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Increased transcription and decreased degradation control the recovery of liver ribosomes after a period of protein starvation

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In the livers of 5-days-protein-depleted mice there is a decrease of 47% of the ribosome mass. When these animals are fed with an adequate diet, ribosome content is restored to the normal value after 1 day of re-feeding. The mechanisms underlying this phenomenon were studied. It was found that: (1) the activity of RNA polymerase I in the nuclei of livers from re-fed animals showed an enhancement of about 2-fold compared with the activity in normal and protein-depleted liver nuclei; (2) ribosome degradation, measured by the disappearance of radioactivity from ribosomal proteins previously labelled by the administration of $NaH^{14}CO_3$ to the mice, stopped during the first day after re-feeding.

In protein-depleted animals there is a great decrease of the liver mass. When these animals are fed with an adequate diet, the liver responds with a rapid restoration of its mass (Addis *et al.*, 1936; Leduc, 1949; Munro, 1964; Short *et al.*, 1974). In mice, liver protein is restored after 36h, and RNA somewhat earlier (Conde & Scornik, 1976). Since rRNA comprises most of the cell RNA, this experimental situation provides a suitable system for studying the control of rRNA mass in normal cells under a condition in which cell division is not pronounced.

The aim of the present paper is to study the mechanisms underlying the dramatic increase of rRNA after protein-depleted mice are fed with an adequate diet.

Experimental

Diets

Purina Rat Chow (pellets) containing 23% (w/w) protein was from Purina de Argentina S.A., Buenos Aires, Argentina. The Protein Depletion Diet (powder) was prepared as indicated in U.S.P. XV, except that dextrin was replaced by the same amount of sucrose. This is a protein-free diet.

Chemicals

[5,6-^{3}H]Uridine 5′-triphosphate (52 Ci/mmol) and $NaH^{14}CO_3$ (53 Ci/mol) were from The Radiochemical Centre, Amersham, Bucks., U.K. The α-amanitin was kindly provided by Professor Th. Wieland of the Max Planck Institute, Heidelberg, W. Germany. All other reagents were of the highest purity available.

Animals

Mice (male, 6 weeks old) were from a white strain bred by the Instituto Nacional de Farmacología, Buenos Aires, Argentina. On arrival, the animals were housed at 25°C in a room illuminated from 7:00 to 19:00h, and fed with rat chow and water *ad libitum*. They were kept for at least 1 week under these conditions. Protein deprivation was started at 19:00h by providing the Protein-Depletion Diet in special holders, in an amount sufficient to maintain feeding *ad libitum*; 5 days later, some of the mice were again fed with Purina Rat Chow.

Analytical procedures

Collection of the livers and determinations of protein, RNA, rRNA and DNA were as described by Scornik (1974). Liver ribosomes were isolated as described by Scornik (1974), but the procedure was adapted to process 3ml of liver homogenate obtained after homogenization of the livers with 3vol. of 0.3M-sucrose, containing 1mM-magnesium acetate and 0.1M-Tris/HCl, pH7.6. In all the experimental conditions tested, the recovery of RNA in the ribosomal fraction was $55 \pm 0.8\%$ (S.E.M.) (30 mice) of the total liver RNA. For radioactivity counting, ribosomal pellets were resuspended in 2ml of water, and 1.5ml of this suspension was mixed with 3ml of 10% (w/w) trichloroacetic acid and heated for

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30min at 90°C. The precipitate, collected by centrifugation for 10min at 2500*g*, was washed first with 3ml of 10% (w/w) trichloroacetic acid and finally with 3ml of ethanol/diethyl ether/chloroform (2:2:1, by vol.). The washed pellet was dissolved in 0.5ml of 90% (w/w) formic acid and mixed with 10ml of a scintillation 'cocktail' containing 33% (v/v) Triton X-100 (Kobayashi & Maudsley, 1974). When indicated, ribosomes were isolated by the 'high-salt' procedure of Blobel & Sabatini (1971) as performed by McConkey (1974). In this case, the recovery of RNA in the ribosomal fraction was 39.8±0.9% (S.E.M.) (20 mice) of the total liver RNA.

Nuclei were isolated as follows: livers were homogenized in 6vol. of a solution containing 0.3M-sucrose, 1.5mM-$MgCl_2$ and 10mM-Tris/HCl buffer, pH7.9 (solution A). A volume of homogenate corresponding to 0.5g of liver was centrifuged for 5min at 800*g*. The pellet was resuspended in 6ml of solution A containing 0.4% (w/v) Triton X-100, layered over 2ml of a solution of 0.88M-sucrose containing 4mM-$MgCl_2$ and 20mM-Tris/HCl, pH7.2, and centrifuged for 20min at 1000*g*. The nuclear pellet was resuspended in the medium to be used for the RNA polymerase I assay. All of these procedures were performed at 4°C. The DNA recovery was 44.5±1% (S.E.M.) of the total tissue DNA for the different experimental conditions.

DNA-dependent RNA polymerase I (RNA nucleotidyltransferase, EC 2.7.7.6) in isolated nuclei was assayed by measurement of the incorporation of [^{3}H]UTP (200–400Ci/mol) into RNA as described by Iapalucci-Espinoza *et al.* (1977). All the assays were performed in the presence of α-amanitin (5μg/ml). No further inhibition of the activity was found when 200μg of α-amanitin/ml was added, indicating that in our nuclei preparation the α-amanitin-insensitive activity corresponds to RNA polymerase I (Weil & Blatti, 1976). Unless indicated, assays were incubated for 3min at 37°C. The reactions were stopped by pipetting a sample of the incubation to Whatman DE 81 paper discs. The papers were washed and counted for radioactivity as described by Cereghini & Franze-Fernández (1974). All assays were performed by triplicate. In preliminary experiments, it was found that the S.E.M. of the RNA polymerase I activity in nuclei from at least five different livers was less than 4.5% for control, protein-depleted or re-fed mice. Thus, in subsequent experiments, each assay was performed with pooled livers from five mice.

Injected solution

$NaH^{14}CO_3$ was dissolved in previously boiled 0.9% (w/v) NaCl to give a radioactivity of 0.4Ci/ml.

Results and discussion

Liver RNA and DNA content after nutritional changes

After 5 days of protein deprivation in adult mice, there is a loss of 47% of the liver RNA (Fig. 1*a*) and

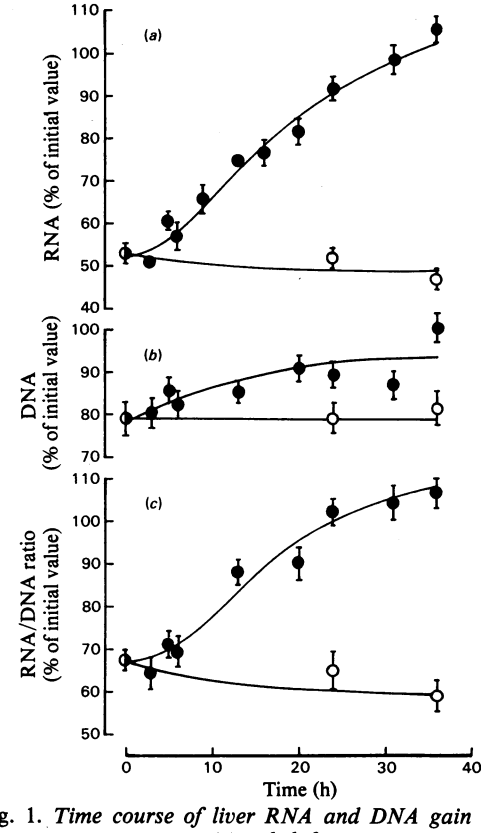

Fig. 1. *Time course of liver RNA and DNA gain after nutritional shifts*

Mice were fed on the Protein-Depletion Diet (U.S.P. XV) for 5 days. At zero time, some animals were returned to Purina Rat Chow (●), whereas others were kept on the same diet (○). The values for total liver RNA (*a*), total liver DNA (*b*) and RNA/DNA ratio (*c*) were expressed as percentages of the values in control animals, killed at the time at which Protein-Depletion Diet was first administered; 100% values were: (*a*) 44mg/100g initial body wt.; (*b*) 15.6mg/100g initial body wt.; (*c*) 2.8. Each point represents at least five animals; the S.E.M. is indicated by the bars, unless too small to be represented. Since the fraction of total RNA that is rRNA is 85% for all the experimental situations (see the text), the rRNA/DNA ratio for normal mice is 2.4. The rate of RNA/DNA gain after the nutritional shift was calculated as follows: the increment of the ratio over a 1-day period was divided by the value of the ratio in the middle of that period, and expressed as a percentage. The value in re-fed mice is 41%/day, equivalent to 1mg of rRNA/mg of DNA per day.

20% of the liver DNA (Fig. 1*b*). Therefore the RNA/DNA ratio falls to 67% of the normal value (Fig. 1*c*). When the protein-depleted mice were re-fed with a meal containing proteins, there was a rapid restoration of total liver RNA content. DNA restoration was rather slower, being completed only after 2–3 days of re-feeding (results not shown). At 24h after re-feeding, the RNA/DNA ratio was that of normal liver, and then tended to increase somewhat above normal values. The data reported above are in agreement with those reported by Conde & Scornik (1976).

The rRNA fraction of the total liver RNA was determined as described by Scornik (1974) in groups of five animals for each experimental condition, and was 86.0±1.3%, 82.0±3.0% and 89.0±3.0% in normal, protein-free and 16h-re-fed animals respectively (values are means±S.E.M.). From these results, we assume that rRNA constitutes 85% of the total RNA, in liver from either control mice or those subjected to nutritional changes. Thus, from the results in Fig. 1 it can be estimated that after 1 day of re-feeding the liver gained 1mg of rRNA/mg of DNA.

DNA-dependent RNA polymerase I activity

Possible mechanisms for the regulation of rRNA content include variations in the rate of rRNA transcription (Emerson, 1971; Green, 1974; Liebhaber *et al.*, 1978), in the efficiency of processing rRNA precursors, in the amount of newly formed rRNA that is discarded ('wastage') (Cooper, 1973), and in the rate of breakdown of mature cytoplasmic ribosomes (Hirsch & Hiatt, 1966; Weber, 1972; Abelson *et al.*, 1974; Melvin *et al.*, 1976). Measurement of transcription rates *in vivo* by the incorporation of a labelled nucleoside precursor into 45S rRNA may not represent the true rate of rRNA synthesis, owing to changes in the nucleotide pool size as consequence of the nutritional changes. This situation is further complicated by the fact that the nucleotide pools may be compartmentalized within the cells (Goody & Ellem, 1975; Dammgen & Scholtissek, 1975; Cortes *et al.*, 1979), and therefore the simultaneous measurement of the specific radioactivity of the total nucleotide pool of a tissue is unlikely to represent the actual specific radioactivity of the precursor pool. On the other hand, isolated nuclei conserve the ability to synthesize RNA. When the measurement of transcription is performed *in vitro* in the presence of an adequate amount of α-amanitin, the endogenous nuclear activity reflects mainly the elongation of RNA chains by RNA polymerase I molecules bound to the template to which the precursor molecules of rRNA are attached (Zylber & Penman, 1971). In this system the problem of determining the specific radioactivity of the nucleotide precursor pools is overcome, and for this reason it is widely used for studying transcription of ribosomal genes (Franze-Fernández & Pogo, 1971; Green, 1974; Grummt & Grummt, 1976; Cox, 1976; Bayley *et al.*, 1976). Fig. 2 shows that the kinetics of the RNA polymerase I activity is similar in nuclei from normal, protein-depleted and re-fed livers. The reaction stops very soon in all cases, suggesting that the elongation of RNA chains is the main activity that isolated nuclei retain. Normal liver nuclei incorporate 70pmol of UMP/mg of DNA in 10min, a value close to the 74pmol of UMP/mg of DNA in 10min reported for rat liver (Goldberg *et al.*, 1977). The initial rate of transcription and final plateau value are increased in nuclei from re-fed livers as compared with normal livers, whereas no difference between the latter and nuclei from depleted livers can be detected. These results were confirmed when heparin was included in the assay (results not shown). This drug prevents non-specific initiations of RNA chains (Iapalucci-Espinoza & Franze-Fernández, 1979) and at the same time suppresses any effect of ribonucleases (Cox, 1976).

The experiments in Table 1 show the RNA polymerase I activity in nuclei isolated at different times after re-feeding. At 5h, the enzyme activity was almost double the original value and was maintained elevated at least up to 31h. No increase in activity was found at 3h after the shift (result not shown).

Ribosome degradation after nutritional changes

It has been reported that rRNA and ribosomal

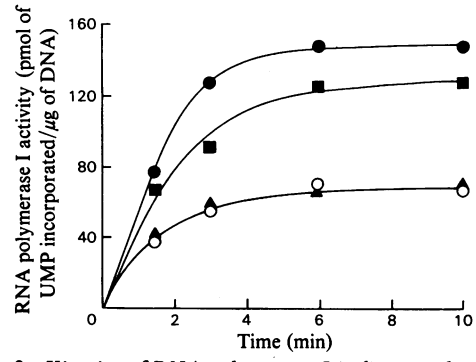

Fig. 2. *Kinetics of RNA polymerase I in liver nuclei after nutritional changes*

Mice were fed with the Protein-Depletion Diet for 5 days. Some of the animals were returned to the normal diet (Purina Rat Chow) for 13h (●) or 23h (■), whereas others were kept on Depletion Diet for 15h (○). (▲) corresponds to normal control animals. RNA polymerase I activity was assayed as indicated in the Experimental section. The points are means of three determinations of one sample of five pooled livers.

Table 1. *RNA polymerase I activity in liver nuclei at different times after the shift to a normal diet*
Protein-depleted mice were either re-fed with normal diet before the experiment, or maintained on the Protein-Depletion Diet. In all the experiments was included a group of normal control animals fed with Purina Rat Chow throughout. RNA polymerase I activity was determined as indicated in the Experimental section. Data are means of three determinations on one sample of five pooled livers.

Expt. no.	Condition	RNA polymerase activity (pmol of UMP/mg of DNA)	(% of normal)
1	Normal	68	100
	Depleted	66	98
	Re-fed, 5h	130	192
	Re-fed, 31h	122	180
2	Normal	70	100
	Depleted	70	100
	Re-fed, 13h	154	220
	Re-fed, 24h	119	170
3	Normal	60	100
	Re-fed, 6h	119	198
	Re-fed, 10h	117	196
	Re-fed, 16h	103	172

proteins from rat liver have the same rate of degradation (Hirsch & Hiatt, 1966). More recently, Tsurugi *et al.* (1974) extended this observation, by analysing the decay of individual proteins obtained from rat liver ribosomes. They found that all but three ribosomal proteins were degraded at the same rate, which was also the same as that of the rRNA molecules.

In HeLa cells and in yeast, most ribosomal proteins have uniformly high stability, with the exception of three or four, two of which appear to exchange with cytoplasmic pools of proteins (Warner & Udem, 1972; Lastick & McConkey, 1976). On the other hand, newly synthesized ribosomal proteins that are not combined in the nucleolus with ribosomal precursor RNA pass out of the nucleus and are unable to return (Craig, 1971; Maisel & McConkey, 1971; Wu *et al.*, 1971). The functional half-life of 'free' ribosomal proteins appears to be less than 5min (Wu *et al.*, 1971). In agreement with these findings is the observation that there is almost no ribosome assembly in the absence of protein synthesis (Warner *et al.*, 1966). These results support the notion that ribosomes are degraded as a unit (Tsurugi *et al.*, 1974) and that the proteins released from them are unable to be reutilized in the formation of new ribosomes (Warner, 1974). Ribosome degradation may thus be followed by measuring the decay of the rRNA or of ribosomal proteins previously labelled with a suitable precursor. When rat liver RNA is labelled *in vivo* with uridine, the half-life of cytoplasmic rRNA varies from 40 to 150h (Hadjiolov, 1966; Hirsch & Hiatt, 1966; Wilson & Hoagland, 1967; Blobel & Potter, 1968; Enwonwu & Munro, 1970; Kawada *et al.*, 1977). These results might reflect a different extent of reutilization of the labelled precursors. On the other hand, liver proteins can be labelled specifically into the guanidinium group of arginine by the administration of $NaH^{14}CO_3$, minimizing the extent of label re-incorporation. The advantage of using $NaH^{14}CO_3$ in studies of the degradation of liver proteins have been discussed extensively by Swick & Ip (1974), Scornik *et al.* (1978) and MacDonald *et al.* (1979). These considerations prompted us to study ribosome breakdown by labelling their proteins with $NaH^{14}CO_3$. Fig. 3(*a*) shows that the decay of ribosomes in normal liver follows first-order kinetics, with a half-life of 3.1 days. This value is almost identical with that reported by Scornik *et al.* (1978). Furthermore, the same decay value was obtained when the ribosomes were prepared by the 'high-salt' method of McConkey (1974), which renders a preparation free of non-ribosomal proteins, or by the method described by Scornik (1974). From Fig. 3(*a*) it was estimated that the fractional rate of degradation of normal liver ribosomes is 22%/day; this represents an absolute rate of 0.53mg of rRNA/mg of DNA per day, calculated by taking a rRNA/DNA ratio of 2.4 (see legend to Fig. 1).

Ribosome breakdown was also studied by the $NaH^{14}CO_3$ method in protein-depleted and re-fed livers (Fig. 3*b*). In the first day after re-feeding, there was a virtual shut-off of ribosome degradation. Radioactivity began to decline afterwards. Ribosomes from protein-depleted animals showed some increased degradation as compared with normal mice. This last result is in agreement with that of Kawada *et al.* (1977) in protein-deficient rats. The fractional rates of degradation of total liver proteins were also estimated (results not shown). For control mice it was 35%/day. Assuming first-order kinetics, for protein-depleted and re-fed mice the fractional rates of degradation were 46 and 19% per day respectively. These results are in agreement with those reported by Scornik & Botbol (1976) and Conde & Scornik (1976).

Since the livers of normal mice show no change in ribosome content, ribosome degradation should be balanced by the synthesis of new ribosomes. Hence the rate of rRNA synthesis (synthesis is meant to imply the production of 28S and 18S cytoplasmic rRNA) in normal animals is 0.53mg of rRNA/mg of DNA per day (Table 2, 'Synthesis' column). If it is assumed that the efficiency of processing and possible 'wastage' of the rRNA precursors are similar in all the experimental conditions studied, synthesis should be proportional to the rate of

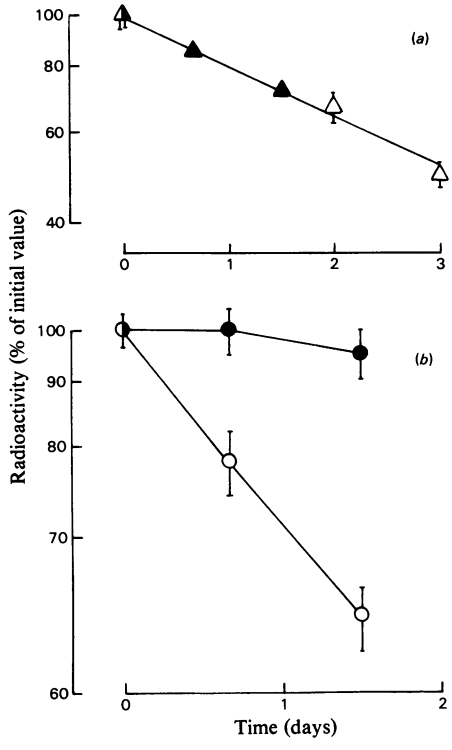

Fig. 3. *Disappearance of protein radioactivity from liver ribosomes labelled by the administration of $NaH^{14}CO_3$*
Each animal received 100μCi of $NaH^{14}CO_3$ 30h before zero time intravenously. At the times indicated on the abscissa, groups of animals were killed and the total liver ribosome radioactivity was determined (see the Experimental section). Results were expressed as percentage of the value at zero time. The bars indicate S.E.M.; the number of animals in each group is five for all points in (*a*) and ten for all points in (*b*). (*a*), Degradation of normal liver ribosomes. (▲), Ribosomes isolated by the 'high-salt' method (see the Experimental section). These ribosomes contained 48% of protein. (△), Ribosomes isolated by the method described by Scornik (1974). The protein content of these ribosomes was 54%. The 100% values were 8.4×10^3 d.p.m. in the 'high-salt' experiment and 7.0×10^3 d.p.m. in the experiment in which ribosomes were isolated by the method used by Scornik (1974). The rate of degradation was 0.22 day^{-1}. (*b*), At zero time, protein-depleted mice were either re-fed with Purina Rat Chow (●) or maintained on the Protein-Depletion Diet (○). Ribosomes were prepared as Scornik (1974). The apparent fractional rates of degradation (k) during the first 1.5 days were calculated on the assumption that the process follows first-order kinetics. For re-fed mice $k = 0.03\,day^{-1}$; for protein-depleted mice $k = 0.29\,day^{-1}$. It should be pointed out that the assumption of first-order kinetics is for comparative purposes. Strictly, the growth process does not follow first-order kinetics, owing to its transient nature.

Table 2. *Estimation of the rates of rRNA degradation from those of synthesis and gain*
Details are given in the text.

Condition	Rate (mg of rRNA/mg of DNA per day)		
	Synthesis	rRNA gain	Degradation
Normal	0.53	0.00	0.53
Re-fed, 24h	1.06	1.00	0.06
Depleted	0.53	−0.15	0.68

transcription. From the relative transcription rates in Table 1 and the rate of rRNA synthesis in normal animals, the synthesis rates for re-fed and protein-depleted mice can be estimated (Table 2, 'Synthesis' column). If the rRNA gain (calculated as indicated in the legend to Fig. 1) is subtracted from these values, it can be estimated that for re-fed animals the rRNA degradation is almost nil (Table 2, 'Degradation' column). This result is in good agreement with the experimental results depicted in Fig. 3, thus suggesting that the assumptions mentioned above on rRNA processing may be correct.

In conclusion, this study shows that liver restores the rRNA lost after 5 days of protein deprivation by re-feeding with an adequate diet for 24h. This dramatic increase can be accounted for by the enhancement of rRNA transcription and the suppression of ribosome degradation.

On the basis of the observation that normal fibroblasts in culture showed cytoplasmic rRNA turnover, whereas virus-transformed fibroblasts and established cell lines did not, Liebhaber *et al.* (1978) proposed that the shut-off of ribosome turnover may be a characteristic property of transformed cells. Liver cells, however, may transiently suppress ribosome degradation. Another example is the curtailment of cytoplasmic rRNA turnover in kidney cells during renal hypertrophy (Melvin *et al.*, 1976).

It is noteworthy that in our experimental conditions the transcriptive capacity of liver nuclei is unimpaired after protein deprivation. A decrease in the RNA polymerase I activity in the isolated nuclei has been reported after food deprivation (Coupar *et al.*, 1978) or partial protein starvation (Andersson & Von Der Decken, 1975) in rats. On the contrary, Bayley *et al.* (1976) found an enhancement of this enzyme activity after 2–3 days of protein depletion. We observed, in some experiments in which mice were starved of proteins for longer periods, that RNA polymerase I activity in liver nuclei increased by 30% or more over the normal value. After the shift to a complete diet, a further increase of about 2-fold over the activity in the protein-starved nuclei was always found. This additional effect suggests that different mechanisms may be involved in the enhancement of RNA polymerase I activity de-

scribed in this study and that observed under severe protein starvation.

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