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The two high molecular weight subunits of cytochrome *c* reductase from potato are immunologically related to the mitochondrial processing enhancing protein

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A soluble heterodimeric enzyme localized in the matrix space of fungal and mammalian mitochondria removes the presequences of mitochondrial precursor proteins. It consists of two subunits, the matrix processing peptidase (MPP) and the so-called processing enhancing protein (PEP). We have used antibodies directed against the matrix processing peptidase from fungi to analyse the corresponding plant enzyme and its submitochondrial localization. Antibodies directed against PEP specifically recognize two polypeptides of 55 kDa and 53 kDa which are exclusively present in the membrane fraction of potato mitochondria. Also the processing activity removing the presequences of precursor proteins synthesized in vitro is found in this fraction suggesting that the immunopositive proteins may be involved in the processing reaction. As it has been shown that in potato MPP forms part of cytochrome *c* reductase, a protein complex of the respiratory chain, we analysed this complex with antibodies against PEP. The 55 kDa and 53 kDa subunits are recognized by antibodies directed against PEP from yeast and *Neurospora*. Incubation of potato cytochrome *c* reductase with plant mitochondrial precursor proteins synthesized in vitro shows that the purified complex itself has processing activity. Molecular and functional analysis of the two immuno-positive subunits may now reveal which of these represents the processing enhancing protein of the mitochondrial processing peptidase from potato.

Introduction

Most nuclear encoded mitochondrial proteins carry the signals which direct them to the mitochondrial compartment at their amino terminal end. These so-called presequences usually consist of 20–80 amino acids and have several chararcteristic features: they are rich in basic residues and often they contain many hydroxylated amino acids but no acidic residues; typically they form amphiphilic alpha helices carrying positive charges on one face and hydrophobic amino acids on the other face of the helix. The presequences specifically interact with components of the mitochondrial protein import apparatus. While the import apparatus has been intensively studied in fungi and mammals, little is known about protein import into plant mitochondria. Only few plant mitochondrial presequences have been characterized and there is limited information about the enzyme which recognizes presequences and cleaves them off upon import of the precursor into the organelle. In *Neurospora* [1], yeast [2] and rat [3,4] this enzyme, termed matrix processing peptidase, has been purified. Two structurally related components, MPP and PEP, co-operate during the endoproteolytic processing event. In *Neurospora* about a quarter of the PEP subunit is found in the soluble fraction of mitochondria and three quarters in the inner mitochondrial membrane where it forms part of the cytochrome *c* reductase complex [5]. In contrast, PEP from yeast is a soluble protein which is homologous but not identical with subunit I of cytochrome *c* reductase. There is limited information on the general processing enzyme from plant mitochondria. It has been reported that antibodies directed against PEP from *Neurospora* deplete mitochondrial lysates from Vicia faba of the processing activity [6]. Eriksson and Glaser [7] found that the processing activity of spinach mitochondria is associated with the mitochondrial membrane fraction. Recently we have shown that MPP is an integral part

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Abbreviations: MPP, matrix processing peptidase; PEP, processing enhancing protein; P_i, inorganic phosphate; PMSF, phenylmethylsulphonyl fluoride.

of cytrochrome *c* reductase from potato [8]. Here we report on an immunological approach towards the identification and submitochondrial localization of potato PEP.

Materials and Methods

Isolation and subfractionation of mitochondria from potato, yeast and Neurospora

Mitochondria were isolated from potato tubers (*Solanum tuberosum L.* var. Bintje) as described in Braun et al. [9]. For the preparation of yeast mitochondria, the method originally described by Daum et al. [10] was used. *Neurospora* mitochondria were prepared according to Zimmermann and Neupert [11] and Schleyer et al. [12]. Mitochondria were lysed in the presence of different octylpolyoxy ethylene (OPOE) concentrations and subfractionated by ultracentrifugation as outlined in Vestweber and Schatz [13] and Schmitz and Lonsdale [14]. The purity of subfractions was analysed with antibodies directed against the matrix enzyme manganese superoxide dismutase and the ADP/ATP translocator [8]. The preparation of cytochrome *c* reductase started from about 150 mg protein from mitochondrial membranes which were solubilized in 3.3% Triton X-100 and briefly centrifuged (10 min $60\,000 \times g$). The respiratory complex was purified by cytochrome *c* affinity chromatography and gel filtration as previously described [15,16].

Preparation of total protein from potato tubers

About 0.1 g of tuber tissue was homogenized in a buffer containing 0.1 M Na-P_i (pH 7.0), 2 mM NaS_2, 2% Triton X-100, 0.1% polyvinyl pyrrolidone. The suspension was centrifuged for 10 min at $18\,000 \times g$ and the pellet was discarded. The protein concentration in the supernatant was determined according to Bradford [17].

In vitro processing assay

Processing extracts were prepared by lysing mitochondria at a protein concentration of 10 mg/ml in a buffer containing 22 mM Tris-HCl (pH 8.0), 0.6% Triton X-100, 25 mM NaCl, 0.1 mM $MnCl_2$, 0.1 mM $MgCl_2$ and 1 mM PMSF. The processing mixture was shaken gently with radiolabelled precursor of the β-subunit of ATPase from tobacco for 1 h at 28°C. In our hands this incubation period gave optimal results without any degradation of the labelled protein. The precursor protein had been synthesized by in vitro transcription of a cDNA of tobacco ATPase [18] and subsequent translation of the transcripts in the presence of [^{35}S]-methionine using rabbit reticulocyte lysate (Amersham) according to the suppliers instructions. The processing reaction was stopped by adding an equal volume of Laemmli buffer (2-fold concentrated, [19]) and incubated for 10 min at 60°C prior to loading onto an SDS/polyacrylamide gel. After separation of polypeptides by SDS/PAGE the gels were incubated for 30 min in Amplify (Amersham) and radiolabelled proteins were visualized by exposure to X-ray film at −70°C.

Analysis of proteins by SDS/PAGE and immunoblotting

Polypeptides were fractionated by electrophoresis in 7.5% SDS/polyacrylamide gels (SDS/PAGE) and either stained with Coomassie Brillant Blue R250 or blotted onto nitrocellulose (Schleicher & Schüll). The blots were washed in a buffer containing 100 mM Tris-HCl (pH 7.4), 150 mM NaCl and 0.1% Tween 20 (Sigma) and incubated overnight in the same buffer containing 1/1000 (v/v) of serum directed against MPP and PEP from *Neurospora* and yeast. The antibodies were kindly provided by W. Neupert, München and G. Schatz, Basel. Immunopositive polypeptides were visualized with biotinylated antibodies, avidin and horseradish peroxidase as recommended by the supplier (Vektor Laboratories).

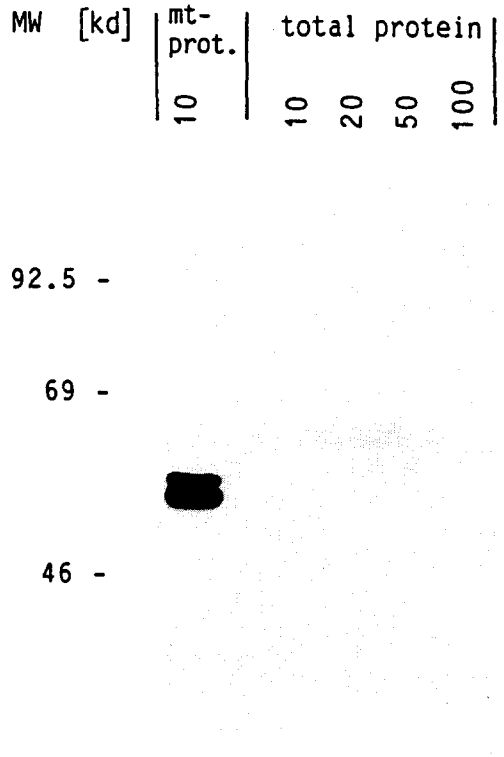

Fig. 1. Immunoblot of proteins from potato tubers separated by SDS/PAGE after incubation with antibodies directed against PEP from *Neurospora*. The first lane from the left contains 10 μg mitochondrial protein; the following four lanes contain 10–100 μg total tuber protein as indicated. The molecular mass of a protein standard is given on the left. As mitochondrial protein represents a very small portion of total tuber protein, the crossreaction is exclusively visible in the fraction containing mitochondrial protein. Unspecific crossreactions with non-mitochondrial proteins seem not to occur.

Results

Mitochondria from potato tubers were purified on Percoll gradients and total mitochondrial proteins were separated by SDS/PAGE. After transfer to nitrocellulose they were incubated with antibodies against PEP and MPP from *Neurospora* and yeast. The antibody against PEP from *Neurospora* strongly crossreacts with two mitochondrial proteins of 55 kDa and 53 kDa but not with cytosolic proteins (Fig. 1). Under the same conditions the antibodies against MPP from *Neurospora* and the *Mas*2 encoded MPP from yeast do not produce a positive signal (not shown). To analyse the submitochondrial localization of the 55 kd and 53 kDa polypeptides, potato mitochondria were subfractionated using the mild detergent octylpolyoxyethylene [13,14]. The purity of subfractions was checked with antibodies directed against specific marker enzymes of the mitochondrial matrix and the membranes, respectively [8]. Antibodies directed against the *Mas*1 encoded PEP from yeast and PEP from *Neurospora* both recognize the 55 kDa and 53 kDa polypeptides in the membrane fraction but not in the matrix of potato mitochondria (Fig. 2). Therefore the subfractionation procedure was further tested by applying it to yeast and *Neurospora* mitochondria. Standard procedures were used to prepare mitochondria from both fungi (see Material and Methods) and they were subfractionated with the method outlined above. Western blots of SDS denatured proteins from fungal mitochondria (Fig. 3) reveal that the *Mas*1 encoded PEP from yeast (48 kDa) is a matrix protein being exclusively present in the soluble fraction as reported earlier [2]. Also for

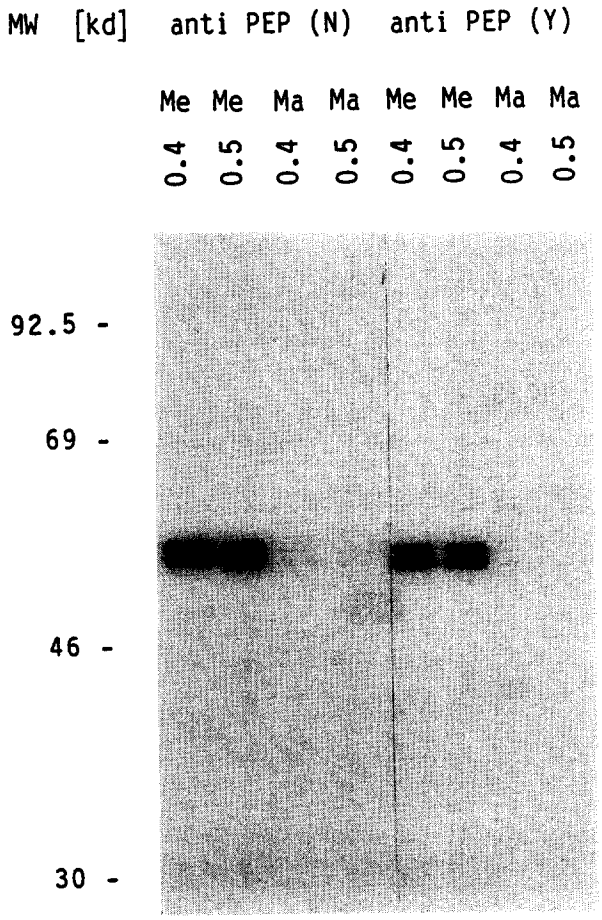

Fig. 2. Immunoblot of subfractionated mitochondrial protein from potato after incubation with antibodies directed against PEP from *Neurospora* (N) and the *Mas*1 encoded PEP from yeast (Y). The subfractionation was carried out with different amounts of octylpolyoxy ethylene (OPOE) as indicated above the lanes. The size of standard proteins is given on the left. The antibodies only recognize two protein bands in the mitochondrial membrane fraction (Me) but not in the matrix (Ma).

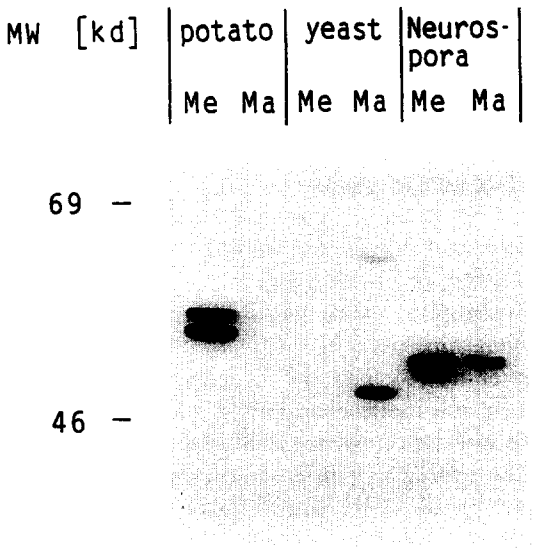

Fig. 3. Immunoblot of mitochondrial protein from potato, yeast and *Neurospora* after incubation with antibodies directed against the *Mas*1 encoded PEP from yeast. Mitochondria from all three species were fractionated into membranes (Me) and matrix (Ma) using 0.5% OPOE and proteins were analysed by SDS/PAGE. The numbers on the left indicate the molecular weight of standard proteins in kDa.

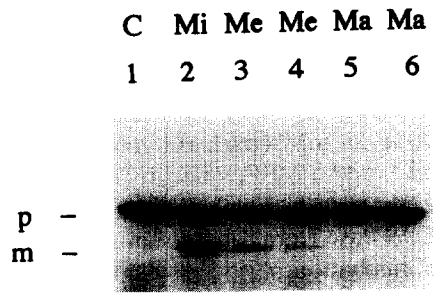

Fig. 4. Submitochondrial localization of processing activity in potato mitochondria. Labelled precursor of the β-subunit of F_1-ATPase from tobacco was incubated with total mitochondrial protein (Mi), mitochondrial membranes (Me) and matrix (Ma). As a control (C) the radiolabelled precursor was incubated without any mitochondrial proteins. The amount of mitochondrial proteins used for the assay was 25 μg (lane 2), 10 μg (lanes 3 and 5) and 5 μg (lanes 4 and 6). The precursor (p) of the β-subunit is not processed to its mature form (m) by the matrix fraction.

Neurospora PEP (52 kDa), our immuno-localization gives the same results as reported by Hawlitschek et al. [1] who showed that in this fungus 75% of PEP are present in the membrane while 25% reside in the matrix. We concluded that our subfractionation procedure is reliable and that at least one or possibly two polypeptides representing the mitochondrial PEP from potato are localized in the membranes.

An analysis of the localization of the mitochondrial processing activity corroborates our immunological results. The β-subunit of the mitochondrial ATPase from tobacco was synthesized in vitro by coupled transcription/translation in the presence of [^{35}S]methionine. The radiolabelled precursor of 56 kDa is processed to the mature form of 52 kDa by total mitochondrial proteins which is in line with the data of Whelan et al. [20]. Processing activity is not detectable in the matrix of potato mitochondria but in the membrane fraction (Fig. 4). For unknown reasons the membrane fraction exhibits generally a lower processing activity than total mitochondrial protein. Like the activity of the matrix processing peptidase from fungi and mammals the membrane bound activity from potato mitochondria is metal dependent and inhibited by chelators of divalent cations like EDTA (not shown).

As it was recently reported that the 51 kDa subunit of cytochrome *c* reductase from potato is identical with MPP [8], we wondered whether PEP is integrated into this respiratory complex as well. Cytochrome *c* reductase from potato mitochondria was purified using a cytochrome *c* affinity column and a gel filtration column as previously described [8,16]. The method yields a highly pure and physiologically intact protein complex comprising ten subunits including three high molecular weight proteins (51 kDa, 53 kDa and 55 kDa). Fractions collected from the gel filtration column were analysed for ubiquinol cytochrome *c* oxidoreductase activity and cytochrome *b* content. The fractions with high cytochrome *b* content, which have been shown to contain the cytochrome *c* reductase complex [16], were separated by SDS/PAGE and blotted onto nitrocellulose. As shown in Fig. 5 antibodies against PEP from fungi crossreact with the 55 kDa and 53 kDa subunits corresponding exactly to the size of the two immunoreactive proteins of the membrane fraction. The amount of immunopositive proteins of the fractions eluted by gel filtration chromatography correlated well with the amount of cytochrome *b* as determined spectrophotometrically using an extinction coefficient of 20 mM^{-1} cm^{-1} for cytochrome *b*. The previously reported crossreaction between an antibody against the *Mas*2 encoded MPP from yeast and the 53 kDa protein [8] was very weak in comparison to the crossreaction shown in Fig. 5 and is probably due to some sequence

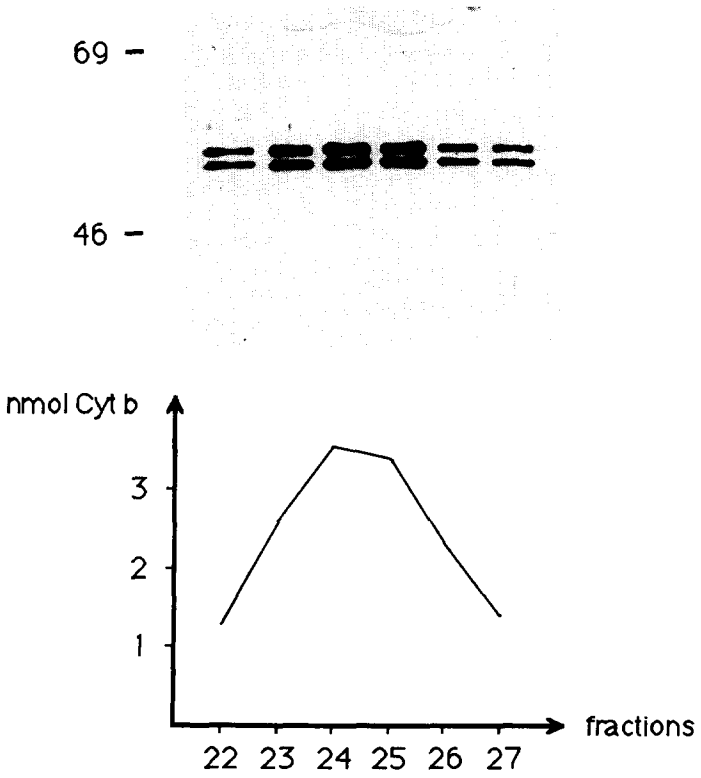

Fig. 5. Immunoblot of purified cytochrome *c* reductase from potato after incubation with antibodies directed against PEP from *Neurospora* and cytochrome *b* content of the corresponding fractions. Equal volumes from six successive protein fractions eluted from the gel filtration column were separated by SDS/PAGE and analysed by immunoblotting. The amount of the cytochrome *b* subunit of each fraction was determined spectrophotometrically as outlined in Braun and Schmitz [16]. The numbers on the left indicate the size of standard proteins.

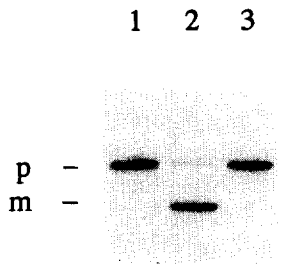

Fig. 6. Potato cytochrome *c* reductase exhibits chelator-sensitive processing activity. The radiolabelled precursor (lane 1) was incubated with 2 μg of purified cytochrome *c* reductase (lane 2). Processing of the precursor (p) to the mature form (m) is inhibited upon preincubation of cytochrome *c* reductase with 1 mM EDTA.

homology between the two subunits of the processing enzyme [21]. It is likely that at least one of the immunopositive proteins represents PEP as the purified complex exhibits strong processing activity towards mitochondrial precursor proteins (Fig. 6). This activity is insensitive towards phenylmethylsulfonyl fluoride (not shown) but sensitive towards metal chelators like EDTA (Fig. 6, lane 3). These properties are typical for the general matrix processing peptidase analysed in fungi and mammals.

Discussion

More than a decade ago, Böhni et al. [22] showed that the enzyme acting on presequences of mitochondrial precursor proteins is localized in the soluble fraction of the organelle. Recent findings of Eriksson and Glaser [7] and our group [8, this report] demonstrate that the corresponding plant enzyme resides in the mitochondrial membranes. In spite of this difference there is an immunological relationship between one of the subunits of the processing peptidase from fungi and mitochondrial proteins from potato. A 55 kDa and a 53 kDa polypeptide are specifically recognized by antibodies against the processing enhancing protein from yeast and *Neurospora*. Both proteins are exclusively present in the membrane fraction which is in line with the notion of a membrane bound plant mitochondrial processing peptidase. Interestingly, both cross-reacting polypeptides form part of cytochrome *c* reductase from potato. Thus this respiratory complex seems not only to contain MPP (51 kDa) as a proteolytically active subunit [8] but also PEP or a homologue of it.

Recently PEP from fungi was found to be a member of a protein family which includes MPP, the so-called 'core' proteins of cytochrome *c* reductase and a subunit of the NADH-dehydrogenase complex [5,23]. The 55 kDa and 53 kDa proteins of potato cytochrome *c* reductase are likely to belong to the same protein family as they crossreact strongly and specifically with the antibodies against PEP from fungi. At least one of them may represent PEP since serum made against *Neurospora* PEP is able to deplete mitochondrial lysates of Vicia of the processing activity [6]. At present it is not possible to predict whether the 55 kDa or the 53 kDa protein is PEP as both are equally well recognized by the antibodies. Determination of the primary structure of these polypeptides by direct protein sequencing or sequencing of corresponding cDNA clones should indicate which of them is homologous with subunits of other cytochrome *c* reductase complexes and which of them might be involved in the proteolytic activity of cytochrome *c* reductase from potato.

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References

1 Hawlitschek, G. Schneider, H., Schmidt, B., Tropschug, M., Hartl, F.-U. and Neupert, W. (1988) Cell 53, 795–806.
2 Yang, M., Jensen, R.E., Yaffe, M.P., Oppliger, W. and Schatz, G. (1988) EMBO J. 7, 3857–3862.
3 Ou, W.-J., Ito, A., Okazaki, H. and Omura, T. (1989) EMBO J. 8, 2605–2612.
4 Kleiber, J., Kalousek, F., Swaroop, M. and Rosenberg, L.E. (1990) Proc. Natl. Acad. Sci. USA 87, 7978–7982.
5 Schulte, U., Arretz, M., Schneider, H., Tropschug, M., Wachter, E., Neupert, W. and Weiss, H. (1989) Nature 339, 147–149.
6 Whelan, J., O'Mahony, P., Harmey, M.A. (1990) Arch. Biochem. Biophys. 279, 281–285.
7 Eriksson, A.C. and Glaser, E. (1992) Biochim. Biophys. Acta (in press).
8 Braun, H.-P., Emmermann, M., Kruft, V. and Schmitz, U.K. (1992) EMBO J. 11, 3219–3227.
9 Braun, H.-P., Emmermann, M., Kruft, V. and Schmitz, U.K. (1992b) Mol. Gen. Genet. 231, 217–225.
10 Daum, G., Böhni, P.C. and Schatz, G. (1982) J. Biol. Chem. 257, 13028–13033.
11 Zimmermann, R. and Neupert, W. (1980) Eur. J. Biochem. 109, 217–229.
12 Schleyer, B., Schmidt, B. and Neupert, W. (1982) Eur. J. Biochem. 125, 109–116.
13 Vestweber, D. and Schatz, G. (1988) J. Cell Biol. 107, 2037–2043.
14 Schmitz, U.K. and Lonsdale, D.M. (1989) Plant Cell 1, 783-791.
15 Weiss, H. and Juchs, B. (1978) Eur. J. Biochem. 88, 17–28.
16 Braun, H.P. and Schmitz, U.K. (1992) Eur. J. Biochem. 208, 761–767.
17 Bradford, M. (1976) Anal. Biochem. 72, 248.
18 Boutry, M. and Chua, N.-H. (1985) EMBO J. 4, 2159–2165.
19 Laemmli, U.K. (1970) Nature 227, 680–685.
20 Whelan, J., Dolan, L. and Harmey, M.A. (1988) FEBS Lett. 236, 217–220.
21 Pollock, R.A., Hartl, F.-U., Cheng, M.Y., Ostermann, J., Horwich, A. and Neupert, W. (1988) EMBO J. 7, 3493–3500.
22 Böhni, P., Gasser, S., Leaver, C. and Schatz, G. (1980) In: The Organization and Expression of the Mitochondrial Genome (Kroon, A.M. and Saccone, C., eds.) pp. 423–433, Elsevier, Amsterdam.
23 Röhlen, D.A., Hoffmann, J., Van der Pas, J.C., Preis, D., Sackmann, U. and Weiss, H. (1991) FEBS Lett. 278, 75–78.