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19 ABSTRACT (Continue on reverse if necessary and identify by block number)

In this research project, techniques for immunoselection of polysomes and cloning of full-length cDNA from the single-celled alga, Chlamydomonas reinhardtii CW15, have been developed. A C. reinhardtii cDNA library has been constructed and initial characterization has shown that the cloned cDNA is the size of full length mRNAs and the cloned cDNA hybridizes to C. reinhardtii cDNA derived from purified polysomal poly(A+) RNA. With these capabilities, immunopurification of specific mRNAs can be combined with efficient cDNA cloning to obtain specific full-copy structural genes.

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**DATE:** 5 February 1989

**FINAL REPORT ON CONTRACT N00014-86-K-0172**

**PRINCIPAL INVESTIGATOR:** Dr. Paul S. Tabor

**CONTRACTOR:** Indiana State University

**CONTRACT TITLE:** Polysome Immunoselection Combined with cDNA Cloning to Obtain Specific Genes from Chlamydomonas reinhardtii

**Start date:** 1 February 1986

**RESEARCH OBJECTIVE:** To develop the capability to obtain full-copy structural genes from C. reinhardtii by immunopurification of specific mRNAs from polysomes combined with efficient cDNA synthesis and cloning.

Specific research aims have included the development of a method for efficient isolation of intact polysomes from C. reinhardtii, immunoselection of isolated polysomes using antibodies against the small subunit of ribulose bisphosphate carboxylase and in vitro translation of immunoselected mRNA, cloning full-length poly(A+) RNA, and construction of a plasmid vector for stable transformation of C. reinhardtii.

**Polysome Isolation**

The ability to isolate and maintain intact polysomes is an essential first step for polysome immunoselection. An improved method for efficient isolation of intact polysomes from the C. reinhardtii cell wallless mutant CW15 has been developed. Different methods were compared on the bases of the yield of polysomes, the integrity of the polysomes determined by analysis of the population of polysomes in density gradients, and the efficiency of purified polysomes in in vitro translation systems. Techniques for lysis of C. reinhardtii CW15 cells that were examined included sonication, homogenization of cells frozen in liquid nitrogen, and gentle swirling in the presence of 0.05% or 2% Nonidet P-40. Homogenization and detergent lysis resulted in yields of crude polysome pellets (obtained by centrifugation of lysates through a 2 M sucrose cushion at 235,00 x g for 2.5 h) that were 2-fold the yield of crude polysomes by sonication (Table 1). However, analysis of the polysomes on sucrose density gradients (15-45%) showed that intact polysomes with higher "mer" numbers were isolated most efficiently when cells were lysed by sonication (Fig. 1).

Buffer systems and RNases inhibitors for use in lysis and purification procedures were examined. The use of a Tris-acetate buffer resulted in increased yield of intact polysomes in comparison with a Tris-chloride buffer system. Polysomes were more stable on storage in Tris-acetate buffer. A pH of 8.4 was required to obtain intact C. reinhardtii polysomes. Inhibition of endogenous RNases is the most critical factor in the ability to isolate of intact polysomes. Dithiothreitol (DTT), heparin and EDTA were used as RNase inhibitors. Heparin at concentrations of 500 mg/ml was essential for isolation of intact polysomes as determined by analysis of polysome populations obtained by sucrose density gradient fractionation and electron microscopy (Fig.1, Fig.2). DTT and heparin acted synergistically to increase the yield of intact polysomes. The results showed that strong lysis processes resulted in higher levels of endogenous RNase activity and lower yields of polysomes.

Thus, the optimal conditions for efficient isolation of intact polysomes from C. reinhardtii CW15 include 1) lysis by sonication in Tris acetate/magnesium acetate/potassium acetate buffer (pH 8.4), 2) amending all buffers with 5 mM dithiothreitol and >0.5 mg/ml heparin (an extremely effective RNase inhibitor), 3) fractionation of a loosely-adhering ribonucleoprotein fraction from the firmly-attached polysome pellet in the initial polysome isolation procedure and 4) the use of a linear 15-45% sucrose gradient for purification of polysomes of discrete "mer" numbers. These results have been published (Tabor and Cho, 1987).

**Polysome immunoselection and in vitro translation**

Polysomes isolated from C. reinhardtii CW15 by the method described above were used in studies to determine the specificity of an available antibody (IgG) against the ribulose bisphosphate carboxylase small subunit (RUBISCO SSU). First, polysomal poly(A+) RNA was obtained by dissociating polysomes with EDTA and using oligo-(d)T cellulose to isolate poly(A+) RNA. This RNA was used in a wheat germ in vitro translation system and a portion of the translation products were immunoselected using the anti-SSU antibody and Protein A-Sepharose columns. The single, 20 kDal peptide immunoprecipitated by the antibody is the same size as the precursor of the RUBISCO SSU (Fig. 3). When intact polysomes were added to the wheat germ in vitro translation system and a portion of these translation products were immunoselected in the same manner, a single immunoselected peptide of 16.5 kDal was found. This immunoselected peptide is identical in size to the peptide obtained when proteins were labeled in vivo by the use of $^{14}$C-acetate and antibodies mixed with the postmitochondrial supernatant for immunoselection (Fig. 4). Finally, intact polysomes and anti-SSU antibodies were mixed and polysomes specifically binding the anti-SSU antibodies were isolated on Protein A-Sepharose columns. The polysomes were dissociated and poly(A+) RNA was isolated by the use of oligo-d(T) columns. The immunoselected polysomal RNA was translated using the wheat germ system and a single, 20 kDal peptide was found (Fig. 5). The intact polysome preparation used in the in vitro translation system apparently contains sufficient endoproteases to process the precursor SSU to the mature form during the in vitro translation procedure. The anti-SSU antibody from G. Schmidt, Univ. of Ga. was exhausted and could not be replaced from this source. The RUBISCO SSU peptide was purified from C. reinhardtii using the procedure of Mishkind and Schmidt (1983) and antibodies were produced in rabbits following the protocol used by Schmidt (personal communication). Antibody of sufficient titer was harvested and the IgG fraction was isolated by the use of Protein A affinity chromatography. Although the antibody showed specificity for the RUBISCO SSU in immunoprecipitation of in vitro translation products from C. reinhardtii RNA, polysomes could not be immunoselected using this antibody.

**Cloning C. reinhardtii full-length poly(A+) RNA**

The vector/primer system of Okayama and Berg (1982) and poly(A+) RNA isolated from C. reinhardtii were used to construct a cDNA library for C. reinhardtii. Yields of 5 mg RNA were obtained from 4 x $10^8$ C. reinhardtii CW15 cells using the guanidine thiocyanate/hot phenol method. Poly(A+) RNA was isolated using mAp, poly(U) paper. Poly(A+) RNA represented from 1.5 to

3.3% of the total RNA. The ability of the isolated poly(A+) RNA to act as a template for cDNA synthesis was assayed using oligo $d(T)_{12-18}$ primer and reverse transcriptase. A 4.5% conversion into cDNA indicated the mRNA could be used readily for reverse transcription. Poly(A+) RNA was annealed to the oligo d(T)-tailed plasmid primer of Okayama and Berg (pSV7186) and cDNA was synthesized using reverse transcripase. The conversion of RNA to DNA was 1.1%. In sequence, oligo d(C) was added to the end of the mRNA:cDNA/plasmid primer using terminal transferase (the addition of d(C) residues was monitored to obtain 20 residues per end) and an oligo d(G)-tailed linker (from pSV1932) was added to promote circularization of the plasmid by using E. coli DNA ligase. mRNA was replaced by second-strand cDNA synthesis using RNase H to selectively induce nicks in the mRNA strand and E. coli Pol I to carry out nick translation. E. coli K12 strain MC 1061 was transformed with the constructed vector. The total number of independent transformants in the cDNA library was calculated to be $1.2 \times 10^5$. Plasmids were isolated from independent transformants and digested with various restriction endonucleases. Different restriction fragment patterns were found for each transformant, indicating that different DNA fragments had been cloned (Fig. 6). Hybridization analyis using labeled cDNA derived from C. reinhardtii poly (A+) RNA showed that 30% of transformants had strong sequence homology with C. reinhardtii mRNA (Fig. 7). Attempts were made to modify the Okayama and Berg method by eliminating the step replacing RNA in the insert molecule, ligating the plasmid and cloning the RNA:DNA hybrid directly. We were not able to recover plasmids with intact inserts (from PstI and PvuII digests) from the transformed population. This indicates that RNA incorporated into the vector significantly decreases the stability of the plasmid in transformed cells.

This study comprised the basis of the Master's research project of a student in my laboratory, T.Z. Lin, who graduated in December 1986.

**Construction of a plasmid vector for transformation of C. reinhardtii**

A third, preliminary study to construct a plasmid vector to transform C. reinhardtii has been accomplished. The E. coli/mouse cell line shuttle vector pSL136 ($kan^r$, and the herpes tk promoter region to allow expression of the gene in eucaryotes as G418 resistance) (a gift from S. Larson, was modified by the insertion of the C. reinhardtii chloroplast Eco RI fragment, Ri3 (a gift from M. Wu), which contains an origin of replication (oriA) and sequences promoting autonomous replication in C. reinhardtii. A plasmid (pGS101) was constructed by ligation of the isolated Ri3 fragment in pSL136. The use of low cation concentrations and high pH were required to obtain transformants. Serial transfers into increasing concentrations of the antibiotic G418 were necessary to isolate stable transformants. Using this method, the transformation frequency was $10^2$ C. reinhardtii transformants per $10^7$ cells. Analysis of plasmid size and restriction enzyme digests of plasmids from C. reinhardtii cultured in selective medium shows that the plasmid undergoes rearrangements, as well as reduction in size. Even though selective pressure by the antibiotic G418 is maintained, plasmid DNA can not be recovered from transformed cultures after 80 generations (a 3-week period).

**Bibliography**

Mishkind, M.L. and G. Schmidt. 1983. Plant Physiol. 72:847.
Okayama, H. and P. Berg. 1982. Mol. Cell. Biol. 2:161.
Tabor, P.S. and Cho, C.-L. 1987. Plant Mol. Biol. Rep. 5:336.

**Publications and Reports**

Tabor, P.S. and C.-L. Cho. 1987. An efficient method for isolation of polysomes from Chlamydomonas reinhardtii CW15. Plant Molecular Biology Reporter 5:336-345.

Tabor, P.S. 1987. Polysome immunoselection combined with cDNA cloning for specific gene isolaiton. Topicvs in Biotechnology: Naval Surface Weapons Center Colloquium. 2 November. Silver Spring, MD.

Cho, C.-L. and P.S. Tabor. 1986. Isolation and in vitro translation of polyribosomes of Chlamydomonas reinhardtii CW15. Ann. mtg. ASM., Washington, D.C., Paper H-57.

Stievenart, G. and P.S. Tabor. 1986. Transformation of Chlamydomonas reinhardtii CW15 with a plasmid containing C. reinhardtii chloroplast sequences. Ann. mtg. ASM., Washington, D.C., Paper H-69.

Lin, Tzong Zen. 1986 cDNA library of Chlamydomonas reinhardtii. Master's Thesis presented to the School of Graduate Studies, Indiana State University. December 1986. 51p.

An ONR progress report was submitted in October 1987.

**Training activities**

Three graduate students have received stipend support and support for their graduate research projects from this ONR contract. T.Z. Lin was supported as a research assistant for Spring '86, Summer '86 and Fall '86 semesters, C.-L. Cho was supported as a research assistant for Spring '86 and Summer '86 semesters, and G. Stievenart was supported during the Spring '86 and Summer '86 semesters. T.Z. Lin is from Taiwan, C.-L. Cho is from Korea and G. Stievenart is an Indiana native.

**Table captions**

Table 1. Yields of polysomes resulting from various methods of lysis. Buffer A is 25 mM Tris-acetate (pH 84.), 2.5 mM magnesium acetate, 50 mM potassium acetate, 5mM dithiotreitol (DTT), 1 ug/ml cycloheximide 0.5 mg/ml heparin (>150 U/mg). Buffer B is 25 mM Tris-HCl (pH 7.5), 25 mM potassium chloride, 25 mM magnesium chloride, 5mM DTT, 1 ug/ml cycloheximide, 0.5 mg/ml heparin. For lysis by homogenization, cell pellets were frozen in liquid nitrogen and ground to a powder. For lysis by sonication, 15 ml aliquots were sonicated three times for 10 sec in an ice bath. Polysomes were pelleted through a 2% sucrose cushion for 2.5 h at 235,000 x g.

**Figure captions**

Fig. 1. Distribution of C. reinhardtii polysomes in sucrose gradients. Polysomes were separated on 15-45% sucrose gradients (centrifuged for 1 h at 227,000 x g) and gradients were scanned at 254 nm. a. acetate buffer with 0.5 mg/ml heparin, b. acetate buffer and no heparin, c. acetate buffer, heparin and 2% Nonidet P-40, d. chloride buffer with heparin, e. loosely-adhering polysome fraction. "m" indicates the monosomal fraction.

Fig. 2. Electron micrographs of pooled polysomes fractionated from density gradients. Magnification is 72,000X. a. buffer containing no heparin, b. buffer with 0.5 mg/ml heparin.

Fig. 3. In vitro translation products of poly(A+) RNA. Left lane, total in vitro products. Right lane, polypeptides selected by anti-RUBISCO (SSU).

Fig. 4. In vitro translation products from intact polysomes. Left lane total translation products. Right lane, immunoselected translation products from polysomes.

Fig. 5. In vitro translation products of poly(A+) RNA isolated from immunoselected polysomes. Left lane, translation products of total poly A+ RNA derived from total polysomes. Right lane, in vitro translation products of total poly(A+) RNA derived from immunoselected polysomes.

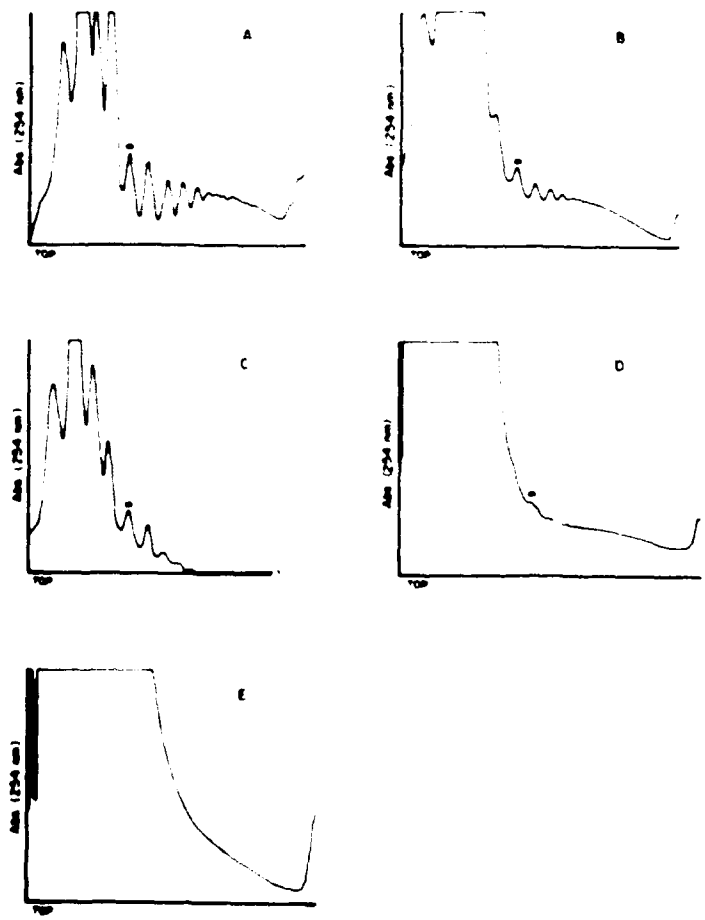
Fig. 6. Agarose electrophoresis of cloned plasmids containing C. reinhardtii cDNA. Lane A, lambda DNA markers; lane B, pBR322; lane C, plasmids isolated from transformation mix and digested with Pst I; lane D, Pst I digestion of pCr1; lane E, Pst I digestion of pCr2; lane F, Pst I digestion of pCr3; lane F, Pst I digestion of Cr4; lane G, Pst I digestion of pCr5; lane H, Pst I digest of cloning vector, pSV7186.

Fig. 7. Dot-blot hybridization detecting cDNA clones. Twenty independent transformants were transferred to nitrocellulose and hybridized with $^{32}$P-labeled cDNA derived from reverse transcription of C. reinhardtii poly(A+) RNA.

# TABLE I. YIELD OF CRUDE PELLETED POLYSOMES FROM VARIOUS EXTRACTION METHODS

| ACETATE BUFFER | HEPARIN | 2% NONIDET P-40 | CHLORIDE BUFFER | $A_{260}$ units/ml | |
|---|---|---|---|---|---|
| | | | | SONICATION | HOMOGENIZATION |
| X | X | | | 42 | 58 |
| X | | | | 12 | 20 |
| X | X | X | | 25 | 43 |
| | X | | X | 18 | 62 |

Figure 1.



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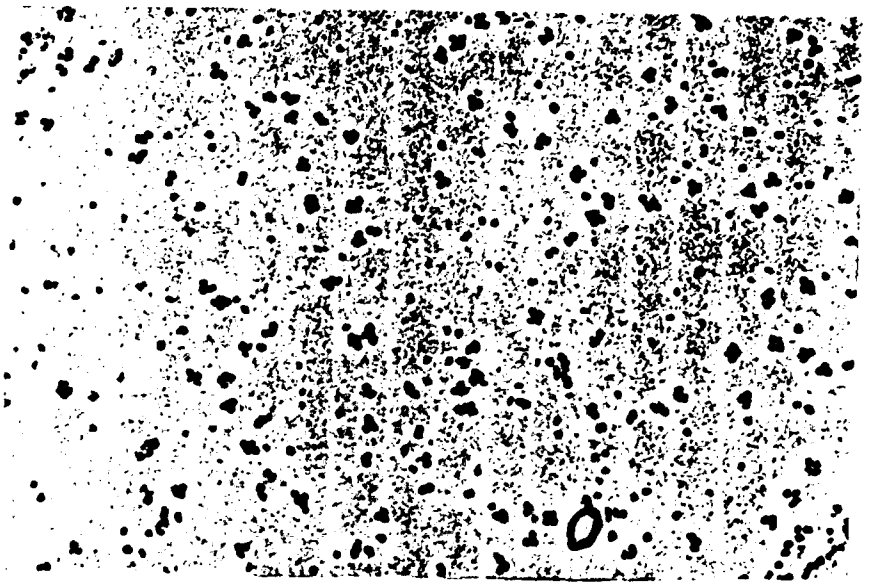

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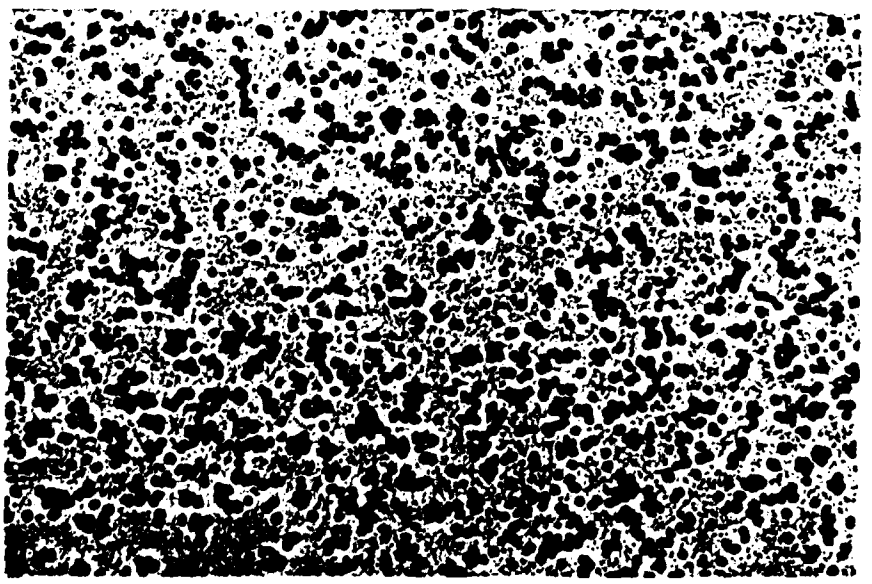

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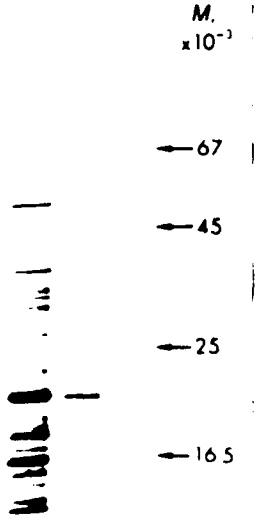


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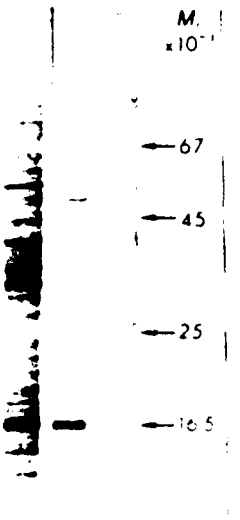


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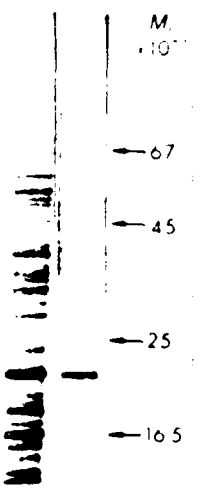


Figure 6.

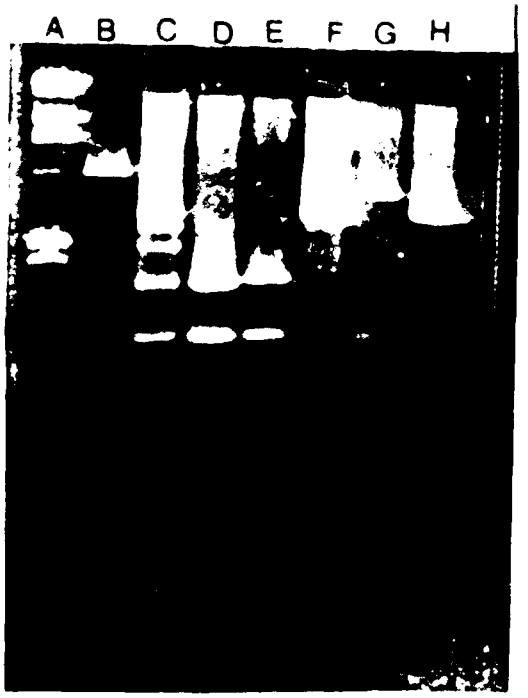


Figure 7.