MOLECULAR AND CELLULAR BIOLOGY, Mar. 1996, p. 1074–1084 Vol. 16, No. 3
0270-7306/96/$04.00+0

Regulation of AP-1 and NFAT Transcription Factors during Thymic Selection of T Cells

MERCEDES RINCÓN[1] AND RICHARD A. FLAVELL[1,2]*

Section of Immunobiology[1] and Howard Hughes Medical Institute,[2] Yale University School of Medicine, New Haven, Connecticut 06510

Received 21 August 1995/Returned for modification 2 November 1995/Accepted 7 December 1995

The ability of thymocytes to express cytokine genes changes during the different stages of thymic development. Although $CD4^-$ $CD8^-$ thymocytes are able to produce a wide spectrum of cytokines in response to a T-cell receptor (TcR)-independent stimulus, as they approach the double-positive (DP) $CD4^+$ $CD8^+$ stage, they lose the ability to produce cytokine. After the DP stage, thymocytes become single-positive $CD4^+$ or $CD8^+$ thymocytes which reacquire the ability to secrete cytokines. In an attempt to understand the molecular basis of this specific regulation, we use AP-1–luciferase and newly generated NFAT-luciferase transgenic mice to analyze the transcriptional and DNA-binding activities of these two transcription factors that are involved in the regulation of cytokine gene expression. Here, we show that both AP-1 and NFAT transcriptional activities are not inducible in the majority of DP cells but that during the differentiation of DP cells to the mature single-positive stage, thymocytes regain this inducibility. Subpopulation analysis demonstrates that this inducibility is reacquired at the DP stage before the down-modulation of one of the coreceptors. Indeed, AP-1 inducibility, just like the ability to express the interleukin-2 gene, is reacquired during the differentiation of DP TcR^{low} $CD69^{low}$ heat-stable antigen $(HSA)^{high}$ thymocytes to DP TcR^{high} $CD69^{high}$ HSA^{high} cells, which is considered to be the consequence of the first signal that initiates positive selection. We therefore propose that the inability of DP thymocytes to induce AP-1 and NFAT activities is one of the causes for the lack of cytokine gene expression at this stage and that this inducibility is reacquired at the latest stage of DP differentiation as a consequence of positive selection. This could be a mechanism to prevent the activation of DP thymocytes before selection has taken place.

The development of T lymphocytes in the thymus involves both negative and positive selective events that eliminate self-reactive cells and promote survival of cells that are capable of recognizing foreign antigens (8, 41). During this process, immature double-negative (DN) $CD4^-$ $CD8^-$ T-cell receptor-negative (TcR^-) thymocytes differentiate into so-called double-positive (DP) $CD4^+$ $CD8^+$ TcR^{low} (DP^{low}) cells. These bipotential precursors differentiate to $CD4^+$ $CD8^-$ TcR^{high} or $CD4^-$ $CD8^+$ TcR^{high} cells which will recognize peptide antigens in the context of major histocompatibility complex (MHC) class II or class I molecules, respectively. Analysis of TcR transgenic mice (3, 32, 33, 45, 55, 57, 62) as well as MHC class II- and class I-deficient mice (10, 16) has shown that this decision is mediated by positive selection that is dictated by the interaction of a specific TcR, associated with a CD4 or CD8 coreceptor, with the appropriate positive-selecting MHC class II or class I, respectively, on epithelial cells of the thymus.

Two models of thymocyte selection have been proposed to explain the decision of a DP cell to commit to the CD4 or CD8 lineage (6, 50, 66), although this question is still open. The instructive model proposes that positive selection occurs at the DP stage of maturation. A $CD4^+$ $CD8^+$ cell specifically interacts with MHC class I or class II molecules through its TcR and CD8 or CD4 coreceptor, respectively. The TcR-mediated signal may differ depending on which coreceptor binds to the MHC molecule, and it dictates whether thymocytes down-modulate CD8 or CD4 to proceed with differentiation. In contrast, the stochastic/selective model originally postulated that positive selection occurs at the single-positive stage of maturation. $CD4^+$ $CD8^+$ cells randomly shut off either CD4 or CD8 independently of the specificity of the TcR. Subsequently, the single-positive cell that can engage an MHC molecule on a thymic stromal cell with its TcR and the appropriate coreceptor will be positively selected; those cells that are not selected die. A more recent version of this model proposes that $CD4^+$ $CD8^+$ thymocytes must engage MHC molecules twice en route to become mature single-positive cells (10, 16). The first TcR-MHC engagement received by DP cells results in the up-regulation of TcR and the progressive random down-modulation of CD4 or CD8. The second engagement, which requires that the chosen coreceptor and the TcR bind to the same MHC molecule, completes positive selection, after which cells become single positive and migrate to the medulla.

In addition to differing in cell surface phenotype, these thymocyte subpopulations are functionally different in terms of their patterns of cytokine production and proliferation. Around days 14 to 15 of gestation, fetal thymocytes are predominantly DN $CD4^-$ $CD8^-$ TcR^- cells that express mRNAs for interleukin-1β (IL-1β), IL-4, IL-5, IL-6, IL-7, gamma interferon, tumor necrosis factor, and IL-2 and also exhibit surface markers of activated T lymphocytes such as CD25 or CD44 (9, 18, 38, 68). Although resting triple-negative thymocytes from adult mice do not express any cytokines, expression can be induced by TcR-independent stimulation with phorbol esters and calcium ionophore (68). However, as these cells approach the DP stage, there is a striking loss in the ability to produce cytokines (11, 36, 48). In contrast, mature single-positive thymocytes regain the capacity to express specific cytokines. The mechanism that ensures the extinction of cytokine gene transcription at the DP stage is not known. It is possible that this is a control mechanism to prevent a potential TcR-

* Corresponding author. Mailing address: Section of Immunobiology, Yale University School of Medicine, P.O. Box 3333, New Haven, CT 06510.

mediated signal from resulting in the activation of DP thymocytes that have not yet been subjected to positive or negative selection.

Since cytokine gene expression is regulated by multiple transcription factors, specific regulation of these factors at the different stages of thymocyte development could explain the pattern of cytokine production. The best-characterized cytokine regulatory region is the IL-2 promoter (for a review see reference 47). This promoter region contains two Oct-1 binding sites called NFIL-2A and NFIL-2D, proximal and distal AP-1 and NFAT binding sites, and a single NF-κB binding site. The AP-1 transcription factor is a complex mixture of different members of the Fos (c-Fos, FosB, Fra-1, Fra-2, and FosB2) and Jun (c-Jun, JunB and JunD) family of proteins (13, 24, 25, 40, 52, 67). NFAT is composed, in part, of a preexisting cytoplasmic component (NFATp or NFATc) (19, 30, 37, 42) that translocates into the nucleus upon T-cell activation, where it associates with a nuclear component that, at least under certain conditions, contains members of the Jun/Fos family (5, 30, 31, 43). Although less characterized, NFAT or AP-1 binding sites have also been identified in the regulatory regions of other cytokine genes such as the IL-4, tumor necrosis factor, and IL-3 genes (46). The specific regulation of these transcription factors has been well studied during T-cell activation, but relatively little is known about the regulation during fetal (29, 68) and adult (11, 48, 56) thymic development. Interestingly, Zúñiga-Pflücker et al. showed that both AP-1 and NFAT are functional in DN thymocytes (68).

We analyze here the regulation of both DNA-binding and transcriptional activities of AP-1 and NFAT transcription factors during thymic development and selection processes. Using transcriptional reporter transgenic mice, we show that DP thymocytes are unable to induce either transcriptional or DNA-binding activities of AP-1 and NFAT upon stimulation. However, during the transition between DP^{low} to DP TcR^{high} (DP^{high}) thymocytes, at a stage when positive selection is believed to occur and before cells acquire the mature single-positive phenotype, thymocytes regain the ability to induce AP-1 and NFAT activity as well as IL-2 gene expression.

MATERIALS AND METHODS

Mice. The TG-B TcR (20) and AP-1–luciferase (AP1-luc) (49) transgenic mice were previously generated in our laboratory. The cytochrome *c* (Cyt c) TcR transgenic mice were kindly provided by S. M. Hedrick and J. Kaye (32). The MHC class II-deficient mice were a gift from D. Mathis and C. Benoist (14). The NFAT-luciferase (NFAT-luc) transgenic mice were generated by microinjection of the 2.8-kb *Bam*HI DNA fragment into fertilized (C57BL/6 × SJL)F_2 eggs as previously described (27). The NFAT-luc transgene contains the firefly luciferase gene driven by a 200-bp fragment of the IL-2 minimal promoter (−326 to −294 and −72 to +47) with three NFAT binding sites inserted in an *Xho*I site between these fragments (17, 65). Three expression-positive founder lines were established and backcrossed onto B10.BR/SGSNJ (Jackson Laboratory, Bar Harbor, Maine) to obtain progeny for these studies. All three lines display the same pattern but different levels of luciferase expression.

Cell preparation and staining. The different thymocyte populations were obtained by staining of total thymocytes with a directly fluorescein isothiocyanate-conjugated anti-TcR (H57-557) and directly Red^{613}-conjugated anti-CD4 (Gibco BRL, Gaithersburg, Md.) and biotinylated anti-CD8 (Becton Dickinson, Mountain View, Calif.) monoclonal antibodies (MAbs), in combination with phycoerythrin-streptavidin (PharMingen, San Diego, Calif.), and subjected to fluorescence-activated cell sorting (FACS) in a Becton Dickinson FACStar Plus cell sorter. The average purity for each population was 98%.

Four-color staining was carried out by using phycoerythrin-conjugated anti-CD8 (Caltag Laboratories, San Francisco, Calif.), Quantum Red-conjugated anti-CD4 (Sigma), fluorescein isothiocyanate-conjugated anti-TcR (H57-557), and biotinylated anti-heat-stable antigen (HSA) (PharMingen) or biotinylated anti-CD69 (PharMingen) MAbs, in combination with Texas red-conjugated streptavidin (Vector Laboratories, Burlingame, Calif.).

IL-2 production assay. Supernatants were collected after 24 h of stimulation from the same thymocyte cultures that were used later for detection of luciferase activity. IL-2 production was analyzed by using the CTL.L line as previously described (21).

TABLE 1. IL-2 production in thymocyte subpopulations

Cell population	Stimulus[a]	IL-2 production (U/ml)[b]	
		Expt 1	Expt 2
Total thymocytes	−	2	1
	P/I	38	26
DP	P/I	4	2
$CD4^+$	P/I	138	228
$CD8^+$	P/I	40	36

[a] Cells (4×10^5/0.5 ml) were incubated for 24 h in the absence (−) or presence (P/I) of PMA (5 ng/ml) plus ionomycin (250 ng/ml), and supernatants were assayed for IL-2 activity.

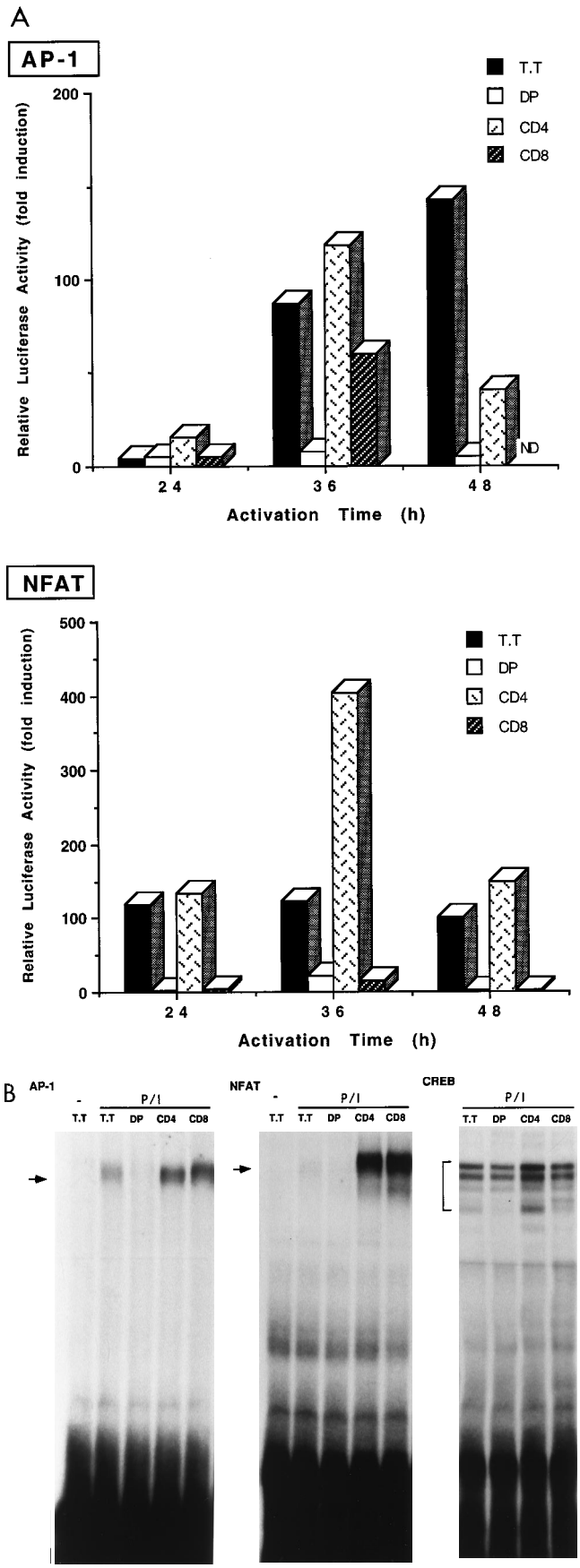
[b] Analyzed as described in Materials and Methods.

Luciferase activity analysis. Cells from either total thymus or individual subpopulations were incubated at 4×10^5 cells per well (48 wells per plate) in the presence or absence of phorbol 12-myristate acetate (PMA) plus ionomycin (Sigma, St. Louis, Mo.). After specific periods of time, cells from each independent well were harvested, washed twice in phosphate-buffered saline, and lysed in lysis buffer (luciferase assay; Promega, Madison, Wis.) for 30 min at room temperature. The lysate was spun down for 2 min, and total supernatant was analyzed by using a luciferase reagent (Promega) and measured in a luminometer (Lumat LB9501) for 10 s (two measurements of each independent sample were made). The background measurement was subtracted from each duplicate, and experimental values are expressed relative to the activity found in extracts from unstimulated cells (equal to the background).

Nuclear extracts and EMSA. Mini nuclear extracts were obtained from a small number of cells (1×10^6 to 4×10^6) by using a previously described procedure (54, 63). Binding reactions were carried out by using 1 to 2 μg of nuclear proteins and 4.5×10^4 cpm of double-stranded oligonucleotides end labeled with ^{32}P as previously described (54, 63). The double-stranded oligonucleotides used in the electrophoretic mobility shift assay (EMSA) contained the human collagenase tetradecanoyl phorbol acetate response element (TRE) (GTCGACGTGAGT CAGCGCGC) (1, 35) for AP-1 binding, the distal NFAT element from the mouse IL-2 promoter (GCCCAAAGAGGAAAATTTGTTTCATACAG) (31) for NFAT binding, and the consensus cyclic AMP response element (CRE) (GATCTCTCTGACGTCAGCCAAGGAGGAGGCG) (39) for the binding of CRE-binding protein (CREBP).

RESULTS

Lack of AP-1 and NFAT inducibility in DP thymocytes correlates with the lack in IL-2 production. We first analyzed the abilities of the various subsets of thymocytes to produce cytokines by analyzing IL-2 secretion upon stimulation. We isolated DN, DP, $CD4^+$, and $CD8^+$ populations from the thymuses of adult mice (8 to 12 weeks old) by cell sorting; under these conditions, cell populations of about 97 to 99% purity were obtained. Since the DP population expresses very low levels of TcR, we used a combination of PMA and calcium ionophore (ionomycin) as a TcR-independent stimulus that mimics the TcR-mediated protein kinase C activation and Ca^{2+} signals, respectively. Since it has been reported that PMA or ionomycin can induce apoptosis in thymocytes under certain conditions (34), we first optimized the concentrations of PMA and ionomycin that could stimulate thymocytes but not induce programmed cell death. The treatment of DP cells with PMA (5 ng/ml) plus high doses of ionomycin (1 μg/ml), a concentration previously used to induce apoptosis in thymocytes, resulted in rapid cell death. However, when the normally employed concentration of ionomycin (250 ng/ml) was used, DP cells remained viable throughout the course of our experiments (up to 48 h in culture at 37°C) (data not shown). We therefore used this concentration of ionomycin, which is the same as that used to activate peripheral T cells (49). As previously reported (11), we could detect some IL-2 production in stimulated DN thymocytes (data not shown) and high levels of IL-2 from activated $CD4^+$ thymocytes (Table 1). $CD8^+$ mature



thymocytes display a helper phenotype, since they are able to secrete IL-2 although to a lesser amount than $CD4^+$ thymocytes. However, DP thymocytes lack the ability to produce IL-2 in response to PMA and ionomycin (Table 1).

To study the molecular basis of the loss of cytokine production during the DP stage, we analyzed the activities of NFAT and AP-1, two inducible nuclear factors involved in the regulation of the expression of IL-2 and other cytokine genes. First, we analyzed the transcriptional activities of these two factors in the different thymic populations. To this end, we used AP1-luc reporter transgenic mice that had been previously generated in our laboratory (49). We have also generated reporter transgenic mice for NFAT carrying a construct in which the firefly luciferase gene is driven by three NFAT binding sites linked to the minimal IL-2 promoter (17, 65). The details of the activation properties of NFAT in peripheral T cells of these mice will be described elsewhere. Analysis by reverse transcriptase PCR have shown that the luciferase transcripts initiate around the predicted 5′ end for these promoters and that there is no significant level of readthrough from any other endogenous promoter in the vicinity of the integration site of the transgene in both transgenic models (data not shown). The luciferase activity in these mice, therefore, is a specific indication of AP-1- or NFAT-mediated transcription. Total thymocytes as well as FACS-sorted thymocyte subpopulations from AP1-luc or NFAT-luc transgenic mice were stimulated with PMA plus ionomycin. Cells were lysed at specified times, and luciferase activity was analyzed. High AP-1 and NFAT transcriptional activities were observed in both $CD4^+$ and total thymocytes; however, little activity was detected in the DP cells (Fig. 1A). Unexpectedly, we also detected lower AP-1 and NFAT transcriptional activities in the single-positive $CD8^+$ population than in the $CD4^+$ population.

The lack of AP-1 and NFAT transcriptional activities in the DP population could be due to the lack of DNA-bound complexes or to the absence of an additional posttranslational modification of the preformed complexes required to mediate transcriptional activity. To assess that possibility, we analyzed the DNA-binding activities for AP-1 and NFAT. Nuclear extracts were prepared from unstimulated total thymocytes or thymocytes from different populations stimulated with PMA plus ionomycin and analyzed for DNA-binding proteins by EMSA. No AP-1 and NFAT DNA binding was detected in unstimulated total thymocytes, but it was inducible upon stimulation (Fig. 1B). However, analysis of extracts from different subpopulations showed the absence of AP-1 and NFAT DNA binding in the DP cells upon stimulation (Fig. 1B), although both transcriptional factors were present in stimulated DN thymocytes (data not shown). After the DP cells differentiate to single-positive cells, both $CD4^+$ and $CD8^+$ thymocytes recover the inducibility of AP-1 and NFAT DNA binding (Fig.

FIG. 1. Transcriptional and DNA-binding activities of AP-1 and NFAT in different stimulated thymocyte populations. (A) Total thymocytes (T.T), DP, single $CD4^+$, or single $CD8^+$ sorted cells were obtained from AP1-luc (for AP-1 transcriptional activity) or NFAT-luc (for NFAT transcriptional activity) transgenic mice and stimulated with PMA (5 ng/ml) plus ionomycin (250 ng/ml). After the indicated periods of time, luciferase activity was analyzed. Luciferase values are expressed as fold activation over the value obtained from unstimulated cells. Results shown are representative of three independent experiments. (B) EMSA of nuclear extracts from different sorted thymocyte populations incubated in the absence (−) or presence of PMA (5 ng/ml) plus ionomycin (250 ng/ml) (P/I) for 6 h. For each assay, 2-μg aliquots of nuclear extracts were incubated with a ^{32}P-end-labeled 21-nucleotide oligomer containing the human collagenase TRE (AP-1 DNA binding), a 30-nucleotide oligomer containing the mouse IL-2 NFAT element (NFAT DNA binding), or a 31-nucleotide oligomer containing the consensus CRE (CREB DNA binding). The arrows show the different complexes.

1B). The AP-1 and NFAT complexes bound specifically since they were competed for by an excess of double-stranded unlabeled oligonucleotide that contained a consensus AP-1 site and the mouse IL-2 promoter NFAT site, respectively, and not by negative controls. In addition, antisera against Fos/Jun family members eliminated almost completely the AP-1 complex, and antisera against NFATp supershifted the NFAT complex (data not shown). As an internal standard for the quality of the extracts made from the different subpopulations, we also analyzed the DNA-binding activity of CREB (39), which is a constitutively expressed transcription factor; the levels of DNA binding of CREB were almost identical in the different subpopulations (Fig. 1B). Together, these data indicated that there was a specific lack of transcriptional as well as DNA-binding activities of AP-1 and NFAT nuclear factors in DP thymocytes, which might account for the inability to produce cytokines such as IL-2.

AP-1 and NFAT activities are inducible in DP thymocytes from TcR transgenic mice. Since mature single-positive thymocytes were able to induce AP-1 and NFAT activities upon stimulation, we next examined at which step during the differentiation of DP to the mature single-positive stage this inducibility is reacquired. The up-regulation of the TcR has been associated with a more mature stage of DP thymocytes, before they become single-positive cells (28, 44, 58, 60). Most DP thymocytes from normal mice express low TcR levels, and only a very small subpopulation express high levels of TcR. These TcR^{high} DP cells are believed to have undergone positive selection. Consistent with this view, it has been found that in TcR transgenic mice, a much higher fraction of DP cells express high TcR levels, but only when the appropriate positively selecting MHC ligand is expressed (6, 32). We were therefore interested in determining whether AP-1 and NFAT were inducible in the DP population from TcR transgenic mice.

TABLE 2. IL-2 production in thymocyte subpopulations from TcR transgenic mice

Cell population	IL-2 production (U/ml)[a]		
	Non-TcR	Cyt c	TG-B
Total thymocytes	27	179	97
DP	3	16	22
$CD4^+$	109	158	ND
$CD8^+$	10	ND	58

[a] Cells were stimulated as described in Table 1, footnote *a*, and IL-2 production was analyzed as described in Materials and Methods. ND, not done.

To address this question, we chose two different TcR transgenic systems: first, TcR transgenic mice (Cyt c TcR) expressing α- and β-chain genes that encode the receptor from a $CD4^+$ T-cell clone specific for pigeon Cyt c in association with $I\text{-}E^k$ class II MHC (32); and second, TcR transgenic (TG-B) mice which express α and β chains of the TcR from a $CD8^+$ T-cell clone specific for the simian virus 40 large T antigen in the context of MHC class I $H\text{-}2K^k$ (20). Figure 2 shows the distribution of the thymic populations (Fig. 2B) as well as the expression of the TcR in each population (Fig. 2A) in non-TcR transgenic mice and in both lines of TcR transgenic mice. As previously described (32, 64), a dramatic skewing toward the CD4 single-positive lineage was observed in thymuses from Cyt c TcR transgenic mice that express the selecting MHC k haplotype (Fig. 2). The skewing toward the CD8 single-positive stage was somewhat less dramatic in MHC class I-restricted

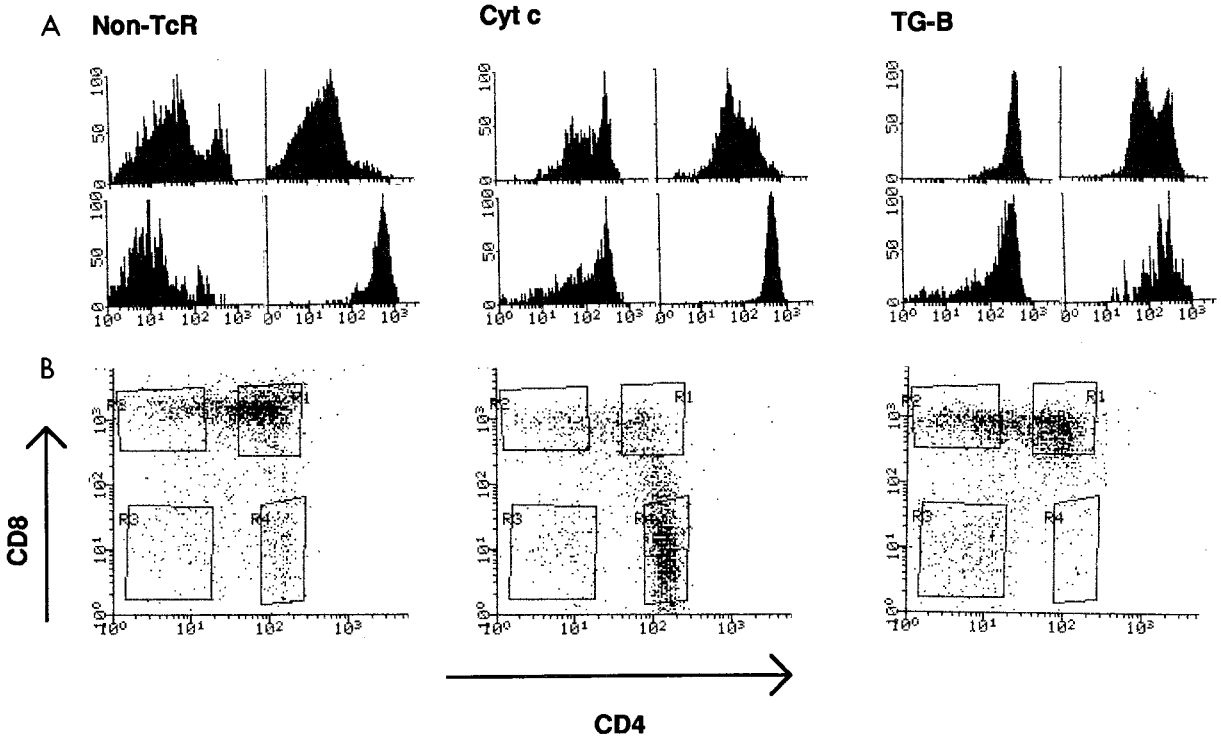


FIG. 2. Up-regulated TcR levels in DP cells from TcR transgenic mice. Thymocytes from non-TcR transgenic mice or Cyt c TcR and TG-B transgenic mice were analyzed by three-color flow cytometry. To set the gates for the four major thymic populations, we used anti-CD4 and anti-CD8 MAbs (B). Panel A represents the TcR expression detected by an anti-TcR MAb within each gate: $CD8^+$ cells (upper left), DP cells (upper right), DN cells (lower left), and $CD4^+$ cells (lower right).

TG-B transgenic mice expressing the H-2^k haplotype, possibly because of differences in TcR affinity for MHC or differences in the ability of the transgenic TcR β chain to associate with the transgenic versus endogenous α chain. DP thymocytes from both Cyt c TcR and TG-B transgenic mice expressed higher TcR levels than non-TcR transgenic mice (Fig. 2), although the levels differed somewhat between the two TcR transgenic systems.

We first compared IL-2 production by DP cells isolated from TcR and non-TcR transgenic mice upon stimulation with PMA plus ionomycin (Table 2). Interestingly, unlike DP cells from control non-TcR transgenic mice, DP cells from both Cyt c TcR and TG-B transgenic mice exhibit inducible IL-2 gene expression by this TcR-independent stimulus. We next analyzed the inducibility of AP-1 and NFAT activities in the TcR transgenic mice. To analyze the transcriptional activity mediated by these factors, we crossed the Cyt c TcR and TG-B transgenic mice with AP1-luc or NFAT-luc reporter transgenic mice. Different thymic subpopulations were isolated by cell sorting from double-transgenic mice (TcR × AP1-luc or TcR × NFAT-luc transgenic mice), stimulated with PMA plus ionomycin, lysed, and analyzed for luciferase activity. Unlike the activity observed in the non-TcR transgenic mice (Fig. 1A), AP-1 and NFAT transcriptional activities are significantly induced in DP cells from both the Cyt c TcR and TG-B transgenic mice (Fig. 3A to D). We also analyzed the DNA-binding activity in the DP population isolated from TcR transgenic mice. Although non-TcR transgenic mice did not show AP-1 and NFAT DNA binding in DP cells (Fig. 1B), DNA binding of both factors was observed in DP cells from TG-B transgenic mice, and lower but significant binding was seen in DP cells from Cyt c transgenic mice (Fig. 3E). The level of AP-1- and NFAT-mediated transcription correlated approximately but not absolutely with the level of IL-2 cytokine recovered from cell supernatants. This is not surprising since several transcription factors in addition to AP-1 and NFAT (NF-κB and Oct-associated protein [OAP]) bind to the IL-2 promoter. It is likely that their levels could also be rate limiting for IL-2 gene transcription. Moreover, numerous posttranslational events can also regulate the level of IL-2 released from cells.

Together, these data suggested that DP thymocytes in a positively selecting environment reacquire the inducibility of AP-1 and NFAT activities before down-modulation of the CD4 or CD8 coreceptor occurs.

The acquisition of AP-1 and NFAT inducibility at the DP stage correlates with the up-regulation of the TcR. Considering that the increased TcR level is the major characteristic that differentiates the DP population from TcR and non-TcR transgenic mice and, as mentioned above, the up-regulation of TcR expression correlates with a more mature phenotype of DP cells (28, 44, 58, 60), this TcR up-regulation could correlate with the acquisition of inducibility. To approach this question, we isolated from TcR transgenic mice two subpopulations of the DP cells on the basis of their TcR levels, DP^{low} and DP^{high}. These two subpopulations were always well defined in DP cells from TG-B transgenic mice (Fig. 4A). However, the definition of both DP^{low} and DP^{high} subpopulations was somewhat less clear in the Cyt c TcR transgenic mice and, perhaps, consequently the intensity and percentage of DP^{high} cells were lower than in TG-B mice. As shown in Table 3, DP^{high} but not DP^{low} cells were able to produce significant amounts of IL-2 in response to PMA plus ionomycin in both TcR transgenic systems.

In addition, we analyzed the AP-1 transcriptional activity in DP subpopulations from both Cyt c × AP1-luc and TG-B × AP1-luc double-transgenic mice. Interestingly, most AP-1 transcriptional activity was detected in the DP^{high} subpopulations; only low activity was seen in DP^{low} cells from Cyt c TcR transgenic mice, and almost no detectable AP-1 activity was found in DP^{low} cells from TG-B transgenic mice (Fig. 4B to E). In contrast, no dramatic difference was observed in the NFAT transcriptional activity from DP^{high} and DP^{low} subpopulations from both TcR transgenic systems, although in most experiments the activity in DP^{high} cells was higher than that in DP^{low} cells at the earliest time points of activation (Fig. 4B to E).

TABLE 3. IL-2 production in DP subpopulations from TcR transgenic mice

TcR transgenic mice	Expt	IL-2 production (U/ml)[a]	
		DP^{low}	DP^{high}
Cyt c	1	1	25
	2	8	34
TG-B	1	2	18
	2	1	16

[a] Cells were stimulated and IL-2 production was detected as described in the footnotes to Table 1.

The analysis of DNA-binding activity by EMSA showed a correlation with transcriptional activity. Most of the AP-1 complexes observed in DP cells from stimulated TG-B transgenic mice were present in the DP^{high} subpopulation, and little DNA binding was observed in DP^{low} thymocytes (Fig. 4F). AP-1 DNA binding was also stronger in DP^{high} than in DP^{low} thymocytes from Cyt c TcR transgenic mice, although, as we expected, the difference was not as notable since the two subpopulations were not as well defined and the TcR levels in DP^{high} cells were lower than in the same subpopulation from TG-B transgenic mice. Analysis of NFAT DNA-binding activity showed more inducible NFAT complexes in DP^{high} than in DP^{low} thymocytes. Nevertheless, in correlation with TcR expression and IL-2 production, the levels of DNA and transcriptional activity detected in the DP^{high} subpopulation never reached the levels observed in the single-positive populations (data not shown).

These data indicate that the reacquisition of AP-1 and NFAT activities occurs at the DP stage after the up-regulation of TcR takes place in the case of AP-1 and, probably, earlier for NFAT.

AP-1 inducibility as a marker for positive selection of thymocytes. Several studies suggest that during successful positive selection, DP^{low} thymocytes increase TcR surface expression (6, 28, 44, 58, 60). More recently it has been proposed that up-regulation of the TcR is the result of the first TcR-MHC engagement that initiates the positive selection program which is completed after the second TcR-MHC engagement and binding to the appropriate coreceptor (10, 16). In that case, DP^{high} cells from TcR transgenic mice should have already been positively selected. Our data, therefore, suggest that AP-1 inducibility is reacquired as a consequence of positive selection at the DP stage. To establish that the DP^{high} subpopulation isolated from TcR transgenic thymuses has been positively selected, we evaluated the expression of additional cell surface markers that discriminate between DP and single-positive thymocytes. The expression of HSA is used as marker of maturity (2, 15), since it is highly expressed in DP thymocytes and is down-modulated only when cells become mature single-positive cells. Thus, when we analyzed HSA expression in DP from TcR transgenic mice, we observed, as expected, that DP^{high} thymocytes, despite the high levels of TcR, were still expressing the same high HSA levels as DP^{low} cells in TG-B transgenic

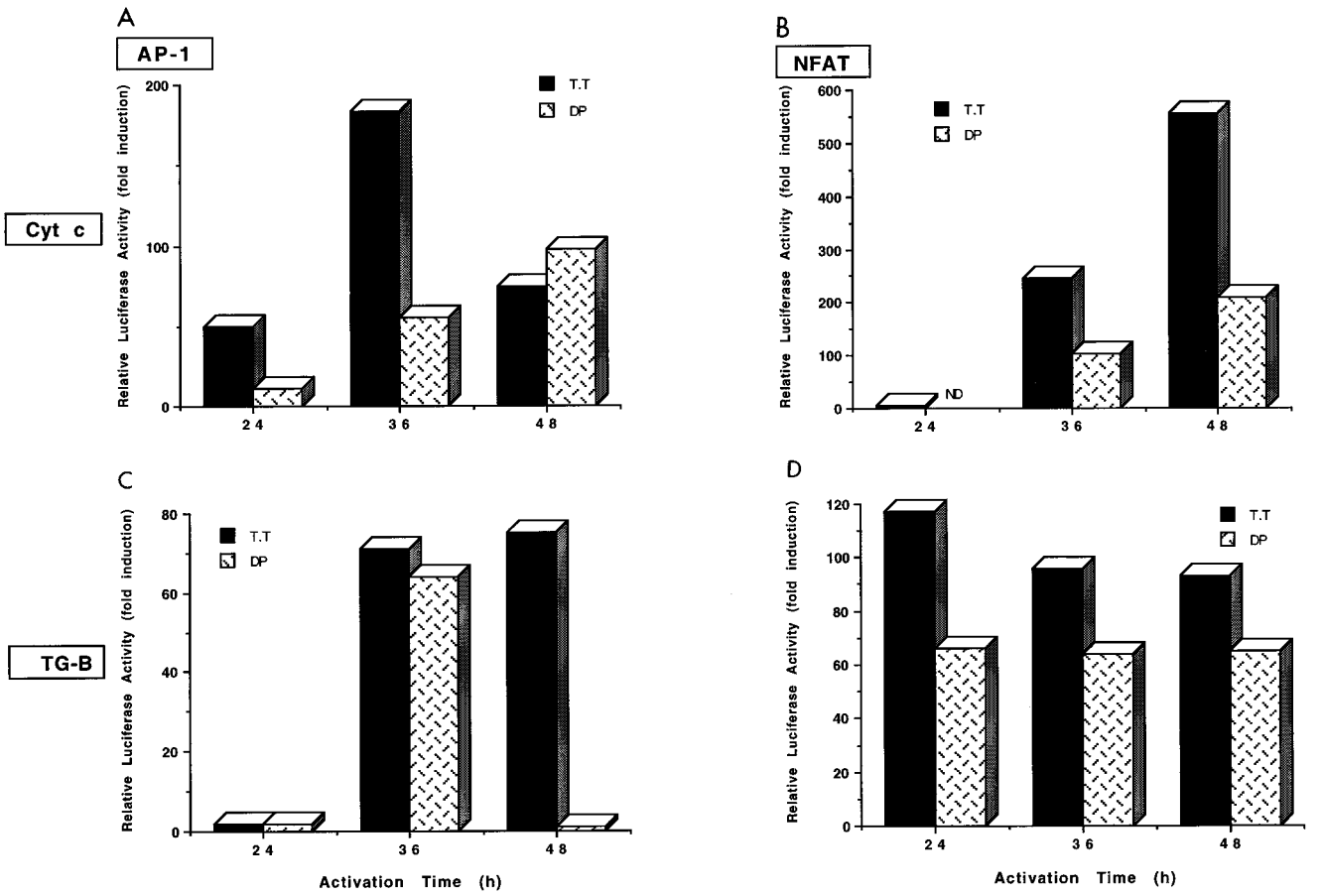


FIG. 3. Analysis of AP-1 and NFAT transcriptional and DNA-binding activities in DP from TcR transgenic mice. (A to D) Transcriptional activity. Total thymocytes (T.T) or DP thymocytes from Cyt c TcR × AP1-luc (A), Cyt c TcR × NFAT-luc (B), TG-B × AP1-luc (C), or TG-B × NFAT-luc (D) double-transgenic mice were stimulated as described for Fig. 1A for different periods of time, and luciferase activity was analyzed as described in Materials and Methods. (E) DNA-binding activity. EMSA of nuclear extracts from total thymocytes (T.T) or DP isolated from Cyt c TcR or TG-B transgenic mice stimulated for 6 h as described for Fig. 1B. For each assay, 2-μg aliquots of nuclear extracts were incubated with a ^{32}P-end-labeled oligonucleotide containing the human collagenase TRE (AP-1) or murine distal NFAT element (NFAT).

mice and that the level was only slightly reduced in Cyt c TcR transgenic mice (Fig. 5). HSA expression was, however, strongly down-modulated in single-positive thymocytes (data not shown). On the other hand, CD69, an early marker of T-cell activation, is also a thymocyte maturation marker since its up-regulation is closely correlated with positive selection occurring at the DP stage (2, 61). We therefore compared levels of CD69 expression in the DP^{high} and DP^{low} populations by four-color cytofluorimetric analysis. DP^{high} thymocytes from both Cyt c TcR and TG-B transgenic mice showed an up-regulation of CD69 compared with the DP^{low} subpopulation (Fig. 5). These results indicated that this DP^{high} subpopulation, although it has not acquired the full mature single-positive phenotype, has undergone positive selection and that therefore AP-1 inducibility can be used as an additional marker of maturation.

It has been previously described that MHC class II-deficient mice have a substantial population of CD4 single-positive thymocytes expressing high levels of TcR (14). These cells are believed to be positively selected on MHC class I molecules, and they are intermediate in maturity between CD4$^+$ CD8$^+$ and end-stage CD4$^+$ CD8$^-$ cells. Indeed, in addition to exhibiting high TcR levels, they are CD69 positive like mature single-positive cells. However, they also show immature characteristics such as low levels of CD8, medium to high levels of

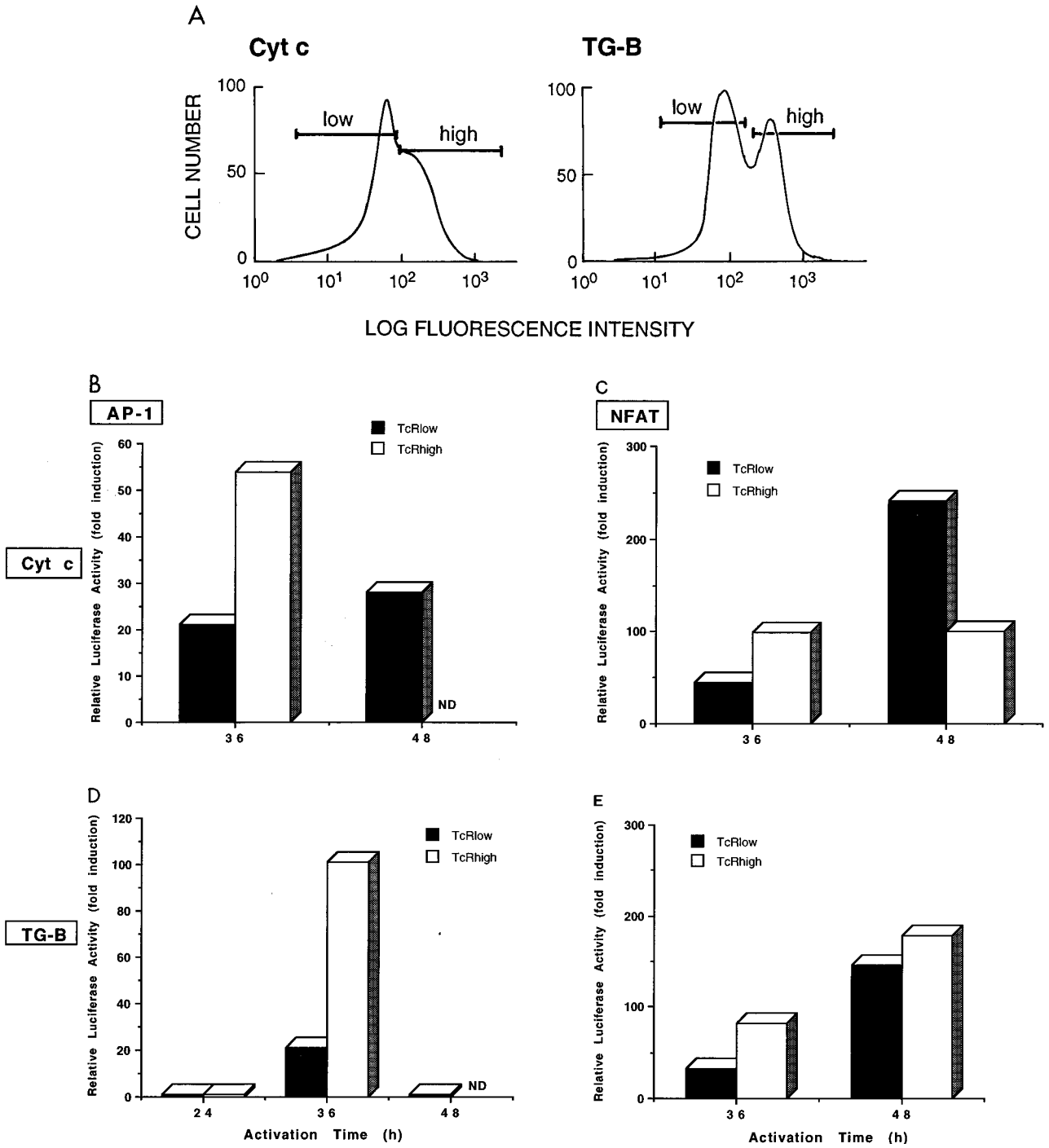


FIG. 4. Comparative analysis of AP-1 and NFAT transcriptional and DNA-binding activities in DP^{low} and DP^{high} cells. (A) Definition of DP^{low} and DP^{high} populations. Three-color flow cytometry analysis was carried out in thymocytes from Cyt c TcR and TG-B transgenic mice as described for Fig. 2. The histograms represent the anti-TcR fluorescence intensities within the DP populations. Cells were then sorted on the basis of the TcR levels in DP^{low} (low) or DP^{high} (high) cells. (B to E) Transcriptional activity. DP^{low} or DP^{high} cells sorted from Cyt c TcR × AP1-luc (B), Cyt c TcR × NFAT-luc (C), TG-B × AP1-luc (D), or TG-B × NFAT-luc (E) double-transgenic mice were stimulated as described for Fig. 1A for different periods of time, and luciferase activity was analyzed as described in Materials and Methods. (F) DNA-binding activity. EMSA of nuclear extracts from DP^{low} or DP^{high} sorted thymocytes from Cyt c TcR or TG-B transgenic mice stimulated for 6 h as described for Fig. 1B. For each assay, 1.5-μg aliquots of nuclear extracts were incubated with a ^{32}P-end-labeled oligonucleotide containing the human collagenase TRE (AP-1) or murine distal NFAT element (NFAT).

HSA, and cortical location (10). The phenotype of this intermediate state of thymocyte differentiation correlates with the phenotype that we observed for the DP^{high} subpopulation obtained from TcR transgenic mice. Therefore, our results indicate these cells could have recovered AP-1 inducibility. To test this possibility, we isolated by cell sorting $CD4^+$ thymocytes from class II-deficient mice (Fig. 6A) and stimulated them with PMA plus ionomycin, and nuclear extracts were then tested for

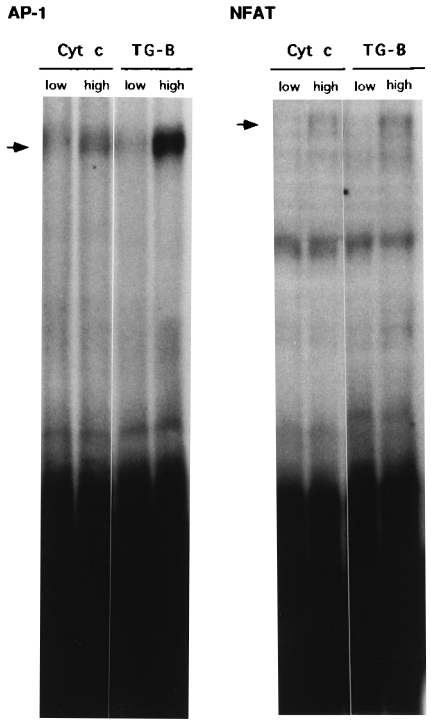


FIG. 4—*Continued.*

AP-1 DNA-binding activity by EMSA. As predicted, we detected AP-1 DNA binding in the $CD4^+$ population from class II-deficient animals at almost the same level as in $CD4^+$ cells from normal mice (Fig. 6B).

Altogether, these data indicate that AP-1 inducibility is reacquired at the DP stage, possibly as a consequence of positive selection but before cells acquire the full mature phenotype of the single-positive stage.

DISCUSSION

Cytokine production and the expression of cytokine receptors are modulated during fetal thymus ontogeny and among the different thymocyte populations in the adult thymus (9). Indeed, DN thymocytes from the adult thymus are able to produce IL-2 and other cytokines in response to a TcR-independent stimulation (36, 68). However, as thymocytes reach the DP stage, they can no longer produce cytokines upon stimulation (9, 11, 48). A further step in thymic development is the maturation of DP thymocytes into single-positive $CD4^+$ or $CD8^+$ cells, which regain the ability to produce specific cytokines in response to a TcR-dependent or -independent stimulus. We were interested first in determining the molecular basis of the loss of cytokine gene expression at the DP stage and then in determining which event triggers the reacquisition of this gene expression during thymic differentiation and at which developmental stage this occurs.

At least two possible mechanisms could explain why DP thymocytes are unable to induce cytokine gene expression. One is the presence of a dominant silencer in the regulatory region of the gene. Such a silencer, similar to the one found in the CD4 gene (53), could be inactive at the DN or single-positive stage but active in DP thymocytes, thereby repressing gene expression despite the presence of inducible positive transcription factors upon stimulation. This kind of mechanism is not likely to apply in the case of the lack of IL-2 gene expression, since no negative regulatory elements have been found within this gene. Alternatively, the lack of IL-2 inducibility could be explained by the absence, at the DP stage, of inducible transcription factors required for the complete transcription of the IL-2 and other cytokine genes. Previously, Chen and Rothenberg (11) scored for the DNA-binding activities of different transcriptional factors in enriched DN, DP, or $CD4^+$ populations from normal mice after stimulation with PMA plus ionomycin, and they described a dramatic reduction of AP-1 and NFAT DNA binding in stimulated DP thymocytes compared with the other populations. Recently, Sen et al. (56) described the presence of nuclear forms of AP-1 and NFAT in freshly isolated thymocytes at all stages of maturation, although, since transcriptional activity was not measured, it is not clear whether these factors are transcriptionally functional; however, the DNA binding of these factors in DP cells (but not other populations) is down-modulated upon incubation at 37°C. In our studies, we analyzed both the DNA-binding and transcriptional activities of these two regulatory factors, which are involved in the expression of several cytokine genes, in purified DP, $CD4^+$, and $CD8^+$ population from non-TcR transgenic mice after stimulation with a TcR-independent stimulus. Our data show that, in correlation with the loss of IL-2 inducibility, stimulation of DP thymocytes does not activate transcription mediated by AP-1 or NFAT. The lack of transcriptional activity of these two factors in DP cells seems to be due to their inability to bind to the specific DNA regulatory element more than to the lack of posttranslational modification of the complex, which is the mechanism that appears to play a key role in the regulation of AP-1 activity in peripheral T cells (49, 59). The absence of DNA-binding and transcriptional activities of these two transcription factors in DP cells is specific and it is not a consequence of a general defect in signal transduction in these cells or of cell death induced upon stimulation. First, the viability of stimulated DP cells is not significantly decreased by activation. Second, nuclear extracts of the cells show binding to a control CRE sequence, establishing that these extracts contain undegraded DNA-binding proteins. Finally, we have generated NF-κB–luciferase reporter transgenic mice, and we observed that stimulation of DP thymocytes, under the same conditions as used here for AP1-luc mice, does up-regulate NF-κB transcriptional activity (data not shown). Therefore, the lack of AP-1 and NFAT in DP thymocytes reflects a specific and intrinsic defect for these factors at this stage of maturation, and it is not a consequence of an impairment of the signalling machinery associated with the TcR. It does, however render DP thymocytes unable to respond to a TcR-specific stimulation.

The differential inducibility of AP-1 and NFAT among the thymic subpopulations suggests the involvement of these nuclear factors in thymocyte development. In this regard, it is noteworthy that somatic chimeric mice generated by injecting homozygous mutant c-*jun*-deficient embryonic stem cells into blastocysts from recombination activating gene 2 (*RAG-2*)-deficient mice have poor restoration of thymocytes (12). Similarly, overexpression of the c-*fos* transgene specifically affects T-cell development in the thymus, by increasing the proportion of mature thymocytes (51). Therefore, some members of the Fos/Jun family, which are present in AP-1 complexes (13, 25, 26, 40, 52, 67) and are believed to also be present in the nuclear component of NFAT (5, 30, 31, 43), seem to play a role in the maturation of thymocytes.

The second question that we address in this study is the nature of the maturation event that restores the ability of thymocytes to activate AP-1 and NFAT before mature thymo-

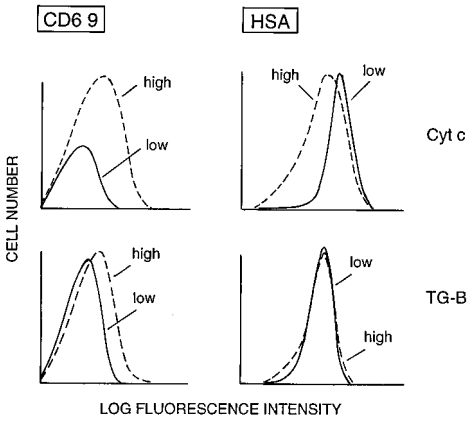


FIG. 5. Expression of CD69 and HSA in DP^{low} and DP^{high} subpopulations. Thymocytes from Cyt c TcR or TG-B transgenic mice were analyzed by four-color flow cytometry after staining with anti-CD4, anti-CD8, anti-TcR and anti-CD69, or anti-HSA. The histograms represent the fluorescence intensities of CD69 or HSA gating in TcR^{low} (low) or TcR^{high} (high) regions in the anti-TcR histogram for $CD4^+$ $CD8^+$ cells.

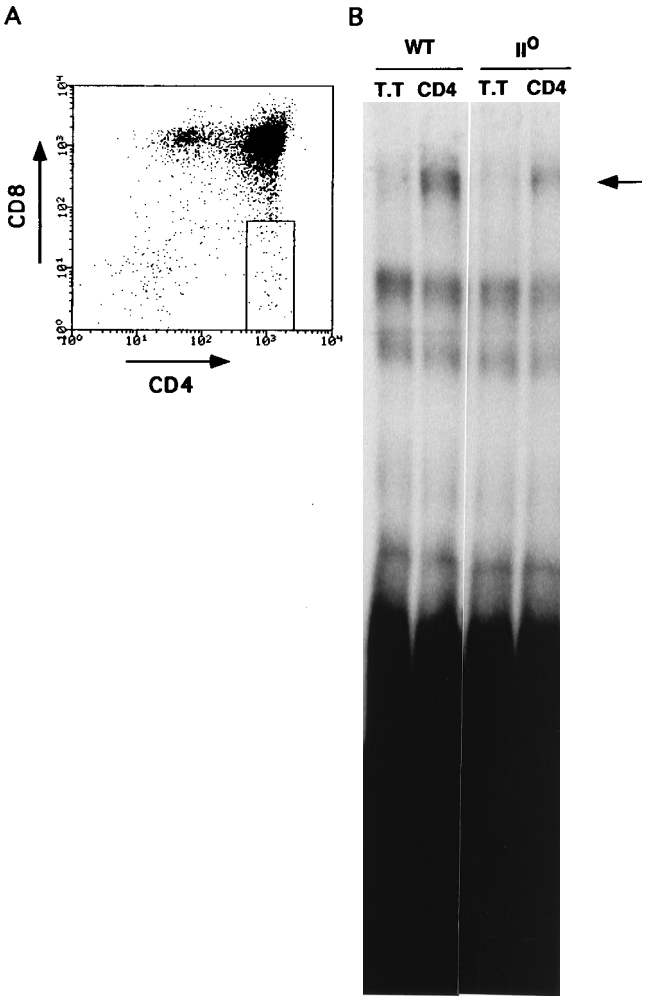


FIG. 6. AP-1 DNA-binding activity in the single-positive $CD4^+$ population from class II-deficient mice. The $CD4^+$ population from class II-deficient mice was obtained by anti-CD4 and anti-CD8 staining and cell sorting (A). (B) EMSA of nuclear extracts from total thymocytes (T.T) and $CD4^+$ cells (CD4) from wild-type (WT) or class II-deficient (II^o) mice stimulated for 6 h as described for Fig. 1B. For each assay, 1.5-μg aliquots of nuclear extracts were incubated with a ^{32}P-end-labeled oligonucleotide containing the human collagenase TRE.

cytes migrate to periphery. Previous studies (11) and our own results indicate that this inducibility has been reacquired by the time full maturation occurs at the single-positive stage. Our results for DP thymocytes from TcR transgenic mice now show that the reacquisition of IL-2 inducibility precedes coreceptor down-modulation. A large subpopulation of DP thymocytes from TcR transgenic mice have up-regulated their TcR levels. Since this up-regulation is generally believed to be a result of positive selection (6, 28, 44, 58, 60) and DP cells from TcR transgenic animals have reacquired the ability to induce AP-1 and NFAT as well as IL-2 production, it appears that positive selection is a prerequisite for this reacquisition. In this regard, the TcR specific for Cyt c has been described to be also weakly positively selected by $I\text{-}A^b$, since most $CD4^+$ T cells express the transgenic receptor (32, 64); however, positive selection is not as efficient as in the E^k background, as evidenced by the fact that most thymocytes are DP, with few single-positive $CD4^+$ cells, and the fact that the DP cells do not express high levels of TcR. Unlike $DP(E^k)$ cells, these $DP(A^b)$ cells do not produce IL-2 and do not induce either NFAT or AP-1 transcriptional activity (data not shown).

Several models of thymocyte selection have been elaborated to explain how the interaction of a TcR with specific MHC dictates the commitment of DP cells to the CD4 or CD8 lineage (6, 50, 66). A recent model, a modification of the original version of the stochastic/selective model (10, 16), proposes that $CD4^+$ $CD8^+$ thymocytes must engage MHC molecules twice en route to becoming completely mature single-positive cells. The first engagement, received by DP cells, results in the up-regulation of TcR and CD69 on the cell surface, reduction of *RAG-1* and terminal deoxynucleotidyltransferase mRNA levels (4, 7), and induction of a stochastic mechanism that gradually down-modulates CD4 or CD8. The second engagement, which requires that a chosen coreceptor and the specific TcR bind to the same MHC molecule, results in down-regulation of surface HSA expression, entry of cells into the medulla, and completion of positive selection, so that cells become truly $CD4^+$ or $CD8^+$ single-positive cells. Our results demonstrate that AP-1 transcriptional and DNA-binding activities, in correlation with IL-2 production, cannot be induced in the DP TcR^{low} $CD69^{low}$ HSA^{high} subpopulation upon stimulation with PMA plus ionomycin (this report) or TcR cross-linking (not shown). In contrast, AP-1 activity and IL-2 expression are normally induced in the DP TcR^{high} $CD69^{high}$ HSA^{high} subpopulation by both stimuli. Therefore, if this model is correct, these results indicate that the IL-2 inducibility that is lost during the transition from DN to DP is reacquired as a consequence of the signal mediated by the first TcR-MHC engagement at the DP stage. Further, this IL-2 inducibility correlates with the acquisition of AP-1 inducibility. Unexpectedly, significant levels of NFAT activity were detected in the DP^{low} subpopulations from both TcR transgenic mice, although the level was higher in the DP^{high} subpopulation. However, the major regulatory component of the NFAT complex is the preexisting cytoplasmic component (19, 30, 37, 42) whose regulation during thymic development remains unknown. In addition, although it has been described that Fos/Jun family members are contained in the nuclear component of NFAT (5, 30, 31, 43), it is not yet clear which Fos/Jun family members are involved and how they participate in NFAT activity. Furthermore, it is possible that factors other than AP-1 can function as the nuclear components of NFAT. It is there-

fore possible that the signal transduced in DP^{low} cells is sufficient to confer a low level of NFAT activity but insufficient to activate AP-1.

In correlation with the stochastic/selective model described above, the $CD4^+$ $CD8^{low}$ population detected in class II-deficient mice (14) represents an intermediate stage of maturation. They seem to have received the first signal mediated by class I-specific TcR engagement and started down-modulating the CD8 coreceptor. However, since they cannot coengage the same MHC molecule with their TcR and CD4 coreceptor, they do not complete full maturation (10). Our experiments show that this intermediate population isolated from class II-deficient animals contains AP-1 complexes after stimulation, which is consistent with our model of how this factor is developmentally regulated. We therefore propose that the acquisition of AP-1 inducibility is an additional marker of thymocyte maturation, helping to discriminate between the DP immature stage, prior to positive selection, and the DP intermediate stage, after the first positive selecting signal has been delivered.

What dictates the loss of cytokine production inducibility during the differentiation of DN to DP thymocytes is a question that remains open and will be a subject of further studies. The signal mediated by the interaction of TcR β chain in association with gp33, expressed at the latest maturation stage of the DN (22–24), with an unknown ligand may be one candidate. Our data suggest that this loss of cytokine gene expression when thymocytes approach the DP stage is not regained until positive selection has occurred. We believe that this could represent a protective mechanism against self-reaction in the thymus. Before positive or negative selection occurs, the majority of DP thymocytes already express low levels of TcR that could mediate a self-antigen-specific activation and proliferation of this population and therefore cause destruction in thymus. To avoid that, DP thymocytes have developed a system to uncouple the expression of activation genes such as cytokines genes. Moreover, because of the inability to induce AP-1, the signal mediated by the TcR during the selection process would not result in the activation of the DP thymocytes but could allow differentiation to mature single-positive thymocytes.

ACKNOWLEDGMENTS

We thank S. Guerder for helpful discussions and critical reading of the manuscript, B. Al-Ramadi for critical reading of the manuscript, T. Taylor for technical assistance with cell sorting, C. Hughes and D. Buktus for generating the transgenic mice, T. Geiger for providing the TG-B TcR transgenic mice and critical reading, and Luika Timmerman and Gerald Crabtree for providing the NFAT-luc construct and helpful discussion.

M. Rincón was supported initially by a fellowship from Ministerio de Educación y Ciencia and in part by National Institutes of Health grant 1R1AI2902. R. A. Flavell is an investigator of the Howard Hughes Medical Institute.

REFERENCES

1. **Angel, P., M. Imagawa, R. Chiu, B. Stein, R. J. Imbra, H. J. Rahmsdorf, C. Jonat, P. Herrlich, and M. Karin.** 1987. Phorbol ester-inducible genes contain a common cis element recognized by a TPA-modulated trans-acting factor. Cell **49:**729–739.
2. **Bendelac, A., P. Matzinger, R. A. Seder, W. E. Paul, and R. H. Schwartz.** 1992. Activation events during thymic selection. J. Exp. Med. **175:**731–742.
3. **Berg, L. J., A. M. Pullen, B. Fazekas de St. Groth, D. Mathis, C. Benoist, and M. M. Davis.** 1989. Antigen/MHC-specific T cells are preferentially exported from the thymus in the presence of their MHC ligand. Cell **58:**1035–1046.
4. **Bogue, M., S. Gilfillan, C. Benoist, and D. Mathis.** 1992. Regulation on N region diversity in antigen receptors through thymocyte differentiation and thymus ontogeny. Proc. Natl. Acad. Sci. USA **89:**11011–11015.
5. **Boise, L. H., B. Petrynyak, X. Mao, C. H. June, C.-Y. Wang, T. Lindsten, R. Bravo, K. Kovary, J. M. Leiden, and C. B. Thompson.** 1993. The NFAT-1 DNA binding complex in activated T cells contains Fra-1 and JunB. Mol. Cell. Biol. **13:**1911–1919.
6. **Borgulya, P., H. Kishi, U. Müller, J. Kirberg, and H. von Boehmer.** 1991. Development of the CD4 and CD8 lineage of T cells: instruction versus selection. EMBO J. **10:**913–918.
7. **Borgulya, P., H. Kishi, Y. Uematsu, and H. von Boehmer.** 1992. Exclusion and inclusion of α and β T cell receptor alleles. Cell **69:**529–537.
8. **Boyd, R. L.** 1991. Towards an integrated view of thymopoiesis. Immunol. Today **12:**71–79.
9. **Carding, S. R., A. C. Hayday, and K. Bottomly.** 1991. Cytokines in T-cell development. Immunol. Today **12:**239–245.
10. **Chan, S. H., D. Cosgrove, C. Waltzinger, C. Benoist, and D. Mathis.** 1993. Another view of the selective model of thymocyte selection. Cell **73:**225–236.
11. **Chen, D., and E. V. Rothenberg.** 1993. Molecular basis for developmental changes in interleukin-2 gene inducibility. Mol. Cell. Biol. **13:**228–237.
12. **Chen, J., V. Stewart, G. Spyrou, F. Hilberg, E. F. Wagner, and F. W. Alt.** 1994. Generation of normal T and B lymphocytes by c-jun deficient embryonic stem cells. Immunity **1:**65–72.
13. **Cohen, D. R., and T. Curran.** 1988. *fra-1*: a serum-inducible, cellular immediate-early gene that encodes a Fos-related antigen. Mol. Cell. Biol. **8:**2063–2069.
14. **Cosgrove, D., D. Gray, A. Dierich, J. Kaufman, M. Lemeur, C. Benoist, and D. Mathis.** 1991. Mice lacking MHC class II molecules. Cell **66:**1051–1066.
15. **Crispe, I. N., and M. J. Bevan.** 1987. Expression and functional significance of the J11d marker on mouse thymocytes. J. Immunol. **138:**2012–2018.
16. **Davis, C. B., N. Killeen, M. E. Casey Crooks, D. Raulet, and D. R. Littman.** 1993. Evidence for a stochastic mechanism in the differentiation of mature subsets of T lymphocytes. Cell **73:**237–247.
17. **Durand, D. B., J. P. Shaw, M. R. Bush, R. E. Replogle, R. Belageje, and G. R. Crabtree.** 1988. Characterization of antigen receptor response elements within the interleukin 2 enhancer. Mol. Cell. Biol. **8:**1715–1724.
18. **Fischer, M., I. MacNeil, T. Suda, J. E. Cupp, K. Shortman, and A. Zlotnik.** 1991. Cytokine production by mature and immature thymocytes. **146:**3452–3456.
19. **Flanagan, W. F., B. Corthesy, R. J. Bram, and G. R. Crabtree.** 1991. Nuclear association of a T-cell transcription factor blocked by FK-506 and cyclosporin A. Nature (London) **352:**803–807.
20. **Geiger, T., L. R. Gooding, and R. A. Flavell.** 1992. T-cell responsiveness to an oncogenic peripheral protein and spontaneous autoimmunity in transgenic mice. Proc. Natl. Acad. Sci. USA **89:**2985–2989.
21. **Gillis, S., M. N. Ferm, and K. A. Smith.** 1978. T cell growth factor: parameters of production and quantitative microassay for activity. J. Immunol. **120:**1109–1113.
22. **Groettrup, M., A. Baron, G. Griffiths, R. Palacios, and H. von Boehmer.** 1992. T cell receptor β chain homodimers on the surface of immature but not mature α, γ and δ chain deficient T cell lines. EMBO J. **11:**2735–2746.
23. **Groettrup, M., K. Ungewiss, O. Azogui, R. Palacios, M. J. Owen, A. C. Hayday, and H. von Boehmer.** 1993. A novel disulfide-linked heterodimer on pre-T cells consists of the T cell receptor b chain and a 33 kd glycoprotein. Cell **75:**283–294.
24. **Groettrup, M., and H. von Boehmer.** 1993. A role of a pre-T-cell receptor in T-cell development. Immunol. Today. **14:**610–614.
25. **Halazonetis, T. D., K. Georgopoulos, M. E. Greenberg, and P. Leder.** 1988. c-Jun dimerizes with itself and with c-Fos forming complexes of different DNA binding affinities. Cell **55:**917–924.
26. **Hirai, S.-I., R.-P. Ryseck, F. Mechta, R. Bravo, and M. Yaniv.** 1989. Characterization of JunD: a new member of jun proto-oncogene family. EMBO J. **8:**1433–1439.
27. **Hogan, B., F. Constantini, and E. Lacy.** 1986. Manipulating the mouse embryo, p. 89–203. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
28. **Huesmann, M., B. Scott, P. Kisielow, and H. von Boehmer.** 1991. Kinetics and efficacy of positive selection in thymus of normal and T cell receptor transgenic mice. Cell **66:**533–540.
29. **Ivanov, V., and R. Ceredig.** 1992. Transcription factors in mouse fetal thymus development. Int. Immunol. **4:**729–737.
30. **Jain, J., P. G. McCaffrey, A. Miner, T. K. Kerppola, J. N. Lambert, G. L. Verdine, T. Curran, and A. Rao.** 1993. The T-cell transcription factor NFATp is a substrate for calcineurin and interacts with Fos and Jun. Nature (London) **365:**352–355.
31. **Jain, J., P. G. McCaffrey, V. E. Valge-Archer, and A. Rao.** 1992. Nuclear factor of activated T cells contains Fos and Jun. Nature (London) **356:**801–804.
32. **Kaye, J., M.-L. Hsu, M.-E. Sauron, S. C. Jameson, N. R. J. Gascoigne, and S. M. Hedrick.** 1989. Selective development of CD4+ T cells in transgenic mice expressing a class II MHC-restricted antigen receptor. Nature (London) **341:**746–749.
33. **Kisielow, P., H. S. Teh, H. Blüthmann, and H. von Boehmer.** 1988. Positive selection of antigen-specific T cells in thymus by restricting MHC molecules. Nature (London) **335:**730–733.
34. **Kizaki, H., T. Tadakuma, C. Odaka, J. Muramatsu, and Y. Ishimura.** 1989. Activation of a suicide process of thymocytes through DNA fragmentation by calcium ionophores and phorbol esters. J. Immunol. **143:**1790–1794.

35. **Lee, W., P. Mitchell, and R. Tjian.** 1987. Purified transcription factor AP-1 interacts with TPA-inducible enhancer elements. Cell **49:**741–752.
36. **Lugo, J. P., S. N. Krishnan, R. Diamond Sailor, and E. V. Rothenberg.** 1986. Early precursor thymocytes can produce interleukin-2 upon stimulation with calcium ionophore and phorbol ester. Proc. Natl. Acad. Sci. USA **83:**1862–1866.
37. **McCaffrey, P. G., C. Luo, T. K. Kerppola, J. Jain, T. M. Badalian, A. M. Ho, E. Burgeon, W. S. Lane, J. N. Lambert, T. Curran, G. L. Verdine, A. Rao, and P. G. Hogan.** 1993. Isolation of cyclosporin-sensitive T cell transcription factor NFATp. Science **262:**750–754.
38. **Montgomery, R. A., and M. J. Dallman.** 1991. Analysis of cytokine gene expression during fetal thymic ontogeny using the polymerase chain reaction. J. Immunol. **147:**554–560.
39. **Montminy, M. R., and L. M. Bilezikijan.** 1986. Binding of nuclear protein to the cyclic-AMP response element of the somatostatin gene. Nature (London) **328:**175–178.
40. **Nakabeppu, Y., K. Ryder, and D. Nathans.** 1988. DNA binding activities of three murine jun proteins: stimulation by fos. Cell **55:**907–915.
41. **Nikolic-Zugic, J.** 1991. Phenotypic and functional stages in the intrathymic development of ab cells. Immunol. Today **12:**65–70.
42. **Northrop, J. P., S. N. Ho, L. Chen, D. J. Thomas, G. P. Nolan, A. Admon, and G. R. Crabtree.** 1994. NF-AT components define a family of transcription factors targeted in T-cell activation. Nature (London) **369:**497–502.
43. **Northrop, J. P., K. S. Ullman, and G. R. Crabtree.** 1993. Characterization of nuclear and cytoplasmic components of the lymphoid-specific nuclear factor of activated T cells (NF-AT). J. Biol. Chem. **268:**2917–2923.
44. **Ohashi, P. S., H. Pircher, K. Bürki, R. M. Zinkernagel, and H. Hengartner.** 1990. Distinct sequence of negative or positive selection implied by thymocytes T-cell receptor densities. Nature (London) **346:**861–863.
45. **Pircher, H., K. Bürki, R. Lang, H. Hengartner, and R. M. Zinkernagel.** 1989. Tolerance induction in double specific T-cell receptor transgenic mice varies with antigen. Nature (London) **342:**559–561.
46. **Rao, A.** 1994. NF-ATp: a transcription factor required for the co-ordinate induction of several cytokine genes. Immunol. Today **15:**274–281.
47. **Riegel, J. S., B. Corthesy, W. M. Flanagan, and G. R. Crabtree.** 1992. Regulation of interleukin-2 gene. Chem. Immunol. **51:**266–298.
48. **Riegel, J. S., E. R. Richie, and J. P. Allison.** 1990. Nuclear events after activation of CD4+8+ thymocytes. J. Immunol. **144:**3611–3618.
49. **Rincón, M., and R. A. Flavell.** 1994. AP-1 transcriptional activity requires both T-cell receptor-mediated and co-stimulatory signals in primary T lymphocytes. EMBO J. **13:**4370–4381.
50. **Robey, E. A., B. J. Fowlkes, J. W. Gordon, D. Kioussis, H. von Boehmer, F. Ramsdell, and R. Axel.** 1991. Thymic selection in CD8 transgenic mice supports an instructive model for commitment to CD4 or CD8 lineage. Cell **64:**99–107.
51. **Rüther, U., W. Muller, T. Sumida, T. Tokuhisa, K. Rajewsky, and E. F. Wagner.** 1988. c-fos expression interferes with thymus development in transgenic mice. Cell **53:**847–856.
52. **Ryder, K., A. Lanahan, E. Perez-Albuerne, and D. Nathans.** 1989. Jun-D: a third member of the Jun gene family. Proc. Natl. Acad. Sci. USA **86:**1500–1503.
53. **Sawada, S., J. D. Scarborough, N. Killeen, and D. R. Littman.** 1994. A lineage-specific transcriptional silencer regulates CD4 gene expression during T lymphocyte development. Cell **77:**917–929.
54. **Schreiber, E., P. Matthias, M. M. Müller, and W. Shaffner.** 1989. Rapid detection of octamer binding proteins with 'mini'-extracts prepared from a small number of cells. Nucleic Acids Res. **17:**6419.
55. **Scott, B., H. Blüthmann, H. S. Teh, and H. von Boehmer.** 1989. The generation of mature T cells requires interaction of αβ T-cell receptor with major compatibility antigens. Nature (London) **338:**591–593.
56. **Sen, J., Y. Shinkai, F. W. Alt, R. Sen, and S. J. Burakoff.** 1994. Nuclear factors that mediate intrathymic signals are developmentally regulated. J. Exp. Med. **180:**2321–2327.
57. **Sha, W. C., C. A. Nelson, R. D. Newberry, D. M. Kranz, J. H. Rusell, and D. Y. Loh.** 1988. Positive and negative selection of an antigen receptor on T cell in transgenic mice. Nature (London) **336:**73–76.
58. **Shortman, K., D. Vremec, and M. Egerton.** 1991. The kinetics of T cell antigen receptor expression by subgroups of $CD4^+CD8^+$ thymocytes: delineation of $CD4^+8^+3^{2+}$ thymocytes as post-selection intermediates leading to mature T cells. J. Exp. Med. **173:**323–332.
59. **Su, B., E. Jacinto, M. Hibi, T. Kallunki, M. Karin, and Y. Ben-Neriah.** 1994. JNK is involved in signal integration during costimulation of T lymphocytes. Cell **77:**727–736.
60. **Swat, W., M. Dessing, A. Baron, P. Kisielow, and H. von Boehmer.** 1992. Phenotypic changes accompanying positive selection of CD4+CD8+ thymocytes. Eur. J. Immunol. **22:**2367–2372.
61. **Swat, W., M. Dessing, and H. von Boehmer.** 1993. CD69 expression during selection aand maturation of $CD4^+CD8^+$ thymocytes. Eur. J. Immunol. **23:**739–746.
62. **Teh, H. S., P. Kisilow, B. Scott, H. Kishi, Y. Uematsu, H. Blüthmann, and H. von Boehmer.** 1988. Thymic major histocompatibility complex antigens and the αβ T-cell receptor determine the CD4/cd8 phenotype of T cells. Nature (London) **335:**229–233.
63. **Tugores, A., M. A. Alonso, F. Sánchez-Madrid, and M. O. de Landázuri.** 1992. Human T cell activation through the activation-inducer molecule/CD69 enhances the activity of transcription factor AP-1. J. Immunol. **148:**2300–2306.
64. **Vasquez, N. J., J. Kaye, and S. M. Hedrick.** 1992. In vivo and in vitro clonal deletion of double-positive thymocytes. J. Exp. Med. **175:**1307–1316.
65. **Verweij, C. L., C. Guidos, and G. R. Crabtree.** 1990. Cell type specificity and activation requirements for NFAT-1 (nuclear factor of activated T-cells) transcriptional activity determined by a new method using transgenic mice to assay transcriptional activity of an individual nuclear factor. J. Biol. Chem. **265:**15788–15795.
66. **von Boehmer, H.** 1986. The selection of α, β heterodimeric T cell receptor for antigen. Immunol. Today **7:**333–336.
67. **Zerial, M., L. Toschi, R.-P. Ryseck, M. Schuermann, R. Müller, and R. Bravo.** 1989. The product of a novel growth factor activated gene, fosB, interacts with JUN proteins enhancing their DNA binding activity. EMBO J. **8:**805–813.
68. **Zúñiga-Pflücker, J. C., H. L. Schwartz, and M. J. Lenardo.** 1993. Gene transcription in differentiating immature T cell $receptor^{neg}$ thymocytes resembles antigen-activated mature T cell. J. Exp. Med. **178:**1139–1149.