Purification of Adenovirus Messenger Ribonucleic Acid by an Aqueous Polymer Two-Phase System†

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ABSTRACT: An aqueous polymer phase system containing 6.3% (w/w) dextran and 3.5% (w/w) poly(ethylene glycol) in 10 mM phosphate buffer (pH 8.0) was developed to select RNA–DNA hybrids from unhybridized RNA. The top phase of this phase system, which contains DNA and the RNA–DNA hybrids, can be used to purify adenovirus messenger RNA both early and late in the infectious cycle. The hybrids can be melted by heat in the top phase and the messenger RNA selected by oligo(dT)cellulose chromatography whereupon the polymers and the DNA percolate and the polyadenylated messenger RNA absorb to the column. The isolated messenger RNA appears to be almost quantitatively recovered at a purity from 70 to 90% depending on the concentration of the specific messenger RNA in the starting material. Early and late viral messenger RNA were selected on the complementary strands of adenovirus DNA according to this procedure.

An efficient method to separate DNA–RNA hybrids from nonhybridized RNA is required in order to select specific messenger RNA (mRNA)[1] by hybridization to defined sequences of DNA. Coupled with an in vitro translation system, the purified mRNA may be used to identify the gene product of a specific DNA sequence. Several gene products expressed early and late in the infectious cycle were recently localized on the adenovirus type 2 (Ad2) genome using specific DNA fragments generated by restriction endonucleases (Lewis et al., 1975, 1976). Hydroxylapatite chromatography (HAP) in a urea phosphate buffer was used in this study to separate the DNA–RNA hybrids from unhybridized RNA (Smith et al., 1974) and oligo(dT)cellulose chromatography was applied to recover the mRNA free from viral DNA (Aviv and Leder, 1972). This HAP system does not in our hands separate hybrids formed between single-stranded DNA and RNA from unhybridized RNA, although separation may be achieved with mixed hybrids containing DNA–DNA and DNA–RNA stretches. We have therefore introduced a different approach for the initial separation of DNA–RNA hybrids from unhybridized RNA. Polymer two-phase systems containing a mixture of dextran and poly(ethylene glycol) have been used to separate high-molecular-weight DNA from RNA (Albertsson, 1965; Öberg et al., 1965). Under certain conditions, the partition coefficient of single-stranded DNA is strikingly different from that of double-stranded DNA (Albertsson, 1962; Alberts, 1967). In this report, we have adopted the dextran–poly(ethylene glycol) two-phase system to separate adenovirus DNA–RNA hybrids from unhybridized RNA. The RNA from the hybrid was then melted by heat and purified by oligo(dT)cellulose chromatography, taking advantage of the fact that the majority of the adenovirus mRNA contains poly(adenylic acid) (Lindberg et al., 1972). The mRNA isolated can readily be translated in vitro in the wheat embryo system.

Materials and Methods

Preparation of RNA. RNA from adenovirus type 2 (Ad2) infected HeLa cells was used throughout. The cultivation and infection of cells in suspension cultures have been described (Lindberg et al., 1972). At appropriate times after infection, cells were labeled with [^{3}H]uridine and cytoplasm was prepared and RNA extracted according to procedures already described (Lindberg et al., 1972; Öberg and Philipson, 1969). To obtain poly(A)-containing [^{3}H]RNA, oligo(dT)cellulose chromatography was used (Aviv and Leder, 1972). In addition to washing with elution buffer containing 0.5 M NaCl, the columns were also washed with 0.25 M NaCl in elution buffer before the poly(A)-containing RNA was eluted with 1 mM EDTA in 10 mM Tris-HCl (pH 7.9).

To obtain viral RNA synthesized before the onset of viral DNA replication (early RNA), cells were infected with Ad2 (10^4 virions/cell) and cycloheximide was added to a final concentration of 25 μg/ml at 2 h after infection. Cells were labeled with [^{3}H]uridine (20 μCi/ml, specific activity 20 Ci/mmol) for 5 h before harvesting the cells at 8 h after infection. To obtain viral RNA synthesized at intermediate times of the productive infection cells were concentrated to about 3×10^6 cells per ml and labeled with [^{3}H]uridine (12.5 μCi/ml) at 6 h after infection. The cells were harvested at 10.5 h after infection. Late viral RNA was prepared by labeling cells infected with a multiplicity of 2000 virions/cell between 17 and 20 h after infection (20 μCi/ml).

Preparation of DNA. Ad2 DNA was obtained from purified virions as previously described (Tibbetts et al., 1973). Complementary strands of Ad2 DNA were separated by binding denatured DNA to poly(U, G) followed by CsCl density gradient centrifugation (Tibbetts et al., 1974). The heavy (h) and the light (l) strands of viral DNA obtained by this technique have been defined (Tibbetts et al., 1974). The strand preparations were dialyzed against 1 mM EDTA–2% formamide in 10 mM Tris-HCl (pH 7.9) in collodium bags before they were used in hybridization experiments. DNA was also isolated from the staphylococcal phage ϕ11 as previously described

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[1] Abbreviations used: mRNA and tRNA, messenger and transfer RNA, respectively; HAP, hydroxylapatite; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; C_0t = concentration of DNA multiplied by the time of hybridization, a parameter used to define the complexity of DNA in hybridization reactions; PEG, poly(ethylene glycol).

(Sjöström et al., 1973) and used to assess unspecific hybridization.

The Aqueous Polymer Two-Phase System. Dextran T500 [16.8% (w/w); Pharmacia] and 9.2% (w/w) poly(ethylene glycol) 6000 (Union Carbide) in 1 mM EDTA were used as stock solution. The mixture was boiled for 10 min and kept agitated in the cold room. Samples of nucleic acids were resuspended or extensively diluted with 10 mM sodium phosphate of varying pH. Appropriate volumes of the two-phase stock solution were added and the mixtures were inverted 40–50 times and the two phases were separated by low-speed centrifugation for 3 min (Alberts, 1967). The amount of radioactively labeled nucleic acid in each phase was determined after Cl_3CCOOH precipitation and collection of the precipitate onto nitrocellulose filters. Since the volumes of the two phases are almost identical, the partition coefficient is expressed as the total amount of radioactivity in the top phase over that of the bottom phase.

Hybridization of Nucleic Acids. The DNA was fragmented either by boiling for 20 min in 0.3 M NaOH followed by neutralization or by sonication and heat denaturation (100 °C for 10 min). All techniques used to fragment and denature adenovirus DNA have been reported previously (Tibbetts et al., 1973, 1974). The denaturation step was omitted with separated strands of adenovirus DNA. The DNA was mixed with oligo(dT) selected [^{3}H]RNA and incubated at 65 °C for 30 min or 24–60 h. All hybridization mixtures contained 1 M NaCl–1 mM EDTA in 10 mM Tris-HCl (pH 7.9). The ratio of RNA to DNA concentration was kept between 10 and 100 and the time period of hybridization corresponded to at least six times the $C_0t_{1/2}$ of adenovirus DNA. In the experiments set up to analyze the urea phosphate–HAP system, hybridization was carried out for 500–1000 times $C_0t_{1/2}$. Isolated RNA was in some cases analyzed by hybridization to DNA strands immobilized on filters by techniques previously described (Pettersson and Philipson, 1975).

Isolation of DNA–RNA Hybrids on Sephadex G-100. Oligo(dT) selected ^{3}H-labeled RNA was hybridized to single- or denatured double-stranded Ad2 DNA for 24–60 h. The sample was diluted to give a NaCl concentration of 0.25 M and digested with RNase A and T1 (25 μg/ml and 25 units/ml, respectively). The RNase-resistant fraction was isolated after addition of sodium dodecyl sulfate to 0.5% by chromatography on Sephadex G-100 (Pettersson and Philipson, 1974). The DNA–RNA hybrid eluting in the void volume was in some cases precipitated with ethanol and resuspended in 1 mM EDTA in 10 mM Tris-HCl (pH 7.9).

Isolation of DNA–RNA Hybrids by the Urea Phosphate–Hydroxylapatite Procedure. A detailed description of this assay was provided by Smith et al. (1974). The hybridization mixtures were brought to 0.05 M sodium phosphate buffer (pH 6.8), 8 M urea, and 0.1% SDS and passed over 1 ml hydroxylapatite columns in the same urea phosphate solution at 40 °C. The nucleic acids were eluted at 40 °C by a linear gradient from 0.05 to 0.5 M phosphate buffer, pH 6.8, containing 8 M urea and 0.1% sodium dodecyl sulfate. A stepwise procedure was used in some experiments with elution in 8 M urea, 0.1% sodium dodecyl sulfate, at 0.2 and 0.4 M phosphate at 40 °C as described by Lewis et al. (1975) or 0.2 M at 40 °C followed by 0.2 M phosphate at 80 °C as described by Smith et al. (1974). Bio-Rad HTP-grade hydroxylapatite was used.

Melting Profile of DNA in the Two-Phase System. ^{32}P-labeled native Ad2 DNA was dissolved in phosphate buffer containing 1 mM NaH_2PO_4 and 9 mM Na_2HPO_4 (pH 8.0) or the upper phase of the two-phase system equilibrated with the same buffer. The solution of DNA was heated for 10 min at different temperatures whereupon 50-μl aliquots were removed and added to 1 ml of buffer containing equimolar amounts (5 mM) of NaH_2PO_4 and Na_2HPO_4 (pH 6.8) to which 0.7 ml of the stock solution for the two-phase system was added. The distribution of the ^{32}P radioactivity in the upper and the lower phases was determined for each temperature.

Experimental Procedure for Preparative Isolation of Messenger RNA. Around 100–300 μg of oligo(dT) selected RNA from adenovirus infected cells was hybridized with 4–50 μg of single- or denatured double-stranded Ad2 DNA for 30 min at 65 °C in 1 M NaCl–1 mM EDTA in 10 mM Tris-HCl (pH 7.9) as indicated in the tables. The hybridization mixture was then diluted in 10 mM Tris-HCl (pH 7.9)–1 mM EDTA to 0.15 M NaCl, the RNA and DNA were precipitated by ethanol, and the precipitate was washed with 80% ethanol. The nucleic acids were dissolved in 2 ml of 1 mM NaH_2PO_4–9 mM Na_2HPO_4 (pH 8.0), 1.2 ml of the two-phase stock solution was added, and the phases were separated as described above. The upper phase was extracted once more with a fresh lower phase and the second lower phase was extracted with fresh upper phase. The upper phase fractions were pooled and EDTA and sodium dodecyl sulfate were added to final concentrations of 1 mM and 0.1%, respectively. The samples were then boiled for 2 min and cooled on ice whereupon 2 ml of 1 mM EDTA in Tris-HCl (pH 7.9) was added. The mixture was then made 0.5 M with regard to NaCl and passed twice through an oligo(dT)cellulose column, about 0.5 ml in volume. The column was washed with 0.5 M NaCl and then with 0.25 M NaCl in elution buffer. The RNA was eluted with 1.5 ml of 1 mM EDTA in 10 mM Tris-HCl (pH 7.9). The isolated RNA was precipitated with ethanol and the precipitate was collected by centrifuging for 60 min at 50 000 rpm in a Beckman SW 50, 1 rotor. The recovery of RNA and the contamination of DNA were continuously followed by using [^{3}H]RNA and [^{14}C]-DNA, respectively.

Results

Analysis of DNA–RNA Hybrids on Hydroxylapatite. Smith et al. (1974) introduced the urea phosphate–HAP system to select large RNA hybridized to denatured double-stranded DNA. A similar system was used by Eron and Westphal (1974) and Lewis et al. (1975) to isolate adenovirus specific messenger RNA by hybridization to denatured and sonicated adenovirus DNA. Since we were interested in selecting viral messenger RNA on both double- and single-stranded DNA probes, we first analyzed the usefulness of the urea phosphate–HAP system. [^{3}H]Uridine-labeled poly(A$^+$) late viral RNA (9 μg) was hybridized to single-stranded or denatured double-stranded DNA (1 μg) in 1 M NaCl–1 mM EDTA–10 mM Tris-HCl (pH 7.9) for periods corresponding to 500 $C_0t_{1/2}$ of Ad2 DNA. Samples of the hybridization mixtures were removed, digested with RNase A and T1, and analyzed for percent of RNA radioactivity in hybrids on Sephadex G-100 as described in Materials and Methods. The hybridization mixture without RNase treatment was also analyzed on urea phosphate–HAP according to the methods used by Smith et al. (1974) and Lewis et al. (1975) which involve 0.2 M phosphate at 40 and 80 °C for elution and 0.2 and 0.4 M phosphate at 40 °C for elution, respectively. Table I shows that 1 μg of Ad2 l strand hybridized around 18% of the RNA by the Sephadex G-100 method but no hybrids were detected with the urea phosphate–HAP systems. Denatured Ad2 DNA (1 μg) hybridized 5.4% of RNA by the Sephadex G-100 method and 8.5 and 9.1% by the stepwise urea phos-

TABLE I: Hybrids between Late Poly(A) + Adenovirus RNA and Single- and Double-Stranded Adenovirus DNA Probes.[a]

DNA Probe	DNA (μg)	RNA (μg)	[^{3}H]RNA (cpm × 10^{-5})	% RNA Radioactivity in Hybrids by: Sephadex G-100	HAP[b,d]	HAP[b,e]	HAP[b] Gradient Elution[f]
Ad2 l strand	1	9	6.5	17.8	ND[c]	ND[c]	ND[c]
Denatured Ad2 DNA	1	9	6.5	5.4	8.5	9.1	1.6

[a] Isolation of RNA late in the infection of adenovirus type 2; selection on oligo(dT)cellulose, hybridization conditions, and analysis of the hybrids are described in Materials and Methods. [b] HAP refers to hydroxylapatite chromatography according to references given. [c] ND: no hybrids detected. [d] Smith et al. (1974). [e] Lewis et al. (1975). [f] Figure 1.

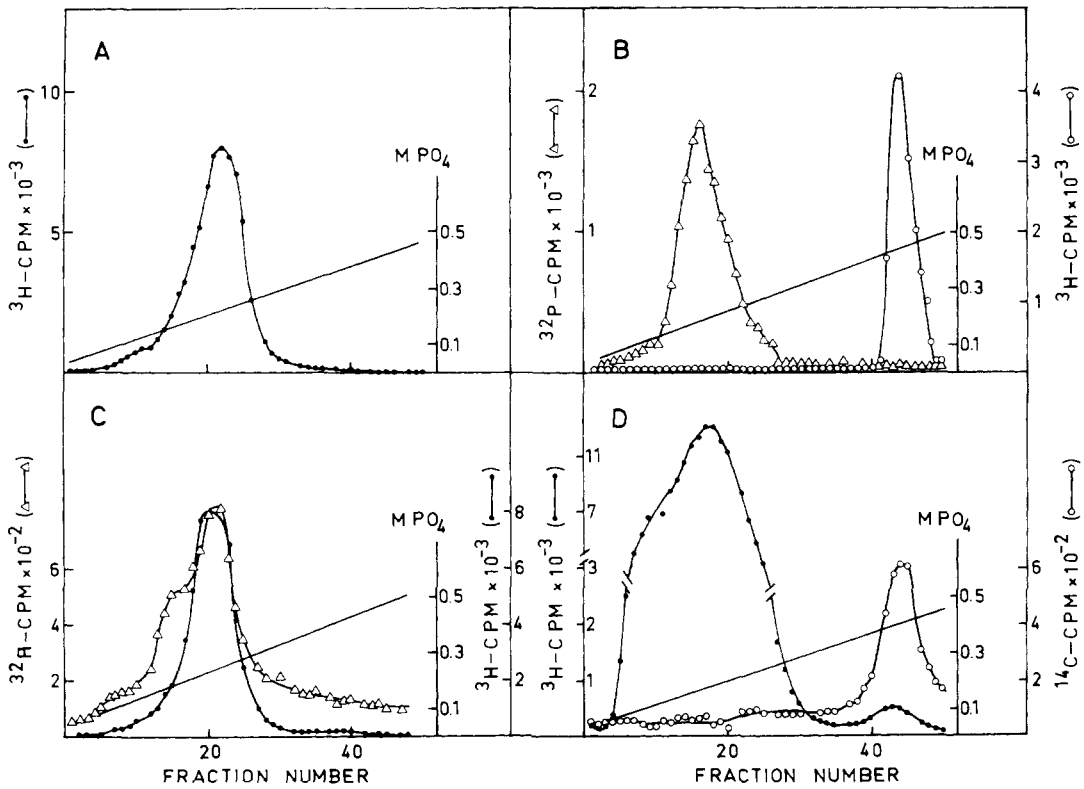

FIGURE 1: Elution of RNA-DNA hybrids in the urea phosphate-HAP system. RNA was extracted 18 h post infection from the cytoplasm of adeno 2 infected HeLa cells. After selection on oligo(dT)cellulose chromatography, RNA was hybridized to fragmented single-stranded and double-stranded adeno 2 DNA. All samples were bound to hydroxylapatite in 8 M urea–0.05 M phosphate buffer–0.1% sodium dodecyl sulfate at 40 °C and eluted with a phosphate gradient in 8 M urea–0.1% sodium dodecyl sulfate. (A) Shows the elution profile of polyadenylated [^{3}H]mRNA from adenovirus infected cells. (B) Shows the elution profile of samples of ^{32}P-labeled l-strand (Δ) adeno 2 DNA and ^{3}H-labeled double-stranded adeno 2 DNA (○) analyzed separately. (C) Shows the elution profile of hybrid formed after incubation of [^{3}H]mRNA (●) with ^{32}P-labeled l-strand DNA (Δ) at an RNA/DNA concentration ratio of 10 and 1 μg of Ad2 l strand. The incubated sample was treated with RNase A and T1 and then fractionated on a Sephadex G-100 column. The material in the void volume was analyzed. (D) Shows the elution profile of hybrids between [^{3}H]mRNA (●) and ^{14}C-labeled adeno 2 DNA (○) at an RNA/DNA concentration ratio of 10 and 1 μg of Ad2 DNA. The DNA was fragmented by boiling in alkali followed by neutralization prior to hybridization. After incubation, the sample was diluted sevenfold in the urea phosphate buffer and analyzed.

phate methods of Smith et al. (1974) and Lewis et al. (1975), respectively.

The hybridization mixtures and control RNA and DNA preparations were also analyzed by the urea phosphate-HAP method using gradient elution with increasing molarity of phosphate (pH 6.8). Since the hybrids between single-stranded DNA and RNA were not resolved by the urea phosphate-HAP method, we analyzed RNase-treated hybrids which were prepurified on Sephadex G-100. Figure 1A shows that late adenovirus RNA elute from HAP between 0.15 and 0.25 M phosphate in the urea phosphate system. The single-stranded adenovirus DNA probe elutes between 0.12 and 0.22 M phosphate and the double-stranded probe between 0.4 and 0.5 M phosphate (Figure 1B). RNase digested hybrids between the l strand of Ad2 DNA and late RNA elute between 0.18 and 0.3 M phosphate (Figure 1C). The single-stranded DNA probe appears to be partially unhybridized since a fraction of it elutes at the position of single-stranded DNA (Figure 1B). In the hybridization mixture between RNA and denatured fragmented adenovirus DNA, 1.6% of the RNA (Table I) elutes together with double-stranded DNA at 0.35–0.45 M phosphate (Figure 1D). The remaining RNA elutes in a broad peak from 0.1 to 0.3 M phosphate. The materials in this peak may contain a fraction of the hybrids composed of single-stranded DNA

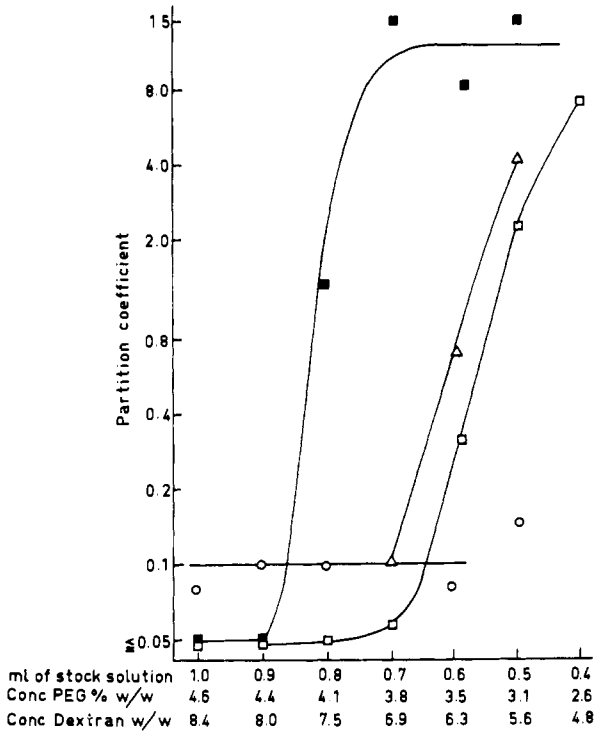

FIGURE 2: The partition of nucleic acids in aqueous polymer phase systems of different composition. Different preparations of ^{3}H- or ^{32}P-labeled nucleic acids in 1 ml of 0.01 M sodium phosphate buffer (pH 6.8) were mixed with varying amounts of the stock solutions of the polymers described in Materials and Methods. The final concentration (w/w) of the polymers in the phase systems formed is indicated on the abscissa and the partition coefficient on the ordinate. (■) ^{32}P-labeled double-stranded adenovirus type 2 DNA; (△) DNA-RNA hybrids labeled with [^{3}H]uridine in the RNA moiety formed by hybridization of single- or denatured double-stranded adenovirus DNA. The hybrids were digested with nucleases and separated on Sephadex G100 prior to analysis. (□) ^{32}P-labeled isolated strands of adenovirus DNA. The partition coefficient was not altered significantly when sonicated or intact DNA was analyzed. (○) [^{3}H]Uridine-labeled cytoplasmic RNA isolated late in the infectious cycle.

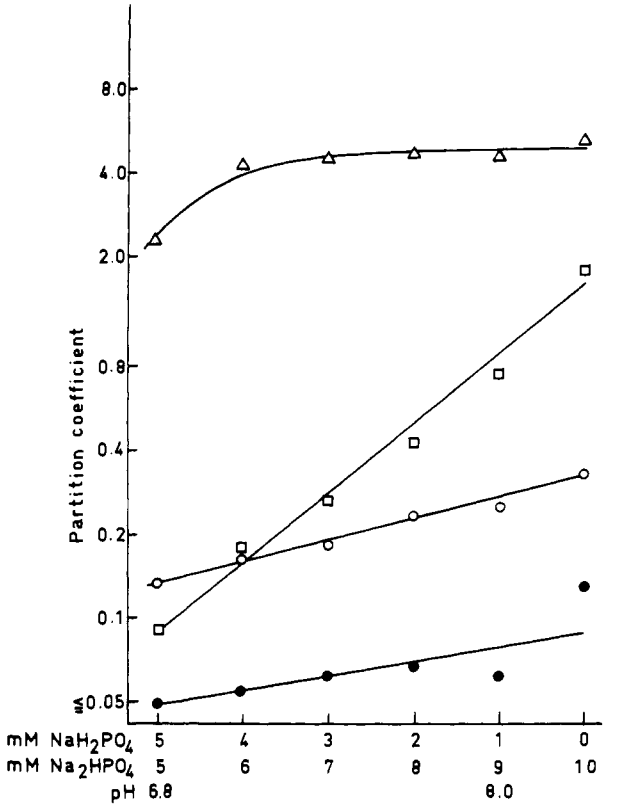

FIGURE 3: The partition of nucleic acids in an aqueous polymer phase system at different pH. One milliliter of nucleic acid solution buffered at 0.01 M with sodium phosphate buffer at varying pH as indicated on the abscissa was mixed with 0.6 ml of the stock solution of the polymer phase system. The mixture thus contained 6.3% dextran (w/w) and 3.5% poly(ethylene glycol) (w/w). (△) DNA-RNA hybrids between single- or double-stranded adenovirus DNA and [^{3}H]uridine-labeled late RNA; (□) ^{32}P-labeled single-stranded adenovirus DNA; (○) [^{3}H]uridine-labeled late cytoplasmic RNA; and (●) [^{3}H]uridine-labeled oligo(dT)-selected late viral RNA.

and RNA as shown in Figure 1C since 5.4% of the RNA should hybridize according to the Sephadex G-100 assay (Table I). These results therefore suggest that the urea phosphate-HAP chromatography cannot be used for selection of RNA by hybridization to single-stranded probes and that selection with double-stranded probes may cause contamination with unselected RNA under the conditions employed (compare Fig. 1A and 1C). An alternative method was therefore developed.

Polymer Concentration and Partition of Nucleic Acids. The polymer concentration was varied by mixing varying amounts of phase stock solution with 1 ml of nucleic acid solutions, both buffered with 5 mM sodium phosphate at pH 6.8. Figure 2 shows the partition of various nucleic acids into the upper and lower phases of the phase system as a function of the polymer concentration. As the polymer concentration is lowered, double-stranded and later single-stranded DNA favor the upper phase, in agreement with the results of Alberts (Alberts, 1967). Cytoplasmic RNA remains in the lower phase independent of the polymer concentration. DNA-RNA hybrids were obtained by hybridizing, unlabeled single or denatured double-stranded adenovirus DNA with excess ^{3}H-labeled RNA followed by RNase digestion and Sephadex chromatography as described in Materials and Methods. These hybrids favor the top phase at low polymer concentrations in a similar way as single-stranded DNA with around a 50-fold difference in distribution between the two phases compared with free RNA. A system composed of 0.6 ml of stock polymer solution per ml of nucleic acid solution was chosen for further studies since it reproducibly gave two distinct phases.

Effects of pH on the Partition of Nucleic Acids. The effect of pH on the partition of nucleic acids was studied next to improve separation since a high pH may favor top phase distribution of DNA (Albertsson, 1965). The nucleic acids were taken up in 1 ml of buffer containing different proportions of NaH_2PO_4 and Na_2HPO_4, 0.6 ml of stock polymer solution was added, and the partition of nucleic acids was examined. Figure 3 shows that DNA-RNA hybrids irrespective of whether they are generated with single- or denatured double-stranded DNA favor the upper phase 25-50-fold compared with both cytoplasmic RNA and oligo(dT) selected RNA which remains to 70 and 90%, respectively, in the lower phases. The hybrids labeled in the RNA moiety show a partition coefficient of 5.0 compared with 0.1 for oligo(dT) selected RNA.

At all pH values, the partition coefficient of cytoplasmic RNA is higher than that for RNA selected by oligo(dT)cellulose chromatography. It is possible that tRNA in the former preparation contributes to the higher values since it has a partition coefficient of approximately unity in a similar two-phase system (Albertsson, 1965). The poly(A) tract in oligo(dT) selected RNA may in a similar way help to keep the nucleic acid in the lower phase since synthetic poly(A) strongly

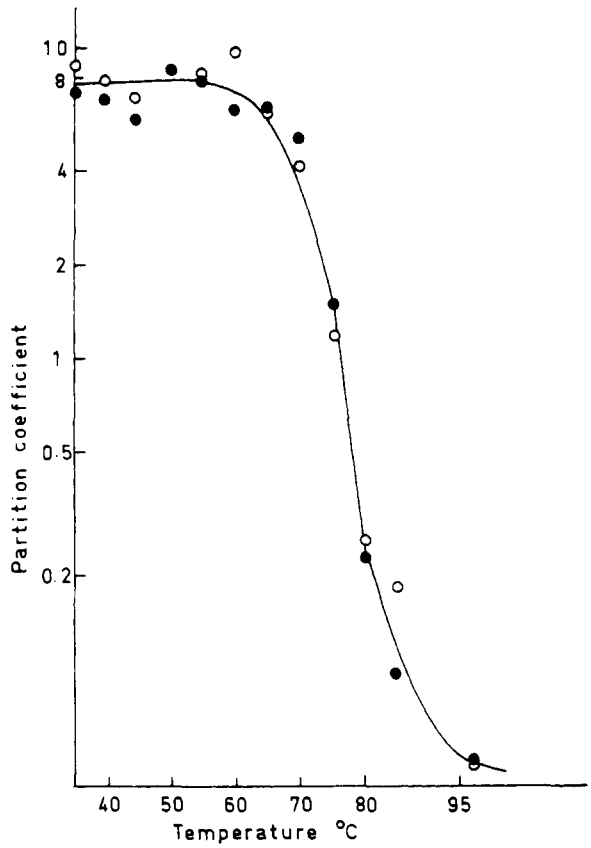

FIGURE 4: Denaturation of adenovirus DNA measured by partition in an aqueous polymer phase system. ^{32}P-labeled adenovirus DNA was heated for 10 min at different temperatures in the presence and absence of the top phase of the selected phase system (Dextran$_{6.3}$-PEG$_{3.5}$ (pH 8.0)) as described in Materials and Methods. Samples were removed and added to a phase system (Dextran$_{6.9}$-PEG$_{3.8}$ (pH 6.8)) giving at least a 100-fold difference in partition between single- and double-stranded DNA and the partition coefficient determined for each temperature. (○) [^{32}P]DNA without added top phase; (●) [^{32}P]DNA with added top phase.

favors the lower phase (Albertsson, 1965). The difference in molecular weight between some classes of RNA in total cytoplasmic and oligo(dT) selected RNA may also be important for partition in the phase systems (Öberg et al., 1965). Single-stranded DNA partition shows a stronger dependence on pH than RNA in this polymer phase system.

Effect of Polymer on DNA Denaturation. In order to purify the messenger RNA, the DNA–RNA hybrid present in the upper phase must be denatured. The top phase of this system contains 6.4% poly(ethylene glycol) and 0.45% dextran. The influence of the polymers on the melting of nucleic acids was studied by determining the melting profile of double-stranded adenovirus DNA in the presence and absence of the polymers of the top phase as described in Materials and Methods. Figure 4 shows that the polymers in the upper phase have no effect on DNA denaturation.

It was also ascertained that RNA can be denatured from the hybrid in the upper phase since boiling (5 min) of DNA–RNA hybrids labeled with [^{3}H]uridine in the RNA moiety caused a change in partition coefficient from 5.0 to 0.25 of the [^{3}H]uridine after melting. Thus 75% of the [^{3}H]RNA partitioned to the lower phase after denaturation.

Removal of the DNA and Polymer. After hybridization, DNA as well as DNA–RNA hybrids was partitioned into the upper phase of the two-phase system and separated 20–50-fold from unhybridized RNA (Figures 2 and 3). After boiling of the upper phase, the polymers and the DNA were separated from the poly(A)-containing messenger RNA by oligo(dT) cellulose chromatography. The polymers, poly(ethylene glycol) and dextran, passed through the oliog(dT)cellulose at 0.5 M NaCl as revealed by cold ethanol precipitation. Table II shows that only between 0.1 and 1.5% of the total DNA eluted with mRNA at low salt. The poly(A)-containing mRNA could thus be purified from the DNA when eluted in the absence of NaCl.

Purity of Isolated Messenger RNA. To test the yield and purity of the isolated RNA in the phase system, two types of experiments were carried out: (a) the yield of [^{3}H]RNA from adenovirus type 2 infected cells after hybridization with double-stranded adenovirus DNA or DNA from a staphylococcal phage (ϕ11) in RNA excess; (b) selection of [^{3}H]RNA from Ad2 infected cells on separated strands of adenovirus DNA and rehybridization to separated strands.

In the first type of experiment 150 μg of oligo(dT) selected ^{3}H-labeled RNA was hybridized with 20 μg of denatured sonicated Ad2 or phage ϕ11 DNA in 2 ml of the hybridization buffer for 30 min at 65 °C. The hybrid was purified by the two-phase polymer system and the ^{3}H-labeled RNA was recovered by oligo(dT)cellulose chromatography. Table III shows that hybridization with Ad2 DNA gave a recovery of around 5% of input RNA. On the other hand, hybridization with phage ϕ11 DNA yielded between 0.2 and 0.5% of the input RNA, indicating that 90% of selected RNA is adenovirus specific. Calculating with a specific activity of 10^4 cpm/μg of RNA and the fact that only 70% of the l strand of adenovirus DNA can hybridize with late ^{3}H-labeled mRNA (Pettersson and Philipson, 1974), around 7 μg of adenovirus RNA can

TABLE II: Removal of Adenovirus DNA from the Top Phase of a Two-Phase Polymer System by Oligo(dT)cellulose Chromatography.[a]

Expt	DNA in Top Phase (cpm)	Radioactivity[b] Eluted at Oligo(dT)cellulose Chromatography: 0.5 M NaCl	0.25 M NaCl	0 M NaCl	Recovery (%)
1	7.6×10^5 (100), M[c]	4.9×10^5 (64)	4.4×10^4 (6)	2.8×10^3 (0.4)	70
2	5.8×10^5 (100), M	3.5×10^5 (61)	1.3×10^4 (3)	5.9×10^3 (0.9)	65
3	3.76×10^5 (100), S	4.06×10^5 (108)	458 (0.1)	348 (0.1)	108
4	3.53×10^5, S, M	3.61×10^5 (102)	4.45×10^3 (1.5)	4.13×10^3 (1.5)	105

[a] ^{14}C-labeled adenovirus DNA was analyzed in a polymer two-phase system used for selection of mRNA as described in Materials and Methods. The top phase was recovered, the DNA melted by heat, and the material analyzed by oligo(dT)cellulose chromatography. Intact adenovirus DNA was used in experiments 1 and 2 and sonicated DNA in experiments 3 and 4. In experiment 3 the DNA was not melted. Sonication was for 2 × 20 s, yielding an average size of around 600 nucleotides of DNA. Values within parentheses give the percentage of radioactivity in the fractions. [b] In cpm. [c] M, melted; S, sonicated.

TABLE III: Selection of ^{3}H-Labeled Late Viral RNA from Adeno 2 Infected Cells on Different Double-Stranded DNA.[a]

Expt	DNA, 20 μg	[^{3}H]RNA (added, cpm)	[^{3}H]RNA Recovered cpm	%
1	Ad2	1.5 × 10^6 (150 μg)	60 700 (6 μg)	4.0
	φ11	1.5 × 10^6 (150 μg)	2 800 (0.3 μg)	0.19
2	Ad2	1.7 × 10^6 (170 μg)	78 400 (7 μg)	4.5
	φ11	1.7 × 10^6 (170 μg)	7 400 (0.7 μg)	0.44

[a] The DNA was sonicated and denatured at 100 °C for 5 min and hybridized with RNA for 30 min at 65 °C. The hybridized mixture was extracted by the two-phase polymer system as described in Materials and Methods. The hybrids in the top phase were melted and subjected to oligo(dT)cellulose chromatography. The last column represents radioactivity eluted from the oligo(dT) column without NaCl. The values within parentheses give the amount of RNA recovered based on the specific activity of the starting material.

theoretically hybridize to the probe, suggesting a recovery of 80%.

To obtain a more unambiguous estimate of specificity, early viral [^{3}H]RNA, labeled before viral DNA synthesis, and intermediate viral [^{3}H]RNA, labeled between 6 and 10.5 h after infection, were selected with separated strands of Ad2 DNA and virus-specific RNA was purified by the selection procedure. The recovered ^{3}H-labeled RNA was tested for its ability to rehybridize with separated strands. Table IV shows that the purified messenger RNA hybridized back to the DNA strand used for selection with efficiencies ranging from 70 to 78% and only 3–4% of the input radioactivity hybridized to the nonhomologous DNA strand. This procedure therefore appears to give a reasonable yield of messenger RNA which obviously hybridizes almost quantitatively back to the DNA used for selection.

Recovery of RNA after Selection of Hybrids in the Phase System. The quantitative recovery of mRNA using this procedure is difficult to assess mainly because of difficulties to determine the specific radioactivity of viral RNA compared with cellular RNA. Large scale experiment utilizing early oligo(dT) selected cytoplasmic RNA and separated strands of adenovirus DNA was, however, carried out to obtain a crude estimate of recovery. Table V shows that oligo(dT) selected RNA with a specific radioactivity of 7 × 10^4 cpm/μg yielded between 1.9 × 10^5 and 2.1 × 10^5 cpm after selection on l and h strand, respectively, with DNA inputs of 8 and 20 μg for l- and h-strand DNA. This would represent around a 100-fold purification with regard to the input RNA. The expected recovery of viral RNA can be calculated to be 2.4 μg from the l strand and 3.0 μg from the h strand, taking into account the amount of DNA used and the fact that 30% of the l and 15% of the h strand are expressed in oligo(dT)-selected cytoplasmic RNA at early times of the adenovirus infection (Tibbetts et al., 1974). The recovery of 2.6 and 2.9 μg for the two strands may suggest almost quantitative recovery for early viral mRNA.

Another approach to evaluate recovery was also tried. The yield of hybrid in the different steps of our procedure was compared with Sephadex G-100 chromatography of RNase-treated hybrids. Sonicated and heat-denatured Ad2 DNA or φ11 DNA (78 μg) was hybridized with oligo(dT)-selected ^{3}H-labeled late adenovirus RNA (60 μg) in 0.7 ml of hybridization buffer for 3 h at 65 °C. The hybrids were partitioned with two transfers in the two-phase systems, melted, and analyzed on oligo(dT)cellulose. Samples of the hybridization mixture were also treated with RNase A and T1 and analyzed by Sephadex G-100 chromatography as described in Materials and Methods. Table VI shows that 15.2% of the RNA radioactivity is recovered in the top phase after phase-system partition when Ad2 DNA was used for hybridization compared with 1.5% with φ011 DNA. After oligo(dT) selection of the messenger RNA 12.2% of the viral RNA was selected after hybridization with Ad2 DNA but only 0.4% with φ11 DNA. The Sephadex G-100 chromatography of the same hybridization mixture yields 12.8% hybrid with Ad2 DNA and 1.3% with φ11 DNA. Our procedure obviously gives a recovery which is comparable to the Sephadex G-100 chromatography assay.

Discussion

The present study reveals that the urea phosphate–hydroxylapatite system cannot be used to purify messenger RNA after selection on single-stranded DNA (Figure 1). Our results also raise the possibility that the current stepwise elution techniques, i.e., elution of hybrids at high temperature (Smith et al., 1974) or high ionic strength (Lewis et al., 1975), may cause appreciable contamination with unhybridized RNA.

The partition of different nucleic acids in a two-phase system

TABLE IV: Rehybridization of Selected ^{3}H-Labeled Viral RNA to the Complementary Adeno 2 DNA Strands.[a]

Source of [^{3}H]RNA	[^{3}H]RNA Input (cpm)	Strand of Ad2 DNA	Resistant to RNase A and T1	Input Resistant to RNase A and T1 (%)
Unselected Intermediate	1.5 × 10^5	h	9160	6.2
h-strand selected	3650	l	139	3.8
Intermediate	4320	h	2980	69.0
h-strand selected	7654	l	248	3.2
Early	5626	h	4045	71.9
l-strand selected	6132	l	4780	78.0
Early	7685	h	296	3.9

[a] Either l or h strand (0.3 or 0.45 μg) was used for hybridization in solution. The mixture was then treated with RNase A and T1, and the RNase-resistant [^{3}H]RNA was determined after Cl_3COOH precipitation.

TABLE V: Recovery of ^{3}H-Labeled Early Viral RNA after Selection of Hybrids in the Polymer Phase System.[a]

Strand of Adenovirus Type 2 DNA	Amount	Vol of Hybridization Mixture	[^{3}H]RNA Input		[^{3}H]RNA Recovered	
			cpm × 10^7	μg	cpm × 10^6	μg
l	8 μg	2 ml	2.2 (100)	312	0.19 (0.9)	2.6
h	20 μg	2 ml	2.2 (100)	312	0.21 (1.0)	2.9

[a] Hybridization of early oligo(dT)-selected RNA and Ad2 DNA strands was for 30 min at 65 °C as described in Materials and Methods. Under the heading "Recovered", the radioactivity refers to that obtained after the oligo(dT)cellulose chromatography step. The values in parentheses give the percentage of radioactivity recovered. The recovery in micrograms was calculated based on the specific radioactivity of the starting material.

TABLE VI: Comparison of the Yields of Hybrids in the Polymer Phase System and in the Sephadex G-100 Assays.[a]

DNA Used for Hybridization	DNA (μg)	RNA (μg)	^{3}H Radioact. in RNA (cpm × 10^{-5})	% [^{3}H]RNA in Hybrid		
				Sephadex G-100	Top Phase of the Phase System	Oligo(dT) selection after Phase System
Adeno 2	78	60	1.92	12.8	15.2	12.2
φ11	78	60	1.92	1.3	1.5	0.4

[a] Preparation of late adenovirus RNA, hybridization, and the methods to score hybrids are described in Materials and Methods.

containing dextran and poly(ethylene glycol) was therefore studied as an alternative method to separate DNA-RNA hybrids from unhybridized RNA. It was found that DNA-RNA hybrids and double-stranded DNA partitioned differently in the two-phase system (Figure 2). Martinson (1973) reported that DNA-RNA hybrid has less affinity for hydroxylapatite than double-stranded DNA. He suggested that the phosphate group in double-stranded DNA is more accessible for interaction with hydroxylapatite than that of the DNA-RNA hybrid. Thus the partition coefficient of double-stranded DNA and that of DNA-RNA hybrid may be expected to be different. DNA-RNA hybrid appears to have a conformation of A type, while double-stranded DNA has a B type conformation (Tunis and Hearst, 1968). This conformational difference may contribute to the difference in partition of DNA-RNA hybrid and double-stranded DNA both on HAP and in the phase system.

This study also illustrates that the two-phase polymer system can be used efficiently to separate DNA-RNA hybrids from poly(A)-containing RNA. Theoretically, with two successive extractions, greater than 65% of the DNA-RNA hybrid and less than 0.5% of the poly(A)-containing RNA are partitioned into the upper phase. Experimentally less than 0.5% of polyadenylated RNA was recovered after one two-phase extraction and oligo(dT)cellulose chromatography (Table III). Comparable amounts of hybrid RNA were recovered with the polymer phase systems and with the Sephadex chromatography assay (Table VI). It should, however, be emphasized that liquid hybridization with denatured double-stranded DNA in moderate RNA excess which was used in some experiments in this study, is less efficient than the hybridization with single-stranded probes, mainly due to competing DNA-DNA hybridization in the reaction mixture (Table I).

It is difficult to determine the recovery of the DNA-RNA hybrids mainly due to the lack of precise methods to determine the specific activity of the different classes of labeled RNA. Aware of these shortcomings only suggestive evidence can be provided that late viral mRNA is almost 90% pure (Table III). Early viral mRNA which only constitutes around 5-10% of the radioactivity in the oligo(dT) selected fraction of cytoplasmic RNA (Lindberg et al., 1972) was also selected with good recovery (Table V) and the purity was probably around 70% judged from rehybridization of strand selected early viral RNA to the homologous strand (Table IV). We have used the selected viral messenger RNA from the two strands of adenovirus DNA for translation in a wheat embryo system (Öberg et al., in preparation) and the results revealed that this method yields for functional mRNA which could be used to map the gene products on the adenovirus genome.

Oligo(dT)cellulose chromatography offers a convenient method to remove both polymers and DNA after melting of the hybrids. This method has already been used to remove DNA from hybrids isolated by hydroxylapatite chromatography (Lewis et al., 1975). One inherent disadvantage of our procedure is the inability to recover messenger RNA lacking poly(A) which appears to be present in some eukaryotic systems (Wilt, 1973; Milcarek et al., 1974) but at least the poly(ethylene glycol) can be removed from the hybrids by hydroxylapatite chromatography (Albertsson, 1971). An enhanced separation of DNA-RNA hybrids from DNA and RNA can probably be achieved by using several transfers in a countercurrent distribution device (Blomquist and Albertsson, 1969).

During the course of this investigation both double- and single-stranded DNA have been covalently linked to a solid support (Noyes and Stark, 1975; Gilboa et al., 1975) and used for selection of SV40 messenger RNA. These techniques have the advantage that the DNA probe may be preserved which also may be achieved if the top phase of the phase system percolating the oligo(dT)cellulose could be reutilized.

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References

Alberts, B. M. (1967), *Biochemistry 6,* 2527–2532.
Albertsson, P.-A. (1962), *Arch. Biochem. Biophys., Suppl. No. 1,* 264–270.
Albertsson, P.-A. (1965), *Biochim. Biophys. Acta 103,* 1–12.
Albertsson, P.-A. (1971), Partition of Cell Particles and Macromolecules, Uppsala, Sweden, Almquist and Wiksell, p 243.
Aviv, H., and Leder, P. (1972), *Proc. Natl. Acad. Sci. U.S.A. 69,* 1408–1412.
Blomquist, G., and Albertsson, P.-A. (1969), *Anal. Biochemistry 30,* 222–229.
Eron, L., and Westphal, H. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 3385–3389.
Gilboa, E., Prives, C. L., and Aviv, H. (1975), *Biochemistry 14,* 4215–4220.
Lewis, J. B., Atkins, J. F., Anderson, C. W., Baum, P. R., and Gesteland, R. F. (1975), *Proc. Natl. Acad. Sci. U.S.A. 72,* 1344–1348.
Lewis, J. B., Atkins, J. F., Baum, P. R., Solem, R., Gesteland, R. F., and Anderson, C. W. (1976), *Cell 7,* 141–151.
Lindberg, U., Persson, T., and Philipson, L. (1972), *J. Virol. 10,* 909–919.
Martinson, H. G. (1973), *Biochemistry 12,* 2737–2746.
Milcarek, C., Price, R., and Penman, S. (1974), *Cell 3,* 1–10.
Noyes, B. E., and Stark, G. R. (1975), *Cell 5,* 301–310.
Öberg, B., Albertsson, P.-A., and Philipson, L. (1965), *Biochim. Biophys. Acta 108,* 173–181.
Öberg, B., and Philipson, L. (1969), *Eur. J. Biochem. 11,* 305–315.
Pettersson, U., and Philipson, L. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 4887–4891.
Pettersson, U., and Philipson, L. (1975), *Cell 6,* 1–14.
Sjöström, J. E., Lindberg, M., and Philipson, L. (1973), *J. Bacteriol. 113,* 576–585.
Smith, M. J., Hough, B. R., Chamberlain, M. E., and Davidson, E. (1974), *J. Mol. Biol. 85,* 103–126.
Tibbetts, C., Johansson, K., and Philipson, L. (1973), *J. Virol. 12,* 218–225.
Tibbetts, C., Pettersson, U., Johansson, K., and Philipson, L. (1974), *J. Virol. 13,* 370–377.
Tunis, M.-J. B., and Hearst, J. E. (1968), *Biopolymers 6,* 1218–1223.
Wilt, F. H. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 2345–2349.

Influence of 5′-Terminal m^7G and 2′-O-Methylated Residues on Messenger Ribonucleic Acid Binding to Ribosomes†

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ABSTRACT: Removal of 80% of the 5′-terminal 7-methylguanosine (m^7G) from methylated reovirus mRNA by β elimination results in a concomitant loss of the ability to bind to wheat germ ribosomes. The mRNA molecules that retain the m^7G account for most of the residual binding. Removal of the m^7G from all molecules in preparations of methylated reovirus and vesicular stomatitis virus mRNA reduces the extent of binding to wheat germ ribosomes from 80% to 5–7%. In the reticulocyte lysate, however, significant binding (17–34%) of the β-eliminated viral RNAs occurs. This m^7G-independent binding appears to be due to recognition by ribosomes of other structural features of the 5′-proximal sequences. Initiation complexes involving β-eliminated animal virus mRNAs and rabbit reticulocyte ribosomes appear to be more stable than the more heterologous combination of the same viral mRNAs and wheat germ ribosomes. In addition, evidence is presented to show that the 2′-O-methylated nucleoside of the 5′-terminal cap has a positive influence on the ribosome binding of viral mRNA and of capped synthetic ribopolymers. A model involving recognition of multiple structural features of the 5′-terminal region of mRNA by ribosomes during initiation of protein synthesis is presented.

The 5′ termini of many eukaryotic mRNAs are blocked with the methylated "cap" structure, $m^7G(5')ppp(5')N$ (Shatkin, 1976). Several lines of evidence have suggested a functional role for the m^7G[1] at the initiation step of capped mRNA translation. In cell-free protein synthesizing extracts derived from wheat germ or *Artemia salina* embryos, translational efficiencies were considerably greater for the methylated forms of reovirus and vesicular stomatitis virus (VSV) mRNAs than their unmethylated counterparts (Both et al., 1975a; Muthukrishnan et al., 1975b). Removal of the m^7G from globin

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[1] Abbreviations used: VSV, vesicular stomatitis virus; m^7pG, 7-methylguanosine 5′-phosphate; m^7G, 7-methylguanosine; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.