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**Purification of specific adenovirus 2 RNAs by preparative hybridization and selective thermal elution**

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**ABSTRACT**

A method is described for the preparative isolation of highly purified adenovirus RNA species. Cytoplasmic RNAs from cells infected with adenovirus 2 were selected by hybridization to viral DNA fragments bound to nitrocellulose membranes. A series of washes at elevated temperatures (50-70<sup>o</sup>) determined conditions at which the true hybrids were stable but non-specific RNA was removed. This temperature has been found to correlate with the base composition of the DNA fragment. After washing at this predetermined temperature, the specific RNA was eluted at 85<sup>o</sup>. The purity of the eluted RNA was greater than 95% as determined by size, sequence specificity, and template activity in an *in vitro* protein synthesizing system. The method described should be generally useful for purification of specific RNAs.

**INTRODUCTION**

Characterization of individual mRNA species has become increasingly important in the study of eukaryotic gene expression and has increased the understanding of the extensive processing mechanisms involved in mRNA synthesis. In light of recent findings that demonstrate the presence of multiple inserts within the coding sequences of a gene (1-4) and show that a specific gene sequence may express different products by synthesizing overlapping mRNAs (5-8), it is often necessary to identify functional genes by purifying the mRNA coding for the gene product.

Several methods have been described for the preparative isolation of specific RNA molecules by hybridization to DNA immobilized on a matrix (9-12), or by hybridization to DNA in liquid and purification of the DNA-RNA hybrid (13). The nitrocellulose membrane technique has several advantages: DNA can be bound to membranes by rapid and efficient methods (14)

and the DNA membranes, which may contain large amounts of valuable DNA, are reusable. In addition, simultaneous selections may be performed on the same RNA preparation with different DNA fragment membranes, and the purified mRNA can be eluted quantitatively from the membranes. In this communication, we present a modification of this technique which allows the isolation of specific mRNAs that remain intact and retain their biological activity. The specificity of the purification is demonstrated by analysis of three different regions of the adenovirus 2 genome. Each DNA region selects unique mRNA species and gives a thermal elution profile characteristic of the G + C content of the DNA. The isolated RNA has been analyzed both by sequence content and size, and by the specificity of polypeptides synthesized when the RNA is used to prime in vitro translation systems.

#### MATERIALS AND METHODS

The labeling and isolation of poly(A)-containing cytoplasmic RNA from adenovirus 2 infected cells has been described previously (15,16,17). Restriction endonuclease fragments of adenovirus 2 DNA were prepared according to published procedures (15,16,18-21). The fragments used in this study were generated by cleavage of adenovirus 2 DNA with restriction endonucleases Eco RI, Sma I, Bal I and Bgl II. DNA was denatured in 0.1X SSC (1X SSC is 0.15 M NaCl, 0.015 M NaCitrate), adjusted to 4X SSC and bound to nitrocellulose membranes (14). The amounts of DNA fragments ranged from 50-100  $\mu$ g equivalents (1  $\mu$ g equivalent is the amount of the fragment derived from 1  $\mu$ g of whole adenovirus 2 DNA) per 27 mm membrane.

To perform hybridization selections, membranes were first soaked in hybridization buffer (0.75 M NaCl; 0.1 M Tris-HCl, pH 7.4; 0.002 M EDTA; 0.5% SDS; 50% formamide, deionized and buffered with 0.02 M NaPhosphate, pH 6.8 (22); and 200  $\mu$ g/ml purified tRNA from E. coli or wheat germ) at 37<sup>o</sup> for 30 min. Labeled poly(A)-containing RNA was resuspended in 5  $\mu$ l H<sub>2</sub>O and then 200  $\mu$ l of hybridization buffer were added. Hybridization was for 4 to 16 hr at 37<sup>o</sup>.

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The supernatant was removed and the membranes were washed at room temperature according to the following protocol:

- i) Three times with buffer 1 (0.01 M Tris-HCl, pH 8.0; 0.002 M EDTA; 0.5% SDS), then transferred to a clean tube.
- ii) Three times with buffer 1, once with buffer 2 (0.01 M Tris-HCl, pH 7.4; 0.002 M EDTA), then transferred to a clean tube.
- iii) Three times with buffer 2 or until a background of about 0.02% of the original input cpm was obtained.

Each wash was for three minutes with 5 ml of buffer; buffer was removed by suction after each wash.

Thermal washes were initially done at increments of 10° between 50° and 70° to establish conditions for each DNA sequence that would elute non-specific RNA without significant loss of the specific hybrids. The membranes were washed twice with 2 ml elution buffer (0.002 M EDTA, pH 7.0; 10 µg/ml tRNA) for 3 min. Between washes the membranes were chilled in ice. Specific RNA was eluted at 85° for 3 min; generally two successive elutions were pooled. Once the optimum for removing non-specific RNA was determined, membranes were washed repeatedly at this temperature until the cpm in the wash were negligible. The 85° elution then followed. For gel electrophoresis the eluted RNA was precipitated with 0.1 M NaCl and 2 volumes of 95% ethanol. RNA to be analyzed by in vitro translation was made 0.5 M in KCl, and poly(A)-containing RNA was reselected on oligo dT-cellulose (23).

Membranes were regenerated by immersion in 0.1 N NaOH in 2X SSC for 30 min. They were then washed 5X with 5 ml of 2X SSC and dried at room temperature. Membranes were used three times.

Selected mRNAs were translated in a nuclease-treated reticulocyte lysate prepared according to published procedure (24). Precipitated RNA was spun down at 25,000 rpm, the pellet dried under nitrogen, and resuspended in 5-10 µl of H<sub>2</sub>O. Translation reactions were in a volume of 25 µl and contained 10 µl of nuclease-treated lysate, 5 µl <sup>35</sup>S-methionine (700-1000 Ci/mmol; Amersham Corp.), 1-2 µl selected RNA,

1 mM MgOAc, 80 mM KOAc, 20  $\mu$ g/ml calf thymus tRNA, 0.5 mM spermidine, 20 mM Hepes, pH 7.6; 1 mM ATP, 0.2 mM GTP, 1 mM DTT, 9 mM creatine phosphate, 35  $\mu$ g/ml creatine phosphokinase, and 60  $\mu$ M each of 19 unlabeled amino acids. Incubation was at 30°C for 1 hr. An aliquot was removed for TCA precipitation, and the remainder processed for gel electrophoresis.

Wheat germ in vitro translations were performed as described (25). Translation reactions were carried out in a 25  $\mu$ l volume containing 12.5  $\mu$ l wheat germ S-30 extract, 2.5  $\mu$ l  $^{35}$ S-methionine, 1-2  $\mu$ l selected RNA, 0.35 mM spermidine, 1.5 mM MgOAc, 110 mM KOAc, 20  $\mu$ g/ml wheat germ tRNA, 20 mM Hepes, pH 7.6, 37.5  $\mu$ M of each of 19 unlabeled amino acids, 1 mM ATP, 0.2 mM GTP, 1 mM DTT, 9 mM creatine phosphate and 35  $\mu$ g/ml creatine phosphokinase. Reactions were carried out at 23°C for 2 hr.

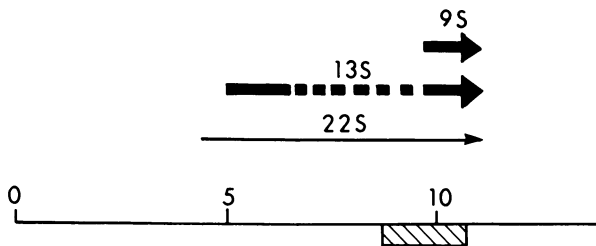
Samples from either reticulocyte or wheat germ translations were prepared for polyacrylamide slab gel electrophoresis in the following manner: Samples were made 5 mM in EDTA and 100  $\mu$ g/ml in RNase A and incubated for 15 min at 30°C. After the addition of 3 volumes of sample buffer (100 mM Tris-HCl, pH 6.8, 2.5 SDS, 12.5% glycerol, 0.7 M mercaptoethanol and 0.012% Bromphenol Blue), samples were heated at 100°C for 3 min. Aliquots were fractionated by electrophoresis in a modified Laemmli gel system (26) with an acrylamide:bisacrylamide ratio of 30:0.174. Slab gels were 1.5 mm thick, with a 5% stacking gel 1.5 cm in length, and an 8 cm resolution gel of 17.5%. Gels were run at 22.5 mA (constant current) for 3-4 hr, and prepared for fluorography as described (27).

## RESULTS

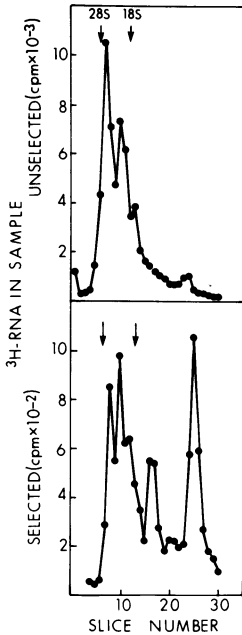
The adenovirus virion contains a double-stranded, linear DNA of  $23 \times 10^6$  daltons. Early cytoplasmic RNA is transcribed from four discrete regions (for review, see 28). Each region accounts for about 10% of the single-stranded coding capacity of the genome. After the onset of viral DNA synthesis, almost all of the genomic capacity is expressed as late RNA. At early times in infection less than 10% of the mRNA synthesized

is viral specific whereas at late times at least 80% is viral in origin. In order to study the structure, metabolism, and function of individual adenovirus mRNAs, we required a method for mRNA purification. For this purpose we tested the filter hybridization and elution procedure developed by Buttner *et al.* (9). Because of our interest in early region 1, the sequences that contain the transforming gene(s) (see 28), we performed initial experiments with a DNA fragment from within this region. By convention, the adenovirus 2 genome is divided into 100 map units, each map unit corresponding to approximately 350 base pairs. Early region 1 is located at the left end of the genome, map positions 0-11. The 8.7-10.7 DNA fragment contains some of the sequences present in three mRNAs (21) (Fig. 1). At late times in infection, the 9S and 13S RNAs are labeled in at least 10-fold excess over the 22S RNA (21).

The method of Buttner *et al.* (9) was applied to selections with 8.7-10.7 DNA. After hybridization the membranes were washed as described and bound RNA was eluted at 85°. The purified and unpurified RNA samples were displayed on 98% formamide-polyacrylamide gels (Fig. 2). The eluted RNA was substantially enriched in 9S and 13S size classes (bottom



**FIGURE 1:** Late cytoplasmic RNAs coded for by the 8.7-10.7 region of the adenovirus 2 genome. The map positions of the RNAs are taken from Spector, McGrogan and Raskas (21). The dashed line represents intervening sequences in the 13S RNA. The thickness of the arrows reflects the relative molar amounts of  $^3\text{H}$ -uridine incorporated into each species in a 2 hr label late in infection (21). The hatched bar indicates the 8.7-10.7 region used for hybridization-selection.



**FIGURE 2:** Late RNA selected by 8,7-10,7 adenovirus 2 DNA without selective thermal elution. Poly(A)-containing cytoplasmic RNA was isolated from adenovirus 2 infected cells labeled with <sup>3</sup>H-uridine from 12 to 14 hr after infection (16). The top panel is an electrophoretic profile of an aliquot of this RNA displayed on a 98% formamide, 3.5% polyacrylamide gel (22). The gel was run for 6.5 hr at 2.5 ma/gel; 2 mm gel slices were eluted in 0.3 ml of concentrated ammonium hydroxide and counted. RNA was selected by hybridization of 1.2x10<sup>7</sup> cpm to 8.7-10.7 DNA (Bal I-G/Sma I-E) for 4 hr. Elution was performed essentially as described by Buttner *et al.* (9). The profile of an aliquot of RNA (total eluted was 6.5x10<sup>5</sup> cpm) eluted at 85° is shown in the bottom panel.

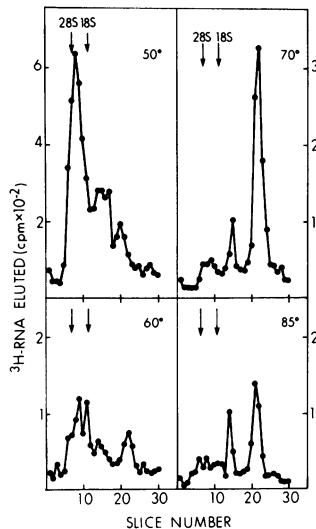
panel), but there was significant contamination with heterogeneous 18S-28S RNAs. The sequence specificity of the labeled eluted RNA was examined by hybridization to viral DNA fragments. The higher molecular weight RNAs annealed to all regions of the genome (data not shown) in amounts predicted by the known distribution of viral species in unfractionated late RNA (16).

Selective thermal elution of specific late adenovirus RNAs

The experiments described above suggested that the conditions used for washing the hybridization membranes were not sufficiently stringent to remove all the non-specific RNA. Some RNA might have remained bound to the filters after extensive washing because of a limited homology. There should then be a temperature at which the mismatched RNA would elute, but the true hybrids would be stable. To test this possibility

the following procedure was utilized: A standardized procedure was used for washing the filters at room temperature (see Methods). The membranes were then incubated in elution buffer (0.002 M EDTA, pH 7.0, 10  $\mu\text{g/ml}$  tRNA) for 3 min at different temperatures.

The RNA eluted at each temperature was displayed on 98% formamide gels. When this procedure was applied to RNA selected with 8.7-10.7 DNA, the results shown in Figure 3 were obtained. The contaminating non-specific RNA, migrating as greater than 13S, eluted primarily at 50° and to a lesser extent at 60°. Most of the lower molecular weight RNA was eluted at 70° and 85°. The profile of material eluted at the higher temperatures compared favorably with the profile of 8.7-10.7 specific RNA revealed by hybridization of late



**FIGURE 3:** Thermal step elution of late RNA selected by 8.7-10.7 DNA. An RNA sample ( $4.1 \times 10^6$  cpm) prepared as described in Figure 2 was hybridized for 4 hr to 8.7-10.7 DNA. Filters were washed at room temperature, as described in Materials and Methods. RNA was eluted sequentially by two washes at each temperature indicated and fractionated on gels as for Figure 2. Electrophoresis was at 2.5 ma/gel for 5.5 hr. The cpm eluted at each temperature were: 50°,  $4.1 \times 10^4$ ; 60°,  $1.2 \times 10^4$ ; 70°,  $2.1 \times 10^4$ ; 85°,  $6.3 \times 10^3$ .

RNA, after fractionation by size (21). In subsequent experiments non-specific RNA was removed by repeated washing at 55° until no more counts eluted. The sequence specificity of RNA eluted at 85° was then examined: After gel electrophoresis, the RNA in each slice was hybridized to viral DNA fragments. The selected RNAs consisted almost entirely of those species illustrated in Figure 1 (data not shown). In this and all the other elutions performed, no evidence could be found of any appreciable degradation of the RNA, as assayed by migration in formamide gels.

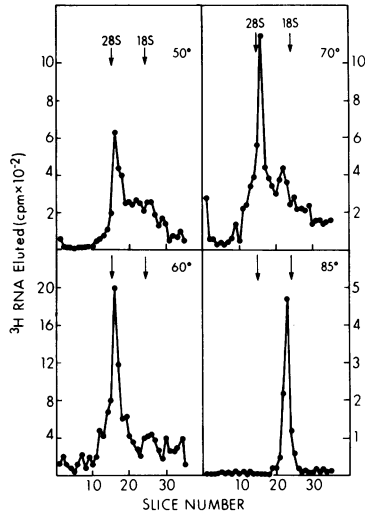
### Dependence of thermal elution on the G + C content of the hybrids

We found that the RNA specified by each region of the adenovirus genome has distinct thermal elution characteristics. These characteristics coincide with the guanosine and cytosine content of the region. An example of this phenomenon is illustrated in Figure 4. Size fractionation of radioactive early cytoplasmic RNA and subsequent hybridization to specific viral DNA fragments has identified a 20S RNA from early region 2 (11,15,19). Labeled early RNA was hybridized preparatively to a 60.2-63.6 DNA fragment which contains the 3' sequences present in this 20S RNA. Specific RNA was not recovered in washes performed at temperatures as high as 70°. The 20S RNA was specifically eluted at 85°. Again, the eluted RNA was essentially free of other labeled species (Goldenberg and Raskas, submitted for publication). The temperature required to remove all of the mismatched RNA is 10° higher than that determined for the 8.7-10.7 region. This corresponds to a 20% difference in G + C content (29). As determined by thermal denaturation mapping, the actual difference in melting temperature between the 8.7-10.7 region and the 60.2-63.6 region is at least 8° (30).

### Purity of eluted RNA as assayed by in vitro translation

To provide an assay for functional purity, eluted RNA was used as a template for in vitro translation. RNAs specified by three regions of the genome were tested (Fig. 5). In addition to early RNA selected by 60.2-63.6 DNA and late RNA

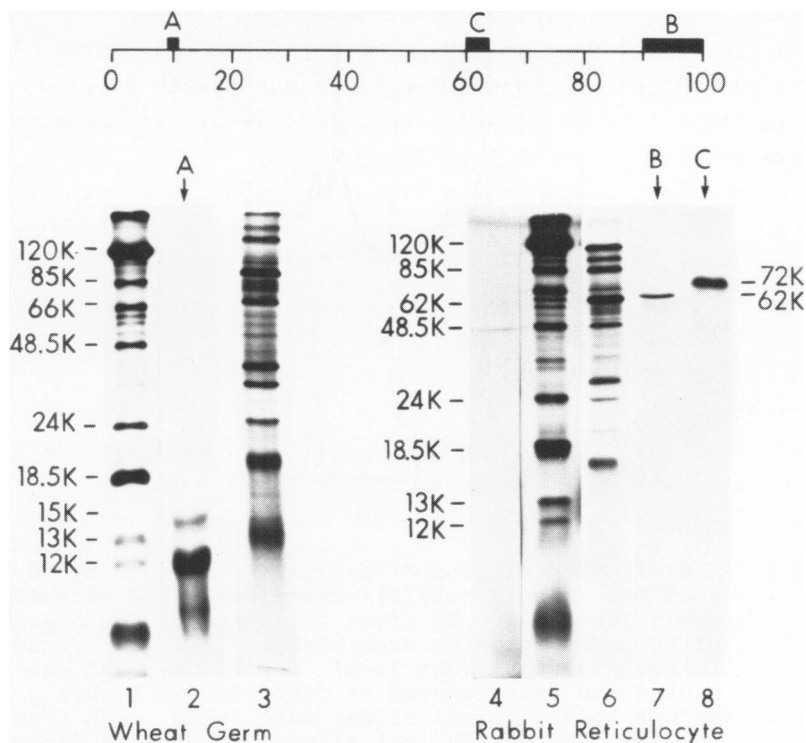




**FIGURE 4:** Thermal step elution of early RNA selected by 60.2-63.6 DNA. Early poly(A)<sup>+</sup> cytoplasmic RNA ( $4.0 \times 10^7$  cpm), labeled 3-6 hr after infection in the presence of 20  $\mu\text{g}/\text{ml}$  cytosine arabinoside (17), was hybridized to 60.2-53.6 DNA (Bgl II-J), for 16 hr. RNA was eluted and fractionated as described in Figure 3 except that 1 mm gel slices were taken. RNA from the 50°, 60° and 70° gel slices was counted directly in  $\text{NH}_4\text{OH}$ . To obtain the 85° profile, RNA was eluted from the gel slices in 0.5  $\mu\text{l}$  of 6X SSC, 0.1% SDS for 48 hr at 66°C; 0.03  $\mu\text{l}$  aliquots of each fraction were assayed. The cpm eluted at each temperature were: 50°,  $2.5 \times 10^5$ ; 60°,  $1.2 \times 10^5$ ; 70°,  $4.0 \times 10^4$ ; 85°,  $2.0 \times 10^4$ .

selected by 8.7-10.7 DNA, late RNA specific for the 89.7-100 region (a gift of D. Carlson) was also translated.

RNA selected with 8.7-10.7 DNA was translated in a wheat germ extract. Proteins of 12K and 15K were synthesized (Lane 2). The smaller polypeptide comigrates with virion polypeptide IX (31-32), which is also present in disrupted virion preparations or translation assays of unfractionated late RNA. Its RNA has been identified as 9S in size in previous studies (33). A 15K protein is present in translation assays of unfractionated late RNA or in labeled extracts of late infected cell proteins. It has been assigned to the 4.4-17.0 region by previous investigators (34). Because very little 22S RNA is labeled at late times, even in preparations labeled for long (10 hr)



**FIGURE 5:** In vitro translation of hybridization-selected viral RNAs. Early poly(A)-containing cytoplasmic RNA was prepared from cells 9 hr after infection in the presence of 20  $\mu\text{g/ml}$  of cytosine arabinoside. Late RNA was prepared 22 hr after infection. Fragment-specific RNA was selected as described in Materials and Methods. In vitro translation was performed in wheat germ (WG) or reticulocyte lysate (R) and products were identified as described. Lanes 1 and 5:  $^{35}\text{S}$ -methionine labeled adenovirus 2 proteins from purified disrupted virions.

Lane 2: Late RNA selected by 8.7-10.7 DNA (Region A in this figure), WG.

Lane 3: Unselected late RNA, WG.

Lane 4: No RNA added, R.

Lane 6: Unselected late RNA, R.

Lane 7: Early RNA selected by 60.2-63.6 DNA (Region B), R.

Lane 8: Late RNA selected by 89.7-100 (Eco RI-C) DNA (Region C), R.

intervals, and since considerable amounts of 13S RNA are labeled late (21), we tentatively assigned the 15K protein to the 13S RNA. Further translation experiments with purified early and late RNAs using RNAs from the 4.4-10.7 region support this assignment (manuscript in preparation).

Two preparations were translated in rabbit reticulocyte lysates. Early region 2 (61.6-75.2) specifies a 72K single-stranded DNA binding protein (34-36). Early RNA selected with a 60.2-63.6 DNA fragment gave a translation product of the expected 72K molecular weight. The translation product of late 89.7-100 specific RNA was a 62K polypeptide (Lane 7). This product comigrated with the 62K polypeptide IV of disrupted virions (Lane 6). A polypeptide of identical size was formed when extracts were provided with unfractionated poly(A) RNA harvested late in infection (Lane 5). This 62K protein is the adenovirus fiber (31,37), a major antigenic determinant which forms the virion spikes. Previous genetic and biochemical experiments have demonstrated that this late protein is coded for, in part, by sequences in the 89.7-100 fragment (13,38).

## DISCUSSION

This communication demonstrates that the nitrocellulose membrane hybridization-elution procedure, when performed with appropriate thermal elution steps, can purify specific messenger RNAs to more than 95% functional homogeneity. The RNAs are structurally and functionally intact: They migrate as discrete peaks on denaturing gels, retain 3' poly(A) sequences as assayed by rebinding to oligo dT-cellulose and direct the synthesis of complete polypeptides in vitro. Rehybridization of the purified RNA is highly specific and very efficient, more than 80% anneals to an excess of the homologous fragment (data not shown). Since selected RNA rehybridizes extremely well, specific RNAs of even greater purity might be obtained by performing a second round of hybridization and elution. We have compared the amounts of selected RNA with the amounts of the same species in unselected samples as determined by direct hybridization. The efficiency of selection generally ranges from twenty to fifty percent. Fragments as

small as 300 nucleotides have been used to select specific RNA, although the efficiency of hybridization is lower with the smallest fragments.

The elution profiles obtained with different regions of the adenovirus 2 genome illustrate the importance of determining the thermal elution characteristics of DNA-RNA hybrids for each set of sequences. There are several other aspects of the technique which should govern its applicability to different systems: 1) Sufficient complementary DNA must be available so that DNA excess conditions are present in the reaction. 2) Although the membranes can be reused several times, we have found that the hybridization efficiency probably decreases after each use. We believe that DNA is most likely lost during the regeneration by 0.1 N NaOH treatment. It is unlikely that DNA is removed under these hybridization conditions (S. Bhaduri, personal communication). 3) Generally a low level of radioactivity in the RNA is desirable to monitor the specificity of the elution. We use a sufficient number of counts in each reaction to allow detection of backgrounds as low as 0.002%.

In addition to the isolation of RNAs for in vitro translation, the modified procedure may be used to map cytoplasmic RNAs. It is especially useful in systems with complicated transcription products such as adenovirus 2, which synthesizes several families of mRNAs that share common 5' and 3' sequences. For the isolation of individual RNA species with common 3' sequences, fractionation of RNA by size can be combined with selection using DNA fragments that encode unique portions of the overlapping RNAs. RNAs that contain common 5' leader sequences may hybridize to DNA sequences that do not code for unique gene products. By rehybridization of "leader selected" RNA to the appropriate DNA fragments, the body of the mRNA can be localized to a unique region. Analysis of nuclear precursors of specific genes also may be performed by selection with DNA fragments containing insertion sequences or sequences restricted to the nucleus (Goldenberg and Raskas, submitted for publication). The fate of specific radiolabeled messenger precursors can then be followed. This

technique should also prove useful in identifying recombinant DNA clones containing specific genes. The DNA from positive clones can be used to select mRNA of the cloned gene, which may then be translated in vitro.

Two other methods of preparative DNA-RNA hybridization are in general use. The solution hybridization procedure described by Lewis et al. (13) has also been used to purify viral mRNAs. In this protocol the DNA is not recoverable, and the isolation of the RNA requires additional purification steps. DNA has also been covalently coupled to a matrix such as cellulose or sepharose, a method which permits extensive reutilization of the DNA (39,40). However, the coupling process requires extensive chemical manipulations and its efficiency is highly variable in our experience. A variant of this procedure involves the synthesis of cDNA using purified specific RNA and oligo dT-cellulose as primer (10). This latter method is useful only if microgram amounts of purified specific RNA are already available. Our results suggest that these methods may be more effective if careful attention is given to the G + C content of the DNA in designing elution conditions.

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