Thyrotropin and cyclic AMP regulation of *ras* proto-oncogene expression in cultured thyroid cells

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We examined the effect of thyrotropin (TSH) on intracellular levels of c-*ras* mRNA in a line of differentiated rat thyroid cells obtained from normal Fischer rat thyroids. These cells are totally dependent on TSH for growth. TSH stimulation of quiescent cells increased c-*ras* mRNA content, with a maximal response (730% of basal) after 6 h, and a decline towards basal levels after 24 h. Dibutyryl cAMP and forskolin mimicked this stimulatory effect of TSH on c-*ras*, but did not enhance β-actin mRNA content. This study demonstrates hormonal and cyclic nucleotide control of c-*ras* expression in a well-differentiated, non-tumorogenic mammalian cell.

Thyroid *Thyrotropin* *Oncogene* *cyclic AMP*

1. INTRODUCTION

Recent data suggest an important role for oncogene expression in cellular growth [1]. Transforming, or viral, oncogenes have come under intense scrutiny as key factors in neoplastic cellular growth. In addition, several lines of evidence implicate cellular (c) proto-oncogenes in the regulation of proliferation in normal cells. Thus c-oncogenes have been highly conserved during evolution and there is increased expression of a number of different oncogenes during critical periods of the cell cycle [2–5]. In addition, certain c-oncogene products bear a striking homology to the epidermal growth factor receptor [6] and platelet-derived growth factor [7,8]. With respect to the *ras* family of oncogenes, much less is known about the c-*ras* proto-oncogenes than the transforming, v-*ras* oncogene. Expression of c-Ki-*ras* is increased in untransformed 3T3 fibroblasts whose transition from the G_0 to G_1 phase of the cell cycle is induced by serum [2]. In rats, liver regeneration is associated with increased c-Ha-*ras* expression [9]. However we are unaware of data indicating specific hormonal regulation of c-*ras* in non-neoplastic cells.

TSH is the primary regulatory hormone of the thyroid gland. Many, but not all, of its diverse effects appear to be mediated by cAMP [10]. While the importance of TSH in enhancing the growth of normal and neoplastic thyroid tissue is well recognized, the mechanism underlying this effect is poorly understood. Even the issue of whether or not cAMP mediates TSH-induced growth of thyroid cells has not been settled conclusively [11–13]. Because of the dominant role of TSH in the regulation of thyroid cell growth, we therefore examined the effect of this hormone on intracellular levels of c-*ras* mRNA in a line of differentiated rat thyroid cells (FRTL-5) obtained from normal Fischer rat thyroids [14].

2. MATERIALS AND METHODS

FRTL-5 cells were grown in Coon's modified Ham's F-12 medium supplemented with insulin (10

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μg/ml), transferrin (5 μg/ml), TSH (1.5 mU/ml), penicillin (125 U/ml), gentamycin (40 μg/ml) and amphotericin B (2.5 μg/ml). The medium was replaced every 3 days. When the 100-mm diameter dishes were approximately half confluent, the cell monolayers were washed twice with Dulbecco's phosphate buffered saline (PBS) and the cells were then allowed to become quiescent by addition of the same medium described above except for the omission of TSH. After 5 days of quiescence, a maximal stimulatory dose of TSH (10 mU/ml) was added to the medium and the incubation was continued for the indicated periods of time. The cells (30–40 dishes per time point) were rinsed with ice-cold PBS and were then solubilized directly on the plate with guanidine monothiocyanate [15]. Total RNA was obtained by centrifugation through 5.7 M $CsCl_2$ in an SW28 Beckman rotor at 23 000 rpm for 20 h. After phenol/chloroform extraction, polyadenylated mRNA was obtained by oligo-dT affinity chromatography [16,17]. Thyroid cell mRNA concentrations were determined spectrophotometrically and equal aliquots (5 μg per lane) were subjected to agarose electrophoresis in the presence of methylmercuric hydroxide [18]. Ribosomal RNA (*E. coli* 16 S and 23 S, and calf liver 18 S and 28 S) and tRNA were used as standards. RNA was transferred by blotting [19] to Transa-Bind diazobenzyloxymethyl-paper (DBM) [20] (Schleicher and Schuell). v-Ha-*ras*(*Sst*I-*Pst*I, 0.7 kb fragment, Pras. 1, sold by Oncor Inc., Gaithersburg, MD) and β-actin DNA probes were labelled with [α-^{32}P]dCTP by nick translation to a specific activity of approx. 2×10^8 cpm/μg [21]. After overnight prehybridization, hybridization was for 42 h. The final washes were in 1 × SSC buffer, 0.1% SDS, at 55°C or 60°C, depending on the background observed on autoradiography after an initial wash at 55°C.

3. RESULTS

FRTL-5 thyroid cells in monolayer culture were made quiescent by the withdrawal of TSH from the medium, all other ingredients remaining the same. After 5 days, during which time cellular growth ceased, TSH (10 mU/ml) was reintroduced. At intervals between 20 min and 24 h thereafter, polyadenylated mRNA was prepared and c-Ha-*ras* mRNA levels were determined by Northern blot analysis. Low levels of c-Ha-*ras* mRNA were detectable in quiescent cells. Within 2 h of TSH stimulation mRNA concentrations increased to 230% of the control, reached a maximum (730% of the control) after 6 h and then

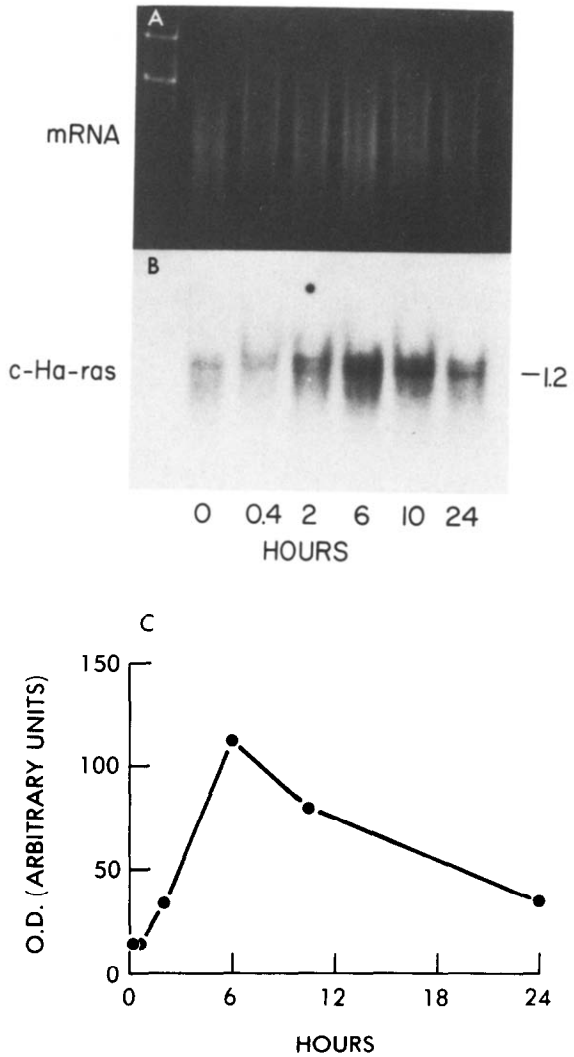

Fig.1. Stimulation by TSH of c-Ha-*ras* mRNA content in FRTL-5 rat thyroid cells. (A) Photograph (302 nm) of mRNA in agarose gel prior to blotting, indicating approximately equal amounts of mRNA in each lane (5 μg in this representative experiment). The lane on the extreme left contains the RNA standards. (B) Autoradiography of radiolabelled Ha-*ras* probe hybridized to the mRNA shown in A following transfer to DBM paper. (C) Quantification by densitometry (E.C. Apparatus Corp., St. Petersburg, FL) of the radiolabelled Ha-*ras* hybridized as shown in B.

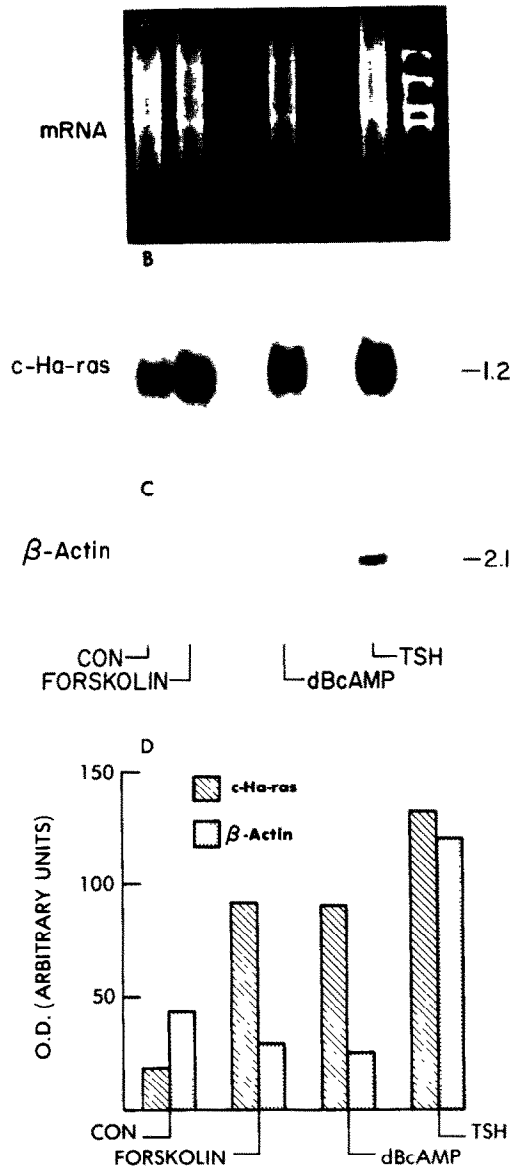

Fig.2. (A) Photograph (302 nm) of mRNA (10 μg/lane) following agarose gel electrophoresis. mRNA was obtained from quiescent (control) cells or from cells exposed to 1 mM dibutyryl cAMP, 75 μM forskolin or 10 mU/ml TSH for 8 h. The lane on the extreme right contains RNA standards. (B) Autoradiograph of radiolabelled Ha-*ras* probe hybridized to DBM paper obtained by blotting the gel shown in A. (C) Autoradiograph of radiolabelled β-actin probe hybridized to DBM paper obtained by blotting the agarose gel shown in A. (D) Quantification by densitometry of the autoradiographs shown in B and C.

declined to 230% of control after 24 h of TSH stimulation (fig.1).

To determine whether TSH enhancement of c-Ha-*ras* mRNA levels in cultured thyroid cells was dependent on cAMP as a second messenger, FRTL-5 cells were first made quiescent by the withdrawal of TSH for 5 days. The cells were then exposed to dibutyryl cAMP or to forskolin, which directly stimulates adenylate cyclase activity [22] independent of interaction with the TSH receptor. Both of these agents mimicked TSH action in increasing the proportion of mRNA transcripts in thyroid cells, although to a lesser degree than that induced by TSH itself (fig.2B,D). Interestingly, while TSH also increased β-actin mRNA levels, this effect was not mimicked by dibutyryl cAMP and forskolin (fig.2C,D). If anything, these agents appeared to decrease slightly the relative proportion of β-actin mRNA.

4. DISCUSSION

This study demonstrates hormonal and cyclic nucleotide control of c-*ras* expression in a well-differentiated, non-tumorogenic mammalian cell. FRTL-5 cells are unable to grow in soft-agar, are non-tumorogenic when injected into animals, contain differentiated functions such as iodide transport and thyroglobulin synthesis, and do not replicate even in full medium containing fetal calf serum unless TSH is present [14]. Stimulation by TSH of c-*ras* expression precedes by approx. 10 h entry into the S phase of the cell cycle, as determined by thymidine incorporation in these cells [12]. TSH and cAMP therefore produce their effect during G_0 or G_1.

Our data contrast with the previously observed effect of cAMP in decreasing the level of the p21 protein product of the *ras* gene in a line of estrogen-dependent mammary carcinoma cells [23]. However this difference may be related to the fact that cAMP stimulates FRTL-5 thyroid cell growth [13] but inhibits growth of these mammary carcinoma cells [23].

It is also of interest that the stimulatory effect of cAMP on c-Ha-*ras* expression is not a general phenomenon for all mRNA types in the thyroid, as is evident from the lack of response of β-actin mRNA to such stimulation. However, expression of at least one other cellular oncogene, *myc*, is also

enhanced by cAMP (and TSH) in cultured thyroid cells [24]. Previous evidence has suggested a role for c-*ras* in the regulation of cAMP generation (reviewed in [25]). This study adds to this remarkable schema by indicating that the reverse situation may also exist in specific hormone responsive cells, namely that cAMP may regulate c-*ras* expression.

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