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Repeating structure of cytoplasmic poly(A)–ribonucleoprotein

(nuclease digestion/reconstitution/nuclear RNA)

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ABSTRACT **A repeating structure of cytoplasmic poly(A)–ribonucleoprotein is revealed by digestion with T2 RNase. A pattern of fragments that are multiples of about 27 residues is obtained. The repeating structure is readily reconstituted from purified poly(A) and cytoplasmic factors. Reconstitution is specific for poly(A), as shown by the lack of competition by poly(G), poly(C), poly(dA), and tRNA. The repeating structure is absent from the nucleus, and so appears to be formed upon transport to the cytoplasm.**

There is a good deal of evidence for the association of proteins with heterogeneous nuclear RNA and mRNA, but little information about the structure of the resulting ribonucleoprotein (RNP) complexes. We report here on the structure of RNP containing poly(A), found at the 3′ end of many RNA molecules.

Poly(A) is revealed by digestion of RNP or naked RNA with pancreatic and T1 RNases, which cleave at U, C, and G but not A residues (1). Newly synthesized poly(A), detected by labeling cells for about 3 hr, followed by extraction of the RNA and digestion with RNase, is fairly homogeneous in size, about 150–250 residues (2). The RNP form of this poly(A) also appears fairly homogeneous when released from polysomes by RNase digestion and analyzed by sedimentation in sucrose gradients. Most of the poly(A) migrates as a band with a sedimentation coefficient of about 12 S (3). One major protein, of molecular weight about 75,000, is found at the same position in the gradient (4). Studies in which poly(A)-containing RNP was isolated by hybridization to oligo(dT)-cellulose or poly(U)-Sepharose have revealed a poly(A)-associated protein of similar size and several additional, smaller ones (5, 6). The 75,000-dalton protein has been suggested to be the poly(A) polymerase (7) and possibly also to play a role in the transport of poly(A)-containing RNA from nucleus to cytoplasm (8). Beyond this, the function of poly(A)-associated proteins and arrangement of components in poly(A)–RNP remain to be established.

We have studied the structure of poly(A)–RNP by digestion with a nonspecific nuclease, T2 RNase. This is analogous to the study of chromatin structure by digestion with micrococcal nuclease or DNase I. Brief digestion reveals a pattern of protection of the nucleic acid by associated proteins.

MATERIALS AND METHODS

Cell Growth, Labeling, and Fractionation. Mouse Friend erythroleukemic cells (line 745-PC4, a gift of David Housman) were grown in minimum Eagle's medium containing 15% fetal calf serum to a density of 10^6 cells per ml and labeled for 4 hr in medium containing dialyzed serum and trace amounts of [^{32}P]orthophosphate (0.2–0.5 mCi/ml; 1 Ci = 3.7×10^{10} becquerels) as the only phosphate. Labeled cells were centrifuged, washed with 0.1× buffer A (*, 9) supplemented with 1 mM $CaCl_2$ and 1 mM $MgCl_2$, suspended in 0.1× buffer A at 2×10^7 cells per ml, and lysed by passage through a 22-gauge needle. The lysate was centrifuged for 3 min at $200 \times g$ and the cytoplasm (supernatant) was removed from the nuclear pellet. Nuclei were washed and suspended in 0.1× buffer A at 2×10^7 per ml. Cytoplasm and nuclei were supplemented with $^1/_{10}$th vol of 10× buffer A before T2 RNase digestion.

[^{32}P]Poly(A). ^{32}P-Labeled cytoplasm was extracted with 1 vol of 0.1 M sodium acetate, pH 5.0/20 mM EDTA/1% sodium dodecyl sulfate and 2 vol of phenol/chloroform/isoamyl alcohol (50:50:2, vol/vol). The aqueous phase was reextracted with the phenol mixture and RNA was precipitated with ethanol. RNA from 5×10^7 cells was dissolved in 0.3 ml of 30 mM Tris·HCl, pH 7.0/30 mM NaCl/2 mM EDTA and digested with pancreatic RNase (0.03 μg) and T1 RNase (6 units, Calbiochem) for 20 min at 37°C. Digestion was terminated by addition of 50 μl of phenol, 50 μl of 4 M NaCl, and 50 μg of yeast tRNA. RNA was precipitated with ethanol, dissolved in 10 M urea/25% (vol/vol) glycerol, subjected to electrophoresis in a 10% acrylamide gel containing 7 M urea (10), and autoradiographed. The band at 150–250 residues was sliced from the gel and eluted as described (11).

Reconstitution. To cytoplasm from 5×10^6 cells (prepared as described except without ^{32}P labeling) was added $^1/_{10}$th vol of 10× buffer A, 1.5 μg of unlabeled polynucleotide (Miles) as indicated, and finally [^{32}P]poly(A) from 5×10^5 cells. The mixture was incubated for 30 min at 37°C.

RESULTS

Whole cytoplasm (postnuclear supernatant) from ^{32}P-labeled mouse (Friend erythroleukemic) cells was treated with T2 RNase, followed by extraction of the RNA, degradation of all but the poly(A) with pancreatic and T1 RNases, and analysis of the poly(A) fragments by gel electrophoresis. The result was a striking pattern of bands, showing the conversion of poly(A) from a continuous distribution of sizes, about 150–250 residues, to a discrete set of fragments (Fig. 1). A band near the bottom of the gel was present both before and after T2 digestion, and may be a product of transcription of poly(dT) sequences rather than of polyadenylylation (12). The identity of the poly(A) and poly(A) fragments was confirmed by elution from the gel, degradation to mononucleotides, and two-dimensional thin-layer chromatography (13). In both cases, AMP was the only nucleotide detected.

Size determination (see below) has shown that the poly(A) fragments produced by T2 RNase digestion are multiples of about 27 residues. On extensive digestion, all fragments are

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Abbreviation: RNP, ribonucleoprotein.

* Buffer A is 60 mM KCl/15 mM NaCl/0.15 mM spermine/0.5 mM spermidine/15 mM 2-mercaptoethanol/15 mM Tris·HCl, pH 7.4.

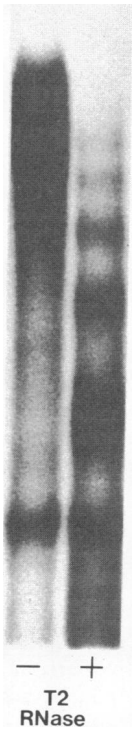

FIG. 1. T2 RNase digestion of cytoplasmic poly(A)–RNP. Distributions of [^{32}P]poly(A) from cytoplasm (*Left*) and from cytoplasm digested with T2 RNase (*Right*) are shown. RNA was extracted from ^{32}P-labeled cytoplasm, either directly or after digestion with T2 RNase (Calbiochem, 10 units/ml for 20 min at 37°C), and then digested with pancreatic and T1 RNases and analyzed by gel electrophoresis. An autoradiogram of the gel is shown.

cleaved to the unit size and further degraded. The transition from large to small fragments is a smooth one, with no indication of preferred intermediates at any stage. We conclude that poly(A)–RNP is organized in a repeating structure with a periodicity of about 27 residues.

Poly(A)–RNP may be reconstituted by the simple mixing of [^{32}P]poly(A) with mouse cell cytoplasm. T2 RNase digestion of the product gives a pattern of bands (Fig. 2, lane 2) nearly identical to that obtained from native poly(A)–RNP. A similar result has been obtained by using a rat liver extract (supernatant from centrifugation of rat liver homogenate at 150,000 × *g* for 20 min) in place of mouse cell cytoplasm. Similar results were also obtained by using micrococcal nuclease instead of T2 RNase. The specificity of reconstitution was tested by the addition of various unlabeled polynucleotides along with the [^{32}P]poly(A). Unlabeled poly(G) (Fig. 2, lane 4) had no effect at a concentration at which unlabeled poly(A) was a potent competitor of the radioactive material (Fig. 2, lane 3). Poly(C), poly(dA), and yeast tRNA (even at a 100-fold higher concentration) also had no effect. Evidently, formation of the 27-residue repeating structure is highly specific for poly(A).

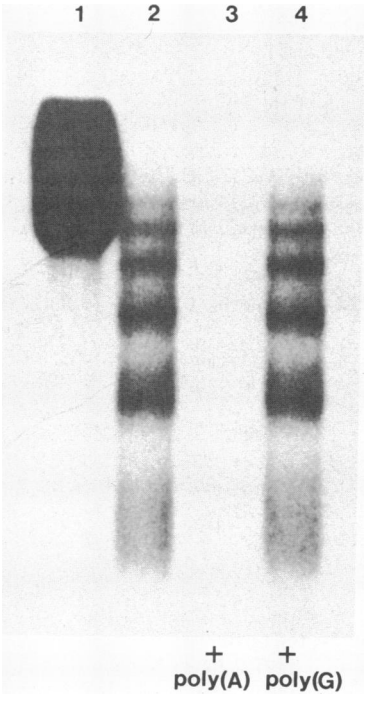

FIG. 2. T2 RNase digestion of reconstituted poly(A)-RNP. [^{32}P]Poly(A) (lane 1) was treated with cytoplasm (lane 2), with cytoplasm and unlabeled poly(A) (lane 3), or with cytoplasm and unlabeled poly(G) (lane 4), followed by T2 RNase digestion (10 units/ml for 20 min at 37°C), extraction, and gel electrophoresis. An autoradiogram of the gel is shown.

There was less background in the T2 digestion pattern of reconstituted poly(A)–RNP (Fig. 2) than in the pattern obtained from native material (Fig. 1), presumably because the [^{32}P]-poly(A) used in reconstitution had been freed of low molecular weight components by gel electrophoresis. The low background made it possible to resolve the T2 pattern into subbands differing in size by a single residue and still observe the main bands as a modulation in intensity of the subbands. Sizes could then be determined by counting subbands from the AMP position to the midpoints of the main bands. The results (Table 1) showed a reduction in size with increasing digestion, but a difference between adjacent bands of about 27 residues, which remained nearly constant.

The reconstitution of poly(A)–RNP was monitored by sedimentation in sucrose gradients, as well as by T2 RNase digestion. The conversion of poly(A) to a rapidly sedimenting complex was observed (Fig. 3). The sedimentation coefficient of the complex was about 12S, in good agreement with the value reported for native poly(A)–RNP (3). Formation of the 12S complex is specific for poly(A), as judged from the absence of an effect of unlabeled poly(G) at a concentration at which unlabeled poly(A) was a potent competitor of the radioactive material. Formation of the 12S complex may be an all-or-none process, because [^{32}P]poly(A) present in excess appears at the top of the gradient and not in intermediate structures.

Experiments similar to those described above for cytoplasmic poly(A)–RNP have also been done on nuclear material. Intact nuclei from ^{32}P-labeled mouse cells were treated with various amounts of T2 RNase and the nucleic acid was then extracted, digested with pancreatic and T1 RNases, and analyzed by gel

Table 1. Sizes of poly(A) fragments produced by T2 RNase digestion of poly(A)–RNP

T2 RNase, units/ml	Band	Size, residues	Difference, residues
10	1	22	
	2	43	26
	3	76	28
	4	106	30
	5	134	28
	6	163	29
20	1	15	
	2	42	27
30	1	12	
	2	36	24
	3	61	25

Procedure was as for Fig. 2, lane 2, except with levels of T2 RNase indicated and electrophoresis in the type of gel used for RNA sequence analysis (14). Sizes were counted up from the position of AMP.

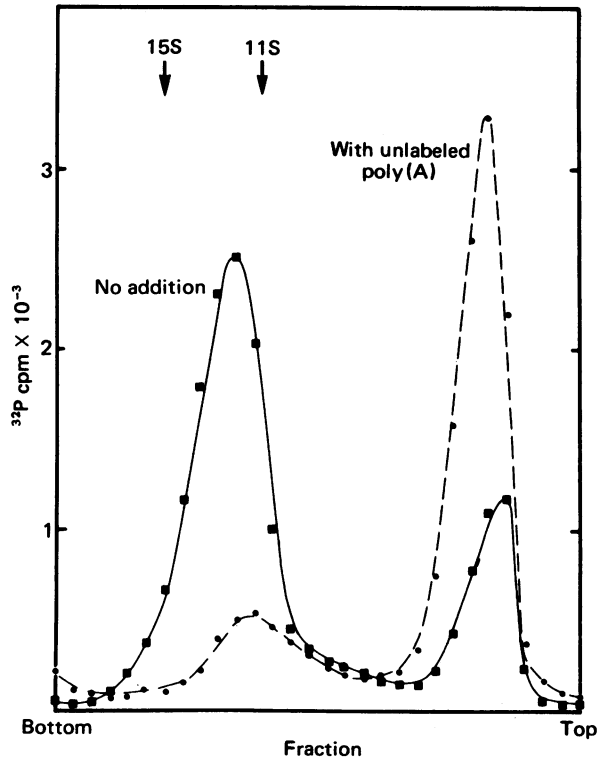

FIG. 3. Sucrose gradient sedimentation of reconstituted poly(A)–RNP. Mixtures of [^{32}P]poly(A) and cytoplasm, with (•– – •) or without (■——■) unlabeled poly(A), were prepared, applied to 5–28.8% isokinetic sucrose gradients (15) containing buffer A and 10 μg of yeast tRNA per ml, and centrifuged in a Beckman SW 41 rotor for 18 hr at 4°C and 40,000 rpm. Arrows indicate the position of nucleosome monomers and dimers from a micrococcal nuclease digest of chromatin run in a parallel gradient.

electrophoresis. In contrast with the band patterns shown above, an essentially continuous distribution of poly(A) fragments was obtained (Fig. 4). Thus the 27-residue repeating structure of cytoplasmic poly(A)–RNP appears to be absent from the nucleus. Nuclear poly(A) does, however, occur in an RNP form, because upon pancreatic and T1 RNase digestion of nuclei, lysis and sedimentation analysis in sucrose, the majority of the poly(A) migrates more rapidly than free poly(A) in the gradient (16).

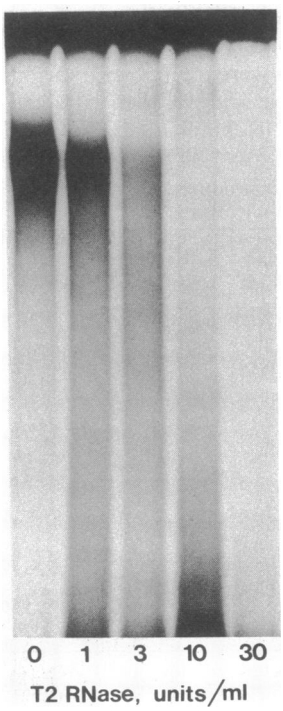

FIG. 4. T2 RNase digestion of nuclear poly(A)–RNP. ^{32}P-Labeled nuclei were digested with T2 RNase at the levels indicated for 20 min at 37°C, and the nucleic acids were extracted and analyzed in a polyacrylamide gel. An autoradiogram of the gel is shown. DNA did not enter the gel, forming the bands at the top.

DISCUSSION

The periodicity of nuclease digestion of poly(A)–RNP may be understood in terms of alternating exposed and protected regions of RNA. Attack by nuclease occurs at any point in an exposed region, producing RNA fragments that are somewhat heterogeneous in size and form broad bands in a gel. During further digestion, exposed RNA is trimmed from the ends of the fragments, causing a reduction in size by the same amount for all bands. When the difference in size between bands is determined, the effect of trimming is eliminated and a value independent of the extent of digestion is obtained (Table 1).

Free poly(A) is digested by T2 RNase in a random fashion, resulting in a continuous distribution of fragments, so the repeating structure of poly(A)–RNP is not a property of poly(A) alone. Rather the protein component of the RNP must be responsible. The protein can be purified from a rat liver extract, with the use of reconstitution with ^{32}P-poly(A) and T2 RNase digestion as an assay. Considerable enrichment has been obtained by chromatography on poly(A)-Sepharose, but further purification is required. With the availability of pure material should come answers to such questions as whether the 75,000-dalton, poly(A)-associated protein (4) or some other polypeptide possesses poly(A)-folding activity, how many protein molecules are bound to a 200-residue stretch of poly(A), what is the arrangement of protein and RNA, and what is the relationship of poly(A)–RNP structure to the transport of poly(A)-containing RNA from nucleus to cytoplasm.

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