TRANSLATION OF TYROSINE AMINOTRANSFERASE mRNA FROM HEPATOMA CELLS IN A WHEAT GERM CELL-FREE SYSTEM

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1. Introduction

Since the initial reports of extraction of mRNA [1,2] and the development of a wheat germ extract [3], the translation of various eukaryotic mRNAs has been examined in several laboratories and the system developed has been applied to a wide variety of translation problems.

In this paper, we show that the RNA prepared from hepatoma tissue culture (HTC) cells directs the synthesis of tyrosine aminotransferase in a cell-free system derived from wheat germ. Immunoprecipitation of cell-free reactions demonstrated the synthesis of a product which was recognized by a specific antityrosine aminotransferase antiserum. Analysis of the immunoprecipitate on sodium dodecyl-sulfate-containing polyacrylamide gels showed the presence of two peaks. The major peak migrates like the enzyme synthesized in vivo or in vitro in cell-free extracts from hepatoma cells; the second peak represents a low molecular weight component which does not appear during in vitro protein synthesis in homologous extracts. The rate of the enzyme synthesized, clearly increased in the presence of total RNA from glucocorticoid-induced cells. Specific TAT messenger RNA from HTC cells is probably not a poly(A)-rich RNA and its translation is strongly dependent on the presence of polyamines and other soluble factors.

2. Materials and methods

2.1. *Reagents*

Wheat germs was purchased from Niblack (Rochester). Oligo(dT)-cellulose was a product from PL Biochemicals Inc. (Milwaukee); creatine phosphate, creatine kinase and spermine were from Sigma (St Louis, Mo.). [^{3}H]Leucine (40 Ci/mmol) and [^{14}C]leucine (280 mCi/mmol) were obtained from CEA (Paris). The synthetic glucocorticoid dexamethasone was kindly provided by Roussel-UCLAF Co. (Romainville).

2.2. *Growth of cells*

HTC cells were grown as in [4]. TAT enzymic activity was assayed by the method in [5]. Protein concentration was determined by the Lowry method [6].

2.3. *RNA preparation*

HTC cells were collected by centrifugation at 1000 × *g* for 10 min at 0°C, washed first with ice-cold NaCl 0.9% and thereafter with Tris buffer (25 mM KCl, 5 mM $MgCl_2$, 25 mM Tris–HCl, pH 7.2). The pellet was resuspended in 1 vol. Tris buffer with 100 μg/ml heparin and homogenised as in [4]. After centrifugation to remove nuclei and mitochondria, the supernatant was added to 2 vol. acetate buffer (1 mM EDTA, 0.5% sodium dodecyl-sulphate, 100 mM sodium acetate, pH 5). This mixture was extracted 3 times with phenol–chloroform (1:1, v/v). The total RNA obtained was ethanol-precipitated twice. After an extraction with 3 M sodium acetate buffer pH 6, 5 mM EDTA, the RNA was dissolved in 100 mM sodium acetate at pH 7 and ethanol precipitated.

Poly(A)-containing RNA was separated from total RNA using oligo(dT)-cellulose as in [2].

2.4. *Translation assays*

Wheat germ cell-free extract was prepared by the method in [7]. In vitro protein synthesis conditions were optimized for Mg^{2+}, K^+ and spermine. A 15 μl portion of wheat germ extract was added to start the reaction in total incubation vol. 50 μl containing 20 mM Hepes, 2 mM dithiothreitol, 1 mM ATP, 25 μM GTP, 8 mM creatine phosphate, 4 μg creatine phosphokinase, 100 μM spermine, 0.2 μCi [^{14}C]-leucine or 10 μCi [^{3}H]leucine, 2.5 mM magnesium acetate, 50 mM KCl, with 10 μg total RNA or 0.5 μg poly(A)RNA, at final pH 7. In order to stabilize TAT during the reaction, 0.1 mM pyridoxal-5-phosphate and 0.5 mM α-ketoglutarate were routinely included in the reaction mixtures. The incubations were performed at 28°C for 90 min. To control the amino acid incorporation, samples were removed and spotted on Whatman 3MM disks which were treated as in [8]. In order to measure TAT synthesis, 3–6 ml reaction mixtures were incubated.

2.5. *Characterization of the translation products*

At the end of incubation, 105 000 × *g* post-ribosomal supernatants were prepared. After partial purification as in [9], the labelled TAT was selectively precipitated from the released soluble proteins using specific anti-tyrosine aminotransferase antibody and carrier TAT. The immunoprecipitates were then characterized by SDS–polyacrylamide gel electrophoresis [10]. The amount of TAT synthesized was determined by quantitation of the ^{3}H radioactivity peak migrating with the mobility of TAT subunit.

3. Results

3.1. *RNA activity*

RNA extracted from HTC cells stimulated incorporation of [^{3}H]leucine into total proteins in the cell-free wheat germ system. Figure 1A shows that the addition of exogenous hepatoma cell total RNA to the assay mixture results in a linear incorporation of [^{3}H]leucine into trichloroacetic acid-precipitable peptide material for about 30 min, with the activity gradually diminishing over the following 90 min period. Increasing the amount of added RNA to 10 μg/ml resulted in a linear increase in amino acid incorporation into protein, but amounts greater than 20 μg/ml caused significant inhibition of protein synthesis (fig.1B). Incorporation was stimulated ~15 times over background. No significant differences were observed comparing the total template activity from hormone induced or uninduced cells.

The products synthesized in the presence of a

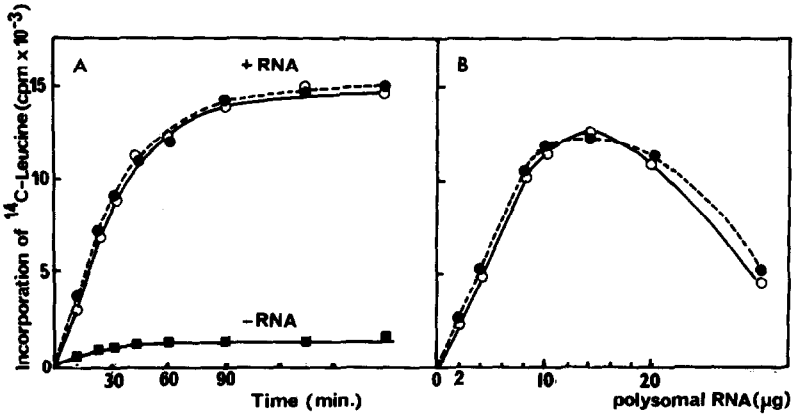

Fig.1. Characterization of the protein synthesis reaction. (A) Time course of protein synthesis. Standard translation assays were performed without (■——■) or with 10 μg total RNA from HTC cells untreated (○——○) or treated with dexamethasone (●- - - -●) for varying lengths of time. The trichloracetic acid precipitable radioactive material in 40 μl was measured. (B) The effect of mRNA concentration on protein synthesis. Standard translation assays were performed for 90 min with varying amounts of total RNA from dexamethasone treated (●- - - -●) or untreated (○——○) HTC cells; the labelled peptides were measured in 40 μl aliquots.

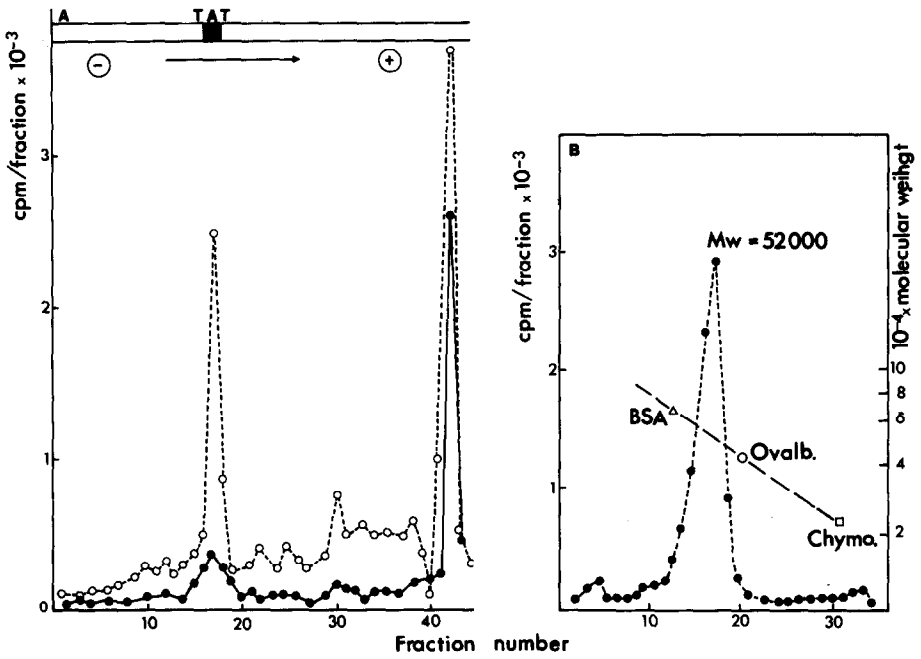

Fig.2. SDS–polyacrylamide gel electrophoresis profiles of immunoprecipitated tyrosine aminotransferase synthesized in vitro. Radioactive TAT was immunoprecipitated from 1 ml release containing polypeptide chains synthesized (A) in the heterologous wheat germ system in the presence of total RNA from control (●——●) or dexamethasone-treated (○- - -○) HTC cells, or (B) in the homologous cell-free extracts from dexamethasone-treated cells (●- - -●). Gels were sliced and counted. The inset represents the subunit Coomassie blue stain observed on a parallel gel for highly purified TAT from rat liver [13]. Molecular weights were determined with parallel gels containing protein standards, (△) bovine serum albumin, (○) ovalbumin, (□) chymotrypsin.

same amount of RNA from dexamethasone-induced or uninduced cells were immunoprecipitated with TAT-antibody and electrophoresed on SDS–polyacrylamide gels.

The electrophoretical patterns presented in fig.2A show the presence of a major peak of the mobility which corresponds to the Coomassie blue-stained subunit of highly purified TAT on a parallel gel. After the addition of RNA from glucocorticoid-treated cells, a 5–7-fold increase in radioactivity was measured in the region corresponding to the 52 000 dalton subunit of TAT. In all experiments, fast-migrating products were observed corresponding to polypeptides which co-precipitate with the TAT antigen-antibody complex; an important low molecular weight peak (of ~10 000) migrates close to blue marker dye front. These additional polypeptides appear at a constant rate with RNA from uninduced or glucocorticoid-induced cells, but they do not exist in the cell-free extracts from homologous HTC cells (fig.2B). These polypeptides might correspond to antibody-reactive fragments which have been commonly observed in the wheat germ cell-free system after the translation of various mRNAs where they represent either the result of endonucleolytically cleaved mRNA [11], premature termination of polypeptide chain synthesis [12] or degraded protein.

Chromatography of total RNA on oligo(dT)-cellulose was used to select for RNA. The non-bound RNA contained rather little total messenger RNA activity but about 50% of the specific mRNA for TAT. So far these experiments suggest that an important part of TAT mRNA contains only a small poly(A) segment. Thus, separation of mRNA from the bulk of ribosomal RNA by chromatography on oligo(dT)-cellulose did not prove to be a valuable step in the purification procedure for TAT mRNA from HTC cells.

3.2. *Sensitivity and dependence on added factors*

The stimulation of cell-free protein synthesis is dependent on the presence of spermine or spermidine. The above results were observed under optimal synergic concentrations of Mg^{2+} (2.5 mM) and spermine (0.1 mM) measured for total amino acid incorporation (fig.3). In absence of spermine, the total [^{3}H]leucine incorporation was only decreased by 30% whereas no TAT synthesis could be measured in the presence of mRNA from dexamethasone-induced or uninduced cells.

We tried to investigate the activity of dialysable compounds prepared from extracts of induced cells as in [4]. In these experiments, the RNA and the dialysate corresponded to a same amount of cells. In absence of spermine, the dialysable compounds can only partially replace the activity corresponding to spermine. When the dialysable compounds are added with spermine in the presence of RNA from induced cells, the counts precipitated during the TAT immunoreaction were equivalent, but the subsequent SDS–polyacrylamide gel electrophoresis revealed a higher amount of counts in the peak corresponding to TAT whereas a lower radioactivity was measured in the low molecular weight peak (fig.4); the 10–12-fold increase in radioactivity measured in the peak corresponding to TAT reflects the increase of TAT enzyme activity measured in the whole cells after glucocorticoid treatment.

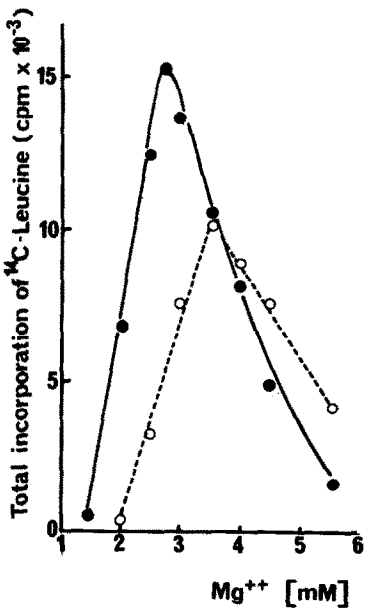

Fig.3. Effect of spermine on protein synthesis. Reactions were carried out for 60 min at optimum K^+ and RNA concentrations, varying Mg^{2+} concentrations, and in the absence (o- - -o) or presence (•——•) of 100 μM spermine. Radioactivity was measured in 40 μl aliquots.

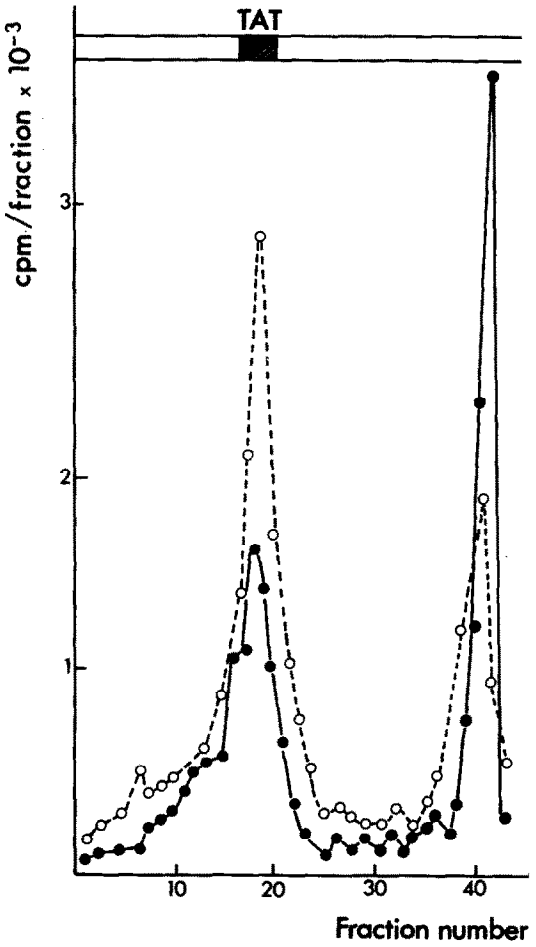

Fig.4. Effect of the dialysable component(s) from dexamethasone-induced HTC cell extracts on the synthesis of tyrosine aminotransferase analysed by SDS–polyacrylamide gel electrophoresis. Radioactive TAT was immunoprecipitated from 1 ml release containing polypeptide chains synthesized in the wheat germ system in optimized conditions, after addition of total RNA from dexamethasone treated HTC cells in the absence (•——•) or in the presence (o- - -o) of the dialysate from induced cell extracts.

4. Discussion

Our results show that the increased rates of tyrosine aminotransferase synthesis during dexamethasone induction can be primarily attributed to an increase of functional mRNA. Here we confirm with mRNA from steroid-sensitive hepatoma cells the observations in [14] where tyrosine aminotransferase mRNA was translated from rat liver in a wheat germ-derived system, and those in [15] in an oocyte system.

In addition, we observed that dialysable components from extracts of induced cells can increase the rate of TAT synthesis also in the heterologous system. These results suggest a post-transcriptional regulation which could be elicited at several steps, mRNA processing, ribonucleoprotein (mRNP)–mRNA interconversion, as well as at initiation, elongation or termination of protein synthesis. The possibility of a shift in the mRNA pool between non-translated mRNP particles and polysomes can be discarded as total cytoplasmic RNA used in our system gave the same results as polysomal RNA [14].

Our experiments suggest that the dialysable component(s) might hinder the formation of incomplete TAT chains, as a shift is observed from the low molecular weight peak to the peak with mobility which corresponds to TAT (fig.3). The polyamine dependency of the wheat germ system reported [12,16,17] combined with a synergic requirement of ion concentrations optima for the maximal translation of different mRNAs does not allow us to propose a precise mechanism for the action of dialysable component(s) from induced cell extracts. Further experiments are needed; these are actually in progress with the more efficient system described in [18]. Our finding of similar results in both the wheat germ and the hepatoma cell-free system indicates that our observations are not an artifact of a single system. The dialysable extract from induced cells might include more than one active component: a polyamine-like component, which could act on elongation by increasing the yield of full-length translation of specific messenger RNA as observed [11] for viral mRNA, as well as other active factors. Phosphorylated sugars have been shown recently [19] to stimulate protein synthesis and Met-tRNA$_f$ binding in mammalian cell extracts. It would be of interest to verify whether phosphorylated sugars influence the rate of cell-free synthesis of TAT. A determinant role of phosphorylation seems attractive as the enzyme TAT has been shown to be a phosphoprotein [20], a result we have confirmed in HTC cells. One could also consider a precise role of the phosphorylated sugars and perhaps of some specific kinase in the process of phosphorylation of nascent aminotransferase chains as proposed [21].

It has been reported that enzyme regulation in hepatomas may not be the same as in normal liver [22]. As compared to rat liver messenger specific RNA for TAT [14,15,18], the corresponding mRNA from HTC cells seems devoid of a large sequence of poly(A). Thus it remains to be shown if the post-transcriptional hypothesis is to be accepted as a general model for steroid regulation of TAT activity in other cell lines, either derived from normal liver or from hepatic tumor cells.

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