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# Multiple Mechanisms of Interference between Transformation and Differentiation in Thyroid Cells

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**Transformation of the thyroid cell line FRTL-5 results in loss or reduction of differentiation as measured by the expression of thyroglobulin and thyroperoxidase, two proteins whose genes are exclusively expressed in thyroid follicular cells. The biochemical mechanisms leading to this phenomenon were investigated in three cell lines obtained by transformation of FRTL-5 cells with Ki-*ras*, Ha-*ras*, and polyomavirus middle-T oncogenes. With the *ras* oncogenes, transformation leads to undetectable expression of the thyroglobulin and thyroperoxidase genes. However, the mechanisms responsible for the extinction of the differentiated phenotype seem to be different for the two *ras* oncogenes. In Ki-*ras*-transformed cells, the mRNA encoding TTF-1, a transcription factor controlling thyroglobulin and thyroperoxidase gene expression, is severely reduced. On the contrary, nearly wild-type levels of TTF-1 mRNA are detected in Ha-*ras*-transformed cells. Furthermore, overexpression of TTF-1 can activate transcription of the thyroglobulin promoter in Ki-*ras*-transformed cells, whereas it has no effect on thyroglobulin transcription in the Ha-*ras*-transformed line. Expression of polyoma middle-T antigen in thyroid cells leads to only a reduction of differentiation and does not severely affect either the activity or the amount of TTF-1. Another thyroid cell-specific transcription factor, TTF-2, is more sensitive to transformation, since it disappears in all three transformed lines, and probably contributes to the reduced expression of the differentiated phenotype.**

The ability of oncogenes to interfere with the differentiated cellular program ultimately lies in their ability to redirect the transcription of various genes. Some oncogenes may themselves be transcription factors (36), while others such as v-*ras* can modify nuclear events by changing the properties of known transcription factors (9, 15). The fact that various oncogenes can inhibit the expression of cellular differentiation markers has been investigated in several cell types (4). Among the various systems studied, the thyroid represents one of the few available models to study the effects of malignant transformation on differentiated epithelial cell lines. Differentiated thyroid cells in culture are distinguished by the markers of thyroglobulin (Tg), thyroperoxidase (TPO), thyroid-stimulating hormone (TSH) dependence for growth mediated by the TSH receptor, and the ability to concentrate iodide from the medium (3). Thyroid-specific expression of the Tg and TPO genes is mediated by transcriptional control (23, 37). This restricted expression is due, at least in part, to the interaction of two thyroid-specific nuclear factors, TTF-1 and TTF-2 (19, 23, 39), with the promoters of both genes. Recently, Pax-8, a paired domain-containing factor, has been demonstrated to be able to activate transcription in cotransfection experiments from the promoters of both Tg and TPO (40). However, the role that Pax-8 may play in thyroid cells has not been assessed yet, and hence, Pax-8 was not included in this study.

The introduction of several viral oncogenes in cultured thyroid cells has been demonstrated to interfere with the expression of cell-type-specific markers (25). With Ki-*ras*, it has also been shown that the expression of Ki-*ras* in thyroid cells correlates with the disappearance of thyroid transcription factor 1 (TTF-1) binding activity (6). In addition, when a temperature-sensitive Ki-*ras* allele was used to transform thyroid cells, transformation was reversible at the nonpermissive temperature, but the differentiated phenotype was permanently lost (20). Tg gene expression could be reactivated in these cells by treatment with the demethylating agent 5-azacytidine and was accompanied by the reappearance of TTF-1 DNA binding activity (8). It has been proposed that Ki-*ras* reduces the capacity of this factor to bind to DNA, via a phosphorylation-dependent mechanism. According to this model, transcriptional shutoff of the TTF-1 gene would be a secondary event and would remain imprinted by DNA methylation (7).

In this study, we compared the effects exerted on thyroid-specific transcription by three oncogenes, v-Ki-*ras*, v-Ha-*ras*, and polyomavirus middle-T antigen. In both cases of *ras* transformation, transcription from transfected thyroid-specific promoters is almost abolished, while in middle-T-transformed cells, a high residual level is observed. These observations correlate well with the expression of the endogenous Tg and TPO genes. However, Ki-*ras* transformation results in the disappearance of TTF-1 mRNA, and the differentiated phenotype may be partially rescued by a TTF-1 expression vector. Conversely, TTF-1 DNA binding activity is present in Ha-*ras*-transformed cells, although the Tg promoter appears unresponsive to its presence. In polyomavirus middle-T-transformed cells, TTF-1 does not seem to be severely affected. TTF-2 DNA binding activity is sensitive to transformation by all three oncogenes, and its absence could entirely account for the lesser disruption of

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thyroid promoter activity observed in the middle-T-transformed cells.

## MATERIALS AND METHODS

**RNA isolation and analysis.** Total RNA was isolated from cultured cells and analyzed by a modification of the guanidine hydrochloride procedure, as described by Adams et al. (1). Poly(A)$^+$ RNA was purified by two rounds of selection over oligod(T)-cellulose, essentially as described by Aviv and Leder (5). RNA was transferred to Hybond nylon (Amersham) and hybridized by the method described by Church and Gilbert (18). Probes were prepared by random oligonucleotide priming.

**cDNA plasmids and eukaryotic expression vectors.** The DNA fragments used as probes in Northern (RNA) analysis were as follows: Tg, plasmid pRTG2 containing a 5′ coding fragment of Tg cDNA (30a); TPO, the *Sal*I-*Eco*RI coding fragment of pTPO 5′c (22); TTF-1, the 0.7-kb *Sac*I fragment of plasmid prTTF-1/4 as described by Guazzi et al. (30); GAPDH (glyceraldehyde-3-phosphate dehydrogenase), a 1.3-kb *Pst*I fragment of plasmid pGAPDH1 containing the coding region of GAPDH (gift from P. Charnay). For transfection assays, the wild-type (pTACAT3) and mutant (pTACAT14) Tg reporter genes used are those described by Sinclair et al. (39); the p420TPOL plasmid is described by Francis-Lang et al. (23). CMV-LUC, containing the luciferase (LUC) gene under the control of the cytomegalovirus (CMV) promoter, was a gift from U. Deuschle, and Rous sarcoma virus promoter-chloramphenicol acetyltransferase (RSV-CAT) was a gift from G. Morrone. Plasmid CMV–TTF-1 contains the entire open reading frame of TTF-1 (30) replacing the LUC cistron in CMV-LUC. Plasmid M1 contains no coding sequence after the CMV promoter and was used as a control.

**Cell culture and transfection assays.** Characteristics of the cell lines described in this study are summarized in references 12, 13, and 25. Additionally, the FRTL-Py cell line was obtained by infecting wild-type FRTL-5 cells with polyoma murine leukemia virus, as described in reference 12. All cells were maintained in Coon's modified F12 medium (Seromed) supplemented with 5% calf serum (GIBCO) and six growth factors as described by Ambesi-Impiombato and Coon (2). Approximately $10^6$ cells were plated for 24 h (FRTL-Ki-*ras*) or 48 h (FRTL-5, FRTL-Py, and FRTL-Ha-*ras*) before transfection in 9-cm tissue culture dishes. At 3 h before transfection, the medium was changed to Dulbecco modified Eagle medium containing 5% serum and the six growth factors. Calcium phosphate precipitates were prepared with 12 μg of DNA as described by Graham and van der Eb (29) and were incubated with the cells for the following times: FRTL-5, 1 h; FRTL-Ki-*ras* and FRTL-Ha-*ras*, 3 h; FRTL-Py, 8 h. The precipitate was removed, and cells were washed with serum-free Dulbecco modified Eagle medium and incubated with 15% glycerol in HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-buffered saline for the following times: FRTL-Ki-*ras*, 15 s; FRTL-Ha-*ras*, 2 min; FRTL-5 and FRTL-Py, 3 min. Cells were washed with Dulbecco modified Eagle medium and incubated for 48 h in Coon's modified F12 medium supplemented with 5% calf serum and six growth factors before harvesting. CAT assays were performed as described previously (28), although the incubation of extracts with substrate was adjusted according to the needs of individual experiments; thus, normalization of TPO promoter activity with RSV-CAT and the relative activity of the Tg-CAT reporter gene in each of the cell lines was performed for 1 h; the transactivation assays of the Tg promoter were performed for 2 h; and the relative activity of wild-type and mutant promoters was observed for 3 h. LUC activity was determined as described previously (21). For Tg-CAT transfections, variability in transfection efficiency between dishes in each cell line was corrected by using a cotransfected CMV-LUC reporter plasmid as a control, whereas TPO LUC activity was normalized to that of a cotransfected RSV-CAT reporter construct.

**Protein preparation and gel retardation assays.** Gel retardation assays were performed with whole-cell extracts prepared as described by Zimarino and Wu (42) or with nuclear extracts prepared as described previously (19). The Tg C probe used to measure TTF-1 binding and the Tg K probe used to measure TTF-2 binding are also described in reference 19. Control probes, Ig 7/8 from the murine immunoglobulin enhancer (5′ ATCCTCAACTTATTTTAGAAATG CAAATTACCCAGGTGGT 3′) and DSE (5′ GTCACAAA GAGGCGGGGCTATGCAAATAGGGTGTGCCGGGG 3′) from the *Xenopus* U2 distal sequence element, contain octamer and dual octamer-Sp1 binding sites, respectively, and were kindly provided by I. Mattaj. Competition was performed by preincubation of the mixes with 50 ng of cold double-stranded oligonucleotides before addition of the probes (0.2 ng).

**Biosynthetic labelling and immunodetection techniques.** For immunoprecipitation, subconfluent cultures of FRTL-5 cells and transformed derivatives were labelled with 0.25 mCi of [$^{35}$S]methionine per ml for 2 h. Alternatively, cells were labelled with 0.3 mCi of $^{32}P_i$ per ml for 3 h. After the labelling, cells were lysed in a buffer containing 10 mM sodium phosphate (pH 7.4), 0.1 M NaCl, 1% Triton X-100, 0.1% sodium dodecyl sulfate (SDS), 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, 10 μg of aprotinin per ml, 10 μg of leupeptine per ml, and 10 μg of pepstatin per ml. Extracts were clarified by centrifugation, a TTF-1 antibody (34) was added, and immune complexes were recovered on protein A-Sepharose beads (Pharmacia). Bound proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE).

## RESULTS

**Different oncogenes alter the expression of thyroid-specific RNAs.** RNA levels for the thyroid markers Tg, TPO, and TTF-1 were examined in two fully differentiated thyroid cell lines (FRTL-5 and Pc) (2, 24) and compared with thyroid cells transformed by various oncogenes (Fig. 1). Figure 1A and B show that Tg and TPO mRNAs were not detected in thyroid cells transformed with the Ki-*ras*, Ha-*ras*, *mos*, or *abl* oncogene (25, 26). With Pc-*mos* and FRTL-Ki-*ras*, expression of TTF-1 was also not detected. However, TTF-1 mRNA was present in FRTL-Ha-*ras* and Pc-*abl* transformed cells, suggesting that transformation can interfere with differentiation by different mechanisms, depending on the oncogene involved. The difference in the dedifferentiated phenotype of Ha-*ras* (Tg$^-$ TPO$^-$ TTF-1$^+$)- and Ki-*ras* (Tg$^-$ TPO$^-$ TTF-1$^-$)-transformed cells is not due to a clonal variation of the transformed phenotype but appears to be strictly related to the oncogene responsible for the transformation event. Consistent phenotypes have been observed in at least three independent isolates of Ha-*ras*-transformed FRTL-5 cells (data not shown). Furthermore, similar phenotypes have been observed in FRTL-5 cells independently transformed by the Ki-*ras* oncogene, either with the Molo-

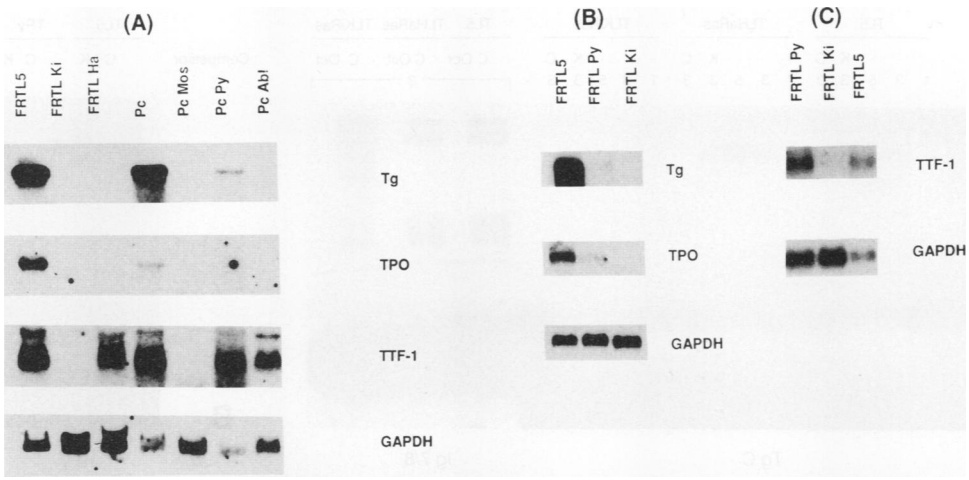


FIG. 1. Levels of Tg, TPO, and TTF-1 mRNAs in normal and transformed thyroid cell lines. (A) Total RNA (30 μg) from normal or transformed thyroid cell lines blotted onto nylon membranes was sequentially hybridized to the cDNA probes indicated to the right of the panel. (B) Poly(A)$^+$ RNA (1 μg) from normal or transformed FRTL-5 cells was hybridized to the probes indicated to the right of the figure. (C) Poly(A)$^+$ RNA (1 μg) from the cells described in panel B was hybridized to the TTF-1 and GAPDH probes as indicated.

ney murine leukemia virus as a helper (referred to as FRTL-Ki-*ras* throughout this report) or with the Kirsten murine leukemia virus as a helper (data not shown). Polyomavirus middle-T-antigen transformation of the thyroid cell lines Pc and FRTL-5 was associated with a clear reduction in the level of Tg mRNA (Fig. 1A and B). TPO mRNA was severely reduced in poly(A)$^+$ RNA isolated from the FRTL-Py cells (Fig. 1B), but was not detectable in total RNA isolated from the Pc-Py line (Fig. 1A) (41). This difference might arise from the increased sensitivity of the hybridization with poly(A)$^+$ RNA in the former case. Since TTF-1 appears to be required for the expression of both Tg and TPO (19, 23, 39), its presence in both middle-T-transformed lines correlates with the residual transcriptional activity of these two genes (Fig. 1A and C).

Three of the above cell lines, each displaying a distinct phenotype, were selected for study in greater detail: the FRTL-Py cell line, which has reduced levels of Tg and TPO RNAs and expresses TTF-1; the FRTL-Ha-*ras* cells, in which Tg and TPO RNAs are absent, yet TTF-1 is expressed; and the fully dedifferentiated phenotype of the FRTL-Ki-*ras* cells.

**Down-regulation of Tg and TPO synthesis occurs at the transcriptional level.** To test whether the decreased levels of Tg and TPO mRNAs were due to a reduction in transcription of the respective genes, we transfected Tg and TPO promoters, linked to the reporter genes for CAT and LUC, respectively, into FRTL-Ki-*ras*, FRTL-Ha-*ras*, and FRTL-Py cells. The transcriptional rate of these two thyroid-specific promoters in the transformed cells was compared with their expression in fully differentiated FRTL-5 cells. The expression of the Tg promoter was measured relative to that of the RSV promoter (RSV-CAT), while TPO promoter activity was monitored by comparison with a CMV promoter (CMV-LUC), in each cell line. Both viral promoters were previously demonstrated to be expressed at equal efficiency in each of the four cell lines (data not shown). The data shown in Fig. 2 demonstrate a drastic reduction in the transcriptional activity of the Tg and TPO promoters in both of the *ras*-transformed cell lines. The reduction in the transcriptional activity of these two promoters in the FRTL-Py line is less marked, Tg being expressed at 21% and TPO at 16% of the level obtained in wild-type cells. To investigate the molecular mechanisms responsible for the reduced thyroid-specific transcription in these transformed cell lines, we examined the DNA binding activity of the thyroid *trans*-acting factor TTF-1 in each of them by gel retardation assay, using a double-stranded oligonucleotide (oligonucleotide C) containing the TATA box-proximal TTF-1 binding site in the Tg promoter (Fig. 3). As expected from the absence of TTF-1 mRNA (Fig. 1) and in accordance with the results of previous studies (6), TTF-1 DNA binding activity was not detected in Ki-*ras*-transformed thyroid cells. Conversely, an

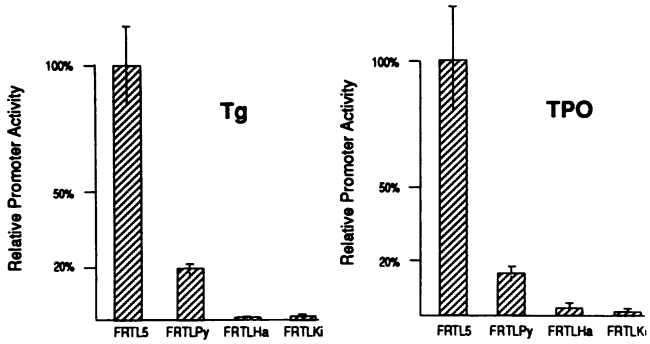


FIG. 2. TPO and Tg promoter activity in normal and transformed thyroid cells. FRTL-5, FRTL-Py, FRTL-Ha-*ras* (FRTLHa), and FRTL-Ki-*ras* (FRTLKi) cells were transfected with either 10 μg of pTACAT3 (panel Tg) or 10 μg of RSV-CAT along with 2 μg of CMV-LUC expression vector. After normalization for LUC activity, the expression of the Tg promoter was measured as the ratio of RSV/pTACAT3 activity in each of the transformed cell lines, relative to the value observed in FRTL-5 cells, which was arbitrarily set to 100%. The error bars show the standard error of the mean. For the TPO promoter (panel TPO), the same cell lines were transfected with either 10 μg of p420TPOL or 10 μg of CMV-LUC along with 2 μg of RSV-CAT expression vector. After normalization for CAT activity, the expression of the TPO promoter was measured as the ratio of CMV/p420TPOL activity in each of the transformed cell lines, relative to the value observed in FRTL-5 cells, which was arbitrarily set to 100%. The error bars show the standard error of the mean.

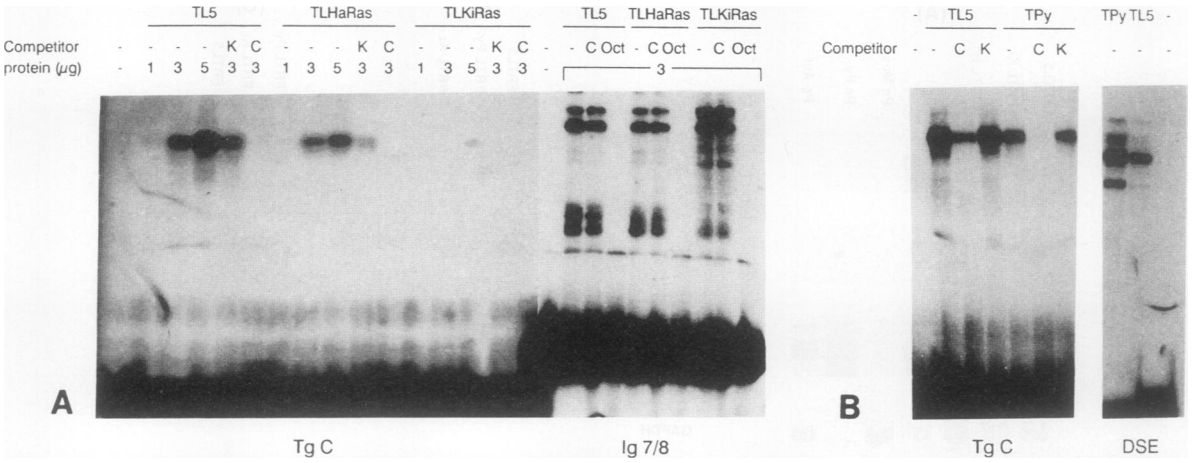


FIG. 3. TTF-1 DNA binding activity in normal and transformed thyroid cells. (A) The indicated amount of nuclear extract from FRTL-5 (TL5), FRTL-Ha-*ras* (TLHaRas), and FRTL-Ki-*ras* (TLKiRas) cells was incubated with [$\gamma$-$^{32}$P]ATP-labelled, double-stranded C oligonucleotide from the Tg promoter (TgC) and electrophoresed through native gels. Extract quality was monitored by binding of 3 μg of each extract to double-stranded Ig 7/8 oligonucleotide containing an octamer binding site. Competitor oligonucleotides (50 ng) were added as indicated. (B) FRTL-5 (TL5) and FRTL-Py (TPy) nuclear proteins (3 μg) were incubated with the same labelled oligonucleotide as in panel A in the absence or presence of 50 ng of the cold competitor. Extract quality was monitored with the control DSE oligonucleotide, containing Sp1 and octamer binding sites.

active DNA binding form of TTF-1 is present, albeit in slightly reduced quantities, in extracts of FRTL-Ha-*ras* (Fig. 3A) and FRTL-Py (Fig. 3B) cells. To test whether the TTF-1 protein present in FRTL-Ha-*ras* and FRTL-Py cells was capable of transcriptional activation, a Tg promoter mutant was transiently introduced in these cell lines and its transcriptional activity was compared with that of the wild-type promoter. Since transcription from the Tg promoter is low in transformed cell lines, the sensitivity of the CAT assay was increased by incubating the extracts of transfected cells for 3 h (with the hourly addition of acetyl coenzyme A). The mutation TACAT14 is unable to bind TTF-1 at the TATA box-proximal site and displays less than 20% of wild-type promoter activity in FRTL-5 cells (39). The transcriptional activity of the promoter bearing the TACAT14 mutation relative to the wild-type promoter in each of the cell lines is shown in Fig. 4. As predicted from the absence of TTF-1, the residual Tg-CAT activity of the FRTL-Ki-*ras* cells was insensitive to the mutation in the TTF-1 binding site. Similarly, the basal Tg-CAT activity in the FRTL-Ha-*ras* line was also insensitive to the pTACAT14 mutation, indicating

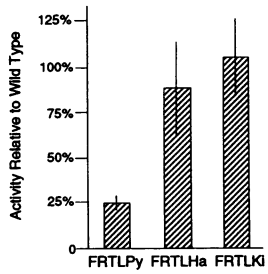


FIG. 4. Activity of wild-type and mutant Tg promoters in normal and transformed thyroid cell lines. The indicated cell lines were transfected with 10 μg of either pTACAT3 (wild-type Tg promoter) or the mutant pTACAT14, along with 2 μg of CMV-LUC. After normalization for LUC activity, the activity of the mutant promoter was measured relative to that of the wild type.

that TTF-1, even if present at nearly wild-type levels and capable of DNA binding, is not competent for activation of Tg promoter transcription. Conversely, an active form of TTF-1 seems to be present in FRTL-Py cells, since in this cell line the residual Tg-CAT activity is sensitive to the mutation in the TTF-1 binding site.

**Overexpression of TTF-1 in FRTL-Ki-*ras* cells, but not in FRTL-Ha-*ras* cells, partially restores Tg promoter activity.** TTF-1 binding activity is lacking in all FRTL-5 cells transformed by Ki-*ras* (6; this study), yet the presence of TTF-1 in the FRTL-Ha-*ras* cell line is not sufficient to confer TTF-1-dependent transcription on a reporter gene. This observation prompted us to investigate the effects of cointroduction of a TTF-1 expression vector with a Tg reporter plasmid in these two cell types. FRTL-Ki-*ras* cells were cotransfected with 8 μg of Tg-CAT reporter plasmid and 0.05 to 2.0 μg of either a control vector (M1) or a CMV promoter-based TTF-1 expression vector (CMV–TTF-1). The introduction of TTF-1 into Ki-*ras* cells stimulated Tg promoter activity in a concentration-dependent manner (Fig. 5A), but failed to increase the activity of a control RSV-CAT reporter (data not shown). Furthermore, this activation was dependent on the integrity of the proximal TTF-1 binding site, since a promoter containing an extensive mutation of this site, C core (19), which is known to severely impair TTF-1 binding, was poorly activated (Fig. 5B). This result suggests that even in the presence of active Ki-*ras*, reintroduction of TTF-1 is capable of activating Tg promoter activity. However, in a similar experiment done in FRTL-Ha-*ras* cells, the introduction of the TTF-1 expression vector failed to stimulate Tg-reporter expression (Fig. 6A), even though an increase in the levels of TTF-1 DNA binding activity could be observed by gel retardation assay (Fig. 6B). TTF-1 also failed to increase the activity of Tg-CAT in FRTL-5 and FRTL-Py cells, suggesting that it is already present in saturating quantities in these cell lines (data not shown).

**TTF-1 is a phosphoprotein and its phosphorylation is reduced in FRTL-Ha-*ras* cells.** To investigate the cause of the inactivity of TTF-1 in FRTL-Ha-*ras* cells, we compared the

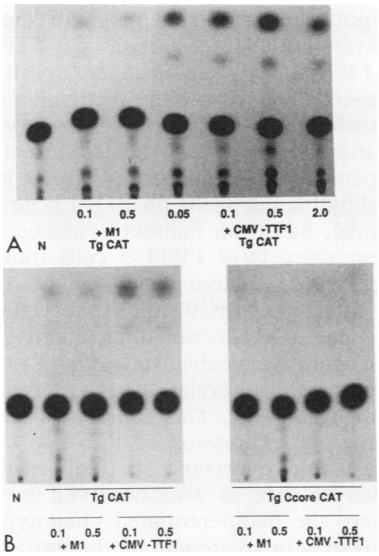


FIG. 5. Activation of the Tg promoter in FRTL-Ki-*ras* cells by TTF-1. (A) Wild-type Tg reporter (Tg-CAT) (8 μg) and 2 μg of the CMV-LUC expression vector were introduced into FRTL-Ki-*ras* cells by transient transfection along with either 0.05 to 2.0 μg of the CMV–TTF-1 expression vector or 0.05 to 2.0 μg of a control vector, M1, as indicated at the bottom of the panel. N refers to mock-transfected cells. (B) The experiment was performed essentially as in panel A, except that Tg C core-CAT was used as a reporter gene in addition to the wild-type Tg-CAT reporter.

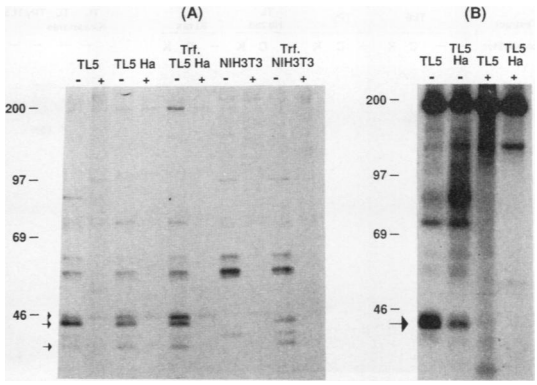


FIG. 7. Pulse-labelling and immunoprecipitation of cellular proteins with an anti-TTF-1 antibody. (A) FRTL-5 (TL5), FRTL-Ha-*ras* (TL5 Ha), and NIH 3T3 cells, mock transfected or transfected with CMV–TTF-1 expression vector (Trf.), were incubated with [$^{35}$S]methionine and immunoprecipitated with an anti-TTF-1 antibody in the presence (+) or absence (−) of an excess of the TTF-1 peptide used for immunization. Arrows indicate the bands identified as TTF-1, as described in the text. An arrowhead indicates the FRTL-Ha-*ras*-specific band. (B) FRTL-5 (TL5) and FRTL-Ha-*ras* (TL5 Ha) cells were incubated with $^{32}P_i$ and immunoprecipitated as described above. The arrow indicates the position of TTF-1. The positions of molecular weight markers ($\times 10^3$) are indicated on the left sides of the autoradiographs.

apparent molecular weight of TTF-1 in these cells with that in FRTL-5 cells. NIH 3T3 cells transfected with the TTF-1 expression vector were used as an additional control, since TTF-1 is capable of activating Tg promoter transcription in these cells (20a). Cells were pulse-labelled with [$^{35}$S]methionine and immunoprecipitated with an anti-TTF-1 antibody. FRTL-5 contains two protein species, of 43 and 39 kDa, which were identified as TTF-1 since (i) they are absent in the immunoprecipitate obtained in the presence of an excess of the TTF-1 peptide used for immunization and (ii) they are present in NIH 3T3 cells only after transfection of the TTF-1 expression vector (Fig. 7A). However, immunoprecipitation of FRTL-Ha-*ras* cell lysates shows an additional protein of 46 kDa (Fig. 7A). The amount of immunoprecipitated 46-kDa protein increased, as did the bands of 43 and 30 kDa, when the cells were transfected with the TTF-1 expression vector (Fig. 7A). This result suggests that the 46-kDa protein is either coimmunoprecipitated with TTF-1 or represents a differentially modified form of TTF-1.

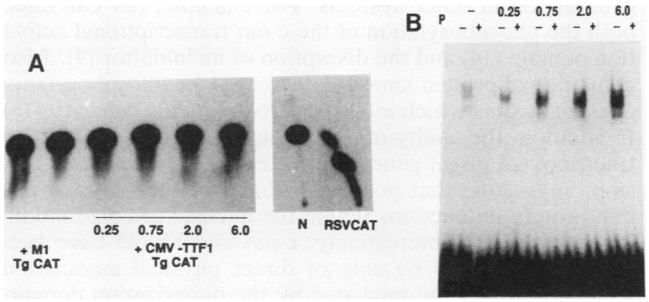


FIG. 6. Overexpression of TTF-1 in FRTL-Ha-*ras* cells fails to increase Tg promoter activity. (A) CMV-LUC (2 μg) and 6 μg of Tg-CAT reporter were transiently expressed in FRTL-Ha-*ras* cells in the presence of increasing concentrations of the CMV–TTF-1 expression vector or the control plasmid M1. Cells were also transfected with 6 μg of RSV-CAT as a positive control. N refers to a mock-transfected sample. (B) Increase in TTF-1 DNA binding activity in the transfected cells was monitored by gel retardation analysis with the Tg C oligonucleotide. The amount of TTF-1 expression vector used in each transfection is indicated (P). +/− refers to the presence on the absence of specific competitor DNA.

Phosphorylation is one of the most frequent posttranslational modifications of transcription factors and has been implicated in the modulation of their transcriptional activating properties in response to extracellular signals (15). In c-*jun*, for example, transformation by v-Ha-*ras* causes an increase in the ability to activate transcription and a parallel increase in phosphorylation (14). Thus, the phosphorylation status of TTF-1 was investigated by labelling FRTL-Ha-*ras* and FRTL-5 cells with $^{32}P_i$ before immunoprecipitation. This experiment shows that in FRTL-5 cells, TTF-1 is a phosphoprotein, with a mobility similar to that observed for the major $^{35}$S-labelled protein on SDS-PAGE. The same band is observed in extracts of $^{32}$P-labelled FRTL-Ha-*ras* cells. However, the intensity of the $^{32}$P-labelled TTF-1 band in this cell line is clearly diminished, suggesting that the presence of an active form of Ha-*ras* could interfere with the phosphorylation of TTF-1. The FRTL5-Ha-*ras*-specific 46-kDa protein is not phosphorylated, as shown by its absence in $^{32}$P-labelled FRTL-Ha-*ras* extracts. If this protein was a nonphosphorylated form of TTF-1, it should have shown an apparent molecular mass smaller than 43 kDa, since phosphate residues decrease the mobility of proteins in SDS-PAGE (see, for example, reference 27). As this was not the case, the Ha-*ras*-specific band could be unrelated to TTF-1. Alternatively, the 46-kDa protein could be the result of the interference exerted by v-Ha-*ras* on a posttranslational modification, different from phosphorylation, of TTF-1 itself.

**TTF-2 is sensitive to transformation.** While in *ras*-trans-

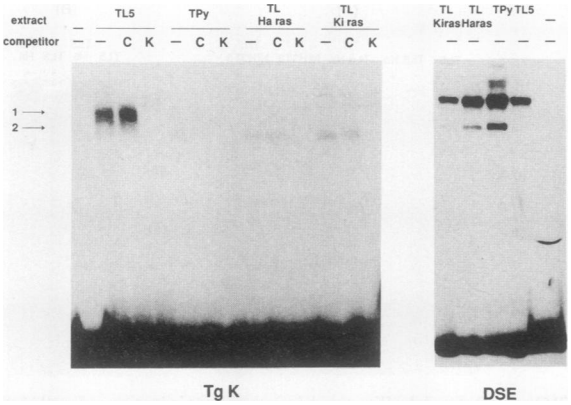


FIG. 8. TTF-2 DNA binding activity is absent in transformed cell lines. Extracts of FRTL-5 (TL5), FRTL-5-Py (TPy), FRTL-Ha-*ras* (TL Ha ras), and FRTL-Ki-*ras* (TL Ki ras) cells were incubated with a $^{32}$P-labelled K oligonucleotide (TgK) containing the TTF-2 binding site from the Tg promoter. Where indicated, 50 ng of cold oligonucleotide K or C was used as a specific or nonspecific competitor, respectively. Extract quality was monitored with the control DSE oligonucleotide, containing Sp1 and octamer binding sites.

formed thyroid cells the dramatic reduction in thyroid-specific promoter activity is associated with the absence or inactivity of TTF-1, the moderate, yet reduced capacity of FRTL-Py cells to transcribe thyroid-specific promoters does not correlate with clear changes in TTF-1. To investigate additional causes of the reduced promoter activity in FRTL-Py cells, we performed a gel retardation assay to detect TTF-2 DNA binding activity in each of the transformed cell lines. TTF-2 binds to a single site within the Tg promoter (19, 39), and TTF-2–DNA complexes comigrate with those formed by a factor with related DNA binding specificity to a homologous sequence within the TPO promoter (23). In both cases, mutation of the binding site reduces promoter activity (23, 39). Incubation of nuclear extracts of FRTL-5 cells with a probe corresponding to the recognition sequence of the TTF-2 binding site within the Tg promoter reveals the complex previously defined as TTF-2 (Fig. 8, complex 1). This complex is highly reduced in FRTL-Py cells and absent in both cell lines transformed by *ras* (Fig. 8). A minor complex of faster mobility is observed in all the transformed cell lines (Fig. 8). The absence of the major TTF-2–DNA complex could therefore explain the moderate reduction in thyroid-specific promoter activity observed in FRTL-Py cells.

## DISCUSSION

The inhibition of cellular differentiation by transformation by various oncogenes is a well-documented phenomenon (4). In this study, we demonstrated that transformation of thyroid cells by three different oncogenes (v-Ki-*ras*, v-Ha-*ras*, and polyomavirus middle-T) results in decreased ability to transcribe the promoters of two thyroid-specific genes, Tg and TPO. As these two genes are dependent on the same tissue-specific factors to regulate their expression, these factors are likely targets for the down-regulation of differentiation mediated by oncogenes.

The absence of TTF-1 RNA in FRTL-5 cells transformed by v-Ki-*ras* or v-*mos* and in other transformed, dedifferentiated thyroid cell lines (data not shown) is always accompanied by the loss of Tg and TPO RNA and is in agreement with the hypothesis that TTF-1 plays an important role in thyroid differentiation. Introduction of a TTF-1 expression vector into FRTL-Ki-*ras* cells leads to a partial recovery of the differentiated phenotype, since it stimulates transcription from a cotransfected Tg promoter. This observation suggests that an important mechanism of the dedifferentiation process that accompanies transformation by Ki-*ras* is the interference exerted by the oncogene on TTF-1 gene expression. A different model, based on indirect evidence obtained with crude nuclear extracts of FRTL-5 cells transformed by a Ki-*ras* temperature-sensitive allele, suggests that this oncogene could interfere with the phosphorylation of TTF-1, which would lead to a reduction of its affinity for DNA (7). If this were the operative mechanism, active Ki-*ras* should also interfere with the phosphorylation of overproduced TTF-1 in the transactivation assay. This apparent discrepancy might arise from the overexpression of TTF-1 in the transfected cells, which could overcome its reduced DNA binding activity. This balance is also observed in the myoblast system, where the dedifferentiated phenotype induced by Ha-*ras* or *fos* may be overcome by the introduction of high levels of MyoD1 (32, 33), yet both oncogenes attenuate the transactivation of muscle-specific promoters by MyoD1 in transient expression assays (33).

In the FRTL-Ha-*ras* cells, near-wild-type levels of TTF-1 DNA binding activity are detectable. However, Tg and TPO promoter activity is severely depressed. Also, the transfected Tg promoter does not respond to TTF-1, as shown by the absence of detectable difference between the activity of a wild-type promoter and a mutant containing several base substitutions in the proximal TTF-1 binding site. Furthermore, as differentiated from the Ki-*ras*-transformed thyroid cells, in the FRTL-Ha-*ras* line, overexpression of TTF-1 protein is not capable of activating transcription from a cotransfected Tg promoter. Immunoprecipitation of $^{35}$S- and $^{32}$P-labelled lysates shows two major differences between wild-type and Ha-*ras*-transformed FRTL-5 cells. The first is the presence of an additional protein band in immunoprecipitates obtained from Ha-*ras*-transformed cells. The second is a quantitative difference in the degree of phosphorylation of TTF-1, which is much lower in Ha-*ras*-transformed cells. Both modification in the phosphorylation of transcription factors and formation of transcriptionally inactive heterodimers have been described as a consequence of oncogene action in other systems. For example, *ras* can affect both the phosphorylation of the c-*jun* transcriptional activation domain (14) and the disruption of its inhibitor (9). Also, activation of protein kinase C decreases the phosphorylation of c-*jun* at sites which inhibit its DNA binding capacity (16). In addition, the ability of exogenously expressed MyoD1 to transactivate target genes is affected by *ras* and *fos* expression, suggesting that posttranslational modification of protein-protein interactions within the nucleus can also modify its activity (33). Interestingly, c-*jun* and MyoD have been recently shown to be able of direct physical association, which appears to be mediated by the dimerization domains of the two proteins (10). Such jun-MyoD heterodimers are inactive in stimulating transcription of target genes, suggesting that in this case the oncogene c-*jun* can directly interfere with muscle differentiation induced by MyoD. In view of the pleiotropic effects of *ras* genes on c-*jun*, a likely possibility is that *ras* can modify TTF-1 activity at several levels. Further studies on the role that phosphorylation may play in TTF-1 transcriptional activity and a better identification of the Ha-*ras*-specific protein, coimmunoprecipitated with TTF-1, are required to determine the relevance of these phenomena.

Considering the great degree of similarity between the Ki-*ras* and Ha-*ras* oncogenes, the observed difference in their biological effects on the differentiated phenotype of the FRTL-5 cell line remains intriguing. However, it has been previously reported that Ha-*ras* shows biochemical effects different from those of Ki-*ras* or N-*ras* upon 12-*O*-tetradecanoyl-phorbol-13-acetate induction of c-*fos* expression in NIH 3T3 cells (17).

Studies on the tissue distribution and the developmental regulation of TTF-1 protein have already indicated that its presence is necessary but not sufficient to activate transcription of the Tg gene, since TTF-1 is present in tissues other than thyroid and it appears in the thyroid bud 5 days before Tg and TPO mRNAs can be detected (34). It is tempting to speculate that Ha-*ras*-transformed cells are blocked at the same step as the thyroid cell precursors in development. Hence, this cell line could provide a valuable tool to further investigate the nature of these processes.

Transformation of two thyroid cell lines with polyomavirus middle-T antigen leads to a reduction in Tg and TPO mRNAs, but does not appear to affect any aspect of TTF-1 function. Consequently, the decrease in transcription of thyroid-specific markers is more moderate. Despite the retention of some thyroid differentiation characteristics, the growth of these cells is stimulated by, but not absolutely dependent on, growth factors which are absolutely required by normal thyroid cells in culture (12). Recently, the loss of TSH-dependent growth in thyroid cells expressing various oncogenes has also been correlated with reduced expression of the TSH receptor (11). Two of the factors essential for thyroid cells growth, insulin and TSH, also affect the accumulation of Tg and TPO mRNAs (31, 38, 41). For Tg, this control undoubtedly occurs at the transcriptional level (31, 35, 38). Interestingly, the more moderate reduction in reporter gene activity in FRTL-Py cells closely parallels the extent of stimulation of insulin–IGF-1 and TSH on Tg transcription (31, 38). Since both transformation (this study) and insulin starvation of normal thyroid cells (38a) leads to the disappearance of TTF-2 DNA binding activity and a reduction in thyroid-specific promoter activity, the two phenomena are possibly related.

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