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Immunology

X-box-binding proteins positively and negatively regulate transcription of the *HLA-DRA* gene through interaction with discrete upstream W and V elements

(class II major histocompatibility complex genes/cis-acting regulatory elements/DNA-binding proteins)

JOHN P. COGSWELL, PATRICIA V. BASTA*, AND JENNY P.-Y. TING

Lineberger Cancer Research Center, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599-7295

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ABSTRACT **Previous reports have identified that the class II box, consisting of the positive regulatory X and Y boxes, is important for expression of all class II major histocompatibility genes. In this paper, we identify additional sequences upstream from the class II box that regulate constitutive transcription of a human class II gene, *HLA-DRA*, in the B-lymphoblastoid cell line Raji. Using 5′ promoter deletions, substitution mutants, and nuclease S1 protection assays, we mapped a positive element, called W, between −135 and −117 base pairs and a negative element, called V, from −193 to −179 base pairs. Sequence comparisons revealed that W and V share homology with the *HLA-DRA* X box situated downstream. Gel-mobility-shift assays confirmed that the Raji nuclear proteins that bound to W and V elements were competed with by an *HLA-DRA* X-box oligonucleotide. These results suggest that X-box-binding proteins mediate both positive and negative effects on transcription by means of interaction with multiple elements (W, V, and X) within the same *HLA-DRA* gene.**

The class II major histocompatibility complex molecules are essential in immune function to restrict activation and differentiation of class II-restricted T cells (1, 2). Class II proteins are particularly suited to this role because they serve both as receptor for peptide antigens and as ligand for the T-cell receptor (3, 4). The class II receptor consists of the noncovalently associated A and B transmembrane proteins (1, 2). Class II molecules are found predominantly on lymphoid cells but not on most other somatic cells (5, 6). Certain immunocompetent cells appear to be more potent T-cell stimulators due, in part, to their higher constitutive class II expression (7, 8) or their capacity to respond to T-cell-derived interleukins that induce class II display (6, 9). These tissue-specific, constitutive, and inducible aspects of class II expression are largely regulated at the transcription level (10, 11).

Our laboratory studies the molecular mechanisms that control transcription of the A-chain gene of human major histocompatibility complex gene *HLA-DR* (*DRA*). Several cis-acting genetic elements have been described that mediate constitutive transcription of the *DRA* gene (10). The octamer sequence in *DRA* that shares the same sequence motif as those of the immunoglobulin, histone 2B, and vertebrate U1 and U2 small nuclear RNA genes (12–15) appears to act as a B-cell-specific element because substitution mutagenesis reduces its transcription in B cells but not in other class II-expressing cells (16). This tissue specificity may be mediated by a B-cell-specific DNA-binding protein that interacts with the *DRA* octamer sequence (15). The class II box, a conserved element present in all human and murine class II genes, consists of two stretches of conserved sequences, X and Y, separated by an 18–20 base-pair (bp) spacer (17, 18). Each X and Y element plays a role in the positive regulation and accuracy of transcription (19–22) and binds multiple proteins (20, 22–26).

This paper defines sequences 5′ to the *DRA* X box that regulate constitutive transcription. Previous studies have defined a pyrimidine tract located immediately 5′ to the class II box (21, 27) and a more 5′ Z element (27), which regulate B-cell-specific transcription. Our functional analyses of the *DRA* promoter 5′ to the X box localize three cis-acting elements: the pyrimidine tract, a positive element W between −135 and −117 bp, and a silencer V from −193 to −179 bp. Sequence comparisons reveal that W and V contain motifs homologous to the X box. A gel-shift assay confirms that W and V elements each bind proteins that also recognize the X box. These results imply that fine control over *DRA* transcription is maintained by complex interactions between X-box-binding proteins and homologous V, W, and X elements.

MATERIALS AND METHODS

Cell Culture. The human B-lymphoblastoid cell line Raji was maintained and transfected as described (19, 28).

Plasmids. Plasmid pSV2CAT, containing the simian virus 40 promoter and enhancer, was used as a positive transfection control (29). pGEM-DRA-CAT used to synthesize antisense RNA for S1 analysis has been described (28). pCH110, which contains the simian virus 40 early promoter fused to the bacterial β-galactosidase gene, was obtained from Pharmacia. pGEM-X36, pGEM-W33, and pGEM-V21 plasmids were constructed by blunt-end ligating complementary X (−110 to −74 bp), W (−141 to −110 bp), and V (−196 to −176 bp) oligonucleotides, respectively, into the pGEM-3Z vector (Promega) via the *Sma* I site of the polylinker region. The deletion mutants and control plasmid pD164-2 lacking *DRA* promoter sequence were constructed as described (28, 30). Substitution mutants pWT-W and pMUT-W are identical to previously described pwt-IRE-γ II and pmutIRE-γII, respectively (28). pMUT-W is identical to pWT-W, except that every base pair between −141 and −111 was mutated to the sequence shown in Fig. 1*B*. The 5′Δ-152 (*Xba* I) CAT vector, which contains an artificial *Xba* I linker placed immediately upstream of the 5′Δ-152 deletion mutant (28), was used to make the WT-V and MUT-V plasmids. Two 41-bp oligonucleotides were ligated into the blunted *Xba* I site. The oligonucleotide cloned into the pWT-V plasmid reconstructs the wild-type DRA promoter sequence from −153 to −193 bp. The oligonucleotide cloned into the pMUT-V is identical, except that every base pair between −180 and −193 bp was randomly mutated to the sequence shown in Fig. 1*B*. All constructs were characterized by dideoxynucle-

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Abbreviations: *DRA*, *HLA-DRA*; CAT, chloramphenicol acetyltransferase.

*Present address: Sphinx, Inc., P.O. Box 52330, Durham, NC 27717.

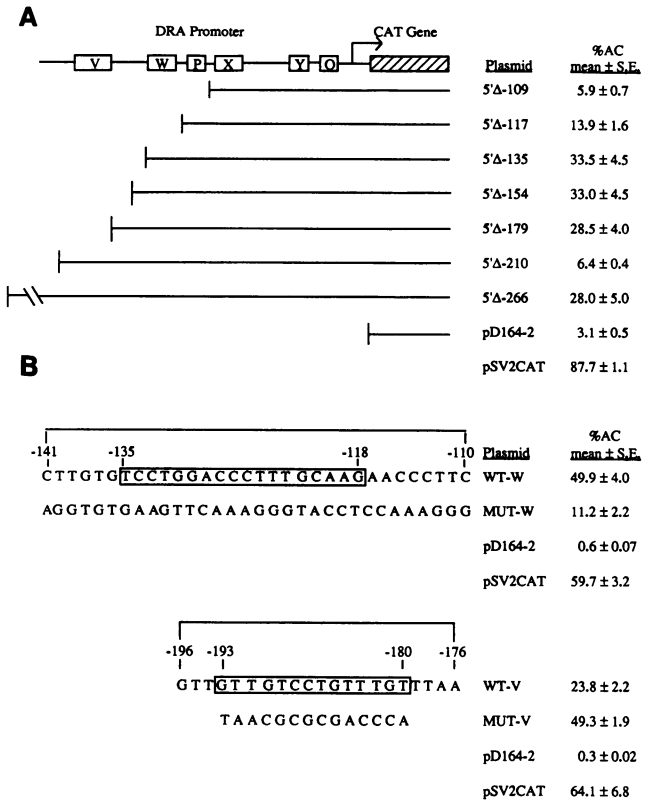

FIG. 1. Map of W and V regions. (*A*) Lines below the *DRA* promoter represent lengths of DRA promoter present in each deletion mutant. ↱, *DRA* transcription start site; ▨, CAT gene; □, known cis-acting regulatory sequences [octamer (O), Y, X, pyrimidine tract (P)] or those that are the focus of this study (W and V elements). (*B*) Relevant wild-type and mutant sequences are at left of their vector. Boxed sequences define functional regions. Brackets above wild-type sequence denote probe used for the gel-mobility-shift assay. %AC, % acetylation.

otide DNA sequencing directly from the double-stranded plasmid DNA.

CAT Assay. CAT activity was assayed 48 hr after transfection, as described (29). After visualization by autoradiography, radioactive spots were cut out of the TLC plate, and radioactivity was counted in scintillation fluid. Percent acetylation was calculated from the counts per min in the acetylated spots compared with the total.

RNA Isolation and S1 Nuclease Protection Analysis. Total cellular RNA was prepared from Raji cells 24–48 hr after transfection and analyzed by S1 nuclease protection assay as described (31–33).

Gel-Mobility-Shift Assay. The X, W, and V probes were cut out of pGEM-X36, pGEM-W21, and pGEM-V21 plasmids, respectively, with *Kpn* I and *Bam*HI restriction enzymes, labeled at the *Bam*HI site with deoxyadenosine [^{32}P]triphosphate, deoxycytidine [^{32}P]triphosphate, and Klenow fragment and then separated from the labeled vector on 12% nondenaturing polyacrylamide gel. Hybridized competitor DNAs were incubated with 2 μg of Raji nuclear proteins (34) at least 5 min before adding labeled probes. Binding conditions and electrophoresis were performed as described (35).

RESULTS

Regulatory Sequences Mapped Upstream from the Class II Box. To map cis-acting regulatory regions of the *DRA* promoter upstream from the X box, we generated a series of 5′ promoter-deletion mutants linked to the chloramphenicol acetyltransferase (CAT) gene and measured their promoter activity by transient expression of CAT in transfected Raji cells (Fig. 1*A*). pSV2CAT was used as a positive transfection control. pD164-2 used as a negative control contains the CAT gene without *DRA* promoter sequence. 5′Δ-109 defines the 5′ end of the X box and contains 109 bp of the *DRA* promoter, including the class II box, the octamer sequence, a putative CCAAT box, the TATA box, and the *DRA* transcription start site. The other deletion plasmids contain additional 5′ sequences of the *DRA* promoter up to the base pairs indicated in the plasmid name.

The means of six representative experiments are listed under the percent acetylation column in Fig. 1*A*. Compared with 5′Δ-109, 5′Δ-117 elicited 2.4 times the enhancement in CAT activity, whereas 5′Δ-135 elicited a 5.7 times increase. These data suggest that two positive regulatory elements exist between positions −109 and −135 bp: the pyrimidine tract included in the 5′Δ-117 plasmid and a second element between −117 and −135 bp that we have named W (according to the nomenclature in ref. 24). Similarly, 5′Δ-210 elicited 22% of the CAT activity of 5′Δ-179, suggesting the presence of a negative regulatory element between positions −179 and −210 bp, which we have named V. pCH110 plasmid cotransfected with each deletion mutant induced equivalent levels of β-galactosidase activity, confirming that transfection efficiencies were equivalent (data not shown). A previous report from this laboratory using similar constructs in a different cell line did not reveal differences in constitutive CAT activity because a less efficient calcium phosphate transfection method was used (28).

W and V Regulate the Authentic CAT Transcript. RNA transcripts initiating at cryptic start sites can cause artifactual changes in the levels of CAT RNA and, hence, CAT protein if they initiate in-frame with the CAT gene. To determine the transcriptional start site and levels of CAT-specific RNA transcribed from the deletion plasmids, total cellular RNAs from transfected Raji cells were subjected to S1 nuclease protection analysis (Fig. 2*A*). The RNA probe hybridized to the deletion-mutant-initiated RNA consisted of 266 bp of *DRA* promoter sequence, the cap site, 27 bp of the *DRA* gene 3′ to the cap site, and 266 bp of CAT structural gene. Fig. 2*B* is a representative experiment using RNA from cells transfected with pD164-2 negative control, basal transcription control plasmid 5′Δ-109, 5′Δ-154 containing the pyrimidine tract and W box, and V-box—containing 5′Δ-210. Each deletion mutant induced an authentic transcript (arrow) that protected a 293-bp fragment of the antisense RNA probe (27 bp of the *DRA* gene 3′ to the cap site and 266 bp of CAT gene). Densitometry revealed that authentic-transcript levels were 4 times higher in the presence of the pyrimidine tract/W element pair (5′Δ-154) and 75% lower when the V element was also included (5′Δ-210). These changes in amount of authentic transcript were proportional to, but less than, the CAT activities (Fig. 2). Similar changes were seen in transcript levels initiating from a cryptic start site situated within the 5′ plasmid sequence (numbered arrowheads). Control lanes included pD164-2, which did not yield an authentic transcript, and RNA from pSV2CAT-transfected cells, which gave a smaller 266-bp-protected fragment, as expected from its lack of any *DRA* sequence. Transfer RNA that does not hybridize to the antisense RNA probe did not yield any bands.

Substitution Mutagenesis Abolishes the Effects of W and V on CAT Activity. The 5′ deletion mutants have inherent problems in that the spacing between plasmid DNA and the sequence of interest has been altered. To confirm authenticity of the W and V elements, we constructed substitution mutants and wild-type control plasmids (described in *Materials and Methods*) and compared their CAT activities after transient transfection into Raji cells. pMUT-W is a substitution mutant in which the entire sequence between −141 and −111 bp, containing W and the pyrimidine tract, is mutated. The pMUT-W plasmid elicited 22% of the CAT activity of its nonmutated pWT-W

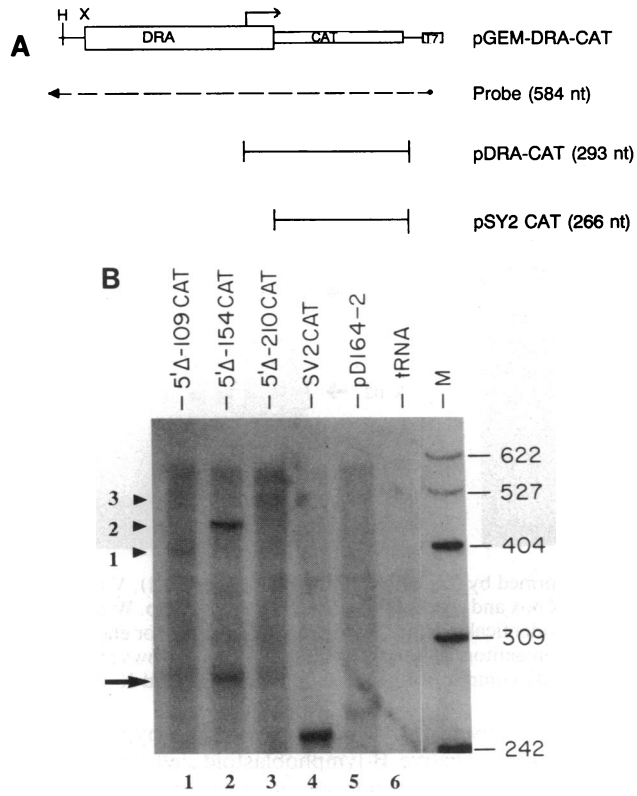

FIG. 2. S1 nuclease protection assay. (*A*) Synthesis of labeled antisense RNA probe from the T7 promoter of the linearized pGEM-DRA-CAT template. H and X, *Hin*dIII and *Xba* I restriction enzyme sites, respectively. Predicted sizes of the correctly initiated deletion mutant and pSV2CAT RNA transcripts are shown relative to the RNA probe. nt, Nucleotide. (*B*) Representative experiment. Plasmids used to transfect Raji cells before RNA isolation are listed at top. tRNA and pD164-2 negative controls are described in text. Markers (M) are end-labeled fragments of *Hpa* II-cut pBR322 plasmid. An arrow marks the authentic transcript. Arrowheads preceded by numbers mark a cryptic start site. CAT levels relative to 5′Δ-109 increased by 12 times for 5′Δ-154 and by 1.2 times for 5′Δ-210.

counterpart. This mutation of the W element had an effect comparable to its deletion, confirming the positive effect of this region on *DRA* transcription. While the deletion analyses had localized the V element between −210 and −179 bp (Fig. 1*A*), a more limited region from −193 to −180 bp was mutated in the pMUT-W plasmid, based upon homology with the *DRA* X box, discussed later (Fig. 4*A*). CAT activity promoted by the wild-type control pWT-V construct was 48.2% (range from 39 to 69%) of that elicited by the pMUT-V construct, suggesting that this 14-bp sequence silences promoter function (Fig. 1*B*). Although the randomly mutated V sequence contains a GACCC pentanucleotide homologous to the W box, our mutational analysis suggests that this sequence is not sufficient for W function (J.P.C., unpublished data). S1 nuclease protection confirmed that the substitution mutants induced levels of authentic transcript that correlated with their CAT activities (data not shown).

W and V Are Homologous to the *DRA* X Box. Sequence comparisons revealed that the W and V elements were homologous to the *DRA* X box (Fig. 4*A*). The homologous sequences within W and V elements are inverted compared with X. Maximal alignment was achieved by adding spaces (dots) between certain residues. Specific homologies exist between all three elements and between W/V and W/X pairs.

W and V Proteins Share X-Like Specificity. DNase I footprinting suggested that Raji nuclear proteins could protect a large region of the *DRA* promoter that included the W and V elements (data not shown). To independently confirm that nuclear proteins bound to the X-like W and V elements, we used a gel-mobility-shift assay. When the W and V probes (brackets above Fig. 1*B* sequences) were separately treated with Raji nuclear extracts, two complexes, B1 and B2, were formed with each probe (Fig. 3 *A* and *B*).

To elucidate whether the homology of V and W elements to X was reflected in the specificity of their binding proteins, an oligonucleotide spanning the *DRA* X box was used to compete in the formation of W–B1, W–B2, V–B1, and V–B2 complexes (Fig. 3 *A* and *B*). The *DRA* X box effectively competed in the formation of both complexes but was somewhat less effective than the homologous competitor at inhibiting the B1 bands (Fig. 3 *A* and *B*). Recognition of the *DRA* X box by the W and V proteins was specific because an unrelated SP1 oligonucleotide (C) did not compete (Fig. 3 *A* and *B*). In addition, W and V oligonucleotides cross-competed, suggesting that their proteins bound with the same specificity. We also examined whether all proteins capable of binding the X box could recognize W and V elements. Unlike W and V, three complexes, X–B1, X–B2, and X–B3, formed with the X probe. X–B1 was effectively inhibited by both W and V competitors. Although not evident in this experiment, W and V DNA competitors sometimes inhibited X-B2 formation at higher concentrations (data not shown). X–B3 was inhibited only with homologous X oligonucleotide, suggesting that this complex was specific to X.

DISCUSSION

We used mutagenesis and S1 nuclease protection to identify cis-acting regulatory elements upstream from the class II box that affect constitutive transcription in B cells. Three regulatory regions were identified: the pyrimidine tract, a positive regulatory element W, and a negative regulatory element V (Figs. 1 and 2). The W and V elements were found to be homologous with the X element of the downstream class II box (Fig. 4*A*). Gel-mobility-shift analyses confirmed that a *DRA* X-box oligonucleotide specifically prevented nuclear proteins from binding to W and V elements (Fig. 3). These data suggest that X-box-binding proteins both positively and negatively regulate *DRA* transcription through interactions with upstream W and V elements.

The pyrimidine tract has been described as a B-cell-specific element that enhances transcription in synergy with the X box (21, 27). Miwa *et al.* (24) described a gel-shift band formed with a large *HLA-DQB* promoter fragment that was inhibited by an oligonucleotide consisting of the pyrimidine tract plus six additional upstream residues. Although this result may indicate a specific protein, more detailed mapping will be necessary to determine whether the *HLA-DQB* W protein binds 5′ (in W) or within the pyrimidine tract sequence. A pyrimidine tract-specific protein has not been identified for *DRA* (36, 37). The *DRA* pyrimidine tract is postulated to affect function by stabilizing binding of certain X proteins that make additional 5′ contacts in this region (36, 37).

The Z element described by Tsang *et al.* (27) was localized by a single-deletion mutant between −136 and −131 bp. Our 5′Δ-135 plasmid may be equivalent to the −136 construct of Tsang because an additional plasmid-derived residue is identical to the promoter sequence at residue −136. Z element maps to the 3′ half of the X-like motif, supporting a critical role for these residues. However, Z element excludes part of the region most homologous to the X box (Fig. 4*A*), suggesting these residues are not critical for promoter function. We speculate that the positive effect of W element may result from cooperative interactions between Z and other downstream elements such as the Servenius sequence, GGACCCT, located from −131 to −125 bp (28, 38). The pyrimidine tract may further stabilize binding by W protein(s) because W

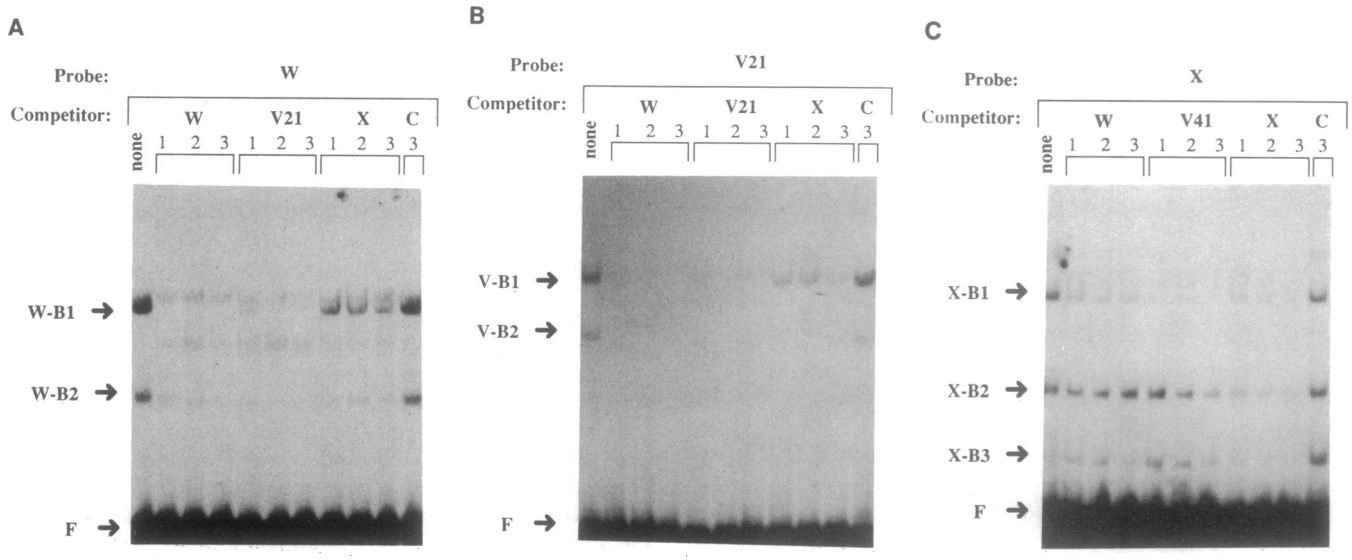

FIG. 3. Gel-mobility-shift assay. The left-most lane shows gel-shifted complexes formed by Raji nuclear proteins on the W (*A*), V (*B*), and X (*C*) elements. Competitor DNAs are listed at top. X probe and competitor contain the X box and spacer region from −110 to −75 bp. W competitor contains the W element and pyrimidine tract from −141 to −110 bp. V21 competitor is identical to V probe (Fig. 1). V41 competitor encompasses bp −193 to −153. Lanes 1, 2, and 3 denote 50-, 100-, and 200-fold molar excesses of competitor, respectively. F, free probe. Arrows marked B1, B2, and B3 identify bound complexes and are distinguished by the probe name (i.e., B1 complex formed with X probe is named X–B1).

plus pyrimidine tract was a more effective competitor than W alone in gel-mobility-shift experiments (data not shown).

The V element is located near an *in vivo* nonspecific nuclease-hypersensitive site around residue −180, which may have important *in vivo* function (39). Gonczy *et al.* (39) suggested that the presence of this hypersensitive site is related to the binding of nuclear factor RF-X because of the simultaneous absence of RF-X binding and this hypersensitive site in class II-negative B-lymphoblastoid cell lines derived from bare lymphocyte syndrome patients. Our characterization of V protein as an X-specific protein appears consistent with this hypothesis and further implies that V protein may be RF-X (40). Further analysis of cloned X proteins should establish whether RF-X specifically recognizes V.

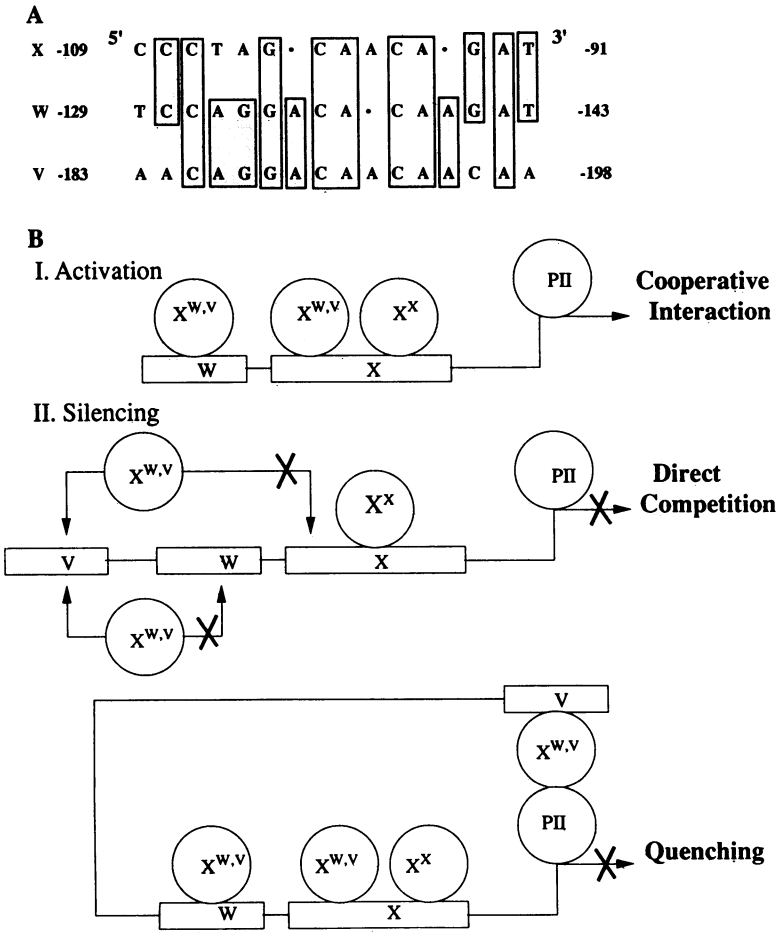

FIG. 4. (*A*) Homologous sequences shared among X, W, and V elements. Dots are spaces added to maximize alignment. Open boxes highlight residues shared among all elements. Stippled boxes distinguish residues shared solely between W/X and W/V pairs. (*B*) Models for how V, W, and X proteins interact. The bent arrow represents active transcription; a crossed-out arrow signifies cessation of binding or of active transcription. Elements are represented by labeled rectangles. Circles signify proteins, and their specificity is identified by an X with a superscript (i.e., X^W is the X-like protein capable of binding the W element). PII, polymerase II.

W and V elements can now be added to the group of X-like sequences upstream from the X element of the class II box that regulates transcription (41). We suggest that the redundant use of X-like sequences leads to a more efficient regulatory system. The W/X pair, similar to the octamer and homologous heptamer pair of the immunoglobulin promoter (42, 43), might confer cooperative function and protein binding to the *DRA* promoter (42, 43). Silencer elements such as V would then be necessary to limit such an efficient system.

Considering that W and V elements exert opposite effects on transcription, it is interesting that their ligands effectively cross-compete in the gel-mobility-shift assay. These data imply that W and V may be the same protein or different proteins of similar specificities. Whether the inhibitory function of V element is determined by its sequence or reflects a specific property of its binding protein remains to be determined. We favor the former explanation because many regulators possess dual function—with the outcome of the interaction being determined by the location or structure of the DNA ligand (i.e., binding of the V protein to the X box, in contrast to the V element, could be functionally positive) (44, 45).

Unlike W and V proteins, the proteins that bind to the X box show a more diverse recognition. X–B1 protein appears most W/V-specific because its binding is best inhibited by W or V. Likewise, W–B2 and V–B2 appear more X-specific because their binding is best inhibited by X. Whether W–B2, V–B2, and X–B1 proteins are the same or different remains to be determined. X–B3 alone appears to have specificity for the X box. These data suggest W and V are members of a family of X-box-binding proteins and parallels recent observations of specificity differences between proteins that bind to X boxes from different genes (25, 26). We suggest that similarly heterogeneous recognition by X-box-binding proteins occurs within a single gene such as *DRA*.

Fig. 4*B* depicts several models as to how W and V elements might interact to regulate *DRA* transcription. The models reflect our own bias and are not meant to exclude other possibilities. Their mechanics are based on established models developed for other systems (46). Each of the proteins has been called X to reflect their X-like recognition. Superscripts designate their ability to bind to a particular element (the X box-binding protein = X^X). Because of their related specificity, X^W and X^V have been combined into a single protein (X^WX^V), although their identity remains to be determined. In the absence of V element, activation is proposed to involve cooperative interaction between W and X. Silencing could occur by one of several mechanisms. The simplest model, direct competition, assumes that the X element–$X^{W,V}$ interaction is functionally positive and that an $X^{W,V}$ protein pool is depleted by binding to a nonfunctional V site. In the squelching model presented, $X^{W,V}$ binds to V element and then loops over and inappropriately contacts polymerase II (although other proteins could be targets). Squelching predicts that silencing occurs without protein displacement. A displacement model (46–48) has not been included because the V and W sites appear not to be sufficiently proximal for V protein to occlude binding by W protein. The availability of cloned X proteins in combination with a homologous cell-free transcription system should help discern which model is most appropriate to how V, W, and X elements modulate *DRA* transcription.

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