FEBS Letters 348 (1994) 228–232

FEBS 14211

Expression of functional *Raphanus sativus* antifungal protein in yeast

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Received 5 May 1994

Abstract

Rs-AFP2 is a 51 amino acid cysteine-rich peptide isolated from radish (*Raphanus sativus*) seeds that exhibits potent inhibitory activity against filamentous fungi. A cDNA clone encoding the Rs-AFP2 preprotein was modified by recombinant DNA methods to allow expression in the yeast *Saccharomyces cerevisiae*. This peptide was expressed in yeast as a fusion protein carrying at its N-terminus the prepro-sequences derived from the precursor of the yeast pheromone mating factor α1. These sequences allow secretion of the biologically active peptide in a correctly processed form. Deletion of the mating factor α1 pro-peptide drastically reduced the expression level of the peptide.

Key words: Mutagenesis; Polymerase chain reaction; *Saccharomyces cerevisiae*; Mating factor

1. Introduction

Recently, we have described a new class of antifungal proteins occurring in seeds of radish [1] and at least four other Brassicaceae species [2]. These proteins specifically inhibit a range of filamentous fungi and have no adverse effects on yeast (*Saccharomyces cerevisiae*), bacteria or cultured human cells [1]. They consist of small 5 kDa polypeptides that are highly basic and rich in cysteine. The primary structures of the two isoforms from radish seeds, Rs-AFP1 and Rs-AFP2, only differ at two positions: the Glu at position 5 in Rs-AFP1 is a Gln in Rs-AFP2, and the Asn at position 27 in Rs-AFP1 is substituted by an Arg in Rs-AFP2. Although both Rs-AFPs are 94% identical at the amino acid sequence level, Rs-AFP2 is two- to thirty-fold more active than Rs-AFP1 on various fungi [1]. This observation suggests that small alterations in the primary structure of these proteins may entail significant changes in their capacity to inhibit growth of filamentous fungi. It would therefore be worthwhile to investigate structure–activity relationships of the Rs-AFPs using site-directed mutagenesis. A prerequisite for such studies, however, is the availability of an efficient expression system allowing production of the proteins in a biologically active form.

The cDNA encoding Rs-AFP1 has recently been cloned [3] and shown to encode a preprotein with a signal peptide followed by the mature protein. Using recombinant DNA methods the cDNA encoding the Rs-AFP2 preprotein was modified to allow expression in *S. cerevisiae*. In this study we show that *S. cerevisiae* can be used as a vector for the production and secretion of Rs-AFP2.

2. Materials and methods

2.1. Strains

Plasmid cloning was done in *Escherichia coli* strain DH5α. The yeast (*S. cerevisiae*) strain c13-ABYS86 (genotype: α *pra*1–1, *prb*1–1, *cps*1–3, *ura3Δ5*, *leu*2–3, 112, *his*–), was used for protein expression. The fungal strains used to test antifungal activity were *Fusarium culmorum* (IMI 180420) and *Ascochyta pisi* (MUCL 20164).

2.2. Plasmid construction

Plasmid pFRG1 is a pBluescript IISK derivative containing a full-length cDNA clone encoding Rs-AFP1 [3]. By PCR-mediated site-directed mutagenesis [4], two mutations were introduced such that the encoded protein is the more active isoform Rs-AFP2) [3]. In this study, a third mutation (CGA to CGT for Arg^{31} of mature Rs-AFP2) was introduced to comply with the codon usage preference in *S. cerevisiae* [5]. The resulting plasmid was called pBluescript/RsAFP*.

The vectors pMFpre/RsAFP2 and pMFprepro/RsAFP2 are based on the yeast/*E. coli* shuttle vector pTG3828 [6]. pTG3828 contains a *URA3-d* selection marker, the origin of replication from the yeast 2 μ plasmid, the prokaryotic ColE1 origin of replication and the ampicillin resistance marker from pBR322. pTG3828 also contains the yeast phosphoglycerate kinase (PGK) terminator preceded by a polylinker with multiple unique restriction sites which facilitate insertion of an expression block.

The expression blocks in pMFpre/RsAFP2 and pMF prepro/RsAFP2 were derived from the M13 phage derivative M13TG5879 [7] which contains the promoter of the yeast mating factor α1 (*MFα1*) gene, the coding region of the *MFα1* pre-sequence with an engineered *Nhe*I site, and the coding region of the *MFα1* pro-sequence with an engineered *Hin*dIII site. The expression cassette of M13TG5879 was amplified by PCR using a sense primer (5'TATCA-GTCGACGCATGCTATTGATAAGATTTAAAGG, *Sal*I site underlined, *Sph*I site double underlined), which introduces a novel *Sal*I site immediately adjacent to the *Sph*I site at the 5' end of the *MFα1* promoter, and the M13 reverse primer as an antisense primer. The resulting PCR product was digested with *Sal*I–*Bam*HI and subcloned into pBluescriptII SK to yield pVD4.

Plasmid pBluescript/RsAFP2* (see above) was used as a template for the amplification of the coding sequence of mature Rs-AFP2 in two separate PCR reactions. In the first PCR reaction the sense primer (5'AATAAGCTTGGACAAGAGACAGAAGTTGTGCCAAAGG, *Hin*dIII site underlined) was designed such that sixteen extra nucleotides (coding for the last five amino acids of the *MFα1* pro-sequence)

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Abbreviations: Rs-AFP, *Raphanus sativus* antifungal protein; IC_{50} protein concentration required for 50% inhibition of fungal growth.

SSDI 0014-5793(94)00597-O

were added upstream of the coding region of mature Rs-AFP2. The *Hin*dIII site allowed in frame cloning into the *Hin*dIII site in the *MFα1* prosequence region of pVD4 [7]. The antisense primer (5'AAGGATCCCTATTAACAAGGAAAGTAGC, *Bam*HI site underlined) introduced a second stop codon and a *Bam*HI site immediately downstream of the stop codon of the coding region of Rs-AFP2. In the second PCR reaction, the same antisense primer was combined with a different sense primer (5'AATGCTAGCTCAGAAGTTGTGCCAAAGG, *Nhe*I site underlined) which added seven extra nucleotides (coding for the last two amino acids of the *MFα1* presequence), including a *Nhe*I site (for in frame cloning into the *Nhe*I site in the *MFα1* presequence region of pVD4) upstream of the coding region of mature Rs-AFP2. The fragments corresponding to the mature domain of Rs-AFP2 obtained by PCR amplification in the first and in the second reaction were digested with *Hin*dIII–*Bam*HI and *Nhe*I–*Bam*HI, respectively, and introduced in the corresponding sites of pVD4 to yield vectors pVD5 and pVD6, respectively. The resulting vectors were digested with *Sal*I–*Bam*HI to isolate the expression blocks, which were then subcloned into *Sal*I–*Bgl*II digested pTG3828 to yield the vectors pMFpre/RsAFP2 and pMFprepro/RsAFP2, respectively (Fig. 1.). These plasmids and plasmid pTG3828 were transformed in yeast by the lithium acetate method as described by Elble [8]. Selection of transformants was done on minimal selective SD medium lacking uracil [9]. Presence of the plasmids in the yeast colonies was verified by PCR as described by Ward [10].

All constructs modified by PCR-based mutagenesis were checked by DNA sequencing using the AutoRead Sequencing Kit (Pharmacia, according to the manufacturer), and a Pharmacia A.L.F. DNA Sequencer.

2.3. Antifungal activity assay

2.3.1. Plate growth inhibition assay. Minimal selective SD medium containing 0.6 g/l of agar was autoclaved, cooled to 40°C and supplemented with spores of *A. pisi* (10^4 spores/ml final spore density) before pouring into petri dishes. A suspension of transformed yeast cells (10^5 cells/ml) was dispersed as 2 μl drops on top of the spore containing agar medium. The plates were incubated for 24 h at 30°C, followed by 2–5 days incubation at room temperature. Inhibition halos indicating zones of suppressed growth of *A. pisi* were viewed macroscopically.

2.3.2. Liquid growth inhibition assay. The supernatant of saturated yeast cultures or different fractions from chromatographic purification steps were filtered (sterile 0.22 μm filter) and serially diluted in sterile water. Diluted samples (20 μl) were incubated in microtiter plate wells with 80 μl of half strength potato dextrose broth (Difco) containing spores (10^4 spores/ml) of *F. culmorum*. Growth of the fungi was monitored by microspectrophotometry as described by Broekaert et al. [11]. Homogenates of yeast cells were prepared by spinning down 1 ml of a saturated yeast culture, suspending the cells in 200 μl of water, vortexing the cells in the presence of 0.2 g of glass beads (425–600 μm), and clearing the homogenate by centrifugation (1 min, 10,000 × *g*).

2.4. Purification of Rs-AFP2 from yeast culture

The supernatant of 100 ml of a saturated culture of transformed yeast (grown on minimal selective SD medium supplemented with 0.5% (w/v) of casamino acids) was centrifuged (4000 rpm, 10 min), and filtered (0.45 μm) to remove yeast cells and debris. Tris-HCl (pH 9) was added to the supernatant to a final concentration of 50 mM. The sample was loaded at a flow rate of 2 ml/min on an anion exchange chromatography column (Q-Sepharose Fast Flow, 20 ml bed volume, Pharmacia), on-line connected with a disposable reversed-phase C8 silica column (Bond Elut, 500 mg solid phase, Varian, Harbor City, USA). The antifungal activity was not retained on the Q-Sepharose matrix but bound to the C8 silica matrix. The C8 silica column was rinsed with 6 ml of 10% (v/v) acetonitrile in 0.1% (v/v) trifluroacetic acid (TFA) and subsequently eluted with 4 ml of 30% (v/v) acetonitrile in 0.1% (v/v) TFA. The latter eluate was dried in a rotating vacuum concentrator, redissolved in 0.5 ml 15% (v/v) acetonitrile containing 0.1% (v/v) TFA, and was loaded on a reversed-phase C2/C18 silica column (Pep-S, 5 μm beads, 0.4 ×25 cm, Pharmacia) connected to a Waters 600 HPLC station. Chromatography parameters are given in the legend to Fig. 2.

2.5. Protein analytical methods

The bicinchoninic acid method [12] adapted to a microplate format was used for protein concentration determination. Bovine serum albumin served as a reference protein. N-terminal amino acid sequence was done by automated Edman degradation in a 477A Protein Sequenator (Applied Biosystems Inc., Foster City, CA) with on-line detection of phenylthiohydanthoin derivatives in a 120-A PTH Analyzer (Applied Biosystems). Prior to amino acid sequence analysis, cysteine residues were modified by S-carboxyamido-methylation as described previously [1]. Digestion of proteins with pyroglutamate aminopeptidase (sequencing grade, Boehringer Mannheim) was done according to the supplier's instructions.

3. Results

3.1. Construction of vectors

Two different plasmids were constructed for testing the ability to express active Rs-AFP2 in the yeast *S. cerevisiae*. These plasmids, pMFpre/RsAFP2 and pMFprepro/RsAFP2, contain the coding region of the mature Rs-AFP2 fused in frame with the predomain or preprodomain, respectively, of the yeast mating factor *MFα1* gene. In these constructs the promoter is the *MFα1* promoter and the plasmid backbone is that of the yeast/*E. coli* shuttle vector pTG3828 [6]. Both pMFpre/RsAFP2 and pMFprepro/RsAFP2 carry the preferred yeast codon (CGA) at arginine 32 of mature Rs-AFP2 instead of the natural codon (CGT) present in the cDNA. All the other codons of the Rs-AFP2 coding region are frequently used by *S. cerevisiae* [5].

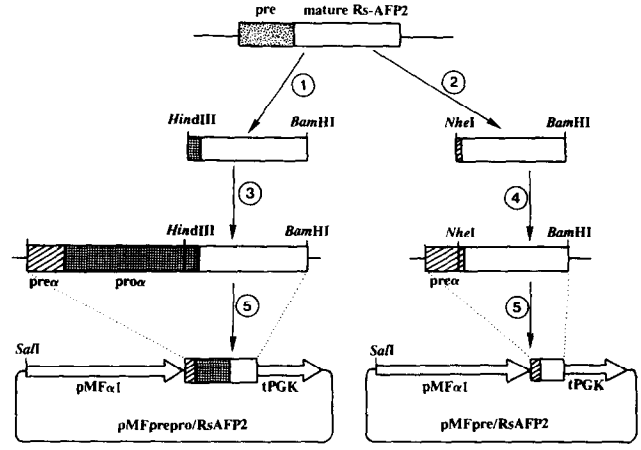

Fig. 1. Schematic representation of the construction of the expression vectors pMFprepro/RsAFP2 and pMFpre/RsAFP2. The different steps in the procedure are: (1) PCR amplification of the coding region of mature RsAFP2 using primers to add a *Hin*dIII site and part of the *MFα1* pro region (5' site) and a *Bam*HI site (3' site); (2) PCR amplification of the coding region of mature RsAFP2 using primers to introduce a *Nhe*I and part of the *MFα1* pre region (5' site) and a *Bam*HI site (3' site); (3) subcloning of the PCR product into *Hin*dIII–*Bam*HI digested pVD4; (4) subcloning of the PCR product into *Nhe*I–*Bam*HI digested pVD4; (5) digestion of the resulting plasmids with *Sal*I–*Bam*HI and subcloning of the insert in *Sal*I–*Bgl*II digested pTG3828. Abbreviations: pre, signal sequence domain of RsAFP1 cDNA; preα, signal sequence domain of the *MFα1* gene; proα, propeptide domain of the *MFα1* gene; pMFα 1, promoter domain of the *MFα1* gene; tPGK, terminator domain of the yeast phosphoglycerate kinase gene.

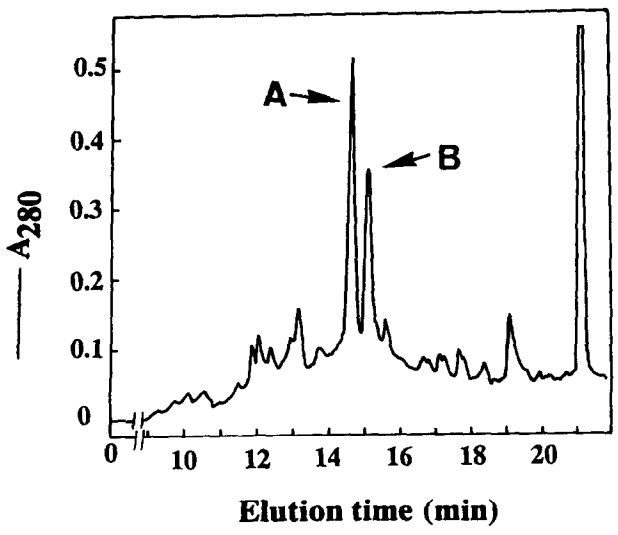

Fig. 2. Reversed-phase chromatography purification of yeast-expressed Rs-AFP2. The sample loaded on the column was derived from 100 ml of culture medium of yeast transformed with pMFprepro/RsAFP2 as described in materials and methods. The column (C2/C18 silica, 5 μm beads, 0.4 × 25 cm, Pharmacia) was equilibrated with 15% acetonitrile containing 0.1% TFA and was rinsed after loading with the same buffer until the absorbance reached background level. The column was subsequently eluted with a 15 min linear gradient from 15% to 50% acetonitrile containing 0.1% TFA at a flow rate of 1 ml/min. The eluate was monitored for proteins by one-line measurement of the absorbance at 280 nm. Peak fractions were collected manually, dried in a rotating vacuum concentrator to remove the solvents, and redissolved in 200 μl of distilled water. Twenty μl fractions were tested in the liquid growth inhibition assay. Only the peak fractions indicated by A and B exhibited antifungal activity. The elution time indicated in the abscissa is relative to the start of the elution gradient.

3.2. *Secretion of the active Rs-AFP2 by yeast*

Plasmids pTG3828, pMFprepro/RsAFP2, pMFpre/RsAFP2 were transferred to yeast. Transformants were selected and tested for their capacity to secrete active Rs-AFP2. A plate assay was developed in which yeast colonies were spotted on a selective medium containing fungal spores. A faint inhibition zone was visible only underneath the yeast colonies transformed with the pMFprepro/RsAFP2 vector (results not shown).

The active material secreted into the culture medium by yeast transformants was subsequently evaluated by a liquid growth inhibition assay using *F. culmorum* as a test fungus. To assess for possible intracellular accumulation of Rs-AFP2, yeast cells were homogenized and the resulting homogenates also assayed using liquid growth inhibition tests. Antifungal activity could only be detected in the culture medium of yeast cells transformed with pMFprepro/RsAFP2, which contained about 2 μg/ml of Rs-AFP2 equivalents. The activity of the homogenate of these cells, as well as that of culture media and cell homogenates of yeast cells transformed with pMFpre/RsAFP2 or pTG3828 transformed yeasts was below the detection limit (about 0.2 μg/ml of Rs-AFP2 equivalents). Hence, only pMFprepro/RsAFP2 seems to convey significant expression of Rs-AFP2 in yeast. We have consequently retained this vector in subsequent experiments.

3.3. *Purification of yeast-expressed Rs-AFP2*

After two initial purification steps consisting of anion-exchange chromatography on Q-Sepharose Fast Flow and passage over a disposable C8 silica column, the partially purified culture medium of pMFprepro/RsAFP2-transformed yeast cells was applied on a reversed-phase chromatography (RPC) column consisting of C2/C18 silica. Fig. 2 shows the RPC profile in which both the main peak (peak A, elution time 14.7 min) and a smaller peak (peak B, elution time 15.2 min) coeluted with antifungal activity. The retention time of peak B was identical to that of authentic Rs-AFP2 (15.2 min).

3.4. *Analysis of yeast-expressed Rs-AFP2*

The amino-terminal amino acid sequence obtained by automated Edman degradation for RPC-purified peak A revealed a sequence of 51 amino acids, all of which being identical to the sequence of Rs-AFP2 (Fig. 3). This sequence includes an N-terminal glutamine which is known to be blocked by cyclisation in authentic Rs-AFP2 [1]. Absence of any contaminating signals in the amino acid sequence analysis indicated that the peak A fraction was essentially homogeneous. No sequence signals could be recorded for RPC-purified peak B material, probably due to blocking of its N-terminus. This protein fraction was treated with pyroglutamate aminopeptidase in order to cleave off the presumed blocked glutamine residue, but also in this case no amino acid sequence could be determined, whereas the same treatment successfully deblocked authenthic RAFP2 [1]. Because of the uncertain identification of the peak B material and because of its lower abundancy relative to peak A material, the peak B material was not further analysed.

The specific antifungal activity of RPC-purified peak A material, as well as that of authentic Rs-AFP2, was determined by measuring the percentage growth inhibition of *F. culmorum* caused by serial dilutions of the protein samples. The IC_{50} values (concentration required for 50% growth inhibition) derived from dose-response curves, was about 3 μg/ml for both protein preparations (Fig. 4A).

Moreover, the type of inhibition caused by RPC-purified peak A material was identical to that caused by

peak A QKLCQRPSGTWSGVCGNNNACKNQCINLEKARHGSCNYVFPAHKCICYFPC

Rs-AFP2 KLCQRPSGTWSGVCGNNNACKNQCINLEKARHGSCNYVFPAHKCICYFPC

Fig. 3. Amino acid sequences of peak A fraction (untreated), and authentic Rs-AFP2 (obtained after digestion with pyroglutamate aminopeptidase [1]). The underlined amino acid sequence is derived from a cDNA clone encoding Rs-AFP2 [3].

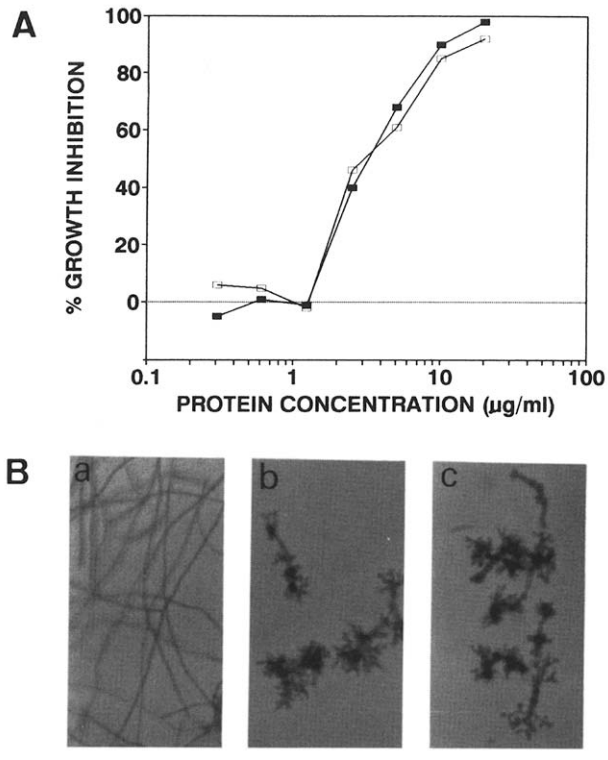

Fig. 4. (A) Dose-response curves for growth inhibition of *Fusarium culmorum* by peak A material (□), and authentic Rs-AFP2 (■). Growth inhibition was measured by microspectrophotometry after 24 h of incubation (B). Microscopic view of *F. culmorum* grown for 24 h in the absence (a) or presence of 10 μg/ml peak A material (b), or 10 μg/ml Rs-AFP2 (c).

authentic Rs-AFP2, showing a characteristic morphological distortion of the fungal hyphae typified by the induction of multiple branches near the tips (Fig. 4B).

4. Discussion

The aim of the present study was to develop an expression system allowing facile production of the antifungal protein Rs-AFP2 for detailed analysis of its active site by mutational analysis. The occurrence of multiple disulfide bridges in authentic Rs-AFP2 [1] prompted us to use an eukaryotic expression system, since it is known that disulfide bridge formation of eukaryotic proteins expressed in prokaryots is often erratic, leading to unproper folding. Yeast was chosen for expression of Rs-AFP2 since, notwithstanding its potent inhibitory effect on filamentous fungi, this protein does not have adverse effects on yeast even at concentrations as high as 500 μg/ml [1].

Two different chimeric constructs were made based on the *MFα1* expression system. In one construct, the Rs-AFP2 coding region was fused immediately behind the pre-sequence of the *MFα1* gene, while in the other construct it was placed behind the prepro-sequence of the *MFα1* gene. In both cases transcription was regulated by the *MFα1* promoter. As shown by the liquid growth inhibition assay, only the prepro-fusion led to secretion of active Rs-AFP2. Deletion of the pro-region resulted in reduction of Rs-AFP2 secretion by at least 10-fold. Furthermore, earlier attempts to use the authentic signal sequence of Rs-AFP2 did not allow efficient secretion of Rs-AFP2 in yeast (Vilas Alves, unpublished results). Reichhart et al. [7] have also observed that production of recombinant insect defensin A in yeast was about 40-fold higher using a *MFα1* prepro-fusion than with a *MFα1* pre-fusion. On the other hand, Achstetter et al. [6] found that expression of leech hirudin in yeast as a *MFα1* prepro-fusion protein was about four times lower than a comparable *MFα1* pre-hirudin fusion, whereas expression of *MFα1* prepro-and pre-human cathepsin S was equally efficient in yeast [13]. Apparently, the effect of the *MFα1* pro-sequence on the secretion of a heterologous protein is dependent on the structure of that protein. It is noteworthy that Rs-AFP2 belongs to a family of plant proteins that comprises the γ-thionins from cereals [1]. γ-Thionins have recently been shown to have a three-dimensional structure similar to that of insect defensins [14]. The striking requirements of the *MFα1* pro-domain for expression of both types of structurally related proteins may be explained by assuming that the pro-domain facilitates correct folding of the mature domain. Alternatively, the absence of the pro-domain may somehow hamper proper co-translational processing in the endoplasmic reticulum of yeast.

During the purification of yeast-expresed Rs-AFP2 by reversed-phase chromatography, two protein fractions exhibiting antifungal activity, called peak A and peak B material, were recovered. Peak A material has exactly the same amino acid sequence as authentic Rs-AFP2 except that its N-terminal glutamine is not cyclisized. This difference is deduced from the observation that amino-terminal sequencing of authentic Rs-AFP2 requires pretreatment with the enzyme pyroglutamate aminopeptidase, whereas that of peak A material does not. Despite of this minor difference between the yeast-expressed Rs-AFP2 and plant-derived Rs-AFP2, both proteins have exactly the same antifungal activity, indicating that N-terminal cyclisation has no effect on the biological properties of the protein. Peak B material could not be further identified since no amino acid sequence signals could be obtained even after pyroglutamate aminopeptidase digestion. This protein is likely to be a processing or amino-terminal derivatisation analog of Rs-AFP2.

Acknowledgements: The first and the second author have contributed equally to the work presented in this article. The authors are grateful to Dr. T. Achstetter (Transgene, Strasbourg, France) for providing the yeast shuttle vector pTG3828 and the expression block vector M13TG5879, and to S. Torrekens and F. Van Leuven (Centre of Human Genetics, Catholic University of Leuven) for amino acid sequence determination. G.W.D.S. and W.F.B. are Research Assistant and Research Associate, respectively, of the Belgian National Fund for Scientific Research. A.L.V.A. acknowledges the receipt of a training

grant from the Commission of the European Communities in the framework of an ECLAIR (AGRE-0005) grant. F.R.G.T. is the recipient of a post-doctoral grant from the Research Council of the Catholic University of Leuven.

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