the intestine constitutes an additional location for this type of molecule, since it was first recorded in cytoplasmic granules of one type of coelomic cells: the granulocytes I (Dhainaut-Courtois et al., 1987; Porchet-Henneré et al., 1987).

At this time, the exact role of these proteins MPII remains unclear. In fact, although ability to bind dioxygen was not determined for the MPII (Demuynck et al., 1993) or for the myohemerythrin (Takagi and Cox 1991), both amino acid sequences contained the hemerythrin signature sequence and the iron ligand residues of *Themiste zostericola* myohemerythrin (Sheriff et al., 1987) or of *Themiste dyscritum* hemerythrin subunit (Stenkamp et al., 1984). In addition, both proteins contain a leucine residue at position 104 that would be necessary for the binding of dioxygen (Xiong et al., 2000). For these reasons in *Nereis diversicolor*, although the main O_2-carrier is a giant extracellular hemoglobin present in a complex vascular system including blood sinus surrounding the gut, MPII could act as a dioxygen supplier. Its activity would perhaps occur in internal compartments of the body (i.e. the intestine or the coelom). Since MPII is devoid of Fe atoms when it is linked to Cd, probably because of competition with Fe and/or a particular interaction with amino acids of this myohemerythrin-like molecule (Demuynck et al., 1993), binding of Cd to the MPII should be considered as detrimental for the organism and so could explain the increased level of MPII in the gut following cadmium treatment of the worms. Thus, Cd binding to this molecule would represent a biological sign of exposure. However, since no significant differences in gut MPII levels were observed between acute and chronically exposed worms, MPII level could not be viewed as a potential biomarker of Cd exposure. Nor does it seems that MPII mRNA is a biomarker since its level was unchanged in non-exposed and Cd-exposed worms.

On another hand, if we consider that this molecule is not able to carry dioxygen, its function could be to serve as a trap for excess essential or non-essential free metal ions in order to protect the integrity of the worm. Thus, the status of this protein would be to play a role in the detoxification of metals, like the metallothioneins. In fact, many invertebrates can react to metallic stress by increasing metallothionein synthesis. However, the mechanisms by which metallothioneins are regulated in invertebrates are still poorly understood (Lemoine and Laulier, 2003) and the primary function of metallothionein remains enigmatic (Vasak and Hasler, 2000). The presence of

metallothioneins was reported in oligochaetes (Furst and Nguyen, 1989), located in the gut (Morgan et al., 1989). However, to date, no metallothioneins have been isolated from Nereids. In fact, metabolic labelling using ^{35}S-cysteine and subsequent separation of the proteins from Cd-exposed *N. diversicolor* by two-dimensional electrophoresis failed to detect metallothioneins in this species (Ruffin et al., 1994). Since *Nereis diversicolor* is characterized by a high resistance to trace metals, the hypothesis of a function for MPII in detoxification of trace metals is possible. In fact, the LC_{50} of Cd is about 100 mg l^{-1} after 192 h of exposure (Bryan, 1976), in contrast to 0.1–10 mg l^{-1} for most marine invertebrates (Cossa and Lassus, 1989). The particular location of MPII or MPII-like protein in the midgut, considered as the main route for metal entry into the body, and the synthesis of this molecule in the case of metal exposure, could be interpreted as a defence mechanism to regulate the uptake of metal *via* the gut. Additional experiments have to be performed to determine which hypothesis is correct.

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