# RhoA Activation Promotes Transformation and Loss of Thyroid Cell Differentiation Interfering with Thyroid Transcription Factor-1 Activity

DIEGO L. MEDINA*, MARCOS RIVAS*, PATRICIA CRUZ, ISABEL BARROSO, JAVIER REGADERA, AND PILAR SANTISTEBAN

*Instituto de Investigaciones Biomédicas "Alberto Sols" (D.L.M., M.R., P.C., I.B., P.S.), Consejo Superior de Investigaciones Científicas, Universidad Autónoma de Madrid, and the Departamento de Morfología (J.R.), Facultad de Medicina, Universidad Autónoma de Madrid, E-28029 Madrid, Spain*

Highly specialized cells, the thyrocytes, express a thyroid-specific set of genes for thyroglobulin (Tg), thyroperoxidase, and the transcription factors TTF-1, TTF-2, and Pax-8. The implication of the small GTPase RhoA in TSH-mediated proliferation of FRTL-5 rat thyroid cells has been previously demonstrated. To further analyze RhoA function in thyroid cell proliferation and differentiation patterns, we combined transient and stable transfection assays to express different mutant RhoA forms in FRTL-5 cells. Constitutively active RhoA (FRTL-5-RhoA QL cells) exhibited a fibroblast-like phenotype with organized actin fibers, whereas cells expressing the RhoA negative dominant phenotype (FRTL-5-RhoA N19 cells) present a rounded morphology and lose normal cytoskeletal architecture. In addition, expression of the constitutively active form of RhoA results in TSH-independent proliferation and anchorage-independent growth and induces tumors when inoculated in nude mice.

Interestingly, FRTL-5-RhoA QL cells express less Tg and TTF-1 than wild-type FRTL-5 (FRTL-5-vector) or FRTL-5-RhoA N19, suggesting a loss at the differentiation stage. This effect is mediated, at least in part, by a decrease in TTF-1 activity, since transient or stable expression of RhoA QL results in a reduction in the activity of the wild-type Tg promoter as well as an artificial promoter the activation of which depends exclusively on TTF-1. The similarity between RhoA effects and thyroid transformation by Ras suggests that RhoA may act as a downstream effector of Ras; in fact, the dominant negative RhoA N19 abolished the down-regulatory effect of Ras V12 over the Tg promoter. Taken together, these results show for the first time that active RhoA is able to transform FRTL-5 cells and that this effect is coupled to a loss of thyroid differentiation due to impaired TTF-1 activity. (*Molecular Endocrinology* 16: 33–44, 2002)

T HE RAS PROTEINS are a group of guanine nucleotide-binding proteins that function as molecular switches in many cellular signaling pathways, interacting with a wide spectrum of regulators and downstream effectors; they produce a broad range of cellular responses such as proliferation, differentiation, or apoptosis (1, 2). The expression of transforming Ras oncogenes interacts with the establishment and maintenance of cellular differentiation in different tissues (3–6), including the thyroid. In this tissue, Ras inhibits the expression of thyroid-specific genes and confers a proliferative advantage over normal thyroid cells (7–11). Ras transformation in the specialized epithelial thyroid cell line FRTL-5 suppresses the expression of thyroid differentiation markers such as thyroglobulin (Tg), thyroperoxidase (TPO), and iodine uptake (12). In parallel, the transcription factors controlling thyroid gene expression, such as TTF-1, TTF-2, or Pax-8, are either not present or inactive (10, 13, 14). In K-ras-

transformed thyroid cells (FRTL-5-K-ras), both TTF-1 and Pax-8 mRNA are undetectable, whereas in H-ras (FRTL-5-H-ras), TTF-1 is present at normal levels and maintains its DNA binding properties, although the cells lack the ability to express Tg and TPO (11). Several proteins have been identified as potential effectors of Ras signaling, including Raf/MAPK kinase/ERK, RalGDS, and PI3K, although the mechanism of Ras-mediated inhibition of thyroid cell differentiation remains essentially unclear.

Another Ras protein family that includes RhoA, Rac1, and Cdc42 plays a pivotal role in controlling many cellular functions, such as cytokinesis, motility, proliferation, and apoptosis (15). These three proteins cooperate with Raf in cell transformation, and the dominant negative forms of RhoA and Rac1 can inhibit Ras-induced transformation, indicating an essential function in this process (16–20). Moreover, Rac1 and RhoA have been implicated in the morphogenic and mitogenic responses to transformation by oncogenic Ras (21, 22). In the context of thyroid cells, the positive effects of RhoA in thyroid cell proliferation have been related to its role in $p27^{Kip1}$ degradation (23). Among other functions, $p27^{Kip1}$ has been implicated in $G_1$

---

Abbreviations: BrdU, Bromodeoxyuridine; CAT, chloramphenicol acetyltransferase; CMV-Luc, cytomegalovirus-luciferase; Tg, thyroglobulin; TPO, thyroperoxidase; TTF-1, thyroid transcription factor.

arrest induced by inhibitors of 3-hydroxy-3-methylglu-taryl-coenzyme-A reductase (23). These inhibitors interfere with cell cycle progression by suppressing the isoprenylation of proteins (24), and RhoA is a class of isoprenylated small GTPases proposed to be involved in $G_1$/S transition in FRTL-5 cells (23, 24). We recently confirmed these results and demonstrated that overexpression of either the dominant negative form RhoA N19 or the specific inhibitor of RhoA activity, the exoenzyme C3, thus inhibits FRTL-5 cell proliferation, causing $G_1$ arrest (25).

In the present study, we combined transient and stable expression of different mutant RhoA forms as tools to study the role of this protein in the differentiation of FRTL-5 thyroid cells. We found that activation of RhoA induces TSH-independent proliferation, morphological transformation, anchorage-independent growth, and tumorigenesis when cells are injected into nude mice. These effects could be mediated by the activation of c-*fos* and c-*jun*, the expression of which is probably central in cell proliferation control and is also necessary for neoplastic transformation by a variety of oncogenes (26, 27). In addition, RhoA activation results in a less differentiated thyroid phenotype, decreasing Tg and TTF-1 gene expression by blocking transactivation of the Tg promoter through inhibition of TTF-1 transcriptional activity. These results demonstrate for the first time that RhoA induces transformation of FRTL-5 thyroid cells.

## RESULTS

### Generation and Analysis of Stable FRTL-5 Cells Expressing Different Mutant Forms of RhoA Protein

The rat thyroid follicular cell line FRTL-5 provides a useful model with which to study growth and differentiation of specialized epithelial cells. To analyze the role of RhoA protein in the differentiation pattern of thyroid cells, FRTL-5 cells were stably transfected with either an AU5-tagged dominant positive form of RhoA (RhoA QL) (28), an AU5-tagged dominant negative form of RhoA (RhoA N19) (29), or with AU5-RhoAQL together with an expression vector for C3 toxin which ribosylates and inactivates RhoA (28, 30). Transfected FRTL-5 clones were tested for exogenous RhoA expression by immunoblotting analysis of total cell extracts using anti-AU5 or -RhoA antibodies. Three representative neomycin-resistant clones referred as FRTL-5-RhoA QL, RhoA N19, and RhoA QL-C3 were selected for further study (Fig. 1A). As has been described extensively, the small GTP-binding protein RhoA regulates the assembly of focal adhesion and actin stress fibers in response to growth factors (15). We thus analyzed the possible morphological changes due to overexpression of the different RhoA mutant forms. Phase contrast photomicroscopy of early passage FRTL-5 clones indicated that, in the presence of

6H complete medium, FRTL-5-RhoA QL cells present a fibroblast-like appearance compared with neomycin-resistant FRTL-5 cells carrying control vector (FRTL-5-vector) (Fig. 1B). In addition, the cellular limits within the colonies are clearly distinguished. Conversely, FRTL-5-RhoA N19 cells present a rounded shape and diffuse cell-to-cell limits within colonies. Interestingly, no fibroblast-like morphology was observed when the RhoA inhibitor, the exoenzyme C3, was expressed together with RhoA QL. In fact, these cells show a rounded morphology similar to those expressing the dominant negative form N19 (Fig. 1B).

We support these results by immunofluorescence using fluorescein-conjugated phalloidin to visualize the actin cytoskeleton. FRTL-5-RhoA QL cells present a more protrusive appearance compared with FRTL-5-vector cells, whereas FRTL-5-RhoA N19 and RhoA QL-C3 cells show a redistribution of the actin fibers, lose their normal cytoskeleton organization and round up (Fig. 1C).

### RhoA Activation Induces a Proliferative Advantage and TSH-Independent Growth

In addition to the morphological changes induced by RhoA, results from several laboratories demonstrate that this protein is involved in TSH-mediated thyroid cell proliferation (23–25). Inhibition of RhoA activity by the C3 exoenzyme or by transient expression of dominant negative RhoA N19 thus decreases FRTL-5 cell proliferation, causing $G_1$ arrest in the cell cycle. To confirm the role of RhoA in proliferation, growth curves were performed in stably transfected cells cultured alone or in the presence of TSH (see *Materials and Methods*).

In the presence of TSH (6H medium), overexpression of constitutively active RhoA (FRTL-5-RhoA QL cells) induces a proliferative advantage compared with controls (FRTL-5-vector cells), whereas expression of the dominant negative mutant form of RhoA (FRTL-5-RhoA N19 cells) decreases cell proliferation (Fig. 2B). Interestingly, FRTL-5-RhoA QL cells grow in the absence of TSH with a growth rate similar to that seen in the presence of the hormone, while the FRTL-5-vector or -RhoA N19 cells barely grow in the absence of TSH (Fig. 2A). These data confirm that RhoA activation induces TSH-independent growth in FRTL-5 cells. Cytometric analysis of FRTL-5-RhoA N19 clones demonstrated a decrease in the S phase of the cell cycle as a consequence of $G_1$ arrest (not shown), confirming previous results (25).

### RhoA Activation Induces Transformation *in Vitro* and Increases the Tumorigenicity of FRTL-5 Thyroid Cells

FRTL-5 cells are considered a normal nontumorigenic cell line, although contradictory results are found throughout the bibliography (12, 31). The tumorigenicity of FRTL-5 cells is thus conditioned by clonal vari-
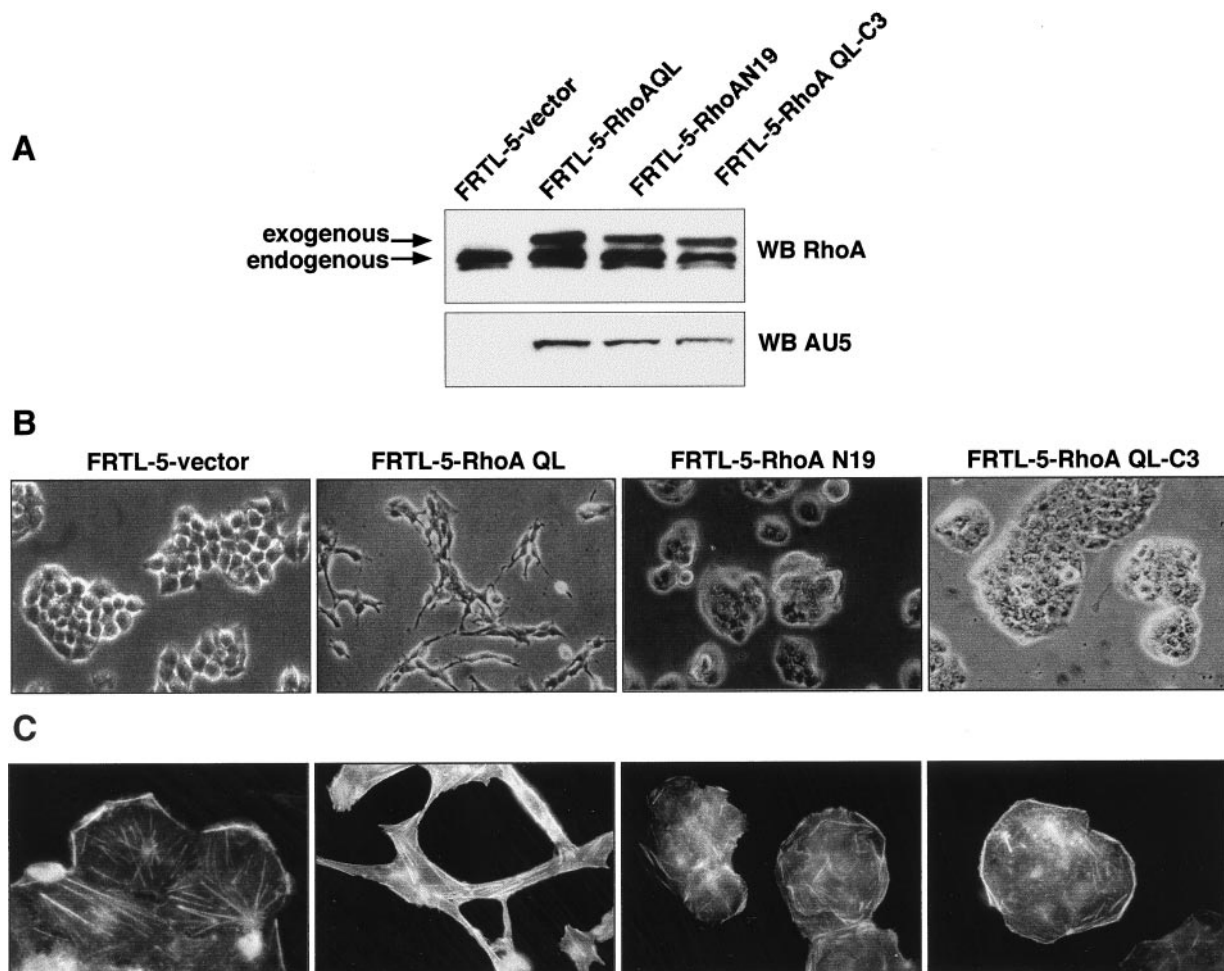
**Fig. 1.** Expression of RhoA Mutants in Thyroid Follicular Cells

A, FRTL-5 cells were stably transfected with vector alone or harboring the AU5-tagged-RhoA QL, AU5-tagged RhoA N19, or exoenzyme C3 plus the AU5-tagged RhoA QL. Neomycin-resistant colonies were isolated and analyzed by immunoblotting, using anti-AU5 or anti-RhoA antibodies. A 30-kDa band corresponding to exogenous RhoA protein was detected in several independent clones. FRTL-5 positive clones studied (vector, RhoA QL, RhoA N19, and RhoA QL-C3) were selected for further studies. B, General morphology of the above clones obtained by phase contrast photomicroscopy, of early-passage cells, 2 d after plating. Magnification, ×100. C, Cells were stained with fluorescein isothiocyanate-phalloidin to visualize the actin cytoskeleton. Magnification, ×100.

ability, TSH levels in nude mice, and cell passage number (12, 31, 32). Rho proteins have transforming and oncogenic potential in a cell type-specific manner (16–20). Cells expressing constitutively active mutants of Rac and RhoA display enhanced growth in low serum, are anchorage independent, and induce tumor formation when inoculated into nude mice (20). In addition, Rac and Rho proteins are essential for transformation by Ras (16, 18).

To assess RhoA-dependent FRTL-5 transformation, the ability of each cell line was tested to form colonies in semisolid medium in the presence or absence of TSH; only cells expressing the active mutant form of RhoA (RhoA QL) were able to grow in soft agar (Table 1). The number of colonies was similar in the absence or presence of TSH, again demonstrating the TSH-independent growth of cells expressing RhoA QL.

To test the role of RhoA protein in FRTL-5 tumorigenicity, we inoculated FRTL-5-RhoA QL and FRTL-5-RhoA N19 cells, as well as FRTL-5-vector cells, into nude mice (2 × 10⁶ cells). Four weeks later, we observed tumors in the FRTL-5-RhoA QL cell-inoculated group, whereas the groups inoculated with FRTL-5-vector cells or FRTL-5-RhoA N19 cells remained normal (Table 1). These results indicate that RhoA induces FRTL-5 cell transformation and tumorigenesis. Excised tumors were analyzed histologically as described in *Materials and Methods*. FRTL-5-RhoA QL-inoculated mice developed large tumors located in the dermis (Fig. 3A), whereas control or FRTL-5-RhoA N19-inoculated mice remained normal. Tumors were highly undifferentiated and infiltrative. Tumor cells were poorly differentiated, showing small cytoplasm and large nuclei with abundant mitosis (Fig. 3B). They
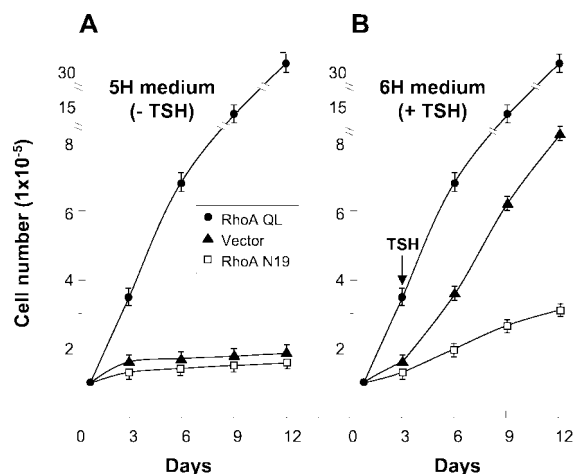
**Fig. 2.** Proliferative Activity of Thyroid Follicular Cells Carrying Constitutively Active RhoA QL or Inactive RhoA N19

FRTL-5 clones were maintained in the absence of TSH (5H medium) for 3 d. From then on, cells were cultured either in the same medium (panel A*)* or in a medium with TSH (6H medium) (panel B). Cell number was monitored every 3 d for 12 consecutive days, and viable cell number is represented. The data are the mean $\pm$ SD of three independent experiments.

**Table 1.** RhoA Activation Induces Transformation *in Vitro* and Tumorigenesis *in Vivo*

| Cell Line | Colonies in Soft Agar (n) | | Tumors in Nude Mice 4 Wk after Injection (n) |
|---|---|---|---|
| | −TSH | +TSH | |
| FRTL-5 vector | 0 | 0 | 0/12 |
| FRTL-5 RhoA QL | 129 $\pm$ 8 | 140 $\pm$ 12 | 12/12 |
| FRTL-5 RhoA N19 | 0 | 0 | 0/12 |

Values for number of colonies are the mean $\pm$ SD obtained from three independent experiments of each cell type. Tumor frequency is expressed as number of mice with tumors/total number of mice.

grew in a solid, diffuse pattern, with neither follicles nor papillary structures. Infiltration was observed mainly as satellite tumor nodules (Fig. 3C). Bromodeoxyuridine (BrdU) immunostaining showed that proliferative cells were located mainly in peripheral regions of the tumor (Fig. 3D); satellite tumor nodules were highly proliferative, mainly in the peripheral area (Fig. 3E). Detailed necropsy of the mice revealed no other relevant effects.

Molecular mechanisms leading to transformation and tumorigenicity are complex and involve the activation of different signaling pathways, as well as the expression of a set of genes the induction of which may play a role regulating these processes. In an attempt to understand some of the mechanisms responsible for FRTL-5 RhoA QL transformation, we analyzed the levels of cAMP as the main signaling pathway controlling thyroid cell growth (33) and because
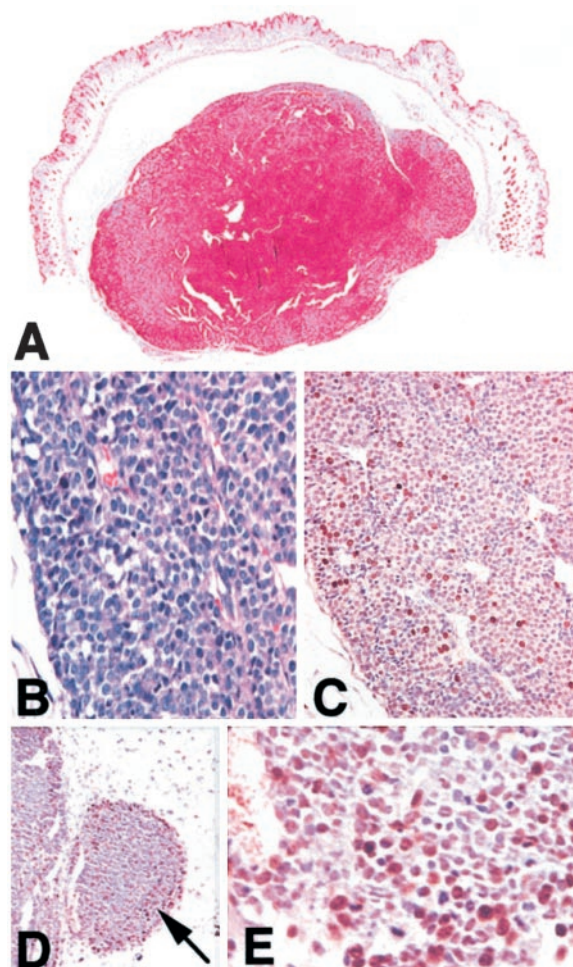


**Fig. 3.** Histological Analysis of Tumors from FRTL-5-RhoA QL-Inoculated Cells into Nude Mice

A, General view of hematoxylin-eosin-stained large nodular tumor derived from FRTL-5-RhoA QL-inoculated cells (8$\times$ magnification). The tumor is well defined, unencapsulated, and localized in the dermis. It presents a solid growth pattern with moderate blood vessel proliferation. B, Hematoxylin-eosin staining of the peripheral area of an undifferentiated, solid tumor derived from FRTL-5-RhoA QL cells, with anaplastic cells with small cytoplasm (125$\times$ magnification). C, Anti-BrdU-hematoxylin staining of the peripheral area of the previous figure showing abundant highly stained, BrdU-positive cells (intense *brown* color) (125$\times$ magnification). D, Anti-BrdU-hematoxylin staining of an infiltrative, satellite nodule formed in the peripheral area of an undifferentiated tumor. The *arrow* indicates the augmented area shown in Fig. 3E (50$\times$ magnification). E, Anti-BrdU-hematoxylin staining of the peripheral area of the infiltrative nodule shown in Fig. 3D. Note that most of the nuclei are highly stained with BrdU (300$\times$ magnification).

most thyroid tumors present increased levels of this second messenger (34). The results obtained show similar levels of cAMP in FRTL-5 vector (370 $\pm$ 25 fmol/well), RhoA QL (374 $\pm$ 20 fmol/well), and RhoA N19 (365 $\pm$ 19 fmol/well) cells maintained in 6H medium, suggesting that the upstream effector cAMP is

not responsible for either growth independence or cell transformation induced by RhoA activation. To identify downstream genes that could explain the above phenomena, we analyzed the levels of early response genes such as c-*fos* and c-*jun*. The election of these genes was based on previous reports demonstrating their involvement in RhoA signaling (35, 36). Western blot assays using nuclear protein extracts detect increased levels of c-Fos and c-Jun in FRTL-5 cells expressing the constitutively active form of RhoA (Fig. 4); the active phosphorylated form of c-Jun (P-Ser 63-c-Jun) is also increased after RhoA QL overexpression. These data suggest that the activation of these oncogenes is involved in RhoA transformation process in FRTL-5 cells.

## Activation of RhoA Is Sufficient to Affect Thyroid Cell Differentiation

Cell transformation is usually coupled to a loss of differentiated phenotype. Transformation of FRTL-5 cells with two oncogenic Ras forms, Harvey (FRTL-5-H-Ras) and Kirnstein Ras (FRTL-5-K-Ras), has been studied extensively. The work of several groups has showed that Ras transformation of FRTL-5 cells results in loss of iodine uptake, as well as in Tg and TPO gene expression (7–11). To study further the effect of RhoA activation in FRTL-5 cells, we measured expression of thyroid-specific mRNA for one of the critical thyroid-specific markers, Tg, and for its transcription factor TTF-1, in FRTL-5 clones carrying the constitutively active RhoA QL or the dominant negative RhoA N19. Tg and TTF-1 mRNA levels were not significantly affected by inactivation of RhoA protein (FRTL-5-RhoA N19 cells), whereas activation of RhoA (FRTL-5-RhoA QL cells) clearly decreased both Tg and TTF-1 mRNA levels (Fig. 5A). To test whether the RhoA-mediated decrease in Tg and TTF-1 mRNA levels correlates with
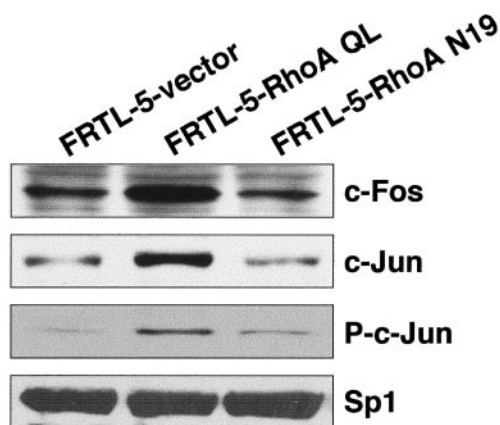
**Fig. 5.** Tg and TTF-1 Levels in Normal and RhoA-Expressing FRTL-5 Cells

A, Total RNA (30 μg) from stable FRTL-5 clones were blotted onto nytran membranes and sequentially hybridized with specific cDNA probes indicated at the *right* of the panels. As a loading control, membranes were hybridized with an actin-labeled probe. B, Total protein extracts (30 μg) from stable FRTL-5 clones were blotted onto protran membranes and incubated with specific anti-Tg antibody. Antiactin antibody was used as a loading control. Nuclear protein extracts (20 μg) from different FRTL-5 clones were immunobloted with anti-TTF-1 antibody. Anti-Sp1 antibody was used as a loading control.

a loss of Tg and TTF-1 protein levels, we performed Western blot assays using total or nuclear protein extracts to detect Tg or TTF-1 protein levels, respectively (Fig. 5B). Total protein extracts from FRTL-5-RhoA QL cells express less Tg than FRTL-5-vector cells or FRTL-5-RhoA N19 cells. Western blot assays using nuclear extracts from FRTL-5 cell clones showed a decrease in TTF-1 protein levels in FRTL-5-RhoA QL cells (Fig. 5B). Figure 5 shows representative Northern and Western blots. Similar results were obtained in all stable clones generated.

## Transient Active RhoA Expression Represses the Activity of the Tg Promoter

Several studies have implicated the protein RhoA in the morphogenic and mitogenic responses to transformation by oncogenic Ras (16, 18). In addition, previous studies of Ras-mediated transformation have shown its negative effect in the activity of thyroid-



**Fig. 4.** c-Fos and c-Jun Expression in Normal and RhoA Expressing FRTL-5 Cells

Nuclear protein extracts (20 μg) from vector, RhoA QL, or RhoA N19 FRTL-5 clones were analyzed by Western blotting by use of anti-c-Fos, anti-c-Jun, and anti-P-c-Jun antibodies. Anti-Sp1 antibody was used as a loading control.
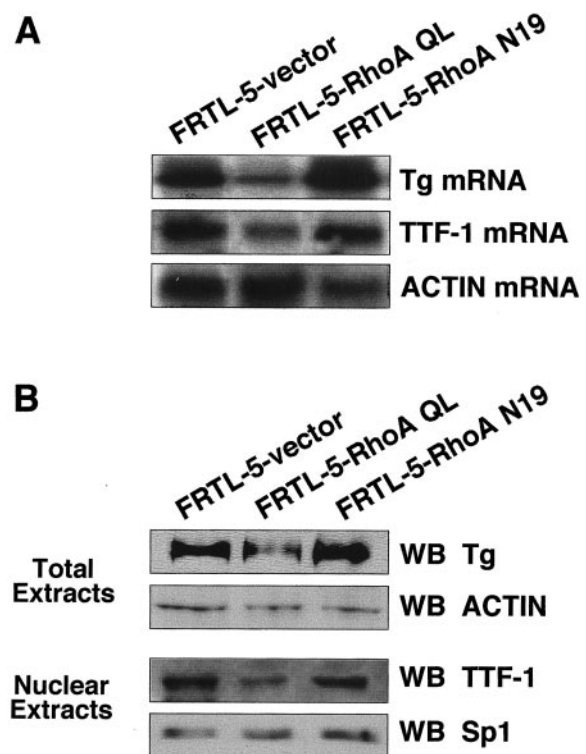
specific promoters (11, 37, 38). Transient expression of Ras V12 in FRTL-5 cells thus decreases the activity of TPO, Tg, and sodium iodide symporter gene promoter (38). Our results show that RhoA activation decreases Tg and TTF-1 mRNA levels, suggesting transcriptional inhibition of thyroid differentiation. To test this hypothesis, FRTL-5 cells were transiently transfected with the Tg promoter (TACAT-3) (39, 40), along with expression vectors encoding for the different RhoA mutant forms or for C3. Plasmid cytomegalovirus-luciferase (CMV-Luc) was transfected to correct for transfection efficiency. Cells were lysed 72 h after transfection, and chloramphenicol acetyltransferase (CAT) and luciferase activity were measured. Expression of the dominant positive RhoA QL resulted in approximately 75% inhibition of Tg promoter activity. The effect was due to RhoA activation, as it was abolished when cotransfected with its negative dominant form RhoA N19 or with the exoenzyme C3 (Fig. 6A). As described by Alberts *et al.* (41), we confirmed activity of transfected RhoA mutants by testing the activity of a construct containing wild-type serum response elements (not shown).

Tg promoter (TACAT-3) contains three binding sites for the homeodomain-containing protein TTF-1, which has been reported as its main transcriptional activator, although it is also regulated by other transcription factors (13, 39, 40). To verify that TTF-1 is involved in the RhoA-induced reduction of the Tg promoter activity, we used a 5C-CAT reporter gene containing an artificial promoter carrying five binding sites for TTF-1 placed in tandem upstream of the TATA box (42). This

promoter is regulated exclusively by TTF-1, showing strong activity in FRTL-5 thyroid cells. In transient transfection assays, 5C-CAT transcription was suppressed by RhoA QL expression to an extent similar to that of the wild-type (TACAT-3) Tg promoter. Again, RhoA N19 or C3 expression reverts RhoA QL inhibition of 5C-CAT promoter (Fig. 6B). As a control for the specificity of the transfected plasmids on the Tg promoter, we used the promoterless TATA-CAT vector (14), the activity of which remained unaffected (not shown).

Due to the parallelism observed in the inhibition of TTF-1 transcriptional activity mediated by Ras (11, 13, 38) and RhoA (present study), we asked whether the dominant negative form of RhoA, the N19 mutant, is able to revert Ras-induced Tg promoter repression. FRTL-5 cells were transiently transfected, as described above, with the wild-type Tg promoter (TACAT-3), along with expression vectors encoding Ras V12, Ras N17, and RhoA N19. Expression of Ras V12 resulted in 80–90% inhibition of the Tg promoter activity, and its dominant negative form Ras N17 reverted the effect. The dominant negative form RhoA N19 also reverted the inhibitory effect of Ras V12 on the Tg promoter (Fig. 7). These results clearly demonstrate that the Ras effect on the Tg promoter is RhoA dependent and suggest that RhoA is downstream of Ras in the control of thyroid differentiated phenotype.
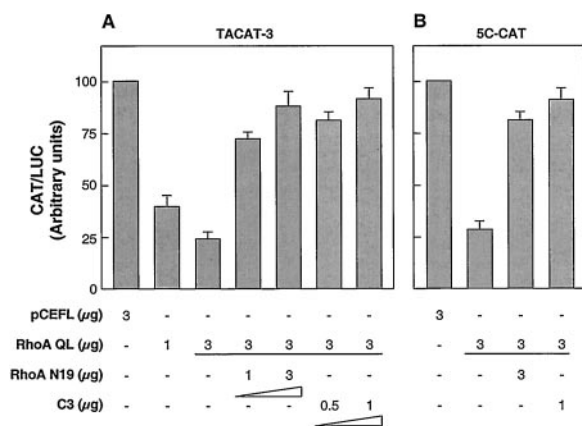


**Fig. 6.** Effects of RhoA Expression on Tg Promoter Activity
  FRTL-5 cells were transiently transfected with a construct containing the wild-type Tg promoter (TACAT-3) (panel A*),* or a construct containing five binding sites for TTF-1 (5C-CAT) (panel B), and with vectors encoding the different mutant RhoA forms or the C3 exoenzyme. Plasmid CMV-Luc was transfected to correct for transfection efficiency. At 72 h after transfection, cells were lysed, and CAT and luciferase activities were measured. Promoter activity is calculated as arbitrary units relative to the cells transfected with vector alone (=100). The data are the mean ± SD of three independent experiments.
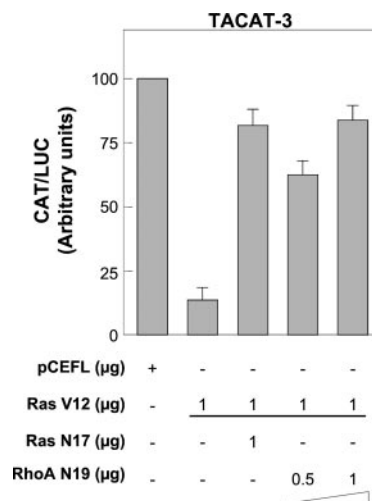


**Fig. 7.** RhoA N19 Reverts Ras-Mediated Inhibition of Tg Promoter Activity
  FRTL-5 cells were transiently transfected with a construct containing the wild-type Tg promoter (TACAT-3) and with expression vectors for Ras V12 alone or together with Ras N17 or RhoA N19. Plasmid CMV-Luc was transfected to correct for transfection efficiency. At 72 h after transfection, cells were lysed, and CAT and luciferase activities were measured. Promoter activity is calculated as arbitrary units relative to the cells transfected with vector alone (=100). The data are the mean ± SD of three independent experiments.

## Stable RhoA QL Expression Represses TTF-1 Transcriptional Activity in FRTL-5 Cells

To confirm the RhoA-mediated decrease in TTF-1 transcriptional activity in FRTL-5 cells, we performed similar assays using FRTL-5 cell clones stably expressing RhoA N19 or RhoA QL. The cells were transfected with 5C-CAT and CMV-Luc; after 72 h cells were lysed and CAT and luciferase activities measured. Expression of the constitutively active RhoA QL clearly inhibits 5C-CAT reporter gene activation, whereas stable expression of the dominant negative RhoA N19 form had no effect on the TTF-1 transcriptional activity (Fig. 8A). As a control, we tested 5C-CAT reporter gene activity in two stable FRTL-5 cell lines expressing the oncogenic Ras forms, H-ras and K-ras (12). As extensively described (13, 14), Ras transfor-
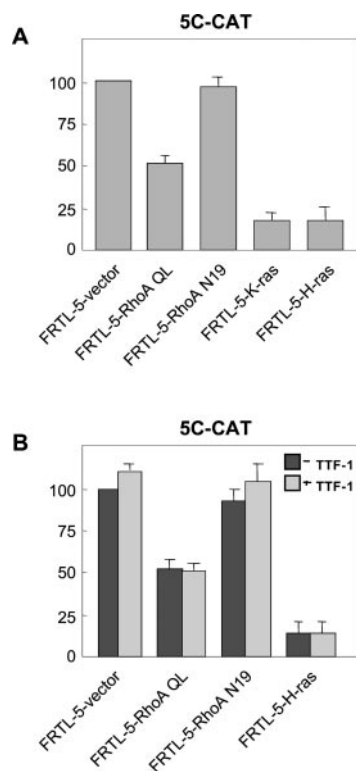
mation inhibits TTF-1 transcriptional activity in these cell lines (Fig. 8A).

TTF-1 transcriptional activity is regulated mainly by posttranslational modifications such as site-specific phosphorylation and redox mechanisms (43–45). In Ras-transformed FRTL-5 cells (FRTL-5-H-ras), Tg expression is absent due to expression of an inactive form of TTF-1 (11, 14). To understand the mechanism of RhoA-mediated dedifferentiation of thyroid cells, we determined whether RhoA activation affects TTF-1 transcriptional activity. The 5C-CAT reporter was transiently transfected with a vector harboring the TTF-1 cDNA into stable FRTL-5 cell clones expressing either RhoA QL or RhoA N19. 5C-CAT activity was not affected either in FRTL-5-vector cells or in FRTL-5 N19 cells when TTF-1 was overexpressed (Fig. 8B). This may be due to the existence of saturated levels of endogenous active TTF-1, as previously reported in FRTL-5 cells (14, 43). Interestingly, overexpression of this transcription factor in FRTL-5-RhoA QL had no effect in reverting RhoA-mediated inhibition of TTF-1 transcriptional activity, supporting the idea that RhoA may decrease Tg expression through some posttranslational modifications of TTF-1 protein (Fig. 8B). A similar phenomenon has been described in FRTL-5-H-ras cells overexpressing TTF-1 (11, 14).



**Fig. 8.** Effect of Stable RhoA Expression on TTF-1 Transcriptional Activity

A, FRTL-5 cells expressing the different mutant RhoA forms were transiently transfected with the 5C-CAT construct containing five specific binding sites for TTF-1. Plasmid CMV-Luc was transfected to correct for transfection efficiency. As a control, we transfected the 5C-CAT construct in FRTL-5 cells stably expressing K-ras and H-ras. B, FRTL-5 cells expressing the different mutant RhoA forms were transiently transfected with 5C-CAT, CMV-Luc, and a TTF-1 expression vector. As a control, we transfected FRTL-5-H-ras. In both panels (A and B), cells were lysed at 72 h after transfection, and CAT and luciferase activities were measured. The promoter activity is calculated as arbitrary units relative to the cells transfected with vector alone (=100). The data are the mean ± SD of three independent experiments.

## DISCUSSION

We show that constitutively active RhoA QL transforms FRTL-5 thyroid cells, rendering them more proliferative, tumorigenic, and undifferentiated. As has been previously demonstrated, RhoA is required for TSH-mediated thyroid cell proliferation (23, 24). Transient expression of dominant negative RhoA N19 or the inhibition of RhoA activity by choleric exoenzyme C3 decreases thyroid cell proliferation as a consequence of $G_1$ arrest (25). Here we present evidence that stable expression of the dominant positive form RhoA QL induces a proliferative advantage in FRTL-5 thyroid cells. Conversely, expression of the dominant negative RhoA N19 decreases FRTL-5 thyroid cell proliferation.

The Rho protein subfamily, including RhoA, Rac1, and Cdc42, are involved in cell shape regulation and actin filament assembly (46, 47). Lovastatin-induced inactivation of these proteins causes cell rounding and actin filament disassembly (48). In several cell types, spread cellular morphology allowed DNA synthesis, whereas rounded cells did not proliferate or undergo apoptosis (49). The analysis of FRTL-5-RhoA QL clones with phalloidin shows that these cells spread in a fibroblast-like fashion and display actin stress fibers. This morphology disappears and becomes rounded, with a distinct actin distribution, in clones generated after transfection of expression vectors for C3 exoenzyme and RhoA QL, demonstrating that the changes observed in the cell phenotype are due to the genes

transfected and not to an indirect action of the transgene used. FRTL-5-RhoA N19 cells also presented a rounded cell shape and diffuse cell-to-cell limits. This altered cell morphology was followed by a decrease in FRTL-5-RhoA N19 cell proliferation. In this regard, Zhu *et al*. (50) reported that for cell cycle progression to occur, the actin cytoskeleton must be assembled. These clones could thus be useful to study in greater detail the links between RhoA-mediated cytoskeletal rearrangement and its positive effects in thyroid cell proliferation.

RhoA activation has transforming and oncogenic potential in some cell lines (21, 22). Fully differentiated FRTL-5 thyroid cells are considered nontumorigenic, although they can be rendered so in a single transformation step by expression of retroviral oncogenes such as H-ras or K-ras (12). As we show here, RhoA QL induces *in vitro* transformation of FRTL-5 cells, assessed by anchorage- and TSH-independent growth and tumors when inoculated into nude mice. Excised tumors revealed highly proliferative infiltrated carcinomas, suggesting that RhoA induces FRTL-5 thyroid transformation *in vivo*. We show that RhoA transformation of FRTL-5 cells is not due to increased cAMP levels, as has been reported in most thyroid tumors (34). The expression of c-Fos and c-Jun is increased in FRTL-5-RhoA QL cells, suggesting that these two oncogenes may be involved in the cell transformation described here. As has previously been reported, the expression of these two proteins is likely to play a role in the control of cell proliferation (26, 51), as they are necessary for cell cycle progression in several cellular systems and for neoplastic transformation by a variety of oncogenes (27). Interestingly, phospho-c-Jun levels are also increased in FRTL-5-RhoA QL transformed cells. These results open new questions about whether RhoA activation increases JNK activity in FRTL-5 cells as has been demonstrated in other systems (28). All together, these data concur with the previous observation of the regulation of early gene expression and cellular transformation elicited by RhoA.

A general effect in thyroid cell transformation is the loss of cell differentiation. Oncogenic Ras thus suppresses thyroid differentiation marker expression, and Ras-transformed cells do not express Tg or TPO, do not respond to TSH, and do not take up iodine (12). In parallel, the transcription factors controlling the expression of thyroid-specific genes are either not present or inactive (10, 11, 13, 14). In K-ras-transformed thyroid cells, both Pax-8 and TTF-1 mRNA are undetectable, whereas in H-ras-transformed cells, TTF-1 is present at normal levels and maintains its binding capacity, although the cells lack the ability to express Tg and TPO (11, 14). Our results show that expression of active RhoA decreases Tg mRNA levels, whereas expression of dominant negative RhoA N19 shows Tg mRNA levels comparable to those of controls (FRTL-5 cells). RhoA expression also decreases Tg protein levels, confirming that RhoA modifies the

thyroid phenotype affecting Tg expression. The RhoA-mediated decrease in Tg gene expression may be due to a lack of activity of TTF-1, a specific transcription factor involved in Tg gene activation. Our results clearly demonstrate that active RhoA decreases TTF-1 expression when compared with FRTL-5-RhoA N19 and FRTL-5-vector cells. Moreover, RhoA activation decreases TTF-1 protein levels in the nucleus, where this factor activates thyroid-specific promoters. The fact that overexpression of exogenous TTF-1 in stable FRTL-5-RhoA QL cells has no effect on Tg promoter construct activity, together with the observation that RhoA QL inhibited the effect of TTF-1 in transient transfection experiments, suggests that RhoA QL affects TTF-1 involving a posttranslational mechanism. This may account not only for the reduction in Tg protein levels, but also for the reduction in TTF-1, as this transcription factor is autoregulated (52). Mechanisms involved in modulating the transcriptional potential of TTF-1 include phosphorylation (43), control of the redox state (44, 45), and interaction with other factors (53). Ras repression of the Tg promoter involves changes in TTF-1 phosphorylation (14). The fact that RhoA N19 reverts the effect of Ras V12 on the wild-type Tg promoter suggests that RhoA is a downstream effector of Ras in this process. Thus, it would be of interest to test the ability of RhoA to modify the phosphorylation state of TTF-1. Interestingly, the protein kinases PKN, MKK3/6, and ERK6 have been proposed as components of a novel signal transduction pathway involved in the regulation of gene expression and cellular transformation elicited by RhoA (36). TTF-1 contains several minimal consensus sequences for ERK phosphorylation and has been reported to be phosphorylated by ERK2 (38). Although ERK2 does not mediate the RhoA effect on NIH-3T3 fibroblasts (36), the role of this family of kinases in response to RhoA and its effect in TTF-1 phosphorylation could be addressed in FRTL-5 cells. However, we cannot rule out the possibility that TTF-1 may be phosphorylated by other RhoA effectors.

The new role for RhoA protein in FRTL-5 thyroid cells increases the complexity of the signal transduction pathways implicated in the control of thyroid cell proliferation and function. FRTL-5 thyroid cells depend mainly on TSH for proliferation (33, 54, 55); this hormone stimulates thyroid cell proliferation through both PKA-dependent and -independent pathways (25, 56). After TSH stimulation, downstream effectors such as Akt (57), Rac1 (38), or RhoA (23–25) are involved in thyroid cell growth and function. Activation of Akt, Rac1, or RhoA increases thyroid cell proliferation, although Akt or Rac1 expression has no effect on the differentiation status of thyroid cells. Here we suggest, for the first time, a role for RhoA in thyroid differentiation. The similarity between Ras-mediated thyroid cell transformation, together with our observation that RhoA N19 reverses the effect of Ras V12 on the Tg promoter, suggests the existence of cross-talk involving both proteins, as described previously for other

cell systems (58). Further studies are needed to explain in detail the role of RhoA in Ras-mediated thyroid transformation.


## MATERIALS AND METHODS

### Cell Culture

Rat thyroid follicular FRTL-5 cells (ATCC CRL 8305; American Type Culture Collection, Manassas, VA) were kindly provided by Dr. L. D. Kohn (Edison Biotech Institute, Athens, OH). The cells had the properties previously described (59, 60), were diploid, and their doubling time with TSH was 24–36 h. Cells were maintained in Coon's modified Ham's F-12 medium (Sigma, St. Louis, MO) supplemented with 5% calf serum (Life Technologies, Inc., Gaithersburg, MD) and six-growth factor (6H complete medium), including TSH (0.5 mU/ml) and insulin (10 $\mu$g/ml) (49). FRTL-5-K-ras and FRTL-5-H-ras cells were maintained as previously described (11, 12).

### Plasmids and Expression Constructs

TACAT-3 corresponds to the wild-type Tg promoter (39, 40). As a negative control, we used the TATA-CAT plasmid construct corresponding to the wild-type Tg promoter minus the *Sal*/I-*Nhe*I fragment (39), resulting in only the Tg TATA box linked to the CAT gene. For detection of TTF-1 transcriptional activity, the C5E1b-CAT construct (Ref. 42; referred to here as 5C-CAT) was transiently transfected into FRTL-5 cells. The 5C-CAT construct contains five tandem repeats of the C binding site for TTF-1 from the Tg promoter and is exclusively dependent on TTF-1 for transactivation (42). The CMV-Luc plasmid was used to correct for transfection efficiency (39). For TTF-1 overexpression assays, we used a vector containing wild-type TTF-1 (41). The role of RhoA was analyzed with expression vectors encoding the dominant positive AU5-tagged-RhoA QL (28), the dominant negative AU5-tagged-RhoA N19 (29), the dominant negative Ras N17 or positive Ras V12 (61), or the botulinum C3 exoenzyme (28).

### Transfection Assays

FRTL-5 cells were stably transfected by the calcium phosphate DNA precipitation method, as described (39, 11). Briefly, calcium phosphate DNA precipitates were prepared with 10 $\mu$g of plasmid DNA containing either constitutively active mutant AU5-tagged RhoA (RhoA QL) (28) or the dominant negative form of AU5-tagged RhoA (RhoA N19) (29). To abolish RhoA activation, a stable cell line was generated transfecting together 5 $\mu$g of AU5-tagged RhoA QL and 5 $\mu$g of the C3 expression vector. In all cases 1 $\mu$g of plasmid DNA containing the neomycin resistance gene under the control of viral long terminal repeat promoter, and 40 $\mu$g of calf thymus genomic DNA as carrier (Roche Molecular Biochemicals) was also cotransfected. Cells were selected with 300 $\mu$g/ml G418 (Sigma). After 3 wk, G418-resistant colonies were isolated and expanded.

For transient transfection assays, cells were plated at a density of $5 \times 10^5$/60 mm diameter tissue culture dish; 48 h later, TACAT-3, 5C-CAT, TATA-CAT (2.5 $\mu$g), or CMV-Luc (1 $\mu$g) reporter plasmids were transfected with the expression vectors as indicated in the figure legends. After 72 h, cell extracts were lysed in lysis buffer (10 mM HEPES, pH 7.9, 40 mM NaCl, 0.1 mM EGTA, 0.5 mM dithiothreitol, 5% glycerol, and 0.5 mM phenylmethylsulfonyl fluoride). Luciferase (Luc) and chloramphenicol acetyltransferase (CAT) activity were measured as described (62, 63).

### Immunoblotting and Immunofluorescence

Nuclear or total extracts were prepared in sample buffer, and protein concentration was determined by the Bradford technique (Bio-Rad Laboratories, Inc., Hercules, CA). Protein samples were resolved in SDS-PAGE and transferred to Protran membranes (Schleicher & Schuell, Inc., Keene, NH). Monoclonal anti-AU5 antibody (0.5 $\mu$g/ml) used to detect AU5-tag was purchased from Badco. Polyclonal anti-RhoA (1 $\mu$g/ml), anti-actin (1 $\mu$g/ml), anti-Sp1 (1 $\mu$g/ml), anti c-Fos (2 $\mu$g/ml), anti-c-Jun (1 $\mu$g/ml), and anti-P-Ser 63-c-Jun (2 $\mu$g/ml) antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-Tg antibody (0.5 $\mu$g/ml) was from DAKO Corp. (Carpinteria, CA), and anti-TTF-1 antibody (1 $\mu$g/ml) from Biopat Immunotechnologies (Italy). Immune complexes were detected with Luminol reagent as indicated by the manufacturer (Santa Cruz Biotechnology, Inc.).

For immunofluorescence assays, FRTL-5-vector, FRTL-5-RhoA QL, FRTL-5-RhoA N19, and FRTL-5-RhoA QL-C3 cells were fixed in 4% formaldehyde for 30 min, and then permeabilized with 0.1% Triton X-100 in PBS. Samples were incubated with fluorescein isothiocyanate-phalloidin (Sigma, 1:40 dilution in PBS) for 1 h at 37 C. Cells were then washed twice with PBS and mounted on microscope slides. Fluorescence was visualized in a photomicroscope (Carl Zeiss, Thornwood, NY) equipped with epifluorescence. Photographs were taken using Kodak 400 ASA film.

### Growth Curve Profiles

To perform growth curves profiles, cells were seeded at a confluence of $10^5$/100 mm dish and maintained 3 d in the absence of TSH (5H medium). From then on, cells were cultured either in medium with TSH, for the 6H curve, or maintained in 5H medium. Fresh medium (6H or 5H, respectively) was added every 3 d. The number of viable cells was determined by cell counting every 3 d for 12 consecutive days. The mean ± SD of three independent experiments is represented.

### Anchorage-Independent Growth

Approximately 9,000 cells were seeded in 60-mm petri dishes in 0.35% noble agar (Sigma) on a 0.5% agar underlayer. Cells were tested to grow in soft agar containing 6H or 5H medium. Plates were incubated for 3 wk, during which time fresh medium (6H or 5H, respectively) was added to the plates every 3 or 4 d. After crystal violet staining, colonies larger than 50 $\mu$m diameter were counted. The mean ± SD of three independent experiments is represented.

### Growth of Tumors in Nude Mice

Tumorigenicity was assayed by injecting $2 \times 10^6$ cells sc into nude mice, which were palpated weekly for tumor development. After 4 wk, the animals were killed and tumors excised for histological analysis under protocols approved by the Human Research Committee (Vanderbilt University, Nashville, TN). For histopathological analysis (64), tissues were fixed in 10% buffered formaldehyde for 48 h, embedded in paraffin, and cut into 6-$\mu$m serial sections that were stained with hematoxylin-eosin (Fig. 3), Mason's trichrome, and periodic acid Schiff methods (not shown). BrdU staining was performed as follows: RhoA QL-inoculated mice received 1% 5-bromo-2′ deoxyuridine (Sigma) ip 2 h before being killed. Tumors were removed and sections prepared as for hematoxylin-eosin staining. Before immunohistochemistry, sections were deparaffined, hydrated, and washed with PBS. PBS was then removed and endogenous peroxidase inhibited using 3% hydrogen peroxidase (10 min, 37 C). Sections

were blocked in goat serum (Zymed Laboratories, Inc., South San Francisco, CA) and incubated overnight with a mouse monoclonal anti-BrdU antibody (Amersham Pharmacia Biotech, Piscataway, NJ). After washing with PBS, sections were incubated with biotinylated goat antimouse antibody (Biocell) in 20% human serum PBS buffer. After washing, sections were incubated with streptavidin-biotin-peroxidase complex (Zymed Laboratories, Inc.) and developed with diaminobenzidine (Sigma). Sections were counterstained with Harris hematoxylin, dehydrated in ethanol, and mounted in DePex (Probus, Barcelona, Spain).

### RNA Isolation and Analysis

Total RNA was isolated by the guanidinium-isothiocyanate-phenol method (65). Total RNA samples were electrophoresed in 1% agarose gels containing formaldehyde. RNA was transferred to Nytran membranes (Schleicher & Schuell, Inc.), and RNA integrity was verified by methylene blue staining of the blots. Hybridization and washing were performed with probes specific for Tg (66), TTF-1 (67), or $\beta$-actin (68) labeled with $[\alpha^{32}P]$-dCTP by random priming.

### cAMP Assays

The Biotrak cAMP competitive enzyme immunoassay system (Amersham Pharmacia Biotech) was used following manufacturer's instructions for the determination of intracellular cAMP. Briefly, cells were cultured in 24-well plates ($10^5$ cells per dish) and then lysed, moved to a donkey antirabbit Ig-precoated microtiter plate and incubated with anti-cAMP antiserum (2 h, 4 C). Samples were then incubated with a cAMP-peroxidase-conjugated antibody (1 h, 4 C) and washed four times with washing buffer. The enzyme substrate was added immediately afterward to all wells and incubated (1 h, room temperature). Before optical density determination in a plate reader at 450 nm, the reaction was terminated by adding 0.1 M sulfuric acid to each well. In parallel, a standard curve with cAMP concentrations from 12.5–3,200 fmol/well was prepared. Each value represents the mean $\pm$ SD of three independent experiments. As control for assay validation, FRTL-5 cells maintained in 5H and 6H medium were used, being the cAMP levels 50 $\pm$ 7 fmol/well and 390 $\pm$ 30 fmol/well, respectively.

## REFERENCES

1. Bourne HR, Sanders DA, McCormick F 1990 The GTPase superfamily: a conserved switch for diverse cell functions. Nature 348:125–132
2. Bos J L 1997 Ras-like GTP-ases. Biochim Biophys Acta 1333:M19–M31
3. Beug H, Palmieri S, Freudenstein SC, Zentgraf H, Graf T 1992 Hormone-dependent terminal differentiation *in vitro* of chicken erythroleukemia cells transformed by mutants of avian erythroblastosis virus. Cell 28:907–919
4. Fiszman MY, Fuchs P 1975 Temperature-sensitive expression of differentiation in transformed myoblasts. Nature 254:429–431
5. Olson EN, Spizz G, Tainsky MA 1987 The oncogenic forms of N-ras or H-ras prevent skeletal myoblast differentiation. Mol Cell Biol 7:2104–2111
6. Schmidt A, Setoyama C, de Crombrugghe B 1985 Regulation of a collagen gene promoter by the product of viral mos oncogene. Nature 314:286–289
7. Gallo A, Benusiglio E, Bonapace IM, Feliciello A, Cassano S, Garbi C, Musti AM, Gottesman ME, Avvedimento EV 1992 v-ras and protein kinase C dedifferentiate thyroid cells by down-regulating nuclear cAMP-dependent protein kinase A. Genes Dev 6:1621–1630
8. Gallo A, Feliciello A, Varrone A, Cerillo R, Gottesman ME, Avvedimento VE 1995 Ki-ras oncogene interferes with the expression of cyclic AMP-dependent promoters. Cell Growth Differ 6:91–95
9. Feliciello A, Giuliano P, Porcellini A, Garbi C, Obici S, Mele E, Angotti E, Grieco D, Amabile G, Cassano S, Li Y, Musti AM, Rubin CS, Gottesman ME, Avvedimento EV 1996 The v-Ki-Ras oncogene alters cAMP nuclear signaling by regulating the location and the expression of cAMP-dependent protein kinase II$\beta$. J Biol Chem 271:25350–25359
10. Avvedimento VE, Musti AM, Ueffing M, Obici S, Gallo A, Sanchez M, DeBrasi D, Gottesman ME 1991 Reversible inhibition of a thyroid-specific trans-acting factor by Ras. Genes Dev 5:22–28
11. Francis-Lang H, Zannini M, De Felice M, Berlingieri MT, Fusco A, Di Lauro R 1992 Multiple mechanisms of interference between transformation and differentiation in thyroid cells. Mol Cell Biol 12:5793–5800
12. Fusco A, Berlingieri MT, Portella G, Di Fiore PP, Grieco M, Vecchio G 1987 One- and two-step transformation of rat thyroid epithelial cells by retroviral oncogenes. Mol Cell Biol 7:3365–3370
13. Missero C, Cobellis G, De Felice M, Di Lauro R 1998 Molecular events involved in differentiation of thyroid follicular cells. Mol Cell Endocrinol 140:37–43
14. Velasco JA, Acebrón A, Zannini M, Martín-Pérez J, Di Lauro R, Santisteban P 1998 Ha-ras interference with thyroid cell differentiation is associated with a down-regulation of thyroid transcription factor-1 phosphorylation. Endocrinology 139:2796–2802
15. Van Aelst L, D'Souza-Schorey C 1997 Rho GTPases and signaling networks. Genes Dev 11:2295–2322
16. Khosravi FR, Solski PA, Clark GJ, Kinch MS, Der CJ 1995 Activation of Rac1, RhoA, and mitogen-activated protein kinases is required for Ras transformation. Mol Cell Biol 15:6443–6453
17. Qiu RG, Chen J, Kirn D, McCormick F, Symons M 1995 An essential role for Rac in Ras transformation. Nature 374:457–459
18. Qiu RG, Chen J, McCormick F, Symons M 1995 A role for Rho in Ras transformation. Proc Natl Acad Sci USA 92:11781–11785
19. Qiu RG, Abo A, McCormick F, Symons M 1997 Cdc42 regulates anchorage-independent growth and is necessary for Ras transformation. Mol Cell Biol 17:3449–3458

20. Lin R, Bagrodia S, Cerione R, Manor D 1997 A novel Cdc42Hs mutant induces cellular transformation. Curr Biol 7:794–797

21. Ridley AJ, Paterson HF, Johnston CL, Diekmann D, Hall A 1992 The small GTP-binding protein rac regulates growth factor-induced membrane ruffling. Cell 70:401–410

22. Ridley AJ, Hall A 1992 The small GTP-binding protein Rho regulates the assembly of focal adhesions and actin stress fibers in response to growth factors. Cell 70:389–399

23. Hirai A, Nakamura S, Noguchi Y, Kitagawa M, Tatsuno I, Oeda T, Hakara, Terano T, Naruyima S, Kohn LD, Saito Y 1997 Geranylgeranylated Rho small GTPase(s) are essential for the degradation of the p27kip1 and facilitate the progression from G1 to S phase in growth-stimulated rat FRTL-5 cells. J Biol Chem 272:13–16

24. Noguchi Y, Nakamura S, Yasuda T, Kitagawa M, Kohn LD, Saito Y, Hirai A 1998 Newly synthesized Rho A, not Ras, is isoprenylated and translocated to membranes coincident with progression of the G1 to S phase of growth-stimulated rat FRTL-5 cells. J Biol Chem 273:3649–3653

25. Medina DL, Toro MJ, Santisteban P 2000 Somatostatin interferes with thyrotropin-induced G1-S transition mediated by PKA and PI3-K: involvement of RhoA and cyclin E-CDK2 complexes. J Biol Chem 175:15549–15556

26. Angel P, Karin M 1991 The role of Jun, Fos and the AP-1 complex in cell-proliferation and transformation. Biochim Biophys Acta 1072:129–157

27. Suzuki T, Murakami M, Onai N, Fukuda E, Hashimoto Y, Sonobe MH, Kameda T, Ichinose M, Miki K, Iba H 1994 Analysis of AP-1 function in cellular transformation pathway. J Virol 68:3527–3535

28. Teramoto H, Crespo P, Coso OA, Igishi T, Xu N, Gutkind S 1996 The small GTP-binding protein Rho activates c-Jun N-terminal kinases/stress-activated protein kinases in human kidney 293T cells: evidence for a Pak-independent signaling pathway. J Biol Chem 271:25731–2573430

29. Crespo P, Bustelo XR, Aaronson DS, Coso OA, Lopez-Barahona M, Barbacid M, Gutkind JS 1996 Rac-1 dependent stimulation of the JNK/SAPK signaling pathway by Vav. Oncogene 13:455–460

30. Lerm M, Schmidt G, Aktories K 2000 Bacterial protein toxins targeting Rho GTPases. FEMS Microbiol Lett 188:1–6

31. Ossendorp FA, Bruning PF, Schuuring EM, Van Den Brink JA, van der Heide D, De Vijlder JJ, De Bruin TW 1990 Thyrotropin dependent and independent thyroid cell lines selected from FRTL-5 derived tumors grown in nude mice. Endocrinology 127:419–430

32. Peter HJ, Gerber H, Studer H, Groscurth P, Zakarija M 1991 Comparison of FRTL-5 cell growth *in vitro* with that of xenotransplanted cells and the thyroid of the recipient mouse. Endocrinology 128:211–219

33. Medina DL, Santisteban P 2000 Thyrotropin-dependent proliferation of *in vitro* rat thyroid cell systems. Eur J Endocrinol 143:161–178

34. Farid NR, Shi Y, Zou M 1994 Molecular basis of thyroid cancer. Endocr Rev 15:202–232

35. Hill CS, Wynne J, Treisman R 1995 The rho family GTPases RhoA, Rac1, and CDC42Hs regulates transcriptional activation by SRF. Cell 81:1159–1170

36. Marinissen MJ, Chiariello M, Gutkind JS 2001 Regulation of gene expression by the small GTPase Rho through the ERK6 (p38$\gamma$) MAP kinase pathway. Genes Dev 15:535–553

37. Cobellis G, Missero C, Di Lauro R 1998 Concomitant activation of MEK-1 and Rac-1 increases the proliferative potential of thyroid epithelial cells, without affecting their differentiation. Oncogene 17:2047–2057

38. Missero C, Pirro MT, Di Lauro R 2000 Multiple ras downstream pathways mediate functional repression of the homeobox gene product TTF-1. Mol Cell Biol 20:2783–2793

39. Sinclair AJ, Lonigro R, Civitareale D, Ghibelli L, Di Lauro R 1990 The tissue-specific expression of the thyroglobulin gene requires interaction between thyroid-specific and ubiquitous factors. Eur J Biochem 193:311–318

40. Civitareale D, Lonigro R, Sinclair AJ, Di Lauro R 1989 A thyroid specific nuclear protein essential for tissue-specific expression of the thyroglobulin promoter. EMBO J 8:2537–2542

41. Alberts AS, Geneste O, Treisman R 1998 Activation of SRF-regulated chromosomal templates by Rho-family GTPases requires a signal that also induces H4 hyper-acetylation. Cell 92:475–487

42. De Felice M, Damante G, Zannini M, Francis-Lang H, Di Lauro R 1995 Redundant domains contribute to the transcriptional activity of the thyroid transcription factor-1. J Biol Chem 270:26649–26656

43. Zannini M, Acebron A, De Felice M, Arnone MI, Martín-Pérez J, Santisteban P, Di Lauro R 1996 Mapping and functional role of phosphorylation sites in the thyroid transcription factor-1 (TTF-1). J Biol Chem 271:2249–2254

44. Arnone MI, Zannini M, Di Lauro R 1995 The DNA binding activity and the dimerization ability of the thyroid transcription factor 1 are redox regulated. J Biol Chem 270:12048–12055

45. Kambe F, Nomura Y, Okamoto T, Seo H 1996 Redox regulation of thyroid-transcription factor, Pax-8 and TTF-1, is involved in their increased DNA-binding activities by thyrotropin in rat FRTL-5 cells. Mol Endocrinol 10:801–812

46. Hall A 1998 Rho GTPases and the actin cytoskeleton. Science 279:509–514

47. Takai Y, Sasaki T, Tanaka K, Nakanishi H 1995 Rho as a regulator of the cytoskeleton. Trends Biochem Sci 20:227–231

48. Fenton RG, Kung HF, Longo DL, Smith MR 1992 Regulation of intracellular actin polymerization by prenylated cellular proteins. J Cell Biol 117:347–356

49. Chen CS, Mrksich M, Huang S, Whitesides GM, Ingber DE 1997 Geometric controls of cell life and death. Science 276:1425–1428

50. Zhu X, Ohtsubu M, Bohmer RM, Roberts JM, Assoian RK 1996 Adhesion-dependent cell cycle progression linked to the expression of cyclin D1, activation of cyclin E-cdk2 and phosphorylation of the retinoblastoma protein. J Cell Biol 133:391–403

51. Kovary K, Bravo R 1991 The jun and fos protein families are both required for cell cycle progression in fibroblasts. Mol Cell Biol 11:4466–4472

52. Oguchi H, Kimura S 1998 Multiple transcripts encoded by the thyroid-specific enhancer-binding protein (T/EBP)/thyroid-specific transcription factor-1(TTF-1) gene: evidence of autoregulation. Endocrinology 139:1999–2006

53. Perrone L, Tell G, Di Lauro R 1999 Calreticulin enhances the transcriptional activity of thyroid transcription factor-1 by binding to its homeodomain. J Biol Chem 274:4640–4645

54. Dumont JE, Lamy F, Roger P, Maenhaut C 1992 Physiological and pathological regulation of thyroid cell proliferation and differentiation by thyrotropin and other growth factors. Physiol Rev 72:667–698

55. Dremier S, Pohl V, Poteet-Smith C, Roger PP, Corbin J, Doskeland SO, Dumont JE, Maenhaut C 1997 Activation of cyclic AMP-dependent kinase is required but may not be sufficient to mimic cyclic AMP-dependent DNA synthesis and thyroglobulin expression in dog thyroid cells. Mol Cell Biol 17:6717–6726

56. Cass LA, Summers SA, Prendergast GV, Backer JM, Birnbaum MJ, Meinkoth JL 1999 Protein kinase A-dependent and -independent signaling pathways contribute to cyclic AMP-stimulated proliferation. Mol Cell Biol 19:5882–5891

57. De Vita G, Berlingieri MT, Visconti R, Castellone MD, Viglieto G, Baldassarre G, Zannini M, Bellacosa A, Tsichlis PN, Fusco A, Santoro M 2000 Akt/Protein kinase B promotes survival and hormone-independent proliferation of thyroid cells in the absence of differentiating and transforming effects. Cancer Res 60:3916–3920

58. Sahai E, Olson MF, Marshall CJ 2001 Cross-talk between Ras and Rho signalling pathways in transformation favours proliferation and increase motility EMBO J 20:755–7666

59. Ambesi-Impiombato FS, Parks LAM, Coon HG 1980 Culture of hormone-dependent functional cells of rat thyroid. Proc Natl Acad Sci USA 77:3455–3459

60. Kohn LD, Valente WA, Grollman EF, Aloj SM, Vitti P 1986 Clinical determination and or quantification of thyrotropin and a variety of thyroid stimulatory and inhibitory factors performed *in vitro* with an improved cell line FRTL-5. US Patent 4:609–622

61. Coso OA, Chiariello M, Yu JC, Teramoto H, Crespo P, Xu N, Miki T, Gutkind JS 1995 The small GTP-binding proteins Rac1 and Cdc-42 regulate the activity of the JNK/SAP signalling pathway. Cell 81:1137–1146

62. Brasier AR, Tate JE, Habener JF 1989 Optimized use of the firefly luciferase assay as a reporter gene in mammalian cell lines. Biotechniques 7:1116–1122

63. Gorman CM, Moffat LM, Howard BH 1982 Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol Cell Biol 2:1044–1051

64. Bancroft JD, Cook HC 1994 Manual of histological techniques and their diagnostic application. London: Churchill Livingstone Editorial

65. Chomczynski P, Sacchi N 1987 Single step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159

66. Di Lauro R, Obici S, Acquaviva AM, Alvino C 1985 The sequence of 967 amino acids at the carboxi-end of rat thyroglobulin. Location and surroundings of two thyroxine-forming sites. Eur J Biochem 148:7–11

67. Guazzi S, Price M, De Felice M, Damante G, Mattei MG, Di Lauro R 1990 Thyroid nuclear factor 1 (TTF-1) contains a homeodomain and displays a novel DNA binding specificity. EMBO J 9:3631–3639

68. Levi A, Eldridge JD, Paterson BM 1985 Molecular cloning of a gene sequence regulated by nerve growth factor. Science 229:393–395