THE EARLY STEPS IN PROTEIN SYNTHESIS AND THEIR REGULATION:
A BACKGROUND STUDY RELATED TO THE BIOLOGICAL EFFECTS OF RADIATION

PROGRESS REPORT

by

The John Collins Warren Laboratories
of the Huntington Memorial Hospital

of

Harvard University

at the

Massachusetts General Hospital

Boston, Massachusetts 02114

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*Paul C. Zamecnik, M.D. - Principal Investigator. Percentage of Effort: 25%

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UNITED STATES ENERGY RESEARCH AND DEVELOPMENT ADMINISTRATION

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July 1, 1975 - June 30, 1976

THE EARLY STEPS IN PROTEIN SYNTHESIS AND THEIR REGULATION:

A BACKGROUND STUDY RELATED TO THE BIOLOGICAL EFFECTS OF RADIATION

Abstract

The proposed program is an interwoven effort to study the details of the mechanism of protein synthesis in normal living systems and their alterations in the presence of oncogenic RNA viruses using the avian myeloblastosis virus as a model. Emphasis will be placed on determining the role of the primary structure of the viral RNA and of other factors required for the production of viral proteins in a cell-free system. Continued studies of the initial steps of protein synthesis where much specificity is determined by the tRNA: tRNA synthetase interactions will be carried out using biochemical and genetic techniques.

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I. Scope of Project - Introduction

This contract has for many years been the principal support for our work on protein synthesis, enabling us to expand and unify various strands of investigation in this area, providing background for increasing understanding of the effect of radiation on the metabolism of living cells.

The program is an interwoven effort of members of these laboratories to study details of the mechanism of protein synthesis in normal living systems and their alteration in the presence of oncogenic viruses. We are using the avian myeloblastosis virus (AMV), an RNA oncogenic virus, as a model and our current emphasis is aimed at the viral RNA and what happens to the normal mechanisms of protein synthesis when the viral 35S RNA inserts itself in the cell. Some individuals among the high molecular weight oncogenic viral RNA molecules must contain the complete signal array for (a) attachment of the reverse transcriptase and other enzymes leading to integration into the genome of the cell; and (b) for initiation, propagation, and termination of major virion proteins synthesized in the in vitro system for which the high molecular weight oncogenic viral RNA serves as messenger. This RNA is thus a focal point for our varied investigations. It is a central product of radiation- or chemically-induced activation of the viral gene contained in the host cell; it is also a messenger RNA with special features enabling it to compete successfully with host mRNA for a portion of protein synthesizing machinery. Our work on tRNA bears on the translation capabilities of the viral messenger RNA, and studies on the recognition reaction between aminoacyl-tRNA ligase and its cognate tRNA will in time help explain the translation of the viral RNA in addition to the normal host RNA translation.

The reports touch on the following areas:

- 1) Alteration of the 5'-terminus of the 35S RNA of AMV by the reaction of pp-RNA with aminoacyladenylate-lysyl synthetase complex in an extension of our earlier studies on the reaction of ATP with the complex.
- 2) Investigation of ppGpp and pppGpp ("magic spots" 1 and 2 of Cashel) in the first steps in protein synthesis as indicated above.
- 3) Establishment of a cell-free wheat germ system to synthesize viral proteins in response to 35S RNA of AMV and investigation of the requirements for such protein synthesis both in the RNA and in other "factors".
- 4) Development of a method for sequential stepwise degradation of oligonucleotides by a post-isolation labeling technique in the subnanomolar range. Automation of such a technique will be helpful in determining the primary structure of the viral RNA.
- 5) Continued studies of the normal recognition reactions in the initial stage of protein synthesis where much specificity is determined by the tRNA: aminoacyl-tRNA synthetase interactions using chemical, biochemical, and genetic techniques.
- 6) Investigation of the primary structure of the AMV 35S RNA. Studies of the internal nucleotides adjacent to the 3'-OH poly(A) sequence of the 35S RNA.

The report covers work carried out during the past year. The investigations described are a result of a consciously interrelated effort to gain insight into factors involved in the biosynthesis of proteins and the regulation of growth. Although not all investigations mentioned receive remuneration directly from the current contract the ERDA (AEC) has been for many years the major source of this interrelated effort (see Proposal).

We are conscious of the fact that there are areas of overlap in the proposal and this annual report. Parts of the latter are better presented with our plans for the future rather than as isolated reporting of work accomplished. In the interest of completeness they are included in both and we are aware that the same wording may appear in both places. In addition, the proposal is referenced more thoroughly than this report of work accomplished during the past year.

II. Specific Reports

A. Investigation of the mechanisms by which an RNA oncogenic virus subverts the normal patterns of growth regulation

1. Characterization of the High Molecular Weight RNA of AMV

a. Introduction

The high molecular weight oncogenic viral RNA has been shown to be implicated in at least two biological functions. To begin with it is now well established that this RNA serves as a template for the oncogenic viral reverse transcriptase to synthesize DNA. The DNA somehow becomes integrated into the host cell DNA so that virus specific mRNA can be transcribed from infected cell DNA. On the other hand there is also ample evidence that the high molecular weight RNA has messenger activity in a cell-free protein synthesizing system. Thus a dual role for this genetic RNA has been well established.

Two years ago we were able to show that the terminal nucleotide at the 3'-OH end of the 35S RNA of AMV was adenosine (1), and last year we presented evidence that this RNA terminated in a run of poly(A) about 30 nucleotides long and that this was the only poly(A) in the 35S molecule (2). These sequencing studies, carried out by Drs. Stephenson, Wirthlin, Scott, and Zamecnik, made use of a tritiated borohydride labeling of nucleosides, combined with the thin layer chromatographic techniques of our former colleagues, The Randeraths. This was the first time that an oncogenic viral RNA was found to terminate with poly(A), although poly(A) in terminal location had been demonstrated in numerous other viral RNAs and eukaryotic mRNAs.

Upon heat, urea, or DMSO treatment the 65-70S RNA complex becomes dissociated and sediments in sucrose gradients as two to four molecules of "35S" RNA plus three or four molecules of 70S-associated 4S RNA.

Because of the diversity of functions of viral RNAs mentioned above, it is of interest to determine the primary nucleotide sequences just internal to the poly(A) terminus of those oncogenic RNA viruses.

To characterize the 3'-OH terminal nucleotide of the viral RNA we have used the periodate-oxidation-tritiated borohydride reduction procedures perfected for microanalytical analysis by the Randeraths, following earlier procedures, and which have more recently been used by others (see refs. 1 and 2). The exposed cis-hydroxyl groups on the 3'-terminal nucleoside of an RNA chain are oxidized to dialdehydes with periodate, and subsequently reduced with tritium labeled borohydride, yielding an RNA containing labeled 3'-termini. Alkaline hydrolysis of the intact RNA yields the terminal nucleoside as a labeled trialcohol, thus identifying the terminal 3'-OH residue of the chain. We have adapted this procedure for terminal labeling of the high molecular weight viral RNA (1). The 3'-OH terminus of intact RNA is labeled and the RNA is hydrolyzed by piperidine. The terminal labeled moiety is released as a labeled nucleoside trialcohol, while the remaining hydrolysate consists of mononucleotides. The nucleoside trialcohols of adenosine, cytidine, guanosine, and uridine are isolated by thin-layer chromatography on cellulose plates using cold carrier trialcohols, while any nucleotides remain at the origin under the conditions used.

We have also used the periodate-[³H]-borohydride labeling technique to determine the total nucleosides of RNA hydrolysates as well as the 3'-OH terminus of the RNA, in order to get ratios of the number and kind of total nucleosides compared with those in the 3'-OH terminus of the same molecule.

b. Procedures to help determine internal nucleotides adjacent to the 3'-OH terminal poly(A) sequence

(1) Isolation of poly(A) containing nucleotide fragments using ribonuclease A and ribonuclease T₁ separately - M.L. Stephenson

Since we have found that all the poly(A) is at the 3'-OH terminus of "35S" RNA we can make use of methods aimed at the isolation of poly(A) (3). Selective digestion of the 35S RNA by specific enzymes, followed by isolation of poly(A)-containing fragments enabled us to isolate selectively nucleotides adjacent to the poly(A) region along with the poly(A). Current studies involve the use of ribonuclease A and ribonuclease T₁. For example, ribonuclease A in high salt concentrations hydrolyzes at U and C residues and should yield runs of A at the 3'-OH end with possibly some G and A nucleotides internal to the poly(A) but no C or U. On the other hand, ribonuclease T₁ (which splits at G residues) should yield the terminal poly(A) plus internal U, C, and A but no G. We have isolated such poly(A)-containing fragments and determined their size and composition by periodate oxidation-¹¹³Borohydride reduction techniques both on terminal and total nucleotides.

Results indicate that when 70S RNA or heated and quick cooled 70S RNA (to generate 35S RNA) are digested in high salt with ribonuclease A or T₁ in order to obtain a 3'-OH poly(A) piece containing a few adjacent nucleotides, the samples are not completely hydrolyzed. When the poly(A)-containing fragments were isolated they contained nucleotides which should not have been there as judged by the specificity of the respective enzymes. In addition, end to total ratios revealed unusually large fragments.

However when 35S RNA isolated from gradients is hydrolyzed by the respective ribonucleases, the RNA appears to correspond to the proper enzyme specificity and gives small fragments containing the proper nucleosides in addition to the poly(A) expected. Evidently the tertiary structure or perhaps other molecules (i.e. 4S RNA) present in 70S RNA units interfere with complete enzymatic splitting by the nucleases whereas when the 35S RNA is isolated free of its associated tRNA as well as possible reaggregation moieties, the RNA is available to the ribonucleases. Electron microscope pictures of 70S "bush" structures, and 35S single stranded linear structures from our AMV RNAs by our colleagues Drs. Weber and Heine of the NCI (unpublished data) and those of other investigators are consistent with this interpretation.

Treatment with ribonuclease T₁ indicates that just interior to the 3'-poly(A) there is a U and a C residue in approximately equal amounts with the possibility of an A residue not ruled out. Results also indicate that a slight amount of G could also be in that segment of the molecule. We could have the following structure:

.....G(U,?G,C,A)-A.....AOH

Results with ribonuclease A indicate that there is a small but significant amount of G adjacent to the poly(A) sequence and that also a small amount of C and U may be present. Therefore we could have the following:

.....C or U(G, traces C,U and A)A.....AOH

The 35S RNA molecules could therefore have C or U in equal amounts plus smaller amounts of G adjacent to the terminal poly(A). These results are somewhat in agreement with those recently published by Rho and Green (4) and who obtained G(C,U)A...A_{OH} using a murine sarcoma-leukemia virus RNA and Quade *et al.* (5) who found 80 percent C adjacent to the poly(A) in Rous sarcoma virus. We do have definite amounts of G adjacent to the poly(A) however.

We are aware of the fact that the principal genetic RNA, the 35S RNA of AMV strain A, is not necessarily a single species of RNA but may consist of more than one type of 35S molecule. The experiment described above indeed gives some indication of heterogeneity in the 35S RNA, or indicates incomplete hydrolysis by one or the other enzyme. Section (2), which describes Dr. Weith's experiments with enzymatic copying to determine sequences, has evidence which does not rule out the possibility of a single 35S species, since he finds C always adjacent to the poly(A) segment under a variety of test conditions. The two 35S RNA preparations were made from different batches of virus. In Dr. Stephenson's experiment described above a broad cut was made of the 35S RNA area while Dr. Weith's 35S RNA represented only the peak area. His results agree with and extend those of Rho and Green (4) and Wang (6).

Although the borohydride, periodate method gives us very reliable results using high molecular weight RNA as discussed in earlier reports, we have run into considerable difficulties when trying to quantitate the nucleotides both total and terminal in poly(A) fragments in the order of 30 plus nucleotides following T₁ or ribonuclease A digestion compared with the 5,000 nucleotides in the 35S RNA fragments. The "high" blank areas of the cellulose plates which are due to reaction of periodate plus borohydride with traces of unknown compounds possibly from the sucrose gradients have been reported before and their presence was reduced to tolerable levels by frequent reprecipitation of the labeled RNA and by isolation of the nucleosides from cellulose plates using longer runs with wicks on the plates. The fragments of poly(A) about 35 nucleotides long from equivalent amounts of 35S RNA (MW $\sim 2.1 \times 10^6$) used in these experiments resulted in a much higher ratio in the contamination of the blank areas so that the sample counts, which are much lower, in some cases were no greater than surrounding "hot spots." We are limited in the amount of viral RNA available to us despite the generosity of the VCP and Dr. and Mrs. Beard and have therefore spent much time and effort in trying to reduce the blank "hot spots" and have only recently partially solved the problem by the prior use of hot aminex columns described by Singhal and Cohn (7) for separation of nucleosides by cation exclusion. We have had to change their elution conditions for nucleosides quite drastically for the corresponding trialcohols, since the latter all emerged near the front of the gradient. Figure 1 indicates the very nice separations we have finally achieved by altering the elution conditions as indicated in the legend.

Even these isolated trialcohols have "hot" spots near them when subsequently run on cellulose or silica gel plates in a single dimension. Fortunately by running the plates in two dimensions we are able to achieve good separations of all the trialcohols from the "hot" spots except for a small amount which travels just under the cytidine trialcohol. These major improvements give us a much greater degree of accuracy in the estimation of the trialcohol.

In a preliminary account of these experiments in last year's report we indicated that there were significant amounts of G residues near the poly(A). The subsequent use of the aminex column cleared up this artifact. We do find a small amount of G present, however, in contrast with results reported by others.

These experiments designed to identify nucleotides just interior to the terminal poly(A) are extremely time consuming and involve: 1) the isolation of 70S RNA, 2) the isolation of 35S RNA, 3) the digestion of 35S RNA by ribonuclease A and T₁, 4) the isolation of poly(A) fragments and for each tube, 5) labeling of the fragments both for total nucleosides and for 3'-OH terminal nucleosides, 6) application of labeled nucleosides to "hot" aminex columns in order to isolate separately the trialcohols of ACG and U, 7) running individual two-dimensional thin-layer chromatograms of each (i.e. ACG and U) using carrier trialcohols, and 8) counting the trialcohol from the cellulose or silica gel plates plus all the surrounding areas to be sure there are no "hot" spots. The experiments described above using the borohydride labeling technique have proved useful in determining the end groups and total nucleosides of the RNA. Other techniques such as the enzymatic copying methods carried out by Dr. Weith are complementary to these borohydride results and should better enable us to determine the sequences of the internal nucleotides. The initial experiments performed by Dr. Weith described later are very promising, especially using the AMV reverse transcriptase. This has been generously supplied to us by Dr. Beard and the Virus Cancer Program.

In our proposal last year we outlined plans for further chemical stepwise sequencing of the RNA from the 3'-end. These all required removal of the terminal poly(A) prior to the proposed sequencing. We have not pursued these proposed experiments to date for two reasons: 1) Dr. Weith's enzyme copying procedure is progressing satisfactorily (see Section A.1.b.(2)), and 2) Dr. Schwartz is perfecting a very sensitive method for post-isolation labeling of oligonucleotides (see Section A.2.a.) which may be amenable to automation. This latter procedure may be capable of sequencing more nucleotide residues than the standard techniques Dr. Stephenson had planned to use. She has therefore turned her efforts establishing a cell-free protein synthesizing system in which to test the template activities of viral RNAs under various conditions and to determine factors necessary for synthesis of the viral proteins (see Section A.4).

FIGURE 1. SEPARATION OF NUCLEOSIDE TRIALCOHOLS USING AMINEX A-25

Figure 1. Separation of nucleoside trialcohols by cation exclusion chromatography using Aminex A-25 column chromatography. The method is essentially that of Singhal and Cohn (7) using Aminex A-25 (Biorad). A column 6 mm x 60 cm was used. Ammonium formate 0.01M pH 4.3 was used for elution of the samples at 55°C, 5 ml/hr and about 30 psi. The sample containing a mixture of nucleoside trialcohols of adenosine, guanosine, uridine, and cytidine was in a volume of 100 μ l.

- (2) Use of an enzymatic copying method to determine the primary structure of AMV 35S RNA - H.L. Weith (joined recently by D. Schwartz)

(a) Introduction

The techniques used for direct sequence analysis of RNA are well developed (8,9). These methods, however, have proven to be of limited application to nucleic acid molecules isolated from various eukaryotic sources and to those nucleic acid molecules which cannot be readily radioactively labeled in vivo.

An alternative approach to sequence analysis of RNA and DNA which may be of general application involves copying the unlabeled nucleic acid with a template-requiring nucleotide polymerizing enzyme such as DNA or RNA polymerase. This approach offers a number of advantages over the direct methods of sequence analysis. First, since radioactive nucleoside triphosphates of high specific activity can be incorporated into the copy by the polymerizing enzyme, only extremely small quantities of the nucleic acid template molecule are required for sequence analysis. Second, the problems imposed by high molecular weight and structural complexity, can be circumvented by allowing the polymerizing enzyme to copy only very short stretches of the template molecule. Finally, it should be possible to select specific regions of the template nucleic acid for sequence analysis by the use of oligonucleotide primers to initiate copying by the polymerizing enzyme at specific sites on the template. The primary disadvantage of this approach to sequence analysis is that it relies on the fidelity of copying by polymerizing enzymes.

(b) Preliminary work accomplished

The presence of a polyadenylic acid sequence at the 3'-hydroxyl terminus of AMV 35S RNA (2) has provided a convenient starting point for sequence analysis of this oncogenic viral RNA molecule by means of enzymatic copying, using oligo thymidylic acid primer to initiate synthesis of DNA complementary to the viral RNA.. Initial experiments using DNA polymerase I from *E. coli* to "reverse transcribe" AMV 35S RNA were compromised by extensive homopolymer synthesis of the type poly(dA) and poly(dT). Similar results have recently been reported by a number of workers (10,11). More recent studies using reverse transcriptase from AMV, however, have proven more fruitful.

It was initially anticipated that specific initiation of synthesis by oligo thymidylic acid on the poly(A) sequence could be accomplished by conducting the polymerization reaction in the absence of dTTP, since only those oligo thymidylic acid primers which hybridize at the 5'-end of the poly(A) sequence in the RNA could initiate synthesis in the absence of T:

Various polymerization reactions have been conducted with [α - 32 P]dATP and [α - 32 P]dCTP, d(pT)₁₀ primer, purified reverse transcriptase, and purified 35S RNA, leaving one or more of the remaining unlabeled deoxytriphosphates out of the reaction mixture. The pattern of incorporation of 32 P into acid insoluble products is summarized in the table below:

Reaction Conditions	pmoles of [32 P] nucleotide incorporated in 20 minutes per 10 μ l reaction	
	dATP	dCTP
Complete System	0.8	1.1
Complete System minus d(pT) ₁₀	0.02	0.04
Complete System minus dTTP	0.03	0.02
Complete System minus dGTP	0.04	0.03
Complete System minus dCTP	0.2	N.A.
Complete System minus dATP	N.A.	0.03

These results indicate that copying of AMV 35S RNA by reverse transcriptase is a highly specific process dependent on the presence of oligo thymidylic acid primer to initiate copying presumably on the poly(A) sequence, and that the 35S RNA preparation used in these studies is free of endogeneous primer molecules. The lack of incorporation of radioactivity in the absence of unlabeled dTTP can be the result of either greatly reducing priming efficiency of oligo thymidylic acid on the poly(A) or more likely the presence of one or more A residues in the RNA template interior to the poly(A) sequence but preceding the first G residue ([α - 32 P]dCTP incorporation experiments) and preceding the first U residue ([α - 32 P]dATP incorporation experiments). Furthermore, the incorporation of [α - 32 P]dATP in the absence of dCTP strongly suggests that the first G residue in the RNA template is interior to the first U residue. These preliminary results permit the following tentative sequence to be written for AMV 35S RNA:

In order to test this possible sequence, polymerization reactions with [α - 32 P]dGTP were conducted. The pattern of incorporation of radioactivity into acid insoluble material is summarized in the table below:

Reaction Conditions	pmoles of [α - 32 P]dGTP incorporated in 20 minutes per 10 μ l reaction	
Complete System	0.45	
Complete System minus d(pT) ₁₀	0.03	
Complete System minus dTTP	0.06*	
Complete System minus dATP	0.1	
Complete System minus dCTP	0.19	

*Reproducible incorporation observed but at reduced levels compared to other reactions minus one triphosphate. This is presumably due to decreased priming efficiency by d(pT)₁₀ on the poly(A).

These results confirm that the first residue adjacent to the poly(A) in AMV 35S RNA is C since dGTP is incorporated into complementary DNA in the absence of the other deoxytriphosphates. The sequence below is possible.

5'G, U, A, CAAAAAAAAA.....A^{3'}OH
 where the number of G,U,A, and C residues is not specified

At the present time this sequence is being tested by means of two dimensional fingerprint analysis of the products produced in larger scale polymerization reactions in the absence of dTTP, using [α -³²]dGTP to label the products. If the above sequence is correct, then only one oligonucleotide product should be observed in the fingerprints and the nearest neighbor analysis should show transfer of ³²P-label only to T, and possibly to G if more than one C residue is present adjacent to the poly(A). If these preliminary results are supported by fingerprinting and nearest neighbor data, a more specific primer oligonucleotide molecule, d(pT)₁₀pG will be synthesized by standard procedures (12). The use of this primer molecule should permit sequence analysis to proceed at least to the next C residue in AMV 35S RNA. It should be noted that these preliminary results are in partial agreement with sequence data recently reported for murine sarcoma-leukemia virus 30-40S RNA (4), the only difference being the possible presence of an A residue between the U and the C residues adjacent to the poly(A) in AMV RNA.

Continued studies of the nucleotide sequences in AMV RNA will be carried out by means of primer-initiated template copying, using purified reverse transcriptase from AMV. Since these proposed studies will be primarily funded from another source (Virus Contract), they have not been discussed in the proposal.

c. Discussion of 35S RNA of AMV

Dr. Weith's results to date suggest that AMV 35S RNA is a homogeneous population of molecules with respect to the first nucleotide residue adjacent to the poly(A) sequence since only dGTP is incorporated in the absence of other deoxytriphosphates. Whether all 35S molecules are identical in sequence awaits more extensive sequence analysis. By this method a definite sequence of C next to the 3'-OH terminal poly(A) has been established. The borohydride results with ribonuclease A fragments do indicate that a small amount of G may be adjacent to the terminal poly(A) in addition to U or C (indicated by the ribonuclease T₁ results). The 35S RNA used in the two experiments was from entirely different virus preparations and the amount of material designated "35S" RNA in pooling the peak material was not the same. We cannot yet say therefore whether all 35S molecules of AMV are identical. It should also be mentioned that the two methods are dependent upon different principals; the borohydride-enzyme digestion procedure is based on binding of poly(A) fragments to millipore filters, while the enzymatic copy method is based on hybridization of oligo dT to poly(A).

It is helpful to have both types of experiments under investigation since the approaches are quite different. Dr. Weith, who joined us from Dr. Sanger's laboratory, has helped to develop some of these procedures and is setting up the method for two-dimensional separation of oligonucleotide fragments used by the Sanger group (13).

The success of both types of experiments described above is dependent upon having good intact 35S RNA and we are all closely cooperating in sharing and comparing various fractions of the viral RNA. Drs. Stephenson and Scott are particularly involved in this aspect of the problem.

We have at the same time been improving our techniques for isolating 35S RNA in μg quantities from AMV and can now routinely obtain fairly pure 35S RNA from sucrose-sarcosyl gradients in OD amounts. DMSO gradients give even sharper peaks. We are still plagued by the fact that even our best RNA preparations stored in liquid nitrogen fail to maintain their integrity for significant lengths of time, as judged in particular by E-M measurements of homogeneity of length of molecules by Drs. Weber and Heine. These vary from preparation to preparation. The quality of the 35S RNA is determined both by how it looks on DMSO-sucrose or sucrose gradients and by its reaction as template for the AMV reverse transcriptase. Fresh 35S absolutely requires a primer in some endogenous reactions carried out by Dr. Weith while older 35S RNA preparations gradually lose their primer requirements indicating either a small loss of poly(A) or a slight fragmentation of the 35S molecule.

2. Other Studies on the Post-Isolation Labeling of Oligonucleotide Aimed at Sequencing RNA

a. A new sensitive method for RNA sequencing by stepwise chemical degradation - D.E. Schwartz

- (1) Introduction
 - (2) Investigation of pathways A and B in order to develop sequencing methods employing post-labeling technique
 - (3) Use of activated charcoal to separate labeled nucleoside dialcohol from salts and other labeled contaminants
 - (4) Fractionation and detection of the labeled dialcohol nucleoside derivatives by thin-layer chromatography
 - (5) Use of DNA oligonucleotides or polynucleotides as carrier for the RNA during sequence analysis
 - (6) Adaptation of the post-labeling method of sequence analysis to a solid support system
 - (7) Uses of the above new method of stepwise sequencing
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(1) Introduction

We are developing a new procedure for the direct analysis of extremely small amounts of unlabeled RNA in which a reactive nucleoside dialdehyde is released from the 3'-terminus of a polynucleotide during each cycle of degradation (14). This dialdehyde is readily reduced with tritiated borohydride to form a stable derivative which may be identified by the combined techniques of thin layer chromatography and autoradiography or liquid scintillation counting. Of particular importance, the method is amenable to automation due to the cyclic nature of the degradations. An automated solid support sequenator has been developed (15) for a similar but much less sensitive sequencing method (16), and it is thought that our procedure can be readily adapted to this machine. The obvious advantage of this automated technique is that the sequence analysis of RNA would be facilitated in the same way that the Edman sequenator (17) has facilitated the sequence analysis of proteins.

The basic steps involved in the stepwise chemical method of degradation were first outlined by investigators in 1953 (18, 19) and involved (i) the periodate-oxidation of the cis-glycol of the polynucleotide, (ii) the removal of the terminal nucleoside residue by a base-catalyzed- β -elimination reaction, and (iii) the removal of the terminal phosphate group so formed by phosphatase, leaving the polynucleotide in a condition suitable for a second cycle of degradation. A subsequent study by Yu and Zamecnik (20.) indicated that primary amines greatly catalyze the β -elimination reaction. Since that time primary amines have been used in the presence of excess periodate to effect the β -elimination, and in all cases the product arising from the oxidized terminal nucleoside has been the purine or pyrimidine base.

In collaboration with Dr. P.T. Gilham (Purdue University), Dr. Schwartz studied the β -elimination reaction for his doctoral thesis. The reaction pathways shown in Figure 2 for the β -elimination of a nucleotide or polynucleotide were elucidated during the course of these studies. Pathway A has been described (14.) and Pathway B and its interrelationship with Pathway A are the subjects of a forthcoming publication (21).

- (2) Investigation of pathways A and B in order to develop sequencing methods employing post-labeling techniques

In these laboratories we have subsequently studied extensively both pathways A and pathway B with the expectation of developing a method for the post-labeling of the released nucleoside derivative. The use of ^{14}C -labeled primary amines (pathway B) to produce labeled derivatives of type IV (Figure 2) has proven to be unsatisfactory since these derivatives are unstable at $\text{pH} < 7$ and are therefore not easily fractionated by chromatographic methods. In contrast, the reaction utilizing tertiary amines (pathway A) proceeds under mild conditions to yield the dialdehyde derivative II (Figure 2) which can be quantitatively reduced with tritiated borohydride to the labeled nucleoside dialcohol III. The overall conversion of I to III takes place in 95-100% yield. The final product III is stable in acidic and basic aqueous media and thus may be readily analyzed by chromatographic methods previously developed for nucleoside fractionations.

- (3) Use of activated charcoal to separate labeled nucleoside dialcohol from salts and labeled contaminants

At the termination of each cycle of degradation the labeled nucleoside dialcohols are separated from salts and radioactive contaminants by adsorption to activated charcoal followed by subsequent desorption in a salt-free form. We have modified an existing method (22) to develop a procedure which (i) separates the dialcohol from >95% of the non-nucleoside contaminants labeled by tritiated borohydride, (ii) produces 85-90% yields of desalted nucleoside dialcohols in a form suitable for direct analysis by thin layer chromatography, and (iii) is rapid and readily automated.

- (4) Fractionation and detection of the labeled dialcohol nucleoside derivatives by thin-layer chromatography

The derivatives of uridine, cytidine, adenosine, and guanosine, having structures corresponding to III (Figure 2), may be fractionated by the thin-layer methods of Randerath (23). The tritiated derivatives are detected by autoradiography or by liquid scintillation counting and consequently analysis can be made in the subnanomolar or picomolar range.

- (5) Use of DNA oligonucleotides or polynucleotides as carrier for the RNA during sequence analysis

It is expected that the sequence analysis of picomolar amounts of oligonucleotide or polynucleotide by the stepwise method will be operationally difficult due to adsorption of the extremely small amounts of nucleotidic material to surfaces such as the walls of the reaction vessel. To circumvent this problem we have prepared a mixture of DNA oligonucleotides which may be used as a carrier with the RNA during its sequence analysis. The DNA mixture, which should ideally consist of oligonucleotides of random composition and length, will be added in great excess to the RNA at the beginning of the first cycle of degradation. The DNA subsequently behaves physically in the same way as RNA but chemically is inert to the cyclic degradations initiated by periodate oxidation. We have found that DNA oligonucleotides resulting from depurinated DNA (24) function ideally as carrier for small RNA oligonucleotides and do not interfere with the RNA sequence analysis. Further studies are being made on the generation of larger and more heterogeneous oligonucleotides and polynucleotides by digestion of DNA with deoxyribonuclease I (25, 26.).

- (6) Adaptation of the post-labeling method of sequence analysis to a solid-support system

We anticipate that the method of stepwise degradation outlined above may be adapted to a solid support system of sequence analysis, recently described by H.L. Weith and P.T. Gilham (15), which allows eight cycles of degradation to be carried out on a polynucleotide in 24 hours. The system consists of reaction vessel connected in a circular closed system to a very small anion exchange column (Dowex 1). The oligonucleotide to be analyzed may be specifically absorbed to the anion exchange resin in a solution of low salt or deabsorbed in a solution of high salt. Thus, at the end of a cycle of degradation the oligonucleotide may be isolated from the reaction mixture by extensive dilution and subsequent reabsorption to the anion exchange resin. A simplified schematic of this procedure is shown in Figure 3 for one cycle of degradation performed on the tetranucleotide ApCpGpU.

- (7) Uses of the above new method of stepwise sequencing

Following the adaptation of this new method of stepwise degradation to the solid support system, we hope to determine the 5' and 3'-terminal sequences of certain oncogenic viruses which contain no terminal polyadenylic acid stretches. This would be done by totally or partially fragmenting the unlabeled high molecular weight RNA with a specific nuclease such as RNase T₁. The resulting fragments would then be enzymatically phosphorylated (³²P-phosphate) with polynucleotide kinase (27.) and the resulting 5'-terminally labeled oligonucleotides would be separated by methods which allow the specific isolation of the 5'-terminal fragments (28.) or the 3'-terminal fragments (29.-31). Following isolation of these oligonucleotide or polynucleotide fragments their sequences would be determined by our post-labeling stepwise method of degradation.

Figure 2

solid support] ~ ApCpGpU ($\sim$ = ionic bond)

↓ high salt concentration

solid support] + ApCpGpU

↓ IO4

↓ tertiary amine

solid support] + ApCpGp +

↓ NABH₄3

solid support] + ApCpGp +

(³H-labeled)

↓ dilution to low salt concentration

solid support] ~ ApCpGp +

↓ elution

³H-labeled nucleoside derivative analyzed by thin-layer chromatography and autoradiography.

Figure 3

b. Labeling and modification of the 5'-P end of high molecular weight RNA by chemical means - E. Rapaport and P.C. Zamecnik

We have developed a new sensitive chemical procedure (32) which introduces high specific activity ^{32}P -label at phosphorylated 5'-ends of low and high molecular weight RNAs. The method is based on the reaction of $[\text{}^{32}\text{P}]$ diimidazolidate of orthophosphate, which reacts exclusively with primary phosphates. The internucleoside phosphate diesters are weak nucleophiles and remain unaltered. The high specific activity ^{32}P -labeled ribonucleic acid can then be analyzed for 5'-end group or 5'-terminal oligonucleotide sequences. This technique should prove useful in identifying terminal oligonucleotides following partial hydrolysis of the RNA. The fragments may then be sequenced using conventional techniques or those mentioned earlier.

It is essential for this labeling procedure that the 5'-end of the molecule terminate in a phosphate, since diimidazolidate of orthophosphate will not label a free 5'-hydroxyl group. Diimidazolidate of orthophosphate has been used as an intermediate in the synthesis of nucleoside-5'-diphosphates and triphosphates from the corresponding monophosphates and diphosphates respectively (33,34). We have succeeded in labeling the 5'-ends of tRNAs and higher molecular weight RNAs, utilizing the diimidazolidate of $[\text{}^{32}\text{P}]$ orthophosphate. The internucleotide diphosphate diester is a weak nucleophile and was shown not to react under these conditions. The reactions of ^{32}P -labeled diimidazolidate of orthophosphate with tRNAs in anhydrous formamide in the presence of tributylamine led to the 5'-labeling of the tRNAs. Excess reagents were dialyzed out and the tRNA (mixed) retained lysine acceptance activity to about 80 percent of its original acceptance activity after chromatography on Sephadex G-100. This was a double labeling experiment using high specific activity ^3H -lysine and $[\beta\text{-}^{32}\text{P}]$ *E. coli* mixed tRNA 5'-diphosphate. The 5'-terminal labeling reaction is indicated in the following figure:

Employing this diimidazole phosphate reagent, we have very recently found the presence of some 5'-phosphorylated ends on 35S AMV-RNA (32). Thus the 35S AMV-RNA appears to consist of 5'-OHRNA, 5'-ppRNA, and 5'-pppRNA. (See also ref. 35)

3. Relationship of the First Step in Protein Synthesis to "Magic Spots" 1 and 2 and to the 5'-end of High Molecular Weight Messenger RNA - P.C. Zamecnik and E. Rapaport

Many years ago in these laboratories, under the auspices of a direct ancestor of this Contract, the first step in protein synthesis was discovered (36,37). This was the reaction by which an amino acid is activated in the presence of ATP and an activating enzyme specific for that amino acid. The reaction is a reversible one, and can be diagrammed as follows (reaction (I)), bearing in mind that our former colleague Robert Loftfield may in the instance of some amino acids be correct in regarding the path from free amino acid to aminoacyl-tRNA to represent a concerted reaction mechanism, without a definitive intermediate.

Some years later, again under the auspices of this Contract, we discovered (38, 39) that in the back reaction of (I) above, a new compound was formed, in an in vitro enzymatic system, as shown in reactions (II) and (III).

This synthesis of Ap₄A (our abbreviation for the compound in reaction (III)) during the course of the usual activation reaction was not restricted to the lysyl synthetase activating enzyme system, but we have found the latter to be a convenient one with which to work, since the enzyme is easily purified, relatively stable over long storage in the frozen state, and that the activation does not require the presence of tRNA, although its rate is accelerated by the latter.

In other words ATP competes with inorganic pyrophosphate in the back reaction. We have indeed found that numerous pyrophosphate compounds will successfully compete with inorganic pyrophosphate in this back reaction, in the in vitro system, and an interesting series of mixed-5'5'-nucleoside tri- or tetraphosphate compounds has been found in our previous work (40,41) to be formed in this way. These may be designated as A-5'-(p)_n-5'-N, where n represents at least three linear phosphoryl moieties, A is adenosine (or deoxyadenosine), and N represents generally a ribo- or deoxyribonucleoside, but certain other moieties as well.

Although these compounds are formed and easily detected in vitro, and their formation has been confirmed in another laboratory recently (Hecht, S.M., personal communication), the question arises as to whether they are test tube curiosities or may be contained also in living cells. It should be mentioned that Finamore and Warner (42) in 1963 discovered the presence of large quantities of the unusual nucleotide P¹, P⁴-diguanosine 5'-tetraphosphate in encysted embryos of the brine shrimp, *Artemia salina*. In some as yet obscure way the energy-rich phosphate of this compound is converted to use in the form of ATP, which then appears to be used in initiating the life processes of this organism when the latter is reintroduced into water. Bearing in mind our in vitro findings and the above example, we searched for the presence of Ap₄A in living *E. coli* and in rat liver. With some difficulty we established that this compound was present in these living forms in a concentration of the order of $1 \times 10^{-8}\text{M}$ (40,41).

We found it difficult to pursue this problem further at that moment due to lack of clear leads. We have, however, watched with great interest the developments which have followed the discovery of the "magic spot" compounds 1 and 2 by Cashel and colleagues (43-45). These compounds are pp-5'-G-3'-pp and ppp-5'-G-3'-pp. They appear in prokaryotic cells under stringent growth conditions (and in unpublished work of which we have heard, in eukaryotic cells as well) and are related in some way to the cessation of RNA synthesis which follows deprivation of an essential amino acid in stringent forms of *E. coli*. They also participate in place of GTP in certain steps in peptide chain elongation. Sy and Lipmann (46) and Sy (47) have recently isolated the usually ribosome-bound enzyme which catalyzes the formation of these two compounds, and have demonstrated that the catalysis occurs according to the following pathway:

Although the reaction is to some extent reversible, it is difficultly so, being inhibited by ATP at $1 \times 10^{-5}M$ concentration. Upon removal of an essential amino acid in growth of a stringent strain of bacteria, there is prompt (10-15 minute) formation of pppGpp. Upon addition of the amino acid there is equally rapid disappearance of this compound, and the pathway for its disappearance is still obscure.

It may be mentioned that many years ago under support of this Contract, we discovered the participation of pppG (GTP) in protein synthesis (48). Over the intervening years we have followed with interest the ramifications of the story, as yet incomplete, of the several roles of GTP in the peptide chain initiation and elongation steps.

We recently wondered whether pppGpp and ppGpp would compete with pp in the back reaction of the amino acid activation step, as diagrammed above, and therefore added ppGpp and pppGpp to the lysine activation system. These two unlabeled compounds were generously furnished by Dr. Michael Cashel. Two unknown new spots appeared in the elution patterns of our PEI-cellulose plates, one when ppGpp was added, and another when pppGpp was added. Because of the position of migration of these ultraviolet absorbing spots, on PEI-cellulose plates in 1M lithium chloride, 0.75M K phosphate, or 1.5M K phosphate, pH 3.4, and in view of our previous experience with pyrophosphate-containing compounds in this reaction, we suspected that these new compounds might be A-5'-ppp-5'-G-3'-pp and A-5'-pppp-5'-G-3'-pp, and the reaction mechanisms involved in their formation to be as shown below. Based on this assumption, the following reactions were carried out to prove or disprove these presumed structures:

- a) Use of 3H -ring-labeled pppA^{*} as a precursor:
The new spots contained the tritium label, indicating that the adenine moiety was part of these compounds.
- b) Use of 3H -ring labeled guanine in the ppGpp^{*} and pppGpp^{*} employed as precursors in the enzymatic reaction (these compounds kindly furnished by Dr. Jose Sy):
The new spots contained the tritium label, indicating that the guanine moiety was a part of these compounds.
- c) Use of $p^{32}Gpp$ and $p^{32}ppGpp$ as precursors (kindly furnished by Dr. Sy as formed in crude mixes, and purified for our use in our laboratories):
The new spots contained ^{32}P radioactivity.

- d) Use of phosphomonoesterase on the new compounds:
Treatment of labeled presumed AppppGpp or ApppppGpp with phosphomonoesterase yielded labeled AppppG and ApppppG respectively. These compounds were identified by their chromatographic properties which were identical with unlabeled AppppG and ApppppG. The two unlabeled markers were in turn prepared by reacting GDP and GTP in the lysine activating system.

Furthermore, in this type of experiment the 5'- α - ^{32}P -label was preserved in the derived compound(s), thus demonstrating that the 5'-pp rather than the 3'-pp moiety of the 5'-ppGpp-3' was involved in the linkage with the adenylyl portion.

- e) Addition of snake venom diesterase to the new compound(s):
With ^3H -ATP as a precursor in the formation of the compound, addition of snake venom diesterase resulted in the liberation of ^3H -AMP (pA) and of ppGpp.

The above reactions and manipulations permit the following formulations to be given:

The new compounds are thus identified as A-5'-ppp-5'-G-3'-pp and A-5'-pppp-5'-G-3'-pp. The question of reversibility of reactions V and VI remains to be studied.

Whether they are also formed in living cells remains a problem for the future, which we intend to pursue in the coming year.

In the case of a number of normal cell messenger RNAs and some viral high molecular weight RNAs as well, the 5'-end terminates as ppRNA or pppRNA. We have therefore wondered whether these pyrophosphoryl moieties might also compete with inorganic pyrophosphate, and participate in the back reaction of the amino acid activating step in protein synthesis, as pictured in reaction (I) above. We have very recently found that the high molecular weight 35S RNA of the Avian Myeloblastosis Virus terminates as 5'-OH-RNA, 5'-ppRNA, or as 5'-pppRNA (32). This is incidentally the first observation that an oncogenic viral high molecular weight RNA may end in part as 5'-pp- or 5'-pppRNA. We have therefore added 35S AMV RNA to the lysine amino acid activating enzyme system depicted in reaction (II) above, in the presence of ^3H -pppA. Preliminary results to date, carried out by Dr. Rapaport, indicate the formation of A*pppA-RNA. This exciting result is being pursued actively. It is especially interesting in view of the recent finding of Shatkin and colleagues (49) of the presence of me7-G-5'-ppp-5'-G-2'Ome-RNA at the 5'-terminus of reovirus and other viral RNAs.

4. Translation Studies in Cell-Free Protein Synthesizing System Using 35S AMV RNA as the Template - M.L. Stephenson and P.C. Zamecnik

Our chemical studies on the 35S RNA and also on the viral tRNA have led us to a point where we would like to investigate the biosynthesis of viral proteins and examine the effects of chemical alteration of the RNA on the normal regulatory mechanisms of the cell. Because RNA sequencing by the enzymatic copying of the RNA as described by Dr. Weith is more promising for determination of longer sequences of nucleotides interior to poly(A), the work described by Dr. Stephenson using borohydride labeling of ribonuclease fragments will not be pursued. She has consequently devoted herself to developing a cell-free protein synthesizing system capable of responding to AMV messenger RNA.

Drs. Stephenson and Zamecnik have had extensive experience in the biosynthesis of proteins in cell-free systems, extending back for many years, mainly under the umbrella of this contract.

A number of pioneer investigations on protein synthesis were carried out by them and others in these laboratories (see Proposal). The studies described in the background section were carried out for the most part on normal tissues and were aimed at elucidating normal mechanisms of protein synthesis (50,51).

The high molecular weight oncogenic viral RNA has been shown to be implicated in at least two biological functions. To begin with, it is now well established that this RNA serves as a template for the oncogenic viral reverse transcriptase to synthesize DNA (52,53). The DNA somehow becomes integrated into the host cell DNA (54) so that virus specific mRNA can be transcribed from infected cell DNA.

On the other hand there is also ample evidence that the high molecular weight RNA has messenger activity in a cell free protein synthesizing system (55,56). This class of RNA in similar viruses (Rauscher) is capable of synthesizing viral group specific-antigen (57) in an *E. coli* cell free system, and Gielkins et al. (58) have also shown that a heated 60S RNA from Rauscher virus in a similar cell-free system can synthesize viral polypeptides. Upon heat, urea, or DMSO treatment the 65-70S RNA complex becomes dissociated and sediments in sucrose gradients as two or three molecules of "35S" RNA plus three or four molecules of 70S associated 4S RNA (59).

Naso, Arlinghaus, et al. (60), using a Rauscher leukemia virus infected cell-free system have recently demonstrated that the 65S RNA binds to ribosomes and forms polysomes, whereas the 35S RNA mainly forms monosomes. The protein product, made in response to 65S RLV-RNA, contained two high molecular weight polypeptides, analogous to the precursor "polyprotein" seen in picornavirus infected cells. See Review by Shatkin for more complete references (61). In cell-free systems these polypeptides are not always cleaved into the lower molecular weight proteins of the mature virion. Several laboratories have shown, however, that *in vivo* there is specific cleavage of these polyproteins into the proteins seen in the mature virus. These have been *in vivo* pulse-chase experiments with subsequent identification of the viral specific proteins by immune precipitation using antisera to the virus under investigation. See Eisenman et al. (62), Baltimore et al. (63), Butterworth et al. (64), and most recently for Rauscher leukemia virus see Naso et al. (65). The cell-free systems so far for these viruses lack enzymatic processes which cleave these polyproteins into smaller mature viral proteins. See very recent paper by Von der Helm and Duesberg using Rous Sarcoma Virus (66).

It has been demonstrated by a number of groups that the viral RNA from Rauscher, Avian Myeloblastosis, Feline leukemia, Moloney Sarcoma, Rous Sarcoma, visna, Mason-Pfizer agent, reo, Eastern Equine Encephalitis, polio, and Sindbis contain sequences of polyadenylic acid (poly(A)). Others have shown that mRNA, both viral and non-viral, contain poly(A) segments and evidence has been presented that the poly(A) of some of these mRNAs contains the 3'-OH terminus of the molecule. In the cases of the RNAs from poliovirus and reovirus there is evidence that poly(A) segments also constitute the 3'-termini (2 for all references). Until our report, however, there had been no definitive evidence that high molecular weight oncogenic viral RNA terminated in a 3'-OH poly(A) sequence. We reported last year that total 4S RNA and the "70S-associated" 4S RNA of AMV were rich in minor bases (67,68) and that both types of 4S RNA of AMV accept amino acids (69). This permits the conclusion that virion tRNA and the "70S-associated" tRNA are not passive inclusions, but play a subtle active role in the life cycle of the virus. Very recent data from Bishop's and Dahlberg's laboratories appear to demonstrate that a particular few individuals from the tRNA family of amino acid acceptor molecules serve as specific primers for Rous sarcoma virus reverse transcriptase (70-72). The 70S-associated tryptophanyl- and/or methionyl-tRNA has been implicated as the primer molecule with conflicting evidence from the two laboratories. The role of these viral tRNAs in protein synthesis has not been delineated aside from the fact that they are able to accept amino acids.

The wheat embryo ribosomes or 30,000 x g supernatant fractions (ribosome plus factors) have been used with success to investigate properties of globin mRNA (73, 74), Satellite Tobacco Necrosis Virus RNA (75), and L cell mRNA under various conditions of deadenylation (76). Efron and Marcus (73) and Roberts and Paterson (74) have demonstrated that the wheat embryo protein synthesizing system plus rabbit globin mRNA produce in vitro products which correspond to their in vivo counterparts from hemoglobin. This system apparently produces accurate translation of the added mRNAs (77,78). We have chosen to use the wheat embryo 23,000 x g supernatant fraction (S-23), which contains factors necessary for protein synthesis as well as ribosomes but is free of endogenous mRNA and ribonuclease.

Other cell-free systems have been shown to produce polyproteins containing viral antigens when incubated with RNA from AMV and from other oncogenic RNA viruses (discussed earlier). None published to our knowledge to date have used wheat embryo ribosomes. We are choosing to do so in part because we have found the AMV 35S RNA or 70S RNA to be exquisitely sensitive to attack by ribonuclease, and because the wheat embryo tissues contain less ribonuclease than some of the other systems investigated. The viral specific proteins produced in response to added viral RNA will be identified in a variety of ways. In these initial studies we plan to look for an increase in the incorporation of labeled amino acids into TCA insoluble material, dependent upon added viral RNA. Preliminary experiments just completed indicate that the 35S RNA from AMV stimulates the incorporation of H^3 -leucine using a wheat embryo S-23 fraction. So far we have been able to achieve a four-fold stimulation by heated 70S RNA over the control values using H^3 -leucine under conditions where we get a 18-fold stimulation using rabbit globin mRNA (a gift from Drs. B. Forget and J. Pritchard). We have run KCl, $Mg(Ac)_2$, amino acid, and RNA concentration curves and are optimizing our incorporation response to the heated 70S or 35S RNA before attempting to identify the proteins produced using the techniques described below. To our knowledge this is the first time oncogenic viral RNA has been used in the embryo system. However, by the time this application is reviewed, such may not be the case. This wheat embryo system has been shown to produce viral specific proteins in response to other viral RNAs (75). See proposal for experiments planned to identify viral specific proteins and to investigate the "factors" necessary for production of such proteins.

B. Normal reactions in the initial stages of protein synthesis, the recognition reaction between tRNA and its cognate aminoacyl-tRNA synthetase

1. A Study of the Molecular Basis for the Specificity of Transfer RNA Aminoacyl tRNA Synthetase Interaction and of the Mechanism of Action of Aminoacyl tRNA Synthetase - D.L. Coleman

a. Introduction

(1) Significance of protein-nucleic acid interactions

(2) Interactions between aminoacyl-tRNA synthetases and tRNA

b. Earlier inhibitor experiments carried out on the methionyl tRNA synthetase

c. Present studies on the recognition by aminoacyl-tRNA synthetase and its cognate tRNA

(1) Crosslinking of the synthetase and tRNA

(a) Introduction

(b) Cross-linking by ultraviolet radiation

(c) Co-crystallization of tRNA and synthetase

(2) Current studies with the methionyl-tRNA synthetases

1. A Study of the Molecular Basis for the Specificity of Transfer RNA Aminoacyl tRNA Synthetase Interaction and of the Mechanism of Action of Aminoacyl tRNA Synthetase - D.L. Coleman

a. Introduction

(1) Significance of protein-nucleic acid interactions

We currently have a meager understanding of how highly specific interactions between proteins and nucleic acids arise, and we are awed by the apparent critical importance of such interactions to the normal and abnormal functioning of the cells. Through the haze of existent observations and theories, it seems that the activity or dormancy of genetic material, the successful subversion of the cell's metabolic machinery by oncogenic viruses, the ordered sequence of events leading to synthesis of proteins and nucleic acids, the involvement of transfer RNAs in the control of amino acid synthesis, the curious patterns of modification of the bases of transfer RNA and their association with transformed cells, the operation of restriction modification enzymes, and many other elements of cell metabolism must involve delicate balances between the association and dissociation of complexes between a spectrum of proteins and nucleic acids in the cell. Various investigators have proposed the importance of a number of different types of interactions such as electrostatic (79) and aromatic stacking (80). Additionally, the possible participation of hydrogen bonding (81) has been recognized. At this time it is not clear whether the interaction between a given protein and nucleic acid might or might not depend on the existence of several types of interactions simultaneously. We have been interested in this type of interaction for many years (51).

Abbreviations Used in this Section: MRS - methionyl tRNA synthetase
PCMB - p-chloromercuribenzoate
DSB - dimethyl-(2-hydroxy-5-nitrobenzyl) sulfonium bromide

(2) Interactions between aminoacyl-tRNA synthetases and tRNA

One of the systems mentioned above has been of continuing interest in the Huntington Laboratories since the discovery of activating enzymes and the implication of soluble RNA in the process of protein synthesis nearly 20 years ago (82). With the demonstration that the identification of the aminoacyl-tRNA at the ribosome is on the basis of the tRNA alone (83) and the elaboration of the genetic code (84) the first evidence was obtained that the different amino acids must be associated with distinctly different tRNAs and that the third element in this triumverate of specificities must be the aminoacyl-tRNA synthetases. Indeed, much attention has been focused on the interactions between the synthetases and their cognate tRNAs, the question being, "Since tRNAs having different specificities share so many structural features in common, what features serve as points of recognition and discrimination?" Nearly all investigations have been directed toward the tRNA (85), but it is clear that the contact surface of the tRNA for the enzyme which embodies the integration of a constellation of recognition points must be matched by a complementary surface on the enzyme. It is to this latter field that we have chosen to direct the attack. For every site on the tRNA which contributes to the binding energy, be it a charged group, aromatic ring, hydrocarbon cluster, or hydrogen bonding donor or acceptor, there is a corresponding complementary group on the enzyme. By chemical and physical means, we are probing the surface of the enzyme, attempting to identify and map the specific amino acids which participate in tRNA binding.

In addition to our interest in the problem of protein-nucleic acid interactions, the question of the mechanism of action of the synthetases continues to be an intriguing one to many investigators. For some time now two models for the mechanism of action of the aminoacyl-tRNA synthetases have vied for acceptance: the concerted mechanism which has been promulgated by Loftfield (86) and the widely accepted sequential mechanism (87). In studying the effect of various site-specific reagents on the activity of the synthetases, it is clear that those which inactivate the enzyme, but do not have a marked effect on the substrate binding properties probably act by blocking the active site directly. As well as stemming from a fundamental interest in the mechanism of action of this class of enzymes, our interest in the mechanism has also been stimulated by the elaboration of the mechanism of synthesis of the peptide antibiotics, gramicidin and tyrocidine, by Lipmann's group (88) and the question of whether the mechanisms of the aminoacyl-tRNA synthetases and the gramicidin and tyrocidine synthetases are similar, possibly reflecting a common ancestral origin for both systems.

b. Earlier inhibitor experiments carried out on the methionyl-tRNA synthetase

Studies carried out with PCMB-inhibited MRS indicate that the initial loss of activity is very rapid being complete in 0.25 minutes at 0°, and occurs to the same extent for both INC and EX activities. A slower inactivation process is also observed, with a rate which does not appear to be directly related to the amount of PCMB when the concentration of the inhibitor is less than that of the enzyme. The fluorescence of MRS is quenched indicating a conformational rearrangement of the enzyme, but the initial rate is much less than the rate of inactivation. This suggests that the initial inactivation of MRS by PCMB is not due to a conformational change large enough to be followed by fluorimetry and thus, that the site(s) of modification by PCMB which lead to a very rapid inactivation may be directly at the active site and/or substrate binding sites of the enzyme. Studies of the binding of methionine and tRNA to the PCMB-inhibited enzyme by equilibrium dialysis and the nitrocellulose filter assay (89), respectively, indicated that the fully inhibited enzyme has a considerably lower affinity for methionine and cannot bind tRNA (90).

The kinetics of inactivation of MRS by DSB have also been investigated and the effect of pH and inhibitor concentration examined. Unlike PCMB inhibition DSB inhibition takes place at a rate which is slow enough to be extrapolated back to the starting point of the experiment. The rate constant is not first order, but this is probably due to the changing concentration of the inhibitor caused by concomitant hydrolysis of the DSB. Further, the extent of inactivation of EX activity is significantly less than the inactivation measured by INC activity, an effect which was not observed for the initial, fast inactivation by PCMB. The presence of methionine and ATP simultaneously during the inactivation reaction results in a lowered rate of inactivation as measured by both activities, but the effect is substantially greater on the EX than on INC activity. These experiments were discussed in last year's report.

c. Present studies on the recognition by aminoacyl-tRNA synthetase and its cognate tRNA

(1) Cross-linking of the synthetase and tRNA

(a) Introduction

In continuing our study of the recognition by aminoacyl-tRNA ligase of its cognate tRNA, we shall be directing more effort towards the use of physical cross-linking of the protein and the nucleic acid. This technique provides direct access to the portions of the molecules which are juxtaposed in the complex. There are several methods which may be used and some have been used with varying degrees of success.

Direct cross-linking resulting from UV radiation offers a promising approach to the study of the sites involved in protein tRNA complex formation. Studies on model systems have shown that ten amino acids can cross-link to uracil, with phenylalanine, tyrosine, and cysteine being the most reactive (91). This technique has been used successfully with the isoleucyl synthetase-tRNA system (92,93) with DNA and DNA-dependent RNA polymerase. The identity of the bases and amino acids involved in cross-linking was not determined in either of these studies.

(b) Cross-linking by ultraviolet radiation

We have carried out some preliminary experiments from which promising results were obtained. Irradiation of tRNA^{met} in the presence of an excess of methionyl-tRNA ligase for 15 minutes using germicidal lamps as a source yielded about 25 percent cross-linking. Schoemaker and Schimmel have observed similar results using the isoleucine system of *E. coli* (92) and find that addition of fresh enzyme to the irradiation reaction produces an additional increment of cross-linked tRNA-ligase. The limitation on the amount of cross-linked tRNA-ligase appears to be due to radiation damage to the enzyme.

We shall continue with these studies in several stages. First, it is desirable to study further the effect of pH, ionic strength and other parameters on the cross-linking process. Indeed, measurement of the rate of cross-linking under defined conditions may serve as a quick and convenient assay for association of enzyme and tRNA. Such an assay would be most useful since there is now evidence that the nitrocellulose filter assay of Yarus (89) may be subject to serious artifacts (94).

Analysis of the sites on the proteins which are cross-linked to the tRNA will be carried out using established techniques of peptide mapping and analysis. We now have a Beckman 120C Amino Acid Analyzer in our laboratory which will be used to identify the residues which cross-link to tRNA.

(c) Co-crystallization of tRNA and synthetase

We have also begun a co-crystallization project. To date we have not obtained any crystals under the conditions which have been used. The use of a simple crystallization-dialysis chamber described by Lagerkvist (95) makes it possible to alter the buffer composition readily and to use very small amounts of protein and tRNA.

(2) Current studies with the methionyl-tRNA synthetases

We are also engaged in further studies on the mechanism of action of the aminoacyl-tRNA ligases. Having prepared a large batch of enzyme (MRL-12, >100 mg) from cultures of the merodiploid strain, EM2001, we found that we have obtained the enzyme in its native form (MW ~ 175,000). Since our previous work was done with enzyme which had been modified by endogenous proteolytic enzymes (90) (MRL-10, MW ~ 60,000), it has been necessary to repeat some of our previous experiments. For example, the sensitivity of MRL-12 to p-chloromercuribenzoate appears to be much lower than that of MRL-10. To determine whether this is in fact due to unshielding of certain parts of the protein, we must convert MRL-12 to the low MW form using trypsin and compare the sensitivity of the modified MRL-12 (MRL-12+) with that of native MRL-12.

We have only recently been able to get the gel filtration equilibrium method (96) working well for the tRNA-MRS system. The determination of the effect of dimethyl-(2-hydroxy-5-nitrobenzyl) sulfonium bromide (DSB) on the ability of MRS to bind tRNA is our immediate objective. Although we have not established whether the inactivation of MRS by DSB is due to reaction with tryptophan or sulfhydryl residues, it is clear from our study of the kinetics of inhibition that PCMB and DSB do not inactivate the enzyme in the same way. The protection which tRNA provides from inactivation by DSB is much more pronounced for aminoacylation activity than for ATP-pyrophosphate activity (97) which strongly suggests that the inhibitor is blocking either binding of tRNA to the ligase or transfer of the activated amino acid to the 3'-terminal of the tRNA.

We have found that DSB-inhibited enzyme seems to have fewer free sulfhydryl groups as measured by titration with dithionitrobenzoic acid. But the measurements were made near the limit of sensitivity of the assay and recognizing that we have yet to look at other residues we have decided to utilize the amino acid analyzer to provide an overall profile of the modification. Utilization of the p-toluene-sulfonic acid method of Liu and Chang (98) avoids the destruction of the tryptophan residues during hydrolysis of the protein.

Having noted the apparent inactivation of MRL by ultraviolet radiation, we are interested in attempting to analyze the nature of the lesion. Although the effects of irradiation on tRNA have been utilized in the study of tRNA-ligase interactions (99), the nature of radiation effects on the enzyme has received no significant attention. The procedures to be utilized include binding studies and the identification of the modified residues.

In the course of the past year, we grew up a 150 liter culture of the merodiploid strain, EM2001, obtained from Dr. McFall of New York University. The yield of MRL from this strain is 4-5 times that of the wild-type. In the course of purifying the MRS from this strain we obtained a significant quantity of valyl-tRNA ligase. We would like to include this ligase in some of our studies and, possibly, lysyl-tRNA ligase which our colleague Dr. Hirshfield has purified, in an effort to determine the generality of the observed effects.

2. Influence of Metabolites on Lysyl-tRNA Synthetase Formation in E. coli - I.N. Hirshfield

In the past year Dr. Hirshfield's efforts have been geared primarily to the purification of lysyl-tRNA synthetase from Escherichia coli K-12, and to the study of the properties of the purified enzyme. In these studies we have purified the enzyme from both wild-type and mutant strains. The cells were grown under carefully controlled conditions in minimal medium with or without 20 mM L-alanine or 3 mM glycyl-L-leucine. The latter were used because they can stimulate the synthesis of lysyl-tRNA synthetase in wild-type and mutant strains of E. coli (100-102). The stimulation is 2- to 3-fold in wild-type and up to 50- to 70-fold in some mutant strains (100,102).

The lysyl-tRNA synthetases from mutant and wild-type cells were purified to 70-90% of homogeneity. Physical and kinetic studies revealed that: 1) The lysyl-tRNA synthetase from the mutant strains differed from the wild-type synthetase; and 2) That the wild-type synthetase isolated from cells grown in the presence of alanine or the peptide differed from the lysyl-tRNA synthetase isolated from cells grown in normal minimal medium. These differences are based on studies of heat and urea denaturation, subunit composition, resistance to trypsin inactivation, and determination of kinetic constants (K_m values) for the substrates ATP, lysine and transfer RNA. These results indicate that the presence of alanine or the leucine dipeptide, glycyl-L-leucine, not only stimulate the synthesis of lysyl-tRNA synthetase, but also modify its structure. Hence there appears to be a structure-function relationship between modification of the synthetase and stimulation of its synthesis. On this basis we have formulated a working hypothesis that lysyl-tRNA synthetase is autoregulatory and can control its own synthesis. See proposal for further discussion.

III. References

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IV. Publications During the Past Year, 1973-1974

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