Gamma-interferon activates a nuclear protein that binds to the gamma-interferon activation site of the thyroglobulin gene

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ABSTRACT
The release of interferon-gamma (IFNγ) has been demonstrated from the infiltrating T lymphocytes of the thyroid gland from patients with autoimmune thyroid disease (AITD). We have shown previously that IFNγ inhibited thyroglobulin (Tg) gene transcription, and that its action was mediated by an increase in intracellular calcium and inositol phosphates. In the present study, we tried to determine the specific site of action of IFNγ on the Tg gene. A 565 bp fragment (position −530 to +34) spanning the transcriptional start site of the human Tg promoter was ligated to the luciferase plasmid and transiently transfected into human thyrocytes. Stimulation with TSH (10 mIU/l) and IFNγ (500 IU/l) resulted in a twofold increase and a 60% reduction in the luciferase activity respectively, similar to the effect observed with the endogenous Tg gene. Deletion studies revealed that the region with the strongest suppression by IFNγ lay between 5′ −388 to −258. Mobility gel shift experiments and DNA footprinting experiments demonstrated that the action of IFNγ was mediated through a trans-acting protein which complexed to position −282 to −262 TTGAGCCTGTTCCCTCAAA. Position −272 to −261 TTCCCTCAAA corresponded to the gamma-interferon activation site (GAS) consensus sequence TTNCC T NNNAA. The turnover time of the nuclear protein lasted for only 4 h although the suppressive effect of IFNγ on Tg gene transcription lasted for 48 h. The effect of IFNγ was lost when the thyrocytes were co-treated with genistein, a specific tyrosine kinase inhibitor.

The presence of the GAS in the promoter sequence of the Tg gene confirms the specific action of IFNγ in thyroid hormone metabolism. In conclusion, apart from its regulatory role in T cell development and perpetuation of the immune response in AITD, IFNγ may also play a role in altering cellular function of the thyrocytes by its action on the Tg gene promoter.

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INTRODUCTION
Autoimmune thyroid disease (AITD) is a common condition affecting between 2% and 4% of the population. The condition is characterized by lymphocytic infiltration of the thyroid gland together with the production of auto-antibodies against thyroid specific antigens, including thyroglobulin, thyroid peroxidase (TPO) and the thyrotropin (TSH) receptor. The intrathyroidal T lymphocytes are very important in the pathogenesis of AITD through thyroid antigen recognition as an essential step to B cell stimulation and for mediating the release of cytokines (Volpe 1993, Weetman & McGregor 1994). The clinical presentation of AITD depends on the spectrum of the cytokines and antibodies produced by the activated lymphoid cells, as these cytokines and auto-antibodies are capable of altering the function and metabolism of the thyroid gland. Among the various cytokines, the action of gamma-interferon (IFNγ) has been the best studied. Functional IFNγ receptors have been identified on the thyrocytes, and thus IFNγ secreted from the infiltrating lymphocytes can exert a direct effect on the thyroid epithelial cells (Nishikawa et al. 1993). The macrophages and lymphocytes infiltrating the thyroid gland from patients with AITD have been shown to release IFNγ into the adjacent thyrocytes (Watson et al. 1994). Enhanced production of IFNγ by thyroid-derived T cell clones from patients with Hashimoto’s thyroiditis has been demonstrated (Del-Prete et al. 1987).
Indeed, in thyroid sections from patients with either Hashimoto’s thyroiditis or Graves’ disease, class II MHC-positive thyrocytes were found immediately adjacent to aggregates of IFNγ-positive lymphocytes (Hamilton et al. 1991), suggesting that class II expression by the thyrocytes was secondary to the release of IFNγ by the infiltrating T cells. Furthermore, IFNγ inhibited proliferation of thyrocytes and altered the expression of the various thyroid specific proteins (Nagayama et al. 1987, Ashizama et al. 1989, Kung & Lau 1990, Kung et al. 1992, Watson et al. 1994). All these findings suggest that, apart from its role in the perpetuation of an autoimmune response in AITD, IFNγ may also have a direct effect on the cellular function of and the hormone production by the thyrocytes.

We have previously demonstrated that IFNγ inhibited thyroglobulin (Tg) gene transcription in human thyrocytes, and that the intracellular signaling pathway of its action was via mobilization of intracellular calcium and increased production of inositol phosphates (Kung et al. 1995). As Tg is the pro-thyroid hormone, inhibition of its production would result in hypothyroidism in AITD. The aim of the present study was to identify the specific site of action of IFNγ on the Tg gene. Understanding the role and action of IFNγ on the Tg gene may enable us to identify specific targets for intervention in AITD.

### MATERIALS AND METHODS

#### Cells

Normal para-adenomatous human thyroid tissues obtained from subjects undergoing hemithyroidectomy were enzymatically digested and cultured in R.p.m.I-1640 medium supplemented with 10% fetal calf serum (Hinds et al. 1981). These cells had been confirmed previously to respond to TSH stimulation by the production of cAMP and release of tri-iodothyronine and Tg. For the stimulation experiment, the cells were resuspended in 1% bovine serum albumin at a concentration of 10⁶/ml. Recombinant human IFNγ (rhIFNγ, 500 IU/ml; Genzyme Diagnostics, Cambridge, MA, USA) was added to the culture for between 2 and 48 h. Bovine TSH (bTSH), forskolin, genistein and other chemicals were obtained from Sigma Chemicals Co. (St Louis, MO, USA).

#### Transient transfection study

A 565 bp fragment (position −530 to +34) spanning the transcriptional start site of the human

| Table 1. Results of transient transfection with p Tg. luc in human thyrocytes |
|--------------------------------------|-------------------|
|                                         | Relative light units |
| p Tg. luc                              | 4359              |
| p Tg. luc+bTSH (10 mU/l)               | 17 330            |
| p Tg. luc+IFNγ (500 IU/l)              | 2695              |
| p Tg. luc+bTSH+IFNγ                   | 2650              |
| p Tg. luc+forskolin (10⁻³ M)           | 8108              |
| Positive pGL-control luciferase plasmid| 13 347            |
| No transfection                       | 191               |

Tg gene was subcloned into the pGL2-enhancer luciferase plasmid (Promega, Madison, WI, USA) between the KpnI and XhoI cloning sites. The sequence contains the cAMP-responsive region which is located within the first 250 bp of the promoter, as well as the TATA box (Christophe et al. 1985, 1989). For the transient transfection experiment, 2·5 µg recombinant p Tg. luciferase (p Tg. luc) plasmid was introduced into the human thyroid cell culture by lipofectum-mediated transfection (Transfectam Assay, Promega). To monitor for transfection efficacy, the cells were co-transfected with 0·5 µg β-galactosidase plasmid. Transfected cells were cultured in the presence or absence of rhIFNγ for 48 h. Cell extracts were prepared and the luciferase and β-galactosidase activities were determined. In each experiment, the results were adjusted for the β-galactosidase activity.

To determine the minimal sequence in the Tg promoter responsible for the action of IFNγ, exonuclease III was used to generate a set of 5’ deletions from the Tg promoter fragment. The deleted DNA was subcloned into the luciferase plasmid for transient transfection experiments. For confirmation, all transfection studies were repeated three times.

#### Cell extracts

Nuclear extracts were prepared according to the method described by Dignam et al. (1983). Cells (10⁹) were harvested from the culture and collected by centrifugation. The cell pellet was suspended in 10 mM Tris–HCl, pH 7·9, 1·5 mM MgCl₂, 10 mM KCl, 0·5 mM dithiothreitol at 4°C for 10 min and again collected by centrifugation and broken down by Dounce homogenization in a small volume of the same buffer. The cell lysate was centrifuged at 25 000 g for 20 min to remove residual cytoplasmic material. The crude nuclei were further broken down by Dounce homogenization in the presence of 25% glycerol. Particulate matter was removed by
centrifugation. The extract was collected for protein content estimation and stored at −80 °C.

Gel retardation assays
DNA fragments of Tg promoter were incubated with nuclear extracts (10 µg) at 37°C for 10 min (Gel Shift Assay Systems, Promega). After 30 min incubation with γ-32P-labeled DNA fragment (5000-10 000 c.p.m.), free and bound DNA were separated by electrophoresis on 6% polyacrylamide gels. For competition experiments, the AP2 consensus oligonucleotide (1·75 pmol) was added as the non-specific competitor.

DNA footprinting assay
The Tg promoter fragment was labeled with T4 polynucleotide kinase and 20 µg nuclear protein extract was incubated with the labeled Tg promoter fragment at 37°C for 3 h. The top strand probe was prepared by removing the label from the 3′ end of the fragment by digestion with XhoI. DNA footprinting was carried out with the Core Footprinting System (Promega). Sequencing was performed using T7 DNA polymerase (Sequenase Version 2·0 DNA Sequencing Kit, United States Biochemical, Amersham, IL, USA) and the DNA was loaded onto a 6% polyacrylamide sequencing gel.

RESULTS
Transfection study
In transient transfection experiments bTSH (10 mIU/l) increased luciferase activity by 2- to 4-fold and IFNγ decreased luciferase activity by 60%. These responses to bTSH and IFNγ were similar to those observed with the endogenous Tg gene (Kung & Lau 1990), suggesting that the minimal promoter element contained the DNA signals necessary for TSH and IFNγ regulation. Forskolin (10−5 M) increased luciferase activity twofold. Table 1 shows the typical result from one set of experiments.

Systematic deletion experiments were performed to determine the minimal DNA sequence necessary for the action of IFNγ. The suppressive effect of IFNγ was lost when cells were transfected with mutant −258 to +34, suggesting that the minimal region required for the IFNγ response lay between position −388 and −258 (Table 2). Transfection with mutant −530 to −171 did not show any luciferase activity, as the regulatory elements of the Tg promoter lay within the first 250 bp of the 5' flanking region.

Gel shift assay
Gel shift assays demonstrated a mobility shift with Tg promoter sequence −316 to −199 (Fig. 1). Specific competition and mobility shift resulting in a retarded band was observed with nuclear extracts prepared from thyrocytes that had been incubated with IFNγ for 4 h (lane d). Nuclear extracts prepared from control thyrocytes did not demonstrate mobility shifting (lane b). Incubation with IFNγ for 2 h revealed a retarded band of weaker intensity (lane c), whereas incubation for 8 h (lane e) or longer (data not shown) did not demonstrate any mobility shift. In the presence of the non-specific competitor AP2 consensus oligonucleotide, the specific retarded band persisted (lane f).

DNA footprinting experiment
This showed that the action of IFNγ was mediated via a trans-acting protein which complexed to the
DNA sequence –282 to –262 TTGAGCCTGT TCCCTCCAAA. Position –272 to –261 TTCCCTCCAA is homologous to the gamma-interferon activation site (GAS) consensus sequence TTNC TNNNAA (Decker et al. 1991). Binding was visualized from nuclear extracts of cells incubated with IFNγ for 2–4 h (Fig. 2). Nuclear extracts from cells incubated with IFNγ for a longer period did not demonstrate binding to the DNA, suggesting that the nuclear protein was turned on for only 4 h or less. Treatment with genistein, a specific tyrosine protein kinase inhibitor, resulted in loss of action of IFNγ (Fig. 3).

**DISCUSSION**

The results of this study demonstrated that the Tg gene contained the specific GAS in its promoter region and that the action of IFNγ was mediated through a trans-acting protein. Among the few thyroid-specific proteins, the Tg gene is the best
characterized (Musti et al. 1986). The molecular mechanisms involved in the tissue-specific and hormone-dependent expression of the Tg gene have been studied both in primary cells and in transgenic animals (Civitareale et al. 1989, Ledent et al. 1990). Using nuclear run-on experiments and measurement of steady state mRNA levels, we had previously demonstrated that IFNγ exerted an inhibitory action on Tg gene transcription (Kung & Lau 1990). However, Nishikawa et al. (1993) suggested that this effect of IFNγ on the Tg gene was probably secondary to the negative effect of IFNγ on TSH receptor expression. The results obtained from the present study confirmed that IFNγ had a direct specific effect on the Tg gene which could be inhibited by genistein, a specific tyrosine protein kinase inhibitor. We further showed that the action of IFNγ on the human Tg gene was mediated by a trans-acting protein, similar to the action of IFNγ on the lymphoid cells. Whether this trans-acting protein is the same gamma-interferon activation factor (GAF) as described for the lymphoid cells remains to be confirmed (Decker et al. 1991). In the lymphoid cell lines, partial purification of GAF revealed a prominent 91 kDa protein, and this GAF activity was inhibited by antibodies to STAT 1α (Khan et al. 1993). The STAT (signal transducers and activators of transcription) proteins are activated through phosphorylation of tyrosine residues of the Jak-1 and -2 kinases (Igarashi et al. 1993, Muller et al. 1993). These proteins are then translocated to the nucleus where, by themselves or in combination with other DNA binding proteins, they bind to the GAS and stimulate transcription (Darnell et al. 1994). We had previously also demonstrated that the effect of IFNγ on the mobilization of intracellular calcium and the production of inositol phosphates in human thyrocytes were inhibited by genistein (Kung et al. 1995), suggesting that the signal transduction mechanism for IFNγ was the same for both lymphoid and non-lymphoid cells. However, whereas IFNγ produced a stimulatory action on the responsive genes in the lymphoid cells, IFNγ exerted an inhibitory effect on the Tg gene. Whether this difference lies in the transcriptional factor that diferrentiates lymphoid cells and the thyroid epithelial cells or whether the Tg gene also binds other proteins which modulate the promoter activity remains to be elucidated. Although the induction of the transcriptional protein in the thyrocytes lasted for only 4 h, the effect of the IFNγ-induced inhibition of Tg transcription continued for much longer and lasted for 48 h as reported previously (Kung & Lau 1990). The same phenomenon was observed in the responsive genes in the lymphoid cells.

Apart from its action on the Tg gene, IFNγ had also been reported to inhibit the transcription of TPO and TSH receptor gene (Nagayama et al. 1987, Ashizama et al. 1989) by a reduction of steady-state mRNA level. The signaling mechanism of IFNγ for these two genes has not been described and it is not clear whether these genes also contain the GAS in their promoter region. Searching the 5’ flanking sequence of the human TPO gene revealed two regions in the distal 5’ area, −2475 to −2466 and −1994 to −1985, showing some degree of homology to the minimal sequence of the GAS (Kimura et al. 1989). Whether these two regions are important in the control of TPO gene transcription and whether they are the sites of IFNγ action remain to be confirmed. No GAS region could be identified from the published sequence of the TSH receptor 5’ flanking region.

At present, the pathogenesis and the initiation of AITD is far from well understood. Although early data suggested that aberrant expression of class II antigen by thyroid epithelial cells may be the initiating process of AITD (Bottazzo et al. 1983), recent studies found that expression of class II antigen alone, without the presence of a co-stimulatory second signal, actually conferred peripheral tolerance and led to T cell anergy (Miller & Morahan 1992, Weetman 1995). Whether IFNγ has a role in the initiation of AITD is uncertain. Nevertheless, the presence of the GAS in the promoter sequence of the Tg gene confirms a specific action of IFNγ in thyroid hormone metabolism. The clarification of the cellular action of IFNγ on the Tg gene enables us to have a better understanding of how these cytokines affect the cellular function of and the hormone production by the thyrocytes. The identification of the activation signals and biological function of these cytokines on the thyroid-specific antigens may enable us to develop alternative immuno-modulatory agents for AITD.

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