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Distal V_β promoters transcribe novel T-cell receptor-β transcripts in early development

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SUMMARY

The transcriptional activation of germline T-cell receptor (TCR) and immunoglobulin (Ig) genes has been proposed to promote the rearrangement of these genes. Here we report the identification of distal TCR promoters (PDs), located upstream of the previously characterized promoters in the mouse $V_{\beta5.1}$ and $V_{\beta8.1}$ gene segments, that are active in germline TCR genes in fetal thymus and liver *in vivo*. We also identified an immature T-cell clone, SL12.4, that expresses both endogenous and transfected PDs in a regulated manner *in vitro*. We propose that the transcription of these distal promoters in germline TCR genes may be important for inducing TCR gene rearrangements during T-cell development. Northern blot, RNase protection and reverse transcription–polymerase chain reaction (RT–PCR) analyses demonstrated that PDs are also transcribed from fully rearranged TCR genes in adult thymus, lymph node, and spleen. Although the functional significance of this expression is not known, our sequence analysis of the 5′ leader in PD-derived $V_{\beta5.1}$ and $V_{\beta8.1}$ transcripts revealed the presence of several open reading frames (ORFs) that may encode novel polypeptides or regulate the efficiency of TCRβ translation.

INTRODUCTION

The differentiation of B and T lymphocytes depends upon the successful rearrangement and expression of T-cell receptor (TCR) and immunoglobulin (Ig) genes containing complete open reading frames (ORFs). Expression of such rearranged TCR genes is driven by the activation of both promoter and enhancer elements.[1] Germline transcripts have also been detected from unrearranged copies of TCR and immunoglobulin genes.[2–5] It has been proposed that activation of the promoters that transcribe these 'germline mRNAs', favour the opening of chromatin structures at TCR and immunoglobulin loci, thus increasing their accessibility to recombination enzymes.[2] Germline promoters that activate gene rearrangement are predicted to exhibit transcriptional activity early in lymphocyte ontogeny and display cell-lineage specificity. Consistent with the first prediction, germline transcripts have been detected by reverse transcription–polymerase chain reaction (RT–PCR) at the earliest stages of T-lymphocyte development, before the onset of TCR gene rearrangement.[5] The cell-lineage specificity of germline V_β expression is evident from the observation that transcripts are detected in lymphoid tissues, such as fetal thymus and bone marrow, but not in non-lymphoid tissues.[5]

Although there have been several reports describing V_β germline transcripts, to our knowledge no studies have identified the promoters used to transcribe these germline transcripts. The only known V_β promoters are located in the leader exon upstream of each V_β element. The transcription initiation sites for these promoters are located typically less than 60 nucleotides from the initiator ATG that defines the TCRβ reading frame.[6] These promoters drive transcription of fully rearranged genes in both immature thymocytes and mature T lymphocytes.[1] In the case of non-rearranged germline genes, it is not clear how such enhancer-dependent V_β promoters could be activated by the TCRβ enhancer, as this positive regulatory element is 3′ of $C_{\beta2}$, far downstream of most V_β elements prior to gene rearrangement. Because germline V_β transcripts are still expressed when the TCRβ enhancer is inactivated by targeted deletion,[7] it is possible that other promoters are important for driving the expression of germline transcripts.

Here we report the identification and characterization of distal promoters (PDs) upstream of the previously described promoters. Because these PDs are enhancer independent and are active early in thymocyte ontogeny, they may be responsible for the transcription of germline V_β genes prior to the activation of the TCRβ enhancer.

MATERIALS AND METHODS

RNA preparation and analysis

RNA was prepared from tissues of BALB/C and SJL/J mice obtained from Jackson Laboratories (Bar Harbor, ME).

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Abbreviations: ORF, open reading frame; p.c., post coitum; PD, distal promoter; PP, proximal promoter; RPA, RNase protection analysis; RT, reverse transcriptase.

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Fetuses were harvested at 14·5, 16·5 and 17·5 days of gestation. Adult mice were killed at 8 weeks of age. Splenocytes from adult mice were incubated with 10 μg/ml concanavalin A (Con-A) in RPMI-1640 media with 10% fetal calf serum (FCS) for 3 days.[8] Total cellular RNA was isolated from tissues and cell lines either by guanidinium isothiocyanate lysis and centrifugation over a CsCl cushion[9] or by a guanidinium thiocyanate/phenol/chloroform extraction procedure.[10] Northern blot hybridization and RNase protection analysis (RPA) were performed as described previously.[11,12] Probes for Northern blot analysis and RPA were generated from mouse $V_{\beta5.1}$ and $V_{\beta8.1}$ cDNAs, $C_{\beta1}$ genomic DNA (provided by E. Palmer, Basel Institute for Immunology, Basel, Switzerland), and $V_{\beta5.1}$ and $V_{\beta8.1}$ genomic clones (provided by D. Loh, Washington University, St. Louis, MO). The $C_{\beta1}$-specific (3′ untranslated region), $V_{\beta8.1}$-specific (coding region), and $V_{\beta5.1}$ PD-specific (genomic region upstream of the PP) probes are 0·4 kb *Eco*RI/*Hind*III, 0·29 kb *Eco*RI/*Pvu*II, and 0·4 kb *Sac*I/*Sca*I fragments, respectively. For RPA, the following probes were used: a 0·2 kb *Sac*I/*Acc*I fragment from the $V_{\beta5.1}$ PD, and a 0·7 kb *Nde*I/*Sty*I fragment from the $V_{\beta8.1}$ PD.

RT-PCR analyses

To remove residual genomic DNA contaminants from the RNA preparations, all RNA samples were treated with 1 U of DNase I for 30 min at 37° in 10 mM Tris-HCl (pH 8·3), 1·5 mM $MgCl_2$, and 50 mM KCl. cDNA was prepared from 2 μg RNA by using an oligo-dT primer and Superscript II reverse transcriptase according to the manufacturer's instructions (GIBCO BRL, Grand Island, NY). All PCR reactions were performed with 1 U Amplitaq polymerase (Perkin Elmer, Foster City, CA), 10 mM Tris-HCl (pH 8·3), 1·5 mM $MgCl_2$, 50 mM KCl, 0·2 mM deoxynucleotides, and 10 pmol of each primer. The cycling conditions for the $V_{\beta5.1}$ primers were 30 seconds at 95°, 30 seconds at 60° and 30 seconds at 72° for 30–40 cycles. The primers used were 5′-TGAAGAGGACTTTCCCACAC-3′ (primer A, forward primer within the PD transcript 5′ untranslated region), 5′-TACATCACCAGCTGGAACAGA-3′ (primer B, reverse primer 30 nt. downstream of $V_{\beta5.1}$); and 5′-GAGAGCTCAACAAGGAGACCTT-3′ (primer C, reverse primer in exon 1 of $C_{\beta1}$). The PCR products were separated on 1% agarose gels and blotted onto Nytran membranes (Schleicher & Schuell, Keene, NH). Southern blot analysis was performed with a 0·20 kb *Eco*RI/*Rsa*I fragment corresponding to the $V_{\beta5.1}$ coding region.

Cell culture and transfection

The cell clone SL12.4 and its culturing requirements have been described.[11] The cells were incubated with 100 μg/ml cycloheximide or 1 μg/ml pactamycin for 6 hr, unless otherwise noted. The $V_{\beta8}$ enhancer$^-$ and $V_{\beta8}$ enhancer$^+$ constructs transfected into SL12.4 cells contained $V_{\beta8.2}C_{\beta1}$ sequences [provided by J. Kaye (The Scripps Research Institute, La Jolla, CA)] and $V_{\beta8.1}C_{\beta2}$ [provided by M. Blackman, P. Marrack and J. Kappler (Howard Hughes Medical Institute, Denver, CO)], respectively. Stable transfection was performed by electroporating 20–40 μg of DNA in 1 × Hepes-buffered saline (140 mM NaCl, 1.5 mM Na_2HPO_4, and 50 mM Hepes, pH 7·005) with a Moonlight Cat Door electroporator (Seattle, WA) at 2900–3200 V. After transfection, the cell suspension was split among the wells of a 24-well plate and G418 (800 μg/ml) was added to the cultures the following day. The cells were selected for G418 resistance for 2–3 weeks, and Northern blot analysis was performed to determine which cell lines expressed the transfected DNA.

DNA sequencing

$V_{\beta5.1}$ and $V_{\beta8.1}$ genomic DNA was sequenced on both strands with Sequenase according to the manufacturer's instructions (USB, Cleveland, OH). The sequence was assembled and analysed with the GCG Wisconsin package (GCG, Madison, WI).

RESULTS

Distal V_β promoters active in fetal and adult lymphoid tissues

Northern blot analysis of TCRβ mRNAs expressed in fetal thymus revealed the presence of 1·7 kb transcripts, in addition to the previously described 1·0 kb and 1·3 kb mRNAs transcribed from partially and fully rearranged TCRβ genes, respectively.[13–19] The 1·7 kb transcripts hybridized with both $C_{\beta1}$- and $C_{\beta2}$-specific probes (Fig. 1a and data not shown). They were expressed at least as early as day 14·5 post coitum (p.c.) and remained at nearly constant levels throughout thymic ontogeny. In contrast, the 1·0 kb and 1·3 kb transcripts rose sharply in levels late in fetal development (Fig. 1a), in agreement with our previous observations and those of others.[8,17–19] To determine whether the 1·7 kb transcripts were derived from rearranged $V_\beta C_\beta$ genes, we assessed whether they hybridized with a $V_{\beta8.1}$ probe known to hybridize with 1·3 kb transcripts from fully rearranged $V_{\beta8}$ genes but not 1·0 kb transcripts from partially rearranged genes that have only undergone D-J rearrangement. The $V_{\beta8.1}$ probe hybridized with the 1·7 kb transcripts (Fig. 1b), suggesting that at least some of these mRNAs were transcribed from fully rearranged genes.

Our subsequent analysis showed that the 1·7 kb mRNAs were derived from distal promoters (PDs) upstream of those that we now define as proximal promoters (PPs) that transcribe the well-characterized 1·3-kb TCR mRNAs. This was shown first using a probe from a region upstream of the previously described $V_{\beta5.1}$ PP promoter. This upstream probe hybridized with the 1·7 kb transcripts in both fetal thymus and ConA-stimulated splenocytes (Fig. 1c).

We used RNase protection analysis (RPA) as an independent assay of the existence of PDs and to map their transcription initiation sites. Analysis with a $V_{\beta5.1}$ genomic probe that spans the previously characterized PP and sequences 0·6 kb upstream (a 0·7 kb *Bgl*II/*Eco*RI fragment) revealed two sets of bands; one corresponding to the PP and the other to a PD (data not shown). The size of the bands corresponded to transcription start sites of ≈ -40 and ≥ -400 for the PP and PD, respectively, relative to the initiator ATG that defines the known TCR reading frame. To more precisely assess the transcriptional initiation sites for the PD, we used a smaller probe for RPA and found multiple bands that correspond to several transcriptional initiation sites in the PD (Fig. 2a). The lengths of the major protected bands in thymus and spleen correspond to PD transcription start sites between approximately -473 and -403 (Fig. 2a). Inspection of the sequence upstream of these initiation sites revealed no TATA box (see

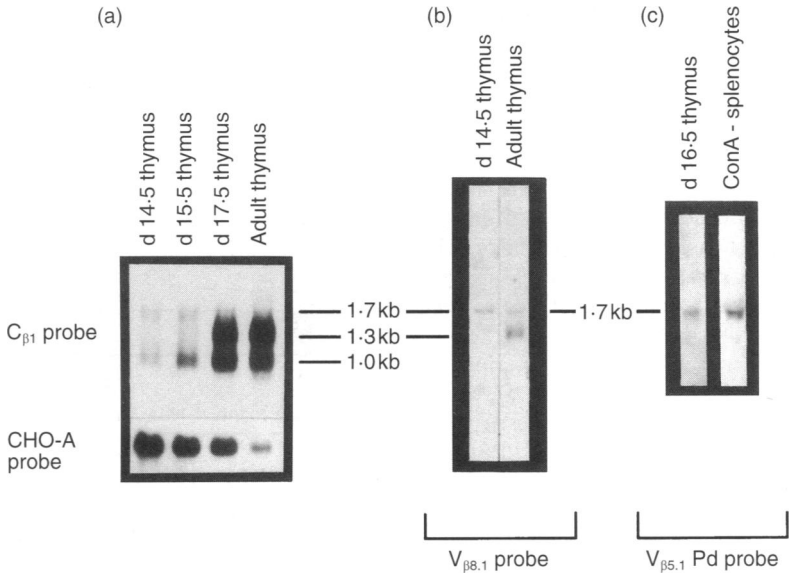

Figure 1. Developmental expression of mRNAs transcribed from proximal and distal V_β promoters. Northern blot analysis of total cellular RNA from thymic tissue (the day of ontogeny for fetal thymus is shown). (a) The 1·0-kb mRNAs are transcripts from D_β and J_β promoters; the 1·3-kb mRNAs are PP V_β-derived transcripts; and the 1·7-kb mRNAs are PD V_β-derived transcripts. Hybridization with the CHO-A probe[29] shows the amount of RNA loaded. All lanes contained 10 μg of RNA except for the adult thymus lane, which contained only 2 μg to permit evaluation of the transcripts at the exposure time shown. (b) The $V_{\beta 8}$-specific probe hybridized with both 1·3 kb and 1·7 kb transcripts because it corresponds to the $V_{\beta 8.1}$ coding region. (c) The $V_{\beta 5.1}$ PD-specific probe (probe B from Fig. 5) hybridized with only 1·7 kb transcripts. All blots were stained with methylene blue to show integrity of RNA and amount of RNA loaded (as described in ref. 30; data not shown).

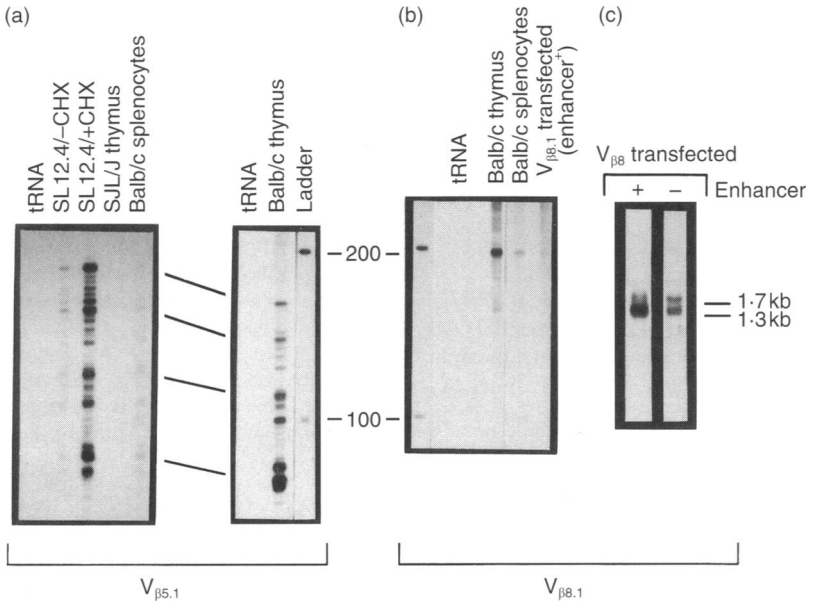

Figure 2. Expression and 5′ mapping of $V_{\beta 5.1}$ and $V_{\beta 8.1}$ mRNAs transcribed from distal promoters. Total cellular RNA (10 μg) from the cell lines and tissues shown was analysed by RPA (a–b) and Northern blot analysis (c). (a) RPA using a $V_{\beta 5.1}$-specific probe. −CHX, without cycloheximide; +CHX, with cycloheximide. (b) RPA using a $V_{\beta 8.1}$-specific probe. Shown is the expression of the enhancer$^-$ $V_{\beta 8}$ construct transfected stably into SL12.4 cells. Non-transfected SL12.4 cells did not express the $V_{\beta 8}$ sequences (data not shown). The integrity and amount of RNA used for RPA in panels (a)-(b) was assessed by Northern blot analysis followed by methylene blue staining of 18S and 28S rRNA (performed as described in ref. 30; data not shown). (c) Northern blot analysis of SL12.4 T cells stably transfected with enhancer$^+$ and enhancer$^-$ $V_{\beta 8}$ constructs (see the Materials and Methods for details). The $V_{\beta 8.1}$ coding region probe hybridized with both PP- (1·3 kb) and PD- (1·7 kb) derived transcripts.

later). Promoters that lack TATA boxes typically transcribe mRNAs from multiple start sites.[20] As a control to show the specificity of the $V_{\beta5.1}$ PD probe, we used the same probe to analyse expression from the SL12·4 T-lymphoma cell clone, which upregulates the levels of $V_{\beta5.1}C_{\beta1}$ transcripts after incubation with the protein synthesis inhibitor cycloheximide.[21] SL12.4 cells used the same multiple transcription start sites as lymphoid tissues, and that expression of transcripts from the multiple initiation sites was increased by cycloheximide. The specificity of the $V_{\beta5.1}$ PD probe was further assessed using a mouse strain that lacks the $V_{\beta5.1}$ gene.[22] As expected, the probe failed to detected transcripts in SJL/J mice (Fig. 2a).

Because we had also detected 1·7 kb $V_{\beta8}$ transcripts by Northern blot analysis, we assessed whether these were also transcribed from a PD. We performed RPA using a probe corresponding to the region upstream of the known PP region in $V_{\beta8.1}$. This analysis revealed that BALB/c thymus and spleen used a PD that contain a transcription initiation site at ≈ -513 nt (Fig. 2b). No signal was seen from SJL/J mice thymus (data not shown), as expected since this strain of mice lacks the $V_{\beta8.1}$ gene segment.[22]

Because 1·7 kb transcripts are expressed early in fetal ontogeny, before transcripts derived from enhancer-dependent promoters, we considered the possibility that PDs may differ from PPs in their requirement for the known enhancer element 3′ of $C_{\beta2}$. To test this, enhancer$^+$ and enhancer$^-$ constructs were stably transfected into SL12.4 T-lymphoma cells and their expression was assessed. Figure 2 shows representative data from two cell lines. The enhancer$^+$ construct expressed higher levels of 1·3 kb transcripts (PP-driven) than 1·7 kb (PD-driven) transcripts, as expected (Fig. 2c). RPA confirmed that the enhancer$^+$ construct was expressing PD-derived transcripts (Fig. 2b). The enhancer$^+$ construct contained $\approx$9 kb upstream of the $V_{\beta8.1}$ element and $\approx$8 kb downstream of the C_β element, demonstrating that such sequences are sufficient to direct both PD and PP transcription. The enhancer$^-$ construct expressed similar levels of 1·7 kb and 1·3 kb transcripts (Fig. 2c), demonstrating that neither the PD nor the PP was absolutely dependent on the TCRβ enhancer for expression.

$V_{\beta5.1}$ distal promoter active in germline genes

To further examine the expression of transcripts from PDs we employed RT–PCR analysis. To detect only those transcripts derived from a PD we designed a sense primer (primer A) that corresponds to the unique 5′ untranslated region of the $V_{\beta5.1}$ PD-derived transcripts (Fig. 3a). This primer was used in combination with primer C (specific for exon 1 of both $C_{\beta1}$ and $C_{\beta2}$) to amplify mRNA transcribed from TCRβ genes which had rearranged $V_{\beta5.1}$. This analysis showed that PD transcripts from fully rearranged genes were present in spleen (Spl), lymph node (LN), fetal thymus (d16.5 Thy), and adult thymus (Ad Thy) (Figs 3b and 3c). As expected, such transcripts were not detected in fetal liver or testis (Fig. 3b and data not shown). Since the Northern blot data suggested that the PD may be transcribed early in lymphocyte development, prior to the activation of the PP, we examined the use of the $V_{\beta5.1}$ PD in germline genes using primers A and B (Fig. 3a). We found that germline transcripts were expressed in both adult and fetal thymus, lymph node, and fetal liver, but not

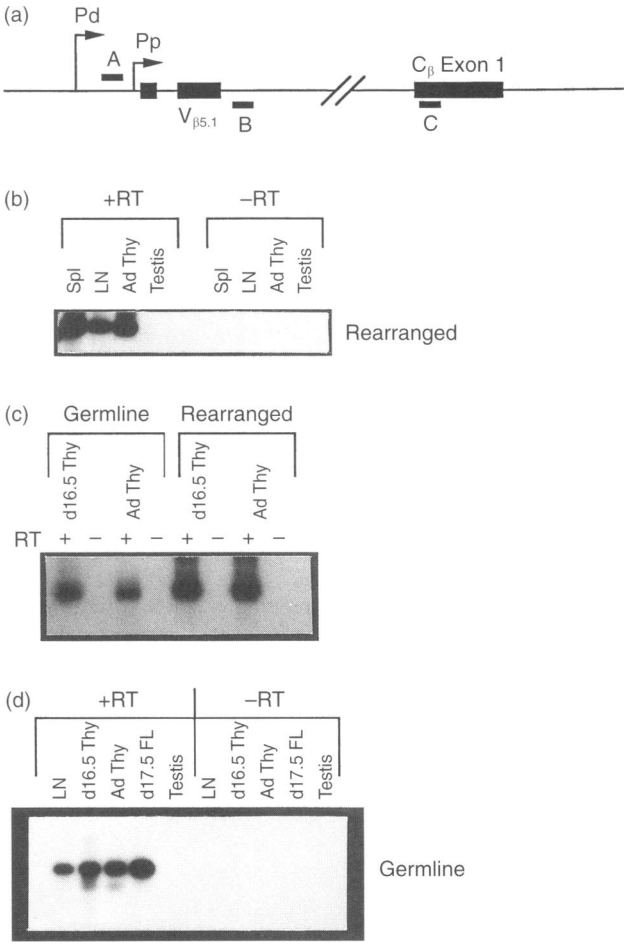

Figure 3. Distal promoter-driven V_β mRNAs transcribed from germline and rearranged genes. RT–PCR was performed to amplify PD-derived $V_{\beta5.1}$ transcripts. After gel electrophoresis, the PCR products were blotted and hybridized with a $V_{\beta5.1}$-specific probe. (a) The relative positions of the primers used for the analysis. The position of primer A is shown in Fig. 4. Primer C is equally complementary with $C_{\beta1}$ and $C_{\beta2}$. (b) Primers A and C were used to selectively amplify transcripts from fully rearranged TCRβ genes. (c) Amplification of transcripts from both germline (primers A and B) and rearranged genes from day 16·5 p.c. fetal and adult thymus. (d) Primers A and B were used to selectively amplify transcripts from germline TCRβ genes. LN, lymph node; Thy, Thymus (fetal and adult); FL, fetal liver; Spl, spleen. All cDNAs generated in the presence of RT (including those from testis) were shown to be capable of being efficiently amplified by PCR, based on amplification with primers specific for mouse β-actin (data not shown).

in the testis (Fig. 3c and d). The failure to observe germline transcripts derived from PD in the testis suggests this promoter possesses lymphoid specificity. The observation that germline transcripts derived from the PD can be detected in the fetal thymus and fetal liver provides evidence that this promoter is activated in the earliest T-lymphocyte progenitors. The presence of germline transcripts in secondary lymphoid tissues is in agreement with a study that described the expression of germline V_β transcripts by mature T cells.[3] Earlier studies on germline transcripts have assumed that such transcripts are derived from the PP. Our observations suggest that germline transcripts are also derived from the PD.

Upstream ORFs

To assess the potential consequences of the extended 5′ leader sequences in PD-transcribed mRNAs we determined the nucleotide sequences of $V_{\beta5.1}$ and $V_{\beta8.1}$ genomic DNA that corresponds to these regions (Fig. 4). The approximate sites of the PD and PP are shown in Fig. 4, along with the conserved decamer sequence known to be important for PP-derived transcription.[23,24] Also shown are significant ORFs defined by ATG initiator codons that closely match the Kozak consensus sequence; i.e. contain the conserved −3 purine and/or +4 G.[25] According to this analysis, two ORFs (encoding 36 and 39 amino acids) are present in the 5′ leader of PD-derived $V_{\beta8.1}$ transcripts. In contrast, $V_{\beta5.1}$ PD-derived transcripts contain only a single short ORF (encoding 7 amino acids) in the 5′ terminus. The significance of these upstream ORFs is not known; they may generate biologically active polypeptides, down-regulate translation from the downstream TCRβ reading frame, or both. Interestingly, $V_{\beta5.1}$ PD-derived transcripts uniquely possess an upstream ATG in-frame with the known $V_{\beta5.1}$ reading frame (Fig. 4). If translation initiates at this ATG it would produce a TCRβ protein with 23 additional amino acids at the amino terminus. This altered amino terminus would thus have an unusual signal peptide that could influence the intracellular trafficking of this TCRβ chain.

We hypothesized that the upstream ORFs in the 5′ leaders of PD-derived transcripts might trigger the rapid degradation of these transcripts as a result of nonsense-mediated down-regulation. Several investigators have shown that premature nonsense codons cause a decrease in mRNA steady-state levels by a post-transcriptional mechanism (for reviews, refs 26 and 27). To investigate the role of premature termination codons in regulating TCR mRNA levels, we used the SL12.4 cell clone that possesses a non-productively rearranged $V_{\beta5.1}J_{\beta1}.6C_{\beta1}$ gene.[11,21] By virtue of the frameshift in the $V_{\beta5.1}J_{\beta1}.6C_{\beta1}$ gene, it contains a premature termination codon in $C_{\beta1}$ exon 1, and thus $V_{\beta5.1}$ transcripts do not accumulate in SL12.4 cells.[11,14,21] Because we have shown previously that protein synthesis inhibitors reverse nonsense-mediated down-regulation,[21] we examined the effect of the protein synthesis inhibitors cycloheximide and pactamycin on the TCRβ transcripts expressed by the SL12.4 cell clone. Northern blot analysis revealed that incubation with these protein synthesis inhibitors up-regulated both PP-and PD-derived transcripts (1·3 kb and 1·7 kb, respectively) (Fig. 5a). To determine if the nonsense codons in the upstream ORFs of PD-derived transcripts were sufficient to trigger nonsense-mediated down-regulation we stably transfected SL12.4 cells with a productively rearranged TCRβ gene (lacking premature nonsense codons in the coding region). This gene construct expressed both PP-derived 1·3 kb transcripts and PD-derived 1·7 kb transcripts; the levels of these two transcripts were not significantly influenced by cycloheximide treatment. The inability of cycloheximide to up-regulate the 1·7 kb mRNA indicates that the upstream ORFs present in this PD-derived RNA do not trigger the nonsense-mediated regulatory pathway. We conclude that at least some PD transcripts are not subject to down-regulation as a result of their upstream ORFs. Therefore, these novel transcripts may be expressed at sufficiently high levels to play an important functional role in lymphocyte development.

DISCUSSION

The generation of germline transcripts from TCR and immunoglobulin variable region gene segments has been proposed to be a consequence of critical events necessary to initiate TCR and immunoglobulin gene rearrangements. The act of transcription may generate a more open chromatin structure to permit access by the recombination machinery. It has generally been assumed that these germline transcripts arise from classical enhancer-dependent TCR and immunoglobulin V promoters. However, germline TCRβ transcripts can still be detected at the earliest stages of thymocyte ontogeny when the TCRβ enhancer may be too distant in germline-configured genes to activate most known V_β promoters. In this study we identified novel TCRβ transcripts derived from a second class of promoters, termed PDs, located upstream of the previously described V_β promoters. PDs are able to drive the transcription of both germline and rearranged V_β genes in a TCRβ enhancer-independent manner. Germline transcripts derived from PDs are found in fetal liver, prior to the activation of the TCRβ enhancer and the onset of TCRβ gene rearrangement events.

Our sequence analysis revealed that at least four classes of proteins could be translated from PD-derived transcripts: (i) full-length TCRβ proteins; (ii) amino terminal-extended TCRβ proteins translated from in-frame start codons in the PD-specific 5′ leader; (iii) novel polypeptides encoded by ORFs in the PD-specific 5′ leader, and (iv) truncated V_β proteins translated from germline transcripts. With regard to this last category, a germline transcript has been identified that encodes a $V_{\beta8.2}$ polypeptide expressed on the cell surface.[4] Interestingly, this germline transcript contains $V_{\beta8.2}$ coding exons spliced to a $V_{\beta5.1}$ leader exon, and thus it may be transcribed from the $V_{\beta5.1}$ PD described here. The function of such V_β polypeptides is not known; they may act as surrogate receptors for immature thymocytes or they may participate in the establishment of tolerance by exposing developing thymocytes to multiple self V_β gene products.

Another consequence of the upstream ORFs in PD-derived transcripts is that these ORFs could inhibit the translation of TCR proteins. Several reports have shown that upstream ORFs depress the rate of translation from downstream reading frames in mammalian transcripts (reviewed, ref. 28). Thus, it is possible that some classes of PD-derived transcripts are infrequently translated and therefore they may represent a new class of 'sterile' transcripts. Other examples of sterile transcripts include those from immunoglobulin genes and the 1·0-kb $(D)J_\beta$ mRNAs transcribed from germline or D-J rearranged TCRβ genes.[2,8,16–19] Therefore, many PD transcripts may not encode functional proteins, but instead their expression is a consequence of the activation of distal promoters necessary to trigger programmed DNA rearrangement events. It could be advantageous that PD-derived transcripts are not efficiently translated, particularly in the case of germline PD transcripts, since these would encode potentially deleterious V_β polypeptides lacking D_β, J_β, and C_β sequences. Although the consequences of the expression of V_β polypeptides is not known, it is reasonable to suspect that their expression might interfere with the normal physiology of immature haematopoietic cells or mature T lymphocytes.

The discovery of distal promoters that drive transcription of germline TCR genes in early development raises at least

(a)

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-543   TCTATCATAG TCAGTAGCGA TCCAATCCAG TGGCTGCACA TTAGGGGGCG CTGCTGATCC
-483   ACTTGTGAGC tgtgggcagt gctaaacaag atggatgcag tgctttgata tgcagcccgc

-423   agaagctgtg gaaccagaaa caacgctggc TTAAGAAGCA CGGGGCTGGG AGGTAAAGCC

-363   TATCATCTAT GCACACACAC CGTGTGTCAC CACAGGGAGC ATGGATTGTA GACTTAAACT
                                                  M  D  C  R   L  K  L
-303   GtagTCTTGG CAATCCAAAA CCCTCTGAAG CTAGAAGAAG AGAAGAAAGC AACTGACTCA
        stp
-243   GAGAAAAACA TGAAGAGGAC TTTCCCACAC CCAGCCCTAT TTACTCAATA CAGCCATCTC
                       Pd primer
-183   CCCTTCTATT GAGAAGGAAA AGGGCTGAGA CTAGGTCCAG TACTACAGAG AGCCTATGGG

-123   AGTAACAGTA TCAACAGATC ACTGACCTCA GAATCTGACA TCACAGGCAA TGAGATGATA
                                          decanucleotide          M  I
-63    AGAGGAGAAG GAGGAGCTGA CTCCTGCTCT CTCACCAAAG AGACCAGTAT CCTGAGAGGA
       R  G  E  G   G  A  D   S  C  S   L  T  K  E   T  S  I   L  R  G
-3     AGCATG
       S  M
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(b)

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-1038  TCCACCGCGG TGGCGGCCGC TCTAGAACTA CTTTGCTGAC TCCCTTTGTC TTTGCTTCTG
-978   CTTGTGAGTC CTTTCTCACC AGGGCAAGGT TATGGAAACT TTCCATCAAG ATCCCTCCAG
-918   GATGTGTAGC CTTAGAAAGG AGGCTTTGTA CTGAGATAGT ATCTGGGTAG GGAGCAACTG
-858   AGCTGTGCTT CACCTAAGAA GGAGCCTTTA CTGCCATTGA GTAGAGGTAA GTGTTCATAG
-798   TAGCAAAGTT ATGAGAAAAG AGTTACAAGA GCTTTGGATG ATTTTGGTAC AATTCCCTGG
-738   GACCCTAGAG GCACCAGATC TGCATATTTC CTGGCAAACA GATCTTTCAC ATTGTTATAA
-678   GGGTTTATTC TTGACAGAAA GTGCAATGGC AAAAACTTAG GTCTGCTATT CACAGCCTCT
-618   CCTGTAACAA ATATAGAGTT CGTATCAAGA AGGAAACATG GAAAGCTGAG CCTAGAAGTT
-558   AGATCTTGTA TCCCTGACGA GGTTGGAGAG ATCTTAACAC aattgttcaa aTCACAATGA

-498   CCTCAACTAT GGTGCTTGAT AAGCTTTCTG TGTGTCAAGC AGTCTTTGTA GCCATAGATA
                M  V  L  D   K  L  S  V   C  Q  A   V  F  V   A  I  D  S
-438   GCACTGAAAT TTTTCTTCAA ATATTTAGCC AAAGTAAAGA ATTAGAAGAA ACCTCAtagA
         T  E  I   F  L  Q   I  F  S  Q   S  K  E   L  E  E   T  S stp
-378   ACTCAGCATG TTGTGAGGGT GCAGCTGAGA CTACAAAAAT AGTAAAGATT GAAACCATGG
                                                                 M  E
-318   AGAGTAAGCA GCAGAATCAT AGTAGGAATA AAATATACTG GCTAAAATCA GAGAAAGAGC
         S  K  Q   Q  N  H   S  R  N  K   I  Y  W   L  K  S   E  K  E  H
-258   ACATGCATCG AGAAATAAAA AACAAAGCGC AGCCCCTTCC CTATCTGAGT TGGtaaTTAC
         M  H  R   E  I  K   N  K  A  Q   P  L  P   Y  L  S   W stp
-198   TGTATATTCA CTTTAAAATT GTTGAAACTA TGAGACTATG CTTGAAAACT GGCTTGCTCA

-138   TGTAGAGAAG TGGTGGAGTG TCTTAACTGT GCAGCTCTAG CATTTTTTAT AGACAGTGAC
                                                              decanucleotide
-78    ATCACTAAGT CACTGAAAGC CCCAGTTCTA ATTTACCAAG CAGGGCTGGA ACATACGCGA

-18    ACCTGCCTTA GTTCTGAGAT G
                         M
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Figure 4. Sequence analysis of the $V_{\beta 5.1}$ (a) and $V_{\beta 8.1}$ (b) distal promoter regions. The sequences are numbered from the initiator ATG defining the known precursor TCRβ protein. PD transcription start site regions (as mapped by RPA) are shown in bold lower case letters. The transcription start site of the $V_{\beta 5.1}$ PP[6] is shown by an arrow; the PP start site for $V_{\beta 8.1}$ is not known. The conserved decanucleotide motif which is important for PP transcription activity is also shown.[1,23,24] The amino acids corresponding to ORFs defined by ATGs (in bold) containing surrounding sequences similar with the Kozak consensus sequence are shown. The position of the sense primer used for detecting $V_{\beta 5.1}$ PD-derived transcripts by RT–PCR (see Fig. 5) is boxed. These sequence data are available from EMBL under accession numbers U82703 and U82704.

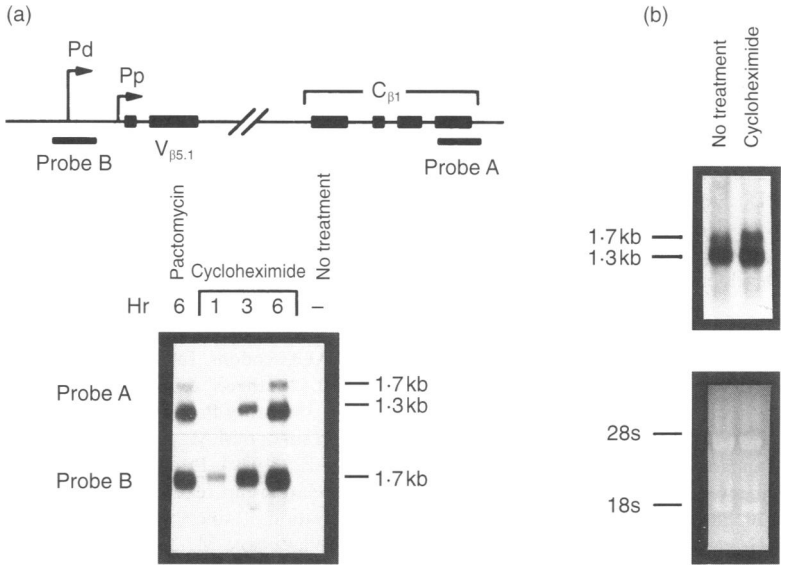

Figure 5. The SL12.4 cell clone expresses both proximal and distal $V_{\beta5.1}$ promoters. (a) Northern blot analysis of total cellular RNA (10 μg) from SL12.4 cells incubated with media alone or media with the translational inhibitors pactomycin or cycloheximide. The same blot was sequentially hybridized with a $C_{\beta1}$-specific fragment (probe A) and a $V_{\beta5.1}$ PD-specific fragment (probe B). All lanes were shown to contain equal amounts of RNA by methylene blue staining of the 18S and 28S rRNA on the blot (as described in ref. 30; data not shown). (b) Northern blot analysis of SL12.4 cells transfected stably with the enhancer$^+$ $V_{\beta8}$ construct. The blot was hybridized with the $V_{\beta8}$-specific probe described in Fig. 1. The lower panel shows methylene blue staining of rRNA to demonstrate equal loading.[30]

three important questions. First, what *cis* elements drive transcription from PDs? The identification of an immature T-lymphoma cell clone, SL12.4, that transcribes a PD will be useful for elucidation of such *cis* elements. Transient transfection experiments have revealed that sequences upstream of the $V_{\beta8.1}$ PD are sufficient to drive modest luciferase expression in SL12.4 cells, but not in non-lymphoid COS-7 cells (K. A. S. & M. F. W., unpublished observations). Second, does transcription from PDs induce TCRβ gene rearrangements? This could be addressed by deletion of PD *cis* elements, followed by examination of the functional consequences in stably transfected T-cell lines or transgenic mice. Third, do the proteins uniquely encoded by PD-derived transcripts possess a functional role? Mutational analysis of the ORFs present in PD transcripts will permit this issue to be addressed. We conclude that the distal promoters described in this report have the potential to contribute an additional level of complexity to the regulation of a set of genes that dictate the development and function of T cells.

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REFERENCES

1. Leiden J.M. (1993) Transcriptional regulation of T cell receptor genes. *Ann Rev Immunol* **11,** 539.
2. Sleckman B.P., Gorman J.R. & Alt F.W. (1996) Accessibility control of antigen-receptor variable-region gene assembly: role of cis-acting elements. *Ann Rev Immunol* **14,** 459.
3. Candéias S., Hardy R.R., Li Y.S. & Staerz U.T. (1994) T cell receptor $V_{\beta8.2}$ gene germ-line transcription: an early event of lymphocyte differentiation. *Eur J Immunol* **24,** 3073.
4. Jolly C.J. & O'Neill H.C. (1995) Expression of a TCR $V_{\beta8.2}$ polypeptide from the unrearranged gene in a murine lymphoid precursor cell line. *Int Immunol* **7,** 1147.
5. Soloff R.S., Wang T.-G., Lybarger L., Dempsey D. & Chervenak R. (1995) Transcription of the TCR-β locus initiates in adult murine bone marrow. *J Immunol* **154,** 3888.
6. Anderson S.J., Chou H.S. & Loh D.Y. (1988) A conserved sequence in the T-cell receptor β-chain promoter region. *Proc Natl Acad Sci USA* **85,** 3551.
7. Bouvier G., Watrin F., Naspetti M., Verthuy C., Naquet P. & Ferrier P. (1996) Deletion of the mouse T-cell receptor β gene enhancer blocks αβ T-cell development. *Proc Natl Acad Sci USA* **93,** 7877.
8. Doskow J. & Wilkinson M.F. (1992) CD3-γ, -δ, -ε,-ξ, T-cell receptor-α and -β transcripts are independently regulated during thymocyte ontogeny and T-cell activation. *Immunol* **77,** 465.
9. Wilkinson M. (1991) Purification of RNA. In: *Essential Molecular Biology: A Practical Approach* (ed. T. A. Brown), p. 69. Oxford University Press, Oxford, UK.
10. Chomczynski P. & Sacchi N. (1987) Single-step method of RNA isolation by acid guanidinium thiocynate-phenol-chloroform extraction. *Anal Biochem* **162,** 156.
11. Qian L., Theodor L., Carter M., Vu M., Sasaki A.W. & Wilkinson M.F. (1993) T cell receptor-β mRNA splicing: regulation of unusual splicing intermediates. *Mol Cell Biol* **13,** 1686.
12. Maiti S., Doskow J., Li S., Nhim R.P., Lindsey J.S. & Wilkinson M.F. (1996) The *Pem* Homeobox Gene. *J Biol Chem* **271,** 17 536.
13. Siu G., Kronenberg M., Strauss E., Haars R., Mak T.W. & Hood L. (1984) The structure, rearrangement and expression of D β gene segments of the murine T-cell antigen receptor. *Nature* **311,** 344.
14. Wilkinson M.F. & MacLeod C.L. (1988) Induction of T-cell receptor-α and -β mRNA in SL12 cell can occur by transcriptional and post-transcriptional mechanisms. *EMBO J* **7,** 101.

15. Davis M.M. (1985) Molecular genetics of the T-cell-receptor β chain. *Ann Rev Immunol* **3,** 537.
16. Calman A.F. & Peterlin B.M. (1986) Expression of T cell receptor genes in human B cells. *J Exp Med* **164,** 1940.
17. Snodgrass H.R., Kisielow P., Kiefer M., Steinmetz M. & Von Boehmer H. (1985) Ontogeny of the T-cell antigen receptor within the thymus. *Nature* **313,** 592.
18. Raulet D.H., Garman R.D., Saito H. & Tonegawa S. (1985) Developmental regulation of T-cell receptor gene expression. *Nature* **314,** 103.
19. Strominger J.L. (1989) Developmental biology of T cell receptors. *Science* **244,** 943.
20. Yiu G.Y., Gu W. & Hecht N.B. (1994) Heterogeneity in the 5′ untranslated region of mouse cytochrome C_T mRNAs leads to altered translational status of the mRNAs. *Nucleic Acids Res* **22,** 4599.
21. Carter M.S., Doskow J., Morris P. *et al.* (1995) A regulatory mechanism that detects premature nonsense codons in T-cell receptor transcripts *in vivo* is reversed by protein synthesis inhibitors *in vitro*. *J Biol Chem* **270,** 28 995.
22. Behlke M.A., Chou H.S., Huppi K. & Loh D.Y. (1986) Murine T-cell receptor mutants with deletions of β-chain variable region genes. *Proc Natl Acad Sci USA* **83,** 767.
23. Lee M.-R., Chung C.-S., Liou M.-L. *et al.* (1992) Isolation and characterization of nuclear proteins that bind to T cell receptor V_β decamer motif. *J Immunol* **148,** 1906.
24. Anderson S.J., Miyake S. & Loh D.Y. (1989) Transcription from a murine T-cell receptor V_β promoter depends on a conserved decamer motif similar to the cyclic AMP response element. *Mol Cell Biol* **9,** 4835.
25. Kozak M. (1989) The scanning model for translation: an update. *J Cell Biol* **108,** 229.
26. Peltz S.W., He F., Welch E. & Jacobson A. (1994) Nonsense-mediated mRNA decay in yeast. *Prog Nucl Acids Res Mol Biol* **47,** 271.
27. Maquat L.E. (1995) When cells stop making sense: effects of nonsense codons on RNA metabolism in vertebrate cells. *RNA* **1,** 453.
28. Geballe A. (1996) Translational control mediated by upstream AUG codons. In: *Translational Control* (eds J. W. B. Hershey, M. B. Mathews & N. Sonenberg), p. 173. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
29. Harpold M.M., Evans R.M., Salditt-Georgieff M. & Darnell J.E. (1979) Production of mRNA in Chinese hamster cells: relationship of the rate of synthesis to the cytoplasmic concentration of nine specific mRNA sequences. *Cell* **17,** 1025.
30. Wilkinson M., Doskow J. & Lindsey S. (1990) RNA blots: staining procedures and optimization of conditions. *Nucleic Acids Res* **19,** 679.