EFFECT OF THYROLIBERIN ON THE BIOSYNTHESIS OF POLY(A)-mRNA SPECIES IN GH_3 CELLS

Direct evidences for alterations in their distribution profiles and increased synthesis of the preprolactin mRNA

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1. Introduction

Continuous cell lines offer homogeneous populations of differentiated cells to study the intracellular mechanisms involved in the hormonal control of specific gene expression. Several 'GH' clonal strains derived from a transplantable rat pituitary tumor [1] have proved to be useful models for studying the secretion of two protein hormones, prolactin and growth hormone [2]. The 'GH' strains differ from each other in their functional characteristics such as basal level of PRL production [3,4] and response to a physiological modulator as thyroliberin (TRH: pGlu–His–$ProNH_2$) [5,6]. TRH induces a biphasic stimulating effect on PRL secretion by GH_3 cells [7] as well as by another pituitary cell line, SD_1 [8]. Previous works allowed us to correlate the level of PRL production by these cells and their intracellular content of PRL mRNA, as indirectly determined by either the PRL mRNA activity of total cytoplasmic poly(A)-RNA in cell-free translation systems [9–12], or the molecular hybridization of the specific sequences to complementary DNA probes [13].

Abbreviations: PRL, prolactin; GH, growth hormone; TRH, thyroliberin; mRNA, messenger RNA; poly(A), polyadenylated; pGlu–His–$ProNH_2$, pyroglutamyl–histidyl–proline amide; pGlu–His–Trp, pyroglutamyl–histidyl–tryptophane; EDTA, ethylene diamino tetraacetic acid; SDS, sodium dodecyl sulfate; poly(U), polyuridylic acid

* Part of these results was reported in the Sixth Meeting of the International Society for Biochemical Endocrinology (1978) [31]

However, the accumulation of a specific mRNA could be accounted for by an increased synthesis and/or a decreased degradation. Thus, we have developed experiments to investigate PRL mRNA turnover in basal conditions and following TRH stimulation. For that purpose, we have used a subclone of the GH_3 line, GH_3B_6, selected in our laboratory for its high level of [^{3}H]TRH binding sites [14]. Here, we provide direct evidence for the stimulation of the biosynthesis rate of the cytoplasmic PRL mRNA after TRH treatment of GH_3B_6 cells for 48 h. Moreover, this effect is specific for PRL mRNA since the synthesis of other poly(A)-mRNA species is only slightly or not at all increased*. These data strongly suggest that the increase of the PRL synthesis induced by TRH is mediated at least partially through a transcriptional effect and/or through the modulation of an early post-transcriptional step of the mRNA maturation.

2. Materials and methods

2.1. *Cell culture*

GH_3B_6 cells were grown in monolayer cultures in Ham F_{10} medium supplemented with 15% horse serum and 2.5% fetal calf serum without antibiotics. Cells were plated at 2×10^6 cells/10 ml per 10 cm-petridish and precultured for 6 days. The medium was renewed each 2 days and TRH (27 nM) was added at the last medium change. In order to get optimum stimulation of PRL synthesis by TRH the medium was not changed during the 48 h incubation (not shown). As control,

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we used a TRH analog (pGlu–His–Trp) inactive on PRL secretion [15].

2.2. *RNA labelling and extraction*

The procedure was essentially that in [16]. To study the rate of synthesis of cytoplasmic RNAs, 100 μCi [^{3}H]uridine (30 Ci/mmol) per dish were introduced, without medium change, for the last 4 h of the 48 h incubation. 2–3 petri dishes were used per experimental group and the experiments were repeated 3 times.

At the end of the pulse-labelling the cells were washed once with Ham F_{10} medium and twice with phosphate-buffered saline. They were harvested and allowed to lyse for 10 min with gently stirring in a solution containing 0.14 M NaCl, 10 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$ and 0.1% NP 40 detergent. After centrifugation to remove the nuclei, cytoplasmic RNAs were extracted and deproteinized ⩾3 times, with an equal volume of mixed chloroform–phenol–isoamyl alcohol (50:49:1, by vol.) in presence of 5 mM EDTA and 0.2% SDS. RNA was precipitated with 2.5 vol. ethanol and stored at –30°C.

2.3. *RNA analysis*

Cytoplasmic RNA precipitate was dissolved in a solution containing 0.1 M NaCl, 10 mM Hepes (pH 7.5), 1 mM EDTA, 0.1% SDS and layered onto 15–30% sucrose linear gradient. A minimum of 2 × 10^7 cells were required for RNA analysis. After centrifugation in SW 41 Ti rotor at 27 000 rev./min for 17 h at 20°C, the absorbance profile at 254 nm was recorded and 0.8–1 ml fractions were collected. Radioactivity incorporated into total and each fraction of cytoplasmic RNA was measured directly on aliquots (100 μl/fraction). Most cytoplasmic mRNAs containing an adenosine-rich sequence (poly(A)-mRNAs) were selectively hybridized (500 μl/fraction) onto poly(U)-glass fiber filters [17] and associated radioactivity was counted.

2.4. *Poly(A)-RNA directed cell-free protein synthesis*

GH_3B_6 cells were cultivated and incubated for 48 h, in the presence or absence of TRH, as described. 5 × 10^8–10^9 cells were harvested and rapidly frozen in liquid nitrogen. They were homogenized by mixing in 3 M LiCl, 6 M urea [18] and RNA allowed to precipitate overnight at 0–4°C. Then, RNA was pelleted by centrifugation at 20 000 × *g* for 30 min and deproteinized by phenol–chloroform extraction. Poly(A)-rich RNAs were further purified by 2 cycles of oligo-(dT)–cellulose chromatography [19] and then analysed on 15–30% sucrose gradient. Total and fractions of the poly(A)-RNAs were used to direct cell-free protein synthesis in a reticulocyte lysate prepared as in [20]. Incorporation of [^{35}S]methionine into total translated proteins was measured by radioactivity of acid-insoluble material.

2.5. *Analysis of cell-free translation products*

The 'in vitro' translation products were analysed by electrophoresis on SDS–20% polyacrylamide slab gels and fluorography as in [21,22]. Furthermore, immunoprecipitations were made by the double antibody procedure. Rabbit anti-rat PRL antiserum (prepared and kindly provided by D. Grouselle) was used as first antibody, and ovine anti-rabbit globulins antiserum as the second one. Immunoprecipitates were pelleted through a layer of 1 M sucrose as in [23] and, after dissociation by heat (5 min at 100°C) in presence of SDS, the proteins were further analysed by gel electrophoresis as above.

3. Results and discussion

3.1. *Effect of TRH on cytoplasmic RNA biosynthesis*

TRH treatment of GH_3B_6 cells for 48 h induced a 2–3-fold decrease of [^{3}H]uridine incorporation into cytoplasmic RNAs, and yet had either no effect or a slight stimulating effect on incorporation into poly-(A)-mRNAs (table 1). Moreover, when the radioactivity incorporated into poly(A)-RNA was expressed as % of total cytoplasmic RNA, a 3-fold increase of this ratio was observed in TRH-treated cells as compared to control cells, whatever was the basal level of RNA synthesis in the experiment (table 1). The decreased incorporation into total cytoplasmic RNA resulted, at least in part, from a reduction of the uridine nucleotide intracellular pool after chronic treatment of the cells by TRH [24], and thus the increase of incorporation into poly(A)-mRNA might be underestimated. Therefore, these data suggest that TRH increases the rate of synthesis of poly(A)-mRNAs whereas apparently decreasing those of most cytoplasmic RNAs.

By sucrose gradient analysis, the radioactivity associated with total cytoplasmic RNA extracted from control cells displayed 2 major peaks corresponding to the ribosomal RNAs (18 S and 28 S) as observed

Table 1
Incorporation of [^{3}H]uridine into total cytoplasmic RNA and poly(A)-RNA of GH_3B_6 cells grown for 48 h in the absence (control) or presence of 27 nM TRH

Treatment (48 h)	[^{3}H]Uridine incorporation into:		Poly(A)-RNA %
	Total cyt. RNA (pmol/A_{254})	Cyt. poly(A)-RNA (fmol/A_{254})	cyt. -RNA
I. Control	6.55	111	1.69
TRH	1.75	101	5.74
II. Control	11.55	59.3	0.51
TRH	5.40	88.5	1.64

Cells were labelled for the last 4 h and RNA were extracted as in section 2. Results of 2 independent expt. Radioactivity was expressed per absorbance unit (A_{254}), corresponding to 40–50 μg RNA

by absorbance. No modification was induced by TRH treatment of the cells (not shown). In contrast, the radioactivity associated with the poly(A)-mRNAs displayed several peaks and shoulders which were modified more or less depending on the duration of the TRH treatment (not shown). When the cells were treated for 48 h, the poly(A)-mRNAs displayed a 5-fold increase of the peak at ~12–13 S, whereas the other peaks (22–30 S) decreased more or less (fig.1). Thus the ratio of incorporated radioactivity into poly-(A)-RNA *vs* total cytoplasmic RNA was 10–15-fold increased for the 12–13 S peak whereas it was not modified or at the best increased 2 times for the other fractions of the gradient (fig.2). Treatment of GH_3B_6 cells (48 h) with pGlu–His–Trp (27 nM) did not significant modify the incorporation of [^{3}H]uridine into the cytoplasmic poly(A)-RNAs as compared to control cells (fig.2), in keeping with its failure to increase the PRL secretion. Thus, these results point out the ability of the TRH peptide to stimulate specifically the rate of biosynthesis of the '12–13 S' poly(A)-RNA. Indeed, the slight enhancement of the relative production rate of other poly(A)-mRNA species (22–30 S) resulted from the lower inhibition of their biosynthesis as compared to that of cytoplasmic RNA (table 1).

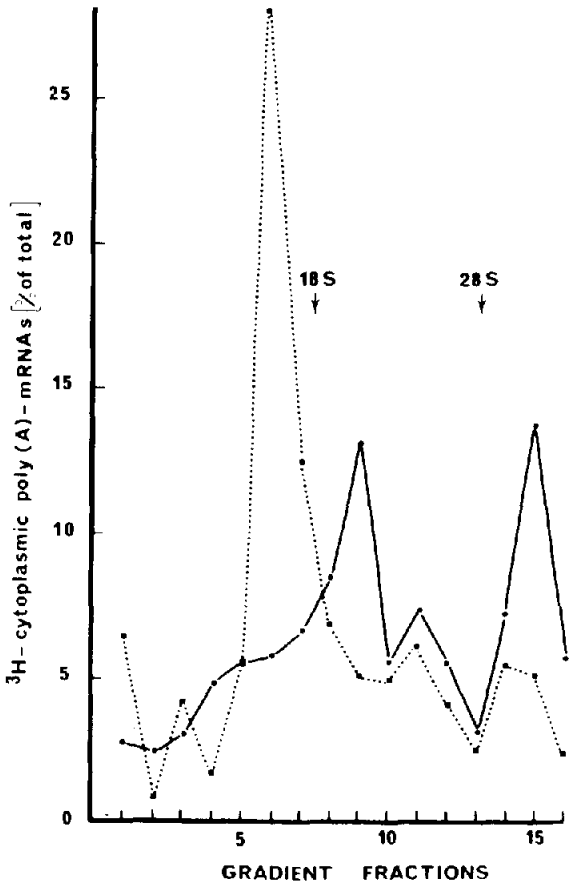

Fig.1. Sedimentation analysis of neosynthesized cytoplasmic poly(A)-mRNAs in GH_3B_6 cells grown for 48 h in the absence (●——●) or presence of 27 nM TRH (■· · ·■). Cells were labelled with [^{3}H]uridine for the last 4 h and RNA was extracted and analysed on 15–30% linear sucrose gradient [4 A_{254} units of total cytoplasmic RNA/gradient] as in section 2. The radioactivity of each poly(A)-RNA fraction was expressed as % of total radioactivity onto the gradient.

3.2. *Characterization of the '12–13 S' poly(A)-RNA*

Total poly(A)-RNA extracted from either control or TRH-treated cells directed the synthesis 'in vitro' of the same many proteins, except one protein synthesized in greater amount after TRH incubation of the cells (fig.3A). This protein has $M_r \gtrsim 25\,000$ as

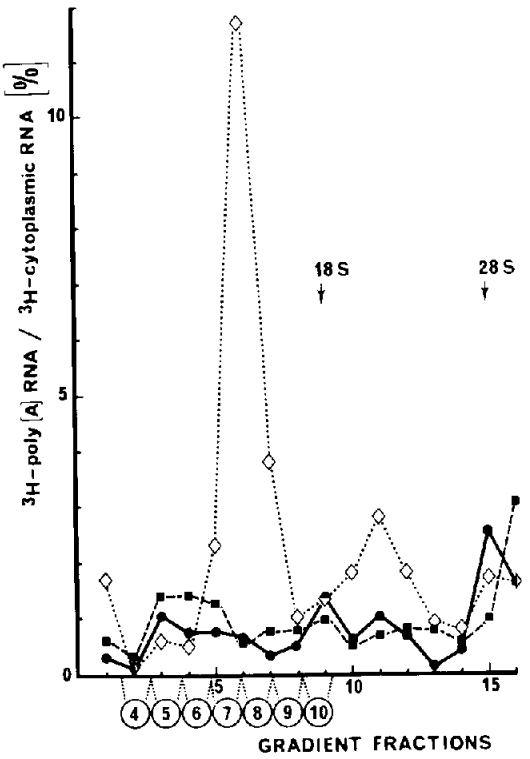

Fig.2. Relative rate of neosynthesis of poly(A)-mRNAs *vs* total cytoplasmic RNAs in each gradient fraction of RNA isolated from GH_3B_6 cells grown for 48 h in control medium (●——●), in medium containing either 27 nM TRH (◇· · ·◇) or 27 nM TRH analog (■– – –■) as in section 2. Circled numbers correspond to poly(A)-mRNA fractions assayed for their translational activity in fig.3.

compared to size markers. Moreover, this protein was the only one to be immunoprecipitated with the anti-rat PRL-specific antiserum (fig.3B). Therefore, it can be identified as the larger precursor form of PRL, the preprolactin (pPRL) already described as the intermediate nascent polypeptide during the biosynthesis of PRL in intact cells [11,25] and, as the initial product of cell-free translation [9,26,27].

The 'in vitro' translational activity has been assayed for every fractions of cytoplasmic RNAs separated by sucrose density gradient. Among the several gradient fractions of the poly(A)-RNAs extracted from TRH-treated cells only the fractions corresponding to the 12–13 S peak (fig.3A,B) displayed the capacity to direct the synthesis of pPRL. The homologous fractions of the poly(A)-RNAs extracted from control cells displayed less capacity of pPRL synthesis (fig. 3A,B).

These data are in agreement with previous work providing evidence that messenger activity coding for PRL lies in a specific mRNA of ~11–13 S purified from GH_3 cells [27] as well as from rat pituitaries [28,29]. Moreover, they provide a direct demonstration of the stimulation of PRL mRNA biosynthesis, and show that the TRH-induced shift concerns pre-

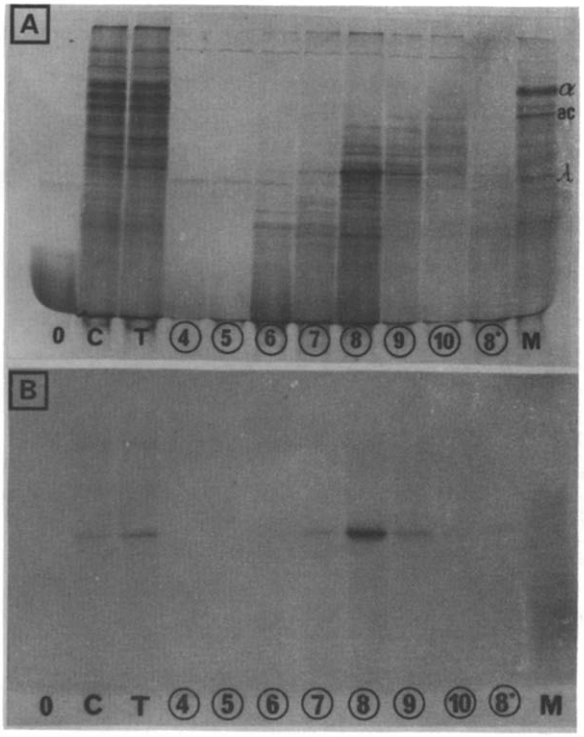

Fig.3(A). Analysis of cell-free translation products: total and fractions of poly(A)-mRNA were used to direct cell-free protein synthesis in a reticulocyte lysate system. The translation products were analysed using 20% acrylamide slab gels in the presence of SDS and fluorographed (see section 2). Products of translation directed by: lane 0, residual endogenous RNA; lane C, total poly(A)-RNA from control cells; lane T, total poly(A)-RNA from TRH-treated cells: lanes 4–10, gradient fractions 4–10 (see fig.2) of poly(A)-RNA from TRH-treated cells; lane 8*, gradient fraction of poly(A)-RNA from control cells, homologous to the lane 8 fraction of TRH-treated cells; lane M, total poly(A)-RNA from myeloma cells: α, α-immunoglobulin chain (M_r 60 000); ac, actin (M_r 43 000; and λ, λ-immunoglobulin chain (M_r 25 000); used as size markers.

Fig.3(B). The translation products were submitted to immunoprecipitation using a highly specific anti-rat prolactin antiserum. Immunoprecipitates were pelleted through a layer of 1 M sucrose, heat-dissociated in presence of SDS, and proteins were analysed by gel electrophoresis as above. The lanes correspond to those in the legend (A).

ferentially this mRNA species. The TRH-mediated regulation of PRL biosynthesis through the control of its specific mRNA production most likely represents the primary step in hormonal stimulation. This may take place at a transcriptional level or at one of the first post-transcriptional events setting in the nucleus, and thus may be related to the presence, already shown, of nuclear specific binding sites for [^{3}H]TRH in GH_3 cells [30].

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