Studies were conducted to characterize the nuclear RNA (nRNA) species that were present in rat liver but absent in the hepatoma. Nuclear RNA was compared between Donryu rat liver and AH136B hepatoma, an azo dye-induced transplantable cell line, by DNA-RNA competitive hybridization. The hepatoma lacked 13-14% of nRNA according to measurements of radioactivity of the hybridized 32P-labeled liver nRNA, and this loss was shown to be due to the failure to transcribe such RNA rather than to the deletion of the relevant DNA in the genome. Characterization of the lost RNA was first attempted by fractionating liver nRNA by density gradient sedimentation and polyacrylamide gel electrophoresis. A comparison of the additive effects of the fractionated RNA's in the competitive hybridization indicated that the pertinent RNA was present in the large RNA molecules (>14S), not in the low molecular weight RNA's. Then poly(A) nRNA was found to show a strong additive effect in the competitive hybridization while nucleolar RNA showed little additive effect, indicating that the pertinent RNA was present in the heterogeneous nRNA, not in the ribosomal precursor. Further characterization was done by fractionating DNA with regard to the repetition in the genome. A comparison of the competitive hybridizations on the fractionated DNA's showed that the loss occurred mostly in RNA transcribed from highly repetitive DNA. In conclusion, the RNA species lost in the hepatoma were components of heterogeneous nRNA transcribed from highly repetitive DNA.

Key words: Nuclear RNA — Loss of nRNA — AH136B hepatoma — DNA-RNA competitive hybridization — 3'-Methyl-4-(dimethylamino)azobenzene

The feeding of carcinogenic azo dyes to rats brings about various kinds of deleterious effects on the liver RNA metabolism, such as inhibition of DNA-dependent RNA polymerase activities in the cell nuclei,1-3) loss of the mechanism for the selective transport of RNA from the nucleus into the cytoplasm,4, 5) and alteration of the processing of nuclear RNA (nRNA).6-8) In addition, several studies of DNA-RNA competitive hybridization indicated that a considerable portion of liver nRNA species were lost or present in very low concentration in the hepatoma,9-13) and cross hybridizations of polysomal polyadenylate RNA with complementary DNA showed that some liver messenger RNA species were missing in the hepatoma.14, 15) Previously we had prepared highly labeled RNA by phosphorylation in vitro with polynucleotide kinase (EC 2.7.1.78) and ATP-[γ-32P], and utilized this 32P-RNA as an indicator for the competitive reaction in DNA-RNA hybridization.9) Competitive reactions were completed, and the loss of nRNA in 3'-methyl - 4 - (dimethylamino)azobenzene (3'-Me-DAB)-induced primary hepatoma or transplantable AH136B hepatoma16) was estimated to be approximately 15% or more according to measurements of radioactivity of the hybridized liver nRNA. The present study utilized the above method of competitive hybridization to characterize the nRNA species that were lost in the hepatoma, and was carried in view of the possible implication of the loss of nRNA in the neoplastic transformation as well as in the distortion of transcriptional control in cancer cells.17-21)

MATERIALS AND METHODS

Liver and Hepatoma Animals used were male Donryu rats obtained from Nippon Rat Co., Sai-
toma. They were maintained on a semisynthetic diet, CE-2, purchased from CLEA Japan Inc., Tokyo. Normal livers were excised from adult rats, more than 12 weeks old, that had been fasted overnight and killed by decapitation. Rat ascites hepatoma AH136B, a 3'-Me-DAB-induced transplantable cell line, was obtained from Dr. Hiroshi Satoh, Sasaki Institute, and utilized for the present study because it grew rapidly in the abdomens of Donryu rats with 100% transplants of massed cancer cells and, therefore, was advantageous for the preparation of a large amount of homogeneous hepatoma nRNA.9

**Preparation of RNA's** Nuclear RNA was prepared from normal rat livers and hepatoma masses as described previously:9: nRNA was extracted from the isolated nuclei by the hot phenol-sodium dodecyl sulfate (SDS) procedure, and purified by incubation with a-amylase and DNase I and also by precipitation with cetyltrimethylammonium bromide. DNase I was purified, when necessary, by passing it through a column of agarose 5-(p-aminophenyl)-uridine 2'(3') phosphate (Miles-Yeda Ltd., Rehovot, Israel). Nucleolar RNA was extracted from the nuclei that had been isolated from liver nuclei by the sonication procedure,4 and purified as described above. Rat liver 28S, 18S, 5.4S and 5S ribosomal RNA's (rRNA's) and transfer RNA (tRNA) were prepared by the methods of Steele et al.,15 and Zubay,26 respectively.

**Fractionation of nRNA** Zone sedimentation was performed in a Hitachi RPS-25 rotor at 5° according to the method of Peacock and Dingman.27 Liver nRNA, prepared by the hot phenol-SDS procedure, was dissolved in 0.1 M NaCl-1 mM EDTA (pH 6.2) at the concentration of approximately 2 mg/ml. One ml of the solution was layered on 29 ml of 5-10% sucrose gradient, and centrifuged in a Hitachi RPS-25 rotor at 25,000 rpm and 20° for 48 hr. The sedimentation coefficients were determined by using the table of McEwen28 and, from them, molecular weights were calculated using the equations given by Studier.30 Fractions of DNA ranging in molecular weight from 7.7 × 10^4 to 2.8 × 10^5 (peak mol. wt. 1.54 × 10^5) were pooled with a recovery of over 70%.

The DNA fragments were then fractionated by incubation to an approximately equivalent C0t followed by separation of double- from single-stranded DNA by hydroxyapatite chromatography based on the methods of Britten and Kohne34 and Holmes and Bonner.35 Single copy DNA was isolated by purifying that remained single-stranded at C0t 10^4, middle repetitive DNA was isolated by separation of DNA that reassociated between C0t 10^3 and Cot 2 × 10^-2, and highly repetitive DNA was isolated by separation of DNA of C0t 2 × 10^-2.

**DNA-RNA Hybridization** DNA-RNA competitive hybridization was carried out as described in the previous study with the use of the nitrocellulose filter technique.38 Labeling of RNA was done by the transfer of 32Pi from ATP[γ-32P] to the 5'-hydroxy terminus of the alkaline phosphatase-treated liver nRNA catalyzed by polynucleotide kinase. ATP[γ-32P] (>5,000 Ci/mmol) was obtained from Amersham International Ltd., Buckinghamshire, England, and the radioactivity was measured by a Packard Tri-Carb model 3255 scintillation counter.
RESULTS

**Loss of nRNA in Hepatoma** DNA-RNA competitive hybridization was conducted on a filter disk containing liver DNA (Fig. 1A) or hepatoma DNA (Fig. 1B). One μg of liver $^{32}$P-RNA was incubated with a filter disk containing approximately 10 μg of DNA. The specific activity of $^{32}$P-RNA was $6.83 \times 10^5$ cpm/μg at the time of hybridization, and the radioactivity that was hybridized in the absence of any competitor RNA was approximately 3,700 cpm. The presence of increasing amounts of unlabeled liver nRNA competed with $^{32}$P-RNA completely while that of unlabeled hepatoma nRNA reached a maximum level of competition and could not compete with approximately 13.5% of the radioactivity of $^{32}$P-RNA that was hybridized.

**Fig. 1.** Comparison of liver and hepatoma nRNA's in competitive hybridization on liver DNA and hepatoma DNA. $^{32}$P-labeled liver nRNA, 1 μg, was incubated with a filter disk containing approximately 10 μg of either liver DNA (A) or AH136B hepatoma DNA (B) in the presence of increasing amounts of unlabeled competing RNA. The numbers of counts in the absence of any competitor RNA (control hybridization) were 3,718 cpm in A and 3,699 cpm in B. Liver nRNA (○); AH136B hepatoma nRNA (△).

**Fig. 2.** Sucrose density gradient profile of liver nRNA (A) and electrophoregram of the low molecular weight RNA's (B). Approximately 2 mg of liver nRNA was placed on top of a 5–20% sucrose density gradient and centrifuged in a Hitachi RPS-25 rotor at 23,000 rpm for 19 hr at 5°C. The RNA was divided at 9 ml (14S) and 18 ml (23S) from the top of the gradient. A part of the <14S RNA was run on 10% polyacrylamide gel slab as described in “Materials and Methods.” Fig. 2B shows a sketch of the methylene blue-stained electrophoregram of the <14S RNA. As standards, 5S rRNA (mol. wt. 40,000), 5.4S rRNA (mol. wt. 45,000) and tRNA (mol. wt. 25,000) were electrophoresed on the same gel slab and a semilog plot of mobility vs. molecular weight was constructed.

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ized with liver DNA (Fig. 1A). A similar reduced competition efficiency of hepatoma nRNA was also seen when hepatoma DNA was used for the hybridization (Fig. 1B), indicating that the hepatoma had failed to transcribe some RNA species but had not deleted the relevant DNA sequence in the genome.

**Search for nRNA Lost in the Hepatoma**

First, the techniques of density gradient sedimentation and polyacrylamide gel electrophoresis were utilized to fractionate liver nRNA. Liver nRNA was sedimented on a 5–20% sucrose density gradient as shown in Fig. 2A. The RNA was collected from the top of the gradient and divided into 3 fractions at 14S and 23S. Each RNA fraction was pooled and then subjected to a 2nd density gradient centrifugation, and the RNA that sedimented in the same fraction was collected. Liver nRNA was then divided in a ratio of approximately 6(<14S):5(14–23S):9 (>23S). A part of the 14S RNA was further divided into 3 subfractions which ran 0–20 mm, 20–40 mm and 40–60 mm on polyacrylamide gel electrophoresis. Fig. 2B shows a sketch of a methylene blue-stained electrophoregram, which is similar to that of Dingman and Peacock. An approximate molecular weight for each band can be calculated on the basis of a semi-log plot of mobility vs. molecular weight with the use of 5S rRNA, 5.4S rRNA and tRNA as standards having reported molecular weights of $4 \times 10^4$, $4.5 \times 10^4$ and $2.5 \times 10^4$, respectively. The <14S RNA was divided in a ratio of approximately $2(0–20\text{mm}):4(20–40\text{mm}):1 (40–60 \text{mm})$. After fractionation, RNA's were examined with respect to their additive effects in the competitive hybridization (Table I). Both the >23S and 14–23S RNA's exhibited efficient additive effects on the competitive reaction, competing almost completely with the portion of the hybridized $^{32}$P-RNA that hepatoma nRNA alone could not compete with. Only a slight additive effect was exhibited by the <14S RNA and little by the 2 fractions which ran 20–40 mm and 40–60 mm on the electrophoresis and contained the majority of the

<table>
<thead>
<tr>
<th>Competitor RNA (μg)</th>
<th>Liver nRNA or subfraction</th>
<th>Noncompeted liver $^{32}$P-RNA (cpm/DNA filter)</th>
<th>% of control hybridization</th>
</tr>
</thead>
<tbody>
<tr>
<td>AH136B hepatoma nRNA</td>
<td>0</td>
<td>4,507</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>1,000</td>
<td>608</td>
<td>13.5</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>40</td>
<td>0.9</td>
</tr>
<tr>
<td>Fraction$^b$</td>
<td>&lt;14S; 500</td>
<td>370</td>
<td>8.2</td>
</tr>
<tr>
<td></td>
<td>14–23S; 500</td>
<td>36</td>
<td>0.8</td>
</tr>
<tr>
<td></td>
<td>&gt;23S; 500</td>
<td>42</td>
<td>0.9</td>
</tr>
<tr>
<td>Subfraction$^c$</td>
<td>0–20 mm; 150</td>
<td>433</td>
<td>9.6</td>
</tr>
<tr>
<td></td>
<td>20–40 mm; 280</td>
<td>514</td>
<td>11.4</td>
</tr>
<tr>
<td></td>
<td>40–60 mm; 70</td>
<td>626</td>
<td>13.9</td>
</tr>
</tbody>
</table>

$^a$ The fractionated liver nRNA's were examined for additive effects in the competitive hybridization. The specific activity of liver $^{32}$P-RNA was $6.56 \times 10^4$ cpm/μg RNA and the number of counts hybridized in the absence of any competitor RNA (control hybridization) was 4,507 cpm/DNA filter.

$^b$ Liver nRNA was sedimented on a 5–20% sucrose density gradient as shown in Fig. 2A and then divided into 3 fractions at 9 ml (14S) and 18 ml (23S) from the top of the gradient. The RNA was divided in a ratio of approximately 6(<14S):5(14–23S):9 (>23S).

$^c$ A part of the <14S RNA was electrophoresed on a 10% polyacrylamide gel slab and divided into 3 subfractions which ran 0–20 mm, 20–40 mm and 40–60 mm. The <14S RNA was divided in a ratio of approximately 2(0–20 mm):4(20–40 mm):1(40–60 mm).
Accordingly, the next examination was conducted on the nucleolar RNA and poly(A) nRNA which contained high molecular weight ribosomal RNA precursor and heterogeneous nRNA (hnRNA), respectively (Table II). Increasing amounts of the nucleolar RNA exhibited little additive effect in the competitive hybridization. On the other hand, poly(A) nRNA, which bound to poly(U) Sepharose and constituted approximately 7% of liver nRNA, exhibited an efficient additive effect, i.e. the addition of 70 μg of poly(A) nRNA competed with ca. 50% of such radioactivity of the hybridized 32P-RNA as could not be competed with by hepatoma nRNA alone. The remainder (93%) of liver nRNA, unbound to poly(U) Sepharose, also showed an efficient additive effect, although a large quantity of this RNA fraction was used in the examination.

Another search was carried out to identify the DNA sequences whose transcripts were lost to the greatest degree in the hepatoma. Liver DNA was fragmented and the DNA fragments were then divided into 3 fractions with regard to the repetition in the genome. An appropriate amount of a DNA fraction was immobilized on a nitrocellulose membrane filter according to the composition in the genome so that a filter disk contained 1 μg of highly repetitive DNA, 2 μg of middle repetitive DNA or 7 μg of single copy DNA. Competitive hybridization was conducted on each of the 3 DNA fractions. The specific activity of 32P-RNA was 6.03 × 10^5 cpm/μg RNA and the number of counts hybridized in the absence of any competitor RNA (control hybridization) was 2,987 cpm/DNA filter.

### Table II. Search for Liver nRNA Lost in AH136B Hepatoma in Nucleolar RNA and Poly(A) nRNA

<table>
<thead>
<tr>
<th>Competitor RNA (μg)</th>
<th>Liver nRNA or its fraction</th>
<th>Noncompeted liver 32P-RNA (cpm/DNA filter)</th>
<th>% of control hybridization</th>
</tr>
</thead>
<tbody>
<tr>
<td>AH136B hepatoma nRNA</td>
<td>Total nRNA</td>
<td>Total nRNA</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>0</td>
<td>2,987</td>
<td>100</td>
</tr>
<tr>
<td>1,000</td>
<td>0</td>
<td>394</td>
<td>13.2</td>
</tr>
<tr>
<td>1,000</td>
<td>1,000</td>
<td>33</td>
<td>1.1</td>
</tr>
<tr>
<td>Nucleolar RNA</td>
<td>75</td>
<td>386</td>
<td>12.9</td>
</tr>
<tr>
<td>1,000</td>
<td>150</td>
<td>353</td>
<td>11.8</td>
</tr>
<tr>
<td>1,000</td>
<td>300</td>
<td>344</td>
<td>11.5</td>
</tr>
<tr>
<td>Poly(A) nRNA</td>
<td>18</td>
<td>305</td>
<td>10.2</td>
</tr>
<tr>
<td>1,000</td>
<td>35</td>
<td>266</td>
<td>8.9</td>
</tr>
<tr>
<td>1,000</td>
<td>70</td>
<td>203</td>
<td>6.8</td>
</tr>
<tr>
<td>Non-poly(A) nRNA</td>
<td>233</td>
<td>209</td>
<td>7.0</td>
</tr>
</tbody>
</table>
| a) Nucleolar RNA, poly(A) nRNA and non-poly(A) nRNA were examined for additive effects in the competitive hybridization. Nucleolar RNA was prepared from the isolated liver nucleoli and poly(A) nRNA was prepared from liver nRNA by binding to poly(U) Sepharose and eluting with formamide. The poly(U) Sepharose affinity chromatography divided liver nRNA into poly(A) nRNA and non-poly(A) nRNA (the unbound fraction) in a ratio of 7:93. The specific activity of liver 32P-RNA was 6.03 × 10^5 cpm/μg RNA and the number of counts hybridized in the absence of any competitor RNA (control hybridization) was 2,987 cpm/DNA filter.

low molecular weight RNA's (Fig. 2B and Table I).

Another search was carried out to identify the DNA sequences whose transcripts were lost to the greatest degree in the hepatoma. Liver DNA was fragmented and the DNA fragments were then divided into 3 fractions with regard to the repetition in the genome. An appropriate amount of a DNA fraction was immobilized on a nitrocellulose membrane filter according to the composition in the genome so that a filter disk contained 1 μg of highly repetitive DNA, 2 μg of middle repetitive DNA or 7 μg of single copy DNA. Competitive hybridization was conducted on each of the 3 DNA fractions. The specific activity of 32P-RNA was 6.03 × 10^5 cpm/μg RNA at the time of hybridization and 1 μg of 32P-RNA was incubated with a filter disk. The radioactivity of 32P-RNA that was hybridized in the absence of any competitor RNA was 600 cpm for highly repetitive DNA, 464 cpm for middle repetitive DNA and 921 cpm for single copy DNA (Fig. 3). The loss of
nRNA in the hepatoma was most clearly shown by the competitive reactions on highly repetitive DNA (Fig. 3A) and was estimated to be approximately 15% with regard to this DNA fraction. On the other hand, unlabeled liver and hepatoma nRNA's competed with 32P-RNA almost equally in the hybridization with middle repetitive DNA or single copy DNA, and the loss of nRNA was as small as 2% or 4%, respectively (Figs. 3B and 3C). These results indicate that the present loss of nRNA occurs mainly in the transcript of highly repetitive DNA.

DISCUSSION

The previous study demonstrated that a considerable portion of nRNA was lost in 3'-Me-DAB induced primary and transplantable AH136B hepatoma but not in the carcinogen-treated liver,9) and the present study indicated that the hepatoma failed to transcribe some RNA species but had not deleted the relevant DNA sequences. Experiments were carried out to characterize RNA that was present in the liver but absent in the hepatoma. The fractionation of liver nRNA by density gradient sedimentation indicated that the pertinent RNA was some common RNA sequence that was present among various sizes of large RNA molecules, since the pertinent RNA was almost all present in either of 2 fractions of large RNA molecules (Table I). Polyacrylamide gel electrophoresis indicated that the loss did not occur in the low molecular weight RNA's40-43) which were recently inferred to be involved in the splicing of RNA.44,45) The examination of nuclear RNA and poly(A) nRNA indicated that the loss occurred in the hnRNA, not in the ribosomal precursor (Table II). It was noted that a large quantity of the non-poly(A) nRNA fraction exhibited an efficient additive effect in the competitive hybridization, suggesting that hnRNA lacking poly(A)43) was also lost in the hepatoma. A comparison of

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**Fig. 3.** Comparison of liver and hepatoma nRNA's in the competitive hybridization on highly repetitive DNA, middle repetitive DNA and single copy DNA. 32P-labeled liver nRNA, 1 µg, was incubated with a filter disk containing approximately 1 µg of highly repetitive DNA (A), 2 µg of middle repetitive DNA (B) or 7 µg of single copy DNA (C) in the presence of increasing amounts of unlabeled competitor RNA. The numbers of counts in the absence of any competitor RNA (control hybridization) were 600 cpm in A, 464 cpm in B and 921 cpm in C. Liver nRNA (○); AH136B hepatoma nRNA (▲).
the competitive hybridizations on 3 DNA fractions indicated that the loss was greatest in the transcript of highly repetitive DNA. The genome is composed of 9% highly repetitive DNA, 19% middle repetitive DNA and 65% single copy DNA, and the repetitive DNA sequences and their transcripts have been inferred to be implicated in the regulation of gene expression. The combination of findings described above indicates that the pertinent RNA lost in the hepatoma consists of sequences of hnRNA transcribed from highly repetitive DNA.

Patel et al. studied a small RNA fraction, termed fraction-3 RNA, that had an approximate molecular weight of 13 thousand. Fraction-3 RNA was found in normal liver nuclei in the form of metabolically active RNA sequences associated with nonhistone chromosomal proteins, and released into the cytoplasm as part of larger RNA molecules after ingestion of 3'-Me-DAB or in the hepatoma. It was proposed that fraction-3 RNA was removed during the processing of the hnRNA and that a distortion of RNA processing occurred during carcinogenesis. Their competitive hybridization study indicated that hepatoma fraction-3 RNA lacked some sequences which were present in liver fraction-3 RNA but contained new sequences which were absent in liver fraction-3 RNA. In contrast, the RNA species in our study were found almost exclusively in the large RNA molecules and hardly at all in the low molecular weight RNA's. Our previous study with the use of 32P-labeled nRNA of AH136B hepatoma indicated that there were unique RNA species in the hepatoma nuclei. Therefore, our RNA species are assumed to be different from or only partly related to theirs. The loss of RNA is thought to be associated with the disruption of gene regulation in the hepatoma and/or failure of this tissue to transcribe some hnRNA's rather than with the difference of growth phase, since no such loss was found in the regenerating liver after partial hepatectomy.

The isolation and chemical analysis of the pertinent RNA and the relevant DNA are expected to allow extension of the present findings to the primary hepatomas induced by 3'-Me-DAB as well as other carcinogens. Further studies on the distortion in RNA synthesis are necessary for elucidation of the mechanism underlying the transformation of normal cells into malignant cells.

ACKNOWLEDGMENTS
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M. AKAO AND K. KURODA


