IDENTIFICATION OF A FUNCTIONAL NF-κB BINDING SITE IN THE MURINE T CELL RECEPTOR β2 LOCUS

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The α and β chains constituting the TCR are expressed specifically on T lymphoid cells after genomic rearrangements have led to the assembly of functional α and β chain genes (1). T cell-specific transcriptional activation of the unrearranged gene segments is considered to be a prerequisite for the recombination process that apparently uses the same enzymatic machinery that rearranges the Ig genes in B lymphocytes (2). Identification of *cis*-acting DNA regulatory sequences that govern transcription of these loci is therefore likely to be important not only for understanding gene expression in mature T cells but also early events during T cell development (3, 4).

Analysis of mice transgenic for a rearranged TCR β chain gene has led to the identification of an enhancer element located 3′ of the exons encoding the constant region (Cβ) of this gene (5). Transient transfections into lymphoid and nonlymphoid cells have allowed its localization to a 550-bp DNA fragment ~5 kb downstream of the Cβ2 exon (6, 7). Although the existence of another regulatory sequence in the TCR-β gene has been suggested by the identification of T cell-specific DNAase 1 hypersensitive sites (8, 9) in the major intron of the murine β2 gene, experiments to functionally define such an element have been unsuccessful thus far (7). We have investigated the interaction of nuclear proteins with DNA fragments derived from this region of the Cβ locus. We report here the identification of a sequence element in the Jβ2-Cβ2 intron that is homologous to the κB element (10) of the Ig κ light chain gene enhancer. We demonstrate by transfection analysis into B and T lymphoid cell lines that this sequence can serve as an inducible T cell-specific regulatory element and thereby provide evidence for the presence of transcription regulatory sequences located within the TCR Jβ2-Cβ2 intron.

Materials and Methods

Cell Lines and Culture. The following cell lines were used for transient transfections: Jurkat, a human T cell line; and S194, a murine myeloma cell line.

The cell lines used for making nuclear extracts were: PD31, Abelson MuLV-transformed pre-B cell line; 70Z, murine pre-B cell line; MPC11, murine myeloma; RL male 11, murine T cell line; EL4, murine T cell line; HeLa, a human cervical carcinoma and Jurkat.

Plasmids. The previously described HTLV-1 *tax* plasmid (11) contains *tax* cDNA transcribed from the SRα promoter (12). Plasmids containing the CAT gene were derived from the pre-

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viously described vector pSPCAT. In pSPCAT, CAT is transcribed from an enhancerless SV40 promoter (13).

$(\kappa 3)_2$CAT: fragment from the murine κ enhancer that contains the NF-κB binding site (κ3 fragment [10]) was cloned 5′ of the SV40 promoter of SPCAT as previously described (13).

(TCR-MX)$_4$CAT: the 60-bp Msp I-Xba I fragment from the major intron of the murine TCR-β2 gene (see map Fig. 1 *A*) was cloned into the Sma I site of SPCAT 5′ of the SV40 promoter.

$(\beta B)_2$CAT: two complementary oligonucleotides containing the TCRβ-B site (GATC-CAAGCAGGGAGATTCCAAGAG and GATCCTCTTGGAATCTCCCTGCTTG) were cloned into the Bam HI site of plasmid pTZ18U. The insert was isolated with Hind II and Sma I, then cloned into the Sma I site of pSPCAT.

DNA Binding Assays. In vitro binding reactions were performed as described previously (10), except that electrophoresis was in Tris-borate buffer (0.045 M Tris-borate, 0.045 M boric acid, 0.001 M EDTA). Typically, each binding reaction contained 8–10 μg protein, 2.5 μg poly d(I-C), and 10,000 cpm of probe (~0.5 ng of DNA). For competition assays, radioactive and nonradioactive DNA fragments were added to the binding reaction first and the nuclear extracts (14) last.

Transient Transfections and CAT Assays. Transient transfections were performed by the DEAE-dextran technique as previously described (13) using 5 μg of each plasmid for 10^7 cells.

CAT assays were performed by the procedure of Gorman et al. (15) using 100 μg of Jurkat or 25 μg of S194 extracts for 4 h. Results were quantitated by liquid scintillation counting.

Results

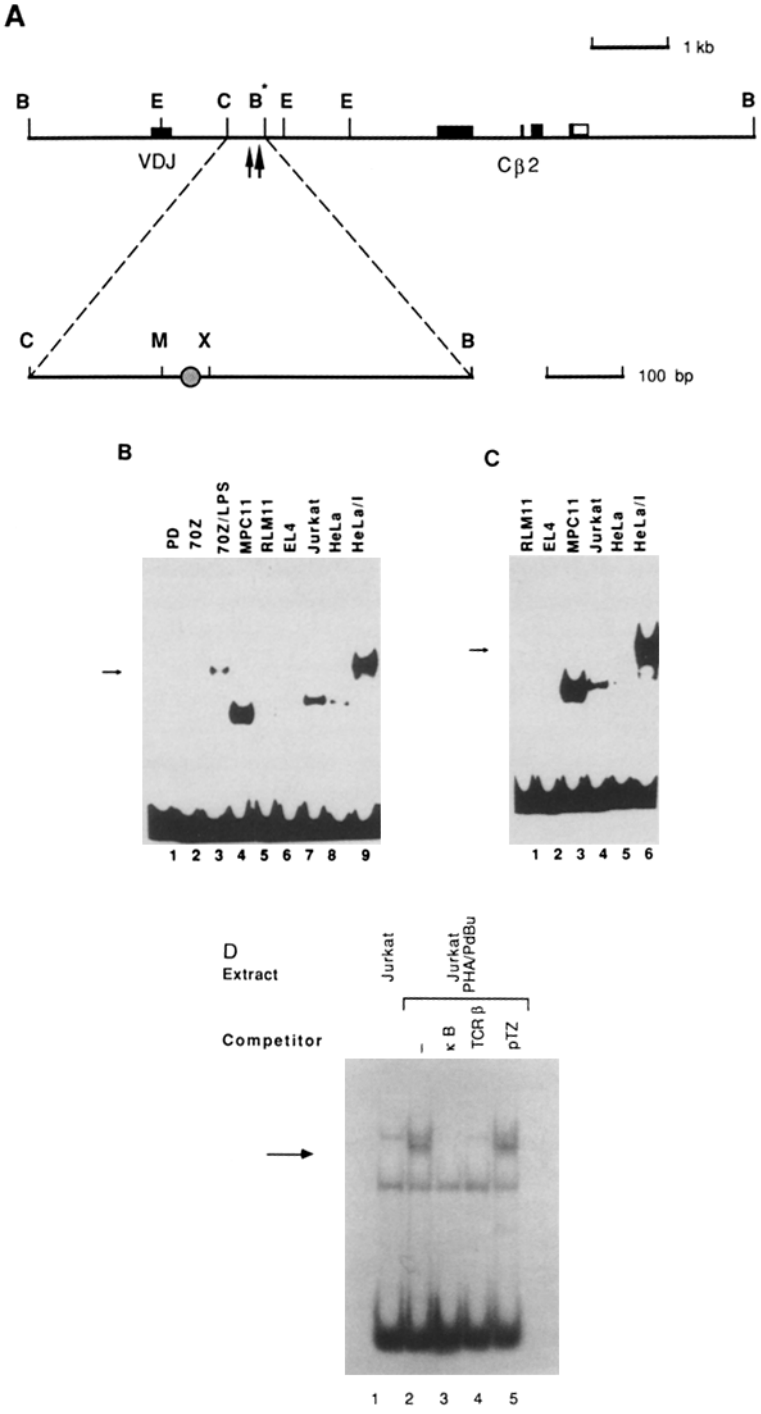
To identify binding sites for putative regulatory proteins in the proximity of the previously identified TCR DNAase 1 hypersensitive sites, 60–120 bp fragments of a 900-bp portion of the intron (Cla I-Eco RI; Fig. 1 *A*) were systematically analyzed by the electrophoretic mobility shift assay (10). One of the fragments bound a protein (the slower migrating bands in Fig. 1 *B*) that was present in extracts derived from a B cell line (MPC11, lane *4*), a pre-B cell line stimulated with bacterial LPS (70Z/LPS, lane *3*), and a nonlymphoid cell line treated with phorbol ester (HeLa/I, lane *9*) but not in extracts derived from unstimulated pre-B cell lines (lanes *1* and *2*), unstimulated T cells (lanes *5–7*), and unstimulated nonlymphoid cells (lane *8*). This distribution was reminiscent of a previously identified nuclear factor, NF-κB,

FIGURE 1. Identification of a NF-κB binding site in the TCRβ2 locus. (*A*) Genomic organization of the Cβ2 locus. Approximate locations of the tissue specific DNaseI hypersensitive sites are shown by the vertical arrows. B* denotes the presence of two Bam HI sites separated by ~120 bp. The portion of the intron that was analyzed by electrophoretic mobility shift assays is shown in detail and the newly identified NF-κB binding site, TCRβ-B, is shown as the shaded circle. Restriction enzyme sites are abbreviated as follows: E, Eco RI; B, Bam HI, C, Cla I; M, Msp I; X, Xba I.

(*B*) Electrophoretic mobility shift analysis using radiolabeled TCR Msp I to Xba I fragment in extracts from lymphoid and nonlymphoid cells as indicated above the lanes. Arrow indicates the position of an inducible nucleoprotein complex in LPS-treated pre-B cells or phorbol ester-treated nonlymphoid cells. Partial proteolysis in murine plasma cell extracts (17) results in the faster migrating band in lane labeled MPC11. Bands in lanes *7* and *8* are nonspecific, as judged by competition assays. Cell lines used are: pre-B cells: PD and 70Z/3; plasma cell: MPC11; T cells: RLM11, EL4, Jurkat; nonlymphoid cells: HeLa.

(*C*) Electrophoretic mobility shift analysis using a radiolabeled κB probe isolated as a Eco RI-Hind III fragment from a plasmid containing the κB oligonucleotide (CAGAGGGGACT-TTCCGAGA) cloned into the Bam HI site in the polylinker of pUC13. Extracts used are the same as in Fig. 1 *B*.

(*D*) In vitro competition assays. Binding and competition assays were carried out using a radioactive TRCβ-B probe isolated as a Eco RI-Hind III fragment from PTZ18U containing a



synthetic oligonucleotide (see Materials and Methods for sequence) cloned into the Bam HI site. (Lane *1*) Uninduced Jurkat cell extracts; (lanes *2–5*) extracts from Jurkat cells treated with PHA 2 μg/ml and phorbol ester (PdBu; 50 ng/ml). Competitor fragments are indicated above each lane.

which binds a 10-base sequence (κB) in the Ig κ light chain gene enhancer (16). In vitro binding experiments with a κB DNA probe confirmed that the pattern of nucleoprotein complex formation on the κB probe was indistinguishable (Fig. 1 *C*) from that on the fragment derived from the TCR locus. Note that the MPC11 extracts result in a faster mobility nucleoprotein complex probably as a result of partial proteolysis often seen with plasma cell nuclear extracts (17). Characterization of this complex by competitions showed it had the same sequence specificity as NF-κB. Sequence analysis of the 60-bp TCR fragment revealed an element, GGGAGATTCC (TCRβ-B), that was homologous to the κB element (GGGACTTTCC) at 8 of 10 base pairs. Further evidence for the presence of an NF-κB binding site in the TCR intron was provided by in vitro competition experiments. NF-κB binding to the κB site was competed away by the κ enhancer derived fragment or the Cla I-Xba I TCR fragment, but not by the Xba I-Bam HI TCR fragment or an Ig μ heavy chain gene enhancer fragment (data not shown). To conclusively establish that the κB homologous sequence in the TCR intron bound induced NF-κB in T cells, an oligonucleotide containing this element was used in binding assays (Fig. 1 *D*). Significant binding activity was only detected in extracts made from Jurkat cells induced with PHA and phorbol ester (PdBu) (Fig. 1 *D*, lane *2*; lane *1* is a binding in an equivalent amount of uninduced Jurkat cell extracts). This nucleoprotein complex could be competed either by an oligonucleotide carrying the κB sequence (Fig. 1 *D*, lane *3*) or the homologous TCRβ-B sequence (Fig. 1 *D*, lane *4*), but not an irrelevant sequence derived from the polylinker of the plasmid pTZ18U (Fig. 1 *D*, lane *5*). We conclude that an NF-κB binding site (TCRβ-B) is present in the Jβ2-Cβ2 intron, within the domain defined as being hypersensitive to DNAase 1 in murine T cells.

Although the NF-κB binding site in the κ enhancer acts in concert with other protein binding sites, it has been shown that multimers of this sequence are sufficient to activate transcription in a B cell-specific manner (13, 18). To investigate whether the newly identified TCR site (TCRβ-B) could also act as a transcriptional activator, a tetramer of the Msp I to Xba I fragment (TCR-MX, see Fig. 1 *A*) was cloned into the vector pSPCAT, in which expression of the bacterial chloramphenicol acetyl transferase (CAT) gene is dependent upon the presence of additional enhancer-like elements, to generate the plasmid p(TCR-MX)$_4$CAT. These plasmids were transfected into B and T lymphoid cells. Surprisingly, in S194 cells that have been previously shown to activate NF-κB-dependent transcription, p(TCR-MX)$_4$CAT showed no activity above the enhancerless control pSPCAT. In contrast, a plasmid, p(κ3)$_2$CAT, containing a dimer of κ3, a 70-bp κ enhancer fragment containing the κB site, showed high activity (Table I, S194 column). Thus, a tetramer of the TCR fragment was not able to activate transcription in a B cell line even though this fragment apparently bound NF-κB in vitro.

To check for transcriptional activity in T cells, we transfected the TCR-derived plasmid into Jurkat cells. Both the vector and the test plasmid were inactive in unstimulated Jurkat cells. However, induction of transfected cells with PHA and phorbol dibutyrate (PdBu) led to an approximately eightfold higher activity of p(TCR-MX)$_4$CAT, while that of pSPCAT remained unchanged (Table I, Jurkat PHA/PdBu column). The TCR fragment therefore acts like an inducible regulatory element in this T cell line.

A κB-like sequence motif in the IL-2 receptor α chain (IL-2Rα) gene is necessary

TABLE I

Functional Analysis of the TCR-β Intron Sequences by Transient Transfection

Plasmid	Cell lines		
	S194 (percent conversion)	Jurkat (-fold induction) plus PHA/PdBu	Jurkat (-fold induction) plus *tax*
pSPCAT	4.7 (± 1.2, 3)	–	1.2 (± 0.7, 8)
p(κ3)$_2$CAT	24.0 (± 3.5, 2)	–	–
p(TCR-MX)$_4$CAT	4.0 (± 0.3, 3)	8.6 (± 1.2, 6)	54.2 (± 7.0, 4)
p(βB)$_2$CAT	–	–	3.8 (± 1.1, 4)

S194 column shows the activity of plasmids measured by the percent conversion of unacetylated chloramphenicol to its acetylated form. Jurkat column shows the induced activity of various plasmids in the presence of either PHA/PdBu or HTLV-1 *tax* compared with the activity in uninduced cells. Standard deviations obtained and the numbers of independent experiments are indicated in the brackets.

for the induction of this gene by the *tax* protein of the type-1 human T cell leukemia virus (HTLV-1) (19–22). To determine whether the TCR-MX sequence was responsive to *tax*, expression of the (TCR-MX)$_4$CAT plasmid was analyzed after co-transfection with a plasmid expressing HTLV-1 *tax*. Whereas expression of the parent plasmid pSPCAT was not stimulated by *tax*, the (TCR-MX)$_4$CAT plasmid was strongly induced (Table I, Jurkat *tax* column). These results showed that although (TCR-MX)$_4$CAT was inactive in B cells, it could be induced by *tax* in Jurkat cells.

To demonstrate that the observed inducible transcriptional activity was dependent upon the TCRβ-B motif, an oligonucleotide carrying only this sequence was cloned upstream of the SV40 early promoter in pSPCAT (see Materials and Methods for sequence). A plasmid carrying a dimer of the oligonucleotide, p(βB)$_2$CAT, was then transfected into Jurkat cells in the presence or absence of *tax*. In the absence of stimulation, background levels of CAT enzyme were expressed, whereas upon co-transfection with the *tax*-expressing plasmid, approximately fourfold induction of p(βB)$_2$CAT was observed (Table I, Jurkat *tax* column). This result strongly suggests that the TCRβ-B element is a component mediating the inducible activity of the TCR-MX fragment.

We have identified and functionally characterized a sequence element in the TCR Jβ2-Cβ2 intron that is homologous to the κB element of the Ig κ light chain gene enhancer. The κB element binds a B cell–specific factor, NF-κB, and monomers or multimers of this sequence function either as a constitutive B cell–specific regulatory sequence or an inducible sequence in T cells and nonlymphoid cells (13, 18). The TCRβ-B sequence, GGGAGATTCC, has an 8 of 10 base pair match to the κ enhancer sequence GGGACTTTCC. Multimerization (4 mer) of a 60-bp fragment containing TCRβ-B from the TCR-β locus generates a regulatory sequence that is inducible in Jurkat cells by the HTLV-1 *tax* gene product or by T cell mitogens (PHA and PdBu). Interestingly, this DNA fragment is part of a region of the Jβ2-Cβ2 intron that has been shown to be hypersensitive to DNaseI in T cell chromatin, a feature that often correlates with important transcription regulatory sequences (23). Our results provide the first direct evidence that sequences important for transcriptional regulation of the TCR genes may be present in the Jβ2-Cβ2 intron.

Surprisingly, the tetramerized TCR Msp I–Xba I fragment is a very inefficient

transcriptional activator in B cells where NF-κB is constitutively present. Perhaps a negative element in the TCR Msp I-Xba I fragment suppresses the activity of the tetramer in B cells.

Several recent reports have demonstrated that transcription of the TCR α and β chain genes is elevated upon treatment of T cells with phorbol esters (24–26). Because transcripts from a partially rearranged (DJβ) allele are also increased, it has been postulated that the critical regulatory sequence is not associated with the classical promoter region (24). Although an enhancer element has been identified 3′ to the Cβ exons, its role in inducibility of TCR gene expression has not been addressed. Our identification of a phorbol ester and mitogen or HTLV-1 *tax*-inducible element in the Jβ2-Cβ2 intron provides a possible explanation for these observations and suggests that under certain conditions of cellular activation TCR-β gene transcription may be induced via these sequence elements.

Summary

We have identified a sequence in the TCR β2 locus that is homologous to the κB site in the Ig κ light chain enhancer. This element, TCRβ-B, is located in the vicinity of previously identified T cell–specific DNaseI hypersensitive sites. Transfection analysis shows that a 60-bp fragment encompassing this site is preferentially active in T cells stimulated with phorbol esters or the HTLV-1 *tax* gene product compared with a B cell line that constitutively expresses NF-κB. Our results provide the first evidence for transcriptional regulatory sequences residing within the Jβ2-Cβ2 intron and suggest the possible involvement of these sequences in modulation of TCRβ gene expression upon cellular activation.

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References

1. Davis, M. M., and P. J. Bjorkman. 1988. T-cell antigen receptor genes and T-cell recognition. *Nature (Lond.).* 336:395.
2. Yancopoulos, G. D., T. K. Blackwell, H. Suh, L. Hood, and F. W. Alt. 1986. Introduced T-cell receptor variable region gene segments recombine in pre-B cells: evidence that B and T cells use a common recombinase. *Cell.* 44:251.
3. Raulet, H. D., D. R. Garman, H. Saito, and S. Tonegawa. 1985. Developmental regulation of T cell receptor gene expression. *Nature (Lond.).* 314:103.
4. Snodgrass, R. H., P. Kieslow, M. Keifer, M. Steinmetz, and H. von Boehmer. 1985. Ontogeny of the T cell antigen receptor within the thymus. *Nature (Lond.).* 313:592.
5. Snodgrass, R. H., Z. Dembic, M. Steinmetz, and H. von Boehmer. 1985. Expression of T cell antigen receptor during fetal development in the thymus. *Nature (Lond.).* 315:232.
6. Krimpenfort, P., R. de Jong, Y. Uematsu, Z. Dembic, S. Ryser, H. von Boehmer, M. Steinmetz, and A. Berns. 1988. Transcription of T cell receptor β-chain genes is controlled by a downstream regulatory element. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:745.
7. McDougall, S., C. L. Peterson, and K. Calame. 1988. A transcriptional enhancer 3′ of Cβ2 in the T cell receptor β locus. *Science (Wash. DC).* 241:205.
8. Bier, E., Y. Hashimoto, M. I. Greene, and A. M. Maxam. 1985. Active T cell receptor

genes have intron deoxyribonuclease hypersensitive sites. *Science (Wash. DC).* 229:528.
9. Gross, D. S., and W. T. Garrard. 1988. Nuclease hypersensitive sites in chromatin. *Annu. Rev. Biochem.* 57:159.
10. Sen, R., and D. Baltimore. 1986. Multiple nuclear factors interact with the immunoglobulin sequences. *Cell.* 46:705.
11. Miyatake, S., S. Motoharu, R. D. Malefijt, T. Heike, J. Fujisawa, Y. Takebe, J. Nishida, J. Shlomai, T. Yokota, M. Yoshida, K. Arai, and N. Arai. 1988. Activation of T cell-derived lymphokine genes in T cells and fibroblasts: effects of a human T cell leukemia type I $p40^x$ protein and bovine papilloma virus encoded E2 protein. *Nucleic Acids Res.* 16:6547.
12. Takebe, Y., M. Seiki, J. Fujisawa, P. Hoy, K. Yokota, K. Arai, M. Yoshida, and N. Arai. 1988. SRα promoter: efficient and versatile mammalian cDNA expression system composed of the Simian virus 40 early promoter and the R-U5 segment of human T-cell leukemia virus Type I long terminal repeat. *Mol. Cell. Biol.* 8:466.
13. Nelsen, B. N., L. Hellman, and R. Sen. 1988. The NF-κB binding site mediates phorbol-ester inducible transcription in non-lymphoid cells. *Mol. Cell. Biol.* 8:3526.
14. Dignam, J. D., R. M. Lebowitz, and R. G. Roeder. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* 11:1475.
15. Gorman, C. M., L. F. Moffat, and B. H. Howard. 1986. Recombinant genomes which express chloramphenicol acetyltransferase in mammalian cells. *Mol. Cell. Biol.* 2:1044.
16. Sen, R., and D. Baltimore. 1986. Inducibility of κ immunoglobulin enhancer-binding protein NF-κB by a post-translational mechanism. *Cell.* 47:921.
17. Mather, E. L. 1988. DNA-binding factors of B lymphoid cells are susceptible to limited proteolytic cleavage during nuclear extract preparation. *Mol. Cell. Biol.* 8:1812.
18. Pierce, J. W., M. Lenardo, and D. Baltimore. 1988. Oligonucleotide that binds nuclear factor NF-κB acts as a lymphoid-specific and inducible enhancer element. *Proc. Natl. Acad. Sci. USA.* 85:1482.
19. Leung, K., and G. J. Nabel. 1988. HTLV-1 transactivator induces Interleukin-2 receptor expression through an NF-κB-like factor. *Nature (Lond.).* 333:776.
20. Ruben, S., H. Poteat, T.-H. Tan, K. Kawakami, R. Roeder, W. Haseltine, and C. A. Rosen. 1988. Cellular transcription factors and regulation of IL-2 receptor gene expression by HTLV-1 *tax* gene product. *Science (Wash. DC).* 241:89.
21. Ballard, D. W., E. Böhnlein, J. W. Lowenthal, Y. Wano, B. R. Franza, and W. C. Greene. 1988. HTLV-1 *tax* induces cellular proteins that activate the κB element in the IL-2 receptor α gene. *Science (Wash. DC).* 241:1652.
22. Böhnlein, E., J. Lowenthal, M. Siekevitz, D. Ballard, B. R. Franza, and W. C. Greene. 1988. The same inducible nuclear proteins regulate mitogen activation of both the Interleukin-2 receptor alpha gene and type I HIV. *Cell.* 53:827.
23. Georgopoulos, K., P. van den Elsen, E. Bier, A. Maxam, and C. Terhorst. 1988. A T-cell specific enhancer is located in a DNaseI-hypersensitive area at the 3' end of the CD3-delta gene. *EMBO (Eur. Mol. Biol. Organ.) J.* 7:2401.
24. Lindsten, T., C. H. June, and C. B. Thompson. 1988. Transcription of T-cell antigen receptor genes is induced by protein kinase C activation. *J. Immunol.* 141:1769.
25. Martinez-Valdez, H., P. J. Doherty, E. Thompson, S. H. Benedict, E. W. Gelfand, and A. Cohen. 1988. Antagonistic effects of calcium ionophores and phorbol esters on T cell receptor mRNA levels in human thymocytes. *J. Immunol.* 140:361.
26. Noonan, D., N. Isakov, A. Theofilopoulos, F. Dixon, and A. Altman. 1987. Protein kinase C-activating phorbol esters augment expression of T cell receptor genes. *Eur. J. Immunol.* 17:803.