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Point mutations destabilizing a precursor protein enhance its post-translational import into mitochondria

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In order to study the role of protein unfolding during post-translational protein import into mitochondria, we destabilized the structure of a mitochondrial precursor protein by site-directed mutagenesis. The precursor consisted of the first 16 residues of the yeast cytochrome oxidase subunit IV precursor fused to mouse dihydrofolate reductase. Labilization of the folded precursor structure was monitored by increased susceptibility to protease and diminished ability of methotrexate to block import of the precursor into isolated yeast mitochondria. On comparing the original precursor with two mutant forms that were destabilized to different degrees, increased labilization correlated with an increased rate and efficiency of import into mitochondria. This supports the view that the precursor must unfold in order to enter the mitochondria.

Key words: site-directed mutagenesis/mitochondria/protein import/unfolding/dihydrofolate reductase

Introduction

Translocation of proteins across biological membranes is essential for the growth and maintenance of living cells. While it is well established that the initial interaction of a precursor protein with its target membrane is mediated by the precursor's signal sequence, the mechanism of the actual translocation process is still unknown (Wickner and Lodish, 1985). However, it has been suggested that protein transport across all transport-competent membrane systems may proceed by very similar mechanisms (Schatz, 1986).

Recent evidence from several laboratories suggests that precursor proteins must be at least partly unfolded in order to cross a membrane (Zimmermann and Meyer, 1986). This implies that post-translational import of a folded precursor protein into mitochondria requires unfolding of the precursor. This was directly shown with an artificial mitochondrial precursor protein containing a mitochondrial presequence fused to mouse dihydrofolate reductase (DHFR); import of this protein into isolated yeast mitochondria is completely blocked by stabilizing its folded structure with folate analogs such as methotrexate (Eilers and Schatz, 1986). Conversely, import of the precursor is dramatically accelerated by presenting it to mitochondria as peptidyl-tRNA chains (Verner and Schatz, 1987) or upon denaturation by urea (Eilers *et al.*, 1988). In both instances, acceleration of import is probably caused by destabilization, or loss, of the precursor's tertiary structure.

In order to establish this point more directly, we have used site-directed mutagenesis to construct two mutant forms of the precursor whose folded structure is destabilized to different degrees. When the original precursor and its two mutant variants were presented to energized mitochondria, rate and efficiency of import increased with decreasing stability of the precursor's structure. However, import of all three precursor forms required ATP in addition to a potential across the mitochondrial inner membrane.

Results

Point mutations destabilize the tertiary structure of a mitochondrial precursor protein

A fusion protein containing the first 16 residues of the yeast cytochrome oxidase subunit IV precursor in front of mouse DHFR behaves like a bona fide mitochondrial precursor protein *in vivo* and *in vitro (Hurt et al.*, 1984, 1985). However, import into mitochondria is not accompanied by proteolytic removal of the presequence since the cleavage site recognized by the matrix-localized processing protease has been deleted. Except for its slightly shorter presequence, this 'wild-type' fusion protein is identical to that which has been extensively studied previously (Hurt *et al.*, 1985; Eilers and Schatz, 1986; Verner and Schatz, 1987).

Two mutants of this precursor protein were constructed by site-directed mutagenesis. In the first mutant (7/42/49), the cysteine in position 7 of the DHFR sequence was changed

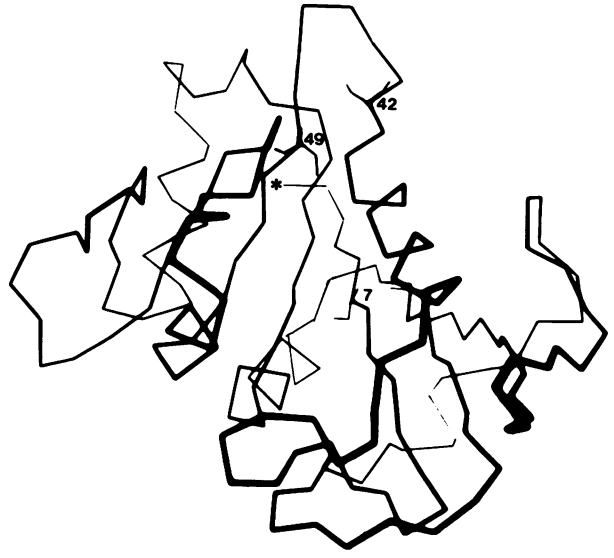

Fig. 1. Crystal structure of chicken liver DHFR (α carbon backbone). The amino acids in positions 7, 42 and 49 are shown with their side chains and represent the changed amino acids in mutant 7/42/49 (see Results). The asterisk marks the amino terminus of the DHFR moiety without the starting methionine. While this methionine is absent in authentic mouse DHFR, it is present in the DHFR moiety of the fusion protein.

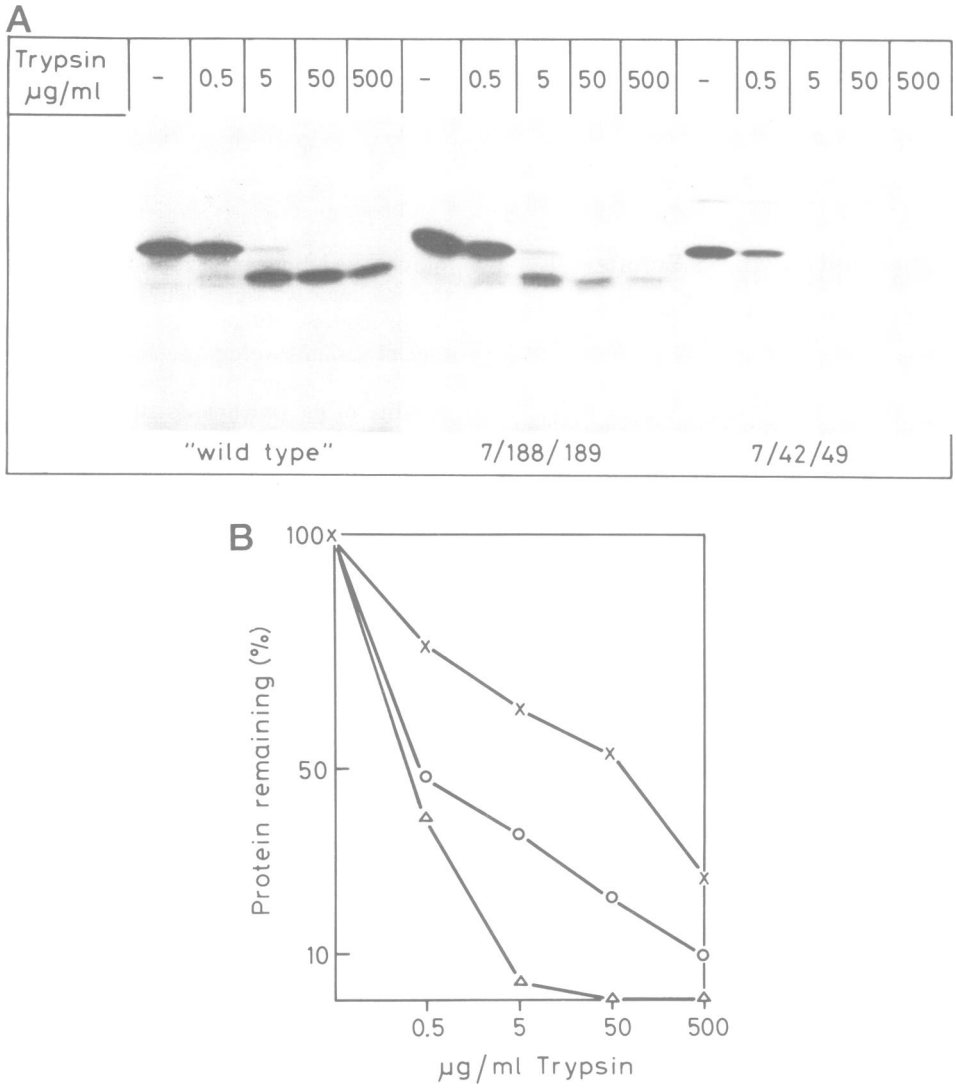

Fig. 2. Trypsin titration of the wild-type precursor and the two mutants. (**A**) Precursors were synthesized in the reticulocyte lysate, diluted 10-fold with 20 mM Hepes−KOH, pH 7.4, and treated with trypsin at the indicated concentrations for 10 min at 0°C. Trypsin was then inhibited by the addition of 10 mM phenyl methyl sulfonyl fluoride (PMSF). Samples were analyzed by SDS−12% PAGE and fluorography. (**B**) The fluorographs shown in (A) were scanned. Aliquots of the synthesized precursor without added trypsin were taken as 100%. ×—× 'wild-type precursor; ○—○ mutant 7/188/189; △—△ mutant 7/42/49.

to serine, and the serine in position 42 as well as the asparagine in position 49 were changed to cysteine. The altered amino acids of this mutant precursor are indicated in the computer image of the highly homologous chicken DHFR (Figure 1). In the second mutant (7/188/189), cysteine at position 7 of the DHFR sequence was again changed to serine while glutamine (188) and cysteine (189) were added at the C terminus [after aspartate (187)].

Authentic mouse DHFR and the DHFR moiety in the previously studied fusion proteins are highly resistant to trypsin unless the folding of the DHFR moiety is perturbed (Hurt *et al.*, 1985; Eilers and Schatz, 1986). To test the stability of the three precursors used in the present study, the proteins were synthesized by coupled transcription/translation *in vitro* in the presence of [^{35}S]methionine and subsequently treated with increasing concentrations of trypsin (Figure 2). As noted with the previously used DHFR-containing fusion protein, the 'wild-type' fusion protein used here exhibited the trypsin resistance typical of enzymically active, folded mouse DHFR: at 5 µg trypsin/ml, it was converted into a fragment that was resistant to even 500 µg trypsin/ml. This fragment presumably represents the presequence-free DHFR moiety (see also Eilers and Schatz, 1986). With mutant 7/188/189 the yield of the protease-resistant fragment was significantly lower. With mutant 7/42/49, no resistant fragment was seen at all, most of the precursor being degraded to smaller fragments even at 50 µg trypsin/ml. By this criterion, lability of the DHFR moieties increases in the order 'wild-type' < 7/188/189 < 7/42/49.

Binding of substrate analogs such as methotrexate is another test to assess proper folding of DHFR (Volz *et al.*, 1982). It was previously shown that methotrexate inhibits mitochondrial import of a DHFR-containing fusion protein at roughly the same concentrations at which it inhibits the enzymic activity of authentic DHFR (Eilers and Schatz, 1986). In contrast, methotrexate does not inhibit import of incorrectly folded DHFR moieties (Verner and Schatz, 1987; Eilers *et al.*, 1988). We therefore tested whether methotrexate would inhibit import of the 'wild-type' precursor and its two mutant variants to different degrees. This was indeed

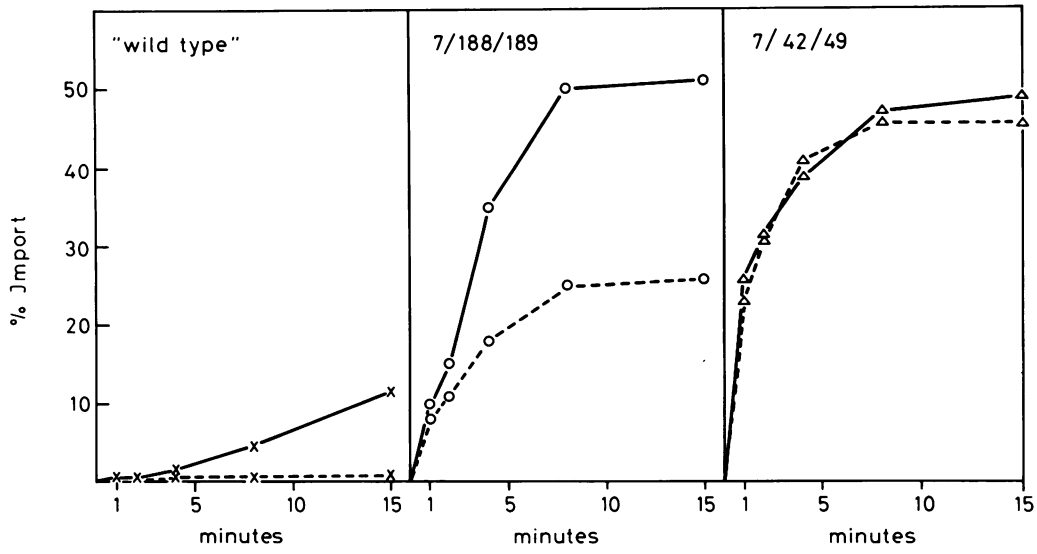

Fig. 3. Time-course of import into mitochondria for the three different precursor proteins in the presence and absence of methotrexate. *In vitro*-translated precursors were incubated with isolated mitochondria for the indicated times at 30°C, either in the absence of methotrexate (—) or in the presence of 400 nM methotrexate (---). Import was stopped by chilling on ice and the addition of 10 μg/ml valinomycin. Mitochondria were subsequently treated with 0.5 mg/ml proteinase K for 15 min at 0°C. The protease was stopped by addition of 2.5 mM PMSF. Mitochondria were reisolated by sedimentation through a 25% sucrose cushion, lysed in gel electrophoresis sample buffer and analyzed by SDS–12% PAGE and fluorography. The precursor band inaccessible to external proteinase K was scanned. No protease-protected material was observed after import assays in the presence of 10 μg/ml valinomycin.

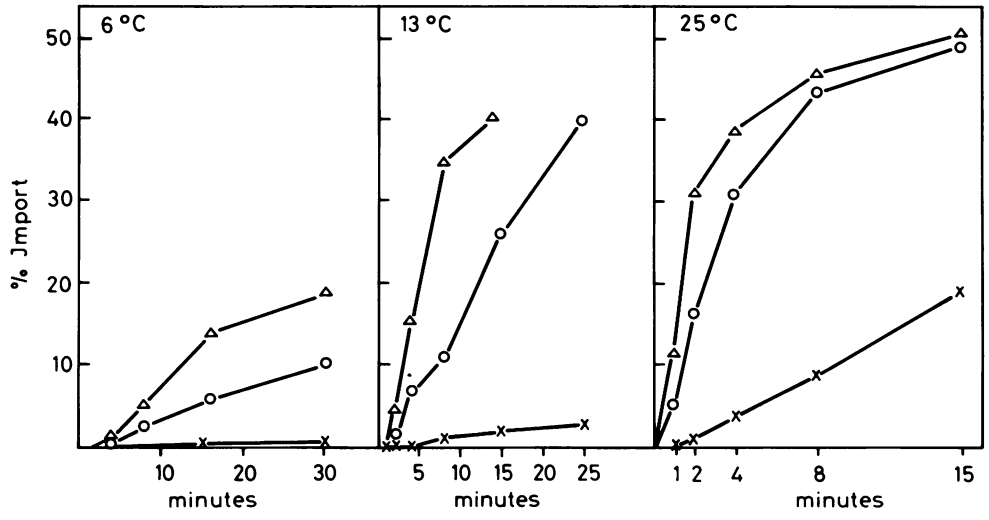

Fig. 4. Time-course of import into mitochondria for the three precursors at three different temperatures. *In vitro*-synthesized radiolabeled precursors were incubated with isolated mitochondria for the indicated periods and at the indicated temperatures. Import was stopped and imported precursor was analyzed as in Figure 3. ×—× 'wild-type' precursor; ○—○ mutant 7/188/189; △—△ mutant 7/42/49.

the case (Figure 3): whereas import of the 'wild-type' precursor was completely blocked, that of mutant 7/188/189 was blocked only partially (by ~2/3) and that of mutant 7/42/49 was not significantly blocked. Resistance to inhibition by methotrexate thus increased in the order 'wild-type' < 7/188/189 < 7/42/49. The same order had been found upon testing susceptibility to trypsin (cf. above). Although neither of the two operational tests yields quantitative data on the tightness of folding, we conclude that the 'wild-type' precursor, mutant 7/188/189 and mutant 7/42/49 contain DHFR moities of increasing labilities.

Destabilization of the precursor protein enhances the rate and the efficiency of its import into mitochondria

Each precursor was synthesized *in vitro* in the presence of [^{35}S]methionine and presented to energized mitochondria at either 6, 13 or 25°C. Figure 4 shows that increased labilization of the precursor not only increased the initial import rate, but also allowed import to occur more efficiently at lower temperatures.

Discussion

In this study we have compared the mitochondrial import of three precursor proteins which differ from each other in only three (out of >200) residues. The DHFR moieties of these three precursors represent three different levels of stability as suggested by their susceptibility to trypsin and their ability to bind methotrexate. Since the three proteins have nearly identical sizes and overall charge, they only

appear to differ from each other by the tightness of folding of their DHFR moieties. They are thus useful tools to assess the role of protein folding in protein translocation across biological membranes.

Unfolded conformers of a closely similar precursor protein have been used before to address this question (Verner and Schatz, 1987; Eilers *et al.*, 1988). These studies had revealed that incomplete folding of the precursor protein accelerated its rate of import into mitochondria and rendered import resistant to methotrexate and low temperature. However, neither of these earlier approaches allowed rigorous comparisons between the folded and incompletely folded form of the precursor: the nascent peptidyl-tRNA chains differ from the folded precursor not only in conformation, but also in size and the presence of a large, highly charged tRNA. A urea-denatured precursor, on the other hand, refolds and rapidly loses import competence as it is diluted out of urea into the mitochondrial suspension, resulting in a race between import and refolding which is difficult to control. The three precursor variants studied here do not suffer from these problems; they should provide a basis for more detailed studies on how a protein's stability affects its translocation across biological membranes. Our results show that decreasing a precursor's stability increases its rate of import into isolated mitochondria. This correlation, obtained with three variants of the same precursor protein, is striking and strongly supports the earlier suggestion (Eilers and Schatz, 1986) that DHFR-containing fusion protein must unfold in order to enter mitochondria post-translationally. This conclusion is also supported by results from other laboratories: mitochondrial precursors trapped during their import into isolated mitochondria were shown to span both mitochondrial membranes with their amino termini present within the matrix, suggesting that at least the amino termini were extended (Schleyer and Neupert, 1985). Similarly, the ability of a bacterial precursor protein to move from the cytoplasm to the periplasmic space was correlated with a loosely folded structure (Randall and Hardy, 1986).

Since DHFR contains at least one 'cryptic' mitochondrial targeting sequence which is inactive unless placed at the amino terminus (Hurt and Schatz, 1987), some of the point mutations described here might act by unmasking one of these cryptic sequences: this possibility is made highly unlikely by the observation that neither urea-denatured nor nascent chains of presequence-free authentic DHFR are imported by mitochondria (Verner and Schatz, 1987; Eilers *et al.*, 1988).

Post-translational import of precursors into mitochondria requires not only an energized inner membrane, but also ATP (Pfanner and Neupert, 1986; Chen and Douglas, 1987; Eilers *et al.*, 1987), whereas post-translational import of incompletely synthesized chains of a precursor was found to be ATP independent (Verner and Schatz, 1987). Since this suggests that ATP may mediate unfolding of precursors during import, we checked the ATP requirement of the three fusion proteins described here. Upon testing import in the presence of increasing levels of apyrase (an ATP-splitting enzyme), import of all three proteins was increasingly inhibited; at high apyrase levels (12.5 U/ml), import was completely blocked. This effect was not caused by any protease contamination in the apyrase preparation, because readdition of an ATP-regenerating system following apyrase treatment restored normal import levels. The ATP dependence of the two mutant proteins probably reflects the fact that they have retained some secondary or tertiary structure. Thus the ATP dependence of the two mutant forms does not invalidate the hypothesis that ATP participates, directly or indirectly, in unfolding of precursors for subsequent translocation across a membrane.

Materials and methods

Oligonucleotide-directed mutagenesis

Mutations were introduced into a fusion protein consisting of the first 16 residues of the yeast cytochrome oxidase subunit IV precursor fused to the amino terminus of mouse DHFR. The fusion gene was carried on a pDS plasmid (Stüber *et al.*, 1984; Hurt *et al.*, 1985). The sequence encoding the fusion protein was excised with *Eco*RI and *Hin*dIII and transferred to the vector M13 mp10. Mutant 7/42/49 was constructed in three successive mutagenesis steps using three different oligonucleotides, essentially according to Zoller and Smith (1983). No selection step was included except that the DNA obtained upon primer extension was transformed into the repair-deficient *Escherichia coli* strain BMH 71-18 mutS (kindly provided by Professor T.Bickle, Biocenter). Mutant 7/188/189 was constructed by first generating a *Bgl*II site within the aspartate codon at the 3′ end of the coding sequence. This site was then cut with *Bgl*II, filled in with the large fragment of *E.coli* DNA polymerase and blunt-ligated to a linker which encoded the two additional amino acids glutamine and cysteine at the C terminus of the mutant protein, as well as a new stop codon. Finally, the mutation in position 7 was introduced by excising the *Eco*RI–*Sac*I fragment (encoding the 5′ half of the fusion protein) and exchanging it for the corresponding fragment from mutant 7/42/49.

Import into isolated yeast mitochondria

Mitochondria were isolated (Daum *et al.*, 1982) from the wild-type *Saccharomyces cerevisiae* strain D273-10B. Import reactions were done in a final volume of 0.2 ml containing 200 μg mitochondria and 15 μl of reticulocyte lysate containing the radiolabeled, *in vitro*-synthesized precursor protein (Hurt *et al.*, 1984). For time-course experiments, import was stopped by adding valinomycin to 10 μg/ml and chilling on ice. Where indicated, methotrexate was present at 400 nM. To deplete ATP levels the translation mixture was treated with varying amounts of potato apyrase (33 000, 3300, 330, 3.3 mU/ml) for 5 min at 30°C before addition of mitochondria. Also, mitochondria were pretreated with 25 μg efrapeptin/ml and varying apyrase levels (12 500, 1250, 125, 12.5, 1.25 mU/ml) for 5 min at 0°C. The membrane potential of these ATP-depleted mitochondria was maintained by adding 10 mM succinate/10 mM L-malate and aerating the mitochondria by vigorous shaking in a water-bath (Eilers *et al.*, 1987).

Other methods

Published methods were used for coupled transcription/translation (Hurt *et al.*, 1984), SDS–12% PAGE (Hurt *et al.*, 1984), DNA manipulations (Maniatis *et al.*, 1982) and protein measurements (BCA method, Technical Bulletin by Pierce Chemical Co., USA). Fluorographs were scanned and quantified with a Camag TLC Scanner II coupled to a Camag SP4280 Integrator.

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References

Chen,W.-J. and Douglas,M.G. (1987) *Cell*, **49**, 651–658.
Daum,G., Böhni,P. and Schatz,G. (1982) *J. Biol. Chem.*, **257**, 13028–13033.
Eilers,M. and Schatz,G. (1986) *Nature*, **322**, 228–232.
Eilers,M., Oppliger,W. and Schatz,G. (1987) *EMBO J.*, **6**, 1073–1077.
Eilers,M., Hwang,S. and Schatz,G. (1988) *EMBO J.*, **7**, 1139–1145.

Hurt,E.C. and Schatz,G. (1987) *Nature*, **325**, 499–503.
Hurt,E.C., Pesold-Hurt,B. and Schatz,G. (1984) *EMBO J.*, **3**, 3149–3156.
Hurt,E.C., Pesold-Hurt,B., Suda,K., Oppliger,W. and Schatz,G. (1985) *EMBO J.*, **4**, 2061–2068.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Pfanner,N. and Neupert,W. (1986) *FEBS Lett.*, **209**, 152–156.
Randall,L.L. and Hardy,S.J.S. (1986) *Cell*, **46**, 921–928.
Schatz,G. (1986) *Nature*, **321**, 108–109.
Schleyer,M. and Neupert,W. (1985) *Cell*, **43**, 339–350.
Stüber,D., Ibrahimi,I., Cutler,D., Dobberstein,B. and Bujard,H. (1984) *EMBO J.*, **3**, 3143–3148.
Verner,K. and Schatz,G. (1987) *EMBO J.*, **6**, 2449–2456.
Volz,K.W., Matthews,D.A., Alden,R.A., Freer,S.T., Hansch,C., Kaufman,B.T. and Kraut,J. (1982) *J. Biol. Chem.*, **257**, 2528–2536.
Wickner,W.T. and Lodish,H.F. (1985) *Science*, **230**, 400–407.
Zimmermann,R. and Meyer,D.J. (1986) *Trends Biochem. Sci.*, **11**, 512–515.
Zoller,M.J. and Smith,M. (1983) *Methods Enzymol.*, **100**, 468–500.

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