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REGULATION OF YEAST MITOCHONDRIAL PROTEIN SYNTHESIS

by

ERIC FINZI

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, Mount Sinai School of Medicine of The City University of New York.

1982

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Abstract

REGULATION OF YEAST MITOCHONDRIAL

PROTEIN SYNTHESIS

by

Eric Finzi

Advisor: Professor Diana S. Beattie

Abstract

Protein synthesis in isolated yeast mitochondria incubated in the presence of optimal amounts of GTP is stimulated 2-fold by addition of dialyzed postpolysomal supernatant (S-150) from either yeast, rat liver, or rat skeletal muscle. S-150's isolated from either glucose-repressed or stationary phase yeast cells had considerably lower stimulatory activity than S-150 isolated from midlog phase cells. Cycloheximide treatment of rats decreased the amino acid incorporation rate by isolated liver mitochondria and lowered the stimulatory activity of the corresponding liver S-150 in comparison with control liver supernatant.

A partial purification of the cytosolic factors which stimulate yeast mitochondrial protein synthesis has been accomplished by chromatography of yeast S-150 on Sephadex G-50. Most of the stimulatory activity eluted in a peak with a molecular weight of 2000 or less. Stimulation of mitochondrial protein synthesis by the low molecular weight activator fraction was insensitive to cycloheximide, sensitive to chloramphenicol and trypsin, and proportional to the concentration of protein added. Mitochondrial protein synthesis in the absence of activator ceased after 20 min, while that in the

presence of activator continued for 40 min. Analysis of the products of the stimulated mitochondrial protein synthesis by dodecylsulfate polyacrylamide gel electrophoresis revealed that the activator increased equally the labeling of all products. These results suggest that cytoplasmic levels of low molecular weight factors present in the cytosol regulate mitochondrial protein synthesis *in vivo*.

Yeast mitochondrial protein synthesis shows biphasic Arrhenius plots both *in vivo* and *in vitro*, with a two-fold increase in the activation energy below the transition temperature, suggesting a functional association between mitochondrial protein synthesis and the inner membrane. Gel electrophoresis of mitochondrial translation products labeled *in vivo* revealed that the same proteins are synthesized and then inserted into the membrane above and below the transition temperature of the membrane.

The rate of leucine uptake into mitochondria was decreased at least five-fold in the presence of chloramphenicol, suggesting that leucine is used mainly for protein synthesis. Under conditions where the membrane potential was dissipated but matrix ATP levels high, uptake was inhibited, suggesting that the membrane potential is required for leucine transport which may occur by an active transport mechanism.

Dedication

To my mother, my father, and my sister, who together provided the encouragement and support which made all this work possible.

Acknowledgments

As I watch the sunset through the 19th floor window and think about my work here in the Biochemistry Department during the last few years, I realize how many generous people have helped. My warmest thanks go to my mentor, Dr. Diana S. Beattie, who not only provided her guidance during this work and her example as a dedicated, top-notch scientist, but who was also understanding and supportive when I was ill. To Marvin Sperling, whose diligent assistance made most of these studies possible, best of luck in your future medical career. May another department be so blessed to have two faculty members as altruistic as Dr. Heng-Chun Li and Dr. Arthur Cederbaum, both of whom were always willing to discuss old experiments and propose new ones. Thanks are extended to Dr. Samuel Waxman, who provided a generous gift of folate-binding protein, technical help, and advice during the folate studies. A hearty handshake to Dr. Kevin Scanlon, for his receptive ear and critical insight into folate experiments. Thanks to Dr. Ronald W. Rinehart for performing experiments involving liver mitochondria. The technical advice often given by Dr. Anthony Scotto and Dr. Arthur Guffanti is also not forgotten. To Sue Morance, thanks for typing this manuscript.

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I. Introduction

A. The Organism

The unicellular fungus, *Saccharomyces cerevisiae*, has provided us with much of what we know about mitochondrial biogenesis. This yeast is a facultative aerobe whose growth conditions can be easily manipulated, and the effects of these changes on mitochondria can be studied. Aerobic growth on nonfermentable carbon sources such as lactate or ethanol leads to the full development of the mitochondrial respiratory chain. However, when *S. cerevisiae* is grown aerobically on a fermentable carbon source such as glucose, cellular respiration and mitochondrial respiratory chain activities are depressed; this phenomen is known as glucose repression (1). The growing culture ferments the glucose, producing ethanol and carbon dioxide; as the glucose supply is exhausted, the cells switch from fermentative to oxidative metabolism, with a rapid increase in components of the respiratory chain (2). This respiratory adaptation (known as glucose derepression) requires protein synthesis in both the cytosolic and mitochondrial compartments.

Anaerobic growth of yeast on glucose in a media supplemented with unsaturated fatty acids and ergosterol /the formation of the two lipid components is oxygen dependent

(3,4)7 leads to marked reductions in mitochondrial respiratory chain components including cytochromes (5-8). Cells grown under these conditions contain mitochondrial-like structures, termed "promitochondria" by Schatz (9). Promitochondria can differentiate into fully respiring mitochondria on transfer of the cells to aerobic conditions (10). The acquisition of full respiratory capacity that occurs when cells undergo glucose derepression or aerobic adaptation has made these two physiologic changes important tools in the study of the formation of the mitochondrial inner membrane.

The easy genetic manipulation of yeast has been instrumental in our understanding of cytoplasmic inheritance (10). Haploid strains, which grow vegetatively by budding, are distinguished sexually by a pair of chromosomal alleles, a and α. Upon mixing of haploid a and α cells, fusion occurs producing diploid zygotes which divide mitotically to yield diploid clones. When diploid cultures are starved for nitrogen, they divide meiotically to produce a tetrad of four haploid cells (ascospores). Mating alleles a and α as well as all other nuclear genes segregate 2:2 in a Mendelian fashion in the four haploid products of each ascus; however, mitochondrial genes preferentially segregate during vegetative growth of diploids prior to meiosis. Therefore, ascospore tetrads generally show a 4:0 or 0:4 pattern of

inheritance of mitochondrial genes (10). Tetrad analysis has been a powerful tool in the investigation of mitochondrial heredity.

Ephrussi and his colleagues (11,12,13) provided the first direct evidence of a cytoplasmically inherited determinant in yeast. They described respiratory deficient mutants which were named petites because of their small colony size. These mutants contained no cytochrome a, a_3, or b, and therefore required fermentable sugars for their growth. Exposure of normal cells to acridine dyes greatly increased the rate of formation of petites. In addition, the petite mutation was induced with equal ease in haploid or diploid cells. Crosses of petite strains with wild-type strains produced only wild-type progeny. Furthermore, repeated backcrosses of the progeny to the petite strains failed to produce petites. They calculated that this could be explained in Mendelian terms only by assuming the presence of 12 recessive genes. Instead they concluded by ascribing "the mutant character to the loss or inactivation of an extranuclear, particulate, and autoreproducing factor required for the normal synthesis of the respiratory enzymes" (12).

Chen et al. (14) described mutants with the petite phenotype but showing a Mendelian (2:2) segregation pattern in crosses to the wild-type. Crosses of the nuclear petites

with cytoplasmic (vegetative) petites gave some wild-type diploids, indicating that the two types of petites "complement" one another; therefore the nuclear and cytoplasmically inherited genes determining petite phenotype are nonidentical (15).

Wright and Lederberg (16) provided direct evidence for the cytoplasmic location of vegetative petite mutations. They took advantage of the ability of certain yeast strains to transiently form, upon mating, heterokaryons which contain unfused parental nuclei along with cytoplasm from both parents. They crossed an α petite strain carrying a nuclear inherited auxotrophy for arginine to an a respiratory competent strain auxotrophic for thiazole. Analysis of haploid clones budded off from heterokaryons /12 arginine$^-$ and 23 thiazole$^-$ mating pair progeny/ revealed that 4 were α arginine$^-$ but respiratory competent while 3 were a thiazole$^-$ petites, indicating that the respiratory activity was segregating independently of the nuclear-inherited auxotrophies and was therefore cytoplasmically inherited.

B. Introduction to Mitochondria

Part of the following summary about mitochondrial structure and function has been adapted from Lehninger (17).

1. Mitochondria vary considerably in size and shape from one species to another. The organelle contains two

membranes: an outer membrane which is generally smooth and an inner membrane which has inward folds. The intermembrane space is between the two membranes, while the matrix is inside the inner compartment. The outer and inner membranes differ in both the total amounts and composition of lipids and proteins. The phospholipid cardiolipin, which is abundant in the inner membrane, is present in only small amounts in the outer membrane, while cholesterol is present mainly in the outer membrane (18,19).

2. The enzymes of respiration and oxidative phosphorylation are located in the inner membrane. Many respiratory chain substrates are oxidized by NAD-linked dehydrogenases. Electrons from NADH are funneled into the electron transport chain via NADH dehydrogenase; the final electron acceptor is oxygen. Electron transport leads to the formation of an electrochemical proton gradient (20) which is used by F_1ATPase in the production of ATP, by metabolite transport systems, and by other processes.

3. The electron transport chain can be separated into four high-molecular weight complexes which catalyze the transfer of electrons as described below (21):

Complex	Electron donor	Electron acceptor
I	NADH	Coenzyme Q
II	Succinate	Coenzyme Q
III	Coenzyme QH_2	Cytochrome C
IV	Cytochrome C	O_2

4. The mitochondrial matrix is the site of the citric acid cycle, fatty acid oxidation, and amino acid oxidation. In addition, mitochondria, in a tissue specific manner, play a role in heme biosynthesis, fatty acid biosynthesis, steroidogenesis, gluconeogenesis, and urea production.

C. Mitochondrial Biogenesis

The first conclusive evidence showing that mitochondria contain their own DNA was provided in the early 1960's. Nass and Nass (22) stained DNA with uranyl and lead ions and observed "fibrils" in the mitochondria under the electron microscope. Pretreatment of the preparation with deoxyribonuclease eliminated the "fibril" staining, suggesting that the mitochondria contained DNA. Definitive proof came from Luck and Reich (23) and Rabinowitz et al. (24); they found that DNA isolated from Neurospora and rat liver mitochondria had a lower buoyant density than nuclear DNA. Subsequent studies using renaturation kinetic analysis (25,26) and restriction fragment digestions have revealed

that the unique gene sequence content of mitochondrial DNA is equal to the molecular weight obtained by electron microscopy (27-29). It is now clear that mitochondria DNA in most species is small ($1x10^7$ to $7x10^7$ daltons), duplex, devoid of histones, and circular (see 10 for review).

In 1964, several groups (23,30,31) showed that isolated mitochondria could incorporate radiolabelled ribonucleotides into an acid-insoluble product. RNA polymerase has since been purified from mitochondria by many groups (32-37). Although reports of protein synthesis in isolated mitochondria appeared over twenty years ago (38), these observations were disputed throughout the 1960's. Mitochondrial protein synthesis was confirmed by many groups (39-42), but reports conflicted on the amino acid and energy requirements for optimal protein synthesis *in vitro*. In a definitive study on rat liver mitochondria, Wheeldon and Lehninger (43) showed that amino acid incorporation could be supported at maximal rates by ATP itself, indicating that the high-energy intermediate generated by electron transport did not energize protein synthesis directly. Wheeldon (44) also observed amino acid incorporation by isolated mitochondria under conditions where bacterial contamination was minimized. Beattie *et al.* (45) definitively excluded bacterial contamination as the cause of the observed *in vitro* incorporation;

they also demonstrated that mitochondrial translation products are predominantly incorporated into the inner membrane (46). Subsequently, Beattie (47) used inhibitors of cytoplasmic and mitochondrial translational systems to demonstrate in rat liver slices that soluble mitochondrial proteins are synthesized outside of the mitochondria and then transferred inside. Greater than 90% of mitochondrial proteins are synthesized on cytosolic ribosomes (see 48 for review); the transport of these proteins into mitochondria is discussed in section D of this chapter.

Luck attempted to determine whether mitochondria arise from preexisting mitochondria or originate de novo (49). He followed the uptake of labeled choline into mitochondrial membranes of a choline requiring Neurospora mutant. Autoradiograms of cell cultures grown after transfer from a labeled to unlabeled medium showed a uniform distribution of the radioactivity in mitochondria. Furthermore, cells shifted from a low to high choline medium showed a change in the density of the entire mitochondrial population; the phospholipid to protein ratio was affected, although the concentration of cytochromes per mg of protein was the same. These results suggest that mitochondria grow by addition of molecules into preexisting membranes of all mitochondria

and that the lipid and protein components can vary independently (49).

As discussed earlier, anaerobically grown cells lack respiratory capacity but acquire it quickly upon exposure to oxygen. Anaerobically grown cells contain promitochondria which lack cytochromes but retain a protein synthesis system (50). Plattner et al. (51) addressed the question of whether mitochondria originate de novo during the aerobic transition or promitochondria differentiate into fully respiring mitochondria. They labeled promitochondrial proteins by growing cells anaerobically with radioactive leucine in the presence of cycloheximide, an antibiotic which inhibits protein synthesis on cytosolic ribosomes but has no effect on mitochondrial translation (52-54). After transfer to unlabeled media and aerobic growth, cells were examined for the presence of label by autoradiography. Plattner et al. (51) demonstrated label in the membranes of mitochondria, indicating that promitochondria do differentiate into mitochondria.

In 1968, Smith and coworkers examined another aspect of mitochondrial biogenesis, DNA replication (55). Using the isotopic transfer method of Meselson and Stahl (56), they demonstrated that yeast mitochondrial DNA, like nuclear

and procaryotic DNA, replicates semiconservatively. The enzymes for this complex process are cytoplasmically synthesized and subsequently transferred into the mitochondria. Mammalian mitochondrial DNA begins replication with synthesis of a new H-strand, leading to displacement of the parental H-strand and formation of a D-loop intermediate (57). A large proportion of DNA molecules are found as D-loop intermediates which contain a 7 S H-strand initiation sequence. Elongation of the H-strand proceeds unidirectionally (58). L-strand synthesis begins only after two-thirds of the new H-strand is completed (57).

The timing of mtDNA replication with respect to nDNA replication appears to vary with the organism. Mammalian mtDNA usually replicates discontinuously at a slightly different time than nDNA, while in some lower eucaryotes it is continuous throughout the cell cycle (see 59 and 60 for review). Mechanisms controlling the initiation of mtDNA replication are largely unknown.

D. Transport of Proteins Into Mitochondria

Mitochondrial protein synthesis accounts for less than 10% of the total protein mass of mitochondria (see 48 and 61 for review). How then are the several hundred mitochondrial proteins which are coded by nuclear genes and translated on

cytoplasmic ribosomes transferred into precisely defined locations within the mitochondrial organelle? In a well-studied example of protein transport, the synthesis of proteins destined to be secreted, ribosomes are attached to the cytoplasmic side of the endoplasmic reticulum. Nascent polypeptide chains are vectorially translocated into the lumen of the rough endoplasmic reticulum. The transmembrane movement of the polypeptide chain is coupled to its elongation, a cotranslational process (62-64). Kellems and Butow (65,66) presented evidence for the binding of cytoplasmic ribosomes to outer mitochondrial membranes and suggested a similar cotranslational mechanism of protein transfer into mitochondria. However, it now appears that the predominant if not sole mode of protein transfer into mitochondria is posttranslational (67).

Hallermeyer et al. (68), using in vivo pulse and pulse-chase labeling experiments, found that the appearance of label into mitochondrial proteins (matrix proteins, ribosomal proteins, cytochrome c, and carboxyatractyloside-binding protein) showed a lag relative to total cellular, ribosomal, microsomal, and cytosolic proteins. Newly synthesized mitochondrial matrix proteins were first detected in the cytosol and then in the mitochondria, suggesting that there is an extramitochondrial pool from which these

proteins are transported into the mitochondria. Furthermore, cycloheximide did not prevent the import of proteins into the mitochondria, indicating that import was posttranslational. Recent studies have indicated that mitochondrial precursors are synthesized on free polysomes and released into the cytosolic space (67).

Import of proteins into mitochondria has since been reconstituted *in vitro*, using the products of cell-free translation systems and isolated mitochondria (69,70). Most but not all cytoplasmically synthesized mitochondrial proteins are made as slightly larger precursors (67), which are transported across mitochondrial membranes and processed to their mature size in the absence of protein synthesis (69). Import of most precursor proteins into mitochondria requires energy; the membrane potential has been shown to be the direct energy source (71).

Hennig and Neupert (72) have shown that the conversion of apocytochrome *c* to holocytochrome *c* by *Neurospora* mitochondria requires the covalent attachment of heme to the protein; an analogue of heme prevented both the translocation of the precursor across the membrane and the attachment of heme. Recently, yeast cytochrome b_2 and c_1 were found to be processed by a two-step import mechanism (73).

In the first import step, the precursor to cytochrome $\underline{c}_1$ is cleaved to an intermediate form by a matrix protease; the second step, the processing of the intermediate to mature cytochrome $\underline{c}_1$, requires heme. Schatz's group have succeeded in partially purifying a matrix-localized o-phenanthroline sensitive protease which cleaves only mitochondrial precursors (74).

The binding of apocytochrome c to Neurospora mitochondria was shown (72) to meet several criteria for specific interaction of a protein with a receptor (75), including: (1) binding is rapid and reversible; (2) saturable binding to a limited number of binding sites; (3) specificity of binding (i.e., apocytochrome c from horse bound with a 10-fold less affinity in comparison with Neurospora apocytochrome c.). Apparently the protein structures required for binding have been relatively conserved during evolution, since Neurospora precursor proteins are imported into rat liver and yeast mitochondria (71,76). Competitive binding experiments with different precursors indicate that there are at least two types of receptors (77). Future work in this area of protein transport and processing should prove exciting.

E. Mitochondrial Genome

A giant step forward in our understanding of the

functional role of mtDNA was made possible by the development of powerful genetic and physical techniques to map the mitochondrial genome. Several classes of mitochondrially inherited mutations have been instrumental in this work. Petites (ϱ^-), whose induction from wild-type cells is greatly increased by acridine dyes (12,13), contain mtDNA from which large segments have been deleted. The retained DNA sequences are amplified to the original wild-type genome size (78-80). Both ϱ^- and ϱ^o (cells devoid of mtDNA) mutants lack mitochondrial protein synthesis (81). Many mutants have been found which are resistant to inhibitors of several mitochondrial functions (protein synthesis, electron transport, and oxidative phosphorylation). Particularly useful inhibitors include: chloramphenicol, erthromycin, and paromycin (protein synthesis); antimycin, diuron (electron transport); and oligomycin (oxidative phosphorylation). Tzagoloff and coworkers isolated and characterized mitochondrial mutants (mit^-) which have defects in the respiratory chain and in oxidative phosphorylation but are capable of mitochondrial protein synthesis (82,83). These mutants are defective in the synthesis of one or more mitochondrial translation products; the mutation is presumably located in the structural gene for these translation products. Syn^- mutants are

non-ρ^- point mutants that are completely blocked in mitochondrial protein synthesis; mutations in mtDNA genes coding for tRNA's and rRNA's are involved (84,85).

The initial approach to mitochondrial gene mapping, classical recombinational analysis of genetic markers to determine linkage patterns, was unsuccessful because mt genes separated by short distances appear unlinked by this analysis (86,87). However, several other genetic mapping techniques have been successfully used. Schweyen et al. (88) examined the frequency of loss of two genetic markers and retention of two others during the generation of ρ^- mutants from a stock strain carrying all four markers (double loss/double retention analysis). Three possible pairs of double-deletion/double retention ρ^- genotypes were possible but only one would result from two independent deletions of mtDNA; the two other genotypes could be explained by a single deletion. The markers were then ordered in a way that yields the rarest complementary pair as a double deletion (88). Molloy et al. also used coretention and codeletion of markers in ρ^- strains to map mutations (89). The analysis assumes that ρ^- clones contain continuous segments of the mitochondrial genome and that closely linked markers will either be coretained or codeleted more often than unlinked markers when a library of ρ^- strains are examined. Slonimski

and Tzagoloff mapped mit$^-$ mutants lacking either cytochrome oxidase or coenzyme QH_2-cytochrome c reductase activity to each other and to drug resistant markers (90). They crossed mit$^-$ strains with isonuclear ϱ^- mutants and assayed recombinants for restoration of respiratory function (growth on a nonfermentable substrate). In crosses leading to restoration of respiration, the mit$^-$ mutant must be within the mtDNA segment retained by the ϱ^-. Furthermore, mit$^-$ x mit$^-$ crosses leading to wild-type progeny led to identification of distinct mutant clusters. Thus the authors localized on mtDNA 3 regions for cytochrome oxidase and one for coenzyme QH_2-cytochrome c reductase which are necessary for the activities of these respiratory chain complexes.

Fine structure mapping of mt genes has proceeded rapidly by classical deletion mapping using libraries of ϱ^- clones (ϱ^- deletion mapping). The principle involved is to locate a point mutation to a given segment of the map by recombinational analysis using ϱ^- clones with different deletion endpoints. If a point mutant recombines with one ϱ^- strain to yield wild-type progeny, but not with another, then the point mutation must lie in the non-overlapping region of their mtDNA's (91). MtDNA can also be isolated and characterized by a variety of physical techniques. These include: (a) mtDNA-mtDNA hybridization (92,93) $\not\varrho^-$ with wild-type and

other ρ^- clones]; (b) restriction endonuclease mapping (94,95) [comparison of restriction fragments between different ρ^- clones]; (c) hybridization of mtDNA with mtRNA (96) [R-looping or heteroduplex analysis]; (d) sequencing of mtDNA (97-99).

Hybridization of tRNA's aminoacylated with labeled amino acids to ρ^- mtDNA has shown that at least 22-23 tRNA's are coded by the yeast mt genome (100,101). The 21 S rRNA, which is a "split" gene containing an intervening sequence (96), and the 15 S rRNA have also been mapped to the yeast genome. The current map of yeast mtDNA, derived through use of all the techniques described above, is shown in Fig. 1. The genes for three subunits of cytochrome oxidase, cytochrome b, subunits 6 and 9 of the proton-translocating ATPase, a ribosomal protein (Var 1), and the two rRNA's are present in the 75 kbp genome. Between the coding regions are long stretches of A-T rich segments whose function is unknown. The A-T content of total yeast mtDNA is 82%, a highly unusual sequence content (102).

The gene coding for cytochrome b has several unusual features. At least four genetically unlinked loci are involved in the expression of cytochrome b. Mutations in some of these box loci affected both cytochrome b and cytochrome oxidase synthesis (103). Correlation of the genetic

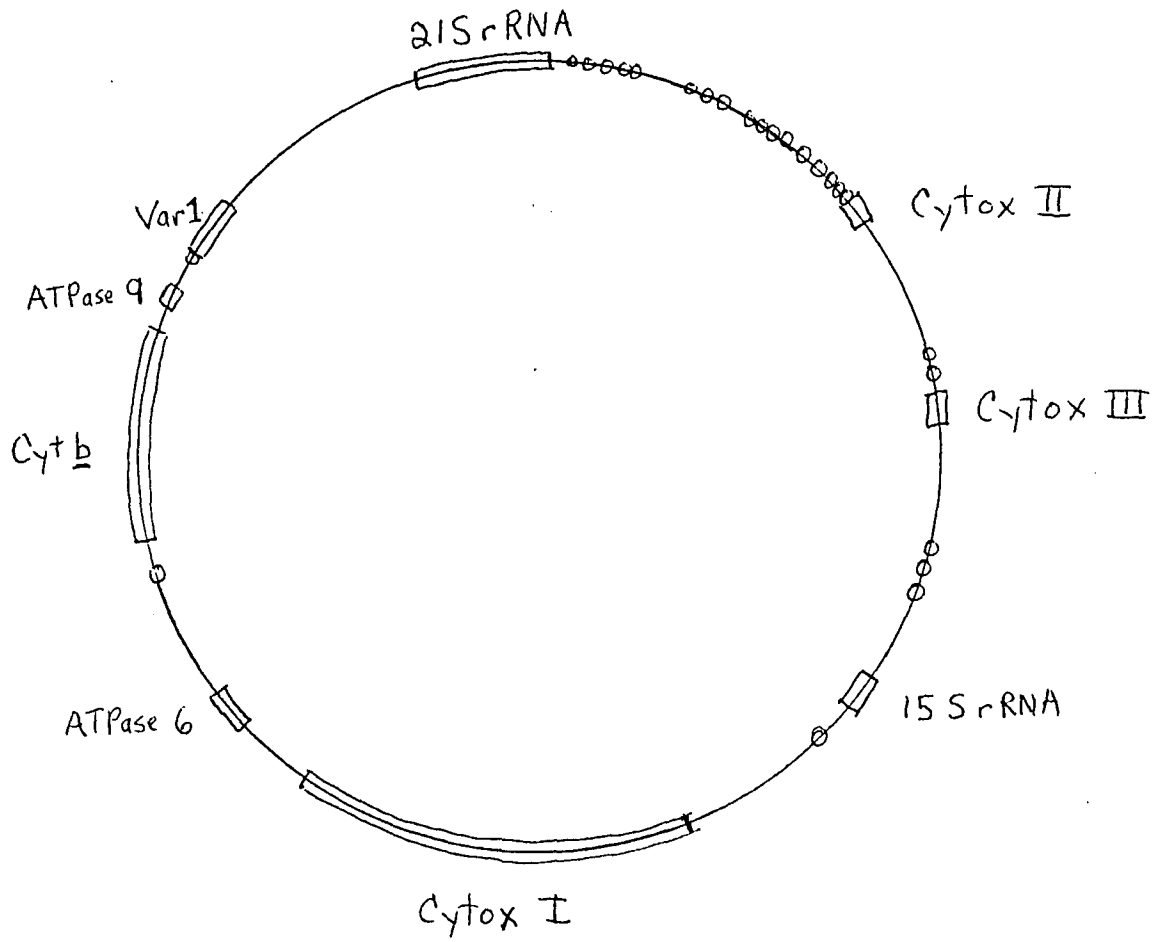

Figure 1

Map of Yeast Mitochondrial Genes.
The open circles represent tRNA genes. Adapted from the Cold Spring Harbor Meeting on Mitochondrial Genes (1981).

and physical maps indicated that the cytochrome b gene is large and mosaic; there are 6 "exons" and 5 "introns" in some strains (104). However, several of the "introns" contain open reading frames; mutations in these segments block cytochrome b mRNA processing and prevent the synthesis of subunit I of cytochrome oxidase. Slonimski and coworkers (105) have proposed that after the initial step in splicing of cytochrome b mRNA, there is translation of an exon-intron coded "maturase" protein which is required for the subsequent splicing of the pre-mRNA for cytochrome b. Another cytochrome b intron-coded maturase helps splice the cytox subunit I transcript, which is also transcribed from a "split" gene. This may explain the pleiotropic nature of certain box mutants (106).

Direct sequence analysis of the gene coding for subunit I of cytox (107) has revealed the presence of 6 introns, 4 of which contain open reading frames. The 4th intron has a 70% homology in predicted amino acid sequences compared to the 1st intron of cytochome b, suggesting that both introns may be translated. However, except for the probable role of two open reading frames in introns of cytochrome b pre-mRNA, the function of other open reading frames, located in areas of the genome where no known mitochondrial translation product maps, is unknown. The presence of similar

sequences (108) in all these open reading frames (high content of lysine, asparagine, and isoleucine), would suggest a common function for these putative proteins. Hopefully, we shall see shortly direct proof for the translation of these open reading frames along with an increased understanding of the function of these proteins.

Macino et al. (109) have shown by sequence analysis of yeast mtDNA that the genetic code in mitochondria does not follow codon rules previously thought to be universal. The UGA codon, normally a terminator codon, is recognized instead by tryptophan tRNA. Threonine tRNA rather than leucine tRNA recognizes CUA (110). Furthermore, mt genes are decoded by only 24 tRNA's (110). This contrasts sharply with Crick's (111) wobble hypothesis which requires a minimum of 32 tRNA's to decipher the genetic code. Mitochondria are able to function with fewer tRNA's because they employ only one tRNA for each unmixed codon family; these tRNA's have a U in the wobble position of their anticodons (110).

The complete sequence of the 16,569 base-pair human mitochondrial genome has recently been determined (112). A comparison between yeast and human mt genomes reveals several important differences. Human mt genes are not spliced, nor are they separated by A-T rich spacer regions. Intact, some adjacent genes contain no nucleotides between

them. Instead, genes coding for mitochondrial proteins and rRNA's are "punctuated" by tRNA genes which probably serve as signals for the processing of the transcripts (112-115). Unlike procaryotic or cytoplasmic eucaryotic mRNA's, human mt mRNA's do not contain non-translated leader sequences but start directly with AUG or AUA initiation codons. In addition, half of the termination codons in the mRNA's are created by post-transcriptional polyadenylation (112). Human mtDNA also contains 8 unidentified reading frames which show 70% homology in DNA sequence compared to bovine mtDNA. A recent report showing that mammalian mitochondria synthesize up to 25 proteins suggests that some of these unidentified reading frames may be translated (116).

Comparisons between mt genomes from different species have turned up the surprising finding that subunit 9 of ATPase (DCCD binding protein) can be coded for by either the mt or nuclear genomes. Subunit 9, a highly hydrophobic protein, is coded for by yeast on mtDNA; however, in Neurospora and humans the gene is located on nuclear DNA and the protein synthesized on cytoplasmic ribosomes (112,117-119). The extensive sequence homology between DCCD binding proteins from these species suggests that gene transfer between mt and nuclear genomes occurred during evolution. Sebald and Viebrock (120) addressed the question of how the nuclear

gene for the protein was modified to enable such a hydrophobic protein to be translated in the cytosol and then transferred posttranslationally into its integral membrane position in the mitochondria. They found that the protein was translated as a precursor containing a hydrophilic 66-residue presequence which balanced the hydrophobic character of the mature protein.

F. Mitochondrial Transcription

RNA polymerase was reported by several groups (32-37) to have been purified from mitochondria. Recently, in careful studies which ruled out nuclear contamination of the mitochondrial preparation, Levens et al. (37) showed yeast mitochondrial RNA polymerase to be a 45,000 dalton protein whose activity is stimulated by poly$\overline{d(AT)}$ templates, inhibited by Mn^{2+}, and unaffected by either rifampicin or α-amanitin. These results distinguish the mitochondrial enzyme from all known nuclear or bacterial RNA polymerases (37).

Edwards et al. (121) have used guanyltransferase, an enzyme which couples GTP to a di- or triphosphate terminated polyribonucleotide, to label primary yeast mitochondrial transcripts. They exploited the knowledge that yeast and mammalian mt mRNA's do not contain the "cap" structure

common to other eucaryotic mRNA's (122) and assumed that primary mt transcripts retain their 5'triphosphate ribonucleotide.

Hybridization of the labeled primary transcripts with ρ^- mtDNA showed 17 separate transcriptional initiation sites on the yeast mt genome. Furthermore, the purified RNA polymerase of Levens et al. (37) initiated transcription of rRNA genes on cloned DNA templates at the same sites as determined by mapping of labeled primary transcripts, indicating some specificity of in vitro transcription by purified RNA polymerase. Nothing is known about the possible interaction of RNA polymerase with regulatory factors. However, it is interesting to note that anaerobically grown cells, which have greatly reduced rates of mitochondrial protein synthesis, contain RNA transcripts indistinguishable from cells grown aerobically (123). These results imply that regulation of mitochondrial protein synthesis in anaerobic cells occurs at the level of translation.

Hybridization of yeast mtDNA with saturating amounts of mtRNA revealed that 35% of the mtDNA hybridized, indicating that about 70% of a single strand equivalent of mtDNA was transcribed (123). Separated strands from a cloned restriction fragment of mtDNA showed large differences in their abilities to hybridize to mtDNA; one strand hybridized 42% but the

other only 2%, suggesting that yeast mt transcription may be highly assymetric.

The physical mapping of yeast mtRNA to mtDNA has shown multiple precursor transcripts, each differing slightly in size, which hybridize to the same gene region (124-126). These results have confirmed genetic and sequencing data which indicate that the genes for cytochrome b and subunit I of cytochrome oxidase contain intervening sequences. The processing enzymes required for the splicing of these RNA's must be nuclear encoded since ρ^- strains are able to produce mature RNA's (124).

In contrast to the multiple promoter sites on yeast mtDNA, mammalian (HeLa cell) mtDNA contains only two, one for H-strand transcription and another for L-strand transcription (127). Attardi et al. (127) have suggested two pathways for H-strand transcription which start near the origin of replication: (1) continuous transcription of nearly the entire H-strand into a single polycistronic molecule, which is then processed by precise endonucleolytic cleavages before and after tRNA sequences; (2) transcription attenuation at the 3' end of the 16 S rRNA in a manner analogous to the bacterial attenuation mechanism described by Yanofsky and coworkers (128). In contrast to yeast, HeLa cell mtDNA

has also been shown to be symmetrically described (129). Mammalian (130-132) and yeast mt (133,134) were reported to contain poly-A additions at the 3' end of their mRNA's that were 60-100 and 20-30 bases long, respectively. However,. the yeast poly-A tail has been disputed (135).

Whether or not all mtRNA was transcribed from the mt genome was a much debated subject several years ago (60). Reijnders et al. (136) provided the first conclusive demonstration that mitochondria did not import RNA. They showed that labeled mtRNA hybridized with nDNA in the absence but not in the presence of a 100 fold excess of unlabeled cytosolic rRNA. This was not due to contamination of the cytosolic rRNA with mtRNA, because cytosolic rRNA had no effect on mtRNA-mtDNA hybridization. Apparently the only RNA isolated from the mt fraction which hybridized to nDNA was contaminating cytoplasmic RNA. The wealth of genetic and physical data on mt genes and translation products which has been amassed over the years makes it improbable that yeast, Neurospora, or human mitochondria import RNA. However, in Tetrahymena, Chiu et al. (137) have found several mt tRNA's which hybridize only to nDNA and not to mtDNA; they concluded that these tRNA's are nuclear transcripts which are imported into the mitochondria. Suyama and Hamada (138) have suggested that mt aminoacyl tRNA

synthetases serve as transport carriers for the imported tRNA's. Whether results reported for Tetrahymena prove to be true for any other organisms remains to be seen.

G. Mitochondrial Translation

The endosymbiotic theory of mitochondrial evolution would suggest that the mitochondrial translation systems possess many similarities to those of procaryotes. However, we have already seen strong contrasts between mitochondria and bacteria in the areas of mRNA processing and genetic code usage. Evidence reviewed below shows that, although there are some similarities between the two systems, the mitochondrial translational apparatus is in many respects unique.

Antibiotics have been important tools in investigations of mitochondrial protein synthesis. Chloramphenicol (CAP), an inhibitor of bacterial protein synthesis (139), was first reported to inhibit amino acid incorporation by isolated mitochondria in 1963 (40). Subsequently, two groups confirmed that CAP was specific for mitochondrial translation; the drug did not affect protein synthesis by isolated microsomes (43,46). By contrast, cycloheximide (Chlx), which had no effect on mitochondrial protein synthesis (46), completely inhibited protein synthesis by isolated microsomes (53,54).

Initial insight into the nature of mitochondrial translation products was provided by Huang et al. (140) who observed that growth of yeast in the presence of CAP inhibited the formation of cytochrome b and cytochrome oxidase. CAP grown cells were respiratory deficient (141), but other mitochondrial enzymatic activities were unaffected (142,143). These results suggested that mitochondrial translation products are probably components of the respiratory chain. Two general approaches have since been used to identify mitochondrial translation products. The first employs in vivo pulse labeling of cells in the presence of Chlx or CAP, followed by gel electrophoresis of isolated mitochondria. The second analyzes mitochondrial translation products synthesized in vitro. These studies have shown that mitochondria synthesize only 5% of all mitochondrial proteins.

In a series of experiments on the biosynthetic origin of F_1ATPase, Tzagoloff et al. (118,144) showed that F_1 and the oligomycin-sensitivity conferring protein (OSCP) are synthesized in the cytoplasm, while four membrane proteins in the ATPase complex are mitochondrial translation products. Glucose derepressing cells were pulse labeled in the presence of Chlx, mitochondria isolated, and immunoprecipitated with antisera to the entire complex. After gel electrophoresis, four radioactive bands were seen,

indicating that these four polypeptides are synthesized on mitochondrial ribosomes. Mason and Schatz (145) employed similar techniques to show that the three largest subunits of cytochrome oxidase, I, II, and III, are synthesized in the mitochondria while subunits IV through VII are made in the cytoplasm. In vivo studies have demonstrated that in N. crassa and S. cerevisiae, cytochrome b is mitochondrially translated (146-148). Similarly, Lambowitz et al. (149) and Groot et al. (150) showed one protein component of the mitochondrial small ribosomal subunit to be of mitochondrial origin. In S. cerevisiae, this protein has been called Var 1 because of the strain dependent variation in its electrophoretic mobility (151).

The second approach to identify mitochondrial translation products, translation in vitro by isolated mitochondria, has confirmed studies in vivo. Isolated mitochondria were shown to synthesize polypeptides similar in mobility on SDS gels to those obtained in vivo (152). Poyton and Groot (153) further refined the in vitro system and demonstrated with specific antisera that isolated mitochondria can synthesize subunits I, II, and III of cytochrome oxidase. As discussed earlier, although we now know the identity of the major mitochondrial translation products, the discovery of numerous unidentified reading frames on the yeast

mitochondrial genome make it probable that additional polypeptides, possibly regulatory factors, are synthesized in small amounts by mitochondria.

The presence of ribosomes in mitochondria was first demonstrated by electron microscopy (154). Subsequently, mitochondrial ribosomes were isolated from rat liver (155), cauliflower (156), HeLa cells (157), N. crassa (158), and S. cerevisiae (159). Mitochondrial ribosomes comprise an extremely diverse class in terms of sedimentation velocity and protein-rRNA composition, with most species lacking a 5 S RNA (see 160 for review). Animal mitochondrial ribosomes have sedimentation velocities of 55-60 S, a result of their extremely low (30%) RNA content (160), while yeast mt contain 70-80 S ribosomes (159).

As is the case with E. coli ribosomes, peptidyltransferase activity of Neurospora, yeast, and rat liver mt ribosomes is inhibited by CAP (161-163). Yeast mitochondrial ribosomes will function with supernatant enzymes (initiation and elongation factors) from E. coli (164,165). However, in general, supernatant factors from the cytoplasm of eucaryotic cells will not function with mt ribosomes (165-167). As mentioned earlier, many human mt mRNA's start directly with initiation codons. It will be interesting to learn how the tertiary structure of mRNA enables it to bind mt ribosomes

without the usual 5' nontranslated leader sequence.

Two polypeptide elongation factors have been isolated from yeast mitochondria, G_{mit} and T_{mit}, which are analogous to peptidyltranslocase and aminoacyl-tRNA binding factors (168). G_{mit} and T_{mit} factors will work with bacterial but not cytosolic ribosomes in a poly-rU system. Antisera can discriminate between the cytosolic and mitochondrial elongation factors. Structural similarities between mt and bacterial G factors are indicated by the inability of diphtheria toxin to ADP-ribosylate either factor (168). However, fusidic acid, which inactivates both bacterial and cytoplasmic G-factors, does not affect the G_{mit} of Neurospora (169.

In 1967, Barnett and Brown (170) reported the first isolation of mitochondrial transfer ribonucleic acids. Several lines of evidence have shown mt tRNA's to be distinct from their cytosolic counterparts. Mitochondrial tRNA will hybridize to mtDNA but neither cytosolic tRNA nor procaryotic tRNA will do so (171). Most mitochondrial tRNA's can also be separated from cytosolic tRNA's by reversed phase column chromatography (172). Recently, DNA sequencing (112) has revealed that most human mt tRNA's lack some features found in all other nonmitochondrial tRNA's, including: (1) the constant seven base length "$T_{\psi}C$" loop; (2) the constant bases A_{14}, G_{15}, G_{18}, and G_{19}. Mitochondria from all species

contain two types of methionyl tRNA, one of which, $tRNA^{fmet}$, is used in the initiation of protein synthesis as in procaryotes (173-177). The $tRNA^{fmet}$, along with the enzyme which formylates the methionine, transformylase, have only been detected in the mitochondria and not in the cytosol (171,177). Mahler et al. (178) utilized the knowledge that yeast mitochondria are deficient in deformylase activity (178,179), to selectively label mitochondrially synthesized proteins with radioactive formate. They showed that initiation of mitochondrial protein synthesis, as measured by f-met-puromycin formation, was much higher in lactate grown cells in comparison with glucose repressed cells, in agreement with the decrease in respiratory chain activity caused by glucose repression.

In bacteria, the formylation of $tRNA^{fmet}$ requires N^{10}-formyltetrahydrofolate as the formyl group donor. Stone and Wilkie (180,181) found that pyrimethamine, an inhibitor of dihydrofolate reductase, also inhibited yeast mitochondrial protein synthesis, indicating that mitochondria require reduced folate pools for the formylation of $tRNA^{fmet}$. It is not known whether the mitochondrial formyl group donor is the same as the bacterial.

An important role for yeast mitochondria in folate coenzyme mediated transfer of one carbon groups and serine-

glycine metabolism has been suggested by the localization of serine transhydroxymethylase, dihydrofolate reductase, N^5,N^{10}-methylene tetrahydrofolate dehydrogenase, N^{10}-formyltetrahydrofolate synthetase, and thymidylate synthetase in mitochondria (182). A yeast mutant was isolated which was auxotrophic for thymidylate, methionine, adenine, and histidine; the mutant was found deficient in only the mitochondrial forms of serine transhydroxymethylase, dihydrofolate reductase, and thymidylate synthetase (182). The anxotrophic requirements could be replaced by added folic acid suggesting that mitochondrial folate coenzymes are involved in yeast folate biosynthesis. The relation between mitochondrial folate pools and regulation of initiation of mt protein synthesis clearly needs much more exploration.

Mitochondrial aminoacyl tRNA synthetases like mt tRNA's differ from their cytosolic counterparts (183-185). Aminoacyl synthetases isolated from mitochondria neither chromatograph identically with cytosolic enzymes nor do they show the same acylation specificities towards different tRNA's (183). Furthermore, antisera raised against the cytosolic form of N. crassa leucyl tRNA synthetase did not cross react with the mitochondrial enzyme, and antisera against the mitochondrial enzyme did not inhibit the cytosolic activity, indicating that the two enzymes are structurally distinct (184).

Interestingly, the genes for both forms of the enzyme are closely linked on the nuclear genome (184), suggesting coordinated regulation.

For years many groups have unsuccessfully attempted to study mitochondrial translation using mt mRNA in heterologous translation systems (186). Only recently, with the help of DNA sequencing data, has it become clear why so many groups failed. The UGA codon is decoded in mitochondria by tryptophan tRNA; however, in all other systems this codon leads to termination of translation. Recently, DeRonde et al. (187) have synthesized the complete polypeptide of subunit II of cytochrome oxidase in a wheat germ extract supplemented with mt mRNA and a UGA-suppressor tRNA. This polypeptide is apparently made as a precursor (188).

Ideally, insight into the mechanisms regulating mitochondrial translation should be gained through studying submitochondrial translational systems which are reconstructed using homologous components. Unfortunately, this approach has so far met with little success. Although Pfisterer and Buetow (189) have recently reported development of a homologous submitochondrial system which synthesizes complete polypeptides, calculations (190) reveal the rate of protein synthesis in their system to be only 0.3 pmol methionine incorp./incubation/mg protein in comparison with

200 or more pmole leucine incorp./incubation/mg protein by isolated, intact mitochondria (191). Clearly, further improvements in the submitochondrial system need to be made before it can be used successfully to study mitochondrial translation.

H. Regulation of Mitochondrial Transcription and Translation

Since most mitochondrial translation products are subunits of respiratory chain complexes which also contain subunits synthesized on cytoplasmic ribosomes, it is clear that mechanism(s) must exist to control the synthesis of proteins at the two intracellular sites such that a functional mitochondrial inner membrane is formed in an orderly manner. Kim and Beattie (192) studied the kinetics of formation of different enzyme complexes of the inner membrane in yeast undergoing glucose derepression. Their results suggested that the various enzyme complexes are assembled into the inner membrane in an asynchronous process. Furthermore, addition of inhibitors of protein synthesis (CAP, Chlx) during derepression revealed that mitochondrial proteins synthesized both in the cytoplasm and in the mitochondria accumulate to a limited and varied extent for each enzyme complex, prior to their integration into functional units in the membrane. Brown and Beattie (193), using similar techniques to examine the induction of cytochrome oxidase

and cytochrome $\underline{b}$-$\underline{c}_1$ complex activities during aerobic adaptation, found that pools of cytoplasmically made proteins accumulate for cytochrome oxidase but not for the cytochrome $\underline{b}$-$\underline{c}_1$ complex when mt protein synthesis is blocked. These results indicated that formation of an active cytochrome $\underline{b}$-$\underline{c}_1$ complex during aerobic adaptation is subject to a different mode of regulation from that observed during glucose derepression (194). Interpretation of these studies is complicated by the difficulty in distinguishing regulation at the level of either assembly or protein synthesis.

The first indication that cytoplasmically synthesized proteins may control mitochondrial protein synthesis came in 1971 (195). Tzagoloff found that addition of either CAP or Chlx to glucose derepressing yeast cells prevented an increase in oligomycin-sensitive ATPase activity. However, a sequential exposure of derepressing cells to CAP followed by Chlx led to a three-fold increase in ATPase activity. Reversing the order of addition of inhibitors led to a much smaller increase in activity. In addition, preincubation of cells in CAP followed by Chlx labeling led to a three-fold increase in net ^{3}H leucine incorporation by mitochondria. Furthermore, the longer the exposure to CAP, the greater the increase in mt protein synthesis. Tzagoloff suggested that cytoplasmic products exert positive control on mitochondrial protein synthesis and that this control is proportional to the accumulation of certain cytoplasmic products. Ibrahim

et al. (196) then tested if preincubation with CAP in vivo could stimulate mitochondrial protein synthesis in vitro. Mitochondria isolated from cells preincubated with CAP incorporated 100% more labeled amino acid into protein than did controls, while Chlx preincubation led to a 50% decrease in mitochondrial incorporation. Studies on mitochondrial polysomes by Ibrahim and Beattie (197) suggested that cytoplasmic proteins may control mitochondrial protein synthesis either by stimulating chain initiation or by causing increased formation of mt messenger RNA's. They found a 2-fold increase in the mitochondrial polysome to monosome ratio when the cells were preincubated in chloramphenicol. Furthermore, the rate of polypeptide chain initiation as judged by formation of f-met-puromycin was also increased both in vivo and in vitro by CAP preincubation. Conversely, both the polysome to monosome ratio and polypeptide chain initiation were decreased 50% by preincubation of cells with Chlx. The rate of amino acid incorporation by isolated mitochondrial polysomes in an elongation system was also increased three-fold by CAP preincubation of cells, indicating that there are indeed a significantly greater number of nascent chains on these polysomes.

Poyton and Kavanagh (198) tested cytoplasmic control of yeast mitochondrial protein synthesis directly, in an

in vitro system. They reported that isolated mitochondria are capable of synthesizing proteins for 30 min, after which time synthesis ceases. Addition of a dialyzed post-polysomal supernatant to mitochondria which had stopped protein synthesis stimulated amino acid incorporation and the stimulation was proportional to the amount of supernatant protein added. They claimed that titration of the S-100 supernatant with antisera to holo-cytochrome oxidase resulted in a loss of the S-100's ability to stimulate synthesis of cytochrome oxidase subunits I, II, and III. A 55,000 dalton precursor protein to cytochrome oxidase subunits IV through VII was reported to exert a specific stimulatory effect on the synthesis of the mitochondrially made subunits of cytochrome oxidase (198-200). However, the attractive hypothesis suggesting control of mt synthesis of subunits I to III of cytochrome oxidase by the cytoplasmically synthesized partner subunits, does not appear to be correct. Lewin *et al.* (201) and Mihara *et al.* (202) have rigorously shown that subunits IV through VII are made as four separate precursor proteins, indicating that the polyprotein precursor probably does not exist. Furthermore, Lewin *et al.* have been unable to confirm the stimulatory properties of the cytoplasmically synthesized cytochrome oxidase subunits.

Genetic studies have provided further evidence for cytoplasmic control of mitochondrial protein synthesis. Ebner et al. (203) isolated a yeast nuclear mutant (pet 494-1) which lacked subunit III of cytochrome oxidase, yet contained normal levels of the cytoplasmically synthesized subunits. Nuclear amber suppressors which affect translation on cytoplasmic ribosomes suppressed the mutant phenotype indicating that the mutated gene locus coded for a cytoplasmically synthesized protein (204). The pattern of cytochrome oxidase subunits in a heme-less mutant (205) was different from that observed with pet 494-1; this argues against the possibility that pet 494-1 carries a lesion in heme a synthesis. Apparently, the mutation is in a cytoplasmic regulatory protein which is necessary for mitochondrial synthesis of subunit III of cytochrome oxidase. Similarly, other nuclear mutants have been isolated that prevent the synthesis of cytochrome b or of subunit 9 of the ATPase (206). Recently, a nuclear mutant which specifically abolishes production of mature cytochrome b mRNA, has been isolated, providing a direct demonstration of cytoplasmic control of mitochondrial gene expression (207).

There are many ways by which cytoplasmically synthesized proteins could regulate mt protein synthesis. Since almost

all the proteins of the mt transcription-translation system are made on cytosolic ribosomes, any one of these proteins could control mt protein synthesis. Prime candidates for a general control mechanism would be RNA polymerase and initiation-elongation factors. On the other hand, as seen above, special regulatory proteins may be involved in the synthesis of either all mt proteins or only specific ones. Alternatively, low molecular weight peptides may be involved. These proteins and/or peptides could work at the level of transcription or translation. It appears probable that multiple regulatory pathways exist.

Feldman et al. (208) showed that 20 min after the shut-off of cytosolic protein synthesis in a temperature-sensitive strain deficient in initiation at non-permissive temperatures, amino acid incorporation into mitochondria ceased. However, incorporation of labeled formate into f-met puromycin was not inhibited at the non-permissive temperature, indicating that cytosolic protein synthesis is required for some step beyond mitochondrial initiation. Given the generation time of yeast, 1½ to 2 hrs, it appears unlikely that the quick shut-off of mt protein synthesis could be due to a lack of RNA polymerase or other factors normally used in transcription-translation. More plausible explanations include: (1) a specific regulatory factor must be synthesized in the cytosol

for mt protein synthesis to continue; (2) when cytoplasmic protein synthesis is shut off, a regulatory factor is produced which specifically inhibits mt protein synthesis. The bulk of this thesis represents work which supports the first hypothesis. The second hypothesis may also have some validity and should be amenable to direct in vitro testing.

Ray and Butow (209) examined the regulation of yeast mt rRNA synthesis using temperature sensitive mutants which are defective at the non-permissive temperature in isoleucine tRNA charging, polypeptide chain elongation, and initiation of protein synthesis. Cytosolic protein synthesis was inhibited by using Chlx or incubating the mutants at the non-permissive temperature. They found a striking correlation between the fraction of cytoplasmic ribosomes "stalled" in polysomes and the extent to which mt rRNA synthesis is inhibited. The greater the fraction of ribosomes in polysomes, the greater the inhibition of mt rRNA synthesis. The mode of inhibition of cytosolic protein synthesis was important, suggesting that specific regulatory signals, not just a lack of cytosolic proteins are required for inhibition of mt rRNA synthesis. Similarly, LaPolla and Lambowitz (210) have found in N. crassa a tight coupling between cytosolic protein synthesis and mt rRNA synthesis. The nature of those regulatory signals is unknown.

Because S 5 in *N. crassa* and Var 1 in *S. cerevisiae* are the only mt ribosomal proteins synthesized intramitochondrially, it is possible that they have special roles in mt protein synthesis. The proportion of mitochondrial protein synthesis devoted to both proteins increases as the rate of mt protein synthesis decreases (150,211,212). Lambowitz (213) has suggested that the intramitochondrial concentration of free S 5 may help coordinate the rates of synthesis of cytosolic and mitochondrial products. Under conditions where the supply of cytosolically-synthesized mt ribosomal proteins became rate-limiting for assembly, free S 5 levels would increase; free S 5 could then decrease mt translation by acting as a repressor of transcription or by inhibiting mRNA translation. Further work in this area will undoubtedly lead to an increase in our understanding of intracellular control mechanisms.

I. Thesis Objectives

An important area in biochemical research today addresses the question of how the growth and development of subcellular organelles is coordinated with overall cell function. Since mitochondrial proteins are synthesized on ribosomes located both inside and outside of the mitochondria, and many of these proteins are assembled into oligomeric respiratory chain complexes, mechanisms must exist to

control the synthesis of proteins at the two sites such that a functional mitochondrial inner membrane is formed. When this thesis work was begun much evidence had already accumulated to suggest that cytoplasmically synthesized proteins may control mitochondrial protein synthesis. In addition, it had recently been reported that a yeast post-polysomal supernatant could restore protein synthetic activity to isolated yeast mitochondria which had stopped synthesis (198). The authors claimed that a polyprotein precursor to subunits IV through VII of cytochrome oxidase exerted a specific stimulatory affect on the mitochondrial synthesis of subunits I to III. In light of these findings, it seemed important to investigate the following:

1. What was the stimulatory activity of the post-polysomal supernatant due to? Could the stimulatory factors be purified and characterized by using a well-defined *in vitro* assay for mitochondrial protein synthesis?

2. Were the cytoplasmically synthesized subunits of respiratory chain complexes involved in the stimulation or did other factors play a role?

3. Could results from *in vitro* experiments be correlated directly with our knowledge of *in vivo* physiological regulation of mitochondrial protein synthesis?

4. How general a phenomenon was the *in vitro*

stimulation? Could it be demonstrated in higher eucaryotes?

Some of the answers to these questions are presented in the following chapters.

J. List of Publications

Portions of the following thesis have been published or submitted for publication:

Abstracts

"Stimulation of Yeast Mitochondrial Protein Synthesis by Postpolysomal Supernatants from Yeast, Rat Liver and E. coli." Everett, T. D., Finzi, E., and Beattie, D. S. XIth International Congress of Biochemistry. (1979)

"Partial Purification of Cytosolic Proteins Essential for Optimal Yeast Mitochondrial Protein Synthesis." Finzi, E. (Intr. by Beattie, D. S.). Biophysical Society Annual Meeting. (1981)

"Isolation of Cytosolic Proteins Which Control Yeast Mitochondrial Protein Synthesis." Finzi, E., Sperling, M., and Beattie, D. S. Cold Spring Harbor Meeting: Mitochondrial Genes. (1981)

"Isolation of Cytosolic Factors Which Control Yeast Mitochondrial Protein Synthesis." Finzi, E., Sperling, M., and Beattie, D. S. EMBO Workshop: Regulatory Mechanisms in Mitochondrial Gene Expression. (1982)

Publications

"Stimulation of Yeast Mitochondrial Protein Synthesis by Postpolysomal Supernatants from Yeast, Rat Liver, and Escherichia coli." Everett, T. D., Finzi, E., and Beattie, D. S. Arch. Biochem. Biophys. (1980)200,467-473.

"Properties of a Partially Purified Fraction Containing Cytosolic Protein(s) Necessary for Yeast Mitochondrial Protein Synthesis." Beattie, D. S. and Finzi, E. in Current Developments in Yeast Research. G. G. Stewart and I. Russell, eds., Pergamon Press (1981), pp. 351-355.

"Partial Purification of Postpolysomal Factors Essential for Optimal Rates of Yeast Mitochondrial Protein Synthesis." Finzi, E. and Beattie, D. S. in The Organization and Expression of the Mitochondrial Genome. A. M. Kroon and C. Saccone, eds., Elsevier/North Holland, Amsterdam (1980), pp. 315-318.

"Partial Purification of Cytosolic Proteins Which Control Yeast Mitochondrial Protein Synthesis." Finzi, E., Sperling, M., and Beattie, D. S. J. Biol. Chem. (1981)256, 11917-11922.

"Control of Yeast and Mammalian Mitochondrial Protein Synthesis by Cytoplasmic Factors." Finzi, E., Rinehart, R. W., Sperling, M., and Beattie, D. S. (1982) FEBS Letters, 137, 314-318.

"Effect of Temperature on Protein Synthesis and Leucine Transport by Yeast Mitochondria." Finzi, E., Clejan, L., and Beattie, D. S. Biochemistry, submitted for publication.

II. Stimulation of Yeast Mitochondrial Protein Synthesis by Postpolysomal Supernatants from Yeast, Rat Liver, and E. coli

A. Introduction

It is now firmly established that mitochondria contain DNA and all necessary components for its transcription and translation. The physical and chemical characterization of many components of the mitochondrial protein-synthesizing system have been reported by numerous investigators for a variety of organisms (48,61,214). Formation of the mitochondrial respiratory chain requires the coordinated synthesis of proteins in both the mitochondria and cytoplasm. Six to eight hydrophobic proteins of the mitochondrial inner membrane are mitochondrial translation products. Three proteins are associated with cytochrome aa_3 (145, 118,215), two to four with oligomycin-sensitive ATPase (118,144,216,217), and one, cytochrome b, with complex III of the respiratory chain (146-148,215,218). The remaining proteins of these enzyme complexes are synthesized by the cytoplasmic system. Previous studies have suggested that cytoplasmic proteins may control mitochondrial protein synthesis either by stimulating chain initiation or by causing increased formation of specific mitochondrial mRNA's (194-198).

Poyton and Kavanagh (198) have reported that mitochondrial protein synthesis *in vitro* ceases when an endogenous pool of cytoplasmically synthesized proteins is depleted. Protein synthetic activity was restored to depleted yeast mitochondria by addition of an S-100 fraction from yeast to the incubation medium. In the current study we have demonstrated that this effect is not species specific and that S-100 fractions obtained from either rat liver or *E. coli* could restore the protein synthetic activity of depleted yeast mitochondria as well or better than the S-100 from yeast. Furthermore, the addition of an S-100 fraction to isolated yeast mitochondria at time zero also stimulated protein synthesis but to a greater extent than when the S-100 fraction was added to depleted mitochondria.

B. Materials and Methods

Pyruvate kinase, puromycin dihydrochloride, cycloheximide, chloramphenicol, and ribonuclease A (bovine pancreas) were obtained from Sigma; trypsin and trypsin inhibitor were from Boehringer Mannheim. Labeled /4,5-^{3}H/ leucine (51.6 Ci/mmol) was obtained from New England Nuclear. Sprague-Dawley rats were obtained from Perfection Breeders.

1. Growth of Yeast and Preparation of Mitochondria

A diploid strain of *S. cerevisiae* used in this

laboratory for several years was grown in the medium previously described (198). All procedures involved sterile solutions and strictly aseptic technique. Mitochondria were isolated from midexponential phase cells, washed once in sterile distilled water, and then once in an MTE (0.6 M mannitol, 0.01 M Tris, pH 7.6, and 0.001 M Na_2 EDTA) buffer. Cells were then suspended in the MTE buffer and shaken for 20 s at 4,000 rpm with glass beads in a Bronwill tissue homogenizer. After cell debris was removed by centrifugation (3 X) for 10 min at 600 x g, the supernatant was centrifuged for 10 min at 12,000 x g to pellet mitochondria. The mitochondrial pellet was washed two times in the MTE buffer, recentrifuged at 12,000 x g, and then suspended in this buffer at a concentration equivalent to 10 mg of protein per ml. All isolation procedures were carried out at 4^{o}. Mitochondria prepared by this method had the same protein synthetic capacity as mitochondria prepared using the glusulase procedure.

2. Protein Synthesis In Vitro

Isolated yeast mitochondria (1.0 mg of protein per ml) were incubated in a protein-synthesizing mixture (198) with the following modifications: 0.003 umol/ml of L-leucine was used and bovine serum albumin was deleted from the mixture. One ml of the protein-synthesizing mixture was

added to a 25 ml Erlenmeyer flask and aerated by gentle shaking at 30°. Protein synthesis was determined as the incorporation of L-[4,5-^{3}H] leucine (0.08 mCi/ml;51.6 Ci/umol) into trichloroacetic acid precipitable protein (219). The filters containing the precipitated protein were counted in a Beckman LS-9000 scintillation counter using Econofluor as the scintillant.

Bacterial contamination of the incubation flasks was determined at the end of each experiment by plating 0.1 ml of incubation medium on blood agar plates. The plates were incubated at 30° for 72 hours and then the colonies were counted. The bacterial contamination varied from 70 bacteria/ml to 1,400 bacteria/ml. Bacterial contamination of this level does not affect the results of mitochondrial protein synthesis *in vitro* (45,220).

3. Preparation of S-100 Fractions

E. coli S-100 fractions were prepared from strain K-12 cells grown on 2% glucose, 2% bactopeptone, 2% yeast extract, and 0.01% $MgSO_4$ in sterile tap water at 30°. The cells were harvested in early log phase and washed once in sterile distilled water and once in 10 mM potassium phosphate buffer, pH 7.0. Cells were then suspended in the 10 mM phosphate buffer containing 0.18 mg of phenylmethylsulfonyl fluoride (PMSF) per ml and shaken 3 X for 30 s at

4,000 rpm in a Bronwill tissue homogenizer with glass beads. The cell homogenate was cleared of cell debris by centrifuging for 10 min at 10,000 x g. The resulting supernatant fraction was centrifuged for 120 min at 105,000 x g to give an S-100 fraction. The upper 3/4 of this fraction was removed from the centrifuge tube with a sterile Pasteur pipette and then dialyzed for 15 h against 10 mM phosphate, pH 7.0.

S. cerevisiae S-100 fractions were prepared from post-mitochondrial supernatants of mid-log phase cells which were processed as described previously, except that the MTE buffer contained 0.18 mg/ml of PMSF. The post-mitochondrial supernatant was centrifuged for 60 min at 105,000 x g to give an S-100 fraction. The upper 3/4 of the S-100 fraction was then dialyzed against 10 mM phosphate, pH 7.0, for 15 h.

To prepare rat liver S-100 fractions Sprague-Dawley rats were decapitated and approximately 50% of the liver removed and placed in STE-PMSF (0.25 M Sucrose, 0.01 M Tris, pH 7.0, 0.001 M Na_2 EDTA, and 0.18 mg PMSF/ml) buffer. The liver tissue was homogenized with a Potter-Elvehjem glass tissue grinder with a Teflon pestle in the STE-PMSF buffer. The homogenate was then centrifuged for 10 min at 600 x g. The resulting supernatant fraction was

centrifuged for 10 min at 10,000 x g to prepare a post-mitochondrial supernatant fraction. This fraction was then centrifuged for 60 min at 105,000 x g. The upper 3/4 of this S-100 fraction was the dialyzed against 10 mM phosphate for 15 h.

Where indicated, the S-100 fractions were treated with ribonuclease A (60 ug/ml) for 45 min at 30° or treated with trypsin (100 ug/ml) for 45 min at 30° and then with lima bean trypsin inhibitor (50 ug/ml) for 10 min at 30° The control (i.e., untreated) S-100 fractions were also incubated for 45 min at 30°. To obtain a control for the trypsin treatment, trypsin was incubated with trypsin inhibitor for 5 min at 30° and then added to the control S-100 fraction before use.

Unless otherwise indicated all steps in the preparation of S-100 fractions were performed at 4°.

4. Electrophoretic Analysis of Mitochondrial Translation Products

Isolated mitochondria (1 mg of protein/ml) were incubated in a protein-synthesizing mixture (198), with the modification that L-methionine was omitted. After a 40 min incubation, S-100 fraction was added to the depleted mitochondria followed by the addition of L-[^{35}S]methionine.

After 20 min, 5 mM unlabeled L-methionine was added and the incubation continued for 15 min. The mixture was then chilled on ice. The incubation mixture was then centrifuged at 12,000 x g for 10 min to pellet mitochondria. The mitochondrial pellet was washed two times in MTE buffer containing 5 mM unlabeled methionine. The pellet was dissolved in dissociating buffer and analyzed by SDS 10% polyacrylamide gel electrophoresis followed by autoradiography (221).

To determine the translational products from mitochondria stimulated by the different S-100 fractions at time zero, an S-100 fraction was added to the protein synthesizing mixture containing L-[^{35}S]methionine. The reaction was begun by the addition of the mitochondria. The mitochondria were incubated for 30 min, then 5 mM unlabeled L-methionine was added, and the incubation continued for an additional 15 min. The mitochondria were then pelleted by centrifugation, and the sample was processed as described above.

C. Results

1. Stimulation of Isolated Mitochondria by Post-polysomal Supernatants Is Not Species Specific

Isolated yeast mitochondria are capable of synthesizing proteins *in vitro* for 30 min, after which time the synthesis ceases. Poyton and Kavanagh (198) previously

reported that the addition of an S-100 fraction from yeast to depleted mitochondria which had stopped protein synthesis fully restored their ability to incorporate amino acids into protein. As indicated in Fig. 2, the stimulatory activity of the S-100 fraction used is not species specific. The addition of an S-100 fraction from either yeast, rat liver, or E. coli all restored the ability of depleted yeast mitochondria to synthesize polypeptides. The data also indicates that the degree of stimulation observed was dependent upon the amount of the S-100 fraction added to depleted mitochondria. In addition, the S-100 fraction by itself did not incorporate amino acids into protein.

To show that the increased rate of amino acid incorporation into protein after addition of the S-100 did indeed represent mitochondrial protein synthesis, choramphenicol (0.2 mg/ml), the specific inhibitor of mitochondrial protein synthesis (40,43,46,140) was added before the S-100 fraction. Under these conditions the stimulatory activity was completely abolished. Similarly, the addition of puromycin (0.2 mg/ml) also blocked the stimulation by the S-100 fraction. The addition of either 0.3 or 0.9 mg of bovine serum albumin to depleted mitochondria failed to restore protein-synthesizing activity, indicating that the stimulation by the S-100 fractions was not just a nonspecific

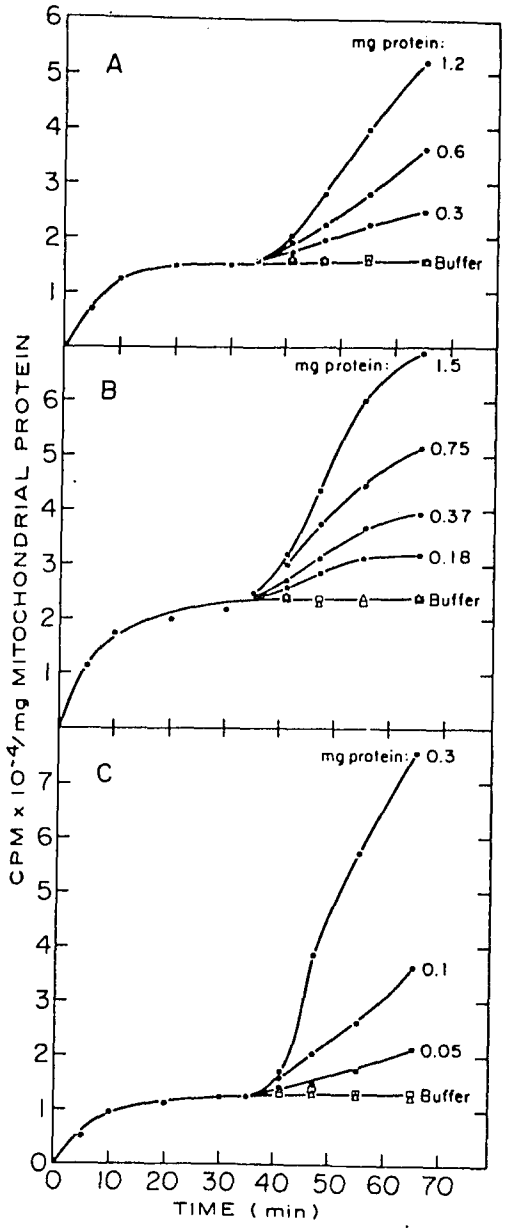

Figure 2

Stimulation of yeast mitochondrial protein synthesis by S-100 supernatants from either yeast (A), rat liver (B), or E. coli (C). Isolated yeast mitochondria were incubated in a protein-synthesizing mixture for 35 min, whereupon a one-tenth volume of dialyzed S-100 supernatant (●) containing the amount of protein indicated or 10 mM phosphate buffer (□) was added. Before the addition of either 1.2 mg of yeast S-100 (A), 1.5 mg of rat liver S-100 (B), or 0.3 mg of E. coli S-100 (C), 0.2 mg/ml of chloramphenicol was added (△) to the protein synthesizing mixture. Aliquots containing 0.1 mg of mitochondrial protein were taken at the times indicated and the amount of newly synthesized protein was determined as described in Section IIB.

effect due to protein.

During these experiments the initial rate of protein synthesis and the degree of stimulation by the S-100 fraction were noted to vary for each mitochondrial preparation. Hence, a comparison of the stimulatory activity of the three different S-100 fractions was made using mitochondria from the same preparation. A one-tenth volume containing 0.5 mg of S-100 protein was added to an incubation mixture containing 1 mg of depleted yeast mitochondria which had stopped protein synthesis. This concentration of yeast and rat liver S-100 fraction stimulated 2-fold the initial activity, while the E. coli S-100 fraction stimulated 6-fold.

The three different S-100 fractions were also added to the incubation mixture at time zero. As shown in Fig. 3, the capacity of the S-100 fractions to stimulate mitochondrial protein synthesis at the beginning of the incubation period is increased greatly over the stimulatory capacity when added to depleted mitochondria. The addition of 1 mg protein of yeast S-100 fraction to isolated yeast mitochondria at time zero stimulated protein synthesis 8-fold, compared to the 2-fold stimulation when the S-100 fraction was added to depleted mitochondria. Similarly, the addition at time zero of 1 mg protein of a rat liver S-100 fraction stimulated mitochondrial protein synthesis 6-fold, while 0.5 mg protein

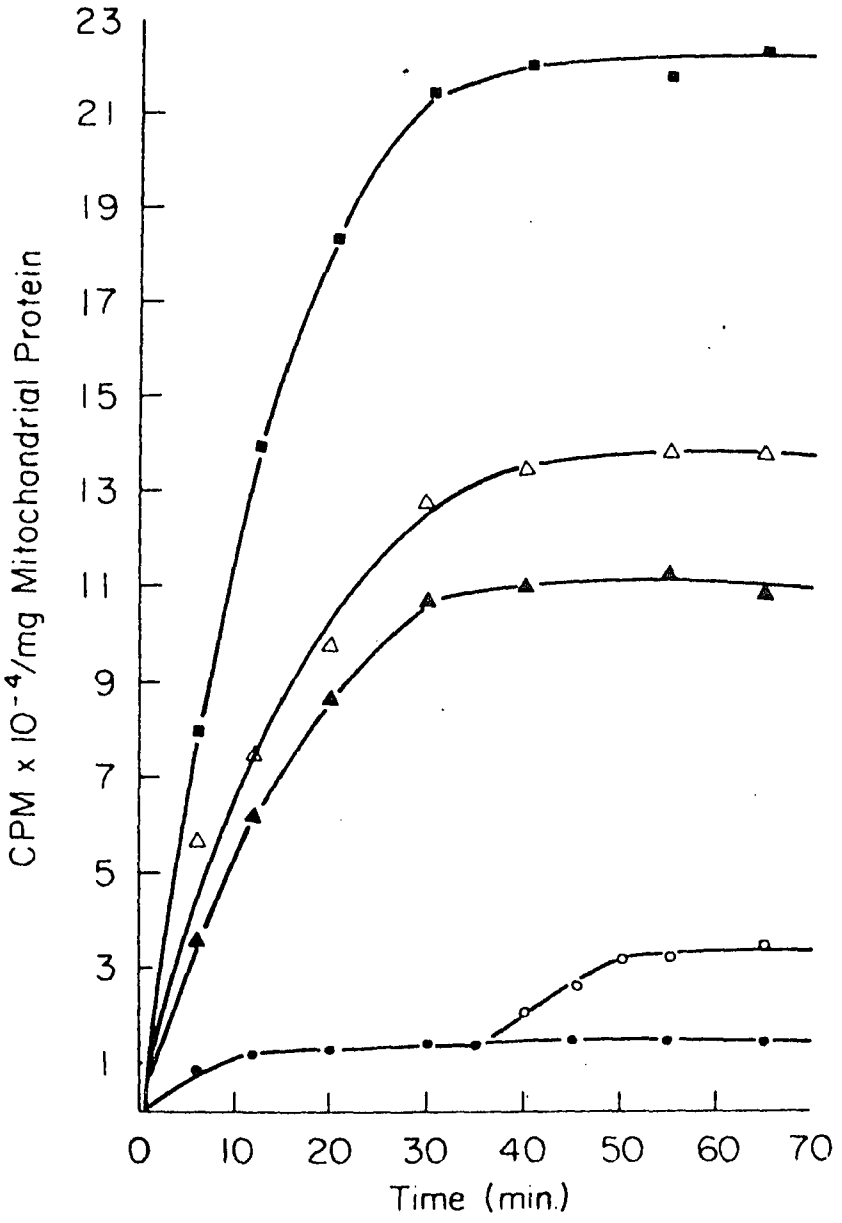

Figure 3

Stimulation of yeast mitochondrial protein synthesis by S-100 supernatants at time zero. Isolated yeast mitochondria were incubated in a protein-synthesizing mixture containing a one-tenth volume of dialyzed S-100 supernatant, (■) 0.5 mg protein from E. coli, (△) 1.0 mg protein from yeast, (▲) 1.0 mg protein from rat liver, (●) 10 mM phosphate buffer. The (0-0) indicate the addition of 1.0 mg protein of yeast S-100 supernatant to depleted mitochondria. Aliquots containing 0.1 mg of mitochondrial protein were taken at the times indicated and the amount of newly synthesized protein was determined as described in Section IIB.

of an E. coli S-100 fraction stimulated mitochondrial protein synthesis 12-fold.

2. Electrophoretic Analysis of Mitochondrial Translation Products

The Mans and Novelli procedure (219) used in these experiments for determination of protein synthesis measures trichloracetic acid precipitable radioactivity which can result from short polypeptides containing only 3 to 5 amino acid residues. Hence, the observed stimulation of mitochondrial protein synthesis might represent an increased rate of initiation but not the synthesis of complete proteins. To evaluate this possibility, isolated mitochondria were labeled with [^{35}S]methionine in the presence of either yeast, rat liver, or E. coli S-100 fractions. The translation products were individually displayed by SDS-polyacrylamide gel electrophoresis followed by autoradiography. As shown in Fig. 4, the gel patterns of the isolated mitochondria, either in the presence or absence of the different S-100 fractions, are very similar to the gel pattern of in vivo mitochondrial translation products. Synthesis of the bands corresponding to subunit III of cytochrome oxidase, cytochrome b, and Var 3 (221) appear to be stimulated to the greatest extent (Fig. 4). These results indicate that the yeast, rat liver, and E. coli

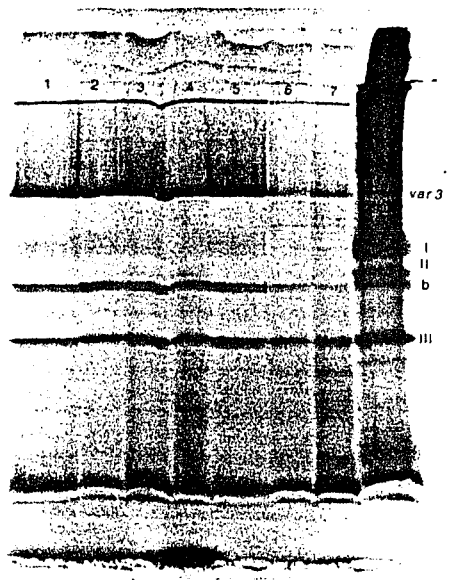

Figure 4

Electrophoretic analysis of mitochondrial translation products. Mitochondria were labeled exactly as described in Section IIB. The L-[^{35}S]methionine was 200 uCi/ml. Lanes 6 and 7 contain 50,000 cpm; all others contain 100,000 cpm. Lanes 1, 2, 3, and 4 contain the products after the zero time addition of yeast, rat liver, E. coli, and 10 mM phosphate buffer pH 7.0, respectively. Lanes 5, 6, and 7 contain the products after the addition of yeast, rat liver, and E. coli to depleted isolated mitochondria. Lane 8 contains the in vivo mitochondrial translation products. The bands corresponding to Var 3, subunits I, II, and III of cytochrome oxidase, and cytochrome b are indicated on the Figure.

S-100 fractions stimulate mitochondria to synthesize complete polypeptides of high molecular weights which correspond to those known *in vivo* mitochondrial translation products.

3. Properties of the Stimulatory Factor(s)

In order to learn more about the composition of the stimulatory factors, the S-100 fractions were treated as follows prior to addition to the incubation mixture; dialysis against 10 mM phosphate, incubation with ribonuclease A or digestion with trypsin. As seen from the data in Table I, the stimulatory activity of both the rat liver and *E. coli* S-100 fractions is nondialyzable, ribonuclease A-resistant, and sensitive to trypsin digestion. These properties are consistent with the hypothesis that the stimulatory factors are proteins. Similar results were previously reported by Poyton and Kavanagh (198) for an S-100 fraction from *S. cerevisiae*.

D. Discussion

The major finding of this paper is that the stimulation of protein synthesis in isolated yeast mitochondria by extramitochondrial proteins is not species specific. The autoradiography data clearly demonstrate that label is incorporated into completed polypeptides indicating that the stimulation of yeast mitochondrial protein synthesis by the

Table I

Properties of the Stimulatory Factors Present in the S-100 Supernatants[a]

Source of S-100 Supernatant	Treatment	Stimulatory Activity (cpm/mg of mitochondrial protein)	% Total Stimulatory Activity
Rat Liver	None	19,280	100
	Dialysis	18,580	96
	RNase	18,320	95
	Trypsin	0	0
E. coli	None	19,580	100
	Dialysis	21,730	111
	RNase	17,460	89
	Trypsin	2,330	12

[a]Mitochondria (1.0 mg/ml) were incubated in 7.5 ml of the protein-synthesizing mixture for 35 min at 30°. The incubating mixture was then partitioned into 1 ml aliquots and 0.1 ml of the indicated S-100 supernatant was added. This mixture was then incubated for an additional 30 min. The stimulatory activity was determined as the difference between those counts present in the mitochondrial protein synthesizing mixture after 65 min of total incubation and those counts present after the initial 35 min of incubation. The *E. coli* S-100 supernatant contained 3 mg of protein/ml and the rat liver S-100 supernatant contained 10 mg of protein/ml. Details of the protein-synthesizing mixture and for the treatments of the S-100 supernatants are given in Section IIB.

yeast, rat liver, and E. coli supernatants reflects a common regulatory function. We have also found that an S-100 fraction prepared from Boston lettuce will also stimulate isolated yeast mitochondrial protein synthesis (data not shown). Hence, cytoplasmically synthesized proteins which stimulate mitochondrial protein synthesis must have retained a similar structure throughout evolution.

Furthermore, the results of this paper clearly show that the supernatants from these three organisms stimulate yeast mitochondrial protein synthesis to an even greater extent when added at the beginning of the incubation period. There are several explanations for this observation. Perhaps, isolated mitochondria lose their integrity during the long incubation or membrane receptors are degraded by protease activity during this time and hence do not achieve maximum rates of protein synthesis. Another suggestion might be that an inhibitor of mitochondrial protein synthesis is produced or activated during the course of the experiment.

Previously, it was suggested that mitochondrial protein synthesis ceases when a pool of cytoplasmically synthesized proteins is depleted (197,198). Our data suggest that freshly isolated mitochondria may also be deficient in proteins necessary for optimal rates of protein synthesis. These protein pools may become limiting if they form

complexes with other protein subunits and then become integrated into the mitochondrial membrane or if they are lost during mitochondrial preparation. Alternatively, these proteins may be degraded after performing a catalytic function. Studies *in vivo* and *in vitro* have shown that synthesis resumes when these protein pools are replenished, indicating the existence of a positive control by cytoplasmically synthesized proteins on mitochondrial protein synthesis. Indeed, we have observed that a dialyzed 100,000 x g supernatant obtained from sonicated yeast mitochondria will also stimulate mitochondrial protein synthesis *in vitro*.

Several categories of interaction may be involved in the regulation of mitochondrial protein synthesis. For example, stimulatory proteins may enter the mitochondria and effect regulation at the level of transcription or translation, or both. Furthermore, the mitochondrial protein-synthesizing system is complicated by the fact that much of its protein-synthesizing machinery is synthesized in the cytoplasm and then transported into the mitochondria. Hence, the transport of the stimulatory proteins may enhance the transport of the components of the mitochondrial protein-synthesizing machinery. Alternatively, the stimulatory proteins may not enter the mitochondria but may act via a membrane receptor which then results in an increase in

transcription, and/or translation, and/or transport.

Yeast mitochondrial ribosomes will function normally when incubated with initiation and elongation factors from E. coli (164,165). Therefore, one might suspect that the initiation and elongation factors in the S-100 fractions are responsible for its stimulatory activity. If this is the case, then one must hypothesize that the initiation and elongation factors of yeast mitochondria are rapidly degraded and that these are the proteins which are depleted in mitochondria when protein synthesis ceases. However, it is unlikely that an S-100 from rat liver provides elongation or initiation factors because rat liver cytoplasm incubated with ribosomes isolated from Neurospora, Euglena, or Xenopus mitochondria fails to have a protein synthetic capacity (166,167,222). Another proposal is that cytoplasmically synthesized polypeptide subunits of certain enzyme complexes containing products of both mitochondrial and cytoplasmic origin interact with the nascent chains present on the mitochondrial polysomes and increase the rate of elongation.

Recently, it has been reported that nuclear gene products regulate the intramitochondrial synthesis of individual subunits of cytochrome oxidase in both N. crassa (223,224) and S. cerevisiae (203,204). These nuclear gene products are not subunits of cytochrome oxidase but regulate either the

transcription or translation of the specific mitochrondrially synthesized protein. By contrast, a general control mechanism may exist in which the synthesis of all of the proteins coded by mitochondrial DNA is stimulated by cytoplasmic protein(s). The stimulatory proteins in the S-100 fractions may possibly be these nuclear gene products which regulate the synthesis of mitochondrial proteins.

III. Partial Purification of Cytosolic Factors Which Control Yeast Mitochondrial Protein Synthesis

A. Introduction

It is now well established that mitochondrial biogenesis requires input from two distinct genetic systems, one mitochondrial and one cytoplasmic, as well as two separate systems for protein synthesis (48,61,85,214). Most mitochondrial translation products are subunits of respiratory chain complexes which also contain subunits synthesized on cytoplasmic ribosomes. Enzyme complexes requiring dual synthesis include cytochrome c oxidase, the cytochrome b-c_1 complex and the oligomycin sensitive ATPase (see 85 and 186 for review). It is generally accepted that a mechanism must exist to control the synthesis of protein at the two intracellular sites such that a functional mitochondrial inner membrane is formed in an orderly manner.

Previous studies with yeast undergoing glucose derepression have suggested that cytoplasmic proteins may control mitochondrial protein synthesis both *in vivo* (194-197) and *in vitro* (196), and that this control may occur by either stimulation of chain initiation or increased formation of specific mitochondrial mRNA's (197). Furthermore, nuclear mutants which regulate the intramitochondrial synthesis of specific subunits of cytochrome oxidase have been described

(203,204,225). The nuclear gene products of these mutants do not appear to be subunits of cytochrome oxidase but are probably regulatory proteins.

Several years ago, Poyton and Kavanagh (198) reported that protein synthesis by isolated yeast mitochondria ceases when an endogenous pool of cytoplasmically synthesized proteins is depleted, and that an 100,000 x g supernatant from yeast can restore protein synthesis to depleted mitochondria. Recently, in this laboratory, we demonstrated that this effect is not species specific as similar fractions from either rat liver or E. coli were equally effective in restoring mitochondrial protein synthesis (226). Furthermore, the addition of the cytosolic fractions to isolated yeast mitochondria at time zero stimulated protein synthesis 6-8 fold. These results suggested that the coordination of the mitochondrial and nuclear genetic systems could be studied by purifying and characterizing the stimulatory proteins using a well-defined in vitro assay for mitochondrial protein synthesis.

In a recent report, Ohashi and Schatz (227) have suggested that the stimulation of yeast mitochondrial protein synthesis by supernatants is due to the presence of non-dialyzable GMP which is converted to GDP (or GTP) during incubation in the protein synthesizing medium. The results

of the current study confirm the observation that GTP stimulates mitochondrial protein synthesis several fold, but demonstrate that the protein synthetic rate is stimulated to an even greater extent by addition of high-speed supernatants from the cytosol. Furthermore, a low molecular weight protein fraction which stimulates mitochondrial protein synthesis independently of GTP has been partially purified. These results suggest that low molecular weight factors present in the cytosol regulate mitochondrial protein synthesis.

B. Materials and Methods

1. Materials

Pyruvate kinase, cycloheximide, and chloramphenicol were obtained from Sigma Chemical Company; trypsin and trypsin inhibitor were from Boehringer-Manheim. Labeled /4,5-^{3}H/leucine (55.9 Ci/mmol) was obtained from New England Nuclear. Sephacryl S-200 Superfine was from Pharmacia. dl-Tetrahydrofolate (TH_4) was prepared from folic acid as described (228,229). The TH_4 was lyophilized and stored with ascorbic acid under N_2 at -20°. N^{10}-formylTH_4 was obtained by neutralizing N^5,N^{10}-methenylTH_4 immediately before use. N^5,N^{10}-methenylTH_4 was prepared by dissolving commercial Leucovorin(N^5-formylTH_4) in 0.2 M potassium phosphate, pH 1.0, 0.15 M ascorbic acid on the day of the experiment. 5-methylTH_4

was generously provided by Dr. Kevin Scanlon. Goat milk folate-binding protein (FBP) was a generous gift from Dr. Sam Waxman.

2. Growth of Yeast and Preparation of Mitochondria for *In Vitro* Protein Synthesis

A diploid strain of *S. cerevisiae* used in this laboratory for several years was grown in medium previously described (198), with the modification that 2% galactose was the energy source. For preparation of mitochondria, cells were grown aerobically and harvested in late log phase of phase of growth. Where indicated, cells were grown in medium (198) containing 8% glucose and harvested in mid-log phase. All procedures involved sterile solutions and strictly aseptic techniques. Unless otherwise noted, all steps up to the breakage of spheroplasts were performed at room temperature. After breakage, all procedures were carried out at 0-4°. Cells were grown aerobically in medium containing 2% galactose until late log phase, when they were harvested by centrifugation and washed once with sterile distilled water. Cells were then suspended in a buffer containing 20 mM Tris-HCl, pH 9.0, 20 mM EDTA, and 50 mM β-mercaptoethanol at a concentration of 1 g wet weight of cells per 3ml of buffer and incubated at 30° for 15 min. Subsequently, the cells were harvested, washed 3 times with

sterile distilled water and then suspended at a ratio of 1 g wet weight of cells per 4 ml of buffer A (1 M sorbitol, 20 mM potassium phosphate, pH 7.4). Three mg of Zymolyase 5000 was added for each g of cells and the suspension incubated at 30° for 60 min, by which time digestion was complete. The spheroplasts were harvested by centrifugation, washed once with buffer A, and carefully resuspended at three times their original culture density in growth medium supplemented with 1 M sorbitol and incubated at 30° for 60 min with gentle shaking. After the incubation, the spheroplasts were harvested by centrifugation at 2000 g for 5 min, and washed once with ice cold buffer A. The spheroplasts were then resuspended in ice cold buffer C (0.6 M mannitol, 1 mM EDTA, 1 mg/ml of bovine serum albumin, 10 mM potassium phosphate, pH 6.8) and lysed at low speed in a Waring Blendor for 25 s. The broken spheroplasts were then diluted 5 to 10 times with buffer C and cell debris removed by three centrifugations at 1200 x g for 10 min. The supernatant was centrifuged for 10 min at 12,000 x g. The mitochondrial pellet was washed two times in buffer, recentrifuged at 16,000 x g, and then resuspended in this buffer at a concentration of 10 mg of protein per ml. Where indicated, the 1 h incubation (recovery step) of spheroplasts in growth media supplemented with 1 M sorbitol was omitted during the preparation of mitochondria for <u>in vitro</u> protein synthesis.

3. Preparation of High Speed Supernatant (S-150)

Fractions were prepared from postmitochondrial supernatants of cells which were processed as described previously, except that buffer C contained 0.18 mg/ml of phenylmethylsulfonylfluoride (PMSF). The postmitochondrial supernatants were centrifuged for 90 min at 150,000 x g to give the S-150 fraction. The upper three-fourths of this fraction was removed from the centrifuge tube with a sterile Pasteur pipet and then dialyzed for 15 h against buffer D (10% glycerol, 50 mM KCl, 1 mM EDTA, 20 mM potassium phosphate, pH 7.0) in Spectrapor 3 tubing which has a 3500 molecular weight cutoff. The dialyzed supernatants were then concentrated by Sephadex G-200 beads to a protein concentration of 30-40 mg/ml and stored at -70° in aliquots. S-150 supernatant from cells grown either in 2% galactose to early stationary phase, or in 8% glucose to midlog phase, were prepared as described above.

4. Partial Purification of Activator Factors from High Speed Supernatant

For each purification experiment, S-150 fractions were prepared fresh from 60 g cells grown to late log phase in 2% galactose, as described above, with the modification that the S-150 fractions were kept at 4°. After Sephacryl S-200 chromatography of the high speed supernatant, the active fractions were pooled and concentrated by Amicon UM2

ultrafiltration. The pooled fractions were either used immediately or brought to 50% glycerol by direct addition of glycerol and then stored at -20°. Prior to use, the pooled low molecular weight activator which had been stored in 50% glycerol was dialyzed for 15 h against buffer D. For determining the trypsin sensitivity of the low molecular weight activator fraction, the pooled activator was dialyzed against 20 mM potassium phosphate, pH 7.0, 50 mM KCl, and 1 mM EDTA for 3 h prior to trypsinization. Where indicated, the molecular weight of the activator was determined by Sephadex G-50 chromatography of S-150 fractions that had been stored at -70°. Folates in the low molecular weight activator fraction were assayed using folate-binding protein as described by Waxman and Schreiber (230).

5. Protein Synthesis In Vitro

Isolated yeast mitochondria (1.0 mg of protein/ml) were incubated in a protein synthesizing mixture (198) with the following modifications: Leucine was added to a final concentration of 20 nmoles per ml, bovine serum albumin (1 mg per ml) was included and amino acid incorporation initiated by addition of mitochondria. In experiments determining the kinetics of protein synthesis, 1 ml of protein synthesizing mixture was incubated in a 25-ml Erlenmeyer flask and aerated by gentle shaking at 30°.

The total reaction volume was reduced to 0.5 ml for all other experiments in which 0.1 ml aliquots were taken 40 min after the start of the incubation and protein synthesis determined by the incorporation of L-[4,5-^{3}H]leucine (0.08 mCi/ml, 55.9 Ci/mmol) into trichloroacetic acid-precipitable protein (219). In some experiments the isolated mitochondrial pellet was preincubated for 3 min at 28^{o} before resuspension and addition of the mitochondria to the flasks.

6. Electrophoretic Analysis of Mitochondrial Translation Products

Isolated mitochondria (1 mg of protein per ml, 0.5 ml) were incubated in a protein synthesizing mixture (198) with the modification that L-methionine was omitted. To determine the translation products from mitochondria stimulated at time zero, the protein-synthesizing mixture was incubated either with S-150, 100 uM GTP, or low molecular weight activator, in the presence of 200 uCi per ml of L-[^{35}S]methionine. The reaction was then started by addition of mitochondria. After a 35 min incubation, 5 mM unlabeled L-methionine was added and the incubation continued for an additional 15 min. The mixture was then centrifuged at 12,000 x g for 10 min to pellet mitochondria. The mitochondrial pellet was analyzed as described previously (221).

To determine the translational products from depleted mitochondria stimulated by an S-150 fraction or low molecular weight activator fraction, mitochondria were incubated in the protein synthesizing mixture for 40 min. Either fraction was then added to the mitochondria, followed by the addition of L-[^{35}S]methionine. After 30 min, 5 mM unlabeled L-methionine was added and the incubation continued for an additional 15 min, after which the mitochondria were processed as described above.

C. Results

1. Stimulation of Mitochondrial Protein Synthesis by Postpolysomal Supernatants

We have recently reported that addition of dialyzed yeast postpolysomal supernatant at time zero to isolated yeast mitochondria stimulated protein synthesis 6-8 fold suggesting that the protein synthetic capacity of mitochondria is apparently limited by extramitochondrial proteins (226). Ohashi and Schatz (226) have confirmed the stimulation by the supernatant but concluded that the stimulatory agent is GDP (or GTP) synthesized during the incubation from non-dialyzable GMP present in the supernatant. As seen in Fig. 5, the addition of GTP to the incubation medium did indeed stimulate protein synthesis by isolated mitochondria several fold; however, addition

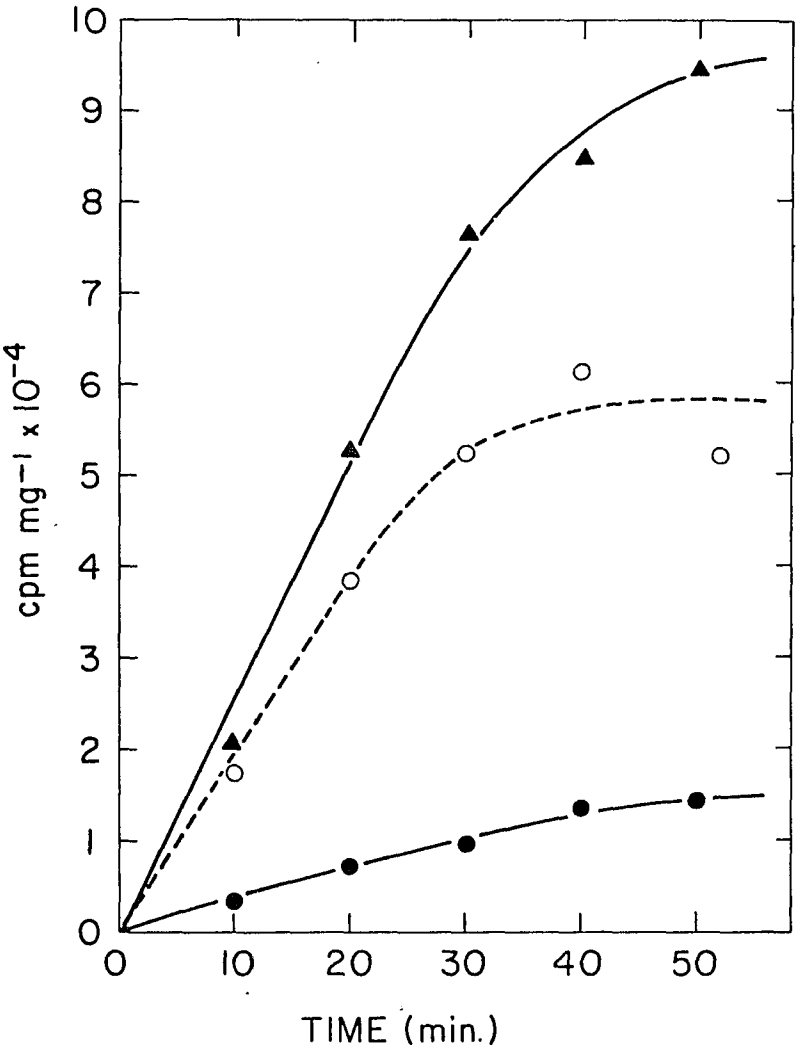

Figure 5

Stimulation of yeast mitochondrial protein synthesis by GTP and S-150 fraction. Isolated yeast mitochondria (1.0 mg per ml) were incubated in a protein-synthesizing mixture containing (▲) 100 uM GTP plus 2.0 mg of dialyzed S-150; (0) 100 uM GTP; (●) no addition. Aliquots containing 0.1 mg of mitochondrial protein was removed at the times indicated and the amount of newly synthesized protein was determined as described under Section IIIB.

of an S-150 fraction at time zero led to a nearly 2-fold stimulation over the GTP supplemented mitochondria when comparing the total incorporation after a 50 min incubation. Addition of GTP in concentrations ranging from 10 uM to 1 mM resulted in the same stimulation of incorporation and did not alter the kinetics, suggesting that GTP does not become rate-limiting for protein synthesis at 100 uM (Fig. 6). For each mitochondrial preparation, the stimulation of protein synthesis by GTP varied from 2- to 7-fold, while that observed after addition of GTP and S-150 varied from 5- to 12-fold; however, in all preparations, addition of an S-150 fraction to the GTP supplemented mitochondria resulted in significant stimulation, in most cases approximately 2-fold.

The differences between these results and those of Ohashi and Schatz (226) may have occurred because their mitochondria were incubated in a protein synthesizing medium supplemented with all amino acids except methionine for 15 min prior to the addition of [^{35}S]methionine. Under these conditions, isolated mitochondria synthesized an incomplete spectrum of mitochondrial translation products, predominantly cytochrome b and subunit III of cytochrome oxidase (226). To evaluate this possibility, the translation products synthesized by mitochondria which either

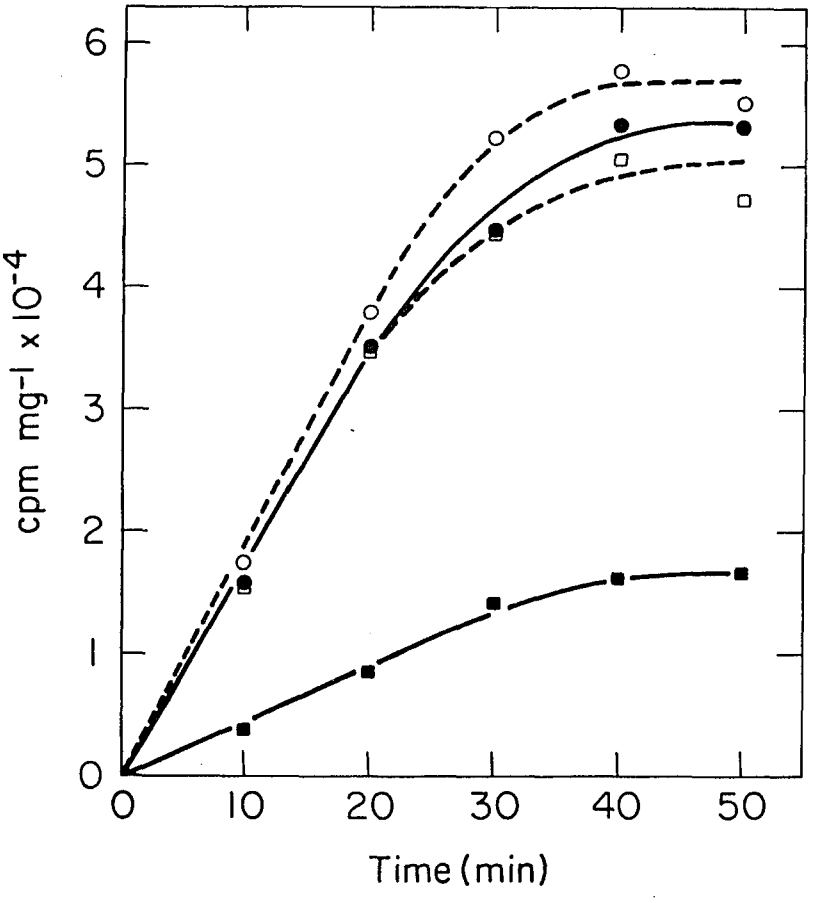

Figure 6

Stimulation of yeast mitochondrial protein synthesis by GTP. Isolated yeast mitochondria (1.0 mg per ml) were incubated in a protein-synthesizing mixture containing (□) 10 uM GTP; (○) 100 uM GTP; (●) 1 mM GTP; and (■) no addition. Aliquots containing 0.1 mg of mitochondrial protein was removed at the times indicated and the amount of newly synthesized protein was determined as described under Section IIIB.

were preincubated for 15 min before addition of [^{35}S] methionine, or were added to incubation medium which already contained [^{35}S]methionine were compared. The gel pattern reveals that preincubation of the mitochondria for 15 min did not prevent synthesis of the complete set of mitochondrial translation products. Furthermore, addition of GTP at this time did not change the relative rates of synthesis of any of the labeled proteins nor did it stimulate the synthesis of any new proteins (Fig. 7).

If the stimulation by S-150 fractions were due solely to conversion of non-dialyzable GMP to GDP (or GTP) during the incubation, then pretreatment of an S-150 fraction with 5'nucleotidase should abolish the stimulation (226). Under our experimental conditions, 5'-nucleotidase pretreatment decreased the stimulatory activity of the S-150 fraction to the same extent as incubating the S-150 at 30° for 30 min in the absence of 5'nucleotidase (Table II).

2. Physiological Correlation of the S-150 Stimulation

To demonstrate that the control of mitochondrial protein synthesis by cytosolic proteins is indeed physiological, S-150 fractions were isolated from cells grown under different conditions, and tested for their ability to stimulate mitochondrial protein synthesis. As shown in Table III, the stimulation of GTP supplemented mitochondria

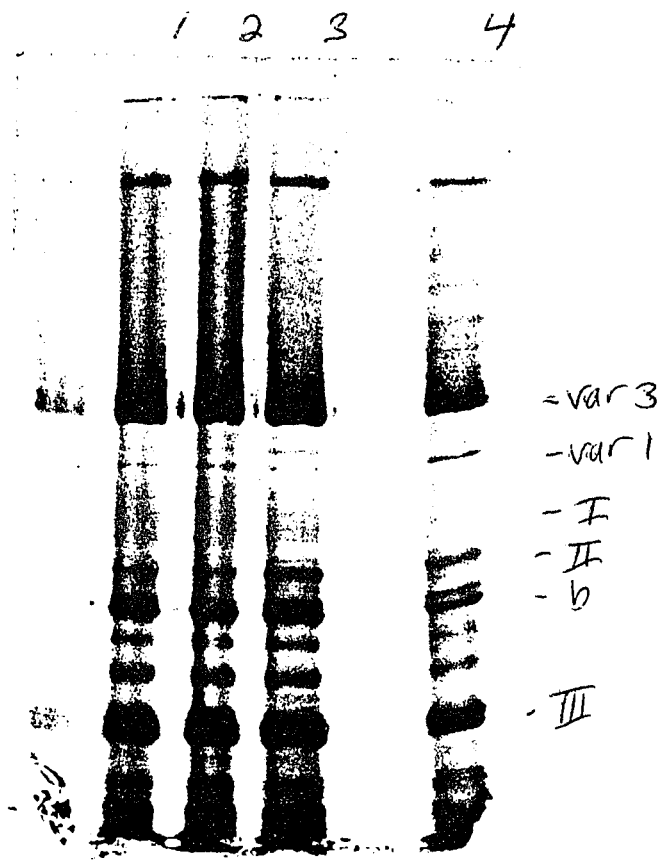

Figure 7

Electrophoretic analysis of *in vitro* mitochondrial translation products. Isolated mitochondria were labeled with 200 uCi per ml of L-[^{35}S]methionine, as described under Section IIIB. In lanes 1 and 2, L-[^{35}S]methionine was added at time zero while in lanes 3 and 4, mitochondria were preincubated for 15 min at 30^{o} in the protein synthesizing mixture before addition of [^{35}S]methionine. Lanes 1 and 4 contain the products synthesized by mitochondria in the absence of GTP. Lanes 2 and 3 contain the mitochondrial translational products after zero time addition of 100 uM GTP. An aliquot containing 50,000 cpm was added to each lane. The bands corresponding to Var 3, Var 1, subunits I, II, and III of cytochrome oxidase, and cytochrome b are indicated on the figure.

Table II

Stimulation of Mitochondrial Protein Synthesis by an S-150 Fraction Is Not Affected by 5'Nucleotidase

Experimental Condition	Incorporation Rate[a]
Mitochondria	21
+GTP	142
+GTP + S-150	227
+GTP + Mock-treated S-150	171
+GTP + 5'Nucleotidase-treated S-150	172

Isolated yeast mitochondria (1.0 mg/ml) were incubated in the protein-synthesizing mixture and the amount of newly synthesized protein determined as described under Section IIIB. The S-150 supernatant (0.87 mg in 0.035 ml) was incubated with or without Crotulus atrox 5'nucleotidase (Sigma, Type IV, 0.04 units) for 30 min at 30°. Mock-treated S-150 supernatant was incubated without 5'nucleotidase. The GTP concentration was 100 uM.

[a]Incorporation is pmol leucine incorporated per mg of mitochondrial protein per 40 min incubation.

Table III

Comparison of Stimulatory Activities of S-150 Fractions Isolated from Yeast Cells Grown Under Different Conditions

Addition to Mitochondria	Incorporation Rate[a]	Percent Stimulation above GTP
none	14	
GTP	101	0
GTP + Midlog S-150	156	100
GTP + Stationary phase S-150	116	22
GTP + Glucose Repressed S-150	128	44

Protein synthesis in isolated mitochondria was assayed as described in the legend to Table I. Where indicated, 0.8 mg of S-150 supernatants isolated as described under Section IIIB, were included in the protein-synthesizing mixture. The GTP concentration was 100 uM.

[a]Incorporation rate is pmol of leucine incorporated per mg mitochondrial protein per 40 min incubation.

by an S-150 fraction from glucose-repressed cells was decreased more than 50% relative to that obtained with an S-150 fraction isolated from cells grown in galactose. This result was anticipated as it was reported previously that mitochondrial protein synthesis is decreased in glucose repressed mitochondria (196). Similarly, an S-150 fraction from cells in stationary phase had less than one-fourth the stimulatory activity of an S-150 isolated from cells grown in mid-log phase. These results indicate that the stimulatory activity of S-150 fractions *in vitro* is well correlated with the mitochondrial protein synthetic rate *in vivo* of cells from which the S-150's supernatants were isolated.

3. Partial Purification of the Stimulatory Factors

Initially, yeast S-150 was fractionated in buffer D by chromatography on Sephacryl S-200. The stimulatory activity eluted mainly in a near void volume peak and in a peak in the 40,000 to 80,000 molecular weight range. In addition, a small amount of stimulatory activity eluted in a peak with a molecular weight less than 10,000 (data not shown). After refractionation of the 40,000 to 80,000 molecular weight peak on Sephacryl S-200, about half of the stimulatory activity was now eluted in the near void volume peak as well as that present in the middle molecular weight range (data not shown). Fig. 8 indicates that

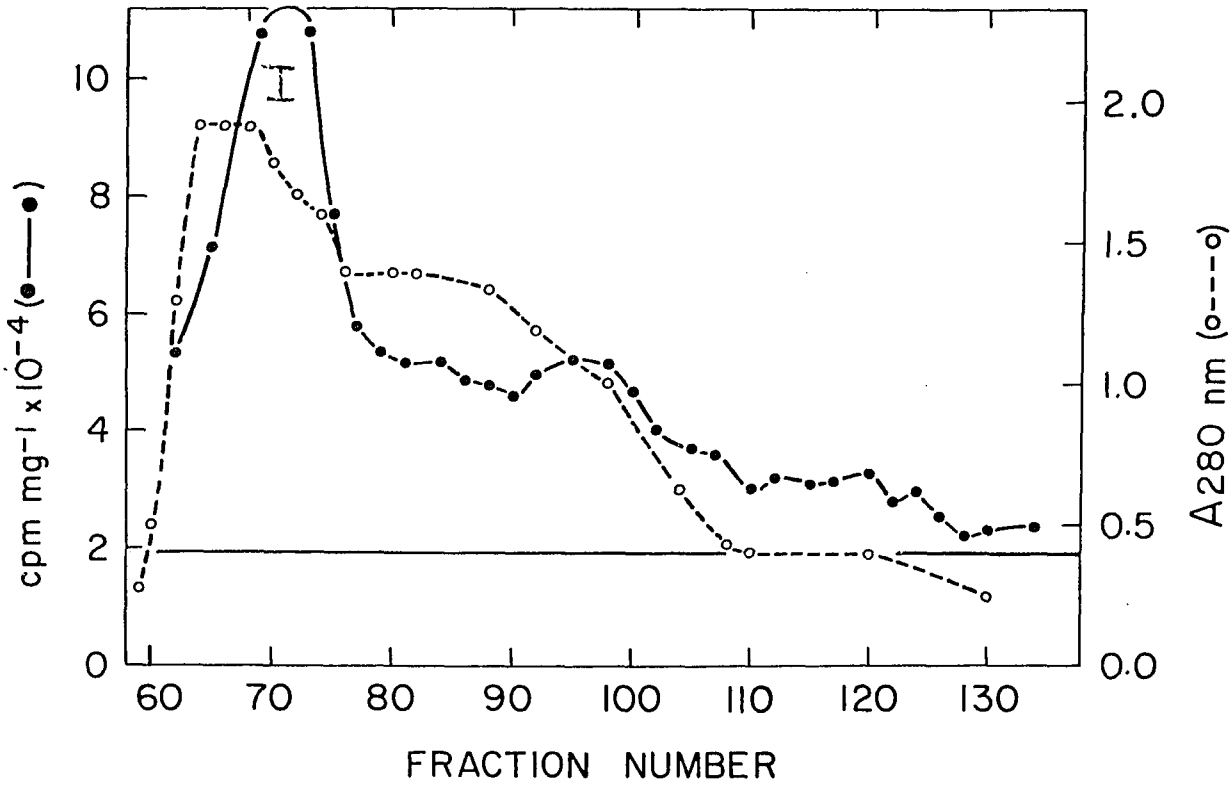

Figure 8

Elution profile of stimulatory activity after Sephacryl S-200 chromatography of S-150 fractions. The S-150 (41.7 mg/ml, 11.1 ml) was loaded onto a 100 x 2.5 cm column and eluted in buffer D at a flow rate of 0.84 ml/min. Three minute fractions were collected and 0.1 ml of fractions were assayed for stimulation of mitochondrial protein synthesis as described in Section IIIB.
The horizontal line represents the incorporation rate by mitochondria alone.

concentrating the S-150 to greater than 40 mg protein per ml before fractionation on Sephacryl S-200 resulted in most of the stimulatory activity appearing in the near void volume (peak I); however, there was a gradual tailing off of activity toward lower molecular weight fractions. These observations suggested that the stimulatory factors present in the S-150 may have a tendency to aggregate to higher molecular weight forms. In order to confirm this suggestion, the activity present in peak I was pooled and dialyzed overnight against buffer D containing 0.5 M potassium phosphate, before refractionation on Sephacryl S-200 in an attempt to disaggregate the stimulatory factors. As seen in Fig. 9, most of the stimulatory activity now eluted in 40,000 to 80,000 molecular weight range (peak II) and the low molecular weight range (peak III) with some spreading of activity occurring throughout the fractions. Further evidence for the suggestion that stimulatory factor(s) may aggregate was obtained when 1.8 M potassium phosphate was added directly to the S-150 to a final concentration of 0.5 M prior to chromatography (Fig. 10). This treatment caused an even greater disaggregation of the stimulatory activity such that half of the initial stimulatory activity was now eluted in peak III. These results suggest that the stimulatory activity eluting in peak I represents the low molecular weight

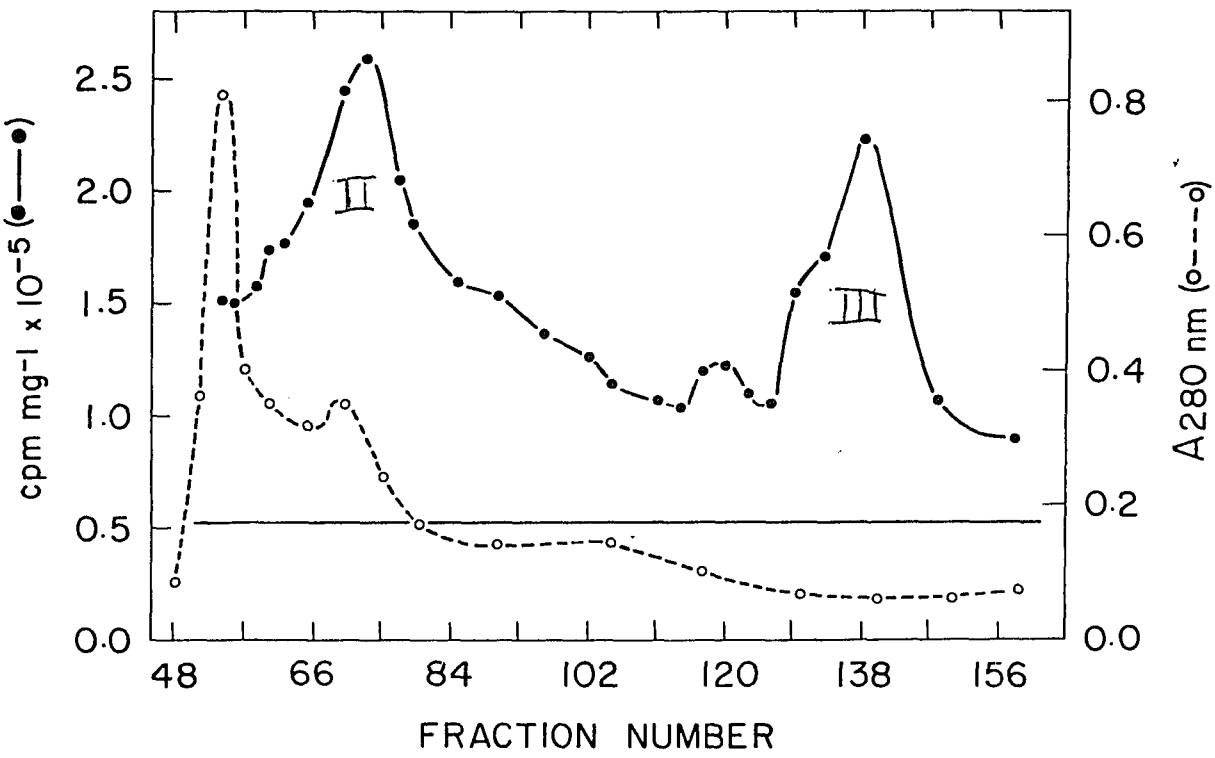

Figure 9

Elution profile of near void volume stimulatory activity after dialysis against high salt and Sephacryl S-200 chromatography. Fractions 62-79 from the elution profile shown in Fig. 8 were pooled and concentrated by Amicon PM 10 ultrafiltration to 5.1 mg per ml. The concentrate (9.2 ml) was dialyzed for 15 h against a buffer containing 10% glycerol, 0.5 M potassium phosphate, pH 7.0, and then loaded onto the column above and eluted with buffer D at a flow rate of 0.78 ml/min. The horizontal line represents the incorporation rate by mitochondria alone. Fractions were assayed for stimulation of mitochondrial protein synthesis as described in the legend to Fig. 8.

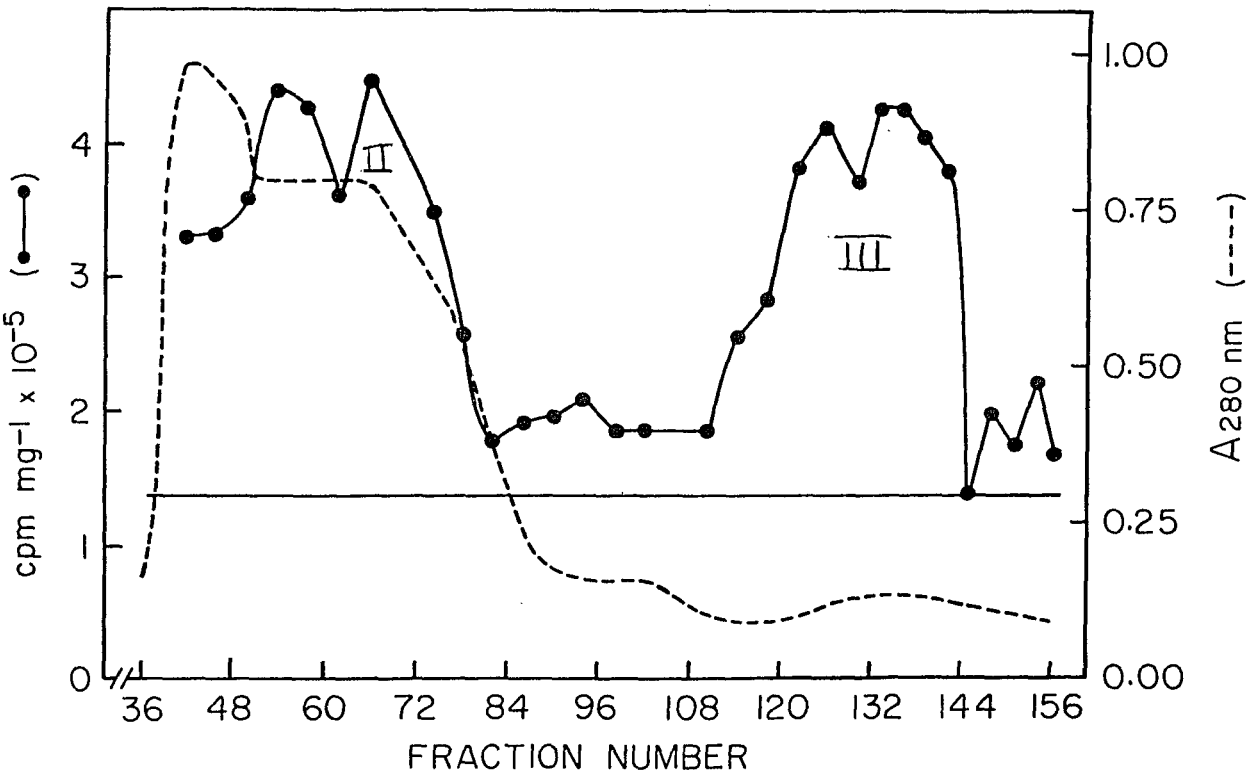

Figure 10

Elution profile of the stimulatory activity of an S-150 fraction after direct addition of high salt. S-150 (11.0 ml, 44.1 mg/ml) was incubated for 15 min with 4.2 ml of a buffer containing 10% glycerol, 1.82 M potassium phosphate, pH 7.0, and then loaded onto a 100 x 2.5 cm Sephacryl S-200 column and eluted in buffer D at a flow rate of 0.80 ml/min. The stimulatory activity was assayed as described in the legend to Fig. 8.

stimulatory factor(s), which have aggregated either with themselves or with other proteins. Consistent with this view was the diffuse elution of the low molecular activator(s) after DEAE-cellulose chromatography possibly because of aggregation with other proteins with different isoelectric points (data not shown).

4. Molecular Weight Estimation of Stimulatory Factor(s)

Chromatography of the S-150 on Sephadex G-50 in the presence of high salt revealed that most of the stimulatory activity coeluted with ^{14}C sucrose, indicating that the low molecular weight activator(s) has a molecular weight of 2000 or less (Fig. 11).

5. Properties of the Low Molecular Weight Activator Fraction

The low molecular weight fraction chosen for further studies contains very little of the total protein and represents a 40-fold purification of the stimulatory activity present in the S-150. The stability of the low molecular weight activator fraction in buffer D varied. Most preparations lost greater than 75% of their activity after 3 days of storage at 4^{o}: however, sometimes less than 30% of the activity was lost after 3 weeks, when the activator fraction was stored in buffer D containing 50% glycerol at -20^{o}. By contrast, the stimulatory activity of

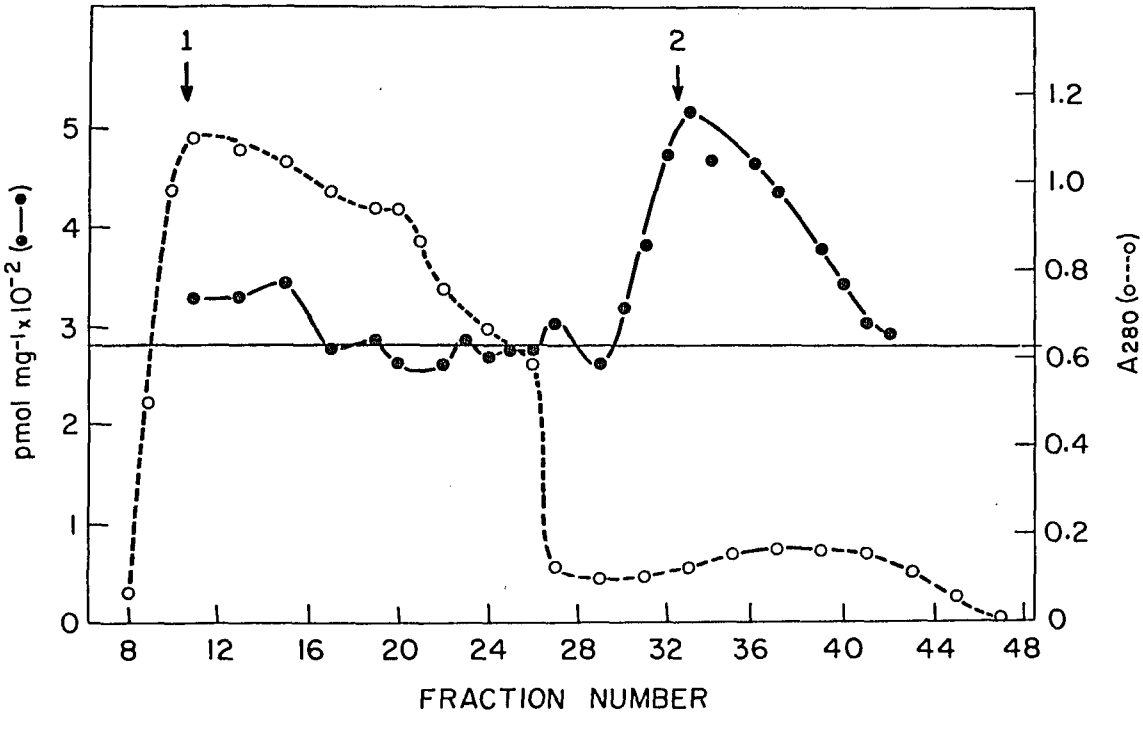

Figure 11

Sephadex G-50 chromatography of S-150 fractions. Elution profile of stimulatory activity. The S-150 (50.0 mg/ml, 3.5 ml) was incubated for 15 min with 1.26 ml of a buffer containing 10% glycerol, 1.82 M potassium phosphate, pH 7.0, and then loaded onto a 48 x 1.6 cm column. The column was eluted with 10% glycerol, 50 mM potassium chloride, 20 mM potassium phosphate, pH 7.0 at a flow rate of 0.4 ml/min. Three minute fractions were collected and 0.1 ml of fractions were assayed for stimulation of mitochondrial protein synthesis as described under Section IIIB. GTP (0.5 mM) was included in all assays. The horizontal line represents the incorporation rate by mitochondria alone. Peaks 1 and 2 represent the elution volumes of folate binding protein (30,000 daltons) and sucrose, respectively.

S-150 fractions was stable for at least 5 months when stored at -70°. As shown in Table IV, stimulation of mitochondrial protein synthesis by the low molecular weight activator(s) was greater than 98% sensitive to chloramphenicol and insensitive to cycloheximide, indicating that the stimulation of amino acid incorporation by the activator(s) represents mitochondrial protein synthesis. Furthermore, stimulation by the activator fraction was trypsin-sensitive, indicating its protein nature.

6. GTP Independence of Low Molecular Weight Activator Fraction

In order to demonstrate that stimulation of mitochondrial protein synthesis by the low molecular weight activator fraction is independent of GTP, mitochondria were incubated with increasing amounts of activator in the presence and absence of GTP (Fig. 12). Addition of different concentrations of the activator fraction stimulated mitochondrial protein synthesis to the same extent whether or not the incubation medium contained GTP. Furthermore, stimulation of mitochondrial protein synthesis by addition of the activator fraction was proportional to the amount of protein added. These results indicate that a low molecular weight fraction purified from the S-150 can stimulate mitochondrial protein synthesis independently of GTP and confirm

Table IV

Properties of the Low Molecular Weight Activator Fraction

Addition to Mitochondria	Incorporation Rate[a]
none	21
GTP (100 uM)	142
GTP + Activator	228
GTP + Activator + Cycloheximide (100 ug/ml)	230
GTP + Activator + Chloramphenicol (200 ug/ml)	3
GTP + Trypsinized Activator	133

Protein synthesis in isolated mitochondria was assayed as described in the legend to Table II. Where indicated, 30 ug of activator fraction was included in the incubation mixture. For trypsin treatment the activator fraction was incubated with trypsin (200 ug per ml) for 20 min at 30° and then with lima bean trypsin inhibitor (400 ug per ml) for 10 min at 30°. The control (i.e., untreated) activator fractions were also incubated for 30 min at 30°. To obtain a control for the trypsin treatment, trypsin was incubated with trypsin inhibitor for 5 min at 30° and then added to the control activator fraction before use.

[a]Incorporation rate is pmol of leucine incorporated per mg mitochondrial protein per 40 min incubation.

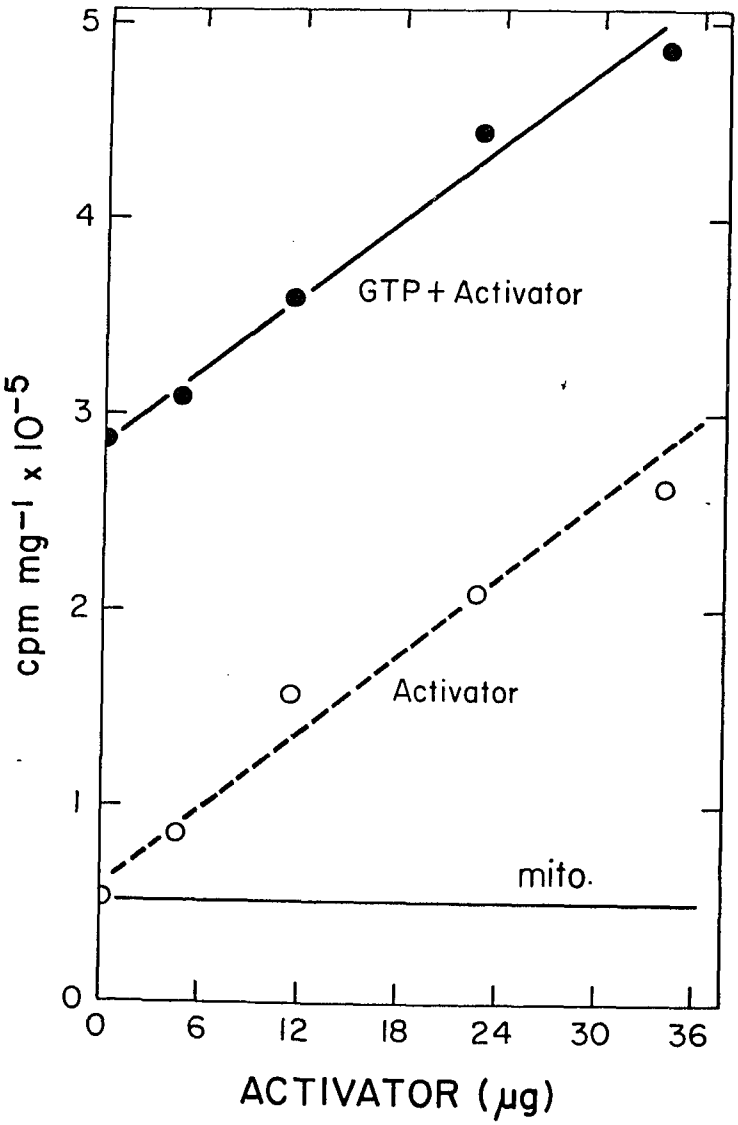

Figure 12

Stimulation of mitochondrial protein synthesis by the low molecular weight activator fraction in the presence and absence of GTP. Isolated mitochondria (1.0 mg/ml) were incubated in the protein-synthesizing mixture with (●) or without (○) 100 uM GTP. Fractions 114-142 from the column chromatography described in Fig. 10 were pooled and concentrated by Amicon UM 2 ultrafiltration to 0.45 mg per ml. The indicated amounts of this low molecular weight fraction were included in the incubation mixture. After 40 min aliquots of 0.1 ml were removed and the amount of newly synthesized protein determined, as described under Section IIIB. The horizontal line represents the incorporation rate by mitochondria alone.

our earlier suggestions (226) that freshly isolated mitochondria are deficient in protein factor(s) necessary for optimal rates of protein synthesis.

Further evidence in support of this suggestion was obtained by studying the kinetics of amino acid incorporation by mitochondria incubated either with GTP, the activator fraction or both (Fig. 13). Addition of 100 uM GTP to isolated mitochondria caused a marked increase in the rate of protein synthesis for 20 min, after which time synthesis ceased. By contrast, in mitochondria incubated with 90 ug of low molecular weight activator, the initial rate of protein synthesis was slower than in GTP supplemented mitochondria; however, synthesis continued at a linear rate for at least 40 minutes. This marked difference in kinetics of protein synthesis between GTP or activator supplemented mitochondria was noted in all experiments. Interestingly, protein synthesis by mitochondria incubated with both GTP and activator had kinetics that exhibited some characteristics of either GTP or activator supplemented mitochondria. The initial rate of protein synthesis when both activator and GTP were added was faster than with either alone, while synthesis continued for 40 min, but was not linear after 20 min. These results strongly suggest that the stimulation of mitochondrial protein synthesis by GTP or the low molecular weight activator

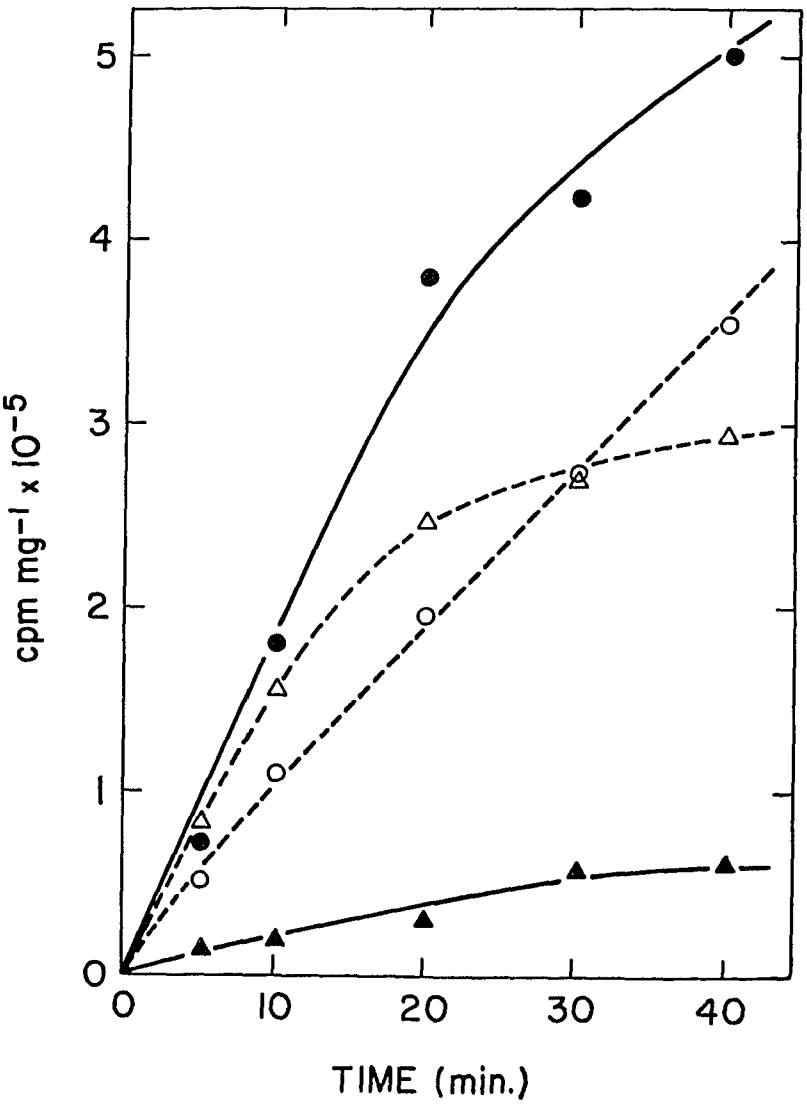

Figure 13

The kinetics of yeast mitochondrial protein synthesis stimulated by GTP or low molecular weight activator fraction. Protein synthesis by isolated mitochondria was assayed as described in the legend in Fig. 5. The low molecular weight activator fraction was prepared as described in the legend to Fig. 12. The incubation mixture contained (●) 100 uM GTP + 90 ug of low molecular weight activator; (○) 90 ug of low molecular weight activator; (△) 100 uM GTP; (▲) no addition.

fraction occurs by different mechanisms.

7. Electrophoretic Analysis of In Vitro Mitochondrial Translation Products

To show that the stimulation of mitochondrial protein synthesis by activator(s) represents synthesis of complete proteins and to determine whether the low molecular weight activator fraction stimulates the synthesis of some or all mitochondrial translation products, isolated mitochondria were labeled with [^{35}S]methionine in the presence or absence of activator. As seen in Fig. 14, the activator fraction stimulated equally the synthesis of all mitochondrial translation products whether added at the beginning of the incubation or after 35 min. The low molecular weight activator(s) and the S-150 from which it was derived stimulated the synthesis of bands corresponding to subunits I, II, and III of cytochrome oxidase, Var 3, cytochrome b, Var 1 and several other unidentified mitochondrial translation products.

8. Effect of Folate Derivatives on Mitochondrial Protein Synthesis

Bianchetti et al. (231) have reported that the formyl group donor N^{10}-formylTH_4 stimulates in vitro yeast mitochondrial protein synthesis. They also found that N^{10}-formylTH_4 stimulated 10-fold the in vitro synthesis of

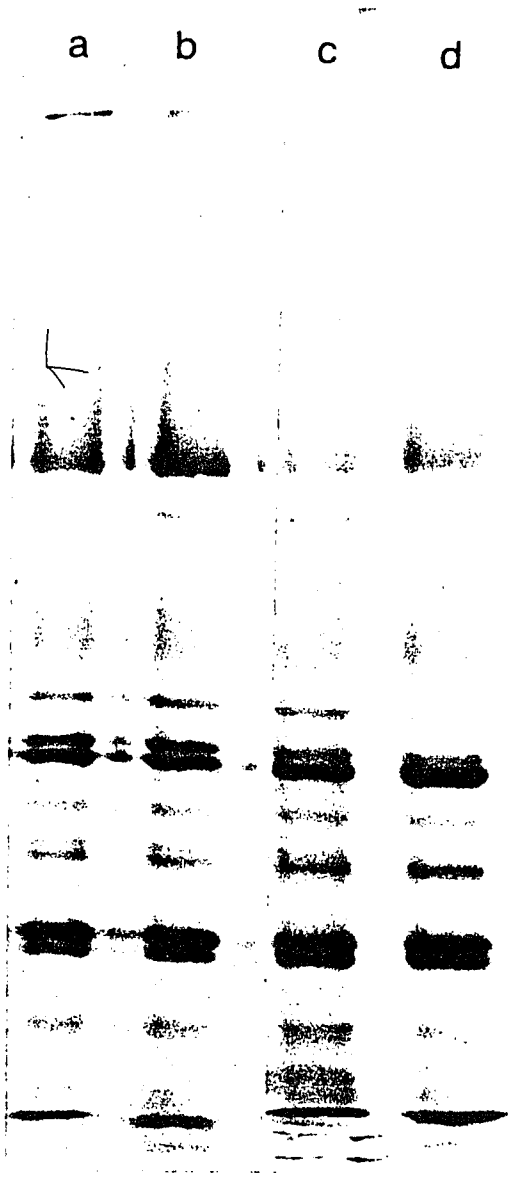

Figure 14

Electrophoretic analysis of *in vitro* mitochondrial translation products. Isolated mitochondria were labeled with 200 uCi per ml of L-[^{35}S]methionine, as described under Section IIIB. Lane a, mitochondria. Lane b, mitochondria plus 37 ug of activator protein. In lanes c and d, 37 ug of activator and 1.0 mg of S-150 supernatant were added after 35 min. To each lane 50,000 cpm were added. The bands corresponding to Var 3, Var 1, subunits I, II, and III of cytochrome oxidase and cytochrome b are indicated in the figure. The low molecular weight activator fraction was prepared as described in the legend to Fig. 12.

initial peptides by mitochondria, as measured by peptidyl puromycin derivatives. They concluded that the endogenous pool of mitochondrial formyl group donors required for the formylation of $tRNA^{fmet}$ becomes exhausted during the course of the incubation. Assay of the low molecular weight activator fraction for folate revealed that it contained large amounts (200 ng/ml) of folate. To test the possibility that the activator was a reduced folate derivative donating formyl groups to the mitochondria, two approaches were taken. First, folates derivatives were synthesized and examined for their ability to stimulate in vitro mitochondrial protein synthesis. As seen in Table V, none of the folates tested stimulated protein synthesis to a significant extent.

9. Effect of Folate-Binding Protein on the Low Molecular Weight Activator Stimulation

The second approach involved the use of purified goat milk folate-binding protein to see if the stimulatory activity of the low molecular weight activator fraction was affected by binding of all the folate. Control experiments using Sephadex G-50 chromatography showed that, under the conditions used in Fig. 15, C^{14}-labeled folic acid was completely bound by folate-binding protein (data not shown). On the basis of assays which indicated the amount of folate in the low molecular weight activator fractions, the fractions

Table V

Effect of Folate Compounds on Protein Synthesis

In Vitro by Yeast Mitochondria

System	Incorporation Rate[a]	% Stimulation
Expt. 1		
GTP (Control)	62 ± 10	
GTP + .1 mM Folic Acid	61 ± 2	- 2
GTP + 0.34 mM Ascorbate + 0.1 mM N^{10}-FormylTH_4	60 ± 3	- 3
GTP + S-150	84 ± 7	+ 35
Expt. 2		
GTP (Control)	383 ± 6	
GTP + S-150	589 ± 42	+ 54
GTP + 5 mM Ascorbate	418 ± 40	+ 9
GTP + 5 mM Ascorbate + 50 uM TH_4	443 ± 18	+ 16
GTP + 5 mM Ascorbate + 50 uM TH_4 + 1 mM Formate	426 ± 49	+ 11
GTP + 5 mM Ascorbate + 100 uM N^{10}-FormylTH_4	363 ± 22	- 5
Expt. 3		
GTP (Control)	89 ± 1	
GTP + 20 uM 5-MethylTH_4	93 ± 1	+ 4
GTP + 60 uM 5-MethylTH_4	96 ± 1	+ 8
GTP + S-150	202 ± 7	+127

[a]Incorporation rate is picomoles of leucine incorporated/mg of mitochondrial protein/40-min incubation. Values shown are the means ±S.D. for each experiment. Protein synthesis in isolated mitochondria was determined as described under Section IIIB. Where indicated, 1.0 mg of S-140 fractions were added to the incubation. The GTP concentration was 0.5 mM. Sodium ascorbate was added as indicated to prevent oxidation of TH_4 and N^{10}-FormylTH_4.

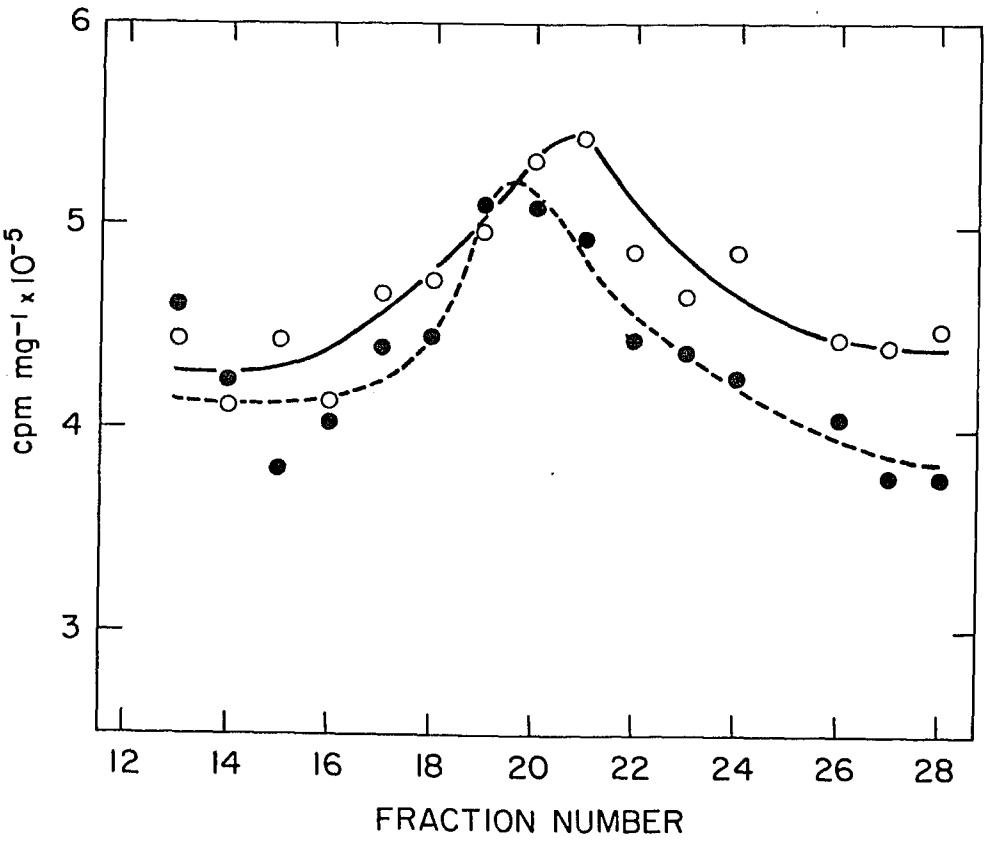

Figure 15

Stimulation of mitochondrial protein synthesis by the low molecular weight activator fraction in the presence and absence of folate-binding protein. The S-150 was fractionated on Sephadex G-50 as described in the legend to Fig. 11. Fractions were then incubated at 4° for 10 min with (●) or without (○) purified goat milk folate binding protein. Folate binding protein was added in a 7-fold excess in binding capacity relative to the amount of free folate in the fractions. Fractions were assayed for stimulatory activity as described in the legend to Fig. 8.

were then incubated with sufficient folate-binding protein to bind all folate. Fig. 15 shows that folate binding protein had no effect on the stimulatory activity of the fractions. It is unlikely that goat milk folate-binding protein can enter the mitochondria since non-mitochondrial proteins are not transported into the mitochondria (67). Therefore, these results suggest that the stimulatory factors in the activator fraction are not folate derivatives.

10. Influence of Ascorbate and Other Respiratory Chain Substrates on Protein Synthesis

Sodium ascorbate, which was added in some experiments to prevent the oxidation of reduced folate derivaties, was found to give a significant but variable stimulation of mitochondrial protein synthesis (Table V and VI). The stimulation by ascorbate was completely inhibited by cyanide, indicating that oxidation of ascorbate via site III of the respiratory chain (232) led to the observed stimulation. D-L lactic acid, oxidation of which involves only site III, and ethanol, which utilizes phosphorylation sites II and III during its oxidation, both stimulated mitochondrial protein synthesis. Succinate, however, was without effect. Although α-ketoglutarate is present in the protein-synthesizing medium, cyanide had no effect on protein synthesis by control flasks (Table VI, expt. 1). These results suggest

Table VI

Effect of Respiratory Chain Substrates on *In Vitro* Yeast Mitochondrial Protein Synthesis

System	Incorporation Rate[a]	% Stimulation
Expt. 1		
GTP (Control)	80 ± 8	
GTP + 1 mM KCN	82 ± 5	+ 3
GTP + 5.5 mM Ascorbate	149 ± 2	+ 86
GTP + 5.5 mM Ascorbate + 1 mM KCN	88 ± 1	+ 10
GTP + S-150	199 ± 11	+149
GTP + S-150 + 1 mM KCN	194 ± 4	+143
GTP + S-150 + 5.5 mM Ascorbate	212 ± 7	+165
Expt. 2		
GTP (Control)	73 ± 4	
GTP + 3 mM Ascorbate	108 ± 9	+ 48
GTP + 3 mM Ethanol	112 ± 7	+ 53
GTP + 1.5 mM Succinate	71 ± 1	- 3
GTP + 3 mM D-L Lactate	95 ± 5	+ 30
GTP + S-150	144 ± 8	+ 97

[a]Incorporation rate is picomoles of leucine incorporated/mg of mitochondrial protein/40-min incubation. Values shown are the means±S.D. for each experiment. The GTP concentration was 0.5 mM. Protein synthesis in isolated mitochondria was determined as described under Section IIIB. Where indicated, 1.0 mg of S-150 fractions were added to the incubation.

that α-ketoglutarate is not readily oxidized by mitochondria under these incubation conditions. The mechanism of action of the low molecular weight activator(s) present in the S-140 fraction is unknown. However, the activator(s) apparently works independently of the respiratory chain since the stimulatory activity is not affected by cyanide (Table VI).

11. Dependence of S-150 Stimulation on Recovery Incubation of Spheroplasts

Ohashi and Schatz (227) did not observe S-140 stimulation of protein synthesis in GTP-supplemented mitochondria. These workers omitted the 1 h "recovery" of Zymolyase prepared spheroplasts in growth media supplemented with 1 M sorbitol during the preparation of mitochondria. To evaluate the possibility that differences between our results and those of these workers may have occurred because they omitted the recovery step, mitochondria isolated from "recovered" and "nonrecovered" spheroplasts were tested for their ability to be stimulated by S-140 fractions. As seen in Table VII, omission of the recovery step sharply reduced the capacity of mitochondria to be stimulated by an S-140 fraction. However, the amino acid incorporation rate in the absence of added S-140 fractions was not influenced by the recovery step.

Table VII

Effect of Recovery of Yeast Spheroplasts on Cytoplasmic S-150 Stimulation of Protein Synthesis *In Vitro* by Yeast Mitochondria

Experimental Condition	Recovery[a]	Incorporation Rate
Mitochondria + GTP	-	301 ± 23
Mitochondria + GTP + S-150	-	389 ± 70
Mitochondria + GTP	+	332 ± 15
Mitochondria + GTP + S-150	+	567 ± 44

[a]Yeast mitochondria were isolated from spheroplasts which were either recovered for 1 h in 1 M sorbitol supplemented with growth media or broken immediately after conversion to spheroplasts. The GTP concentration was 0.5 mM. Where indicated, 1.0 mg of S-150 was added to the incubation. Protein synthesis was assayed as described under Section IIIB. The incorporation rate represents the picomoles of leucine incorporation/mg of mitochondrial protein/40 min incubation. Values shown are the means±S.D. of a representative experiment which was repeated three times.

D. Discussion

Previously it was suggested that mitochondrial protein synthesis ceases when a pool of cytoplasmically synthesized proteins is depleted (198). Studies *in vivo* and *in vitro* have provided evidence that synthesis resumes when these protein pools are replenished, indicating the existence of a positive control by cytoplasmically synthesized proteins on mitochondrial protein synthesis (194-198,221,226). By contrast, Ohashi and Schatz (227) recently concluded that the stimulation of mitochondrial protein synthesis by cytoplasmic extracts is due to guanyl nucleotides. A major finding of the current study is that the stimulation of yeast mitochondrial protein synthesis by postpolysomal supernatants is due in part to low molecular weight cytoplasmic factors of a protein nature which function independently of GTP. This conclusion is supported by the following evidence: (1) mitochondrial protein synthesis studied in the presence of optimal concentrations of GTP can still be stimulated by addition of an S-150 fraction; (2) stimulation by the S-150 fraction is not affected by incubation with 5'nucleotidase; (3) growth conditions which decrease the rate of mitochondrial protein synthesis *in vivo* also decrease the observed stimulation by the S-150 fraction; (4) stimulation of GTP supplemented mitochondria

by a partially purified activator fraction is trypsin-sensitive; (5) assay in the presence of GTP of fractions from Sephadex G-50 chromatography of the S-150 revealed that almost all of the stimulatory activity eluted in a peak with a molecular weight of 2000 or less.

The differences between our results and those of Ohashi and Schatz (227) might be explained by two major differences in the methods used to prepare mitochondria for protein synthesis in vitro. First, these workers prepared mitochondria by suspending spheroplasts in a thick paste with glass beads and then breaking them by stirring with a glass rod. We have found breakage of spheroplasts to be a critical step in mitrochondrial preparation. Mitochondria isolated from spheroplasts broken in more concentrated suspensions had the same protein synthetic capacity as those lysed with more dilute buffer; however, mitochondria isolated from concentrated suspensions of spheroplasts could not be stimulated by S-150 fractions when GTP was included in the assay medium. Furthermore, mitochondria isolated from spheroplasts broken for longer than 25 s also lost their capacity to be stimulated by S-150 fractions when GTP was present, although the unstimulated protein synthetic capacity remained the same. Apparently, stimulation of mitochondrial protein synthesis by cytoplasmic factors is

dependent on the isolation of mitochondria of the highest integrity, while this may not be as important for GTP stimulation. Second, these workers omitted the 1 h recovery incubation of spheroplasts in growth medium supplemented with 1M sorbitol. We found that stimulation of mitochondrial protein synthesis in vitro by yeast S-150 was dependent upon the recovery of spheroplasts. Omission of the recovery step sharply reduced the stimulatory activity. We would like to suggest the possibility that some component of the outer or inner membrane is required for stimulation of mitochondrial protein synthesis by cytoplasmic factors. This component, perhaps a membrane receptor, might easily be damaged by certain breakage techniques used to isolate mitochondria and during the cell wall digestion incubation step. In support of the former idea, Ades and Butow (233) have recently reported that the transport of cytoplasmically synthesized proteins into the mitochondria is highly dependent on the method used to break spheroplasts and prepare mitochondria.

Amino acid incorporation by isolated yeast mitochondria was stimulated by ascorbate, lactate, and ethanol. Cyanide blocked ascorbate stimulation, indicating that electron transport by the respiratory chain was involved. Surprisingly, neither α-ketoglutarate nor succinate increased the

incorporation rate. In contrast to these results, Grivell and Roodyn (234) and Ibrahim et al. (152) observed increased incorporation rates in the presence of succinate, while other workers (198) have found α-ketoglutarate to be a good respiratory substrate for mitochondrial protein synthesis. No explanation for these differences is apparent.

A partial purification of cytoplasmic factor(s) which stimulate mitochondrial protein synthesis has been achieved in this study, and a low molecular weight protein fraction has been found to stimulate the synthesis of all mitochondrial translation products independently of guanine nucleotides. Furthermore, the activator protein fraction doubles the length of time isolated mitochondria synthesize proteins, suggesting that protein synthesis by isolated mitochondria incubated with GTP may cease after 20 min because the low molecular weight activator factor(s) are rapidly degraded when their catalytic function has been completed.

The observation that the synthesis of all mitochondrial translation products is stimulated by the activator factor(s) indicates a general control mechanism by which mitochondrial protein synthesis could be coordinated with the cytoplasmic synthesis of mitochondrial proteins. By regulating the levels of low molecular weight activator(s), yeast cells

could increase or decrease the synthesis of proteins on mitochondrial ribosomes according to need. Such a mechanism would represent a form of unidirectional control by the nuclear genetic system on the mitochondrial genetic system. Support for this view is provided by the data indicating a strong correlation between the rates of mitochondrial protein synthesis in vivo and the presence of activator(s) in S-150 supernatant (Table III). Furthermore, these results suggests suggest that cytoplasmic levels of activator factor(s) may directly regulate mitochondrial protein synthesis in vivo; however, it seems unlikely that the stimulatory factor(s) are cytoplasmically synthesized partner subunits of respiratory chain complexes as was previously suggested (198-200). The activator factor(s) do not appear to function via the respiratory chain since cyanide has no affect on S-150 stimulation.

The low molecular weight activator(s) may regulate mitochondrial protein synthesis in a variety of ways. The activator(s) may enter the mitochondria and stimulate transcription, translation, or both. Alternatively, the activator(s) may act indirectly by binding to a membrane receptor which leads to an increase in transcription and/or translation. These possibilities are currently being investigated in our laboratory.

IV. Control of Yeast and Mammalian Protein Synthesis by Cytoplasmic Factors

A. Introduction

The biogenesis of the mitochondrial inner membrane requires the synthesis of proteins at 2 intracellular sites: 1 cytoplasmic; 1 mitochondrial. A mechanism must exist to coordinate the synthesis of proteins at the 2 distinct intracellular sites such that mitochondrial formation occurs in an orderly manner. Previous studies in yeast using selective inhibitors of cytoplasmic and mitochondrial protein synthesis have suggested that cytoplasmically synthesized proteins may control mitochondrial protein synthesis both *in vivo* (118,194-196) and *in vitro* (194) and that this control may occur by either stimulation of chain initiation or increased formation of mitochondrial mRNA's (197). Protein synthesis by yeast mitochondria *in vitro* was reported stimulated by addition of a high-speed cytoplasmic supernatant from yeast (198). This effect is not species-specific, as protein synthesis *in vitro* by isolated yeast mitochondria could be stimulated markedly by post-polysomal supernatants from either *E. coli*, rat liver, or yeast (226). However, stimulation of yeast mitochondrial protein synthesis by all of these high speed supernatants may be due to the presence of non-dialyzable GMP which is

converted to GDP (or GTP) during the incubation in the protein-synthesizing medium (227). We have confirmed that GTP stimulates yeast mitochondrial protein synthesis *in vitro*, but found that addition of a post-polysomal supernatant from yeast doubles the rate of mitochondrial protein synthesis in the presence of optimal concentrations of GTP (235). Data presented here indicate that high-speed supernatants from rat skeletal muscle and liver also stimulate yeast mitochondrial protein synthesis in the presence of GTP. In addition, evidence has been obtained to suggest the control of protein synthesis in liver mitochondria by cytoplasmic proteins.

B. Methods

1. Preparation of Mitochondria

Yeast mitochondria were prepared as in Section IIIB2. Skeletal muscle mitochondria were prepared as in (236) or from mechanically homogenized muscles (237). Rat liver mitochondria were prepared in 0.2 M mannitol, 0.07 M sucrose, 10 mM Hepes (pH 7.7), and 2 mM EDTA as in (238).

2. *In Vitro* Mitochondrial Protein Synthesis

The incubation mixture for yeast mitochondrial protein synthesis *in vitro* was modified (198) to contain 20 nmol/ml of L-leucine, 1 mg/ml of bovine serum albumin and, where indicated, 0.5 mM GTP. Protein synthesis was

initiated by addition of mitochondria and was determined by the incorporation of L-[4,5-^{3}H]leucine (0.04 mCi/ml, 55.9 Ci/mmol) into trichloroacetic acid-precipitable protein (219). Amino acid incorporation by either skeletal muscle or liver mitochondria was carried out as in (237). Where indicated, cycloheximide (0.5 mg/ml in 0.85 g%NaCl) was administered intraperitoneally from 2-24 h before sacrifice.

3. Preparation of Cytosolic Postpolysomal Supernatants (S-140)

Rat skeletal muscles were excised and homogenized with a Brinkmann Polytron homogenizer in buffer D (10 mM potassium phosphate, pH 7.4, 1 mM EDTA, 10% glycerol, 1 mM PMSF, and 1 mM TLCK). Immediately after sacrifice, rat livers were perfused via the vena cava with ice-cold 10 mM potassium phosphate, pH 7.4, 130 mM NaCl, 1 mM EDTA to remove erythrocytes, followed by homogenization in buffer D. The post-mitochondrial supernatant from the homogenates was then centrifuged for 90 min at 140,000 x g. The upper 60-70% of the supernatant was dialyzed 15 h in Spectrapore tubing (M_r-cutoff 3500) against 10 mM potassium phosphate, pH 7.4, 10% glycerol. The dialyzed S-140 was concentrated by treating the intact dialysis bag with Sephadex G-50, then frozen with liquid nitrogen, and stored in aliquots at -70°. Yeast S-140 fractions were prepared as in Section IIIB3.

C. Results and Discussion

1. Effect of GTP on Protein Synthesis In Vitro by Yeast and Mammalian Mitochondria

The effect of exogenous GTP on the rate of protein synthesis in vitro was compared in yeast and mammalian mitochondria (Table VIII). Addition of 10-1000 uM GTP to the incubation medium stimulated yeast mitochondrial protein synthesis $>$ 3-fold. A marked effect was observed at 10 uM of GTP with the maximum stimulation observed at 100 uM. By contrast, addition of these same concentrations of GTP had almost no effect on the rate of protein synthesis by either rat liver or skeletal muscle mitochondria. At most, a marginally significant increase was observed. Perhaps, mammalian mitochondria have a larger endogenous pool of guanine nucleotides in comparison with yeast mitochondria. Alternatively, yeast mitochondria may suffer a greater loss of nucleotides during preparation.

2. Effect of Postpolysomal Supernatants from Yeast, Rat Liver, and Skeletal Muscle on Protein Synthesis In Vitro by Yeast and Rat Mitochondria

In (226) the stimulation of yeast mitochondrial protein synthesis by addition of postpolysomal supernatant fractions isolated from E. coli, rat liver, and yeast was observed in a medium lacking exogenous GTP. As shown in

Table VIII

Effect of GTP on Protein Synthesis *In Vitro* by Yeast and Mammalian Mitochondria

Source of Mitochondria	Protein Synthesis[a]			
	Control	10 uM GTP	100 uM GTP	1 mM GTP
Yeast	58	181	227	192
Rat Skeletal Muscle	5.5	--	6.1	--
Rat Liver	10.0	10.2	11.2	12.5

[a]Incorporation rate is picomoles of leucine incorporated/mg of mitochondrial protein/40-min incubation. Protein synthesis in isolated mitochondria was determined as described in Section IVB. Values shown are representative of an experiment which was repeated two times.

Table IX, addition of an S-140 from either rat skeletal muscle, rat liver, or yeast significantly stimulated yeast mitochondrial protein synthesis in the presence of 0.5 mM GTP. S-140 from rat liver increased the incorporation rate *in vitro* more effectively than the S-140 from yeast. Protein synthesis in the presence of added S-140 fractions was 97% chloramphenicol-sensitive, indicating its mitochondrial origin.

While most efforts have focused on the cytoplasmic control of yeast mitochondrial protein synthesis, some studies have suggested a similar control of protein synthesis in rat liver mitochondria. Therefore, we investigated the effect of addition of cytoplasmic S-140 fractions on the rate of mammalian mitochondrial protein synthesis. In contrast to the above data, addition of an S-140 fraction from either *E. coli*, yeast, rat skeletal muscle, or rat liver failed to stimulate rat liver mitochondrial protein synthesis *in vitro* (not shown). Similarly, addition of an S-140 fraction isolated from regenerating rat liver, in which the rate of mitochondrial protein synthesis is increased, was without effect. All attempts to stimulate rat skeletal muscle mitochondrial protein synthesis by addition of S-140 fractions were unsuccessful.

Table IX

Cytoplasmic S-140 Fractions from Yeast, Rat Liver, and Skeletal Muscle Stimulate Protein Synthesis *In Vitro* by Yeast Mitochondria

Addition to Mitochondria	Incorporation Rate[a]					
	Expt. 1		Expt. 2		Expt. 3	
	Activity	% Stim.	Activity	% Stim.	Activity	% Stim.
GTP (Control)	192 ± 10.9		141 ± 6.4		91.5 ± 2.6	
GTP + S-140 (Yeast)	379 ± 33.2	97	204 ± 21.6	45		61
GTP + S-140 (Skeletal Muscle)	265 ± 14.8	38	--		--	
GTP + S-140 (Rat Liver)	--		243 ± 7.4	72	223 ± 9.0	144

[a]Incorporation rate is picomoles of leucine incorporated/mg of mitochondrial protein/40-min incubation. Protein synthesis in isolated mitochondria was determined as described in Section IVB. Values shown and the means±S.E. for each experiment. Where indicated, 1.0 mg of S-140 fractions were added to the incubation.

3. Rat Liver Mitochondrial Protein Synthesis *In Vitro* After *In Vivo* Cycloheximide Treatment

The effects of cycloheximide administration to rats upon RNA and protein synthesis provided another approach to the cytoplasmic control of mammalian mitochondrial protein synthesis (239-242). After administration of cycloheximide, the synthesis of total mitochondrial protein, $>$ 90% of which is cytoplasmically synthesized, was inhibited at 2 h followed by a recovery phase during which an actual stimulation was observed at 24 h. Here, the rates of liver mitochondrial protein synthesis *in vitro* were measured at several time intervals after the administration of cycloheximide *in vivo* (Fig. 16). A 30-40% decline in the incorporation rate was evident 2 h after injection and was still observed after 16 h; however, by 24 h after cycloheximide treatment, the ability of liver mitochondria to synthesize protein *in vitro* had increased slightly above the control level. These results suggest that inhibition of cytoplasmic translation may lead to a decrease in the rate of mitochondrial protein synthesis. Restoration of the rate of cytoplasmic protein synthesis after the metabolism and excretion of cycloheximide may then be followed by return of the rate of mitochondrial protein synthesis to control levels or higher.

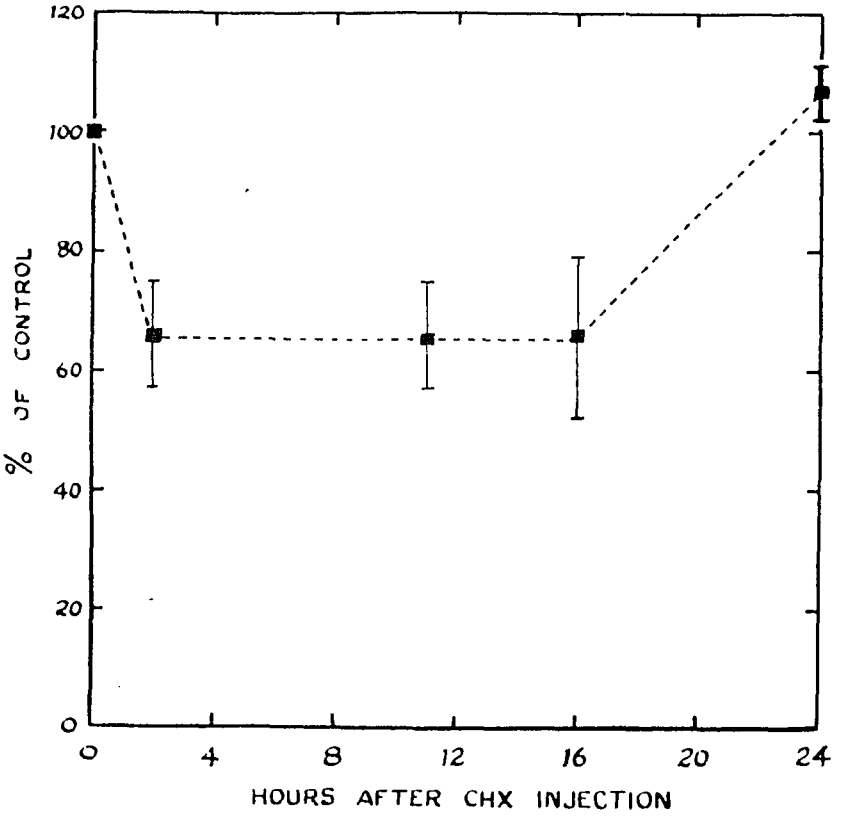

Figure 16

Effect of cycloheximide administration *in vivo* upon liver mitochondrial protein synthesis *in vitro*. Cycloheximide (2.5 mg/kg) was injected intraperitoneally at the indicated times prior to sacrifice. Protein synthesis *in vitro* by rat liver mitochondria was determined as in Section IVB. The data shown represent the mean values and ranges of 2 separate experiments.

4. Dose Dependent Effects of Cycloheximide Treatment

The effect of cycloheximide administration *in vivo* was dose-dependent when the rate of liver mitochondrial protein synthesis *in vitro* was examined 2 h and 18 h after injection (Table X). A maximum inhibition of 30-40% was observed at either time.

5. Effect of Liver Cytoplasmic S-140 Fractions from Control and Cycloheximide-Treated Rats upon Protein Synthesis by Isolated Yeast Mitochondria

The above experiments with cycloheximide suggest a control of rat liver mitochondrial protein synthesis by cytoplasmically synthesized proteins; however, no direct stimulation of mammalian mitochondrial protein synthesis could be demonstrated after addition of S-140 fractions. Thus it was of interest to determine the stimulatory effect of high-speed supernatants from cycloheximide-treated rats on the rate of protein synthesis *in vitro* by isolated yeast mitochondria. The S-140 fractions were prepared from rat livers after intraperitoneal injection of cycloheximide at the times indicated in Fig. 16 and tested for their ability to stimulate protein synthesis (Table XI). All the S-140 fractions tested increased the incorporation rate in isolated yeast mitochondria; however, the stimulatory

Table X

Effect of Dosage Level of Cycloheximide Administered *In Vivo* Upon Liver Mitochondrial Protein Synthesis *In Vitro*

Cycloheximide (mg/kg)	Hours After Injection	Incorporation Rate[a]	% of Control
0	2	26.7 ± 1.1	100.0
2.0	2	22.0 ± 0.8	82.3
10.0	2	21.3 ± 2.1	79.6
100.0	2	15.6 ± 1.6	58.3
0	18	30.4 ± 3.5	100
1.25	18	26.1 ± 0.8	85.8
2.50	18	21.3 ± 1.3	70.1

[a]Incorporation rate is picomoles of leucine incorporated/mg of mitochondrial protein/incubation. The indicated dose of cycloheximide dissolved in physiological saline was injected intraperitoneally at 2 or 18 h prior to sacrifice. Protein synthesis by isolated yeast mitochondria was determined as described in Section IVB. Values shown are the means±S.E. of a representative experiment which was repeated two times.

Table XI

Effect of Liver Cytoplasmic S-140 Fractions From Control and Cycloheximide-Treated Rats Upon Protein Synthesis *In Vitro* by Yeast Mitochondria

Treatment of Yeast Mitochondria	Incorporation Rate[a]	% Stimulatory Activity
No addition	91.5 ± 2.2	--
Plus S-140	223 ± 7.6	100
Plus S-140 2 h after Cycloheximide	185 ± 6.1	70
Plus S-140 16 h after Cycloheximide	185 ± 8.8	70
Plus S-140 24 h after Cycloheximide	168 ± 5.5	57

[a]Incorporation is picomoles of leucine incorporated/mg mitochondrial protein/40-min incubation. Protein synthesis *in vitro* by isolated yeast mitochondria was determined as described in Section IVB. Rat liver S-140 fractions (1.0 mg) were prepared from animals sacrificed at the stated time after intraperitoneal administration of cycloheximide (2.5 mg/kg). Values shown are the means±S.E. of a representative experiment which has been repeated three times.

ability of S-140 fractions isolated from rats 2-24 h after ycloheximide treatment was decreased compared with the control. These results suggest that the inhibition of cytoplasmic translation in rat liver by cycloheximide may result in decreased levels of factors present in the cytosol which stimulate yeast mitochondrial protein synthesis *in vitro*.

These results indicate that high-speed supernatant fractions obtained from both rat liver and skeletal muscle can significantly stimulate protein synthesis by isolated yeast mitochondria in the presence of optimal concentrations of GTP. Furthermore, these results do not support the conclusion that stimulation of protein synthesis in isolated yeast mitochondria by high-speed supernatant from different organisms is solely due to guanine nucleotides (227). Moreover, cycloheximide administration *in vivo* resulted in decreased rates of protein synthesis *in vitro* by rat liver mitochondria. This decrease could be correlated with a diminution in the ability of the supernatant fractions to stimulate yeast mitochondrial protein synthesis. Despite these indications of a cytoplasmic regulation of mammalian mitochondrial protein synthesis, no stimulation of the latter by addition of S-140 fractions was observed. Perhaps, isolated liver mitochondria contains a sufficient pool of the

stimulatory factor(s) and hence do not require additional stimulatory factor(s) during the course of the incubation. In support of this, rat liver mitochondria synthesize proteins at a linear rate of 40-60 min *in vitro* (243); while protein synthesis by yeast mitochondria *in vitro* ceases after 20-30 min (198,226,235).

We have purified a low molecular weight fraction from yeast that stimulates protein synthesis by isolated yeast mitochondria (235). The possibility that a similar stimulatory factor(s) is present in rat liver supernatants is intriguing.

V. Effect of Temperature on Protein Synthesis and Leucine Transport by Yeast Mitochondria

A. Introduction

Studies on the temperature dependence of membrane-bound enzymes and transport systems, in a variety of organisms, have shown discontinuities in the Arrhenius plots with an increase in activation energy at lower temperatures (244-250). The breaks in the Arrhenius plots have been shown by many physical techniques including electron spin resonance (251, 252), fluorescence probing (253,254), differential scanning calorimetry (255,256), and X-ray diffraction (257) to be paralleled by thermotropic phase transitions in the lipid bilayer. Studies in which the fatty acid composition of membrane lipid in *E. coli* (244,245,250,258,259) and *S. cerevisiae* (260-262) have been manipulated have shown that the break temperature in Arrhenius plots correlated well with the degree of lipid unsaturation.

The activities of mammalian and yeast mitochondrial respiratory chain complexes such as NADH oxidase, succinate dehydrogenase, and the cytochrome b-c_1 complex have also been reported to show discontinuities in Arrhenius plots (248,250,260-265). Towers *et al.* (266) reported breaks in the Arrhenius plots of *in vitro* rat liver mitochondrial protein synthesis from which they concluded that there is a

physical association between mitochondrial ribosomes and the inner membrane. Similar results for *in vitro* yeast mitochondrial protein synthesis were subsequently reported by Marzuki *et al.* (264). Uptake of the radiolabeled amino acid into isolated mitochondria was not investigated in either study. Hence, it is possible that the transport of the amino acid was rate-limiting for protein synthesis and that changes in this process with temperature led to the observed breaks in the Arrhenius plots.

We have investigated the temperature dependence of yeast mitochondrial protein synthesis both *in vivo* and in an optimized *in vitro* system. Similar breaks in the Arrhenius plots have been observed in both cases. Furthermore, mitochondrial translation products labeled *in vivo* below the apparent transition temperature of the inner mitochondrial membrane were shown to be inserted into the membrane. Leucine uptake does not appear rate-limiting for mitochondrial protein synthesis; however, the rate of leucine uptake is greatly stimulated by protein synthesis. In contrast to the reported energy-independent uptake of neutral amino acids by rat liver mitochondria (267-270), yeast mitochondria take up leucine rapidly only in the presence of an energy source. Valinomycin and carbonyl cyanide m-chlorophenyl hydrazone (CCCP) both inhibit uptake under

conditions where matrix ATP is high, suggesting that leucine import requires a membrane potential and may occur by active transport.

B. Materials and Methods

1. Growth of Yeast and Amino Acid Incorporation Studies

The haploid strains, KL14-4A and D273-10B, were grown aerobically in 3% galactose and harvested in the late log phase of growth, as described previously (271). Yeast mitochondria were prepared from strain KL14-4A as in (235) for *in vitro* protein synthesis experiments and amino acid uptake studies. Isolated yeast mitochondria (1.0 mg of protein/ml) were incubated in a protein-synthesizing mixture (272) with the following modifications. The incubation mixture, pH 7.2, contained 20 nmol/ml of leucine, 0.3 M mannitol, bovine serum albumin (1 mg/ml), and 0.5 mM GTP. In experiments determining the kinetics of protein synthesis, 1 ml of protein-synthesizing mixture was incubated in a 25 ml Erlenmeyer flask and aerated by gentle shaking at the indicated temperature. Protein synthesis was initiated by addition of mitochondria and aliquots containing 0.1 mg of protein were removed from the incubation at indicated times and protein synthesis determined by the incorporation

of L[4,5-^{3}H]leucine (55.9 Ci/mmol) into trichloracetic acid (TCA) precipitable protein, as previously described (219). To determine the temperature dependence of protein synthesis, the incubations were usually run at 4 different temperatures for each mitochondrial preparation, using Gyrotory shakers with an accuracy of $\pm 0.5^{o}$. The protein synthetic rate was calculated from the linear part of the incorporation curve. To combine the results of several individual experiments in one figure, the results have been normalized to the incorporation rate of 28^{o} of a representative experiment. The actual incorporation rate at 28^{o} ranged in different experiments from 47 to 107 pmoles per 10 min per mg of mitochondrial protein. The curves of Figures 20 and 22 were obtained by using the method of least squares to fit the data.

2. Leucine Uptake Into Mitochondria

The uptake of radioactive leucine into isolated mitochondria was studied in the same incubation medium used for protein synthesis. Aliquots containing 50 to 100 ug of mitochondrial protein were removed at various time intervals and filtered on Millipore filters (2.5 cm, 0.45 uM pore size) which had been presoaked in 10 mM unlabeled leucine to minimize nonspecific binding to the filters. The filters were immediately washed 2 times with

5 ml of ice cold 0.3 M KCl, before air drying and counting (273). The zero time values were taken as blanks and subtracted from all other values. The nonspecific binding did not exceed 0.04% of the total radioactivity added to the filter. Studies on the temperature dependence of leucine uptake were performed as described for protein synthesis.

Respiratory chain substrates, ATP, phosphoenolpyruvate (p-enolpyruvate) and inhibitors were added in the concentrations indicated in the legend of Table XIII. For studies on the energy requirements of leucine uptake, mitochondria were preincubated for 2 min at 28° in the incubation medium, before starting the reaction by addition of radioactive leucine. Chloramphenicol (200 ug/ml) was added to block completely protein synthesis. Aliquots were removed after 8 min and uptake determined as described above.

3. Estimation of the Mitochondrial Free Leucine Pool

Isolated mitochondria were incubated at either 7° or 28° in the protein synthesis incubation medium described above. After 9 min of incubation chloramphenicol in an ethanolic solution was added to a final concentration of 200 ug per ml. The incubation was continued for another minute and 100 ul aliquots were then withdrawn and processed for leucine uptake or TCA precipitable protein. At the same

time, to estimate the free intramitochondrial leucine pool, 100 ul aliquots were removed, filtered on Millipore filters and washed twice with 5 ml of ice cold 0.3 M KCl. Filters were then immediately placed into 20 ml glass beakers containing 1 ml of ice cold 0.2 N perchloric acid (PCA). The beakers had been treated previously with Glas-Treet (Alltech Associates) to reduce binding of leucine to the glass. The filters were then cut into small pieces; after 30 minutes, the PCA was poured off and the filter pieces washed with 1 ml of 0.2 N PCA. The combined acid extracts were centrifuged for 10 minutes at 15,000 x g, and aliquots of the acid supernatant counted. Nonspecific binding of leucine to the Millipore filters, as calculated from zero time points at 7^{o} and 28^{o} was subtracted from the free leucine pool and total leucine uptake measurements.

4. Protein Synthesis In Vivo

To determine the temperature dependence of mitochondrial protein synthesis in vivo, yeast cells were pulse labeled with [^{35}S]methionine in presence of cycloheximide (221) for 4 and 8 minutes at 0^{o}, 5^{o}, 10^{o}, 15^{o}, 20^{o}, 25^{o}, and 30^{o}. The rate was calculated from the linear slope obtained. Mitochondria and submitochondrial particles were isolated after breaking the cells in a Bronwill shaker (221) and TCA-precipitable radioactivity determined (219). Sodium

dodecylsulfate gel (SDS) electrophoresis was performed in 10% acrylamide, gels stained with Coomassie Blue, dried and autoradiographed as described previously (221).

5. Materials

Pyruvate kinase, chloramphenicol, cycloheximide ATP, and p-enolpyruvate were obtained from Sigma Chemical Company. Labeled L-[4,5-^{3}H]leucine (55.9 Ci/mmol) and L-[^{35}S]methionine (1000 Ci/mmol) was obtained from New England Nuclear. Acrylamide, bisacrylamide, and TEMED (N,N,N',N'-tetramethylethylenediamine) were from Eastman. Other chemicals used were of the highest purity commercially available.

C. Results

1. Temperature Dependence of Mitochondrial Protein Synthesis In Vivo

The temperature dependence of yeast mitochondrial protein synthesis *in vitro* has been used to study indirectly possible associations between the inner mitochondrial membrane and the protein synthetic system (264). To study *in vivo* mitochondrial protein synthesis, yeast cells were pulse labeled in the presence of cycloheximide at various temperatures, mitochondria isolated, and the rate of protein synthesis determined. As seen in Figure 17, an Arrhenius plot of protein synthetic activity shows a clear

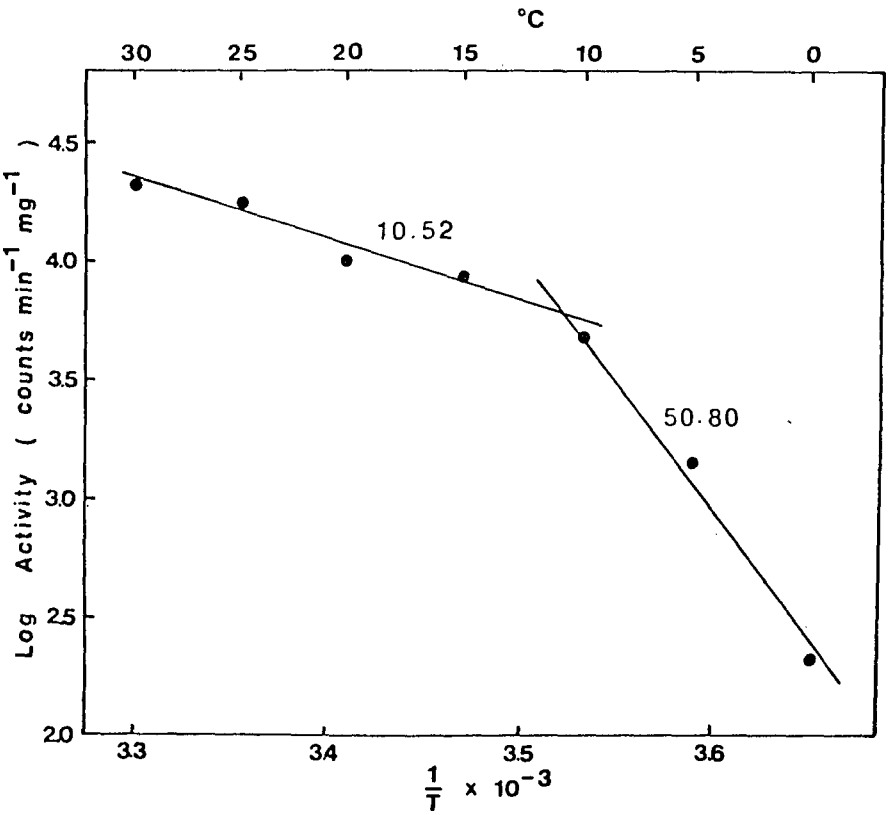

Figure 17

Arrhenius plot of mitochondrial protein synthetic activity *in vivo*. Yeast cells were pulse labeled for 4 and 8 minutes with [^{35}S]methionine in the presence of cycloheximide, mitochondria isolated, and the rate of TCA precipitable radioactivity determined as described in Section V_B. Each value represents the mean of two experiments. The numbers on the graph are activation energies expressed as Kcal/mole.

break at 10-12°.

2. Electrophoretic Analysis of Mitochondrial Translation Products Labeled Below the Transition Temperature of the Membrane

It was possible that only incomplete polypeptides were synthesized by mitochondria *in vivo* at temperatures below the transition temperature of the membrane. To investigate this possibility, yeast mitochondrial translation products were labeled *in vivo* at 7° and 30°, then analyzed by SDS polyacrylamide gel electrophoresis which was followed by autoradiography. The gel pattern obtained reveals that the two strains of yeast have the ability to synthesize exactly the same translation products at 7° as at 30° (Fig. 18).

To determine if the mitochondrial translation products synthesized at low temperatures were indeed inserted into the membrane, mitochondria were isolated from cells labeled at 5°, sonicated, and separated into membrane and soluble fractions. SDS gel electrophoresis of the membrane fraction revealed the normal mitochondrial translation products; the small percentage of TCA precipitable counts present in the supernatant did not migrate as complete polypeptides after gel electrophoresis (data not shown). These results suggest that yeast mitochondria can synthesize complete

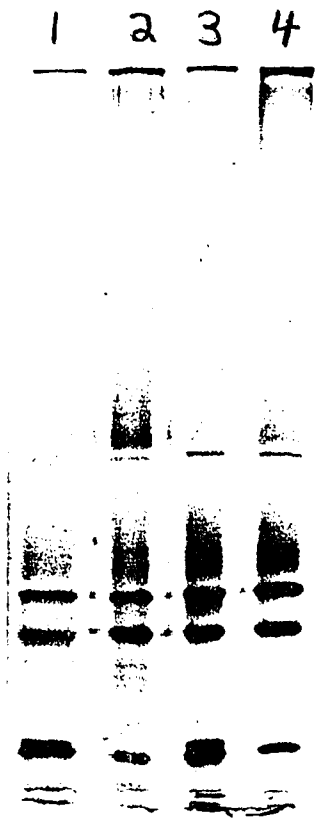

Figure 18

Electrophoretic analysis of mitochondrial translation products labeled at different temperatures. Yeast cells were labeled in the presence of cycloheximide at 7^{o} and 30^{o} for the times indicated as described in Section VB. Lane 1, strain KL14-4A labeled at 7^{o} for 7 min. Lane 2, strain D273-10B labeled at 7^{o} for 7 min. Lane 3, strain KL14-4A labeled at 30^{o} for 3 min. Lane 4, strain D273-10B labeled at 30^{o} for 3 min.

translation products and insert them into the membrane at temperatures below the transition temperature.

3. Temperature Dependence of Mitochondrial Protein Synthesis In Vitro

Since uptake of the radiolabeled methionine into whole yeast cells occurs via a membrane transport system, it is possible that changes in the specific activity of the cytosolic methionine pool with temperature may have affected the observed rate of in vivo mitochondrial protein synthesis. Therefore, we investigated the temperature dependence of mitochondrial protein synthesis using an optimized in vitro system. Figure 19 shows that amino acid incorporation at 28° was linear for about 8 min, after which time the rate was slower (226,235). The observed incorporation rate at 28° was 10.7 pmoles leucine per min per mg of mitochondrial protein and was inhibited 97% by chloramphenicol indicating its mitochondrial origin. At lower temperatures there was a short time lag before the incorporation rate became linear. This lag may reflect the time required at lower temperatures for the uptake and equilibration inside the mitochondrial matrix of externally supplied nucleotides and amino acids. The Arrhenius plot of amino acid incorporation shows a clear break at 11-13° with an increase in activation energy from 19.50 Kcal/mole

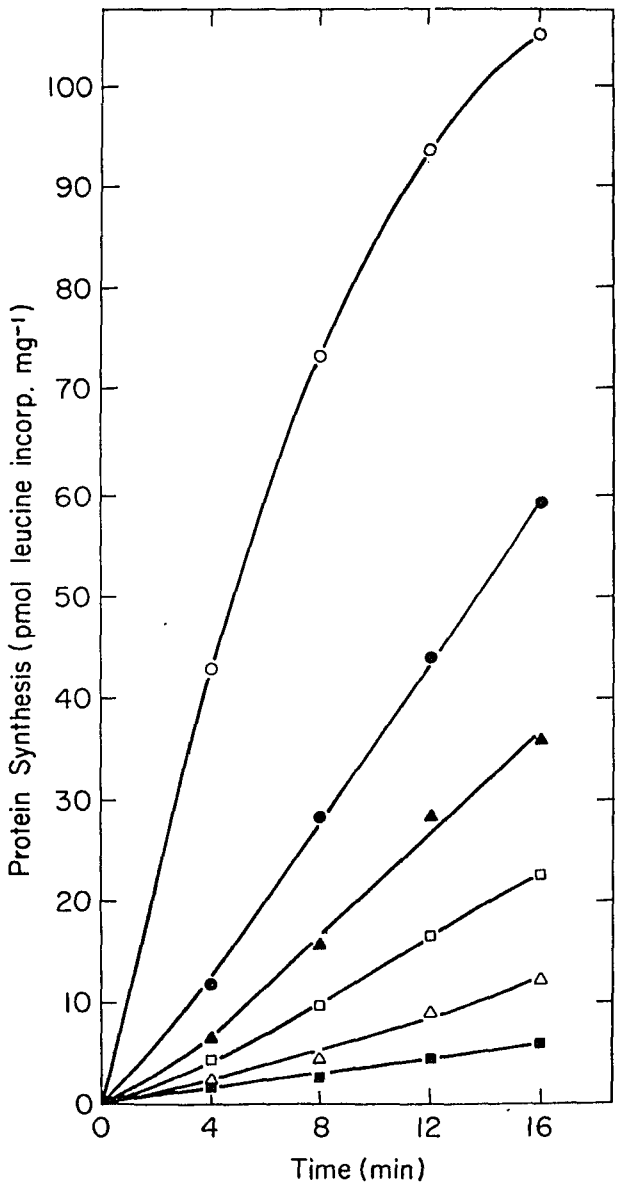

Figure 19

The kinetics of *in vitro* yeast mitochondrial protein synthesis at different temperatures. Isolated yeast mitochondria (1.0 mg per ml) were incubated in a protein-synthesizing mixture at (○) 28°; (●) 20°; (▲) 16°; (□) 13°; (△) 10°; (■) 7°. Aliquots containing 0.1 mg of mitochondrial protein were removed at the times indicated and the amount of newly synthesized protein was determined as described under Section VB.

above the break to 41.94 Kcal/mole below (Fig. 20). Therefore, yeast mitochondrial protein synthesis shows biphasic Arrhenius plots both *in vitro* as well as *in vivo* with an identical transition temperature.

4. Temperature Dependence of Leucine Uptake Into Mitochondria

To calculate protein synthetic rates in isolated mitochondria, one usually assumes that the added labeled amino acid equilibrates rapidly with the intramitochondrial pool of the amino acid. If the external pool of the amino acid is much larger than the intramitochondrial pool, then incorporation rates are derived from the specific activity of the added labeled amino acid. At lower temperatures, however, the uptake of the label, in this case leucine, may be rate limiting for protein synthesis. Therefore, breaks in Arrhenius plots of mitochondrial protein synthesis *in vitro* might reflect membrane mediated transport of leucine into mitochondria rather than protein synthesis. To investigate this possibility, the rate of leucine uptake into isolated mitochondria was studied at various temperatures.

The uptake of [^{3}H]leucine was linear for almost 8 min at 28° with an observed rate of 14.2 pmoles per min per mg of protein (Fig. 21). At lower temperatures, the uptake

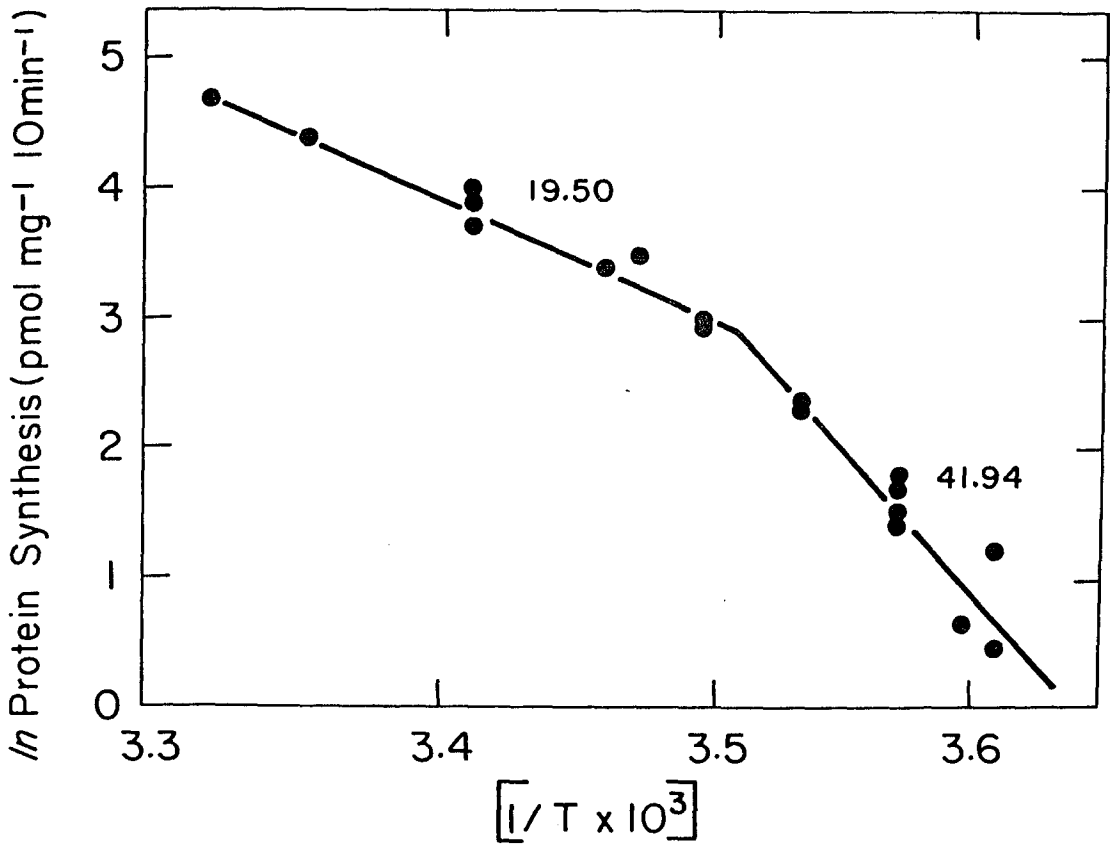

Figure 20

Arrhenius plot of in vitro mitochondrial protein synthesis. Isolated mitochondria (1.0 mg/ml) were incubated in a protein-synthesizing mixture and the incorporation rate determined as described under Section VB. To combine the results of several experiments, the rates were normalized to 28°. The numbers on the graph are activation energies expressed as Kcal/mole.

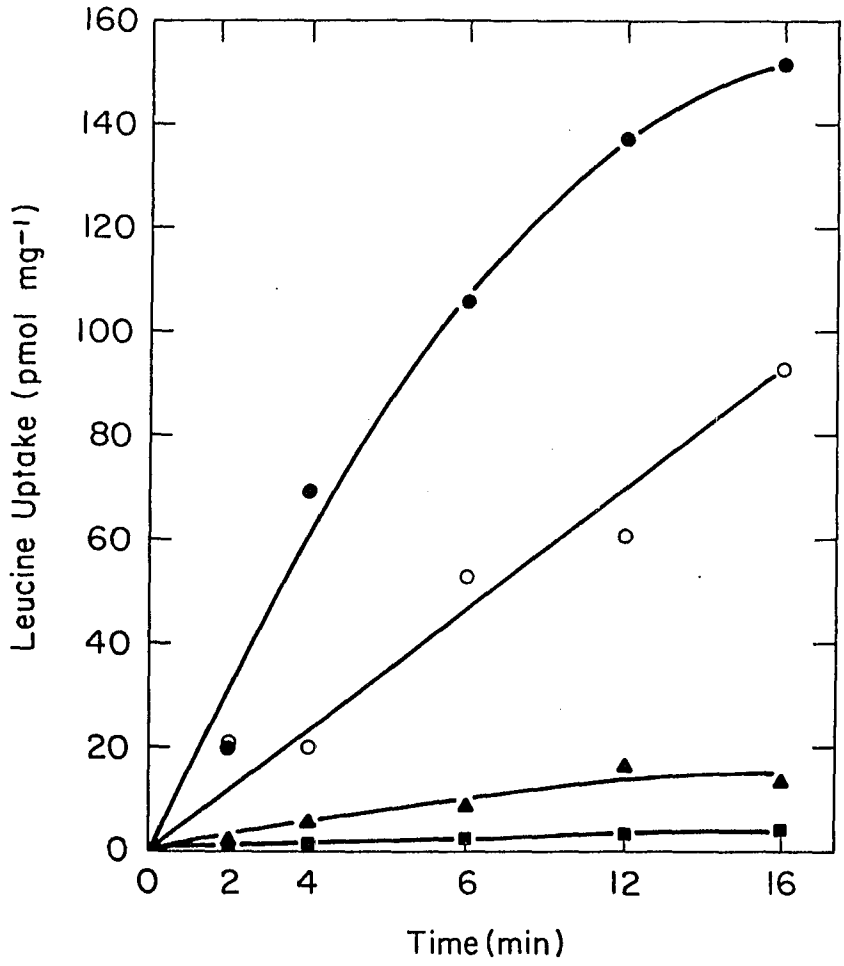

Figure 21

Kinetics of uptake of [^{3}H]leucine into mitochondria at different temperatures. Isolated mitochondria were incubated at (●) 28°; (○) 20°; (▲) 10°; and (■) 4°, as described in Section VB. Aliquots were removed at the indicated times and filtered on Millipore filters prior to counting as described in Section VB.

was linear for at least 16 min; however, no lag in uptake was observed at any temperature. In each experiment, the rate of leucine uptake at 28° was usually 30-60% higher than the rate of leucine incorporation into TCA-precipitable protein. Figure 22 shows the Arrhenius plot of leucine uptake rates. Accurate measurements of uptake were more difficult to obtain at lower temperatures because of the low rates of uptake; however, the data suggest a break at 10-13°. This temperature range agrees well with the observed break in both *in vitro* and *in vivo* mitochondrial protein synthesis.

5. Leucine Uptake Is Dependent on Protein Synthesis

One cannot determine from the data in Figures 21 and 22 whether or not leucine uptake regulates protein synthesis. Figure 23 shows, however, that the rate of leucine uptake is markedly dependent on protein synthesis. Uptake was decreased at least 5-fold in the presence of sufficient chloramphenicol to block 97% mitochondrial protein synthesis. These results suggest that the intramitochondrial pool of leucine is very small relative to the amount of leucine incorporated into protein during the incubation.

The data of Table XII indicate that, although the rate of uptake of leucine at 7° is 10% of that at 28°,

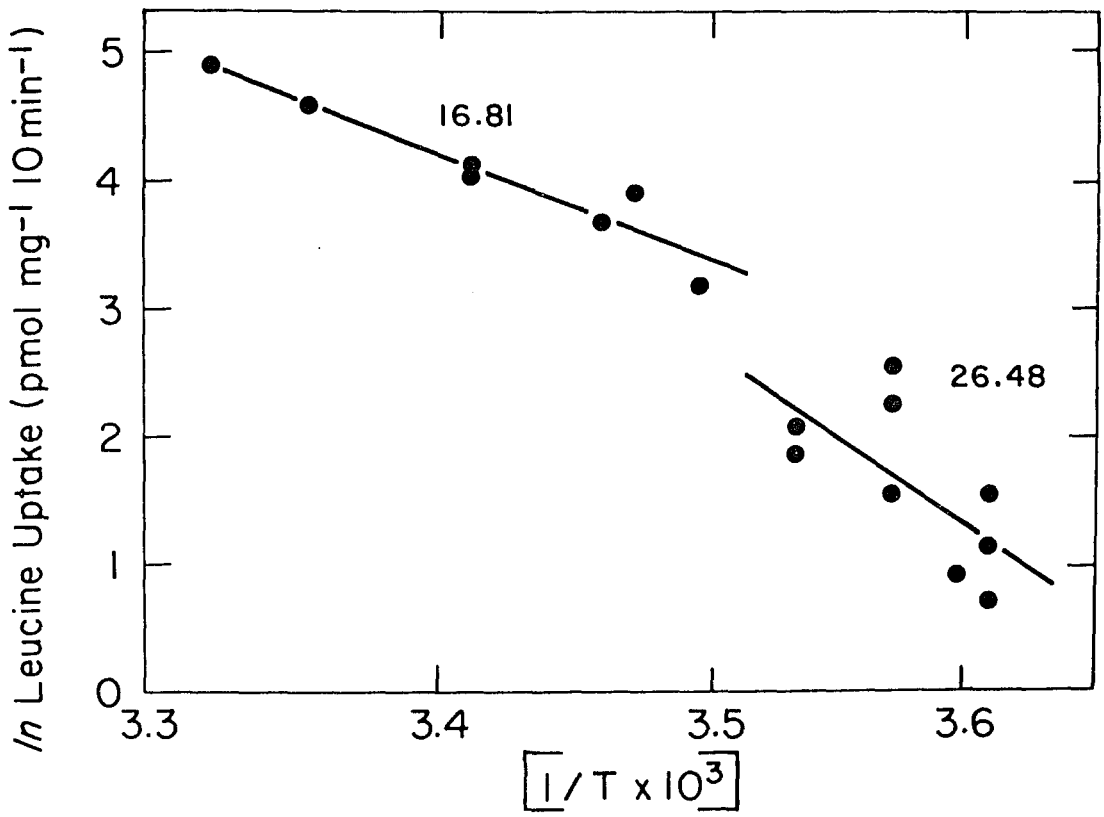

Figure 22

Arrhenius plot of [^{3}H]leucine uptake into mitochondria. Isolated mitochondria were incubated and the rate of uptake determined as described under Section VB. To combine the results of several experiments, the rates were normalized to 28°. The numbers on the graph are activation energies expressed as Kcal/mole.

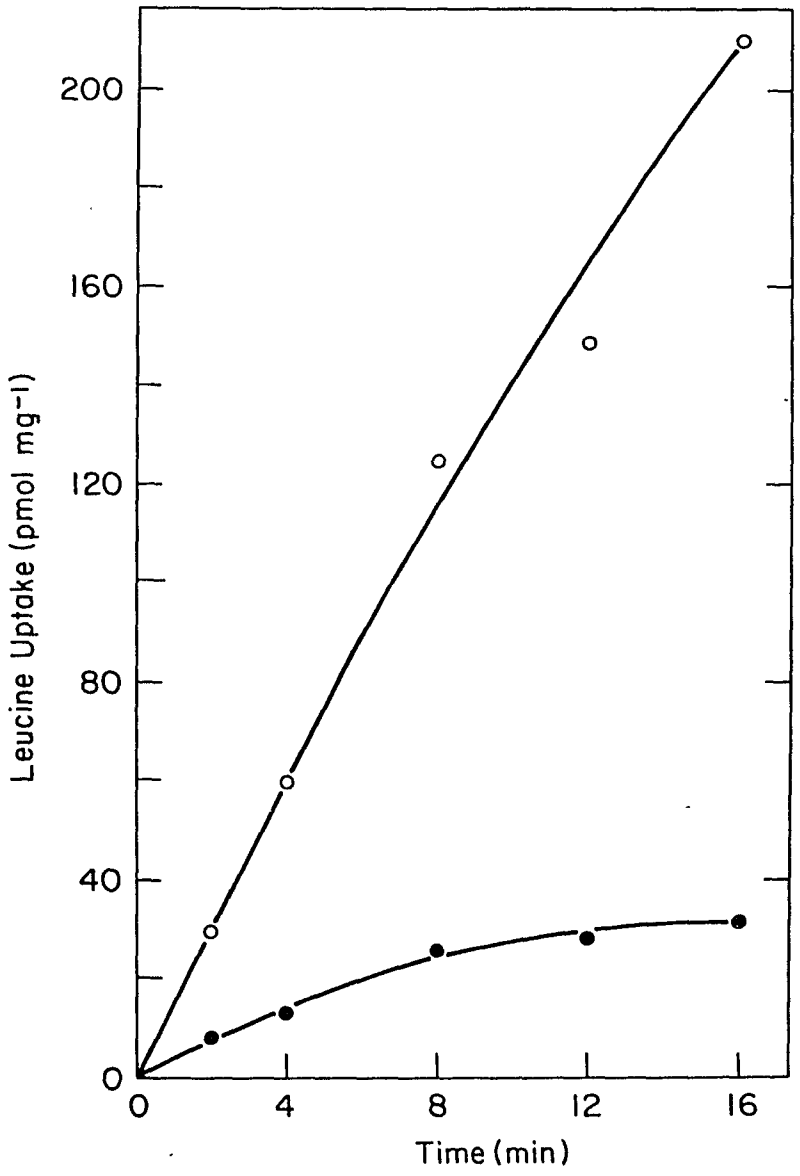

Figure 23

Kinetics of [^{3}H]leucine uptake into mitochondria in the presence (200 ug/ml) and absence of chloramphenicol. Isolated mitochondria were incubated at 28° with (●) or without (○) chloramphenicol as described in Section VB. Aliquots were removed at the indicated times and filtered on Millipore filters prior to counting as described in Section VB.

Table XII

Comparison of Leucine Uptake, Amino Acid Incorporation, and Leucine Pools as a Function of Temperature[a]

Measurements at Different Temperatures	Counts $min^{-1}mg^{-1}$	
	7^{o}	28^{o}
Leucine Uptake	32,700 ± 5820	330,000 ± 19400
Free Leucine Pool	22,100 ± 9610	33,300 ± 13700
TCA-Precipitable Protein	10,800 ± 1020	257,000 ± 15200
Total Leucine (calculated)	32,900	290,000

[a]Yeast mitochondria were incubated as described under Section VB. Values shown are the means±S.D. of a representative experiment which was repeated 3 times. Total leucine was calculated by adding free leucine pool and TCA-precipitable protein measurements.

the free leucine intramitochondrial pool, as estimated by recovery of non-TCA-precipitable radioactive leucine, is equivalent at the two temperatures. The observed differences in the free leucine pool are not statistically significant. The sum of leucine incorporated into TCA-precipitable protein and the free leucine pool is close to the total uptake value measured by Millipore filtration, as expected. These results suggest that during the incubation the specific activity of intramitochondrial leucine pools is similar above and below the transition temperature of the membrane.[a] Therefore, it is unlikely that breaks in Arrhenius plots of mitochondrial protein synthesis in vitro are caused by the temperature dependence of leucine uptake. If one assumes that after 9 min of incubation at 28° there has been equilibration of the intramitochondrial leucine pool with external leucine, then the internal pool size is in the range of 14-30 pmoles per mg of mitochondrial protein. The lower limit is estimated from the free leucine pool measurements; the upper limit from the difference between total leucine uptake and leucine incorporated into TCA-precipitable protein. These values are consistent with

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[a]Attempts were made to directly measure the leucine pools of mitochondria isolated from the incubation medium by Millipore filtration, using a sensitive fluorometric amino acid analyzer; results were inconclusive apparently because of the low leucine content of mitochondria relative to the background leucine levels.

the 30 pmoles of leucine per mg of mitochondrial protein equilibrated within the mitochondria during the incubation in chloramphenicol (Fig. 23).

6. Leucine Uptake Into Mitochondria Is Inhibited by Valinomycin and CCCP

Addition of the ionophore valinomycin and the uncoupler CCCP to the incubation medium inhibited leucine uptake into mitochondria when protein synthesis was completely blocked by chloramphenicol (Table XIII). At a 5 uM concentration of CCCP uptake was inhibited 36%; at 15 uM there was 79% inhibition. The high concentration of CCCP required for inhibition may result from the presence in the incubation medium of 100 uM cysteine which has been reported to activate CCCP (274). The ionophore valinomycin, which causes breakdown in the membrane potential in the presence of K^+, inhibited leucine uptake 76%. Furthermore, omission of respiratory chain substrates and the ATP regenerating system led to a 93% inhibition of uptake.

Inhibition of leucine uptake by valinomycin and CCCP suggests that the transport of this amino acid into the mitochondria requires a membrane potential. However, the inhibition might result from a decrease in intramitochondrial ATP due to the increased activity of the oligomycin-sensitive ATPase (275) after the breakdown of the membrane

Table XIII

Effect of Energy and Inhibitors on Uptake of [^{3}H]leucine Into Mitochondria[a]

System	pmoles of leucine uptake $min^{-1}mg^{-1}$
Expt. 1	
Complete	1.33 ± 0.29
Complete + Valinomycin	0.251 ± 0.19
Complete minus ethanol, p-enolpyruvate, and ATP	0.096 ± 0.12
Expt. 2	
Complete	1.67 ± 0.07
Complete + Valinomycin	0.40 ± 0.16
Complete + Valinomycin + Oligomycin	0.44 ± 0.18
Complete + CCCP (5 uM)	1.08 ± 0.26
Complete + CCCP (15 uM)	0.35 ± 0.19

[a]The complete system consisted of the protein-synthesizing mixture described under Section VB, mitochondria (1.0 mg/ml), 5 mM ethanol, and 200 ug/ml of chloramphenicol. Mitochondria were incubated for 8 min at 28°, aliquots removed, and uptake determined as described under Section VB. The concentration of various substances were: valinomycin, 5 uM; oligomycin, 10 uM; ATP, 2 mM; p-enolpyruvate, 5 mM. Values shown are the means±S.D. of representative experiments.

potential. Therefore, if ATP was the direct energy source for uptake, oligomycin should reverse the inhibition by preventing hydrolysis of ATP that enters the mitochondria. Furthermore, since ADP/ATP exchange is electrogenic, ATP entry into mitochondria will be favored by the absence of a membrane potential such that ATP levels in the matrix are higher in the presence of valinomycin and oligomycin than in their absence (276). Table XIII shows that oligomycin and valinomycin inhibited the rate of leucine uptake to the same extent as valinomycin alone, suggesting that the membrane potential is the direct energy source for leucine uptake and that leucine transport may occur via an active transport mechanism.

D. Discussion

Mitochondrial membrane-bound enzymes and transport systems are directly influenced by phase transitions in the phospholipid bilayer, as seen by changes in activation energies below the transition temperature of the membrane. Isolation of mitochondrial ribosomes in good yield requires conditions that partially solubilize mitochondrial membranes (197,277,278), suggesting that ribosomes are physically associated with the inner membrane. Marzuki et al. (264) observed the transition temperature of Arrhenius plots of in vitro yeast mitochondrial protein synthesis to vary with

the unsaturated fatty acid content of mitochondria. They concluded that mitochondrial ribosomes are associated with a lipid environment similar to that of the respiratory chain complexes. The results reported in this study also indicate that yeast mitochondrial protein synthesis exhibits a break in the Arrhenius plot at 10-13°, both *in vivo* and *in vitro*. Furthermore, leucine transport into mitochondria does not appear to be rate-limiting for protein synthesis, suggesting that the uptake of the labeled leucine did not cause the observed temperature dependence of protein synthesis. However, other membrane-associated processes such as the uptake of externally supplied ATP into the mitochondrial matrix or alternately the synthesis of ATP by the respiratory chain may be rate-limiting for protein synthesis and contribute to the observed temperature dependence. Hence, we can only conclude that there is a functional association between the inner membrane and mitochondrial protein synthesis.

The results presented here indicate that yeast mitochondria *in vivo* can insert complete mitochondrial translation products into the inner membrane at 5°, albeit at a slower rate. No labeled peptides were observed in the supernatant after sonication of mitochondria previously labeled at 5°, suggesting that the translation products are inserted

directly into the membrane. By contrast, transfer *in vitro* of cytoplasmically synthesized precursor proteins into a protease-resistant location in the inner mitochondrial membrane of *Neurospora crassa* mitochondria is temperature-dependent (279). At 0-4^{o} the precursor to the ATP/ADP carrier was observed bound to the mitochondria but sensitive to protease digestion. After the incubation temperature was raised, this protein was inserted into a protease-resistant location in the membrane (279). The differences between our *in vivo* results and those of these workers may result from the difficulty in observing low rates of protein insertion into membranes *in vitro* at temperatures below the phase transition. Alternatively, the insertion of mitochondrial translation products into the membrane may occur by a different mechanism than that of cytoplasmically synthesized proteins.

The present study indicates that leucine transport into yeast mitochondria is tightly regulated by mitochondrial protein synthesis. The rate of uptake was decreased at least 5-fold in the presence of chloramphenicol, while in the absence of chloramphenicol, the rate of uptake was always slightly higher but comparable to the incorporation rate of leucine into TCA-precipitable protein. Apparently, the leucine utilized for protein synthesis is immediately

replaced by transport of more leucine into the mitochondria. Although the rates of protein synthesis differ greatly with temperature, the intramitochondrial leucine pools are small and similar at temperatures above and below the transition temperature. Therefore, leucine transport does not appear to be rate limiting for protein synthesis. Estimates show that intramitochondrial free leucine pools are quite small, 14-30 pmoles per mg of mitochondrial protein. Small pool sizes would be expected from the strict dependence of uptake on protein synthesis which suggests that intramitochondrial leucine is used mainly for protein synthesis.

A uniport mechanism for neutral amino acid uptake into rat liver mitochondria was suggested by the observation that mitochondria swell in isosmolar solutions of neutral amino acids (267,269,270). No apparent energy requirement for swelling was observed. In addition, ATP, uncouplers, and inhibitors of the respiratory chain had no effect on the uptake of radioactive L-leucine into rat liver mitochondria (268). By contrast, the studies presented here on leucine uptake into yeast mitochondria indicate that this process requires energy. Uptake was inhibited in the presence of CCCP or valinomycin, conditions under which the membrane potential is dissipated. When the membrane potential was low but matrix ATP levels high, uptake was also inhibited,

suggesting that the membrane potential and not ATP is required for transport of leucine. Moreover, omission of respiratory chain substrates and ATP from the incubation led to a complete inhibition, suggesting that leucine uptake may occur via an active transport mechanism. The energy requirement for yeast mitochondrial leucine transport may be due to the relatively high uptake rate, 14 pmoles per mg per min compared to that reported for rat liver mitochondria (280). The faster rates of yeast mitochondria leucine transport may be due in turn to the much higher rates of protein synthesis in yeast mitochondria compared with those of rat liver mitochondria (281).

E. Concluding Remarks

Protein synthesis in eucaryotes is a complex multi-step process which may be regulated in a variety of ways. Experiments reported here have shown that cytoplasmic S-150 fractions from yeast, rat liver, and rat skeletal muscle can stimulate yeast mitochondrial protein synthesis *in vitro* in the presence of optimal concentrations of GTP. Physiological relevance of this *in vitro* stimulation is suggested by the correlation of *in vivo* mitochondrial protein synthetic activity in yeast and rats with S-150 stimulatory activity *in vitro* (Tables III, XI). Furthermore, a low molecular weight ($\leq$ 2000) protein fraction purified from yeast

postpolysomal supernatants has been found to stimulate the synthesis of all mitochondrial translation products.

The mechanism by which the activator factor(s) work is unknown; however, the cyanide insensitivity of the S-150 stimulatory activity suggests that mitochondrial electron transport is not involved (Table VI). A possible mechanism is suggested by evidence which indicates that protein synthesis in reticulocytes in regulated by heme (see 282 for review). When reticulocyte lysates are incubated in the absence of added hemin, protein synthesis is shut off after 5 min. The inhibition is caused by the activation of a heme-controlled repressor which blocks polypeptide chain initiation by phosphorylating subunit 2 of the initiation factor eIF-2. Heme prevents the activation of the repressor. In this regard, we have found that protein synthesis by isolated mitochondria ceases after 20 min; however, in the presence of the low molecular weight activator fraction amino acid incorporation continued for 40 min. Perhaps the low molecular weight yeast factor activates a phosphatase which dephosphorylates a mitochondrial initiation factor, thereby stimulating protein synthesis. Recently, low molecular weight (1000-4000) peptide-like factors have been implicated in insulin action (283-286). Seals and Czech (285) have shown that binding of insulin

to its receptor on isolated adipocyte plasma membranes leads to production of a peptide-like second messenger which activates pyruvate dehydrogenase in mitochondria. Kiechle et al. have isolated a similar factor from insulin-treated adipocytes and shown it to stimulate pyruvate dehydrogenase by activating a phosphatase which dephosphorylates the enzyme (286).

A possible role for heme in regulating mitochondrial translation was first suggested by Beattie (287), who found that inducers of heme biosynthesis stimulated mitochondrial translation. Saltzgaber and Schatz (288) have observed that heme-deficient yeast mitochondria synthesize normal amounts of subunits II and III but greatly decreased quantities of subunit I, of cytochrome oxidase. In vivo studies by Clejan et al. have shown a 33% decrease in mitochondrial protein synthesis by heme-deficient yeast mutants (289). Similarly, Kumar and Padmanaban (290) have found that heme-deficiency in Neurospora crassa decreased mitochondrial protein synthesis both in vivo and in vitro. In the absence of hemin, N. crassa mitochondrial lysates ceased protein synthesis within 5 min; as in reticulocyte lysates, hemin addition led to a restoration of synthesis. Whether heme regulation of mitochondrial protein synthesis occurs by a heme-controlled repressor mechanism remains to be seen.

The GTP stimulation of yeast mitochondrial protein synthesis reported here (Figures 5, 6) and by Ohashi and Schatz (227) is intriguing. No carrier protein which can transport GTP into mitochondria is known to exist. The possibility that GTP exerts its stimulatory effect by binding to a regulatory membrane receptor warrants further investigation.

Recently, Dieckmann et al. (207) have utilized advances in cloning and yeast transformation techniques to isolate a nuclear gene which controls production of mature cytochrome b mRNA. Similarly, O'Malley et al. cloned the nuclear gene for the yeast mitochondrial adenine nucleotide translocator (291). Rapid isolation and characterization of nuclear genes coding for mitochondrial proteins should greatly facilitate our understanding of mitochondria and make this field an interesting one to follow.

Abbreviations

ATP	adenosine 5'triphosphate
CAP	chloramphenicol
CCCP	carbonyl cyanide m-chlorophenylhydrazone
Chlx	cycloheximide
Cytox	cytochrome oxidase
EDTA	ethylenediaminetetraacetic acid
GMP	guanosine 5'phosphoric acid
GDP	guanosine 5'diphosphate
GTP	guanosine 5'triphosphate
Kbp	kilobase pairs
mRNA	messenger RNA
mt	mitochondria(1)
mtDNA,RNA	mitochondrial DNA,RNA
MTE	mannitol-Tris-EDTA
NADH	nicotinamide adenine dinucleotide
nDNA	nuclear DNA
PCA	perchloric acid
PMSF	phenylmethylsulfonyl fluoride
rRNA	ribosomal RNA
SDS	sodium dodecylsulfate
S-150	150,000 x g supernatant
STE	sucrose-Tris-EDTA
TCA	trichloroacetic acid

TH_4	tetrahydrofolate
TLCK	N-α-p-tosyllysine chloromethyl ketone
tRNA	transfer RNA

References

1. Slonimski, P. P. (1953) Formation des Enzymes Respiratoires chez la Levure, Masson, Paris
2. Linnane, A. W., and Haslam, J. M. (1970) Curr. Top. Cell. Regul. 2,101-172
3. Andreasen, A. A., and Stier, T. J. B. (1953) J. Cell. Comp. Physiol. 41,23-36
4. Bloomfield, D. K., and Bloch, K. (1960) J. Biol. Chem. 235,337-345
5. Linnane, A. W., Haslam, J. M., Lukins, H. B., and Nagley, P. (1972) Annu.Rev. Microbiol. 26,163-198
6. Paltauf, F., and Schatz, G. (1969) Biochemistry 8,335-338
7. Criddle, R. S., and Schatz, G. (1969) Biochemistry 8,322-334
8. Plattner, H., and Schatz, G. (1969) Biochemistry 8,339-343
9. Schatz, G. (1965) Biochim. Biophys. Acta 96,342-345
10. Gillham, N. W. (1978) Organelle Heredity, Raven Press, New York
11. Ephrussi, B., and Hottinguer, H. (1950) Nature (London) 166,956
12. Ephrussi, B., and Hottinguer, H. (1951) Cold Spring Harbor Symp. Quant. Biol. 16,75-85

13. Slonimski, P. P., and Ephrussi, B. (1949) Ann. Inst. Pasteur, Paris 77,47-63

14. Chen, S-Y., Ephrussi, B., and Hottinguer, H. (1950) Heredity 4,337-351

15. Benzer, S. (1959) Proc. Natl. Acad. Sci. U.S.A. 45,1607-1620

16. Wright, R. E., and Lederberg, J. (1957) Proc. Natl. Acad. Sci. U.S.A. 43,919-923

17. Lehninger, A. L. (1975) Biochemistry, 2nd ed., New York, Worth Publishers

18. Parsons, D. F., Williams, G. R., and Chance, B. (1966) Ann. N. Y. Acad. Sci. 137,643-666

19. Parsons, D. F., and Yano, Y. (1967) Biochim. Biophys. Acta 135,362-364

20. Mitchell, P. (1966) Biol. Rev. Camb. Phil. Soc. 41, 445-502

21. Hatefi, Y. (1978) Meth. Enzymol., Vol. LIII, 3-5

22. Nass, N. M. K., and Nass, N. (1963) J. Cell. Biol. 19,593-629

23. Luck, D. J. L., and Reich, E. (1964) Proc. Natl. Acad. Sci. U.S.A. 52,931-938

24. Rabinowitz, M., Sinclair, J., DeSalle, L., Haselkorn, R., and Swift, H. H. (1965) Proc. Natl. Acad. Sci. U.S.A. 53,1126-1133

25. Wetmur, J. G., and Davidson, N. (1968) J. Mol. Biol. 31,349-370

26. Britten, R. J., and Davidson, N. (1968) Science 161, 529-540

27. Hollenberg, C. P., Borst, P., and Van Bruggen, E. F. J. (1970) Biochim. Biophys. Acta 209,1-15

28. Petes, T. P., Byers, B., and Fangman, W. L. (1973) Proc. Natl. Acad. Sci. U.S.A. 70,3072-3076

29. Locker, J., Rabinowitz, M., and Getz, G. S. (1974) J. Mol. Biol. 88,489-507

30. Wintersberger, E. (1964) Z. Physiol. Chem. 336,285-288

31. Kalf, G. F. (1964) Biochemistry 3,1702-1706

32. Rogall, G., and Wintersberger, E. (1974) FEBS Lett. 46,333-336

33. Eccleshall, T. R., and Criddle, R. S. (1974) in Biogenesis of Mitochondria (Kroon, A. M., and Saccone, C., eds.), pp. 31-46, Academic Press, New York

34. Tzai, M-J., Michaelis, G., and Criddle, R. S. (1971) Proc. Natl. Acad. Sci. U.S.A. 68,473-477

35. Scragg, A. H. (1976) Biochim. Biophys. Acta 442,331-342

36. Levens, D., Morimoto, R., and Rabinowitz, M. (1981) J. Biol. Chem. 256,1466-1473

37. Levens, D., Lustig, A., and Rabinowitz, M. (1981) J. Biol. Chem. 256,1474-1481

38. McLean, J. R., Cohn, G. L., Brandt, I. K., and Simpson, M. V. (1958) J. Biol. Chem. 233,657-663

39. Roodyn, D. B., Reis, P. J., and Work, T. S. (1961) Biochem. J. 80,9-21

40. Kroon, A. M. (1963) Biochim. Biophys. Acta 72,391-402

41. Wintersberger, E. (1965) Biochem. Z. 341,401-419

42. Campbell, M. K., Mahler, H. R., Moore, W. J., and Tewari, S. (1966) Biochemistry 5,1174-1184

43. Wheeldon, L. W., and Lehninger, A. L. (1966) Biochemistry 5,3405-3418

44. Wheeldon, L. (1966) Biochem. Biophys. Res. Commun. 24,407-411

45. Beattie, D. S., Basford, R. E., and Koritz, S. B. (1967) J. Biol. Chem. 242,3366-3368

46. Beattie, D. S., Basford, R. E., and Koritz, S. B. (1967) Biochemistry 6,3099-3106

47. Beattie, D. S. (1968) J. Biol. Chem. 243,4027-4033

48. Beattie, D. S. (1971) Sub-Cell. Biochem. 1,1-23

49. Luck, D. J. L. (1963) J. Cell.Biol. 16,483-499

50. Groot, G. S. P., Rouslin, W., and Schatz, G. (1972) J. Biol. Chem. 247,1735-1742

51. Plattner, H., Salpeter, M. M., Saltzgaber, J., and Schatz, G. (1970) Proc. Natl. Acad. Sci. U.S.A. 66,1252-1259

52. Beattie, D. S., Basford, R. E., and Koritz, S. B. (1967) Biochemistry 6,3099-3106

53. Ennis, H. L., and Lubin, M. (1964) Science 146,1474-1476

54. Siegel, M. R., and Sisler, H. D. (1965) Biochim. Biophys. Acta 103,558-567

55. Smith, D., Tauro, P., Schweizer, E., and Halvorson, H. O. (1968) Proc. Natl. Acad. Sci. U.S.A. 60,936-942

56. Meselson, M., and Stahl, F. W. (1958) Proc. Natl. Acad. Sci. U.S.A. 44,671-682

57. Robberson, D. L., Kasamatsu, H., and Vinograd, J. (1972) Proc. Natl. Acad. Sci. U.S.A. 69,737-741

58. Robberson, D. L., Clayton, D. A. (1972) Proc. Natl. Acad. Sci. U.S.A. 69,3810-3814

59. Nass, M. M. K. (1976) Handbook of Genetics, Vol. 5, (King, R. C., ed.), Chapter 17, Plenum Press, New York

60. Borst, P. (1972) Annu. Rev. Biochem. 41,333-376

61. Schatz, G., and Mason, T. L. (1974) Annu. Rev. Biochem. 43,51-87

62. Sabatini, D. D., and Kreibich, G. (1976) in The Enzymes of Biological Membranes (Martonosi, A., ed.), Vol. 2, pp. 531-579, John Wiley & Sons, London, New York

63. Blobel, G., and Dobberstein, B. (1975) J. Cell. Biol. 67,835-851

64. Blobel, G., and Dobberstein, B. (1975) J. Cell. Biol. 67,852-862

65. Kellems, R. E., and Butow, R. A. (1972) J. Biol. Chem. 247,8043-8050

66. Kellems, R. E., and Butow, R. A. (1974) J. Biol. Chem. 249,3304-3310

67. Neupert, W., and Schatz, G. (1981) Trends Biochem. Sci. 6,1-4

68. Hallermayer, G., Zimmermann, R., and Neupert, W. (1977) Eur. J. Biochem. 81,523-532

69. Maccecchini, M.-L., Rudin, Y., Blobel, G., and Schatz, G. (1979) Proc. Natl. Acad. Sci. U.S.A. 76,343-347

70. Neher, E.-M., Harmey, M. A., Hennig, B., Zimmermann, R., and Neupert, W. (1980) in The Organization and Expression of the Mitochondrial Genome (Kroon, A. M., and Saccone, C., eds.), pp. 413-422, Elsevier/North Holland Biomedical Press, Amsterdam, New York, Oxford

71. Schleyer, M., Schmidt, B., and Neupert, W. (1982) Eur. J. Biochem. 125,109-116

72. Hennig, B., and Neupert, W. (1981) Eur. J. Biochem. 121,203-212

73. Gasser, S. M., Ohashi, A., Daum, G., Böhni, P. C., Gibson, J., Reid, G. A., Yonetani, T., and Schatz, G. (1982) Proc. Natl. Acad. Sci. U.S.A. 79,267-271

74. Boehni, P., Gasser, S., Leaver, C., and Schatz, G. (1980) in The Organization and Expression of the

Mitochondrial Genome (Kroon, A. M., and Saccone, C., eds.), pp. 423-434, Elsevier/North Holland Biomedical Press, Amsterdam, New York, Oxford

75. Kahn, C. R. (1975) Methods Membrane Biol. 3,81-146

76. Teintze, M., Slaughter, M., Weiss, H., and Neupert, W. (1982) J. Biol. Chem. 257,10364-10371

77. Zimmermann, R., Hennig, B., and Neupert, W. (1981) Eur. J. Biochem. 116,455-460

78. Faye, G., Fukuhara, H., Grandchamp, C., Lazowska, J., Michel, F., Casey, J., Getz, G. S., Locker, J., Rabinowitz, M., Bolotin-Fukuhara, M., Coen, D., Deutsch, J., Dujon, B., Netter, D., and Slonimski, P. P. (1973) Biochemie (Paris) 55,779-792

79. Hollenberg, C. D., Borst, P., Flavell, R. A., Vankreijl, C. F., Van Bruggen, E. F. J., and Arnberg, A. C. (1972) Biochim. Biophys. Acta 277,44-58

80. Locker, J., Rabinowitz, M., Getz, G. S. (1974) Proc. Natl. Acad. Sci. U.S.A. 71,1366-1370

81. Linnane, A. W., Haslam, J. M., Lukins, H. B., and Nagley, P. (1972) Annu. Rev. Microbiol. 26,163-198

82. Tzagoloff, A., Akai, A., and Needleman, R. B. (1975) Proc. Natl. Acad. Sci. U.S.A. 72,2054-2057

83. Tzagoloff, A., Akai, A., Needleman, R. B., and Zulch, G. (1975) J. Biol. Chem. 250,8236-8242

84. Tzagoloff, A., Foury, F., and Akai, A. (1976) in The Genetic Function of Mitochondrial DNA (Kroon, A. M., and Saccone, C., eds.), pp. 155-161, North Holland, Amsterdam

85. Tzagoloff, A., Macino, G., and Sebald, W. (1979) Annu.Rev. Biochem. 48,419-441

86. Netter, P., Petrochilo, E., Slonimski, P. P., Bolotin-Fukuhara, M., Coen, D., Deutsch, J., and Dujon, B. (1974) Genetics 78,1063-1100

87. Wolf, K., Dujon, B., and Slonimski, P. P. (1973) Mol. Gen. Genet. 125,53-90

88. Schweyen, R. J., Steyrer, U., Kaudewitz, F., Dujon, B., and Slonimski, P. P. (1976) Molec. Gen. Genet. 146,117-132

89. Molloy, P. L., Linnane, A. W., and Lukins, H. B. (1975) J. Bacteriol. 122,7-18

90. Slonimski, P. P., and Tzagoloff, A. (1976) Eur. J. Biochem. 61,27-41

91. Saccone, C., and Kroon, A. M. (eds.) (1976) The Genetic Function of Mitochondrial DNA, North Holland, Amsterdam

92. Southern, E. M. (1975) J. Mol. Biol. 98,503-517

93. Linnane, A. W., Lukins, H. B., Molloy, P. L., Nagley, P., Rytka, J., Sriprakash, K. S., and Trembath, M. K. (1976) Proc. Natl. Acad. Sci. U.S.A. 73,2082-2085

94. Nathans, D., and Smith, H. O. (1975) Annu.Rev. Biochem. 44,273-312

95. Morimoto, R., Lewin, A., Hsu, H.-J., Rabinowitz, M., and Fukuhara, H. (1975) Proc. Natl. Acad. Sci. U.S.A. 72,3868-3872

96. Merten, S., Synenki, R. M., Locker, J., Christianson, T., and Rabinowitz, M. (1980) Proc. Natl. Acad. Sci. U.S.A. 77,1417-1421

97. Maxam, A. M., and Gilbert, W. (1977) Proc. Natl. Acad. Sci. U.S.A. 74,560-564

98. Sanger, F., and Coulson, A. R. (1975) J. Mol. Biol. 94,441-448

99. Nobrega, F. G., and Tzagoloff, A. (1980) J. Biol. Chem. 255 9828-9837

100. Martin, N. C., and Rabinowitz, M. (1978) Biochemistry 17,1628-1634

101. Wesolowski, M., and Fukuhara, H. (1979) Mol. Gen. Genet. 170,261-275

102. Bernardi, G., Piperno, G., and Fouty, G. (1972) J. Mol. Biol. 65,173-189

103. Kotylak, Z., and Slonimski, P. P. (1976) in The Genetic Function of Mitochondrial DNA (Saccone, C., and Kroon, A. M., ed.), pp. 143-153, North Holland, Amsterdam

104. Slonimski, P. P., Pajot, P., Jacq, C., Foucher, M., Perrodin, G., Kochko, A., and Lamouroux, A. (1978) in Biochemistry and Genetics of Yeast (Bacilla, M., Horecker, B. L., and Stoppani, A. O. M., eds.), pp. 339-368, New York, Academic Press

105. Jacq, C., Lazowska, J., and Slonimski, P. P. (1980) in The Organization and Expression of the Mitochondrial Genome (Kroon, A. M., and Saccone, C., eds.), pp. 139-152, Elsevier/North Holland Biomedical Press, Amsterdam, New York, Oxford

106. Perlman, P. S., Mahler, H. R., Dhawale, S., Hanson, D., and Alexander, N. J. (1980) in The Organization and Expression of the Mitochondrial Genome (Kroon, A. M., and Saccone, C., eds.), pp. 161-172, Elsevier/North Holland Biomedical Press, Amsterdam, New York, Oxford

107. Bonitz, S. G., Coruzzi, G., Thalenfeld, B. E., Tzagoloff, A., and Macino, G. (1980) J. Biol. Chem. 255 11927-11941

108. Corruzzi, G., Bonitz, S. G., Thalenfeld, B. E., and Tzagoloff, A. (1981) J. Biol. Chem. 256,12780-12787

109. Macino, G., Coruzzi, G., Nobrega, F. G., Li, M., and Tzagoloff, A. (1979) Proc. Natl. Acad. Sci. U.S.A. 76,3784-3785

110. Bonitz, S. G., Berlani, R., Coruzzi, G., Li, M., Macino, G., Nobrega, F. G., Nobrega, M. P., Thalenfeld, B. E., and Tzagoloff, A. (1980) Proc. Natl. Acad. Sci. U.S.A. 77,3167-3170

111. Crick, F. H. C. (1966) J. Mol. Biol. 19,548 - 555

112. Anderson, S., Bankier, A. T., Barrell, B. G., De Bruijn, M. H. L., Coulson, A. R., Drouin, J., Eperon, I. C., Nierlich, D. P., Roe, B. A., Sanger, F., Schreier, D. H., Smith, A. J. H., Staden, R., and Young, I. G. (1981) Nature 290,457-465

113. Battey, J., and Clayton, D. A. (1980) J. Biol. Chem. 255,11599-11606

114. Ojala, D., Montoya, J., and Attardi, G. (1981) Nature 290,470-474

115. Montoya, J., Ojala, D., and Attardi, G. (1981) Nature 290,465-470

116. Ching, E., and Attardi, G. (1982) Biochemistry 21,3188-3195

117. Sebald, W. (1977) Biochim. Biophys. Acta 463,1-27

118. Tzagoloff, A., Rubin, M. S., and Sierra, M. F. (1973) Biochim. Biophys. Acta 301,71-104

119. Tzagoloff, A., Akai, A., and Foury, F. (1976) FEBS Lett. 65,391-395

120. Sebald, W., and Viebrock, A. (1982) in EMBO Workshop: Regulatory Mechanisms in Mitochondrial Gene Expression, Abstract, p. 65

121. Edwards, J. C., Christianson, T., Levens, D., Locker, J., Mueller, D., Ticho, B., and Rabinowitz, M. (1982) in EMBO Workshop: Regulatory Mechanisms in Mitochondrial Gene Expression, Abstract, p. 26

122. Shatkin, A. (1976) Cell 9,645-653

123. Jakovcic, S., Hendler, F., Halbreich, A., and Rabinowitz, M. (1979) Biochemistry 18,3200-3205

124. Morimoto, R., Locker, J., Synenki, R. M., and Rabinowitz, M. (1979) J. Biol. Chem. 254,12461-12470

125. Van Ommen, G.-J. B., Boer, P. H., Groot, G. S. P., de Haan, M., Roosendaal, E., Grivell, L. A., Haid, A., and Schweyen, R. J. (1980) Cell 20,173-183

126. Grivell, L. A., Arnberg, A. C., Boer, P. H., Borst, P., Bos, J. L., Van Bruggen, E. F. J., Groot, G. S. P., Hecht, N. B., Hensgens, L. A. M., Van Ommen, G. J. B., and Tabak, H. F. (1979) ICN-UCLA Symp. Mol. Cell. Biol. 15,305-324

127. Attardi, G., Chomyn, A., Mariottini, P., and Montoya, J. (1982) in EMBO Workshop: Regulatory Mechanisms in Mitochondrial Gene Expression, Abstract, p. 2

128. Yanofsky, C. (1981) Nature 289,751-758

129. Murphy, W. I., Attardi, B., Tu, C., and Attardi, G. (1975) J. Mol. Biol. 99,809-814

130. Avadhani, N. G., Lewis, F. S., and Rutman, R. J. (1974) Biochemistry 13,4638-4645

131. Ojala, D., and Attardi, G. (1974) J. Mol. Biol. 88,205-219

132. Hirsch, M., and Penman, S. J. (1973) J. Mol. Biol. 80,379-391

133. Coopers, C. S., and Avers, C. J. (1974) in The Biogenesis of Mitochondria (Kroon, A. M., and Saccone, C., eds.), pp. 289-303, Academic Press, New York

134. Hendler, F., Padmanaban, G., Patzer, J., Ryan, R., and Rabinowitz, M. (1975) Nature 258,357-359

135. Groot, G. S. P., Flavell, R. A., Van Ommen, G. J. B., and Grivell, L. A. (1974) Nature 252,167-169

136. Reijnders, L., Kleisen, C. M., Grivell, L. A., and Borst, P. (1972) Biochim. Biophys. Acta 272,396-407

137. Chiu, N., Chiu, A., and Suyama, Y. (1975) J. Mol. Biol. 99,37-50

138. Suyama, Y., and Hamada, J. (1976) in Genetics and Biogenesis of Chloroplasts and Mitochondria (Bücher, T., Neupert, W., Sebald, W., and Werner, S., eds.), pp. 763-770, North Holland, Amsterdam

139. Vasquez, D. (1966) Symp. Soc. Gen. Microbiol. 16,169

140. Huang, M., Biggs, D. R., Clark-Walker, G. D., and Linnane, A. W. (1966) Biochim. Biophys. Acta 114, 434-436

141. Clark-Walker, G. D., and Linnane, A. W. (1967) J. Cell. Biol. 34,1-14

142. Lamb, A. J., Clark-Walker, G. D., Linnane, A. W. (1968) Biochim. Biophys. Acta 161,415-427

143. Mahler, H. R., and Perlman, P. S. (1971) Biochemistry 10,2977-2990

144. Tzagoloff, A., and Meagher, P. (1972) J. Biol. Chem. 247,594-603

145. Mason, T. L., and Schatz, G. (1973) J. Biol. Chem. 248,1355-1360

146. Weiss, H. (1976) Biochim. Biophys. Acta 456,291-313

147. Katan, M. B., Van Harten-Loosbroek, N., and Groot, G. S. P. (1976) Eur. J. Biochem. 70,409-417

148. Lin, L.-F. H., Clejan, L., and Beattie, D. S. (1978) Eur. J. Biochem. 87,171-179

149. Lambowitz, A. M., Chua, N.-H., and Luck, D. J. L. (1976) J. Mol. Biol. 107,223-253

150. Groot, G. S. P., Mason, T. L., and Van Harten-Loosbroek, N. (1979) Mol. Gen. Genet. 174,339-342

151. Douglas, M. G., and Butow, R. A. (1976) Proc. Natl. Acad. Sci. U.S.A. 73,1083-1086

152. Ibrahim, N. G., Stuchell, R. N., and Beattie, D. S. (1973) Eur. J. Biochem. 36,519-527

153. Poyton, R. O., Groot, G. S. P. (1975) Proc. Natl. Acad. Sci. U.S.A. 72,172-176

154. Andre, J., and Marinozzi, V. (1965) J. Microscopie (Paris) 4,615-626

155. O'Brien, T. W., and Kalf, G. F. (1967) J. Biol. Chem. 242,2172-2179

156. Pollard, C. J., Stemler, A., and Blaydes, D. F. (1966) Plant Physiol. 41,1323-1329

157. Vesco, C., and Penman, S. (1969) Proc. Natl. Acad. Sci. U.S.A. 62,218-225

158. Rifkin, M. R., Wood, D. D., and Luck, D. J. L. (1967) Proc. Natl. Acad. Sci. U.S.A. 58,1025-1032

159. Wintersberger, E. (1967) Z. Physiol. Chem. 348,1701-1704

160. O'Brien, T. W., and Matthews, D. E. (1976) in Handbook of Genetics, Vol. 5 (King, R. C., ed.), Chapter 16, Plenum Press, New York

161. deVries, H., and Kroon, A. M. (1974) in The Biogenesis of Mitochondria (Kroon, A. M., and Saccone, C., eds.), pp. 357-365, Academic Press, New York

162. Datema, R., Agsteribbe, E., and Kroon, A. M. (1974) Biochim. Biophys. Acta 335,386-395

163. Moorman, A. F. M., and Grivell, L. A. (1975) in Molecular Biology of Nucleocytoplasmic Relationships (Puiseux-Dao, S., ed.), pp. 61-67, Elsevier, Amsterdam

164. Grivell, L. A., Reijnders, L., and Borst, P. (1971) Biochim. Biophys. Acta 247,91-103

165. Scragg, A. H. (1971) FEBS Lett. 17,111-114

166. Küntzel, H. (1969) FEBS Lett. 4,140-142

167. Swanson, R. F. (1973) Biochemistry 12,2142-2146

168. Richter, D., and Lipmann, F. (1970) Biochemistry 9,5065-5070

169. Grandi, M., Helms, A., and Küntzel, H. (1971) Biochem. Biophys. Res. Commun. 44,864-871

170. Barnett, W. E., and Brown, D. H. (1967) Proc. Natl. Acad. Sci. U.S.A. 57,452-458

171. Halbreich, A., and Rabinowitz, M. (1971) Proc. Natl. Acad. Sci. 68,294-298

172. Martin, N. C., and Rabinowitz, M. (1978) Biochemistry 17,1628-1634

173. Galper, J. B., and Darnell, J. E. (1969) Biochem. Biophys. Res. Commun. 34,205-214

174. Galper, J. B., and Darnell, J. E. (1971) J. Mol. Biol. 57,363-367

175. Bianchetti, R., Lucchini, G., and Sartirana, M. L. (1971) Biochem. Biophys. Res.Commun. 42,97-102

176. Epler, J. L., Shugart, L. R., and Barnett, W. E. (1970) Biochemistry 9,3575-3579

177. Smith, A. E., and Marcker, K. A. (1968) J. Mol. Biol. 38,241-243

178. Mahler, H. R., Dawidowicz, K., and Feldman, F. (1972) J. Biol. Chem. 247,7439-7442

179. Feldman, F., and Mahler, H. R. (1974) J. Biol. Chem. 249,3702-3709

180. Stone, A. B., and Wilkie, D. (1974) J. Gen. Microbiol. 83,283-293

181. Stone, A. B., and Wilkie, D. (1975) Biochim. Biophys. Acta 390,133-136

182. Zelikson, R., and Luzzati, M. (1977) Eur. J. Biochem. 79,285-292

183. Schneller, J. M., Schneller, C., Martin, R., and Stahl, A. J. C. (1976) Nucleic Acids Res. 3,1151-1165

184. Beauchamp, P. M., Horn, E. W., and Gross, S. R. (1977) Proc. Natl. Acad. Sci. U.S.A. 74,1172-1176

185. Barnett, W. E., Brown, D. H. and Epler, J. L. (1967) Proc. Natl. Acad. Sci. U.S.A. 57,1775-1781

186. Borst, P., and Grivell, L. A. (1978) Cell 15,705-723

187. De Ronde, A., Van Loon, A. P. G. M., Grivell, L. A., and Kohli, J. (1980) Nature 287,361-363

188. Sevarino, K. A., and Poyton, R. O. (1980) Proc. Natl. Acad. Sci. U.S.A. 77,142-146

189. Pfisterer, J., and Buetow, D. E. (1981) Proc. Natl. Acad. Sci. U.S.A. 78,4917-4921

190. Finzi, E. (1982) Unpublished observations

191. Finzi, E., Rinehart, R. W., Sperling, M., and Beattie, D. S. (1982) FEBS Lett. 137,314-318

192. Kim, I.-C., and Beattie, D. S. (1973) Eur. J. Biochem. 36,509-518

193. Brown, G. G., and Beattie, D. S. (1978) Biochim. Biophys. Acta 538,173-187

194. Lin, L.-F. H., Kim, I. C., and Beattie, D. S. (1974) Arch. Biochem. Biophys. 160,458-464

195. Tzagoloff, A. (1971) J. Biol. Chem. 246,3050-3056

196. Ibrahim, N. G., Stuchell, R. N., and Beattie, D. S. (1973) Eur. J. Biochem. 36,519-527

197. Ibrahim, N. G., and Beattie, D. S. (1976) J. Biol. Chem. 251,108-115

198. Poyton, R. O., and Kavanagh, J. (1976) Proc. Natl. Acad. Sci. U.S.A. 73,3947-3951

199. Poyton, R. O., and McKemmie, E. (1979) J. Biol. Chem. 254,6763-6771

200. Poyton, R. O., and McKemmie, E. (1979) J. Biol. Chem. 254,6772-6780

201. Lewin, A. S., Gregor, I., Mason, T. L., Nelson, N., and Schatz, G. (1980) Proc. Natl. Acad. Sci. U.S.A. 77,3998-4002

202. Mihara, K., and Blobel, G. (1980) Proc. Natl. Acad. Sci. U.S.A. 77,4160-4164

203. Ebner, E., Mason, T. L., and Schatz, G. (1973) J. Biol. Chem. 248,5369-5378

204. Ono, B.-I., Fink, G., and Schatz, G. (1975) J. Biol. Chem. 250,775-782

205. Saltzgaber-Müller, J., and Schatz, G. (1978) J. Biol. Chem. 253,305-310

206. Tzagoloff, A., Akai, A., and Needleman, R. B. (1975) J. Biol. Chem. 250,8228-8235

207. Dieckmann, C. L., Pape, L. K., and Tzagoloff, A. (1982) Proc. Natl. Acad. Sci. U.S.A. 79,1805-1809

208. Feldman, F., and Mahler, H. R. (1974) J. Biol. Chem. 249,3702-3709

209. Ray, D. B., and Butow, R. A. (1979) Molec. Gen. Genet. 173,239-247

210. LaPolla, R. J., and Lambowitz, A. M. (1977) J. Mol. Biol. 116,189-205

211. Collins, R. A., Bertrand, H., LaPolla, R. J., and Lambowitz, A. M. (1979) Mol. Gen. Genet. 177,73-84

212. Terpstra, P., Zanders, E., and Butow, R. A. (1979) J. Biol. Chem. 254,12653-12661

213. Lambowitz, A. M. (1980) in The Organization and Expression of the Mitochondrial Genome (Kroon, A. M., and Saccone, C., eds.), pp. 291-300, Elsevier/North Holland Biomedical Press, Amsterdam, New York, Oxford

214. Buetow, D. E., and Wood, W. M. (1978) Subcell. Biochem. 5,1-85

215. Foury, F., and Tzagoloff, A. (1976) Mol. Gen. Genet. 149,43-50

216. Clavilier, L. (1976) Genetics 83,227-233

217. Foury, F., and Tzagoloff, A. (1976) Eur. J. Biochem. 68,113-119

218. Tzagoloff, A., Foury, F., and Okai, A. (1976) Mol. Gen. Genet. 149,33-42

219. Mans, R. J., and Novelli, G. D. (1961) Arch. Biochem. Biophys. 94,48-53

220. Kroon, A. M., Saccone, C., and Botman, M. J. (1967) Biochim. Biophys. Acta 142,552-554

221. Beattie, D. S., Chen, Y. S., Clejan, L., and Lin, L.-F. H. (1979) Biochemistry 18,2400-2406

222. Avadhani, N. G., and Buetow, D. E. (1972) Biochem. Biophys. Res. Commun. 46,773-781

223. Narang, F. E., Bertrand, H., and Werner, S. (1978) J. Biol. Chem. 253,6364-6369

224. Bertrand, H., and Werner, S. (1977) Eur. J. Biochem. 79,599-606

225. Cabral, F., and Schatz, G. (1978) J. Biol. Chem. 253,4396-4401

226. Everett, T., Finzi, E., and Beattie, D. S. (1980) Arch. Biochem. Biophys. 200,467-473

227. Ohashi, A., and Schatz, G. (1980) J. Biol. Chem. 255,7740-7745

228. Blakley, R. L. (1969) in The Biochemistry of Folic Acid and Related Pteridines (Neuberger, A., and Tatum, E. L., eds.), pp. 58-105, Elsevier, New York

229. Gupta, V. S., and Huennekens, F. M. (1967) Biochemistry 6,2163-2177

230. Waxman, S., and Schreiber, C. (1980) Methods Enzymol. 66,468-483

231. Bianchetti, R., Lucchini, G., Crosti, P., and Tortora, P. (1977) J. Biol. Chem. 252,2519-2523

232. Lloyd, D. (1974) in The Mitochondria of Microorganisms, pp. 99-104, Academic Press, London

233. Ades, I. Z., and Butow, R. A. (1980) J. Biol. Chem. 255,9918-9924

234. Grivell, L. A., and Roodyn, D. B. (1970) Biochem. J. 116,26P

235. Finzi, E., Sperling, M., and Beattie, D. S. (1981) J. Biol. Chem. 256,11917-11922

236. Mockel, J. J., and Beattie, D. S. (1975) Arch. Biochem. Biophys. 167,301-310

237. Rinehart, R. W., Roberson, J., and Beattie, D. S. (1982) Arch. Biochem. Biophys. 213,341-352

238. Beattie, D. S. (1968) Biochem. Biophys. Res. Commun. 31,901-907

239. Rothblum, L. I., Devlin, T. M., and Ch'ih, J. J. (1976) Biochem. J. 156,151-157

240. Ch'ih, J. J., Procyk, R., and Devlin, T. M. (1977) Biochem. J. 162,501-507

241. Ch'ih, J. J., Pike, L. M., and Devlin, T. M. (1977) Biochem. J. 168,57-63

242. Ch'ih, J. J., Froman, P. A., and Devlin, T. M. (1978) Proc. Soc. Exp. Biol. Med. 159,288-293

243. Beattie, D. S., and Ibrahim, N. (1973) Biochemistry 12,176-180

244. Overath, P., Schairer, H. U., and Stoffel, W. (1970) Proc. Natl. Acad. Sci. U.S.A. 67,606-612

245. Esfahani, M., Limbick, A. R., Krutton, S., Okal, T., and Wakil, S. (1971) Proc. Natl. Acad. Sci. U.S.A. 68,3180-3184

246. Engelman, D. M. (1971) J. Mol. Biol. 58,153-165

247. Steim, J. M., Tourtellote, M. E., Reinert, J. C., McElhaney, R. N., and Rader, R. L. (1969) Proc. Natl. Acad. Sci. U.S.A. 63,104-109

248. Raison, J. K., Lyons, J. M., Thomson, W. W. (1971) Arch. Biochem. Biophys. 142,83-90

249. Eletr, S., and Keith, A. D. (1971) Proc. Natl. Acad. Sci. U.S.A. 69,1353-1357

250. Wilson, G., Rose, S. P., and Fox, C. F. (1970) Biochem. Biophys. Res.Commun. 38,617-623

251. Raison, J. K., Lyons, J. M., Mehlhern, R. J., and Keith, A. D. (1971) J. Biol. Chem. 246,4036-4040

252. Hubbell, W. L., and McConnell, H. M. (1971) J. Amer. Chem. Soc. 93,314-326

253. Overath, P., and Trauble, H. (1973) Biochemistry 12,2625-2634

254. Lussan, C., and Fancon, J. F. (1971) FEBS Lett. 19,186-188

255. Reinert, J. C., and Steim, J. M. (1970) Science 165,1580-1582

256. Melchior, D. L., Morowitz, H. J., Sturtevant, J. M., and Tsong, T. Y. (1970) Biochim. Biophys. Acta 219,114-122

257. Luzzati, V. (1968) in Biological Membranes (Chapman, D., ed.), p. 71, New York, Academic Press

258. Schairer, H. U., and Overath, P. (1969) J. Mol. Biol. 44,209-214

259. Rosen, B. P., and Hackette, S. L. (1972) J. Bacteriol. 110,1181-1189

260. Janki, R. M., Aithal, H. N., McMurray, W. C., and Tustanoff, E. R. (1974) Biochem. Biophys. Res. Commun. 56,1078-1085

261. Watson, K., Bertoli, E., and Griffiths, D. E. (1975) Biochem. J. 146,401-407

262. Watson, K., Houghton, R. L., Bertoli, E., and Griffiths, D. E. (1975) Biochem. J. 146,409-416

263. Ainsworth, P. J., and Tustanoff, E. R. (1972) Biochem. Biophys. Res.Commun. 47,1299-1305

264. Marzuki, S., Cobon, G. S., Crowfoot, P. D., and Linnane, A. W. (1975) Arch. Biochem. Biophys. 169, 591-600

265. Lenaz, G., Sechi, A. M., Parenti-Castelli, G., Landi, L., and Bertoli, E. (1972) Biochem. Biophys. Res. Commun. 49,536-542

266. Towers, N. R., Kellerman, G. M., Raison, J. K., and Linnane, A. W. (1973) Biochim. Biophys. Acta 299,153-161

267. Cybulski, R. L., and Fisher, R. R. (1977) Biochemistry 15,5116-5120

268. King, M. J., and Diwan, J. J. (1973) Arch. Biochem. Biophys. 159,166-173

269. Gamble, J. G., and Lehninger, A. L. (1973) J. Biol. Chem. 248,610-618

270. Cybulski, R. L., and Fisher, R. R. (1976) Biochemistry 15,3183-3187

271. Brown, G. G., and Beattie, D. S. (1977) Biochemistry 16,4449-4454

272. McKee, E. E., Sevarino, K. A., Bellus, G., and Poyton, R. O. (1981) in Current Developments in Yeast Research (Steward, G. G., and Russell, I., eds.), p. 357-361, Pergamon Press,

273. Brawand, F., and Walter, P. (1974) Analytical Biochemistry 62,485-498

274. Heytler, P. G. (1979) Methods Enzymol. 55,462-472

275. Stigall, D. L., Galante, Y. M., and Hatefi, Y. (1979) Methods Enzymol. 55,308-315

276. Klingenberg, M. (1970) in Essays in Biochemistry, Vol. 6 (Campbell, P. N., and Dickens, F., eds), p. 119, New York, Academic Press

277. O'Brien, T. W., and Kalf, G. F. (1967) J. Biol. Chem. 242,2180-2185

278. Grivell, L. A., Reijnders, L., and Borst, P. (1971) Biochim. Biophys. Acta 247,91-103

279. Neher, E. M., Harmey, M. A., Hennig, B., Zimmerman, R., and Neupert, W. (1980) in The Organization and Expression of the Mitochondrial Genome (Kroon, A. M., and Saccone, C., eds.), pp. 413-422, Elsevier/North Holland, Amsterdam

280. Buchanan, J., Popovitch, J. R., and Tapley, D. F. (1969) Biochim. Biophys. Acta 173,532-539

281. Finzi, E., Rinehart, R. W., Sperling, M., and Beattie, D. S. (1982) FEBS Lett. 137,314-318

282. Kramer, G., and Hardesty, B. (1980) in Cell Biology, Vol. 4, Gene Expression: Translation and the Behavior of Proteins (Prescott, D. M., and Goldstein, L., eds.), pp. 69-96, Academic Press, New York

283. Larner, J., Huang, L. C., Brooker, G., Murad, F., and Miller, T. B. (1974) Fed. Proc. 33,261 abstr.

284. Larner, J., Galasko, G., Cheng, K., De Paoli-Roach, A. A., Huang, L., Daggy, P., and Kellog, J. (1979) Science 206,1408-1410

285. Seals, J. R., and Czech, M. P. (1981) J. Biol. Chem. 256,2894-2899

286. Kiechle, F. L., Jarett, L., Kotagal, N., and Popp, D. A. (1981) J. Biol. Chem. 256,2945-2951

287. Beattie, D. S. (1971) Arch. Biochem. Biophys. 147, 136-142

288. Saltzgaber-Müller, J., and Schatz, G. (1978) J. Biol. Chem. 253,305-310

289. Clejan, L., Beattie, D., Gollub, E. G., Lin, K. P., and Sprinson, D. P. (1980) J. Biol. Chem. 255,1312-1316

290. Kumar, C. C., and Padmanaban, G. (1980) J. Biol. Chem. 255,11130-11134

291. O'Malley, K., Pratt, P., Robertson, J., Lilly, M., and Douglas, M. G. (1982) J. Biol. Chem. 257,2097-2103