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# Cloned Complementary Deoxyribonucleic Acid Probes for Untranslated Messenger Ribonucleic Acid Components of Mouse Sarcoma Ascites Cells†

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ABSTRACT: Mouse sarcoma ascites cells contain several abundant mRNA species that occur to a large extent in an untranslated state. RNA preparations enriched in these species were used as starting material to construct recombinant plasmids. Cloned plasmids bearing sequences homologous to four of the untranslated mRNA species were identified by translation of hybrid-selected material. These plasmids, as well as a recombinant plasmid derived from chick α-actin mRNA, were used as probes for the estimation of mRNA levels in polyribosomes and in small ribonucleoprotein (RNP) particles of the ascites cells. Considerable amounts of the mRNA molecules belonging to the untranslated species were present in polyribosomes as well as in mRNPs. The actin mRNA, on the other hand, was present almost exclusively in polyribosomes. The distributions obtained by the hybridization assay resembled those estimated by translation of the same RNA preparations in cell-free systems. This indicates that the mRNA molecules of a given species engaged in translation in the cells and those present as untranslated RNP particles are equally effective in cell-free translation systems.

Some mRNA species in mouse sarcoma ascites cells appear to be subject to a control process that determines their degree of utilization for protein synthesis. They occur to a large extent as small cytoplasmic ribonucleoprotein particles not engaged in translation, even though the RNA is functional when used in cell-free systems (Geoghegan et al., 1979). The translation assay has been used to identify the major mRNA species in this category (Geoghegan et al., 1979). However, the limitations of this assay, particularly when applied to complex mRNA mixtures, have made it difficult to obtain precise data on the distribution of mRNA components in polysomes and untranslated mRNPs.[1] Such data would be important for the understanding of the intracellular signals that may be involved in determining the degree of mRNA utilization.

We report on the preparation of recombinant plasmids with cDNA inserts homologous to the major untranslated mRNA components of mouse sarcoma ascites cells. The cloned plasmids served as effective probes for the quantitative estimation of the corresponding mRNAs in polysomes and in mRNP particles. The results show that the mRNA species considered to be untranslated can also occur to a considerable extent as polysomal mRNA and that the mRNA molecules derived from polysomes and from RNP particles are translated with similar efficiencies in cell-free systems.

## Materials and Methods

*Materials.* The restriction endonucleases *Hin*cII, *Pvu*II, *Hae*III, and *Eco*RI, the deoxynucleotidyltransferase, φX174 DNA, and formamide were purchased from Bethesda Research Labs (Gaithesburg, MD), and the restriction enzyme *Pst*I was purchased from New England Biolabs (Beverly, MA). All restriction enzymes were used according to the manufacturer's specifications.

The enzyme nuclease S1 from *Aspergillus oryzae* was obtained from Worthington Biochemicals (Freehold, NJ), and the reverse transcriptase was kindly supplied by Dr. J. Beard of Life Sciences, Inc. (St. Petersburg, FL). Actinomycin D and oligo(dT)$_{8-10}$ were obtained from Calbiochem (La Jolla, CA). The unlabeled deoxyribonucleoside triphosphates, calf thymus DNA, yeast RNA, Pipes [piperazine-*N*,*N*'-bis(2-ethanesulfonic acid)], Mops [3-(*N*-morpholino)propanesulfonic acid], and EDTA (ethylenediaminetetraacetic acid) were purchased from Sigma Chemical Co. (St. Louis, MO). The labeled nucleotides were from New England Nuclear (Boston, MA), and poly(A), *Escherichia coli* tRNA, and the agarose used for gel electrophoresis were from Miles Laboratories (Elkhart, IN). Nitrobenzyloxymethyl-paper (NBM) was purchased from Schleicher & Schuell (Keene, NH), and

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[1] Abbreviations: RNP, ribonucleoprotein; mRNP, mRNA-containing ribonucleoprotein; poly(A+) RNA, poly(A)-containing RNA; cDNA, DNA complementary to mRNA sequences; Tris, tris(hydroxymethyl)-aminomethane; NaDodSO$_4$, sodium dodecyl sulfate; oligo(dT), oligo-(deoxythymidylate).

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converted to the diazobenzyloxymethyl (DBM) form by the procedure of Alwine et al. (1977).

The yeast RNA, *E. coli* tRNA, and poly(A) were purified by repeated phenol extraction to remove residual protein.

*Preparation of RNA Used for Cloning.* Messenger ribonucleoprotein particles highly enriched in untranslated mRNA components were obtained from cytoplasmic extracts of mouse sarcoma ascites cells that were exposed briefly to low levels of cycloheximide. The latter treatment maximizes mRNA uptake into polysomes but leaves some major species in the mRNP fraction (Geoghegan et al., 1979). The procedures for cell incubation in the presence of the inhibitor and for the preparation of the cytoplasmic extracts were as described previously (Geoghegan et al., 1979) except for the use of 130 mM NaCl instead of the KCl during cell lysis. For the isolation of the mRNP particles, 12-mL samples of the cytoplasmic extract were layered over 12 mL of 30% sucrose in 50 mM Tris-HCl, pH 7.6, 100 mM NaCl, and 1 mM $MgCl_2$ in 25-mL centrifuge tubes and subjected to centrifugation at 30000 rpm for 150 min in the Spinco fixed-angle type 30 rotor at 4 °C. The lower 7-mL portion of the tube contents was discarded, and the next 9-mL portion was collected. This procedure permitted the isolation of particles from large volumes of cell lysate. The mRNP fraction was supplemented with $NaDodSO_4$ to a final concentration of 0.5% and with 2.5 volumes of ethanol. The material precipitated overnight was deproteinized by extraction with phenol and phenol–$CHCl_3$ (Geoghegan et al., 1978).

Poly(A)-containing RNA was obtained from the above preparation by adsorption on oligo(dT)–cellulose at room temperature in the presence of 50 mM Tris-HCl, pH 7.6, 500 mM NaCl, and 0.1% $NaDodSO_4$ and elution with 0.1% $NaDodSO_4$. After precipitation by ethanol in the presence of 0.1 M NaCl, the RNA was subjected to zone centrifugation at 15 °C in the Spinco SW 41 rotor through a linear 5–30% sucrose gradient in 20 mM Tris-HCl, pH 7.6, 10 mM NaCl, and 0.1% $NaDodSO_4$. The centrifugation was for 5 h at 40000 rpm. The fractions sedimenting in the 12–16S and the 17–20S ranges were pooled separately and collected by ethanol precipitation. They were designated as L and H, respectively.

*Construction of Recombinant Plasmids.* Samples of 2.5 μg of poly(A+) RNA fractionated by zone centrifugation were incubated in a total volume of 50 μL with 650 units/mL reverse transcriptase in the presence of 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 12 mM dithiothreitol, 5 mM $MgCl_2$, 40 μg/mL actinomycin D, 12 μg/mL oligo(dT), 1 mM each of dATP, dGTP, and TTP, and 60 μCi of [$^3$H]dCTP (2.2 Ci/mmol). After incubation at 46 °C for 20 min, EDTA was added to a concentration of 20 mM, the chilled mixture was deproteinized by shaking with $CHCl_3$ (Buell et al., 1978), and the resulting aqueous phase was subjected to gel filtration through Sephadex G-100 in $H_2O$. The material in the excluded fraction was collected by ethanol precipitation in the presence of 10 μg of *E. coli* transfer RNA used as carrier. The recovered material was incubated in 0.3 N NaOH at 45 °C for 4 h to destroy the RNA, the incubation mixture was neutralized with acetic acid, and the cDNA was collected by ethanol precipitation overnight followed by centrifugation at 15000 rpm. It was redissolved in 10 μL of 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA.

For the synthesis of the second strand, the cDNA was incubated in a total volume of 50 μL with 520 units/mL reverse transcriptase in the presence of 50 mM Tris-HCl, pH 8.3, 12 mM dithiothreitol, 5 mM $MgCl_2$, 0.5 mM each of dATP, dGTP, and dCTP, and 10 μCi of [$^{32}$P]TTP (0.8 Ci/mmol). The incubation was for 3 h at 46 °C. The reaction product was deproteinized by extraction with $CHCl_3$, subjected to gel filtration as described above, and recovered by ethanol precipitation in the presence of 10 μg of poly(A) used as carrier.

The precipitated material was dissolved in a minimal volume of 10 mM Tris-HCl, pH 7.6, and 5 mM EDTA and then incubated for 10 min at 45 °C with 10 units of S1 nuclease in 70 μL of 0.2 M sodium acetate, pH 4.5, 0.4 M NaCl, and 2.5 mM $ZnSO_4$ to remove single-stranded material. The reaction product was deproteinized with $CHCl_3$ and collected again by ethanol precipitation in the absence of carrier. The resulting double-stranded DNA was tailed at the 3′ termini by incubation at 37 °C for 20 min with 24 units of deoxynucleotidyltransferase in 50 μL of 0.1 M potassium cacodylate and 20 mM Tris (final pH 7.5), 1 mM $CoCl_2$, 1.7 mM β-mercaptoethanol, and 30 μM [$^3$H]dCTP (25 Ci/mmol). The plasmid PBR322 was cleaved at the *Pst*I restriction site and incubated with deoxynucleotidyltransferase as described above, except that [$^3$H]dGTP (30 Ci/mmol) was used as the substrate, at a concentration of 50 μM, and that the time of incubation was 35 min. The reaction mixtures were then supplemented with an equal volume of 10 mM Tris-HCl, pH 8.0, and 5 mM EDTA and deproteinized by phenol extraction. Samples of 7 ng of tailed double-stranded cDNA were mixed with 35 ng of tailed PBR322, and the mixtures were precipitated with ethanol in the presence of 0.1 M NaCl. The material collected by centrifugation was resuspended in 50 μL of 10 mM Tris-HCl, pH 7.6, 200 mM NaCl, and 1 mM EDTA. The mixtures were next incubated at 60 °C for 5 min, then allowed to return to room temperature over a 3-h period, and finally stored overnight at 4 °C prior to transformation.

*Cloning and Colony Hybridization. Escherichia coli*, strain MM294, was transformed according to the procedure of Cohen et al. (1972), in accordance with NIH guidelines for recombinant DNA research. Transformants, identified by their resistance to tetracycline and sensitivity to ampicillin, were screened by in situ colony filter hybridization according to the procedure of Grunstein & Hogness (1975). The cDNA probe used for the hybridizations was prepared by incubating 0.2-μg samples of the poly(A+) RNA from mRNP particles, derived from cycloheximide-treated cells, with reverse transcriptase essentially as described above. Actinomycin D was omitted from the reaction mixture, and 20 mCi/mL [$^{32}$P]dATP (400 Ci/mmol) was used as the labeled nucleotide.

Positive colonies were used for the isolation of plasmid DNA, using the following procedure. The cells were grown in L-broth culture medium containing 10 μg/mL tetracycline to a density corresponding to an $A_{590}$ of 0.85, and the plasmids were amplified by an overnight exposure of the culture to 230 μg/mL chloroamphenicol. The bacterial cells were collected by centrifugation and incubated in the presence of lysozyme as described by Clewell & Helinski (1970), and the resulting spheroplasts were lysed by the Hirt procedure (1967). Plasmid DNA was obtained by buoyant density centrifugation in CsCl–ethidium bromide gradients, using the procedure of Clewell & Helinski (1970), modified as follows. The DNA samples were dissolved in 10 mM Tris-HCl, pH 8.0, and 5 mM EDTA instead of in Tes buffer, and the centrifugation was in the vertical Ti65 Spinco rotor at 48000 rpm for 20 h at 15 °C.

*Hybrid Selection and Cell-Free Translation.* Samples of plasmid DNA were cleaved by using the restriction endonuclease *Eco*RI, then denatured by alkali, and bound to nitrocellulose as described by Kafatos et al. (1979). The filters were prehybridized for 90 min at 58 °C in 0.1 M Pipes buffer,

pH 6.4, 50% formamide, 0.6 M NaCl, and 100 μg/mL poly(A). They were next incubated with 50 μL of the same solution lacking the poly(A) but including 30 μg of RNA isolated from mRNP particles. RNA not subjected to oligo(dT)–cellulose fractionation was used in this case. The hybridization was for 150 min at 55 °C. The filters were then washed 6 times at 60 °C with 50 mM Pipes, pH 6.4, 0.2 M NaCl, and 0.5% $NaDodSO_4$ and 3 times with the same solution lacking the detergent. Bound RNA was next eluted from the filters with 100 μL of $H_2O$ kept at 100 °C for 90 s. *E. coli* tRNA (2 μg) was added as carrier, and the mixture was precipitated with ethanol in the presence of 0.1 M NaCl. The precipitated material was dissolved in 7 μL of $H_2O$, and portions were used for translation in the reticulocyte lysate. The conditions for cell-free translation and for identification of products by polyacrylamide gel electrophoresis were as described previously (Cereghini et al., 1979).

*Quantitative Estimation of Specific mRNAs.* RNA samples in 7 μL of $H_2O$ were dotted onto cellulose nitrate strips as described by Thomas (1980). The strips were prehybridized at 55 °C for 5–12 h in 50% formamide, 5 × SSC (SSC: 0.15 M NaCl and 0.015 M sodium citrate), 50 mM sodium phosphate, pH 6.8, 500 μg/mL denatured calf thymus DNA, 1% glycine, and 0.02% each of bovine serum albumin, ficoll, and polyvinylpyrrolidone. The hybridization was carried out in the same solution lacking glycine and containing 1.5 μg of the plasmid DNA probe labeled with $^{32}P$ by nick translation according to Rigby et al. (1975). The specific activity of the probe ranged from $30 \times 10^6$ cpm/μg of DNA to $60 \times 10^6$ cpm/μg of DNA. The labeled probe was denatured at 100 °C prior to inclusion in the hybridization buffer. After incubation at 55 °C for about 18 h, the strips were transferred to a large volume of 2 × SSC and 0.1% $NaDodSO_4$ at room temperature. They were placed next on a Büchner funnel and washed thoroughly with the above solution followed by $NaDodSO_4$ in 0.1 × SSC, using gentle suction. The washed strips were subjected to autoradiography in order to locate the dots. These were cut out from the cellulose nitrate strips and the amounts of radioactivity determined in the scintillation counter.

*Northern Blots.* Samples of cytoplasmic poly(A+) RNA (1 μg) were denatured at 60 °C for 5 min in 50% formamide, 6% formaldehyde, 20 mM Mops, pH 7.0, 5 mM sodium acetate, and 1 mM EDTA and subjected to electrophoresis at 100 V for 6 h in 1% agarose containing the same buffer without formamide (Crain et al., 1981). The gel was washed quickly 4 times in $H_2O$ and twice in 0.2 M sodium acetate, pH 4.0 (10 min each wash), and then transferred to DBM-paper by electrophoresis in the same acetate buffer, as described by Stellwag & Dahlberg (1980), using 0.2 M NaOAc as the electrode buffer. Electrophoresis was at 150 mA for 2 h, after which the DBM-paper was either stored overnight in 50 mM sodium phosphate, pH 6.8 at 4 °C, or processed immediately for prehybridization and hybridization as described above.

## Results

*Identification of Recombinant Plasmids.* The starting material used for the synthesis of the radioactive cDNA probe for in situ colony hybridization, although enriched in the desired mRNA species, represented a heterogeneous population. The detection of the recombinant plasmids was based on the expectation that the abundant species in the $^{32}P$-labeled cDNA would react rapidly with the colonies containing inserts homologous to these species. The hybridizations were carried out for two time periods, and the colonies reacting most rapidly were selected. Figure 1 shows the results of a typical in situ hybridization, carried out for 5 and 17 h. About 15% of the colonies tested showed rapid reaction with the cDNA. A high proportion of the cloned recombinant plasmids selected in this fashion carried sequences specific for the desired mRNA components (Table I). Figure 2 shows the translation products of individual mRNA species selected by hybridization to cloned plasmids. The polypeptide P40 obtained by translation of mRNP RNA tended to migrate as a doublet in some experiments (see left panel in Figure 2). Translation of the hybrid-selected P40 mRNA also showed this tendency (data not shown).

A recombinant plasmid specific for chick α-actin mRNA, pAC269 (Schwartz et al., 1980), kindly supplied to us by Dr. Robert Schwartz, was also used in this study. Although its sequence homology to the mouse actin mRNA must be limited,


FIGURE 1: Detection of clones bearing DNA sequences homologous to abundant cDNA species. Infected *E. coli* colonies transferred to cellulose nitrate and subjected to in situ hybridization with [$^{32}P$]cDNA derived from poly(A+) mRNP RNA. Duplicate sets of colonies were reacted for 5 and 17 h. For details, see Materials and Methods.

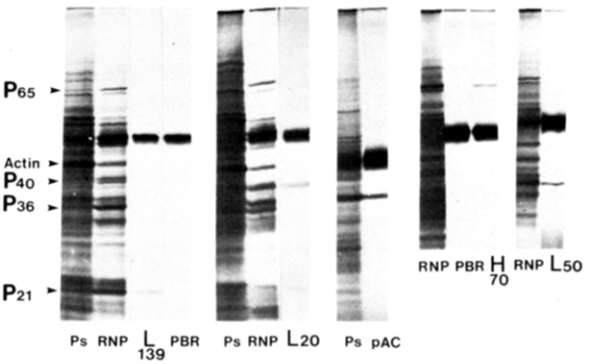


FIGURE 2: Translation products from mRNAs selected by hybridization to cloned recombinant plasmids. RNA isolated from mRNP particles was annealed to DNA from indicated plasmids, and from PBR322. The RNP particles used in these experiments were from ascites cells not subjected to any incubation or cycloheximide treatment. Polysomal RNA, instead of mRNP RNA, was used in the reaction with the plasmid pAC296 (pAC). Translation products from the RNAs recovered from hybrids and from total polysomal (Ps) and mRNP (RNP) RNAs were analyzed by gel electrophoresis followed by autoradiography. For details, see Materials and Methods. Polypeptides of interest are identified in the panel at left.

Table I: Yields of Cloned Plasmids Specific for Untranslated mRNP Species[a]

| RNA fraction[b] | total clones tested | mRNA species selected by cloned plasmids | | | | | |
|---|---|---|---|---|---|---|---|
| | | P65 | P40 | P36 | P21 | others | ND[c] |
| L | 16 | 0 | 5 | 3 | 4 | 0 | 4 |
| H | 11 | 1 | 0 | 2 | 1 | 2 | 5 |

[a] Data show numbers of transformants bearing DNA sequences homologous to indicated mRNA species. Recombinant plasmids used for the transformation were constructed with cDNAs derived from mRNP RNA fractions H and L (see Materials and Methods). Category "others" stands for mRNAs other than the major untranslated species under study. [b] RNA fraction used for cloning. [c] ND, none detected.

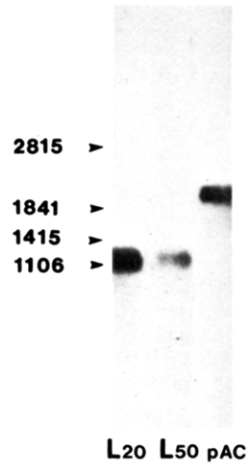


FIGURE 3: Gel electrophoresis of poly(A+) mRNA species homologous to recombinant plasmids. Poly(A+) RNA from mouse sarcoma cells was subjected to Northern blot analysis as described under Materials and Methods. Labeled plasmids L20, L50, and pAC269, which represent the P40, P36, and actin mRNAs, respectively, were used for the hybridizations. Size markers were obtained by digesting PBR322 samples with mixtures of *Pst*I and *Pvu*II or *Pvu*II and *Hinc*II and subjecting the denatured restriction fragments to the same analysis.

it could be used under hybridization conditions stringent enough to yield highly purified mRNA, as judged by examination of its translation products (Figure 2).

Plasmids specific for the P40 and P36 mRNAs, as well as the chick pAC269, were used for a Northern blot analysis of the mRNA sizes in poly(A)-containing polysomal RNA. In each case, a single radioactive band was observed (Figure 3). The sizes, estimated by comparison with the mobilities of denatured restriction fragments of PBR322, were 1200, 1170, and 2030 nucleotides for the P36, P40, and actin mRNAs, respectively.

*Size of DNA Inserts.* Digestion of the plasmids with *Pst*I released DNA fragments representing presumably the full length of the inserts. Figure 4 shows the migration of the digestion products on polyacrylamide gel electrophoresis. The plasmids H70, L20, and L139, representing the mRNAs for P65, P40, and P21, respectively, yielded a single fragment each. The cloned plasmid L50, representing the P36 mRNA, yielded two fragments. Another cloned plasmid specific for P36 mRNA also yielded two *Pst*I fragments, of somewhat different sizes. It appears, therefore, that the DNA sequence corresponding to the P36 mRNA contains a *Pst*I restriction site. A *Pvu*II site was also observed in this sequence. The DNA inserts coding for P40 and P21 were found to contain a *Hinc*II site. No systematic attempts to construct restriction maps were made.

The sizes of the fragments shown in Figure 4 are 600, 650, 240 + 130, and 260 base pairs for the P65, P40, P36, and P21 cloned cDNAs, respectively.

*Messenger RNA Levels in Polyribosomes and mRNPs.* For determination of levels of individual mRNA species in various preparations, RNA samples were fixed onto nitrocellulose and allowed to react with the appropriate labeled probe. This procedure proved effective for quantitative mRNA assays. The relation between amounts of labeled pAC269 hybridized and amounts of poly(A+) RNA present in the dots is shown in Figure 5. In this experiment, yeast RNA (6 μg) used as a nonspecific control led to the binding of only 15 cpm above the value for the blank. This high degree of specificity was achieved only when the reactions were carried out in the absence of dextran sulfate, an ingredient that is commonly used in this type of hybridization assay.

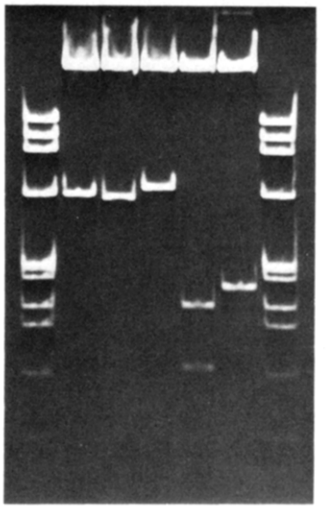


FIGURE 4: Electrophoretic mobility of fragments released from recombinant plasmids by digestion with *Pst*I restriction endonuclease. Samples of plasmids digested with *Pst*I were subjected to polyacrylamide gel electrophoresis (6% gels) in Tris–borate–EDTA buffer according to the procedure of Peacock & Dingman (1968). Gels were stained with ethidium bromide and photographed. Lanes c, d, e, and f represent digests of plasmids H70, L20, L50, and L139, respectively. Lane b represents the digest of an as yet unidentified plasmid, H153. Lanes a and g display an *Hae*III digest of φX174, used as size markers.

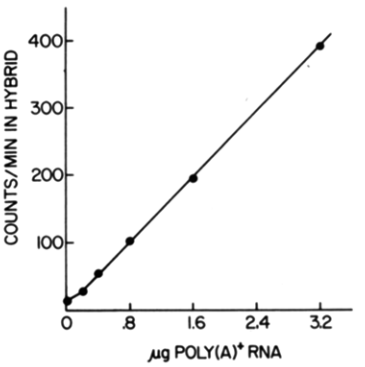


FIGURE 5: Dot hybridization assay for the estimation of actin mRNA. Samples of 6 μg of RNA, containing indicated amounts of mouse sarcoma poly(A+) RNA and yeast RNA as the balance, were applied to cellulose nitrate and subjected to hybridization with pAC269 labeled with $^{32}P$ by nick translation. For details, see Materials and Methods. The blank value (radioactivity bound in the absence of any RNA) was 3 cpm.

The assay was applied to the polysomal and mRNP RNA fractions obtained from ascites cells incubated for 1 h in culture medium. The amount of polysomal RNA used in these measurements, 6 μg, was within the linear range of the assay for this RNA fraction. The RNA from the mRNP fraction showed a linear response only with amounts up to 2 μg, and 1.2-μg samples were used in this case. Table II shows the distribution of some of the untranslated mRNA species and of the actin mRNA, as assayed by hybridization to the nick-translated probes. The data are expressed as cpm hybridized to the RNA sample present in each dot. Yeast RNA used as control caused virtually no hybridization. The results show that most of the actin mRNA is present in polysomes, while 70–80% of the P40, P36, and P21 mRNA molecules occur as free mRNPs in the incubated cells (Table III). About 50% of the RNA molecules that hybridize with the probe for P65 remain untranslated in these cells.

The above distributions are similar in a general way to those estimated by the translation assay. The latter assay, when

Table II: Levels of Individual mRNA Species in Polyribosomes and Free RNP Particles of Ascites Cells Incubated in Culture Medium[a]

| RNA samples | nick-translated probes | | | | |
|---|---|---|---|---|---|
| | H70 (P65) | pAC269 (actin) | L20 (P40) | L50 (P36) | L139 (P21) |
| polysomal | 180 | 557 | 99 | 133 | 255 |
| RNP | 104 | 65 | 180 | 169 | 406 |
| yeast | 13 | 9 | 10 | 13 | 10 |
| none | 10 | 4 | 8 | 8 | 4 |

[a] Ascites cell samples were incubated in complete medium for 1 h. RNA preparations were obtained from polyribosomes and from small RNP particles isolated by zone centrifugation from cytoplasmic extracts of incubated cells. The RNP fraction consisted of material sedimenting in the 20–70S range, and the polyribosome fraction comprised the components larger than the ribosomal dimer. The procedures for cell incubation and isolation of RNA fractions have been described previously (Geoghegan et al., 1979). Amounts of RNA used for hybridization were 6 μg for the polysomal and yeast RNAs and 1.2 μg for the RNP RNA. Data show amounts of labeled plasmid DNA hybridized to RNA samples, expressed as cpm. The RNA preparations used in this experiment were not fractionated by oligo(dT)-cellulose chromatography. Total amounts of RNA present in the polysomal and RNP RNA preparations were 970 and 370 μg, respectively.

Table III: Distribution of mRNA Components in Untranslated Fraction of Cells Incubated in Culture Medium[a]

| type of assay | messenger RNA species | | | | |
|---|---|---|---|---|---|
| | P65 | actin | P40 | P36 | P21 |
| hybridization | 52 | 18 | 79 | 72 | 76 |
| translation | | | | | |
| wheat germ | | 6 | 65 | 73 | 45 |
| reticulocyte | 95 | 17 | 49 | 81 | 49 |

[a] RNA preparations described in Table II were used for hybridization assays as shown in Table II and for translation assays as described previously (Cereghini et al., 1979). Data represent amounts of each mRNA species present in mRNP fraction, expressed as percent of total amounts present in combined polysomal and RNP fractions. Translation assays were carried out both in wheat germ and in reticulocyte cell-free systems.

applied to the same polysomal and RNP RNA preparations, yielded the results shown in Table III. The values for the P36 and P40 mRNAs obtained by translation are the least reliable, because the corresponding polypeptides are resolved very poorly from other translation products of polysomal RNA preparations (see Figure 2). Yet these values agree reasonably with those obtained by the hybridization assay. The translation data for the P65 mRNA, on the other hand, should be more accurate, since P65 is usually well resolved on one-dimensional polyacrylamide gels. The large discrepancy between the values for P65 mRNA distribution obtained by the two assays (Table III) was observed consistently and is difficult to understand.

## Discussion

The present results confirm the existence in mouse sarcoma ascites cells of several abundant mRNA species that occur to a large extent as untranslated molecules. Brief exposure of the cells to a low level of cycloheximide tends to drive mRNA into polysomes and leaves the free mRNP fraction highly enriched in these mRNA species (Geoghegan et al., 1979). This was first shown by the translation assay and is confirmed by the fact that recombinant plasmids derived from the DNA complementary to the mRNA of free RNPs yield a relatively high proportion of clones with sequences homologous to the untranslated mRNAs. The same cDNA served as an effective probe for the initial screening by in situ hybridization, when used under conditions that reveal colonies hybridizing rapidly with the probe.

The dot hybridization assay proved effective for the quantitative estimation of individual mRNA levels. It shows clearly that the mRNAs for P21, P36, P40, and P65 occur both in polyribosomes and in small RNP particles. Further studies with the P36 and P40 mRNAs, to be presented in a separate report, show that the polyribosomal component of these species exhibits a typical sedimentation profile for mRNA engaged in translation. They also show that mRNA molecules in the mRNP fraction are not shifted to polysomes when the capacity of the cells for polypeptide chain initiation is enhanced. These studies indicate that the unusual distribution of these mRNA species cannot be attributed to low initiation efficiency. The P40, P36, and P21 chains derived from polysomes and from RNPs appear to be equally effective in in vitro translation. This is indicated by the similarity in the values for mRNA levels obtained by the translation and hybridization assays. It seems unlikely, therefore, that the difference in in vivo behavior is due to some alteration in mRNA structure that affects "translatability". The functional characteristics of the isolated mRNP particles in the reticulocyte cell-free translation system suggest that proteins associated with the particles may be responsible for the untranslated state (Geoghegan et al., 1979; Bergmann et al., 1982).

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