DIFFERENTIAL EFFECT OF ATP ON RNA AND DNA RELEASE FROM NUCLEI OF NORMAL AND NEOPLASTIC LIVER

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SUMMARY

Nuclei isolated from differentiated normal liver, moderately differentiated Hepatoma 5123 and poorly differentiated Novikoff hepatoma have been compared with respect to ATP-dependence of messenger-like RNA release and resistance to lysis (DNA release) in a cell-free system containing homologous cytosol. The release of RNA from the nuclei of liver, Hepatoma 5123D and the Novikoff hepatoma was totally ATP-dependent, partially ATP-dependent and ATP-independent, repectively. The sensitivity of the nuclear RNA transport in the presence of ATP to beryllium nitrate, an inhibitor of a nuclear pore phosphatase, paralleled their ATP-dependence. Although RNA release from the nuclei of both liver and Novikoff hepatoma has an absolute requirement for cytosol proteins, the structural integrity of liver, but not Novikoff hepatoma nuclei in the presence of ATP, is dependent on macromolecules in the cytosol.

The release of messenger-like RNA from the isolated nuclei of both liver (1,2) and hepatoma (2,3) to surrogate cytoplasm is dependent both quantitatively and qualitatively, on soluble cytoplasmic macromolecules. RNA sequences released to fortified homologous cytosol are very similar to those released *in vivo*, while those released to cytosol from heterologous tissue are not (2,4). Labeled RNA released from nuclei prelabeled *in vivo* for 30 minutes has several properties in common with messenger, but not transfer or ribosomal RNA (1-5). Evidence was also presented that RNA release from normal liver nuclei is energy (ATP)-dependent (1-3) and the specific cytosol proteins are a necessary component of the incubation medium to prevent nuclear lysis (6,7). Other workers have reported an apparent ATP-independent release of RNA from tumor cell nuclei (8,9) which was accompanied by a parallel release of DNA (8).

The present comparative study was designed to clearly define the energy-

dependence of RNA release and the stability requirements in vitro of nuclei isolated from normal and the corresponding neoplastic cell.

MATERIALS AND METHODS

Preparation of nuclei and cytosol: Liver was obtained from 250 gm male rats and the ascitic form of the rapidly growing dedifferentiated Novikoff was carried intraperitoneally in 200 gm female rats of the Sprague Dawley strain. The moderately differentiated (cf. 3) Morris hepatoma 5123D was carried intramuscularly in the hind legs of Buffalo strain rats. The transplant generation times of the Novikoff and 5123D hepatomas was 7 days and 1.5 months, respectively.

Liver nuclear RNA was prelabeled in vivo for 30 minutes after the intraperitoneal injection of 40 μCi per 250 gm of [6-^{14}C] orotic acid (S.A. = 323 μCi/mg). Nuclear RNA of the Novikoff hepatoma ascites cells, or the 5123D hepatoma, after gently dispersing the loosely attached cells, was prelabeled by incubating the cells (10^7/ml) in Earles minimal essential medium (Schwartz-Mann, N.Y.) containing 2 μCi/ml of [5,6-^{3}H] uridine (S.A. = 39.3 Ci/nmole) for 20 minutes at 30° with gentle agitation. Before homogenization the cells were washed once with 0.9% NaCl, then once with 2.0 volumes of the appropriate homogenization medium specified below.

Nuclei were isolated from rat liver and the 5123D hepatoma by homogenization in 10 volumes of 2.3M sucrose-3.3mM calcium acetate as previously described (3,6). In order to disrupt the Novikoff hepatoma cells, it was necessary to suspend them in one volume of special homogenizing medium composed of 300mM sucrose, 2.0mM $MgCl_2$, 3.0mM $CaCl_2$, 10mM Tris-HCl (pH 8.0), 0.1% Triton X-100 and 0.5mM dithiothreitol (10), then shearing the cells with 20 strokes of a tight-fitting Dounce homogenizer. The homogenate was layered over 20 ml of 2.0M sucrose-3.3mM calcium acetate and the nuclei were sedimented by centrifuging for 60 minutes at 34,000g. After washing the nuclei with 1.0M sucrose-1.0mM calcium acetate at 3,000g x 5 minutes, the nuclei were resuspended in the latter medium for assay in the transport system.

The cytosol (105,000g supernatant) fraction was prepared (1,3) from a 1:3 homogenate of liver, or 5123D hepatoma. For the Novikoff hepatoma cytosol the ascites cells were swelled for 10 minutes at 0° in 2 volumes of TKM buffer (50mM Tris-HCl, pH 7.5, 25mM KCl, 2.5mM $MgCl_2$), concentrated to a thick slurry by centrifugation at 500g for 10 minutes, then lysed with a Dounce homogenizer.

All cytosol fractions were prepared by centrifuging the homogenate at 105,000g for 90 minutes, then dialyzing the supernatants against TKM buffer for 15 hours at 4°.

Cell-free system for RNA transport: The standard medium for RNA transport (1,2) contained 50mM Tris-HCl (pH 7.6), 25mM $MgCl_2$, 0.5mM $CaCl_2$, 0.3mM $MnCl_2$, 5.0mM NaCl, 2.5mM dithiothreitol, 300 μg/ml of yeast RNA, 10 mg/ml of dialysed cytosol protein from the same tissues as the nuclei and an energy supply consisting of 2.5mM ATP, 2.5mM phosphoenolpyruvate and 35 units of pyruvate kinase. After addition of nuclei (5 x 10^6 nuclei/ml) the mixture was incubated for the designated times at 30°.

Estimation of labeled RNA release: RNA was precipitated from the nuclei-freed supernatant in the presence of carrier yeast RNA with 0.1 volumes of 50% trichloroacetic acid then dissolved in solubilizer and counted in liquid scintillant (1).

Estimation of labeled DNA release: Liver nuclei with labeled DNA were prepared (11) from 250 gm rats 24 hours after the intraperitoneal injection of [^{3}H]thymidine (200 μCi/250gm; S.A. = 20 μCi/nmole) and 48 hours after partial hepatectomy to induce DNA synthesis. Prelabeled Novikoff hepatoma nuclei were obtained by collecting the tumor cells 18 hours after the injection of 200 μCi of [^{3}H]thymidine into the tumor-bearing rat. The nuclear isolation procedures for these tissues were identical to those outlined above. The DNA release to the transport medium was estimated by determining the trichloroacetic acid - precipitable counts in the nuclei-freed medium as described above for RNA release.

Protein determination: The protein content of the cytosol fraction was estimated by the Biuret reaction (12).

RESULTS

Differential energy-dependence: In the following experiments the transport of labeled RNA from nuclei prelabeled 30 minutes *in vivo*, was estimated during a 30 minutes incubation at 30°. Under these conditions the release of this labeled RNA, which has the properties of messenger (1-5), is completed within this incubation period. Detectable amounts of labeled ribosomal RNA are not released until the incubation time is in excess of 45-50 minutes due to the prolonged processing time of these components. Furthermore, energy is added only at the beginning of the incubation for messenger release, while maximal rRNA processing and transport requires the addition of further energy at 10 minute intervals (6,7).

Shown in Fig. 1(a) are the time-courses of the release of labeled RNA from liver, Novikoff hepatoma and 5123D hepatoma nuclei in the presence and absence of an energy source (i.e., ATP, phosphoenol pyruvate and pyruvate kinase). In order to avoid extensive overlap of the curves only the 20 and 30 minute points of the 5123D hepatoma are shown. The results show that the RNA transport from liver nuclei has essentially an absolute requirement for energy. In contrast RNA transport from the 5123D hepatoma and Novikoff hepatoma nuclei are 25% energy-dependent and totally energy-independent, respectively.

This marked difference between liver and hepatoma nuclei relative to the energy-dependence of RNA transport is further emphasized by the effect of beryllium nitrate on this process as illustrated diagrammatically in Fig.

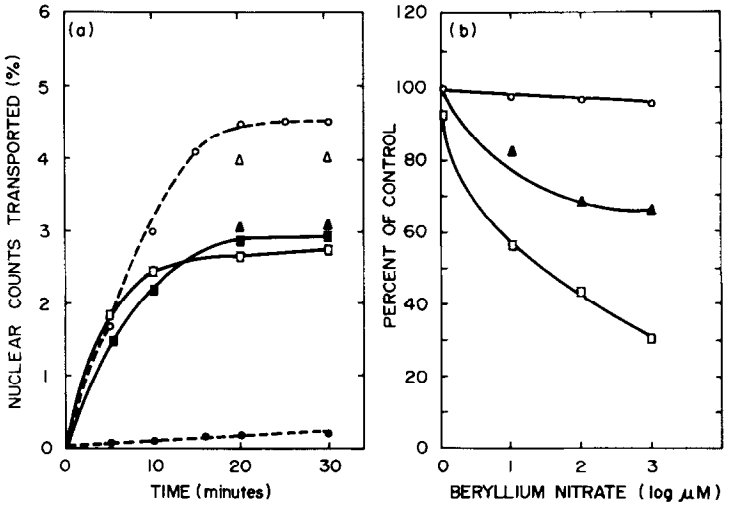

Fig. 1. Energy-dependence of RNA release from nuclei of normal and neoplastic liver to homologous cytosol. (a) RNA transport from liver nuclei with (--o--) and without (--●--) energy, the 5123D hepatoma with (Δ) and (▲) without energy (only the 20 and 30 minute points are shown) and the Novikoff hepatoma with (-□-) and without (-■-) energy. Duplicate experiments agreed to within 0.2% of the nuclear counts transported. (b) Ratio of the amount of RNA transported to medium containing beryllium nitrate, to that transported in the control medium, (both in the presence of energy) by nuclei from liver (-□-), 5123D hepatoma (-▲-) and Novikoff hepatoma (-o-). Nuclei were preincubated for 15 minutes at 0° in 1.0M sucrose - 1.0mM calcium acetate medium with or without beryllium nitrate, before adding the standard transport medium and further incubation for 30 minutes at 30°. Other conditions were as for Fig. 1(a).

1(b). This salt was tested as a possible inhibitor of RNA transport in view of its specific inhibitory effect on a nuclear pore phosphatase (13). Beryllium ions drastically inhibited the ATP-dependent release of RNA from the liver nuclei, had a partial effect on release from 5123D hepatoma nuclei, but had essentially no effect on transport from Novikoff hepatoma nuclei.

Differential nuclear stability: Previous studies (2,6,7,11) provided evidence for the existence in the cytosol fraction of proteins which, together with spermidine and calcium ions, are necessary for the stabilization of the membranes of liver nuclei. More specifically, a reduction in the concentration of cytosol protein in the assay below 3 mg/ml resulted in the unregulated release of RNA by gross nuclear lysis as determined microscopically; furthermore, this lysis was shown to be enhanced by ATP (6). The standard assay

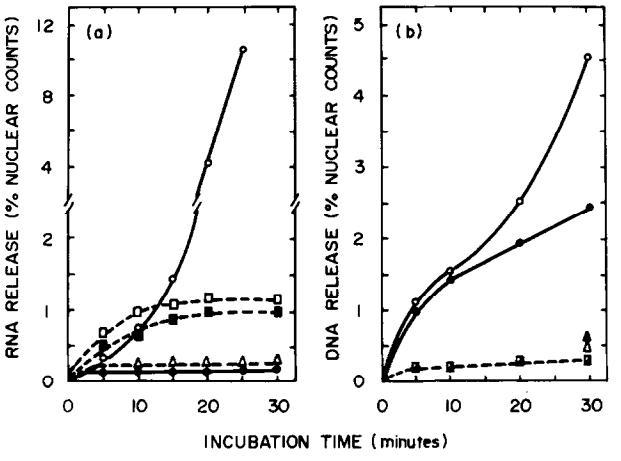

Fig. 2. Nuclear RNA and DNA release to transport medium modified to contain 1.0 mg/ml of cytosol protein. (a) Labeled RNA release from liver nuclei in the presence (-o-) and absence (-●-) of energy and from Novikoff hepatoma nuclei in the presence (-□-) and absence (-■-) of energy. The release of labeled RNA from Novikoff hepatoma nuclei in the complete absence of added cytosol protein but with energy present is also shown (-Δ-). (b) Labeled DNA release from liver nuclei in the presence (-o-) and absence (-●-) of energy and from Novikoff hepatoma nuclei in the presence and absence of energy (-◪-). DNA release from liver nuclei to medium containing 10 mg/ml of cytosol in the presence (Δ) and absence (▲) of energy is also shown. Other conditions were as for Fig. 1(a).

medium outlined under Materials and Methods supports the regulated transport of RNA from isolated nuclei in the absence of detectable nuclear lysis.

Fig. 2 shows the time course of release of (a) labeled RNA and (b) labeled DNA from prelabeled nuclei to incubation media containing 1.0 mg/ml instead of 10 mg/ml of cytosol protein. In the absence of energy, there is minimal RNA release from liver nuclei. Although the initial RNA transport in the presence of energy is also minimal due to a limited amount of transport factors, within 5-10 minutes an exponential release of labeled RNA ensues. Furthermore, as shown in Fig. 2(b) there is a limited early release of DNA from liver nuclei in the absence of energy and during the first 15 minutes in the presence of energy, followed by a linear late release of DNA in the presence of energy at incubation times greater than 15 minutes. The late,

but not the early DNA release correlates with the unregulated RNA release; some of the early DNA release may be due to the desorption of contaminating DNA from the outer nuclear membrane during incubation. The results in Fig. 2(b) also demonstrate that in the presence of 10 mg/ml of cytosol protein there is only minimal release of DNA from the nuclei, both in the presence and absence of energy; this release thus bears no relation to the controlled RNA release (cf. Fig. 2a).

The Novikoff hepatoma nuclei appear to be more resistant to nuclear lysis than do liver nuclei. As indicated in Fig. 2(a) the time course of release of labeled RNA from the hepatoma nuclei though much less at 1.0 mg/ml of cytosol protein than at 10 mg/ml (cf. Fig. 1), due to limiting transport factors, shows similar kinetics in both the absence and presence of ATP. Furthermore, there is no evidence of uncontrolled RNA release due to nuclear lysis, even when the cytosol protein concentration is reduced to zero. The ability of Novikoff hepatoma nuclei to maintain their structural integrity at low cytosol protein concentration (i.e., 1.0 mg/ml) is also reflected in the DNA release data shown in Fig. 2(b). The DNA release in this case is similar in magnitude as that exhibited by liver nuclei in standard transport medium.

DISCUSSION

The results of the present comparative study emphasizes certain biochemical and apparent structural differences between the nuclei of normal and neoplastic cells, which not only explain apparent discrepancies in the recent literature (8,9), but also have particular relevance to the problem of modified nuclear RNA restriction in the cancer cell (cf. 3).

The striking lack of an energy requirement for messenger-like RNA release by nuclei from the Novikoff hepatoma cells, is comparable to the apparent ATP-independence of RNA release from myeloma (8) and HeLa (9) cell nuclei and contrasts with the absolute energy requirement of liver nuclei. Furthermore, it is of interest that the energy-dependence of more highly differentiated tumors (e.g., the 5123D hepatoma) is intermediate between that

of liver and the Novikoff hepatoma. Despite the excellent correlation between sensitivity of the nuclei to beryllium ions and their energy-dependence for RNA transport, it can only be assumed at this time that a nonspecific phosphatase is involved in the ATP-dependent transport of informosomes through the nuclear pores.

Additional evidence for changes in the nuclear membrane upon neoplastic transformation comes from estimates of the structural stability of the nuclei as measured by DNA release, in medium containing low cytosol protein. In the presence of energy liver nuclei were stable in the complete transport medium (cf. Materials and Methods), but lysed when the cytosol protein concentration was reduced from 10.0 to 1.0 mg/ml. In contrast, Novikoff hepatoma nuclei were stable, i.e., showed no DNA release, when the cytosol protein in the complete transport medium was reduced to zero. The refractoriness of the Novikoff hepatoma nuclei to lysis is probably not characteristic of all tumor nuclei. For example, 5123D nuclei shows varying degrees of lysis at low cytosol protein concentrations (D. E. Schumm, unpublished observations). DNA release is also reported (8) to occur during the incubation of myeloma nuclei in medium containing 0.2 mg/ml of cytosol protein, although the lack of spermidine and calcium ion and the relatively high concentration of ATP in this system, as well as several other differences, precludes a strict comparison with the present study.

Despite the observed differences in energy-dependence of RNA transport and stability of nuclei, RNA transport from the nuclei of both normal and neoplastic cells depends qualitatively and quantitatively on the transport proteins in the cytosol fraction (2,3). This is most dramatically illustrated in Fig. 2(a) of the present study, which indicates that there is virtually no RNA release from the Novikoff hepatoma nuclei at 1.0 mg/ml or zero cytosol protein, either in the absence or presence of energy. While previous studies (2,3) suggested that differences in the spectrum of putative transport proteins could account for modified nuclear RNA restriction in the cancer cell,

i.e., the modified selection process which determines which messengers are to be transported to the cytoplasm, the differences identified in the present study may also contribute to this phenomenon. The possible relationship of altered RNA processing and transport to oncornavirus origin and evolution has also been emphasized (14).

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REFERENCES

1. Schumm, D.E. and Webb, T.E. (1972). Biochem. Biophys. Res. Commun. 48, 1259-1265.
2. Schumm, D.E., McNamara, D.J. and Webb, T.E. (1974). Nature (N.B.), 245, 201-202.
3. Schumm, D.E., Morris, H.P. and Webb, T.E. (1973). Cancer Res. 33, 1821-1828.
4. Schumm, D.E. and Webb, T.E. (1974). Biochem. Biophys. Res. Commun. 58, 354-360.
5. Schumm, D.E. and Webb, T.E. (1974). Biochem. J. 139, 191-196.
6. Yu, L.C., Racevskis, J. and Webb, T.E. (1972). Cancer Res. 32, 2314-2321.
7. Racevskis, J. and Webb, T.E. (1974). Eur. J. Biochem. 49, 93-100.
8. Stuart, S.E., Rottman, F.M. and Patterson, R.J. (1975). Biochem. Biophys. Res. Commun. 62, 439-447.
9. Chatterjee, N.K. and Weissbach, H. (1973). Arch. Biochem. Biophys. 157, 160-166.
10. Marzluff, W.F., Murphy, E.C. and Huang, R.C.C. (1973). Biochem. 12, 3440-3446.
11. Schumm, D.E. and Webb, T.E. (1975). J. Nat. Cancer Inst. 54, 123-127.
12. Gornall, A.G., Bardawill, C.J. and David, M. (1949). J. Biol. Chem. 177, 751-766.
13. Cutler, L.S., Chaudhry, A. P. and Montes, M. (1974). J. Histochem. and Cytochem., 1113-1117.
14. Gillespie, D. and Gallo, R.C. (1975). Science 188, 802-811.