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Mitochondrial Biogenesis: Recent Developments and Insights

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Mitochondrial biogenesis: recent developments and insights

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Biosynthesis of a functional mitochondrion requires the coordinate expression of genes in both mitochondrial and nuclear DNAs. In yeast, three mitochondrial genes are split and RNA splicing plays a pivotal role in their expression. The recent finding that some introns are capable of self-splicing activity *in vitro* has permitted analysis of the mechanisms involved in RNA catalysis and may eventually shed light on the evolution of splicing mechanisms in general.

Most mitochondrial proteins are encoded by nuclear genes, synthesized in the cytoplasm and imported by the organelle. The availability of cloned genes coding for several constituent subunits of the ubiquinol–cytochrome *c* reductase, which are imported by mitochondria, has allowed study of selected steps in the addressing of proteins to mitochondria and their intercompartmental sorting within the organelle. Recent developments are discussed.

1. Introduction

Biogenesis of a functional mitochondrion depends on the coordinate expression of genes in both nuclear and mitochondrial DNAs. It has been suggested that the retention of a separate genetic system by this one-time endosymbiont is an evolutionary frozen accident (Borst 1971). Be that as it may, the study of the mechanism of gene expression, as practised by the mitochondrial genomes of various organisms, has not only told us much about mitochondrial biogenesis; it has also led to insight into the ways in which components of the transcriptional and translational machinery function and in which genetic systems evolve. Alongside this, the study of nuclear genes coding for mitochondrial components has provided vital information on the ways in which synthesis is coordinated and the products specifically addressed to the organelle.

The study of mitochondrial genes has even turned up an entirely new step in gene expression, namely the process of RNA editing as exercised during expression of genes in kinetoplast DNA in trypanosomes (Benne *et al.* 1986). During editing, which may occur during or immediately after transcription, nucleotides are added to a short segment of the transcript, thereby permitting the correction of frameshifts, or creation of new 5′ leader sequences in mRNA (Feagin *et al.* 1987). Why kinetoplast DNA should take the trouble to maintain what in other DNAs would be pseudogenes, and why it should have adopted what appears to be such an error-prone way of circumventing the problems posed in their expression, is as yet unknown. One possibility is that RNA editing copes with restrictions imposed by strict DNA sequence constraints in this curious mitochondrial DNA.

A detailed review of all facets of mitochondrial biogenesis is beyond the scope of this contribution and is in any case unnecessary, given recent excellent reviews (Clayton 1984; Tzagoloff & Myers 1986). Findings in two areas have implications that extend beyond the bounds of the organelle itself. These are examined below.

2. Self-splicing of yeast mitochondrial RNAs; mechanisms of RNA catalysis

From first encounters with yeast mitochondrial introns, it was clear that they were unusual (see Grivell *et al.* (1983) for review). All had the potential to form extensive and complex secondary structures and many contained long reading frames. A few gave rise to circular RNA forms on excision. The significance of these features has since fallen into place, with the discovery that folding is required to make ends meet and to align exons during splicing (Waring & Davies 1984). The reading frames encode a family of nucleic-acid-wielding proteins, many of which are required for excision of the intron that encoded them. Finally, in several cases, the 'circular' RNAs have turned out to be branched and they accumulate as products of a splicing reaction that can be achieved *in vitro* in the absence of protein (Peebles *et al.* 1986; Schmelzer & Schweyen 1986; van der Veen *et al.* 1986).

The introns in yeast mitochondrial DNA can in fact be divided into two distinct groups, on the basis of their sequence, their predicted secondary structures and, more recently, the mechanism of self-splicing *in vitro* (reviewed by Tabak & Grivell (1986)). Group I introns share a set of short sequence elements and a common folding pattern. The self-splicing intron present in the *Tetrahymena* large rRNA gene is of the group I type and the transesterification theme first elucidated for this intron (Cech *et al.* 1981) also appears to hold for its mitochondrial counterparts (Garriga & Lambowitz 1984; Van der Horst & Tabak 1985). Nucleophilic attack by a guanosine nucleotide on the 5′-exon–intron junction is required to trigger the reaction; *in vitro*, both initial and subsequent bond openings can occur at a variety of sites, thus generating a number of aberrant RNAs, including lariats and interlocked circles (Arnberg *et al.* 1986; Tabak *et al.* 1987). These aberrant reactions conjure up a picture of a highly reactive RNA molecule, which, having bound guanosine at its active site, is capable of breaking and re-forming a variety of phosphodiester bonds. *In vivo*, reactivity may be subject to stringent controls by proteins, because such products do not appear to be formed.

Of the four group II introns in yeast mtDNA, two have been demonstrated to possess self-splicing activity. There are three points of interest in this.

(i) The introns are predicted to possess a secondary structure that differs radically from that of group I introns (Michel & Dujon 1983). They also lack the characteristic set of conserved sequence elements. Activity is thus likely to be mediated by a catalytic centre, which differs in its architecture from that of group I. Its characterization will be of value for insight into the molecular basis of RNA catalysis and evolution of the catalytic mechanism.

(ii) Splicing is not dependent on exogenous nucleotides. The 2′-OH group of an intron-internal adenosine fulfils the role played by guanosine in group I splicing, with the result that cleavage of the 5′-exon is accompanied by formation of a lariat.

(iii) An intron lariat is formed as a product of the reaction. Lariat formation is a characteristic of splicing reactions undergone by nuclear pre-RNAs. In both mitochondrial and nuclear RNAs, the 2′–5′ linkage is made to an adenosine residue embedded in a short consensus sequence with similar features. RNA catalytic mechanisms operative in the splicing of mitochondrial group II introns may therefore form the basis for the spliceosome–snRNP-associated reaction in the nucleus (Cech 1986; Padgett *et al.* 1986).

Although in theory any of the intron-internal ribose 2′-OH groups could act as attacking nucleophile in lariat formation, this probably does not occur in practice because such groups

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are far less reactive than OH^- or the terminal 2′-, 3′-OH groups in RNA. Additional factors, including bond stressing and 2′-OH activation by neighbouring proton-abstracting groups, must therefore also contribute to the reactivity of the adenosine used to form the branch. As part of a study aimed at identification of sequences making up this reaction centre, Van der Veen *et al.* (1988) have carried out a mutagenic analysis of the branchpoint region. Two mutants, containing either a deletion of the branchpoint adenosine (ΔA_{880}), or an addition of a U residue at a position 25 nucleotides upstream of it ($+U_{856}$), are of particular interest and are shown in figure 1. Under conditions normally used for splicing, both mutants are inactive. However, at high ionic strength and in the presence of raised concentrations of Mg^{2+}, efficient exon–exon ligation occurs in either the total absence (ΔA_{880}) or with only low levels of lariat accumulation ($+U_{856}$). The simplest explanation for this activity, and one which we prefer (see Van der Veen *et al.* (1988) for full discussion), is that the role of the branchpoint adenosine is fulfilled by free OH^-, which has gained access to the active centre and which catalyses a site-specific hydrolysis, analogous to the action of the guanosine nucleotide in group I splicing. The implications are as follows.

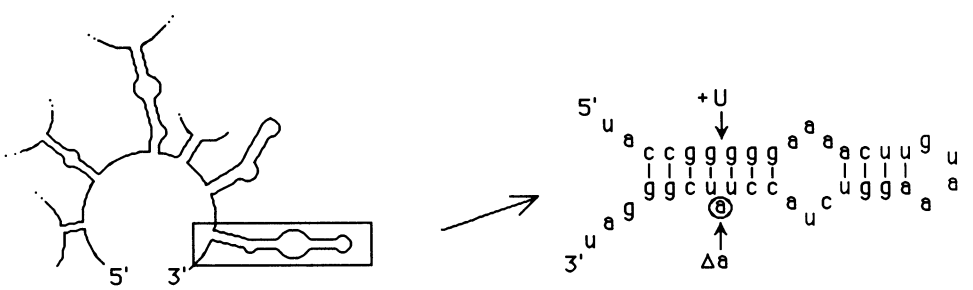

FIGURE 1. Sites of mutations affecting self-splicing of a mitochondrial group II intron. Schematic representation of predicted conserved secondary structure in intron aI5 of yeast mtDNA. Mutations in the branchpoint region, which lead to altered splicing behaviour, are shown in an expanded view of the 3′-hairpin. (See text for explanation.)

(i) Efficient splicing in the absence of lariat formation implies that OH^- does not compete with the 5′-exon in its attack on the intron–exon border in the second stage of the reaction. How this is achieved is not clear. If the same active centre is used for both stages, a conformational change may limit access of OH^-, thus preventing total uncoupling. Alternatively, separate centres may catalyse the individual steps.

(ii) Efficient exon–exon ligation in the absence of lariat formation raises the question of why group II introns should have maintained this step as part of their splicing mechanism. One possibility is that lariat formation is required to stabilize an intron configuration that promotes attack of the 5′-exon on its 3′-counterpart; differences in efficiency between linear and branched forms may not be evident at the lower rates of catalysis operative *in vitro*, or if a high Mg^{2+} concentration stabilizes the appropriate conformation. Alternatively, proteins may contribute to activity by binding to the branchpoint region, effectively excluding water via electrostatic shielding. In such a situation, branchpoint mutations will be unconditionally splicing-defective *in vivo*, because access of OH^- will be prevented. Furthermore, it may be

impossible to achieve the configuration required for the uncoupled reaction under the ionic conditions prevailing in mitochondria. It may be significant in this respect that in many other RNAs unpaired adenosine residues are often involved in protein binding (Peattie *et al.* 1981).

3. Mitochondrial protein import

Functional domains in mitochondrial presequences

Most mitochondrial proteins are encoded by nuclear genes, synthesized in the cytoplasm and imported by the mitochondrion (Eilers *et al.*, this symposium; Grivell 1988). Given that several hundred proteins have to follow this route in order to construct a functional organelle, mitochondrial protein import probably represents one of the major highways for protein routing within the eukaryotic cell. Most such proteins are synthesized in precursor form, with an extension at the N-terminus. Although diversity is the hallmark of such presequences, with wide variation in length and sequence being possible, common features are present; the most obvious of these is a predominance of positively charged and neutral hydroxyl amino acids, and absence or scarcity of negatively charged residues. However, the most important shared feature may be the ability of the presequences to form helices with an asymmetric distribution of charged and hydrophobic residues on their surfaces, when placed in a suitable environment (Roise *et al.* 1986; Von Heijne 1986). Such a structure suggests a model for import in which one face of the helix interacts with the mitochondrial surface, either through electrostatic interaction with negatively charged phospholipid head groups or the membrane potential, while the hydrophobic face buries itself in the lipid bilayer. Subsequently, action of the membrane potential could lead to re-orientation of the helix within the membrane, either by interaction with the helical dipole or by electrophoresis of positive charge.

The amphiphilic helix model takes account of the observation that one chemically synthesized presequence of an import protein can form an amphiphilic helix capable of perturbing both natural and artificial phospholipid bilayers (Roise *et al.* 1986). It also provides ready explanations for the findings that amphiphilic α-helical synthetic peptides can specifically block import of precursors by isolated mitochondria (Ito *et al.* 1985), and that prokaryotic, eukaryotic cytoplasmic, or even quasi-random synthetic sequences conforming to the general structure of a mitochondrial presequence have targeting activity (Allison & Schatz 1986; Baker & Schatz 1987). On the other hand, a number of problems remain (see Grivell (1988) for discussion).

(i) The model offers no satisfactory explanation for the specificity of targeting and import; it seems unlikely that sufficient specificity can be achieved on the basis of presequence–lipid interactions alone.

(ii) There is a frequent lack of correlation between predicted surface-active properties of a presequence and its known import ability (see, for example, Roise *et al.* (1986) and below).

(iii) The model stresses the importance of overall amphiphilic character; it is often unable to account for the effects of mutations on specific amino acid residues in the presequence.

A number of these points can be illustrated by results obtained with the presequence of the 40 kDa core II subunit of complex III (figure 2). The core II protein is located in the mitochondrial inner membrane, with the bulk of its mass protruding into the matrix space. Its 16-amino-acid presequence displays features typical of many other matrix and inner membrane proteins, although amphiphilicity of either the complete sequence, or its N-terminal region, is

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not predicted to be particularly high (Oudshoorn *et al.* 1988). The presequence or an N-terminal 10-residue segment of it can, however, efficiently direct other proteins to the mitochondrion. Using a set of synthetic oligonucleotides, P. Oudshoorn (unpublished work) has studied the effects of single amino acid substitutions on import both *in vitro* and *in vivo* and on processing by a partly purified matrix protease preparation. The results shown in figure 2 can be summarized as follows.

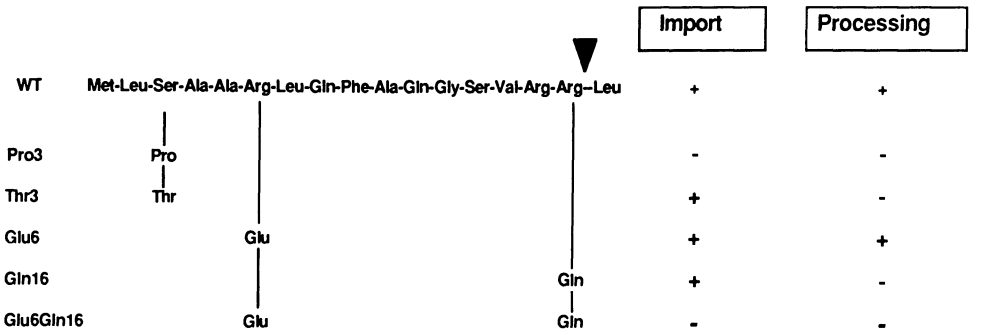

FIGURE 2. Presequence mutations affecting protein import by isolated mitochondria. A set of synthetic oligonucleotides were used to construct the DNA sequence coding for the first 18 amino acids of the precursor to the core II subunit of the yeast ubiquinol–cytochrome c reductase, manipulation of the sequence being facilitated by provision of *Eco*RI and *Bam*HI restriction sites at the 5′- and 3′-termini respectively. Creation of mutant forms of the presequence was achieved by substitution of the appropriate oligonucleotides by versions carrying the desired point mutation. Changes made were designed to have a minimal effect on amphiphilicity, as predicted from the hydrophobic moment (Eisenberg *et al.* 1984). The mutant presequences were then tested for their ability to transport attached mouse DHFR into mitochondria, import being measured as the extent of resistance to proteinase K digestion after incubation for 5′ and 20′ (see legend to figure 3 for further details).

(i) Some of the mutations introduced have drastic effects on *in vitro* import; others have little or no detectable effect. All mutants are predicted to have highly similar hydrophobic moments as calculated according to Eisenberg *et al.* (1984). Although it must be realized that the predictions of secondary structure are no more than that, and that too little is known of the factors which influence the structure of short peptides, the results suggest strongly that amphiphilicity is not the only factor governing import, nor is it the most important one.

(ii) Various of the mutations reduce the overall positive charge of the presequence. This reduction is tolerated, as is also the presence of a negatively charged residue (e.g. Glu_6). A double mutant in which two of the three Arg residues have been replaced and which therefore carries a net charge of +1 is, however, inactive in import.

(iii) A Ser–Pro substitution at position 3 reduces both import and processing to extremely low levels (figure 3, less than 5% wild type), despite the fact that hydrophobic moment and potential for α-helix formation are not predictably altered. Such behaviour is difficult to interpret in any terms other than a requirement of the import machinery for a specific structure or sequence, determined by a specific residue. Serine is relatively strongly conserved at this position, occurring in nine of 30 yeast sequences examined and in a further five out of 16 sequences in other organisms, whereas proline is rare (two occurrences in a total of 46 presequences (Grivell 1988)). The inhibitory effect of the mutation on processing is also somewhat unexpected, in view of the distance of the change from the processing site. As far as is known, processing is the responsibility of a single enzyme, a chelator-sensitive protease (Böhni *et al.* 1983; Yaffe *et al.* 1985), which, although it can recognize a wide range of

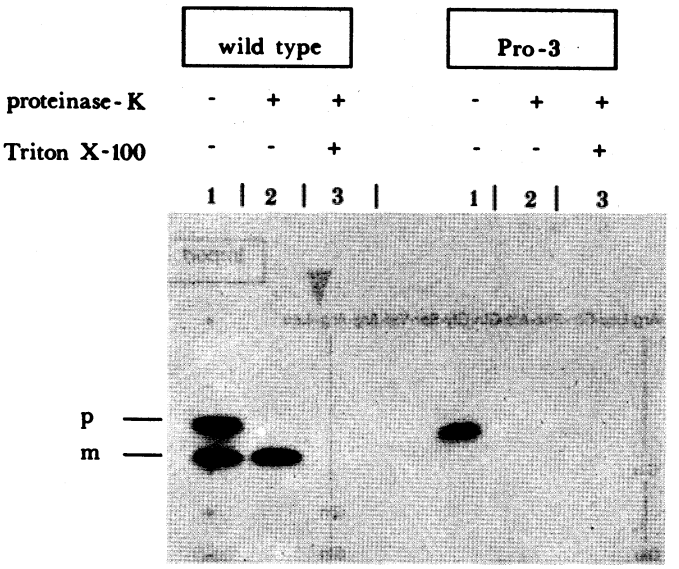

FIGURE 3. A Ser-Pro substitution in the core II presequence blocks *in vitro* import. *In vitro* mitochondrial import of mouse DHFR fused to either a wild-type presequence of the core II protein, or one in which serine at position 3 has been replaced by proline, was monitored essentially as described by Van Steeg *et al.* (1986) with incubation being carried out for 20 min. Proteinase K treatment was for 30 min at 0 °C at a concentration of 50 μg ml^{-1}; digestion was stopped by addition of phenyl methyl sulphonylfluoride to a final concentration of 5 mM. The effectiveness of protease treatment was checked by incubation of a sample of the incubation mixture with proteinase K in the presence of 1% Triton X-100. The mitochondria were then analysed for radiolabelled proteins by SDS–15% polyacrylamide gel electrophoresis and fluorography.

presequences and even act across species barriers, must depend on common structural elements for its action.

The requirement for serine is evidently strict, because even a Ser–Thr substitution at this site results in a block of processing. Such an effect can only be interpreted in terms of altered primary structure.

An unusual presequence

Subunit VI of the yeast ubiquinol–cytochrome *c* reductase is a highly acidic protein with sequence homology to the so-called hinge protein of the beef-heart enzyme (Van Loon *et al.* 1984; Wakabayashi *et al.* 1982). Analogy with the hinge protein suggests that this subunit is located on the outer face of the inner membrane, where it may be involved in promoting interaction between cytochromes *c* and c_1. The exceptional character of the subunit raises the questions of whether it makes use of a special mechanism to cross membranes, whether its addressing sequence has adapted in any way to accommodate the unusual features of the protein and, if adaptation has taken place, whether addressing of other proteins to the same location in the mitochondrion is possible. Recent sequence analysis of the N-terminus of the mature subunit (M. Schmitt and B. Trumpower, personal communication) shows that the transient presequence is N-terminally located and is 25 amino acids long. It differs from most other mitochondrial presequences in carrying a net negative charge, but is nevertheless capable of forming a clear amphiphilic α-helix with its negative charges clustered on one face (figure 4). To test the addressing capabilities of this presequence, a DNA fragment encoding the first 21 amino acids was fused to sequences encoding the mature portions of cytochrome *c* oxidase subunit IV (coxIV) and Mn-superoxide dismutase (Mn-SOD) respectively. Import of the

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fusion proteins into mitochondria in transformed cells was tested either directly, by fractionation and Western blotting, or by means of a complementation assay based on restoration of enzyme activity to cells containing a disrupted version of the gene for coxIV or Mn-SOD. In this assay, complementation is assumed to imply targeting to the correct mitochondrial compartment: the matrix space for Mn-SOD and the inner membrane for coxIV. Additionally, for Mn-SOD, the location of the fusion protein was verified by immunoelectron microscopy (data not shown).

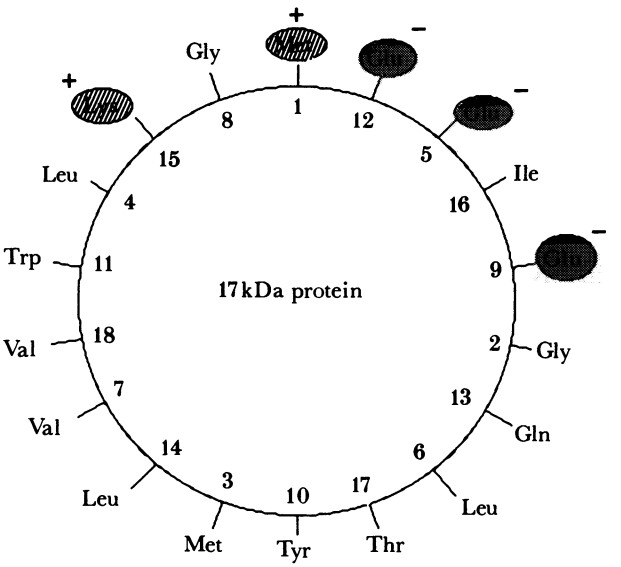

FIGURE 4. The precursor extension of subunit VI of yeast ubiquinol–cytochrome *c* reductase can form a negatively charged amphiphilic helix. Helical wheel projection of the first 18 residues of the presequence of the 17 kDa subunit VI of the yeast ubiquinol–cytochrome c reductase. The sequence shown is taken from Van Loon *et al.* (1984) with a single nucleotide correction to the published sequence at position +5. This results in a glycine residue at amino acid position 2, rather than aspartate as originally determined.

Results obtained are summarized in figure 5. Both proteins display essentially similar behaviour in that both are targeted to mitochondria, where they are recovered in the intermembrane space (IMS). In neither case is complementation observed; this result implies either that the proteins have not attained their normal locations, or that, having attained them, they have undergone rapid export to the IMS. We favour the first alternative, which therefore suggests that the presequence of the 17 kDa protein achieves targeting to the IMS as the result of its inability to cross the mitochondrial inner membrane. We are currently testing this hypothesis, together with the idea that the negative charges within the presequence are responsible for this behaviour. The route followed by the 17 kDa protein into the IMS may turn out to be the simplest of all those leading to this particular mitochondrial compartment, contrasting with that for several haemoproteins, which involves membrane-bound intermediates (see Hay *et al.* 1984), and that for the Rieske iron–sulphur protein, which involves matrix import, followed by export to the IMS (Hartl *et al.* 1986). These latter routes may therefore reflect needs for specific post-translational modifications of the various proteins.

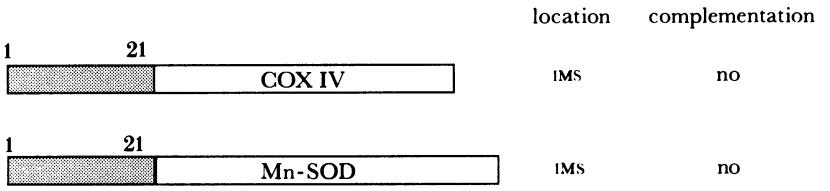

FIGURE 5. The precursor extension of subunit VI of yeast ubiquinol–cytochrome *c* reductase can direct other proteins to the mitochondrion. Schematic representation of fusion proteins used to test the targeting properties of the presequence of the 17 kDa subunit VI of the yeast ubiquinol–cytochrome *c* reductase. Details of the constructs, together with a description of the subfractionation and complementation assays, will be presented elsewhere (Oudshoorn *et al.* 1988).

Mutational analysis of proteins involved in mitochondrial protein import

Although the main emphasis of the amphiphilic helix model for insertion of presequences into the mitochondrial membrane lies in direct interactions with the lipid bilayer, several lines of evidence point to involvement of proteins in unfolding of the precursor protein and its binding to the mitochondrial surface (Eilers *et al.*, this symposium). So far, however, little is known of such proteins.

Results of a mutational approach, which may be of value in the identification of receptors and components of the translocational machinery, are shown in figures 6 and 7. In this, use is made of yeast cells lacking Mn-SOD as a result of gene disruption. As shown by Van Loon *et al.* (1986), such cells grow normally on non-fermentable substrates in air, but are inviable in 100% oxygen. Resistance to oxygen can be conferred by transformation of the cells with a plasmid, carrying information for either the wild-type SOD precursor or a fusion protein, in which the mitochondrial topogenic signal is provided by all or part of the presequence of the 40 kDa core II protein of complex III. As shown in figure 6, fusions formed with either 35 or 18 amino acids from the N-terminus of the 40 kDa precursor protein confer oxygen resistance, even though the SOD molecules that arise after processing by the matrix protease still carry extra sequences, consisting of 19 or 2 amino acids respectively of the N-terminus of the 40 kDa protein. In contrast, fusions formed with either 10, or 3 amino acids of the 40 kDa precursor do not confer oxygen resistance. In the case of the 3 amino acid fusion, this result is explicable in terms of lack of sufficient addressing information. The lack of complementation in the case of the 10-amino-acid fusion is of more interest; however, previous work (Oudshoorn *et al.* 1988) has shown that this segment of the presequence does contain sufficient information for addressing of proteins to the mitochondrion. Although it is possible that this fusion, which is not processed by the matrix protease because the cleavage site is absent, is enzymically inactive as a result of the presence of 10 additional amino acids, results obtained with the longer fusions make such an explanation unlikely. At this stage we therefore prefer the alternative, that lack of complementation is due to incorrect localization of the fusion protein as a consequence of an inability to translocate across the inner membrane.

To identify components of the translocational machinery, the SOD^- host, carrying the 10-SOD fusion, has been subjected to mutagenesis and the surviving cells screened for oxygen resistance. Positive colonies were found; after plasmid curing, these were re-transformed with the plasmid, to distinguish between chromosomal and plasmid-borne mutations. Results obtained for three isolates are shown in figure 7, which demonstrates that in each case the

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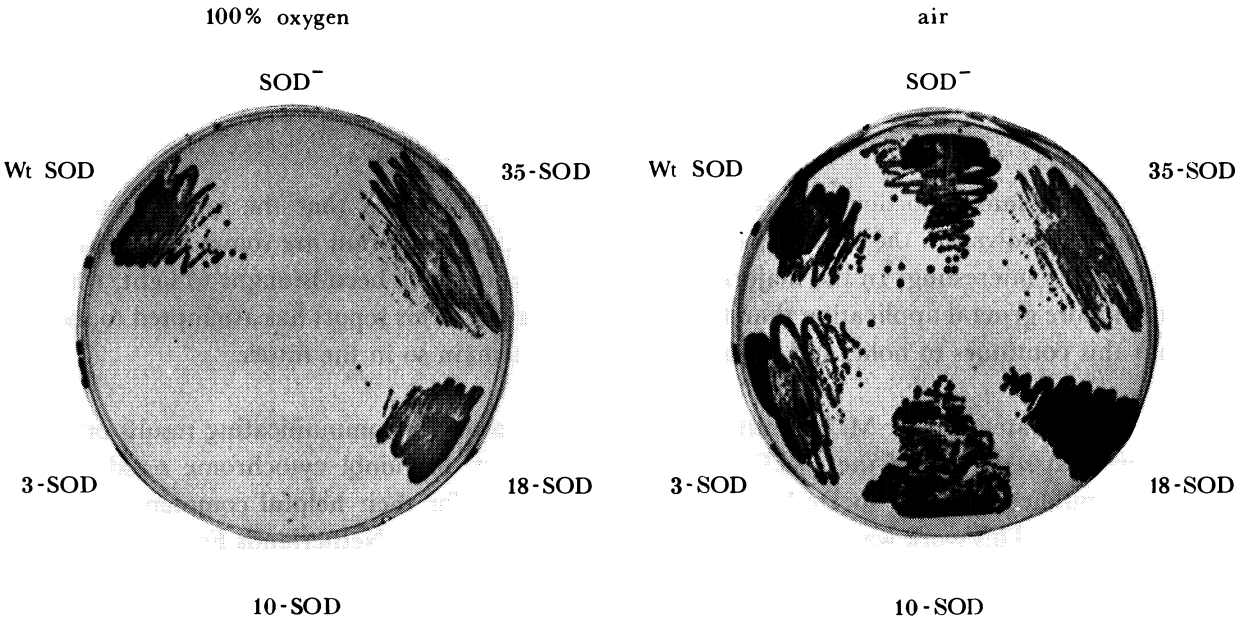

FIGURE 6. Complementation assay for mitochondrial protein import directed by N-terminal segments of the presequence of core II. The ability of N-terminal segments of the precursor of the core II subunit of ubiquinol–cytochrome c reductase to direct Mn-SOD to the matrix space of the mitochondrion was tested by a complementation assay based on the sensitivity of SOD^- cells to 100% oxygen when grown on glycerol (Van Loon *et al.* 1986). Of the transformants shown, two failed to display complementation, owing either to a failure to target the fusion protein to the mitochondria (3-SOD), or an inability to target the fusion correctly within the mitochondria (10-SOD). (See text for further discussion.)

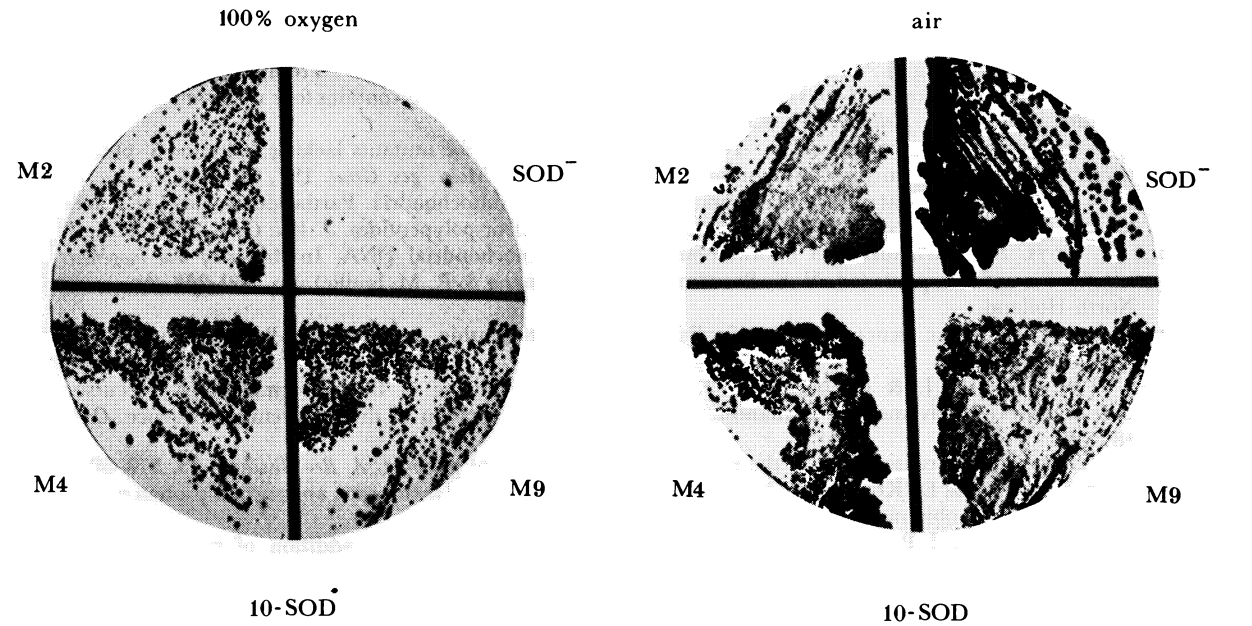

FIGURE 7. Nuclear mutations compensate for the import deficiency caused by truncation of the core II presequence. Mn-SOD deficient yeast cells harbouring a plasmid-borne 10-SOD fusion gene were subjected to ethyl methanesulphonate mutagenesis and survivors were screened for oxygen sensitivity. Resistant colonies were selected and cured of plasmid by growth in the presence of fluoro-orotic acid (Boeke *et al.* 1984). The mutants M2, 4 and 9 thus isolated displayed oxygen resistance when transformed with fresh plasmid; this result indicates that resistance is chromosomally encoded (Oudshoorn *et al.* 1988).

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mutation is chromosomal. Further characterization of these mutants, including assay of their ability to import other fusion proteins carrying import-defective presequences, are now in progress.

4. Conclusions and prospects

Research into mitochondrial biogenesis has repeatedly shown that the solutions to the problems involved in the construction of a functional mitochondrion are sometimes unusual, and always interesting. In the majority of cases, principles have been brought to light, which are of more general application than to the organelle alone. This report has attempted to show that this continues to hold, and we hope that it will remain so in the future.

We are grateful to Dr M. Schmitt and Dr B. Trumpower for communicating results on the N-terminal sequence of the 17 kDa subunit VI of yeast ubiquinol–cytochrome *c* reductase before publication, and to Dr R. Benne and Dr P. Sloof for their helpful comments on the manuscript. This work was supported in part by grants from the Netherlands Foundations for Chemical Research (SON) and for Technical Sciences (STW) with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO).

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PHILOSOPHICAL TRANSACTIONS OF THE ROYAL SOCIETY B BIOLOGICAL SCIENCES

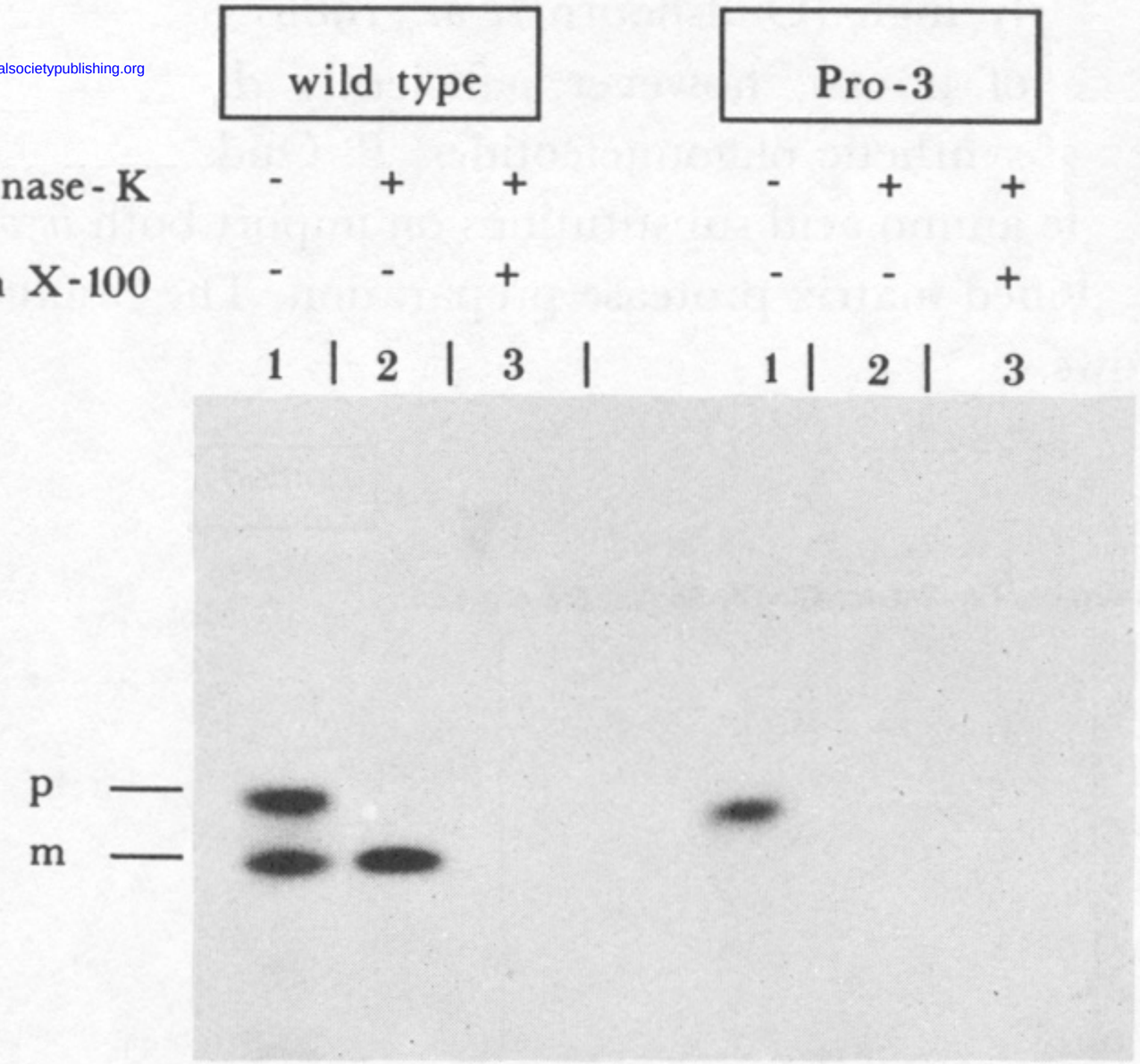

FIGURE 3. A Ser-Pro substitution in the core II presequence blocks *in vitro* import. *In vitro* mitochondrial import of mouse DHFR fused to either a wild-type presequence of the core II protein, or one in which serine at position 3 has been replaced by proline, was monitored essentially as described by Van Steeg *et al.* (1986) with incubation being carried out for 20 min. Proteinase K treatment was for 30 min at 0 °C at a concentration of 50 μg ml^{-1}; digestion was stopped by addition of phenyl methyl sulphonylfluoride to a final concentration of 5 mM. The effectiveness of protease treatment was checked by incubation of a sample of the incubation mixture with proteinase K in the presence of 1% Triton X-100. The mitochondria were then analysed for radiolabelled proteins by SDS–15% polyacrylamide gel electrophoresis and fluorography.

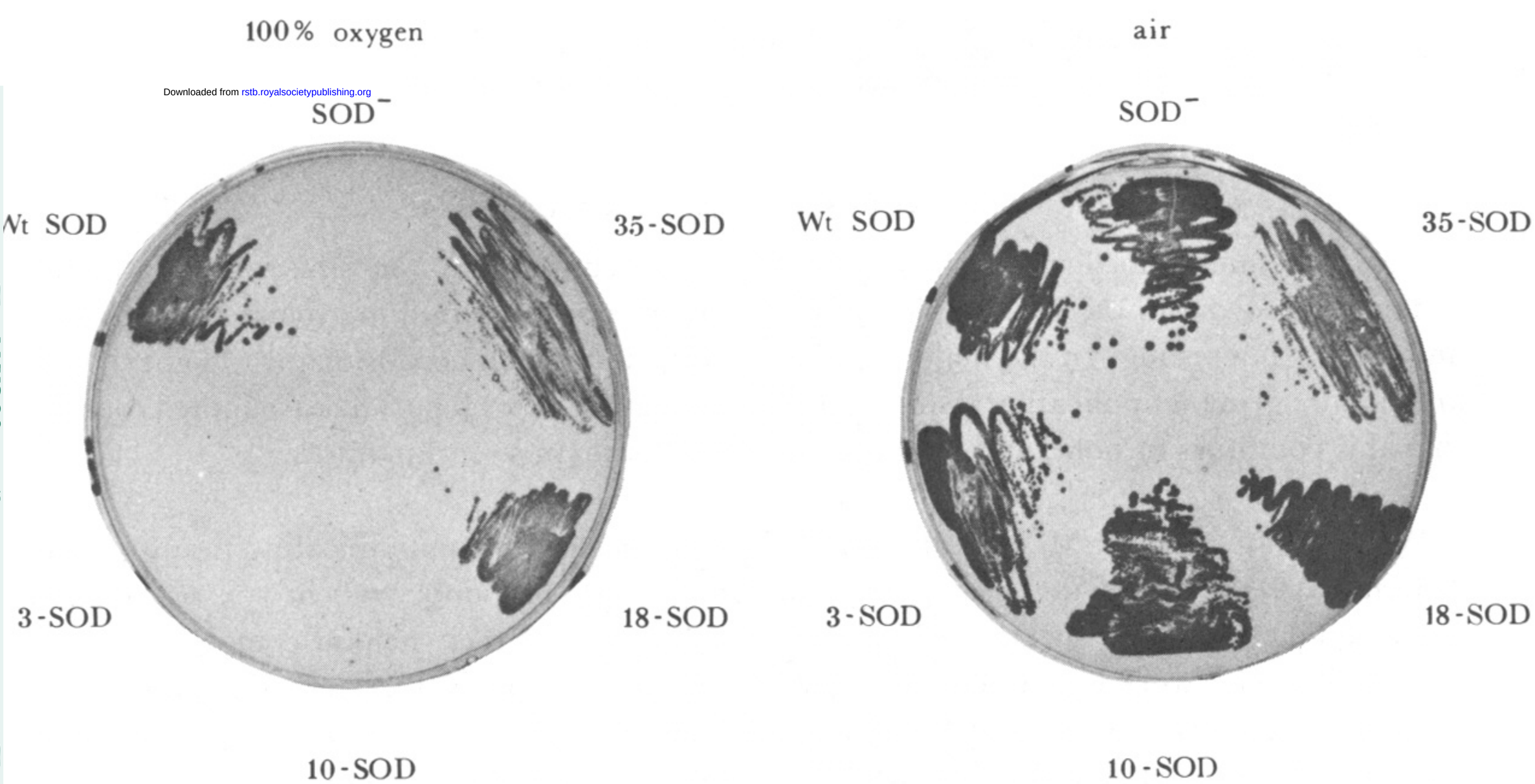

FIGURE 6. Complementation assay for mitochondrial protein import directed by N-terminal segments of the presequence of core II. The ability of N-terminal segments of the precursor of the core II subunit of ubiquinol–cytochrome c reductase to direct Mn-SOD to the matrix space of the mitochondrion was tested by a complementation assay based on the sensitivity of SOD^- cells to 100% oxygen when grown on glycerol (Van Loon *et al.* 1986). Of the transformants shown, two failed to display complementation, owing either to a failure to target the fusion protein to the mitochondria (3-SOD), or an inability to target the fusion correctly within the mitochondria (10-SOD). (See text for further discussion.)

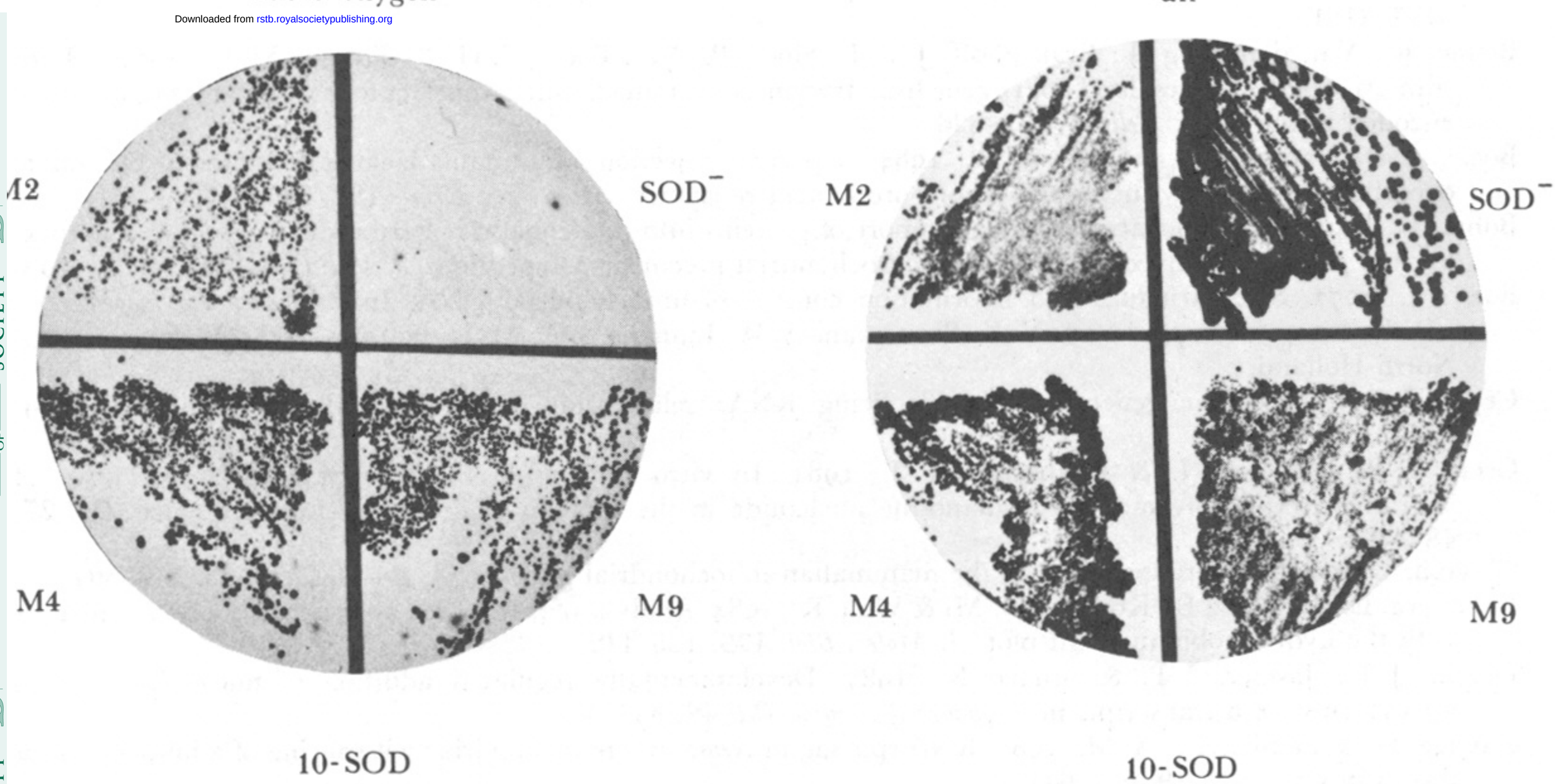

FIGURE 7. Nuclear mutations compensate for the import deficiency caused by truncation of the core II presequence. Mn-SOD deficient yeast cells harbouring a plasmid-borne 10-SOD fusion gene were subjected to ethyl methanesulphonate mutagenesis and survivors were screened for oxygen sensitivity. Resistant colonies were selected and cured of plasmid by growth in the presence of fluoro-orotic acid (Boeke *et al.* 1984). The mutants M2, 4 and 9 thus isolated displayed oxygen resistance when transformed with fresh plasmid; this result indicates that resistance is chromosomally encoded (Oudshoorn *et al.* 1988).