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Prolonged Transcription in a Cell-Free System Involving Nuclei and Cytoplasm

(transcription nuclei/RNA synthesis/protein synthesis)

GUANG-JER WU AND GEOFFREY ZUBAY

Department of Biological Sciences, Columbia University, New York City, N.Y. 10027

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ABSTRACT **A cell-free system for nuclear-directed transcription has been developed that gives prolonged synthesis in the presence of cytoplasm. The nuclear and cytoplasmic components have been prepared from Krebs II ascites tumor cells for most experiments but further observations indicate that components prepared from other cell types may be used. After an initial 5- to 10-min period of relatively rapid RNA synthesis a linear rate ensues for 2–3 hr. In the absence of cytoplasm no net RNA synthesis occurs after the initial 10-min period. Experiments with α-amanitin suggest that about half of the cell-free synthesized RNA is made by RNA polymerase II, the enzyme believed to be responsible for messenger synthesis *in vivo*.**

The conditions used for RNA synthesis were derived from conditions found to be optimal for protein synthesis that proceeds linearly for 2–3 hr. It has not yet been possible to demonstrate the synthesis of protein from cell-free synthesized RNA in this system. A major problem here is that isolated nuclei, even when carefully washed, contain a great deal of translatable RNA.

We consider the development of cell-free systems that reflect normal *in vivo* conditions to be of paramount importance for the analysis of transcription and translation, particularly for questions concerned with regulation. Whereas such systems are highly developed for bacteria like *Escherichia coli* (1), comparable systems derived from animal cells remain to be proven. We have set our sights on the development of a cell-free system, with components derived from animal cells, in which the regulation of transcription and translation can be studied. Since most regulating processes operate at the point of initiation, it is important that we seek conditions that lead to initiation at normal *in vivo* sites. Experience with cell-free systems derived from bacteria has taught us that initiation at abnormal sites is a frequent occurrence in cell-free systems that can be minimized by carefully controlled conditions empirically derived. With cell-free systems made from animal cells there are very few guidelines for developing a system that synthesizes *in-vivo*-like RNA from the proper initiation point. By contrast good criteria exist for mRNA-directed synthesis of protein, as complete protein can be synthesized and adequately characterized. Since transcription and translation occur side-by-side in the whole cell, it seemed like a good possibility that conditions which are optimal for high fidelity translation might also be optimal for high fidelity transcription. This is a brief report of the transcription behavior of intact nuclei in a cytoplasmic extract under conditions that are optimal for translation.

Abbreviations: DB, a Tris·HCl–KCl–$Mg(OAc)_2$–mercaptoethanol buffer, pH 7.5; S-*n*, supernatant from cell-sap after centrifugation at $n \times g$; H-nuclei, nuclei prepared in hypotonic medium; TH-nuclei, H-nuclei washed with Triton X-100; EDTA, ethylenediaminetetraacetic acid; NET, a Tris·HCl–EDTA–NaCl buffer, pH 9.0.

MATERIALS AND METHODS

1. *Chemicals.* α-Amanitin was obtained from Dr. T. Wieland. Actinomycin D was purchased from Cal. Biochem. Calf-thymus DNA was purchased from Sigma Chem. Co. Ribonuclease-free sucrose was purchased from Schwarz-Mann. [8-^{3}H]GTP (5.62 Ci/mmol, 0.5 mCi/ml) and uniformly labeled [^{14}C]leucine (304 Ci/mol, 0.1 mCi/ml) were purchased from New England Nuclear Corp. Whatman Cellulose Powder CC-41 was purchased from Reeve Angel. $(dT)_{10}$-Cellulose was purchased from Collaborative Res. Inc. Krebs II ascites tumor cells were obtained from Dr. A. Burness and maintained and collected as described by Dr. A. Burness (personal communication).

2. *Preparation of Cell-Sap from Ascites Tumor Cells.* All operations under sections 2–4 were carried out at 0°. Cells were washed once with five volumes of DB (30 mM Tris·HCl, pH 7.5, 120 mM KCl, 5 mM $Mg(OAc)_2$, 7 mM 2-mercaptoethanol), suspended in two volumes of RB (10 mM Tris·HCl, pH 7.5, 10 mM KCl, 1.5 mM $Mg(OAc)_2$), allowed to swell for 5 min, homogenized with four strokes using a B-type Dounce homogenizer, followed by addition of 0.1 volumes of a buffer containing 0.23 M Tris·HCl, pH 7.5, 1.27 M KCl, 0.04 M $Mg(OAc)_2$, 0.07 M 2-mercaptoethanol. Ninety percent of the cells were broken under these conditions and the nuclei were still intact as observed by phase microscopy. Cell-sap prepared in this way contains 1.5–1.8 mg/ml of DNA.

3. *Preparation of Other Cell-Free Extracts.* S-1 is the supernatant from cell-sap after centrifugation of cell-sap at 270 × *g* for 2 min. S-30 is the supernatant from cell-sap which is centrifuged at 30,000 × *g* for 10 min and dialyzed against two changes of one liter of DB at 4° for 6 hr. Preincubated S-30 was prepared as described previously (2). S-100 is the supernatant from cell-sep which is centrifuged at 100,000 × *g* for 3.5 hr.

4. *Preparation of Nuclei.* Both methods are modifications of procedures of Zylber and Penman (3).

(*a*) Hypotonic nuclei (H-nuclei). Nuclei were sedimented from cell-sap by centrifugation at 270 × *g* for 2 min. After decantation the sediment was resuspended in five volumes of DB and washed once more in the same buffer. The final sediment was suspended in either DB or one of the cytoplasmic extracts (S-1, S-30, or S-100 preincubated or not preincubated) to a DNA concentration of 1.5–1.8 mg/ml.

(*b*) Triton-washed hypotonic nuclei (TH-nuclei). Nuclei were sedimented from cell-sap by centrifugation at 270 × *g* for 2 min. After decantation the sediment was resuspended, washed once in DB containing 0.1% Triton X-100, washed once with DB and finally resuspended in either preincubated S-30 or DB to a DNA concentration of 1.5–1.8 mg/ml.

5. *Conditions for Cell-Free RNA Synthesis.* The incubation mixture contains per 100 μl: 45–54 μg of DNA as supplied by nuclei or cell-sap, 5 mM $Mg(OAc)_2$, 82 mM KCl, 30 mM Tris·HCl, pH 7.5, 7 mM 2-mercaptoethanol, 1 mM ATP, 0.25 mM CTP, 0.25 mM UTP, 0.055 mM GTP, 0.0018 mM [^{3}H]GTP (0.96 μCi, 5.62 Ci/mmol, 0.5 mCi/ml), 10.3 mM trisodium phospho*enol*pyruvate, 40 μM all 20 amino acids. Cell-free RNA synthesis was done at 29°. After the appropriate time the incubation is stopped with cold 5% trichloroacetic acid. The resulting precipitate is washed four times with cold trichloroacetic acid and finally dissolved in formic acid, dry plated, and counted on a windowless gas flow counter.

6. *Conditions for Cell-Free mRNA-Directed Protein Synthesis.* Same as in section 5 except for omission of nuclei, radioactive GTP, and L-leucine and addition of nonradioactive GTP to 0.25 mM and [^{14}C]leucine (0.12 μCi, 304 Ci/mol, 0.1 mCi/ml) to 4 μM. Cell-free mRNA-directed protein synthesis was done at 29°.

7. *Conditions for Nuclear-Directed Protein Synthesis.* Same as in section 6 except for inclusion of nuclei.

8. *Determination of RNA Content of Nuclei.* Orcinol test used (4). *E. coli* ribosomal RNA was used as the standard. H-nuclei contain 44 μg of RNA per 100 μg of DNA. TH nuclei contain 35 μg of RNA per 100 μg of DNA.

9. *Determination of DNA Content of Nuclei and Cell-Sap.* The diphenylamine test of Dische was used (5). Calf-thymus DNA was used as the standard.

10. *Preparation of RNA for RNA-Directed Protein Synthesis.* To 30 ml of packed Krebs II ascites tumor cells (500 × *g* for 2 min) was added 90 ml of ice-cold NET buffer (0.14 M NaCl 1 mM ethylenediaminetetraacetate (EDTA), 10 mM Tris·HCl, pH 9.0). The cells were lysed by homogenization with 40 strokes in a B-type Dounce homogenizer at 0°. The homogenate was centrifuged at 12,000 × *g* for 10 min at 2° to remove mitochondria and chromatin. To the supernatant was added EDTA to 5 mM and sodium dodecyl sulfate to 0.5%. This suspension was shaken with an equal volume of 88% phenol–chloroform–isoamyl alcohol (100:100:1) at 25° for 5 min. The phases were separated by centrifugation at 12,000 × *g* for 5 min. To the interphase and lower phase were added 20 ml of ice-cold NET buffer followed by shaking at 25° for 5 min. The phases were separated by centrifugation and the aqueous-rich phases were pooled. The pooled aqueous phase was shaken twice with an equal volume of 88% phenol. To the pooled aqueous phases was added 0.1 volumes of 2 M potassium acetate, 10 mM EDTA (pH 5.1), and 2.5 volumes of cold 95% ethanol. RNA was precipitated at −20° overnight. The RNA pellet was collected by centrifugation and extracted twice with 50 ml of ice-cold 1 M NaCl. The supernatant contains mainly tRNA. The pellet, containing mostly rRNA and mRNA, was dissolved in water. Both fractions were dialyzed against water and frozen at −70° until used.

For preparation of partially purified RNA, the ascites RNA was purified either by $(dT)_{10}$-cellulose column chromatography (6) or by Whatman CC-41 cellulose column chromatography (7, 8).

11. *Preparation of RNA for Gradient Analysis and Conditions of Gradient Analysis.* To [^{3}H]GTP-containing RNA, synthesized for different periods at 29° as described above, was added two volumes of ice-cold NET buffer followed by addition of EDTA to 5 mM and sodium dodecyl sulfate to 0.5%. This was extracted with an equal volume of 88% phenol on a vortex shaker at room temperature for 5 min. The phases were separated by centrifugation. The interphase and lower phase were re-extracted twice with one volume of NET buffer and shaken on a vortex mixer at room temperature for 2 min. The pooled aqueous-rich phases were extracted twice with equal volumes of 88% phenol on a vortex mixer at room temperature for 1 min. To the aqueous phase was added 0.1 volumes of 2 M potassium acetate–10 mM EDTA (pH 5.1) followed by addition of 2.5 volumes of ice–cold 95% ethanol. RNA was precipitated at −20° overnight. The RNA pellets were collected by centrifugation, washed twice with ice-cold 70% ethanol and twice with ice-cold 95% ethanol, and dissolved in 0.6 ml of water. To 0.5 ml of the RNA solution was added sodium dodecyl sulfate to 0.2%, NaCl to 0.1 M, EDTA to 1 mM and Tris·HCl to 10 mM, pH 7.5, and layered on top of 11 ml of a 10–70% (w/v) linear sucrose gradient containing 0.2% sodium dodecyl sulfate, 0.1 M NaCl, 10 mM Tris·HCl, pH 7.5, and 1 mM EDTA. The gradient was centrifuged at 25,000 rpm for 19 hr at 23° in a Spinco SW 41 rotor. The gradient was collected in 0.5-ml fractions. Each fraction (0.2 ml) was pipetted onto a sheet of Whatman no. 3 filter paper, dried and washed as described by Bollum (9). Each filter was put into a scintillation vial, covered with 5 ml of toluene fluor and counted in a Packard liquid scintillation counter.

RESULTS AND DISCUSSION

1. *The Cell-Free System for mRNA-Directed Protein Synthesis Used Here is Very Similar to That Developed by Others.* Following the pioneering work of Lockard and Lingrel on the synthesis of hemoglobin (10), several groups have demonstrated the synthesis of complete proteins in animal-derived cell-free systems (see ref. 2 for a recent review). We have used a cell-free system made from Krebs II ascites tumor cells under conditions that are very similar to those used by Aviv, Boime, and Leder (11). This system contains a cell-free extract, crude mRNA prepared from whole cells, and those small molecules needed for protein synthesis (see *Materials and Methods*). Protein synthesis measured by the alkali-stable, trichloroacetic acid-precipitable [^{14}C]leucine is linear for 2–3 hr at 29°. The main changes we have introduced have been adjustments in the concentrations of $Mg(OAc)_2$ and KCl, the use of phospho*enol*pyruvate in place of creatine phosphate as the energy source, synthesis at 29° rather than 36–37°, and storage of the extracts at liquid nitrogen temperatures prior to use. These modifications have been made because they lead to a more reproducible and stable system with greater rates of peptide synthesis. It is well known that adjustments in the concentrations of $Mg(AOc)_2$ and KCl are often necessary when using different types of messenger. When using phospho*enol*-pyruvate it is not necessary to add pyruvate kinase, as the cell-free extracts contain this enzyme. If one uses creatine phosphate instead it is necessary to add creatine kinase. This is inconvenient, as the activity of commercially available creatine kinase varies appreciably from batch to batch. Synthesis at 29° is linear for longer periods than at 36° and

TABLE 1. *Protein synthesis in different types of extracts*

Extract used	[^{14}C]Leucine incorporation (cpm) after 2 hr
Cell-sap	3010
S-1	3227
S-30 (NP)*	2487
S-30 (P)†	123
S-30 (P)† + mRNA‡	2264

One thousand cpm is equivalent to the apparent incorporation of 10 pmol of leucine per 100 μl of incubation mixture. This is a lower limit, since the contribution of unlabeled leucine from the cell extracts and from impurities in the other amino acids added has not been considered.

* NP refers to nonpreincubated extract.

† P refers to preincubated extract.

‡ Crude ascites RNA (500 μg/ml) used; this amount has been found to give optimal incorporation.

results in greater peptide synthesis. Normally all four ribonucleoside triphosphates are added, since our ultimate goal is to use this system for transcription as well as translation. However, UTP and CTP may be removed without any effect on translation. It has also been found that added tRNA is not necessary; evidently the extracts have an adequate supply of this. Most of our measurements of the amount of translation have been made using crude ascites RNA. On occasion partially purified ascites mRNA (prepared by the procedures referred to in *Materials and Methods*) has been used. The same conclusions have been reached using crude or partially purified mRNA and the former has been used out of convenience. Also a number of messengers from other sources have been tested and found to be efficiently translated (for example, see ref. 2). The cell-free extract used for protein synthesis is usually an S-30 extract (see *Materials and Methods*) that has been preincubated for 45 min at 36° and dialyzed for 6 hr prior to use. The object of preincubation is to remove endogenous messenger so that the S-30 can be programmed by added messenger. Other extracts containing endogenous messenger that have been examined include a crude whole cell lysate called cell-sap, an S-1 extract, and an S-30 extract without preincubation. The cell-sap is a crude cell lysate including some unbroken cells, the S-1 is the cell-sap with the nuclei and possible unbroken cells removed and the S-30 without preincubation has had in addition mitochondria and other possible cell organelles removed. The capacity of the different extracts for peptide synthesis is shown in Table 1.

2. *The System Used for Transcription Is Similar to That Used for Translation Except for the Inclusion of Nuclei.* Various systems have been used by others to study cell-free RNA synthesis. Some have used fractionated preparations of deoxynucleoprotein called chromatin (for example see ref. 12), whereas others have used intact nuclei (see ref. 13 for further references). We have chosen to concentrate our efforts on intact nuclei, since they represent a situation closer to the whole cell and therefore one less likely to result in artifacts. The conditions we have used for studying transcription are very nearly the same as those we have used for studying messenger-directed translation except for the addition of nuclei and the substitution of [^{14}C]leucine by [^{3}H]GTP. RNA synthesis is measured in terms of gross [^{3}H]GTP counts incorporated into cold trichloroacetic acid-insoluble substance. When the cell-sap extract is used RNA synthesis proceeds rapidly for 5–10 min and then at about half this rate for the next 2–3 hr (see Fig. 1). We have not made measurements beyond 3 hr. About 0.5 μg of RNA is synthesized per 100 μg of input nuclear DNA in 2 hr. Leaving the cytoplasm out of this system eliminates the incorporation after the first few minutes (see Fig. 1 and Table 2), which demonstrates that some cytoplasmic components are essential for maintaining high rates of synthesis. Extracts prepared from human HeLa and Chinese hamster ovary (CHO) cells give similar results.

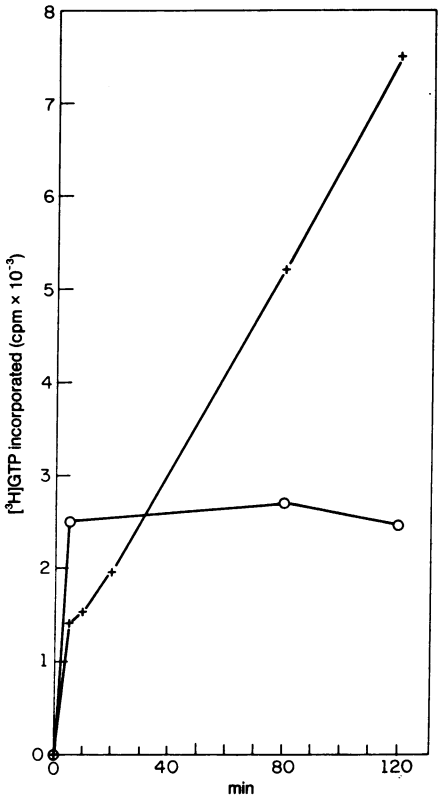

FIG. 1. RNA synthesis as a function of time of synthesis. Synthetic system contains either nuclei (O——O) or a crude cell lysate with the same concentration of nuclei (cell-sap) (+——+), radioactive GTP, and a complex mixture described in *Materials and Methods*. When nuclei alone are used to stimulate synthesis there is no pool of GTP; when cell-sap is used there is an appreciable pool of GTP so that the real incorporation is 2 to 3 times the apparent incorporation (see *text* for further explanation). In the situation with nuclei alone 1000 cpm is equivalent to 36 pmol of GTP incorporated per 100 μl of incubation mixture. Here and in Fig. 2 the experimental data have been multiplied by the indicated factor to obtain the numbers on the *ordinates*.

Only preliminary attempts have been made to determine the cytoplasmic components repsonsible for stimulating transcription. The stimulating effect of cytoplasm is not replaceable by surface-active agents such as Ficoll, polyethylene glycol, glycerol, or bovine-serum albumin. Various types of cytoplasmic extracts have been used to stimulate transcription. A preincubated S-30 and nonpreincubated S-1 and S-30 extracts (see *Materials and Methods*) have all been found to stimulate about the same level of synthesis from nuclei as far as gross counts are concerned. However, the gross counts incorporated is only a rough measure of the actual synthesis, since it does not take into account the possibility of pools of

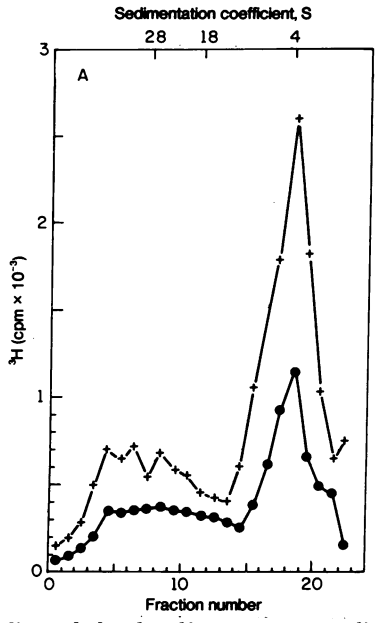

FIG. 2. Sodium dodecyl sulfate–sucrose gradient of RNA synthesized in the cell-free system. RNA synthesized in the system containing cell-sap from 0 to 10 min (●——●) and from 0 to 30 min (+——+).

unlabeled GTP substrate in the extracts. Attempts to estimate the pool size have been made by measuring the effect of extra additions of unlabeled GTP on the incorporated counts. If there is no pool then the incorporated counts should be inversely proportional to specific activity of the added GTP. Such measurements have only been made for incubation mixtures containing cell-sap or nuclei in the absence of cytoplasm. There is no significant pool when nuclei alone are used, since the gross radioactivity incorporated is lowered by a factor of 3 when the specific activity of the added GTP is lowered by that amount. A comparable experiment with cell sap gives a lowering in gross radioactivity incorporated of only 30%, suggesting an appreciable pool of unlabeled GTP or GTP precursor in the cell-sap. Controls in which UTP and CTP have been left out and in which pancreatic RNase has been included show that whole cells, present in small amounts in a cell-sap preparation, make no more than a small contribution to the radioactive RNA synthesized (see Table 2). Some of the residual GTP incorporation in the absence of added UTP and CTP is probably due to pools of these triphosphates in the cell-sap extracts. When these two triphosphates are left out of the system containing nuclei alone no GTP incorporation is obtained.

TABLE 2. *Characteristics of the cell-free system for RNA synthesis using cell-sap*

Conditions	Percent normal amount of [^{3}H]-GTP incorporated after 2 hr
Complete system	100
− nuclei	3
− cytoplasm	20
+ 2 μg/ml of actinomycin D	25
+ 10 μg/ml of actinomycin D	5
+ 10 μg/ml of α-amanitin	50
− CTP, − UTP	20
− CTP, − UTP, + 20 μg/ml of pancreatic RNase	11

See *Materials and Methods* for a description of the complete system and details of the assay.

Table 2 also shows that synthesis is completely dependent upon the presence of nuclei and highly sensitive to the presence of actinomycin, as would be expected for DNA-directed transcription. The levels of actinomycin tested do not inhibit messenger-directed protein synthesis under similar conditions.

Preliminary attempts have been made to characterize the RNA made in a complete system. About 50% of the transcription is inhibited by α-amanitin. The α-amanitin-sensitive synthesis is believed to correspond to that fraction of the RNA synthesized by RNA polymerase II that probably makes the messenger RNA (14). The gross RNA product resulting from cell-free synthesis has been characterized by ultracentrifugation in a sodium dodecyl sulfate–sucrose gradient and found to be very polydisperse (see Fig. 2) with a prominent peak in the 4S region. The transfer of newly synthesized RNA from the nucleus to the extra-nuclear fluid has been measured by separating the nuclei from the rest of the incubation mixture at various times and determining the cold trichloroacetic acid-precipitable [^{3}H]GTP counts in both fractions. In the complete system about one-third of the radioactively labeled RNA is transferred to the cytoplasm after 1 hr. Centrifugation of this cytoplasmic extract on a sucrose gradient shows that about 15% of the labeled RNA is located in the ribosome–polysome region. The remainder sediments at substantially lower rates. Labeled RNA from the ribosome–polysome region has been isolated by phenol extraction and found to sediment between 4 and 28 S on an sodium dodecyl sulfate-sucrose gradient. All of these data on RNA sizes are of limited significance, since the RNA is vulnerable to nuclease action during the synthesis step and again during gradient analysis of the cytoplasmic extract.

Most of the previously described systems using nuclei are relatively free of cytoplasm and are short-lived with no net RNA synthesis occurring after the first 10 min of incubation. An interesting exception to this has recently been reported by Marzluff, Murphy, and Huang (13). These investigators have found conditions under which the synthesis of RNA from

TABLE 3. *Cell-free protein synthesis directed by nuclei*

System	[^{14}C]leucine incorporation after 2 hr (cpm)	
	− P·RNase*	+ P·RNase
H-nuclei in S-30	947	152
H-nuclei in S-100	917	227
H-nuclei	152	125
TH-nuclei in S-30	417	52
TH-nuclei in S-100	312	43
TH-nuclei	26	21
S-30	52	
S-30 + mRNA (500 μg/ml)	1097	
S-100	21	
S-100 + mRNA (500 μg/ml)	19	

* When pancreatic RNAse (P·RNase) is used 20 μg/ml is present.

purified mouse myeloma nuclei is linear for 1 hr at 25°. The low temperature is critical to the longevity of the system. Whereas this system is active for longer periods than most cell-free systems, it is not one in which transcription and translation can be studied side-by-side. Another possible drawback of this system is that it uses Mn^{+2}, 15% glycerol, and rather high KCl. We are concerned that such conditions may encourage initiation at abnormal sites, although no certain criteria for measuring this are currently available. Our system gives about the same level of RNA synthesis at incubation temperatures between 25 and 36°. Addition of Mn^{+2}, glycerol, or Ca^{+2}, or moderate alterations in the KCl or $Mg(OAc)_2$ concentrations do not improve our system either for RNA or protein synthesis.

3. *Although Nuclei Stimulate Protein Synthesis in the Cell-Free System, It Has Not Been Possible to Distinguish Between the Synthesis Catalyzed by De Novo Synthesized Messenger and Already Present Messenger.* The cell-free system is reasonably stable for 2–3 hr for both transcription and translation. Furthermore, a good deal of the cell-free synthesized RNA is transferred from the nucleus to the cytoplasm. Our next concern was to see if some of this cell-free synthesized RNA is translated after being transferred to the cytoplasm. To test for this the system of choice would appear to be one involving nuclei and an S-30 that has been preincubated, since such an S-30 gives a low background incorporation in the absence of added messenger. One could then look for nuclear-stimulated peptide synthesis. However, there is one serious difficulty in using intact nuclei for such a study, since they contain about 40% as much RNA as DNA. If this RNA is translatable it might obscure the translation catalyzed by the cell-free synthesized RNA. Indeed, we have found that the system of nuclei and preincubated S-30 extract is quite effective in catalyzing peptide synthesis. This peptide synthesis appears to result mostly from preformed messenger, as it is insensitive to 2 μg/ml of actinomycin D, which inhibits RNA synthesis. Much to our surprise, either preincubated S-30 or S-100 both stimulate peptide synthesis 5- to 10-fold (see Table 3). The fact that S-100, which does not contain ribosomes (shown in Table 3 by the inability of S-100 to support messenger-directed protein synthesis), is about as effective as S-30 shows that these nuclear preparations must contain sufficient ribosomes for peptide synthesis. The possibility that whole cell contamination is a factor is excluded by pancreatic RNase controls, which show very low incorporation. This is true of nuclei prepared by the hypotonic method (H-nuclei, see *Materials and Methods*) as well as Triton-washed nuclei (TH-nuclei). A probable location of these ribosomes is the outer nuclear membrane but no attempt has been made to directly determine their localization.

4. *The Cell-Free System May Be Valuable for Studying the Regulation of Transcription.* The most important attribute of the transcription system described here is that transcription occurs for long periods under mild conditions designed to simulate the intracellular environment. We hope that with fuller characterization of the RNAs made this system will be useful for gene regulation studies. Nuclei and cytoplasm from different cell types of the same organism can be combined to see if the nuclear expression is controlled by the cytoplasm. Various small molecules that are candidates for gene activators, such as steroid hormones and cyclic nucleotides, can be tested for their effect on the synthesis of particular RNAs.

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