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Molecular Basis for Developmental Changes in Interleukin-2 Gene Inducibility

DAN CHEN AND ELLEN V. ROTHENBERG*

Division of Biology, 156-29, California Institute of Technology, Pasadena, California 91125

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At least three stages in the intrathymic development of pre-T cells are demarcated by differences in the competence to express the interleukin-2 (IL-2) gene as an acute response to stimulation. IL-2 inducibility appears to be acquired relatively early, prior to T-cell receptor (TcR) gene rearrangement. It is then abrogated during the stage when cells are subject to positive and negative selection, i.e., the fate determination processes that select cells for maturation or death. IL-2 inducibility finally reappears in mature classes of thymocytes that have undergone positive selection. To provide a basis for a molecular explanation of these developmental transitions, we have examined the representation in different thymocyte subsets of a set of DNA-binding proteins implicated in IL-2 gene regulation. As the DNA-binding activities of many factors are elicited only by inductive stimuli, the cells were cultured in the presence or absence of the calcium ionophore A23187 and phorbol ester. Our results separate these factors into four regulatory classes: (i) constitutive factors, such as Oct-1 and probably Sp1, that are expressed in thymocytes at all stages; (ii) inducible factors, such as NF-κB and complexes binding to the region of a CD28 response element, that can be activated in all thymocytes, including those cells ($CD4^+$ $CD8^+$ TcR^{low}) that can undergo selection; (iii) inducible factors, such as NF-AT and AP-1, that can be activated in mature ($CD4^+$ $CD8^-$ TcR^{high}) and immature ($CD4^-$ $CD8^-$ TcR^-) thymocytes alike but not in the transitional stages when the cells ($CD4^+$ $CD8^+$ TcR^{low}) are subject to selection; and (iv) a factor containing CREB, which can be activated in thymocytes of all developmental stages by culture but does not require specific induction. These results verify that inducible transcription factors are targets of intrathymic developmental change. They also identify NF-AT and AP-1 as factors that are particularly sensitive to the mechanism altering thymocyte responses during the stages when thymocytes may undergo positive and negative selection.

T-cell precursors undergo a cascade of developmental changes in the thymic microenvironment which ultimately endow them with the recognition specificities, signaling mediators, and capacity for functional responses of mature T lymphocytes (reviewed in references 10, 36, and 49). Recognition specificity is the result of the rearrangement of T-cell receptor (TcR) genes and the cell surface expression of their products. The TcR complex becomes effective in triggering appropriate signaling pathways through the expression of the coreceptor molecules, CD4 and/or CD8, at the cell surface, together with a network of cytoplasmic protein kinases (1, 8). The acquisition of functional response capability, however, is more complex. Mature T-cell function is exercised only in response to antigen stimulation, through the abrupt and transient transcriptional activation of specific response genes such as the gene encoding interleukin-2 (IL-2). The competence to express IL-2 upon stimulation is largely, if not exclusively, a property of T lymphocytes. Accordingly, one of the changes induced in precursor cells by passage through the thymus is acquisition of IL-2 inducibility (39, 40).

Analysis of the competence to express IL-2 at various stages of intrathymic development has revealed a series of transitions distinct from those recognized on the basis of cell surface phenotype and TcR expression (reviewed in reference 36). In particular, immature thymocytes before TcR expression exhibit a form of IL-2 inducibility that seems to be extinguished in most of their descendants, while a different form of IL-2 inducibility is found in the minority of cells that go on to full maturation (9, 17, 24, 38, 39). Neither the early inducibility nor the extinction of inducibility is well understood in relation to the generation of mature IL-2 producers. However, these findings suggest provocative links between responsiveness to activation signals and thymocyte fate determination, as we have discussed elsewhere (36, 39). The lymphostromal interactions responsible for the changes in IL-2 inducibility may or may not be the same as those that trigger quite different effects on TcR expression. However, an advantage of using IL-2 inducibility to monitor developmental processes is that this property is relatively well understood at a molecular level. Thus, it is possible to define the impact of each transition, and gain a clue as to its mechanism, by analysis of the IL-2 gene-regulatory proteins that it affects.

Therefore, we have taken advantage of an extensive literature characterizing the minimal enhancer sequence of the IL-2 gene and many of the DNA-binding proteins that interact with it (reviewed in reference 47). Both inducible and constitutively expressed *trans*-acting factors are involved in initiating IL-2 gene transcription, including NF-κB or TcF-1 (18, 32, 42), AP-1 (20, 27), Oct-1 and other octamer-binding proteins (21), and two uncloned factors termed NF-AT (41) or Pu-box factor III (5, 33) and CD28RC or the CD28 response element (CD28RE)-binding factor (11, 48). We have used electrophoretic mobility shifts to score for the DNA-binding activities of these and other IL-2 DNA-binding factors in thymocyte nuclear extracts after in vitro stimulation. For those factors which do not require activation to reside in nuclei and bind DNA, these assays allow us to score the presence of factor proteins. For those which require specific signaling events to display DNA-binding

* Corresponding author.

activity, these assays further allow us to score cells for the existence of the signaling pathway that controls the factor modification. Our results demonstrate that inducible IL-2-regulatory factors are expressed noncoordinately in T-cell development. We further show that a full panoply of IL-2-regulatory proteins is present in immature thymocytes. Finally, our results show that the transitional $CD4^+$ $CD8^+$ TcR^{low} cortical thymocytes that are the main targets of selection are highly activatable even though functionally incompetent, responding to stimulation with intense induction of a specific but incomplete set of IL-2 DNA-binding activities.

MATERIALS AND METHODS

Reagents. Tetradecanoyl phorbol acetate (TPA) and the calcium ionophore A23187 (both from Sigma) were dissolved in dimethyl sulfoxide to the stock concentrations of 10 μg/ml and 0.7 mM, respectively. Recombinant human IL-1α was purchased from Genzyme. Antisera against Fos and Jun family proteins were kindly provided by Rodrigo Bravo. These reagents react specifically with all known members of the Fos and Jun families, respectively (23). Antiserum 244, against the W39 peptide of CREB, was a generous gift from M. R. Montminy (13). It is highly specific for recognition of CREB (26). In addition to the published documentation of the specificity of these antisera, we carried out controls for specificity under the conditions of our assays. Supershifted bands were not produced when 1 μl of a control antibody, RL172.4 (anti-CD4 ascites fluid), was added to the reactions. Furthermore, anti-CREB, anti-Fos, and anti-Jun did not affect the mobility of complexes binding the NF-κB oligonucleotide. We therefore consider the supershifted complexes to be specific.

Cells. Freshly isolated thymocytes from 4- to 6-week-old C57BL/6 mice were fractionated by complement-dependent cytolysis or panning. To isolate immature TcR^- $CD4^-$ $CD8^-$ cells, we used complement-dependent lysis with anti-CD4 (RL172.4), anti-CD8 (3.155), and anti-CD5 (CG-15) (39), or we used cells taken directly from the thymus of immunodeficient C.B-17 *scid/scid* (SCID) mutant mice, which are naturally arrested at this stage (>98% TcR^- $CD4^-$ $CD8^-$) (2). Cortical $CD4^+$ $CD8^+$ TcR^{low} thymocytes were either enriched by adherence to peanut agglutinin-coated plates or isolated by complement-dependent lysis with anti-$H\text{-}2K^b$ (28-13-33), taking advantage of their anomalously low class I major histocompatibility complex expression (4). By either method, the fraction contained over 92% $CD4^+$ $CD8^+$ cells. Mature thymocytes were either enriched by nonadherence to peanut agglutinin-coated plates (at 4°C) or isolated by complement-dependent lysis (at 37°C) with anti-CD8 antibody. By the latter method, over 70% $CD4^+$ $CD8^-$ and less than 30% $CD4^-$ $CD8^-$ cells were obtained; the fraction contained no cortical-type $CD4^+$ $CD8^+$ thymocytes. Flow cytometric analysis was used routinely to confirm the phenotypes of the fractions. Freshly isolated SCID thymocytes were put into culture directly, without prefractionation. All cells were grown in RPMI 1640 supplemented with 5% fetal bovine serum (HyClone, Logan, Utah), 2 mM L-glutamine, 50 μM 2-mercaptoethanol, and antibiotics, with or without TPA (17 nM, 10 ng/ml) plus A23187 (70 to 120 nM). In some experiments, the stimulated cells were also supplemented with IL-1 (20 or 50 U/ml).

IL-2 bioassay. Supernatants were collected from 20-h cultures with or without stimulation under the indicated conditions. The IL-2 bioassay was performed as described previously (39).

Nuclear extracts. Nuclear extracts were made from cells cultured for 2 to 4 h according to the procedure of Stein et al. (45). Briefly, 1×10^7 to 5×10^7 thymocytes were washed twice with ice-cold phosphate-buffered saline and resuspended in 100 μl of lysis buffer (10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES; pH 7.9], 10 mM Tris, 60 mM KCl, 1 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride), and then the nuclei were pelleted at $1,200 \times g$ for 5 min at 4°C. The nuclei were washed with lysis buffer without Nonidet P-40, then resuspended in nuclear resuspension buffer (250 mM Tris [pH 7.8], 60 mM KCl, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride), and disrupted with three cycles of freezing and thawing. After clarification by centrifugation at $7,000 \times g$ for 15 min, the supernatant was collected, and the nuclear extract was stored at −80°C until use. Protein concentrations in the extracts were determined by the Bradford assay (Bio-Rad protein assay; Bio-Rad Laboratories, Richmond, Calif.).

Oligonucleotides. Most double-stranded oligonucleotides were synthesized at the Microchemical Facility in our Division of Biology. Unless specified, the sequences are found in the 5'-flanking region of the mouse IL-2 gene (28) at distances from the cap site as shown below:

Spl consensus (Stratagene)	5' GATCGATCGGGGCGGGGCGATC 3' 3' CTAGCTAGCCCCGCCCCGCTAG 5'
NFI/CTF (4) (Stratagene)	5' ATTTTGGCTTGAAGCCAATATG 3' 3' TAAAACCGAACTTCGGTTATAC 5'
AP-2 consensus (Stratagene)	5' GATCGAACTGACCGCCCCGCGGCCCGT 3' 3' CTAGCTTGACTGGCGGGGCGCCGGGCA 5'
AP-1 (CREB) (−161 to −143)	5' AATTCCAGAGAGTCATCAG 3' 3' GGTCTCTCAGTAGTC 5'
NF-κB (−211 to 192)	5' AAGAGGGATTTCACCT 3' 3' TTCTCCCTAAAGTGGATTTA 5'
NFAT-1 (−289 to 260)	5' AAGAGGAAAATTTGTTTCATACAGAAGGCG 3' 3' TTCTCCTTTTAAACAAAGTATGTCTTCCGCTTAA 5'
Spl (−300 to −278)	5' gatcTCTCCACCCCAAAGAGGAAAATT 3' 3' AGAGGTGGGGTTTCTCCTTTTAActag 5'
CD28RE (−174 to 156)	5' gatcGGGGGTTTAAAGAAATTCC 3' 3' CCCCCAAATTTCTTTAAGGctag 5'
NF-IL2A (−100 to −69)	5' gatcTCTTTGAAAATATGTGTAATATGTAAAACAT 3' 3' AGAAACTTTTATACACATTATACATTTTGTActag 5'

Lowercase letters indicate bases not found in the natural sequence. Oligonucleotides were annealed with their complements and labeled by polymerization with α-^{32}P-labeled deoxynucleoside triphosphates to fill in the 5' overhanging ends.

Gel mobility shift assay. The gel shift assays were performed as described previously (6), with minor modifications. Nuclear extracts (2.5 to 10 μg of protein) were preincubated with 0.5 to 2 μg of poly(dI · dC) at 25°C for 15 min and then incubated with 5 fmol of ^{32}P-end-labeled oligonucleotides (2×10^4 to 4×10^4 dpm) for another 15 min in the final volume of 10 to 20 μl. Reaction mixtures were electrophoresed in 6% acrylamide gels, using 0.5× Tris-borate-EDTA as running buffer. Gels were dried and ex-

posed to Kodak XAR-5 film overnight at −70°C with an intensifying screen. For competition or antibody supershifting experiments, the indicated molar excess of specific competitor DNA or 0.5 to 1 μl of antibody was added at the beginning of the preincubation, before addition of the labeled oligonucleotide. Under these conditions, the complexes scored here (indicated by arrows or brackets in the figures) were all sequence specific and able to be competed for by homologous oligonucleotides.

Data analysis. To compare the representation of a given DNA-binding protein in different extracts, as in Fig. 3 through 5, samples containing equal amounts of nuclear protein were analyzed in parallel by electrophoretic mobility shift assay with the same preparation of labeled oligonucleotide probe. The amounts of radioactivity in the shifted complexes and, where applicable, in the unbound fraction of the probe were determined by analysis with a Molecular Dynamics PhosphorImager. The levels of binding activity in the various cell types were compared after being normalized to the levels of Oct-1 protein determined in parallel in aliquots of the same extracts. Results reported were all based on at least three (usually six or more) analyses using at least two (usually four or more) independent cell preparations.

The introduction of antibodies to identify complexes containing CREB, Jun, and Fos significantly perturbs quantitation. In our hands, the antibody against CREB appears to enhance the DNA-binding affinity of the complexes that it reacts with, so that the total amount of complex (shifted plus supershifted by reaction with antibody) appears greater than the amount of complex formed in the absence of antibody. Conversely, the antibodies against Jun and Fos individually reduce the DNA-binding affinity of the complexes that they react with and appear to reduce the overall amount of complex formed when they are used together in supershifting experiments (data not shown). We have therefore estimated the relative amounts of AP-1 (Fos/Jun) complexes only from the level of complex formed that is not supershifted by anti-CREB.

TABLE 1. IL-2 inducibility in thymocyte subpopulations

Cell population[a]	IL-2 activity (U/ml/5 × 10^6 cells)[b]		
	Exp 1	Exp 2	Exp 3
Total thymus			
Unstim.	<4[c]		
Stim.	52		33
Stim. + IL-1	104		
$CD4^+$ $CD8^-$[d]			
Unstim.			
Stim.			323
$CD4^+$ $CD8^+$[e]			
Unstim.			<4
Stim.			3.5
TcR^- $CD4^-$ $CD8^-$[f]			
Unstim.		<4	
Stim.		<4	
Stim. + IL-1		355	
SCID			
Unstim.	<4		
Stim.	99	37	
Stim. + IL-1	1,581	848	

[a] Cells were prepared as described in the text and cultured as indicated for 20 h. Unstim., unstimulated; Stim., in the presence of 100 nM A23187 and 17 nM TPA; Stim. + IL-1, as for Stim. plus IL-2 at 20 U/ml for experiment 1 and 50 U/ml for experiment 2.

[b] Measured by bioassay on CTLL-2 cells. Units are defined in a given experiment as described in reference 39. Where no value is shown, that sample was not included in a particular experiment. Results from experiments with 3 independent cell preparations, representative of over 10 independent experiments, are given (see also references 24 and 38 to 40).

[c] Undetectable.

[d] Prepared by cytolysis with anti-CD8. This fraction also contained ~30% $CD4^-$ $CD8^-$ cells.

[e] Prepared by cytolysis with anti-H-$2K^b$. This fraction consisted of 92% $CD4^+$ $CD8^+$ cells with <5% each $CD4^+$ $CD8^-$, $CD4^-$ $CD8^+$, and $CD4^-$ $CD8^-$ cells.

[f] Prepared from normal mice.

RESULTS

Nuclear extracts from cells in defined stages of T-cell development. To define the IL-2-regulatory proteins present at various stages of thymocyte development, we prepared nuclear extracts from isolated subsets of thymocytes representing the immature ($CD4^-$ $CD8^-$ TcR^-), transitional (cortical $CD4^+$ $CD8^+$ TcR^{low}), and mature helper-type ($CD4^+$ $CD8^-$ TcR^{high}) stages of development. The nuclear extracts from these populations were then used in electrophoretic mobility shift assays to detect factors capable of binding to any of a panel of double-stranded synthetic oligodeoxynucleotide probes derived from the murine IL-2 5′ flanking sequence. The sequences of these probes are listed in Materials and Methods. Previous reports and our own preliminary studies had indicated that the appearance of certain critical binding activities depended on cellular activation. Therefore, we prepared nuclear extracts from thymocyte subsets that were cultured for 2 to 4 h with or without stimulation with the phorbol ester TPA (17 nM) plus the Ca^{2+} ionophore A23187 (70 to 120 nM). These stimuli mimic the second messengers induced by TcR ligands but do not require the presence of the TcR complex and therefore allow cells from a broad range of developmental stages to be assayed for responsiveness.

Each population of cells analyzed was prepared by two different methods to control for any perturbations in cell physiology that might be caused by a particular method of fractionation (see Materials and Methods). For any given thymocyte subset, the different methods of isolation in fact yielded essentially indistinguishable profiles of DNA-binding proteins. Thus, the differences between fractions discussed below reflect intrinsic cellular characteristics and not simply artifacts of the isolation procedure.

The immature $CD4^-$ $CD8^-$ TcR^- cells, cortical-type $CD4^+$ $CD8^+$ TcR^{low} cells, and mature $CD4^+$ $CD8^-$ TcR^{high} cells differ significantly in the ability to express IL-2 upon induction (Table 1). In all cases, IL-2 protein secretion (Table 1) agrees well with IL-2 RNA expression, as measured by in situ hybridization and by quantitative RNase protection assays, as described elsewhere (25, 39, 40). The $CD4^+$ $CD8^-$ TcR^+ cells (initially ~10% of total thymus) are about 10-fold enriched for the ability to express IL-2 relative to unfractionated thymocytes, whereas cells in the $CD4^+$ $CD8^+$ TcR^{low} fraction (initially 80% of total thymus) are at least 20-fold depleted of this ability. The immature cells from the SCID thymus produce modest amounts of IL-2 under normal stimulation conditions and very high levels if IL-1 is added. In this intense IL-1-dependent response, they resemble normal immature thymocytes, which have the capacity to express high levels of IL-2 but do so only when IL-1 is present (Table 1). By contrast, addition of IL-1 does not

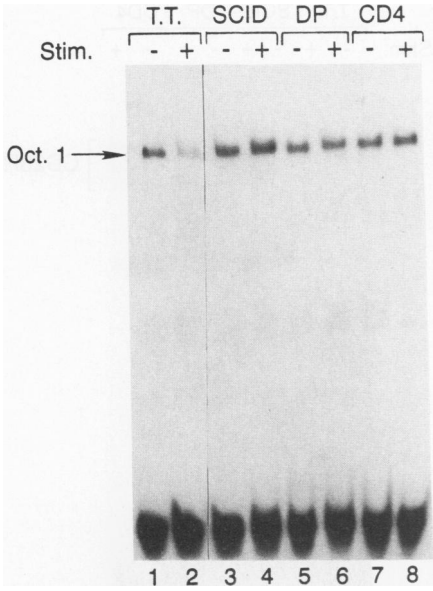

FIG. 1. NF-IL2A (Oct-1) binding activity in thymocytes. The NF-IL2A oligonucleotide (−100 to −69) was used as a probe to test the binding activity in nuclear extracts from unfractionated thymocytes (total thymocytes [T.T.]; lanes 1 and 2), SCID thymocytes (lanes 3 and 4), $CD4^+$ $CD8^+$ TcR^{low} cortical thymocytes (double positive [DP]; lanes 5 and 6), and a population enriched for mature $CD4^+$ $CD8^-$ TcR^{high} thymocytes (CD4; lanes 7 and 8). The cells were cultured in the absence (−) or presence (+) of A23187 plus TPA (Stim.) for 4 h prior to lysis. Aliquots estimated to represent equal amounts of nuclear protein from each sample were compared; in fact, the sample in lane 2 contained less protein than did the other samples. The electrophoretic mobility shift assay was performed as described in Materials and Methods.

reveal any cryptic IL-2 inducibility in the $CD4^+$ $CD8^+$ TcR^{low} cells (38; data not shown). These results agree well with previously published data (16, 24, 34, 38, 39) indicating that these fractions are functionally and phenotypically consistent.

DNA-binding factors present in developmental thymocyte subsets. The results of the electrophoretic mobility shift experiments revealed that the factors capable of binding to IL-2 gene flanking sequences can be divided into four classes on the basis of their developmental regulation.

(i) Constitutive activities. As shown in Fig. 1, all thymocyte fractions possess a nuclear protein with binding activity for the NF-IL2A element (−100 to −69), with or without induction by TPA plus A23187. Its mobility compared with those of the NFIL-2A-binding proteins in EL4.E1 cells suggests that this protein is Oct-1 (data not shown). In over 10 independent experiments, there was no consistent difference between fractions or between stimulated and unstimulated samples in the amount of this binding factor relative to total nuclear protein.

Another factor that appeared to be expressed constitutively was one that binds to the oligonucleotide containing a sequence upstream of the NF-AT site, namely, the region from −300 to −278 (Fig. 2a). Despite the lack of any conventional Sp1 sites in the IL-2-regulatory region (29), the −300/−278 oligonucleotide includes a sequence, CCAC CCC, which is similar to a reported variant Sp1-binding site (44). In fact, the −300/−278-binding factor binds with much higher affinity to a consensus Sp1 oligonucleotide than to the −300/−278 oligonucleotide itself, as demonstrated by the >10-fold difference between the molar efficiencies of these oligonucleotides as competitors (Fig. 2b). Binding of the thymocyte factor could not be significantly competed for by

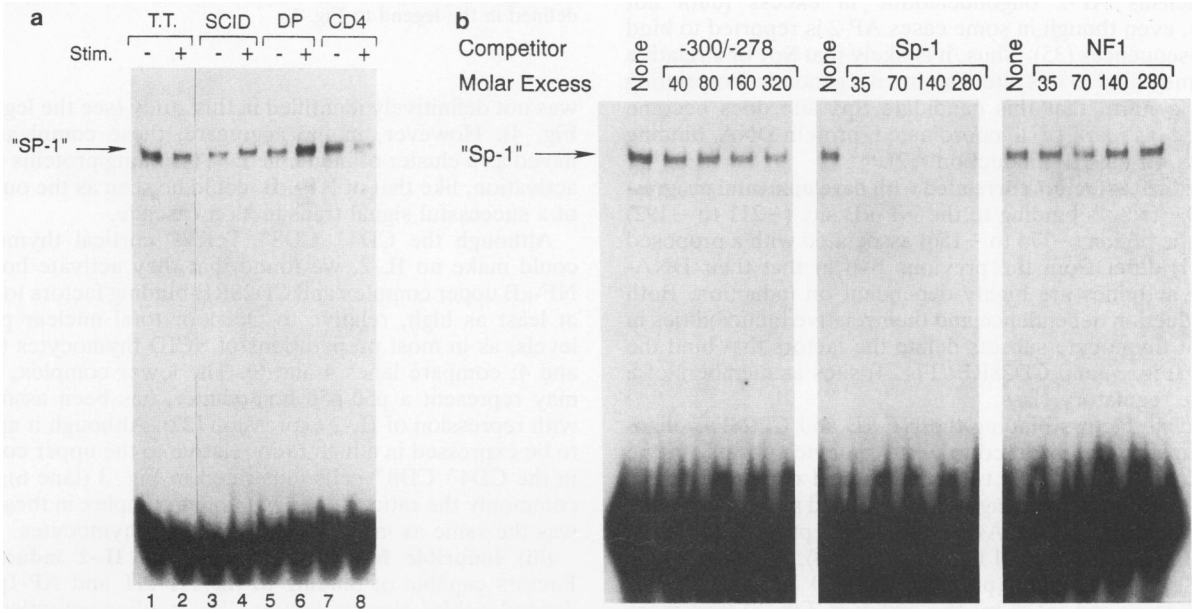

FIG. 2. Analysis of a constitutive DNA-binding activity in thymocytes that interacts specifically with the −300/−278 sequence. (a) Protein binding study. Nuclear extracts from samples of thymocytes that were cultured with (+) or without (−) stimulation were analyzed as for Fig. 1 to measure proteins binding to the −300/−278 oligonucleotide. Symbols and abbreviations are as defined in the legend to Fig. 1. The arrow indicates the prominent specific complex that may correspond to Sp1. (b) Results of a competition experiment suggesting that the major complex formed with the −300/−278 oligonucleotide may be closely related to Sp1. The indicated competitor oligonucleotides were added to an unfractionated thymocyte extract in the molar excesses over the labeled probe that are indicated above the lanes. The relatively poor self-competition by the −300/−278 oligonucleotide and the strong competition by the consensus Sp1 oligonucleotide indicate that the factor binds to the −300/−278 site with relatively low affinity. No competition is observed with the unrelated NF1 oligonucleotide.

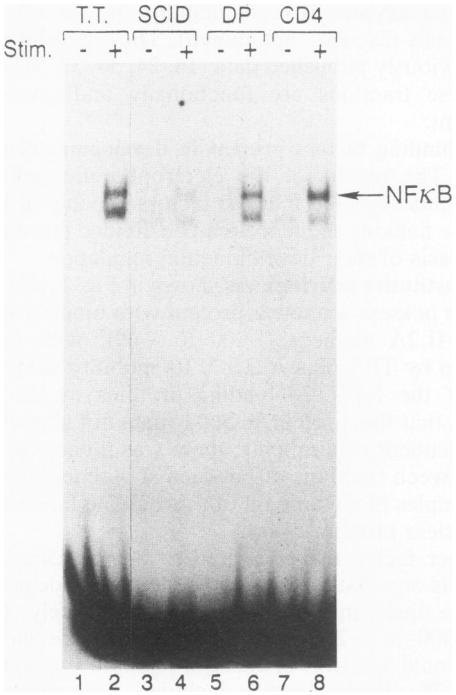

FIG. 3. Comparison of inducible NF-κB site-binding activity in different thymocyte subsets. Samples of the extracts analyzed in Fig. 1 and 2 were incubated with an oligonucleotide spanning from mouse IL-2 positions −211 to −192 to test for specific NF-κB site-binding activity. The specific inducible NF-κB-binding complex is indicated by the arrow. Abbreviations are as defined in the legend to Fig. 1.

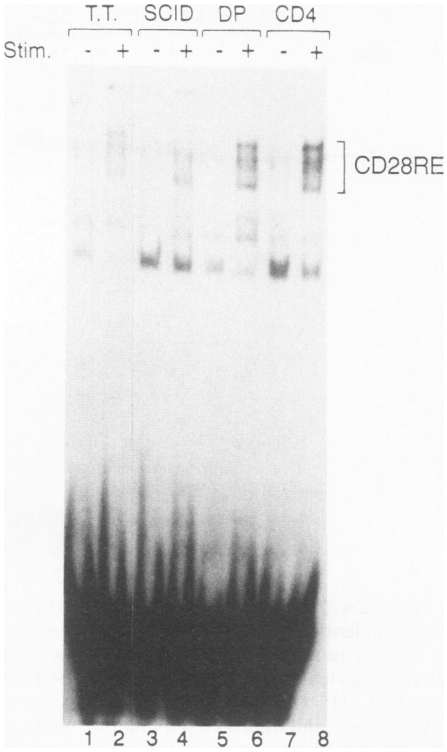

FIG. 4. Comparison of CD28RE-binding activity in different thymocyte subsets. The oligonucleotide spanning from IL-2 positions −174 to −156 was used in a binding assay with the panel of extracts analyzed in Fig. 1 through 3. The bracketed bands indicate inducible specific complexes. The complexity of this pattern, relative to that reported previously (11), may be due to the inclusion in our CD28RE oligonucleotide of five G residues that are omitted in the oligonucleotide used by Fraser et al. (11). Abbreviations are as defined in the legend to Fig. 1.

a consensus AP-2 oligonucleotide in excess (data not shown), even though in some cases AP-2 is reported to bind similar sequences (35). Thus, it is likely that Sp1 or a relative of Sp1 may bind to this site. Our in vivo genomic footprinting studies confirm that this candidate Sp1 site does become occupied as part of a coordinated protein-DNA binding complex during IL-2 induction (12).

(ii) Inducible factors correlated with developmental progression. The factors binding to the NF-κB site (−211 to −192) and to the region (−174 to −156) associated with a proposed CD28RE differ from the previous two in that their DNA-binding activities are highly dependent on induction. Both their induction dependence and their relative inducibilities in different thymocyte subsets define the factors that bind the NF-κB (Fig. 3) and CD28RE (Fig. 4) sites as members of a separate regulatory class.

Inducible factors binding the NF-κB and CD28RE oligonucleotides were observed in all thymocyte subsets. In the case of the NF-κB site, two well-defined complexes were seen, possibly representing the p65-p50 and p50-p50 forms of this transcription factor. As we have noted previously for the model IL-2-inducible cell line EL4.E1 (28), the appearance of the upper complex (putative p65-p50 form or T-cell-specific TCF; indicated by the arrow in Fig. 3) was more stringently induction dependent. We provisionally refer to this factor as NF-κB in the text that follows. The factors binding the CD28RE oligonucleotide formed multiple retarded complexes that were distinctively enhanced upon induction (bracket in Fig. 4). The specific type of complex reported to be induced in human Jurkat cells by CD28 engagement (11) may correspond to one of these bands but was not definitively identified in this study (see the legend to Fig. 4). However, in the aggregate, these complexes behaved as a cluster of inducible DNA-binding proteins whose activation, like that of NF-κB, could be seen as the outcome of a successful signal transduction cascade.

Although the $CD4^+$ $CD8^+$ TcR^{low} cortical thymocytes could make no IL-2, we found that they activate both the NF-κB upper complex and CD28RE-binding factors to levels at least as high, relative to Oct-1 or total nuclear protein levels, as in most preparations of SCID thymocytes (Fig. 3 and 4; compare lanes 4 and 6). The lower complex, which may represent a p50-p50 homodimer, has been associated with repression of IL-2 expression (22). Although it appears to be expressed in a high ratio relative to the upper complex in the $CD4^+$ $CD8^+$ cells illustrated in Fig. 3 (lane 6), more commonly the ratio of lower to upper complex in these cells was the same as in the other subsets of thymocytes.

(iii) Inducible factors correlated with IL-2 inducibility. Factors capable of binding to the NF-AT and AP-1p sites defined a third class of factors. The binding activities associated with these factors were highly induction dependent, like the factors in the second group. However, in contrast to the second group, the NF-AT and AP-1 (see below) factors appeared to be inducible mainly or exclusively in cells competent to express IL-2, and in both mature cells and immature thymocytes alike.

Figure 5 shows the results of assaying thymocyte extracts

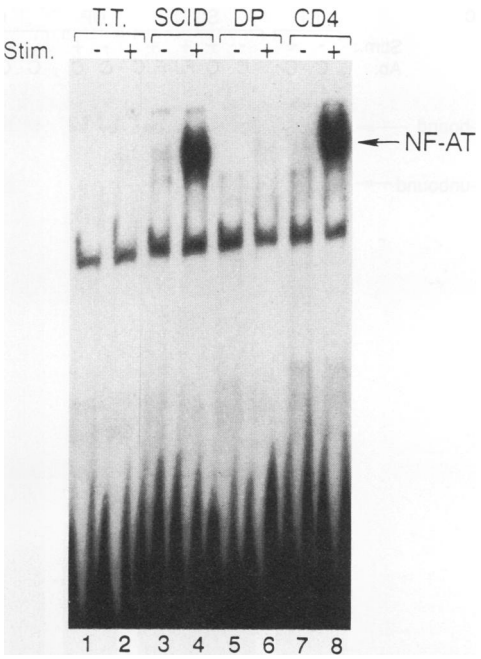

FIG. 5. Comparison of NF-AT-binding activity in different thymocyte fractions. The figure shows a typical result when the oligonucleotide from mouse IL-2 positions −289 to −260 was used in the binding assay with the panel of extracts used for Fig. 1 through 4. The inducible NF-AT complex is indicated by an arrow. Abbreviations are as defined in the legend to Fig. 1.

for NF-AT activity. NF-AT binding was highly inducible in mature $CD4^+$ cells but low or undetectable in unfractionated and $CD4^+$ $CD8^+$ cortical thymocytes (Fig. 5, lanes 2, 6, and 8). These results are in full agreement with the report by Riegel et al. (34). The inducibility of NF-AT-binding activity was not restricted to cells in the final stage of T-cell differentiation and maturation, however. SCID thymocytes (as well as $CD5^{low}$ $CD4^-$ $CD8^-$ TcR^- thymocytes isolated from normal mice; data not shown) were able to mobilize NF-AT-binding activity to levels quite comparable with those of mature $CD4^+$ $CD8^-$ TcR^{high} cells. A comparative titration of nuclear extracts from stimulated $CD5^{low}$ $CD4^-$ $CD8^-$ TcR^- immature cells from normal mice and stimulated $CD4^+$ $CD8^+$ TcR^{low} cortical cells showed that the immature cells contained at least 10 times more NF-AT DNA-binding activity than do the cortical cells (data not shown). Thus, NF-AT inducibility appears to be acquired at an early developmental stage.

(iv) Developmentally distinct factors binding to the AP-1 site. Our results indicate that the major AP-1 site at −161 to −143 in the IL-2 5′ flanking region ($AP\text{-}1_p$) can be engaged by at least two differentially regulated factors, one conventionally inducible factor consisting entirely of Fos and Jun family members (AP-1 complex) and one which includes at least one CREB subunit (CREB complex). The two complexes were similar in electrophoretic mobility and could be resolved only by use of antibodies.

All thymocyte subsets contained factors capable of binding to the putative AP-1 site, and surprisingly, most did so with or without in vitro stimulation (Fig. 6a). In total thymocytes overall and in isolated $CD4^+$ $CD8^+$ TcR^{low} cells, essentially all of the $AP\text{-}1_p$ site-binding activity was attributable to the CREB complex, whether or not the cells were stimulated (Fig. 6b, lane 2; Fig. 6c, lanes 1, 2, 7, and 8). CREB complexes were present in unstimulated cells of all subsets (Fig. 6c, lanes 1, 3, 7, and 9). Levels of the CREB complex appeared to be increased by exposure of the thymocytes to culture at 37°C (data not shown) but were not enhanced by TPA plus A23187. In $CD4^+$ $CD8^-$ TcR^{high} and SCID thymocytes specifically, however, activation with TPA plus A23187 led to strong induction of an AP-1 complex (Fig. 6c, lanes 3, 4, 9, and 10). Thus, we conclude that (i) all thymocytes can activate a leucine zipper family factor including at least one CREB subunit simply in response to stimuli such as a temperature shift and (ii) immature and mature thymocyte subsets alike can activate a canonical AP-1 factor in response to Ca^{2+} ionophore and protein kinase C stimulation, but (iii) cortical $CD4^+$ $CD8^+$ TcR^{low} thymocytes are specifically disabled in their activation of the inducible AP-1 factor despite their ability to activate other factors such as the CD28RE-binding complexes and NF-κB.

DISCUSSION

Summary of developmental changes in IL-2 DNA-binding factors. The pattern of inducibility of the DNA-binding proteins studied here is summarized qualitatively in Fig. 7. As a framework for the following discussion, the data are presented as a hypothetical developmental progression, in which the average cell types in each of the fractions studied here are assumed to be linked in a precursor-product continuum. The discontinuities in the inducibility of AP-1 (Fos/Jun) and NF-AT, both at the immature-to-cortical transition and, presumably, at the cortical-to-mature transition (see below) contrast sharply with the gradual, monotonic increases in the inducibility of NF-κB and at least the majority of the complexes that bind to the CD28RE oligonucleotide. Each thymocyte subset has its own characteristic profile of these factors. The combinatorial requirement for these factors in assembling IL-2 transcription complexes accounts for the ability of immature thymocytes and the inability of their cortical thymocyte descendants to express the IL-2 genes. The most interesting aspect of these results, however, is the light that they shed on the kinds of physiological changes imposed on developing thymocytes during intrathymic processing.

Reverse maturation of function in the immature-to-cortical thymocyte transition. The results presented here show that cells in the immature population can already deploy a set of transcription factors that includes all those known to be needed for IL-2 expression in mature cells. Even NF-AT, initially described as a mature T-cell-specific factor, is already highly inducible in these cells. Any developmental changes needed to turn on NF-AT expression must therefore already have occurred at this stage, before TcR gene rearrangement. Furthermore, the signaling pathways needed to activate NF-AT and other regulatory factors already appear to be effectively indistinguishable in immature cells from those in mature T cells. Both normal $CD4^-$ $CD8^-$ TcR^- populations (data not shown) and SCID thymocytes could activate NF-AT, the AP-1 complex, and other regulatory factors comparably to mature cells in response to A23187 plus TPA. Surprisingly, costimulation with IL-1 did not appear to be required for induction of any of these activities. The only difference in DNA-binding protein activation observed in the immature cell samples (normal or SCID) when IL-1 was added was a modest enhancement of the induction of the NF-κB binding activity (data not shown). The basis for the strong observed effect of IL-1 on IL-2 RNA and protein

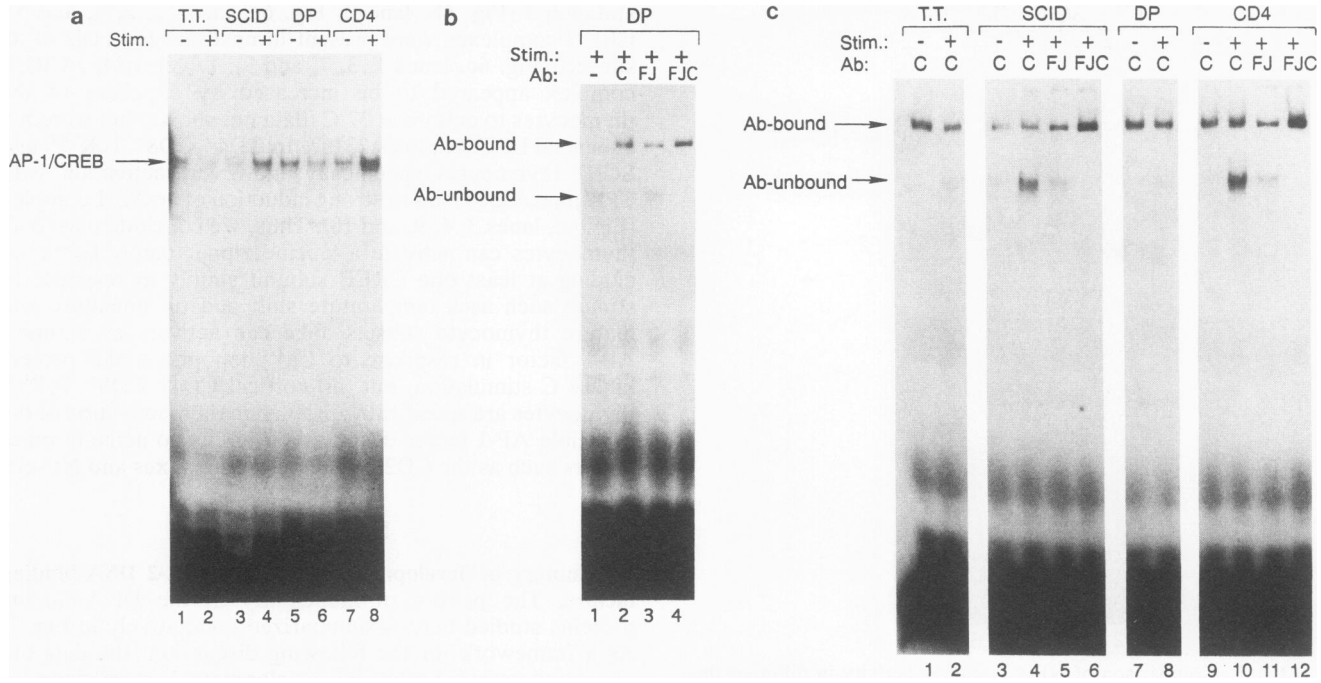

FIG. 6. AP-1/CREB proteins of thymocytes. (a) Comparison of AP-1_p site-binding activity in different thymocyte fractions. Nuclear extracts from the indicated thymocyte samples were analyzed for complex formation with the AP-1_p oligonucleotide. Abbreviations for thymocyte samples and culture conditions are as given in the legend to Fig. 1. (b) Reactivity of AP-1_p site-binding factors in cortical thymocytes with different antibodies. An extract from stimulated $CD4^+$ $CD8^+$ TcR^{low} thymocytes was used for electrophoretic mobility shift assays with the AP-1_p oligonucleotide, with the following antibodies (Ab) added: lane 1, none; lane 2, anti-CREB; lane 3, anti-Fos and anti-Jun family; lane 4, all three antibodies. Anti-CREB shifts a much larger fraction of the initial, unperturbed binding activity than does anti-Jun plus anti-Fos. The material reactive with anti-CREB defines the CREB complex. (c) Differential expression in thymocyte subsets of Fos/Jun and CREB complexes with binding activity for the AP-1_p site. Extracts from the thymocyte samples shown in panel a were analyzed for binding to the AP-1_p oligonucleotide in the presence of the following antibodies: anti-CREB (C), anti-Fos family plus anti-Jun family (FJ), and a cocktail of all three antibodies (FJC). As CREB-like factors are present in all samples, all C lanes contain supershifted (antibody-bound) complexes. The Fos/Jun or AP-1 complex referred to in the text is the material in the lanes treated with anti-CREB that remains in the antibody-unbound position. Lanes 6 and 12 verify that all this material is in fact reactive with anti-Jun and/or anti-Fos.

expression (9, 17, 39) must therefore reside elsewhere and remains under investigation. However, the results presented here clearly show that the molecular prerequisites for IL-2 producer function that we can score have already been achieved through extremely early events in T-cell development, in a process completely independent of T-cell recognition specificity.

In this context, the transition to $CD4^+$ $CD8^+$ TcR^{low} cortical thymocyte status is confirmed as a loss of response function. However, the loss is surprisingly specific. Previous work had shown that murine $CD4^+$ $CD8^+$ TcR^{low} thymocytes fail to express known response genes, like the IL-2 and IL-2 receptor alpha-chain genes, even when the TcR is bypassed with strong pharmacological stimuli (4, 8, 9, 16, 24). A priori, the cells might have been found to lack basic molecular transducers of signals such as calmodulin or appropriate isoforms of protein kinase C. Instead, the results described here show that these cells are capable of strong though selective transcription factor activation. For example, the inducibility of NF-κB in these cells makes it likely that a fully operational protein kinase C-dependent pathway is present. Thus, the dissociation of one ubiquitous phorbol ester-inducible response from another, in the selective loss of AP-1 induction while NF-κB induction is preserved, implies a precise, factor-specific mechanism rather than a cataclysmic, global change in cell physiology. There are two implications of these results. First, the reverse maturation process in which AP-1 and NF-AT inducibility are lost defines a novel transition in thymocyte development. Its possible timing and significance are discussed further below. Second, the cells in this functionally incompetent population remain highly responsive, with an ability, in principle, to integrate signals from protein kinase C, cyclic AMP/protein kinase A (50), and possibly also CD28 (14, 46) for transcription factor mobilization. Their potential to carry out efficient, if specialized, patterns of gene expression is further supported by our recent evidence that they can serve as preferential targets for transient expression of certain exogenous DNAs in transfection experiments (30). Thus, the cortical thymocytes need not be beyond rescue; in fact, the repertoire of inducible transcription factors that they preserve might participate in the poorly understood processes of positive and negative selection.

Selection and the selectable state. A most interesting possibility raised by these results is that changes in NF-AT and AP-1 inducibility may contribute to the mechanisms that (i) make thymocytes subject to selection and (ii) transform them into mature thymocytes via positive selection. Figure 7 indicates a possible temporal correlation between these important developmental transitions and the discontinuities in the use of NF-AT and AP-1. At present, this hypothesis is speculative, because it is not yet known whether the "typical" subset members depicted in Fig. 7 are truly related in a precursor-product continuum. However, the striking simi-

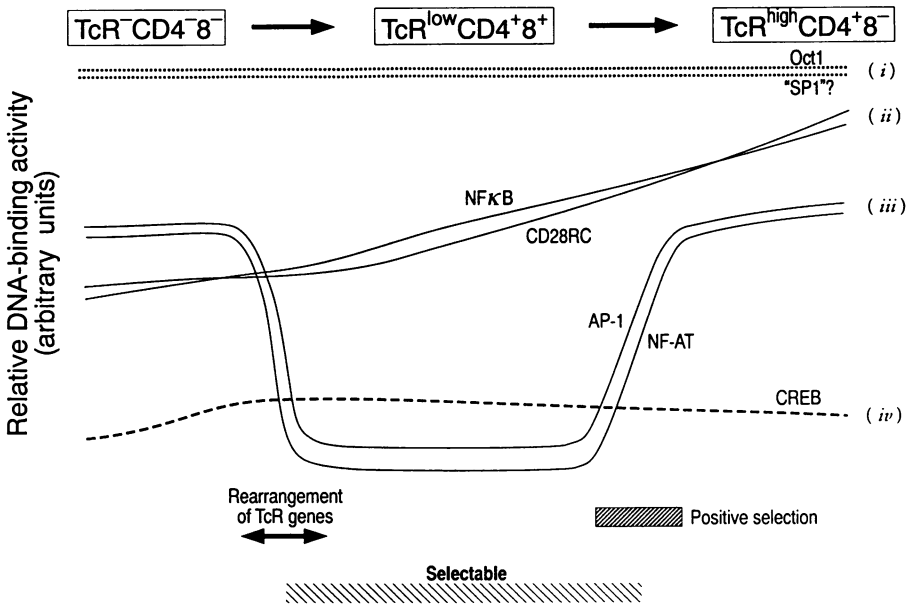

FIG. 7. IL-2 DNA-binding factors in thymocytes: a proposed developmental progression. This summary figure juxtaposes the results from Fig. 1 through 6 on the abundance of various IL-2 DNA-binding factors in three broad stages of thymocyte differentiation. The data are arranged along a horizontal axis that represents the differentiative progress of thymocytes toward maturity, with the key events that mark transitions and states of interest indicated. While the characteristics of the three stages are established by our results, the proposed connections between them are hypothetical, as discussed in the text. The vertical axis represents relative levels of DNA-binding activities of various factors, expressed on an arbitrary scale, with the results for different classes of factors offset. As the true copy numbers of different factors are not known, the values along the vertical axis in this figure cannot be used to compare one factor with another. (i), (ii), (iii), and (iv) refer to the four regulatory classes of factors distinguished in the text. · · · ·, true constitutive factors. – – –, CREB, the binding activity of which is increased by culture but is not dependent on Ca^{2+}/protein kinase C activation; ——, factors whose DNA-binding activity is detectable only after Ca^{2+}/protein kinase C activation.

larities between the IL-2 DNA-binding activities available in immature and mature thymocytes suggest two possible kinds of linkages between transcription factor regulation and thymocyte fate determination. One, an alternative to the scheme shown in Fig. 7, is that the downregulation of NF-AT and AP-1 may occur only in cells that have failed positive selection and are committed to die. In this case, cortical thymocytes that remain susceptible to positive selection could be identified as a subset specifically protected from NF-AT and AP-1 downregulation, bypassing the typical cortical cell state. The other possibility, illustrated in Fig. 7, is that a relatively simple, reversible molecular switch may shut off AP-1 and NF-AT inducibility prior to any selection and restore it as a consequence of selection. In this case, the mechanism controlling NF-AT and AP-1 inducibility would define one of the molecular targets of the positive selection process. The choice between these alternative interpretations depends on the precise timing of NF-AT and AP-1 downregulation relative to TcR gene rearrangement and surface expression. As of this writing, the possibility depicted in Fig. 7 is favored by two kinds of evidence. We and others have already shown that thymocytes lose a variety of inducible gene expression responses at a stage well before their acquisition of a selectable TcR (3, 9, 37, 51). Also, our own preliminary results (7) indicate that NF-AT and AP-1 inducibility has already been lost by cortical thymocytes that are still actively in cycle, a population that clearly includes cells with full precursor activity that may yet be positively selected (15, 19, 31, 43). Thus, no evidence yet available suggests loss of activatability as a late event. If further investigation confirms the early timing of NF-AT and AP-1 downregulation, then this process must be reversible, as a direct or indirect consequence of positive selection. Work to define the biochemical basis of the changes in AP-1 and NF-AT availability is now under way.

In summary, the data from this study identify NF-AT and the AP-1 complex described here as potentially valuable probes to define the mechanisms regulating the varying responses of thymocytes to activation in the course of their development. A full understanding of their changes in activity must await the cloning of the genes encoding NF-AT components and the identification of AP-1 family members that actually participate in IL-2 regulatory complexes. However, the inducibility of these factors is at least correlated with the abrupt changes in cellular responses to signaling that define both onset of and rescue from the selectable state. Furthermore, their pleiotropic roles in response gene regulation can explain the functional effects of the regulatory changes. Whether or not their function is also implicated in the fate determination events per se, they provide likely molecular targets of the switch mechanism that transforms cell physiology to convert simple activation signals into the arbiters of irreversible developmental choice.

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