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A Targeted Mutation at the T-Cell Receptor α/δ Locus Impairs T-Cell Development and Reveals the Presence of the Nearby Antiapoptosis Gene *Dad1*

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Locus control regions are *cis* gene regulatory elements comprised of DNase I-hypersensitive sites. These regions usually do not stimulate transcription outside of a chromosomal context, and therefore their ability to regulate the expression of genes is thought to occur through the modification of chromatin accessibility. A locus control region is located downstream of the T-cell receptor (TCR) α/δ locus on mouse chromosome 14. This locus control region is known to drive T-cell-specific TCRα transcription in transgenic mice. In this report, we describe a targeted deletion of this locus control region and show that this mutation acts at a critical checkpoint in αβ T-cell development, between the TCR-intermediate and TCR-high stages. Our analysis further reveals that the antiapoptosis gene *Dad1* is at the 3′ end of the TCR α/δ locus and that *Dad1* is required for embryogenesis. We show that mouse *Dad1* has a broader expression pattern than the TCR genes, in terms of both tissue and temporal specificity. Finally, we report that the chromatin between TCRα and *Dad1* is DNase I hypersensitive in a variety of cell types, thus correlating with *Dad1* expression and raising the possibility that *Dad1* regulatory sequences reside in this region.

Study of the genomic organization of eukaryotic genes has, in many cases, revealed an unexpected degree of proximity between genes (26). These multigene loci sometimes arise as products of gene duplication, but many examples of nonhomologous genes which occupy overlapping or abutting regions of DNA also exist. In the case of gene families being encoded in a complex, the expression of these genes is often coordinated through shared regulatory sequences. The β-like globin gene complex represents a paradigm for such multigene loci: in this locus, all five globin genes have proximal transcriptional promoters and enhancers while a locus control region (LCR) upstream of the genes participates in organizing the chromatin configuration of the entire locus (for a recent discussion, see reference 21). In the case of nonhomologous genes encoded in neighboring DNA, the issue of regulation is less clear. Often the genes are not expressed in the same tissue type or developmental window (3, 20, 33). Furthermore, one gene may be tightly regulated at the transcriptional level while the other is expressed at a so-called "housekeeping" level (1). In these situations, the existence of boundary sequences is usually inferred. Finally, there may be nonhomologous genes which share regulatory elements and therefore have coordinated expression, although these have yet to be described.

The T-cell receptor (TCR) α/δ gene locus encodes two homologous genes. However, the genomic organization of this locus is quite unusual: the δ gene is embedded within the α gene (see Fig. 1A). In T-cell precursors, variable (V), diversity (D), and joining (J) gene segments are somatically rearranged to either the α or the δ constant region to give rise to a TCRα chain or a TCRδ chain. The regulation of this locus is of great interest because expression of the TCR on the cell surface is required for proper T-cell development (16, 22, 23). Furthermore, TCRα and TCRδ are found on mutually exclusive populations of T cells, indicating that tight regulation of the locus is necessary. It is thus an excellent system in which to study complex gene regulation. In transient transfection assays, transcriptional enhancers were found proximal to each of the constant regions (12, 14, 17, 28, 35). While the δ enhancer has not been shown to be sufficient to drive transcription in vivo, it can promote VDJ rearrangement (19). Conversely, the α enhancer has been shown to require a downstream LCR to stimulate transcription of a linked transgene (6), but its role in rearrangement awaits confirmation. Like the β-globin LCR, the TCRα LCR contains DNase I-hypersensitive sites (HS) which do not have significant transcriptional enhancer activity in transient transfection assays.

The advent of gene targeting in mice has revolutionized the field of transcriptional regulation. Previously, the strictest test for in vivo function of regulatory sequences was the transgenic approach. While of great usefulness, transgenes have the disadvantage of integrating into heterologous regions of what is usually uncharacterized chromatin. Moreover, tandem integration of multiple copies of transgenes could influence the level of expression and make interpretation difficult (13). By using gene targeting, we can now test the importance of regulatory elements by mutating them in their natural context. Because targeted deletions allow a direct comparison with the activity of the endogenous locus, it is the more valid approach when trying to test the necessity of a particular regulatory element and may reveal unexpected redundancy. For example, targeting experiments with the β-globin LCR have contributed significant understanding to that gained from transgenic data (9, 15). Here we describe a deletion of the endogenous TCRα LCR by gene targeting which leads to incomplete TCRα expression. In the course of these studies, we discovered that the mouse TCR α/δ gene locus has a third gene encoded 12 kb away from the α constant region. The expression pattern of this gene, *Dad1*, is wider than that of the TCR genes, and the dynamic chromatin structure of the LCR correlates with this

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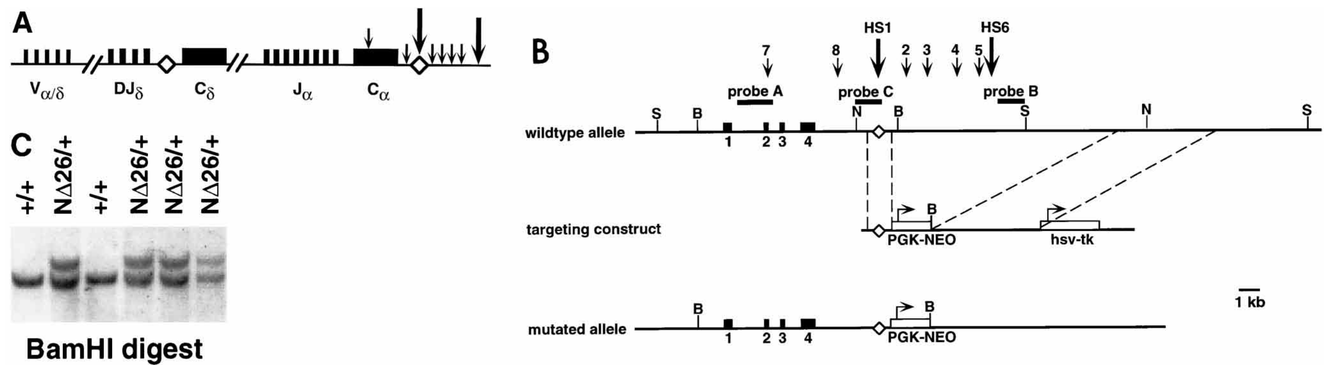

FIG. 1. (A) Schematic diagram of the genomic TCR α/δ locus. The variable (V), joining (J), diversity (D), and constant (C) regions are shown, as are transcriptional enhancers (diamonds) and the DNase I HS (arrows) found by Diaz et al. (6). (B) Map of the wild-type LCR, the targeting construct used to generate NΔ26, and the resultant targeted allele. Restriction enzyme sites: B, *Bam*HI; N, *Nhe*I; S, *Sac*I. hsv-tk, herpes simplex virus thymidine kinase. (C) Southern blot showing the detection of wild-type versus mutant alleles. Genomic DNA was digested with *Bam*HI and hybridized with a *Hin*dIII fragment (probe A in panel B).

fact. These findings indicate that the regulation of this locus is much more complex than previously appreciated.

MATERIALS AND METHODS

Generation of the NΔ26 mutation. Cosmid clones of the TCR α/δ locus from mouse strain 129SV were isolated from a genomic library provided by M. Koshland (University of California [UC] Berkeley). The parental targeting vector pPNT has already been described (32). The gene targeting construct was constructed by subcloning a 1.1-kb *Xmn*I-*Sca*I genomic fragment into the blunted *Xho*I site of pPNT and a 4.8-kb *Eco*RI fragment into the *Eco*RI site. The construct was linearized with *Not*I and electroporated into R1 embryonic stem (ES) cells by using protocols described by Ramirez-Solis et al. (27). Two correctly targeted clones were isolated from 300 colonies screened. ES cell injection into C57BL6 embryonic day 3.5 blastocysts was performed by standard procedures at the UC Berkeley pathogen-free facility.

Flow cytometry. TCR Cα and Cδ knockout mice were purchased from Jackson Laboratories (Bar Harbor, Maine) and bred in microisolator cages. The antibodies and reagents used were anti-CD4–phycoerythrin, anti-CD8–fluorescein isothiocyanate, anti-CD3–biotin, anti-Thy 1.2–biotin, strepavidin-Tricolor (all from Caltag), and anti-γδ–fluorescein isothiocyanate (Pharmingen).

TCR RNase protection assays. All riboprobes were prepared by in vitro transcription with [^{32}P]GTP. The TCRα constant-region probe was transcribed by using SP6 RNA polymerase on *Eco*RI-digested template CαdH3, which was pGEM-Cα from which *Hin*dIII fragments had been removed by digestion and religation. pGEM-Cα is a 500-bp *Sau*3AI fragment from the Cα cDNA cloned into the *Bam*HI site of pGEM-3. The TCRδ constant-region probe and the γ-actin probe have been described previously (6), as has the guanidinium-phenol RNA isolation method (37).

***Dad1* gene organization and expression.** An oligo(dT)-primed, 22D6 pre-B-cell line cDNA phage library was constructed in λgt11 and screened by using a 3.5-kb *Hin*dIII-*Eco*RI genomic DNA probe (see probe D in Fig. 4A). Positive clones were subcloned into the *Eco*RI site of pBluescript(KS)+ (Stratagene) and sequenced by the UC Berkeley automated sequencing facility. The *Dad1* subclone was named 18BSA. To map the precise genomic organization of *Dad1*, cDNA was used as a probe against genomic cosmid DNA and appropriate segments of genomic DNA were subcloned and sequenced. The *Dad1* riboprobe was transcribed by using T7 RNA polymerase on the *Bsp*EI-digested template 18dHS, which was 18BSA from which an *Hpa*I-*Sma*I fragment had been removed by digestion and religation. The mouse embryonic Northern blot (Clontech) was probed with the full-length *Dad1* cDNA by following the manufacturer's instructions.

DNase I hypersensitivity assays. Due to the fragility of thymocytes, we used 0.05% saponin to lyse the cells (7) and then followed the procedures described by Siebenlist et al. (29). All other tissues were homogenized, and nuclei were purified on a sucrose cushion (36).

Nucleotide sequence accession numbers. The GenBank accession numbers for the mouse *Dad1* gene are U81050, U81051, and U81052.

RESULTS

A 10-kb deletion downstream of the TCRα enhancer impairs α expression. A set of DNase I HS downstream of the TCRα constant region has been shown to confer strong tissue-specific expression on a TCR transgene in mice (6). Integration site-independent and copy number-dependent expression has been observed in all transgenic founders, whereas weak and variable expression has been observed for a construct in which the HS downstream of the α enhancer (HS2 through HS6 in Fig. 1B) were not included. Thus, HS2 through HS6 are required for LCR activity. To determine whether these same HS are needed for regulation of the TCR α/δ locus in vivo, we deleted the region downstream of the α enhancer from the endogenous locus via ES cell gene targeting (Fig. 1B). We refer to this mutation as NΔ26, since it replaced HS2 through HS6 with the neomycin resistance gene. This mutation was confirmed by Southern blotting; Fig. 1C shows a Southern blot of genomic DNA probed with a 5′ fragment (probe A in Fig. 1B) to reveal the expected 10-kb fragment for the wild-type allele and an 11.7-kb fragment for the NΔ26 allele. Other probes 5′ and 3′ of the region deleted also gave us the expected patterns (data not shown). The mutation was passed through the germ line to yield mice heterozygous for the NΔ26 allele. Unexpectedly, when such heterozygotes were intercrossed, no homozygous progeny were recovered ($n > 200$ mice; data not shown), although heterozygous progeny were obtained at the expected Mendelian ratio. The lethality was not due to a defect in TCR expression, as null mutations in the TCR genes produce mice which, although immunocompromised, can survive in barrier mouse facilities. Since this early lethality precluded analysis of T cells homozygous for the NΔ26 allele, we used two alternative approaches to study the expression of TCR from the NΔ26 allele. First, to circumvent the lethality of the NΔ26 homozygous mutant embryos, we crossed NΔ26 heterozygotes to mice homozygous for null alleles of TCR genes: a mutation of TCRα was created by inserting the selection marker into the first exon of the constant region ($C\alpha^-$) (23), and a similar mutation was created by disrupting the constant region of TCRδ ($C\delta^-$) (16). Resulting compound heterozygous progeny develop normally, but only the NΔ26 allele can lead to surface TCR gene expression. By using flow cytometry, we could assess levels of TCR protein expression on a single-cell basis. Mature thymocytes express high levels of CD3 and are positive for either CD4 or CD8, but not both. High levels of TCRα proteins are required to reach this mature state (23). By antibody staining for developmental markers in the thymus, we detected roughly 50% fewer mature thymocytes in $C\alpha^{-/N\Delta26}$ mice relative to their phenotypically wild type $C\alpha^{-/+}$ littermates (Fig. 2A and B), and therefore the NΔ26 allele is impaired in high-level TCRα expression. Wild-type levels of TCRα were present on immature and mature T cells, as judged by surface levels of stoichiometrically associated CD3. Total

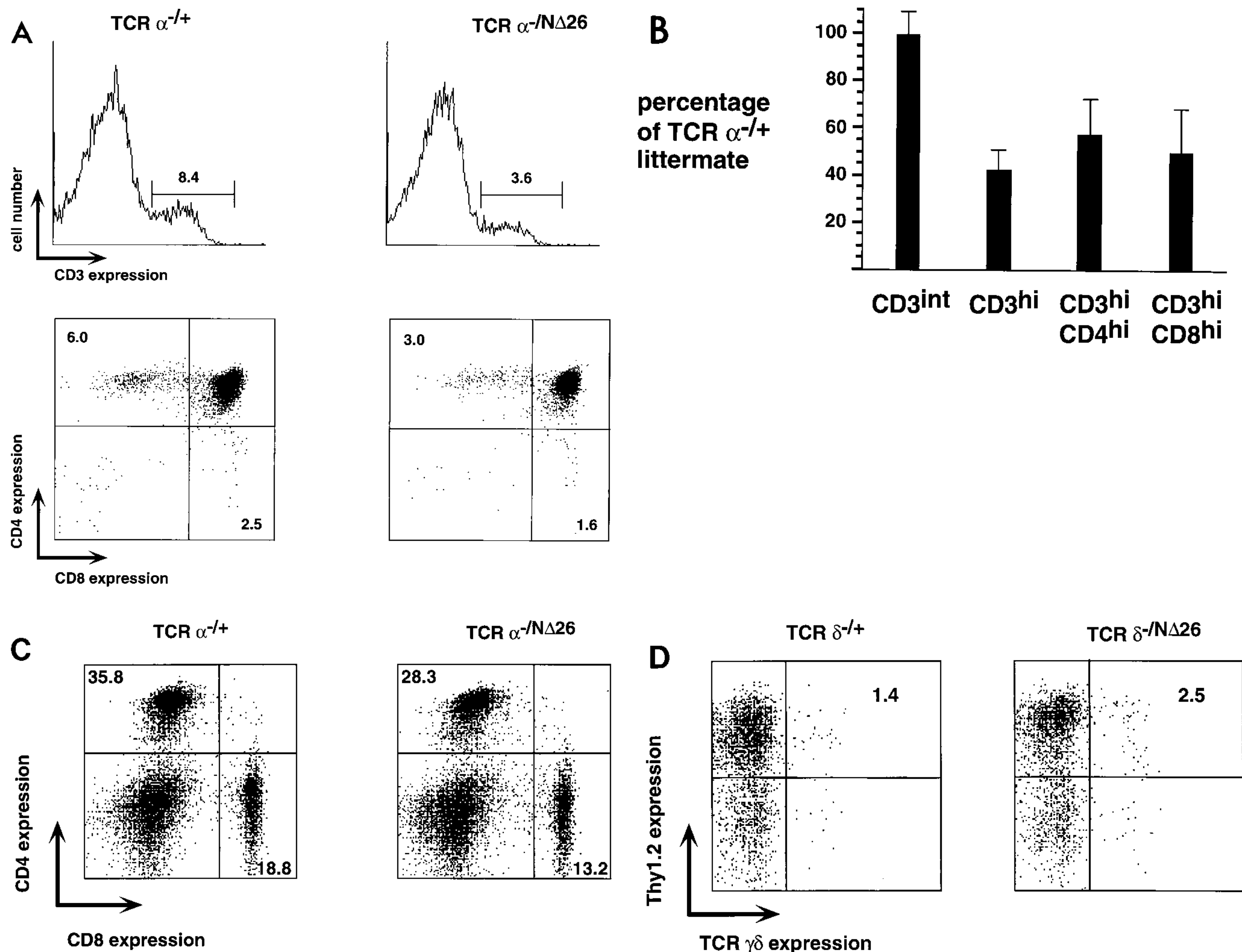

FIG. 2. Flow cytometry analysis of mice derived from crosses of NΔ26 heterozygous mice with either Cα$^{-/-}$ or Cδ$^{-/-}$ mice. (A) Representative analysis of thymocytes from a Cα$^{-/NΔ26}$ mouse (right) compared to those from a Cα$^{-/+}$ littermate (left). The top panels show staining for CD3 with the percentage of high-expressing cells indicated. The bottom panels show staining for CD4 and CD8 with the percentage of total cells indicated. (B) Data from multiple experiments as in panel A combined to generate a graph quantifying the effect of the NΔ26 allele on numbers of mature (CD3 high) cells versus immature (CD3 intermediate) cells and CD4 single-positive cells versus CD8 single-positive cells. The cell numbers from phenotypically wild-type Cα$^{-/+}$ littermates were set as 100%. A total of 13 compound heterozygotes were analyzed. (C) Representative analysis of lymph node cells from Cα$^{-/+}$ (left) and Cα$^{-/NΔ26}$ (right) mice. (D) Splenocytes from Cδ$^{-/+}$ and Cδ$^{-/NΔ26}$ mice were stained for the T-cell marker Thy1.2 and the γδ TCR. The percentage of cells positive for both (γδ T cells) is indicated.

thymocyte numbers were also normal (data not shown), as expected, since the majority of thymocytes are immature (intermediate for both TCRα and CD3 and high for both CD4 and CD8) and not dependent on TCRα expression for their formation. The reductions in mature-thymocyte numbers were equal in the CD4 and CD8 lineages of mature T cells. A less severe phenotype was observed in the peripheral compartments of mature T cells: staining of lymph node cells usually showed more than 50% of wild-type numbers of mature T cells (Fig. 2C). A similar analysis of Cδ$^{-/NΔ26}$ mice showed that TCRδ surface expression from the mutant allele was not reduced compared to the wild type in the spleen (Fig. 2D).

RNase protection assays specific to the NΔ26 allele. The second approach we took to study TCR expression from the NΔ26 allele was RNase protection. To distinguish gross levels of transcription from the wild-type allele and the NΔ26 allele in TCR-expressing tissues, we analyzed RNA from TCR Cα$^{-/NΔ26}$ mice. The transcript from the Cα^{-} allele leads to a truncated protected fragment (referred to as TCRα/neo in Fig. 3), and thus we can differentiate messages from individual alleles. There was no difference in levels of Cα RNA from either allele in whole thymus tissue (Fig. 3, left panel, lane 2). Furthermore, there was no difference in the RNA level from this NΔ26 allele compared to a littermate with a single wild-type allele (compare lanes 1 and 2). We also tested TCRδ transcription by using a probe which would not detect messages from the TCR Cδ^{-} allele. Comparing levels of TCRδ in RNA from TCR Cδ$^{-/NΔ26}$ and TCR Cδ$^{-/+}$ mice, we again saw no differences (Fig. 3, right panel). These results agree with the flow cytometry data, which implied that the levels of transcription in surviving cells are unaffected by the NΔ26 mutation. To further investigate expression from the NΔ26 allele, we looked for deregulated expression of TCRα in heterozygous NΔ26 mice. By using +/NΔ26 RNA in RNase protection assays, we saw that transcripts containing the TCRα constant region from the NΔ26 allele were still T cell specific (see Fig. 5A). In particular, we did not observe significant expression of TCRα in the bone marrow, which is rich in developing B cells that express the

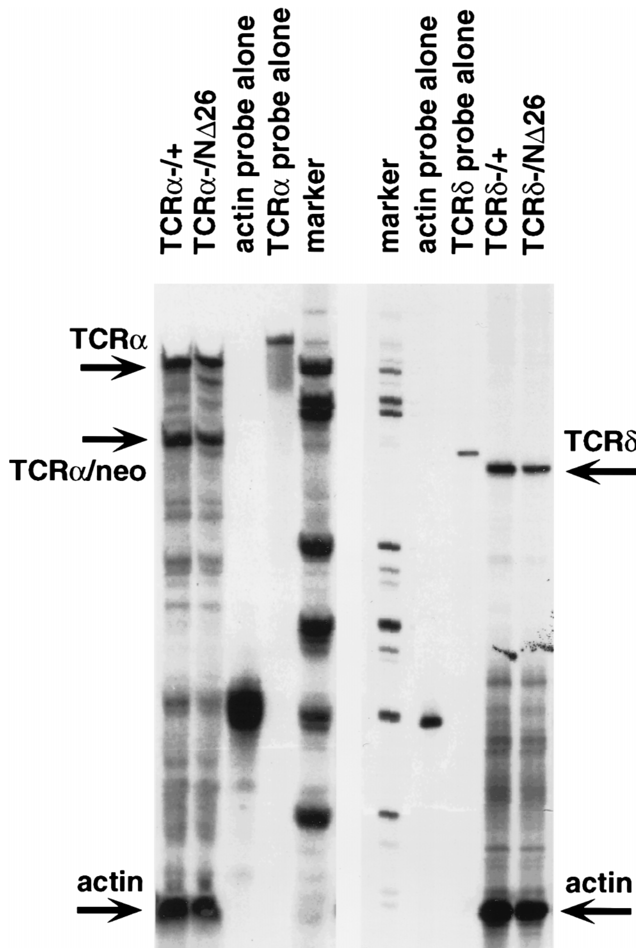

FIG. 3. RNase protection analysis of transcription from the NΔ26 allele. (Left) Analysis of TCR Cα transcription in $C\alpha^{-/N\Delta26}$ and $C\alpha^{-/+}$ mice. The Cα riboprobe differentiated the TCR $C\alpha^{-}$ message (TCRα/neo) from the full-length message (TCRα). (Right) Comparison of TCR Cδ transcription in $C\delta^{-/N\Delta26}$ and $C\delta^{-/+}$ mice. The TCR Cδ riboprobe did not detect the message from the $C\delta^{-}$ allele. The message from the γ-actin probe was used as the internal standard to quantitate TCR message levels.

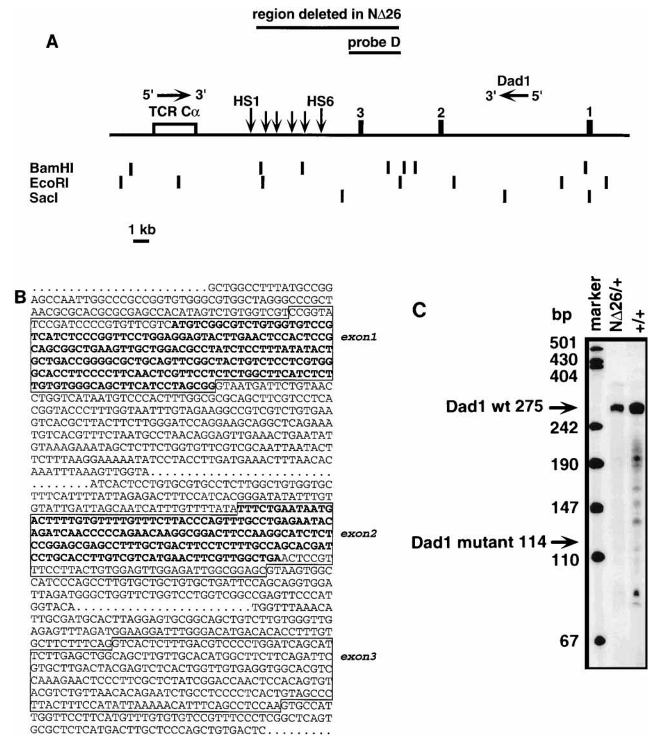

FIG. 4. (A) Genomic organization of the *Dad1* gene. Restriction sites are indicated (B, *Bam*HI; E, *Eco*RI; S, *Sac*I), as are the probe used to screen the cDNA library (probe D) and the region deleted on the NΔ26 allele. (B) Genomic sequences of the mouse *Dad1* gene. Exon boundaries (boxes) and coding sequences (boldface) are indicated. We based the outer ends of the 5′ and 3′ untranslated sequences on the cDNA clone we obtained from our screen. (C) RNase protection assay of NΔ26 heterozygous liver RNA with a probe against exons 2 and 3 of the *Dad1* gene. The NΔ26 allele encodes a transcript containing exon 2 but not exon 3, which would yield a protected fragment of 114 bp which we did not observe.

RAG genes required for rearrangement of immunoglobulin and TCR genes (25). This indicates that the LCR regulatory sequences are not required for tissue-specific TCR recombination. Together with the flow cytometry data, these results suggest that the LCR is required to enhance TCRα transcription in a fraction of immature T cells and deletion of this LCR leads to their inability to upregulate TCRα upon positive selection and their subsequent elimination. In contrast, in other immature T cells and in non-T cells, the LCR is not required for proper regulation of TCRα in the context of our mutant allele. Therefore, the NΔ26 allele is only partially defective in TCRα gene regulation.

Antiapoptosis gene *Dad1* is found 12 kb downstream of the TCRα constant region. The early lethality of NΔ26 mice suggested the presence of another gene in the deleted region, more likely in the sequences downstream of the HS than in the HS sequences themselves. By using a 3.5-kb genomic DNA probe (probe D in Fig. 4A) which encompasses such DNA, we detected a single message in various RNA samples on preliminary Northern blots (data not shown). Based on this result, we used this probe to screen a cDNA library. This led to the isolation of a clone encoding antiapoptosis protein DAD1 (24). This cDNA clone was the same size as the signal detected on the previous Northern blots and contained the entire *Dad1* open reading frame and the 3′ untranslated region (UTR). We mapped the genomic organization of the *Dad1* gene by using the *Dad1* cDNA as a probe against digested genomic DNA downstream of TCRα and by sequencing such DNA which flanked *Dad1* exons. Our analysis revealed that *Dad1* consists of three dispersed exons oriented in the opposite transcriptional direction from TCR α/δ, with the third exon lying less than 3 kb away from HS6. Our mutation deleted the third exon of *Dad1*, which encodes the 3′ UTR (Fig. 4A and B). This mutated *Dad1* allele does not lead to a detectable transcript, as shown by RNase protection assays using a probe which would protect a 114-bp mutant fragment (Fig. 4C). The characterization of the developmental phenotype of the *Dad1* null mutants will be described elsewhere.

Expression of mouse *Dad1* is distinct from that of the TCR genes. *Dad1* was originally identified as a temperature-sensitive mutation which resulted in the disappearance of the DAD1 protein and subsequent apoptosis of a hamster cell line (24). The human *Dad1* gene is expressed at high levels in many adult tissues. To determine its expression pattern in mice, we first employed RNase protection on various adult tissues with the *Dad1* probe and a γ-actin standard (Fig. 5A). This assay revealed *Dad1* expression in all of the cell types tested, with some degree of variation in levels in different tissues. We next looked for the presence of *Dad1* RNA in whole embryos (Fig.

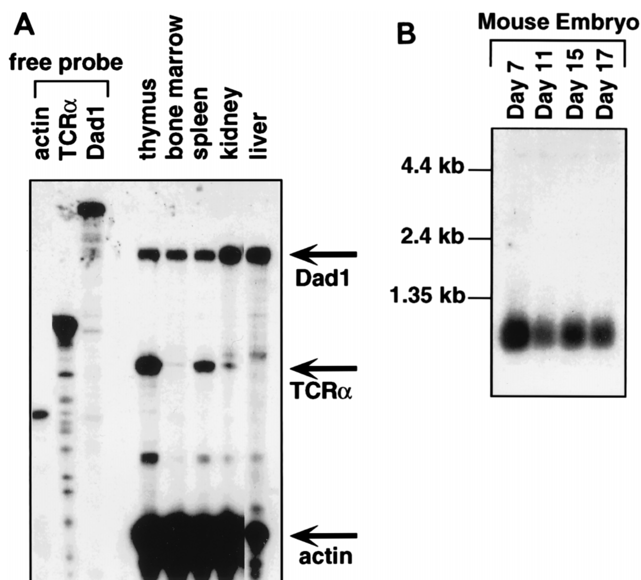

FIG. 5. Analysis of mouse *Dad1* expression patterns. (A) RNase protection assay showing *Dad1* levels in adult mouse tissues. We used NΔ26 heterozygous RNA so we could simultaneously examine TCR Cα levels from the NΔ26 allele in non-T-cell tissues. A γ-actin probe was also used in this assay. (B) Northern blot of mouse embryo poly(A)$^+$ RNA with a *Dad1* cDNA probe.

5B). The expected 0.9-kb *Dad1* message was present at all stages of mouse development examined (embryonic days 7, 11, 15, and 17). Significantly, this indicates that *Dad1* is turned on much earlier during embryogenesis than TCRδ (day 14) or TCRα (day 16) (11).

DNase hypersensitivity of the LCR changes in different tissues. To further investigate the regulation of the TCR α/δ and *Dad1* genes in vivo, we performed DNase I hypersensitivity assays with nuclei from various mouse tissues (Fig. 6). In contrast to a previous analysis of this region which did not detect HS in a B-cell line (6), ours detected HS in all of the cell types examined. In thymocyte chromatin (left panel), multiple HS were detected in a 10-kb region 3′ of the TCRα constant region, with the strongest sites mapping to the previously defined α enhancer and HS6, as was observed in the T-cell line.

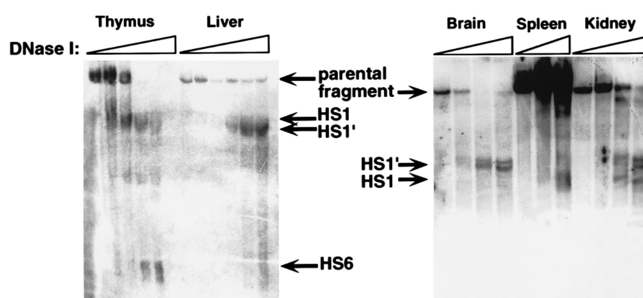

FIG. 6. DNase I hypersensitivity of the DNA between TCRα and *Dad1*. (A) Thymocyte and liver nuclei subjected to limited DNase I digestion and subsequent Southern blotting. This Southern blot was generated by digesting DNA to completion with *Sac*I and probing it with a 0.8-kb *Sma*I genomic fragment (probe B in Fig. 1A). The probe is at the TCR Cα-distal end of the parental fragment. (B) DNase I hypersensitivity assays performed on nuclei of brain, spleen, and kidney cells. These samples were digested to completion with *Nhe*I and probed with an *Nhe*I-*Xho*I probe from the TCRα enhancer region (probe C in Fig. 1A). Since the probe is at the TCR Cα-proximal end of the parental fragment, this blot indicates that HS1′ is farther from Cα than is HS1.

Strikingly, in other tissues, such as brain, liver, and kidney, hypersensitivity was also detected. The positions and intensities of these HS varied between tissues. For example, the hypersensitivity around the α enhancer (right panel) is clearly different between T-cell-containing tissues (HS1) and non-T-cell-containing tissues (HS1′). However, no simple correlation between the positions of these HS and the level of *Dad1* RNA emerged in specific tissues. The presence of these HS in non-T cells thus does not correlate with TCR expression, which is limited to T cells but, instead, correlates with *Dad1* expression. This strongly suggests that the *Dad1* gene is regulated by *cis*-acting sequences which overlap those regulating TCRα.

DISCUSSION

Because the TCRα and TCRδ genes have distinct expression patterns during T-cell development, it is interesting to consider how they are regulated within the same locus. The presence of an LCR downstream of these two genes suggests that they could share this regulatory element in a manner similar to the well-studied interactions between the β-globin genes and their LCR. Deletions of the human β-globin LCR which leave the proximal promoters and enhancers intact lead to a transcriptional shutdown of all globin genes in the deleted locus and greatly reduced chromatin sensitivity to DNase I (10). Our NΔ26 mutation, which deletes the LCR while leaving the transcriptional enhancer intact, did not lead to a complete shutdown of transcription of TCRα and had no gross negative effects on TCRδ. Instead, we observed a consistent decrease in the number of cells expressing high levels of TCRα. In light of recent data about the role of enhancers and LCRs in increasing the likelihood of high-level transcription (8, 34), it could be that in the wild-type locus, the presence of an LCR increases the probability of transcription such that all immature T cells receiving the positive selection signal express high levels of TCRα. However, when the LCR is deleted, high levels of TCRα transcription are still stochastically activated in a fraction of immature T cells in response to positive selection. No defect in high-level TCRα transcription was observed in NΔ26 heterozygous mice (data not shown); thus, the Dad1 gene product is not haploinsufficient.

Because the NΔ26 allele retains the neomycin resistance gene and its linked promoter, derived from the phosphoglycerate kinase 1 gene, it is possible that the partial phenotype we observed is due to actions of the strong promoter of this selection marker. The selectable marker is known to be expressed when integrated into most chromosomal locations, hence its utility in a variety of gene-targeting experiments. It must therefore have a fairly strong ability to overcome negative chromatin effects and could partially rescue TCRα (and perhaps TCRδ) expression by substituting as an LCR-like activity. While the selection marker has been observed in many cases to inhibit transcription or rearrangement, this inhibition most likely involves competition between the selection marker promoter and the endogenous promoters-enhancers for residual or complete LCR activity on the mutant allele (5, 9). In contrast, our mutation has most likely removed all TCRα LCR activity. Our confidence in this statement is bolstered by the presence of the gene *Dad1* just downstream of TCRα. We have removed all HS between the TCRα enhancer and *Dad1*, and therefore any compensating activity on the mutant allele most likely arises from outside the domain of TCRα proper. It will be quite informative to compare the effects of the NΔ26 mutation to those of a targeted mutation of the LCR from which the selectable marker has been removed. In the meantime, the tissue specificity of TCRα expression is intact on the NΔ26

allele, implying that the LCR is not required for this aspect of regulation. $C\alpha^{-/N\Delta 26}$ mice, with their impaired T-cell numbers, may be a useful model for studying immune responses to antigens.

The homozygous lethality of the NΔ26 mutation directly led to our identifying the *Dad1* gene downstream of the TCRα gene. This finding came as a surprise, considering that the TCR α/δ locus is one of the best-characterized segments of DNA in the entire genome. Over 100 kb have been sequenced in this locus in both mouse and human genomic DNAs, leading to insights into chromosomal organization (18). However, sequencing did not originally extend to the region containing *Dad1* exons. Because *Dad1* plays a role in preventing apoptosis, the precise genomic location of this gene has been of interest to those studying developmental diseases and cancer. Previous mapping studies of both the human (2, 38) and mouse (2) *Dad1* genes are consistent with our finding, although the proximity of *Dad1* to TCRα was not revealed by these approaches. The coding sequences of the *Dad1* gene are highly conserved between species (2, 24, 30, 31), even in organisms which do not have TCR genes. Therefore, the extent to which the location of *Dad1* next to TCR genes has been conserved throughout evolution would be an interesting aspect to investigate, especially as it relates to the question of gene regulation.

In our present study, we showed that *Dad1* expression starts earlier during mouse embryogenesis than that of the TCR genes and is wide and variable in adult cell types. Because *Dad1* is expressed in pattern so distinct from that of the TCR genes, it will be important to understand how the regulation of *Dad1* with respect to the TCR genes is accomplished. In our initial effort to understand this regulation, we have tested the DNase I hypersensitivity of the DNA between *Dad1* and TCRα from nuclei of multiple tissues. We detected various patterns of HS in all samples, suggesting that the B-cell line previously shown to lack HS (6) is a rare exception. The strong correlation of hypersensitivity with regulatory sequences makes it likely that these ubiquitous HS are involved in regulating *Dad1* transcription. The fact that the DNA spanning HS2 through HS6 can promote expression of a β-globin reporter gene in all tissues further supports this notion (26a). A deletion of the regulatory sequences between *Dad1* and TCRα which does not disturb the 3′ UTR of *Dad1* will definitively show whether or not these HS are required for *Dad1* transcription.

The data from previous (6) and current work has shown that these HS are required for aspects of TCRα expression; therefore, by extrapolation, we suggest that *Dad1* and TCRα share the same LCR. While it would be unusual for nonhomologous genes expressed in such different patterns to share regulatory sequences, it has been shown that an LCR can interact with more than one promoter at a time (4), so there is a precedent for the possibility that this type of regulatory element simultaneously drives the expression of *Dad1* and TCRα in T cells. The question would then become how TCR expression in non-T cells is prevented. A dynamic boundary element may exist in the cluster of non-T-cell-specific DNase I HS which separate mouse TCR α/δ and *Dad1*. This boundary element would function to keep TCRα chromatin shut in non-T cells, while in T cells, the α enhancer may be involved in disrupting this boundary such that the LCR can open chromatin in both directions, allowing TCR recombination and transcription, as well as *Dad1* transcription. Alternatively, the specificity of chromatin accessibility could arise as a result of interactions between the LCR and the T-cell-specific TCRα enhancer in T cells and the LCR and the *Dad1* promoter-enhancer in all cells. A DNA-binding protein complex able to disrupt chromatin in the appropriate region would be formed only in cells in which the LCR-binding and promoter-enhancer-binding proteins are coexpressed. We are currently testing these two models via the combined approaches of gene targeting and transgene analysis.

Finally, the proximity of *Dad1* to TCRα becomes more intriguing when we consider that *Dad1* was originally cloned by its ability to protect hamster cells from apoptosis (24). *Dad1* has also been shown to confer some protection from developmental programmed cell death when overexpressed in *Caenorhabditis elegans* (31), and its homolog in yeast is essential for viability (30). Our preliminary analysis of homozygous *Dad1* null mutants indicates that *Dad1* has a vital role early in embryogenesis (data not shown), and future studies of the homozygous mutants will shed light on this role. However, because apoptosis is a crucial process during the development of T cells, it will be especially interesting to understand the role of *Dad1* during T-cell development, as this will address the question of whether the location of the *Dad1* gene near TCRα has functional significance.

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REFERENCES

1. **Apel, T., A. Schere, T. Adachi, D. Auch, M. Ayane, and M. Reth.** 1995. The ribose 5-phosphate isomerase-encoding gene is located immediately downstream from that encoding murine immunoglobulin kappa. Gene **156:**191–197.
2. **Apte, S. S., M.-G. Mattei, M. F. Seldin, and B. R. Olsen.** 1995. The highly conserved defender against the death 1 (DAD1) gene maps to human chromosome 14q11-q12 and mouse chromosome 14 and has plant and nematode homologs. FEBS Lett. **323:**304–306.
3. **Bennani-Baiti, I. M., B. K. Jones, S. A. Liebhaber, and N. E. Cooke.** 1995. Physical linkage of the human growth hormone gene cluster and the skeletal muscle sodium channel α-subunit gene (SCN4A) on chromosome 17. Genomics **29:**647–652.
4. **Bresnick, E. H., and G. Felsenfeld.** 1994. Dual promoter activation by the human beta-globin locus control region. Proc. Natl. Acad. Sci. USA **91:**1314–1317.
5. **Chen, J., F. Young, A. Bottaro, V. Stewart, R. K. Smith, and F. W. Alt.** 1993. Mutations of the intronic IgH enhancer and its flanking sequences differentially affect accessibility of the JH locus. EMBO J. **12:**4635–4645.
6. **Diaz, P., D. Cado, and A. Winoto.** 1994. A locus control region in the T cell receptor α/δ locus. Immunity **1:**207–217.
7. **Enver, T., A. Brewer, and R. Patient.** 1985. Simian virus 40-mediated cis induction of the Xenopus β-globin DNase I hypersensitive site. Nature **318:**680–683.
8. **Festenstein, R., M. Tolaini, P. Corbella, C. Mamalaki, J. Parrington, M. Fox, A. Miliou, M. Jones, and D. Kioussis.** 1996. Locus control region function and heterochromatin-induced position effect variegation. Science **271:**1123–1125.
9. **Fiering, S., E. Epner, K. Robinson, Y. Zhuang, A. Telling, M. Hu, D. I. K. Martin, T. Enver, T. J. Ley, and M. Groudine.** 1995. Targeted deletion of 5′HS2 of the murine beta-globin LCR reveals that is not essential for proper regulation of the beta-globin locus. Genes Dev. **9:**2203–2213.
10. **Forrester, W. C., E. Epner, M. C. Driscoll, T. Enver, M. Brice, T. Papayannopoulou, and M. Groudine.** 1990. A deletion of the human beta-globin locus activation region causes a major alteration in chromatin structure and replication across the entire beta-globin locus. Genes Dev. **4:**1637–1649.
11. **Fowlkes, B. J., and D. M. Pardoll.** 1989. Molecular and cellular events of T cell development. Adv. Immunol. **44:**207–264.
12. **Gill, L. L., D. Zaninetta, and K. Karjalainen.** 1991. A transcriptional enhancer of the mouse T cell receptor delta gene locus. Eur. J. Immunol. **21:**807–810.
13. **Hanscombe, O., D. Whyatt, P. Fraser, N. Yannoutsos, D. Greaves, N. Dillon, and F. Grosveld.** 1991. Importance of globin gene order for correct developmental expression. Genes Dev. **5:**1387–1394.
14. **Ho, I. C., and J. M. Leiden.** 1990. Regulation of the human T-cell receptor alpha gene enhancer: ubiquitous and T-cell-specific nuclear proteins interact

with four hypomethylated enhancer elements. Mol. Cell. Biol. **10:**4720–4727.
15. **Hug, B. A., R. L. Wesselschmidt, S. Fiering, M. A. Bender, E. Epner, M. Groudine, and T. J. Ley.** 1996. Analysis of mice containing a targeted deletion of β-globin locus control region 5′ hypersensitive site 3. Mol. Cell. Biol. **16:**2906–2912.
16. **Itohara, S., P. Mombaerts, J. Lafaille, J. Iacomini, A. Nelson, A. R. Clarke, M. L. Hooper, A. Farr, and S. Tonegawa.** 1993. T cell receptor delta gene mutant mice: independent generation of alpha beta T cells and programmed rearrangements of gamma delta TCR genes. Cell **72:**337–348.
17. **Joulin, V., D. Bories, J. F. Eleouet, M. C. Labastie, S. Chretien, M. G. Mattei, and P. H. Romeo.** 1991. A T-cell specific TCR delta DNA binding protein is a member of the human GATA family. EMBO J. **10:**1809–1816.
18. **Koop, B. F., and L. Hood.** 1994. Striking sequence similarity over almost 100 kilobases of human and mouse T-cell receptor DNA. Nat. Genet. **7:**48–53.
19. **Lauzurica, P., and M. S. Krangel.** 1994. Enhancer-dependent and -independent steps in the rearrangement of a human T cell receptor delta transgene. J. Exp. Med. **179:**43–55.
20. **Lerner, A., L. D'Adamio, A. C. Diener, L. K. Clayton, and E. L. Reinherz.** 1993. CD3 zeta/eta/theta locus is colinear with and transcribed antisense to the gene encoding the transcription factor Oct-1. J. Immunol. **151:**3152–3162.
21. **Martin, D. I., S. Fiering, and M. Groudine.** 1996. Regulation of β-globin gene expression: straightening out the locus. Curr. Opin. Genet. Dev. **6:**488–495.
22. **Mombaerts, P., A. R. Clarke, M. L. Hooper, and S. Tonegawa.** 1991. Creation of a large genomic deletion at the T-cell antigen receptor beta-subunit locus in mouse embryonic stem cells by gene targeting. Proc. Natl. Acad. Sci. USA **88:**3084–3087.
23. **Mombaerts, P., A. R. Clarke, M. A. Rudnicki, J. Iacomini, S. Itohara, J. J. Lafaille, L. Wang, Y. Ichikawa, R. Jaenisch, and M. L. Hooper.** 1992. Mutations in T-cell antigen receptor genes alpha and beta block thymocyte development at different stages. Nature **360:**225–231.
24. **Nakashima, T., T. Sekiguchi, A. Kuraoka, K. Fukushima, Y. Shibata, S. Komiyama, and T. Nishimoto.** 1993. Molecular cloning of a human cDNA encoding a novel protein, DAD1, whose defect causes apoptotic cell death in hamster BHK21 cells. Mol. Cell. Biol. **13:**6367–6374.
25. **Oettinger, M. A., D. G. Schatz, C. Gorka, and D. Baltimore.** 1990. RAG-1 and RAG-2, adjacent genes that synergistically activate recombination. Science **248:**1517–1523.
26. **Olson, E. N., H. H. Arnold, P. W. Rigby, and B. J. Wold.** 1996. Know your neighbors: three phenotypes in null mutants of the myogenic bHLH gene MRF4. Cell **85:**1–4.
26a. **Ortiz, B. D., D. Cado, and A. Winoto.** Unpublished data.
27. **Ramirez-Solis, R., A. Davis, and A. Bradley.** 1993. Gene targeting in embryonic stem cells. Methods Enzymol. **225:**855–878.
28. **Redondo, J. M., S. Hata, C. Brocklehurst, and M. S. Krangel.** 1990. A T cell-specific transcriptional enhancer within the human T receptor delta locus. Science **247:**1225–1229.
29. **Siebenlist, U., L. Henninghausen, J. Battey, and P. Leder.** 1984. Chromatin structure and protein binding in the putative regulatory region of the c-myc gene in Burkitt lymphoma. Cell **37:**381–391.
30. **Silberstein, S., P. G. Collins, D. J. Kelleher, and R. Gilmore.** 1995. The essential *OST2* gene encodes the 16-kD subunit of the yeast oligosaccharyltransferase, a highly conserved protein expressed in diverse eukaryotic organisms. J. Cell Biol. **131(2):**371–383.
31. **Sugimoto, A., R. R. Hozak, T. Nakashima, T. Nishimoto, and J. H. Rothman.** 1995. dad-1, an endogenous programmed cell death suppressor in Caenorhabditis elegans and vertebrates. EMBO J. **14:**4434–4441.
32. **Tybulewicz, V., C. Crawford, P. Jackson, R. Bronson, and R. Mulligan.** 1991. Neonatal lethality and lymphopenia in mice with a homozygous disruption of the c-abl proto-oncogene. Cell **65:**1153–1163.
33. **Vyas, P., M. A. Vickers, D. J. Picketts, and D. R. Higgs.** 1995. Conservation of position and sequence of a novel, widely expressed gene containing the major human α-globin regulatory element. Genomics **29:**679–689.
34. **Walters, M. C., W. Magis, S. Fiering, J. Eidemiller, D. Scalzo, M. Groudine, and D. I. K. Martin.** 1996. Transcriptional enhancers act in cis to suppress position-effect variegation. Genes Dev. **10:**185–195.
35. **Winoto, A., and D. Baltimore.** 1989. Alpha-beta lineage-specific expression of the alpha T cell receptor gene by nearby silencers. Cell **59:**649–655.
36. **Wu, C.** 1989. Analysis of hypersensitive sites in chromatin. Methods Enzymol. **170:**269–289.
37. **Xie, W. Q., and L. I. Rothblum.** 1991. Rapid, small-scale RNA isolation from tissue culture cells. BioTechniques **11:**324, 326–327.
38. **Ylug, I. G., C. G. See, and E. M. C. Fisher.** 1995. The DAD1 protein, whose defect causes apoptotic cell death, maps to human chromosome 14. Genomics **26:**433–435.