Cadmium accumulation and its effect on gene expression have been investigated at sublethal cadmium concentrations in the soil oligochaete Enchytraeus buchholzi. This worm is capable of accumulating cadmium to large amounts, which coincides with the induction of a mRNA isolated as a cDNA clone by differential screening of a cDNA library constructed from cadmium-treated enchytraeids. The cDNA clone designated CRP1 is 1474 base pairs in length and contains a 753-base pair open reading frame, encoding a novel Cys-rich non-metallothionein protein. In vitro translation of the in vitro transcribed CRP1 results in a protein with a molecular mass of 25 kDa and an pI of approximately 7.5. These values are consistent with those predicted from the deduced amino acid sequence. The CRP protein contains 27% Cys, most of them arranged in Cys-X-Cys and Cys-Cys segments. The sequence is also characterized by a 31-amino-acid motif, which is tandemly repeated along the sequence. Northern blot analysis reveals that the CRP gene is not constitutively expressed in untreated worms, but rather it is rapidly induced by cadmium. The CRP gene may be a promising candidate gene for monitoring bioavailable cadmium at subtoxic levels in terrestrial environments.

The anthropogenic cadmium pollution of the environment is increasingly realized as a hazard for ecosystem dynamics and even for human health (1). Fortunately, many organisms are capable of accumulating and detoxifying cadmium to a variable extent (2). A well-known mechanism of cadmium detoxification is the binding of cadmium to metallothioneins, which are cytosolic low molecular mass cadmium-binding proteins, present in a wide variety of organisms (3). Often metallothioneins occur in parallel with other cadmium-binding proteins of higher molecular masses. Their characterization, however, has been largely neglected to date (4).

Much interest has focused on oligochaetes of the genus Lumbricus. These earthworms are able to accumulate cadmium by binding to metallothionein-like proteins (5). Currently, they are used for monitoring the bioavailability of cadmium in terrestrial environments (6). Only little attention has been paid to enchytraeid oligochaetes, although they may be capable of accumulating cadmium to a greater extent than many lumbricids.
Concentrations in fluid medium for induced mortality. The worms were incubated at different cadmium concentrations in fluid medium for 6 days. The cadmium content of worms was measured by atomic absorption spectroscopy. The data represent the mean of two different experiments. Bars indicate maximal variations. For the determination of cadmium effects on mortality, at least 20 worms were examined at each cadmium concentration. No mortality occurred in controls.

Northern Analysis—Total RNA (15 µg) or poly(A)+ RNA (5 µg) were separated in 1% agarose formaldehyde gels, blotted onto a nylon membrane filter (Nytran, Schleicher and Schuell), and hybridized for 16 h at 65 °C in 6 x SSC, 0.5% SDS, 100 µg/ml salmon sperm DNA with 32P-labeled DNA probes synthesized by random priming from CRP1-cDNA. After washing in 0.1 x SSC, 0.1% SDS for 1 h at 65 °C, the filters were exposed to Kodak X-OMat films.

DNA Sequencing—Dideoxy sequencing of cDNA in pBluescript SK vector was performed on the ALF DNA Sequencer using the AutoRead DNA Sequencing kit (Pharmacia, Freiburg, Germany). For sequencing of the total cDNA insert, sequential deletions were generated by exonuclease III digestion as described recently (14). Sequence data, which were obtained from both strands, were analyzed with PC/Gene software (Intelligenetics, Mountain View, CA). EMBL data bases were used to compare DNA and deduced protein sequences.

In Vitro Transcription and Translation—Approximately 1 µg of CRP-cDNA was transcribed in vitro using the Promega transcription in vitro system and T3 RNA polymerase (Promega, Heidelberg, Germany). The RNA was extracted with phenol-chloroform and then in vitro translated using the Promega rabbit reticulocyte lysate system and the ICN translase mixture containing [35S]methionine and [35S]cysteine (>1000 Ci/ mmol, ICN). Samples of 35S-labeled translation products were separated on 10–15% SDS-PAGE according to Chamberlain (18). The gels were fluorographed according to Chamberlain (18).

RESULTS

Cadmium Accumulation—Mature worms of E. Buchholzi were incubated at different cadmium concentrations for 6 days. Fig. 1 shows the effect of cadmium on mortality. The median lethal concentration (LC50) was 7.2 (range, 6.4–8.1) mg cadmium/liter. No mortality occurred at and below 4 mg cadmium/liter. Under such sublethal conditions, the worms still accumulated considerable amounts of cadmium (Fig. 1), but they did not reveal any detectable injuries such as inhibition of reproduction, disintegrations, or loss of segments. We therefore decided to investigate cadmium-induced gene expression in worms exposed to the subtoxic cadmium concentration of 3 mg cadmium/liter.

Identification of Cadmium-induced Transcripts—In order to detect cadmium-inducible mRNAs, we constructed a cDNA library from cadmium-treated E. Buchholzi. Differential screening of 3 x 106 recombinants of the amplified library resulted in the identification of five cDNA clones hybridizing specifically to cadmium-induced 32P-labeled cDNA probes. Subsequent sequencing of the 5' and 3' terminus of these five cDNAs revealed that they were presumably derived from the same mRNA. We therefore focused our studies on the largest clone, designated CRP1. This had a size of approximately 1.5 kilobases. A mRNA species of approximately the same size was detected in cadmium treated but not in untreated worms by Northern blot analysis (Fig. 2a). Obviously, CRP1 is specifically induced by cadmium.

Nucleotide Sequence of the CRP1 cDNA—Fig. 3 shows the complete sequence (1474 bp) of the CRP1-cDNA. The putative open reading frame of 580 bp is very rich in AT and contains 5 ATTTA motifs. The polyadenylation signal AATAAA is located 16 bp upstream of the poly(A) tail. EMBL data base searches do not detect any significant homologies between the CRP1-cDNA and other hitherto known sequences.

Kinetics of CRP1 Gene Expression—Mature E. Buchholzi were exposed to 3 mg cadmium/liter for different periods. The time course of CRP1-mRNA induction in E. Buchholzi was monitored by Northern blot analysis (Fig. 2b). In untreated worms, there is no expression of CRP1 at all. However, CRP1-mRNA was already detectable after 6 h of cadmium treatment. Maximal expression occurred after 12 h of cadmium exposure and remained rather constant during the following 58 h.

Deduced Amino Acid Sequence of CRP1—The cadmium-induced CRP protein, predicted from the nucleotide sequence, contains 251 amino acids and has a molecular mass of 25 kDa. There is a high prevalence of Cys residues (27%), whereas aromatic amino acids are absent. About 70% of the Cys residues are arranged in Cys-X-Cys and Cys-Cys segments. The protein shows a tandem repeat structure which extends from the first Cys residue at position 11 to the carboxyl terminus (Fig. 4a). Seven repeats contain 31 amino acid residues, whereas the carboxyl-terminal repeat lacks the last 7 amino acids. The structural identity in the repeats is based upon a motif of 9 Cys residues, although there are also several conservations of the non-Cys residues. The amino acid sequence of the CRP protein differs from any other known proteins as revealed by comparison with the sequences of the SWISSPROT data base.

In Vitro Translation of CRP1—The CRP1-cDNA was transcribed in vitro, and the resulting RNA was then translated in vitro in a rabbit reticulocyte lysate system in the presence of [35S]methionine and [35S]cysteine. SDS-PAGE reveals the
Novel Cys-rich, Non-metallothionein Protein

FIG. 3. Full-length CRP1-cDNA sequence and deduced amino acid sequence. Underlined sequences indicate the Kozak motif at position 139-145, the polyadenylation signal at position 1453-1458, and the ATTAA segments in the 3' non-coding region.

Novel Cys-rich, Non-metallothionein Protein

a

b

CRP1 translation products as one broad band at about 35 kDa (Fig. 5b). However, this molecular mass can be shifted to 25 kDa after blocking the sulfhydryl residues by carboxymethylation. This molecular mass is identical to that predicted from the deduced amino acid sequence of the CRP protein. Two-dimensional gel electrophoresis reveals a slightly basic isoelectric point (pI) of approximately pH 7.5 for the CRP1 translation products (Fig. 5b). This fits the predicted pI of 7.8 for the CRP protein, as calculated by computer-assisted analysis of the deduced amino acid sequence.

**DISCUSSION**

This study shows that the soil oligochaete *Enchytraeus* accumulates cadmium at sublethal concentrations to consider-
able amounts, and this is associated with the induction of a gene encoding a novel cysteine-rich protein designated CRP. Indeed, a cadmium-induced cDNA clone was isolated from a cDNA library constructed from cadmium-treated Enchytraeus using the differential screening method. This clone very likely represents the full-length copy of the CRP-mRNA coding region since an open reading frame cannot be deduced upstream from the starting ATG codon, and the size of CRP1 is matching very well with that of the corresponding mRNA as revealed by Northern blot analysis. The molecular mass of the deduced CRP protein is 25 kDa. However, an apparent molecular mass of about 35 kDa was obtained, when the translation products of in vitro transcribed CRP1-cDNA were separated by SDS-PAGE. This discrepancy obviously reflects an interaction of the sulfhydryl groups by carboxymethylation.

The cDNA deduced amino acid sequence of CRP exhibits an organization, which is normally typical for heavy metal-binding proteins. (i) The amino acid composition of the CRP protein is nearly identical with that of a recently reported metallothionein from the mussel Mytilus edulis (20). (ii) The high cysteine content and the arrangement of the cysteine residues in Cys-X-Cys and Cys-Cys motifs strongly resembles the structure of metallothioneins. Also, the heavy metal-binding phytochelatins in plants reveal such Cys-X-Cys motifs derived from the γ-GluCys portion of glutathione (21). (iii) The CRP protein largely resembles the structure of metallothioneins and Cys-Cys motifs strongly resembles the structure of metallothioneins, which is normally typical for heavy metal-binding phytochelatins in plants revealing such Cys-X-Cys motifs derived from the γ-GluCys portion of glutathione (21). (iv) The CRP protein contains histidine, which is always lacking in metallothionein. Moreover, metallothionein and CRP differ with respect to the expression pattern, thus presumably indicating a different physiological significance. Normally, metallothioneins are constitutively expressed in tissues undergoing rapid growth and development and are widely believed to play an important role in macromolecular synthesis, so as to provide copper and zinc to newly synthesized apoenzymes (23). In contrast to metallothionein, the CRP gene is not constitutively expressed under normal physiological conditions but is rather rapidly induced under cadmium stress. Indeed, the expression of CRP mRNA begins at 6 h after cadmium treatment reaching its maximum level after 14 h. Moreover, it is likely that the CRP mRNA is rapidly degraded in the worms. This can be deduced from the 3' non-coding nucleotide sequence, which contains several ATTATA segments, usually found in mRNA species with a short metabolic half-life (24, 25).

Such a high turnover rate is characteristic for mRNAs encoding proteins which are only needed under special conditions because it permits both a rapid induction and a rapid cessation of protein synthesis (26).

Finally, our data support the view, that the CRP protein is involved in the cadmium detoxification process in enchytraeids. Although the functional significance of CRP in this process remains to be elucidated, the CRP gene is a promising candidate gene to indicate a beginning cadmium intoxication in soil oligochaetes.

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