Vol. 96, No. 2, 1980 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
September 30, 1980 Pages 812-816

ON THE NATURE OF NUCLEAR ENVELOPE-ASSOCIATED RNA

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Received August 14,1980

SUMMARY

Nuclear envelopes were isolated from rat-liver nuclei. Nuclear envelope-associated RNA was isolated and hybridized to filter-bound DNA in the presence of competing RNA populations. Cytoplasmic RNA did not effectively compete for DNA binding sites, while nuclear RNA did. The results indicate a high degree of complexity for nuclear envelope-associated RNA, and are compatible with the idea that hnRNA may be processed after attachment to the nuclear envelope (or nuclear matrix).

INTRODUCTION

The nuclear envelope (NE)[1] is unique among intracellular membranes, consisting of a double-membrane and two singular components, the nuclear matrix and nuclear pores. The nuclear matrix confers structural rigidity upon the nucleus, and consists mainly of acidic protein (1,2) and associated RNA (3). The nuclear pores are complex structures which appear to provide hydrophilic channels connecting nucleoplasm with cytoplasm; they have a diameter of about 9 nm available for diffusion (4), and are thought to be the sites of nucleocytoplasmic exchange of macromolecules. In spite of advances in delineating these structural features, the role played by the nuclear envelope in controlling selective passage of macromolecules remains obscure. The ability to restrict passage of large molecules (such as proteins) depends upon the continuity of the NE, but the localization of specific proteins to the nuclear interior appears to arise from preferential binding (5). Of particu-

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[1]Abbreviations: NE, nuclear envelope; hnRNA, heterogeneous nuclear RNA; Agarose-UMP, Agarose 5'-(p-aminophenyl phosphoryl) Uridine 2'(3') Phosphate; TKM buffer, 50 mM Tris-HCl (pH 7.6), 25 mM KCl, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol; NP buffer, 2 mM $NaHCO_3$, 1 mM phenylmethylsulfonyl fluoride; TES buffer, 50 mM Tris-HCl (pH 8.4), 100 mM EDTA, 0.5% Sarkosyl; SSC buffer, 150 mM NaCl, 15 mM sodium citrate; rRNA, ribosomal RNA.

0006-291X/80/180812-05$01.00/0

lar interest is the possible role of NE in the metabolism of heterogeneous nuclear RNA (hnRNA) and transport of RNA to the cell sap. Rapidly-labeled nuclear RNA is preferentially attached to the nuclear matrix (3), and the attachment is not mediated by divalent cations (6), a fact which allows adventitiously-adsorbed cytoplasmic and nuclear RNA to be removed with the use of chelating agents. We have proposed that RNA may be translocated along the inner nuclear envelope (or nuclear matrix elements) in route to the cytoplasm (7), and others have suggested that nuclear RNA (at least rRNA) may be processed as it moves along the nuclear matrix (8). Understanding the conversion of nuclear RNA into cytoplasmic RNA requires an examination of the nature of the population of RNA associated with the NE. If the NE-associated RNA consists of nuclear RNA being "processed" to mature cytoplasmic RNA, then cytoplasmic RNA should not effectively compete for DNA sequences complementary to all the sequences present (in NE-associated RNA). Alternatively, if NE-associated RNA is processed RNA, transiently associated with the NE in route to the cytoplasm, then cytoplasmic RNA should effectively compete NE-associated RNA for DNA binding sites. We employed competition hybridization to examine the ability of cytoplasmic RNA to compete NE-associated RNA for binding sites on filter-bound DNA.

MATERIALS AND METHODS

Male, Sprague-Dawley rats with body weights between 300 and 400 g were used in these experiments. RNA was radiolabeled *in vivo* by i.p. injection of 4 mCi 5-^{3}H-orotic acid (20 Ci/mmol; New England Nuclear, Boston, MA) 30 min prior to sacrifice.

Preparation of RNase-free DNase. DNA ("RNase-free"; Worthington Biochemical, Freehold, NJ) was freed of RNase activity by chromatography on Agarose-UMP (Miles-Yeda Ltd., Rehovot, Israel) columns and subsequent treatment with prepared bentonite (Fisher Scientific, Santa Clara, CA), as described by Grady et al. (9).

Preparation of Nuclear Envelopes. Livers were removed and homogenized in 0.25 M sucrose-TKM buffer (50 mM Tris-HCl, pH 7.6; 25 mM KCl; 5 mM $MgCl_2$; 5 mM 2-mercaptoethanol) with a teflon-glass homogenizer. The homogenate was filtered through 100-mesh nylon bolting cloth, mixed with two volumes of 2.3 M sucrose-TKM buffer, and the nuclei were purified by sedimentation through a 2.3 M sucrose-TKM cushion (10). Purified nuclei were resuspended in NP buffer (2 mM $NaHCO_3$ plus 1 mM phenylmethylsulfonyl fluoride (PMSF)) and were allowed to swell on ice for 10 min. The nuclei were then pelleted by centrifugation, and resuspended in NP buffer. RNase-free DNase (prepared as described) was then added to 50 µg/ml, $MgCl_2$ was added to 0.1 mM, and digestion was allowed for 25 min at 37 C. The digested nuclei were then harvested by centrifugation at 17,500 rpm for 10 min in an SS34 rotor and Sorval centrifuge at 0 C. They were resuspended in NP buffer, RNase-free DNase was added to 25 µg/ml, $MgCl_2$ was added to 0.1 mM, and digestion was allowed for 5 min at 37 C. The digested nuclei were then harvested by centrifugation, and resuspended in and rinsed with NP buffer. The resulting crude envelopes were then resuspended in 20 mM Tris-EDTA (pH 7.6) by homogenization, and pelleted by centrifugation; resuspension and centrifugation were repeated. This technique effectively removes adsorbed ribosomes from the nuclear surface. The

nuclear envelopes were then resuspended in TKM buffer, collected by centrifugation, and resuspended in TKM buffer. The nuclear envelopes were then centrifuged at 500 rpm for 5 min in an SS34 rotor at 0 C; the supernatant was carefully removed, and the centrifugation was repeated. This procedure results in purified nuclear envelopes identical with those obtained from sedimentation through discontinuous sucrose gradients, as described by Harris and Milne (11).

Isolation of NE-Associated RNA. Nuclear envelopes were resuspended in extraction buffer (0.3% SDS; 100 mM NaCl; 50 mM sodium acetate,pH 5.2; 10 mM EDTA) and extracted for 10 min at 60 C with buffer-saturated phenol (recrystallized) containing 0.1% 8-hydroxyquinoline. The aqueous phase was decanted, and the phenol phase was reextracted with one-half volume of extraction buffer for 10 min at 60 C. The mixture was then cooled on ice, and one volume of chloroform:isoamyl alcohol (24:1) was added. The phases were separated by centrifugation, the aqueous phase was withdrawn and combined with the first, and the RNA was precipitated by addition of two volumes of 95% EtOH. The RNA was harvested by centrifugation, resuspended in SSC (150 mM NaCl, 15 mM sodium citrate), and reextracted two additional times. RNA was then quantitated by absorbance at 260 nm, and was used in hybridization reactions when it had an A_{260}/A_{280} ratio more than 1.95.

Competing RNA populations were extracted as above. Cytoplasmic RNA was extracted from the supernatant fluid of liver homogenates after they had been centrifuged at 10,000 rpm for 10 min at 4 C. Competing nuclear RNA was extracted from purified nuclei. Competing cytoplasmic and nuclear RNA populations had A_{260}/A_{280} ratios more than 2.0.

Preparation of DNA. Liver DNA was isolated from purified nuclei by a method derived from that of Endow et al. (12). Nuclei were resuspended in TES buffer (50 mM Tris-HCl, pH 8.4; 100 mM EDTA; 0.5% Sarkosyl). A pronase solution was prepared and auto-digested for 2 h at 37 C prior to use. Auto-digested pronase was then added to the nuclear suspension to a concentration of 200 µg/ml, and digestion was allowed for 3 h at 37 C. The DNA was then precipitated by addition of two volumes of 95% EtOH, and harvested by centrifugation at 12,000 rpm for 15 min at 0 C in an SS34 rotor. The crude preparation was then resuspended in SSC buffer, RNase T_1 and α-amylase (both from Worthington Biochemical) were each added to 100 Units/ml, and digestion was allowed for 2 h at 37 C. Auto-digested pronase was then added to 200 µg per ml, and digestion was allowed for 2 additional hours. The aqueous phase was then extracted twice with chloroform:isoamyl alcohol (24:1) and precipitated by addition of two volumes of 95% EtOH. DNA was then quantitated by absorbance at 260 nm and had an A_{260}/A_{280} ratio of 2.05.

Hybridization Reactions. Purified DNA was resuspended in 0.01 x SSC, and heated to 100 C for 10 min, and rapidly cooled. The denatured DNA was applied to 14-mm diameter filters (type HA, 0.45-µm pore diameter; Millipore Corporation, Bedford, MA) using gentle suction. Filters were then dried overnight at 60 C; they contained 44 µg DNA. Hybridization reactions were performed at 68 C for 18 h in 2 x SSC buffer also containing 0.1% SDS; total volume was 1 ml.

RESULT AND DISCUSSION

We isolated NE via a procedure which employs DNase digestion, and which yields NE associated with a substantial amount of nuclear RNA and nuclear matrix. Prerequisite to a study of NE-associated RNA is elimination of protease and RNase activities during NE preparation. Phenylmethylsulfonyl fluoride is effective in blocking protease activities (6). For strict elim-

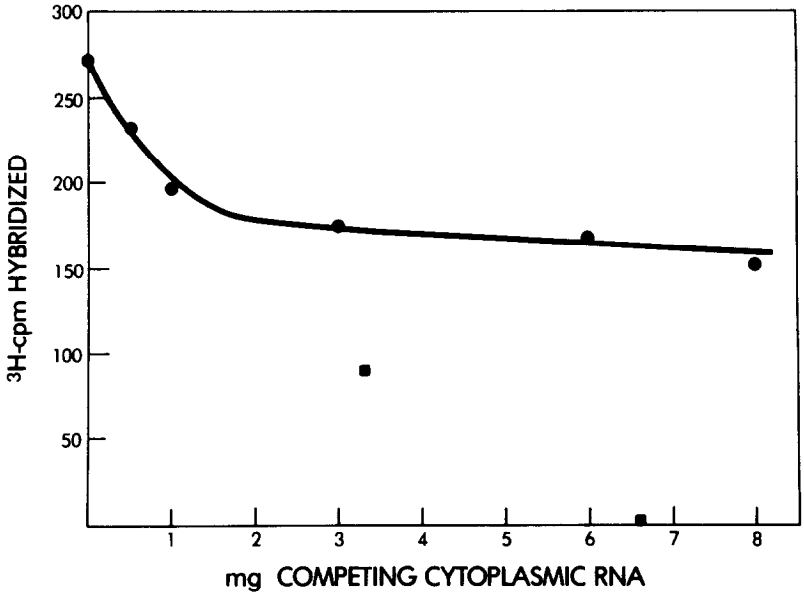

Figure 1. Hybridization of nuclear envelope-associated RNA with filter-bound DNA in the presence of unlabeled competing cytoplasmic or nuclear RNA. RNA was prelabeled in vivo, nuclear envelopes were purified as described, and nuclear envelope-associated RNA was isolated. 10.4 µg of nuclear envelope-associated RNA (specific activity 8300 cpm/µg) was hybridized to 44 µg of filter-bound rat-liver DNA in the presence of varying amounts of unlabeled cytoplasmic RNA (●) or unlabeled nuclear RNA (■). Hybridization reactions were for 18 h at 68 C in 2 x SSC also containing 0.1% SDS. Points represent means of duplicate or triplicate determinations. Binding to blank filters was 23.0± 1.4; this value was subtracted from experimentally obtained cpm to determine the ^{3}H-cpm hybridized.

ination of contaminant RNase activity from commercial DNase preparations, we employed the procedure of Grady et al. (9), using Agarose-UMP and bentonite. This procedure results in DNase free of detectable RNase and appears to be superior to treatment of DNase with iodoacetate (9). The composition of NE isolated using this technique was 69% protein, 18% phospholipid, and 13% RNA (DNA was not detected in NE preparations isolated under these conditions). Polyacrylamide gel electrophoresis (2.5% acrylamide-0.75% bis-acrylamide) using the method of Loening (13) showed that the rapidly-labeled NE-associated RNA migrated heterogeneously with a molecular size range of 28-16S (about 90% of the radioactivity was in this range), in good agreement with the results of Faiferman and Pogo (3).

Labeled NE-associated RNA (specific activity 8300 cpm/µg) was hybridized to filter-bound rat-liver DNA in the presence of increasing amounts of unlabeled cytoplasmic or unlabeled nuclear RNA (see legend to Figure 1). While nuclear RNA competed effectively against the NE-associated RNA, cytoplasmic RNA was not able to compete for most of the DNA binding sites for NE-associ-

ated RNA (Figure 1), even at ratios of nearly 800:1 (solubility limits precluded extension of the curve). Also of interest was the apparent complexity of the RNA population; approximately 0.3% of the RNA hybridized under these conditions, as opposed to 10 times this amount for total nuclear RNA (14,15) or for RNA transported from isolated nuclei *in vitro* (16).

It is clear from the results that the RNA sequences associated with the NE are not all present in cytoplasmic RNA, and that the population is much more complex than total nuclear RNA. These results support models which propose that nuclear envelope-associated RNA may be a "precursor" to cytoplasmic RNA (3) or that processing of nuclear RNA may occur during movement of RNA along the nuclear envelope or associated matrix elements (8).

ACKNOWLEDGEMENT

This research was supported by USPHS NIH grants CA-21141 and AM-19843

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