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ALTERED RESTRICTION OF NUCLEAR RNA DURING INCUBATION IN VITRO

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SUMMARY

Nuclei were isolated from rat liver and incubated in vitro in two commonly employed RNA transport assays. Released [^{14}C] RNA was isolated and hybridized with filter-bound DNA in the presence of competing cytoplasmic RNA. A significant portion of RNA which was transported to either medium was not represented in cytoplasmic RNA. These results indicate that the restriction of some sequences to the nucleus in vivo is not maintained in vitro.

INTRODUCTION

In eukaryotic cells, nuclear RNA populations are more complex than the cytoplasmic populations (1,2), and the majority of nuclear RNA is catabolized within the nucleus (3). Most RNA molecules appear to be synthesized as larger precursor molecules. Ribosomal RNA is synthesized as a 45S nucleolar precursor (4); the precursor is methylated (5,6) and becomes associated with 80S nucleolar RNP[1] (7,8). The 18S and 28S ribosomal RNA are drived from this 45S precursor and are found in nucleoplasmic particles (9,10) and subsequently in cytoplasmic ribosomal subunits. This maturation proceeds with fidelity in vitro (10) when ribosomal proteins are included in the incubation mixture. A recent report has demonstrated that the synthesis, processing, and appearance in the cytoplasm of rRNA proceeds with fidelity even when the nuclear envelope is grossly damaged (11).

The maturation of mRNA species has been more difficult to define. In general, heterogeneous nuclear RNA (hnRNA) appears to represent the

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[1]Abbreviations: RNP, ribonucleoprotein particle; hnRNA, heterogeneous nuclear RNA; snRNP, small nuclear RNP; STKM buffer, sucrose (as specified), 50 mM Tris-HCl (pH 7.6), 25 mM KCl, 5 mM $MgCl_2$, and 5 mM 2-mercaptoethanol; SSC, 0.15 M NaCl and 0.015 M sodium citrate; SDS, sodium dodecyl sulfate; EDTA, ethylenediamminetetraacetic acid; rRNP, ribosomal RNP; mRNP, messenger RNP.

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precursor to cytoplasmic mRNA (12,13); specifically, globin (14,15), albumin (16), and histone (17) mRNA are found in the nucleus as part of larger precursor molecules. Recent work has suggested a role for small nuclear RNP (snRNP) in this processing (18). Since only a small percentage of nuclear RNA is destined for transport to the cytoplasm, isolated nuclei might provide a potentially useful system to study mRNA maturation. Transported RNA could be obtained free from cytoplasmic RNA. Such in vitro systems have provided insight into the energetics of the nucleocytoplasmic RNA translocation process (19-21); however, the specificity of selection of sequences for transport is unknown. Since some RNA sequences are found only in the nucleus (22,23), a significant test of the reliability of the in vitro system is whether this sequestration is maintained; that is, are all sequences transported in vitro represented in cytoplasmic RNA. To address this question, we have isolated nuclei from rat liver, and incubated them in vitro in two commonly employed assays; the transported RNA was isolated and hybridized to filter-bound DNA in the presence of increasing amounts of cytoplasmic RNA.

MATERIALS AND METHODS

Livers were removed from male, Sprague-Dawley rats, homogenized in 0.25 M STKM buffer (0.25 M RNase-free sucrose, 50 mM Tris-HCl (pH 7.6), 25 mM KCl, 5 mM $MgCl_2$, 5 mM 2-mercaptoethanol), mixed with two volumes of 2.3 M STKM buffer, and nuclei were purified by sedimentation through a 2.3 M STKM buffer cushion (24). The purified nuclei were rehomogenized in 50 mM Tris-HCl (pH 8.4), 100 mM EDTA, and 0.5% Sarkosyl, and DNA was purified by the method of Endow et al. (25). DNA was then suspended in 0.01 x SSC (SSC is 0.15 M NaCl and 0.015 M sodium citrate), heated to 100°C for 10 min, and rapidly cooled on ice. Washed 14-mm filters (type HA, 0.45-µm pore diameter; Millipore Corporation, Bedford, Mass.) were added to the solution containing denatured DNA, and the mixture was incubated for 4 h at room temperature with continuous mixing. Filters were removed and rinsed individually with 3 ml of 0.01 x SSC plus 0.5% Triton X-100 and 0.5% sodium deoxycholate, and further rinsed with 2 x SSC containing 0.1% sodium dodecyl sulfate (SDS). Filters (150 mm^2) contained 11.4 µg DNA, as measured with the diphenylamine reaction (26), with color development overnight at room temperature. For labeling of transported RNA, rats were given 250 µCi [14C]-orotic acid (61 mCi/mmol; Amersham, Arlington Heights, Ill.) via i.p. injection 90 min prior to sacrifice and an additional 250 µCi 45 min prior to sacrifice, and liver nuclei were purified as described. Two in vitro assays were employed. Medium I was as described (20) with ATP at 2 mM, and incubation was for 10 min at 30°C; medium II was as described (27), and incubation was for 40 min at 30°C, with the addition of phosphoenolpyruvate at 10-min intervals. After incubation, the mixtures were cooled on ice, and the nuclei were pelleted by centrifugation. The supernatant fluid was mixed with two volumes of extraction buffer containing SDS (0.3% SDS, 100 mM NaCl, 50 mM sodium acetate (pH 5.2), and 10 mM EDTA) and extracted at 60°C for 10 min with buffer-saturated phenol (recrystallized) containing 0.1% 8-hydroxyquinoline. The aqueous phase was decanted, and the RNA precipitated by addition of two volumes of 95% EtOH. RNA was resuspended

in SSC and centrifuged at 16,000 rpm for 40 min to pellet glycogen. Extraction and precipitation were repeated at least twice. RNA was quantitated by absorbance at 260 nm, and was used in hybridization reactions when it had an A_{260}/A_{280} ratio more than 2.0. The specific activity RNA transported to medium I was 2120 cpm/µg, and that of total RNA extracted from medium II was 566 cpm/µg. However, RNA extracted from medium II also contained cytoplasmic RNA from the added cytosol. Control extractions were therefore performed (see legend to Figure 1), and they indicated that the actual specific activity of RNA transported to medium II was 1300 cpm/µg. Competing cytoplasmic RNA was extracted from the supernatant fluid of liver homogenates after a centrifugation at 10,000 rpm for 10 min at 4°C. Competing nuclear RNA was extracted from purified nuclei after a 10-min incubation at room temperature with DNase I (RNase-free, Worthington Biochemical, Freehold, N.J.) at 20 µg/ml in TKM buffer. Cytoplasmic and nuclear RNA preparations with A_{260}/A_{280} ratios of 1.9 or higher were used.

For hybridization reactions, filters containing 11.4 µg DNA were used with varying amounts of unlabeled cytoplasmic RNA (up to 3 mg). In hybridization reactions we used 5.9 µg of the [14C] RNA transported to medium I; or 17 µg of the [14C] RNA transported to medium II, accompanied by 24 µg of cytoplasmic RNA from the cytosol included in the medium. The reactions were carried out in 1 ml of 2 x SSC containing 0.1% SDS, for 17 h at 70°C. After hybridization, filters were rinsed with 30 ml of 2 x SSC containing 0.1% SDS, and radioactivity was assessed in Aquasol. The binding of [14C] RNA to blank filters was only slightly above background (44 cpm with RNA released to medium I and 49 cpm with RNA released to medium II, vs. 26 cpm for counter background), and was subtracted from the data from hybridization reactions.

RESULTS AND DISCUSSION

The results (Figure 1) demonstrated that a significant portion of the RNA which was transported to either medium I or medium II was not represented in cytoplasmic RNA species. In contrast, nuclear RNA completely blocked the hybridization reaction at concentrations greater than 1 mg (see Figure 1).

The biological ramifications of the results are complex. While the incubation conditions exert considerable influence on the types of RNA transported (28) (predominantly mRNP are transported in medium I and rRNP are transported in medium II), a lack of restriction occurred under both sets of conditions. One explanation of the results is that the repeated, intervening sequences of rRNA and mRNA precursors are normally catabolized in the nucleus, but somehow leak out *in vitro*. However, the specific energetics of transport *in vitro* (19,20) are difficult to reconcile with non-specific leakage. Furthermore, while rRNA processing proceeds with apparent fidelity in medium II, 32S rRNA accumulates intranuclearly in medium I (data not shown), as reported *in vivo* (29).

Another possibility is that snRNP, which often associate with larger nuclear RNP structures (18), may somehow be transported *in vitro* but not *in vivo*. In this regard, we have consistently found small RNA species in transported 45S RNP along with rapidly-labeled larger RNA (data not shown).

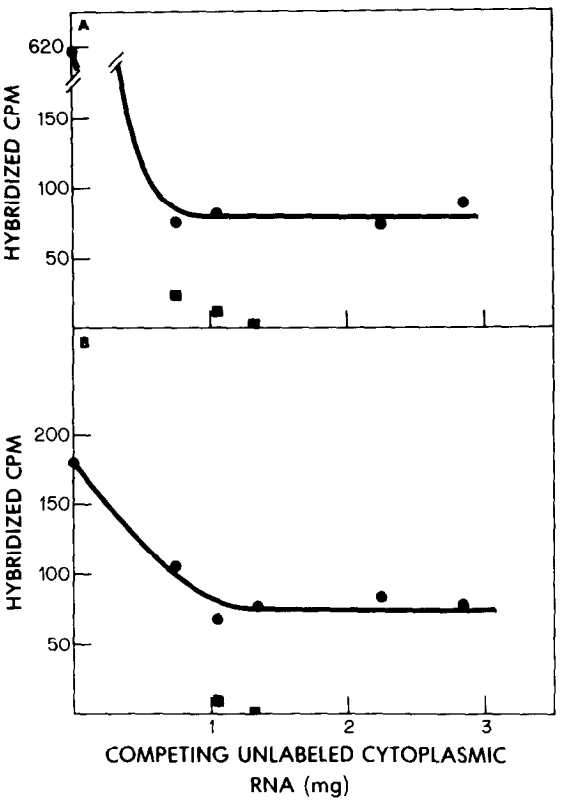

Figure 1. Hybridization of [14C] RNA transported in vitro to filter-bound DNA in the presence of competing unlabeled cytoplasmic RNA. Filters (150 mm^2) contained 11.4 µg DNA. A) 41.5 µg of total RNA extracted from medium II (overall specific activity of 566 cpm/µg) was hybridized to filter-bound DNA in the presence of competing cytoplasmic RNA. As noted, medium II contained cytosol; quantitative controls demonstrated that the cytosol used in the assay medium contributed 24 µg of RNA to the total of 41.5 µg. Correcting for this amount of cytoplasmic RNA, the amount of transported RNA used in the hybridizations reactions was 17 µg, with an approximate specific activity of 1300 cpm/µg. Binding to blank filters was 49 cpm with RNA released in medium II (counter background was 26 cpm), and this amount was subtracted from the hybridized cpm. B) 5.9 µg of RNA transported to medium I (specific activity of 2120 cpm/µg) was hybridized to filter-bound DNA in the presence of various amounts of competing cytoplasmic RNA. Binding to blank filters was 44 cpm with RNA released to medium I, and this amount was subtracted from the hybridized cpm. ■ points in A and B represent hybridized cpm when [14C] transported RNA competed with the specified amounts of nuclear RNA, after subtraction of binding to blank filters.

An interesting possibility is that some mRNA precursors are synthesized in the nucleus, processed to mature mRNA species, but not transported in vivo, and that incubation in vitro short-circuits this post-transcriptional control. Support for this contention can be derived from studies which demonstrate that the nuclear RNA populations of normal and carcinogen-treated cells contain the same sequences (30), even though additional sequences are transported to the cytoplasm in carcinogen-treated cells (23).

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