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# Nuclear Polyadenylate-Binding Protein

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**Polyadenylate-binding activity can be detected in eluates from sodium dodecyl sulfate gels by a nitrocellulose filter-binding assay. Nuclear extracts from rat liver show a single peak of binding activity at 50 to 55 kilodaltons; cytoplasmic extracts show a single peak at 70 to 80 kilodaltons, corresponding to a 75-kilodalton protein previously described. Similar results are obtained with yeast and mouse fibroblasts, indicating a high degree of conservation of both nuclear and cytoplasmic polyadenylate-binding proteins. The activity from rat liver nuclei has been purified 125-fold on the basis of specific binding to polyadenylate and shows two main bands in sodium dodecyl sulfate gels at 53 and 55 kilodaltons.**

The structure of ribonucleoproteins differs markedly between the nucleus and cytoplasm. Diverse RNA sequences in the nucleus are associated with a set of 35- to 40-kilodalton (kd) proteins, and the RNA-protein complex (RNP) is released from nuclei, on brief nuclease digestion, in the form of 30 to 40S particles (10–12, 15). Diverse sequences in the cytoplasm, on the other hand, are found largely in polysomes and are readily digested to acid-soluble form (17). Polyadenylate [Poly(A)] at the 3′ end of RNA molecules is organized in a distinctive structure that also differs between nucleus and cytoplasm. Nuclear poly(A) is released in 15S rather than 30 to 40S particles and is susceptible to digestion by nucleases essentially at random (1, 18). By contrast, poly(A) in the cytoplasm is associated with a 75-kd protein in a periodic fashion, as shown by nuclease digestion to multiples of ca. 27 residues (1, 2). The question arises of whether a modified form of the 75-kd protein or an altogether different one occurs in 15S nuclear poly(A)-RNP, or even, in view of the featureless digestion pattern of nuclear poly(A), whether it is associated with a specific protein at all. Previous studies have revealed a 60-kd protein cross-linked to poly(A) upon UV irradiation of a nuclear extract (16). Here we describe a method of assaying poly(A)-specific binding proteins and its use to detect 53- to 55-kd proteins from the nuclei of a range of organisms. This approach further permits the isolation of the 53- to 55-kd proteins for biochemical characterization and for studies of their physiological role.

## MATERIALS AND METHODS

**Assay of poly(A)-binding activity.** Assay mixtures contained 2 ng of $^{32}$P-poly(A) [poly(A) 180 to 220 (Miles Scientific, Naperville, Ill.), 5′ end labeled with T4 polynucleotide kinase and [$\gamma$-$^{32}$P]ATP, specific activity of ca. $10^8$ cpm/$\mu$g], 100 $\mu$g of polycytidylate [poly(C); P-L Biochemicals, Madison, Wis.], and protein [0.05 to 0.2 U of specific poly(A)-binding activity] in 100 $\mu$l of buffer A (0.1 M NaCl, 30 mM Tris-hydrochloride [pH 8.0], 15 mM $\beta$-mercaptoethanol, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride). Mixtures were kept for 10 min at 25°C, diluted to 1 ml with ice-cold buffer B (60 mM KCl, 10 mM Tris-hydrochloride [pH 8.0], 1 mM EDTA), and passed through 13-mm nitrocellulose filters (HAWP; Millipore Corp., Bedford, Mass.) at 5 ml/min. The filters were washed with 5 ml of ice-cold buffer B, dried, and counted. Background was determined by substituting unlabeled poly(A) for poly(C). Specific poly(A) binding refers to $^{32}$P-poly(A) bound in the presence of poly(C) minus background. A unit of specific poly(A)-binding activity retains 1 ng of poly(A) on the filter.

**Recovery of poly(A)-binding activity from SDS-polyacrylamide gels.** Crude extracts (100 $\mu$g of protein) and more-purified protein fractions [1,500 U of specific poly(A)-binding activity] were analyzed in 1.5-mm and 0.5-mm-thick sodium dodecyl sulfate (SDS)–10% polyacrylamide gels (9), respectively, calibrated with SDS-polyacrylamide gel electrophoresis low-molecular-weight standards (Bio-Rad Laboratories, Richmond, Calif.). Protein was eluted from gel slices and precipitated as described (5). Pellets were resuspended in 20 $\mu$l of 6 M guanidine-hydrochloride–30 mM Tris (pH 8.0) diluted to 1 ml with buffer A and kept for 1 h at 25°C to allow renaturation before assays of poly(A)-binding activity. Approximately 75% of the activity in crude extracts was recovered from gels in this way.

**Crude extracts.** Nuclear and cytoplasmic extracts from rat liver were prepared as described below and previously (2) up to the first ammonium sulfate precipitation. Yeast extracts were prepared as described (3). Mouse fibroblast whole-cell extract refers to LA9 cells (provided by D. Chang, Department of Pathology, Stanford University) lysed directly in SDS: $3 \times 10^7$ cells were washed twice in 137 mM NaCl–3 mM KCl–8 mM $Na_2HPO_4$–1.5 mM $KH_2PO_4$–1 mM $CaCl_2$–0.5 mM $MgCl_2$, sonicated twice for 1 min with a Kontes microultrasonic cell disrupter in 20 mM Tris-hydrochloride (pH 8.0)–5% $\beta$-mercaptoethanol–1% SDS–1 mM phenylmethylsulfonyl fluoride–1.4 $\mu$g of pepstatin per ml–400 ng of leupeptin per ml, heated for 10 min at 100°C, and clarified by centrifugation at 13,000 $\times$ $g$ for 10 min.

**Fractionation of nuclear poly(A)-binding activity.** Nuclei from 60 g of rat liver (6) were suspended in 38 ml of buffer C (pH 7.0) (0.1 M NaCl, 30 mM Tris-hydrochloride, 15 mM $\beta$-mercaptoethanol, 1 mM $CaCl_2$, 1 mM phenylmethylsulfonyl fluoride), 2 ml of 20% Nonidet P-40 was added, and the mixture was kept for 10 min in ice and centrifuged at 5,000 $\times$ $g$. The pellet was washed three times with 15 ml of buffer C (pH 7.0), resuspended in 7 ml of buffer C (pH 8.3), adjusted to pH 8.3 if necessary by the addition of 1 N NaOH, turned end-over-end in a 15-ml tube for 4 h (13), and centrifuged in a Beckman Ti50 rotor at 48,000 rpm for 45 min. The supernatant (nuclear extract) was made 5 mM in EDTA (pH 8.0) and 80 $\mu$g/ml in poly(A), kept for 10 min at 4°C, and applied to a column (height, 3.25 cm; diameter, 1.75 cm) of oligodeoxythymidylate [oligo(dT)]-cellulose (type 3; Collaborative Research, Lexington, Mass.) in buffer D

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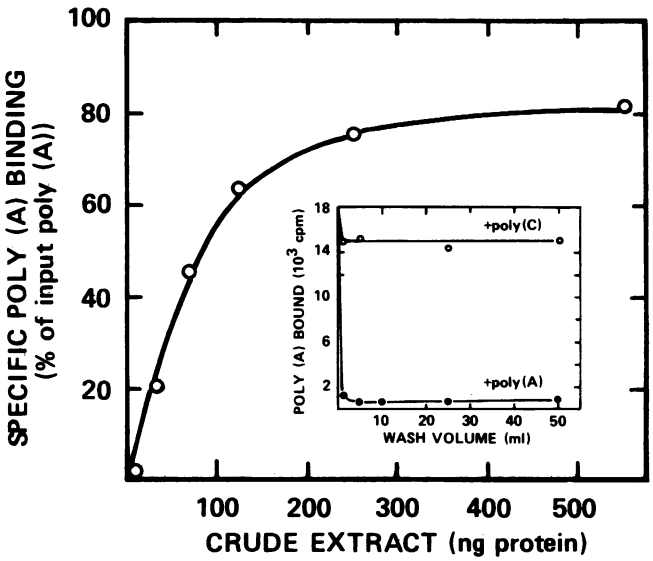


FIG. 1. Poly(A)-binding activity in nuclear extract from rat liver. The inset shows primary data for assay mixtures containing 30 ng of protein and excess unlabeled poly(C) or poly(A) as described in the text.

(buffer C [pH 8.0] containing 5 mM EDTA). The column was washed with 40 ml of buffer D containing 1 mg of poly(C) per ml, 40 ml of buffer D containing 1 mg of poly(C) per ml and 0.3 M NaCl, and 4 ml of buffer D. Poly(A)-binding activity was eluted with 12 ml of 5 mM NaCl–5 mM Tris (pH 8.0) at 45°C (7), adjusted to the composition of buffer D, centrifuged at 10,000 × *g* for 15 min to remove insoluble material, and applied to a column (height, 0.75 cm; diameter, 2.2 cm) of Affi-Gel blue (Bio-Rad) in buffer D. The column was washed with 12 ml of buffer D containing 4 M NaCl, 4 ml of buffer D, and 12 ml of buffer D containing 0.5 M guanidine-hydrochloride. Poly(A)-binding activity was eluted with 4 ml of buffer D containing 1.3 M guanidine-hydrochloride, supplemented with 100 μg of poly(A) and 4 mg of poly(C), dialyzed against 1 liter of buffer D for 3.5 h, and fractionated on an oligo(dT)-cellulose column (height, 0.75 cm; diameter, 2.2 cm) as described above except with 10 ml of each of the wash buffers and 2.2 ml of the elution buffer. Columns were run at 6 ml/h, and procedures were performed at 4°C unless otherwise indicated.

## RESULTS

**Nuclear and cytoplasmic poly(A)-binding proteins.** For the assay of poly(A)-binding activity, $^{32}$P-poly(A) and a 5,000-fold excess of an unlabeled polynucleotide were mixed with the protein fraction of interest. The mixture was passed through nitrocellulose, and the filter-bound radioactivity was determined. Binding that was abolished by competition with unlabeled poly(A) but not poly(C) was judged to be poly(A) specific. This assay revealed the release of poly(A)-binding activity from rat liver nuclei during several hours at elevated pH (Fig. 1), as previously described for the release of 15S and 30 to 40S RNP particles. The activity was probably in RNP form but nonetheless detectable in the assay due to exchange between the RNP and free poly(A). Indeed the rate of exchange, measured by the addition of unlabeled poly(A) to the assay mixture for various times, was very rapid (half-life, ~1 min). Exchange was inhibited by binding to nitrocellulose, as shown by the stability of the $^{32}$P-poly(A)-protein complex on the filter (Fig. 1, inset).

The nitrocellulose filter-binding assay revealed poly(A)-binding activity not only in RNP preparations but also in eluates from SDS gels. SDS was removed by acetone precipitation, and protein renaturation was effected by addition of 6 M guanidine-hydrochloride and dilution (5). Approximately 75% of the poly(A)-binding activity in a nuclear extract could be recovered from a single peak in the 48- to 54-kd region of a gel (Fig. 2). Similar analysis of a rat liver cytoplasmic extract revealed a single peak in the 71- to 81-kd region. The following conclusions may be drawn. First, the nuclear poly(A)-binding activity differs from the cytoplasmic one; the nuclear extract is not simply contaminated with the 75-kd cytoplasmic poly(A)-binding protein previously described. Second, the 48- to 54-kd nuclear and 75-kd cytoplasmic poly(A)-binding proteins are unique; no additional poly(A)-specific, renaturable components are present in crude extracts but lost during fractionation (see below and reference 2 for fractionation procedures). Finally, the nuclear and cytoplasmic binding proteins are well localized; the absence of cross-contamination between nuclear and cytoplasmic extracts suggests a corresponding compartmentalization of the binding proteins in vivo.

**Evolutionary conservation of poly(A)-binding proteins.** Remarkably, assays of poly(A)-binding activity across SDS gels of yeast and mouse fibroblast extracts gave results similar to those with rat liver extracts (Fig. 3 and 4). Peaks of binding activity were detected in the same two regions of the gels, and the lower-molecular-weight component was enriched in a yeast nuclear fraction. Evidently both nuclear and cytoplasmic poly(A)-binding proteins are conserved among tissues and organisms, indicative of their involvement in essential functions. The only disparate findings were an apparent absence of the nuclear protein from the yeast whole-cell extract and the presence of the cytoplasmic protein in the yeast nuclear fraction. The likely explanation

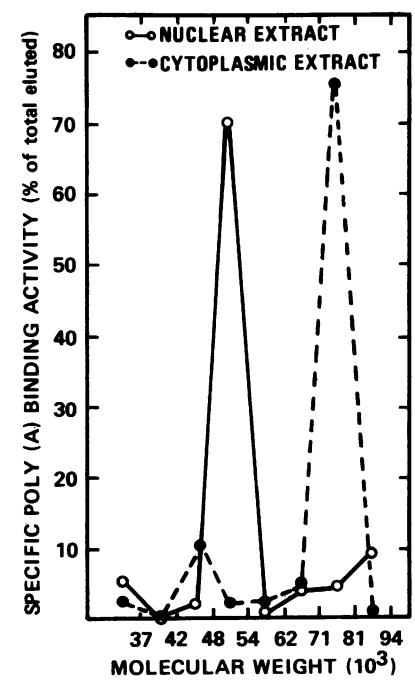


FIG. 2. Distribution of poly(A)-binding activity in SDS gels of nuclear and cytoplasmic extracts from rat liver. The divisions on the abscissa correspond to gel slices.

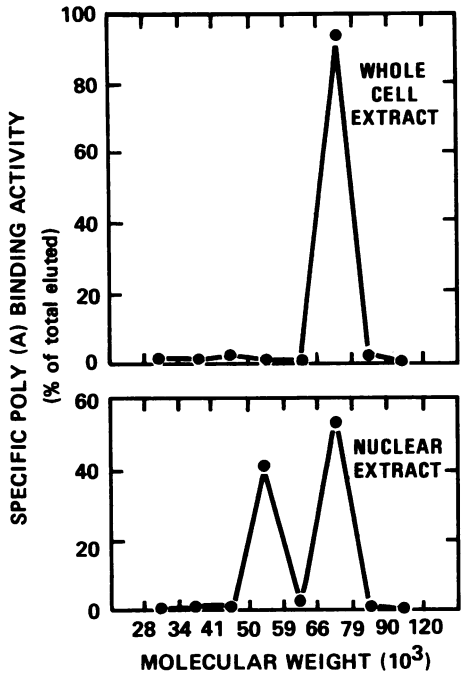


FIG. 3. Distribution of poly(A)-binding activity in SDS gels of yeast whole-cell and nuclear extracts.

is that yeast nuclei are small and the cytoplasm predominates; therefore, the level of nuclear protein is too low to detect in a whole-cell extract, and the great abundance of cytoplasmic protein results in contamination of a crude nuclear fraction. Mouse fibroblasts, on the other hand, have large nuclei filling much of the cell, so the levels of nuclear and cytoplasmic proteins were expected and found to be comparable.

**Purification of nuclear poly(A)-binding proteins.** A purification procedure was devised that exploits specific binding to poly(A) and avoids a loss of activity due to aggregation in the absence of poly(A) or a denaturant. First, poly(A) was added to rat liver nuclear extract to convert any free binding

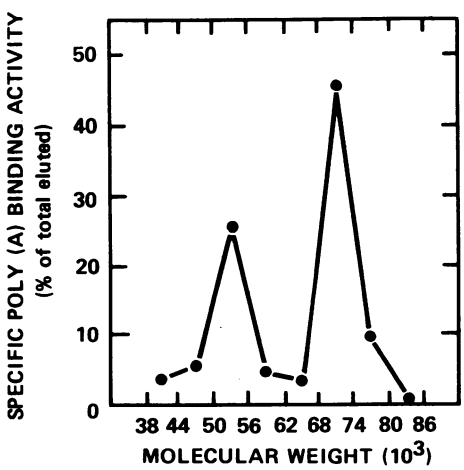


FIG. 4. Distribution of poly(A)-binding activity in SDS gels of mouse fibroblast whole-cell extract.

TABLE 1. Fractionation of nuclear poly(A)-binding activity

| Step | Vol (ml) | Total units | Protein (μg/ml) | Yield (%) | Sp act (U/μg) |
|---|---|---|---|---|---|
| Crude extract | 6.7 | 50,000 | 1,250 | 100 | 6 |
| Oligo(dT)-cellulose | 7.4 | 12,300 | 10 | 27 | 166 |
| Affi-Gel blue | 4.1 | 10,000 | 6.5 | 20 | 375 |
| Oligo(dT)-cellulose | 1.7 | 2,500 | 2 | 5 | 735 |

activity to RNP form, and the mixture was applied to oligo(dT)-cellulose. Extraneous polynucleotide-binding proteins were removed by extensive washing with poly(C), and the ionic strength was reduced and the temperature was raised to elute poly(A)-RNP. The eluate was applied to Affi-Gel blue, which dissociated the RNP, releasing poly(A) into solution and adsorbing the binding activity, as shown by its subsequent elution with guanidine-hydrochloride. Finally, the activity was reconstituted with poly(A) in the presence of excess poly(C), and poly(A)-RNP was again selected on oligo(dT)-cellulose. This procedure gave a 125-fold enrichment of the activity in a 5% yield from the nuclear extract (Table 1). The purified material showed two main bands in an SDS gel (barely resolved in Fig. 5), with apparent masses of 53 and 55 kd. These bands contained over 90% of the poly(A)-binding activity applied to the gel (Fig. 6) and accounted for ca. 80% of the Coomassie blue staining intensity in the gel.

## DISCUSSION

Nuclear poly(A)-binding proteins were fractionated here on the basis of a difference in affinity for poly(A) and

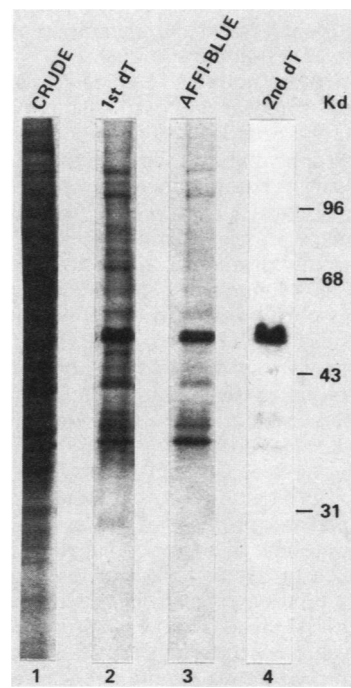


FIG. 5. SDS gels of nuclear extract from rat liver (crude) and of eluates from oligo(dT)-cellulose and Affi-Gel blue columns. A photograph of the Coomassie blue-stained gels is shown.

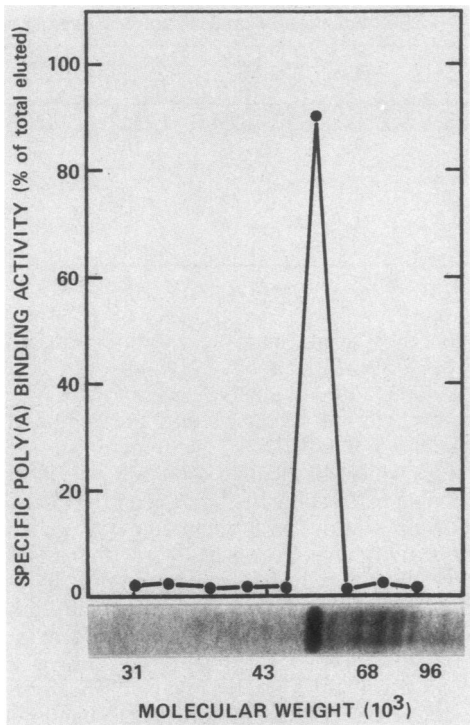


FIG. 6. Distribution of poly(A)-binding activity in an SDS gel of the second oligo(dT)-cellulose eluate (see Fig. 5 and text). Data points for binding activity are centered over regions sliced from the gel shown below.

poly(C). Most of the purification was achieved by adsorption of poly(A)-RNP on a solid support and extensive washing with poly(C). The same procedure has been used to purify the cytoplasmic poly(A)-binding protein (unpublished data), and a similar approach may be applicable to other sequence-specific, nucleic acid-binding proteins.

Preliminary peptide mapping studies reveal considerable homology of yeast nuclear and cytoplasmic poly(A)-binding proteins, raising the possibility that the nuclear proteins are related to the cytoplasmic one. This seems more likely to be due to a processing event in vivo than to proteolysis in vitro, since a whole-cell extract from mouse fibroblasts, prepared under conditions that should inhibit proteolysis, contains comparable amounts of nuclear and cytoplasmic proteins.

The 53- and 55-kd nuclear poly(A)-binding proteins described here may correspond with the 60-kd protein reported to be cross-linked to poly(A) in a nuclear extract (16). The discrepancy in molecular masses could be due to a residual nucleotide cross-linked to the apparent 60-kd species (4). Other reports of a 75-kd nuclear poly(A)-binding protein can be questioned (8, 14). On applying the published procedures to nuclear extracts, we obtained no binding protein at all. The same procedures applied to cytoplasmic extracts gave the 75-kd cytoplasmic protein, so the evidence for a 75-kd nuclear species may be due to cytoplasmic contamination.

The occurrence of 53- to 55-kd nuclear and 75-kd cytoplasmic poly(A)-binding proteins in yeast opens the way to molecular genetic studies. The yeast proteins are immunogenic, and with antisera the genes are readily cloned. Future studies will be directed towards mutagenesis and analysis of the physiological roles of the proteins.

## ACKNOWLEDGMENTS

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