Role of the forkhead transcription family member, FKHR, in thymocyte differentiation

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While performing a large-scale analysis of mRNA transcripts in the murine thymus, our attention was drawn to the forkhead family transcription factor FKHR. Here we demonstrate that FKHR is expressed in thymocytes, most prominently in those that are undergoing positive selection. Interestingly, FKHR transcripts show a highly regionalized pattern of expression, concentrated in the innermost areas of the medulla. We define the FKHR binding site as (G/C)(A/C)N(G/a)T(A/c)AA(T/c) A(T/g)(T/g)(G/c), a sequence found in the regulatory elements of many genes, including certain that encode molecules crucial for thymocyte differentiation. To study the function of FKHR, we engineered mice expressing a dominant-negative mutant specifically in T cells in a tetracycline-regulatable fashion. In these animals, T cell differentiation appeared quite normal; however, total thymocyte numbers were decreased, owing to reductions in all four of the CD4/CD8 subsets, and incorporation of the thymidine analogue bromo-deoxyuridine was increased, again in all four subsets. These data suggest that, in thymocytes, FKHR may be involved in cell survival and/or cycling.

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1 Introduction

$\alpha\beta$ T cell progenitors undergo an elaborate program of differentiation in the thymus before attaining full maturity and emigrant status [1]. Differentiative changes include the gradual display of antigen-specific receptors, alterations in the expression of a multitude of other cell-surface molecules, modulations in cell-cycle status, fluctuations in life expectancy, alterations in thymic localization, and the acquisition of effector functions. The end result is a repertoire of $CD4^+8^-CD3^{hi}$ helper cell precursors and $CD4^-8^+CD3^{hi}$ killer cell precursors residing in the medulla.

An interesting feature of thymocyte differentiation is the long time that mature helper and killer cell precursors are retained in the thymic medulla, estimated at about 1 or 2 weeks, or about 1/3 of their total residence time in the thymus [2–4]. It is known that $CD4^+8^-$ and $CD4^-8^+CD3^{hi}$ cells undergo gradual changes in the expression of several cell-surface molecules, including heat-shock antigen, Qa-2, CD69 and the peanut agglutinin (PNA) receptor [3–6]. At the same time, they acquire an enhanced capacity to respond to antigen challenge or other TCR cross-linking [6–8], and to survive and expand in the peripheral lymphoid organs [8]. In addition, it was recently demonstrated that a significant fraction of mature medullary thymocytes cycle just prior to their exit to the periphery [9, 10]. However, in general, we remain surprisingly ignorant of what takes place during this extended period.

With this in mind, we were intrigued to discover that the distribution of a transcription factor, FKHR, exhibits clear regionalization within the thymic medulla, being concentrated in the innermost areas (see below). Our interest in FKHR was first piqued while inventorying RNA expressed in the thymus, in particular in the medulla (H. Leenders et al., unpublished data). FKHR is a member of the forkhead family of transcription factors, whose original member, forkhead, was identified as controlling developmental processes in *Drosophila* [11–13]. The first mammalian members to be recognized were hepatocyte transcription factors, the HNF. The different factors had in common a conserved stretch of 110 amino acids, required for DNA binding, now known as the forkhead domain. More than 80 members of the forkhead transcription factor family have been identified in species as

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Abbreviations: **B6:** C57BL/6 **BrdU:** Bromo-deoxyuridine **DN:** $CD4^-8^-$ or double-negative **DP:** $CD4^+8^+$ or double-positive **GST:** Glutathione S-transferase **ISP:** $CD4^-8^+$ $CD3^-$ or immature single-positive **IRS:** Insulin response sequence **PKB:** Protein kinase B **PNA:** Peanut agglutinin **SP:** $CD4^+8^-$ or $CD4^-8^+$ or single-positive **tet:** Tetracycline

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diverse as *Caenorhabditis elegans* and *Homo sapiens*. These fall into several subfamilies, FKHR being grouped with FKHR2, AFX and others; this particular subfamily has been implicated in tumorigenesis and, most recently, control of cell survival and cycling through the upstream mediation of protein kinase B (PKB) [13–15]. Several of the forkhead family members, notably trident, winged-helix-nude (Whn) and FKHR, are expressed in the thymus [16, 17].

Because of the implication of forkhead family members in general, and FKHR and its homologues in particular, in critical cellular processes that also underlie T cell maturation, and because of FKHR's intriguing central medullary localization, we wondered whether this transcription factor might play a role in thymocyte differentiation. Here, we address this issue from multiple perspectives.

2 Results

2.1 Expression of FKHR in lymphoid compartments

The FKHR gene is transcribed in multiple tissues of the adult and fetus ([18] and our unpublished data). To generate hints about its function in the immune system, we detailed its pattern of expression in various lymphoid compartments. First, we asked whether the FKHR gene is transcribed primarily in thymocytes, thymic stroma, or both. RNA was prepared from whole thymus, a suspension of thymocytes, and the non-suspendable stromal cells. The purity of the thymus fractions was assessed by RT-PCR using T cell-specific (TCR-β) and stromal cell-specific (Whn) probes. We found it impossible to prepare an immediately *ex vivo* stroma fraction devoid of thymocyte contamination, no matter how extensively we washed it, and so decided to rely on subtraction (expression in whole thymus minus that in the thymocyte fraction) to estimate expression levels in the stroma. As indicated in Fig. 1, this procedure produced the expected results with the control probes: expression of TCR-β was very similar in thymocytes and the whole thymus; Whn expression was much lower in the former than the latter. By this criterion, both FKHR and its most related subfamily member, FKHR2, are expressed primarily in thymocytes.

Next, we studied regionalization of FKHR expression in the thymus. Sections were hybridized *in situ* with antisense probes for RAG-1 (to delineate the cortex and medulla), for FKHR, for FKHR2 and trident (other members of the forkhead family known to be expressed in the thymus), and with control sense probes. Fig. 2A shows that FKHR transcripts were found in both the cortex and

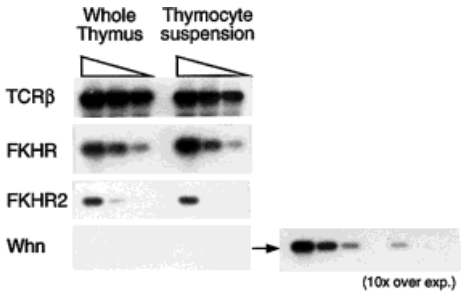

Fig. 1. Expression of FKHR in the thymus. RT-PCR for TCRβ, FKHR, FKHR2 and Whn was performed on fivefold serial dilutions of cDNA reverse-transcribed from RNA isolated from whole thymus and thymocyte cell suspension. The right panel of the bottom row was exposed ten times longer.

medulla, but were more concentrated in the latter. This pattern was clearly different from that for the other two forkhead family members, which were expressed equally or more intensely in the cortex; in particular, trident transcripts were most abundant in the subcapsular region, consistent with its known association with cycling cells. Interestingly, examination of the RAG-negative and FKHR-positive regions on several sections suggested that FKHR might not be expressed throughout the medulla. To explore this issue, we cut multiple sequential thymus sections in three layers of the thymus, and compared the presence of FKHR transcripts with the absence of transcripts for RAG-1. On the initial, external, sections (Fig. 2B, sections 49 and 50), FKHR was expressed in only a portion of the medulla; in later, more internal, sections (58 and 59), the RAG-1-negative and FKHR-positive regions were more coincident; in even later, again external, sections (69 and 70), the FKHR hybridization pattern was again more restricted. This suggested that cells with high FKHR expression were concentrated in a subregion of the medulla, located centrally, thus defining two distinct anatomical compartments. The same conclusion was reached by comparing the expression of FKHR and MHC class II molecules on adjacent sections (data not shown). Fig. 2C confirms the medullary expression of FKHR by demonstrating its absence in thymi of class I/class II double-deficient mice, which have only a rudimentary medulla composed essentially entirely of stromal cells. On the other hand, thymi of class I or class II single-deficient animals expressed FKHR in the medulla, indicating that TCR engagement of neither class I nor class II molecules is absolutely required, and that FKHR expression does not partition differentially in cells of the $CD4^+$ or $CD8^+$ lineage.

The next issue we focused on was which thymocyte subsets express FKHR. In several independent experiments, different thymocyte populations were sorted

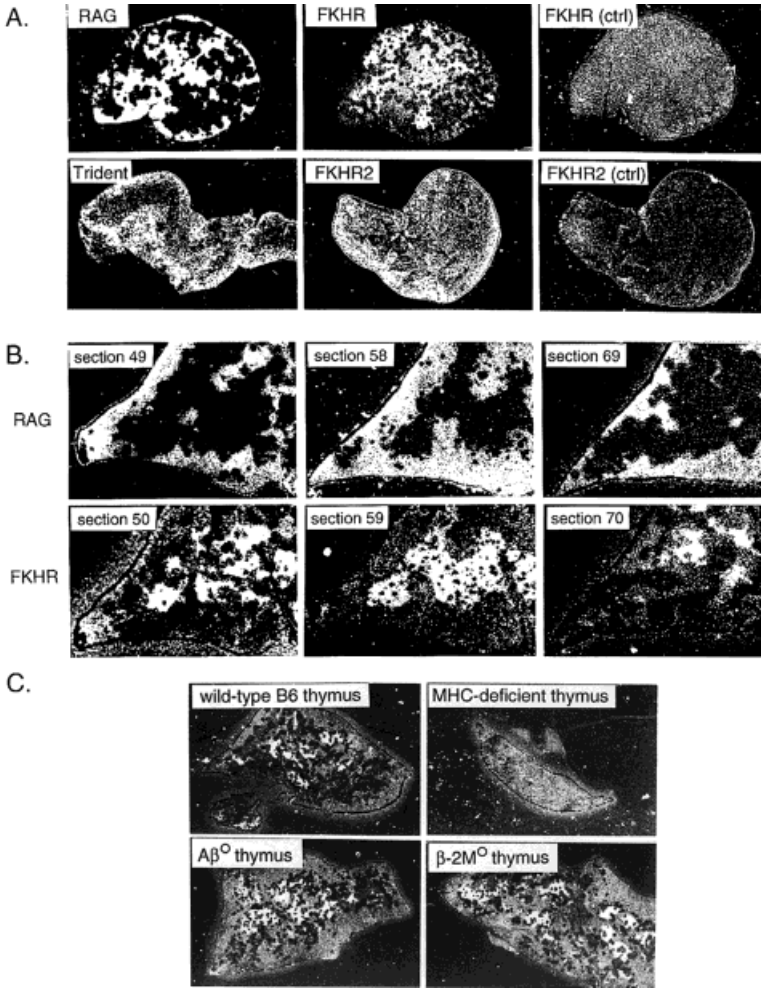

Fig. 2. Regionalization of FKHR expression in the thymus. (A) *In situ* hybridization for a wild-type B6 thymus. Neighboring sections of the same thymus were hybridized with RAG-1 anti-sense, FKHR anti-sense and control FKHR sense probes in the upper panel, and trident anti-sense, FKHR2 anti-sense and FKHR2 sense probes in the lower panel. (B) Magnification of thymus sections in different layers of thymus tissue. The top and bottom panels present hybridization patterns with RAG-1 and FKHR anti-sense probes, respectively. At three levels of the thymus (left, center, right) two adjacent sections were taken. (C) Thymic FKHR expression in wild-type B6, β-2 $M^{0/0}$, $A\beta^{0/0}$ and β-2 $M^{0/0}$, $A\beta^{0/0}$ mice.

cytofluorimetrically according to their expression of CD4, CD8 and CD3. FKHR and HPRT transcript levels were estimated by semi-quantitative RT-PCR, the latter as a control for RNA concentration and quality. Fig. 3, panel A presents results from a typical experiment, while panel B summarizes data from several such studies. $CD4^-CD8^-$ (DN) cells (not shown) and the bulk of $CD4^+CD8^+$(DP) cells, which are immature and display only low levels of CD3, expressed little FKHR. However, subsets that were undergoing or had undergone positive selection, including the small population of DP $CD3^{hi}$ cells, expressed substantially higher levels. Transcripts were found approximately equally in mature $CD4^+8^-$ and $CD4^-8^+$ (SP) cells (not shown), and routinely appeared to increase with the degree of SP thymocyte maturation, being lower in the most immature $CD4^+8^{int}$ cells and highest in the most mature $CD4^+8^-PNA^-$ cells.

Finally, we assessed FKHR expression in peripheral lymphocytes. According to RT-PCR analysis of sorted cells, FKHR was expressed in peripheral $CD4^+$ and $CD8^+$ T cells at levels similar to those of mature SP thymocytes, and was not further elevated upon activation (not shown). Regionalization of FKHR in peripheral lymphoid organs was assessed by *in situ* hybridization of sections of mesenteric lymph node and spleen, delineating regions of interest by staining adjacent sections with PNA and an anti-IgM mAb. Abundant FKHR transcripts

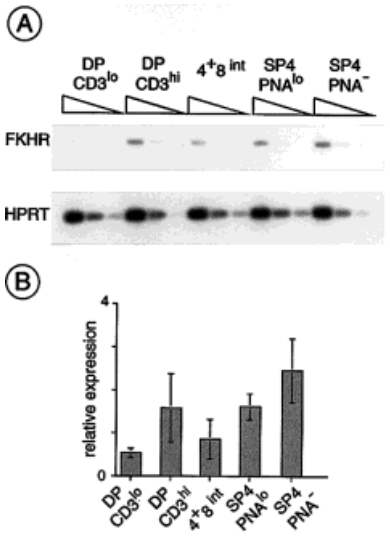

Fig. 3. FKHR mRNA expression in sorted thymocyte populations. (A) A representative experiment using RT-PCR to estimate FKHR and HPRT transcript levels, performed on fivefold dilutions from $CD4^+CD8^+CD3^{lo}$, $CD4^+CD8^+CD3^{hi}$, $CD4^+CD8^{int}CD3^{hi}$, $CD4^+CD8^-PNA^{lo}$, and $CD4^+CD8^-PNA^-$ thymocyte RNA. (B) Results from four to five independent experiments are plotted. The bars represent the mean value of FKHR mRNA levels standardized according to HPRT expression levels (arbitrary units), and the standard errors are indicated.

were found in both the B and T cell areas (Fig. 4). In the lymph node (left panel), germinal centers stood out because of their high expression levels, while the B cell follicles and paracortical T cell areas showed a lower level of expression, equally intense in the two cases. In the spleen (right panel), transcripts were concentrated in the white pulp, occurring at about the same level in the B and T cell areas, and showing no increase in the germinal centers.

2.2 The FKHR DNA-binding site

Other clues to FKHR function might come from identifying genes whose promoter it binds to and regulates. As a first step towards this goal, we set out to define the FKHR DNA-binding site. The overall strategy, described in [19] and diagrammed in Fig. 5A, was to: (1) produce in *E. coli* a fusion protein between bacterial glutathione S-transferase (GST) and the DNA-binding forkhead domain of FKHR (Fig. 5B); (2) allow this protein to bind a population of degenerate double-stranded oligonucleotides; (3) isolate bound oligonucleotides via glutathione-Sepharose; (4) amplify the bound sequences by PCR; (5) repeat the series of enrichment steps multiple times; and (6) clone and sequence the resulting mix of putative high-affinity binding sites. The same procedure was performed in parallel with the HNF3β forkhead domain, to validate our assay (as its binding site has already been defined [20]) and to provide a comparison.

As detailed in Fig. 5C, the degenerate double-stranded oligonucleotide was a 13mer with fixed or partially variable "core" nucleotides and completely variable flanking nucleotides. The sequences were chosen according to the generic forkhead domain DNA-binding motif [12]. To evaluate the true degeneracy of the starting material, we cloned and sequenced it (upper panel). Some biases introduced during oligonucleotide synthesis were detected, most apparent at a few flanking positions.

After five rounds of selection, a putative high-affinity binding site for FKHR was defined (middle panel). Amongst the 36 sequences analyzed, only 3 were identical, indicating that the PCR amplification steps did not artificially enrich particular sequences. Flanking sequences were quite variable, with only a few nucleotide exclusions: A and T at position –3, A at positions 9 and 10. Within the core, the most striking biases were that T and A were highly favored at positions 2 and 3, respectively. We further noted that whenever the core sequences were the most favored nucleotides GTAAATA,

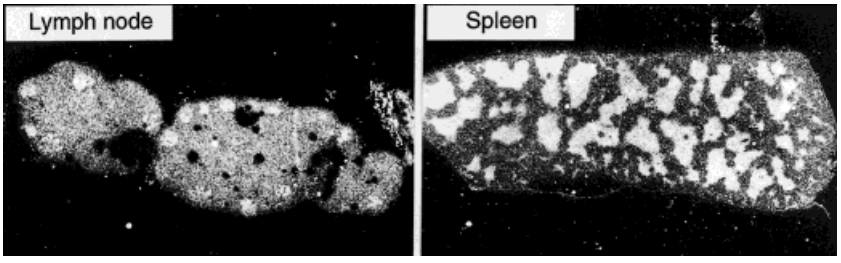

Fig. 4. FKHR expression in lymph node and spleen. Sections of mesenteric lymph nodes and spleen from mice kept under specific-pathogen-free conditions were hybridized with an FKHR anti-sense probe.

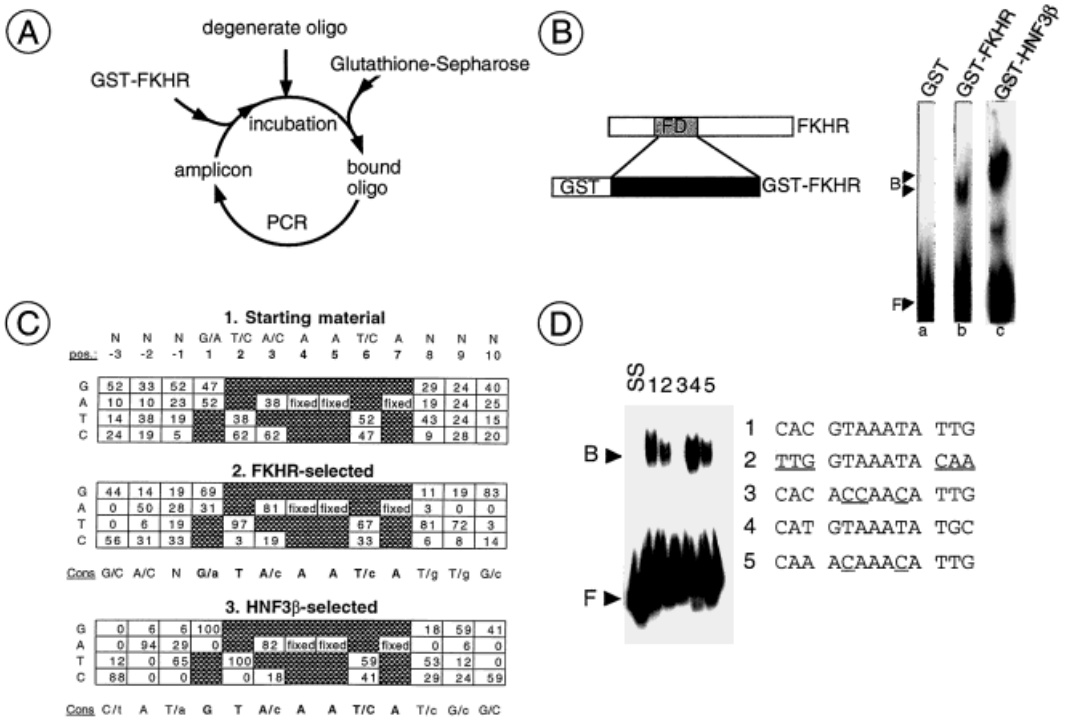

Fig. 5. The FKHR binding site. (A) Schematic overview of the selection procedure. (B) Schematic representation of fulllength FKHR protein and construction of a GST-FKHR fusion protein (left panel). Gel retardation of bacterially expressed GST, GST-FKHR and GST-HNF3β with a high-affinity HNF3β oligonucleotide probe [20] (right panel). The F and B triangles indicate the positions of free probe and bound oligonucleotides, respectively. (C) Summary of sequenced starting material and selected binding sites for FKHR and HNF3β. Numbers represent the frequency of each nucleotide at each position. The numbers of sequences employed were: 21 for the starting material, 36 for FKHR and 17 for HNF3β. (D) GST-FKHR gel retardations. Double-stranded oligos corresponding to indicated sequences were used for protein complex formation with 50 ng purified GST-FKHR. GST-FKHR does not bind to single-stranded oligo 1 (leftmost lane).

the following flanking nucleotides were TTG, without exception, suggesting that the decamer GTAAATATTG might be optimum for FKHR binding.

A putative high-affinity HNF3β DNA-binding site was also defined (lower panel). In general, the flanking sequences were less variable than for the FKHR binding site, with common nucleotide exclusions: G and A at position –3, T and C at position –2, C at position –1, A at position 8, and T and A at position 10. Sequence preferences at partially variable positions within the core were very similar to those at the same positions of the FKHR binding site. A notable exception was position 1, where only G seemed acceptable.

These observations were substantiated by gel retardation analysis of the recombinant fusion proteins to oligonucleotide target sequences (Fig. 5D). GST-FKHR bound effectively to the experimentally determined consensus binding site (lane 1); it still bound, but less efficiently, when the central "core" sequences were conserved but the flanking sequences altered (lane 2). Changing the core nucleotides abolished GST-FKHR binding (lane 3). GST-FKHR also bound to the experimentally determined consensus binding site for HNF3β (lane 4), as well as to a previously published HNF3β binding site (lane 5, [20]).

2.3 Role of FKHR in thymocyte differentiation

To evaluate FKHR function during the maturation of thymocytes, we attempted to express a dominant-negative FKHR variant specifically in the T cell lineage in a regulatable fashion. The strategy we chose is based on the tetracycline (tet)-responsive system pioneered by Bujard and colleagues [21, 22].

Thus, we generated one mouse line, LTH1, expressing a tet-regulatable transcriptional activator (tTA) under the dictates of the T-lineage-specific lck proximal promoter (lck-tTA construct; Fig. 6A, left panel). This line has been extensively characterized as part of another study and

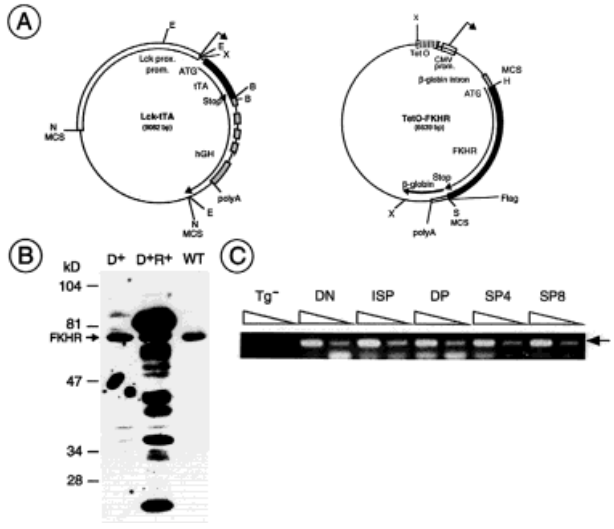

Fig. 6. Construction of vectors and distribution of transgene expression. (A) The Lck-tTA transactivator construct is diagrammed in the left panel. The plasmid contains the Lck proximal promoter [38] (white bar), the TetR-VP16 transactivator (solid bar), the human growth hormone gene (exons are shown as filled gray bars) and the plasmid backbone. MCS, multiple cloning site; E, EcoRI; X, XbaI; B, BamHI, N, NotI; polyA, polyadenylation site. The TetO-FKHR reporter construct is shown in the right panel. The plasmid contains the C-terminal-truncated FKHR sequence (solid bar), seven Tet operator sequences (boxes) and a minimal CMV promoter (white bar), the rabbit β-globin gene elements and a plasmid backbone (solid line). H, HindIII; S, SpeI. (B) Transgene-encoded FKHR overexpression. Extracts were made from thymi of mice containing the transactivator alone (D^+, lane 1), mice containing both the transactivator and reporter (D^+R^+, lane 2) and wild-type B6 mice (WT, lane 3). Protein expression was detected with a rabbit anti-FKHR antiserum. The position of endogenously encoded FKHR is indicated by an arrow, and the transgene-encoded dominant-negative mutant migrates just above. (C) Transgenic reporter expression in sorted thymocytes. RT-PCR was performed on two concentrations of cDNA (fivefold dilutions) from sorted $CD4^-CD8^-CD3^-$ (DN), $CD4^-CD8^+CD3^-$ (ISP), $CD4^+CD8^+CD3^+$ (DP), $CD4^+CD8^-CD3^+$ (SP4) and $CD4^-CD8^+CD3^+$ (SP8) thymocytes. A non-transgenic $CD4^-CD8^+CD3^+$ thymocyte population was included (lanes 1 and 2). A transgene-specific PCR product spanning different exons was amplified to detect reporter mRNA expression.

will be described in detail elsewhere (Whitfield et al., in preparation). For the present experiments, it should be noted that tTa is efficiently transcribed, that transcripts are indeed confined to cells of the T lineage, and that similar transcript levels are found in the four CD4/CD8 thymocyte subsets. A second mouse line carries sequences encoding a truncated FKHR protein, capable of binding to DNA but incapable of transactivation, downstream of a promoter that requires tTA for its activity (tetO-FKHR construct; Fig. 6A, right panel). More specifically, the FKHR mutant lacks the 60 C-terminal amino acids that constitute the transcriptional activation domain, and only those, and therefore should act as a potent dominant-negative mutant of FKHR and perhaps related forkhead family members [23–26]. Mating the two lines should generate double-transgenic offspring that express the truncated FKHR protein specifically in T cells in the absence of tet administration.

As illustrated in Fig. 6C, in double-transgenic mice never exposed to tet, FKHR transcripts were found in all of the major thymocyte subsets, at approximately equal levels, indicating that tTA activity nicely paralleled the activity of the lck promoter [27]. These transcripts resulted in significant levels of the transgene-encoded FKHR protein, as demonstrated by the Western blots of thymocyte extracts shown in Fig. 6B. Indeed, the trangene-encoded FKHR mutant was substantially more abundant than the natural, endogenously encoded protein.

This system should allow us to study the role of FKHR in diverse aspects of thymocyte differentiation. A first assay was to perform a standard cytofluorimetric analysis of thymocyte subsets. Total thymocyte numbers were reduced in most mice expressing the truncated FKHR molecule (Fig. 7A). However, the ratios of DN, DP and SP cells appeared normal (Fig. 7B), so the reduction was a general phenomenon, not limited to populations, *e.g.* DP cells, characterized by a rapid turnover.

A second assay evaluated thymocyte cycling by quantitating incorporation of the thymidine analogue bromodeoxyuridine (BrdU). Fig. 7C shows BrdU incorporation in CD4/CD8 subsets in a typical experiment. BrdU incorporation was significantly increased in all subsets. This result was observed in several independent experiments.

3 Discussion

3.1 FKHR expression

FKHR had an intriguing pattern of expression in the murine thymus. It was found mainly in thymocytes rather than stromal cells, in particular those thymocytes that were undergoing or had undergone positive selection, expression increasing in parallel with the display of maturity markers. This cell-type distribution fit well with the anatomic compartmentalization of expression, as FKHR transcripts were found at higher levels in the medulla than in the cortex. The fact that transcripts were concentrated in the innermost regions of the medulla may indicate that the most mature SP cells reside in the

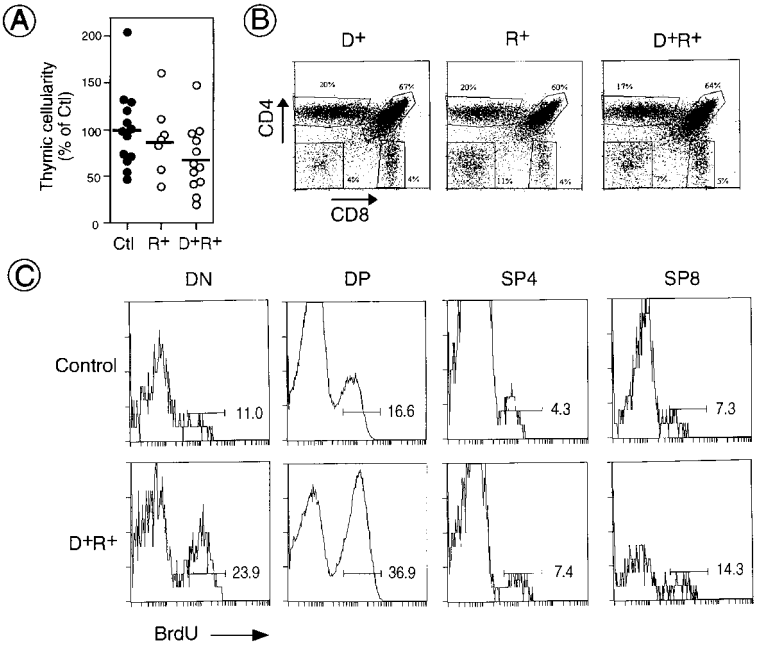

Fig. 7. Thymocyte numbers and cycling in double-transgenics. (A) Relative number of thymocytes in dominant-negative FKHR mice (double transgenic, D^+R^+) as compared with reporter-only animals (R^+) and control littermates (Ctl). The mean of the number of thymocytes in control littermates is taken as 100%. (B) CD4/CD8 profiles of thymocytes from driver-only (D^+), reporter only (R^+) and double-transgenic animals. (C) BrdU incorporation in $CD4^-CD8^-CD3^-$ (DN), $CD4^+CD8^+CD3^{lo}$ (DP), $CD4^+CD8^-CD3^+$ (SP4) and $CD4^-CD8^+CD3^+$ (SP8) thymocyte populations. Values represent the proportion of cells positive for BrdU incorporation. A representative experiment, of four, is shown.

most central location. We know of no other structural or functional marker that differentiates medullary zones in this manner — it will certainly be interesting to see whether other phenotypic features of SP thymocytes partition with FKHR transcripts. In the meantime, given the implication of forkhead factors in control of the cell cycle [13], it is tempting to speculate that there is some relationship with the recently highlighted population of cycling, fully mature, soon-to-emigrate SP thymocytes [9, 10].

3.2 The FKHR binding site

We used a powerful oligonucleotide selection procedure to define the FKHR binding site. Parallel determination of the binding site for HNF3β provided a technical control as this site has been defined previously using a similar method. The reported high-affinity HNF3β binding site contained an ACAAACA core motif, while two weak-affinity sites contained GTAAATG and ACCAATA cores [20]. Our selected consensus was GTAAA(T/C)A, which resembles the high-affinity binding site except for the first two nucleotides. All of our selected HNF3β binding sites (*n*=17) contained a GT dinucleotide at the first two positions. The discrepancies observed between our and the published data might be explained by the following two considerations. First, Overdier et al. [20] employed completely random 12mer oligonucleotides as starting material, whereas we used partially random 13mers that forkhead proteins probably already had some affinity for. Secondly, in the study of Overdier et al., many of the selected sequences had exactly the same core and flanking sequences. Only 3 different DNA-binding sequences were obtained out of 33 sequenced clones, whereas in our case only 3 identical sequences out of 36 were obtained. This suggests that in the prior study, preferential PCR amplification of certain oligonucleotides may have taken place. However, a consensus HNF binding site has been deduced from liver-specific promoter sequences [28], and this (G/a)(C/t)AAA(C/T)A consensus motif suggests that a GT or an AC dinucleotide may be amenable to HNF3β binding.

Differences in the selected HNF3β and FKHR binding sites were observed only in the flanking sequences. HNF3β demonstrated a strong preference for a CAT 5'-flanking sequence, while FKHR had a preference for a TTG 3'-flanking sequence. 5'-flanking nucleotides have already been observed to play a role in the binding of

several forkhead transcription factors [20, 28, 29]; however, 3' flanking sequences have not been previously reported to strongly influence binding.

Recently, FKHR was shown to bind to an insulin response sequence (IRS) and to promote transcription of a downstream reporter [30, 31]. This sequence consists of a CAAAA(C/T)AA motif. Single-point mutations at position 3 to 6 of this motif prevented reporter activity [31], indicating that these nucleotides are important for FKHR binding and reporter stimulation. Positions 1 to 7 of our selected GTAAATA motif very likely correspond to the first 7 nucleotides of the IRS. These data indicate that single-nucleotide substitutions in the first two nucleotides (GT) of our selected DNA binding site by CA does not inhibit in vivo DNA binding. Note that the degenerate primers we used did not contain the CA nucleotide possibilities at positions 1 and 2.

In summary, our and published data indicate that FKHR can bind the general forkhead binding site (G/C/A)(T/C/A)AAA(T/C)A. Substitutions in core positions 1, 2 and 6 are allowed as long as the indicated nucleotides are used. Flanking sequences have less of an influence on DNA binding *in vitro*, but the *in vivo* situation could be different. Interestingly, we found close matches to the consensus sequence within the CD4 proximal enhancer [32] and the CD8 single-positive enhancer ([33] and HL, W. Ellmeier, D. Littman, CB and DM, unpublished).

3.3 FKHR function

The first issue to address is whether the truncated FKHR molecule expressed specifically in thymocytes of the double-transgenic mice actually did act in a dominant-negative fashion, and whether expression levels were high enough to effectively inhibit FKHR function. Unfortunately, it is not possible to definitively resolve this issue because no downstream targets of FKHR in thymocytes have so far been identified. However, a dominant-negative effect in our experiments was highly likely. Certainly, there is plenty of precedent that this type of truncated molecule, capable of binding to DNA but incapable of transactivating its targets [23–25], has potent dominant-negative activity, in particular as concerns the very related HNF3β [25, 26]. And certainly the transgene-encoded FKHR mutant is expressed at levels substantially higher than those of the endogenously encoded wild-type molecule (Fig. 6). So one does expect inhibition of FKHR and perhaps its closest relatives. Indeed, functional consequences were detected in the double transgenics.

On the one hand, overexpression of the truncated FKHR molecule in thymocytes resulted in a reduction in their numbers. As discussed below, this reduction would probably have been even higher had there not also been an increase in the fraction of cycling cells. A similar reduction was seen in the four CD4/CD8 subsets, probably reflecting the fact that the blocking agent was expressed, under the dictates of the lck promoter, in essentially all thymocytes. Similarly, incubation of double-transgenic thymocytes *in vitro* resulted in greater cell death than for control thymocytes, and this was true of all four CD4/CD8 subsets (not shown). Hence, in the natural context, one would anticipate that FKHR promotes survival, above all in the most mature SP cells. FKHR [30] and its close relative AFX [34] have recently been implicated in apoptosis, promoting death in cells where PKB has not been mobilized. Such a role would appear to be in opposition to our observations of a pro-survival function in thymocytes, but it is possible that there are cell-type particularities, perhaps reflecting the degree of engagement of the PKB and intersecting pathways. It is also possible that the interpretation of our results is complicated by effects of the truncated FKHR molecule on the activity of other forkhead family members. We have directly checked apoptosis induction by dexamethasone and anti-CD3 mAb in our system, and have not seen significant abnormalities in the double-transgenics (not shown).

On the other hand, overexpression of the FKHR mutant resulted in a doubling of the number of thymocytes found to be cycling in the steady-state double-transgenic thymus. Again, this was an across-the-board effect, and one must keep in mind that in the natural context, FKHR might exert its strongest influence in the most mature thymocyte populations found in the medulla. This result fits well with the recent suggestion that forkhead factors whose activity is inhibited via PKB-mediated phosphorylation, such as FKHR and AFX, may inhibit the progression of cells through the cell cycle at the G1 phase [13]. Perhaps FKHR and related proteins play some role in the elaborately orchestrated changes in cell cycling that occur throughout the process of thymocyte differentiation [1].

In short, our results suggest a subtle role for FKHR in the crucial processes of cell survival and cycling that take place during the differentiation of T cell progenitors in the thymus.

4 Materials and methods

4.1 RNA analysis

RNA was prepared from whole mouse thymus and thymocyte cell suspensions by the LiCL/Urea technique as described [35]. After reverse transcription with AMV reverse

transcriptase (Boehringer), PCR amplification was performed on fivefold serial dilutions of the cDNA with the following primers: TCRβ; CCCAAGGTCTCCTTGTTTGA and CTATGGCCAGGGTGAAGAAC; FKHR: TTCAAGGATAAGGGCGACAG and ACTCGCAGGCCACTTAGAAA; FKHR2 AGCCGTGTACTGTGGAGCTT and CCTGGATAGTCTGCATGGGT (based on an EST clone: AA238456); Whn: CCACGGTGCCAAGCTGATG and GCTGAGGTGCATGTCTCCCA. Amplified products were quantitated by Fuji imaging after Southern blot hybridization with internal primers. Sorted thymocyte sub-populations (50,000 cells) were directly collected in 2 ml Trizol RNA preparation reagent (Gibco BRL), and RNA was isolated according to the manufacturer's instructions. Reverse transcription and PCR amplification were performed as described above, with HPRT primers as controls: GTAATGATCAGTCAACGGGGGAC and CCAGCAAGCTTGCAACCTTAACCA.

4.2 *In situ* hybridization

Sections were hybridized with ^{35}S-labeled cRNA probes as described previously [36]. The following linearized plasmids containing cDNA sequences were used as templates. Clone MTA.A08.095 contains the C-terminal 1.1 kb coding sequence of FKHR. Mouse EST clones 697745 and 532253 (IMAGE) contain 488 bp of FKHR2 coding sequence downstream of the forkhead domain or approximately 1.5 kb Trident 3'UTR, respectively. Exposure times ranged from 7 days for RAG hybridization to 3 months for trident hybridization.

4.3 GST-FKHR fusion protein

GST-FKHR fusion protein was synthesized by using the pGEX-4T3 expression vector (Pharmacia), and contains amino acids 145–295 of murine FKHR. The GST-HNF3β fusion protein has been described previously and contains amino acid 95–279 of the wild-type HNF3β protein [20]. Recombinant proteins were prepared in *E. coli* BL21(DE3)pLysS induced with IPTG, and were affinity-purified with glutathione-Sepharose 4B (Pharmacia).

4.4 Gel retardation assay

Gel retardation assays were performed basically as described previously [37]. A double-stranded oligo containing a high affinity site for HNF3β, AGTCCTCCAAACAAACATTGACGAGTC (binding site is underlined) [20] was used as control. Binding reactions contained 20,000 cpm (10 fmol) of this ^{32}P end-labeled oligo, approximately 50 ng of purified protein, and 100 ng poly dI:dC.

4.5 DNA binding site selection

Selection of the optimal DNA binding site for FKHR and control HNF3β was performed as described by Pierrou et al. [19]. Total soluble bacterial extract containing GST-FKHR or GST-HNF3β protein was incubated with the following 51 mer: GTATCGATAAGCTTCGCTCNNN*(A/G)(C/T)(A/C)AA(C/T)*ANNNACGTGCTCTAGACTAGTAC (the core DNA binding site is indicated in Italics). Of the partially random primers 50 μg (22 pmol) were gel-purified before annealing to a ^{32}P kinased complementary 19-mer primer. Extension with Klenow DNA polymerase was for 1 h at 37°C and successful extension was checked by running an aliquot on a denaturing acrylamide gel. The double-stranded 51-mer was precipitated and resuspended in 1× binding buffer (20 mM Hepes, pH 7.9; 50 mM KCl; 2 mM $MgCl_2$; 0.5 mM EDTA; 10% glycerol; 0.1 mg/ml BSA; 2 mM DTT; 0.5 mM PMSF). Of the 51-mer 22 pmol were mixed with 10 μg poly dI:dC. Total bacterial extract containing either 10–20 ng GST-FKHR or GST-HNF3β protein was added last and the binding reaction was incubated for 15 min at room temperature. Of glutathione-Sepharose 50 μl (a 10% slurry in 1× binding buffer) was added and carefully flicked for 2 min. The glutathione-Sepharose beads with bound protein-DNA complexes were pelleted by centrifugation and washed four times in binding buffer. One third of the washed glutathione-Sepharose pellet was used in a 30 μl PCR reaction. Amplification was performed with 0.66 μM each of GTATCGATAAGCTT CGCTC and GTACTAGTCTAGAGCACGT primers. Glutatione-Sepharose was pelleted and the supernatant was precipitated and used for a second round of binding site selection in the same conditions as above. Five rounds of binding and amplification were performed. Too much starting material and/or too many cycles resulted in the appearance of a smear of higher molecular size. So, in the fourth round of selection, the number of PCR cycles was reduced to 25 cycles and in the fifth round to 15 cycles. Products from the last amplification step were gel purified, eluted and precipitated. The material was digested with HindIII and XbaI and the individual fragments concatenated by ligation and cloned between the corresponding sites in pBluescript.

4.6 FKHR antiserum

Two rabbits were immunized with 100 μg purified GST-FKHR in CFA by intradermal multipoint injections. Antisera were screened by Western blot for anti-GST-FKHR activity 4 weeks after immunization, and the rabbit was finally bled at 7 weeks after immunization.

4.7 Constructs for transgenesis

The plasmid for expression of tTA under the control of the Lck proximal promoter was made as follows: a 1035 bp EcoRI/BamHI tTA fragment was excised from pUHD15–1 [21] and subcloned between the EcoRI and BamHI sites of pBluescript (pBS-tTA).

The SpeI-EcoRV fragment of pBS-tTA was blunt-end cloned into the BamHI-filled site of p1017, which provides the 3.2 kb Lck proximal promoter and the splicing and polyadenylation signals from the human growth hormone gene [38]. For the reporter construct, to engineer regulated expression of a deletion mutant of FKHR, the TetO-hCMV minimal promoter sequence was provided by the recently described TetO-Eα construct [39]. The EcoRI/EcoRI fragment (Eα-fragment) was excised and replaced by a SalI-BssHII polylinker. Screening of a P19 embryonal carcinoma cDNA library with an FKHR probe provided several clones with the entire mouse FKHR coding sequence (EMBL acc. no. AJ252157). The 60 C-terminal coding amino acids of FKHR were replaced by a PCR product containing an in-frame FLAG sequence followed by a stop codon. In order to also remove most of the 5'UTR, the BsmBI site 34 bp upstream of the translation start site was used. A BsmBI (end-filled) SpeI fragment containing the modified FKHR sequence was then subcloned between the polylinker sites EcoRV and SpeI sites of TetO-hCMV. The resulting plasmid (TetO-FKHRDC-Flag; see Fig. 6A, right panel) contained the TetO sequences, minimal CMV promoter and the C-terminal truncated FKHR cDNA with a rabbit β-globin intron 5' of this gene.

4.8 Generation of transgenic mice

Lck-tTA (as a 6.4 kb NotI fragment) and TetO-FKHR C (as a 3.5 kb XhoI fragment) were injected into fertilized B6xSJL F2 eggs. Several Lck-tTA and TetO-FKHR founder mice were obtained after PCR typing and backcrossed (at least once) to B6 mice before intercrossing.

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