MOLECULAR AND CELLULAR BIOLOGY, Aug. 1991, p. 4217–4227
0270-7306/91/084217-11$02.00/0

Vol. 11, No. 8

A Complex Regulatory DNA Element Associated with a Major Histocompatibility Complex Class I Gene Consists of Both a Silencer and an Enhancer

JOCELYN D. WEISSMAN AND DINAH S. SINGER*

Experimental Immunology Branch, Building 10, Room 4B-17, National Cancer Institute, Bethesda, Maryland 20892

Received 4 March 1991/Accepted 13 May 1991

A novel regulatory element which contributes to the regulation of quantitative, tissue-specific differences in gene expression has been found between −771 and −676 bp upstream of the major histocompatibility complex (MHC) class I gene, PD1. Molecular dissection of this element reveals the presence of two overlapping functional activities: an enhancer and a silencer. Distinct nuclear factors bind to the overlapping enhancer and silencer DNA sequence elements within the regulatory domain. The levels of factors binding the silencer DNA sequence in different cell types are inversely related to levels of class I expression; in contrast, factors binding the enhancer DNA sequence can be detected in all cells. In cultured cell lines, inhibition of protein synthesis leads to the rapid loss of silencer complexes, with a concomitant increase in both enhancer complexes and MHC class I RNA. From these data, we conclude that a labile silencer factor competes with a constitutively expressed, stable enhancer factor for overlapping DNA-binding sites; the relative abundance of the silencer factor contributes to establishing steady-state levels of MHC class I gene expression.

The immune responses of all vertebrate species are mediated by molecules encoded within the major histocompatibility complex (MHC) (19). The MHC class I molecules, which serve as the targets of cellular immune responses and allograft rejection, are receptors for peptide antigens (3). These molecules are transmembrane glycoproteins consisting of a polymorphic heavy chain of 42 to 45 kDa and a nonpolymorphic light chain (β_2-microglobulin) of 12 kDa. Consistent with their pivotal role in immune surveillance, the class I molecules are expressed on nearly all somatic tissues (28). However, their level of expression varies markedly among the tissues. The highest levels of cell surface expression of class I antigens occur in the lymphoid tissues: lymph node, peripheral blood lymphocytes, and spleen. Markedly lower levels are found on other somatic tissues, such as kidney and liver; there is little detectable cell surface class I antigen expression on germ line tissue or brain (28). In general, these differences reflect tissue-specific regulation of gene expression.

Analysis of the 5′ flanking regions of a number of class I genes has revealed the presence of a series of DNA elements capable of participating in the regulation of class I gene expression (Fig. 1A). Among these is a major enhancer, enhancer A, located at bp −180 to −170, which overlaps an interferon response element (15, 20, 27). A functional enhancer A is necessary for optimal expression of the downstream class I gene (13, 15, 18). A variety of *trans*-acting factors which bind to enhancer A have been identified, and their genes have been cloned (17, 21). These factors act as inducers of class I expression. Thus, KbF1 and RIIBP, both of which bind enhancer A, are associated with high levels of expression. Factors which bind specifically to the interferon response element and are induced by interferon have also been described (4, 6).

In addition to these DNA sequence elements, which are predominantly positive regulators, a negative regulatory element (silencer) has recently been described (7). This silencer was originally identified within a region of 589 bp located between 503 and 1,091 bp upstream of the promoter of the swine MHC class I gene, PD1. Removal of this DNA segment from the 5′ flanking region of PD1 resulted in increased expression from the downstream promoter by 5- to 10-fold. Furthermore, placement of the DNA segment containing the silencer upstream of the heterologous simian virus 40 promoter also reduced expression from that promoter. In vivo competition studies demonstrated that the activity of the silencer was mediated by *trans*-acting factors (7).

In this study, we extend these initial observations by reporting that the silencer is contained within a 96-bp region which consists of the silencer and an overlapping positive regulatory element (enhancer). Each DNA sequence element is recognized by a complex of cellular *trans*-acting factors. The tissue levels of silencer-binding factors vary inversely with the level of class I expression, whereas enhancer-binding factors are present constitutively. The silencer-binding factors are further shown to be labile. On the basis of these results, a model for the active negative regulation of MHC class I genes is proposed and discussed.

MATERIALS AND METHODS

Cells and cell culture. COS-1 and F9 cell lines were maintained in Dulbecco's modified Eagle's medium (Mediatek, Reston, Va.) supplemented with 10% (vol/vol) fetal calf serum (Biofluids, Rockville, Md.). M12-4.1 cells were maintained in RPMI 1640 (GIBCO) supplemented with 10% fetal calf serum, nonessential amino acids, and 2-mercaptoethanol. L(PD1) cells (stably cotransfected with the herpes simplex virus thymidine kinase gene and a 17.8-kb swine DNA fragment containing PD1) were maintained in Dulbecco's modified Eagle's medium–hypoxanthine-aminopterin-thymidine supplemented with 10% fetal calf serum. Treat-

* Corresponding author.

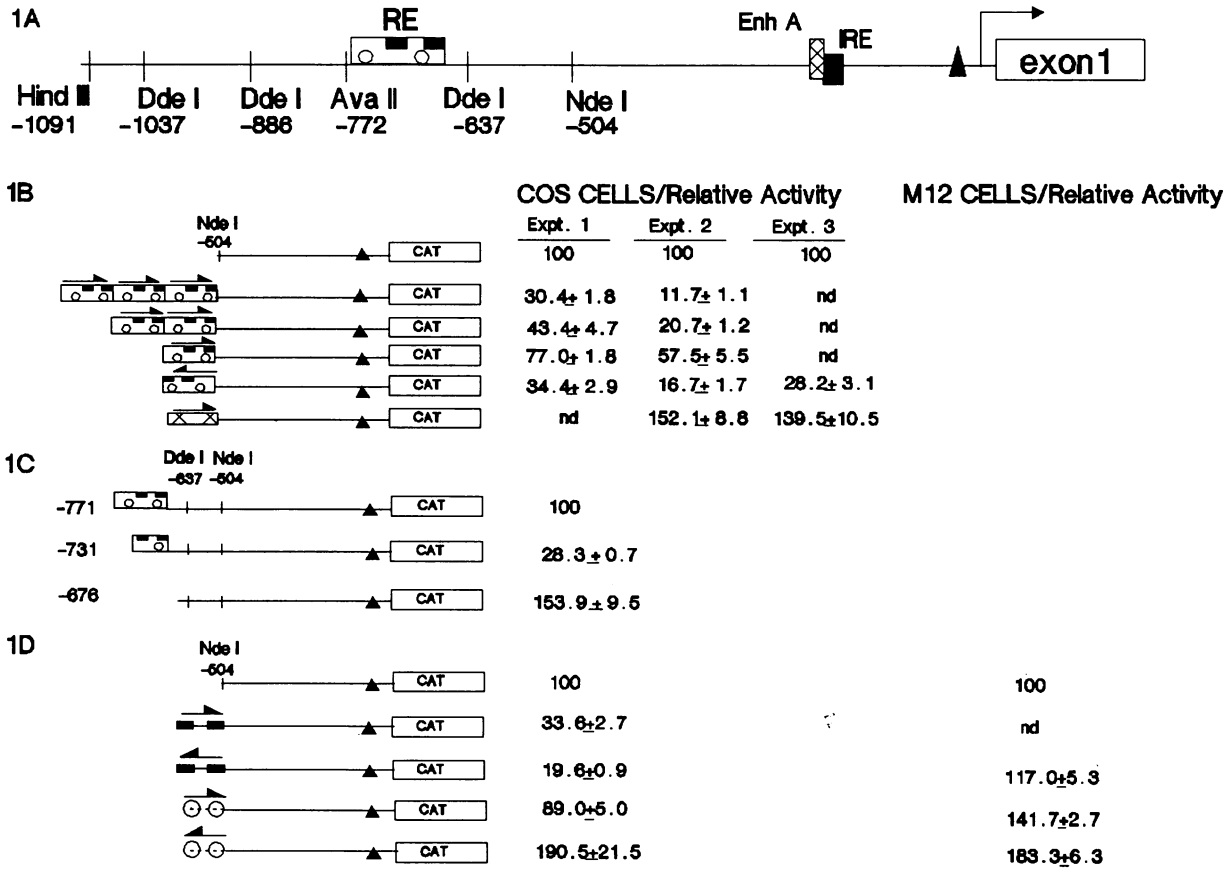

FIG. 1. (A) 5′ flanking region of PD1. The upstream region of the PD1 gene contains the promoter elements CCAAT and TCTAA (a variant of the TATAA box common in class I genes) (▲). Transcription initiates approximately 20 bp downstream of the TCTAA box (↱). Further upstream are the interferon response element (IRE; ▮) and the overlapping enhancer A (Enh A; ▮). Between bp −772 and −637 is the complex regulatory element (RE), consisting of the enhancer (○——○) and silencer (■——■), as defined below. (B) Function of the 140-bp fragment as a silencer element. A 140-bp *Ava*II-*Dde*I fragment spanning the regulatory element was cloned into the expression vector pN(−38). pN(−38) contains the core PD1 promoter and 503 bp of 5′ flanking sequences upstream of the CAT gene, terminating at the *Nde*I site. Constructs containing between one and three copies of the 140-bp insert (▮▮▮) were isolated and tested for their relative abilities to generate CAT activity following transfection into COS cells. A 151-bp fragment (⊠) derived from bp −1036 to −885 of PD1, with no known function or factor-binding capacity (data not shown), was used as a control for nonspecific reduction of CAT activity. The results represent averages of three independent transfections in three separate experiments and are expressed as the means ± standard errors of the triplicates within each experiment. (C) Use of deletion mutations to map an enhancer element between bp −771 and −731 and a silencer between bp −731 and −676. Deletion mutants generated by BAL 31 digestion (see text) were tested for their relative abilities to generate CAT activity following transfection in COS cells. The results are expressed as the means ± standard errors of three independent transfections, relative to the activity of the preceding deletion. nd, not determined. (D) Independent functions of isolated silencer and enhancer elements on a downstream MHC class I promoter. A silencer ds-oligonucleotide (see Materials and Methods) was synthesized and introduced into the *Nde*I site of pN(−38) (■——■). An enhancer ds-oligonucleotide, in which the silencer-binding site was mutated, was synthesized and introduced into the *Xba*I site of pN(−38) (○——○). The relative ability of each silencer-containing construct to generate CAT activity was assessed following transfection into COS and M12-4.1 cells. The enhancer-containing constructs were introduced into both COS and M12-4.1 cells. All results represent averages of three independent transfections.

ment with protein synthesis inhibitors was for 16 h with cycloheximide (5 μg/ml; Sigma) or anisomycin (100 μg/ml; Sigma). Preliminary experiments showed that under these conditions, cycloheximide completely blocked protein synthesis but did not measurably reduce cell viability (data not shown).

Plasmid constructions, DNA probes, and oligonucleotides. The pN(-38) expression vector contains the chloramphenicol acetyltransferase (CAT) reporter gene downstream of the PD1 promoter (Fig. 1) (7). A subclone of the PD1 5′ flanking sequence (254-17) was generated by cloning of a 254-bp *Dde*I (−886 to −637 bp) DNA fragments into pUC18; a 140-bp DNA fragment containing the entire regulatory DNA element was isolated from 254-17 by digestion with *Ava*II-*Kpn*I. Another upstream *Dde*I fragment of 151 bp (−1037 to −886 bp) was also subcloned into pUC18; the 151-bp insert was isolated by *Bam*HI-*Eco*RI digestion. The 151-bp fragment does not contain silencer sequences and was used as a negative control. BAL 31 deletion mutants used in Fig. 1C were generated as previously described and were prepared by Jean E. Maguire (NIH) (7). Plasmid DNA was prepared by standard alkaline lysis with centrifugation on CsCl gradients or by Qiagen Maxicolumn (Diagen) isolation according to the manufacturer's instructions, followed by CsCl gradi-

ent centrifugation. Sequences were verified by the dideoxynucleotide termination method, using the Sequenase 2.0 kit (U.S. Biochemical).

All oligonucleotides were synthesized on an Applied Biosystems model 381A DNA synthesizer. Complementary strands were annealed and purified on Elutip-d columns (Schleicher & Schuell). Figure 3 lists sequences of competitor double-stranded oligonucleotides (ds-oligonucleotides). For functional studies, the 140-bp and 151-bp fragments and ds-oligonucleotides corresponding to the silencer (CCAAAA TTATCTGAAAAGGTTATTAAAA) and enhancer (with mutated silencer residues) (GGTCCACATTCAAAATAAC CTTTGAGAATTACCATAATGATAGCACTCAAAGGT TATTTTGAATACATGTGGACC) motifs were subcloned separately in the *Nde*I or *Xba*I site, respectively, of pN (−38).

Cell transfections. COS-1 cells were transiently transfected by the calcium phosphate technique (11). CAT enzymatic activities of the transfected constructs were measured 48 h after transfection. Activities were equalized on the basis of protein concentrations and were normalized as mentioned in text.

Cellular extracts. Cell extracts were made by a modification of the method of Dignam et al. (5). Briefly, COS-1 and F9 cells were harvested by EDTA (GIBCO) treatment, washed in cold phosphate-buffered saline (PBS), and pelleted. M12-4.1 cells were collected by centrifugation, washed in cold PBS, and pelleted. The pellet was resuspended in 1 cell volume of Dignam buffer C (20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.9], 1.5 mM $MgCl_2$, 0.42M NaCl, 25% glycerol, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonylfluoride). The final NaCl concentration was adjusted on the basis of cell pellet volume to 0.42 M, and cells were lysed by repeated cycles of freezing and thawing. After brief, light vortexing, the extract was centrifuged at 35,000 rpm (100,000 × *g*), 4°C, for 20 min. The supernatant was recovered, aliquoted, and stored at −70°C. Extracts from the swine tissues were kindly provided by Jeffrey M. Gimble (Oklahoma Medical Research Foundation, Oklahoma City, Okla.), prepared as reported previously (9). Mouse tissue extracts were prepared according to the method of Gimble et al. (9), aliquoted, and stored at −70°C. Protein concentrations were determined by using the Micro BCA protein assay kit (Pierce).

Gel mobility shift assays. Binding reactions were carried out in a volume of 20 μl for 30 min at room temperature. Typical reaction mixtures contained 1.5 fmol of [^{32}P]DNA, 3 μg of cell extract, and 3 μg of poly(dI-dC) in 10 mM Tris-Cl (pH 7.9)–1 mM $MgCl_2$–1 mM dithiothreitol–1 mM EDTA–5% glycerol–75 to 250 mM NaCl. Mobility shifts of the tissue extracts contained 6 μg of protein. Where indicated, unlabeled competitor fragment or ds-oligonucleotide (10^4 M excess) was also added to the binding reaction prior to the addition of protein. In assays in which extracts were treated, EDTA (10 mM final), 1,10-phenanthroline (8 mM final), or ethanol solvent was added to the binding reaction mixture containing the protein, buffer, and poly(dI-dC); DNA probe was added subsequently. Heat pretreatment of the COS extract was at 70°C for 10 min; treated extracts were kept on ice until added to the binding reaction mixture. Binding reactions of the heparin-agarose column fractions (individual or mixed) were done as stated in the text. Following incubations, reaction mixes were subjected to electrophoresis on 4% polyacrylamide gels for 90 min at 160 V in 0.5× TBE (1× TBE is 89 mM Tris, 89 mM borate, and 2 mM EDTA). Gels were dried, autoradiographed, and where indicated scanned on a Molecular Dynamics model 300 scanning densitometer. DNA fragments were end labeled by T4 polynucleotide kinase following dephosphorylation by bacterial alkaline phosphatase.

RNA analysis. Cytoplasmic RNA was prepared by the method of White and Bancroft (30). Briefly, cells were harvested by EDTA treatment, washed with cold PBS, and pelleted. They were resuspended in 0.5% Nonidet P-40–10 mM Tris-Cl (pH 7.0)–150 mM NaCl–2 mM $MgCl_2$, incubated on ice for 3 min, and centrifuged at 1,000 × *g*, 4°C, for 10 min. Sodium dodecyl sulfate (0.5% final) and EDTA (2.5 mM final) were then added to the postnuclear supernant and mixed. Following sequential extractions with phenol-chloroform, the RNA was precipitated and quantitated by spectrophotometric absorption.

Northern (RNA) analysis was done as previously described (8). Filters (Immobilon NC; Millipore) were sequentially probed with the swine MHC class I probe PD1A and actin (8). Hybridizations were carried out overnight at 42°C in a solution containing 40% formamide, 4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 1× Denhardt's solution, 10% dextran sulfate, and 200 μg of salmon sperm DNA per ml. Final washes, for 30 min, were as follows: PD1A, 52°C, 1.0× SSC; and actin, 55°C, 0.1× SSC. Densitometric analysis was made on a Molecular Dynamics model 300 scanning densitometer.

Protein fractionation. Whole cell extract (25 mg) from COS-1 cells was applied in modified Dignam buffer C (0.1 M NaCl) to a prewashed heparin-agarose column at 4°C. Fractions (1 ml) were collected by gravity flow. Proteins were eluted stepwise in modified Dignam buffer C with increasing concentrations of NaCl. Fractions were aliquoted and stored at −70°C. Protein concentrations were determined by Micro BCA Protein assay kit (Pierce).

RESULTS

The MHC class I silencer domain contains two functional activities. Truncation analysis of the 5′ flanking region of the MHC class I gene, PD1, originally revealed the presence of a silencer element contained within a 589-bp segment, located between 503 and 1,091 bp upstream of transcription initiation (Fig. 1A) (7). To delineate the silencer element further, DNA segments spanning the region between −503 and −1,091 bp were tested for silencer activity by introducing them into the vector pN(−38), which contains the PD1 promoter and 503 bp of proximal upstream sequences (Fig. 1A). Among these, only a 140-bp *Ava*II-*Dde*I DNA fragment (Fig. 1A, labeled RE), was found to have silencer activity (Fig. 1B). The magnitude of the silencer effect varied from about 10- to <2-fold, depending on the number and orientation of the 140-bp inserts. This is the same range of effect that was originally noted for the region from −503 to −1,091 bp and suggests that the entire silencer activity of that region is contained within the 140-bp fragment.

To localize the silencer element further, we used a set of 5′ deletion mutants traversing the 140-bp segment (Fig. 1C). These constructs were generated by BAL 31 deletion of the PD1 5′ flanking upstream region and share a common 3′ boundary which includes the PD1 promoter. Analysis of cell extracts for CAT activity following transient transfection of COS cells mapped the silencer element to the segment from −731 to −676 bp (Fig. 1C). Surprisingly, an enhancer activity was detected in the region between −771 and −731 bp (Fig. 1C). Inspection of the DNA sequence in this region reveals that an inverted repeat spans the entire region

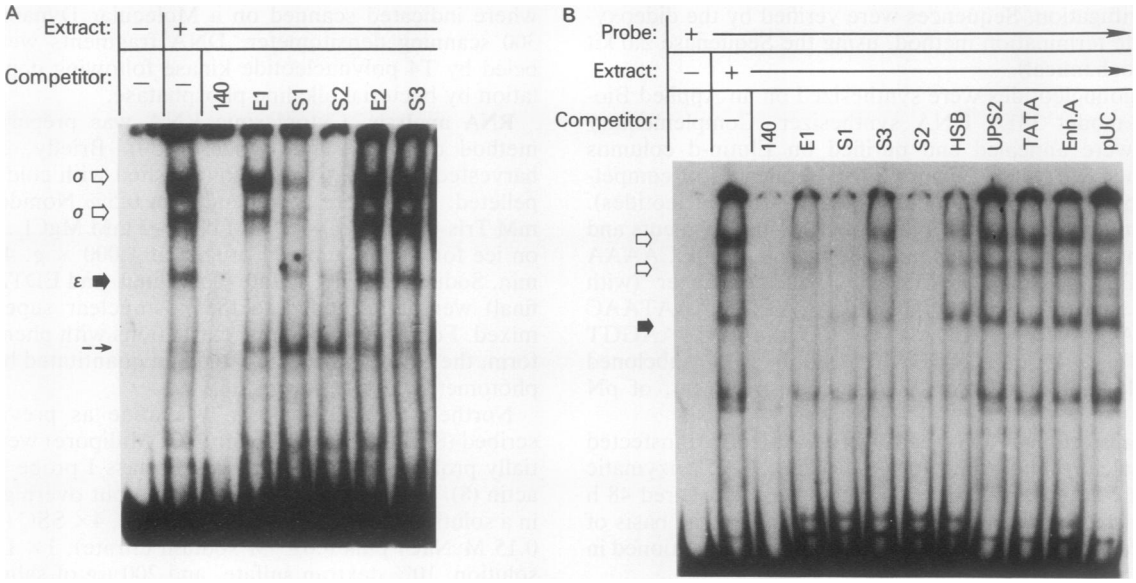

FIG. 2. Evidence that the three complexes formed between regulatory region DNA and COS cell extract derive from two distinct DNA sequence elements. (A) Complexes are differentially inhibited by ds-oligonucleotides derived from different DNA sequences within the regulatory region. Gel mobility shift assays were performed with the 140-bp probe and COS cell extracts as described in Materials and Methods. ds-Oligonucleotides were added as cold competitors at a molar excess of 10^4. ds-Oligonucleotides E1, E2, and E3 are derived from enhancer sequences, while S1, S2, and S3 are derived from the silencer region (Fig. 3). (B) Silencer complexes are not competed for by ds-oligonucleotides derived from other regulatory sequences of the PD1 gene. ds-Oligonucleotides corresponding to the TATA box for UPS2 and enhancer A (Enh.A) were added to the binding reaction mixtures at a molar excess of 10^4. UPS2, upstream unrelated sequence; HSB, high-salt buffer; pUC, vector DNA. Solid arrows, enhancer complex (ε); open arrows, silencer complexes (σ); dotted arrow, nonspecific complexes.

between −770 and −674 bp (Fig. 3); removal of the 5′ half of the inverted repeat abolished enhancer activity (Fig. 1C). The silencer activity overlaps the 3′ half of the inverted repeat. Thus, it appears that the 96-bp segment between −770 and −674 bp actually consists of two overlapping activities: the silencer and an enhancer.

To characterize the enhancer and silencer activities independently of one another, ds-oligonucleotides corresponding to subregions of the regulatory domain were synthesized and cloned into pN(−38) (Fig. 1D). Introduction of the isolated silencer unit, in either orientation, resulted in reduced expression from the PD1 promoter relative to the pN(−38) vector in COS cells. Characterization of the enhancer domain was complicated by the overlapping silencer. A ds-oligonucleotide was synthesized in which residues specific to the silencer were mutated, leaving the inverted repeat intact (see below and Materials and Methods). Introduction of this construct into pN(−38) increased expression from the PD1 promoter in COS cells; however, the enhancement was observed in only one orientation. The B-lymphoblastoid line M12-4.1 expresses high levels of class I (see below). In M12-4.1 cells, the enhancer, but not the silencer, was observed to function. As discussed below, the differential ability of these elements to function in M12-4.1 cells reflects the paucity of silencer-binding factors in these cells.

The MHC class I regulatory domain forms distinct silencer and enhancer complexes with *trans*-acting factors. Earlier in vivo studies demonstrated that the silencer activity is mediated by *trans*-acting factors (7). To assess the complexity of interactions occurring between such *trans*-acting factors and the regulatory DNA sequence, in vitro gel shift assays were performed with COS cell extracts and the 140-bp *Ava*II-*Dde*I fragment spanning the 96-bp domain (Fig. 2). Three distinct and specific bands are reproducibly generated (Fig. 2, unbroken arrows). Other bands are occasionally seen in different extract preparations (Fig. 2A, dotted arrow); these appear to be nonspecific complexes since their appearance is variable and not inhibited by unlabeled, specific DNA fragments. To localize the DNA sequence elements involved in the formation of the three specific complexes, ds-oligonucleotides spanning the entire 140-bp DNA segment were used as cold competitors. All of the ds-oligonucleotides capable of inhibiting the complexes corresponded to two distinct regions of sequence within the functionally defined 96-bp regulatory domain (Fig. 2; summarized in Fig. 3). Some ds-oligonucleotides, such as E1 and E3, were able to inhibit formation of the most rapidly migrating complex but not formation of the other two complexes. Conversely, other ds-oligonucleotides, such as S1, preferentially inhibited the upper two bands (Fig. 2 and 3). Thus, there are at least two distinct binding sites contained within the 140-bp segment. Comparison of the locations of these binding sites with the functionally defined sequence elements (Fig. 1 and 3) indicated that ds-oligonucleotides E1 and E3 span the functional enhancer elements, whereas ds-oligonucleotides S1 and S7 span the functional silencer element. From these studies, we conclude that the slower-migrating bands derived from complexes with the silencer element (σ complexes), whereas the fastest-migrating band derives from complexes with the enhancer element (ε complex).

Among the other ds-oligonucleotides tested for their ability to inhibit complex formation, several, such as S2, S5, and E8, competed with all three bands. As noted above, the functionally defined enhancer element (−771 to −730 bp) spans a DNA sequence containing half of a palindromic site. The other half-site is located within the functionally defined

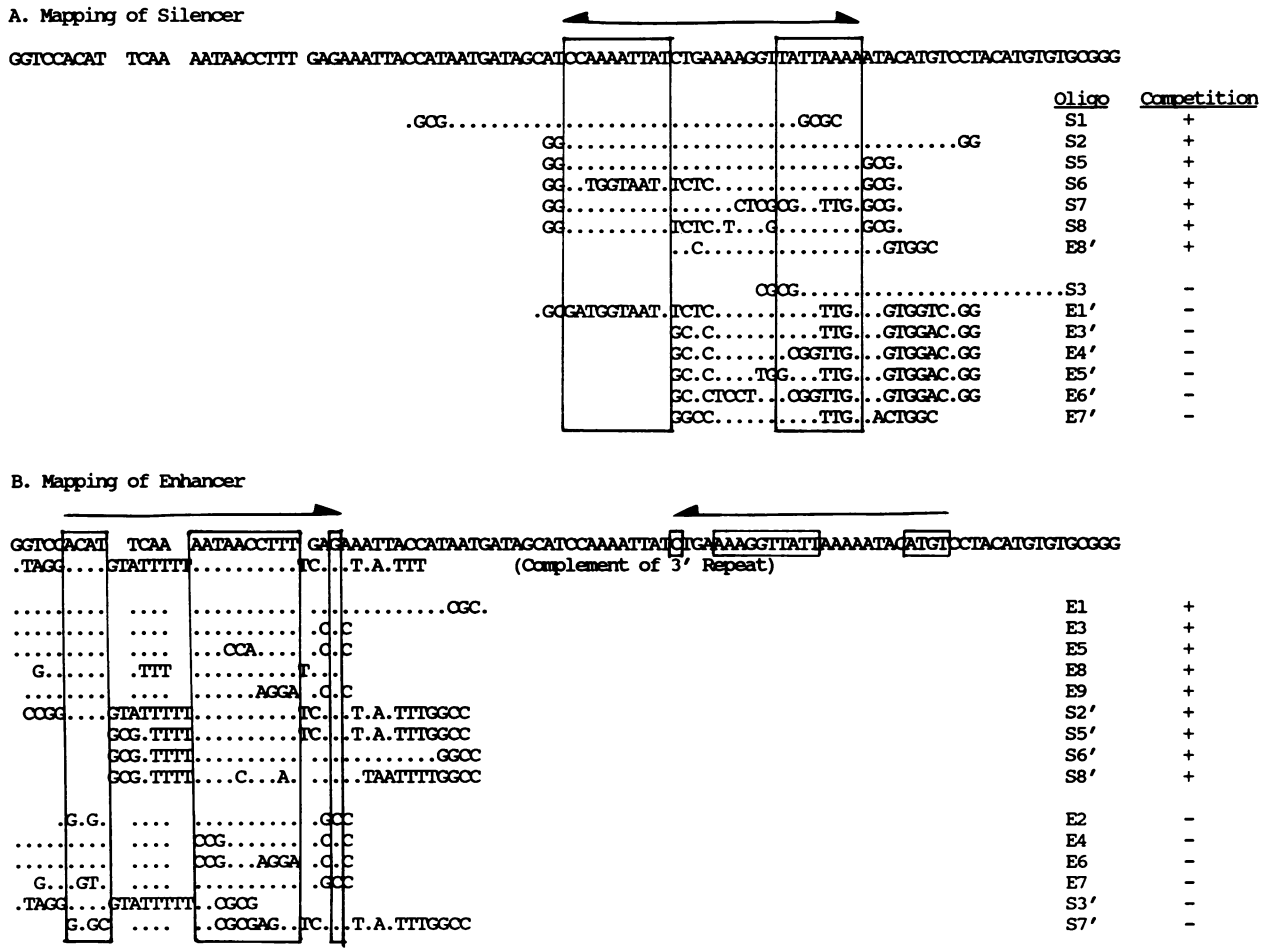

FIG. 3. Mapping of silencer- and enhancer-binding sites by inhibition of complex formation by various ds-oligonucleotides. A series of ds-oligonucleotides spanning the 140-bp *AvaII-DdeI* DNA fragment was tested for the ability to compete against enhancer and silencer complexes. Of these, the only ones that competed were those contained within the 96-bp segment shown. To determine important residues for binding, variant ds-oligonucleotides were synthesized and tested for their abilities to inhibit silencer and enhancer complex formation. (A) Mapping of the silencer-binding site. Arrows delineate boundaries of the silencer element. (B) Mapping of the enhancer-binding site. Boxed regions represent sequences determined by the inhibition studies to be critical for complex formation. Arrows in panel B delineate the interrupted, inverted repeat of the enhancer. +, inhibition of complex; −, no inhibition; ', complementary strand; ., residue identity with native sequence. For simplicity, only one strand of the ds-oligonucleotide sequence is shown.

silencer region. ds-Oligonucleotides which display some capacity to compete both enhancer and silencer complexes contain this 3' half-palindromic sequences. Taken together, these data suggest that there are distinct factors associated with the enhancer and silencer elements and that the binding sites for these factors overlap. ds-Oligonucleotides derived from other regulatory elements, such as enhancer A and the TATA box, or from pUC vector sequences do not compete with any of the complexes (Fig. 2B).

To determine which nucleotides in the DNA-binding sites interact with the *trans*-acting factors, ds-oligonucleotides containing mutated sequences of either the enhancer or silencer DNA elements were tested for the ability to inhibit complex formation in the gel shift assay (Fig. 3). Each ds-oligonucleotide was assessed for its ability to inhibit formation of either the slower-migrating silencer complexes or the faster-migrating enhancer complex. Within the silencer DNA element, two critical domains were identified (Fig. 3A). Mutation of either one alone was not sufficient to abrogate inhibition of the upper two silencer bands (S6 and S7). However, mutation of both or deletion of one and mutation of the other eliminated inhibitory activity (S3 to E7'). Thus, two sites contribute to the generation of the silencer complexes. Furthermore, the two silencer complexes are related, since both are competed against by the same set of ds-oligonucleotides (i.e., S1 and S2 in Fig. 2). Because the silencer element DNA sequence is A+T rich (75%), we considered the possibility that the DNA-binding factors were specific for single-stranded DNA; however, single-stranded DNA was unable to inhibit either of the complexes (data not shown). Unrelated A+T-rich sequences also did not compete (Fig. 2B). Furthermore, transcription does not initiate around the silencer element, which bears some resemblance to a promoter (7). Thus, the silencing effect is not simply the result of upstream competition for the proper transcription initiation site.

Within the enhancer region, essential nucleotides mapped within the inverted repeat segments (Fig. 3B). Base substitutions outside of the inverted repeat segments did not affect the ability of the sequence to inhibit enhancer complex

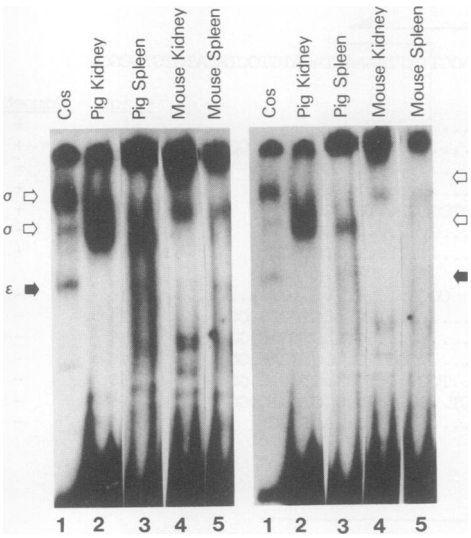

FIG. 4. Correlation of levels of silencer (σ) and enhancer (ε) complexes in different tissues with levels of MHC class I expression. Extracts were prepared from kidney and spleen of mouse and pig as described in the text. Equivalent amounts (6 μg) of each tissue extract were combined with a radiolabeled 140-bp probe and analyzed by mobility shift assays. Arrows represent the three major complexes generated with COS cell extracts. Open arrows, silencer complexes; solid arrow, enhancer complex. Left panel, 3-day exposure; right panel, 24-h exposure.

formation; only substitutions within the repeat segments eliminated this ability. (Attempts to precisely map contact residues by various footprinting techniques were unsuccessful, possibly because of the extensive region of binding and the presence of overlapping complexes.) These oligonucleotide competition studies suggest that the enhancer binding site appears to consist of an interrupted inverted repeat. The two halves of the repeat differ in the length of their interruption: 4 bp separate the repeat components in the 5′ segment, and 8 bp separate them in the 3′ segment.

The ratio of silencer to enhancer complexes correlates with levels of in vivo expression. The levels of MHC class I gene expression in vivo vary in different tissues (28). For example, kidney and liver cells contain low steady-state levels of class I RNA, whereas high levels occur in cells of the spleen and lymph node. If the complexes observed in the gel shift assay correlate with functional activities, then there should be a correlation between the level of class I gene expression and the level of DNA-binding factors. Cell types expressing high levels of class I, such as splenocytes, should be relatively enriched for enhancer factors, while cell types expressing low levels of class I, such as kidney cells, should be relatively enriched for silencer factors. Extracts prepared from spleen and kidney were tested for the ability to generate the two forms of complexes with the 140-bp probe. Extracts of kidney from either pig or mouse generated a major complex which comigrated with the one of the silencer complexes (Fig. 4, lanes 2 and 4). ds-Oligonucleotides spanning the silencer element, such as S1 (Fig. 3), inhibited this binding (data not shown). Neither kidney extract generated detectable enhancer complex (Fig. 4, lanes 2 and 4). Although splenic extracts also gave rise to silencer complexes (Fig. 4, lanes 3 and 5), the level of silencer-associated complex from spleen was markedly lower than that from kidney extracts. The spleen extracts additionally generated a complex with a mobility indistinguishable from that of the enhancer complex. Competition with ds-oligonucleotides derived from the enhancer region (E1) specifically inhibited this band (data not shown). Minor bands were seen in all of the extracts examined; these either were nonspecific (not able to be inhibited competitively) or, in some cases, may represent degradation products of the relevant factors. Thus, there is a correlation between the level of class I gene expression and the complement of DNA-binding factors within a given tissue. Kidney cells, which express low levels of class I, contain high levels of silencer factor but undetectable enhancer factor. Extracts from spleen, which contains a population of cells expressing high levels of class I, are enriched for enhancer factor. Furthermore, because extracts from mouse and pig generate indistinguishable complexes, these DNA-binding factors appear to have been conserved between the species.

To extend the correlations between the level of class I RNA and the level of DNA-binding factors, we examined three cell lines that differ in class I expression. COS cells, which are derived from a kidney fibroblast line, express moderately low levels of class I RNA (see Fig. 6B). As shown above, extracts from COS cells form both silencer and enhancer complexes with the 140-bp probe. The B-cell line, M12-4.1, expresses very high levels of class I RNA. In gel shift assays, extracts from M12-4.1 cells generate a major band with a mobility corresponding to that of the enhancer complex (Fig. 5). That this band contains the enhancer complex is demonstrated by its ability to be competed against by the enhancer ds-oligonucleotide, E9 (Fig. 5). Barely detectable amounts of silencer complex form with M12-4.1 extracts under any conditions tested. In contrast, extracts from the teratocarcinoma F9 cell, which expresses virtually no class I RNA, form large amounts of a silencer complex which is specifically inhibited by a silencer ds-oligonucleotide, S1 (Fig. 5). Thus, both in vivo in tissues and in homogeneous cell lines, an inverse correlation is observed between the level of class I gene expression and silencer factors.

Class I gene expression is negatively regulated. The paucity or absence of enhancer complexes generated by kidney, COS, and F9 cell extracts could be due to either the absence of enhancer factor itself or the inability of such a factor to bind DNA in the presence of the silencer factor. If silencer factor is competitively inhibiting the binding of enhancer factor, then removal of silencer factor should reveal the enhancer factor. Preliminary studies had shown that silencer complexes from COS cells form optimally at salt concentrations between 50 and 100 mM. In contrast, enhancer complexes were stable even at higher salt concentrations. To test for the possible presence of enhancer factors in F9 cell extracts, binding reactions were done in 500 mM salt, in which enhancer factors, but not silencer factors, would bind to the 140-bp probe. As shown in Fig. 5, at 500 mM salt, silencer complexes are no longer observed with F9 extracts. However, enhancer complexes can now be generated. Again, these complexes are specifically inhibited by the appropriate ds-oligonucleotides (Fig. 5). It is concluded that enhancer factors are constitutively expressed in F9 cells, but their binding to enhancer DNA is prevented by the preferential binding of silencer factors to the overlapping silencer DNA sequences. Thus, class I gene expression appears to be negatively regulated by levels of silencer factor.

The class I silencer factor is labile. Since class I gene expression can increase rapidly in response to immunomod-

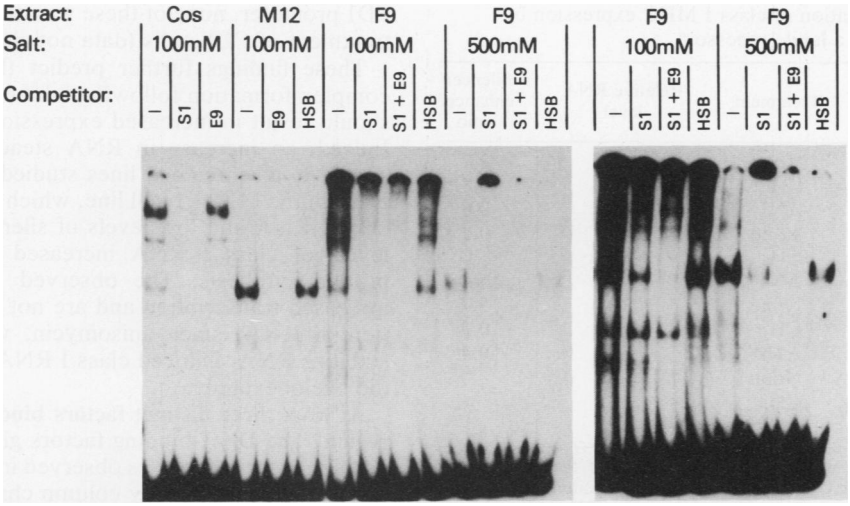

FIG. 5. Levels of silencer and enhancer complexes vary among different cell lines. Extracts from COS, M12-4.1 (B lymphoma), and F9 (teratocarcinoma) cells were analyzed for the ability to form complexes with the 140-bp fragment. Complexes were formed either under standard (100 mM salt) or high-salt (500 mM) reactions conditions. ds-Oligonucleotide competitors were added at a molar excess of 10^4. Solid arrow, silencer complex; open arrow, enhancer complex.

ulators such as interferon, we speculated that the silencer factors would be labile. If so, inhibition of cellular protein synthesis would reveal decay of the silencer factor. Treatment of COS cells with either cycloheximide or anisomycin, two inhibitors of protein synthesis that have different mechanisms of action, resulted in a relative decrease in silencer factors and a concomitant increase in enhancer factors (Fig. 6A and Table 1). Similarly, enhancer factor binding was induced in F9 cells following treatment with these agents. In M12-4.1 cells, the small amount of silencer factors present in untreated extracts completely decayed following either cycloheximide or anisomycin treatment. Quantitation of the levels of silencer and enhancer complexes indicates that there is a relative increase of enhancer complex of 4-fold in COS cells and 14-fold in F9 cells following inhibition of protein synthesis (Table 1). This may be an underestimate of

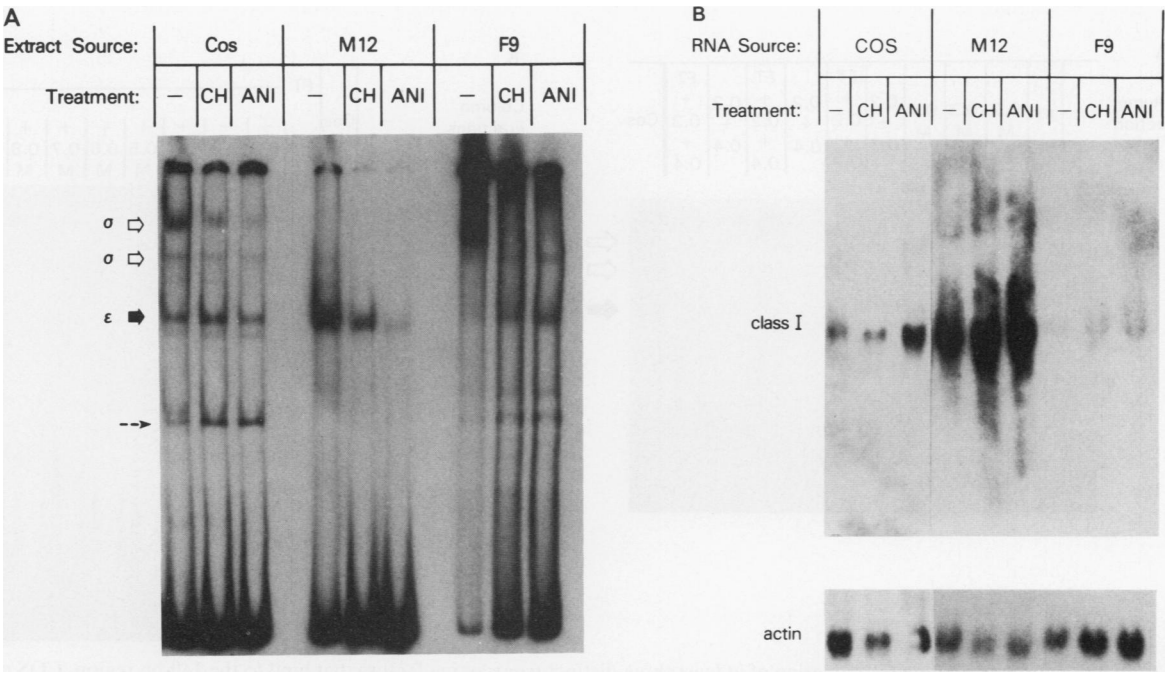

FIG. 6. Evidence that the silencer (σ) complex contains labile repressor factors. COS, M12-4.1, and F9 cells were grown in the absence or presence of inhibitors of protein synthesis as described in the text. Cells were harvested, and aliquots were used for the preparation of cell extracts or RNA. (A) Gel mobility shift assay of the 140-bp fragment with cell extracts; (B) class I RNA levels in cells grown in the presence and absence of protein synthesis inhibitors. The PD1 coding region probe, PD1A, was used in this hybridization. CH, cycloheximide; ANI, anisomycin. ε, enhancer complex; ⇢, nonspecific complexes.

TABLE 1. Regulation of class I MHC expression by a labile repressor[a]

Cell	Treatment	Relative RNA level	Silencer/ enhancer ratio
Kidney (COS)	None	1.0	2.2
	CH	2.0	0.8
	ANI	5.2	0.6
B cell (M12-4.1)	None	1.0	0.2
	CH	3.0	ND
	ANI	2.4	ND
Teratocarcinoma (F9)	None	1.0	5.7
	CH	2.0	0.5
	ANI	3.2	0.4
Fibroblast (L cell)	None	1.0	
	CH	2.3	
	ANI	4.4	

[a] RNA and cell extracts were prepared from each of the cell lines as described in the text. RNA levels were determined by densitometric analysis following hybridization with a class I probe; the intensity of hybridization was normalized to the level of hybridization with an actin probe. Levels of enhancer and silencer complexes relative to those of untreated cells were determined by densitometric analysis of retarded bands in a gel mobility shift assay. CH, cycloheximide; ANI, anisomycin; ND, no silencer factor detected.

the actual relative change, since there is a concomitant decay of the enhancer complex during the treatment. This is seen most clearly in the extracts from untreated M12-4.1 cells, in which there is little silencer complex. Since silencer complexes decay more rapidly than enhancer complexes, we conclude that the silencer-binding factor is labile relative to the enhancer-binding factor. Although a number of other complexes are observed in gel shift assays using other DNA segments which span 1.1 kb of 5′ flanking sequences of the PD1 promoter, none of these was affected by cycloheximide treatment of COS cells (data not shown).

These findings further predict that increased enhancer complex formation following inhibition of protein synthesis should result in increased expression of class I transcripts. Indeed, an increase in RNA steady-state levels was observed in all three cell lines studied (Fig. 6B and Table 1). Even in the M12-4.1 cell line, which expresses high levels of class I RNA and low levels of silencer factor, steady-state levels of class I RNA increased following inhibition of protein synthesis. The observed increases likely reflect increased transcription and are not due simply to stabilization of RNA, since anisomycin, which is not known to stabilize RNA, induced class I RNA levels as effectively as did cycloheximide.

At least three distinct factors bind to the regulatory DNA region. The DNA-binding factors giving rise to the silencer and enhancer complexes observed in the gel shift assay were further characterized by column chromatography. COS cell extract was fractionated on a heparin-agarose column by binding of extract in buffer at 0.1 M NaCl and subsequent step elution of column fractions at increasing salt concentrations. Column fractions were then assayed by gel shift with either the 140-bp fragment or a ds-oligonucleotide corresponding to the downstream enhancer A (Fig. 1A). Whereas enhancer A-binding activity was readily detected (data not shown), none of the individual column fractions generated any complexes with the 140-bp probe (Fig. 7). However, mixing of the flowthrough fraction with the 0.2 M eluate regenerated the enhancer complex as well as the faster migrating of the two silencer complexes (Fig. 7). Mixing of the flowthrough fraction with the 0.3 or 0.4 M eluates restored the slower-migrating silencer complex. None of the higher-salt eluates contained detectable DNA-binding activity. The flowthrough fraction was necessary to restore

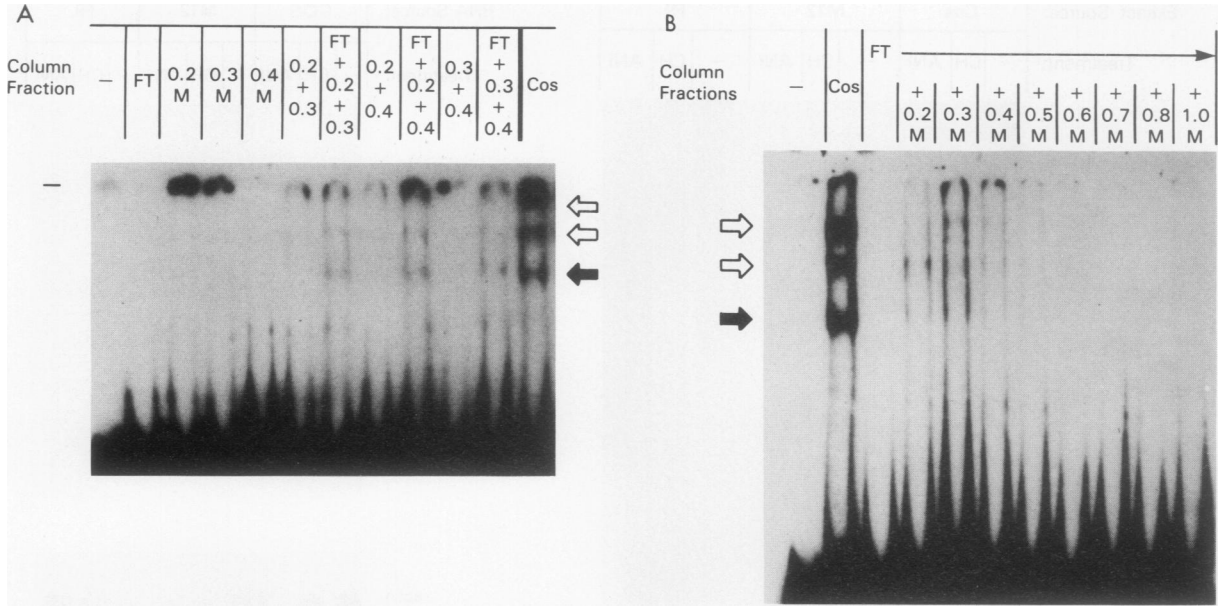

FIG. 7. Identification by column fractionation of at least three distinct *trans*-acting factors that bind to the 140-bp region. COS cell extract (25 mg) was applied to a heparin-agarose column as described in Materials and Methods. Step salt fractions were collected and assayed by gel mobility shift assay. In samples in which column fractions were mixed, equal amounts of each were combined prior to incubation with the probe. (A) Gel mobility shift assay of selected individual column fractions (flowthrough [FT]; 0.2, 0.3, and 0.4 M) and various combinations of these fractions; (B) gel mobility shift assays of different fractions with flowthrough fraction. –, probe alone; open arrows, silencer complexes; closed arrows, enhancer complex; Cos, unfractionated COS cell extract.

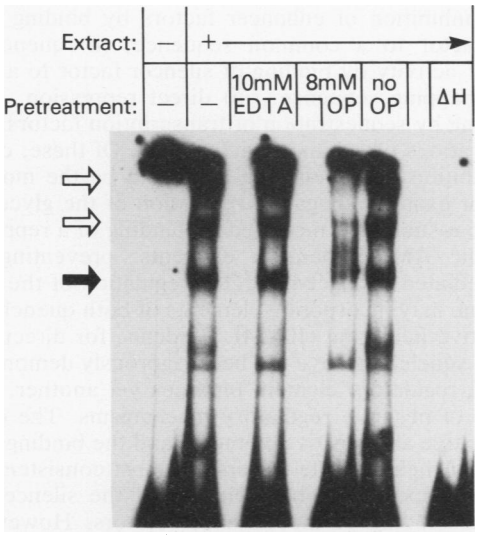

FIG. 8. Evidence that silencer- and enhancer-binding activities are EDTA resistant but heat and metal chelator sensitive. Gel shift assays of COS extracts (3 μg) were performed in the presence of either 10 mM EDTA or 8 mM *o*-phenanthroline (OP), using the 140-bp probe. Heat-treated extracts were tested in a gel mobility shift assay using the 140-bp probe. No OP, treated with solvent alone; open arrows, silencer bands; solid arrow, enhancer band. Lower, unmarked, bands are nonspecific bands that are not reproducibly observed.

complex formation, since no combination of the other eluted fractions fully regenerated the complexes (Fig. 7). A combination of the flowthrough fraction with 0.2 and 0.3 M elution completely restored the pattern obtained with unfractionated cell extract. Thus, there must be at least three factors responsible for the gel shift pattern normally observed: a factor(s) found in the flowthrough which contributes to both the enhancer and silencer complexes, a factor enriched in the 0.2 M eluate which contributes to the enhancer complex, and a factor enriched in the 0.3 M eluate which contributes to the silencer complex.

As an initial attempt to characterize the factors binding to the regulatory domain biochemically, cell extracts were subjected to various treatments (Fig. 8). EDTA treatment did not alter the binding profile of the extract, indicating that none of the three factors binding to this region requires Mg^{2+} or Ca^{2+}. In contrast, the heavy metal chelator *o*-phenanthroline markedly reduced complex formation of all three bands, suggesting that at least one of the factors requires zinc or possibly copper. Finally, heat treatment completely eliminated all DNA-binding activity, indicating that at least one of the factors is sensitive to heat denaturation.

DISCUSSION

Variations in levels of class I MHC gene expression are achieved by the cumulative effects of a series of negative and positive regulatory DNA sequence elements associated with the 5′ flanking sequences (28). Among the elements which have been studied previously, none had the ability to confer tissue specificity. In this study, we have defined a complex regulatory element associated with a swine MHC class I gene, PD1, whose properties are consistent with a role in the regulation of tissue-specific levels of expression. This element consists of two overlapping functional DNA sequences: an enhancer element and a silencer element.

The enhancer element consists of an interrupted inverted repeat; the element itself does not display DNA sequence homology with other known enhancers. A synthetic enhancer element, consisting of an inverted repeat in which silencer-specific nucleotides have been mutated, functions to augment expression from a downstream class I promoter, both in COS cells and in the lymphoblastoid line M12-4.1. Under standard assay conditions, the concentration of enhancer-binding factors is proportional to the level of class I gene expression in the cell from which the extract derived. However, enhancer factors which specifically associate with the enhancer sequence can be detected in all cell types examined. In cells expressing very low levels of class I RNA, the binding of enhancer factors may be prevented by the preferential binding of silencer factors. The enhancer-binding factors are relatively stable, as demonstrated by their resistance to cycloheximide and anisomycin, and consist of a complex of at least two proteins, neither of which can bind DNA independently.

The silencer element spans 28 bp, consisting of a 10-bp 5′ segment and an 8-bp 3′ segment separated by 10 bp. It overlaps the 3′ moiety of the enhancer element inverted repeat (Fig. 3). A synthetic silencer element consisting of the 28-bp unit decreases expression from a downstream class I promoter as effectively as does the native, intact DNA segment of 140 bp in COS cells. In M12-4.1 cells, which contain very low levels of silencer-binding factors, the synthetic silencer does not affect expression. The only DNA sequence homology of the silencer element to any known regulatory element is to the yeast α2 operator, which binds the yeast mating-type repressor, alpha-2; homology of 59% between the PD1 silencer and conserved yeast operator residues can be generated by alignment of these elements (29). Indeed, these two regulatory elements are functionally homologous, since the yeast α2 operator binds PD1 silencer-associated factors and functions as a silencer element in mammalian cells (29).

Cellular factors which bind the silencer element are distinct from those which bind the enhancer. The abundance of silencer factors is inversely proportional to the level of class I gene expression within a cell. Thus, high levels of silencer factors are detected in class I-negative murine embryonic teratocarcinoma cells, but very low levels are found in the B-cell line M12-4.1, which expresses high class I levels. Similarly, higher levels of silencer-binding factors were detected in extracts prepared from kidney than in those prepared from spleen. In contrast, enhancer factors are constitutively expressed in all cells. However, the binding of enhancer factors to the enhancer DNA sequence is diminished in the presence of silencer factors. The silencer factors are labile, as demonstrated by their decay following treatment with inhibitors of protein synthesis. As in the case of the enhancer factors, the silencer factor consists of a complex of at least two proteins, neither of which binds DNA independently.

The relationship of the silencer and enhancer factors to one another is not yet clear. Each factor consists of a complex of proteins which are resolved by column chromatography. However, there may be a common subunit shared by the two factors: the flowthrough fraction from the heparin-agarose column is able to reconstitute both the enhancer and silencer factors, but in distinct column fractions. Neither silencer nor enhancer factor is sensitive to EDTA treatment, but both are sensitive to the heavy metal chelator *o*-phenan-

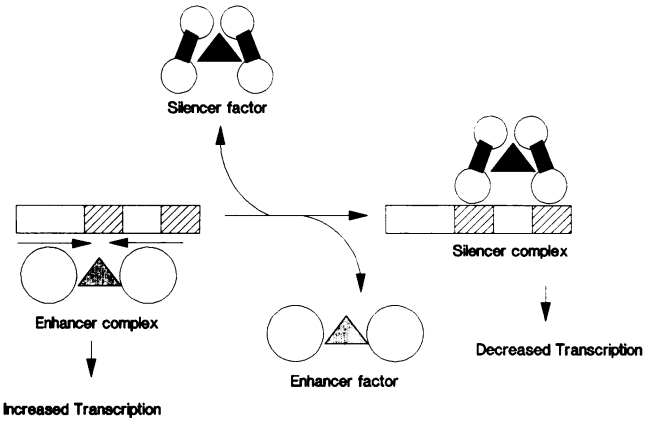

FIG. 9. Model for regulation by overlapping silencer and enhancers. Both silencer and enhancer factors consist of a complex of protein, none of which bind DNA independently. In the absence of silencer factor, enhancer factor binds to the enhancer elements, defined by an interrupted inverted repeat (arrows), leading to increased transcription from the downstream promoter. This configuration would be found in cells expressing high levels of class I, such as the M12-4.1 cells. Silencer factor displaces the enhancer factor and binds to the discontinuous binding site of the silencer element (shaded boxes). The silencer factor thus quenches the enhancer but also actively decreases transcription from the downstream promoter. In cells which express low levels of class I, such as F9 teratocarcinoma cells, silencer factor binding predominates. In other cell types with intermediate levels of expression, there is an equilibrium between the two complexes.

throline and to heat treatment. These observations are consistent with the interpretation that a common, zinc- or copper-binding subunit reconstitutes the activity of the two complexes.

These studies suggest a model of class I gene expression in which the level of expression of the gene is negatively regulated (Fig. 9). In this model, all cells contain enhancer factors, independent of their level of class I gene expression. But the ability of the enhancer factors to bind enhancer and promote class I gene expression is determined by the presence of silencer factors, which prevent binding of the enhancer factors. Thus, the level of transcription is inversely proportional to the cellular concentration of silencer factor.

The silencer factor also actively reduces transcription, since introduction of isolated silencer elements upstream of the PD1 promoter reduces expression. Since the silencer factor is labile, as indicated by its sensitivity to cycloheximide, the negative regulation that it mediates is an active process which requires continued synthesis of silencer factor. This active negative regulation allows for rapid increases in transcription in response to immunomodulators or various conditions of stress. Indeed, numerous examples exist in which MHC class I expression increases in response to these situations, including treatment with interferons, tumor necrosis factor, or alcohol (23–26).

Although enhancers have been extensively described, the role of negative regulation in gene expression is beginning to be appreciated as more examples are described among eukaryotic gene systems. Among the various genes for which negative regulation has been observed are those for yeast a mating type (16), various drosophila homeoboxes (2, 12), glycoprotein hormone α subunit (1), and β-interferon (10, 14). The molecular mechanisms underlying them have recently been divided into four distinct categories: (i) competitive inhibition of enhancer factors by binding of a silencer factor to a common sequence, (ii) quenching of enhancer activity by binding of silencer factor to a distinct but overlapping sequence, (iii) direct repression, and (iv) squelching by sequestration of transcription factors by high concentrations of enhancer factors (22). Of these, competitive inhibition and quenching appear to be the most common. For example, negative regulation of the glycoprotein hormone α subunit is mediated by binding of a repressor to the cyclic AMP-responsive elements, preventing cyclic AMP-mediated enhancement (1). Regulation of the β-interferon gene may incorporate elements of both quenching and competitive inhibition (10, 14). Evidence for direct repression and squelching have not been rigorously demonstrated. The PD1 regulatory element provides yet another, distinct example of negative regulatory mechanisms. The overlapping negative and positive elements and the binding characteristics of their cognate factors are most consistent with a quenching model, in which binding of the silencer factor precludes binding of the enhancer factors. However, the silencer element also mediates direct repression, since the isolated silencer reduces downstream promoter activity.

In conclusion, these studies demonstrate that levels of class I gene expression are achieved by the complex interactions of enhancer and silencer factors, in which the dominant mechanism is negative regulation.

ACKNOWLEDGMENTS

We thank Jeffrey Gimble for the pig extracts and Shelby Berger and Stuart Rudikoff for critical reading of the manuscript. J.D.W. gratefully acknowledges Roland Christy for nurturing initial scientific curiosity.

REFERENCES

1. **Akerblom, I. E., E. P. Slater, M. Beato, J. D. Baxter, and P. L. Mellon.** 1988. Negative regulation by glucocorticoids through interference with a cAMP responsive enhancer. Science **241:** 350–353.
2. **Biggin, M. D., and R. Tjian.** 1989. A purified Drosophila homeodomain protein represses transcription in vitro. Cell **58:**433–440.
3. **Bjorkman, P., M. Saper, B. Samraoui, W. Bennett, J. Strominger, and D. Wiley.** 1987. The foreign antigen binding site and T cell recognition regions of class I histocompatibility antigens. Nature (London) **329:**512–518.
4. **Blanar, M., E. Boettger, and R. Flavell.** 1988. Transcriptional activation of HLA-DR alpha by interferon gamma requires trans-acting protein. Proc. Natl. Acad. Sci. USA **85:**4672–4676.
5. **Dignam, J., R. Lebovitz, and R. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11:**1475–1489.
6. **Driggers, P. H., D. L. Ennist, S. L. Gleason, et al.** 1990. An interferon gamma-regulated protein that binds the interferon-inducible enhancer element of major histocompatibility complex class I genes. Proc. Natl. Acad. Sciences USA **10:**3743–3747.
7. **Ehrlich, R., J. Maguire, and D. Singer.** 1988. Identification of negative and positive regulatory elements associated with a class I major histocompatibility complex gene. Mol. Cell. Biol. **8:**695–703.
8. **Ehrlich, R., S. Sharrow, J. Maguire, and D. Singer.** 1989. Expression of a class I MHC transgene: effects of in vivo α/β-interferon treatment. Immunogenetics **30:**18–26.
9. **Gimble, J. M., D. Levens, and E. E. Max.** 1987. B-cell nuclear proteins binding in vitro to the human immunoglobulin kappa enhancer: localization by exonuclease protection. Mol. Cell. Biol. **7:**1815–1822.
10. **Goodbourn, S., and T. Maniatis.** 1988. Overlapping positive and negative regulatory domains of the human beta-interferon gene.

Proc. Natl. Acad. Sci. USA **85**:1447–1451.
11. **Gorman, C., L. Moffat, and B. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
12. **Han, K., M. Levine, and J. Manley.** 1989. Synergistic activation and repression of transcription by Drosophila homeobox proteins. Cell **56**:573–583.
13. **Handy, D., P. Burke, K. Ozato, and J. Coligan.** 1989. Site-specific mutagenesis of the class I regulatory element of the Q10 gene allows expression in non-liver tissues. J. Immunol. **142**: 1015–1021.
14. **Harada, H., T. Fujita, M. Miyamoto, et al.** 1989. Structurally similar but functionally distinct factors, IRF-1 and IRF-2, bind to the same regulatory elements of IFN and IFN-inducible genes. Cell **58**:729–739.
15. **Israel, A., A. Kimura, A. Fournier, M. Fellous, and P. Kourilsky.** 1986. Interferon response sequence potentiates activity of an enhancer in the promoter region of a mouse H-2 gene. Nature (London) **322**:743–746.
16. **Keleher, C., C. Goutte, and A. Johnson.** 1988. The yeast cell-type-specific repressor alpha-2 acts cooperatively with a non-cell-type specific protein. Cell **53**:927–936.
17. **Kieran, M., V. Blank, R. Logeat, et al.** 1990. The DNA binding subunit of NNF-κB is identical to factor KbF1 and homologous to the rel oncogene product. Cell **62**:1007–1018.
18. **Kimura, A., A. Israel, O. LeBail, and P. Kourilsky.** 1986. Detailed analysis of the mouse H-2Kb promoter: enhancer-like sequences and their role in the regulation of class I gene expression. Cell **44**:261–272.
19. **Klein, J.** 1975. Biology of the mouse histocompatibility complex. Springer-Verlag, New York.
20. **Korber, B., N. Mermod, L. Hood, and I. Stroynowski.** 1988. Regulation of gene expression by interferons: control of H-2 promoter responses. Science **239**:1302–1306.
21. **Levi, B. Z., S. Hirschfeld, E. Appella, and K. Ozato.** 1989. H-2RIIBP, a member of the nuclear hormone receptor superfamily that binds to both the regulatory element of major histocompatibility class I genes and the estrogen response element. Proc. Natl. Acad. Sci. USA **86**:8289–8293.
22. **Levine, M., and J. Manley.** 1990. Transcriptional repression of eukaryotic promoters. Cell **59**:405–408.
23. **Lindahl, P., I. Gresser, P. Leary, and M. Tovey.** 1976. Interferon treatment of mice: enhanced expression of histocompatibility antigens on lymphoid cells. Proc. Natl. Acad. Sci. USA **73**: 1284–1287.
24. **Parent, L., R. Ehrlich, L. Matis, and D. Singer.** 1987. Ethanol: an enhancer of major histocompatibility complex antigen expression. FASEB J. **1**:469–473.
25. **Pfizenmaier, K., P. Scheurich, C. Schluter, and M. Kronke.** 1987. Tumor necrosis factor enhances HLA-A,B,C and HLA-DR gene expression in human tumor cells. J. Immunol. **138**:975–980.
26. **Satz, M., and D. Singer.** 1984. Effect of mouse interferon on the expression of a porcine major histocompatibility gene introduced into mouse L cells. J. Immunol. **132**:496–501.
27. **Shirayoshi, Y., P. Burke, E. Appella, and K. Ozato.** 1988. Interferon-induced transcription of a major histocompatibility class I gene accompanies binding of inducible nuclear factors to the interferon consensus sequence. Proc. Natl. Acad. Sci. USA **85**:5884–5888.
28. **Singer, D., and J. Maguire.** 1990. Regulation of the expression of class I MHC genes. Crit. Rev. Immunol. **10**:235–257.
29. **Weissman, J., and D. Singer.** 1991. Striking similarities between the regulatory mechanisms governing yeast mating-type genes and mammalian major histocompatibility complex genes. Mol. Cell. Biol. **11**:4228–4234.
30. **White, B. A., and F. C. Bancroft.** 1982. Cytoplasmic dot hybridization. Simple analysis of relative mRNA levels in multiple small cell or tissue samples. J. Biol. Chem. **257**:8569–8572.