MONTANA STATE UNIVERSITY

The partial purification and characterization of a ribosomal wash factor required for the translation of polycytidylate
by Charles Alexander Roessner

A thesis submitted in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Genetics
Montana State University
© Copyright by Charles Alexander Roessner (1976)

Abstract:
The partial purification and characterization of a ribosomal wash factor (P factor) from E. coll ribosomes is described. P factor is required for the translation of poly C but not poly A or poly U and is assayed during purification in a cell-free poly C directed 14C polyproline synthesizing system.

P factor has been partially purified by ammonium sulfate fractionation and DEAE cellulose column chromatography of the 1.0 M NH4CI wash of E. coli ribosomes. Other techniques utilized in the study of P factor include phosphocellulose column chromatography, Sephadex gel filtration, analytical polyacrylamide gel electrophoresis, poly C-cellulose affinity chromatography, sucrose density gradient ultracentrifugation, and nitrocellulose filter binding assay for detection of 3H poly C binding to ribosomes.

P factor is a heat labile, acidic protein and can be distinguished from other factors required for translation by its physical and chemical properties. P factor may function by preventing the non-functional binding of poly C to the 50S subunit of DEAE cellulose purified ribosomes.

THE PARTIAL PURIFICATION AND CHARACTERIZATION OF A RIBOSOMAL WASH FACTOR REQUIRED FOR THE TRANSLATION OF POLYCYTIDYLATE

by

CHARLES ALEXANDER ROESSNER

A thesis submitted in partial fulfillment
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Genetics

Approved:

Helen J Cameron
Chairperson, Graduate Committee

P D Skean
Head, Major Department

Henry L Parsons
Graduate Dean

MONTANA STATE UNIVERSITY
Bozeman, Montana

December, 1976

ACKNOWLEDGEMENTS

The author would like to express his deepest appreciation to the following:

Judith Evelyn Roessner for the support, encouragement, and love she supplied throughout this study.

Dr. Helen Cameron for her guidance, confidence, and assistance during this study.

The staff of Chemistry Station, third floor Gaines Hall, especially:

Dr. Gordon Julian and Dr. Samuel Rogers for laboratory space, supplies and materials, and for helpful advice on biochemical techniques.

Drs. Guylyn Warren, Kenneth Hapner, and John Robbins for helpful discussions and advice on technical matters.

Dr. P. David Skaar and Ernest Vyse for advice and assistance in preparation of this manuscript.

The Biology Department for financial assistance in purchasing chemicals and supplies.

Carrie Dixon for technical assistance during most of this study.

TABLE OF CONTENTS

	Page
INTRODUCTION	1
Poly C Directed ^{14}C-polyproline Synthesis	1
Factors Involved in Translation	3
Rationale	12
MATERIALS AND METHODS	14
Materials	14
Buffers	14
Growth of Cells	15
Preparation of Ribosomes	16
Preparation of pH 5 Enzyme	17
Standard Incorporation Procedure	18
Plating Procedure for the ^{14}C-polyproline Peptides	18
Basic Polyacrylamide Gel Electrophoresis	19
Construction of a Poly-C-Cellulose Column	19
Ribonuclease Assay	20
Preparation of Ribosomal Subunits	21
Sucrose Density Gradient Centrifugation	21
EXPERIMENTAL RESULTS	23
Partial Purification of P Factor	23
Characterization of P Factor	33
Kasugamycin	49
DISCUSSION	52
Initiation Factors	52
Elongation Factors	53
Release Factors	53
S1	54
Host Factor and Factor X	54
Conclusion	55
Mechanism of Action of P Factor	55
LIST OF REFERENCES	59

LIST OF TABLES

Table		Page
1.	Comparison of P Factor With Other Factors Involved in Translation	53

LIST OF FIGURES

Figure		Page
1.	Scheme for Cycling Factors in the Initiation of Protein Synthesis of Prokaryotes	5
2.	Scheme for Polypeptide Chain Elongation in Prokaryotes .	8
3.	Stimulation of Poly C Directed Polyproline Synthesis by $(NH_4)_2SO_4$ Fractions of the 1.0 M NH_4Cl Ribosomal Wash	24
4.	DEAE-Cellulose Column Chromatography of the 50-75% $(NH_4)_2SO_4$ Fraction	26
5.	Phosphocellulose Column Chromatography of the 50-75% $(NH_4)_2SO_4$ Fraction	28
6.	Polyacrylamide Gel Electrophoresis of Post-DEAE Protein .	29
7.	Activity of Polyacrylamide Gel Fractions	31
8.	Poly-C-Cellulose Affinity Chromatography	32
9.	Progress of Synthesis of Polyproline with Time	34
10.	Titration of Poly C	36
11.	Mg^{++} Titration	37
12.	Heat Lability of P Factor	38
13.	Assay for Prolyl-t-RNA Synthetase Activity in P Factor .	40
14.	Ribonuclease Activity of P Factor Preparation	41

Figure		Page
15.	DNA and t-RNA Protection of ^{3}H Poly C from Ribonuclease Activity of P Factor Preparation	42
16.	Sucrose Density Gradient Analysis of the Effect of P Factor on Poly C Binding to Ribosomes	44
17.	Binding of ^{3}H Poly C to Ribosomal Particles	45
18.	The Effect of DNA on Synthesis of Polyproline	46
19.	The Effect of Different Preincubations on the Synthesis of Polyproline in a Standard Incorporation	48
20.	The Effect of Kasugamycin on the Synthesis of Polyproline	50

Abstract

The partial purification and characterization of a ribosomal wash factor (P factor) from E. coli ribosomes is described. P factor is required for the translation of poly C but not poly A or poly U and is assayed during purification in a cell-free poly C directed ^{14}C polyproline synthesizing system.

P factor has been partially purified by ammonium sulfate fractionation and DEAE cellulose column chromatography of the 1.0 M NH_4Cl wash of E. coli ribosomes. Other techniques utilized in the study of P factor include phosphocellulose column chromatography, Sephadex gel filtration, analytical polyacrylamide gel electrophoresis, poly C-cellulose affinity chromatography, sucrose density gradient ultracentrifugation, and nitrocellulose filter binding assay for detection of ^{3}H poly C binding to ribosomes.

P factor is a heat labile, acidic protein and can be distinguished from other factors required for translation by its physical and chemical properties. P factor may function by preventing the non-functional binding of poly C to the 50S subunit of DEAE cellulose purified ribosomes.

INTRODUCTION

Protein synthesis is a process which occurs in all living organisms. Proteins are synthesized via that process termed translation which has been shown to be similar in its basic mechanisms in all organisms from bacteria to mammals. This basic similarity has allowed researchers to develop cell free study systems from bacteria and apply knowledge gleaned from these systems directly to systems from higher organisms. One of the earliest *in vitro* systems, in which polyuridylic acid (poly U) is translated into polyphenylalanine, was developed from the bacterium *Escherichia coli* by Nirenberg and Matthaei (1961). This system, or modifications of it, has been and still is widely used to study protein synthesis *in vitro*.

Poly C Directed ^{14}C-polyproline Synthesis

Shortly after Nirenberg and Matthaei (1961) described the *in vitro* poly U directed synthesis of polyphenylalanine, polycytidylate (poly C) was reported to direct the synthesis of polyproline in a similar system (Wahba, et al., 1963).

Poly C has not been as popular as a messenger as polyadenylate (poly A) or poly U in study systems because it is not nearly as effective in supporting peptide synthesis (see for example, Speyer, et al., 1963, and Schiff, et al., 1973). This poor messenger capability has been attributed to a low affinity of poly C for the 30S ribosomal

subunit due to the secondary structure of poly C (Takanami and Okamoto, 1963, Haselkorn and Fried, 1964). One molecule of poly C reportedly binds to each ribosome (Moore, 1966), and poly U can competitively displace the bound poly C. Purified ribosomal RNA, however, is able to bind several molecules of poly C per RNA molecule, most of which bind to the 23S species. Poly U also binds to ribosomal RNA but not competitively with poly C. Poly A fails to bind at all (Moore, 1966a, Hayes, et al., 1966).

Two cell free systems have been described that use poly C as a messenger somewhat efficiently. The first involves a supernatant fraction (S100) that has been concentrated by bringing it to a 76 percent saturation with $(NH_4)_2SO_4$ and dissolving the resulting precipitate in a small amount of buffer (Irvin, 1970, Irvin and Julian, 1970). The second system is that described by Cameron et al. (1972) in which a pH 5 fraction of S100 that is sufficient for the translation of poly A and poly U must be supplemented with the proteins removed from ribosomes by 1.0 M NH_4Cl for the translation of poly C. In this respect, the poly C system is similar to natural mRNA systems, e.g. bacteriophage RNA's, in that they, too, require 1.0 M NH_4Cl ribosomal wash factors.

The poly C system is also similar to natural mRNA systems in that both are inhibited by the same low concentrations of chloramphenicol (10^{-5} M), whereas the poly A and poly U systems require much higher

concentrations (10^{-3} - 10^{-4} M) (Hahn, 1967). This differential effect of chloramphenicol on the artificial messengers can be partially overcome by adding the 1.0 M NH_4Cl ribosomal wash required for translating poly C to the poly A and poly U systems (Cameron, et al., 1972). It is not known if the factor (s) required to translate poly C is the same factor (s) that enhances chloramphenicol inhibition of translation of poly A and poly U.

Factors Involved in Translation

Translation, that process wherein messenger-RNA is read to produce a polypeptide, involves several protein factors which interact with ribosomes. These factors are generally named according to the stage of translation in which they function, i.e., initiation factors, elongation factors, termination factors, and regulation factors such as the interference factors.

Initiation Factors

The initiation factors are isolated from the 1.0 M ribosomal salt wash and normally function in the initiation of translation of natural messenger RNA's. The process of initiation results in the formation of a complex consisting of 70S ribosomes, messenger RNA, and n-formylmethionine-t-RNA.

1. IF-1. A small (M.W. 9,000; Wahba et al., 1969) protein that is thought to have as its major function the catalytic reconversion of

the complex IF-2 GDP to IF-2 GTP (Benne et al., 1973).

2. IF-2. This factor has been separated into two proteins, IF-2 α and IF-2 β, with molecular weights of 98,000 and 83,000, respectively, either of which performs the function attributed to IF-2 equally well (Wahba and Miller, 1974). IF-2 primarily directs the binding of f-met-t-RNA and GTP to the 30S ribosomal subunit (Salas et al., 1967).

3. IF-3. This factor has also been separated into two proteins, IF-3α and IF-3β, with molecular weights of 23,500 and 21,500, respectively (Lee-Huang and Ochoa, 1974). IF-3 binds mRNA to the 30S subunit, and each IF-3 may recognize different messengers. IF-3α functions in translation of E. coli mRNA, MS-2 RNA, and early T4RNA. IF-3β functions with late T4 RNA (Lee-Huang and Ochoa, 1971). IF-3 is also the dissociation factor (Sabol et al., 1970). It causes the dissociation of 70S ribosomes into 50S and 30S subunits, and as long as IF-3 is bound to the 30S subunit, 70S ribosomes cannot be formed.

IF-3 has been shown to stimulate the translation of synthetic polynucleotides (Schiff et al., 1973). This stimulation was most pronounced in high magnesium (18-20 mM) and was non-specific in that translation of poly A and poly U were stimulated as well or better than translation of poly C.

The initiation of protein synthesis and the function of the initiation factors may be diagrammed as in figure 1 (from Haselkorn and

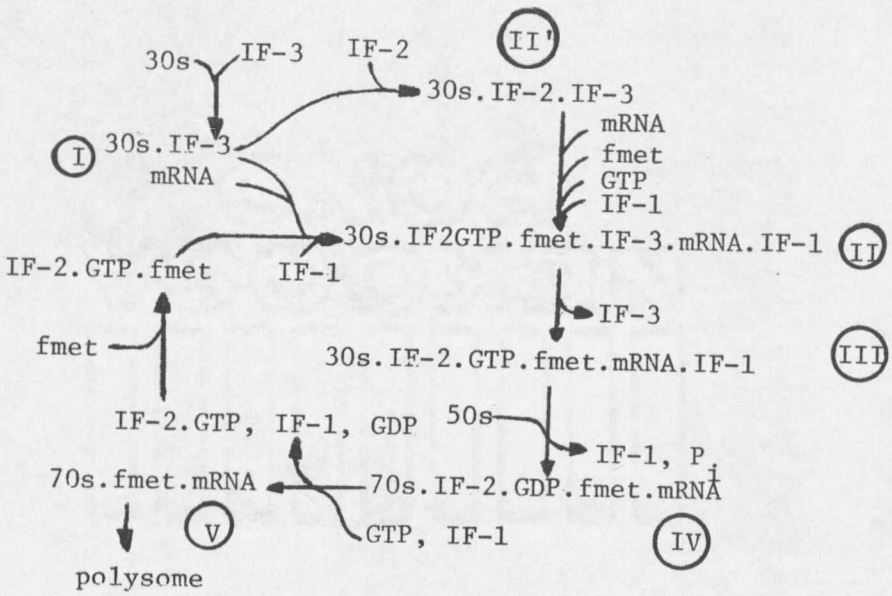

Figure 1. Scheme for cycling factors in the initiation of protein synthesis of prokaryotes. IF-1, IF-2, and IF-3 are the initiation factors; fmet is formylated met-$tRNA_F$. IF-1 is probably needed only for the release of IF-2 in step IV→V, but it has been observed to associate with complexes like II and III in vitro, so it is included there as well. (from Haselkorn and Rothman-Denes, 1973).

Rothman-Denes, 1973). There is some question as to the steps leading to the formation of complex II. For example, Jay and Kaempfer (1974) have recently isolated an f-met-t-RNA-30S complex and believe this complex may be formed first, directing the binding of IF-3-m-RNA.

Elongation Factors

The elongation factors are normally isolated from S-100 and function in the elongation step of translation. Elongation is the step by step addition of the amino acids in the peptide chain following the initiating formylmethionine until the chain is completed.

1. EF-Tu. This is a highly unstable protein with a molecular weight of 42,000 (Miller and Weissbach, 1970). It forms a complex with aminoacyl-t-RNA and GTP and directs their binding to the 70S ribosome during elongation (Ravel, 1967).

2. EF-Ts. EF-Ts is a stable protein (M.W. 28,500-35,000) (Hachmann et al., 1971) that functions in a fashion similar to IF-1. It catalytically converts EF-Tu-GDP to EF-Tu-GTP thus helping recycle EF-Tu (Beaud and Lengyel, 1971).

3. EF-G (translocase). EF-G is a stable protein (M.W. 72,000-84,000) (Lucas-Lenard and Lipmann, 1971) that is responsible for the translocation step of elongation. This step includes three processes: (1) release of t-RNA from the P site, (2) movement of the peptidyl-t-RNA from the A site to the P site so that a new aminoacyl-t-RNA can

bind, and (3) movement of the ribosome by one codon along the message (Haselkorn and Rothman-Denes, 1973).

EF-G has also been reported to inhibit initiation in the absence of active aminoacylation of t-RNA (Lee-Huang et al., 1974a).

The elongation cycle may be diagrammed as in figure 2 (from Haselkorn and Rothman-Denes, 1973).

Termination Factors (Release Factors)

The termination factors are isolated from S-100 and function in the release of the completed polypeptide chain from the ribosome (termination).

1. RF-1 and RF-2. These release factors are proteins of molecular weight 44,000 and 47,000, respectively (Klein and Capecchi, 1971). RF-1 recognizes the termination codons UAA or UAG but not UGA, and RF-2 recognizes UAA or UGA but not UAG (Scolnick et al., 1968).

2. RF-3 (S). This protein has no release factor activity by itself but rather stimulates the activity of RF-1 and RF-2 (Goldstein et al., 1970).

Interference Factors

Several ribosomal wash factors have recently been described that stimulate or inhibit the IF-3 directed binding of different mRNA's to the ribosome. These factors, termed interference factors, represent a possible control mechanism operating at the translation level.