

## **Induction of a Diverse T Cell Receptor $\gamma/\delta$ Repertoire by the Helix-Loop-Helix Proteins E2A and HEB in Nonlymphoid Cells**

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### **Abstract**

During specific stages of thymocyte development, the T cell receptor (TCR) locus is assembled from variable (V), diversity (D), and joining (J) gene segments. Proper TCR  $\gamma$  and  $\delta$  V(D)J rearrangement during thymocyte development requires the presence of the E2A proteins. Here we show that E2A and a closely related protein, HEB, in the presence of recombination activating gene (RAG)1 and RAG2, each have the ability to activate TCR  $\gamma$  and  $\delta$  rearrangement in human kidney cells. The coding joints are diverse, contain nucleotide deletions, and occasionally show the presence of P nucleotides. Interestingly, only a subset of V, D, and J segments are targeted by the E2A and HEB proteins. Thus, E2A and HEB permit localized accessibility of the TCR  $\gamma$  and  $\delta$  loci to the recombination machinery. These data indicate that a distinct but diverse TCR repertoire can be induced in nonlymphoid cells by the mere presence of the V(D)J recombinase and the transcriptional regulators, E2A and HEB.

Key words: E2A • HEB • recombination activating gene • T cell receptor  $\gamma$  and  $\delta$  rearrangements • chromatin accessibility

### **Introduction**

The ability of lymphocytes to recognize a large array of antigens is dependent on the successful rearrangement of the Ig and TCR loci (1, 2). This is accomplished by the joining of V(D)J segments during specific stages of thymocyte development. Conserved sequence motifs, known as recombination signal sequences (RSSs), flank one or both sides of the coding regions. Rearrangement is mediated by the two recombination activation gene (RAG) products, RAG1 and RAG2, which recognize the RSSs and introduce double-stranded DNA breaks at the coding signal sequence boundaries (3–5). These cleavage products are joined and processed by the DNA repair machinery to generate loci that have the ability to encode antigen-specific B and T cell receptors (6).

V(D)J recombination is controlled by several regulatory mechanisms. Ig V(D)J rearrangement is completed only in cells committed to the B cell lineage, whereas TCR V(D)J recombination occurs exclusively in thymocytes (7–9). Within the lymphoid lineages, V(D)J recombination is ordered and developmentally regulated (10). In differentiating

B cells, Ig heavy chain gene rearrangement generally precedes Ig light chain gene recombination (11, 12). Likewise, during thymocyte development, TCR  $\gamma$ ,  $\delta$ , and  $\beta$  V(D)J joining is completed before the onset of TCR- $\alpha$  rearrangement (7). TCR- $\beta$  gene rearrangement is ordered as well: D and J regions are joined before the initiation of V to DJ joining. In contrast, TCR- $\delta$  rearrangement is unordered (13).

Ig V(D)J recombination is regulated by transcriptional activators that bind to sequence elements present in the Ig enhancers. Among the Ig enhancer binding proteins are members of the basic helix-loop-helix (bHLH) family, including E12 and E47 (14, 15). E12 and E47 are encoded by the E2A gene and arise through differential splicing. E12 binds with relatively low affinity to its optimal binding site, the E2 box, whereas E47 binds with high affinity to the same site. Both E12 and E47 belong to a subset of bHLH proteins, named E-proteins (15). The family of E-proteins also include E2-2, HEB, and the *Drosophila* gene product, daughterless (15). E-proteins have the ability to bind their target sites either as homo- or heterodimers (15). In B lineage cells, both E12 and E47 exist mainly as homodimers (16). In contrast, the E2A proteins form heterodimers with HEB, another member of the bHLH family in developing thymocytes (15).

The E2A proteins are required for proper B lineage development, as B lymphocyte development in E2A-defi-

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cient mice is arrested at a stage before the initiation of Ig gene rearrangement (17–19). E2A-deficient mice also show a block, albeit incomplete, at a similar stage during T lineage maturation (20). HEB functions during a later stage of thymocyte development, specifically during the maturation of CD4<sup>-</sup>CD8<sup>-</sup> into CD4<sup>+</sup>CD8<sup>+</sup> T lineage cells (21).

Recent evidence has indicated that TCR  $\gamma$  and  $\delta$  V(D)J recombination in E2A-deficient mice is severely perturbed, specifically those rearrangements used predominantly during adult thymocyte maturation (22). E2A proteins are also required during adult thymocyte development to inhibit V(D)J rearrangements that normally recombine exclusively during fetal thymocyte maturation (22). How E2A proteins both regulate TCR  $\gamma$  and  $\delta$  rearrangement remains an open question.

Here we demonstrate that both E2A and HEB, as homo- and heterodimers, have the ability to activate TCR  $\gamma$  and  $\delta$  V(D)J rearrangements in nonlymphoid cells. Activation of TCR  $\gamma$  and  $\delta$  V(D)J rearrangement is restricted to a subset of V, D, and J regions. The TCR coding joints are diverse, contain P nucleotides and show nucleotide deletions. We demonstrate that the induction of TCR  $\gamma$  and  $\delta$  rearrangements correlates with the activation of TCR V $\gamma$  and V $\delta$  germline transcription. These data suggest that E2A and HEB have the ability to regulate TCR  $\gamma$  and  $\delta$  recombination by promoting localized accessibility of the RSSs to the recombination machinery.

## Materials and Methods

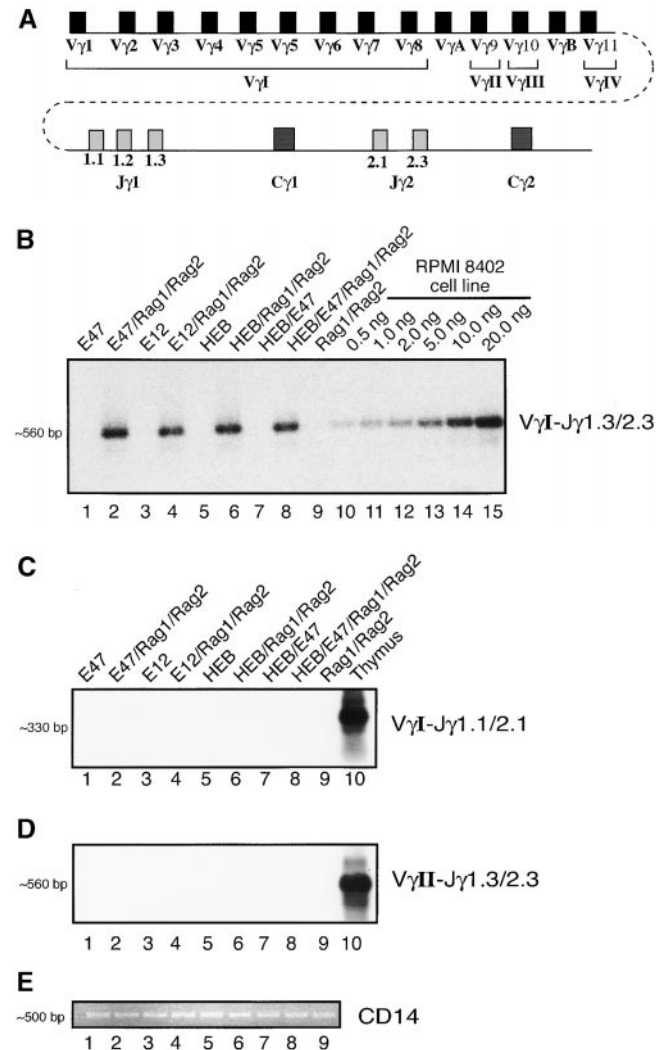
**Cell Culture.** BOSC 23 cells were grown in DMEM mixed with 10% fetal bovine serum at 37°C as described earlier (23, 24).

**DNA Constructs and Transfection Protocol.** The pEBB-Rag1 and pEBB-Rag2 expression vectors expressed wild-type proteins (25). The E2A transcription factors E12 and E47 were cloned into the pH $\beta$ APneo vector, which has been described previously (26). The HEB cDNA was cloned into the pXS vector, which is a derivative of the pcDL-SR $\alpha$ 296 vector. Calcium phosphate precipitation transfections were carried out essentially as reported previously (23, 24). BOSC 23 cells were plated 12–16 h before transfection at  $2 \times 10^6$  cells per 6-cm dish. The following day, 9–12  $\mu$ g of total DNA, comprised of 3  $\mu$ g of each expression vector or carrier DNA, was used for each transfection. The cells were harvested  $\sim$ 3 d after transfection.

**PCR and TCR Rearrangements.** 200 ng of genomic DNA, isolated from BOSC 23 cells after transfection, was analyzed by PCR in a 25- $\mu$ l reaction volume. Each of these reaction mixtures contained 10 mM Tris, pH 8.3, 50 mM KCl, 2 mM MgCl<sub>2</sub>, 100 ng of each primer, 200  $\mu$ M dNTPs, and 1 unit of Amplitaq Gold (PE Biosystems). PCR reactions were performed as follows: 10 min preactivation at 94°C; 26–35 cycles of 45 s at 94°C, 1 min at 51–61°C, as indicated by the figure legends, 80 s at 72°C; followed by a 10-min extension at 72°C (24). The primers used to detect TCR  $\gamma$  and  $\delta$  rearrangements are listed in the Table I (27, 28). 15  $\mu$ l of each PCR product was analyzed on a 1.5% agarose gel followed by ethidium bromide staining and/or Southern blotting.

**Reverse Transcription PCR and Germline Transcription.** cDNA was prepared from BOSC 23 cells as described previously (24). The efficiency of each cDNA reaction was checked by amplifica-

tion of the  $\beta$ -actin transcripts with human specific primers: huBact-for and huBact-rev using the following conditions: 3 min at 94°C; 28 cycles of 94°C for 15 s, 58°C for 15 s, 72°C for 45 s; and 10 min at 72°C. PCR to detect V $\delta$  germline transcripts was performed as described above using the primers V $\delta$ 2 and V $\delta$ 2-rev and the PCR protocol, 10 min preactivation at 94°C; 26 cycles of 45 s at 94°C, 60 s at 53°C, 80 s at 72°C; followed by a 10-min extension at 72°C. Similarly, V $\gamma$ I germline transcripts were de-



**Figure 1.** Induction of TCR- $\gamma$  rearrangement in BOSC 23 cells by the bHLH transcription factors E2A and HEB. (A) Schematic representation of TCR- $\gamma$  locus. (B) BOSC 23 cells were transfected with expression vectors for E12, E47, HEB, RAG1, and RAG2 and analyzed by PCR for V $\gamma$ 8-J $\gamma$ 1.3/2.3 recombination. DNA derived from a cell line, RPMI 8402, was included to quantitate the level of rearrangement (lanes 10–15). PCR products were separated on a 1.5% agarose gel, transferred to Nytran Plus, and hybridized with a cloned V $\gamma$ 8-J $\gamma$ 2.3 fragment. (C) Transfected BOSC 23 cells were analyzed by PCR for V $\gamma$ I-J $\gamma$ 1.1/2.1 rearrangement. PCR products were separated by agarose gel electrophoresis, transferred to Nytran Plus, and hybridized with a radiolabeled V $\gamma$ 8 probe. (D) Transfected BOSC 23 cells were analyzed by PCR for V $\gamma$ II-J $\gamma$ 1.3/2.3 rearrangement. PCR products were separated by agarose gel electrophoresis, transferred to Nytran Plus, and hybridized with a radiolabeled V $\gamma$ II probe. (E) Genomic DNA was analyzed with primers to the CD14 locus to demonstrate that all samples contained comparable amounts of DNA.

tected with primers V $\gamma$ 1 and V $\gamma$ 1-rev using the PCR protocol 10 min preactivation at 94°C; 26 cycles of 45 s at 94°C, 60 s at 59°C, 80 s at 72°C, followed by a 10-min extension at 72°C. V $\gamma$ II PCR conditions were as follows: 45 s at 94°C, 60 s at 62°C, and 60 s at 72°C.

## Results

*Activation of TCR- $\gamma$  VJ Rearrangement in Embryonic Kidney Cells by the E2A and HEB Proteins.* Previously we demonstrated that TCR- $\gamma$  VJ rearrangements in E2A-deficient thymocytes are perturbed (22). To determine whether the E2A and its heterodimeric partner, HEB, have the ability to directly activate TCR- $\gamma$  VJ rearrangement, we employed a strategy described previously (24). In brief, either E12, E47, or HEB were transfected in the absence or presence of wild-type RAG1 and RAG2, in BOSC 23 cells, a human embryonic kidney cell line. Genomic DNA from the transfected cells was harvested 3 d after transfection and amplified by PCR using the appropriate primers. The TCR- $\gamma$  locus contains a limited set of V segments as well as two clusters of J segments, each located upstream of a distinct constant region (Fig. 1 A). To examine for the presence of V $\gamma$ -J $\gamma$  rearrangements, forward degenerate primers were used that recognize specific V regions, be-

longing to several of the V $\gamma$  gene families (Table I). The reverse primers contained degenerate sequences with specificity for the J $\gamma$ 1.3 and J $\gamma$ 2.3, which differ from each other by a single nucleotide. As a probe, a V $\gamma$ 8-J $\gamma$ 2.3 fragment was used, which has the ability to hybridize with various members of the V $\gamma$ I family. When DNA was analyzed from BOSC 23 cells transfected with either E12 or E47 or RAG1 and RAG2 alone, rearrangements were not detectable (Fig. 1 B). Interestingly, in the presence of RAG1 and RAG2 and either E12 or E47, V $\gamma$ I-J $\gamma$ 1.3/2.3 rearrangements were readily formed (Fig. 1 B, lanes 2 and 4).

V $\gamma$ I-J $\gamma$ 1.3/2.3 rearrangements were also present in cells transfected with HEB, RAG1, and RAG2 (Fig. 1 B). To determine whether heterodimers of E47 and HEB have the ability to promote VJ rearrangement, both proteins were introduced into BOSC 23 cells in conjunction with RAG1 and RAG2. Nuclear extracts were derived from the transfected cells and analyzed by electrophoretic mobility shift assay (EMSA) to ensure that heterodimers of E47 and HEB were formed (unpublished observations). E47/HEB heterodimers showed a significant induction of V $\gamma$ I-J $\gamma$ 1.3/2.3 rearrangements in the transfected cells at levels comparable to E47 or HEB alone (Fig. 1 B, lane 8). Thus, in the presence of the V(D)J recombinase, both E2A and HEB, both as homo- and as heterodimers, have the ability to activate TCR- $\gamma$  VJ rearrangement in nonlymphoid cells.

To obtain an estimate of the frequency of TCR V $\gamma$ I-J $\gamma$ 1.3/2.3 recombination, the levels of rearrangements of the transfected cells was compared with that of DNA isolated from a lymphoid cell line, RPMI 8402, which harbors endogenous V $\gamma$ I-J $\gamma$ 1.3/2.3 joints. By comparing the hybridization signal of genomic DNA (100 ng) isolated from the transfectants to that of DNA isolated from the RPMI 8402 cell line, we estimate that up to 5–10% of the transfected cells contain V $\gamma$ I-J $\gamma$ 1.3/2.3 joints (Fig. 1 B, lanes 10–15). Thus, rearrangement involving V $\gamma$ I-J $\gamma$ 1.3/2.3 occurs with a relatively high frequency in BOSC 23 cells that overexpress either E2A or HEB and RAG1 and RAG2.

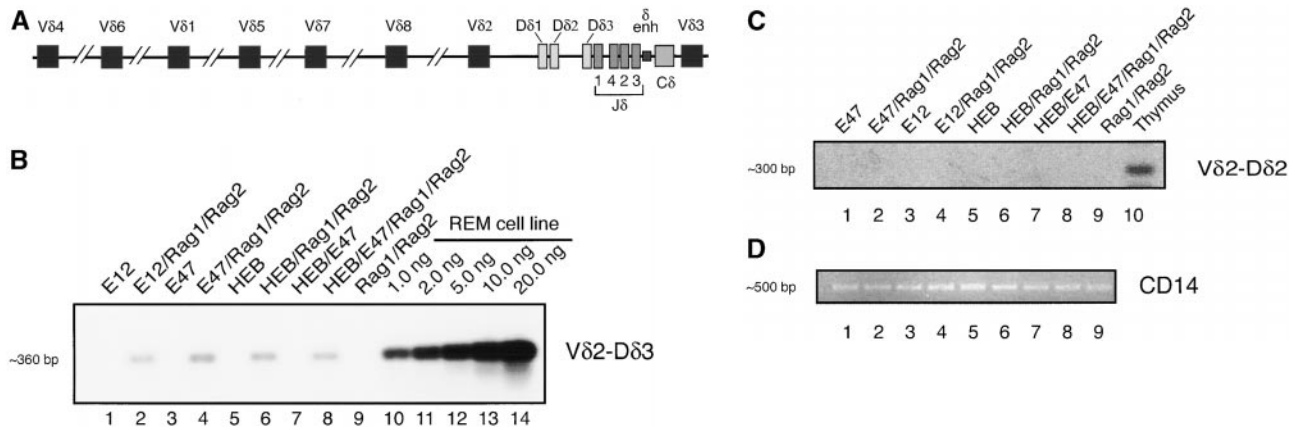
To determine whether the E2A proteins also have the ability to activate rearrangements using the J $\gamma$ 1.1 and J $\gamma$ 2.1 segments, genomic DNA from the transfectants was analyzed by PCR using the appropriate primers (Table I). Interestingly, rearrangements involving J $\gamma$ 1.1, J $\gamma$ 1.2, and J $\gamma$ 2.1 were not detectable in cells transfected with either E2A or HEB (Fig. 1 C). Additionally, V $\gamma$ II-J $\gamma$ 1.3/2.3 rearrangements were not activated by the overexpression of E2A and HEB (Fig. 1 D). Taken together, these data indicate that E2A and HEB promote joining of a subset of V regions to a selected group of J $\gamma$  segments.

*Both E2A and HEB Have the Ability to Activate TCR- $\delta$  VD Rearrangement in BOSC 23 Cells.* In addition to the defects in TCR- $\gamma$  VJ joining, TCR- $\delta$  rearrangements in E2A-deficient thymocytes are affected as well (22). To determine whether E2A proteins have the ability to promote TCR- $\delta$  rearrangement, we employed the same strategy as described above. Interestingly, V $\delta$ 2-D $\delta$ 3, but not V $\delta$ 2-D $\delta$ 2 joints, were present in cells expressing E2A or HEB in

**Table I.** Nucleotide Sequences of PCR Primers

Primer	Sequence
TCR- $\delta$ gene	
V $\delta$ 1	CTCAAGCCCAGTCATCAGTATCC
V $\delta$ 2	CGCGTCGACCAACAGTGCCTGTGTCAATAGG
V $\delta$ 3	CGCGTCGACCCAGACGGTGGCGAAGTGGC
D $\delta$ 1	CGCGTCGACTCCATGTTCAAATAGATATAGTATT
D $\delta$ 2	GTAGATCTAGAAGAGGGTTTTTATACTGATGTG
D $\delta$ 3	GTAGATCTAGAAATGGCACTTTTTGCCCTGCG
J $\delta$ 1	CACGGGATCCTTTTTCCAAGGATGAG
V $\delta$ 2-rev	GGTCAGTGGTTTTTGAGCTGC
TCR- $\gamma$ gene	
V $\gamma$ I	GAAGATCTAGACAGGCCGACTGGGTCATCTGC
V $\gamma$ II	GAAGATCTAGACAGCCCGCTGGAATGTGTGG
V $\gamma$ IV	CCTGAAAGATCTATTTCTAGACCAGC
J $\gamma$ 1.1/2.1	GAAGATCTAGACTTACCAGGTGAAGTTACTATAAGC
J $\gamma$ 1.2-3	AAGAAAACCTTACCTGTAATGATAAGC
J $\gamma$ 1.3/2.3-3	CTAGTCTAGACCGTATATGCACAAAGCCAGATC
V $\gamma$ I-rev	GTCACAAGGCAGATTTTTCAGTGTGG
V $\gamma$ II-rev	GCACACTGGTGGTAACTGTGGCTTCC
$\beta$ -actin	
hu $\beta$ act-for	GGATGATGATATCGCCGCG
hu $\beta$ act-rev	GGATAGCAACGTACATGGCTGGG
CD14	
huCD14-for	CAGAGGTTCCGAAGACTTATCGAC
huCD14-rev	GTTATCTTTAGGTCCTCGAGCGTC

The primer sequences are listed 5' to 3'.



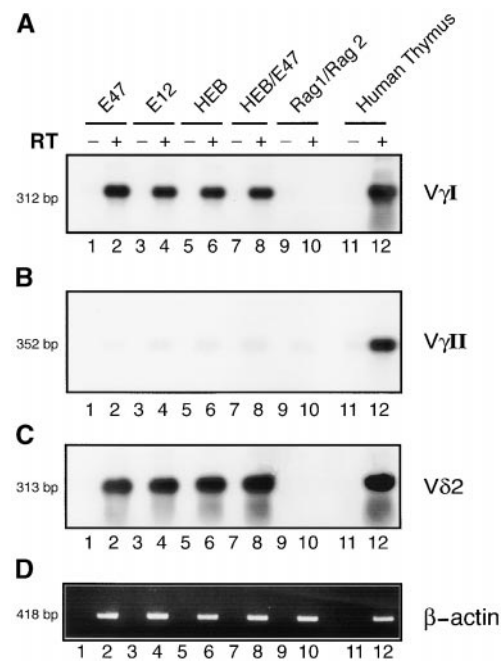
**Figure 2.** E2A and HEB promote TCR- $\delta$  rearrangement in BOSC 23 cells. (A) Schematic representation of TCR  $\delta$  locus. (B) BOSC 23 cells were transfected with expression vectors for E12, E47, HEB, RAG1, and RAG2. 100 ng of DNA was amplified by PCR for V $\delta$ 2-D $\delta$ 3 recombination using the V $\delta$ 2 and D $\delta$ 3 primers. PCR products were separated on a 1.5% agarose gel, transferred to Nytran Plus, and hybridized with a V $\delta$ 2 probe. The REM cell line contains endogenous V $\delta$ 2-D $\delta$ 3 rearrangements. (C) Transfected BOSC 23 cells were analyzed by PCR for V $\delta$ 2-D $\delta$ 2 rearrangement. PCR products were separated by agarose gel electrophoresis, transferred to Nytran Plus, and hybridized with a V $\delta$ 2 probe. (D) Genomic DNA was analyzed with primers to the CD14 locus to demonstrate that all samples contained comparable amounts of DNA.

the presence of RAG1 and RAG2 (Fig. 2). However, we note that in contrast to TCR- $\gamma$  VJ rearrangement, the frequency of TCR V $\delta$ 2-D $\delta$ 3 rearrangements is relatively low (<1%) in the transfected BOSC 23 cells (Fig. 2 B). We also have examined genomic DNA derived from the various transfectants for the presence of rearrangements involving V $\delta$ 1-D $\delta$ 1, V $\delta$ 3-D $\delta$ 1, D $\delta$ 2-D $\delta$ 3, and D $\delta$ -J $\delta$ 1 segments. None of these rearrangements were detectable in genomic DNA derived from transfected cells (data not shown). These data indicate that E2A and HEB have the ability to promote accessibility of distinct V $\delta$  and D $\delta$  regions to the recombination machinery.

**Both E2A and HEB Activate V $\gamma$  and V $\delta$  Germline Transcription.** To examine how E2A and HEB proteins promote accessibility, transfected cells were analyzed for the presence of V $\gamma$ I and V $\delta$ 2 germline transcripts. RNA was isolated from the transfected cells, examined by reverse transcription (RT)-PCR using the appropriate primers and analyzed by Southern blotting. RT-PCR using  $\beta$ -actin-specific primers was used as a control for RNA integrity and cDNA synthesis (Fig. 3 D). V $\gamma$ I and V $\delta$ 2 transcripts were absent in cells transfected with expression vectors encoding the RAG proteins alone (Fig. 3, A and C; lane 10). In contrast, the presence of either E12, E47, or HEB, as homodimers and as heterodimers, promoted the induction of V $\gamma$ I- and V $\delta$ 2-specific germline transcription (Fig. 3, A and C; lanes 2, 4, 6, and 8). Interestingly, V $\gamma$ II germline transcription was not induced upon overexpression of the E-proteins (Fig. 3 B). In summary, E2A and HEB have the ability to activate V $\gamma$ I and V $\delta$ 2 but not V $\gamma$ II germline transcription. The data indicate a close relationship between the ability of E2A and HEB to activate germline transcription and to induce rearrangement.

**Analysis of the Coding Joints Isolated from Embryonic Kidney Cells Expressing the RAG Proteins and either E12 or E47 or HEB.** To examine the nature of the TCR coding joints, rearrangements were amplified by PCR and analyzed by

DNA sequencing. The V $\gamma$ I-J $\gamma$  joints showed significant diversity. Over 20 recombinant clones were analyzed (Table II). Distinct V $\gamma$  regions, interspersed throughout the V $\gamma$ I locus, were used in the presence of either E2A or HEB (Table II). Interestingly, only a selected group of V $\gamma$  regions, including V $\gamma$ 3, V $\gamma$ 4, and V $\gamma$ 8 and one pseudogene,



**Figure 3.** Induction of V $\gamma$  and V $\delta$  germline transcription by E2A and HEB in BOSC 23 cells. (A) Transfected BOSC 23 cells were analyzed by RT-PCR for the presence of V $\gamma$ I germline transcripts. (B) Transfected BOSC 23 cells were analyzed by RT-PCR for the presence of V $\gamma$ II germline transcripts. The blots were hybridized with a V $\gamma$ II-specific probe. (C) Transfected BOSC 23 cells were analyzed by RT-PCR for the presence of V $\delta$ 2 germline transcripts as indicated. The blots were hybridized with a V $\delta$ 2-specific probe. (D) RT-PCR was performed on the same cDNA samples with primers to amplify  $\beta$ -actin transcripts.

**Table II.** Sequences of V $\gamma$ -J $\gamma$  Coding Joints in Transfected BOSC 23 Cells

V $\gamma$	J $\gamma$ 2.3	V segment used
ACC TGG GAT AGG*	G AAT TAT TAT AAG*	
ACC TGG GAC AGG	<u>C</u> - - - T TAT TAT AAG	V3
ACC TGG GAC AGG	<u>C</u> - - - - - T TAT AAG	V3
ACC TGG GAC AGG	- - AT TAT TAT AAG	V7
ACC TGG GAT GGG	<u>C</u> - - - T TAT TAT AAG	V4
ACC TGG GAT GGG	<u>C</u> - - AT TAT TAT AAG	V4
ACC T - - - - -	- AAT TAT TAT AAG	V8
ACC TGG GAC AGG	- - AT TAT TAT AAG	V7
ACC TGG GAT GGG	<u>CC</u> - gAT TAT TAT AAG	V4
ACC TGG GA - - -	G AAT TAT TAT AAG	V7
ACC TGG GAT GGG	<u>C</u> - - - T TAT TAT AAG	V4
ACC TGG GAC AGG	- - - - - AT TAT AAG	V7
ACC TGG GAT GGG	<u>CC</u> - gAT TAT TAT AAG	V4
ACC TGG GAC - - -	- AAT TAT TAT AAG	V7
ACC TGG GAC AGG	- - AT TAT TAT AAG	V7
ACC TGG GAC AGG	- - - - - AT TAT AAG	V7
ACC T - - - - -	- AAT TAT TAT AAG	V8
ACC TGG GAT GGG	<u>CC</u> - gAT TAT TAT AAG	V4
ACC TGG GAT AG -	G AAT TAT TAT AAG	V8
ACC TGG GAT GGG	<u>C</u> - - - T TAT TAT AAG	V4
ACC TGG GAC AGG	- - AT TAT TAT AAG	V7
ACC TGG GAT AG -	- AAT TAT TAT AAG	V8
ACC T - - - - -	- AAT TAT TAT AAG	V8

Coding joints in BOSC 23 cells transfected with either E2A or HEB and RAG1 and RAG2, are diverse and resemble those found in T lineage cells. Genomic DNA from transfected cells was amplified with the V $\gamma$ 1 family and J $\gamma$ 1.3/2.3 primers. The resulting fragments were cloned and sequenced. Dashes represent bases that have been deleted. Underlined bases indicate P nucleotide additions. Bases in lowercase lettering could not be matched to the germline DNA sequence.

\*The germline sequences of V $\gamma$ 8 and J $\gamma$ 2.3 for comparison.

V $\gamma$ 7, were isolated (Table II). Rearrangements involving segments of other V $\gamma$  gene families were not detected. Of the J $\gamma$  regions, only the most distal J segment, J $\gamma$ 2.3, was used (Table II). All coding joints showed nucleotide excisions of variable sizes (Table II). Several of the sequences showed P nucleotide additions (Table II). Interestingly, several nucleotide residues could not be matched to germline sequences and may reflect N nucleotide additions, possibly caused by the presence of DNA polymerase mu in kidney cells (29; Table II). DNA sequencing also showed considerable V $\delta$ 2-D $\delta$ 3 junctional diversity (Table III). Specifically, V $\delta$ 2-D $\delta$ 3 coding joints showed nucleotide deletions and occasionally the presence of P nucleotide additions (Table III). Thus, the expression of E2A or HEB in conjunction with the V(D)J recombinase is sufficient to generate a diverse repertoire of V $\gamma$ J and V $\delta$ D joints, resembling those of  $\gamma\delta$  T lineage cells.

**Table III.** Sequences of V $\delta$ -D $\delta$  Coding Joints in Transfected BOSC 23 Cells

V $\delta$ 2	D $\delta$ 3
GCC TGT GAC ACC*	ACT GGG GGA TAC G*
GCC TGT GAC AC -	- CT GGG GGA TAC G
GCC TGT GAC - - -	ACT GGG GGA TAC G
GCC TGT GAC AC -	- CT GGG GGA TAC G
GCC TGT GAC ACC	ACT GGG GGA TAC G
GCC TGT GAC - - -	ACT GGG GGA TAC G
GCC TGT GAC ACC	- - T GGG GGA TAC G
GCC TGT GAC - - -	ACT GGG GGA TAC G
GCC TGT GAC ACC	- CT GGG GGA TAC G
GCC TGT GAC AC -	- CT GGG GGA TAC G
GCC TGT GAC - - -	ACT GGG GGA TAC G
GCC TGT GAC ACC	- - - - GG GGA TAC G
GCC TGT GAC ACC	- - T GGG GGA TAC G
GCC TGT GAC ACC	- CT GGG GGA TAC G
GCC TGT GAC - - -	- CT GGG GGA TAC G
GCC TGT GAC - - -	ACT GGG GGA TAC G
GCC TGT GAC ACC	<u>G</u> t - - T GGG GGA TAC G
GCC TGT GAC ACC	- - T GGG GGA TAC G
GCC TGT GAC - - -	- - - - - - - - AC G

DNA sequences of V $\delta$ 2-D $\delta$ 3 coding joints isolated from BOSC 23 cells transfected with E2A or HEB and RAG1 and RAG2 are diverse and resemble those found in T lineage cells. Genomic DNA from transfected cells was amplified with V $\delta$ 2 and D $\delta$ 3 primers. The resulting fragments were cloned and sequenced. Dashes indicate bases that have been deleted. Underlined bases represent P nucleotide additions. Bases in lowercase lettering could not be matched to the germline DNA sequence.

\*The germline sequences of V $\delta$ 2 and D $\delta$ 3 for comparison.

## Discussion

The data presented here demonstrate that the bHLH transcription factors E2A and HEB act in concert with RAG1 and RAG2 to activate a subset of TCR- $\gamma/\delta$  rearrangements in nonlymphoid cells. The striking finding is that E2A and HEB permit only a limited repertoire of rearrangements. This raises the question how the presence of E2A and HEB allow only a subset of rearrangements. It is possible that other factors affecting recombination efficiency influence the repertoire of rearrangements. For example, coding and RSS sequences may effect the effect the efficiency of TCR rearrangements in the transfectants. Thus, E2A and HEB may modestly promote general TCR  $\gamma$  and  $\delta$  locus accessibility allowing for detection only of those V regions that normally rearrange more efficiently. Such a scenario cannot be excluded for the rearrangements that we observe for the TCR- $\delta$  locus. However, we note that recombination at the TCR- $\gamma$  locus is efficient, suggesting that in the transfectants, the accessibility of this TCR locus to the recombination machinery is localized. Further support for localized accessibility is provided by the data indicating a close relationship between the ability of

E2A and HEB to activate germline transcription and to induce rearrangement.

A role for E2A in TCR  $\gamma$  and  $\delta$  rearrangement is not unexpected. Our previous data using E2A-deficient mice showed that the E2A proteins are required for proper  $\gamma\delta$  T lineage development (22). Specifically, the number of  $\gamma\delta$  T cells found in the secondary lymphoid tissues and the intraepithelial layers of the intestine are significantly reduced in E2A-null mutant mice (22). Both TCR- $\gamma$  VJ and TCR- $\delta$  VD rearrangements are perturbed. In contrast, TCR- $\delta$  DJ joining is not affected in E2A-deficient thymocytes (22). These observations are consistent with our data indicating that E2A proteins have the ability to promote V $\delta$ -D $\delta$  but are not involved in D $\delta$ -J $\delta$  rearrangement. Selective targeting of the recombinase by either E2A or HEB was also observed in the TCR- $\gamma$  locus. The VJ and VD joints that were formed showed significant diversity. All of the rearrangements showed deletions of various sizes and many showed P nucleotide additions. Interestingly, a few bases could not be assigned to germline sequences (Tables II and III). TdT is not expressed in kidney cells, but a related enzyme, DNA polymerase  $\mu$ , is detectable in kidney cells (29). It is conceivable that the additions of these nucleotides in the transfectants are caused by the activity of DNA polymerase  $\mu$  (29).

Remarkably, rearrangement involving the most distal J $\gamma$  region (J $\gamma$ 2.3) was readily detectable in cells expressing E2A or HEB, whereas the J $\gamma$ 1.3 was not used. These data raise the question how E2A and HEB permit localized accessibility. Both E2A and HEB belong to a distinct group of HLH proteins, termed class I HLH or E-proteins. They contain highly conserved activation domains, designated AD1 and AD2. AD1 has been shown to recruit a group of proteins, termed the SAGA complex which contains histone acetyl transferase (HAT) activity (30). Both AD1 and AD2 have also been shown to interact with p300, which also has HAT activity (31). It is conceivable that E2A and HEB permit localized opening of chromatin surrounding the RSSs by modification of histones through the recruitment of complexes containing HAT activity. Consistent with these observations are the findings that the level of germline transcripts of V $\gamma$  and V $\delta$  region genes in E2A-deficient mice correlates precisely with the level of rearrangements (22).

E2A and HEB may also directly recruit RAG1 and RAG2 to the RSSs. E box sites in the proximity of the V and J regions have been identified and it will be interesting to determine by mutational analysis if these sites are required for proper V(D)J rearrangement (unpublished observations). As both E2A and HEB have the ability to promote rearrangement and to activate germline transcription, the protein domains and amino acids that permit recruitment must be conserved between these two proteins. Identification of the domains and residues that promote accessibility may provide further insight as to how E-proteins regulate both Ig and TCR gene rearrangement.

In summary, the data described here demonstrate that a diverse TCR  $\gamma$  and  $\delta$  repertoire can be induced in non-

lymphoid cells by the mere presence of a transcriptional activator and the VDJ recombinase. The striking observation is that E2A and HEB do not promote accessibility throughout the TCR  $\gamma$  and  $\delta$  loci. Rather, E2A and HEB permit chromatin opening to only a selected set of V, D, and J region genes. Recent data have demonstrated a tight correlation with regulated changes in histone H3 acetylation and VDJ recombination at the TCR- $\delta$  locus (32). It will be particularly interesting to determine if E2A-deficient thymocytes show abnormalities in histone H3 acetylation along the TCR- $\delta$  locus.

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## References

1. Mombaerts, P., J. Iacomini, R.S. Johnson, K. Herrup, S. Tonegawa, and V.E. Papaioannou. 1992. RAG-1-deficient mice have no mature B and T lymphocytes. *Cell*. 68:869-877.
2. Shinkai, Y., G. Rathbun, K.P. Lam, E.M. Oltz, V. Stewart, M. Mendelsohn, J. Charron, M. Datta, F. Young, A.M. Stall, and F.W. Alt. 1992. RAG-2-deficient mice lack mature lymphocytes owing to inability to initiate V(D)J rearrangement. *Cell*. 68:855-867.
3. Schatz, D.G., M.A. Oettinger, and D. Baltimore. 1989. The V(D)J recombination activating gene, RAG-1. *Cell*. 59: 1035-1048.
4. Oettinger, M.A., D.G. Schatz, C. Gorka, and D. Baltimore. 1990. RAG-1 and RAG-2, adjacent genes that synergistically activate V(D)J recombination. *Science*. 248:1517-1523.
5. van Gent, D.C., J.F. McBlane, D.A. Ramsden, M.J. Sadofsky, J.E. Hesse, and M. Gellert. 1995. Initiation of V(D)J recombination in a cell-free system. *Cell*. 81:925-934.
6. Weaver, D., N. Boubnov, Z. Wills, K. Hall, and J. Staunton. 1995. V(D)J recombination: double-strand break repair gene products used in the joining mechanism. *Ann. NY Acad. Sci.* 764:99-111.
7. Willerford, D.M., W. Swat, and F.W. Alt. 1996. Developmental regulation of V(D)J recombination and lymphocyte differentiation. *Curr. Opin. Genet. Dev.* 6:603-609.
8. Alt, F.W., G.D. Yancopoulos, T.K. Blackwell, C. Wood, E. Thomas, M. Boss, R. Coffman, N. Rosenberg, S. Tonegawa, and D. Baltimore. 1984. Ordered rearrangement of immunoglobulin heavy chain variable region segments. *EMBO (Eur. Mol. Biol. Organ.) J.* 3:1209-1219.
9. Chen, J., F. Young, A. Bottaro, V. Stewart, R.K. Smith, and F.W. Alt. 1993. Mutations of the intronic IgH enhancer and its flanking sequences differentially affect accessibility of the JH locus. *EMBO (Eur. Mol. Biol. Organ.) J.* 12:4635-4645.
10. Schatz, D.G., M.A. Oettinger, and M.S. Schlissel. 1992. V(D)J recombination: molecular biology and regulation. *Annu. Rev. Immunol.* 10:359-383.

11. Sleckman, B.P., J.R. Gorman, and F.W. Alt. 1996. Accessibility control of antigen-receptor variable-region gene assembly: role of cis-acting elements. *Annu. Rev. Immunol.* 14:459–481.
12. Schlissel, M.S., and P. Stanhope-Baker. 1997. Accessibility and the developmental regulation of V(D)J recombination. *Semin. Immunol.* 9:161–170.
13. Sleckman, B.P., B. Khor, R. Monroe, and F.W. Alt. 1999. Assembly of productive T cell receptor  $\delta$  variable region genes exhibit allelic inclusion. *J. Exp. Med.* 188:1465–1471.
14. Murre, C., G. Bain, M.A. van Dijk, I. Engel, B.A. Furnari, M.E. Massari, J.R. Matthews, M.W. Quong, R.R. Rivera, and M.H. Stuiver. 1994. Structure and function of helix-loop-helix proteins. *Biochim. Biophys. Acta.* 1218:129–135.
15. Massari, M.E., and C. Murre. 2000. Helix-loop-helix proteins: regulators of transcription in eucaryotic organisms. *Mol. Cell. Biol.* 20:429–440.
16. Bain, G., S. Gruenwald, and C. Murre. 1993. E2A and E2-2 are subunits of B-cell-specific E2-box DNA-binding proteins. *Mol. Cell. Biol.* 13:3522–3529.
17. Bain, G., E.C. Maandag, D.J. Izon, D. Amsen, A.M. Kruisbeek, B.C. Weintraub, I. Krop, M.S. Schlissel, A.J. Feeney, M. van Roon, et al. 1994. E2A proteins are required for proper B cell development and initiation of immunoglobulin gene rearrangements. *Cell.* 79:885–892.
18. Sun, X.-H. 1994. Constitutive expression of the Id1 gene impairs mouse B cell development. *Cell.* 79:893–901.
19. Zhuang, Y., P. Soriano, and H. Weintraub. 1994. The helix-loop-helix gene E2A is required for B cell formation. *Cell.* 79:875–884.
20. Bain, G., I. Engel, E.C. Robanus Maandag, H.P. te Riele, J.R. Volland, L.L. Sharp, J. Chun, B. Huey, D. Pinkel, and C. Murre. 1997. E2A deficiency leads to abnormalities in  $\alpha\beta$  T-cell development and to rapid development of T-cell lymphomas. *Mol. Cell. Biol.* 17:4782–4791.
21. Zhuang, Y., P. Cheng, and H. Weintraub. 1996. B-lymphocyte development is regulated by the combined dosage of three basic helix-loop-helix genes, E2A, E2-2, and HEB. *Mol. Cell. Biol.* 16:2898–2905.
22. Bain, G., W.J. Romanow, K. Albers, W.L. Havran, and C. Murre. 1999. Positive and negative regulation of V(D)J recombination by the E2A proteins. *J. Exp. Med.* 189:289–300.
23. Pear, W.S., G.P. Nolan, M.L. Scott, and D. Baltimore. 1993. Production of high-titer helper-free retroviruses by transient transfection. *Proc. Natl. Acad. Sci. USA.* 90:8392–8396.
24. Romanow, W.J., A.W. Langerak, P. Goebel, I.L.M. Wolvers-Tettero, J.J.M. van Dongen, A.J. Feeney, and C. Murre. 2000. E2A and EBF act in synergy with the V(D)J recombinase to generate a diverse immunoglobulin repertoire in nonlymphoid cells. *Mol. Cell.* 5:343–353.
25. Roman, C.A., S.R. Cherry, and D. Baltimore. 1997. Complementation of V(D)J recombination deficiency in RAG-1<sup>-/-</sup> B cells reveals a requirement for novel elements in the N-terminus of RAG-1. *Immunity.* 7:13–24.
26. Kee, B.L., and C. Murre. 1998. Induction of early B cell factor (EBF) and multiple B lineage genes by the basic helix-loop-helix transcription factor E12. *J. Exp. Med.* 188:699–713.
27. Breit, T.M., I.L. Wolvers-Tettero, K. Hahlen, E.R. van Wering, and J.J. van Dongen. 1991. Extensive junctional diversity of gamma delta T-cell receptors expressed by T-cell acute lymphoblastic leukemias: implications for the detection of minimal residual disease. *Leukemia.* 5:1076–1086.
28. Kneba, M., I. Bolz, B. Linke, and W. Hiddemann. 1995. Analysis of rearranged T-cell receptor beta-chain genes by polymerase chain reaction (PCR) DNA sequencing and automated high resolution PCR fragment analysis. *Blood.* 86:3930–3937.
29. Dominguez, O., J.F. Ruiz, T. de Lera, M. Garcia-Diaz, M.A. Gonzalez, T. Kirchoff, C. Martinez-A, A. Bernad, and L. Blanco. 2000. DNA polymerase mu (Pol  $\mu$ ) homologous to TdT, could act as a DNA mutator in eucaryotic cells. *EMBO (Eur. Mol. Biol. Organ.) J.* 19:1731–1742.
30. Massari, M.E., P.A. Grant, M.G. Pray-Grant, S.L. Berger, J.L. Workman, and C. Murre. 1999. A conserved motif present in a class of helix-loop-helix proteins activates transcription by direct recruitment of the SAGA complex. *Mol. Cell.* 4:63–73.
31. Qui, Y., A. Sharma, and R. Stein. 1998. p300 mediates transcriptional stimulation by the basic helix-loop-helix factors of the insulin gene. *Mol. Cell. Biol.* 18:2957–2964.
32. McMurry, M.T., and M.S. Krangel. 2000. A role for histone acetylation in the developmental regulation of VDJ recombination. *Science.* 287:495–498.