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HYDROPHOBIC INTERACTION OF RETROVIRAL DNA POLYMERASES WITH ALKYL-AGAROSE MATRICES

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SUMMARY: The chromatographic behavior of DNA polymerase activity in solubilized preparations of Rauscher murine leukemia virus (R-MuLV), murine mammary tumor virus (MuMTV), Mason-Pfizer monkey virus (MP-MV), and avian myeloblastosis virus (AMV) was examined on agarose derivatives containing hydrocarbon arms of various lengths. Ethyl-(C_2)- and hexylimino-(C_6)-agarose failed to bind significant quantitites of DNA polymerase activity. R-MuLV DNA polymerase bound to octylimino-(C_8)- and decylimino-(C_{10})-agarose, and could be quantitatively recovered from these matrices by elution with buffers containing ethylene glycol alone and in the presence of detergents, respectively. The other viral polymerases demonstrated equal or greater affinities for these matrices. The potential for hydrophobic interaction by viral DNA polymerases revealed by our studies provides a mechanism whereby the specific complexing of reverse transcriptase with retroviral structural proteins in the absence of RNA may be explained.

INTRODUCTION

The interaction of proteins with immobilized hydrocarbon chains of various lengths has been termed "hydrophobic chromatography" (1,2). Recent studies (2,3) have shown that only those hydrocarbon chains terminating in apolar head groups provide optimal conditions for true hydrophobic interaction of such insolubilized ligands with certain proteins. In addition to providing a new method for the purification of these polypeptides (1,3), such experiments may also be used to infer the presence of surface hydrophobic regions on proteins, and have recently provided indications that similar regions may be present on interferon molecules from different species (3). We have recently shown (4) that the structural proteins of type B and type C mammalian retroviruses possess several different degrees of hydrophobicity. By examining the

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chromatographic behavior of these proteins on alkyl-agarose matrices, we were able to define four categories of hydrophobic interaction (relative hydrophobicity) which could be correlated with the structural roles played by such proteins. In this paper we describe the first study of the hydrophobic interaction of DNA polymerases from the mammalian type C Rauscher murine leukemia virus (R-MuLV), the avian type C myeloblastosis virus (AMV), the type B murine mammary tumor virus (MuMTV), and the type D Mason-Pfizer monkey virus (MP-MV) with alkyl-agarose matrices. Although these DNA polymerases are unrelated on the basis of immunologic cross-reactivity (5,6), they appear to possess degrees of hydrophobicity equal to or greater than virion structural ectoproteins (7). This observation may be significant in light of recent findings of protein-protein interactions among retroviral DNA polymerases and structural proteins.

MATERIALS AND METHODS

Reagents. Purified preparations of R-MuLV, MP-MV, and AMV were obtained through the courtesy of the Virus Cancer Program. MuMTV purified from tissue culture supernatant fluid was the generous gift of Dr. Nurul H. Sarkar of this Institute. (^{14}C)-amino acids mixture was purchased from New England Nuclear, Inc., and R-MuLV uniformly labeled with the (^{14}C)-amino acids was prepared as described previously (4). (^{3}H)-dTTP was purchased from Amersham, Inc. Alkyl-agarose matrices were obtained from Miles Laboratories, Inc. Different lots of each matrix were found to yield identical results.

Hydrophobic Chromatography of Solubilized Virions. Alkyl-agarose derivatives were poured into Bio-Rad disposable glass columns to yield a final bed volume of 1.5 - 2 mls. Columns were equilibrated at 4°C with wash buffer containing 0.05 M Tris-HCl, pH 7.8, 1 mM dithiothreitol, 10% (v/v) glycerol, and 1 M KCl. Unlabeled virus or (^{14}C)-labeled virions to which 250 µg of unlabeled virus protein had been added were solubilized by the addition of an equal volume of 2X concentrated buffer to yield a solution with the following final concentrations of components: 1% (v/v) nonionic detergent P-40 (NP-40), 0.5% (w/v) sodium deoxycholate, 10 mM dithiothreitol, 0.4 M KCl, 10% (v/v) glycerol and 50 mM Tris-HCl, pH 7.8. The mixture was then allowed to stand at 0°C for approximately 15 minutes and then centrifuged at 10,000 xg at 4°C for 20 minutes. The solubilized virus solution was then diluted 10-fold with column wash buffer and applied to the alkyl-agarose columns at a flow rate of approximately 10 mls/hour. All column chromatography steps were performed at 4°C. While the material was entering the column and during subsequent elution steps or washes, fractions of 2 ml volume were collected. The first elution buffer contained 8.5 M ethylene glycol in wash buffer, and column flow rates were increased to approximately 18 mls/hour. Following this elution step, an additional elution was carried out with detergent-containing buffer (1% NP-40, 0.5% sodium deoxycholate) in 8.5 M ethylene glycol elution buffer. The

quantity of protein present in those column runs containing (^{14}C)-amino acids-labeled virus was monitored by taking 50 µl aliquots of each fraction and determining trichloroacetic acid-precipitable radioactivity, as previously described (4).

DNA Polymerase Assays. Reactions were carried out in a total volume of 0.1 ml and consisted of 50 mM Tris-HCl, pH 7.8, 1 mM dithiothreitol, and 10 µg of bovine serum albumin monomer together with poly (A)·$(dT)_{12-18}$ as template-primer at a concentration of 5 µg/ml. Divalent cation concentrations for reactions catalyzed by AMV, MuMTV, and MP-MV DNA polymerases were 10, 2, and 5 mM $MgCl_2$, respectively, while for R-MuLV DNA polymerase 0.5 mM $MnCl_2$ was used (8,9). (^3H)-dTTP was added together with unlabeled substrate to yield a final concentration of 22 µM with a specific activity of approximately 1,000 cpm/pmole. Reactions were initiated by the addition of reaction mixture to enzyme fractions, and terminated by the addition of cold trichloroacetic acid solution containing sodium pyrophosphate following a 30-minute incubation at 37°C. Acid-insoluble material was collected by vacuum filtration onto Whatman glass fiber filters (GF/B). After drying, the filters were placed into toluene-based scintillation fluid and counted in a Packard counter.

RESULTS

Alkyl-agarose derivatives were used for chromatography of solubilized virions rather than ω-amino alkyl-agarose derivatives, since the presence of the terminal amino group in the latter matrix introduces the possibility of ionic interactions (2). In an attempt to further minimize ionic interactions which might produce nonspecific adsorption to matrices, all buffers contained 1 M KCl. Under the conditions which were used, significant quantities of R-MuLV polypeptides (4) or DNA polymerase activity failed to adsorb to ethylimino (C_2)-agarose or hexamethylimino (C_6) agarose (data not shown). Agarose derivatives containing longer hydrocarbon chains did, however, bind significant quantities of R-MuLV proteins, and representative chromatographic profiles are shown in Figure 1. All of the applied R-MuLV DNA polymerase bound to the (C_8) agarose column, while 15% of the labeled viral protein was found in flow through fractions. The proteins present in the (C_8) agarose column wash fractions are the core shell protein, p30, and the low molecular weight structural proteins, p10 and p12 (4). By washing the column with buffer containing 8.5 M ethylene glycol, ≈ 90% of the adsorbed DNA polymerase activity was recovered, together with the virion ectoprotein gp 69/71 and the p15 polypeptide (4). Further washing of the column with buffer containing 8.5 M

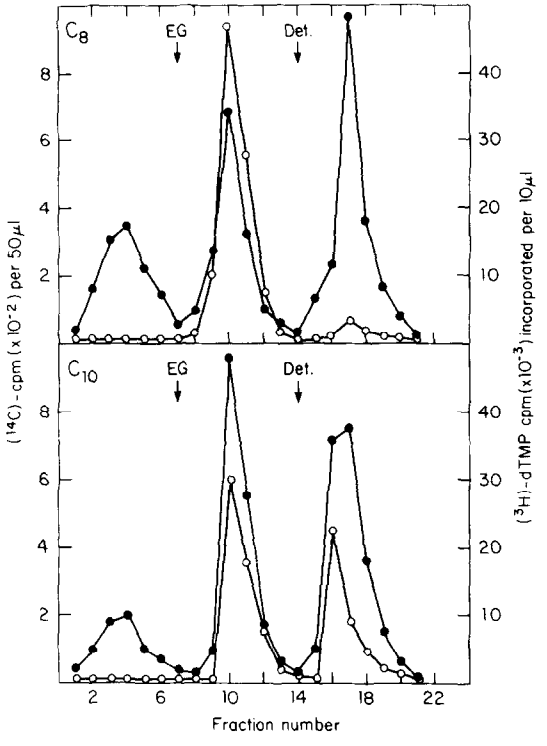

Figure 1: Binding of (^{14}C)-R-MuLV polypeptides (•) and DNA polymerase activity (o) to (C_8)-agarose and (C_{10})-agarose. Approximately 1.5 to 2 X 10^6 (^{14}C)-cpm were applied to each column. Chromatography was carried out and the amount of labeled protein and DNA polymerase activity in flow-through and eluate fractions determined as described in Materials and Methods. Arrows indicate the addition of buffers containing: EG, 8.5 M ethylene glycol, or Det, 1% (v/v) NP-40 + 0.5% (w/v) sodium deoxycholate in column wash buffer + 8.5 M ethylene glycol.

ethylene glycol together with detergents removed an additional quantity of polymerase activity $\leq$ 5% of the total input activity. The detergent-containing buffer has proven effective for the removal of strongly hydrophobic virion structural proteins, such as the intramembrane protein p15(E), from alkyl-agarose matrices (4). The strength of adsorption of a protein to alkyl-agarose matrices has been found to increase as the hydrocarbon arm extending from the matrix is lengthened (1,3). In the case of R-MuLV protein interaction with (C_{10})-agarose, it may be seen that less structural protein is present in this flow-through than was observed with the (C_8)-agarose matrix. Additional-

ly, the 8.5 M ethylene glycol wash removed only 50 - 55% of adsorbed DNA polymerase acticity. Detergent together with ethylene glycol was necessary to accomplish removal of the remaining adsorbed enzyme activity.

In order to further examine possible differences in the "tightness" of R-MuLV DNA polymerase binding to the (C_8)- and (C_{10})-agarose matrices, we tested the ability of different concentrations of ethylene glycol to remove adsorbed enzyme activity from both matrices (Figure 2). Columns were run and elution attempted using step gradients of ethylene glycol in column wash buffer, the concentration of which was increased in 2 M increments up to a maximum of 8M. The lowest concentration of ethylene glycol tested (2 M) was sufficient to remove 80 - 85% of bound polymerase activity from the (C_8)-agarose column, although 5 - 10% of the activity was removed by a final wash with detergent. Surprising results were obtained from the same study carried out with (C_{10})-agarose. No significant elution of adsorbed enzyme activity occurred at any concentration of ethylene glycol added to the column, but elution was accomplished by the use of ethylene glycol + detergent-containing buffer. This experiment clearly shows that R-MuLV DNA polymerase bound with greater avidity to (C_{10})-agarose than to (C_8)-agarose. The enzyme activity eluting from (C_{10})-agarose following the 8.5 M ethylene glycol wash (Fig. 1) might result from "shocking" adsorbed enzyme by immediate exposure to a high concentration of apolar solvent. A gradual increase in ethylene glycol concentration, as shown in Figure 2, therefore, may not allow for a sufficiently drastic environmental change to effect elution.

Using methods identical to those described above, we have examined the chromatographic behavior of DNA polymerases from solubilized MuMTV, MP-MV, and AMV on (C_8)- and (C_{10})-agarose. Although they represent different retroviral types, the DNA polymerases from these sources behaved in a similar manner (Table 1). None of the DNA polymerase activities bound to (C_6)-agarose to any significant degree, although all bound to (C_8)- and (C_{10})-agarose. For all these other viral polymerases, $\leq$ 50% of input activity was recovered from

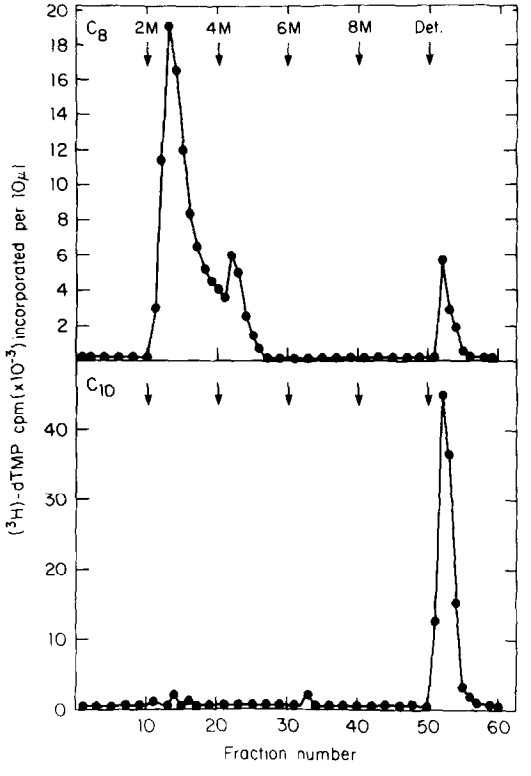

Figure 2: Elution of R-MuLV DNA polymerase activity from (C_8)-agarose and (C_{10})-agarose columns by increasing concentrations of ethylene glycol. Approximately 200 µg of solubilized viral protein were applied to each column. Arrows indicate the addition of buffer containing the indicated concentrations of ethylene glycol in column wash buffer. Fractions of 1 ml volume were collected, and assayed as described in Materials and Methods.

the alkyl-agarose columns following adsorption. Furthermore, buffer containing detergent did not remove more enzyme activity from the columns than had been eluted by ethylene glycol-containing buffer. In these two respects, the chromatographic behavior of MuMTV, MP-MV, and AMV DNA polymerases differed from that of R-MuLV. Control experiments using solubilized virus as well as purified enzymes established that the decreased recovery observed was not due to inhibition by buffer contents at the final concentrations found in the assays. Additional DNA polymerase activity was not recovered following elution with higher ethylene glycol concentrations, extremely low ionic-strength

Table 1

Interactions of Retroviral DNA Polymerases With Alkyl-Agarose Matrices

Matrix	Fractions	Per Cent Recovery of Input DNA Polymerase Activity[a] From: R-MuLV	MuMTV	MP-MV	AMV ($\alpha\beta$)
(C_6)-agarose	Flow-through	100	100	100	100
(C_8)-agarose	Flow-through	< 1	< 1	< 1	< 1
	Ethylene glycol	88	32	50	12
	Detergent + Ethylene glycol	5	< 1	< 1	< 1
(C_{10})-agarose	Flow-through	< 1	< 1	< 1	< 1
	Ethylene glycol	55	23	30	1.8
	Detergent + Ethylene glycol	35	< 1	< 1	< 1

[a]One unit of enzyme activity is defined as that amount of enzyme catalyzing the incorporation of 1 picomole of (^{3}H)-dTMP into acid-insoluble material in poly (A)·$(dT)_{12-18}$-directed synthesis in 30 minutes at 37°, using conditions given in Materials and Methods.

Input units of enzyme activity: R-MuLV = 22,000; MuMTV = 30,000; MP-MV = 10,000; AMV ($\alpha\beta$ enzyme form) = 100,000. Column runs and assays were carried out as described in Materials and Methods.

buffer (10), or tetraalkylammonium salts (3). As with the R-MuLV enzyme, the other retroviral DNA polymerases bound tighter to (C_{10})-agarose than to (C_8)-agarose.

DISCUSSION

The interaction of retroviral DNA polymerases with alkyl-agarose matrices has been examined for the first time. That binding, when it was observed, was dependent upon hydrophobic interactions is suggested by the following criteria: a) binding took place in the presence of high salt (1 M KCl) concentrations; b) increasing the length of the hydrocarbon chain increased

the strength of the observed binding; and c) hydrophobic eluting agents, such as ethylene glycol and/or detergents, could be used to recover enzyme activity as well as virion structural proteins. We have recently described the interaction of R-MuLV and MuMTV structural proteins with alkyl-agarose matrices (4). By determining the behavior of viral proteins binding to (C_8)- and (C_{10})-agarose columns, and the conditions needed to elute bound proteins, criteria were set up to determine the potential for hydrophobic interaction (relative degrees of hydrophobicity) of the viral polypeptides. Four degrees of apparent hydrophobicity, from least hydrophobic to strongly hydrophobic, could be determined by this procedure. The least hydrophobic proteins of R-MuLV were found to be the p10 and p12 polypeptides, while the strongly hydrophobic protein was found to be the membrane-associated polypeptide, p15(E). The ectoprotein of R-MuLV, gp 69/71, was found to be moderately hydrophobic. By our criteria, the R-MuLV DNA polymerase exhibits an even greater potential for hydrophobic interaction than the virion ectoprotein, suggesting a greater number of available surface hydrophobic regions. This is in contrast to the other virion RNA-binding proteins p10 and p12 (11), which are the least hydrophobic of the virion polypeptides (4). Although the degree of hydrophobicity of virion structural proteins appeared to correlate with their structural roles, it would at first appear difficult to account for the surface hydrophobic regions apparently present on virion DNA polymerase.

The major internal R-MuLV polypeptide, p30, has recently been shown to bind specifically to virion DNA polymerase *in vitro* (12), forming a heteropolymeric complex. Gerwin and Levin (13) have also very recently shown that MuLV DNA polymerase is packaged normally into virions lacking genomic RNA molecules. These two reports suggest that, in the virion, reverse transcriptase may not be functionally associated with virion RNA, but may recognize structural proteins and participate in virion assembly through protein-protein interactions. Our analysis of the hydrophobic interactions of R-MuLV DNA polymerase with alkyl-agarose matrices yielded results which suggest that it

may interact with virion structural proteins via such a hydrophobic attraction.

Results similar to those obtained with R-MuLV DNA polymerase, except for lower degrees of recovery, were observed for the MuMTV, MP-MV, and AMV DNA polymerases. Of these enzymes, the lowest recovery from the hydrophobic matrices was observed with the AMV polymerase. It is interesting to note that this is the only one of the retroviral polymerases known to be a two-subunit enzyme (6), and is also the largest (in molecular weight) among those enzymes we have studied. For these reasons, it may possess more accessible hydrophobic regions than the other enzymes. Preliminary studies carried out in our laboratory with the purified α and $\alpha\beta$ forms of AMV polymerase indicate that even the low-molecular-weight α subunit (60,000 daltons) is very strongly hydrophobic, and may be associated with the β subunit through apolar bonding. Our studies, therefore, support recent evidence that retroviral DNA polymerase is capable of interaction with structural proteins (12,13) and provides a mechanism whereby such interaction may occur.

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