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Characterization of a Protein Species Isolated from HeLa Cell Cytoplasm by Affinity Chromatography on Polyadenylate–Sepharose

(tetrameric structure/phosphorylated protein)

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ABSTRACT **Chromatography of different soluble extracts from HeLa cells on poly(A)–Sepharose columns has allowed the isolation of a protein fraction eluted by 0.2 M NaCl and localized predominantly in the cytoplasmic supernatant and in the 0.5 M KCl ribosomal wash. This fraction is present in large amounts (around 3% of total cytosolic proteins) and appears to contain a major protein species that is acidic on electrofocusing (pI around 4.5) and phosphorylated. It runs on glycerol gradients and Sephadex G-200 chromatography close to the aldolase marker (158,000 daltons) and dissociates into apparently identical subunits of 38,000 ± 2,000 daltons on sodium dodecyl sulfate–acrylamide gels, suggesting a tetrameric structure.**

There is now unambiguous evidence for the presence of poly (A) sequences about 200 nucleotides long in both mRNAs and their nuclear precursors of all eukaryotic organisms, as was originally found in HeLa (1, 2) and mouse sarcoma 180 ascites cells (3). These sequences are located at the 3′ end (4–6) of both RNA species to which they are added after transcription (7–9). They have also been found in the virus-specific nuclear and polysomal RNA of oncogenic viruses like adenovirus (9) and simian virus 40 (10), whose DNA is integrated into the host genome. The finding that 3′-deoxyadenosine (cordycepin), a specific inhibitor of poly(A) synthesis, blocks the cytoplasmic appearance of mRNA without significantly affecting heterogeneous nuclear RNA (HnRNA) synthesis (7, 8, 11) strongly supports the idea that poly(A) sequences might be involved in the nucleocytoplasmic transfer of mRNA (7, 8). On the other hand, the occurrence of poly(A) in mRNA from vaccinia virus that replicates exclusively in the cytoplasm (4) as well as in the genome of many RNA viruses (12–17) suggests a possible role at some step of the translation mechanism. Of course, these two hypotheses are neither limitative nor mutually exclusive.

It is also well established that mRNA is transferred from the nucleus to the cytoplasm in association with proteins, as HnRNA and mRNA have been found in, respectively, nuclear and cytoplasmic ribonucleoprotein particles. Such particles have been observed in a wide variety of eukaryotic organisms including HeLa cells (18–25). One could therefore anticipate that any physiological role of poly(A) would involve poly(A)–protein interactions. After RNase digestion of cytoplasmic ribonucleoprotein particles from mouse sarcoma 180 ascites cells, Kwan and Brawerman were able to isolate a poly(A)–protein complex (26) although no information about its protein moiety has been presented yet. Using the same kind of approach with rat liver or mouse L cells, Blobel found a 78,000-dalton protein likely to be associated with the poly(A) segment of polysomal mRNA (27).

As an alternative approach, we set out to make a systematic investigation of poly(A)-binding proteins by affinity chromatography of different cellular extracts on poly(A)–Sepharose columns. The present paper reports on the isolation and partial characterization of a cytoplasmic protein from HeLa cells that exhibits *in vitro* affinity for poly(A).

Abbreviations: HnRNA, heterogeneous nuclear RNA; SDS, sodium dodecyl sulfate; CS, cytoplasmic supernatant or cytosol; NS, nuclear supernatant; RW, ribosomal wash; F 0.2 M, fraction eluted by 0.2 M NaCl.

MATERIALS AND METHODS

Growth and Labeling of HeLa Cells. Conditions for growth, labeling with [^{3}H]leucine, and collection of HeLa cells (S_3) have been described (25).

Cellular Fractionation. All operations were carried out at 0°–4° except when otherwise stated. The washed cell pellet was first suspended in 7 volumes of water, centrifuged 5 min at 1100 × *g*, and then resuspended in the hypotonic buffer RSB [10 mM Tris·HCl, pH 7.4 (25°)–1.5 mM $MgCl_2$–10 mM NaCl]. The swollen cells were homogenized with a Teflon–glass homogenizer, and when most of the cells were broken, as seen under the phase microscope, nuclei were separated by another 5-min centrifugation at 1100 × *g*. After removal of mitochondria by centrifugation for 20 min at 18,000 rpm (no. 870 rotor of the IEC B-20 centrifuge), the ribosome–polysome fraction was pelleted from the supernatant by a 2-hr spin at 52,000 rpm (A-269 angle rotor of the IEC B-60 Ultracentrifuge). The upper 4/5 of the resulting supernatant in RSB buffer was adjusted to the composition of buffer A [10 mM Tris·HCl, pH 7.4 (25°)–1.5 mM $MgCl_2$–10 mM NaCl–1 mM 2-mercaptoethanol–5% glycerol]. This fraction will be referred to as cytosol or cytoplasmic supernatant (CS).

The pelleted nuclei were resuspended in RSB, homogenized again, and recentrifuged. After resuspension in buffer A, nuclei were disrupted by sonication with a microtip-equipped Branson model B-12 sonifier. Samples kept in ice were given 10-sec pulses of sonic power (50 W, 20 KHz) separated by 1-min cooling intervals. Centrifugation of nuclear debris and chromatin for 1.5 hr at 52,000 rpm (in rotor A-269) yielded a supernatant fraction referred to as nuclear sap (NS).

The ribosome–polysome pellet was resuspended and kept overnight in buffer containing 0.5 M KCl, then centrifuged for 2 hr at 52,000 rpm (A-269). The upper 4/5 of the supernatant was withdrawn and dialyzed against buffer A (without

KCl). The resulting protein precipitate was eliminated by a 10-min spin at 10,000 × g and the supernatant was collected. This fraction is called ribosomal wash (RW).

Poly(A)–Sepharose Chromatography. Poly(A) was covalently bound to cyanogen bromide-activated Sepharose 4B according to the procedure initially described by Wagner *et al.* (28) for binding poly(rI·rC) and extensively washed with buffer A. Quantitation of bound poly(A) was achieved by spectrophotometric determination of adenylic acid residues released by overnight incubation in 0.5 N NaOH at 37°. The binding of 0.2–0.6 mg of poly(A) per ml of packed Sepharose was routinely obtained.

The three different cellular extracts in buffer A were loaded onto a 2-ml column of poly(A)–Sepharose packed in a Pasteur capillary pipette. After the column was washed with the same buffer until the effluent contained no detectable material, elution was carried out with two successive steps of 0.2 M and 2 M NaCl in buffer A. In all these steps, including loading of the column, the flow rate was adjusted to 6 ml/hr by means of a peristaltic pump collecting 1-ml fractions. These were assayed either for their absorbance at 280 nm or for radioactivity. In this latter case, 100-μl aliquots were precipitated with 10% (w/w) cold trichloroacetic acid in the presence of 50 μg of bovine-serum albumin as a carrier. After they cooled in ice for 15 min, precipitates were collected by filtration on Whatman GF/C glass filters, washed with 5 ml of 5% trichloroacetic acid and 2 ml of ethanol, then dried and counted in a toluene-based scintillator in a Packard-Tricarb spectrometer. Protein concentrations of particular fractions were also determined according to Linn and Lehman (29).

Sedimentation Analyses on Glycerol Gradients. About 1 mg of bovine-serum albumin was dissolved in a 0.3-ml aliquot of the peak fraction eluted from poly(A)–Sepharose by buffer A containing 0.2 M NaCl, layered on top of a 11.5-ml 10–30% (w/v) glycerol gradient in buffer A containing 0.1 M NaCl, and centrifuged for 37 hr at 38,000 rpm in the SB-283 rotor of the IEC B-60 ultracentrifuge at 4°. Fractions of 0.5 ml were collected from the bottom of the tubes by means of a peristaltic pump, and their absorbance at 280 nm was determined. Radioactive fractions were precipitated, filtered, and counted. Calibration of these gradients was achieved by running on parallel gradients several marker proteins like cytochrome *c*, ovalbumin, bovine-serum albumin, aldolase and catalase, all purchased from Boehringer. The following molecular weight and S values, specified by the manufacturer, were used: 13,500 and 2 S for cytochrome *c;* 45,000 and 3.6 S for ovalbumin, 68,000 and 4.5 S for bovine-serum albumin, 160,000 and 7.5 S for aldolase, 240,000 and 11.1 S for catalase. Two markers of widely different sizes were run on the same gradient.

Sephadex G-200 Chromatography. Sephadex G-200 (40–120 μm) was obtained from Pharmacia and swollen by heating in a boiling water bath for 5 hr as recommended by the manufacturer. After cooling and removing of the fine particles by successive settlings and decantations, it was equilibrated by several changes of buffer A containing 0.1 M KCl. The column (1.6-cm internal diameter) was packed under gravity, then washed overnight in the same buffer with a constant hydrostatic pressure of 25 cm. The final height of the column was 81 cm, corresponding to 155 ml packed volume. All samples (1–2 mg of protein) were loaded in 0.5 ml of buffer A containing 0.1 M KCl and a few crystals of both sucrose and Dextran blue.

The same marker proteins were used as for glycerol gradient sedimentation. Each marker was run separately on the same column. The sample was run together with 1 mg of bovine-serum albumin. Fractions of 1.15 ml were collected at a flow rate of 12 ml/hr. Marker proteins were detected by their absorbance at 280 nm or at 230 nm. The radioactive sample protein was counted as trichloroacetic acid-precipitable material.

Electrophoresis on Sodium Dodecyl Sulfate–Acrylamide Gels. Ten percent gels containing 0.1% sodium dodecyl sulfate (SDS) were prepared according to Matringe and Jacob (30), using 0.5-ml stacking gel and 3-ml separation gel in 6-mm diameter glass tubes. Aliquots of the chromatographic fractions were heated for 5 min at 100° in the presence of 1% SDS and 1% 2-mercaptoethanol.

Samples up to 100 μl were layered on top of the stacking gel along with a few sucrose crystals and 4 μl of 0.01% bromphenol blue.

Electrophoresis was carried out at 5 mA per gel until the dye had reached 0.5 cm above the lower end of the gel. Gels were either stained for 2 hr in a 0.25% solution of Coomassie brilliant blue in methanol–acetic acid–water (5:1:5), then destained by diffusion in the same solvent, or cut into 1-mm slices which were depolymerized overnight in 0.5 ml of hydrogen peroxide (110 volumes) and counted in 10 ml of a Triton-containing scintillator.

Molecular weights were estimated according to Weber and Osborn (31), with the same protein standards as for glycerol gradient sedimentation.

Electrofocusing was performed according to the principles of Vesterberg and Svensson (32), with the analytical 110-ml column and pH 3–10 ampholines from LKB. All experiments were run for 20 hr at 300 V under refrigeration with running tap water (15°). Up to 1-ml aliquots of the chromatographic fractions were directly included in the stabilizing sucrose gradient (0.5–47%, w/v) containing 1% ampholines. Fractions of 2 ml were collected at a flow rate of 100 ml/hr, assayed for pH at 25°, precipitated with 10% trichloroacetic acid, filtered, and counted.

RESULTS

Poly(A)–Sepharose Chromatography of HeLa Cellular Extracts. Three soluble fractions, i.e., cytoplasmic supernatant (CS), nuclear supernatant (NS), and ribosomal wash (RW), from a single [^{3}H]leucine-labeled HeLa cell preparation were successively chromatographed on the same poly(A)–Sepharose column (Fig. 1*a*). The same cellular fractions from a similar nonradioactive preparation were also analyzed under identical conditions (Fig. 1*b*). After extensive washing of the column, a very sharp peak of both radioactivity and absorbance at 280 nm is eluted by 0.2 M NaCl in the case of CS and RW. Although there appears to be a small but detectable peak also in the nuclear supernatant, it is obvious that this 0.2 M NaCl fraction (to be referred to as F 0.2 M) is by far more abundant in the cytoplasmic and ribosomal extracts in terms of absolute quantity. Its relative proportion in the cytoplasm is very high, amounting to about 3% and 6% of total input for, respectively, CS and RW when assayed either by the Lowry method or by radioactivity. RW seems, therefore, to be enriched in this fraction. A second quantitatively smaller

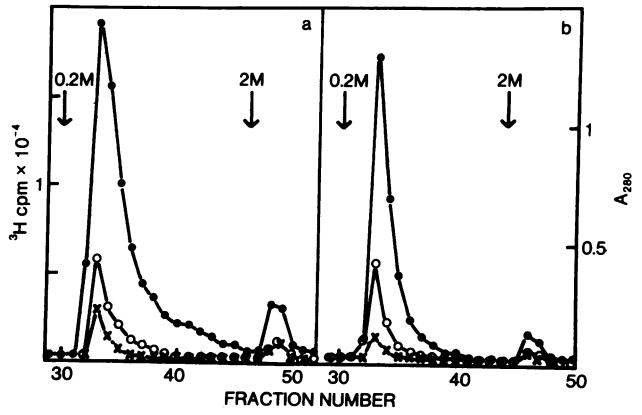

FIG. 1. Poly (A)—Sepharose chromatography of different HeLa cell extracts. (*a*) 2.5 × 10^9 cells labeled for 24 hr with [^{3}H]leucine were fractionated as described in *Methods* to yield cytoplasmic supernatant (CS), nuclear supernatant (NS), and ribosomal wash (RW) fractions containing, respectively, 60 mg (2.5 × 10^7 cpm), 9 mg (1.9 × 10^6 cpm), and 9 mg (1.8 × 10^6 cpm) of total protein. The whole of each of these three fractions was successively chromatographed on the same poly(A)–Sepharose column and analyzed as described in *Methods*. (●) CS; (○) RW; (+) NS. Values for cpm have been multiplied by 10^{-4}. (*b*) Nonradioactive cells (5.2 × 10^9) were fractionated to yield CS, NS, and RW fractions containing, respectively, 145, 20, and 23 mg of total protein. The whole of NS and RW fractions and only half of CS fraction was chromatographed as in (*a*) and the absorbance profile at 280 nm recorded. For sake of comparison between the three fractions, the *ordinate* values of CS were multiplied by a factor of 2 in order to compensate for the loading of only half the total fraction. (●) CS; (○) RW; (+) NS. For sake of simplicity, the flow-through fractions of the column were not represented.

fraction eluted by 2 M NaCl is also consistently observed in the cytoplasmic extracts albeit in a much less reproducible yield. Further studies to be reported here will only be concerned with F 0.2 M.

SDS–Acrylamide Gel Analysis of F 0.2 M from Cytosol (CS). Fig. 2 represents the electrophoretic distribution of either unlabeled or [^{3}H]leucine-labeled F 0.2 M from HeLa cytoplasmic supernatant in 10% acrylamide gels containing 0.1% SDS. In both cases, the presence of a discrete species corresponding to a molecular weight of about 38,000 is observed. Although some impurities appear as very faint bands in the stained gel, F 0.2 M protein turns out to be nearly homogeneous (above 90%) according to this analytical criterion. Similar analyses, not presented here, show that F 0.2 M from either RW or NS also contains a major polypeptide of the same size.

Electrofocusing Analysis of F 0.2 M from CS. When analyzed by electrofocusing under nondissociating conditions, most of F 0.2 M from CS is found as an essentially monodisperse, sharply focused band at pH $\simeq$ 4.5 as shown in Fig. 3. The presence of a shoulder on the acidic side of the peak (towards the lower electrode) may be a result of rapid settling of some precipitated material during fraction collection and, therefore, is not significant. Indeed, although not performed in this particular case, reversal of electrode polarity does not change the position of such a shoulder in several similar cases as observed in our laboratory (Ch. Ducamp and Ph. Jeanteur, unpublished results).

When the same experiment was carried out in the presence of 6 M urea in order to dissociate possible aggregates (either

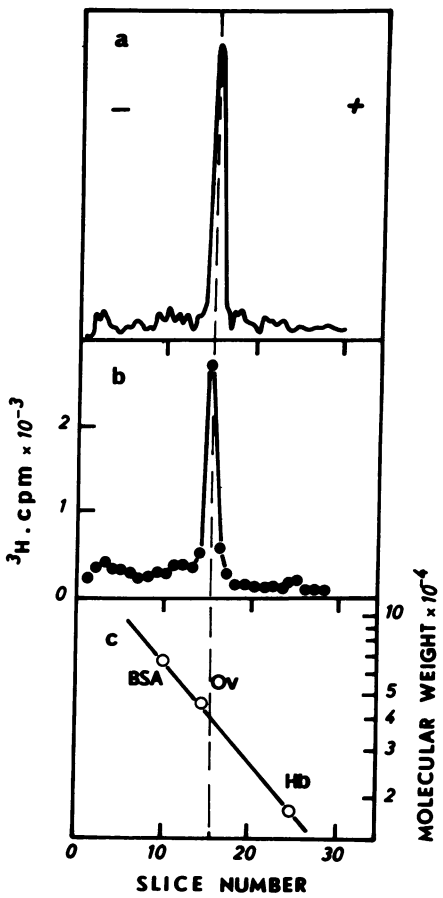

FIG. 2. SDS–acrylamide gel electrophoresis of F 0.2 M from CS. (*a*) Densitometer tracing of a stained gel loaded with 20 μg of F 0.2 M protein from unlabeled cytoplasmic supernatant obtained as in Fig. 1*b*. (*b*) Radioactivity profile of F 0.2 M from [^{3}H]leucine-labeled CS obtained as in Fig. 1*a*. (*c*) Molecular weight estimation of denatured F 0.2 M protein, using the calibration curve made with the following markers: bovine-serum albumin (*BSA*), ovalbumin (*Ov*), and rabbit hemoglobin (*Hb*).

of natural or artificial origin), a very sharp and unique peak is also observed confirming the charge homogeneity. As is to be normally expected from the presence of urea (33), the pH was slightly shifted towards higher value (4.7).

Size Estimation of Native F 0.2 M from CS. The sedimentation profile in glycerol gradient of F 0.2 M labeled with [^{3}H]-leucine is presented in Fig. 4*a* along with the calibration curve obtained with several markers of known S values (Fig. 4*b*). Under these conditions, F 0.2 M appears to migrate at a position very close to that of the aldolase marker which is a tetrameric enzyme of 158,000 daltons.

In a gel filtration experiment, the same F 0.2 M elutes from a Sephadex G-200 column as a single, highly symmetrical peak slightly behind that of aldolase (Fig. 5*a*). Its molecular weight can be estimated to be about 150,000 from the calibration curve obtained with different size markers as shown in Fig. 5*b*. Comparing this value with that obtained above in SDS–acrylamide gel (38,000 daltons), we can conclude that F 0.2 M protein is likely to be a tetramer made of four subunits indistinguishable from the point of view of size and electrical charge.

Chemical Analysis of F 0.2 M from CS. Chemical analysis of this protein was undertaken in order to explain its acidic

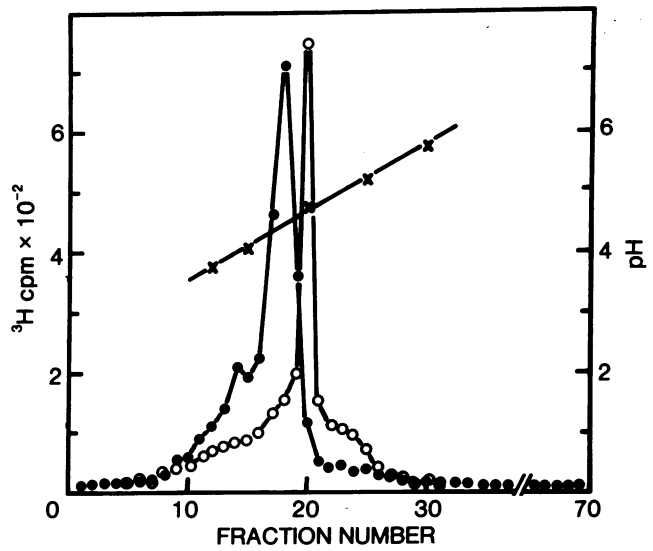

FIG. 3. Electrofocusing of F 0.2 M from CS. Cpm (2×10^4) of F 0.2 M from CS labeled with [^{3}H]leucine were analyzed as described in *Methods* in the absence (●——●) and in the presence (○——○) of 6 M urea.

characteristics, which could be accounted for in three different ways: (*a*) excess of acidic over basic amino acids; (*b*) presence of phosphoryl groups; or (*c*) presence of acidic sugars.

Results presented in Table 1 show that this protein contains inorganic phosphate and has a high excess of acidic amino acids. However, this latter feature may not be significant if, in the native protein, these acidic groups were masked by amide functions that are released as ammonia during the hydrolytic process. Indeed, enough ammonia was released by mild acid hydrolysis (in order to minimize the contribution of degraded amino acids) to account for the neutralization of most of the excess of acidic over basic residues. It seems, therefore, that phosphoryl groups should be mainly responsible for the acidic character of F 0.2 M protein. The existence of such groups has been confirmed by recent experi-

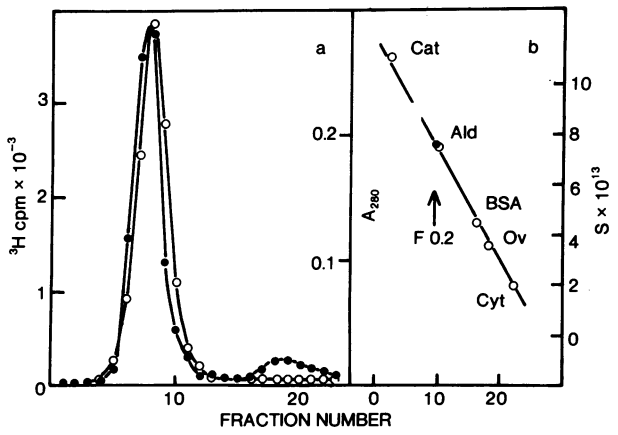

FIG. 4. Glycerol gradient analysis of F 0.2 M from CS. (*a*) Sedimentation pattern of a sample containing 1.5×10^4 cpm of tritiated F 0.2 M protein (●——●). The absorbance profile at 280 nm of the aldolase marker (○——○) run on a parallel gradient was plotted on the same diagram. Sedimentation conditions were described in *Methods*. (*b*) Sedimentation coefficient estimation of native F 0.2 M protein using the calibration curve made with the following markers: catalase (*Cat*), aldolase (*Ald*), bovine-serum albumin (*BSA*), ovalbumin (*Ov*), and cytochrome *c* (*Cyt*).

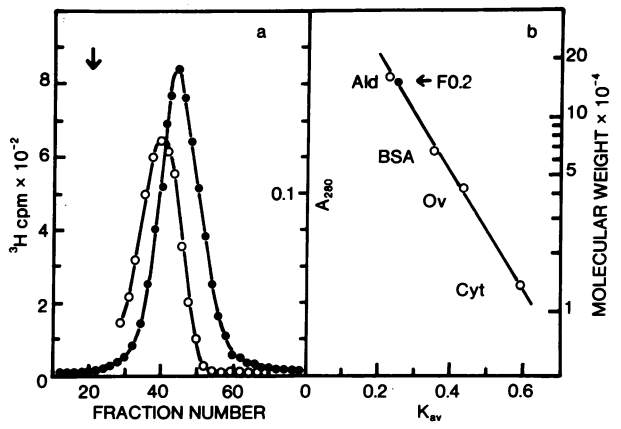

FIG. 5. Sephadex G-200 chromatography of F 0.2 M from CS. (*a*) Elution pattern of a sample containing 2.3×10^4 cpm of tritiated F 0.2 M protein (●——●). The absorbance profile at 280 nm of the aldolase marker (○——○), run in a separate experiment, was plotted on the same diagram. Conditions of chromatography were described in *Methods*. The *arrow* indicates the position of the blue dextran marker. (*b*) Molecular weight estimation of native F 0.2 M protein using the calibration curve made with the same markers as in Fig. 4 except for the absence of catalase. $K_{av} = V_e - V_0/V_t - V_0$; with V_0 = void volume as measured by dextran blue, V_e = elution volume of a given protein, and V_t = column bead volume.

TABLE 1. *Chemical composition of F 0.2 M from cytosol*

Amino acids*	Residues (%)	Residues per assumed monomeric unit of 38,000 daltons
Asp	10.03	33
Thr	5.04	17
Ser	5.87	19
Glu	11.84	39
Pro	4.55	15
Gly	9.50	31
Ala	8.46	28
Val	7.48	25
1/2 Cys	Presence	—
Met	1.53	5
Ile	5.24	17
Leu	8.59	28
Tyr	3.95	13
Phe	3.98	13
Trp†	Presence	—
His	2.88	10
Lys	6.87	23
Arg	4.23	14
Asp + Glu	21.87	72
Lys + Arg	11.10	37
amide NH_3‡	10.0	33
PO_4§	—	2–3

* Amino-acid analyses were performed with a Beckman Multichrom analyzer after acid hydrolysis in 6 N HCl at 110° under reduced pressure. Figures given are averages of four experiments using two different times of hydrolysis (20 and 70 hr).

† Presence suspected from spectral data. No quantitative determination has been achieved yet.

‡ Estimated after mild acid hydrolysis (2 hr in 2 N HCl at 110°).

§ Estimated by the method of Ames (34) modified for microquantities.

ments in our laboratory demonstrating the presence of hot trichloroacetic acid-precipitable radioactivity in F 0.2 M after ^{32}P labeling. Acidic sugars have not yet been assayed.

DISCUSSION

In order to elucidate the role of poly(A) in the nucleocytoplasmic transport of mRNA or/and in its translation in polysomes, we hypothesized that any of these possible functions should involve poly(A)–protein interactions. Along this line, we set out to make a systematic investigation of cellular proteins able to bind to poly(A) *in vitro*. We report here the results of affinity chromatography on poly(A)–Sepharose columns of different soluble extracts from HeLa cells.

The presence of a protein fraction adsorbed to and eluted from poly(A) by 0.2 M NaCl was demonstrated in both the cytoplasmic supernatant and the 0.5 M KCl ribosomal wash. This protein fraction is present in large quantities in the cytoplasm, amounting to about 3% of the total protein content of cytosol and 6% of that of ribosomal wash. The small amount of this material found in the nuclear supernatant might be due to cytoplasmic contamination. In terms of absolute quantity, it is found mostly in the cytoplasmic supernatant. Although preliminary experiments seem to indicate that proteins present in the 0.2 M NaCl eluates of the three types of cellular extracts are similar, the present study has only been concerned with that originating from the cytoplasmic supernatant.

Analysis of this F 0.2 M fraction on SDS–acrylamide gels revealed the presence of a unique band corresponding to a molecular weight of about 38,000. From its behavior on glycerol gradient and on Sephadex G-200, a molecular weight of about 150,000 was inferred for the native protein, suggesting a tetrameric structure which in turn may be indicative of possible regulatory properties. By electrofocusing, this protein turned out to be acidic, and chemical analyses demonstrated its phosphorylation.

Relevance of this protein to any physiological function of poly(A) is by no means established. However, it should be noticed that polypeptides of about the same size have been found in HeLa nuclear ribonucleoprotein particles in our lab (25) as well as in rat liver (35) and rat brain (30). In this latter case, this polypeptide was found to be phosphorylated (36). On the other hand, the protein described here bears no resemblance with Blobel's 78,000-dalton species supposedly bound to poly(A) (27) nor with any of those found in the cytoplasmic mRNA-containing particles from various tissues.

Salas and Green (37) have described in 3T6 cells a 40,000-dalton protein (P6) that binds to single-stranded DNA and whose synthesis is correlated with that of DNA. Relevance of the present protein to the latter as well as to other known RNA-binding factors (38) remains to be established.

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