Regulation of Pre-T Cell Receptor (pTα-TCRβ) Gene Expression during Human Thymic Development

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Summary

In murine T cell development, early thymocytes that productively rearrange the T cell receptor (TCR) β locus are selected to continue maturation, before TCRα expression, by means of a pre-TCRα- (pTα-) TCRβ heterodimer (pre-TCR). The aim of this study was to identify equivalent stages in human thymocyte development. We show here that variable-diversity-joining region TCRβ rearrangement and the expression of full-length TCRβ transcripts have been initiated in some immature thymocytes at the TCRα/β$^-$ CD4$^+$CD8$^-$ stage, and become common in a downstream subset of TCRα/β$^-$ CD4$^+$CD8$^+$ thymocytes that is highly enriched in large cycling cells. TCRβ chain expression was hardly detected in TCRα/β$^-$ CD4$^+$CD8$^-$ thymocytes, whereas cytoplasmic TCRβ chain was found in virtually all TCRα/β$^-$ CD4$^+$CD8$^+$ blasts. In addition, a TCRβ complex distinct from the mature TCRα/β heterodimer was immunoprecipitated only from the latter subset. cDNA derived from TCRα/β$^-$ CD4$^+$CD8$^+$ blasts allowed us to identify and clone the gene encoding the human pTα chain, and to examine its expression at different stages of thymocyte development. Our results show that high pTα transcription occurs only in CD4$^+$CD8$^-$ and CD4$^+$CD8$^+$ TCRα/β$^-$ thymocytes, whereas it is weaker in earlier and later stages of development. Based on these results, we propose that the transition from TCRα/β$^-$ CD4$^+$CD8$^-$ to TCRα/β$^-$ CD4$^+$CD8$^+$ thymocytes represents a critical developmental stage at which the successful expression of TCRβ promotes the clonal expansion and further maturation of human thymocytes, independent of TCRα.

During thymocyte development in normal mice, rearrangement and expression of the TCRβ chain gene precedes that of the α chain (1). The first TCRβ gene rearrangements are detectable in a subset of CD4$^-$CD8$^-$ double negative (DN)[1] thymocytes that express IL-2Rα (CD25) (2, 3). However, most rearrangements are out-of-frame at this stage (4). In contrast, the V-D-J rearrangements of the immediate progeny of these cells (i.e., CD25$^-$ DN thymocytes) (3) are predominantly in-frame, indicating that cells within the CD25$^-$ DN population have been selected on the basis of productively rearranged β chain genes (4). The CD25$^+$ to CD25$^-$ transition is accompanied by a strong cellular proliferation, which is consistent with the fact that CD25$^-$ DN thymocytes include a high proportion of large cycling cells (5). Proliferating CD25$^-$ blasts rapidly acquire (or express already) low levels of CD4 and CD8 and, therefore, represent cells in transit to the CD4$^+$CD8$^+$ double positive (DP) stage (6, 7). At this stage, proliferation comes to an abrupt halt, TCRα/β is expressed for the first time, and DP thymocytes are subsequently selected to develop into mature CD4$^+$ or CD8$^+$ single positive (SP) thymocytes (5, 8, 9). Therefore, the transition from CD25$^+$ to CD25$^-$ DN thymocytes appears to be the critical stage at which cells with productive β chain expression gain a selective advantage to mature further, independently of TCRα (3, 4).

Evidence supporting such a regulatory role of the TCRβ protein in early T cell development was originally obtained in SCID mice, and later confirmed in rearrangement (RAG-1 or RAG-2)-deficient mice, and in mice with a mutated TCRβ gene. Both the severe drop in total thymocyte numbers and the blockade at the CD25$^+$ DN stage observed in these animals were fully restored upon introduction of a productively rearranged TCRβ transgene (9–

[1] *Abbreviations used in this paper:* DN, double negative; DP, double positive, pTα, pre-TCRα; RAG-1, recombinase activating gene 1; RT, reverse transcriptase; SP, single positive.

The first two authors contributed equally to this work.

11). Therefore, successful expression of β, independent of the α chain, is sufficient for driving $CD25^+$ DN thymocytes to the DP stage, and results in the expansion of the DP thymocyte pool. In contrast, TCRα expression appears irrelevant to these processes, but its role seems to be confined to further differentiation of DP thymocytes to the SP stage (11).

The biochemical form of the $TCR\beta^+/\alpha^-$ complex involved in early thymocyte development has recently been resolved by the identification on murine pre-T cell lines of a disulphide-linked heterodimer (referred to as pre-TCR) composed of TCRβ and a novel 33-kD transmembrane glycoprotein termed pre-TCRα (pTα) chain or gp33 (12, 13). This complex has also been found on large DP thymocytes from $TCR\alpha^{-/-}$ mice (12). Recent analysis of pTα gene-deficient mice (14) provided formal proof that expression of the pre-TCR is physiologically required for the transition of $CD25^+$ DN T cell precursors, through rapidly dividing $CD25^-$ DN cells, into small $CD4^+CD8^+$ thymocytes (4, 5, 15).

Evidence for an equivalent pre-TCR complex in human thymocyte development is still lacking. Furthermore, although TCRβ rearrangement also precedes that of TCRα in humans (16), no direct experimental evidence has been provided that resolves the timing of TCRβ and TCRα gene expression during the development of human thymocytes. In the present study we have approached this issue by direct examination of the transcriptional activation of both TCRβ and TCRα genes in individual subsets of TCR-negative thymocytes representative of the earliest human intrathymic stages (17). This analysis allowed us to identify a novel subset of large-sized $TCR\alpha/\beta^-$ $CD4^+CD8^+$ cycling thymocytes that express a functionally rearranged TCRβ locus but lack TCRα gene expression. Using cDNA derived from these cells we could identify and clone the human pTα cDNA. Comparison of the developmental regulation of pTα, TCRβ, TCRα, and RAG-1 gene expression provided a clear picture of the maturational progression of early human intrathymic stages and led us to identify the transition from $CD4^+CD8^-$ to large $CD4^+CD8^+$ thymocytes as a key control point in early human T cell development.

Materials and Methods

Cell Samples. Fetal thymus, liver, and bone marrow tissue were obtained from 18–22-wk fetuses after legal termination of pregnancy. Umbilical cord blood samples were collected immediately after delivery. Adult bone marrow was obtained from puncture of the iliac crest of healthy donors. Peripheral blood was obtained from patients subjected to chemotherapy and treatment with G-CSF. $CD34^+$ cells (>99% purity upon reanalysis) were isolated from the indicated sources by Ficoll-Hypaque (Nycomed, Oslo, Norway) centrifugation (18) followed by immunomagnetic sorting with anti-CD34–coupled magnetic beads (Dynabeads; Dynal Corp., Oslo, Norway).

Postnatal thymus samples were removed during corrective cardiac surgery of patients aged 1 mo to 3 yr. Thymocyte suspensions were depleted of small- and medium-sized $TCR\alpha/\beta^+$ DP thymocytes by centrifugation on stepwise Percoll (Pharmacia LKB, Uppsala, Sweden) density gradients as previously described (19). Large thymocytes recovered from the 1.068 density layer were further depleted (>99% purity) of α/β and γ/δ mature T cells, as well as of B, NK, and myeloid cells, by two rounds of depletion with anti-CD3– and anti-CD19–coated magnetic beads (Dynal), and with anti-CD56 (Leu-19; Becton Dickinson & Co., San Jose, CA) and anti-CD11b (MO1; Coulter Corp., Hialeah, FL) mAbs indirectly coupled to sheep anti–mouse IgG-coated magnetic beads (Dynal). $CD4^+CD8^+$ thymocytes were magnetically sorted from the remaining pool with anti-CD8–coated magnetic beads (Dynal). $CD4^+CD8^-$ thymocytes were then isolated from the CD8-depleted pool by treatment with anti-CD4–coated magnetic beads (Dynal). The remaining $CD4^-CD8^-$ thymocytes were finally sorted into the $CD34^+CD1^-$ and $CD34^+CD1^+$ subsets by treatment with an anti-CD1a (Na1/34) mAb (20) indirectly coupled to magnetic beads (Dynal), as described (21). Mature $TCR\alpha/\beta^+$ SP cells were isolated from CD1-depleted large thymocytes by treatment with anti-CD3–coated magnetic beads. Conventional $TCR\alpha/\beta^+$ $CD4^+CD8^+$ small thymocytes (referred to as DP) were isolated from the 1.09 Percoll density layer by magnetic sorting with anti-CD1a.

Flow Cytometry Analysis. Directly labeled mAbs against the following antigens were used: CD4 (Leu3a-PE), CD34 (HPCA-2-PE), and CD8 (Leu2a-FITC) from Becton Dickinson & Co.; CD1a (T6-RD1 and T6-FITC), and CD71 (T9-FITC) from Coulter Corp.; CD28 (CD28-FITC) from Serotec Ltd., Oxford, UK; and CD44 (CD44-FITC) from Caltag Laboratories (South San Francisco, CA). Unlabeled mAb against CD8β (2ST8-5H7, kindly provided by Dr. E.L. Reinherz, Dana-Farber Cancer Institute, Boston, MA) (22), as well as mAb recognizing monomorphic determinants of TCRα/β (BMA031, generously provided by Dr. R. Kurrle, Behringwerke AG, Marburg, Germany) were used in combination with goat anti–mouse FITC- or PE-coupled $F(ab)'_2$ Ig (Southern Biotechnology Associates, Inc., Birmingham, AL). Isotype-matched irrelevant mAbs (Caltag Laboratories) were used as negative controls. For detection of cytoplasmic TCRβ, cells were treated with 0.5% saponin (Sigma Chemical Co., St. Louis, MO), incubated with the anti-TCRβ chain βF1 mAb (generously provided by Dr. M Brenner, Brigham and Women's Hospital, Boston, MA) (23), and labeled with PE-coupled goat anti–mouse IgG1 (Southern Biotechnology Associates, Inc.). Stained cells were analyzed in an Epics XL flow cytometer (Coulter Corp.) as previously described (21). Cell cycle analyses were performed by flow cytometry in cells treated with 0.05% digitonin, washed, and stained with 50 μg/ml of propidium iodide (both from Sigma Chemical Co.).

TCRβ Gene Rearrangement Analysis. TCRβ gene rearrangements were analyzed by using a modification of the genomic DNA-PCR assay described elsewhere (24). Genomic DNA (1 μg) was amplified by PCR for 30 cycles (1 min at 95°C, 2 min at 68°C, and 5 min at 72°C) in 50 μl reaction buffer (Perkin-Elmer Cetus, Norwalk, CT.) containing 1.25 U AmpliTaq polymerase (Perkin-Elmer Cetus) and 1 μM each of 5′ and 3′ primers. The primers used to detect $D\beta_2$ to $J\beta_2$ rearrangements were as follows: $5'D\beta_2$, 5′-GGAGGGGGACTAGCAGGGAGG-3′; and $3'J\beta_2$, 5′-CACCCAGCTCCTCCAGCTCCGG-3′ For V-D-J rearrangements, Vβ-specific primers (25) were used in concert with the 3′ $J\beta_2$ primer. PCR products (16 μl) were electrophoresed, blotted onto nylon membranes (Zeta Probe, Bio-Rad Laboratories, Hercules, CA), and probed with a ^{32}P-labeled $3'J\beta_2$ oligonucleotide (5′-GGCTGGAAGGTGGGGAGACGCCCG-3′) located immediately 5′ of the 3′ $J\beta_2$ primer used for amplification.

Northern Blot Analysis. Preparations of total RNA (10 μg) isolated as described elsewhere (18) were run on 1% agarose-formaldehyde gels, trasferred to nylon membranes, and hybridized as previously described (26) with ^{32}P-labeled cDNA probes corresponding to the TCR Cα (PY1.4) (27) or Cβ (Jurβ$_2$) (28) regions (kindly provided by Dr. T.W. Mak, The Ontario Cancer Institute. Toronto, ON, Canada). The human pTα cDNA probe was derived in this study (see below). The same blot was subsequently stripped and hybridized with a β-actin probe (26).

Reverse Transcriptase–PCR Analysis. Total RNA (1 μg) was reverse transcribed into cDNA according to the manufacturer's protocol (Boehringer Mannheim, Mannheim, Germany). Equivalent amounts of cDNA among different samples was estimated by reverse transcriptase (RT) PCR carried out for 18, 21, and 25 cycles with β-actin primers, as previously described (18). Titration of cycle number allowed us to perform densitometric analyses (Bio-imaging BAS 1500; Fujifilm, Kanagawa, Japan) under nonsaturating conditions. The β-actin control shown in Fig. 4 *A* also applies to PCR analyses shown in Figs. 4 *B*, and 5 *A*. Vα and Vβ degenerate primers (29) used in combination with either Cα or Cβ primers, respectively, enabled the amplification of all known human Vα and Vβ segments, as described (18, 29). Vβ-specific transcription was assessed by using Vβ$_2$-, Vβ$_6$-, and Vβ$_{13.1}$-specific primers in concert with a Cβ primer, as previously reported (25). Specific amplifications were detected by Southern blot hybridization with Cα (27) or Cβ (28) cDNA probes.

Amplifications of pTα cDNA were performed for 35 cycles, at an annealing temperature of 55°C, using the following primers: 5′pTα, 5′-CCCATCTCTCCCTGCCTTCTG-3′; and 3′pTα, 5′-GGAGCAGGTCAAACAGCAGC-3′. Amplified products were analyzed by Southern blotting with a cDNA pTα probe derived in this study (see below). RAG-1 transcription was assessed by RT-PCR carried out for 30 cycles at an annealing temperature of 55°C, followed by Southern blotting using an oligonucleotide probe. As control for the sensitivity of the RAG-1 RT-PCR assay, amplifications were performed simultaneously on serial dilutions of a plasmid vector containing the RAG-1 full-length cDNA (30), mixed with a fixed quantity of irrelevant cDNA. Signal intensity was quantitated by densitometric analyses (Bio-imaging BAS 1500, Fujifilm). The following primers were used: 5′RAG-1, 5′-CAGCGTTTTGCTGAGCTCCT-3′; 3′RAG-1, 5′-CTAGGAGAAGCCCTCAATGC-3′; and RAG-1 probe, 5′-AAGTATAGGTATGAGGGAA-3′.

Cloning of the Human pTα cDNA. cDNA derived from TCRα/β$^-$ CD4$^+$CD8$^+$ thymocytes was amplified for 40 cycles at an annealing temperature of 50°C by using a combination of primers recognizing sequences in the Ig-like (5′-CCTGGCCTTGACAACCCTGTCTG-3′) and in the transmembrane (5′-GTGGTGGCTGGAGGTGCTGGCC-3′) domains of the reported mouse pTα cDNA (13). Amplified products were then fractionated, blotted, and probed with an internal oligonucleotide (5′-GCTGCAGGTCAGGAGCACATCG-3′) specific for the transmembrane domain of the mouse pTα. A 380-bp hybridizing product was purified by standard procedures, cloned in a pMOS Blue-T vector (Amersham International, Amersham, Bucks, UK), and sequenced using a T7 DNA polymerase (Sequenase II, United States Biochemical Corp., Cleveland, OH). Sequence analysis revealed an 82% homology with the corresponding fragment of the murine pTα cDNA. The cloned fragment was then used as a probe for Northern blot and RT-PCR analyses (see above), as well as to probe a cDNA human thymus library (Clontech, Palo Alto, CA.). Three independent positive clones out of 2 × 10^5 plated clones were isolated by replating and amplified by PCR using two λgt11 primers flanking the EcoRI cloning site. PCR reactions were carried out for 30 cycles at an annealing temperature of 55°C, and amplification products were cloned and sequenced as described above.

Immunoprecipitation and Western Blot. 7 × 10^7 Cells were lysed in 1 ml of lysis buffer (2% Triton X–100 [Sigma], 20 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1 mM $MgCl_2$, 10 mM iodoacetamide [Merck, Darmstadt, Germany], 1 mM PMSF, and 1 μg/ml each of leupeptin, pepstatin, and aprotinin [Sigma Chemical Co.]). After removal of their nuclei, supernatants were precleared three times with normal mouse serum (NMS) Igs coupled to either protein A– or protein G–Sepharose beads (Pharmacia LKB). Immunoprecipitation was carried out overnight with βF1 (anti-TCRβ) (23) and αF1 (anti-TCRα) (T Cell Diagnostics, Cambridge, MA) mAbs covalently coupled to either protein G– or protein A–Sepharose beads, respectively, as described (31). The precipitates were separated by SDS-12% PAGE under nonreducing conditions, and transferred onto nitrocelulose membranes (Bio-Rad Laboratories). Blots were blocked with 5% skim milk TBS, and the TCRβ and TCRα precipitates were detected by hybridization with either βF1 ascites diluted 1:2,000 (23), or with 2.5 μg/ml αF1 mAb, respectively. After three washes with 0.2% Tween-20 (Sigma Chemical Co.) TBS blots were incubated with goat anti–mouse Igs coupled to peroxidase (Amersham International), washed four times, and revealed by the enhanced chemiluminescence method (ECL; Amersham International).

Results

Characterization of a TCR-negative subset of CD4$^+$CD8$^+$ Large Cycling Human Thymocytes. Recent studies have shown that the differentiation of the the most immature human T cell precursors, identified as CD34$^+$ DN thymocytes (32), proceeds throughout several TCR-negative stages characterized by the progressive loss of CD34 and the sequential acquisition of CD1, CD4, and CD8 (32–34). Thus, human T cell precursors acquire CD4 before CD8 (22, 34) as proposed in the following maturation sequence: CD34$^+$CD1$^-$CD4$^-$CD8$^-$→ CD34$^+$CD1$^+$CD4$^-$CD8$^-$→ CD34$^{+/-}$CD1$^+$CD4$^+$CD8$^-$→ CD34$^-$CD1$^+$CD4$^+$CD8$^+$. The latter subset seems to include the immediate progenitors of the major intrathymic subpopulation of small DP thymocytes that express the TCRα/β complex and then differentiate to the SP stage (17).

To identify the developmental stage at which the successful expression of TCRβ may be of selective advantage to human thymocytes independent of the TCRα chain, we examined β and α chain gene status in individual subsets of TCRα/β$^-$ early T cell progenitors isolated from the postnatal thymus, according to the proposed maturation sequence (17). To this end, large thymocytes recovered from Percoll gradients were depleted of mature T, B, NK, and myeloid cells. As shown in Fig. 1 *A*, expression of CD4 and CD8 defined three different subsets of TCR-negative thymocytes within the remaining pool: CD4$^-$CD8$^-$, CD4$^+$CD8$^-$, and CD4$^+$CD8$^+$, representing 9.5 ± 4.5%, 29 ± 10%, and 60 ± 10%, respectively. Immunomagnetic sorting allowed the independent isolation of the three subsets that showed a >99% purity upon reanalysis (Fig. 1 *A*). By forward scatter (FSC) analysis, each TCR-negative sub-

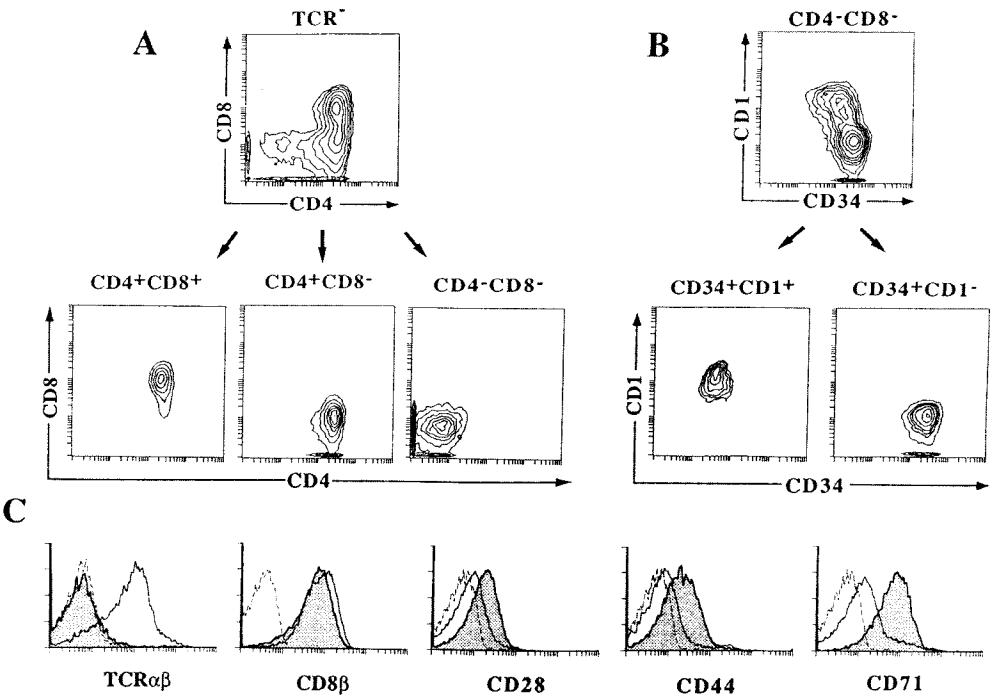

Figure 1. Cell surface phenotype of individual subsets of large TCR⁻ human thymocytes. (*A*) CD4 vs. CD8 expression on Percoll-separated large TCR⁻ thymocytes defines three distinct subsets of $CD4^-CD8^-$, $CD4^+CD8^-$, and $CD4^+CD8^+$ thymocytes. Each subset was reanalyzed for CD4 vs. CD8 expression after immunomagnetic sorting. (*B*) Expression of CD34 vs. CD1 in the $CD4^-CD8^-$ population shown in *A* defines two subsets of $CD34^+CD1^+$ and $CD34^+CD1^-$ thymocytes. Representative profiles of CD34 vs. CD1 expression on both isolated subsets are shown. (*C*) Expression of TCRα/β, CD8β, CD28, CD44, and CD71 on either $TCR\alpha/\beta^-$ $CD4^+CD8^+$ large thymocytes shown in *A* (*shaded areas*), or $TCR\alpha/\beta^+$ $CD4^+CD8^+$ small thymocytes separated on Percoll gradients (*unshaded areas*). (*Dashed histograms*) Background values obtained with isotype-matched irrelevant mAbs.

set was shown to include relatively large-sized cells, as compared with the subset of conventional $TCR\alpha/\beta^+$ DP cortical thymocytes isolated from the Percoll fraction of small cells (Table 1). As expected (32), nearly all cells within the $CD4^-CD8^-$ pool express CD34, whereas CD1 defines two distinct subpopulations of $CD34^+CD1^+$ (52.5 ± 6%) and $CD34^+CD1^-$ (47.5 ± 6%) thymocytes that were independently isolated (>99% pure) by immunomagnetic sorting (Fig. 1 *B*). Based on the expression of CD34, CD1, CD4, and CD8 molecules, the four isolated subsets of TCR-negative large-sized thymocytes will be hereafter referred to as $CD34^+CD1^-$, $CD34^+CD1^+$, $CD4^+CD8^-$, and $CD4^+CD8^+$.

We next examined in more detail the phenotypic profile displayed by $TCR\alpha/\beta^-$ $CD4^+CD8^+$ large thymocytes, as compared with that of common cortical $TCR\alpha/\beta^+$ $CD4^+CD8^+$ small thymocytes (Fig. 1 *C*). It is notable that both subsets coexpressed CD8α and CD8β (Fig. 1 *C*) and displayed similar expression levels of CD1, CD2, CD4, CD5, and CD7 T-lineage molecules, whereas both lacked expression of CD11b, CD13, CD14, CD16, CD25, CD33, CD56, and CD69 (not shown). However, markers such as CD28, CD44, and CD71 (transferrin receptor), which were weakly expressed on small $TCR\alpha/\beta^+$ $CD4^+CD8^+$ thymocytes, were consistently found at higher expression levels on large $TCR\alpha/\beta^-$ $CD4^+CD8^+$ thymocytes (Fig. 1 *C*). More importantly, both subsets were shown to differ dramatically in their cell cycle status. As shown in Table 1, the proportion of $TCR\alpha/\beta^-$ $CD4^+CD8^+$ thymocytes engaged in DNA synthesis and mitosis (up to 45%) was much higher than the proportion of the $TCR\alpha/\beta^+$ $CD4^+CD8^+$ pool (up to 3%), or any other subset of thymocytes (up to 12%). To avoid confusion, conventional $TCR\alpha/\beta^+$ $CD4^+CD8^+$ small thymocytes will be hereafter referred to as DP thymocytes.

Table 1. *Forward Scatter and Cell Cycle Analysis of Early Human Thymocyte Subsets*

Subset	Mean FSC ± SD	Percent cycling cells ± SD	Percent total thymocytes ± SD
$CD4^-CD8^-TCR\alpha/\beta^-$	439 ± 20	11.0 ± 1.5	0.69 ± 0.3
$CD4^+CD8^-TCR\alpha/\beta^-$	404 ± 24	10.5 ± 3.6	2.21 ± 1.3
$CD4^+CD8^+TCR\alpha/\beta^-$	422 ± 12	42.0 ± 4.5	6.57 ± 4.3
DP $TCR\alpha/\beta^+$	311 ± 3	2.2 ± 0.9	10.10 ± 4.5

Postnatal thymocytes were fractionated into populations enriched for large or small cells on Percoll density gradients. Subsets of $TCR\alpha/\beta^-$ thymocytes and $TCR\alpha/\beta^+$ DP thymocytes were purified either from the large or from the small cell fractions, respectively. Medium-sized cells including both SP and DP $TCR\alpha/\beta^+$ thymocytes were not analyzed in this study.

TCRβ Chain Gene Rearrangement during Human Thymocyte Development. Each individual subset of $TCR\alpha/\beta^-$ thymocytes was next examined for the rearrangement status of their TCRβ loci by using a PCR genomic assay (24). As shown schematically in Fig. 2 *A*, the combination of a primer located immediatly 5′ to $D\beta_2$ with a primer positioned 3′ to the $J\beta_2$ cluster, allowed us to detect amplifications of a germline fragment, as well as of a set of smaller

fragments corresponding to partial rearrangements of $D\beta_2$ to each of the seven $J\beta_2$ ($J\beta_{2.1}$-$J\beta_{2.7}$) gene segments (35). As expected from the physical map, Vβ-specific primers (25) used in combination with the $3'J\beta_2$ primer enabled the amplification of complete V-D-J rearrangements, but not of germline fragments (35). The resulting PCR products were analyzed by Southern blot hybridization with an internal primer positioned immediately 3′ to the $J\beta_2$ cluster (Fig. 2 *A*).

Partial rearrangements of $D\beta_2$ to $J\beta_2$ were consistently negative in the most early $CD34^+CD1^-$ thymocytes, in which a sharp germline band equivalent to that detected in non-T (K562) cells was observed (Fig. 2 *B*). A conspicuous germline amplification product was also readily detectable in the two downstream subsets of $CD34^+CD1^+$ and $CD4^+CD8^-$ thymocytes, indicating that they were essentially in germline configuration. However, very faint bands of rearrangement, particularly those corresponding to short-sized products whose amplification is favored by this technique, were eventually detected in both subsets upon prolonged exposure. In contrast, multiple rearrangements of $D\beta_2$ to the $J\beta_{2.1}$-$J\beta_{2.7}$ elements, as extensive as those found in $TCR\alpha/\beta^+$ (DP or SP) thymocytes, were detected at the next developmental stage of $TCR\alpha/\beta^-$ $CD4^+CD8^+$ large thymocytes (Fig. 2 *B*). Rearrangements involving Vβ segments were essentially negative at both the $CD34^+$ $CD1^-$ and the $CD34^+CD1^+$ developmental stages. Weak bands corresponding to complete V-D-J rearrangements were first detected at the $CD4^+CD8^-$ stage. In contrast, the $TCR\alpha/\beta^-$ $CD4^+CD8^+$ large thymocytes displayed a pattern of multiple rearrangements similar to that observed in more mature TCRα/β-expressing thymocytes. As an example, the $V\beta_3$-$J\beta_2$ rearrangement patterns observed are shown in Fig. 2 *C*. Identical results were obtained for rearrangements involving $V\beta_1$, $V\beta_9$, and $V\beta_{19}$ genes (not shown).

Timing of TCRβ and TCRα Gene Transcription during Human Thymic Development. To next examine the transcriptional activation of TCRα and TCRβ genes during early thymocyte development, total RNA isolated from each individual TCR-negative subset was analyzed by Northern blotting. As shown in Fig. 3, hybridization with a Cβ probe revealed that $TCR\alpha/\beta^-$ $CD4^+CD8^+$ large thymocytes displayed a pattern of abundant 1.3-kb mature and 1.0-kb immature TCRβ transcripts that resembled that found in more mature DP or SP $TCR\alpha/\beta^+$ thymocytes. In contrast, mature transcripts were weakly detected in their immediate precursors (i.e., $CD4^+CD8^-$ $TCR\alpha/\beta^-$ thymocytes) and were absent in the most early $CD34^+CD1^-$ and $CD34^+CD1^+$ thymocytes. All these subsets, however, expressed 1.0-kb immature TCRβ transcripts (Fig. 3), and

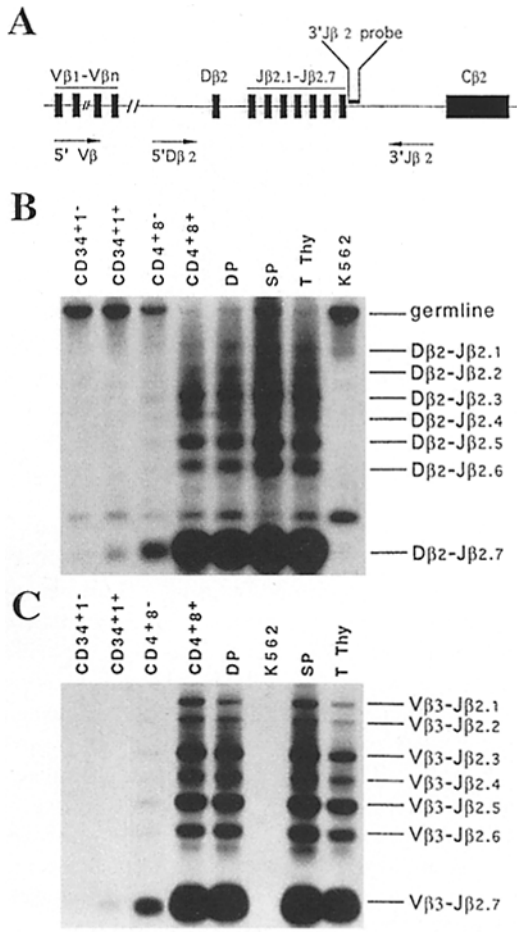

Figure 2. Analysis of D-J and V-D-J TCRβ gene rearrangements during human thymocyte development. (*A*) Schematic representation of the DNA-PCR strategy showing the positions of the primers used either for amplification (*arrows*) or for hybridization (*solid bar*) of $D\beta_2$-$J\beta_2$ and Vβ-$D\beta_2$-$J\beta_2$ rearrangements. (*B*) Southern blot analysis of $D\beta_2$-$J\beta_2$ rearrangements in the indicated thymocyte subsets. (*C*) Southern blot analysis of $V\beta_3$-$D\beta_2$-$J\beta_2$ rearrangements in DNA samples identical to those shown in B. Total thymocytes (*T Thy*) and K562 cells served as positive and negative controls, respectively. (*Right*) Positions of the germline fragment and the specific rearrangements of $D\beta_2$ (*B*) or $V\beta_3$ (*C*) to the $J\beta_{2.1}$-$J\beta_{2.7}$ elements.

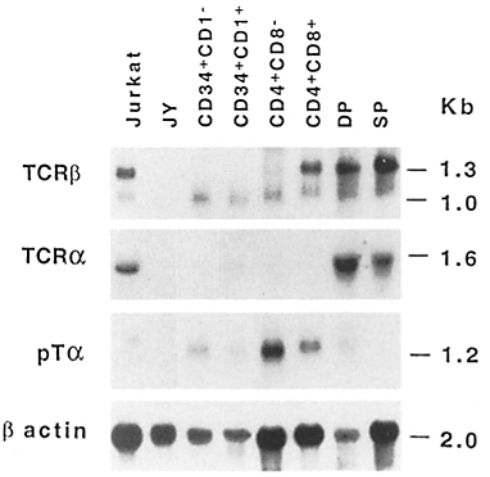

Figure 3. Regulation of TCRβ, TCRα, and pTα gene transcription during human thymic development. Northern blots of total RNA isolated from the indicated cell sources were hybridized with TCR-Cβ, TCR-Cα, or pTα cDNA probes. β-actin mRNA expression served as internal control. Sizes of the bands are indicated at the right (*Kb*).

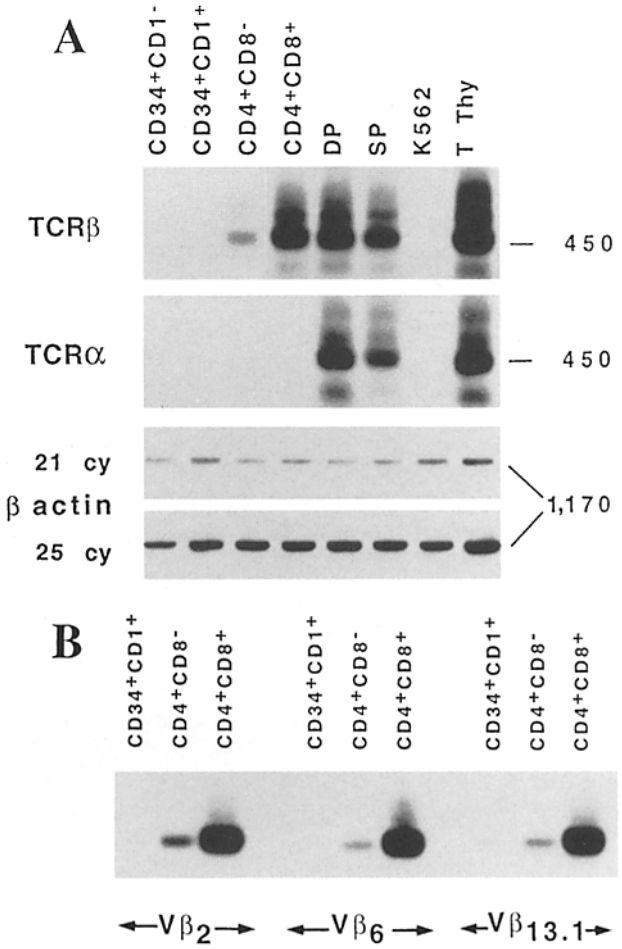

Figure 4. RT-PCR analysis of TCRβ and TCRα gene transcription in early human thymocyte subsets. (*A*) cDNA samples prepared from the indicated cell sources were amplified by using pan-Vβ and pan-Vα primers in concert with Cβ- or Cα-specific primers, respectively. Amplified products were analyzed by Southern blotting with either Cβ or Cα cDNA probes. Equivalence of cDNA among different samples was assessed by RT-PCR using β-actin primers under nonsaturating conditions (21 and 25 cycles). Sizes of amplified products are indicated at the right (bp). (*B*) Expression of full-length TCRβ transcripts was analyzed by RT-PCR using $V\beta_2$-, $V\beta_6$-, and $V\beta_{13.1}$-specific primers in concert with a Cβ primer. cDNA samples and β-actin controls are identical to those shown in *A*.

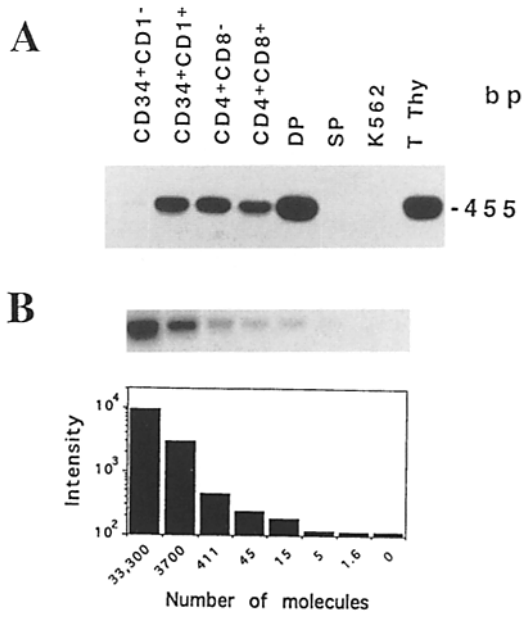

Figure 5. Regulation of RAG-1 expression during human intrathymic development. (*A*) RAG-1 trancription was assessed by RT-PCR and Southern blotting in the indicated cell samples. Sizes of amplified products are indicated at the right (*bp*). The β-actin control is shown in Fig. 4 *A*. (*B*) The relative intensity values of amplifications corresponding to a RAG-1 cDNA titration curve (*top*) were plotted against the number of RAG-1 cDNA molecules in a logarithmic scale (*bottom*).

low levels of 1.6-kb germline transcripts could also be detected upon prolonged exposure (not shown). Subsequent hybridization with a Cα probe showed that expression of 1.6-kb mature-length TCRα transcripts only occurred in the DP or SP stages, at which the TCRα/β is already expressed on the cell surface (Fig. 3). Expectedly, mature TCRα and TCRβ transcripts were present in Jurkat T cells, but not in JY B cells.

To ascertain unambiguously the expression of mature TCRβ message at the $CD4^+CD8^-$ stage, as well as to confirm the lack of TCRα transcription in both the $CD4^+CD8^-$ and the $CD4^+CD8^+$ subsets, further studies were performed by using a sensitive RT-PCR technique (18, 29) proved to detect transcription of all known Vβ and Vα TCR genes. As shown in Fig. 4 *A*, the combination of panVβ primers with Cβ primers revealed that TCRα/β^- $CD4^+CD8^-$ thymocytes had readily detectable amounts of TCRβ message. There was a higher expression in TCRα/β^- $CD4^+CD8^+$ blasts, whereas no amplified fragments were detected in $CD34^+CD1^-$ and $CD34^+CD1^+$ thymocytes. Expression of V-D-J-Cβ transcripts at the $CD4^+CD8^-$ stage was independently confirmed by using Cβ primers in combination with Vβ-specific primers (25). Representative results corresponding to Vβ2-, Vβ6-, and Vβ13-specific amplifications are shown in Fig. 4 *B*. In addition, pan-Vα primers used in combination with Cα primers confirmed the absence of TCRα transcripts in all thymocyte subsets shown to lack surface TCRα/β (Fig. 4 *A*). Collectively, our data suggest that, in contrast to mice, the onset of V-D-J TCRβ gene rearrangement and transcription occurs in humans in immature thymocytes already expressing CD4, and is essentially completed during the transition to the next TCRα/β^- $CD4^+CD8^+$ developmental stage.

RAG-1 Gene Transcription Is Intrathymically Upregulated during the Transition from the $CD34^+CD1^-$ to the $CD34^+CD1^+$ Stage. Previous studies in mice have shown that the expression of RAG-1 and RAG-2 genes usefully traces the emergence of TCR gene rearrangement and transcription during T cell development (36). To approach this issue in humans, RAG-1 transcription was analyzed by RT-PCR in the thymocyte subsets previously examined for their TCRβ and TCRα gene status (Fig. 5 *A*). Comparative

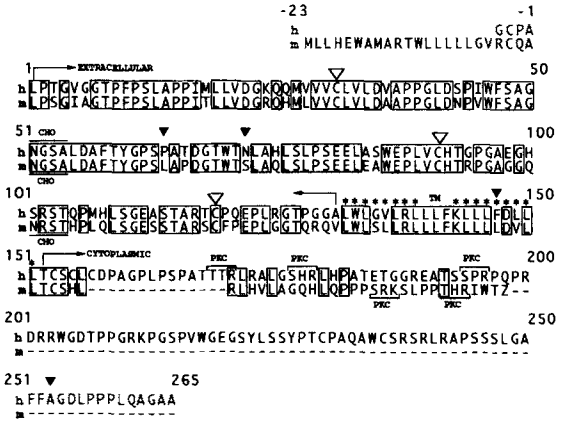

Figure 6. Predicted human pTα amino acid sequence. Comparison and alignment of the predicted amino acid sequence of human pTα with the corresponding murine pTα sequence is shown. (*Boxed*) Conserved residues; (*dashes*) gaps at the cytoplasmic tail. (▽) The three cysteines conserved in positions 31, 91, and 119. Locations of the potential N-linked glycosilation sites (*CHO*) and protein kinase C (*PKC*) phosphorylation sites are indicated. (▼) Mismatches with respect to a recently reported human pTα sequence (37). Amino acid sequence analysis was done with the PROSITE pattern database (45). The nucleotide sequence data of the cloned human pTα cDNA are available from EMBL/GenBank/DDBJ, under accession number U38996.

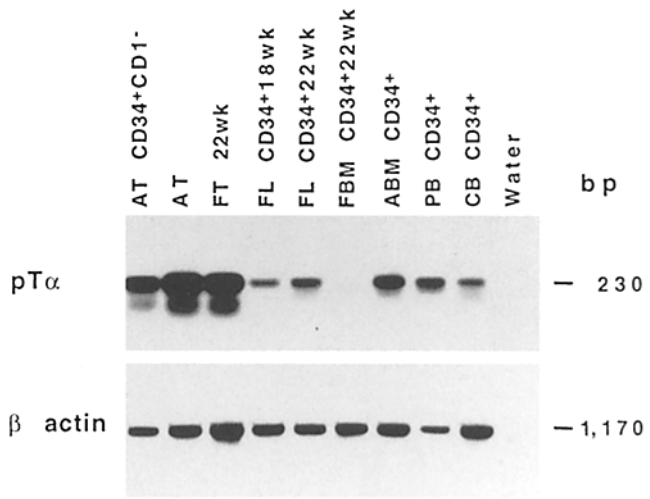

Figure 7. Regulation of pTα gene expression in human T cell ontogeny. pTα mRNA expression was analyzed by RT-PCR and Southern blotting in CD34$^+$ precursors isolated from fetal liver (*FL*), fetal bone marrow (*FBM*), adult bone marrow (*ABM*), mobilized peripheral blood (*PB*), or cord blood (*CB*). CD34$^+$CD1$^-$ thymocytes and unseparated thymocytes obtained from the adult (1-yr-old) thymus (*AT*), as well as total thymocytes from a 22-wk-old fetal thymus (*FT*), were included as positive controls. Omission of template cDNA (*water*) served as negative control. Integrity of cDNA samples was assessed by amplification with β-actin primers after 25 cycles. Sizes of the PCR products are indicated at the right (*bp*).

densitometric analyses were performed by using a RAG-1 cDNA titration curve (Fig. 5 *B*). As shown in Fig. 5 *A*, expression of RAG-1 was hardly detected in the most early CD34$^+$CD1$^-$ thymocytes, but it increased abruptly (~500-fold) at the next CD34$^+$CD1$^+$ and CD4$^+$CD8$^-$ developmental stages. A slightly weaker (about threefold) expression was found in TCRα/β$^-$ CD4$^+$CD8$^+$ blasts. However, the amount of RAG-1 message increased again (about 20-fold) in the more mature TCRα/β$^+$ DP small thymocytes, coincident with the onset of TCRα trancription, whereas no message was detected in SP thymocytes (Fig. 5 *A*). Because of the high sensitivity of the RAG-1 PCR assay that allowed us to detect as few as 15 transcripts (Fig. 5 *B*), it is very likely that the signal displayed by CD34$^+$CD1$^-$ thymocytes (corresponding to ≤75 RAG-1 cDNA molecules) may reflect the amplification of RAG-1 transcripts present in a minute contaminating subset of CD1$^+$ cells. These data suggest that transcriptional activation of RAG-1 begins during the transition from CD34$^+$CD1$^-$ to CD34$^+$CD1$^+$ thymocytes, slightly before the onset of TCRβ rearrangement, and is shut down after TCRα expression in cells in transit to the SP stage.

Cloning and Developmental Expression of the Human pTα Gene. The identification of a subset of TCRα/β$^-$ CD4$^+$CD8$^+$ cycling thymocytes that express mature TCRβ message, although lacking TCRα transcripts, prompted us to search for the possible expression in these cells of a gene homologous to the murine pTα (13). cDNA derived from TCRα/β$^-$ CD4$^+$CD8$^+$ blasts allowed us to clone a human cDNA fragment spanning 985 nucleotides that encodes a protein with 87% amino acid identity to murine pTα at the extracellular and transmembrane domains, but containing a longer cytoplasmic tail (Fig. 6). These data were considered reasonable evidence that we had identified the gene encoding the human pTα chain. In addition, after submission of this manuscript, another group independently cloned a human cDNA (37) with a predicted amino acid sequence essentially identical to that reported here, except for positions 64, 72, 147, and 253 (Fig. 6).

Next, Northern blot analyses provided evidence that pTα transcription is tightly regulated during human T cell development. As shown in Fig. 3, we found that the largest amount of pTα transcripts (1.2 kb) corresponded to CD4$^+$CD8$^-$ and CD4$^+$CD8$^+$ TCRα/β$^-$ immature thymocytes. There was a weaker expression in earlier (CD34$^+$CD1$^-$ and CD34$^+$CD1$^+$) and later (TCRα/β$^+$ DP) developmental stages, whereas no pTα transcription was detected in mature SP thymocytes. These results were confirmed by RT-PCR (not shown).

Expression of murine pTα has been reported to be exquisitely T-lineage specific (38). Accordingly, we could not detect pTα transcription in human non-T cells including B, NK, myeloid, and dendritic cells (not shown). To investigate whether pTα transcription is specifically induced in early pro-T cells after homing to the thymus, expression of pTα mRNA was examined by RT-PCR in highly purified CD34$^+$ hematopoietic progenitors from distinct postnatal and fetal extrathymic tissues. Total thymocytes derived from either fetal (22-wk-old) or adult (1 mo–3 yr) thymus, as well as CD34$^+$CD1$^-$ precursors from adult thymus were included in the study as positive controls. As shown in Fig.

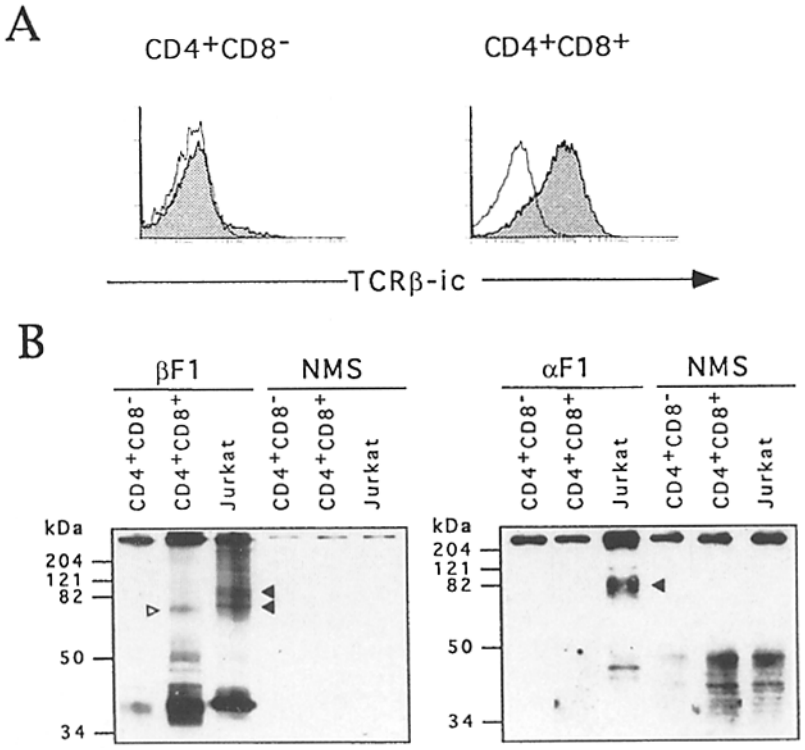

Figure 8. TCRβ chain expression in TCRα/β⁻ CD4⁺CD8⁺ large cycling thymocytes. (*A*) Flow cytometry analysis of TCRα/β⁻ CD4⁺CD8⁻ and TCRα/β⁻ CD4⁺CD8⁺ thymocytes for cytoplasmic TCRβ (*βF1*) expression (*shaded histograms*). (*Unshaded histograms*) Background fluorescence. (*B*) Expression of an immature TCRβ complex distinct from the mature TCRα/β heterodimer in TCRα/β⁻ CD4⁺CD8⁺ blasts. Western blots of TCRβ (*βF1*) and TCRα (*αF1*) immunoprecipitates from the indicated cell samples were revealed with mAbs to TCRβ or TCRα, respectively. Precipitations with normal mouse serum (*NMS*) served as negative controls. Molecular sizes are indicated on the left (*kDa*). Bands corresponding to the intracellular and surface forms of the TCRα/β heterodimer precipitated from Jurkat T cells (▶), as well as the position of the immature TCRβ complex precipitated from TCRα/β⁻ CD4⁺CD8⁺ blasts (▷), are indicated.

7, transcription of pTα was easily detected in mobilized CD34⁺ cells from peripheral blood, as well as in CD34⁺ precursors isolated from umbilical cord blood or from adult bone marrow. More importantly, pTα gene expression also occurred in fetal life in CD34⁺ progenitors present in the liver at 18 wk of gestation, although it was absent in CD34⁺ precursors from fetal bone marrow at any developmental age up to 22 wk. These results suggest that transcription of the pTα gene is activated early in ontogeny in CD34⁺ progenitors, before their entry into the thymus.

TCRβ Chain Is Expressed in CD4⁺CD8⁺ Blasts: Identification of an Immature TCRβ Complex Distinct from the TCRα/β Heterodimer. The coincident expression of large amounts of TCRβ and pTα transcripts in TCRα/β⁻ CD4⁺CD8⁺ blasts raised the possibility that, at this stage, cells have already been selected by means of a putative human pre-TCR complex consisting of a functional TCRβ chain associated with the pTα chain. Flow cytometric analysis shown in Fig. 8 *A* revealed that cytoplasmic TCRβ was first expressed in a minor proportion of CD4⁺CD8⁻ thymocytes (5–10% in different experiments). Consistent with the prediction in mice of >56% for a population in which cells have been selected on the basis of TCRβ expression (39), cytoplasmic TCRβ protein was found in most (80–90%) TCRα/β⁻ CD4⁺CD8⁺ human blasts (Fig. 8 *A*). Therefore, nearly all cells at the TCRα/β⁻ CD4⁺CD8⁺ stage have been selected based on productive β gene rearrangement.

To characterize the molecular form of TCRβ chains involved in this selection process, lysates from the TCRα/β⁻ CD4⁺CD8⁻ and CD4⁺CD8⁺ thymocytes, as well as from the TCRα/β–expressing Jurkat T cell line, were precipitated with mAbs against constant regions of either the TCRβ (βF1) or the TCRα (αF1) chains, and the immunoprecipitated proteins were then analyzed in Western immunoblots with mAbs to TCRβ or TCRα, respectively. As shown in Fig. 8 *B*, the anti-TCRβ mAb precipitated three β chain monomeric forms (36–40 kD) from both thymocyte subsets as well as from Jurkat T cells, although a very faint signal was observed in the CD4⁺CD8⁻ thymocytes (Fig. 8 *B*, *left*). As expected from previous studies (40), two different bands corresponding in size to the intracellular (70 kD) and the cell surface expressed (80–90 kD) TCRα/β heterodimer were detected in anti-TCRβ precipitates from Jurkat T cells, but were absent in both the CD4⁺CD8⁻ and the CD4⁺CD8⁺ thymocyte subsets. In contrast, a single band, with a slightly lower molecular mass than the 70-kD intracellular TCRα/β heterodimer, was detected in TCRβ precipitates from CD4⁺CD8⁺ thymocytes, but was completely absent in CD4⁺CD8⁻ thymocytes (Fig. 8 *B*, *left*). Strikingly, the anti-TCRα mAb failed to immunoprecipitate this TCRβ complex, or any other protein complex, from CD4⁺CD8⁺ thymocyte lysates, whereas an 80–90 kD TCRα/β heterodimer was precipitated from Jurkat T cells with this reagent (Fig. 8 *B*, *right*). Therefore, our data provide evidence that TCRα/β⁻ CD4⁺CD8⁺ blasts express an immature TCRβ complex composed of the TCRβ chain associated with another chain different from TCRα. Although the molecular mass of this complex fits with that expected for a human pTα-TCRβ heterodimer, direct evidence for this notion must

still await the development of suitable reagents against the human pTα chain.

Discussion

In this study, we have tried to identify the critical stages in early human intrathymic differentiation where most of the developmental decisions are made. In particular, we have focussed on the developmental point at which TCRβ may confer on intrathymic precursors a selective advantage to mature further in the absence of TCRα expression. These studies have been greatly facilitated by the observation that the DN to DP transition occurs in humans through a $CD4^+CD8^-$ intermediate (32–34). Our results show that it is precisely at this $CD4^+CD8^-$ developmental stage that complete V-D-Jβ gene rearrangement and full-length TCRβ transcription are first detected, although many of these cells still display a TCRβ germline configuration. In contrast, extensive V-D-Jβ gene rearrangement and mature-length TCRβ message are common in $TCR\alpha/\beta^-$ $CD4^+CD8^+$ thymocytes, known to represent the immediate progeny of $CD4^+CD8^-$ cells (32–34). As predicted for a population in which cells have been selected on the basis of successful β gene rearrangement (39), cytoplasmic TCRβ protein was found in nearly all $TCR\alpha/\beta^-$ $CD4^+CD8^+$ thymocytes. These data allowed us to propose a model for the early stages of human intrathymic development (Fig. 9) that envisages the $CD4^+CD8^-$ to $CD4^+CD8^+$ transition as the critical point at which human thymocytes are selected based on productive TCRβ expression. According to this model, the onset of V-D-J TCRβ gene rearrangement and transcription occurs relatively late in human thymocyte development, as $CD34^+CD1^+$ pro-T cells downregulate CD34 and acquire the CD4 coreceptor molecule, and it is preceded by activation of the recombinase machinery (i.e., transcription of RAG-1) during the $CD34^+CD1^-$ to $CD34^+CD1^+$ transition. These results seem in conflict with those recently reported by Ktorza et al. (41) showing RAG-1 and mature TCRβ transcription at earlier intrathymic stages. However, no quantitative analyses were reported in that study to rule out the possibility that amplified transcripts do correspond to downstream cell contaminants.

The proposal that productive TCRβ expression occurs at the $CD4^+CD8^-$ to $CD4^+CD8^+$ transition is further supported by our observation that $TCR\alpha/\beta^-$ $CD4^+CD8^+$ thymocytes include a high proportion of cycling cells, an expected finding considering that functional expression of TCRβ results in clonal expansion of murine thymocytes at the equivalent developmental point (5, 7, 8). Based on what is already known in the mouse about the implication of the pTα-TCRβ complex in this maturation step, we performed experiments designed to provide evidence for the existence of an equivalent complex in humans. We identified and cloned a human cDNA highly homologous to the murine pTα (13), with a predicted amino acid sequence that is essentially identical to that recently reported by others as the human pTα chain (37). As described in mice (13), pTα gene expression was also shown to be developmentally regulated in humans. We found that pTα message is highly expressed by both the $CD4^+CD8^-$ and the $CD4^+CD8^+$ $TCR\alpha/\beta^-$ thymocyte subsets, whereas there is a weaker expression in earlier and later stages of development. The coincident expression of TCRβ protein and pTα transcripts (in the absence of TCRα, γ, and δ proteins) in $TCR\alpha/\beta^-$ $CD4^+CD8^+$ blasts, strongly suggests that cells in transit to this stage monitor the occurrence of productive TCRβ rearrangements through a human pTα-TCRβ heterodimer. Supporting this possibility, a TCRβ complex of the expected size, and different from the mature TCRα/β heterodimer, was immunoprecipitated from $TCR\alpha/\beta^-$ $CD4^+CD8^+$ blasts. However, we have been unable as yet to biochemically resolve this putative pTα-TCRβ heterodimer in reducing gels, probably because the pTα chain escapes detection by current labeling techniques, as reported in mice (12). Though pTα protein expression studies still await the development of suitable anti-pTα reagents, the results reported here allow us to propose that the earliest direct action of the putative human pre-TCR is to induce rapid clonal expansion, and si-

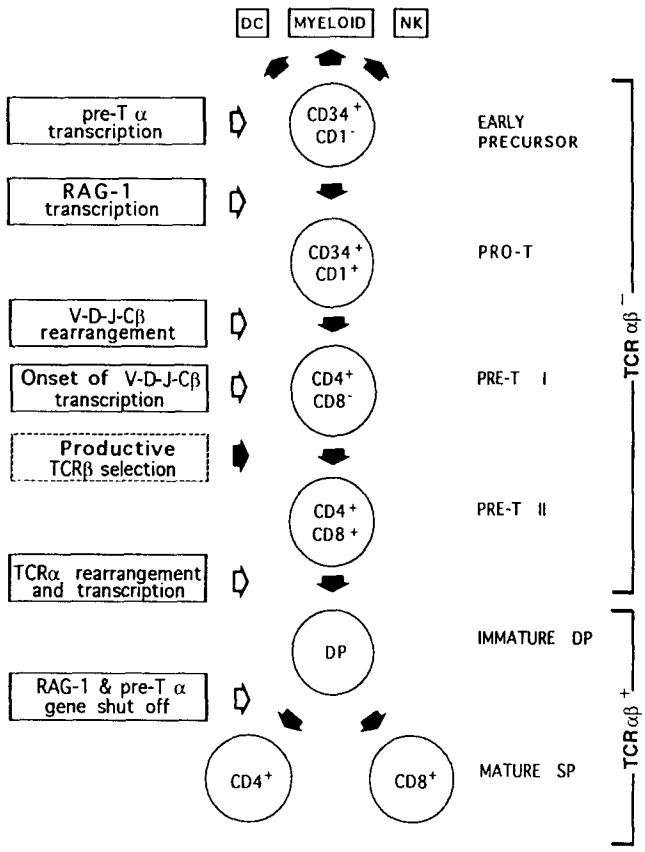

Figure 9. Proposed model for the early T cell developmental stages in the human postnatal thymus. The figure summarizes the ordered progression of early human T cell development, focusing on the expression of pTα and fully rearranged TCRβ genes encoding a putative pre-TCR complex. Selection based on productive TCRβ expression is placed at the transition between $CD4^+CD8^-$ and $CD4^+CD8^+$ immature $TCR\alpha/\beta^-$ thymocytes. The model purposely mirrors the recently reviewed analyses of early T cell (3) and B cell (44) development in the adult mouse.

multaneous transition of thymocytes from the $CD4^+CD8^-$ to the $CD4^+CD8^+$ stage.

Analysis on the developmental expression of the human pTα gene provided evidence that pTα is also expressed outside the thymus in $CD34^+$ progenitors isolated from distinct adult hematopoietic tissues. More importantly, pTα transcription was also detected early in fetal life in $CD34^+$ precursors present in the liver, whereas no pTα expression was found in fetal bone marrow $CD34^+$ cells. Although the precursor potential of the latter $CD34^+$ subset needs to be analyzed before further conclusions can be drawn, the fact that $CD34^+$ fetal liver cells do contain TCRα/β precursor potential (17, 18) may suggest that there is a coincidence of pTα expression and precursor activity for TCRα/β T cells in hematopoietic precursors before their entry into the thymus. Alternatively, pTα-expressing extrathymic $CD34^+$ cells may represent recirculating pro-T cells derived from the thymus. This possibility appears very unlikely considering that pTα gene expression has also been found in the bone marrow of athymic mice (38). Finally, we provide evidence that pTα RNA is expressed by the most immature $CD34^+CD1^-$ thymocytes that still have the potential to develop into TCRγ/δ, dendritic, myeloid, and NK cells (17, 21). Therefore, pTα transcription precedes the onset of TCRβ rearrangement and RAG-1 expression inside the thymus. An intriguing feature of these early intrathymic precursors, also found in their murine counterparts (2, 42), is that they express 1.0- and 1.6-kb germline TCRβ transcripts before the onset of D-J TCRβ rearrangement (our present study). It is believed that TCR gene segments are selectively rendered accessible to the recombinase machinery by developmentally regulated transcription while they are still in their germline configuration (43). Whether the coincident expression of pTα and germline TCRβ transcripts identifies TCRα/β-committed precursors within the $CD34^+CD1^-$ immature pool of thymocytes deserves further investigation. However, given that both the onset of full-length TCRβ transcription and the upregulation of pTα gene expression are downstream events coincident with the acquisition of surface CD4, we favor the view that the $CD4^+CD8^-$ stage represents the critical point at which intrathymic precursors restrict their developmental potential towards the TCRα/β lineage.

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