THE IN VIVO EQUIVALENCE OF A CELL-FREE SYSTEM FOR RNA PROCESSING AND TRANSPORT

Dorothy E. Schumm and Thomas E. Webb

Department of Physiological Chemistry, Ohio State University
College of medicine, Columbus, Ohio 43210

Received March 26,1974

SUMMARY

The equivalence of messenger RNA released (transported) from isolated rat liver nuclei to three selected media, with messenger RNA normally released to liver cytoplasm in vivo, has been evaluated by competitive DNA: RNA hybridization. Near normal nuclear restriction was exhibited by nuclei in media fortified with ATP, salts, spermidine and dialyzed cytosol. The RNA transport in the latter system was markedly inhibited by colchicine as was also the transport of RNA in vivo. Both nuclear restriction and sensitivity of the RNA transport to colchicine in media lacking spermidine and cytosol deviated significantly from the in vivo norm. The results emphasize the importance of establishing the in vivo equivalence in cell-free systems designed to study RNA synthesis, processing and transport.

The importance of post-transcriptional events in the regulation of the flow of genetic information from the nucleus to the cytoplasm of eukaryotic, cells has been widely recognized in recent years (1-9). The facilitate the identification of these controls, a cell-free system was developed (10-12) which permits a study of the release of RNA from isolated nuclei to a defined medium under conditions approximating the in vivo environment. In contrast to several comparable cell-free systems (13-15), it shows a requirement for spermidine and dialyzed cytosol. Furthermore, the cytosol-mediated modulation of RNA release in this system is due to the presence of cytoplasmic macromolecules. An attempt has been made to evaluate the reliability of three systems derived from rat liver (11,13,14). One criterion is the sequence homology of RNA released from resting liver nuclei to the various media, with that normally present in resting liver cytoplasm. A second criterion involved measurement of the effect of colchicine on RNA transport in the cell-free system, since evidence is also presented that this agent inhibits the nucleocytoplasmic transport of RNA in vivo. The rationale for the use of colchicine is the

observation that structures resembling microtubules reside in the vicinity of nuclear pores (16) and that colchicine disrupts microtubules (17).

MATERIALS AND METHODS

Nuclei: The nuclei were purified (7,10) from the livers of 250 gm male Sprague Dawley rats, 30 minutes after the intraperitoneal injection of 60 μCi of orotic acid -6-^{14}C (S.A.=323 μCi 1 mg). All injections were I.P. Each assay mixture for RNA transport contained 5 X 10^6 nuclei in the media described below.

Incubation media: Transport medium A (11,12) contained 50 mM Tris HCl (pH 7.6), 25 mM KCl, 2.5 mM $MgCl_2$, 0.5 mM $CaCl_2$, 0.3 mM $MnCl_2$, 5.0 mM NaCl, 2.5 mM Na_2HPO_4, 5.0 mM spermidine, 2.0 mM dithiothreitol, 2.0 mM ATP, 2.5 mM phosphoenolpyruvate, 35 units of pyruvate kinase, 300 μg of yeast RNA/ml, and 15 mg/ml of dialyzed cytosol protein, in a total volume of 15 ml. After adding nuclei the mixture was incubated at 30° for 30 minutes with addition of 1.0 mM phosphoenol pyruvate at 10 and 20 minutes (11,12).

Transport medium B (13) contained 5 mM $MgCl_2$, 1.0 mM ATP, 0.88 M sucrose, 0.025 mM KCl and 50 mM Tris HCl (pH 7.5). As specified (13), the incubations were performed for only 5 min at 20° C, then terminated by adding 1 vol. of cold buffer containing 5 mM $MgCl_2$, 50 mM Tris, 0.88 M sucrose and 25 mM KCl.

Transport medium C (14) consisted of 50 mM Tris-HCl (pH 7.5), 250 mM sucrose, 25 mM KCl, 2 mM KCl, 2 mM β-mercaptoethanol, 6 mM ATP and 4 mM $MgCl_2$. As specified (14) the incubations were performed for 25 minutes at 30°, then terminated by the addition of one volume of ice-cold buffer containing 50 mM Tris-HCl (pH 7.5), 250 mM sucrose, 25 mM KCl and 4 mM $MgCl_2$.

Following incubation the nuclei were collected by centrifuging the mixtures at 1,200 g for 10 minutes. Radiolabeled RNA was purified from the supernatant by use of chloroform-phenol (12).

Where indicated the nuclei were preincubated for 15 minutes at 0° in media A,B, or C with or without (controls) the addition of 1 mg/ml of colchicine

(i.e. 2.5 mM) prior to further incubation at 30° or 20° as specified above. The RNA in the nuclei-freed supernatants was precipitated in the presence of carrier yeast RNA with 50% trichloroacetic acid, dissolved in solubilizer and counted in liquid scintillant (cf. 11).

Competitive Hybridization: Unlabeled competitor RNA was obtained by incubating unlabeled nuclei in media A,B or C, then purifying the released RNA from the nuclei-freed supernatant with phenol-chloroform. Alternatively, 60 minute *in vivo* labeled (60 µCi of orotic acid-6-^{14}C/250 gm body weight) or unlabeled cytoplasmic RNA was purified directly from rat liver (19). For liquid hybridization the purified RNA fractions were dissolved in buffer (0.1 M Na_2HPO_4, 0.4 M NaCl, pH 7.2), then incubated for 24 hours at 60°C with 40 µg of DNA and the indicated amounts of unlabeled competitor RNA. After dilution with 4.0 ml of cold 0.14 M sodium phosphate containing 50% formamide the incubated samples were applied to 1.0 ml packed columns of hydroxylapatite (Biogel, HTP, DNA grade) for the separation of the single stranded and hybrid nucleic acids (20).

In vivo Effect of Colchicine: Following a 15 minute prelabeling of liver nuclear RNA with orotic acid-6-^{14}C (30 µCi/250 gm), rats either received saline (controls) or colchicine (4 mg/kg body weight) and the livers were removed for processing (18) 3 hours later. The specific activity of the cytoplasmic RNA, purified by phenol-chloroform extraction, was estimated by radioassay and A_{260} measurements.

RESULTS

Labeled RNA released during the first 30 minutes incubation in medium A (cf. Materials and Methods) of nuclei prelabeled *in vivo* for 30 minutes or less, is messenger-like by a number of criteria and is released in informosome-like nucleoprotein particles (11,12,21,22).

Shown in Fig. 1 are the results of competitive hybridization experiments in which RNA-^{14}C from normal liver cytoplasm was hybridized with liver DNA in the presence of increasing amount of unlabeled competitor-RNA released from

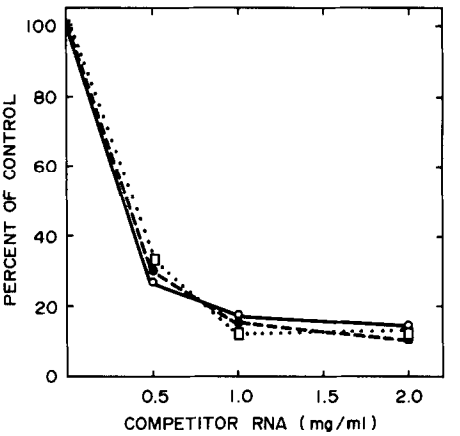

Fig. 1. DNA:RNA competitive hybridization between labeled RNA isolated directly from liver cytoplasm and increasing amounts of unlabeled RNA released from liver nuclei in vitro to medium A (-O-), B (--●--) or C (·· ■ ··). The hybridization mixtures contained 100 µg of RNA-^{14}C, 40 µg of DNA and the designated amounts of RNA released in the cell-free systems. The hybridization was carried out at 60° for 24 hours before analysis of the products by hydroxylapatite chromatography.

normal liver nuclei to media A,B, or C (cf. Materials and Methods). The similarity of the saturation curves indicates that essentially all (90-95%) of the families of RNA transcribed from reiterated genes and which are released to the cytoplasm in vivo, are also released by liver nuclei in the three incubation media. However, the results indicate little about the specificity of the release, and are not incompatible with nonspecific nuclear leakage, or even release of RNA by gross nuclear lysis.

A measure of the in vivo equivalence of RNA released in the cell-free systems involves the reverse of the above competitive hybridization procedure. Shown in Fig. 2 are the results obtained when RNA-^{14}C released from isolated nuclei to media A,B, or C is hybridized to DNA in the presence of increasing amounts of unlabeled cytoplasmic RNA. Although 78% of the labeled RNA released to medium A competed with (i.e. is similar to) the cytoplasmic RNA released in vivo, only 50% and 32% of the RNA released to media B and C, respectively, competes under the same conditions. As further indicated in Fig. 2 the

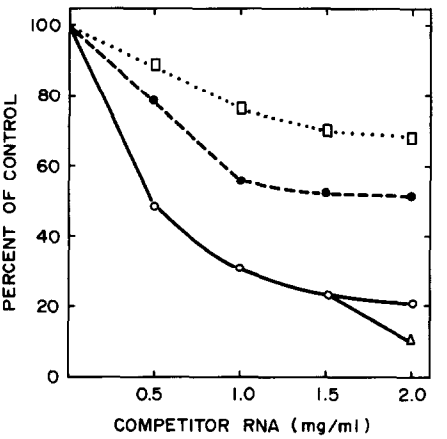

Fig. 2. DNA:RNA competitive hybridization between labeled RNA released from prelabeled liver nuclei to the 3 incubation media (symbols as for Fig. 1) and increasing amounts of unlabeled RNA isolated directly from liver cytoplasm. The results obtained by competing the RNA released to medium A against a mixture of 2.0 mg of unlabeled cytoplasmic RNA and 1 mg of the unlabeled RNA present in the nuclear preparation is also shown (-Δ-). Other conditions were as in Fig. 1.

Table 1. Inhibition of nuclear RNA transport by colchicine. In the in vivo experiments the rats received either saline (controls) or colchicine 15 minutes after the administration of orotic acid-6-^{14}C and the cytoplasmic RNA was isolated 3-hours later. The specific acitivity of the RNA was determined in terms of cpm/A_{260}. In the cell-free experiments prelabeled nuclei were pre-incubated at 0° for 15 minutes in media A,B, or C in the presence or absence of colchicine before assay for RNA transport. The results, which were reproducible to with 10%, are presented as percent of nuclear counts released in RNA. Further details are given under Materials and Methods.

System	Colchicine	S.A. (cpm/A_{260})	% Nuclear Counts Transported	Percent Inhibition
1) In vivo	-	4560		64
	+	1250		
2) Cell-free				
Medium A	-		4.5	77
	+		1.0	
Medium B	-		7.5	22
	+		5.9	
Medium B	-		31.2	5
	+		29.4	

disparity between the types of RNA released *in vivo* and to medium A is reduced to approximately 10% by applying a correction for RNA-^{14}C which is an adsorbed contaminant in the nuclear preparation. This RNA, which is released to all media from nuclei held at 0°, amount to approximately 1.0% of the total nuclear counts. The correction is obtained by testing the ability of the combination of unlabeled contaminant RNA and normal cytoplasmic RNA to compete with the RNA-^{14}C released from nuclei *in vitro*, and may be applied to all three systems.

As indicated in Table 1, colchicine reduces the accumulation of labeled RNA in the cytoplasm of liver cells by 64%. Pretreatment of the isolated nuclei with colchicine for 15 minutes at 0° in media A, before incubation at 30° reduced RNA-^{14}C transport by 77%. This pretreatment gave corresponding reductions of only 22% and 5% in media B and C, respectively.

DISCUSSION

On the basis of tests of nuclear restriction and response to an agent which inhibits RNA transport *in vivo*, the cell-free system (A) of Schumm and Webb (11,12) appears to provide a reliable model system for studying messenger RNA processing and transport in general and the phenomenon of nuclear restriction in particular. A correspondence of RNA transported *in vivo* and *in vitro* to with -in 10-20% seems most adequate since the dialyzed cytosol lacks certain potential regulatory components such as hormones or cyclic nucleotides. Nuclei in media lacking spermidine and dialyzed cytosol exhibit a distinct loss of nuclear restriction.

The RNA transport from nuclei to medium A was blocked by colchicine, which also blocks nucleocytoplasmic RNA transport *in vivo*. Colchicine does not inhibit RNA synthesis in Hela cells (23), but rather appears to affect RNA processing or transport. However, this inhibition cannot be unequivocally attributed to a disruption of microtubules (17) since colchicine appears to inhibit nucleoside transport in Hela cells independent of its effect on microtubules (23). Nevertheless, the fact that RNA transport in the cell-free system of Schumm and Webb (11,12) and in vivo are inhibited to the same extent by colchicine offers further support for the reliability of this system.

ACKNOWLEDGEMENT

This work was supported by U.S.P.H.S. Grant CA-12411.

REFERENCES

1. Drews, J., Brawerman, G. and Morris, H. P., Eur. J. Biochem. 3, 284 (1968).
2. Stevenin, T., Mandel, P. and Jacob, M., Proc. Nat'l Acad. Sci, U.S.A 62,
3. Shearer, R. W. and Smuckler, E. A., Cancer Res. 32, 339 (1972).
4. Garrett, C. T., Moore, R. E., Katz, C. and Pitot, H. C., Cancer Res. 33, 2469 (1973).
5. Levitan, I. B. and Webb, T. E., Science 167, 283 (1970).
6. Rizzo, A. J., Heilpern, P. and Webb, T. E., Cancer Res. 31, 876 (1971).
7. Rizzo, A. J. and Webb. T. E., Biochem. Biophys. Acta 195, 109 (1969).
8. Cooper, H. L., J. Biol. Chem. 244, 1946 (1969).
9. Chiarugi, V. P., Biochem. Biophys. Acta 179, 129 (1969).
10. Yu, L. C., Racevskis, J. and Webb, T. E., Cancer Res. 32, 2314 (1972).
11. Schumm, D. E. and Webb, T. E., Biochim. Biophys. Res. Commun. 48, 1259 (1972).
12. Schumm, D. E., Morris, H. P. and Webb, T. E., Cancer Res. 33, 1821 (1973).
13. Ishikawa, K., Kuroda, C. and Ogata, K., Biochim. Biophys. Acta 179, 316 (1969).
14. Chatterjee, N. K. and Weissbach, H., Arch. Biochem. Biophys. 157, 160 (1973).
15. Raskas, H. J., Nature (N.B.) 233, 134 (1974).
16. Schjeide, O. A., in Cell Differentiation, O. A. Schjeide and J. De Vellis eds. Van Nostrand Reinhold Co., N. Y. 1970 P. 169.
17. Wilson, L., Bamburg, J., Mizel, S. B., Grisham, L. M. and Creswell, K. M., Fed. Proc. 33, 158 (1974).
18. Shearer, R. W. and Smuckler, E. A., Cancer Res. 31, 2104 (1971).
19. Ono, T., Kawamara, M., Hyodo, M. and Wakabayashi, K., Gann 62, 31 (1971).
20. Goodman, N. C., Anal. Biochem. 52, 286 (1973).
21. Schumm, D. E., McNamara, D. J. and Webb, T. E., Nature (N.B.) 245, 201 (1973).
22. Schumm, D. E. and Webb, T. E., Biochem. J., in press.
23. Mizel, S. B. and Wilson, L., Biochem. 11, 2573 (1973).