Electrophoretic behavior of mouse liver 40 S ribosomal subunits after treatment with dimethylnitrosamine

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SUMMARY

The effect of DMN, a potent carcinogen, on the mouse liver ribosomes was analyzed in a TBM buffer by the 2.0% polyacrylamide-0.5% agarose composite gel electrophoresis. The untreated mouse liver ribosomes were separated from the 40 S subunit to the tetramers of the 80 S ribosomes. The DMN-treated mouse liver ribosomes were separated mainly from the 80 S ribosomes and the 40 S subunit. The free 40 S ribosomal subunits (S-40 S) from mice treated with DMN were observed migrating slower than those from untreated mice. This was also confirmed by the experiment using the artificial mixture of both ribosomes. When the DMN-treated mice liver polysomes were analyzed under the condition that the ribosomal subunits were separated in a TBE buffer, S-40 S from mice treated with DMN migrated slower than those from untreated mice, whereas the 60 S ribosomal subunits did not change after treatment with DMN. This was also confirmed by the analysis of radioactive DMN. These results suggested that the main effect of DMN on the mouse liver ribosomes was the modification from the 40 S subunits to S-40 S.

Key words: composite gel, ribosome, conformation, DMN.

INTRODUCTION

Ribosomes are an essential component in the translation of mRNA into protein. How ribosomes participate in this complex mechanism has been the underlying question in studies on ribosome structure and function. Comparative sequence analysis, and genetic and biochemical studies provide increasing evidence that rRNA has evolved into a precisely tuned structure at both the primary sequence level and higher order structure levels. These experiments reportedly used chemical modification to determine the conformation of nucleotides in rRNA. Ketohexal, used in these studies, is known to be guanine-specific reagent. Dimethylnitrosamine (DMN) is known to cause tumors in rodents. Radioactive DMN is also reported to exist as methylguanine in DNA and RNA. Dahlberg et al. used composite gels to characterize bacterial ribosomes with two dimen-
sional electrophoresis. We also reported the electrophoretic characterization of mouse liver ribosomes using the composite gels.

Keeping this in view, I studied the effect of DMN on the ribosome through the analysis of the electrophoretic mobility of the liver ribosomes treated with DMN. Here, I am reporting the electrophoretic characterization of liver ribosomes after treatment with DMN.

MATERIALS AND METHODS

Materials

DMN was obtained from Aldrich Chemical Co.; acrylamide, bis, TEMED and 1-ethyl-2-(3-(1-ethynaphtho-(1,2d)-thiazolin-2-ylidene)-2-methyl-propenyl)-naphtho(1,3d)-thiazolium bromide ("stains-all") from Eastman Kodak Co.; SeaKem agarose from Marine Colloid Inc.; [14C]-DMN (4.82 mCi/mmol) and [3H]-orotic acid (17.8 Ci/mmol) from New England Nuclear. The electrophoretic cell and elution apparatus were the products of E-C Apparatus Co.

Methods

Isolation of mouse liver ribosomes

The procedure used for the isolation of mouse liver ribosomes has essentially been described in previous studied. Male C3H/HeN mice were fasted for 24 h before sacrifice. Three hundreds µg of DMN dissolved in 0.9% NaCl was injected intraperitoneally at 2 h or 4 h. To examine the incorporation of DMN into the liver ribosome, either 1 mCi of [3H]-orotic acid was intraperitoneally injected 16 h prior to sacrifice or 160 µCi of [14C]-DMN was injected intraperitoneally 2 h prior to sacrifice. The mice were then sacrificed by cervical dislocation and about 1 g of liver was rapidly removed and cooled in an ice-cold 0.25 M sucrose-TKM buffer. Sodium deoxycholate was added to the supernatant to make a final concentration of 1.3%. The resulting solution was overlaid on a 1.0 M sucrose-TKM buffer and centrifuged at 150,000×g in a 50 Ti rotor for 3 h with the Beckman L2-65 centrifuge. The pellet fraction was then suspended in the TKM buffer and used as a ribosome fraction. An aliquot of ribosome suspension was mixed well with an equal volume of water-saturated phenol and centrifuged at 2,000 rpm for 2 min with the Spinco Microfuge. The resultant aqueous layer was directly applied to electrophoresis as a ribosomal RNA fraction. The pellet fraction was then suspended in the TKM buffer and used as a ribosome fraction.

In the case of polysome preparation, the homogenate was centrifuged at 15,000×g in a Sorvall RC-2 for 20 min. Sodium deoxycholate was added to the supernatant to make a final concentration of 1.3%. The resulting solution was overlaid on a 2.0 M sucrose-TKM buffer and centrifuged at 150,000×g in a 50 Ti rotor for 1 h with the Beckman L2-65 centrifuge. The pellet fraction was then suspended in the TKM buffer and used as a polysome fraction.

The RNA concentration of ribosomes was determined by measuring absorption at 260 nm (1 A 260 unit=50 µg/ml).

Preparation of 1.8% polyacrylamide-0.5% agarose composite gels

The gels used were prepared as described, except for a 20% acrylamide stock solution composed of 18.5 g of acrylamide and 1.5 g of bis in 100 ml. The 3 mm thickness of the composite slab gel (160 ml) was prepared as follows. Agarose (0.8 g) was mixed with 114.6 ml of water in a 300 ml Erlenmeyer flask. The flask was attached to the condenser, stirred rapidly on the hot plate, and boiled without scorching the agarose solution. While the agarose solution was refluxing for 15 min, all other liquid reagents required for the polyacrylamide gel except for the ammonium persulfate (16 ml of 900 mm Tris-900 mm boric acid, pH 8.3, 10 ml of 0.5% TEMED
and 14.4 ml of 20% acrylamide stock solution) were combined in a small flask and heated to 38-40°C under running water at about 40°C. After 15 min, the agarose solution was cooled to 40°C with running water at about 37°C, and mixed with the above warmed liquid reagents. This mixture was then adjusted to 37-38°C. Within 15-20 s after the addition of 5 ml of 1.6% ammonium persulfate, the mixture was slowly poured into the assembled cell warmed at 20°C. The cooled slot former was inserted into the slot area. A tightly sealed tube of ice was laid in front of the slot former while the cell was in a horizontal position, and the resultant composite gel was left standing at 20°C overnight.

Application of samples to composite gels

RNA prepared from ribosomes was mixed with an equal volume of 40% sucrose and 0.01% bromphenol blue as a dye marker and 2 to 10 μg of RNA was applied to the gel slot. Ribosomes were mixed with a 1/4 volume of pre-warmed (35-40°C) 0.5% agarose in 10 mM Tris-HCl, pH 7.0 containing 40% sucrose and 0.01% bromphenol blue as a dye marker, and 20 to 150 μg of the ribosomes were applied to the gel slot.

Composite gel electrophoresis

After gelation, while the cell was still horizontal, the gel block in front of the slot former was blotted, carefully removed, and discarded. The cell was then placed in the vertical running position, and a TBM buffer was added to the cell in sufficient quantity in both the lower and upper reservoirs to cover the electrodes and the slot area (about 2 l total volume). The electrodes were thus set by placing the anode side of the plug down when connecting the power supply to the E-C cell. The slot former was carefully removed. The temperature of the circulating water was gradually reduced to 0°C. All gels were pre-run for 2 h at 300 V and the pre-run buffer was replaced with a freshly prepared TBM buffer.

Two dimensional gel electrophoresis

The "turned gel" method was used in the situation when the gel concentration was the same for the second run, and only when the electrophoretic buffer had to be changed. A three-place slot former, with the slots to one side, was used in the first gel, allowing spaces for the application of ribosome samples (slot 1 and 2). After the first run was carried out for 90 min at 0°C and 300 V, slot 1 was sliced off and stained. Slot 2 was left intact in a piece of gel approximately 10-12 cm square. The gel in slot 2 was then re-assembled in a clean and dry cell, turning 90° from the first run to come with slot 2 to the top. The gel was supported with a 1.25% agarose solution made up in the TBE buffer. After gelation of the supporting gel, the cell buffer (TBE buffer) was added. After the RNA marker was applied to slot 3, the second electrophoretic run was carried out for 60 min at 0°C using 300 V.

Staining procedure

After the electrophoretic run, all gels were stained with 0.005% "Stains-all" in a 50% formamide overnight and gradually destained in running tap water. When the background was clear, the gel was placed on a lighted viewer and photographed with a green filter. After obtaining the densitometer reading, the gels were cut into 1-mm sections and the counts in the sections were determined.

RESULTS

Electrophoresis of mouse liver ribosomes after treatment with DMN

When the ribosomes mixed with 0.5% agarose were applied to the gel slots, it was found that the ribosomes had separated into several bands according to the size of the particles, with 40 S subunits moving the fastest and the larger polysomes moving slower in the composite gel. When the electrophoresis of the mouse liver ribosomes was carried out for 240 min in a TBM buffer as shown in Fig. 1, ribosome bands from the 40 S subunits to the tetramers were observed (slot 1).

The electrophoresis of the mouse liver ribosomes after treatment with DMN mainly separated the 80 S ribosomes band and the 40 S subunits band.
Furthermore, the 40S subunits band from the DMN-treated mice liver was observed to migrate slower than that from the untreated mice liver (slot 2). Thus, it was suggested that DMN caused the disruption of the polysomes to the 80S ribosomes.

**Electrophoresis of ribosomal subunits after treatment with DMN by using the two dimensional technique**

To examine whether free 40S subunits (S-40S) from the mice treated with DMN, do in fact, migrate slower than those (F-40S) from the untreated mice liver, ribosomes from the mice treated with DMN were mixed with the control ribosomes from the untreated mice and analyzed in the electrophoresis using the two dimensional technique as shown in Fig. 2. When the first electrophoresis was carried out for 90 min in the TBM buffer, the dimers band of the 80S ribosomes and the 80S ribosome band were separated. Furthermore, two free 40S subunits bands and some possible degradation bands migrating faster than F-40S were separated (Fig. 2 slot 1).

Thus, it was suggested that DMN caused the modification of the free 40S subunits to S-40S in the TBM buffer. The artificial mixture of ribosomes was applied to slot 2 and the first electrophoresis was carried out for 90 min in the TBM buffer, and then the gel was turned 90° at the first run, and RNA used as a marker was applied to slot 3. When the second electrophoresis was carried out for 90 min in a TBE buffer, two...
free 40S subunits bands and some possible degradation bands migrating faster than F-40S were also separated. S-40S also migrated slower than F-40S in the TBE buffer. Furthermore, the 40S subunits bands migrated slower than the 18S RNA (Fig. 2 slot 2). Thus, it was indicated that the free S-40S separated in the TBM buffer also migrated slower than F-40S in the TBE buffer.

Furthermore, the 80S ribosome band in the TBM buffer was observed to separate the weakly stained S-40S band in the TBE buffer. Thus, it was suggested that the 40S subunits in the ribosomes from the mice treated with DMN were separated as S-40S in the TBE buffer.

**Electrophoretic characterization of the effect of DMN on the mouse liver polysomes**

To study whether the 40S subunits in the polysomes contain S-40S, the polysomes were prepared and then the electrophoresis of the polysomes was carried out for 120 min in the TBE buffer. The 60S subunits band migrated at 28S RNA, whereas the 40S subunits bands also migrated slower than the 18S RNA as shown in Fig. 3.

Although the 60S subunits were not affected by the treatment with DMN, the 40S ribosomal subunits from the mice treated with DMN for either 2 h or 4 h migrated slower than those from the untreated mice liver. The results suggested that DMN modified the polysomal 40S subunits to S-40S, whereas the polysomal 60S subunits did not change after treatment with DMN.

**Radioactive distribution of ribosomal subunits separated by gel electrophoresis**

The labeled DMN was used to examine the binding of RNA with DMN. In the case of the 40S ribosomal subunits in polysomes from untreated mice injected with [3H]-orotic acid, radioactivity had mostly accumulated in F-40S (Fig. 4 lane A). In the case of the 40S ribosomal subunits in polysomes from mice injected with [14C]-DMN, radioactivity had mostly accumulated in the S-40S (Fig. 4 lane B). The radioactive peak in the 60S ribosomal subunits showed almost the same migration between untreated and DMN-treated polysomes. In the case of polysomal RNA from mice injected with [14C]-DMN, radioactivity was found in 28S RNA and 18S RNA (Fig. 4 lane C).

The radioactive analysis suggested the following results. DMN usage was approximately three hundred μg of DMN, and this amount of DMN had a strong effect on the mouse liver polysomes. DMN was incorporated into polysomal RNA and the ribosomal subunits. DMN modified the 40S subunits to S-40S. The 60S subunits appeared unchanged after treatment with DMN.

**DISCUSSION**

The data presented above suggest that the binding of DMN with ribosomes occurs specifically rather than randomly, with respect to their synthesis and localization.
The result in Fig. 1 suggested that DMN caused the destruction of the polysomes into the single 80S ribosomes. DMN has been reported to inhibit the hepatic protein synthesis.\(^{12,13}\) These reports may support the above possibility. In Fig. 1, the free 40S subunits (S-40S) from mice treated with DMN migrated slower than those (F-40S) from untreated mice. The 60S subunits in the polysomes were not changed after treatment with DMN.

These results in Fig. 1-4 suggested the following possibilities; DMN was incorporated into the 28S and 18S RNA and caused the destruction of the polysomes into the single 80S ribosomes. The 40S subunits (S-40S) from the mice treated with DMN migrated slower than those (F-40S) from the untreated mice, whereas the 60S subunits appeared unchanged after treatment with DMN.

This suggests that the effect of DMN on the 60S subunits can not be detected under the conditions used. It is supposed that DMN affects the 40S subunits in the polysomes, converts the 40S subunits into S-40S, and produces free S-40S and possibly smaller degradation products.

The results in Fig. 1-4 showed the following observations. DMN was incorporated into the polysomal RNA and the ribosomal subunits. The 40S subunits in the polysomes from the mice treated with DMN migrated slower than those from the untreated mice. The 60S subunits in the polysomes were not changed after treatment with DMN.

Fig. 4. Radioactive distribution of ribosomal subunits separated by gel electrophoresis. Untreated polysomes were prepared from mice injected with \(^{[\text{3H}]}\) orotic acid (A). DMN-treated polysomes were prepared from mice injected with \(^{[\text{14C}]}\)-DMN-treated polysomal RNAs were prepared from mice injected with \(^{[\text{14C}]}\)-DMN (C). Sample: (A): twenty-three µg of \(^{[\text{3H}]}\)-polysome. (B): twenty-seven µg of \(^{[\text{14C}]}\)-polysome. (C): seven µg of \(^{[\text{14C}]}\)-polysomal RNA. The electrophoresis on a 1.8% acrylamide-0.5% agarose gel in the TBE buffer was carried out for 120 min at 300 V and 0°C as described in Fig. 3. After the electrophoresis, the gel was stained, scanned, sliced, and counted as described under Materials and methods. Figure on left side shows the absorbance at a wavelength of 570 nm, and that on right side the radioactivity. \(^{[\text{3H}]}\)-radioactivity (filled circle); \(^{[\text{14C}]}\)-radioactivity (open circle); \(A_{570}\) nm (line); ordinate: migration (mm).

The result in Fig. 1 suggested that DMN caused the destruction of the polysomes into the single 80S ribosomes. DMN has been reported to inhibit the hepatic protein synthesis.\(^{12,13}\) These reports may support the above possibility. In Fig. 1, the free 40S subunits (S-40S) from mice treated with DMN migrated slower than those (F-40S) from untreated mice.

The result in Fig. 2 indicated that the effect of DMN on the ribosomes may produce free S-40S and possibly smaller degradation products.

The results in Fig. 3 and 4 showed the following observations. DMN was incorporated into the 28S and 18S RNA and caused the destruction of the polysomes into the single 80S ribosomes. The 40S subunits (S-40S) from the mice treated with DMN migrated slower than those (F-40S) from the untreated mice, whereas the 60S subunits appeared unchanged after treatment with DMN.

The reason for the modification from the 40S subunits to S-40 is not clear under the conditions of the present studies. This change in mobility of the 40S subunits may suggest the following possibilities; 1) The increase in molecular weight of the 40S subunits by the binding of DMN with RNA. 2) The conformational change in the 40S subunits by the binding of DMN with RNA. However, when the molecular weight of the 40S subunits was assumed to be 1.5 \(\times\) 10\(^6\) daltons, the molecular weight of S-40S was approximately 1.65 \(\times\) 10\(^6\) daltons by the calculation in Fig. 3. Thus, this change in the mobility of the 40S subunits by itself cannot explain the increase in molecular weight of the 40S subunits by the binding of DMN with RNA.

Additionally, in our earlier report,\(^{14}\) the 18S rRNA from HeLa cell separated as three bands in the composite gel and the conformational change of each band in the 18S rRNA occurred at 40°C. Recent evidence has shown that there was a change in the conformation of the 30S subunits accompanying the conformational change of the 16S rRNA.\(^{15,16}\)
Thus, this evidence has suggested that the change in the mobility of the 40S subunits may reflect the conformational change of the 40S subunits rather than the increase in molecular weight of the 40S subunits by the binding of DMN with RNA.

In this view, it could be concluded that the primary effect of DMN on the mouse liver ribosomes causes the destruction of the polysomes into the single 80S ribosomes and the conformational changes of the 40S subunits into S-40S and finally possibly causes the production of the smaller degradation materials.

Studies are in progress to elucidate these possibilities.

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REFERENCES
TBE 緩衝液中で電気泳動すると、DMN 処理のマウスの 40S サブユニット (S-40S) は未処理のマウスの 40S サブユニットより遅く移動することが観察された。60 S サブユニットは、DMN 処理後も変化しなかった。このことは、放射能の DMN を用いた分析でも確かめられた。これらの結果は、DMN のマウス肝リポソームに対するおもな影響は 40 S サブユニットを修飾して、S-40 S にすることを示唆した。