The Tα2 Nuclear Protein Binding Site from the Human T Cell Receptor α Enhancer Functions as Both a T Cell–specific Transcriptional Activator and Repressor

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Summary

T cell–specific expression of the human T cell receptor α (TCR-α) gene is regulated by the interaction of variable region promoter elements with a transcriptional enhancer that is located 4.5 kb 3′ of the TCR-α constant region (Cα) gene segment. The minimal TCR-α enhancer is composed of two nuclear protein binding sites, Tα1 and Tα2, that are both required for the T cell–specific activity of the enhancer. The Tα1 binding site contains a consensus cAMP response element (CRE), and binds a set of ubiquitous nuclear proteins. The Tα2 binding site does not contain known transcriptional enhancer motifs. However, it binds at least two nuclear protein complexes, one of which is T cell specific. We now report that although the Tα2 nuclear protein binding site displays transcriptional activator activity in the context of the TCR-α enhancer, this site alone can function as a potent, T cell–specific transcriptional repressor when positioned either upstream, or downstream of several heterologous promoter and enhancer elements. These results demonstrate that a single nuclear protein binding site can function as a T cell–specific transcriptional activator or repressor element, depending upon the context in which it is located.

The process of eukaryotic development involves the tissue-specific and temporally regulated expression of specific subsets of genes. The molecular mechanisms that positively and negatively regulate gene expression during mammalian development are complex, and involve the interaction of ubiquitous, and tissue-specific nuclear proteins with *cis*-acting transcriptional regulatory sequences known as promoter, enhancer, and silencer elements (1–3). Mammalian T lymphocyte development in the thymus is an excellent model system for studies designed to better elucidate the molecular mechanisms that control developmentally regulated gene expression. Two subsets of mammalian T cells have been identified that are distinguished by their cell surface expression of heterodimeric antigen receptor molecules (4, 5). All cells of the helper and cytotoxic phenotype express the TCR-α/β molecule, whereas a second set of T cells, of unknown function, express the related, but distinct TCR-γ/δ. These two T cell subsets appear to belong to separate developmental lineages, in that the TCR-α gene is rearranged and expressed only in TCR-α/β^+ cells, whereas the TCR-δ gene is rearranged and expressed exclusively in TCR-γ/δ^+ cells (5).

To better understand the molecular mechanisms that regulate the development of these two T cell subsets, we have attempted to delineate the *cis*-acting sequences and *trans*-acting factors that control the rearrangement and expression of the TCR genes. Previous studies have demonstrated that human and murine TCR-α gene expression is regulated by a transcriptional enhancer located 3–4.5 kb 3′ of the single TCR-α Cα gene segment (6, 7). This enhancer is required for high level transcription from a TCR Vα promoter, and is active only in TCR-α/β^+ T cells (7). Deletion and in vitro mutagenesis analyses, along with DNaseI footprint experiments, demonstrated that the minimal TCR-α enhancer is composed of two nuclear protein binding sites, Tα1 and Tα2, that must be separated by more than 15, and less than 85 nucleotides (7, 8). The Tα1 binding site contains a consensus cAMP response element (CRE),[1] and has been shown to bind a set of ubiquitously expressed CRE binding proteins (CREBs). In contrast, the Tα2 binding site does not contain previously identified transcriptional enhancer motifs. However, it binds at least two nuclear protein complexes, one of which is T cell specific (8). In the studies described in this paper, we demonstrate that the Tα2 nuclear protein binding site can function as both a T cell–specific transcriptional activator and repressor, depending upon the context in which it is located.

[1] *Abbreviations used in this paper:* CAT, chloroamphenicol acetyl transferase; CRE, consensus cAMP response element; CREB, CRE binding protein.

 J. Exp. Med. © The Rockefeller University Press • 0022-1007/90/11/1443/07 $2.00
Volume 172 November 1990 1443–1449

Tα2 functions as a transcriptional activator domain in the context of the intact TCR-α enhancer. However, this binding site, alone functions as a T cell–specific transcriptional repressor when positioned either upstream or downstream of a variety of heterologous promoter and enhancer elements. To our knowledge, this is the first report of a single mammalian nuclear protein binding site that can function as a cell lineage-specific transcriptional activator and repressor, and, as such, suggests a novel mechanism for positively and negatively regulating mammalian gene expression from a single transcriptional enhancer element that is composed of multiple nuclear protein binding sites.

Materials and Methods

Cells and Media. Human T cell lines, Jurkat (TCR-α/β^+) and Peer (TCR-γ/δ^+), as well as the EBV-transformed human B cell line, clone 13, and the chronic myelogenous leukemia cell line K562 were grown in RPMI 1640 medium supplemented with 10% FCS (Gibco Laboratories, Grand Island, NY) and 1% penicillin/streptomycin (Gibco Laboratories).

Plasmids. The pSPCAT plasmid containing the minimal SV40 promoter immediately 5′ of the bacterial chloramphenicol acetyl transferase (CAT) gene, and the pSPI1CAT plasmid which is pSPCAT that contains the 4F2 heavy chain (4F2HC) enhancer 5′ of the minimal SV40 promoter have been described previously (9, 10) as have the pRSVCAT (11) and p4F2-589CAT (12) plasmids in which CAT transcription is under the control of the Rous sarcoma virus long terminal repeat (LTR) and 4F2HC promoters, respectively. The pSPœnhCAT plasmid which is pSPCAT that contains the 116-bp BstXI/DraI human TCR-α enhancer fragment 3′ of the CAT gene has also been described previously (7). The promoterless pCAT-Basic plasmid was obtained from Promega-Biotec (Madison, WI).

Synthetic Oligonucleotides and In Vitro Mutagenesis. Complementary synthetic oligonucleotides corresponding to the wild-type

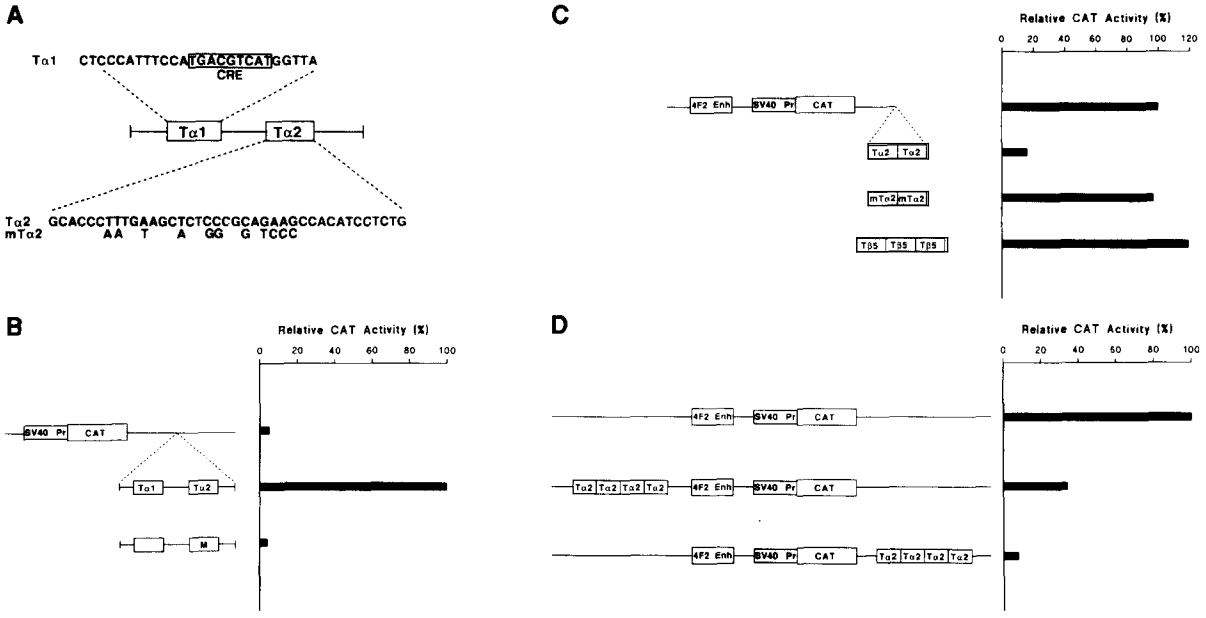

Figure 1. The Tα2 nuclear protein binding site of the human TCR-α enhancer can function as both a transcriptional activator and repressor. (*A*) A schematic representation of the 116-bp minimal human TCR-α transcriptional enhancer (7). The nucleotide sequences of the previously described (7) Tα1 and Tα2 nuclear protein binding sites are shown above and below the map, respectively. The CRE within the Tα1 binding site is boxed and labeled. The sequence of a mutant Tα2 oligonucleotide (mTα2) that fails to bind nuclear proteins (8) is shown in the bottom line of the panel. Synthetic Tα2 and mTα2 oligonucleotides corresponding to the sequences shown were synthesized with BamHI and BglII overhanging ends, and used in the functional studies described in *C* and *D* below. (*B*) The effects of mutations of the Tα2 binding site on the activity of the TCR-α enhancer. The wild-type TCR-α enhancer or a mutant enhancer (M) containing 11 nucleotide substitutions in the Tα2 binding site (mTα2, *A* above) were cloned into the BamHI site 3′ of the minimal SV40 promotor/CAT cassette in the pSPCAT vector (9) and 10-μg of each of the resulting plasmids along with 2 μg of the pRSVβgal reference plasmid were transfected into human Jurkat T cells. The data are displayed as CAT activities relative to that produced by the plasmid containing the wild-type TCR-α enhancer that resulted in 34% acetylation. (*C*) The effects of the Tα2 nuclear protein binding site on transcription from the minimal SV40 promoter and 4F2HC enhancer. Two copies of synthetic oligonucleotides corresponding to the wild-type (Tα2) or mutant (mTα2) Tα2 binding sites (see *A* above) or three copies of the Tβ5 nuclear protein binding site of the human TCR-β enhancer (TAGAACTTCAGAGGGGAGGG) were cloned into the BamHI site 3′ of the minimal SV40 promoter and the 500 bp 4F2HC enhancer (4F2 Enh) in the pSPI1CAT plasmid (10). 10 μg of each of the resulting plasmids along with 2 μg of the pRSVβgal plasmid was transfected into Jurkat T cells and CAT, and β-galactosidase activities were determined as described in Materials and Methods. The data are displayed as CAT activities relative to that produced by the pSPI1CAT plasmid that produced 3% acetylation. (*D*) The effects of position on the transcriptional repressor function of the Tα2 nuclear protein binding site. Four copies of the synthetic Tα2 oligonucleotide (see *A*) were cloned into the BamHI site 3′ of the 4F2HC enhancer and minimal SV40 promoter/CAT gene or the SacI site 5′ of the 4F2HC enhancer and SV40 promoter/CAT gene in pSPI1CAT, and 10 μg of the resulting plasmids along with 2 μg of the pRSVβgal plasmid were transfected into Jurkat cells. The data are shown as CAT activities relative to those produced by the pSPI1CAT control plasmid that produced 7% acetylation.

Tα1 and Tα2 nuclear protein binding sites, and a mutant Tα2 nuclear protein binding site (mTα2, see Fig. 1 *A*) were synthesized with BamHI/BglII overhanging ends on a DNA synthesizer (model 380B; Applied Biosystems, Foster City, CA), and annealed before cloning into the pSPCAT, pSPI1CAT, p4F2CAT, and pRSVCAT plasmids. The copy number cloned into each plasmid was confirmed by dideoxy DNA sequence analysis. A minimal human TCR-α enhancer containing 11 nucleotide substitutions within the Tα2 nuclear protein binding site (see Fig. 1 *A*) was produced by oligonucleotide-mediated gapped heteroduplex mutagenesis as described previously (10) and verified by dideoxy sequence analysis.

Transfections and CAT Assays. Jurkat, K562, and clone 13 cells were transfected using a modification of the DEAE-dextran method as described previously (7). Peer cells were transfected by electroporation (7). To control for differences in transfection efficiencies, all transfections contained 2 μg of the pRSVβgal reference plasmid. Cells were harvested 48 h after transfection, and cell extracts normalized for protein content using a commercially available kit (Biorad, Richmond, CA) were assayed for both β-galactosidase and CAT activities as described previously (7).

Results

The Tα2 Nuclear Protein Binding Site Can Function as a Transcriptional Activator and Repressor. To determine whether the Tα1 and Tα2 binding sites by themselves were necessary, or sufficient for TCR-α enhancer activity, two types of experiments were performed. First, the Tα1 and Tα2 binding sites were mutated so as to abolish nuclear protein binding as assayed by electrophoretic mobility shift analyses. Mutation or deletion of either the Tα1 (8) or Tα2 (reference 8, and Fig. 1 *B*) binding sites essentially abolished enhancer activity, indicating that both the Tα1 and Tα2 sites function as transcriptional activator elements in the context of the TCR-α enhancer. In a reciprocal set of experiments, synthetic oligonucleotides corresponding to the Tα1 and Tα2 binding sites were cloned into the BamHI site of the pSPCAT reporter plasmid (9), 3′ of the minimal SV40 promoter and the bacterial CAT gene, and transfected into Jurkat TCR-α/β^+ T cells. In the absence of stimulation of protein kinase A, two copies of the Tα1 oligonucleotide had no significant effect on CAT transcription (data not shown). Surprisingly, however, two copies of the Tα2 oligonucleotide appeared to completely suppress CAT transcription (data not shown), although the magnitude of this repression was difficult to quantitate because of the low basal levels of transcription from the minimal SV40 promoter in this plasmid. To better assess the negative regulatory activity of the Tα2 element, two copies of the synthetic Tα2 oligonucleotide were cloned into the BamHI site of the pSPI1CAT vector (10) that contains the previously described 4F2 heavy chain (4F2HC) enhancer 5′ of the minimal SV40 promoter/CAT cassette (Fig. 1 *C*). This plasmid was chosen as a reporter for Tα2 transcriptional repressor activity because we have demonstrated previously that the 4F2HC enhancer is highly active in a wide variety of lymphoid and non-lymphoid human tumor cell lines (10). As shown in Fig. 1 *C*, two copies of the Tα2 binding site resulted in an 84% reduction in CAT activity in Jurkat cells as compared with the pSPI1CAT control plasmid. This effect was specific for the Tα2 oligonucleotide because it was not observed when two copies of a mutant Tα2 (mTα2) oligonucleotide (Fig. 1 *A*) that fails to bind T cell–specific nuclear proteins (8), or three copies of the Tβ5 oligonucleotide that corresponds to a nuclear protein binding site from the human TCR-β transcriptional enhancer (13) were cloned into the BamHI site of pSPI1CAT (Fig. 1 *C*).

Tα2 Transcriptional Repressor Activity Is Position Independent. We have demonstrated previously (7) that the transcriptional activator properties of the TCR-α enhancer are independent of the position and orientation of the enhancer element. To determine whether the transcriptional repressor activity of the Tα2 binding site was similarly position independent, we compared the activity of four copies of Tα2 cloned 5′ of the 4F2HC enhancer and minimal SV40 promoter/CAT cassette to the activity of the same sites cloned 3′ of the CAT gene in this vector (Fig. 1 *D*). The ability of Tα2 to repress transcription was slightly reduced when the Tα2 sites were cloned 5′ of the promoter and enhancer as compared with 3′ of the CAT gene (66% reduction vs. 92% reduction). Nevertheless, the transcriptional repressor effect was relatively independent of the position of the Tα2 binding sites.

Tα2 Represses Transcription from Multiple Heterologous Promoters and Enhancers. Previous studies (7) have demonstrated that the TCR-α enhancer is able to confer T cell–specific transcriptional enhancement upon both a Vα promoter and several heterologous promoters. To determine whether the Tα2 element alone was capable of repressing transcription from multiple distinct promoter and enhancer elements in Jurkat cells, four copies of a synthetic Tα2 oligonucleotide were cloned 3′ of CAT reporter genes that were under the control of the Rous sarcoma virus (RSV) promoter/enhancer, or the 4F2 heavy chain (4F2HC) promoter, a G + C rich "housekeeping" promoter that lacks a TATA box and initiator sequence (14), but contains multiple SP1 binding sites (15). These plasmids were transfected into human Jurkat T cells, and the resulting CAT activities were compared with those produced by control plasmids lacking the Tα2 binding sites (Fig. 2). Four copies of Tα2 significantly reduced transcription from each of these promoter/enhancer combinations (>90% reduction), demonstrating that the transcriptional repressor activity of Tα2 was not unique to the SV40 promoter or to the 4F2HC enhancer, and that this activity was not restricted to TATA-containing promoters. In additional experiments, four copies of the Tα2 binding site also inhibited transcription from a minimal SV40 promoter/TCR-α enhancer by >98% (data not shown).

The Transcriptional Repressor Activity of Tα2 Is T Cell–specific. The TCR-α enhancer is active only in TCR-α/β^+ T cells (7), and previous studies have demonstrated that the Tα2 enhancer motif binds at least two nuclear protein complexes, one of which is present only in TCR-α/β^+ and TCR-γ/δ^+ T cells (8). To determine whether the transcriptional repressor activity of the Tα2 binding site was restricted to a specific cell lineage, plasmids containing four copies of the Tα2 binding site cloned 3′ of the 4F2HC enhancer and SV40 promoter/CAT cassette were transfected into Jurkat TCR-α/β^+

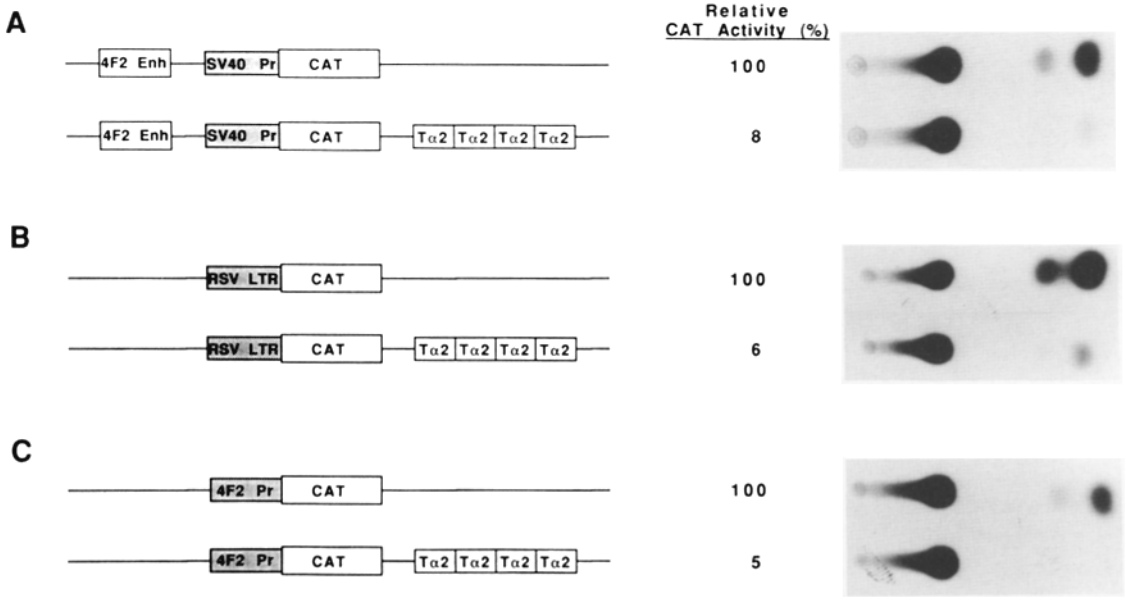

Figure 2. The Tα2 element can repress transcription from multiple heterologous promoter and enhancer elements. Four copies of a synthetic Tα2 oligonucleotide (see Fig. 1 *A*) were cloned into the BamHI site 3′ of the 4F2HC enhancer and minimal SV40 promoter/CAT cassette in pSPI1CAT (10) (*A*), the RSV LTR/CAT cassette in pRSVCAT (11) (*B*), or the 589-bp 4F2HC promoter (4F2 Pr)/CAT cassette in p4F2-589CAT (12) (*C*). 10 μg of each of the resulting plasmids along with 2 μg of the pRSVβgal reference plasmid were transfected into Jurkat T cells, and CAT and β-galactosidase activities were determined as described in Materials and Methods. The data are displayed as CAT activities relative to those produced in transfections with the appropriate control plasmids lacking the Tα2 elements.

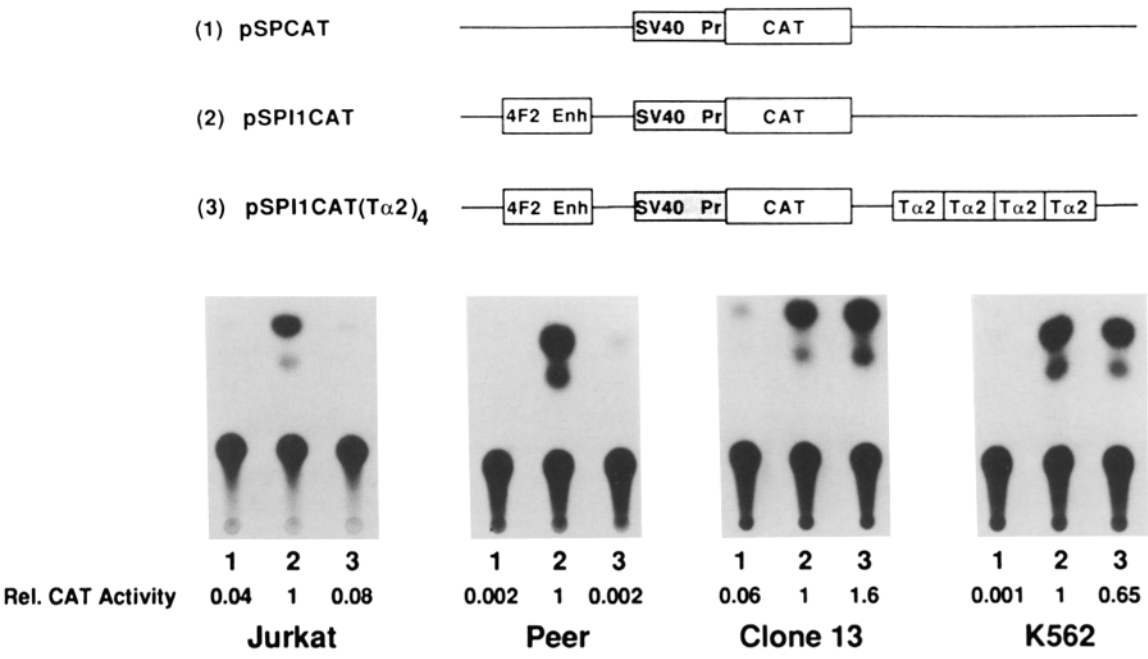

Figure 3. The Tα2 element functions as a T cell–specific transcriptional repressor. 10 μg of the pSPCAT, pSPI1CAT, and pSPI1CAT(Tα2)$_4$ plasmids shown schematically in the top panel along with 2 μg of the pRSVβgal reference plasmid were transfected into Jurkat TCR-α/β$^+$ T cells, Peer-γ/δ$^+$ T cells, clone 13 EBV-transformed B cells, and K562 chronic myelogenous leukemia cells, and cell extracts were assayed for both CAT and β-galactosidase activities as described in Materials and Methods. The data are displayed as CAT activities relative to those produced by the pSPI1CAT plasmid that were 7, 8, 3, and 6% total acetylation, respectively, in the Jurkat, Peer, clone 13, and K562 cell lines.

T cells, Peer TCR-γ/δ^+ T cells, clone 13 EBV-transformed B cells, and K562 chronic myelogenous leukemia cells, and their activities were compared with those produced by the same plasmids lacking the Tα2 binding sites (Fig. 3). Significant repression of transcription (92 and 98% reductions, respectively) was observed in the Jurkat and Peer T cells. In contrast, no repression was observed in either the K562 or clone 13 cells. A similar pattern of T cell–specific repression was observed after transfection of a plasmid containing four copies of the Tα2 binding site cloned 3′ of an RSV promoter/enhancer/CAT cassette (data not shown). Taken together, these results demonstrated that the transcriptional repressor activity of the Tα2 element is T cell specific, and suggested that this activity may be mediated by the ability of this element to bind one or more T cell–specific nuclear proteins.

The Transcriptional Repressor Activity of Tα2 is a cis-acting Effect and Is Proportional to the Number of Copies of the Tα2 Binding Site. The transcriptional activator function of many enhancer elements is directly proportional to the number of copies of these elements that are present in a given reporter plasmid. To determine whether the transcriptional repressor activity of the Tα2 element was similarly proportional to its copy number, one to four copies of the synthetic Tα2 oligonucleotide were cloned into the BamHI site 3′ of the RSV promoter/enhancer/CAT cassette, and these plasmids were transfected into Jurkat T cells (Fig. 4). These experiments demonstrated that the transcriptional repressor activity of the Tα2 element was directly proportional to the number of copies of Tα2 with 94% repression of transcription observed with four copies of Tα2.

One mechanism that could account for the transcriptional repressor activity of the Tα2 element is that this element is able to compete efficiently for the binding of generalized transcription factors, thereby causing an overall decrease in transcription in the transfected cells. To test this possibility, all transfections contained 2 μg of the pRSVβgal reference plasmid in which β-galactosidase gene expression is under the control of the RSV promoter/enhancer. As shown in Fig. 4, β-galactosidase activity was essentially identical in all of the transfections, regardless of the number of copies of Tα2 contained within the CAT reporter plasmid. Similar results were obtained in all of the transfections shown in Figs. 1–3 (data not shown). Thus, the transcriptional repressor activity of Tα2 is a *cis*-acting effect, and is not simply the result of generalized decreases in transcriptional activity in cells transfected with Tα2-containing plasmids.

A second mechanism that could account for the apparent transcriptional repressor activity of the Tα2 binding site is that this enhancer element, when polymerized, can also function as a promoter, thereby artifactually reducing CAT transcription in our transient transfection assays by a mechanism of promoter interference. To rule out this possibility, four copies of the Tα2 binding site were cloned in both orienta-

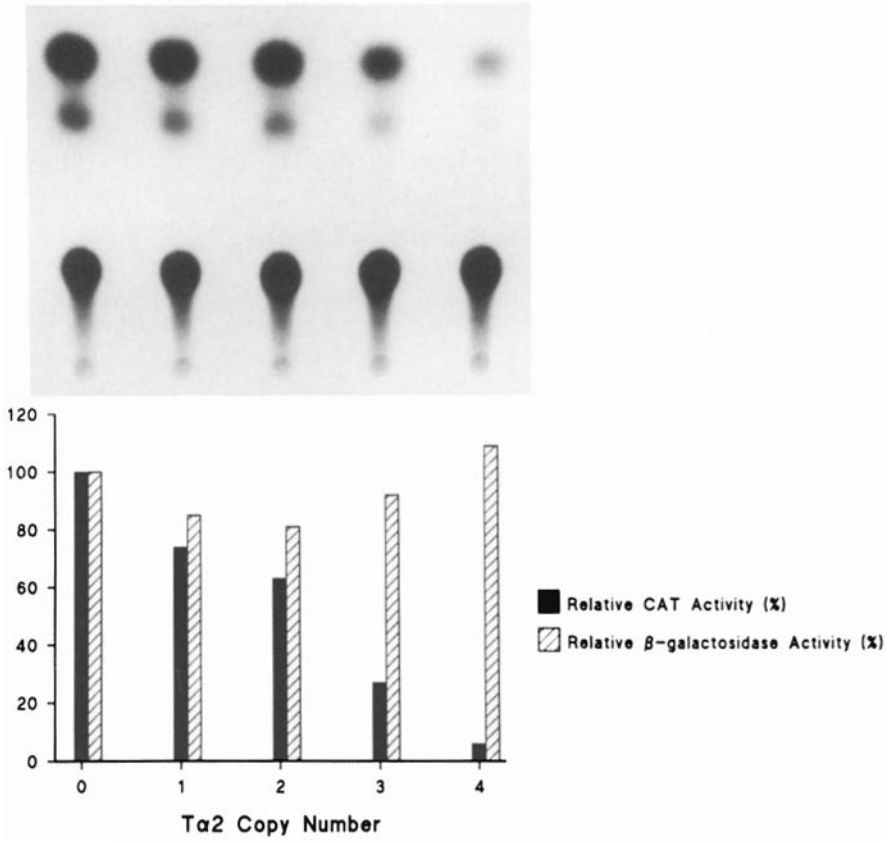

Figure 4. The effect of copy number on the transcriptional repressor activity of the Tα2 element. One to four copies of the synthetic Tα2 oligonucleotide were cloned into the BamHI site of pRSVCAT (see Fig. 2 *B*) 3′ of the RSV LTR/CAT cassette and 10 μg of the resulting plasmids, along with 2 μg of the pRSVβgal reference plasmid, were transfected into Jurkat T cells. CAT (*solid bars*) and β-galactosidase (*slashed bars*) activities were determined as described in Materials and Methods. The CAT activities were normalized to that produced by the pRSVCAT control plasmid that produced 27% total acetylation.

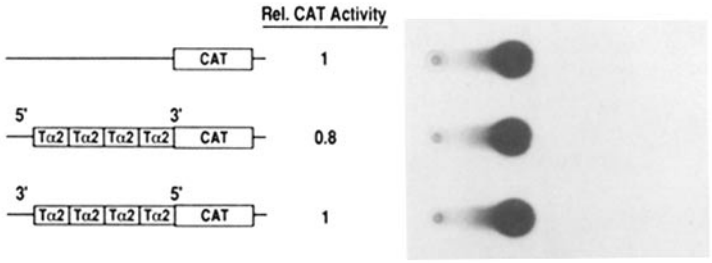

Figure 5. Lack of promoter activity of the Tα2 element. Four copies of the synthetic Tα2 oligonucleotide were cloned in both orientations into the HindIII site immediately 5′ of the CAT gene in the promoterless pCAT-Basic plasmid. 10 μg of the resulting plasmids along with 2 μg of the pRSVβgal reference plasmid were transfected into Jurkat T cells. The data are shown as CAT activities relative to that produced by the promoterless pCAT-Basic control plasmid (which produced 0.17% acetylation) following correction for differences in transfection efficiencies. The pSPœnhCAT plasmid was included as a positive control in a parallel transfection and produced 24% total acetylation (data not shown).

tions immediately 5′ of a promoterless CAT gene in the pCAT-Basic plasmid, and the resulting plasmids were transfected into Jurkat T cells (Fig.5). Four copies of the Tα2 binding site had no effect on CAT activity as compared with the promoterless pCAT-Basic control plasmid. Thus, the element does not display significant promoter activity in this system.

Discussion

The studies described in this report have demonstrated that a single nuclear protein binding site from the human TCR-α enhancer can function as both a T cell–specific transcriptional activator and repressor, depending upon the context in which this element is located. The finding that Tα2-mediated transcriptional repressor activity is T cell specific, when taken together with previous observations (8) that the Tα2 element binds at least one T cell–specific nuclear protein complex, suggests that nuclear protein binding may be responsible for the Tα2 transcriptional repressor activity. This hypothesis is supported by the finding that Tα2 mutations that abolished nuclear protein binding also abolished transcriptional repressor activity. The fact that the Tα2 element is able to repress transcription from multiple, apparently unrelated, enhancer elements suggests that repression may be mediated by direct effects of Tα2 and its cognate nuclear proteins on the ability of the transcription complex to form at the promoter, or to initiate transcription once formed. These effects apparently do not require either a functional TATA box, or an initiator sequence in that Tα2 significantly repressed transcription from the 4F2HC promoter that lacks these elements.

Previous studies in other systems have demonstrated that a single nuclear protein can either activate or repress transcription by binding to distinct *cis*-acting regulatory elements (16, 17). In addition, one previous report (18) has demonstrated that two different sequence motifs that are involved in silencing transcription of the yeast silent mating type loci can activate transcription when linked to heterologous promoters. To our knowledge, however, this is the first report of a single *cis*-acting element that can both activate and repress transcription in a tissue-specific fashion in a mammalian system. Our results raise two questions concerning the general phenomena of transcriptional repression and activation. First, what is the molecular mechanism that accounts for the bifunctional transcriptional regulatory potential of the Tα2 element in T cells? And second, what is the physiologic significance of these findings in terms of developmentally regulated gene expression?

One mechanism that could account for the positive and negative regulatory potential of the Tα2 nuclear protein binding site is that different sets of proteins are able to bind to this site, depending upon the context in which it is located. Thus, one or more transcriptional activator proteins might bind to Tα2 in the context of the wild-type TCR α enhancer, while a distinct set of transcriptional repressor proteins might bind preferentially to the Tα2 element alone. There is an equally tenable, and perhaps more interesting possibility that could account both for the ability of a single nuclear protein to activate and repress transcription when bound to different *cis*-acting sequence elements, and for the ability of a single *cis*-acting sequence element to mediate both transcriptional activation and repression. Specificially, some transcriptional regulatory proteins might be capable of binding to *cis*-acting regulatory sequences with two different conformations, one of which mediates transcriptional activation, and the other of which causes transcriptional repression. Interconversions between these two conformations could be caused either by the binding of such bifunctional regulatory proteins to different sequence motifs, or alternatively, by interactions of such bifunctional proteins with additional proteins bound to adjacent *cis*-acting elements. Thus, for example, interactions between proteins bound to the Tα2 and Tα1 sites of the TCR-α enhancer might result in a Tα2 conformation that activates transcription, while binding of the same proteins to Tα2 elements alone might result in a transcriptional repressor conformation. Our ability to distinguish between these different potential mechanisms requires a better understanding of the Tα1 and Tα2 binding proteins.

Finally, it is worth noting that, regardless of the molecular mechanisms that account for the dual transcriptional regulatory activities of the Tα2 element, our results suggest that a transcriptional enhancer that is composed of multiple nuclear protein binding sites can be converted to a transcriptional silencer by altering the levels of expression of the relevant transcriptional activator proteins. Thus, for example, the

activity of the human TCR-α enhancer could be changed from that of a transcriptional activator to that of a transcriptional repressor by reductions in the levels of expression or binding activities of the Tα1 binding proteins, resulting in an enhancer element that contains only a functional Tα2 binding site. Such changes might play an important role in regulating TCR-α gene expression in different T cell subsets during thymic ontogeny.

This work was supported in part by US Public Health Service grant AI-29673 (J. M. Leiden).

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Received for publication 6 April 1990 and in revised form 10 July 1990.

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