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Detection of mRNA sequences in nuclear 30S ribonucleoprotein subcomplexes

[poly(A) + mRNA/complementary DNA/nuclear particles]

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ABSTRACT **RNA from nuclear 30S ribonucleoprotein (RNP) complexes of mouse ascites cells has been shown to contain sequences homologous to poly(A) + mRNA by its ability to hybridize with complementary DNA prepared from poly(A) + mRNA template. Analysis of the hybridization kinetics of poly(A) + mRNA with its own complementary DNA revealed several abundancy classes. The total complexity of poly(A) + mRNA from ascites cells was estimated to be approximately 30,000 sequences of average molecular weight (6×10^5). When the hybridization reaction of 30S RNP-RNA with mRNA-specific cDNA was compared to the homologous reaction the majority, and most probably all, of the poly(A) + mRNA sequences were found to be present in the RNA. The kinetics of hybridization suggest that 10–15% of the RNA in this RNP complex is homologous to poly(A) + mRNA. The 30S RNP subcomplexes therefore contain nuclear poly(A) + mRNA sequences as well as the bulk of heterogeneous RNA.**

The heterogeneous nuclear RNA of eukaryotes (hnRNA) is found complexed with proteins (1–4). In many cell types, the predominant extractable form is a 30S subcomplex of the larger hnRNP, presumably due to endogeneous nuclease activity during RNP isolation (1, 2, 5). In many cell types, the 30S RNP subcomplex contains multiple copies of two polypeptides; a 37,500 dalton and a 40,000 dalton species (6, 7). The RNA component consists of several small-RNA molecules which are presumably derived by fragmentation of a single hnRNA molecule. Approximately 50–70% of the nuclear RNA pulse-labeled with uridine or adenosine is found in this subcomplex (2, 6, 8). Poly(A) is not found in 30S RNP but in a distinct 15–17S ribonucleoprotein (RNP) form (7). The 30S RNP subcomplex therefore contains a large proportion of the total hnRNA and hnRNA-associated proteins. This model does not preclude the possibility that a smaller fraction of the hnRNA sequence is associated with other proteins.

The bulk of hnRNA turns over in the nucleus with a small proportion entering the cytoplasm as mRNA (9, 10). Since most hnRNA is found in a 30S RNP subcomplex form, it seems likely that mRNA sequences in the nucleus would be found in an RNP form that consists, in part, of 30S RNP subunits. Preliminary evidence from filter hybridization-competition studies could demonstrate nucleus-restricted RNA sequences but could not prove the presence of sequences found in the cytoplasm (2). In the cytoplasm, mRNA is found associated with several proteins of discrete molecular weights (11–13); however, proteins which are similar (in terms of molecular weight and immunological crossreactivity) to the two major hnRNA associated proteins have not been found in mRNP (14–16). It is possible, therefore, that only those hnRNA sequences which are destroyed in the nucleus are retained in the 30S nuclear subcomplex. We have now attempted to assess the role, if any, of 30S RNP in mRNA processing by assaying 30S RNP-RNA for mRNA sequences in hybridization studies using complementary DNA (cDNA) synthesized from a cytoplasmic poly(A) + mRNA template. We find the majority, and most likely all, of the cytoplasmic poly(A) + mRNA sequences are present in the 30S RNP subcomplex RNA.

Abbreviations: RNP, ribonucleoprotein; hnRNA, heterogeneous nuclear RNA; SSC, standard saline citrate buffer, 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0; × SSC means that the concentration of the solution used is times that of the standard saline-citrate solution; R_0t, the product of RNA concentration (in moles of nucleotide/liter) and time in seconds; $R_0t_{1/2}$, the R_0t value at which one-half of the cDNA probe is hybridized.

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MATERIALS AND METHODS

RNA Extraction and Purification. Cytoplasm was prepared from Taper ascites cells as previously described (17) and RNA extracted by the chloroform:phenol method of Perry (18). Poly(A) + mRNA was prepared from this RNA by oligo-(dT)-cellulose chromatography (19). The final cytoplasmic RNA precipitate was resuspended in binding buffer (500 mM KCl, 10 mM Tris at pH 7.5), at a concentration of 100–200 μg/ml, and passed through a 3- to 4-g column of oligo(dT)-cellulose (T-3, Collaborative Research). The column was washed with a volume of binding buffer equal to the original RNA volume. Poly(A) + RNA was eluted with glass distilled H_2O. The oligo(dT)-cellulose chromatography was repeated and the final poly(A) + RNA eluant was precipitated with 2 volumes of ethanol after addition of potassium acetate to 2% (wt/vol).

Globin mRNA was prepared from mouse reticulocyte polysomes (17). Polysomes were pelleted and RNA extracted with chloroform:phenol. The polysomal RNA was dispersed on sucrose gradients and the 9S region collected and further purified by oligo(dT)-cellulose chromatography.

The 30S RNP were prepared as previously described (6). RNA from 30S RNP was isolated from pelleted complexes by extraction with chloroform:phenol, and ethanol precipitation. All RNA concentrations were estimated by absorbance at 260 nm, and assumed that 24 A_{260} units equal 1 mg/ml of RNA.

Complementary DNA Preparation. We prepared cDNA from cytoplasmic poly(A) + RNA by the procedure of Verma *et al.* (20) with the reverse transcriptase (RNA-dependent nucleotidyltransferase) from avian myeloblastosis virus (a kind gift of Dr. J. Beard, Life Sciences, Inc.) with the following differences: (*i*) The labeled nucleoside triphosphate was d[^{3}H]CTP (>15 Ci/mmol) which was concentrated 5-fold and added (1/10 vol) without carrier dCTP. (*ii*) Oligo$(dT)_{12-18}$ primer was added to a final concentration of 7.5 μg/ml, poly(A) + RNA to 20 μg/ml, and reverse transcriptase to 300–500 units/ml (21). One unit of activity is expressed as the incorporation of 1 nmol of dTMP into acid-insoluble material in 10 min utilizing a poly(rA)·dT template. (*iii*) Reaction volumes were 200–400 μl. The cDNA was purified as described (20) and stored at −20° in 1 × standard citrate buffer (0.15 M NaCl, 0.015 M sodium

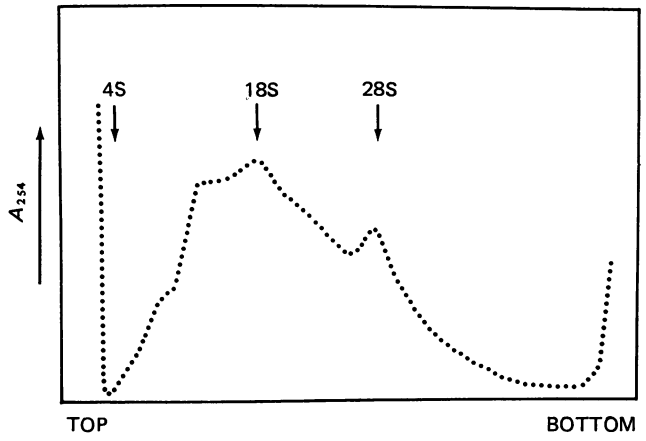

FIG. 1. Sucrose gradient centrifugation of cytoplasmic poly(A) + mRNA from mouse ascites cells. Twenty micrograms of poly(A) + RNA in distilled H_2O was layered on a 5 ml, 5–20% sucrose gradient containing 10 mM Tris at pH 7.0, and centrifuged for 2.5 hr at 49,000 rpm in a Beckman SW 50.1 rotor. The gradient was analyzed on an ISCO density gradient fractionator and the absorbance at 254 nm was monitored. Arrows indicate positions of 4S and rRNA species from a sucrose gradient containing poly(A)–RNA which was run in parallel.

citrate at pH 7.0) (SSC). The cDNA specific activities were 25–30 × 10^6 cpm/μg and its mean size 800 to 900 nucleotides, as estimated by acrylamide gel electrophoresis.

Hybridization and S_1 Nuclease Digestions. RNA and cDNA were mixed at 0° in siliconized tubes and drawn into 5 or 10 μl Corning glass pipettes which were then sealed. Pipettes were prepared for hybridization by soaking in 5 M H_2SO_4 overnight, exhaustively rinsing with glass distilled H_2O, and heating at 200° for 2 hr. Pipettes treated in this manner gave quantitative recoveries of cDNA. Hybridization was performed in a water bath at 67° in 1 × or 6 × SSC. RNA was always present at a 5000-fold, or greater, excess over cDNA. To assay the extent of hybridization, samples were removed from the 67° bath, quenched on ice, and expelled into 400 μl of S_1 nuclease digestion buffer (30 mM sodium acetate, 20 mM NaCl, 0.6 mM $ZnSO_4$, 100 μg/ml of heat denatured *Escherichia coli* or mouse DNA at pH 4.5) (22). The samples were incubated with 50 units of S_1 nuclease (Miles Laboratories) for 2 hr at 37°. Under these conditions 0–5% of native *E. coli* DNA was solubilized and 90–96% of either heat denatured *E. coli* DNA or cDNA was solubilized. After digestion, samples were precipitated with trichloroacetic acid, collected on Whatman GF/A glass fiber filters, and the radioactivity was assayed with toluene-liquifluor. The percentage of cDNA which was S_1 nuclease resistant was calculated by subtracting background radioactivity (nonhybridized samples digested with S_1 nuclease) from each data point. All hybridization points were performed in duplicate.

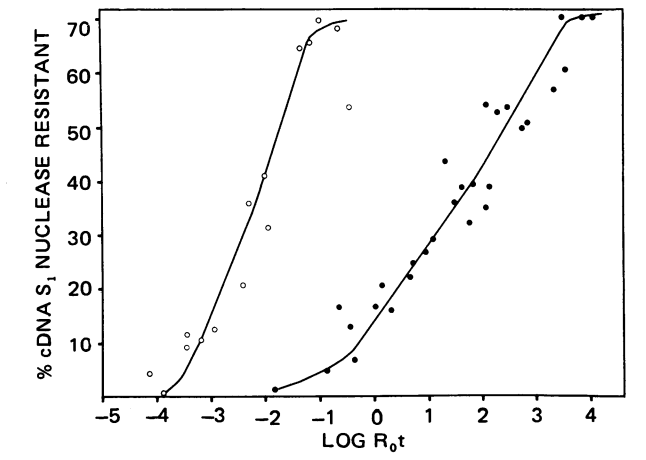

FIG. 2. Hybridization kinetics of poly(A) + mRNA with homologous cDNA and globin mRNA with globin cDNA. Samples were prepared, hybridized, and assayed with S_1 nuclease as described in *Materials and Methods*. Poly(A) + mRNA with homologous cDNA, ●—●; globin mRNA with cDNA, o—o.

Table 1. Analysis of poly(A) + mRNA abundancy classes

Abundancy class	% mRNA*	$R_0t_{1/2}$	$R_0t_{1/2}$ corrected†	No. of 6 × 10^5 dalton sequences per class‡
I	28	0.2	0.056	6
II	43	31	13.3	1,500
III	29	900	261	29,000

* Corrected to 100% hybridization as a saturation value. Data are taken from Fig. 2.

† Corrected for the abundancy class being 100% of reacting RNA.

‡ Based on a $R_0t_{1/2}$ for globin mRNA of 6 × 10^{-3} M sec and a complexity of 4 × 10^5 daltons using the formula:

$$\frac{R_0t_{1/2}\ \text{mRNA class}}{R_0t_{1/2}\ \text{globin mRNA}} \times \frac{4.5 \times 10^5\ \text{daltons}}{6 \times 10^5\ \text{daltons}} = \text{no. of sequences}$$

Polyacrylamide Gel Electrophoresis. The procedure of Loening (23) was used. RNA (4S and 5S) were used as standards in estimating RNA size. Bromphenol blue was used as tracking dye.

RESULTS

Oligo(dT)-cellulose purified cytoplasmic poly(A) + RNA from mouse Taper ascites cells sediments in sucrose gradients between 10 S and 30 S (Fig. 1). We estimate the mean molecular weight of ascites cell poly(A) + RNA to be 6 × 10^5. Our preparations are usually contaminated by a small amount of 18S and 28S rRNA, which we estimate to compose no more than 15% of the poly(A) + RNA fraction. This RNA can be efficiently translated in a cell-free protein synthesizing system (U. Storb, personal communication) and we will refer to it as poly(A) + mRNA.

When cDNA prepared from a poly(A) + mRNA template is hybridized in 1 × SSC to template RNA, the reaction is observed to take place over a 4- to 5-fold logarithm range of R_0t [product of RNA concentration (in moles of nucleotide/liter) and time in seconds] in comparison globin mRNA·cDNA hybridization occurs within a 2-fold logarithm range of R_0t, as expected for a simple pseudo-first order reaction (Fig. 2). The broad kinetics of poly(A) + mRNA is due to different abundancies of various mRNA sequences, as shown by Bishop and others (24–27). To gain some impression of the diversity of ascites cell mRNA, we have divided our hybridization curve into three regions and have calculated $R_0t_{1/2}$ (the R_0t value at which one-half of the cDNA probe is hybridized) values for each and from these the number of different mRNA sequences in the three abundancy classes (Table 1). We have calculated the values by using the globin mRNA·cDNA $R_0t_{1/2}$ as a standard, and the fact that the nucleotide sequence diversity is proportional to $R_0t_{1/2}$ (24). Similar estimates for poly(A) + mRNA have been reported for other mouse cell types (25–27), though our estimate of 30,000 sequences for the least abundant mRNA class is somewhat larger than these authors' estimates (8000–12,000). Our data are very similar to estimates that Bishop *et al.* (24) have made for HeLa cell poly(A) + mRNA.

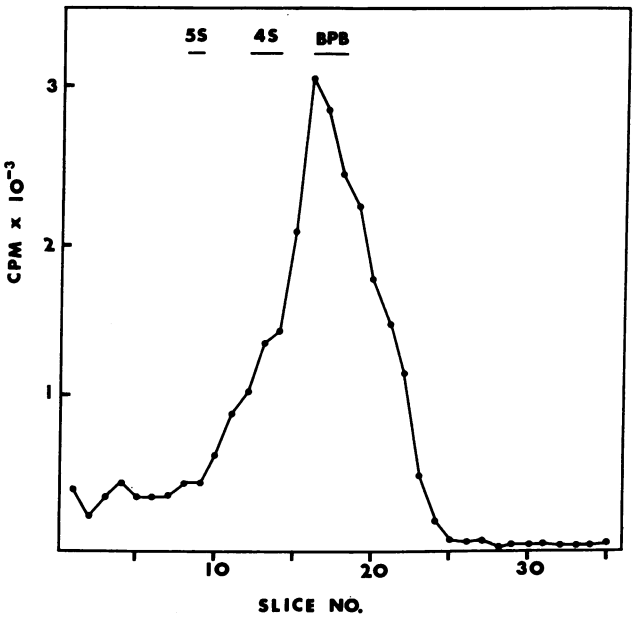

FIG. 3. Polyacrylamide gel electrophoresis of 30S RNP-RNA. Cells from 10 ml of ascites fluid were labeled, as previously described (2), for 25 min with 10 μCi/ml of [^{3}H]adenosine. The 30S RNP complexes were prepared (6) and RNA was extracted as detailed in *Materials and Methods*. Approximately 20,000 cpm of 30S RNP-RNA was electrophoresed on 12% polyacrylamide gels for 2 hr at 5 mA per gel. Gels were sliced into 2.2 mm sections, the RNA solubilized from these fractions, and the radioactivity was assayed (40). BPB, bromphenol blue.

As a preliminary to the hybridization studies with 30S RNP-RNA, we have examined its size distribution on polyacrylamide gels since size may influence hybridization kinetics (28, 29). We estimate 30S RNP-RNA as extracted and purified to be approximately 50 to 90 nucleotides in length (Fig. 3). This is similar to size estimates for 30S RNP-RNA from rat liver nuclei as estimated by sucrose gradient sedimentation (1). We next hybridized crude 30S RNP-RNA to poly(A) + mRNA specific cDNA in 1 × SSC. The reaction occurs at a rate which is 100 times slower than the homologous reaction (Fig. 4). At R_0t values where the homologous reaction has reached a plateau, the crude 30S RNP-RNA can hybridize only 40% as much poly(A) + mRNA specific cDNA. cDNA alone showed no increased nuclease resistance when reacted for a time equal to that of the highest R_0t values, which indicates that homologous RNA is necessary for hybridization. When 30 RNP is purified by sedimentation through a second sucrose gradient after pelleting and resuspension (6), the RNA recovered in 30S RNP (20–30% of the original RNA) is similar in size to crude 30S RNP-RNA (data not shown). The loss of RNA during the purification of RNP is due in part to trailing of RNA not associated with the particle from the 10 to 30S region of our RNP gradients, and the inability to completely resuspend the pellets of crude 30S RNP. Purified 30S RNP-RNA reacts with poly(A) + mRNA specific cDNA faster than when crude 30S RNP-RNA is used (Fig. 4). The actual increase, 4- to 5-fold, is consistent with the retention of all mRNA homologous sequences by purified 30S RNP with the loss of only nonhomologous sequences during the purification procedure. The more rapid kinetics also suggest that the presence of poly(A) + mRNA homologous sequences is not a spurious association of these sequences with 30S RNP subcomplexes since these sequences are enriched rather than reduced by purification of the complexes.

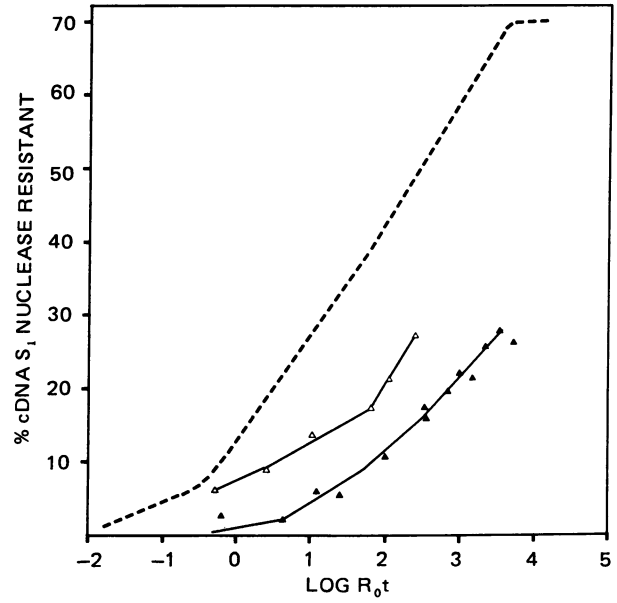

FIG. 4. Hybridization kinetics of crude and purified 30S RNP-RNA with poly(A) + mRNA specific cDNA in 1 × SSC. Samples were prepared, hybridized, and assayed as described in *Materials and Methods*. Crude 30S RNP-RNA with poly(A) + mRNA specific cDNA, ▲—▲; purified 30S RNP-RNA with poly(A) + mRNA specific cDNA, △—△; poly(A) + mRNA homologous reaction replotted from Fig. 2, - - - -.

Since 30S RNP-RNA cannot be hybridized to poly(A) + mRNA specific cDNA to the level achieved in the homologous reaction at reasonable times and RNA concentrations, further hybridizations were carried out in 6 × SSC at 67° to increase the rate of reaction. Under these conditions much more of the cDNA is driven into hybrid by 30S RNP-RNA (Fig. 5). The reaction of crude 30S RNP-RNA with poly(A) + mRNA specific cDNA reaches 63% of the homologous reaction plateau

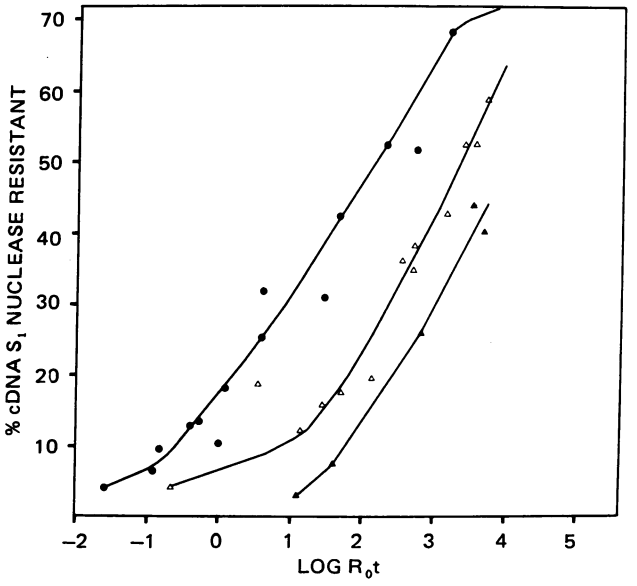

FIG. 5. Hybridization kinetics of crude and purified 30S RNP-RNA and poly(A) + mRNA with poly(A) + mRNA specific cDNA in 6 × SSC. Samples were prepared, hybridized, and assayed as described in *Materials and Methods*. Poly(A) + mRNA homologous reaction, ●—●; crude 30S RNP-RNA with poly(A) + mRNA specific cDNA, ▲—▲; purified 30S RNP-RNA with poly(A) + mRNA specific cDNA, △—△.

level at the highest R_0t values attained (44% absolute reactivity). At similar R_0t values the purified 30S RNP–RNA forms a hybrid with 85% as much of the cDNA as the homologous reaction (59% absolute reactivity). Again the purified 30S RNP subcomplexes yield RNA which hybridizes more rapidly (3-fold) than that of crude 30S RNP. The kinetics indicate no large differences in relative abundancies of the poly(A) + mRNA homologous sequences, as compared to the poly(A) + RNA homologous reaction. Although purified 30S RNP–RNA failed to reach hybridization levels comparable to the poly(A) + mRNA homologous reaction, no indication of a plateau at high R_0t values has been observed (Fig. 5). Higher R_0t values have not been attained due to the difficulty in obtaining the relatively large quantities of 30S RNP–RNA needed to drive this reaction. However, despite this limitation we can conclude that a majority, if not all, of the cytoplasmic poly(A) + mRNA sequences represented in the cytoplasm are present in the RNA found associated with the nuclear 30S RNP subcomplex. If the difference in hybridizable cDNA between 30S RNP–RNA and poly(A) + mRNA were taken as an absolute difference and attributed to sequences in each abundancy class, only approximately 15% of the poly(A) + mRNA sequences could be absent from the 30S RNP–RNA.

It is more difficult to determine precisely the proportion of the nuclear 30S RNP–RNA that is homologous to cytoplasmic poly(A) + mRNA since the effect of a smaller driver RNA size (30S RNP–RNA) on the rate of hybridization is not clear. We have attempted to resolve this difficulty by cleaving poly(A) + mRNA by alkali hydrolysis (30) and hybridizing it to its own cDNA. For a 4-fold reduction in the size of our poly(A) + mRNA, we find an approximate 2-fold reduction in the rate of hybridization of the same cDNA used in the RNP–RNA hybridization experiments (data not shown). This value is close to the square-root-length effect observed for second-order reactions of DNA–RNA hybridization (29). With this correction for 30S RNP–RNA hybridization with poly(A) + mRNA specific cDNA, we would expect the reaction to be approximately 5-fold faster if the molecules were the same size as mRNA. In this case, the poly(A) + mRNA homologous sequences would constitute about 15% of the RNA sequences. Similar calculations for crude 30S RNP–RNA indicate 5% of the RNA sequences are homologous to poly(A) + mRNA. These estimates should be regarded as approximate values since the effect of using RNA all of which is shorter in length than the cDNA probe on the hybridization rate is unclear.

DISCUSSION

There is substantial evidence for a precursor-product relationship between large hnRNA and mRNA (31–36) although opposing evidence has been reported for specific mRNAs (37, 38). Certainly mRNA represents a subpopulation of the rapidly synthesized hnRNA sequences whether it is cleaved from a much larger precursor or not. The exact steps in the processing of hnRNA to functional cytoplasmic mRNA are, at present, unknown. An important aspect of this process are the organization of the mRNA sequences in hnRNP complexes. Our data indicate that sequences homologous to cytoplasmic poly(A) + mRNA sequences of mouse ascites cells are found in the nucleus in structures containing 30S RNP subcomplexes. Since the total amount of RNA in a single 30S RNP is not sufficient to represent an entire sequence of most mRNA molecules (1), a given mRNA sequence must span more than one 30S RNP subcomplex. The majority of the sequences present in 30S RNP subcomplexes however are not homologous to cytoplasmic poly(A) + mRNA and are most likely nucleus-restricted sequences (2). A small proportion of these sequences may be poly(A)–mRNA sequences (39). These observations taken together with the fact that 30S RNP contain the bulk of the hnRNA suggests that the 30S RNP proteins may bind to all hnRNA transcripts which includes mRNA as they are synthesized or immediately following release from the chromatin template, and that this large hnRNA containing RNP complex is the true substrate for the processing of nuclear RNA to functional cytoplasmic mRNA. The only identifiable hnRNA sequence known not to be found in a 30S RNP form is the nuclear poly(A) which is found complexed with other proteins to form a 15–17S RNP subcomplex (5, 7, 40, 41, and T. J. Quinlan, A. Kinniburgh, and T. E. Martin, submitted for publication).

The hybridization kinetics of poly(A) + mRNA with homologous cDNA indicates several abundancy classes of poly(A) + mRNA exist in the cytoplasm of mouse ascites cells. Comparing the hybridization kinetics of purified 30S RNP–RNA (Figs. 4 and 5) with the homologous kinetics suggests that there may be no major abundancy differences between poly(A) + mRNA sequences in 30S RNP and the cytoplasm. More detailed studies are needed to substantiate this conclusion. If correct this would imply, however, that for poly(A) + mRNA sequences in 30S RNP, relative abundancies are the result of differences in transcriptional yield rather than RNA turnover since mammalian hnRNA seems to have a single short half-life (10).

The 30S RNP subcomplex contains the two major hnRNA-associated proteins and few other polypeptides (6). Although other proteins may be present on intact hnRNA (3, 16), these two proteins are associated with the bulk of the hnRNA. Since these polypeptides are not found in mRNP (14, 16) it seems likely that they are replaced on the poly(A) + mRNA during, or just after, transport from the nucleus to the cytoplasm. Some proteins associated with cytoplasmic mRNA could be present on nuclear mRNA sequences since our data do not exclude the possibility that some fraction of the mRNA sequences are contained in other RNP forms in the nucleus. At present, however, we presume that an association of mRNA with the 30S RNP proteins is one phase of the maturation of these sequences in the nucleus.

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