1977

Dissociation and isolation of the subunits of avian myeloblastosis virus RNA-directed DNA polymerase

Mae Joanne Rosok
The University of Montana

DISSOCIATION AND ISOLATION OF THE SUBUNITS OF AVIAN MYELOBLASTOSIS VIRUS RNA-DIRECTED DNA POLYMERASE

By

Mae Joanne Rosok

B.A., University of Montana, 1973

Presented in partial fulfillment of the requirements

for the degree of

Master of Science

UNIVERSITY OF MONTANA

1977

Approved by:

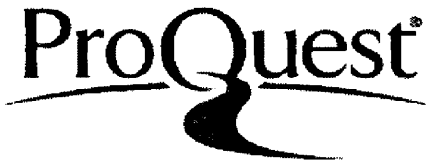
Kenneth F. Watson

Chairman, Board of Examiners

Dean, Graduate School

June 23, 1977

Date



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Rosok, Mae Joanne, M.S. June, 1977 Chemistry

Dissociation and Isolation of the Subunits of Avian Myeloblastosis Virus RNA-Directed DNA Polymerase

Director: Kenneth F. Watson

RNA tumor viruses constitute a group of animal viruses capable of inducing oncogenesis in the host. Contained within the virions is an RNA-directed DNA polymerase, reverse transcriptase, which utilizes the viral RNA as template to produce a DNA product. This is a required step for viral infection and replication. The DNA polymerase from avian myeloblastosis virus (AMV) has been purified and is composed of two polypeptide subunits, alpha and beta. To understand the mechanism of reverse transcription, it is necessary to examine the role of each subunit. Therefore, the purpose of this investigation was to develop a method for dissociation and fractionation of the two subunits. Dissociation was achieved by urea treatment and the two polypeptides were fractionated using phosphocellulose chromatography in the presence of urea. The best separation of the alpha and beta subunits was obtained by pretreating the AMV DNA polymerase in 8 M urea, and then chromatographing the enzyme on phosphocellulose in 8 M urea at 25°C.

ACKNOWLEDGEMENTS

I wish to express deepest gratitude to Dr. Kenneth Watson, my adviser, whose training, encouragement, and support during the years of my graduate education have been of great value to me. The suggestions given and interest shown by my colleagues John Olsen, Brian McGiffert, Len Ramsey, and Dr. Peter Schendel and by Dr. Walter Hill have been greatly appreciated. I also wish to acknowledge Ming Tam for his guidance during the two-dimensional gel electrophoresis experiment.

And I am grateful to Gary, whose ongoing concern has been a constant source of strength.

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ABBREVIATIONS

A list of abbreviations used throughout this paper are: RNA, ribonucleic acid; DNA, deoxyribonucleic acid; BUdR, 5-bromodeoxyuridine; NP-40, Nonidet P-40; AMV, avian myeloblastosis virus; SDS, sodium dodecyl sulfate; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; $tRNA^{trp}$, tryptophanyl transfer RNA; RNase H, ribonuclease H; poly rA·oligo dT_{12-18}, polyriboadenylate-oligodeoxythymidylate; NaCl, sodium chloride; BSA, bovine serum albumin; EDTA, ethylenediamine-tetraacetic acid; DEAE-cellulose, diethylaminoethyl-cellulose; KCl, potassium chloride; DTT, dithiothreitol; CM-Sephadex, carboxymethyl Sephadex; $(NH_4)_2SO_4$, ammonium sulfate; dATP, deoxyadenosine 5'-triphosphate; dTTP, deoxythymidine 5'triphosphate; Tris, tris-(hydroxymethyl) aminomethane; PPO, 2,5-diphenyloxazole; bis-MSB, p-bis (o-methylstyryl); bis acrylamide, N',N'-methylene bis acrylamide; H_3PO_4, phosphoric acid; KOH, potassium hydroxide; TEMED, N',N',N',N'-tetramethyl ethylenediamine; cpm, counts per minute; ma, milliamp.

Chapter I

INTRODUCTION

The relationship between tumorogenesis and viruses was first realized by Peyton Rous in the early part of this century. While studying avian sarcomas, cancers of the connective tissue, Rous found that inoculation into healthy chickens of cell-free and bacteria-free filtrates prepared from the tumors induced sarcoma formation at the site of inoculation (Rous, 1911). This demonstrated the viral etiology of this cancer.

In a similar manner, Ellermann and Bang (1908,1909) determined that chicken leukemias, which were later designated as cancers, were due to viral infections. Avian sarcoma and leukemia viruses are now classified as ribonucleic acid (RNA) tumor viruses, a group of oncogenic animal viruses which contain single-stranded RNA as the carrier of viral genetic information. Included in the leukosis group of RNA tumor viruses is avian myeloblastosis virus (AMV) which causes acute myeloblastic leukemia in chickens by inducing production of a large number of immature white cells.

A description of AMV has been obtained by various physical and chemical studies and by electron microscopy. The particles have a density of 1.15-1.19 gm per cm^3 in sucrose (Duesberg, 1970), are roughly spherical in shape with a diameter of approximately 100 nm (Sharp et al., 1952; Bernhard et al., 1958), have a sedimentation

value of 693S (Beard, 1963), and have a molecular weight of 3-4 x 10^8 (Bellamy et al., 1974; Salmeen et al., 1975). The spherical shape is given by the envelope which has projecting spikes on the outer surface (Eckert et al., 1963). The envelope encompasses the nucleoid core structure containing ribonucleoprotein.

The major component of AMV is protein, which comprises 61.5% of the weight of the virus. Other components are lipids (36.1%), which are present in the outer envelope; carbohydrates (3%), and RNA (2.4%) (Stromberg et al., 1973). A very small amount of deoxyribonucleic acid (DNA) is present (Levinson et al., 1970; Biswal et al., 1971), and is believed to be of cellular origin (Varmus et al., 1971).

The RNA of the virus is enclosed within the core and can be isolated by phenol extraction and fractionation in glycerol or sucrose density gradients (Robinson and Baluda, 1965). The two major species of RNA found are 60-70S, the viral genome, and 4-5S, which is primarily cellular transfer RNA molecules (Bauer, 1966; Bonar et al., 1967; Bishop et al., 1970). Two minor RNA species that are present sediment similarly to 18S and 28S RNAs of eucaryotic ribosomes (Bonar et al., 1967; Obara et al., 1971). Another minor RNA isolated from the virus, 7S RNA, is of cellular origin (Erikson and Erikson, 1976).

REPLICATION OF RNA TUMOR VIRUSES

Before 1970, the mechanism of replication of the RNA of RNA tumor viruses was very unclear. Other RNA viruses appear to replicate their RNA by an RNA to RNA mechanism utilizing a virus-coded RNA-directed RNA polymerase (Baltimore et al., 1970; Huang et al., 1970; Howatson, 1970; see review by Bukrinskaya, 1973). However, studies of the mechanism of genetic information transfer and replication in RNA

tumor viruses indicated that DNA was somehow involved in infection and replication. This would suggest a reversal of information flow, which was contradictory to the dogma which stated that genetic information was transferred from DNA to RNA to protein.

The first evidence suggesting DNA involvement in an infection by an RNA tumor virus was given by Temin (1963) who showed that production of Rous sarcoma virus, a virus causing tumors in chickens, is inhibited by the addition of actinomycin D to cells normally producing Rous sarcoma virus in tissue culture. Several others supported this finding (Bather, 1963; Bader, 1964; Vigier and Golde, 1964). DNA-directed RNA synthesis is inhibited by actinomycin D which interacts with the DNA to effectively block the elongation process of transcription.

From these observations, Temin (1964) hypothesized that DNA was a replicative intermediate in the genetic flow of viral information carried by the RNA genome. Temin suggested that the DNA copy, the provirus, could be integrated into the host genome, and viral genetic information could then be expressed through host cellular mechanisms.

Further support for DNA involvement in RNA tumor virus replication was given by Bader (1965a) who used antagonists of DNA synthesis, such as cytosine arabinoside, to demonstrate that DNA synthesis was required for virus production. Inhibition of DNA synthesis also prevents cell transformation[1] by the virus (Bader, 1965b; Nakata and Bader, 1968).

Studies with 5-bromodeoxyuridine (BUdR) (Balduzzi and Morgan, 1970; Boettiger and Temin, 1970) indicated that viral DNA synthesis, not cellular DNA synthesis, is required for virus replication and transformation. To prevent cell death due to BUdR treatment, tissue

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[1]Cell transformation is an *in vitro* phenomenon analogous to tumorogenesis in the host; although the cellular changes are not identical in both events, transformation is an informative model.

cultures were deprived of serum which prevented the cells from entering S phase, which in turn caused a cessation of cellular DNA synthesis. Cells infected by an avian sarcoma virus in the presence of BUdR and then light-treated, causing light inactivation of DNA, had aborted infections and showed no evidence of cell transformation. Because no cellular DNA synthesis was occurring, these studies demonstrated that infection and transformation are somehow associated with viral-related DNA synthesis.

Boettiger and Temin (1970) further supported the above conclusion by titrating the virus added to cells. Those infected with greater amounts of virus, introducing more viral genomes per cell, had a lower incidence of abortive infection. If cellular DNA were the affected nucleic acid, titration of the viral genome would not alter the extent of abortive infection and transformation.

The evidence of direct involvement of DNA synthesis in viral replication prompted investigators to search for an RNA-directed DNA polymerase. Temin (1970) examined the possibility that such an enzyme was produced in infected cells. He inoculated cells in tissue culture with an avian tumor virus and then added inhibitors of protein synthesis. A viral infection resulted in the cells and the infections were not aborted but proceeded as in control cultures. This indicated that protein synthesis is not required following infection. Thus, the necessary enzyme(s) was already present, either in the infected cell or carried in by the virus particle. That such an enzyme would be located in the virus was not totally new, as other animal RNA viruses had been shown to contain RNA transcriptase (Baltimore et al., 1970; Howatson, 1970), assuring that viral messenger RNA was synthesized immediately subsequent to infection, in turn guaranteeing the synthesis of necessary viral proteins.

The crucial discovery of an RNA-directed DNA polymerase, or reverse transcriptase, in RNA tumor viruses was made independently by Baltimore (1970) and Temin and Mizutani (1970). By treating the virus with Nonidet P-40 (NP-40), a nonionic detergent, Temin and Mizutani (1970) found that the virus coat was disrupted and polymerase activity was observed when the following were added: the four deoxyribonucleoside triphosphates, including tritiated thymidine triphosphate to monitor the reaction; a divalent cation, magnesium or manganese, and a sulfhydryl reducing agent. Since synthesis of DNA was sensitive to pretreatment with ribonuclease (Baltimore, 1970; Temin and Mizutani, 1970) it was concluded that the resident RNA was acting as template, and this reaction was termed the endogenous reaction. In other words, the DNA strand that is polymerized contains complementary deoxynucleotides to the viral RNA bases.

To substantiate that viral RNA from the tumor viruses was serving as a template for DNA synthesis, Spiegelman et al. (1970) and Taylor et al. (1972) examined the product of the endogenous reaction. Analysis by alkali stability, buoyant density, susceptibility to deoxyribonuclease, base composition, and nearest neighbor analysis confirmed the DNA nature of the product. Hybridization of the DNA product to the viral RNA genome conclusively established that DNA was synthesized utilizing the resident RNA as a template.

The existence of DNA as an intermediate in tumor virus replication has important implications for oncogenesis. The DNA may be incorporated into the host genome, causing a permanent infection in the cell. This occurs with certain DNA viral systems, such as adenovirus and simian virus-40 (Doerfler, 1975). The oncogenic potential of RNA tumor viruses is further supported by the presence of the *src* gene, or transforming gene, in the genome of avian sarcoma viruses

(Stehelin et al., 1976). The expression of this portion of the RNA results in neoplastic transformation of fibroblasts.

The studies cited above indicate an important relationship between carcinogenesis and reverse transcriptase suggesting that the enzyme has an essential role in virus-host interaction. Therefore, further characterization of the enzyme is necessary to understand its function in virus replication and tumor cell formation.

PURIFICATION AND PROPERTIES OF THE RNA-DIRECTED DNA POLYMERASE

Purification

The RNA-directed DNA polymerase from AMV has been extensively purified (Kacian et al., 1971; Grandgenett et al., 1973). The isolation procedure involves disrupting the virus particles in a detergent-high salt solution in which the polymerase is solubilized. It is then applied to a series of two ion-exchange chromatography columns; first, an anion exchanger, and secondly, a cation exchanger. This separates the DNA polymerase from the bulk of the viral proteins. The final purification step is glycerol gradient centrifugation of the pool of DNA polymerase activity from cation exchange chromatography.

Template-Primer Properties

A template-primer nucleic acid complex is required by the reverse transcriptase for synthesis of DNA. The template-primers can be a variety of nucleic acid molecules including synthetic polyribonucleotides and polydeoxyribonucleotides, natural 60-70S viral RNA, and natural DNAs. A synthetic deoxyribo-oligomer hybridized to a polyribohomopolymer template is often used to monitor reverse transcriptase activity.

The primer portion is a nucleic acid segment complementary to the template and has a free 3'-hydroxyl end which provides a starting point for polymerization. Primers are required by all known DNA polymerases.

Since the purified AMV DNA polymerase utilizes isolated 60-70S viral RNA as a template for polymerization, a primer must be associated with the viral RNA. Heating 60-70S RNA releases a low molecular weight RNA, and at the same time the ability of the viral RNA to act as a template for polymerization decreases (Canaani et al., 1972; Dahlberg et al., 1974). Analysis of the polymerase reaction product which showed that a covalent bond existed between a deoxyribonucleotide and ribonucleotide (Flugel and Wells, 1972; Verma et al., 1972) supported that a small RNA species was acting as a primer with the 60-70S RNA. Further analysis revealed that the small RNA, acting as a specific primer, is tryptophanyl transfer RNA ($tRNA^{trp}$) (Waters et al., 1975; Panet et al., 1975; Folk and Faras, 1976), and is probably of cellular origin (Faras and Dibble, 1975).

Protein Composition of AMV DNA Polymerase

The pure polymerase consists of two polypeptide subunits as demonstrated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The larger subunit termed beta (β) has an estimated molecular weight varying from 91,000 (Stromberg et al., 1974) to 105,000-110,000 (Kacian et al., 1971; Grandgenett et al., 1973). The smaller subunit, alpha (α), has a molecular weight of approximately 65,000-69,000 (Kacian et al., 1971; Grandgenett et al., 1973; Stromberg et al., 1974). A molecule of the AMV DNA polymerase is composed of one alpha subunit and one beta subunit as analyzed by glycerol gradient centrifugation (Kacian et al., 1971; Grandgenett et al., 1973).

Ribonuclease H

Associated with the two-subunit polymerase is a second enzymatic activity, ribonuclease H (RNase H) (Moelling et al., 1971). Ribonuclease H degrades the RNA moiety of an RNA-DNA hybrid. Magnesium is required for the RNase H reaction, which produces mono- and oligo-ribonucleotides with 5'-phosphate termini (Keller and Crouch, 1972; Leis et al., 1973).

Keller and Crouch (1972) demonstrated that the enzyme acts as an exoribonuclease by its inability to nick a circular RNA-DNA hybrid. A second property of the RNase H is that it is processive (Leis et al., 1973); once the enzyme has begun degrading one hybrid molecule, it will complete the degradation of that molecule before moving to another. The specific function of RNase H in reverse transcription is not yet clear.

Summary

The RNA-directed DNA polymerase has been isolated and purified from AMV. The enzyme requires a template and a primer for polymerase activity. The DNA polymerase can utilize isolated 60-70S viral RNA as a template if a primer is associated with the RNA. A cellular transfer RNA molecule, $tRNA^{trp}$, acts specifically in this role.

Coinciding with polymerase activity is RNase H activity which degrades the RNA portion of an RNA-DNA hybrid. The protein associated with the two enzymatic activities is composed of two subunits, alpha and beta.

PARTIAL CHARACTERIZATION OF DNA POLYMERASE SUBUNITS

In order to fully understand the mechanism of reverse transcription, the role of each subunit of the polymerase and its relationship with the other needs to be determined. Work in this area has led to several interesting observations.

By using phosphocellulose as the cation exchanger in the second step of purification of AMV DNA polymerase, Grandgenett and coworkers (1973) separated the smaller alpha subunit from αβ where alpha eluted at a lower salt concentration. No pure beta was obtained.

In order to achieve more complete dissociation of the subunits of the enzyme, Grandgenett (1976) treated the enzyme with organic solvents such as dimethyl sulfoxide and 1,4-dioxane prior to chromatography on phosphocellulose. Alpha and αβ eluted from the ion-exchange column as described above (Grandgenett et al., 1973). At higher salt concentrations more enzyme eluted in a series of peaks, termed the beta-enriched fraction, which contained varying amounts of alpha associated with the beta subunit.

Examination of the properties of the three fractions, α, αβ, and the β-enriched fraction, has introduced some possible roles for alpha and beta. Differences in nucleic binding properties of α and of αβ and the β-enriched fraction suggest that beta has a very important role in nucleic acid binding. The holoenzyme has a strong affinity for nucleic acids (Grandgenett and Green, 1974; Panet et al., 1974; Grandgenett and Rho, 1975; Panet et al., 1975; Grandgenett, 1976; Grandgenett et al., 1976). Both αβ and the β-enriched fraction bind to polyriboadenylate-oligodeoxythymidylate (poly rA·oligo dT_{12-18}), a commonly used synthetic template-primer, 3-4 times more avidly than does the α subunit (Grandgenett, 1976). A more pronounced difference

in binding affinities of the three enzyme fractions was observed with murine leukemia virus RNA (Grandgenett, 1976); alpha has 30-40 times less affinity for the murine viral RNA than does αβ or the β-enriched fraction. With regard to the ability of the three enzyme forms to bind to the primer, $tRNA^{trp}$, Panet et al. (1975) found that AMV DNA polymerase binds to $tRNA^{trp}$ as does the β-enriched fraction (Grandgenett et al., 1976). In comparison, alpha has little affinity for the primer molecule (Grandgenett et al., 1976).

The varying binding abilities of α, αβ, and the β-enriched fraction may be partially responsible for enzymatic differences. All three fractions have polymerase activity, however alpha is less active with most template-primers than the two other enzyme forms. αβ and the β-enriched fraction have comparable activities with 60-70S RNA whereas alpha is 5-10 times less active with viral RNA (Grandgenett et al., 1973). The polymerase activity of alpha is also less with natural DNAs and synthetic template-primers. In contrast, alpha is able to copy rabbit globin 9S messenger RNA primed with oligo dT at the 3'-poly A terminus as efficiently as αβ or the β-enriched fraction (Grandgenett, 1976).

The RNase H activity of alpha is also different in that it acts as a random exoribonuclease, moving from one hybrid molecule to the next before completely degrading the first (Grandgenett and Green, 1974). The β-enriched fraction and αβ both act as processive exoribonucleases (Grandgenett, 1976). Although alpha possesses both enzymatic activities, the efficiency and specificity of enzyme action appears to depend upon the presence of beta.

Presently, there is good evidence that beta is a precursor to

alpha. Moelling (1974) first suggested that alpha was formed by proteolytic cleavage of beta when she observed that with increasing storage time, the molar concentration of alpha increased and the $\beta:\alpha$ ratio became less than one as shown by SDS-PAGE. Stronger support for a precursor role for beta was given by tryptic and chymotryptic digestion of the subunits. Denatured alpha and beta, labelled with radioactive iodine, were isolated after SDS-PAGE and treated with trypsin (Gibson and Verma, 1974) or trypsin and chymotrypsin (Rho et al., 1975). The proteolytic digest maps showed that the peptide groups present in alpha were among those present in beta. With this information, the relationship of the two subunits becomes more complex since it is apparent that they are structurally interdependent as well as enzymatically interdependent.

PURPOSE OF STUDY

Complete dissociation and purification of the subunits has not yet been accomplished, and only assumptions can be made regarding the role of beta in reverse transcription. Therefore, characterization of the individual subunits is a critical preliminary step in understanding reverse transcription and cannot be determined without isolated subunits. The goal of this study was to isolate the individual subunits by a method which would not irreversibly destroy enzymatic activity.

The experimental technique employed for dissociation involved denaturation of the AMV DNA polymerase in urea. The subunit nature of other enzyme systems has been examined using urea as the dissociating agent (Frieden, 1971). Urea dissociates multi-subunit proteins by disrupting non-covalent interactions, and upon removal of the urea,

renaturation of the subunits into the original functional enzyme is possible (Tanford, 1968; Frieden, 1971). Work performed by Deal and coworkers (Johnson et al., 1969; Deal et al., 1969) on dissociation and reassociation of glycolytic enzymes demonstrated that urea would not permanently alter enzyme function.

The feasibility of using urea to dissociate AMV DNA polymerase was further supported by research with the DNA-dependent RNA polymerase of Escherichia coli. The RNA polymerase has been dissociated into its subunits by pretreatment at 25°C in a 6-8 M urea solution followed by electrophoresis or ion-exchange chromotography at 4°C (Burgess, 1969; Lill and Hartmann, 1970; Heil and Zillig, 1970; Ishihama and Ito, 1972; Yarbrough and Hurwitz, 1974; Palm et al., 1975). Renaturation and reassociation of the subunits was accomplished by diluting the urea (Yarbrough and Hurwitz, 1974) or removing the urea by dialysis (Lill and Hartmann, 1970; Heil and Zillig, 1970; Ishihama and Ito, 1972; Yarbrough and Hurwitz, 1974; Lill et al., 1975; Palm et al., 1975). The reconstitution buffer used for dilution or dialysis was 0.2-0.3 M salt, high sulfhydryl reducing agent concentration and 10-20% glycerol. The reconstituted enzyme regained its catalytic properties (Burgess, 1969; Lill and Hartmann, 1970; Ishihama and Ito, 1972; Yarbrough and Hurwitz, 1974; Palm et al., 1975) and behaved like the untreated RNA polymerase on sucrose gradients (Heil and Zillig, 1970) and during gel electrophoresis (Lill and Hartmann, 1970).

Dissociation of the AMV DNA polymerase into its subunits by treatment in urea, isolation of alpha and beta, and renaturation of both proteins after removal of urea appears promising in view of the above experiments. The subsequent characterization of alpha and beta should lead to a more comprehensive understanding of reverse transcription.

Chapter II

MATERIALS AND METHODS

MATERIALS

Unlabelled deoxyribonucleoside triphosphates were purchased from P-L Biochemicals and tritiated deoxythymidine was obtained from Schwarz-Mann and ICN Chemical and Radioisotope Division. Polyriboadenylate (poly rA) was purchased from Miles Laboratories and oligodeoxythymidylate (oligo dT_{12-18}) from Collaborative Research, Inc. NP-40 was obtained from Particle Data Laboratories, LTD. Coomassie blue R, DTT, bovine serum albumin (BSA), riboflavin, and Trizma base were purchased from Sigma Chemical Company. Ammonium sulfate (special enzyme grade), urea (ultra-pure), and sodium deoxycholate were obtained from Schwarz-Mann. DEAE cellulose (DE-52), phosphocellulose (P-11), and DE-81 filter circles were obtained from Whatman, Inc. CM-Sephadex C-50 was purchased from Pharmacia Fine Chemicals. Polyacrylamide and bis acrylamide were obtained from BioRAD Laboratories; TEMED from Aldrich Chemical Company, Inc.; beta-mercaptoethanol from Eastman Kodak Co.; and SDS from Mallinckrodt Chemical Works. Beta-alanine, trichloroacetic acid, glycerol, and toluene were purchased from J. T. Baker Chemical. NCS tissue solubilizer was obtained from Amersham/Searle. 2,5-diphenyloxazole (PPO) and p-bis (o-methylstyryl) benzene (bis-MSB) were from ICN Chemical and Radioisotope Division. All general chemicals were reagent grade.

METHODS

PURIFICATION OF AVIAN MYELOBLASTOSIS VIRUS

AMV (BAI strain A) was purified as described (Carnegie et al., 1969; Kacian et al., 1971) from frozen chick plasma supplied through the office of Program Resources and Logistics, Viral Cancer Program, National Cancer Institute, Bethesda, MD 20014. The virus was first pelleted at 59,000xg onto a 100% glycerol pad; as a final step, it was resuspended and centrifuged one hour at 59,000xg through a 20% glycerol layer onto a 100% glycerol pad. The virus was resuspended in 0.01 M Tris-HCl (pH 7.4), 0.1 M NaCl, 1 mM EDTA and then disrupted. Viral protein concentration was determined by the method of Warburg and Christian (1942) in the presence of 0.5% (wt/vol) SDS.

PURIFICATION OF AMV DNA POLYMERASE

The virus, 5 mg per ml of viral protein, was disrupted as outlined by Kacian et al. (1971) in 6.7% (vol/vol) NP-40, 0.67% (wt/vol) sodium deoxycholate, and 0.8 M KCl. An average preparation consisted of 150 mg viral protein. After the solution incubated at 0°C for 45 minutes, it was centrifuged 20 minutes at 20,000xg. The supernatant was diluted 10-fold with 0.01 M potassium phosphate (pH 7.2), 5% glycerol, 1 mM DTT and applied at a rate of 1 ml per minute to a DEAE cellulose column (2 x 15 cm) previously equilibrated in 0.01 M potassium phosphate (pH 7.2) and 5% glycerol. The pellet was washed by resuspension with 3 ml of a solution containing 0.01 M Tris-HCl (pH 7.4), 0.1 M NaCl, 1 mM EDTA, 6.7% NP-40, 0.8 M KCl. After 15 minutes at 0°C, the suspension was centrifuged 20 minutes at 20,000xg. The supernatant was diluted

10-fold as above and applied to the DEAE column. After loading the sample, the column was washed with 150-200 ml of 0.05 M potassium phosphate (pH 7.2), 5% glycerol, and 1 mM DTT and then eluted with 0.3 M potassium phosphate (pH 7.2), 5% glycerol and 1 mM DTT. Fractions (2 ml) were collected and the enzyme was located by assaying for polymerase activity.

Fractions containing the bulk of activity were pooled and diluted four-fold with 0.01 M potassium phosphate (pH 8.0), 5% glycerol and 1 mM DTT and applied at a rate of 0.3-0.4 ml per minute to a CM Sephadex C-50 column (0.9 x 8 cm) previously equilibrated in 0.01 M potassium phosphate (pH 8.0) and 5% glycerol. The column was washed with 50-100 ml of 0.1 M potassium phosphate buffer (pH 8.0) containing 5% glycerol and 1 mM DTT. Fractions (0.5 ml) were collected after application of the 0.3 M potassium phosphate (pH 8.0) buffer.

The region of enzyme activity was pooled and precipitated with $(NH_4)_2SO_4$ (pH 7), 4°C, at a final concentration of 65% of saturation. After 1.5 hours in ice, the enzyme was pelleted by centrifuging 45 minutes at 23,500xg. The pellet was resuspended in 0.3-0.5 ml 0.3 M potassium phosphate (pH 8.0), and 1 mM DTT and layered onto 10-30% glycerol gradients (12 ml) in 0.3 M potassium phosphate (pH 8.0) and 1 mM DTT. The gradients were centrifuged 40-45 hours at 0.5°C at 125,000xg in a Spinco SW 41 rotor. Fractions were collected by puncturing the tubes and collecting drops. The fractions containing polymerase activity were diluted with 50% glycerol and stored at -20°C. The purity of the enzyme from each preparation was determined by SDS-PAGE. Kacian et al. (1971), who obtained a 35-fold increase of the specific activity of the AMV reverse transcriptase, estimated that the enzyme represented 0.5% of the total protein in the virus particle based upon the molecular weights of the polymerase and the virus.

DNA POLYMERASE ASSAYS

The standard reaction mixture (100 ul) contained the following components in umoles: Tris-HCl (pH 8.2 at 20°C), 5.0; potassium chloride, 10.0; magnesium chloride, 0.8; DTT, 0.1; deoxyadenine trinucleotide (dATP), 0.02; tritiated deoxythymidine triphosphate (^{3}H-TTP), 0.01 (specific activity, 20 counts per minute per picomole). To complete the reaction 0.9 ug of previously annealed poly rA·oligo dT_{12-18} and AMV polymerase were added. Poly rA·oligo dT_{12-18} was prepared by mixing poly rA and oligo dT_{12-18} to final concentrations of 100 ug per ml and 80 ug per ml, respectively, in 0.01 M Tris-HCl (pH 8.2), 0.1 M NaCl, and 1 mM EDTA. Annealing was promoted by heating the solution to 40°C for 30 minutes followed by cooling to room temperature.

Reaction mixtures were incubated at 40°C for 10 minutes and terminated by spotting onto DE-81 filter circles (2.4 cm diameter) as described (Blatti et al., 1970). The filters were dried under a heat lamp and placed in liquid scintillation vials containing three ml toluene-base scintillation cocktail, 0.39% (wt/vol) PPO, 0.008% (wt/vol) bis-MSB, 0.35% (vol/vol) water, and 2.5% (vol/vol) NCS tissue solubilizer. The extent of deoxynucleotide incorporation was determined by measuring radioactivity of the samples in a Beckman LS-230 scintillation spectrometer.

SODIUM DODECYL SULFATE POLYACRYLAMIDE GEL ELECTROPHORESIS

SDS-PAGE was performed following the procedure of Shapiro et al. (1967) as modified by Kacian et al. (1971). Briefly, the protein to be analyzed was precipitated 30 minutes at 0°C with 5% trichloroacetic acid and resuspended in a denaturing solution of 0.01 M

sodium phosphate (pH 7.8), 1% (wt/vol) SDS, and 1% (vol/vol) beta-mercaptoethanol. Bromophenol blue, 0.01% (wt/vol), the indicator and marker dye, was added to the samples prior to an incubation period of 60 minutes at 37°C. Glycerol was then added to 20%. The tray buffer contained 0.1 M sodium phosphate (pH 7.8) and 0.1% (wt/vol) SDS. Electrophoresis was performed at 10 ma per gel until the dye had entered the gel, at which time the current was increased to 15 ma per gel for 90 minutes. The gels were stained in 0.25% (wt/vol) Coomassie blue in water, glacial acetic acid, ethanol (5:1:5) and destained in 7.5% (vol/vol) glacial acetic acid, 5% (vol/vol) ethanol.

Homogeneity of the AMV DNA polymerase and the individual subunits after isolation was determined by SDS-PAGE. Although no contaminating protein may be visible in an SDS polyacrylamide gel, the limitations of the technique (approximately 5%) may not completely reflect the protein content of the sample. However, for these studies such a minor contaminant would not be a critical factor in the analysis of results.

MICRO-LOWRY PROTEIN DETERMINATION

Purified AMV DNA polymerase was analyzed for protein content by a modified procedure of Lowry et al. (1951). To 0.5 ml aliquots containing 1-10 ug of the standard, BSA, or AMV DNA polymerase, 0.25 ml copper sulfate reagent, prepared as described by Lowry et al. (1951), was added. After a ten minute incubation at room temperature, the phenol reagent was mixed into the protein solutions followed by incubation of the standards and the polymerase sample for 30 minutes at room temperature. The absorbancy of each solution was then measured at 650 nm. As BSA was the standard, the polymerase protein content can only be compared from one preparation to the next in relative terms.

Correlating protein to DNA polymerase activity, that is, giving a specific activity, was useful in estimating the protein content of some preparations. The specific activity of purified AMV DNA polymerase was 15,000 picomoles ^{3}H-TMP incorporated per ug enzyme protein per ten minute reaction.

DISCONTINUOUS POLYACRYLAMIDE GEL ELECTROPHORESIS

Basic System for Neutral or Acidic Proteins

Gel preparation. The gels were prepared as outlined by Davis (1964) and Jovin et al. (1964). The composition of the gel solutions was as follows. For the running gel, small pore gel, solution R-1 contained 2.0 gm acrylamide, 0.056 gm bis acrylamide, 8 ml 10 M urea; R-2 (pH 8.9), 0.907 gm Trizma base, 1.2 ml 1 N HCl, 0.012 ml TEMED, 8 ml 10 M urea; R-3 0.028 gm ammonium persulfate, 8 ml 10 M urea. The latter solution was prepared fresh just prior to use. The stacking gel, large pore gel, solutions were: S-1, 0.5 gm acrylamide, 0.125 gm bis acrylamide, 8 ml 10 M urea; S-2 (pH 6.7), 1.28 ml 1 M H_3PO_4, 8 ml 10 M urea; S-3, 0.2 gm riboflavin, 8 ml 10 M urea. All solutions were taken to 10 ml with water. Each running and stacking gel solution was also prepared without urea, and water was used to dilute the solutions to 10 ml. The running gel solutions, R-1, R-2, R-3, were mixed in a ratio of 1:2:1 and poured to 7 cm in 5 mm inner diameter tubes. After the running gels polymerized, the stacking gels containing a 2:1:1 ratio of S-1, S-2, S-3, were poured to a length of 1.5 cm above the running gel. They were polymerized under fluorescent light.

Sample preparation. The polymerase was removed from its storage environment (50% glycerol, 0.15 M potassium phosphate (pH 8.0), 0.5 mM DTT) by adding an equal volume of water and then adding saturated

$(NH_4)_2SO_4$ to 65%; the sample (approximately 0.04 mg per ml) was precipitated in ice for 2.5 hours. After centrifuging 30 minutes at 23,500xg, samples were resuspended in 50 mM Tris-HCl (pH 8.5), 2 mM EDTA, 10 mM DTT with or without 8 M urea. The samples in urea were incubated 2 hours at room temperature prior to electrophoresis. Samples not containing urea were resuspended in buffer just before electrophoresis; 20% glycerol was added to increase sample density.

Electrophoresis. The tray buffer (pH 8.4) contained 0.6 gm Trizma base and 2.88 gm glycine in 1000 ml water. Current was applied in the direction of the anode at 3 ma per gel initially, and then increased to 5 ma per gel. Total electrophoresis time was approximately 1.5 hours. Gels were stained and destained as described for SDS-PAGE.

Acidic System for Neutral or Basic Proteins

Gel preparation. The procedure of Reisfield et al. (1962) was basically followed. All stock solutions were made to 10 ml. Stock solutions R-1, R-3, S-1, and S-3 were prepared identically to those of the basic system. R-2 contained 0.27 gm KOH, 1.72 ml glacial acetic acid, 0.4 ml TEMED, 8 ml 10 M urea, and glacial acetic acid to 10 ml to bring the pH to 4.5. The stacking gel solution, S-2, was composed of 0.27 gm KOH, 0.29 ml glacial acetic acid, 0.046 ml TEMED, 8 ml 10 M urea. It was brought to 10 ml at pH 5.2 with glacial acetic acid and water. A second set of stock solutions were prepared without urea.

The running gel solutions, mixed in a ratio of 2:1:4, were poured to 7 cm in 5 mm inner diameter glass tubes. After two hours, 1.5 cm stacking gels were poured and polymerized by fluorescent light.

The stacking gel solution contained 4 parts S-1, 1 part S-2, 2 parts S-3 and 1 part 8 M urea. The gels sat for 2-3 hours before being used.

Sample preparation. The polymerase (approximately 0.04 mg per ml) was precipitated with 65% saturated $(NH_4)_2SO_4$ and then resuspended in 8 M urea, 50 mM potassium acetate (pH 5), 10 mM DTT, 2 mM EDTA and incubated for 2 hours at room temperature. Samples not incubated under denaturing conditions were resuspended in 50 mM potassium acetate (pH 5), 10 mM DIT, 20% glycerol. THe tracking dye, pyronine G, was added to 0.005% (wt/vol).

Electrophoresis. One liter of tray buffer (pH 4.5) contained 31.2 gm beta-alanine and 8 ml glacial acetic acid. The samples were layered onto the gels, and then a current of 3 ma per gel was applied until the tracking dye was into the running gel when the amperage was increased to 4 ma per gel. Current was applied toward the cathode until the dye was approximately 1 cm from the bottom of the gel. The gels were stained and destained as previously described.

TWO-DIMENSIONAL POLYACRYLAMIDE GEL ELECTROPHORESIS

First Dimension

Acidic disc electrophoresis in urea. Preparation of the cylindrical gels follows the format previously discussed except the dimensions of the gels were 0.3 x 5.5 cm. The running gel was 5 cm in length and the stacking gel was 0.5 cm.

AMV DNA polymerase was denatured in the urea solution described previously. The sample was divided into two aliquots and layered onto two gels. A current of 2 ma per gel was applied for 2.5 hours. One gel was stained in Coomassie blue and destained. The second gel

was dialyzed in an SDS-sodium phosphate (pH 7.8) environment to remove the acid and urea and to charge the protein for electrophoresis in the second dimension. The method, as developed by Martini and Gould (1971), was to first dialyze against a 0.1 M sodium phosphate and 1% (wt/vol) SDS buffer for 30 minutes. The buffer for the second dialysis step (20 minutes) contained 1% SDS and 0.01 M sodium phosphate, and the final dialysis step was in a 0.1% SDS and 0.01 M sodium phosphate buffer for 25 minutes. Dialysis was accomplished by laying the gel on a plastic mesh screen suspended above the bottom of a beaker. The buffer was stirred continuously and maintained at a temperature of 37-40°C.

SDS-PAGE. An identical amount of polymerase precipitated for the first dimension urea disc gels was prepared for SDS-PAGE. The sample was layered onto two 5% SDS polyacrylamide gels, 0.3 x 5.5 cm. Electrophoresis was performed at 5 ma per gel for 45 minutes and then increased to 6 ma per gel until the marker dye, 0.02% bromophenol blue, was at the bottom of the gel. One gel was stained for protein and the other was used for electrophoresis in the second dimension.

Second Dimension

The slab gel was prepared according to the technique of Howard and Traut (1973). The cylindrical urea disc gel and SDS polyacrylamide gel were laid with upper ends together between two glass plates to form the upper edge of the slab. The gel solution, 5% (wt/vol) acrylamide, 0.25% (wt/vol) bis acrylamide, 0.1 M sodium phosphate (pH 7.8), 0.1% (wt/vol) SDS, 6% (vol/vol) TEMED, 0.3% (wt/vol) ammonium persulfate, was poured through the bottom edge of the glass plates and allowed to polymerize for approximately one hour. The dimensions

of the slab were 0.2 x 9 x 12 cm. The glass plates were clamped into a simplified apparatus built for two-dimensional electrophoresis. Bromophenol blue, 0.08% (wt/vol), in 30% glycerol in tray buffer (0.1 M sodium phosphate (pH 7.8) and 0.1% (wt/vol) SDS) was layered onto the top edge of the slab.

A current of 50 ma was applied for two hours and then decreased to 25 ma for 15 hours. The gel was removed from the glass plates and stained in Coomassie blue for 2.5 hours and then destained.

PREPARATION OF P-11 CELLULOSE

Ten grams of Whatman P-11 cellulose was resuspended and treated in 2 volumes, 500 ml each, of 0.5 N KOH for 1.5 hours. The resin was washed to neutrality with water and then treated twice with 2 volumes, 500 ml each, of 0.5 N HCl for a total time of 1.5 hours. After washing to neutrality with water, the resin was washed 2-3 times with 0.01 M potassium phosphate (pH 7.2) and stored in that buffer at 4°C.

P-11 CHROMATOGRAPHY OF UREA-TREATED POLYMERASE

Column Preparation

Columns were built in Pasteur pipettes (0.5 x 2.5 cm) using glass wool as the bottom filter, or in Pharmacia columns (0.9 x 1 cm). The P-11 columns were equilibrated in 0.01 M potassium phosphate (pH 7.2), 6 or 8 M urea, 5% glycerol, and 1 mM EDTA.

Sample Preparation

AMV DNA polymerase was either precipitated with $(NH_4)_2SO_4$ and resuspended in the denaturing solution or it was diluted to denaturing conditions to remove the enzyme from its storage environment. The concentration of protein in these solutions varied from 0.01-0.1 mg per ml. The enzyme was incubated at room temperature for two hours in denaturation buffer, 8 M urea, 50 mM potassium phosphate (pH 7.2), 10 mM DTT, and 2 mM EDTA.

Elution

After applying the sample to the column, elution was carried out by a stepwise increase of concentrations of potassium phosphate. All buffers contained either 6 or 8 M urea, 5% glycerol, 1 mM DTT, and 1 mM EDTA. The elution volumes were precipitated with trichloroacetic acid and analyzed by SDS-PAGE.

Chapter III

RESULTS

DISSOCIATION OF AVIAN MYELOBLASTOSIS VIRUS DNA POLYMERASE BY UREA

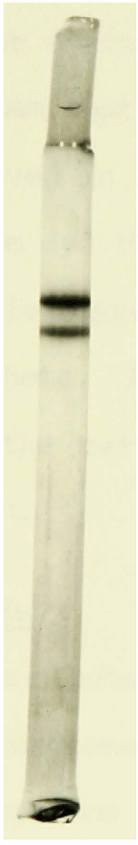
Discontinuous Polyacrylamide Gel Electrophoresis

Disc gel electrophoresis is often employed as an analytical technique for determining the size and charge properties of proteins. An advantage of the method is that urea can be added to the system allowing the study of proteins under denaturing conditions. Combining this technique with urea treatment, it was possible to determine whether urea would act as a dissociating agent for the AMV DNA polymerase.

Urea disc gel electrophoresis of the enzyme was performed under two conditions: (1) a basic environment for acidic or neutral proteins, and (2) an acidic environment for basic or neutral proteins. Polymerase treated with 8 M urea at pH 8.5 and subjected to electrophoresis in a basic environment did not enter the urea disc gels as $\alpha\beta$ or dissociated subunits, as indicated by the absence of any stained protein in the gels (data not shown). However, incubation of polymerase in an acidic-urea environment (pH 5, 8 M urea) resulted in the appearance of two protein bands in the disc gel as shown (Figure 1).

To learn more about the effect of urea in both the basic and acidic gel systems, two experiments were performed in which urea was omitted from the preincubation solution. First, polymerase in 50 mM Tris-HCl (pH 8.5), 10 mM DTT, and 2 mM EDTA was subjected to electrophoresis in a basic environment in two disc gels, one containing

Figure 1. Polyacrylamide Disc Gel (pH 5, 8 M Urea) of Urea-treated AMV DNA Polymerase. Enzyme (24 ug) was incubated in 8 M urea, 0.05 M potassium acetate (pH 5), 2 mM EDTA, and 10 mM DTT at 20°C for 2 hours. The sample (0.05 ml) was applied to a polyacrylamide disc gel (acidic system) containing 8 M urea prepared as described in Methods. Current was applied in the direction of the cathode at 3 ma per gel for 50 minutes and then increased to 4 ma per gel for approximately 60 minutes. Following electrophoresis, the gel was stained in 0.25% Coomassie blue and destained.



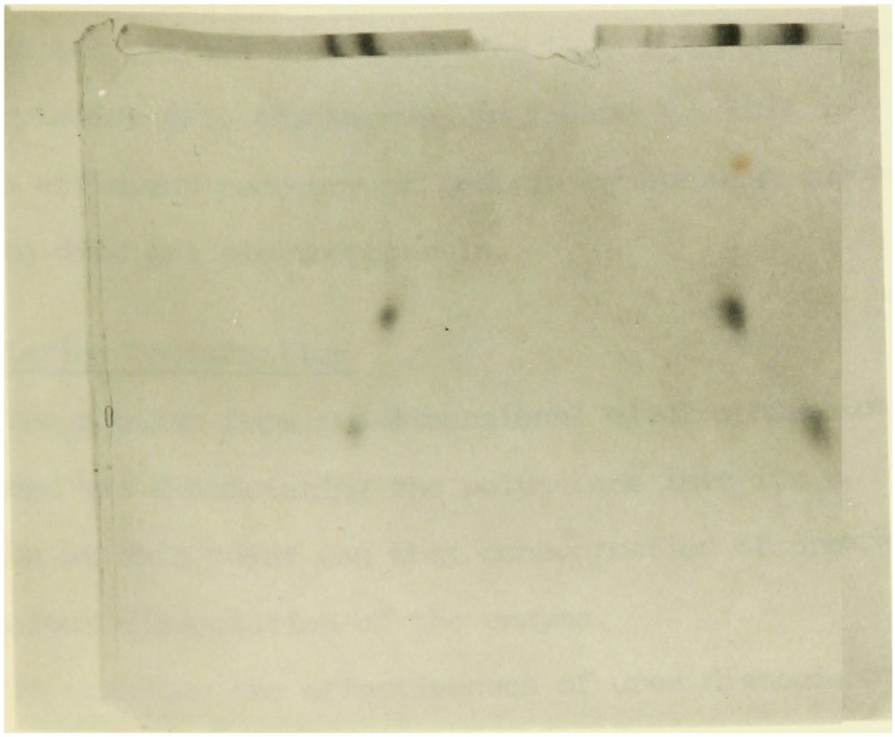
8 M urea and one without urea. Second, enzyme in 50 mM potassium acetate (pH 5), 10 mM DTT, and 2 mM EDTA was applied to two acidic disc gels; one contained 8 M urea and the second gel did not. Protein did not penetrate any gel under any of the above conditions. These results suggested that urea was responsible for dissociating the polymerase to some extent.

As seen in Figure 1, the staining intensity of the upper band in the disc gel is greater than that of the lower band. This staining intensity difference is observed in SDS polyacrylamide gels, where beta binds more Coomassie blue dye than alpha due to the greater molecular weight of beta. This suggested that the two bands in the disc gels might be alpha and beta. However, more conclusive evidence was required to be sure that the two observed bands were alpha and beta and not some other combination.

Two-Dimensional Electrophoresis

The positions of alpha and beta in SDS polyacrylamide gels are known (Kacian et al., 1971; Grandgenett et al., 1973; Stromberg et al., 1974), so removing the proteins from the urea gel and subjecting them to electrophoresis in an SDS environment with controls would identify the bands. An applicable procedure was developed by Martini and Gould (1971) who were studying ribosomal proteins. The first step involved separation of proteins in the first dimension in a cylindrical disc gel (pH 5) containing urea. The urea gel was then dialyzed against decreasing levels of SDS and sodium phosphate (pH 7.8) to remove the acid and urea and to charge the proteins with SDS, thus permitting electrophoresis of the proteins as anions in the second dimension in an SDS environment.

Figure 2. Two-Dimensional Gel Electrophoresis of AMV DNA Polymerase Subunits. Two aliquots of AMV DNA polymerase (18 ug) were prepared for first dimension disc gels (pH 5, 8 M urea) and SDS polyacrylamide gels as described in Methods. After electrophoresis in the first dimension, the cylindrical gels were laid with upper ends together to form the upper edge of the slab gel, which was prepared according to a modified method of Howard and Traut (1973) (see Methods). Electrophoresis in the second dimension was performed by applying a current in the direction of the anode at 50 ma for 2 hours and then 25 ma for 15 hours. The slab gel was stained for 2.5 hours in 0.25% Coomassie blue and then destained. The upper and lower spots on the left side of the slab gel represent the upper and lower bands, respectively, of the first dimension disc gels (pH 5, 8 M urea); and the upper and lower spots on the right side of the slab represent beta and alpha, respectively, of the first dimension SDS polyacrylamide gels. Also shown in the figure are the respective stained cylindrical gels obtained from first dimension electrophoresis.



By simultaneously subjecting the proteins present in the disc gel and alpha and beta from an SDS cylindrical polyacrylamide gel to electrophoresis in an SDS polyacrylamide slab gel, the two proteins present in the cylindrical disc gel were identified as beta, upper band, and alpha, lower band (Figure 2). The alpha and beta subunits from the urea gel stained less intensely than alpha and beta from the SDS polyacrylamide gel, as observed in Figure 2. This is probably a result of less efficient recovery of protein by ammonium sulfate precipitation prior to disc gel electrophoresis.

Dissociation Optimization

The results from two-dimensional electrophoresis confirmed that urea was dissociating the polymerase into its subunits. The question at this point was what concentration of urea was necessary for maximum dissociation of the enzyme.

To consider the effectiveness of urea dissociation at concentrations less than 8 M, three aliquots of polymerase (24 ug) were incubated at pH 5 with varying levels of urea and were layered onto 8 M urea gels. Electrophoresis was then performed in an acidic environment. As only dissociated subunits penetrate and migrate in the urea gel, the staining intensity of the protein bands present in the gels is a measure of dissociation. Figure 3 illustrates that dissociation is not as complete in 4 M or 6 M urea as in 8 M urea; the results are summarized in Table 1. Thus, it appeared that 8 M urea would provide the most efficient dissociation condition during pretreatment for preparative isolation of DNA polymerase subunits.

Figure 3. Disc Gel Electrophoresis (pH 5, 8 M Urea) of AMV DNA Polymerase Pretreated in Decreasing Concentrations of Urea. Three aliquots of enzyme (24 ug) were pretreated in 8 M, 6 M, or 4 M urea solutions, containing 50 mM potassium acetate (pH 5), 2 mM EDTA, and 10 mM DTT, for two hours at room temperature prior to disc electrophoresis in an acidic environment. The disc gels containing 8 M urea were prepared as described in Methods. Electrophoresis was performed as described in the legend of Figure 1. The disc gels were stained for one hour in 0.25% Coomassie blue and destained. The gels represent polymerase pretreated in (A) 8 M urea; (B) 6 M urea; (C) 4 M urea.

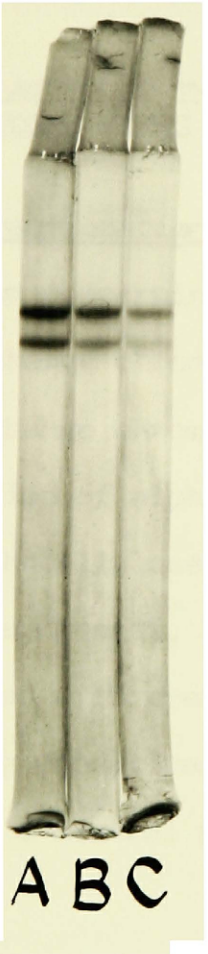

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Table 1. Percentage Dissociation of AMV DNA Polymerase Subunits in 4 M and 6 M Urea Compared to 8 M Urea.

Urea Concentration	% Dissociation
8 M	100%
6 M	65
4 M	36

Percentage dissociation was determined by densitometric scanning of Coomassie blue-stained protein in the acidic disc gels containing 8 M urea by measuring absorbance at 560 nm. Dissociation by pretreatment in 8 M urea was assumed to be 100%.

PREPARATIVE ISOLATION OF THE SUBUNITS OF AVIAN MYELOBLASTOSIS VIRUS DNA POLYMERASE

Methods and Conditions for Fractionation

Due to information known concerning the behavior of AMV DNA polymerase on certain ion-exchange chromatography resins, as previously described, ion-exchange chromatography was the method chosen for preparative isolation of alpha and beta. In view of Grandgenett's application of phosphocellulose chromatography to dissociation (Grandgenett et al., 1973; Grandgenett, 1976) aliquots of purified DNA polymerase were dissociated with urea and chromatographed on this cation-exchanger under varying conditions of urea concentration and temperature while maintaining a neutral pH (7.2) during all fractionation experiments.

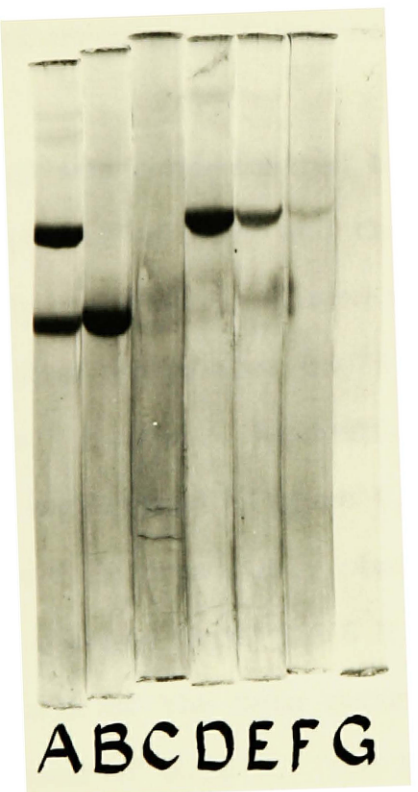
Phosphocellulose Chromatography (P-11)

In the initial experiment, enzyme was dissociated in 8 M urea at 25°C and chromatographed in 6 M urea at 4°C. The lower urea concentration was necessary due to the insolubility of 8 M urea at 4°C. These conditions are similar to those described by Lill et al. (1975) for dissociation of *E. coli* RNA polymerase, in which separated subunits were subsequently reassociated to an active form.

Polymerase (150 ug) was precipitated in 65% saturated $(NH_4)_2SO_4$ and treated at room temperature with urea at pH 7.2 as described in Methods. After application of the sample to the P-11 column, it was eluted step-wise with 0.05 M, 0.075 M, 0.15 M, 0.25 M, 0.4 M potassium phosphate buffers all containing 6 M urea, and 0.6 M potassium phosphate with 4 M urea. Each wash was collected separately and analyzed by SDS-PAGE. Figure 4 shows that alpha eluted with the lowest potassium phosphate concentration, 0.05 M, with a slight amount washing through with the 0.075 M salt. Since the potassium phosphate concentration in the dissociation solution was 0.05 M, it was impossible to determine from the results of this experiment whether alpha would bind to phosphocellulose. The bulk of beta eluted at 0.15 M potassium phosphate with more eluting at 0.25 M and 0.4 M potassium phosphate. A very small amount of alpha is present with beta in the 0.15 M and 0.25 M washes, as shown in Figure 4, indicating that either the polymerase was not completely dissociated or that the column was not being washed with adequate volumes of the lower concentration phosphate solutions. A second contaminating band can be seen above alpha in the gels of the 0.075 M, 0.15 M, and 0.25 M washes. This may be a contaminating protein in the enzyme pool used for the study or a proteolytic product of beta produced during storage. Others have shown that breakdown occurs during storage of the purified polymerase (Moelling, 1974; Papas et al., 1976; Verma, 1977).

To determine whether alpha would bind to phosphocellulose at a potassium phosphate concentration less than 0.05 M or whether alpha had no binding affinity at all for P-11 in the presence of 6 M urea, a second sample was treated in 8 M urea, diluted two-fold with 6 M urea in 0.01 M potassium phosphate (pH 7.2) to decrease the salt

Figure 4. Separation of AMV DNA Polymerase Subunits by P-11 Chromatography (pH 7.2) in 6 M Urea at 4°C. Polymerase (150 ug) was precipitated with 65% saturated $(NH_4)_2SO_4$ and then pretreated in 8 M urea, 0.05 M potassium phosphate (pH 7.2), 10 mM DTT, and 2 mM EDTA at 25°C for 2 hours. The treated enzyme (100-120 ug in 0.6 ml) was applied to a P-11 column (0.5 x 2.5 cm) equilibrated in a 6 M urea buffer, and then the column was eluted with increasing concentrations of potassium phosphate in 6 M urea. The experiment was performed at 4°C. Each salt wash (6 ml) was collected and analyzed by SDS-PAGE as described in Methods. The quantity of protein present in the control gel of this and subsequent figures was determined by measurement, and the amounts indicated as present in the column washes were estimated by inspection of the staining intensity of the proteins. The gels represent: (A) AMV DNA polymerase (40 ug); (B) 0.05 M potassium phosphate wash (40 ug); (C) 0.075 M elution; (D) 0.15 M potassium phosphate wash (30-40 ug); (E) 0.25 M elution (5 ug); (F) 0.4 M potassium phosphate elution (2 ug); (G) 0.6 M potassium phosphate wash (4 M urea).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concentration, and chromatographed at 4°C. After application of the sample to the column in 0.025 M potassium phosphate, the column was washed with 0.01 M and then 0.05 M potassium phosphate in 6 M urea. As shown in Figure 5, alpha bound to the column at the lower salt and eluted with 0.05 M potassium phosphate.

Since each subunit of the polymerase must be isolated without contamination by the other in order to accurately determine the properties of each, and since 8 M urea was shown to optimize dissociation of the subunits, modifications were made to include 8 M urea in the chromatographic conditions. Concurrently, it was necessary to raise the temperature to 25°C.

Polymerase (175 ug) was concentrated by $(NH_4)_2SO_4$ precipitation, treated in 8 M urea at pH 7.2 at 20°C and then applied to a P-11 column at room temperature. The column was eluted with 8 M urea solutions containing 0.05 M potassium phosphate, 0.075 M, and 0.20 M, and finally with 0.40 M phosphate in 5 M urea. Separation of the subunits appeared to be excellent in this experiment (Figure 6). Alpha eluted with 0.05 M potassium phosphate as previously observed. However, the elution pattern of beta changed from the 6 M urea, 4°C experiment (Figure 4) in that the bulk of the beta subunit eluted with 0.075 M potassium phosphate instead of 0.15 M. The higher urea concentration seems to affect the binding properties of the protein.

To further optimize dissociation, separation, and recovery of purified subunits, an attempt to decrease manipulation and to avoid losses due to insufficient precipitation by ammonium sulfate was made. Polymerase (45 ug) in storage buffer (50% glycerol, 0.15 M potassium phosphate (pH 8.0), 0.5 mM DTT) was diluted with 0.01 M

Figure 5. Binding of Alpha Subunit to P-11 (pH 7.2) in 0.025 M Potassium Phosphate and 6 M Urea at 4°C. Polymerase (150 ug) was treated as described in Methods. After preincubation, the enzyme solution (100-120 ug in 0.6 ml) was diluted two-fold with 0.01 M potassium phosphate (pH 7.2) in 6 M urea buffer and applied to an equilibrated P-11 column (0.5 x 2.5 cm). The column was eluted with 6 M urea buffers containing 0.01 M and 0.05 M potassium phosphate. Both were analyzed by SDS-PAGE as described in Methods. The SDS gels represent: (A) AMV DNA polymerase (40 ug); (B) 0.01 M potassium phosphate elution; (C) 0.05 M potassium phosphate wash (10-15 ug).

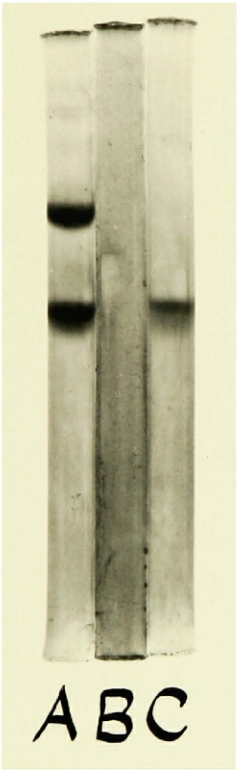
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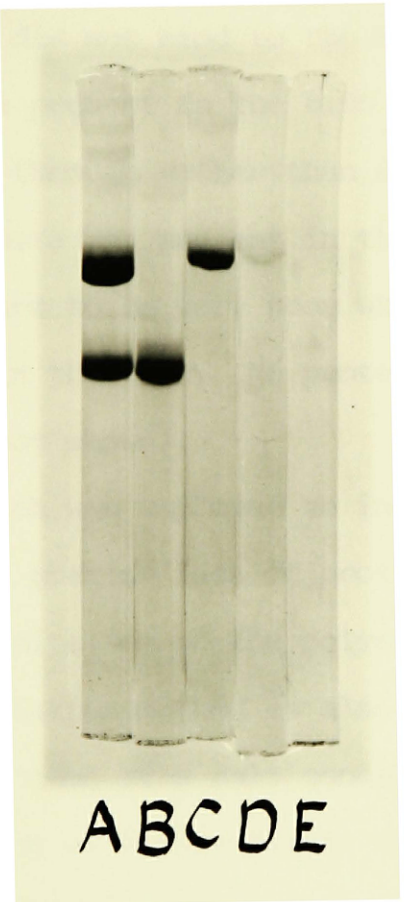
Figure 6. Isolation of the Subunits of AMV DNA Polymerase by P-11 Chromatography (pH 7.2) in 8 M Urea at 20°C. The polymerase (175 ug) was concentrated by $(NH_4)_2SO_4$ precipitation and then was pretreated in an 8 M urea solution. The enzyme sample (120 ug in 0.6 ml) was applied to a P-11 column (0.9 x 1 cm) equilibrated with an 8 M urea buffer and then eluted with buffers containing 8 M urea and increasing concentrations of potassium phosphate. Each wash was collected separately and analyzed by SDS-PAGE. The gels represent: (A) AMV DNA polymerase (50-60 ug); (B) 0.05 M potassium phosphate wash (10 ml) (30-40 ug); (C) 0.075 M potassium phosphate elution (20 ml) (20-25 ug); (D) 0.15 M elution (10 ml) (2 ug); (E) 0.4 M potassium phosphate (5 M urea) wash (10 ml).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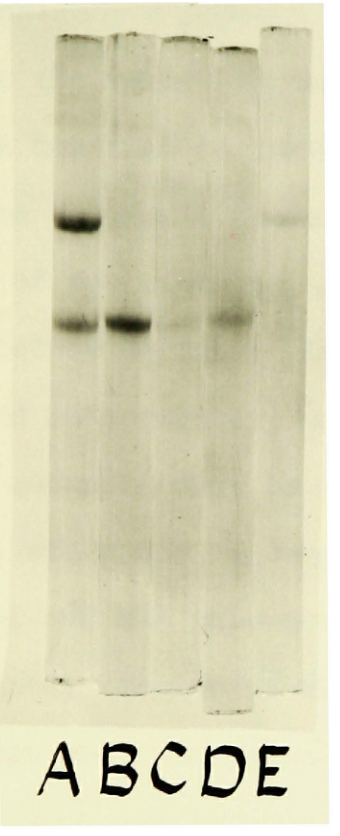
potassium phosphate in 8 M urea to decrease the glycerol and salt concentrations. After preincubation, the solution was applied to a P-11 column equilibrated in 8 M urea at pH 7.2 and 20°C. The initial eluate (flow-through) was collected during application of the sample and then the column was washed with buffers containing 0.05 M, 0.075 M, and 0.25 M potassium phosphate. Figure 7 shows that alpha was present in the flow-through as well as the 0.05 M phosphate wash indicating that alpha did not bind to the P-11 resin at low salt in 8 M urea. The protein present in the 0.05 M wash may represent the remainder of the flow-through rather than a separate elution at that salt concentration. Beta was present in the 0.075 M wash; however, recovery of beta appears to be very poor when compared to the quantity of alpha represented in the gels. No protein was present in the 0.25 M phosphate wash (data not shown).

This method, which was employed to increase protein recovery, actually resulted in a greater loss of protein than the method involving ammonium sulfate precipitation of the polymerase. A second disadvantage of the dilution method is also apparent in that alpha does not bind to the column at a salt concentration as low as 0.02 M. This results in recovery of a more dilute concentration of this subunit, which may lead to complications when trying to recover enzyme activity.

Figure 7. Dissociation of AMV DNA Polymerase in Storage Buffer by Dilution with 8 M Urea. Polymerase (45 ug) in storage buffer (50% glycerol, 0.15 M potassium phosphate (pH 8.0), 0.5 mM DTT) was diluted with urea to give a solution (4 ml) containing 8M urea, 0.02 M potassium phosphate, 10 mM DTT, 2 mM EDTA, and 10% glycerol. After incubating at room temperature for 1.5 hours, the sample was applied to a P-11 column (0.9 x 1 cm) equilibrated in 8 M urea and 0.01 M potassium phosphate (pH 7.2). The column was eluted with 8 M urea washes containing potassium phosphate at concentrations of 0.05 M, 0.075 M, and 0.25 M. Aliquots of each wash were analyzed by SDS-PAGE. The SDS polyacrylamide gels represent: (A) AMV DNA polymerase (5 ug); (B) flow-through (3 ml) (5 ug); (C) first 0.5 ml of 0.05 M potassium phosphate wash (1 ug); (D) remainder of 0.05 M wash (15 ml) (2 ug); (E) 0.075 M potassium phosphate elution (20 ml) (1 ug).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Chapter IV

DISCUSSION

The RNA genome of AMV, a chicken leukemia virus, is expressed by reverse transcription, a process by which the virus-specific polymerase utilizes the viral RNA as template to produce a DNA copy. The transcribed DNA can then be integrated into the host genome, thus maintaining the oncogenic state. The RNA-directed DNA polymerase of AMV is composed of two subunits, alpha and beta; the roles of each have not been completely defined. In order to determine the functions of each subunit, and subsequently, understand the mechanism of reverse transcription, the subunits must be isolated individually. Therefore, the purpose of this study was to develop a method by which the alpha and beta subunits of AMV DNA polymerase could be dissociated and preparatively isolated, keeping in mind that the dissociation technique could not be so harsh that the subunits' activities would be permanently destroyed. Because urea has been used successfully as a dissociation agent for multi-subunit enzymes (Burgess, 1969; Lill and Hartmann, 1970; Heil and Zillig, 1970; Frieden, 1971; Ishihama and Ito, 1972; Yarbrough and Hurwitz, 1974; Palm et al., 1975), and has not permanently altered their functions (Johnson et al., 1969; Deal, 1969; Ishihama and Ito, 1972; Yarbrough and Hurwitz, 1974; Lill et al., 1975; Palm et al., 1975), it was employed for dissociation studies of AMV DNA polymerase.

The effectiveness of urea as a dissociation agent of the polymerase subunits was first examined analytically by disc gel electrophoresis. When the polymerase was pretreated in 8 M urea at pH 5 and analyzed in an acidic gel electrophoresis system, two proteins entered the disc gel (Figure 1). In contrast, no migration of protein occurred in a basic disc gel system in which the polymerase was pretreated at pH 8.5 and subjected to electrophoresis in a basic environment. The presence of two proteins in the acidic polyacrylamide gel indicated that dissociation of the polymerase was occurring to some degree and that the proteins were behaving as basic molecules. Causes for the absence of protein migration in the basic system could be that the basic pH environment was not appropriate for the proteins to enter the gel, or that the basic pH was not appropriate for subunit dissociation. The first possibility is more likely since subsequent experiments suggested that an acidic pH was not necessary for dissociation.

The important factor in causing dissociation of the polymerase was shown to be urea. When the enzyme was not preincubated in urea prior to electrophoresis, migration of the two proteins was not observed, and the presence of urea in the disc gel was not sufficient to cause dissociation. These results strongly suggest that the preincubation step with urea was crucial for enzyme dissociation.

The two proteins migrating in the disc gels (pH 5 and 8 M urea) were identified as alpha and beta by two-dimensional electrophoresis. This experiment demonstrated that in an SDS polyacrylamide slab gel, alpha and beta obtained by SDS-PAGE migrated the same distance as the lower and upper protein bands, respectively, obtained from the disc

gel. Identifying the two proteins as alpha and beta demonstrated that urea would be suitable agent for dissociation of the subunits of the polymerase.

To determine whether 8 M urea was required for optimum dissociation or whether a lower concentration would provide comparable dissociation, aliquots of polymerase were pretreated in varying concentrations of urea prior to disc gel electrophoresis (pH 5). The results demonstrated that 65% and 36% dissociation of the enzyme occurred in 6 M and 4 M urea, respectively, compared to 8 M urea (Table 1 and Figure 3), assuming that 8 M urea caused complete dissociation. The results of this experiment emphasized the need for dissociation of the polymerase in 8 M urea in order to achieve optimum separation and recovery of the subunits. For this reason the concentration of urea in all pre-incubation solutions was 8 M.

With the information that urea would dissociate the polymerase and that 8 M urea provided maximum dissociation, the next task was to develop a procedure for preparative isolation of alpha and beta. Phosphocellulose chromatography was chosen as the preparative isolation method, primarily due to the work of Grandgenett and his coworkers (Grandgenett et al., 1973; Grandgenett, 1976). They found that alpha separated from αβ on a phosphocellulose column under non-denaturing conditions; alpha eluted with 0.11 M potassium phosphate (pH 8) and αβ eluted with 0.22 M potassium phosphate. After treating AMV DNA polymerase with dimethyl sulfoxide or 1,4-dioxane, Grandgenett (1976) applied the sample to a phosphocellulose column. In this experiment, alpha and αβ had the same elution patterns as described above. In addition, a beta-enriched fraction eluted with salt concentrations greater than 0.25 M potassium phosphate. Because Grandgenett achieved

fractionation of the polymerase using phosphocellulose chromatography after treatment of the polymerase by denaturing agents, the method was applied to this study and was found to be very useful.

In order to achieve the best separation of the polymerase subunits, the experimental conditions employed during chromatography needed to be considered, particularly, pH, temperature, and urea concentration. Although disc gel electrophoresis was performed at pH 5, and the polymerase was dissociated, a higher pH was more desirable for chromatography, primarily because the pH optimum for enzymatic activity is above pH 7, and lengthy exposure to an acidic pH may affect enzyme stability. Another reason for maintaining a neutral pH was that the elution profile of alpha, $\alpha\beta$, and a beta-enriched fraction has been described for phosphocellulose chromatography at a pH above neutrality (Grandgenett, 1976). With this information as a guideline, less time and material were expended trying to determine the salt concentrations required to elute each component separately.

A second experimental parameter considered was temperature. The conditions established by Lill et al. (1975) for dissociation of RNA polymerase from E. coli were incubation in 6.5 M urea at 25°C followed by phosphocellulose chromatography at 4°C in 7 M urea. Upon removal of the urea, the subunits were mixed stoichiometrically and the holoenzyme was reconstituted into an enzymatically functional form. Because renaturation of the subunits of the AMV DNA polymerase is essential for subsequent characterization studies, it was necessary to consider chromatography at 4°C, even though optimum dissociation of the polymerase was obtained with 8 M urea (Table 1 and Figure 3), which is insoluble at 4°C. To determine whether adequate separation of alpha and beta could be obtained by preincubating the polymerase in 8 M urea

and then chromatographing it at a lower urea concentration, or whether 8 M urea was required throughout the experiment, purified enzyme was pretreated with urea and chromatographed under varying conditions of urea concentration and temperature.

By employing 6 M urea during chromatography at 4°C, alpha was cleanly separated from beta, but the beta fraction contained a small amount of alpha (Figure 4). The presence of alpha in the beta fraction may have represented partial reassociation of the subunits due to the lower urea concentration (6 M) present during chromatography. A second explanation for incomplete separation could be that since the column was washed with small volumes of the elution buffers, elution of alpha with the 0.05 M potassium phosphate wash may have been incomplete. If more extensive washing of the column would eliminate the problem of isolating impure beta, chromatography at pH 7.2 and 4°C in 6 M urea would be a workable dissociation technique.

Better separation resulted when the urea concentration in the column buffers was increased to 8 M in which case beta was isolated without contamination by alpha (Figure 6). As both of the above described conditions for separation of the subunits appear very workable, the choice of which system to use will ultimately depend upon the results of renaturation studies in which enzyme activity must be recovered.

In an attempt to further optimize the yields of alpha and beta from phosphocellulose chromatography in 8 M urea, the ammonium sulfate precipitation of polymerase prior to urea dissociation was omitted, since, on the average, only 65-75% of the enzyme was recovered in the precipitate. In this case, the polymerase in storage buffer was placed directly into the dissociating preincubation solution by diluting with 8 M urea, which was then applied directly to the phosphocellulose

column. Results of the experiment show that alpha did not bind to the column (see Figure 7), even though the potassium phosphate concentration in the dissociation solution was less than 0.05 M. Beta eluted in the 0.075 M potassium phosphate wash. Both subunits were pure, but the percentage protein recovered compared to the amount originally applied was only 10-15%. In contrast, in the preceding experiments, 70-80% of the AMV DNA polymerase that was applied to the phosphocellulose columns in 6 M and 8 M urea for the isolation of the subunits (Figures 4 and 6) was recovered. The reasons for these differences are not yet understood.

From the four chromatography studies it is evident that the elution pattern of the alpha subunit of AMV DNA polymerase on phosphocellulose is different from that obtained by Grandgenett and coworkers (1973), in that alpha eluted at 0.05 M potassium phosphate, or less, depending upon the urea concentration, as compared to 0.11 M potassium phosphate. The binding properties of beta also changed with urea concentration; beta eluted with 0.15 M potassium phosphate when the buffers contained 6 M urea and with 0.075 M potassium phosphate when 8 M urea was present in the column buffers. The binding changes are likely due to the unfolding of the proteins by the denaturing action of urea, which would affect the overall charge properties of the molecule.

The denaturation of the AMV DNA polymerase in 6 M and 8 M urea resulted in a loss of enzymatic activity. In the case of E. coli DNA-dependent RNA polymerase treated with urea, a similar loss of enzymatic function was observed. However, RNA polymerase activity was restored after renaturation and reassociation of the subunits. In a similar manner, activity of the AMV DNA polymerase may be recovered, but the possibility must be recognized that the alpha and beta subunits may have been irreversibly denatured by urea, or that a factor necessary for activity may have been lost due to the urea treatment.

One must also consider that a non-protein factor, such as nucleic acid, carbohydrate, or phospholipid might exist as an integral part of the DNA polymerase. However, indirect evidence suggests that none of the above three components are part of the functional polymerase as demonstrated by the lack of incorporation of corresponding labeled precursors into the DNA polymerase being synthesized in virus-infected cells (Davis and Rueckert, 1972). Nonetheless, until the AMV DNA polymerase itself has been examined, it must be kept in mind that a non-protein component essential for enzymatic activity may be associated with the enzyme.

A variety of studies may be undertaken if renatured subunits are obtained. Essential primary experiments would be to determine whether beta has any binding affinity for nucleic acids and whether it has any enzymatic activity. If the beta subunit had polymerase or RNase H activity or both, examination of the mechanism of reverse transcription by beta would be important for characterization of the subunit. Particular aspects of such a study would include the determination of the ability of beta to utilize various natural and synthetic template-primers and the analysis of the size and secondary structure of the DNA product. Understanding the role of beta would require comparison of its properties to those of alpha and $\alpha\beta$. Specifically, one could examine the dependency of alpha and beta upon the presence of certain ions and functional groups that have been shown to affect the polymerase activity of $\alpha\beta$. For example, removal of zinc from the enzyme results in a loss of polymerase activity (Poiesz et al., 1974). Both sulfhydryl and phosphate groups may have an important role in enzymatic function. Studies in our laboratory with sulfhydryl groups have shown that by treating the AMV DNA polymerase with sulfhydryl blocking reagents,

polymerization is inhibited (McGiffert, personal communication). Lee et al. (1975) found that by phosphorylating the Rous sarcoma virus DNA polymerase, which is similar to the AMV DNA polymerase, with a protein kinase, polymerase activity is stimulated. With isolated subunits, the effect of each of the above phenomena on the enzymatic properties, binding, and structure of alpha and beta can be determined. This, in turn, should result in a better definition and understanding of reverse transcription.

Chapter V

SUMMARY

Urea was shown to dissociate the two subunits of the AMV DNA polymerase by disc gel electrophoresis and two-dimensional electrophoresis. Optimum dissociation of the polymerase occurs with 8 M urea present during the preincubation step compared to 6 M and 4 M urea. In accordance with optimum dissociation in 8 M urea, the alpha and beta subunits were best separated and isolated by phosphocellulose chromatography in 8 M urea at 25°C, although better separation may be possible by chromatography in 6 M urea at 4°C if experimental conditions are altered.

REFERENCES

Bader, J. P. 1964. The role of DNA in the synthesis of Rous sarcoma virus. Virol. 22: 462.

_____1965a. The requirement for DNA synthesis in the growth of Rous sarcoma and Rous-associated virions. Virol. 26: 253.

_____1965b. Transformation by Rous sarcoma virus: A requirement for DNA synthesis. Science 149: 757.

Balduzzi, P. and H. R. Morgan. 1970. Mechanisms of oncogenic transformation by Rous sarcoma virus. II. Intracellular inactivation of cell-transforming ability of Rous sarcoma virus by 5-bromodeoxyuridine and light. J. Virol. 5: 470.

Baltimore, D. 1970. RNA-dependent DNA polymerase in virions of RNA tumour viruses. Nature (London) 226: 1209.

Baltimore, D., A. S. Huang, M. Stampfer. 1970. Ribonucleic acid synthesis of vesicular stomatitis virus. II. An RNA polymerase in the virion. Proc. Nat. Acad. Sci. USA 66: 572.

Bather, R. 1963. Influence of 5-fluorodeoxyuridine and actinomycin D on the production of Rous sarcoma virus in vitro. Proc. Amer. Assoc. Cancer Res. 4:4.

Bauer, H. 1966. Untersuchungen uber das myeloblastosevirus des huhnes (BAI stamm A): II. Isoleirung und charakterisierung der im virus enthaltenen nukleinsaure. Z. Naturforsch. 21b: 453.

Beard, J. W. 1963. Avian virus growths and their etiologic agents. Adv. in Cancer Res. 7: 1.

Bellamy, A. R., S. C. Gillies, J. D. Harvey. 1974. Molecular weight of two oncornavirus genomes: Derivation from particle molecular weights and RNA content. J. Virol. 14: 1388.

Bernhard, W. R., R. A. Bonar, D. Beard, J. W. Beard. 1958. Ultrastructure of viruses of myeloblastosis and erythroblastosis isolated from plasma of leukaemic chickens. Proc. Soc. Exp. Biol. Med. 97: 48.

Bishop, J. M., W. E. Levinson, N. Quintrell, D. Sullivan, L. Fanshier, J. Jackson. 1970. The low molecular weight RNAs of Rous sarcoma virus. I. The 4S RNA. Virol. 42: 182.

Biswal, N.,B. McCain, M. Benyesh-Melnick. 1971. The DNA of murine sarcoma-leukemia virus. Virol. 45: 706.

Blatti, S. P., C. J. Ingles, T. J. Lindell, P. W. Morris, R. F. Weaver, F. Weinber, W. J. Rutter. 1970. Cold Spring Harbor Symp. Quant. Biol. 35: 649.

Boettiger, D. and H. M. Temin. 1970. Light inactivation of focus formation by chick embryo fibroblasts infected with avian sarcoma virus in the presence of 5-bromodeoxyuridine. Nature 228: 622.

Bonar, R. A., L. Sverak, D. P. Bolognesi, A. J. Langlois, D. Beard, J. W. Beard. 1967. Ribonucleic acid componenets of BAI strain (myeloblastosis) avian tumor virus. Cancer Res. 27: 1138.

Bukrinskaya, A. G. 1973. Nucleocapsids of large RNA viruses as functionally active units in transcription. Adv. in Virus Res. 18: 195.

Burgess, R. R. 1969. Separation and characterization of the subunits of ribonucleic acid polymerase. J. Biol. Chem. 244: 6170.

Canaani, E. and P. Duesberg. 1972. Role of subunits of 60 to 70S avian tumor virus RNA in its template activity for the viral DNA polymerase. J. Virol. 10: 23.

Carnegie, J. W., A. O'C. Deeney, K. C. Olson, G. S. Beaudreau. 1969. An RNA fraction from myeloblastosis virus having properties similar to transfer RNA. Biochim. Biophys. Acta 190: 274.

Dahlberg, J. E., R. C. Sawyer, A. J. Faras, W. E. Levinson, H. M. Goodman, J. M. Bishop. 1974. Transcription of DNA from the 70S RNA of Rous sarcoma virus. I. Identification of a specific 4S RNA which serves as primer. J. Virol. 13: 1126.

Davis, B. J. 1964. Disc electrophoresis-II Methods and application to human serum proteins. Ann. J. J. Acad. Sci. 121: 404.

Davis, M. L. and R. R. Rueckert. 1972. Properties of a ribonucleo-protein particle isolated from Nonidet P-40-treated Rous sarcoma virus. J. Virol. 10: 1010.

Deal, W. C., Jr. 1969. Metabolic control and structure of glycolytic enzymes. IV. Nicotinamide adenine dinucleotide dependent in vitro reversal of dissociation and possible in vivo control of yeast glyceraldehyde 3-phosphate dehydrogenase synthesis. Biochem. 8: 2795.

De-The, G., C. Becker, J.W. Beard. 1964. Virus of avian myeloblastosis (BAI strain A): XXV. Ultracytochemical study of virus and myeloblast phosphatase activity. J. Nat. Cancer Inst. 32: 201.

Doerfler, W. 1975. Integration of viral DNA into the host genome. Current Topics in Microbiology and Immunology 71: 1.

Duesberg, P. H. 1970. On the structure of RNA tumor viruses. Current Topics in Microbiology and Immunology 51: 79.

Eckert, E. A., R. Rott, W. Schafer. 1963. Myxovirus-like structure of avian myeloblastosis virus. Z. Naturforsch. 18b: 339.

Ellermann, V. and O. Bang. 1908. Experimentelle leukamie bei huhnern. Zentralbl. Bakteriol. Abt. I (orig) 46: 595.

_____1909. Experimentelle leukamie bei huhnern. A. Hyg. Infekt. 63: 231.

Erikson, E. and R. L. Erikson. 1976. The origin of 7S RNA in virions of avian sarcoma viruses. Virol. 72: 518.

Faras, A. J. and N. A. Dibble. 1975. RNA-directed DNA synthesis of Rous sarcoma virus: Structural and functional identification of 4S RNA primer in uninfected cells. Proc. Nat. Acad. Sci., USA 72: 859.

Flugel, R. M. and R. D. Wells. 1972. Nucleotides at the RNA-DNA covalent bonds formed in the endogenous reaction by the avian myeloblastosis virus DNA polymerase. Virol. 48: 394.

Folk, W. and A. J. Faras. 1976. Initiation of DNA synthesis by an avian oncornavirus RNA-directed DNA polymerase: Tryptophan tRNA as the major species of primer RNA. J. Virol. 17: 1049.

Frieden, C. 1971. Protein-protein interaction and enzymatic activity. Ann. Rev. of Biochem. 40: 653.

Gibson, W. and I. M. Verma. 1974. Studies on the reverse transcriptase of RNA tumor viruses. Structural relatedness of two subunits of avian tumor viruses. Proc. Nat. Acad. Sci., USA 71: 4991.

Grandgenett, D. P. 1976. Dissociation of αβ DNA polymerase of avian myeloblastosis virus by dimethyl sulfoxide. J. Virol. 17: 950.

Grandgenett, D. P., G. F. Gerard, M. Green. 1973. A single subunit from avian myeloblastosis virus with both RNA-directed DNA polymerase and ribonuclease H activity. Proc. Nat. Acad. Sci., USA 70: 230.

Grandgenett, D. and M. Green. 1974. Different mode of action of ribonuclease H in purified α and αβ ribonucleic acid-directed deoxyribonucleic acid polymerase from avian myeloblastosis virus. J. Biol. Chem. 249: 5148.

Grandgenett, D. P. and H. M. Rho. 1975. Binding properties of avian myeloblastosis virus DNA polymerases to nucleic acid affinity columns. J. Virol. 15: 526.

Grandgenett, D. P., A. C. Vora, A. J. Faras. 1976. Different states of avian myeloblastosis virus DNA polymerase and their binding capacity to primer tRNAtrp. Virol. 75: 26.

Heil, A. and W. Zillig. 1970. Reconstitution of bacterial DNA-dependent RNA polymerase from isolated subunits as a tool for the elucidation of the role of the subunits in transcription. FEBS Letters 11: 165.

Howard, G. A., and R. R. Traut. 1973. Separation and radioautography of microgram quantities of ribosomal proteins by two-dimensional polyacrylamide gel electrophoresis. FEBS Letters 29: 177.

Howatson, A. F. 1970. Vesicular stomatitis and related viruses. Adv. Virus Res. 16: 195.

Huang, A. S., D. Baltimore, M. Stampfer. 1970. Ribonucleic acid synthesis of vesicular stomatitis virus. III. Multiple complementary messenger RNA molecules. Virol. 42: 946.

Ishihama, A. and K. Ito. 1972. Subunits of RNA polymerase in function and structure. II. Reconstitution of Escherichia coli RNA polymerase from isolated subunits. J. Mol. Biol. 72: 111.

Johnson, G. S., M. S. Kayne, W. C. Deal, Jr. 1969. Metabolic control and structure of glycolytic enzymes. VIII. Reversal of the dissociation of rabbit muscle pyruvate kinase into unfolded subunits. Biochem. 8: 2455.

Jovin, T., A. Chrambach, M. A. Naughton. 1964. An apparatus for preparative temperature-regulated polyacrylamide gel electrophoresis. Anal. Biochem. 9: 351.

Kacian, D. L., K. F. Watson, A. Burny, S. Spiegelman. 1971. Purification of the DNA polymerase of avian myeloblastosis virus. Biochim. Biophys. Acta 246: 365.

Keller, W. and R. Crouch. 1972. Degradation of DNA-RNA hybrids by ribonuclease H and DNA polymerases of cellular and viral origin. Proc. Nat. Acad. Sci., USA 69: 3360.

Lee, S. G., M. V. Miceli, R. A. Jungmann, P. P. Hung. 1975. Protein kinase and its regulatory effect on reverse transcriptase activities of Rous sarcoma virus. Proc. Nat. Acad. Sci., USA 72: 2945.

Leis, J. P., I. Berkower, J. Hurwitz. 1973. Mechanism of action of ribonuclease H isolated from avian myeloblastosis virus and Escherichia coli. Proc. Nat. Acad. Sci., USA 70: 466.

Levinson, W.J., J. M. Bishop, N. Quintrell, J. Jackson. 1970. Presence of DNA in Rous sarcoma virus. Nature 227: 1023.

Lill, I. J. and G. R. Hartmann. 1970. Reactivation of denatured RNA polymerase from Escherichia coli. Biochem. Biophys. Res. Commun. 39: 930.

Lill, U. J., E. M. Behrendt, G. R. Hartmann. 1975. Hybridization in vitro of subunits of the DNA-dependent RNA polymerase from Escherichia coli and Micrococcus luteus. Eur. J. Biochem. 52: 411.

Lowry, O. H., N. J. Rosebrough, A. L. Farr, R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193: 265.

Martini, O. and H. Gould. 1971. Enumeration of rabbit reticulocyte ribosomal proteins. J. Mol. Biol. 62: 403.

Moelling, K. 1974. Reverse transcriptase and RNase H: Present in a murine virus and in both subunits of an avian virus. Cold Spring Harbor Symp. Quant. Biol. 34: 969.

Moelling, K., D. P. Bolognesi, H. Bauer, N. Busen, H. W. Plassmann, P. Hausen. 1971. Association of the viral reverse transcriptase with an enzyme degrading the RNA moiety of RNA-DNA hybrids. Nature New Biol. 234: 240.

Nakata, Y. and J. P. Bader. 1968. Studies on fixation and development of cellular transformation by Rous sarcoma virus. Virol. 36: 401.

Obara, T., D. P. Bolognesi, H. Bauer. 1971. Ribosomal RNA in avian leukosis virus particles. Int. J. Cancer 7: 535.

Palm, P., A. Heil, D. Boyd, B. Grampp, W. Zillig. 1975. The reconstitution of Escherichia coli DNA-dependent RNA polymerase from its isolated subunits. Eur. J. Biochem. 53. 283.

Panet, A., I. M. Verma, D. Baltimore. 1974. Role of the subunits of the avian RNA tumor virus reverse transcriptase. Cold Spring Harbor Symp. Quant. Biol. 39: 919.

Panet, A., W. A. Haseltine, D. Baltimore, G. Peters, F. Harada, J. E. Dahlberg. 1975. Specific binding of tryptophan transfer RNA to avian myeloblastosis virus RNA-dependent DNA polymerase (reverse transcriptase). Proc. Nat. Acad. Sci., USA 72: 2535.

Papas, T. S., D. J. Marciani, K. Samuel, J. G. Chirikjian. 1976. Mechanism of release of active α subunit from dimeric $\alpha\beta$ avian myeloblastosis virus DNA polymerase. J. Virol. 18: 904.

Poiesz, B. J., G. Seal, L. A. Loeb. 1974. Reverse transcriptase: Correlation of zinc content with activity. Proc. Nat. Acad. Sci., USA 71: 4892.

Reisfield, R. A., U. J. Lives, D. E. Williams. 1962. Disk electrophoresis of basic proteins and peptides on polyacrylamide gels. Nature 195: 281.

Rho, H. M., D. P. Grandgenett, M. Green. 1975. Sequence relatedness between the subunits of avian myeloblastosis virus reverse transcriptase. J. Biol. Chem. 250: 5278.

Robinson, W. S. and M. A. Baluda. 1965. The nucleic acid from avian myeloblastosis virus compared with the RNA from the Bryan strain of Rous sarcoma virus. Proc. Nat. Acad. Sci., USA 54: 137.

Rous, P. 1911. A sarcoma of the fowl transmissible by an agent separable from the tumor cells. J. Exp. Med. 13: 397.

Salmeen, I., L. Rimai, L. Liebes, M. A. Rich, J. J. McCormick. 1975. Hydrodynamic diameters of RNA tumor viruses. Studies by laser beat frequency light scattering spectroscopy of avian myeloblastosis and Rauscher murine leukemia virus. Biochem. 14: 134.

Shapiro, A. L., E. Vinueala, J. V. Maizel. 1967. Molecular weight estimation of polypeptide chains by electrophoresis in SDS-polyacrylamide gels. Biochem. Biophys. Res. Commun. 28: 815.

Sharp, D. G., E. A. Eckert, D. Beard, J. W. Beard. 1952. Morphology of the virus of avian erythromyeloblastic leukosis and a comparison with the agent of Newcastle disease. J. Bacteriol. 63: 151.

Spiegelman, S., A. Burny, M. R. Das, J. Keydar, J. Schlom, M. Travnicek, K. F. Watson. 1970. Characterization of the products of RNA-directed DNA polymerases in oncogenic RNA viruses. Nature 227: 563.

Stehelin, D., R. V. Guntaka, H. E. Varmus, J. M. Bishop. 1976. Purification of DNA complementary to nucleotide sequences required for neoplastic transformation of fibroblasts by avian sarcoma viruses. J. Mol. Biol. 101: 349.

Stromberg, K., R. Gantt, S. Wilson. 1973. Structural studies on avian myeloblastosis virus: Conditions for isolation and biochemical characteristics of the core component. Biochim. Biophys. Acta 304: 1.

Stromberg, K. N. E. Hurley, N. L. Davis, R. R. Rueckert, E. Fleissner. 1974. Structural studies of avian myeloblastosis virus: Comparison of polypeptides in virion and core components by dodecyl sulfate polyacrylamide gel electrophoresis. J. Virol. 13: 513.

Tanford, C. 1968. Protein denaturation. Adv. in Protein Chemistry. 23: 121.

Taylor, J. M., A. J. Faras, H. E. Varmus, W. E. Levinson, J. M. Bishop. 1972. RNA-directed DNA synthesis by the purified DNA polymerase of Rous sarcoma virus: Characterization of the enzymatic product. Biochem. 11: 2343.

Temin, H. 1963. The effects of actinomycin D on growth of Rous sarcoma virus in vitro. Virol. 20: 577.

_____1964. Nature of the provirus of Rous sarcoma. Nat. Cancer Inst. Monogr. 17: 557.

_____1970. Malignant transformation of cells by viruses. Perspect. Biol. Med. 14: 11.

Temin, H. M. and S. Mizutani. 1970. RNA-dependent DNA polymerase in virions of Rous sarcoma virus. Nature (London) 226: 1211.

Varmus, H. E., W. E. Levinson, J. M. Bishop. 1971. Extent of transcription by the RNA-dependent DNA polymerase of Rous sarcoma virus. Nature New Biol. 233: 19.

Verma, I. M. 1977. The reverse transcriptase. Biochim. Biophys. Acta 473: 1.

Verma, I. M., N. L. Meuth, D. Baltimore. 1972. The covalent linkage between RNA primer and DNA product of the avian myeloblastosis virus DNA polymerase. J. Virol. 10: 622.

Vigier, P. and A. Golde. 1964. Effects of actinomycin D and of mitomycin C on the development of Rous sarcoma virus. Virol. 23: 511.

Warburg, O. and W. Christian. 1942. Isolierung und kristallisation des garungsferments enolase. Biochem Zeit 310: 384.

Waters, L. C., B. C. Mullin, T. Ho, W. K. Yang. 1975. Ability of tryptophan tRNA to hybridize with 35S RNA of avian myeloblastosis virus and to prime reverse transcription in vitro. Proc. Nat. Acad. Sci., USA 72: 2155.

Yarbrough, L. R. and J. Hurwitz. 1974. The reversible denaturation of deoxyribonucleic acid-dependent ribonucleic acid polymerase of Escherichia coli. J. Biol. Chem. 249: 5394.