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Regulation of N-region diversity in antigen receptors through thymocyte differentiation and thymus ontogeny

(T-lymphocyte maturation/T-cell repertoire/T-cell receptor/terminal deoxynucleotidyltransferase)

MOLLY BOGUE, SUSAN GILFILLAN, CHRISTOPHE BENOIST, AND DIANE MATHIS

Laboratoire de Génétique Moléculaire des Eucaryotes du Centre National de la Recherche Scientifique et U.184 de l'Institut National de la Santé et de la Recherche Médicale, Institut de Chimie Biologique, 11 rue Humann, 67085 Strasbourg Cédex, France

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ABSTRACT **The random addition of "N nucleotides" by terminal deoxynucleotidyltransferase (TdT) is an important component of the diversity of T-cell receptor genes. We have investigated the expression of TdT during thymocyte differentiation and thymus ontogeny. TdT gene transcripts are confined to immature thymocytes of the cortex, being down-regulated concomitantly with recombination-activating gene transcripts after positive selection of mature medullary T cells. According to *in situ* hybridization, TdT RNA is absent from the neonatal thymus, but it appears 3 to 5 days after birth, just before the appearance of significant N-region diversity in T-cell receptor junctional sequences but clearly after the thymus attains competence at clonal deletion.**

A major component of the impressive diversity of B-cell antigen receptors and the major component of T-cell receptor (TCR) diversity is amino acid variability in the third complementarity determining region, or CDR-3. This variability derives from multiple sources: the particular diversity (D) or joining (J) segments selected by the rearranging variable (V) gene segment, exonuclease nibbling of the exposed V, D, and J termini and template-dependent (P) or template-independent (N) addition of nucleotides at the V–D and D–J junctions.

A large body of evidence has demonstrated that some of these processes are developmentally regulated (for review, see ref. 1 and references therein). In particular, many reports have described a deficiency in the N-region diversity of antigen receptors from fetal and newborn animals, as compared with those from adults. This striking deficiency was found true of immunoglobulin heavy chains (2–8), $\gamma\delta$ TCRs (9–14) and $\alpha\beta$ TCRs (15–18).

The developmental regulation of N-region diversity would seem of some importance because it is phylogenetically conserved. Not only mammals, but also birds (17) and amphibians (7), use N nucleotide addition in an age-dependent manner. Certain functional studies also argue for its significance. Perinatal B-cell receptors are thought to be more polyspecific and autoreactive than adult receptors and are considered to be generally of lower affinity (19, 20). Such a reactivity pattern has been suggested to be useful in establishing the initial repertoire and/or in providing a first defense against common bacterial and parasitic pathogens. Interestingly, the dearth of N-region diversity in some (although not all) neonatal immunoglobulin heavy-chain molecules appears to be actively selected (8), as does its paucity in $\gamma\delta$ (11, 13) and $\alpha\beta$ (16) TCRs.

The enzyme implicated in N nucleotide addition is terminal deoxynucleotidyltransferase (TdT). Initially, the evidence for its role was purely correlative: it was observed that those cells that express TdT have antigen receptors with N-region diversity, whereas those that do not express TdT have receptors devoid of this source of third complementarity determining region variability (21, 22). In addition, it was noted that TdT preferentially catalyzes the addition of guanine residues to artificial templates and that N nucleotides are most commonly guanine or cytosine residues (23). An early attempt to provide direct support for the role of TdT proved somewhat disappointing. Introduction of a retrovirus expressing a TdT cDNA into a pre-B-cell line lacking this enzyme led to the assembly of immunoglobulin genes with N nucleotide addition, but the average number of nucleotides per gene was significantly lower than that observed for receptors derived from adult animals (24). However, in a more recent study, introduction of the recombination-activating genes *RAG-1* and *RAG-2* into a fibroblast cell line produced receptors that lack N-region diversity, but cotransfection of the TdT gene led to receptors with adult levels of N-region addition (25).

Given the large influence of N-region diversity on the B- and T-cell repertoires, we felt it important to know in detail how TdT expression and N nucleotide addition are regulated. In this report, we present information on regulation in the thymus—through the life of a thymocyte and during the lifetime of the animal.

MATERIALS AND METHODS

Mice. (C57BL/6 × DBA/2) F_1 mice (B6D2) were bred in our animal facility. Newborn mice (10–16 hr old) were designated day 1.

Organ and Cell Preparations. The thymus and heart of newborn or young mice were dissected from developmentally staged mice. For *in situ* hybridization, these tissues were embedded in Tissue-Tek (Miles) and frozen immediately at −80°C.

For cell sorting, suspensions of thymocytes from individual mice were stained with anti-CD3, anti-CD4, and anti-CD8 monoclonal antibodies as described (26). Cells were sorted after gating on two or three fluorescent parameters, using an ATC 3000 flow cytometer. A minimum of 10,000 cells was collected to prepare RNA for sequencing or quantitative PCR analysis.

To detect $CD4^+CD8^-$ thymocytes expressing TCR $V_\beta 6$, cell suspensions were incubated before anti-CD4/anti-CD8 treatment with monoclonal antibody 44.22.1 (27), as described (26).

Templates for RNA Probes. Templates for RNA probe synthesis were made by cloning PCR-generated fragments of TdT and RAG-1 into the *Eco*RV site of pBluescript. The substrate for all amplifications was cDNA synthesized from C57BL/6 thymus RNA. For TdT, a 972-base-pair (bp) frag-

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Abbreviations: TdT, terminal deoxynucleotidyltransferase; RAG, recombination-activating protein; D, J, and V, diversity, joining, and variable regions, respectively.

ment spanning exons 2–9 was amplified by using the oligonucleotides 5′-CTCAGGGTCAGACGTCCTGGAGT-3′ (288–310) and 5′-TTCTGGAAATGGTCGAGAGCATC-3′ (1237–1259) [numbers in parentheses correspond to base pairs as designated by Koiwai *et al.* (28)]. A fragment of RAG-1 cDNA (32–593) was amplified by using oligonucleotides (F3 and R6) and PCR conditions identical to those described by Chun *et al.* (29).

RNA Probe Synthesis and *in Situ* Hybridization. Template plasmids were linearized with *Eco*RI or *Hin*dIII, and both sense and antisense probes were synthesized by using T3 or T7 RNA polymerase. One microgram of template DNA was incubated in a 20-μl reaction containing 40 mM Tris (pH 7.9), 6 mM $MgCl_2$, 10 mM NaCl, 2 mM spermidine, bovine serum albumin at 100 μg/ml, 10 mM dithiothreitol, 500 μM (each) ATP, GTP, and UTP, 25 μM CTP, 200 μCi (8.75 μM; 1 Ci = 37 GBq) cytosine [α-[^{35}S]thio]triphosphate, 20 units of RNasin, and 30 units of the appropriate polymerase. RNA synthesis was done at 37°C for 1–2 hr, and the template DNA was then digested with RNase-free DNase (Boehringer Mannheim) for 10 min at 37°C. After extraction with phenol/chloroform and precipitation in ethanol, the probes were resuspended in cold (0°C) 0.1 M NaOH and allowed to hydrolyze for 40–60 min in an ice slurry (until the average fragment length was 150–400 nucleotides). The probes were again precipitated, resuspended in 10 mM dithiothreitol, then diluted to 25,000 cpm/ml in hybridization buffer [50% (vol/vol) formamide/300 mM NaCl/20 mM Tris·HCl, pH 7.5/1× Denhardt's solution/5 mM EDTA, pH 8/10% (wt/vol) dextran sulfate/10 mM dithiothreitol/tRNA at 500 μg/ml]. *In situ* hybridization was done as described by Hafen *et al.* (30). Slides were exposed from 1 to 3 weeks.

RNA Preparation, PCR Sequencing, and Quantitation. Details of these procedures have been described elsewhere (31, 32). Briefly, RNA was prepared from sorted cell populations by a standard Nonidet P-40 lysis method, and first-strand cDNA was synthesized by avian myeloblastosis virus reverse transcriptase. For TCR sequence analysis, V_β-specific cDNA was amplified by two rounds of PCR with nested sets of C-region primers on the 3′ end and a mixture of three degenerate V-region oligonucleotides at the 5′ end. PCR products were digested, purified, and ligated into M13. M13 plaques were screened with a V_β6-specific oligonucleotide (NP-34: ACATCTGCCCAGAAGAACGAGATGGCCGTT). Positive clones were sequenced by the dideoxynucleotide chain-termination method.

For TdT mRNA quantitation, 5-fold serial dilutions of the cDNAs were used as templates for PCR amplifications, using the primers ACAGCGAGAAGAGCGGCCAGCAG (1298–1320) and CCATGGTTCAATGTAGTCCAGTC (1604–1626) for 25 cycles. Amplification of TdT-specific fragments was detected by Southern blot analysis of the PCR products using an internal oligonucleotide (1432–1455) as a probe.

RESULTS AND DISCUSSION

As with RAG mRNA, TdT Transcripts Are Expressed in Thymocytes Until Positive Selection. To obtain a global view of TdT expression in mouse thymocytes, we performed *in situ* hybridization on thymus sections using TdT and RAG-1 probes, the latter because its hybridization pattern is well-characterized (33) and, therefore, serves as a convenient geographical marker. As expected, and as illustrated in Fig. 1*e*, RAG-1 transcripts are detected in the thymic cortex but not in the medulla. Fig. 1*b* shows that TdT transcripts are delimited in an identical fashion.

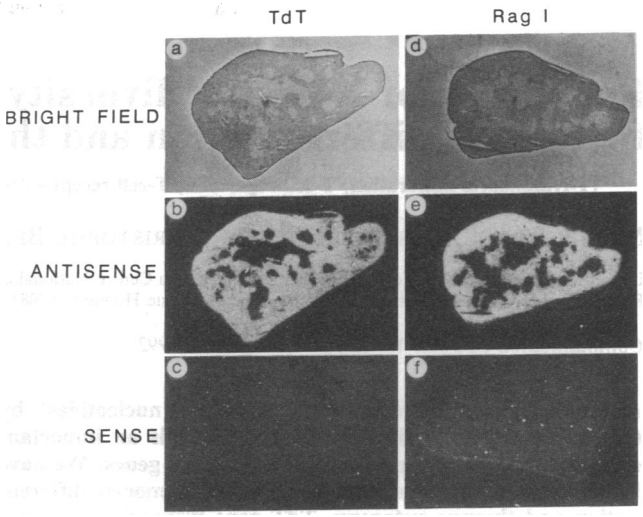

FIG. 1. Location of TdT and RAG-1 transcripts in thymus sections from a 12-day-old mouse. Thymus sections were hybridized with TdT and RAG-1 RNA probes, as described, and stained with toluidine blue. Sections hybridized with antisense probes were photographed by using both bright-field (*a* and *d*) and dark-field (*b* and *e*) optics at ×17.5. Serial sections hybridized with sense probes (*c* and *f*) were photographed at the same magnification using dark-field optics.

That expression of TdT is confined to cortical thymocytes has been suggested (34–36). But all of these studies analyzed protein expression or activity, and most relied on crude thymocyte fractionation techniques—necessary at the time but now known to achieve only partial subset separations. Our *in situ* hybridization results confirm these previous suggestions and further establish that the regulation of TdT expression is pretranslational.

Given the striking geographical delimitation of TdT transcripts, we wondered whether their "turn-off" might coincide with positive selection of thymocytes into the mature $CD4^+CD8^-$ and $CD4^-CD8^+$ compartments, as has recently been established for RAG-1 and RAG-2 (37). Thus, we sorted thymocytes on the basis of CD4, CD8, and CD3 expression and quantitated the amount of TdT RNA by PCR titration under nonsaturating conditions. As illustrated in Fig. 2, the TdT gene has the same pattern of expression as the *RAG* genes: it is well expressed in immature $CD4^-CD8^-CD3^-$, $CD4^-CD8^+CD3^-$, and $CD4^+CD8^+CD3^{lo}$ cells but is expressed very poorly in mature $CD4^+CD8^-CD3^+$, $CD4^-CD8^+CD3^+$, and $CD4^-CD8^-CD3^+$ cells; in fact, the

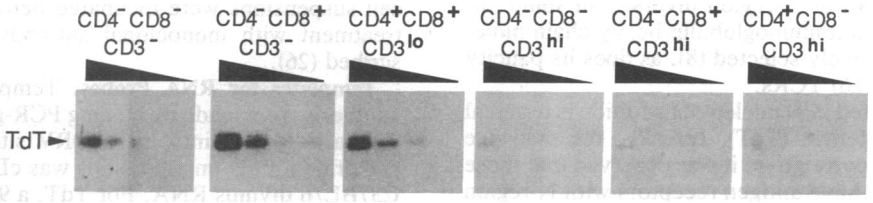

FIG. 2. TdT mRNA quantitation by PCR. A constant number of sorted thymocytes (98% purity) were mixed with carrier HeLa cells, and RNA was prepared. Five-fold serial dilutions of the cDNA derived from each preparation were used as templates for PCR amplification (25 cycles) with the described primers. The amplified TdT fragment was detected by Southern blotting with a TdT oligonucleotide probe. The experiment shown corresponds to cDNA from 3×10^4 sorted cells.

small amount of TdT mRNA we detect in mature cells (roughly 1/25th that observed in immature cells) may correspond to slight contamination with immature cells in the sorted samples. This result has been obtained in multiple titrations with independent RNA preparations.

It has recently become evident that, while allelic exclusion at the TCR β locus is quite strict, a TCR α gene can undergo multiple, successive rearrangements (for review, see ref. 1). Borgulya *et al.* (37) have suggested that *RAG* gene expression is maintained in $CD4^+CD8^+$ thymocytes until positive selection has been achieved to allow cells multiple chances to match an appropriate α chain with their particular β chains. Our results indicate that the successively rearranged TCR α genes will have the potential for N-region diversity. A successful positive selection event then leads to the coordinate shut-off of *RAG* and TdT gene expression.

Unlike RAG mRNA, TdT Transcripts Are Regulated Throughout Ontogeny. To assess TdT transcript levels during ontogeny, we performed *in situ* hybridization of thymus sections from embryos and newborn mice of known gestational age, ranging from day 14 fetal to day 18 newborn. Heart tissue and sense probes served as negative controls. RAG-1 transcripts were monitored in parallel.

RAG-1 RNA was detected at high levels in the cortex of all thymi examined (data not shown). In contrast, TdT transcripts were only detected in the cortical regions of thymi from late postnatal mice. This initial experiment indicated that the onset of expression occurs somewhere between days 2 and 6. Focusing on this transition period, we examined a second series of thymi for both TdT expression (Fig. 3*A*) and N-region diversity of the TCR β chain (see below). Significant levels of TdT RNA were not seen until day 4, and the highest levels were seen only after day 5. These findings are in accord with older studies showing that expression of TdT protein or activity in thymocytes is very limited or nonexistent until after birth in mice (35, 38), rats (38), and humans (34). Our studies offer more precision to the determination of timing and further show that TdT regulation is pretranslational.

Right from its onset, TdT gene expression is detectable throughout the thymic cortex, indicating that a substantial fraction of cortical thymocytes synthesize this enzyme (Fig. 3*B*). In addition, PCR analysis of sorted day 4 thymocytes demonstrated that $CD4^-CD8^-$ and $CD4^+CD8^+$ cells express TdT transcripts at similar levels (data not shown). These findings, in context with the observation that perinatal thymocytes readily express high levels of TdT when placed in suspension culture (35), suggest that some change in the thymic environment permits increased transcription between days 3 and 5 after birth. Had the onset of TdT expression in $\alpha\beta$ T cells coincided with recruitment of a new population of precursors, as has been suggested for expression in some $\gamma\delta$ T cells (39), one might have expected that thymocytes expressing TdT transcripts would be more focalized at first detection.

Appearance of N-Region Diversity in Thymocyte TCRs Throughout Ontogeny. Having determined the onset of detectable TdT gene transcription, we next sought to chart the appearance of N-region diversity in thymocyte receptors. We elected to concentrate on receptors carrying the $V_\beta 6$ region because, of those V segments expressed by B6D2 mice, we possessed the largest data base on $V_\beta 6$; this particular strain combination was chosen because it permits studies on clonal deletion of self-reactive cells, as discussed below. Mice were taken on successive days after birth, their thymi were removed, the $CD4^+CD8^+$ and $CD4^+CD8^-$ thymocyte populations were sorted, and $V_\beta 6^+$ TCR transcripts were amplified and cloned for sequencing. Often, the very same thymi were used for these sequencing experiments, for the *in situ* hybridization studies described above, and for the clonal deletion experiments discussed below.

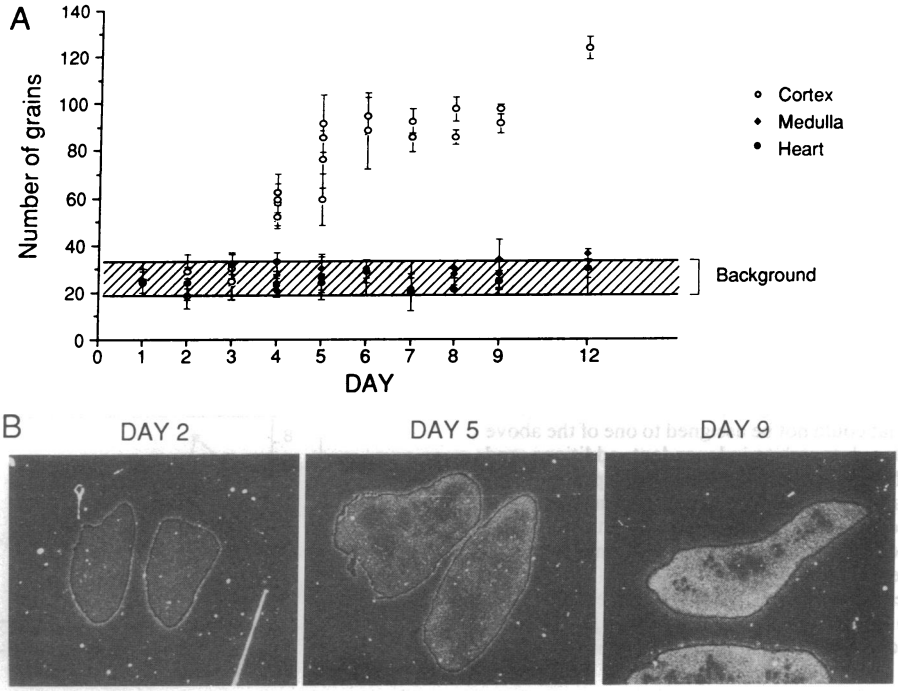

FIG. 3. Timing of onset of TdT expression in the thymus. (*A*) Grain counts. Sections from thymi taken from newborn mice at the days indicated (day of birth designated day 1) were hybridized with an antisense TdT RNA probe. Silver grains were counted to assess specificity and relative levels of hybridization. Each symbol represents the average number of grains in an equal area of tissue (thymic cortex, thymic medulla, and heart), and the shaded region indicates the number in an equal area of slide (background); grains in three to nine distinct areas of each region were counted. (*B*) Dark-field photographs (×17.5) of representative sections used for the grain counts in *A*.

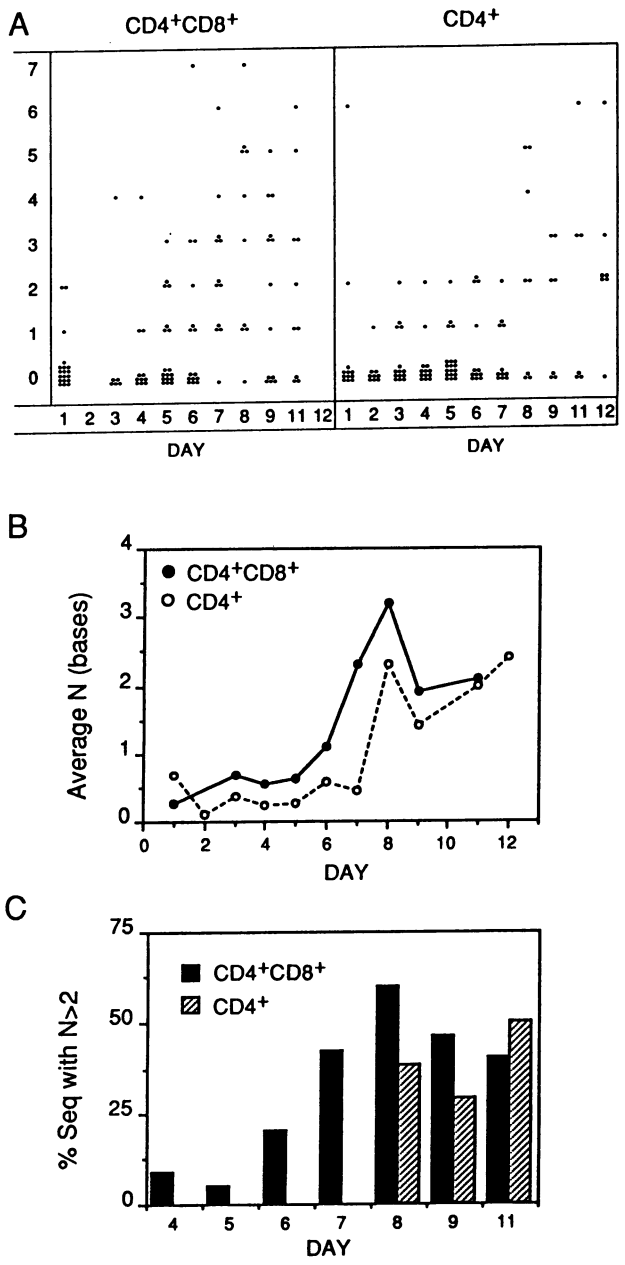

FIG. 4. Time course of N nucleotide addition. N additions to $V_\beta 6^+$ TCRs from $CD4^+CD8^+$ and $CD4^+CD8^-$ thymocytes from postnatal mice. $V_\beta 6^+$ sequences were obtained from sorted cells from developmentally staged B6D2 mice. Nucleotides were assigned to V, D, and J segments that compose the junctional region, as described (16). Mono- or dinucleotides representing inverted repeats of undigested germ-line termini were grouped as P nucleotides (10). Remaining nucleotides that could not be assigned to one of the above categories were considered template-independent additions and were, thus, designated N nucleotides. (*A*) Each dot represents one sequence, the position along the *y* axis indicating the number of N nucleotides in that sequence, and the *x* axis indicating the day after birth the sample was taken. Repeats are represented only once; the few out-of-frame sequences obtained are omitted. (*B*) Average number of N nucleotides per sequence. Averages were determined from the data in *A*. (*C*) Percentage of sequences (Seq) with >2 N nucleotides per sequence. Percentages were determined from the data in *A*.

Fig. 4 represents the time course of N nucleotide addition to $V_\beta 6$ segments in different ways. In each representation, the "flow" of diversity is readily seen: an increase in N addition is first evident in $CD4^+CD8^+$ cells at day 6, and the maximum level is attained by day 8; there is an additional lag of 1 to 2 days for the more mature $CD4^+CD8^-$ cells, an increase in N addition being first evident at day 8. The representation in Fig. 4*C* emphasizes the abrupt shift from neonatal to adult-type TCR β sequences. We feel that this representation is particularly relevant for two reasons: (*i*) any change of >2 nucleotides will add an amino acid to the junctional region and (*ii*) TdT-independent nucleotide additions have been seen in yeast, and these are almost always 1 or 2 nucleotides. In addition, our previous results showed that sequences with >2 N nucleotides were almost never found in neonatal thymocytes but were frequent in adult populations (16).

Two points are noteworthy from these data. (*i*) There is a delay of 1 to 2 days between the time that TdT genes synthesize detectable levels of RNA and the time when significant levels of N-region diversity are found in the receptors of $CD4^+CD8^+$ thymocytes. This lag must represent the period required for accumulation of sufficient levels of TdT protein in $CD4^-CD8^-$ cells undergoing TCR β gene rearrangement, as well as the period needed for these cells to differentiate into $CD4^+CD8^+$ cells. (*ii*) There is another delay of 1 to 2 days before significant levels of N-region diversity are found in the receptors of $CD4^+CD8^-$ cells. This lag should represent the time required for a double-positive cell to differentiate into a more mature single-positive cell. Interestingly, this time is shorter than the estimate of 3 to 4 days for this transition in adult animals, obtained from either [^{3}H]thymidine- (40) or 5-bromodeoxyuridine- (41) labeling experiments. That perinatal thymocytes might spend less time in the $CD4^+CD8^+$ stage has been invoked as an explanation for the fact that TCR α chains in very young animals tend to use more proximally situated V–J combinations than do α chains from adults, keeping in mind that TCR α genes successively rearrange in double-positive cells until they are selected into the single-positive compartment (see discussion in ref. 1).

N-Region Diversity and Clonal Deletion of Self-Reactive Thymocytes. TCRs carrying the $V_\beta 6$ variable region recognize a product of the 3′ open reading frame of the mouse mammary tumor virus presented in the context of major histocompatibility complex-encoded E molecules (42, 43). Being self-reactive, then, thymocytes that carry $V_\beta 6^+$ TCRs are eliminated in thymi of adult mice expressing this endogenous virus and displaying E complexes (41). However, elimination does not occur in fetal or neonatal animals (44–46). We wondered whether this defect might somehow be related to the lack of N-region diversity in perinatal receptors: perhaps germ-line-encoded $V_\beta 6^+$ TCRs are less self-reactive and, thus, thymocytes expressing them are less susceptible to deletion.

Therefore, we performed a careful comparison of the time of deletion of $V_\beta 6^+$ T cells and the timing of acquisition of N-region diversity in $V_\beta 6^+$ TCRs from B6D2 mice. It is

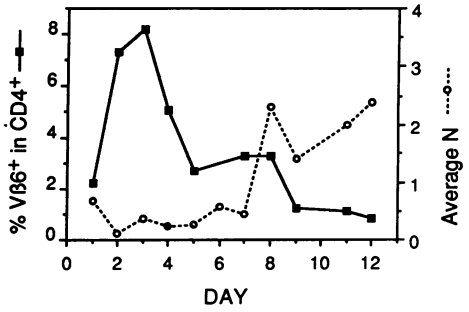

FIG. 5. Comparison of timing of clonal deletion of $V_\beta 6^+$ CD4 T cells and of amplification of N-region diversity in $V_\beta 6^+$ TCRs. ■, Proportion of $V_\beta 6^+$ $CD4^+CD8^-$ cells expressed as percentage of total $CD4^+$ cells; ○, average number of N nucleotides (sequence data of Fig. 4 are superimposed for clarity).

important to note that the two assays were performed concurrently on thymi from the same animals—the number of $V_\beta 6^+$ cells in the $CD4^+CD8^-$ compartment was assessed by triple-staining and cytofluorimetry; the level of N-region diversity was determined by amplification of $V_\beta 6^+$ transcripts from sorted $CD4^+CD8^-$ cells. The data are plotted in Fig. 5. Clearly, the defect in clonal deletion of $V_\beta 6^+$ T cells in perinatal animals is not due to the lack of N-region diversity in perinatal receptors because significant deletion precedes the increase in N diversity by 3 or 4 days.

This finding is in accord with recent results from the $V_\beta 17a$ system. Mice expressing a $V_\beta 17a^+$ TCR transgene derived from a T cell of an adult animal and having ample N-region diversity did not delete T cells expressing this receptor during the perinatal period (47).

CONCLUSIONS

Because N-region diversity is a critical determinant of TCR variability, we have studied the regulation of TdT expression and N nucleotide addition in thymocytes. We find that TdT expression is regulated at the RNA level during the differentiation of a thymocyte and during the ontogeny of the thymus. TdT "turn-on" is evident at the earliest stage of T-cell differentiation examined, and "turn-off" seems to coincide with positive selection from the $CD4^+CD8^+$ to $CD4^+CD8^-$ or $CD4^-CD8^+$ compartments. This pattern closely resembles that of the *RAG* genes, as mentioned above, as well as that of the *lck* proximal promoter (48). These genes (and others) may constitute a family slated for "turn-off" as an early step in the positive selection program. It may be significant, then, that the TdT and proximal *lck* promoters have been found to bind the same sequence-specific DNA-binding protein, LyF-1, which is not yet well-characterized but is apparently enriched in the lymphoid lineage (49). On the other hand, the TdT and *RAG* genes are not coordinately regulated during ontogeny: the former is turned-on only several days after birth, whereas the latter are already "on" at the earliest fetal ages examined. This lack of coordination suggests that the paucity of N-region diversity in TCRs of perinatal animals may not be just a remnant of evolution but rather may be of some biological importance. This hypothesis was suggested by the observation that the lack of N-region diversity in neonates is exaggerated by thymic selection events (16).

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