Regulation of thymosin β10 expression by TSH and other mitogenic signals in the thyroid gland and in cultured thyrocytes

Giuseppe Viglietto, Daniela Califano, Paola Bruni, Gustavo Baldassarre, Maria Teresa Vento, Barbara Belletti, Monica Fedele, Giovanni Santelli, Angelo Boccia, Giovanni Manzo, Massimo Santoro[2] and Alfredo Fusco[1]

Istituto Nazionale dei Tumori di Napoli, Fondazione Senatore Pascale, Via M. Semmola, 80131 Napoli, Italy, [1]Dipartimento di Medicina Sperimentale e Clinica, Facoltà di Medicina e Chirurgia di Catanzaro, Università di Reggio Calabria, via Tommaso Campanella, 88100 Catanzaro, Italy and [2]Dipartimento di Biologia e Patologia Cellulare e Molecolare c/o Centro di Endocrinologia ed Oncologia Sperimentale del CNR, Facoltà di Medicina e Chirurgia di Napoli, Università degli Studi di Napoli 'Federico II', via Pansini, 5, 80131 Napoli, Italia

(Correspondence should be addressed to A Fusco, Dipartimento di Biologia e Patologia Cellulare e Molecolare, Facoltà di Medicina e Chirurgia di Napoli, Università degli Studi di Napoli 'Federico II', via Pansini, 5, 80131 Napoli, Italy)

Abstract

Objective: To investigate the expression of thymosin β10 – a small conserved acidic protein involved in the inhibition of actin polymerization – in human and experimental thyroid goiters as well as the regulation exerted by TSH on thymosin β10 expression in thyroid follicular cells both *in vivo* and *in vitro*.

Design: To this aim, we have used 5 bioptic specimens from patients affected by thyroid goiter, a well known experimental model of thyroid goitrogenesis (rat fed with the drug propylthiouracil) and a cultured rat thyroid cell line (PC Cl 3 cells) as a model system.

Results: We report that the mRNA expression of thymosin β10 is markedly enhanced in human goiters compared with normal thyroid. *In vivo* results showed that the steady-state level of thymosin β10 mRNA is up-regulated in the thyroid gland of propylthiouracil-fed rats in parallel with follicular cell proliferation: iodide administration to goitrous rats, which induced a marked involution of thyroid hyperplasia, reduced the mRNA level of thymosin β10. Finally, *in vitro* studies showed that in cultured rat thyrocytes, the expression of thymosin β10 mRNA is induced in a time- and dose-dependent manner by the activation of pathways which are mitogenic for thyroid cells (i.e. the protein kinase (PK) A and PKC pathways).

Conclusion: Taken together, the findings reported here demonstrate that thymosin β10 expression is regulated by extracellular signals that stimulate growth of thyroid cells both *in vitro* and *in vivo*, and suggest a role for this protein in thyroid diseases characterized by proliferation of follicular cells.

European Journal of Endocrinology **140** 597–607

Introduction

Enlargement of the thyroid gland during the development of goiters is characterized by extensive follicular cell hyperplasia associated with the occurrence of an abnormal hypervascularization (1).

Previous studies performed with experimental induction of iodide deficiency and/or antithyroid drugs (such as propylthiouracil) treatment of animals, demonstrated that increased pituitary secretion of thyrotropin (TSH) induced thyroid cell hyperplasia and a marked hypervascularization (2–4). The development of thyroid goiters involves dramatic modifications of the morphology and of the functions of both parenchymal and endothelial cells. The initial response to an increase of serum TSH consists essentially of follicular cell hypertrophy, loss of colloid and endothelial cell proliferation.

The mitotic activity of thyrocytes dramatically increases with a peak after 7–10 days from the beginning of the treatment; as a consequence, a huge increase in the total thyroid cell mass with development of a highly vascularized goiter occurs within a few weeks (2–4). Subsequent iodide administration to goitrous rats induces a rapid and marked involution of thyroid hypervascularity, followed by the regression of thyroid cell hyperplasia (5).

Such striking modifications of the morphology and of the functions of thyroid parenchymal or endothelial cells involves extensive remodeling of the cytoskeleton (6). Most molecules involved in the remodeling of cell skeletal structures remain to be defined; however, actin is likely to play a pivotal role in the maintenance of the structure and function of cells, since the dynamic equilibrium between monomeric

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and polymerized actin is crucial for cell motility and mitosis (7–9).

The β-thymosin family comprises small, highly conserved acidic proteins, which bind monomeric G-actin *in vitro* (10). Originally, thymosins were proposed to have immunomodulatory functions (11). However, subsequent studies suggested that β-thymosins may also have functions common to most cells (12). A recently proposed role for β-thymosins is the regulation of the cytoskeleton. In fact, thymosins $\beta4$ and $\beta10$ were shown to bind monomeric G-actin *in vitro* and *in vivo*, and thus to inhibit actin polymerization into stress fibers (13, 14) which, in turn, determine cell shape, exocytosis and the formation of adhesion plaques.

A number of investigations indicate that the expression of β-thymosins is relatively high in actively proliferating cells, and that terminal differentiation provokes changes in the relative abundance of these proteins (15–21). Accordingly, the expression of thymosin $\beta10$ has been shown to be enhanced in different human tumors (22, 23) whereas the expression of thymosin $\beta15$ is enhanced in prostate cancer (24). In particular, it seems that thymosins $\beta4$ and $\beta10$ may play a role in the process of thyroid tumor development, since we have recently shown that thymosin $\beta10$ is highly over-expressed in human and experimental thyroid tumors (25), and other groups have observed increased expression of thymosin $\beta4$ in autoimmune thyroid diseases (Graves' and Hashimoto's disease) and in medullary thyroid carcinomas (26, 27).

In this study we report that the mRNA expression of thymosin $\beta10$ is markedly enhanced in human goiters compared with normal thyroid. Furthermore, we show that thymosin $\beta10$ expression is up-regulated by propylthiouracil feeding of rats and is down-regulated by iodide administration to goitrous rats. *In vitro* studies showed that the activation of the protein kinase A (PKA)-dependent pathway through TSH and forskolin, of the protein kinase C (PKC)-dependent pathway by 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) and of the receptorial tyrosin kinase (RTK) pathway by insulin, exerted positive regulatory effects on the mRNA expression of thymosin $\beta10$ in cultured rat thyrocytes. Taken together, these findings demonstrate that the expression of the thymosin $\beta10$ gene is regulated by signals that stimulate the growth of follicular cells of the thyroid gland, and thus suggest a role for thymosin $\beta10$ during goiter development.

Materials and methods

Tissue samples

Thyroid goiter samples were obtained after resection from patients who had undergone surgery both at the National Cancer Institute 'Fondazione Pascale', Naples, Italy and at the Laboratoire d'Histologie et de Cytologie, Centre Hospitalier, Lyon Sud, France. Bioptic specimens were divided into two equal representative parts: one was immediately frozen in liquid nitrogen until RNA extraction was performed; the other was fixed in 4% paraformaldehyde, embedded in paraffin and then processed for pathologist diagnosis. Patients were diagnosed as having nodular goiters. All patients had elevated serum TSH levels.

Cell lines and treatments

The rat thyroid cell line PC Cl 3 used in this study is a thyroid epithelial cell line derived from 18-month-old Fisher rats, and it is described by several authors (28–30). PC Cl 3 cells were grown in F12 medium (Sigma, St Louis, MO, USA) supplemented with 5% calf serum (Flow Laboratories, Costa Mesa, CA, USA) and a mix of six hormones: TSH, somatostatin, insulin, growth hormone, glycyl-hystidyl-lysine and transferrin (Sigma). Experiments with PC Cl 3 cells were performed by growing cells to semiconfluence and subsequently starving them in serum-free medium supplemented with 0.5% BSA for three days. Bovine TSH (Sigma), forskolin (Sigma), TPA (Sigma), insulin (Sigma), 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) (Sigma) and cycloheximide (Sigma), were diluted at the concentrations indicated in the Results section in serum-free F12 medium supplemented with 1% calf serum or 0.5% BSA. PC Cl 3 cells were analyzed for DNA content by flow cytometry. Cells were collected and washed in PBS. DNA was stained with propidium iodide (50 μg/ml) and analyzed with a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA) interfaced with a Helwett Packard computer (Palo Alto, CA, USA). Cell cycle data analysis was performed by the CELL-FIT program (Becton Dickinson).

In vitro *studies in proylthiouracil-fed and iodide-refed rats*

Thirty 8-week-old Fisher rats were fed with rat chow having a low iodide content. The goitrogen agent, propylthiouracil (PTU) was added to the drinking water at a concentration of 2 mg/ml. Thirty 8-week-old Fisher rats fed with normal chow were used as controls. After 1, 3, 8, and 16 days, the rats were anesthetized with ether, blood samples were taken from the jugular vein and rats were killed. TSH, tri-iodothyronine (T_3) and thyroxine (T_4) were evaluated by radioimmuno-assay using a rat-specific kit (Amity Amersham, Little Chalfont, Bucks, UK). Thyroid glands were removed and divided into two equal representative parts: one was weighed and subsequently frozen in liquid nitrogen until RNA extraction was performed, the other was fixed in 4% paraformaldehyde for 15 h at 4 °C, embedded in paraffin and then processed for immunoperoxidase staining.

To study the effects of iodide on thyroid hyper-plasia *in vivo*, twenty 8-week-old Fisher rats were fed

with rat chow having a low iodide content. PTU, at a concentration of 2 mg/ml, was added to the water for 16 days. Subsequently, PTU treatment was suspended and rats were given iodide in the water at a concentration of 3 μg/ml. As controls, 20 rats drinking normal water were used. After 1, 5 and 10 days, the rats were anesthetized with ether, blood was taken and animals were killed. Thyroid glands were removed and divided into two equal representative parts: one was immediately frozen in liquid nitrogen until RNA extraction was performed, the other was fixed in 4% paraformaldehyde for 15 h at 4 °C, embedded in paraffin and then processed for immunoperoxidase staining.

RNA extraction and Northern blotting hybridization

Total cellular RNA was isolated from cultured cell lines as described previously (31). RNA was extracted from frozen specimens by the CsCl cushion method (32) with minor modifications. Northern blots were performed essentially as described (33) using nylon Hybond-N membranes (Amersham Inc.), according to the instructions provided by the manufacturer. All cDNA probes were radiolabeled with a random primed synthesis kit (Multi-Prime, Amersham Inc.). Hybridization reactions were performed at 42 °C, in 50% formamide, 5% Denhardt's solution, $5 \times$ SSPE, 0.2% SDS and 100 μg/ml denatured sonicated salmon sperm DNA, with 2×10^6 c.p.m./ml hybridization solution. Filters were washed at 60 °C twice in $2 \times$ SSC, 0.2% SDS for 30 min and subsequently, for the stringent washes, twice for 30 min each in $0.2 \times$ SSC, 0.1% SDS. Filters were air-dried and exposed to autoradiographic film for 4 days in the case of thymosin β10 probe. The thymosin β10 probe and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) plasmid used in this study have been described elsewhere (25).

The homology between rat thymosin β4 and thymosin β10 nucleotide sequences in the region spanning the probe used is 61%. In order to be certain that the human thymosin β10 probe used discriminated between thymosin β4 and thymosin β10, we amplified either rat thymosin β4 or thymosin β10 from RNA extracted from PC Cl 3 cells using the following primers: 5′-TCGTCTCTCCTTGTTCG-3′ and 5′-CAATGCTCGTGGAATGT-3′ (for rat thymosin β4); 5′-GATCT-CGGGCTCGGAACGAG-3′, 5′-TTAGGAAATT-TCACCGC-3′ (for rat thymosin β10).

Amplified DNA fragments were cloned into pCR II vectors and sequenced to determine their identity. Subsequently, 0.01, 0.1 and 1 ng thymosin β4- or thymosin β10-specific PCR products, respectively, were loaded onto a 2% agarose gel, transferred to a nylon membrane and hybridized with the human thymosin β10 probe used in this study, in the same experimental conditions as described for Northern blot experiments.

Under these conditions the thymosin β10 probe used in this study recognized the rat thymosin β10 but not the rat thymosin β4.

Nuclear RNA isolation

The run-on *in vitro* assay was used on nuclei isolated from the rat PC Cl 3 thyrocytes. PC Cl 3 cells were grown to semiconfluence and starved for 48 h in F12 medium supplemented with 0.5% BSA. Exogenous TSH was added to starved PC Cl 3 cells at a dose of 1 mU/ml in F12 with 0.5% BSA. After 24 h stimulation with TSH, approximately 3×10^7 cells were collected by centrifugation at 2000 r.p.m. for 5 min, resuspended in lysis buffer (10 mmol/l Tris–HCl pH 7.4, 10 mmol/l NaCl, 3 mmol/l MgCl$_2$, 0.5% NP40) and incubated in ice for 20 min. Nuclei were isolated by centrifugation at 1600 r.p.m., counted and stored in storage buffer (50 mmol/l Tris–HCl pH 8.3, 40% (v/v) glycerol, 5 mmol/l MgCl$_2$ and 0.1 mmol/l EDTA) at a concentration of 10^8 nuclei/ml in liquid nitrogen. *In vitro* transcription was performed using 5×10^6 nuclei in a reaction buffer containing 10 mmol/l Tris–HCl pH 8.0, 300 mmol/l KCl and 5 mmol/l MgCl$_2$, and 150 μCi [^{32}P]UTP incubated for 60 min at 28 °C. Nuclear RNA was prepared as previously described (34).

Hybridization to immobilized plasmid DNA

Fifteen micrograms of the plasmid pGEM4Z (Promega Inc., Madison, WI, USA) and of the plasmids carrying human thymosin β10 and rRNA 18S were linearized, denatured with 2 mol/l NaOH, neutralized and applied to slot blot apparatus. Nylon filters (Hybond-N, Amersham Inc.) were subsequently fixed by UV irradiation. Hybridization reactions were performed at 42 °C, in 50% formamide, 5% Denhardt's solution, 50 mmol/l Na phosphate pH 7.0, $5 \times$ SSC, 0.1% SDS and 100 μg/ml denatured sonicated salmon sperm DNA, and 2.5 μg/ml pGEM 4Z plasmid, with 5×10^6 c.p.m./ml hybridization solution. Filters were washed at room temperature in $2 \times$ SSC, 0.2% SDS twice for 30 min and subsequently, for the stringent washes, twice for 30 min each in $2 \times$ SSC, 0.1% SDS at 60 °C, and for 30 min at 37 °C in 10 μg/ml RNAse in $2 \times$ SSC. Filters were air-dried and exposed to autoradiographic film for 15 days in the case of pGEM 4Z and of thymosin β10 probe, and for 1 day in the case of the rRNA 18S probe.

Immunoperoxidase staining and proliferation index in experimental goiters

Immunohistochemistry was performed using purified anti-proliferating cell nuclear antigen (PCNA) antibodies (DAKO, Glostrup, DK) at a concentration of 1 μg/ml. Control reactions were performed using control normal rabbit immunoglobulin G (IgG) at concentrations of 1 μg/ml. Incubation with anti-rabbit IgG and

avidin-biotin-peroxidase complex was carried out according to the supplier's conditions (Vectastain, Burlingame, CA, USA) followed by counterstain with Harris' hematoxylin. To determine the labeling index after PTU and PTU followed by iodide treatments, the number of follicular cell nuclei stained by PCNA antibodies and the total number of nuclei were calculated. In each of 2 separate experiments, nuclei were counted in 5 representative fields from 2 different sections. A minimum of 200 cells per field were counted. The percentage of nuclei that were labeled, the labeling index, was determined for each treatment. The averages with standard errors are reported in Fig. 2.

Results

Analysis of thymosin β10 mRNA expression in human thyroid goiters

To investigate the role of thymosin β10 in the development of thyroid goiters, we analyzed 5 human goiters for thymosin β10 expression by Northern blot hybridization. The results are reported in Fig. 1. Normal thyroid tissue expressed low levels of thymosin β10 transcript of 0.9 kb (Fig. 1). An increase of approximately 2- to 5-fold in the mRNA expression of thymosin β10 was observed in all (5/5) goiters analyzed, as measured by autoradiographic film scanning, indicating that over-expression of thymosin β10 is a frequent event in human thyroid goiters. Filters were normalized by hybridization with GAPDH.

Effect of PTU and iodide treatment on rat thyroid gland in vivo *and on thymosin β10 mRNA expression*

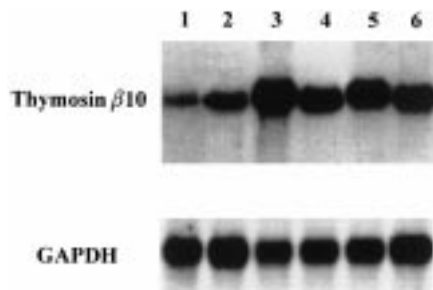To investigate the role of thymosin β10 during thyroid goiter development, we used PTU-fed rats as an *in vivo*

Figure 1 Analysis of thymosin β10 gene expression in human thyroid goiters by Northern blot. Twenty micrograms total RNA derived from normal thyroid (lane 1) and goiters from patients with Graves' disease (lanes 2–6) were loaded in each lane. The bottom portion of the figure shows hybridization with GAPDH to ensure RNA uniform loading and integrity.

model of thyroid goitrogenesis. In PTU-fed rats the serum levels of TSH significantly increased (day 0, 0.70 ± 0.12 ng/ml; day 16, 5.6 ± 0.11 ng/ml), and were accompanied by a marked enlargement of the thyroid glands, as demonstrated by the increase in the weight of the glands (day 0, 16.7 ± 1.8 mg; day 16, 48.2 ± 3.6 mg), and a marked reduction in the levels of the thyroid hormones T_3 (day 0, 0.7 ng/ml; day 16, 0.2 ng/ml) and T_4 (day 0, 5.1 mg/dl; day 16, 1.1 mg/dl). During the first weeks of goitrogen administration, there was a rapid change in the morphology of the gland: thyroglobulin was rapidly readsorbed, and this was accompanied by a corresponding decrease in the follicular lumen, since the epithelial cell compartment increased steadily and a marked increase in vascularity occurred (data not shown). To assess the extension and the timing of cell proliferation during the development of thyroid hyperplasia in PTU-fed rats, thyroids were analyzed by hematoxylin-eosine staining and by immunohistochemistry with anti-PCNA antibodies, which stain proliferating cells. Immunohistochemical analysis with anti-PCNA antibodies showed that a proliferation of thyroid follicular cells started from day 5 and peaked between days 8 and 16. The labeling index was calculated as described in Materials and methods and was expressed as a percentage of PCNA-positive cells compared with total cell number. The results are plotted in Fig. 2. As expected, follicular cells in the normal thyroid gland showed no staining for PCNA whereas about 7% and 13% of thyrocytes stained for PCNA after 8 and 16 days of treatment respectively.

Iodide refeeding to goitrous rats determined a marked involution of thyroid hyperplasia (5). In iodide refed rats, serum levels of TSH remained high for 1–5 days and decreased steadily after 10 days of treatment (day 0, 6.6 ± 0.15 ng/ml; day 10, 1.2 ± 0.16 ng/ml). The decrease in the TSH levels was accompanied by a dramatic reduction in the weight of the thyroid gland. The serum values of the thyroid hormones T_3 and T_4 returned to normal (0.7 ng/ml and 4.7 mg/dl respectively) on day 10 of iodide refeeding.

Thyroid glands of control rats expressed almost undetectable levels of thymosin β10 (Fig. 3, first lane) mRNA. Thymosin β10 mRNA expression started to increase on day 3 of PTU treatment with a peak at days 8–16 (Fig. 3, lanes 2–4). The kinetics of thymosin β10 up-regulation induced by increased levels of TSH in PTU-fed rats perfectly overlapped the timing of PCNA-labeling of thyroid follicular cell nuclei, suggesting that thymosin β10 may play a pivotal role in the proliferation of TSH-stimulated thyroid cells.

We also investigated the effects of refeeding goitrous rats with iodide on the mRNA expression of thymosin β10. Upon iodine refeeding to 16-day PTU-treated rats, thymosin β10 mRNA expression gradually decreased from day 1 to return to basal levels by day 10 (Fig. 3, lanes 5, 6), in parallel with the decreased proliferation
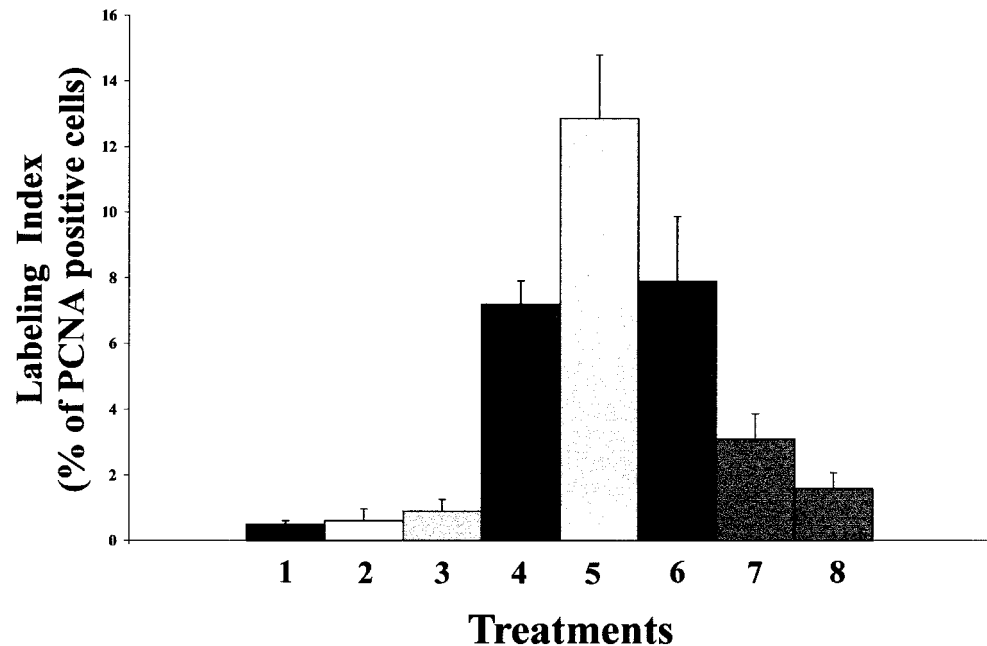
Figure 2 Comparison of the labeling index in follicular cells in the thyroid glands of rats treated with PTU or with PTU followed by iodide. The labeling index for each treatment was calculated as described. Paraffin sections from thyroids derived from normal rats (bar 1), from rats treated with PTU for 1, 3, 8 and 16 days (bars 2, 3, 4 and 5 respectively) or with PTU for 16 days followed by iodide administration for 1, 5 and 10 days (bars 6, 7 and 8 respectively) were used.

of thyrocytes (Fig. 2) and the consequent regression of thyroid hyperplasia induced by iodide.

The activation of the PKA-dependent pathway up-regulates mRNA expression of thymosin β10 in cultured thyrocytes

Our *in vivo* data suggested that the expression of thymosin β10 may be associated with the proliferation of thyroid follicular cells. Since TSH represents the major physiological modulator of thyroid growth and

Figure 3 Analysis of the mRNA expression of thymosin β10 in the thyroid gland of PTU-treated and iodide-refed goitrogenous rats, by Northern blot. Lane 1: normal thyroid glands from control rats; lanes 2–4: thyroid glands from rats treated with PTU for 3, 8 and 16 days respectively; lanes 5 and 6: thyroid glands from rats treated with PTU for 16 days and subsequently refed with iodide for 1 and 10 days respectively. Twenty micrograms total RNA were loaded in each lane. The bottom portion of the figure shows hybridization with GAPDH to ensure RNA uniform loading and integrity. The ratios of thymosin β10 mRNA to 18S RNA in PTU-fed rats compared with control rats (1 at day 0) were 1.8-, 6.4-, and 7.1-fold respectively on days 3, 8 and 16.

function, we first investigated the molecular effects of TSH on thymosin β10 mRNA expression, using the rat thyroid cell line PC Cl 3 as an *in vitro* model. PC Cl 3 cells are cultured rat thyroid cells which represent a suitable model to investigate the relationship between cell growth and differentiation, since they express all the molecular differentiative thyroid markers such as thyroglobulin, TSH receptor and thyroperoxidase (27–29) and are able to take up iodide from the culture medium.

RNA prepared from untreated cultures of PC Cl 3 cells contains small amounts of a single thymosin β10 transcript of 0.9 kb. Treatment of cells with 1 mU/ml TSH resulted in a rapid increase in the level of thymosin β10 mRNA, which peaked at 3–6 h and remained elevated until 48 h (Fig. 4). The increased expression of thymosin β10 mRNA induced by TSH was dose-dependent (cells were treated for 3 h with 0.01, 0.1 and 1 mU/ml TSH) as shown in Fig. 4. In a first set of experiments, we added TSH to the culture medium in the absence of serum to avoid possible interference of serum on TSH effects. However, similar results were obtained when TSH was administered in the presence of 1% calf serum (data not shown).

TSH modulates thyroid functions by binding to a high affinity receptor of the 'seven-spanned transmembrane domains' (TSHR) on the thyrocyte cell surface, whose activation increases intracellular cAMP levels, which in turn activate PKA as well as phosphatidyl inositol turnover in thyroid cells (6). To investigate whether the TSH-regulated induction of thymosin
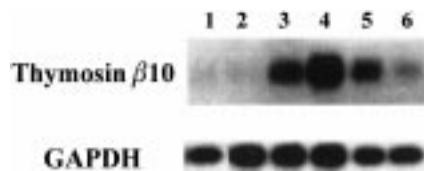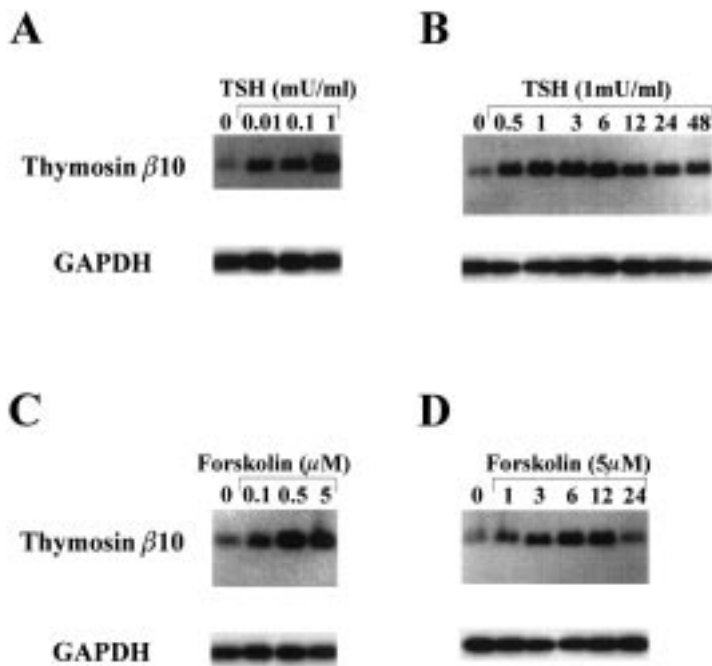
Figure 4 Regulation of thymosin $\beta10$ mRNA expression by the PKA-dependent pathway in the cultured rat thyroid cell line, PC Cl 3. Dose-dependent and time-course analyses of TSH regulation of thymosin $\beta10$ mRNA expression in rat thyroid cells are shown in the upper panels. (A) The levels of thymosin $\beta10$ mRNA are strongly induced by treatment of 3 day-starved PC cells with increasing amounts of TSH (0.01, 0.1, and 1 mU/ml) for 3 h (lane 1, untreated PC cells; lanes 2–4, treatment with the indicated concentration of TSH). (B) Thymosin $\beta10$ steady-state mRNA levels are markedly increased by treatment of PC cells with 1 mU/ml TSH (lane 1, untreated PC cells; lanes 2–7, TSH treatment for the indicated time (h)). Dose-dependent and time-course analyses of forskolin regulation of thymosin $\beta10$ mRNA expression in rat thyroid cells are shown in the lower panels. (C) The levels of thymosin $\beta10$ mRNA are strongly induced by treatment of 3 day-starved PC cells with increasing amounts of the PKA activator, forskolin (0.1, 0.5, and 5 μmol/l) for 3 h (lane 1, untreated PC cells; lanes 2–4, treatment with the indicated concentration of forskolin). (D) Thymosin $\beta10$ steady-state mRNA levels are markedly increased by treatment with forskolin (5 μmol/l) with a peak at 3–6 h (lane 1, untreated PC cells; lanes 2–4, forskolin treatment for the indicated time (h)). The bottom portions of the figure show hybridization with GAPDH to ensure RNA uniform loading and integrity.

$\beta10$ expression could be mediated by PKA, we evaluated whether thymosin $\beta10$ mRNA expression was induced by treatment with forskolin, which increases the intracellular level of cAMP. As shown in Fig. 4, treatment of PC Cl 3 cells with forskolin stimulated thymosin $\beta10$ mRNA expression in a dose-dependent manner. Time-course experiments (Fig. 4) at a dose of 5 μg/ml showed that the kinetics of induction of thymosin $\beta10$ mRNA induced by forskolin was similar to TSH, suggesting cAMP mediation in the induction of thymosin $\beta10$ mRNA expression exerted by TSH.

To correlate the induction of thymosin $\beta10$ mRNA by TSH and forskolin in PC Cl 3 cells with the extent of cell proliferation, we have also performed a flow cytometry analysis of TSH- or forskolin-stimulated PC Cl 3 cells. Cells were starved in medium containing 0.5% BSA and subsequently they were stimulated with 1 mU/ml TSH and with 5 μg/ml forskolin. At different times after stimulation, cells were collected

and analyzed by flow cytometry. Results are reported in Table 1.

The activation of different mitogenic pathways up-regulates mRNA expression of thymosin $\beta10$ in cultured thyrocytes

The activation of the PKC pathway by phorbol esters (TPA) has been shown to be mitogenic for cultured thyroid cells *in vitro*; however, in contrast to TSH, TPA also exerts dedifferentiating effects on thyroid cells (6). Therefore, to determine whether thymosin $\beta10$ mRNA expression was also regulated through pathways other than the TSHR/PKA cascade, we performed Northern blot analysis of TPA-stimulated PC Cl 3 cells. Analysis of TPA-stimulated thyrocytes showed that the steady-state level of thymosin $\beta10$ transcript increased in response to TPA stimulation in a dose-dependent manner, with maximal stimulation occurring at a concentration of 10 ng/ml (Fig. 5A). Furthermore, time-course analysis of TPA-induced thymosin $\beta10$ stimulation showed that thymosin $\beta10$ transcript began to accumulate as early as 1 h and peaked after 3–6 h from the beginning of the treatment (Fig. 5B). These results demonstrated that thymosin $\beta10$ mRNA expression was regulated by the activation of the PKC transduction pathway in a time- and dose-dependent manner.

Activation of membrane receptors with tyrosine kinase activity (RTK) is a common event in growth factor-stimulated, proliferating cells (34). In the case of thyroid cells, insulin has been shown to be mitogenic for cultured thyrocytes, possibly by interacting with

Table 1 Percentage of S phase PC Cl 3 cells. Cells were starved on 0.5% BSA for 3 days and subsequently treated with TSH (1 mU/ml), forskolin (5 μg/ml) and insulin (10 μg/ml) for the indicated times in the presence of 1% calf serum.

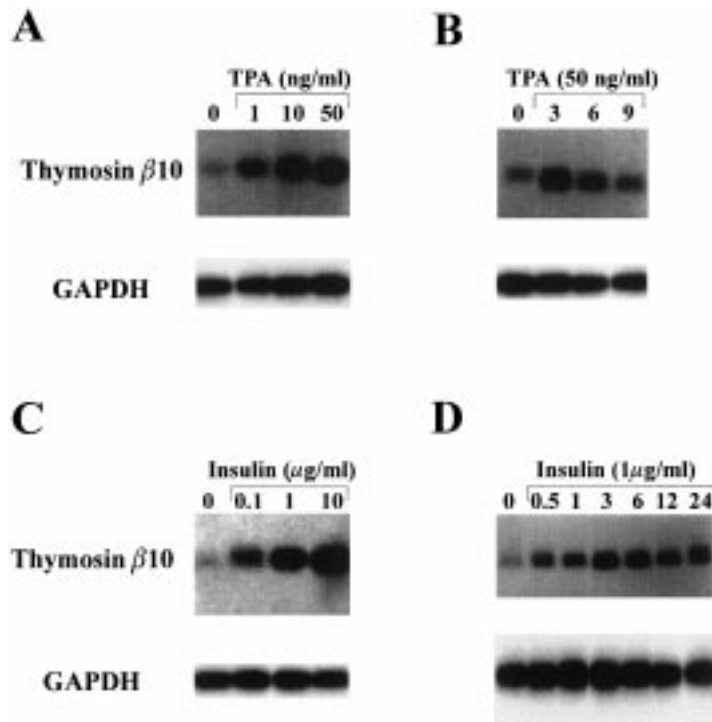| Treatment	**Time** (h)			
	0	12	24	48
TSH	6	11	28	14
Forskolin	6	8	19	10
Insulin	6	6	21	8

Figure 5 Regulation of thymosin β10 mRNA expression by mitogenic pathways in the cultured rat thyroid cell line, PC Cl 3. Dose-dependent and time-course analyses of TPA regulation of thymosin β10 mRNA expression in rat thyroid cells are shown in the upper panels. (A) The levels of thymosin β10 mRNA are strongly induced by treatment of 3 day-starved PC cells with increasing amounts of TPA (1, 10 and 50 ng/ml) for 3 h (lane 1, untreated PC cells; lanes 2–4, treatment with the indicated concentration of TPA). (B) Thymosin β10 steady-state mRNA levels are markedly increased by treatment of PC cells with TPA (50 ng/ml) with a peak at 3 h (lane 1, untreated PC cells; lanes 2–4, TPA treatment for 3, 6 and 9 h respectively). Dose-dependent and time-course analyses of insulin regulation of thymosin β10 mRNA expression in rat thyroid cells are shown in the lower panels. (C) The levels of thymosin β10 mRNA are strongly induced by treatment of 3 day-starved PC cells with increasing amounts of insulin (0.1, 1, and 10 μg/ml) for 3 h (lane 1, untreated PC cells; lanes 2–4, treatment with the indicated concentration of insulin). (D) Thymosin β10 steady-state mRNA levels are markedly increased by treatment of PC Cl 3 cells with 1 μg/ml insulin (lane 1, untreated PC cells; lanes 2–6, insulin treatment for the indicated time (h)). The bottom portion of each panel shows the hybridization with GAPDH, which accounts for uniform loading and integrity of RNAs.

the insulin-like growth factor (IGF)-I receptor, thus mimicking the mitogenic effects of IGF-I (6). To investigate the role of RTK activation on the expression of thymosin β10, we determined the effects exerted by insulin on thymosin β10 mRNA expression in cultured thyroid cells. As shown in Fig. 5C and D, the level of the steady-state of thymosin β10 transcript was dramatically augmented in response to insulin stimulation in a dose-dependent manner. Insulin treatment of PC Cl 3 cells induced the accumulation of the thymosin β10 transcript as early as 1 h, with a peak occurring at 1–3 h after the beginning of the treatment, demonstrating that thymosin β10 expression may be modulated by the activation of the insulin pathway.

To correlate the induction of thymosin β10 mRNA by insulin in PC Cl 3 cells with the extent of cell proliferation, we have also performed a flow cytometry analysis of insulin-stimulated PC Cl 3 cells. Cells were starved in medium containing 0.5% BSA and subsequently stimulated with 10 μg/ml insulin. At different times after stimulation, cells were collected and analyzed by flow cytometry. Results are reported in Table 1.

Effects of RNA synthesis inhibitors and protein synthesis inhibitors on TSH-induced regulation of thymosin β10 mRNA

TSH represents the major modulator of thyroid growth, differentiation and function (6). To further study the molecular mechanisms whereby thymosin β10 mRNA expression is regulated by the PKA pathway, we studied

the effects of the RNA polymerase inhibitor DRB (at a dose of 25 μg/ml) on the transcription of thymosin β10 mRNA exerted by TSH (Fig. 6A). In the presence of DRB, the TSH-induced increase in the steady-state levels of thymosin β10 (Fig. 6A) was strongly reduced, suggesting that TSH stimulation of thymosin β10 may occur, at least in part, at the transcriptional level. The rapid increase of thymosin β10 mRNA induced by TSH in thyrocytes suggests a rapid induction of thymosin β10 mRNA similar to that of early genes such as c-fos. Further to characterize the mechanism for TSH up-regulation of thymosin β10, we examined the effect of the protein synthesis inhibitor cycloheximide (Fig. 6B). In PC Cl 3 cells, the increase in thymosin β10 mRNA after 1 or 3 h of treatment with TSH was not substantially modified by cycloheximide (5 μg/ml) pretreatment of PC Cl 3 cells, suggesting that protein synthesis may not be necessary for thymosin β10 gene transcription. Conversely, control experiments using the vascular endothelial growth factor (VEGF) probe which is superinduced by TSH in the presence of cycloheximide, confirmed that cycloheximide (5 μg/ml) had no gross effect on thymosin β10 mRNA expression (data not shown).

Transcriptional analysis of thymosin β10 in cultured thyrocytes

The effects exerted by the RNA polymerase inhibitor DRB on the steady-state level of thymosin β10 mRNA level induced by TSH suggest that TSH is able to stimulate the transcriptional rate of the thymosin β10
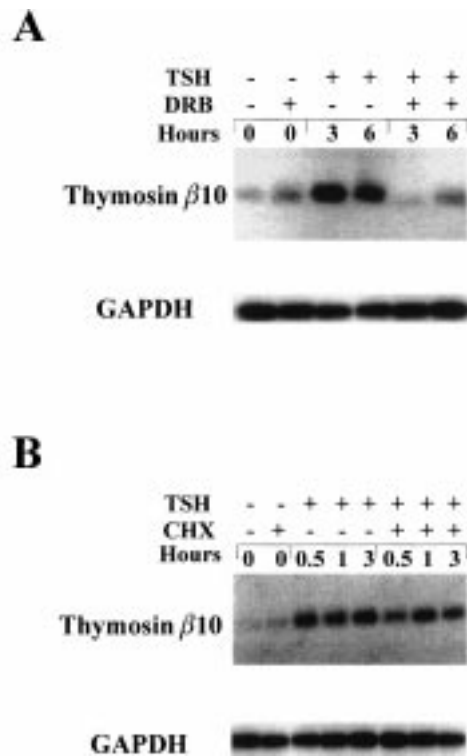
Figure 6 The effect of RNA polymerase and protein synthesis inhibitor on thymosin β10 mRNA expression induced by TSH. (A) DRB on TSH induction of thymosin β10 mRNA expression in PC Cl 3 thyroid cells. PC Cl 3 cells were treated with 1 mU/ml TSH for 1 and 3 h in the presence (+) and absence (−) of DRB (25 μg/ml). Total RNA (20 μg/lane) was subjected to Northern blot analysis using thymosin β10 as probe. Lane 1, untreated PC cells; lane 2, PC cells treated with 25 μg/ml DRB; lanes 3 and 4: TSH treatment for the indicated time in the absence of DRB; lanes 5 and 6: TSH treatment for the indicated time in the presence of 25 μg/ml DRB. The bottom portion of the figure shows the ethidium bromide staining of the gel. (B) The effect of cycloheximide (CHX) on TSH induction of thymosin β10 mRNA expression in PC Cl 3 thyroid cells. PC Cl 3 cells were treated with 1 mU/ml TSH for 0.5, 1 and 3 h in the presence (+) or absence (−) of CHX (5 μg/ml). Total RNA (20 μg/lane) was subjected to Northern blot analysis using thymosin β10 as probe. Lane 1, untreated PC cells; lane 2, PC cells treated with 5 μg/ml CHX; lanes 3–5: TSH treatment for 0.5, 1 and 3 h in the absence of CHX; lanes 6–8: TSH treatment for 0.5, 1 and 3 h in the presence of 5 μg/ml CHX. The bottom portion of the figure shows hybridization with GAPDH to ensure RNA uniform loading and integrity.

gene. To determine whether TSH exerts a regulatory control on the transcriptional rate of thymosin β10, we performed run-on transcription assays on nuclei isolated from cultured rat thyrocytes treated with TSH (Fig. 7). As shown in Fig. 7, PC Cl 3 cells express a basal amount of thymosin β10 transcript. However, the transcriptional rate of the thymosin β10 gene increased 2- to 3-fold after TSH treatment, thus confirming that TSH may regulate thymosin β10 gene expression by controlling the transcriptional rate. However, since the increase in the transcriptional rate did not account
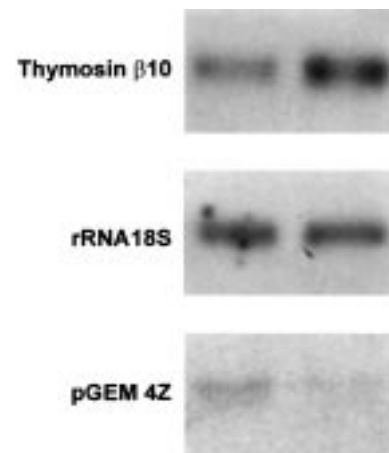

Figure 7 Transcriptional activation of thymosin β10 gene after stimulation of thyroid cells by TSH. Plasmids carrying the thymosin β10 or rRNA 18S inserts bound to nylon filters were hybridized with ^{32}P-labeled run-on transcripts from nuclei isolated after 24 h from the beginning of TSH stimulation of PC Cl 3 cells. Filters were exposed to autoradiographic film for 15 days in the case of pGEM 4Z and thymosin β10 probes, and for 12 h in the case of rRNA 18S probe. Increased transcription rate induced by TSH in PC Cl 3 cells was observed in the case of thymosin β10 (2- to 3-fold), whereas no change in the transcription rate of rRNA 18S was observed after TSH stimulation.

completely for the increase in the steady-state mRNA level induced by TSH, it is likely that, in addition to a transcriptional control, TSH may modulate thymosin β10 mRNA expression through post-transcriptional regulatory mechanisms. Control experiments (using pGEM 4Z and the plasmid carrying the insert for rRNA 18S) demonstrated that the increase in the hybridization signal observed in the lane containing the plasmid carrying the thymosin β10 cDNA, hybridized with nuclear RNA extracted from TSH-treated cells compared with untreated cells, was not due to an aspecific effect. Altogether, these data suggest that the regulation of the expression of thymosin β10 induced by TSH in thyroid cells occurs, at least in part, at the transcriptional level.

Discussion

In this study we provide evidence that the mRNA expression of thymosin β10 is markedly enhanced in human goiters compared with normal thyroid. It has been suggested that increased proliferation of thyrocytes results from the abnormal constitutive activation of the TSH receptor (TSHR) pathway. Theoretically several mechanisms may account for chronic signaling of TSHR in thyroid cells: (i) increased TSH production by pituitary gland; (ii) increased susceptibility of thyrocytes to TSH induced by iodide deficiency as in goitrous patients; (iii) chronic stimulation of TSHR by autoimmune antibodies as in Graves' disease (1, 6). To

investigate the molecular mechanisms regulating thymosin β10 in thyroid cells, we made use of a well-known model of animal goitrogenesis: rats fed with the goitrogen drug, PTU. Propylthiouracil treatment of rats mimics the constitutive chronic activation of the TSH receptor pathway (1). Upon feeding rats with PTU we observed a marked reduction in the levels of the thyroid hormones T_3 and T_4 accompanied by a significant increase in the serum TSH level and by a marked enlargement of the thyroid glands. PCNA staining of thyroid sections demonstrated that thyroid follicular cells started to proliferate after 5 days and reached a maximal level of proliferation by days 8–16. Iodide refeeding to goitrous rats resulted in a steady decrease in the serum values of T_3 and T_4 and in TSH levels, accompanied by a marked involution of thyroid hyperplasia.

The mRNA expression of thymosin β10 in the thyroid gland of PTU-fed rats increased markedly, with a time course overlapping the increase in the TSH level, suggesting that thymosin β10 expression may be regulated *in vivo* by TSH. Furthermore, since the up-regulation of thymosin β10 preceded the response of thyrocytes to TSH, thymosin β10 might play an important role in the hyperplasia induced by PTU. Such a conclusion was supported by the finding that subsequent iodide administration to goitrogenous rats reduced the steady-state levels of thymosin β10 mRNA in parallel with a marked regression of thyroid hyperplasia and the involution of the thyroid vasculature. All together these findings suggest that thymosin β10 expression is regulated – either directly or indirectly – by increased serum TSH levels and that increased thymosin β10 expression may be associated with the remodeling of the thyroid gland induced by chronic TSH stimulation.

The finding that thymosin β10 expression is up-regulated in human and experimental thyroid goiters, which are characterized by increased proliferation of thyrocytes and by extensive morphological and functional modifications, suggests that thymosin β10 may have a role in the regulation of cellular processes such as cell growth and the cytoskeletal assembly. Thymosin β10 may modify the equilibrium between monomeric and polymerized G-actin in proliferating thyrocytes. In fact, although an increase in the free G-actin pool may only be inferred on the basis of enhanced mRNA expression, it is likely that increased expression of thymosin β10 in thyroid cells may have a role in the reorganization of the cytoskeleton occurring in TSH-stimulated cells (35–37).

In the absence of TSH, thyroid cells undergo drastic morphological and biochemical changes: they become flat, pseudopods and microvilli disappear and actin becomes organized into stress fibers (35–37). As a result of TSH or forskolin stimulation, the actin stress fibers disassemble in parallel with changes in the shape and increased numbers of pseudopods and apical microvilli (35–37). All these modifications appear to have a specific functional significance since it is known that actin cytoskeleton integrity is related to the ability of the cell to secrete thyroid hormone. In this regard, it is conceivable that by decreasing the amount of polymerized actin, thymosin β10 may contribute to the disruption of subcortical actin filament which, in turn, is responsible for processes such as vesicle exocytosis and pseudopod formation (38, 39).

The mechanisms that regulate thymosin β10 mRNA expression have been studied in osteosarcoma and hematopoietic cells (15, 16). However, the molecular basis of thymosin β10 expression in endocrine tissue is poorly understood. To gain a more detailed knowledge of the regulatory pathways controlling thymosin β10 expression in thyroid cells, the cultured rat thyroid cell line PC Cl 3 was used as an *in vitro* model system. Here, we show that exogenously added TSH up-regulates thymosin β10 mRNA expression in a dose-dependent manner. TSH-induced up-regulation of thymosin β10 mRNA expression occurs, at least in part, at the transcriptional level, as demonstrated by experiments performed with the RNA polymerase inhibitor, DRB. Further confirmation of the transcriptional effects exerted by TSH on the mRNA expression of thymosin β10 was obtained by nuclear run-on, which showed a TSH-dependent increase in the transcription rate of the thymosin β10 gene. However, time-course studies and experiments with the protein synthesis inhibitor cycloheximide indicated that TSH-induced thymosin β10 mRNA expression did not require protein synthesis, since induction of thymosin β10 mRNA by TSH was not modified by cycloheximide treatment, in agreement with the rapid induction of thymosin β10 mRNA exerted by TSH.

TSH modulates thyroid functions by activating the cAMP-dependent pathway (6), since activation of the PKA-dependent pathway by the use of forskolin induced a dose- and time-dependent increase in the thymosin β10 expression, suggesting that the effects exerted by TSH on thymosin β10 mRNA expression were mediated through the cAMP-dependent protein kinase A. Previous work has shown that activation of the cAMP-dependent pathway in neuroblastoma cells down-regulates thymosin β10 mRNA expression. Such results are only in apparent contrast with our findings. In fact, in neuroblastoma cells, cAMP acts as a differentiating agent which blocks cell proliferation, whereas in thyroid cells the activation of the PKA pathway by TSH and/or forskolin leads to both proliferation and differentiation.

In the present investigation we also demonstrate that thymosin β10 expression in thyroid cells is also regulated by signals, other than TSH, involved in the growth of the thyroid cells, such as phorbol esters and insulin. Activation of the PKC pathway by TPA has been shown to induce thyroid cell proliferation and dedifferentiation (6). Insulin induces thyroid cell proliferation, possibly by mimicking the mitogenic effects of IGF-I through activation of the IGF-I receptor (6). Here,

we report that both TPA and insulin were able to up-regulate thymosin $\beta10$ expression in thyroid cells in a dose-dependent manner, suggesting that in thyroid cells the up-regulation of thymosin $\beta10$ expression is associated with the activation of multiple proliferative pathways. However, it remains to be determined whether thymosin $\beta10$ expression in thyroid cells is strictly required for growth or, alternatively, if its expression is necessary for other cellular processes that accompany cell proliferation (i.e. cytoskeleton remodeling).

In conclusion, our findings demonstrate that in the thyroid cell, thymosin $\beta10$ expression is regulated by TSH and other mitogenic pathways such as the PKC and insulin pathways. However, since the biological function of thymosin $\beta10$ has yet to be defined, further studies are necessary to determine the role of thymosin $\beta10$ in the thyroid cell during the development of hyperplastic goiters, although the observation that thymosin $\beta10$ mRNA is over-expressed in human thyroid goiters suggests that it may play a key role in the development of such hyperproliferative disease.

Acknowledgements

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