Similarity of Ribosomal and Ribosomal Precursor RNA's from Rat Liver and the Novikoff Ascites Hepatoma¹

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SUMMARY

The 32P-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 32P-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. 32P-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 U2 and T1 RNase digestions did not reveal differences in the primary sequences of 28 S rRNA from normal and malignant tissues. In addition, 28 S rRNA-32P 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 32P-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 32P 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 32Pi (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 7000 x g for 10 min at 0°.

Normal rat livers were labeled by a single i.p. injection of 32P 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

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was extracted by a SDSphenol method at 20° or 65° (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 SDSphenol method (28) at 65° 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°.

**Isolation of DNA.** DNA was isolated by 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°, and nuclease-free Pronase (predigested at 2 to 5 mg/ml at 37° 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° and then was placed in a 37° 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° 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°) at a concentration of 100 µg/ml and 50 units/ml, respectively, for 1 hr at 37°. 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° until loaded on filters. The A280:A260 ratios of these preparations were 1.87.

**Loading DNA on Filters.** The DNA was denatured in 0.5 M NaOH for 0.5 hr at 37° to ensure denaturation and as an additional purification step (15). This solution was mixed with 300 ml of 6x 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° 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 Meis and Schlipper (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% (2x SSC; 5 mM morpholinopropane sulfonic acid, pH 7.0) in a volume of 0.3 ml at 45°. RNA samples were adjusted to the appropriate concentrations and percentage formamide, heated at 70° for 5 min, and then rapidly chilled (24). Then the reactants were allowed to reach 45° 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° in 30% formamide:70% (2x 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° in fresh buffer, then twice in 2x 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°) at concentrations of 20 units and 40 µg/ml, respectively, at 37° in 2x SSC for 0.5 hr. The filters were washed twice with 2x SSC, dried in the vacuum oven at 80°, 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°) 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 the nucleotides in RNA samples from the DNA bound to filters by Meijs and Schilperoot (9), with the use of formamide to lower the temperature of hybridization (2, 10, 20). Experiments were performed in 30% formamide:70% (2x SSC; 5 mM morpholinopropane sulfonic acid, pH 7.0) in a volume of 0.3 ml at 45°. RNA samples were adjusted to the appropriate concentrations and percentage formamide, heated at 70° for 5 min, and then rapidly chilled (24). Then the reactants were allowed to reach 45° and the DNA and blank filters were added.

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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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*A comparison of hybridization in 2x SSC, 65°, and 30% formamide:70% 2x SSC, 45°, was made using 28 S rRNA. Both conditions gave similar RNase-resistant percentage hybrids; however, the rate of the reaction in formamide at 45° was about one-half the rate at 65°. The percentage hybrid was also the same when intact or partially degraded (1 N NaOH, 90 sec, 0°, 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 T1 RNase followed by T2 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 in 7 M urea in 0.05 M Tris-Cl, pH 7.5 (total volume, 600 ml/gradient); 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 18S and 28S rRNA was competed with either unlabeled 28S or 18S rRNA. Both procedures demonstrated lack of significant competition with the other species of RNA (Charts 2 and 3). When 45S nucleolar RNA-32P was competed with 18S plus 28S 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 if 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 45S 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 18S 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 28S rRNA from both tissues demonstrated the specificity of hybridization and purity of the RNA molecules. This lack of difference in liver and hepatoma 18S rRNA was shown previously by Wikman et al. (31). In Chart 3, 28S 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 28S 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 18S rRNA from hepatoma cells and unlabeled 18S and 28S 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, 18S RNA; r28S, 28S 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.
and normal liver, and no significant differences were noted. In 3 other preparations of labeled and unlabeled RNA, no significant difference was found.

The $^{32}$P-labeled 28 S and 45 S nucleolar RNA's from hepatoma cells were competed with unlabeled RNA from hepatoma and normal liver. Both species were competed equally well by the liver and hepatoma RNA's (Charts 5 and 6). Repetition of this experiment with different preparations of labeled and unlabeled RNA produced similar results. The absolute level of competition was low, perhaps due to contamination from nonribosomal RNA or even due to $^{32}$P-labeled DNA. There were no significant differences between the nucleolar ribosomal precursor RNA's from hepatoma and liver as determined by simultaneous and sequential competitive hybridization.

Structural Analyses of $^{32}$P-labeled RNA's. Earlier studies on the nucleotide-$^{32}$P compositions of ribosomal and pre-ribosomal RNA's from rat liver and various tumors (6) reported large differences in the AMP and CMP content. In contrast, similar studies in which UV absorbance (6) or a nucleoside derivative method (22) was used suggest no significant differences. To explain the discrepant nucleotide-$^{32}$P compositions, the specific activities of labeled nucleotides were measured in rRNA's and their precursors. RNA's labeled under standard conditions were completely digested with venom phosphodiesterase-liberating 5'-nucleotides, the natural precursors for these RNA's. The amount of each nucleotide was determined with the Picker Nuclear nucleotide analyzer, and the $^{32}$P content was measured by electrophoresis at pH 3.5. Table 1 summarizes the specific activities of the nucleotides relative to adenylic acid and indicates there are significant differences in the degree of labeling for rat liver and hepatoma RNA's. While the hepatoma rRNA approaches uniform nucleotide labeling at 18 hr, that of the rat liver RNA was not uniformly labeled even after 24 hr.

Studies on the distribution of $^{32}$P-labeled nucleotides in short oligonucleotides, particularly dinucleotides, after pancreatic (6, 13, 27, 31, 32) or Tt RNase (30) digestion have also indicated sequential differences in ribosomal preribosomal RNA's. In nucleolar RNA's, the GU content was about 50% higher in malignant tissues (32), and a somewhat lower 30% differences has been found in 28 S rRNA (13, 27). To evaluate the effects of nonuniform labeling on the oligonucleotide distribution, the differences were reexamined by means of absorbance measurements. Chart 1 compares the separation of dinucleotides after complete digestion with pancreatic RNase. Table 2 summarizes the data for both ribosomal and preribosomal RNA's. Since no differences in GU content were observed, these RNA's appear to be the same from this point of view as well.
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 \(^{32}\)P-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 \(^{32}\)P-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>
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</tbody>
</table>

Purified RNA's were completely digested with venom phosphodiesterase, and the nucleotides were determined by UV absorbance after liquid chromatography, or by radioactivity after paper electrophoresis. Specific activities, which are tabulated relative to adenylic acid, are averages of 4 to 6 determinations.
Ribosomal and Precursor RNA's

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 unlabelled 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.

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