Messenger RNA species partially in a repressed state in mouse sarcoma ascites cells

Richard Yenofsky, Ingrid Bergmann*, and George Brawerman†

Department of Biochemistry and Pharmacology, Tufts University School of Medicine, 136 Harrison Avenue, Boston, Massachusetts 02111

Communicated by Hamish N. Munro, July 19, 1982

ABSTRACT Four major mRNA species of mouse sarcoma ascites cells, coding for polypeptides designated P65, P40, P36, and P21, occur predominantly as untranslated messenger ribonucleoprotein particles. Cloned cDNA probes were used to study their distribution in cytoplasmic extracts of these cells. A considerable portion of the mRNA molecules sedimented as small particles, whereas the rest was present in polyribosomes. In contrast, the actin mRNA was present almost exclusively in polyribosomes. Incubation of the ascites cells in culture medium, particularly after a starvation treatment, caused an enhancement in polypeptide chain initiation relative to elongation in these cells, as evidenced by a shift of ribosomes into the polyribose fraction and by an increase in polyribose size. Exposure of the cells to a low concentration of cycloheximide, an inhibitor of the elongation step, had a similar effect. The actin mRNA and the active P65, P40, P36, and P21 mRNA molecules were shifted to larger polyribosomes in the treated cells, but no shift of molecules from small particles to polyribosomes was observed. The incubation in culture also led to considerable increases in the proportion of P65 and P40 mRNA molecules in the untranslated state. The results indicate that the untranslated state cannot be attributed to poor initiation efficiency. It is suggested that a portion of the mRNA molecules is maintained in a repressed state and that mRNA repression may represent an important translation control process.

mRNA molecules not engaged in translation have been observed in a variety of eukaryotic cells (1). They occur in the cytoplasm as small ribonucleoprotein (RNP) particles (2) and are active in cell-free translation systems after deproteinization. The untranslated messenger ribonucleoprotein (mRNP) particles present in oocytes (3) appear to represent material stored for use after fertilization. Stage-specific activation of individual mRNA species has been observed in the course of early embryonic development (4). The significance of untranslated mRNAs in somatic cells is less obvious. It is also not clear whether the untranslated state in these cells is due to the affected mRNA species being inefficient in the translation initiation process or whether these RNA molecules are associated with agents that prevent their translation. Exposure of cells to cycloheximide at low concentrations has been shown to cause the transfer of RNAs from the small RNP fraction to polyribosomes (5–7). This compound, by slowing down polypeptide chain elongation, enhances ribosome packaging on mRNA chains and tends to promote the uptake into polyribosomes of mRNA molecules that have a low rate of initiation. The observed effect of cycloheximide has been taken as an indication that the untranslated mRNP fraction contains active but inefficient mRNA species. This view is also supported by the fact that α-globin mRNA, which occurs to some extent as free mRNP in reticulocytes (8), behaves like an inefficient species both in vivo and in vitro (9, 10).

Some mRNA species of mouse sarcoma 180 ascites cells appear to occur in a masked state. They remain as small mRNPs in cells exposed to cycloheximide (7). Moreover, the free mRNP particles from the treated cells are translated poorly in the reticulocyte cell-free system, under conditions that permit effective translation of mRNPs derived from polyribosomes (7, 11). Untranslated globin and histone mRNPs isolated from other cell types have also been shown to be inactive in vitro (12, 13), an indication that they may represent repressed mRNA as well. Myosin heavy chain mRNPs, isolated from induced chicken myoblasts, appear to be maintained in an untranslated state when introduced into the unduced cells, whereas the free mRNA is taken up into polysomes in these cells (14). It has been suggested that ferritin mRNA, which occurs to a considerable extent as untranslated mRNP in rat liver, could be maintained in a repressed state through interaction with its own translation product, apoferitin (15).

In the present report, we examine four major mRNA species that occur to a considerable extent as free mRNP particles in mouse sarcoma ascites cells. These species code for polypeptides designated P65, P40, P36, and P21. The availability of cloned cDNA probes for these mRNA species (16) permitted us to make precise measurements of mRNA levels in polyribosomes and in mRNPs. mRNA molecules of each species were found both in mRNPs and in polyribosomes. The distributions of individual mRNA species were compared in cells subjected to treatments that cause the accumulation of ribosomes on mRNA, either by enhancing polypeptide chain initiation or by inhibiting elongation. These treatments increased ribosome packing on the active mRNA molecules but did not decrease the proportion of the molecules in the untranslated state. Our results indicate that mouse tumor cells contain mRNA species that are utilized to a limited extent for protein synthesis and that the untranslated molecules of these species are maintained in a "repressed" state in the cells. mRNA repression appears to be a means to regulate the rate of synthesis of some polypeptides in the cells.

EXPERIMENTAL PROCEDURES

Cell Incubations. Mouse sarcoma 180 ascites cells, maintained by weekly transfer into the peritoneal cavity of albino mice, were incubated with gentle shaking at a density of 2 × 10⁶ cells per ml in a culture medium consisting of Krebs bicarbonate buffer supplemented with glucose, amino acids, and serum, as described (17). For the starvation–recovery experi-

Abbreviations: RNP, ribonucleoprotein; mRNP, messenger RNP.
* Present address: Centro de Virologia, Serrano 661, 1414 Buenos Aires, Argentina.
† To whom reprint requests should be addressed.
ments, the ascites cells were incubated in unsupplemented Krebs bicarbonate buffer for 60 min and in complete medium for an additional 15 min (18). Cells subjected to the cycloheximide treatment were first incubated for 20 min in the culture medium, and the incubation was continued for an additional 15 min in the presence of the drug at 0.75 \( \mu \)g/ml (7). The cells were collected by centrifugation after very rapid cooling of the cultures to avoid ribosome runoff. Ascites cells not subjected to incubation prior to cell lysis were transferred rapidly from the animals into ice-cold glucose/citrate buffer (7).

Fractionation of Cytoplasmic Extracts and RNA Preparation. The cells were subjected to hypotonic swelling followed by lysis in the presence of 0.1% Triton X-100, 0.1 M NaCl, and 10% sucrose, as described (19). Particulate matter was removed from the lysates by centrifugation at 12,000 \( \times \) g for 10 min, and the supernatant fraction was subjected to zone centrifugation in the SW 27 Spinco rotor. Samples were layered over a 30-ml 10–40% sucrose gradient in 50 mM Tris-HCl, pH 7.6/100 mM NaCl/1 mM MgCl\(_2\). A 5-ml cushion of 2 M sucrose in the same buffer was included at the bottom of the tubes. Centrifugation was at 4°C for 3.5 hr at 25,000 rpm. Fractions of about 1.8 ml were collected, and they were supplemented immediately with sodium dodecyl sulfate to a final concentration of 0.5%. Individual fractions, or pooled fractions, were precipitated with 2.5 vol of ethanol. The precipitates were dissolved in 0.5% sodium dodecyl sulfate/0.1 M Tris-HCl, pH 9.0, and subjected to deproteinization by extraction with phenol as described (20).

Hybridization and Translation Assays. RNA samples were fixed as dots onto cellulose nitrate (Millipore) sheets and subjected to hybridization with 32P-labeled recombinant plasmids bearing cDNA inserts, as described (16). The amounts of RNA used for hybridization were 6 \( \mu \)g for polysomal RNA and 0.8–1.6 \( \mu \)g for the RNA from small particles. The amount of radioactivity bound to the dots was measured by scintillation counting.

RNA samples prepared by deproteinization of the small RNP particle fraction were translated in the reticulocyte cell-free system and the translation products were separated by one-dimensional polyacrylamide gel electrophoresis as described (7, 11, 21). The relative amounts of radioactivity in individual bands were determined by autoradiography followed by densitometry tracing.

RESULTS

Sedimentation Profiles of Polyribosomes Bearing Individual mRNA Species. Fig. 1 shows the sedimentation characteristics of the P40 and P36 mRNAs in cytoplasmic extracts of cells incubated for 5 hr in culture medium. A large portion of these mRNA molecules is in particles sedimenting more slowly than the ribosomal subunits, while the remainder cosediments with the polyribosomes. The actin mRNA, on the other hand, is present almost exclusively in the polyribosome fraction. It can also be seen in Fig. 1 that a considerable portion of the P40 and P36 mRNAs is in small polyribosomes (dimers and trimers) as well as in the ribosomal monomer peak. In contrast, most of the actin mRNA is in large polyribosomes. The fractions near the top of the gradient were not used for the assays, but it is unlikely that these fractions contained significant amounts of the mRNA molecules under study. Previous studies using the translation assay had shown that the RNP particles bearing the P36 and P40 mRNAs have a sedimentation coefficient of about 26 S (11).

The distribution of the actin and P36 mRNAs in cells exposed briefly to cycloheximide was also determined (Fig. 2). Nearly all the ribosomes were in polyribosomes in extracts of the treated cells, and the overall polyribosome size was considerably greater than in cells not exposed to the drug (compare Figs. 1 and 2). Thus cycloheximide, which preferentially inhibits polypeptide chain elongation, promoted the accumulation of ribosomes on mRNA. Both the actin and the P36 mRNAs were in polyribosomes larger than those shown in Fig. 1, although a substantial amount of P36 mRNA was also present in small polyribosomes. The portion of the P36 mRNA in untranslated

![Fig. 1. Sedimentation characteristics of mRNA components in cytoplasmic extracts of ascites cells incubated in culture medium for 5 hr. Extracts were subjected to zone centrifugation and RNA obtained from individual fractions was used for hybridization assays with cDNA probes for actin mRNA (○), P36 mRNA (■), and P40 mRNA (□). The solid continuous line represents absorbance at 257 nm. Data are expressed as amount of radioactivity from nick-translated probes that bound to the RNA samples. Direction of sedimentation is from right to left.](image1)

![Fig. 2. Sedimentation characteristics of actin and P36 mRNA components in cytoplasmic extract of ascites cells exposed to cycloheximide. See Experimental Procedures and Fig. 1 for details. ○, Actin mRNA; ■, P36 mRNA.](image2)
particles, however, was at least as high in the drug-treated cells as in cells not exposed to cycloheximide.

Extent of Utilization of mRNA Species in Relation to Efficiency of Ribosome Loading. Incubation of the ascites cells in culture medium affects their capacity for utilizing ribosomes for translation. Cells removed from the animals 6–7 days after incubation are relatively inefficient in this respect, as shown by the high proportion of ribosomal monomers in these cells (Fig. 3). Incubation in culture medium for 75 min increases ribosome utilization to some extent. Incubation for 60 min in the absence of nutrients followed by 15 min in complete medium leads to highly effective uptake of ribosomes into polyribosomes. The starvation treatment appears to inhibit polypeptide chain initiation selectively and causes the release of the ribosomes from mRNA (17). Addition of nutrients to the starved cells leads to rapid formation of large polyribosomes (17, 18), an indication that the cells have become highly efficient with respect to the initiation step of translation.

The distributions of individual mRNA species in polyribosomes and small RNP particles were compared in the three types of cells. In addition, changes in mRNA distribution in small and large polyribosomes were also followed, in order to see whether the active mRNA components showed the expected increases in ribosome packing in the incubated cells. The mRNA in the 80S peak was considered to be associated with a ribosome and was included in the small polyribosome values in this analysis. The untranslated mRNA was prepared from particles with sedimentation coefficients ranging from about 20 to 60 S (see Fig. 3). The P65, P40, and P36 mRNPs are known to sediment in this range (11), and it was assumed that the bulk of the P21 mRNP would be in the same fraction. The data in Table 1 show that the actin mRNA is nearly fully utilized in ascites cells not subjected to incubation, in spite of the limited capacity for polypeptide chain initiation relative to elongation in these cells. Incubation of the cells in culture medium led to some shift of this mRNA species to larger polyribosomes. A considerably greater shift occurred in the cells subjected to the starvation–recovery treatment, an indication that this treatment leads to accumulation of ribosomes on this RNA species. There was, however, little change in the extent of utilization of actin mRNA in the treated cells. The four other mRNA species were utilized to extents of 30–60% in the untreated ascites cells (Table 1). The active components of these RNA species showed shifts from small to large polyribosomes in the incubated cells and in the starved–recovered cells. The extent of ribosome packing on these mRNA species, however, remained considerably lower than that of the actin mRNA. In spite of the observed increases in ribosome loading on the active mRNA molecules, no increase in the proportion of molecules engaged in translation was observed for any of these species.

Changes in the Extent of mRNA Utilization. Incubation of the ascites cells outside the animals led to increases in the proportions of untranslated P65, P40, and P21 mRNAs (Table 1). This effect was most prominent in the case of the P40 mRNA. The proportion of active molecules belonging to this species decreased from 40% to 20% in the starved–recovered cells. The proportion of active P36 mRNA molecules, on the other hand, showed no change upon short-term incubation of the cells. The effect of prolonged incubation of the cells on the distribution of individual mRNA species was not examined systematically, but decreases in the proportion of untranslated P36 mRNA have been observed occasionally in cells incubated for about 5 hr. This may account for the large difference in the relative amounts of untranslated P40 and P36 mRNAs shown in Fig. 1. The conditions leading to the changes in P36 mRNA distribution have not yet been defined.

Extent of Translation of Deproteinized mRNA in the Reticulocyte Cell-Free System. The relatively low extent of ribosome packing on the active P65, P40, P36, and P21 mRNA molecules seemed to indicate that the rate of initiation on these molecules was particularly low. In order to determine whether this behavior was due to some structural feature of these mRNA species, the translation of the deproteinized RNAs in the reticulocyte cell-free system was examined. RNA isolated from the mRNP fraction was added in increasing amounts to the translation system, and the synthesis of individual polypeptides was determined. Actin synthesis was nearly maximal at a level of 1 μg of RNA, whereas the synthesis of P65 and P36 increased substantially at RNA inputs between 1 and 4 μg (Fig. 4). The changes in P40 synthesis were similar to those of actin. The RNA concentrations used in this experiment were approaching saturation, as shown by the data on total polypeptide synthesis (Fig. 4). Under these conditions, the actin mRNA appeared to compete poorly with some of the untranscribed mRNA species. Insofar as the above experimental conditions provide a test for mRNA initiation efficiency, the results indicate that the un-

---

**Table 1. Levels of untranslated mRNAs in mouse sarcoma ascites cells before and after incubation in culture medium**

<table>
<thead>
<tr>
<th>mRNA</th>
<th>No incubation</th>
<th>Culture medium, 75 min</th>
<th>Starvation-recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Large/ small</td>
<td>Large/ small</td>
<td>Large/ small</td>
</tr>
<tr>
<td></td>
<td>% mRNPs*</td>
<td>poly-</td>
<td>mRNPs*</td>
</tr>
<tr>
<td></td>
<td>poly-</td>
<td>somes*</td>
<td></td>
</tr>
<tr>
<td>Actin 16</td>
<td>3.1</td>
<td>18</td>
<td>3.9</td>
</tr>
<tr>
<td>P65 38</td>
<td>0.7</td>
<td>51</td>
<td>0.9</td>
</tr>
<tr>
<td>P40 61</td>
<td>0.6</td>
<td>74</td>
<td>1.4</td>
</tr>
<tr>
<td>P36 66</td>
<td>0.5</td>
<td>69</td>
<td>1.2</td>
</tr>
<tr>
<td>P21 54</td>
<td>0.4</td>
<td>55</td>
<td>0.6</td>
</tr>
</tbody>
</table>

As the ascites cells were analyzed after removal from the animal (no incubation), after incubation in complete culture medium for 75 min, and after incubation for 60 min in the absence of nutrients followed by 15 min in complete medium (starvation-recovery). Cytoplasmic extracts were fractionated into large polyribosomes (>trimers), small polyribosomes (trimers to monomers), and RNP particles (20–60S), as indicated in Fig. 3. mRNA levels were analyzed by hybridization to DNA probes.

* Values represent percent of mRNA molecules of each species present in untranslated RNP particles.

† Values represent distribution in large and small polyribosomes expressed as ratio of amounts in the two fractions. These values provide a measure of relative ribosome packing on individual mRNA species.

---

![Fig. 3. Effects of incubation conditions on polyribosome profiles in cytoplasmic extracts of ascites cells. Extracts from cells lysed directly after harvesting from the peritoneal cavity (A), from cells incubated for 75 min in culture medium (B), and from cells subjected to the starvation–recovery treatment (C) were subjected to some centrifugation as in Fig. 1. Arrows indicate fractions pooled for measurements of mRNA levels.](image-url)
translated mRNA species are at least as effective as the actin mRNA in this respect.

DISCUSSION

The present study shows that four major mRNA species of mouse sarcoma 180 ascites cells, which predominate in the untranslated rRNA fraction of these cells, are also engaged in translation. This unusual distribution could be due to association of mRNA molecules with factors that prevent their interaction with ribosomes. Alternatively, the rate of initiation on these molecules could be particularly low, so that a considerable portion of the molecules would be devoid of ribosomes at the steady state. The latter possibility was examined by altering the activity of cells with respect to either polyribosome chain initiation or elongation. Increase in initiation activity without concomitant acceleration in polyribosome chain elongation should have resulted in the accumulation of ribosomes on mRNA. As a consequence, the proportion of untranslated mRNA molecules should have decreased. Preferential decreases in elongation rates should have had the same effect.

The desired state of increased ribosome loading on mRNA was achieved to a small extent by incubation of the ascites cells in culture medium, and to a much greater extent by first incubating the cells in the absence of nutrients and continuing the incubation for about 15 min in complete medium. These treatments resulted in the increased uptake of ribosomal monomers into polyribosomes, as well as in shifts of the active mRNA molecules into larger polyribosomes. These treatments, however, did not cause any decrease in the proportion of untranslated molecules. These results tend to rule out inefficient initiation as the cause for the occurrence of large amounts of untranslated P65, P40, P36, and P21 mRNA molecules. The distribution of the mRNA species in extracts of cycloheximide-treated cells leads to the same conclusion.

Our results show that the portion of the mRNA molecules not present as small mRNP's is associated with ribosomes engaged in translation. This conclusion is derived from the fact that the ribosome packing on these molecules is increased in cells with higher initiation capacity. This packing, however, remains considerably lower than that on the actin mRNA. This would tend to indicate that these RNA molecules are relatively inefficient in initiation. We have examined this possibility by comparing the translation of the different mRNA species in the reticulocyte cell-free system. The conclusion from these experiments, based on the assumption that inefficient species would compete poorly under conditions of RNA excess, is that the four untranslated mRNA species are at least as effective as the actin mRNA in the initiation process. The apparent contrast between the in vivo and in vitro behaviors raises the possibility that the cytoplasmic environment may affect the rate of initiation on these mRNA species as well as their utilization for translation.

The transfer of the sarcoma cells from the peritoneal cavity to the culture medium appears to trigger changes in the extent of utilization of some of the mRNA species. The proportion of active P40 mRNA molecules is decreased considerably in the cells subjected to the starvation–recovery treatment. The P65 mRNA is affected to a lesser extent, and the P36 and P21 species show little or no change in degree of utilization. These changes suggest that shifts of mRNA molecules between the two functional states can take place when the cells are placed in a different environment. However, the present results could also be explained in terms of selective degradation of some polyribosomal mRNA. Precise measurements of mRNA levels in cells before and after incubation would be required to verify the latter possibility.

The ability of cells to segregate a portion of the mRNA molecules of some species into a "repressed" state appears to provide a means for the regulation of synthesis of the corresponding polypeptides. Thus the present results suggest the occurrence of a translational control process that may operate through the repression and activation of mRNA molecules. The mRNA species described in this report also occur as untranslated mRNP's in mouse erythroleukemia cells. Further studies should indicate whether mRNA repression is a general process in mammalian cells and whether the four mRNA species in the present study are subject to repression in a wide variety of cell types.

This work was supported by grants from the National Institutes of Health (GM 17973) and from the National Science Foundation (PCM 8020712).