Increased Activity of Rat Liver Messenger RNA and of Albumin Messenger RNA Modulated by Thioacetamide

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ABSTRACT

Administration of thioacetamide to rats was found to increase the activity of liver messenger RNA in the wheat germ protein synthesis assay. The synthesis of total protein was increased about 2.5-fold after 4 days of treatment. The treatment was associated with an increase in the relative quantity of polyadenylic acid-containing RNA. Immunoprecipitation studies showed that albumin synthesis directed by the messenger RNA was increased disproportionately, reaching a level 5 to 6 times the control after 4 days of thioacetamide administration.

INTRODUCTION

A unique system for the biochemical study of protein synthesis and its regulation is provided by albumin, the major protein of blood serum, and the liver, where serum albumin is made (10, 11, 16, 24). This system has been studied extensively, and recent studies have shown that an albumin mRNA, sedimenting at about 17S and containing poly(A)$^1$ sequences, can be isolated from liver (19, 21, 22). In our investigation concerning the regulation of protein synthesis in the early stages of chemically induced carcinogenesis of liver, we elected to study the effect of thioacetamide because this compound is a drug that is effective in the induction of tumors (1, 5) after prolonged treatment. The nucleic acid was precipitated at $-20^\circ$ following the addition of 2 volumes of ethanol containing 0.3 M LiCl. The precipitate was washed at least 4 times with 3 m sodium acetate and 5 mM EDTA, pH 5.5, as described (3), to remove DNA and small-molecular-weight RNA's. The mRNA preparation obtained in this way is referred to as "total RNA." Following the method of Aviv and Leder (2), isolation of poly(A)-containing RNA (mRNA) was accomplished by passage of the total RNA through a column of oligo(dT)-cellulose after heat denaturation at 65$^\circ$ for 5 min followed by rapid cooling at 4$^\circ$. The intermediate salt wash step was omitted. The mRNA was eluted with low-ionic-strength buffer. The starting material and the eluates were assayed for RNA with absorbance at 260 nm, and the assumption that 1 mg RNA per ml equals 20 A$_{260}$ units. The percentage of total RNA retained by the column was calculated on the basis of these assays.

In Vitro Translation. Assays for in vitro synthesis of protein were performed with wheat germ extracts prepared as described by Roberts and Patterson (14) and the omission of the preincubation step. KCl and magnesium acetate concentrations were adjusted to 100 and 2.5 mM, respectively, except as noted. Five $\mu$l of $[^{3}H]$leucine (0.5 mCi/ml, 58 Ci/mmol) were used as the radioactive amino acid precursor in the assay. Fifty-$\mu$l assays were used with a 2-hr incubation at 25$^\circ$. Following incubation a 20-$\mu$l aliquot was removed, and total mRNA activity was measured after TCA precipitation (3).

Immunoprecipitation. Immunoprecipitation of the in vitro translation product with an anti-rat serum albumin IgG was performed as a measure of albumin mRNA activity. Following protein synthesis a 20-$\mu$l aliquot was diluted 10-fold in an immunoprecipitation buffer containing 0.05 M sodium phosphate (pH 7.4), 0.1 M NaCl, 10 mM EDTA, 1 mg gelatin per ml, 10 mM leucine, 1% Triton X-100, 1% sodium deoxycholate, and 2 $\mu$l rabbit anti-rat albumin IgG. After a 1-hr

Materials and Their Sources. [3H]Leucine (Amersham/Searle Corp., Arlington Heights, Ill.); wheat germ (General Mills, Vallejo, Calif.); oligo(dT)-cellulose, T-2 (Collaborative Research, Waltham, Mass.); rabbit anti-rat albumin, IgG (Cappel Laboratories, Inc., Cochranville, Pa.); and thioacetamide (Fisher Scientific Company Pittsburgh, Pa.) were obtained.

Animals. Male Sprague-Dawley rats, weighing about 200 g, were given i.p. injections of 0.2 ml of thioacetamide dissolved in 0.9% NaCl solution (50 mg/ml) every 24 hr. Food and water were provided ad libitum. Control animals were sacrificed after overnight starvation.

RNA Isolation. The minced livers were homogenized in 15 volumes of buffer containing 0.1 M Tris (pH 9), 10 mM EDTA, 0.5% SDS, 0.01 M dithiothreitol, and heparin, 0.5 mg/ml. A second homogenization was performed immediately with an equal volume of phenol:chloroform (1:1). The resulting emulsion was centrifuged to separate the phases. The aqueous phase was reextracted 4 more times with phenol:chloroform until deproteinization was complete.

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Footnotes:

1 The abbreviations used are: poly(A), polyadenylic acid; oligo(dT), oligo-deoxythymidylic acid; SDS, sodium dodecyl sulfate; TCA, trichloroacetic acid.
incubation at 30°, the antigen:antibody complex was precipitated with 40 μl of IgG fraction of goat anti-rabbit IgG at 30° for 1 hr and then at 4° overnight. The immunoprecipitates were pelleted by sedimenting through a 1-ml cushion of 1 M sucrose made in the immunoprecipitation buffer. The pellets were dissolved in 0.05 n NaOH and then reprecipitated with an equal volume of cold 20% TCA and collected on GF/C glass fiber filters. The filters were dried and counted in 5 ml of Aquasol (New England Nuclear, Boston, Mass.).

**Electrophoresis of Immunoprecipitates.** After reprecipitation with TCA as described above, the immunoprecipitates were collected by centrifugation and washed with alcohol and acetone. The precipitates were dissolved in 0.05 M Tris (pH 6.8), 1% SDS, and 1 mM dithiothreitol by boiling in a water bath for 5 min, and SDS:7% polyacrylamide gel electrophoresis was performed as described by Laemmli (6). Following electrophoresis the bands were visualized by staining with Coomassie blue (Eastman Organic Chemicals, Rochester, N.Y.), and then the gel was sliced. Radioactivity of the slices was determined by extracting the slices overnight at 37° with 1 ml of NCS (Amersham/Searle):H₂O (9:1).

**RESULTS**

**Characterization of the Translation System.** Rat liver RNA markedly stimulated the incorporation of [³H]leucine in the wheat germ translation system. As shown in Chart 1, the addition of liver mRNA to the assay mixture resulted in a linear incorporation of [³H]leucine into synthesized total protein as well as albumin for at least 60 min. The activity then gradually leveled off. Endogenous activity (no added RNA) remained essentially constant throughout the period of incubation. Approximately 7% of the product was found to be albumin on the basis of immunoprecipitation (Chart 1). Standard assays were performed for 2 hr to allow for the completion of protein synthesis.

**Translation of Total RNA.** Initially, total RNA's isolated from livers of thioacetamide-treated and control animals were tested for their ability in directing total protein and albumin synthesis in vitro (Table 1). The incorporation of [³H]leucine into total protein and albumin was found to be linear up to a concentration of 200 μg/ml of assay mixture, whereupon the synthesis plateaued. Increasing the amount of RNA added to the assay mixture caused a greater stimulation of albumin synthesis as detected by immunoprecipitation than of total protein synthesized. RNA obtained from thioacetamide-treated animals stimulated higher levels of incorporation into synthesized protein as compared to the RNA from control animals. RNA from rats given thioacetamide for 4 days was about 2.5 times as active as the RNA from control rats in total protein synthesis. A 5-fold higher synthesis of albumin was observed with RNA from 4-day thioacetamide-treated animals as with RNA from control animals (Table 1).

**Translation of Poly(A)-containing RNA.** Poly(A)-containing RNA's obtained from separate groups of untreated animals as well as animals treated with thioacetamide for 2, 4, and 6 days were also tested for their capacity for protein synthesis in vitro (Chart 2). An optimum concentration of the mRNA (60 μg/ml) caused a 6- to 16-fold stimulation of protein synthesis above the background level, depending on the source of the message. Increasing the mRNA concentration beyond this level progressively inhibited protein synthesis.

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**Table 1**

<table>
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<tr>
<th>Source of RNA</th>
<th>RNA added (μg/50 μl)</th>
<th>Total protein (cpm/20 μl)</th>
<th>Albumin (cpm/20 μl)</th>
<th>% cpm as albumin</th>
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<td>0</td>
<td>0</td>
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<td>2</td>
<td>15,600</td>
<td>1,020</td>
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<td>41,200</td>
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<td>Treated</td>
<td>15</td>
<td>136,000</td>
<td>25,620</td>
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</table>

* Determined by TCA precipitation technique (3).
* Determined by a double antibody immunoprecipitation technique.
Thioacetamide Modulation of mRNA in Liver

Poly(A)-containing RNA-directed in vitro total protein synthesis. Poly(A)-containing RNA's were isolated from rat livers treated with thioacetamide for 0 day (A, a), 2 days (B, b), 4 days (C, c), and 6 days (D, d). Standard translation assays were performed for 2 hr with varying amounts of RNA.

Messenger RNA's obtained from treated animals stimulated higher incorporation of [3H]leucine in total protein than did mRNA from control animals. This confirms the assay data obtained with total RNA's. Poly(A)-RNA from rats given injections of thioacetamide for 4 days caused the highest incorporation and was 3 times as active as the RNA from control animals in total protein synthesis (Chart 2). Immunoprecipitation of synthesized protein showed that mRNA from 4-day-treated animals caused a 6-fold higher incorporation of [3H]leucine into albumin above the control. By the sixth day of thioacetamide treatment, mRNA activity decreased considerably and reached a level attained with 2 days of treatment.

Level of Poly(A)-containing RNA. An examination of level of poly(A)-containing RNA revealed an exposure time-dependent linear increase in the quantity of mRNA (Chart 3). The percentage of mRNA as measured by absorbance at 260 nm was about 4% of the total RNA isolated from control animals. By the sixth day of treatment, 9% of the total RNA was retained by the oligo(dT)-cellulose column. No significant difference in the amount of total RNA was observed between control and treated livers.

Effect of Potassium and Magnesium Ion Concentration on the Translation of RNA. The optimum K+ and Mg2+ concentrations required for the translation of RNA's isolated from control and treated animals were examined because albumin mRNA has been reported to have higher K+ and Mg2+ concentration optima than did the total mRNA (12, 23). With total RNA from either control animals or 4-day-treated animals, optimum stimulation of total protein synthesis in the wheat germ assay was observed at 75 mM K+ and 1.5 mM Mg2+, while about 87 mM K+ and 2.0 mM Mg2+ were required to optimize albumin synthesis. However, for all the K+ concentrations tested, ranging from 50 to 100 mM, RNA from thioacetamide-treated rats consistently caused a higher level of albumin synthesis compared to the RNA from control animals. This was also true for the Mg2+ concentrations tested, ranging from 1 to 4 mM. These data exclude the possibility of a difference in K+ and/or Mg2+ requirement as being responsible for the difference in translation of RNA's from control and treated animals.

Specificity of Immunoprecipitation. To check the specificity of indirect immunoprecipitation, we explored the ability of increasing amounts of pure rat serum albumin to displace the radioactive synthesized product in a competition assay. About 29% of the total protein synthesized in vitro directed by mRNA from 4-day-treated rats was specifically precipitated by the addition of rabbit anti-rat albumin IgG (Chart 4). The addition of 15 μg of pure rat serum albumin reduced the radioactivity in the precipitate to 8%. No further reduction in the radioactivity could be obtained by increasing the amount of unlabeled albumin to 50 μg. With the translation product of control RNA, only about 10% of the radioactivity was precipitable with the anti-IgG. The addition of 50 μg of albumin reduced the radioactivity in the precipitate to 3% (data not shown). It seems that about one-third of the radioactivity precipitable with anti-albumin IgG is not displaceable by the addition of unlabeled albumin. In separate control experiments only 2% of the total radioactivity was precipitated with normal serum, and 4% of the translation products of HeLa cell mRNA was precipitated with anti-albumin IgG.

Analysis of Immunoprecipitates. The immunoprecipitates formed were further characterized by SDS-polyacrylamide gel electrophoresis. After in vitro protein synthesis assays with control RNA or RNA from treated animals, 20 μl of the assay mixture were immunoprecipitated with anti-albumin IgG, and the precipitates were subjected to electrophoresis on SDS-gel (Chart 5). In all cases 1 major peak of...
DISCUSSION

These studies have demonstrated that thiacetamide, a hepatocarcinogen, can increase the translational activity of liver mRNA. In vitro translation of the oligo(dT)-cellulose-purified mRNA and the total RNA substantiate the above conclusion. The maximum translational activity was obtained after 4 days of thiacetamide treatment. The reasons for the modulations of translational activity of mRNA due to in vivo exposure of the animal to thiacetamide are not clear. No difference has been observed in the translational requirement of K⁺ and/or Mg²⁺ for the RNA's obtained from control and treated animals. The possible mechanisms for the change in the activity could be a modulation of the secondary structure of mRNA (7) or a difference in the level of poly(A) inhibitory to peptide synthesis (9).

An interesting finding of this investigation was the increase in the apparent percentage of poly(A)-containing RNA of thiacetamide-treated animals without a parallel increase in the amount of total RNA. After 1 treatment of thiacetamide, an acute and prolonged increase in the cytoplasmic poly(A)-containing RNA was noted by Smuckler and Koplitz (17), which they interpreted as due to increased release of poly(A)-containing RNA into cytoplasm. This experiment suggests that this may also be due to increased synthesis of poly(A)-containing RNA, since unfractonated whole-cell RNA has been used in the present studies.

Since previous studies (3, 4, 13, 15) indicate that mRNA activity measured in the wheat germ assay is directly proportional to the mRNA concentration, the results in Table 1 show that the total mRNA in a 4-day thiacetamide-treated animal is increased 2.5-fold and that the albumin mRNA is increased 5-fold. More accurate estimations of the mRNA would require preparations of complementary DNA; experiments in this direction are in progress.

REFERENCES


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