IN VITRO SYNTHESIS AND STABILITY OF RNA IN ISOLATED NUCLEI FROM BOVINE LYMPHOCYTES

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SUMMARY: Nuclei from Concanavalin A-stimulated lymphocytes (30 hr after Con A addition) incorporate up to 5 times more (3-H)UTP into RNA than nuclei from resting lymphocytes. The incorporation kinetics is linear for almost 60 min. 14-20% of the in vitro labeled RNA is polyadenylated. Poly(A)(-)RNA from both types of nuclei sediments from 4-5S up to more than 30S on sucrose gradients. Nuclei from stimulated cells synthesize about double the amount of RNA larger than 18S than nuclei from resting cells. The same holds for poly(A)(+)RNA. Poly(A)(-) RNA labeled during 10 min in both types of nuclei is stable during a 30 min chase. Under the same conditions poly(A)(+)RNA in nuclei from resting cells is degraded to about 50% during the chase whereas it is stable in nuclei from stimulated cells.

INTRODUCTION

Isolated nuclei offer the possibility to study and manipulate the various reactions involved in RNA synthesis and maturation, e.g. initiation of RNA chains and processing steps like modification of the 5' end ("capping"), methylation, polyadenylation and endonucleolytic cleavages (1). It can be expected that many of these reactions are affected by a change in the proliferative state of the cell. We have previously shown that the RNA synthesizing capacity of isolated nuclei reflects the physiological state of the cell since nuclei from cells which are actively synthesizing RNA in vivo are more active in in vitro transcription than nuclei from cells which are less active in in vivo RNA synthesis (2).

Lymphocytes provide a natural example for a resting cell population.Upon stimulation by some plant lectins they can be induced

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to procede from a dormant into a metabolically more active state. This change apparently leads to the expression of a cell-specific genetic program (3, 4).

In this communication we compare the RNA synthesizing capacity of isolated nuclei from resting lymphocytes with that of nuclei prepared from Concanavalin A (Con A)-stimulated cells. In lymphocytes from bovine lymph nodes which are used in this work the rate of (^{3}H)uridine incorporation increases only 4-6 hours after addition of Con A (5). Consequently, no increase in the RNA synthesizing capacity of isolated nuclei was observed during this initial period (6). In the experiments to be reported below nuclei were prepared from lymphocytes 30 hours after Con A activation when the morphological changes (lymphoblast formation) and the increased (^{3}H)uridine incorporation rates (6) indicate efficient transcription activity in vivo.

MATERIALS AND METHODS

Cells: Lymphocytes from bovine retropharyngeal lymph nodes were prepared and cultivated as described (5, 6). After 20 hours in a humid CO_2 (5%) atmosphere, the cells were stimulated with 5 µg/ml Con A (Boehringer, Mannheim. The cells were harvested 30 hours after Con A addition. At this time, up to 60% of all lymphocytes were converted to blast cells. An unstimulated control culture was kept under identical conditions for the same time period before the cells were collected.

Isolation of nuclei (7): The cells were collected by centrifugation (5 min, 480 g) at room temperature and washed once with a 0.15 M sucrose solution containing 25mM HEPES buffer (pH 8) and 5mM $CaCl_2$ (buffer A). The pellet was then cooled on ice and resuspended in cold 0.25 M sucrose containing 25mM HEPES and 5mM $CaCl_2$ (buffer B) to give a concentration of 4 x 10^8 cells/ml. To this suspension was added an equal volume of buffer A containing 0.5% Brij 58 (Serva, Heidelberg). The suspension was gently shaken on ice for 5 min before 25 volumes of buffer B containing 2% Dextran (Sigma) were added. The nuclei were then collected by centrifugation (6 min, 480 g). These nuclei still show some cytoplasmic material adhering to the nuclear envelope when inspected microscopically. Experiments with L-cell nuclei have shown that this together with the high concentration of serum albumin in the incubation buffer minimizes loss of enzymes, e.g. RNA polymerase (K.P.Schäfer, unpublished results).

RNA synthesis in isolated nuclei: Nuclei were resuspended in incubation buffer which contains: 50mM Tris-HCl (pH 7.4); 5mM $MgCl_2$; 60mM KCl; 1mM dithioerythritol; 10% (v/v) glycerol and 1% (w/v) bovine serum albumin (Sigma, lot A 4378). CTP, GTP and ATP were added in final concentrations of 0.3mM; (^{3}H)UTP (1.2 Ci/mmole) was used as radioactive tracer in a final concentration of 0.03mM. The standard reaction mixture also contains 3.2mM phosphoenolpyruvate and 20 µg/0.25 ml pyruvate kinase. If not stated otherwise, 5 x 10^6 nuclei were

incubated in a 0.25 ml volume at 25°C.
The reaction was stopped by addition of 1 ml ice-cold DB buffer (30mM Tris-HCl (pH 7.5), 5mM magnesium acetate, 120mM KCl, 7mM 2-mercaptoethanol) and 2 ml of 10% TCA; the precipitates were collected on glass fiber filters, washed with TCA and counted in toluene containing 0.5% (w/v) PPO.
If the in vitro synthesized RNA was to be extracted for further analysis 2×10^7 nuclei were incubated in a 1 ml volume of the reaction mixture containing final concentrations of 0.5mM CTP, GTP and ATP, respectively, as well as 0.05mM of (^{3}H)UTP. After incubation for the appropriate time at 25°C, the sample was quickly frozen in liquid nitrogen.

Isolation of RNA: The procedure used follows that of Kirby (8). Briefly, nuclei were lysed by anionic detergents. The lysate was shaken in a phenol-cresol-hydroxychinoline-mixture at room temperature. The aqueous phase was extracted several times with chloroform; the nucleic acids were precipitated with ethanol. The dried precipitate was resuspended and treated with RNAase-free DNAase (lot.no. 15469, Boehringer, Mannheim) and proteinase K (Merck, Darmstadt). The enzymes were removed by chloroform extraction.
Glassware was freed of RNase impurities by heating at 180°C. Plastic material was treated with 15% H_2O_2. Whereever necessary, buffers were treated with diethylpyrocabonate and then autoclaved.

Preparation of poly(A) containing RNA (9): RNA in binding buffer (700mM NaCl; 50mM Tris-HCl, pH 7.5; 10mM EDTA; 20% (v/v) formamide and 5 µg/ml potassium polyvinyl sulfate) was added to a poly(U) Sepharose column (0.7 ml), equilibrated with binding buffer. The sample was recycled several times to allow for a complete adsorption to the column. Flow through material (poly(A)(-)RNA) was then collected in 5 ml, and the column washed with 5 ml of binding buffer. The adsorbed poly(A)(+)RNA was then eluted with 10mM potassium phosphate buffer (pH 7.5) containing 10mM EDTA, 90% (v/v) formamide and 5 µg/ml potassium polyvinyl sulfate. The eluted RNA was dialysed versus DB buffer at 4° and concentrated by ethanol precipitation.

Other techniques: RNA was analysed by sedimentation through 17 ml linear sucrose gradients (10%-60%) for 19 hours at 21°C and 25,000 rpm in the SW 27 rotor of the Spinco centrifuge. The sucrose solution was buffered by 10mM Tris-HCl (pH 7.5) and contained 100mM NaCl and 10mM EDTA (10). The gradients were collected from the bottom using the LKB-Uvicord and a LKB fraction collector.

RESULTS

Characterization of the system: In all experiments described in this communication, nuclei from activated lymphocytes, prepared 30 hours after addition of Con A, are compared with nuclei from unstimulated cells which were cultivated for the same length

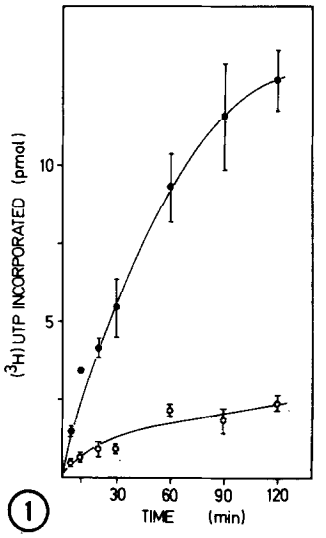
of time in the absence of Con A.

Under our conditions of incubation the rate of RNA synthesis depends linearly on the number of nuclei in the range of 1-10 x 10^6 nuclei/0.25 ml incubation buffer. If not stated otherwise, 5 x 10^6 nuclei were used in a standard incubation assay.

Optimum KCl concentration was between 50-100mM which is comparable to optimum salt concentrations found for the RNA synthesis in nuclei from other cells (10, 11). Maximal RNA synthesis was observed at a concentration of 0.2-0.3mM for GTP, CTP and ATP, respectively, when (^{3}H)UTP was used in a concentration of 0.03mM.

Fig. 1 shows the amount of radioactivity incorporated into acid precipitable material as a function of incubation time. It can be seen that nuclei from Con A-stimulated lymphocytes incorporate 5-6 times more (^{3}H)UTP than nuclei from resting cells incubated under the same conditions. It is also apparent that the incorporation rate of nuclei from Con A-stimulated cells remains approximately constant for about one hour. The rate declines afterwards but incorporation continues for at least 120 min.

17 $\pm$ 3% (mean of six experiments) of the RNA labeled during the first 30 min binds to poly(U) Sepharose columns and therefore most probably contains poly(A) sequences. The percentage of poly(A)(+)RNA synthesized in nuclei from Con A-stimulated cells is not significantly different from that synthesized in nuclei from unstimulated cells. We have found that nuclei kept for several weeks at -70° synthesize RNA at similar rates as freshly prepared nuclei. The nuclei stored at -70°C loose, however, their capacity to polyadenylate RNA since the radioactive RNA extracted from these nuclei did not bind to poly(U) Sepharose.



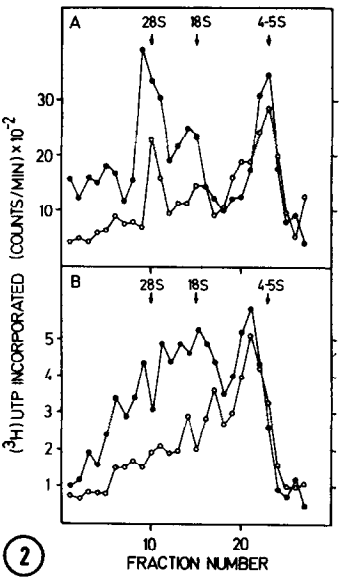


Fig. 1: TIME COURSE OF RNA SYNTHESIS IN ISOLATED NUCLEI
Nuclei (5×10^6/250 µl) were incubated under standard conditions (including 6mM KCl) as described. Incorporated radioactivity ((3-H)UTP) was converted to pmol (3-H)UTP.
● nuclei from Con A-stimulated cells; o nuclei from resting cells.

Fig. 2: SEDIMENTATION ANALYSIS OF IN VITRO LABELED POLY(A)(-)RNA AND POLY(A)(+)RNA
10^8 nuclei from either Con A-stimulated cells or resting cells, respectively, were incubated for 30 min under standard conditions. Total RNA was extracted, separated on poly(U) Sepharose columns in poly(A)-free and poly(A)(+)RNA. Each class of RNA was analyzed separately on 10-60% sucrose gradients.
A poly(A)(-)RNA B poly(A)(+)RNA
● nuclei from Con A-stimulated cells; o nuclei from resting cells. - It should be mentioned that our experimental conditions during gradient analysis do not discriminate against aggregation of RNA.

Centrifugation analysis of the RNA synthesized in isolated nuclei: Nuclei from Con A-stimulated cells as well as from resting cells, respectively, were incubated in incubation buffer for 30 min at 25°C. The RNA was extracted and passed through a poly(U) Sepharose column as described. Unbound as well as the poly(U)-bound RNA were then sedimented

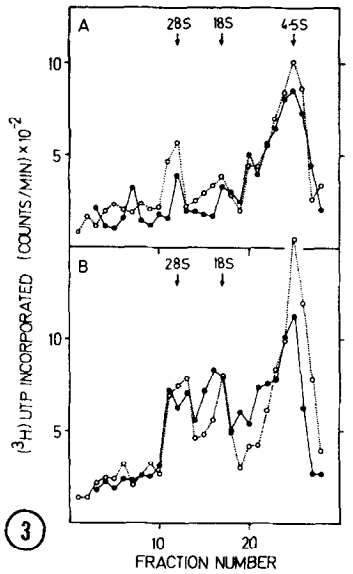
through sucrose gradients. Fig. 2 shows the sedimentation profile of RNA which does not bind to poly(U) Sepharose. It can be seen that considerably more RNA sedimenting with 28S and 18S was produced in nuclei from Con A-stimulated cells compared to the nuclei from untreated control cells. It also appears that labeled material which sediments faster than 28S is preferentially synthesized in nuclei from Con A-stimulated cells. Small molecular weight RNA (4-5S), however, appears in similar quantities under both conditions.

In Fig. 2B the sedimentation profile of poly(A)(+)RNA is shown. It is apparent that, in nuclei from Con A-stimulated cells, poly(A)(+)RNA with sedimentation coefficients of larger than 18S is produced in high quantities (see legend to Fig. 2). Again, poly(A)(+)RNA of low sedimentation coefficient ($\sim$ 6S) is synthesized in approximately equal amounts, in both types of nuclei.

Stability of in vitro labeled RNA: Under the conditions used our nuclear preparations did not show unspecific breakdown of RNA which would be revealed by an accumulation of small molecular weight fragments.

It therefore seemed possible to study the fate of RNA pulse labeled *in vitro* under conditions of an excess of unlabeled substrate ("chase"). Nuclei from resting and Con A-stimulated cells were labeled with (^{3}H)UTP for 10 min under standard conditions. Half of the assay was then withdrawn and the RNA extracted. The remaining part of the assay was supplied with a 50-fold excess of UTP and incubation continued for 30 more min. RNA was then extracted, too.

After separation into poly(A)(-)RNA and poly(A)(+)RNA on poly(U) Sepharose columns both classes of RNA were analyzed on sucrose gradients (Fig. 3 and 4).



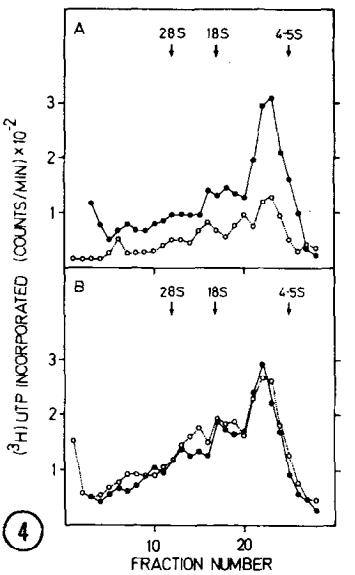


Fig. 3: FATE OF POLY(A)(-)RNA SYNTHESIZED IN VITRO
2 x 10^8 nuclei were incubated under standard conditions (2 ml total volume). After 10 min 1 ml was withdrawn and RNA extracted. The remaining ml of the assay was made 2.5mM in unlabeled UTP and incubated for 30 min. RNA was then extracted as well. The poly(A)(-)RNA was isolated from both preparations as flow-through of the poly(U) Sepharose columns. (The poly(A)(+) RNA was used to obtain the gradients shown in Fig. 4.)
A poly(A)(-)RNA from nuclei from resting cells.
B poly(A)(-)RNA from nuclei from Con A-stimulated cells.
Gradient conditions (10-60%) were as described under Methods.
●—● 10 min pulse label; o---o 10 min pulse label followed by a 30 min chase.

Fig. 4: FATE OF POLY(A)(+)RNA SYNTHESIZED IN VITRO
The poly(A)(+)RNA from the experiment of Fig. 3 was analyzed on 10-60% sucrose gradients.
A poly(A)(+)RNA from nuclei from resting cells.
B poly(A)(+)RNA from nuclei from Con A-stimulated cells.
●—● 10 min pulse label; o---o 10 min pulse label followed by a 30 min chase.

The pulse labeled poly(A)(-)RNA clearly shows the peaks of the ribosomal RNA species and a prominent peak in the 4-5S region (Fig. 3A and B). Again, we see that nuclei (Fig. 3B) from Con A-stimulated cells synthesize more RNA than control nuclei (Fig. 3A).

Poly(A)(+)RNA is pulse labeled to about the same extent in both types of nuclei. After the 30 min chase, however, more than 50% of the poly(A)(+)RNA has disappeared in resting nuclei whereas it has remained unchanged in nuclei from Con A-stimulated cells (Fig. 4A and B).

DISCUSSION

Under optimum conditions, 5 x 10^6 nuclei from Con A-stimulated lymphocytes polymerize 25 pmol nucleotides/30 min at 25°. Nuclei from mouse myeloma cells incorporate about 80 pmol/10^6 nuclei/30 min under similar conditions (12). If we take into account, however, that only about 40-60% of our cells (T-cells) respond to the mitogen and at 30 hours after stimulation have only reached 2/3 of their maximum transcription activity (6) we arrive at a figure of about 15 pmole/10^6 nuclei/30 min for nuclei from stimulated lymphocytes. Part of the remaining difference may reflect the state of the transformed myeloma cell as compared to the untransformed lymphocyte.

As mentioned above, 14-20% of all RNA synthesized in isolated nuclei binds to poly(U) Sepharose and therefore contains probably poly(A) sequences. The percentage is the same in nuclei from resting as well as from activated cells although the absolute number is higher in the latter case. It is interesting to note that a fraction of poly(A)(+) RNA with sedimentation coefficients of 5-10S is labeled equally well in nuclei from resting and from Con A-stimulated cells. The nature of this class of RNA molecules is unknown. A partial degradation of longer primary transcription products cannot be excluded.

It would be interesting to compare the poly(A)-containing RNA which we observe in isolated nuclei with that synthesized *in vivo*.

The RNA species synthesized in isolated nuclei, however, should be compared to nascent in vivo RNA. Nascent RNA in vivo was investigated by Derman et al. (13) in HeLa cells. These workers found that more than 50% of all RNA, labeled during a 45 sec pulse, sedimented with S-values smaller than 32. These results, although obtained with a different cell type, are comparable with the data presented in this work.

We see that clearly more poly(A)-free RNA is synthesized in nuclei from Con A-stimulated cells than in nuclei from dormant lymphocytes. Most of these RNA species appear to be rRNA as judged from their characteristic sedimentation pattern. Again, a set of poly(A)-free RNA molecules with S-values around 5 seems to be synthesized in almost equal quantities in nuclei from resting and Con A-stimulated lymphocytes.

With respect to poly(A)(+)RNA one striking difference is the obvious instability of this class of RNA in nuclei from resting cells during the chase period (Fig. 4). It seems that there is a uniform degradation throughout all size classes of RNA chains with no accumulation of smaller species or fragments. S.Berger has determined the decay pattern of poly(A)(+)hnRNA in human lymphocytes (personal communication). She found that processing of poly(A)(+)hnRNA to yield poly(A)(+)RNA is rapid and proceeds to completion without accumulation of intermediates.

But she also found that the apparent half-life of poly(A)(+) hnRNA did not change upon lectin stimulation. This apparent half-life is, however, the result of several interrelated processes namely (i) degradation of poly(A)(+)hnRNA within the nucleus, (ii) processing of poly(A)(+)hnRNA to poly(A)(+)mRNA and its export to the cytoplasm, and (iii) the reutilization of labeled precursors after intranuclear breakdown of primary

transcription products. We have shown that the transfer of poly(A)(+)RNA from the nucleus to the cytoplasm is enhanced in vivo after lectin stimulation (5). Hence the channeling of part of the poly(A)(+)hnRNA into the pathway (i) or (ii) may be an important means of the cell in regulating mRNA abundance in the cytoplasm. It has to be determined, however, to what degree the functioning of this channeling machinery is disturbed by the isolation of the nuclei.

What is preserved in isolated nuclei are the enzymes and/or their relative activities, respectively, which distinguish resting and Con A-stimulated cells. It is known that RNA polymerase levels in lymphocytes increase drastically after lectin stimulation (14). Our preparation procedure for nuclei reduces loss of nuclear proteins as much as possible. The increase in RNA synthesizing capacity in nuclei from Con A-stimulated cells should therefore be the result of an increased number of RNA chains being transcribed. In addition, it may be possible to use nuclei to probe the qualitative differences in the set of RNA molecules transcribed as well as their processing in resting and Con A-stimulated cells.

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