Identification of a Putative Regulator of Early T Cell Activation Genes

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Molecules involved in the antigen receptor–dependent regulation of early T cell activation genes were investigated with the use of functional sequences of the T cell activation-specific enhancer of interleukin-2 (IL-2). One of these sequences forms a protein complex, NFAT-1, specifically with nuclear extracts of activated T cells. This complex appeared 10 to 25 minutes before the activation of the IL-2 gene. Studies with inhibitors of protein synthesis indicated that the time of synthesis of the activator of the IL-2 gene in Jurkat T cells corresponds to the time of appearance of NFAT-1. NFAT-1, or a very similar protein, bound functional sequences of the long terminal repeat (LTR) of the human immunodeficiency virus type 1; the LTR of this virus is known to be stimulated during early T cell activation. The binding site for this complex activated a linked promoter after transfection into antigen receptor–activated T cells but not other cell types. These characteristics suggest that NFAT-1 transmits signals initiated at the T cell antigen receptor.

INTERACTION OF ANTIGEN WITH THE antigen receptor of T lymphocytes initiates an ordered series of phenotypic changes resulting in cell division and immunologic function. The role of the antigen receptor in this process appears similar to that of many hormone receptors: antigen binding leads to hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP_2) (*1*), generation of diacylglycerol and inositol 1,4,5-trisphosphate (IP_3) (*2*), an increase in the concentration of intracellular calcium ion (*3*), and phosphorylation of membrane and intracellular proteins (*4*). These early events appear to be mediated through or at least require the T3 molecule, which is physically associated with the antigen receptor in the Ti-T3 complex (*5*). A second signal is provided by macrophages that can, in part, be replaced by tumor promoters that activate protein kinase C (*6*). Relatively little is known about how these membrane and cytoplasmic signals result in the activation of genes essential for T cell proliferation and immunologic function.

The interleukin-2 (IL-2) gene is physiologically active only in T cells that have been stimulated through the antigen receptor or its associated molecules and plays a major role in T cell proliferation (*7*). This induction of IL-2 appears to be mediated by a transcriptional enhancer that extends from 52 to 319 bp upstream of the IL-2 gene (*8*). This sequence can activate a linked promoter in response to an antibody to the antigen receptor but not to antibodies to other cell surface molecules. By deletion analysis, four functional sequences have been found within the IL-2 enhancer (*9*). Oligonucleotides produced to two of these sites activate a linked promoter in response to signals from the antigen receptor but not to less specific stimuli such as tumor promoters. Hence they are named antigen receptor response elements, or ARRE-1 and -2 (*9*). One of these functional oligonucleotides bound a protein complex, NF-IL2-E, that was present only in activated cells. We have now found that this protein appears to be restricted to activated T cells and have renamed it nuclear factor of activated T cells, or NFAT-1. The oligonucleotide to which it binds activates a linked promoter in activated T cells, and the binding activity appears just before the activation of the IL-2 gene.

The two ARREs within the IL-2 enhancer are approximately 200 bp apart and are the binding sites for different protein complexes, NFAT-1 and NF-IL2-A. Binding sites for other proteins lie between these two functional elements but are not discussed here. The sequences on the coding and noncoding strands of the NFAT-1 site (ARRE-2) that are protected from deoxyribonuclease I (DNase I) digestion by nuclear extracts of activated Jurkat cells extend from −288 to −267 and −263 to −290, respectively (Fig. 1, A and B).

To investigate the tissue distribution of NFAT-1 we used a double-stranded, blunt-ended oligonucleotide corresponding to the protected nucleotides (Fig. 1A). Since DNase I protection requires virtually complete occupancy of a site and, hence, is insensitive to low levels of DNA binding proteins, nuclear extracts of various cells and tissues were examined with the gel mobility shift assay (*10*). NFAT-1 was not detected in nonstimulated Jurkat cells, KB cells (a HeLa derivative), Hep G2 human hepatoma cells, TEPC murine B cells, Faza rat hepatoma cells, EL-4 murine T cells, or C2C12 murine myoblast cells (Fig. 1C). A faint band, approximately 1/50 as intense as the NFAT-1 band, was present in extracts of nonstimulated Jurkat cells. The nature of this band is not clear since it does not comigrate precisely with the NFAT-1 band. Because several transcriptional factors, including NF κB, can be activated by phorbol 12-myristate 13-acetate (PMA) in a variety of cell lines (*11*), we examined the nuclear extracts of cells stimulated with PMA (Fig.

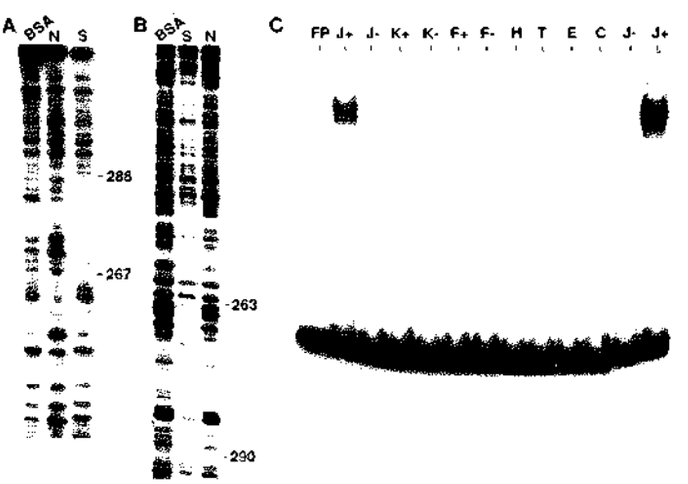

Fig. 1. (**A**) The position of ARRE-2 within the IL-2 enhancer and the sites of DNase I protection by NFAT-1. DNase I protection (*23*) of the coding (**A**) and noncoding (**B**) strands of the ARRE-2 site extending from −290 to −263. Nuclei were extracted (*24, 25*) with 0.3*M* $(NH_4)_2SO_4$ and the fraction that contained the nuclear proteins was precipitated with $(NH_4)_2SO_4$ (0.2 g/ml) and dialyzed at 4°C for 4 hours. For the DNase footprinting assay (*23*) we used poly(dI-dC) as a nonspecific competitor; BSA, bovine serum albumin alone; N, 20 μg of nuclear-extract from nonstimulated Jurkat cells; S, 50 μg of nuclear extract from Jurkat cells stimulated with PMA (50 ng/ml) and PHA (2 μg/ml) for 2 hours. (**C**) Representation of NFAT-1 in different cell lines. Nuclear extracts from: J, Jurkat cells; K, KB cells (a derivative of HeLa); F, Faza cells (a rat liver cell line); H, Hep G2 cells (a human hepatocyte line); T, TEPC murine B cell line; E, EL-4 murine T cell line; C, C2C12 murine myoblasts. Lanes labeled "+" are the complexes formed with nuclear extracts from cells treated with PHA (2 μg/ml) and PMA (50 ng/ml) for 2 hours.

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1C). Neither the nuclear extracts of these stimulated cells or the extracts of rat brain, kidney, spleen, or liver formed a complex with ARRE-2, although the α-globin CAAT-binding protein and NF-IL2-A could be detected. The inability to detect NFAT-1 in rodent tissues is not related to an inability of the rodent factor to bind the human sequence since NFAT-1 could be readily detected in PMA- and ionomycin-stimulated EL-4 and C6VL-B cells.

To examine the function of the binding site for NFAT-1 we prepared a plasmid in which three copies of ARRE-2 were placed upstream of a truncated γ-fibrinogen promoter (*12*). This promoter contains a TATA box and a functional Sp-1 site and initiates transcription correctly both in vivo and in vitro when truncated (*13*). This construct was cotransfected into the cell lines shown in Table 1 using Rous sarcoma virus luciferase (*14*) to control for efficiency of transfection (*15*). The ARRE-2 and the SV40 constructs function with similar efficiency in stimulated Jurkat cells (Table 1). In contrast, the pSV construct (*15*) functioned about 50- to 100-fold better than the ARRE-2 construct in PMA- and PHA (phytohemagglutinin)-stimulated KB cells, human Raji B cells, murine L cells, and Hep G2 cells (Table 1). Thus three copies of the ARRE-2 sequence can confer T cell–specific function upon the normally uninduced fibrinogen promoter.

A role of NFAT-1 in the activation of the IL-2 gene is further suggested by a comparison of the kinetics of appearance of binding activity for the NFAT-1 protein and the activation of the IL-2 gene. Jurkat cells were stimulated with PHA and PMA at the times shown in Fig. 2, and nuclear extracts as well as whole-cell RNA were prepared. The appearance of NFAT-1 was measured as the ability of the nuclear extract to form a complex with the ARRE-2 region on low ionic strength gels (*10*). By 20 min after stimulation, NFAT-1 was detectable and the amount produced steadily increased for 2 hours. The level of a CAAT-binding protein for the human α-globin promoter did not change during the time course shown in Fig. 2A; therefore the changes in NFAT-1 binding were not due to degradation or a nonspecific PHA effect on nuclear DNA-binding proteins. In Jurkat cells, IL-2 mRNA, measured by ribonuclease protection (*16*), was first detectable between 30 and 45 min after exposure to PHA and PMA (Fig. 2, B). The appearance of NFAT-1 10 to 25 min before IL-2 mRNA is consistent with a role of NFAT-1 in IL-2 gene activation.

We investigated the requirements for NFAT-1 binding activity in Jurkat cells using anisomycin, which inhibits ribosomal subunit association and blocks 98% of protein synthesis of Jurkat cells at 100 μ*M* within 5 min (*17*). When nuclear extracts from Jurkat cells were stimulated with PHA and PMA in the presence or absence of 100 μ*M* anisomycin, we found that anisomycin inhibited the NFAT-1 binding activity by about 95% without affecting NF-IL2-A binding (Fig. 3A). This small induction in the presence of anisomycin may represent leakage of protein synthesis or may be due to residual protein synthesis from a superinduced mRNA. When the same extracts were examined by DNase I protection, the sequences between −263 and −290 were not protected with the uninduced extracts or with extracts from Jurkat cells activated in the presence of 100 μ*M* anisomycin. Using DRB (5,6-dichloro-1-D-ribofuranosylbenzimidazole), a rapidly acting inhibitor of RNA synthesis, we found that RNA synthesis was also essential for the appearance of NFAT-1 (*18*), indicating that a gene must be activated to obtain NFAT-1 binding activity. Although this gene is likely to be the one encoding NFAT-1, another possibility includes a gene required for synthesis of a protein necessary for the development of NFAT-1 binding activity.

If protein synthesis is required for NFAT-1 binding activity and NFAT-1 is required for IL-2 gene activation, then protein synthesis should be required for IL-2 gene activation. We tested this prediction by examining the requirements for IL-2 gene activation using short periods of exposure to anisomycin (less than 1 hour) to avoid many secondary effects commonly encountered when longer periods of exposure are used. As shown in Fig. 3B, anisomycin added as early as 20 min after stimulation inhibited the appearance of nearly all IL-2 mRNA (Fig. 3B). In these same mRNA samples anisomycin did not inhibit *c-fos* gene activation or the constitutive expression of *c-myc* mRNA (*19, 20*). These controls indicate that the failure to induce IL-2 mRNA in the anisomycin-treated samples was not due to a nonspecific effect on mRNA production. Since anisomycin at 100 μ*M* takes only a few minutes to inhibit protein synthesis by 98% (*17*), these results indicate that the protein required for IL-2 gene activation first appears at or before 20 min. This result is consistent with the first appearance of NFAT-1 at 20 min (Fig. 2) and strengthens the notion that NFAT-1 is involved in IL-2 gene activation.

Table 1. Tandem repeats of the antigen receptor response element activate expression of a linked gene only in T cells activated through the antigen receptor. Values shown are percent conversion of chloramphenicol to the acetylated form. A cotransfected RSV-luciferase plasmid (*15*) was used to normalize for transfection efficiency. Results represent the means of two to three determinants.

Cell type	Stimulus	Activity	
		SV40	ARRE-2
Jurkat	Anti-Ti + TPA	91.6	75.0
	TPA alone	60.5	2.3
KB	PHA + TPA	11.6	0.07
L cells	Iono + TPA	89.0	0.08
Raji	Iono + TPA	9.8	0.09
Hep G2	PHA + TPA	89.1	1.86

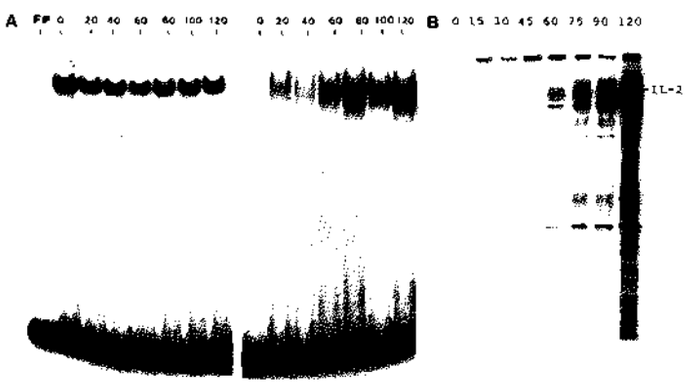

Fig. 2. (**A**) Time course of the appearance of NFAT-1 binding activity. Nuclear extracts were prepared from Jurkat cells at the indicated times in minutes after PMA and PHA addition and the gel mobility shift assay performed with a probe derived from −93 to −63 (ARRE-1) to measure NF-IL2-A and −255 to −285 (ARRE-2) to measure NFAT-1. (**B**) Time-course of the activation of the IL-2 gene. Jurkat cells were stimulated with PHA (5 μg/ml) and PMA (50 ng/ml), and RNA was prepared at the indicated times (shown in minutes). IL-2 mRNA was measured with a uniformly labeled RNA probe (*16*) derived from the IL-2 gene. The 330-bp probe was hybridized to Jurkat RNA in 60% formamide at 42°C and then digested with ribonuclease as described (*16*). The positions of correctly initiated IL-2 transcripts (280 bp) are indicated at the right.

We examined the regulatory sequences of the long terminal repeat (LTR) of the human immunodeficiency virus type 1 (HIV-1) for a binding site for NFAT-1, since previous workers had found that the lag time until appearance of the HIV-1 mRNA exceeds 30 min but is less than 2 hours (*21, 22*) after activation of HIV-1–infected T cells. Thus regulation of the HIV-1 LTR appears similar to that of an early T cell activation gene. A fragment from the HIV-1 LTR extending from −342 to −154 competed for binding of NFAT-1 to the ARRE-2 sequence at a 20-fold molar ratio (Fig. 4A). The increase in binding observed with a tenfold molar excess is typical of an effective competitor and may reflect quantitatively greater binding associated with a concentration of binding sites closer to the dissociation constant of the interactions. Similar concentrations of the HIV-1 LTR did not influence complex formation with NF-IL2-A. We localized the site of this protein's interaction within the HIV-1 LTR using DNAse I protection (Fig. 4B). With stimulated nuclear extracts, we found a region of DNAse-1 protecion between −216 and −254 and a weak footprint between −288 and −303 (Fig. 4B, lane 2). The −216 to −254 region was not seen with extracts from nonstimulated cells or from cells stimulated in the presence of anisomycin (lanes 3 and 7). When the incubation was carried out in the presence of a 100-fold excess of several fragments of the IL-2 gene, only the −285 to −255 region was an effective competitor (lanes 4 to 6). The specificity of these interactions was internally controlled by a region of protection (−303 to −288) not affected by any of the treatments. The binding site between −216 and −254 in the HIV-1 LTR was not related to a region of similarity between IL-2 at −254 to −275 and HIV-1 at −216 to −254. These results indicate that either NFAT-1 or a protein with similar biologic characteristics and interacting with similar sequences binds to the HIV-1 LTR at a region outside of the enhancer that is required for optimum activation of the viral LTR (*21*). More definitive evidence for a role of NFAT-1 in the activation of the IL-2 gene and the HIV-1 LTR will require purification of NFAT-1 and examination of its function in vitro.

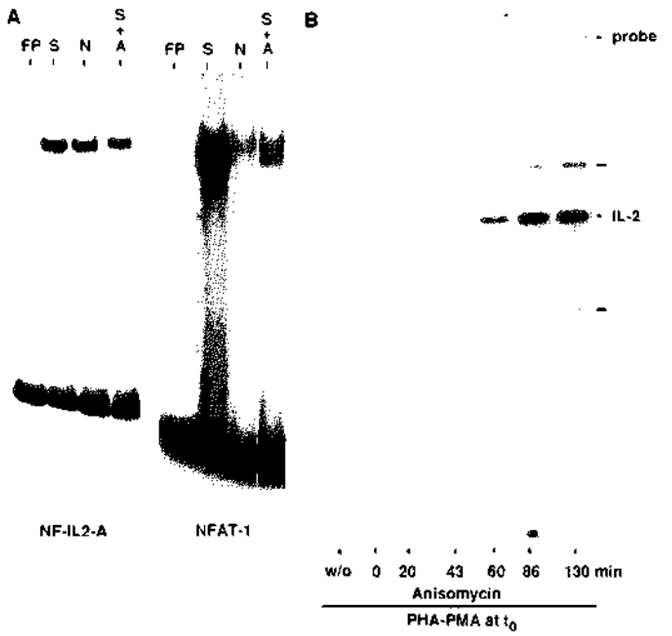

Fig. 3. (**A**) The appearance of NFAT-1 binding activity and IL-2 gene activation are inhibited with anisomycin. Jurkat cells were stimulated with PHA and PMA in the presence (lanes labeled S+A) and absence (lanes labeled S) of anisomycin at 100 μ*M*. After 2 hours nuclear extracts were prepared and assayed. NF-IL2-A was assayed with the −63 to −93 oligonucleotide, and NFAT-1 with the −285 to −255 oligonucleotide. Lanes N: results with unstimulated cell extracts. (**B**) Jurkat cells were activated with PHA and PMA and at the indicated times; anisomycin was added at 100 μ*M* final concentration. RNA was harvested at 130 minutes and IL-2 transcripts were determined by ribonuclease mapping (*16*). The position of the IL-2–specific protected fragments are indicated at the right of the figure.

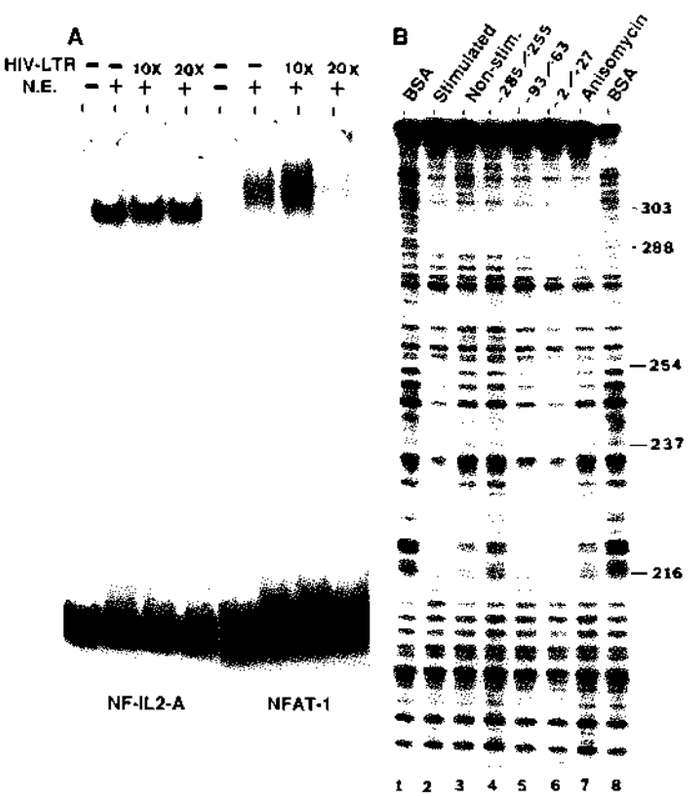

Fig. 4. The HIV-1 LTR interacts with NFAT-1 or a similar factor. (**A**) Gel mobility shift assays showing that the HIV-1 LTR competes for the formation of the NFAT-1 complex but not the NF-IL2-A complex. Nuclear extracts from stimulated Jurkat cells were incubated with the −285 to −255 end-labeled oligonucleotide representing the NFAT-1 binding site and the −63 to −93 oligonucleotide representing the NF-IL2-A binding site in the presence and absence of the HIV-1 LTR competitor (−342 to −154) at a 10- and 20-fold molar excess. Complexes were then separated on low ionic strength gels (*10*). (**B**) Localization and characterization of the putative NFAT-1 site in the HIV-1 LTR. A probe extending from −342 to −154 was prepared from the HIV-1 LTR and end-labeled at −154. This probe was incubated with nuclear extracts from: lane 2, stimulated Jurkat cells; lane 3, nonstimulated Jurkat cells; lanes 4 to 6, stimulated Jurkat cells in the presence of a 100-fold molar excess of the sequences indicated at the top of the gel; lane 7, Jurkat cells stimulated in the presence of 100 μ*M* anisomycin. Lanes 1 and 8 contain the DNase digestion products of the probe incubated with BSA and no nuclear extract.

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18. NFAT-1, CBP, and NF-IL2-A were measured by the gel shift assay in Jurkat cells stimulated with PHA and TPA in the presence and absence of 100 μ*M* DRB, which inhibits 95% of RNA synthesis within 5 minutes. While no effect of DRB was observed on NF-IL2-A and CBP the appearance of NFAT-1 was inhibited by more than 90% [B. Sehgal and I. Tamm, *Bio. Pharm.* **27**, 2475 (1978)].
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A Transcriptional Enhancer 3′ of $C_{\beta 2}$ in the T Cell Receptor β Locus

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Run-on transcription experiments were used to demonstrate that transcription of T cell receptor β chain V genes is activated by DNA rearrangement, in a manner similar to immunoglobulin genes. A transcriptional enhancer likely to be involved in this activation has been identified. A 25-kilobase region from $J_{\beta 1}$ to $V_{\beta 14}$ was tested for enhancer activity by transient transfections, and an enhancer was found 7.5 kilobases 3′ of $C_{\beta 2}$. The β enhancer has low activity relative to the simian virus 40 viral enhancer, does not display a preference for V_β promoters, has a T cell–specific activity, and binds two purified immunoglobulin heavy chain enhancer factors.

GENE PRODUCTS OF THE TWO MOST closely related members of the immunoglobulin gene superfamily, immunoglobulin (Ig) genes and T cell receptor (TCR) genes, perform the antigen recognition function in the humoral and cellular immune responses, respectively (*1, 2*). When Ig genes undergo joining of the variable (V), diversity (D), and joining (J) gene segments a transcriptional enhancer located between the J and constant (C) segments is brought within functional proximity of the rearranged V gene promoter and activates transcription (*3*). Like the Ig heavy chain locus, the TCR β locus is composed of V, D, J, and C gene segments (*4, 5*). Since TCR β VDJ joining is similar to Ig VDJ joining, it seemed possible that an enhancer might be located between TCR J_β and C_β gene segments that would activate rearranged V_β promoters. This possibility is strengthened by the recent report of an enhancer in the TCR α gene locus (*6*). However, it may be that TCR genes are never transcribed at as high a rate as Ig genes and an enhancer is not necessary for TCR β chain gene regulation. If so, TCR V_β promoters would be constitutively active and T cell–specific VDJ joining would be sufficient to ensure T cell–specific expression.

To distinguish these possibilities experimentally, we used run-on transcription in isolated nuclei, which quantitates polymerase loading on a particular region of DNA regardless of subsequent processing or degradation of the transcript (*7*). In initial experiments BO4H.H.9.1 was used. This hybridoma has rearranged its β-chain genes and expresses $V_{\beta 3}$ (*8*); genomic DNA blots showed that unrearranged $V_{\beta 1}$ was present (*9*). Quantitation of two experiments (Table 1) confirms that $V_{\beta 3}$ and $C_{\beta 1}$ are transcribed at similar levels but that transcription from the unrearranged $V_{\beta 1}$ gene is undetectable. Similarly, in the T lymphoma cell line SL3, which by RNA blot analysis expresses functional β mRNA but not $V_{\beta 3}$ (*9*), there is only a low level of transcription detected from the unrearranged $V_{\beta 3}$ gene, a level substantially below the $C_{\beta 1}$ level in this cell line (Table 1). Low transcription of $V_{\beta 3}$ may reflect enhancer-independent activity that precedes VDJ joining in the IgH locus (*10*). The unrearranged $V_{\beta 3}$ gene was shown to be transcriptionally silent in the NIH 3T3 fibroblasts as well as in the plasmacytomas S107 and P3X63-Ag8, although appropriate controls were positive (Table 1). Therefore, the run-on transcription experiments indicate the following. (i) Although there may be a low level of transcription from unrearranged V_β genes in some T cells, rearrangement is necessary to activate transcription fully. (ii) Unrearranged V_β gene segments are transcriptionally silent in non-T cells. Thus we searched the β locus for a transcriptional enhancer.

We considered it possible that a putative β chain enhancer might act preferentially on V_β promoters in a manner analogous to the Ig κ enhancer (*11*). Therefore, two vectors were used for this study: (i) pA10CAT2, which contains the SV40 early promoter 5′ of the chloramphenicol acetyl transferase (CAT) gene but no enhancer (*12*), and (ii) pV_βCAT, which contains the $V_{\beta 3}$ gene promoter 5′ of the CAT gene and no enhancer (*13*). The entire region from $D_{\beta 1}$ to $V_{\beta 14}$ was subcloned into one or both vectors and tested by transient transfection into T cells for enhancer activity. Both orientations of most inserts were tested. We found that the efficiency of T cell transfection was 15 times higher when electroporation was used rather than DEAE dextran (*9*); thus all the results presented here were obtained from electroporation of EL4 T cells (*14*). To correct for differences in transfection efficiency we cotransfected a plasmid expressing β-galactosidase (*15*).

There was no detectable enhancer activity in pA10CAT2 constructs in the region between $J_{\beta 1}$ and $C_{\beta 1}$ (Fig. 1A). Since a T cell–specific deoxyribonuclease (DNase) I hypersensitive site has been reported in the $J_{\beta 2}$ to $C_{\beta 2}$ intron (*16*) and enhancer regions are usually hypersensitive to DNase I, we tested this region by using both vectors. No enhancer activity was detected in any of our constructs after multiple transfections. Three additional T cell lines were also transfected with constructs containing this re-

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