PRECIPITATION OF NUCLEIC ACIDS WITH CETYLTRIMETHYLAMMONIUM BROMIDE: A METHOD FOR PREPARING VIRAL AND CELLULAR DNA POLYMERASE PRODUCTS FOR CESIUM SULFATE DENSITY GRADIENT ANALYSIS*

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SUMMARY

Precipitation with cetyltrimethylammonium bromide followed by precipitation with ethanol:salt provides a quick, simple, and effective method for preparing labelled nucleic acid products of DNA polymerase reactions for analysis by Cs_2SO_4 equilibrium density gradient centrifugation. Results obtained using this treatment show that this approach is superior to other methods of preparing products of endogenous DNA polymerase reactions from relatively crude particulate fractions such as disrupted virions.

The detection of RNA-DNA hybrid molecules from endogenous DNA polymera reactions constitutes one good indication of the presence of an RNA-directed DNA polymerase (reverse transcriptase). However, in analyzing radiolabelled nucleic acids produced in "endogenous" DNA polymerase reactions, we have foun it difficult in many cases (especially with crude enzymes) to obtain meaningf data with Cs_2SO_4 equilibrium density gradients, a technique routinely employe in the analysis of products of the virion DNA polymerase system. Endogenous DNA polymerase systems are, by their nature, not highly purified. Our results have been frequently obscured by the presence of artifacts which are of two main types. The first is the complete or partial disappearance of bands with a density characteristic of DNA (~1.43 g/cc) after heat or alkali treatment, neither of which should degrade DNA. The second consists of material banding in the density regions of RNA (~1.60 g/cc) or RNA-DNA hybrids (>1.5 g/cc) which is unaffected by heat or alkali. Any DNA attached

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to an RNA molecule should of course be shifted to the density region of DNA after alkaline hydrolysis of the RNA. We have been unable to eliminate these anomalies by either phenol:cresol extractions alone (1) or in combination with precipitation in 70% ethanol: 0.3 M NaCl. We have also found numerous other treatments, such as incubation with pronase plus SDS or purification of DNA product by glycerol velocity gradients, to be ineffective.

These artifacts probably arise from contamination with proteins, particularly basic proteins. We therefore sought a method which would separate basic proteins from nucleic acids. We find that DEAE-cellulose chromatography effectively removes "artifacts" from DNA polymerase products, supporting the idea that the presence of basic proteins is responsible for these artifacts. However, DEAE-cellulose binds high molecular weight nucleic acids (e.g., 70S RNA from RNA tumor viruses) too tightly for subsequent recovery except by methods which result in at least partial destruction (alkali or high concentrations of urea). A more applicable method which separates basic proteins from nucleic acids was needed.

One method for nucleic acid purification involves coprecipitation with the quarternary amine, cetyltrimethylammonium bromide (CTAB) (2). Ralph and Bellamy (3,4) claim that coprecipitation with CTAB effectively removes nucleases and other basic proteins; the CTAB can then be removed by dissolving the precipitate in 1 M NaCl and adding three volumes of ethanol. The free nucleic acids are collected as a precipitate.

Products of crude or partially purified DNA polymerases from two RNA tumor viruses were purified by the CTAB method or by repeated phenol:cresol extraction (with and without ethanol:salt precipitation). In this report we show by comparison of Cs_2SO_4 equilibrium density gradient centrifugation analyses of these products that the CTAB method is much superior to the alternative method.

MATERIALS AND METHODS

Preparation of DNA Polymerases

Chicken plasma infected with avian myeloblastosis (AMV) was kindly

supplied by Dr. J. Beard, Duke University. The virus was purified as described previously (5). The purified pelleted virus was suspended in Triton-KCl buffer (0.05 M Tris·HCl, pH 7.9, 0.05 M NaCl, 0.25% Triton X-100, 0.5 M KCl, 0.02 M DTT, and 0.001 EDTA), stirred at 0° for two hours, and centrifuged (30,000 x g; 30 minutes). The pellet was resuspended in Triton-KCl buffer, sonicated as above, and again centrifuged (30,000 x g; 30 minutes). The 30,000 x g supernatant solutions were combined, dialyzed against buffer (0.05 M Tris·HCl pH 7.9, 0.001 M EDTA, 0.001 M DTT and 5% glycerol) and stored at -20°. The dialysate was the enzyme source. Rauscher leukemia virus (RLV) (10^{11}-10^{12} particles/ml) was purchased from Electro-Nucleonics, Bethesda, and made 0.03% in Triton X-100. This was used as the enzyme source without further purification.

DNA Polymerase Reactions

AMV DNA polymerase products were prepared in 0.5 ml of a reaction mixture containing 0.25 ml enzyme preparation and 0.05 M Tris·HCl, pH 7.8, 0.005 M DTT, 0.005 M $MgCl_2$, 0.04 M KCl, 8 x 10^{-4} M dATP, dCTP, and dGTP, and containing 0.6 mC ^{3}H-TTP (New England Nuclear, 14.6 C/mM). The reaction was terminated after 30 minutes. RLV DNA polymerase products were prepared similarly, except 1 ml of enzyme was used in a total volume of 2 ml and the reaction terminated after 10 minutes.

Preparation of DNA Products

Reactions were terminated by addition of sodium dodecyl sulfate to a concentration of 1% and adjustment of the NaCl concentration to 0.12 M. These solutions were twice extracted with a half volume of phenol:cresol (50 g phenol, 7 ml m-cresol, 5 ml H_2O, 0.1% 8-quinolinol) (1) and twice with two volumes diethyl ether. Residual ether was removed by bubbling air through the final aqueous phase. DNA purified to this step is hereafter referred to as phenol:cresol-treated product.

A portion of each product was further treated by addition of 0.5 mg yeast tRNA (Worthington) and 150 µg calf thymus DNA (Worthington) and precipitation overnight at -20° in 70% ethanol:0.3 M NaCl. The precipitate was collected by centrifugation (25,000 x g; 10 minutes) and dissolved in buffer (0.01 M Tris·HCl, pH 7.2, 0.4 M NaCl, and 0.001 M EDTA). This is referred to as ethanol:salt-treated product.

Another portion of each product was treated by adding carrier DNA and RNA as above, followed by slow addition of 25 µl 0.1 M cetyltrimethylammonium bromide (Eastman Organic Chemicals), precipitation on ice for 20 minutes, and collection by centrifugation (15,000 x g; 10 minutes). The pellet was dissolved in 1 ml 1 M NaCl, three volumes of ethanol were added, and the nucleic acids precipitated for at least 2 hours at -20° and collected by centrifugation (25,000 x g; 10 minutes). The precipitate was dissolved in buffer (0.01 M Tris·HCl, pH 7.2, 0.001 M EDTA, and 0.4 M NaCl). This preparation is referred to as CTAB-treated product.

Cs_2SO_4 Density Gradient Centrifugation

Samples were diluted to 1 ml with TNE buffer (0.01 M Tris·HCl, pH 7.2, 0.001 M EDTA, and 0.1 M NaCl) and added along with 75 µg calf thymus DNA to 4 ml of a solution of 1 g Cs_2SO_4 (Schwarz/Mann, optical grade) per ml TNE buffer. These solutions were centrifuged in polyallomer tubes in a SW 50.1 rotor for 64-68 hours at 35,000 rpm. Approximately 30 fractions were collected from each gradient and the densities measured by refractive index with a Bausch and Lomb Abbe refractometer. Fifty µg yeast tRNA was added to each fraction and the nucleic acids precipitated with 2 ml 10% trichloroacetic acid. The samples were then collected on Millipore membrane filters (HAWP 025), dried, and counted in Liquifluor:toluene (40 ml/l) scintillation fluid.

RESULTS

Endogenous DNA Product of AMV Reverse Transcriptase

Phenol:cresol-treated endogenous product of AMV DNA polymerase

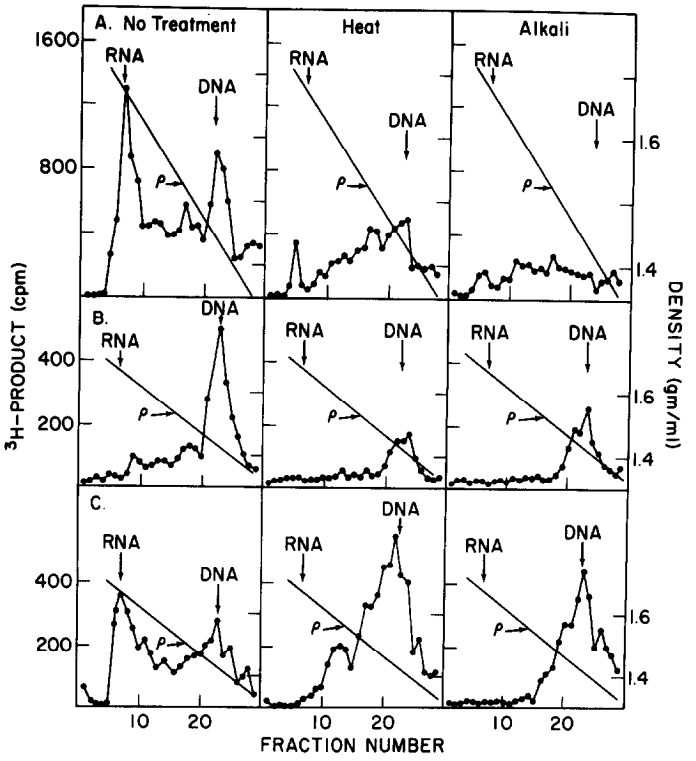

Figure 1. Cs_2SO_4 density gradient analysis of endogenous DNA product from AMV reverse transcriptase. Product was prepared and purified as described under Materials and Methods. Left panels show native product, center panels show heat-denatured product, and right panels show alkali-denatured product. A., phenol:cresol-treated product; B., ethanol:salt-treated product; C., CTAB-treated product.

contains radiolabelled material banding as a RNA-DNA hybrid (Figure 1a). This material, however, is not shifted to the DNA density region after heat and alkali treatment; most of the acid-precipitable label simply disappears. It is impossible to interpret these results. Ethanol:salt-treated product apparently contains no hybrids (Figure 1b). It is unclear why this product should lack the "hybrid" peak found in phenol:cresol-treated product. This peak is either artifactual and removed by ethanol precipitation or a true hybrid destroyed during the ethanol treatment. Heat and alkali treatment still drastically reduce the total acid-precipitable radioactivity. In contrast, CTAB-treated product contains a band of labelled material in the density region of RNA (Figure 1c) which is shifted completely to the DNA

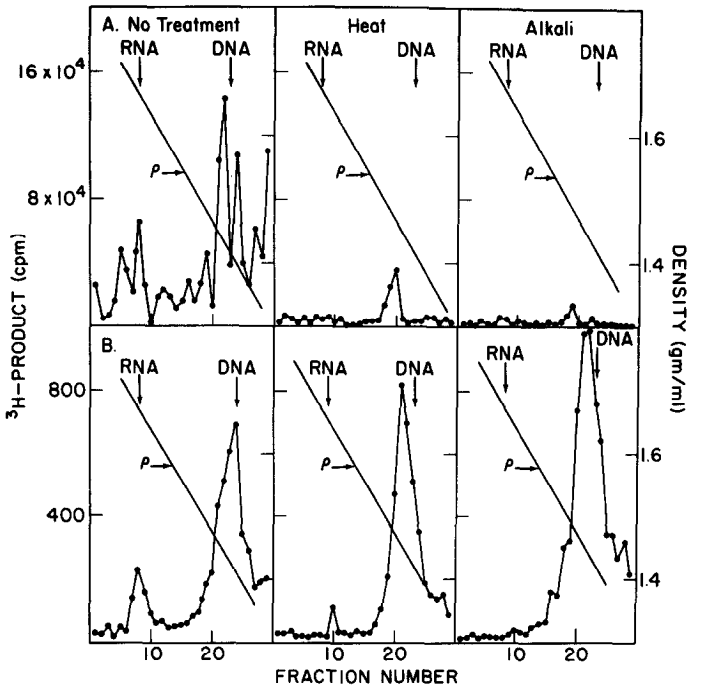

Figure 2. Cs_2SO_4 density gradient analysis of endogenous DNA product from RLV reverse transcriptase. Product was prepared and purified as described under Materials and Methods. Left panels show native product, center panels show heat-denatured product, and right panels show alkali-denatured product. A., ethanol:salt-treated product; B., CTAB-treated product.

region by alkali treatment, and moved to a density intermediate between DNA and RNA by heat treatment. This agrees with the results presented by Verma et al. (6) indicating a covalent link between RNA primer and DNA product of AMV reverse transcriptase.

Endogenous DNA Product of RLV Reverse Transcriptase

Endogenous RLV product, analyzed after ethanol:salt precipitation, gives an uninterpretable pattern from Cs_2SO_4 gradients (Figure 2a). Most acid-precipitable radioactivity is again lost upon treatment by heat or alkali; furthermore, the peak remaining after alkali treatment is denser than DNA. However, CTAB-treated product (Figure 2b) clearly contains an RNA-DNA hybrid, as evidenced by a band with the density of RNA which is partly shifted to the DNA region by heat treatment and is completely

alkali-sensitive. Furthermore, no acid-precipitable label is lost upon heat or alkali treatment of CTAB-treated RLV product. CTAB treatment yields far less acid precipitable label than does ethanol:salt treatment from equivalent fractions. It seems likely that this difference represents artifacts removed from DNA product by CTAB treatment.

DISCUSSION

Endogenous DNA polymerase product from two RNA tumor viruses (AMV and RLV) contains artifacts which are removed by CTAB treatment. Most of the acid-precipitable radioactivity disappears from phenol:cresol- or salt: ethanol-treated AMV product upon heat or alkali treatment. Only after CTAB treatment is the presence of a RNA-DNA hybrid (and evidence of reverse transcriptase) clearly evident. Furthermore, alkali and heat treatment bring little or no loss of radioactivity. Ethanol:salt-treated RLV product, although containing apparent hybrids, lost most acid-precipitable radioactivity upon heat or alkali treatment; furthermore, the material that remains is denser than DNA. The presence of a RNA-DNA hybrid is again evident after CTAB treatment, and the label is heat and alkali stable. From these results, it is obvious that treatment of DNA polymerase products with CTAB gives much clearer results from Cs_2SO_4 density gradient analysis than the other treatments tested. We have also found this to be true in the analysis of an endogenous RNase sensitive DNA polymerase system present in a cytoplasmic particulate fraction of human leukemic cells (7).

CTAB treatment probably minimizes loss of acid-precipitable label by elimination of contaminating nucleases. Our results therefore are in keeping with the finding of Ralph and Bellamy (3,4) that precipitation with CTAB is effective in reducing nuclease contamination from nucleic acids. CTAB precipitation also eliminates labelled material banding as RNA-DNA hybrids which are alkali-insensitive. The exact nature of these artifacts is not clear, but they may be due to coprecipitation of protein, nucleic

acids, and labelled nucleoside triphosphates and short oligonucleotides. Ralph and Bellamy show that CTAB precipitation also separates nucleoside phosphates from nucleic acids (3,4), and we have found CTAB treatment to remove over 99.5% of non-precipitable label from DNA polymerase products (data not shown).

Another method for purification of DNA polymerase products which also appears to give satisfactory results is gel filtration with Sephadex G-50 (6,8,9). However, the CTAB method offers several advantages over gel filtration, namely that more samples may be processed at one time, less equipment is required, and deproteinization is more complete. Where very high purification is needed, a combination of the two methods should be the best approach. CTAB treatment, because of its simplicity and effectiveness, should prove of general value in preparing DNA polymerase products for density gradient analysis, and particularly in screening crude endogenous DNA polymerase systems for RNA-DNA hybrids.

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