MOLECULAR HYBRIDIZATION USED TO CHARACTERIZE
THE RNA SYNTHESIZED BY ISOLATED BOVINE
THYMUS NUCLEI*

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In vitro synthesis of RNA by nuclei isolated from various tissues has been observed in many laboratories.1–3 Several investigators have realized the usefulness of isolated nuclei for studying the regulation of RNA synthesis. The effects of nuclear proteins4 and various hormones5–7 for example, on the incorporation of various precursors into RNA have been analyzed recently. However, if the full potential of isolated nuclei as an assay system for studying the regulation of RNA synthesis is to be realized, then it is necessary that the characterization of the synthesized RNA be more complete. In the present studies, we have used the technique of DNA-RNA hybridization in an attempt to characterize the RNA synthesized in vitro by thymus nuclei. We have shown that the isolated thymus nucleus is capable of synthesizing a population of RNA molecules that is specific to thymus. The intracellular distribution of RNA species was also investigated and differences were found between nuclear RNA and cytoplasmic RNA. The isolated nucleus was shown to synthesize RNA similar to that found in the nucleus and in the cytoplasm.

Materials and Methods.—(1) Isolation of nuclei: Nuclei were isolated from thymus glands of freshly killed steers. Steer thymus was found to yield more reproducible preparations of nuclei than calf thymus. The glands were obtained at a local slaughter house and transported to the laboratory in ice. The extraction procedure was begun within 30 min after removal of the gland from the animal. Isolation of nuclei was performed by a modification of the isotonic sucrose procedure.8 All steps of the isolation were carried out at 0–4°C. Minced tissues were homogenized in 0.25 M sucrose, 6 mM MgCl₂, in a Waring Blender at 40 v for 3 min. The purity of nuclear preparations was checked by phase-contrast microscopy, by light microscopy of specimens stained with hematoxylin, and by electron microscopy. Nuclear preparations were contaminated 3–4% with whole cells. Representative examples of our nuclear preparations are shown in Figure 1.

(2) Incubation for RNA synthesis: Nuclei were incubated at a concentration of 0.1 ml of packed nuclei per milliliter of medium. This is equivalent to 0.45 μg DNA per milliliter. The medium was: 0.25 M sucrose, 6 mM MgCl₂, 2 mM MnCl₂, 0.02 M Tris HCl, pH 8.2. Penicillin G (50 units/ml) and streptomycin sulfate (50 μg/ml) were added to control bacterial growth. The mixture contained 20 μC/ml of uridine-5-H³ (Schwarz BioResearch, Inc.), spec. act. 20 c/m mole. Incubations were carried out for 30 min at 37°C, with back and forth shaking. RNA synthesis was assayed by removing 0.2 ml from the mixture and precipitating with 5% cold trichloroacetic acid (TCA). The precipitate was collected on Millipore filters (type RA, 1.2-μm pore size), washed, dried, and counted in a liquid scintillation spectrometer using a toluene–PPO–POPOP scintillation cocktail.9

(3) Extraction of RNA for characterization: RNA was extracted by the hot phenol–SDS method.10 An equal volume of a solution of 0.28 M LiCl₂, 2% sodium dodecyl sulfate (SDS), 0.02 M sodium acetate, pH 5.1, was added to the suspension of nuclei. The viscosity, due to the large amount of DNA present, was reduced by sonication for 2–3 min. The sonicated mixture was combined with an equal volume of redistilled phenol,
saturated with water, buffered at pH 5.1 with sodium acetate. The mixture was shaken at 60°C for 10 min, cooled, centrifuged, and the aqueous phase removed. The interphase was removed, sonicated for 2 min, and re-extracted as before. The resulting aqueous phase was combined with the initial aqueous phase, and extraction of the combined aqueous phases were repeated until little or no interphase material remained upon centrifugation. The final aqueous phase was combined with 2 vol of ethanol and the RNA precipitated at −20°C. The RNA was resuspended and digested with DNase (electr. purif. Worthington Biochemical Corp.) at 20 μg/ml for 30 min at 37°C. It was then digested with pronase (Calbiochem) at 20 μg/ml for 30 min at 37°C. The mixture was deproteinized by extraction with phenol. The RNA was ethanol-precipitated and resuspended in 0.01 M PO₄, pH 6.8. The RNA was further purified by stepwise elution from a 2.4 × 10-cm column of hydroxylapatite equilibrated with 0.01 M PO₄, pH 6.8 (Bio-Gel HTP, Calbiochem). Elution was effected by successive applications of: 25 ml of 0.01 M PO₄, 50 ml of 0.05 M PO₄, 50 ml of 0.2 M PO₄, and 25 ml of 0.5 M PO₄. As is seen in Figure 2, the purified RNA eluted in 0.2 M PO₄. The fractions containing radioactivity were combined, dialyzed free of PO₄, adjusted to 0.1 M KCl, and the RNA precipitated by adding 2 vol of cold ethanol. The RNA was then resuspended and dialyzed to 4 × SSC (SSC is 0.15 M NaCl, 0.015 M sodium citrate) and used for hybridization studies. The RNA so purified had a spec. act. of 200–250 cpm/μg, was free of DNA as judged by a negative diphenylamine test,11 and had an OD 260/280 of 2.0. RNA purified for use as competitor was purified in the manner described above.

(4) DNA-RNA hybridization: DNA-RNA hybridization was performed by the procedure of Gillespie and Spiegelman12 as modified by Church and McCarthy.13 Calf thymus DNA (Sigma) was further purified by digestion with pronase at 10 μg/ml for 30
min at 37° C. It was deproteinized by shaking with phenol at pH 8.0. The resulting aqueous phase was mixed with 2 vol of cold ethanol and the DNA spooled, dried, and resuspended in 0.1 × SSC. DNA was denatured and immobilized on membrane filters18 (Schleicher and Schuell, type B6). Disks of appropriate size were cut from the filter so that each disk contained 100 μg of DNA. Hybridization was carried out between 40 μg of H3-uridine-labeled RNA and 100 μg of DNA in 1 ml of 4 × SSC at 65°C. The kinetics of hybridization were determined. Maximum hybridization was obtained in 16 hr and no loss of hybrid was observed up to 24 hr.

Results and Discussion.—RNA synthesis in isolated nuclei: Conditions for the optimal rate of RNA synthesis by isolated thymus nuclei were investigated. The pH optimum for the reaction was 8.2. The reaction was stimulated by Mg ions with an optimum at 6 mM. Calcium ions were inhibitory. Incorporation was linear for 20–30 minutes. After 30 minutes of incubation, an aliquot from the reaction was precipitated with cold 5 per cent TCA and hydrolyzed in 0.3 N KOH for 12–16 hours. The hydrolysate was acidified with perchloric acid and the precipitate removed. The supernatant was neutralized, combined with a mixture of ribonucleotides, and analyzed by Dowex-1 formate chromatography14 (Fig. 3). Essentially all of the radioactivity was recovered from the column. The radioactivity recovered from the column was 87 per cent as uridine monophosphate (UMP), 6 per cent as cytidine monophosphate (CMP), and 7 per cent

Fig. 2.—Preparative hydroxylapatite-column chromatography.

Fig. 3.—Dowex-1 formate-column chromatography.
as nucleosides, probably uridine. An aliquot of the extracted RNA was analyzed by sucrose gradient centrifugation and found to be of low molecular weight (Fig. 4). The specific radioactivity along the gradient was relatively constant, which indicates that degradation had occurred during the extraction procedure.

Nuclei incubated under the above conditions were not able to incorporate P\textsuperscript{32}-orthophosphate into RNA. Incorporation of phosphate did occur into an acid-precipitable, alkali-labile material. Dowex-1 formate chromatography showed that most of the radioactivity released by alkali was in the form of orthophosphate. The nature of this product was not further investigated. However, it has been shown that isolated thymus nuclei are capable of incorporating orthophosphate into a phosphoprotein fraction, and this may account for the incorporation we have observed.

RNA synthesis by free nuclei, whole cells, and bacteria: Since our nuclear preparations were contaminated with whole cells by 3–4 per cent, it became necessary to investigate whether the observed RNA synthesis was due to free nuclei or, alternatively, to a few but very active whole cells. Liquid emulsion autoradiographs of the nuclear suspension were prepared after 30 minutes of incubation. It was found that 69 per cent of the free nuclei showed silver grains and 62 per cent of the contaminating whole cells were also labeled. The degree of labeling was not noticeably different in whole cells and free nuclei. Therefore it is reasonable to assume that not more than 3 or 4 per cent of the synthesized RNA could be due to whole cells.

A serious problem encountered in these studies was the contribution to RNA synthesis by contaminating microorganisms. It was found that the measurement of RNA synthesis by isolated nuclei without sterilization and the use of antibiotics became completely obscured by bacterial synthesis. For this reason the following precautions were taken. All glassware and solutions were autoclaved before use; penicillin G (50 units/ml) and streptomycin sulfate (50 µg/ml) were added to the incubation mixtures. Under these conditions 10\textsuperscript{4} to 10\textsuperscript{5} bacteria per milliliter were found in our incubation mixtures. In the presence of antibiotics this number dropped by 50 per cent during a 30-minute incubation. In order to estimate the contribution of bacteria to RNA synthesis, we deter-
mined the titer of bacteria necessary to influence the measured RNA synthesis. Bacteria from a nuclear suspension of mined the observed. Using must reach approximately 10⁸/ml before a significant contribution can be observed. Using the conditions previously mentioned, we were able to keep the bacterial titer in our incubations below 2 × 10⁸/ml, which is well below the titer at which bacterial synthesis can be observed.

**Table 1. Contribution of bacteria to nuclear RNA synthesis.**

<table>
<thead>
<tr>
<th>Sample</th>
<th>Cpm incorporated in 30 min/mg nuclear DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nuclei + residual bacteria</td>
<td>7,089</td>
</tr>
<tr>
<td>Nuclei + 1.4 × 10⁷ added bacteria/ml</td>
<td>6,811</td>
</tr>
<tr>
<td>Nuclei + 1.4 × 10⁸ added bacteria/ml</td>
<td>11,022</td>
</tr>
</tbody>
</table>

**DNA-RNA hybridization:** The specificity of the hybridization reaction was determined by measuring the amount of labeled RNA that could be bound to filters containing nonhomologous DNA. The results of these experiments are shown in Table 2.

**Table 2. Specificity of DNA-RNA hybridization.***

<table>
<thead>
<tr>
<th>Source of DNA†</th>
<th>Per cent of input RNA hybridized</th>
<th>Source of DNA†</th>
<th>Per cent of input RNA hybridized</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine</td>
<td>3.85</td>
<td>E. coli</td>
<td>0.60</td>
</tr>
<tr>
<td>Mouse</td>
<td>0.40</td>
<td>Sea urchin</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Blank filter†</td>
<td>0.39</td>
</tr>
</tbody>
</table>

* Reaction was between 40 μg H-labeled RNA and 100 μg of DNA in 4 × SSC for 18 hr at 65°C.
† Nonhomologous DNA's were the generous gift of Dr. Kirby D. Smith.
‡ Filter of the same size, prepared in the same manner as those with DNA but with no DNA.

It may be seen that significant binding occurs only on filters containing bovine DNA.

To determine whether the RNA synthesized by isolated thymus nuclei was characteristic of the population of RNA found in the thymus gland, experiments were conducted in which unlabeled RNA from various tissues was used to compete for binding sites on the DNA with H-labeled RNA synthesized by isolated thymus nuclei. Figure 5 shows the results of these experiments. RNA purified from thymus is the most efficient competitor, indicating a closer degree of similarity of labeled RNA with thymus RNA than RNA from other tissues. The fairly close similarity between RNA populations of the thymus and spleen is to be expected because of the lymphoid cells which are common to both tissues. These experiments confirm the results of McCarthy and Hoyer,‡ who found differences in the RNA populations discovered in different tissues, and furthermore show that a large proportion of the RNA made by isolated nuclei is similar to that found in thymus cells. Similar results have been obtained by Paul and Gilmour,§ who found that isolated chromatin synthesized some organ-specific RNA in vitro. In our experiments complete competition is not attained by thymus RNA. There are two possible reasons for this. The isolation procedure might cause the nuclei to synthesize new species of RNA that are not
normally present in thymus. This possibility cannot be ruled out, but this new species could involve only about 0.5 per cent of the RNA that is synthesized, for this is the percentage of the input RNA that continues to bind when close to maximal competition is reached. Alternatively, the differences between the labeled RNA and unlabeled thymus RNA may be quantitative, i.e., a large difference in the relative abundance of various populations may exist between total thymus RNA and that synthesized in 30 minutes. A quantitative difference of this sort should be expected since different populations of RNA in animal cells are known to have different turnover rates.19, 20 The concentration in a cell of a rapidly turning-over fraction at any one time could be low but would make up a large percentage of the fraction labeled with a 30-minute pulse. Therefore

the amount of whole-cell competitor RNA necessary to compete away this fraction would be extremely high and may not have been reached in our experiments. This second explanation probably accounts at least partially for the lack of complete competition.

Recent experiments by Shearer and McCarthy19 have shown different populations of RNA in the nucleus and cytoplasm. We have carried out experiments which confirm these results and indicate that isolated nuclei are able to synthesize RNA similar to that found in the nucleus and that found in the cytoplasm. RNA was prepared from liver and thymus nuclei and from liver and thymus cytoplasm. Cytoplasmic RNA was prepared from the supernatant resulting from a 1000 × g, 15-minute centrifugation of a 10 per cent w/v homogenate in 0.25 M sucrose, 6 mM MgCl₂, of the respective tissues. Figure 6 shows that thymus nuclear RNA is the most effective competitor. Since thymus

![Graph](image-url)
cytoplasmic RNA does not compete as effectively as nuclear RNA, it can be concluded that there is a population of molecules present in the nucleus that is not found in the cytoplasm. Blackburn and Klemperer\textsuperscript{21} have obtained similar results. They found that isolated rat liver nuclei synthesize RNA which can be competed far more effectively by nuclear RNA than by ribosomal RNA. Figure 6 also shows that liver nuclear RNA does not compete as well as thymus nuclear RNA. However, there is only a small difference between the two nuclear RNA's, indicating that the thymus nuclear RNA contains a few species not found in the liver nucleus but that many of the sequences in the two preparations are similar. This means that there is a greater degree of homology between the RNA extracted from nuclei of these two different cell types than between the nucleus and cytoplasm of the same cell. It may be that messages that are specific for nuclei are responsible for directing the synthesis of proteins of general metabolic significance, such as enzymes of nucleic acid metabolism or histones and the acidic nuclear proteins, as suggested by Shearer and McCarthy.\textsuperscript{19}

Liver cytoplasmic RNA is a relatively poor competitor for the RNA synthesized by thymus nuclei. The difference in competitive abilities is greater between liver cytoplasm and liver nuclei on the one hand than thymus cytoplasm and thymus nuclei on the other. This may indicate that the tissue-specific populations seen in Figure 5 may be cytoplasmic in origin. To show the similarity between liver and thymus nuclear RNA's more conclusively, it must be demonstrated that the competition seen in Figure 6 is for the same species of RNA. This same approach can be used to decide whether there are any RNA's in the cytoplasm that are not detectable in the nucleus. Figure 7 shows the results of an experiment in which various RNA’s were used in the presence of thymus nuclear RNA at a concentration which gave close to maximal competition. If the added RNA competed for different sequences in the labeled population than thymus nuclear RNA, one would expect to see additional competition in this mixing experiment. Such an additive effect is not observed, however, with either liver nuclear RNA or thymus cytoplasmic RNA. One must conclude, therefore, that liver nuclear RNA and thymus nuclear RNA have many sequences in common and both compete for the same fraction of labeled molecules. Relatively few sequences found in the thymus nucleus are not found in the liver nucleus. The difference that we have observed, although reproducible, is probably a maximum value. By electron microscopic observations, it is clear that the nuclear preparations have a small amount of contaminating cytoplasm. This contamination would enrich the liver nuclear RNA in poorly competing species.
Shearer and McCarthy \(^1\) were unable to find any RNA in the cytoplasm which was not also present in the nucleus. They used cultured cells which are rapidly growing and relatively unspecialized. We felt that the cytoplasm of a differentiated, stable cell such as the thymocyte might contain a population of RNA molecules that is accumulating slowly and is below detectable levels in the nucleus and in this sense could be said to be specific for cytoplasm. However, as can be seen in Figure 7, thymus cytoplasmic RNA does not add to the competitive ability of thymus nuclear RNA when the thymus nuclear RNA concentration is close to maximal competitive ability. Therefore we must conclude that if there is such a cytoplasmic RNA fraction, it is undetectable due to the fairly low specific activity of our labeled RNA, or that it is not synthesized in any appreciable amount during 30 minutes of incubation.

**Summary.**—RNA synthesis by isolated bovine thymus nuclei was studied. It was found that the possibility of bacterial contamination must be controlled in order to measure actual nuclear RNA synthesis. The RNA synthesized was found to be similar to that found in thymus tissue. A proportion of these molecules were found to be specific to the thymus gland. Some of the RNA molecules synthesized in vitro were found to be specific to the nucleus and were not found in the cytoplasm. No sequences could be detected in the cytoplasm that were not also present in the nucleus. A high degree of similarity was found between the nuclear RNA's from thymus and liver, while the RNA populations in the cytoplasm of these tissues had very few sequences in common.

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