Regulation of pTα Gene Expression by a Dosage of E2A, HEB, and SCL*

Mathieu Tremblay‡‡§§, Sabine Herblot‡‡§§, Eric Lécuyer‡‡§§, and Trang Hoang‡‡***‡‡

From the ‡‡Clinical Research Institute of Montréal, Montréal, Québec H2W 1R7, Canada and the **Departments of Pharmacology and Biochemistry and the Molecular Biology Program, University of Montreal, Montréal, Québec H3C 3J7, Canada

The expression of the pTα gene is required for effective selection, proliferation, and survival of β T-cell receptor (βTCR)-expressing immature thymocytes. Here, we have identified two phylogenetically conserved E-boxes within the pTα enhancer sequence that are required for optimal enhancer activity and for its stage-specific activity in immature T cells. We have shown that the transcription factors E2A and HEB associate with high affinity to these E-boxes. Moreover, we have identified pTα as a direct target of E2A-HEB heterodimers in immature thymocytes because they specifically occupy the enhancer in vivo. In these cells, pTα mRNA levels are determined by the presence of one or two functional E2A or HEB alleles. Furthermore, E2A/HEB transcriptional activity is repressed by heterodimerization with SCL, a transcription factor that is turned off in differentiating thymocytes exactly at a stage when pTα is up-regulated. Taken together, our observations suggest that the dosage of E2A, HEB, and SCL determines pTα gene expression in immature T cells.

The development of αβ T cells from multipotent progenitors is a complex and multistep process that is critically dependent on genetic recombination and extracellular signals. Maturation of recent thymic immigrants from bone marrow derived precursors is characterized by the sequential expression of the pre-TCR complex and of several surface markers including CD4 and CD8. Within the CD4/CD8 double-negative (DN) population, cell survival, cell proliferation, and β allelic exclusion as well as the subsequent transition to the more mature CD4/CD8 double-positive (DP) stage are critically dependent on pre-TCR signaling. The pre-TCR is formed by the association of a correctly rearranged βTCR chain with the invariant pTα chain and signaling molecules of the CD3 complex. Since expression of the pre-TCR is critical for αβ T-cell differentiation, dissecting the molecular program that drives the expression of its components is of particular interest to understand the transcriptional regulation of early T-cell development.

Recently, the promoter and the enhancer sequences of the pTα gene have been cloned (4) and partially characterized (5–8). A 250-bp enhancer element located 4-kb upstream of the initiation site is necessary and sufficient for specific expression of the pTα gene in transgenic DN thymocytes in transgenic mice (5). Several transcription factors have been shown to regulate enhancer activity, including c-Myb, and the activated form of Notch (5–7). We have previously observed that the basic helix-loop-helix (bHLH) transcription factor, HEB, plays a critical role in the regulation of pTα expression (9). The E2A and HEB genes encode class I bHLH transcription factors, also called E-proteins (E12-E47 and HEB, respectively), which bind specific DNA sequences (E-box, CANNTG) as homo- or heterodimers. E-protein function is essential for T- and B-cell development as revealed by gene-targeting experiments or expression of dominant-negative molecules, such as Id factors or the SCL oncoprotein (9–15). E2A- and HEB-deficient mice, as well as Id or SCL-LMO1 transgenic mice display a T-cell differentiation defect characterized by a partial differentiation block at the DN to DP transition associated with thymic atrophy (9, 16–18). Our previous study revealed that the partial differentiation block of SCL-LMO1 transgenic thymocytes is, at least in part, due to decreased pTα gene expression in immature DN thymocytes (9). SCL is a tissue-specific bHLH transcription factor that forms heterodimers with E-proteins and acts as a transcriptional activator or repressor, depending on the cellular context (9, 18–23). SCL expression is detected in primitive DN thymocytes, and it is normally shut off during T-cell differentiation (9). Enforced SCL expression, in combination with its nuclear partner LMO1, inhibits E-protein function during early thymopoiesis (9) and subsequently leads to leukemogenesis (18, 24–26).

The mechanism through which bHLH factors regulate pTα expression remains to be documented. Here, we provide evidence that E2A-HEB oligomers determine pTα mRNA levels in DN thymocytes in vivo, through direct binding to two conserved E-boxes of the upstream enhancer. The effect is tightly dosage-dependent and is disrupted by heterodimerization with SCL.

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‡‡ To whom correspondence should be addressed: Institut de Recherches Cliniques de Montréal, Montréal, Québec H2W 1R7, Canada Tel.: 514-987-5588; Fax: 514-987-5757; E-mail: heang@ircm.qc.ca.

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†† Recipient of a studentship from CIHR.

1 The abbreviations used are: TCR, T-cell receptor; pTα, pre-TCRα; DN, double-negative; DP, double-positive; ISP, immature single positive; bHLH, basic helix-loop-helix; β-gal, β-galactosidase; HPRT, hypoxanthine phosphoribosyltransferase; RbgG, rabbit IgG; ChIP, chromatin immunoprecipitation assay; EMSA, electrophoretic mobility shift assay; RIPA, radiolmmune precipitation assay buffer.

12680 This paper is available online at http://www.jbc.org
and stable transfectants were kept under neomycin selection (1 mg/ml). For the detection of SCL protein, nuclear extract were analyzed by Western blot using the anti-SCL mouse monoclonal antibodies BTL73 and BTL136 (generously provided by Dr. D. Mathieu-Mahul, Institut de Génétique Moléculaire, Montpellier) and an anti-Sp1 rabbit polyclonal antibody (Geneka Biotechnology Inc., Montreal), as control for loading.

E2A+ and HEB− mice were kindly provided by Dr. Y. Zhuan (Duke University Medical Center, Durham, NC) (27–29) and bred with C57Bl6/J mice for more than three generations. Heterozygous animals were crossed to get homozygous knockout mice, and all the litters were genotyped by PCR as described (27, 28). CD3ε−/− mice (C57Bl6/J) were kindly provided by Dr. B. Malissen (Centre d’Immunologie INSERM-CNRS de Marseille-Luminy, Marseille) (30). Animals were maintained under specific pathogen-free conditions according to institutional animal care and use guidelines.

**FACS Analysis and Cell Sorting**—Thymi were removed from newborn, 1-week, or 2-month-old mice. Single cell suspensions and immunostaining were performed as previously described (9). Thymocytes were stained with anti-CD4 (H129.19), anti-CD8 (53–6.7), anti-CD25 (7D4), anti-CD44 (IM7), anti-CD6 (145–2C11), anti-PTP-1D mouse monoclonal antibodies BTL73 and BTL136 (provided by Dr. D. Mathieu-Mahul, Institut de Génétique Moléculaire, Montpellier), and a control anti-Myc antibody. Protein-DNA complexes were resolved by 4% PAGE in 0.5× Tris borate-EDTA at 150 V, at 4 °C. All EMSA experiments were performed with an excess of probe. Oligonucleotide sequences used in EMSA experiments are available upon request.

**CHIP Assays**—Chromatin immunoprecipitations were performed essentially as described previously (19, 32, 33). Twenty million AD10.1-MSCV, AD10.1-SCL, or CD3ε−/− primary thymocytes cells were fixed by adding 1% formaldehyde to the culture media for 10 min at room temperature. Formaldehyde was then quenched by addition of 0.125 M glycine. Subsequent steps were performed at 4 °C. Fixed cells were pelleted by centrifugation, washed twice in cold phosphate-buffered saline, then washed once in Triton buffer for 15 min (10 mM Tris-HCl, pH 8.0, 10 mM EDTA, 0.25% Triton X-100) and once in NaCl buffer for 15 min (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.5 mM EGTA, 200 mM NaCl). Cells were pelleted, resuspended in RIPA buffer (10 mM Tris-HCl, pH 8.0, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% SDS) and sonicated (7–10 bursts) to make soluble chromatin ranging in size from 500 to 1000 bp. Cellular debris were removed by centrifugation (16,000 × g for 10 min), and protein concentrations were determined by Bradford staining. Aliquots were reserved for isolation of input DNA, while 1 mg of chromatin extract was incubated overnight at 4 °C with specific antibodies: anti-E47 rabbit polyclonal antiserum (Santa Cruz Biotechnology Inc.), anti-HEB rabbit polyclonal antiserum (Santa Cruz Biotechnology Inc.), anti-SCL mouse monoclonal antibodies BTL73 and BTL136 (provided by Dr. D. Mathieu-Mahul, Institut de Génétique Moléculaire, Montpellier), anti-rabbit IgG (Sigma) and anti-PA10 mouse monoclonal antiserum (Co- vance). DNA-protein complexes were immunoprecipitated with pan-sorbin antibodies (Calbiochem, San Diego) for 30 min at 4 °C, then, pan-sorbin antibodies were sequentially washed twice with 1 ml of RIPA buffer containing 500 mM of NaCl, twice with 1 ml of LiCl buffer (10 mM Tris-HCl, pH 8.0, 250 mM LiCl, 1 mM EDTA, 1% Nonidet P-40, 1% deoxycholate) and twice with 1 ml of TE buffer. Chromatin samples were then eluted by heating for 10 min at 65 °C in 10 μl of elution buffer (50 mM Tris-HCl, pH 8.0, 10 mM EDTA, 1% SDS). After centrifugation, supernatants were diluted by addition of 300 μl of TE buffer and heated overnight at 65 °C to reverse cross-links. RNA and proteins were sequentially degraded by addition of 30 μg of RNase A for 30 min at 37 °C, and 120 μg of proteinase K for 2–3 h at 37 °C. DNA was then phenol/chloroform-extracted and ethanol-precipitated in the presence of 10 μg of rRNA as a carrier. DNA samples were resuspended in 30 μl of water, serially diluted, and 30 cycles of amplification were performed using specific primers for pTα enhancer, pTα promoter, and the hypoxanthine phosphoribosyltransferase (HPRT) promoter. Oligonucleotide sequences are available upon request. One-fifth of PCR products were loaded on a 1.2% agarose gel, transferred on Bio-Dyne B membrane (Pall Corporation, Ann Arbor), hybridized with internal oligonucleotide probes, and analyzed on a PhosphorImager apparatus.

**RESULTS**

**E2A- and HEB-deficient Mouse Expres Decreased Level of pTα mRNA**—The essential role of E-proteins during T-cell development has been revealed by gene-targeted disruption of the E2A and HEB genes in mice (9,14–18,34,35). As shown in Fig. 1B, E2A- and HEB-deficient mice have an increased percentage of immature DN thymocytes and a decreased number of total thymocytes indicating a partial block of T-cell differentiation at the DN to DP transition step, a critical checkpoint controlled by the pre-TCR. Differentiation of TCRγδ lineage thymocytes were analyzed in E2A−/− and HEB−/− mice and wild-type littermates. We show that the percentage TCRγδ thymocytes

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E2A/HEB/SCL Regulate pTα Gene Expression

12681
in the immature DN subsets of E2A or HEB heterozygote mice is similar to that of wild-type littermates (Fig. 1C). Thus, differentiation in the γδ lineage is unaffected, despite reduced levels of both E2A and HEB in heterozygous littermates (Fig. 2A). We have previously reported that HEB deficiency is associated with a decreased level of pTa mRNA in DN thymocytes (9). Since E2A also controls the DN to DP transition, we assess here the role of E2A in vivo in regulating pTa expression, as compared with that of HEB. We therefore used semiquantitative and real-time RT-PCR to investigate pTa mRNA level in E2A- and HEB-deficient mice, as well as in heterozygous and wild-type littermates. Immature DN and DP thymocyte populations were purified by flow cytometry according to their surface expression of CD4, CD8, βTcR, and Thy1.2 markers. Within the DN compartment, we observed a 3- and 5-fold decrease of pTa mRNA level in E2A-deficient thymocytes as compared with wild-type controls by semiquantitative RT-PCR and real-time PCR, respectively (Fig. 2, C and D). Similarly, HEB-deficient DN thymocytes show a 2–3-fold decrease in pTa, as described previously (9). Interestingly, E2A and HEB heterozygous DN thymocytes expressed, on average, intermediate levels of pTa mRNA as compared with wild-type and null DN thymocytes, despite some variations between animals. This decrease is in direct correlation with the decreased E2A or HEB mRNA levels observed in heterozygous mice (Fig. 2A) and reveals that E2A and HEB are haploinsufficient in the thymus. Finally, E2A deficiency did not affect pTa levels in the more mature DP thymocytes, while HEB deficiency consistently impaired its expression. Together, these results indicate that E2A and HEB gene dosage plays an
using cross-linked CD3

E2A and HEB specifically associate with the pTa enhancer and promoter in primary thymocytes. A, schematic diagram of the pTa locus; arrows show the position and orientation of primers used for ChIP assays. B, chromatin immunoprecipitation assays were performed as described under “Experimental Procedures” using cross-linked CD3ε−/− thymocytes nuclear extracts. Anti-HA and RbIgG were used as isotype-matched controls for immunoprecipitations. 5-fold serial dilutions of immunoprecipitated DNA were used for amplification with specific primers for pTa enhancer and promoter regions. The HPRT promoter region was amplified as a negative control. Input chromatin served as a positive control for PCR amplification. The PCR products were analyzed by agarose gel electrophoresis, trans-located within the pTa promoter sequence (5, 8), we also used oligonucleotides flanking the promoter region (Fig. 3A). As shown in Fig. 3B, anti-E47 and anti-HEB antibodies efficiently immunoprecipitated the pTa enhancer sequence as well as the pTa promoter sequence, whereas little or no background was observed with a control antibody. The specificity was further confirmed by the absence of amplification of an irrelevant promoter sequence, the HPRT promoter that is not regulated by E2A or HEB.

Functional Importance of Two E-box Binding Sites within the pTa Enhancer—It has been previously reported that the pTa enhancer element is essential for specific pTa expression in immature DN thymocytes (4, 5). This enhancer element contains potential binding sites for different transcription factors (YY1, ZBP-89, Sp1, c-Myb, and CSL) and particularly four E-boxes (Fig. 4A). We therefore cloned the pTa enhancer sequence upstream of a minimal TATA promoter, and the luciferase reporter gene and confirmed that the enhancer is active in the pTa+ “immature” T-cell line AD10.1 but not in the “mature” T-cell line Jurkat nor in fibroblast cell lines (data not shown). In order to determine the importance of E-boxes in driving enhancer activity in the immature DN AD10.1 cell line, each of the four E-box sites was mutated individually or in combination. As shown in Fig. 4B, mutation of either E2 or E3 sites induced a 3-4-fold decrease of pTa enhancer activity, while simultaneous mutations of these two sites abolished enhancer activity. The E1 site seems to play a weaker role in transcriptional activity since its mutation modestly affected enhancer activity when E2 and E3 were intact. However, E1 mutation combined with E2 or E3 mutations further decreased enhancer activity, revealing its potential role as a positive regulator when E2 or E3 were mutated. Interestingly, mutation of the E4 site induced a 2-3-fold increase of enhancer activity, either alone or in combination with E1 or E3 muta-

mature DN thymocytes (4, 5). Thus, chromatin immunoprecipitation assays were performed as described under “Experimental Procedures” using cross-linked CD3ε−/− thymocytes nuclear extracts. Anti-HA and RbIgG were used as isotype-matched controls for immunoprecipitations. 5-fold serial dilutions of immunoprecipitated DNA were used for amplification with specific primers for pTa enhancer and promoter regions. The HPRT promoter region was amplified as a negative control. Input chromatin served as a positive control for PCR amplification. The PCR products were analyzed by agarose gel electrophoresis, transferred onto Biodyne B membrane, and hybridized with an internal oligonucleotide. C, Western blot of nuclear extract of wild-type and CD3ε−/− thymus, wild-type bone marrow, and AD10.1 cell lines was performed using specific antibodies against E47, HEB, and PTP-1D, the latter as control for loading.

important role in regulating pTa expression specifically at the immature DN stage and suggest that E2A and HEB might have overlapping as well as distinct function during T-cell development.

E2A and HEB Gene Products Bind the pTa Enhancer in Vivo in DN3 Thymocytes—The expression of the pTa gene is directed by a promoter and a 5′ enhancer, both containing multiple E-protein binding sites (E-box) (4, 5). Since E2A and HEB deficiency leads to decreased pTa expression, we addressed the question of whether these proteins associate with pTa regulatory sequences in primary thymocytes in vivo. We therefore performed ChIP using primary thymocytes. Within the DN subset, thymocyte maturation can be followed according to the sequential expression of CD44 and CD25 molecules (1), thus identifying 4 subpopulations (DN1 to DN4), as described in Fig. 1. Since the pTa gene is expressed at the DN3 and DN4 stages, we used thymocytes from CD3ε−/− mice that are arrested at the DN3 stage because of the lack of a pre-TCR signal (30, 36, 37).

E2A and HEB protein expression assessed by Western blotting were higher in these thymocytes as compared with wild-type immature DN3 stage because of the lack of a pre-TCR signal (30, 36, 37). Cross-linked chromatin extracts were prepared and subjected to immunoprecipitation using specific antibodies against E2A and HEB, as well as an isotype-matched control antibody (rabbit IgG). After immunoprecipitation, decross-linking, and purification, serial dilutions of DNA templates were used for PCR amplification using oligonucleotide primers flanking the pTa enhancer regions (Fig. 3A). Since, gel shift assays revealed that HEB could bind E-boxes located within the pTa promoter sequence (5, 8), we also used oligonucleotides flanking the promoter region (Fig. 3A). As shown in Fig. 3B, anti-E47 and anti-HEB antibodies efficiently immunoprecipitated the pTa enhancer sequence as well as the pTa promoter sequence, whereas little or no background was observed with a control antibody. The specificity was further confirmed by the absence of amplification of an irrelevant promoter sequence, the HPRT promoter that is not regulated by E2A or HEB.

Fig. 4. The integrity of two E-boxes is critical for pTa enhancer activity. A, schematic representation of the pTa enhancer showing its different binding sites. B, point mutations of specific E-box sites impair pTa enhancer activity. The AD10.1 DN T-cell line was transfected with wild-type or mutant pTa enhancer constructs as shown. Results are expressed as luciferase activity relative to the minimal TATA promoter and represent the average ± S.D. of replicate determinations and are representative of (n) independent experiments. Luciferase reporter activities were normalized to that of an internal control (CMV-Luc). C, sequences of the different E-box sites within the pTa enhancer. Shown in bold are residues that match the core E47 (38) or HEB (39) consensus.
activities were calculated as in Fig. 4. Furthermore, this lower mRNA level is in
PCR (Fig. 5 C), and this decrease was confirmed by semiquantitative RT-PCR.

To test whether SCL-mediated repression depends on E-protein function, we transiently expressed reporter constructs containing wild-type or mutated E-box sites together with the SCL expression vector. We therefore performed EMSA using nuclear extracts prepared from AD10.1 cells and specific oligonucleotides (lanes 3–10 and 19), or E1, E2, and E4 wild-type oligonucleotides (lanes 13–16 and 20–27). Arrows point to the different complexes formed on the E3 probe.

On the opposite, SCL overexpression was still able to repress enhancer activity when E4 was mutated. Together, these results demonstrate that SCL-mediated repression of the pTa enhancer activity requires the integrity of the two E-box binding sites, E2 and E3, suggesting that SCL directly represses E-protein function.

**E2A and HEB Gene Products as Well as SCL-containing Complexes Bind E-box Elements of the pTa Enhancer in Vitro—**

E-box mutations or SCL-induced repression of the pTa enhancer activity indicate the crucial role of E-proteins in regulating pTa gene expression. Since E2A or HEB occupy the pTa enhancer in DN thymocytes, we addressed the question of whether E2A or HEB gene products directly bind E-boxes within the pTa enhancer. We therefore performed EMSA using nuclear extracts prepared from AD10.1 cells and specific oligonucleotide probes that cover the E2 or E3 binding sites. Fig. 6A illustrates the binding of a slowly migrating complex (C1) on both the E2 and E3 probes. Supershift assays using specific antibodies identified the E2A and HEB gene products as components of this C1 complex (Fig. 6B, lanes 2–4 and 7–9), while an isotype-matched control antibody did not affect the mobility of the E2 and E3 binding complex (Fig. 6B, lanes 5 and 10). In addition, when nuclear extracts from SCL-expressing AD10.1 cells were used, we observed a faster migrating complex (C2, Fig. 6A) containing E2A-SCL and HEB-SCL heterodimers as revealed by supershift assays using specific antibodies against E2A, HEB, and SCL (Fig. 6B, lanes 11–20). Together, these results demonstrate that E2A-HEB heterodimers bind in vitro the E2 and E3 sequences and suggest that SCL repression of E-protein activity is mediated by DNA binding of E2A-SCL or
HEB-SCL heterodimers to the same sites on the pTa enhancer sequence.

The specificity and the relative affinities of E2A-HEB and SCL-containing complexes for E-box binding sites were tested by competition assays using increasing amounts of unlabeled double-stranded oligonucleotides (Fig. 6C). Both E2 and E3 competitors efficiently displaced E2A-HEB heterodimers and SCL-containing complexes binding to DNA (lanes 3–6 and 13–16). In contrast, mutations within E3 (lanes 7–10) and E2 (data not shown) sequences disrupted DNA binding as these competitors failed to displace E-protein complex formation. Interestingly, higher concentrations of E1 or E4 competitors are required to displace E2A-HEB heterodimer formation on either E3 (lanes 20–27) or E2 probes (data not shown). The relative levels of binding of E2A-HEB or SCL-containing complexes to these E-box sequences were E3 > E2 > E1 > E4. Analysis of dissociation constants indicates a 70-fold difference in binding affinities between E3 and E4 sequences. Interestingly, both E3 and E2 sequences conform to E47 (38) and HEB (39) consensus sequences while E4 does not (Fig. 4C). These relative binding affinities are in agreement with our transactivation assays showing that E1 has a weaker contribution to enhancer activity as compared with E2 and E3, and that E4 is a negative regulator (Fig. 4).

SCL-containing Complexes Bind the pTa Enhancer in Situ—To test whether SCL associates with pTa regulatory sequences in situ, we performed chromatin immunoprecipitation assays (ChIP) on stable AD10.1 transfectants, expressing either the empty MSCV vector or the MSCV-SCL-encoding vector (Fig. 7). Using specific antibodies against E47 (E2A), HEB, and SCL, as well as isotype-matched control antibodies (anti-HA and rabbit IgG), we were able to show that in addition to anti-E47 and anti-HEB, the anti-human SCL antibody efficiently immunoprecipitated both the pTa enhancer, and promoter sequences when chromatin extracts from SCL-expressing AD10.1 cells were used. Combined, our observations indicate that the pTa enhancer and promoter are direct targets of E2A-HEB heterodimers in immature T cells. Moreover, SCL-induced repression of E2A-HEB transcriptional activity is not due to an Id-like titration of these factors, but rather is mediated by a DNA binding-dependent mechanism.

Relative Levels of E2A, HEB, and SCL Determines pTa Gene Expression in Immature Thymocytes—During thymocyte maturation, T-cell commitment at the DN2 stage is marked by the initiation of pTa gene expression that reaches maximal levels at the DN3 stage (Fig. 8). Interestingly, pTa starts to be expressed at the DN2 stage, coinciding with an elevation of both E2A and HEB mRNA (Fig. 8). This elevation, also observed at the protein level (40), is however not sufficient for optimal pTa gene expression since both E2A and HEB remain constant at the DN3 stage while pTa levels abruptly increase. We therefore investigated SCL expression in purified thymocyte populations together with E2A, HEB, and pTa mRNA levels using semiquantitative RT-PCR. As shown in Fig. 8, SCL and pTa exhibit opposite expression patterns, i.e. SCL is expressed at the DN1 and DN2 stages and is down regulated at the DN3 stage, coinciding exactly with an elevation in pTa gene expression. Taken together, our observations suggest that the relative dosage between E2A-HEB and SCL determines pTa gene expression in maturing thymocytes.

DISCUSSION

In the present study, we show that the bHLH factors E2A and HEB drive pTa enhancer activity in immature thymocytes through high affinity binding to two conserved E-boxes, E2 and E3. Moreover, SCL inhibits E2A-HEB activity through binding to the same regulatory elements.

Importance of E2A-HEB in Driving Stage-specific Activity of the pTa Enhancer—During thymocyte differentiation, E47 increases at the mRNA and protein levels, determined by flow cytometry analysis, from the DN1 to DN4 stage, and start to decrease as the cells progress to the DP stage (9, 40). HEB mRNA also follows the same pattern (9). Our Western blot analysis of CD3ε−/− thymocytes (consisting of more than 90% DN3 cells) and wild-type thymocytes (more than 80% DP cells) suggests that E47 and HEB protein levels decrease at the DP stage. This expression pattern is in agreement with the stage-specific expression of the pTa gene in immature T cells, which is maximal at the DN3 and DN4 stages and then decreases after β selection (41), and the finding that the pTa gene is a target of transcription regulation by E2A and HEB (7–9). More importantly, this correlation suggests that pTa levels are determined by E2A/HEB levels. In the present study, we provide direct evidence that E2A and HEB gene dosage determines pTa mRNA levels.

At the molecular level, the regulatory elements of the pTa gene have been identified and Reizis and Leder (4, 5) have shown that the pTa upstream enhancer is necessary and sufficient to drive stage and tissue-specific expression of the pTa gene in immature thymocytes. Indeed, this regulatory sequence is inactive in transient transfection assays in non-T cells or in mature T-cell lines that do not express the endogenous pTa gene (data not shown) (4). Furthermore, reporter...
transgenes driven by the pTα enhancer element are preferentially expressed in immature thymocytes, in a pattern closely resembling that of the endogenous pTα gene (5, 42). Analysis of the pTα enhancer sequence revealed potential binding sites for several transcription factors, including ZBP89, YY1, c-Myb, CSL, and E-proteins. Previous reports have shown that pTα enhancer activity depends on the integrity of binding sites for c-Myb and CSL, the latter a downstream effector of the Notch1 pathway (5, 6). However, mutation of either c-Myb or the CSL binding site did not abolish enhancer activity in transgenic reporter experiments, suggesting that these transcription factors, otherwise important for optimal and stage-specific pTα expression, are not absolutely required for enhancer activity.

Here we demonstrate that in addition to c-Myb and the CSL-NICD complex, E2A and HEB are critical determinants of pTα enhancer activity. Moreover, 80% of enhancer activity in a DN cell line is determined by two E-box binding sites, E2 and E3, which are conserved between mouse and man (5). Finally, we show that E2A and HEB associate with these E-boxes with high affinity in vitro and in vivo, while E4 that is not conserved (5) has a low affinity for these proteins. We therefore propose that E2A and HEB serve as nucleation factors for the assembly of a multimeric complex into an enhanceosome-like structure.

There is substantive controversy with regards to the contribution of E-boxes to pTα enhancer activity. Indeed, Reizis and Leder (5) and Takeuchi et al. (8) suggested that mutations of these E-boxes did not affect enhancer activity, while Petersson et al. (7) reported the opposite. This discrepancy may be attributable to different cellular contexts used for transient transactivation assays or alternatively, to the reporter vector itself used in these experiments. Indeed, our previous work indicated that the promoterless pXpII vector, as well as other luciferase reporter vectors (pGL3 basic), contains E-boxes within or near their multiple cloning sites (19). The use of the modified pXpII vector (19) where all E-boxes in the vicinity of the multiple cloning site were mutated has permitted us to reveal the functional importance of the conserved E-box sites on pTα enhancer activity.

Transient transactivation assays in heterologous cells indicate that E47 can activate the pTα enhancer and promoter, the latter containing a tandem E-box site previously shown to be required for full promoter activity (5, 8). However, a role for E2A in activating the pTα gene in primary thymocytes has not been assessed. Using two complementary approaches, we show by chromatin immunoprecipitation that E47 binds both pTα regulatory sequences in vitro. Furthermore, the activity of a single E2A allele is not sufficient for full pTα gene expression, indicating that HEB does not compensate for E2A haploinsufficiency, at least with regards to the transcriptional activity of the pTα locus. Hence, E2A−/− mice exhibit a 30% lower level of pTα mRNA in DN thymocytes, with variable penetrance, and a modest decrease in thymocyte numbers. Interestingly, loss of one E2A allele in the context of the HEB−/− genotype exacerbates T-cell differentiation defect caused by HEB haploinsufficiency, resulting in an increase in the DN and ISP populations (28). These results revealed the importance of E2A and HEB in T-cell development (28). We show here that both E2A and HEB bind the pTα regulatory sequences in chromatin and that full E2A and HEB loci activities are required for proper pTα expression. We therefore conclude that the combined dosage of E2A and HEB controls pre-TCR levels and, consequently, determines cell fate in the thymus.

SCL-E2A/HEB Complexes Occupy E-box Elements within the pTα Enhancer and Promoter—SCL is a tissue-specific bHLH transcription factor that heterodimerizes with E47 and HEB and binds DNA at consensus E-box sequences (43, 44). Furthermore, SCL shows an opposite expression profile to that of E proteins (9) and decreases from the DN1 to DN3 stage, exactly when pTα mRNA increases. We and others have previously shown that ectopic SCL expression in the thymus, in combination with its nuclear partners LMO1 or LMO2, down-regulates E-protein target genes such as the Cd4 and pTα genes (9, 18, 45). However, the molecular mechanism through which SCL represses E-protein function in thymocytes remains to be determined. SCL could either sequester E2A-HEB factors into complexes that do not bind DNA in the same way as Id proteins, HLH factors lacking a DNA binding domain. Alternatively, SCL-containing complexes could bind DNA on E-protein target sites and prevent E-protein transcriptional activity. Here we show that SCL associates with E2A or HEB and binds in vitro the pTα E-box sites, E2 and E3, with the same affinity as E2A-HEB heterodimers and that SCL occupies the pTα enhancer and promoter sequences in vivo, as revealed by chromatin immunoprecipitation assays. Together, these results suggest that the repression induced by SCL is mediated by a DNA binding-dependent mechanism rather than a sequestration effect. It remains to be determined whether SCL disrupts the formation of a transcription factor complex required for optimal enhancer activity or alternatively, whether SCL recruits new cofactors that repress pTα gene transcription. In B cell development, E2A recruits a chromatin remodeling complex at target DNA (46–48) and drives the transcription of immunoglobulin genes (29, 34, 49–52). It is possible that high levels of the SCL transgene are sufficient to form inactive SCL-E2A or SCL-HEB heterodimers, hampering the formation of this complex (the present study) (45). Alternatively, since SCL genetically interacts with LMO1 or LMO2 to inhibit T-cell development at the DN-DP transition point controlled by the pre-TCR, it is possible that SCL recruits new cofactors that actively repress pTα gene transcription. Further protein–protein interaction studies are warranted to distinguish between these two possibilities.

In summary, we unambiguously identify the pTα enhancer and promoter sequences as direct targets of E2A-HEB heterodimers in the thymus, as well as direct targets of repression by the SCL oncogene. Moreover, we show that pTα and SCL exhibit opposite expression patterns in DN1 to DN3 subsets, indicating that SCL may regulate E-protein activity during early thymocyte development. These results, together with previous reports, suggest that the pTα enhancer is regulated by a complex combination of transcription factors including c-Myb, CSL-NICD complex, and E-proteins. This unique combination may determine the tissue and stage-specific expression of an essential component of the pre-TCR, the pTα chain, required for αβ T-cell development.

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12686
Regulation of \(pT\alpha\) Gene Expression by a Dosage of E2A, HEB, and SCL
Mathieu Tremblay, Sabine Herblot, Éric Lécuyer and Trang Hoang

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