A conserved sequence block in the murine and human T cell receptor Jα loci interacts with developmentally regulated nucleoprotein complexes *in vitro* and associates with GATA-3 and Octamer-binding factors *in vivo*

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A highly conserved sequence block (CSB) located in the mouse and human T cell receptor (TCR) Jα loci is recognized by tissue-specific factors and up-regulates TCR α enhancer activity. In this study, the properties of CSB-interacting factors were further explored to decipher the function of this *cis*-acting element. Thymocytes corresponding to different developmental stages were found capable of forming differential CSB-nucleoprotein complexes. Pronounced changes in the CSB-complex-forming activity were observed during the transition from double-negative to double-positive thymocytes. Furthermore, we showed that transcription factors Oct-1, Oct-2 and GATA-3 interacted with CSB both *in vitro*, as evidenced by electrophoretic mobility shift assays, and *in vivo*, as demonstrated by chromatin immunoprecipitation assays in mouse thymus. Importantly, we also demonstrated that GATA-3 associated *in vivo* with TCR α enhancer, the activity of which is known to be required in regulating chromatin accessibility to the V(D)J recombinase. Thus, CSB may temporally regulate local chromatin structure and help to spread TCR α enhancer activity over the entire 70-kb Jα locus by forming developmentally regulated CSB-nucleoprotein complexes and by interacting with other *cis*-regulatory element-nucleoprotein complexes present within the TCR α/δ locus.

Key words: V(D)J recombination / T cell development / Transcription factor / Chromatin accessibility / Conserved sequence block

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1 Introduction

In the vertebrate specific recognition of foreign antigens is accomplished by two major cell lineages: B cells and T cells. B cells express immunoglobulin (Ig) to recognize soluble antigens, whereas T cells express cell surface T cell receptor (TCR) molecules to recognize antigenic peptides bound to the surface of antigen-presenting cells (reviewed in [1]). Based on expression of the heterodimeric $\alpha\beta$ or $\gamma\delta$ TCR, T cells can be divided into $\alpha\beta$ T cells, the major subset of peripheral blood T cells, and $\gamma\delta$ T cells, a minor population of T cells [1, 2].

The variable regions of Ig and TCR genes are structurally related. Each is composed of variable (V), diversity (D) and joining (J) gene segments in the case of Ig heavy chain and TCR β- and δ-chain genes, or of V and J gene segments in the case of Ig light chain and TCR α and γ chain genes [1, 2]. During lymphocyte ontogeny a site-specific DNA recombination process, referred to as V(D)J recombination, assembles antigen variable region genes. Despite the fact that a common enzymatic complex, referred to as the V(D)J recombinase, is responsible for both Ig and TCR variable region gene rearrangements [3, 4], the assembly and expression of these genes is tightly regulated and lineage specific. Accordingly, complete rearrangement and expression of TCR gene is limited to T cells and complete Ig rearrangement limited to B cells. Moreover, during T cell ontogeny the rearrangement and expression of the TCR genes are both developmental stage and lineage specific. For instance, rearrangements at the TCR β, γ and δ locus occur several days before rearrangement at the α locus. Moreover, TCR α chain gene expression is limited to $\alpha\beta$ T cells whereas δ chain gene expression is limited to $\gamma\delta$ T cells [5, 6]. This differential regulation of recombination may be explained by the differential accessibility of TCR or Ig genes to the

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Abbreviations: CSB: Conserved sequence block **EMSA:** Electrophoretic mobility shift assay

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V(D)J recombinase during lymphocyte development [7]. The exact mechanism by which the accessibility of variable region gene segments is controlled during development is not known. However, it probably involves interactions between *trans*-acting factors, whose expression and/or activity is under tight regulation, and *cis*-acting elements located in TCR and Ig gene loci [5–8].

CSB, a block of 125 nucleotide in length with no detectable transcript, may contribute to the regulation of TCR α gene rearrangement/expression. It is located in the TCR 3' Jα region, 5' to the Cα gene, and its sequence is highly conserved between human and mouse [9, 10]. Within this CSB block, there are only six differences over 125 nucleotide positions (95 % similar). The similarity is much higher than the approximately 71 % overall sequence similarity found between human and mice in the noncoding regions within the TCR Cα/Cδ locus [11]. This similarity is also higher than that obtained when TCR α enhancer region sequences from human and mouse are compared [10–14]. By transfection assays, we demonstrated that CSB increased TCR α enhancer activity by twofold, while no enhancer activity was detected when CSB alone was assayed. Moreover, electrophoretic mobility shift assay (EMSA) results indicated that an intricate network of interactions between CSB and *trans*-factors, including lymphoid- as well as T lineage-specific nuclear factors, was involved in forming CSB-bound complexes *in vitro* [8]. Based on these results, we, therefore, hypothesized that CSB may be involved in shaping chromatin structure in the TCR α/δ locus so that the accessibility of V(D)J recombinase to this region could be regulated [8].

Following V to DδJδ rearrangements, TCR α chain gene rearrangements in the early developing thymus fell largely to the 5' side of Jα locus. In adult mice the pattern changes and all Jα gene segments, including the 3' Jα are used [15, 16]. Furthermore, the rearrangement appeaers to occur in blocks and discrete regions of the Jα locus are open at a given time. It was also shown that at the α locus, both alleles rearrange simultaneously usually to the same region along the Jα locus [17, 18]. How the progressive rearrangement to the Jα gene segments is regulated remains an open question. Since CSB displays such a complex pattern of interactions with nuclear factors, it is possible that it plays a role in controlling the progressive rearrangement along the Jα cluster.

In this study we analyzed the expression/regulation of CSB-interacting factors. These results are discussed with regards to the role CSB plays in regulating the accessibility to the V(D)J recombinase at the chromatin level of the TCR Jα locus during thymocyte development.

2 Results

2.1 Fetal thymus, liver and brain contained factors which formed differential CSB-nucleoprotein complexes *in vitro*

To address the developmental regulation and tissue specificity of CSB-interacting factors, nuclear extracts from mouse thymus as well as liver and brain at various embryonic stages were analyzed for the presence of factors capable of forming CSB-bound complexes. Fetal liver was analyzed because it is the organ where hematopoietic/lymphoid progenitor cells reside at the embryonic stage.

As shown in Fig. 1 A, changes in the patterns of CSB-nucleoprotein complexes were observed among thymi at different embryonic stages. For example, the slowest-migrating complex (FT15-1) formed at embryonic day 14 (E14) and E15 became barely detectable in extracts from E16 through E18 as well as at the adult stage. In contrast, from E16 through E18 a complex denoted FT2 with mobility similar to the adult thymic T2 complex became detectable. Moreover, different patterns of CSB-complexes were observed between E14 and E15 and the FT2-forming activity is always lower than the adult T2-forming activity. Thus, differential CSB-complex-forming activities exist in the developing thymus at different embryonic stages, especially from E14 through E16, and between the fetal and adult stage.

Interestingly, differential CSB-complex-forming activities also exist in fetal liver extracts as evidenced by the appearance of a complex, denoted FL15-2, in extracts from E15 through E18, and the absence of the slowest-migrating complex, denoted FL16-1, in E14 and E15. The adult livers however contained barely detectable CSB-complex-forming activity as observed previously ([8], and not shown). As for the fetal brain, extracts of E14 and E16 unexpectedly displayed CSB-binding activity, in contrast to the adult brain that showed hardly detectable CSB-complex-forming activitiy as described previously [8] (Fig. 2 A and not shown). Together, these results clearly suggested that developmentally regulated factors are involved in forming these tissue-specific CSB-nucleoprotein complexes *in vitro*.

2.2 Mapping the sites on CSB that interact with nuclear factors in thymus, liver and brain extracts

Our results shown in Fig. 1 A indicated that tissue- and/or stage-specific factors are likely involved in interacting with CSB. To map the subsite(s) on CSB which may be

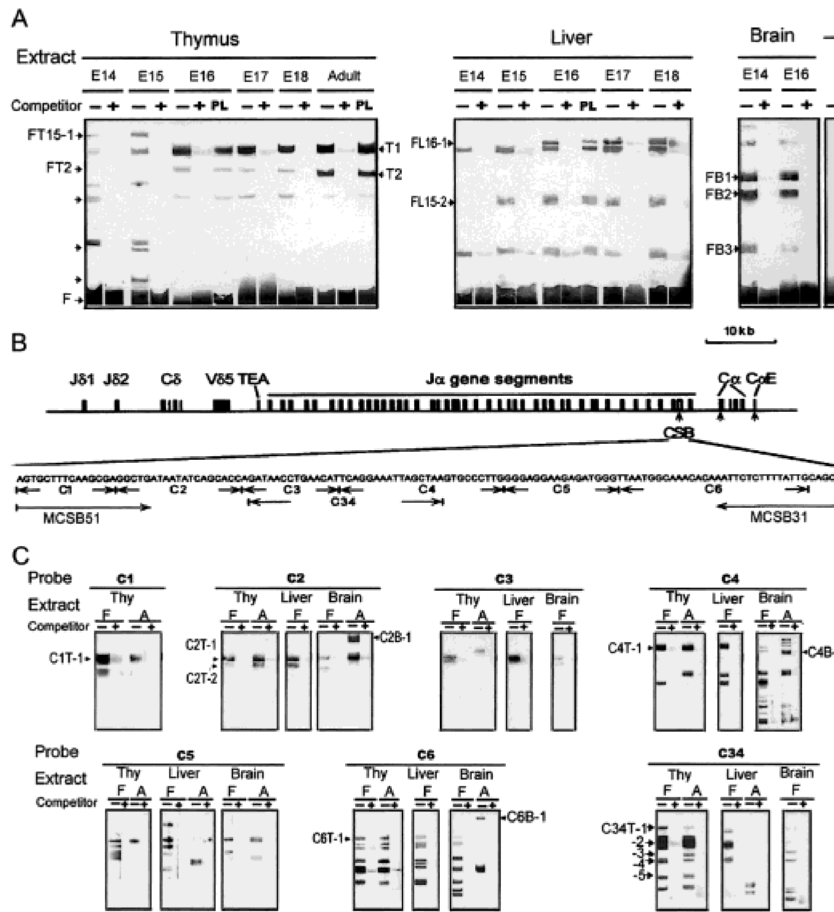

Fig. 1. Stage- and/or tissue-specific CSB-/CSB subsite-bound complex formation. (A) ^{32}P-labeled CSB was incubated with nuclear extracts of fetal thymi, livers or brains at embryonic days from E14 to E18 or from adult mice to perform EMSA. Adult liver and brain contained undetectable binding activity (not shown). Assays were done in the absence (–) or presence (+) of 50-fold excess of unlabeled CSB as specific competitor. Unlabeled pUC19 polylinker sequences (PL) were used as nonspecific competitors. In all cases the bindings competed by the specific competitors were not competed by PL. Some PL competition results are shown as examples. Arrows marked differentially formed complexes. FB1 and FB2, the major fetal brain complexes detected at E14 and E16 whereas FB3 detected preferentially at E14; F, free probe. (B) Diagram of the mouse TCR α/δ locus. The vertical arrows locate segments analyzed by chromatin immunoprecipitation shown in Fig. 4. The arrowhead-lines below the CSB sequence represent subsites analyzed by EMSA shown in Fig. 1 C. (C) CSB subsite-complex formation. Annealed, labeled complementary oligonucleotides C1 to C6 and C34 were used as probes to detect subsite-binding activity in extracts of various tissues at E16 (denoted F) and at the adult stage (denoted A). Only extracts exhibiting complex-forming activity are shown. + and –, with or without specific competitor, respectively. C1T-1, C2T-1, -2, C4T-1, C6T-1 and C34T-1 to -5 are detected only in the thymus, not in liver or brain, of adult mouse. C2B-1, C4B-1 and C6B-1 were detected in brain only.

important for interacting with these factors, extracts of the thymus, liver and brain at E16 and at the adult stage were subjected to EMSA with annealed double-stranded oligonucleotides representing various subsites of CSB (C1 to C6 and C34, Fig. 1 B and ref. [8]). The differential fetal C1-, C4-, C5- or C34-complexes formed between different tissues (Fig. 1 C, lanes F) suggest that these sequences are probably involved in forming tissue-specific CSB-complexes at E16. The differential patterns of subsite-complexes observed between different adult tissues (Fig. 1 C, lanes A) indicate the entire CSB is likely responsible for forming tissue-specific CSB-complexes at the adult stage observed in Fig. 1 A.

Along the same line, the C1, C3, C4, C5 and C34 subsites appear important for interacting with developmentally regulated factors during thymocyte development, whereas in the liver and brain tissues the C2 to C6 and C34 subsites were shown to interact with factors differentially expressed between the fetal and adult stages

(Fig. 1 C). Thus, almost the entire CSB sequence appears important for the developmentally regulated CSB-complex-formation as observed in the thymus, liver and brain between E16 and the adult stage (Fig. 1 A).

2.3 Correlation between CSB-complex-forming activity and rearrangement status of the TCR α locus in developing thymocytes

The differential CSB-binding activities observed in developing thymi from day E14 to E16 suggested the existence of a correlation between the onset of TCR α rearrangements and the formation of certain CSB-nucleoprotein complexes. Since TCR δ rearrangement occurs in CD4$^-$CD8$^-$ double-negative (DN) and TCR α rearrangement in CD4$^+$CD8$^+$ double-positive (DP) thymocytes, we studied CSB-factor interactions in these thymocyte subsets to directly assess the possible relationship between changes in CSB-complex-forming activity and the onset of TCR α rearrangement during thymocyte development.

Anti-CD4 antibody (Ab)-linked magnetic beads were added to thymocytes from four weeks old mice to isolate both CD4$^+$ and DP thymocytes (DP+CD4 in Fig. 2). Anti-CD8 Ab-linked magnetic beads were then added to the unbound fraction to separate CD8$^+$ cells (CD8 in Fig. 2) from DN thymocytes. Extracts from these isolated populations were assessed for CSB-binding activity by EMSA (Fig. 2 A). Because the DP+CD4 fraction contains less than 10 % CD4$^+$ cells and the rearrangement status of Vα-Jα is the same for CD4$^+$ and CD8$^+$, the CSB-nucleoprotein complexes detected in the DP+CD4 fraction and different from that in the CD8 fraction were considered to be essentially contributed by the DP thymocytes. We observed very weak CSB-complex-forming activity in the DN thymocytes. DN1 to DN5 complexes were detected only after long exposure. Interestingly, a pronounced activity in forming a major CSB-bound complex (DP3) and three minor CSB-bound complexes (DP1, 2 and 4) marked the transition from the DN to the DP+CD4 (or DP as explained above) population. CD8 cells contained activity resulting in one major CSB-bound complex (CD8-2) and three less apparent complexes (CD8-1, 3 and 4). Thus the CSB-complex-forming activity is developmentally regulated at the DN to DP transition, a feature reminiscent of the regulation of TCR α enhancer activity [19, 20].

To document further the occurrence of a developmentally- and/or lineage-regulated CSB-binding factor(s), gel overlay assays were performed. Nuclear extracts of various cell lines or of tissues at various developmental stages were separated by SDS-PAGE, followed by probing the gel with radiolabeled CSB. As expected, many proteins bound radiolabeled CSB (Fig. 2 B, left panel). As a control, *E. coli* extracts were also used and no CSB-binding factors were detected under the assay condition (not shown). Importantly, CSB-binding factors appeared to include ubiquitously expressed molecules, as judged by the molecular mass, and molecules with restricted expression patterns. Moreover, in adult thymocytes the transition from DN to DP+CD4 (or DP) is apparently marked by the appearance of several CSB-binding factors with molecular mass around/higher than 106 kDa. These ~106-kDa CSB-binding factors were also prefer-

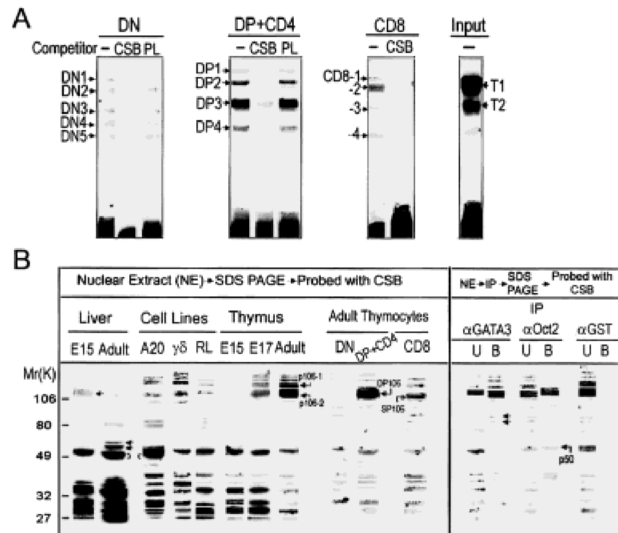

Fig. 2. (A) Regulated CSB-complex-forming activity in developing thymocytes. EMSA were performed with nuclear extracts of CD4$^-$CD8$^-$ (DN), DP+CD4, CD8$^+$, or total adult thymocytes (input), and ^{32}P-labeled CSB was used as probe in the absence (–) or presence of indicated competitors. Arrows marked the complexes competed by unlabeled CSB, not by PL. (B) Gel overlay analysis of factors with CSB-binding activity. In the left panel, nuclear extracts from various cell lines or tissues at E15, E17 or adult stage were separated by SDS-PAGE. The gel was renatured and probed with overlaid ^{32}P-labeled CSB. RL, a T leukemia; A20, a B lymphoma; γδ, a TCR γδ-expressing hybridoma. Arrows in the liver extracts mark differentially expressed CSB-binding factors between E15 and the adult stage. The brackets mark CSB-binding fractions preferentially detected in adult liver and the B cell line. p106-1/-2, DP106 and SP106, predominant CSB-binding fractions in the adult thymus, DP+CD4 and CD8$^+$ thymocytes, respectively. In the right panel the adult thymocyte nucleoproteins immunoprecipitated (IP) by anti-GATA3 (αGATA3), anti-Oct2 (αOct2) or anti-GST (αGST) Ab were separated by SDS-PAGE in lanes denoted B whereas the Ab-unbound components were separated in lanes denoted U. ^{32}P-labeled CSB was overlaid on the gel to detect CSB-binding factors. No CSB-binding factor was detected in the αGST-bound fraction.

entially expressed in developing fetal thymocytes at E17 as compared to that at E15 (Fig. 2 B). Therefore, in addition to lineage specificity, there are changes in the expression of CSB-binding factors in thymocytes with active Vα-Jα rearrangement versus thymocytes at the stage before the onset of Vα-Jα rearrangement.

2.4 Transcription factors Oct-1, Oct-2 and GATA-3 interact with CSB *in vitro*

The CSB subsites C2 and C3 contain binding motifs for transcription factor(s) of the GATA family (Fig. 1 B). Our subsite mapping results clearly indicated that there were factors binding to C2 and C3 in the thymus, brain and fetal liver (Fig. 1 C), suggesting that members of the GATA family may be involved in forming the complexes. The subsites C4 and C6 do not contain any apparent binding motif. However, the oligonucleotides Oct1x and Oct2x, containing one- or two-copy of canonical octamer sequence (ATGCAAAT) respectively, could unexpectedly compete for the C4- or C6-complexes formation in the adult thymic extracts. The C1, C2, C3, C5 or oligonucleotide with SP1-binding site had no effect on C4- or C6-complexes formation. Reciprocally, C4 and C6 could also compete for the Octamer-complexes formation, although to a much less extent (Fig. 3 A, [8] and not shown). These results suggest that certain factors may be involved in forming both the Octamer- and C4-/C6-complexes, but with different kinetics. We thus included antibodies in EMSAs to determine if GATA-3, a T cell-specific GATA family member, and Octamer-binding factors were involved in forming the thymic CSB-complexes *in vitro*. Both anti-Oct1 and anti-Oct2 Ab exerted an inhibitory effect on the thymic CSB-complexes formation: anti-Oct1 Ab exhibited a stronger effect on T2 whereas anti-Oct2 Ab displayed a stronger inhibitory effect on T1 formation. Because anti-Oct2 Ab also caused supershift/inhibition on the slowest migrating thymic Oct2x-bound complex and, usually, the slowest migrating Oct1x-bound complex is occupied by Oct-1 (Fig. 3 B, [21] and not shown), these results suggest that both Oct-1 and Oct-2 are involved in forming a DNA-protein complex *in vitro* if the DNA contains two copies of Octamer site, such as Oct2x, or two sites which could be competed by the Octamer sequences, such as C4 and C6 on CSB. Addition of anti-GATA3 Ab affected the mobility/formation of CSB-complexes whereas the control anti-GST Ab did not (Fig. 3 B). When the same anti-GATA3 Ab, recognizing chicken GATA-3 as well, was used in EMSA with chicken thymic nuclear extracts the CSB-complexes were supershifted (unpublished results). Therefore, the partial inhibition observed in mouse thymic extract suggests that the Ab binds at a site that reduces the ability of GATA-3 to engage in the CSB-protein complex interactions, possibly by affecting the GATA-3 to interact with other factor(s) and such factor(s) may be absent in chickens. Together, these results suggested that transcription factors Oct-1, Oct-2 and GATA-3 were differentially involved in the process of forming CSB-nucleoprotein complexes *in vitro*.

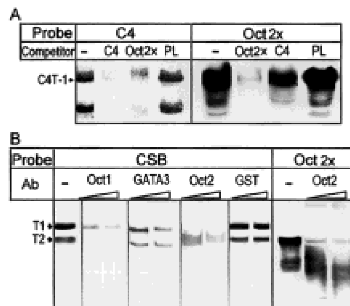

Fig. 3. (A) Octamer-binding factors interact with C4 on CSB. EMSA was performed with adult thymic nuclear extracts using ^{32}P-labeled C4 or oligonucleotide containing two copies of Octamer sequences (Oct2x) as probes in the absence (–) or presence of indicated competitors. (B) Effect of anti-Oct-1, -Oct-2, or -GATA-3 antibody on CSB-nucleoprotein complex formation. Mouse thymic nuclear extracts were incubated with ^{32}P-labeled CSB or Oct2x as probes to perform EMSAs. Assays were done in the absence (–) or presence of increasing amount of indicated antibody (Ab).

We analyzed next the distribution of CSB-binding factors in the Ab-bound fraction versus the unbound fraction by gel overlay assays to provide further support for the association of GATA-3/Oct-2 with CSB in normal thymocytes. Fractions pulled-down by anti-GATA3 or anti-Oct2 Ab were shown to contain CSB-binding factors as evidenced by binding to ^{32}P-labeled CSB overlaid on the gel (Fig. 2 B, right panel, lanes B). These factors include p106-1/-2 which were scarcely expressed, if any, in the DN thymocytes and appear to be pulled-down more efficiently by anti-GATA3 Ab than anti-Oct2 Ab. Moreover, the anti-Oct2 Ab precipitated a CSB-binding fraction p50 showing molecular mass comparable to that deduced from Oct-2 cDNA sequence and that of *in vitro* translated Oct-2 (unpublished results), whereas anti-GATA3 Ab precipitated ~80-kDa CSB-binding fractions (Fig. 2 B, marked by arrows in αGATA3, lane B) more efficiently. The control anti-GST Ab did not precipitate any factors with detectable CSB-binding activity, confirming the specificity of this analysis. Although we can not presently pinpoint directly the band(s) with CSB-binding activity as GATA-3 or Oct-2 in the thymic extracts, our results suggested GATA-3 and Oct-2 (or molecules which could cross-react with the respective Ab) involvement in interacting with CSB *in vitro*.

2.5 *In vivo* association of Oct-1 and Oct-2 with CSB and of GATA-3 with both CSB and the TCR α enhancer

Dedon et al. [22] used formaldehyde to covalently link proteins in close proximity to DNA to study protein-DNA interactions *in vivo*. Recently a similar approach was used to show that transcription factor CREB is associated with the MHC class II X2 box *in vivo* [23]. We adopted this technique to determine if the observed *in vitro* interactions between CSB and Oct-1/-2 and GATA-3 do reflect the situation *in vivo*. Chromatin was prepared from adult mice thymocytes, cross-linked with formaldehyde and immunoprecipitations were performed with the relevant Ab. After collecting the complexes with magnetic beads, cross-links were reversed. DNA was purified and PCR was performed with primers for CSB, for the downstream region of exon 1 of the TCR Cα segment, and for the further downstream region encompassing Tα1, Tα2, Tα3 and Tα4 elements of the TCR α enhancer, as diagramed in Fig. 1 B. A PCR product for CSB was obtained when Ab to GATA-3, Oct-1 or Oct-2 were used in the immunoprecipitation (Fig. 4). However, these Ab appeared to immunoprecipitate CSB with different efficiency since semi-quantitative PCR results indicated that anti-Oct2 Ab precipitated the smallest amount of CSB (not shown). In contrast, none of the Ab tested immunoprecipitated DNA specific for exon 1 of the TCR Cα segment located about 4.3 kb downstream of CSB, indicating the specificity of the reaction. Moreover, a PCR product for the TCR α enhancer located approximately 7.5 kb downstream of CSB was obtained when anti-GATA3 Ab was used in the immunoprecipitation (Fig. 4). These results indicate that GATA-3, Oct-1 and Oct-2 occupy the CSB *in vivo* and demonstrate that *in vivo* the TCR α enhancer interacts with GATA-3, but not with Oct-1, nor Oct-2.

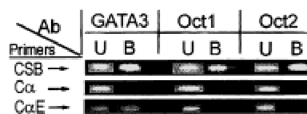

Fig. 4. In vivo association of GATA-3, Oct-1 and Oct-2 with CSB and of GATA-3 with the TCR α enhancer. Chromatin immunoprecipitation assays on thymocytes of 4-week-old mice were used to determine if these three factors were associated with CSB *in vivo*. Chromatin was cross-linked, isolated, sheared and immunoprecipitated with the indicated antibodies. Both the immunoprecipitated DNA (denoted B) and DNA not precipitated by the indicated antibody (denoted U) were purified and used as the respective template in PCR with primers for CSB (CSB), the exon 1 of the TCR α gene constant region (Cα), or the TCR α enhancer (CαE).

3 Discussion

Comparison of the complete nucleotide sequence of a 97.5-kb region spanning the TCR α/δ loci in both mouse and human revealed an extraordinary degree of conservation [9–11]. This contrasts with comparisons made between other homologous large sequenced regions. While most of the known structural (*e. g.* Jα gene segments) and regulatory (*e. g.* TCR α enhancer) elements in the TCR α/δ locus were shown to be conserved, additional blocks of highly conserved sequence were unexpectedly found in the non-coding regions. One of these blocks, which we named conserved sequence block (CSB), showed a much higher interspecies homology (95 % similar) than the known regulatory elements found in the TCR α/δ locus, and may constitute an unknown regulatory element [9]. We subsequently demonstrated that an intricate network of CSB-protein/protein-protein interactions were involved in forming CSB-nucleoprotein complexes *in vitro* [8]. The observation that CSB could enhance TCR α enhancer activity and its ability to differentially bind nuclear factors suggested that CSB may function in regulating locus accessibility for TCR α/δ gene rearrangements/expression [8].

In this study we showed that CSB-complex-forming activity was regulated in a developmental stage-/tissue-specific manner. The detection of CSB-complexes in the fetal liver and brain, but not in the adult liver or brain, suggested that progenitor cells of hematopoietic/lymphopoietic lineage in the fetal liver and cells in early developing brain contain CSB-interacting factors and/or CSB-complex-forming regulators. The presence of differential CSB-complexes in the thymus at different developmental stages further suggested that in a cell CSB might interact with a nucleoprotein complex, the components of which vary as the cell differentiates. Using gel overlay assays, the CSB-binding factors were visualized and indicated that developmentally regulated as well as cell type-specific factors may play a role in directing CSB-nucleoprotein complex formation. Moreover, the profiles of both the CSB-complex-forming activity and the expression of CSB-binding factors were markedly different in DN thymocytes, in which TCRδ rearrangement occurs, as compared with that in DP, in which TCRα rearrangement is actively in progress, or even with that in SP in which the process of TCRα rearrangements is shut down, thus echoing the changing patterns of chromatin accessibility of α/δ locus to RAG proteins during thymocyte maturation [19, 20].

By combining Ab in EMSA, we then showed that GATA-3, Oct-1 and Oct-2 were differentially involved in the process of forming thymic CSB-nucleoprotein complexes *in vitro*. To eliminate the possibility that results obtained *in*

vitro may not reflect what occur *in vivo*, we employed a chromatin immunoprecipitation assay to extend our data *in vivo*. This technique provides strong *in vivo* evidence that a factor is indeed bound at the site as previously characterized *in vitro* [22, 23]. Consistent with *in vitro* data, GATA-3, Oct-1 and Oct-2 were found to associate with CSB *in vivo*. The interpretation of the results was supported by the observation that only GATA-3 was found to bind at the TCR α enhancer *in vivo*, and none of the tested Ab immunoprecipitated DNA in exon 1 of the TCR Cα segment indicating the specificity of the reaction. Together, these results have allowed us to refine the role of CSB in the developmentally regulated and lineage-specific process of TCRα rearrangement/expression as follows.

The *in vivo* association of GATA-3 with CSB and TCR α enhancer may provide a functional link between these two *cis*-regulatory elements. TCR α enhancer knockout mice showed that the α enhancer activity is critical at the DP stage for accessibility over the 70-kb Jα region [19, 20]. Recently, it was demonstrated that TCR α and δ enhancers are long-range developmental regulators of histone H3 acetylation, which was in turn proposed to play a primary role in establishing α/δ locus accessibility for V(D)J recombination [24]. However, the precise mechanism by which the enhancer could regulate the long-range changes in histone acetylation is not known. We showed previously that CSB could augment TCR α enhancer activity by transient transfection assays [8] and here we demonstrated that GATA-3 is associated with both CSB and TCR α enhancer *in vivo*. Therefore, we propose that GATA-3 may serve as one of the bridging molecules between TCR α enhancer and CSB/other regulatory elements in the TCR α locus (described below), with the purpose of relaying the α enhancer function over the entire 70-kb Jα region and contributing to the shaping of the local chromatin structure flanking CSB for accessibility to the V(D)J recombinase.

GATA-3, a member of the GATA family, was identified as a T lineage-specific transcription factor binding to TCR α, β and δ enhancers *in vitro* [25–27], and is required for development of the T cell lineage [28]. Recently, it was demonstrated that ectopic expression of GATA-3 in a Th1 clone induced chromatin remodeling of the IL-4 locus [29]. Notably, GATA-1 was associated with/acetylated *in vivo* by CREB-binding protein at, at least, two lysine-rich motifs highly conserved among members of the GATA family, including GATA-3 [30]. Therefore, it will be interesting to document if GATA-3 is associated with protein(s) with intrinsic and/or associated histone acetyltransferase activity to provide molecular evidence for the observed TCR α enhancer-modulated acetylation [24]. By Western blot analyses we noted that anti-GATA3 Ab reacted to molecules around/above 80 kDa (unpublished results), i. e. the same size as molecules precipitated by anti-GATA3 Ab and showing CSB-binding activity by gel overlay assays (Fig. 2 B). The value is higher than the mass deduced from the GATA-3 cDNA sequence, suggesting that the detected GATA-3 may be posttranslationally modified to various forms in the thymic environment.

The POU family members Oct-1/Oct-2 were shown to interact specifically with a B cell-specific coactivator Bob 1 and this interaction was the main determinant for B cell-specific activation of Ig promoters [31]. It was also shown that expression of the Oct-2 gene is involved in T cell activation and thymocyte development [32–34]. In this study we detected differential interactions of Oct-1 and Oct-2 with CSB both *in vivo* and *in vitro* in the thymus. Furthermore, the results of gel overlay assays on the fraction precipitated by anti-Oct2 Ab indicated that other factors, such as p106-1/-2 which were scarcely expressed, if any, in DN thymocytes, were associated with Oct-2 *in vitro*. Therefore, we propose that as shown for Ig genes, Oct-1/Oct-2 may interact with developmentally regulated and/or T lineage-specific factors that exert their function in regulating TCR α gene rearrangement/expression in thymocytes.

TCR α enhancer activity was also required for transcription from the T early alpha (TEA) promoter [35, 36]. TEA is located immediately 5' to the most upstream Jα gene segment (Jα 61) and the modified Jα usage observed in TEA knockout mice directly linked TEA to the control of recombinational accessibility in the 5' Jα cluster. TEA was thus suggested to act as a "rearrangement-focusing" element targeting the primary waves of Vα-Jα rearrangement to the most 5' Jα in an ongoing TCR Jα rearrangement model [37]. In fact, we observed a few more "conserved sequence blocks" within the TCR Cδ and Cα interval [9–11]. The sequence of individual blocks is highly conserved between mouse and human (around or higher than 90 % similar) but different from each other. Among them, the block most proximal to the Cδ is located in the TEA region, immediately 5' to the Jα61. Interestingly, the block immediately 3' to the TEA is located at the junction between two Jα clusters, one being underrepresented and the other being significantly represented in TEA knockout mice. Further downstream, another conserved block is located in the region 5' to the Jα cluster in which the Jα were overtly represented in TEA knockout mice. Our preliminary studies indicated that differentially expressed nuclear factors were involved in interacting with these blocks. Therefore, these conserved sequence blocks may function in a similar way as CSB and regulate the regional Jα accessibility to V(D)J recombinase.

Consistent with the proposed CSB function are recent results obtained using CSB knockout mice [38]. Following deletion or replacement with a neomycin resistance gene of a DNA fragment encompassing CSB, a very local effect was noted on the expression of the $J\alpha$ flanking CSB. The most significant effect was an approximately twofold reduction in expression of $J\alpha4$ [38], interestingly echoing the twofold increase in TCR α enhancer activity imposed by the CSB on the reporter gene expression [8]. Therefore, by recruiting developmental stage- and/or tissue-specifically expressed regulatory factors (such as p106, GATA-3) as well as ubiquitous factors, and cooperating with other *cis*-regulatory elements (most likely the TCR α enhancer, TEA and/or other "conserved sequence blocks" described above), CSB may regulate the chromatin accessibility in the region flanking CSB so that $V\alpha$ to $J\alpha$ rearrangement would proceed in temporal oder along the $J\alpha$ locus. Thus, recombination to the most 3' $J\alpha$ flanking the CSB would occur "late" during T cell development as observed previously [15, 16]. In other words, we propose that CSB may function similarly to the TEA as a "rearrangement-focusing" element [37].

In conclusion, our demonstration that CSB interacts with developmentally regulated factors in thymocytes and that GATA-3 associates *in vivo* with both the CSB and the TCR α enhancer suggests a mechanism explaining how recombination along the TCR $J\alpha$ locus is temporally regulated and how the TCR α enhancer may modulate the chromatin structure over the entire 70-kb $J\alpha$ locus during T cell development [19, 20, 24]. Further characterization of the differentially expressed CSB-interacting factors would provide a detailed molecular basis for understanding how the chromatin accessibility to the recombinase in TCR α/δ locus is regulated.

4 Materials and methods

4.1 Nuclear extract preparations and DNA binding substrates and EMSA

Nuclear extracts from mouse tissues and cell lines were prepared by using slight modifications of the procedure by Schreiber et al. [39] as described in ref. [8].

The annealed complementary oligonucleotides and CSB substrate, derived by PCR with primers MCB51 and MCB31, were end-labeled with [γ-^{32}P] ATP by T4 polynucleotide kinase. The labeled DNA substrate was then incubated with nuclear extract, in the presence of sonicated salmon sperm DNA [39] as described in [8]. Where indicated, the nonlabeled competitor DNA was added before the protein extracts. Complexes were resolved in nondenaturing 6 % polyacrylamide gel, dried and exposed to X-ray film [8]. When antibody was included, the extracts were incubated with specific antibodies against Oct-1, Oct-2, or GATA-3 (Santa Cruz Biotechnology, Inc.) or with control anti-GST antibody, a gift from Dr. W.-J. Syu, at 4 °C for 1 h before the addition of the radiolabeled probe.

4.2 Gel overlay analysis

Gel overlay analysis was carried out essentially as described in [40]. Briefly, following SDS-PAGE the gel was placed in acetone to remove SDS. The gel was then denatured, renatured, followed by addition of ^{32}P-labeled CSB probe and binding was carried out at 4 °C in binding buffer containing sonicated salmon sperm DNA and BSA [40]. Following binding, the gel was washed with four to six changes binding buffer over 24–48 h at 4 °C. The gel was then dried and exposed to film.

4.3 Chromatin immunoprecipitation

Chromatin immunoprecipitation assays were performed essentially as described in [23]. Immunoprecipitations were performed at 4 °C on the cross-linked chromatin samples with 5 μg of primary antibody (anti-Oct1, anti-Oct2 and anti-GATA3 from Santa Cruz Biotechnology, Inc.). Immune complexes were harvested with secondary antibodies linked to magnetic beads (Dynal, Lake Success, NY), washed, disrupted with elution buffer and covalent links reversed as described [23]. DNA was ethanol precipitated and purified by proteinase K digest, phenol/chloroform extraction, and ethanol precipitated. DNA pellets were dissolved in water and used as template for PCRs. The primer sets used in the PCR are as followings: $mC\alpha$ (5'-ACCCTCTGCCTGTTCAC-3') and $amC\alpha$ (5'-AAGCTTGTCTGGTTGCTCC-3') for a 152-bp fragment located in the first exon of TCR $C\alpha$ segment, $mC\alpha E$ (5'-CCCACTTCCATGACGTC-3') and $amC\alpha E$ (5'-CTTTTCTGCACCTGTGGTTG-3') for a 190-bp fragment encompassed in the TCR α enhancer region, MCB51 and MCB31 for the 125-bp CSB element.

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