MOLECULAR AND CELLULAR BIOLOGY, Sept. 1990, p. 4720–4727
0270-7306/90/094720-08$02.00/0

Vol. 10, No. 9

# Regulation of the Human T-Cell Receptor α Gene Enhancer: Multiple Ubiquitous and T-Cell-Specific Nuclear Proteins Interact with Four Hypomethylated Enhancer Elements

I-CHENG HO AND JEFFREY M. LEIDEN*

*Howard Hughes Medical Institute and Departments of Internal Medicine and Microbiology/Immunology, University of Michigan Medical Center, MSRBI Room 4510, 1150 West Medical Center Drive, Ann Arbor, Michigan 48109-0650*

Received 12 February 1990/Accepted 26 June 1990

**Transcription of human T-cell receptor (TCR) α genes is regulated by a T-cell-specific transcriptional enhancer that is located 4.5 kilobases 3′ of the Cα gene segment. Previous studies have demonstrated that this enhancer contains at least five nuclear protein-binding sites called Tα1 to Tα5. In the studies described in this report, we have determined the molecular requirements for human TCR α enhancer function. In vitro mutagenesis and deletion analyses demonstrated that full enhancer activity is retained in a 116-base-pair fragment containing the Tα1 and Tα2 nuclear protein-binding sites and that both of these sites are required for full enhancer function. Functional enhancer activity requires that the Tα1 and Tα2 binding sites be separated by more than 15 and fewer than 85 base pairs. However, the sequence of this spacer region and the relative phase of the two binding sites on the DNA helix do not affect enhancer function. Deletion and mutation analyses demonstrated that the Tα3 and Tα4 nuclear protein-binding sites are not necessary or sufficient for TCR α enhancer activity. However, a fragment containing these two sites was able to compensate for Tα1 and Tα2 mutations that otherwise abolished enhancer activity. Electrophoretic mobility shift analyses of the TCR α enhancer binding proteins revealed that the Tα1, Tα3, and Tα4 binding proteins are expressed in a variety of T-cell and non-T-cell tumor cell lines. In contrast, one of the two Tα2 binding activities was detected only in T-cell nuclear extracts. The activity of the TCR α enhancer does not appear to be regulated solely at the level of DNA methylation in that the enhancer sequences were found to be identically hypomethylated in B and T cells as compared with fibroblasts. Taken together, these results suggest that TCR α enhancer activity is regulated by the interaction of multiple T-cell-specific and ubiquitous nuclear proteins with partially redundant *cis*-acting enhancer elements that are hypomethylated in cells of the lymphoid lineage.**

Human B and T lymphocytes express a set of related but distinct cell surface antigen receptors. Mature B cells express cell surface immunoglobulin which is able to recognize and bind soluble antigens, while human T cells express membrane-bound T-cell receptor (TCR) molecules that recognize antigenic peptides bound to major histocompatibility molecules on the surfaces of antigen-presenting cells (reviewed in reference 16). Thus far, two types of TCR molecules have been identified (reviewed in reference 5). The majority of peripheral blood T cells, including all cells of the helper and cytotoxic phenotypes, express the disulfide-linked, heterodimeric α/β TCR. In contrast, 1 to 10% of peripheral blood T cells express the heterodimeric γ/δ TCR (reviewed in reference 22). The precise function of these $\gamma/\delta^+$ T cells remains unknown. Each of the immunoglobulin and TCR genes is composed of multiple germ line gene segments that are rearranged and expressed in a cell lineage-specific fashion (reviewed in references 1 and 22). Thus, immunoglobulin genes are generally rearranged only in B lymphocytes, while TCR genes are rearranged and expressed predominantly in T cells. In addition, the TCR α gene is generally rearranged and expressed only in TCR $\alpha/\beta^+$ cells, while TCR δ-gene rearrangement and expression is limited to $\gamma/\delta^+$ T cells. Previous studies have suggested that immunoglobulin and TCR gene rearrangements are mediated by a single recombinase activity (25) and that the lineage specificity of rearrangement is regulated at the level of transcription of the unrearranged immunoglobulin and TCR loci (4, 26; reviewed in reference 1). Therefore, a molecular analysis of the transcriptional regulation of the immunoglobulin and TCR genes may help to elucidate the fundamental mechanisms that control B- and T-cell development.

Transcription of each of the immunoglobulin genes as well as the TCR α and β genes has been shown to be regulated by the interaction of variable gene segment promoters with intragenic transcriptional enhancers (2, 7, 8, 11, 13, 17, 20, 21, 23). Human and murine TCR α gene expression is controlled by highly related transcriptional enhancers that are located 3 to 4.5 kilobases (kb) 3′ of the Cα gene segments in both species (8, 23). The human TCR α enhancer is necessary for transcription from TCR Vα promoters and is active in TCR $\alpha/\beta^+$ T cells but not in TCR $\gamma/\delta^+$ T cells, B cells, or fibroblasts (8). DNase I footprint analyses have demonstrated that this enhancer contains at least five nuclear protein-binding sites called Tα1 to Tα5 (8). The Tα1 binding site contains a consensus cyclic AMP response element, while the Tα3 site includes overlapping AP-2 and κE2-like enhancer motifs (8). The Tα2, Tα4, and Tα5 binding sites do not display sequence identity with previously described enhancer elements (8). Previous deletion analyses have suggested that the Tα5 nuclear protein-binding site does not play an important role in determining TCR α enhancer activity (8).

While these previous studies have defined the cellular activity and potential *cis*-acting elements of the human TCR α enhancer, the importance of each of the nuclear protein-

* Corresponding author.

binding sites for enhancer activity and the identity and cell lineage-specific pattern of expression of the TCR α enhancer-binding proteins remain unknown. In the studies described in this report, we have utilized in vitro mutagenesis and deletion analyses to define the molecular requirements for human TCR α enhancer function. In addition, electrophoretic mobility shift experiments have been used to study the *trans*-acting factors that regulate this enhancer. Finally, we have determined the methylation status of the enhancer in a variety of T-cell and non-T-cell tumor cell lines. Taken together, the results suggest that the TCR α enhancer is composed of multiple redundant *cis*-acting elements that bind a set of T-cell-specific and ubiquitous nuclear proteins. The organization of these nuclear protein-binding sites is constrained by specific spatial requirements. While hypomethylation may be necessary for enhancer function, it is not sufficient for activity, as the enhancer is identically hypomethylated in TCR $\alpha/\beta^+$ and TCR $\gamma/\delta^+$ T cells as well as in cells of the B-lymphocyte lineage.

## MATERIALS AND METHODS

**Cells and media.** Human T-cell lines Jurkat, Peer, Molt-13, Molt-4, and CEM as well as the Epstein-Barr virus-transformed B-cell lines Clone 13 and JY and the chronic myelogenous leukemia cell line K562 were grown in RPMI 1640 medium supplemented with 10% fetal calf serum (GIBCO Laboratories, Grand Island, N.Y.) and 1% penicillin-streptomycin (GIBCO). HeLa cells were grown in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum and 1% penicillin-streptomycin. CEM cells were treated with phorbol myristate acetate (50 ng/ml) for 16 h prior to use in the preparation of nuclear extracts. This treatment resulted in at least 50% of the cells expressing the $CD3^+$ TCR $\alpha/\beta^+$ phenotype (data not shown).

**Transfections and CAT assays.** Jurkat T cells were transfected by a modification of the DEAE-dextran method as previously described (8). To control for differences in transfection efficiencies, all transfections contained 2 μg of the pRSVβgal plasmid, in which the β-galactosidase gene is under the control of the Rous sarcoma virus long terminal repeat. Cells were harvested 48 h after transfection, and cell extracts normalized for protein content with a commercially available kit (Bio-Rad Laboratories, Richmond, Calif.) were assayed for both β-galactosidase and chloramphenicol acetyltransferase (CAT) activities as previously described (12).

**Site-directed mutagenesis.** Mutations were produced by using an oligonucleotide-mediated, gapped heteroduplex mutagenesis protocol as previously described (12). All mutants were confirmed by dideoxy-DNA sequence analysis with commercially available Sequenase reagents (U.S. Biochemical Corp., Cleveland, Ohio). Oligonucleotides were synthesized on a model 380B DNA synthesizer (Applied Biosystems, Inc., Foster City, Calif.). In order to produce an enhancer fragment with a 5-base-pair (bp) insertion in the spacer region between the Tα1 and Tα2 binding sites, a cloned synthetic oligonucleotide containing the wild-type Tα1 and Tα2 sites separated by the wild-type spacer region was digested with *Bst*EII, the overhanging ends were filled in with the Klenow fragment of DNA polymerase, and the resulting blunt ends were religated together. To produce enhancer fragments containing 85- and 145-bp spacer regions, one and two copies of a 60-bp *Dra*I-*Hpa*I fragment from pSPCAT were cloned into the *Bst*EII site of the wild-type synthetic enhancer fragment that had been filled in with the Klenow fragment of DNA polymerase. A deletion mutant lacking 41 bp from the 5′ end of the *Bst*XI-*Dra*I enhancer fragment (including a deletion of 18 bp from the 5′ end of Tα1) (see Fig. 1) was produced by digestion of the 116-bp *Bst*XI-*Dra*I enhancer fragment with *Aat*II. An enhancer fragment lacking 45 bp from the 5′ end of the 275-bp *Bst*XI-*Apa*I enhancer fragment (including 22 bp from the 5′ end of Tα1) was produced by digestion of this fragment with *Bst*EII. All deletion and insertion mutants were confirmed by restriction enzyme and dideoxy-DNA sequence analyses.

**Electrophoretic mobility shift assays.** Nuclear extracts were prepared according to the methods of Dignam et al. (6) or Osborn et al. (19). Synthetic oligonucleotides corresponding to the Tα1 to Tα4 nuclear protein-binding sites (see Fig. 1) were synthesized with *Bam*HI-*Bgl*II overhanging ends, annealed, and labeled with [α-$^{32}$P]dCTP and [α-$^{32}$P]dGTP by using the Klenow fragment of DNA polymerase as previously described (12). Electrophoretic mobility shift experiments were performed as previously described (12) using 20,000 cpm of labeled oligonucleotide, 0.25 to 0.5 μg of poly(dI:dC) (Pharmacia, Piscataway, N.J.), and 2 to 5 μg of nuclear extract per binding reaction. Assays using Tα1 and Tα3 oligonucleotides contained 100 mM NaCl. Assays using Tα2 and Tα4 oligonucleotides contained 10 mM NaCl. The DNA-protein complexes were fractionated by electrophoresis on 4% polyacrylamide gels in Tris-glycine buffer as previously described (12).

**Southern blotting.** High-molecular-weight DNA was isolated from the Jurkat, Peer, CEM, Clone 13, and HeLa cell lines, and 10-μg samples were digested overnight at 37°C with a fivefold excess of the appropriate restriction endonucleases according to the instructions of the manufacturer (Boehringer Mannheim, Indianapolis, Ind). The DNA samples were fractionated by electrophoresis in 1 to 1.5% agarose gels, transferred to nitrocellulose, and hybridized to the 1.4-kb *Kpn*I-*Bam*HI fragment containing the human TCR α enhancer (see Fig. 4) that had been labeled with [α-$^{32}$P]dCTP by random hexanucleotide priming as previously described (12).

## RESULTS

**Molecular analyses of the minimal TCR α enhancer.** We have previously localized the minimal human TCR α transcriptional enhancer to a 116-bp *Bst*XI-*Dra*I fragment that contains the Tα1 and Tα2 nuclear protein-binding sites (Fig. 1) (8). In order to better understand the functional importance of these two binding sites, deletion and in vitro mutagenesis analyses of the 116-bp *Bst*XI-*Dra*I enhancer fragment were performed. Deleted and mutated forms of the enhancer fragment were cloned into the *Bam*HI site 3′ of the minimal simian virus 40 promoter and bacterial CAT reporter gene in the pSPCAT plasmid (14) (Fig. 2A) and transfected into human Jurkat T cells. Mutations of the Tα1 and Tα2 binding sites (Fig. 1) that were shown in electrophoretic mobility shift assays to abrogate nuclear protein binding (Fig. 3) decreased enhancer activity by 71 and 96%, respectively (Fig. 2A). Similarly, deletion of 41 bp from the 5′ end of the *Bst*XI-*Dra*I fragment (including 18 bp from the 5′ end of Tα1) reduced enhancer activity by 90% (Fig. 2A). Therefore, we concluded that both the Tα1 and Tα2 binding sites are required for enhancer function.

In order to determine whether the Tα1 and Tα2 binding sites alone or in combination were sufficient to confer transcriptional enhancer activity, synthetic oligonucleotides corresponding to these two binding sites were ligated into the *Bam*HI site of the pSPCAT plasmid, either alone or in

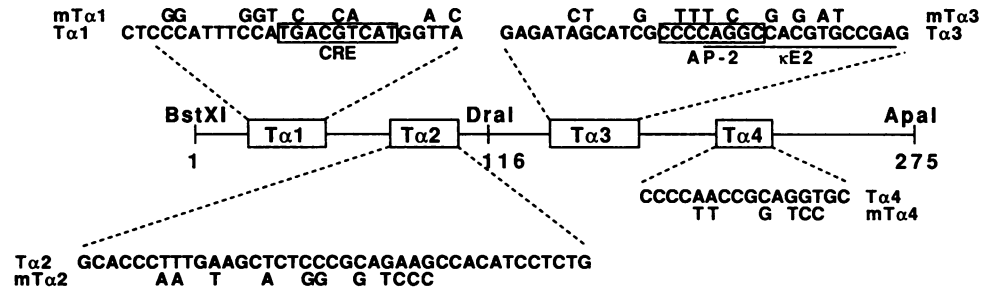


FIG. 1. Schematic representation of the human TCR α transcriptional enhancer. The nucleotide sequences of the Tα1 to Tα4 nuclear protein-binding sites (8) are shown above and below a partial restriction endonuclease map. Oligonucleotides corresponding to the wild-type Tα1 to Tα4 sequences were synthesized with *Bam*HI and *Bgl*II overhanging ends and used in electrophoretic mobility shift experiments. Mutant oligonucleotides (mTα1 to mTα4) containing the nucleotide substitutions shown in the top and bottom lines of the figure were synthesized as controls. The cyclic AMP response element (CRE) and AP-2 binding sites are boxed. The κE2-like sequence is underlined.

combination with each other, and the resulting plasmids were transfected into Jurkat cells. No combination of Tα1 and Tα2 oligonucleotides displayed significant enhancer activity (data not shown). These results led us to investigate further the function of the 20-bp spacer region located between the Tα1 and Tα2 binding sites. Specifically, we were interested in determining whether the sequence of this spacer region was important for enhancer function and whether the 20-bp spacing which corresponds to two turns of the DNA helix was necessary to position the Tα1 and Tα2 binding sites on the same side of the DNA helix. Mutation of all of the 20 bp composing the spacer region had no significant effect on enhancer activity (Fig. 2B), indicating that the precise sequence of the spacer was irrelevant to enhancer function. In contrast, alterations in the size of the spacer had profound effects on enhancer activity. The deletion of 5 bp from the spacer sequence reduced enhancer function by 87% (Fig. 2B). This effect appeared to be due to the reduction in spacer size rather than changes in the phasing of the Tα1 and Tα2 binding sites, because the addition of 5 bp to the spacer region resulted in only a 16% reduction in enhancer function. Further additions of 65 and 125 bp to the spacer sequence reduced enhancer activity by 71 and 82%, respectively (Fig. 2B). Taken together, these results demonstrated that the minimal TCR α enhancer is composed of two necessary nuclear protein-binding sites (Tα1 and Tα2) that must be separated by more than 15 bp. While the relative phase of these two binding sites on the DNA helix is not critical for enhancer function, the finding that increasing the distance between the two sites by more than 85 bp significantly reduced enhancer activity suggested that interactions between the Tα1 and Tα2 nuclear binding proteins may be important in generating enhancer activity.

**The Tα3 and Tα4 binding sites can compensate for Tα1 and Tα2 mutations and deletions.** Previous studies (8) have demonstrated that the *Dra*I-*Apa*I fragment containing the Tα3 and Tα4 nuclear protein-binding sites (Fig. 1) neither displays significant enhancer activity alone nor increases the enhancer activity of the *Bst*XI-*Dra*I enhancer fragment that contains the Tα1 and Tα2 binding sites (herein termed the Tα1-Tα2 enhancer). In order to study further the function of the Tα3 and Tα4 binding sites, we compared the effects on enhancer activity of Tα1 and Tα2 deletions and mutations made in the context of the *Bst*XI-*Apa*I fragment (herein termed the Tα1-Tα4 enhancer) with the same mutations and deletions made in the context of the Tα1-Tα2 enhancer (Fig. 2A and 2C). As described above, deletion of the 5′ end of the Tα1 binding site in the context of the Tα1-Tα2 enhancer resulted in a 90% reduction in enhancer activity (Fig. 2A). In contrast, this same deletion made in the context of the Tα1-Tα4 enhancer reduced activity by only 22% (Fig. 2C). Similarly, mutation of the Tα2 binding site in the context of the Tα1-Tα2 enhancer reduced activity by 96%, while the same mutation in the context of the Tα1-Tα4 enhancer resulted in only a 12% reduction in enhancer function (Fig. 2C). In order to determine whether the Tα3 and Tα4 binding sites were compensating for the Tα2 mutations, Tα2+Tα3 and Tα2+Tα4 double mutations were assayed for enhancer activity following transfection into Jurkat cells (Fig. 2C). These double mutations reduced enhancer activity by 87 and 94%, respectively. Thus, the Tα3 and Tα4 binding sites can partially compensate for Tα2 mutations. In contrast, the same mutations of the Tα3 or Tα4 binding sites further reduced the activity of the Tα1 deletion mutant by only 27 and 36%, respectively (Fig. 2C). Taken together, these results suggested that full enhancer activity requires either the presence of functional Tα1, Tα3, and Tα4 binding sites or the presence of the Tα2 binding site along with any one of the Tα1, Tα3, or Tα4 sites. Thus, the TCR α enhancer contains multiple redundant enhancons that can function together in different combinations to produce transcriptional enhancer activity.

**Multiple T-cell-specific and ubiquitous nuclear proteins bind to the TCR α enhancer.** While previous studies (8) have identified four important nuclear protein-binding sites within the human TCR α enhancer, the specificity of binding of these proteins, as well as their number and lineage specificity of expression, remained unknown. In order to address these questions, synthetic oligonucleotides corresponding to the Tα1 to Tα4 binding sites were used in electrophoretic mobility shift experiments with nuclear extracts from a variety of T-cell and non-T-cell tumor cell lines (Fig. 3 and 4). Each of the oligonucleotides was shown to bind between one and three nuclear protein complexes, as evidenced by their different mobilities following nondenaturing gel electrophoresis. The specificities of these binding reactions were confirmed in cold competition experiments using mutated or unrelated oligonucleotides (Fig. 3) (see Fig. 1 for the sequences of the wild-type and mutant oligonucleotides).

In order to examine the cellular distribution of expression of the TCR α enhancer binding activities, the electrophoretic mobility shift experiments were repeated using nuclear extracts prepared from a variety of T-cell and non-T-cell tumor cell lines, including the TCR $\alpha/\beta^+$ cell lines Jurkat and CEM, the TCR $\gamma/\delta^+$ T-cell lines Peer and MOLT-13, the Epstein-Barr virus-transformed B-cell line JY, the chronic myeloge-

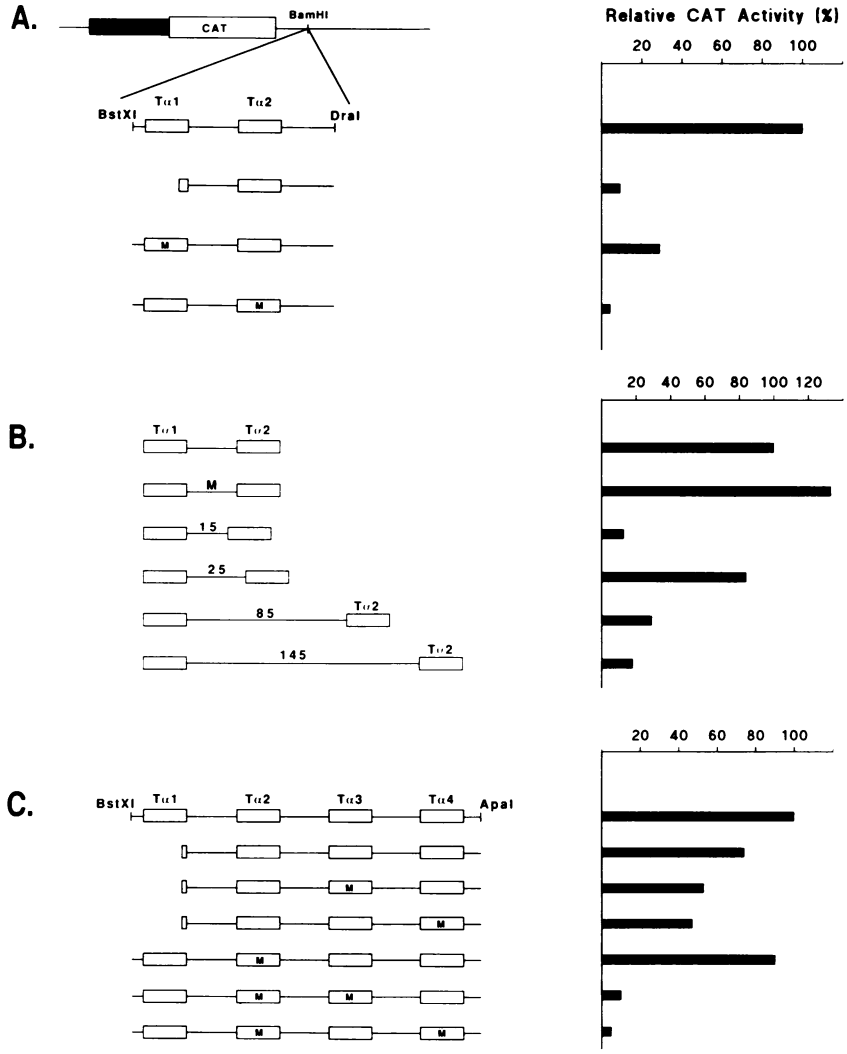


FIG. 2. In vitro mutagenesis and deletion analysis of the human TCR α transcriptional enhancer. (A) Effects of deletion and mutation of the Tα1 and Tα2 binding sites on enhancer activity. The 116-bp *Bst*XI-*Dra*I fragment containing the Tα1 and Tα2 nuclear protein-binding sites (Fig. 1) was digested with *Aat*II to remove 41 nucleotides from the 5′ end. In addition, the Tα1 and Tα2 binding sites were individually subjected to in vitro mutagenesis with synthetic oligonucleotides corresponding to the mutant Tα1 and mutant Tα2 sequences shown in Fig. 1. Mutated nuclear protein-binding sites are denoted by boxed M's. These deleted and mutated enhancer fragments were cloned into the *Bam*HI site of the pSPCAT vector, and the resulting plasmids were transfected into Jurkat T cells. To normalize for differences in transfection efficiencies, all transfections contained 2 μg of the pRSVβgal reference plasmid. Data are shown graphically in the right panel as relative CAT activity following correction for transfection efficiency and are normalized to the CAT activity produced by transfection of the wild-type enhancer (top line) fragment which produced 34.1% acetylation. SV40Pr, Minimal simian virus 40 promoter. (B) Effects of alterations in the spacer region on enhancer activity. An 89-bp synthetic oligonucleotide corresponding to the wild-type Tα1 and Tα2 binding sites along with the wild-type 20-bp spacer was synthesized with *Bam*HI-*Bgl*II overhanging ends and cloned into the *Bam*HI site of the pSPCAT vector. In addition, synthetic oligonucleotides containing wild-type Tα1 and Tα2 binding sites but a mutated 20-bp spacer region (AACCTCTTTTACCT GCTTTA) or a wild-type spacer lacking 5 bp (CCAAGAGGGTAGGGC) were cloned into the *Bam*HI site of this same vector. Finally, enhancer fragments containing 5-, 65-, and 125-bp insertions in the spacer region (denoted as 25, 85, and 145) were constructed as described in Materials and Methods and cloned into the *Bam*HI site of pSPCAT. The resulting plasmids were transfected into Jurkat cells as described for panel A, and CAT activities normalized to those produced by the wild-type oligonucleotide are shown graphically in the right panel. (C) Effects of deletions and mutations of the Tα1 to Tα4 nuclear protein-binding sites on enhancer activity. The wild-type 275-bp *Bst*XI-*Apa*I enhancer fragment containing the Tα1 to Tα4 nuclear protein-binding sites (Fig. 1) was subjected to in vitro mutagenesis with synthetic oligonucleotides corresponding to the mutant Tα2, mutant Tα3, and mutant Tα4 sequences shown in Fig. 1, as depicted schematically in the left panel. Mutated binding sites are denoted by boxed M's. In addition, a deletion mutant lacking 45 bp of 5′ sequence was constructed by digestion with *Bst*EII (lines 2, 3, and 4, left panel). The resulting fragments were cloned into the *Bam*HI site of pSPCAT and transfected into Jurkat cells. CAT activities normalized to those produced by the wild-type *Bst*XI-*Apa*I enhancer fragment are shown graphically in the right panel. All transfections were repeated at least twice with similar results.

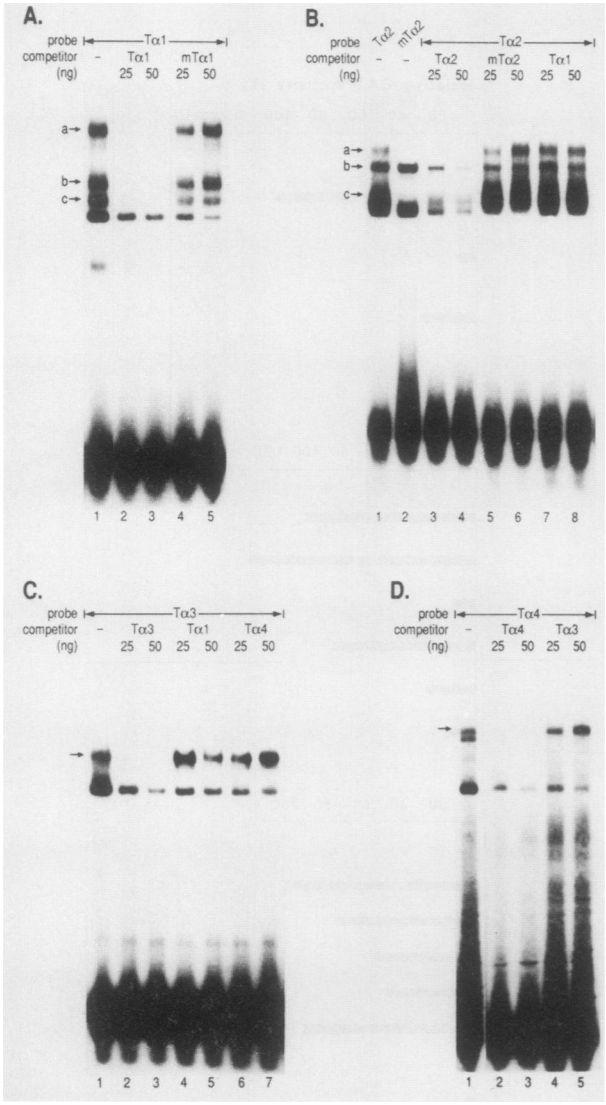


FIG. 3. Electrophoretic mobility shift analysis of the human TCR α enhancer binding proteins. Radiolabeled synthetic oligonucleotides corresponding to the Tα1 to Tα4 binding sites (Fig. 1) were used in electrophoretic mobility shift analyses with Jurkat nuclear extracts as described in Materials and Methods. In each case, the specificity of nuclear protein binding was confirmed by performing cold competitor experiments using unlabeled mutant or control oligonucleotides. Specific bands of altered mobility are shown by arrows. As previously described (12), the common band of relatively high mobility that is present in each lane represents nonspecific binding, as it is present with each labeled oligonucleotide and is partially inhibited by both specific and nonspecific unlabeled competitors. In panel B, lane 2, nuclear protein binding to a radiolabeled mutant Tα2 (mTα2) oligonucleotide is shown in order to better demonstrate the specificity of binding of the Tα2c protein.

nous leukemia cell line K562, the human cell line HeLa, and the MOLT-4 T-cell tumor cell line that expresses the TCR β but not the TCR α gene. These experiments (summarized in tabular form in Fig. 4B) demonstrated that the Tα1, Tα3, and Tα4 binding activities were expressed in all of the cell lines examined. In contrast, the Tα2a binding activity was detected only in the T-cell lines and was not observed in the B-cell or fibroblast nuclear extracts (Fig. 4A). It should be noted that the specificity of the Tα2b binding activity remains somewhat uncertain in that the Tα2b protein complex appeared to bind equally well to the wild-type and mutant Tα2 oligonucleotide probes (Fig. 3B, lanes 1 and 2). In addition, the binding of the Tα2b complex to a wild-type Tα2 probe was inhibited completely by unlabeled Tα2 oligonucleotide competitor and partially by mutant Tα2 or Tα1 cold competitor oligonucleotides (Fig. 3B, lanes 3 to 8).

**The TCR α enhancer is hypomethylated in cells of the lymphoid lineage.** The expression of a number of eucaryotic genes has been correlated with the hypomethylation of their transcriptional regulatory sequences (reviewed in reference 3). In order to determine whether the activity of the TCR α enhancer could be correlated with its methylation status, a Southern blot analysis of the TCR α enhancer was performed with methylation-sensitive isoschizomers (Fig. 5). In these experiments, genomic DNAs from the Jurkat and CEM TCR $\alpha/\beta^+$ cell lines, the Peer TCR $\gamma/\delta^+$ cell line, and the Clone 13 B-cell and HeLa cell lines were digested with the *Kpn*I and *Bam*HI restriction enzymes either alone or in combination with *Msp*I or its methylation-sensitive isoschizomer, *Hpa*II. In addition, these DNA samples were digested with a combination of *Kpn*I and *Bam*HI and the methylation-sensitive enzyme *Hha*I. Somewhat surprisingly, digestion of Jurkat DNA with *Kpn*I and *Bam*HI alone (Fig. 5, lane 1) revealed two bands of 1.4 and 1.8 kb, rather than the expected single band of 1.4 kb. Further restriction enzyme mapping of this region in Jurkat and HPB-ALL DNAs demonstrated that one allele in Jurkat cells lacks the expected *Kpn*I site at the 5′ end of this fragment, giving rise to the larger 1.8-kb band. This restriction fragment length polymorphism also gave rise to a 0.6-kb band in the *Kpn*I-*Bam*HI-*Msp*I (Fig. 5, lane 2) and *Kpn*I-*Bam*HI-*Hpa*II (lane 3) digests of Jurkat DNA, in addition to the expected 0.2-kb fragment 1. Taken together, the results of these experiments demonstrated that all of the *Msp*I and *Hha*I restriction enzyme sites surrounding the TCR α enhancer (Fig. 5, circled restriction enzyme sites) were unmethylated in the T-cell lines and in the Clone 13 B cells. Thus, digestion of these DNA samples with *Hpa*II or *Msp*I produced an identical band pattern composed of *Msp*I fragments 1 to 4 (Fig. 5, lanes 2, 3, 6, 7, 10, 11, 14, and 15). Similarly, digestion of these samples with *Hha*I produced bands 5 to 8, which were expected if all of the *Hha*I sites in this region were unemthylated (Fig. 5, lanes 4, 8, 12, and 16). In contrast, these sites were methylated in the HeLa cell line, producing an *Hpa*II band composed of *Msp*I fragments 1 to 3 (Fig. 5, lane 19) and an *Hha*I band composed of *Hha*I fragments 5 to 8 (lane 20). The finding that both the *Hpa*II and *Hha*I sites were methylated and uncut by *Hpa*II and *Hha*I in HeLa DNA while digestion with *Msp*I was complete essentially rules out the possibility that these results were due simply to partial digestion of this DNA sample. Moreover, these same sites were found to be methylated in normal human placental DNA (data not shown), indicating that this methylation was not an artifact of the HeLa tumor cell. Finally, it should be noted that these experiments did not allow us to precisely localize the 5′ border of hypomethylation in lymphoid cells, as the probe terminated at its 5′ end in an area that was still entirely hypomethylated in these cells. Thus, the region of hypomethylation shown in Fig. 5 represents the minimum region involved in this process. Taken together, these data suggest that while hypomethylation may be necessary for TCR α enhancer activity, it is not sufficient, as the enhancer is active in the

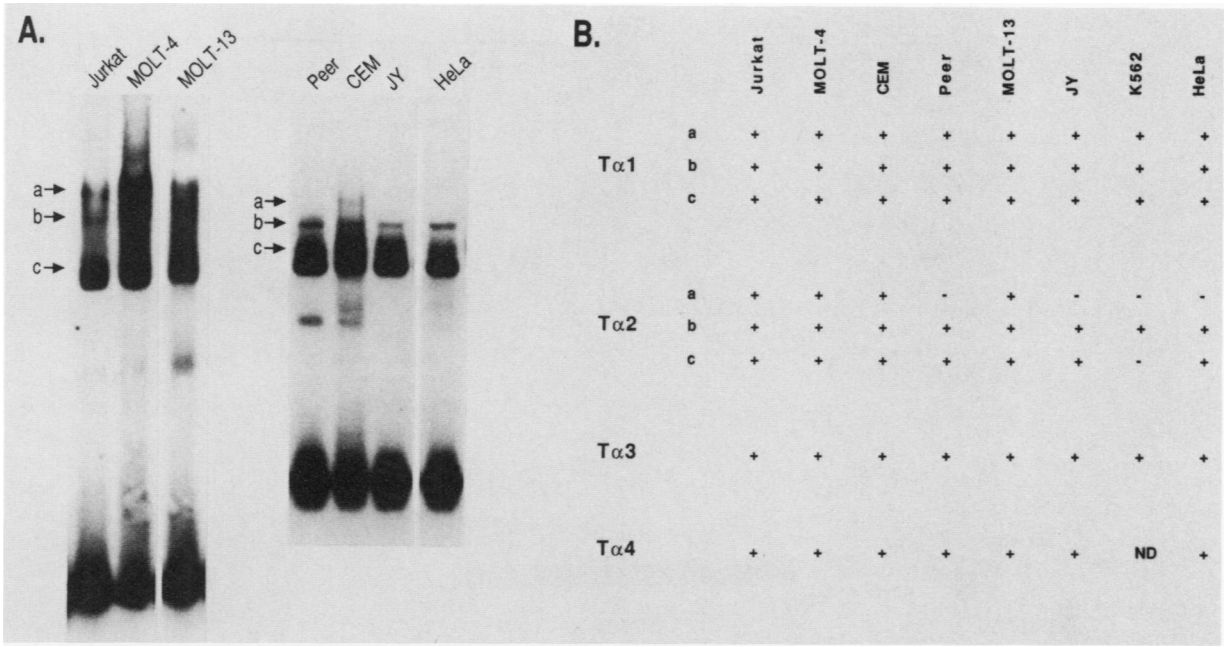


FIG. 4. Identification of TCR α enhancer binding activities in different human tumor cell lines. Gel mobility shift analyses similar to those shown in Fig. 3 were used to identify TCR α enhancer binding activities in nuclear extracts prepared from a variety of human tumor cell lines. (A) Electrophoretic mobility shift analysis of Tα2 binding proteins in TCR $\alpha/\beta^+$ Jurkat, MOLT-4, and CEM cells, TCR $\gamma/\delta^+$ Peer and MOLT-13 cells, Epstein-Barr virus-transformed JY B cells, and HeLa cells. Arrows denote specific bands of altered mobility. (B) Summary of the Tα1 to Tα4 binding activities in a variety of human tumor cell lines. The Tα1 and Tα2 a, b, and c binding activities correspond to those shown in Fig. 3A and B. ND, Not determined.

Jurkat and CEM cell lines but is inactive in the Peer, Clone 13, and HeLa cell lines (8).

## DISCUSSION

The studies described in this report have demonstrated that the human TCR α enhancer is composed of four important, partially redundant *cis*-acting enhancon sequences, Tα1 to Tα4, that bind a set of ubiquitous and T-cell-specific nuclear proteins. The minimal enhancer is composed of the Tα1 and Tα2 nuclear protein-binding sites. These sites must be separated by at least 15 nucleotides, but their enhancer activity rapidly decreases as they are separated by more than 85 bp. While the Tα3 and Tα4 binding sites do not themselves possess intrinsic enhancer activity (8), they are able to compensate for the loss of either the Tα1 or Tα2 sites. Finally, while hypomethylation of the enhancer sequences may be required for their activity, such hypomethylation is not, in itself, sufficient to activate the enhancer.

These results raise a number of questions concerning the molecular mechanisms that regulate TCR α gene expression during T-cell development in the thymus. The first question concerns the role of the Tα3 and Tα4 nuclear protein-binding sites in regulating the enhancer. Although these elements are clearly not required for enhancer function in Jurkat cells, there are several pieces of evidence that suggest that they might, in fact, play an important role in regulating enhancer activity during some stage of T-cell development. First, the finding that Tα3 and Tα4 can both compensate for Tα2 mutations suggests that they are able to bind important transcriptional regulatory proteins. In addition, the finding that the Tα3 binding site contains a κE2-like motif is significant in that all of the antigen receptor enhancers have now been shown to contain Ephrussi motifs. This suggests that Ephrussi box-binding proteins may play an important role in regulating the expression of multiple members of the immunoglobulin supergene family. Recent studies (18) have suggested that there is a family of such Ephrussi box-binding proteins, some of which might be expressed in a cell lineage-specific fashion. Finally, it is possible that the Tα3 or Tα4 binding sites or both are also involved in regulating TCR α gene expression in response to specific stimuli, such as T-cell activation. In this regard, it is interesting to note that the Tα3 binding site contains a consensus AP-2 enhancer motif which has been shown to confer phorbol ester responsiveness on a number of eucaryotic genes (10). Previous studies have demonstrated that the TCR α gene is phorbol ester responsive (15), raising the possibility that Tα3 may be involved in mediating this effect.

What are the molecular mechanisms that regulate the lineage-specific expression of the human TCR α gene? Previous studies have suggested that the enhancer itself is active only in TCR $\alpha/\beta^+$ cells (8). And a recent report has suggested that transcriptional silencer elements located between the Cα gene and the TCR α enhancer are responsible for down regulating enhancer activity in non-TCR $\alpha/\beta^+$ cells (24). Our data suggest that the enhancer may, in fact, be regulated at several levels. First, the enhancer is methylated in nonlymphoid as compared with lymphoid cells. Such methylation has been shown to be correlated with transcriptional inactivity in a number of other systems (reviewed in reference 3). Interestingly, in one case, methylation of a cyclic AMP response element similar to that found within the Tα1 binding site has been shown to prevent nuclear protein binding to that site (9). A second level of regulation may involve the expression of the Tα2a binding activity that

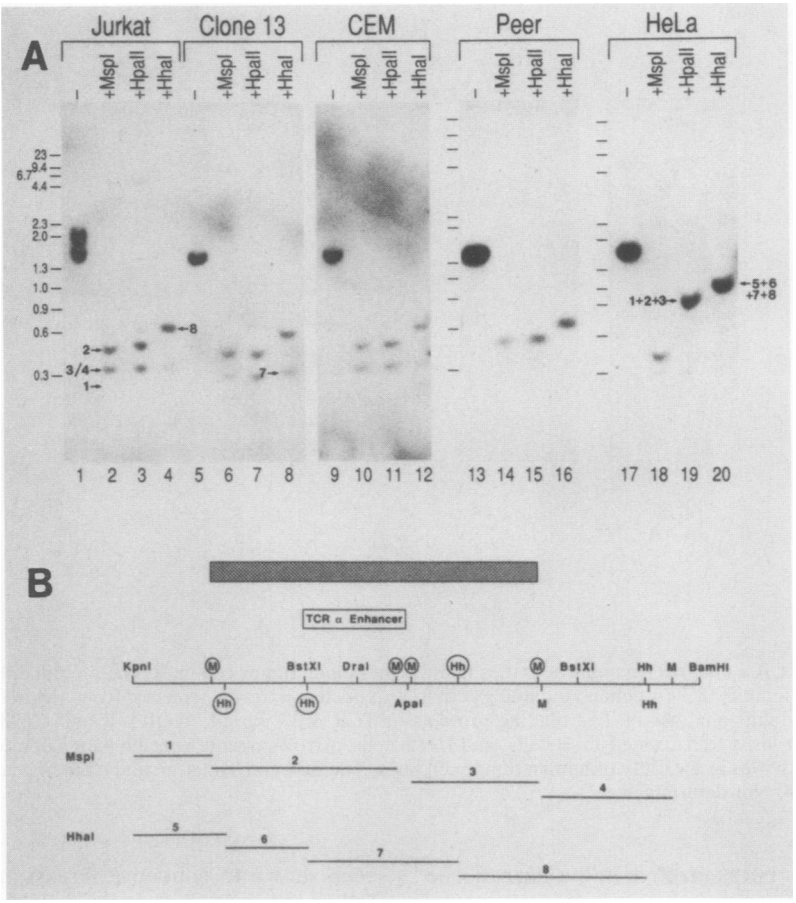


FIG. 5. Southern blot analysis of the pattern of methylation of the TCR α enhancer in different human tumor cell lines. (A) Southern blots. High-molecular-weight DNA from a variety of human tumor cell lines was digested with *Kpn*I-*Bam*HI either alone (−) or in combination with the *Msp*I, *Hpa*II, or *Hha*I restriction endonucleases and subjected to Southern blot analysis with a radiolabeled 1.4-kb *Kpn*I-*Bam*HI genomic fragment probe that contained the TCR α enhancer (shown schematically in panel B). Size markers in kilobases are shown to the left of each blot. Fragments are labeled according to the restriction enzyme map shown in panel B. (B). Partial restriction enzyme map of the 1.4-kb *Kpn*I-*Bam*HI genomic fragment containing the TCR α enhancer. M, *Msp*I; Hh, *Hha*I. The predicted *Msp*I and *Hha*I restriction enzyme fragments are shown schematically and numbered below the map. The position of the TCR α enhancer is denoted above the map. Circled restriction enzyme sites are sites that were shown to be hypomethylated in cells of the lymphoid lineage as compared with nonlymphoid cells. The hatched box represents the minimal region of hypomethylation detected in these studies in lymphoid cells.

we have shown is expressed in a T-cell-specific fashion. This lineage-specific pattern of expression might be involved in limiting TCR α enhancer activity to TCR $\alpha/\beta^+$ and TCR $\gamma/\delta^+$ cells. However, several findings suggest that additional mechanisms must be important in restricting the activity of the enhancer to TCR $\alpha/\beta^+$ T cells. First, although the Tα2a binding activity was observed only in T cells, it was expressed at equivalent levels in TCR $\alpha/\beta^+$ and TCR $\gamma/\delta^+$ T cells (Fig. 4A). Thus, the differential expression of the Tα2a binding activity cannot, by itself, account for the cell lineage specific activity of the enhancer. Similarly, the enhancer containing the mutant Tα2a binding site remained active in T cells (Fig. 2C) but was inactive in B cells (data not shown), even though both cell types were shown to express identical Tα1, Tα3, and Tα4 binding activities as assessed by electrophoretic mobility shift assays (Fig. 4B). Taken together, these results suggest that the cell lineage-specific activity of the TCR α enhancer may be mediated by additional T-cell-specific transcriptional activator proteins that are not detected in our DNase I footprint and electrophoretic mobility shift assays or by the activity of the previously described transcriptional silencer elements (24) or both. Ongoing studies designed to clone the TCR α enhancer binding proteins and to study their expression and function during thymic ontogeny should lead to further insights into the regulation of TCR α gene expression during T-cell development.

## ACKNOWLEDGMENTS

We thank Jeanelle Pickett for expert secretarial assistance and Beverly Burck for the preparation of the illustrations.

This work was supported in part by Public Health Service grant AI-29673 from the National Institutes of Health.

## LITERATURE CITED

1. **Alt, F. W., T. K. Blackwell, and G. D. Yancopoulos.** 1987. Development of the primary antibody repertoire. Science **238:** 1079–1087.
2. **Banerji, J., L. Olson, and W. Schaffner.** 1983. A lymphocyte-specific cellular enhancer is located downstream of the joining region of immunoglobulin heavy chain genes. Cell **33:**729–740.

3. **Bird, A. P.** 1986. CpG-rich islands and the function of DNA methylation. Nature (London) **321**:209–213.
4. **Blackwell, T. K., M. W. Moore, G. D. Yancopoulos, H. Suh, S. Lutzker, E. Selsing, and F. W. Alt.** 1986. Recombination between immunoglobulin variable region gene segments is enhanced by transcription. Nature (London) **324**:585–589.
5. **Davis, M. M., and P. J. Bjorkman.** 1988. T-cell antigen receptor genes and T-cell recognition. Nature (London) **334**:395–401.
6. **Dignam, J. D., R. M. Lebovitz, and R. G. Roeder.** 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Res. **11**:1475–1489.
7. **Gillies, S. D., S. L. Morrison, V. T. Oi, and S. Tonegawa.** 1983. A tissue-specific transcription enhancer element is located in the major intron of a rearranged immunoglobulin heavy chain gene. Cell **33**:717–728.
8. **Ho, I.-C., L.-H. Yang, G. Morle, and J. M. Leiden.** 1989. A T-cell-specific transcriptional enhancer element 3′ of Cα in the human T-cell receptor α locus. Proc. Natl. Acad. Sci. USA **86**:6714–6718.
9. **Iguchi-ariga, S. M. M., and W. Schaffner.** 1989. CpG methylation of the cAMP-responsive enhancer/promoter sequence TGACG abolishes specific factor binding as well as transcriptional activation. Genes Dev. **3**:612–619.
10. **Imagawa, M., R. Chiu, and M. Karin.** 1987. Transcription factor AP-2 mediated induction by two different signal-transduction pathways: protein kinase C and cAMP. Cell **51**:251–260.
11. **Kadesch, T., P. Zervos, and D. Ruezinsky.** 1986. Functional analysis on the murine IgH enhancer: evidence for negative control of cell-type specificity. Nucleic Acids Res. **14**:8209–8221.
12. **Karpinski, B. A., L.-H. Yang, P. Cacheris, G. D. Morle, and J. M. Leiden.** 1989. The first intron of the 4F2 heavy-chain gene contains a transcriptional enhancer element that binds multiple nuclear proteins. Mol. Cell. Biol. **9**:2588–2597.
13. **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 gene is controlled by a downstream regulatory element. EMBO J. **7**:745–750.
14. **Leung, K., and G. J. Nabel.** 1988. HTLV-1 transactivator induces interleukin-2 receptor expression through an NF-κB-like factor. Nature (London) **333**:776–778.
15. **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**:1769–1774.
16. **Marrack, P., and J. Kappler.** 1987. The T cell receptor. Science **238**:1073–1079.
17. **McDougall, S., C. L. Peterson, and K. Calame.** 1988. A transcriptional enhancer 3′ of Cβ2 in the T cell receptor β locus. Science **241**:205–208.
18. **Murre, C., P. S. McCaw, and D. Baltimore.** 1989. A new DNA binding and dimerization motif in immunoglobulin enhancer binding, daughterless, MyoD, and myc proteins. Cell **56**:777–783.
19. **Osborn, L., S. Kunkel, and G. J. Nabel.** 1989. Tumor necrosis factor α and interleukin 1 stimulate the human immunodeficiency virus enhancer by activation of the nuclear factor κB. Proc. Natl. Acad. Sci. USA **86**:2336–2340.
20. **Picard, D., and W. Schaffner.** 1984. A lymphocyte-specific enhancer in the mouse immunoglobulin kappa gene. Nature (London) **307**:80–82.
21. **Queen, C., and D. Baltimore.** 1983. Immunoglobulin gene transcription is activated by downstream sequence elements. Cell **33**:741–748.
22. **Strominger, J. L.** 1989. Developmental biology of T cell receptors. Science **244**:943–950.
23. **Winoto, A., and D. Baltimore.** 1989. A novel inducible and T cell-specific enhancer located at the 3′ end of the T cell receptor α locus. EMBO J. **8**:729–733.
24. **Winoto, A., and D. Baltimore.** 1989. αβ lineage-specific expression of the α T cell receptor gene by nearby silencers. Cell **59**:649–655.
25. **Yancopoulos, G., 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**:251–259.
26. **Yancopoulos, G., and F. W. Alt.** 1985. Developmentally controlled and tissue-specific expression of unrearranged VH gene segments. Cell **40**:271–281.