

Brief Definitive Report

Two Waves of Recombinase Gene Expression in Developing Thymocytes

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Summary

During T cell development in the thymus, T cell receptor (TCR) α, β, γ, and δ genes are rearranged and expressed. TCR rearrangement strictly depends upon the coordinate activity of two recombinase activating genes, Rag-1 and Rag-2. In this study we have followed the expression of these genes at different stages of intrathymic development. The results indicate that there are two periods of high Rag-1 and Rag-2 mRNA expression. The first wave peaks early at the CD25⁺CD4⁻CD8⁻CD3⁻ stage of development and coincides with the initial appearance of transcripts derived from fully rearranged TCR β, γ, and δ genes, whereas the second wave occurs later at the CD4⁺CD8⁺ stage coincident with full-length TCR α mRNA expression. Active downregulation of Rag-1 and Rag-2 mRNA expression appears to occur in vivo between the two peaks of recombinase activity. This phenomenon can be mimicked in vitro in response to artificial stimuli such as phorbol myristate acetate and calcium ionophore. Collectively our data suggest that recombinase expression is actively regulated during early thymus development independently of cell surface expression of a mature heterodimeric TCR protein complex.

T lymphocytes recognize foreign antigen via their clonally distributed heterodimeric α/β or γ/δ TCRs. In the thymus, developing T cells differentiate through several phenotypically distinct stages, during which time the genes coding for the α, β, γ, and δ chains of the TCR rearrange and are eventually expressed in conjunction with the CD3 complex on the cell surface. The genomic assembly of these TCR genes from the component V, (D), and J segments during development of T cells is mediated by a site-specific recombination event (reviewed in 1 and 2) and is achieved by either inversion or deletion of the intervening DNA segments, ultimately producing transcribable genes. The joint expression of two unrelated genes, Rag-1 and Rag-2, located within a single locus, have been shown to be necessary for activation of the V(D)J recombinase machinery (3, 4). It is, however, not known whether these two genes directly encode the components of the recombinase or whether they act via other genes or proteins. When either of these genes is rendered nonfunctional by homologous recombination, no complete TCR molecules can be assembled, hence no mature T cells are produced (5, 6).

In the T cell lineage, Rag-1 and Rag-2 are expressed in immature thymocytes, principally CD4⁺CD8⁺ (double positive or DP) cells, but not in mature T cells (7). It is believed that during T cell differentiation in the thymus, these genes are transcriptionally active while TCR gene rearrangement is occurring; however the mechanisms controlling their expression are still poorly understood. In vivo stable downregulation of recombinase expression occurs as a developmental consequence of upregulation of the TCR after positive selection (8). In addition, transient downregulation of recombinase occurs as a result of specific engagement of the TCR during the positive selection process (9). The latter phenomenon can be mimicked in vitro by TCR cross-linking or activation of protein kinase C by phorbol esters (10). However, so far recombinase expression has not been assessed in more immature thymocyte populations (before the DP stage) where many of the developmental decisions are made. We have used a combination of in situ hybridization and Northern blot analysis to investigate both recombinase and TCR gene expression in immature and mature thymocyte subsets. Our data indicate that there are two distinct waves of recombinase activity during thymus development corresponding to initial expression (and hence presumably rearrangement) of first, TCR β, γ, and δ and second, TCR α genes. Furthermore, downregulation of recombinase activity can occur even at very early stages of T cell development.

Materials and Methods

Mice and Cell Suspensions. C57BL/6 female mice were purchased from Harlan Olac (Bicester, UK) and maintained in the Swiss Institute for Experimental Cancer Research animal facility (Epalinges, Switzerland). Immature and mature T cell subsets were prepared as previously described (11). A combination of complement-mediated cytolysis and magnetic bead depletion using Goat anti-rat Ig coated M-450 Dynabeads (Dynal, Oslo, Norway) was used to negatively select subsets from the thymus and peripheral lymphoid organs.
of 4-6-wk-old mice. Further positive selection for up to three different cell surface markers was done by immunofluorescence staining and cell sorting. The mAbs used for these deletions and immunofluorescence have all been previously described (11). Cells were cultured in 24-well plates (Costar Corp., Cambridge, MA) in the presence of 10 ng/ml of PMA and 250 ng/ml calcium ionophore (ionomycin), both purchased from Calbiochem (La Jolla, CA), or in culture medium alone for the times indicated. The culture medium was Hapes-buffered DMEM supplemented with glutamine, 10\(^{-4}\) M 2-ME, and 5% FCS.

**Flow Microfluorometry and Cell Sorting.** Immunofluorescent analysis and sorting was performed as previously described (11). Briefly, cell suspensions were labeled with various combinations of mAbs conjugated with FITC, PE, or biotin, the latter in combination with streptavidin-tricolor or streptavidin-PE, (both supplied by Caltag Laboratories, San Francisco, CA) for two- or three-color analysis or sorting. All immunocjugates used were prepared in this laboratory. Analysis was done on a FACSScan\textsuperscript{\textregistered} flow cytometer, while sorting was performed on a FACSStar\textsuperscript{\textregistered} cell sorter (both supplied by Becton Dickinson & Co., Mountain View, CA). The LYSYS II program was used for both analysis and sorting. The purity of each population was determined by reanalysis after sorting and was found to be 97-99% for all subsets.

**Probes.** RNA probes for the constant regions of the four TCR genes have been described previously (12) and are as follows: TCR Ca, a 493-bp EcoRI/PstI fragment inserted into pSP5671; TCR CB\(_2\), a 434-bp EcoRI/HindIII fragment inserted in pSP5671, which recognizes both CB\(_1\) and CB\(_2\); TCR C\(_{\gamma}\), a 1.6-kb BamHI fragment covering the constant region and the 3'-untranslated region, inserted into pSP665; and TCR C\(_{\delta}\), a 580-bp EcoRI fragment inserted into pgEM2. The Rag-1 probe is a 580-bp EcoRI/HindIII fragment inserted into pSP665 (7), and was the kind gift of Dr. Pierre Vassalli (University of Geneva, Geneva, Switzerland). The Rag-2 probe is a 559-bp fragment of the Rag-2 cDNA (from 308-905) cloned into XbaI/EcoRI of the pBluescript KS\(\textsuperscript{+}\) polylinker using PCR-primerings containing these restriction sites, and was the kind gift of Dr. Ulf Grawunder (Basel Institute of Immunology, Basel, Switzerland). The mouse β-actin probe, a 200-bp PstI/BglIII insert in pSP65 was kindly provided by Dr. Markus Nabholz (Swiss Institute for Experimental Cancer Research, Epalinges, Switzerland). After linearization of these plasmids with the appropriate restriction endonucleases, antisense RNA probes were generated and labeled with α\(^{-35}\)SJUTP for Northern hybridization or α\(^{-35}\)SJUTP for in situ hybridization (both purchased from NEN-DuPont de Nemours, Bad Homburg, Germany), by transcription using SP6, T3, or T7 RNA polymerases (Boehringer Mannheim, Mannheim, Germany). The DNA template was removed by treatment with DNase (Boehringer Mannheim). This was followed by successive ethanol precipitations to purify the probe and to remove unincorporated nucleotides. For in situ hybridization, the \(^{35}\)S-labeled probes were size-reduced by alkaline hydrolysis to fragments of an average size of 150 bp and 25 μg of yeast t-RNA (Sigma Chemical Co., St. Louis, MO) per 2 \(\times\) 10\(^6\) cpm was added as carrier RNA for the hybridization.

**Northern Hybridization.** Total cellular RNA was prepared from FACS\textsuperscript{\textregistered} sorted cell populations by guanidinium isothiocyanate extraction, followed by CsCl\textsubscript{2} gradient centrifugation and phenol/chloroform extraction. 4 μg of RNA was separated on a 1.2% formaldehyde agarose minigel and subsequently transferred onto nylon membranes (Hybond-N; Amersham International, Amersham, UK) using an electro-blot apparatus (Bio-Rad Laboratories, Richmond, CA). After UV fixation for 5 min, the filters were sealed in plastic wrap and stored at -20°C until use. Hybridization was done overnight at 55°C in a solution of 50% formamide/2.5 \(\times\) Denhardt\textdagger{'}s/50 mM sodium phosphate buffer/10 mM EDTA/0.2% SDS/5 \(\times\) SSC containing 200 μg/ml denatured herring sperm DNA (Boehringer Mannheim), after prehybridization under the same conditions for at least 6 h. After labeling, 15-20 \(\times\) 10\(^6\) cpm of the appropriate probe was added to 6 ml hybridization solution for each 6 cm\(^2\) membrane. After hybridization, the filters were washed in 2 \(\times\) SSC/0.2% SDS at room temperature for 10 min, and then 3-4 times for 20 min each in 0.1 \(\times\) SSC/0.1% SDS at 65°C. Exposure to x-ray film (Eastman Kodak, Rochester, NY), in the presence of enhancing screens, was at -70°C for the times indicated.

**In Situ Hybridization.** Detection of Rag-1 and TCR mRNA in various T cell subpopulations was performed as previously described (12). Briefly, cells were stained as for immunofluorescence, lightly fixed with PBS containing 1% paraformaldehyde, and 10,000 cells of each selected population were sorted directly onto prewashed, Poly-L-lysine (Sigma Chemical Co.) coated microscope slides. After air drying, the slides were fixed in PBS containing 4% paraformaldehyde. The slides were pretreated with proteinase K (Sigma Chemical Co.) and postfixed in paraformaldehyde before acetylation. Hybridization solution (50% deionized formamide/300 mM NaCl/5 mM EDTA/20 mM Tris-HCl, pH 7.5/1 \(\times\) Denhardts/10% wt/vol dextan sulphate/100 mM dithiothreitol [DTT]), containing 2 \(\times\) 10\(^6\) cpm \(^{35}\)S-labeled RNA probe/μl was placed onto the cells, covered with a siliconized glass coverslip, and sealed with rubber cement. Hybridization was performed in a humidified atmosphere at 48°C for 12-18 h. After digestion of single stranded nonhybridized RNA with RNase A and T1 (Boehringer Mannheim) and extensive washing in 50% formamide/2 \(\times\) SSC/0.1% SDS at 54°C, the slides were dehydrated, dried, and then dipped in NTB-2 nuclear track emulsion (Eastman Kodak) at 42°C. Hybridized slides were exposed at -20°C for 10-20 d before development, and counterstained with nuclear fast red. For every individual experiment, two identical slides, each containing two different cell populations (in some cases a positive and a negative control), were hybridized with each probe and 500-1,000 cells from each population evaluated. Negative controls were mature T cells for Rag-1 and P815 mastocytoma cells for the TCR gene probes. Thymocytes from TCR β-deficient mice (in which both β loci are deleted, reference 13) were also included as a negative control for the TCR Cβ probe. Less than 1% mRNA\textsuperscript{-} cells were detected in negative control slides.

**Results and Discussion.**

Two Waves of Rag-1 Expression During Thymus Development. The most immature thymocyte yet identified resembles the pluripotent bone marrow stem cell, is present in very small numbers, and is characterized by low levels of expression of CD4, Thy1, and heat-stable antigen (HSA), and high levels of CD44 and H-2K (14). Over a period of 11–15 d under the influence of the thymic microenvironment, these CD4\textsuperscript{o} precursor cells proliferate and differentiate through a series of CD4\textsuperscript{+} CD8\textsuperscript{+} (DN) surface TCR \textsuperscript{-} populations (15, 16, 18) before acquiring CD4 and CD8 and subsequently a functional TCR (19). The DN TCR\textsuperscript{-} compartment, comprising 2-4% of total thymocytes can be further divided on the basis of differential expression of several cell surface markers (15, 16, 17, 18). These DN TCR\textsuperscript{-} subsets are all HSA\textsuperscript{+} and progress in a developmental sequence defined by expression of CD44 and CD25 (IL-2Rα) as follows: from
CD44<sup>+</sup>CD25<sup>−</sup> to CD44<sup>−</sup>CD25<sup>+</sup> to CD44<sup>−</sup>CD25<sup>−</sup> (17). This last DN population then acquires either CD4 or CD8 in the absence of cell surface TCR, becoming an immature single positive (ISP) cell which then rapidly becomes DP (reviewed in 20). During this differentiation from CD4<sup>+</sup> precursor cell to DP, most of the TCR gene rearrangement occurs. (For more comprehensive reviews of thymus development see 21, 22.)

To investigate recombinase activity in the developing thymus, we performed in situ hybridization with a Rag-1 probe on purified cells from each of the thymocyte subsets described above. Some representative examples are shown in Fig. 1 A and the complete data are shown in Fig. 1 B. In addition, where sufficient numbers of cells were obtained (i.e., from the CD44<sup>−</sup>CD25<sup>−</sup> DN subset onwards), RNA was prepared for Northern blot analysis of Rag-1 and Rag-2 expression (Fig. 1 C). As expected from previous studies (7, 10) the highest percentage of Rag-1 mRNA positive cells and the largest amount of Rag-1 and Rag-2 message were found in the DP population, whereas no message for either of these two genes was detectable in mature CD4<sup>+</sup> or CD8<sup>+</sup> single positive (SP) thymocytes or peripheral T cells. Interestingly, Rag-1 mRNA positive cells were detected in all of the immature (surface TCR negative) subsets tested; however the percent of positive cells and the steady state levels of mRNA varied considerably. A small number of Rag-1 mRNA positive cells were detected in the CD4<sup>+</sup> precursor cell population presumably in preparation for the first TCR gene rearrangements, as this population has been shown to be predominantly in germline status with regard to TCR β and γ loci (13). The percent Rag-1 mRNA positive cells increased in the subsequent subsets to peak at the CD44<sup>−</sup>CD25<sup>−</sup> DN stage. This subset (comprising ~60% of total DN thymocytes in C57BL/6 mice) was the earliest population investigated by Northern blot analysis and had readily detectable amounts of Rag-1 message. However the more mature CD44<sup>−</sup>CD25<sup>−</sup> DN population had considerably (20-fold) less Rag-1 message and fewer Rag-1 mRNA positive cells. It thus appears that Rag-1 mRNA is downregulated in many of the cells in this latter population. The ISP subset (next in the developmental sequence), had an intermediate level of Rag-1 message and Rag-1 positive cells, consistent with it being an immediate precursor of DP thymocytes. Analysis of Rag-2 mRNA by Northern blot confirmed a similar pattern of expression as for Rag-1 in the various subsets (Fig. 1 C), although the overall level of expression of Rag-2 was lower than Rag-1. Taken together these results indicate that there are at least two waves of recombinase activity during T cell development. One wave peaks at the CD44<sup>−</sup>CD25<sup>−</sup> DN stage and the other at the DP stage.

**TCR Gene Expression in Developing Thymocytes.** Since it is known that during fetal thymic development TCR α gene expression occurs later than expression of the other TCR genes (23), one explanation for our results is that recombinase activity could correlate with periods of TCR gene rearrangement. To investigate this possibility we have evaluated the expression of TCR α, β, γ, and δ mRNA by in situ hybridization in each of these subsets. As shown in Fig. 2 A, some TCR β<sup>+</sup> mRNA cells could be detected in the early CD44<sup>−</sup>CD25<sup>−</sup> DN subset. At the subsequent CD44<sup>−</sup>CD25<sup>−</sup> DN stage (corresponding to the first peak of recombinase expression; see Fig. 1, B and C), TCR β mRNA<sup>+</sup> cells were much more abundant, and both TCR γ mRNA<sup>+</sup> and TCR δ mRNA<sup>+</sup> cells were first detected. These data suggest that rearrangement of the TCR β, γ, and δ genes may be temporally correlated to the transcriptional activation of the recombinase locus at the CD44<sup>−</sup>CD25<sup>−</sup> DN stage.

Since in situ hybridization with TCR constant region probes does not distinguish between completely rearranged and shorter (i.e., partially rearranged and germline) transcripts, we also performed Northern blot analysis on the total cellular RNA isolated from CD44<sup>−</sup>CD25<sup>−</sup> DN thymocytes (Fig. 2 B). These data indicated that full-length transcripts corresponding to TCR β (VDJ<sub>CB</sub>), TCR γ (V<sub>Jγ</sub>γ), and TCR δ (VDJ<sub>Cδ</sub>) were present. In addition large amounts of shorter transcripts, presumably derived from partially rearranged or germline loci were detected for all three TCR genes, as would be expected for an immature (and heterogeneous) population in the process of undergoing rearrangement.

As noted before (12), TCR α mRNA<sup>+</sup> cells were first detected in very low numbers at the ISP stage and increased dramatically in DP thymocytes (Fig. 2 A), coinciding with the second wave of Rag-1 and Rag-2 activity. These TCR α transcripts were exclusively full length (V<sub>Jα</sub>Cα) by Northern blot analysis (data not shown), confirming that they originated from rearranged genes.

Taken together, these results suggest that the two waves of recombinase gene expression during thymus development coincide with two distinct periods of TCR gene rearrangement and expression. The first wave corresponds to the TCR β, γ, and δ genes and the second to the TCR α gene.

**Regulation of Rag-1 mRNA Expression in DN Thymocytes.** It has been previously shown that recombinase gene expression in DP thymocytes can be downregulated by both specific, (antigen presented by MHC molecules) (8, 9) and nonspecific (TCR, cross-linking and treatment with phorbol esters) stimuli (10). A more recent study (24) showed that phorbol esters could also downregulate Rag-1 and Rag-2 expression in fetal thymic ISP (CD4<sup>+</sup>CD8<sup>+</sup>CD3<sup>−</sup>) cells. These latter results, taken together with the data presented here, raise the possibility that expression of Rag-1 and Rag-2 (and hence recombinase activity) could be differentially regulated at different stages of T cell development. To further investigate this hypothesis, we cultured total DN, CD44<sup>−</sup>CD25<sup>−</sup> DN, or total thymocytes in medium alone or in the presence of PMA and calcium ionophore. After 3 h at 37°C, the cells were harvested and total cellular RNA extracted and analyzed by Northern blot. As shown in Fig. 3, Rag-1 mRNA was completely downregulated in all subsets tested after treatment with PMA and calcium ionophore. Similar results were observed for Rag-2 (data not shown). This effect was extremely rapid, since identical results were observed after only 1 h of exposure to these agents (data not shown). These data show that Rag-1 and Rag-2 expression can be regulated in DN cells despite the absence of a mature TCR α/β-CD3 complex on
Figure 1. Recombinase gene expression in developing thymocytes. (A) Examples of in situ hybridization with Rag-1 probe. Populations are (top from left to right) DP thymocytes and LN T cells (bottom from left to right) CD44-CD25+CD3- DN and CD44-CD25-CD3- DN thymocytes. (B) Analysis of Rag-1 mRNA expression in T cell subsets by in situ hybridization. Subsets are ordered according to developmental sequence (see text). ISP and SP thymocytes were CD4-CD8-CD3- and CD4-CD8+CD3+ cells, respectively. Results are expressed as mean percent positive cells ± SD of three separate experiments, each one analyzing 500-1,000 cells/slide and 2-4 individual slides for each population. Differences between each subset and its immediate precursor (or progeny) are statistically significant (p <0.05) except for the two earliest subsets (p = 0.18). (C) Northern blot analysis of Rag-1 and Rag-2 expression in T cell subsets. Independent blots for Rag-1 and Rag-2 were washed and rehybridized with a β-actin probe. Rag-1 and Rag-2 signals were quantitated by densitometry and normalized to the β-actin signal. This ratio is indicated for each subset (in arbitrary units). Exposure times were 18 h for Rag-1, 5 d for Rag-2, and 18 or 5 h for β-actin.

the cell surface. It is interesting to note that Rag-1 message was upregulated 10-fold in the CD44-CD25+ DN cells (the last DN stage) cultured in medium alone (Fig. 3). Under these conditions this subset (and not the earlier CD44-CD25+ DN) has been previously shown to differentiate spontaneously in vitro into DP thymocytes within 12-18 h (25, 26). This suggests that in vivo, this late DN population is subject to some sort of negative regulation of Rag-1. Indeed, the earlier CD44-CD25+ DN subset, which has more abundant Rag-1 message and a higher percentage of Rag-1 positive cells (Fig. 1, B and C), does not appear to be negatively regulated in this way (Fig. 3, and data not shown).

In conclusion, our data demonstrate that recombinase gene expression occurs in two distinct waves during thymus development and that these waves temporally distinguish rearrangement and expression of first TCR β, γ, and δ and subsequently TCR α genes. Furthermore, we show that Rag-1 and Rag-2 expression is subject to downregulation (at least by phorbol esters) in DN subsets not expressing a mature TCR α/β complex. Based on these and other recent studies (8, 9, 10, 24), we would speculate that regulation of recombinase gene expression in immature T cells occurs on at least two levels. Transcriptional activation of the recombinase locus begins very early in development and shuts down at the late DP stage after positive selection has been completed. During this time frame, when the recombinase locus is open for transcription, transient downregulation of recombinase expression can presumably also occur as a result of specific signaling events. In DP thymocytes it is clear that engagement of the TCR by self-Ag/MHC ligands turns off recombinase expression. In contrast the putative physiological signal responsible

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Figure 2. TCR gene expression in developing thymocytes. (A) Analysis of TCR mRNA expression in T cell subsets by in situ hybridization with TCR constant region probes. (B) Northern blot analysis of TCR transcripts in CD44^-CD25^+CD3^- DN thymocytes. Control populations are P815 mastocytoma cells in lane 1 and P1, a TCR α/β CTL clone in lane 2. The same blot was hybridized sequentially with the indicated TCR probes, as well as for β-actin. The sizes of transcripts in kilobases are noted. Exposure times were as follows: Cot and C~/ for 48 h; C~ for 18 h. The same blot was hybridized sequentially with the indicated TCR probes, as well as for β-actin. The sizes of transcripts in kilobases are noted. Exposure times were as follows: Cot and C~/ for 48 h; C~ for 18 h.

Figure 3. Regulation of Rag-1 mRNA expression in thymocyte subsets. Total thymocytes are compared to total DN thymocytes and CD44^-CD25^-CD3^- DN thymocytes. For each population, PMA/calcium ionophore-treated cells are compared with untreated and medium alone controls. The blots were washed and rehybridized with a β-actin probe to allow normalization of the Rag-1 signals. (Ratio calculated as in Fig. 1 C). Exposure times were as follows: for Rag-1, 4 h for total thymus; 18 h for total DN; 24 h for CD44^-CD25^- DN; and for β-actin 6 h for all populations.

for recombinase downregulation in CD44^-CD25^- DN thymocytes is unknown. Since DN thymocytes arrest at the CD44^-CD25^- stage in mutant mice deficient for recombinase activity (5, 6), and several recent studies have implicated expression of the TCR β chain (in the absence of TCR α) as being necessary for progression of DN thymocytes to the DP stage (13, 19), it could be speculated that engagement of an immature β chain TCR complex on CD44^-CD25^- DN thymocytes is responsible for the transiently reduced levels of recombinase expression observed in these cells. Downregulation of recombinase activity may be necessary at this stage of development in order to allow expansion and further differentiation of TCR β+ cells before TCR α rearrangement. Thus far we have not been able to demonstrate downregulation of Rag-1 or Rag-2 expression upon exposure of DN thymocytes to immobilized anti-CD3 or anti-TCR β antibodies (data not shown). Nevertheless we cannot exclude the possibility that physiological recombinase expression may be regulated in vivo by ligation of an immature TCR complex, perhaps present on only a minority of DN cells. Alternatively, other ligand–receptor complexes may be involved in the control of recombinase expression during this critical developmental period.
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