Development 122, 23-30 (1996)
Printed in Great Britain © The Company of Biologists Limited 1996
DEV2025

Pax1 is expressed during development of the thymus epithelium and is required for normal T-cell maturation

Johan Wallin[1], Hermann Eibel[2], Annette Neubüser[1], Jörg Wilting[3], Haruhiko Koseki[1] and Rudi Balling[1,4]

[1]Department of Developmental Biology, Max-Planck Institute of Immunobiology, 79108 Freiburg, Germany
[2]Clinical Research Unit for Rheumatology, University of Freiburg, 79108 Freiburg, Germany
[3]Department of Anatomy, University of Freiburg, 79104 Freiburg, Germany
[4]Institut für Säugetiergenetik, GSF-Forschungszentrum für Umwelt und Gesundheit, 85764 Oberschleißheim, Germany

SUMMARY

***Pax1* is a transcriptional regulatory protein expressed during mouse embryogenesis and has been shown to have an important function in vertebral column development. Expression of *Pax1* mRNA in the embryonic thymus has been reported previously. Here we show that *Pax1* protein expression in thymic epithelial cells can be detected throughout thymic development and in the adult. Expression starts in the early endodermal epithelium lining the foregut region and includes the epithelium of the third pharyngeal pouch, a structure giving rise to part of the thymus epithelium. In early stages of thymus development a large proportion of thymus cells expresses *Pax1*. With increasing age, the proportion of *Pax1*-expressing cells is reduced and in the adult mouse only a small fraction of cortical thymic stromal cells retains strong *Pax1* expression. Expression of *Pax1* in thymus epithelium is necessary for establishing the thymus microenvironment required for normal T cell maturation. Mutations in the *Pax-1* gene in *undulated* mice affect not only the total size of the thymus but also the maturation of thymocytes. The number of thymocytes is reduced about 2- to 5-fold, affecting mainly the $CD4^+8^+$ immature and $CD4^+$ mature thymocyte subsets. The expression levels of major thymocyte surface markers remains unchanged with the exception of Thy-1 which was found to be expressed at 3- to 4-fold higher levels.**

Key words: *Pax1*, thymus, T-cell maturation, Thy-1, pharyngeal endoderm, mouse

INTRODUCTION

Development of the thymic epithelium and T cell development are intimately linked by multiple interactions between thymic stromal cell and developing thymocytes (van Ewijk, 1991; Ritter and Boyd, 1993; Boyd et al., 1993). For T cell maturation (Faas et al., 1993; Jenkinson et al., 1992; Hueber et al., 1992; Muller et al., 1993) as well as positive and negative selection of thymocytes (Jenkinson et al., 1992; Kaye and Ellenberger, 1992; Vukmanovic et al., 1992, 1994; Tanaka et al., 1993; Hugo et al., 1994) interactions between the immature thymocytes and different stromal cell lines are required. During this process the stroma provides adhesive sites by expression of specific adhesion molecules (Sawada et al., 1992; Lannes Vieira et al., 1993; Patel and Haynes, 1993), present (self-) antigenes via MHC molecules (Anderson et al., 1993; Markowitz et al., 1993) and secretes cytokines, growth and differentiation factors required for T cell maturation (Moll et al., 1992; Palacios and Nishikawa, 1992; Faas et al., 1993, Moore et al., 1993; Farr et al., 1993; Wolf and Cohen, 1992; Kasai et al., 1993; Takahama, 1994). The maturation pattern of thymocytes is precisely defined by characteristic precursor-offspring relationships and controlled by sequential interactions between the thymocytes and different stromal cell types (van Ewijk, 1991). The intrathymic migration route of thymocytes mirrors these interactions. Starting at the cortico-medullary junction, prothymocytes ($CD2^{lo}$, thy-1^{lo}, $CD4^-8^-$ TCRαβ) reach the outer cortex through thymic parenchyma. Then, they turn back and, while moving through the cortex, express new surface proteins ($CD4^+8^+$TCRαβ^{lo}). After acquiring the capacity to recognize self-MHC ($CD4^+8^+$TCRαβ^{hi,} $CD69^+$) they finally complete their developmental journey as mature T cells ($CD4^+$ or $CD8^+$ TCRαβ^{hi}) in the medulla.

In a reciprocal fashion, thymocytes also influence the development of the thymic stroma. However, much less is known about the origin and the maturation pattern of the different epithelial cell types during thymic ontogeny and in the adult thymus. The thymus stroma is composed of epithelial cells of both ectodermal and endodermal origin and of bone marrow-derived cells and has been extensively characterized both at the morphological level and for the expression of distinct cell surface markers (van Ewijk, 1991; Boyd et al., 1993). During embryogenesis, endoderm of the third pharyngeal pouch fuses with ectoderm of the third branchial cleft to form the early epithelial thymic primordium (van Ewijk, 1991; Boyd et al., 1993). The development of this primordium depends on interactions with the surrounding neural crest-derived mesenchyme

(Bockman and Kirby, 1984). Between days 11 and 12 of gestation in the mouse, the thymus primordium is populated by lymphoid progenitor cells. As a result of interactions between these cells and the thymic epithelium, cortical and medullary regions can be distinguished from day 13 p.c. onwards (Boyd et al., 1993). Recent results have demonstrated that the differentiation of both cortical and medullary cell types depends on interactions with thymocytes of specific maturation stages (Holländer et al., 1995). Towards the end of gestation most of the stromal components of the thymus are thought to be fully developed (van Ewijk, 1991; Boyd et al., 1993).

Thymus development is affected at different stages in a variety of mouse mutants and human disorders. In the mouse mutant strain *nude* a mutation in the forkhead domain containing transcription factor *whn* results in a defective ectodermal contribution to the thymus primordium, leading to a cystic thymus rudiment which is unable to attract lymphocytes (Nehls et al., 1994; Cardier and Haumont, 1980). Human patients with DiGeorge syndrome completely lack a thymus or possess only a small defective rudiment, probably caused by a neural crest defect interfering with the normal interaction between the epithelial thymus primordium and its surrounding neural crest-derived mesenchyme (Bockman and Kirby, 1984; DiGeorge, 1968). In *scid* mice and several transgenic strains with an early block in T cell development missing interactions between thymocytes and thymus epithelium result in impaired differentiation of thymus epithelial cell types (Boyd et al., 1993). So far no mutation which specifically affects the endoderm-derived cells of the thymus epithelium has been described.

In this report we describe the expression pattern of the transcriptional regulatory protein Pax1 in thymus epithelial cells of endodermal origin and show that mutations in *Pax1* result in reduction of thymus size and altered T cell development. The murine *Pax1* gene was originally identified by homology to the *Drosophila paired* and *gooseberry* genes (Deutsch et al., 1988). During mouse embryogenesis the *Pax1* gene product plays an important role in the development of the vertebral column and the pectoral girdle as has been determined by analyzing an allelic series of mouse mutants with alterations in the *Pax1* gene (Deutsch et al., 1988; Balling et al., 1988; Koseki et al., 1993; Wallin et al., 1994; Dietrich and Gruss 1995). Here we show, that the *Pax1* gene product is expressed first by endodermal precursor cells of the thymus epithelium in the pharyngeal pouches. It is further maintained in thymic epithelial cells during ontogeny and later on in the adult thymus by a small subset of cortical epithelial cells. Analyzing the thymus and T cell development in *Pax1* mutant mice we demonstrate that *Pax1* is required for proper thymus development.

MATERIALS AND METHODS

Mice

undulated (un) mice were purchased from the Jackson Laboratory. *Undulated short-tail* (*Un*^{s}) and *undulated-extensive* (*un*^{ex}) mutant mice were kindly provided from Dr A. M. Malashenko, Krosnogorsk, Russia and Dr J. L. Cruickshank, Leeds, England, respectively. These mutants were backcrossed onto the C57BL/6 strain. N1 to N5 generations were used for analysis. Embryos were recovered on days 9.5 to 18.5 p.c. where day 0.5 was 12 a.m. on the day of detection of the vaginal plug. On average, litters were born on day 19 p.c. As controls, +/+ or *un*/+ littermates were used.

Genotyping

Pax1 mutant embryos were genotyped by PCR analysis on embryonic DNA prepared from yolk sacs or the embryo proper as described by Wallin et al. (1994).

RNase protection assay

Total cellular RNA was isolated from adult mouse hematopoetic organs and day 12.5 p.c. embryos by the guanidine/caesium chloride method. RNase protection assays were performed as described by Koseki et al. (1993).

In situ hybridization

Pax1 riboprobes were generated from a *Hin*cII-*Sac*I paired box fragment using either ^{35}S-UTP or digoxigenin-11-UTP labelling for in situ hybridization on sections or in whole embryos, respectively. Fixation, hybridization and subsequent detection procedures were essentially as described by Kessel and Gruss (1991); Rosen and Beddington (1993).

Immunohistochemistry

For immunostainings, embryos or isolated thymi were fixed in 3% acetic acid in absolute ethanol at 4°C over-night, incubated first in absolute ethanol and then in xylene, both twice for 30 minutes, followed by a 1:1 mixture of xylene:paraffin for 30 minutes at 55°C once, infiltrated with paraffin by three incubations for 1 hour at 55°C and embedded. The generation of the Pax1-specific antiserum has been described previously (Chalepakis et al., 1991).

Pax1 immunostaining was done on deparaffinized sections that were first bleached in 0.3% H_2O_2 in methanol for 30 minutes, washed in PBS, blocked with 10% normal goat serum in PBS for 60 minutes and then incubated with the antiserum diluted 1:200 in the blocking solution. Detection was made with peroxidase-conjugated goat-anti-rabbit IgG (Sigma), and DAB was used as chromogen.

Histology

For histological analysis, thymi were fixed in Bouin's solution and embedded in paraffin. 7 μm sections were stained in haematoxylin-eosin.

FACS analysis

Thymocyte suspensions were prepared by squeezing the cells through a fine metal grid. Aggregates were removed by filtration through a nylon mesh. Flow cytometry was performed by using the following biotinylated or FITC or PE-labelled mAbs: H57-597 (from Pharmingen) GK1.5 for CD4; 53-6.7 for CD8 (both from Becton-Dickinson, Palo Alto, CA); RM2-5 for CD2; H1.2F3 for CD69 (Pharmingen); 53.7.3 for thy-1.2 (Becton Dickinson); ER-TR4, ER-TR5 and anti-IA^{b} (AF6-120.1, Pharmingen, San Diego, CA) to analyze thymic stroma. Biotinylated mAbs were counterstained with a streptavidin-PE conjugate (Southern Biotechnology Ass., Birmingham, AL) for two-color fluorescence or with streptavidin-TRI-color (MEDAC, Hamburg, Germany). The analyses were performed using a Becton-Dickinson FACScan and LYSYS II software.

RESULTS

Pax1 is expressed during thymus development and in cortical stroma cells of the adult mouse thymus

Expression of *Pax1* RNA in the embryonic thymus has been described previously (Deutsch et al., 1988). Northern blot analysis of RNA isolated from adult thymus also demonstrated the presence of *Pax1* transcripts in the adult organ (data not shown). In order to determine whether *Pax1* expression could also be detected in other lymphatic organs, RNAse protection

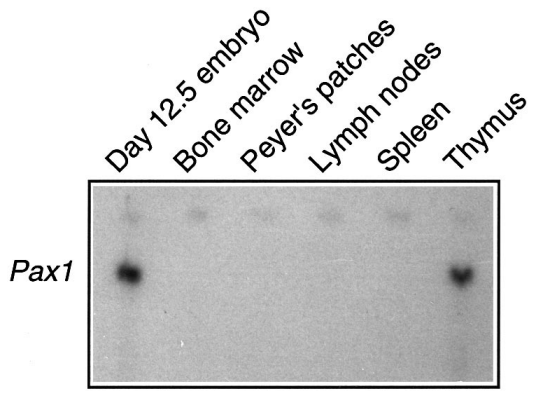

Fig. 1. *Pax1* RNA levels in different hematopoetic organs of adult mice and in day 12.5 p.c. embryos. 40 µg of total RNA of each sample was analyzed by RNase protection. The thymus was the only organ in which *Pax1* RNA could be detected.

assays were performed. As shown in Fig. 1, only the thymus but not bone marrow, lymph nodes, spleen or Peyer's patches contained detectable levels of *Pax1* RNA.

To localize the site of *Pax1* expression in the thymus more precisely and to study the expression pattern of the *Pax1* gene during thymic ontogeny we performed a series of in situ hybridization and immunohistochemical analyses. By whole-mount in situ hybridization *Pax1* expression could be detected in the endoderm of the foregut region as early as day 8.5 (data not shown). At day 9.5, transcripts were present in the first two pharyngeal pouches and by day 10.5 p.c., clear expression was also observed in the third pharyngeal pouch (Fig. 2).

Immunostaining demonstrated that Pax1 protein is present in the endoderm of the first, second and third pharyngeal pouches at day 10.5 (Fig. 3a). The third domain is known to fuse with ectodermal cells of the third branchial cleft to form the early thymic epithelial primordium which is then from day 11 onwards populated by lymphoid progenitor cells (van Ewijk, 1991; Boyd et al., 1993). Immigration of these cells did not change the *Pax1* expression pattern since a large number of epithelial cells are *Pax1*$^+$ in the thymus anlage of day 12.5 embryos (d12.5; Fig. 3b). At this stage the *Pax1*$^+$ cells are evenly distributed throughout the thymus primordium. At day 14.5 the two major *Pax1* expression domains are found in the vertebral column and the thymus (Fig. 3d). At this stage, a distinction between cortical and medullary regions can be made, indicating for the first time that the *Pax1*-expressing cells become confined to the developing thymic cortex (Fig. 3c). *Pax1* expression by cortical epithelial cells continues to be maintained up to the adult thymus. In sections of thymi from 6 week old mice, expression could still be detected immunohistochemically in a subpopulation of cortical stromal cells, whereas the medulla did not contain any *Pax1* positive cells (Fig. 4). The *Pax1*$^+$ cells were found to be dispersed throughout the cortex and also in subcapsular locations. The main difference in *Pax1* expression pattern between the developing thymus of day 14 and the adult thymus resides in the relative number of *Pax1*$^+$ cells: at day 14, a significant fraction of thymic epithelial cells show *Pax1* expression. In adult mice however, *Pax1* expression is seen only in a small number of cortical epithelial cells (Fig. 4) that neither express MHC II molecules nor the thymic epithelial cell surface protein stained

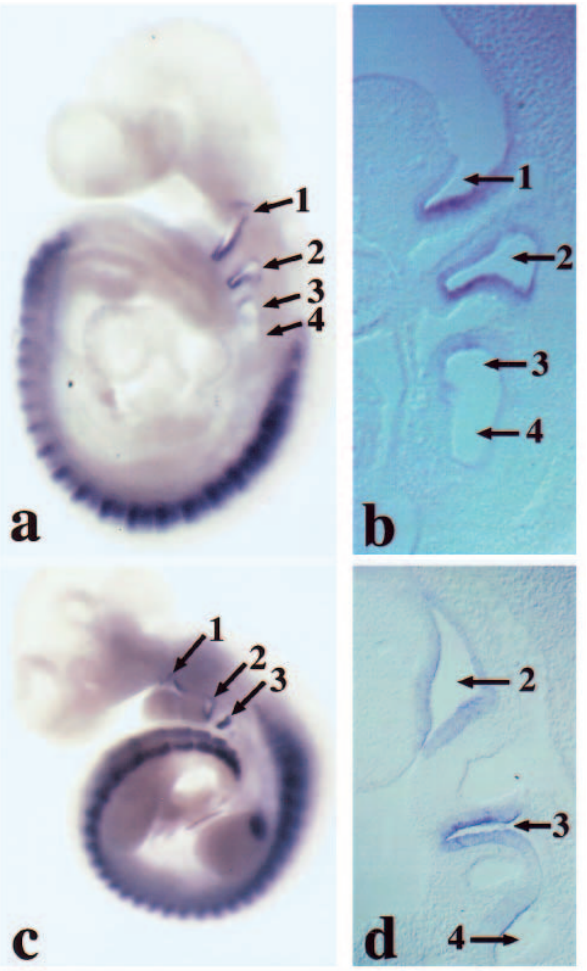

Fig. 2. Whole-mount in situ hybridizations of day 9.5 and 10.5 p.c. embryos and sections through the pharyngeal region of the stained embryos showing *Pax1* expression in pharyngeal pouches. (a) At day 9.5, *Pax1* is expressed in the first and second pharyngeal pouch and only very weakly in the third. (b) Section through the pharyngeal region of the embryo shown in A. *Pax1* is expressed in the endodermal epithelium of the pharyngeal pouches. (c) At day 10.5 strong *Pax1* expression is also present in the third pharyngeal pouch. (d) Section through the pharyngeal region of the embryo shown in C. 1, 2, 3 and 4 label the first, second, third and fourth pharyngeal pouch, respectively.

by the mAb ER-TR4 (data not shown). These results suggest that *Pax1* is expressed mainly by thymic epithelial precursor cells and not by their more differentiated offspring.

Thymic development is altered in *Pax1* mutant mice

To investigate the contribution of the *Pax1* gene product to thymic development we studied three different *Pax1* mouse mutants, *undulated* (*un*), *undulated-extensive* (*un*^{ex}) and *Undulated short-tail* (*Un*^{s}). *un* and *un*^{ex} are recessive mutations and homozygotes are viable and fertile, whereas *Un*^{s}, with a complete deletion of the *Pax1* gene, is a perinatal homozygous lethal. All *undulated* mutants have a smaller thymus which in adult *un/un* and *un*^{ex}/*un*^{ex} mice is about two thirds of a normal thymus. In the more strongly affected genotypes, *Un*^{s}/*un* and *Un*^{s}/*Un*^{s}, the reduction of the thymus was more apparent, and

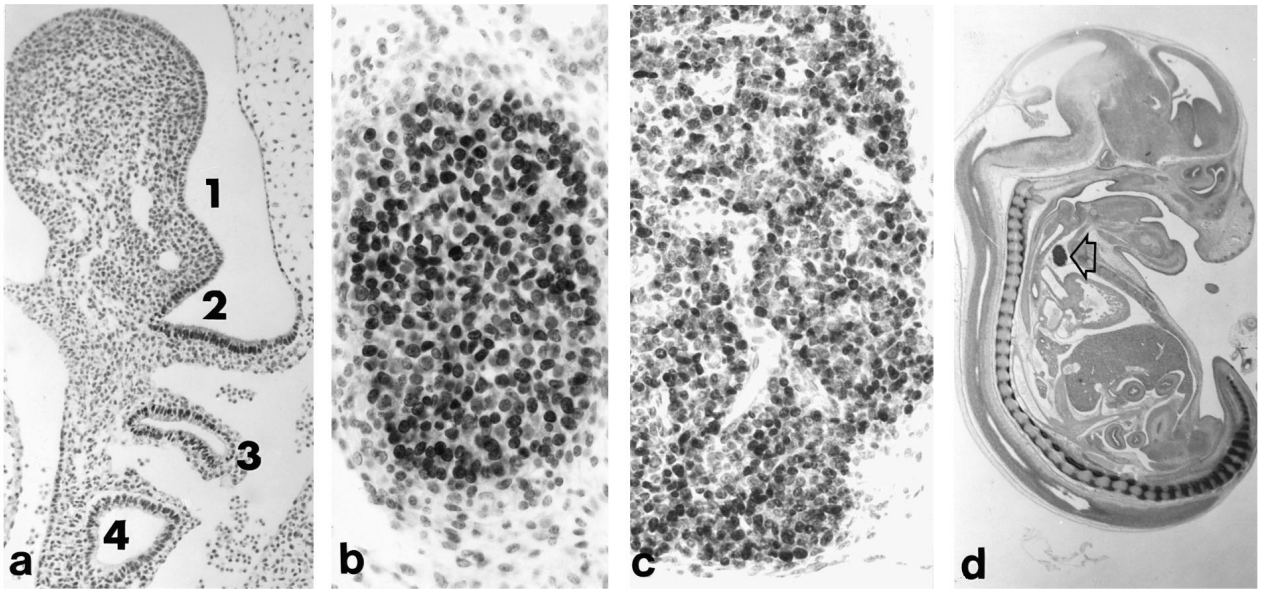

Fig. 3. *Pax1* immunoperoxidase staining of the pharyngeal pouch endoderm and of early stages in thymus development. (a) Parasagittal section of a day 10.5 p.c. embryo showing *Pax1* expression in the first three pharyngeal pouches. (b) Thymus primordium at day 12.5 p.c. A large proportion of the cells are *Pax1*-positive. (c) At day 14.5 p.c., small regions that are devoid of *Pax1*-staining cells can be detected. (d) A mid-sagittal section of a day 14.5 p.c. embryo. Strong *Pax1* expression domains are found in the vertebral column and the thymus.

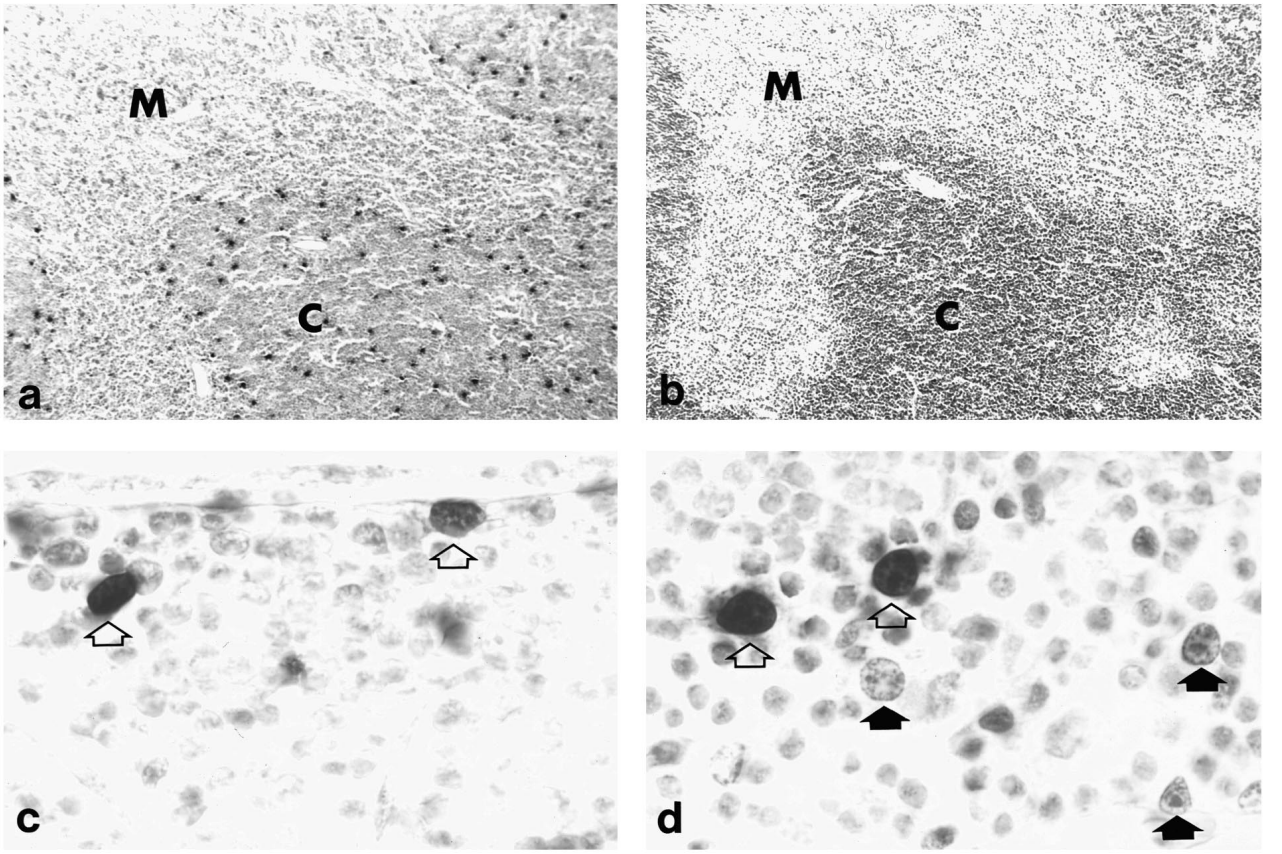

Fig. 4. Immunoperoxidase stainings of thymi from 6 week old mice using a Pax1 antiserum. (a) *Pax1*-positive cells constitute a subpopulation of cortical stromal cells. (b) Hematoxylin-eosin staining of a section adjacent to that in A. (c) *Pax1*-positive subcapsular cells are indicated by open arrows. (d) *Pax1*-positive cells have characteristically large nuclei with several nucleoli, known as ‘reticular cells’ (open arrows). Also shown are similar cells that are *Pax1*-negative (solid arrows).

the organs did not reach more than half the normal size. Histological examination did not reveal any obvious abnormalities except the appearance of large cysts (Fig. 5) and hematoxylin-eosin stainings showed an apparently normal gross morphology with cortical and medullary regions (data not shown).

Thymic epithelial cells and developing thymocytes are in a close symbiotic relationship (van Ewijk, 1991; Boyd et al., 1993). Since the altered or lacking Pax1 protein resulted in a smaller thymus, we analyzed, by flow cytometry, whether these changes would also affect thymocyte maturation. Thymi of the +/+, Un^s/+, un^{ex}/+, un^{ex}/un^{ex} and Un^s/Un^s genotypes were isolated from embryos at different stages of development (day 17.5, 18.5, 19 and 21 p.c.) and from adult +/+ *un*/+ un^{ex}/+, *un/un* and un^{ex}/un^{ex} mice. Since Un^s/Un^s mice are not viable after birth and since adult *un/un* and un^{ex}/un^{ex} mice might be stressed because of their defects in vertebral column development, we focussed our analysis on thymic development during embryogenesis and in d19.5 Un^s/Un^s and wild-type mice. At day 17.5, thymocyte numbers were found to be reduced by a factor of 5 in the Un^s/Un^s homozygous mutants (Table 1) and by a factor of 2 in Un^s/+ embryos. A twofold reduction was also found in *un/un* and un^{ex}/un^{ex} embryos and mice (data not shown). Phenotypic analyses of the major thymocyte subsets of all *undulated* mutants at day 17.5 revealed a slight, however not significant, increase in the contribution of the $CD4^-8^-$ thymocyte compartment, which consists mainly of the most immature T cells of the whole thymocyte population (Table 1). The contribution of the more mature $CD4^+8^+$, $CD4^+$ and $CD8^+$ subsets was equivalently decreased (Table 1). In d19.5 Un^s/Un^s mice, the contribution of the $CD4^-8^-$ compartment was significantly increased to 33.5% as compared to 13.1% in wild-type

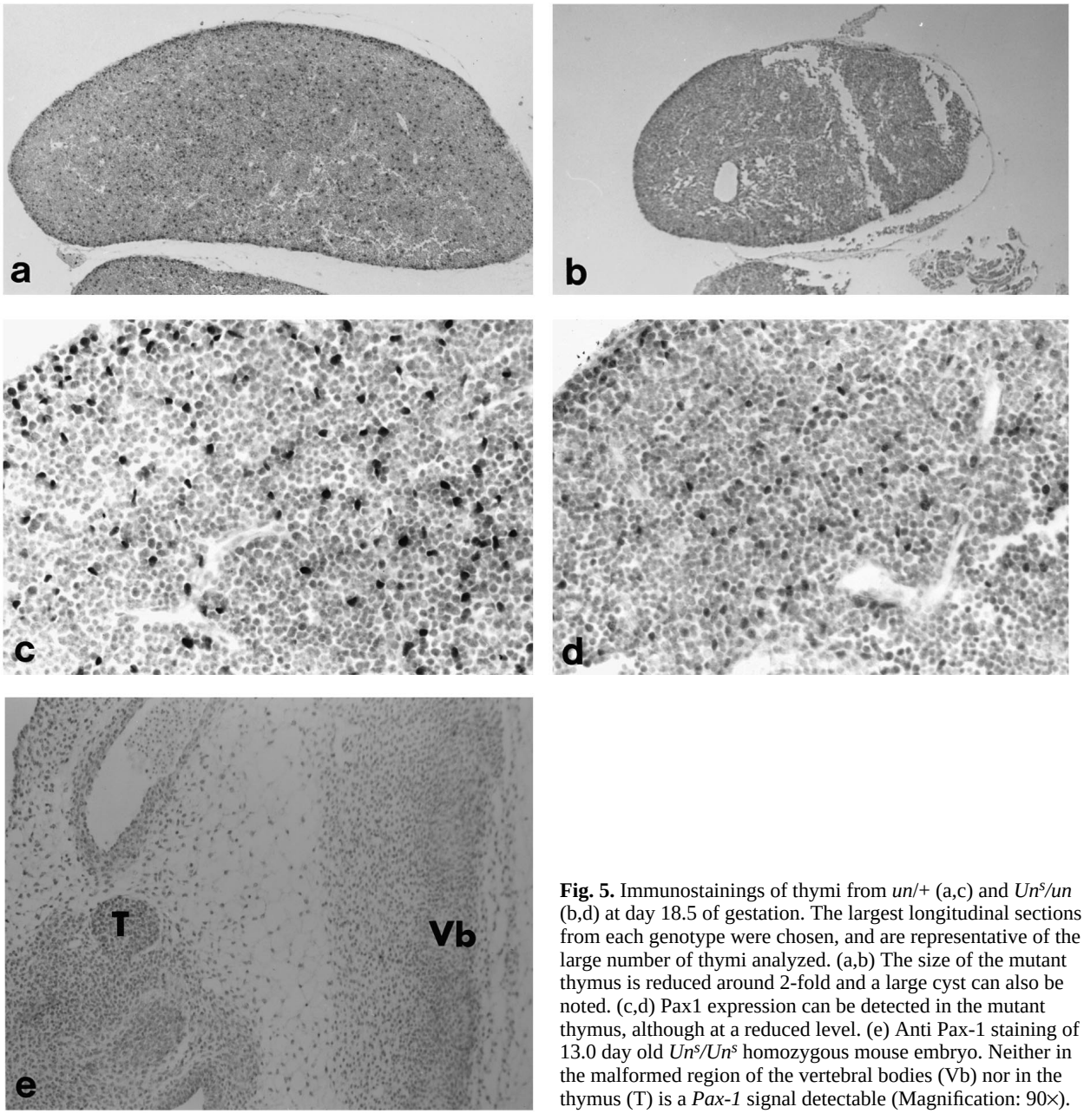

Fig. 5. Immunostainings of thymi from *un*/+ (a,c) and Un^s/un (b,d) at day 18.5 of gestation. The largest longitudinal sections from each genotype were chosen, and are representative of the large number of thymi analyzed. (a,b) The size of the mutant thymus is reduced around 2-fold and a large cyst can also be noted. (c,d) Pax1 expression can be detected in the mutant thymus, although at a reduced level. (e) Anti Pax-1 staining of a 13.0 day old Un^s/Un^s homozygous mouse embryo. Neither in the malformed region of the vertebral bodies (Vb) nor in the thymus (T) is a *Pax-1* signal detectable (Magnification: 90×).

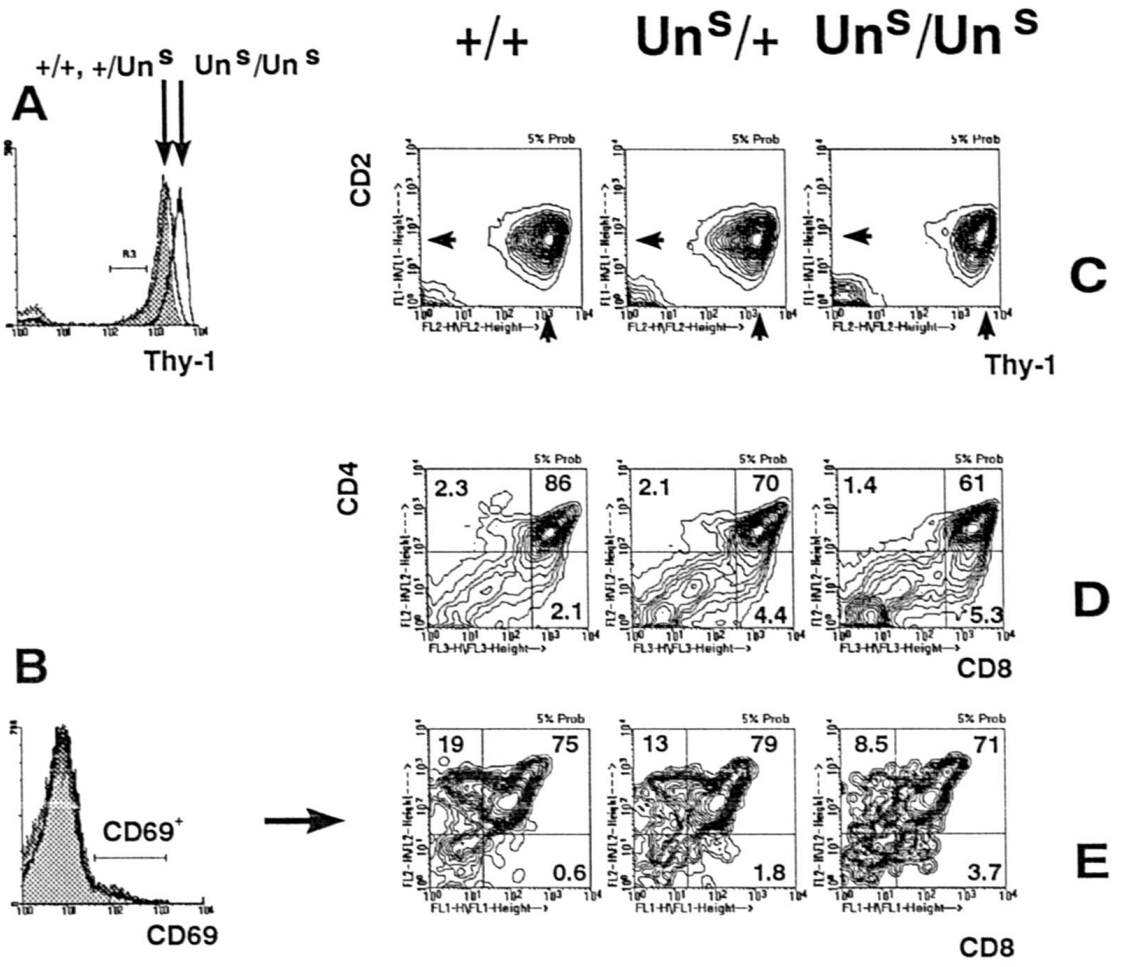

Fig. 6. Thymocyte populations in *Un^s/Un^s* compared to *Un^s/+* and to wild-type mice at day 19.5. During analysis, cell populations similar in size were defined by a forward/side scatter gate. Cells were stained for CD2 and thy-1 (A,C). Arrows indicate the elevated Thy-1 expression in thymocytes from *Un^s/Un^s* mice and the unchanged CD2 expression. In B,D,E, thymocytes were triple stained using anti-CD4, anti-CD8 and anti-CD69 antibodies. B shows CD69 staining of total thymocytes as an overlay of the individual (wt, *Un^s/+*, *Un^s/Un^s*) histogram plots; D shows CD4 and CD8 staining of total thymocytes. Thymocytes from *Un^s/Un^s* embryos had less cells of CD4$^+$CD8$^+$ and CD4$^+$ subsets which resulted in a relative increase of double negative and of CD8$^+$ subsets. In E cells were gated during analysis for CD69$^+$ subsets and as in D their CD4 and CD8 staining is shown. *Un^s/Un^s* thymocytes consistently contained less cells in the CD4$^+$CD8$^+$ and CD4$^+$ subsets.

Table 1. Thymocyte subsets

		wt		*Un^s/+*			*Un^s/Un^s*		
Age	Subset	% of cells	Cell number $\times 10^6$	% of cells	Cell number $\times 10^6$	% of wt cell number	% of cells	Cell number $\times 10^6$	% of wt cell number
d 17.5			*n*=2		*n*=3			*n*=2	
	Total		1.70±0.60		0.98±0.23	58		0.33±0.07	19
	CD4$^-$8$^-$	41.2	0.70±0.16	48.0	0.47±0.10	67	57.6	0.19±0.04	27
	CD4$^+$8$^+$	58.8	1.00±0.47	53.0	0.52±0.12	52	42.4	0.14±0.03	14
d 19.5			*n*=6		*n*=4			*n*=5	
	Total		3.20±0.90		2.70±0.50	84		1.70±0.72	53
	CD4$^-$8$^-$	13.1	0.42±0.14	15.2	0.41±0.09	98	33.5	0.57±0.20	136
	CD4$^+$8$^+$	81.3	2.60±0.77	81.5	2.20±0.41	85	64.7	1.10±0.53	42
	CD4$^+$	4.1	0.13±0.02	2.7	0.07±0.02	57	1.3	0.02±0.01	18
	CD8$^+$	6.0	0.19±0.1	5.2	0.14±0.04	73	4.51	0.08±0.02	40
			n=3		*n*=2			*n*=3	
	CD69$^+$	5.4	0.17±0.11	2.6	0.07±0.01	40	1.18	0.02±0.007	12

Thymocytes were isolated and the samples were analyzed individually by flow cytometry as described in the material and methods section. For each sample, 2-4×10^4 cells were acquired. The relative size of each subset (double negative, double positive CD4$^+$ CD8$^+$ or CD69$^+$ cells) is shown in the '% of cells' columns. The absolute number of cells/subset including its range was calculated from the total cell number/thymus and it is shown in the 'cell number' columns. The changes in cell numbers in comparison to wt samples are indicated by '% of wt cell number'. The number of individual mice tested is given by *n*.

mice. At the same time the contribution of the more mature CD4$^+$8$^+$ subset was reduced to 64.7% instead of 81.3% in control littermates (Table 1). Thymocytes are positively and negatively selected by interacting with thymic epthelial cells and we therefore analyzed whether the changed *Pax1* expression also affects T cell education. Positive selection is

phenotypically characterized by the expression of the early activation marker CD69 (Swat et al., 1993). Triple staining with anti-CD4, anti-CD8, and CD69 antibodies in d19.5 p.c. Un^s/Un^s embryos displayed a twofold reduction in the relative numbers of positively selected $CD4^+8^+CD69^+$ subsets (Table 1, Fig. 6D,E) corresponding to a 2- to 8-fold reduction in absolute numbers (Table 1). Thus, in 19-day old Un^s/Un^s embryos the composition of the different thymocyte subsets was more similar to d17.5 p.c. wild-type embryos than to their 19- to 21-day old normal counterparts. This result shows that the generation of mature thymocytes is impaired in $Pax1^-$ mice. This impairment could be due to lower numbers of thymocyte precursor cells which are characterized by low surface levels of thy-1. To our surprise, we could not identify these cells in the thymus of d19.5 Un^s/Un^s mice since all thymocytes expressed elevated levels of thy-1 (Fig. 6A,C). The upregulation of thy-1 expression was reproducibly found in Un^s/Un^s mutants where thymocytes expressed about 2-4× higher levels of this glycoprotein than control littermates (Fig. 6A).

DISCUSSION

Expression of *Pax1* in epithelial cells of the thymus primordium up to the adult stage, and thymus defects in *Pax1* mutant mice indicate an important role for *Pax1* in thymus development and thymus function in the adult. Early on, $Pax1^+$ cells are found in the pharyngeal pouch endoderm. After fusion with ectoderm from the third branchial cleft, *Pax1*-expressing cells are evenly distributed within the developing thymus epithelial primordium and later on become restricted to the cortical region. As in other parts of the embryo *Pax1* is excluded from the ectoderm, its expression in the developing thymus is probably in cells of endodermal origin. This expression pattern therefore suggests an intimate mixing of ectodermal and endodermal cells within the early thymus primordium and also that endoderm contributes to the cortical region, whereas earlier histological studies of thymus development had proposed a purely ectodermal origin for the cortex (Cardier and Haumont, 1980). Likewise the observed phenotype of *Pax1* mutant mice suggests a defect in all or part of the endoderm-derived cells within the thymus epithelium.

The *Pax1* gene product functions as a transcriptional activator. The genes regulated by the Pax1 protein during thymus development and in adult cortical cells are so far not known. Neither is the specific role of the cortical subpopulation expressing Pax1 for thymus function understood. One might, however, speculate that Pax1 expression marks a set of precursor cells that can differentiate further into specific thymic stroma cell types. To our knowledge no antibody marker with an identical staining pattern has been described. The only expression pattern showing some similarity is the one of *t*, a SV40 T antigen controlled by the growth hormone releasing factor promoter (Moll et al., 1992a,b). In this case, the transgene-positive cells are – like the $Pax1^+$ cells – found exclusively in the cortex, but also the transgene-positive cells are clustered at the cortico-medullary junction. In axial skeleton development *Pax1* expression is required for early steps of sclerotome development and is downregulated when sclerotome cells differentiate into chondrocytes. By analogy, in cortical thymic epithelial cells *Pax1* expression could also promote early steps of the differentiation of cortical thymic epithelial cell subsets. Due to a lack of *Pax1* function mutant mice could suffer from a deficiency in the differentiation of one or more cortical stromal cell types.

This hypothesis was corroborated by the analysis of a series of *Pax1* mutants in which a decrease in thymus size as well as a reduced number of thymocytes was found in mutant animals. Since thymic stromal cells foster T cell development (van Ewijk, 1991; Boyd et al., 1993), this phenotype might reflect a corresponding decrease in the number of thymic stromal cells that originate from $Pax1^+$ precursors or changes in the expression of proteins that interact with T cells during thymocyte maturation. Each of these defects would cause a reduced capacity of the thymus to support T cell development in the cortical phase. Since we were unable to find another marker that would identify $Pax1^+$ cells, this hypothesis remains to be proven. Whatever the defects in the thymic stroma of *Pax1* mutants are, they lead to an accumulation of immature $CD4^-8^-$ cells and an increased expression of thy-1. As Un^s/Un^s homozygotes die at birth it remains unknown, whether the observed delay in T cell maturation would be corrected postnatally. Two functions are linked to the glycoprotein thy-1: thy-1 is involved in adhesion of thymocytes to thymic epithelial cells (Hueber et al., 1992; Irlin and Peled, 1992; Kroczek et al., 1986) and, thy-1 crosslinking induces, in combination with signalling through the T cell receptor, cell death of $CD4^+8^+$ thymocytes (Kroczek et al., 1986; Gunter et al., 1987). The latter function has been linked to negative selection of T cells (He et al., 1991). Thus, the increased expression of thy-1 in $Pax1^-$ mice might make thymocytes more susceptible to cell death, which would result in lower numbers of more mature thymocyte subsets.

In conclusion our studies demonstrate that the expression of the *Pax1* gene is not only important for sclerotome (Koseki et al., 1993 Wallin et al., 1994) but also for thymus development. A dual involvement in thymus and skeletal development, similar to that of *Pax1,* has also been established for another transcriptional activator, the c-*fos* gene product. In the c-*fos* case, however, the long bones and not the vertebral column are affected besides the thymus.

We thank Rüdiger Fritsch for the Pax1 antibody, Petra Fiedler and Bärbel Strack for excellent technical assistance, and Uli Birsner for oligonucleotide synthesis. J. Wallin was supported by an EMBO fellowship and by the Swedish Medical Research Council. H. K. was supported by the Human Frontier Science Program. The work was supported by the Max-Planck-Society and the Deutsche Forschungsgemeinschaft.

REFERENCES

Anderson, G., Jenkinson, E. J., Moore, N. C. and Owen, J. J. (1993). MHC class II-positive epithelium and mesenchyme cells are both required for T-cell development in the thymus. *Nature* **362**, 70-73.

Balling, R., Deutsch, U. and Gruss P. (1988). undulated, a mutation affecting the development of the mouse skeleton, has a point mutation in the paired box of Pax 1. *Cell* **55**, 531-535.

Bockman, D. E. and Kirby, M. L. (1984). Dependence of thymus development on the derivatives of the neural crest. *Science* **223**, 498-500.

Boyd, R. L., Tucek, C. L., Godfrey, D. I., Izon, D. J., Wilson, T. J.,

Davidson, N. J., Bean, A. G. D., Ladyman, H. M., Ritter, M. and Hugo, P. (1993). The thymic microenvironment. *Immunol. Today* **14**, 445-459.

Cardier, A. C. and Haumont, S. M. (1980). Development of thymus, parathyroids, and ultimobranchial-bodies in NMRI and *nude* mice. *Am. J. Anat*. **157**, 227-263.

Chalepakis, G., Fritsch, R., Fickenscher, H., Deutsch, U, Goulding, M. and Gruss, P. (1991). The molecular basis of the undulated/Pax1 mutation. *Cell* **66**, 873-884.

Deutsch, U., Dressler, G.R. and Gruss, P. (1988). Pax-1, a member of a paired box homologous murine gene family, is expressed in segmented structures during development. *Cell* **53**, 617-625.

Dietrich, S. and Gruss, P. (1995). *undulated* phenotypes suggest a role of *Pax-1* for the development of vertebral and extravertebral structures. *Dev. Biol.* **167**, 529-548.

DiGeorge, A. M. (1968). Congenital absence of the thymus and its immunological consequences: Concurrence with congenital hypothyroidism. *Birth Defects Orig. Artic. Ser*. **4**, 116.

Faas, S. J., Rothstein, J. L., Kreider, B. L., Rovera, G. and Knowles, B. B. (1993). Phenotypically diverse mouse thymic stromal cell lines which induce proliferation and differentiation of hematopoietic cells. *Eur. J. Immunol.* **23**, 1201-1214.

Farr, A., Nelson, A., Hosier, S. and Kim, A. (1993). A novel cytokine-responsive cell surface glycoprotein defines a subset of medullary thymic epithelium in situ. *J. Immunol.* **150**, 1160-1171.

Gunter, K. C., Germain, R. N., Kroczek, R. A., Saito, T., Yokoyama, W. M., Chan, C., Weiss, A. and Shevach, E. M. (1987). Thy-1 mediated T-Cell activation requires co-expression of CD3/Ti complex. *Nature (London)* **326**, 505.

He, H.-T., Naquet, P., Caillol, D. and Pierres, M. (1991). Thy-1 supports adhesion of mouse thymocytes to thymic epithelial cells through a Ca^{2+} independent mechanism. *J. Exp. Med.* **173**, 515-518.

Holländer G.A., Wang, B., Nichogiannopoulou, A., Platenburg, P.P., van Ewijk, W., Burakoff, S.J., Gutierrez-Ramos, J.-C. and Terhorst C. (1995). Developmental control point in induction of thymic cortex regulated by a subpopulation of prothymocytes. *Nature* **373**, 350-353.

Hueber, A. O., Pierres, M. and He, H. T. (1992). Sulfated glycans directly interact with mouse Thy-1 and negatively regulate Thy-1-mediated adhesion of thymocytes to thymic epithelial cells. *J. Immunol.* **148**, 3692-3699.

Hugo, P., Kappler, J. W., Godfrey, D. I. and Marrack, P. C. (1994). Thymic epithelial cell lines that mediate positive selection can also induce thymocyte clonal deletion. *J. Immunol.* **152**, 1022-1031.

Irlin, Y. and Peled, A. (1992). Thy-1 antigen-mediated adhesion of mouse lymphoid cells to stromal cells of haemopoietic origin. *Immunol. Lett.* **33**, 233-237.

Jenkinson, E. J., Anderson, G. and Owen, J. T. (1992). Studies on T cell maturation on defined thymic stromal cell populations in vitro. *J. Exp. Med.* **176**, 845-853.

Kasai, M., Shirasawa, T., Kitamura, M., Ishido, K., Kominami, E. and Hirokawa, K. (1993). Proenzyme form of cathepsin L produced by thymic epithelial cells promotes proliferation of immature thymocytes in the presence of IL-1, IL-7, and anti-CD3 antibody. *Cellular Immunology* **150**, 124-136.

Kaye, J. and Ellenberger, D. L. (1992). Differentiation of an immature T cell line: a model of thymic positive selection. *Cell* **71**, 423-435.

Kessel, M. and Gruss, P. (1991). Homeotic transformations of murine vertebrae and concomitant alteration of Hox codes induced by retinoic acid. *Cell* **67**, 98-104.

Koseki, H., Wallin, J., Wilting, J., Mizutani, Y., Kispert, A., Ebensperger, C., Herrmann, B. G., Christ, B. and Balling, R. (1993). A role for Pax-1 as a mediator of notochord signals during the dorsoventral specification of vertebrae. *Development* **119**, 649-660.

Kroczek, R. A., Gunter, K. C., Germain, R. N. and Shevach, E. M. (1986). Thy-1 functions as a signal transduction molecule on T lymphocytes and transfected B lymphocytes. *Nature (London)* **322**, 181-185.

Lannes Vieira, J., Chammas, R., Villa Verde, D. M., Vannier dos Santos, M. A., Mello Coelho, V., de Souza, S. J., Brentani, R. R. and Savino, W. (1993). Extracellular matrix components of the mouse thymic microenvironment. III. Thymic epithelial cells express the VLA6 complex that is involved in laminin-mediated interactions with thymocytes. *Int. Immunol.* **5**, 1421-1430.

Markowitz, J. S., Auchincloss, H. Jr., Grusby, M. J. and Glimcher, L. H. (1993). Class II-positive hematopoietic cells cannot mediate positive selection of CD4+ T lymphocytes in class II-deficient mice. *Proc. Natl. Acad. Sci. USA* **90**, 2779-2783.

Moll, J., Eibel, H., Schmid, P., Sansig, G., Botteri, F., Palacios, R. and Van der Putten, H. (1992a). Thymic hyperplasia in transgenic mice caused by immortal epithelial cells expressing c-kit ligand. *Eur. J. Immunol.* **22**, 1587-1594.

Moll, J., Eibel, H., Botteri, F., Sansig, G., Regnier, C. and Van der Putten, H. (1992b). Transgenes encoding mutant simian virus 40 large T antigens unmask phenotypic and functional constraints in thymic epithelial cells. *Oncogene* **7**, 2175-2187.

Moore, N. C., Anderson, G., Smith, C. A., Owen, J. J. and Jenkinson, E. J. (1993). Analysis of cytokine gene expression in subpopulations of freshly isolated thymocytes and thymic stromal cells using semiquantitative polymerase chain reaction. *Eur. J. Immunol.* **23**, 922-977.

Muller, K. P. and Kyewski, B. A. (1993). T cell receptor targeting to thymic cortical epithelial cells in vivo induces survival, activation and differentiation of immature thymocytes. *Eur. J. Immunol.* **23**, 1661-1670.

Nehls, M., Pfeifer, D., Schorpp, M., Hedrich, H. and Boehm, T. (1994). New member of the winged-helix protein family disrupted in mouse and rat *nude* mutations. *Nature* **372**, 103-108.

Palacios, R. and Nishikawa, S. (1992). Developmentally regulated cell surface expression and function of c-kit receptor during lymphocyte ontogeny in the embryo and adult mice. *Development* **115**, 1133-1147.

Patel, D. D. and Haynes, B. F. (1993). Cell adhesion molecules involved in intrathymic T cell development. *Semin. Immunol.* **5**, 282-292.

Ritter, M. A. and Boyd, R. L. (1993). Development in the thymus: it takes two to tango. *Immunol. Today* **14**, 462-469.

Rosen, B. and Beddington, R. S. P. (1993). Whole-mount in situ hybridization in the mouse embryo: gene expression in three dimensions. *Trends Genet.* **9**, 162-167.

Sawada, M., Nagamine, J., Takeda, K., Utsumi, K., Kosugi, A., Tatsumi, Y., Hamaoka, T., Miyake, K., Nakajima, K., Watanabe, T. and et al. (1992). Expression of VLA-4 on thymocytes. Maturation stage-associated transition and its correlation with their capacity to adhere to thymic stromal cells. *J. Immunol.* **149**, 3517-3524.

Swat, W., Dessing, M., von Boehmer, H. and Kiesielow, P. (1993). CD69 expression during selection and maturation of $CD4^+8^+$ thymocytes. *Eur. J. Immunol.* **23**, 739-746.

Takahama, Y., Letterio, J. J., Suzuki, H., Farr, A. G. and Singer A. (1994). Early progression of thymocytes along the CD4/CD8 developmental pathway is regulated by a subset of thymic epithelial cells expressing transforming growth factor β. *J. Exp. Med.* **179**, 1495-1506.

Tanaka, Y., Mamalaki, C., Stockinger, B. and Kioussis, D. (1993). *In vitro* negative selection of αβ T cell receptor transgenic thymocytes by conditionally immortalized thymic cortical epithelial cell lines and dendritic cells. *Eur. J. Immunol.* **23**, 2614-2621.

van Ewijk, W. (1991). T-cell differentiation is influenced by thymic microenvironments. *Annu. Rev. Immunol.* **9**, 591-615.

Vukmanovic, S., Grandea, A. G., Faas, S. J., Knowles, B. B. and Bevan, M. J. (1992). Positive selection of T-lymphocytes induced by intrathymic injection of a thymic epithelial cell line. *Nature* **359**, 729-732.

Vukmanovic, S., Stella, G., King, P. D., Dyall, R., Hogquist, K. A., Harty, J. T., Nikolic-Zugic, J. and Bevan, M. J. (1994). A positively selecting thymic epithelial cell line lacks costimulatory activity. *J. Immunol.* **152**, 3814-3823.

Wallin, J., Koseki, H., Wilting, J., Ebensperger, C., Christ, B. and Balling R. (1994). The role of *Pax-1* in the development of the axial skeleton. *Development* **120**, 1109-1121.

Wolf, S. S. and Cohen, A. (1992). Expression of cytokines and their receptors by human thymocytes and thymic stromal cells. *Immunology* **77**, 362-368.

(Accepted 6 September 1995)