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The Role of Gata3 in the Regulation of T Cell Receptor Gene Expression

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By

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Abstract

Determining the molecular mechanisms by which T cell receptor (TCR) genes are regulated is a central problem in current immunology. The enhancers of all four TCR genes (α, β, γ, and δ) have been localized and well-characterized; accordingly, current research has focused on isolating individual enhancer-binding proteins, characterizing their interaction with DNA and other nuclear factors, and determining how these transcription factors cooperate to cause the many interesting phenomena of TCR expression observed in vivo. The research described in this thesis involves the isolation of a human Gata3 cDNA and the initial characterization of the interaction of its protein product with several TCR gene enhancers.

Other investigators have previously shown that Gata3 is a member of a multigene family of three zinc-finger DNA-binding proteins which recognize the consensus sequence WGATAR (W=T or A; R=G or A). Gata1 is specific to erythroid and myeloid cell lineages while Gata2 has a wide tissue distribution. Gata3, however, was found to be expressed most abundantly in the T cells of chickens, the species in which it was first isolated. Because WGATAR consensus sites are present in all of the TCR gene enhancers, we set out to isolate and characterize the human Gata3 cDNA.

Using oligonucleotides corresponding to a portion of the zinc finger domain of human Gata1, we succeeded in isolating a full-length human Gata3 cDNA from a HUT78 T-cell library. Nucleotide sequencing and analysis of the predicted amino acid sequence revealed striking homology between chicken and mouse Gata3 throughout the coding region. RNA blot analysis revealed that human Gata3 is abundantly expressed in $\alpha\beta$ and $\gamma\delta$ T-cell lines, but not in B, macrophage, or HeLa cell lines. Gel-shift

analysis using oligos corresponding to WGATAR sequences present in all four TCR enhancers showed that in-vitro-translated Gata3 binds to the TCR α, β, and δ enhancers, but not to the NF-γ1 site in the γ enhancer. Additional gel-shift studies using whole nuclear extracts showed Gata3-like binding activity predominantly in mature T and pre-T cell lines. Finally, the in-vitro activity of Gata3 was correlated with in-vivo activity of a WGATAR site in the TCR β enhancer using transient transfection assays. Taken together, these results indicate that Gata3 is a vital component of the transcriptional machinery which regulates TCR expression in both αβ and γδ T cells. Future studies will be required to determine how Gata3 cooperates with other enhancer- and promoter-binding proteins to regulate TCR transcription in a lineage-specific manner.

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INTRODUCTION AND BACKGROUND

The mammalian immune response has classically been divided into two components, the humoral response and the cell-mediated response. The humoral response is initiated by stimulation of B-cell immunoglobulin (Ig) receptors by soluble antigen, which results in clonal expansion of those B-cells stimulated. Some stimulated cells differentiate into plasma cells, which secrete antibody and do not express surface Ig, while others differentiate into memory cells, which maintain surface Ig. Secreted antibody then serves the host by a variety of means, including antibody-dependent cell-mediated cytotoxicity, activation of the complement pathway, agglutination of soluble antigen, and neutralization of surface molecules of infectious agents. The in-vivo function of T cells in the cell-mediated response is less well understood, but certainly includes killing of infected and transformed host cells (by T-cytotoxic cells) and secretion of interleukins (by T-helper cells) which then initiate and potentiate the activities of other effector cells (such as macrophages, natural killer cells, T-cytotoxic cells, and B cells).

The T-cell antigen receptor (TCR) is an $\alpha\beta$ or $\gamma\delta$ heterodimer which differs from Ig in a number of important respects (10, 11). First, it is expressed only on the cell surface and never as a secreted molecule. Second, in $\alpha\beta$ T-cells, it requires a co-receptor, either CD4 or CD8, in order to elicit a full response. Third, it is expressed on the cell surface only in association with the CD3 complex, which consists of γ, δ, and ε chains and either a ζ-ζ or a ζ-η dimer. Finally, the $\alpha\beta$ TCR recognizes antigen as a peptide in the context of self major histocompatability (MHC) molecules, a phenomenon known as MHC-restriction (10). CD8-bearing T-cells recognize peptide antigen in a groove formed by the $\alpha1$ and $\alpha2$ domains of class I MHC molecules while CD4-bearing T-cells are thought to recognize peptides in a similar groove formed by the

α1 and β1 domains of class II MHC heterodimers. The ligands for γδ TCR are less well understood, but may include Mycobacterial antigens and host heat-shock proteins (45). γδ T-cells, which form about 5% of peripheral blood T-cells but are more abundant in epithelial tissues, generally do not express CD4 or CD8 and generally do not show MHC restriction (1).

Much recent immunological research has focused on elucidating the mechanism by which T-cells become tolerant to self antigens and, in the case of αβ T-cells, become restricted to self-MHC (59). While some very recent evidence suggests that mature T-cells may become tolerant to self in secondary lymphoid organs and peripheral tissues, most research into this subject has focused on the development of immature T-cells in the thymus. According to the current paradigm, T-cell precursors migrate to the thymus from the bone marrow when they express neither TCR, CD4, nor CD8 (13, 14). Once in the thymus, they are induced to express either TCR αβ or TCR γδ; T-cell precursors expressing TCR αβ also express both CD4 and CD8. During this so-called double-positive stage, TCR αβ+ thymocytes undergo the poorly-understood processes of positive selection (selecting for T-cells with TCR's which recognize self-MHC) and then negative selection (selecting against T-cells with TCR's which recognize self antigens). The vast majority (>95%) of thymocytes do not survive the selection process and die by apoptosis. Those thymocytes which do survive then repress the expression of either CD4 or CD8 and emerge from the thymus as mature, single-positive T-cells.

Development of TCR γδ+ thymocytes is not well-understood, but appears substantially different from that of αβ T cells (reviewed in ref. 1). Thymocytes expressing the γδ TCR are expressed several days earlier than those expressing αβ TCR in murine fetal thymic ontogeny, and TCR γδ+ thymocytes do not express any known surface markers which vary during the course of intrathymic development. The

first γδ thymocytes appear in coordinated waves of expression in the murine fetal thymus (1). Those expressing Vγ3 appear first, followed sequentially by Vγ4, Vγ2, and Vγ5; these early γδ thymocytes display almost no junctional diversity and are thus termed "invariant" or "canonical" γδ TCR's (1). It is not known whether these invariant γδ thymocytes arise by programmed rearrangement or by intrathymic selection; evidence for each possibility exists (1, 24). In contrast, γδ thymocytes in the adult show a wide usage of V(D)J combinations and extensive junctional diversity. Additional experimental differences between αβ and γδ thymocytes have been noted. For example, treatment of thymic organ cultures with anti-CD3 or anti-TCR antibodies or cyclosporin A will block the appearance of mature αβ T cells, a phenomenon corresponding to negative selection; however, treatment with these reagents does not block the appearance of mature γδ T cells (1). A more complete understanding of γδ T cell development will probably depend on the discovery of developmental surface markers and further elucidation of the nature of the γδ TCR ligand(s).

Like the Ig heavy and light chains, all four TCR chains consist of N-terminal variable domains, which participate in antigen and MHC recognition, and C-terminal constant domains. The variable domains are encoded by a number of variable (V), diversity (D), and/or joining (J) segments which undergo somatic rearrangement early in thymic development (10). The putative recombinase machinery which mediates somatic rearrangement is believed to have multiple activities (11). First, it must recognize a highly conserved heptamer-nonamer recombination signal sequence (RSS) which flanks V, D, and J regions. It must have endonuclease activity to cleave the DNA specifically at that site. In some TCR loci, the recombinase has exonuclease and terminal deoxynucleotidyl transferase activities which mediate deletion and addition of nucleotides from the cleaved ends. A DNA polymerase activity would be required to fill in the opposite strand when random nucleotides are added. Finally, the

recombinase would be required to ligate the cleaved end together. TCR diversity, then, is accomplished by utilizing various V(D)J combinations as well as through variation in the sites of V-D, V-J, and D-J joining; in all, a potential repertoire of 10^{15} - 10^{18} theoretically possible TCR's has been proposed (10). The process of T-cell rearrangement and expression is crucial in determining the ultimate fate of a given T-cell, because many V(D)J recombinations will not survive positive and negative selection and because thymocytes which do not express a functional TCR will also die in the thymus.

In addition to the relatively unique process of somatic recombination, TCR genes are also regulated at the transcriptional level by cis-acting DNA elements, namely promoters and enhancers. As in other genes, promoter elements are believed to increase transcription by binding nuclear proteins which stabilize the interaction of RNA polymerase II with the transcriptional start site on the DNA (35). Enhancers, which increase transcription in a distance- and orientation-independent fashion, act through an unknown mechanism but are clearly required for the full expression of many genes. A third type of cis-acting regulatory DNA is the locus control region (LCR), which has been definitively identified in only two gene loci: β-globin and CD2 (18, 17). These regions are defined by two criteria: they are hypersensitive to DNase I and they confer position-independent high-level expression of transgenes in transgenic mice. While an LCR has not been positively identified in any TCR gene locus, the large size and complexity of these genes suggest the need for regulation beyond simple promoter-enhancer interaction. Furthermore, at least one group has found several DNase I hypersensitive sites 3' of the Cα gene segment in the TCR α-δ locus and transgenic studies of these sites are currently in progress (Diaz and Winoto, unpublished results).

Although aspects of TCR transcriptional regulation are likely to be shared with many other gene loci, a thorough understanding of these cis-acting DNA sequences is likely to yield important insights into immunologic phenomena. For example, one central question in T-cell development is the relationship of transcriptional regulation to TCR rearrangement. Several groups have shown that low-level germline transcription precedes rearrangement and class-switching in the Ig heavy-chain locus and a similar phenomenon may take place in T-cells (76, 77, 32). One explanatory hypothesis is that the transcriptional regulatory elements of these loci are first activated, thus making the locus accessible to the recombinase machinery (76). Alternatively, the locus might first be opened by an independent mechanism which then makes the region accessible to both transcriptional and recombinatorial elements. Finally, the observation of germline transcripts may have no physiologic significance and the recombinatorial event may indeed precede and/or potentiate transcriptional activation.

A second interesting transcriptional phenomenon is that during thymic ontogeny, CD4+8+ thymocytes express relatively low levels of TCR (about 2,000 heterodimers per cell), whereas in mature, single-positive T-cells, TCR expression is markedly upregulated (to about 40,000 per cell, ref. 11). This finding suggests that some TCR transcriptional elements are inducible; it is intriguing to speculate that this induction might be tied to cell-surface events involving positive and negative selection, which is believed to occur at this same stage. The notion of TCR inducibility is further supported by work on the T-cell line CEM (53, 70). These cells have functionally rearranged α and β TCR loci but transcribe only the β chain mRNA. When these cells are stimulated with the phorbol ester TPA, however, they subsequently express both α and β chain mRNA and also express the $\alpha\beta$ TCR heterodimer on the cell surface. Other work has shown that TCR expression is transiently down-regulated in mature T-

cells treated with anti-CD3 antibody and phorbol myristate acetate (PMA) and that this phenomenon is mediated in part by a change in TCR transcriptional levels (39).

Understanding TCR transcriptional regulation may also yield insight into the question of αβ vs. γδ T-cell lineage divergence (48). Because of its unusual location within the TCR α locus, the TCR δ locus is deleted in the course of any functional α chain rearrangement. This fact raises the possibility that αβ T-cells arise from γδ T-cells. Supporting evidence includes the delayed rearrangement and expression of TCR α in thymic ontogeny, the fact that γδ T-cells and αβ T-cell precursors are both CD4-8-, and the observation that some αβ T-cells have non-productively rearranged TCR γ chains. Studies of the circular DNA deletion products of TCR gene rearrangement give conflicting results. Okazaki and Sakano (36, 37) made a circle library from 3-week old Balb/c thymuses and found only 5 Jδ1+ clones versus more than 200 Jα1+ clones; furthermore, several Jδ+ and Jα+ clones were analyzed and found to contain additional recombination structures, consistent with a progressive rearrangement model of the α-δ locus. These results, however, could also be explained by a progressive recombination within a given α or δ locus, with no δ to α progression; alternatively, a recombination event could take place within the deletion circle itself. Takeshita et. al. (61) obtained similar results, although their methods were flawed by use of a splenocyte circle library of unclear physiologic significance and a λ phage vector which excluded inserts larger than 7 kilobases (kb) and thus biased the library toward non-germline DNA fragments. Winoto and Baltimore (70), however, found in two independent thymocyte circle libraries that δ+ clones were at least as abundant as Jα+ clones and that the vast majority of δ+ clones were in germline configuration, consistent with a model separating α-δ locus rearrangement from lineage commitment. If this model is correct, then an understanding of the differential regulation of TCR α and TCR δ gene loci may give insight into how αβ / γδ lineage commitment occurs.

Transcriptional regulation may explain how certain TCR loci are expressed at different times in the murine fetal thymus. TCR β chain, for example, is rearranged and expressed about two days before TCR α chain, yet it seems likely that the same basic recombinase machinery mediates the rearrangement of each (11). In addition, the finding of waves of rearrangement and expression of particular Vγ gene segments in the fetal thymus suggests that Vγ promoter elements are involved in this differential activity of the recombinase (1). Furthermore, after V(D)J recombination occurs at any TCR locus, some mechanism must exist to repress transcription from upstream V-segment promoters. Understanding transcriptional regulation may also explain the phenomenon of allelic exclusion; that is, why only one clonotypic TCR is expressed per T cell despite the presence of two alleles.

Finally, it is likely that TCR transcriptional elements are involved in several important human diseases. Numerous studies have found leukemias in which oncogenes, such as c-myc, are translocated to the TCR α locus where they may be placed under TCR α enhancer control (71). In addition, the regulatory elements in the long terminal repeats (LTR's) of several human T-lymphotropic retroviruses (HIV-1 and -2 and HTLV-1 and -2) would be expected to parasitize T-cell specific transcription factors. In the case of HIV-1, for example, Waterman and Jones have clearly demonstrated that the transcription factor TCF-1α binds to both the TCR α enhancer and the HIV-1 LTR (66, 67). Although the evidence is less conclusive, the proto-oncogene c-ets-1 may regulate both the TCR α enhancer and the HTLV-1 LTR (6, 22). In summary, then, a thorough understanding of the cis-acting DNA elements which regulate TCR α, β, γ, and δ-chain transcription and of the nuclear proteins which bind to them is likely to lead to a better understanding of TCR rearrangement in particular and T-cell development in general.

The enhancers of all four TCR genes have recently been identified and are depicted in Figures 1 and 2 in the context of their respective loci. The murine TCR α enhancer was first localized by Winoto and Baltimore (71) to a region 3 kb 3' of the Cα constant region. Cloning progressively smaller fragments of a cosmid clone spanning the region into a chloramphenicol acetyl transferase (CAT) vector, they localized enhancer activity to a 237 bp segment of DNA. The enhancer was found to be T-cell specific, being active in CAT assays using both αβ and γδ T cell lines, but not in B cells or fibroblasts (73). In addition, they found that the pre-T cell line CEM showed little α enhancer activity, but that activity was induced 10- to 15-fold with PMA stimulation. This finding correlates well with previous work showing that CEM has a functionally rearranged TCR α chain, but normally does not express TCR α mRNA; α chain mRNA is espressed, however, following PMA stimulation (53). This correlation may explain modulation of TCR αβ expression in response to cell-surface stimuli observed in vivo both in developing thymocytes and in mature T cells (11, 39).

Winoto and Baltimore went on to characterize the α enhancer in detail with nucleotide sequencing and DNase I footprinting (71, 73). They found five footprinted regions within the 237 bp minimal enhancer (NFα1-5). NFα1 could be deleted without a large drop in enhancer activity, while NFα2-5 were found to be essential. Footprinting showed that NFα1 and 2 were ubiquitous while NFα3 and 4 appeared to be T-cell specific. Finally, several regions of strong sequence homology were found with HTLV-1 and -2 and with HIV-2.

Later work by this group established the existence of silencer elements in the region between the α enhancer and Cα (ref. 71 and Fig. 1). This finding began with the observation that the minimal 237 bp enhancer was fully active in both αβ and γδ T cell lines while a 7 kb fragment containing extensive flanking sequences was active only in αβ T cells. By adding subfragments of this 7 kb region back to the minimal

enhancer, they narrowed silencing activity to two 0.3 kb segments, one just 3' of Cα and the other just 5' of the α enhancer. These segments, called Sil I amd Sil II, were found to silence the activity of either the α enhancer or a heterologous enhancer in γδ T cells, and not in αβ T cells. The silencers were active both alone and in tandem and functioned in a distance- and orientation-independent manner. Silencer activity was also found in a number of non-T cells, including B-cell, macrophage, and HeLa cell lines. These results suggest that progression along the αβ T-cell developmental pathway involves specific relief in αβ T-cell precursors from silencer activity, perhaps mediated by DNA-binding proteins. The work also reinforces the idea that αβ and γδ T cells arise from separate lineages.

Using similar techniques, Ho et. al. isolated a 700 bp enhancer located 4.5 kb 3' of the human Cα locus (20). Interestingly, their enhancer showed activity in Jurkat αβ T cells, but not in Peer γδ T cells or any other cell line tested. DNaseI footprinting revealed five protected sites, Tα1- Tα5. Tα1 showed strong homology to NFα2 and to a consensus cAMP-responsive-element (CRE) binding site; the Tα2 footprint showed homology to NFα354. Similar to the murine α enhancer, initial deletion studies showed that Tα1 and Tα2 alone were sufficient for full enhancer activity, while Tα3, Tα4, and Tα5 could be deleted with little loss of enhancer function. More detailed mutagenesis experiements, however, revealed that actual enhancer function is more complicated (21). Ho and Leiden showed first that both footprints were required for Tα1-Tα2 minimal enhancer activity and that the length of the spacer between them, but not its nucleotide sequence, was critical for its function. They also showed, however, that the Tα3 and Tα4 elements could at least partially compensate for mutations in either Tα1 or Tα2; constructs containing wild-type Tα2-Tα3-Tα4, Tα2-Tα3, Tα2-Tα4, and Tα1-Tα3-Tα4 retained at least 40% of full enhancer activity while Tα1-Tα4 and Tα1-Tα3 constructs were inactive. Gel-shift studies using oligonucleotides corresponding

to individual DNaseI footprints revealed multiple protein-binding activites in Tα1 and Tα2 while Tα3 and Tα4 showed single specific shifted bands. Interestingly, only the Tα2a band was shown to be T-cell specific and no band was specific to either the αβ or γδ T-cell lineage; this finding apparently conflicts with their observation that the 700 bp human α enhancer was active only in αβ T cells. The authors concluded that lineage specificity was mediated by additional undiscovered binding sites, a silencer element, and/or differential methylation of enhancer elements.

Other groups using similar methods have succeeded in isolating and characterizing the TCR β, γ, and δ enhancers. Working with a human genomic clone, Gottschalk and Leiden localized a TCRβ enhancer to 480 bp of DNA, 5.5 kb 3' of the Cβ2 locus (ref. 16 and fig. 2). This enhancer directed high-level transcription from both a TCRβ promoter and an SV40 promoter in both αβ and γδ T cell lines; furthermore, moderate enhancer activity was present in Clone13 B cells and K562 erythroleukemia cells, consistent with previous reports showing activity of the murine TCRβ enhancer in both B and T cell lines (28). No TCRβ enhancer activity was present in HeLa cells. DNaseI footprinting revealed five protected regions, Tβ1-Tβ5 (fig. 2); mutation studies established that Tβ1 and Tβ5 were not required for full activity in Jurkat T cells. Gel-shift assays using Tβ2, Tβ3, and Tβ4 oligonucleotides showed two protein/DNA bands, named A and B, for each oligo tested. No band was completely T-cell specific, but Tβ2B was the only one not present using JY B cell nuclear extracts; it was restricted to αβ and γδ T cells and K562 erythroleukemia cells. All non-T cells did lack at least one of the six binding activities, indicating that T-cell specific expression of TCRβ is mediated by the interaction of multiple ubiquitous and lineage-restricted transcription factors. Furthermore, they showed with gel-shift and cross-competition experiments that Tβ2 and Tα1 both bind at least one common nuclear

protein, as do Tβ5 and Tα2, suggesting that TCRα and TCRβ are coordinately regulated through the action of common enhancer-binding proteins.

TCR γ and δ enhancer elements have been only recently identified and are not as well-characterized as those for TCR α and β. Spencer et. al. localized a TCRγ enhancer to 1000 bp of DNA located 3kb 3' of Cγ1 in the mouse (55 and fig. 2). This enhancer was initially detectable only in Peer γδ T cells, but when multimerized was active in several αβ and γδ T cell lines and not in several B or nonlymphoid cell lines. DNaseI footprinting detected six protected regions, NFγ1-NFγ6 (fig. 2). A deletion mutant containing only NFγ2-NFγ4, however, retained full enhancer activity; furthermore, a tetramer of the NFγ3 site was as active as a tetramer of the NFγ2-NFγ4 region, suggesting a crucial role for this site. Transgenic studies by another group have indicated that a silencer element located 3' to Cγ1 may play an important role in restricting TCRγ expression to γδ T cells, but this silencer has not yet been positively identified (4, 23).

The human TCRδ enhancer is the only one yet identified within an intron. Redondo et. al. have localized it to a 370 bp region between Jδ3 and Cδ (46 and fig. 1). It is strongly active, however, in only one αβ and one γδ T cell line; no activity was seen in B or HeLa cells, and only weak activity was present in several other T cell lines tested. DNaseI footprinting revealed seven protected sites, δE1-δE7. One of these footprints, δE4 contains a 7 bp internal repeat. Several other internal homolgies, as well as homologies to TCRα and E-box motifs, are noted by the authors, but no gel-shift or cross-competition studies have yet been done to assess their significance.

Now that a number of putative binding sites for TCR enhancer-binding proteins (EBPs) have been identified, a number of workers have focused on isolating the cDNA's encoding these proteins. Once isolated, the cDNA can be sequenced and the predicted amino acid can be determined and analyzed. Homology with other known transcription factors can give insight into their method of action. The cDNA can also be

used to assess tissue distribution by Northern blot analysis and in-situ hybridization. Timing of expression can also be analyzed and used to infer in-vivo function. Mutations of the cDNA allow investigators to determine the various domains of the protein and their relative importance in DNA binding and protein-protein interaction. The action of the protein in vivo can be assessed by transient transfection assays. Finally, the cDNA can be used to isolate genomic clones which allow assessment of the transcription factor's own cis-acting DNA elements (i.e., promoters, enhancers, and silencers). Ultimately, the goal is to dissect at the molecular level the sequence of events that leads to TCR rearrangement and expression in thymoctyes.

A number of methods have been used to isolate enhancer-binding protein cDNA's. If the EBP is suspected to be homologous, in part, to a cDNA which has been isolated, then a probe can be made from the homologous region (by PCR or oligonucleotide synthesis) and used to screen a cDNA library under low-stringency conditions (75). Alternatively, the cDNA can be isolated on the basis of the function of its respective protein. The λgt11 phage, for example, can be induced to express its cDNA insert as a fusion protein which can then be screened on the basis of its ability to bind to an oligonucleotide containing the EBP binding site (51). Success using this method requires that the EBP binds to DNA as a monomer or homodimer, that the protein does not require eukaryotic post-translational modification for activity, that the protein folds properly in E. coli, and that it can survive the screening process. An alternative method of expression cloning involves screening cDNA clones expressed in mammalian cells based on their ability to gel-shift a radiolabeled oligonucleotide binding site similar to the native protein (47, 62a).

Tsai and coworkers first employed this latter method to isolate the cDNA encoding the mouse Gata1 (previously called Eryf1, NF-E1, or GF-1), an erythroid-specific transcription factor which binds to the consensus sequence WGATAR (W=A

or T, R=A or G) present in several globin enhancers and promoters (62a). They made a mouse erythroleukemia (MEL) cDNA expression library which they transfected in pools into COS cells. Minilysates of transfected cells were prepared and assayed for gel-shift activity. If a pool showed activity matching that of MEL cell extract activity, that pool was subjected to sib selection and rescreening until single clones were isolated. They found that murine Gata1 is a 413-amino acid polypeptide which contains two similar C_X class zinc fingers, a motif found in other proteins known to bind DNA (35, 25). The zinc finger motif in Gata1 is unusual in that it is repeated within the protein and in that there is a 17 amino acid spacing between the cysteine pairs, as opposed to 12-14 amino acids reported for most other zinc fingers.

Using a PCR fragment corresponding to the mouse Gata1 zinc finger domain, Yamamoto and coworkers isolated three distinct cDNA's from a chicken erythroid cell library (75). The three clones (now called Gata1, Gata2, and Gata3) showed 90% amino acid sequence homology within the finger domain, but differed markedly outside of it. All three clones were shown to bind to the WGATAR consensus sequence with high affinity and to strongly transactivate multimers of the sequence in cotransfection assays, yet Northern blot analysis revealed a distinct pattern of tissue distribution for each. Gata1 is expressed exclusively in cells of the erythroid, mast cell, and megakaryocyte lineages (33). Gata2 is found not only in erythroid cells but also in embryonic brain, liver, and cardiac muscle and in adult kidney, fibroblasts, and epithelial cells; it is absent in T-cells (69). Gata3 is also present in small amount in mature erythroid cells, but is abundantly expressed in a T-cell line and somewhat less in embryonic brain. The finding of Gata3 message in chicken T-cells together with the observation of the WGATAR consensus sequence within DNase I footprint sites in all four TCR enhancers (see figures 1 and 2) encouraged us to isolate the human Gata3 cDNA and to begin to characterize its activity in T cells.

MATERIALS AND METHODS

Oligonucleotides and cDNA Library Screening

In order to isolate the human Gata3 cDNA, the following oligonucleotides were synthesized at the UC Berkeley Cancer Research Lab :

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huGF1.5  5'ACAGGACAGGCCACTACCTATGCAACGC                  3'
huGF1.6  3'              TGATGGATACGTTGCGGACGCCGGAGAT    5'
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The two oligos were annealed by cooling from 65^0C to 37^0C to 20^0C to 4^0C for 15 minutes each. The resulting oligo corresponds to a forty base pair (bp) segment in the zinc-finger region of the human Gata1 cDNA which is highly conserved between the chicken Gata1 and Gata3 cDNA's (39 out of 40 bp identical). Assuming that the human Gata1 and Gata3 would have comparable identity at this segment, we used the annealed huGF1.5/1.6 oligo to screen a λgt11-HUT78 (human T-cell line) cDNA library. This library was chosen because, in the chicken, only Gata3 and not Gata1 or Gata2 mRNA is found in T-cells; hence, any clone from the HUT78 library which hybridized to the huGF1.5/1.6 probe would likely encode human Gata3. The probe was radiolabeled by filling in with Klenow fragment of E. coli DNA polymerase I and ^{32}P dCTP and then by end-labeling with T4 polynucleotide kinase and γ ^{32}P ATP. Approximately 5 X 10^5 plaque-forming units of the HUT78 cDNA library were plated out, lifted onto nylon filters (Colony/Plaque Screen, DuPont), and screened with the probe under low-stringency conditions. Hybridization was done at 42^0C using a hybridization mixture containing 6X SSC, 5X Denhardt's, 20% formamide, and 100 μg/mL salmon sperm DNA. The filters were washed with 6X SSC / 0.1% SDS at 42^0 C and exposed overnight using Kodak X-OMAT AR film. Three positive clones (R1, R7, and R11) were identified and plaque-purified for further study.

Analysis of cDNA inserts

Inserts of the R1, R7, and R11 clones were subcloned into the pSP72 vector (Promega) and named pR1, pR7, pR11. The pSP72 vector contains transcriptional start sites for the SP6 and T7 polymerases at opposite ends of the insert cloning site. The pR1, pR7, and pR11 clones were restriction-enzyme-mapped and the coding region was sequenced using the Sanger dideoxy chain termination technique using Klenow. pR1 was found to contain a full length cDNA with an AUG translational start codon and was oriented in pSP72 such that transcription using the SP6 polymerase gives the sense RNA whereas transcription using the T7 polymerase gives the antisense RNA. Transcription of pR1 was carried out in the presence of GpppG using 2.5 μg of template. A tenth volume of the RNA product was translated using a rabbit reticulocyte lysate (Promega) following directions given by the maufacturer. One reaction was conducted in the presence of ^{35}S methionine and a sample of the product was sized on a 15% SDS-PAGE gel using a rainbow-color protein marker (Amersham). The gel was dried and subjected to autoradiography. Subsequent translation reactions for use in gel-shift analysis were performed using cold L-methionine; two percent (one μL) of the reaction product was used in each gel-shift reaction.

Gel-shift analysis

To assess the binding activity of the in-vitro translated cDNA product and to compare it to that of whole nuclear extracts, a number of oligonucleotides corresponding to T-cell receptor enhancer regions containing a putative Gata3 binding site (WGATAR) were synthesized by the UC Berkeley Cancer Research Lab; putative binding sites and mutated sites are underlined (see figures 1 and 2):

βT -A 5'TCGAGTGTTTATCTCTGAGTCACATCAG 3'
-B 3' CACAAATAGAGACTCAGTGTAGTCAGCT 5'

βT mut-A 5'TCGAGTGTTGCGATCTGAGTCACATCAG 3'

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      -B 3'    CACAACGCTAGACTCAGTGTAGTCAGCT      5'

γT -A    5'TCGAGGACAGAGAGATAGAAAGGCTTTG          3'
   -B    3'    CCTGTCTCTCTATCTTTCCGAAACAGCT      5'

δT -A    5'TCGACTAGAGTTATCACTTTCTGTTATCAAGTGGCTTCA      3'
   -B    3'    GATCTCAATAGTGAAAGACAATAGTTCACCGAAGTAGCT 5'

NFα4/5-A 5'TCGACTCTCCCGCAGAAGCCACATCCTCTGGAAAG         3'
      -B 3'    GAGAGGGCGTCTTCGGTGTAGGAGACCTTTCAGCT     5'
```

For use in gel-shift experiments, the A and B oligonucleotides (oligos) of each set were annealed at 65^0C, 37^0C, 20^0C and 4^0C for 15 minutes each. The annealed βT oligonucleotide corresponds to the Tβ2 site in the human TCR β enhancer (16), whereas γT and δT correspond to the NFγ1 site (55) and δE4 site (46) in the mouse TCR γ and human TCR δ enhancers, respectively. βT, γT, and δT oligonucleotides all contain putative Gata3 binding sites (there are two putative Gata3 binding sites in the δT oligo). βT mut is the same as βT with a mutation at the GATA nucleotides. NFα4/5 corresponds to the NFα4/5 site in the mouse TCR α enhancer (73) and contains no WGATAR site; it was used as a non-specific competitor in gel-shift experiments.

In addition to the above oligonucleotides, two restriction fragments containing elements of the TCR γ and TCR α enhancers were also used in gel-shift experiments. J21BgD50 (a gift from Yun-Hui Hsiang and Dr. Dave Raulet) is a plasmid containing a 50 bp Dde I fragment from the mouse TCRγ enhancer which corresponds to the NFγ1 site in the γT oligo above (55). This fragment was cut from a Bgl II linker site and purified on low-melt agarose. A 510 bp Bgl II fragment containing the entire mouse TCR α enhancer (71) (kindly provided by Chris Kingsley) was isolated by similar methods.

Gel-shift analysis was performed essentially as described (51, 52). The βT oligo (corresponding to the Tβ2 site) was cloned into the J21 vector at a Sal I site and a 48 bp Sal I/ HindIII fragment containing the βT oligo was end-labeled with ^{32}P dATP

and Klenow for use as a probe. Each experiment used 2 X 10^4 counts per minute (cpm) probe and 4 μg poly (dIdC). The binding buffer contained 10 mM Hepes-KOH pH 7.9, 50 mM NaCl, 5mM Tris-HCl pH 7.5, 15 mM EDTA, 1 mM DTT, and 10% glycerol. Four to eight μg of nuclear extract (or 1μL of the in-vitro translated cDNA product) were added and then incubated for 20-30 minutes. For competition experiments, anywhere from 10- to 200-fold molar excess of the oligonucleotides and restriction fragments described above were included in the reaction. The reaction mixture was then run on a 4% polyacrilamide gel in a Tris-glycine buffer (25mM Tris-HCl pH 8.5, 1mM EDTA, 190 mM glycine), dried, and exposed overnight.

Methylation interference was performed using the same Sal I/ HindIII fragment containing the βT oligo. The fragment was end-labeled with either ^{32}P dATP (sense strand) or ^{32}P dCTP (anti-sense strand) and methylated at A and G residues with dimethyl sulfate according to the Maxam-Gilbert sequencing reaction. Approximately 1 X 10^6 cpm of methylated DNA was then used in a gel-shift experiment with 25 μL of in-vitro translated pR1 cDNA product. The free and bound DNA's were cut from the gel and eluted on to NA45 membrane. Following purification, the DNA's were cleaved with piperidine, lyophilized with water twice, and run on a 8% polyacrilamide / 8M urea sequencing gel. The wet gel was then exposed overnight.

Plasmids and Gene Transfection

In order to correlate the in vitro binding activity of the pR1 cDNA product with the in vivo activity of one of its TCR binding sites, a number of transient transfection experiments were performed. Multimers (1-, 2-, 3-, and 6-mers) of the βT oligonucleotide (which contains the WGATAR site) were cloned into the Sal I site of the J21 vector, which contains a minimal -71 to +109 c-fos promoter driving the CAT (chloramphenicol acetyl transferase) gene. Transfections of J21-derived plasmids into Jurkat (human αβ T cell), Molt 13 (human γδ T cell), or U937 (human macrophage)

cell lines were performed as described using Deae-dextran with an equal molar amount of plasmid DNA (71). Cells were incubated for 40-48 hours following transfection and then harvested. Protein extracts of transfected cells were made by rapid freeze-thawing and heat-treatment at 60^0C for seven minutes. 100-250 μg of protein extracts (depending on the cell line used) were assayed for CAT activity by incubation with ^{14}C-chloramphenicol for 1-2 hours and analysis on a thin-layer chromatography (TLC) plate. At least three independent transfection experiments were performed for each construct and cell line.

RESULTS AND DISCUSSION

Isolation and characterization of the human Gata3 clone.

Initial attempts in isolating the human Gata3 cDNA focused on amplifying a fragment from the zinc-finger domain of the human Gata1 gene using polymerase chain reaction (PCR) with human genomic DNA as a template. Numerous attempts to generate a probe by this method failed, perhaps because the double zinc-finger domain in the gene may be divided into two exons (19). After observing extremely high homolgy between the chicken Gata1 and Gata3 cDNA's in a forty base-pair segment within the zinc-finger domain, we used the oligonucleotides huGF1.5/1.6 to screen a HUT78 human T-cell cDNA library under low-stringency conditions. Three cDNA clones, R1, R7, and R11, were isolated and were found to have insert sizes of 2.6 kb, 1.5 kb, and 1.3 kb, respectively. Restriction enzyme mapping analysis established that all three clones encode the same gene; the R7 and R11 clones are thus contained within the larger R1 clone (Fig. 3A). The R1 clone nucleotide sequence was then determined using the dideoxy chain-termination method.

The full coding region contains sequences homologous to the huGF1.5/1.6 probe (65% homology) as well as to the remainder of the human Gata1 Zn-finger

domain (Fig.3B). The largest open reading frame predicts a protein of 444 amino acids, identical in size to the chicken Gata3 protein. Comparison of the predicted human Gata3 (R1 cDNA) amino acid sequence with that of the chicken Gata3 reveals 90% conservation between the two species throughout the entire protein (Fig.4). In contrast, strong homology between human Gata1 and chicken Gata1 proteins is evident only within the Zn-finger domain (75, 79). The striking degree of amino acid and nucleotide sequence conservation between species which diverged several hundred million years ago argues strongly that Gata3 performs a basic and vital function in cells which express it. Furthermore, the strong homology in areas outside the zinc-finger domain suggests that these areas participate as domains of critical importance for the function of the protein. Because the zinc-finger domain in all other proteins studied is a DNA-binding domain, the areas outside of it in Gata3 may participate in protein-protein interactions.

The predicted Gata3 amino acid sequence was analyzed by the MacVector 3.5 sequence analysis software kit (IBI/ Kodak) The predicted 48kD protein is highly hydrophilic when analyzed on the Kyte-Doolittle scale, and it contains 64% polar or charged amino acid residues. The amino acid sequence is remarkable for a high content of proline (51 of 444 residues). Serine and threonine together make up 21% of the protein. There are five cysteine residues in addition to the four pairs found in the zinc finger domain; four of these five are conserved between chicken and human, suggesting that they might participate in forming the tertiary structure of the protein. There is little extended secondary structure (α helices or β-sheets) predicted, consistent with the high proline content scattered throughout the protein.

To assess the tissue distribution of the Gata3 message, a Northern blot analysis was performed. A Gata3-specific probe (0.8 kb HincII fragment of the R11 insert) was used in order to avoid potential cross-hybridization with other Gata gene

messages. The Northern blot contained 1X polyA$^+$ RNA isolated from several T (Jurkat, Molt13, Peer), B (Bjab, Namalwa), macrophage (U937), and HeLa cell lines. As a control, we used a cytochrome-c oxidase probe in a Northern blot analysis with the same RNA samples (Fig.5). Interestingly, the Gata3 probe hybridized to a 3.6 kb message (and to no other message) which was found only in αβ (Jurkat) and γδ (Peer, Molt13) T cells and not in the other cell lines tested (Fig.5 and data not shown). The apparent T-cell specificity of the Gata3 message supported the possibility that it might regulate transcription of one or more T-cell specific genes.

The Gata3 gene product binds to the TCR β and δ enhancer elements.

To test the possibility that the Gata3 gene product regulates T-cell specific genes, we have analyzed several of the T-cell receptor (TCR) enhancer elements containing WGATAR sequences for binding to the Gata3 in-vitro translated protein (see Fig. 1 and 2). The putative Gata3 recognition site is present in the enhancers of all four TCR genes, namely the TCR α (Tα3 in ref.20 = nucleotides 227-232 in ref. 71), TCR β (Tβ2 in ref.34 = βE1 in ref.16), TCR γ (NFγ1 site in ref.55), and TCR δ (δE4 in ref. 46). The putative Gata3 enhancer elements in the TCR α, β, and δ have previously been shown to be important for their respective enhancer function (21, 16, 46), whereas the putative Gata3 binding site in the TCR γ enhancer can be deleted without severely affecting the corresponding enhancer activity in transient transfection experiments using T cell lines (71, 55).

The full-length pR1 clone was transcribed in vitro and the resulting RNA's were translated using a rabbit reticulocyte lysate in the presence of ^{35}S methionine. The labeled protein product was analyzed on a SDS-PAGE gel as shown in figure 6. Only the sense RNA (transcribed using SP6 polymerase) gave a dominant protein band; it lies between the 46kD and 69kD markers, consistent with the size of the predicted Gata3 protein (48kD). For binding experiments, the in-vitro translation reaction was

performed again without ^{35}S methionine and the reaction product was used in a gel-shift analysis with the βT oligonucleotide as a probe (=Tβ2 of the TCR β enhancer). A dominant band was detected with the translated Gata3 protein that binds specifically to the βT oligonucleotide (Fig.7). The band could be competed away with an increasing molar excess of cold βT (10, 25, and 50 fold); interestingly, the δT oligo (=δE4 of the human TCR δ enhancer) competed just as well as the βT oligo. No competition was observed with a non-specific competitor nor with a mutant βT oligo which was altered only in the GATA sequence (Fig.7 and 8). Competition with the α enhancer fragment was less dramatic, but still indicative of specific competition (Fig. 7). Surprisingly, the γT oligo, which also contains a WGATAR site, did not compete with the βT Gata3 binding site, indicating that other specific DNA sequences may be required for the Gata3 protein to bind (Fig.7). Finally, a methylation interference experiment indicated that the in vitro Gata3 protein indeed binds to the GA nucleotides of the GATA site in the βT oligo (data not shown). These data indicate strongly that Gata3 protein binds to both the TCR β and δ enhancers and probably to the α enhancer as well.

To further strengthen the binding studies, we have also examined the βT protein- binding activity using total nuclear extracts. The βT DNA bound predominantly to a protein in nuclear extracts from EL4, YAC-1, and RLM11 mouse mature T-cell lines as well as from RS4.2 pre-T cells (Fig.8). The DNA/protein band detected runs at a position similar to the in-vitro translated human Gata3 protein, suggesting that they represent the same protein. Furthermore, the complex could be competed away with the wild type but not with the mutant βT oligo (Fig. 9). No binding activity was observed in WEHI 231 B-cell or 22D6 and 38B9 preB-cell extracts (Fig.8). A faint band was detected using S194 B-cell extract which runs at a different position than the in vitro Gata3 protein. As shown in figure 9, the S194 band

is competed equally well by the native and mutant βT oligos, suggesting a non-specific protein-DNA interaction. These data further indicate that the Gata3 gene product which binds to the βT oligo is T-cell specific, although its presence in some B cell lines can not be entirely ruled out. The B-cell specific Oct2 enhancer-binding protein, for example, is present in one T-cell line (56). Alternatively, the activity detected in the S194 extract could be due to the activity of the Gata2 protein, which is capable of binding to the same consensus sequence as the Gata3 protein. In chicken, the Gata2 message has been shown to be present in fibroblasts, liver, and kidney, but not in T cells (75). It is not clear if Gata2 mRNA is also present in some B-cell lines. The molecular weight of the predicted chicken Gata2 protein is very similar to that of the chicken Gata3 protein and it runs at a position similar to the Gata3 protein in a gel-shift analysis (18).

The same gel-shift experiments were performed using nuclear extracts from Jurkat, Molt13, and Peer human T-cell lines; a protein in these extracts bound to the βT oligo at a position similar to that using the in-vitro Gata3 product (arrow in Fig. 10). The protein/DNA band could be competed with excess of cold βT oligo but not with an irrelevant NFα4/5 oligo which contains no WGATAR sequence. Although a less prominent protein/DNA band also appeared at a similar position in U937 macrophage nuclear extracts, it could not be competed with even 200-fold molar excess of the βT oligo, suggesting that it is an abundant non-specific protein (Fig. 10). The same non-specific protein seemed to be present in the other human nuclear extracts as shown by the residual band present at 200-fold molar excess specific competition. The same protein/DNA band using BJA-B nuclear extract did compete away somewhat at 100 and 200-fold molar excess of βT oligo but not nearly to the extent seen with the T-cell extracts. In summary, the similarity in gel-shift activity between the in-vitro translated

Gata3 gene product and the total nuclear extracts suggests that the Tβ2-binding protein in vivo is the Gata3 protein.

The TCR β enhancer element Tβ2 confers enhancer activity in T cells

To test whether the βT (=Tβ2 element in human TCR β enhancer) oligonucleotide alone can confer enhancer function, we cloned the βT oligo as a monomer or multimer into a CAT vector (J21) containing a minimal c-fos promoter. These constructs were then transfected into T and macrophage cell lines. As shown in Table I and Figure 11, a dimer of the βT oligo transfected into Molt13 γδ T cells conferred 34- to 48-fold enhanced signal over J21 background CAT activity; a hexamer of the βT site gave up to 79-fold induction. Transfection of the same constructs gave similar results in Jurkat αβ T-cells, although the magnitude of the effect was less (Fig. 12). No induction of CAT activity could be detected in the U937 macrophage cell line, even with the construct containing a hexamer of the βT oligo; if anything, the βT site seemed to repress background CAT activity (Fig. 13). The in-vivo pattern of βT enhancer activity in Molt13 and Jurkat and the absence of activity in U937 again correlates well with the binding data shown above, the most abundant Gata3 protein being present in Molt13 γδ T cells, somewhat less in Jurkat αβ T cells, and none in the U937 macrophage cell line.

In summary, we have cloned and partially characterized the human Gata3 cDNA. We have showed that the human Gata3 gene product is present in both αβ and γδ T cells and binds to at least three T-cell receptor enhancer elements (those of the TCR α, β, and δ genes). Although Gata3 does not bind to the TCR γ enhancer element NFγ1, it may bind to other as yet uncharacterized γ regulatory elements. Our data showing the involvement of Gata3 with regulation of α,β, and δ TCR genes indicate that Gata3 is important for both αβ and γδ T-cell development and is consistent with the previous finding that a minimal TCR α enhancer is active in both T cell lineages with

lineage-specificity achieved through the action of silencers (72). Furthermore, the presence of Gata3 binding activity in the pre-T cell line RS4.2 supports the notion that Gata3 might be a regulatory gene which is turned on early in T-cell development.

Gata3 joins a small list of identified TCR transcription factors which is growing rapidly. Waterman and Jones succeeded in purifying a T-cell-specific factor, TCF-1α, which binds to the HIV-1 LTR and to the CD3δ gene promoter (66). Subsequent footprinting studies revealed that TCF-1α binds with high affinity to an oligonucleotide containing a CTTTG core motif found in the 5' half of the Tα2 element of the human TCRα enhancer, which corresponds to the murine NFα3 footprint. This binding was also detected using the entire minimal TCRα enhancer. Binding was also observed using oligos containing a similar motif in the $p56^{lck}$ and CD3γ promoters and in the TCRδ enhancer. Binding studies were correlated with transfection experiments which showed that a double point mutation in the TCF-1α binding site abolished TCRα enhancer activity in T-cell lines. Interestingly, multimers of the TCF-1α binding site in Tα2 were not sufficient to direct transcription in transfection studies, suggesting that TCF-1α interacts with proteins binding to the other two identified sites within the minimal enhancer, called CRE (Tα1) and TCF-2α (3' half of Tα2).

Cloning of the cDNA encoding TCF-1α revealed that it is a member of a family of high mobility group (HMG) proteins containing a pair of extended α helices at the C-terminal end (67). Deletion studies established that this HMG motif is sufficient to bind DNA. Northern blot analysis showed that TCF-1α mRNA expression is restricted to thymus; curiously, no mRNA was detected in spleen, even though that organ contains an abundance of T cells. An interesting finding was that TCF-1α mRNA was induced three- to five-fold in the immature T cell line CEM by phorbol ester treatment. Finally, the authors report that TCF-1α transactivates the minimal TCRα enhancer when cotransfected into HeLa cells, despite their previous observation that the TCF-1α site

alone can not act as an enhancer. They speculate that TCF-1α might be modified in T-cells such that interaction with the CRE and TCF-2α elements is required in T-cells but not in non-lymphoid cells; clearly more study will be required to determine the precise role of TCF-1α in TCRα expression.

The proto-oncogene c-ets-1 has been implicated in TCRα transcriptional regulation by several lines of evidence. First, Bhat et. al. showed that c-ets-1 and ets-2 mRNA is strongly enriched in thymocytes and in mature T cells, particularly CD4+ T cells (3). Bosselut et. al. later showed that c-ets-1 and ets-2 can both transactivate the the HTLV-1 LTR in HeLa and NIH3T3 cotransfection assays (6). Finally, while trying to isolate the TCF-2α transcription factor from a λgt11-Jurkat T-cell expression library, Ho et. al. found that a clone which bound to a Tα2 probe was identical to c-ets-1 (22). Gel-shift assays revealed that their in-vitro-translated c-ets-1 clone binds specifically to an oligo corresponding to Tα2 and that the protein/DNA complex is specifically supershifted by an anti- c-ets-1 antibody. The authors neglected, however, to compare the gel-shift activity of in-vitro-translated c-ets-1 with that of native nuclear extracts; in particular, it is curious that they did not try to demonstrate that an anti-c-ets-1 antibody can supershift the protein/Tα2 complex using Jurkat nuclear extracts. Furthermore, there are other reasons to question the physiologic relevance of c-ets-1/ Tα2 binding. First, it has been shown that c-ets-1mRNA is not detectable in murine thymus until day 18, at least one day too late to be involved in early TCRα mRNA expression (3). Second, c-ets-1 mRNA is expressed in ten-fold higher amounts in CD4+ T cells than in CD8+ T cells, even though both have comparable levels of surface αβ TCR expression (3). Finally, while c-ets-1 is expressed in both B and T cells, Tα2 binding activity appears to be restricted to T cells (67, 22). More work will be needed to demonstrate how these findings are compatible with a role for c-ets-1 in TCRα gene regulation and whether or not addition factors also bind to the Tα2 site.

Much work also remains to be done to fully charcterize Gata3 activity in T cells. One important study which remains to be done is to discover whether Gata3 can transactivate the Tβ2 footprint in cotransfection assays. Isolation of a murine Gata3 cDNA would allow studies of mRNA expression in fetal thymus. Introducing mutations into the Gata3 cDNA should allow determination of the domains important for DNA binding and protein-protein interaction. Development of polyclonal antisera or monoclonal antibodies against Gata3 can be used to supershift Gata3/DNA complexes in gel-shift assays using whole nuclear extracts. In addition, these antibodies may bring down proteins which bind to Gata3 in immunoprecipitation. A better understanding of how Gata3 interacts with DNA may follow from a recent report of the X-ray crystallographic structure of a zinc-finger/DNA complex (43a). It will also be interesting to learn why Gata3, like RAG-1 and Oct-2, is expressed in embryonic brain tissue (8). Finally, the Gata3 cDNA can be used to study the organization of the Gata3 gene locus and its associated cis-acting DNA elements and trans-acting factors; the Gata1 gene, for example, has been shown to regulate it own activity due to the presence of Gata binding sites in its own promoter (19). Ultimately, the most complicated and the most fascinating problem will be to piece together a coherent picture of how all these ubiquitous and lineage-specific transcription factors interact with cis-acting DNA elements and with each other to regulate the expression of TCR and other T-cell-specific genes in the precise fashion observed in life.

Addendum: While this manuscript was in the final draft stage, two other reports describing work on Gata3 were published. Ho et. al. isolated a human Gata3 cDNA from a Jurkat T-cell library and confirmed our finding that it binds to the TCRα enhancer; they localized this binding activity to the Tα3 footprint and further demonstrated that Gata3 can transactivate a dimer of the Tα3 site when cotransfected into HeLa cells (22a). Ko et. al. isolated both murine and human Gata3 cDNA's from T cell libraries and showed that each can transactivate WGATAR sites present in the chicken β-globin enhancer and the human δE4 enhancer footprint (27).

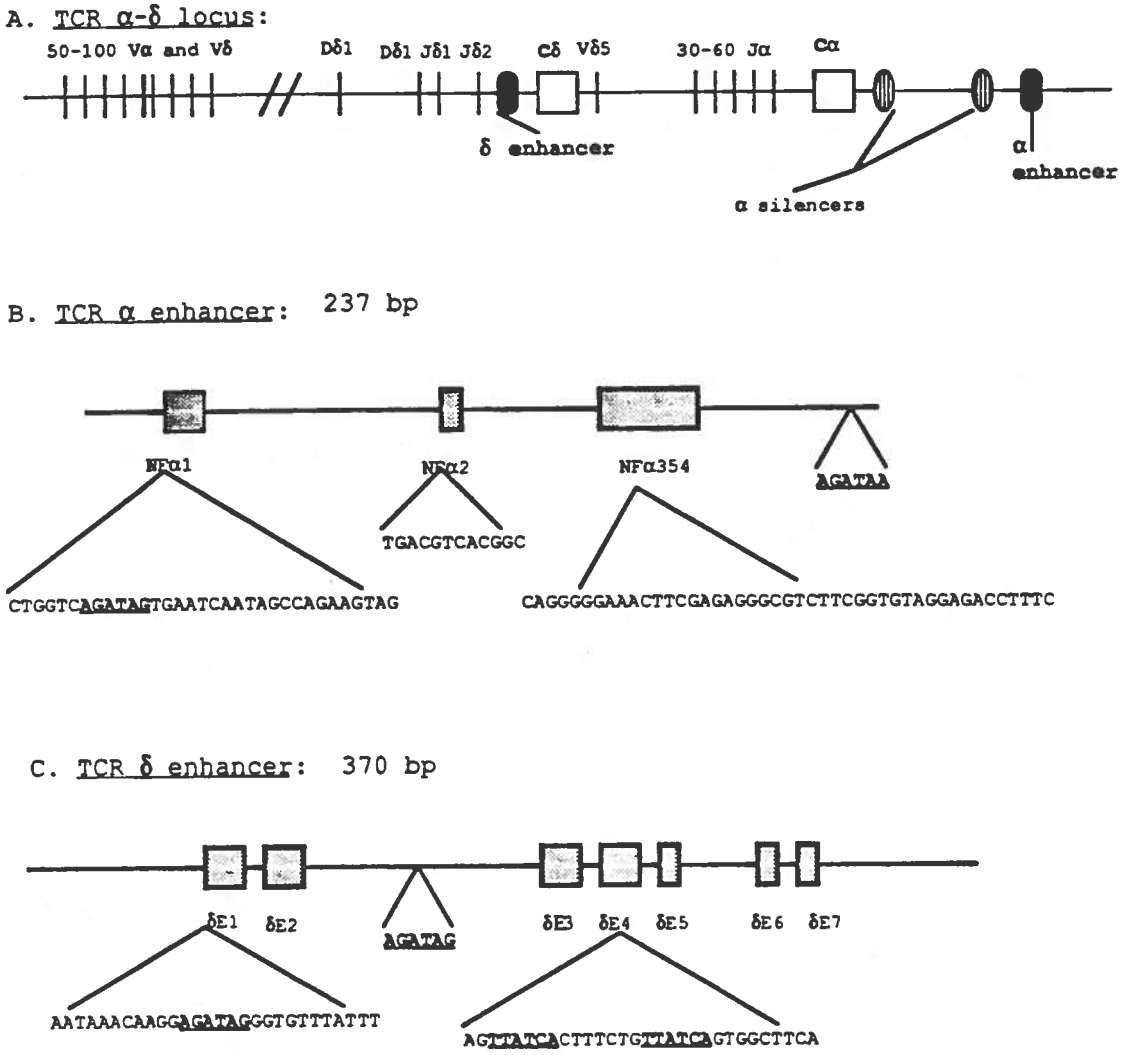
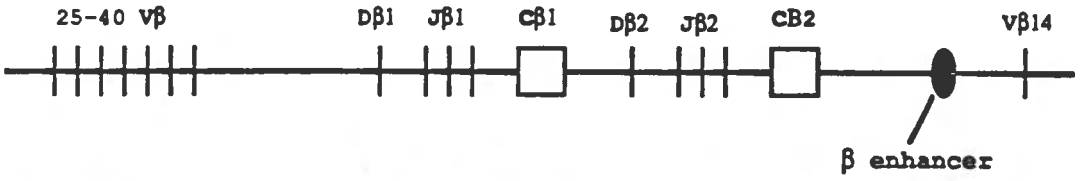
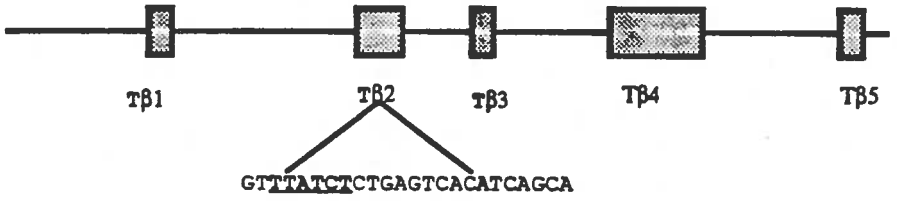


Figure 1: Diagrammatic representation of the T-cell receptor (TCR) α-δ locus and its respective enhancers. A. TCR α-δ locus, not drawn to scale, showing the approximate locations of its respective enhancers and silencers (refs. 25, 35, 67). B. and C. Scale diagrams of the murine TCRα (ref. 45) and human TCR δ (ref. 56) enhancers; blocks represent DNase I footprints. WGATAR sequences are indicated in bold underline.

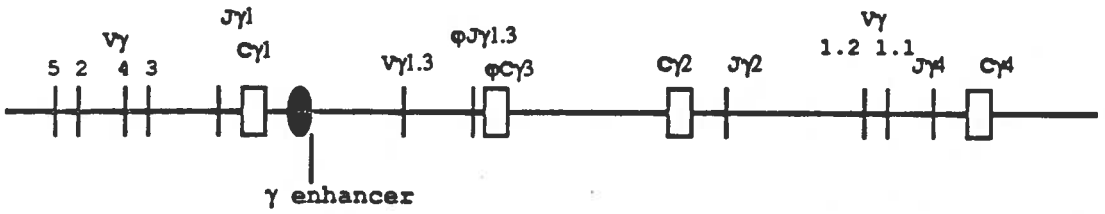
A. TCR β locus:



TCR β enhancer: 481 bp



B. TCR γ locus:



TCR γ enhancer: 1000 bp

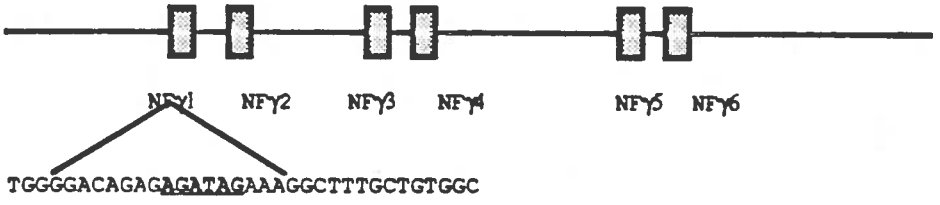
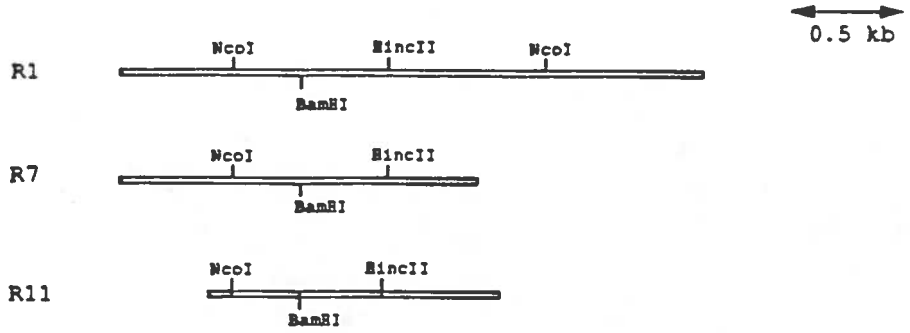


Figure 2: Diagrammatic representation of the human TCR β (ref. 89) and murine TCR γ (ref. 78) loci and their respective enhancers (ref. 34, 45). Genomic loci are not drawn to scale, while the enhancer diagrams are. DNase I footprints are indicated by dotted blocks; WGATAR sequences are indicated in bold underline.

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M  E  V  T  A  D  Q  P  R  W  L  S  H  H  H  P  A  V  L  N  G  Q  H  P  D  T  H  H  P  G  L  S  H  S  Y  M  D  A  A  Q>
                                                                                                                    360
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V  S  P  D  P  S  L  S  T  P  G  S  A  G  S  A  R  Q  D  E  K  E  C  L  K  Y  Q  V  P  L  P  D  S  M  K  L  E  S  S  H>
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S  R  G  S  M  T  A  L  G  G  A  S  S  S  T  H  H  P  Y  T  T  Y  P  P  T  C  P  E  Y  S  S  G  L  F  P  P  S  S  L  L>
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                                                                                                                   1440
TGTCCTCCCTGAGCCACATCTCGCCCTTCAGCCACTCCAGCCACATGCTGACCACGCCCACGCCGATGCACCCGGCATCCAGCCTGTCCTTTGGACCACACCACCCCTCCAGCATGGTCA
M  S  S  L  S  H  I  S  P  F  S  H  S  S  H  M  L  T  T  P  T  P  M  H  P  A  S  S  L  S  F  G  P  H  H  P  S  S  M  V>

CCGGCATGGGTTAGAG
T  G  M  G  *>
```

Figure 3: The human Gata3 cDNA map and sequence.

A. Selected restriction enzyme map of the three Gata3 cDNA clones isolated from the λgt11-HUT78 human T-cell library, R1, R7, and R11.

B. The human Gata3 cDNA nucleotide sequence and predicted amino acid sequence. DNA sequencing was performed using the dideoxy sequencing technique with deletion clones generated based on the R1 clone restriction enzyme map.

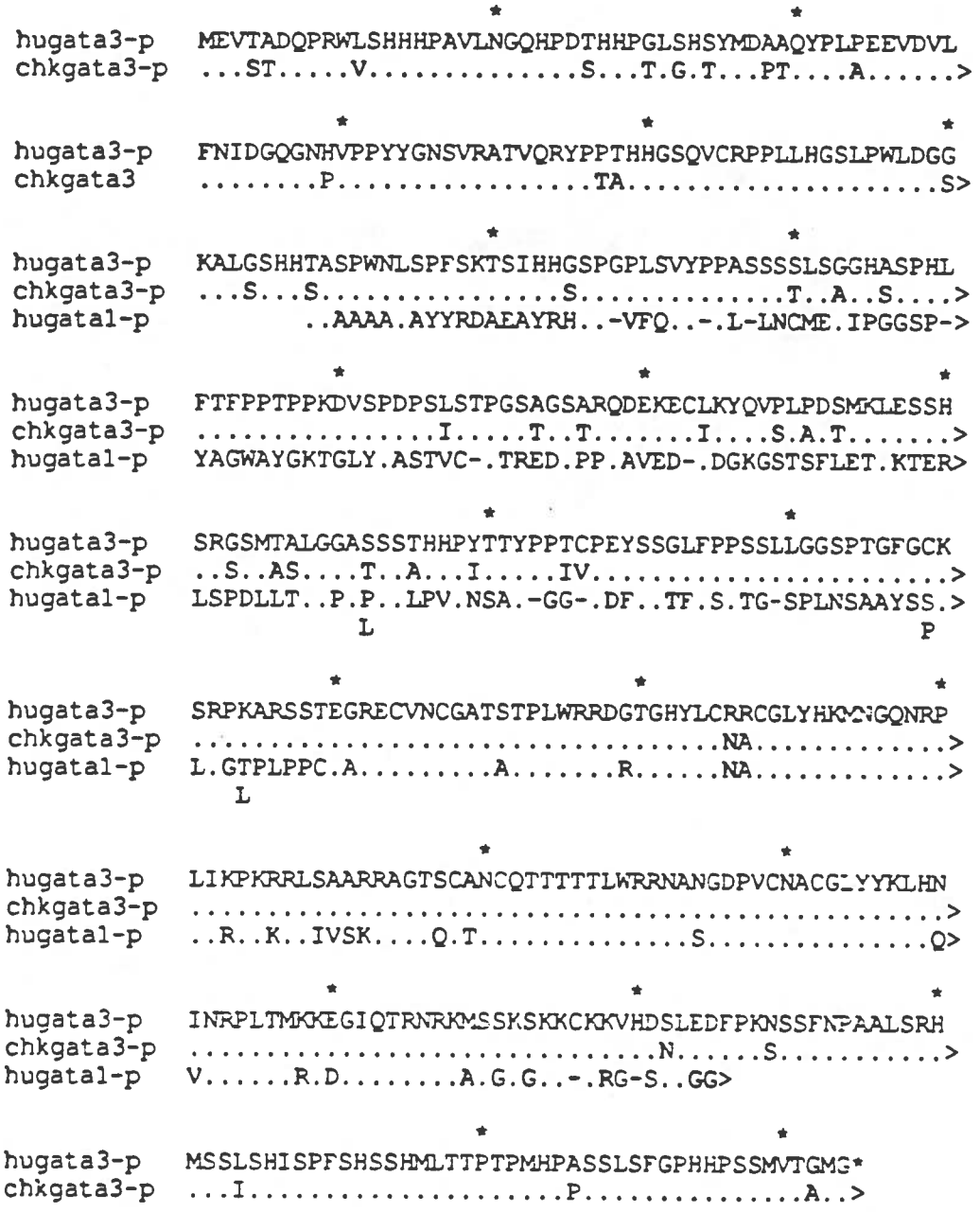

Figure 4: Comparison of the amino acid sequence of human and chicken Gata3 and human Gata1. Dots indicate identity. Note the extensive homology between human and chicken Gata3 throughout the entire coding region; homology between human Gata3 and Gata1 is limited to the Zn-finger domain.

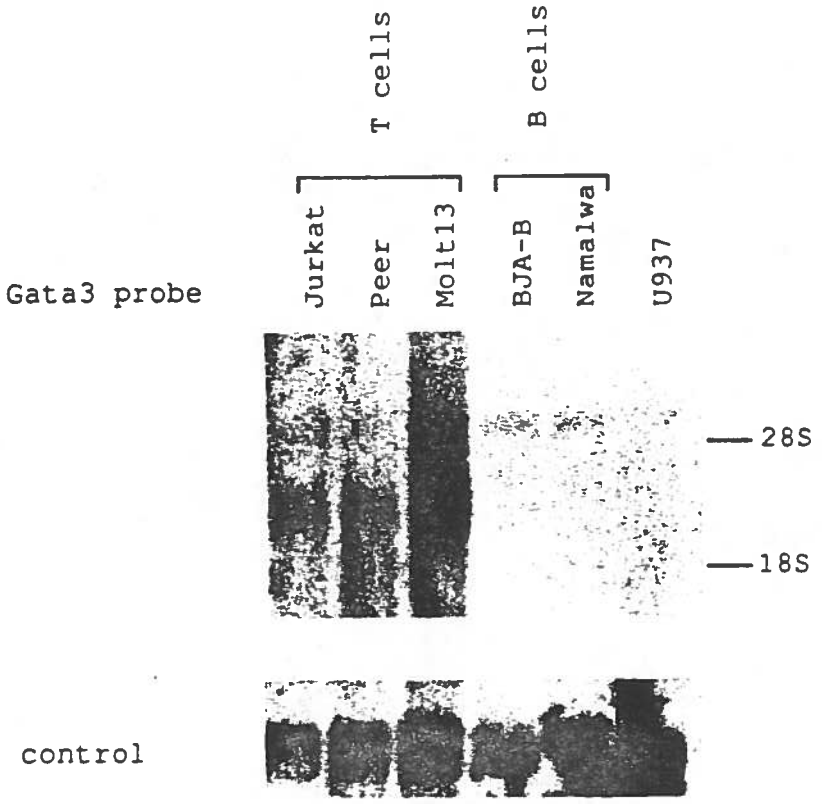

Figure 5: Northern blot analysis of the human Gata3 clone. A Gata3-specific probe (NcoI/HincII fragment of the R11 clone) was generated for use in a Northern blot analysis of 1X polyA+ RNA from T (Jurkat, Molt13, Peer), B (BJA-B, Namalwa), and macrophage (U937) cell lines. A control cytochrome-c oxidase probe was used to screen the same RNA samples (lower band). The blot was washed with 2X SSC, 0.1% SDS at 65^0C .

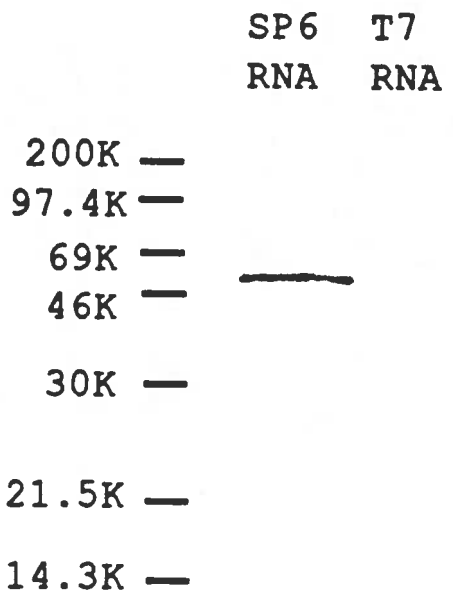

Figure 6: SDS-PAGE gel analysis of the in-vitro translated Gata3 cDNA product. RNA was transcribed from either the SP6 (sense) or T7 (antisense) promoter of the pR1 Gata3 clone and then translated using a rabbit reticulocyte lysate (Promega) in the presence of ^{35}S methionine. The reaction mixture was then run on a 15% SDS-PAGE gel with an Amersham rainbow protein marker (MW_r shown on left). After drying, the gel was exposed to X-ray film (Kodak X-OMAT).

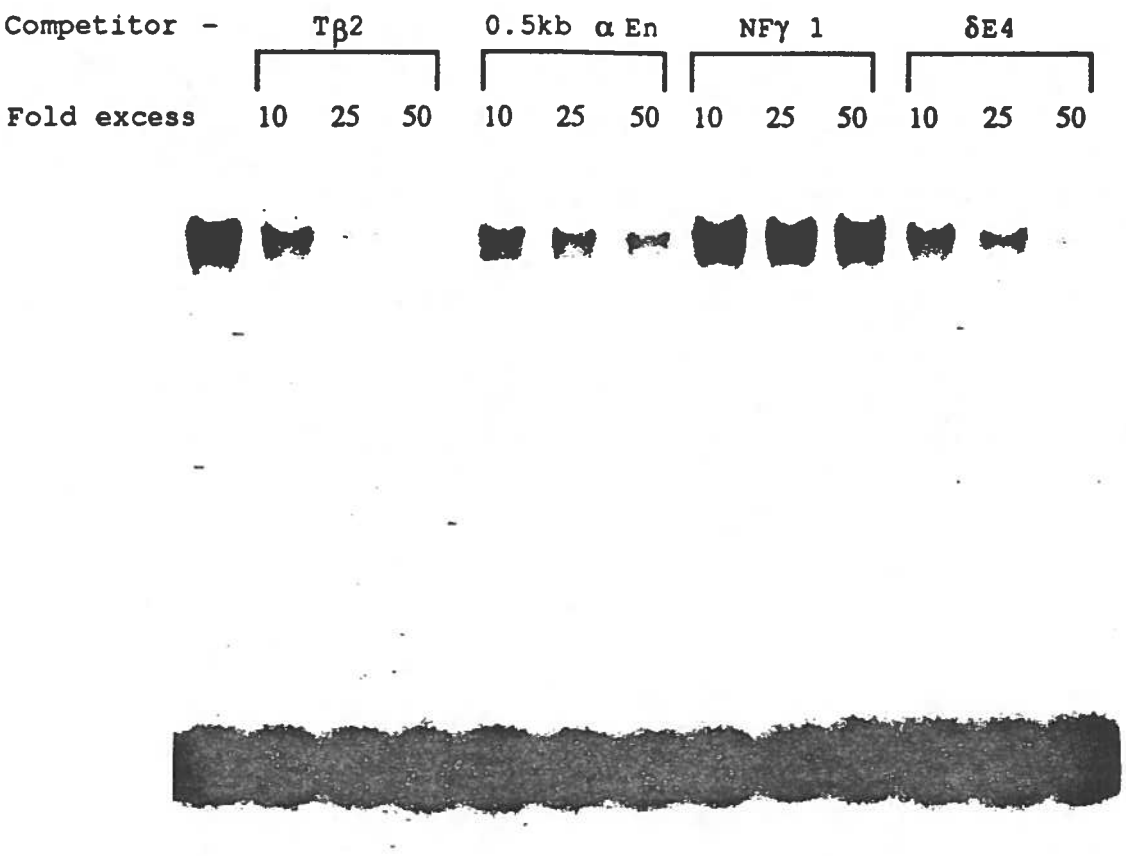

Figure 7: Gel-shift analysis of the in-vitro translated Gata3 protein. A portion (2%) of unlabeled in-vitro translated Gata3 protein was tested for binding to the βT oligonucleotide probe (=Tβ2), which contains a WGATAR site. Specificity was established by competition using cold βT oligo (=Tβ2) in the indicated 10, 25, and 50 fold molar excess. A 0.5 kb PvuII TCR α enhancer fragment, a NFγ1 fragment from the TCRγ enhancer (competition with the γT oligo has been done with the identical result), and the δT oligo (=δE4 of the TCR δ enhancer), which all contain WGATAR sequences, were each tested for competition in binding to the Gata3 in-vitro translated protein using the same molar excess as for cold Tβ2.

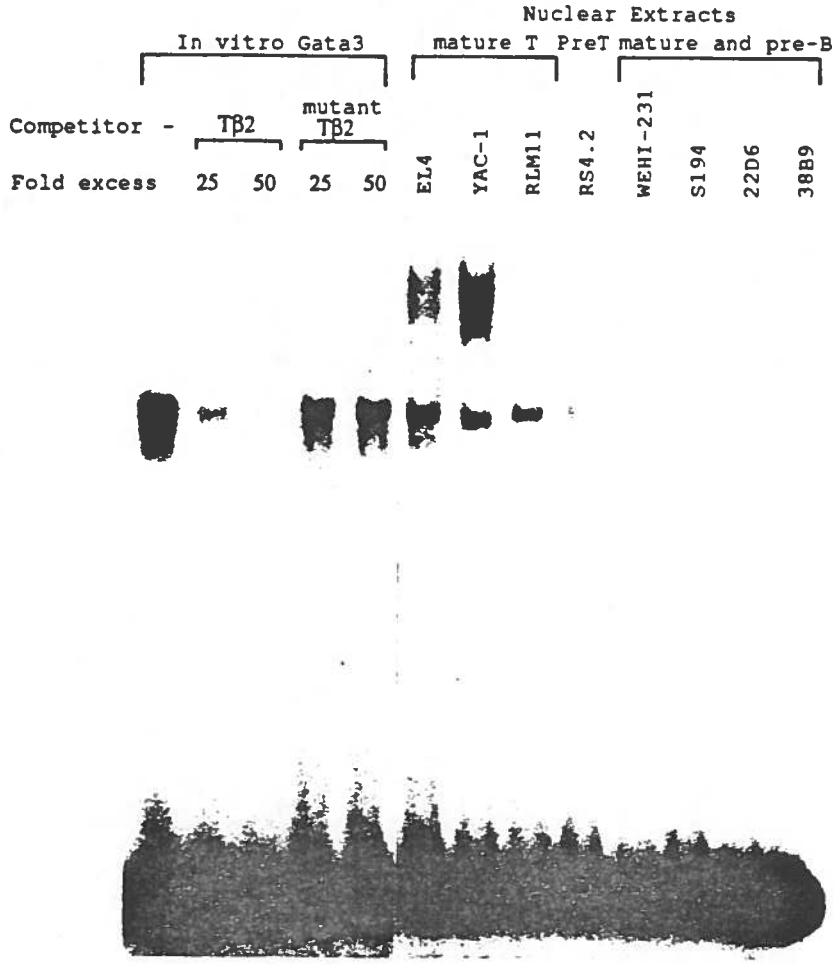

Figure 8: Gel-shift analysis of the protein-binding activity of βT oligo comparing in vitro translated Gata3 with murine total nuclear extracts. Gel-shift analysis was performed using ^{32}P dATP-labeled βT oligo and proteins from: In-vitro translated Gata3 with or without competition of 25- and 50-fold molar excess of the wild-type βT (=Tβ2, lanes 2-3) and mutant Tβ2 (=mutant Tβ2, lanes 4-5) oligos and nuclear extracts from the indicated mouse T, pre-T, B and pre-B cell lines (lanes 6-13).

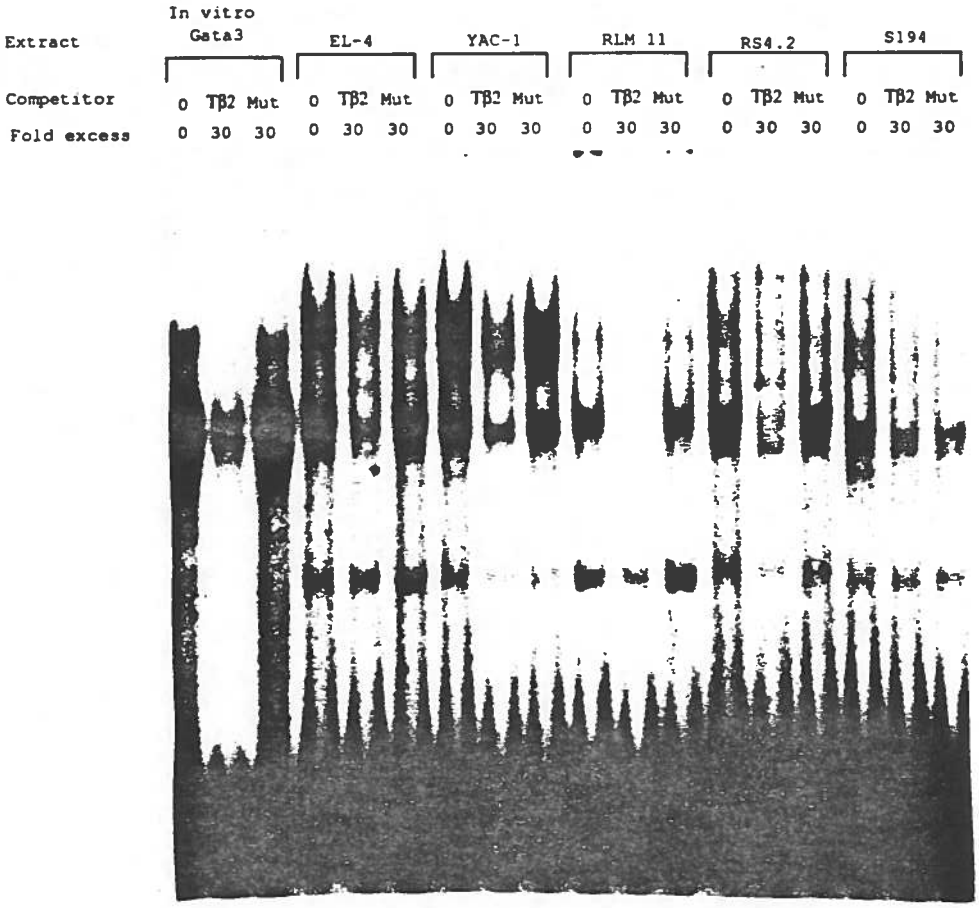

Figure 9: Gel-shift competition using murine total nuclear extract and either cold βT or mutant βT oligonucleotides. Gel-shift analysis was performed using ^{32}P-labeled βT oligo and proteins from either in-vitro translated Gata3 or the mouse nuclear extracts indicated. Cold competition was performed with the indicated molar excess of either wild-type βT (=Tβ2) or mutant βT (=Mut) oligos.

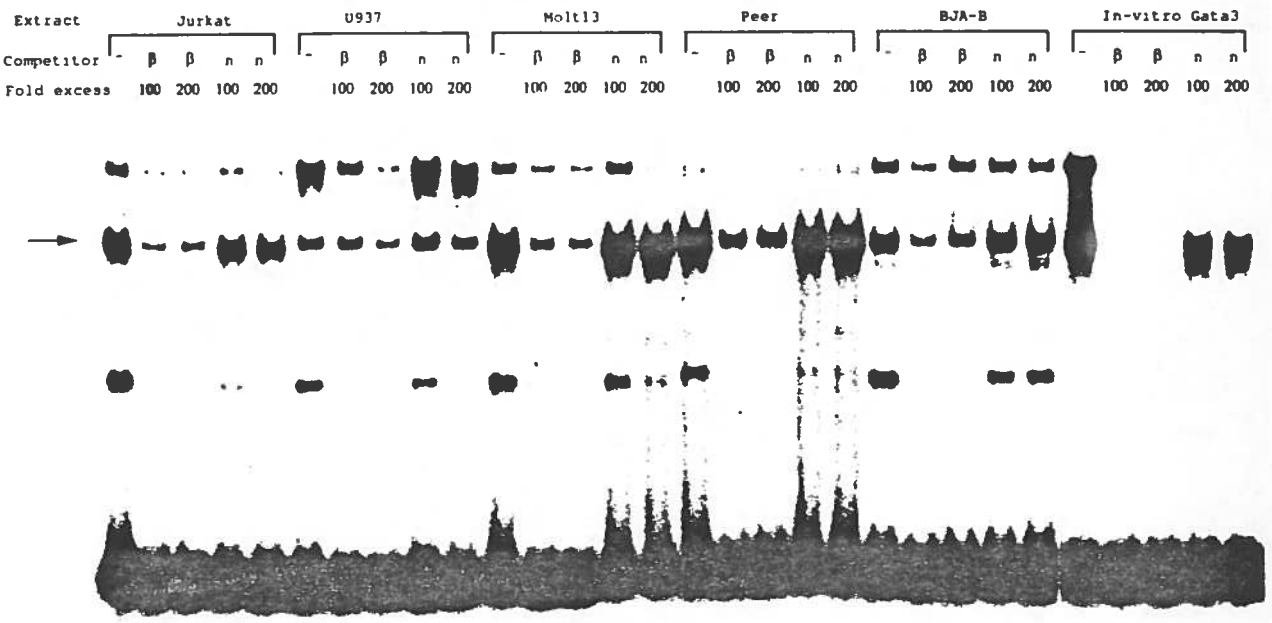

Figure 10: Gel-shift analysis of the βT oligo using human total nuclear extracts. Gel-shift analysis of ^{32}P-labeled βT oligo with nuclear extracts from αβ (Jurkat) and γδ (Molt13, Peer) T-cell lines, a B-cell line (BJA-B), and a macrophage cell line (U937). Competition with 100- and 200-fold molar excess of cold βT oligo (marked β) or an irrelevant NFα4/5 which contains no WGATAR sequence (marked n) was performed to assess specificity. For comparison, the in-vitro translated Gata3 product was also included in the gel-shift experiment. The arrow indicates the band containing the Gata3 protein.

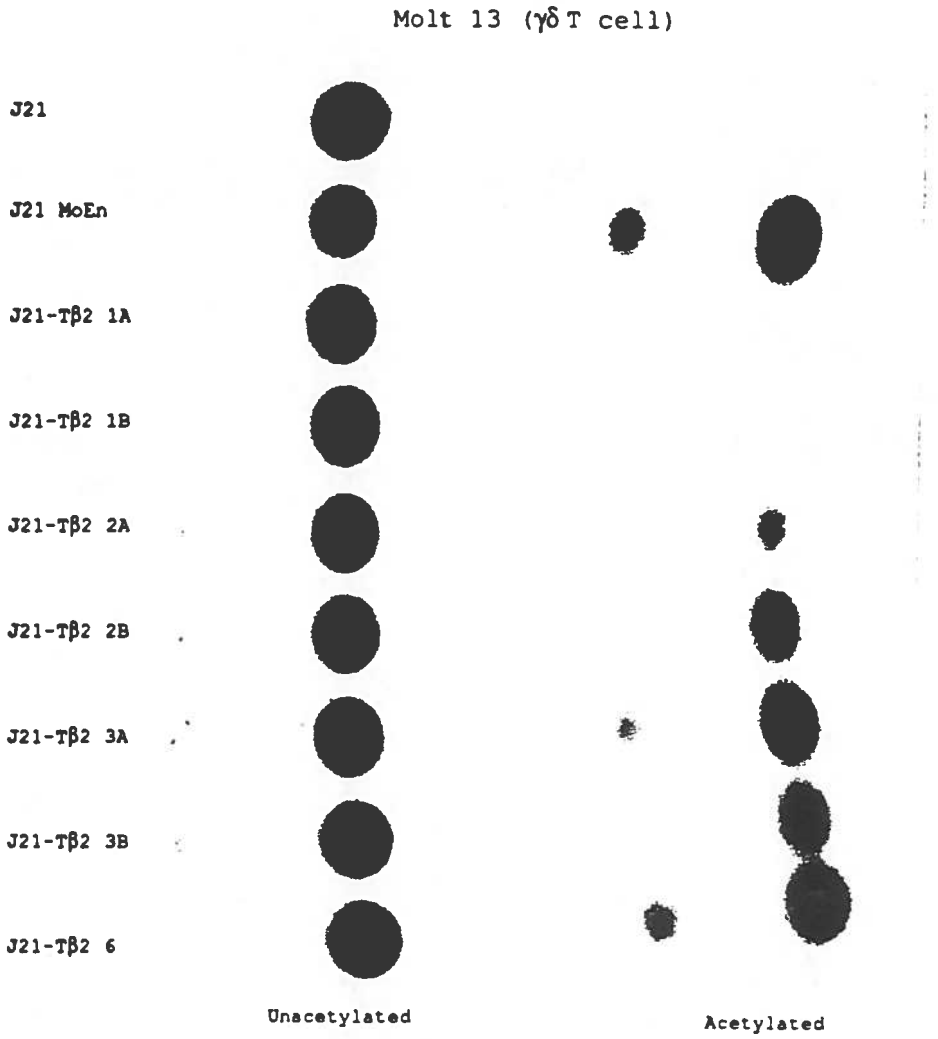

Figure 11: The Tβ2 footprint has enhancer activity in Molt13 γδ T cells. Molt13 cells were transfected with the indicated plasmid constructs containing a monomer or multimers of the βT (=Tβ2) oligonucleotide and then assayed for CAT activity as described in the text. J21 MoEn, which contains a Moloney murine leukemia virus enhancer, was used as a positive control. A and B indicate opposite orientations of the oligonucleotides relative to the J21 vector. Each multimerized oligo was cloned in a tandem head-to-tail orientation except for J21-Tβ2-6, which has 5 copies in a head-to-tail fashion and one copy in the opposite orientation.

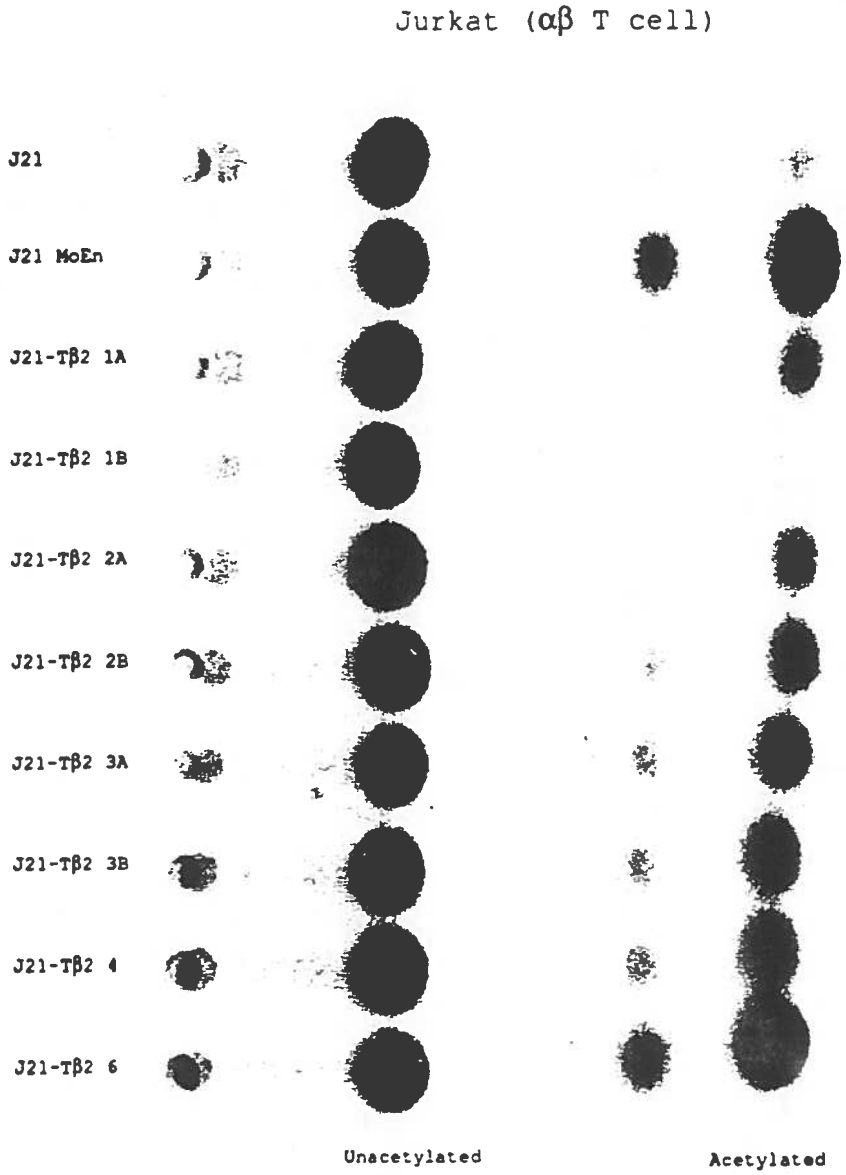

Figure 12: The Tβ2 footprint has enhancer activity in Jurkat αβ T cells. Jurkat cells were transfected with the indicated plasmid constructs containing a monomer or multimers of the βT (=Tβ2) oligonucleotide and then assayed for CAT activity as described in the text. J21 MoEn, which contains a Moloney murine leukemia virus enhancer, was used as a positive control. A and B indicate opposite orientations of the oligonucleotides relative to the J21 vector. Each multimerized oligo was cloned in a tandem head-to-tail orientation except for J21-Tβ2-6, which has 5 copies in a head-to-tail fashion and one copy in the opposite orientation.

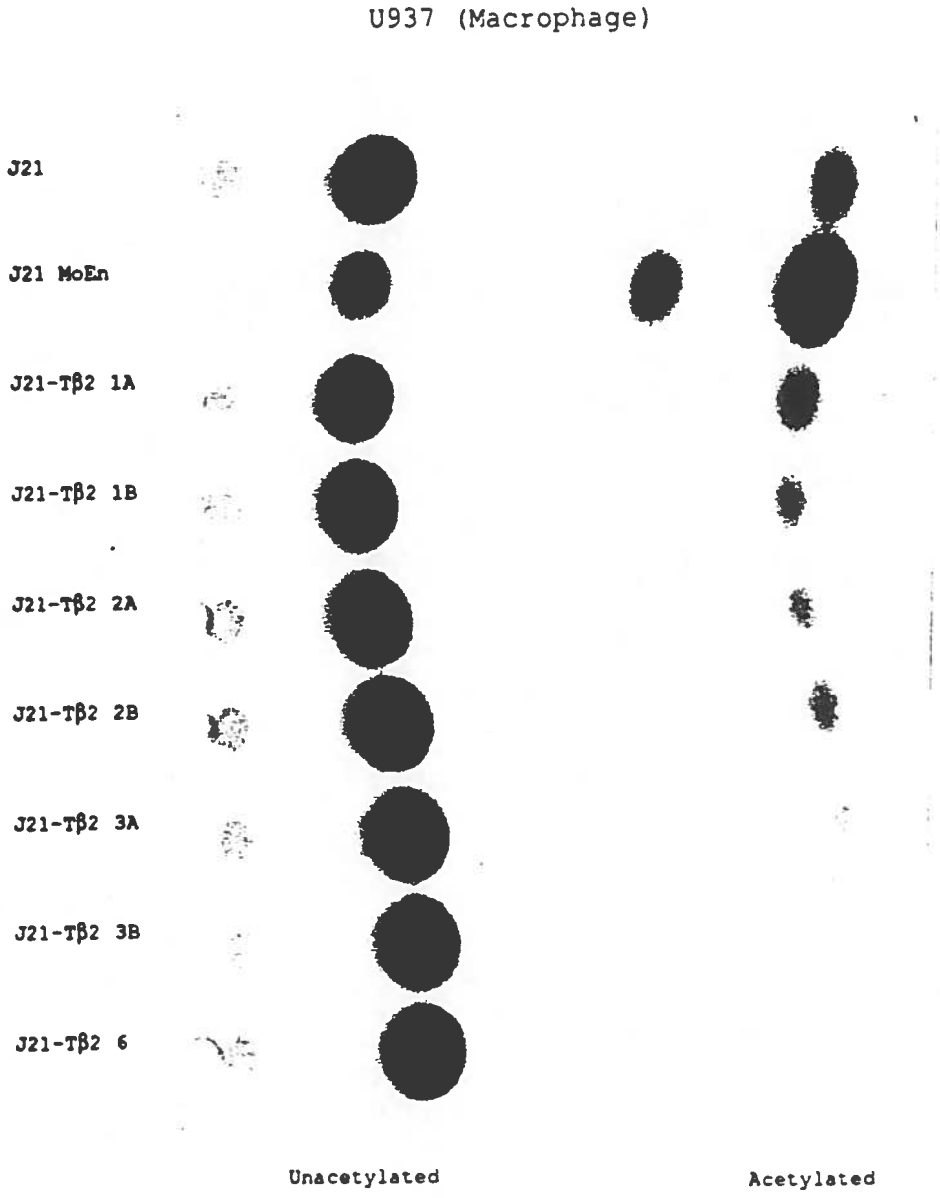

Figure 13: The Tβ2 footprint has no enhancer activity in U937 macrophage cells. U937 cells were transfected with the indicated plasmid constructs containing a monomer or multimers of the βT (=Tβ2) oligonucleotide and then assayed for CAT activity as described in the text. J21 MoEn, which contains a Moloney murine leukemia virus enhancer, was used as a positive control. A and B indicate opposite orientations of the oligonucleotides relative to the J21 vector. Each multimerized oligo was cloned in a tandem head-to-tail orientation except for J21-Tβ2-6, which has 5 copies in a head-to-tail fashion and one copy in the opposite orientation.

Table I. In vivo activity of the multimerized βT oligonucleotides

	Molt13		Jurkat		U937	
	exp.1*	exp.2	exp.1	exp.2	exp.1	exp.2
J21	0.23 (1X)	0.36 (1X)	4.06 (1X)	2.36 (1X)	4.58 (1X)	10.75 (1X)
J21MoEn	28.30 (120X)	47.21(130X)	83.30 (20X)	35.11 (15X)	29.94 (7X)	86.17 (8X)
J21-βT- 1A	1.10 (5X)	2.30 (6X)	11.04 (3X)	7.81 (3X)	4.00 (0.8X)	10.75 (1X)
1B	0.28 (1X)	0.51 (1X)	3.84 (1X)	2.07 (1X)	2.90 (0.6X)	6.37 (0.6X)
2A	3.82 (16X)	6.77 (18X)	13.76 (3X)	7.94 (3X)	1.19 (0.3X)	4.63 (0.4X)
2B	8.02 (34X)	17.6 (48X)	20.65 (5X)	11.14 (5X)	1.50 (0.3X)	5.10 (0.5X)
3A	11.12 (47X)	24.53 (67X)	23.3 (6X)	15.95 (7X)	0.83 (0.2X)	4.48 (0.4X)
3B	9.88 (42X)	16.90 (46X)	24.36 (6X)	13.79 (6X)	0.52 (0.1X)	2.91 (0.3X)
6	17.90 (76X)	28.91 (79X)	45.24 (11X)	37.43 (16X)	0.95 (0.2X)	2.12 (0.2X)

* Exp.1: experiment #1, exp.2: experiment #2. Molt13, Jurkat, and U937 cells were transfected with the indicated plasmid constructs and harvested after a two-day incubation. Numbers correspond to the percent acetylation of ^{14}C Chloramphenicol following incubation with the indicated protein extract. Numbers in parentheses represent n-fold induction over J21 background CAT activity. J21 MoEn, which contains a Moloney murine leukemia virus enhancer, was used as a positive control. A and B indicate opposite orientations of the oligonucleotides relative to the J21 vector. Each multimerized oligo was cloned in a tandem head-to-tail orientation except for J21-βT-6, which has 5 copies in a head-to-tail fashion and one copy in the opposite orientation.

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