Regulation of Mammalian Protein Synthesis *in vivo*

STIMULATED TRANSPORT OF NUCLEAR RIBONUCLEOPROTEIN COMPLEXES TO THE CYTOPLASM AFTER CYCLOHEXIMIDE TREATMENT

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By studies *in vivo* with purified nuclei from rat liver, it was shown that a non-lethal dose of cycloheximide causes a decrease in the content of total nuclear ribonucleoprotein complexes by 2 h after treatment. Analysis of the complex by sucrose-density-gradient centrifugation substantiated this observation for the faster-sedimenting complex, but showed an increase in the content of a smaller complex. Radioisotope incorporation studies showed that the overall decrease in nuclear ribonucleoprotein content was not due to a decreased synthesis, but rather to an increased transport to the cytoplasm. The results of a double-radioisotope technique support the conclusion that, during the inhibitory phase of protein synthesis brought on by cycloheximide, gene transcription continues and the gene product is transported to the cytoplasm for subsequent translation.

In mammalian nuclei, mRNA and its precursor hnRNA exist in the form of RNP complexes with sedimentation coefficients up to 300S (Samarina *et al.*, 1968; Niessing & Sekeris, 1970; Stevenin & Jacob, 1972). These complexes have been extracted from a variety of tissues, including rat liver, Ehrlich-ascites-tumour cells and other eukaryotic cells (Martin & McCarthy, 1972; Pederson, 1974; Karn *et al.*, 1977). A sub-complex with a sedimentation coefficient of 10–20S has been identified as a component of mammalian nuclear RNP. This sub-complex contains poly(A)-containing mRNA and appears to be the precursor to cytoplasmic mRNA (Quinlin *et al.*, 1974; Kish & Pederson, 1975; Quinlin *et al.*, 1977; Karn *et al.*, 1977).

As a means to study adaptive mechanisms in mammalian protein synthesis we have utilized rats treated with a non-lethal dose of cycloheximide (i.e. 2 mg/kg body wt.). Under these conditions the appearance of radioactively labelled cellular and plasma proteins is reversibly inhibited so that the inhibitory and recovery periods can be studied (Ch'ih & Devlin, 1974; Rothblum *et al.*, 1976; Ch'ih *et al.*, 1977a). Furthermore, the adaptive mechanism of protein synthesis during and after the inhibition not only involves translational events, but also is associated with an increased transcriptional activity resulting in enhanced synthesis and accumulation of cytoplasmic poly(A)-containing mRNA as well as rRNA. During the inhibitory phase of protein synthesis, however,

enhanced synthesis and accumulation of only the cytoplasmic poly(A)-containing mRNA have been noted (Ch'ih *et al.*, 1977b).

In the present paper we report results concerning the fate of the nuclear RNP complex during the inhibition of protein synthesis *in vivo*, and extend our previous observations that the increase of cytoplasmic mRNA is due to an enhanced processing and transport of nuclear RNP complexes containing mRNA. Some of the observations described in the present paper have appeared in preliminary form (Duhl *et al.*, 1978).

Experimental

Materials

Cycloheximide, calf thymus DNA, yeast RNA and Triton X-100 were supplied by Sigma Chemical Co. (St. Louis, MO, U.S.A.). Miles Laboratories (Kankakee, IL, U.S.A.) was the source of [³H]rRNA (16S and 23S) and crystalline bovine serum albumin. Amersham (Arlington Heights, IL, U.S.A.) provided [³H]leucine (40–53 Ci/mmol), [¹⁴C]leucine (354 mCi/mmol), [³H]orotate (19–23 Ci/mmol) and NCS solubilizer. Ribonuclease-free sucrose was purchased from Schwartz–Mann (Orangeburg, NY, U.S.A.). All other chemicals were of the highest purity available commercially.

Animals

The experiments were performed on male Wistar rats (200 ± 10 g). Maintenance of the animals, treat-

Abbreviations used: hnRNA, heterogeneous nuclear RNA; RNP, ribonucleoprotein; DNP, deoxyribonucleoprotein.

ment with cycloheximide and removal of livers were carried out as described previously (Ch'ih *et al.*, 1977a).

Isolation of subnuclear components

Nuclei were first isolated by the method of Blobel & Potter (1966) with the use of 0.1% Triton X-100 as previously described (Rothblum *et al.*, 1976). The isolated nuclei were further purified by sedimenting through 1.75M- and 2.4M-sucrose at 82500g for 60min each. The purified nuclei were used for the subsequent isolation of nuclear nucleoprotein complexes.

Nuclear sap, RNP complexes and DNP complex were sequentially fractionated as described by Umansky *et al.* (1976) with minor modifications. The nuclear-sap proteins were isolated from purified nuclei by washing successively with 0.14M-NaCl/0.01M-Tris/HCl (pH 7.6), 0.075M-NaCl/0.024M-EDTA (pH 8.0) and 0.01M-Tris/HCl (pH 7.6). The extracts were combined and centrifuged for 60min at 105000g. The sediment was RNP complex, and the supernatant contained nuclear-sap proteins. The pellet after the 0.01M-Tris/HCl (pH 7.6) extraction step was used to isolate the DNP complex by the method of Huang & Huang (1969).

A linear 15–30% (w/v) sucrose gradient in 0.1M-NaCl/1mM-MgCl₂/5mM-phosphate buffer (pH 8.0) was used to separate the various RNP complexes (Spinco SW 27 rotor, 23000 rev./min for 15h). The gradient was removed from the top, and monitored continuously at 260nm with the use of a flow cell (1cm path length) in a Gilford spectrophotometer attached to a recorder. Fractions were collected with an ISCO fraction collector.

Isotopic-labelling studies

Incorporation in vivo of [³H]orotate into nuclear RNP complexes. Groups of two to four animals were injected intramuscularly with [³H]orotate (100μCi/100g body wt.) 60min before being killed. At the time indicated, nuclear RNP complexes were isolated from cycloheximide-treated and untreated rat livers, and radioactivities were determined and expressed as d.p.m./A₂₆₀ unit or d.p.m./mg of DNA. Incorporation *in vivo* of [³H]leucine (100μCi/100g body wt.) into nuclear RNP complexes of normal and cycloheximide-treated rats was carried out as described for [³H]orotic acid.

The effect of cycloheximide on the turnover of nuclear RNP complexes was determined as follows. Animals in groups of two or three were pre-labelled with [³H]leucine (100μCi/100g body wt.) for 16h before injection of cycloheximide. At 0, 30, 60 and 120min after cycloheximide administration, nuclear RNP complexes were isolated and radioactivities were determined.

The relative turnover rate of the nuclear RNP 60min after cycloheximide administration was measured by using a dual-labelling technique similar to the procedure described by Ch'ih *et al.* (1977b).

Radioactive leucine ([³H]leucine, 100μCi/100g body wt; [¹⁴C]leucine, 20μCi/100g body wt.) was injected intramuscularly 16h before injection of cycloheximide. The experimental design involved two sets of animals, each containing one control and one experimental. In set I, the treated animal was injected with [³H]leucine, and the control animal with [¹⁴C]leucine, whereas in set II these radioisotopes were injected in reverse. At 1h after cycloheximide treatment, the animals were killed and equal amounts of liver in each set were pooled; nuclear RNP was prepared.

Analytical methods

Proteins were determined by the method of Lowry, *et al.* (1951), with crystalline bovine serum albumin as the standard. DNA and RNA were determined as described previously (Ch'ih *et al.*, 1977a). Radioactivities were determined as described previously (Ch'ih *et al.*, 1977b).

U.v.-absorption spectra were obtained with a Perkin-Elmer 124 double-beam spectrophotometer. Sedimentation coefficients were determined by the equation given by McEwen (1967), by using ³H-labelled 16S and 23S RNA of *Escherichia coli* or 40S and 60S ribosomal subunits as markers.

Results

Characterization of nuclei and subnuclear fractions

When selecting methods for the preparation of nuclei, it was considered that the method of choice should be rapid, with a minimum damage to nuclei, and relatively free of contamination by cytoplasmic RNP complexes. A low recovery (16–20%) of relatively 'clean' nuclei was accepted in preference to a high yield of contaminated nuclei. Therefore nuclei isolated by the method of Blobel & Potter (1966) in the presence of 0.1% Triton X-100 were purified further by centrifugation in 1.75M- and 2.4M-sucrose. Examination by phase-contrast microscopy indicated that over 90% of the nuclei were intact, although a limited amount of nuclear damage was suggested by the presence of some free nucleoli. Furthermore, nuclei from cycloheximide-treated rat liver isolated under identical conditions showed no differences from controls. There were no statistical differences between control and treated livers in total cellular DNA, RNA and protein, and nuclear DNA and RNA. Nuclear protein content of the treated liver, however, decreased significantly (Table 1).

To examine the change in nuclear protein content after cycloheximide treatment, the procedures described by Umansky *et al.* (1976) and Huang & Huang (1969) were combined to fractionate nuclear-sap proteins, RNP complexes and DNP complex sequentially. These subnuclear fractions exhibited typical u.v. spectra. The contents of the subnuclear fractions (Table 2) showed no change in DNP complex between the control and treated livers. A significant decrease of RNP complexes (66%) and sap proteins (44%) was noted with the cycloheximide-treated animals, which is comparable with the decrease of total nuclear protein (Table 1).

Effect of cycloheximide on the synthesis of nuclear ribonucleoprotein complexes

When the incorporations of [³H]leucine into nuclear RNP complexes were compared (Table 3), the specific radioactivity (d.p.m./*A*₂₆₀ unit) of the

treated was similar to that of the control. When the change of nuclear RNP-complex content was considered by expressing the amount per mg of DNA, a significant difference was observed. These data suggest that greater isotope dilution occurs in the control animal. On the other hand, when [³H]orotate was used as the labelled precursor, the radioactivity (d.p.m./*A*₂₆₀ unit) showed a 6-fold increase in the treated animal. When calculated per mg of DNA, the rate of incorporation of [³H]orotate was stimulated by 32%. This is in agreement with what we have reported previously, that cycloheximide inhibited protein synthesis (Rothblum *et al.*, 1976; Ch'ih *et al.*, 1977a), but not the synthesis of poly(A)-containing mRNA found in the cytoplasm (Ch'ih *et al.*, 1977b). The different radioactivities shown in Table 3 suggest that, during the first hour of cycloheximide treatment, RNP complexes have been transported out of the nucleus before the injection of radioisotope.

Table 1. *Cellular and nuclear content of DNA, RNA and protein in the treated and untreated animals*
Data presented are means $\pm$ s.d., for the numbers of experiments shown in parentheses. **P* < 0.05. Treated animals received 2mg of cycloheximide/kg body wt. 2h before death.

	Content (mg/g of liver)			
	Untreated		Treated	
	Whole homogenate	Nuclear fraction	Whole homogenate	Nuclear fraction
DNA	1.73 $\pm$ 0.47 (12)	0.28 $\pm$ 0.12 (12)	1.85 $\pm$ 0.77 (6)	0.38 $\pm$ 0.11 (4)
RNA	7.58 $\pm$ 0.89 (32)	0.05 $\pm$ 0.01 (4)	7.77 $\pm$ 1.07 (13)	0.06 $\pm$ 0.02 (4)
Protein	157.7 $\pm$ 11.7 (10)	2.08 $\pm$ 0.19 (8)	148.8 $\pm$ 7.0 (3)	1.23 $\pm$ 0.12 (3)*

Table 2. *Nuclear content of deoxyribonucleoprotein complex, nuclear-sap proteins and ribonucleoprotein complexes of treated and untreated rat liver*

Data presented and experimental conditions are as described in Table 1.

Condition	Deoxyribonucleoprotein complex (mg of protein/g of liver)	Nuclear-sap proteins (<i>A</i> ₂₈₀ /g of liver)	Ribonucleoprotein complexes (<i>A</i> ₂₆₀ /g of liver)
Untreated	0.69 $\pm$ 0.22 (7)	1.42 $\pm$ 0.49 (3)	1.78 $\pm$ 0.87 (14)
Treated	0.57 $\pm$ 0.06 (3)	0.80 $\pm$ 0.39 (3)*	0.61 $\pm$ 0.19 (14)*

Table 3. *Effects of cycloheximide on the incorporation of [³H]leucine and [³H]orotate into nuclear ribonucleoprotein complexes*

Data presented and experimental conditions are as described in Table 1. Radioisotopes were given 1 h after the injection of cycloheximide, and animals were killed 60min later.

Condition	Incorporation of			
	[³ H]Leucine		[³ H]Orotate	
	(d.p.m./ <i>A</i> ₂₆₀ unit)	(d.p.m./mg of DNA)	(10 ⁻³ $\times$ d.p.m./ <i>A</i> ₂₆₀ unit)	(10 ⁻³ $\times$ d.p.m./mg of DNA)
Untreated	548 $\pm$ 154 (6)	3355 $\pm$ 846 (6)	3.1 $\pm$ 1.1 (4)	17.9 $\pm$ 6.2 (4)
Treated	614 $\pm$ 102 (4)	672 $\pm$ 132* (4)	19.1 $\pm$ 10.7* (4)	23.7 $\pm$ 8.6 (4)

To examine the sequence of formation, processing and transport of the nuclear RNP complexes, sucrose-density-gradient profiles of the nuclear RNP complexes isolated from the untreated and treated rat liver were compared. As shown in Fig. 1, untreated rat liver nuclear RNP complexes exhibited an absorbance profile containing particles with s values in the range 10–80S, which is similar to the profile reported by Louis & Sekeris (1976). On treatment with cycloheximide, however, complexes with higher sedimentation coefficients decreased, accompanied by an increase of a complex sedimenting at less than 20S. When [3 H]orotate was used to study the synthesis of RNA (Fig. 2), the 30S and 10–20S RNP complexes showed the highest amount of incorporation in the untreated rat liver, similarly to the report of Gros *et al.* (1977). In contrast with the normal, cycloheximide-treated rat liver showed a lower amount of label present in the larger complex. These results suggest the possibility of an enhanced rate of processing of the hnRNA to poly(A)-containing mRNA. The similar amount of radioactivity present in the 10–20S RNP complex exhibited by the untreated liver suggests that, once the subcomplex was formed, it may have been transported to the cytoplasm. These data are in agreement with the stimulated synthesis as shown in Table 3 and the increase of cytoplasmic poly(A)-containing mRNA (Ch'ih *et al.*, 1977b).

Fig. 1. Separation of ribonucleoprotein complexes of control and cycloheximide-treated rats by sucrose-density-gradient centrifugation

Equal amounts of untreated (----) and treated (—) nuclear RNP complexes were applied to a 15–30% sucrose gradient and centrifuged in the SW27 rotor at 23000 rev./min for 15 h. Sedimentation coefficients were determined with ribosomal subunits as markers and are indicated by arrows.

Fig. 2. Effect of cycloheximide on the incorporation of [3 H]orotate into the various nuclear RNP complexes. The experimental conditions were similar to those described in Fig. 1, except that [3 H]orotate (100 μ Ci/100 g body wt.) was injected 1 h before death. Sedimentation coefficients were determined by the method of McEwen (1967) and are indicated by arrows. ----, Untreated; —, cycloheximide-treated.

Effect of cycloheximide on the transport of the nuclear ribonucleoprotein complexes

As presented in Table 2, 2 h after cycloheximide treatment the nuclear RNP complexes were decreased drastically. In order to substantiate this decrease, the amount of nuclear RNP at 30, 60 and 120 min after the injection of cycloheximide was investigated. The approaches used were (1) a determination of nuclear RNP content on the basis of A_{260} /g of liver, (2) a determination of the radioactivity of the nuclear RNP pre-labelled with [3 H]leucine for 16 h, and (3) a dual-labelling technique to verify the processing and transport of the nuclear RNP complexes.

As shown in Fig. 3, the change in RNP content (A_{260} /g of liver) and radioactivity (d.p.m./g of liver) occurred within the first 60 min. There was no further decrease between 60 and 120 min after the injection of cycloheximide. This decrease seen in cycloheximide-treated rat liver may be due to activation of degradation processes, or to the isolation and purification

procedures. These possible artifacts were ruled out when dual-label experiments were conducted.

In Table 4, data obtained from dual-label experiments indicated a 33% decrease of nuclear RNP complexes, whereas the liver cellular protein content was only decreased slightly. These data further suggest that within the first hour of the cycloheximide treatment the nuclear RNP complexes were transported to the cytoplasm at a higher rate.

Fig. 3. Effect of cycloheximide on the content of nuclear ribonucleoprotein complexes

The animals were labelled with [³H]leucine for 16h before the administration of cycloheximide. At the times indicated, nuclear RNP was isolated and radioactivity determined as described in the Experimental section. Data presented were averages of at least two experiments. Δ, A₂₆₀/g of liver; ○, d.p.m./g of liver.

Furthermore, the relative amounts of nuclear RNP complexes of various sizes determined from sucrose-gradient fractions (Table 4) showed a 45% decrease of complexes greater than 40S, whereas the 30–40S complexes decreased by 18% and the amount of 10–20S complex was similar to that of the control. Preliminary results obtained from cycloheximide-treated cytoplasmic RNP complexes dissociated with EDTA *in vitro* (14–45mM) showed that a higher amount of RNP sedimented at about 20S. These data strongly suggest that, although the mRNA-containing RNP complex (10–20S) was transported out to the cytoplasm, there was concomitant conversion of larger RNP complexes containing hnRNA into the subcomplex where poly(A)-containing mRNA has been found (Quinlin *et al.*, 1974; Kish & Pederson, 1975; Quinlin *et al.*, 1977).

Discussion

There are extensive, but unfortunately inconsistent, reports dealing with the effect of cycloheximide on macromolecular synthesis. Some of the reported differences are due no doubt to the variety of different experimental designs used, including lethal doses versus non-lethal doses, short- and long-term observations, and studies *in vivo* versus *in vitro*. In our previous studies, we demonstrated that after the injection of a non-lethal dose of cycloheximide, which did not cause severe damage to the liver (Ch'ih *et al.*, 1976), the inhibition of cellular protein synthesis was reversible (Ch'ih & Devlin, 1974; Rothblum *et al.*, 1976) and was followed by an activation of the protein-synthetic system. A significant stimulation of plasma fibrinogen synthesis was observed as early as 12h after the injection of cycloheximide (Ch'ih *et al.*, 1977a). Furthermore, the adaptive mechanism involved not only the translational activity but also transcriptional events (Ch'ih *et al.*, 1977b), so that

Table 4. Relative amount of labelled protein in whole homogenate, nuclear ribonucleoprotein complexes and the complexes separated by sucrose density gradients

The dual-labelling technique was used in this experiment; animals were pre-labelled with either [³H]leucine or [¹⁴C]leucine for 16h before the injection of cycloheximide. In Set I, the [³H]leucine-labelled rat received cycloheximide, whereas in Set II the [¹⁴C]leucine-labelled rat was given cycloheximide. At 1h after the cycloheximide, the animals were killed and samples were processed as described in the Experimental section. Relative amount is calculated from the equation:

Relative amount = 100{([³H]/[¹⁴C])_{Set I} × ([¹⁴C]/[³H])_{Set II} }[†]

Fraction	Set I [³ H]/[¹⁴ C]	Set II [¹⁴ C]/[³ H]	Relative amount treated/control (%)
Whole homogenate	4.39	0.20	94
Nuclear RNP	2.37	0.19	67
From sucrose gradient			
10–20S	3.50	0.28	99
30–40S	3.36	0.20	82
>40S	3.07	0.10	55

cytoplasmic poly(A)-containing mRNA content and synthesis were stimulated during the inhibitory phase of protein synthesis.

The transport of mRNA out of the nucleus, a necessary event preceding protein synthesis, remains a poorly understood phenomenon. As mRNA is synthesized it is immediately complexed with protein while still associated with chromatin (Augenlicht & Lipkin, 1976). It is not clear if the role of protein is to effect this transport, or is restricted to other aspects of RNA maturation (Niessing & Sekeris, 1970, 1973; Blanchard *et al.*, 1975).

A difficulty inherent in the study of mRNA transport in the steady-state condition of the mammalian system is to identify the transport form and amounts of the nuclear ribonucleoprotein complexes. Results in the present paper show that the use of cycloheximide (2mg/kg body wt.) 1 h before death results in not only a decrease in total nuclear ribonucleoprotein complexes, but also a shift in the relative amounts of subcomplexes corresponding to the increase of cytoplasmic poly(A)-containing mRNA (Ch'ih *et al.*, 1977b). The stimulated transcriptional activity observed in the present work and the earlier report (Ch'ih *et al.*, 1977b) is not related to the activation of rRNA synthesis, which eliminates the use of additional inhibitors such as actinomycin D or α -amanitin to differentiate mRNA synthesis from that of rRNA. Thus cycloheximide can be valuable to study the transport of mRNA-containing nuclear ribonucleoprotein complexes, particularly the role of proteins involved in the transport process.

The protein and RNA moieties are known to have different metabolisms (Busch, 1975; Niessing & Sekeris, 1970; Lukanidin *et al.*, 1972). Studies *in vitro* have shown a sensitivity of ribonucleoprotein synthesis to cycloheximide (Biswas, 1978). The sensitivity of the protein component of the complex to cycloheximide reported in the present paper is in agreement with our previous work (Rothblum *et al.*, 1976), as well as the lack of sensitivity in the RNA component (Ch'ih *et al.*, 1977b). These findings suggest that, during the inhibitory phase of protein synthesis, the major effect on RNA metabolism is its lessened ability to complex with protein to form a functional ribonucleoprotein complex.

The heterogeneity in size and sedimentation coefficient of the nuclear ribonucleoprotein complex has been described extensively (Samarina *et al.*, 1968; Niessing & Sekeris, 1970; Faiferman *et al.*, 1971; Stevenin & Jacob, 1972; Karn *et al.*, 1977). Our data substantiate the existence of rapidly sedimenting particles, and suggests that cycloheximide affects their cellular amounts. Labelling studies reveal that these particles incorporate limited amounts of radioactive precursors, whether labelled for a short period (1h) or a long period (16h). We consider this consistent with what is known about some very slowly

turning-over protein and RNA components of the complex (Busch, 1975; Louis & Sekeris, 1976).

To eliminate artifacts caused by the observed isotope dilution in the control ribonucleoprotein complex, the complexes were labelled *in vivo* for 16h with [³H]- and [¹⁴C]-leucine, enabling us to evaluate the content of the complexes by using labelled proteins as a marker. Since the cycloheximide treatment caused a decrease in the labelled complexes greater than 40S, less of a decrease in the 30–40S complex and no change in the 10–20S complex, we believe that the complex progressively matured to the 10–20S form, which has been shown to contain poly(A)-containing mRNA (Karn *et al.*, 1977), and was transported to the cytoplasm. It is not likely that the lack of change in content of the 10–20S complex is due to a degradation process preceding its formation, since previous work has shown an increase in poly(A)-containing RNA in the cytoplasm at this time (Ch'ih *et al.*, 1977b).

Although the precise relationship between the nuclear and cytoplasmic RNP complexes is still unknown, it is now possible to gain some insight by using a protein-synthesis inhibitor such as cycloheximide to uncouple RNA synthesis from protein synthesis, allowing studies of mRNA biogenesis and transport in an intact animal system instead of experimental conditions restricted to mammalian cells in tissue culture. We are aware that the use of cycloheximide as a perturbing agent *in vivo* has limitations, but believe the system has promise as an animal model to study the nucleo-cytoplasmic interactions.

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