Similarity of Ribosomal and Ribosomal Precursor RNA's from Rat Liver and the Novikoff Ascites Hepatoma

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SUMMARY

The $^{32}$P-labeled ribosomal RNA's and their precursors in rat liver and the Novikoff hepatoma have been examined by two competitive hybridization techniques and by nucleotide analyses. Significant differences were not found between the corresponding RNA of these tissues by competition of labeled RNA from the hepatoma with unlabeled 18 and 28 S ribosomal RNA and 28 and 45 S nucleolar RNA of liver and hepatoma by sequential or simultaneous competition hybridization. Differences were found in the specific activity of $^{32}$P-labeled nucleotides, but no significant differences were found by ultraviolet light absorbancy in the distribution of short oligonucleotides after digestion with pancreatic ribonuclease. These data provide evidence that the previously reported differences in nucleotide composition and oligonucleotide distribution were probably the result of nonuniform labeling rather than differences in the primary sequences. Accordingly, the primary structures of ribosomal RNA's and their precursors appear to be conserved in oncogenesis.

INTRODUCTION

A number of reports from this and other laboratories suggested that there are significant differences, in tumors and other cells (6, 13, 18, 31, 32), between the primary structures of rRNA's and their precursors. $^{32}$P-Labeled nucleolar precursor RNA's in several transplantable tumors were found to contain less adenylic and more cytidylic acid than does normal or regenerating rat liver (6). This was also true for a primary hepatoma induced by 3'-methyl-4-dimethylaminoazobenzene (18) and, although considerably less pronounced, for 28 S rRNA of Novikoff hepatoma and normal liver (30). In contrast, analyses of polypurines (1) and the longer polypyrimidines (23) after complete pancreatic or combined U$_2$ and T$_1$ RNase digestions did not reveal differences in the primary sequences of 28 S rRNA from normal and malignant tissues. In addition, 28 S rRNA-$^{32}$P from HeLa cells was competed equally well by simultaneous competitive hybridization with unlabeled HeLa 28 S rRNA and RNA from various normal human tissues (1).

To resolve these conflicting reports, in this study $^{32}$P-labeled rRNA's and ribosomal precursor RNA's were competed with unlabeled RNA from normal liver and hepatoma cells. Both simultaneous and sequential competitive hybridizations were used to preclude artifacts that might result from the use of a single method. The effects of $^{32}$P labeling also were examined in structural comparisons between normal and tumor tissues.

MATERIALS AND METHODS

Labeling and Preparation of rRNA. Novikoff hepatoma ascites cells were maintained in male Holtzman rats for 6 days and then labeled in vitro for 18 hr with $^{32}$P (Union Carbide, Tuxedo, N. Y.) essentially as described by Mauritzen et al. (19). At the end of the incubation or when unlabeled RNA was prepared, the cells were pelleted by centrifugation at 0°. Normal rat livers were labeled by a single i.p. injection of $^{32}$P into fasted Holtzman rats 24 hr prior to sacrifice. The rats were killed by decapitation and bled; the livers were removed, minced, and washed directly in cold 0.25 M sucrose (27).

Cells were homogenized and polysomes were isolated at 0–4° essentially as described by Rich (25). Ribosomal subunits were prepared by homogenization of the polysomes in 8 mM EDTA; 5 mM Tris (pH 7.5) and centrifugation on 10 to 40% linear sucrose gradients in 10 mM Tris (pH 7.5) (Spinco SW-27 rotor for 18 hr at 25,000 rpm). The RNA
was extracted by a SDS:phenol method at 20°F or 65°F (28) and purified on 5 to 40% sucrose gradients (12, 24).

**Labeling and Preparation of Nucleolar RNA.** Cells were prepared and labeled for 6 hr as described above. Nuclei were prepared by methods described by Chauveau et al. (8), and the nucleoli were isolated after sonic treatment (5). RNA was extracted by the SDS:phenol method (28) at 65°F and purified by repeated sucrose density gradient centrifugation in a SW-27 rotor at 26,000 rpm for 16 hr (12, 24). The RNA species were precipitated with 2% potassium acetate in ethanol and desalted with ethanol at -20°F.

**Isolation of DNA.** DNA was isolated by means of a modified Marmur (17) procedure, with extensive digestion with nuclease-free Pronase (Calbiochem, San Diego, Calif.) in the initial stage of the isolation. The pellet of nucleoli or nuclei was suspended in about 100 volumes of 1 x SSC (0.15 M NaCl:0.015 M sodium citrate):0.1 M EDTA, pH 8.0, at 4°F, and nuclease-free Pronase (predigested at 2 to 5 mg/ml at 37°F for 0.5 hr) was added to a concentration of 0.5 mg/ml. If this initial Pronase stage was eliminated, the percentage hybrid of the nucleolar DNA with 28 S rRNA was reduced by one-half the normal value, indicating a selective loss of rDNA genes into the interphase upon deproteinization with chloroform:isoamyl alcohol. This mixture was kept at room temperature until it reached 15°F and then was placed in a 37°F H2O bath, with the addition of SDS to 0.5%, for 1 hr. SDS was added to a concentration of 1%, and sodium perchlorate was added to a concentration of 1 M. This mixture was shaken gently for 1 hr at 37°F and then was combined with an equal volume of chloroform:isoamyl alcohol (24:1) with shaking for another hour. After centrifugation to separate the phases, the aqueous layer was collected and reextracted twice with chloroform:isoamyl alcohol. The DNA was spooled out of the aqueous phase with the addition of 1 volume of 100% ethyl alcohol. The DNA was dissolved in 1/100 SSC:1 mM EDTA at a concentration of 0.5 mg/ml and treated with pancreatic and T1 RNase (previously heated at pH 5.1 for 10 min at 90°F) at a concentration of 100 µg and 50 units/ml, respectively, for 1 hr at 37°F. Pronase, nuclease-free and predigested, was added at a concentration of 50 µg/ml for 1 hr. SDS, sodium perchlorate, and chloroform:isoamyl alcohol were added as described above. The DNA was spooled, dissolved in 1/100 SSC:1 mM EDTA, digested again with Pronase, and deproteinized as before. The DNA was spooled from the aqueous phase, dissolved in 1/100 SSC:1 mM EDTA and frozen at -20°F until loaded on filters. The A260:A280 ratios of these preparations were 1.87.

**Loading DNA on Filters.** The DNA was denatured in 0.5 N NaOH for 0.5 hr at 37°F to ensure denaturation and as an additional purification step (15). This solution was mixed with 300 ml of 6 x SSC, neutralized with HCl, and then filtered through a 142-mm, 0.45-µm Millipore filter. The discs (0.6 cm in diameter) were punched out, stored in a desiccator and baked in a vacuum oven at 80°F for 4 hr before use. The filters were uniformly loaded with DNA (10 to 15 µg/0.6-cm disc).

**DNA Content of the Filters.** The DNA content of the filters was analyzed by the diphenylamine method (4) as modified for DNA bound to filters by Meigs and Schipperoot (21). Highly polymerized Novikoff hepatoma nuclear and calf thymus DNA (Worthington Biochemical Corp., Freehold, N. J.) were used as standards.

**RNA-DNA Hybridization.** RNA-DNA hybridization was carried out essentially by the procedure of Gillespie and Spiegelman (9), with the use of formamide to lower the temperature of hybridization (2, 10, 20). Experiments were performed in 30% formamide:70% (2 x SSC; 5 mM morpholinopropionate sulfonic acid, pH 7.0) in a volume of 0.3 ml at 45°F. RNA samples were adjusted to the appropriate concentrations and percentage formamide, heated at 70°F for 5 min, and then rapidly chilled (24). Then the reactants were allowed to reach 45°F and the DNA and blank filters were added.

Simultaneous competition experiments were run by adding RNA-32P at saturating amounts with increasing concentrations of unlabeled competing RNA. In the sequential competition procedure, increasing inputs of unlabeled RNA in excess of saturation were hybridized. The filters were then washed at 45°F in 30% formamide:70% (2 x SSC); each set was incubated under hybridization conditions for 1 hr. These filters were then transferred to vials containing the RNA-32P and incubated for the same length of time. At the end of the hybridization the filters were incubated for 1 hr at 45°F in fresh buffer, then twice in 2 x SSC at room temperature for 10 min, and then treated with T1 RNase and pancreatic RNase (previously heated at pH 5.1 for 10 min at 90°F) at concentrations of 20 units and 40 µg/ml, respectively, at 37°F in 2 x SSC for 0.5 hr. The filters were washed twice with 2 x SSC, dried in the vacuum oven at 80°F, and then counted in a toluene fluor (4 g PPO and 100 mg POPOP per liter of toluene).

**Specific Activities by Phosphodiesterase Digestion.** The specific activities of nucleotides in RNA's were determined by complete digestion (2.5 mg of enzyme per ml for 24 hr at 37°F) of the 32P-labeled RNA's with venom phosphodiesterase (Worthington) and determination of their nucleotide compositions by both 32P and absorbance measurements. Routinely, the 32P composition was measured by separating RNA samples at 65°F and 30% formamide:70% (2 x SSC, 45°C) using 28 S RNA's as standards.

**The abbreviations used are:** SDS, sodium dodecyl sulfate; rDNA, ribosomal DNA; SSC, 0.15 M sodium chloride:0.015 M sodium citrate.

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4 A comparison of hybridization in 2 x SSC, 65°C, and 30% formamide:70% 2 x SSC, 45°C, was made using 28 S rRNA. Both conditions gave similar R Nase-resistant percentage hybrids: however, the rate of the reaction in formamide at 45°C was about one-half the rate at 65°C. The percentage hybrid was also the same when intact or partially degraded (1 N NaOH, 90 sec, 0°C, 8 to 18 S in size) 28 S rRNA was used.
Oligonucleotide Analysis by UV Absorbance. Purified nucleolar or cytoplasmic RNA (1 to 10 mg) was digested with pancreatic RNase (26) or with U1 RNase followed by T1 RNase (23) and applied to a DEAE-Sephadex A-25 column (Chart 1). The oligonucleotides were separated first according to chain length on DEAE-Sephadex A-25 (Pharmacia Fine Chemicals, Piscataway, N. J.) as described by Tener (29). Columns (0.7 x 10 cm) were eluted with a linear sodium chloride gradient from 0.05 to 0.2 M NaCl in 7 M urea and 0.05 M Tris-Cl, pH 7.5. Dinucleotide fractions were pooled and further separated on acidic columns (right) eluted with 400-ml gradients of 0 to 0.3 M NaCl in 0.003 M HCl. 3-ml fractions were collected every 5 min and their absorbance was measured at 260 nm. The mono- and dinucleotides were further separated according to base composition on columns (0.7 x 15 cm) eluted with a linear sodium chloride gradient from 0 to 0.3 M in 0.003 M HCl (total volume, 400 ml/gradient). Three-ml fractions were collected every 5 min and their absorbance was measured at 260 nm. For nucleolar RNA's, the dinucleotides were analyzed by the Picker Nuclear nucleotide analyzer at 254 nm.

RESULTS

Competitive Hybridization. To demonstrate specificity of competitive hybridization by both the simultaneous and sequential techniques, 32P-labeled 18 S RNA was competed with 18 S plus 28 S rRNA by the sequential technique, its binding was reduced by 50% if the temperatures of the reaction and the wash were maintained at 45° or the filters were treated with RNase after the 1st stage. However, when the reactions with unlabeled RNA were allowed to reach room temperature and then washed at 45°, the 45 S RNA was competed by 95% (Chart 4). Specificity was maintained in the sequential technique in these experiments by never allowing the temperature to drop below 45° during the wash.

When 32P-labeled 18 S rRNA from hepatoma cells was competed with unlabeled RNA from hepatoma and normal liver, no differences were demonstrated (Chart 2). The lack of significant competition by 28 S rRNA from both tissues demonstrated the specificity of hybridization and purity of the RNA molecules. This lack of difference in liver and hepatoma 18 S RNA was shown previously by Wikman et al. (31). In Chart 3, 28 S rRNA-32P from hepatoma cells was competed with unlabeled RNA from both hepatoma

Chart 1. DEAE-Sephadex A-25 column chromatography of pancreatic RNase digestion products from 28 S rRNA. Neutral columns (left) were eluted with 600-ml gradients of 0.05 to 0.2 M NaCl in 7 M urea and 0.05 M Tris-Cl, pH 7.5. Dinucleotide fractions were pooled and further separated on acidic columns (right) eluted with 400-ml gradients of 0 to 0.3 M NaCl in 0.003 M HCl.

Chart 2. Competition experiments between 5 μg of 32P-labeled 18 S rRNA from hepatoma cells and unlabeled 18 S and 28 S rRNA from hepatoma and normal liver. The competition in the upper graph was performed by the simultaneous technique with a 100% value of 392 cpm (r18S, 18 S RNA; r28S, 28 S RNA; NHAC, Novikoff hepatoma ascites cells). The competition in the lower graph was performed by the sequential procedure with a 100% value of 300 cpm.

3314 CANCER RESEARCH VOL. 33

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Ribosomal and Precursor RNA's

Simultaneous

Liver-r28S
Liver-r18S
Hepatoma-r28S
Hepatoma-r18S

UNLABELED-RNA

RNA – 32P

24 6 8 10

μg UNLABELED-RNA

μg RNA - 32P

NHAC-r28S - 32P
24 Hr

Simultaneous

% OF CONTROL

Liver-r28S
Liver-r18S
Hepatoma-r28S
Hepatoma-r18S

Sequential

48 Hr : 48 Hr

% OF CONTROL

μg UNLABELED-RNA

45S-nRNA-32P

0 2 4 6 8 10

μg UNLABELED 18S AND 28S rRNA

Chart 3. Competition experiment between 5 μg ³²P-labeled 28 S rRNA from hepatoma cells and unlabeled 18 S rRNA and 28 S rRNA from hepatoma and normal liver. The competition in the upper graph was performed by the simultaneous procedure with a 100% value of 250 cpm (28 S RNA, r28S; 18 S RNA, r18S; Novikoff hepatoma ascites cells (NHAC). The competition in the lower graph was performed by the sequential technique with a 100% value of 214 cpm.

Chart 4. Sequential competition between 10 μg ³²P-labeled 45 S nucleolar RNA (nRNA) from hepatoma cells and unlabeled hepatoma 18 S plus 28 S rRNA. After unlabeled 18 S plus 28 S rRNA was hybridized on 3 sets of filters, 1 set was maintained carefully at 45° and washed; the other 2 sets were allowed to reach room temperature. Of the filters allowed to reach room temperature, one set was treated with RNase and Pronase while the other was washed at 45°.
DISCUSSION

In this study, no significant differences were found by simultaneous and sequential competitive hybridization between liver and hepatoma rRNA and their nucleolar precursors. Arnaldi and Attardi (1) also found no significant difference in 28 S rRNA from HeLa cells and various normal human tissues when compared by simultaneous competition. All the hybridization experiments were performed with tumor DNA and 32P-labeled tumor RNA that would detect additional sequences in the tumor RNA. However, the sequence (23) and base composition (6, 22) data would suggest no large deletions or additions in the rRNA's from normal and malignant tissues. Recently, Hashimoto and Muramatsu (11) reported the presence, in 18 S rRNA of normal mouse liver, of a tetranucleotide and a hexanucleotide that were absent from mouse hepatoma. Such limited differences would not be detected by the techniques used in this paper.

Table 1
Specific activities of 5'-nucleotides in 32P-labeled RNA's from normal livers and the Novikoff hepatoma

<table>
<thead>
<tr>
<th>RNA</th>
<th>Nucleotide</th>
<th>Liver (24 hr)</th>
<th>Novikoff (18 hr)</th>
</tr>
</thead>
<tbody>
<tr>
<td>18 S rRNA</td>
<td>pU</td>
<td>0.64 ± 0.07</td>
<td>1.19 ± 0.07</td>
</tr>
<tr>
<td></td>
<td>pA</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>pC</td>
<td>0.66 ± 0.03</td>
<td>0.96 ± 0.04</td>
</tr>
<tr>
<td></td>
<td>pG</td>
<td>0.67 ± 0.02</td>
<td>0.93 ± 0.02</td>
</tr>
<tr>
<td>28 S rRNA</td>
<td>pU</td>
<td>0.68 ± 0.06</td>
<td>1.07 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>pA</td>
<td>1.00</td>
<td>1.00</td>
</tr>
<tr>
<td></td>
<td>pC</td>
<td>0.67 ± 0.07</td>
<td>0.94 ± 0.03</td>
</tr>
<tr>
<td></td>
<td>pG</td>
<td>0.59 ± 0.03</td>
<td>0.76 ± 0.02</td>
</tr>
</tbody>
</table>
Table 2

Distributions of oligonucleotides in nucleolar and rRNA's from rat liver and the Novikoff hepatoma after digestion by pancreatic RNAse.

Fragments were separated by DEAE-Sephadex A-25 column chromatography (Chart 1) at neutral or acid pH, and the elution profiles were monitored by the absorbance at 260 nm for the rRNA's or at 254 nm for the precursors. The values are averages of 2 determinations.

<table>
<thead>
<tr>
<th>RNA</th>
<th>Oligonucleotide</th>
<th>Liver</th>
<th>Novikoff hepatoma</th>
</tr>
</thead>
<tbody>
<tr>
<td>45 S nucleolar RNA</td>
<td>A-Cp</td>
<td>9.4</td>
<td>9.2</td>
</tr>
<tr>
<td></td>
<td>G-Cp</td>
<td>12.5</td>
<td>12.2</td>
</tr>
<tr>
<td></td>
<td>A-Up</td>
<td>8.7</td>
<td>8.9</td>
</tr>
<tr>
<td></td>
<td>G-Up</td>
<td>4.7</td>
<td>4.6</td>
</tr>
<tr>
<td>28 S rRNA</td>
<td>A-Cp</td>
<td>5.6</td>
<td>5.5</td>
</tr>
<tr>
<td></td>
<td>G-Cp</td>
<td>10.5</td>
<td>10.3</td>
</tr>
<tr>
<td></td>
<td>A-Up</td>
<td>2.6</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>G-Up</td>
<td>5.1</td>
<td>4.8</td>
</tr>
<tr>
<td>18 S rRNA</td>
<td>A-Cp</td>
<td>6.7</td>
<td>6.2</td>
</tr>
<tr>
<td></td>
<td>G-Cp</td>
<td>9.8</td>
<td>9.8</td>
</tr>
<tr>
<td></td>
<td>A-Up</td>
<td>3.2</td>
<td>3.3</td>
</tr>
<tr>
<td></td>
<td>G-Up</td>
<td>3.8</td>
<td>3.9</td>
</tr>
</tbody>
</table>

Although previous reports had suggested that additional sequences were present in hepatoma 28 S rRNA and its nucleolar precursors (31, 32), the nucleotide analyses reported here indicate that at least some of these previously reported differences in nucleotide composition and oligonucleotide distribution probably were the result of nonuniform labeling rather than actual differences in the primary sequences. Earlier comparisons of polypyrimidines (23) would support this conclusion.

The phenomenon of "super" competition was observed with the sequential technique when reactants were allowed to reach room temperature and then were washed at 45°. This interference probably results from "tails" of unlabeled RNA that are sticking off the DNA and that then bind to adjacent regions when the temperature is lowered. Lucas and Ginsberg (16) reported that this interference was eliminated by RNase or RNA partially degraded by NaOH. The short time required for the interference to occur would indicate that it arose from RNA already bound to the surface. Once this nonspecific binding occurs, reheating to hybridization temperature does not remove it. In the experiments reported using the sequential method, the hybridization temperature of 45° was carefully maintained during the wash, and nonspecific interference or "super" competition did not arise.

The high degree of similarity between rRNA's from normal rat liver and those from the Novikoff hepatoma cell line that has been maintained by serial transplants for many generations suggests a high degree of fidelity in replication and transcription of the approximately 200 gene copies of rDNA and also a high degree of precision in processing these rDNA transcripts. This high degree of fidelity has also been demonstrated in HeLa cell rRNA and normal human tissues (1). Perhaps a model such as the one proposed by Howell (14) for Chlamydomonas, which is a variation on the master gene concept (7), might explain the lack of heterogeneity. Brown et al. (3) found, in 2 different species of Xenopus, that their 18 S and 28 S rRNA's were identical but that a difference existed in the 40 S precursor molecules. In contrast, the nonconserved regions of 45 S nucleolar RNA in Novikoff hepatoma cells appear to be maintained in oncogenesis and in subsequent cell growth.

REFERENCES


18. Matsuoka, T., Higashi, S., Gotoh, S., and Sakamoto, Y. Changes in the Nucleotide Compositions of Nucleolar 45 S RNA of Azote


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