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Thyrotropin Receptor Gene Expression in Oncogene-Transfected Rat Thyroid Cells: Correlation Between Transformation, Loss of Thyrotropin-dependent Growth, and Loss of Thyrotropin Receptor Gene Expression

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Rat FRTL-5 and PC-Cl-3 thyroid cells are continuously cultured, clonal lines which require thyrotropin to grow and function. Both can be efficiently transformed when infected with RNA or DNA viruses carrying oncogenes or when directly transfected with activated oncogenes. Transformation, assayed by the appearance of cell growth in agar and by tumorigenicity in syngeneic rats or nude mice, is associated with the loss of thyrotropin-dependent cell division and thyrotropin-regulated functions such as thyroglobulin synthesis. In 16 clones of FRTL-5 or PC-Cl-3 cells transformed with different oncogenes, we show that loss of thyrotropin-dependent growth and function correlates with the loss of thyrotropin receptor gene expression, measured with a rat thyrotropin receptor cDNA probe.

FRTL-5 (1) and PC-Cl-3 (2, 3) are clonal lines of rat thyroid cells in continuous culture which retain their ability to express typical markers of thyroid differentiation such as thyrotropin (TSH)-enhanced thyroglobulin synthesis or iodide trapping. In addition, both require TSH to grow (1-3). In analogy with other cells in culture, each can be transformed by oncogenes such as v-ras, v-raf, v-mos, c-myc, Py middle T, and v-abl (3-6). In the case of FRTL-5 cells, complete transformation can be achieved by transfection with a single oncogene as in the case of infection with Kirsten murine sarcoma virus carrying the v-ras oncogene (4). In the case of PC-Cl-3 cells, complete transformation requires the cooperation of two oncogenes such as v-ras and c-myc (3). One consequence of the

Abbreviation used: TSH, thyrotropin.

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transformation of fibroblasts and epithelial cells by activated oncogenes is a loss of their growth response to exogenously added serum or specific growth factors (7-10). In both FRTL-5 and PC-Cl-3 cells, complete transformation, assayed by the acquisition of the ability to grow in soft agar and form tumors in syngeneic rats or nude mice (3-6, 10), is associated with loss of TSH-dependent DNA synthesis, growth, and function.

Loss of TSH responsiveness could result from a TSH receptor defect or a defect in a post receptor element important for signal transduction (11). The present report describes the interesting result that in all cases of oncogene transformation of FRTL-5 or PC-Cl-3 cells thus far studied, loss of TSH-responsiveness correlates with a loss in TSH receptor gene expression.

MATERIALS AND METHODS

Cell Culture - The FRTL-5 (ATCC CRL 8305) (1) and PC-Cl-3 (2, 3) rat thyroid cells are derived, respectively, from 3 wk and 18 mo old normal Fisher rats; both are passaged and grown in Coon's modified Ham's F-12 medium supplemented with, 5% heat inactivated, mycoplasma-free calf serum and a six hormone (6H) mixture containing the following: TSH, $1x10^{-10}$ M; insulin, 10 μg/ml; hydrocortisone, $1x10^{-8}$M; human transferrin, 5 μg/ml; somatostatin, 10 ng/ml; and glycyl-L-histidyl-L-lysine acetate, 10 ng/ml (1-3). The isolation and growth of FRTL-5 or PC-Cl-3 cells transfected with different oncogenes have also already been described (2-6).

Assay of the Transformed State- Tumorigenicity of the cell lines was tested by injecting $2x10^6$ cells into syngeneic rats or athymic mice (2, 3, 10). Soft agar colony assays were performed as described by McPherson et al. (12).

Assays of TSH-dependent Growth and Function - Assays measuring [^{3}H]thymidine (40 Ci/mmol, Amersham) incorporation into DNA and immunoprecipitable thyroglobulin were performed in cells maintained in medium with and without added TSH (3-6, 13, 14). All assays were performed in duplicate, on more than one batch of cells, and on more than one occasion.

RNA Isolation and Northern Analysis - RNA was purified from cultured cells by a modification of the guanidine hydrochloride extraction method (15). Northern analyses using Nytran membranes (Schleicher & Schuell) were performed as described (3, 5, 16). The rat TSH receptor probe used was the purified insert from clone T8AFB which represents residues -54 to 2780 of the nucleotide sequence and encodes the full length TSH receptor (16). ß-Actin was kindly provided by Dr. B. Paterson (National Cancer Institute).

RESULTS AND DISCUSSION

FRTL-5 rat thyroid cells transformed with Kirsten murine sarcoma virus (Ki Ki, Ki Mol) or Harvey murine sarcoma virus (FRTL-5-Ha), both carrying v-ras oncogenes, acquire a completely malignant or transformed phenotype as evidenced by growth in soft agar and tumor formation when injected into syngeneic rats or nude mice (3-5). The transformed cells also completely lose their dependence on TSH as a specific growth factor, whether assayed as increased [^{3}H]thymidine incorporation into DNA or cell number (3-5, Fig. 1). These properties cannot be explained by the ability of sarcoma virus

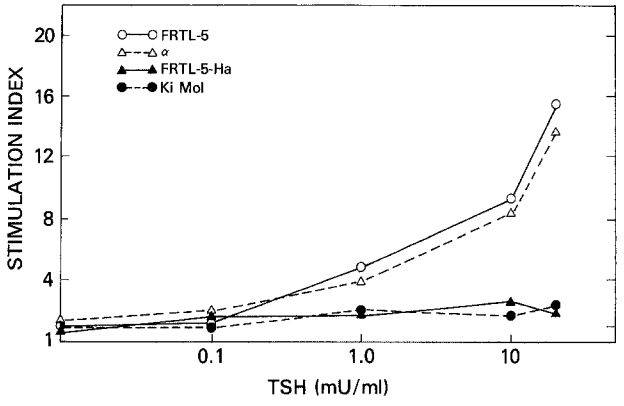

Figure 1. Ability of TSH to increase [^{3}H]thymidine incorporation into DNA of FRTL-5 rat thyroid cells transfected with v-ras oncogenes (Ki Mol or HaMSV) or transfected with a plasmid expression vector containing TGF-α (α) by comparison to control FRTL-5 cells. Stimulation index defines the fold increase of thymidine incorporation into DNA in the presence of TSH by comparison to cells grown in no TSH. Cells were maintained for 2 days without TSH by withdrawing the 6 hormone mixture from the culture medium. Different concentrations of TSH were added to the culture medium together with $1x10^6$ cpm/ml [^{3}H]thymidine; incorporation of radiolabel into DNA was measured 72 hours later after trichloroacetic acid precipitation of repeatedly washed cells (7). Ki Mol cells are transformed with a wild type strain of Kirsten murine sarcoma virus, KiMuLV (2-6, 14); and FRTL-5-Ha cells are those transfected with Harvey murine sarcoma virus containing the h-ras oncogene (2-6).

transformed cells to produce and secrete large amounts of transforming growth factor-α (TGF-α) (17). Thus, FRTL-5 cells transfected with a plasmid expression vector containing TGF-α (Clones α and α1) do not acquire a malignant phenotype despite their ability to produce and secrete TGF-α (18). Like normal FRTL-5 cells, they maintain their TSH responsiveness as measured by either thyroglobulin synthesis (2-6) or [^{3}H]thymidine incorporation into DNA (Fig. 1).

A previous study (14), concerned with the fact that v-ras transformed cells contain ras *p21* proteins with GTP-binding and GTPase activities, showed that Ki Ki and Ki Mol cells had the same basal cAMP levels as FRTL-5 cells as well as apparently normal G proteins and adenylate cyclase catalytic activity, as measured by their responsiveness to cholera toxin, pertussis toxin, and forskolin. It suggested (14), therefore, that the loss of TSH-dependent growth and differentiation might reflect an abnormality at the receptor level rather than a defect in a post receptor element important in signal transduction. FRTL-5 rat thyroid cells contain two species of mRNA, 3.3 and 5.6 kb, which encode the full length protein and can be identified with a rat TSH receptor cDNA probe (Ref. 16 and Fig. 2A). Both species are absent in the v-ras transformed FRTL-5 cells, Ki Ki, Ki Mol, and FRTL-5-Ha, which lose TSH-responsiveness and acquire the transformed phenotype (Fig. 2A). In contrast, both species of TSH receptor mRNA are present in TGF-α transfected cells (α and α_1) just as in normal FRTL-5 cells (Fig. 2A). These data indicate

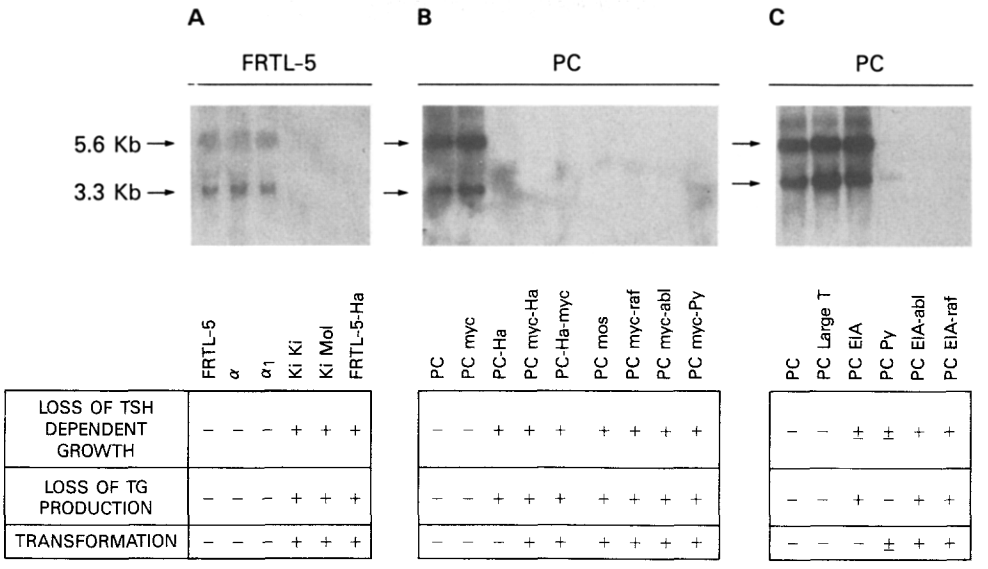

Figure 2. Expression of TSH receptor gene in FRTL-5 (A) and PC-Cl-3 (B, C) rat thyroid cells transformed with different oncogenes by comparison to their state of transformation and their ability to exhibit TSH-dependent DNA synthesis and growth as well as TSH-dependent thyroglobulin synthesis. Total RNA, 20 μg, was applied to each lane; TSH receptor mRNA was measured by Northern analysis using the purified insert from clone T8AFB which represents residues -54 to 2780 of the nucleotide sequence and encodes the full length TSH receptor (16). Transformation was measured by the appearance of cell growth in agar and tumorigenicity in syngeneic rats or nude mice; a (+) represents positivity in both assays, a (-) represents negativity in both assays, a (±) represents weak positive results in one assay only. TSH-dependent DNA-synthesis was measured as described in the Figure 1 legend; TSH-dependent cell growth was measured by cell number. A (+) represents loss of TSH-dependent DNA synthesis and/or cell growth, a (-) represents retention of TSH-dependent DNA synthesis and/or cell growth, a (±) represts a cell which retains TSH-dependent DNA synthesis and cell growth but can also grow in serum. TSH-dependent thyroglobulin synthesis was measured after cells were maintained in TSH- and serum-depleted medium for 2 days, incubated with 10 mU/ml TSH for 2 days, and exposed to radiolabeled [^{35}S]methionine during the last 12 hours of this incubation (23). The medium was evaluated by radioimmunoassay using anti-rat thyroglobulin (2-6, 23); a (+) represents loss of TSH-dependent thyroglobulin synthesis and secretion into the medium measured by the radioimmunoassay whereas a (-) represents a cell which retains TSH-dependent thyroglobulin synthesis and secretion. In (A) the v-ras oncogene or TGF-α transfected FRTL-5 cell lines are those described in Figure 1. In (B) and (C), the cells are control PC-Cl-3 rat thyroid cells (2) or PC cells transfected or infected, as appropriate, with the PMCGM1 plasmid carrying the human c-myc oncogene (PC myc); with HaMSV containing the v-ras-Ha oncogene (PC-Ha); with both of the aforementioned transfected in different order (PC myc-Ha, PC-Ha-myc); with myc and the v-raf oncogene obtained by infection of PC myc with the murine sarcoma virus 3611 (PC myc-raf); with myc and the v-abl oncogene obtained by infection of PC myc with the Abelson murine leukemia virus (PC myc-abl); with the polyoma murine leukemia virus carrying the polyoma virus middle T antigen alone (PC Py); with Py plus myc (PC myc-Py); with the myeloproliferative sarcoma virus carrying the v-mos oncogene (PC mos); with the plasmid R15 carrying the adenovirus E1A gene alone (PC E1A) or with v-raf (PC E1A-raf) or v-abl (PC E1A-abl); and with the p214LT plasmid carrying the polyoma large T gene (PC Large T). The basic methodologies to obtain these transformants and their characterization has been described (2-6, 10). Blots were washed and rehybridized with ß-actin to insure that equal amounts of nondegraded RNA were applied to each lane; this was true in each case except that of PC Py in (C) wherein the amount of hybridizable ß-actin was reduced by two-thirds compared to other lanes.

that the transformation-associated loss of TSH-dependent growth and function correlates with the loss of expression of the TSH receptor gene.

Interestingly, this correlation is not specific to FRTL-5 cells nor is it oncogene specific. Thus, PC-Cl-3 rat thyroid cells differ from FRTL-5 cells in that more than one oncogene is usually required to express the complete malignant phenotype (3). For example, transfection with c-myc (PC myc) or v-ras (PC Ha) does not result in transformation of PC-Cl-3 as assayed by agar growth or tumorigenicity, whereas sequential transfection with both, in any order (PC myc-Ha or PC-Ha-myc), does induce the malignant phenotype (Fig. 2B and Refs. 3, 6). Similarly, complete transformation is induced by combinations of myc with v-raf (PC myc-raf), v-abl (PC myc-abl), or polyoma murine leukemia virus carrying the middle T oncogene (PC myc-Py). In each instance, Northern blot analysis using the rat TSH receptor cDNA probe (Fig. 2B) reveals there is a correlation between loss of both species of TSH receptor mRNA and loss of TSH-dependent growth and function. Even in a case wherein the presence of a single oncogene is associated with transformation, i.e. infection with myeloproliferative sarcoma virus carrying the v-mos oncogene (PC mos), loss of TSH-dependent growth, measured as [^{3}H]thymidine incorporation into DNA and cell number, correlates with the loss of TSH receptor mRNA by Northern analysis with the rat TSH receptor cDNA probe (Fig. 2B).

Clone PC-Ha in Figure 2 B illustrates the case wherein loss of TSH-dependent growth and function is associated with loss of TSH receptor mRNA despite incomplete transformation. Transfection of PC-Cl-3 with plasmid (R15) carrying the adenovirus E1A (PC E1A) and infection with polyoma murine leukemia virus carrying the middle T oncogene (PC Py) illustrate situations wherein loss of TSH receptor function as well as transformation is incomplete (Fig. 2 C). In both cases cells retain TSH-dependent growth measurable in the absence of serum although they can also grow in serum without TSH (3-6); in one case (PC Py) the ability of TSH to increase the synthesis of thyroglobulin is retained whereas in the other (PC E1A) it is lost. In both cases, TSH receptor mRNA is retained with the retention of one or more TSH-dependent properties (Fig. 2C). If, however, EIA transfection is combined with v-abl or v-raf (PC E1A-abl, PC E1A-raf), there is once again direct correlation of loss of TSH receptor mRNA with loss of TSH-dependent function (Fig. 2C).

In sum, in every instance thus far examined, with either complete transformation or incomplete transformation, the loss of TSH-dependent growth was associated with the loss of measurable TSH-receptor mRNA. No example has thus far been found of a post-receptor abnormality in signal-coupling wherein TSH receptor mRNA was retained in association with loss of TSH-dependent growth. These data are consistent with initial

studies showing a normal G-protein/adenylate cyclase complex responsive to cholera toxin, pertussis toxin, and forskolin in v-ras transformed FRTL-5 rat thyroid cells (14).

Inhibition of TSH receptor gene expression can result from multiple mechanisms, i.e. it can represent the action of oncogenes on posttranscriptional mechanisms on primary transcript processing, on export of mature mRNA from the nucleus, or on the stability of cytoplasmic transcripts. Alternatively, it can result from altered gene transcription. Since studies of thyroglobulin gene have shown that its expression requires a thyroid specific transcription factor, TTF1 (19), since this factor is lost in neoplastic transformation (20), and since, as evidenced in Fig. 2, loss of TSH-dependent growth is usually associated with a loss in thyroglobulin formation and secretion, we currently speculate that expression of the thyroglobulin and TSH receptor genes may be coordinately repressed and related to TTF1 expression. Preliminary results using a temperature sensitive ras transformant which can be reversibly dedifferentiated support this conclusion (21, 22); studies to directly support this possibility in the multiplicity of transformants evaluated herein are in progress.

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