Polyadenylate-containing RNA of Polyribosomes Isolated from Rat Liver and Morris Hepatoma 7800

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SUMMARY

The fraction of polyribosomal RNA that contains polyadenylate sequences was examined in rat liver and the Morris hepatoma 7800. Sequential sodium dodecyl sulfate-phenol extraction at pH 7.6 and pH 9.0 followed by polyacrylamide cellulose chromatography of the fraction extracted at pH 9.0 was used to isolate the polyadenylate-containing fraction of polyribosomal RNA. This fraction from either tissue appeared to be the same with respect to mobility on acrylamide gels, sensitivity to ribonuclease digestion, and 32P base composition analysis. There was no difference in the absolute amount of polyadenylate-containing RNA in polyribosomes from either liver or hepatoma.

The time course of the decay in specific activity of the polyadenylate-containing RNA was determined following the administration of a single dose of 32P. From these data, a half-life of approximately 9 to 12 hr could be calculated for the polyadenylate-containing RNA fraction from both liver and the hepatoma polyribosomes.

INTRODUCTION

Recent reports (2, 5, 6) from other laboratories have demonstrated that some mRNA's from eukaryotic cells contain a terminal sequence of polyadenylate homopolymer. These sequences are also found in these cells in the heterogeneous nRNA fraction which is believed to be the precursor of cytoplasmic mRNA (4, 18, 19). A previous report from this laboratory (26) has shown that 5-fluoroorotic acid inhibits the formation of mature cytoplasmic RNA but has little effect on the synthesis of a class of RNA that has the characteristics of mRNA. Further studies (8) have revealed that, while 5-fluoroorotic acid strongly inhibits the formation of ribosomal 28 S and 18 S RNA, it has very little effect on the incorporation of 32P, or orotate-3H into polyadenylate-containing RNA.

The occurrence of a sequence of polyadenylate in a fraction of RNA that exhibits the properties expected of mRNA has facilitated the direct isolation of this material uncontaminated by other classes of RNA. The determination of the specific activity of polyadenylate-containing RNA at various times after the administration of a labeled RNA precursor should permit an estimation of the rate of turnover of this presumptive mRNA fraction. Previous methods for the investigation of mRNA in eukaryotic cells have involved indirect methods for the determination of the amount of label present in mRNA (7, 22-25).

In view of the probability that the heterogeneous, rapidly labeled RNA fraction in the nucleus is the precursor of mRNA in the cytoplasm (4, 19), several investigators have suggested that, since polyadenylate sequences are found in both types of RNA, these sequences may be involved in the maturation of heterogeneous nRNA in the nucleus or in its subsequent transport to the cytoplasm. Any derangement of these processes would necessarily be manifested by a loss of, or an alteration in, the expression of those functions that were specified by the mRNA involved. In most of the transplantable hepatomas there is a loss of several enzymatic functions expressed in normal liver (16). For this reason we undertook an investigation of the characteristics of the polyadenylate-containing RNA from polyribosomes of normal rat liver and of the transplantable Morris hepatoma 7800.

MATERIALS AND METHODS

Animals. Livers were obtained from male Holtzman albino rats fed a standard laboratory chow diet ad libitum and fasted for 18 hr prior to sacrifice. The Morris 7800 tumor was maintained by transplantation, at 20-day intervals, into the hind legs of male Buffalo rats (Texas Inbred Rat Co., Houston, Texas), which were also maintained on the standard chow diet. Administration of radioisotope was by i.p. injection in the case of rats not bearing tumors. Radioisotope was given to tumor-bearing rats by i.v. injection into the penile vein under light ether anesthesia.

Preparation of Total Liver and Hepatoma Polyribosomes. Total liver and hepatoma polyribosomes were prepared by a modification of the procedure of Blobel and Potter (1). Livers and hepatomas were rapidly excised from decapitated and exsanguinated rats. In the case of hepatoma tissue, all necrotic and hemorrhagic areas were trimmed away. The tissues were rinsed in ice-cold TKM buffer containing 0.44 M sucrose. After being minced with scissors, the tissues

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Chart 1. Polyacrylamide-agarose gel electrophoresis of RNA fractions obtained from °P-labeled polyribosomes from rat liver (A and B) and from the Morris hepatoma 7800 (C and D). Rats were given 1 mCi of °P, and were sacrificed 6 hr later. The various RNA fractions were prepared as described in “Materials and Methods.” The approximate S values for rRNA’s and RNA’s are given at the top of the charts and the numbers along the ordinate are in hundreds of counts. A and C, radioactivity profile of RNA extracted from polyribosomes at pH 9.0 and fractionated on polythymidylate-cellulose. B and D, RNA fractions as in A, but after treatment with pancreatic RNase A (2.0 μg/ml, 37° for 10 min).

were homogenized in 2 volumes of the same buffer with 5 strokes of a loose-fitting Teflon-glass Potter-Elvehjem homogenizer. The homogenate was centrifuged at 12,000 rpm for 10 min in the Sorvall SS-34 rotor, and 1 volume of 20% Triton X-100 was added to 10 volumes of the resulting postmitochondrial supernatant. Seven ml of Triton-treated supernatant were layered over a discontinuous sucrose gradient consisting of 3 ml of 2.0 M sucrose in TKM buffer overlaid with 2 ml of 1.4 M sucrose in TKM. The gradients were centrifuged at 50,000 rpm for 5 hr in a Spinco type 50 Ti rotor. Polyribosome pellets were either used immediately or stored at -70° for up to 3 days before use.

Extraction of Polyadenylate-containing RNA. Polyadenylate-containing RNA was obtained from polyribosomes by a sequential sodium dodecyl sulfate-phenol extraction, first at pH 7.6 and then at pH 9.0, essentially as described by Lee et al. (15). Both extractions were performed at 0°. Polyadenylate-containing RNA was found exclusively in the aqueous phase from the pH 9.0 extraction (8).

Polythymidylate-cellulose Column Chromatography of RNA. Polythymidylate-cellulose was prepared by the procedure of Gilham (9). Small columns (0.8 cm high: 1 sq cm cross-sectional area) of polythymidylate cellulose were poured in water-jacketed Pyrex columns in the cold room and washed with “high-salt” buffer (10 mM Tris, pH 7.5–0.1 M NaCl) until the A260 of the eluate was below 0.01 absorbance unit. RNA samples (up to 12 A260 units) were diluted with at least 5 volumes of high-salt buffer and applied to the column. The column was washed with 10 ml of high-salt buffer. The material not retained by the column was eluted in the 1st 3 ml of eluate. The temperature of the column was raised to 30° by means of a circulating water bath, and elution was continued with 10 ml of deaerated high-salt buffer. A small amount of material absorbing at 260 nm was eluted in this fraction but it was found not to be enriched in adenylate. Polyadenylate-rich RNA emerged in the 1st 3 ml of eluate after elution was started with 10 mM Tris, pH 7.5, at 30° (8).

Gel Electrophoresis of RNA. RNA samples were applied to gels consisting of 2.6% acrylamide–0.6% agarose prepared and run according to previously described procedures (26). The RNA concentration in the gels was determined by scanning at 260 nm in a Gilford 2400 spectrophotometer with a linear transport attachment. For determination of the distribution of radioactivity, the gels were cut into 2-mm slices, the slices were solubilized overnight in 30% H2O2 at 80°, and their radioactivity was determined by liquid scintillation counting.

32P-Labeled Base Composition Analysis. Samples of
$^{32}$P-labeled RNA were hydrolyzed in 0.3 M KOH and the base composition of the hydrolysate was determined by a modification of the procedure of Greenman et al. (12) as described previously (8). The determination of $^{32}$P radioactivity was made by utilizing the Cherenkov radiation effect of this isotope. The $^3$H channel of a Packard Tri-Carb liquid scintillation spectrometer was used for this, as described by Haviland and Bieber (14).

**Materials.** Carrier-free $^{32}$PO$_4$ was obtained from Schwarz/Mann, Orangeburg, N. Y. Sucrose, RNase-free, was obtained from the Mann Research Laboratories, New York, N. Y. Powdered cellulose was obtained from H. Reeve Angel Co., Clifton, N. J., and Tris was purchased from the Sigma Chemical Co., St. Louis, Mo.

**RESULTS**

Characterization of Polyadenylate-containing RNA from Liver and Hepatoma Polyribosomes. RNA fractions from normal rat liver and from hepatoma polyribosomes were examined by gel electrophoresis on polyacrylamide-agarose gels. The animals were each given injections of 1 mCi of $^{32}$P$_1$, either i.v. (hepatoma) or i.p. (liver), 6 hr prior to sacrifice. Results described earlier (8) demonstrated that 6 hr after administration of $^{32}$PO$_4$ almost all of the radioactivity in the pH 7.6 RNA fraction was located in the 28 S and 18 S ribosomal subunits, with a small amount at a position corresponding to 4 S and 5 S RNA.

The RNA extracted from polyribosomes at pH 9.0 was first separated into polyadenylate-containing and nonpolyadenylate-containing fractions by polythymidylate-cellulose chromatography. The radioactivity in the nonpolyadenylate-containing fraction (Chart I, A and C) was located largely in the 28 S and 18 S ribosomal subunits that were present in this fraction. In addition there was a considerable portion of the label in a heterogeneous fraction of RNA with mobilities corresponding to S values of 7 S to 30 S. The $^{32}$P in the polyadenylate-containing fraction (Chart I, A and C) was located only in the heterogeneous RNA of 7 to 30 S size distribution.

When the 2 fractions obtained by polythymidylate-cellulose chromatography were treated with pancreatic RNase A before application to the gels, the results shown in Chart I, B and D, were obtained. In the polyadenylate-containing fraction, a substantial proportion of the radioactivity was located in a fraction that was resistant to RNase A and had a mobility that suggested a size of 10 to 12 S, which was comparable to that described by Hadjivassiliou and Brawerman (13). This fraction of RNase-resistant RNA was not observed in any other fraction after enzymatic hydrolysis. A similar nuclease-resistant fraction has been observed by several groups in HeLa cell polyribosomes and has been shown to consist almost exclusively of adenylate residues (2, 4, 5).

The $^{32}$P base composition of the various RNA fractions were reported previously (8). The fraction extracted at pH
7.6 had a low (A + U/G + C) ratio characteristic of rRNA. The material extracted at pH 9.0 had an (A + U/G + C) ratio of 1.0. This value is similar to that obtained from rat liver polyribosomes labeled with $^{32}$P in the presence of either actinomycin D or 5-fluoroorsotic acid (8). After separation on a column of polythymidylate-cellulose, this latter RNA was shown to consist of a fraction high in adenylate [(A + U/G + C) ratio of 1.4 to 1.5] and a fraction that had a base composition similar to that of the RNA extracted at pH 7.6.

The data of Chart 1 show that there were no major differences between the corresponding RNA fractions from normal liver and hepatoma polyribosomes. The possibility remains, however, that minor differences do occur but were not revealed by the methods used in this investigation.

### Amount of Polyadenylate-containing RNA in Polyribosomes from Liver and Hepatoma

Polyribosomes were obtained from 10 normal livers and Morris 7800 hepatomas as described in “Materials and Methods.” The various RNA fractions were separated and the amount of RNA in the polyadenylate-containing fraction from the polythyminidylate-cellulose column was determined by its absorbance at 260 nm. Preliminary experiments, in which polyadenylate-$^3$H (Schwarz/Mann) was added to a total liver polyribosome suspension before extraction revealed that over 90% of the added polyadenylate was found in the fraction bound by polythymidylate-cellulose.

Table 1 shows that, while there were differences in the amount of polyadenylate-containing RNA in the 2 tissues on a total tissue-weight basis, there was no difference when the data was expressed as µg polyadenylate RNA per mg of total polyribosomal RNA. The difference found on a total-weight basis probably represents differences in the amount of polyribosomes obtained from the 2 tissues by the procedure used.

### Turnover of Polyadenylate-containing RNA in Liver and Hepatoma Polyribosomes

The time course of incorporation of $^{32}$P into the various RNA fractions in both liver and hepatoma is shown in Chart 2. A single dose of isotope was administered at zero time. Animals were sacrificed at the times shown and the RNA fractions were prepared. The polyadenylate-rich fraction eluted from the polythymidylate-cellulose column was rapidly labeled in both normal liver and hepatoma, although the rate of incorporation into polyadenylate-rich material was slightly faster in liver than in hepatoma. However, this difference may be a reflection of the relative availability of $^{32}$P-labeled nucleotides due to the different route of administration in each case (i.p. versus i.v.) or the different vasculature of the 2 tissues, or both.

In liver and hepatoma the specific activity of the polyadenylate-rich fraction reached a maximum at 3 to 6 hr after administration of the label. The pH 7.6 RNA fraction (rRNA) and the nonpolyadenylate-containing fraction of the pH 9.0 RNA were labeled to a far lesser degree and their specific activity had still not reached a peak 15 hr after administration of the isotope.

In order to investigate the turnover of the various RNA fractions, the labeling period was extended and the loss of $^{32}$P from the pH 9.0 fraction was plotted in a semilogarithmic manner (Chart 3). In this experiment there was considerable variation between animals in the absolute values for the specific activities of corresponding RNA fractions. For this reason the data have been plotted as the ratio of the specific activity of each pH 9.0 RNA fraction to the specific activity of the pH 7.6 RNA fraction at each time point. As Chart 3 shows, there appears to be an exponential decay of the activity of the polyadenylate-rich RNA in both liver and hepatoma. The half-life calculated from the slopes of these lines differed for liver and hepatoma (11.6 and 9.6 hr, respectively). This small difference in half-life may not be significant due to possible differences in reutilization of the isotope in each tissue. If there were greater reutilization of $^{32}$P into RNA in liver, this could account for the longer half-life of the polyadenylate-rich fraction in this tissue.

The relative specific activity of the nonpolyadenylate-containing pH 9.0 RNA remained fairly constant throughout the time period shown. However, gel electrophoretic examination of this RNA fraction at each time point showed that there was an increase in the proportion of radioactivity in rRNA subunits and a decrease in the proportion in heterogeneous “messenger-like” material as the length of time after labeling increased. Thus the decay curve observed for this fraction is likely to represent a composite of the slow increase in label into rRNA, together with a more rapid decay of activity in those messenger-like RNA molecules that do not have a substantial polyadenylate sequence and that are not retained by polythymidylate-cellulose.

### DISCUSSION

The data presented in this paper show that much of the messenger-like RNA from polyribosomes of rat liver and Morris hepatoma 7800 contains a segment of polyadenylate. Similar sequences have been demonstrated previously in the polydisperse RNA component of polyribosomes from HeLa cells (6), mouse sarcoma ascites cells (15), and in the RNA of several of the RNA tumor viruses (10, 11). Polyadenylate sequences have also been reported in rat liver cytoplasm (13), but their location in polyribosomal RNA was not established in that investigation.
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in vivo. More recently, an attempt has been made to use the sensitivity to RNase action of mRNA in polyribosomes to examine mRNA turnover (22, 23).

The finding that polyadenylate-containing mRNA was rapidly labeled after a single dose of $^{32}$P, (Chart 2) suggested a rapid rate of turnover for this fraction. In liver, the maximum specific activity of the polyadenylate-containing RNA was reached at 3 to 5 hr after labeling. This phase was followed by a slow decline in specific activity. The decay portion of the labeling curve (Chart 3) showed an exponential decrease in activity with a calculated half-life of 10 to 12 hr. This was slower than expected considering the rapid increase in specific activity shown by this fraction in the early stages after administration of the label. A possible explanation for this is that a proportion of the mRNA may have been turning over more rapidly and that the turnover of this material was not apparent in the experiment shown in Chart 3 in which the early time points (0.5 to 6 hr) were not examined. The half-life for rat liver polyribosomal mRNA found in these studies (9 to 11 hr) is longer than the value of 5 hr obtained for this tissue by Endo et al. (7). These authors used a procedure in which the RNA from polyribosomes was examined on sucrose gradients before and after RNase treatment during which mRNA was pre-

Polyadenylate sequences have also been found in the heterogeneous nRNA fraction of eukaryotic cells (4). Since this fraction of nRNA has been postulated to be the precursor of cytoplasmic mRNA (4), it has been suggested that cytoplasmic RNA which contains a polyadenylate sequence represents a large proportion of the cytoplasmic mRNA (2). Sheiness and Darnell (21) have recently shown that, in HeLa cells in culture, the polyadenylate sequences in the cytoplasm shorten with increasing age of the cell. However, to date the only class of mRNA shown to have no polyadenylate sequences is the presumptive mRNA for histone synthesis (20).

Trakatellis et al. (24) studied the turnover of mRNA in mouse liver by determining the increase in specific activity of the 18 S to 5 S RNA region isolated from a sucrose gradient after a single pulse of label. However, this region of the gradient includes 18 S rRNA as well as some 4 S and 5 S RNA, and it is difficult to determine the contribution of these types of RNA to the total radioactivity in this region. These workers (25) have used a short pulse of label followed by administration of actinomycin D to inhibit further RNA synthesis. If the time between the administration of label and actinomycin D is short, very little labeled rRNA appears in the cytoplasm. However, the use of actinomycin D has been criticized because of its effects other than on RNA synthesis. Endo et al. (7) have suggested that actinomycin D prolongs the lifetime of mRNA in rat liver
sumed to have been degraded. Recently, Murphy and Attardi (17) have used polythymidylylate-cellulose to isolate an mRNA fraction from HeLa cells and showed that the half-life of this RNA was 2 to 3 days. This is much longer than the value of 3 hr obtained for mRNA from mouse L-cells by means of a procedure that involved the examination of the protein synthetic capacity of polyribosomes isolated at various times after actinomycin D treatment (3).

There were no significant differences observed between the polyadenylate-containing RNA fraction from liver or Morris hepatoma 7800 in any of the parameters studied. There were slight differences in the time course of the labeling pattern from each tissue, but it was not possible to attach any significance to them. It is almost certain that there are differences in mRNA between the 2 tissues in view of the obvious differences in growth rates and enzyme patterns observed (16). These differences do not appear to be related to the metabolism of the polyadenylate-containing mRNA under the conditions of our experiments.

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REFERENCES


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