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The basic helix-loop-helix (bHLH) transcription factors HEB and E2A are critical mediators of gene regulation during lymphocyte development. We have cloned a new transcription factor, called HEBAlt, from a pro-T cell cDNA library. HEBAlt is generated by alternative transcriptional initiation and splicing from the HEB gene locus, which also encodes the previously characterized E box protein HEBCan. HEBAlt contains a unique N-terminal coding exon (the Alt domain) that replaces the first transactivation domain of HEBCan. Downstream of the Alt domain, HEBAlt is identical to HEBCan, including the DNA binding domain. HEBAlt is induced in early thymocyte precursors and down-regulated permanently at the double negative to double positive (DP) transition, whereas HEBCan mRNA expression peaks at the DP stage of thymocyte development. HEBAlt mRNA is up-regulated synergistically by a combination of HEBCan activity and Delta-Notch signaling. Retroviral transduction of HEBAlt or HEBCan into hemopoietic stem cells followed by OP9-DL1 coculture revealed that HEBAlt-transduced precursors generated more early T lineage precursors and more DP pre-T cells than control transduced cells. By contrast, HEBCan-transduced cells that maintained high level expression of the HEBCan transgene were inhibited in expansion and progression through T cell development. HEBB–/– fetal liver precursors transduced with HEBAlt were rescued from delayed T cell specification, but HEBCan-transduced HEBB–/– precursors were not. Therefore, HEBAlt and HEBCan are functionally distinct transcription factors, and HEBAlt is specifically required for the efficient generation of early T cell precursors.


The generation of T cells from hemopoietic stem cells requires the activity of successive combinations of specific transcriptional regulators, including members of the class I basic helix-loop-helix (bHLH)1 transcription factor family (1). In vertebrates, the class I bHLH family includes E2A (Tcf2a), HEB (Tcf12), and ITF-2 (Tcf4/E2–2). Each of these bHLH factors shares a C-terminal bHLH (DNA binding and dimerization) domain and conserved N-terminal activation domains (AD1 and AD2) (2). bHLH factors act as dimers to control transcription of their target genes, and their activity can be inhibited by Id (inhibitor of DNA binding) factors (3). E2A homodimers are essential for early B cell development, whereas HEB/E2A heterodimers are dominant in T cell development (4, 5). Ectopic expression of Id factors disrupts T cell differentiation, consistent with essential roles for bHLH factors in T cell development (6, 7).

The basic helix-loop-helix (bHLH) transcription factors HEB and E2A are critical mediators of gene regulation during lymphocyte development. We have cloned a new transcription factor, called HEBAlt, from a pro-T cell cDNA library. HEBAlt is generated by alternative transcriptional initiation and splicing from the HEB gene locus, which also encodes the previously characterized E box protein HEBCan. HEBAlt contains a unique N-terminal coding exon (the Alt domain) that replaces the first transactivation domain of HEBCan. Downstream of the Alt domain, HEBAlt is identical to HEBCan, including the DNA binding domain. HEBAlt is induced in early thymocyte precursors and down-regulated permanently at the double negative to double positive (DP) transition, whereas HEBCan mRNA expression peaks at the DP stage of thymocyte development. HEBAlt mRNA is up-regulated synergistically by a combination of HEBCan activity and Delta-Notch signaling. Retroviral transduction of HEBAlt or HEBCan into hemopoietic stem cells followed by OP9-DL1 coculture revealed that HEBAlt-transduced precursors generated more early T lineage precursors and more DP pre-T cells than control transduced cells. By contrast, HEBCan-transduced cells that maintained high level expression of the HEBCan transgene were inhibited in expansion and progression through T cell development. HEBB–/– fetal liver precursors transduced with HEBAlt were rescued from delayed T cell specification, but HEBCan-transduced HEBB–/– precursors were not. Therefore, HEBAlt and HEBCan are functionally distinct transcription factors, and HEBAlt is specifically required for the efficient generation of early T cell precursors.


1 Abbreviations used in this paper: bHLH, basic helix-loop-helix; DP, double positive; DN, double negative; ISP, immature single positive; SCF, stem cell factor.

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positive (ISP), CD8<sup>+</sup>CD4<sup>−</sup>CD3<sup>−</sup>) cells, and then express CD4<sup>+</sup> to become double positive (DP, CD4<sup>+</sup>CD8<sup>+</sup>) thymocytes. HEB<sup>−/−</sup> thymuses accumulate ISP cells and show a decrease in the percentage of DP cells, indicating that loss of HEB results in a second partial block at the ISP to DP transition (11). In normal thymocyte development, positive selection leads to the production of mature SP (CD4<sup>+</sup> or CD8<sup>+</sup>) thymocytes, whereas negative selection deletes potentially autoreactive T cells. E2A<sup>−/−</sup> thymocytes exhibit perturbations in the CD4 to CD8 ratios of SP thymocytes, and a propensity toward malignant transformation into thymic lymphomas (9). No reports have been published thus far on the ability of HEB<sup>−/−</sup> thymocytes to become cancerous, perhaps because of the more severe neonatal lethality in the HEB<sup>−/−</sup> mice (12).

Class I HHLH factors and Id factors are intimately involved in the control of cell cycle proteins in addition to their roles in activating lymphocyte-specific genes (4, 5, 21). Therefore, understanding the integrated control of differentiation and proliferation by HEB and E2A during lymphocyte development will be essential for understanding both normal T cell development and the potential for aberrant differentiation that can lead to cancer when the functions of these regulatory proteins are perturbed. In this report we describe a new form of HEB called HEBAlt, which is expressed specifically in early T cell precursors. The distinctive expression pattern of this factor in thymocytes has already prompted us to use it as a reference sample in a few studies (22–24). In this study, however, we present the first detailed description of HEBAlt, and show that HEBAlt is specifically required for efficient generation of T cell precursors.

Materials and Methods

Animals

The animals used for the RT-PCR studies were C57BL6/Jc mice, C57BL6/ E129 Rag-2<sup>−/−</sup> mice, C57BL6/Jc Scid mice, C57BL6/Jc MHC<sup>−/−</sup> mice, and C57BL6/Jc-TCR<sup>β</sup>−/−<sup>β</sup>−/− mice. HEB<sup>−/−</sup> mice were generated by Y. Zhuang (Duke University Medical Center, Durham, NC) (12) and provided to us by T. Hoang, Université de Montreál (Montreál, Quebec, Canada). Thymus samples were taken from 3- to 5-wk-old mice, and spleenocytes and bone marrow from mice 12 wk or older. For O9-DL1 cocultures, E14.5 embryos were obtained from either National Institutes of Health Swiss timed matings (National Cancer Institute) or from C57BL/6 wild-type or C57BL6/Jc-HEB<sup>−/−</sup> timed matings at Sunnybrook Research Institute. The studies described within this report have been reviewed and approved by institutional review committees at California Institute of Technology or Sunnybrook Research Institute.

Sequence analysis

The HEBAlt cDNA sequence, obtained by sequencing a cDNA from a SCID thymocyte arrayed library (25), matches a RIKEN 12-kD male wolfish duct full-length cDNA (GenBank accession no. AK078415) (26). The Alt-specific (does not match HEBCan accession no. NM_011544) coding nucleotide sequence was run against the nonredundant GenBank database, the chicken EST database, and the Fugu genome using the Blastx and tBlastn programs (27). GenBank accession nos. for (Sca-1 C57BL6/Jc-HEB<sup>−/−</sup> mice) were obtained from either National Institutes of Health Swiss timed matings (National Cancer Institute) or from C57BL/6 wild-type or C57BL6/Jc-HEB<sup>−/−</sup> timed matings at Sunnybrook Research Institute. The studies described within this report have been reviewed and approved by institutional review committees at California Institute of Technology or Sunnybrook Research Institute.

Western blot analysis

C57BL6/Jc (CD4<sup>+</sup>CD8<sup>+</sup>) thymocytes were purified using anti-CD4-biotin and anti-CD8-biotin Abs, streptavidin microbeads, and a MIDI-MACS magnetic column (Milenyi Biotec). Cell lysates were run on acrylamide gels and transferred to membrane as previously described (22). Abs used to probe the protein blots were rabbit polyclonal anti-HEB (A-20, sc-537) and rabbit anti-β-tubulin (H-235, SC-9104) from Santa Cruz Biotechnology.

Expression constructs

Full-length coding sequences were assembled and cloned into the pBk-CMV plasmid backbone using cDNAs from an arrayed SCID thymocyte library (25). These cDNAs do not contain the alternative “Ank” exon found in some other reported HEB cDNAs (29) (see Fig. 1) because they were naturally absent from the HEB cDNA clones obtained from the SCID thymocyte library. Recombinant plasmids were sequenced and used as templates in PCRs (Platinum Pfx DNA polymerase; Invitrogen Life Technologies) to amplify the coding region for insertion into the Zero Blunt TOPO vector (Invitrogen Life Technologies). XhoI restriction fragments amplified by Pfx from TOPO clone DNA were then cloned into the MIGR1 retroviral expression vector at the XhoI site. Primers were as follows: HEBAlt 5′-gactcgagcaccatgtactgtgcttatcct; HEBCan-1 5′-gatgcagagacacatggtaattct (see Fig. 1), and first-strand cDNA was generated from total RNA using SuperScript RT III. All values were calculated relative to β-actin or GAPDH as indicated.

Gel shift analysis

Nuclear extracts were prepared from transfected HeLa cells according to the reported procedure (18). Nuclear cell extract (5 μg of protein) was incubated with 0.5 fmol of 32P-labeled oligonucleotide at 0°C for 20 min in a 10-μl reaction mixture (20 mM HEPES buffer (pH 7.9), 100 mM KCl, 12.5 mM MgCl2, 1 mM EDTA, 20% (v/v) glycerol, 2 mM DTT, 0.5 mM PMSF, and 1 μg of poly(dI-dC)-poly(dI-dC)) in the presence or absence of unlabeled oligonucleotide. DNA protein complexes were resolved by electrophoresis on 5% polyacrylamide gels at 4°C for 1 h at 120 V in TGE
buffer (25 mM Tris-HCl (pH 8.0), 192 mM glycine, and 2 mM EDTA). Supershift was done by following a published protocol (18). One microdotel of anti-HEB Ab (sc-357) or 3 μl of anti-FLAG Ab (Sigma-Aldrich) were used for Supershift analysis. Wild-type E box oligonucleotide sequence GGGTTA (18). Bold type indicates wild-type or mutant E box binding sites.

**Isolation of fetal liver precursors**

Fetal livers were dissected from day 14.5 embryos and made into single cell suspensions by pipetting up and down in 1 ml of HBSS (Invitrogen Life Technologies) plus 0.25% BSA and 2 mM EDTA per liver. Cells were enriched for multipotent precursors by magnetic bead depletion with biotinylated Abs (lineage (Lin): anti-Gr-1, anti-Ter119, anti-F4/80, and anti-CD19) and streptavidin microbeads using the Midi-MACS system. Fetal livers obtained from HEB<sup>+/−</sup> X HEB<sup>+/−</sup> timed matings were individually genotyped by PCR and then pooled according to genotype for MACS enrichment before tranduction and plating on OP9-DL1 monolayers.

**Retroviral transduction**

Retroviral DNA was prepared using Endo-Free DNA extraction reagents and columns (Qiagen). The DNA was cotransfected into <i>ϕ</i>NX-Eco packaging cells with the <i>ϕ</i>Eco plasmid using a standard calcium phosphate transfection method. Supernatants were collected after 24 and 48 h, titered using NIH3T3 cells, and frozen in aliquots at −70°C until use. Cells were transduced by modified spin infection (22) in the presence of either polybrene (8 μg/ml) or Lipofectamine (Invitrogen Life Technologies).

Fetal liver Lin<sup>−</sup> cells were purified, transduced, and cultured overnight in OP9-DL1 medium (30) plus 5 ng/ml IL-7, 5 ng/ml stem cell factor (SCF), and 5 ng/ml Flt3 ligand (R&D Systems). The next day (16–24 h after infection) the Sca-1<sup>−</sup>CD117<sup>+</sup> GFP<sup>+</sup> fraction was sorted and plated on OP9-DL1 monolayers. Results were similar whether input cells were sorted for LSK GFP<sup>+</sup> populations. OP9-DL1 coculture

OP9-DL1 cells, which support the in vitro development of T cells, have been previously described (30). For coculture, 3,000–10,000 transduced precursors were placed on OP9-DL1 monolayers in OP9-DL1 medium in the presence of IL-7, SCF, and Flt3 ligand (all 5 ng/ml; some experiments omitted SCF after day 7 and lowered IL-7 to 1 ng/ml). OP9-DL1 medium consisted of high glucose DMEM (Invitrogen Life Technologies) supplemented with 10 mM HEPES, 1 mM sodium pyruvate, 1× PSG (penicillin-streptomycin-glutamine; Invitrogen Life Technologies), 16% FBS (HyClone), and 55 μM 2-ME. Cultures were split every 3–5 days and placed on freshly plated subconfluent OP9-DL1 monolayers. Cultures were analyzed every 3–4 days after plating by flow cytometry using a FACSCalibur (BD Biosciences), and data were analyzed using CellQuest Pro (BD Biosciences).

**Results**

**Discovery of HEBAlt, an evolutionarily conserved isoform of HEB that includes a novel N terminus and lacks the AD1 domain**

During a gene discovery project for transcription factors expressed at the pro-T cell stage of T cell development (25), we discovered a new form of the bHLH factor HEB. This transcript, termed HEBAlt, includes the bHLH domain and the AD2 domain of the previously described HEB transcript (which we call HEBCan, for canonical HEB), but replaces AD1 with a novel sequence we refer to as the Alt (alternative) domain (Fig. 1). The predicted 23 aa Alt domain is homologous to the N-terminal region of ITF-2A, which represents an alternatively spliced form of the related bHLH factor ITF-2 (31) (Fig. 1, A and C). The predicted amino acid sequence of Alt domain of HEB is well conserved throughout vertebrate phylogeny (Fig. 1C), indicating conserved function. We assembled a map of the HEB locus by comparing the HEBCan and HEBAlt cDNAs with the mouse genome sequence. The HEB locus is very large, spanning over 200 kb in the mouse, and the Alt exon is located between exons 8 and 9 (Fig. 1B). The transcriptional initiation site of HEBAlt mRNA is just upstream of the Alt exon. In the HEBAlt transcript, the Alt domain replaces exons 1–8 of HEBCan; in the HEBCan transcript, the Alt domain is spliced out.

**FIGURE 1.** Structure, organization, and conservation of an alternatively initiated and spliced form of HEB called HEBAlt. A, cDNA structures of vertebrate class I bHLH factor isoforms. Domains (▏) AD1, activation domain 1; AD2, activation domain 2; and bHLH, basic helix-loop-helix DNA binding and dimerization domain (▏). Alt, Alt domain are shown. B, Genomic organization of the mouse HEB locus. Location and splicing of the Alt exon are indicated between exons 8 and 9. Arrows indicated transcriptional start sites. Black vertical boxes indicate exons, and numbers below the line indicate intron size in kilobase (only indicated for large introns). The black horizontal box below the line indicates the location of the bHLH domain. Ank, alternatively spliced exon (29), can be included or excluded. The Ank exon is excluded from the HEB transgenes used in this study due to their absence in SCID thymocyte cDNA library clones. C, Amino acid sequences of Alt domains present in ITF-2 or HEB cDNAs in different vertebrate species. Stars indicate identity, colon indicates conserved types of amino acids, and dashes indicate gaps. GenBank accession numbers: MITF-2A (mouse, <i>Mus musculus</i>) U16321.1; TsHEBAlt (green spotted pufferfish, <i>Tetraodon nigroviridis</i>) CAAE01014581.1; DrHEBAlt (zebrafish, <i>Danio rerio</i>) NP_999981; HsHEBAlt (human, <i>Homo sapiens</i>) NP_999623; FrHEBAlt (Japanese pufferfish, <i>Fugu rubripes</i>) CAA01000314.1; FrITF2A CAAB01001447.1 and GgHEBAlt (chicken, <i>Gallus gallus</i>) BU441064.1.
HEBAlt is expressed only in the DN stages of thymocyte development

To define HEBAlt expression in the context of other bHLH factors during T cell development, RT-PCR was performed on primary cells representing successive stages of T cell differentiation (Fig. 2A). HEBAlt exhibits a strikingly unique expression pattern among the genes surveyed, in that it is the only bHLH factor that is restricted to the DN stages of T cell development (Fig. 2A). HEBCan, by contrast, is expressed in all thymocyte and splenocyte subsets analyzed in this study, and peaks in relative message level of c-Kit, pre-T cell, Rag-2, pre-T cell, and with the onset of TCR rearrangement. To estimate HEBCan and HEBAlt protein levels during T cell development, Western blot analysis indicates that HEBAlt and HEBCan levels are similar in all thymocyte and splenocyte subsets. DN1 (c-Kit"CD44"CD25"), DN2 (c-Kit"CD44"CD25"), DN3 (c-Kit"CD44"CD25"), and DN4 (c-Kit"CD44"CD25"). The CD4"CD8" DP subset was sorted directly from C57BL/6 thymus. D, Quantitative real-time PCR analysis of E14.5 fetal thymocytes sorted according to expression of c-Kit, CD44, and CD25 as in E. Western blots for HEB expression on cell lysates isolated from DN cells enriched by MACS purification (lane 1), DN3 (CD44"CD25"Thy-1") cells (lane 2), and DN4 (CD44"CD25"Thy-1") cells (lane 3) isolated by sorting, and unfractionated wild-type thymocytes (lane 4), which are composed primarily (85%) of DP cells. An anti-β-tubulin Ab was used as a loading control.

FIGURE 3. HEBAlt binds to an E box motif. A, Structure of the Flag-tagged HEBAlt construct (3XF-HA; Met, methionine). B, Western blot showing that the protein expressed from the 3XF-HA construct was detected by both the anti-flag Ab and anti-HEB Ab. C and D, Gel shift experiments in which a radiolabeled probe containing two E box oligonucleotides (E box oligo) (18) was incubated with nuclear extracts (NE) or in vitro transcribed and translated (TnT) proteins, then run on acrylamide gels. Specificity controls include: untransfected HeLa cells (Control-NE), mutant E box oligonucleotides (mE box oligo), unlabeled 10-fold E box oligonucleotide competitor (cold E box), anti-HEB Ab, anti-Flag epitope Ab, and unlabeled mutant E box competitor (cold mE box). Arrow indicates bound probe; free probe is evident at the bottom of the gel in D but ran off the gel in C.
are ~85% DP cells, consistent with the mRNA expression pattern. HEBCan protein levels do not appear to be higher in DP cells than in DN cells, however, despite the increase in mRNA. This result indicates that HEBCan may be subject to the same types of post-translational control that have been reported for E2A during T cell development (10).

**HEBAlt can specifically bind an E box motif**

Although HEBAlt contains all of the domains necessary for dimerization and DNA binding, it remained possible that the Alt domain could cause a conformational change that would prohibit binding to DNA. To determine whether HEBAlt-containing dimers could bind relevant E box sites from the pre-Tα locus (18), gel shift analyses were performed. Because HEBAlt-specific Abs were not available, two protein sources were used in standard gel shift assays to detect HEBAlt-specific binding: 1) in vitro transcribed and translated HEBAlt protein, and 2) Flag-tagged HEBAlt protein (3XF-HEBAlt) expressed in HeLa cells by transient transfection (Fig. 3A). Immunoblotting of HeLa cells transfected with 3XF-HEBAlt vs control plasmid DNA showed specific expression of Flag-tagged HEBAlt on a background of minimal endogenous HEB protein (Fig. 3B). HEBAlt from both sources exhibited binding to the probe. The specificity of the interaction was confirmed in two ways: 1) by preincubation with anti-HEB Ab (Fig. 3C) or anti-Flag Ab (Fig. 3D), each of which blocked binding of HEBAlt to the labeled DNA, and 2) by incubation with mutant E box oligonucleotides, which did not induce a gel shift when labeled (Fig. 3C) or inhibit binding to the labeled E box probe when used as unlabeled competitor (Fig. 3D).

**Synergistic regulation of HEBAlt transcription by HEBCan and Delta-Notch signaling**

To understand the role of HEBAlt in early T cell development, we generated retroviral expression constructs in the MIGR1 (MSCV-IRES-GFP) backbone that express HEBAlt (MIGR1-HEBAlt) or express HEBCan (MIGR1-HEBCan) (Fig. 4A). These constructs were used to transduce the adh.2C2 pro-T cell line (24, 32). Two days after transduction, GFP+ cells were sorted and subjected to quantitative real-time PCR analysis of HEBAlt and HEBCan mRNA levels (Fig. 4B). Total HEBAlt mRNA levels increased relative to the control in cells transduced with the MIGR1-HEBAlt retroviral construct, whereas HEBCan levels remained unchanged.

**FIGURE 4.** HEBCan and Delta-Notch signaling synergistically up-regulate HEBAlt transcription. A. Retroviral expression constructs for HEB gene transfer. HEB isoforms were cloned into the MIGR1 retroviral backbone, which contains a multiple cloning site upstream of an IRES (internal ribosome entry site) and GFP (green fluorescent protein). The MSCV retroviral promoter (depicted by arrows) drives transcription of the bicistronic HEB-IRES-GFP mRNA; separate HEB and GFP proteins are generated from the transcripts. MIGR1-HEBAlt (M-HA) and MIGR1-HEBCan (M-HC) are shown. B. Adh.2C2 DN3/DN4-like pro-T cells (24) were transduced with MIGR1-based constructs, sorted for GFP+ populations 48 h later, and analyzed by quantitative real-time PCR to measure total (retroviral plus endogenous) HEBAlt and HEBCan mRNA levels. Quantitative real-time PCR measurements are normalized to β-actin values and shown on a log scale. C. Adh.2C2 pro-T cells were transduced with MIGR1-based constructs and analyzed by Western blot analysis 48 h later for total (retroviral plus endogenous) HEB protein levels. Anti-β-tubulin Ab was used as a loading control. D. Adh.2C2 pro-T cells cultured under three conditions: 1) in medium, 2) OP9-GFP coculture, or 3) OP9-DL1 coculture for 48 h, without retroviral transduction. HEBAlt and HEBCan mRNA levels were measured relative to β-actin by quantitative real-time PCR and shown on a linear scale. No retrovirus was used in this study; mRNA levels reflect endogenous gene expression only. E. Adh.2C2 cells were transduced with MIGR1 or MIGR1-HEBCan, and transduced cells were cocultured on either OP9-GFP or OP9-DL1 cells for 48 h, and then analyzed for HEBAlt mRNA levels relative to β-actin by quantitative real-time PCR. No HEBAlt retrovirus was used in this study; HEBAlt mRNA measurements reflect endogenous levels only. F. Model for the induction of HEBAlt by combinatorial inputs from stromal signals and HEBCan activity. HEBCan protein (gray oval) generated from the HEB locus feeds back directly or indirectly (question mark) on the promoter that drives HEBAlt expression within the HEB locus, and synergistically up-regulates HEBAlt with stromal signals provided by OP9-DL1 cells.
In contrast, both HEBCan and HEBAlt total mRNA levels increased in HEBCan-transduced cells. These results suggested that increased levels of HEBCan up-regulated endogenous HEBAlt transcription in adh.2C2 cells. This up-regulation was confirmed at the protein level in a separate set of experiments (Fig. 4C).

The timing of normal endogenous HEBAlt up-regulation in DN thymocytes suggested it may be a target of Delta-Notch signaling. To test the influence of Delta-Notch signaling on HEBAlt expression, we cocultured adh.2C2 pro-T cells on OP9-GFP (Delta-1 negative) or OP9-DL1 (Delta-1 positive) monolayers and analyzed the levels of HEBAlt mRNA relative to adh.2C2 cultured without stroma. Coculture on either OP9-GFP or OP9-DL1 cells induced up-regulation of both HEBAlt and HEBCan mRNA, suggesting the influence of signals common to both stromal lines (Fig. 4D). However, OP9-DL1 coculture induced slightly higher levels of HEBAlt than OP9-GFP coculture. Strikingly, the combination of retroviral expression of HEBCan plus Delta-Notch signaling in the adh.2C2 cell line caused a synergistic increase in HEBAlt levels far above the increase caused by either inducer alone (Fig. 4E and F). It remains to be determined whether these factors bind directly to HEBAlt regulatory regions or whether this induction is indirect.

Retroviral expression of HEB isoforms in hemopoietic multipotent precursors

To test whether expression of HEBAlt can influence the ability of multipotent progenitors to adopt a T cell fate, we used the same retroviral vectors shown in Fig. 4 to transduce fetal liver-derived hemopoietic precursors (Fig. 5A). HEBAlt is distinguished from HEBCan both by the absence of exons 1–8 and by the presence of the Alt exon. To determine which of these domains are important for differences in functional impact, we also transduced cells with an artificial variant of HEB that contains exons 9–21 plus a start codon. This construct, HEBTr, is identical with HEBCan except that it lacks the Alt domain, and thus allows discrimination between functions that require the Alt domain and those that do not.

Fetal liver cells enriched for Lin<sup>-</sup>/H11002 cells were transduced and cultured overnight to allow expression of GFP before sorting. MIGR1-transduced control cells always had higher percentages and levels of GFP<sup>+</sup> cells than either MIGR1-HEBAlt- or MIGR1-HEBCan-transduced samples (Fig. 5B). Furthermore, MIGR1-HEBCan-transduced samples were routinely GFP<sup>+</sup> compared with either control or HEBAlt-transduced samples; this was also true of MIGR1-HEBTr-transduced samples (data not shown). Importantly, a clear GFP<sup>+</sup> population and a clear GFP<sup>-</sup> population were always observed in the MIGR1-transduced control cells, whereas the GFP levels in HEB-transduced cells appeared as a continuum from GFP<sup>+</sup> (above nontransduced levels) to GFP<sup>-</sup>, instead of two separate GFP<sup>+</sup> and GFP<sup>-</sup> populations. HEBTr-transduced fetal liver cells were very similar to the HEBCan-transduced cells in terms of percentage and levels of GFP detected by FACS analysis (data not shown).

Immunoblot analysis of unsorted cells 24 h after transduction confirmed that fetal liver cells transduced with HEBAlt or HEBCan expressed elevated levels of HEBAlt or HEBCan protein, respectively, and that control transduced fetal liver cells do not express detectable protein levels of either HEB isoform (Fig. 5C).

**FIGURE 5.** Retroviral gene transfer of HEBAlt, HEBCan, or HEBTr into fetal liver-derived hemopoietic stem cells. A, Experimental design for transduction of fetal liver (FL) cells transduced with MIGR1-based constructs and cocultured with OP9-DL1 cells. Open circle indicates untransduced cells; gray circle indicates transduced transgenic cells. B, FACS plots of Lin<sup>-</sup> fetal liver cells 20 h after transduction with retroviral constructs. This plot is representative of multiple experiments. Numbers designate the percentage of GFP<sup>+</sup> cells in the gate. C, Western blot of fetal liver cells cultured in IL-7, SCF, and Flt3 ligand for 24 h after transduction with MIGR1-based constructs. HEBTr (Tr, truncated) is identical with HEBAlt except that the Alt domain is replaced with a methionine. D and E, Quantitative real-time PCR analysis of HEBAlt and HEBCan mRNA levels in fetal liver cells transduced with MIGR1-based constructs, cultured for 48 h in IL-7, SCF, and Flt3 ligand, and sorted for the Sca-1<sup>-</sup> c-kit<sup>+</sup> GFP<sup>+</sup> population or the Sca-1<sup>-</sup> c-kit<sup>+</sup> GFP<sup>-</sup> population 48 h after transduction with MIGR1-based constructs. Values are shown on a log scale.
In a separate experiment, transduced fetal liver cells were sorted for GFP+ and GFP− subsets 48 h after transduction and analyzed by quantitative real-time PCR (Fig. 5, D and E). In agreement with the up-regulation of HEBAlt by HEBCan in adh.2C2 cells, HEBAlt mRNA was also induced in fetal liver cells expressing retroviral HEBCan (Fig. 5D). This was not seen at the protein level at 24 h, suggesting that more time (up to 48 h) is required for the induction of HEBAlt at the protein level in response to ectopic HEBCan expression, in addition to the decreased sensitivity of the Western blot assay as compared with the quantitative real-time PCR assay. Total levels of HEBCan mRNA in the HEBCan-transduced cells did not increase, indicating that high levels of HEBCan were not tolerated. Like the GFP− sorted fractions, the GFP− sorted fractions had higher levels of HEBAlt mRNA in both HEBAlt-transduced and HEBCan-transduced cells (Fig. 5E), indicating that low level expression of the GFP from the transgene was not detectable by flow cytometry, as has been previously reported (33).

Transcription expression of retroviral HEB transgenes generates a high frequency of GFP− cells from sorted GFP+ transduced precursors

To assess the effects of expressing HEBAlt or HEBCan in hematopoietic stem cells, we placed transduced fetal liver precursors in OP9-DL1 coculture, which induces T cell development, or OP9-GFP coculture, which allows development of B cells (34). Introduction of HEB into precursors did not induce T cell development in OP9-GFP coculture, indicating that Notch signaling was still necessary in the presence of HEB (data not shown). T cell development did occur in OP9-DL1 coculture, as assessed by the appearance of CD4+CD8+ DP pre-T cells. In cell cultures that were seeded with sorted GFP+ HEB-transduced precursors, the majority of the surviving cells down-regulated retroviral transgene expression to appear GFP+ by FACS analysis by day 14 of coculture. This was a specific response to HEB expression because virtually no GFP− cells were generated by the MIGR1-transduced controls. Consistent with their origin from HEBAlt-transduced cells, the GFP− cells in the HEBAlt cultures were similar in phenotype to the cells that remained detectably GFP− (Fig. 6A).

Different effects of transient vs sustained expression of HEBAlt

Strikingly, HEBAlt-transduced precursors generated higher percentages (Fig. 6A) and higher numbers (Fig. 6B) of CD4+CD8− DP T cell precursors than control MIGR1-transduced precursors. This increase was seen in multiple experiments, as plotted in Fig. 6C. The GFP− cells in the HEBAlt cultures were similar in phenotype to the GFP+ cells, with an expanded DP population. However, the numbers of GFP− cells were much higher than the numbers of GFP+ cells for HEBAlt-transduced cultures. To determine the time at which these GFP− cells first arose, the percentage of GFP+ cells at was tracked every 3–4 days over the 2-wk period of coculture (Fig. 7A). This time course suggested that the drop in the percentage of GFP+ cells occurred during the DN stages, and that the initial appearance of these cells, there was a steady ratio of GFP+ to GFP− cells in each culture.

Fig. 7B shows representative data from a separate set of experiments in which hemopoietic stem cells were sorted, transduced, and placed in OP9-DL1 coculture without first purifying the GFP+ cells, which yielded enough cells for day 4 analysis but not enough cells for a day 0 analysis (Fig. 7B). By day 4, a substantial number of GFP− cells were already present in the HEB-transduced cultures, and this ratio remained constant throughout the remainder of the culture period. The initial transduction rate was unknown, but expected to be quite high from previous titering of the supernatants (similar to that shown in Fig. 5B). Survival of these cells (and maintenance of GFP− cells) was clearly increased when cells were immediately placed in OP9-DL1 coculture after transduction rather than overnight culture with IL-7, SCF, and Flt3 ligand followed by sorting of GFP+ cells before OP9-DL1 coculture. These data are consistent with the appearance of GFP− cells at the DN1

FIGURE 6. HEBAlt-transduced cells have increased numbers of DP T cells. MIGR1, MIGR1-HEBAlt (M-HA), MIGR1-HEBCan (M-HC), MIGR1-HEBTr (M-HTr) transduced fetal liver-derived LSK GFP+ cells were cocultured on OP9-DL1 cells for 14 days and then analyzed by FACS for GFP, CD8, and CD4 expression. A, Plots (upper row) are analyzed for GFP expression vs CD8 expression, gated on a live lymphocyte scatter gate. Plots (middle row) (CD4 vs CD8) are gated on the GFP+ cells, whereas plots (lower row) (CD4 vs CD8) are gated on the GFP− cells. Very few GFP− cells were present in the MIGR1 control transduced sample, so those are not shown (N/A = not applicable). B, Total cell numbers for the GFP+ and GFP− DP cells for each construct. The numbers shown are from a different experiment than that shown in A but are representative in relative scale for multiple experiments. C, Compilation of percentage of DP cells in the GFP− populations for four different experiments analyzed at approximately day 14 of OP9-DL1 coculture.

A

B

C

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to DN2 transition (Fig. 7C), at the time of or before T cell specification.

The detection of low levels of transgene message in GFP− cells sorted from day 7 cocultures confirmed that at least some of these cells had arisen from HEBAlt-transduced precursors which had down-regulated the transgene (Fig. 7D). Inclusion of CD25+ as a sorting criteria ensured that OP9-DL1 stromal cells, which also express GFP, were excluded from analysis. Furthermore, the GFP− cells in the HEBAlt-transduced cultures expanded more rapidly than either the control cells or the HEBAlt-transduced GFP+ cells (Fig. 7, E and F). These data suggest retroviral HEBAlt causes an initial increase in T cell precursors, and that subsequent down-regulation of the HEBAlt transgene confers a proliferative advantage over those that maintain HEBAlt expres-
sion during T cell development. Because HEBAlt can be induced by HEBCan in early precursors, it also suggests that the early influence of HEBCan on T cell precursor expansion may be due to up-regulation of HEBAlt. Cells transduced with HEBTr, which is identical with HEBAlt except for the deletion of the Alt domain, were even more severely inhibited in their ability to become DP T cell pre-
cursors, indicating that the Alt domain was required for the T cell-promoting activity of HEBAlt.

### Figure 7: Early transient expression of the HEBAlt but not the HEBCan retroviral transgene confers a proliferative advantage on developing T cell precursors

A and B. Percentage of GFP+ cells in each culture as a function of time; one representative experiment is shown but results were consistent over multiple experiments. Cells were sorted for the GFP+ fraction before OP9-DL1 coculture without sorting for GFP in B. The earliest measurement in these samples was at day 4 due to technical constraints. C. The time at which cells at each stage of T cell development emerged in these experiments. D. Transduced GFP+ LSK cells were cocultured with OP9-DL1 cells for 7 days and then CD25+ cells were sorted back out to assess whether the GFP− cells that arose during coculture had detectable low levels of transgene by quantitative real-time PCR analysis of GFP-containing mRNAs. GFP levels are shown relative to β-actin levels on a log scale. E. Total cell numbers (GFP− and GFP+ cells in a lymphocyte scatter gate) for OP9-DL1 cocultures containing transduced precursors at day 7 and day 11. Results are representative of multiple experiments. F, GFP+ cell numbers for OP9-DL1 cocultures containing transduced precursors at day 7 and day 11. Results are representative of multiple experiments. G. Model for the mechanism by which HEBAlt-transduced precursors achieve an increased number of GFP+ T cell precursors.

### Different effects of transient vs sustained expression of HEBCan

The appearance of GFP− cells in the HEB-transduced cultures provided the ability to distinguish between early and late effects of transgene expression during T cell development. Sustained expression of HEBCan did not increase the numbers or percentages of DP cells, but was instead inhibitory for proliferation (Fig. 6B). By contrast, cells that transiently expressed HEBCan exhibited a similar increase in the percentages of DP T cells as the HEBAlt-trans-
duced cells. Unlike the HEBAlt-transduced cultures, cells that expressed HEBCan transiently did not expand in number in OP9-DL1 coculture. Because HEBAlt can be induced by HEBCan in early precursors, it also suggests that the early influence of HEBCan on T cell precursor expansion may be due to up-regulation of HEBAlt. Cells transduced with HEBTr, which is identical with HEBAlt except for the deletion of the Alt domain, were even more severely inhibited in their ability to become DP T cell precursors, indicating that the Alt domain was required for the T cell-promoting activity of HEBAlt.

### HEBAlt rescues delayed T cell specification in HEB−/− precursors and enhances the generation of T cell precursors from wild-type multipotent progenitors

Analysis of the percentage of GFP+ cells at each time point in culture indicated that a brief burst of transgene expression within the first 4 days of culture was responsible for the increase in T cell precursors seen in the HEBAlt-transduced cultures (Fig. 7, A and B). FACS analysis at day 4 confirmed that HEBAlt-transduced cultures had increased percentages of CD25+Thy-1+ pro-T cells at this early time point in both GFP+ and GFP− populations. These results were consistent among multiple experiments (Fig. 8D). HEBCan-transduced GFP− cells also displayed a higher percentage of pro-T cells (Fig. 8A). To distinguish between the intrinsic ability of HEBCan to influence early T cell development vs its ability to up-regulate endogenous HEBAlt in early T cell precursors (Fig. 8C), we transduced HEB−/− fetal liver precursors with the same retroviral constructs used in the wild-type experiments. Control-transduced HEB−/− precursors showed a delay in the production of DN2 cells by day 4 of coculture relative to the wild-type MIGR1-transduced controls. Strikingly, only HEBAlt
increased the percentage of DN2 cells from HEB−/− precursors. The inability of other HEB isoforms, which have the ability to form bHLH heterodimers to increase pro-T cell generation, suggests that HEBAlt plays a specific positive function rather than a simple dominant negative function by inhibiting other bHLH proteins such as E2A. Therefore, HEBAlt rescues efficient generation of pro-T cells from hemopoietic stem cells, whereas HEBCan does not.

**Discussion**

We have discovered a new transcription factor, HEBAlt, that is up-regulated as cells become specified to the T cell lineage, and that is required for efficient generation of T cell precursors. The timing of endogenous HEBAlt expression suggests that it may be downstream target of Notch and/or IL-7R signaling, which are critical for thymocyte specification and survival (30, 35, 36). Our results support a developmental model in which HEBCan collaborates with Notch and other stromal signals to up-regulate HEBAlt in multipotent precursors. Based on our analysis of HEBAlt-transduced and HEBCan-transduced HEB−/− fetal liver precursors, HEBAlt appears to be an important component of the regulatory machinery that drives the generation of early T cell precursors. HEBAlt is not, however, sufficient to rescue later stages of T cell development (D. Wang, C. Claus, and M. Anderson, unpublished observations). E2A is also specifically required at the DN1 to DN2 transition (10), suggesting that HEBAlt-E2A heterodimers may be important during this transition. This possibility remains to be formally tested. HEBAlt cannot rescue early T cell development in the absence of Notch signaling (data not shown), but it is likely to act collaboratively with Notch and E2A to specify the T cell fate.

The transient high level expression and down-regulation of vector to generate GFP+ cells in HEB-transduced populations provided the opportunity to assess the effects of transient vs sustained expression of the HEBAlt and HEBCan transgenes on T cell development. Analyses of RNA (Fig. 7) and DNA (data not shown) from GFP+ populations developing in OP9-DL1 cocultures confirmed that at least some of the GFP+ cells that arose in these cultures were derived from GFP− cells that down-regulated the retroviral transgene. Analysis of these two populations in HEBAlt-transduced cultures showed that once the early increase in T cell precursors was achieved, the accelerated rate of development was independent of continued high level expression of HEBAlt, whereas sustained HEBAlt expression appeared to inhibit proliferation but not differentiation. By contrast, T cell development was...
inhibited by sustained high level expression of HEBCan, but not by transient expression of HEBCan. Our results are most consistent with a model involving a transient burst of HEBAlt expression that caused increased production of T cell precursors, followed by expansion of cells that subsequently down-regulate HEBAlt expression (Fig. 7). The increase in T cell precursors could be due to increased proliferation of DN2/3 cells and/or to enhanced entry into the T cell lineage. Future experiments will focus on cell cycle analysis during the first 4 days of OP9-DL1 coculture to distinguish between these possibilities.

Although transient high level expression of HEBCan enhanced the percentages of DP cells in OP9-DL1 coculture, no increase in total cell numbers occurred. Different ratios of HEBAlt-containing dimers and HEBCan-containing dimers would be expected in the HEBAlt-transduced vs the HEBCan-transduced cells, and this could impact differently on target genes required for differentiation vs those required for growth control. Previous work in other laboratories has indicated that overexpression of any full length class I bHLH factor (E2A, HEBCan, or IFT-2B) is generally detrimental to cell growth and survival (37), whereas Id expression is associated with cell proliferation (38). Other studies have shown that E2A expression can inhibit tumor formation in developing thymocytes (39). This is due in part to direct regulation of cell cycle regulation genes (21, 37, 40), but may also involve induction of apoptosis (39). At least some of these effects appear to be dependent on the presence of the AD1 domain (41, 42), which is not present in HEBAlt. Therefore, although HEBAlt was induced in HEBCan-transduced cells, the different ratios of each factor in HEBAlt- vs HEBCan-transduced cells decoupled their effects on differentiation and proliferation.

Developing HEB−/− cells in OP9-DL1 coculture exhibited delayed up-regulation of CD25, and a complete block at the DN to DP transition, whereas in HEB+/− mice, thymocytes were partially blocked at the DN3 and CD8+ ISP stage with reduced numbers of DP and SP cells (11). It is possible that more profound effects on early T cell development would be seen in the absence of both HEBAlt and ITF-2A because these homologs are both expressed during this time of development and are structurally very similar to each other. However, even in the presence of ITF-2A, the OP9-DL1 system enabled a kinetic analysis of the appearance of each developmental stage over time, which revealed specific potential roles for HEBAlt that were not apparent in the steady state HEB−/− thymus.

The failure of HEB−/− cells to develop to the DP stage was not rescued by the transduction of HEBAlt or HEBCan at the hematopoietic stem cell stage (data not shown). However, preliminary studies in our laboratory using wild-type transduced fetal thymocytes and DN2/3 precursors generated on OP9-DL1 cocultures suggest that once precursors are committed to the T cell lineage, elevated levels of HEBCan and HEBAlt have quite different effects than the effects they have on hematopoietic stem cells (G. Vaccarelri, C. Claus, D. Wang, and M. Anderson, unpublished observations). Therefore, the highly regulated expression of HEBAlt and HEBCan during T cell development is likely to reflect stage-specific roles for each factor that shift at major developmental checkpoints.

In conclusion, our results indicate that HEBAlt is a functionally distinct transcription factor that plays a specific role in guiding hematopoietic stem cells into the T cell lineage. We have linked the regulation of HEBAlt to Delta-Notch signaling and HEBCan function, and shown that transient expression of HEBAlt has long lasting effects on the generation of T cell precursors. It will be interesting to see how germline deletion of the Alt exon affects T cell development, in work that is now under way.

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Disclosures

The authors have no financial conflict of interest.

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