Redox Effector Factor-1 Regulates the Activity of Thyroid Transcription Factor 1 by Controlling the Redox State of the N Transcriptional Activation Domain*

Received for publication, January 18, 2002, and in revised form, February 5, 2002
Published, JBC Papers in Press, February 7, 2002, DOI 10.1074/jbc.M200582200

Gianluca Tell‡§, Alex Pines‡, Igor Paron‡, Angela D’Elia‡, Alessia Biscà‡, Mark R. Kelley‡, Giorgio Manzini‡, and Giuseppe Damante‡

The modulation of a TF activity is achieved by different means: (i) at a translational level, through the regulation of the expression of the TF itself, and (ii) at a post-translational level, through modifications such as phosphorylation and glycosylation, occurring at specific residues of the TF. During the last few years, another kind of post-translational regulation has become increasingly evident (i.e. redox regulation). This control is exerted through the modulation of the oxidation/reduction state of the thiol groups of cysteine residues usually present in the DNA-binding domain of TFs themselves. It has been previously demonstrated that the DNA binding activity and the dimerization ability of TTF-1 are redox-regulated in vitro (7). It was shown that a reducing environment is required, in vitro, for a proper DNA-binding activity and that oxidation promotes (i) the formation of disulfide bond(s) between two specific cysteine residues (87 and 363) located outside the homeodomain and (ii) the formation of higher order oligomers of the protein itself.

Ref-1 has been identified as a protein capable of both apurinic/apyrimidinic endonuclease DNA repair activity and nuclear redox activity, being able to induce the DNA binding activity of AP-1, NF-kB, Myb, members of the ATF/cAMP-response element-binding protein family, hypoxia-inducible factor (HIF-1α) (8), and Pax proteins (9). Moreover, recent developments have pointed out a primary role for Ref-1 in

Thyroid transcription factor 1 (TTF-1), also named Nkx2.1, is a tissue-specific transcription factor (TF) that controls the expression of some thyroid- and lung-specific genes (1). However, it is not well ascertained how TTF-1 is able to activate the transcription of thyroid-specific genes (such as those of thyroglobulin and thyroperoxidase) only in the follicular thyroid cell (2) and the transcription of lung-specific genes (such as those encoding for surfactant proteins) exclusively in epithelial lung cells (3). Thus, unknown regulatory mechanisms must control the TTF-1 transcriptional function. TTF-1, similarly to the large majority of eukaryotic promoter-specific TFs, displays a modular nature. In fact, the two basic molecular functions (i.e. specific DNA binding and transcriptional activation) are performed by distinct domains. The DNA-binding function is brought on by the homeodomain (HD) that recognizes, with high affinity, DNA sequences containing the 5’-CAAG-3’ core motif (4). Moreover, TTF-1 exhibits two independent transcriptional activation domains, located at the N-terminal (N domain) and at the C-terminal (C domain) regions with respect to the HD (Fig. 1 and Ref. 5). It has been previously demonstrated that the N domain plays a leading role in the activation of the transcriptional machinery, being able to squelch both its own transcriptional activity and the C domain one (5). Moreover, the N domain directly interacts with the TATA-binding protein TBP (6). Therefore, regulatory mechanisms, acting upon the N domain, could control the activity of the whole molecule.

Thyro transcription factor 1 (TTF-1,1 also named Nkx2.1) is a tissue-specific transcription factor (TF) that controls the expression of some thyroid- and lung-specific genes (1). However, it is not well ascertained how TTF-1 is able to activate the transcription of thyroid-specific genes (such as those of thyroglobulin and thyroperoxidase) only in the follicular thyroid cell (2) and the transcription of lung-specific genes (such as those encoding for surfactant proteins) exclusively in epithelial lung cells (3). Thus, unknown regulatory mechanisms must control the TTF-1 transcriptional function. TTF-1, similarly to the large majority of eukaryotic promoter-specific TFs, displays a modular nature. In fact, the two basic molecular functions (i.e. specific DNA binding and transcriptional activation) are performed by distinct domains. The DNA-binding function is brought on by the homeodomain (HD) that recognizes, with high affinity, DNA sequences containing the 5’-CAAG-3’ core motif (4). Moreover, TTF-1 exhibits two independent transcriptional activation domains, located at the N-terminal (N domain) and at the C-terminal (C domain) regions with respect to the HD (Fig. 1 and Ref. 5). It has been previously demonstrated that the N domain plays a leading role in the activation of the transcriptional machinery, being able to squelch both its own transcriptional activity and the C domain one (5). Moreover, the N domain directly interacts with the TATA-binding protein TBP (6). Therefore, regulatory mechanisms, acting upon the N domain, could control the activity of the whole molecule.

Thyroid transcription factor 1 (TTF-1) is a homeodomain-containing transcriptional regulator responsible for the activation of thyroid- and lung-specific genes. It has been demonstrated that its DNA binding activity is redox-regulated in vitro through the formation of dimers and oligomeric species. In this paper, we demonstrate that the redox regulation mainly involves a Cys residue (Cys87), which resides out of the DNA binding domain, belonging to the N-transactivation domain. In fact, the oxidized form of a truncated TTF-1 (containing the N-transactivation domain and the DNA-binding domain, here called TTF-1N-HD) looses specific DNA binding activity. Since most of the oxidized TTF-1N-HD is in a monomeric form, these data indicate that the redox state of Cys87 may control the DNA-binding function of the homeodomain, suggesting that Cys87 could play an important role in determining the correct folding of the homeodomain. By using gel retardation and transient transfection assays, we demonstrate that the redox effector factor-1 (Ref-1) mediates the redox effects on TTF-1N-HD binding and that it is able to modulate the TTF-1 transcriptional activity. Glutathione S-transferase pull-down experiments demonstrate the occurrence of interaction between Ref-1 and TTF-1N-HD. Having previously demonstrated that Ref-1 is able to modulate the transcriptional activity of another thyroid-specific transcription factor (Pax-8), our data suggest that Ref-1 plays a central role in the regulation of thyroid cells.
pathways of activation of p53, through redox mechanism (10), together with a direct interaction with p53 itself in vivo (11). As for its apurinic/apyrimidinic endonuclease activity, Ref-1 is better known by the acronym APE, which accounts for the role it plays in repairing of DNA damage. This process is due to reactive oxygen species, such as superoxide anion (O_2^-), H_2O_2, and the hydroxyl radical (\cdotOH), which are by-products of respiration. Ref-1 protein expression is selectively induced by non-toxic levels of a reactive oxygen species variety. This is thought to be due to a translational induction, being inhibited by treating cells with cycloheximide (12). Moreover, we have recently demonstrated that reactive oxygen species are able to induce Ref-1 nuclear translocation in B-cells (13) as well as in thyroid cells (14). It is largely known that, in thyroid cells, the production of reactive oxygen species occurs after thyrotropin (TSH) stimulation and plays a key role during thyroid hormone synthesis (15–18). We have concordantly demonstrated that cytoplasm to nucleus translocation of Ref-1 occurs in thyroid cells upon TSH stimulation. These findings suggest that the Ref-1-mediated mechanism may constitute a major switch by which TSH controls thyroid cells. This view is supported by the observation that Ref-1 controls the DNA-binding function of Pax-8, which is another thyroid-specific TF. Pax-8 recognizes the DNA by means of a conserved DNA-binding domain called the paired domain. A conserved Cys residue at position 37 of the Pax-8 paired domain is responsible for the redox regulation of the DNA binding activity (9). The Cys37 residue is required to be in a reduced state in order to allow the structural transition of the Prd domain required for a proper DNA binding. This feature is accomplished, in vivo, through Ref-1, which is able to induce the Pax-8-driven transactivation potential of the thyroid-specific thyroglobulin promoter, as we have demonstrated by co-transfection assays in HeLa cells (19). In this paper, we show that the redox control of DNA binding activity demonstrated for TTF-1 (7) is exerted by a unique Cys residue (Cys87), which represents the "redox sensor" of the molecule. Differently from Pax-8 and other TF studied up to now, this Cys residue, involved in the redox control, resides outside the DNA binding domain, mapping in the N transcriptional activation domain of TTF-1.
Together with those previously reported by us and other authors (20), our data suggest a master role for Ref-1 in the control of the thyroid cell physiology.

**EXPERIMENTAL PROCEDURES**

**Oligodeoxynucleotide Synthesis and Purification**—Oligodeoxynucleotides were synthesized with an automated Applied Biosystems DNA synthesizer, model 380B, according to standard procedures and purified by fast protein liquid chromatography using a Mono-Q column (Amersham Biosciences) eluted with an ammonium bicarbonate gradient. The purity of oligonucleotides was controlled on a 20% polyacrylamide, 7 M urea gel electrophoresis.

**DNA Constructs**—DNA encoding for recombinant TTF-1 N-domain protein was obtained by PCR using the primer TH1 (5'-GGCGCGATCCATGTCGATGAGTCCAAAGCACACG-3') and primer TH2 (5'-CCGCGGATCCCTTGTCCTTCGCCTGGCGCTTCAT-3') and, as template, the plasmid CMV-TTF1 (5). The PCR product was BamHI-digested and cloned into the bacterial expression vector pQE12 (Qiagen).

Plasmid pTACAT3 contains the wild-type Tg promoter linked to the chloramphenicol acetyltransferase (CAT) gene, and it is described in Ref. 21. Plasmid with the promoter C5E1b as well as plasmids expressing proteins TTF-1 and Δ14 were described elsewhere (5). Plasmids CMV-CAT, RSV-CAT, and Ki-Ras-CAT containing the CMV, RSV, and Ki-Ras promoters linked to the CAT gene (19, 22, 23) together with the plasmid PGL-2 (Promega), containing the promoter and enhancer sequences of SV40 linked to the luciferase gene (LUC), were used in cotransfection studies with Ref-1-expressing plasmid.

The mutant C87S of the Δ14 construct (C87SΔ14) and of the recombinant TTF-1N-HD cloned in pQE12 plasmid were created by the QuickChange site-directed mutagenesis kit (Stratagene), using as template the plasmid CMV-TTF1 and the following oligonucleotides: C87Sa (5'-GCC GGC GGG GGC TAC TCT AAC GGC AAC-3') and C87Sb (5'-GCC CAG GTT GCC GTT AGA GTA GCC CCC CAC GGC-3'). The introduced mutation was verified by

**FIG. 3. Oxidation decreases TTF-1N-HD DNA-binding affinity.** A, EMSA of the reduced (lanes 2–4) and oxidized (lanes 5–7) forms of TTF-1N-HD incubated with 32P-labeled oligonucleotide C14 in the presence of different amounts of calf thymus competitor DNA, as indicated. In each lane, except the first, which represents the oligonucleotide probe alone, DNA binding was obtained by incubating 80 ng of purified TTF-1N-HD with 100 fmol of 32P-labeled oligonucleotide C14. The reduced forms of the recombinant TTF-1N-HD protein were obtained by treatment with 5 mM DTT for 5 min at room temperature, and the oxidized forms were obtained by treatment of the protein with 5 mM diamide for 5 min at room temperature. At the end of the treatments, each sample was incubated with the 32P-labeled oligonucleotide C14 for 20 min at room temperature and loaded onto a native 10% polyacrylamide gel for EMSA analysis. The arrow labeled with B indicates the protein-DNA complex. B, the densitometric scannings relative to the bound complexes with respect to the bound signal obtained at the lowest concentration of competitor DNA. Open bars represent data obtained in reducing conditions, while solid bars represent data obtained in oxidizing conditions. C, the band intensity ratios of the bound complexes obtained in reducing conditions, relative to oxidizing conditions at the different concentrations of competitor DNA.
**Fig. 4.** rRef-1 is a stimulator of TTF-1N-HD DNA binding activity. A, DNA binding by 80 ng of recombinant TTF-1N-HD with the 32P-labeled oligonucleotide C14 (100 fmol) in the absence (lane 2) or presence of 2 μg (lane 5) of rRef-1 or of the reducing agent DTT 5 mM (lane 3) was analyzed by EMSA. Lane 1 represents probe alone. The oxidized forms of the recombinant TTF-1N-HD were obtained by treatment of the protein with diamide 5 mM for 5 min at room temperature (lanes 4 and 5). B, DNA binding by 5 ng of recombinant TTF-1 HD with the 32P-labeled oligonucleotide C14 (100 fmol), in the absence (lane 2) or presence of 2 μg (lane 5) of rRef-1 or of the reducing agent DTT (5 mM) (lane 3) was analyzed by EMSA. Lane 1 represents probe alone. The oxidized forms of the recombinant TTF-1 HD were obtained by treatment of the protein with diamide 5 mM for 5 min at room temperature (lanes 4 and 5). The arrow labeled with B indicates the protein-DNA complex; the arrow labeled with F indicates the free oligonucleotide. Note that this experiment was performed with the highest concentration of calf thymus DNA (50 μg/ml) as competitor. C, cytochrome c (Cyt c), bovine serum albumin, and ovalbumin (OVA) are not able to rescue the loss of TTF-1N-HD DNA binding activity obtained by oxidation with diamide. DNA-binding activity by 80 ng of TTF-1N-HD with the 32P-labeled oligonucleotide C14 (100 fmol) in the absence (lane 3) or presence of rRef-1 (lanes 4 and 5) or the proteins cytochrome c (lane 6), bovine serum albumin (BSA) (lane 7), or ovalbumin (lane 8). The specificity of the retarded complex is tested with the α-His antibody (lanes 9 and 10). Lane 11 contains the rRef-1 alone. The specificity of the supershifted complex is tested by incubating the TTF-1N-HD/C14 sample with equal amounts of preimmune serum (lane 12).
nucleotide sequencing of the entire constructs.

Recombinant Ref-1 His-tagged expressing plasmid pDS56Ref-1 was kindly provided by Dr. T. Curran (St. Jude Children's Research Hospital, Memphis, TN) together with the eukaryotic expression vector CMV-Ref-1.

**Protein Expression and Purification**—The cDNA coding for amino acids 1–230 of rat TTF-1 was cloned into the vector pQE12 (Qiagen) in frame with the coding region of six histidine residues. Thus, the expressed protein (TTF-1N-HD) contains an extra hexahistidine sequence at the C terminus, allowing for protein purification by nickel/nitrilotriacetic acid affinity chromatography. The TTF-1N-HD wild type protein (named TTF-1N-HDWT) and its C87S mutant (named TTF-1N-HDCTS7S) were expressed in M15 Escherichia coli cells. Overnight cultures were inoculated into Luria-Bertani (LB) medium supplemented with 50 μg/ml Ampicillin and grown at 37 °C to an OD of 0.6–0.7, and then they were induced by 1 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h. At the end of the induction phase, bacteria expressing recombinant TTF-1N-HDWT or TTF-1N-HDCTS7S proteins were pelleted and resuspended in 10 ml of lysis buffer A (20 mM Tris, pH 8.0, 250 mM NaCl, 0.1% Tween 20, 1 mM β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 0.8 mM imidazol) for each gram of bacterial pellet and centrifuged at 10,000 × g for 20 min at 10 °C. The supernatants were loaded onto a nickel/nitrilotriacetic acid column, equilibrated with buffer A, and washed with 10 volumes of buffer A. The protein was eluted with buffer B (20 mM Tris, pH 8.0, 500 mM NaCl, 0.1% Tween 20, 1 mM β-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 500 mM imidazol). Sample concentrations were determined either spectrophotometrically (using ε at 23,470 M⁻¹cm⁻³ calculated as described previously (24)) or by Bradford colorimetric assay (25). The purified proteins gave a single band on an overloaded SDS-PAGE. Fractions containing purified proteins were dialyzed against water and then stored at −80 °C.

Recombinant Ref-1 protein (rRef-1) was obtained as a hexahistidine tag fusion protein from overexpression in E. coli and then purified by nickel-chelate chromatography from bacterial extracts and treated as previously described (26). Glutathione S-transferase (GST)-Ref-1 protein was obtained as previously described (27).

**Electrophoresis**—Double-stranded oligodeoxynucleotides, labeled at the 5′-end with 32P, were used as probes in gel retardation assays. The C14 oligonucleotide is a 14-mer whose C14 oligonucleotide is a 14-mer whose 14-base pair sequence is −80 to +60 from the transcription start site of the CAGTCAAGTGTTCT-3′ (28). The gel retardation assay was performed by incubating protein and DNA in a buffer containing 20 mM Tris-HCl, pH 7.6, 75 mM KCl, 0.25 μg/ml bovine serum albumin with or without calf thymus DNA (50 μg/ml) as reported in the figure legends, 10% glycerol for 30 min at room temperature. Protein-bound DNA and free DNA were separated on a native polyacrylamide gel run in 0.5× TBE for 1.5 h at 4 °C. The gel was dried and then exposed to an x-ray film at −80 °C. When required, in vitro protein oxidation was obtained by prolonged air exposure or by diimide treatment.

**Protein-DNA UV Cross-linking**—The nonmonocyclic binding of TTF-1N-HDWT and the C87S mutant on the C14 sequence were determined by UV cross-link analysis, following the procedure of Molnar et al. (29). Briefly, proteins and DNA were incubated as described for EMSAs but without glycerol. After 30 min, aliquots were either loaded onto a native polyacrylamide gel to detect the protein-DNA complexes or subjected to UV cross-linking analysis (300 nm, 50 W) for 10 min. After UV exposure, 4X Laemmli sample buffer with or without β-mercaptoethanol was added, and samples were loaded onto a 12% SDS-PAGE. Following electrophoresis, the gel was dried and exposed to autoradiography. Prestained protein molecular markers were from MBI Fermentas. Detection of the association between TTF-1N-HD and rRef-1 through GST pull-down assay. For pull-down experiments, Ref-1 was expressed as GST fusion protein. For the interaction between the purified TTF-1N-HD and GST-Ref-1, 100 ng of the former and 500 ng of the latter were mixed for 30 min at room temperature, coprecipitated with 30 μl of GST-agarose (Amersham Biosciences), washed three times with PBS in the presence of 0.1% Nonidet P-40, and eluted by 50 μl of 10 mM GSH in PBS. Eluted samples were subjected to 12% SDS-PAGE and immunoblot analysis using α-His (Amersham Biosciences) or α-TTF-1 antibodies or to EMSA analysis with the radiolabeled oligonucleotide C14. The blots were developed using the ECL chemiluminescence method (Amersham Biosciences).

**RESULTS**

**Oxidation Reversibly Inactivates TTF-1N-HD DNA Binding Activity**—It has been previously demonstrated that a reducing environment is required for a proper DNA-binding activity by the TTF-1 whole molecule (7). Two of the four Cys residues of TTF-1 (Cys87 and Cys363) had turned out to be the targets of the redox control, which is mainly exerted, in vitro, through the modulation of the oligomerization state of TTF-1. The authors proposed that a role for this kind of regulation may occur in vivo, hypothesizing the presence of an unknown co-factor for the control of the redox state of TTF-1. However, our previous data demonstrated that Cys87, on its own, is sufficient for controlling the dimerization state of TTF-1, therefore suggesting a master role for this residue in the TTF-1 redox control (6). To test if the reducing environment may affect the DNA binding function of TTF-1 HD, through the modulation of the redox state (3 μM), we tested the activity of the reduced form of TTF-1, TTF-1N-HD (Fig. 1), containing only the N domain and the HD, to specifically recognize the high affinity DNA-binding site (oligonucleotide C14). The specific TTF-1N-HD binding activity to the site C14, assessed by EMSA and in the presence of a specific competitor DNA from calf thymus, was abolished after the addition of the oxidizing agent diamide (1,1-azobis(N,N-dimethylformamide)) (see Fig. 2A, lane 3 versus lane 2). This effect was observed at a concentration as low as 0.1 mM diamide
and was completely reversible by the addition of an excess of the reducing agent dithiothreitol (DTT) (Fig. 2A, lane 4), thus underlying a possible functional role in vivo. As has been previously demonstrated by Arnone et al. (7), the loss of DNA binding activity is not directly attributable to a loss of binding by the HD, because it does not contain Cys residues and it is not sensible to modulation of redox conditions (Fig. 2B). To assess whether the DNA binding inhibition seen for TTF-1N-HD could be related to the dimerization state of the protein, we performed SDS-PAGE analysis of the samples used in the EMSA experiments described above. The monomeric state of the TTF-1N-HD protein was dependent on redox conditions. The protein was totally present as a single monomeric form only under reducing conditions (data not shown). Following a treatment with 5 mM diamide, dimeric species were present. However, it should be noticed that a considerable amount (about 80% of the total protein) of monomeric protein is still available in the oxidizing environment (data not shown). Therefore, dimerization does not account for the complete loss of specific DNA binding activity demonstrated in Fig. 2.

Oxidation of TTF-1N-HD Decreases Its DNA Binding Specificity—To characterize in a better way the reasons why the oxidized form of TTF-1N-HD was unable to recognize the C14 sequence, the specific binding activity of this protein was evaluated in the presence of decreasing amounts of competitor DNA (genomic calf thymus DNA) and in reducing and oxidizing conditions (Fig. 3A). As is evident from lanes 5–7 of Fig. 3A, the oxidized form of TTF-1N-HD was still able to interact with the oligonucleotide C14 sequence. However, the affinity of this interaction was lower if compared with that observed when the protein was present in the reduced form (Fig. 3A, lanes 2–4). From the relative densitometric evaluation of the retarded bands present in A, and then reported in B, the loss of specific DNA binding by the oxidized protein is strikingly evident in the presence of genomic DNA. Absolute quantitation of the retarded bands in oxidizing conditions revealed that the amount of the reduction in DNA binding affinity can be estimated at 3 orders of magnitude (Fig. 3C). These results suggest that the weakening of the TTF-1N-HD/oligonucleotide C14 interaction by oxidation would be due to (i) absolute loss of binding activity or (ii) inability to discriminate between different DNA sequences with following subtraction of protein involved in nonspecific bonds.

Ref-1 Mediates the Redox Regulation Acting on the TTF-1N-HD—Ref-1 is the major cofactor involved in redox regulation of several TFs, and it also plays a primary role in thyroid cells (14). To test whether Ref-1 is also able to control the DNA binding activity of the isolated TTF-1N-HD, we performed an EMSA analysis with the oxidate form of the TTF-1N-HD protein and the Ref-1 protein. As we have previously demonstrated, the oxidized form of the TTF-1N-HD was unable to show any kind of specific DNA-biding activity (Fig. 4A, lane 4). However, the presence of Ref-1 was sufficient per se to reconstitute the complete binding activity (lane 5) as in the presence of DTT (lane 3). The role of Ref-1 is not directed to the HD, since, as demonstrated in Fig. 4B, the addition of Ref-1 to the sample does not affect the HD DNA binding affinity (lane 5). To test the specificity of the redox control played by Ref-1 over TTF-1N-HDWT, we performed EMSA analysis of the oxidized form of TTF-1N-HDWT in the presence of three unrelated proteins: cytochrome c (Fig. 4C, lane 6), bovine serum albumin (lane 7), and ovalbumin (lane 8). As is evident from Fig. 4C, the three proteins are not able to rescue the DNA binding activity of the oxidized TTF-1N-HDWT with respect to Ref-1 (lanes 4 and 5).

In Vitro Association between TTF-1N-HD and Ref-1—Ref-1 has been shown to be the redox regulator of different TFs (8–10, 32). However, there has been direct evidence that documents a physical interaction between the two proteins (33) only in the case of p53. To test this possibility in the case of TTF-1, we used a GST pull-down approach. After expression and purification of Ref-1 as GST fusion protein (27), we applied GST pull-down for testing the interaction with TTF-1N-HD (see “Experimental Procedures” for details). As is evident from Fig. 5, TTF-1N-HD specifically interacts with GST-Ref-1 but not with GST alone, demonstrating the occurrence of the interaction between the two proteins.

Ref-1 Increases the TTF-1N Domain Transcriptional Activity in Vivo—To test if the stimulatory effect of Ref-1 on the TTF-1 N-domain activity could have relevance in vivo (Fig. 6), a cell transfection approach was used. The thyroglobulin promoter (Tg) is not functional when transfected in HeLa cells, and its activity can be reconstituted by the forced expression of the construct Δ14, which encodes for the partial TTF-1 protein containing the transactivating N-domain and the DNA binding domain (HD) (5). We wondered if the co-transfection of a Ref-1 expression vector was able to modify the Δ14 effect on this promoter. Results are shown in Fig. 6A. As expected, the Tg promoter is inactive in HeLa cells, and the expression of Δ14 is able to activate it. The co-transfection of a Ref-1 expression vector increases the Δ14-induced activation of Tg construct by 2-fold (Fig. 6A). The activity of Ref-1 is specific, since the activities of the CMV, RSV, Ki-Ras, and SV40 promoters are not modified by the co-transfection of the Ref-1 expression vector (Fig. 6C). Similar data have been obtained in the case of the TTF-1 whole molecule (Fig. 6A), suggesting that the redox control exerted by Cys87 in the transactivation domain plays a pivotal role over the other Cys residues of the molecule and that, in terms of redox regulation, the transcriptional activity of Δ14 recapitulates that of the whole molecule. The redox control is specific for the promoters activated by TTF-1. In fact, similar effects are observable by using an artificial promoter (called C5) obtained through polymerization of five C sites (Fig. 6B) recognized by TTF-1. To exclude the possibility that the stimulatory effect of Ref-1 over TTF-1 could be due to an altered expression of TTF-1 itself, Western blot analysis of transfected cells was always performed in order to measure the TTF-1 protein levels before and after Ref-1 expression. Data not shown clearly demonstrate the absolute independence of the TTF-1 protein levels from those of Ref-1.

The redox regulation mediated by Cys87 in the activation domain seems to be due to the control of the binding activity of TTF-1. However, at the moment we cannot exclude a stimulatory effect of Ref-1 directly on the transcriptional activation domain of TTF-1, involved in possible interactions with proteins of the basal transcriptional machinery.

Cys87 Is Responsible for the Ref-1-mediated Redox Control over TTF-1 Transcriptional Activity—To test if the Cys87-mediated oxidation specifically controls the transcriptional activity of TTF-1, a Δ14 mutant was constructed, in which a Ser residue replaces the Cys at position 87. The transcriptional activity of the C87SΔ14 mutant is reported in Fig. 7. A couple of transcriptional features of this mutant are evident: (i) higher constitutive basal transcriptional activity with respect to the wild type protein and (ii) complete redox insensitivity toward Ref-1 action. These results greatly support the importance of Cys87 for the redox regulation of TTF-1 activity, suggesting that it is necessary and sufficient for the redox control. The increase in the basal level of transcription obtained in the case of the mutant, with respect to the wild type protein, would suggest that at least two forms of TTF-1 are present in a cell: a reduced one, which is active in terms of specific DNA binding
and an oxidized form bearing lower specific activity.

Cys\textsuperscript{87} Controls TTF-1 DNA Binding Activity—To test the effect, at the molecular level, of Cys\textsuperscript{87} to Ser mutation and therefore to determine whether this residue is involved in controlling the DNA binding activity of TTF-1N-HD through redox modulation, the C87S mutant (here called TTF-1N-HDC87S) was expressed, as His\textsubscript{6} fusion protein, in bacteria and purified to homogeneity by using the nickel/nitrilotriacetic acid-agarose resin, as described under "Experimental Procedures." Then its DNA binding activity was tested by EMSA analysis, and results are reported in Fig. 8A. In striking contrast to the wild-type protein, oxidative conditions by the addition of diamide do not affect at all the DNA binding activity of the C87S mutant (Fig. 8A, lane 8). Moreover, the addition of the recombinant Ref-1 protein has no effect on the affinity of the C87S-C14 complex (Fig. 8A, lane 9). Therefore, we can conclude that the substitution of the Cys\textsuperscript{87} with a Ser residue confers redox insensitivity to the TTF-1N-HD protein. It is interesting to note that the C87S-C14 complex displays a significantly higher mobility with respect to wild type-C14 complex. Moreover, 4-fold more wild-type TTF-1N-HD must be used, in the EMSA analysis, to have the same protein-DNA complex signal as that obtained with the C87S mutant. This evidence further suggests an important role for residue Cys\textsuperscript{87} in controlling DNA binding affinity. The EMSA mobility patterns, although suggestive, do not allow us to exclude the possibility either that the wild type-C14 interaction occurs with the protein in a dimeric form or that the C87S form is a truncated form of TTF-1N-HD. To exclude this last possibility, Western blot analysis was performed on samples of TTF-1N-HDWT and TTF-1N-HDC87S. SDS-PAGE analysis of the purified proteins clearly demonstrates the presence of a single species of TTF-1N-HDC87S showing exactly the same electrophoretic mobility of the wild type form (Fig. 8B). Moreover, electrospray mass spectrometric analysis on the sample of TTF-1N-HDC87S confirmed this evidence (data not shown). To demonstrate the monomeric state of the wild type-C14 and C87S-C14 complexes, a protein-DNA cross-linking procedure was utilized. TTF-1N-HDWT and TTF-1N-HDC87S were incubated with the C14 sequence to allow for the interaction. The

Fig. 6. Effect of Ref-1 on the activity of Tg, C5, RSV, Ki-Ras, SV40, and CMV promoters. Plasmids were transfected in HeLa cells at the indicated amounts (see "Experimental Procedures" for details). 48 h after transfections, cells were harvested, and CAT, LUC, and \(\beta\)-galactosidase activities were measured. A, effect of Ref-1 on Δ14- and entire TTF-1-induced activity of Tg promoter; B, effect of Ref-1 on Δ14- and entire TTF-1-induced activity of C5 promoter; C, effect of Ref-1 on the CMV, RSV, Ki-Ras, and SV40 promoters. Open bars represent data of transfections without the Ref-1 construct, whereas solid bars represent data of transfections with the Ref-1 construct. In all panels, bars indicate the mean value ± S.D. of at least five independent experiments.
mixture was subjected to UV cross-linking (29), and samples were run onto a 12% SDS-polyacrylamide gel (Fig. 8C). The free DNA migrates as a band of about 9 kDa. As expected, due to the monomeric nature of the TTF-1N-HDWT/DNA interaction, the TTF-1N-HD-DNA complex migrates between 30 and 40 kDa (Fig. 8, lane 2). This result is in agreement with the sum of the molecular masses of the TTF-1N-HDWT protein, 25 kDa, in addition to a single molecule of the oligonucleotide C14 (about 9 kDa). These results allow us to conclude that the TTF-1N-HDWT interacts with the C14 sequence in a monomeric form. Similarly, the interaction of the C87S mutant is stoichiometric, since the molecular mass of the complex with the C14 oligonucleotide is exactly the same as that obtained with the WT (Fig. 8, lane 4).

To exclude the possibility that the retarded band obtained by EMSA analysis of the WT form could be due to dimerization of the protein after DNA binding, the UV cross-linking experiment was analyzed in oxidizing conditions: after treatment of the UV-cross-linked samples with diamide and following running in SDS-PAGE in oxidizing conditions without adding β-mercaptoethanol to the Laemmli sample buffer. As is evident from Fig. 8, lanes 5–8, the protein-DNA complexes show exactly the same molecular masses of that obtained in reducing conditions (lanes 1–4). Therefore, we can conclude that the altered mobility obtained by EMSA analysis of the C87S mutant with respect to the WT protein is due to a different mode of interaction with the DNA, suggesting a major role for residue Cys87 in controlling DNA binding.

**DISCUSSION**

Redox regulation of cellular functions is mainly due to ultimate effects in gene expression. In the last few years, a great body of experimental evidences suggested that these outcomes are achieved through modulations of TF activity. Up to now, several TFs, containing specific Cys residues, have been demonstrated to be the target of redox regulation. However, in every case, this regulation directly occurs on the DNA binding domain of these proteins. Only in a recent paper, Morel and Barouki (34) suggested that also the transactivation domain could be a target of the redox regulation. Oxidative stress elicits inhibitory functions toward the NFI/CTF transcriptional ability in the HepG2 hepatoma cell line, and it is mediated by the redox state of a Cys residue (Cys-427) present in the trans-
FIG. 8. The Cys87 residue is responsible for the redox regulation of the DNA binding activity of TTF-1N-HD. A, EMSA analysis of the TTF-1N-HDC87S DNA-binding activity. 20 ng of the TTF-1N-HDC87S purified protein were assayed for DNA binding activity with the 32P-labeled oligonucleotide C14 (100 fmol) after treatment with 5 mM diamide (lanes 8 and 9) and with (lane 9) or without (lane 8) the addition of rRef-1. The specificity of the complex is tested by incubation with the α-His antibody (lane 10) or a preimmune serum (lane 11). Lanes 2–6 represent the same kind of experiment, and it was performed with 80 ng of the TTF-1N-HD wild type form. In each case, at the end of the treatments, oxidized and reduced forms of the purified proteins were incubated with 100 fmol of 32P-labeled oligonucleotide C14 for 20 min at room temperature in the presence of competitor DNA (50 μg/ml) and loaded onto a native polyacrylamide gel for EMSA analysis. Lane 1 represents the oligonucleotide probe alone. B, SDS-PAGE analysis of the purified TTF-1N-HD WT and TTF-1N-HDC87S recombinant proteins. The two recombinant proteins, used in the present study, were expressed in bacteria and purified by nickel/nitrilotriacetic acid affinity chromatography, as described under "Experimental Procedures." After purification, proteins were analyzed by 12% SDS-PAGE and subsequently stained with Coomassie Blue or silver staining (not shown). In each lane, 3 μg of purified proteins were loaded. C, UV cross-linking analysis of TTF-1N-HD WT and TTF-1N-HDC87S recombinant proteins with the 32P-labeled oligonucleotide C14. Protein-DNA cross-linking analysis was performed as described by Molnar et al. (29) (see "Experimental Procedures" for details). Analyses were made in reducing and oxidizing conditions. 80 ng of the purified TTF-1N-HD wild type form or 20 ng of the purified TTF-1N-HDC87S protein were incubated with 100 fmol of 32P-labeled oligonucleotide C14 for 20 min at room temperature, and UV cross-linking was performed. After UV cross-linking, samples were added with 4× Laemmli sample buffer with (lanes 1–4) or without β-mercaptoethanol (lanes 5–8). To force the oxidizing conditions, 5 mM diamide was added before incubating in Laemmli sample buffer (lanes 5–8).
activation domain of the TF. Unfortunately, the authors did not provide data regarding the cellular factors involved in that kind of regulation. Our data add new important insights into the redox control of gene expression. In fact, we demonstrate that Ref-1 also plays a role in the control of the TTF-1 DNA binding activity, through the control of the redox state of a Cys residue located outside the DNA-binding domain in the transcriptional activation domain (TAD). We must remember that Ref-1 has already been demonstrated to control, in a redox-dependent manner, the DNA binding activity of different important TFs (such as p53, c-Myb, AP-1, Pax proteins, NF-κB, and members of the ATF/cAMP-response element-binding protein family) by modulating the redox state of the DNA-binding domain. These interesting outcomes suggest that the redox control of TTF-1 DNA-binding activity, played at the level of Cys\textsuperscript{87}, is exerted in an indirect manner. The lowering of DNA binding activity is obtained through an absolute loss of binding activity together with a reduction in the ability to specifically recognize its target among different DNA sequences, with the following subtraction of protein involved in nonspecific binding. At present, we do not know if the redox regulation exerts through the control of the dimeric/oligomeric state of TTF-1 as it had previously suggested (6, 7). Data not shown, demonstrating the presence of a considerable amount of monomeric form of TTF-1N-HD in oxidizing conditions, would suggest that the thiol moiety of Cys\textsuperscript{87} undergoes an oxidation, with a gain of an oxygen atom, and it possibly generates a sulfonic group. It is tempting to speculate that the oxidation of Cys\textsuperscript{87} could affect the formation of the TAD of TTF-1 so as to alter the DNA binding activity of the HD. Up to now, there is no evidence regarding a possible structural role of the TAD over the TTF-1 HD. However, we cannot exclude a possible TAD involvement in controlling some structural requirements of the HD itself, which are necessary for high specific DNA binding activity. Otherwise, the N domain could be able to transiently contact DNA in a manner to correctly position the HD domain for a productive recognition of the C site. This hypothesis could explain the different footprints on the C site of the Tg promoter, obtained when the whole TTF-1 molecule is assayed with respect to the HD alone (35). In this regard, the altered mobility obtained in the case of the C87S mutant and the oligonucleotide C14 would be suggestive of a role of the Cys\textsuperscript{87} residue in controlling the DNA-binding properties of the HD.

An additional level of control, played over the transcriptional activity of TTF-1, by the redox status of Cys\textsuperscript{87} could be due to the intrinsic properties of the N transactivation domain as a classical transcriptional activation domain. The oxidation of Cys\textsuperscript{87} could affect the conformation of the N domain, preventing the productive protein-protein interactions with the transcriptional apparatus, which are required for the transcriptional activation. In this regard, it should be noted that the TTF-1 N domain exerts its activities by binding to the TATA-box-binding protein TBP (6). Therefore, together with an effect on the DNA-binding specificity, the redox status of the Cys\textsuperscript{87} could also play an important role in the control of the transcriptional activity of TTF-1 by modulating the recruitment of TBP.

As has been previously demonstrated for Pax-8 and now for TTF-1, the redox control in the thyroid cell model seems to be of primary importance in regulating cell type-specific gene expression. In fact, it is largely known that TSH exerts its functions in the thyroid cell through many different mechanisms: from activation through cAMP signaling cascades to production of large amounts of H\textsubscript{2}O\textsubscript{2} in response to TSH for the synthesis of thyroid hormone (37, 38). We and other researchers have recently demonstrated that the cellular redox status controls the levels and subcellular localization of Ref-1 (13, 20). In particular, in FRTL-5 cells, TSH is able to control Ref-1 by two major mechanisms involving neosynthesis (late times) and cytoplasm to nucleus translocation of the protein (early times) (14). Therefore, we suggest that Ref-1 plays a central role as a regulator of thyroid-specific gene expression in response to physiological stimuli. Interestingly, the same stimuli elicited by TSH (i.e. H\textsubscript{2}O\textsubscript{2} and cAMP production) are able to strongly control the expression level and the subcellular localization of Ref-1 (14, 39).

In conclusion, our data support two major ideas. First, a Ref-1-mediated mechanism may constitute a major switch to the control of thyroid cells. In fact, Ref-1 is controlled by TSH, and, in turn, it modulates both Pax-8 and TTF-1 functions. Second, the DNA-binding function of the TTF-1 HD is controlled by redox regulation occurring outside of it. Therefore, it is possible to argue that the N transcriptional activation domain acts not only as a classical transactivator, involved in contacting proteins of the transcriptional machinery apparatus but could possibly modulate a structural transition of the HD required for an optimal DNA-binding specificity by TTF-1. Further studies are required to investigate such a hypothesis by more sophisticated structural techniques such as NMR or x-ray crystallography.

Acknowledgments—We thank R. Acquaviva for help with TTF-1N-HD purification, S. Formisano for a gift of anti-TTF-1 polyclonal antibody, Tom Curran for the CMV-Ref-1 and His-tagged-Ref-1 plasmids, and Cristiana Campa for the mass analysis of the recombinant proteins used in this study. The help of E. Tell during manuscript preparation is greatly appreciated. This work was performed within the Center of Excellence of Biocrytallography at the University of Trieste.

REFERENCES
1. Damante, G., Tell, G., and Di Lauro, R. (2000) Prog. Nucleic Acids Res. 66, 307–356
2. Francis-Lang, H., Price, M., Polycarpou-Schwarz, M., and Di Lauro, R. (1992) Mol. Cell. Biol. 12, 576–588
3. Bruno, M. D., Bohinski, R. J., Huelsman, K. M., Whitsett, J. A., and Korfhagen, T. R. (1995) J. Biol. Chem. 270, 6531–6536
4. Damante, G., Fabbro, D., Pellizzari, L., Civitareale, D., Guarzi, S., Polycarpou-Schwarz, M., Canu, S., Madri, G., Formisano, S., and Di Lauro, R. (1994) Nucleic Acids Res. 22, 3075–3083
5. De Felice, M., Damante, G., Zannini, M., Francis-Lang, H., and Di Lauro, R. (1995) J. Biol. Chem. 270, 26649–26654
6. Tell, G., Perrone, L., Fabbro, D., Pellizzari, L., Pucillo, C., De Felice, M., Acquaviva, R., Formisano, S., and Damante, G. (1998) Biochem. J. 329, 395–403
7. Arnone, M. I., Zannini, M., and Di Lauro, R. (1995) J. Biol. Chem. 270, 12048–12055
8. Xanthoudakis, S., Miao, G., Wang, F., Pan, Y. C., and Curran, T. (1992) EMBO J. 11, 3323–3335
9. Tell, G., Scaloni, A., Pellizzari, L., Formisano, S., Pucillo, C., and Damante, G. (1998) J. Biol. Chem. 273, 25062–25072
10. Jayaraman, L., Murthy, K. G. K., Zhou, C., Curran, T., Xanthoudakis, S., and Pribbs, C. (1997) Genes Dev. 11, 558–570
11. Meira, L. B., Cheo, D. L., Hammer, R. E., Burns, D. K., Reis, A., and Friedberg, E. C. (1997) Nat. Genet. 17, 145
12. Ramana, C. V., Boldogh, I., Irimi, T., and Mitra, S. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 5061–5066
13. Tell, G., Zecca, A., Pellizzari, L., Spessotto, P., Colombo, A., Kelley, M. R., Damante, G., and Pucillo, C. (2000) Nucleic Acids Res. 28, 1109–1115
14. Tell, G., Pellizzari, L., Pucillo, C., Pagliusi, F., Cesselli, D., Kelley, M. R., Di Loreto, C., and Damante, G. (2000) J. Mol. Endocrinol. 24, 383–390
15. Kimura, T., Okajima, F., Shio, K., Kobayashi, I., and Kondo, Y. (1995) Endocrinology 136, 116–123
16. Bjorkman, U., and Ekholm, R. (1992) Endocrinology 130, 393–399
17. Kimura, T., Okajima, F., Kikuchi, T., Kuwabara, A., Tomura, H., stop, K., Kobayashi, I., and Kondo, Y. (1997) Am. J. Physiol. 273, E639–E643
18. Lesney, A. M., Deme, D., Legue, O., Ohayon, R., Chanson, P., Sales, J. P., Pires de Carvalho, D., Dupay, C., and Virion, A. (1999) Biochimie (Paris) 81, 373–380
19. Tell, G., Pellizzari, L., Cimarrosti, D., Pucillo, C., and Damante, G. (1998) Biochem. Biophys. Res. Commun. 252, 178–183
20. Kambe, F., Nomura, Y., Okamoto, T., and Seo, H. (1996) Mol. Endocrinol. 10, 801–812
Ref-1 Controls TTF-1 Transcriptional Activity

21. Sinclair, A. J., Lonigro, R., Civitareale, D., and Di Lauro, R. (1990) Eur. J. Biochem. 193, 311–318
22. Sassone-Corsi, P., and Verma, I. M. (1987) Nature 326, 507–510
23. Hoffman, E. K., Trusko, S. P., Freeman, N., and George, D. L. (1987) Mol. Cell. Biol. 7, 2592–2596
24. Gill, S. C., and von Hippel, P. H. (1989) Anal. Biochem. 182, 319–326
25. Bradford, M. M (1976) Anal. Biochem. 72, 248–254
26. Abate, C., Patel, L., Rauscher, F. J., and Curran, T. (1990) Science 249, 1157–1161
27. Huq, I., Wilson, T. M., Kelley, M. R., and Deutsch, W. A. (1995) Mutat. Res. 337, 191–199
28. Civitareale, D., Lonigro, R., Sinclair, J., and Di Lauro, R. (1989) EMBO J. 9, 2537–2542
29. Molnar, G., O'Leary, N., Pardee, A. B., and Bradley, D. W. (1995) Nucleic Acids Res. 23, 3318–3326
30. Graham, F. L., and van der Eb, A. J. (1973) Virology 52, 456–467
31. Francis-Lang, H., Zannini, M., De Felice, M., Berlingieri, M. T., Fusco, A., and Di Lauro, R. (1992) Mol. Cell. Biol. 12, 5793–5800
32. Walker, L. J., Robson, C. N., Black, E., Gillespie, D., and Hickson, I. D. (1993) Mol. Cell. Biol. 13, 5370–5376
33. Gaidon, C., Moorthy, N. C., and Prives, C. (1999) EMBO J. 18, 5609–5621
34. Morel, Y., and Barouki, R. (2000) Biochem. J. 348, 235–240
35. Guazzi, S., Price, M., De Felice, M., Damante, G., Mattei, G. I., and Di Lauro, R. (1999) EMBO J. 18, 3631–3639
36. Marshall, H. E., Merchant, K., and Stamler, J. (2000) FASEB J. 14, 1889–1900
37. Carvalho, D. P., Dupuy, C., Gorin, Y., Legue, O., Pommier, J., Haye, B., and Virion, A. (1996) Endocrinology 137, 1007–1012
38. Corvailain, B., van Sande, J., Laurent, E., and Dumont, J. E. (1991) Endocrinology 128, 779–785
39. Asai, T., Kambe, F., Kikumori, T., and Seo, H. (1997) Biochem. Biophys. Res. Commun. 236, 71–74