

# Transcription factors in mouse fetal thymus development

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## Abstract

**T cell precursors from murine fetal liver enter the fetal thymus where they proliferate, differentiate, and mature. These processes are accompanied by changes in the pattern of transcription factors known to control the expression of specific genes. We have monitored the expression of five different transcription factors during mouse fetal thymus ontogeny: nuclear factor (NF)- $\kappa$ B, cAMP-response-element binding protein (CREB), NF-IL-2A, msNF-AT1, and hNF-AT1. NF- $\kappa$ B binding activity was not detected in extracts from fetal liver but was present in the thymus at day 14 of embryogenesis. Thereafter, NF- $\kappa$ B expression was biphasic, being maximal at 14–16 days gestation and in newborn mice, and decreased during the intermediate gestational stages and in the adult. An inverse correlation was observed between NF- $\kappa$ B binding activity in the nuclei and levels of its inactive precursor in the cytoplasm of all samples analyzed. In contrast, CREB activity was uniform throughout thymus development. Similarly, NF-IL-2A activity was detected in fetal liver and thymic extracts from different gestational stages, in approximately equivalent amounts. However, band shift experiments revealed three distinct NF-IL-2A–DNA complexes, whose relative abundance is altered during thymic ontogeny. Likewise, NF-AT1 transcription factor appears to be heterogeneous and includes representatives which are differentially (msNF-AT1) or stably (hNF-AT1) expressed during thymic development. These results are discussed in the context of present knowledge about T cell development within the thymus.**

## Introduction

Complex cell–cell interactions, hormones, growth factors, and other extracellular inducers are known to activate specific patterns of genes in target cells during development or in response to the environment. Regulation of gene expression occurs at several different levels, but the activation of *trans*-acting protein factors of transcription (which mediate differential gene transcription by binding to their cognate *cis*-acting elements in gene promoters and enhancers) is perhaps the most fundamental (1,2). Regulatory regions of eukaryotic genes contain specific combinatorial patterns of *cis*-acting elements, which are frequently similar and bind ubiquitous transcription factors. However, some transcription factors have a more restricted tissue or cell distribution and mediate tissue-specific gene expression. The best examples of such are the transactivator MyoD, a product of the master regulatory gene for myogenesis *myoD*, the transcription factor GATA-1, which is restricted to hematopoietic cells, and the

transcription factor HNF1/LFB1 that is enriched in the liver (3–5)

Until now, relatively little information has been available regarding T cell specific or T cell associated transcription factors. Possible candidates include TCF1 $\alpha$ /LEF1, which is essential for T cell receptor  $\alpha$  chain (TCR- $\alpha$ ) and CD4 gene transcription (6,7), and GATA-3, which binds to several TCR regulatory elements (8,9). Much more is known about ubiquitous transcription factors such as nuclear factor (NF)- $\kappa$ B and cAMP-responsive-element binding protein (CREB). NF- $\kappa$ B activity has been found in nuclear fractions of many different cell types, but particularly within nuclei of cells of the immune system. Most target genes for this factor can be divided into three groups: (i) genes encoding immunomodulatory cytokines such as IL-2, IL-6, tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), and granulocyte macrophage colony stimulating factor (GM-CSF), (ii) genes encoding immunoregulatory surface receptors such as IL-2 receptor  $\alpha$  chain (IL-2R $\alpha$ ), the MHC class

I proteins (MHC I) and the invariant chain of MHC class II; and (iii) acute phase proteins including serum amyloid A precursor (for reviews, see refs 10 and 11).

CREB is responsible for transcriptional activation of different cAMP-inducible genes (for review, see ref. 12). CREB, or related proteins, activate the murine TCR- $\beta$  chain transcription, binding to the *cis*-element CRE found in the  $V_{\beta}$  promoter and TCR- $\beta$  enhancer regions (13,14). In addition, the transactivator CREB is known to bind to both human and mouse 3' TCR- $\alpha$  enhancers (15) and a CRE motif has been found in the 3' enhancer of the human CD2 gene (16).

There are at least two other important regulators of transcription in T lymphocytes, i.e. factors NF-AT1 and NF-IL2A. These factors recognize cognate elements which were originally described within the IL-2 gene regulatory region (17–20). Recently, it has been shown that the NF-AT1 binding site has a complex structure and also contains an Ets cognate sequence (21). In addition, Ets binding sites have been found in the regulatory regions of many genes having T cell specific expression, i.e. CD2, CD3 $\delta$ , CD3 $\epsilon$ , TCR- $\alpha$ , TCR- $\beta$ , and TCR- $\delta$  (21).

Most studies dealing with transcription factors and the genetic regulatory events in T lymphocytes have been restricted to examining events in mature T cell activation (22). Relatively little is known about transcriptional control in developing thymocytes. However, because complex cell–cell interactions between stroma and thymocytes are known to control development within the thymus, it seems likely that transcription factors mediate at least some of these events. T cell precursors derived from fetal liver enter the fetal thymus anlage via the blood stream and then undergo a complex series of differentiation and maturation events. During this process, rapidly dividing immature CD4<sup>-</sup>CD8<sup>-</sup> cells differentiate through an intermediate stage (CD4<sup>+</sup>CD8<sup>+</sup>) expressing both CD4 and CD8 before finally giving rise to cells expressing either CD4<sup>+</sup> or CD8<sup>+</sup>. These latter 'mature' thymocytes express clonally elaborated TCR molecules and are immunologically competent (for review, see ref. 23). During mouse fetal thymus development, CD4<sup>+</sup>CD8<sup>+</sup> (double positive) and cells expressing either CD4 or CD8 (single positive) appear at days 16 and 18–19 respectively (24). In this study, we investigated the changes in transcription factor expression that accompany these complex intrathymic events. In the first instance, we examined nuclear and cytoplasmic extracts prepared from fetal thymocytes starting at day 14 of embryonic development for a variety of transcription factors. The results obtained showed that transcription factor levels are tightly regulated during thymic development.

## Methods

### Oligonucleotides

Synthetic oligonucleotides were end-labeled with [ $\gamma$ -<sup>32</sup>P]ATP using T4 polynucleotide kinase (25). Sequences of double-stranded oligonucleotides used in this study are shown in Table 1.

### Thymocyte preparation

Cell suspensions were prepared from the thymuses of fetal, newborn, or 4 week old adult (C57Bl/6  $\times$  DBA/2)F<sub>1</sub> mice. Fetal mice were obtained from timed matings of DBA/2 female and

C57Bl/6 male mice, and the date of finding the vaginal plug was taken as day 0 of development. Fetal thymuses were removed using a dissecting microscope and cell suspensions prepared by passing thymus lobes through hypodermic needles of decreasing size as previously described (26).

### Nuclear and cytosol extract preparation and electrophoretic mobility-shift assay (EMSA)

Nuclear and cytoplasmic extracts were prepared from fresh thymuses using a modification of the technique described by Dignam *et al.* (27,28). Buffers contained the protease inhibitors phenylmethylsulfonyl fluoride (PMSF) (0.5 mM), and 5  $\mu$ g/ml each of leupeptin, aprotinin, pepstatin, chymostatin, and antipain. Proteins were quantified by the method of Bradford (29). Binding reactions were carried out by incubating the end-labeled DNA (20 000 c.p.m.) with 1–2  $\mu$ g of nuclear or 5–10  $\mu$ g cytoplasmic proteins and 2  $\mu$ g of poly(dI–dC) in a buffer containing 10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), pH 7.9, 60 mM KCl, 4% Ficoll, 1 mM EDTA (30). After 30 min at room temperature, the reaction mixtures were loaded onto a 4% polyacrylamide gel in 0.25  $\times$  TBE buffer (TBE: 89 mM Tris, 89 mM boric acid, 2 mM EDTA) and electrophoresed at 10 V/cm for 1.5 h at room temperature. For competition experiments, a 100- to 400-fold molar excess of unlabeled oligonucleotide was added to the reaction mixture prior to the addition of labeled DNA. The treatment of cytosolic fractions with 0.6% deoxycholate (DOC) in the presence of 1.2% NP-40 was performed during the binding reaction as described by Baeuerle and Baltimore (31).

### Photo-affinity labeling of sequence-specific proteins (UV crosslinking)

DNA–protein UV crosslinking was performed using a modification of the method reported by Wu *et al.* (32). The first stage of the binding reaction was the same as that for EMSA except for an increased concentration (up to 60 000 c.p.m.) of the labeled DNA probes. After 30 min at room temperature, the reaction mixtures (in open tubes) were placed on ice 5 cm from an inverted 254 nm UV transilluminator and irradiated for 20 min. Then, an equal volume of 2% sodium dodecylsulfate (SDS), 10% 2-mercaptoethanol, 0.1 M Tris–HCl, pH 7.0, 20% glycerol, and Bromphenol blue dye was added. The samples were boiled for 2 min and subjected to electrophoresis in 0.1% SDS–10% polyacrylamide gels followed by autoradiography.

## Results

### NF- $\kappa$ B binding activity in fetal and adult thymus

To probe for NF- $\kappa$ B binding activity in nuclear extracts of thymocytes, EMSA was used with three different labeled probes: a  $\kappa$ B oligonucleotide from the human immunodeficiency virus (HIV) type 1 enhancer ( $\kappa$ B), a mutated variant of this oligonucleotide with three point mutations in the binding site ( $m\kappa$ B), and a palindromic variant of the  $\kappa$ B element from the MHC class I gene promoter region (MHC- $\kappa$ B) (Table 1). Incubation of the radiolabeled  $\kappa$ B oligonucleotide with nuclear extracts of thymocytes from newborn and adult mice revealed two discrete, electrophoretically retarded DNA–protein complexes (b1 and b2) (Fig. 1). These two bands were observed more clearly after

**Table 1.** Oligonucleotide with binding sites for transcription factors

Oligonucleotides <sup>a</sup> (5' – 3')	Binding sites and transcription factors	Gene promoters or enhancers
AGCTTGGGGACTTTCAGCCG	$\kappa$ B/NF- $\kappa$ B	HIV
AGCTTGGGGATTCCCATCTG	MHC- $\kappa$ B/NF- $\kappa$ B	MHC I (H-2K <sup>b</sup> )
AGCTT <b>GCT</b> CACITTCAGCCG	m $\kappa$ B <sup>b</sup>	
AGCTTTTGGAACTCTATCCAAGTCG	NF1-/NF1	MMTV-LTR
AGCTTGATGAGTCAGCCG	AP1-/AP1	human collagenase
AGCTCCATGACGTCATGG	CRE/CREB	TCR- $\alpha$
GGAGGAAAAACTGTTTCATACAGAAGGCGT	AT1 <sup>c</sup> /hNF-AT1	human IL-2
GGAGTAAAAA <b>ATTTT</b> TAATACAGAAGGCGT	mAT1 <sup>b</sup>	
-GAGGAAAA <b>TTT</b> GTTTCATACAGAAG	msAT1 <sup>b</sup> /msNF-AT	murine IL-2
GAAAAATATGTAAATATGTAAAAACATTTTG	IL-2A <sup>d</sup> /NF-IL2A	human IL-2
AGCTTGATTTGCATTTCTG	Oct/OTF	Ig $\kappa$ chain

<sup>a</sup>Known binding sites of transcription factors are underlined.

<sup>b</sup>Mutated nucleotides are shown in bold.

<sup>c</sup>Ets-binding site is shown in italic.

<sup>d</sup>Non-canonical Oct-binding site is shown in italic.

shorter exposure of autoradiograms (Fig. 2, panel B). The formation of these complexes was inhibited by preincubation with a 200-fold molar excess of unlabeled  $\kappa$ B oligonucleotide but was not inhibited by excess unlabeled m $\kappa$ B oligonucleotide (Fig. 1, lanes 2, 5 and 3, 6 respectively). Incubation of the radiolabeled m $\kappa$ B oligonucleotide with nuclear extracts from newborn or adult thymocytes did not produce any significant DNA-protein complexes (Fig. 1, lanes 7 and 8). Hence, NF- $\kappa$ B binding activity was specific in nuclear extracts from mouse thymocytes.

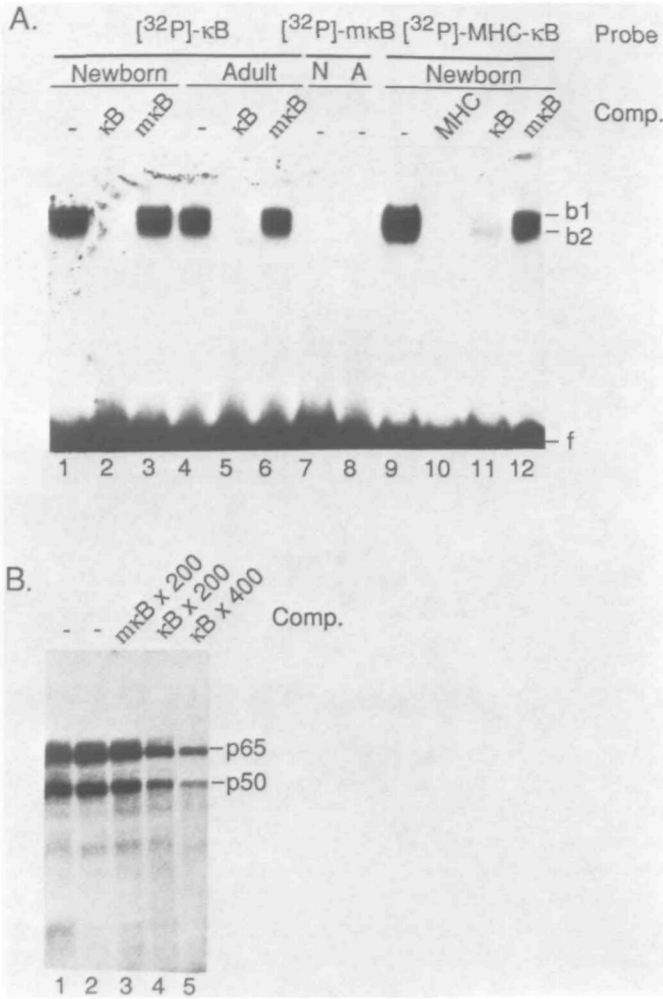
Using labeled MHC- $\kappa$ B oligonucleotides, EMSA produced the same retarded DNA-protein complexes as with the  $\kappa$ B probe (Fig. 1, lane 9, bands b1 and b2). However, two differences were noted between EMSA experiments with the  $\kappa$ B and MHC- $\kappa$ B oligonucleotides. Firstly, a shorter exposure of EMSA showed that with the MHC- $\kappa$ B oligonucleotide (Fig. 2C, lanes 10–12), the two bands b1 and b2 were of approximately equal intensity for newborn thymus extracts, whereas with the  $\kappa$ B oligonucleotide (Fig. 2B) band b1 was consistently more intense than band b2. Secondly, in competition experiments, a 200-fold excess of unlabeled  $\kappa$ B-oligonucleotide failed to completely inhibit the appearance of band b2 (Fig. 1, lane 11). Taken together, these results would seem to agree with the recent reports of Ghosh *et al.* (33) and Urban and Baeuerle (34) who suggested that the upper band b1 may result from the interaction of the heterodimeric p65-p50 form of NF- $\kappa$ B with the  $\kappa$ B oligonucleotide, while the lower band b2 was the result of the interaction of the homodimeric p50-p50 form of NF- $\kappa$ B with the same probe. These results suggest that the palindromic MHC- $\kappa$ B oligonucleotide may bind equally well to both isoforms of NF- $\kappa$ B, whereas the non-palindromic  $\kappa$ B oligonucleotide has a higher affinity for the p65-p50 NF- $\kappa$ B complex.

We used UV crosslinking of the  $\kappa$ B probe to NF- $\kappa$ B-like proteins from newborn thymocyte extracts to determine the subunit composition of these proteins (Fig. 1B). Taking into account the molecular weight of the oligonucleotide probe, SDS-PAGE showed that newborn thymus NF- $\kappa$ B-like protein had the

canonical subunit composition for NF- $\kappa$ B factor (35) with apparent molecular weights of 65 and 50 kDa. Labeling of these proteins was inhibited by a excess of  $\kappa$ B oligonucleotide but not of m $\kappa$ B oligonucleotide (Fig. 1B), again showing the specificity of the interaction being measured.

These experiments established the specificity of NF- $\kappa$ B detection. NF- $\kappa$ B binding activity during mouse thymus ontogeny was then determined. Results of these experiments are shown in Fig. 2. The first time point studied was day 14 of embryonic development. For these experiments, nuclear extracts of thymocytes were prepared from a pool of 40–50 mice, equivalent to  $1.5-2 \times 10^6$  cells, and in all experiments equal amounts of protein were used in each reaction. These studies showed that NF- $\kappa$ B binding activity was high on days 14–16 of development with a significant decrease during days 17 and 18. After birth, NF- $\kappa$ B levels increased slightly before reaching the adult thymus level (Fig. 2). Importantly, the intensity of the upper b1 band, which corresponds to the p65-p50 complex of NF- $\kappa$ B, was greater early in fetal thymus development (Fig. 2B and C, lane 1–3). In the newborn thymus (lanes 10–12) the intensity of bands b1 and b2 were approximately equal.

It is well known that in many different tissues there is a cytoplasmic precursor pool of NF- $\kappa$ B factor. The heterodimeric complex of p65-p50 NF- $\kappa$ B interacts with the inhibitory protein I $\kappa$ B which keeps NF- $\kappa$ B in the cytoplasm in an inactive form (36,37). Treating cytoplasmic extracts with DOC dissociates NF- $\kappa$ B from I $\kappa$ B and thereby reveals the presence of an inactive cytoplasmic pool of NF- $\kappa$ B. Mouse fetal thymocyte cytoplasmic extracts were therefore exposed to DOC to measure their cytoplasmic NF- $\kappa$ B content. Our results showed that between days 14 and 18 of development there was a significant increase in the cytoplasmic pool of NF- $\kappa$ B binding activity (Fig. 3, *cf.* lanes 3, 7 and 11). This binding activity was competed by excess unlabeled  $\kappa$ B oligonucleotide (Fig. 3, lanes 8 and 12). Hence, during fetal thymus development, an inverse correlation between the levels of the nuclear NF- $\kappa$ B and of the cytoplasmic NF- $\kappa$ B

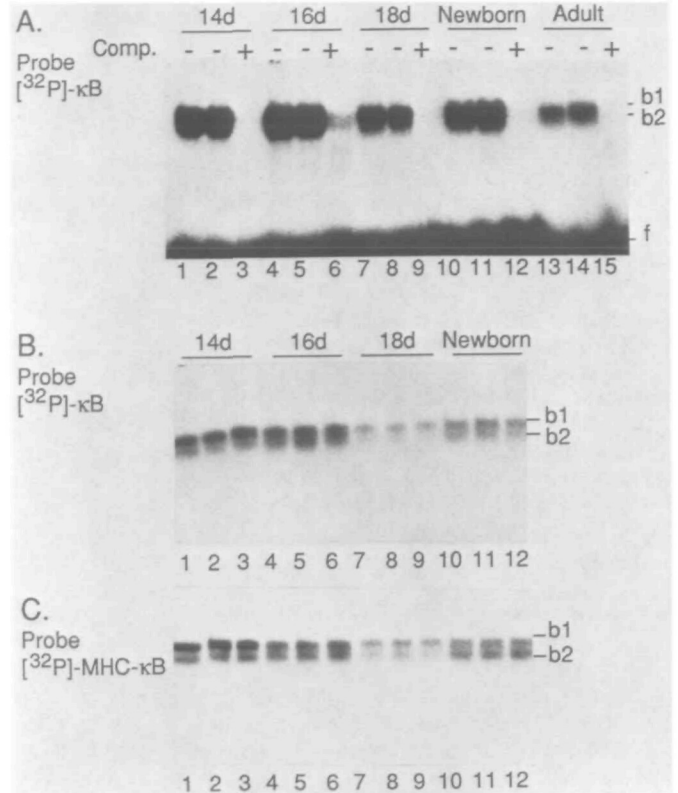


**Fig. 1.** NF- $\kappa\text{B}$  binding activity in nuclear extracts of thymus of newborn and adult mice. (A) EMSA was done using three labeled oligonucleotides.  $\kappa\text{B}$ ,  $\text{m}\kappa\text{B}$ , and  $\text{MHC-}\kappa\text{B}$  (see Table 1 for oligonucleotide sequences). A 200-fold molar excess of each unlabeled oligonucleotide was used in the competition experiments. N, newborn; A, adult mouse. The arrows b1 and b2 indicate the mobility of NF- $\kappa\text{B}$ -DNA complexes. (B) Detection of NF- $\kappa\text{B}$  protein subunits by UV cross linking to  $[^{32}\text{P}]\text{-}\kappa\text{B}$  oligonucleotide and SDS - PAGE. DNA-binding reactions were performed with total protein extract of thymuses of newborn mice without competitors (lanes 1 and 2) or in the presence of 200-fold molar excess of  $\text{m}\kappa\text{B}$  oligonucleotide (lane 3), or 200- or 400-fold excess of  $\kappa\text{B}$  oligonucleotide (lanes 4 and 5).  $\kappa\text{B}$  oligonucleotide was crosslinked after DNA-binding reaction with sequence-specific proteins using UV treatment. Then labeled proteins were analyzed by 0.1% SDS - 10% polyacrylamide gel electrophoresis with protein markers. The main protein subunits of NF- $\kappa\text{B}$  were designated as p50 and p65.

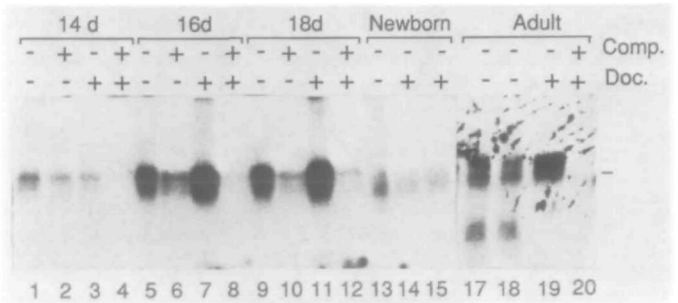
precursor pool was evident. A similar inverse correlation was seen between nuclear and cytoplasmic NF- $\kappa\text{B}$  in samples obtained from newborn and adult thymocytes (Fig. 3, lanes 14 and 19).

*CREB binding activity during thymus ontogeny*

CREB activity in fetal and adult mouse thymocytes was determined using labeled CRE oligonucleotide (which contains a binding site for the CREB transcription factor) (Fig. 4). Competition experiments with different unlabeled oligonucleotides confirmed the specificity of CREB binding activity (Fig. 4, panel

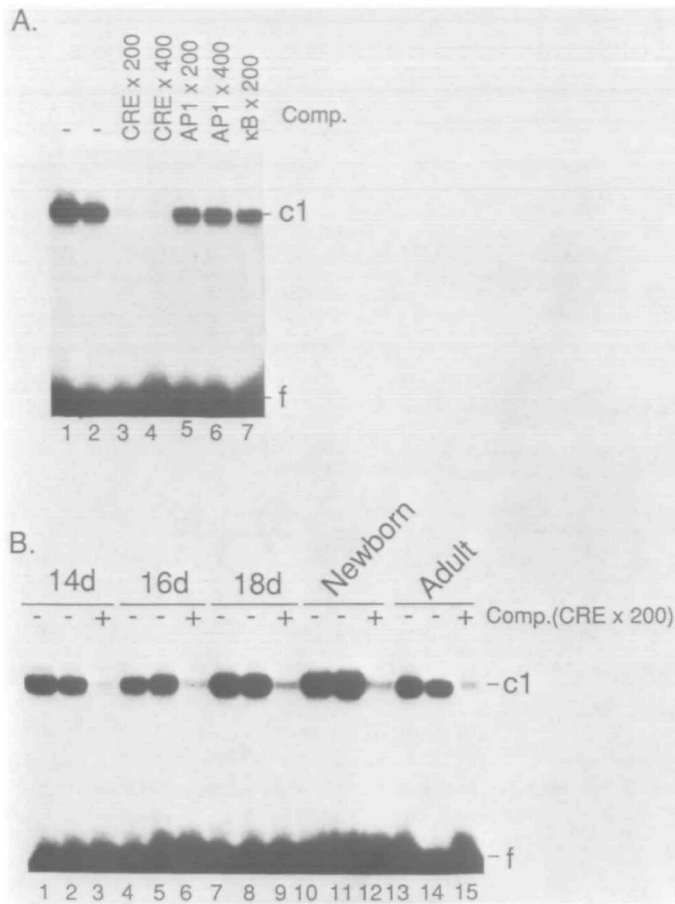


**Fig. 2.** NF- $\kappa\text{B}$  binding activity in nuclear extracts of mouse thymus from different stages of development. Stages of development (14 days, 16 days, 18 days of embryonic development, and newborn and adult) are shown on the top. EMSA was done using two labeled probes.  $[^{32}\text{P}]\text{-}\kappa\text{B}$  oligonucleotide (panels A and B) and  $[^{32}\text{P}]\text{-MHC-}\kappa\text{B}$  oligonucleotides (panel C). A 200-fold molar excess of unlabeled  $\kappa\text{B}$  oligonucleotide were used as competitor (Comp. +). The arrows b1 and b2 indicate the mobility of NF- $\kappa\text{B}$  - DNA complexes, the arrow f indicates the free DNA probe



**Fig. 3.** Presence and specificity of the precursor of NF- $\kappa\text{B}$  transcription factor in the cytoplasmic extracts of mouse thymus from different stages of development. EMSA was done using labeled  $\kappa\text{B}$  oligonucleotide probe. Cytoplasmic extracts were treated with 0.6% sodium deoxycholate (Doc. +) prior to DNA binding reactions or not treated (Doc. -) and then analyzed on 4% non-denaturate polyacrylamide gel. The presence of competitor, 200-fold molar excess of unlabeled  $\kappa\text{B}$ -oligonucleotide is designated as Comp. + .

A). Interestingly, an excess of the AP-1-binding oligonucleotide (which differs from the CREB sequence by only one base) did not inhibit CREB binding activity (Fig. 4A, lanes 5 and 6). The

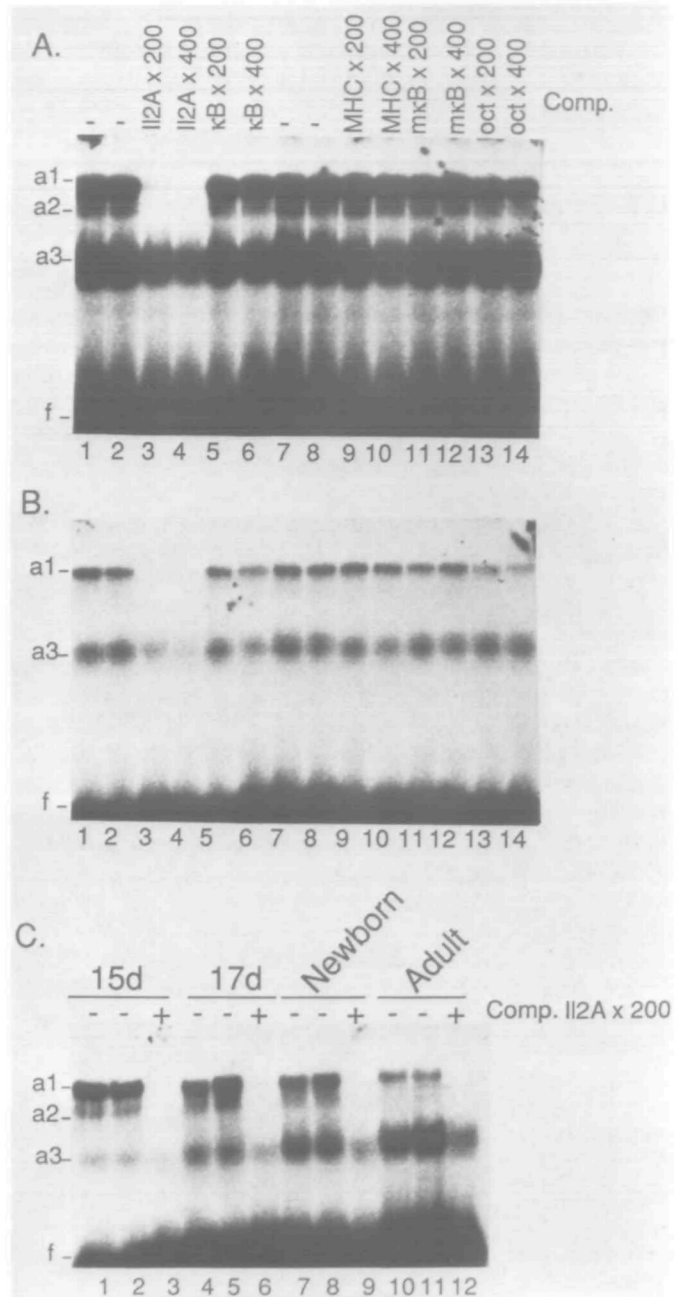


**Fig. 4.** CREB binding activity in nuclear extracts of thymus from different stages of development (A). Specificity of CREB binding activity EMSA was done using labeled CRE oligonucleotide without competitions (lanes 1 and 2) or in the presence of 200- or 400-fold excess of unlabeled CRE oligonucleotide (lanes 3 and 4 respectively), or 200- or 400-fold excess of unlabeled AP1-binding oligonucleotide (lanes 5 and 6), or 200-fold excess of x̄B oligonucleotide (lane 7). The arrow c1 indicates the mobility of the CREB – DNA complex, f indicates the free oligonucleotide probe. (B). CREB activity during thymus development. The presence of 200-fold molar excess of unlabeled CRE oligonucleotide as competitor is designated Comp +.

level of CREB binding activity increased slightly during fetal thymus development, peaking at birth and then decreased to the adult level (Fig 4B). The main band of retardation was designated c1 and longer exposure revealed some additional minor bands of retardation (not shown).

*NF-IL-2A and NF-AT1 transcription factors during thymus ontogeny*

The presence and specificity of NF-IL-2A binding activity in mouse thymocytes was determined by EMSA with a labeled IL-2A oligonucleotide containing the cognate sequence for the NF-IL-2A transcription factor (Table 1). Specificity analyses using competitor oligonucleotides are shown in Fig. 5(A and B). Three distinct DNA – protein complexes were observed. Bands a1 and a2 were specific, while a3 was only partly inhibited by unlabeled IL-2A oligonucleotide (Fig. 5A, lanes 3 and 4). Shorter exposures (6 h) of the same autoradiogram (panel B) revealed that unlabeled x̄B



**Fig. 5.** NF-IL-2A binding activity in nuclear extracts of mouse thymus from different stages of development. (A) and (B) Specificity of NF-IL-2A binding activity. Panel (B) is the same as panel (A) but after a short-time exposure of the autoradiogram (6 h). EMSA was done using labeled IL-2A oligonucleotide probe without any competitor (lanes 1, 2, 7 and 8) or in the presence of 200- or 400-fold molar excess of different oligonucleotides (see Table 1 for oligonucleotide sequences). The arrows at a1, a2, and a3 indicate mobilities of different NF-IL-2A – DNA complexes, f indicates the free oligonucleotide probe. (C) NF-IL-2A activity during thymus development. The presence of 200-fold molar excess of unlabeled IL-2A oligonucleotide as competitor is designated Comp. +.

oligonucleotide, MHC x̄B-oligonucleotide, but not M̄x̄B oligonucleotide, partly reduced the intensity of the a3 band. In addition, a 400-fold molar excess of x̄B oligonucleotide partly inhibited labeling of band a1 (Fig. 5B).

Similarity between human NF-IL-2A factor and the ubiquitous Oct1 protein has recently been established (22,38). We used an oligonucleotide with a canonical motif as a competitor for NF-IL-2A binding activity in EMSA experiments (Fig. 5A and B, lanes 13 and 14). We observed only partial inhibition of mouse NF-IL-2A binding activity (bands a1 and a2, but not for band a3) by excess unlabeled octamer oligonucleotide. This partial inhibition was seen more clearly after shorter exposure of autoradiograms (panel B). It is possible that bands a1 and a2 represent Oct1 and Oct2 factors whereas band a3 represents a complex between Oct1 and a 40 kDa Oct1-associated protein as recently suggested by Ullman *et al.* (39).

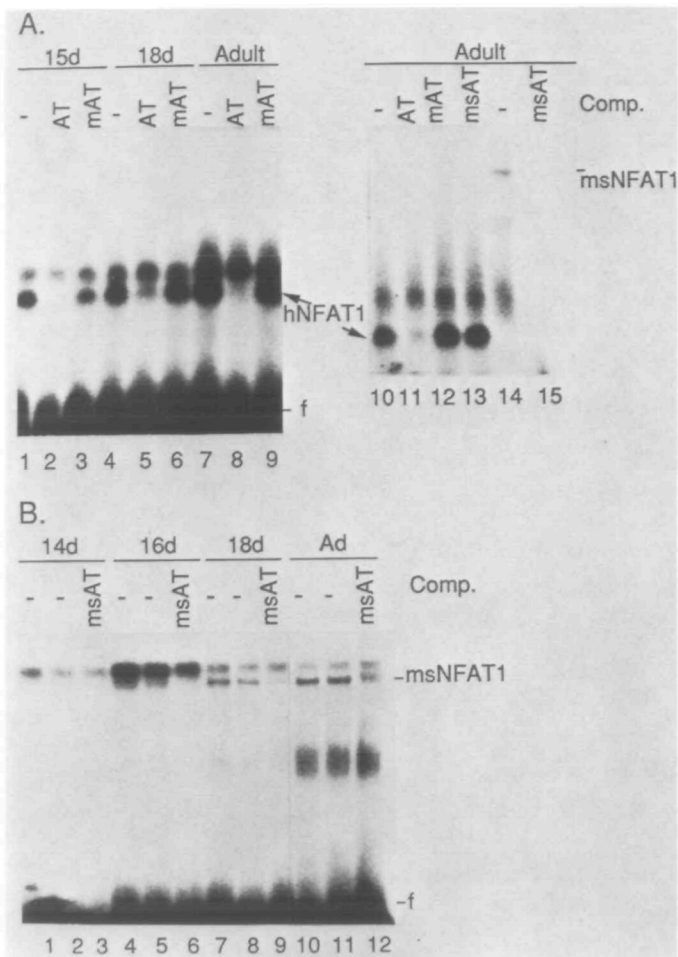
The ontogeny of NF-IL-2A binding activity in the thymus is shown in Fig. 5(C). The intensity of bands a1 and a2 were high initially but then decreased in the adult thymus. Band a2 seems to be absent in the adult. In contrast, the intensity of band a3 dramatically increased during development.

A complex picture emerged when the ontogeny of NF-AT1 factor binding activity was analyzed. Using a labeled oligonucleotide with the canonical NF-AT1 binding site from the human IL-2 gene enhancer (17) we detected two DNA-protein retarded complexes (Fig. 6). From competition experiments with homologous and mutated oligonucleotides, only the lower of these two bands was specific (Fig. 6, lanes 10–13). When we used as competitor an oligonucleotide with the equivalent mouse NF-AT1 (msAT) binding site, which differs from the human by only two nucleotides (40) (see Table 1), we saw no inhibition of binding to the human NF-AT1 oligonucleotide (Fig. 5A, lane 13). Furthermore, when the mouse probe was used as the labeled oligonucleotide, several bands were observed only one of which was specific (msNF-AT1). The upper, non-specific band (revealed using human NF-AT1 probe) (lanes 10–13) was also recognized using the mouse probe (lanes 14 and 15). From these experiments, it seemed that the human and mouse probes were detecting specifically two different proteins which we have designated hNF-AT1 and msNF-AT1 respectively.

The ontogeny of hNF-AT1 and msNF-AT1 appeared to be different. Thus, significant levels of hNF-AT1 activity were observed in the fetal thymus from day 14 (Fig. 7, lanes 16–18; Fig. 6A, lanes 1–9) and this activity continued to increase with age. However, we were unable to detect specific msNF-AT1 (the lower band) at day 14, but by day 16 this became barely detectable, and thereafter increased in concentration to that seen in the adult thymus (Fig. 6, Panel B).

#### *Fetal liver transcription factor*

Having established the pattern of expression of several ubiquitous transcription factors in the 14 day fetal thymus, we wanted to compare the pattern seen in thymocytes with a tissue known to be a rich source of precursor cells, i.e. the fetal liver. Results of these experiments are shown in Fig. 7. Fetal liver cell suspensions were enriched for hemopoietic cells by centrifugation over a Hypaque-Ficoll gradient prior to nuclear protein purification. No difference in NF-IL-2A or hNF-AT1 binding activity between fetal liver and thymocytes was observed. CREB activity was significantly increased in the thymus compared with fetal liver extracts. However, the most dramatic difference was seen with NF- $\kappa$ B, which was practically absent in fetal liver cells but present at a high level in thymocytes.

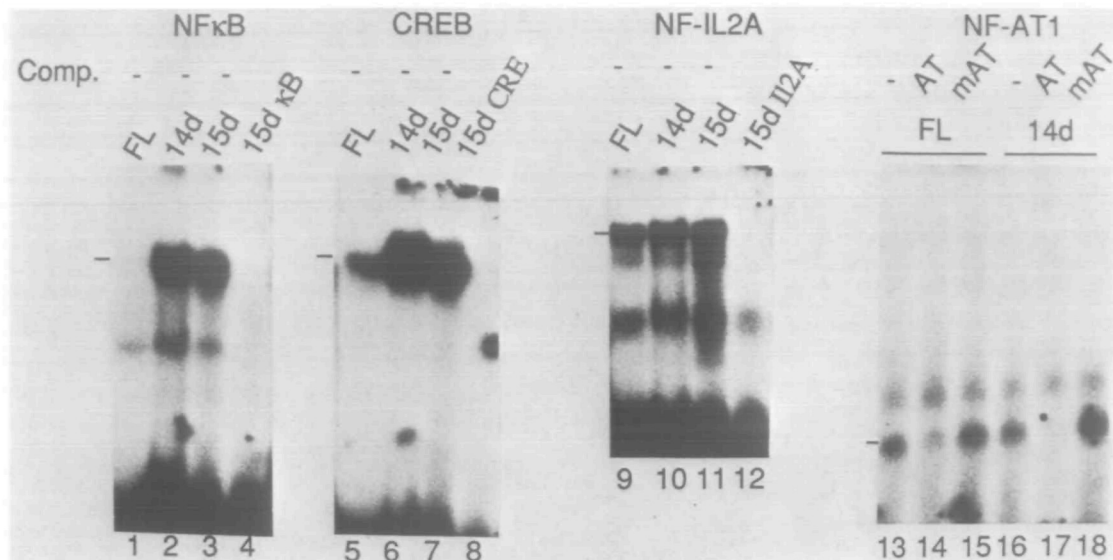


**Fig. 6.** NF-AT1 binding activity in nuclear extracts of mouse thymus from different stages of development. EMSA was done using two labeled probes: human NF-AT1 binding oligonucleotide (panel A, lanes 1–13) or mouse NF-AT1 binding oligonucleotide (panel A, lanes 14 and 15, and panel B). A 200-fold molar excess of three different oligonucleotides, i.e. human NF-AT1 binding oligonucleotide (AT), a mutated variant of this oligonucleotide (mAT), and mouse NF-AT1 binding oligonucleotide (msAT), was used as competitor. The arrow hNF-AT1 indicates the position of the NF-AT1–DNA complex revealed by the human probe and msNF-AT1 indicates the mobility of the DNA–protein complex revealed by the mouse probe.

#### **Discussion**

In this report, EMSA experiments have been carried out using nuclear and cytoplasmic extracts of fetal mouse thymocytes in order to determine qualitative and quantitative aspects of transcription factor expression. The transcription factors studied included NF- $\kappa$ B, CREB, NF-IL-2A, NF-AT1, NF-1, and AP-1, and these were chosen because of their known critical involvement in the regulation of genes active in mature, immunocompetent T lymphocytes.

At day 10.5 of development, the mouse thymus is a cluster of epithelial cells which becomes colonized by blood-borne, fetal liver-derived hemopoietic cells. Over the next few days, migration of fetal liver cells continues and, by day 14, the thymus contains  $1–2 \times 10^4$  hemopoietic cells and an equal number of epithelial



**Fig. 7.** Transcription factor binding activity in fetal liver and thymus (14 and 15 days of gestation). EMSA was done using labeled probe for the detection of NF- $\kappa$ B, CREB, NF-IL-2A, and NF-AT1 binding activities. DNA binding reactions were performed without competitor (Comp -) or in the presence of 200-fold molar excess of unlabeled  $\kappa$ B oligonucleotide (lane 4), CRE oligonucleotide (lane 8), IL-2A oligonucleotide (lane 2), human NF-AT1 binding oligonucleotide (AT, lanes 14 and 17), or a mutated variant of this oligonucleotide (mAT; lanes 15 and 18). The dashed lines indicate the positions of specific DNA-protein complexes.

cells. Very little is known about the interaction(s) between epithelial cells and hemopoietic precursors at this critical time of thymocyte development. Most hemopoietic cells in the 14 day thymus do not express cell surface TCRs.

However, beginning at day 14, surface expression of gamma/delta TCR can be detected on about 3% of cells and a truncated form of TCR- $\beta$  chain transcripts can be identified. In addition, ~50% of cells express on their surface IL-2R $\alpha$  chains although IL-2 is not thought to play a role in the growth of fetal thymocytes at this time (41). By day 16 of development, cells expressing both CD4 and CD8, double positive thymocytes appear, followed soon after by single positive CD4<sup>+</sup> and CD8<sup>+</sup> cells. Coincident with the appearance of double positive cells, surface expression of the TCR- $\alpha/\beta$  can be detected (for review, see ref. 23). During this time, the thymus is growing exponentially, reaching ~5  $\times$  10<sup>8</sup> cells at day 20 (birth) and ~2  $\times$  10<sup>8</sup> cells in the 4 week adult organ. It can be shown using organ cultures that this differentiation process is independent of external influences; the 14 day fetal thymus contains all the cells and factors necessary to carry out this complex differentiation process (26).

Expression of functional TCR- $\alpha/\beta$  in association with the co-receptor molecules CD4 and CD8 are known to be crucial for cell signalling processes involved in TCR- $\alpha/\beta$  receptor selection. Although at the phenotypic level much is known about the ontogeny of thymocyte subpopulations and of TCR receptor gene rearrangements, the mechanisms by which transcription factors regulate the expression of these important events are largely unknown. The experiments reported herein were designed to provide information regarding the baseline expression of transcription factors during thymus ontogeny.

Starting with NF- $\kappa$ B, EMSA experiments revealed that there was a distinct pattern of expression during thymus development.

Whereas fetal liver cells from day 14 embryos contained no detectable NF- $\kappa$ B binding, thymocyte nuclei at this time contained abundant activity. As pointed out above, 14 day fetal thymocytes are heterogeneous, as are the fetal liver cells isolated herein. Indeed, the frequency among FicolI-separated fetal liver cells capable of recolonizing the fetal thymus is of the order of 1/10<sup>4</sup> (42). In the experiments reported herein, no attempt has been made to purify subpopulations of fetal liver cells or thymocytes. Nevertheless, distinct differences were noted between fetal liver and thymocytes from 14 day embryos, and we therefore consider the increase in NF- $\kappa$ B expression between fetal liver and thymus to be significant especially since other transcription factors remained unchanged.

By the criteria of subunit composition and binding specificity, the binding activity to the NF- $\kappa$ B specific oligonucleotide of mouse thymus extracts corresponds to NF- $\kappa$ B which is composed of distinct DNA binding (p50) and transactivation (p65) subunits (35,43).

During thymus development, an inverse correlation between nuclear and cytoplasmic concentrations of NF- $\kappa$ B was noted. It is well known that NF- $\kappa$ B resides in the cytoplasm in an inactive form, complexed to an inhibitor I $\kappa$ B, and that cellular activation results in the dissociation of I $\kappa$ B from NF- $\kappa$ B with consequent releases of active NF- $\kappa$ B (36). The dissociation of I $\kappa$ B from NF- $\kappa$ B can be regulated in many ways including free radicals (44) and phosphorylation of I $\kappa$ B by protein kinase (45). It is also known that the p50 subunit of NF- $\kappa$ B is initially synthesized as a precursor of 105 kDa which requires processing to generate active binding protein p50 (33,46). Thus the rate of synthesis of the p50 subunit will also regulate the effective concentration of NF- $\kappa$ B.

The p50 and p65 subunits of NF- $\kappa$ B transcription factor have been recently cloned and shown to belong to the Rel-related transcription factor family (33,46-48). Using EMSA and UV

crosslinking with SDS-PAGE, we have shown that nuclear extracts of developing thymocytes contained both the heterodimeric (p65 – p50) and homodimeric (p50 – p50) NF- $\kappa$ B complexes. The ratio of these complexes changed during thymus ontogeny with the highest p65 – p50 activity being found in the day 14 fetal thymus. As the p65 – p50 NF- $\kappa$ B complex is a much more effective transactivator than the p50 – p50 complex (49) these changes in the ratio of p65 – p50 to p50 – p50 probably reflect changes in target gene expression mediated by NF- $\kappa$ B during thymus ontogeny. Presumably, the increase in NF- $\kappa$ B expression by fetal liver cells entering the thymus must be regulated by a TCR-independent mechanism and is thus different from that seen following TCR-mediated stimulation of mature T lymphocytes. Because NF- $\kappa$ B is known to be involved in the control of IL-2R (50) and IL-2 (51) gene expression in mature T cells, it is possible that alterations in subunit composition combined with the high nuclear concentrations of NF- $\kappa$ B in the day 14 fetal thymus may be involved in regulating IL-2R expression by fetal mouse thymocytes. Thus, NF- $\kappa$ B may play a crucial role in early fetal thymus development.

For NF-AT1, the pattern of expression during thymus development was also complex. Using oligonucleotides corresponding to either the human IL-2 gene enhancer or the equivalent mouse sequence, EMSA experiments suggested that the mouse thymus contains at least two different NF-AT1 proteins which we have designated hNF-AT1 and msNF-AT1. These two proteins had different patterns of expression during ontogeny. Thus, whereas hNF-AT1 appeared to be an ubiquitous factor expressed at a constant level throughout ontogeny (a pattern also seen with CREB and NF-1) msNF-AT1 appeared to be developmentally regulated being first detectable on day 16 of thymus development. The timing of msNF-AT1 expression is significant since by polymerase chain reaction (PCR) analysis, IL-2 transcription could be initially detected only at day 16 of thymus development (52). This result differs from a previous report using *in situ* hybridization in which two waves of IL-2 transcription at days 14 and 16 could be detected (53). Thus our EMSA experiments suggest that msNF-AT1 may be involved in IL-2 gene expression during thymus development, beginning at day 16. It should be pointed out that the human NF-AT1-binding oligonucleotides used in this study contained an Ets binding site whereas in the mouse oligonucleotide this sequence was truncated and modified (Table I). The presence of this Ets cognate sequence may account for the differences observed in EMSA using either mouse or human oligonucleotides.

NF-IL-2A was present at a high level throughout thymus development. Recently, it has been shown that NF-IL-2 and the 90 kDa Oct1 factor are proteins with related sequence specificity (38). A possible interpretation of multiple bands of retardation formed by thymic nuclear extracts with labeled IL-2A oligonucleotide in EMSA is that band a1 and a2 represent Oct1 and Oct2 factors whilst band a3 is a heteromeric complex between NF-IL-2A and Oct1-associated protein (39). The dramatic increase in the intensity of band a3 during thymus development suggests that this type of interaction between different transcription factor subunits increases during thymus development.

The thymus is the primary organ where T lymphocytes are generated. Studies to date have concentrated on the phenotypic

and functional description of events that take place in the developing thymus. However, we remain largely ignorant as to the molecular mechanisms governing thymocyte growth and survival, particularly during the early stages of fetal thymus development. Ultimately, the complex series of events that guides the differentiation of precursor T cells to functional mature T lymphocytes must be regulated at the gene transcriptional level. The recent cloning of genes encoding transcription factor proteins means that attempts can now be made, using transgenic mouse technology, to manipulate the availability of such transcription factors in developing thymocytes. By this means, the roles of transcription factors in controlling thymocyte differentiation can be investigated.

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### Abbreviations

CREB	c-AMP-response-element binding protein
EDTA	ethylenediamine tetra-acetic acid
EMSA	electrophoretic mobility-shift assay
DOC	deoxycholate
GM-CSF	granulocyte macrophage colony stimulating factor
HIV	human immunodeficiency virus
IL-2R $\alpha$	IL-2 receptor $\alpha$ chain
NF- $\kappa$ B	nuclear factor $\kappa$ B
NF-AT1	nuclear factor of activated T cells 1
PMSF	phenylmethylsulfonyl fluoride
PCR	polymerase chain reaction
SDS	sodium dodecylsulfate
TCR- $\beta$	T cell receptor $\beta$ chain
TNF- $\alpha$	tumor necrosis factor $\alpha$

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