Enrichment of special Novikoff hepatoma and regenerating liver mRNA by hybridization to cDNA-cellulose

cancer genes/gene control mechanisms/tissue phenotypes

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ABSTRACT Total polyosomal poly(A)+ RNA of Novikoff hepatoma and of 18-hr regenerating rat liver were compared by analysis of their in vitro translational products on two-dimensional isofocusing/sodium dodecyl sulfate gels. This technique resolved the translated proteins sufficiently to permit detection of quantitative and some qualitative differences between the two mRNA populations. Excess cDNA from regenerating liver or Novikoff hepatoma, covalently linked to cellulose, was used to adsorb the complementary mRNA sequences from Novikoff hepatoma or regenerating liver. As shown by two-dimensional gel electrophoresis, the translated products of the bound mRNA fractions contained proteins common to both tissues. Novikoff hepatoma mRNA which did not bind to regenerating liver cDNA was enriched in sequences encoding for proteins 11/5.1, 15/6.8, 40/8.2, and 65/5.1 (shown as molecular weight/PI). These polypeptides were not detectable in the translational products of regenerating liver mRNA. Regenerating liver mRNA that was not bound to Novikoff hepatoma cDNA was enriched in sequences coding for proteins 12.5/4.9, 13.5/7.4, 17/8.2, 24/5.5, and 46/6.4; these proteins were not found in the translational pattern from Novikoff hepatoma. These results show that adsorption of mRNA to solid-phase cDNA provides a valuable technique for differentiating mRNA species in related tissues and for corresponding enrichment of these specific mRNAs.

cDNAs transcribed from purified mRNA species have been widely used as specific hybridization probes for studies on gene frequency, transcription of chromatin in vitro, and mRNA metabolism (1-4). Comparisons of different mRNA populations by hybridization to nonfractionated cDNA largely discriminate classes of mRNA (5-7); however, it is virtually impossible to detect distinct mRNA species that contribute less than 1% of the total mRNA population. Detection of mRNA species present in small amounts can be achieved when the hybridization of mRNA to solid-phase cDNA, as described by Venetianer and Leder (8), is combined with the analysis of the translational products of the hybridized mRNA. Recent improvements in two-dimensional gel electrophoresis (9, 10) were utilized in these studies to achieve a high resolution of the translated proteins.

MATERIALS AND METHODS

Enzymes. Reverse transcriptase, isolated from avian myeloblastosis virus, was generously supplied by J. Beard (Life Science, Inc., St. Petersburg, FL) and J. Gruber (National Cancer Institute, Bethesda, MD). Unlabeled deoxyribonucleoside triphosphates, oligo(dT)12-18, oligo(dT)-cellulose, and oligo(A) were purchased from P-L Biochemicals (Milwaukee, WI); [8-3H]dGTP and [35S]methionine were purchased from Amersham/Searle (Arlington Heights, IL).

Isolation of mRNA. Total polyosomal poly(A)+ RNA was prepared by a procedure (11) essentially as described by Kabat (12) and Krystosek et al. (13).

In Vitro Synthesis of cDNA-Cellulose. cDNA was transcribed from poly(A)+ RNA with reverse transcriptase. The reaction mixture included 50 mM Tris-HCl (pH 8.3), 20 mM diithiothreitol, 6 mM MgCl2, 100 mM potassium acetate, 600 μM dTTP, 140 μM dATP, dCTP, and dGTP, 36 μg of actinomycin D per ml, 1% ethanol, 15 mg of oligo(dT)-cellulose per ml, 25 μg of mRNA per ml, and 55 units of reverse transcriptase per ml. After a 5-min incubation on ice, the sample was incubated at 42° for 60 min and agitated in a shaking water bath (70 strokes per min) to suspend the cellulose. [8-3H]dGTP (10 μCi/ml) was used to label the cDNA. cDNA synthesis was approximately 45% with respect to input mRNA (14).

Hybridization of mRNA to Oligo(dT)-Cellulose cDNA. Twenty micrograms of mRNA was hybridized to a 10-fold excess of cDNA (approximately 200 μg), covalently linked to 0.3 g of cellulose, at 40° for approximately 50 hr in a total volume of 3 ml containing 50% formamide, 0.6 M NaCl, 0.1% sodium dodecyl sulfate, 10 mM Tris-HCl (pH 7.6), 1 mM EDTA, and 0.5 A260 unit of oligo(A) per ml (14, 15). After the unbound mRNA was washed off the cellulose, the bound mRNA was eluted with 50% formamide/0.1% sodium dodecyl sulfate/10 mM Tris-HCl, pH 7.6, at 60° (14). To the eluted fraction, 0.5 A260 unit of oligo(A) per ml was added. The mRNA of both fractions was precipitated by addition of 2.5 volumes of 100% ethanol saturated with sodium acetate. Bound and unbound mRNA were then translated in the wheat germ cell-free system (16, 17) in the presence of [35S]methionine.

Two-Dimensional Gel Electrophoresis. The in vitro translated proteins were analyzed by isoelectric focusing/sodium dodecyl sulfate gel electrophoresis (9). A miniature system was used, with one-half of a 200-μl disposable pipet (10) serving as a tube for the isoelectric focusing. The slab gels for the sodium dodecyl sulfate dimension were poured between two Kodak projector slide glass sheets (8.3 × 8.3 × 1.2 mm); five slab gels were electrophoresed simultaneously in a specially constructed apparatus. After electrophoresis the gels were dehydrated in dimethyl sulfoxide for 2 hr, impregnated with 15% 2,5-diphenyloxazole in dimethyl sulfoxide for 3 hr, soaked in water for 30 min, and vacuum dried for autoradiography (18).

RESULTS

Translational Products of mRNA. With improved protein separation, it was possible to resolve approximately 100 products translated in the wheat germ system with Novikoff hepatoma or regenerating rat liver mRNA (Fig. 1). After longer exposure (not shown), approximately 300 components could be detected on the autoradiogram from the gel shown in Fig. 1A. It was
sufficient to translate only 0.5 μg of mRNA to synthesize an adequate amount of labeled proteins for these studies.

Comparison of the translational products of mRNA from Novikoff hepatoma and regenerating liver shows common spots (marked with an arrow) with similar amounts of isotope: e.g., 12/8.2,* 15.5/4.5, 19/8.0, 42/7.5, and 58/5.1. However, many common proteins had different amounts of isotope in one gel or the other. In addition, other proteins could only be detected in the translational products from either Novikoff hepatoma or regenerating liver mRNA.

Analysis of Hybridized mRNA. In order to clarify the complex picture of similarities and differences between the translated products of regenerating liver and Novikoff hepatoma, cDNA-cellulose was used as an affinity matrix for the binding of mRNA sequences. When Novikoff hepatoma mRNA was hybridized to Novikoff cDNA-cellulose, essentially all spots present in the pattern obtained from the bound mRNA fraction (Fig. 2B) were also present in the control pattern (Fig. 2A).

Hybridization of Novikoff hepatoma mRNA to cDNA-cellulose from regenerating rat liver produced different results. Some of the hepatoma mRNAs were apparently completely adsorbed to the cDNA-cellulose (arrow, Fig. 3A). The proteins that were translation products of the partially bound mRNAs are shown by arrowheads; these proteins are designated 21/4.8, 22/7.5, 50/5.3, and 53/7.2. Proteins that were translational products of the unbound mRNA fraction but not of the bound mRNA are marked with a square. Corresponding proteins, however, were translated from the bound fraction when Novikoff hepatoma mRNA was hybridized to its homologous cDNA (Fig. 2B). As shown in Fig. 2A, the proteins marked with a square were minor components of the translated products from unfractionated Novikoff hepatoma mRNA. Because they were absent from the translational readouts of regenerating liver mRNA (Fig. 1B), it appears that these mRNA species are specific to Novikoff hepatoma. Table 1 presents molecular weights and isoelectric points of these tumor proteins. The relative amount of these proteins in the unbound fraction increased 4- to 6.6-fold compared to the original material.

In the experiment shown in Fig. 4, regenerating liver mRNA was hybridized to cDNA-cellulose from Novikoff hepatoma. The unbound mRNA, which in this case should contain the regenerating liver messengers that are absent from Novikoff hepatoma, was rehybridized to the homologous cDNA-cellulose from regenerating liver. Several translational products (12.5/4.9, 13.5/7.4, 17/8.2, 24/5.5, and 46/6.4) were present in regenerating liver (Fig. 1B) but not in Novikoff hepatoma.

DISCUSSION

By using improved conditions for reverse transcription of mRNA with oligo(dT)-cellulose primer (14, 19, 20), approximately 200 μg of cDNA was synthesized per 0.3 g of cellulose. This cDNA-cellulose matrix hybridized homologous mRNA sequences efficiently when present in roughly 10-fold excess. When Novikoff mRNA was hybridized to regenerating liver cDNA, the most complete binding was obtained for those mRNAs that were present in comparable amounts in regener-
FIG. 2. Wheat germ translational products. Novikoff hepatoma mRNA was hybridized to excess cDNA-cellulose from Novikoff hepatoma. The bound mRNA fraction (B) and the original material (A) were translated and the products were separated on two-dimensional gels. Symbols and axes as in Fig. 1. Exposure time was 24 hr.

FIG. 3. Wheat germ translational products. Novikoff hepatoma mRNA (Fig. 2A) was hybridized to cDNA-cellulose from regenerating liver. Bound (A) and unbound (B) mRNA were translated and the products were separated on two-dimensional gels. Symbols and axes as in Fig. 1. Exposure time was 24 hr.
Specific proteins translated from the unbound hepatoma mRNA (Fig. 3B) were characterized by molecular weight and isoelectric point. For quantitative radioactive analysis, proteins 1-3 and the corresponding proteins of the control pattern (Fig. 2A) were cut out of the gel and solubilized in 1 ml of 30% H2O2 at 50° for 48 hr. The remaining gel was cut into small pieces (5 x 5 mm) and dissolved in 60 ml of H2O2 at 50° for 72 hr. Percentage radioactivity of proteins 1-3 in the gels was calculated from direct determination of isotope in the spots divided by the amount of isotope in the whole gel. There were 89,100 cpm in the control gel (Fig. 2A) and 32,200 cpm in the gel shown in Fig. 3B. Inasmuch as protein 4 was not easily separated from a protein with slightly lower molecular weight, calculation of enrichment was not included.

not present in regenerating liver, the maximum purification of an individual mRNA would be 10-fold. An even higher enrichment would be possible if only a few components were different, as may be the case between normal and regenerating liver. This could be an important additional purification step for mRNA species that are not highly abundant if, for example, separation according to size or immunoprecipitation techniques only results in incomplete purification of the corresponding mRNA. Such enrichment of some mRNA species is also shown for regenerating liver in Fig. 4.

Table 1. Tumor proteins

<table>
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<th>Protein</th>
<th>Mr</th>
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<th>% of total control</th>
<th>Fold enrichment</th>
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<td>1</td>
<td>11,000</td>
<td>5.1</td>
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<td>0.86</td>
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<tr>
<td>2</td>
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<td>6.8</td>
<td>0.12</td>
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<tr>
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<td>8.2</td>
<td>0.05</td>
<td>0.3</td>
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<tr>
<td>4</td>
<td>65,000</td>
<td>5.1</td>
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Novikoff hepatoma mRNAs that did not bind in the presence of a 10-fold excess of cellulose-fixed cDNA from regenerating liver (Table 1) might be associated with the malignancy of Novikoff hepatoma. However, because the very malignant, rapidly growing Novikoff hepatoma was compared to the comparably slower growing regenerating liver, considerable differences in the translational products of the unbound mRNA populations (Figs. 3B and 4) might be due to the different states of differentiation. It will be useful to apply the same methods to comparisons of closely related tissues such as normal and regenerating liver and various minimal deviation Morris hepatomas. It is recognized that the population of mRNA translated by the wheat germ system may not reflect the total mRNA population of these tissues. Only the products of the more abundant species are present in sufficient amounts to be visible in these autoradiograms and, furthermore, some "inactive" or less active mRNA may not be translated. These possibilities require further analysis.

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