Regulators of mitochondrial translation in Saccharomyces cerevisiae

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Chapter IV

The translational activators Mss51p and Pet309p play distinct roles in the synthesis of Cox1p in mitochondria of Saccharomyces cerevisiae

Michel Siep, Leslie A. Grivell and Hans van der Spek.
Summary

Mitochondrial synthesis of subunit I of cytochrome c oxidase (Cox1p) in Saccharomyces cerevisiae is dependent on membrane-associated translational activators encoded by MSS51 and PET309. Although the similarity of phenotype of mss51 and pet309 disruption mutants and the apparent proximity in intra-mitochondrial localization of the proteins might suggest some kind of physical or functional interaction, we now present evidence that these proteins act at distinct levels in the translation of COX1 mRNA. First, neither two-hybrid analysis, nor 2D Blue Native Gel electrophoresis, provided any indication that Pet309p and Mss51p have a physical interaction. Second, the respiratory-deficient phenotype of a mss51-null strain is not suppressible by multicopy expression of PET309, and vice versa. Third, mitochondrial genome rearrangements which phenotypically suppress a pet309-null strain as a result of fusion of the COX1 gene to a different 5'UTR do not suppress a mss51-null strain. Additionally, as also reported by other groups, we find that the mss51-null mutant does not give rise to mitochondrial revertants that frequently have been observed to arise in strains disrupted for other translational activator genes (including PET309), indirectly suggesting that Mss51p either does not interact with the 5'-UTR COX1 mRNA, or that unlike Pet309p, it interacts with a region that cannot be readily separated from the Cox1p coding sequence.

Introduction

In the yeast Saccharomyces cerevisiae the mitochondrial genome encodes seven mRNAs, six of which encode subunits of the respiratory chain (COX1, COX2, COX3, COB, ATP6, and ATP9). The seventh mRNA encodes VAR1, a subunit of the mitochondrial ribosome. A highly unusual feature of the translation of these mRNAs is their specific dependence on a set of translational activators, many of which are either firmly associated with the mitochondrial inner membrane, or display interaction with the mitochondrial ribosome (Fox, 1996). In the majority of cases, translational activation appears to be dependent on interaction with specific regions of the 5'-UTR of the mRNA involved. Association of the proteins with the mitochondrial inner membrane has led to the hypothesis that mitochondrially encoded subunits of complexes of the respiratory chain need to be translated near the site of their of assembly into the respiratory chain (Michaelis et al., 1991; Fox, 1996). In the case of COX3 mRNA translation is specifically activated by Pet54p, Pet122p and Pet494p. All three proteins are associated with the inner mitochondrial membrane (McMullin and Fox, 1993). Activation occurs through the interaction of these translational activators with the 5' leader of the mRNA (Muller et al., 1984; Costanzo and Fox, 1988; Costanzo and Fox, 1993; Wiesenberger et al., 1995; Costanzo and Fox, 1995). Genetic evidence and two-hybrid studies have given support to a model in which a complex containing Pet54p, Pet122p and Pet494p mediates the interaction of the COX3 mRNA with the mitoribosome at the surface of the inner membrane (Brown et al., 1994). Translation of apocytochrome b (Cobp) is dependent on Cbs1p and Cbs2p (Rödel, 1997). Activation of translation occurs through interaction with the 5'-UTR (Rödel, 1986; Rödel, 1987). Both translational activators are associated with the mitochondrial inner membrane, and Cbs2p has been suggested to associate with the mitoribosome (Michaelis et al., 1991). These data led to the formulation of a model for the translation of the COB mRNA involving interactions between the 5'-UTR of the mRNA, Cbs1p, Cbs2p and the mitochondrial ribosome at the surface of the mitochondrial inner membrane. Synthesis of cytochrome c oxidase subunit 1 (Cox1p) depends on Mss51p and Pet309p. We have recently shown that Mss51p is firmly associated with the mitochondrial inner membrane (Siep et al., 2000). Pet309p was previously shown to be an integral protein of the mitochondrial inner membrane (Manthey et al., 1998). Using two-hybrid analysis, genetic complementation studies and 2D Blue-Native-gel electrophoresis we
searched for possible physical or functional interactions between Mss51p and Pet309p. None of these approaches provided any evidence for such an interaction. We therefore conclude that these two translational activators play distinct roles in the synthesis of Cox1p.

Material & Methods

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<th>Mitochondrial genome</th>
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Strains and media

Yeast were grown at 30 °C on minimal medium, YPGlyc or YPD plates. Minimal medium plates: 0.67% YNB, 2% glucose, 2.2% agar, supplemented with amino acids as necessary. Rich media: 1% bacto-peptone, 1% yeast-extract, 2% glycerol (YPGlyc) or 2% glucose (YPD), 2.2% agar. To isolate mitochondria, yeast were grown at 30 °C in YPGlyc medium or minimal medium with the appropriate amino acids. Strain W303(167) has the nuclear genotype MATalpha, ade2-1, his3-11,-15, trp1-1, ura3-1, leu2-3,-112, the mitochondrial genome is derived from KAR167. In W303(167) Δmss51 the MSS51 gene has been disrupted by insertion of the URA3 gene (Table I). Strain GM10 has the nuclear genotype MATalpha, his4-580, leu2-3, -112, trp1-289, ura3-52, the mitochondrial genome is derived from KAR167. In GM11 the PET309 gene has been disrupted by insertion of the URA3 gene (Table I). These strains were a kind gift of prof. dr J. McEwen. The Mss51HA and Pet309c-myc fusion proteins were expressed in these strains by transforming the strains with the desired plasmids: pMS3 and pMS7 (Table II) for Mss51HA, pGM1 and pGM2 (Table II) for Pet309c-myc. E. coli strain DH5alpha was used in all DNA manipulations requiring E. coli. E. coli cells were grown in 2YT (1.6% Bacto tryptone, 1% yeast extract, 0.5% NaCl), supplemented with ampicillin (100 mg/l) as necessary. For complementation studies yeast were transformed with plasmid DNA by the one step method (Chen et al., 1992). E. coli were made competent and transformed with plasmid DNA by electroporation.
Plasmid DNA was isolated from *E. coli* according to the CTAB method (Del Sal et al., 1988).

**Constructs**

Constructs pMS3 and pMS7 contain the MSS51HA fusion in single copy vector YCplac111 or in multicopy vector YEplac181, respectively (Table II). The plasmids pGM1 and pGM2 contain a fusion of the PET309 gene and the c-myc tag, in single copy vector YCpmyd11 and multicopy vector YEpmyc181, respectively (Table II). These constructs were a kind gift of prof. dr. J. McEwen. To perform two-hybrid analysis, the MSS51 coding sequence was fused with the Gal4 DNA-binding domain. Using PCR, an EcoRV restriction site was introduced directly upstream of the start codon of the MSS51 orf in construct pMS3 (Table II). A 1453 bp EcoRV/Smal fragment was cloned in Smal digested pAS2-1 (Clontech). This resulted in construct pMS14 (Table 2), an in-frame fusion of the DNA-binding domain of Gal4, MSS51 and the HA-tag. In order to search for an interaction with Mss51p, the C-terminal part of Pet309p was fused to the Gal4 DNA-activator domain. A 745 bp EcoRV/Smal fragment of pGM2 was cloned in the Smal site of pACT2 (Clontech), resulting in construct pMS17 (Table II), containing an in-frame fusion of the AD-domain of Gal4 and the C-terminal 35 kD of Pet309. In the two-hybrid screen the FRYL genomic DNA library was used (Fromont-Racine et al., 1997). The original vector is a modified pACTII vector and the genomic DNA is derived from strain Ym955. The *E.coli* FRYL library contained $5 \times 10^6$ clones, 72.5% with insert i.e. $3.6 \times 10^6$ clones. The mean length of DNA fragments was 700 bp.

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**Two-Hybrid screening**

For screening the FRYL library with Gal4AD-MSS51 as bait construct, a mating strategy was used (Fromont-Racine *et al.*, 1997). The Y187 strain (Table I) was transformed with the FRYL library DNA using the high efficiency transformation protocol (Gietz and Schiestl, 1995). The bait construct pMS14 (Table II) was transformed to strain CG1945 (Table I) using one-step transformation (Chen *et al.*, 1992). In order to eliminate cells displaying growth based on weak, a specific interactions that activate the HIS3 gene, we used -LWH plates containing 20 mM 3-Amino-1,2,4-triazole (3-AT). 3-AT dose dependently inhibits imidazoglycerol-phosphatase (IGP), an enzyme involved in histidine biosynthesis. [His\(^{+}\)] colonies were screened for blue-colouring using an X-Gal overlay assay. After partial lysis of the cells with chloroform, 10 ml X-Gal overlay (0.5% agar, 100 mM KPi pH 7.4, 0.04% X-gal) was poured on the plates, plates were incubated at 30 °C for 30 minutes to 18 hours. Blue colonies were streaked on new -LWH plates and checked for blue-colouring again. [His\(^{+}\), X-Gal\(^{+}\)] colonies were grown in -L to select for the prey plasmid. From these cultures plasmids were isolated and the inserts were determined by sequencing the Gal4BD-insert fusions with a Gal4 domain specific primer. To look at a possible interaction between the C-terminal part of Pet309p and Mss51p another two-hybrid screening method...
was used. In this system the host strain PJ69-4A (Table I) contains three different reporter genes, driven by different promoters: HIS3, ADE2 and LacZ (James, 1996). Both plasmids pMS14 and PMS17 (Table II), containing Gal4ADMSS51 fusion and Gal4BDPET309-C-terminus, respectively, were transformed to strain PJ69-4A using one-step transformation (Chen et al., 1992). Double transformants were subsequently screened on -A and -H plates, [A⁺/H⁺] positives were checked for blue-colouring using the X-Gal overlay analysis, as described previously.

**Preparation of mitochondrial fractions for BN-PAGE**

Yeast cells were grown on 20 ml YPGal medium (1% yeast extract, 1% peptone, 2% galactose) overnight to an OD600 of approximately 2. Cells were harvested at 4,000 rpm and washed once in cold H₂O. The pellet (± 0.2 mg wet weight) was resuspended in MTE (0.65 M mannitol, 20 mM Tris pH 7.1, 1 mM EDTA). After addition of 0.4 ml of glass beads this suspension was vortexed for 5 minutes at 4°C. The lysate was put in another tube, and the glass beads were washed in 0.5 ml MTE. The pooled supernatants were centrifuged at 4,000 rpm for 3 minutes at 4°C to remove the unbroken cells. Mitochondria were collected by centrifugation at 13,000 rpm for 15 minutes at 4 °C. Pellets were stored at -70 °C.

**Electrophoresis and Western blotting**

Standard SDS-PAGE was performed according to Laemmli (Laemmli et al., 1970) and 2D Blue-Native gel electrophoresis was carried out according to the method of Schägger and Von Jagow (Schägger and von Jagow, 1991; Schägger et al., 1994). Following electrophoresis, proteins were blotted to nitrocellulose (Towbin et al., 1979). Immunoreactive material was visualised using chemoluminesence (ECL TM, Amersham), according to the instructions of the manufacturer.

**Results**

Translation of subunit 1 of the cytochrome c oxidase complex (Cox1p) in mitochondria of Saccharomyces cerevisiae is regulated by the nuclear encoded proteins Mss51p and Pet309p. Disruption of either MSS51 or PET309 leads to loss of Cox1p synthesis, causing respiratory growth deficiency. In pulse-labelling experiments with strains disrupted for either MSS51 or PET309, highly similar phenotypes were observed (Fig. 1, panel B and D). In both disruptant strains no synthesis of Cox1p could be detected. Additionally, the level of the other mitochondrial translation products was observed to be lower than in a wild-type strain. In combination with previously published work (Manthey and McEwen, 1995; Siep et al., 2000), this result indicates that both Mss51p and Pet309p are involved in an early step in the synthesis of Cox1p.

Pet309p is an integral protein of the mitochondrial inner membrane (Manthey et al., 1998). As judged from the results of sub-fractionation and carbonate extraction, Mss51p appears to be firmly associated with the inner membrane (Siep et al., 2000). This similarity in intra-mitochondrial location suggests that interaction between the two proteins might occur. We employed two-hybrid analysis (Fromont-Racine et al., 1997) to investigate whether such an interaction is possible. The MSS51 ORF was used as bait to screen the FRYL yeast genomic DNA library (Fromont-Racine et al., 1997). In this screening 20 million diploids were obtained of which 10,000 were HIS3 positive. This relatively high level is, to a large extent, ascribable to the ability of Mss51p alone to weakly activate this marker gene. After more stringent selection on plates lacking histidine but containing 20 mM 3-AT, only 144 diploids were able to grow. These were screened further for activation of the lacZ gene by X-Gal overlay, a procedure which left only 7 diploids all of which displayed weak blue colouring. Sequence analysis of the inserts revealed proteins, which as a consequence of
their cellular location would be unlikely to interact with Mss51p. Therefore, we regarded them as false-positives. Significantly, PET309 was not picked up in this screen. The FRYL library contains $5.10^6$ clones, and complete coverage of the library requires the screening of 15 million interactions (Fromont-Racine et al., 1997). So, the number of diploids tested in this study should have been sufficient to detect all possible interactions. Although there may be several reasons why two interacting proteins fail to be scored in a two-hybrid assay, we interpret this observation as an indication that there is no interaction between Mss51p and Pet309p. Similar results were obtained in a comparable study in which PET309 was used as bait (Manthey, personal communication).

Figure 1: Disruption of MSS51 or PET309 leads to loss of Cox1p synthesis. In vitro pulse chase experiment with mitochondria of W303(167), Δmss51, GM10 (PET309+) and GM11 (Δpet309). Equal amounts of mitochondria (500 µg) were incubated with $^{35}$S labelled methionine, after 10 and 30 minutes pulse samples were taken, at 30 minutes the labelling reaction was stopped by addition of excess of methionine, to follow the turnover of the synthesized proteins. During the chase, samples were taken after 10, 30 and 60 minutes. Samples (100 µg) were taken at the indicated time points and analyzed on 11% SDS-PAGE. Bands were visualized using a phosphorimager (Molecular Dynamics) and quantified using ImageQuant. Positions of the mitochondrial translation products are indicated on the right.

We pursued this question further by combining the MSS51 ORF and the Pet309 C-terminal domain (the matrix part; 250 amino acids) in a two-hybrid screen. As in the library screen, weak activation of the HIS3 gene in the Mss51p control samples was observed. However, no significant activation of the HIS marker and the lacZ marker were found. Therefore, we conclude that no physical interaction exists between Mss51p and Pet309p.

We then examined the possibility of a functional interaction between Mss51p and Pet309p by looking for phenotypic complementation of respiratory deficiency when each of the genes was overexpressed in the other disruptant strain. A mss51-null strain was transformed with the PET309 gene on single and multicopy plasmids (pGM1 and pGM2 respectively; Table II), and a pet309-null strain was transformed with single- and multicopy plasmids containing the MSS51 gene (pMS3 and pMS7 respectively; Table II). Growth of the transformants on minimal medium supplemented with adenine, histidine and tryptophane, indicates the presence of the plasmid (Fig. 2). The ability to grow on both YPD and YPGlyc plates was determined (Fig. 2). While all strains displayed similar growth on YPD, growth on YPGlyc was found to occur only with the respective wild-type strains (W303(167) and GM10), and the disruptant strains transformed with the corresponding
wild-type gene. We conclude that Mss51p and Pet309p fulfill independent functions in the activation of Cox1p translation.

Figure 2: Complementation of Δmss51 and Δpet309 with MSS51 or PET309. Δmss51 and Δpet309 were transformed with single (s.c.) and multi copy (m.c.) constructs containing the wild type MSS51 gene (pMS3 and pMS7: Table 2) or the wild type PET309 gene (pGM1 and pGM2: Table 2). Growth of transformants was determined on MM+AHW, YPD and YPGlyc. Pictures were taken after 4 days of growth at 30 °C.

Respiratory deficiency caused by the absence of individual translational activators can often be phenotypically suppressed by mitochondrial DNA rearrangements which fuse the affected mitochondrial gene to a 5'-UTR of a mitochondrial gene responsive to another activator (Rödel, 1986; Costanzo et al., 1986; Dunstan et al., 1997). Such phenotypic revertants have indeed been found for a pet309-null strain (Manthey and McEwen, 1995). They contain Cox1p coding sequences fused downstream of the COB 5'-UTR, and Cox1p synthesis is Cbs1p/Cbs2p dependent. This mitochondrial revertant is unable to complement a mss51-null nuclear background (Fox, personal communication). Additionally, we (this report, data not shown) and others have looked extensively for the appearance of phenotypic suppressors of respiratory growth using a mss51-null strain. These attempts have all been negative. Taken together, these results strongly suggest that Mss51p either does not directly interact with the COX1 5'-UTR, or that it interacts with a region of the 5'-UTR that is not easily separable from the coding sequence.
A mitochondrial protein was analyzed on 8-18% gradient BNE-gel electrophoresis. After Western blotting, complex IV was detected with antibody 11D6-B7 against the Cox1 subunit and Mss51HA was detected with antibody 12CA5 against the HA-epitope. Both antibodies were obtained from Boehringer Mannheim. For 2D electrophoresis lanes 1 and 4 of the BNE-gel were run in 10% SDS-PAGE. Arrows indicate the direction of BNE and SDS-PAGE electrophoresis. The positions of Mss51HA and Cox1 are indicated on the left.

Figure 3: Mss51p is in a higher order complex. A: 30 μg mitochondrial protein was analyzed on 8-18% gradient BNE-gel electrophoresis. After Western blotting, complex IV was detected with antibody 11D6-B7 against the Cox1 subunit and Mss51HA was detected with antibody 12CA5 against the HA-epitope. Both antibodies were obtained from Boehringer Mannheim. B: For 2D electrophoresis lanes 1 and 4 of the BNE-gel were run in 10% SDS-PAGE. Arrows indicate the direction of BNE and SDS-PAGE electrophoresis. The positions of Mss51HA and Cox1 are indicated on the left.

As a final approach to the detection of a possible interaction between Mss51p and Pet309p, the molecular organization of Mss51p was investigated by 2D Blue Native gel electrophoresis (Schägger et al., 1994) under conditions that have allowed detection of several other mitochondrial complexes and intermediates in the assembly of cytochrome c oxidase (Nijtmans et al., 1998). The location of cytochrome c oxidase (about 250 kDa) was identified by incubation of the blot with antibodies directed against Cox1p. Application of this technique to mitochondrial extracts containing C-terminally HA-tagged Mss51p allowed detection of the protein at a position corresponding roughly to a molecular mass of approximately 150 kDa (Fig. 3A). We do not yet know whether this complex consists of a multimer of Mss51p, or Mss51p in association with another protein. If the latter, the other component is unlikely to be limiting in amount, since increased expression of Mss51p is directly reflected in the amount of the complex. The other component of this complex is unlikely to be Pet309p, as migration is unaffected by disruption of the PET309 gene (Fig. 3B). Taken together, these observations are highly suggestive of a lack of strong physical interaction between Mss51p and Pet309p. However, existence of weak interactions, disrupted by the extraction or separation techniques used, cannot be entirely ruled out. Additionally, since we do not know where the bulk of Pet309p migrates in this system, we cannot exclude the remote possibility that a minor amount of Mss51p is complexed with Pet309p at this position in the gel.
Discussion

Synthesis of cytochrome c oxidase subunit 1 (Cox1p) in S. cerevisiae mitochondria requires the products of MSS51 and PET309 genes. Mutants lacking either one of these genes resemble each other in being both Cox1p- and respiratory-deficient. We have recently shown (Siep et al., 2000) that lack of Cox1p in mss51 disruptants is indeed due to loss of synthesis of the subunit, rather than to destabilisation of the protein as a result of impaired modification or assembly into holo-cytochrome c oxidase. Similar observations have been made for pet309 null mutants (Manthey et al., 1995). mss51 and pet309 mutants were initially characterized in strains containing mitochondrial introns and both display complex changes in the levels of COX1 derived transcripts. Whether the proteins exert their effects entirely through translation (for example via the synthesis of intron-encoded RNA maturases as well as of Cox1p), or whether they combine roles in both translation and RNA processing is currently unknown. Neither protein exhibits obvious hydrophobic regions, yet Mss51p is firmly associated with the mitochondrial membrane from which it can be eluted by carbonate extraction and Pet309p behaves as an integral membrane protein.

The fact that both proteins localize to the mitochondrial inner membrane raises the question whether they might either physically or functionally interact. This appears not to be the case. Lack of physical interaction is indicated by the combined results of two-hybrid analysis and 2D Blue-Native gel electrophoresis. Lack of functional interaction is indicated by the absence of cross-complementation between the two genes in either single or multiple copies and by the lack of phenotypic suppression of the mss51-null mutant by a 5'-UTR-COß/COX1 fusion that restores respiratory function to a pet309-null mutant. This fact and the observation that similar mitochondrial rearrangements have been observed to arise in a pet309-null strain indicates that Pet309p acts through an interaction with the 5'-UTR of the COX1 mRNA. This interaction may well be responsible for the tethering of COX1 mRNA to the mitochondrial inner membrane.

In line with the conclusion that Pet309p may interact directly with RNA is the recent observation that the protein contains several tandem copies of a PPR (pentatricopeptide) motif (Small and Peeters, 2000). This 35-amino-acid repeat is prevalent in members of a large family of plant and fungal proteins that either localise to mitochondria or chloroplasts, or are predicted to do so. Like the related TPR (tetratricopeptide) motif, the PPR motif consists of a pair of anti-parallel α-helices. Tandem arrays of the motif are expected to form a superhelix enclosing an extended groove or tunnel which is lined with hydrophilic and positively charged residues and which is wide enough to bind a single RNA strand.

Compared with Pet309p, few clues to a possible function can be gleaned from examination of the sequence of Mss51p. The only close relatives of Mss51p are proteins of unknown function in S. pombe and Arabidopsis thaliana. The former displays similarity over its whole length and most probably represents the S. pombe orthologue of Mss51p. The latter only displays similarity over its C-terminal 200 amino acids, the remainder of the protein being homologous to a family of proteins with similarity to S. cerevisiae spliceosomal protein Prp19.

Other features also distinguish PET309 and MSS51 genes. In analysis of DNA micro-array expression profiling data (data not shown; Eisen et al., 1998), MSS51 clusters together with a group of about 40 other genes encoding proteins involved in mitochondrial assembly or respiratory chain function, 20 of which are either proteins of the mitochondrial ribosome, aminoacyl-tRNA synthetases, or other factors closely involved in the translation process. In
contrast, the cluster containing PET309 contains only 6 other genes with roles in respiratory chain function or assembly and PET309 itself displays a similar expression profile to a tightly linked cluster of genes involved in RNA processing, splicing (PRP24, PRP19, SEN15, SMD3, YSH1) and/or translational activation (MSS116, AEP1). To what extent this implies distinct functions in Cox1p synthesis is unclear, however, as apart from PET111 and PET494 which cluster closely together, other mitochondrial translational activators also display distinct expression patterns that cause them to be assigned to different groups.

If Mss51p activates Cox1p synthesis by a mechanism other than interaction with the 5'-UTR of COX mRNA, what might its site of action be? An interaction with the mitochondrial ribosome has been suggested, based on the observation that the respiratory-deficient phenotype of the mss51-3 mutation is exacerbated by the presence of a P<sub>R454</sub> mutation in the mitochondrial SSU rRNA (a C to G change at position 1514; Li et al., 1982). The mss51-3 mutation reduces the level of Mss51p by more than 100-fold and the negative effects this might have on an interaction with the mitochondrial ribosome might plausibly be amplified by a concomitant change in the conformation of SSU rRNA. Sensitivity to the status of the 1477 site is also a feature of mutations in MSS1 and MTO1 genes, whose products together form a complex that has been suggested to influence the fidelity of mitochondrial translation (Colby et al., 1998). In the case of Mss1p/Mto1p, ribosomal interaction would appear to be weak or transient, since the complex does not co-fractionate with mitochondrial ribosomes in gradient separations (Colby et al., 1998). For Mss51p, data on this point have so far been inconclusive (Chapter V). Mss51p may function at a stage of translational initiation subsequent to interaction of the ribosome with the 5'-UTR of the COX1 mRNA. Alternatively, it may promote efficient elongation of the nascent Cox1p subunit, or, in analogy with bacterial ribosomal trigger factor (Hesterkamp et al., 1996 and 1997) folding of the nascent polypeptide as it exits the ribosome.

Acknowledgements

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