Human neuronal cells express neither major histocompatibility complex (MHC) class I RNA nor cell surface molecules but can be induced to do so by various cytokines. In the present studies, we report that expression of MHC class I in a neuroblastoma cell line, CHP-126, is actively repressed. This repression is mediated by the combined effects of a series of upstream silencer elements. Removal of the silencers reveals not only an active promoter element but also the presence of an active enhancer. Four silencers have been identified and shown to have distinct sequences, binding factors, and patterns of function. One element is located between −724 and −697 base pairs (bp) and corresponds to a silencer involved in tissue-specific regulation of class I gene expression. Three additional elements occur between −503 and −402 bp. One of these corresponds to a c-jun responsive element. Neither of the remaining elements corresponds to DNA sequences known to regulate expression of other genes. These data demonstrate that MHC class I expression normally is actively repressed in neuronal cells and suggest a model of rapid and specific triggering of class I in neuronal cells in response to infection.

Major histocompatibility complex (MHC) class I molecules function as cell-surface receptors for intracellularly derived peptides and provide immune surveillance by targeting those cells infected with intracellular pathogens for elimination by cytotoxic T cells (1). Inappropriate high expression of class I has been associated with autoimmune disease and destruction of normal cells (2). Consistent with their role in immune surveillance, MHC class I molecules are expressed constitutively on nearly all normal somatic tissues, although the level of expression varies (3). Thus, the highest levels of class I expression are observed in lymphoid tissues, with lower but varying levels in peripheral tissues. The question of whether MHC class I expression occurs in the central nervous system has been controversial. Original attempts to assess MHC class I in the brain did not detect expression in any cell types. Human neuronal tissue and normal neuronal cell lines do not have detectable levels of class I RNA or protein, suggesting that normally class I genes are not transcribed in these cells (4–6).

Tissue-specific levels of class I expression are achieved by active transcriptional regulation which is necessary to maintain a normal immune response. Molecular analysis of the extended class I promoter revealed the presence of a series of negative and positive regulatory elements that can be broadly divided into domains governing tissue-specific and hormonal regulation. Thus, a complex regulatory element is responsible for maintaining tissue-specific levels of expression (the tissue-specific element, or TSE) (7). It consists of an overlapping enhancer and silencer, extending over 78 bp (−768 to −689 bp).

The constitutive activity of the class I promoter in any given tissue is in part determined by the relative activities of the enhancer and silencer in that tissue (7). Additional constitutive silencers, such as a c-jun element (−420 to −413 bp) also contribute to this regulation (8). MHC class I promoters also respond to a variety of cytokines (9). Activated transcription is regulated by elements broadly centered around an enhancer, enhancer A, located between −180 and −170 bp and an overlapping interferon response sequence (10, 11). The cognate transcription factors, NFκB and IRF-1, are induced by TNF and interferon, resulting in a concomitant enhancement of expression of class I genes (11). Thus, distinct regulatory elements determine constitutive and activated transcription of the promoter; their activities are integrated at the basal promoter.

The molecular basis for the failure to express basal levels of class I genes in neuronal cells has not been established. Two possible mechanisms can be considered. The generally assumed mechanism is that the class I promoter is simply inactive in neuronal cells and requires activation by cytokine-induced transcription factors (4). However, the alternative possibility is that the class I promoter is constitutively functional but actively repressed by upstream elements. The first suggestion that active repression of class I expression occurs in neuronal cells originally came from studies of transgenic mice constructed with class I promoter constructs. In transgenic mice with a full-length class I promoter region, containing all of the upstream regulatory elements, there was no detectable promoter activity in the brain (12). However, moderate transgene promoter activity was observed in brains of transgenic mice containing a class I promoter truncated to within 500 bp of transcription initiation (13). It is also now clear that cells of glial origin do express low levels of class I, which can be further induced by cytokines (14). Furthermore, expression of class I in the brains of pigs is readily detectable (12).

To distinguish between these two possibilities, the ability of a class I promoter to function in a human neuroblastoma line was studied. The human neuroblastoma line, CHP-126, which has been used as a model for the study of class I regulation, does not express class I under normal growth conditions (9). Here we report that the class I promoter is active in CHP-126 cells but that the failure to express class I is the result of active repression.
**EXPERIMENTAL PROCEDURES**

**Cell Lines—**HeLa cells were obtained from ATCC and were grown in Dulbecco's modified Eagle's medium, supplemented with 4 mM glutamine, 100 μg/ml gentamicin, 10% fetal calf serum. CHP-126 neuroblastoma cells were kindly provided by Drs. Lois Lampson (Brigham and Women's Hospital, Boston, MA) and Henry McFarland (NINDS, NIH, Bethesda, MD) and were grown in Dulbecco's modified Eagle's medium supplemented with 4 mM glutamine, 0.1 mM nonessential amino acids, gentamicin sulfate (10 μg/ml), and 10% fetal calf serum. Both cell lines were maintained in a humidified incubator at 37 °C, 7.5% CO₂.

**Promoter Constructs—**The 5'-truncation series was derived from the MHC class I promoter, PD1, as described previously by targeted polymerase chain reaction amplification; all constructs terminate at an inserted 3' HindIII site at +1 (8). The deletion of the cjun binding site, to generate the construct of ~500XCAT, was described in Howcroft et al. (8). The construct ~503/TSEsilCAT was constructed by inserting a double-stranded oligonucleotide corresponding to the TSE silencer (~724 to ~697 bp) with NdeI ends into the NdeI site within the vector of ~503CAT (7), a single copy of the silencer element inserted in antisense orientation. Double-stranded oligonucleotides A1, A2, B1, and B2 (see Table II for DNA sequences) were synthesized with XbaI ends and cloned into the XbaI site of ~503CAT. All constructs were sequenced across the insert to determine orientation and to verify the DNA sequence.

**Transfections and CAT Assays—**Transfection of DNA was using the CaPO₄ method (15), with 10 μg of plasmid DNA per 10⁶ cells, in 100 mm dishes. 1 × 10⁶ CHP-126 cells or 7.5 × 10⁵ HeLa cells were plated 24 h prior to transfection. Cells were fed 24 h after transfection and were harvested after an additional 24 h.

**CAT assay was as described by Gorman et al. (15). For HeLa cell transfections, enzymatic activity was normalized to either total protein or to a co-transfected RSV-luciferase control, with equivalent results. For CHP-126 cell transfections, none of the transfection control vectors tested, which use viral promoters, was expressed. Therefore, all transfections were normalized to total protein but were repeated with at least three different plasmid preparations, in at least three independent transfections. Furthermore, all constructs in a deletion series were assayed in parallel in a single experiment, allowing direct comparison of relative promoter efficiency.

**Gel Mobility Shift Assays—**DNA fragments used in the gel shift assay were a 105-bp NdeI/Xbal fragment spanning 502 to 398 bp, a 254-bp DdeI fragment spanning 890 to 637 bp, and a 140-bp AvaI fragment derived from the 254-bp fragment (7). DNA fragments to be used as probes were prepared by end labeling with [³²P]ATP by T4 kinase or by fill-in using [³²P]dCTP, using Klenow fragment.

**Cell extracts were prepared by a modification of the Dignam procedure, as described previously (7). For gel shift assays, 3 μg of cell extract was incubated with 1.5-fmol probe, in binding buffer, 3 μg of poly(dI-dC), and 5% glycerol for 30 min. The binding buffer consisted of 10 mM Tris, pH 7.9, 1 mM EDTA, and 1 mM MgCl₂. All gel shift reactions with the various DNA fragments were performed under the following conditions: 140-bp fragment was at room temperature in 125 mM NaCl, 105-bp fragment on ice at 105 mM NaCl, and 254-bp fragment at room temperature in 150 mM NaCl. For either cold competition with double-stranded oligonucleotides or antibody supershifts, cell extracts were preincubated with the competitor or antibody for 30 min on ice before addition of radiolabeled probe. Competitor double-stranded oligonucleotides were added at a 10⁶ molar excess. All antibodies were purchased from Santa Cruz Laboratories and used at the recommended concentrations.

**Flow Cytometry—**Flow cytometric analysis of CHP-126 cells was as described previously (16). Briefly, CHP-126 cells were harvested and stained with either fluorescein isothiocyanate-conjugated monoclonal antibody to human MHC (W6/32), kindly provided by Julie Titus, NIDDK, or with a control fluorescein isothiocyanate-conjugated antibody, anti-IA4 (Pharmingen, catalog number 0627AD). Cells (10,000) were analyzed by flow cytometry on a Becton Dickinson FACScan.

**RESULTS**

**MHC Class I Genes Are Not Expressed in CHP-126 Cells—**Under normal growth conditions, CHP-126 cells express neither cell surface MHC class I (Fig. 1) nor class I RNA (data not shown). Introduction into CHP-126 cells of an exogenous full-length class I promoter construct, consisting of 1090 bp of 5'-flanking sequences ligated to the CAT reporter gene, results in levels of CAT activity that are about 100-fold less than observed in HeLa cells (data not shown). The following studies were undertaken to determine whether the class I promoter is inactive in neuronal cells or actively repressed.

**Class I Expression Is Actively Repressed in CHP-126 Neuroblastoma Cells—**The 5'-flanking region of the MHC class I promoter contains an array of silencer and enhancer elements that regulate its expression (7, 17). To determine whether any of these regulatory elements functions in CHP-126 cells, 5'-truncation constructs derived from the ~1090CAT promoter construct were assessed for their activity when transfected into CHP-126 cells. As shown in Fig. 2A, successive truncations between ~1090 and ~402 bp increasingly activate the class I promoter in CHP-126 neuroblastoma cells, indicative of the presence of multiple silencer elements. Within the series, the ~402CAT construct displays maximal promoter activity, achieving a level nearly 10 times greater than the full-length ~1090CAT construct. Thus, the class I promoter is constitutively active but repressed by upstream silencer elements.

Further truncation of the class I promoter region from ~402 to ~294 bp results in a marked decrease in promoter activity, demonstrating the presence of an active enhancer element in this region. We have identified this novel enhancer as an element that spans a USF-binding E-box located between ~309 and ~314 bp; it is distinct from the previously described enhancer A (18, 19) which is located further downstream between ~170 and ~180 bp.

Taken together these data demonstrate that in CHP-126 cells, the class I promoter is capable of high levels of activity but is actively repressed. The remaining studies were directed toward the characterization of the silencer elements that repress constitutive class I promoter activity in CHP-126 neuroblastoma cells. We describe four such silencer elements, as summarized in Fig. 2B.

**A Silencer Element Is Contained within the Tissue-specific Regulatory Element—**Repression of the class I promoter in CHP-126 cells is partially mediated by a silencer element(s) contained between ~1090 and ~503 bp (Fig. 2). Previous analysis of the class I promoter in this region demonstrated the presence of a complex regulatory element, consisting of overlapping enhancer (~768 to ~689 bp) and silencer elements.

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**Fig. 1.** CHP-126 cells do not express detectable levels of MHC class I molecules. CHP-126 cells (left panels) or HeLa cells (right panels) were stained with either a control antibody or an anti-class I antibody (W6/32) that binds all human class I molecules and were analyzed by fluorescence-activated cell sorting as described under “Experimental Procedures.”
Fig. 2. A series of silencer elements decreases downstream class I promoter activity in CHP-126 cells. A, a nested series of truncation mutants with a common 3′ terminus at +1, but differing 5′ termini (as indicated), ligated to the CAT reporter, were transfected into CHP-126 cells. Promoter activity is relative to the −503/CAT construct. B, map of the class I promoter region, indicating locations of silencer and enhancer elements and some of the oligonucleotides used in analyses. TSE, tissue-specific element that consists of overlapping enhancer (inverted arrows) and silencer (hatched boxes) that regulates constitutive expression; Sil-A, silencer that functions in CHP-126 cells but not HeLa cells; Sil-B, element that functions in CHP-126 cells as a silencer and in HeLa cells as an enhancer; c-jun, silencer that functions in both CHP-126 and HeLa cells as a silencer and binds c-jun; E-box, enhancer element that functions in CHP-126 cells; A, IRE, enhancers in activated transcription.

The TSE silencer reduces class I promoter activity in CHP-126 and HeLa cells

The silencer component (−724 to −697 bp) of the TSE was inserted into the −503 truncation construct of the class I promoter to generate the −503/TSEsil construct. Both the parent and derivative constructs were transfected into either CHP-126 or HeLa cells; promoter activity was assessed by CAT assay, as described under “Experimental Procedures.” The results are expressed relative to the parent construct. Transfections were repeated six times for CHP-126 cells and eight times for HeLa cells. The level of repression resulting from the TSEsil was significant in both CHP-126 (p < 0.0005) and HeLa cells (p < 10−4). There was no significant difference in the level of repression between the two cell lines (p < 0.2).

| Promoter construct | Relative promoter activity |
|--------------------|---------------------------|
| CHP-126            | HeLa                      |
| −503CAT            | 1                         |
| −503/TSEsilCAT     | 0.404 ± 0.064             |

TABLE I

The factors that associate with the TSE silencer are not known. In gel mobility supershifts with antibodies directed against known transcription factors, none of the following antibodies had any effect on the complexes: SAP, SRF, YY1, E2F, c-fos, c-jun, n-myc, p53, and NF-κBp65 (data not shown). This result was not surprising, since the DNA sequences of the silencer and enhancer do not correspond to the binding of any known mammalian transcription factor. Surprisingly, antibodies directed against ATP2, Max, and TAF1/250 were able to shift the TSE silencer complexes in both HeLa and CHP-126 extracts (data not shown); none of these factors is known to

(−724 to −697 bp) (Fig. 2B) (7). Levels of class I expression in a variety of tissues were shown to be governed by this element (TSE, see Fig. 2B). To determine whether the silencer component of the TSE also functions in neuronal CHP-126 cells, its effect on class I promoter activity was assessed. Truncation of the class I promoter to −503 resulted in about a 3-fold enhancement of promoter activity in CHP-126 cells (Fig. 2A). Insertion of the isolated silencer segment (−724 to −697 bp) upstream of the truncated −503 promoter construct reduced promoter activity in both CHP-126 and HeLa cells (Table I). Thus, the TSE silencer functions to repress promoter activity in CHP-126 cells, as well as in HeLa cells. The magnitude of this repression is comparable with that mediated by the segment between −503 and −1090 bp, suggesting that the TSE is the major silencer element in this region of the promoter.

To characterize the DNA-binding factors in CHP-126 cells that interact with the TSE, gel shift assays were performed using a radiolabeled fragment that spans the entire TSE (254-bp probe in Fig. 2B). CHP-126 extracts generate three bands with this probe (Fig. 3). In contrast, extracts from HeLa cells generate only two of the three bands (Fig. 3). Previous studies of HeLa extracts identified the upper band as associated with the silencer element (thin arrow), whereas the lower band reflects binding to the enhancer moiety (dashed arrow) (7). To identify the target DNA binding sites of the three complexes in CHP-126 cells, oligonucleotides containing the binding sites for either the enhancer (E9), the enhancer and silencer (S2), or a control (S3) were used as cold competitors (see Fig. 2B). The enhancer-specific oligonucleotide, E9, eliminated only the lower band, indicating that it corresponds to enhancer binding. The two remaining bands were both inhibited by S2 but not by either E9 or the control, mapping their target site to the silencer (Fig. 3). Thus, both CHP-126 and HeLa cell extracts appear to contain common binding factors
demonstrating that the c-deletion resulted in markedly increased promoter activity, segmentsof theregionbetween
silencer elements Sil-A, Sil-B, and the c-promoter in CHP-126 cells was shown by deleting the c-blasts(8)(Fig.2
bp that has been shown previously to also function in fibro-
elements tothese silencers are specific forCHP-126 cells. The third silencer
results in increased activity of the downstreampromoter, indi-
ation in CHP-126 cells.
503 bp there are additional silencer elements that func-
tioned in CHP-126 cells but as an enhancer in HeLa cells. Relative activities were compared with the paren-
tal −402CAT construct.

Fig. 3. CHP extracts form a novel complex with the TSE. Gel
shifts using a radiolabeled 254-bp probe, which includes the 100-bp TSE
element with either CHP-126 (left panel) or HeLa (right panel) extracts,
in the presence or absence of 100-fold excess of unlabeled double-
stranded oligonucleotide competitors corresponding to the silencer (S2),
the enhancer (E9), and a flanking, control sequence (S3) are shown.

Taken together, these data indicate that one of the silencers
regulating MHC class I promoter activity in neuronal CHP-126
cells is the silencer component of the tissue-specific element,
TSE, which has previously been demonstrated to span 28 bp
and to consist of a discontinuous binding site of 10 and 8 bp,
separated by 10 bp.(7). These studies also provide further evi-
dence of the role of the TSE in regulating tissue-specific ex-
pression of class I.

The DNA Segment −503 to −402 bp Contains Three Silencer
Elements That Function in CHP-126 Cells—As shown in Fig.
2A, removal of DNA sequences between −503 and −402 bp
results in increased activity of the downstream promoter, indi-
cating that this DNA segment contains additional silencer el-
ements. In the following experiments, we demonstrate that
three silencer elements are contained in this region. Two of these silencers are specific for CHP-126 cells. The third silencer
element is a c-jun binding site located between −420 and −413
bp that has been shown previously to also function in fibro-
blasts(8) (Fig. 2B). That this latter element regulates the class
I promoter in CHP-126 cells was shown by deleting the c-jun
binding site from the −503 construct to generate −503X. This
deletion resulted in markedly increased promoter activity,
demonstrating that the c-jun site functions as a silencer in
CHP-126 cells (Fig. 4A). Although the −503XCAT construct
was more active than the parental −503CAT construct, it was
still significantly less active than the −402CAT construct.
These data suggest that in the DNA segment between −402
and −503 bp there are additional silencer elements that func-
tion in CHP-126 cells.

To identify these additional silencers, double-stranded oligo-
nucleotides corresponding to the 5′ (A1, A2) and central (B1)
segments of the region between −402 and −503 bp (see Fig. 2B)
were cloned upstream of the −402CAT construct, and their
effects on promoter activity were examined (Fig. 5). In CHP-
126 cells, all of the constructs (402A1, 402A2, and 402B1) had
lower promoter activity than the parental −402CAT construct
(Fig. 5A); this effect was independent of orientation of the DNA
fragment (data not shown). These data demonstrate that the
interval between −402 and −503 bp contains three functional
silencer elements Sil-A, Sil-B, and the c-jun silencer (summa-
ized in Fig. 2B and Table II).

Whereas all three elements function as silencers in CHP-126
cells, they differ in their activities in HeLa cells (Fig. 4B and
5B). In HeLa cells, deletion of the c-jun binding site in the
−503XCAT construct rendered the promoter more active than
in the parental −503CAT construct, demonstrating its function
as a strong silencer. However, the −503XCAT construct was
also significantly more active than the −402CAT construct,
suggesting that the segment between −503 and −402 contains
an enhancer element whose activity is normally obscured by
the c-jun silencer (Fig. 4B). This enhancer is identified as Sil-B,
since introduction of oligonucleotide B1 upstream of −402CAT
increases promoter activity in HeLa cells (Fig. 5B). In contrast,
the Sil-A element (402A1 and 402A2) had no significant effect
on promoter activity in HeLa cells (Fig. 5B). These effects were
also independent of orientation (data not shown). Taken to-
gether, these studies defined three types of elements, the c-jun
binding site that acts as a repressor in both cell types, the Sil-A
element that functions as a silencer in CHP-126 but not HeLa
cells, and the Sil-B element that functions as a silencer in CHP-
126 cells but as an enhancer in HeLa cells.

Discrete Protein-DNA Complexes Correlate with Functional
Silencer Activity in the −503 to −402-bp DNA Segment—The

Fig. 4. The c-jun binding site functions as a downstream sil-
cencer in CHP-126 cells (A) and HeLa cells (B). The constructs
−402CAT and −503CAT differ in the lengths of their 5′-flanking se-
quence. −503CATwas derived from −503XCATby deletion of the c-jun
binding site (8). Results are expressed relative to the −402CAT
construct.

Fig. 5. Two other sequences between −503 and −402 bp function as downstream silencers in CHP-126 cells (A) but not in
HeLa cells (B). The double-stranded oligonucleotides A1, A2, B1,
and B2 were introduced into a unique XbaI site at the 5′ terminus of the
−402CAT construct. Relative activities were compared with the paren-
tal −402CAT construct.
above studies mapped the CHP-126-specific silencer, Sil-A, to a 25-bp segment (−503 to −479 bp) and the tissue-specific enhancer/silencer Sil-B to a 28-bp segment (−440 to −433 bp). To determine whether any protein complexes associate with these functionally defined regions, a radiolabeled 105-bp DNA fragment, extending from −439 to −503 bp (see Fig. 2B), was used as a probe in gel shift assays with cell extracts from either CHP-126 cells or HeLa cells (Fig. 6A). Both extracts formed a low mobility complex that was competed by an unlabeled oligonucleotide corresponding to the 5′ silencer (A4), suggesting that both cell types contain factors that specifically interact with this sequence (thin arrow, Fig. 6A). Fine mapping of this common complex using a series of oligonucleotide competitors localized the recognition sequence to the sequence ATGGAT-TAT (summarized in Table II, lines A1 through A7). To identify the protein components of these complexes, gel supershift assays were performed with a variety of antibodies to known transcription factors (Fig. 6B). Only antibodies to Max and ATF2 affected complex formation, and only the low mobility complex common to the CHP-126 and HeLa extracts was affected. Thus, although the Sil-A element functioned as a silencer in CHP-126 but not in HeLa cells (Fig. 6A), both cell types formed common complexes in both extracts. It is likely that the complexes differ either (a) by the presence of additional factors or (b) in post-translational modifications of the component factors.

CHP-126 extracts also generated a novel complex with the 105-bp probe (thick arrow, Fig. 6A). By competition with unlabeled oligonucleotides, this complex mapped to the central enhancer/silencer region (B3 in Fig. 6A, summarized in Table II, lines B1 through B7) to the sequence CTATTATTTATATA. A core sequence of TATTATTT was shown to be necessary for stable complex formation in gel shift, since an oligonucleotide (B2) mutated in this sequence was unable to compete the complex. However, this core sequence is not sufficient for complex formation, since an oligonucleotide consisting only of the core sequence (B4) did not compete, whereas an extended oligonucleotide (B5) did (Table II). Consistent with the conclusion that the functional element consists of an extended sequence, the mutated B2 oligonucleotide still functioned as an enhancer/silencer when placed upstream of the −402CAT construct (Fig. 5, 402B2). This complex was not supershifted by any of the antibodies to known transcription factors (Fig. 6B and data not shown). Furthermore, a scan of the existing transcription factor data bases did not reveal similarities between either Sil-A or Sil-B and known binding sites of characterized transcription factors.

Although the recognition sequences of Sil-A and Sil-B share a common core of ATTAT (see Table II), they do not bind common factors. Thus, an oligonucleotide that specifically competes complexes associated with Sil-A (A4) does not affect Sil-B complexes. Conversely, oligonucleotide B3 competes Sil-B, but not Sil-A, complexes (Fig. 6A, Table II).

We have previously noted that c-jun in L cell extracts does not form complexes with the 105-bp segment that are detected by the gel shift assay (8). Similarly, neither the HeLa nor the CHP-126 extracts generated complexes with the c-jun binding site, as evidenced by the finding that an oligonucleotide spanning the c-jun binding site (B7) did not compete any of the complexes (Fig. 6A).

**Upstream and Downstream Silencer Elements Bind Common**
Factors in CHP-126 Extracts—Analysis of the factors that bind to the silencer elements by antibody supershift revealed that anti-Max and anti-ATF2 interacted both with complexes formed with the upstream TSE and with the downstream silencer Sil-A (Figs. 3 and 6). This raised the possibility that common factors might associate with the various silencers. To test this, complexes formed with the radiolabeled 105-bp probe containing Sil-A and Sil-B were competted with the unlabeled 140-bp fragment containing the upstream tissue-specific element (see Fig. 2B). As shown in Fig. 7 (left), the upstream TSE effectively competted both the upper Sil-A complex as well as the lower Sil-B complex associated with the radiolabeled 105-bp fragment. In contrast, an unrelated fragment derived from a downstream 178-bp DNA segment did not compete either (Fig. 7, left). To further delineate the common binding site(s), oligonucleotides derived from the upstream TSE silencer were used as cold competitors with the labeled 105-bp probe (Fig. 2B and 7A, right). The oligonucleotide S2, which contains the TSE silencer sequence, was able to compete both the silencer bands associated with the downstream 105-bp fragment (Sil-A and Sil-B). In contrast, neither oligonucleotide E9, which is specific for enhancer factors, nor S3, an irrelevant control, inhibited complex formation with the 105-bp probe. These data indicated that common factors bound to the upstream TSE silencer and to the downstream silencers Sil-A and Sil-B.

Sil-A and Sil-B bind distinct factors, as evidenced by the finding that they do not compete with one another for complex formation (see Fig. 5A and Table II, where oligonucleotide A4 competed only the upper complex and B3 competed only the lower complex). However, the TSE inhibited both complexes (Fig. 7A), indicating that the upstream TSE silencer contains two distinct binding sites for silencer factors, corresponding to Sil-A and Sil-B factors. To confirm this conclusion, gel shift competition assays were performed with a radiolabeled 140-bp probe containing the TSE in the presence of oligonucleotides A4 and B5 derived from the two downstream silencers, Sil-A and Sil-B, respectively (Fig. 7B). As predicted, each of the downstream silencer oligonucleotides inhibited only one of the two silencer-associated complexes. The B5 oligonucleotide, which corresponds to the CHP-specific downstream silencer, Sil-B, competed the CHP-specific complex formed by the upstream silencer (thick arrow). The A4 oligonucleotide, which corresponds to Sil-A, competed the common complex formed by the upstream silencer (thin arrow). Alignment of these oligonucleotide sequences with the TSE does not reveal any homologies that clearly define common binding sites. Although all three sequences contain the sequence motif TTATT, this sequence also occurs in the oligonucleotide E9 which fails to compete any of the silencer complexes. These findings suggest the possibility that distinct factors directly bind the DNA sequences and that the common factors associate indirectly, through protein interactions.

DISCUSSION

The human neuroblastoma cell line, CHP-126, does not normally express either class I RNA or cell surface protein (5, 6). In the present studies, we have demonstrated that the absence of class I gene expression is due to active repression of a functional class I promoter. This repression is accomplished through the additive effects of a series of upstream silencer elements. Removal of these silencers reveals not only an active promoter element but also the presence of a functioning enhancer. The four silencers responsible for repression have been characterized and shown to have distinct sequences, binding factors, and patterns of function. One, located between −724 and −697 bp, functions in all cell types as a constituent of a tissue-specific regulatory element that serves to maintain tissue-appropriate levels of class I expression (17). A second element, located between −309 and −314 bp, is also a constitutive silencer in all cell types and is regulated by c-jun (8). Two additional elements (Sil-A and Sil-B in Fig. 2B), both located between −402 and −503 bp, function as silencers in CHP-126 cells but not in HeLa cells.

The downstream silencers display differing functions in CHP-126 cells and HeLa cells. In particular, Sil-B is a silencer element in CHP-126 cells but an enhancer in HeLa cells. Although it functions in HeLa cells, no specific DNA-binding protein complex could be identified. In contrast, Sil-A, which forms indistinguishable protein complexes in CHP-126 and HeLa extracts, functions as a silencer in CHP-126 cells but has no detectable function in HeLa cells. The relationship between the various complexes detected by gel shift and the functioning of the elements remains to be determined. One possibility is that post-translational modifications determine function but...
not binding. Alternatively, binding of proteins may occur in cell extracts that do not occur in vivo.

The silencer elements coordinate repression of constitutive class I transcription through a complex set of interactions. The two CHP-126-specific silencer sequences, Sil-A and Sil-B, do not bind factors in common, as demonstrated by their failure to compete complexes formed by the other in gel shift assays (Fig. 6A). But each does bind factors in common with the tissue-specific upstream silencer (TSE in Fig. 2B) (Fig. 7). The helix-loop-helix transcription factor Max was identified as a component of the CHP-126-specific complexes associated with Sil-B and TSE. Since Max most often binds DNA in association with other helix-loop-helix factors, it is likely that each of these complexes contains additional factors. In addition, since the DNA sequences of the upstream and downstream silencers have little homology to one another, it is also probable that the associated complexes are distinct from one another. Because neither element contains a canonical E-box motif (CACGTG), which is the recognition site for Max (20), it is possible that Max is not binding the DNA sequence directly but rather associates with proteins that are. Similarly, the ATF-2 transcription factor is a component of the silencer complexes common to CHP-126 and HeLa cell extracts. The compositions of these complexes are also likely to differ. It is intriguing to consider the possibility that these factors link the complexes bound to the upstream and downstream silencers, to create higher-order interactions that regulate the promoter in concert. Similar structures, termed enhanceosomes, have recently been described and postulated to allow integrated regulation of the downstream promoter (21). Further characterization of these complexes and their mode of action will require biochemical purification and analysis.

In CHP-126 cells, the class I promoter is regulated not only by the series of silencers located between −402 bp and −1090 bp but also by an enhancer that maps between −309 and −416 bp. This enhancer is critical for constitutive promoter function in CHP-126 cells, since its removal results in a complete loss of promoter activity. This is in contrast to the situation in HeLa cells, where the enhancer is not required for class I promoter activity, since promoter constructs with as little as 50 bp of 5′ sequence are functional (16). Taken together, these data indicate that class I promoter activity is simultaneously negatively and positively regulated in CHP-126 cells. These data also resolve an apparent discrepancy with earlier studies, which failed to detect either constitutive class I promoter activity or promoter activity of class I promoter/CAT constructs transfected into CHP-126 cells (4). However, in those studies, the promoter constructs used were either shorter than 300 bp or longer than 1.4 kilobase pairs and would thus either lack an enhancer or encompass silencer elements, respectively.

The transcription factor, NFκB, is not constitutively expressed in CHP-126 cells. However, it is induced following treatment of the cells with TNF and binds to an enhancer in the class I promoter, enhancer A (see Fig. 2) (10, 11). This binding has been correlated with cytokine-mediated induction of class I transcription. The present studies demonstrate that although NFκB may be necessary for cytokine-induced class I expression, it is not necessary for constitutive expression. An upstream enhancer is able to activate the promoter in the absence of either NFκB or IRF-1. The present studies clearly distin-
guish for the first time the requirements for constitutive and activated transcription.

Although many human neuroblastomas do not express class I, many murine neuroblastomas express significant levels (14). The present findings suggest that those lines that express class I may have lost the constitutive active repression mechanism, revealing the presence of an active promoter. Inhibition of the NF-κB component p50 by the oncogene n-myc has been proposed to cause the deficiency of class I expression in the rat B104 neuroblastoma line (22). Such a mechanism is unlikely to account for the failure of human CHP-126 cells to express class I, since they are devoid of p50 (10); p50 is only induced after activation of the CHP-126 neuroblastomas by cytokines (or TNF), suggesting that B104 represents a cell line that was transformed after activation. This latter example appears to reflect a viral strategy for avoiding immune surveillance rather than a normal regulatory mechanism.

The importance of minimizing class I expression in the normal neuronal cell is likely due to the necessity of avoiding deleterious inflammatory responses in the central nervous system. Furthermore, induction of class I in neuronal cells is associated with reduced bioelectrical activity (23). On the other hand, it is equally important that the neuronal cells be able to quickly identify intracellular pathogens and flag the immune system, which would require a rapid induction of class I. One way to achieve both needs would be to maintain an active class I promoter, whose expression is normally repressed but readily inducible in response to appropriate stimuli. The present studies have demonstrated the existence of such an active repression mechanism and have identified a series of both general and CHP-126-specific silencers that effect it.

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