Heating RNA before cell-free translation is essential for the efficient and reproducible synthesis of several peroxisomal proteins

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Total RNA, extracted with guanidinium thiocyanate from liver of clofibrate-treated rats, was translated *in vitro*. Heating the RNA at 60°C for 5 min before translation increased the synthesis of three peroxisomal polypeptides 10–100-fold. Preproalbumin synthesis increased 10-fold. Total incorporation of [³⁵S]methionine into proteins merely doubled. Heating is essential for reproducible and adequate translation of mRNAs coding for peroxisomal and some other proteins.

Although heat denaturation of mRNA causes a doubling in the translational efficiency of ovalbumin mRNA (Payvar & Schimke, 1979) and of mouse myeloma mRNA (Leinwand & Ruddle, 1977), no selectivity towards individual mRNAs has been described. While investigating the cause of low and variable rates of synthesis of peroxisomal proteins *in vitro*, we found that heating total rat liver RNA before translation led to large increases in the translation of some mRNAs, including several coding for peroxisomal proteins. Some of these results have been communicated in abstract form (Mortensen *et al.*, 1984).

Materials and methods

Clofibrate [ethyl 2-(4-chlorophenoxy)-2-methylpropionate] was a gift from Ayerst Laboratory (New York, NY, U.S.A.). Male F-344 rats (Charles River, Wilmington, MA, U.S.A.) were fed on chow containing 0.5% (w/w) clofibrate for 3 weeks in order to cause peroxisomal proliferation (Hess *et al.*, 1965) and selective increases in peroxisomal proteins (Lazarow *et al.*, 1982). Total RNA was isolated from liver by the procedure of Chirgwin *et al.* (1979). The RNA was heated in Microfuge tubes in a water bath for 5 min (routinely at 65°C unless stated otherwise) and then rapidly cooled in an ice/water bath. The RNA (200 µg/ml, final concentration) was translated at 29°C for 90 min, in a micrococcal-nuclease-treated (Pelham & Jackson, 1976) rabbit reticulocyte-lysate system (Scheele *et al.*, 1980) with [³⁵S]methionine as label (1000–1300 Ci/mmol; New England Nuclear Corp., Boston, MA, U.S.A.).

Some peroxisomal translation products were

immunoprecipitated from 50 µl of translation mixture with goat antiserum prepared against rat liver peroxisomes (50 µl) and carrier peroxisomes (50 µg of protein) as described previously (Robbi & Lazarow, 1978). The immunoprecipitates were analysed by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis (Fujiki *et al.*, 1982) and fluorography on pre-flashed Kodak XAR-5 X-ray film (Laskey & Mills, 1975). The peroxisomal polypeptides isolated by this procedure include catalase, fatty acyl-CoA oxidase and the bifunctional protein possessing enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities (Lazarow *et al.*, 1982). The last-mentioned three activities are involved in peroxisomal β -oxidation of fatty acids (Lazarow, 1978).

Results

Heating rat liver RNA at 60°C for 5 min caused 84% increase in protein synthesis *in vitro* (Table 1) that was not uniformly distributed among the translation products (Fig. 1a). Several large [³⁵S]polypeptides (M_r 40000–80000) increased strikingly, including preproalbumin [M_r 68000 (Strauss *et al.*, 1977a,b)], which increased to 9.5 times the control value (Table 1). Most smaller bands showed little if any effect (arrows).

A number of peroxisomal polypeptides were immunoprecipitated (Fig. 1b). Heating caused large increases in the cell-free synthesis of several of these, including catalase (10-fold), acyl-CoA oxidase (41-fold) and the bifunctional hydratase/dehydrogenase (130-fold) (Table 1). Polypeptides that co-migrate with these three peroxisomal products are visible among the total translation

Table 1. Effect of heating RNA immediately before cell-free translation on its template activity

Polypeptide	Molecular mass* (kDa)	Radioactivity incorporated		
		Control (c.p.m./ μ l of translation)	Heated (c.p.m./ μ l of translation)	Ratio (heated/control)
Total translation products†		30000	55000	1.8
Immunoprecipitated peroxisomal polypeptides‡		(arbitrary units)§		
Hydratase/dehydrogenase	77	~ 2	260	~ 130
Acyl-CoA oxidase subunit A	72	11	450	41
Catalase	66	30	300	10
Band 1	44	99	220	4.4
Band 2	27	23	60	2.6
Preproalbumin¶	68	26	250	9.5

* Apparent molecular mass estimated by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, with standards of Lazarow *et al.* (1982), except for preproalbumin, for which the mass is taken from the published sequence data (Strauss *et al.*, 1977a,b). Rat liver preproalbumin migrates between hydratase/dehydrogenase and acyl-CoA oxidase subunit A (as does bovine serum albumin); this migration is anomalously slow for its size.

† Trichloroacetic acid-precipitable radioactivity after subtraction of incorporation in the absence of added exogenous RNA (7200 c.p.m./ μ l of translation mixture).

‡ Fig. 1, lanes 3 and 4.

§ Scanning densitometry of fluorogram of Fig. 1; area under peaks expressed in mm².

¶ Fig. 1, lanes 1 and 2.

Table 2. Effect of reheating RNA on its template activity *in vitro*

RNA was isolated by the guanidinium thiocyanate/CsCl/sodium dodecyl sulphate procedure of Ullrich *et al.* (1977) from free polyribosomes of clofibrate-treated rats. Batch I was divided into two portions at the end of the isolation procedure, and one was heated at 65°C for 5 min. Both portions were divided into smaller samples and frozen at -80°C overnight or for 3 weeks. These samples were thawed and translated *in vitro*, with and without heating immediately before translation. Batch II was frozen at -80°C for 9 months, and on numerous occasions it was thawed, samples were taken for translation, and it was refrozen. This RNA required heating for good translation, as usual. After 9 months of storage, a sample of this RNA was heated at 65°C for 5 min, refrozen overnight or for 3 weeks, and then translated, with and without reheating.

RNA	Radioactivity incorporated into total products*		
	Not heated immediately before translation (c.p.m./ μ l of translation)	Heated immediately before translation (c.p.m./ μ l of translation)	Ratio (heated/not heated)
Batch I			
A. Not heated at end of isolation procedure			
1. Frozen overnight	4220	6680	1.58
2. Frozen 3 weeks	3850	9140	2.37
B. Heated at end of isolation procedure			
1. Frozen overnight	7220	8300	1.15
2. Frozen 3 weeks	9410	8000	0.85
Batch II			
Heated 9 months after isolation			
1. Refrozen overnight	8700	9560	1.10
2. Refrozen 3 weeks	8060	9490	1.18

* Trichloroacetic acid-precipitable radioactivity after subtraction of incorporation in the absence of added exogenous RNA (8200 c.p.m./ μ l).

products if the RNA is heated before translation (Fig. 1, lane 2). Smaller immunoprecipitated translation products increased less (Table 1).

Maximal heat stimulation occurred at 60°C, and 5–10 min was required (Fig. 2). The 3 min lag

observed at 50°C may reflect the time required to warm through the Microfuge tubes. Individual translation products increased in parallel with time of heating (results not illustrated).

The heat-induced increase in mRNA translat-

Fig. 1. Translation products of guanidinium thiocyanate-extracted (Chirgwin *et al.*, 1979) RNA heated (+) or not heated (-) at 60°C for 5 min immediately before cell-free translation

Fluorograms of sodium dodecyl sulphate/polyacrylamide-gel-electrophoretic analysis of (a) total products (0.2 μ l) and (b) products immunoprecipitated from 50 μ l of translation mixture with antiserum against peroxisomes are shown. Identification of bands was as indicated by Lazarow *et al.* (1982) and unpublished work by R. M. Mortensen and by Y. Fujiki. Key: Alb, preproalbumin; Glo, globin; HD, hydratase/dehydrogenase; AOX, acyl-CoA oxidase subunit A; CAT, catalase. Fluorograms were exposed for 2 weeks.

Fig. 2. Incorporation of [35 S]methionine into total translation products as a function of (a) time and (b) temperature of heating RNA before cell-free translation

Endogenous incorporation of radioactivity into trichloroacetic acid-precipitable products without added RNA has been subtracted. (a) Heating at 50°C (●) or 65°C (○); (b) heating for 5 min.

ability is not abolished by freezing the RNA overnight or for 3 weeks at -80°C (Table 2). Peroxisomal proteins are synthesized equally well whether the RNA is heated 3 weeks before translation, 1 day before translation, immediately before translation or twice (sodium dodecyl sulphate/polyacrylamide-gel electrophoresis and fluorography; results not illustrated). These results suggest that, if RNA is heated for the preparation of poly(A)⁺ RNA, no further heating should be required.

The heating effect apparently depends on the method of RNA isolation. Total RNA prepared by the guanidinium thiocyanate/guanidinium chloride procedure of Chirgwin *et al.* (1979) consistently

required heating, although the magnitude of the heat-stimulation varied from 1.6 to 4 times the control value. On the other hand, in one experiment, RNA prepared from a postmitochondrial supernatant by extraction with phenol and chloroform (modification of Shore & Tata, 1977) did not require heating for good synthesis of peroxisomal proteins, although heating modestly improved (approximately doubled) their yield (results not illustrated).

The results reported here were obtained with RNA isolated from rats that had been treated with clofibrate to increase their formation of peroxisomal proteins (Mortensen & Lazarow, 1984). Similar results (not shown) have been obtained with RNA isolated from normal rats.

Discussion

Our findings agree with previous results (Payvar & Shimke, 1979; Leinwand & Ruddle, 1977) in that total cell-free protein synthesis doubles if the RNA is heated before translation. Unexpectedly, much larger increases of 10–100-fold occurred in the translatability of individual mRNAs, in particular ones coding for peroxisomal proteins.

The molecular basis of this heat effect is unknown. Heat might disrupt intramolecular secondary structure of the mRNAs that otherwise interfere with translation. Although 'snapback' of RNA structures is rapid at suitable salt concentrations, it is uncertain that snapback would occur under these conditions of rapid cooling in distilled water. Alternatively, heat may disrupt artifact intermolecular aggregates, e.g. with ribosomal RNA. Such aggregation, if random, would increase with mRNA size, explaining the observed size effect. Heat could also abolish interactions of mRNAs with other types of molecules (contaminants?) surviving the purification procedures, or there might be traces of a heat-labile nuclease.

From a practical viewpoint, heating RNA isolated by the guanidinium thiocyanate procedure (Chirgwin *et al.*, 1979; Ullrich *et al.*, 1977) before cell-free translation is essential to investigations that involve synthesis of peroxisomal proteins *in vitro*.

From a theoretical viewpoint, the fact that several mRNAs for peroxisomal proteins are far more responsive to heating than is the average mRNA raises the possibility that mRNAs of this class possess structural features of biological significance that are sensitive to thermal denaturation. Further experimentation is necessary to evaluate this possibility.

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