Analysis of Loss of Nuclear RNA in Azo Dye-induced Hepatoma by DNA-RNA
Competitive Hybridization

Mitsutaro Akao and Keiko Kuroda
Department of Toxicology and Experimental Pathology, Research Institute for Chemobiodynamics, Chiba University, Inohana, Chiba 280, Japan

ABSTRACT

DNA-RNA hybridization studies, using nuclear RNA's (nRNA's) labeled in vivo and in vitro with high specific radioactivities, were performed to compare the nRNA populations of normal rat liver, livers treated with 3'-methyl-4-dimethylaminoazobenzene (3'-Me-DAB), and 3'-Me-DAB-induced hepatomas. The study with normal liver nRNA labeled by i.p. injection of [3H]orotic acid indicated that the nuclei of a 3'-Me-DAB-induced transplanted hepatoma, AH136B, lacked some RNA species present in normal liver nuclei. No qualitative difference in the RNA populations was seen between normal liver and the livers of rats fed a carcinogenic amount of 3'-Me-DAB, either alone or in combination with 4-nitrostilbene which enhanced the azo dye carcinogenesis. Then, nRNA's of both normal liver and AH136B hepatoma were labeled in vitro by phosphorylation with polynucleotide kinase and adenosine 5'-[γ-32P]triphosphate. The competitive hybridization with 32P-labeled normal liver nRNA was completed, and the deletion of RNA in the nuclei of AH136B hepatoma or 3'-Me-DAB-induced primary hepatoma was estimated to be 15% or more in the measure of radioactivity of the hybridized normal liver nRNA. 32P-labeled AH136B hepatoma nRNA was competed completely by liver nRNA's, suggesting that no unique RNA species were present in the hepatoma nuclei.

INTRODUCTION

In our previous studies (1-3), the feeding of azo dyes to rats was found to cause reductions of DNA-dependent RNA polymerase (EC 2.7.7.6) activities of rat liver nuclei, which were associated with the progress of hepatocarcinogenesis. In view of the possibility that such deleterious alteration of RNA synthesis would exert influences on the genome activity for the transcription of DNA, the present study was undertaken to determine the effects of 3'-Me-DAB on the RNA population in the cell nucleus by DNA-RNA hybridization.

Competitive hybridization is the most sensitive method for determining the difference in RNA populations and has been utilized in studies on 3'-Me-DAB carcinogenesis for elucidating the altered regulation in the transport of RNA from the nucleus into the cytoplasm (21, 22, 25, 26). With regard to the difference in the RNA populations in the cell nuclei, Ono et al. (18) showed that nRNA of a 4-dimethylaminoazobenzene-induced transplanted hepatoma AH130 was less efficient than that of normal liver in competing the pulse-labeled normal liver nRNA, and they suggested the deletion of nRNA in the hepatoma. On the other hand, Amano et al. (4) reported that AH130 hepatoma seemed to synthesize the same RNA species as did normal liver. The specific activities of labeled RNA's used in their studies were low, and the competitive reactions were not completed. In the present study, we prepared highly labeled RNA's not only in vivo by i.p. injection of [3H]orotic acid but also in vitro by phosphorylation with polynucleotide kinase (EC 2.7.1.78) which catalyzed the transfer of 32P, from [γ-32P]ATP to the 5'-hydroxyl termini of the purified nRNA, and we determined the differences of RNA populations in the cell nuclei among normal liver, 3'-Me-DAB-treated livers, and 3'-Me-DAB-induced transplanted and primary hepatomas.

MATERIALS AND METHODS

Materials. Animals used were male Donryu rats obtained from Nippon Rat Co., Saitama, Japan. The basal diet was CE-2 (16), a semisynthetic diet purchased from CLEA Japan, Inc., Tokyo, Japan. A diet containing 0.06% 3'-Me-DAB was fed to rats, 8 to 9 weeks old at the start, for 50 days. Then rats were divided into 3 groups. One group was subsequently fed basal diet for 7 to 8 weeks. The livers treated with 3'-Me-DAB was taken from this group. The second group was continued on basal diet. Ten of 19 animals of this group died of hepatomas. The tissues of primary hepatoma were taken from 3 animals sacrificed at 1 year after the start of 3'-Me-DAB feeding. The third group was subsequently fed a diet containing 0.05% 4-nitrostilbene for 40 days, which enhanced 3'-Me-DAB carcinogenesis (1), and were then fed basal diet for 2 to 3 weeks. Liver tissues were also taken from this group. Rat ascites hepatoma AH136B, a 3'-Me-DAB-induced transplanted hepatoma forming cell islands in the ascites (17), was obtained from Dr. Hiroshi Sato, Sasaki Institute, Tokyo, Japan. The ascites fluid was inoculated i.p. in 4- to 5-week-old rats (1 ml/animal). Ten to 12 days after the inoculation, a large tumor mass (3 to 5 g) was seen in the vicinity of stomach and liver and was used for the preparation of nRNA of the hepatoma. For histological examination, pieces of liver and hepatoma were fixed in 10% formaldehyde solution, and paraffin sections were stained with hematoxylin and eosin.

Isolation of Nuclei. Liver tissues were freed from visible nodules and cholangioloblastic lesions, and hepatoma tissues were freed from liver tissues and necrotic masses. The cell nuclei of both tissues were isolated by the same procedure at 0-4°C. Two to 3 g of either tissue was minced with scissors and homogenized with 27 ml 0.25 M sucrose-50 mM Tris-HCl (pH 7.6)-70 mM KCl-1 mM MgCl2 by 15 strokes of a loose pestle in a Dounce homogenizer. The homogenate was filtered through a 200 mesh nylon cloth (Izumi Co., Ltd., Tokyo, Japan), and the filtrate was centrifuged for 10 min at 2500 rpm. The sediment was suspended with a nylon rod in 30 ml of 2.0 M sucrose-3.3 mM CaCl2. The suspension was layered over 8 ml
of 2.2 M sucrose-3.3 mM CaCl₂ and centrifuged for 1 hr at 50,000 × g in a RPS-25-2 rotor of a Hitachi ultracentrifuge. The nuclear pellet was used for the isolation of nRNA.

Isolation of nRNA. nRNA was isolated by a modification of the method of Shearer and McCarthy (24). The nuclear pellet from 3 to 4.5 g tissue was suspended in 20 ml of 0.01 M sodium acetate buffer (pH 5.1) and mixed with 0.3 ml of 20% sodium dodecyl sulfate and an equal volume of H₂O-saturated phenol. The mixture was heated at 60° with occasional shaking for 10 min, cooled to room temperature, and centrifuged at 4000 rpm for 10 min. The aqueous phase was removed; then the phenol phase and interface were combined with 10 ml sodium acetate buffer. The mixture was shaken at 60° for 5 min, cooled, and centrifuged. The aqueous phase was combined with the previous one and reextracted with an equal volume of fresh H₂O-saturated phenol. The aqueous phase was mixed with 0.25 volume of 1 M LiCl and 2.5 volumes of ethanol and was stored at −20° overnight. The precipitate was collected by centrifuging at 4000 rpm for 10 min and was dissolved in 0.01 M Tris buffer (pH 7.8) containing 10 mM MgCl₂ (10 ml per approximately 50 g of original tissue). DNase (RNase free; Worthington Biochemical Corp., Freehold, N. J.) and α-amyrase (type II-A from Bacillus subtilis; Sigma Chemical Co., St. Louis, Mo.) were added to the final concentrations of 20 and 50 µg/ml, respectively, and the mixture was incubated for 1 hr at 37°. The solution was then treated with hot phenol extraction as described above, and RNA was precipitated by ethanol. The RNA precipitate was washed with ethanol and ether and dried in an evacuated desiccator under CaCl₂. The average yields of nRNA’s from liver and AH136B hepatoma were 42 and 58%, respectively. The RNA preparations were negative for DNA by diphenylamine assay (12) and had a high ratio of the absorbance at 260 nm to that at 280 or 230 nm (25). The quantitative analysis of RNA was made by the method of Cericotti (7).

Isolation of DNA. DNA was prepared from normal liver of a rat that had been fasted overnight and killed by decapitation. The liver was minced, washed, and homogenized in a Dounce homogenizer with ice-cold 0.1 M NaCl solution containing 0.1 M EDTA (pH 8.0). DNA was isolated and purified according to the method of Marmur (15).

DNA-RNA Hybridization. DNA-RNA competitive hybridization was carried out by procedures similar to those of Garret et al. (11) with the use of the nitrocellulose filter technique (13). The procedures, including some modifications, are briefly described below.

The hybridization reaction was conducted in a total volume of 0.5 ml in a screw-capped glass vial: an appropriate amount of unlabeled RNA in 0.25 ml of 1/100 × SSC; a fixed amount of labeled RNA in 0.05 ml of 1/100 × SSC; 0.2 ml of 10 × SSC; and an 8-mm filter disc containing approximately 10 µg of liver DNA. The incubation for annealing was carried out at 67° for 20 hr. Each filter disc, after removal of unhybridized RNA, was placed into a scintillation vial, immersed in 0.2 ml of 1 M perchloric acid, and heated at 70° for 20 min. The contents in the vial were neutralized with 1 M NaOH and 0.125 M HCl using phenolphthalein as an indicator and were mixed with 10 ml of Aquasol (New England Nuclear, Boston, Mass.). When the filter was dissolved, the sample was counted in a Packard Tri-Carb Model 3255 scintillation counter. A filter disc containing no DNA was incubated with labeled RNA in the absence of any competitor to determine background counts. The background counts were 4 to 7 cpm above machine background with the use of [³H]RNA and were 10 to 15 cpm with the use of [³²P]RNA. Competition data were presented as percentages of the total counts bound in the absence of competitor.

Preparation of Labeled RNA’s. [³H]RNA was prepared from normal liver of a rat that had been given an i.p. injection of 5 mCi of [5-³H]orotic acid (26 Ci/mmol; The Radiochemical Centre, Amersham, England) 50 min prior to sacrifice (11).

Labeling with [³²P] was done by the transfer of [³²P] from [γ-³²P]ATP to the 5'-hydroxyl termini of isolated nRNA’s of normal liver and AH136B hepatoma under the catalysis of polynucleotide kinase (23). Prior to labeling, isolated nRNA preparations were further purified by precipitation with CTA bromide to remove any remaining polysaccharides and polynucleotides (14) and were then treated with alkaline phosphatase. RNA was dissolved in 0.01 M Tris buffer (pH 7.5) containing 10 mM MgCl₂ (1 mg RNA per ml), mixed with 0.1 volume of 1% CTA bromide, and stored at 4° overnight. The precipitate was collected by centrifuging at 4000 rpm for 10 min and washed with 0.1% CTA bromide buffer. The pellet was dissolved in the Tris buffer containing 1 M NaCl (2 mg RNA per ml) and reprecipitated by being mixed with an equal volume of ethanol. This procedure was repeated 3 times to convert CTA-RNA to sodium RNA. The final precipitate was washed with 67% ethanol containing 0.1 M NaCl, ethanol, and ether and dried. A small amount (2 mg) of the purified RNA was dissolved in 5 ml of 0.1 M Tris buffer (pH 8.0) containing 10 mM MgCl₂. Ten units of alkaline phosphatase (from calf intestine, grade I; Boehringer and Söhne GmbH, Mannheim, West Germany) were added, and the mixture was incubated at 37° for 30 min. RNA was isolated by hot phenol extraction and ethanol precipitation. The phosphorylation reaction was conducted in a total volume of 5 ml in a 50-ml centrifuge tube; 1 mg of phosphatase-treated nRNA in 3.9 ml of 0.05 M Tris buffer (pH 7.6) containing 50 mM MgCl₂ and 5 mM 2-mercaptoethanol; 1 mCi of [γ-³²P]ATP (>3000 Ci/mmol; The Radiochemical Centre) in 1 ml of 50% ethanol; and 100 units of polynucleotide kinase (Boehringer and Söhne GmbH) in 0.1 ml of the Tris buffer. The mixture was incubated at 37° for 1 hr. RNA was isolated by hot phenol extraction and ethanol precipitation. The RNA was washed 3 times by being dissolved in 5 ml of 0.01 M sodium acetate buffer (pH 5.1) containing 0.2 M NaCl and precipitated with 2 volumes of ethanol. The RNA was washed with ethanol and ether, dried, dissolved in the acetate buffer, and passed through Sephadex G-25 (medium grade). The RNA in the eluate was precipitated with 2 volumes of ethanol and stored at −20° overnight. The RNA was again dissolved in the acetate buffer, precipitated with ethanol, washed with ethanol and ether, and dried in an evacuated desiccator under CaCl₂. At the time of preparation, the specific activities of [³²P]RNA’s of normal liver and AH136B hepatoma were 260,000 and 180,000 cpm/µg RNA, respectively.

RESULTS

The liver and hepatoma tissues that were utilized for the preparations of nRNA’s were examined histologically. The livers of the rats that had been fed 0.06% 3'-Me-DAB diet for 50 days and then maintained on basal diet for a brief period (7 to 8 weeks) showed no tumor nodules but spots of cholangiofi-
Deletion of nPNA in Azo Dye-induced Hepatoma

brosis, cirrhosis, and cysts (Fig. 1). On the other hand, nodules of hyperplastic proliferation of hepatocytes and trabecular carcinomas (Ref. 27; Fig. 2), 1 to 2.5 cm in diameter, were seen in the livers of one-half of the rats that had been fed 3'-Me-DAB followed by 0.05% 4-nitrostilbene diet for 40 days. The lumps of AH136B transplanted hepatoma consisted mostly of the cancer cells with a slight intrusion of fibrous cells and contained few necrotic areas (Fig. 3). The primary hepatoma lumps, which were found in 3 rats that had been fed 3'-Me-DAB and then maintained on basal diet until being sacrificed at 1 year after the start of 3'-Me-DAB feeding, were trabecular carcinomas (Fig. 4) and contained some glandular and fibrous cells.

The i.p. injection of 5 mCi of [3H]orotic acid was able to label normal liver nRNA with as high a specific activity as $4.07 \times 10^6$ cpm/µg RNA (1.04 × 10^5 dpm/µg RNA). The [3H]nRNA (2 µg) was incubated with a filter disc containing approximately 10 µg of DNA. The radioactivity of nRNA that was hybridized in the absence of any competitor RNA was approximately 1000 cpm. The [3H]nRNA was competed by nRNA's from 3'-Me-DAB-treated livers and AH136B hepatoma as well as from normal liver. Chart 1 shows that there is no significant difference in the competition efficiencies of nRNA's between normal liver and the livers that were treated with either 3'-Me-DAB or both 3'-Me-DAB and 4-nitrostilbene. The slight difference at the lower levels of competitor RNA's suggests the quantitative difference in the frequencies of various RNA components. In contrast, Chart 2 shows that AH136B hepatoma nRNA has a deficient competition efficiency in comparison with that of normal liver nRNA. The definite and nearly constant difference at any level of competitor RNA suggests a qualitative difference in the species of RNA components, i.e., that the nuclei of AH136B hepatoma lack some RNA species present in normal liver nuclei.

In order to demonstrate more definitely the deletion of RNA species in the hepatoma nuclei and to examine whether unique RNA species were present in the hepatoma nuclei, isolated normal liver and AH136B hepatoma nRNA's were labeled by the transfer of [32P]ATP of much higher specific activity. The specific activity of normal liver nRNA was 177,000 cpm/µg RNA, and that of AH136B was 148,000 cpm/µg RNA.

![Chart 1](chart1.png)

**Chart 1.** Hybridization of 3H-labeled normal liver nRNA for comparison of normal liver and 3'-Me-DAB-treated liver nRNA's. 3H-labeled normal liver nRNA (2.0 µg) was incubated with a filter containing approximately 10 µg of liver DNA in the presence of increasing amounts of unlabeled competing RNA. The number of counts hybridized in the absence of competitor (control hybridization) was 978 cpm. ○, normal liver; ●, liver treated with 3'-Me-DAB; ◇, AH136B transplanted hepatoma; ▲, primary hepatoma.

![Chart 2](chart2.png)

**Chart 2.** Hybridization of 3H-labeled normal liver nRNA for comparison of normal liver and AH136B hepatoma nRNA's. Conditions were as in Chart 1. The control hybridization was 993 cpm. ○, normal liver; ▲, AH136B hepatoma.

![Chart 3](chart3.png)

**Chart 3.** Hybridization of 32P-labeled normal liver nRNA. 32P-labeled normal liver nRNA (1.0 µg) was incubated with a filter containing approximately 10 µg of liver DNA in the presence of increasing amounts of unlabeled competing nRNA. The control hybridization was 881 cpm. ○, normal liver; ●, liver treated with 3'-Me-DAB; ◇, AH136B transplanted hepatoma; ▲, primary hepatoma.

as at the time of hybridization; 1 µg of each [32P]nRNA was incubated with a filter disc. The radioactivity of normal liver RNA that was hybridized in the absence of any competitor RNA was 881 cpm, and that of AH136B hepatoma was 535 cpm. The 32P-labeled normal liver RNA was competed by nRNA's from normal liver, 3'-Me-DAB-treated liver, AH136B hepatoma, and primary hepatoma that was found in rats fed 3'-Me-DAB. Chart 3 shows that 3'-Me-DAB-treated liver RNA competes as completely as does normal liver RNA, whereas AH136B hepatoma RNA reaches its maximum in the competition efficiency and cannot compete approximately 15% of the radioactivity of labeled normal liver RNA that is hybridized with DNA. It is also seen that RNA of primary hepatoma has a weaker competing efficiency. This figure indicates clearly that there is a considerable deletion of RNA species in the hepatoma nuclei. Then, 32P-labeled AH136B hepatoma RNA was competed by nRNA's from normal liver, 3'-Me-DAB-treated liver, and AH136B hepatoma. Chart 4 shows that normal and 3'-Me-DAB-treated liver RNA's compete as completely as does AH136B hepatoma RNA. This figure does not support the supposition that unique RNA species are present in the hepatoma nuclei.

![Chart 4](chart4.png)

**Chart 4.** Hybridization of 32P-labeled normal liver nRNA. 32P-labeled normal liver nRNA (1.0 µg) was incubated with a filter containing approximately 10 µg of liver DNA in the presence of increasing amounts of unlabeled competing nRNA. The control hybridization was 881 cpm. ○, normal liver; ●, liver treated with 3'-Me-DAB; ◇, AH136B transplanted hepatoma; ▲, primary hepatoma.

DISCUSSION

The deletion of RNA in the nucleus of hepatoma was the common conclusion of several DNA-RNA hybridization studies.
M. Akao and K. Kuroda

that the nuclei of both transplanted and primary azo dye-transplanted hepatomas (8, 10, 11, 18). However, the competitive hybridizations of these studies were not completed, and each experimental curve did not reach an end point. The design of the present study to favor an end point was to prepare the experimental curve was not able to reach an end point. The use of polynucleotide kinase reaction and \( [\gamma-32P]ATP \) for labeling was effective for the preparation of \( ^{32}P \)-labeled RNA's which had severalfold higher specific activities. The competitive hybridization was completed, and it was definitely concluded that the nuclei of both transplanted and primary azo dye-induced hepatomas lacked some RNA species. The deletion was estimated to be 15% or more in the measure of radioactivity of \( ^{32}P \)-labeled normal liver mRNA that was hybridized with DNA. The competitive hybridization with \( ^{32}P \)-labeled AH136B hepatoma was also completed, and the results supported the proposition that the hepatomas do not synthesize unique RNA (10, 25).

Although both studies with \textit{in vivo} and \textit{in vitro} labeled RNA's indicated that the azo dye-induced hepatomas lacked some RNA species in the nuclei, they are supposed to assay different RNA species. The study with \textit{in vivo} labeled RNA assayed the RNA species that were being actively synthesized in the cell nuclei. The \textit{in vitro} labeling by phosphorylation of the 5'-terminus of a RNA molecule is expected to label all RNA species with the same specific activities per mol, i.e., labeling all RNA species with the specific activities per unit weight that are inversely proportional to molecular weights. Therefore, the study with \( ^{32}P \)RNA assayed more of small and stable RNA species. It is expected that these RNA species saturated their complementary DNA sequences at the low labeled RNA:DNA ratio and were competed effectively by the increase of the unlabeled RNA:labeled RNA ratio. The loss of ability of hepatomas to transcribe these small and stable RNA species could account for the achievement of the completed competitive reactions in the study with \( ^{32}P \)RNA.

The mRNA from the liver that had been subjected to the carcinogenic effect by the ingestion of 3'-Me-DAB, either alone or in combination with 4-nitrostilbene, showed a competing efficiency which was qualitatively similar to that of normal liver mRNA. This finding suggests that there is a great difference in the transcription or presence of mRNA between the cancer cells and the carcinogen-treated liver cells.

The RNA's that were measured by the DNA-RNA hybridization technique were inferred to be the transcripts from the repetitive codes of DNA (6, 19), the roles of which were suggested to be transcriptional control, differentiation, and transportation of RNA (5, 9, 20). The deletion of such RNA is supposed to be closely associated with the transformation of a normal cell into a cancer cell. The present method for labeling is expected to contribute to the isolation and characterization of the pertinent RNA.

ACKNOWLEDGMENT

We thank Dr. Hiroshi Sato, Sasaki Institute, for the supply of AH136B hepatoma cells.

REFERENCES

17. Odashima, S. Comparative studies on the transplantability of liver cancers induced in rats fed with 3'-methyl-4-(dimethylamino)azobenzene for 3-6 months. Gann, 52: 325-345, 1962.
20. Parsons, L. T., and McCarty, K. S. Rapidly labeled messenger ribonucleic
Deletion of nRNA in Azo Dye-induced Hepatoma


Fig. 1. Liver of a rat fed 0.06% 3'-Me-DAB diet for 50 days and then maintained on basal diet for a brief period (8 weeks). Cirrhosis and vacuolar degeneration of hepatocytes are seen at the surface of the liver. H & E, x 25.

Fig. 2. Trabecular carcinoma in the liver of a rat fed 3'-Me-DAB and followed by 0.05% 4-nitrosothylene diet for 40 days. A marked proliferation of cancer cells is seen. H & E, x 40.

Fig. 3. Nodule of a AH136B transplanted hepatoma. The nodule is composed mostly of massed cancer cells. A slight intrusion of fibrous cells is seen at the right. H & E, x 100.

Fig. 4. Primary hepatoma found in a rat fed 3'-Me-DAB and then maintained on basal diet until sacrifice at 1 year. Anisocytosis of cancer cells is moderate, but mitotic figures are numerous. H & E, x 100.
Analysis of Loss of Nuclear RNA in Azo Dye-induced Hepatoma by DNA-RNA Competitive Hybridization

Mitsutaro Akao and Keiko Kuroda