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Isolation of protein synthesis initiation factors using Volvox Carteri: a clue to the translational control of cytodifferentiation

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THE ISOLATION OF PROTEIN SYNTHESIS INITIATION FACTORS USING *VOLVOX CARTERI:*

A Clue to the Translational Control of Cytodifferentiation

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ABSTRACT

Volvox carteri, a multicellular green algae, possesses two distinct cell types--the gonidia and somatic cells. This simple germ-soma dichotomy is ideal for the study of the control of cytodifferentiation. The control of cytodifferentiation in this organism has been identified as a posttranscriptional mechanism, which suggests translation. Initiation factors have been shown to play an important role in controlling translation in many other developmental systems. Therefore, study of the initiation factors in *Volvox* may shed light on a translational control mechanism. In order to study any modifications of these factors, they must be isolated, possibly by following an adapted wheat germ protocol. Further studies elucidating the phosphorylation state of these factors should be conducted to determine their role in controlling translation in *Volvox* and thus their role in the divergence of two distinct phenotypes by the descendents of a single cell.

INTRODUCTION

Volvox carteri presents the biological researcher with an ideal instrument with which to study the control of cytodifferentiation. This multicellular green algae possesses two distinct cell types--the gonidia or reproductive line and the somatic or vegetative line. There is a complete division of labor between these two cell types. During the early 1900s biologists realized the value of this germ-soma dichotomy to developmental studies. Powers wrote in 1904 that "we have in this simple *Volvox* aggregate a perfect example of the continuity of germ cells" (1). But it was not until the 1960s, when the asexual reproduction of the algae could be controlled in the laboratory, that developmental studies of *Volvox* were initiated. Studying the control of

cytodifferentiation using the controlled asexual life cycle of *Volvox* may reveal what controls the regular development of divergent phenotypes by the descendents of a single cell.

Each adult *Volvox* individual contains an outer shell of approximately 2000 biflagellate somatic cells. Inside this shell is a transparent glycoprotein-rich extracellular matrix which separates and stabilizes approximately sixteen large gonidia cells. During embryogenesis the gonidia give rise to juveniles which contain the full complement of somatic and gonidia cells.

The asexual life cycle of *Volvox* can be synchronized by a 24-hour light/dark cycle so that large cultures of synchronously developing clones can be maintained. During two periods of 16 hours light and 8 hours dark the organism reproduces every 48 hours. At the onset of one light period the juveniles hatch from the parental cells. These juveniles swim away and mature into adults while the parental cells undergo senescence and die. Each mature gonidium of the prior juveniles begin cleavage divisions after approximately seven hours of illumination. Cleavage is the process by which the gonidium undergoes 11-12 rapid cell divisions, producing a miniature version of the adult. The sixth cleavage is asymmetric, resulting in large and small cells. The large cells undergo 2 more small cleavage divisions which result in somatic cells. The small cells of the sixth cleavage undergo 3-4 more cleavage divisions for a total of 11-12 divisions for somatic cells. The large cells at this stage become the gonidia initials and the small cells become the somatic initials. At the end of cleavage these presumptive gonidia and somatic cells remain undifferentiated cytologically and biochemically. They are distinguishable only by size and are connected by a cytoplasmic bridge.

Also, the fully cleaved embryo is inside out--the presumptive gonidia are at the outside and the presumptive somatic cells are at the inside of the hollow embryonic sphere. At the end of the dark period the embryo undergoes inversion to correct this problem, creating a juvenile which resembles the adult configuration--gonidia located at the interior of the sphere surrounded by the monolayer of somatic cells. Throughout the next light and dark periods the cytoplasmic bridges break and the gonidial and somatic initials expand and differentiate into their mature forms and are called a juvenile (1). At the onset of the next light period these juveniles are hatched and swim free of the parental cells, starting the life cycle again. A diagram of the lifecycle cycle is shown in Figure 1.

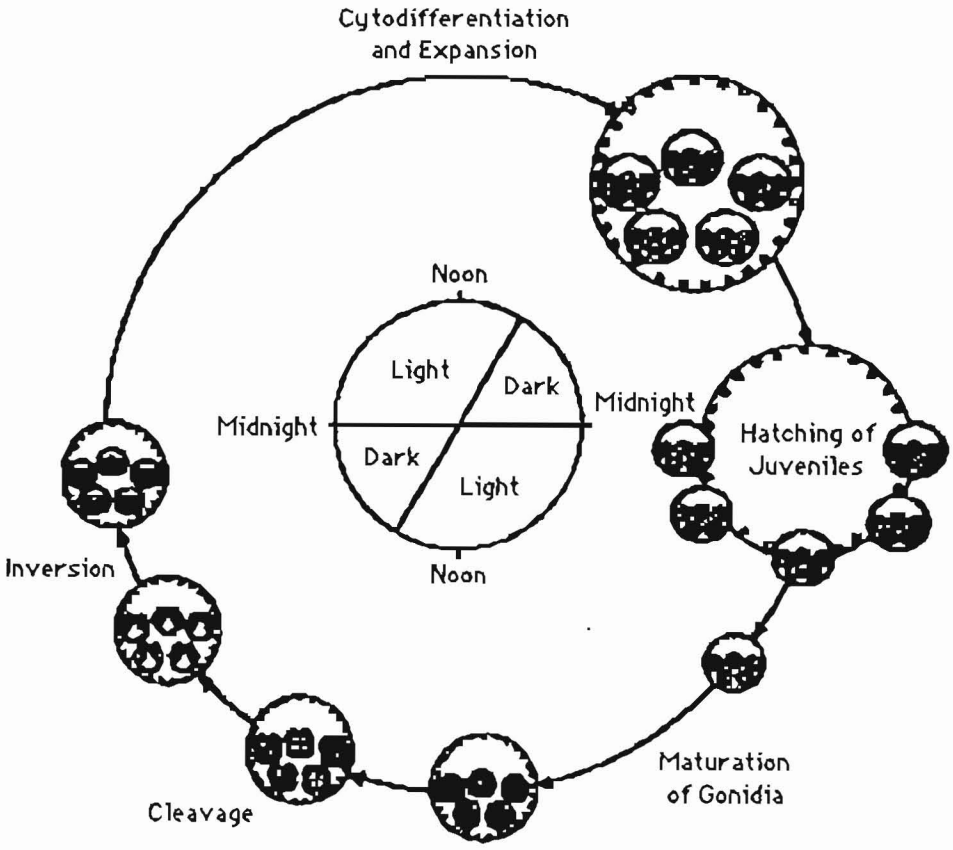


Figure 1. The 48 hour asexual life cycle of *Volvox carteri*, synchronized by a 24 hour light/dark cycle (reproduced from reference 2).

Polypeptide synthetic patterns during these stages of embryogenesis shed light on possible control mechanisms involved in the cytodifferentiation of *Volvox*. After inversion is completed the presumptive gonidia and somatic cells remain in an arrested, undifferentiated state. Upon illumination the cells undergo a rapid change in polypeptide synthesis altering the types of polypeptides present. This polypeptide synthesis correlates with breaking of the cytoplasmic bridges followed by subsequent cytodifferentiation into mature gonidia and somatic cells. Studies have shown that mRNA transcripts are similar during the dark and light period cells. This suggests that the change in cytodifferentiation is a result of a posttranscriptional process such as translation.

The rate-limiting step of translation is during the initiation phase and so this may be the most likely step at which translational control is exerted. Initiation factors appear to be responsible for control during this phase. They are involved in this control by phosphorylation/ dephosphorylation reactions. Thus, the control mechanisms of the light-dependent change in polypeptide synthesis of *Volvox* and the accompanying cytodifferentiation could possibly be elucidated by a search for the role of the initiation factors during this period. But first it must be shown that these initiation factors can be isolated from *Volvox* cultures.

PROTEIN SYNTHETIC PATTERNS IN *VOLVOX*

As discussed above, embryogenesis in *Volvox* is completed in the dark but little differentiation takes place until the the culture is illuminated. Within a few hours of light the cytoplasmic bridges are broken, gonidia and somatic cells have different polypeptide patterns, and

they assume their different physical characteristics. The studies that describe these results and other studies which lead us to study translational control are described below. A brief discussion of the steps of translation prepare us for specific examples observed in *Volvox*.

Translation is the mRNA-directed biosynthesis of polypeptides. Proteins are template manufactured through the specific interactions of numerous molecules. In a process called transcription, DNA codes for mRNA. The mRNA molecule binds to a ribosome. Ribosomes read mRNA in the 5'->3' direction in order to translate the nucleotide code of mRNA into the amino acid code of proteins, a process called translation. Nucleotide triplets on mRNA code for tRNA molecules, which carry amino acids to the ribosome. Active translation occurs on polysomes (numerous ribosomes attached to the same mRNA molecule at gaps of about 80 nucleotides). The ribosome starts to read the mRNA molecule upon recognition of the start codon AUG (the nucleotides adenine, uracil, and guanine). Initiation is the process by which a ribosome binds to a preexisting polysome. It requires four steps, in addition to initiation factors: 1) at completion of a previous event the ribosome dissociates into the 40S and 60S subunits; 2) initiation tRNA binds to the 40S ribosomal subunit to form the 40S preinitiation complex; 3) mRNA binds to the 40S initiation complex; and 4) the 40S initiation complex binds with the 60S subunit to form an 80S initiation complex (3). Elongation is the addition of amino acids to the C-terminal end of a nascent polypeptide chain. Elongation also requires four steps, in addition to elongation factors: 1) aminoacyl-tRNA binds to the A site of the ribosome, catalyzed by an elongation factor; 2) GTP is hydrolyzed and the the elongation factor is ejected; 3) the peptide bond is formed; and 4) the peptidyl-tRNA in the A site is transferred to the P site

of the ribosome, which opens the A site (3). Translation is halted when the codons UAA, AGA, or UAG--which have no corresponding tRNAs but bind with a release factor--are encountered in the reading frame and so the polypeptide is freed from the ribosome.

The scanning model of translation explains how the 40S subunit recognizes the AUG codon and so how reading for translation starts. There appear to be three steps: 1) the 40S subunit threads onto the 5' end of the mRNA; 2) the ATP-required linear scanning of the mRNA by the 40S subunit; and 3) migrating 40S subunit stops at the first AUG codon and translation progresses (4). This model fits into the translation scheme by explaining how the ribosome chooses a reading frame, which establishes in which order the mRNA triplets are translated.

Kirk and Kirk established by 1983 that the polypeptide incorporation of ^{35}S labels increased rapidly upon illumination after a dark period (4). They showed that the change in polypeptide synthesis was due to illumination and not a coincidence of some stage-dependent process by using premature and delayed illumination. The cultures subjected to premature illumination showed an increase of polypeptides at the time of illumination rather than the usual time. The delayed cultures did not produce the polypeptides at the usual time but waited until the lights were turned on (5). Changes in the polypeptide labeling are reversible during the first 2 hours of illumination but by the fourth hour the two cell types have divergent polypeptide labeling patterns and are irreversible (6).

In 1985 Kirk and Kirk proved that ^{35}S labeling was a reliable method for determining polypeptide labeling patterns. Studies with ^{3}H-arginine confirmed the conclusions drawn from the ^{35}S studies: the rate of

polypeptide synthesis in illuminated cultures is greater than in unilluminated cultures (6). Thus, difference in efficiency of incorporation of the ^{35}S label did not account for the differences observed between the light and dark periods.

Pulse-chase experiments showed that the polypeptide pattern differences between dark and light periods are not due to unilluminated cells rapidly degrading protein that cannot be incorporated into chloroplasts or light causing a breakdown of proteins, which would result in an exaggerated amount of proteins (6). Pulse-chase experiments at 5 minutes of label incubation for light and dark cultures showed similar results to the 1 hour label incubation. These results proved that protein turnover or breakdown over a one hour period was not the cause for the differences observed between dark and light polypeptide patterns.

The relative rates of label incorporation for five specific proteins were compared. Three chloroplast-related proteins-- one of which is manufactured in the chloroplast (the large subunit of RuBP-carboxylase), and the others in the cytosol (the small subunit of RuBP-carboxylase and the chlorophyll a/b binding protein)--were found in abundance during the light period relative to the dark period. Proteins not associated with the chloroplast (the α and β tubulins) were also found in abundance during the light period, although less abundant than the chloroplast-related proteins (6). This suggests that the change in types of polypeptides during the light period is not due to the chloroplast's response to light but rather a global response to the light which promotes the differentiation of the cells from presumptive gonidia and somatic cells to fully differentiated juveniles.

Kirk and Kirk also established that the change in polypeptide patterns is not due to the photosynthetic activity of chloroplasts. They reasoned that the action spectra of the polypeptide labeling should resemble the action spectra observed for photosynthesis in other green algae--a maxima in purple and red light and a minima in green light. The study consisted of illuminating the *Volvox* at different wavelengths and monitoring incorporation. The efficiency of incorporation was maximized in green light (6). These results appear to refute the possibility that the increase in proteins is due to photosynthetic activity because the maxima action spectra for *Volvox* occurs during the minima for other green algae. In addition, an inhibitor of photosynthetic electron transport (DCMU) and an uncoupler of photophosphorylation (CCCP) have no effect on the pattern of protein synthesis of the light period cells (5, 6, 7). This again supports the idea that an increase in polypeptide labeling is due to differentiation of embryos into juveniles rather than a simple increase in photosynthetic activity upon illumination.

To determine whether the change in polypeptide synthesis upon illumination is due to altered transcriptional activity, cultures were exposed to actinomycin D (an inhibitor of transcription) either before or after illumination. The differences in polypeptide labeling patterns were negligible with and without actinomycin D treatment when compared to the differences observed with dark and light period polypeptide patterns. To insure that transcription was completely inhibited, heat shock (known to be a transcriptionally controlled process) was used as a control. Cultures that were first exposed to actinomycin D treatment then heat shock and then light and label showed that the heat-shock effect is almost completely inhibited but the light effect is unaffected (7). These results

prove that the increase in polypeptide synthesis in response to light is not due to increased transcription and thus must be posttranscriptional.

The *in vitro* translation of mRNAs isolated from light and dark period cells confirmed this result. Total cellular RNAs were isolated from both dark and light period sibling cultures and translated through *in vitro* techniques, using a reticulocyte lysate system. The *in vitro* translation products were extremely similar for the dark period and the light period, after one hour of illumination. Both sets of RNA products were more similar to the *in vivo* illuminated polypeptides than the *in vivo* unilluminated polypeptides. Heat shock was again used as a control during the *in vitro* translation to insure that any difference in the translatable messages of the mRNAs would be detectable (6, 7). The results were similar to those already discussed. This shows that the same pool of mRNA is available for translation in both light and dark period cells but the mRNAs are utilized differently. Thus, a translational control mechanism seems to be operating during the latter part of the dark period to prevent the synthesis of many proteins. Upon illumination, the translational repression is relieved.

MECHANISMS OF TRANSLATIONAL CONTROL

Translational control can be characterized according to when it takes place--whether it is during the initiation phase, elongation phase, or if it is generalized repression. Numerous mechanisms appear to account for the control of translation including polyadenylation of mRNAs, mRNA untranslated sequences, secondary structure, elongation controls, mobilization of inactive mRNAs into the active mRNA pool, and initiation factors.

The structure of specific mRNAs appears to have a role in the regulation of translation rates. The polyadenylation of mRNAs after transcription appears to stimulate mRNA translation. A detailed study concluded that poly(A)$^-$ mRNAs are recruited less efficiently into polysomes than poly(A)$^+$ mRNAs (3). In tissue plasminogen activator mRNA the poly(A) tail is lengthened by 400-600 adenylate residues during mouse oocyte maturation which results in strong activation of translation (3). But genetic studies in yeast suggest that the poly(A) binding protein (PABP) and the poly(A) tails may be involved in establishing the distribution of mRNAs between mRNPs (inactive mRNA molecules) and polysomes rather than the actual initiation rate on polysomes. This was discovered by deleting the PABP gene from yeast which resulted in a decreased amount of RNA in polysomes but not the number of polysomes per mRNA (3). Reticulocyte lysate *in vitro* assays showed that deadenylation reduced initiation rates by 40% and that this was not caused by mRNA degradation. In a nuclease-treated reticulocyte lysate system, VSV mRNAs were translated at 1.5-3.0 times greater efficiency if they were polyadenylated (8). Thus, polyadenylation may play a role in increasing the efficiency of translation of specific mRNAs.

The regulation of ferritin mRNAs and transferrin receptor mRNAs by iron presents a novel mechanism for translational control (9). Ferritin is a protein that sequesters excess iron whereas transferrin receptor controls iron uptake. Common RNA sequences in the 5' and 3' noncoding regions of these mRNAs appear to be the modulator in these interactions. The location of these common sequences, called iron responsive elements (IRE), may be related to the opposite effects exerted on the two proteins. When the IREs are in the 5'- untranslated region of ferritin mRNA,

translation is enhanced by excess iron; whereas the presence of the IREs in the 3' untranslated region of the transferrin mRNA leads to iron-dependent degradation. A protein which binds IRE sequences and blocks translation of ferritin mRNA, but does not appear to be an iron-binding protein, has been isolated from liver and red blood cells. This protein, called FRP, apparently works by oxidation/reduction mechanisms, possibly a disulfide-sufhydryl switch that is regulated by iron (3). This type of translational regulation by common mRNA sequences may be a global control mechanism for coordinating the synthesis of metabolically related proteins (9).

It appears that the elongation phase is not an important control site in most organisms because: 1) the initiation phase is usually the rate-limiting step; 2) the same mechanism for elongation is used for all mRNAs so any specific control seems unlikely; 3) most cells function at near maximum rates of elongation so that any control exerted would be limited; and 4) ribosomes would not be used efficiently if they were tied up in an inhibited elongation phase (3). But elongation control has been observed in several organisms. HeLa cells that are deprived of serum for 24 hours have an elongation rate that is one-half that of serum-fed cells. Also, *Drosophila* cells stressed by heat shock have severely inhibited elongation rates as do HeLa cells that are stressed by amino acid analogues. In similar case, the HSP 70 mRNA of chicken reticulocytes in nonstressed cells is present in polysomes but translated slowly whereas upon heat shock the elongation block is removed and translation increases 10-fold. Frameshifting and readthrough of a stop codon can also increase elongation of a polypeptide. Frameshifting is caused by slippage of a tRNA derivative from one codon to the next or the one before it. This creates a

new reading frame. Stimulators of frameshifting include secondary structure elements, an adjacent stop codon, and the upstream Shine/Dalgarno region (3). Readthrough allows a ribosome to continue translation past a stop codon and so translate distal regions of mRNA. This phenomenon is observed in murine leukemia virus, Sindbis virus, and feline leukemia virus. Stimulators of readthrough may include secondary structure elements and the insertion of selenocysteine into glutathione oxidase as coded by UGA in mammalian cells (3). Elongation control mechanisms do participate in controlling the rate of protein synthesis but apparently to a lesser degree than initiation control mechanisms.

The mobilization stage of initiation is the binding of the first ribosome to an mRNP to begin the formation of a polysome (3). Regulation of the proportion of active to non-active mRNAs is a means of translational control. When mouse lymphosarcoma cells are treated with dexamethasone a shift of mRNA from polysomes into mRNPs occurs, thus inhibiting translation. Also, in early development of sea urchins and *Xenopus* oocytes there is a shift from mRNAs of the mRNP pool into mRNAs of polysomes. Also, in the presence of low glucose insulin mRNAs are found in mRNPs but in the presence of high glucose concentrations they are mobilized into polysomes. Proteins that are associated with the mRNPs appear to be responsible for sequestering mRNAs in the inactive form yet the mechanism is unknown (3). Thus, the proportion of active to inactive mRNA is also a mechanism of translational regulation.

Secondary structure of mRNA appears to be a control of specific mRNA translation. Studies show that denaturation of ovalbumin and conalbumin mRNAs enhanced their translation *in vitro.* Also, reovirus mRNA with substitution of guanisine for inosine, which would disrupt

secondary structure, showed that these mRNAs bind to ribosomes more efficiently than did the native reovirus mRNA. The 5' capped region structure mediates its function through the cap-binding protein (CBP), which was later found to be the initiation factor eIF-4E. The cap-binding process is ATP-dependent in order to provide energy to facilitate the CBP-mediated destabilization of secondary structure at the 5' region. Subsequently, the eIF-4A component of the initiation factor was found to bind and hydrolyze ATP. This "unwinding" model of mRNAs suggests that translational control is exerted by conformational manipulations of the mRNA itself (10).

Another possible mechanism for global control is exhibited by poliovirus when it infects mammalian cells by inhibiting the host's translation. The initiation factor eIF-4F is proteolytically cleaved at the γ–subunit which inactivates the factor, blocking initiation and so reducing the translation of all capped mRNAs. Viral mRNA is uncapped and initiates translation internally rather than by initiation factors and thus is unaffected. Also, the viral infection appears to activate an eIF-2α kinase which phosphorylates eIF-2 and thereby inhibits translation (through a mechanism discussed in the next section) (3). Thus, translational control could be exerted upon blocking the initiation phase by activating one initiation factor (in this case eIF-2a) while simultaneously inactivating another (in this case eIF-4F).

The initiation phase is recognized as the rate-limiting step in protein synthesis because mRNA must bind to the preinitiation complex (10). Global inhibition of translation at the initiation phase can be observed during mitosis in HeLa cells and in cells treated with heat shock and serum deprivation (3). Control during this phase is most likely

affected by phosphorylation and dephosphorylation of initiation factors. The roles of these factors are discussed in the following section.

INITIATION FACTORS

Initiation factors appear to be responsible for the control of the initiation phase and thus the rate of translation for a specific mRNA. These factors are named according to function. Eukaryotic initiation factor 1 (eIF-1) stimulates the formation and stabilization of the initiation complex assembly. The designation of factors as eIF-2 means that these factors facilitate initiator tRNA binding to ribosomal subunits through energy derived from their components involved in GTP-GDP exchange. Designation of factors as eIF-3 means they aid in the formation of the native ribosomal subunits. Factors designated as eIF-4 are involved in facilitating the binding of mRNA to ribosomes. Factors designated as eIF-5 facilitate the binding of ribosomal subunits and the positioning of the Met-tRNA for the synthesis of the first peptide bond (11). The addition of upper case letters after the abbreviation for the initiation factor represents newly identified factors that are related to already classified factors. The addition of a Greek letter after the abbreviation refers to the subunit of the factor.

Covalent modification of specific initiation factors by phosphorylation correlates with activation of translation *in vivo* and/or increased factor activity *in vitro.* The cap-binding protein, eIF-4Fα, is dephosphorylated in cells during mitosis or at high temperatures, which correlates with inhibition of protein synthesis. The phosphorylation site of this factor *in vivo* is Ser53. The factor is phosphorylated by treatment of quiescent cells with serum, insulin, tumor necrosis factor α, and

phorbol ester, a mitogen. The phosphorylated protein binds to the 40S initiation complex as demonstrated by *in vitro* studies using radiolabeled protein expressed from eIF-4Fα cDNAs. Overexpressed wild-type eIF-4Fα cDNAs cause 3T3 cells and rat 2 fibroblasts to become tumorigenic. The γ-subunit of eIF-4F is also phosphorylated in 3T3-L1 cells treated by insulin and phorbol esters which correlates with activation of translation. Protein kinase C and protease-activated kinase I and II phosphorylate this subunit *in vitro*. Since the α- and γ- subunits are both phosphorylated by kinase C it is unknown which one exerts the activation effect (3 and 12). Thus, phosphorylation of this factor, whether at the α- or γ- subunits, results in activation of translation whereas dephosphorylation results in inhibition of protein synthesis.

The level of phosphorylation of other initiation factors also correlates with the stimulation of translation. Activation of protein synthesis results from phosphorylation of eIF-4B. Inhibition of protein synthesis correlates with dephosphorylation of this factor upon heat shock and serum deprivation (12). These correlations are suspect due to difficulty in measuring the activity *in vitro* and to rapid changes in the phosphorylation states of active, crude assay systems.

Repression of translation through phosphorylation of initiation factors is a reciprocal way of controlling translational regulation. The phosphorylation of eIF-2α results in inhibition of the translation rate. In rabbit reticulocyte lysate systems this phosphorylation results from deprivation of hemin. The absence of hemin results in activation of a protein kinase, which is called the hemin regulated inhibitor (HRI). Another eIF-2α kinase, called double-stranded RNA activated inhibitor (DAI), phosphorylates the HRI also at Ser51. Strong inhibition of protein

synthesis is achieved by phosphorylation of only 25-30% of the subunit. The phosphorylation impedes the GTP->GDP reaction, thus preventing the factor from recycling and preventing numerous rounds of initiation. In a reaction catalyzed by eIF-2B the GDP is replaced by GTP. Phosphorylated eIF-2 does not undergo the eIF-2B catalyzed guanine nucleotide exchange reaction but sequesters eIF-2B and thus inhibits it. Studies to prove that eIF-2 is responsible for translational control were conducted by substituting Asp (which resembles phosphoserine) for the Ser51 residue. After small amounts of this mutant protein accumulated severe inhibition of global protein synthesis resulted (3, 12, 13). Also, improved translation mediated by the expression of a nonphosphorylated eIF-2α mutant (Ser51 to Ala51) was specific to plasmid-derived mRNA and did not affect global mRNA translation thus the nonphosphorylated state did not repress translational mechanisms (13).

Additional controls of initiation rates have been found *in vitro*. Increasing the tonicity of the growth medium inhibits the initiation process. In HeLa cells about 95% of amino acid incorporation are inhibited by excess NaCl. Also, phosphorylation of eIF-2α and eIF-4B were indistinguishable from a control when hypertonically shocked. Increasing the pH of external growth medium from 8.8 to 9.0 caused inhibition of protein synthesis by about 90% as well as resulting in dephosphorylation of eIF-4B and phosphorylation of 30-50% of eIF-2α. But no effects were observed for pH changes between 5.0 and 8.0. Also, serum depletion over 4 days of cell growth and complete removal of serum within 16 hours resulted in phosphorylation of eIF-2α and dephosphorylation of eIF-4B. Chemical agents--such as ethanol, dimethyl sulfoxide, sodium azide, sodium fluoride, and sodium selenite--cause inhibition of translation

(14). Translational control appears to be sensitive to various factors of the cellular environment.

Initiation factors appear to influence the control of translation in numerous developmental organisms. The phosphorylation/ dephosphorylation states of the factors play a role in both the activation and inhibition of protein synthesis; thus, in order to study one of the possible mechanisms of translational control it follows that the state of the initiation factors in the organism should be elucidated.

ISOLATION OF INITIATION FACTORS FROM *VOLVOX*

A protocol adapted from the *Purification and Properties of Protein Synthesis Initiation and Elongation Factors from Wheat Germ* by Lax *et al* was used (15). Although wheat germ and *Volvox* are not similar organisms, wheat germ is the only photosynthetic organism for which isolation of initiation factors has been done. Solutions used are:

1) 10X Standard *Volvox* Media: autoclave 1536 ml of H_2O and then add the following solutions-

200 ml HEPES (12g/200 ml), autoclaved
20 ml Na_2glycerophosphate (5g/100ml), autoclaved
20 ml KCl (5g/100ml), autoclaved
20 ml of $Ca(NO_3)_2$ $4H_2O$ (16.97g/100ml), autoclaved
20 ml $MgSO_4$ (4g/100ml), autoclaved
20 ml of Na_2CO_3 (2g/100ml), autoclaved
20 ml of urea (3g/100ml), filtered
20 ml of thiamine (0.1g/100ml), filtered
2 ml Biotin (0.25mg/100ml), filtered
2 ml of Cobalamin (0.15 mg/100ml), filtered
120 ml of the trace metal solution

pH to 8.0 with 10 M NaOH

Add 10 ml of bacteriostat solution

Store at 4°C, dilute to 1X for use in cultures

2) Trace Metal Solution: add to 1 L-
Na_2EDTA (O.75g), let dissolve
$FeCl_3$ $6H_2O$ (0.097 g), let stir 15 min
$MnCl_2$ $4H_2O$ (0.041g)
$ZnCl_2$ (0.005 g)
$CoCl_2$ H_2O (0.002g)
Na_2MoO_4 (0.004g)

fiter, sterilize, and store at room temperature in the dark

3) Bacteriostat Solution:
50 ml chlorobenzene
50 ml 1,2 dichloroethane
100 ml 1-chlorobutane

4) Buffer B:
20 mM HEPES KOH, pH 7.6
5 mM $Mg(OAc)_2$
1 mM dithiothreitol (DTT)
10% glycerol
KCl as indicated (e.g., Buffer B-40 contains 40 mM KCl)

5) Buffer E:
20 mM HEPES KOH, pH 7.6
1 mM $Mg(OAc)_2$
2 mM $CaCl_2$
6 mM 2-mercaptoethanol (BME)
120 mM KCl

6) Standard translation assay reaction mixture contains in 100 µl (can be prepared in advance and frozen at -80°C):
24 mM HEPES KOH, pH 7.6
2.4 mM DTT
0.1 mM spermine
7.8 mM creatine phosphate
3 µg creatine kinase; 35 mM KCl
95 mM KOAc; 2.5 mM $Mg(OAc)_2$
1 mM ATP
0.2 mM GTP
25 units of ^{3}H-leucine
50µM of the other 19 amino acids

Maintenance of cultures: *Volvox* cultures are grown in sterilized 500 ml Erlenmeyer flasks containing 300 ml of sterile Standard *Volvox* Medium. To start a new culture, about 1 ml of dense, synchronized *Volvox* culture is injected into each flask using a long Pasteur pipet under sterile conditions. The cultures are grown in a water bath at 32°C. The original synchronous culture is achieved within seven days using a 24 hour light-dark cycle, 16 hours of light followed by 8 hours of dark.

Collection of Proteins: *Volvox* individuals are harvested on the seventh day of growth by aspiration through an 80 µm nylon monofilament screening fabric. The organisms are transferred to a Kontes 40 ml tissue grind tube with a rubber policeman and Pasteur pipet. The organisms are broken open with 20 strokes of a tight-fitting Kontes tissue grind pestle. The cells are collected by centrifugation at 1000 rpm for 2 min at 4°C. The supernatant is discarded and the pellet is suspended in an equal amount of Buffer E. The gonidia are broken open by sonication using the Sonic Dismembrator Model 300 at a setting of 50 for 20 seconds. The sample is clarified to remove cell debris by centrifugation at 20,000 rpm for 20 min at 4°C. The supernatant is recovered and the volume is measured, the absorbance at 260 nm is measured, and the Lowry protein assay is conducted. Ribosomes are isolated from the supernatant by centrifugation at 40,000 rpm for 4 hours at 4°C. The supernatant (S 150) is recovered and the volume is measured and the Lowry protein assay is conducted.

Ammonium Sulfate Fractionation of Proteins: The S 150 is brought to 40% of saturation by the gradual addition of 22.6 g/100 ml of ammonium

sulfate with continuous stirring at 8°C. After stirring for 20 minutes, the precipitate is collected by centrifugation at 15,000 g for 15 min at 4°C. The supernatant is then brought to 70% saturation by the gradual addition of 18.2 g/ 100 ml of ammonium sulfate while stirring at 8°C. The precipitate is collected as described above. Each of the precipitates is suspended in about one-eighth the initial volume of Buffer B-40 and is dialyzed against Buffer B-40 overnight with the temperature kept at 8°C. The volume of the 0-40% fraction and 40-70% fraction are measured and the protein content determined by the Lowry assay.

Chromatographic Separation of Initiation Factors: The 0-40% fraction (3 ml containing 9.6 mg protein) is applied to a 30-ml DEAE-cellulose column (3 X 3 cm) equilibrated in Buffer B-40. The column is washed with Buffer B-40 until the absorbance at 280 nm is less than 0.2 and then developed with a 24 ml KCl gradient (40-140 mM) in Buffer B. The column is washed with excess Buffer B-150 and then developed with a second 24 ml linear KCl gradient (150 to 300 mM) in Buffer B. The column is washed with excess Buffer B-300. The gradient is maintained by a peristaltic pump pulling the gradient through the column. Fractions of 2 ml are collected. The absorbance at 280 nm is monitored. The protein content of the high absorbance fractions are determined by Lowry.

Identification of Initiation Factors: A translational assay is conducted to measure the ability of the isolated initiation factors to support natural mRNA-directed polypeptide synthesis. A control assay was conducted according to Promega *in vitro* translation using the supplied wheat germ

extract. Other reaction components are added to 100 µl of the standard translation assay reaction mixture when the assay is conducted:

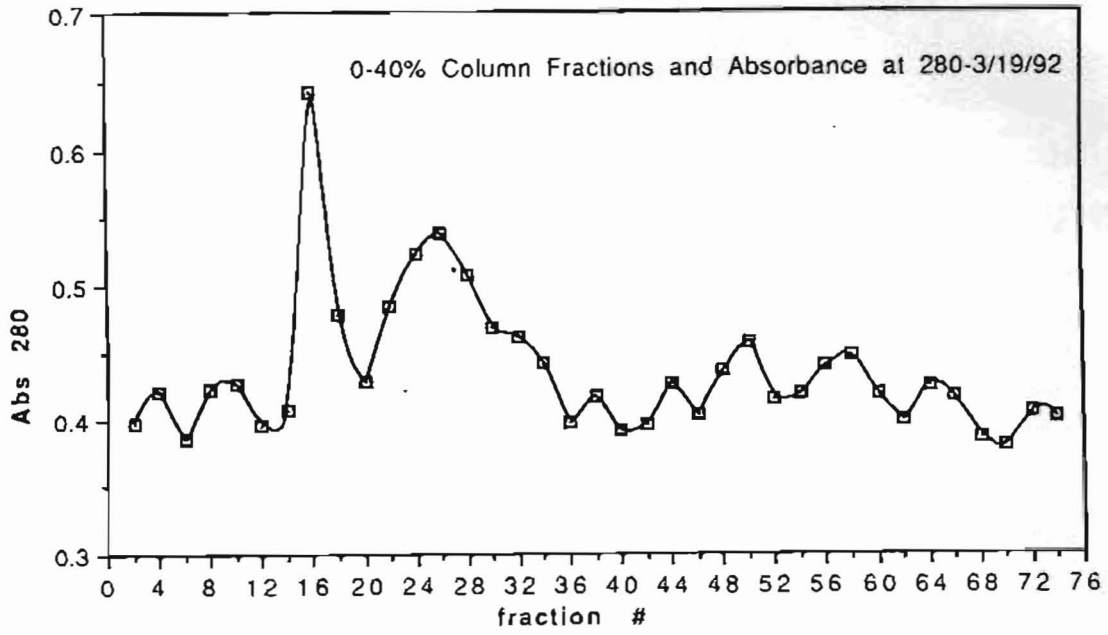
2 µl of 1x salt-washed wheat germ polysomes at 0.64 A_{260}/µl
2 µl Brome Mosaic Virus mRNA heated at 67 C for 10 min before adding
2 µl wheat germ tRNA at 0.75 A_{260}/2 µl
100 µg of 0-40% fraction (36 µl)
100 µg of 40-70% fraction (31 µl)

The mixture is incubated at 25°C for 30 min. After incubation about 1 ml of 5% trichloroacetic acid is added. The reaction mixture is heated at 90°C for 10 min and passed through a glass fiber filter upon aspiration. The filter is washed with 5% TCA twice and dried by an acetone wash. The filter is placed in a vial and 5 ml of scintillation fluid is added. The radioactivity is measured in a liquid scintillation counter.

RESULTS

A comparison between the column chromatography activity peaks of wheat germ to *Volvox* shows a similarity in the peak configuration which suggests that initiation factors may be present (Fig. 2). The translational assay control resulted in significant amounts of radioactivity recovered and so translation was supported by this assay. The translational assay using the isolated protein did not result in any significant incorporation and thus it appears translation was not supported by the assay.

A) *Volvox*



B) Wheat Germ (reproduced from reference 15)

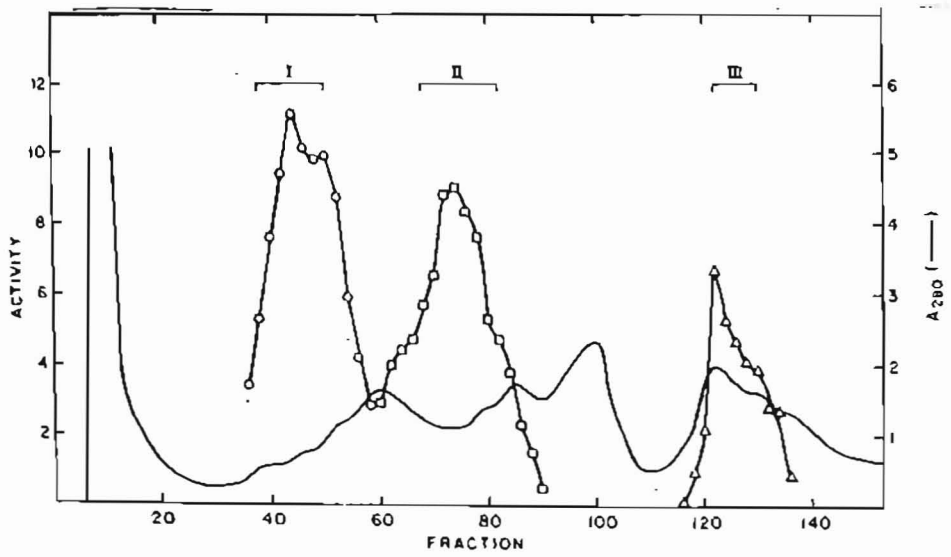


Fig. 2: Abs_{280} vs. fraction number after column chromatography

DISCUSSION

In wheat germ extracts, the first activity peak of the column chromatography has been identified as eIF-4B and the second peak as eIF-4F. The comparison with our results shows a similarity suggesting that these initiation factors may have been purified from *Volvox* successfully. However, with translational assay which did not support *in vitro* translation there is no way to tell if these are indeed initiation factors. The third peak of the wheat germ fractions has been identified as eIF-3. Our fractions show no peak correlating to this position. This is possibly due to the difficulty in maintaining the KCl gradient near the end of the column run. Due to our small sample volume we had to decrease the size of the gradient that Lax *et al* used. This may have caused difficulties because the chromatography was not run under the same conditions as the wheat germ protocol. Problems with maintaining cultures of *Volvox* were circumvented by keeping the water bath constant at 32°C, maintaining sterile conditions, and accurately preparing the Standard *Volvox* Media (which is often difficult due to the low concentrations of trace metals). Our biggest difficulty was maintaining a constant temperature in what turned out to be temperature-instable incubators.

No significant radioactive incorporation resulted from the translational assay thus the isolated proteins did not support translation *in vitro*. Problems with the assay itself may account for this result. First, the optimal RNA concentration must be determined by serially diluting the RNA template first and then adding the same volume of RNA to each reaction to ensure that other variables are kept constant, which we did not do. Second, the template mRNA should be heated at 67°C for 10

min and immediately cooled on ice in order to increase the efficiency of translation by destroying local regions of secondary structure, which we did. Third, the optimum potassium concentration varies with the mRNA used from 30-200 mM, which we did not do. Fourth, the magnesium concentration may need to be adjusted for the specific mRNA used by varying it between 1.0-2.5 mM, which we did not do (16). Finally, additional eIF-3 was added to the translational assay in the wheat germ protocol but both the 0-40% and 40-70% fractions were added in order to supercede this addition. It is possible that the amount of eIF-3 present was not sufficient to aid in the formation of the native ribosomal subunits. Any of these problems may have caused the failure of the proteins isolated by this method to support natural mRNA-directed polypeptide synthesis.

CONCLUSIONS

It appears that the proteins isolated from *Volvox* may be initiation factors, due to similarities in activity peak configuration to those established for wheat germ. But translation was not supported by the addition of these proteins to the *in vitro* translation assay and so no conclusive evidence exists to show that these proteins are initiation factors. Problems with the assay need to be worked out in order to further test the ability of the proteins isolated by this method to support polypeptide synthesis and so establish their identity.

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