er phonology and orthography are dependent systems that arose together or whether the two are independent, as would be expected if one is biologically determined and one is a human invention. This case illustrates that the neural substrate that supports writing processes can be separate from that which supports spoken speech—the result that would be expected if writing is an independent skill, an invention of the human species, that develops from, but is not part of, the inherited basis of spoken language. The innateness of our ability for spoken language guarantees that this skill develops with a well-established pattern and time course (*10*). Writing (and reading) are more problematic. Both skills require explicit instruction and practice and are a major focus of the public educational process. Many people grow to adulthood without mastering these functions. The percentage of functionally illiterate adults in the United States has been placed as high as 20% of the population (*11*). Spoken language may be innate, an "instinct" if you will, but writing does not develop without instruction. The pattern of lateralization observed here suggests it can have a more varied neural representation as well.

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13. Informed consent was obtained from V.J. in accordance with the regulations of the UC Davis Human Subjects Committee. We thank V.J. and her husband for their patience and cooperation. We also thank S. Nutik, E. Austin, J. Walker, and R. Berwald for sharing information regarding V.J.'s medical care. We express our appreciation to A. Caramazza and M. Miozzo for sharing the Johns Hopkins Dysgraphia Battery with us. Supported by the National Institute of Neurological Disorders and Stroke (grant P01 NS 17778) to the University of California at Davis.

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Thymocyte Development in the Absence of Pre-T Cell Receptor Extracellular Immunoglobulin Domains

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Immature thymocytes express a pre–T cell receptor (pre-TCR) composed of the TCRβ chain paired with pre-Tα. Signals from this receptor are essential for passage of thymocytes through a key developmental checkpoint in the thymus. These signals were efficiently delivered in vivo by a truncated form of the murine pre-TCR that lacked all of its extracellular immunoglobulin domains. De novo expression of the truncated pre-TCR or an intact αβTCR was sufficient to activate characteristic TCR signaling pathways in a T cell line. These findings support the view that recognition of an extracellular ligand is not required for pre-TCR function.

Mature αβ T lymphocytes bear on their surface a heterodimeric T cell receptor (TCR) that contains the protein products from rearranged TCRα and TCRβ loci. During development, rearrangement of the TCRβ locus occurs first, allowing immature $CD4^-CD8^-$ [double-negative (DN)] thymocytes to synthesize the TCRβ protein and express it on the surface in association with the pre-Tα (pTα) protein (*1*, *2*). Signals from this "pre-TCR" then induce the cells to differentiate into $CD4^+CD8^+$ [double-positive (DP)] cells and to undergo a rapid series of cell divisions. In the absence of pre-TCR function, this differentiation is blocked, and thymic cellularity is severely reduced (*3*–*12*).

Signaling by the pre-TCR and the mature receptor involves the CD3/ζ chains and depends on the concerted action of Src family and Syk/ZAP-70 tyrosine kinases (*4*–*6*). No ligand for the pre-TCR has yet been identified, but one might be expected, given the surface expression of the pre-TCR (*11*, *13*, *14*) and its structural resemblance to antigen receptors. Alternatively, surface expression of the TCRβ-pTα heterodimer might be sufficient to initiate the pre-TCR signaling process.

To distinguish between these possibilities, we generated transgenic mice expressing a truncated form of the pre-TCR heterodimer that lacked all of its extracellular immunoglobulin (Ig) domains. Truncated forms of TCRβ and pTα (β_T and $pT\alpha_T$, respectively) were constructed (Fig. 1A) (*15*) that have their normal extracellular Ig domains replaced with Flag or Myc epitope tags but still retain the cysteine residues and transmembrane domains required for heterodimer formation and assembly with the signal-transducing CD3 and ζ chains (*16*). To confirm that the modifications had not adversely affected assembly of the receptor (*17*), we transiently transfected the truncated chains, individually or together, by electroporation into a TCR-deficient thymoma (*18*). Because the cells do not express TCRβ, the expression of $pT\alpha_T$ alone did not rescue CD3 on the cell surface (Fig. 1B). In contrast, the introduction of β_T alone resulted in a slight but reproducible increase in surface expression of CD3, presumably through the formation of heterodimers with endogenous pTα. The expression of β_T and $pT\alpha_T$ together resulted

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in rescue of surface CD3, showing that a pre-TCR complex could be assembled with the ectodomain-deficient heterodimer.

Transgenic mice were generated that expressed either β_T or $pT\alpha_T$ in thymocytes under the control of the *Lck* proximal promoter (*19*). For analysis of thymocyte development in the absence of endogenous TCR gene rearrangements, these mice were interbred and back-crossed to mice with no *recombinase activating gene–1* (*Rag-1*) (*10*). Most thymocytes in wild-type mice were $CD4^+CD8^+$ DP cells with a minor fraction of cells expressing either CD4 or CD8 (mature single-positive cells) (Fig. 2). In contrast, the *Rag-1*$^{-/-}$ thymus was blocked at an early developmental stage and contained no DP or mature thymocytes. The *Rag-1*$^{-/-}$ developmental block was partially corrected in mice that expressed the β_T transgene; most of the thymocytes in these mice were $CD4^+CD8^+$, and their thymic cellularity was 10-fold greater than in nontransgenic *Rag-1*$^{-/-}$ mice (Fig. 2). However, these mice still had only 10% of the number of thymocytes in wild-type mice. The DP cells also retained CD25 expression, a characteristic that has previously been associated with impaired development at this stage (*3*, *20*) (Fig. 3A). Although surface expression of CD3, and thus of the pre-TCR, was undetectable on these thymocytes (Fig. 3B), intraperitoneal injection of a monoclonal antibody (mAb) to the NH_2-terminal epitope tag on β_T or of an mAb to CD3ε restored both the differentiation and expansion of the DP compartment (*21*–*23*). Thus, small amounts of the truncated TCRβ were, indeed, expressed at the cell surface in association with CD3.

Rag-1$^{-/-}$ mice with the $pT\alpha_T$ chain had no DP thymocytes (Fig. 2), as was expected given the absence of endogenous TCRβ rearrangement in these mice (*10*, *12*). In contrast, *Rag-1*$^{-/-}$ mice expressing both the β_T and $pT\alpha_T$ transgenes had DP cells that were phenotypically similar to their wild-type counterparts in abundance, forward scatter, and expression of surface molecules, such as CD25 (Fig. 3A) and CD2 (*21*). A minor population of large thymocytes in these mice expressed low but detectable amounts of surface CD3, similar to what has been previously reported for cells that express the pre-TCR (*11*, *13*); almost all of these were DP cells (*21*). $CD25^+$ DN cells had no detectable pre-TCR by flow cytometry. Thus, the observed developmental effects were not caused by pre-TCR overexpression.

We also confirmed the function of the truncated pre-TCR by transfecting the $pT\alpha_T$ and β_T transgenes into *Rag-1*$^{-/-}$ embryonic stem (ES) cells (*19*, *24*) and making chimeric mice by microinjection into *Rag-1*$^{-/-}$ blastocysts. Almost all of the ES cell–derived thymocytes ($Ly9.1^+$) in the chimeric mice were $CD25^-$ DP cells (*21*). Thus, as in the transgenic mice, the trun-

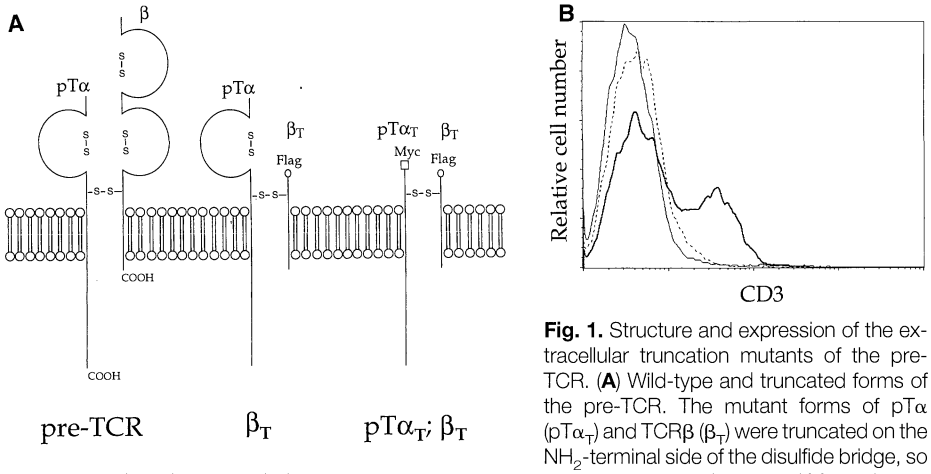

Fig. 1. Structure and expression of the extracellular truncation mutants of the pre-TCR. (**A**) Wild-type and truncated forms of the pre-TCR. The mutant forms of pTα ($pT\alpha_T$) and TCRβ (β_T) were truncated on the NH_2-terminal side of the disulfide bridge, so that only nine ($pT\alpha_T$) and four (β_T) residues of the native sequences were retained. Flag and Myc epitopes were added as indicated, but these were difficult to detect by fluorescence-activated cell sorting (FACS), perhaps because they were obscured by the presence of the CD3 chains. (**B**) FACS profiles (*45*) showing surface expression of the truncated pre-TCR after transient transfection (*25*) into a *Rag-1*$^{-/-}$;*p53*$^{-/-}$ thymoma (*18*). Constructs encoding $pT\alpha_T$ (thin line), β_T (dashed line), or both (thick line) were electroporated into the cells 1 day before staining for surface expression of CD3ε with mAb 145-2C11. A plasmid encoding human CD8 was transfected as a control to establish the amount of background staining (which was indistinguishable from that observed after introduction of $pT\alpha_T$ alone) (*21*).

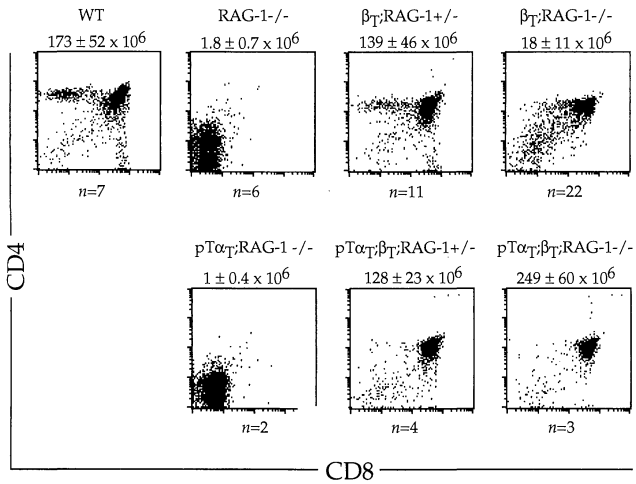

Fig. 2. Rescue of $CD4^+$-$CD8^+$ thymocyte development in *Rag-1*$^{-/-}$ mice expressing truncated pre-TCRs. FACS profiles show the relative expression of CD4 and CD8 for representative wild-type (WT), *Rag-1*$^{-/-}$, and transgenic mice expressing the indicated mutant pre-TCRs. Thymocytes were stained with mAbs specific for CD4 and CD8 and then analyzed by flow cytometry (*45*). The FACS profiles are representative of data acquired from multiple experiments; the associated figures for thymic cellularity are averages generated from figures for the indicated number of mice between the ages of 5 to 8 weeks. Older mice showed the same phenotypes as those presented here but were not used for calculating averages (*21*).

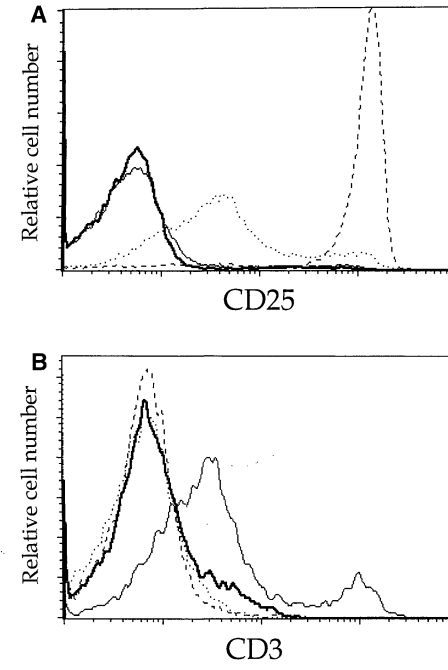

Fig. 3. Cell surface phenotype of thymocytes from transgenic mice expressing the indicated mutant pre-TCRs. Relative expressions of CD25 (**A**) and CD3ε (**B**) on unseparated thymocytes from the following mice: *Rag-1*$^{-/-}$ (dashed line), β_T;*Rag-1*$^{-/-}$ (dotted line), $pT\alpha_T$;β_T;*Rag-1*$^{-/-}$ (thick solid line), and C57BL/6–wild type (thin solid line).

cated pre-TCR was proficient in promoting differentiation from the DN to DP stages.

Coexpression of both truncated molecules, but not β_T alone, in *Rag-1*$^{-/-}$ mice allowed efficient development of thymocytes to the DP stage (Fig. 2). We also observed that the fully truncated heterodimer prevented the efficient emergence of single-positive thymocytes and expression of αβ TCRs in mice expressing *Rag-1*, whereas β_T alone did not (Fig. 4A). This blockage of αβ TCR expression resulted from suppression of rearrangements at the endogenous TCRβ locus (Fig. 4B). Thus, pre-TCRs containing endogenous full-length pTα paired with β_T were not efficient in either promoting the differentiation of DP cells or mediating allelic exclusion; that is, endogenous pTα did not play a substantial role in promoting the development of thymocytes that expressed the truncated heterodimer.

Immunofluorescence experiments indicated that the truncated pre-TCR was distributed on the surface of transfected cells in a manner indistinguishable from that of a wild-type TCR (*21*). We also employed an in vitro assay to examine signaling from the truncated receptor and to determine whether the truncations had caused it to be constitutively activated (*25*). To do so, we transiently transfected either the truncated pre-TCR or a wild-type αβ TCR into a TCRβ-deficient Jurkat T cell line (*26*). Receptor-dependent signaling was then monitored with a luciferase assay (*27*) that reports on the relative transcriptional activity of the nuclear factor of activated T cells (NFAT) (*28*). To increase the sensitivity of the system, we cotransfected the cells with an activated form of ras, which provided some (*29*) but not all (*30*) of the signal needed to activate the NFAT cassette.

Transfection of plasmids encoding either an irrelevant protein (cre recombinase) or β_T alone into the TCRβ-deficient Jurkat line did not induce changes in NFAT activity, as detected with the luciferase reporter assay (Fig. 5A), and did not reconstitute appreciable amounts of surface CD3 (Fig. 5B). In contrast, ligand-independent signal transduction was detected within hours of transfection of either TCRβ or $pT\alpha_T;\beta_T$. The signal was transient and had subsided by 40 to 50 hours, despite sustained expression of the receptors (Fig. 5B). The unmodified αβ receptor reproducibly signaled more robustly than the truncated receptor even though the former was less abundant on the cell surface. Thus, the truncations had not resulted in a constitutively activated signaling phenotype. Further support for this view came from control experiments in which the same cells were transfected with a constitutively active mutant of *Lck*, a kinase essential for pre-TCR function (*5–8*), or a constitutively ligated CD8/ζ chimeric receptor (*31*), which is ligated by endogenous major histocompatibility complex class I molecules on the Jurkat cells. In both cases, by 7 hours after transfection, the luciferase signal was already roughly 500 times greater than the response of the transfected TCR (*21*). The ligand-independent signal could also be observed in the TCR-deficient mouse thymo-

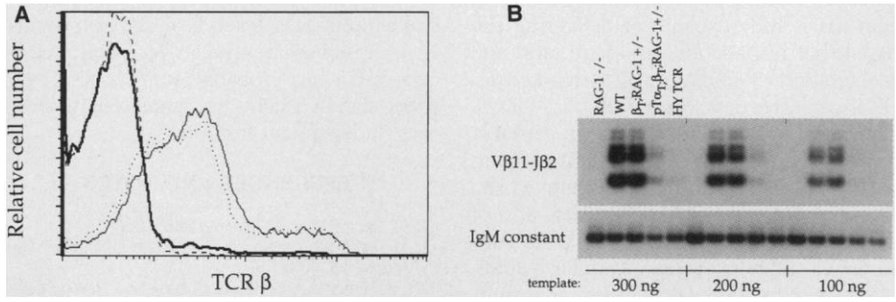

Fig. 4. Allelic exclusion mediated by the fully truncated pre-TCR. (**A**) Relative expression of TCRβ on thymocytes from the following mice: *Rag-1*$^{-/-}$ (dashed line), β_T;*Rag-1*$^{+/-}$ (dotted line), $pT\alpha_T;\beta_T$;*Rag-1*$^{+/-}$ (thick solid line), and C57BL/6–wild type (WT) (thin solid line). (**B**) Allelic exclusion at the endogenous TCRβ locus in thymocytes from mice of the indicated genotypes. Genomic DNA was isolated from thymocytes, and rearrangements at the TCRβ locus were detected with a semiquantitative PCR assay and primers specific for Vβ11 and Jβ2, as previously described (*46*). PCR primers that amplify a region of the IgM constant domain were used as a control. PCR products were detected by Southern blot with primers that hybridize in the amplified regions. Consistent results were obtained with a similar PCR assay with primers specific for Vβ12 and Jβ2 or by Southern blot with a probe from the intron upstream of Dβ1 (*47*).

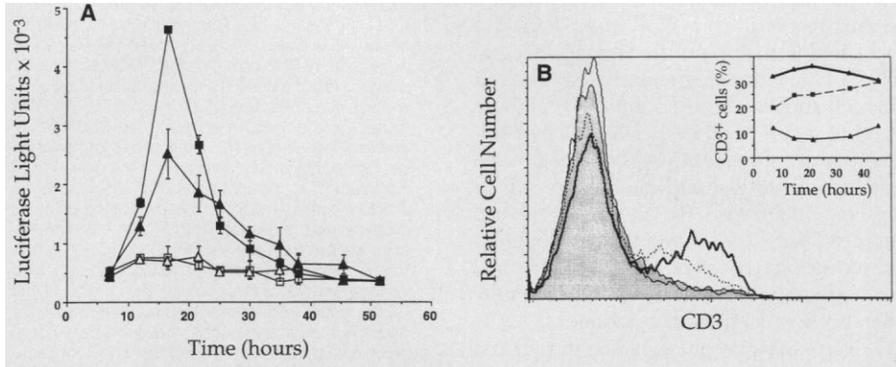

Fig. 5. Ligand-independent signaling by TCRs. (**A**) Luciferase activity after transfection of a TCRβ-deficient Jurkat cell line with plasmids encoding cre recombinase (control; open squares), β_T (open triangles), the Jurkat TCRβ chain (closed squares), or $pT\alpha_T;\beta_T$ (closed triangles), all under the transcriptional control of the EF-1α promoter (*25, 30*). An NFAT-luciferase reporter plasmid (*44*) and a plasmid encoding v-*Ras* were included in all transfections. Transfection efficiency was equivalent between samples, as determined by comparing responses to ionomycin alone. (**B**) Surface CD3ε expression was assessed at 14.5 hours after transfection in TCRβ-deficient Jurkat cells transfected with cre recombinase (shaded histogram, with next-to-highest peak), β_T (thin solid line and highest peak), TCRβ (dotted line), or $pT\alpha_T;\beta_T$ (thick solid line). The inset figure plots the percentage of $CD3\epsilon^+$ cells over time (line designations as in the accompanying histogram). The data are representative of three such extended kinetic experiments (10 time points) and are consistent with the results from many experiments analyzing NFAT activity at various single time points after transfection. (**C**) TCRβ-deficient Jurkat cells were cotransfected with the indicated constructs together with the v-*Ras* and NFAT-luciferase plasmids. At 7 hours after electroporation, cells were either left untreated (black bars) or incubated with anti-CD3 mAb Leu 4 (4 μg/ml) (hatched bars). The cells were incubated at 37°C for 5 hours and then lysed and analyzed for luciferase activity. Transfection efficiency was assessed by comparing responses to ionomycin alone.

ma (as used in Fig. 1) (*18*, *21*). Finally, when comparable amounts were expressed, both the αβ and $pT\alpha_T;\beta_T$ receptors responded equivalently to ligation by mAb to CD3ε (Fig. 5C).

Our data show that a TCRβ;pTα heterodimer lacking all of its extracellular Ig domains is fully capable of delivering the signal that initiates the differentiation and proliferation of immature DN thymocytes. DP thymocytes developed in *Rag-1*$^{-/-}$ mice expressing a TCRβ that lacks the variable domain but retains the constant domain (*32*). Our truncated pre-TCR removed the potential for interactions between any of the Ig domains of the TCRβ;pTα heterodimer and putative extracellular ligands. Moreover, the expression of either a truncated pre-TCR or a wild-type αβ TCR was sufficient to activate TCR signaling pathways in vitro. Thus, ligand binding by the extracellular portion of the TCRβ;pTα heterodimer is probably not essential for the pre-TCR signal to be generated in vivo.

If the extracellular domain of the pTα molecule is not involved in ligand binding, then its primary purpose may be to provide the necessary structural stability for assembly with the TCRβ and the CD3/ζ chains. In this respect, a pre-TCR composed of a truncated β chain and the endogenous pre-TCRα chain was inefficiently expressed at the cell surface (Figs. 1B and 3B), perhaps because it was improperly assembled or unstable, and consequently was retained in the endoplasmic reticulum. The coexpression of a $pT\alpha_T$ chain with β_T efficiently rescued both cell surface CD3 and the developmental transition; therefore, the fully truncated heterodimer may be more stable structurally than an asymmetrical heterodimer.

Ligand-independent signaling in response to de novo expression of a cell surface receptor has a precedent in the receptor-dependent but ligand-independent activation of heterotrimeric G proteins in lipid vesicles reconstituted with α-adrenergic receptors (*33*). Low-level signaling may be a general response to the acquisition of a cell surface receptor and the coincident redistribution of signaling enzymes and their substrates (*34*). The DN to DP transition of thymocytes is a unique developmental checkpoint that may depend on this type of low-grade signaling activity. Unlike most other developmental checkpoints, this one tests for the productive outcome of an autonomous genomic rearrangement process and therefore need not necessarily derive its differentiation cue from an extracellular interaction.

The pre-B cell receptor is essential for a stage of B cell development analogous to that of the thymic cells studied here, the transition from pro- to pre-B cells (*35*). Pre-B cells can develop in the absence of the IgM variable domain and independently of association with surrogate light chains (*36*, *37*). Thus, like the DN to DP transition, the pro- to pre-B cell transition also may be regulated by ligand-independent signaling from a precursor form of the antigen receptor. Finally, the survival of peripheral lymphocytes depends on signals provided by cell surface antigen receptors; for B cells (*38*), but probably not T cells (*39*), these signals might be generated in a ligand-independent fashion.

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45. Single-cell suspensions of thymocytes were stained with the following antibodies (purchased from Caltag, Pharmingen, Becton-Dickinson, or VWR) in phosphate-buffered saline plus 0.3% bovine serum albumin: antibody to CD4 (anti-CD4) (CT-CD4-Tricolor), anti-CD8 [53-6.7–fluorescein isothiocyanate (FITC)], anti-TCRβ [H57-597–phycoerythrin (PE)], anti-CD25 (7D4-FITC), anti-Flag (M2), anti–Myc epitope (9E10), and anti-CD3ε (145-2C11-PE). Jurkat transfectants were stained with Leu 4 (anti-human CD3ε), followed by FITC-conjugated goat antibody to mouse IgG. Stained cells were analyzed with a Becton-Dickinson FACScan and CellQuest software. Dead cells were excluded by gating on the forward and side scatter.
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