Received 6 June; accepted 26 September 1989.

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ACKNOWLEDGEMENTS. We thank Elaine Pickett, Denise Edger and Richard Trezza for technical assistance, Jonathan Katz for computer skills and Iris Sheldon for secretarial help. We also thank Susan Frank and Dr Raymond Apple for critical reading of the manuscript. This work was supported by research grants from the NIH and the American Cancer Society.

Complex lymphoid and epithelial thymic tumours in *Thy*1-*myc* transgenic mice

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T-LYMPHOCYTE development takes place mainly in the thymus, where stromal cells of epithelial and haemopoietic origin are involved in inductive and selective mechanisms, which enable specific lymphocyte populations to migrate to the periphery and establish a network of immune responses. Experiments with intact animals have clarified the precursor–product relationships between thymocyte subpopulations, but the molecular mechanisms of cell interactions in the thymus are difficult to study *in vivo*. In an attempt to expand thymic cell populations *in vivo* and maintain them *in vitro* for such studies, we directed high levels of expression of the murine c-*myc* proto-oncogene in transgenic mice by inserting it into the mouse Thy-1 transcriptional unit. Such mice develop thymic tumours which contain proliferating thymocytes and, interestingly, expanded populations of epithelial cells. Both cell types can be maintained *in vitro*.

Thy-1 is a surface glycoprotein expressed in a variety of mouse tissues, notably thymus and brain[1], whereas the product of the c-*myc* proto-oncogene is a nuclear protein which has been associated with malignancies in a variety of tissues[2]. Deregulation of *myc* expression can lead to cell proliferation and immortalization/transformation[3–8] and has been used for experimental tissue directed oncogenesis[9–15]. In some cases cells from such tumours were able to grow *in vitro*[11,16].

The expression of c-*myc* was directed to the thymus[17] by placing exons 2 and 3 of the mouse c-*myc* gene into the first intron of the mouse Thy-1 gene (Fig. 1*a*). This transcription unit does not have the *myc* attenuation sequences[18,19] and results after splicing in a hybrid messenger RNA containing coding sequences for the complete c-*myc* gene product only. Fertilized mouse eggs were injected and 13 transgenic mice were obtained, containing 1–20 copies of the transgene. Three of the animals (TM1, TM28 and TM29) died in the first week after birth before detailed analysis was carried out. The other mice, except TM7 and TM6L (a low copy (1–2) number offspring of TM6), developed breathing difficulties at the age of 8 to 12 weeks, caused by large thymic tumours and three had metastasis in peripheral lymphoid organs. Two of six tumours from founder mice tested caused subcutaneous tumours in histocompatible animals. Three of eight tumours from founder mice tested were established as long-term (>6 months) cell cultures. Two of the founder mice (TM6 and TM25) were bred extensively and all of the descendants developed large thymic tumours by 9–14 weeks.

To determine whether the normal *Thy-1* transgene expression pattern, in brain and thymus, is observed for the hybrid gene, RNA was analysed from brain, thymus and spleen. All thymic tumours expressed the *Thy-myc* gene at levels several-fold higher than the endogenous c-*myc* gene (Fig. 1*b*). Hybrid mRNA levels varied, even within a mouse line, reflecting the variability of tumour phenotype (compare lane TM25 with TM25.32 and TM25.49). Lines without tumours showed background signal (TM6L, TM7). The only tumour (TM25.49) that expressed low levels also contained mostly mature $CD4^+$ single positive cells (Table 1). As reported[11,13], high levels of c-*myc* transgene expression reduce the endogenous c-*myc* mRNA levels (T and E in Fig. 1*b*). Background signal was detected in other tissues, except in the lymphoid organs which appeared enlarged in some of the animals (Fig. 1*b*; TM3, TM14 and TM25.120). As expected[17], the transgene is switched off in cells progressing to the single positive stage (Fig. 1*b*, all except TM3, TM14 and TM25.120).

In the brain, unexpectedly low levels of transcription were observed, as shown by RNase protection and run-on assays (Fig. 1*b*, *d*). Expression of the *myc* gene is efficiently directed by a downstream thymocyte specific enhancer (our unpublished data), nevertheless, we have no explanation for the low levels in brain, as none of the sequences thought to be important for brain specific expression were interrupted (ref. 20; and our unpublished data).

Cell lines were established from thymomas that were of an adherent epithelial and a non-adherent thymocyte phenotype. Cultured cells were maintained in 10% fetal calf serum without any further growth factor addition. All the thymocyte cell lines are of a double positive character and express high levels of the transgene (Fig. 1*c*, lane *Thy*, panel *myc*). By contrast, only one out of four adherent cell lines expressed the hybrid gene at levels above background (Fig. 1*c*, lane D, panel *myc*).

Histological analysis also showed that both stromal and lymphoid cell populations had expanded. The tumours were composed of two main areas separated by a clear junction (Fig. 2*a*). Lymphocytes and macrophages were distributed throughout both areas, whereas the main blood vessels were predominantly located in the central zone. These were sometimes parts of islands of large lymphoblasts and macrophages surrounded by bands of expanded epithelial cell populations (Fig. 2*a*, *b*). The lymphoblasts were larger than normal and many mitotic figures could be seen.

Immunohistochemistry revealed that essentially all lymphoid populations were Thy-1.2^+, although the intensity of the Thy-1.2 labelling varied considerably in some tumours. The majority also stained positively for both CD4 and CD8 (data not shown). All tumours stained strongly for keratin in the central zone (Fig. 2*b*), whereas the outer zone was essentially negative although cells with epithelial morphology were clearly visible in this region. Dual immunofluorescence with keratin and either IVC4 (ref. 21) or 4F1 monoclonal antibodies showed that some

keratin$^+$ cells in the central zone had medullary (IVC4$^+$) and some had cortical (4F1$^+$) characteristics (Fig. 2*c*; panel 3, IVC4$^+$ and panel 1, 4F1$^+$). In other tumours a large proportion of keratin$^+$ cells stained for both markers, indicating double-positive epithelial cells similar to the neoplastic epithelial cells in human thymomas. It has been suggested that these cells may be an epithelial stem cell[22,23]. In the keratin$^-$ outer region some 4F1$^+$ (but not IVC4$^+$) cells were also seen.

The expanded epithelial cells, as well as the thymocytes (see below), seem to be transformed as they can establish long-term, fast growing *in vitro* cultures and cause tumours in histocompatible animals. Epithelial transformation was unexpected, as Thy-1 appears normally to be confined to rodent lymphocytes and has only been reported to appear on thymic epithelial cells *in vitro*[24] (see also Fig. 1*c*). Possibly, *Thy-1* and, by extension, the *Thy-myc* gene are expressed transiently during epithelial maturation. Another possibility is that the epithelial cells respond to signals produced by the over-proliferating lymphocytes in these tumours and expand, thereby increasing the probability of the occurrence of a transforming event that is unrelated to the expression of *Thy-myc* gene. Complementary situations have been described in c-*fos*[25] and hGRF-SV40 transgenic mice[26], where hyperplasia of the epithelial component of the thymus leads to over proliferation of lymphocytes. In these cases, however, neither the epithelial nor the lymphoid cells exhibited a transformed phenotype and the authors did not describe long-term cultures *in vitro*.

To further characterize the lymphocyte suspensions from large thymic tumours, the progeny of the TM6 and TM25 mice were analysed by FACS (Table 1) using reagents against Thy-1 and HSA (heat stable antigen) which appear early in haematopoietic differentiation[27–29], the T-cell subset differentiation markers CD4 and CD8, and components of T-cell antigen receptors Vβ8 and the CD3 ε chain which are expressed highly only later in differentiation (for review see ref. 30). Thymocytes of young transgenic animals were normal in terms of CD3, CD4, CD8 and HSA expression (data not shown). This favours the idea that the tumours arise in a population of phenotypically normal cells, although *Thy-myc* mRNA levels in these thymuses were comparable to those in fully grown tumours (data not shown). Most tumours contained mainly CD4$^+$CD8$^+$ cells, which is also the predominant phenotype in normal thymus[31]. On forward light scatter, however, more than 80% of the CD4$^+$CD8$^+$ tumour

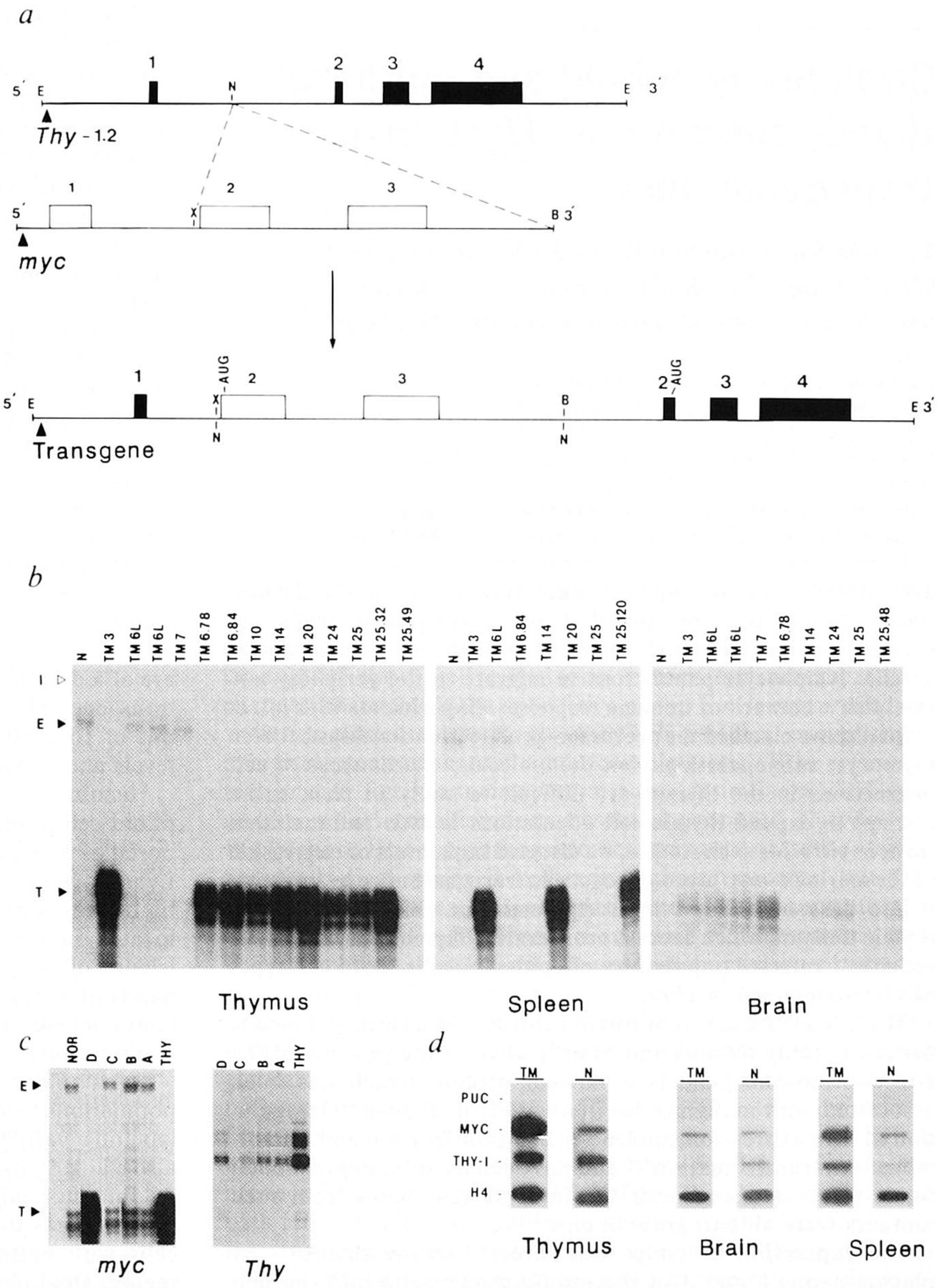

FIG. 1 *a*, Transgene construction. The *Xba*I–*Bam*H1 4.8 kb fragment of the mouse *myc* gene containing the coding sequences (exons 2 and 3) and the polyadenylation site (4) was inserted into the *Nru*I site of the first intron of the mouse *Thy*-1.2 gene (*Eco*RI–*Eco*RI 8.2 kb fragment). The hybrid construct was microinjected as a 13.0 kb *Eco*RI–*Eco*RI fragment into fertilized oocytes from (CBA × C57BL/10)F$_1$ mice[17]. *b*, Steady-state mRNA levels of the hybrid transgene analysed by RNase protection[36]. A *Sac*–*Pst*I 200 base pair (bp) fragment from the mouse *myc* cDNA[37] covering part of exon 1 and 2 was cloned in a pGEM4 vector in the *Pst*I–*Sac*I sites of the polylinker. A 220 bp RNA probe was generated with T7 polymerase and hybridized to 30 μg of total RNA isolated from brain, thymus and spleen of transgenic mice[38]. The probe protects 200 bp of the endogenous *myc* transcripts (E) and 145 bp of the hybrid transgene transcripts (T). Triangles indicate the protected fragments from transgenic (TM) and non-transgenic (N) mice. *c*, The first panel (*myc*) shows the steady-state mRNA levels of the *Thy*-1–*myc* transgene in established thymic epithelial cell lines. The riboprobe was prepared as described above and hybridized to 30 μg of total RNA. Lanes A, B, C and D represent different thymic epithelial cell lines, derived from thymic tumours and established in culture. Lanes NOR and THY represent RNA isolated from non-transgenic thymus and a thymoma, respectively. The second panel (*Thy*-1) shows expression of the endogenous *Thy*-1.2 mRNA in the same epithelial cell lines, detected by S1 analysis[38]. A 680 bp *Sac*I–*Tth*111I probe from the mouse *Thy*-1.2 gene covering the third intron and part of the fourth exon, was labelled with T_4 polynucleotide kinase and hybridized to 30 μg of total RNA. The probe protects a 244 bp fragment of the fourth exon. Lanes A, B, C and D, RNA from the epithelial cell lines as above, lane THY, RNA from transgenic thymus. *d*, Run-on assay of nuclei from brain, thymus and spleen of TM25 (TM) and non-transgenic mice (N). The assay was performed as described elsewhere[38]. Equal counts of the nascent RNA were hybridized to the following DNA fragments: linearized pUC vector molecules as a negative control, the 4.8 kb *Xba*I–*Bam*H1 fragment of the *myc* gene detecting both the endogenous gene and the transgene, an *Apa*I–*Apa*I 1.3 kb fragment covering the 3′ untranslated part of the *Thy*-1 gene and finally a histone H4 probe.

cells were large blasts, whereas most of these cells are normally smaller than resting peripheral lymphocytes[28]. Several of the tumours were mainly $CD4^+CD8^-$ HSA-low and CD3-high (for example, TM25.49; Table 1), which is normally the phenotype of a minor population of functionally mature thymocytes[32,33]. Other tumours contained cells with the abnormal phenotype $CD4^+CD8^+$, HSA-low (for example TM6.78; Table 1) which probably result from abnormal differentiation, although they could represent the expansion of a normally very rare cell type. In tumours with both $CD4^+CD8^-$ and $CD4^+CD8^+$ cells, Southern blots derived from FACS-sorted cells of Cβ rearrangement suggested that both cell populations were (mono-) oligoclonal and part of the tumour (data not shown). The expression of Vβ8 in some tumours was similar to that on normal thymocytes[34]. Some tumours, however, were clearly $CD3^+$ but completely $V\beta8^-$, whereas others showed disproportional overexpression of Vβ8, indicating that these tumours were oligoclonal. This was confirmed by Southern blots with a TCR β-chain constant region probe, to detect TCR β-chain rearrangements[35]. Compared with liver DNA, only one or two new bands are observed due to rearrangements of the Cβ1 or Cβ2 locus in any given tumour (Fig. 3), indicating that the tumours were formed by clonal expansion from one or a very small number of cells.

The tumour cells were also tested for their ability to propagate in histocompatible $(CBA/CA \times C57BL/10)F_1$ mice after subcutaneous injection. After injection of 10^6 to 10^7 tumour cells, subcutaneous tumours developed in 7 cases out of 8 for TM25 and in 2 out of 5 for TM6. No correlation could be made, however, between the ability to form subcutaneous tumours in F_1 mice and metastasis in the original thymoma-bearing animals (Table 1) whose lymphoid organs were invaded by $CD4^+CD8^+$ T lymphocytes.

These results indicate that oncogenic transformation has taken place at the $CD4^+CD8^+$ cell stage in a stochastic manner and after the TCR β-chain has rearranged. In some of these tumours the cells continue to differentiate to single positive cells, and in a few cases double positive cells escaped the thymus and gave rise to metastases.

Although the mechanism of tumour formation in these transgenic mice remains unknown, it is the first time that the expression of c-*myc* has been deliberately targeted in the thymus

TABLE 1 Summary of FACS analysis on tumours in TM6 and TM25 offspring

Offspring	$CD4^+CD8^+$	$CD4^+$	$CD8^+$	$CD4^-CD8^-$	HSA^+	Metastasis	s.c. passage	Cell-lines: Lymph.	Cell-lines: Adher.
TM6.2	40	50	5	5	0	–	ND	–	–
.3	74	18	3	4	ND	–	ND	–	–
.75	42	54	2	2	0	–	ND	+	+
.78	74	20	1	4	1	–	+	+	+
.84	90	8	1	1	5	–	+	–	+
.137	59	12	3	26	7	–	–	–	–
.140	53	29	1	17	3	–	–	–	–
.141	56	15	3	27	34	–	–	–	–
.158	90	5	1	4	63	–	ND	–	–
.159	95	3	1	2	16	–	ND	–	–
.161	86	6	2	6	44	–	ND	–	–
.163	92	3	1	3	30	–	ND	–	–
.165	71	14	2	13	29	–	ND	–	–
.166	87	9	1	3	2	–	ND	–	–
Total:						0/14	2/5	2/14	3/14
25.2	86	0	8	0	88	–	ND	–	–
.3	33	46	8	12	13	+	ND	+	–
.4	59	28	3	10	84	–	+	–	+
.5	60	18	5	16	68	+	+	+	+
.8	63	30	2	3	ND	–	ND	+	+
.9	74	20	3	3	30	–	ND	–	–
.26	80	16	2	2	80	+	ND	–	–
.32	94	3	1	1	80	–	ND	+	+
.45	76	20	2	1	93	–	ND	+	+
.48	28	46	8	16	ND	–	ND	+	+
.49	2	72	10	17	0	–	ND	–	–
.103	50	38	1	10	ND	–	–	+	+
.105	87	4	2	7	18	–	+	–	+
.106	72	8	5	14	0	+	+	–	+
.112	96	3	0	0	ND	–	+	–	–
.114	43	29	2	26	ND	–	+	+	+
.120	85	11	0	3	ND	+	+	+	+
Total:						5/17	7/8	9/17	11/17

The columns indicate the percentage of $CD4^+CD8^+$ and HSA^+ cells. The next two columns indicate which of the animals showed metastasis, or gave tumours when passaged subcutaneously (s.c.) in histocompatible recipients. The last two columns show which of the tumours could be established in tissue culture as non-adherent (Lymph.) or adherent cells (Adher.). The cell lines were established in RPMI medium supplemented with 10% FCS, at a density of $\sim 10^7$ tumour cells per ml. No additional growth factors were added and the established cultures were maintained at a density of 10^5–10^6 cells per ml. Our analysis does not distinguish between normal cells and neoplastic tissue which is the result of oligoclonal expansion of a few transformed cells. It is possible, therefore, that the phenotype we describe (by FACS and Southern blot analysis) is partly due to the presence of normal cells in the enlarged thymus. Expression of cell-surface antigens was analysed by flow-cytometry on a FACS II Unit (Becton Dickinson) using monoclonal antibodies specific for mouse: CD4, CD8, Thy-1.2, CD3ε, Vβ8 and HSA(J11d). Antibodies were purchased from Becton Dickinson. For mouse CD4 and CD8, antibodies were directly conjugated to fluorescein (αCD8, green) or phycoerythrin (αCD4, red), therefore, no second layer antibody was required. For CD3ε and HSA, anti-hamster and anti-rat antibodies were used, respectively, with a second layer antibody.

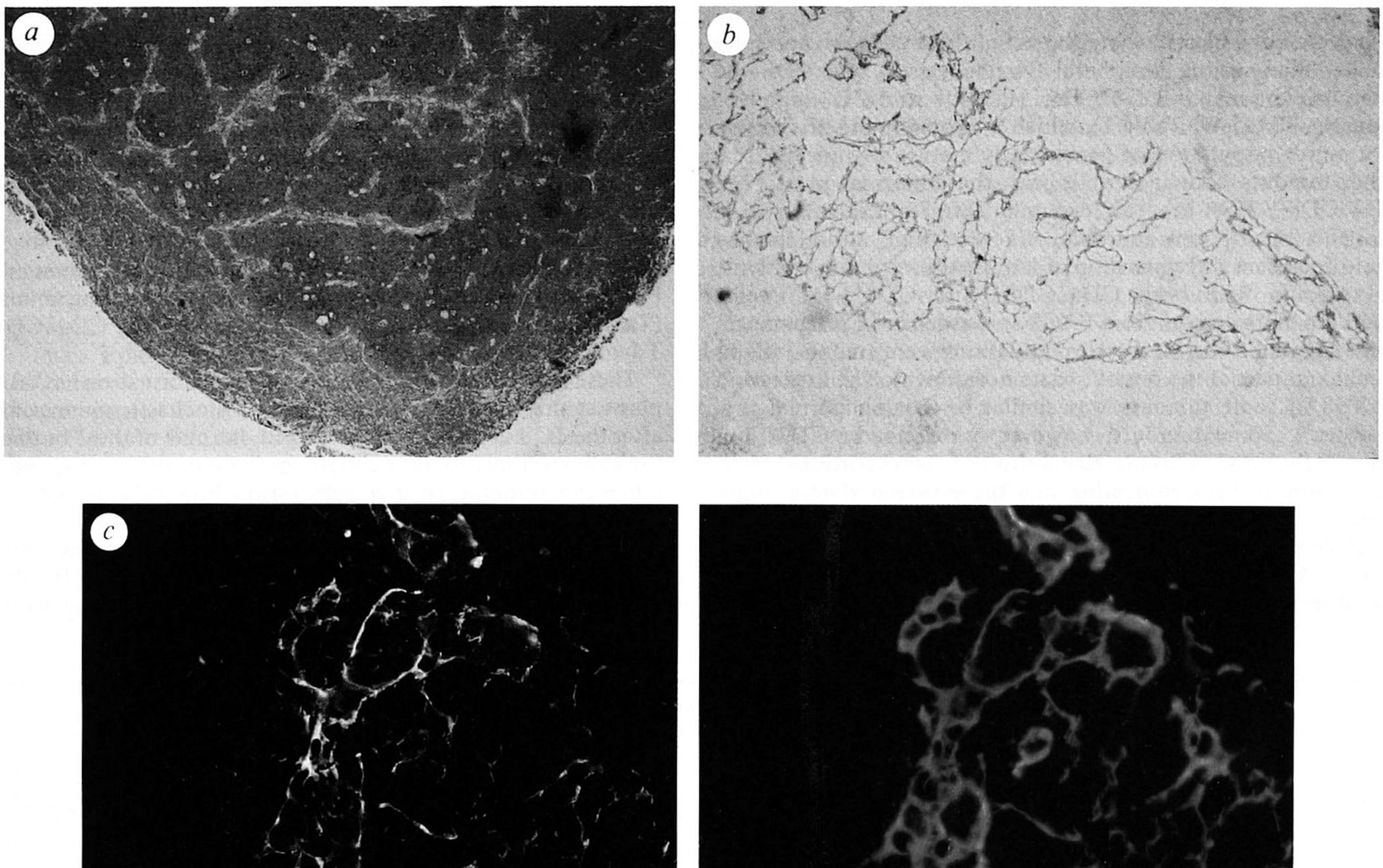

FIG. 2 Histological analysis of thymic tissues from mice TM6 and 25. *a*, TM25 tumour showing inner and outer zones. Haematoxylin stain; final magnification ×148. *b*, TM25 tumour showing keratin-positive epithelial cells in the central zone. Frozen section labelled with polyclonal rabbit anti-keratin antibody by indirect immunoperoxidase technique. Final magnification ×370. *c*, Dual immunofluorescence analysis of a TM6 tumour. Monoclonal antibodies 4F1 and IVC4 (green) each label a subpopulation of keratin positive (red) epithelial cells in the central zone. 1, 4F1; 2, the same field with keratin; 3, IVC4; 4, the same field with keratin. Final magnification ×1,850. This TM6 immunofluorescence is a good example of most TM6 and TM25 tumours. METHODS. Tissue preparation. Thymuses were either snap-frozen for immunohistochemistry or fixed in buffered formalin, embedded in paraffin wax and sections stained with Haematoxylin/Eosin. Primary antibodies for immunohistochemistry were: rat IgM monoclonal antibodies 4F1 and IVC4, neat supernatant, to detect cortical and medullary type thymic epithelial cells respectively[21]; rat IgG monoclonal anti-*Thy*-1.2 (H30-12), the gift of Dr A. F. Williams, supernatant, 1:30; rat IgG monoclonal antibodies anti-CD4 (YTS191.1) and CD4 (YTS169.4) (Seralab), supernatant, 1:5; polyclonal rabbit anti-keratin, purified immunoglobulin used at 1:200 (Dakopatts). Secondary antibodies were: peroxidase conjugated swine anti-rabbit Ig, 1:20 (Dakopatts); biotinylated sheep anti-rat Ig, 1:100, species specific, (Amersham); fluorescein isothiocyanate (FITC) conjugated streptavidin, 1:100, (Amersham); rhodamine tetramethyl isothiocyanate (TMRITC) conjugated swine anti-rabbit immunoglobulin 1:20 (Dakopatts). Immunolabelling was done by standard indirect immunoperoxidase and immunofluorescence.

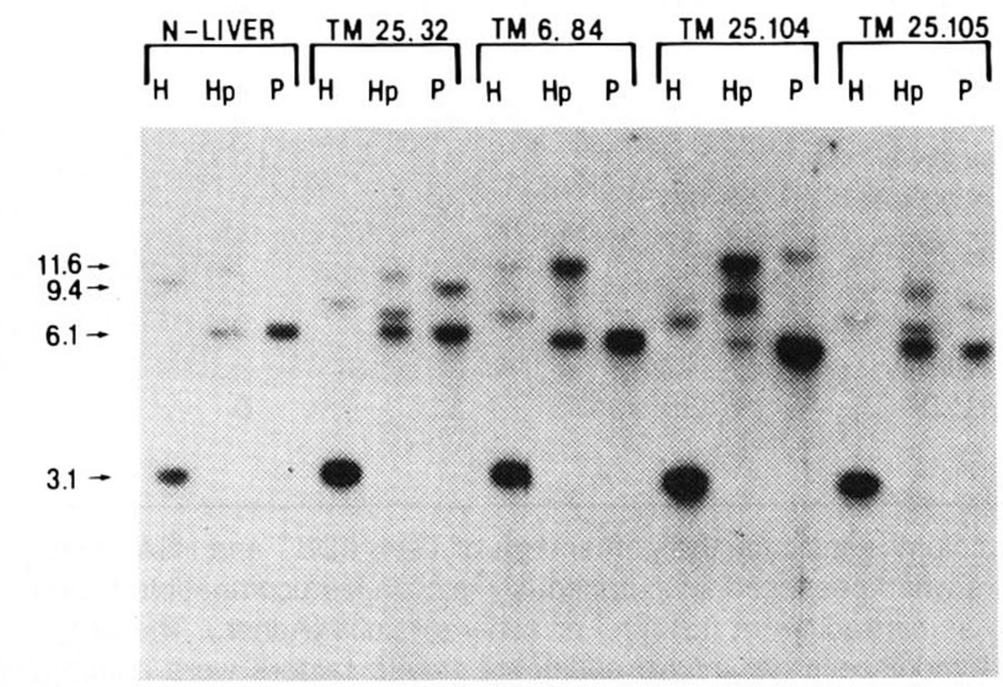

FIG. 3 Samples (10 μg) of DNA extracted from liver or thymus were digested with *Hin*dIII, *Hpa*I or *Pvu*II and separated on a 0.7% agarose gel. After Southern transfer[38], the filter was hybridized with a TCR β constant region probe[39] and labelled with ^{32}P by the mixed primer method[38].

and has caused tumours of thymocyte and thymic epithelial origin. Thus, one of the important aspects of this investigation is the successful establishment of *in vitro* cultures of adherent cells with thymic epithelial phenotypes. In combination with thymocyte cultures from normal or *Thy-myc* transgenic mice, it may be possible to reconstruct *in vitro* a thymic microenvironment in order to study T-cell differentiation. □

Received 19 June; accepted 28 September 1989.

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ACKNOWLEDGEMENTS. We thank H. Land and M. Cole for the gifts of plasmids, Chris Atkins for technical assistance and Cora O'Carroll for secretarial assistance. E.S. was supported by the Greek State Scholarship Foundation. This work was supported by the MRC.

Ionomycin-regulated phosphorylation of the myeloid calcium-binding protein p14

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Two associated calcium-binding proteins (CaBPs) have recently been identified specifically in cells of myeloid origin. These proteins have relative molecular masses (M_r) of 8,000 and 14,000 and are variously referred to as the cystic fibrosis antigen[1], the L1 light chain[2], MRP-8 (ref. 3) or p8, and the L1 heavy chain[2], MRP14 (ref. 3) or p14, respectively. The expression of p8 and p14 seems to be confined to a specific stage of myeloid cell differentiation, because both proteins are expressed in circulating neutrophils and monocytes but not in normal tissue macrophages[4,5]. In chronic inflammatory conditions, however, such as rheumatoid arthritis, macrophages in affected tissues express both p8 and p14 (refs 4, 6, 7). These proteins are members of a family of CaBPs of low M_r (ref. 8), which include S-100 α and β proteins[9,10], calcyclin (2A9)[11], intestinal CaBP[12] and p11 (ref. 13). All the proteins have an M_r of ~10,000 with the exception of p14 which has a longer C-terminal sequence after the second calcium-binding domain. Little is known about their function, although by analogy with calmodulin they could be molecules involved in intracellular signalling that are activated by an increase in the intracellular Ca^{2+} concentration ($[Ca^{2+}]_i$) (ref. 14). Here we report that p14 is phosphorylated in both monocytes and neutrophils. The level of p14 phosphorylation can be increased by elevating the $[Ca^{2+}]_i$ using the ionophore ionomycin, but is not affected by activation of protein kinase C using phorbol 12,13-dibutyrate. The phosphorylated residue is threonine at position 113, which is the penultimate amino acid in p14 and contained in the longer 'tail' sequence. Part of this sequence is identical[15] to the neutrophil immobilizing factors NIF-1 and NIF-2 (ref. 16), indicating that the phosphorylation event could have a role in the generation of NIF activity in the p14 protein.

We have recently described a monoclonal antibody, designated mAb 5.5, that recognizes an epitope present in p8 and p14 (refs 5, 6; J.E. *et al.*, manuscript in preparation). *In vivo* the two proteins are noncovalently associated as a heterodimeric complex, indicating that they function as a dimer (J.E. *et al.*, manuscript in preparation). We have now separated the p14 protein by two-dimensional gel electrophoresis into four distinct isoforms (Fig. 1*a*), all of which react with mAb5.5 after western blotting (Fig. 1*b*). Thus the p14 proteins, differing slightly in apparent M_r, are characteristically present as two abundant proteins with isoelectric points (pI) of 5.4 and 5.6, and two less abundant proteins with pIs of 5.4 and 5.3. By densitometric scanning, the two most abundant forms comprise 80–95% of the p14 molecule. The detection of two principal forms of p14 is consistent with a previous observation that p14 is heterogeneous[17]. Because p14 is a product of a single gene, as is p8 (ref. 8), our findings indicate that some form of post-translational modification occurs, which is thought not to be glycosylation[3]. By contrast, the p8 protein, which was localized by analysis of the purified molecule (J.E. *et al*, manuscript in preparation), runs electrophoretically as a single entity (Fig. 1*a*).

We investigated whether the p8–p14 complex is involved in signal transduction. Stimulation of myeloid cells by various factors causes an elevation of $[Ca^{2+}]_i$ and activation of protein kinase C (PKC), resulting in several specific phosphorylation events believed to be important in the regulation of complex effector functions[19]. We therefore assessed whether the p8–p14 complex is phosphorylated in neutrophils and monocytes after their stimulation with either ionomycin, which increases the $[Ca^{2+}]_i$, or phorbol 12,13-dibutyrate (PdBu), which activates PKC. In the absence of any specific treatment, neutrophils showed a low level of radiolabel in two proteins, corresponding to the two less abundant forms of p14 (Fig. 2*a*). Stimulation with PdBu for 10 min did not affect the level of phosphorylation (Fig. 2*b*) at any dose tested (10–1,000 ng ml^{-1}), or after shorter stimulation periods (1, 3, 5 and 7 min). By contrast, ionomycin treatment caused various increases in the phosphorylation of both p14 proteins up to 10 times the level observed in unstimulated cells (Fig. 2*c*). Similar results were obtained for monocytes, although only a twofold to threefold increase in phosphorylation observed after stimulation with ionomycin (Fig. 2*d*–*f*). The higher level of constitutive monocyte p14 phosphorylation could be due to cell activation caused during the purification procedure. Similar increases in phosphorylation were obtained using the calcium ionophore A23187. Under no circumstances was p8 found to be phosphorylated.

The phosphorylation site(s) in p14 was determined by phosphoamino-acid analysis and identification of a ^{32}P-labelled CNBr cleavage peptide. Purified p14, immunoprecipitated from ionomycin stimulated ^{32}P-labelled neutrophils and monocytes, was hydrolysed for 2 h and the phosphoamino acids were separ-