NUCLEAR RESTRICTION OF NUCLEIC ACIDS IN THE PRESENCE OF ATP

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SUMMARY: Analysis of the release of RNA from isolated myeloma nuclei in vitro has shown that, in contrast to other systems, release of RNA was not ATP-dependent. Further, when ATP was added to the reaction mixture DNA was released concomitantly. Analysis of synchronized cells indicated that the release of DNA from nuclei in the presence of ATP could not be attributed solely to fragile mitotic nuclei.

Transport of newly synthesized RNA from the nucleus to the cytoplasm is an important step in the expression of genetic potential in eukaryotic cells. In recent years significant contributions have been made toward the understanding of the molecular maturation of RNA molecules in the nucleus of mammalian cells. The observations of poly A tracts (1) and methylation (2,3) in mRNA transported from the nucleus to the cytoplasm have renewed interest in development of a system in which the transcription, processing, selective stabilization and degradation, as well as transport of RNA can be studied in vitro. As early as 1959, the transport of RNA from mammalian nuclei was studied using isolated nuclei (4). Within the past four years several systems for assaying release of pre-labeled RNA from isolated nuclei, each with distinct and different advantages, have been developed (5,6,7,6). In all of these systems the release of RNA or RNP particles from nuclei appears to depend on temperature and on an exogenous source of ATP and/or an ATP generating system. Further study has revealed that the ATP requirement is not a requirement for energy, and release can be induced to varying degrees by the addition of nucleoside diphosphates (9) or EDTA (7). These results have led to the proposal that ATP stimulates release of RNA from isolated nuclei by its ability to deplete Mg^{++} (4,7) or to relax or dissociate some structure which retains RNA in the nucleus (6). The ratio of Mg^{++} to ATP has an influence on the efficacy of release as does the absolute

concentration of divalent cation (9). A further requirement for the presence of cytoplasmic protein(s) has been described (8).

In developing an in vitro system to study processing and nuclear transport of mRNA we have found that the effect(s) of ATP and other chelators on isolated nuclei is not selective for RNA release, nor is the release of RNA dependent on the presence of a chelator in the reaction mixture.

MATERIALS AND METHODS

Cell maintenance and isotopic labeling: MOPC-21 mouse myeloma tissue culture cells were maintained in Dulbecco's modified medium (GIBCO) supplemented with 10% fetal calf serum. Logarithmically growing cells were incubated 30 min with 5,6-^{3}H-uridine (Amersham/Searle, 44 Ci/mmole, 4 μCi/ml, 0.2 femtomoles/cell) when release of RNA was to be followed. When release of both RNA and DNA was to be followed MOPC-21 cells at 3 X 10^{5} cells/ml were labeled for 18 h with 2-^{14}C-thymidine (New England Nuclear, 54.7 MCi/mmole, 0.05 μCi/ml) and then pulsed for 30 min with 5,6-^{3}H-uridine immediately prior to isolation of nuclei.

Isolation of nuclei: Isotopically labeled cells were harvested by centrifugation at 4° C and washed with saline. Washed cells were resuspended in hypotonic buffer (10 mM NaCl, 3 mM $MgCl_2$, 10 mM Tris-HCl, pH 7.4) and lysed by Dounce homogenization. Isolated nuclei were washed with transport buffer (3 mM $MgCl_2$, 8 mM KCl, 4 mM NH_4Cl, 10 mM Tris-HCl, pH 7.6, 10 mM 2-mercaptoethanol, 250 mM sucrose) and examined by phase microscopy. Nuclei appeared free of cytoplasmic contamination.

Preparation of cytosol: Dialyzed cytosol was prepared from the 105,000 x g supernatant of MOPC-21 tissue culture cells following Dounce homogenization. The 105,000 x g supernatant was dialyzed overnight against transport buffer and frozen at -80° C in 1 ml aliquots. All manipulations were preformed at 0 to 4° C.

Induction of synchrony: MOPC-21 cells at 3 X 10^{5} cells/ml were labeled for 18 h with 2-^{14}C-thymidine and synchronized by two 2 mM thymidine blocks 6 h apart. Synchrony was followed by viable cell counts and incorporation of thymidine (Methyl-^{3}H) (New England Nuclear, 57 Ci/mmole) into samples of the cultured cells during a 1 h pulse. An aliquot of the synchronized cells was labeled with ^{3}H-uridine for 30 min at 3.5, 8, and 12 h following release from the second thymidine block corresponding to S, M, and G_1 phases of the cell cycle, respectively.

Assay procedure: One ml reaction mixtures were prepared containing 10^{6} nuclei, 0.3 ml dialyzed cytosol (0.15-0.18 mg protein as determined by the method of Lowry (7), equivalent to cytoplasmic protein from 10^{6} cells), and 4 mM ATP or EDTA in transport buffer. Each reaction contained approximately 2 X 10^{5} cpm of TCA precipitable ^{3}H-RNA. Release of isotopically labeled RNA from isolated nuclei at 37° C was monitored for 30 min. Buffer containing 10^{8} unlabeled MOPC-21 nuclei and 100 μl labeled nuclei were pelleted by centrifugation at 3000 x g for 5 min in a Sorvall RC2-B. The post-nuclear supernatant was withdrawn and carrier RNA added before precipitation with an equal volume of ice cold 10% TCA. The TCA precipitate was then collected on a Whatman GF/C filter. The filters were dried and counted by liquid scintillation. Percent release was calculated as the percentage of the total TCA precipitable radioactivity which appeared in the post nuclear supernatant.

RESULTS

We have examined the release of pre-labeled RNA from intact isolated MOPC-21 mouse myeloma nuclei under various conditions. As in other systems (5,6,7), release of pre-labeled TCA precipitable RNA was dependent on temperature and was greatly affected by the ratio of divalent cations, particularly Mg^{++}, to ATP in the system.

Pre-labeled RNA was rapidly released from MOPC-21 nuclei at concentrations of 3 mM Mg^{++} and 4 or 5 mM ATP in isotonic transport buffer, a ratio of divalent cation to chelator which is optimum or near optimum for release in other systems (6,7). RNA was also released in the presence of 4 mM EDTA or AMP-PNP supporting the observation that release of RNA by ATP is not an "active" transport phenomenon. Release of RNA was stimulated by a dialyzed cytosol fraction prepared from a 105,000 x g supernatant. These results are in agreement with those of Schumm and Webb (8).

In contrast to other *in vitro* assay systems, we found that if no chelator was added to a reaction mixture containing 3 mM Mg^{++} and dialyzed cytosol, release of pre-labeled TCA precipitable RNA paralleled release stimulated with 4 mM ATP or EDTA (see Figure 1). Addition of 0.5 to 1 mM Ca^{++} to the reaction mixtures to stabilize the nuclear envelope did not alter the release pattern either with or without added chelator (data not shown). Further, the absolute concentration of Mg^{++} influences the release of pre-labeled RNA from isolated MOPC-21 nuclei. When release of RNA was assayed in the presence of increasing concentrations of Mg^{++}, there was a progressive decrease in percent RNA release when no chelator was present. However, RNA was released from isolated nuclei when ATP was present with Mg^{++} in a ratio of 4:3 until the absolute concentration of Mg^{++} was extremely high (data not shown). At 8 mM Mg^{++} without added chelator, release of RNA at 37° C was precluded and this reaction mixture was chosen as a negative control.

Since our results indicated that release of pre-labeled RNA from MOPC-21 nuclei at 3 mM Mg^{++} was not dependent on the presence of a chelator in the re-

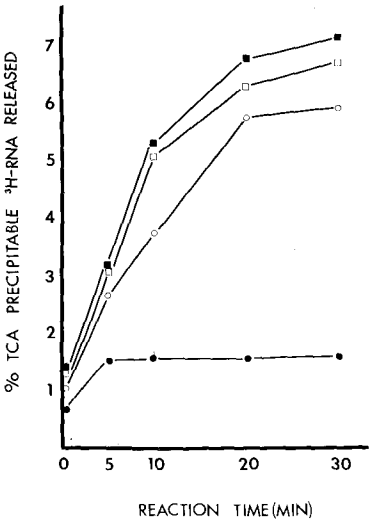

Figure 1.
Release of TCA precipitable ^{3}H-RNA from isolated nuclei. MOPC-21 cells were labeled with 5,6-^{3}H-uridine for 30 min immediately prior to isolating nuclei. Release is shown in the presence of ■—■, 4 mM ATP; □—□, no additions; ○—○, 4 mM EDTA; ●—●, 8 mM $MgCl_2$.

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action mixture, as appeared to be the case under similar conditions in other systems, we have explored some physiological parameters of this system. To monitor the biochemical integrity of the nuclear membrane a double-label procedure to assay the release of both RNA and DNA was initiated. MOPC-21 cells at 5 X 10^5 cells/ml were labeled for 18 h with ^{14}C-thymidine and then pulsed for a 30 min period with ^{3}H-uridine immediately prior to isolation of nuclei. Steady state labeling of the RNA was insured by monitoring incorporation of ^{3}H-uridine into TCA precipitable material. Incorporation of ^{3}H-uridine at 0.2 femtomoles per cell was found to be linear over a 60 min period (data not shown). Results of such double-label release experiments indicated that when ATP or EDTA was present in the reaction mixture, significant quantities of TCA precipitable ^{14}C-thymidine as well as ^{3}H-uridine were released to the post-nuclear supernatant. Figure 2 shows the change in ratio of released RNA to DNA with time. When EDTA was present, the ratio of RNA to DNA remained constant indicating that the nuclear envelope was allowing both RNA and DNA to pass through. Addition of ATP to the

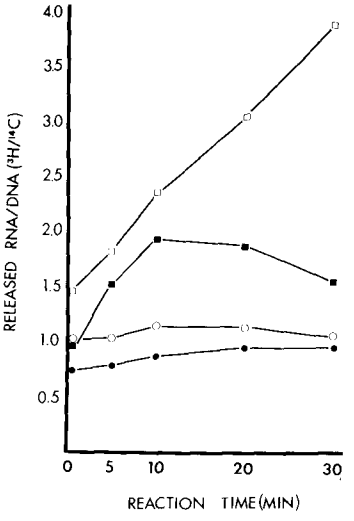

Figure 2.
Ratio of released ^{3}H-RNA to released ^{14}C-DNA. MOPC-21 cells at 3 X 10^{5} cells were labeled for 18 h with 2-^{14}C-thymidine and then pulsed for 30 min with 5,6-^{3}H-uridine immediately prior to isolation of nuclei. Composition of reaction mixtures: ■—■, 4 mM ATP; □—□, no additions; ○—○, 4 mM EDTA; ●—●, 8 mM Mg^{++}.

reaction mixture stimulated selective release of RNA for only the first ten minutes of incubation (ratio of RNA to DNA increased) before significant quantities of DNA were released. At 20 min when 6.5% of the total TCA precipitable ^{3}H-uridine had been released, 4.7% of the total TCA precipitable ^{14}C-thymidine had been released in the presence of 4 mM ATP. The released ^{14}C-thymidine labeled material was stable to digestion with alkali. When no chelator was present, the ratio of released RNA to DNA increased with time indicating that RNA was differentially released. The reaction containing 8 mM Mg^{++} showed no time dependent release of RNA (see Figure 1) and the ratio of released RNA to DNA did not change indicating that there was release of neither RNA or DNA. The percent release of RNA for each reaction mixture was analogous to that shown in Figure 1.

At any one time in a randomly dividing culture approximately 2-3% of the cells are in some phase of mitosis. To determine whether the release of DNA resulted from disruption of mitotic nuclei, cells were synchronized and release

of both RNA and DNA assayed during various phases of the cell cycle. MOPC-21 cells at 3 X 10^5 cells/ml were pre-labeled with ^{14}C-thymidine as before and synchronized by double thymidine blockage (10). Synchrony was followed by incorporation of ^{3}H-thymidine into samples of the culture, viable cell counts and percent mitotic figures observed. Correlation of cell counts and the percent cells in mitosis with incorporation of thymidine into TCA precipitable material indicated that most of the cells were synchronized during the first mitotic wave.

Figure 3 shows the percent release of pre-labeled RNA from nuclei isolated

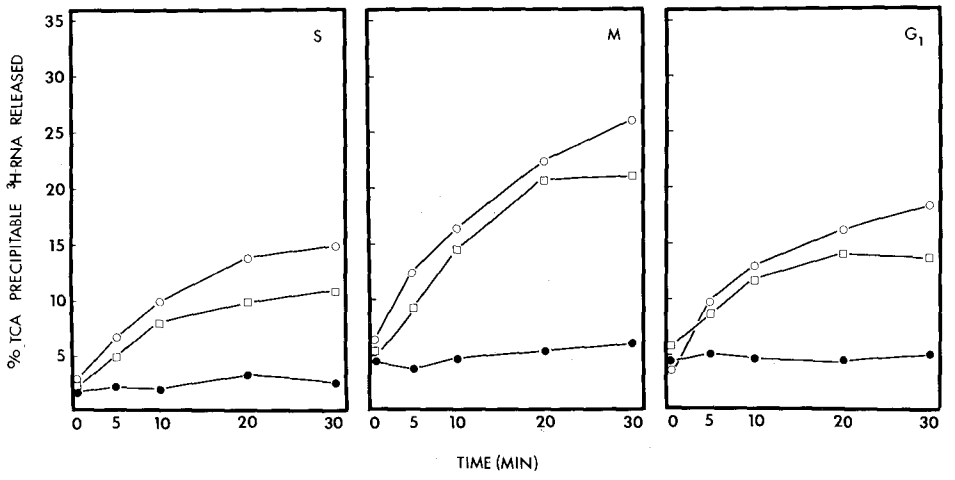

Figure 3.
Release of ^{3}H-RNA from synchronized cells in S, M, and G_1 phases of growth. Frame S, S phase; frame M, M phase; frame G_1, G_1 phase. In each frame ○—○, 4 mM ATP; □—□, no additions ●—●, 8 mM $MgCl_2$.

in S, M, and G_1 phases of the cell cycle. Release of RNA and DNA was assayed as before. In all three phases of the cell cycle monitored, pre-labeled RNA was released to the post-nuclear supernatant whether or not ATP was present. Nuclei isolated from cells in mitosis, however, released more RNA than those isolated from cells in other phases of the cell cycle.

Figure 4 shows the ratio of RNA to DNA released during the three monitored phases of the cell cycle. It is obvious that nuclei isolated from cells in M (frame M) release RNA differentially only if no chelator is present. Nuclei

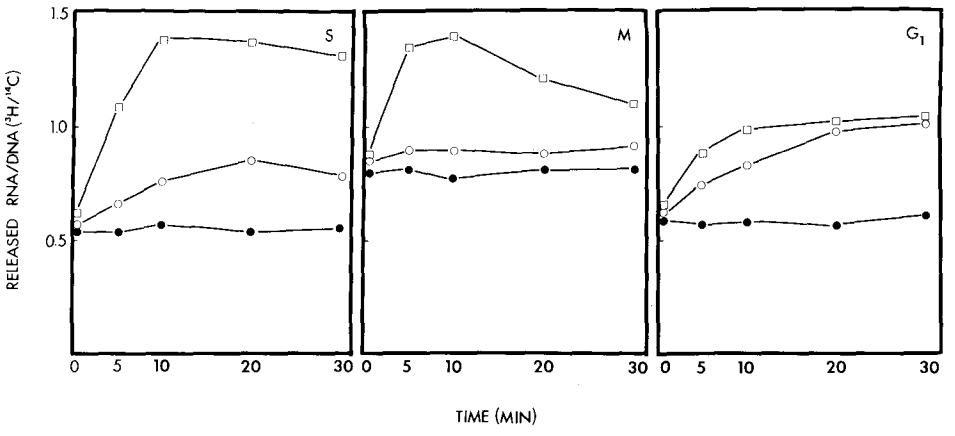

Figure 4.
Ratio of ^{3}H-RNA released to ^{14}C-DNA released by nuclei from synchronized MOPC-21 cells. Frame S, nuclei from cells in S; frame M, nuclei from cells in M; frame G_1, nuclei from cells in G_1. In each frame ○—○, 4 mM ATP, □—□, no additions; ●—●, 8 mM $MgCl_2$.

isolated from cells in either S or G_1, however, released RNA differentially (ratio of RNA to DNA increases) when 4 mM ATP was present in the reaction mixture. This effect was most pronounced in G_1. The reactions without ATP appear to differentially release RNA more efficiently than the ATP containing reactions, but it should be noted that the presence of ATP did slightly increase the percent RNA released in these two phases of the cell cycle (see Figure 3, frame S and G_1).

We have partially characterized the RNA released from isolated MOPC-21 nuclei. Linear 5-20% sucrose gradient profiles indicated that the released RNA was heterogeneous in size with a broad peak at 16 S. Discrete peaks corresponding to rRNA were not observed and the size distribution of the released RNA resembled the profile of purified MOPC-21 polysomal mRNA. A significant proportion of the ^{3}H-labeled TCA precipitable RNA released from MOPC-21 nuclei in the first 20 min of incubation bound to poly U impregnated glass fiber filters or to unsubstituted cellulose columns. Experiments are in progress to determine differences in RNA released in the presence or absence of chelators.

IMPLICATIONS

The immediate attractiveness of an *in vitro* system which would allow manipulation of parameters affecting the molecular events of transcription, processing, stabilization, and transport of RNA must be tempered by extreme caution in assessing the physiological validity of the system. Recent attempts to ascertain the possible role of poly A in nuclear transport (11), the physiological integrity of *in vitro* transport systems (12), and role of ATP in nuclear transport (9) have ignored the importance of molecular restriction of the nuclear envelope. Any conclusions based on manipulation of a biological membrane system in which biochemical restriction of macromolecules is not monitored must remain suspect. Our data indicates that gentle preparation of nuclei, careful attention to reconstitution of a system with physiological concentrations of cytoplasmic protein(s) and maintenance of the physical integrity of nuclei do not insure selective release of RNA. Results from double label assays of nuclear release with synchronized cells suggest that the release of DNA from nuclei in the presence or absence of ATP cannot be explained solely by the presence of mitotic nuclei. It appears that there is some ATP-induced "leakiness" or dilation of the nuclear membrane permitting DNA exit in all examined phases of the cell cycle under conditions of optimum RNA release in other *in vitro* assay systems. Since the nuclei remain physically intact throughout incubation, one possible explanation for DNA release might involve loops of chromatin which project through the nuclear envelope. These strands of DNA would then be vulnerable to the action of nucleases in the cytosol fraction as well as mechanical shear during centrifugation. Our data suggest that the selective nuclear restriction of macromolecules is altered in the presence of ATP.

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