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PROGRESS REPORT

TO

THE UNITED STATES ATOMIC ENERGY COMMISSION

CONTRACT AT(30-1)910

BIOLOGICAL EFFECTS OF RADIATION AND RELATED BIOCHEMICAL

AND PHYSICAL STUDIES

Proposal #6: Physical Chemical Studies on Nucleic Acids
and Derivatives

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Investigators: Mary L. Petermann, Ph.D., Member
Liebe F. Cavalieri, Ph.D., Member

Department: Divisions of Biological Chemistry and Genetics

The Sloan-Kettering Institute for Cancer Research
410 East 68th Street, New York, New York 10021

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Key.

Section 5702

Mary L. Petermann, Ph.D., Head

1. During the past year we have continued to study active subunits from rat liver ribosomes.

A. Isolation of subunits (Dr. M. L. Petermann)

As described previously, when adult male rats were pre-treated with phenobarbital to decrease the RNase content of their livers, highly active subunits were obtained by dissociating ribosomes with urea. Both small and large subunits could be stored in 10% sucrose at -20° , if $MgCl_2$ was added to the small-subunit solution before freezing. Activity is now tested in this laboratory, by measuring polyphenylalanine synthesis in the presence of polyuridylic acid and the liver pH 5 fraction.

In preliminary experiments active 40S and 55S subunits were also obtained by dissociating ribosomes with 6.6 M formamide.

B. Properties of Subunits (Dr. M. G. Hamilton, Dr. Petermann)

The 28S RNA from the large subunit had a molecular weight of 1.65×10^6 daltons, measured by sedimentation equilibrium.

The active small subunit, 40S, is a complex particle, containing the 18S RNA (600,000 daltons) and 850,000 daltons of assorted proteins, attached with varying degrees of firmness. Thus when whole ribosomes were dissociated by various treatments, and the subunits were isolated by sedimentation in sucrose gradients, 9 M formamide removed 28% of the small-subunit proteins; EDTA removed 31%; and 5 M urea removed 38%. Exposure of 40S subunits to EDTA, without the sucrose gradient fractionation, removed varying amounts of proteins. As previously reported, the 40S subunit also contained about 50,000 daltons of rapidly labeled RNA. This RNA was detached by 5 M urea as well as by EDTA.

2. Unfolded Subunits. (Dr. Petermann, Dr. Hamilton)

Both subunits were converted to slowly sedimenting forms in 9 M formamide. The large subunit, 42S, resembled the 5 M urea particle (39S) described previously; it still had a molecular weight of 3×10^6 daltons and contained 28S RNA, but had lost the 5S RNP described below. The small subunit, 27S still contained 18S RNA, but had lost protein, as discussed above.

3. KCl Subunits (Dr. Hamilton)

The 40S subunit isolated by the 0.88 M KCl method of Martin and Wool (J. Mol. Biol. 43:151, 1969) had a molecular weight and gross protein content similar to those of the urea subunit, but was much less stable.

4. The 5S RNA-protein complex (5S RNP).

When the 5S RNA was detached from ribosomes or large subunits by EDTA, 5 M urea, or 9 M formamide, and the extract was analyzed by polyacrylamide gel electrophoresis, three bands appeared: tRNA, 5S RNA, and a slower band containing both 5S RNA and protein. Formamide gave the best yield of this material. It has been purified by fractionation on G-200 Sephadex, where its elution volume corresponded to a molecular weight of about 80,000 daltons. When it was fixed with formaldehyde and centrifuged in a CsCl gradient it banded at a density of 1.568 g/ml, corresponding to an RNA content of 48.5%. Combining this value with the molecular weight of the 5S RNA, 40,000, gave 83,000 daltons for the complex. Since the protein had a molecular weight of 41,000, measured by gel electrophoresis in sodium dodecyl sulfate (SDS gel), the RNP complex must contain only one molecule of protein. The complex was readily dissociated by 0.5 M KCl. In 5 mM K phosphate buffer at pH 7.8 it was most stable in 20 mM KCl, 0.2 mM MgCl₂ or in 40 mM KCl with no Mg; at lower cation or higher Mg concentrations² the stability decreased.

5. Ribosomal proteins (Dr. Hamilton, Dr. Petermann)

As described previously, the 50S large subunit obtained by EDTA treatment contained many different proteins. Sedimentation equilibrium measurements on isolated proteins gave molecular weights of 29,000 or less. Molecular weight analysis of the total protein mixture, by electrophoresis in SDS gels, has now revealed small amounts of additional components with molecular weights of 45,000 to 58,000. Altogether there were about 73 molecules of protein, with a number average molecular weight of 19,000 daltons.

The 600,000 daltons of protein remaining on the small subunit after EDTA treatment showed complex patterns on gel electrophoresis, both in 6 M urea at pH 4.6 and in SDS gels. The latter patterns indicated the presence of about 28 molecules of protein. Their molecular weights ranged from 10,000 to 35,000, with a number average of 21,000 daltons. In the acid-urea gel the total proteins of the 40S subunit showed increased amounts of several components, plus one extra slow-moving protein. In SDS gels, however, the 30S and 40S patterns showed only minor quantitative differences, so the extra 40S proteins do not have unique sizes.

For both subunits, the amounts of the various proteins indicate that, unlike the bacterial ribosome, the animal ribosome probably contains multiple copies of many of its proteins.

On SDS gel electrophoresis the proteins of whole ribosomes showed a slow band, of molecular weight about 70,000, that was not found in either the 40S or 59S subunit.

Plans for Continuation of Present Objectives:

Since we now have methods for preparing active ribosomal subunits in milligram quantities, we plan to investigate the structure of each subunit in detail. Changes in conformation will be correlated with activity (ability to synthesize polyphenylalanine), and we will try to reconstitute active large subunits by combining the unfolded 42S form with the 5S RNP.

The role of sulfhydryl groups in maintaining the structure of the 40S subunit will be examined, and the subunit will be extracted with agents, such as concentrated salts, that remove initiation factors. The treated particles will be characterized and their proteins will be analyzed by gel electrophoresis. The released proteins will also be examined electrophoretically, and their possible functions will be investigated. Similar studies will be made on the large subunit.

The rapidly labeled RNA attached to the 40S subunit in vivo will be studied further. We plan to compare its binding to that of the labeled peptidyl or aminoacyl tRNA that we find on this subunit after the in vitro incorporation of ^{14}C -amino acids.

The 70,000-dalton protein from whole ribosomes will be characterized, and we will try to determine its function.

Section 6601

Liebe F. Cavalieri, Ph.D., Head

1. DNA Polymerase Studies

We had shown previously by means of acrylamide gel electrophoresis, that DNA polymerase exists as three molecular species. In order to establish this more firmly, the purification was extended to gel filtration. Two active fractions were isolated using this method: one in the molecular weight range 120,000-140,000 daltons, the other in the range 65,000-78,000 daltons.

In a continuation of studies on the isolation of various forms of DNA polymerase we have used a third new method which involves DNA-acrylamide gel column chromatography. Acrylamide monomer is polymerized in the presence of DNA and the resulting gel is pressed through a wire mesh. The gel particles are then used in the usual way for chromatography. Crude polymerase is applied to the column and after one passage the specific activity is increased 200-fold. The purified enzyme has been characterized by sucrose density gradient sedimentation, gel filtration, acrylamide electrophoresis, and sedimentation equilibrium. All methods show that the active polymerase is heterogeneous with respect to size. The molecular weight classes are: 10,000-20,000; 40,000-50,000; 75,000-85,000; 100,000-120,000. The existence of the various species has been corroborated using still another isolation procedure in the presence of the proteolytic inhibitor phenylmethyl sulfonyl fluoride. A combination of DEAE cellulose chromatography and Sephadex G-200 gel filtration reveal the same general molecular weight classes of active polymerase. There is a marked tendency for the lower molecular weight species to aggregate as the concentration increases. It is not yet established whether these active smaller molecules are subunits or are fragments arising from proteolysis.

Following an experiment done many years ago with synthetic ribopolymers as templates in the *E. coli* polymerase system, we have investigated the use of 5S and ribosomal RNAs from rat liver as possible templates in this DNA polymerase system. A DNA product is formed and we suspect that the RNA is acting both as a template and a primer; the question is still unresolved. Gradient centrifugation in Cs_2SO_4 shows a variety of products which change as a function of the extent of reaction. These products include: a DNA-RNA hybrid, DNA and poly dAT. Both crude as well as highly purified polymerase behave similarly. We have found that added traces of DNA to ribosomal RNA results in a striking increase in synthesis. That is, there is a synergistic effect: the extent of synthesis is greater than for the sum of DNA and RNA alone. Here too, a variety of products are formed as shown by gradient centrifugation and these also vary with the extent of reaction.

2. Rous Sarcoma Virus RNA

RNA has been isolated from Rous sarcoma virus in order to determine the length and nature of the homopolymer segments; i.e. of the polyadenylate and polyuridylylate stretches. Cs_2SO_4 density gradient centrifugation was the

method of analysis. If, in fact, there are long lengths of poly-A and poly-U in the viral RNA, then the addition of synthetic poly-A and poly-U to this viral RNA should lead to complexes poly (A+U). The density of the viral RNA was shifted to lower values, as expected, on addition of either poly-A or poly-U. Therefore, the viral RNA has both types of stretches in it.

3. In vivo DNA Replication Studies in E. coli Membrane Attachment of DNA

We have shown that shear can denature as well as degrade DNA. There is a fraction of cell DNA which is very sensitive to mild shearing forces. When cells are lysed at 60° while gently stirring, a small fraction of the DNA is denatured. We have demonstrated that this DNA is shear-sensitive because it is bound to the cellular membrane. It then became of interest to ask if the attachment point is the growing point of the chromosome. An experiment was carried out in which *E. coli* chromosomes were aligned at the origin. The origin was pulsed, then chased and the DNA examined with time for the location of the pulse. The experiment was to look for the distribution of the pulse between the denatured and native DNA bands in a CsCl gradient. The distribution was the same as for a random pulse, and there was no change in this distribution with time. Since the growing point of the chromosome was at the origin at the time of the pulse, the experiment shows that neither the origin nor the growing point are preferentially associated with the shear-sensitive (i.e., membrane-bound) DNA. We conclude that there are many attachment points of the DNA to the membrane, not just one or two; some of the attachment points may be at the loci of RNA synthesis.

3a. The Control Mechanism of DNA replication

The relation between protein synthesis and the initiation of DNA replication has been investigated using thymine starvation as a means of initiating a new cycle of DNA replication synchronously in a culture of *E. coli* 15T-any-try-met-. The amounts of protein synthesis during thymine starvation was correlated with the amount of DNA synthesis following the re-addition of thymine and the removal of required amino acids. It was found that a new cycle is initiated whenever the total protein of the cell doubles.

When the newly-initiated cycles are allowed to go to completion in the absence of amino acids, DNA synthesis comes to a halt until protein synthesis is allowed to resume. However, paradoxically, reinitiation of a second synchronous round of replication can occur, if chloramphenicol is added. The addition of methionine has the same effect, and we conclude that chloramphenicol acts by increasing the methionine pool (because of protein breakdown in the absence of protein synthesis). It thus appears that methionine is required for DNA initiation, at least under these unusual circumstances which allow initiation without precedent protein synthesis. (Note that neither chloramphenicol nor methionine is effective unless a "premature" round of replication has been

initiated, e.g., by thymine starvation.) The second round of replication is not initiated until the first has been completed, and methionine is not required until this time. However, methionine must be present when the first cycle is completed, or the ability to reinitiate will be lost. Exposure to methionine at an earlier time is not sufficient. We are currently investigating whether the role of methionine involves modification-restriction, using appropriate mutants.

The amount of DNA synthesis observed after initiation of the second round is sufficient to account for a new cycle on every chromosome (i.e., a doubling of the number of growing points), and perhaps even a third initiation in some cases.

4. SV 40-Specific RNA

The virus SV 40 productively infects DVI cells, an established line of monkey kidney cells in tissue culture. The "late" RNA transcribed from the viral DNA can be identified by hybridization with SV 40 DNA. Such RNA, when pulse-labeled, has a molecular weight which is several times bigger than its template. Studies have been carried out to determine whether these RNA molecules also contain cellular RNA, or contain multiple copies of the viral information. Cellular RNA, if attached to viral RNA, will be associated with viral DNA after hybridization but will be RNase-sensitive. Viral RNA is also partially RNase-sensitive (i.e., incompletely hybridized) under certain circumstances. Procedures for eliminating the RNase sensitivity of viral RNA have been developed, and preliminary results indicate that there is non-viral information in the molecule; this may arise if the viral DNA is integrated into the cell genome before transcription.

5. Induction of Lambda Prophage

Treatments which stop bacterial DNA synthesis have two consequences which are probably related: when DNA synthesis is allowed to proceed again, (1) there is premature initiation of new DNA replication cycles, and (2) any prophage present in the cell is induced to start replicating. Similarly, uv treatment of the sex factor, F, before transferring it into a Lambda lysogen, both prevents replication of the F DNA and induces Lambda. We have studied this phenomenon ("indirect induction") to see whether the mechanism is the same as for direct induction by stopping bacterial DNA synthesis. An F factor whose replication is temperature-sensitive was introduced into a lysogenic cell. At the temperature restrictive for F there was an increase in the rate of Lambda induction, suggesting that indirect induction is caused by stopping F replication rather than by a specific uv effect.

6. Genetic Control of Phage T4 DNA Synthesis

In studies utilizing bacteriophage T4, we investigated the role of DNA ligase in DNA repair and replication in vivo. DNA ligase is known to repair single-strand breaks (nicks) in native DNA in vitro. Apparently, the repair of nicks by ligase in vivo is essential for phage survival since defects in the gene for this enzyme (gene 30) are lethal.

We have found that the major function of the T4 gene 30 ligase is to repair DNA damage caused by the phage rII gene product (probably an endonuclease). When the rII gene is defective, the gene 30 ligase becomes dispensable. Phages carrying defects in both, the rII gene and gene 30 exhibit normal abilities to repair ultraviolet (uv)-irradiated DNA in vivo. This probably means that the gene 30 ligase specifically recognizes rII-mediated damage. The repair of nicks, which are presumably generated during the repair of UV-irradiated DNA in vivo probably involves an alternate ligating activity.

Studies on gene 30-rII double mutants showed that these phages replicate their DNA at a slower rate than normal. The slow rate of replication is caused by a slow repair of "gaps" which are generated in newly made DNA during replication. The repair of these gaps requires the phage DNA polymerase (gene 43). Thus, two types of DNA synthetic processes seem to be involved in normal DNA replication: (a) synthesis at the replication fork, and (b) synthesis behind the fork. These two processes may involve different enzymatic functions.