

REGULATION OF TYROSINE AMINOTRANSFERASE SYNTHESIS IN VITRO BY mRNA AND SOLUBLE FACTORS FROM HEPATOMA TISSUE CULTURE CELLS

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1. Introduction

In the last few years, evidence has been accumulated showing that gene expression in eukaryotic cells is not only regulated at the level of transcription, but also at post-transcriptional steps (reviewed [1]).

Tyrosine aminotransferase (TAT, EC 2.6.1.5.) has several distinctive features which have made this enzyme a convenient model for investigation of protein synthesis in hepatoma cells [2,3]. As an approach to the problem of regulation of TAT by glucocorticoids, we investigated the synthesis of the enzyme in cell-free systems where the translational control of specific mRNAs and the mechanisms in post-translational processing could be studied in detail.

In this paper, we have examined the *in vitro* synthesis of TAT in a homologous cell-free system from dexamethasone-induced or uninduced hepatoma tissue culture (HTC) cells. Since the rate of TAT synthesis is greater in extracts prepared from induced cells than from uninduced cells, we tried to determine which cellular components are required for the expression of this increased rate of synthesis. Our results show that induction enhances largely the TAT synthetic activity of the polysomal fraction; in addition, the soluble fraction from induced cells contains a dialysable component which optimizes TAT specific mRNA translation. These results are consistent with the observations made in whole cells which suggest that enzyme induction results from an increase in enzyme-specific mRNA and that TAT synthesis is also regulated at the translational level [4].

2. Materials and methods

2.1. Reagents

The synthetic glucocorticoid, dexamethasone, was kindly provided by Roussel-UCLAF Co. (Romainville). [³H]Leucine (40 Ci/mmol) was purchased from CEA (Paris). Pyruvate kinase, aurointricarboxylic acid and spermine were products from Sigma (St Louis, Mo.). Tissue culture media were obtained from Grand Island Biological Co.

2.2. Growth of cells

Suspension cultures of HTC cells were grown in S-77 media with 10% calf-serum [5]. For hormone induction, dexamethasone was added to final conc. 10⁻⁶ M, 12 h before harvesting the cells. TAT enzymic activity was assayed by the method in [6]. Protein concentration was determined by the Lowry method [7].

2.3. Preparation of 'S-20' 20 000 × g supernatant

The cells were harvested by centrifugation at room temperature at a density of 4–6 × 10⁵/ml and washed twice, once with ice-cold 0.14 M NaCl and then with buffer A (2.5 mM Tris-HCl, pH 7.6, 25 mM KCl, 5 mM MgCl₂). The cell pellet was resuspended in 1 vol. buffer and subsequent steps were performed at 4°C. After hypotonic swelling for 10 min, the cells were homogenised with 20 strokes in a Potter-Braun tissue grinder with a tight-fitting pestle. The homogenate was brought to 0.25 M sucrose (by adding about 0.3 vol 2 M sucrose solution), and centrifuged at 20 000 × g for 20 min. The overlaying lipid layer was removed and the remaining supernatant 'S-20'

fraction used in the amino acid incorporating system as in [8].

2.4. Preparation of dialysable factors

The pellet of induced cells was homogenised in 10% strength buffer A without sucrose and centrifuged at $105\,000 \times g$ for 60 min. The supernatant was dialysed (Visking tubing 18/32) under vacuum (400 mm Hg pressure). The dialysable material was concentrated to 0.1 vol. by lyophilisation. Mg^{2+} , Ca^{2+} , K^+ , Na^+ and Cl^- concentrations have been measured; they represented less than 5% optimal ionic concentrations used for cell-free incubation where 0.1 vol. the concentrated dialysate was added to 1 vol. freshly prepared 'S-20' supernatant.

2.5. Characterization of the translation products

In order to measure TAT synthesis, 5–10 ml reaction mixture were incubated before being centrifuged at $100\,000 \times g$ for 90 min. The labelled TAT was selectively precipitated from the released soluble proteins using specific anti-tyrosine aminotransferase antibody and carrier TAT as in [8]. To control the amino acid incorporation, 25 μ l samples were removed and spotted on Whatman 3MM disks, and the acid-precipitable radioactivity was determined by the method in [9]. Synthesis of ferritin was measured by radioimmunoprecipitation as in [10].

3. Results

3.1. Influence of the origin of the polysomes

The 'S-20' extracts prepared from dexamethasone-induced or uninduced cultures were centrifuged in order to separate the polyribosomes from the $105\,000 \times g$ supernatant fraction. The polyribosomes from both types of cells were resuspended in the supernatant layer obtained from either induced or uninduced cells. The 'recombined S-20' were incubated and assayed for total amino acid incorporation, released polypeptides, TAT and ferritin synthesis. The amount of radioactivity incorporated into total acid precipitable material by the 'recombined S-20' was ~66% that incorporated by unfractionated extracts (results not shown); this decrease is probably due to damage to polyribosomes or to their incomplete resuspension.

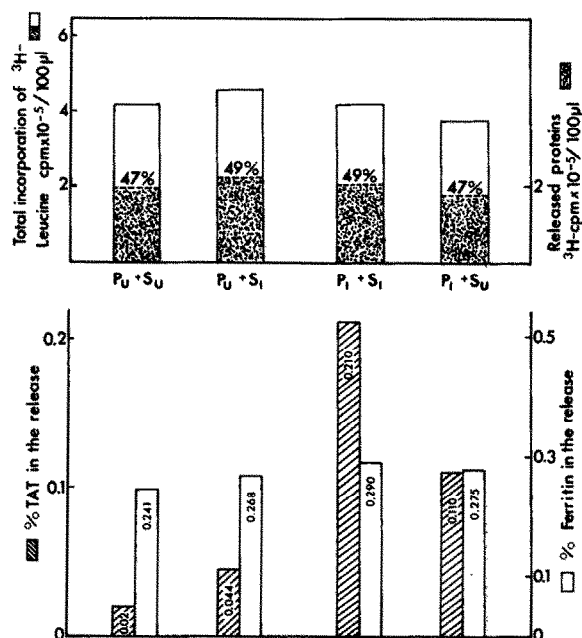


Fig. 1. Influence of the polyribosome fraction (P) and the $105\,000 \times g$ supernatant (S) from uninduced (U) and dexamethasone-induced (I) cells on the amino acid incorporation into proteins. TAT and ferritin synthesis were estimated by immunoprecipitation in 1 ml or 2 ml samples of the release as reported in section 2. The values are the mean of duplicate measurements.

As shown in fig. 1, for any recombination, the labellings were very similar in total proteins, in the released proteins and in ferritin which was chosen as an internal uninducible protein control [10]. The incorporation into TAT was 10-times greater in the homologous recombined extract prepared from induced cells ($P_I + S_I$) than in the corresponding preparation from uninduced cells ($P_U + S_U$). The activity of the cell-free systems reported here reflects the rate of TAT enzymic activity measured in whole cells at the time the extracts were made. TAT synthesis carried out by heterologous recombinations was determined largely by the origin of the polyribosome fraction. Nevertheless, the source of supernatant is not negligible. The addition of the supernatant from induced cells (S_I) to polysomes from uninduced cells (P_U) led to a 2-fold increase of the basal level; this result could be due to some messenger molecules in the supernatant from induced cells or to a difference

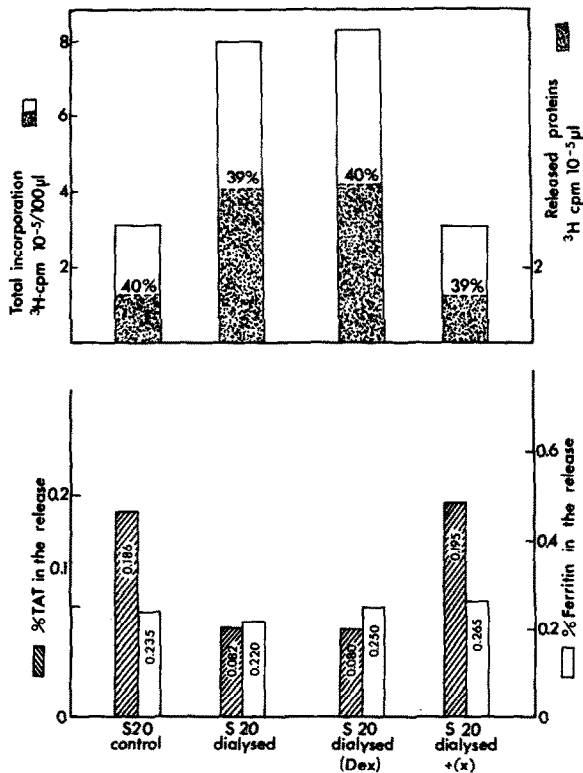


Fig.2. Influence of dialysis of the 'S-20' 20 000 X g supernatant extract. The extracts from dexamethasone-induced cells were dialyzed twice for 30 min (Visking tubing 18/32) against buffer A in the presence or in absence of hormone (Dex) prior incubation with [^3H]leucine. In the experiment represented in the last column, the concentrated dialysate (X) was added for incubation as described in section 2.

in the concentration of some unknown supernatant component. When the supernatant from uninduced cells was incubated with the polysomal fraction from induced cells, the amount of TAT synthesized diminished by 40–50% relative to optimal synthesis (P_I-S_I). Even so, TAT synthesis remains 4–5 times higher than the basal level.

3.2. Influence of dialysis

The dialysis of the induced 'S-20' extract diminished the internal pool of amino acids and consequently led to a higher (2-fold) incorporation of labelled amino acids (fig.2). In these conditions the synthesis of ferritin remained constant whereas TAT synthesis diminished significantly as compared to the control

(50–60%). As the maintenance of specific protein synthesis might depend on the continuous presence of the inducing hormone, dialysis was conducted in the presence of dexamethasone. Even in this case, TAT synthesis decreased in the same proportions. This result cannot be explained by a phenomenon of rapid degradation of the specific mRNA for TAT in the absence of the inducing hormone as observed [11] for the ovalbumin mRNA after withdrawal of oestrogen.

We next incubated the dialyzed 'S-20' extract from induced cells with a dialysate from induced cells. In these conditions, the specific rate of TAT was restored to the induced level (fig.2). When the dialysate was added to the 'S-20' extract from uninduced cells, we observed the same 2–3-fold increase in specific rate of TAT as that measured by adding the supernatant from induced cells to the polysomes from uninduced cells (P_U-S_I , fig.1).

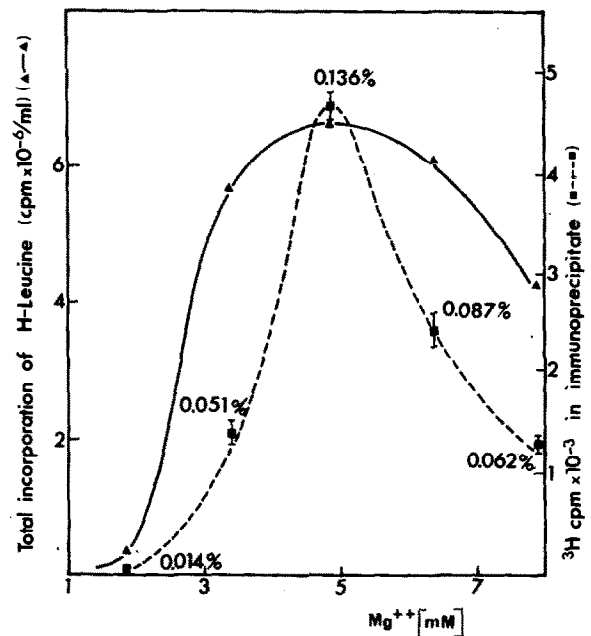


Fig.3. Dependence of TAT synthesis on Mg^{2+} concentration. Total protein synthesis (Δ — Δ) was measured by the amount of [^3H]leucine incorporated. Specific synthesis of TAT (\blacksquare — \blacksquare) was measured by immunoprecipitation and is expressed as % total protein synthesis for each given Mg^{2+} concentration (mean value of 3 different determinations).

Table 1
Effect of spermine on protein synthesis

Cellular origin of extract	[Spermine] (mM)	Radioactivity in released polypeptides (cpm/mg protein $\times 10^{-5}$)	% Total released radioactivity in TAT $\left(\frac{\text{cpm in TAT} \times 100}{\text{cpm in total released proteins}} \right)$
S20-I	—	2.6	0.20
S20-I dialyzed	—	5.1	0.11
	0.100	5.0	0.17
	0.200	3.7	0.16
S20-U	—	2.4	0.02
	0.100	2.3	0.04
	0.200	1.7	0.03

'S20' extracts prepared from dexamethasone-induced (I) and control (U) cells were harvested with [^3H]leucine and the radioactivity into the released protein fraction was determined as indicated in section 2. The radioactivity incorporated into TAT was measured by immunoprecipitation as in [9]

3.3. Nature of the dialysable component

Only circumstantial evidence can be provided about the real nature of the active component. It could not be precipitated with ethanol even in the presence of carrier RNA. The component resisted heating for 5 min at 80°C. Mixing with Norite [12], which normally binds nucleotides, did not retain the dialysable activity; these observations weigh against an involvement of small molecular weight RNAs as described [13,14].

Since the unidentified component was dialysable, its molecular weight must be relatively low. In view of these results and in connection with reports [15,16] dealing with the stimulatory activity of polyamines in cell-free protein biosynthesis, we investigated the effect of spermine in our system. Spermine was assayed at the Mg^{2+} concentration yielding maximum synthesis of TAT which also yields maximum incorporation of leucine into total proteins (fig.3). Table 1 shows that 1 mM spermine restored almost the activity of the dialyzed induced 'S-20' extract. Nevertheless spermine was less active than the dialysable component. Over 0.1 mM, spermine inhibited total protein synthesis.

4. Discussion

In vivo experiments had suggested that an accumulation of TAT-specific RNA [17] and an increase in the concentration of ribosomes engaged in TAT synthesis [18] occur on induction. These results imply that the concentration of active TAT mRNA is elevated in the induced state. The experiments described in the present paper are in accord with this idea, since the polyribosome fraction from induced cells is more active in cell-free TAT synthesis than the same fraction from uninduced cells, irrespective of supernatant content.

Our results indicate less translation (40–50%) of mRNA coding for TAT in the polysomes of HTC cells in the presence of 105 000 $\times g$ supernatant from uninduced cells or from induced HTC cells when the supernatant was pre-dialyzed. A similar lack of stimulation was observed with the supernatant from Zajdela hepatoma cells which are not inducible [19]. It is possible to restore the activity of the dialyzed extracts by adding back the concentrated dialysate to the incubation mixture. It therefore appears that the level of TAT synthesis may be regulated by compo-

nents other than the level of the specific mRNA for TAT. A positive translational stimulation of the TAT-mRNA superimposed on the transcriptional control might explain these observations.

The active soluble component(s) could be linked to the activity of polyamines. However, spermine, which is the most effective and acts at the lowest concentration for the *in vitro* synthesis of albumin [20], did not appear as active as the dialysable component itself. But spermine could require different Mg^{2+} concentrations for optimal TAT synthesis and total protein synthesis as observed for various proteins in other systems [20–22]. Involvement of polyamines in hormonal effects have been shown not only *in vitro* but also *in vivo*, especially during milk protein synthesis [23]. Further experiments would be necessary to investigate whether a hormonal control of polyamine synthesis (spermine, spermidine or putrescine) exists in HTC cells under glucocorticoid treatment.

Another question remains to be solved: at which step is the soluble component operative? The translational control of TAT synthesis could be elicited theoretically at initiation, elongation or termination. The synthesis of most major proteins is limited by initiation. In our post-mitochondrial hepatoma cell system, essentially completion of nascent TAT chains was occurring as aurin-tricarboxylic acid used at 0.1 mM, where it inhibits only initiation [24], leads to only a 10–15% inhibition of the endogenous protein synthesis. Thus, the active dialysable component probably acts at a step other than initiation. In order to clarify this point, we measured more complete TAT-mRNA translation in a heterologous cell-free system which is described in [25].

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