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Characterization of the Ribonucleic Acid Synthesized in an Isolated Nuclear System from Rat Heart Muscle*

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ABSTRACT: Ribonucleic acid (RNA) synthesized by a purified nuclear preparation from rat heart muscle was examined by sucrose density gradient analysis and by nearest neighbor frequency studies of the bases. Examination of the RNA sedimentation profile after a short incubation period revealed some high molecular weight material. This was particularly so in the presence of a high concentration of ammonium sulfate. Nearest neighbor analyses showed that the species of RNA synthesized under conditions of low ionic strength had a base composition similar to ribosomal ribonucleic acid (rRNA) or its precursor. With ammonium sulfate at high ionic strength (0.6 M) the predominant species was deoxyribonucleic acid-like RNA.

Whereas the importance of DNA-dependent RNA polymerase (EC 2.7.7.6) is fairly well established in the genetic control of protein synthesis, and its properties have been extensively studied in bacterial and mammalian systems (Weiss, 1960; Hurwitz *et al.*, 1962; Chamberlin and Berg, 1962; Nakamoto *et al.*, 1964; Tsukada and Lieberman, 1964; Widnell and Tata, 1964a,b), the nature of the RNA synthesized by mammalian nuclear preparations has not yet been clearly defined.

Stimulation of mammalian nuclear RNA polymerase activity upon the addition of various salts, notably ammonium sulfate, to the assay medium was first described by Goldberg (1961). Recently it has been stressed by Breuer and Florini (1966) that the stimulatory effect of $(NH_4)_2SO_4$ may be due to increased activity of the primer component of the nuclear system. A similar conclusion was reached by Pogo *et al.* (1966), from their experiments involving regenerating liver cell nuclei. So far, it has been assumed on the basis of indirect evidence that the RNA produced under standard reaction conditions involving Mg^{2+} ions and low ionic strength is like rRNA and that in the presence of ammonium sulfate (with high ionic strength) the product more closely resembles complementary RNA. Furthermore, both Tata and Widnell (1966) and Liao *et al.* (1966) have suggested that differential synthesis

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of RNA in mammalian nuclei may be followed by studying the nearest neighbor frequency of the RNA synthesized under varying conditions.

It is clear that before definitive statements are made about selective gene transcription under conditions of hormonal stimulation or other influences, a more precise definition of the RNA synthesized during varying circumstances should be available. A similar objection applies to the suggestion that there might be two DNA-dependent RNA polymerases in liver nuclei (Widnell and Tata, 1964b). Indeed, the authors themselves offered no conclusions and suggested the use of nearest neighbor frequency analysis to further specify the nature of the RNA synthesized in the reactions carried out under conditions with and without high ionic strength.

The objective of this paper is to present data concerning the base composition of the RNA synthesized by a purified nuclear system from rat heart using the technique of nearest neighbor frequency analysis (Josse *et al.*, 1961). A similar approach has been taken by Widnell using a rat liver nuclear system and his results with labeled GTP-α-^{32}P[1] and ATP-α-^{32}P have been reported (Widnell, 1965; Widnell and Tata, 1966).

Materials and Methods

Materials. Female rats of the Sprague–Dawley strain weighing 180–200 g and maintained on Purina Chow *ad libitum* were used throughout. Nuclei from heart muscle were isolated in a pure state by minor modifications of the methods described in the papers by Widnell (1963) and Widnell and Tata (1964a).

Details of the Isolation Procedure. Five to ten rats were killed by decapitation and their hearts were excised and immediately plunged into a beaker containing ice-cold 0.32 M sucrose–3 mM $MgCl_2$. All subsequent procedures were performed at 0–4°. After the pooled hearts were weighed, the tissue was minced finely and twice passed through a Latapie tissue grinder which was fitted with a sieve of pore diameter 1.16 mm. The procedure was repeated using a sieve of pore diameter 0.95 mm. The tissue was then homogenized in three volumes of 0.32 M sucrose–3 mM $MgCl_2$ using a MSE homogenizer at slow speed (2000 rpm) for 45 sec and then at top speed for another 45 sec. The homogenate was filtered through two layers of nylon bolting cloth (110 mesh; Blaw-Knox Co., Chicago, Ill.) and rinsed with 10 ml of medium. The unfilterable residue was rehomogenized and filtered in order to obtain a maximum yield of nuclei. Aliquots (20 ml) of homogenate were diluted with 7 ml of distilled water and carefully layered over 17 ml of homogenizing medium and spun for 10 min at 700*g* using a swing-out bucket in an International refrigerated centrifuge. The pellet was suspended in a heavy sucrose solution containing 2.4 M sucrose–1 mM $MgCl_2$ and centrifuged at 20,000 rpm (40,000*g*) for 80 min in the SW 25.1 rotor of a Model L-2 Spinco ultracentrifuge. The volume of heavy sucrose solution used and its molarity are critical if the yield of nuclei is to be high and at the same time free from contamination. In our experience, the initial wet weight of tissue (in g × 3.8) gave the correct volume in milliliters of 2.4 M sucrose–1 mM $MgCl_2$ in which to suspend the crude nuclear pellet. The recovery of DNA in the purified nuclear pellet averaged 50–60% of that in the whole homogenate. A very pure preparation could be obtained by resuspending the pellet and centrifuging it in 2.2 M sucrose–1 mM $MgCl_2$ under the same conditions as the initial ultracentrifugation. This was not done for routine assays of DNA-dependent RNA polymerase activity. The final nuclear pellet which appears starch white in color was resuspended in 1–2 ml of 0.24 M sucrose containing 1 mM $MgCl_2$.

Nuclear preparations were studied by phase contrast microscopy and were found to be mainly undamaged and relatively free from contamination with myofibrils, red cells, and intact muscle cells. The DNA content of nuclei was estimated by Burton's (1952) modification of the diphenylamine method and RNA by the method of Fleck and Munro (1963).

RNA Polymerase Assays. Assays of DNA-dependent RNA polymerase activity in nuclear preparations were carried out essentially according to the methods of Weiss (1960) and Widnell *et al.* (1967). For the Mg^{2+}-activated reaction the incubation mixture contained in a final volume of 0.5 ml: 50 μmoles of Tris-HCl buffer (pH 8.5), 2.5 μmoles of $MgCl_2$, 10 μmoles of neutralized glutathione, 5 μmoles of PEP and 10 μg of pyruvate kinase, 3 μmoles of NaF, 0.4 μmole each of GTP, CTP, and ATP, 0.024 μmole of UTP-α-^{32}P (sp act. 200–300 μc/μmole) (Schwarz BioResearch Inc., Orangeburg, N. Y.), and 0.1 ml of nuclear suspension containing 100–200 μg of DNA. In nearest neighbor frequency studies, the incubation mixtures contained one of the α-^{32}P -labeled nucleotides and the remaining three unlabeled nucleotide triphosphates. The specific activity of the labeled nucleotide was adjusted to 200–300 μc/μmole from the original value of 600–890 μc/μmole.

The incubation mixture for the Mn^{2+}–$(NH_4)_2SO_4$-activated reaction contained in a final volume of 0.5 ml: 50 μmoles of Tris-HCl buffer (pH 7.5), 2 μmoles of $MnCl_2$, 300 μmoles of $(NH_4)_2SO_4$ adjusted to pH 7.5 with NH_4OH, 5 μmoles of PEP, 10 μg of pyruvate kinase, 0.4 μmole each of GTP, CTP, and ATP, 0.024 μmole of UTP-α-^{32}P with the same specific activity as mentioned above and 0.1 ml of nuclear suspension containing 100–200 μg of DNA.

All incubations were carried out in a Dubnoff shaker at 37° in duplicate or triplicate with suitable blanks containing 5 ml of 0.5 N PCA added at the zero time. The incubation time for the Mg^{2+}-activated reaction was 15 min and for the Mn^{2+}–$(NH_4)_2SO_4$ reaction was 45 min. Unlike Widnell and Tata (1964a,b), we did not preincubate the mixture in the latter reaction.

[1] Abbreviations used: ATP, GTP, UTP, and CTP, adenosine, guanosine, uridine, and cytidine triphosphates; AMP, GMP, UMP, and CMP, adenosine, guanosine, uridine, and cytidine monophosphates; PEP, phosphoenolpyruvate; PCA, perchloric acid.

Reactions were terminated by the addition of 5 ml of 0.5 N $HClO_4$ at 0°. Pure yeast RNA (1 mg) (type XI purified RNA, Sigma Chemical Co., St. Louis, Mo.) in aqueous solution at pH 7 was added as carrier. The precipitate was collected after standing for 1 hr at 0° by centrifugation at 2500 rpm (1000*g*) for 10 min in the Model PR-2 International refrigerated centrifuge and washed three times with 5 ml of ice-cold 0.2 N PCA and twice with ethanol–ether (3:1, v/v). RNA was then extracted from the precipitate with 2 ml of 10% NaCl in 0.05 M Tris-HCl (pH 7.5) containing 0.5 mg of carrier yeast RNA/ml at 100° for 30 min. The precipitate was reextracted for 15 min with 2 ml of the above solution and the pooled supernatants were cooled to 0°. The RNA was precipitated with two volumes of cold 95% ethanol (−20°) and after 2 hr at 0°, the RNA was pelleted for 10 min at 2500*g* and washed twice with cold 0.2 N PCA and then once with cold 95% ethanol. The dry RNA pellet was hydrolyzed with 0.3 N KOH for 18 hr at 37°. The hydrolysate was neutralized with PCA and the precipitate was removed by centrifugation. The hydrolyzed RNA was then subjected to column chromatography by the method of Katz and Comb (1963). The fractions were recovered by flash evaporation and counted in a dioxane-based solution using liquid scintillation spectrometry (Bray, 1960). The recovery of radioactivity varied from 98 to 104% in all instances. Further validation of the method was achieved by obtaining a similar base composition of the carrier yeast RNA in every determination. In 33 analyses the percentage values with standard deviations of the base composition of the yeast RNA used as an internal check were: AMP, 26.2% ±1.21; UMP, 22.3% ±1.13; GMP, 31.2% ±1.40; and CMP, 20.3% ±1.10. The base composition of the radioactive RNA was calculated by nearest neighbor frequency analysis according to the method of Josse *et al.* (1961).

Suitable aliquots of labeled RNA were also analyzed by sucrose density gradient centrifugation. The sucrose solutions contained 0.01 M KCl–0.01 M Tris-HCl (pH 7.5)–0.001 M EDTA (pH 7.5). Fractions were analyzed for radioactivity by drying a 0.2-ml aliquot onto a piece of Whatman 3MM paper (6.5 × 2.5 cm). The papers were washed eight to nine times in ice-cold 0.25 N PCA containing 0.9% sodium pyrophosphate, two times in absolute ethanol, two times in ethanol–ether (1:1), and finally with ether. They were then dried in air, inserted in polyethylene vials containing Bray's (1960) solution and counted by liquid scintillation spectrometry.

Results

The RNA synthesized by the Mg^{2+}-activated reaction appears to undergo marked degradation during incubation. At 2 min, however, some high molecular weight RNA is still present with sedimentation coefficients ranging from 18 to 50 S as judged by the position of the peaks of reticulocyte rRNA used as a marker.

In the presence of high concentrations of ammonium sulfate one observes by sucrose density gradient analysis a substantial amount of radioactivity in the heavy layers. Similar results were obtained by Widnell and Tata (1966) in a rat liver nuclear system. In contrast to the Mg^{2+}-activated reaction, therefore, there is considerably less breakdown of high molecular weight RNA.

The results of the nearest neighbor frequency analyses of the radioactive RNA synthesized in the above two reactions are given in Tables I and II. The order of the base frequencies with regard to proximity are quite different in these two reactions. Table III gives the base compositions of the RNA which were calcu-

TABLE I: Nearest Neighbor Frequency Analysis, Mg^{2+}-Dependent Reaction.

Radioactive Nucleoside Triphosphate	Percentage of Total ^{32}P Cpm Recovd in 2′3′-Nucleoside Monophosphate			
	UMP	AMP	CMP	GMP
UTP-α-^{32}P	28.4	12.1	26.8	32.7
	25.0	13.1	29.8	32.0
GTP-α-^{32}P	19.1	11.7	32.2	37.0
	19.6	13.6	30.2	36.6
ATP-α-^{32}P	18.3	21.3	23.6	36.8
	17.3	21.7	25.0	36.0
CTP-α-^{32}P	24.4	10.5	35.2	29.9
	22.0	11.5	35.8	30.7

TABLE II: Nearest Neighbor Frequency Analysis, Mn^{2+}–NH_4^+-Stimulated Reaction.

Radioactive Nucleoside Triphosphate	Percentage of Total ^{32}P Cpm Recovd in 2′3′-Nucleoside Monophosphate			
	UMP	AMP	CMP	GMP
UTP-α-^{32}P	30.5	19.3	27.7	22.6
	32.6	18.9	27.9	20.6
	31.0	18.3	25.9	24.8
GTP-α-^{32}P	34.7	27.3	10.5	27.5
	33.2	29.1	8.7	29.0
	31.0	26.0	12.4	30.6
ATP-α-^{32}P	21.6	25.2	24.8	28.4
	20.1	27.2	26.9	25.6
	21.0	26.1	27.1	25.8
CTP-α-^{32}P	29.0	19.8	26.8	24.3
	28.6	20.2	26.3	24.7
	28.4	19.1	26.5	26.0

TABLE III: Comparative Values Obtained for RNA Base Composition.

Source	Percentage of 2'3'-Nucleoside Monophosphate				Reference
	UMP	AMP	CMP	GMP	
Mg^{2+} reaction, heart nuclei	22.6	14.5	29.9	33.0	This study (nnf analysis)[a]
Rat liver ribosomal	23.0	19.0	25.0	33.0	Stirewalt and Rabinowitz[b]
28S RNA, rat heart	23.6	15.8	24.9	35.7	Posner and Fanburg (1966)
18S RNA, rat heart	33.9	16.2	21.5	28.0	Posner and Fanburg (1966)
Mn^{2+}–NH_4^+ reaction heart nuclei	29.9	24.5	23.4	22.2	This study (nnf analysis)[a]
Complementary RNA	28.6	28.4	21.4	21.6	Calculated from Chargaff's (1955) data for rat DNA

[a] nnf, nearest neighbor frequency. [b] W. S. Stirewalt and M. Rabinowitz, unpublished observations.

lated from these frequency values. It can be readily seen that the RNA synthesized in the Mg^{2+}-activated reaction is rich in guanine and cytosine and its over-all composition is very similar to that of 28S RNA. Of note is the somewhat low value for AMP compared to that obtained for total rRNA. Posner and Fanburg (1966) and I. G. Wool, W. S. Stirewalt, and A. N. Moyer (personal communication) have analyzed 28S RNA from rat heart muscle and consistently obtain a value of 15.4–15.8% for AMP. Whether any precursor of 28S and 18S RNA is synthesized or not cannot be deduced from these data.

With high concentrations of ammonium sulfate the RNA has a base composition similar to that of complementary RNA (Table III). The values for AMP and UMP are much higher than that for rRNA and the GC content is distinctly lower. Therefore, it is highly likely that a much larger segment of the DNA primer is being copied under these circumstances than in the absence of high ionic strength.

Discussion

Highly purified nuclear preparations from mammalian tissues provide a system for the assay of DNA-dependent RNA polymerase activity and possibly a means for evaluating the genetic control of protein synthesis. For instance, Tata and Widnell (1966) have shown the stimulation of nuclear RNA polymerase activity during the latent period of action of thyroid hormones and Liao *et al.* (1966) have assayed RNA polymerase activity in prostatic nuclei following the administration of androgens. That an increase in rRNA should precede increased protein synthesis in the cytoplasm is not entirely unexpected. However, of considerable importance is the mechanism by which differential synthesis of RNA is obtained and a knowledge of the species of RNA synthesized at different time intervals. Characterization of the RNA produced by the cell nucleus under varying conditions may be made by sucrose density gradient centrifugation, column chromatography, nearest neighbor frequency analysis, and hybridization studies.

Sucrose density gradient centrifugation analysis of the RNA synthesized in the polymerase reaction is not very helpful in identifying the various species of RNA because of the marked breakdown of RNA that occurs in the cell-free system. The resolution of the RNA peaks into distinct species is also dependent on divalent ion concentration and ionic strength. Artifactual aggregations of RNA with RNA, DNA, and other molecules may also occur.

The nearest neighbor frequency analysis method allows us to compare the base composition of the RNA synthesized in the nuclear system under varying conditions with known species of RNA obtained from the tissue in question. The variations in nearest neighbor frequency of the four bases have been used as a means of studying selective gene transcription under hormonal stimulation (Tata and Widnell, 1966; Liao *et al.*, 1966) and during inhibition with actinomycin D (Liao *et al.*, 1966).

In the Mg^{2+}-activated reaction the over-all composition of the synthesized RNA resembles that of rRNA and more particularly 28S RNA. However, there is also some similarity to the base compositions of 35S and 45S RNA as reported by Floyd *et al.* (1966), and the likelihood of ribosomal precursor RNA being synthesized in this reaction cannot be overlooked. Under conditions involving stimulation by NH_4^+ ions the product has a base composition very similar to complementary RNA. It is quite possible that more than one species of RNA is being synthesized in these reactions and that the over-all composition merely reflects that of the predominant species. Small amounts of RNA varying in composition from the predominant species may go undetected and caution must therefore be exercised in interpreting the variations in nearest neighbor sequences of the bases in RNA.

We have not yet confirmed our findings by hybridization studies. However, Blackburn and Klemperer (1966), using rat liver nuclear DNA have shown that about 4% of the RNA synthesized by nuclei in the

presence of Mn^{2+} and $(NH_4)_2SO_4$ form hybrids with homologous DNA compared to 1% hybridization under conditions of low ionic strength. The data of these authors and of Shenkin and Burdon (1966) seem to indicate that this DNA-like RNA is single stranded and represents a copy of one of the chains of DNA. So far, RNA–RNA hybridization studies according to the method of Hayward *et al.* (1966) have not yet been carried out in mammalian nuclear preparations with the object of identifying the different species of RNA synthesized in the DNA-dependent RNA polymerase system.

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