A RAPID METHOD FOR THE ISOLATION AND PARTIAL PURIFICATION OF SPECIFIC EUCARYOTIC MESSENGER RNA'S.

G. C. Rosenfeld, J. P. Comstock, A. R. Means and B. W. O'Malley

Departments of Obstetrics and Gynecology,
Physiology, Medicine and Biochemistry

Vanderbilt University School of Medicine
Nashville, Tennessee 37232

Received March 21, 1972

SUMMARY - Nitrocellulose filters in the presence of high salt are demonstrated to selectively retain 2½% of a filtered total nucleic acid preparation obtained from hen oviduct. The bound RNA can be extracted from the filters, in the presence of 0.5% SDS, and subsequently shown to code for the in vitro synthesis of two hormone-dependent proteins (ovalbumin and avidin) in a heterologous rabbit reticulocyte lysate system. This technique allows for the rapid bulk isolation and partial purification (≈50-fold) of distinct species of messenger RNA from the same tissue.

There is increasing evidence that a number of mRNA's isolated from eucaryotic cells contain adenylate-rich sequences (1,2,3,4). It has been shown that the presence of these residues can be used to advantage in the selective isolation of mRNA from other species of polysomal RNA since polyadenylic sequences bind preferentially to nitrocellulose filters (Millipore) (5). Although it appears that mRNA can be prepared using this technique (5,6), it has not yet been demonstrated that the RNA recovered from the filter is still capable of initiating in vitro synthesis of specific proteins.

We have recently reported the isolation of an estrogen dependent mRNA from chick and hen oviduct which codes for the synthesis of ovalbumin in a heterologous cell-free system (6,7). Preliminary experiments demonstrated that this mRNA contains poly (A) segments. On the assumption that ovalbumin mRNA could be preferentially bound to nitrocellulose filters and subsequently eluted, we have devised a rapid technique for the bulk isolation and partial purification of specific mRNA's. These mRNA molecules are then capable of

coding for the in vitro synthesis of ovalbumin and avidin in a heterologous rabbit reticulocyte lysate system.

MATERIALS AND METHODS - Young laying hens were used for all studies. Total nucleic acid was prepared from hen oviduct as previously described (7). For estimation of RNA mass one $A_{260/ml}$ was assumed to equal 40 µg. RNA samples were stored at -196° C until used.

Total nucleic acid samples were either used directly in translation studies or applied to a nitrocellulose filter essentially as described by Lee et al. (4). Four mg of total nucleic acid was diluted 1:10 (v/v) in a cold buffer containing 10 mM Tris (pH 7.6), 500 mM KCl and 1 mM $MgCl_2$. This mixture was filtered at room temperature through a 25 mm (0.45 µ) Millipore nitrocellulose filter (pre-soaked in buffer) at a rate of 1 drop per second and washed with an additional 5 volumes of buffer. The filter was cut into small pieces and placed in a centrifuge tube. Two mls of buffer containing 100 mM Tris (pH 9.0) and 0.5% sodium dodecyl sulfate was added and the tube shaken at room temperature for 30 minutes. The fluid was removed and the filter washed with one ml of 100 mM Tris (pH 9.0). This wash was combined with the fluid. The RNA solution was chilled to 4° C and repeatedly centrifuged to remove all visible traces of SDS. The supernatant liquid was made 200 mM with NaCl and the RNA precipitated with 2 volumes of 95% ethanol. After 12 hours at -20° C the RNA was pelleted, washed twice with 95% ethanol, vacuum dried, dissolved in distilled water and stored at -196° C.

The rabbit reticulocyte lysate system used in these studies for in vitro translation of mRNA has been previously described (6) as have the procedures for immunologic detection of ovalbumin and avidin (6,8,9,10).

RESULTS AND DISCUSSION - The data in Table 1A reveals the relative ovalbumin mRNA activity of oviduct RNA extracted from a total cell homogenate or a preparation of polysomes. Comparing equivalent concentrations of hen total nucleic acid and an 8-17S fraction of hen polysomal RNA, we could demonstrate a two-fold purification of ovalbumin mRNA activity. Following filtration of

TABLE 1

THE ABILITY OF VARIOUS OVIDUCT RNA PREPARATIONS TO DIRECT THE SYNTHESIS OF OVALBUMIN AND AVIDIN IN A CELL-FREE SYSTEM

A. OVALBUMIN

Source	Total Acid-Insoluble cpm	Ovalbumin cpm
No message	113,660	0
Total Nucleic Acid (100 µg)	53,960	1220
Polysomal RNA (100 µg)	59,900	2680
Filter RNA (6 µg)	111,380	4610

B. AVIDIN

Source	Total Acid-Insoluble cpm	Avidin cpm
No message	94,620	0
Total Nucleic Acid (100 µg)	37,760	0
Polysomal RNA (100 µg)	41,000	0
Filter RNA (6 µg)	92,860	668

The preparations of total nucleic acid and polysomal RNA have been previously described (6,12,13) as have the conditions for protein synthesis using lysate of rabbit reticulocytes (6,7). The final incubation volume was 0.5 ml. An aliquot (25 µl) was utilized for determination of total acid-insoluble radioactivity and the remainder (475 µl) was reacted with either ovalbumin or avidin antisera. The details of the antibody reaction for ovalbumin have also been described (6). Determination of newly synthesized avidin was performed precisely as described for ovalbumin except that avidin standard (8.0 µg) and avidin antiserum (100 µl) were used. Immunological identification of avidin has been documented previously (8,9,10). All values represent the average of triplicate determinations which varied less than 50 cpm. A background determined by substituting anti-BSA for the specific antisera ranged between 300-400 cpm and has been subtracted from the specific protein data shown in the Table. The identical preparations of RNA were used as the message for ovalbumin (A) or avidin (B).

---

the total nucleic acid preparation, the nitrocellulose bound RNA was extracted with SDS (pH 9.0) and assayed in the cell-free protein synthesizing system for mRNA activity. It is evident that utilization of the RNA from the filter caused no depression in the total protein synthesizing capacity of the reticulocyte system while resulting in a 100-fold purification of the ovalbumin mRNA activity.

Since avidin comprises a much smaller proportion of the total oviduct protein, it is consequently more difficult to demonstrate in vitro synthesis of this protein using nucleic acid preparations from whole tissue homogenates and polysomes. Although we often detect a small amount of avidin synthesis using the total nucleic acid of hen, we present in Table 1B, a more dramatic example of the ability of nitrocellulose filters to selectively bind specific mRNA. In this case, avidin mRNA activity of total cell or polysomal RNA preparations could not be demonstrated until we effected a major purification of specific mRNA sequences relative to the bulk of stable RNA species present in the oviduct extracts.

Figure 1A and 1B show the dependence of ovalbumin and avidin synthesis

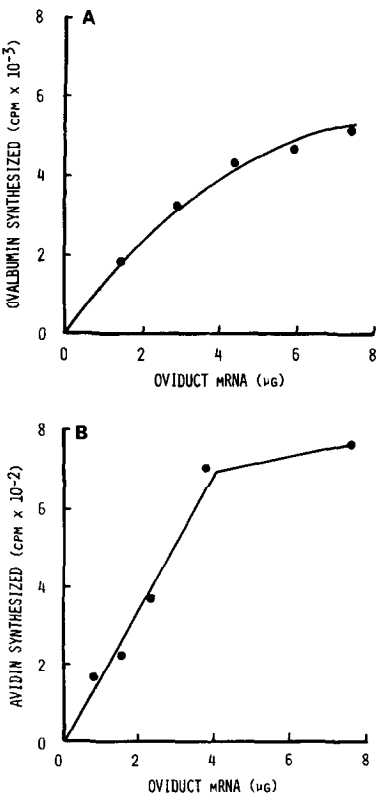

Figure 1 - Synthesis of specific oviduct proteins as a function of the amount of Millipore filter-prepared mRNA added to the reticulocyte system. Incubations were at 37° for 60 minutes. All reactions were performed in triplicate. Backgrounds varied from 250-320 cpm and have been substracted. A. Ovalbumin B. Avidin

on the amount of filter-bound hen RNA added to the reticulocyte lysate system. There was a linear increase, in both instances, when up to 6 μg of RNA was added. At this point 4% of the total protein synthesized was ovalbumin and 0.7% was avidin.

We have routinely been able to bind and recover approximately 2½% of the total nucleic acid passed through the filter. Thus from 4 mg of applied RNA we can extract 80-120 μg of filter-bound RNA. In a previous report (6) using adenosine labeled RNA, we have shown that the contamination of the filter with oviduct cytoplasmic rRNA and tRNA is less than 1%. This is based on the resistance of polyadenylate residues of mRNA to RNase treatment.

Since mRNA usually comprises approximately 2-3% of total cellular RNA (11) we would theoretically expect a maximum 50-fold purification in our ovalbumin mRNA species using this technique if all cell mRNA was uniformally retained by the Millipore filter. We have found, however, that the mRNA activity of the purified RNA for certain specific proteins can on occasion be 100 times better than the total nucleic acid preparation. This discrepency may be due to the presence in the total nucleic acid fraction of an inhibitor(s) of *in vitro* protein synthesis which is not retained on the nitrocellulose filter. It is also possible that all mRNA's do not possess poly A sequences and that we are selectively retaining certain species of oviduct mRNA.

As evidenced by our attempts to detect avidin mRNA this procedure can permit the detection of certain animal cell mRNA species which are otherwise undetectable in extracts of total cell RNA. Our results agree with the recent publication of Brawerman *et al.* (5) demonstrating the utilization of this technique in the purification of a specific messenger RNA.

This technique has permitted the rapid simultaneous isolation, partial purification and translation of two specific mRNA's from a hormone responsive target tissue and may prove to be of major benefit for attaining bulk isolation and purification of many other biologically active species of mRNA.

ACKNOWLEDGMENTS

Supported by American Cancer Society P-576, Ford Foundation 630-0141, NIH HD-04473 and the Vanderbilt Center for Population Research (NIH HD-05797).

REFERENCES

1. Edmonds, M., and Caramela, M. G., J. Biol. Chem. 244, 1314, 1969.
2. Lim, L., and Canellakis, E. S., Nature 227, 3710, 1970.
3. Darnell, J. E., Wall, R., and Tushinski, R. D., Proc. Natl. Acad. Sci. U.S.A. 68, 1321, 1971.
4. Lee, S. Y., Mendecki, J., and Brawerman, G., Proc. Natl. Acad. Sci. U.S.A. 68, 1331, 1971.
5. Brawerman, G., Mendecki, J., and Lee, S. Y., Biochemistry 11, 637, 1972.
6. Means, A. R., Comstock, J. P., Rosenfeld, G. C. and O'Malley, B. W., Proc. Natl. Acad. Sci. U.S.A. (in press).
7. Rosenfeld, G. C., Comstock, J. P., Means, A. R., and O'Malley, B. W., Biochem. Biophys. Res. Commun. 46, 1695, 1972.
8. O'Malley, B. W., and Korenman, S. G., Life Sci. 6, 1953, 1967.
9. Korenman, S. G., and O'Malley, B. W., Endocrinology 83, 11, 1968.
10. O'Malley, B. W., Bichemistry 6, 2546, 1967.
11. Darnell, J. E., Bact. Rev. 32, 262, 1968.
12. Means, A. R., Hall, P. F., Nicol, L. W., Sawyer, W. H., and Baker, C. A., Biochemistry 8, 1488, 1969.
13. Lockard, R. E., and Lingrel, J. B., Biochem. Biophys. Res. Commun. 37, 204, 1969.