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PROGRESS REPORT

TO

THE UNITED STATES ATOMIC ENERGY COMMISSION

CONTRACT AT(11-1)-3521

BIOLOGICAL EFFECTS OF RADIATION AND RELATED BIOCHEMICAL AND PHYSICAL STUDIES

Proposal #5:	Physical and Chemical Studies on Nucleic Acids and Derivatives
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DISCLAIMER

SUMMARY

In vitro DNA synthesis has been studied using a number of RNA templates, both natural and synthetic and a variety of DNA polymerases. We have shown that E. coli polymerase I can produce faithful DNA copies of rabbit hemoglobin m-RNA and of avianmyeloblastosis virus RNA. We have studied the kinetics of DNA synthesis as a function of polymerase concentration and have discovered that the reaction takes place in a cooperative manner with E. coli Pol I; sigmoid kinetics are observed. We have also discovered the existance of allosteric effects in which the precursor, thymidine-5'-triphosphate, can act as a modifier in reverse transcription. A study of the heat inactivation of a number of DNA polymerases (bacterial, viral and mammalian) shows that the extent of inactivation observed depends on the primer-template used in the assay. The results imply a) the existence of different subsites on the enzyme for different templates and that these are differentially affected by heat and/or b) that the primer-templates produce conformational changes of the polymerase on interacting with it and that this effect is changed on heating the enzyme; i.e. a different, or no, conformational change is induced by the interaction.

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I. TITLE

"Physical and Chemical Studies of Nucleic Acid Derivatives."

II. 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.

III. METHODS AND MATERIALS

1. Kinetic and heat inactivation studies. The procedures for carrying out the kinetic studies have been detailed in references 1, 2 and 3. 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° after the heat treatment and later when incubating the reaction mixture at 37°.

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.

2. Sources of enzymes. Seven cell lines from which enzymes are isolated are currently in use. The first three have been obtained from Dr. Clarkson of Sloan-Kettering Institute (4): SKL7, originating from a patient with acute lymphocytic leukemia; SKLN, originating from a normal donor; RCS-1, originating from a patient with reticulum cell sarcoma; WiL_2, originating from a patient with hereditary spherocytic anemia (5); CV-1 is an established line of green monkey kidney cells. FL-1 is the Fogh-Lane line originating from human amnion tissue. CLM-7 is a Chinese hamster fibroblast line originating from normal bone marrow, obtained from Dr. Biedler of Sloan-Kettering Institute. In addition to these, we have been and will continue to use white cells from leukemia patients in Memorial Hospital. These are: ALL (acute lymphoblastic leukemia), AML (acute myelocytic leukemia), CLL (chronic lymphatic leukemia) and CML (chronic myelogenous leukemia). Normal blood lymphocytes will be obtained from normal donors.

3. Tissue Culture facility. Part of our laboratory space is a tissue culture room containing within it three separate sterile cubicles to minimize

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cross-contamination. Adjoining this area is glassware washing facility. The Walker Laboratory has a common sterilization laboratory which serves one floor of the building. The same laboratory provides media for ordinary tissue culture lines. We make our own media for special needs.

IV. RESULTS

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 (2)

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.

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 (6,7,8) 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 (9) 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 (10). The exact disposition of the A-rich stretches is unknown. The oligo dT primer probably couples to the short poly A stretch 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.

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Title: Evidence for Template-Specific Sites in DNA Polymerases (3)

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.

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Title: Evidence for Allosterism in In Vitro DNA Synthesis on RNA Templates (1)

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 (11)

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). Poly (dT) which has been synthesized using poly $(dA) \cdot (dT)_{10}$ 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 $(dA) \cdot (dT)$ by E. coli DNA polymerase I. The specific dTTP-dependent incorporation of (^3H)-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

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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 re-initiation 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.

Title: Purification of Avian Myeloblastosis Virus DNA Polymerase by Affinity Chromatography on Polycytidylate-Agarose (12)

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 polyribonucleotide 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.

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