Regulation of Major Histocompatibility Complex Class I Gene Expression in Thyroid Cells

ROLE OF THE cAMP RESPONSE ELEMENT-LIKE SEQUENCE*

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Motoyasu Saji‡, Minho Shong‡, Giorgio Napolitano‡, Lisa A. Palmer‡, Shin-Ichi Taniguchi‡, Masayuki Ohmori‡, Masanori Ohta‡, Koichi Suzuki‡, Susan L. Kirshner‡, Cesidio Giuliani‡, Dinah S. Singer‡, and Leonard D. Kohn‡**

From the Cell Regulation Section, Metabolic Diseases Branch, NIDDKD and ¶Experimental Immunology Branch, NCI, National Institutes of Health, Bethesda, Maryland 20892

The major histocompatibility complex (MHC) class I gene cAMP response element (CRE)-like site, −107 to −100 base pairs, is a critical component of a previously unrecognized silencer, −127 to −90 bp, important for thyrotropin (TSH)/cAMP-mediated repression in thyrocytes. TSH/cAMP induced-silencer activity is associated with the formation of novel complexes with the 38-base pair silencer, whose appearance requires the CRE and involves ubiquitous and thyroid-specific proteins as follows: the CRE-binding protein, a Y-box protein termed thyrotropin receptor (TSHR) suppressor element protein-1 (TSEP-1); thyroid transcription factor-1 (TTF-1); and Pax-8. TTF-1 is an enhancer of class I promoter activity; Pax-8 and TSEP-1 are suppressors. TSH/cAMP decreases TTF-1 complex formation with the silencer, thereby decreasing maximal class I expression; TSH/cAMP enhance TSEP-1 and Pax-8 complex formation in association with their repressive actions. Oligonucleotides that bind TSEP-1, not Pax-8, prevent formation of the TSH/cAMP-induced complexes associated with TSH-induced class I suppression, i.e. TSEP-1 appears to be the dominant repressor factor associated with TSH/cAMP-decreased class I activity and formation of the novel complexes, TSEP-1, TTF-1, and/or Pax-8 are involved in TSH/cAMP-induced negative regulation of the TSH receptor gene in thyrocytes, suppression of MHC class II, and up-regulation of thyroglobulin. TSH/cAMP coordinate regulation of common transcription factors may, therefore, be the basis for self-tolerance and the absence of autoimmunity in the face of TSHR-mediated increases in gene products that are important for thyroid growth and function but are able to act as autoantigens.

Thyrotropin (TSH)3 suppresses major histocompatibility (MHC) class I gene expression in association with its action to increase the growth and function of rat FRTL-5 thyroid cells in continuous culture (1, 2). Since enhanced class I expression has been demonstrated in thyrocytes from patients with autoimmune thyroid disease (ATD) (3), we proposed (1, 2, 4–6) that TSH suppression of class I levels might be a normal mechanism to preserve self-tolerance in the face of increases in gene products associated with growth and function and that its loss or attenuation might cause ATD. The importance of suppressing class I to preserve self-tolerance and prevent autoimmunity is becoming clear in multiple disease states. For example, methimazole and iodide, agents used to treat patients with Graves’ disease, one form of ATD, act in part by suppressing class I levels in thyrocytes (2, 7), also methimazole prevents the development of a systemic lupus erythematosus syndrome or autoimmune blepharitis in experimental models in mice (8, 9). Class I-deficient mice are resistant to developing these experimental diseases (9, 10), and the action of methimazole mimics the class I-deficient state in these experimental diseases (8–10).

TSH/cAMP coordinately decrease expression of the TSH receptor (TSHR) and class I genes (5, 6), while increasing thyroglobulin (TG) and thyroid peroxidase (TPO) gene expression. We suggested that TSH-decreased MHC class I and TSHR gene expression might involve common transcription factors and that this allowed the cross-talk necessary for preserving self-tolerance to gene products increased during TSHR-directed function and growth. Similarly, we considered the possibility that transcription factors involved in TSH/cAMP-increased TG and TPO gene expression might suppress class I, since TG and TPO are major thyroid autoantigens in ATD.

Transcription factors involved in TSH/cAMP regulation of TSHR gene expression (11–20) include CREB, which binds to the CRE in the TSHR minimal promoter and is necessary for efficient TSHR expression (11–13). Thyroid transcription factor-1 (TTF-1), which requires a double-strand element, and the single-strand binding protein, SSBP-1, which binds to a non-coding strand element overlapping the 5′-end of the TTF-1 site, are enhancers that work together with CREB to maximize TSHR gene expression. TSH/cAMP decreases the RNA levels of each, decreases complex formation with the TSHR promoter.

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¶ Current address: Cattedra di Endocrinologia, Universita degli Studi “G. Annunzio”-Chieti, Faculty of Medicine and Surgery, Palazzina Scuola di Specializzazione, Via dei Vestini, 66100 Chieti, Italy.

** To whom correspondence should be addressed: Metabolic Diseases Branch, NIDDK, Bldg. 10, Rm. 9C101B, NIH, Bethesda, MD 20892-1360. Tel.: 301-496-3564; Fax: 301-496-0200; E-mail: lenk@bdg10.niddk.nih.gov.

* The abbreviations used are: TSH, thyrotropin; MHC, major histocompatibility complex; TSHR, thyrotropin receptor; CRE, cAMP response element; CREB, CRE binding protein; EMSA, electrophoretic mobility shift assay; CAT, chloramphenicol acetyltransferase; bp, base pairs; PCR, polymerase chain reaction; TG, thyroglobulin; TPO, thyroid peroxidase; ATD, autoimmune thyroid disease; hGH, human growth hormone.

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and decreases TSHR promoter activity (15–17, 20). TSHR suppressor element protein-1 (TSEP-1), a Y-box protein, is a suppressor of the enhancer activity of the TSHR CRE (19); TSH/cAMP-induced phosphorylation of TSEP-1 is implicated in its suppressor activity (19). Pax-8 is a positive regulator of TG and TPO gene expression (21, 22); it interacts with some TTF-1 sites on those promoters but does not interact with TTF-1 sites on the TSHR promoter (14–16). TSH/cAMP decrease TTF-1 but increase Pax-8 complex formation and action, accounting for TSH/cAMP-positive regulation of the TG and TPO genes, despite TSH/cAMP-induced negative regulation of TSHR (15, 16).

In this report, we show that the CRE-like site (TGACCGCA) at −107 to −100 bp in the class I promoter, which is homologous to a consensus CRE (TGACGTCGA) (23, 24), is critical for the activity of a hitherto unrecognized 38-bp (−127 to −90 bp) constitutive silencer of the class I promoter. TSH/cAMP induce the formation of specific and novel protein-DNA complexes with the silencer; the induced complexes reflect the ability of TSH/cAMP to regulate the interaction of multiple transcription factors with the silencer, the net result of which is suppression of class I gene expression. We show that TSH/cAMP-induced suppression of the class I, TG, and TSHR genes involves common transcription factors, as hypothesized (1, 2, 4–6).

EXPERIMENTAL PROCEDURES

Materials—High-purity bovine TSH was obtained from the hormone distribution program, NIDDKD, National Institutes of Health (NIDDK-BSH; 30 units/mg) or was a previously described preparation, 26 ± 3 units/mg, homogenous by ultracentrifugation, about 27,500 in molecular weight, with the amino acid and carbohydrate composition of TSH (25). [α-32P]Deoxy-CTP (3000 Ci/mmol) and [14C]chloramphenicol (50 mCi/mmol) were from NEN Life Science Products and [γ-32P]ATP from Amersham Corp. Anti-CREB-327 or -activating transcription factor-2 (ATF-2), and their preimmune counterparts, was the gift of Dr. James P. Hoeffler, Invitrogen, San Diego, CA. Anti-CREB-2 was from Dr. J. M. Leiden (University of Michigan Medical Center, Ann Arbor, MI) and anti-mXR from Dr. L. H. Glimcher (Harvard School of Public Health and Department of Medicine, Harvard University Medical School, Boston). The TTF-1 expression vector, pRcCMV-TTF-1, was that used previously (15, 16, 26) and was the kind gift of Roberto Di Lauro (Stazione Zoologica A. Dohrn, Villa Comunale, Naples, Italy); the pRcCMV plasmid used in its construction was from Invitrogen. The Y-box and Pax-8 expression vectors, pRcCMV-TSE-1 and pRcCMV-Pax-8, were constructed by ligating their full-length coding sequences with the pRcCMV vector (19, 21). All other materials were from the Sigma unless otherwise noted.

Cell Culture—FRTL-5 rat thyroid cells (Interphy Research Foundation, Baltimore, MD; ATCC No. CRL 8305) were a fresh subclone (F1) that had all properties previously detailed (1, 2, 11–15, 18–20). They were grown in 6H medium consisting of Coon’s modified F12 supplemented with 5% heat-treated, mycoplasma-free calf serum (Life Technologies, Inc.), 1 mM nonessential amino acids (Life Technologies, Inc.), 10 ng/ml histidyl-L-lysine acetate (10 ng/ml), and somatostatin (10 ng/ml) (27).

Electrophoretic mobility shift assays were performed as described (11–13, 15–20, 32, 37). In the second procedure, FRTL-5 cells were grown to 80% confluency (12, 13, 15–20, 32, 37), using 10–30 μg of cell lysate and a 130-μl assay volume. Incubation was at 37 °C for 4 h with acetyl-CoA supplementation (20 μl of a 3.5 mg/ml solution) after 2 h. Acetylated chloramphenicol was separated by thin layer chromatography and autoradiographed; spots were quantitated in a scintillation spectrometer. Protein concentration was determined by Bradford’s method (Bio-Rad); recrystallized bovine serum albumin was the standard.

Electrophoretic Mobility Shift Assays (EMSA)—Oligonucleotides used for EMSA were synthesized or were purified from 2% agarose gel using QIAEX (Qiagen, Chatsworth, CA) following restriction enzyme digestion. Twelve hours later, fresh 5H medium with 5% calf serum was added, supplemented or not with 10−10 M TSH or 10 μM forskolin. CAT activity was assayed 36 h later and conversion rates were normalized to hGH levels and protein.

CAT activity was measured as described (1, 11–13, 15–20, 32, 37), using 10–30 μg of cell lysate and a 130-μl assay volume. Incubation was at 37 °C for 4 h with acetyl-CoA supplementation (20 μl of a 3.5 mg/ml solution) after 2 h. Acetylated chloramphenicol was separated by thin layer chromatography andautoradiographed; spots were quantitated in a scintillation spectrometer. Protein concentration was determined by Bradford’s method (Bio-Rad); recrystallized bovine serum albumin was the standard.

Cellular Extracts—Cell extracts were prepared by a modification of the method of Dignam et al. (38). Briefly, FRTL-5 cells were harvested by scraping, after being washed twice in ice-cold phosphate-buffered saline and pelleted. The pellet was resuspended in 2 volumes of Dignam buffer C (20 mM HEPES at pH 7.9, 1.5 mM MgCl2, 0.42 mM NaCl, 25% glycerol, 0.5 mM dithiothreitol, 0.5 mM phosphomethylsulfonyl fluoride, 1 μg/ml leupeptin, 1 μg/ml pepstatin). The final NaCl concentration was adjusted on the basis of cell pellet volume to 0.2 M. Cells were lysed by repeated cycles of freezing and thawing. Extracts were centrifuged at 100,000 × g and at 4 °C for 20 min. The supernatant was recovered, aliquoted, and stored at −70 °C.

Electrophoretic mobility shift assays were performed as described previously (12, 13, 14–20, 32, 40). Binding reactions, in a volume of 20 μl, were for 20 min at room temperature. Reaction mixtures contained 1.5 fmol of [32P]DNA, 3 μg of cell extract, and 0.5 or 3 μg of poly(dI-dC) in 10 mM Tris-Cl at pH 7.9, 1 mM MgCl2, 1 mM dithiothreitol, 1 mM EDTA, and 5% glycerol. Where indicated, unlabeled double- or single-stranded oligonucleotides were added to the binding reaction as competitors and incubated with the extract for 20 min prior to the addition of labeled DNA. In experiments using antisera, extracts were incubated with each antisera for 4 h at 4 °C after being processed as above. Reaction mixtures were electrophoresed on 4 or 5% native polyacrylamide gels at 160 V in 1 × TBE at 4 °C. Gels were dried and autoradiographed.
The 1,10-Phenanthroline-Copper Ion Footprinting Procedure—Footprinting, using 1,10-phenanthroline-copper ion, was carried out essentially as described (41). After a scaled-up EMSA using an end-labeled fragment, Fr168, comprising −168 through −1 bp of the PD1 promoter, the gel was immersed in 200 ml of 50 ml Tris-Cl at pH 8.0 and 20 ml of the following solutions were added: 2 ml 1,10-ortho-phenanthroline, 0.45 mM CuSO₄, and 58 mM 3-mercaptopropionic acid. After 15 min at room temperature, 20 ml of 28 ml 2.9-dimethyl ortho-phenanthroline was used to quench the reaction; 2 min later, the gel was rinsed in distilled H₂O and autoradiographed for 40 min at 4 °C, until the retarded bands were visible. Bands were excised and eluted overnight at 37 °C in 0.5 M ammonium acetate containing 0.1% sodium dodecyl sulfate and 10 mM magnesium acetate. The eluted DNA was ethanol-precipitated and resuspended in distilled H₂O. Equal numbers of counts from each sample were dried, resuspended in 98% formamide containing 10 mM EDTA, 0.025% bromphenol blue, and 0.025% xylene cyanol, and then separated on an 8% sequencing gel along with G + A and C + T Maxam-Gilbert sequence reactions (42) performed using the same probe. Autoradiography was at −80 °C overnight.

Statistical Significance—All experiments were repeated at least three times with different batches of cells. Values are the mean ± S.E. of these experiments where noted. Significance between experimental values was determined by two-way analysis of variance and are significant if p values were <0.05 when data from all experiments were considered.

RESULTS
The CRE-like Sequence between −107 and −100 bp Functions as a Constitutive Silencer and Is a Target for TSH/cAMP-mediated Repression of the Class I Promoter
The ability of TSH/cAMP to repress MHC class I transcription has been mapped to within 127 bp of initiation of transcription (1). Examination of this 128-bp DNA segment revealed the presence of an 8-bp sequence, −107 to −100 bp (Fig. 1A), with homology to characterized CREs (23, 24). To determine whether this element functioned to regulate class I promoter activity, a set of derivative constructs was generated from a parental construct containing 127 bp of 5′-flanking sequence p(−127)CAT. In one derivative, the 8-bp CRE-like sequence was deleted; in the other, we substituted a nonpalindromic mutation of the CRE-like octamer (Fig. 1B). Both constructs displayed increased promoter activity, relative to the parental construct, when transfected into FRTL-5 cells maintained in the absence of TSH (Fig. 1B).

The ability of the CRE-like element to silence a heterologous promoter was also assessed by introducing a 38-bp DNA segment, spanning −127 to −90 bp, downstream of an SV40 minimal promoter (Fig. 1C). The choice of a 38-bp segment was derived from the experiments described below. When placed in a 5′ to 3′ orientation, a single copy of this DNA segment was able to significantly reduce SV40 promoter activity, and the magnitude of the effect increased with the number of copies of the 38-bp segment inserted (Fig. 1C). When placed in a 3′ to 5′ orientation, two copies of this DNA segment were also able to significantly reduce SV40 promoter activity (Fig. 1C). Derivatives of the 38-bp segment, containing either a deletion (∆CRE1 (±)) or nonpalindromic (NP CRE 1 (±)) mutation of the CRE-like element, did not significantly decrease SV40 promoter activity (Fig. 1C).

TSH or forskolin significantly decreased the activity of the p(−127)CAT construct in FRTL-5 cells relative to untreated controls (Table I, A, columns 3–5), i.e. TSH enhanced the silencer activity. Deletion or mutation of the CRE, which increases the constitutive level of constitutive activity in the absence of TSH, diminished the repressive response to TSH (Table I, A, columns 3–5). The role of the CRE-like site in conferring TSH/cAMP responsiveness was confirmed in studies using the 38-bp silencer linked to the heterologous promoter (Table I, B). Although the SV40 promoter alone did not respond to TSH or forskolin, the promoter activities of a construct containing a single copy of the CRE 1 in a sense orientation (CRE 1 (+)) or of constructs containing one or two copies of CRE 1 in a 3′ to 5′ orientation (CRE 1 (−)), CRE 2 (−)), but not their nonpalindromic mutations, were significantly reduced by TSH or forskolin (Table I, B, columns 3–5).

From these data, it is concluded that the 8-bp CRE-like site is important for the function of a constitutive silencer located in a 38-bp fragment of the class I 5′-flanking region, −127 to −90 bp from the start of transcription. The 38-bp silencer is responsive to TSH or its cAMP signal; the CRE-like element within it is necessary for this functional response. The residual suppressive effect of TSH in p(−127)CAT chimeras containing a CRE deletion or mutation (Table I, A) suggests, nevertheless, that this may not be the sole site of TSH/cAMP action and that additional sites downstream of −90 bp might be TSH/cAMP-responsive.
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**TABLE I**

| Promoter Construct | Control | +TSH (1 x 10^-10 M) | +Forskolin (10 μM) | Relative effect (+/-TSH) | (+/-Fsk) |
|--------------------|---------|---------------------|-------------------|-------------------------|---------|
| A.                 |         |                     |                   |                         |         |
| p(-127)CAT control | 100     | 35 ± 1.5            | 33 ± 2.5          | 0.35                    | 0.35    |
| p(-127)ΔCRE CAT    | 160 ± 11 | 120 ± 6.0^a         | 134 ± 5.0^a       | 0.75^b                  | 0.84^a  |
| p(-127) NPCRE      | 220 ± 15 | 165 ± 15^b          | 176 ± 9.0^c       | 0.75^d                  | 0.8^e   |
| B.                 |         |                     |                   |                         |         |
| pCAT promoter control | 100 | 100 ± 5             | 162 ± 4           | 1.0                    | 1.02    |
| pCAT promoter CRE 1 (+) | 68 ± 7 | 27 ± 8              | 32 ± 6^d          | 0.4^c                   | 0.35^e  |
| pCAT promoter CRE 1 (-) | 103 ± 5 | 26 ± 7^e            | 31 ± 5^d          | 0.27^d                  | 0.30^d  |
| pCAT promoter CRE 2 (±) | 53 ± 4 | 20 ± 5^f            | 22 ± 7^f          | 0.38^g                  | 0.42^h  |
| pCAT promoter NPCRE 1 (+) | 172 ± 8 | 169 ± 9              | 181 ± 8           | 0.95                    | 1.05    |
| pCAT promoter ΔCRE 1 (+) | 167 ± 9 | 170 ± 8              | 170 ± 4           | 1.02                    | 1.02    |

^a Increased activity relative to p(-127)CAT control, p < 0.05 or better.
^b Decreased activity relative to control cells in the absence of TSH, p < 0.05.
^c Decreased activity relative to pCAT promoter control in the absence of TSH, p < 0.05.
^d Decreased activity induced by TSH or forskolin, p < 0.05 or better.

**TSH/cAMP Induces Novel Complexes Whose Formation Depends on the CRE-like Element**

When a DNA fragment encompassing the silencer, -168 to -1 bp (termed Fr168 (Fig. 2A, bottom), was used in gel mobility shift assays with extracts derived from FRTL-5 cells cultured with or without TSH, a multiplicity of protein-DNA complexes was formed with either extract (Fig. 2A). Whereas protein-DNA complexes A to E were common to both extracts, TSH-treatment of the FRTL-5 cells induced the appearance of two novel complexes, F and G (Fig. 2A, lane 2 versus 1). Formation of the TSH-induced complexes was specific, since their appearance could be prevented by unlabeled Fr168 (Fig. 2A, lane 3). More importantly, formation of only the F and G complexes could be prevented by the 38-bp silencer fragment, -127 to -90 bp, containing the CRE-like site and termed CRE-1 (Fig. 2A, lane 4).

Forskolin (10 μM) could substitute for TSH to induce the formation of the F and G complexes with the Fr168 probe (Fig. 2B). Moreover, with either forskolin- (Fig. 2B) or TSH-treated cells (data not shown), formation of the F and G complexes could be prevented by a derivative 38-bp fragment, -127 to -90 bp, in which a consensus CRE sequence (CON CRE) was substituted for the native CRE-like sequence (Fig. 2B, lanes 3 and 4 versus 2) but not by derivative oligonucleotides from the CRE-like element that had been deleted (ΔCRE) or mutated to a nonpalindromic (NP CRE) substitution (Fig. 2B, lanes 5 and 6, respectively, versus lane 2). The region of the 38-bp silencer 5’ to the CRE (termed 5’ CRE) did not inhibit formation of the TSH/cAMP-induced complex (Fig. 2B, lane 7 versus 2) nor did a shortened form of CRE-1, termed CRE-2, with only 6 base pairs on either side of the CRE octamer (Fig. 2B, lane 8 versus 2).

Phenanthroline-copper ion footprint analysis of the TSH-induced F or G complex identified a protected region, -131 to -95 bp, bounded by two strong hypersensitive sites (Fig. 3) that encompasses the CRE-like site, -107 to -100 bp. A less prominent hypersensitive band at -110 bp suggests this region may bind more than one factor as will be shown below and in a separate report. Although not the only protected region in the footprint, these data, together with the data in Fig. 2, established that coincident with TSH/forskolin-induced suppression of class I RNA levels (1, 2) and TSH/forskolin-activated silencer activity (Fig. 1, Table I), TSH/forskolin-induced the appearance of novel complexes with the class I promoter, whose formation required the CRE-like sequence, as did silencer activity. The data (Fig. 2) additionally suggested that sequences flanking the CRE-like site are involved in complex formation, consistent with the extended footprint (Fig. 3).

The 38-bp DNA Fragment with CRE-dependent Silencer Activity Forms CRE-dependent Complexes with Multiple Proteins: the Effect of TSH on These Complexes and the Functional Role of the Proteins Involved

Identification of a Multiplicity of Transcription Factors That Interact with the 38-bp DNA Fragment Exhibiting CRE-dependent Silencer Activity—To characterize proteins capable of interacting with the 38-bp silencer element, we radiolabeled a double-stranded oligonucleotide spanning the segment -127 to -90 bp (CRE-1) and used it in gel shift assays with extracts from FRTL-5 cells maintained in the absence of TSH (Fig. 4); subsequently (see below), we evaluated the effect of TSH on the characterized complexes.

Four sets of complexes were observed (Fig. 4, A–D) with extracts from cells without TSH, all of which appeared to be specific, since their formation was prevented by competition with unlabeled CRE-1 (Fig. 4A, lanes 3–6 versus 2), albeit with different affinities. More importantly, formation of all the complexes was dependent on the CRE-like element. Thus, whereas their formation was inhibited by the native 38-bp CRE-1 silencer, no comparable competition was evident using the silencer fragment in which the CRE was deleted (Fig. 4A, ΔCRE-1, lanes 7–9, versus 2). An oligonucleotide derived from the somatostatin receptor (Promega CRE), which contains a

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consensus CRE but otherwise unrelated flanking nucleotides, was unable to duplicate the action of CRE-1 as a competitor (Fig. 4A, lanes 10–12 versus 2–6). These data are consistent with the interpretation that multiple proteins interact with the 38-bp silencer, that the CRE is a critical element required for their binding, but that sequences flanking the CRE are involved in the binding of most.

As is the case for the TSHR CRE (11–13), one protein interacting with the CRE-like site in the 38-bp silencer is CREB-327 or an immunologically related CRE binding protein (23, 24, 43–45). Thus, the CRE-containing oligonucleotide derived from the somatostatin receptor, which contains a consensus CRE but otherwise unrelated flanking nucleotides, appeared to inhibit the formation of one component within the A complex (Fig. 4A, lanes 10–12 versus 2). Moreover, one of the components in the A complex could be super-shifted (Fig. 4B, lane 4) by an antibody to CREB-327 (43) but not by anti-CREB2, anti-mXBP, or anti-activating transcription factor-2 (ATF2-BR).

Three points should be noted with respect to these data. First, in the A complex region, proteins other than CREB are likely to interact with the CRE-like sequences, given the limited effect of anti-CREB antibody in supershifting the complex. One will be identified as Pax-8 below; however, other CRE binding protein analogs of CREB (23, 24) may be components of the A complex and remain to be identified. Second, the CRE-like element is a functional CRE, since it interacts with CREB and is TSH/forskolin-responsive (11–13, 23, 24, 43–45). Third, the consensus CRE from Promega, while decreasing the formation of a component in the A complex region, appears to enhance the formation of one or more components in the C region (Fig. 4, lane 12). This observation may be relevant to data that reveal a similar action of anti-CREB (see below).

We pursued the identity of other factors forming complexes with the 38-bp silencer. As noted in the introduction, we had hypothesized that transcription factors that negatively regulate class I promoter activity might be some in involved in TSH/cAMP-induced negative regulation of the TSHR and/or positive regulation of the TG or TPO promoters (4–6). Two obvious candidates are the tissue-specific factors, TTF-1 and Pax-8. Maximal TSHR gene expression in the absence of TSH is associated with the binding of TTF-1; additionally, TTF-1 acts synergistically with proteins such as CREB, which bind the CRE-like sequence of the TSHR (15). In the TG and TPO promoters, some TTF-1 sites also interact with Pax-8, i.e., the oligo(C) site in the TG promoter (21, 22, 26). TSH/cAMP decreases TTF-1 complex formation with the TSHR and TG promoters but increases Pax-8 complex formation with the TG promoter, coincident with increased TG gene expression (15,
We evaluated the possibility that TTF-1 and/or Pax-8 might interact with the class 1 38-bp silencer.

DNA binding sites for both TTF-1 and Pax-8 are contained within oligonucleotide C derived from the Tg promoter (Fig. 5C). Oligonucleotide C is able to completely inhibit formation of complex B and reduce the amount of complex A, whereas a mutant of oligonucleotide C, which loses the ability to bind TTF-1 or Pax-8 (21, 22, 26), also loses its inhibitory effect (Fig. 5A, lanes 4 and 5 versus 2). This suggests that either TTF-1 or Pax-8 (or related factors) contribute to these complexes.

To distinguish between TTF-1 and Pax-8 binding, an oligonucleotide that binds only TTF-1, oligonucleotide TTF-1 from the TSH receptor promoter, or its mutated counterpart (Fig. 5C) which loses TTF-1 binding and activity (15, 16), was substituted in the competition assays (Fig. 5B). Whereas oligonucleotide C affected both complexes A and B, oligonucleotide TTF-1 completely inhibited complex B but did not affect complex A (Fig. 5B, lane 5 versus 2–4). In contrast, the mutant derivative of oligonucleotide TTF-1 does not compete for complex formation (Fig. 5B, lane 6 versus 2–4). Taken together, these data suggest that complex B contains TTF-1 and that Pax-8 is a component of complex A, in addition to CEB.

Two additional points should be noted in these experiments. First, both the Pax-8 and TTF-1 complexes require CRE-dependent interactions with the 38-bp silencer. Thus, in the same experiment (Fig. 5A) the 38-bp CRE-1 oligonucleotide, but not its ΔCRE-1 derivative, prevented the formation of both A and B complexes (Fig. 5A, lanes 1 and 3, respectively, versus 2).

Second, the concentration of poly(dI-dC) in the assay could change the appearance of the complexes (Fig. 5, A versus B). Higher poly(dI-dC) concentrations (3 μg/assay) were observed to significantly enhance formation of the A and B complexes (Fig. 5, A versus B) but attenuate formation of the C complexes (Fig. 5, B versus Fig. 4). Since TTF-1, CREB, and Pax-8 are double strand-specific in their DNA interactions (12–16, 21, 22, 26), one possibility to explain this phenomenon was that higher concentrations of poly(dI-dC) were suppressing the binding of proteins with lesser affinity or specificity, such as single strand DNA-binding proteins, and that these comprised the protein/DNA adducts in complex C.

To assess this possibility, each of the component single strands of the 38-bp silencer was tested for its ability to inhibit complex formation in low poly(dI-dC) conditions (Fig. 6A). Neither strand affected the formation of complexes A or B; however, both single strand oligonucleotides reduced the formation of complex C, the non-coding strand sequence more efficiently than the coding strand (Fig. 6A). Thus, whereas the A and B complexes involved double strand DNA interactions, formation of the components of complex C appeared to result from the interaction of factors that can bind to individual strands of the 38-bp CRE-1 silencer.

Two proteins involved in the regulation of the TSH receptor gene are known to be single-strand DNA binding proteins. One is a single strand binding protein (SSBP-1) which was identified by its ability to bind to the noncoding strand of the TSH receptor promoter, immediately 5’ to and contiguous with the TTF-1 binding site (17, 20). The second is a Y-box protein that we cloned and termed TSEP-1 (TSH suppressor element protein-1), because of its ability to suppress the constitutive enhancer activity of the TSH receptor CRE (19). TSEP-1, like other Y-box family proteins, binds to both single or double strand DNA (13, 19, 46–48). The ability of the cognate DNA binding sites of these two factors on the TSHR (Fig. 6C) to inhibit formation of complex C bands was assessed (Fig. 6B).

An oligonucleotide corresponding to the SSBP binding site, −194 to −169 bp on the non-coding strand of the TSHR minimal promoter, competed for complex C entirely, without affecting either complexes A or B (Fig. 6B, lanes 3 and 4 versus 2). A second oligonucleotide containing the TSEP-1 binding site de-
Fig. 5. The 38-bp silencer region containing the CRE-like sequence forms complexes with both TTF-1 and Pax-8 in addition to CREB. The double-stranded, radiolabeled 38 bp DNA fragment, 127 to 90 bp (CRE-1), was incubated with extracts from FRTL-5 cells maintained in 5% medium plus 5% calf serum for 6 days; complexes were analyzed by EMSA in 3.0 μl, and analyzed as described under “Experimental Procedures.” A, complex formation was evaluated in the presence or absence of the noted unlabeled, double-stranded oligonucleotides: CRE-1, ΔCRE-1 with the CRE-like sequence deleted, an oligonucleotide (oligonucleotide C) containing the TTF-1/Pax-8 element in the TG promoter, and a mutant thereof which loses TTF-1/Pax-8 binding and activity in the FRTL-5 cell. The sequence of oligonucleotide C and its mutant, with the TTF-1/Pax-8 site underlined, is presented in C; their properties have been characterized previously (15, 16, 21, 22, 28). Mutated nucleotides are starred and included within the boxed area. B, complex formation was again evaluated in the presence or absence of CRE-1, an oligonucleotide containing the TTF-1 element in the TSHR (15–18, 20), and a mutant thereof which loses TTF-1 binding and activity in the FRTL-5 cell (15–18, 20), in addition to oligonucleotide C and its mutant. The TSHR TTF-1 site does not bind Pax-8 (14–16). The sequences of the oligonucleotides containing the TSHR TTF-1 element and its mutant are presented in C; their properties have been characterized previously (15–18, 20). Mutated nucleotides are again starred and included within the boxed area. The amount of each competitor was 100-fold in excess of probe. Letters represent groups of complexes formed by the extract; the TTF-1 and Pax-8 containing complexes with the 38-bp silencer, which are noted, are based on the inhibition data as discussed in the text.

Thus, TSH treatment of the cells results in markedly diminished formation of the A and B complexes, which contain CREB, Pax-8, and TTF-1 (Fig. 7, lane 4 versus lane 2). The decreased CREB interaction is evidenced by a diminished ability of anti-CREB-327 to supershift the A complex (Fig. 7, lane 5 versus 3, dashed arrow). The residual component of the A complex involves Pax-8, as evidenced by inhibition of its for-
Fig. 6. The C complexes formed with the 38-bp silencer appear to involve proteins able to bind either its coding or noncoding strands (A); these appear to involve two single strand binding proteins important in TSH/cAMP suppression of TSHR gene expression in FRTL-5 thyroid cells: TSEP-1 and SSBP-1 (17, 19, 20). The double-stranded radiolabeled 38-bp DNA fragment, –127 to –90 bp, termed CRE-1, was incubated with extracts from FRTL-5 cells maintained in 5H medium plus 5% calf serum for 6 days; complexes were analyzed by EMSA in 0.5 μg/ml poly(dI-dC) as described under "Experimental Procedures." A, complex formation was evaluated in the presence or absence of a 100-fold excess over probe of the unlabeled single strand oligonucleotides comprising the coding and noncoding strand of CRE-1: B, complex formation was evaluated in the presence or absence of a single strand oligonucleotide from the noncoding strand of the TSHR, which binds a single strand binding protein termed SSBP-1 (17, 20), and a single strand oligonucleotide from the coding strand of the TSHR which binds a Y-box protein termed thyrotropin receptor suppressor element protein-1 (TSEP-1) (19). These are termed oligonucleotide SSBP and oligonucleotide TSEP-1, respectively; their sequences are presented in C. The amount of each unlabeled oligonucleotide, in fold-excess over probe, is noted.

Fig. 7. TSH treatment of FRTL-5 cells decreases CREB and TTF-1, but not Pax-8, binding within the A and B complexes formed by the class I 38-bp silencer containing the CRE-like sequence; it also causes an increase in C complex formation, which includes protein-DNA complexes with TSEP-1 and the SSBP-1. The radiolabeled double-stranded 38-bp DNA fragment, –127 to –90 bp, termed CRE-1, was incubated with extracts from FRTL-5 cells maintained in 5H medium plus 5% calf serum for 6 days and then treated for 16 additional h with the same medium or the same medium plus 1 × 10^-17 M TSH. Incubations were performed in the presence of 3 μg/ml poly(dI-dC) and in the presence or absence of 2 μl of rabbit antisera against CREB-327. Complexes were analyzed by EMSA, as described under "Experimental Procedures." The A, B, and C complex areas are noted (see Figs. 4–6); the A region contains complexes with CREB and Pax-8, the B with TTF-1, and the C with TSEP-1 and SSBP-1 (Figs. 4–6).

Oligonucleotide TIF from the TSHR insulin response element (18), like oligonucleotide TSEP-1, contains a CCTC motif and TSEP-1 binding site (Fig. 8C). In the presence of excess, unlabeled single strand oligonucleotide TIF(–), formation of the TSH-induced complexes with either double strand Fr168 or Fr127 is inhibited (Fig. 8A, lane 1 versus 2 and Fig. 8B, lane 3 versus 2, respectively), as is the case for the positive control, double strand oligonucleotide CRE-1, the 38-bp silencer itself (Fig. 8B, lane 7 versus 2). In contrast, a single strand form of oligonucleotide TIF(–), with a mutation in the CCTC motif that is important for TSEP-1 binding (mutant 2), did not compete (Fig. 8A, lane 3 versus 2; Fig. 8B, lane 5 versus 2), whereas the oligonucleotide TIF(–)-derivative with a mutation that does not involve the CCTC motif, mutant 1, remained a competitor (Fig. 8A, lane 4 versus 2; Fig. 8B, lane 6 versus 2). These results were duplicated by wild type and mutant single strand oligonucleotide TSEP-1(+) and oligonucleotide S-box(+) from the TSHR (Fig. 8C), which also have TSEP-1 sites (data not shown).
Binding activity because of a mutation in the CCTC binding motif, does not act as a competitor (Mut. 2) oligonucleotide binds TSEP-1, whereas the mutant 2 (noted. The dark bars S-box(1,9)); the sequences of the competitor oligonucleotides and their location in the TSHR 5'-flanking region are shown). Oligonucleotide TIF, one of the TSEP-1 binding sites on the TSHR (C), on the formation of the TSH-induced protein-DNA complexes with radiolabeled Fr168, −168 to −1 bp (A) or radiolabeled Fr127, −127 to −1 bp, of the 5'-flanking region. FRTL-5 cells were maintained 6 days in 5H medium with 5% calf serum at which time fresh 5H medium or 5H medium containing 1 × 10^−6 M TSH (6H) was added for 36 h. Cell extracts were prepared, incubated with [32P] radiolabeled Fr168(A) or Fr127(B) of the class I 5'-flanking region, and evaluated by EMSA. Fr 168 is diagrammatically depicted in Fig. 2; Fr127 deletes nucleotides 168 through 128. Incubations were additionally performed in the presence of single strand oligonucleotide TIF(−) (A, lane 1; B, lane 3), a TSEP-1 binding site on the TSHR, or mutants thereof. Oligonucleotide TIF is one of three TSEP-1 binding sites of the TSHR, termed oligonucleotide TSEP-1(+), oligonucleotide S-box(+), and oligonucleotide TIF(−) (C); the sequences of the competitor oligonucleotides and their location in the TSHR 5'-flanking region are noted. The dark bars represent the CCTC motif which is important for TSEP-1 binding in the TSHR (19). In each case the mutant 1 (Mut. 1) oligonucleotide binds TSEP-1, whereas the mutant 2 (Mut. 2) form loses binding activity (19). Oligonucleotide TIF Mut-2, which loses TSEP-1 binding activity because of a mutation in the CCTC binding motif, does not act as a competitor (A, lane 3; B, lane 5), whereas oligonucleotide TIF Mut-1, which does not affect TSEP-1 binding activity, retains the competitive effect of the wild type oligonucleotide (A, lane 4; B, lane 6). We additionally show that double strand oligonucleotide C from the TG promoter, which can bind TTF-1 or Pax-8, does not prevent formation of the TSH-induced complex, whereas double strand CRE-1 does inhibit formation of the TSH-induced complexes.

Figure 8. Effect of oligonucleotide TIF, one of the TSEP-1 binding sites on the TSHR (C), on the formation of the TSH-induced protein-DNA complexes with radiolabeled Fr168, −168 to −1 bp (A) or radiolabeled Fr127, −127 to −1 bp, of the 5'-flanking region. FRTL-5 cells were maintained 6 days in 5H medium with 5% calf serum at which time fresh 5H medium or 5H medium containing 1 × 10^−6 M TSH (6H) was added for 36 h. Cell extracts were prepared, incubated with [32P] radiolabeled Fr168(A) or Fr127(B) of the class I 5'-flanking region, and evaluated by EMSA. Fr 168 is diagrammatically depicted in Fig. 2; Fr127 deletes nucleotides 168 through 128. Incubations were additionally performed in the presence of single strand oligonucleotide TIF(−) (A, lane 1; B, lane 3), a TSEP-1 binding site on the TSHR, or mutants thereof. Oligonucleotide TIF is one of three TSEP-1 binding sites of the TSHR, termed oligonucleotide TSEP-1(+), oligonucleotide S-box(+), and oligonucleotide TIF(−) (C); the sequences of the competitor oligonucleotides and their location in the TSHR 5'-flanking region are noted. The dark bars represent the CCTC motif which is important for TSEP-1 binding in the TSHR (19). In each case the mutant 1 (Mut. 1) oligonucleotide binds TSEP-1, whereas the mutant 2 (Mut. 2) form loses binding activity (19). Oligonucleotide TIF Mut-2, which loses TSEP-1 binding activity because of a mutation in the CCTC binding motif, does not act as a competitor (A, lane 3; B, lane 5), whereas oligonucleotide TIF Mut-1, which does not affect TSEP-1 binding activity, retains the competitive effect of the wild type oligonucleotide (A, lane 4; B, lane 6). We additionally show that double strand oligonucleotide C from the TG promoter, which can bind TTF-1 or Pax-8, does not prevent formation of the TSH-induced complex, whereas double strand CRE-1 does inhibit formation of the TSH-induced complexes.

Functional Role of Proteins That Bind to the 38-bp DNA Fragment with CRE-dependent Silencer Activity—We evaluated the functional effect of TSEP-1, TTF-1, and Pax-8 on the activity of the construct containing 127 bp of 5'-flanking sequence, p(−127)CAT, and on a derivative, in which the 8-bp CRE-like sequence was deleted p(−127 Δ-CRE)CAT. Each was cotransfected into FRTL-5 cells, maintained in the absence of TSH, along with plasmids containing the TSEP-1, TTF-1, and/or Pax-8 cDNAs: pRCMV-TSEP-1, pRCMV-TTF-1, pRCMV-Pax-8, respectively (Fig. 9). Transfections with pRCMV, the vector's only construct, served as the control in each case. Cotransfection of pRCMV-TSEP-1 decreased the promoter activity of p(−127)CAT but not the activity p(−127 Δ-CRE)CAT (Fig. 9A); the same result was obtained comparing the effect of pRCMV-TSEP-1 on p(−127)CAT versus p(−127 NP CRE)CAT, which has a nonpalindromic mutation of the CRE as described in Fig. 1B. In contrast to pRCMV-TSEP-1, cotransfection of pRCMV-TTF-1 increased the promoter activity of p(−127)CAT but not the activity p(−127 Δ-CRE)CAT (Fig. 9B). pRCMV-Pax-8 decreased the promoter activity of p(−127)CAT but not the activity p(−127 Δ-CRE)CAT (Fig. 9C), and its presence prevented the enhancer activity of pRCMV-TTF-1 (Fig. 9D). The effect of Pax-8 to reduce p(−127)CAT activity in Fig. 9C is ascribed to its action on endogenous TTF-1, whose levels are maximally expressed in FRTL-5 cells maintained in 5H medium with no TSH (15). In each case, the activity was not duplicated by the vector control, pRCMV.

These data indicated that TSH, which increased the binding
The present experiments were aimed at characterizing the role of the CRE as a MHC Class I Gene Silencer. We show, for the former, we show, for the first time, that a CRE-like sequence, −107 to −100 bp from the start of transcription, functions as a constitutive silencer of the class I gene. Thus, not only does its deletion or mutation in the homologous class I promoter result in increased expression, its insertion within a 38-bp surrounding region into a heterologous promoter decreases promoter activity as a function of copy number, so long as the CRE is intact. With respect to the latter, we also show that TSH activates the silencer, that the TSH action requires an intact CRE-like sequence, that this is one mechanism by which the hormone decreases class I expression, and that the action of TSH is cAMP-mediated, since it is duplicated by forskolin.

The CRE-like sequence appears to be a functional CRE and can bind CREB, although the binding of other CRE binding proteins as homo- or heterodimers is not excluded and is even likely. The ability of TSH to decrease CREB interactions with the silencer, in association with decreased class I transcription, suggests that CREB binding to the silencer normally functions as an enhancer of class I gene activity, in accord with its known mode of action in other genes (23, 24). One possibility for the action of TSH to decrease CREB binding is to alter its homo- or heterodimer structure. This is evidenced in our separately submitted report. The ability of anti-CREB to alter the CREB complex, and thereby duplicate the in vivo effect of TSH/cAMP-treatment of cells to increase the CRE-dependent formation of complex C and its component TSEP-1 suppressor, supports the important role of CREB binding. The decrease in CREB binding appears to be an important component of the TSH-increased binding of TSEP-1 and the TSH/cAMP-induced class I repressive effect. Since CREB and TSEP-1 are ubiquitous proteins, this result may be relevant to other tissues where hormones regulate the growth or function of a cell via the cAMP signal transduction system.

TSH/forskolin treatment of FRTL-5 cells induce the formation of a novel complex with the class I promoter, whose existence is dependent on the 38-bp silencer and its CRE. Thus, its formation with class I promoter fragments containing 168 or 127 bp of 5′-flanking region is prevented by an unlabeled oligonucleotide with the sequence of the 38-bp silencer containing the CRE but not by the same unlabeled oligonucleotide wherein the CRE is mutated or deleted. We show that in the absence of TSH, the 38-bp silencer binds a multiplicity of proteins in a CRE-dependent fashion in addition to CREB: TTF-1; a Y-box protein termed TSEP-1 which is homologous to human YB-1; a single strand binding protein, SSBP-1 (or a related protein), which binds to the TSHR; and Pax-8 which binds to the TG and TPO promoters. In addition to decreasing the interaction of CREB with the silencer, TSH treatment of the cells decreases the formation of a complex which we associate with the binding of TTF-1, but retains or enhances, at least relatively, the binding of complexes associated with TSEP-1 and Pax-8 binding. At this time, we have not determined its effect on complexes associated with SSBP-1 binding, although we anticipate these might decrease, consistent with TSH/cAMP-induced decreases in SSBP-1 RNA levels and SSBP-1 complex formation with the TSHR.

In these experiments, we show that TTF-1 is an enhancer of CRE-dependent class I promoter activity, that TSEP-1 is a suppressor, and that Pax-8 acts as a suppressor of TTF-1-dependent enhancer activity. Additionally, we show that oligonucleotides that interact with TSEP-1, but not those interacting with Pax-8, inhibit the formation of the TSH/forskolin-induced novel complexes with the class I promoter. TSEP-1 is, therefore, the dominant component of the TSH-induced novel complex; since TSEP-1 is a suppressor, it is not surprising class I expression is decreased.

Together with previous observations, the above results can be incorporated into a reasonable model of TSH/cAMP control of class I gene expression via the novel silencer described herein. In previous experiments in FRTL-5 cells, we have shown (15, 16) that TSH/cAMP decrease TTF-1 RNA levels and
that this decrease can account for a decrease in complex formation with TTF-1 binding sites. Similarly, we have previously shown (17, 20) that TSH/cAMP decreases SSBP-1 RNA levels and that this decrease can account for a decrease in complex formation with SSBP binding sites. We have shown in FRTL-5 cells that TSH/cAMP, in contrast to decreasing TTF-1 complex formation with the TTF-1 sites, increases Pax-8 complex formation to sites that bind both TTF-1 and Pax-8 (15, 16). Finally, we have shown in FRTL-5 cells that TSEPi binding can be increased by protein kinase A pretreatment of extracts from cells incubated without TSH, i.e. phosphorylation can increase TSEPi binding (19). This is consistent with studies of Y-box proteins in oocyte development (55).

A reasonable model to interpret the data in this and our previous reports is, therefore, as follows. The 38-bp silencer binds a multiplicity of proteins. In the absence of TSH, TTF-1 and CREB form prominent complexes and are enhancers that maximize class I expression in the cells. In the presence of TSH and its activated cAMP signal, CREB and TTF-1 binding are decreased, the former by an unknown mechanism possibly related to altered heterodimer formation, the latter reflecting a TSH-induced decrease in TTF-1 RNA and protein levels. This results in decreased maximal class I expression. We suggest that TSH/cAMP, simultaneously with decreased maximal class I expression, cause a protein kinase A-mediated phosphorylation of TSEPi. TSEPi acts as a suppressor of class I activity. Pax-8 binding is preserved or relatively enhanced, thereby additionally minimizing TTF-1 enhancer binding and activity. The net result of the changes in the interaction of these proteins (CREB, TTF-1, Pax-8, and TSEPi) with the 38-bp silencer is the formation of new complexes with the class I promoter and class I suppression. Formation of the complexes is dependent on the CRE-like element, -107 to -100 bp, and appears to reflect a dominant role of TSEPi and an ancillary one for Pax-8.

TSH up-regulates the function and growth of the thyroid cell; during the early stages of the cell cycle, it coordinate decreases TSHR and MHC class I gene expression (1, 2, 56). We have hypothesized that this is necessary to maintain self-tolerance in the face of the increase in proteins associated with TSH-induced growth and function (1, 2, 4–6). The present data are consistent with this hypothesis. First, Pax-8 suppresses TTF-1-induced class I promoter activity, while simultaneously acting as an enhancer of TG or TPO gene expression (21, 22, 26). Pax-8 is, therefore, a transcription factor whose binding activity is increased by TSH/cAMP (15, 16), whose activity is associated with increased thyroid function, TG synthesis, or TPO activity, and yet whose activity simultaneously helps suppress class I gene expression. Second, human Y-box homologs of TSEPi are able to bind to c-myc (46, 47, 57, 58) and to other genes associated with activation of growth: the epidermal growth factor receptor and c-Ki-ras (59–61). Sabath et al. (62) have suggested that TSEPi might stimulate the transcription of numerous growth-associated genes. TSEPi, therefore, functions not only in the TSH/cAMP-induced suppression of TSHR and MHC class I, but also, simultaneously, is likely to be a positive regulator of the growth of FRTL-5 cells.

In sum, common transcription factors are involved in TSH/cAMP-induced negative regulation of class I and the TSHR as hypothesized (1, 2, 4–6) and in TSH/cAMP-induced positive regulation of TG and TPO regulation. The net result is cross-talk which suppresses class I and preserves self-tolerance, while allowing TSH-induce thyroid growth and function to proceed.

Y box proteins are known to suppress MHC class II gene expression and are important in cAMP-induced down-regulation of class II (63–65). Since aberrant class II, as well as increased class I expression, is associated with thyroid autoimmunity (3, 6), coordinate suppression of class I and class II by a common transcription factor would additionally preserve self-tolerance in the thyroid cell challenged with TSH (6).

There is a feature of the Y-box protein family members that may be relevant to understanding the break down of tolerance and the development of autoimmunity. Infections by hepatitis C, retro-, and foamy viruses have been associated with the appearance of thyroid autoantibodies and frank thyroid autoimmunity of various types (66–73). All are single strand RNA viruses; foamy is a retrovirus family member with a similar long terminal repeat (74). We have identified a binding site for TSEPi on the hepatitis C virus upstream region, which is important in strand replication.4 10 Studies by Kashanchi et al. (75) reveal the involvement of Y-box proteins in the transcription of human T-cell lymphotrophic virus type-1. Thus, the downstream regulatory element 1 in the long terminal repeat of human T-cell lymphotrophic virus type-1, as well as the site A region of human immunodeficiency virus, both contain Y-box elements. In addition, cotransfection of Jurkat T-cells with a YB-1 expression vector and wild type or mutant viral promoter-CAT constructs demonstrated that the Y-box sequence was essential for efficient transactivation. Kashanchi et al. (75) suggest that interleukin-2 induction of YB-1, as described earlier for T-cell proliferation, might lead to stimulation of viral gene expression and viral replication (75). We speculate that autoimmunity may be precipitated by the activation of positive strand RNA viruses infecting cells, “capturing” TSEPi, and using it, and/or other single strand binding proteins such as SSBP-1 which also regulates class I and the TSHR, to promote viral replication. This results in the loss of normal negative regulation of host genes important for self-tolerance.

Concern can be expressed that the present data and studies in thyrocytes in culture are not relevant to the whole animal. In this respect, previous work in transgenic animals has shown that downstream sequences including the silencer region can influence the pattern of tissue-specific class I expression and the ability of the gene to respond to γ-interferon (76). The present report is consistent with the former point; our preliminary report,5 which shows the CRE is a critical element in the γ-interferon response, further supports this.

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