A simple method for selection of RNA-DNA hybrids has been developed and applied to the purification of adenovirus-specific messenger RNA. Cytoplasmic RNA prepared from adenovirus type 2 (ad2)-infected HeLa cells or from an ad2-transformed rat cell line was hybridized in solution to the complementary strands of ad2 DNA. The hybridization mixture was subsequently fractionated by chromatography on a Sepharose 2B column. The intact probe DNA as well as the RNA-DNA hybrids are excluded from the gel matrix and elute with the void volume. Nonhybridized RNA, in contrast, is included into the gel matrix and elutes as a broad peak well separated from the excluded fractions. Fractions corresponding to the void volume, were collected and the RNA-DNA hybrids were denatured in 90% formamide. The selected RNA was separated from the DNA by affinity chromatography on poly(U)-Sepharose. Restriction endonuclease fragments of DNA with a large enough size to make them excluded from the agarose column were also used for hybridization. In these experiments hybridizations were carried out under conditions which would allow R-loop formation (Thomas, M., White, R. L., and Davis, R. W. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 2294-2298) and the hybridized RNA was separated from unhybridized RNA by Sepharose chromatography. The validity of the method was demonstrated by programming an in vitro protein-synthesizing system with selected RNA.

Methods to separate RNA-DNA hybrids from unhybridized RNA are of great importance for mapping of genes for different polypeptides on viral or bacterial genomes. Programming an in vitro protein-synthesizing system with mRNA that has been selected by hybridization makes it possible to identify the gene product which is specified by a given DNA sequence. Several methods designed for this purpose have previously been described; RNA hybridized to denatured double-stranded DNA can be isolated from unhybridized RNA by hydroxyapatite chromatography in the presence of formamide. The selected RNA was separated from the DNA by affinity chromatography on poly(U)-Sepharose. Restriction endonuclease fragments of DNA with a large enough size to make them excluded from the agarose column were also used for hybridization. In these experiments hybridizations were carried out under conditions which would allow R-loop formation (Thomas, M., White, R. L., and Davis, R. W. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 2294-2298) and the hybridized RNA was separated from unhybridized RNA by Sepharose chromatography. The validity of the method was demonstrated by programming an in vitro protein-synthesizing system with selected RNA.

The coupling of DNA to a solid support is time-consuming and usually requires large quantities of DNA (5). All the described systems are capable of selecting messenger RNA but an unspecific background hybridization makes them less useful for mRNA species which are present in small quantities. Exclusion chromatography on agarose permits the fractionation of high molecular weight nucleic acids and thus provides a simple alternative method to achieve separation between RNA-DNA hybrids and nonhybridized RNA. In the present communication we describe the purification of RNA-DNA hybrids by exclusion chromatography on agarose (Sepharose 2B).

**EXPERIMENTAL PROCEDURES**

**Cell and Virus—**Ad2 was propagated in suspension cultures of HeLa S3 cells in Eagle’s spinner medium supplemented with 7% calf serum as described before (6). Suspension cultures of one ad2-transformed rat cell line (A,T,C,, see Ref. 7) were cultivated in Eagle’s spinner medium supplemented with 10% calf serum.

**Preparation of Restriction Endonuclease Fragments and Complementary Strands of ad2 DNA—**Restriction endonuclease fragments of ad2 DNA were prepared as previously described (6). Before use, the purity of the fragments was documented by agarose gel electrophoresis. Intact viral l- or r-strands were prepared by equilibrium centrifugation in CsCl of denatured ad2 DNA in the presence or ribopoly(U,G) as described by Tibbetts et al. (8). The viral r-strand refers to the strand of ad2 DNA which is transcribed in the rightward direction. The leftward transcribed strand of ad2 DNA is referred to as the l-strand (see Ref. 9 concerning the nomenclature of the complementary strands from ad2 DNA).

**Extraction of RNA—**HeLa cells were infected with purified ad2 at an input multiplicity of 20,000 virus particles/cell. Cycloheximide (25 μg/ml) was added at 2 h postinfection, the cells harvested at 7 h postinfection, and “early” cytoplasmic RNA was prepared. Late-adenovirus cytoplasmic RNA was prepared from cells harvested 18 h postinfection with an input multiplicity of 2000 virus particles/cell. RNA was in both cases extracted by the method of Brawerman et al. (19) and precipitated twice with ethanol.

**Liquid Hybridization—**The RNA was melted in 80% formamide at 65°C for 15 min immediately before hybridization. The formamide concentration was adjusted to 50% and the RNA hybridized to separated strands of ad2 DNA in a buffer which contained: 0.4 M NaCl, 0.1% sodium dodecyl sulfate, 1 mM ethylenediaminetetraacetic acid (EDTA) and 0.02 M piperazine-N,N'-bis(2-ethanesulfonic acid), pH 6.4. Usually 1 to 3 μg of DNA was hybridized with 200 to 300 μg of cytoplasmic RNA in a total volume of 1 ml at 50°C for 16 h. Restriction endonuclease fragments of ad2 DNA (1 to 2 μg) were denatured in 0.3 M sodium hydroxide for 10 min at room temperature, neutralized, and hybridized to a cytoplasmic RNA as described above except that the formamide concentration was increased to 80%. Formamide for the hybridization experiments was purchased from Merck, Darmstadt, West Germany, and purified as described previously (11).

**Sephadex 2B Chromatography—**A column (0.9 x 20 cm) of Sepharose 2B (Pharmacia Fine Chemicals) was prepared in a buffer which recovery from the phase system. Simian virus 40 (SV40) DNA, both double- and single-stranded, has been covalently linked to a solid support and used for the selection of SV40 mRNA.
contained 0.5 M LiCl, 0.5% SDS, 1 mM EDTA, and 0.05 M Tris-HCl, pH 7.9. All of the radioactivity was excluded from the column due to the large size of adenovirus DNA (Fig. 1A). Fractionation of $^{32}$P-labeled cytoplasmic RNA, on the other hand, showed one broad peak, apparently well separated from the excluded fractions (Fig. 1B). These experiments suggest that it should be possible to separate RNA which is hybridized to the complementary strands of ad2 DNA from nonhybridized RNA because the former would elute in the void volume together with the strands whereas the latter would be included into the gel matrix.

Late $^{32}$P-labeled cytoplasmic ad2 RNA was hybridized in solution to the viral r-strand, and the hybridization mixture was subsequently fractionated on a Sepharose 2B column. A well defined peak of radioactivity accounting for around 6% of the total input radioactivity was found in the void volume (Fractions 7 to 9, Fig. 1C). These fractions were well separated from the nonhybridized RNA which was found, as a broad peak, among the fractions included into the gel matrix. Chromatography of a cytoplasmic RNA prepared from mock-infected cells and hybridized to the complementary strands of ad2 DNA, gave, as expected, no peak of radioactivity in the excluded fractions (data not shown). These results suggest that chromatography on Sepharose 2B may constitute a rapid and simple method to separate RNA-DNA hybrids from nonhybridized RNA. The recovery of RNA is 95 to 100%. It should, however, be emphasized that quick denaturation of the RNA, before hybridization, seems to be important since

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**RESULTS AND DISCUSSION**

**Selection of RNA-DNA Hybrids by Exclusion Chromatography**—Several exclusion chromatography matrices are presently available for fractionation of large macromolecules, such as nucleic acids. Among those we decided to investigate whether 2% agarose (Sepharose 2B) would be useful for separation of RNA-DNA hybrids from unhybridized RNA. Complementary strands of the ad2 genome (11.5 x 10^6 daltons) labeled with $^3H$ thymidine were first fractionated on a Sepharose 2B column in a buffer which contained 0.5 M LiCl, 0.5% SDS, 1 mM EDTA, and 0.05 M Tris-HCl, pH 7.9. All of the radioactivity was excluded from the column due to the large size of adenovirus DNA (Fig. 1A). Fractionation of $^{32}$P-labeled cytoplasmic RNA, on the other hand, showed one broad peak, apparently well separated from the excluded fractions (Fig. 1B). These experiments suggest that it should be possible to separate RNA which is hybridized to the complementary strands of ad2 DNA from nonhybridized RNA because the former would elute in the void volume together with the strands whereas the latter would be included into the gel matrix.

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The abbreviation used is: SDS, sodium dodecyl sulfate.
The synthesis of a completely different set of polypeptides: hybridization to the complementary strands of ad2 DNA. Early viral mRNA selected on the viral r-strand directed the synthesis of two predominant polypeptides: a set of polypeptides in the molecular weight range of 42,000 to 50,000 (E42-50K) and a 15,000 polypeptide. In agreement with others (19) we observed that the E42,000 to 50,000 polypeptides are heterogeneous in SDS-polyacrylamide gels and split up into several components with molecular weights around 42,000 to 50,000 in SDS-polyacrylamide electrophoresis. Early viral mRNA selected by hybridization to the viral l-strand directed the synthesis of a completely different set of polypeptides: E75,000, E26,000, E18,500, and E11,000, as well as four minor polypeptides: E60,000, E17,500, E16,000, and E12,500. Early adenovirus mRNA selected by hybridization to the viral r-strand thus showed little or no contamination with early-viral mRNA selected by the l-strand of the viral genome. Furthermore, mRNAs for polypeptides of cellular origin which are present in large amounts early after ad2 infection were not observed when selected mRNAs were translated. Consequently the selection methods show high specificity with little or no contamination of unhybridized mRNA. The ad2 coded early-polypeptides synthesized in response to the selected mRNAs corresponds in number and approximate molecular weights to the ones previously described using the urea phosphate-hydroxyapatite system for selection of mRNAs (2, 19).

Selection of mRNA from ad2 transformed Cells—Cell lines transformed by ad2 contain variable amounts of the viral genome integrated into cellular DNA (7). Different sequences transcribed early during productive infection are transcribed in the transformed cells (20) although the amount of viral specific RNA only amounts to around 10% of the amount present early during productive infection (21). To demonstrate the resolving power of our hybrid purification procedure we selected viral specific mRNA from an ad2-transformed rat cell line, A.T.C. Cytoplasmic RNA extracted from this cell line which contains around 90% of the ad2 genome (7) was hybridized in solution to the complementary strands of ad2 DNA.

Fractions corresponding to the void volume (Fractions 7 to 9) were pooled and precipitated with ethanol. The RNA-DNA hybrids melted in formamide followed by poly(U)-Seph-

Isolation of Hybrids between Early ad2 RNA and the Separated Strands of Adenovirus DNA—The purity of the selected mRNAs was tested by in vitro translation. Fig. 2 shows the result when “early” ad2 RNA was hybridized to the complementary strands of the ad2 genome. Early adenovirus mRNA selected on the viral r-strand directed the synthesis of two predominant polyepptides: a set of polyepptides in the molecular weight range of 42,000 to 50,000 (E42-50K) as well as an E15,000 polyepptide. In agreement with others (19) we observed that the E42,000 to 50,000 polyepptides are heterogeneous in SDS-polyacrylamide gels and split up into several components with molecular weights around 42,000 to 50,000 in SDS-polyacrylamide electrophoresis. Early viral mRNA selected by hybridization to the viral l-strand directed the synthesis of a completely different set of polyepptides:
Hybridized RNA was isolated as described above and subsequently translated in an in vitro protein-synthesizing system (Fig. 3). Viral specific messenger RNA selected by hybridization to the viral r-strand directed the synthesis of two polypeptides E42,000 to 50,000 and E15,000, whereas l-strand-selected mRNA directed the synthesis of three polypeptides, E18,500, E17,500, E16,000. One additional polypeptide around 38,000 in molecular weight was also selected by the viral l-strand. It is of interest that early adenovirus mRNA selected by the viral l-strand gives no evidence for such an early polypeptide. The origin of this 38,000 polypeptide is therefore at present unknown. No other polypeptides were synthesized in response to the added RNA suggesting that our method for selection of mRNA gives little contamination of unhybridized RNA even when the selected RNA constitutes a minor fraction of the total mRNA. Consistent results have also been obtained by selection of mRNA from several other ad2-transformed rat and hamster embryo cell lines.

Use of Restriction Enzyme Fragments for Hybrid Selection—Restriction endonuclease fragments with a size large enough to make them excluded from Sepharose 2B were also used as probes for hybridization. Experiments using [3H]thyridine-labeled restriction enzyme fragments showed that a minimum size of around 7 x 10^6 daltons was required to make double-stranded DNA elute in the void volume of a Sepharose 2B column. The DNA was first melted at an alkaline pH, neutralized, and hybridized to the RNA in high formamide (80%) in order to favor RNA-DNA hybridization as described by Thomas et al. (22). The R-loops which are formed between an mRNA and its complementary DNA sequences under these conditions (11, 22), were isolated on the column. Fractions which eluted in the void volume of the column were pooled, precipitated with ethanol, collected, and divided into two equal aliquots. One aliquot was directly precipitated with ethanol while the other was melted by heating in 90% FA buffer followed by ethanol precipitation. Both samples were reprecipitated with ethanol dissolved in sterile water and subsequently translated in vitro. The unmelted sample served as a control for the hybridization since RNA in RNA-DNA hybrids will not be translated in a cell-free system (23). Polypeptides which were synthesized in vitro exclusively in response to the melted RNA were considered as selected by the appropriate DNA fragment. A low background level of translation was sometimes observed when the unmelted RNA was used for translation. This was most frequently observed when DNA fragments with a molecular weight close to the minimum size required for exclusion from Sepharose 2B, i.e., around 7 x 10^6 daltons, were used for selection and was most likely due to aggregated mRNAs which occasionally contaminated the selected RNA-DNA hybrids.

Fig. 4 shows the result of one experiment in which two different restriction endonuclease fragments of ad2 DNA were used as probes for hybridization. Ad2 early-cytoplasmic RNA hybridized to the Eco RI A fragment (map position 0-58.5, 2 Unpublished information.
molecular weight $13.6 \times 10^3$) selected two ad2 early mRNAs encoding polypeptides E42,000 to 50,000 and E15,000 (Fig. 4A). Hybridization of a late-cytoplasmic ad2 RNA to fragment Bam HI B of ad2 DNA (map position 0-29.1, molecular weight 6.7 $\times$ 10^4) selected three ad2 mRNAs encoding polypeptides IVa_, 15,000, and IX (Fig. 4B). Polypeptide IX which is not readily visible in Fig. 4B due to the endogenous globin synthesis, was revealed by immunoprecipitation with a monoclonal antibody against virion polypeptide IX (14) (data not shown). The 15,000 polypeptide synthesized in response to the selected mRNA was immunoprecipitated with an antiserum raised in syngeneic rats against an ad2-transformed rat cell line (A,T,C,) (16), suggesting that the mRNA encoding the selected mRNA was immunoprecipitated with an antiserum against virion polypeptide IX (14) (data not shown). The 15,000 polypeptide synthesized in response to the selected mRNA was immunoprecipitated with an antiserum raised in syngeneic rats against an ad2-transformed rat cell line (A,T,C,) (16), suggesting that the mRNA encoding the late 15,000 polypeptide is identical with the mRNA encoding the early 15,000 polypeptide (E15,000). Large scale purification of mRNAs by purification of R-loops on the Sepharose 2B column is, however, hampered by the low efficiency of hybridization between the RNA and its complementary DNA sequence under R-loop conditions.

In conclusion, our present results demonstrate that chromatography on Sepharose 2B is a simple and efficient method for isolation and purification of RNA-DNA hybrids. The selection of early adenovirus mRNA by l- and r-strand DNA clearly shows that the specificity which is obtained with this method is equal to or better than what can be achieved with other methods. Furthermore selection of adenovirus mRNA from an ad2-transformed rat cell line, in which the viral mRNA constitutes less than 1% of the total RNA content of the cell, shows that the method is valid also for systems in which the RNA species to be selected is present in very low amounts.

The size of the DNA probe used for hybrid selection and the possibility to separate its complementary strands appear at present to be the major limitation of the method. This problem can in many cases be circumvented by cloning fragments in a suitable bacterial vector. The complementary strands of bacteriophage $\lambda$, a commonly used cloning vehicle, are easy to separate and of sufficient size to make our present method applicable. Recombinant DNA research is progressing at a very rapid pace and libraries of cloned fragments from different organisms are becoming available. We thus expect that this simple method for isolation and purification of specific mRNA species should have a wide application.

In a recent communication we used this method in combination with the hybrid arrested cell-free translation method (23) in order to map a late-ad2 minor mRNA encoding a viral maturation protein on the ad2 genome (24).

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