Ha-*ras* Interference with Thyroid Cell Differentiation Is Associated with a Down-Regulation of Thyroid Transcription Factor-1 Phosphorylation*

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ABSTRACT

Mechanisms responsible for the lack of thyroid-specific differentiation markers in Ha-*ras* transformed FRTL-5 cells have been investigated. *In vivo* cell labeling and immunoprecipitation demonstrate that phosphorylation of the thyroid transcription factor-1 (TTF-1) is clearly reduced in thyroid cells transformed with the Ha-*ras* oncogene. Fingerprinting analysis of phosphotryptic peptides from FRTL-5 and Ha-*ras*-FRTL-5 cells also reveals a heterogeneous pattern of TTF-1 phosphorylation in the transformed cell line. This heterogeneity is localized in the amino terminal cluster of phosphoserines, as determined by transfection of HeLa cells with TTF-1 mutants in which serine residues have been replaced by alanines. Amplification and nucleotide sequence of the 5′-coding region of the TTF-1 gene in Ha-*ras*-FRTL-5 cells rule out the possibility that differences in phosphorylation were the consequence of any mutational event affecting residues within the N-terminal protein sequence. Hypophosphorylated TTF-1 is still able to bind its DNA consensus sequence within the thyroglobulin promoter, although a reporter construct whose expression is exclusively dependent on TTF-1 is not transactivated. Transfection of Ha-*ras*-FRTL-5 cells with an expression vector encoding the cAMP dependent protein kinase A (PKA) catalytic subunit partially reestablishes TTF-1 transcriptional activity. Taken together, these results indicate that the lack of specific thyroid gene expression in Ha-*ras*-FRTL-5 cells could be a direct consequence of the inability of TTF-1 to promote transcription. (*Endocrinology* **139:** 2796–2802, 1998)

THYROID-DIFFERENTIATED phenotype is defined by the expression of specific genes, thyroglobulin (Tg), thyroperoxidase (TPO), TSH receptor (TSH-R), and the Na^+/I^- symporter (NIS) for iodide trapping (1, 2). So far, three tissue-specific transcription factors have been isolated: thyroid transcription factor (TTF) 1 and 2, homeo- and fork-head domain-containing proteins, and the paired-box factor Pax-8 (3–5). Binding sites for TTF-1 have been identified and functionally characterized within the Tg, TPO, and TSH-R gene promoters (6–9) and also recently in the rat NIS promoter (10, 11), attributing to TTF-1 a main role in the maintenance of the thyroid differentiated phenotype.

Loss of differentiation as a result of cellular transformation is a well characterized phenomenon (12). In many thyroid tumors, expression of molecular markers such as Tg and TPO and their corresponding transcription factors TTF-1 and Pax-8 is lost, particularly in late stages of thyroid carcino-

genesis (13). Oncogenic transformation of thyroid cells in culture mimics this loss of differentiation, and experiments with FRTL-5 thyroid cells demonstrate that Ha- and Ki-*ras* transformation also abolish Tg and TPO expression completely. However, while in Ki-*ras* transformed cells TTF-1 is undetectable, Ha-*ras*-FRTL-5 cells display normal levels of TTF-1 messenger RNA (mRNA) and protein (14).

Previous results from our laboratory showed that TTF-1, encoded by the *titf*-1 locus, is a phosphoprotein required for specific expression of Tg and TPO (15). Precise mapping of the TTF-1 phosphorylation sites demonstrated that this transcription factor is phosphorylated on a maximun of seven serine residues distributed in three different phosphotryptic peptides (15). Therefore, as described for many other transcription factors, phosphorylation appears to be a key regulatory mechanism by which TTF-1/DNA binding and/or transactivation could be modulated. Several observations indicate that phosphorylation of TTF-1 may be relevant only in specific cell types. Previous works in thyroid cells proposed that phosphorylation is a critical step for TTF-1 binding to the Tg promoter (16), whereas studies in TTF-1-transfected HeLa cells indicated that phosphorylation does not play an important role in the regulation of TTF-1 DNA binding activity (15). Therefore, the biological significance of TTF-1 phosphorylation remains to be elucidated.

In this study, we have searched for regulatory mechanisms affecting TTF-1 activity that could be altered upon Ha-*ras* transformation. Using a construct containing a specific TTF-1 responsive element within the Tg promoter (17), we dem-

onstrate that the ability of TTF-1 to promote transcription is lost in cells transformed with the Ha-*ras* oncogene. This ability is partially reestablished when the catalytic subunit of the protein kinase A (PKA) is expressed in these cells. *In vivo* cell labeling and immunoprecipitation of TTF-1 from Ha-*ras*-FRTL-5 cells show a clear decrease in the overall extent of phosphorylation, along with a heterogeneity in the distribution of the phosphorylated serines. This heterogeneous phosphorylation is more prominent within the amino terminal region of the protein.

Materials and Methods

Cell culture

FRTL-5 thyroid cells (18) (ATCC CRL 8305; American Type Culture Collection, Rockville, MD) were grown in Coon's modified Ham's F-12 medium (Sigma Chemical Co., St. Louis, MO) supplemented with 5% donor calf serum (GIBCO, Life Technologies, Inc., Gaithersburg, MD) and a hormone mixture including 0.5 mU/ml TSH and 10 μg/ml insulin (Sigma). Ha-*ras* transformed FRTL-5 cells (19) were maintained in the same conditions. HeLa cells were grown in DMEM supplemented with 10% FCS (Gibco Life Technologies).

TTF-1 protein analysis

FRTL-5 or transformed Ha-*ras*-FRTL-5 cells were labeled in methionine-free medium containing 0.5 mCi of [^{35}S] methionine/ml for 2 h. Cells were lysed in a buffer containing 10 mM sodium phosphate (pH 7.4), 0.1 M NaCl, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 2 mM phenylmethylsulfonil fluoride, 1 μg/ml pestatin, 1 μg/ml leupeptin, 1 μg/ml aprotinin, 50 mM sodium fluoride, 10 mM sodium pyrophosphate, and 5 mM sodium vanadate. Extracts were incubated with an specific TTF-1 antibody (15) coupled to protein A sepharose beads (Pharmacia Biotech Inc., Piscataway, NJ). Immune complexes were washed extensively with lysis buffer, boiled in SDS-Laemmli sample buffer, and resolved by SDS 8%-PAGE. For Western blot, nuclear extracts were obtained as described (20) and subjected to SDS-PAGE. Proteins were then transferred to nitrocellulose membranes and probed with the same TTF-1 specific antibody (15). After incubation with a secondary antibody, immunoreactive bands were visualized by enhanced chemiluminescence ECL (Amersham Corp., Arlington Heights, IL).

Electrophoretic mobility shift assays

DNA binding assays were performed as described (21) using ^{32}P-labeled double-stranded oligonucleotide C (5'-CACTGCCCAGT-CAAGTGTTCTTGA-3') derived from the −92 to −68 region of the Tg promoter and shown to recognize the transcription factor TTF-1 (6). Nuclear proteins (3 μg), prepared as described (20), were preincubated 15 min on ice in a binding reaction mixture containing 40 mM HEPES, pH 7.9, 200 mM KCl, 0.5 mM DTT, 0.2 mM EDTA, 5% Ficoll, and 3 μg poly(dI-dC). In competition and supershift experiments, 100-fold excess of unlabeled competitor or 1 μg of TTF-1 antibody were added, respectively. Then 50 pg of labeled C probe were added to the mixture and incubated for 30 min at room temperature. The resulting DNA-protein complexes were separated from free DNA on a 5% polyacrilamide gel.

Plasmids and transfections

For detection of TTF-1 transcriptional activity, the constructs C5E1b-CAT and E1b (17), referred in this work as 5C-CAT and TATA-CAT, respectively, were transiently transfected by the calcium phosphate precipitation technique (22) into FRTL-5 and Ha-*ras*-FRTL-5 cells. The 5C-CAT construct contains five tandem repeats of the C binding site from the Tg promoter, and it is exclusively dependent on TTF-1 for transactivation (17). TATA-CAT was used as a negative control. The plasmid CMV-Luc (17) was used to correct for transfection efficiency. Forty eight hours after transfection, cell extracts were prepared and CAT (22) and luciferase activities (23) determined. For PKA overexpression assays, vectors containing either a wild-type or a mutated PKA catalytic subunit (24) were transfected in Ha-*ras*-FRTL-5 cells. TTF-1 mutants carrying

Ser/Ala substitution were generated by PCR as previously described (15). Expression vectors for wild-type and mutant TTF-1 complementary DNAs were transfected into HeLa cells 36 h before *in vivo* labeling.

In vivo labeling and fingerprinting analysis of phosphopeptides

Exponentially growing FRTL-5, their Ha-*ras* derivatives and transfected HeLa cells were maintained in phosphate-free medium for 30 min, then labeled with 0.5 mCi of [^{32}P] orthophosphate/ml for 3 h. Cell extracts and TTF-1 immunoprecipitation were performed as described above. Immunoprecipitated, *in vivo* phosphorylated TTF-1 and the different mutant proteins were separated in SDS 8% polyacrilamide gel electrophoresis and electroblotted to nitrocellulose. After autoradiography of the membranes, the bands were excised and digested with trypsin-TPCK (Worthington) as described (15). The resulting solubilized peptides were collected, oxidized, and loaded onto TLC plates (25). First dimension separation was performed by electrophoresis at 1,000 V in 0.13 M (NH$_4$)$_2$CO$_3$, pH 8.9, for 30 min. TLC plates were then subjected to chromatography in isobutiric: pyridine: acetic acid: n-butanol:water (62.5: 4.8: 2.9: 1.9: 27.9). When necessary, phosphopeptides were recovered from the TLC plate and subjected to chemical cleavage with cyanogen bromide (CNBr) in the conditions described (26).

TTF-1 mutational analysis

A 200-bp DNA fragment containing the N-terminal region of the TTF-1 protein was amplified from FRTL-5 and Ha-*ras*-FRTL-5 genomic DNA by PCR, using the primers 5'-AGTAGCGAGGCTTCGCCTTC-3' and 5'-TGTCTGTAAGCTGCGAGCGG-3' for forward and reverse annealing, respectively. Amplification was performed following standard protocols using *Taq* polymerase (Perkin Elmer Co., Foster City, CA). Amplified DNA fragments were subcloned in the pCRII vector (Invitrogen, Carlsbad, CA), and a representative number of positive clones were sequenced (Applied Biosystems, Foster City, CA). Sequences were aligned using the Pileup program (GCG package, University of Wisconsin DNA software) and compared with the previously published TTF-1 sequence (27).

Results

TTF-1 phosphorylation is clearly reduced in Ha-ras transformed FRTL-5 cells

The presence of detectable TTF-1 mRNA and protein, together with the lack of thyroid-specific genes Tg and TPO in Ha-*ras*-FRTL-5 cells (14), prompted us to analyze posttranscriptional mechanisms affecting TTF-1 function in this transformed cell line. We focused our attention in potential changes in TTF-1 phosphorylation.

The levels of TTF-1 phosphorylation in thyroid FRTL-5 cells and their Ha-*ras* transformed counterpart were analyzed by *in vivo* ^{32}P labeling and immunoprecipitation. Equal number of exponentially growing cells were incubated with radiolabeled orthophosphate. After protein extraction, immunoprecipitated TTF-1 was separated by electrophoresis (Fig. 1A). A marked decrease in TTF-1 phosphorylation was observed in Ha-*ras* transformed FRTL-5 cells, as determined by the amount of ^{32}P incorporated into the transcription factor. Comparison with the parental FRTL-5 cells and quantitation of the signal by densitometric scanning indicated up to a 5-fold reduction in the amount of radiolabeled ^{32}P in TTF-1 immunoprecipitated from Ha-*ras*-FRTL-5 cells. We ruled out the possibility that differences in radioactive labeling were due to the presence of different amount of TTF-1 protein in both extract preparations. For that purpose, steady-state levels and *de novo* protein synthesis were determined by Western blot and immunoprecipitation of ^{35}S-me-

thionine-labeled TTF-1 (Fig. 1B), respectively. In both cases, comparable levels of immunoreactive TTF-1 signal was observed, unambiguously indicating that differences in ^{32}P labeling were exclusively due to a decrease in the amount of tracer incorporation.
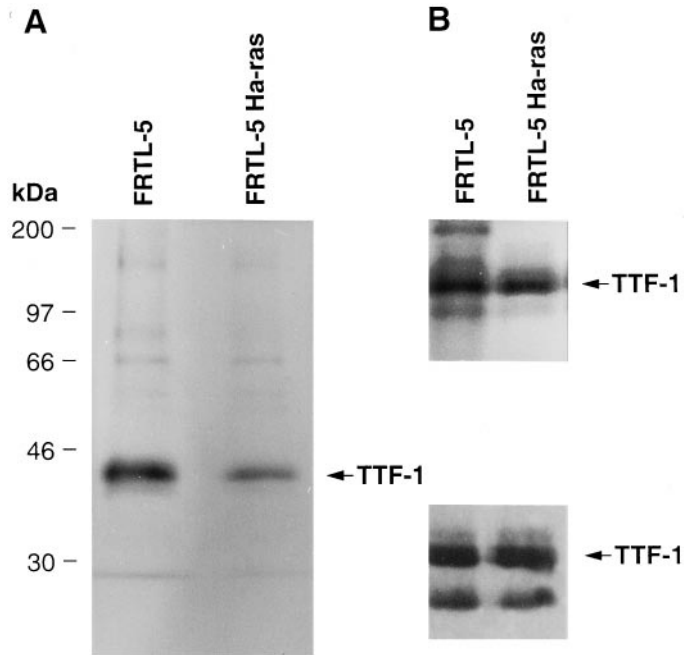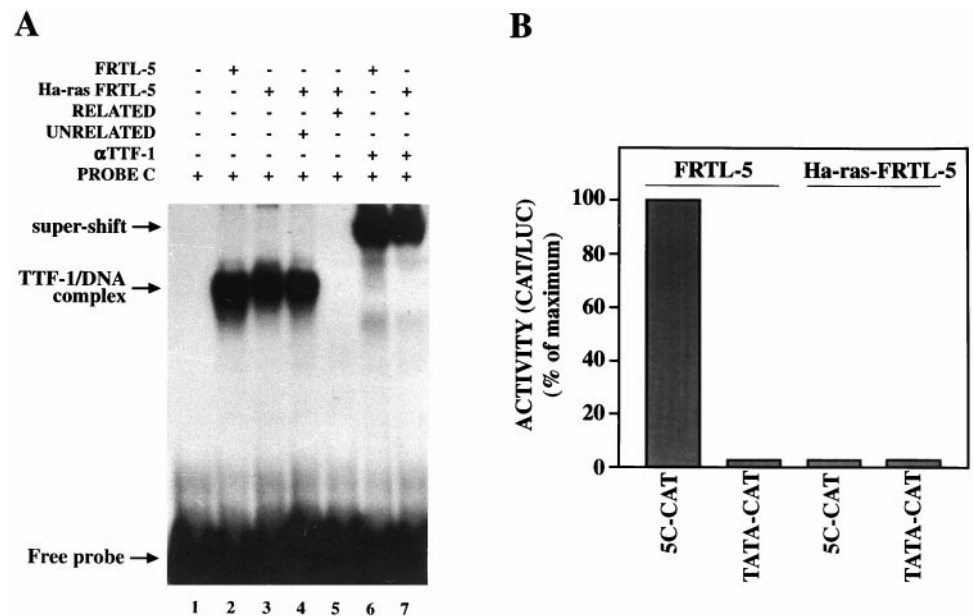

FIG. 1. Phosphorylation of TTF-1 in FRTL-5 and Ha-*ras*-FRTL-5 cells. A, The same number of thyroid FRTL-5 and Ha-*ras* transformed FRTL-5 cells were *in vivo* labeled with ^{32}P before TTF-1 immunoprecipitation. Proteins were resolved in a SDS-8% PAGE and detected by autoradiography. B, In the same experimental conditions, TTF-1 was immunoprecipitated from ^{35}S methionine-labeled cell cultures (*upper panel*), or immunodetected by Western blot (*lower panel*).

TTF-1 retains DNA binding capacity, although it is unable to promote gene transcription in Ha-ras-FRTL-5 cells

We then analyzed whether the TTF-1 protein in Ha-*ras*-FRTL-5 exhibits any alteration in either DNA binding capacity or transactivation activity. Figure 2A shows the results of the electrophoretic mobility shift assay performed to detect the presence of protein-DNA complexes in crude nuclear extracts from FRTL-5 and Ha-*ras*-FRTL-5 cells. Incubation of equal amounts of nuclear proteins with a ^{32}P-labeled C oligonucleotide and separation by nondenaturing polyacrylamide gel electrophoresis yielded the same TTF-1/DNA complexes in both cell types (lanes 2 and 3). Specificity of the complex in Ha-*ras*-FRTL-5 was assayed using a 100-fold excess of an unrelated oligonucleotide (lane 4) or unlabeled oligonucleotide C (lane 5). The identity of the complex in FRTL-5 and Ha-*ras*-FRTL-5 was further confirmed by supershift assays using an anti-TTF-1 specific antibody (lanes 6 and 7).

From the above experiments we concluded that the low levels of TTF-1 phosphorylation observed in Ha-*ras*-FRTL-5 do not modify the capacity of the transcription factor to bind its DNA recognition sequence. Therefore, we analyzed the ability of TTF-1 to promote transcription in transformed and nontransformed thyroid cells, using a construct exclusively dependent on TTF-1 for transactivation. This construct, containing five tandem repeats of the oligonucleotide C, or a minimal promoter TATA-CAT control vector (17), were transiently transfected in both FRTL-5 and Ha-*ras*-FRTL-5 cells. The activity was determined assaying crude cell extracts for CAT activity, whereas luciferase activity derived from the CMV-Luc plasmid was used to correct variability in transfection efficiencies. Consistent with a functional TTF-1 protein in normal cells, activity of the 5C-CAT construct in FRTL-5 was clearly observed (Fig. 2B). However, when this construct was transfected in Ha-*ras*-FRTL-5 cells, CAT activity values were comparable with those obtained in the negative control, indicating that TTF-1 is unable to promote transcription in Ha-*ras*-transformed FRTL-5 cells.

FIG. 2. Binding and transcriptional activity of TTF-1 in FRTL-5 and Ha-*ras*-FRTL-5 cells. A, TTF-1 binding to oligonucleotide C was assayed as described in *Materials and Methods*. Nuclear extracts were prepared from FRTL-5 or Ha-*ras* transformed FRTL-5 cells (lane 2 and 3). The specificity of the retarded complex was established by competition with a 100-fold excess of an unrelated oligonucleotide (lane 4) or unlabeled oligonucleotide C (lane 5). The identity of the complex was demonstrated by supershift with an anti-TTF-1 antibody (lanes 6 and 7). B, Ten micrograms of the construct containing five tandem repeats of the TTF-1 binding site (5C-CAT) and the promoterless vector TATA-CAT were transiently transfected in FRTL-5 and Ha-*ras*-FRTL-5 cells. CAT activity was normalized for transfection efficiency and expressed relative to the activity of the 5C-CAT construct (100%) in control FRTL-5 cells. The data are from three independent experiments.

Changes in TTF-1 phosphorylation pattern in Ha-ras transformed FRTL-5 cells

The results described above provide a direct correlation between the absence of the thyroid differentiation marker Tg and the inability of TTF-1 to promote transcription in Ha-*ras*-FRTL-5 cells. The fact that this functional alteration is associated with a decrease in ^{32}P-radiolabeled TTF-1 in these transformed cells prompted us to analyze alterations of TTF-1 phosphorylation in more detail. We investigated whether the reduction of phosphorylation either targets to any particular serine residue/s or affects the overall phosphorylation extent of the TTF-1 molecule. To address this question, cultures from FRTL-5 and Ha-*ras*-FRTL-5 cells were *in vivo* labeled with ^{32}P-orthophosphate. Immunoprecipitated TTF-1 was separated by electrophoresis and transferred to nitrocellulose. Membrane-bound TTF-1 was digested with tripsin-TPCK and resulting peptides were resolved by two dimensional electrophoresis-chromatography, as described in *Materials and Methods*. Representative experiments, where the same number of counts were loaded, are shown in Fig. 3. TTF-1 fingerprinting analysis from FRTL-5 cells shown the migration of three phosphotryptic peptides (designated 1, 2, and 3), consistent with the previously described phosphorylation pattern (15). Analysis from Ha-*ras* transformed cells revealed a similar pattern with tryptic phosphopeptides 1 and 2 migrating at the bottom of the TLC place. However, a heterogeneous mobility of phosphopeptide 3 was observed. This peptide, clearly defined in FRTL-5 cells, migrates as a doublet in the transformed cell line, and two phosphopeptides were identified (3a and 3b). Mixing experiments of both preparations indicated that spot 3 of FRTL-5 cells corresponds to spot 3a in Ha-*ras*-FRTL-5 cells (not shown), and, therefore, 3a and 3b are different phosphorylation stages of the same peptide. The faster mobility of peptide 3b indicates a higher amount of ^{32}P incorporation into this phosphopeptide.
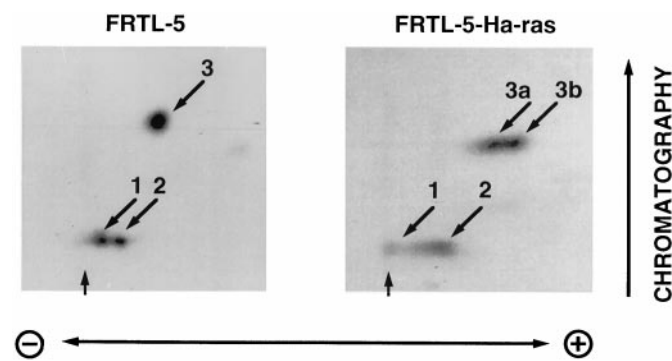
Alteration of TTF-1 phosphorylation in Ha-ras-FRTL-5 cells affects the N-terminal cluster of phosphoserines

To localize tryptic peptide 3 within the TTF-1 protein, HeLa cells were initially transfected with either wild-type or different serine phosphorylation mutants and then labeled with ^{32}P-orthophosphate. We reasoned that this approach, in which specific phosphorylation sites are artificially removed from the TTF-1 protein sequence (15), would allow us to determine the relative position of the phosphopeptides in the TTF-1 protein, by comparison of the tryptic maps. After immunoprecipitation and digestion of the TTF-1 protein, phosphopeptides were resolved by fingerprinting analysis (Fig. 4). As for the endogenous TTF-1, three major phosphopeptides were identified in HeLa cells transfected with wild-type TTF-1 (panel A). Construct S1–24, bearing four alanines replacing serines in the N-terminal region produced a peptide map in which peptide 3 completely disappeared (panel B). Tryptic map of ^{32}P labeled S64 mutant where the two serines in the C-terminal has been replaced with alanines shows peptide 3 migration at the *upper right corner* of the chromatography plate, whereas peptide 1 is missing at the *bottom*. Double mutant S61 (panel C) indicates that peptide 2 derives from the TTF-1 central region, and confirms the identity of peptide 3 with the N-terminal.

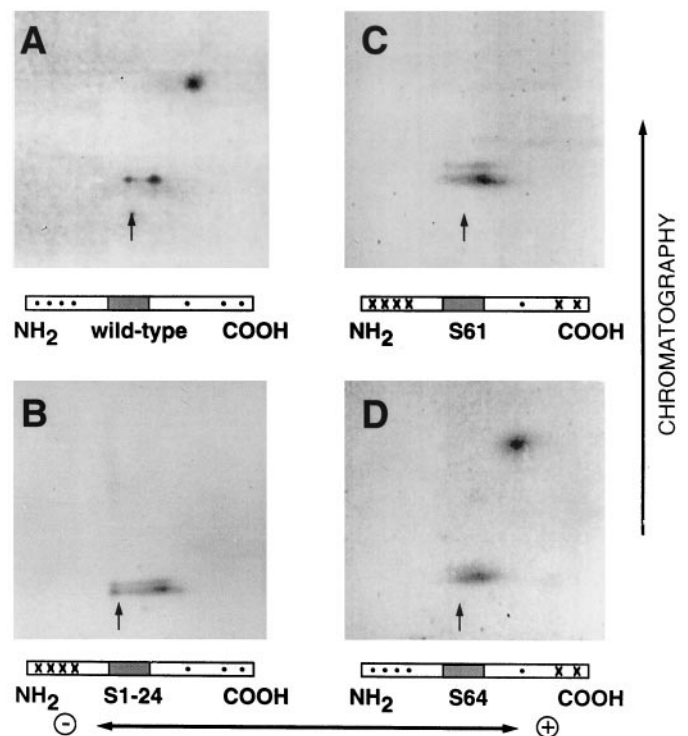Once peptide 3 was localized in the parental cell line, we

FIG. 4. Identification of tryptic phosphopeptides within the TTF-1 protein. HeLa cells were transfected with a wild-type TTF-1 expression vector (A) or constructs carrying Ser/Ala substitutions in different positions (B–D). After transfection, cell cultures were labeled with ^{32}P and TTF-1 immunoprecipitated for phosphotryptic peptide mapping. Position of phosphoserines (·) are indicated within the TTF-1 schemes. Ser/Ala substitutions in the different mutant constructs (S1–24, S61 and S64) are also indicated (X). The homeodomain of the TTF-1 protein sequence is *boxed*.

FIG. 3. Heterogeneity of TTF-1 phosphorylation in Ha-*ras*-FRTL-5 cells. ^{32}P-labeled-TTF-1 was immunoprecipitated from FRTL-5 cells and Ha-*ras*-FRTL-5 cells, subjected to SDS-PAGE, and transferred to nitrocellulose. Membrane-bound TTF-1 was digested with trypsin and resulting phosphopeptides resolved by two dimensional electrophoresis-chromatography. Representative autoradiographs are shown. The position of sample application are indicated at the bottom of the autoradiographs.

confirmed the observation that peptides 3a and 3b found in Ha-*ras*-FRTL-5 cells correspond to different phophorylation stages of the same peptide. After analyzing the amino acid sequence of the N-terminal in detail, we chose a chemical cleavage with CNBr (26), which would digest peptide 3 at amino acid positions Met^3-Ser^4 and Met^{29}-Glu^{30}, leaving the four phosphoserines in one unique subpeptide, whose migration could be easily and precisely compared by one dimension chromatography. Peptides corresponding with spots 3, 3a, and 3b were recovered from the TLC plate and digested with CNBr. Samples were then separated on a new TLC plate and migration compared. Under these conditions, the three reactions yielded a unique radiolabeled peptide (designated CN-3) showing equal mobility (Fig. 5B). These results allowed us to conclude that the three radiolabeled peptides correspond to the same TTF-1 protein fragment and also demonstrated that no other residue is phosphorylated in peptide 3 in Ha-*ras*-FRTL-5 cells.

We considered the possibility that differences in phosphorylation detected in the peptides maps could be attributable to the presence of an specific mutation affecting the amino acid sequence of the N-terminal region of TTF-1. To test this possibility, we used a PCR approach with specific primers designed from the previously published TTF-1 sequence (27). A unique 200 bp PCR product, containing coding sequence from amino acid 1 to 25, was amplified from FRTL-5 and Ha-*ras*-FRTL-5 genomic DNA. These PCR products were subcloned and sequenced. The translation of the DNA sequence obtained from Ha-*ras*-FRTL-5 is shown (Fig. 5A) and corresponds to that obtained in FRTL-5 cells and to the previously published TTF-1 gene (27). These results ruled out the possibility that differences in phosphorylation could be the consequence of mutations affecting residues at the

N-terminal TTF-1 region, supporting the notion that as a consequence of the Ha-*ras* transformation, specific kinase pathways are altered and lead to modifications of TTF-1 phosphorylation.

PKA overexpression partially reestablishes TTF-1 transcriptional activity in Ha-ras-FRTL-5 cells

To gain further insights in the mechanisms responsible for the differential phosphorylation of TTF-1 in Ha-*ras*-FRTL-5 cells, we explored the participation of signal transduction pathways that could be involved in the regulation of TTF-1 transcriptional activity. Because previous works have correlated Ha-*ras* transformation with alteration of different kinase pathways (28) and considering a recent report describing and characterizing a functional PKA phosphorylation site within the N-terminal motif of TTF-1 (29), we analyzed whether exogenous expression of PKA in Ha-*ras*-FRTL-5 cells modulates transcriptional activity of TTF-1. Ha-*ras*-FRTL-5 cells were cotransfected with different concentrations (0.5, 1, 2, 4, and 6 μg) of an expression vector containing either the wild-type or the mutated PKA catalytic subunit (24), along with the 5C-CAT or the TATA-CAT constructs. CAT and luciferase activities were determined in cell extracts (Fig.6). Overexpression of PKA in Ha-*ras*-FRTL-5 cells yielded a moderate increase (2-fold) in 5C-CAT activity, whereas no increase was found with the TATA-CAT construct. The maximum activity was obtained when 2 μg of the wild-type PKA vector was cotransfected, whereas no increase in CAT activity was found when the mutated PKA was used. These data indicate that PKA is able to partially reestablish transcriptional activity of TTF-1. Nevertheless, CAT activity values in Ha-*ras*-FRTL-5 cells in the presence of PKA were significantly lower than those obtained in normal FRTL-5 (Fig. 2B), indicating that other kinase pathways, pre-
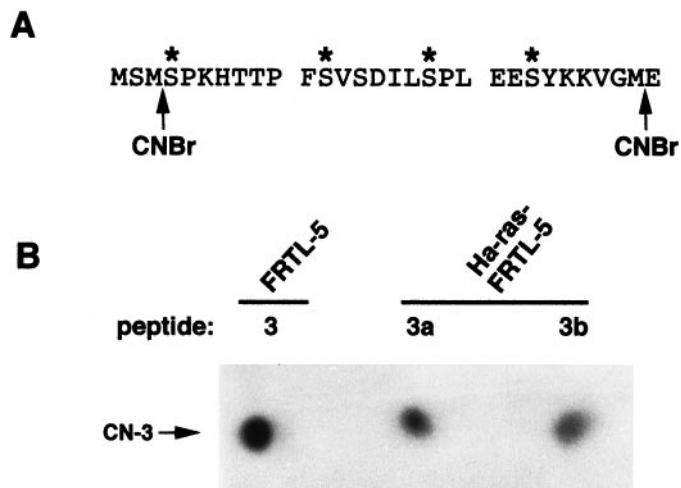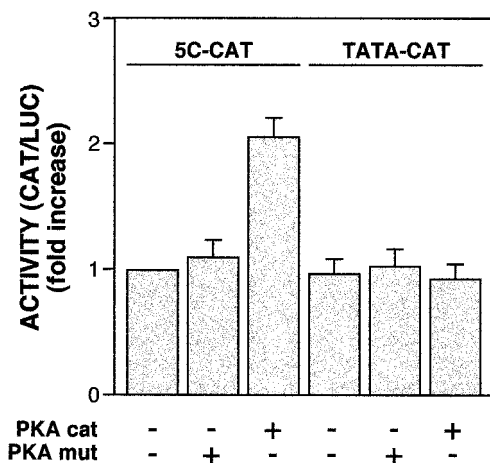

FIG. 5. Sequence analysis and chemical cleavage of the N-terminal region of TTF-1. A, Nucleotide sequence corresponding with the TTF-1 amino terminal protein in Ha-*ras*-FRTL-5 was obtained by PCR using specific primers designed according to the previously published TTF-1 sequence (27). Translation of the nucleotide sequence is shown and demonstrates the lack of mutations affecting TTF-1 primary structure. The position of the previously identified phosphoserines (15) and CNBr cleavage sites are indicated. B, Chromatography of peptide CN-3, originated by chemical cleavage of peptides 3 (FRTL-5), 3a and 3b (Ha-*ras*-FRTL-5) with CNBr.

FIG. 6. PKA partially reestablishes TTF-1 activity in Ha-*ras*-FRTL-5 cells. Ten micrograms of the construct containing five TTF-1 binding sites (5C-CAT) alone or in combination with 2 μg of a plasmid containing either the catalytic subunit or the mutated PKA were transiently cotransfected to Ha-*ras*-FRTL-5 cells. Same experimental approach was performed with the TATA-CAT promoterless construct. CAT activity was normalized for transfection efficiency and expressed as fold induction relative to the activity of 5C-CAT transfected alone (value 1). The data are from three independent experiments.

sumably altered in Ha-*ras*-FRTL-5 cells, may be responsible for the final phosphorylation stage of TTF-1.

Discussion

It is well established that TTF-1 plays an important role in thyroid cellular processes such as differentiation, proliferation, and development (1, 13, 30–32), promoting the expression of specific thyroid genes. These genes are actively expressed in the differentiated cell line FRTL-5 under normal conditions. Transformation of FRTL-5 cells by different oncogenes frequently leads to the loss of the thyroid specific markers Tg and TPO, along with the absence of TTF-1 mRNA (14). An exception to this mechanism is observed in the Ha-*ras*-FRTL-5 cell line. These cells have lost the expression of Tg and TPO, although they exhibit normal levels of TTF-1 protein (14), suggesting a subtle posttranscriptional mechanisms of regulation.

One of the most common mechanisms affecting transcription factor activity is phosphorylation, so we focused our attention in changes in orthophosphate incorporation into TTF-1. Because this factor is inactive in Ha-*ras* transformed FRTL-5 cells, we also explored whether the transcription factor was able to bind its DNA recognition site and/or whether its transcriptional activity was impaired. These two possibilities, along with changes in the subcellular distribution, are the most common ways by which phosphorylation alters the activity of a transcription factor (33, 34). The detection of equal amount of TTF-1 protein in nuclear extracts from both normal and transformed cells ruled out the latter possibility. In addition, our results indicate that hypophosphorylated TTF-1 in Ha-*ras*-FRTL-5 cells is able to bind DNA, although its ability to transactivate is lost. Modification of phosphorylation upon oncogenic transformation of some transcription factors have been described in other cell systems (35, 36). This modification normally affects DNA binding activity of the targeted transcription factor. Interesting for our studies is the fact that *ras* affects phosphorylation of c-*jun* transcriptional activation domain (37). Activation of protein kinase C by *ras* transformation also decreases phosphorylation of c-*jun* at sites that inhibit its DNA binding capacity (38). Due to the pleiotropic effect of *ras*, there is a possibility that it could also modify TTF-1 transactivation activity.

In a previous work, transfection in HeLa cells of TTF-1 mutant derivatives carrying Ser/Ala substitutions determined that phosphorylation is not involved in controlling TTF-1/DNA binding (15). However, other studies indicate that phosphorylation of this transcription factor is essential for DNA binding in thyroid cells (16), suggesting that regulation of TTF-1 function is likely to be specific in the thyroid context. Our results of phosphopeptide mapping of TTF-1 in Ha-*ras*-FRTL-5 cells, along with a transfection approach with mutant derivatives, indicate that the reduction of TTF-1 phosphorylation is accompanied by an heterogeneous pattern of the phosphopeptides affecting the N-terminal domain of the protein. The higher mobility of the spot 3b, only present in Ha-*ras* transformed cells, indicates that this peptide corresponds to a highly phosphorylated form of peptide 3a. We interpret these results considering that, although the

equilibrium between phosphorylated and nonphosphorylated forms of the TTF-1 protein is displaced to the latter form, some population of highly phosphorylated TTF-1 molecules are present in those cells transformed by Ha-*ras*. This observation may indicate that a precise equilibrium between kinases and phosphatases responsible for TTF-1 phosphorylation is necessary for the correct function of this transcription factor.

The fact that the N-terminal stretch of the TTF-1 protein is involved in phosphorylation alterations in Ha-*ras*-FRTL-5 cells may be of special interest because previous results have designated this N-terminal region as one of the TTF-1 transactivation domain (17). In the N-terminal site of the TTF-1 protein, several consensus sequences for protein kinases have been observed. The sites that we have identified by computer analysis show homology to casein kinase II (CKII), protein kinase C (PKC), and mitogen-actived protein kinase (MAPK) (15), and we have previously demonstrated that PKC is able to *in vitro* phosphorylate TTF-1 (15). A recent report also localizes and functionally characterizes a PKA phosphorylation site within this N-terminal motif of TTF-1 in lung cells (29). Our results cotransfecting the catalytic subunit of the PKA into Ha-*ras*-FRTL-5 cells show a moderate increase in a reporter construct depending on TTF-1, suggesting that although PKA may participate in the regulation of TTF-1 phosphorylation, it is not sufficient to reestablish basal levels of TTF-1 transcriptional activity in Ha-*ras* transformed cells. Taken together, these observations indicate that phosphorylation of TTF-1 at the N-terminal site is a rather complex mechanism in which different kinases are involved. We have to consider that Ha-*ras* induces cellular transformation by activating several signal transduction pathways including the Raf-MEK-MAPK and the PKC cascades (28), and also a modest reduction in PKA activity has been reported in Ha-*ras* transformed FRTL-5 cells (39). How these pathways integrate to precisely modulate TTF-1 function remains to be elucidated.

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