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Phil. Trans. R. Soc. Lond. B 1988 **319**, 107-120
doi: 10.1098/rstb.1988.0035

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Phil. Trans. R. Soc. Lond. B **319**, 107–120 (1988)
Printed in Great Britain

Mutational analysis of the yeast coenzyme QH_2–cytochrome *c* reductase complex

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The synthesis of cytochrome *b* in yeast depends on the expression of both mitochondrial and nuclear gene products that act at the level of processing of the pre-mRNA, translation of the mRNA, and maturation of the apoprotein during its assembly with the nuclear-encoded subunits of coenzyme QH_2–cytochrome *c* reductase. Previous studies indicated one of the nuclear genes (*CBP2*) to code for a protein that is needed for the excision of the terminal intervening sequence from the pre-mRNA. We show here that the intervening sequence can promote its own excision in the presence of high concentrations of magnesium ion (50 mM), but that at physiological concentrations of the divalent cation (5 mM), the splicing reaction requires the presence of the *CBP2*-encoded product. These results provide strong evidence for a direct participation of the protein in splicing, most likely in stabilizing a splicing competent structure in the RNA.

The conversion of apocytochrome *b* to the functional cytochrome has been examined in mutants lacking one or multiple structural subunits of the coenzyme QH_2–cytochrome *c* reductase complex. Based on the phenotypes of the different mutants studied, the following have been concluded. (i) The assembly of catalytically active enzyme requires the synthesis of all except the 17 kDa subunit. (ii) Membrane insertion of the individual subunits is not contingent on protein–protein interactions. (iii) Assembly of the subunits occurs in the lipid bilayer following their insertion. (iv) The attachment of haem to apocytochrome *b* is a late event in assembly after an intermediate complex of the structural subunits has been formed. This complex minimally is composed of apocytochrome *b*, the non haem iron protein and all the non-catalytic subunits except for the 17 kDa core 3 subunit.

Introduction

Mitochondria are composed of some 250–300 different proteins of which only a relatively small fraction is encoded in mitochondrial DNA (Tzagoloff & Myers 1986). Most of the genetic information responsible for the biogenesis of this organelle is therefore derived from nuclear DNA. In the yeast *Saccharomyces cerevisiae*, the nuclear genes required for the expression of respiratory competence are referred to as *PET* genes. These genes code for the catalytic components of mitochondria, the regulatory factors that affect transcription and translation of proteins essential for respiration, and other protein constituents that influence the respiratory capacity of mitochondria by more indirect means.

As a result of several extensive mutant screens, we have isolated a collection of respiratory deficient *pet* strains which, based on genetic analyses, defines at least 200 different complementation groups. Although the collection encompasses most of the nuclear gene products directly involved in the oxidative metabolism of mitochondria, certain types of mutants are either under-represented or absent. Stringent mutations in components necessary

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for mitochondrial DNA replication, for example, would be expected to induce a loss of mitochondrial DNA. Such strains would be scored as cytoplasmic petite mutants in the screening protocol. Similarly, mutations in genes whose products function in mitochondrial transcription or translation also lead to a secondary instability in mitochondrial DNA (Myers *et al.* 1985). This rather substantial class, inclusive of the genes for the amino acyl-tRNA synthetases and ribosomal proteins, is represented by mutants with partial lesions which express a clear growth phenotype but do not totally prevent maintenance of the wild type mitochondrial genome.

In this article we will describe studies in which the *pet* mutants have been used to clarify some facets of the biosynthesis of cytochrome *b* and its parent respiratory complex, coenzyme QH_2–cytochrome *c* reductase.

Mutants blocked in the synthesis of apocytochrome *b* mRNA

Cytochrome *b* is encoded by a mitochondrial gene which has been shown to consist of either three or six exons depending on the strain of yeast (Nobrega & Tzagoloff 1980; Lazowska *et al.* 1980). Transcription of the gene is initiated from a promoter located upstream of the glutamyl-tRNA gene (Christianson *et al.* 1983). The dicistronic primary transcript undergoes multiple processing steps to yield the mature apocytochrome *b* mRNA and the tRNA (figure 1). The processing reactions include excision of the tRNA, cleavage at a site 943 nucleotides upstream of the initiation codon to form the mature 5′ end, and excision of the intervening sequences. There is probably an additional cleavage at the 3′ end of the transcript, although the transcription termination site has not yet been established. These processing events are catalysed by mitochondrial as well as nuclear-encoded proteins and cofactors.

Because most of the work described here has been done with a strain containing the short form of the gene, the discussion will be confined to those genetic elements involved in processing of this particular variant. The essential difference between the two genes is the requirement of additional protein factors or maturases for excision of the intervening sequences from the pre-mRNA transcribed from the long gene (Pillar *et al.* 1983). In other respects the maturation pathways are probably identical. The processing reactions illustrated in figure 1 are catalysed by two types of enzyme: those specific for the apocytochrome *b* transcripts, and those having general functions in processing of other mitochondrial transcripts as well. Among the latter are the enzymes that excise the tRNAs (Martin & Underbrink-Lyon 1981) and cleave the precursor RNAs at consensus sequences near their 3′ termini (Thalenfeld *et al.* 1983). There are at least three different processing enzymes that are specific to the apocytochrome *b* pre-mRNA. One of these is encoded by the nuclear gene *CBP1*. This protein effects a cleavage of the transcript at the −943 site corresponding to the mature 5′ end of the mRNA (Dieckmann *et al.* 1984). The second specific factor is also a product of a nuclear gene (*CBP2*); it is required for excision of the terminal intervening sequence (McGraw & Tzagoloff 1983). The function of this protein will be discussed in more detail in the following section. The third processing enzyme is encoded in the first intervening sequence of the pre-mRNA. This mitochondrially translated protein (BOX7 maturase) promotes excision of the first intervening sequence (Dhwahle *et al.* 1981).

The efficiency with which the different processing steps take place can be gauged from the steady-state concentrations of the cytochrome *b*-specific transcripts. Northern analyses of

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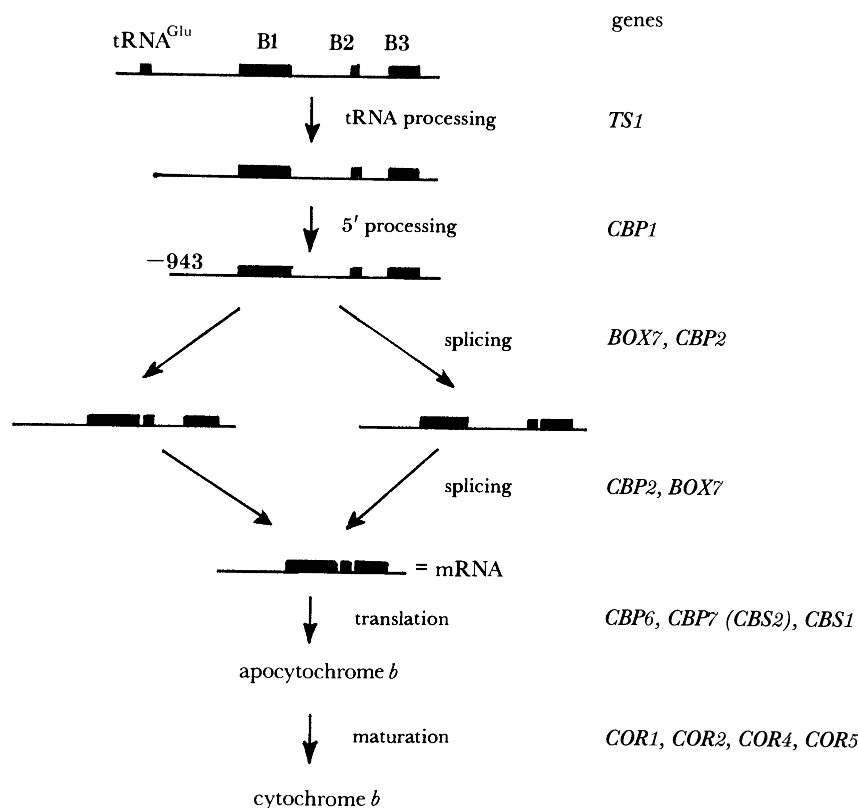

FIGURE 1. Processing of the apocytochrome *b* pre-mRNA. The three exons (B1, B2, B3) of the short cytochrome *b* gene are depicted by the solid bars. *TS1* is a mitochondrial gene coding for an RNA cofactor involved in processing of tRNAs at their 5′ ends. *BOX7* is located in the first intron of the cytochrome *b* gene. Its product is required for the excision of the first intervening sequence. All the other genes listed have a chromosomal location; *CBP1* and *CBP2* function in 5′ end processing and excision of the terminal intervening sequence, respectively, and *COR1, 2, 4, 5* are the structural genes for the non-catalytic subunits of coenzyme QH_2–cytochrome *c* reductase. Mutations in this set of genes block haem attachment to apocytochrome *b*.

mitochondrial RNA indicate the most abundant RNA to be a 2.1 kilobase (kb) transcript corresponding to the fully processed mRNA (Bonitz *et al.* 1982). Approximately 10–20% of the total RNA is a 2.9 kb intermediate containing only the terminal intervening sequence. Although larger transcripts containing only the first or both intervening sequences are also detected, they are present at much lower concentrations. These observations suggest that the excision of the tRNA and the secondary cleavages at the 5′ and 3′ termini occur at faster rates than the splicing reactions.

EXCISION OF THE TERMINAL INTERVENING SEQUENCE

The short and long cytochrome *b* genes have an identical terminal intron of 733 nucleotides (Nobrega & Tzagoloff 1980; Lazowska *et al.* 1980). Based on its sequence and predicted secondary structure, this intron has been classified as a group I intron (Michel *et al.* 1982) and therefore should be capable of catalysing its own excision by the mechanism first described for the self-splicing of the ribosomal RNA intron of *Tetrahymena* (Cech *et al.* 1981) and subsequently shown to operate for a number of mitochondrial group I introns (Garriga &

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Lambowitz 1984; Van der Horst & Tabak 1985). This has been experimentally verified. A substrate capable of protein-independent self-splicing has been synthesized from a template containing a fragment of the cytochrome *b* gene cloned in a vector with an SP6 promoter. This substrate contains 103 nucleotides from the 3′ terminal region of the first intervening sequence, the entire second exon and terminal intervening sequence plus part of the third exon (figure 2). When incubated in the presence of GTP, Mg^{2+} and salt, the synthetic RNA is converted to two smaller products (figure 3). The larger of the products has been shown by sequence analysis to be the excised linear intervening sequence (Gampel & Tzagoloff 1987). The second shorter product consists of the ligated exons. The self-splicing reaction is absolutely dependent

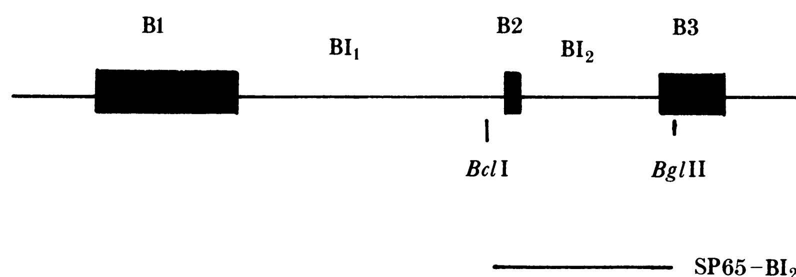

FIGURE 2. SP6-derived substrate used for the splicing assay *in vitro*. The region of wild-type mitochondrial DNA spanning the *Bcl*I and *Bgl*II sites in the cytochrome *b* gene was ligated to the *Bam*H1 site of the vector pSP65. The resultant plasmid, SP65–BI_2 was linearized at a site downstream of the insert and used as a template for transcription. This precursor RNA was used in the self-splicing assay *in vitro*. The identical region of DNA was also cloned from a mutant (M6-200) previously shown to have a mutation in the terminal intron that blocks splicing of the pre-mRNA *in vivo* (Tzagoloff *et al.* 1976)

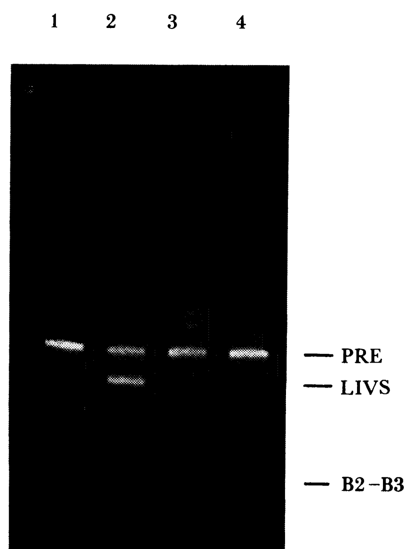

FIGURE 3. Self-splicing of the terminal intervening sequence from the SP6-derived substrate. Precursor RNA synthesized from the wild-type and mutant templates described in figure 2 were incubated for 30 min at 37 °C in the presence or absence of 0.2 mM GTP in a reaction mixture containing 50 mM Tris–HCl, pH 8, 100 mM $(NH_4)_2SO_4$, and 50 mM $MgCl_2$. The reaction products were separated by electrophoresis on a 1% agarose gel and visualized with ethidium bromide. Lane 1, wild-type substrate without GTP; lane 2, wild-type substrate with GTP; lane 3, mutant substrate without GTP; lane 4, mutant substrate with GTP. The migrations of the substrate (PRE), linear intervening sequence (LIVS), and ligated exon product (B2–B3) are indicated in the margin.

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on the presence of the guanosine nucleotide; the optimum concentraton is approximately 0.2 mM, a value consistent with the concentration in mitochondria. The self-splicing reaction exhibits a fairly sharp dependence on the concentration of Mg^{2+} with an optimum at 50 mM. This concentration is at least one order of magnitude higher than the reported concentration of the divalent metal in mitochondria (Corkey *et al.* 1986). It is also important to note that, at more physiologically relevant concentrations of Mg^{2+}, splicing is not observed.

The mechanism of splicing *in vivo* probably involves the same two transesterifications responsible for self-splicing in the *in vitro* reaction. This is supported by the finding that the native 2.9 kb precursor is also capable of self-splicing *in vitro* in the presence of Mg^{2+} and GTP. Furthermore, a mutation in the terminal intron that blocks splicing *in vivo* completely abolishes self-splicing in the *in vitro* assay (Gampel & Tzagoloff 1987).

The ability of the native precursor and of the synthetic RNA substrate to self-splice in the absence of protein factors is at variance with earlier observations that mutations in the nuclear gene *CBP2* prevent excision of the terminal intervening sequence from the apocytochrome *b* pre-mRNA *in vivo* (McGraw & Tzagoloff 1983). The accumulation of the 2.9 kb precursor in *cpb2* mutants suggested that the protein encoded by this nuclear gene was in some manner required for *in vivo* processing (McGraw & Tzagoloff 1983; Hill *et al.* 1985).

To clarify the function of the CBP2 product, we have attempted to purify the protein to test its effect on the splicing reaction *in vitro*. The extremely high RNase activity of yeast mitochondria has precluded the use of a functional assay as a means of monitoring the

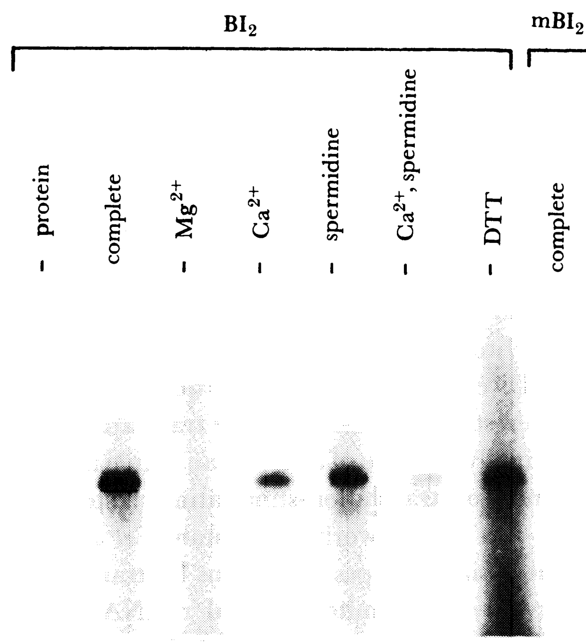

FIGURE 4. Protein-dependent splicing. The wild type (BI_2) and mutant (mBI_2) substrates were incubated under the conditions described in the figure. The complete reaction assay contained 0.1 µg of substrate, 50 mM Tris–HCl, pH 8, 5 mM $MgCl_2$, 5 mM spermidine, 5 mM $CaCl_2$, 5 mM dithiothreitol (DTT), 10 µCi†α [^{32}P]GTP, carrier RNA, and a partly purified preparation of the CBP2 protein. After incubation at 37 °C for 30 min, the reaction was centrifuged through a column of Sephadex G-50. The material recovered in the excluded volume was precipitated with alcohol and separated by electrophoresis on a 1% agarose gel. The radioactive product visualized by autoradiography corresponds to the excised linear intervening sequence with a guanosine nucleotide covalently linked to the 5′ end.

† 1 Ci = 3.7×10^{10} Bq.

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purification of the protein. Instead we have had to rely on the use of an antibody to follow its fractionation. Although a substantial degree of enrichment has been achieved, the best preparations at present are still contaminated by other mitochondrial proteins. The partly purified preparations, none the less, have been useful in assessing the role of the protein in splicing. As already mentioned, no protein-independent splicing of the terminal intervening sequence occurs at physiological concentrations of Mg^{2+}. This is shown in the experiment of figure 4, in which the concentration of Mg^{2+} was 5 mM. In the same experiment the addition of a fraction enriched for the CBP2 product promotes the incorporation of radioactively labelled GTP into an RNA which migrates identically to the linear intervening sequence formed at higher Mg^{2+} concentrations in the absence of protein. The protein-dependent excision of the intervening sequence is stimulated by the inclusion of low concentrations of spermidine and Ca^{2+}. Neither spermidine, Ca^{2+}, or the addition of both have any significant effect on self-splicing at high Mg^{2+} concentrations.

The results of these *in vitro* assays indicate that the CBP2 protein is directly involved in splicing of the apocytochrome *b* pre-mRNA. The precise function of the protein still needs to be determined but a reasonable interpretation is that it stabilizes a splicing-competent secondary or tertiary structure in the RNA. In the self-splicing reaction this structure may be maintained in the absence of protein by providing Mg^{2+} at a much higher concentration than that present in mitochondria.

Translation of apocytochrome *b* mRNA

The failure of yeast to synthesize cytochrome *b* can also result from mutations in nuclear genes whose products facilitate translation of the apocytochrome *b* transcripts. At present three such genes have been identified. Two of the genes, *CBS1* and *CBS2* (*CBP7*) code for proteins that probably interact with the 5′ untranslated leader of the pre-mRNA and mRNA (Rodel *et al.* 1986; Muroff & Tzagoloff 1988). This is evidenced by the fact that the respiratory deficiency of *cbs*1 and *cbs*2 mutants is suppressed by a mitochondrial DNA rearrangement resulting in the substitution of the apocytochrome *b* 5′ leader by sequences normally found upstream of other mitochondrial genes (Rodel 1986). Mutations in the third gene (*CBP6*) also block the synthesis of apocytochrome *b* but in this instance the mitochondrial rearrangements have no suppressor activity (Dieckmann & Tzagoloff 1985).

The details of how these interesting proteins stimulate translation of this single transcript will be difficult to ascertain, principally because of a lack of an active mitochondria-free translation system *in vitro*. The existence of translation-stimulating factors is not confined to the apocytochrome *b* transcript. Fox and coworkers (Costanzo *et al.* 1985; Muller *et al.* 1984) have described a requirement of similar types of proteins for translation of subunits 2 and 3 of cytochrome oxidase. Conceivably, each mitochondrial mRNA may need to complex with specific proteins in order to interact with mitochondrial ribosomes.

Assembly of coenzyme QH_2–cytochrome *c* reductase

Cytochrome *b* is an integral component of a larger complex that catalyses the transfer of electrons from coenzyme QH_2 to ferricytochrome *c*. This enzyme consists of at least eight distinct subunit polypeptides of which only cytochrome *b*, cytochrome c_1, and the non-haem

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iron protein have electron carrier properties. The other subunits of the complex have no recognizable prosthetic groups and probably do not participate directly in the catalytic mechanism.

Most of the nuclear genes coding for the subunits of the yeast complex have been cloned, thereby allowing the primary sequences of the proteins to be derived from the gene sequences (Tzagoloff *et al.* 1986; Van Loon *et al.* 1983*a*, 1984; De Haan *et al.* 1987; Maarse & Grivell 1987; Sadler *et al.* 1984; Beckman *et al.* 1987). We have recently identified mutants in the structural subunits in the *pet* collection (Crivellone *et al.* 1988). In addition the cloned genes have been used to construct strains with disruptions or deletions in the genes. With the exception of the 17 kDa subunit (core 3) mutant, all the other mutants examined are respiratory deficient and in most instances exhibit the absence of mature cytochrome *b*. The exceptions are the non-haem iron and cytochrome c_1 mutants, which have reduced but still spectrally detectable levels of the cytochrome. These phenotypes are summarized in table 1.

Table 1. Phenotypes of coenzyme QH_2–cytochrome *c* reductase mutants

group	gene	subunit	enzyme activity	cytochrome *b*[a]
—	*COB*	cytochrome *b*	−	absent
G7	*COR1*	core 1 (44 kDa)	−	absent
G144	*COR2*	core 2 (40 kDa)	−	absent
—	*COR3*	core 3 (17 kDa)	+	normal
G67	*COR4*	core 4 (14 kDa)	−	absent
G153	*COR5*	core 5 (11 kDa)	−	absent
G12	*RIP1*	non-haem iron subunit	−	normal
G101	*CYT1*	cytochrome c_1	−	reduced

[a] Refers to the mature cytochrome.

Mutants with lesions in the structural subunits of the complex have been used to examine some aspects of the assembly of this inner membrane enzyme. The first question of interest was whether mutations in single subunits influence the synthesis or stability of other subunits of the complex. In these experiments total mitochondrial proteins from single mutants were probed with subunit-specific antibodies by the Western blot technique. The results of such analyses, shown in figure 5, indicate that the concentrations of a subset of structural subunits are markedly affected in some mutants. The most severe decreases are seen in apocytochrome *b*, the non-haem iron protein, and the 14 kDa and 11 kDa subunits. In contrast, the 44 kDa and 40 kDa core proteins and cytochrome c_1 appear to be present in amounts similar to those found in the wild type, independent of the mutant examined. Although the observed pleiotropic effects could be the result of decreased rates of synthesis, a more likely explanation is that mutations preventing normal assembly of the enzyme cause higher rates of turnover of some proteins. According to this interpretation the absence of subunits such as cytochrome *b* or the core proteins leads to a less structured complex in which some subunits are more susceptible to proteolytic degradation. It is of interest to note that mutations in the non-haem iron protein have the least deleterious effect on the other constituents of the enzyme.

Apocytochrome *b* is synthesized on mitochondrial ribosomes that are bound to the matrix side of the inner membrane. This protein is probably co-translationally inserted into the

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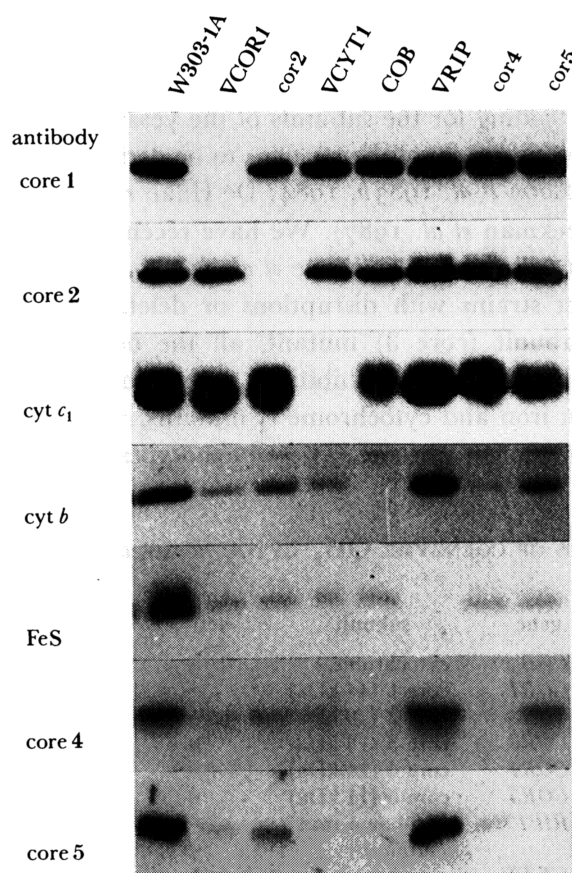

FIGURE 5. Concentrations of subunit polypeptides of coenzyme QH_2–cytochrome *c* reductase in single mutants. Total mitochondrial protein (40 μg) from each mutant strain was separated by eletrophoresis on 12% SDS–polyacrylamide gels. The proteins were transferred to nitrocellulose paper and the blots reacted with the indicated subunit-specific antibodies. The antibody–antigen complex was visualized by autoradiography after a second reaction of the blot with ^{125}I-labelled protein A. Mitochondria were prepared from wild type (W303-1A) and from mutants carrying a disruption in the *COR1* (∇COR1), cytochrome c_1 (∇CYT1), or the non-haem iron protein (∇RIP) genes. The other strains studied had point mutations in the genes for cytochrome *b* (COB), the 14 kDa core 4 subunit (cor4), and the 11 kDa core 5 subunit (cor5). Ab FeS refers to the anitbody against the non-haem iron protein.

membrane. The remaining subunits are synthesized on cytoplasmic ribosomes, mostly as precursors with amino terminal extensions, and are transported into mitochondria where they are processed to their mature forms (Ohashi *et al.* 1982; Sen & Beattie 1985; Van Loon *et al.* 1983*b*). At least two of the proteins (non-haem iron protein and cytochrome c_1) have been shown to undergo two separate proteolytic cleavages, the first in the matrix and the second in the intermembrane space (Hartl *et al.* 1986; Ohashi *et al.* 1982). The insertion of the subunits into the inner membrane could occur independently of protein–protein interactions or could be coupled to the assembly of the complex. To gain insights into this process we have examined the membrane distribution of individual subunits of the enzyme in mutants lacking all but one of the subunits (Crivellone *et al.* 1988). Mitochondria disrupted by sonic irradiation in the presence or absence of salt were separated into two fractions, one consisting of membrane vesicles and the other of the proteins released as a result of the sonic treatment. Each fraction was probed with the subunit-specific antibodies following electrophoresis on SDS–poly-

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acrylamide gels. The results of these experiments indicate that with the exception of the 40 kDa core 2 subunit, all the other subunits are recovered quantitatively in the membrane fraction (figure 6). The core 2 subunit, however, is distributed equally in the two fractions. This component therefore appears to be least stably associated with the membrane. These results suggest that the insertion of the subunits into the holoenzyme takes place in the lipid phase subsequent to their assembly in the inner membrane.

The assembly of the yeast complex has also been studied by analysing the sedimentation properties of the subunits in mutants with lesions in single and multiple subunits. Centrifugation of the wild-type enzyme through a sucrose gradient column containing 0.1% Triton X-100

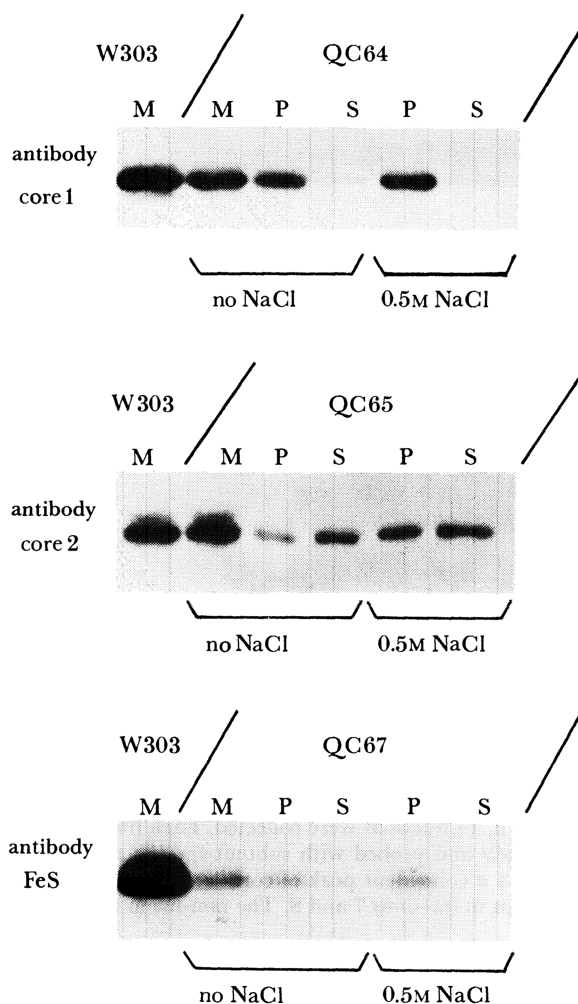

FIGURE 6. Intramitochondrial distribution of core 1, core 2, and the non-haem iron protein in strains with multiple mutations. Mitochondria (M) were sonically irradiated either in the absence or presence of 0.5 M NaCl, and were centrifuged at 156000 *g* for 30 min to separate submitochondrial membrane particles (P) from the soluble proteins (S). Equivalent volumes of each fraction were separated on a 12% SDS–polyacrylamide gel. The proteins were transferred to nitrocellulose and probed with subunit specific antibodies as described in the legend to figure 5. W303 is a respiratory-competent strain of yeast. QC64 is a respiratory-deficient strain with mutations in all the structural subunits except core 1 and core 3. QC65 is a multiply marked strain with the wild-type genes for core 2 and core 3. QC67 has only the wild-type genes for the non-haem iron protein and core 3.

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yields three distinct size fractions (figure 7). The largest subcomplex consists of the two high molecular weight core proteins probably in a stoichiometry of one molecule of core 1 to two molecules of core 2. The second subcomplex is composed of cytochrome b, cytochrome c_1, the 14 kDa subunit and the 11 kDa subunit. The non-haem iron protein sediments as monomer and is recovered in the low-molecular-mass region of the gradient. At present it has not been possible to devise conditions under which the wild-type complex is maintained in a monomeric form without dissociation into the three subfractions.

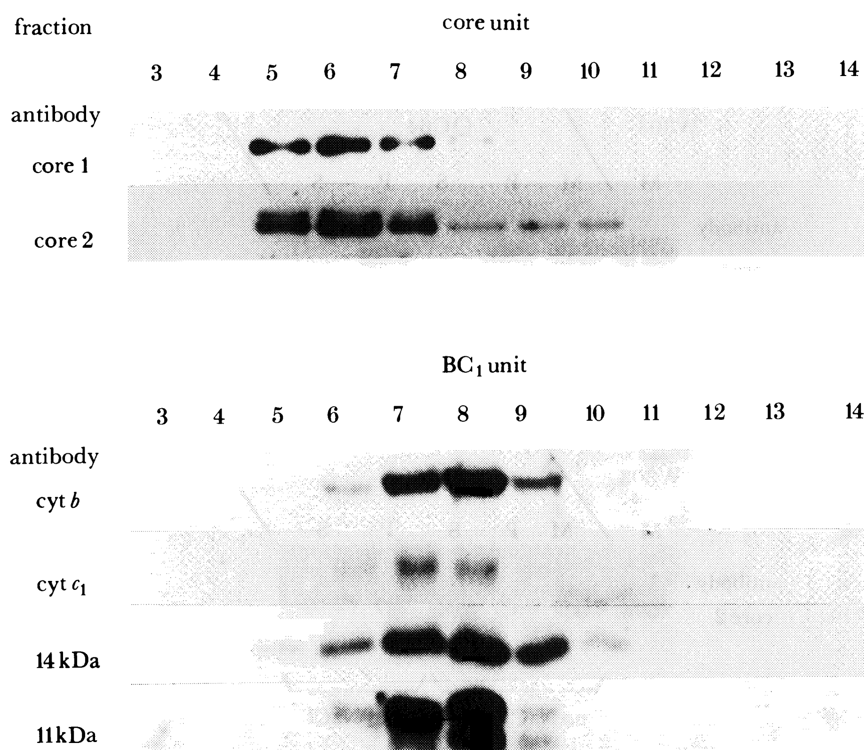

FIGURE 7. Sedimentation of coenzyme QH_2–cytochrome c reductase of yeast in sucrose gradients. The yeast enzyme was solubilized by treatment of wild-type mitochondria with 0.5% deoxycholate in the presence of 1 M KCl. The extract was layered on a 5 ml column of 6–25% sucrose containing 0.1% Triton X-100. After centrifugation at 256000 g for 6 h, 14 fractions were collected. Each fraction was separated by electrophoresis on 12% SDS–polyacrylamide gels and probed with subunit-specific antibodies as detailed in the legend to figure 5. The Western blot shows a coincident peak of core 1 and core 2 in fraction 6 and of cytochromes b, c_1, and the two small core proteins in fractions 7 and 8. The non-haem iron protein peaked in fraction 13 (not shown).

Several conclusions can be drawn from sedimentation analyses of mutant enzymes. First, the association of the core 1 and core 2 subunits with one another occurs even when all the other subunits of the complex are absent. As shown in figure 8, the two proteins co-sediment as a high molecular weight complex in mitochondrial extracts of mutants lacking all the subunits except core 1 and core 2. That the sedimentation properties of the two proteins are not due to artifactual aggregation or interaction with other proteins is confirmed by the results obtained with mutants having additional mutations in core 1 or core 2. Core 1 sediments as a monomeric

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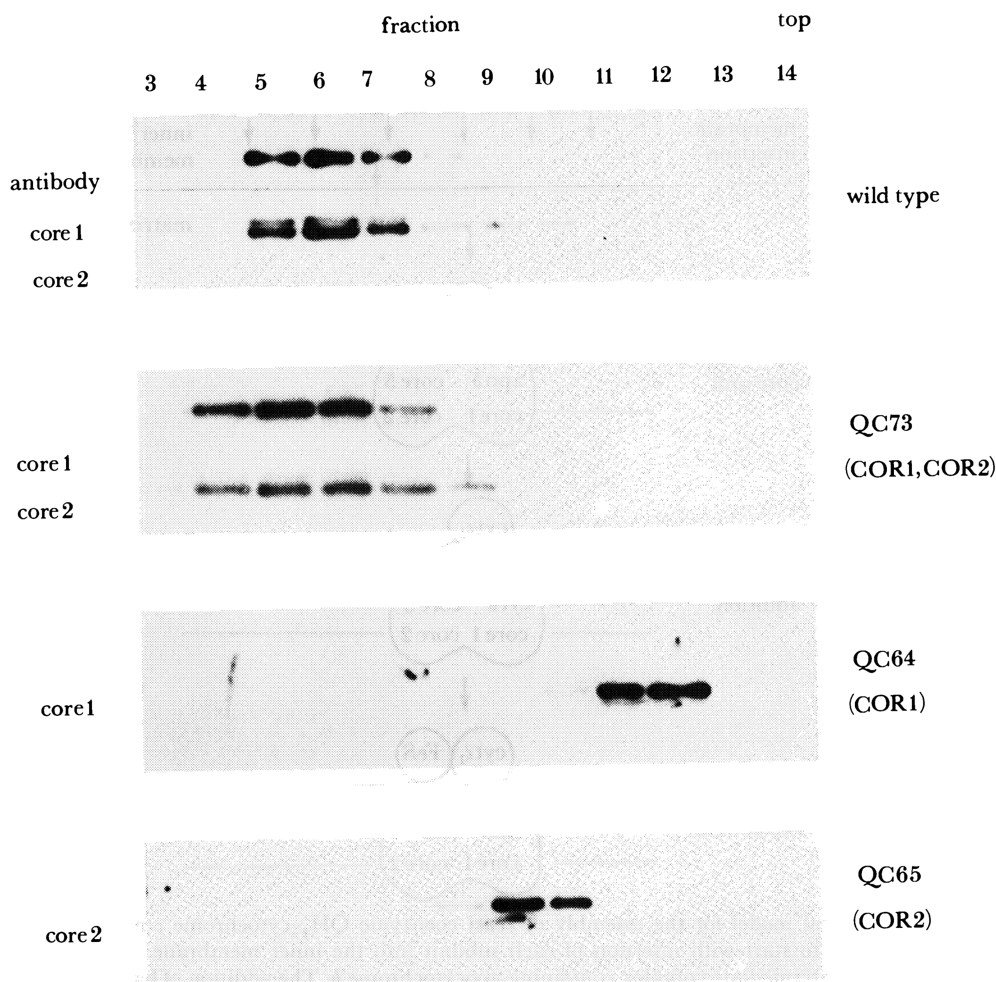

FIGURE 8. Sedimentation of the core 1 and core 2 subunits extracted from mutant mitochondria. The conditions of extraction and sucrose gradient centrifugation were identical to those described in the legend to figure 7. QC73 is a mutant with lesions in all the subunits of the complex except for core 1, core 2, and core 3. QC64 is identical to QC73 except for an additional mutation in core 2. The mutant QC65 is also identical to QC73 except that it has a mutation in core 1.

protein when extracted from a mutant containing only this subunit of the complex. Similarly, core 2 is recovered in the low-molecular-mass region of the gradient in an extract of a mutant lacking core 1. The somewhat higher sedimentation value of core 2 is probably due to its tendency to form a dimer, as was previously reported for the *Neurospora* protein (Karlsson *et al.* 1983). Secondly, we have found that the stability of the subcomplex consisting of the two cytochromes and the 14 kDa and 11 kDa subunits is affected by mutations in the non-haem iron protein as well as some of the non-catalytic components of the enzyme. For example, mutations in the non-haem iron protein cause cytochrome c_1 to sediment as a monomer but do not prevent the association of the low-molecular-mass subunits with cytochrome *b* (data not shown). Even though these studies are still incomplete they point out that the assembly of the enzyme is likely to proceed through a defined temporal sequence of subunit interactions.

The studies of the *pet* mutants allow some inferences to be drawn about the assembly of the yeast complex. These are summarized below and are illustrated schematically in figure 9.

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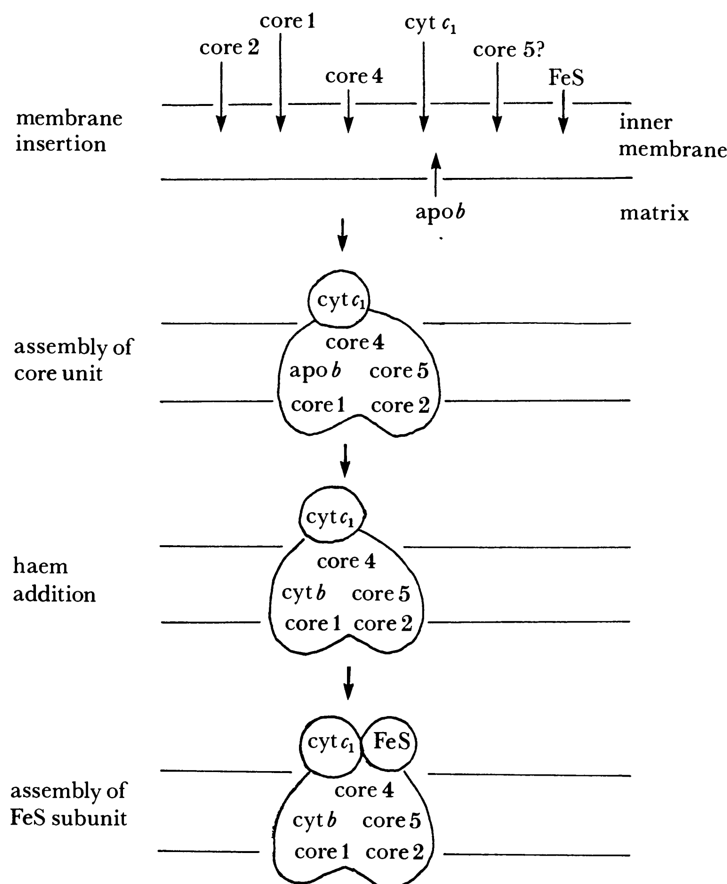

FIGURE 9. Hypothetical model for the assembly of yeast coenzyme QH_2–cytochrome *c* reductase. The assembly process is shown to start with insertion of each subunit into the inner membrane. This is followed by the formation of an intermediate complex containing apocytochrome *b*. The addition of haem is indicated to occur only after the apoprotein has associated with the non-catalytic subunits. Because the non-haem iron protein is not required for the attachment of haem its interaction with the complex can occur at a later stage. The structure and orientation of the complex is based on the studies of Weiss and co-workers (Leonard *et al.* 1981).

(i) Membrane insertion of the catalytic and non-catalytic subunits of the complex does not depend on subunit contacts. Therefore, the assembly of the complex probably occurs entirely in the membrane milieu. The fact that individual subunits are capable of integrating into the membrane even when other components of the enzyme are absent also argues that they all have membrane-anchoring domains.

(ii) Even though assembly of the holoenzyme is likely to involve a temporal sequence in which partner subunits interact with each other, some protein contacts are established independently. This applies to the formation of a stable complex of the core 1 and core 2 subunits. Other interactions, however, hinge on the presence of specific subunits. For example the association of cytochrome c_1 with cytochrome *b* and the two low-molecular-mass subunits is either blocked or is made labile in mutants lacking the non-haem iron protein.

(iii) Mutations in all the subunits except for the 17 kDa core 3 protein, the non-haem iron protein and cytochrome c_1 block maturation of apocytochrome *b*. The addition of haem must occur at a stage when the apoprotein is complexed to some of the non-catalytic subunits and therefore is probably a fairly late event in the assembly of the enzyme.

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(iv) Mutations in every subunit of the complex, except the 17 kDa core 3 subunit and the non-haem iron protein, cause substantial reductions in the concentrations of apocytochrome *b*, non-haem iron protein, and the two low-molecular-mass subunits. The most likely explanation for this phenomenon is that in the partly assembled enzyme some of the subunits are more exposed and consequently susceptible to proteolytic degradation. The fact that this effect is not seen in mutants lacking either the 17 kDa subunit or the non-haem iron protein suggests that these may be more peripherally associated with the core structure of the enzyme and do not appreciably affect most of the subunit interactions. This in turn implies that association of the 17 kDa subunit and of the non-haem iron protein with the rest of the complex occurs late in assembly.

This research was supported by Research Grant HL22174 from the National Institutes of Health, United State Public Health Service.

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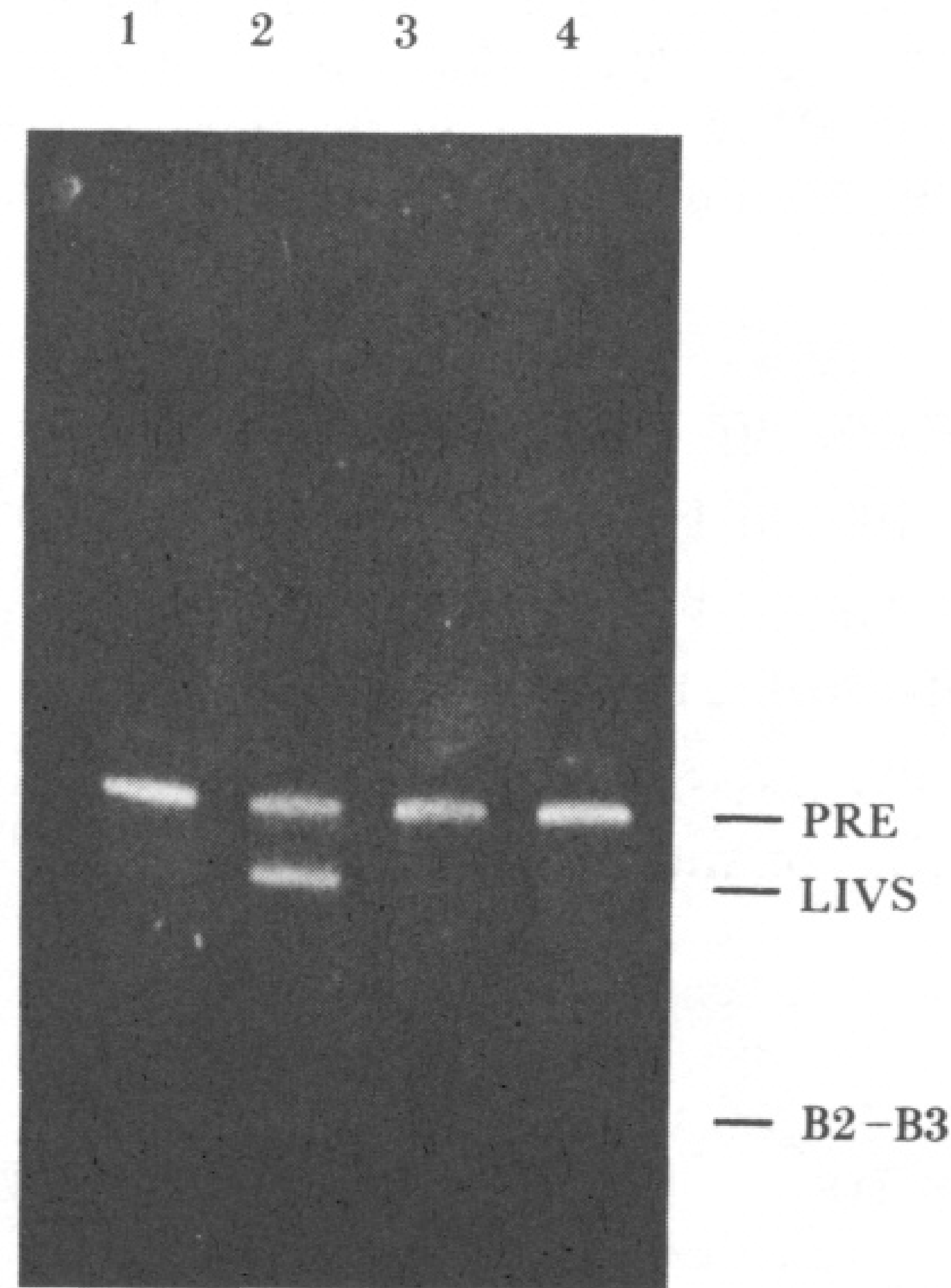

FIGURE 3. Self-splicing of the terminal intervening sequence from the SP6-derived substrate. Precursor RNA synthesized from the wild-type and mutant templates described in figure 2 were incubated for 30 min at 37 °C in the presence or absence of 0.2 mM GTP in a reaction mixture containing 50 mM Tris–HCl, pH 8, 100 mM $(NH_4)_2SO_4$, and 50 mM $MgCl_2$. The reaction products were separated by electrophoresis on a 1% agarose gel and visualized with ethidium bromide. Lane 1, wild-type substrate without GTP; lane 2, wild-type substrate with GTP; lane 3, mutant substrate without GTP; lane 4, mutant substrate with GTP. The migrations of the substrate (PRE), linear intervening sequence (LIVS), and ligated exon product (B2–B3) are indicated in the margin.

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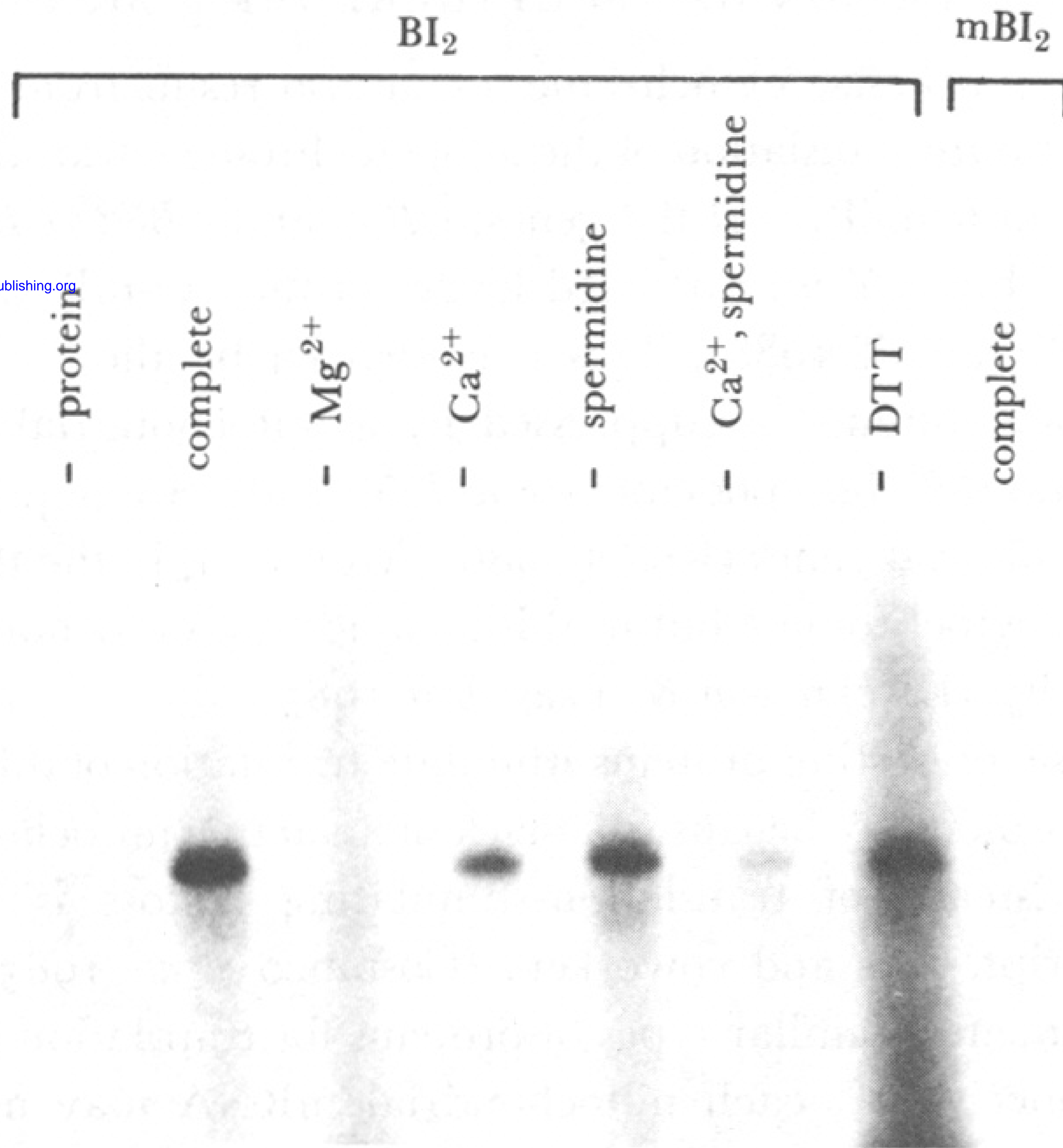

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FIGURE 4. Protein-dependent splicing. The wild type (BI_2) and mutant (mBI_2) substrates were incubated under the conditions described in the figure. The complete reaction assay contained 0.1 μg of substrate, 50 mM Tris–HCl, pH 8, 5 mM $MgCl_2$, 5 mM spermidine, 5 mM $CaCl_2$, 5 mM dithiothreitol (DTT), 10 μCi†α [^{32}P]GTP, carrier RNA, and a partly purified preparation of the CBP2 protein. After incubation at 37 °C for 30 min, the reaction was centrifuged through a column of Sephadex G-50. The material recovered in the excluded volume was precipitated with alcohol and separated by electrophoresis on a 1% agarose gel. The radioactive product visualized by autoradiography corresponds to the excised linear intervening sequence with a guanosine nucleotide covalently linked to the 5′ end.

† 1 Ci = 3.7×10^{10} Bq.

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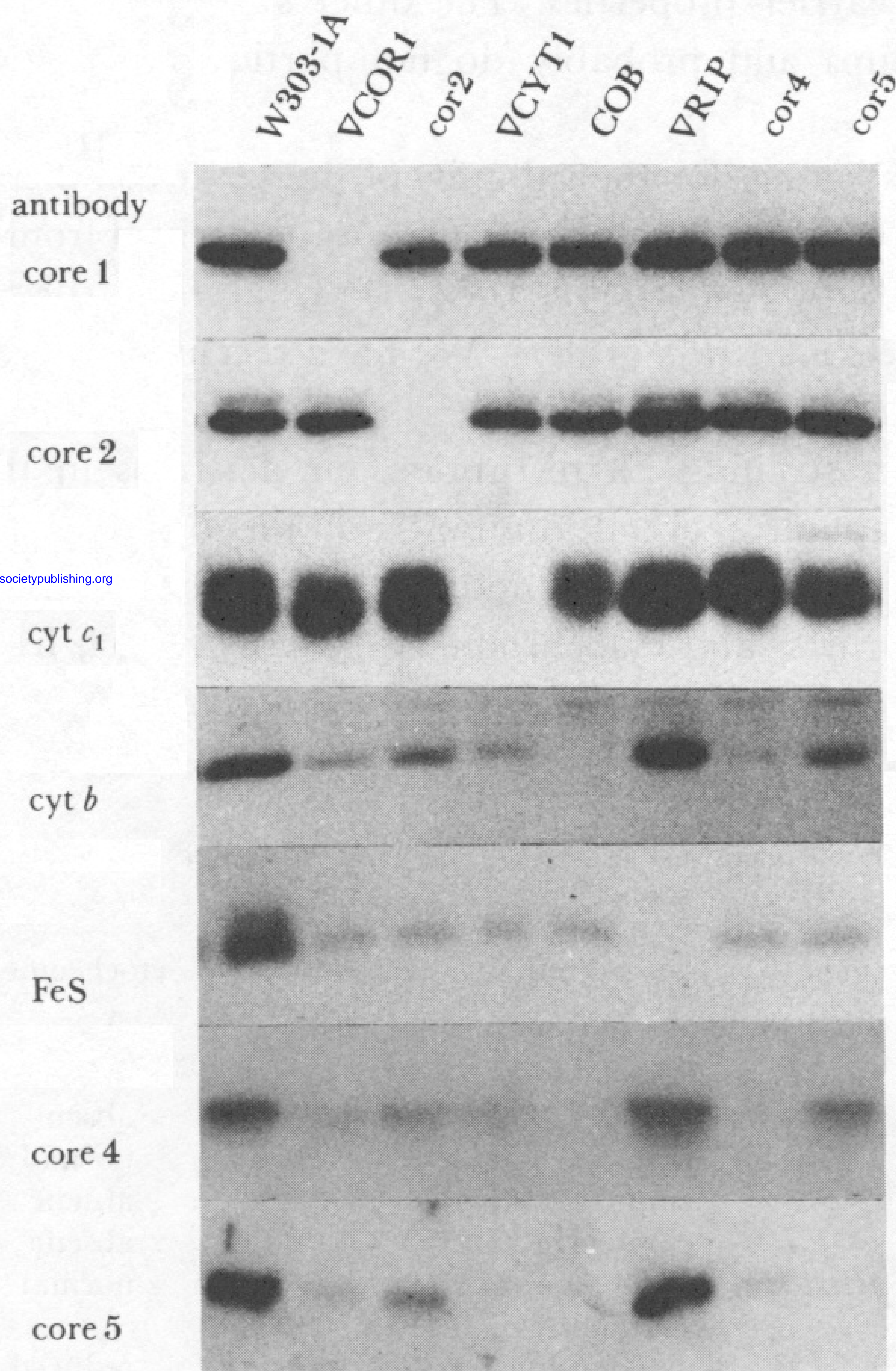

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FIGURE 5. Concentrations of subunit polypeptides of coenzyme QH_2–cytochrome *c* reductase in single mutants. Total mitochondrial protein (40 μg) from each mutant strain was separated by eletrophoresis on 12% SDS–polyacrylamide gels. The proteins were transferred to nitrocellulose paper and the blots reacted with the indicated subunit-specific antibodies. The antibody–antigen complex was visualized by autoradiography after a second reaction of the blot with ^{125}I-labelled protein A. Mitochondria were prepared from wild type (W303-1A) and from mutants carrying a disruption in the *COR1* (∇COR1), cytochrome c_1 (∇CYT1), or the non-haem iron protein (∇RIP) genes. The other strains studied had point mutations in the genes for cytochrome *b* (COB), the 14 kDa core 4 subunit (cor4), and the 11 kDa core 5 subunit (cor5). Ab FeS refers to the anitbody against the non-haem iron protein.

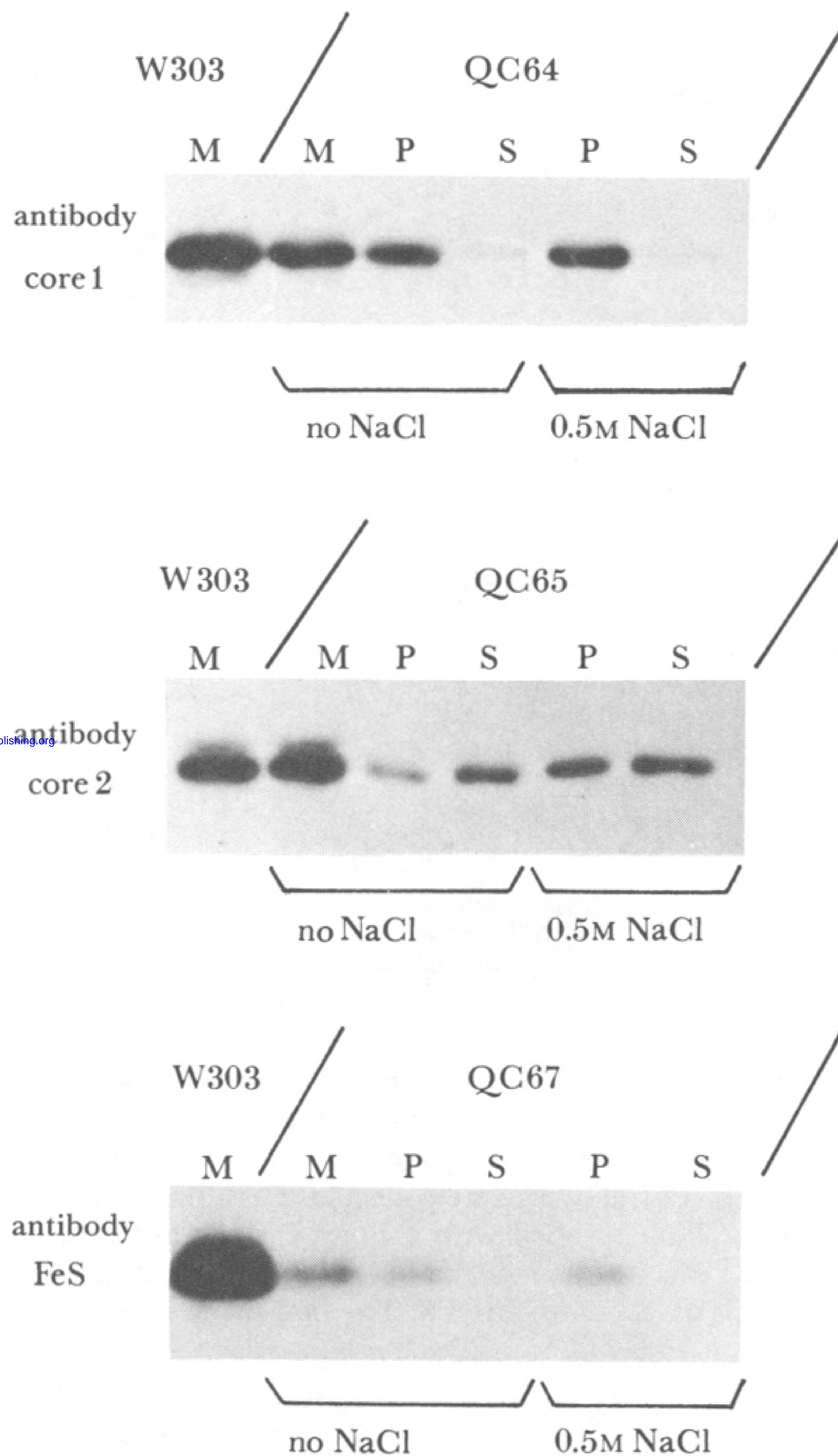

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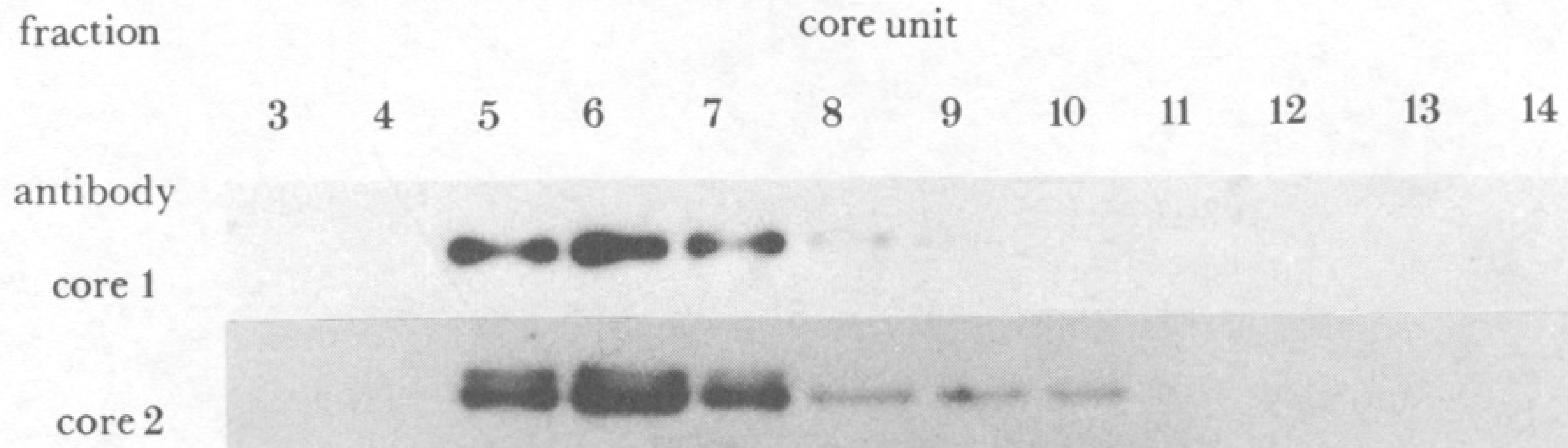
FIGURE 6. Intramitochondrial distribution of core 1, core 2, and the non-haem iron protein in strains with multiple mutations. Mitochondria (M) were sonically irradiated either in the absence or presence of 0.5 M NaCl, and were centrifuged at 156000 g for 30 min to separate submitochondrial membrane particles (P) from the soluble proteins (S). Equivalent volumes of each fraction were separated on a 12% SDS–polyacrylamide gel. The proteins were transferred to nitrocellulose and probed with subunit specific antibodies as described in the legend to figure 5. W303 is a respiratory-competent strain of yeast. QC64 is a respiratory-deficient strain with mutations in all the structural subunits except core 1 and core 3. QC65 is a multiply marked strain with the wild-type genes for core 2 and core 3. QC67 has only the wild-type genes for the non-haem iron protein and core 3.



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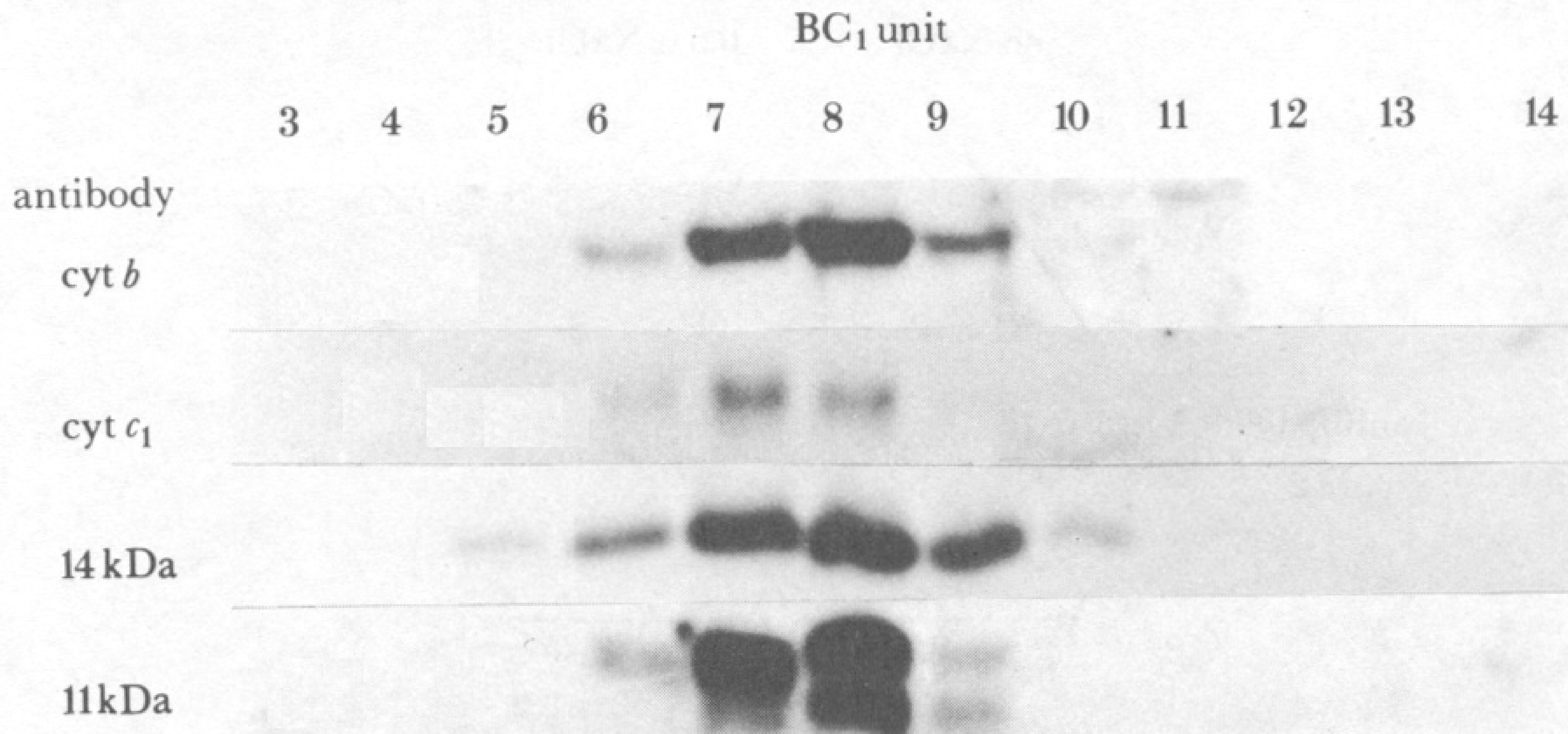

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FIGURE 7. Sedimentation of coenzyme QH_2–cytochrome *c* reductase of yeast in sucrose gradients. The yeast enzyme was solubilized by treatment of wild-type mitochondria with 0.5% deoxycholate in the presence of 1 M KCl. The extract was layered on a 5 ml column of 6–25% sucrose containing 0.1%Triton X-100. After centrifugation at 256000 *g* for 6 h, 14 fractions were collected. Each fraction was separated by electrophoresis on 12% SDS–polyacrylamide gels and probed with subunit-specific antibodies as detailed in the legend to figure 5. The Western blot shows a coincident peak of core 1 and core 2 in fraction 6 and of cytochromes *b*, c_1, and the two small core proteins in fractions 7 and 8. The non-haem iron protein peaked in fraction 13 (not shown).

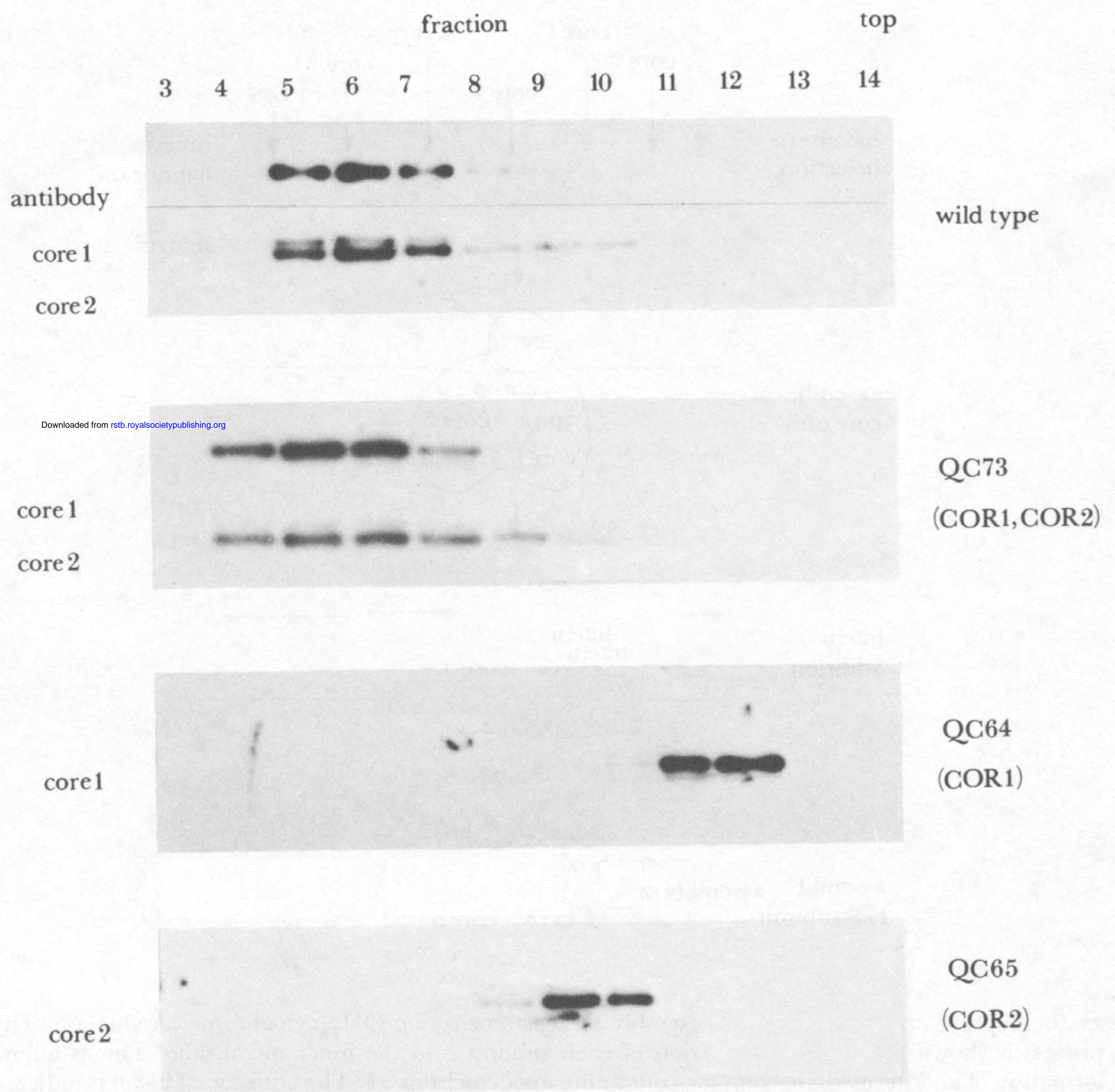

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FIGURE 8. Sedimentation of the core 1 and core 2 subunits extracted from mutant mitochondria. The conditions of extraction and sucrose gradient centrifugation were identical to those described in the legend to figure 7. QC73 is a mutant with lesions in all the subunits of the complex except for core 1, core 2, and core 3. QC64 is identical to QC73 except for an additional mutation in core 2. The mutant QC65 is also identical to QC73 except that it has a mutation in core 1.