

Cloning and expression of *Saccharomyces cerevisiae* copper-metallothionein gene in *Escherichia coli* and characterization of the recombinant protein

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The gene sequences for intact and truncated forms of copper-binding metallothionein from *Saccharomyces cerevisiae* were cloned and overexpressed in *Escherichia coli* BL21(DE3)pLysE cells. In contrast to several other genes, the intact and truncated metallothionein genes are amplified in the polymerase chain reaction when Mg^{2+} is replaced by Co^{2+} . The recombinant truncated protein binds copper *in vivo* and *in vitro*. A ratio of 8 Cu/12 cysteines was determined from atomic absorption, X-ray fluorescence and amino acid analysis. Extended X-ray absorption spectroscopy indicates that all Cu is in Cu(I) form and coordinated to three S atoms.

Metallothioneins (MT) are low-molecular-mass proteins with high cysteine contents that bind metals through thiolate bonds and appear to be involved in metal homeostasis and heavy-metal detoxification in eucaryotic organisms, fungi and yeast (for reviews see Kägi and Kojima, 1987; Kägi and Schaeffer, 1988; Riordan and Vallee, 1991). The Cu-MT which mediates copper resistance in the yeast *Saccharomyces cerevisiae* is a class-II MT, having no evolutionary relationship to the mammalian MT. It is the gene product of the CUP1 locus (Fogel and Welch, 1982), which regulates its own expression by sequestering the free Cu required by its ACE1 positive transcription factor to bind DNA (Fürst et al., 1988).

The purified yeast Cu-MT is a 53-residue protein containing eight Cu(I) atoms ligated to 12 cysteines and lacking the first eight amino acids from the amino terminal (truncated Cu-MT) coded for in the CUP1 locus (Winge et al., 1985; Wright et al., 1987).

In the absence of crystallographic data, models of Cu-S coordination in yeast MT (reviewed in Winge, 1991) have been based on spectroscopic and biochemical analyses. Isolated yeast Cu-MT was first studied using extended X-ray absorption fine structure (EXAFS) by Bordas et al. (1982, 1983), but the protein was erroneously assumed to contain 4 Cu atoms/mol and the Cu/S ratio was taken as 1 : 2. A more recent investigation (George et al., 1988), based on a Cu/S ratio of 8 : 12, led to a model in which a single solvent-excluding Cu(I)-S cluster, consists of a distorted cube with a Cu(I) ion at each corner, connected trigonally by doubly

bridging cysteinyl thiolates. This model is, however, not unique and models with two or more equivalent metal centres and/or with triply bridging sulphurs are equally possible. These models should be seriously considered, since Weser and Hartmann (1991) have reported that two of the eight Cu ions are more reactive, less tightly bound and silent in circular dichroism.

Obviously, the elucidation of the structure of the metal centre(s) in yeast MT will require detailed crystallographic and/or NMR studies besides the X-ray spectroscopic observations. The absence of sufficient amounts of purified material has, to date, precluded these approaches.

In this study, cloning and overexpression of the yeast metallothionein gene (mt gene) are reported together with EXAFS data from the isolated recombinant truncated protein. Conditions for optimum expression, detection, isolation and characterization of the truncated Cu-MT were developed. The structure of the metal-binding site of the recombinant protein, which binds, Cu *in vitro* and *in vivo*, was probed by X-ray absorption spectroscopy. In the present state of progress with the purification, EXAFS is one of the few, if not the only method available to characterize the metal centre(s). The copper is present as Cu(I), indicated by the position of the absorption edge, and each Cu(I) is coordinated with low symmetry to three sulphurs. Quantitative X-ray fluorescence combined with amino acid analysis give a Cu/S ratio of 8 : 12, in agreement with atomic absorption spectroscopy.

MATERIALS AND METHODS

Cell cultures and media

Escherichia coli strains used were JM109 (Sambrook et al., 1989), INVαF' (TA-cloning kit, Invitrogen Corporation), HMS174, BL21(DE3), BL21(DE3)pLysE, BL21(DE3)pLysS (Studier et al., 1990). Cells were grown at 37 °C in Luria-Bertani medium and plates were prepared with 1.5 % bacto agar (Difco) in this medium. Ampicillin (100 µg/ml, Sigma), chloramphenicol (50 µg/ml, Sigma) and kanamycin (50 µg/

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Abbreviations. EXAFS, extended X-ray absorption fine structure; IPTG, isopropyl-β-thiogalactopyranoside; MT, metallothionein; PCR, polymerase chain reaction; [³⁵S]ATP[S], ³⁵S-labelled adenosine 5'-[α-thio]triphosphate, X-gal 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside.

Enzymes. NdeI, BamHI (EC 3.1.21.4).

ml, Sigma) were added to liquid and solid media as indicated below. Minimum medium contained 5 mg/ml thiamine (Sigma), 1 mM MgSO₄, 0.4% glucose, 18.7 mM NH₄Cl, 22 mM KH₂PO₄ and 33.7 mM Na₂HPO₄.

Molecular cloning techniques

DNA manipulations and analyses were carried out basically according to the procedures given in Sambrook et al. (1989).

Restriction enzymes *Nde*I and *Bam*HI (Boehringer Mannheim) were used for screening for the cloned mt gene. The 5' ends were dephosphorylated using calf intestinal alkaline phosphatase (Stratagene). Enzymic digestions were terminated by extraction with Strataclean resin (Stratagene) and ligations were carried out using a T4 ligation system (Amersham).

DNA was sequenced by dideoxy-mediated chain termination (Sanger et al., 1977) using the Sequenase Version 2.0 Sequencing kit (United States Biochemical) with the following modifications. Approximately 2 µg double-stranded DNA was used as template and the sequencing primers were added to the plasmid pellet prior to drying. For sequencing, the pellet was resuspended in 8 µl distilled H₂O and 12 µCi ³⁵S-labelled adenosine 5'-[α-thio]triphosphate ([³⁵S]ATP[S]; Amersham) was used for each sequencing reaction. The 5.7% acrylamide/0.3% bis-acrylamide in 8 M urea sequencing gel was prepared according to Maxam and Gilbert (1977) and run at 65 W constant power. Gels fixed in 15% acetic acid were exposed to a Kodak X-omat AR film.

In vitro gene amplification by the polymerase chain reaction

Yeast genomic DNA, used as template in the reaction, was purified from wild-type *S. cerevisiae*, following the method of Rose (1987) without the final sucrose and CsCl gradient steps. The polymerase chain reaction (PCR) was carried out using an *in-vitro* DNA amplification system (Perkin Elmer Cetus) with 10 ng/µl template DNA and the temperature cycle was 1 min at 94 °C, 1 min at 55 °C and 1.5 min at 72 °C, repeated 30 times using the Hybaid (Biometra) temperature-controlled block. The purified primers used in the reaction were obtained from the Oligonucleotide Synthesis Service at the EMBL in Heidelberg, FRG.

The effect of cations on the PCR was tested by replacing MgCl₂ by CoCl₂, CuCl₂ or NiCl₂ in the PCR mix. Conditions were as described above, except that beside yeast DNA constructs of pET-3a vector with mt gene, the gene for the ribosomal protein L9 from *Bacillus stearothermophilus* (Vorgias et al., 1991), genes for α and β subunits of HU histone-like DNA-binding protein from *E. coli* (Padas et al., 1992), or genes for cell-division control proteins from yeast (kindly provided by P. Wagner, Homburg, FRG) were also used as templates. As these genes had been inserted between the *Nde*I and *Bam*HI sites of the pET-3a vector, the same primers could be used in all cases.

Subcloning, cloning and expression

PCR products, resuspended in distilled H₂O after ethanol precipitation, were directly subcloned using the TA cloning kit (Invitrogen Corporation). Transformed *E. coli* INVαF' cells harbouring the PCR1000-mt plasmid, were selected on Luria-Bertani agar plates containing 5-bromo-4-chloro-3-in-

dolyl-β-D-galactopyranoside (X-gal) (Applied Genetic Systems) and kanamycin. Large-scale preparations of the construct from the positive cells were carried out using Qiagen-tip100 ion-exchange columns (Diagen Inc.). The *Nde*I-*Bam*HI restriction fragment, corresponding to the mt gene, was purified from the isolated construct using the Mermaid kit (BIO101) and stored at -20 °C. In some experiments, pUC19 (Sigma) vector was used for subcloning the mt gene in *E. coli* JM109 cells.

The pET-3a expression vector (Studier et al., 1990) was maintained, propagated in and purified from *E. coli* HMS174 cells. Extraction of the plasmid was carried out according to the protocol (Qiagen) or using Elutip-d ion-exchange columns (Schleicher and Schuell). The mt gene fragment was inserted between the *Nde*I and *Bam*HI restriction sites and ampicillin plates were used to select positively transformed *E. coli* HMS174 cells. This construct, purified from HMS174 cells, was used to transform the *E. coli* BL21(DE3), BL21(DE3)pLysE and BL21(DE3)pLysS expression cells. Solid and liquid media used with transformed BL21(DE3) cells were supplemented with ampicillin, while that for BL21(DE3)pLysE and BL21(DE3)pLysS cells also contained chloramphenicol for selection of the pLys(S/E) plasmid.

Conditions for [³⁵S]cysteine pulse labelling were determined on cells grown and induced with 0.5 mM isopropyl-β-thiogalactopyranoside (IPTG; Applied Genetic Systems), in minimum medium. At different time intervals after induction, the absorbance of the culture was monitored and simultaneously 100 µl culture was incubated with 3.0 µl [³⁵S]cysteine (10 Ci/l, Amersham) for 3 min. Labelled cells were subsequently pelleted and kept at -20 °C for gel electrophoresis. The following procedure was finally adopted as optimum for cell growth and induction. A 5-ml Luria-Bertani medium culture with the appropriate antibiotic(s) was inoculated with frozen clones and grown shaking overnight at 37 °C. From this, a fresh culture was grown in Luria-Bertani medium with 0.5 mM CuSO₄ to about A₆₀₀ = 1.0 or 10⁸ cells/ml. Expression of the mt gene was induced with 0.5 mM IPTG for 1 h. Cells were harvested by low-speed centrifugation and the pellets were frozen at -80 °C.

Site-directed mutagenesis

The mt gene was modified using the oligonucleotide-directed mutagenesis system V2.1 (Amersham) with oligonucleotides designed to replace the cysteines by histidines at positions 24 and 26 of the truncated Cu-MT. The mutated mt gene was cloned and expressed using the pET-3a vector in BL21(DE3)pLysE cells, and the gene product is designated Cu-MT_{24,26}.

Isolation of the recombinant Cu-MT from *E. coli*

Unless otherwise stated, all preparations were carried out at 4 °C and under Ar. All buffers were degassed and saturated with Ar.

E. coli pellets from 1-l culture were lysed by thawing and pipetting in 7 ml lysis buffer consisting of 20 mM Tris/HCl, pH 8.0, 1 mM phenylmethylsulfonyl fluoride and 0.2% 2-mercaptoethanol. The viscous lysate was made 1.5 mM in MgCl₂, sonicated for about 1 min and centrifuged at 100 000×g for 30 min. This step was followed by extracting the pellet once more with 1 ml lysis buffer. The combined supernatant was heated to 60 °C for about 2 min until a heavy white precipitate appeared, and the insoluble material

was separated by centrifugation as above. The final soluble extract was loaded on a 35-ml DEAE-cellulose (Sigma) column equilibrated with 10 mM Tris/HCl, pH 8.0, 0.2% 2-mercaptoethanol (buffer A). The column was washed and run at 2.8 ml/min and 4.2 ml fractions were collected. Proteins bound to the column were eluted with a KCl gradient of 0–0.4 M in buffer A. Cu-containing fractions were determined spectrophotometrically as described below, pooled and dialysed overnight against buffer A without 2-mercaptoethanol (buffer B). This material was concentrated by covering the dialysis tube with Aquacide II (Calbiochem) and subsequently loaded on a Sephadex G-50 gel-filtration column (89 cm×2.5 cm). The column was equilibrated and run at 0.5 ml/min with buffer B and 5.0 ml fractions were collected. Cu-containing fractions were pooled and lyophilized. Material thus prepared was further purified by reverse-phase HPLC for amino acid analysis at the EMBL Heidelberg, FRG protein service group.

Detection of Cu-MT

Recombinant Cu-MT, labelled with [³⁵S]cysteine, was detected by autoradiography of non-denaturing 15% polyacrylamide and 15% SDS/polyacrylamide gels run according to Laemmli (1970). With non-denaturing gels, the soluble cellular extract was loaded either directly or after carboxymethylation. For denaturing gels, cells were lysed and carboxymethylated as described by Berka et al. (1988), but the loading buffer contained 0.2% SDS. Electrophoresis was carried out at 25 mA constant current until 5 min after the blue stain had run out of the gel. The radioactive signal was enhanced with Amplify solution (Amersham) and autoradiograms were recorded on Kodak X-omat AR film, exposed at –20 °C for variable times. Where necessary, proteins other than Cu-MT in the preparation were visualised by staining with Coomassie blue (Merck) or silver (Amersham).

Cu-MT was also detected by monitoring copper using diethyldithiocarbamic acid (Sigma) as a staining agent and for spectrophotometric analysis. Protein solutions were analysed without carboxymethylation on non-denaturing 15% polyacrylamide gels as described above, and the gels were stained with 0.2% diethyldithiocarbamic acid (mass/vol.) which produced a brown band when complexed with Cu (Lo et al., 1982). Spectrophotometric scans over 800–200 nm were performed on samples made 0.05 M in HCl and 0.2% in diethyldithiocarbamic acid using a double-beam spectrophotometer (Uvikon 810, Kontron). The acidified protein solution was used as reference and the sample was the same solution containing, additionally, 0.2% diethyldithiocarbamic acid. The peaks at 280, 450 and 650 nm are indicative of Cu. In the case of untreated protein in buffer B, Cu(I)-S bonding is detected as a shoulder at 280 nm (Winge et al., 1985; Byrd et al., 1988).

Finally, MT was also monitored by amino acid analysis and Cu was determined by atomic absorption spectroscopy at the Institut für Angewandte Analytik an der Universität Hamburg GmbH, FRG.

EXAFS measurements

X-ray absorption data were collected at 20 K on lyophilized samples of truncated recombinant Cu-MT on the EXAFS beamline (Hermes et al., 1984; Pettifer and Hermes, 1985, 1986) of the EMBL Outstation at the Deutsches Elektronen Synchrotron, FRG. X-ray fluorescence and trans-

mission data were measured, the former using a multielement solid-state detector system (Cramer et al., 1988). X-ray absorption spectroscopy data were collected both at the Cu and Zn absorption edges. Curve-fitting analysis was carried out using EXCURVE (Gurman et al., 1984, 1986).

RESULTS

In-vitro amplification, cloning, expression and isolation of *S. cerevisiae* Cu-MT

Fidelity of copying of the mt gene from *S. cerevisiae* genomic DNA during PCR amplification was established by sequencing the constructs PCR1000-mt and pUC19-mt. Comparable results were obtained using the wild-type or Cu-resistant *S. cerevisiae* genomic DNA as template.

A comparison of the effects of Co²⁺, Ni²⁺ and Cu²⁺ on the amplification of the mt gene indicated that only Co²⁺, at concentrations similar to Mg²⁺, resulted in amplification. This effect was observed using both yeast genomic DNA or the pET3a-mt construct as templates and appears to be specific since out of the seven different genes tried (see Materials and Methods), only mt was amplified in the presence of Co²⁺. The increase in the A₂₆₀ value after the reaction was 10 ± 3% (10 determinations) higher with Co²⁺ than with Mg²⁺ at a concentration of 1.5 mM. The fidelity of amplification with Co²⁺ was checked by sequencing PCR1000-mt₈ constructs.

Primers for *in-vitro* amplification, illustrated in Fig. 1, were based on the yeast mt gene sequence (Butt et al., 1984). *Nde*I and *Bam*HI restriction sites were built into the 5'-end and 3'-end primers, respectively, to facilitate further cloning. Two different sequences were chosen for the 5'-end primers. The M1(N), 33 b long, was designed to copy the entire mt gene, the other designated M13(N), 39 b long, could hybridize to the sequence region coding for the ninth amino acid, Gln, of the protein facilitating copying of the gene fragment corresponding to the truncated MT. The latter had two stop codons in front of the *Nde*I restriction site to avoid amplification of the entire mt gene. The 3'-end primer M2(C), 48 b long, was designed for the complementary DNA strand. The complete mt gene, amplified using M1(N) and M2(C), was labelled mt₀ and the gene fragment corresponding to truncated Cu-MT, copied using M13(N) and M2(C) primers, was labelled mt₈. Minor side products of the PCR were also detected.

Sequencing of the PCR1000-mt₀(mt₀) and pUC19-mt₀ constructs verified the agreement between mt₀ and mt₈ sequences and the published mt gene sequence (Butt et al., 1984). Both in mt₀ and mt₈ the C at positions 102 and 135 were exchanged for T, but without consequence for the amino acid sequence.

The pET-3a expression vector and the *E. coli* used as host cells for expression are described in detail by Studier et al. (1990). Both mt₀ and mt₈ fragments were inserted between the unique *Nde*I and *Bam*HI sites of the vector and the *gene10* initiation codon at the *Nde*I site resulted in the expression of the proteins without any additional amino acids. In the three types of expression cells, synthesis of the soluble recombinant protein started about 20 min after induction. Some basal level of expression was observed, even without induction in BL21(DE3) and to a lesser extent in BL21(DE3)pLysS cells. BL21(DE3)pLysE cells were chosen as the host for mt gene expression and subsequent Cu-MT purification. Autoradiograms in Fig. 2 A–D depict a time-

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|-------------------------|--|
| 5' end mt gene sequence | TCATCACATAAA ATG TTC AGC GAA TTA ATT MET Phe Ser Glu Leu Ile |
| | Nde1 |
| M1(N) primer sequence | TCATCACATAAA <u>CAI</u> ATG TTC AGC GAA TTA ATT MET Phe Ser Glu Leu Ile |
| | Nde1 |
| M13(N) primer sequence | <u>IGATAG CAI</u> ATG CAA AAT GAA GGT CAT GAG TGC CAA TGC MET Gln Asn Glu Gly His Glu Cys Gln Cys |
| 3' end mt gene sequence | AAG AAG TCA TGC TGC TCT GGG AAA TGA AACGAATAGGTCCTTA Lys Lys Ser Cys Cys Ser Gly Lys |
| M2(C) primer sequence | TTC TTC AGT ACG ACG AGA CCC TTT ACT <u>CCT AGG</u> TTGCTTATCAGAAAT BamHI |

Fig. 1. *S. cerevisiae* mt gene sequence at the 5'-end and 3'-end regions and primers used in the PCR. 5'-end primers were M1(N) for amplification of the complete gene (mt_0) and M13(N) for the gene lacking the first eight codons (mt_8). The 3'-end primer was M2(C). Sequences not complementary to the CUP1 locus sequence are underlined.

course analysis of soluble extracts from controls and from cells expressing mt_0 and mt_8 genes. Contrary to common practice, cellular extracts were analysed on SDS/polyacrylamide gels, to allow visualization of all soluble proteins incorporating [35 S]cysteine, including the low-molecular-mass peptide which appears after induction (Fig. 2, lanes 12, 14, 16). This peptide is absent in untransformed cells (Fig. 2, lanes 1–4) and those transformed only with the vector (lanes 5–8). The position of this peptide is where MT is expected to be and, as the arrows show (Fig. 2), enhanced signals from at least two bands are detected. Continued synthesis of the recombinant protein results in a decrease in the 35 S signal from all other proteins and the strongest MT signal is observed at about 60 min after induction. The conventional method of analysis of yeast MT on a non-denaturing gel is depicted in Fig. 2 E; among the radiolabelled peptides, only the low-molecular-mass recombinant MT migrates into the gel and is visualized as a broad band.

Comparison of Fig. 2 A and C with B and D indicates that, within the time course of these experiments, i. e. about 2 h, the recombinant MT is stable regardless of whether Cu is present in the medium or not. Including 0.5 mM CuSO_4 in the culture medium significantly increases the [35 S]cysteine signal from all proteins in the cellular extract. Cu-binding by the recombinant protein is demonstrated by the non-denaturing gel in Fig. 3 A. The diethyldithiocarbamic acid band indicating bound copper in lane 2 (Fig. 3 A) coincides with the [35 S]cysteine signal in lane 1.

As a first step in the purification of Cu-MT from *E. coli*, Cu-containing material was fractionated from the heat-resistant cellular lysate on a DEAE-cellulose column. Cu-rich fractions, determined by the spectrophotometric analysis with diethyldithiocarbamic acid, eluted over 30–100% of the KCl gradient. Atomic absorption measurements yielded a total of about 2 mg Cu in the heat-resistant lysate loaded on this column which, assuming 8 Cu atoms/mol, would correspond to about 20 mg Cu-MT in the cell extract.

Cu-MT was purified from the combined DEAE-cellulose eluted fractions on a Sephadex G-50 gel-filtration column. Most of the Cu-containing material eluted at approximately ($V_{\text{elution}}/V_{\text{total}} = 0.6-0.7$). Spectrophotometric analysis of this material, shown in Fig. 4 A, displays the features due to Cu-diethyldithiocarbamic acid interaction and in Fig. 4 B the shoulder seen at about 280 nm is attributed to Cu(I)-S bonds

(Winge et al., 1985; Byrd et al., 1988). Atomic absorption measurements yield a total of 0.219 mg Cu in this pool. The protein thus purified was analysed by non-denaturing gel electrophoresis and stained for Cu and for impurities as depicted in Fig. 3 A. A major band corresponding to protein with bound Cu is seen in lane 2 (Fig. 3 A); however, when the same material is stained with silver (lane 3) impurities are detected. Note that in Fig. 3 A, lane 3, Cu-MT cannot be visualized with silver stain.

Amino acid analysis was carried out after further purification by reverse-phase HPLC. The results obtained were as follows: P 2.52 (2), G 4.73 (4), S 6.84 (7), T 2.48 (2), C 12.49 (12), D 7.53 (7), E 8.57 (10), K 7.06 (7), H 0.79 (1). They agree well with those expected from the truncated yeast MT sequence, shown in parenthesis. The yield, based on amino acid analysis, was estimated to be about 0.5 mg recombinant protein/litre *E. coli* culture. Calculations based on this yield and Cu determined from atomic absorption measurements, give a Cu/protein ratio of 8.4 ± 0.3 .

X-ray absorption spectroscopy measurements of recombinant Cu-MT

At this stage, further purification of the recombinant protein could be achieved only by reverse-phase HPLC, which yielded denatured apoprotein unsuitable for structural studies. Since none of the impurities in the preparation stained for Cu, the structure of the metal centre can be probed by X-ray absorption spectroscopy which is sensitive only to the immediate environment of the metal.

A quantitative X-ray fluorescence analysis and an amino acid analysis on the lyophilized sample confirmed the Cu/S ratio as determined from atomic absorption and amino acid analysis measurements, but, additionally, about 1 Zn/mol was also detected. The high metal concentration allowed simultaneous recording of transmission and fluorescence data at the Cu edge. A curve-fitting analysis of the EXAFS, derived from the transmission spectra shown in Fig. 5, indicates that each Cu is coordinated by three S atoms at 0.224 nm. A short Cu-Cu interaction at 0.269 nm and additional scatterers in higher shells are also observed. At this stage, it is not possible to define the Cu-S cluster unambiguously, but it appears clear that it does not have a highly symmetrical structure.

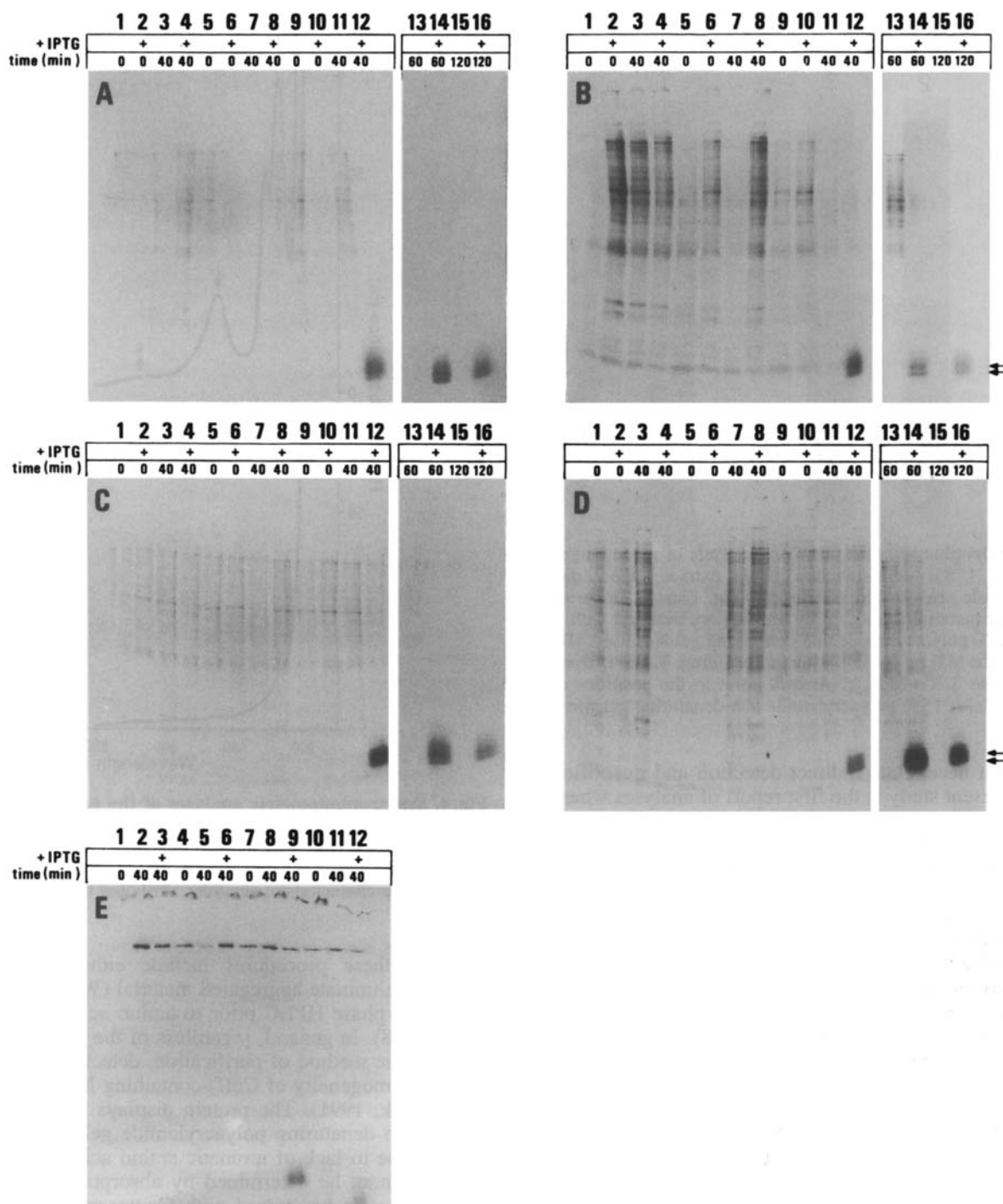


Fig. 2. Autoradiographs of electropherograms of expression. [^{35}S]cysteine-labelled lysates from non-induced and induced (+ IPTG) BL21(DE3)pLysE cells were analysed on 15% SDS/polyacrylamide gels. Induction was at $t = 0$. In all cases, cell lysate corresponding to about $0.007 A_{600}$ cells was loaded onto the gel. (B) and (D) show induction in Luria-Bertani medium, (A) and (C) show induction in Luria-Bertani medium + 0.5 mM CuSO_4 . In (A–D), lanes 1–4 show untransformed cells. Lanes 5–8 show transformation with pET-3a. In (A) and (B), lanes 9–16 transformation with pET3a- mt_6 is shown. In (C) and (D), lanes 9–16 transformation with pET3a- mt_0 is shown. The presence of Cu enhances the radiolabelling of all proteins. Films used for (A) and (C) were exposed for 2 h and films for (B) and (D) for 24 h. Arrows indicate the two bands with enhanced signals upon induction. (E) Analysis of the recombinant Cu-MT by non-denaturing gel electrophoresis. Lanes 1–3, untransformed cells. Lanes 4–6, transformation with pET-3a. Lanes 7–9, transformation with pET3a- mt_0 . Lanes 10–12, transformation with pET3a- mt_6 .

DISCUSSION

In this study, an efficient system for cloning and expression of the *S. cerevisiae* *mt* gene fragments, corresponding to both the intact and truncated forms of Cu-MT in *E.*

coli, is reported. Preparative and analytical procedures had to be developed to characterize the recombinant truncated protein, since detailed descriptions are lacking in the literature. Air sensitivity and the unusual amino acid sequence of

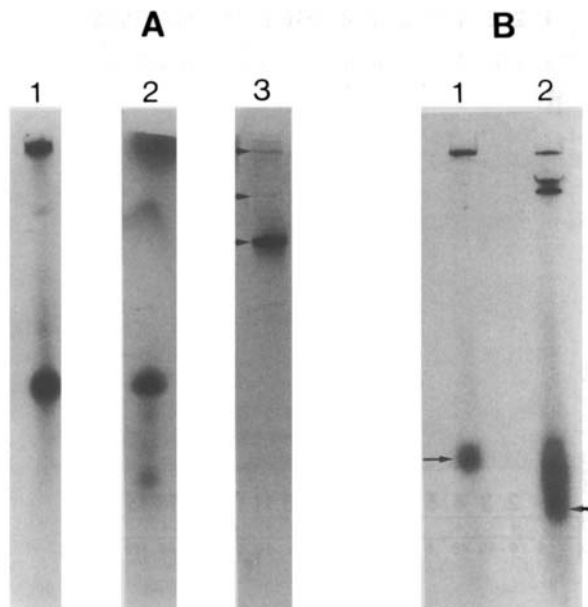


Fig. 3. Electrophoretic analysis. (A) Analysis of recombinant Cu-MT. Lane 1, [^{35}S]cysteine-labelled cellular extract. Lane 2, diethyl-dithiocarbamic-acid-stained purified protein. Lane 3, impurities in the purified material (indicated by arrows) are detected with silver stain. A 15% polyacrylamide non-denaturing gel was used. (B) Detection of Cu-MT_{24,26} by [^{35}S]cysteine labelling. Lane 1, truncated Cu-MT. Lane 2, Cu-MT_{24,26}. Arrows point to the positions of the major signals. A 15% polyacrylamide non-denaturing gel was used.

this protein necessitate indirect detection and quantification and the present study is the first report of analyses where the Cu-content, homogeneity and quantity of Cu-MT are simultaneously monitored and documented. EXAFS data probing the metal-binding characteristics of this protein are also presented.

Both sequences, coding for the intact and truncated forms of *S. cerevisiae* Cu-MT, could be efficiently amplified by PCR. For the latter, the 5'-end primer contained stop signals upstream of the point of truncation to avoid copying the complete gene. A specific feature of *in-vitro* amplification of the mt gene was that, among different genes tested, it was the only one for which the PCR took place in the presence of Co^{2+} as well as Mg^{2+} . The efficiency and yield of our expression system may be compared with that of Berka et al. (1988). These authors have cloned the gene corresponding to the intact form of *S. cerevisiae* Cu-MT in *E. coli* using a vector with the transcriptional and translational signals from bacteriophage λ . Expression was heat induced in the absence of Cu and the recombinant protein was shown to be functional by binding Cu, Cd and Zn to the induced cell extracts. The half-life of the recombinant protein was 15 min, compared to the 2 h reported here. The yield of expression was estimated to be about 1% of total cellular protein, based on the total amount of Cu recovered in a crude preparation. This material had to be further purified for amino acid analysis and the subsequent yield was not given. Yield, with our expression system, of an equivalent crude preparation would be similar, however, for the isolated protein, amino acid analysis results would correspond to about 0.5 mg Cu-MT/litre *E. coli* culture.

Procedures for purification of yeast Cu-MT found in the literature are based on conventional gel-filtration chromatography (e. g. Winge et al., 1985; Berka et al., 1988). The final

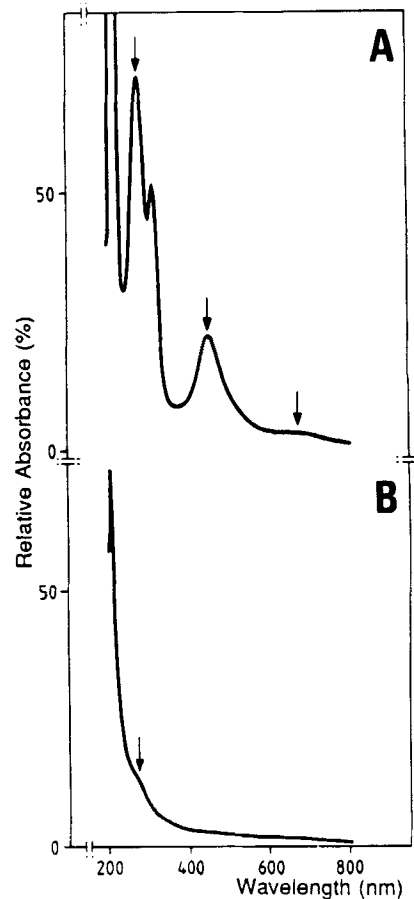


Fig. 4. Spectrophotometric analyses of the isolated Cu-MT. (A) Analysis in the presence of 0.2% diethyldithiocarbamic acid; arrows indicate peaks at 280, 450 and 650 nm, showing the presence of Cu in the solution. (B) Analysis in 10 mM Tris/HCl, pH 8.0; arrow indicates the characteristic shoulder attributed to Cu(I)-S bonds.

steps of these procedures include either gel-permeation HPLC to eliminate aggregated material (Wright et al., 1987) or reverse-phase HPLC prior to amino acid analysis (Berka et al., 1988). In general, regardless of the source of the protein and the method of purification, detecting and determining the homogeneity of Cu(I)-containing MT has been difficult (Vasak, 1991). The protein displays an anomalous behaviour on denaturing polyacrylamide gels because of oxidation. Due to lack of aromatic amino acids, the amount of protein cannot be determined by absorption at 280 nm nor can the protein be stained with Coomassie blue. Published procedures for detection of protein are analysis of [^{35}S]cysteine-labelled material on non-denaturing gels (e. g. Hamer et al., 1985; Berka et al., 1988) or detection of spectroscopic features of the bound Cu in the isolated protein (Winge et al., 1985; Byrd et al., 1988; Thrower et al., 1988; Weser and Hartmann, 1991). Cu is quantified by atomic absorption spectroscopy and purity is monitored by amino acid analysis (e. g. Winge et al., 1985; Byrd et al., 1988). Unfortunately, the yield of preparations is usually expressed in terms of their Cu content without a comparison with that expected from amino acid analysis of purified material.

For isolation of recombinant Cu-MT, a procedure similar to those given in the literature (e. g. Winge et al., 1985) was followed, except that the first step in fractionation was carried out on an ion-exchange column. In contrast to some

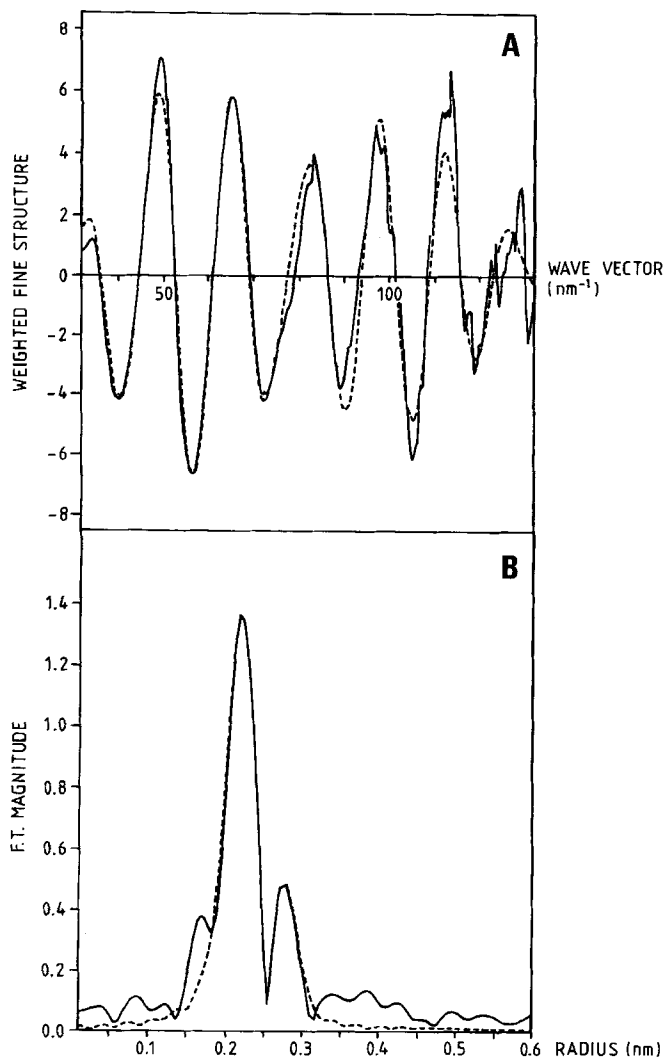


Fig. 5. Curve-fitting analysis of yeast Cu-MT EXAFS data. (A) k^3 -weighted EXAFS, where k is the magnitude of the wave vector. (B) Corresponding Fourier transforms (F. T.).

reports on yeast Cu-MT (e. g. Weser and Hartmann, 1991), there was no evidence for heterogeneity of the Cu content of the preparations. The use of diethyldithiocarbamic acid was introduced to allow rapid determination of the Cu-containing material during isolation and visualization of Cu-MT on non-denaturing polyacrylamide gels. Cu incorporation into the recombinant truncated protein *in vivo* is demonstrated in Fig. 3 by the coincidence of the positions of the [35 S]cysteine signal and the Cu-diethyldithiocarbamic acid signal. Fig. 3 also illustrates that, although on native gels analysis of cell extracts may display a single [35 S]cysteine band or the Cu-MT isolated according to conventional procedures may yield a single Cu band, the preparations still contain impurities which are detected by silver staining. Methods need to be developed for further purification of the isolated material.

X-ray absorption spectroscopy data reported here demonstrate the similarity between the native and the recombinant yeast Cu-MT. X-ray fluorescence analysis combined with amino acid analysis gives a Cu/S ratio of 8 : 12, in line with the results of atomic absorption spectroscopy. All the Cu is in Cu(I) form and coordinated to three sulphurs. The Cu-S and the short Cu-Cu distances agree well with those reported by George et al. (1988) for the native protein. More specific

conclusions on the structure of the metal site, however, requires EXAFS data from an extensive series of model compounds. These findings also imply that the interpretation of the EXAFS study of Bordas et al. (1982) on Cu-MT extracted from yeast needs revision, since it is based on an incorrect Cu/S ratio.

Currently studies to improve the yield and purity of the preparation are being carried out, in parallel, to those to characterize products of mutated mt genes. The Cu-MT_{24,26}, in which the Cys24 and Cys26 have been replaced by two histidines, still binds Cu as indicated by diethyldithiocarbamic acid analysis, but the mobility of the protein on non-denaturing polyacrylamide gels, depicted in Fig. 3 B, is significantly altered. The production of such mutants aim at changing the electronic properties of the metal-binding site in order to probe its specificity.

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