Gene Transcription in Differentiating Immature T Cell Receptor^{neg} Thymocytes Resembles Antigen-activated Mature T Cells

By Juan Carlos Zúñiga-Pflücker, Heather L. Schwartz, and Michael J. Lenardo

From the Laboratory of Immunology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Maryland 20892

Summary

Early in ontogeny thymocytes have a surface marker phenotype that resembles activated mature T cells but they lack expression of the T cell receptor (TCR) complex. We have made preparations of day 14/15 triple negative fetal thymocytes that exhibit the activated T lymphocyte markers CD25, intercellular adhesion molecule 1, Ly-6A/E, CD44, and heat stable antigen and are rapidly proliferating as evidenced by flow cytometric examination of BrdU incorporation. We found that binding activities of the gene regulators nuclear factor (NF)-κB, the NF-κB p50 homodimer complex, nuclear factor of activated T cells (NF-AT), oct-1, oct-2, activator protein 1 (AP-1), and serum response factor (SRF), are all present in these early thymocytes. Whereas the octamer factors and SRF persist during ontogeny, NF-κB, NF-AT, and AP-1 decrease and are undetectable in the adult thymus. Transfection of disaggregated thymocytes by electroporation or intact thymic lobes by gold-particle bombardment revealed that reporter constructs for NF-κB, NF-AT, AP-1, octamer factors and, to a small extent, the TCR-α enhancer were active in early thymocyte development. We rigorously eliminated the possibility that these transcriptional events were due to minor populations of TCR$^+$ cells by showing that these reporter constructs were also active in recombinase activating gene (RAG)$^{-/-}$ thymocytes that are incapable of completing TCR gene rearrangement, and predominantly contain cells that have an activated phenotype. Thus, transcriptional events that are usually triggered by antigen stimulation in mature T cells take place early in thymic ontogeny in the absence of the TCR. Our analysis suggests that there are striking regulatory similarities but also important differences between the activation processes that take place in antigen-stimulated mature T cells and thymic progenitor cells.

During thymic ontogeny, fetal liver derived pre-thymocytes undergo a complex process of differentiation and expansion (1). The commitment to the T lineage and some of the expansion in cell number occurs before TCR rearrangement and in cells that lack the surface expression of CD3, CD4, and CD8 (triple negative, TN)[1] (2). Because TN thymocytes have not yet rearranged their TCR genes and lack expression of the TCR–CD3 complex, they are incapable of an activation response through the TCR (3). Thymocyte development follows a stage- and time-specific set of maturational events. Around gestational day 12, the murine fetal thymus is seeded by hematopoietic precursors from the fetal liver. Days 13–15 thymocytes are predominantly TN. TCR rearrangement and expression of CD4 and CD8 begin by days 16 and 17. At a later time in ontogeny (days 18 and 19), thymocytes expressing antigen receptor, CD4 and CD8 undergo TCR-mediated positive and negative selection, and emerge as self-restricted, self-tolerant, and functional T cells (4). Despite the lack of TCR, TN cells exhibit several characteristics that resemble activated mature TCR$^+$ T cells: (*a*) increased cell volume; (*b*) brisk cellular expansion; (*c*) expression of activated T cell surface markers, such as lymphokine receptors and adhesion molecules; and (*d*) production of several lymphokines (5, 6). For example, TN thymocytes at days 14 and 15 of fetal development have been shown to express mRNA for IL-1β, IL-4, IL-5, IL-6, IL-7, IFN-γ, and TNF (7–9). Also expressed are the cell surface molecules: Thy-1, CD2, CD16, CD44, heat stable antigen (HSA), thymic shared antigen (TSA-1), CD18 (LFA-1), CD54 (ICAM-1), Ly-6, CD26 (THAM-1) and the α chain of the IL-2 receptor (CD25) (10–17). Markers such as CD25, ICAM-1, CD44, and Ly-6

[1] *Abbreviations used in this paper:* AP-1, activator protein-1; CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; NF-AT, nuclear factor of activated T cells; RAG, recombinase activating gene; SRF, serum response factor; tk, thymidine kinase; TN, triple negative.

are prominent on mature T lymphocytes only after TCR activation (15, 18, 19). Thus, some aspects of the activation program that are triggered in mature T cells by TCR engagement are found in T cell progenitors in the absence of TCR signaling.

This report addresses the regulatory molecules that may mediate this TCR-independent, activation-like process in immature TN thymocytes. Recently, other groups have reported initial analyses of DNA-binding proteins in different thymic subsets (20, 21). Our analysis has concentrated on the functional and binding properties of elements that may control the expression of IL-2 and the α chain of the IL-2 receptor in TN thymocytes. In mature T cells the elements controlling these genes have been well established (for a review see reference 22). Our results suggest that although the same gene regulatory factors appear to be responsible for the "activated" phenotype in both immature and mature T cells, a TCR-independent pathway leads to the induction of gene regulatory factors early in T cell development.

Materials and Methods

Mice. Timed pregnant and 6-wk-old C57Bl/6 female mice were obtained from the National Cancer Institute Frederick Research Facility. Recombinase activating gene (RAG) 2−/− mice were obtained from breeding stocks at the Howard Hughes Medical Institute, Children's Hospital (Boston, MA), and bred in National Institutes of Health (NIH) animal facilities (23).

Fetal Thymus Organ Culture (FTOC). Intact fetal thymic lobes were isolated from day 14 embryos and placed in culture as previously described (24). Briefly, six individual fetal thymic lobes were placed on the surface of 0.8-μm Nucleopore filters. Filters were supported by a Gelfoam gelatin sponge (20 × 60 × 7 mm). Each sponge was placed in a well of a tissue culture dish (6 wells/plate) and soaked in 4 ml of culture medium. The FTOC culture medium used was EHAA (Click's) medium supplemented with 10% FCS, 2 mM glutamine, 10 U/ml penicillin, 100 μg/ml streptomycin, 100 μg/ml gentamicin, 0.11 mg/ml sodium pyruvate, 5×10^{-5} M 2-ME and 10 mM Hepes, pH 7.4. Cultures were incubated at 37°C in a humidified incubator containing 5% CO_2 in air. At the end of culture period, the thymus lobes were mechanically disrupted and prepared for flow cytometry analysis or cellular extracts were prepared for chloramphenicol acetyltransferase (CAT) assays.

Flow Cytometry. Cell suspensions were prepared in HBSS (without phenol red) containing 1% BSA and 0.1% sodium azide (FACS® buffer). Cells (10^6/100 μl buffer) were incubated on ice for 30 min with 10 μl of appropriate antibodies and washed twice before analysis. Analysis was performed using a FACScan® (Becton Dickinson & Co., Mountain View, CA) flow cytometer with the Lysis II software; data was size and propidium iodide/live gated. All antibodies used for flow cytometry were obtained from Pharmingen (San Diego, CA). For in vivo BrdU labeling, day 15 timed pregnant mice were injected intraperitoneally with 2 mg/mouse BrdU in PBS. Mice were killed at different times, and fetal thymus cell suspensions were prepared for flow cytometry analysis as recommended by the manufacturer (Becton Dickinson & Co.).

RNA Analysis. RNA from freshly isolated fetal thymocytes or cultured adult lymph node T cells was isolated using RNAzol-B (Tel-Test, Inc., Friendswood, TX) according to the manufacturer's instructions, and Northern blot analysis was performed using standard procedures (25).

Nuclear Extracts and Electrophoretic Mobility Shift Assay (EMSA). Nuclear extracts from freshly isolated fetal thymocytes, cultured adult lymph node T cells, or established antigen-specific T cell clones were prepared as described previously (26). Protein concentrations were determined using an assay kit (Bio Rad Laboratories, Hercules, CA). EMSAs were performed essentially as described (27), with a custom-designed mini-gel apparatus (Owl Scientific, Cambridge, MA), using modified conditions. Briefly, 6–10-μl reactions were set up in the wells of a Terasaki plate (Nunc Intermed, Roskilde, Denmark) and contained 2–5-μg nuclear extract protein, 0.5 μg poly[dI:dC], and 40,000 cpm of ^{32}P-end-labeled oligonucleotide probe in a solution of 10 mM Tris-Cl, pH 7.5, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, and 10% glycerol. The samples were incubated at room temperature for 20 min and loaded with a tapered micropipette tip into the wells of the mini-gel apparatus. For competitions, 20–200-fold excess of unlabeled oligonucleotides were added to the binding mix before the addition of the nuclear extract protein. Electrophoresis was carried out on 4% polyacrylamide gels (30:1) in 50 mM Tris, 380 mM glycine, 2 mM EDTA, pH 8.5, or 6.7 mM Tris-Cl, pH 7.5, 3.3 mM sodium acetate, and 1 mM EDTA with buffer recirculation, at 11 V/cm for 90 min at room temperature. Gels were dried and autoradiographed. Oligonucleotide probes have been previously described (28).

In Vitro Transfections. Cell suspensions were washed and placed in RPMI 1640 medium supplemented with 20% FCS. Cells were transfected by electroporation using a BTX (San Diego, CA) Electro Cell Manipulator 600 apparatus with each sample containing 5–10 × 10^6 cells/cuvette (4-mm gap) in 250 μl medium. Samples were incubated on ice for 10 min with 10–15 μg CsCl purified CAT reporter plasmid DNA and were electroporated at 250 V, 1900 μFD, 186 ohm, with an ~100 millisecond time constant. After electroporation, samples were incubated on ice for 10 min, and then cultured for 24–36 h in FTOC medium. CAT reporter plasmids used in the analysis have been previously described (28, 29).

In Vivo Transfection. In vivo transfections were carried out using an accelerated DNA/particle bombardment (biolistic) delivery system. Intact fetal thymic lobes from FTOC were used as targets for DNA-covered gold particles (0.5–2 μm) using modifications of the technique previously described (30). After 1 d in culture, intact thymic lobes from day 14 mice on nucleopore filters were briefly removed from their Gelfoam sponge support and subjected to bombardment by accelerated DNA/gold particles in a self-constructed apparatus as follows: 30 μg of CAT reporter plasmid DNA and 1 mg gold (Aldrich Chemical Co., Milwaukee, WI) particles were mixed at room temperature in a solution of 1.2 M $CaCl_2$ and 100 mM spermidine at a final volume of 30 μl for 20 min. The DNA-gold suspension was microcentrifuged, decanted, and resuspended in 70% ethanol (wash), microcentrifuged, decanted, and resuspended in 100% ethanol. DNA/gold particles were spotted onto a mylar film and allowed to air dry. DNA/gold particles were accelerated by the force of a 10–12-kV electric discharge from a 2-μF capacitor. The discharge apparatus was contained within a vacuum chamber. The mylar film was positioned in between the discharging electrodes and a steel screen (100 mesh) (7 mm between the mylar film and the screen), which stopped the film but allowed the particles to travel to their target (5 mm distance between the screen and the fetal thymic lobes). The fetal thymic lobes were coated with DNA/gold particles that penetrated to a depth of 8–10 cell layers, as determined by microscopic examina-

tion of frozen sections. No gross disruption of the thymic capsule was apparent, and the fetal lobes remained viable. The entire procedure lasted <1 min and the lobes were placed back onto the sponge for a 24–48-h culture.

CAT Assays. CAT assays were performed using standard procedures (31) and extracts were normalized by protein concentration. Chloramphenicol conversion was quantitated directly after thin layer chromatographic separation by using a Fast Scan Computing Densitometer (Molecular Dynamics, Inc., Sunnyvale, CA).

Results and Discussion

Day 14/15 Fetal Thymocytes Have Phenotypic Features of Activated Mature T Lymphocytes. Day 14/15 fetal thymocytes were prepared by removing thymic lobes by microscopic surgery and disrupting the organ structure by gentle homogenization. Thymocytes were purified by fine mesh filtration that trapped stomal supporting cells. Preparations were >98% free of stromal cells as determined by flow cytometry. Such preparations yielded fetal thymocytes negative for CD4 and CD8 (data not shown). Though day 14 double-negative cells may contain a very small fraction of T cells bearing Vγ3/Vδ1 receptors (32), the vast majority are progenitor cells that have not yet begun rearrangement of the α and β TCR loci. Scollay et al. (5), and other groups have used several markers to subdivide and determine lineages using analytical flow cytometry of adult $CD4^-/CD8^-$ thymocytes (33). Flow cytometric analysis shows that our preparation of fetal thymocytes express cell surface markers that are characteristically present or upregulated on activated mature TCR^+ T cells including CD25 (IL-2Rα) (22), CD54 (ICAM-1) (18), CD59 (Ly-6A/E) (15), HSA (Zúñiga-Pflücker, J. C., unpublished observations) and CD44 (Pgp-1) (19) (Fig. 1*a*). The cells are <1% positive for CD3 (Fig. 1 *a*). These cells are also undergoing rapid DNA synthesis as evidenced by the high percentage (>25%) incorporating the nucleoside analog BrdU into their DNA within the first 2 h of in vivo exposure (Fig. 1 *b*). Adult unfractionated thymocytes do not show a high incorporation of BrdU in such a short time (<10%; data not shown) (34). Early thymocyte resemblance to activated mature T cells is also apparent in a Northern blot probed for IL-2 mRNA (Fig. 2). IL-2 message is present early in ontogeny (day 14), peaked by day 15, decreased by day 16, and virtually absent later in development when the percentage of immature TN thymocytes is very low. The apparent transcription of the IL-2 gene must be initiated early in thymocyte development since there is a high expression by day 14 and no expression in fetal liver. Previous studies have provided evidence both for and against the notion that the IL-2 produced by fetal thymocytes contributes to the cellular proliferation that occurs at this early stage (6, 35–38). Nevertheless, the expression of the many lymphokines and their receptors can be interpreted as evidence of an inductive or activation event. Taken together with previous reports, these results indicate that our preparations of early TN thymocytes contain cell types that appear to mimic the phenotype of activated mature T cells.

Molecular Control of Activation in Immature Thymocytes. The functional phenotype and precursor potential of TN thymocytes implies that highly regulated molecular events lead to the pattern of gene expression observed in these cells. We prepared nuclear extracts from fetal thymocytes at different stages of development and analyzed DNA-binding transcription factors by EMSA. We studied the factors implicated in the control of IL-2 and IL-2Rα genes, which are expressed by mature T cells after TCR-mediated activation (22, 28, 29). Fig. 3 *a* shows that early in development (day 14–16), nuclear extracts from fetal thymocytes contain the nuclear factor (NF)-κB (20), which governs many genes with immunoregulatory function (39). The complex can be competed by using unlabeled oligonucleotides containing the Ig-NF-κB recognition sequence (Fig. 3 *a*, +, ++), but not by an irrelevant oligonucleotide (Fig. 3 *a*, *n*). The NF-κB heterodimer (p50/p65) participates in the transcription of the IL-2 and IL-2Rα genes in antigen-activated mature T cells, but is lower in abundance in resting T lymphocytes (28). Though NF-κB was evident in early development (days 14–16), the complex was not detected at later times (day 18 to adult) when $CD3^+$ cells were predominant.

Resting mature T cells contain large amounts of a faster migrating p50 homodimer complex, NF-κC, which is downregulated after antigen activation (28). The IL-2κB site can be repressed by NF-κC (28). We observed significant quantities of the NF-κC complex throughout thymic ontogeny (Fig. 3 *a*), suggesting that the IL-2κB site and the IL-2 gene may be modestly active in some early cells and completely shut off at later times. These results are consistent with IL-2 mRNA (Fig. 1 *b*), which is present at day 14/15 but then undetected from day 17 onward. Moreover, the early presence of significant levels of the NF-κC repressor complex may explain previous in situ hybridization data showing that many thymocytes even at day 14/15 do not express IL-2 mRNA and positive cells have significantly fewer IL-2 transcripts than other genes expressed at this stage (40; Zúñiga-Pflücker, J. C., unpublished observations) (see below). Thus, two forms of κB complex are present in immature TN thymocytes including the active form of the NF-κB complex that upregulates IL-2 and IL-2Rα gene expression in mature T cells.

Day 14/15 thymocytes, but not adult thymocytes, also express the DNA-binding complex activator protein (AP) 1 (Fig. 3 *b*). The AP-1 transcription factor is comprised of the products of the c-*fos* and c-*jun* oncogenes (41), and found to be expressed early during the activation of T cells (22). Functional AP-1 sites are present in the IL-2 gene (29). The protein–DNA complex that we detect can be specifically competed by a consensus AP-1 recognition sequence (Fig. 3 *b*, +, ++), but an irrelevant oligonucleotide (Fig. 3 *b*, *n*) fails to compete. We also found that another oncogene product, c-*myc*, that may control cell cycle progression and is induced upon activation (22), is present in the early thymocytes (day 14–15) (data not shown).

Recently, it was shown that the nuclear factor of activated T cells, (NF-AT), is comprised of the oncogene products of c-*fos* and c-*jun* and an unidentified novel subunit, creating a protein complex that specifically recognizes the NF-AT element (42). The NF-AT complex is an inducible DNA-binding

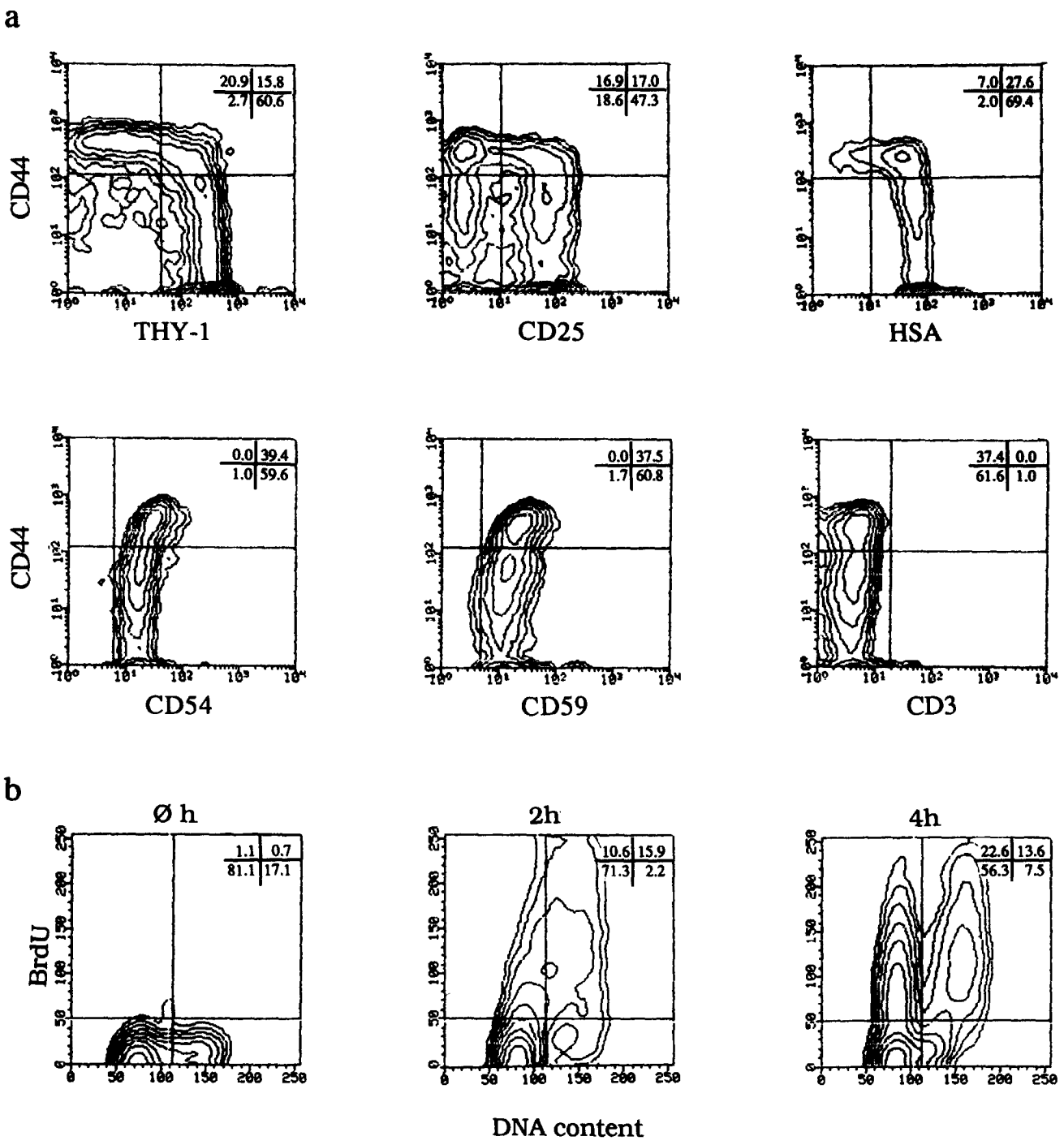

Figure 1. (*a*) Two parameter flow cytometry analysis of cell surface molecules expressed on day 15 fetal thymocytes of C57Bl/6 mice, CD44 (Pgp-1) vs. THY-1, CD25 (IL-2R), HSA, CD54 (ICAM-1), CD59 (Ly-6A/E), and CD3. Freshly isolated fetal thymocyte suspensions were prepared for flow cytometry as described in Materials and Methods. Thymocytes were >98% free from stroma elements as determined by forward- vs. side-scatter. (*b*) Freshly isolated day 15 fetal thymocytes from pregnant mice treated intraperitoneally with 2 mg/mouse of BrdU were isolated at the indicated times and fixed with ice-cold ethanol. Fixed cells were stained with anti-BrdU antibody and propidium iodide. Antibodies used for flow cytometry were obtained from Pharmingen (San Diego, CA). All plots display 10,000 events; the events contained in each quadrant are given as percent of total in the upper right corner.

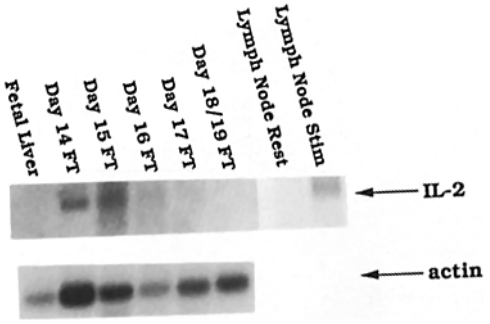

Figure 2. Northern blot analysis of RNA from thymocytes at different stages of development and lymph node cells that were freshly isolated or stimulated for 6 h with 5 μg/ml Con A. The Northern blot was successively hybridized with mouse IL-2 and actin cDNA probes. Probes were obtained from restriction fragments of plasmids containing the mouse IL-2 or mouse actin cDNAs. Restriction fragments were labeled using a random primed DNA labeling kit (Boehringer Mannheim, Indianapolis, IN). Preparation of lymph node (*Rest/Stim*) RNA showed equal loading and integrity as determined by ethidium bromide staining. (*Arrows*) mRNA corresponding to the appropriate molecular weight.

complex responsible for IL-2 gene control in activated T cells (22). NF-AT is expressed at low levels by the earliest thymocytes (day 14), increases to high levels at day 15 and 16, and then is undetectable in the adult thymus (Fig. 3 *c*). The NF-AT complex can be specifically competed with the NF-AT recognition sequence (data not shown). Thus, the expression of IL-2 mRNA early in development (days 14–16), but not in adult thymus, correlated with the presence of three activators of the IL-2 gene: NF-κB, AP-1, and NF-AT. The delayed appearance of the NF-AT complex may account for the IL-2 RNA peaking by day 15 instead of day 14 when NF-κB and AP-1 are highest, moreover NF-AT reaches its maximum level by day 16 when NF-κB and AP-1 are decreasing. Thus, day 15 may constitute the moment when all three factors reach sufficient levels for the induction of IL-2, whereas in day 14 NF-AT is limiting, and in day 16 NF-κB and AP-1 are limiting. This intricate pattern also suggests that more than one activation/induction event is acting upon these early thymocytes.

We also detected the presence of the homeo-domain proteins oct-1 and oct-2 (43) and the serum response factor (SRF) (44) throughout thymic development (Fig. 3, *d* and *e*). In adult thymus, oct-2 is present at comparatively low amounts. Therefore, our results show the binding activities of certain inducible T cell transcription factors, NF-κB, AP-1, NF-AT, and oct-2 that control T cell activation genes such as IL-2 or the IL-2 receptor α chain gene are largely restricted to a stage early in thymocyte development. At the later $CD4^+/CD8^+$ (double positive) stage of differentiation, which comprises >80% of adult thymocytes, these factors are apparently switched off (45). By contrast, factors that are constitutively expressed in mature T cells such as oct-1 and SRF are present early on and maintained throughout thymic development.

Functional Presence of Key Regulatory Factors in Immature Thymocytes. We next sought evidence that these nuclear factors were functional. We transfected several CAT-reporter constructs (Fig. 4 and 28, 29) into fetal thymocytes. The CAT-reporter constructs contained tandem repeats of the recognition sequences for the various nuclear factors driving minimal promoters from either the thymidine kinase (tk) or c-*fos* genes (Fig. 4). Fig. 5 shows that day 14 fetal thymocytes transfected with a plasmid containing two copies of the Ig-NF-κB site display a high level of CAT enzyme activity. The activity observed indicates that the NF-κB present in early thymocyte nuclei can effectively drive the expression of a CAT reporter gene. However, the thymocytes in this experiment were tested in cell suspension and therefore not in their natural thymic micro-environment.

To preserve the structural integrity of the thymus during gene analysis, a novel approach, biolistics, allows for the introduction of plasmid DNA into intact cells, tissues, or organs, using the penetration of accelerated DNA-coated gold microspheres (30). We adapted biolistics to study gene expression in intact fetal thymic lobes. Initial control experiments showed that biolistic DNA transfer did not appreciably disrupt the fetal thymic structure, presumably allowing the transfected cells to respond to the normal environmental stimuli that the thymic architecture imparts (46). Histochemical analysis showed that DNA/gold particles could effectively penetrate as deep as six to eight cell layers into the subcapsular and outer cortical region of the fetal thymic lobe and could transfect a wide distribution of immature thymocytes, therefore obtaining a representative transfected population (data not shown). As with the electroporated day 14 fetal thymocytes (Fig. 5), the fetal lobes transfected by biolistics showed strong transcriptional activity of an NF-κB binding element (Fig. 6). A construct with mutated NF-κB sites gave no CAT activity by this approach (data not shown). This confirms that the presence of active NF-κB apparent in previous experiments (Fig. 5) is not an artifact of our cell preparations. Although the biolistic method provided successful transfections, it was less efficient than transfection by electroporation of cell suspensions.

Biolistic transfections also revealed that reporter constructs driven by the binding sequences for NF-AT, AP-1, and the octamer factors, as well as the TCR-α enhancer (47), were activated above the minimal level of activity of the tk or c-*fos* minimal promoter alone. The NF-κB and the AP-1 reporter constructs consistently gave the strongest signal whereas the octamer reporter was the weakest. Control experiments with either mock CAT reactions or extract from thymuses bombarded with DNA-free gold particles confirmed that there was no nonspecific acetylation of chloramphenicol (Fig. 6). Because each of these reporter constructs is activated in mature T cells stimulated by peptide antigen (29), immature thymocytes are capable of transcriptional processes that are characteristic of activated mature T cells. Consistent with the EMSA results showing an abundance of the inhibitory NF-κC p50 homodimer complex, the IL-2κB motif or the entire IL-2 promoter which contains a site with high affinity

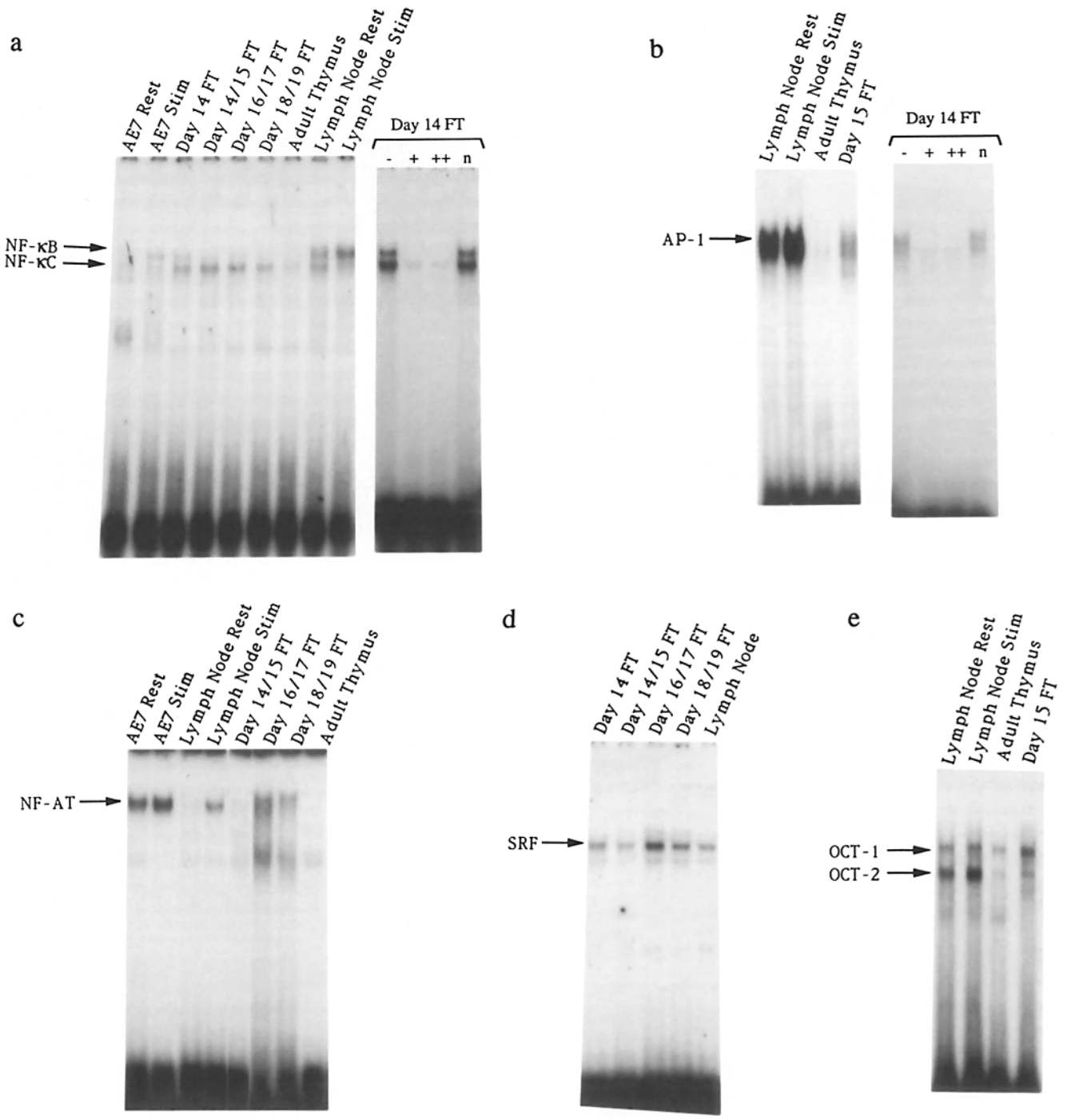

Figure 3. EMSA of nuclear extracts from thymocytes at different stages of development. Nuclear extracts were prepared from freshly isolated thymocytes, lymph node cells, or CD4+ T_H1 cell clone (AE7) (28, 29) as described in Materials and Methods. Lymph node cells were stimulated with 5 μg/ml of Con A for 6 h and A.E7 cells were stimulated with 10 μM peptide antigen presented by APCs (29). The extracts were mixed with ^{32}P-oligonucleotide probe containing binding sequences as previously described (29) for the (*a*) NF-κB, (*b*) AP-1, (*c*) NF-AT, (*d*) SRF, and (*e*) octamer. (*a* and *b*) Day 14 FT nuclear extracts were treated as above (−), or with the addition of a cold oligonucleotide probe competitor, either specific (+, ++) (4 or 8 ng, respectively) or nonspecific (*n*, 8 ng). Note that the AP-1 autoradiograph is overexposed so that the difference between day 15 and adult is clear though induction in the lymph node is obscured. The NF-κB oligo contains the NF-κB consensus site from the mouse Ig κ light chain enhancer; the AP-1 oligo contains the AP-1 consensus site from the human collagenase enhancer; the NF-AT oligo contains the NF-AT consensus site from the mouse IL-2 enhancer; the SRF oligo contains the consensus SRF site from the human IL-2Rα enhancer; and the octamer oligo contains the consensus octamer site from the mouse IL-2 enhancer.

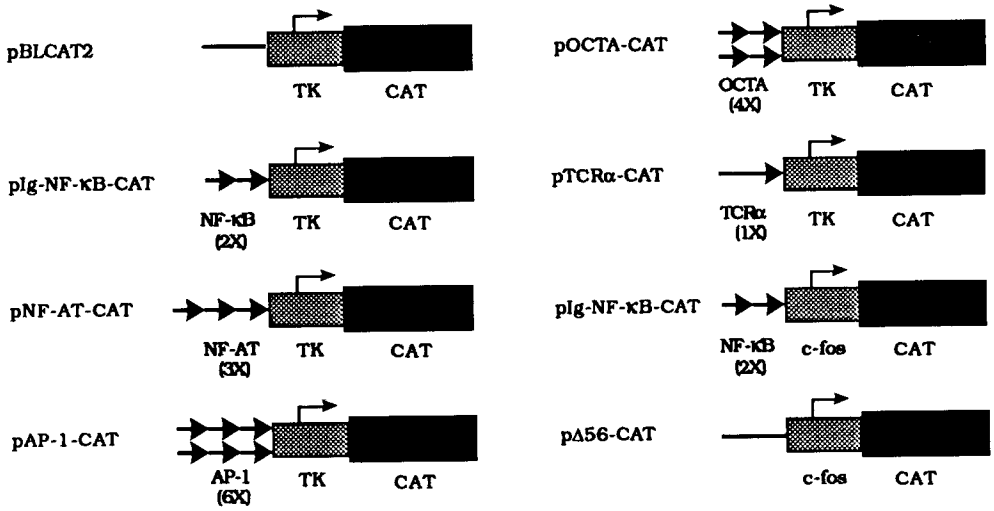

Figure 4. Diagram of the CAT reporter plasmid used for transfectants. The backbone plasmid was either the pBLCAT2 plasmid or the Δ56-*c-fos*-CAT plasmid (28, 29). Oligonucleotides containing the various *cis*-acting sequences, as indicated in the Fig. 3 legend, were cloned in front of the TK or *c-fos* minimal promoters, as monomers or in tandem repeats as indicted by the number of arrows. The plasmid constructs were previously verified by DNA sequencing (29).

for the p50 complex yielded low activity in a biolistics assay (data not shown). These results reinforce the notion that IL-2 is synthesized at low levels by a fraction of early thymocytes. Because most day 14 thymocytes lack a TCR–CD3 complex, the transcriptional events we observe may be governed by a novel and distinct pathway of activation.

Functional Expression of Key Enhancer Element Factors in RAG2$^{-/-}$ Thymocytes. Although the vast majority of day 14/15 fetal thymocytes do not bear TCRs, there exists a small percentage of either TCR$^+$-γ/δ and TCR$^+$-α/β thymocytes (1). These TCR$^+$ thymocytes may influence the state of activation of the other thymocytes, or themselves display the gene regulatory molecules of activated mature T cells, which are detected by our sensitive EMSA and CAT reporter transfection assays. To directly address this issue, we used mutant mice lacking the RAG2 that do not undergo DNA recombination in the joining regions of antigen receptor genes (23). These thymocytes cannot produce a TCR and remain arrested at the TN stage of development (23). Thus, thymuses of RAG2$^{-/-}$ mice serve as source of TN thymocytes without contamination by TCR$^+$ (γ/δ–α/β) cells. Fig. 7 *a* shows that thymocytes prepared from young adult RAG2$^{-/-}$ mice are very similar to day 14/15 fetal thymocytes by flow cytometry analysis, because they both have high percentages of Thy-1, HSA, CD25 (IL-2R), CD54 (ICAM-1), and CD59 (Ly-6A/E), positive cells. They differ in that the RAG2$^{-/-}$ thymocytes have relatively fewer CD44 bright cells than day 14/15 fetal thymocytes. Moreover, the RAG$^{-/-}$ thymocyte

Electroporation of Day 14 FT

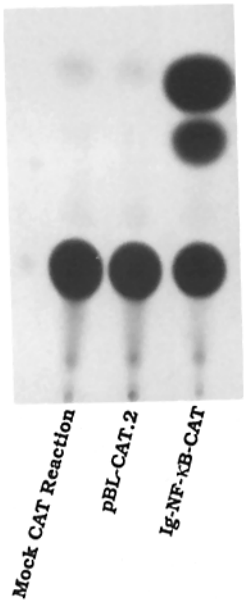

Figure 5. NF-κB is functionally active in day 14 fetal thymocytes. 24 h after transfection by electroporation with the Ig-NF-κB-CAT construct or the pBL-CAT2 backbone, thin layer chromatographic analysis of CAT enzyme activity of cellular extracts from transfected day 14 fetal thymocytes (6 × 10^6 cell/sample) were performed. Mock CAT reaction was used on extracts from thymocytes that were not electroporated. CAT assays were normalized for protein content of the cellular extract. 25 μg of protein were used in each assay. Percent acetylation of 6.0, 6.1, and 61.1% were observed for mock, pBL-CAT2, and p-Ig-NF-κB-CAT transfections, respectively.

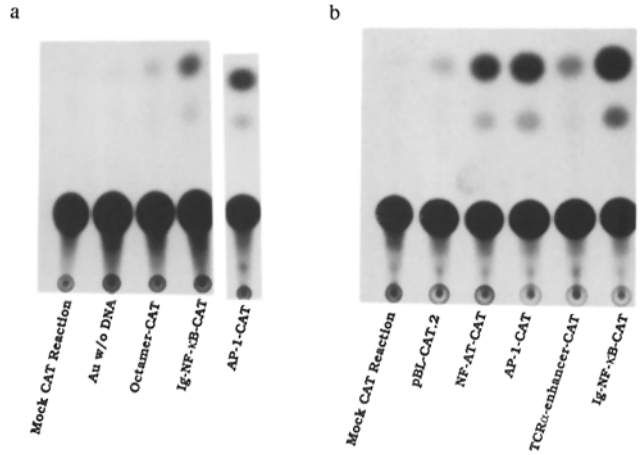

Figure 6. Biolistic transfection demonstrates the transcriptional functions of intact day 14 fetal thymic lobes. FTOC were set up as described (23) and served as targets for the accelerated gold/DNA particles, 48 h later CAT assays of cellular extracts were performed. Biolistic transfections are detailed in the Materials and Methods section. Two independent experiments (*a* and *b*) are shown that are representative of six experiments. CAT assays were normalized for protein content of the cellular extract. 20 μg of protein were used in each assay. 30–48 fetal lobes were used for each bombardment. Induction of CAT activity as determined by percent acetylation over backbone control: (*a*) octamer 1.7X, Ig-NF-κB 4.2X, AP1 5.8X; (*b*) NF-AT 2.5X, AP-1 2.8X, TCR 1.4X, Ig-NF-κB 3.6X.

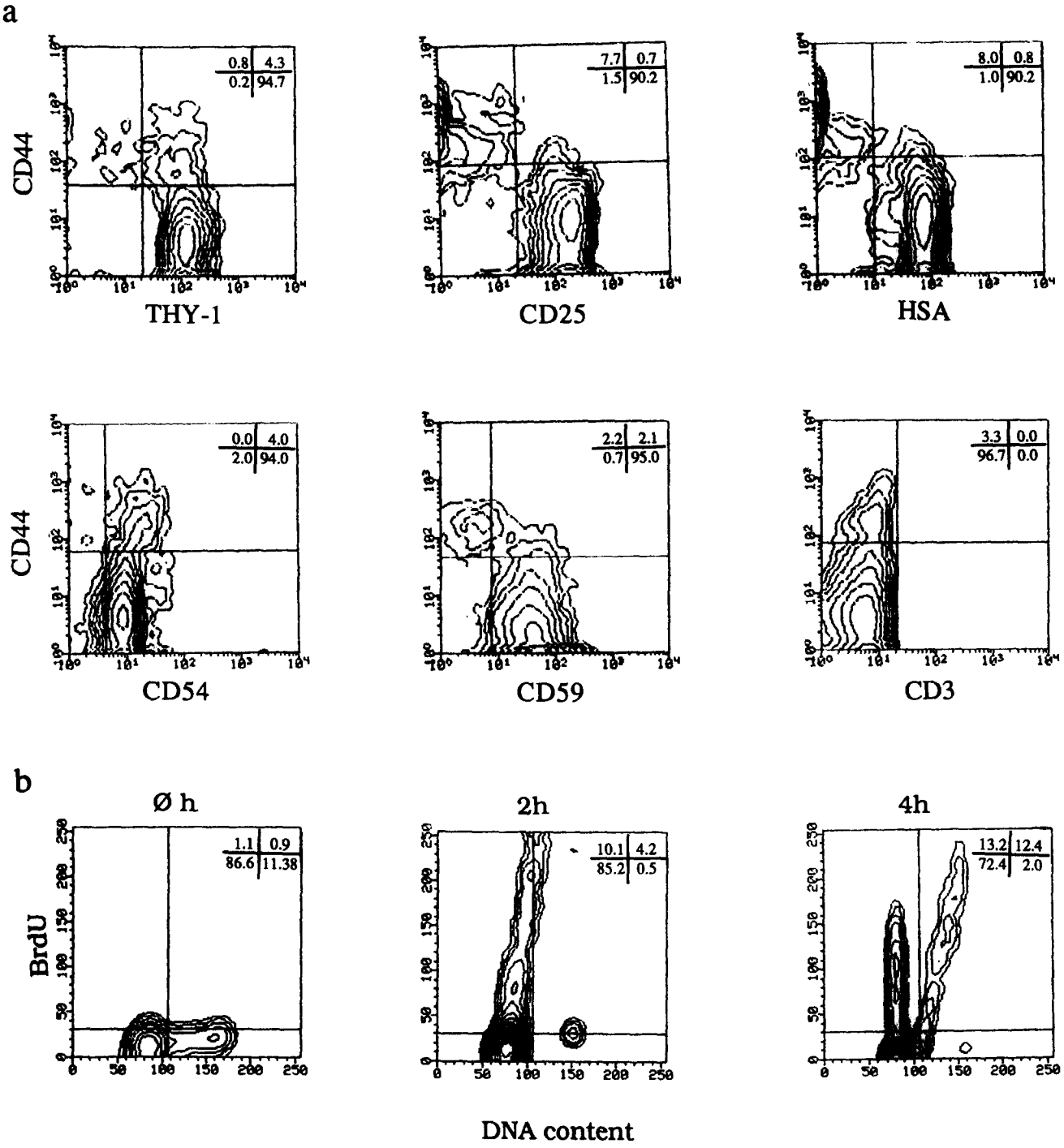

Figure 7. (*a*) Two parameter flow cytometry analysis of cell surface molecules expressed on thymocytes from RAG2$^{-/-}$ mice, CD44 vs. THY-1, CD25 (IL-2R), HSA, CD54 (ICAM-1), CD59 (Ly-6A/E), and CD3. Freshly isolated young adult thymocyte suspensions were prepared for flow cytometry as described. Thymocytes were >98% free from stroma elements as determined by forward- vs. side-scatter. (*b*) Freshly isolated young adult thymocytes from mice treated intraperitoneally with 0.5 mg/mouse of BrdU were collected at the indicated times and fixed with ice-cold ethanol. Fixed cells were stained with an anti-BrdU antibody and propidium iodide. All plots display 10,000 events. The events contained in each quadrant are given as percent total (*top right corner*).

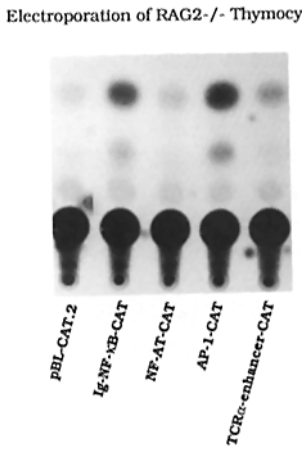

Figure 8. Several transcriptional elements are functionally active in thymocytes from RAG2$^{-/-}$ mice. After a 24-h incubation, CAT assays of cellular extracts from freshly isolated thymocytes from 4-wk-old RAG2$^{-/-}$ mice transfected by electroporation (10×10^6 cells/sample) were performed. CAT assays were normalized for protein content of the cellular extract. 25 μg of protein was used in each assay. Shown is a representative experiment from two independent trials. Average induction of CAT activity as determined by percent acetylation over backbone control: Ig-NF-κB 6.3X, NF-AT 2.4X, AP-1 9.2X, TCRα 1.5X.

population is almost entirely encompassed by the IL-2R$^+$ stage of thymic development (>90%), allowing us to better assess this specific stage in T cell development.

Thymocytes from in vivo BrdU treated RAG$^{-/-}$ mice showed a high percentage of cells incorporating the nucleoside analog in a 4-h period (>25%) (Fig. 7 *b*), similar to that seen in fetal thymocytes. Surprisingly, this data suggests that RAG2$^{-/-}$ thymocytes are proliferating despite the fact that the thymus is <10% the size of a normal adult mouse (23).

As shown in Fig. 8, electroporation of freshly isolated RAC2$^{-/-}$ thymocytes with NF-κB, AP-1, NF-AT, and TCR-α enhancer plasmid CAT reporter constructs reveals that, as in the fetal immature thymocytes, each is active. Again, the NF-κB and the AP-1 constructs consistently give the strongest activity. Thus, the activation-like molecular features of fetal thymocytes are apparently not due to the presence of small numbers of TCR$^+$ thymocytes. These data further support the view that the early TN thymocytes may employ a novel, TCR-independent, activation pathway which is intact in the RAG2$^{-/-}$ thymocytes.

Conclusion. Our data show that day 14/15 TN fetal thymocytes contain a large population of cells that have the phenotype of antigen-activated mature T cells. On their surface, these cells express IL-2Rα chain, HSA, Ly-6A/E, and ICAM-1, but have no TCR–CD3 complex. These cells were also proliferating and expressed IL-2 mRNA. At the molecular level, we detected the presence of the DNA-binding transcription factors NF-κB, NF-κC, AP-1, NF-AT, SRF, oct-1, and low levels of oct-2. These binding activities are functional since transfected CAT reporter constructs dependent on their respective *cis* elements were active. In adult thymocytes, the NF-κB, NF-AT, and AP-1 binding complexes were undetectable, and it was not possible to test transfected DNA in these cells because they are quiescent (48). Finally, RAG2$^{-/-}$ thymocytes which were genetically devoid of any TCR$^+$ cells, had a similar cellular, proliferative, and molecular phenotype to day 14/15 TN thymocytes. In contrast to activated mature T cells, the IL-2 promoter, the octamer element, and the TCR-α enhancer exhibit only weak activity in day 14/15 TN fetal thymocytes. Thus, early thymocytes differ from activated mature T cells in that they have high expression of the IL-2Rα gene, but apparently low expression of the IL-2 gene itself.

Newly arriving thymic progenitors come to thymus and encounter a nurturing atmosphere that commits them to a path of T cell differentiation. Our results suggest that upon commitment to this path, early thymocytes are "stimulated" and enter an activation-like state. The transcription events we observed involving NF-κB, AP-1, and NF-AT are probably not simply a reflection of the proliferating nature of early thymocytes. In mature T cells, the activity of these *trans*-activators are induced by specific signaling pathways, cause IL-2 production and proliferation, and then disappear when the process is underway (22, 28, 29).

We envision that the activation state of early thymocytes may serve two purposes. First, the primary role of the activation state may be to promote cell proliferation to expand the pool of potential thymic precursors. DNA replication at this stage may facilitate the process of TCR gene rearrangement. The fetal thymus grows in size exponentially during gestational days 13–17. This can be attributed, in part, to a constant influx from the fetal liver, but also to a rapid proliferation of new arrivals, as shown in this report (Fig. 1 *b*). Previous reports (49) show that thymic precursors lie within the IL-2R$^+$ population, indicating that up to this stage in thymic development, precursor potential is retained. A second role of the activation state may be to indicate successful commitment to the T lymphoid lineage by a transient display of differentiated functions. We favor the speculative idea that early thymocytes that fail to display the "activated" phenotype would be selected against during ontogeny and may even undergo elimination within the thymus.

Early thymocytes actively express lymphoid-specific inducible genes that are recapitulated later in the life of the T cell. In other instances genes expressed early in development later take on a new or related role in the adult organism (50, 51). It is important also to consider that the transcriptional phenotypes we detected in TN thymocytes and antigen-activated mature cells were not identical. Many day 14/15 thymocytes are strongly IL-2Rα^+ and express significant amounts of IL-2Rα mRNA (data not shown), but IL-2 expression is quite low. Moreover, whereas NF-κB and AP-1 elements were highly active, NF-AT was intermediate, and the octamer motif was weakly expressed. Thus, early thymocyte IL-2Rα^+ stage may reflect a modified subset of the gene activation events found in antigen-activated mature T cells. However, because day 14 TN thymocytes are likely to contain a variety of subpopulations (1–3), it is difficult to precisely delineate their differences with mature cells.

The notion that immature thymocytes undergo TCR-independent activation implies the existence of a signaling pathway independent of the TCR–CD3 complex that is important for early thymic development. The fact that RAG2$^{-/-}$ thymocytes show a similar pattern of cellular, proliferative, and transcriptional features also suggests some form of TCR-independent activation. The most likely source for an activation stimulus is the thymic stroma. However, the molecular signals between fetal liver (bone marrow) immigrants and thymic stromal cells that govern the activation

process are as yet obscure. Recent evidence indicated that thymocyte differentiation requires interactions with more than one kind of stromal cell (52, 53).

It is intriguing that RAG2$^{-/-}$ thymocytes rapidly incorporate the nucleoside analog BrdU in vivo, suggesting that they are actively proliferating (Fig. 7 *b*). By our analysis and that of others (23), the thymus in RAG2$^{-/-}$ mice only achieves a size less than one tenth that of a normal mouse. Cell counts confirm the smaller organ in RAG$^{-/-}$ mice contains <10% of the normal number of cells in the adult. This is about the same number of TN thymocytes present in a normal adult thymus. It is puzzling that the TN thymocytes are incapable of occupying the "space" that typically accommodates TCR$^+$ cells. Recent evidence suggests that survival into the next step of differentiation may be mediated through the expression of a rearranged TCR β chain homodimer (54). The similarity of the persisting phenotype of RAG2$^{-/-}$ thymocytes to that of fetal thymocytes before TCR rearrangement would be consistent with a limited thymic lifespan if TCR is not available to allow thymocyte differentiation to proceed.

Taken together, our data suggests that immature thymocytes appear to undergo a process of TCR-independent activation that might increase the number of thymocyte precursors as well as stimulate the differentiation process and lymphoid lineage commitment. The activation events, when examined molecularly, partly resemble those seen in mature antigen-activated T cells.

We are grateful to Frederick W. Alt for providing us with the RAG2$^{-/-}$ mice and B. J. Fowlkes and Ron Germain for critical comments and advice on the manuscript. We also thank Stefen Boehme, Kevin Bruhn, Jeffrey Critchfield, and Di Jiang for their constant support.

This work was supported in part by a Cancer Research Institute Investigator Award to M. J. Lenardo. J. C. Zúñiga-Pflücker is a Jane Coffin Childs Memorial Fund for Medical Research Fellow.

Address correspondence to Juan Carlos Zúñiga-Pflücker, NIH, NIAID, Laboratory of Immunology, Bldg 10, Rm 11D09, Bethesda, MD 20892. Heather L. Schwartz is currently at the University of California, Berkeley, CA 94708.

Received for publication 6 April 1993 and in revised form 20 May 1993.

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