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# Identification of Negative and Positive Regulatory Elements Associated with a Class I Major Histocompatibility Complex Gene

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**Regulatory DNA sequence elements were functionally identified in the 5'-flanking region of a gene, *PD1*, which encodes a porcine classical transplantation antigen. Both a positive regulatory element and a novel negative regulatory DNA element were mapped within 1.1 kilobases upstream of exon 1. The negative regulatory element reduced the activity of both the homologous *PD1* promoter and a heterologous simian virus 40 promoter. In vivo competition experiments indicated that the functions of the *PD1* positive and negative regulatory elements are mediated by distinct cellular *trans*-acting factors. The *PD1* positive regulatory element interacted with cellular factors in common with those binding to the simian virus 40 enhancer. Finally, the negative regulatory element required the presence of a positive regulatory element to function. This interaction between positive and negative regulatory elements represents a novel mechanism for regulating gene expression.**

The class I genes of the major histocompatibility complex (MHC) comprise a family of homologous DNA sequences, some of which encode the heavy-chain moiety of classical transplantation antigens found on the surface of all somatic cells. The major functions of the transplantation antigens are to mediate graft rejection and to serve as restriction elements for antigen recognition (18–20). Although class I molecules are expressed on all nucleated mammalian cells, with the exception of neurons and mature trophoblasts (9, 24), their expression is quantitatively regulated. The highest levels of expression of class I antigens are found on lymphoid cells, while markedly lower levels occur in other somatic tissues, such as kidney. Various endogenous and exogenous factors are known to modulate class I gene expression. For example, interferons, both alpha/beta and gamma, and tumor necrosis factor increase the levels of class I mRNA (3, 6, 15, 25, 36; D. S. Singer, R. Ehrlich, H. Golding, L. Satz, L. Parent, and S. Rudikoff, *in* C. Warner, M. Rothschild, and S. Lamont, ed., *Molecular Biology of the Major Histocompatibility Complex of Domestic Animals*, in press). In contrast, certain oncogenic viruses can dramatically reduce these levels (29, 38).

DNA sequence elements involved in the regulation of class I MHC genes are beginning to be identified. Transcriptional promoters and enhancers and DNA sequence elements involved in the interferon response have been identified in a number of species (11, 16, 17, 21, 43; Singer et al., in press). In the murine $L^d$ gene, a negative regulatory element was found which functions only in undifferentiated embryonal teratocarcinoma cells (30). Other regulatory DNA elements and the mechanisms by which they function remain largely unknown.

In both mice and humans, the class I MHC gene families contain between 17 and 40 homologous sequences (5, 32, 41, 47). In contrast, the class I MHC gene family of miniature swine consists of only seven members (39) which, although homologous, can be readily distinguished. One of these genes, *PD1*, encodes a major transplantation (SLA) antigen. In vivo, *PD1* expression is regulated. The gene is expressed preferentially in lymphoid tissues and at higher levels in B cells than in T cells (R. Ehrlich and D. S. Singer, manuscript in preparation). Introduction of *PD1* into transgenic mice results in expression of this gene in a variety of tissues and at different levels (10). The pattern of expression of *PD1* in the transgenic mouse parallels that observed in swine in vivo, indicating that its expression is regulated in the mouse. Furthermore, in vivo alpha/beta interferon treatment of *PD1* transgenic mice induces increased expression of SLA antigen in a variety of tissues, including spleen and thymus (Ehrlich and Singer, in preparation). *PD1* is also expressed in transfected mouse L cells, in which its transcription is enhanced by alpha/beta interferon (36). Using a series of deletion mutants of the 5'-flanking segment of *PD1* fused to a reporter gene, that for chloramphenicol acetyltransferase (CAT) (the *cat* gene), the transcriptional promoter and an interferon response element have been mapped (Singer et al., in press).

In the present paper, we identify by function positive and negative regulatory DNA sequence elements. The negative regulatory element reduces *PD1* expression by 5- to 10-fold and also functions, although less efficiently, on a heterologous promoter. The activity of the negative regulatory element is dependent on the presence of an enhancer element. In vivo competition experiments demonstrated that the functions of both positive and negative regulatory elements are mediated by *trans*-acting factors.

## MATERIALS AND METHODS

**Recombinant plasmids.** The CAT vectors pSV0, pSV1, and pSV2 were described by Gorman et al. (14). pSV3 is similar to pSV1, except that the 3' *Bam*HI site has been removed and a multiple cloning site polylinker has replaced the *Acc*I-*Sph*I fragments of pSV2. Briefly, pSV0 does not contain eucaryotic promoters or enhancers, pSV1 and pSV3 contain only the simian virus 40 (SV40) promoter, and pSV2 contains both the SV40 promoter and enhancer.

The construction of *Bal* 31 *PD1* deletion mutants was as follows. A *PD1* DNA fragment containing part of intron 1, exon 1, and 1.1 kilobases of 5'-flanking sequences was digested with 5 units of *Bal* 31 exonuclease per μg from a

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unique *Sac*I site for periods of time between 7 and 15 min. The deleted fragments were isolated and subcloned into the *Hin*dIII site of pSV0 (27). The deletion endpoints were all determined by DNA sequence analysis of the deletion fragments. 5′ deletion mutants were constructed by removal of *Nde*I-*Nde*I fragments from position −528 to the unique *Nde*I site in the pSV0 vector. Competitor plasmids were constructed by deletion of the *Bam*HI-*Hin*dIII fragment containing the *cat* gene.

**DNA transfections.** Cells were transfected with plasmid DNA by the calcium phosphate method (14). Efficiency of transfection was quantitated by isolation of DNA and hybridization with appropriate probes (27).

**Assays for CAT expression.** CAT assays were performed 48 to 72 h after transfections as described previously (14). Protein concentrations of the extracts were determined by a Bio-Rad protein assay. Unless otherwise indicated, identical amounts of protein, usually corresponding to 20 to 50 μl of the extracts, were added to the reaction mix.

**Preparation of RNA.** RNA samples from cell suspensions were prepared by the guanidinium isothiocyanate-CsCl procedure of Maniatis et al. (27).

**Hybridization probes.** The *cat* probe used for filter hybridization was the 1.7-kilobase *Hin*dIII-*Bam*HI fragment derived from the pSV0 vector. The *cat* probe used for S1 nuclease analysis extended from the *Bgl*II site of *PD1* to the *Eco*RI site in the *cat* gene (Fig. 1B). DNA fragments were radiolabeled by nick translation (27) and purified by passage over Elutip D columns (Schleicher & Schuell, Inc., Keene, N.H.).

**S1 nuclease analysis.** End-labeled (27) DNA probe was hybridized to 60 μg of total cellular RNA for 16 h at 51°C. S1 treatment and gel electrophoresis were as described previously (1, 46).

**Filter hybridization.** Analysis of total cellular RNA was performed in formaldehyde agarose gels (27, 44). Hybridization was performed at 42°C in 40% formamide–10% dextran sulfate–4× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–10× Denhardt–10 mM Tris hydrochloride (pH 7.5)–800 μg of denatured salmon sperm DNA–800 μg of denatured *Escherichia coli* DNA–100 ng of radiolabeled probe. The final wash was in 0.1× SSC at 65°C. Filters to be used for rehybridization were stripped by the method of Thomas (44). Quantitation of specific RNA hybridization was performed by densitometric analysis of exposed X-ray film.

**DNA sequence analysis.** DNA sequence analysis was by M13 cloning and dideoxy sequencing (35). Sequence comparisons were by either the NUCALN program (49) or the SEQ program (33).

## RESULTS

**DNA sequence of 5′-flanking region of *PD1*.** The DNA sequence of the 5′-flanking region of *PD1* was determined (Fig. 1A). DNA sequence elements necessary for *PD1* promoter activity are located between positions −38 and −220 base pairs (bp) (Singer et al., in press). (Because the precise transcriptional initiation site has not yet been mapped for any class I gene, all numbering of 5′-flanking sequences is relative to the translational initiation site, ATG, with A as +1.) Between 55 and 154 nucleotides upstream of the translation initiation site, the *PD1* sequence contains the sequence ACCC as well as the canonical promoter elements CCAAT separated by 29 bp from the sequence TCTAA (a variant of a TATAA box). The elements ACCC and CCAAT have been functionally associated with promoter activity in other gene systems (8), whereas the ability of TCTAA to function as a promoter element has been demonstrated only for *PD1* (Singer et al., in press), although it is also found in HLA class I genes (42). The initiation of transcription of *PD1* has been mapped by S1 nuclease protection to approximately 25 bp downstream of the TCTAA element (data not shown). Comparison of this region with a variety of other class I MHC genes, including $K^b$ (48), *A3* (42), HLA12.4 (26), RLA RG19 (28), $Q7^b$ (7), and $Q7^d$ (40), revealed that the tetranucleotide sequence TCAG is conserved in all these genes at the same position relative to the TATAA box; transcription is presumed to begin within this sequence.

DNA sequence elements with homology to known enhancer elements are found within the 5′-flanking region. The region −373 to −364 bp is homologous to the core enhancer sequence [GGTGTGG(A/T)(A/T)(A/T)G], as defined by Laimins et al. (23), differing in only two positions (underlined in Fig. 1A). The region −118 to −106 bp is homologous to the $K^b$ enhancer B, with 10 of 13 positions identical, and occurs in approximately the same position relative to the initiation of transcription. The segment −203 to −214 bp (TGG GAGTCCCCG) is homologous to a region in the $K^b$ gene which binds a nuclear factor (16a). Finally, the stretch between −447 and −462 bp shows homology with a segment in the $K^b$ gene described by Kimura et al. (17) as sequence VII.

The *PD1* interferon-responsive element has been functionally mapped to the region between −38 and −220 bp (Singer et al., in press). Within this region, between −180 and −209 bp (underlined in Fig. 1A), is a sequence which is 69% homologous to the extended consensus sequence of the interferon-responsive element described by Friedman and Stark (11). However, within the invariant core consensus sequence of 7 bp, *PD1* differs in two positions. The $H\text{-}2K^b$ interferon response element (17) is 72% homologous to the *PD1* sequence and is located in the same position relative to the first codon. A similar degree of homology (79%) was noted between *PD1* and analogous region of the murine *Q10* gene, which does not respond to interferon. Comparison of the three class I sequences revealed no consistent substitutions which can predict interferon responsiveness. Upstream sequences flanking the interferon response element, which were suggested to function as enhancer sequences necessary for interferon responsiveness of $K^b$ (16, 17), showed no significant homology between *PD1* and $K^b$. No greater homology was observed comparing the sequences of *PD1* and HLA-A3 (42). Therefore, it remains to be established whether this particular sequence within the *PD1* segment constitutes the interferon response element for the SLA gene and whether its function is dependent on the existence of other 5′-flanking sequences.

**Identification of enhancer element in 5′-flanking region of *PD1*.** Positive regulatory elements have been identified in the 5′-flanking regions of a variety of genes, including class I genes in other species (30; Israel et al., in press). In mouse class I genes, the enhancer element overlaps the interferon-responsive element. To test the possibility that an enhancer element exists in the 5′-flanking segment of *PD1*, a 308-bp fragment of *PD1* extending from −220 to −528 bp (Fig. 1) was introduced 5′ to the SV40 promoter in the vector pSV3, which contains the SV40 promoter ligated to the bacterial *cat* gene, but does not contain any viral enhancer sequences. The ability of this construct to direct CAT synthesis in transfected L cells relative to that of the pSV3 vector is shown in Table 1. The presence of the *PD1* 5′ DNA segment

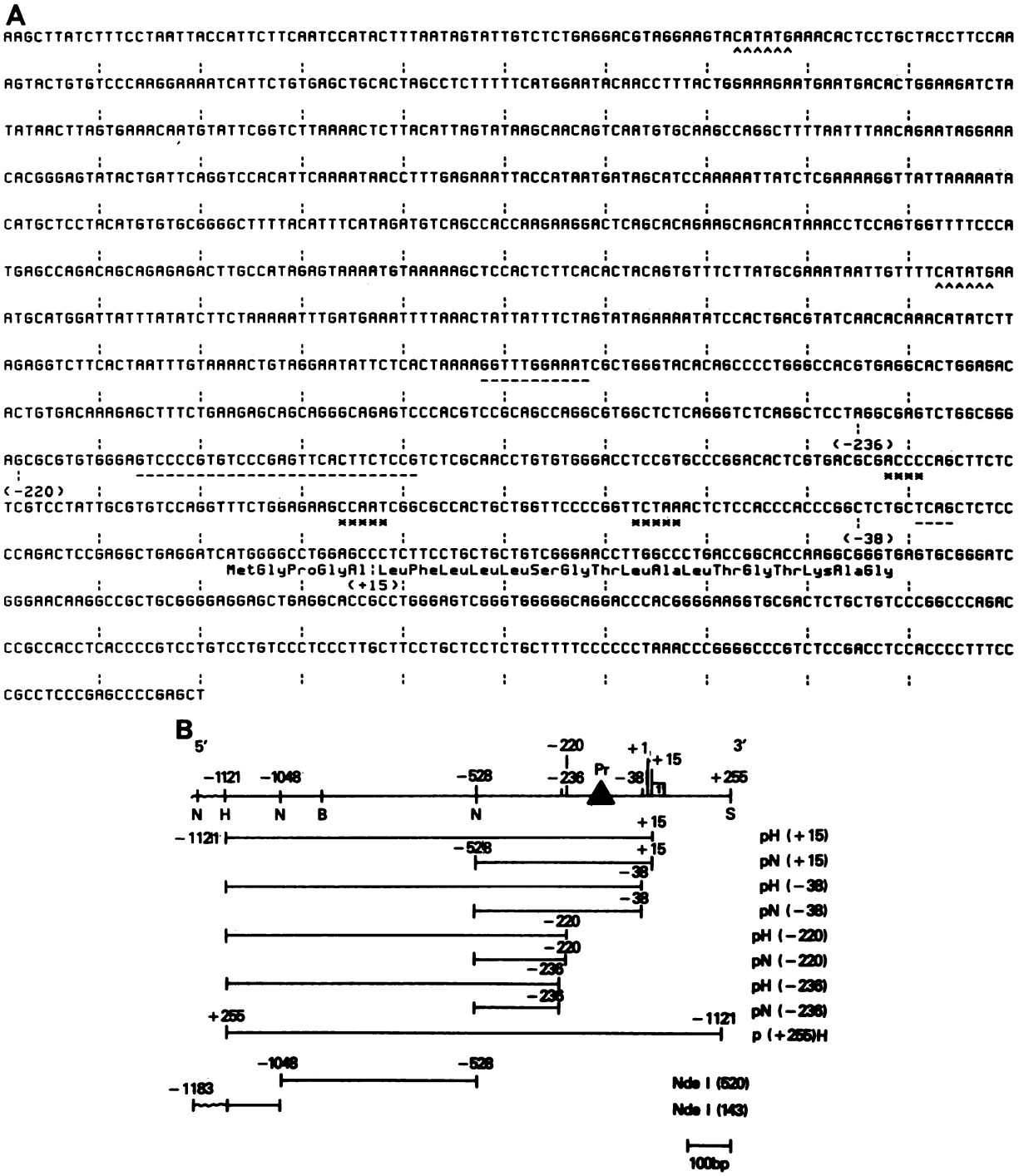


FIG. 1. (A) DNA sequence of *PD1* 5′-flanking region. The 1,420-bp DNA sequence contains 5′-flanking sequence, exon 1, and part of intron 1. All numbering of deletions and sequence elements is relative to the translational initiation site. DNA sequence elements are indicated as follows: *, TCTAA, CCAAT, ACCC; – – – –, −373 to −364 bp, a sequence homologous to core enhancer sequence (53); – – – –, −180 to −209 bp sequence homologous to interferon consensus sequence (10); – – – –, −32 to −29 bp, transcription initiation. ^, *Nde*I restriction sites. Numbers indicate precise endpoints of deletions from panel B. The sequence of the DNA segment −251 to +297 is reproduced from Satz et al. (37). (B) DNA fragments from the 5′-flanking region of *PD1* used to construct *cat* fusion genes. DNA deletion fragments containing *PD1* 5′-flanking sequences (solid line) were inserted into the *Hin*dIII site of pSV0 (see Materials and Methods). Except for p(+255)H, all fragments were inserted in their correct orientation relative to *cat*; p(+255)H was in the opposite orientation. *Nde*I (520) and *Nde*I (143) fragments were inserted into a unique *Nde*I site in pSV1 and pSV2 vectors. Restriction sites used for sequencing and subcloning: H, *Hin*dIII; B, *Bgl*II; N, *Nde*I; S, *Sac*I.

TABLE 1. *PD1* 5'-flanking sequence contains a positive regulatory element[a]

| DNA concn (μg) | Relative CAT activity generated by: | | |
|---|---|---|---|
| | pSV3 | pSV3[*PD1*(−528→−220)] | pSV2 |
| 2.5 | 1.0 | 4.4 ± 3.0 | 15.8 ± 9.4 |
| 5.0 | 1.0 | 4.4 ± 1.2 | 10.8 ± 3.5 |

[a] A 308-bp (−528→−220) fragment derived from the 5'-flanking region of *PD1* (Fig. 1B) was introduced into pSV3 at a unique *Bam*HI site (see Materials and Methods). The activities of the vectors pSV3, pSV3[PD1(−528→−220)], and pSV2 were assessed after transient transfection of mouse L cells by assaying CAT activity. The results were normalized to the activity of pSV3 to allow for comparison between different experiments. The results represent the summary of three experiments.

in the pSV3 vector enhanced the level of CAT activity by four- to fivefold in transfected cells over the range of concentrations tested. This augmentation is specific for this DNA segment since insertion of a segment of DNA from another region of the *PD1* gene (spanning exon 2 through intron 3) did not affect the level of CAT activity (data not shown). Thus, the *PD1* gene contains an enhancer as well as an interferon-responsive element in the 5'-flanking region extending from −220 to −528 bp.

**Identification of negative regulatory element in 5'-flanking region of *PD1*.** To identify functionally any other regulatory elements within the 5' end of *PD1*, we constructed a series of deletion mutants (Fig. 1B). The 5' termini of the mutants were located either at a *Hin*dIII site at −1121 (pH series) or at an *Nde*I site at −528 (pN series) (Fig. 1B). A nested set of deletions (spanning nucleotides +15 to −236) was generated by *Bal* 31 exonuclease digestion from a unique *Sac*I site located within intron 1 (Fig. 1B). The precise locations of the deletion endpoints were determined by DNA sequence analysis and are indicated in Fig. 1A. The deletion mutants were cloned into the promoter-assay vector pSV0CAT, which lacks eucaryotic promoters or enhancers, and tested for their ability to promote CAT synthesis after transfection into Cos cells (Fig. 2). The 5'-deleted derivative consistently gave 5- to 10-fold-higher CAT activity than the parental construct. A similar difference was found between pH(+15) and pN(+15). The same results were obtained with mouse L cells or pig fibroblast cell lines (data not shown). A variety of technical explanations for these observations could be eliminated. The differences between the pN and pH series are not due to different efficiencies of transfection since transfected cells contained equal amounts of plasmid DNA (see Materials and Methods). Furthermore, the differences cannot be attributed to the difference in length of the *PD1* insert in the *cat* vector (1.1 kb versus 0.5 kb) or to any effect of the insert on a cryptic promoter present within the pBR322 DNA sequences since no difference in CAT activity was seen between pH(−236) and pN(−236), neither of which has eucaryotic promoter activity (Fig. 2). Therefore, the results suggest that the DNA segment −1121 to −528 bp, which had been removed from the pN series of constructs, contained a negative regulatory element.

To investigate the molecular basis for the differences in CAT activity, we analyzed RNA from cells transiently transfected with either pH(−38) or pN(−38) for the presence of *cat* mRNA (Table 2). Both direct Northern (RNA) and S1 nuclease analyses of RNA from transfected Cos cells gave comparable results, namely, that pN(−38) transfectants generated three- to fourfold more steady-state levels of *cat*-specific mRNA than pH(−38) transfectants. Furthermore, the S1 nuclease analysis revealed only a single correctly initiated transcript in both transfectants (data not shown). Northern analysis of RNA from L cells transfected with the two constructs gave similar results (Table 2). Thus, the presence of the negative regulatory element in the 5'-flanking region of the *PD1* gene results in reduced levels of *PD1* transcripts in transfected cells.

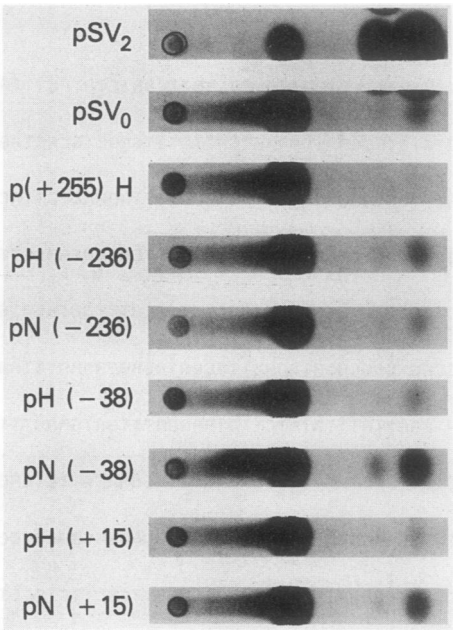


FIG. 2. A DNA segment in the 5'-flanking region of *PD1* reduces CAT activity. Cos cells were transiently transfected with 1 μg of the indicated deletion constructs; cell extracts were assayed for CAT activity. The densitometric values of the acetylated chloramphenicol spots on the X-ray film were as follows: pSV0, 1.37; p(+255)H, 0; pH(−236), 2.0; pN(−236), 1.2; pH(−38), 1.0; pN(−38), 8.9; pH(+15), 0.8; pN(+15), 4.5.

The deletion constructs used to identify the negative regulatory element mapped its location to the region −1120 to −528 bp, which spans two *Nde*I sites (Fig. 1B). To further map the position of the negative regulatory element, we tested the ability of the larger, 520-bp *Nde*I fragment to decrease *PD1* promoter activity. The 520-bp *Nde*I fragment was reintroduced in both orientations into the pN(−38) construct. The amounts of CAT activity generated by the pN(−38) constructs containing the 520-bp *Nde*I fragment

TABLE 2. Negative regulatory element decreases the activity of the *PD1* promoter

| DNA | *cat* RNA[a] | | |
|---|---|---|---|
| | Expt 1 | Expt 2 | Expt 3 |
| pN(−38) | 3.6 | 3.2 | 4.7 |
| pH(−38) | 1 | 1 | 1 |
| pSV2 | 13.8 | ND[b] | ND |

[a] Values represent relative levels of *cat* RNA among the constructs. These numbers were derived by densitometric analysis; in experiments 1 and 3, values were normalized to an internal actin control to correct for variations in the amount of RNA loaded on the gel. Experiment 1, Northern analysis of RNA from transiently transfected Cos cells. Experiment 2, S1 nuclease analysis of 60 μg of RNA derived from transiently transfected Cos cells. Hybridization was with an excess of probe. Experiment 3, Northern analysis of RNA from transiently transfected L cells. Probes and protocols were as described in Materials and Methods.

[b] ND, Not determined.

TABLE 3. Negative regulatory element functions on both homologous and heterologous promoters[a]

| Insert (bp) | Relative CAT activity | | |
|---|---|---|---|
| | pN(−38) | pSV2 | pSV1 |
| None | 1.0 | 1.0 | 1.0 |
| NdeI(F) (520) | 0.08 ± 0.02 | 1.5 ± 0.03 | 1.6 ± 0.03 |
| NdeI(R) (520) | 0.08 ± 0.06 | 0.5 ± 0.03 | 1.4 ± 0.06 |
| NdeI(F) (143) | | 1.5 ± 0.03 | 1.0 ± 0.01 |
| NdeI(R) (143) | | 1.3 ± 0.03 | |

[a] The large 520-bp and small 143-bp *Nde*I fragments derived from the 5'-flanking regions of *PD1* (Fig. 1B) were introduced into pSV1 and pSV2 at the unique *Nde*I site in the vector; the 520-bp fragment was also reintroduced into the pN(−38) vector from which it was derived. The orientation of the inserts is denoted by F (forward) or R (reverse). The activity of the vectors containing the fragments was assessed after transient transfection into mouse L cells by assaying CAT activity. DNA concentrations in each of the transfections were monitored and were approximately equal. The results have been normalized to the appropriate vector control (in the absence of insert) to allow for comparison between different experiments. The absolute level of CAT activity was highest in the pSV2 series and lowest in the pSV1 series. The results represent the compilation of three experiments.

were compared with that generated by pN(−38) alone (Table 3). Reinsertion of the 520-bp *Nde*I fragment into pN(−38) in either orientation reduced CAT activity by greater than 10-fold, which was the level observed with the native pH(−38). Therefore, the negative regulatory element resides within the region −1048 to −528 bp.

The ability of the negative regulatory element to function on a heterologous promoter was examined by introducing the 520-bp *Nde*I fragment of *PD1* into the unique *Nde*I site upstream of the SV40 promoter in the pSV1CAT and pSV2CAT constructs. Both of these vectors contain the SV40 promoter fused to the *cat* gene. They differ in that pSV2 retains the 72-bp enhancer elements, whereas pSV1 does not. The 143-bp *Nde*I fragment of pH(−38) (Fig. 1B) was also inserted to serve as a control. Each of these constructs was transfected into L cells, and the amount of CAT activity relative to that of the parental construct (pSV2 or pSV1) was determined (Table 3). Transfection of Cos 7 cells gave comparable results (data not shown). The control 143-bp *Nde*I fragment did not decrease the relative amount of CAT activity directed by either pSV1 or pSV2. The 520-bp *Nde*I fragment, to which the negative regulatory element was mapped, did not reduce activity of the pSV1 construct. However, a threefold reduction of CAT activity relative to that of the other constructs was observed when this fragment was introduced into the pSV2CAT construct, which contains the viral enhancer. Thus, the *PD1* negative regulatory element functions in association with a heterologous SV40 promoter, but only in the presence of the viral enhancer element. The negative regulatory element is less efficient in conjunction with a heterologous promoter, for which a 3-fold reduction is seen, than in conjunction with the homologous *PD1* promoter, for which a 10-fold reduction is seen (Table 3). Furthermore, whereas the negative regulatory element functions in either orientation in association with the *PD1* promoter, it functions in only one orientation in association with the SV40 promoter.

**Mediation of the functions of *PD1* regulatory elements by cellular *trans*-acting factors.** The functional requirement of the negative regulatory element for the presence of the SV40 enhancer suggested that the interaction of these two elements was mediated by cellular *trans*-acting factors. To test this hypothesis, we performed a series of in vivo competition assays. Test plasmids, either pH(−38) which contains both the *PD1* positive and negative regulatory elements and promoter or pN(−38) which contains only the *PD1* positive regulatory element and promoter (Fig. 1B), were cotransfected into monkey Cos 7 cells with one of a variety of competitor DNAs containing different regulatory elements (Fig. 3). Included among these were the *PD1* negative regulatory element, either alone or in association with the SV40 enhancer, and the SV40 enhancer alone. In all cases, the competitors contained not only the regulatory DNA element of interest and the SV40 promoter, but also an SV40 origin of replication. Since Cos cells constitutively express T antigen, the competitor DNA, but not the test DNA, was able to replicate. The competitor DNAs were derived from the plasmids described above by removal of the *cat* gene (referred to as ΔCAT). In all these experiments, a plasmid containing only the SV40 origin of replication and promoter with no other regulatory elements (pSV1ΔCAT) was used as a control and as a carrier in titration experiments. Preliminary experiments documented that this construct did not affect CAT activity generated by the test plasmids; none of the competitor constructs alone generated CAT activity (data not shown).

That the in vivo competition assay works was demonstrated by the fact that cotransfection of pSV2 with the competitor pSV2ΔCAT, containing the viral enhancer elements, reduced the level of CAT activity generated by pSV2 (Fig. 3A). This figure also demonstrates that the presence of the *Nde*I fragment (containing the negative regulatory element) in the construct did not alter the ability of the SV40 enhancer to compete for enhancer-binding factors. This competition assay was therefore suitable for testing *trans*-acting factors.

The ability of the test plasmids to direct the synthesis of CAT in the presence of various amounts of competitor DNA was assessed. A representative competition experiment in which 1 μg of test plasmid DNA was cotransfected with 10 μg of competitor DNA is shown in Fig. 3B; a summary of all the competition assays done is given in Fig. 4. The construct pH(−38), which contains both negative and positive regulatory elements of *PD1*, was cotransfected with a number of different competitors. Cotransfection with pSV1ΔCAT, pSV2ΔCAT, or pSV1NdeΔCAT had no marked effect on the level of CAT activity generated by the pH(−38) test DNA, indicating their inability to compete for factors associated with the negative regulatory element. Cotransfection with pSV2NdeFΔCAT or pSV2NdeRΔCAT resulted in significantly increased levels of CAT activity (Fig. 3B and 4A). Two conclusions can be made from these results. (i) *Trans*-acting factors mediate the function of the negative regulatory element and (ii) the ability of the negative regulatory element to interact with these factors is dependent on the presence of an enhancer, as evidenced by the fact that pSV2NdeΔCAT, which contains the enhancer, was able to compete, whereas pSV1NdeΔCAT did not. Furthermore, although it was found that the negative regulatory element was able to function in association with the SV40 enhancer and promoter only in one orientation (Table 3), it was able to act as a competitor in either orientation (Fig. 4A). Thus, the criteria for function and competition are not identical.

When pN(−38), which contains only the *PD1* promoter and positive regulatory element, was used as the test DNA, pSV1ΔCAT again had no effect on the resulting level of CAT activity. However, in the presence of pSV2ΔCAT as competitor, there was a decrease in the amount of CAT generated from the pN(−38) construct, indicating the ability of the SV40 enhancer in pSVΔCAT to compete with the *PD1*

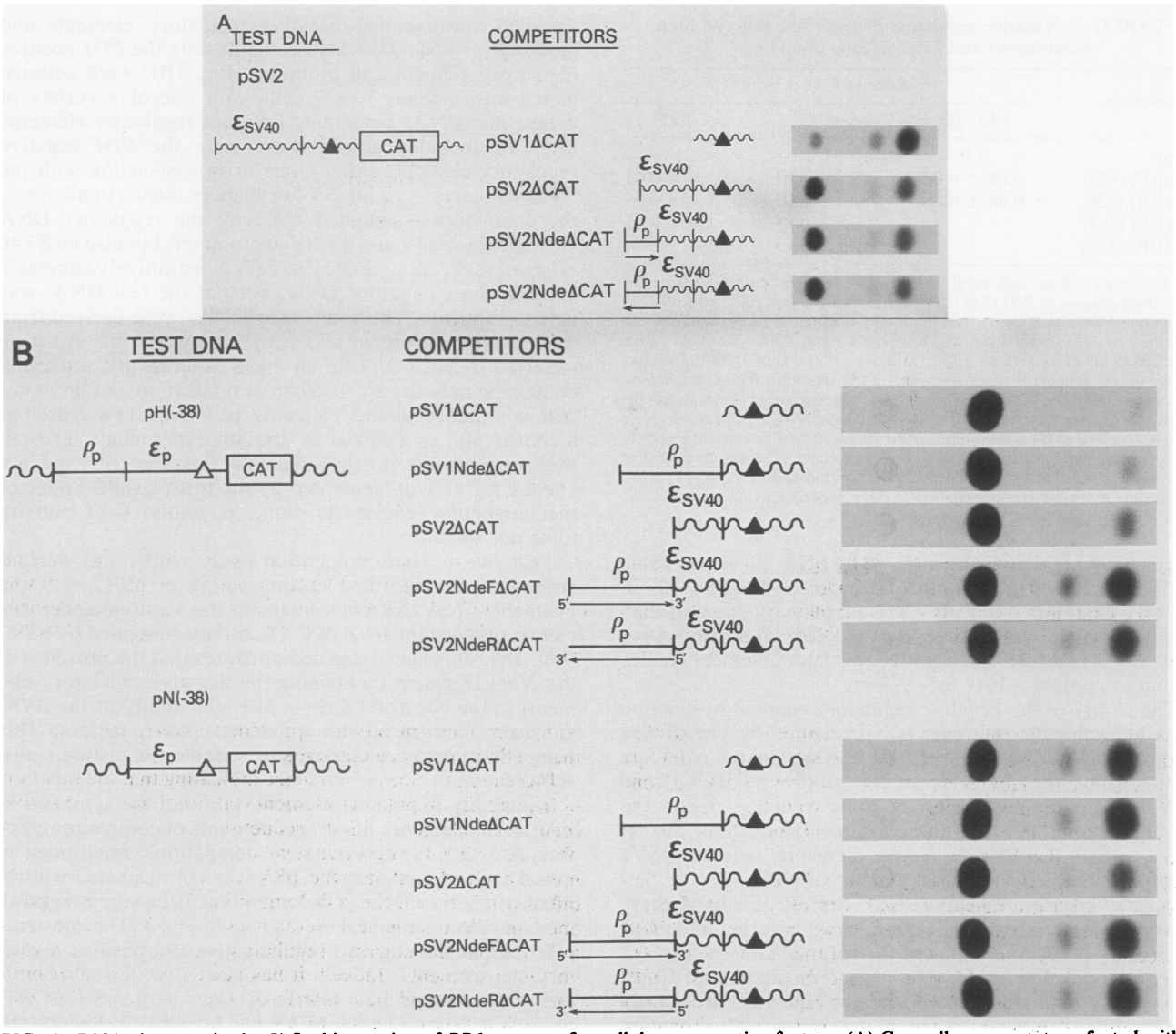


FIG. 3. DNA elements in the 5′-flanking region of *PD1* compete for cellular *trans*-acting factors. (A) Cos cells were contransfected with 1 μg of pSV2 test DNA and 10 μg of pSV1ΔCAT, pSV2ΔCAT, pSV2NdeFΔCAT, or pSV2NdeRΔCAT competitor DNA. CAT activity was assayed as described in the text. (B) Cos cells were cotransfected with 10 μg of the competitor DNAs and 1 μg of either pH(−38) test DNA or pN(−38) test DNA. CAT activity was assayed as described in the text. $\rho_p$, *PD1* negative regulatory element; $\varepsilon_p$, PD1 positive regulatory element; $\varepsilon_{SV40}$, SV40 enhancer; Δ, PD1 promoter; ▲, SV40 promoter. Solid lines represent *PD1* sequences; wavy lines represent SV40 sequences. Arrows indicate the orientation of the *PD1* negative regulatory element within the vector.

positive regulatory element for *trans*-acting factors (Fig. 3B and 4B). Thus, the viral enhancer and the class I positive regulatory element must share at least a subset of common binding factors which are essential for enhancer function.

## DISCUSSION

In the present study, we identified both negative and positive regulatory elements within 1.1 kilobase of initiation of transcription in the 5′-flanking regions of the swine class I MHC gene *PD1*. Cellular *trans*-acting factors were shown to mediate the functions of these elements. The ability of the negative regulatory element to function appeared to be dependent on a positive regulatory element. This kind of interaction between regulatory elements has not been previously described.

The positive regulatory element is located in the region −220 to −528 bp and is able to enhance activity of a heterologous (SV40) promoter by four- to fivefold. Enhancer elements have been described for the murine class I gene $K^b$ (16, 45) and have been mapped to a region between −213 and −165 bp upstream of initiation of transcription. Comparison of this region of the $K^b$ gene with the *PD1* sequence in the region −528 to −220 bp revealed a region between −106 and −118 which is 77% homologous to the $K^b$ enhancer B. In addition, the complement of the *PD1* sequence CCCTGGGC (−347 to −340 bp) is also found within the enhancer segment of $K^b$. Whether this element has enhancer activity remains to be established. Also contained within this region of *PD1* (between −361 and −373 bp) is a sequence with homology to the core enhancer sequence, as defined by Laimins et al. (23). In addition, there are various direct and inverted repeats. The functions, if any, of these elements remain to be demonstrated. Israel et al. (in press) have recently identified a binding site for a *trans*-acting factor at positions −170 to

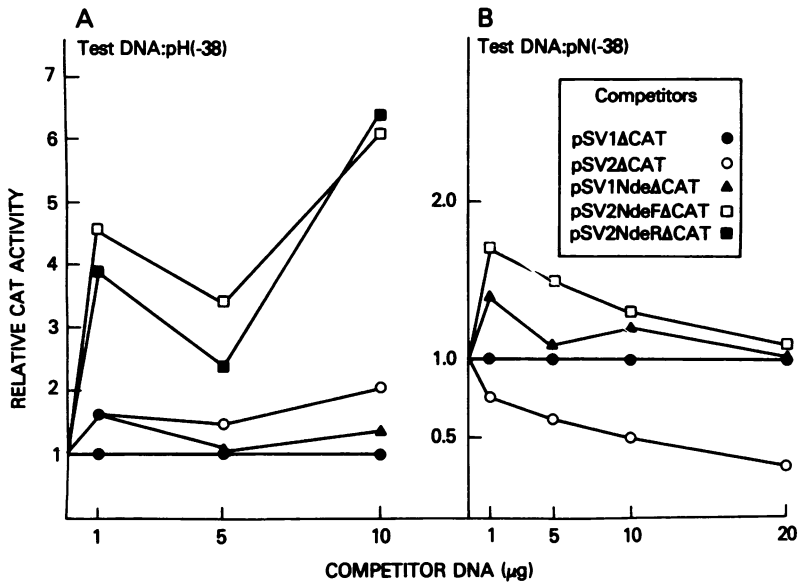


FIG. 4. DNA elements in the 5'-flanking sequences of *PD1* compete for cellular *trans*-acting factors. Cos cells were cotransfected with the various competitor DNA constructs (see Fig. 3) and 1 μg of either pH(−38) (A) or pN(−38) (B). pSV1ΔCAT was used as carrier DNA to adjust the total amount of DNA to 20 μg in all transfections. CAT activity was determined by densitometric analysis. For each test plasmid, all CAT activity was normalized to that obtained with pSV1 as the competitor. The results are compiled from five independent titration experiments for each test DNA.

−158 bp in the $K^b$ gene. A sequence with homology to this binding site is found in a similar position (−214 to −203 bp) in the *PD1* gene. However, the *PD1* sequence differs from the $K^b$ in a position which has been shown to be essential for binding. The ability of the *PD1* sequence to bind *trans*-acting factors remains to be established.

The present study showed that the *PD1* gene also has associated with it a negative regulatory element. The presence of this DNA segment, located between −528 and −1048 bp, reduces by 10-fold the efficiency of the homologous *PD1* promoter and by 3-fold that of the heterologous SV40 promoter. The function of the negative regulatory element is orientation independent when acting on the *PD1* promoter and positive regulatory element, but orientation dependent in the heterologous system. The reason for this difference is not clear. However, a similar effect was noted for the negative regulatory element associated with the beta interferon gene (12), suggesting that the distance separating the element and the heterologous promoter is critical.

DNA sequence elements have been shown to be important in the negative regulation of a variety of procaryotic genes. Recently, such elements have also been found associated with a number of eucaryotic genes, such as human beta interferon (12, 51), rat insulin I (22, 31), c-*myc* (34), c-*mos* (50), retinol-binding protein (4), and p53 (2). In addition, a negative regulatory element has recently been identified in the 5'-flanking region of the murine class I $L^d$ gene (30). This latter element has been demonstrated to be active only in undifferentiated F9 teratocarcinoma cells. After retinoic acid induction of F9 differentiation, this element appears to function as an enhancer. The negative regulatory element of *PD1* differs from the murine element in that it is active in both differentiated and undifferentiated F9 cells, as well as in a variety of other somatic cell types (data not shown). Thus, the negative regulatory element of *PD1* is unlike other regulatory elements which have been described for class I MHC genes. The *PD1* DNA segment containing the negative regulatory element has no apparent homology with any of these DNA sequence elements.

There are two lines of evidence indicating that the function of the negative regulatory element is dependent on a positive regulatory element. First, reduction in CAT activity is observed when the negative regulatory element is introduced into the pSV2, but not the pSV1, vector. Since the only difference between the pSV2 and pSV1 vectors is the presence of SV40 enhancer sequences in pSV2, these data indicate that the negative regulatory element interacts with enhancer elements. Indeed, it has been suggested that both the rat insulin and beta interferon negative regulatory elements function by acting on associated enhancer elements (13, 22). Second, in vivo competition studies strengthen the interpretation that the *PD1* negative regulatory element interacts with the positive regulatory element and that their functions are mediated by *trans*-acting cellular factors. The positive regulatory element mediates binding of factors common to those binding to the SV40 enhancer, since competition with the SV40 enhancer diminishes CAT activity directed by the *PD1* promoter in cotransfection assays. The factors whose binding is governed by the negative regulatory element can be competed by the autologous element, but only if that element is linked to a positive regulatory element, such as the SV40 enhancer. The ability to compete, unlike its ability to function, in association with the SV40 enhancer is orientation independent.

The molecular mechanism by which the regulatory elements and their factors function is not known. However, the present data suggest a model in which the negative and positive regulatory elements each define an independent binding site for distinct factors (Fig. 5). (It should be noted that none of the data demonstrate direct binding to either site or the number of factors associated with the elements. For simplicity in discussion, we will assume the binding of a factor to the element itself.) The binding to the positive element of its cognate factor (positive factor) occurs inde-

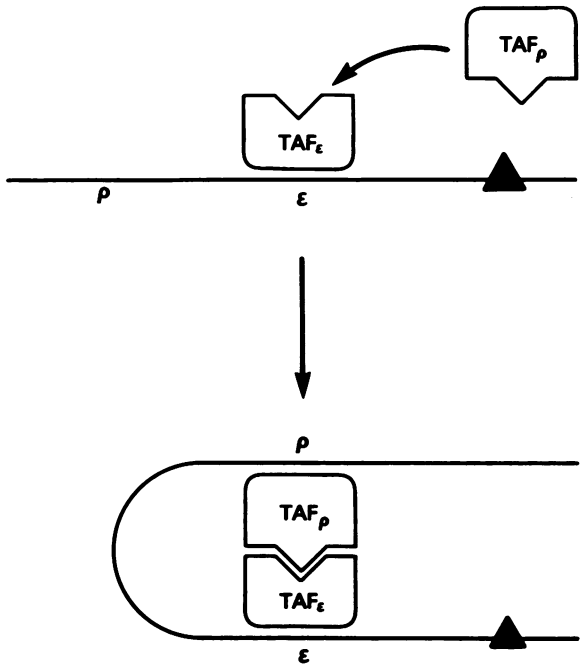


FIG. 5. Model for the interaction of the positive and negative regulatory elements of *PD1*. The enhancer-specific *trans*-acting factors (TAF$_\varepsilon$) first binds the enhancer (ε). This is followed by the association of the TAF$_\rho$, which in turn associates with the negative regulatory element, allowing a ternary complex to form. ▲, PD1 promoter.

pendently of the negative element. This is supported by the findings that the positive element functions in the absence of the negative element and is competed by the SV40 enhancer alone. The negative factor binds to the negative regulatory element; however, this interaction must be stabilized by a simultaneous interaction with the positive element. This interaction would be most likely to occur by complexing of the positive and negative factors. We speculate that a complex is formed between the factors binding to each of the elements, resulting in a change in conformation of the DNA segments which reduces the efficiency of RNA polymerase II binding. This model would explain the ability of pSV2Nde, but not pSV1Nde, to increase activity of pH(−38) in competition assays. An alternative model in which positive and negative factors compete for a common binding site can be eliminated by the data. The fact that pSV2Nde can compete for the enhancer-binding factor of SV40 demonstrates that it binds these factors. Thus, the presence of a negative regulatory element does not result in the displacement of a positive factor.

The nature of the cellular *trans*-acting factors remains to be determined. It is unlikely that the factors binding to the negative regulatory element are identical to those binding to the SV40 enhancer since the enhancer alone is unable to compete for the negative regulatory element binding factor. The ability to observe this competition also suggests that negative factors are either limiting relative to positive factors or bind more weakly. This conclusion is supported by the facts that (i) more pSV2 DNA is required to compete the positive regulatory element in pN(−38) than pSV2Nde for competing the negative regulatory element in pH(−38) and (ii) competition of pH(−38) with pSV2Nde results in only an increase in CAT activity. The decrease in activity corresponding to competition for positive factors is not observed within the dose range of this assay. Studies are currently under way to isolate these factors and determine their interactions with one another and the regulatory DNA elements.

The in vivo function of the *PD1* regulatory elements is unknown. The effects of the negative and positive regulatory elements are within the range of variation of class I expression observed in vivo among tissues. It can be speculated that the cumulative activities of the positive and negative regulatory elements as well as the promoter modulate the level of *PD1* expression in different tissues. Further detailed analysis of the 5′-flanking region of *PD1*, as well as of other SLA genes which display distinct patterns of expression, should contribute significantly to an understanding of the mechanisms regulating class I gene expression.

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## LITERATURE CITED

1. **Berk, A., and P. Sharp.** 1977. Sizing and mapping of early adenovirus mRNA's by electrophoresis of S1 endonuclease digested hybrids. Cell **12**:721–732.
2. **Bienz-Tadmor, B., R. Zakut-Houri, S. Liberesco, D. Givol, and M. Oren.** 1985. The 5′ region of the p53 gene: evolutionary conservation and evidence for a negative regulatory element. EMBO J. **12**:3209–3213.
3. **Burrone, O. H., and C. Milstein.** 1982. Control of HLA-A, B, C synthesis and expression in interferon treated cells. EMBO J. **1**:345–349.
4. **Calantuoni, C., A. Pivozzi, C. Blance, and R. Cortese.** 1987. Negative control of liver specific retinol binding protein gene is repressed in HeLa cells. EMBO J. **6**:631–636.
5. **Cohen, D., P. Paul, M. Font, O. Cohen, B. Sayagh, A. Marcadet, M. Busson, G. Majory, H. Cann, and J. Dausset.** 1983. Analysis of HLA class I genes with restriction endonuclease fragments. Implications for polymorphism of the human major histocompatibility complex. Proc. Natl. Acad. Sci. USA **80**:6289–6292.
6. **Collins, T., L. A. Lapierre, W. Fiers, J. L. Strominger, and J. S. Pober.** 1986. Recombinant human tumor necrosis factor increases mRNA levels and surface expression of HLA-A, B antigens in vascular endothelial cells and dermal fibroblasts in vitro. Proc. Natl. Acad. Sci. USA **83**:446–450.
7. **Devlin, J., E. H. Weiss, M. Paulson, and R. A. Flavell.** 1985. Duplicated gene pairs and alleles of class I genes in the Qa2 region of the murine MHC: a comparison. EMBO J. **12**:3203–3207.
8. **Dierks, P., A. Van Ooyen, M. D. Chochran, C. Dobkin, J. Reiser, and C. Weissman.** 1983. Three regions upstream from the cap site are required for efficient and accurate transcription of the rabbit beta globin gene in mouse 3T6 cells. Cell **32**:695–706.
9. **Faulk, W. D., A. R. Sanderson, and A. Temple.** 1977. Distribution of MHC antigens in human placental chorionic villi. Transplant. Proc. **9**:1379–1384.
10. **Frels, W. I., J. A. Bluestone, R. Hodes, M. Capecchi, and D. S. Singer.** 1985. Expression of a microinjected porcine class I MHC gene in transgenic mice. Science **228**:577–580.
11. **Friedman, R. L., and G. R. Stark.** 1985. Alpha interferon induces transcription of HLA and metallothionein genes which have homologous upstream sequences. Nature (London) **314**: 637–639.
12. **Goodbourn, S., H. Burstein, and T. Maniatis.** 1986. The human beta interferon gene enhancer is under a negative control. Cell **45**:601–610.
13. **Goodbourn, S., K. Zinn, and T. Maniatis.** 1985. Human beta

interferon gene expression is regulated by an inducible enhancer element. Cell **41**:509–520.
14. **Gorman, C., L. F. Moffat, and B. H. Howard.** 1982. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. Mol. Cell. Biol. **2**:1044–1051.
15. **Imai, K., M. A. Pellegrino, A. K. Ng, and S. Ferron.** 1981. Role of antigen density in immune lysis of interferon-treated human lymphoid cells. Scand. J. Immunol. **14**:529–535.
16. **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.
16a. **Israel, A., A. Kimura, M. Kieran, O. Yano, J. Kanellopoulis, O. Le Bail, and P. Kourilsky.** 1987. A common positive trans-acting factor binds to enhancer sequences in the promoters of mouse H-2 and beta2-microglobulin genes. Proc. Natl. Acad. Sci. USA **84**:2653–2657.
17. **Kimura, A., A. Israel, O. LeBail, and P. Kourilsky.** 1986. Detailed analysis of the mouse H-2K$^b$ promoter: enhancer-like sequences and their role in the regulation of class I gene expression. Cell **44**:261–272.
18. **Klein, J.** 1975. Biology of the mouse histocompatibility complex. Springer-Verlag, New York.
19. **Klein, J.** 1979. The major histocompatibility complex of the mouse. Science **203**:516–521.
20. **Klein, J., and E. Gotze (ed).** 1977. Major histocompatibility complex. Springer-Verlag, New York.
21. **Korber, B., L. Hood, and I. Stroynowski.** 1987. Regulation of murine class I genes by interferons is controlled by regions located both 5′ and 3′ to the transcriptional initiation site. Proc. Natl. Acad. Sci. USA **84**:3380–3384.
22. **Laimins, L., M. Holmgren-Konig, and G. Khoury.** 1986. Transcriptional "silencer" element in rat repetitive sequences associated with the rat insulin 1 gene locus. Proc. Natl. Acad. Sci. USA **83**:3151–3155.
23. **Laimins, L., G. Khoury, C. Gorman, B. H. Howard, and P. Gruss.** 1982. Host-specific activation of transcription by tandem repeats from SV40 and Moloney murine sarcoma virus. Proc. Natl. Acad. Sci. USA **79**:6453–6457.
24. **Lampson, L. A., C. A. Fisher, and J. P. Whelan.** 1983. Striking paucity of HLA-ABC and beta$_2$-microglobulin on human neuroblastoma cell lines. J. Immunol. **130**:2471–2478.
25. **Lindhal, P., P. Leary, and I. Gresser.** 1973. Enhancement by interferon of the expression of histocompatibility antigens of mouse lymphoid cells. Eur. J. Immunol. **4**:779–784.
26. **Malissen, M., B. Malissen, and B. Jordan.** 1982. Exon/intron organization and complete nucleotide sequence of an HLA gene. Proc. Natl. Acad. Sci. USA **79**:893–897.
27. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Marche, P., M. Tykocinski, E. Max, and T. J. Kindt.** 1984. Structure of a functional rabbit class I MHC gene: similarity to human class I genes. Immunogenetics **21**:71–82.
29. **Meruelo, D., R. Kornreich, A. Rossomondo, C. Pampeno, A. L. Mellor, E. H. Weis, R. A. Flavell, and A. Pellicer.** 1984. Murine leukemia virus sequences are encoded in the murine MHC. Proc. Natl. Acad. Sci. USA **81**:1804–1808.
30. **Miyazaki, J., E. Appella, and K. Ozato.** 1986. Negative regulation of the MHC class I gene in undifferentiated embryonal carcinoma cells. Proc. Natl. Acad. Sci. USA **83**:9537–9541.
31. **Nir, U., M. D. Walker, and W. J. Rutter.** 1986. Regulation of rat insulin I gene expression. Evidence for negative regulation in nonpancreatic cells. Proc. Natl. Acad. Sci. USA **83**:3180–3184.
32. **Orr, H., R. Bach, H. Ploegh, J. L. Strominger, P. Kavathas, and R. DeMars.** 1982. Use of HLA loss mutants to analyse the structure of the human MHC. Nature (London) **296**:454–456.
33. **Queen, C., and L. J. Korn.** 1984. A comprehensive sequence analysis program for the IBM personal computer. Nucleic Acids Res. **12**:591–599.
34. **Remmers, E. G., J. Q. Yang, and K. B. Marcu.** 1986. A negative transcriptional control element located upstream of the murine c-myc gene. EMBO J. **5**:899–904.
35. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
36. **Satz, M., and D. S. Singer.** 1984. Effect of mouse interferon on the expression of the porcine MHC gene introduced into mouse L-cells. J. Immunol. **132**:496–501.
37. **Satz, M., L. C. Wang, D. S. Singer, and S. Rudikoff.** 1985. Structure and expression of two porcine genomic clones encoding class I MHC antigens. J. Immunol. **135**:2167–2175.
38. **Schrier, P., R. Bernard, M. J. Vaseen, A. H. Howeling, and A. J. van der Eb.** 1983. Expression of class I MHC switched off by highly oncogenic adenovirus 12 in transformed rat cells. Nature (London) **305**:771–775.
39. **Singer, D. S., M. Camerini-Otero, M. Satz, B. Osborne, D. Sachs, and S. Rudikoff.** 1982. Characterization of a porcine genomic clone encoding a major transplantation antigen; expression in mouse L-cells. Proc. Natl. Acad. Sci. **79**:1403–1407.
40. **Steinmetz, M., K. W. Moore, J. Frelinger, B. T. Sher, F. Shen, E. A. Boyse, and L. Hood.** 1981. A pseudogene homologous to mouse transplantation antigens: transplantation antigens are encoded by eight exons that correlate with protein domains. Cell **25**:683–692.
41. **Steinmetz, M., A. Winoto, K. Minard, and L. Hood.** 1982. Clustering of genes encoding mouse transplantation antigens. Cell **28**:489–498.
42. **Strachan, T., R. Sodoyer, M. Damotte, and B. Jordan.** 1984. Complete nucleotide sequence of a functional class I HLA gene, HLA-A3; implications for the evolution of HLA genes. EMBO J. **3**:887–897.
43. **Sugita, K., J. Miyazaki, E. Appella, and K. Ozato.** 1987. Interferons increase transcription of a major histocompatibility complex class I gene. Mol. Cell. Biol. **7**:2625–2630.
44. **Thomas, P.** 1980. Hybridization of denatured RNA and small DNA fragments transferred to nitrocellulose. Proc. Natl. Acad. Sci. USA **77**:5201–5205.
45. **Vogel, J., M. Kress, G. Khoury, and G. Jay.** 1986. A transcriptional enhancer and an interferon-responsive sequence in major histocompatibility complex class I genes. Mol. Cell. Biol. **6**: 3550–3554.
46. **Weaver, R., and C. Weissman.** 1979. Mapping of RNA by modification of the Berg-Sharp procedure: the 5′ termini of 15S globin mRNA, precursor, and 10S globin have identical map coordinates. Nucleic Acids Res. **6**:1175–1192.
47. **Weiss, E. H., L. Golden, K. Fahrner, A. Mellor, H. Devlin, H. Tiddens, H. Bud, and R. A. Flavell.** 1984. Organization and evolution of the class I gene family in the MHC of the C57BL/10 mouse. Nature (London) **310**:650–655.
48. **Weiss, E. H., L. Golden, R. Zakut, A. Mellor, K. Fahrner, S. Kvist, and R. A. Flavell.** 1983. The DNA sequence of the H-2K$^b$ gene: evidence for gene conversion as a mechanism for the generation of a polymorphism in histocompatibility antigens. EMBO J. **3**:453–462.
49. **Wilbur, W., and D. Lipman.** 1983. Rapid similarity searches of nucleic acid and protein data banks. Proc. Natl. Acad. Sci. USA **80**:726–730.
50. **Wood, T. G., M. L. McGeady, B. M. Baroudy, D. G. Blair, and G. F. Vande Woude.** 1984. Mouse c-mos oncogene activation is prevented by upstream sequences. Proc. Natl. Acad. Sci. USA **81**:7817–7821.
51. **Zinn, K., and T. Maniatis.** 1986. Detection of factors that interact with human beta interferon regulatory regions in vivo by DNase I footprinting. Cell **45**:611–618.