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B-Lymphocyte Development Is Regulated by the Combined Dosage of Three Basic Helix-Loop-Helix Genes, *E2A*, *E2-2*, and *HEB*†

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B-lymphocyte development requires the basic helix-loop-helix proteins encoded by the E2A gene. In this study, the control mechanism of *E2A* was further explored by disruption of the E2A-related genes, *E2-2* and *HEB*. In contrast to *E2A*, *E2-2* and *HEB* are not essential for the establishment of the B-cell lineage. However, both *E2-2* and *HEB* are required for the generation of the normal numbers of pro-B cells in mouse embryos. Breeding tests among mice carrying different mutations revealed that *E2-2* and *HEB* interact with *E2A* in many developmental processes including the generation of B cells. Specifically, mice transheterozygous for any two mutations of these three genes produced fewer pro-B cells than the singly heterozygous littermates. This study indicates that B-cell development is dependent not only on an essential function provided by the E2A gene but also on a combined dosage set by *E2A*, *E2-2*, and *HEB*.

B-lymphocyte development is a tightly regulated differentiation process which includes at least three major steps: (i) commitment of lymphoid precursor cells to become B-lineage cells, (ii) maturation into immunoglobulin M-positive B cells, and (iii) antigen-dependent differentiation and proliferation to become effector and/or memory cells. This entire differentiation process is controlled by a complex array of regulatory molecules, and mutations in any key step in the regulatory pathway may cause abnormal B-cell development such as leukemias or lymphopenias seen in patients. Recent studies on the E2A gene and the Id genes indicate that helix-loop-helix (HLH) proteins play critical roles in the first step of B-cell development (1, 28, 35).

E2A is a founding member of a large HLH gene family, members of which are often involved in lineage determination and cell type differentiation (12, 13, 18). Most HLH proteins (with some exceptions) can be grouped into three classes (3, 16). The first class is known as E proteins, characterized by a broad expression pattern and the capability of forming homodimers as well as heterodimers with the other two classes of HLH proteins. E2A, E2-2, and HEB belong to this class (10, 11). The second class consists of tissue-specific HLH proteins which often play tissue-specific roles. Most tissue-specific HLH proteins have a stronger potential to form heterodimers with the E proteins than to form homodimers by themselves (14, 16). The function of this class of HLH proteins is demonstrated by the studies of MyoD, a muscle-specific HLH protein. MyoD can activate myogenesis when expressed in nonmuscle cells, indicating that MyoD functions as a master switch for myogenesis (32, 33). Other tissue-specific HLH proteins such as MASH1, Add1, Scl1, and SCL are found to play critical roles in controlling differentiation of sympathetic neurons, adipocytes, the skeleton, and erythrocytes, respectively (5, 8, 26, 30). The third class is commonly known as Id, i.e., inhibitors of DNA binding (3). Id proteins are different from the rest of HLH proteins by lacking the DNA binding domain. Consequently, the heterodimers formed by Id and other HLH proteins cannot bind to DNA. In general, Id proteins dimerize efficiently with E proteins and less efficiently with tissue-specific HLH proteins (3, 29).

Detailed biochemical analysis, particularly on myogenesis, has led to the following model. In most cell types, a differentiation event is controlled by a heterodimer formed between a tissue-specific HLH protein and an E protein (14). This event is negatively regulated by Id proteins, which exert their effect by competitive dimerization with the E protein. High-level expression of Id favors proliferation and inhibits differentiation (3). At differentiation, a transient increase of tissue-specific HLH protein can override this inhibitory effect by activating a positive feedback loop which leads to a cascade of differentiation events (32). For most cell types including muscle, each class of HLH proteins is often represented by several structurally and functionally related genes. For example, four myogenic HLH proteins including MyoD were identified in muscle. Studies show that these myogenic HLH proteins play similar but nonidentical roles in regulating myogenesis (21). Thus, the actual interaction scheme among HLH proteins can be far more complex.

In contrast to myogenesis, B-cell development cannot be simply explained by this model. Thus far, no B-cell-specific HLH gene has been isolated by various approaches including a two-hybrid screen (unpublished data) (2a, 13a). The collective evidence indicates that E47, a product of the E2A gene, is the major player in regulating B-cell differentiation. First, the E47 homodimer can be easily detected in B cells but not in other cell types (25). Second, this B-cell-specific homodimerization of E47 appears to be regulated by posttranslational modifications such as phosphorylation (25) and disulfide bond formation (2). Third, it was demonstrated by transfection assay that E47 is able to activate immunoglobulin gene rearrangement in T cells (24). Finally, recent gene knockout studies demonstrated that the E2A gene is essential for B-cell development (1, 35).

The E2-2 and HEB genes are structurally and functionally

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† While the manuscript was in preparation, H.W. died in his fight against brain cancer. Y.Z. and P.C. dedicate their part of this work to his memory.

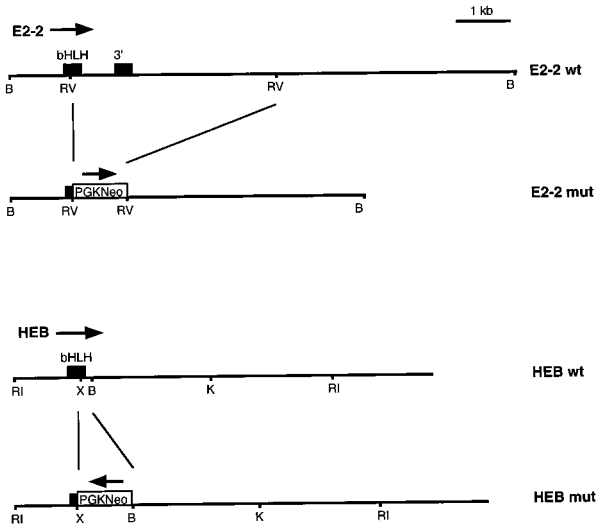

FIG. 1. Disruption of the E2-2 and HEB genes by homologous recombination. Wild-type (wt) and mutant (mut) alleles of *E2-2* and *HEB* are shown with the regions used in making the targeting constructs. Abbreviations: B, *Bam*HI; RV, *Eco*RV; RI, *Eco*RI; X, *Xba*I; K, *Kpn*I. All the restriction sites shown are sites mapped in the 129/sv mouse genome. Gene orientations are indicated with arrows. In the E2-2 mutation, the sequence between the two RV sites was substituted with a PGKNeo marker. The RV site on the left is located within the first helix of the bHLH domain of the E2-2 gene. In the HEB mutation, the sequence between the *Xba*I site and the *Bam*HI site was substituted with a PGKNeo gene which is in the reverse orientation relative to the E2A gene. The *Xba*I site is located in the second helix of the bHLH domain of the HEB gene, whereas the *Bam*HI site is located in the intron 3′ to the bHLH-encoding exon.

related to the E2A gene (10, 11). Like *E2A*, both *E2-2* and *HEB* have broad expression patterns, both can recognize the E-box sequences, and both can form homodimers as well as heterodimers with other classes of HLH proteins (11). Although evidence indicated that *HEB* is involved in regulating the expression of the CD4 gene in T cells (23), the primary roles of *HEB* and *E2-2* in mammalian development are largely unknown. The fact that both genes are coexpressed with *E2A* in B cells suggests that *E2-2* and *HEB* may also play roles in B-cell development.

In this report, we investigated the function of *E2-2* and *HEB* by gene targeting. In contrast to the E2A knockout, we found that mice homozygous for E2-2 or HEB mutations can make B cells, indicating that both genes are not essential for the establishment of the B-cell lineage. However, the numbers of pro-B cells made were reduced in both E2-2 and HEB mutant homozygous mice. By breeding tests, we found that both *E2-2* and *HEB* can enhance the defect of *E2A*, suggesting that three genes have a common role in B-cell development. These results argue that B-cell development requires not only a functional E2A gene but also a combined E-protein dosage set by three E-protein genes.

TABLE 1. Breeding data for E2-2 and HEB mutations

Cross and stage (no. of litters)	No. of pups[a]		
	wt	Het	Mut
$E2\text{-}2^{+/-} \times E2\text{-}2^{+/-}$			
E16.5–term (5)	8	25	11
1–2 wk postpartum (11)	25	47	0
$HEB^{+/-} \times HEB^{+/-}$			
E18.5–term (3)	4	15	7
1–2 wk postpartum (18)	47	86	1[b]

[a] wt, wild type; Het, heterozygous; Mut, mutant.
[b] Eight pups died before weaning.

MATERIALS AND METHODS

Cell culture and mice. Genomic clones for both *E2-2* and *HEB* were isolated from a phage library prepared from 129/sv genomic DNA (a gift from P. Soriano). Gene targeting constructs were built on the basis of the pPNT vector (31), which contains PGKNeo and PGKTK as positive and negative selection markers, respectively. Gene targeting was carried out in two different ES cell lines, EFC-1 (a gift from S. Fiering and A. Smith) for the E2-2 construct and AB-1 (a gift from P. Soriano) for the HEB construct. Both cell lines were originally derived from the 129/sv inbred strain. Homologous recombination events were screened by PCR and confirmed subsequently by Southern blotting. The recombinant clones were then injected into C57BL/6 hosts, and the resulting chimeric mice were backcrossed to C57BL/6. Three HEB clones and one E2-2 clone produced germ line transmitted progenies. All the subsequent analyses were carried out on the hybrid background, which is C57BL/6 and 129/sv. Mouse colonies were bred in a clean facility free of infections.

Fluorescence-activated cell sorter (FACS) analysis. Fetal liver, spleen, and thymus samples were prepared as described elsewhere (35). For each sample, 10,000 cells were scored on a Becton Dickinson FACScan analyzer and then analyzed by Reproman (True FACS Software Inc.) or Cell Quest (Becton Dickinson). The following antibodies were used in the experiment: B220-fitc, B220-pe, BP1-fitc (clone 6C3), CD43-pe, CD4-pe, CD8-fitc, CD5-pe, TCRβ-fitc, and CD3-pe (Sigma or Pharmingen). 7AAD (from Molecular Probe) was included in each staining as a third color to mark the dead cells. β-Galactosidase (β-Gal) expression was detected by fluorescein di-β-D-galactopyranoside (17, 35).

The quantitative analysis of fetal liver B cells was carried out as follows: first, the percentages of $CD43^+$ $B220^+$ pro-B cells in the total population were calculated by using a fixed gate for each batch of samples; second, the relative amount of B cells for each individual sample was calculated by normalizing against the average value of the wild types within the batch; finally, data from different litters but the same type of cross were compiled into a histogram, with the vertical axis indicating the numbers of samples analyzed and the horizontal axis indicating the relative amount of B cells. The Student *t* test was used to evaluate the significance of the mean values.

Fetal liver transfusion assay. Fetal liver samples were prepared from 18.5-day-old (E18.5) embryos by disrupting the tissues with repeated pipetting. Cell suspensions were then treated with 150 mM NH_4Cl once and then washed with phosphate-buffered saline (PBS) containing 5% bovine calf serum. Cells were kept on ice, while the genotypes of the samples were determined by PCR, which took about 4 h. Right before transfusion, cells were washed with plain PBS twice and resuspended at 2×10^6/ml in PBS. Approximately 10^6 cells were delivered into each host through tail vein injection. Recipient mice (C57BL/6) were prepared by irradiation with 11 Gy 1 day before the transfusion. B cells were analyzed by eye bleeding 4 weeks later and then by sacrificing the mice 7 weeks posttransfusion.

RESULTS

Characterization of the E-protein gene family by gene targeting. Mutations in the E2-2 and HEB genes were generated by homologous recombination in ES cells. The E2-2 gene was disrupted by replacing the C terminus of the gene starting from the first helix of the basic HLH (bHLH) domain with a PGKNeo marker (Fig. 1). The HEB gene was disrupted by replacing part of the second helix of the bHLH domain with the same PGKNeo marker (Fig. 1). Both mutations were intended to eliminate the dimerization (and therefore the DNA binding) activity of the proteins. Homologous recombinants were obtained at a frequency of 10 to 20% for both genes.

Mouse strains were derived from the ES cells carrying the E2-2 or HEB mutation. Mice heterozygous for the E2-2 or HEB mutation appeared normal and were indistinguishable from their wild-type littermates. E2-2 mutant homozygous mice were born with extremely low frequency and did not survive more than 1 week after birth. Mice homozygous for the HEB mutation were born with close-to-expected frequency and normal appearance but died within the first 2 weeks. Thus far, only one HEB mutant homozygous mouse of 142 genotyped F_2 offspring has survived to adulthood (Table 1). These postnatal lethalities are very similar to the phenotype of the

TABLE 2. Postnatal lethality in transheterozygous mice

Cross and stage (no. of litters)	No. of pups				
	wt[a]	E2A$^{+/-}$	HEB$^{+/-}$	E2-2$^{+/-}$	Transhet[b]
E2A$^{+/-}$ × HEB$^{+/-}$					
E16.5–18.5 (6)	10	9	17		11
1–2 wk postpartum (2)	5	5	4		0 (2)
E2A$^{+/-}$ × E2-2$^{+/-}$					
E16.5–18.5 (4)	10	9		7	11
1–2 wk postpartum (5)	10	18		12	3 (11)
HEB$^{+/-}$ × E2-2$^{+/-}$					
E16.5–17.5 (6)	10		17	12	8
1–2 wk postpartum (7)	7		15	11	0 (6)

[a] wt, wild type.
[b] Transhet, transheterozygous. The numbers of pups that died before weaning are given in parentheses.

E2A mutant homozygous mice (1, 35), except that E2A-null mice have a slightly higher chance to survive to adulthood (unpublished data).

To compare the functions of *E2-2*, *HEB*, and *E2A*, mutant heterozygous mice were crossed. Surprisingly, postnatal lethality was observed in every transheterozygous combination (Table 2). In nearly all cases, the transheterozygous mice appeared normal at birth but exhibited slow growth and a high rate of mortality before weaning. Some E2A/E2-2 transheterozygous mice survived to adulthood, albeit at low frequencies. Although the cause of death is still a subject of investigation, these genetic interactions suggest that these three E-protein genes share common roles during postnatal development.

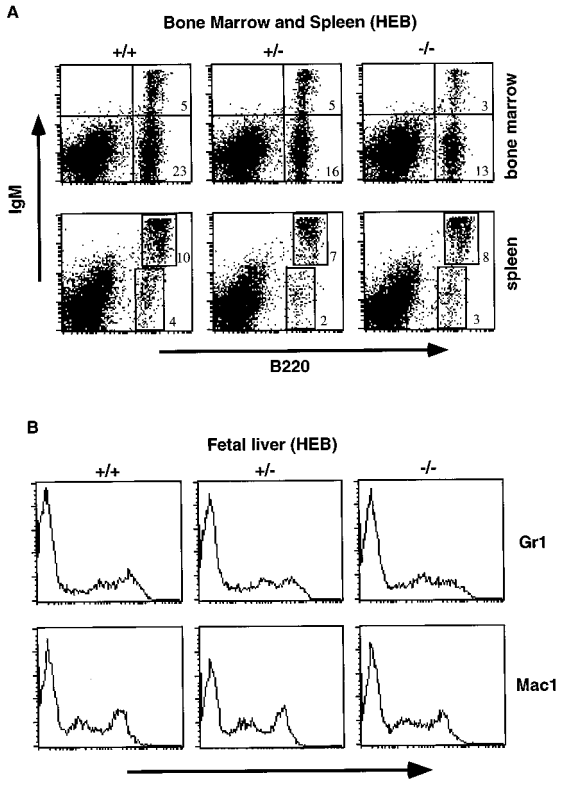

FIG. 2. FACS analysis of B cells and myeloid cells in the HEB knockout mice. (A) Detection of B cells in HEB mutant homozygous mice. The bone marrow and spleen tissues of the HEB knockout mice were analyzed by B220 and immunoglobulin M double staining. Representative samples of 2-week-old mice are shown. Gates were set for live cells by 7AAD exclusion. (B) Fetal liver samples of HEB mice were analyzed by single-color staining with Gr-1 and Mac-1, which detect granulocytes and monocytes, respectively.

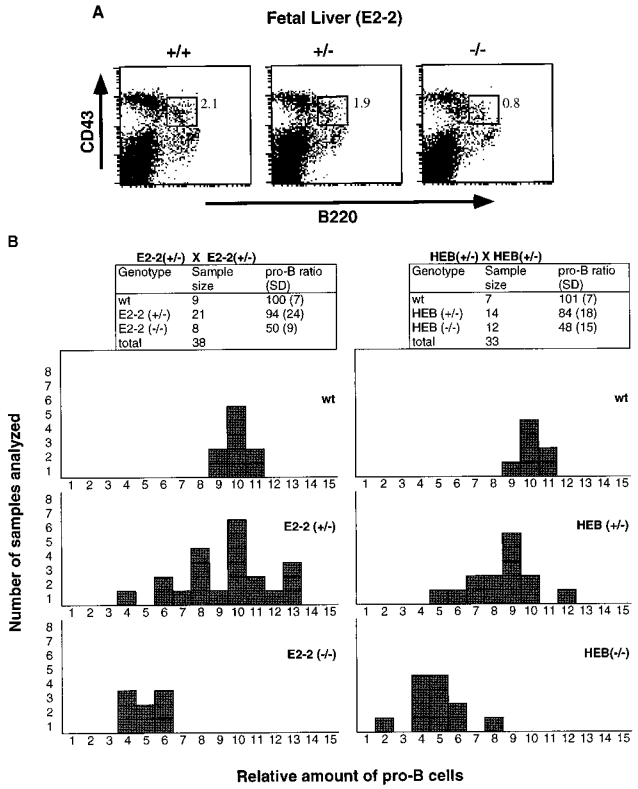

FIG. 3. FACS analysis of pro-B cells in E2-2 and HEB knockout mice. (A) Detection of B cells in E2-2 mutant homozygous mice. The fetal livers of E2-2 knockout mice were analyzed by B220 and CD43 double staining. 7AAD was used for excluding the dead cells. Representative samples of E18.5 embryos are shown. B220^{dull} CD43^{+} pro-B cells are boxed. This type of analysis was used for generating primary data shown in Table 3. (B) The numbers of pro-B cells are reduced in fetal livers of E2-2 and HEB mutant mice. Fetal liver samples were collected from heterozygous intercrosses of either E2-2 or HEB mutations. The percentages of pro-B cells for each individual were scored by using a fixed gate for each batch of samples (e.g., Table 3). To compare the numbers of pro-B cells among various genotype groups, the relative amount of pro-B cells in each individual sample was calculated by normalizing against the wild types (wt). Sample sizes and the relative ratio of B220^{dull} CD43^{+} pro-B cells for each genotype are summarized at the top. Standard deviations (SD) are in parentheses. The histograms were compiled by plotting the relative amount of pro-B cells against the number of samples analyzed.

***E2-2* and *HEB* are involved in B-cell development.** The postnatal lethality in transheterozygous mice indicates that *E2-2* and *HEB* play roles similar to that of *E2A* in mouse development. Given that the E2A knockout mice have a specific defect in the B-cell lineage, we asked if the E2-2 or HEB mutations produce a similar effect. Analysis of spleen and bone marrow samples showed that HEB mutant homozygous mice produced mature B cells (Fig. 2A). Other hematopoietic lineages including myeloid (Fig. 2B) and erythroid (data not shown) are also detected in HEB mutant homozygous mice. Because of the difficulty in obtaining viable E2-2 pups, fetal livers were analyzed for the effect of the E2-2 mutation. B-lineage cells marked with B220 and CD43 surface antigens were detected in the E2-2 mutant homozygous fetuses (Fig. 3A). Both myeloid and erythroid cell lineages are detected in E2-2 homozygous

TABLE 3. Pro-B cells in fetal livers of E2-2 mice

Stage	% $B220^+$ $CD43^+$ cells		
	Wild type	E2-$2^{+/-}$	E2-$2^{-/-}$
E16.5 (litter 1)	2.9	3.0, 3.8, 3.7, 2.3, 3.5	1.5, 1.9, 1.4, 1.8
E17.5			
Litter 2	2.1	2.2, 2.0, 1.7, 1.9, 2.0, 2.7	0.8
Litter 3	2.9, 2.6, 2.3, 2.8	1.6, 2.0, 1.8, 2.0, 2.8	1.0, 1.5
E18.5			
Litter 4	14.9, 12.9, 17.0	8.8, 6.2, 14.2, 14.8, 16.8	5.3
Litter 5	4.1	6.7, 5.1, 5.4, 5.1, 3.4, 5.0	

mutant mice (data not shown). These results indicate that neither *E2-2* nor *HEB* is essential for lineage establishment of B lymphocytes.

Although E2-2 and HEB are not essential for B-cell formation, a slight decrease in pro-B cell numbers was frequently observed in both E2-2 and HEB mutant homozygous mice. To further investigate this subtle phenotype, a quantitative analysis of fetal liver B cells was performed. The population of $B220^{dull}$ $CD43^+$ pro-B cells (9) was counted for each genotype group generated from either the E2-2 heterozygous cross (Table 3) or the HEB heterozygous cross. Histograms were used to display the relative ratios of pro-B cells among various genotype groups (Fig. 3B). On average, mice with homozygous mutations for either *E2-2* or *HEB* produced approximately 50% the wild-type level of pro-B cells. These results indicate that both the E2-2 and HEB genes are required for the generation of normal numbers of pro-B cells.

A combined dosage of three E-protein genes is required for B-cell development. We have previously reported that the numbers of pro-B cells are regulated by the dosage of the E2A gene (35). This common effect of *E2A*, *E2-2*, and *HEB* on the generation of pro-B cells suggests that three E-protein genes play a common role in B-cell development. To compare the function of these E-protein genes, mice transheterozygous for different E-protein gene mutations were analyzed for the content of fetal liver pro-B cells. This analysis revealed that a transheterozygous combination of any two E-protein gene mutations generated fewer pro-B cells than the single heterozygous littermates (Fig. 4). Overall, there was about a 70% reduction of $B220^{dull}$ $CD43^+$ pro-B cells in mice transheterozygous for *E2A*/*E2-2* and *E2A*/*HEB* and a 50% reduction for *E2-2*/*HEB*. These nonallelic interactions among different E-protein genes support the idea that these three E-protein genes are all involved in a common pathway in B-cell development.

B-cell deficiency in E2A knockout mice is cell autonomous. B-cell development requires external signals provided by the stromal environment. For example, physical contact between the pro-B cells and the stromal cells is crucial in the early stages of B-cell development (34). Thus, the B-cell deficiency

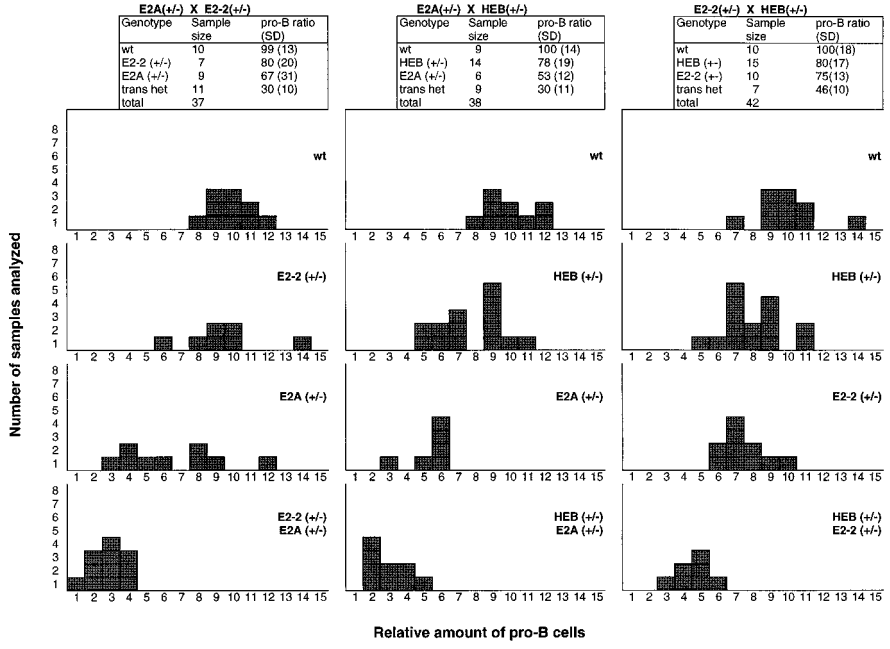

FIG. 4. Generation of pro-B cells requires a common role played by *E2A*, *E2-2*, and *HEB*. Transheterozygous (trans het) embryos were generated by breeding mice carrying single heterozygous mutations. Liver samples were collected from E16.5 to E18.5 stage embryos. The relative numbers of $B220^{dull}/CD43^+$ pro-B cells present in each genotype group were analyzed. Three types of heterozygous mating are shown: E2A/E2-2, E2A/HEB, and E2-2/HEB. Analyses were done as for Fig. 3. wt, wild type; SD, standard deviation.

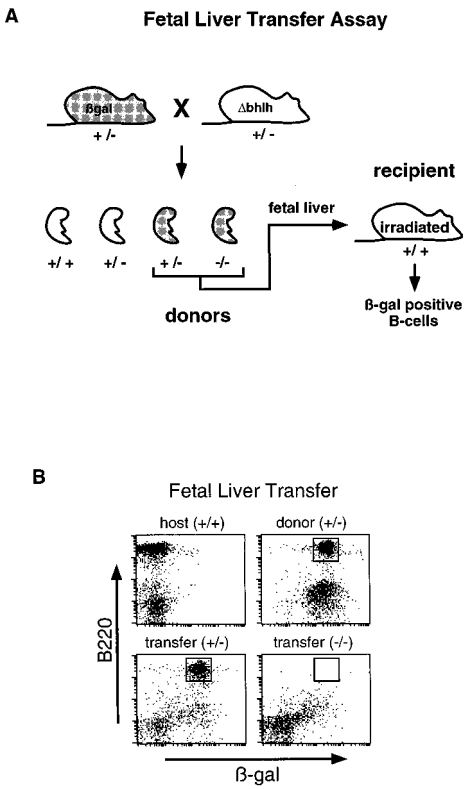

FIG. 5. The B-cell defect of E2A-null mice cannot be rescued by fetal liver transfusion. (A) Diagram of the assay procedure. Donor cells were obtained from a cross between an $E2A^{\beta gal}$ heterozygous mouse (shaded) and an $E2A^{\Delta bhlh}$ heterozygous mouse. Control cells ($+/E2A^{\beta gal}$) and E2A mutant cells ($E2A^{\Delta bhlh}/E2A^{\beta gal}$) were transferred to lethally irradiated C57BL/6 recipients. Peripheral blood cells and spleen cells were analyzed by β-Gal and B220 double staining 4 and 7 weeks, respectively, posttransfusion. (B) E2A mutant cells cannot be rescued by transfusion. Peripheral blood cells were used in this assay. The upper panels show wild-type (left) and $E2A^{\beta gal}$ heterozygous (right) cells from unmanipulated control mice. The lower panels show cells recovered from transfusion using either heterozygous (left) or mutant (right) donors. The $\beta\text{-Gal}^+$ $B220^+$ B-cell population is boxed.

in E2A knockout mice may result from a defect in the B cells or a defect in the stromal cells. To address this issue, a transfusion assay was carried out by transferring E2A-null fetal liver cells into wild-type hosts. In this experiment, we would expect to see a rescue of the B-cell lineage if the defect was environmental but would not see a rescue if the defect was B cell autonomous.

The β-Gal marker present in the previously characterized $E2A^{\beta gal}$ allele (35) provided the lineage tracer in this transfusion assay. Donor cells were obtained from mating between $E2A^{\beta gal}$ heterozygous mice and $E2A^{\Delta bhlh}$ heterozygous mice (Fig. 5A). Both E2A transheterozygous mutant cells ($E2A^{\Delta bhlh}/E2A^{\beta gal}$) and heterozygous control cells ($+/E2A^{\beta gal}$) were labeled with a single copy of the β-gal marker for equal staining. As shown in Fig. 5B and Table 4, the heterozygous donors gave rise to β-Gal-positive B cells while the E2A-null donors did not. On the basis of this result, we conclude that the E2A knockout causes an intrinsic defect in the B lineage.

***HEB* and *E2A* but not *E2-2* are involved in T-cell development.** Sawada and Littman have previously shown that *HEB* is directly involved in regulating CD4 expression in T cells (23). In agreement with this report, we found that the amount of CD4 antigen on thymocytes is reduced in HEB mutant homozygous mice (Fig. 6). In addition to the CD4 phenotype, we found that CD5, a pan-T-cell surface antigen, is also down regulated in thymocytes of HEB-deficient mice. These $CD8^{high}$ $CD4^{low}$ $CD5^{low}$ cells were determined to be immature thymocytes on the basis of the fact that they stain negative for T-cell receptor β and CD3 markers (data not shown). The impact of the HEB mutation on both CD4 expression and CD5 expression appears to be limited to the thymocytes. Mature T cells present in the spleen express normal levels of CD4 and CD5 (Fig. 6C).

TABLE 4. Failure of transfusion to rescue the B-cell lineage of E2A-null mice

Host or donor	No. of B cells[a]	
	β-Gal negative	β-Gal positive
Control		
Host[b]	1,567	32
Donor[c]	5	749
+/−		
Donor A (female)		
Expt 1	8	693
Expt 2	3	751
Donor B (male)		
Expt 3	5	1,076
Expt 4	28	869
Expt 5	15	886
−/−		
Donor C (female)		
Expt 6	2	16
Expt 7	2	2
Donor D (male)		
Expt 8	1	9
Expt 9	7	19
Expt 10	0	2

[a] Number of $B220^+$ cells per 10,000 blood cells.
[b] C57BL/6 wild-type mouse.
[c] $E2A^{\beta gal}$ heterozygous mouse.

In findings similar to those for the B-cell phenotype, we found that CD4 and CD5 expression is also dependent on the dosage of different E-protein genes. Mice transheterozygous for E2A and HEB mutations show a reduction in CD4 and CD5 expression similar to that of HEB mutant homozygous mice (Fig. 7), indicating that E2A is also involved in regulating CD4 and CD5 expression. In contrast to B cells, in which three E-protein genes are involved, *E2-2* is apparently not involved in regulating CD4 and CD5 expression in T cells. *E2-2* when tested as a mutation either homozygous or transheterozygous with *HEB* has no effect on the expression levels of CD4 and CD5 antigens (data not shown). The absence of E2-2 activity in T cells is apparently not due to transcription silencing, since E2-2 RNA was detected in the thymus (data not shown). These results demonstrated once again that these three E-protein genes play not only overlapping but also unique roles in mouse development.

DISCUSSION

The functions of *E2-2* and *HEB* in B-cell development were analyzed by gene disruption. In contrast to the E2A gene, both *E2-2* and *HEB* are not essential for the establishment of the B-cell lineage. However, during embryonic development the

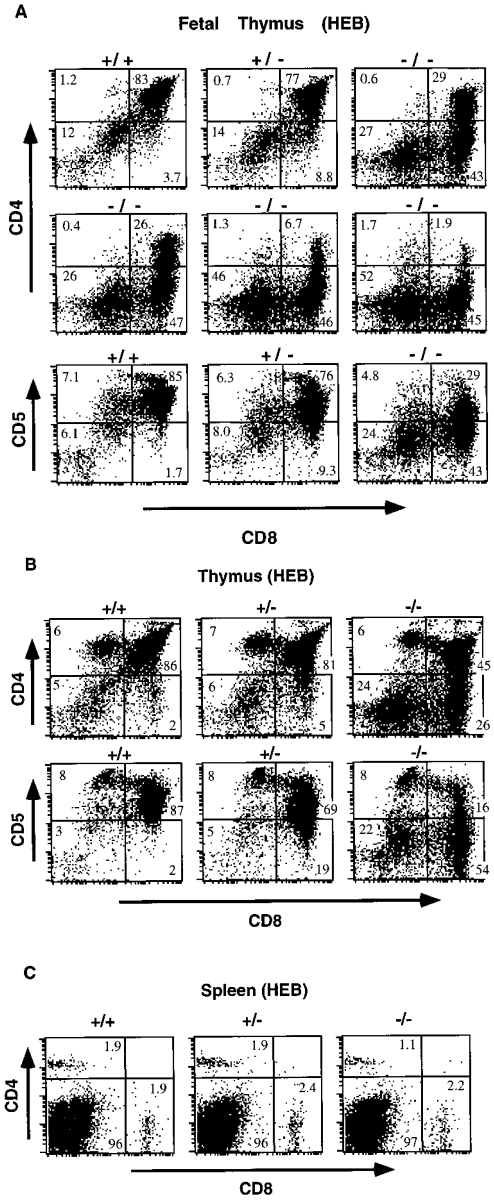

FIG. 6. CD4 expression and CD5 expression are impaired in HEB knockout mice. (A) Analysis of fetal thymus by CD4-CD8 and CD5-CD8 staining. Fetal thymuses were collected at stage E18.5 and used immediately for FACS analysis. A total of 18 embryos were analyzed, among which 7 were HEB mutant homozygotes. Samples shown in the upper and middle panels are from the same litter. The impairment in CD4 expression in the HEB mutants (−/−) varies among individuals but is always significantly different from their heterozygous (+/−) and wild-type (+/+) littermates. The same samples were used in the upper and lower panels. The results of CD5-CD8 staining are highly representative. (B) Analysis of postnatal thymus by CD4-CD8 and CD5-CD8 staining. Thymocytes were prepared from 1-week old littermates. The data are highly representative on the basis of analyses of 18 samples, among which 4 were $HEB^{-/-}$. (C) Analysis of splenic T cells by CD4-CD8 staining. Spleen cells were prepared from 2-week-old littermates. Live cells were analyzed after deletion of erythrocytes.

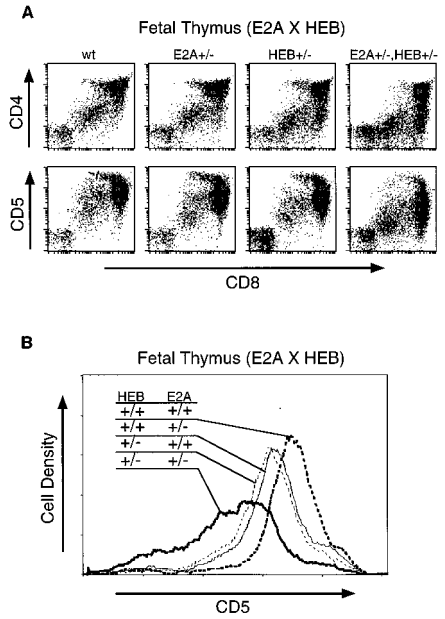

FIG. 7. CD4 expression and CD5 expression are dependent on the dosage of E2A and HEB. Thymocytes in the E2A and HEB transheterozygotes were analyzed for the gene dosage effect. The data represent the analysis of E18.5 fetal thymus. The results are highly repeatable on the basis of analyses of five litters (E17.5 to 2 weeks postpartum) generated by HEB and E2A heterozygous intercrosses. (A) Dot plots of CD4-CD8 and CD5-CD8 double staining of fetal thymocytes. (B) The samples in the lower plots of panel A were plotted with one parameter to highlight the levels of CD5 expression. Only CD8-positive cells were included in this single-parameter plot.

presence of both *E2-2* and *HEB* is important for the generation of the normal numbers of pro-B cells. Analysis of mice transheterozygous for different E-protein gene mutations demonstrated that the function of *E2-2* and *HEB* overlaps with but does not equal that of *E2A*. This study combined with our previous analysis of the E2A-null mice (35) indicates that three E-protein genes are differentially involved in two developmental events, i.e., the lineage commitment and the expansion of the pro-B-cell population. It appears that all three E-protein genes are involved in the control of the pro-B-cell population whereas only *E2A* is required for the establishment of pro-B cells.

***E2A* plays a greater role than *E2-2* and *HEB* during B-cell development.** The quantitative analysis of fetal liver B cells in various mutant backgrounds indicates that all three E-protein genes are required for producing the normal numbers of pro-B cells. If the numbers of pro-B cells are used to measure the dosage contribution of each E-protein gene, one can roughly estimate that one copy of *E2A* is equivalent to two copies of *E2-2* or *HEB*. However, it is important to exercise caution in extrapolating this conclusion from the genetic level to the biochemical level, since many variables, including the timing and levels of gene expression, RNA and protein stability, and dimerization and DNA binding affinity are yet to be determined. Nonetheless, the quantitative difference in dosage contribution suggests that *E2A* plays a greater role than *E2-2* and *HEB* in regulating the numbers of pro-B cells.

The major difference between *E2A* and *E2-2*/*HEB* is that *E2A* is absolutely essential for the establishment of the B-cell lineage whereas *E2-2* and *HEB* are not. If the proteins encoded by these three E-protein genes were functionally identical, one would expect to see a certain degree of functional complementation by *E2-2* and *HEB* in the E2A-null mice. However, no B cells were detected in the E2A-null mice even after they had reached adulthood (unpublished data). This dramatic difference between *E2A* and *E2-2*/*HEB* cannot be simply explained by the difference in the dosage contribution. It suggests that *E2A* may provide a unique function which cannot be replaced

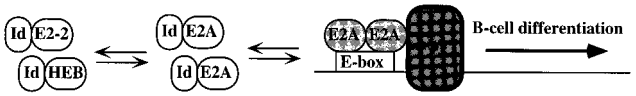

FIG. 8. Unique and common roles of *E2A* in B-cell development. This model postulates that E2A homodimers are directly involved in initiating B-cell differentiation. E2-2, HEB, and Id proteins are indirectly involved in this process by modulating the amount of available E2A proteins.

by either *E2-2* or *HEB* and this function must be critical for the establishment of the B-cell lineage.

In many ways, the activities of E-protein genes in B-cell development are remarkably similar to the regulation of *Drosophila* sex types by a set of bHLH genes located in the *achaete scute* complex. In early embryogenesis, this complex is involved in regulating the *Sex-lethal* gene, which is the master switch in sex determination. Expression of *Sex-lethal* is required in the female flies but is lethal in the male flies (4, 20). When several *achaete scute* complex genes including *T3*, *T4*, and *T5* were analyzed by ectopic expression in the males, it was found that only *T4* on its own can cause male lethality whereas *T3* and *T5* can enhance the phenotype of *T4* (19). The similarities between sex determination in the fly and B-cell development in the mouse suggest a conserved mechanism used by various HLH proteins.

A working model. On the basis of our studies and other accumulated knowledge about the HLH proteins, we propose a working model in which *E2A* is the central player in regulating both the commitment and expansion of the B-cell lineage. This model (Fig. 8) assumes that E2A homodimers control a single downstream event (or very few) which leads to B-cell formation. In a noncommitted precursor cell, the level of the E2A proteins determines the probability of this cell to become a pro-B cell. Both *E2-2* and *HEB* can modulate the pool size of E2A homodimers through a competitive dimerization with Id. Deletion of any E-protein gene or addition of an Id transgene will drive the equilibrium towards the depletion of E2A homodimers, resulting in a decrease in the number of pro-B cells. However, none of these can fully block B-cell formation unless E2A is completely depleted in the system. This model predicts that (i) the B-cell-deficient phenotype in E2A-null mice can be rescued by ectopic expression of *E2A* but not by *E2-2* or *HEB* and (ii) the dosage defect can be rescued by ectopic expression of any E-protein gene or by targeted deletion of particular Id genes. We are currently testing these possibilities.

Since *E2A* is not a B-cell-specific gene, the presence of E2A protein may not be sufficient to trigger B-cell differentiation. Two mechanisms have been proposed to explain how *E2A* achieves its B-cell-specific function. The first one suggests that other factors such as B-cell-specific coactivators may be involved in restricting E2A function in B cells (6, 22). Interaction between transcription factors and coactivators has been shown to be important for the B-cell-specific transcription of the POU domain proteins (7, 15, 27). The second one suggests that the tissue specificity of *E2A* may be controlled by posttranslational modification of the E2A proteins. It appears that the formation of E47 homodimers is dependent on site-specific phosphorylation and/or disulfide bond formation, which occur exclusively in B cells (2, 25). Clearly, more detailed investigations are required to resolve the issue of tissue specificity.

T-cell phenotypes, a direct measurement of the endogenous E-protein activity. The T-cell phenotype provides a simple readout for the endogenous E-protein activities. It is possible that levels of CD4 and CD5 expression are controlled by a direct binding of the E proteins to their cognate E-box sites. It has been shown that two E-boxes present in the promoter region of the CD4 gene are occupied predominantly by the HEB proteins rather than E2A (23). This may explain why *HEB* seems to play a greater role than *E2A* in determining the expression level of the CD4 gene. E-box sequences are also found in the promoter regions of the CD5 gene, although these sites have not been tested for enhancer activities (30a).

It remains to be determined what the impact of HEB or E2A mutations is on T-cell development in addition to their effect on CD4 and CD5 expression. In the thymus, T-cell development can be roughly divided into three consecutive stages based on the expression of the CD4 and CD8 surface antigens, i.e., the double negative (DN) stage, the double positive (DP) stage, and the single positive stage. As thymocytes maturing from the DP stage to the single positive stage, they start to express T-cell receptors on the cell surface. The $CD8^{high}$ $CD4^{low}$ $CD5^{low}$ cells in the mutant mice do not express T-cell receptors; therefore, they are in a transition stage between DN and DP. However, the exact nature of these cells remain to be determined by further investigation.

In addition, we have consistently observed that the DN cell population is proportionally larger in HEB mutant homozygous mice than in their wild-type or heterozygous littermates (Fig. 5A). This may suggest another developmental delay in the transition from the DN stage to the DP stage. If this phenotype is related to the E-protein dosage, a more severe defect may be induced when more copies of E-protein genes are deleted.

Pleiotropic roles played by the E-protein genes in mouse development. The postnatal lethality in the homozygous and transheterozygous mutants indicates another developmental role played by the E proteins. This unknown function should be independent of the B- and T-cell phenotypes, since both cell types are not essential for mice to survive in clean facilities. Embryonic development, although not affected by deletion of any two copies of E-protein genes, is sensitive to a further reduction of E-protein gene copy numbers. Using the few surviving E2A E2-2 transheterozygous mice in mating with the E2-2 single-heterozygous mice, we found that the resulting $E2A^{+/-}$ $E2\text{-}2^{-/-}$ embryos failed to develop to term (data not shown). Although the exact timing and nature of this embryonic lethality are unclear at the moment, this phenotype confirms that the E proteins are also involved in embryonic development.

The diversified phenotypes observed in this study are consistent with the idea that E proteins are involved in dimeric interactions with many tissue-specific HLH proteins. Further genetic dissection is needed for determining the function of individual E-protein genes in developmental events other than B lymphopoiesis. Although the exact function of E proteins in each case may be different, the underlying molecular mechanism should be similar. In practice, studies of B-cell development provide a general model for understanding the pleiotropic roles played by these E-protein genes.

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