# Transcriptional regulation of major histocompatibility complex class I gene by insulin and IGF-I in FRTL-5 thyroid cells

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## Abstract

Increased major histocompatibility complex (MHC) class I gene expression in nonimmune cell 'target tissues' involved in organ-specific diseases may be important in the pathogenesis of autoimmune diseases. This possibility in part evolves from studies of cultured thyrocytes where properties appear relevant to the development of thyroid autoimmune disease. In FRTL-5 rat thyroid cells in continuous culture, hormones and growth factors that regulate cell growth and function specifically decrease MHC class I gene expression. We hypothesized that this could reflect a mechanism to preserve self-tolerance and prevent autoimmune disease. The mechanisms of action of some of these hormones, namely TSH and hydrocortisone, have been already characterized. In this report, we show that IGF-I transcriptionally downregulates MHC class I gene expression and that its action is similar to that of insulin. The two hormones have a complex effect on the promoter of the MHC class I gene, PD1. In fact, they decrease the full promoter activity, but upregulate the activity of deleted mutants that have lost an upstream, tissue-specific regulatory region but still retain the enhancer A region. We show that insulin/IGF-I promotes the interactions of the p50/p65 subunits of NF-κB and AP-1 family members with these two regions, and that the tissue-specific region acts as a dominant silencer element on insulin/IGF-I regulation of promoter activity. These observations may be important to understand how MHC class I gene transcription is regulated in the cells.

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## Introduction

Evidence has accumulated that major histocompatibility complex (MHC) class I molecules, as well as class II, are important in the development of autoimmunity. Thus, there is an increased expression of MHC class I and II molecules in pancreatic β islet cells of patients with type 1 diabetes mellitus and in thyrocytes from patients with autoimmune thyroid diseases (Hanafusa *et al*. 1983, Bottazzo *et al*. 1985, Kohn *et al*. 2000). The importance of MHC class I overexpression is suggested by the observation that MHC class I-deficient mice do not develop autoimmunity in different experimental models: systemic lupus erythematosus (SLE), diabetes and autoimmune blepharitis (Mozes *et al*. 1993, Serreze *et al*. 1994, Chan *et al*. 1995, Singer *et al*. 1999). Furthermore, treatment of mice with methimazole (MMI), a drug that decreases MHC class I gene expression in thyroid cells, reduces cell-surface expression of class I molecules and prevents the development of experimental SLE, blepharitis and experimental autoimmune uveitis (Singer *et al*. 1994, Chan *et al*. 1995, Wang *et al*. 2003). Downregulation or absence of MHC class I expression is also considered the hallmark of tissue immune privilege (Ito *et al*. 2004).

FRTL-5 rat thyrocytes are a functional clone of cells that largely behave as normal thyrocytes *in vitro* and have been used as one model to study thyroid cell functions relevant to human pathophysiology (Capen 1996). FRTL-5 cells are the most preferred and frequently used thyroid cell lines for their simplicity and accessibility, and because they allow permanent transfections (Medina & Santisteban 2000, Kimura *et al*. 2001).

In FRTL-5 cells, hormones and growth factors that regulate cell growth and function – thyroid-stimulating hormone (TSH), hydrocortisone, insulin, insulin-like growth factor (IGF)-I and iodide – coordinately and


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specifically decrease MHC class I expression (Saji *et al.* 1992*a*, 1992*b*, 1997, Giuliani *et al.* 1995, Taniguchi *et al.* 1998). We hypothesized that hormonal suppression of class I levels might be a physiologic mechanism to preserve self-tolerance and prevent autoimmune thyroid disease in the face of the hormonal increase of thyroid-specific genes, which are potential autoantigens (Saji *et al.* 1992*a*, 1992*b*, 1997, Giuliani *et al.* 1995, Taniguchi *et al.* 1998, Kohn *et al.* 2000). Other growth factors and cytokines, such as transforming growth factor (TGF)-β1, thymosin α1 and α,γ-interferons, also regulate MHC class I expressions in thyroid cells (Saji *et al.* 1992*b*, Giuliani *et al.* 2000, Napolitano *et al.* 2000, Grassadonia *et al.* 2004).

We have previously defined the 5′ flanking region of the PD1 gene (Giuliani *et al.* 1995, Saji *et al.* 1997, Taniguchi *et al.* 1998, Giuliani *et al.* 2000, Napolitano *et al.* 2000, 2002), a classical MHC class I gene, whose properties are maintained when transfected into cells from different species. It has two main regions that control the MHC class I level in a particular cell. The 'tissue-specific region' is –771 to –676 bp from the start of transcription; it sets the constitutive level of class I expression in each tissue. The 'hormone-sensitive region', –203 to –56 bp, is responsible for the regulation of class I expression within each tissue and is modulated by drugs, cytokines and hormones (Singer *et al.* 1997). For many of these substances, we described a transcriptional regulation and identified the transcription factors and the *cis*-elements on the 5′-flanking regions of the class I gene involved (Saji *et al.* 1992*a*, 1992*b*, 1997, Giuliani *et al.* 1995, Taniguchi *et al.* 1998, Giuliani *et al.* 2000, Napolitano *et al.* 2000, 2002).

In this report, we show that IGF-I regulates MHC class I expression in FRTL-5 thyroid cells at transcriptional level, and its action is similar to that of insulin. IGF-I decreases MHC class I gene expression and, like insulin, has a complex action on MHC class I promoter activity with a disparate response between the full length of the promoter (1100 bp) and the region –203 bp from the start site of the gene. We show that this response is a consequence of the interaction of thyroid-restricted as well as ubiquitous transcription factors with two *cis*-acting regulatory elements: enhancer A, –180 to –170 bp, and a dominant negative element located between –1100 and –416 bp that has a tissue-specific region spanning –771 to –679 bp. The similar effect of both ligands, insulin and IGF-I, supports the importance of these regulatory elements.

## Materials and Methods

### *Materials*

Insulin was from Sigma-Aldrich (St Louis, MO, USA); IGF-I was from Peprotech EC (London, UK); [α-$^{32}$P]deoxy-CTP (3000 Ci/mmol) and [γ-$^{32}$P]ATP (3000 Ci/mmol) were from MP Biomedicals Europe, (Asse-Relegen, Belgium); [$^{14}$C]chloramphenicol (50 mCi/mmol) was from Amersham. Antibodies against the p50 and p65 subunits of NF-κB, fra-2 and c-jun were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The source of all other materials was Sigma-Aldrich, unless otherwise specified.

### *Cell culture*

FRTL-5 rat thyroid cells (Interthyr Research Foundation, Baltimore, MD, USA) were a fresh subclone (F1) that had all properties previously detailed (Ambesi-Impiombato 1986, Kohn & Valente 1989, Saji *et al.* 1992*a*, 1992*b*, 1997, Giuliani *et al.* 1995, Taniguchi *et al.* 1998, Giuliani *et al.* 2000, Napolitano *et al.* 2000, 2002, Grassadonia *et al.* 2004). Their doubling time with TSH was 36 ± 6 h; they were diploid and between their 5th and 25th passage. Cells were grown in 6H medium consisting of Coon's modified F12 (Sigma-Aldrich) supplemented with 5% calf serum, 1 mM nonessential amino acids (Gibco Invitrogen) and a mixture of six hormones: bovine TSH ($1 \times 10^{-10}$ M ), insulin (10 μg/ml), cortisol (0·4 ng/ml), transferrin (5 μg/ml), glycyl-L-histidyl-L-lysine acetate (10 ng/ml) and somatostatin (10 ng/ml). Fresh medium was added every 2–3 days, and cells were passaged every 7–10 days. In individual experiments, cells were shifted to 3H medium with no TSH, insulin or hydrocortisone and only 0·2% calf serum, where noted insulin 10 μg/ml or IGF-I 10–100 ng/ml was added.

### *Plasmids*

The chloramphenicol acetyltransferase (CAT) chimeras of the PD1 promoter have been already described (Weissman & Singer 1991, Giuliani *et al.* 1995, Saji *et al.* 1997). Briefly, different lengths of the 5′-flanking region of the MHC class I swine gene (PD1), 1100, 416, 203 and 90 bp, were inserted into pSV0-based CAT constructs. Termed p(–1100)CAT, p(–416)CAT, p(–203)CAT and p(–90) CAT respectively, they are numbered from the nucleotide at the 5′- end to +1bp, the start of transcription. The mutated construct p(–1100)EnhA[–] has the enhancer A site, –180 to –170 bp, deleted. It was created by two-step recombinant PCR methods (Vallette *et al.* 1989, Higuchi 1990, Giuliani *et al.* 1995). In the first step, two PCR reaction products were formed using overlapped primers containing the mutation; in the second, these were templates, and DNA sequences at their 5′- or 3′- ends were primers. PCR products were then inserted into the multi-cloning site of the pSVO-based CAT construct (Weissman & Singer 1991). The sequences of all constructs were confirmed by a standard method (Sanger *et al.* 1977).

### *Stable transfectants of FRTL-5 cells containing class I promoter-CAT chimeras*

Using a DEAE-dextran procedure (Lopata *et al.* 1984), we stably transfected FRTL-5 cells with the PD1/CAT



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chimeras. Near confluent cells in 6H medium were cotransfected with 20 μg plasmid DNA and 2 μg pMAMneo (Clontech). After 2 days, 400 μg/ml G418 (Invitrogen) were added to the medium, and after 3 weeks the G418-resistant colonies were pooled and used for experiments herein. CAT assays were performed as described previously (Lopata *et al.* 1984, Giuliani *et al.* 1995, 2000).

### *Flow cytometry analysis*

Single-cell suspensions were prepared and stained as previously described (Saji *et al.* 1992*a*, Giuliani *et al.* 2000), using a directly fluoresceinated monoclonal antibody (mAb) against the rat MHC class-I antigen, FITC-anti-rat FT1A (Pharmigen, San Diego, CA, USA). Leu-4 was used as background control.

### *Cell extracts*

FRTL-5 cells were grown in the presence of complete 6H medium until 60% confluent, and then shifted in 3H medium with 0·2% calf serum with or without insulin 10 μg/ml or IGF-I 100 ng/ml, for 8 days. Cellular extracts were prepared by a modification of previously described methods (Dignam *et al.* 1983). In brief, cells were washed twice in cold PBS (pH 7·4), scraped and centrifuged (500 ***g***). The cell pellet was resuspended in two volumes of Dignam buffer C (25% glycerol, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES-KOH) (pH 7·9), 1·5 mM $MgCl_2$, 0·42 M NaCl, 0·5 mM dithiothreitol, 1 μg/ml leupeptin, 1 μg/ml pepstatin and 0·5 mM phenylmethylsulfonyl fluoride). The final NaCl concentration was adjusted on the basis of cell pellet volume to 0·42 M. Cells were lysed by repeated cycles of freezing and thawing. The extracts were centrifuged (100 000 ***g***) at 4 °C for 20 min. The supernatant was recovered, aliquoted and stored at −70 °C.

### *Electrophoretic mobility shift assays (EMSA)*

DNA probes were created by restriction enzyme treatment of the chimeric CAT constructs described above and purified from 2% agarose gel using QIAEX (Qiagen). Oligonucleotides were synthesized by Operon Biotechnologies (Huntsville, AL, USA). They were labeled with [α-$^{32}$P]dCTP, using Klenow (New England BioLabs, Beverly, MA, USA), or with [γ-$^{32}$P]ATP, using T4 polynucleotide kinase (New England BioLabs), and then purified on an 8% native polyacrylamide gel.

EMSA were performed as previously described (Giuliani *et al.* 1995). Binding reactions in low salts and no detergent included 1·5 fmol [$^{32}$P]DNA, 3 μg cell extract and 3 μg poly(dI-dC) in 10 mM Tris–Cl (pH 7·9), 1 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 1 mM ethylendiaminotetraacetic acid (EDTA) and 5% glycerol in 20 μl total volume. Incubations were at room temperature for 30 min. Where indicated, antibodies were added to the binding reaction and incubated with the extract for 20 min before adding labeled DNA. After incubation, reaction mixtures were electrophoresed on 4·5% native polyacrylamide gel at 160 V in 0·5 TBE at room temperature. Gels were dried and autoradiographed.

### *Immunofluorescence studies*

FRTL-5 cells were seeded on tissue culture treated-glass chamber slides (BD Falcon; BD Biosciences, Erembodegem, Belgium) in the presence of complete 6H medium with 5% calf serum until they reached 60% confluency. Thereafter cells were shifted in 3H medium with 0·2% calf serum, with or without insulin 10 μg/ml or IGF-I 100 ng/ml, for 8 days. Cells were then fixed with periodate-lysine-paraformaldehyde (PLP), permeabilized with saponin 0·2% in PBS and incubated with a mouse anti-rat MHC class I antibody, RT1Aa class I polymorphic (Serotec, Kidlington, Oxford, UK) at a dilution 1:35 for 1 h at room temperature. Cells incubated without primary antibody served as a negative control. After being washed, cells were incubated with an anti-mouse fluorescein-conjugated secondary antibody, Alexa 488 (Invitrogen). Propidium iodide (50 μg/ml) was used to stain nuclei. The slides were visualized with a Leica DM IL microscope through a Leika × 100 oil-immersion lens.

### *Other assays*

Protein concentration was determined by the BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA); crystalline BSA was the standard.

### *Statistical analysis*

All experiments were repeated at least three times with different batches of cells. Values are the mean ± S.D. Significance between experimental values was determined by two-way analysis of variance (ANOVA) and was $P<0·05$ or better when data from all experiments were considered.

## Results

### *IGF-I-like insulin decreases MHC class I transcription and acts through a dominant negative element located between −1100 and −416 bp*

Insulin and IGF-I have been shown to decrease endogenous FRTL-5 cells class I RNA levels (Saji *et al.* 1992*b*). Furthermore insulin has been shown to act transcriptionally on the class I promoter where it has a disparate response, suggesting the existence of two insulin response elements: a dominant negative element located between



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–1100 and –203 bp and a positive element contained below –203 bp (Giuliani *et al.* 1995). Thus, the addition of IGF-I (100 ng/ml) to FRTL-5 cells stably transfected with –1100 bp of class I promoter 5′-flanking sequence ligated to the CAT reporter gene (p(-1100)CAT) significantly reduced CAT activity, whereas a significant increase was measured in a construct truncated to contain only 203 bp of upstream sequences (p(–203)CAT) (Fig. 1A). A significant change in each was seen at 10 ng/ml with a maximal effect at 100 ng/ml (Fig. 1B and C), similar to that of 10 μg/ml insulin. The maximal decrease of the full-length promoter activity is about 50% (±7%) of control, matching the decrease of mRNA previously described (~46% of control) (Saji *et al.* 1992*b*).

The decrease in class I RNA and promoter activity of the p(–1100) full-length construct was accompanied by a decrease in class I surface antigen expression evaluated by flow cytometry analysis (Fig. 2A) and immunofluorescence (Fig. 2B and C). To map the response elements for insulin/IGF-I, FRTL-5 cells were transfected with several 5′-deletion mutants of the class I gene: p(–416)CAT, p(–203)CAT, p(–90)CAT (Fig. 3A). Despite decreasing the expression of the full promoter, insulin/IGF-I caused a significant increase in CAT activity of the p(–416)CAT and p(–203)CAT chimeras (Fig. 3B). No effect was seen on the p(–90)CAT construct, which contains a minimal promoter with CCAAT and TATA-like boxes (Fig. 3A and B); thus, a negative regulatory elements maps between –1100 bp and –416 bp, whereas a positive element is located between –203 bp and –90 bp.

*EMSA indicates that the functional effects of IGF-I to increase promoter activity in p(–416) and p(–203) chimeras, like those of insulin and serum, correlate with changes in binding of the Mod-1 complex to DNA*

We performed EMSA with a $^{32}$P-labeled probe corresponding to the 5′-flanking region between –203 and –130 bp – therefore called 74 bp – which contains the enhancer A and the interferon response element (IRE). Cellular extracts from IGF-I (100 ng/ml)-treated cells showed an increase of the binding of a slow-moving complex that has the same migration and characteristics of Mod-1 (Fig. 4, lane 3 vs 1). Mod-1 is a complex between enhancer A and the heterodimer of the p50 subunit of

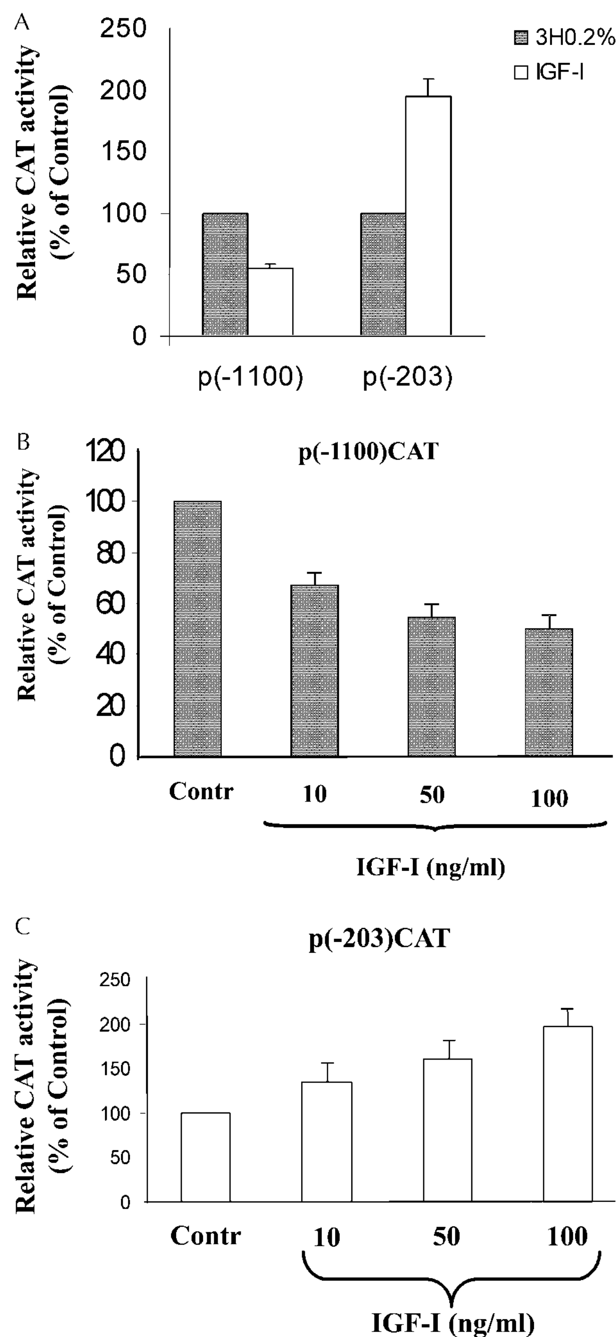


**Figure 1** (A) Effect of IGF-I on exogenous class I promoter activity of FRTL-5 cells stably transfected with the p(–1100)CAT and p(–203)CAT chimeras. The pool of G418-resistant p(–1100)CAT- and p(–203)CAT-transfected cell colonies was grown to near confluence, and then maintained in 3H 0·2% medium ± IGF-I 100 ng/ml for 7 days. CAT activity is expressed as percent control activity. Data are the mean of three separate experiments. 3H 0·2%: medium without TSH, insulin, hydrocortisone and only 0·2% calf serum; IGF-I: same medium plus 100 ng/ml IGF-I. (B) Effect of IGF-I on exogenous class I promoter activity of FRTL-5 cells stably transfected with the p(–1100)CAT chimeras. (C) Effect of IGF-I on exogenous class I promoter activity of FRTL-5 cells stably transfected with the p(–203)CAT chimeras. G418-resistant FRTL-5 cells stably transfected with p(–1100)CAT or p(–203)CAT were grown to near confluence, and then maintained in 3H 0·2% medium + IGF-I 10, 50, and 100 ng/ml for 7 days. CAT activity is expressed as percent control activity. Data are the mean of three separate experiments. Contr: cells maintained in 3H 0·2% medium (no TSH, insulin, hydrocortisone, 0·2% calf serum).



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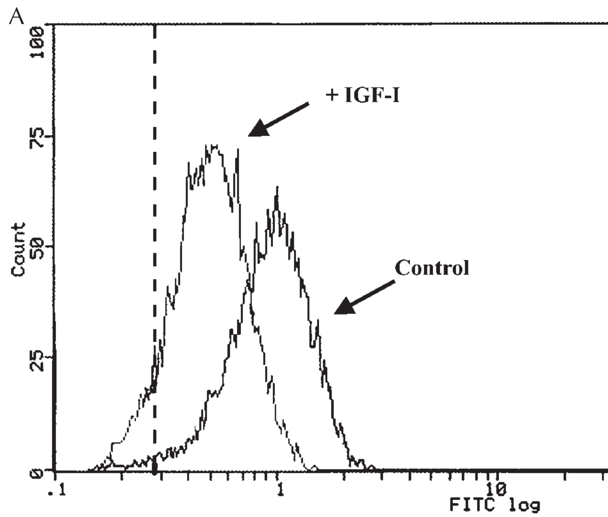


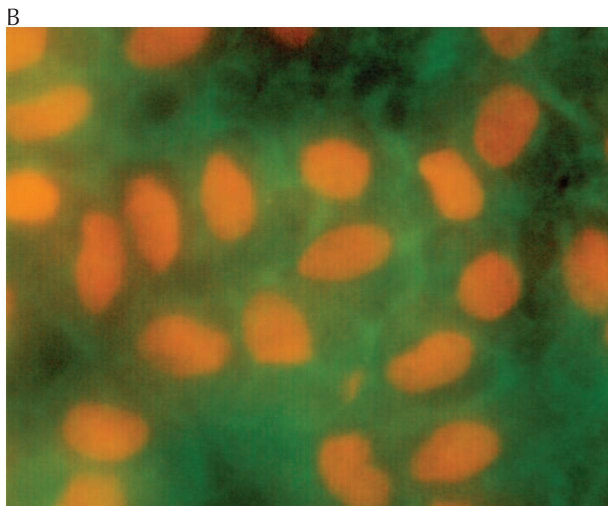


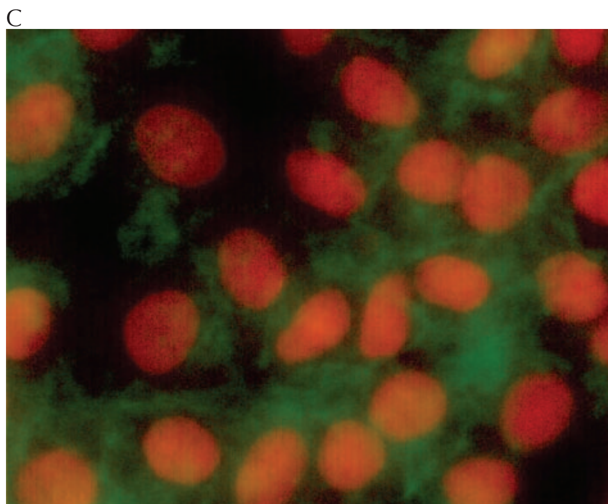


NF-κB and fra-2 (a c-fos family member) (Giuliani *et al.* 1995). Previous studies have shown that this complex is downregulated by hydrocortisone, iodide, TGF-$\beta_1$ and phorbol esters, and increased by insulin, serum, α- and γ-interferons, thymosin α1 and glucose (Giuliani *et al.* 1995, 2000, Taniguchi *et al.* 1998, Napolitano *et al.* 2000, 2002). A decrease in Mod-1 has been associated with decreased class I expression, whereas its increase is associated with increased class I expression (Giuliani *et al.* 1995, 2000, Saji *et al.* 1997, Taniguchi *et al.* 1998, Napolitano *et al.* 2000, 2002). In cellular extracts from cells maintained in 3H 0·2% calf serum, IGF-I 100 ng/ml increased Mod-1 like insulin 10 μg/ml and 5% calf serum (Fig. 4, lanes 3, 2 and 4 vs 1). To confirm that the complex was Mod-1, we preincubated the extracts with antibodies against the p50 and p65 subunit of NF-κB and against fra-2. Formation of the Mod-1 complex was super-shifted by anti-p50 (Fig. 5, lane 3 vs 2) and anti-fra-2 (Fig. 5, lane 7 vs 5), but not by antibodies against the p65 subunit of NF-κB (Fig. 5, lane 6 vs 5) or other c-fos family members (data not shown) (Giuliani *et al.* 1995). The increase in Mod-1 binding to the 74 region of the PD1 promoter agrees with the functional data from the CAT assay that show an increase of the CAT activity of the p(−203)CAT vector.

*IGF-I and insulin also regulate a tissue-specific element, −771 to −676 bp, which controls constitutive tissue levels of MHC class I*

As noted above, although insulin/IGF-I increase the activity of the p(−203)CAT vector, the effect on the full-length PD1 gene is downregulation (Figs 1A and B, and 3B). These data correlate with the decrease of the endogenous MHC class I gene by insulin/IGF-I and serum, as shown by flow cytometry analysis and immunofluorescence (Fig. 2), and in a previous report by

**Figure 2** (A) Flow cytometry analysis of the expression of surface MHC class I antigen on FRTL-5 cells. Cells grown to near confluence were maintained in 3H 0·2% medium in the absence (control) or presence of IGF-I 100 ng/ml for 7 days and then stained with a fluorescinated mAb that specifically reacts with epitope S of the rat MHC class I antigen. Dashed line represents the Leu-4 background control. (B and C) Immunofluorescence analysis of MHC class I expression on the surface of FRTL-5 cells. FRTL-5 cells were seeded on tissue culture chamber slides in the presence of complete 6H medium with 5% calf serum until they reached 60% confluency. Thereafter, cells were shifted and maintained for 8 days in 3H medium with 0·2% calf serum (B) or the same medium plus IGF-I 100 ng/ml (C). Cells were then fixed and incubated with a mouse anti-rat MHC class I antibody and an anti-mouse, fluorescein-conjugated secondary antibody. The slides were visualized with a Leica DM IL microscope through a Leika ×100 oil-immersion lens. Propidium iodide was used for nuclear counterstain (red). Controls for each study included cells receiving no primary antibody (data not shown). All data represent four experiments with similar results. The same results were obtained from cells treated with insulin 10 μg/ml (data not shown).



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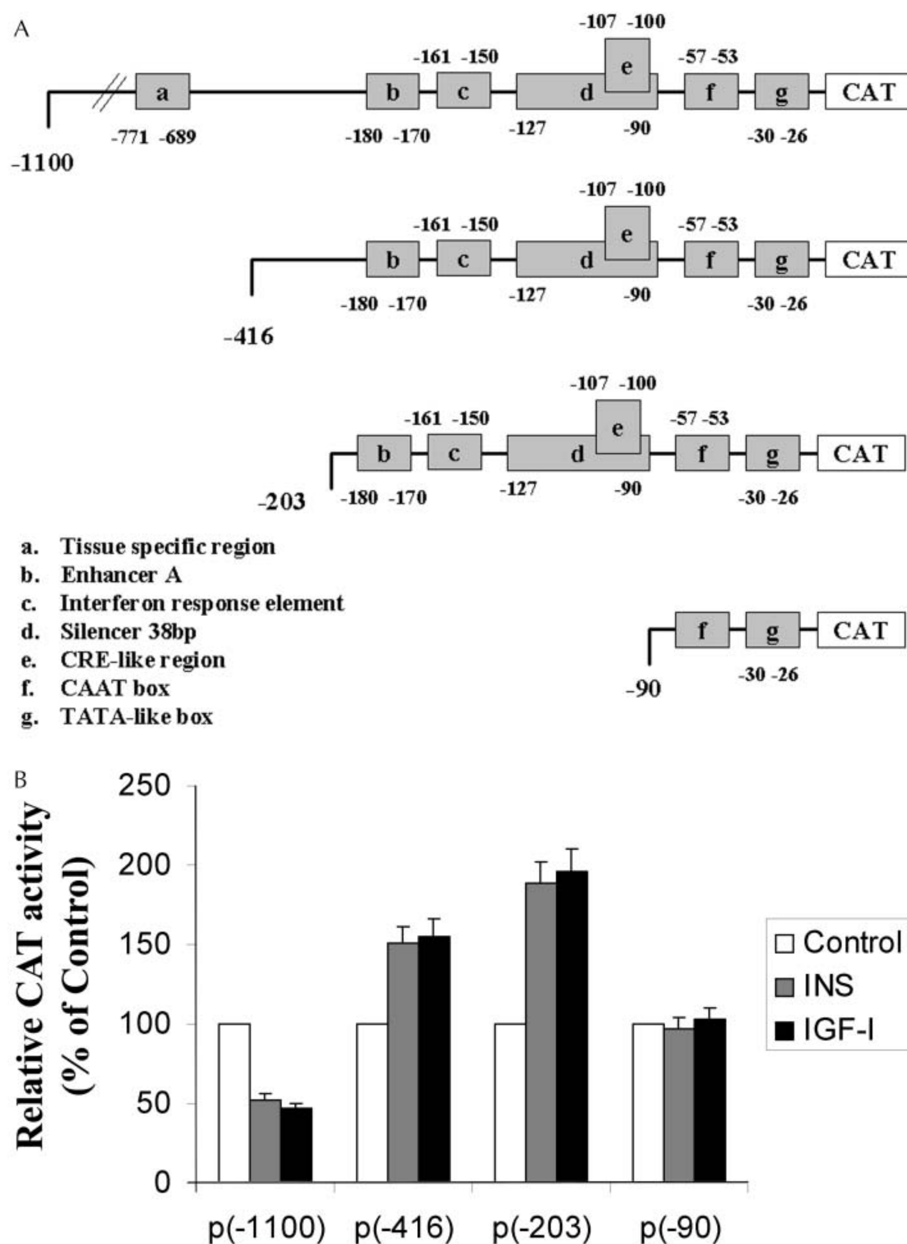


**Figure 3** Effect of insulin and IGF-I on exogenous class I promoter activity in FRTL-5 cells stably transfected with PD1 promoter-CAT chimeras having different lengths of the 5′ flanking region. (A) Diagrammatic representation of the different class I chimeras. Some of the different regulatory *cis*-elements are noted. (B) G418-resistant FRTL-5 cells, stably transfected with p(–1100)CAT, p(–416)CAT, p(–203)CAT and p(–90)CAT, were grown to near confluence, and then maintained for 7 days in 3H 0·2% medium+insulin 10 µg/ml or IGF-I 100 ng/ml. CAT activity is expressed as percent control activity. Data are the mean of three separate experiments. Control: cells maintained in 3H 0·2% medium (no TSH, insulin, hydrocortisone and 0·2% calf serum).

Northern analysis and *in vitro* transcription extension assays (Saji *et al*. 1992*b*). The functional data from the CAT assay analysis showed that the negative dominant region responsive to insulin and IGF-I is located between –1100 and –416 bp of the PD1 gene. The region between –771 and –676 bp contains a tissue-specific element that controls constitutive class I expression in different cells. In this region, two *cis*-acting elements overlapping each



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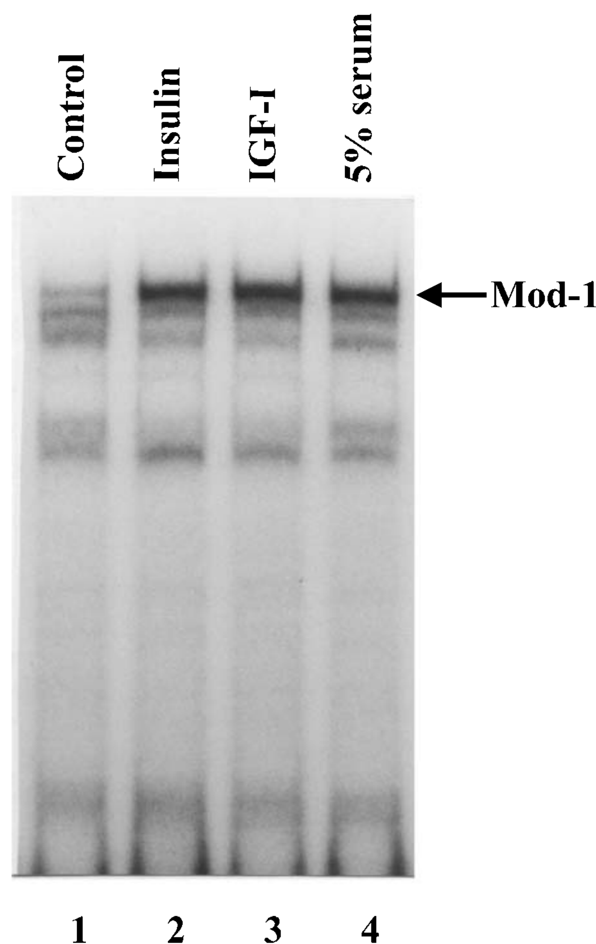


**Figure 4** Ability of IGF-I, insulin or serum to increase formation of the Mod-1 complex with enhancer A. Cells were maintained for 7 days in 3H medium (no TSH, insulin, hydrocortisone) containing 0·2% calf serum (lane 1, control) or in the same medium with insulin 10 μg/ml (lane 2), IGF-I 100 ng/ml (lane 3) or 5% calf serum (lane 4). EMSA was performed, as detailed in Materials and Methods, using cellular extracts and the –74 bp probe, which spans the region between –203 and –130 bp from the transcription start site. The arrow indicates the Mod-1 complex.

other have been identified: a silencer element and an enhancer element (Weissman & Singer 1991, Murphy *et al.* 1996). Using a radiolabeled probe with a sequence encompassing the class I promoter between –771 and –637 bp, termed 'the 140 fragment' because of its total length, including nucleotides from restriction sites on either end, we performed EMSA experiments with cell extracts from FRTL-5 cells in 3H 0·2% plus IGF-I (100 ng/ml) or insulin (10 μg/ml).

As shown in Fig. 6 (lane 2 vs 1), IGF-I (100 ng/ml) increased the formation of a slowing complex, with the 140 fragment (indicated with an arrow), that is similar, based on its mobility near the top of the gel, to the silencer complex previously identified (Weissman & Singer 1991). The identification of this complex as the silencer was confirmed by EMSA experiments using as competitor a double-strand oligonucleotide spanning the functional silencer element previously identified and named S2 (Weissman & Singer 1991) (data not shown). This was specific since other oligonucleotides spanning different portions of the region, such as S3 and E9 (Weissman & Singer 1991), did not decrease the complex (data not shown). High levels of the silencer complex are consistent with the low levels of class I expression found in different cells (Weissman & Singer 1991, Murphy *et al.* 1996, Howcroft *et al.* 2005).

Preincubations of the cell extracts with antibodies against the p65 subunit of NF-κB showed a decrease of this band (Fig. 6, lane 4 vs 2), as did preincubation with antibodies against c-jun (Fig. 6, lane 5 vs 2). No effect was seen with antibodies against p50 (Fig. 6, lane 3 vs 2). The same results were obtained in extracts from cells treated with insulin 10 μg/ml (data not shown).

Although it was not possible to see any super shift, the decrease of the complex by the antibodies against p65 and c-jun suggest that these proteins may either bind directly to the *cis*-acting element or interact with other transcription factors involved in the silencer complex formation.

These data suggest that the protein-DNA complex increased by insulin/IGF-I on the 140 fragment of the PD1 promoter involves a *cis*-acting element included between –731 and –674 bp that functions as silencer on PD1 promoter, and *trans*-acting factors including or related to the p65 subunit of NF-κB and c-jun.

*Lack of IGF-I and effect of insulin on the −1100 bp construct with a deletion of the enhancer A site: p(–1100)EnhA[-]*

Enhancer A, the site of the upregulatory effect of IGF-I/insulin to class I expression (Fig. 3A), was deleted in the full-length construct. The addition of IGF-I (100 ng/ml) or insulin (10 μg/ml) to FRTL-5 cells stably transfected with the p(–1100)EnhA[-]CAT results in a loss of their ability to decrease promoter activity (Fig. 7). These data demonstrate the need of an intact enhancer A site for the functional effect of insulin/IGF-I on the promoter and suggest a functional relationship between the enhancer A site and the upstream, tissue-specific element region, as indicated by the EMSA experiments.

## Discussion

In the present study, we show that IGF-I decreases MHC class I expression in thyroid cells, acting similarly to insulin at the promoter level. This effect is not specific to thyroid



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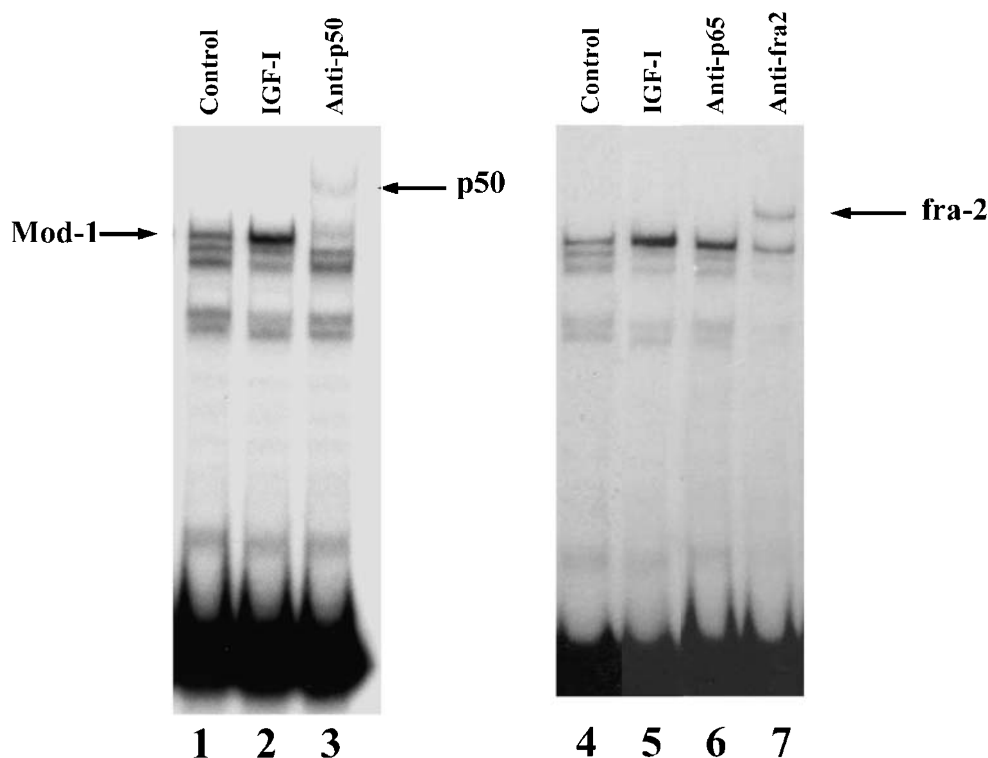


**Figure 5** Ability of antisera specific for the p50 or p65 subunits of NF-κB and fra-2 to inhibit formation of Mod-1 complex. EMSA was performed, as detailed in Materials and Methods, using cellular extracts from FRTL-5 maintained for 7 days in 3H medium (no TSH, insulin, hydrocortisone) containing 0·2% calf serum (lanes 1 and 4), or in the same medium with IGF-I 100 ng/ml (lanes 2, 3 and 5–7) and using the –74 bp probe, which spans the region between –203 and –130 bp from the transcription start site. The arrows show the complex which is super-shifted by antibodies against p50 and fra-2, and the Mod-1 complex.

cells, and it has been shown also in human scalp hair follicles, where it has demonstrated the role of MHC class I downregulation by IGF-I in preserving immune privilege (Ito *et al.* 2004). Mapping the site of IGF-I/insulin action on the PD1 promoter, we observed a paradoxical effect: IGF-I/insulin decreased the transcription of the full promoter (1100 bp) but increased expression of the truncated mutant p(–203) (–203 to +1bp). This mutant includes the enhancer A region, the IRE and a downstream silencer, including a CRE-like element.

The functional data observed with the p(–203)CAT construct activity were confirmed by EMSA experiments that showed an increase by IGF-I/insulin of the Mod-1 complex with enhancer A (–180 to –170 bp from the start of transcription). In a previous report, we showed that Mod-1 is formed by the binding of the p50 subunit of NF-κB and fra-2, a c-fos family member, with the enhancer A region (Giuliani *et al.* 1995). An increase of Mod-1 is associated with an increased activity of the promoter, whereas its decrease is associated with a decreased expression of the PD1 promoter (Giuliani *et al.* 1995, 2000, Saji *et al.* 1997, Taniguchi *et al.* 1998, Napolitano *et al.* 2000, 2002).

The EMSA experiment performed with a fragment of the PD1 promoter spanning the tissue-specific region explained the discrepancy between the activity of the p(–203)CAT construct and the full promoter. We demonstrate that IGF-I/insulin increased the binding of a protein complex with the tissue-specific region. This complex had all the characteristics of a previously identified silencer element (Weissman & Singer 1991). Previous studies have shown that the formation of the silencer complex is enhanced in tissues or cells where MHC class I expression is low, and is decreased in tissues or cells where the expression is high (Weissman & Singer 1991, Murphy *et al.* 1996, Howcroft *et al.* 2005). These data suggest that insulin/IGF-I increases the formation of the two complexes, Mod-1 and the silencer element. Insulin/IGF-I thus regulates two different regions of the PD1 promoter (the tissue-specific region and the enhancer A). The regulation is consistent with functional action associated with these elements, and the silencer element acts as a dominant region, whereby the insulin/IGF-I regulates the promoter tissue-specific activity.

In this study, we further show that in FRTL-5 thyroid cells the transcription factors involved in the silencer



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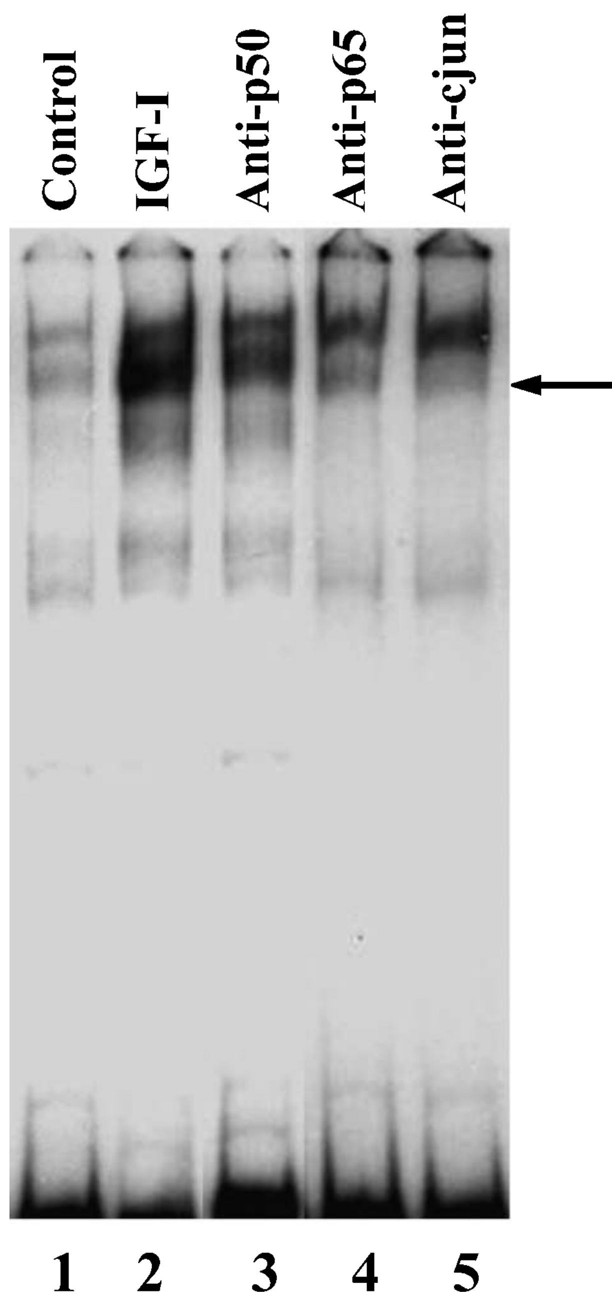


**Figure 6** EMSA was performed, as detailed in Materials and Methods, using cellular extracts from FRTL-5 maintained for 7 days in 3H medium (no TSH, insulin, hydrocortisone) containing 0·2% calf serum or in the same medium with IGF-I 100 ng/ml and using the 140 bp probe, which spans the region between –771 and –637 bp from the transcription start site. Lane 1 is the incubation of the radioactive probe with extracts from control cells (3H 0·2% medium), lane 2 is the incubation of the radioactive probe with extracts from cells exposed to 100 ng/ml IGF-1, lanes 3–5 show the ability of antibodies directed against the p65 and the p50 subunit of NF-κB and against c-jun to inhibit the formation of the silencer element. The arrow shows the protein-DNA complex increased by IGF-I and insulin, identified as the silencer element.

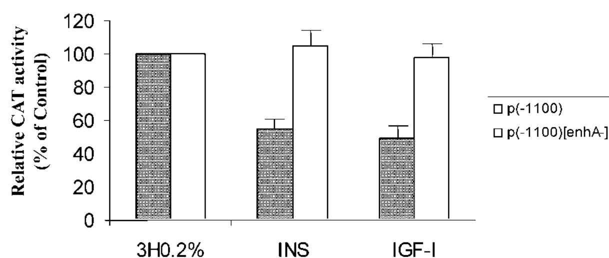


**Figure 7** Effect of insulin and IGF-I on exogenous class I promoter activity of FRTL-5 cells stably transfected with the p(–1100)CAT and p(–1100)[enhA-]CAT chimeras. The pool of G418-resistant transfected cell colonies was grown to near confluence, and then maintained in 3H 0·2% medium+insulin 10 μg/ml or IGF-I 100 ng/ml for 7 days. CAT activity is expressed as percent control activity. Data are the mean of three separate experiments. 3H 0·2%: medium without TSH, insulin, hydrocortisone and only 0·2% calf serum; INS: same medium plus 10 μg/ml insulin; IGF-I: same medium plus 100 ng/ml of IGF-I.

complex formation are the p65 subunit of NF-κB and the AP-1 family member: c-jun. In fact, there was a significant decrease of the complex when the cellular extracts were incubated with antibodies against c-jun or p65. We postulate that IGF-I/insulin stimulates the formation of the two distinct heterodimers, p50/fra-2 and p65/c-jun, resulting in the increased binding of Mod-1 to enhancer A and in an increased binding or interaction of p65/c-jun complex to the silencer element on the tissue-specific region of the promoter. To date, a direct binding of c-jun to the PD1 promoter has been demonstrated only in a TRE-like sequence element located downstream of the tissue-specific region, between –440 and –431 bp from the transcription start site (Howcroft *et al.* 1993).

It is intriguing to note that the two primary regions that regulate MHC class I transcription, that is, the tissue-specific element and the enhancer A element, interact with different members of the same family of transcription factors, NF-κB and AP-1.

The data also suggest that in FRTL-5 cells the tissue-specific region acts as a dominant regulatory element, such that the increase of the silencer complex overcomes the effect of Mod-1 complex on enhancer A and downregulates the MHC class I gene expression. Mod-1 and the tissue-specific region interact with different NF-κB subunits. Thus, it is possible that a regulatory effect in one subunit can affect the other and alter the way in which factors regulate the promoter. It can be hypothesized that insulin/IGF-I actions on MHC class I expression vary among different cell types, according to the cell-type-specific factors that bind to the tissue-specific region (Maguire *et al.* 1992). Further studies are underway to investigate the action of insulin/IGF-I in different types of cells where the silencer/enhancer constitutive activity of the tissue-specific region is different.

The surprising results in the past years linking over-expression of TLR-3 signals to both islet cells and



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thyrocytes in insulitis/diabetes and Hashimoto's thyroiditis are striking, given the importance of insulin/IGF-I to regulate islet- and thyroid cell-specific functions. We are investigating whether MHC class I overexpression is part of a reaction to a primary insult to the cell that activates the TLR3 system and produces an innate immune response mimicking that of a dendritic cell (Harii *et al.* 2005).

The present study has been performed in an *in vitro* system, the FRTL-5 cells, which have the advantage of being suitable for permanent transfections, and which allow study in a well-defined and reproducible model of single hormone or growth factor actions on gene expression (Takahashi *et al.* 1990, Medina & Santisteban 2000). The pathophysiologic relevance of these data needs to be confirmed with studies on different systems. However, the reliability of FRTL-5 cells as a model to study MHC gene regulation has been validated by studies conducted in animal models (Mozes *et al.* 1993, Singer *et al.* 1994, 1999, Chan *et al.* 1995, Wang *et al.* 2003) and human tissues (Schuppert *et al.* 1996, Ito *et al.* 2004). Of particular interest is the study of Ito *et al.* (2004) conducted in a organ-cultured human system, the scalp hair follicles, where the MHC class I downregulation by IGF-I and TGF-β1, initially reported on FRTL-5 cells (Saji *et al.* 1992*b*, Napolitano *et al.* 2000), has been confirmed.

In summary, our data show that IGF-I/insulin downregulates MHC class I gene expression transcriptionally through a silencer element located in the tissue-specific region that acts as a dominant regulator of MHC class I genes in FRTL-5 thyroid cells. These observations help us to understand the regulation of MHC class I gene expression. Knowledge of the mechanisms involved in insulin/IGF-I regulation of MHC class I transcription is important not only to understand the development of autoimmunity but also to find new strategies for cancer immunotherapy (Ly *et al.* 2001, Yang & Meyskens 2005).

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