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Initiation Factors in Protein Synthesis of Liver and Skeletal Muscle of Rats Force-Fed a Threonine-Devoid Diet[1] (37751)

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In earlier studies (1) young rats force-fed for 3–7 days a purified diet devoid of the single essential amino acid threonine developed many of the pathologic changes that resembled those found in children with kwashiorkor (2). The experimental animals developed morphologic alterations (3, 4) such as fatty liver with increased glycogen, atrophy of the pancreas, submaxillary gland, stomach, spleen and thymus, and a number of biochemical changes including enhanced protein synthesis in the liver (5–7) and decreased protein synthesis in the skeletal muscle (7, 8). The increase in hepatic protein synthesis both *in vivo* and *in vitro* in rats force-fed a threonine-devoid diet was associated with a shift of the average polyribosome size toward heavier aggregates (6, 9–11), whereas the decrease in skeletal muscle protein synthesis was accompanied by a decrease in the aggregates of polyribosomes with an increase in monomers (unpublished data). Further studies revealed that the increased protein synthesis in the liver and decreased protein synthesis in the skeletal muscle of rats force-fed a threonine-devoid diet were related predominantly to altered activities of the ribosomes (7, 9–11).

Recently evidence has been presented which shows that the initiation factors associated with ribosome preparations may in part regulate protein synthesis in several mammalian systems (12–18). Therefore, in view of the altered protein synthetic activities of hepatic and skeletal muscle ribosomes in rats force-fed a threonine-devoid diet in comparison with those force-fed a complete diet (6, 7, 9–11), this study was designed to investigate whether differences in activities of initiation factors could be involved.

Initiation factors were prepared from the ribosomes of livers and skeletal muscles of control and experimental animals and the relative effectiveness of these initiation factors on the stimulation of polyphenylalanine synthesis at low Mg^{2+} concentrations as well as on the formation of initiation complex were studied. The results indicate that the *in vitro* activities of initiation factors of livers increased and of initiation factors of skeletal muscles decreased in rats force-fed a threonine-devoid diet compared to those force-fed a complete diet.

Materials and Methods. Female Sprague–Dawley rats weighing 50–70 g were force-fed for 3 days a purified complete diet or one devoid of threonine as described previously (6). Each animal received three feedings (totaling 7 g) of diet daily. On the morning of the fourth day, the rats were sacrificed 16 hr after the last feeding. The livers and skeletal muscles (predominantly from thigh and leg muscles) were removed and ribosomes were prepared from these tissues as described earlier (6, 7, 9). Postmitochondrial supernatant (PMS) was obtained by centrifugation of homogenates for 15 min at 15,000 rpm in a Spinco 30 rotor. Ribosomes were prepared by centrifugation of deoxycholate-treated PMS for 3 hr at 30,000 rpm using a Spinco 30 rotor in a Model L3-40 Spinco ultracentrifuge.

Initiation factors and salt-washed ribosomes were prepared from hepatic and skeletal muscle ribosomes as described earlier (19).

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Ribosomal pellets prepared as described above were rinsed and resuspended in 2.5 ml Buffer A (containing 20 m*M* Tris–HCl (pH 7.4), 5 m*M* Mg^{2+}, 100 m*M* KCl, 1 m*M* dithiothreitol, 0.25 m*M* EDTA and 10% glycerol) and the ribosomal suspension was treated with a high-salt buffer (20 m*M* Tris–HCl (pH 7.4), 15 m*M* Mg^{2+}, 2 *M* KCl, 1 m*M* dithiothreitol, 0.25 m*M* EDTA and 10% glycerol) to a final concentration of 1 *M* KCl. The suspension was maintained at 0° for 1 hr with occasional stirring and then the ribosomes were pelleted at 48,000 rpm in a Spinco 50.1 rotor for 2 hr. The supernatant which contained initiation factors was removed, excess KCl was removed by overnight dialysis against buffer containing 20 m*M* Tris–HCl (pH 7.4), 5 m*M* Mg^{2+}, 200 m*M* KCl, 1 m*M* dithiothreitol, 0.25 m*M* EDTA and 15% glycerol, and then stored at —20°. To prepare salt-washed ribosomes, the pellets, obtained above after centrifugation of 1 *M* KCl-treated ribosomes, were resuspended in a buffer [containing 20 m*M* Tris–HCl (pH 7.4), 10 m*M* Mg^{2+}, 500 m*M* KCl, 1 m*M* dithiothreitol, 0.25 m*M* EDTA] and then centrifuged through 5 ml of 25% sucrose containing the above buffer for 5 hr at 40,000 rpm. The pellets were resuspended and dialyzed against Buffer A.

Labeled mouse hepatic phenylalanine-tRNA and methionyl-tRNA were prepared by the method of Moldave (20) and Skogerson and Moldave (21). pH 5 enzyme was prepared from mouse liver and was used as a source of tRNA and of aminoacyl-tRNA synthetases. tRNA was charged with either ^{14}C-(U)-L-phenylalanine (513 mCi/mmole) or L-methionine (methyl-^{14}C) (59 mCi/mmole) along with 19 unlabeled amino acids. A mixture of elongation factors I and II was prepared from pH 5 supernatants of livers and of skeletal muscles by the method of Moldave (20).

The assay for poly-(U)-directed polyphenylalanine synthesis was carried out as described earlier (19). The assays for the binding of ^{14}C-phe-tRNA and ^{14}C-met-tRNA to salt-washed ribosomes in the presence of initiation factors were carried out by the methods of Shafritz and Anderson (12) and Nirenberg and Leder (22).

Results. Polyphenylalanine synthesis. Polyuridylic acid directed polyphenylalanine synthesis by salt-washed mammalian ribosomes at low Mg^{2+} concentrations (3–5 m*M*) has been shown to require several protein factors which are recovered from 0.5–1.0 *M* KCl washings (12–19, 23). Several studies from our laboratory have shown that polymerization of ^{14}C-phe-tRNA is dependent upon salt-washed ribosomes, initiation factors, elongation factors, poly-(U) and GTP (19, 23). Under our experimental conditions, addition of initiation factors to the complete assay system caused a severalfold stimulation in the rate of polypeptide synthesis (19, 23). Initiation factors, elongation factors and salt-washed ribosomes were prepared from livers and skeletal muscles of rats force-fed a complete diet or a threonine-devoid diet for 3 days and assayed for their activities in polypeptide synthesis.

The results in Table I show that the complete incorporating system using components of livers of experimental rats stimulated polyphenylalanine synthesis to a greater degree than did the complete system using components of livers of control rats. However, in the case of skeletal muscle, the complete incorporating system using components of experimental rats stimulated polypeptide synthesis to a lesser degree than did the complete system of the control group. In order to investigate whether the initiation factors, ribosomes or elongation factors were responsible for the increased activity in the livers and the decreased activity in the skeletal muscles of the experimental animals in the stimulation of polyphenylalanine synthesis (Table I), these components from control and experimental animals were interchanged in the assay system and their activities in the stimulation of polyphenylalanine synthesis were compared. The results in Table I demonstrate that in the case of the liver, the higher activity of the experimental group in the polymerization of ^{14}C-phe-tRNA was attributed to the initiation factors of the experimental group. Initiation factors of the livers of the experimental animals exhibited 48% higher activity in initiation and sub-

TABLE I. Initiation Factor-Dependent Stimulation of Polyphenylalanine Synthesis in Livers and Skeletal Muscles of Rats Force-Fed a Complete Diet (C) or a Threonine-Devoid Diet (TD).

Ribosomes	Elongation factors	Initiation factors	^{14}C-Phenylalanine incorporation into protein from ^{14}C-phe-tRNA (%)[a] Liver	Muscle
C	C	C	100	100
C	C	TD	148	67
C	TD	C	106	76
C	TD	TD	152	42
TD	TD	TD	159	50
TD	TD	C	114	89
TD	C	TD	154	53
TD	C	C	102	101

Summary

Group	% Activity[b] Ribosomes	Elongation factors	Initiation factors
C	100	100	100
TD liver	104 ± 2.35	106 ± 3.17	148 ± 4.49[c]
TD muscle	106 ± 8.12	76 ± 4.10[c]	57 ± 2.17[c]

[a] The activity of the control assay containing components of livers or skeletal muscles of rats force-fed the complete diet was arbitrarily set at 100. The values represent a mean of 6 experiments. In each experiment, tissues from 10 to 15 rats of each group, were pooled.

[b] The activity of each fraction of the experimental group was compared with the corresponding fraction of the control group (100%). The values represent mean ± standard error of 12 to 16 observations.

[c] $P < 0.001$.

sequent synthesis of polyphenylalanine chains when compared to the initiation factors of livers of control animals. On the other hand, ribosomes and elongation factors from the livers of the experimental group exhibited similar activities as those of the control group. The results in Table I also show that in the case of skeletal muscle, the lower activity of the experimental group in polypeptide synthesis appeared to be due to initiation factors as well as to elongation factors. Initiation factors and elongation factors of skeletal muscle of the experimental group exhibited significantly lower activities in the synthesis of polyphenylalanine chains compared to those of the control group.

Binding of ^{14}C-phe-tRNA and ^{14}C-met-tRNA. Several laboratories have demonstrated that the initiation factors specifically promote the binding of phe-tRNA and met-tRNA to salt-washed ribosomes in the presence of a suitable template (12–18). In our laboratory the binding of poly-(U)-dependent ^{14}C-phe-tRNA and AUG-dependent ^{14}C-met-tRNA to salt-washed ribosomes in liver and hepatoma, which requires the presence of initiation factors, has been characterized (23).

Table II shows activities of initiation factors of livers and skeletal muscles of rats force-fed a threonine-devoid or a complete diet on the binding of ^{14}C-phe-tRNA and ^{14}C-met-tRNA to ribosomes. The results indicate that the initiation factors of the livers of the experimental rats promoted the binding of phe-tRNA and met-tRNA to a greater extent (activity increases of 60 and 92%, respectively) than did the initiation factors of the livers of control rats. On the other hand, in the case of skeletal muscle, the initiation factors of the experimental group exhibited significantly lower activity in similar binding studies compared to the initiation

TABLE II. Initiation Factors-Dependent Binding of ^{14}C-phe-tRNA or ^{14}C-met-tRNA to Salt-Washed Ribosomes of Livers and Skeletal Muscles of Rats Force-Fed a Complete Diet (C) or a Threonine-Devoid Diet (TD).

		^{14}C-phe-tRNA Bound (%)[a]		^{14}C-met-tRNA Bound (%)[a]	
Ribosomes	Initiation factors	Liver	Muscle	Liver	Muscle
C	C	100	100	100	100
C	TD	158	51	181	53
TD	TD	163	53	201	63
TD	C	104	106	99	99

Summary

	% Activity[b]			
	^{14}C-phe-tRNA Binding		^{14}C-met-tRNA Binding	
Group	Ribosomes	Initiation factors	Ribosomes	Initiation factors
C	100	100	100	100
TD liver	103 ± 6.37	160 ± 15.76[c]	105 ± 5.19	192 ± 8.97[d]
TD muscle	104 ± 7.93	48 ± 6.43[d]	109 ± 4.69	52 ± 3.55[d]

[a] The activity of the control assay containing components of livers or skeletal muscles of rats force-fed a complete diet was arbitrarily set at 100. The values represent a mean of 2–4 experiments. In each experiment tissues from 10–12 rats of each group were pooled.

[b] The activity of each fraction of the experimental group was compared with corresponding fraction of the control group (100%). The values represent mean ± standard error of 4 to 8 observations.

[c] $0.05 > P > 0.02$.

[d] $P < 0.001$.

factors of the control group. The data in Table II further demonstrate that in both livers and skeletal muscles of the control and experimental groups, salt-washed ribosomes did not exhibit any significant differences in the binding of the phe-tRNA and met-tRNA.

In previous studies (6, 9, 11) preparations of crude ribosomes obtained from the livers of rats force-fed a threonine-devoid diet showed increased *in vitro* protein synthesis in comparison to those of the livers of rats force-fed a complete diet. Such was also the case in the present study. However, in two experiments when salt-washed ribosomes instead of crude ribosomes were examined for cell-free protein synthesis, no differences were observed in the protein synthetic activity of the control and experimental groups. On the other hand, while the crude ribosome preparations of skeletal muscles of the experimental rats showed marked decreases in cell-free protein synthesis (7), the differences in the protein synthetic activity between control and experimental animals were diminished when salt-washed ribosomes were used. Sucrose density gradient studies of the salt-washed ribosomes of the livers and skeletal muscles of control and experimental rats revealed disaggregated polyribosomes of similar degrees in all cases, which were markedly different from the dissimilar patterns reported earlier using unwashed ribosomal preparations (6, 9–11).

Discussion. The results of this study indicate that the initiation factors of livers of young rats force-fed a threonine-devoid diet for 3 days show enhanced activity while the initiation factors and elongation factors of skeletal muscles of the same animals show decreased activities. Earlier studies have mainly stressed the importance of the alterations in the ribosomes or polyribosomes of the livers and skeletal muscles of the experi-

mental animals in enhancing or diminishing the protein synthetic mechanism (6, 7, 9–11). In this study it has been demonstrated that part of the dietary induced alterations in the activity of the crude ribosomal preparations may be related to nonribosomal factors, such as initiation factors, which can be removed from ribosomes by salt washes and which show altered activities in the experimental animals.

Alterations in the rate of protein synthesis at the translational level have been reported in response to nutritional changes (24). Studies with Ehrlich ascites cells by Van Venrooij, Henshaw and Hirsch (25) suggested that the rate of protein synthesis may be modulated by nutritional factors at the level of peptide-chain initiation and elongation in addition to substrate limitation. Alexis, Basta and Young (26) have reported a marked decrease in the protein synthetic activity of ribosomes of skeletal muscles of rats fed a low-protein diet and this decrease was attributed to nonribosomal factors associated with the ribosomes. Morgan *et al.* (27) and Jefferson *et al.* (28), using perfused heart preparations where they altered the supply of amino acids, have also concluded from their studies that peptide-chain initiation and elongation are likely points of control in the regulation of protein synthesis. In view of the findings of this study, it has been established that the activities of nonribosomal factors are altered in the livers and skeletal muscles of rats force-fed a threonine-devoid diet.

Based upon earlier studies using the livers of rats force-fed a threonine-devoid diet in comparison to the livers of rats force-fed a complete diet, we speculated that the transcriptional level of control may be responsible for the enhanced hepatic protein synthesis in the experimental animals (24). Evidence for this evolved from data indicating increases in the livers of total RNA, nuclear and ribosomal RNA, increased DNA-dependent RNA polymerase activity, increased *in vivo* incorporation of ^{32}P- or ^{14}C-orotate into ribosomal RNA, nuclear RNA and nucleolar RNA, and increased mRNA in the cytoplasm of the experimental animals (1, 24). Now, based upon the data of the present study, there is evidence that translational control may also be involved since initiation factors appear to have enhanced activities in the experimental animals. This may also play a contributory part in the overall enhancement of hepatic protein synthesis in rats force-fed a threonine-devoid diet.

Summary. Young rats were force-fed for 3 days a complete purified diet or one devoid of threonine. Initiating factors prepared from the ribosomes of livers of experimental animals stimulated polyphenylalanine synthesis and the binding of ^{14}C-phe-tRNA and ^{14}C-met-tRNA to a greater extent than those from the livers of control animals. In contrast, initiation factors and also elongation factors showed decreased activities in the skeletal muscles of experimental animals in comparison to those from control animals.

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