COO-3521-5

PROGRESS REPORT

TO

UNITED STATES ENERGY RESEARCH AND DEVELOPMENT ADMINISTRATION

CONTRACT E(11-1)-3521

BIOLOGICAL EFFECTS OF RADIATION AND RELATED BIOCHEMICAL AND PHYSICAL STUDIES

Proposal #6:	Physical and Chemical Studies on Nucleic Acids and Derivatives
Contract Period:	January 1, 1976 to December 31, 1976
Report Period:	September 1, 1974 to August 31, 1975 including a comprehensive review of the last three years
Investigators:	Liebe F. Cavalieri, Ph.D., Member Laboratory 6601

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

DISCLAIMER

Section 6601

Liebe F. Cavalieri, Ph.D., Head

TABLE OF CONTENTS

Section 6601

Liebe F. Cavalieri, Ph.D., Head

I. OBJECTIVE

Our long range goal, growing out of the research which we have pursued in bacterial systems over a number of years, is to define and characterize the DNA polymerases of mammalian cells, both normal and neoplastic, applying the procedures that have been successful with bacterial enzymes.

II. METHODS AND MATERIALS

a) In addition to conventional chromatographic methods for the isolation and purification of polymerases we use DNA-acrylamide gel chromatography, a type of affinity chromatography. We have developed such columns (19) and we find them efficient and convenient. In addition to their specificity they have a major advantage in that DNA is entrapped in the gel matrix and is not leached out.

b) Because of its extremely high resolving capability we still study polymerases by electrofocusing in gel beds. The method has already been used for DNA polymerases (20) with a conventional liquid system, and the results warrant its application in our work.

c) We plan to study Polymerase II (the 6-8S enzyme) isolated from WiL$_2$ cells which appears heterogeneous, to see if a monomer-dimer situation exists. We will examine the main peak and its shoulder (sometimes two peaks are discernable) as a function of: a) rotor speed, which may result in resolution of the incipient peak at higher speeds; b) ionic strength. If dimerization (or oligomerization) of the enzyme depends only on ionic strength, the association-dissociation may be due to a simple charge interaction between the species. This type of interaction can in general be distinguished from association due to hydrophobic forces by examining the amounts of each species present at different temperatures. Although there are technical difficulties (such as determining the area under the activity peak as a measure of its mass) the experiments are worth developing since the 6-8S enzyme(s) is ubiquitous in eukaryotic cells and obviously needs to be characterized.

d) In this discussion we have primarily referred to sedimentation velocity as the means of analysis. However, the same general theory for associating-dissociating systems holds for gel filtration and has been applied in a number of cases (21-23) in the concentration range 5-900 µg/ml of proteins (21,23). Using sephadex G-100 and determining that the gel matrix does not perturbe the associating-dissociating system, it is possible to calculate the degree of polymerization of the protein (up to a ten-mer) and the respective equilibrium constants. The latter requires that the initial solute zone is sufficiently large that a plateau region is present in the effluent zone. If we cannot accumulate enough polymerase to do this, we can in any event determine whether or not an associating-dissociating system exists by analyzing the trailing zone.

Section 6601

Liebe F. Cavalieri, Ph.D., Head

e) Kinetic and heat inactivation studies. The procedures for carrying out the kinetic studies have been detailed in references 9, 15 and 16. In brief, after the enzyme has been treated for a given time at a given temperature, aliquots are added to standard assay mixtures containing template, substrates and buffer, are incubated and the rates of synthesis are calculated from the time course of incorporation of acid-insoluble material. They are initial rates taken from the initial tangent of the incorporation versus time curve. This procedure eliminates variables arising from long reaction times.

Strictly speaking, these are not classical heat inactivation studies. To be so, the rate of synthesis should be examined at the elevated temperature. We cannot do this since the template would also be changing at the higher temperature, introducing another (unknown) variable. In our work, we make the assumptions that no renaturation of the enzyme occurs in cooling to 0^0 after the heat treatment and later when incubating the reaction mixture at 37^0.

To determine the influence of the primer on the kinetics of synthesis, a series of oligonucleotides, dT_{10}-dT_{100}, will be prepared by partial hydrolysis of dT_{1000}. After fractionation, these will be annealed to various templates such as poly rA, poly dA and hemoglobin mRNA and the resulting primer-templates used in kinetic studies in the same way as in the oligo dT_{10} series.

III. RESULTS AND CONCLUSIONS for years 1973 - 1975

RESULTS 1975

Membrane Associated DNA Polymerase Activity.

The enclosed reprint describes some of our recent work in this area. A brief summary of this paper follows. Membranes have been implicated in DNA synthesis for a long time, but it is clear from the background material presented in the Proposal that their role is still uncertain. While it is certain that DNA polymerase activities can be found associated with various types of membranous structures, the nature of the interaction is not known. If the membrane is to play a role in replication it is reasonable to assume an intimate type of interaction among the various components of the system - nucleic acids, polymerase(s) and membranes. Such interactions would include both polar (hydrophilic) and non-polar (hydrophobic) forces, which are present in all of the components.

Changes in membrane structure can be determined by fluorescence spectroscopy to an exquisitely sensitive degree. For example, if the fluorophore changes from a hydrophobic (lipid) environment to a hydrophilic (aqueous) one there will be a decrease in the fluorescence intensity. We have used this basic idea in observing the endogenous DNA polymerase activity associated with our membrane preparations. We reasoned that if the nucleic acid were intimately associated with the membrane then its use as a template for synthesis would dislodge it from its original binding position and this could induce a conformational change in the membrane giving rise to a fluorescence change. This expectation has been borne out.

Section 6601

Liebe F. Cavalieri, Ph.D., Head

Smooth membranes were prepared from WiL_2, a human lymphoid continuous cell line (1). The membranes have been characterized both morphologically and biochemically. Examination by electron microscopy reveals only smooth membranes with some small vesicles. There is no evidence of mitochondria or ribosomes. The levels of various enzyme markers have been determined and they indicate that there is no detectable contamination by endoplasmic reticulum or inner or outer mitochondrial membranes. Therefore, most of the smooth membrane in the $\rho = 1.16$ fraction is probably plasma membrane, with perhaps some nuclear membrane from nuclei accidentially ruptured during cell lysis.

This membrane fraction synthesizes DNA, in the presence of the four deoxynucleoside triphosphates. When the four deoxynucleoside triphosphates are added to the membrane fraction in assay buffer at 37^0, fluorescence changes also occur. Since, at the wavelength used, most of the fluorescence is due to tryptophan, we conclude that conformational changes must be occurring in membrane proteins. When DNA synthesis is prevented by omitting dCTP or dGTP from the reaction mixture, fluorescence changes are nearly absent and therefore we conclude that DNA synthesis produces the conformational changes in the membrane proteins. Our data show that the ratio of DNA synthesized to protein present in the membrane fraction studied is $1/10^{11}$. That is, remarkably little DNA synthesis is required to produce measurable changes in membrane conformation.

In order to learn whether smooth membranes from other sources possess a similar endogenous DNA polymerizing system, we examined membranes from SKL-N (a lymphoid line originating from a normal donor (2)) and SKL-7 (a lymphoid line originating from peripheral blood of an acute myleomonocytic leukemia (2)). We observed both RNase-sensitive and -insensitive activity in both cases. But CV-1 (an established line of monkey kidney cells), CLM-7 (a mouse fibroblast line) and Fl-1 (a human amnion line) showed no endogenous activity. These results suggest that endogenous DNA-synthesizing activity in smooth membranes may be characteristic only of lymphocytes. Since some cell types lack this activity, it seems unlikely that the binding of nucleic acid and polymerases to membrane is artifactual in the case of lymphocytes.

In addition to studying the smooth membrane fraction discussed above, we have begun a study of nuclear membranes (preprint No. 1, enclosed). Nuclei were prepared from WiL_2 cells using the double detergent (NP-40, DOC) method (3), which strips off the outer nuclear membrane. After removal of the nuclei by centrifugation the supernatant material showed no endogenous DNA polymerase activity; however, brief sonication of the nuclei just after the double detergent treatment yielded an endogenous RNase-sensitive activity in the supernatant; about 80-90 percent of this endogenous activity could be eliminated by RNase treatment. The activity liberated by sonication has been shown to be membrane-associated and is presumably on the inner nuclear membrane. It is of particular interest that the nuclear pellet itself possesses a marked endogenous DNA polymerase activity which is <u>not</u> sensitive to RNase. Thus two types of activity, and perhaps two different polymerases, have been separated by this simple procedure. We have begun to examine the DNA product of this reaction. The native product from a 30 minute incubation had a density of 1.42 in Cs_2SO_4 whether the label was H^3-dTTP or H^3-dCTP.

Section 6601

Liebe F. Cavalieri, Ph.D., Head

Heat denaturation yielded two peaks: one at $\rho = 1.44$ and a smaller one at $\rho = 1.55$. We are examining the latter in more detail. Alkaline treatment yielded only one peak of denatured DNA at $\rho = 1.44$ in Cs_2SO_4. We have done velocity sedimentation on these products and the results are given in the preprint.

We have also examined the extent of endogenous DNA synthesis in the nuclear membrane fraction as a function of $(NH_4)_2SO_4$ and ethanol concentration. The former decreases the reaction markedly while the latter (at 30% (v/v)) increased the rate of reaction 3-fold. This may have implications regarding the membrane association of the polymerase. We have carried out preliminary fluorescence measurements with these nuclear membrane fractions and find that changes do occur on addition of the four deoxynucleoside triphosphates; very small changes occur when only three are present.

It is known that isolated mammalian DNA polymerases (4,5,6) produce some type of polymer synthesis when only 3 deoxynucleoside triphosphates are present. It has been shown for a nuclear DNA polymerase obtained from KB cells (4) that the synthesis is not due to terminal transferase. We have studied this situation using the lymphocyte endogenous nuclear membrane system. The results are summarized below. Other results are given in the preprint.

System	pmoles dCMP incorp/mg membrane protein
Complete (H^3-dCTP, dGTP, dATP, TTP)	20
Complete + RNase pretreatment	2.6
Complete -dATP	9.0
Complete -dATP + RNase pretreatment	1.4
Complete -dATP, -dGTP	2.7
Complete -dATP, -dGTP + RNase pretreatment	1.4
Complete -dATP, -dGTP, -TTP	2.7
Complete -dATP, -dGTP, -TTP + RNase pretreatment	1.5
Complete (H^3-TTP, dCTP, dGTP, dATP)	15.5
Complete + RNase pretreatment	2.6

We feel that these results are important and we plan to explore this situation; qualitatively similar results have been obtained with the plasma membrane system. Future work will include a study of the products obtained with various combinations of the precursors; a search for deoxynucleoside diphosphate kinase activity; a detailed search for terminal transferase.

Section 6601

Liebe F. Cavalieri, Ph.D., Head

In normal fresh human peripheral lymphocytes the cytoplasmic particulate fraction appears to contain an endogenous DNA polymerase activity which is RNA-primed but DNA directed (7). We are examining a similar fraction from WiL_2 cells and comparing the results to those obtained with isolated enzymes. Thus, in an on-going study using isolated partially purified (RNA-dependent) polymerases III and IV (8) (kindly supplied by Dr. A. Weissbach) we have studied the use of various oligonucleotide-polynucleotide pairs as primer-templates with these enzymes. These R-DNA polymerases are known to be capable of copying ribostrands of ribopolynucleotide-oligonucleotide pairs. In a collaborative effort we have extended his work using other primer-templates, and we find that in the presence of Mn^{++} both polymerase III and IV can use an RNA template with an RNA primer ($rArU_{10}$); this is not the case when Mg^{++} is the metal ion. Also, neither enzyme can use a DNA template with an RNA primer ($dArU_{10}$) in the presence of Mn^{++}; there is a small amount of synthesis when using Mg^{++}. These results show that reverse transcription can take place using an RNA primer, and that metal ions may be important as a control. A study of the effects of different metal ions in the endogenous system may help to clarify the type of reaction present.

RESULTS 1974

The following are summaries and conclusions of recently published papers which are pertinent to this proposal.

TITLE: DNA Complementary to Rabbit Globin mRNA made by E. coli Polymerase I (9).

Summary:

E. coli DNA polymerase I has been used to synthesize DNA complementary to rabbit globin mRNA. In addition to the heteropolymeric DNA, poly (dT) and poly (dA)·(dT) are also synthesized. The extent of synthesis of these three products decreases at different rates upon heat inactivation of the polymerase, suggesting that the sites of synthesis on the enzyme are either entirely or partially separate. The use of polymerase I for copying RNAs makes possible the ready availability of complementary DNAs.

Conclusion:

We have shown that E. coli DNA polymerase I uses rabbit hemoglobin mRNA as a template to produce poly (dT), poly (dA)·(dT) and anti-mRNA DNA. The extent of homopolymer synthesis is greater than that of the anti-messenger. Heat inactivation of the polymerase shows the greatest decrease for poly (dA)·(dT) synthesis, followed by poly (dT) and anti-messenger. This suggests distinct areas of the enzyme for each type of synthesis; the areas may be separate or may overlap. Although the yield of product is lower using the heated enzyme, the proportion of anti-messenger is greater. We plan to examine in the manner described here the products formed from the same mRNA using the reverse transcriptases of the oncogenic RNA viruses.

Section 6601

Liebe F. Cavalieri, Ph.D., Head

The size of the anti-messenger formed (S=3.5) is significantly smaller than that of the template (S=10). Other investigators using viral reverse transcriptases (10,11,12) have obtained larger DNA products. The small size may be due to destruction of the product by the nucleolytic action of polymerase I or to incomplete transcription of the RNA or both. It has been shown (13) that globin mRNA has poly A-rich stretches 50-70 bases long containing about 70% A and that the 3'-terminus contains 5 or 6 A residues (14). The exact disposition of the A-rich stretches is unknown. The oligo dT primer probably couples to the short poly A stretch as well as to the A-rich regions some of which must be internal. Synthesis originating at the 3'-end would give complete complementary strands which might be nicked once or twice, reducing the size of the final product. Synthesis originating internally would lead directly to shorter complements. Since the heteropolymeric DNA product has been shown to hybridize completely and specifically to the template mRNA, it therefore represents complementary strand synthesis.

TITLE: Evidence for Template-Specific Sites in DNA Polymerases (15).

Summary:

Using rabbit hemoglobin messenger RNA as template, E. coli polymerase I produces poly (dT), poly (dA)·(dT) and antimessenger DNA products. Mild heating of the enzyme causes a differential loss in activity as indicated by three rates of inactivation for the three types of synthesis. Heat inactivation studies have also been carried out with DNA polymerases from oncogenic RNA viruses and mammalian sources using various homopolymeroligomer pairs as primer-templates. In general, for any given enzyme these synthetic primer-templates reveal different extents of inactivation of the polymerase. These findings may be interpreted to suggest a) that the binding of DNA polymerase to various primer-templates produces conformational changes in the enzyme which are dependent on the type of template bound, or b) that many, if not all, DNA polymerases have different subsites for different templates.

Conclusion:

We have found that inactivation of DNA polymerases produced by heating depends upon the type of primer-template used to measure that inactivation. The data indicate that the apparent binding constants of polymerase to both primer-template and deoxynucleoside triphosphates are probably decreased by partial heat inactivation and there may be a change in the catalytic site as well. A decrease in the apparent binding constant between the polymerase and the primer-template could be explained by either an actual decrease in binding or by a decreased ability of the enzyme to undergo a conformational change induced by the primer-template to produce the active form. The differential heat inactivation observed with different primer-templates when the enzyme concentration is rate-limiting indicates either that the active site on the enzyme differs with different templates - an unlikely possibility - or that the template binding site is actually a collection of subsites with different specificities, which can be differentially inactivated. The results can be interpreted in either of two ways. The binding of the enzyme

to the template may result in a conformational change of the enzyme which, in turn, results in the active form of the enzyme. We suggest that the ability to undergo this conformation change depends on the primer-template inducing it and the state of the enzyme. Partial heat inactivation could alter the enzyme such that it could no longer undergo a conformational change to the active form. An alternative interpretation of our results is that there exist subsites within the active center of the polymerase molecule and that they recognize specific bases and the type of sugar-phosphate backbone of the primer-template. Heat inactivation of the enzyme may be affecting these specific subsites in some fashion and cause the observed differences in the utilization of specific primer-templates. Clearly, however, not all of the results are explained by these interpretations, suggesting other subtleties within the active centers of DNA polymerases which await further clarification.

TITLE: Evidence for Allosterism in In Vitro DNA Synthesis on RNA Templates (16).

Summary:

Hemoglobin mRNA and $(rA)_n \cdot (dT)_{10}$ have been used as primer-templates in a kinetic study of DNA synthesis with Escherichia coli DNA polymerase I and Mason-Pfizer monkey virus reverse transcriptase (RNA-directed DNA polymerase). The rate versus enzyme concentration curve is sigmoidal and is consistent with a cooperative phenomenon. The results could be interpreted in terms of the formation of an active complex containing enzyme dimers (or oligomers) on the primer-template. We have also observed sigmoidal kinetics in rate versus deoxynucleotide triphosphate concentration. These results are consistent with an allosteric mechanism in which the triphosphates act as both modifiers and DNA precursors. In the critical range, a 6- to 8-fold increase in both enzyme and triphosphate concentrations can lead to a 1500-fold increase in the rate of synthesis on an RNA template. Thus, small changes in enzyme and precursor concentrations could play a regulatory role in vivo.

TITLE: A New Sensitive Method for Detecting Poly Adenylate in Viral and Other RNAs Using Escherichia Coli DNA Polymerase I (17).

Summary:

In the presence of messenger or viral RNA containing poly (A) stretches as a template and oligo $(dT)_{10}$ as a primer, DNA polymerase I from Escherichia coli synthesizes poly (dT) using dTTP as the sole precursor. No synthesis occurs when dATP is present as the sole precursor, although dATP is incorporated into acid-insoluble product in the presence of dTTP. Identical results were observed using the synthetic homopolymeric template poly (rA) with oligo $(dT)_{10}$ as added primer. Thus, poly (dT) synthesized on poly (A) stretches appears to serve as a template for the synthesis of poly (dA).

Section 6601

Liebe F. Cavalieri, Ph.D., Head

Poly (dT) which has been synthesized using poly (dA)·$(dT)_{10}$ as template-primer does not allow the incorporation of dATP in the presence of dTTP. We have utilized these observations as a basis for the development of an extremely sensitive and precise technique for the detection of poly (A) stretches in any RNA sample. This technique can also be used in the presence of DNA but the sensitivity is decreased. Poly (A) can be detected in as little as 2 nanograms of mRNA.

Conclusions:

We have shown that the poly (A) tracts present in mRNA and viral RNA genomes, when complexed to $(dT)_{10}$, can serve as effective template-primers for the synthesis of poly (dA)·(dT) by E. coli DNA polymerase I. The specific dTTP-dependent incorporation of (^{3}H)-dATP into acid-insoluble product does not occur with poly (dA) templates, thus providing a specific enzymatic detection technique for the presence of poly (A) stretches in any RNA sample. The addition of exogenous RNA lacking poly (A) tracts to the reaction at concentrations up to 50-fold that of mRNA has no effect on product synthesis. DNA at increasing concentrations does inhibit the product synthesis, although at DNA concentrations 50-fold that of input mRNA, significant product synthesis is still observed. The inhibition of product synthesis by added DNA appears to be due to the binding of DNA polymerase to the free 3'-hydroxyl groups on the DNA normally used by the enzyme to initiate DNA synthesis. As only two of the four DNA precursors are present in the poly (A) detection reaction mixture, heteropolymeric DNA synthesis does not occur and enzyme bound to the DNA apparently remains as inactive "dead-end complexes." The inhibition of (^{3}H)-dAMP incorporation which is dependent on dTTP by contaminating DNA can be overcome by the use of excess enzyme, although the detection of poly (A) tracts by this method in total cellular nucleic acid extracts would more easily be accomplished by first removing most of the contaminating cellular DNA.

Although several methods exist for the detection of poly (A) stretches in RNA samples, all require the use of radioisotopically labelled RNA when conditions prohibit the isolation of large quantities of RNA. A distinct advantage of the use of E. coli DNA polymerase I for the detection of poly (A) stretches in RNA samples is that labelled RNA is not required. The sensitivity of this technique is such that poly (A) stretches could be detected using as little as 2 nanograms of input mRNA. The synthesis of large quantities of labelled product relative to the small amount of poly (A) present in the RNA samples may be accomplished through an amplification mechanism in which newly synthesized product is removed from the poly (A) stretch through synthesis of the poly (dA)·(dT) duplex followed by annealing of fresh $(dT)_{10}$ to the poly (A) tract, allowing reinitiation of poly (dT) synthesis. The sensitivity of the assay can be further increased through the use of higher enzyme concentrations or by raising the specific activity of the (^{3}H)-dATP used in the reaction. Examination of radioisotopically-labelled RNA samples for the presence of poly (A) stretches may be performed in an identical manner, although the synthesis of alkali -insensitive, acid-precipitable material would be determined in this case.

Section 6601

Liebe F. Cavalieri, Ph.D., Head

TITLE: Purification of Avian Myeloblastosis Virus DNA Polymerase by Affinity Chromatography on Polycytidylate-Agarose (18).

Summary:

Polycytidylic acid (poly rC) covalently linked to cyanogen bromide-activated agarose is an effective affinity matrix for the RNA-dependent DNA polymerase from Avian Myeloblastosis virus (AMV). Poly rC-agarose is capable of binding large quantities of AMV DNA polymerase, which is then eluted using a linear KCl gradient of increasing concentration. The DNA polymerase isolated from crude, detergent-disrupted virions by a single pass through columns of poly rC-agarose appears nearly homogeneous (approximately 90% pure) as determined by sodium dodecyl sulfate-polyacrylamide disc gel electrophoresis. Complete recovery of input enzymatic activity was obtained. Results suggest that poly-ribonucleotide columns may provide a high-yield, rapid method for the purification of oncornaviral DNA polymerase. This is the first instance in which RNA-dependent DNA polymerase isolated using affinity chromatography has been shown to be nearly homogeneous by biophysical in addition to catalytic criteria.

RESULTS 1973

1. DNA Polymerase from E. Coli

We have obtained circumstantial evidence that both the DNA-dependent and the RNA-dependent activities reside in one molecule by observing that the activities co-purify using several different types of procedures. For this, we used as starting material the 60% ammonium sulfate cut described in a previous publication. First: when the 60% cut was subjected to acrylamide gel electrophoresis, the DNA-dependent (DD) and RNA-dependent (RD) activities appeared together at an R_m value of 0.48. When this fraction was applied to a glycerol gradient, the activities appeared together at an $S_{20,w}$ value of 4.5. The gel electrophoresis was repeated at a lower pH value (7.2) with identical results. Similarly, when the gel electrophoresis was carried out at a higher gel concentration (7%) or lower (4%) there was no separation of the DD and RD activities although, of course, the R_m values were lower (0.33) and higher (0.52), respectively. Second: when the 60% fraction was applied to a phosphocellulose column, the DD and RD activities eluted together at an ionic strength of 0.12 using a linear KCl gradient. Third: when this peak fraction was applied on a glycerol gradient (SW 50.1, 45,000 rpm, 15 hrs 4°C) both activities again appeared in the same fractions corresponding to an $S_{20,w}$ value of 4.2.

In order to show that one molecule is capable of carrying out both DD and RD synthesis, the enzyme has to be used at a limiting concentration. We therefore determined the amount of $dA \cdot dT_{10}$ which would saturate a fixed amount of enzyme and then added increasing amounts of $rA \cdot dT_{10}$. If the same molecule were carrying out both synthetic reactions, we would observe poly rA·dT synthesis at the expense of poly dA·dT synthesis. This is, in fact, what we observed when the products of the reaction were examined on a Cs_2SO_4 gradient. There is not only a shift in density toward the higher density value of poly rA·dT when the input amount of $rA \cdot dT_{10}$ is increased but there is also a concomittant decrease in the amount of poly dA dT synthesized. We conclude that one molecule carries out both functions. There is another

finding which leads to the same conclusion. We have found that the Pol A mutant H560, which lacks polymerase I, shows no activity with $rA \cdot dT_{10}$. Since the Pol A mutant arises from a point mutation, it seems certain that the activity responsible for DD and RD synthesis resides in the same molecule.

In order to determine whether there are one or two active sites on the molecule, we carried out three types of experiments: a) Heat Inactivation: this was carried out in two ways. First the enzyme was heated for varying times at 45° C. The $rA \cdot dT_{10}$ reaction is very sensitive while the $dA \cdot dT_{10}$ is virtually unaffected. The effect of varying the temperature gives the same result; each aliquot was maintained at the stated temperature, for 3 minutes. Again, the $rA \cdot dT_{10}$ reaction is exquisitely sensitive. b) Effect of pH. The pH optimum was determined for each reaction. There is a gross difference in behavior. c) Effect of KCl concentration. We have shown that at 0.1 M KCl the $dA \cdot dT_{10}$ reaction is totally unaffected but that the $rA \cdot dT_{10}$ is completely inhibited. We have shown that this effect is reversible: on diluting the KCl, full activity is restored.

We conclude from these independent measurements that the sites for DD and RD synthesis differ. We know nothing about their exact configuration; they may be totally separated physically or they may partially overlap.

2. Endogenous RNase-sensitive DNA-polymerizing Activity from Tissue Culture Cells

Cell lines used: SK-L7 (myelomonocytic leukemia); SK-LN1 (hemophiliac) were used for the majority of all experiments discussed.

Cells were grown in McCoy's Medium. They were harvested and pelleted and washed. The cells were suspended in buffer and homogenized. The supernatant from a low-speed spin was then spun at 45,000 rpm to obtain a pellet.

All cell lines examined were found to possess RNase-sensitive endogenous DNA-polymerizing activity. All pellet fractions were 70-90% RNase-sensitive with respect to endogenous DNA-polymerizing ability.

The great majority of RNase-sensitive endogenous DNA-polymerizing activity from the gel runs is situated atop the gel and apparently does not enter the gel, but as much as 20% of such activity (relative to the activity on gel top fractions) was found to migrate into the 5% acrylamide gels. Assays of gel fractions after slicing and elution of material from gels generally show a single peak of "endogenous reverse" activity migrating into the gels with a mobility of 0.1 relative to the distance moved by the bromophenol blue tracking dye.

The gel top fractions containing a high amount of RNase-sensitive endogenous DNA-polymerizing activity. After collection, the flocculent precipitate was spun down at 1000 x g for 5 minutes. All of the activity was found in the slightly turbid supernatant. This activity was stable for at least one week at 4° C while pellet fractions appear to lose their endogenous reverse activity after 72 hours at 4° C. The greatly increased stability may be due to the loss of nucleases or proteases into the gel during electrophoresis. The gel top fraction supernatant was placed atop fresh 5% gels and rerun. Again, a band of endogenous reverse activity was observed

Section 6601

Liebe F. Cavalieri, Ph.D., Head

at R_M = 0.1 but unlike the fresh pellet fraction, most of the endogenous reverse activity of the gel top fractions could be run into the gel and recovered. This suggests that the process of electrophoresis may be instrumental in cleaving an RNA-transcriptase complex into reproducibly-sized moieties capable of entering the gels. RNase H activity has been observed in the gel top fractions but has not yet been observed in peak gel fractions.

The RNase-sensitive endogenous DNA polymerizing activity migrating into the 5% gels were recovered and run on 10-30% glycerol gradients to ascertain the number of species (according to size) which entered the gels. The results of two runs show the endogenous reverse activity to exist as two and possibly three species of 5 and 10S.

Section 6601

Liebe F. Cavalieri, Ph.D., Head

REFERENCES

1. Levy, J.A., Virolainen, M. and Defendi, V.: Cancer 22: 517 (1968).

2. Southam, C.M., Burchenal, J.H., Clarkson, B., Tanzi, A., Mackey, R. and McComb, V.: Cancer 24: 211 (1969).

3. Vaughan, M.H., Warner, J.R. and Darnell, J.E.: J. Mol. Biol. 25: 235 (1967).

4. Sedwick, W.D., Want, T.S.F. and Korn, D.: J. Biol. Chem. 249: 5809 (1974).

5. Greene, R. and Korn, D.: J. Biol. Chem. 245: 254 (1970).

6. Goulian, M.: Ann. Rev. Biochem. 40: 855 (1971).

7. Reitz, M.S., Smith. R.G., Roseberry, E.A. and Gallo, R.C.: Biochem. Biophys. Res. Commun. 57: 934 (1974).

8. Spadari, S. and Weissbach, A.: J. Biol. Chem. 249: 5809 (1974).

9. Modak, M.J., Marcus, S.L. and Cavalieri, L.F.: Biochem. Biophys. Res. Commun. 55: 1 (1973).

10. Verma, I.M., Temple, G.F., Fan, H. and Baltimore, D.: Nature New Biol. 235: 163 (1972).

11. Kacian, D.L., Spiegelman, S., Bank, A., Terada, M., Metafora, S., Dow, L. and Marks, P.A.: Nature New Biol. 237: 167 (1972).

12. Ross, J., Aviv, H., Scolnick, E. and Leder, P.: Proc. Natl. Acad. Sci. USA 69: 268 (1972).

13. Lim, L. and Camellakis, E.S.: Nature 227: 710 (1971).

14. Burr, H. and Lingrel, J.B.: Nature New Biol. 233: 41 (1971).

15. Marcus, S.L., Modak, M.J. and Cavalieri, L.F.: Biochem. Biophys. Res. Commun. 56: 516 (1974).

16. Cavalieri, L.F., Modak, M.J. and Marcus, S.L.: Proc. Natl. Acad. Sci. USA 71: 858 (1974).

17. Modak, M.J., Marcus, S.L. and Cavalieri, L.F.: J. Biol. Chem. 249: 7373 (1974).

18. Marcus, S.L., Modak, M.J. and Cavalieri, L.F.: J. Virol. 14: 853 (1974).

19. Cavalieri, L.F. and Carroll, E.: Proc. Natl. Acad. Sci. USA 67: 807 (1970).

20. Cohen, L.H., Penner, P.E. and Loeb, L.A.: Annal. N.Y. Acad. Sci. 209: 354 (1973).

Section 6601

Liebe F. Cavalieri, Ph.D., Head

21. Ackers, G.K. and Thompson, T.E.: Proc. Natl. Acad. Sci. USA 53: 342 (1965).

22. Winzor, D.J. and Scheraga, H.A.: Biochem. 2: 1263 (1973).

23. Winzor, D.J. and Scheraga, H.A.: J. Phys. Chem. 68: 338 (1964).

Liebe F. Cavalieri

DIVISION OF BIOMEDICAL AND ENVIRONMENTAL RESEARCH COMPREHENSIVE REPORT

As requested in letter of March 11, 1975 by George D. Duda

#1. The main research accomplishments, with special reference to the originally stated objectives.

Our originally stated objectives have been concerned with the control and mechanism of DNA replication; the work has been carried out and is reported in summary herein. Much of our work has been concerned with *in vitro* DNA synthesis using purified DNA polymerases. In the early work we used primarily the DNA polymerase (Pol I) from E. coli. Using this system we made two new and potentially biologically important observations. First, we showed that RNA templates (which we had shown for the first time in 1963 could be utilized by DNA polymerase) exhibit an unusual pattern of DNA synthesis when it is observed as a function of polymerase concentration. The results indicate that a cooperative mechanism is operative when DNA polymerase uses RNA templates; i.e. when the first polymerase attaches itself to the RNA there is a low rate of synthesis but when more polymerase molecules attach the rate increases exponentially and finally levels off. These results are manifested as a sigmoid curve. Such a mechanism, if operating *in vivo* could serve as a control for reverse transcription.

Secondly, in this same system we observed a classical allosteric effect brought about by the precursors themselves, i.e. the deoxynucleoside-5'-triphosphates. When DNA synthesis was observed as a function of precursor concentration, following a lag period, DNA synthesis was enhanced markedly. This effect too represents a control mechanism for *in vitro* reverse transcription and would be of significance, if operating *in vivo*.

In the last several years our efforts have been directed more toward the mammalian DNA polymerase with, however, the same goals in mind as stated in the first paragraph above. The state of the art of polymerase isolation and purification is in a much more primitive state in this area and much of our early effort has been directed to improving methods of isolation and purification. We had developed earlier a new type of affinity chromatography using DNA entrapped in acrylamide gel beads to separate DNA binding proteins from those that do not. This was followed with affinity chromatography using various types of agarose matrices. We have studied DNA synthesis on DNA templates using mammalian enzymes purified in this manner and so far have not observed the unusual patterns described above for the bacterial polymerase.

In attempts to try to determine the functions of the mammalian DNA polymerases we have spent some time on organelle (mainly membranous structures) isolation with the hope of locating a given polymerase in known regions of the cell. So far we have found DNA polymerase activity associated with the (inner) nuclear membrane. The DNA polymerase(s) appears to be one not heretofore described and has the interesting property that it utilizes poly $rAdT_{10}$ as a primer template and not poly $dAdT_{10}$. However, it is premature to assign an *in vivo* function to this enzyme(s).

As indicated, the polymerase is associated with a membrane; but the membrane also contains DNA and/or RNA, for on adding the four deoxynucleoside precursors, DNA synthesis takes place. Therefore this is an endogenous DNA-

synthesizing "complex." We plan to use it as a model system for in vivo DNA synthesis; i.e. we will add back components of cellular extracts to see if we can find Okazaki pieces and semi-conservative DNA replication, for example. We have already isolated the products of this endogenously synthesizing system and analyzed them in sucrose velocity gradients as well as Cs_2SO_4 equilibrium density gradients. The sizes of the products vary somewhat but they have sedimentation constants in the 9-11S range. Their densities correspond to those of double-stranded DNAs.

Using this same endogenous system we have found that polymer synthesis occurs in the presence of only three, or two or one deoxynucleoside-5'-triphosphate(s). The reaction appears not to be due to terminal transferase. These analyses are continuing.

#2. Plans for the continuation of present objectives and possible new objectives in consideration of past results.

The results outlined above have proved so encouraging that we plan to continue working on the original aims. That is, we plan to extensively purify the various DNA polymerases both from normal and neoplastic cells and to attempt to localize them in the cell so as to gain clues as to their function(s). These experiments will be carried out with cells under various physiological states (i.e., S-phase, G-phase, etc.).

We have also discovered that the endogenous DNA synthesis which occurs on membranes (the "complex" noted above) can be monitored by observing the fluorescence changes of the tryptophan of the membrane proteins. This method represents a new and potentially valuable approach to the role of membranes in DNA synthesis. This represents a new goal not heretofore mentioned in previous proposals.

Another new approach to be applied to the study of structure and function of DNA polymerases involves the use of non-aqueous media for the preparation of cell organelles and then the isolation of DNA polymerases from them; this will permit a comparison with the aqueous systems and give information concerning possible translocation of polymerases during isolation.

#3. Graduate students trained, degrees granted, and post-doctoral tenures completed.

Drs. S. Marcus and M. Modak spent two years training in my laboratory learning methodology and techniques used in the nucleic acids of molecular biology. At present Dr. I. Yamaura is a post-doctoral fellow learning the same techniques.

#4. Bibliography, with titles, of publications associated with this contract.

Cavalieri, L.F. and Carroll, E.: RNA-dependent DNA Polymerase from E. coli: an Effect produced by Traces of Added DNA. Nature 232: 254 (1971).

Modak, M.J., Marcus, S.L. and Cavalieri, L.F.: DNA Complementary to Rabbit Globin mRNA Made by E. coli Polymerase I. Biochem. Biophys. Res. Commun. 55: 1 (1973).

Modak, M.J., Marcus, S.L. and Cavalieri, L.F.: Synthesis of DNA Complementary to AMV RNA Using E. coli Polymerase I. Biochem. Biophys. Res. Commun. 56: 247 (1974).

Marcus, S.L., Modak, M.J. and Cavalieri, L.F.: Evidence for Template-specific Sites in DNA Polymerases. Biochem. Biophys. Res. Commun. 56: 516 (1974).

Marcus, S.L., Modak, M.J. and Cavalieri, L.F.: Purification of Avian Myeloblastosis Virus DNA Polymerase by Affinity Chromatography on Polycytidylate-Agarose. J. Virol. 14: 853 (1974).

Cavalieri, L.F.: Extent of Double Strandedness in Avian Myeloblastosis Virus RNA. J. Virol. 14: 1458 (1974).

Cavalieri, L.F., Modak, M.J. and Marcus, S.L.: Evidence for Allosterism in In Vitro DNA Synthesis on RNA Templates. Proc. Nat. Acad. Sci. USA 71: 858 (1974).

Modak, M.J., Marcus, S.L. and Cavalieri, L.F.: A New Sensitive Method for Detecting Polyadenylate in Viral and Other Ribonucleic Acids Using Escherichia coli Deoxyribonucleic Acid Polymerase I. J. Biol. Chem. 249: 7373 (1974).

Cavalieri, L.F., Sonenberg, M. and Cronin-Sheridan, A.P.: In Vitro DNA Synthesis on Smooth Membranes Observed by Fluorescence. Acta Hematologica 54: 248 (1975).

Cavalieri, L.F. and Carroll, E.: New DNA Polymerase Activity Associated with Nuclear Membranes from a Human Lymphoid Cell Line. Biochem. Biophys. Res. Commun. Submitted for publication.

#5. Your opinion as to the present state of knowledge in this area of research, its significance in the fields of biology and medicine, and needed future investigation.

Our research is concerned primarily with the mechanism and control of DNA replication. While palpable progress in this area is clear in bacterial systems, there is much less progress in mammalian systems. Although there is much effort being expended in the general area of DNA polymerases, the field is indeed in a primitive state, both with respect to the structures and functions of the DNA polymerases. It is a truism to state that a knowledge of the functions (one of which is DNA replication) of normal cells is a prerequisite to the understanding of growth in cancer cells. Therefore intensive study in the molecular biology of nucleic acids is a must both for biology and medicine. In my opinion future investigations of the type just alluded to should be expanded in all directions.

#6. The present division of federal support for your overall research program.

Current:

1. NCI 08748-09, Core Grant to Sloan-Kettering Institute for Cancer Research, for salary and benefits of the principal investigator and certain core services.

2. Source: AEC
   Title: Physical-chemical studies on nucleic acids and derivatives
   Principal Investigator: Liebe F. Cavalieri
   Period: January 1, 1975 to December 31, 1975
   Amount: Direct Cost - $29,276

3. Source: NCI #CA 17085-01
   Title: Molecular Biology - Cancer Program Project Grant
   Principal Investigator: Liebe F. Cavalieri
   Period: January 1, 1975 to December 31, 1979
   Amount: Direct Cost - $30,810

4. Source: NCI #1 RO1CA18383-01
   Title: The Mechanism of the reverse transcription of oncornaviral RNA
   Principal Investigator: Liebe F. Cavalieri
   Period: June 30, 1975 to June 30, 1978
   Amount: Direct Cost - $34,270

Future:

1. NCI 08748-09, Core Grant to Sloan-Kettering Institute. These funds will cover the salary and benefits of the principal investigator, plus certain core services. Support is now being sought only for research expenses that will no longer be covered by Sloan-Kettering Institute funds.

2. AEC - see above.