Base Compositions of RNA of Nuclear Fractions Obtained by Sequential Extraction with Saline Solutions

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SUMMARY

Nuclear preparations of the Walker 256 carcinosarcoma, Novikoff hepatoma, Morris hepatoma 7794, and Morris hepatoma 9108 were successively extracted with 0.15 M and 2 M NaCl solutions to obtain the “nuclear sap” and “DNA” or chromatin fractions, respectively; the residue fraction contains much of the nuclear RNA as well as other RNA of the nuclear ribonucleoprotein network (24). The RNA prepared from these extracts was characterized by density gradient sedimentation; nucleotide compositions were determined by optical density and $^{32}$P base analysis. The orthophosphate-$^{32}$P was injected 20 minutes before the animals were sacrificed.

The $^{32}$P base compositions of the RNA of the DNA-RNA fraction derived from the “DNA” or chromatin fraction differed from that of the nuclear residue fraction in their higher content of adenylic acid and lower content of guanylic acid. Although the UV base compositions of the DNA-RNA and nuclear residue fractions were similar, the former had a slightly higher content of adenylic acid. In the tumors, both the $^{32}$P base compositions of the RNA of the residue fractions and the DNA-RNA fractions differed from those of normal liver. In both, the values for adenylic acid were significantly lower than those found in normal liver. In the residue fraction, the guanylic acid content was lower in the normal liver than in the tumors. These results are in agreement with the data reported in earlier studies on isolated nuclei and nucleoli and indicate that in the tumors the base composition of RNA synthesized in the extranucleolar chromatin, as well as that of the nucleolar RNA, differs from that of the normal liver.

INTRODUCTION

Studies have been made in this and other laboratories on the base compositions of whole nuclear RNA of tumors and other tissues (10, 13, 16, 22) and on the base compositions of RNA from preparations of isolated nucleoli (11, 12, 14, 15, 21).

Remarkable differences have been found in the base compositions of the nucleolar RNA fractions of the tumors and other tissues (11, 15, 17, 21). Among the difficult problems in interpreting data on the whole nuclear preparations has been the fact that the nucleoli of tumors contribute a substantially larger proportion to the total nuclear RNA than the nucleoli of resting tissues, particularly the liver which has been generally used as a reference in such studies. It seemed particularly desirable to ascertain whether the contribution of the extranucleolar fraction differed in tumors and other tissues.

In attempts to utilize procedures for separation of the newly synthesized nucleolar RNA from that of the RNA of the chromatin, the procedure reported by Sibatani et al. (20) was modified so as to include appropriate buffers and polyvinyl sulfate in the two sequential extraction stages, i.e., with 0.15 M NaCl and 2 M NaCl (23, 27). With the exception of the difficulties of controlling the activity of the nuclear RNases (7, 26), these procedures were generally satisfactory for studies on the nuclei of liver cells, both normal and those treated with thioacetamide.

The procedures employed with the liver cells were of somewhat questionable utility when used with some transplantable tumors in view of the fact that in the tumor preparations, substantial amounts of cytoplasm adhered to the nuclear membrane. Since the presence of RNA in these adherent cytoplasmic tags might have affected the compositions of the fractions isolated by sequential saline extractions, the problems of analysis of the products in the nuclei of tumors might have been insurmountable in view of the problems attending the use of more severe extraction procedures (2, 6, 9, 10).

Fortunately for such studies, the Morris hepatomas have become available. As has been noted elsewhere (5), the nuclear products obtained from these tumors are morphologically the same as those of the normal liver with respect to purity and the absence of adherent cytoplasmic tags.

In the present study, two Morris hepatomas, i.e., hepatomas 7794 and 9108 were employed along with the Walker 256 carcinosarcoma and Novikoff hepatoma for comparative purposes. Significant differences were found between the tumors and normal liver with respect to the $^{32}$P base compositions of the RNA of the residue fraction which contains much of the newly synthesized nucleolar RNA (24, 25). As noted earlier (24, 25), this fraction also contains other nuclear products. In addition, significant differences were found in the base compositions of the newly synthesized RNA in the “chromatin” fraction extracted with 2 M sodium chloride; the differences in the base compo-
sitions of the RNA's of this fraction were not as great as those found for the residue fraction. In each instance, the adenyllic acid content of the RNA was greater in the liver than in the tumor. These results conform with the data reported in earlier studies on isolated nuclei and nucleoli (12, 15, 21).

MATERIALS AND METHODS

Animals. Male albino rats weighing approximately 175-250 gm were obtained from the Charles-Jones Co. (Houston, Texas). They were fed ad libitum on Purina laboratory chow. The Walker 256 carcinoma was transplanted subcutaneously, and the Novikoff hepatoma cells were transplanted intraperitoneally from ascitic suspensions.

The Morris hepatomas 9108 and 7794 were provided by Dr. Harold P. Morris. At 6-7 days after implantation of the tumors, rats bearing Novikoff hepatoma ascites were injected i.p. with 2 ml orthophosphate-$a^32P$ (carrier-free and adjusted to pH 7). Rats bearing the Walker and other tumors were injected intravenously with 1.5-2.0 ml orthophosphate-$a^32P$ via the jugular vein 20 minutes prior to sacrifice. The isotope was obtained from the Cheek-Jones Co. (Houston, Texas).

They were fed ad libitum on Purina laboratory chow. The animals were housed in metal cages in a temperature-regulated room with a 12-hour light/dark cycle. The rats were fasted for 24 hours prior to sacrifice. The animals were anesthetized with diethyl ether anesthesia.

Nuclear Preparations. The solid tumors were excised, placed in ice cold 0.25 M sucrose, and transferred to the cold laboratory (4°C) for further treatment. The tissue was minced, weighed, pressed, and homogenized in 2.0 M sucrose containing 3.3 mm calcium acetate (16 ml sucrose solution/gm wet weight of original tissue). The Novikoff ascitic fluid was harvested from the rats and immediately centrifuged at 12,000 x g (10,000 rpm, SS-34 rotor, Servall centrifuge) for 15 minutes. The supernate was discarded and the pellet containing the packed hepatoma cells was transferred to a Teflon-glass homogenizer. Care was taken to avoid contamination with the erythrocyte pellet. The Novikoff hepatoma cells were homogenized in the 2.0 M sucrose solution. The homogenates were centrifuged at 40,000 x g (17,000 rpm, #19 rotor, Spinco ultracentrifuge) for 75 minutes. The supernate was discarded and the nuclear pellets were subjected to sequential extraction with salt solutions. As noted earlier (9, 10), the nuclear preparations of the Novikoff and Walker tumors contain cytoplasmic contaminants. The nuclear preparations of the Morris hepatomas (5) were of a quality essentially undistinguishable from those of normal liver (Fig. 1).

Salt Fractionation. The nuclear pellet was initially suspended in 0.15 M NaCl (2 ml/gm containing 0.05 M Tris pH 7.6), 1 mM magnesium acetate, 100-200 µg polyvinyl sulfate/ml (24, 27). The suspension was centrifuged at 4000 x g for 10 minutes; the extraction of the pellet was repeated with a fresh volume of Tris-saline, and the supernates were pooled.

The pellet remaining after two extractions with Tris-saline was suspended in a small volume of Tris-saline (0.5 ml/gm) for transfer to a Teflon-glass homogenizer with a tightly fitting pestle. To obtain the "chromatin" or "DNA" fraction, the suspended pellet was then homogenized in 2 M NaCl (4 ml/gm) containing 100-200 µg polyvinyl sulfate/ml. The suspension was centrifuged at 40,000 x g (18,500 rpm, #30 rotor, Spinco) for 30 minutes. The supernate was saved for further treatment. The pellet was extracted a second time with a fresh volume of 2 M NaCl solution, centrifuged, and the second supernate was saved. The pellet remaining after two extractions in the 2 M NaCl is the residue fraction.

Preparation of RNA. The pooled supernate from the Tris-saline extractions was adjusted to 0.3-0.5% sodium dodecyl sulfate and homogenized with an equal volume of aqueous phenol solution consisting of phenol:meta-cresol:water (30: 9:4: v/v) and containing 0.1% 8-hydroxyquinoline. The suspension was heated to 65°C and the RNA was extracted as described previously (16, 25). The RNA was precipitated at 4°C by addition of 2.5 volumes of ethanol containing 2% potassium acetate. The precipitate was dissolved in a small volume of 0.1 M sodium acetate (pH 5.1) and stored at -15°C.

Fractionation of 2 M NaCl Extract. The RNA bound to DNA was separated from other RNA in the 2 M NaCl extract by addition of 0.5 volumes of ethanol to the extract (25). The precipitate of fibrous DNA and chromatin was removed and pressed free of entrapped liquid. The fibrous DNA and chromatin were placed in 1 M NaCl (1.3 ml/gm) containing 100-200 µg polyvinyl sulfate/ml and shaken 1 hr at 4°C in an Equipose shaker. The suspension was then centrifuged at 31,000 x g (16,000 rpm, Servall RC-2 centrifuge) for 10 minutes, and the supernate was decanted. The extraction with 1 M NaCl was repeated once. The RNA prepared from this material is referred to as the DNA-RNA fraction (25). The RNA in the supernate remaining after addition of one-half volume of ethanol to precipitate the DNA is referred to as the "deoxyribonucleoprotein-RNA" of the chromatin. To precipitate the RNA in this fraction, an additional one and one-half volumes of cold ethanol was added; the solution was stirred and allowed to precipitate 2-3 hr at 4°C. The precipitate was collected by centrifugation. The residue fraction was the pellet remaining after the extractions with 2 M NaCl.

For extraction of RNA, the procedure employing treatment with sodium dodecyl sulfate and hot phenol was used as previously described (8, 13, 18, 19).

Sucrose density gradients were prepared and fractionated as described previously (27). The UV and $a^32P$ base compositions were also determined (11, 27). For the hepatomas 9108, 7794 and Novikoff, the rapidly sedimenting RNA (45 S and above) was separated from the remainder of the RNA for analysis of $a^32P$ and the UV base composition. The values reported in the tables for these RNA's represent the compositions of the more rapidly sedimenting RNA's (Tables 2, 3).

RESULTS

Sedimentation Profiles of Nuclear RNA Fractions. The density gradient sedimentation profiles for the fractions obtained by the sequential extractions of the Walker tumor nuclear preparations are shown in Chart 1. Table 1 presents the yields of RNA, expressed as percentages of the total RNA recovered by successive saline extractions from the Walker tumor. The fractions which contained most of the RNA were the Tris-saline, DNA, and residue fractions. The distribution...
of optical density and radioactivity were similar in the other tumors studied.

The optical density pattern for the Tris-saline fraction is similar to that reported earlier for whole nuclear RNA. Little rapidly labeled RNA was present in this extract. The DNA-1 and DNA-2 RNA fractions obtained from the first and second extraction with 2 M NaCl contained smaller amounts of 6 S RNA and larger amounts of 18 S, 28 S and more rapidly sedimenting RNA. The first extraction contained the bulk of the RNA, i.e., approximately 95%. The radioactivity from the pulse label employed distributed in a broad peak in the 45 S, 55 S and >55 S regions. As noted previously (3), the DNA in the two extracts made with 2 M NaCl constituted 99% of the total DNA in the Walker tumor and liver nuclear preparation, respectively.

The profile for the RNA of the residue fraction is shown at the bottom of Chart 1. In addition to the 18 S and 28 S RNA in this fraction, there is a substantial amount of more rapidly sedimenting RNA. The pulse-labeled RNA was found largely in the normal liver had a markedly higher content of adenylic acid. The RNA of the DNA-RNA fraction in the normal liver was solubilized in 0.3% sodium dodecyl sulfate, 0.14 M NaCl, 0.05 M Tris, 0.15 M NaCl, 0.001 M magnesium acetate, pH 7.5. The DNA extracts contained the RNA soluble in 2 M NaCl, 100-200 μg polyvinyl sulfate/ml and subsequently precipitated by addition of one-half volume of ethanol. The deoxyribonucleoprotein extracts contained RNA soluble in the 2 M NaCl solution and precipitated by 2 volumes of ethanol after prior removal of the DNA fractions. The residue fraction contained the RNA remaining in the nuclear pellet after two successive extractions with Tris-saline and two successive extractions with 2 M NaCl. The residue was solubilized in 0.3% sodium dodecyl sulfate, 0.14 M NaCl, 0.05 M sodium acetate, pH 5.1 before treatment with phenol. The values in parentheses are standard errors for 3-7 experiments.

Base Composition of RNA in the DNA-RNA Fraction. For studies on the base composition of the RNA, the "rapidly sedimenting RNA" in tubes numbered 17-24 was separated from the remaining RNA's for analysis. As shown in Table 2, the 32P base composition of this RNA in the DNA fraction or 2 M NaCl extract was significantly different in the tumors as compared to values obtained for rapidly labeled RNA of this fraction from normal liver, particularly with respect to the content of adenylic acid. The RNA of the DNA-RNA fraction in the normal liver had a markedly higher content of adenylic acid. The pulse-labeled RNA was found largely in these regions.

### Table 1

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-saline RNA</td>
<td>45.1 (±1.0)</td>
</tr>
<tr>
<td>DNA-RNA</td>
<td>25.4 (±0.8)</td>
</tr>
<tr>
<td>DNP-RNA</td>
<td>5.4 (±0.5)</td>
</tr>
<tr>
<td>Residue RNA</td>
<td>25.0 (±0.5)</td>
</tr>
</tbody>
</table>

Average percent yield of RNA in nuclear fractions of the Walker tumor. The Tris-saline extract contained RNA soluble in 0.05 M Tris, 0.15 M NaCl, 0.001 M magnesium acetate, pH 7.5. The DNA extracts contained the RNA soluble in 2 M NaCl, 100-200 μg polyvinyl sulfate/ml and subsequently precipitated by addition of one-half volume of ethanol. The deoxyribonucleoprotein extracts contained RNA soluble in the 2 M NaCl solution and precipitated by 2 volumes of ethanol after prior removal of the DNA fractions. The residue fraction contained the RNA remaining in the nuclear pellet after two successive extractions with Tris-saline and two successive extractions with 2 M NaCl. The residue was solubilized in 0.3% sodium dodecyl sulfate, 0.14 M NaCl, 0.05 M sodium acetate, pH 5.1 before treatment with phenol. The values in parentheses are standard errors for 3-7 experiments.

<table>
<thead>
<tr>
<th>A</th>
<th>U</th>
<th>G</th>
<th>C</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morris hepatoma 9108</td>
<td>20.5</td>
<td>23.2</td>
<td>28.9</td>
<td>27.2</td>
</tr>
<tr>
<td>Morris hepatoma 7794</td>
<td>22.6</td>
<td>25.1</td>
<td>27.3</td>
<td>25.4</td>
</tr>
<tr>
<td>Walker 256 carcinoma</td>
<td>20.0</td>
<td>28.5</td>
<td>24.6</td>
<td>22.4</td>
</tr>
<tr>
<td>Novikoff hepatoma</td>
<td>22.6</td>
<td>28.6</td>
<td>29.5</td>
<td>21.4</td>
</tr>
<tr>
<td>Liver (24, 25)</td>
<td>30.2</td>
<td>22.6</td>
<td>24.2</td>
<td>23.9</td>
</tr>
</tbody>
</table>

Base composition of the rapidly sedimenting RNA in the DNA-RNA fraction. The precipitate was hydrolyzed in 0.3 N KOH for 18 hr at 37°C, adjusted to pH 7, and applied to a Dowex 1-X8 (formate) column. The 2'-(3')-mononucleotide peaks were lyophilized, taken up in 0.1 N HCl, and either counted in a liquid scintillation spectrometer or analyzed for optical density at 260 millimicrons as described previously (13, 26). The values for the Walker tumor are averages of 7 experiments; for the Novikoff tumor, 4 experiments; for the Morris hepatoma 9108, 3 experiments; and for the Morris hepatoma 7794, 2 experiments. The values for liver are for the whole fraction (24, 25). A, adenylic acid; C, cytidylic acid; G, guanylic acid; U, uridylic acid; r, A+U/G+C; UV, ultraviolet analysis.

The pulse-labeled RNA was found largely in these regions.
acid than that of the tumors studied. The values for the adenylie acid content of the Walker and Novikoff tumors were not significantly different from those of the two Morris hepatomas studied. For the other nucleotides, the ranges of the values for the tumors included the values for normal liver.

The UV base composition of the rapidly sedimenting RNA of the DNA-RNA fraction obtained by extraction with 2 M NaCl is presented in Table 2. The composition of this RNA in the tumors was different from that of the DNA-RNA fraction of the normal liver (24, 25). Presumably because of degradation by ribonucleases, rapidly sedimenting RNA was not obtained from normal liver in this fraction.

Base Composition of RNA in Residue Fraction. The $^{32}$P base composition of the rapidly sedimenting RNA of the nuclear residue fraction contained lower amounts of adenylie and uridylic acid in most of the tumors than was found in the DNA-RNA fraction (Table 3). The guanylic acid content was generally higher in the RNA from the tumors, indicating the presence of preponderant amounts of newly synthesized nucleolar RNA (11, 15, 21) in this fraction. Lesser differences were found in the UV base composition of this fraction by comparison with the DNA-RNA fraction, although the adenylie acid content was slightly lower in this fraction than in the DNA-RNA fraction. However, in the residue fraction the differences between the normal liver and the tumor with respect to $^{32}$P base composition were even more marked than those found in the DNA-RNA fraction extracted with 2 M NaCl. The adenylie acid content of the newly synthesized RNA of this fraction was significantly higher in the normal liver, and the guanylic acid content was significantly lower in the normal liver than that of the tumors studied.

The nucleotide composition of newly synthesized RNA of the DNP fraction of the Walker tumor (Table 4) was similar to that of the DNA-RNA and that of the RNA in the Tris-saline extract. Both of these RNA fractions contained only small amounts of radioactivity by comparison with the other fractions studied, i.e., the Tris-saline fraction and DNP fractions contained 7% and 5%, respectively, of the total radioactivity in the Walker tumor RNA fractions as compared to 32 and 56% in the DNA-RNA and residue fractions, respectively.

### DISCUSSION

An extensive series of studies in this and other laboratories (11, 15, 17, 21) has shown that the base composition of newly synthesized RNA of nuclei of tumors that have been transplanted for long periods and tumors that are "minimal deviation" tumors differs from that of normal and regenerating liver in the lower content of adenylie and higher content of cytidylie acid in the tumors (4, 5). As noted earlier (24, 25), the nucleolar elements are largely insoluble in the saline solutions utilized for the sequential extraction of saline soluble fraction and the deoxyribonucleoprotein fraction of the nucleus which is extracted with 2 M NaCl. Accordingly, it is not surprising that the base composition of the newly synthesized RNA of the "residue fraction" was markedly different from that of the liver and more like that of the nucleolus than that of the fraction obtained by extraction with 2 M NaCl (25). On the other hand, little information has been available on the base compositions of the newly synthesized RNA of the "chromatin" fraction extracted with 2 M NaCl from tumors and other tissues.

By the term "chromatin" most authors (3) refer to the deoxyribonucleoprotein complex of the cell nucleus with its associated components. Operationally, virtually none of the deoxyribonucleohistone is soluble in 0.15 M NaCl solutions and it is virtually all soluble in 2 M NaCl (3). In the most highly purified preparations of deoxyribonucleoprotein extracted from cells or nuclei with 2 M NaCl both RNA and acidic protein were present (3). Although the assumption of a relationship is reasonable, it has not been proven that these latter components are juxtaposed to DNA and histone in the nuclei nor has it been shown that they are either coordinatey or coevally bound to deoxyribonucleohistone. As the present study shows, however, the base compositions and labeling of the RNA of this fraction differs from that of other nuclear RNA fractions of...
the tumours as they did in normal and thioacetamide-treated liver. The fact that the RNA rich in adenylic and uridylic acids is present in the DNA-RNA fraction suggests that this RNA is either bound to or associated with the DNA, but it is possible that it was simultaneously extracted and precipitated.

Preparations of RNA from the chromatin fraction (2 M NaCl extract) of control rat liver and liver of rats treated with thioacetamide did not contain rapidly sedimenting RNA (25). In the present study it was found that the chromatin fraction of nuclear preparations of tumors extracted by similar procedures contained substantial amounts of rapidly sedimenting RNA. The preservation of this RNA in the tumor nuclei may be related to the low levels of ribonucleases in the tumors (7). In both the chromatin and residue fractions of the tumors, the major localization of the "newly synthesized" RNA was in the rapidly sedimenting regions. The RNA from the "chromatin" fraction (2 M NaCl extract) was different in base composition from that of normal or thioacetamide-treated liver (24, 25). In view of the difficulties that exist for fractionation of these rapidly sedimenting RNA's by chromatographic procedures (21), it has not been possible to determine whether the differences between the tumors and liver reflect qualitative differences in the genes being transcribed or quantitative differences (2) in the amounts of RNA being produced at the same gene loci, i.e., quantitative differences in rates of transcription. The critical need that exists in this area is for development of methods for fractionation of the rapidly sedimenting RNA's.

A number of reports of very rapidly sedimenting RNA, i.e., 55-80 S, have appeared (1, 22, 28, 29). In HeLa cells (22, 28, 29) labeled RNA was found which sedimented at approximately 75 S. Attardi et al. (1) reported the presence of 80 S RNA in immature duck erythrocytes. The data of this study show that there are at least two classes of the very rapidly sedimenting RNA, one associated with chromatin and the other with the residue fraction which contains the bulk of the nucleolar RNA. The $^{32}$P base compositions of the most rapidly sedimenting RNA fractions from HeLa cells (22) were similar to the RNA isolated in the present experiments from the chromatin fractions of the Walker and Novikoff hepatomas.

REFERENCES


Fig. 1. Nuclear preparation of hepatoma 9108. × 2,000.
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