The Tissue-specific Pathways Regulating Cell Proliferation Are Inherited Independently in Somatic Hybrid between Thyroid and Liver Cells

Bianca Maria Veneziani,* Giovanni Villone, Rossana Romano, Angelina Di Carlo, Corrado Garbi, and Donatella Tramontano*

* Dipartimento di Medicina Sperimentale, Università degli Studi di Reggio Calabria, Via T. Campanella, Catanzaro; Centro di Endocrinologia ed Oncologia Sperimentale del Consiglio Nazionale delle Ricerche, Naples; and Dipartimento di Biologia e Patologia Cellulare e Molecolare, Università degli Studi di Napoli, Via S. Pansini, 5, 80131 Naples, Italy

Abstract. Thyroid stimulating hormone (TSH) and insulin-like growth factors type 1 (IGF-I) regulate the proliferation and differentiation of cultured thyroid cells but not of cultured liver cells. We have examined the influence of TSH and IGF-I on the metabolic functions and proliferation of somatic hybrids obtained by fusing rat thyroid cells (FRTL5) with rat liver cells (BRL).

While IGF-I is able to stimulate the proliferation of the hybrid cells (TxL) TSH fails to induce their growth. However, the hybrid TxL cells have surface TSH receptors with normal ligand characteristics. The addition of TSH to TxL cells led to typical enhancement of cAMP production and depolymerization of actin filaments. Yet, TSH failed to stimulate iodine uptake in the hybrid cells. Interestingly, iodine inhibited TxL proliferation induced by IGF-I but not by serum.

It is concluded that the hybrid TxL cells inherited from the parental thyroid cells several important differentiated traits including mitogenic pathways induced and used by IGF-I, functional TSH receptors, and sensitivity to the inhibitory action of iodine.

In the thyroid gland the control of cell proliferation depends upon the balance between stimulatory and inhibitory signals. At least two mechanisms are involved in the stimulation of thyroid cell proliferation, one cAMP dependent, triggered by thyroid stimulating hormone (TSH), and one cAMP independent, triggered by insulin-like growth factors (IGFs), insulin, or EGF (Bacharach et al., 1985; Dere and Rapoport, 1986; Ingbar et al., 1986; Jin et al., 1986; Roger and Dumont, 1982; Roger et al., 1983, 1988; Smith et al., 1986; Tramontano et al., 1986b, 1988; Westermak and Westermak, 1982; Westermak et al., 1983). In the FRTL5 cells, a line of normal rat thyrocytes in culture, it has been demonstrated that TSH interacts synergistically with IGFs to modulate mitogenesis (Tramontano et al., 1986b), suggesting that only the concomitant activation of the two pathways allows the full expression of the proliferative response of the thyroid follicular cells.

More importantly, recent data indicate that the interaction between TSH and IGFs observed in cells in culture may have a physiological role since it has been shown that IGF-like peptides are produced by the thyrocytes of human, porcine, rat, and ovine origin in vivo and in vitro (Brown et al., 1986; Clemmons and Van Vyk, 1985; D’Ercole et al., 1983, 1984; Errick et al., 1986; Han et al., 1987; Maciel et al., 1988; Mak et al., 1985; Stiles et al., 1985). Iodine is one of the most important regulators of the thyroid gland functions and it is well known that iodine inhibits proliferation of thyroid follicular cells both in vivo and in vitro (Bray, 1968; Chapman, 1941; Gartner et al., 1985; Halmi, 1971; Ingbar, 1972; Nagataki and Ingbar, 1986). In the FRTL5 cells iodine inhibits growth induced through both the cAMP dependent and the cAMP independent pathways (Becks et al., 1988; Tramontano et al., 1989).

This complex network of interaction regulating thyroid cell proliferation represents a distinctive "trait" of thyrocytes and it can be analyzed as a differentiated function. Thus, we have investigated how the various pathways regulating thyroid cell growth are inherited by hybrid cells, TxL (Ambesi-Impiombato et al., 1985), obtained by fusing FRTL5 cells (Ambesi-Impiombato et al., 1982; Ambesi-Impiombato and Villone, 1987), a line of normal rat thyroid follicular cells whose growth is dependent upon and regulated by TSH and IGF (Tramontano et al., 1986b, 1987; Tramontano and Ingbar, 1986), and BRL-30E cells (BRL), a line of normal rat liver cells, whose growth is regulated by serum (Nissley et al., 1977).

We have investigated the effect of serum, TSH, IGFs, and iodine on the proliferation and differentiated functions of the TxL cells. The results presented here provide evidence that the hybrid TxL clones have inherited the mechanisms con-
trolling growth from both liver and thyroid cells, in that, they respond to serum as the BRL cells, and to IGF-I as the FRTL5 cells. In addition, similarly to the thyroid parental cells, growth of TxL cells is inhibited by iodine. On the other hand, the growth pathway dependent upon TSH is lost in the TxL cells, in spite of the presence of biologically active TSH receptors on the plasma membrane of TxL cells.

**Materials and Methods**

**Materials**

Materials were purchased from the following sources: Cocoon's modified Ham F-12 tissue culture medium from Hazleton Research Products, Inc. (Denver, CO); calf serum and L-glutamine from Gibco Laboratories (Grand Island, NY); DME from Flow Laboratories, Inc. (Irvine, Scotland); BSA from Reheis Chemical Co (Phoenix, AZ); biosynthetic IGF-I (Thr51-IGF-I) from AMGen Biologicals (Thousand Oak, CA); tissue culture dishes (100 × 20 and 6 × 15-mm style) and centrifuge tubes (15 ml) from Falcon Labware, Becton, Dickinson & Co. (Oxnard, CA); 24-well culture plates from Costar (Cambridge, MA); isobutyl methylxanthine (MIX), bovine TSH (bTSH), and RITC-conjugated phalloidin from Sigma Chemical Co. (St. Louis, MO); highly purified bovine TSH (TSH) was kindly provided by the National Pituitary Program, National Institutes of Health (Bethesda, MD), [methyl-3H]thymidine (1.0 mCi/ml) and 125I-TSH from Amersham Corp. (Arlington Heights, IL); 125I from Du Pont-New England Nuclear Corp. (Boston, MA). All other chemicals and reagents were obtained from commercial sources and were of reagent grade or higher.

**Culture Techniques**

In these experiments FRTL5 clone 2 cells (Ambesi-Impiombato et al., 1982; Ambesi-Impiombato and Villone, 1987), FRTL5 HGPRt+, BRL 30E TK− and several hybrid clones TxL (Ambesi-Impiombato et al., 1985) were used. FRTL5 HGPRt− cells were obtained by chemical mutagenesis of FRTL5 cells as already reported elsewhere (Ambesi-Impiombato et al. 1985). BRL 30E TK− cells are a line of Buffalo rat liver cells bromo deoxyuridine resistant kindly provided by Dr. H. G. Coon (National Institutes of Health). Techniques and methods for cell fusion and hybrid cells selection have been extensively described elsewhere (Ambesi-Impiombato et al. 1985). All the cell strains were routinely cultured in Coon's modified Ham F-12 (mFl2) medium supplemented with 5% calf serum, in 100-mm tissue culture plates or 24-well Costar plates at 37°C in an atmosphere of 95% air/5% CO2 in humified incubator. The medium of the FRTL5 and the FRTL5 HGPRt− was supplemented with TSH (1 mU/ml), insulin (1 μg/ml), and transferrin (5 μg/ml) (3H medium). Techniques for the subculture of cells have been described in detail previously (Ambesi-Impiombato et al., 1982). Techniques and methods for cell fusion and hybrid cells selection have already been reported (Ambesi-Impiombato et al., 1985). In some studies, cells were maintained in the absence of TSH, insulin, or transferrin (H-free medium).

**Growth Curves**

For long-term experiments, cells were plated in 60-mm dishes at a concentration of 1 × 105/dish, in medium supplemented with serum as indicated in the legend to the figure. Replicate dishes were periodically (every 2–3 d), trypsinized, and counted in a Neubauer chamber (Saaringa, Federal Republic of Germany).

**[3H]Thymidine Incorporation**

For studies of [3H]thymidine incorporation into DNA, cells were sparsely seeded in H-free medium in 24-well culture plates, 5 d later, medium was removed, and cells were washed twice with mFl2 medium devoid of serum and then they were incubated in the same medium for 24 h. The medium was removed and 500 μl of the same medium containing 0.1% BSA and the appropriate concentrations of the agents to be tested were added. After the appropriate incubation time, medium was removed, [3H]methyl-thymidine (5 μCi/ml) was added in 250 μl of DME, and cells were incubated for an additional 2 h. The labeling was stopped by aspirating the medium and washing the cells twice with PBS and three times with 10% ice-cold TCA. TCA-precipitable material was solubilized with 500 μl of 2% SDS. Cell-associated radioactivity was then counted in a scintillation spectrometer.

**Radioreceptor Assay**

TSH was labeled by a gentle chloramine-T technique as previously described (Goldfine et al., 1974). Final specific activity of 125I-TSH was 150 μCi/μg. Final specific activity of 125I-IGF-I was 280 μCi/μg. Binding studies were performed as previously described (Tramontano and Ingbar, 1986). Briefly, for studies of the binding of 125I-IGF-I and 125I-TSH, cells were grown to confluence in 24-well Costar plates in 3H medium replaced for an additional 5 d by H-free medium. Immediately before the binding studies, media were aspirated, and monolayers were washed three times with medium, pH 7.3; TSH or 125I-TSH and varying concentrations of unlabeled peptides were then added to each well in 250 μl of modified Krebs-Ringer bicarbonate buffer (KRB) containing 0.1% BSA (Tramontano and Ingbar, 1986). Steady-state binding experiments were carried out overnight at 4°C. At the end of the incubation cells were washed three times with ice-cold modified KRB. Cells were then solubilized in 1 N NaOH and 125I content of the cell lysates was determined. Protein concentration was measured in aliquot of cell lysate by the method of Lowry et al. (1951). Non-specific binding was determined in the presence of 26 nM IGF-1 or 1 μM TSH.

**Measurements of Cyclic AMP Concentration**

Measurements of the effects of TSH on AMP generation were performed as previously described (Tramontano and Ingbar, 1986). Hybrid and BRL cells were grown to confluence in H-free medium in 24-well Costar plates. FRTL5 cells were grown to confluence in 3H medium in 24-well Costar plates and were then maintained in H-free medium for 7 d. Monolayers were then washed three times with KRB containing 0.1% BSA, pH 7.2, and cells were then incubated at 37°C in 250 μl of the same buffer containing the appropriate concentrations of TSH. After 30 min, the supernatants were collected and frozen at −20°C. To extract intracellular cAMP, monolayers were treated overnight at −20°C with 500 μl of ice-cold absolute ethanol. Extracts were then dried, and each pellet was reconstituted with its original supernatants. Aliquots of appropriate volume were then taken for RIA of cAMP concentration, and results were expressed as pmol/105 cells. Cells were counted in a Neubauer chamber after release from plates by trypsinization. In all experiments, quadruplicate wells were studied for each experimental point.

**Staining of Actin Filaments**

Cells were sparsely seeded on glass coverslips. After 3 d, appropriate concentrations of the agents to be tested were added, and cells were cultured for additional 24 h. At the end of the incubation time, cells were fixed for 15 min at room temperature with a solution of 3.7% formaldehyde in PBS in the presence of 2% sucrose to reduce the background. Coverslips were then washed five times with PBS; the cells were permeabilized with 0.1% Triton X-100 for 5 min in PBS and then washed five times in PBS. Cells were then incubated in the RITC-conjugated phalloidin (5 μg/ml) and washed five times with PBS. Coverslips were mounted on microscope slides using a 50% solution of glycerol in PBS and examined with a Zeiss photofluor microscope.

**Iodide Trapping**

Studies of radioiodine uptake were performed as previously described (Lombardi et al., 1988). Cells were brought to confluence and maintained for 4–6 d in H-free medium. Then they were incubated for additional 48 h in medium supplemented with 0.1% BSA in the presence or in the absence of TSH (0.01–10 nM) and IGF-I (0.01–1 nM). Cells were washed once with KRB and incubated with Na125I (0.1 μCi/well) and 10 μM NaI in 250 μl of KRB 37°C for 20 min. The reaction was stopped by washing cells with ice-cold KRB. Intracellular 125I was extracted with 1 ml TCA 10% for 20 min at room temperature and the extract was counted in a gamma scintillation counter.

**Statistical Analyses**

In all studies at least three separate samples were studied for each experimental point. Analysis of differences in the values obtained in the various experimental groups was conducted by analysis of variance followed by Neuman-Keuls test for the significance of differences among multiple ex-
Results

Effect of Serum on the Proliferation of TxL Clones

Among several clones obtained by fusing FRTL5 cells and BRL, four have been chosen for this study. Clones TxL6, TxL13(oH), and TxL20 have been selected in HAT medium supplemented with 5% calf serum, whereas TxL13(wH) have been selected in HAT medium supplemented with 5% calf serum and the 3H medium (see Materials and Methods). The average number of chromosomes for the above mentioned clones was 78 vs. 42 of the parental liver and thyroid cells. To ascertain whether serum could substan the growth of TxL hybrid clones, cells were seeded in the presence of 0.5 or 5% calf serum. Similarly to the BRL cells, TxL hybrid clones grew vigorously in the presence of either 0.5 or 5% calf serum (Fig. 1). As it has already been reported (Ambesi-Impiombato et al., 1982; Tramontano et al., 1986a), FRTL5 cells did not proliferate in the presence of serum alone at any of the concentrations tested (data not shown).

Figure 1. Growth curves of liver parental and hybrid cells performed in mF12 culture medium supplemented with 0.5 (●) or 5% (▲) calf serum. SD ≲ 2% per each experimental point.

Effect of TSH and IGF on the Mitogenesis of TxL Hybrids

Although the proliferation of TxL clones is not dependent upon the presence of hormones in their culture medium, experiments were performed to determine whether their proliferation was still sensitive to hormones or growth factors. TxL cells were cultured in the presence of TSH and IGF-I, alone or in combination, for 36 h, then [3H]thymidine incorporation into DNA was measured. TSH was totally ineffective on the proliferation of all hybrid clones tested as it was on the BRL cells, while it did stimulate the incorporation of [3H]thymidine into the DNA of FRTL5 cells (Fig. 2). Conversely, IGF was able to induce DNA synthesis in all the hybrid clones and in FRTL5 cells but not in the BRL cells (Fig. 2). The mitogenic effect of IGF-I was dose dependent in the TxL cells as it is in the FRTL5 cells. However, as it

Figure 2. [3H]Thymidine incorporation into DNA of parental and hybrid cells performed in mF12 culture medium supplemented with 0.1% BSA and TSH (●) or IGF-I (▲). TxL13 (wH) clones have been selected and subcultured in HAT medium without hormones, while TxL13 (wH) clones have been selected and subcultured in HAT medium supplemented with 3H.
Figure 3. \[^{3}H\]thymidine incorporation into DNA of parental and hybrid cells performed in mFl2 culture medium supplemented with 0.1% BSA and TSH alone (○) or in combination with 1.3 nM IGF-I (●).

is shown by the different scale on the y axis (Fig. 2), the magnitude of the stimulation of the \[^{3}H\]thymidine incorporation into DNA induced by IGF-I in the FRTL5 cells was by far greater than in the hybrid clones (Fig. 2). This difference can be ascribed to the high basal level of \[^{3}H\]thymidine incorporation observed in the hybrid cells (TxL6 80,601 ± 5,300; TxL13(w/oH) 17,574 ± 4,658; TxL13(wH) 11,444 ± 3,544; TxL20 6,975 ± 1,890; cpm mean ± SD of three separate experiments performed in triplicates) compared to the parental cells (FRTL5 257 ± 20; BRL 452 ± 39; cpm mean ± SD of three separate experiments performed in triplicates).

When cells were concomitantly treated with increasing concentrations of TSH and one single concentration of IGF-I, the expected synergistic effect induced by the two mitogens in the FRTL5 cells was not observed in the TxL cells, while the effect of IGF-I alone was maintained and unaffected by TSH (Fig. 3).

**Binding of \(^{125}\text{I}-\text{TSH} and ^{125}\text{I}-\text{IGF-I to TxL Cells**}

To ascertain whether the lack of effect of TSH on the proliferation of the TxL cells was due to a lack of receptors for TSH, steady-state and competition binding studies were performed (Fig. 4). \(^{125}\text{I}-\text{TSH} binds the TxL cells with kinetic characteristics similar to that of the FRTL5 cells (Table I). Analysis of saturation studies revealed an upward concave configuration of the Scatchard plots (Scatchard, 1949), suggesting that TxL cells contain more than one binding site for TSH as do the thyroid parental cells. When the analyses were based on the assumption that two classes of binding sites were present and data were resolved in two linear functions, the affinities of the two sites differed widely as did their respective binding capacities. Specific binding of \(^{125}\text{I}-\text{TSH} to the BRL cells could not be demonstrated. \(^{125}\text{I}-\text{IGF-I} binds to specific membrane receptors of both the hybrid clones and the parental cells (Fig. 5). Scatchard analyses of saturation studies conducted on the hybrid clones and on the parental cells (Table II), revealed a single apparent binding site whose affinity is consistent with that of type 1 IGF receptor found in other tissues (Beguinot et al., 1985; Pilistine et al., 1984).

**TSH-stimulated Production of cAMP in TxL Cells**

Since the lack of mitogenic effect of TSH could not be ascribed to lack of TSH receptors, it has been investigated whether the TSH receptors present on the membrane of TxL cells were biologically active. In two separate experiments it was observed that the basal level of cAMP in the TxL cells was as low as that of quiescent FRTL5 cells, suggesting that in the hybrid cells the adenylate cyclase is not constitutively activated. More importantly, TSH did significantly increase the intracellular levels of cAMP into the hybrid cells in a dose-dependent fashion, although the increase of cAMP in the TxL cells was by far lower than that in the FRTL5 cells (Fig. 6).

**Cytoskeletal Organization in Hybrid Cells**

In FRTL5 cells, as in other thyroid cells (Westermark and Porter, 1982), TSH profoundly influences cell morphology and the organization of cytoskeletal elements (Tramontano et al., 1982). The latter effect is manifested by the depolymerization of actin filaments, and is mediated by cAMP (Tramontano et al., 1982; Westermark et al., 1983). As

Figure 4. Steady-state binding of \(^{125}\text{I}-\text{TSH} and competition study with unlabeled bTSH in parental and hybrids clones.
shown in Fig. 7 the addition of TSH (1 nM) to quiescent FRTL5 cells induced the expected disruption of microfilaments. In the BRL cells actin is organized in microfilaments spanning the whole cell and this pattern is not influenced by the addition of TSH (Fig. 7, c and d). The organization of the cytoskeletal elements in the hybrid clones was examined in basal conditions or under the stimulation of TSH. Only in clone TxL13(w/oH), where microfilaments were organized similarly to the FRTL5 cells, was the effect of TSH evident (Fig. 7, e and f).

As for the other hybrids (Fig. 8), clone TxL20 was composed of cells with no polymerized actin, clone TxL6—included cells with microfilaments and cells with depolymerized actin and clone TxL13(wH) displayed well defined microfilaments, and TSH did not influence cell morphology and cytoskeletal organization (data not shown).

**Effect of Iodine on the Proliferation of TxL Clones**

It is generally accepted that iodine transport into thyroid cells is stimulated by TSH via cAMP and that the inhibitory effects of iodine on thyroid cell functions is to be ascribed to its ability to inhibit adenylate cyclase system. On the other hand it has been recently reported that iodine inhibits thyroid cell growth stimulated by either cAMP dependent and cAMP independent pathways.

Since TSH stimulated increase of cAMP intracellular levels in the TxL clones, the effect of TSH on iodine uptake in the hybrid clones was studied. Cells were treated for 48 h with increasing concentrations of TSH or IGF-I and then 125I uptake was measured. In the absence of TSH a small amount of iodine was present in all the cells tested. TSH failed to increase basal uptake of radiolabeled iodine in the TxL cells or in the liver parental cells at any concentration, whereas TSH stimulated a dose-dependent increase of iodine uptake in the FRTL5 cells. Finally, IGF-I did not influence iodine uptake in any of the cells tested (Table III).

Experiments have been performed to investigate whether

---

**Table I. Kinetic Properties of the TSH Receptor in the Hybrids**

|          | Low affinity binding sites | High affinity binding sites |
|----------|----------------------------|----------------------------|
|          | \( K_* \) \( MBC^\dagger \) | \( K_* \) \( MBC^\dagger \) |
|          | \( M^{-1} \) \( M \)       | \( M^{-1} \) \( M \)       |
| FRTL5    | \( 1.0 \times 10^7 \) \( 3.9 \times 10^{-4} \) | \( 4.9 \times 10^4 \) \( 9.2 \times 10^{-11} \) |
| TxL6     | \( 2.3 \times 10^7 \) \( 1.5 \times 10^{-4} \) | \( 1.5 \times 10^4 \) \( 1.8 \times 10^{-10} \) |
| TxL13 (w/oH) | \( 7.1 \times 10^6 \) \( 8.3 \times 10^{-9} \) | \( 5.0 \times 10^8 \) \( 1.1 \times 10^{-10} \) |
| TxL13 (wH)  | \( 1.2 \times 10^7 \) \( 3.2 \times 10^{-9} \) | \( 5.6 \times 10^8 \) \( 7.3 \times 10^{-11} \) |
| TxL20    | \( 8.1 \times 10^6 \) \( 3.4 \times 10^{-9} \) | \( 3.4 \times 10^8 \) \( 4.4 \times 10^{-11} \) |

Scatchard analysis of data shown in Fig. 4. The values reported are the mean of those obtained in three separate experiments. Standard deviation is < 2%.

\( * \) \( K_* \), affinity constant. \( \dagger \) \( MBC \), maximum binding capacity.

---

**Table II. Kinetic Properties of the IGF-I Receptor in the Hybrids**

|          | \( K_* \) \( MBC^\dagger \) |
|----------|-----------------------------|
|          | \( M^{-1} \) \( M \)       |
| BRL      | \( 1.4 \times 10^6 \) \( 9.3 \times 10^{-11} \) |
| FRTL5    | \( 1.4 \times 10^6 \) \( 2.7 \times 10^{-10} \) |
| TxL6     | \( 1.1 \times 10^6 \) \( 2.4 \times 10^{-10} \) |
| TxL13 (w/oH) | \( 1.4 \times 10^6 \) \( 1.5 \times 10^{-10} \) |
| TxL13 (wH)  | \( 1.3 \times 10^6 \) \( 1.6 \times 10^{-10} \) |
| TxL20    | \( 1.2 \times 10^6 \) \( 1.8 \times 10^{-10} \) |

Scatchard analysis of data shown in Fig. 5. The values are the mean of those obtained in three separate experiments. Standard deviation is > 2%.

\( * \) \( K_* \), affinity constant. \( \dagger \) \( MBC \), maximum binding capacity.
Figure 7. TSH effect on the organization of actin cytoskeleton in the FRTL5 and BRL parental cells and in the hybrid clone TxL13 (w/oH).

Cells were cultured on glass coverslips, fixed, and stained with RITC-phalloidin. Actin filaments are prominent in untreated FRTL5 (a), BRL (c), and TxL13 (w/oH) (e) cells. Addition of TSH to the culture medium induced actin depolymerization in the FRTL5 parental cells (b) and in the hybrid cells (f), but not in the BRL parental cells (d). Bar, 10 μm.

the basal level of iodine present in the TxL cells was able to inhibit their proliferation. Hybrid clones and the parental cells were treated for 36 h with IGF-I (1.3 nM) in the presence or the absence of iodide (1 mM) (Fig. 9). As it did for the parental FRTL5 cells, iodine inhibited the incorporation of [3H]thymidine into the DNA of the TxL clones stimulated by IGF-I, while it did not affect the synthesis of DNA of the liver parental cells.

Discussion

The relationship between TSH and IGFs and the inhibitory effect of iodine on the proliferation of thyroid cells is a distinctive "trait" of the thyrocytes and can be looked at as a differentiated function of thyroid follicular cells.

To continue along this line of reasoning, experiments have been performed to examine the question of whether the mechanisms regulating thyroid cells proliferation are so strictly intertwined that they must be inherited as a single functional property or they can be inherited independently. To address this question, somatic hybrid clones, TxL, derived by fusing FRTL5 cells and BRL cells, have been used. At first the proliferative response of TxL clones to calf serum and to TSH or IGF, alone or in combination, has been examined. TxL hybrid cells respond to the mitogenic stimulation of calf serum in that they vigorously proliferate in the presence of serum alone even at concentrations as low as 0.5%. These data indicate that the hybrid clones inherited from the liver parental the ability to grow in the presence of serum, without added hormones, while the thyroid parental are unable to do so.

When examined for their ability to respond to specific factors able to stimulate the proliferation of thyroid cells, the TxL clones showed a differential response to TSH and IGF. Thus IGF, unable to induce the proliferation of BRL cells, did stimulate the DNA synthesis in the hybrid clones in a fashion similar to that of FRTL5 cells. On the contrary, TSH
Figure 8. Actin staining in clones TxL6, TxLI3 (wH), and TxL20. Clone TxL6 was composed of cells with no polymerized actin and cells containing microfilaments (a). In clone TxLI3 (wH) cells contained well-developed microfilaments (b). In clone TxL20 all cells had no organized microfilaments (c). Bar, 10 μm.

was completely ineffective in promoting the proliferation of the hybrid cells, although TxL cells possess, on their plasma membrane, receptors for TSH. Steady-state binding studies and Scatchard analysis demonstrated that the TSH receptors present in the TxL cells were quantitatively and qualitatively similar to those of the FRTL5 cells. In addition, they were able to transduce the appropriate postreceptoral signal as it is demonstrated by the observation that, although TSH increases intracellular levels of cAMP in all hybrid cells tested, it depolymerizes actin only in clone TxL13(w/oH).

As for proliferation, it cannot be excluded that TxL cells are independent from TSH because the TSH-induced pathway became constitutively activated downstream from the adenylate cyclase. In addition, since the TxL clones similarly to the BRL cells are able to proliferate even in the absence of serum, it cannot be excluded that autocrine factors are being produced and involved in the control of the proliferation. Preliminary observations from our laboratory indicate that the medium conditioned by the TxL clones stimulate [PH]thymidine into the DNA of FRTL5 cells.

Studies on somatic cell hybrids have shown that the fusion of a cell expressing a specific differentiated function with a nonexpressing cell results in the loss of the specific function in the stable hybrid (Chin and Fournier, 1987; Davidson, 1974; Fourgere and Weiss, 1978; Killary and Fournier, 1984; Lincheseiner et al., 1987). In respect to thyroid functions, it has already been reported that the TxL clones lost the ability to produce thyroglobulin and to concentrate iodine (Ambesi-Impiombato et al., 1985), suggesting that they follow the general rule. However, the presence of a biologically active TSH receptor together with the ability of iodine to inhibit the proliferation of TxL clones and finally the ability of IGF-I to induce cell growth in the hybrid cells, all properties of the thyroid cells, indicate that the hybrid clones have inherited and maintained important markers of thyroid differentiation.

The data presented here indicate that in the FRTL5 cells, the two pathways leading to thyroid cell proliferation, one triggered by TSH and one triggered by IGF, are totally independent and they can be independently inherited. That is, the hybrid clones have inherited from the parental FRTL5 cells only the IGF-stimulated pathway but not the TSH-stimulated one. Since the growth pathway regulated by TSH is preferentially lost, an intrinsic difference between the pathway induced by TSH and that induced by IGF-I have to be supposed. In an hypothetical hierarchy of the pathways controlling cell proliferation, the TSH-dependent one represents, in the thyroid cells, the highest level of sophistication in the control of growth.
Cells were incubated with TSH and IGF-I for 48 h. I\textsuperscript{−} uptake and protein concentration were measured as described in Materials and Methods. Values are the mean of triplicate samples per each experimental point; standard deviation is <5%.

|                  | FRTL5 | BRL |TxL6 |TxL13 (w/oH) |TxL13 (w/H) |TxL20 |
|------------------|-------|-----|-----|-------------|-------------|------|
| **Basal**        | 125   | 60  | 75  | 47          | 67          | 140  |
| TSH (0.01 nM)    | 1,522 | 60  | 75  | 47          | 62          | 180  |
| TSH (0.1 nM)     | 5,000 | 70  | 100 | 65          | 80          | 110  |
| TSH (1 nM)       | 4,200 | 50  | 100 | 35          | 52          | 180  |
| TSH (10 nM)      | 3,000 | 110 | 67  | 37          | 37          | 130  |
| IGF-I (0.01 nM)  | 120   | 62  | 80  | 48          | 92          | 120  |
| IGF-I (1 nM)     | 110   | 57  | 95  | 52          | 65          | 120  |
| IGF-I (1.3 nM)   | 180   | 47  | 65  | 47          | 12          | 110  |
| IGF-I (1.3 nM) + NaI, P < 0.001 |        |     |     |             |             |      |

of growth, in that it behaves as a "luxury function", and it is lost in hybrid clones. The IGF stimulated pathway behaves more like an "housekeeping" pathway and then it is retained by the hybrids.

Finally, it has already been reported (Becks et al., 1988; Tramontano et al., 1989) that iodine specifically inhibits thyroid cell proliferation stimulated by TSH and IGFs; it is to be noted that neither IGFs nor insulin are able to induce iodine uptake above basal level. Nevertheless, the iodine taken up by the FRTL5 cells in basal condition (i.e., in the absence of TSH) is sufficient to inhibit the IGF-I-stimulated FRTL5 cell proliferation. This effect of iodine, like other autoregulatory effects, is blocked by compounds with antithyroid activity, such as methimazole, that are inhibitors of thyroid peroxidase-catalyzed iodination. This observation suggests that the inhibitory effect of iodine, at least on thyroid cells proliferation, does not depend upon the concentration of iodine within the thyrocytes but upon iodine organification. Thus, the tissue specificity of the inhibitory effect of iodine, manifested by its failure to inhibit the growth of myoblasts or fibroblasts, may reflect a fundamental difference between the mitogenic pathways in the FRTL5 cells and those in the other cell types with respect to their sensitivity to iodine such as a failure of these cells to carry out the requisite organification (Tramontano et al., 1989). In this view, the observation that in hybrid cells iodine inhibits the IGF-stimulated growth but not the serum-stimulated one, the latter being inherited by the hybrid from the liver parental cells, suggests that iodine can inhibit only the thyroid-inherited arm of mitogenic regulation. In addition, it raises the intriguing possibility that in the thyroid cells, along the way leading to cell proliferation that is triggered by IGF-I, thyroid-specific signals are present and they may be the target of the inhibitory action of iodine.

This work was partially supported by a grant from Associazione Italiana per la Ricerca sul Cancro, Italy and from Progetto Finalizzato Oncologia, Consiglio Nazionale delle Ricerche, Italy.

Received for publication 15 May 1990 and in revised form 15 August 1990.

**References**

Ambesi-Impimombo, F. S., and G. Villone. 1987. The FRTL5 thyroid cell strain as a model for studies on thyroid cell growth. *Acta EndocrinoL Suppl.* 281:242-245.

Ambesi-Impinmbato, F. S., R. Picone, and D. Tramontano. 1982. Influence of hormones and serum on growth and differentiation of the thyroid cell strain FRTL. In Growth of Cells in Hormonally Defined Media. D. A. Sibasalko, G. H. Sato, and A. Pardee, editors. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York. 483-492.

Ambesi-Impinmbato, F. S., P. Curcio, G. Perrella, R. Picone, D. Tramontano, B. M. Veneziani, and G. Villone. 1985. Fate of thyroglobulin synthesis in hybrid cells. *Thyroglobulin: The Prothoroid Hormone.* Vol. 2. 225-233.

Bacharach, L. K., M. C. Eggo, W. W. Mak, and G. N. Burrow. 1983. Phorbol esters stimulate growth and inhibit differentiation in cultured thyroid cells. *Endocrinology.* 116:1603-1609.

Becks, G. P., M. C. Eggo, and G. N. Burrow. 1988. Organic iodine inhibits a deoxyribonucleic acid synthesis and growth in FRTL5 thyroid cells. *Endocrinology.* 123:545-551.

Brown, A. L., D. E. Graham, S. P. Nisley, D. J. Hill, A. J. Strain, and M. M. Rechler. 1986. Developmental regulation of insulin-like growth factor II mRNA in different rat tissues. *J. Biol. Chem.* 261:13144-13150.

Beguinot, F., C. R. Kahn, A. C. Moses, and R. L. Smith. 1985. Distinct biologically active receptors for insulin, insulin-like growth factor I, and insulin-like growth factor II in cultured skeletal muscle cells. *J. Biol. Chem.* 260:15892-15897.

Bray, G. A. 1968. Increased sensitivity of the thyroid in iodine depleted rats to the goitrogenic effect of thyrotropin. *J. Clin. Invest.* 47:16-46.

Chapman, A. 1941. The regulation of the thyroid and pituitary glands to iodine metabolism. *J. Endocrinol.* 29:680-691.

D'Ercole, A. J., G. T. Applewhite, and L. F. Underwood. 1980. Evidence that somatomedin is synthesized by multiple tissues in the fetus. *Dev. Biol.* 75:315-328.

Endocrinology. *Acta Endocrinol. Suppl.* 8:195-218.

Figure 9. Effect of NaI (1 mM) on \(^{3}H\)thymidine incorporation into DNA of parental and hybrid cells. In the parental liver cells, IGF-I (1.3 nM) vs. IGF-I (1.3 nM) + NaI (1 mM), \(P > 0.5\). In all other cells tested, IGF-I vs. IGF-I + NaI, \(P < 0.001\).
crine or autocrine mechanisms of action. Proc. Natl. Acad. Sci. USA. 81:935-939.

Dere, W. H., and B. Rapoport. 1986. Control of growth in cultured rat thyroid cells. Mol. Cell Endocrinol. 44:195-199.

Errick, J. A., K. W. A. Ing, M. C. Eggo, and G. N. Burrow. 1986. Growth and differentiation in cultured human thyroid cells: effects of epidermal growth factor and thyrotropin. In Vitro Cell. Dev. Biol. 22:28-36.

Fourgere, C., and M. C. Weiss. 1978. Phenotypic exclusion in mouse melanoma rat hepatoma hybrid cells: pigment and albumin production are not expressed in simultaneously.

Gartner, L., W. Greil, R. Dembarker, and K. Horn. 1985. Involvement of cyclic AMP, iodide and metabolites of arachidonic acid in the regulation of cell proliferation of isolated porcine thyroid follicles. Mol. Cell. Endocrinol. 42:145-155.

Goldfine, I. D., S. H. Amir, A. W. Peterson, and S. H. Ingbar. 1974. Preparation of biologically active TSH. Endocrinology. 95:1228-1233.

Halmi, N. S. 1961. Thyroidal iodide transport.

Fourgere, C., and M. C. Weiss. 1978. Phenotypic exclusion in mouse melanoma rat hepatoma hybrid cells: pigment and albumin production are not expressed in simultaneously.

Gartner, L., W. Greil, R. Dembarker, and K. Horn. 1985. Involvement of cyclic AMP, iodide and metabolites of arachidonic acid in the regulation of cell proliferation of isolated porcine thyroid follicles. Mol. Cell. Endocrinol. 42:145-155.

Goldfine, I. D., S. H. Amir, A. W. Peterson, and S. H. Ingbar. 1974. Preparation of biologically active TSH. Endocrinology. 95:1228-1233.

Halmi, N. S. 1961. Thyroidal iodide transport. Vitam. Horm. 19:133-142.

Han, V. K. N., A. J. D'Ercole, and P. K. Lund. 1987. Cellular localization of somatostatin (insulin-like growth factor) messenger RNA in the human fetus. Science (Wash. DC). 236:193-197.

Ingbar, S. H. 1972. Autoregulation of the thyroid: the response to iodide excess and depletion. Mayo Clin Proc. 47:814-827.

Ingbar, S. H., D. Tramontano, and F. S. Ambesi-Impiombato. 1987. Observations on the regulation of thyroid cell growth. In Molecular Biological Approaches to Thyroid Research. International Symposium Reisenburg. Thieme Medical Publisher, Inc., New York. 43-50.

Jin, S., D. Hornicek, M. Neylan, M. Zakkarja, and G. M. McKenzie. 1986. Evidence that adenine 3',5', monophosphate mediates stimulation of thyroid growth in FRTL5 cells. Endocrinology. 119:802-810.

Killary, A. M., and R. E. Fournier. 1984. A genetic analysis of extinction: trans-dominant loci regulate the expression of liver-specific traits in hepatoma hybrid cells. Proc. Natl. Acad. Sci. USA. 81:935-939.

Dere, W. H., and B. Rapoport. 1986. Control of growth in cultured rat thyroid cells. Mol. Cell Endocrinol. 44:195-199.

Errick, J. A., K. W. A. Ing, M. C. Eggo, and G. N. Burrow. 1986. Growth and differentiation in cultured human thyroid cells: effects of epidermal growth factor and thyrotropin. In Vitro Cell. Dev. Biol. 22:28-36.

Fourgere, C., and M. C. Weiss. 1978. Phenotypic exclusion in mouse melanoma rat hepatoma hybrid cells: pigment and albumin production are not expressed in simultaneously.

Gartner, L., W. Greil, R. Dembarker, and K. Horn. 1985. Involvement of cyclic AMP, iodide and metabolites of arachidonic acid in the regulation of cell proliferation of isolated porcine thyroid follicles. Mol. Cell. Endocrinol. 42:145-155.

Goldfine, I. D., S. H. Amir, A. W. Peterson, and S. H. Ingbar. 1974. Preparation of biologically active TSH. Endocrinology. 95:1228-1233.

Halmi, N. S. 1961. Thyroidal iodide transport. Vitam. Horm. 19:133-142.

Han, V. K. N., A. J. D'Ercole, and P. K. Lund. 1987. Cellular localization of somatostatin (insulin-like growth factor) messenger RNA in the human fetus. Science (Wash. DC). 236:193-197.

Ingbar, S. H. 1972. Autoregulation of the thyroid: the response to iodide excess and depletion. Mayo Clin Proc. 47:814-827.

Ingbar, S. H., D. Tramontano, and F. S. Ambesi-Impiombato. 1987. Observations on the regulation of thyroid cell growth. In Molecular Biological Approaches to Thyroid Research. International Symposium Reisenburg. Thieme Medical Publisher, Inc., New York. 43-50.

Jin, S., D. Hornicek, M. Neylan, M. Zakkarja, and G. M. McKenzie. 1986. Evidence that adenine 3',5', monophosphate mediates stimulation of thyroid growth in FRTL5 cells. Endocrinology. 119:802-810.

Killary, A. M., and R. E. Fournier. 1984. A genetic analysis of extinction: trans-dominant loci regulate the expression of liver-specific traits in hepatoma hybrid cells. Proc. Natl. Acad. Sci. USA. 81:935-939.