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Differential regulation of a Thy-1 gene in transgenic mice

(gene regulation/brain/lymphoid tissue)

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ABSTRACT **We have generated Thy-1.1-transgenic Thy-1.2 mice to study the developmental expression of the Thy-1 gene in detail by transcriptional and immunological methods. In brain, the expression of the injected gene was identical to that of the endogenous gene in a tissue- and development-specific manner. In lymphoid tissue, the transferred gene was also expressed correctly in the early phases of T-cell lineage development; however, as the T cells matured, the transcription of the transferred gene, but not the endogenous gene, was suppressed. This result shows that different regulatory elements are used to express the Thy-1 gene in early and late lymphoid development.**

Specific changes in the composition of the cell surface are thought to play a crucial role in development. It is therefore important to understand how the expression of surface molecules is regulated. A good example is Thy-1, a small glycoprotein that appears on a number of cell types at particular stages of development (see ref. 1 for review). It is the simplest well-characterized member of the immunoglobulin superfamily of molecules (2–4). No biological role has yet been demonstrated for Thy-1, although it is capable of modulating intracellular Ca^{2+} levels (5). It appears at high levels on nervous tissue and is a substantial component of the neuronal surface (1). In other tissues, it shows marked differences between species; e.g., in mice Thy-1 is a marker for mature T lymphocytes (6), whereas in humans it is not found on these cells (7).

The Thy-1 gene from mouse (8, 9) and humans (10, 11) has been cloned and completely characterized. The mouse gene contains two promoters with two alternative exons, which are spliced onto the same second exon (Fig. 1). Both promoters are G+C-rich, lack the "TATA box" (8, 12), and are part of a methylation-free "island" (unpublished data). Such islands are characteristic of "housekeeping" genes but have also been found in some tissue-specific genes (13).

The pattern of expression of the Thy-1 gene suggests that different regulatory elements may be involved in its tissue- and development-specific regulation. In this paper we describe Thy-1 expression in transgenic mice and show that the gene is under the control of different regulatory elements in brain and lymphoid tissue.

MATERIALS AND METHODS

Microinjection and Nucleic Acid Analysis. F_2 fertilized eggs of CBA × C57BL/10 parents were injected with ≈2 pl of 10 mM Tris Cl, pH 7.5/0.1 mM EDTA containing DNA at ≈2 μg/ml. The microinjected eggs were transferred into pseudopregnant females (14, 15). Ten days after birth, the pups were analyzed by Southern blot analysis of tail DNA (16). RNA isolation and nuclease S1-protection analysis were carried out as described (15). Transcription assays in nuclei from transgenic (Tg12) brain, thymus, spleen, and liver and normal (CBA × C57BL/10)F_1 thymus were performed according to Linial *et al.* (17).

Immunological Assays. Thy-1.1 was detected by means of monoclonal antibody OX7 (18), and Thy-1.2, by means of monoclonal antibody 30-H12 (19). Radioimmunoassays were done (20) with tissue homogenates (brain, kidney, liver) or suspensions of viable cells (thymocytes), with the same tissues from age-matched A/Thy-1.1 mice (for Thy-1.1) or nontransgenic CBA × C57BL littermates (for Thy-1.2) as controls. In some experiments, thymocytes were divided into two aliquots, to one of which 15% Triton X-100 was added to 1% (wt/vol), and the lysed cells were diluted in 0.1% Triton X-100 in phosphate-buffered saline for assay (21). For immunohistochemistry, acetic acid/alcohol-fixed sections of brain (22) and acetone-fixed cryostat sections of lymphoid tissue were used, with horseradish peroxidase (HRP)-conjugated OX7 F(ab')$_2$ to visualize Thy-1.1 or 30-H12 followed by HRP-conjugated rat immunoglobulin-specific antibodies as a second layer for Thy-1.2. Endogenous peroxidase was blocked by a 30-min preincubation in 0.5% H_2O_2 in methanol. T-lymphocyte subsets were identified by using rat monoclonal antibodies YTS 191 (to L3T4) and YTS 169.4 (to Lyt-2) (23). B lymphocytes were identified using HRP-conjugated rabbit anti-mouse IgG (22). Immunofluorescence-labeled lymphoid cells were counted under a fluorescence microscope, fixed in 1% formalin in phosphate-buffered saline at 4°C, and analyzed on an EPICS-C fluorocytometer (Coulter). For purification of T lymphocytes, a spleen-cell suspension in RPMI 1640 medium was centrifuged over Histopaque-1077 (Sigma), cultured on plastic for 2 hr to remove adherent cells, and then passed down a nylon wool column.

RESULTS

Production and Characterization of Transgenic Mice. The Thy-1 gene (Fig. 1) that we introduced by microinjection into pronuclei of mouse zygotes was "marked" in two ways to enable us to distinguish its nucleic acid and protein products from those of the host. First, we used the Thy-1.1 gene, since the host mouse strain (CBA × C57BL) is Thy-1.2 in type. This allelic difference arises from a single amino acid substitution (4) and allows distinction by monoclonal antibodies. To produce a larger difference recognizable at the nucleotide level without changing the mature protein product (avoiding possible problems from a hybrid protein) a 6-kb *Eco*RI–*Apa* I fragment of the mouse Thy-1.1 gene containing the first four exons (including exons Ia and Ib) and part of exon IV was linked to a 3.1-kb *Apa* I–*Eco*RI human DNA sequence containing the remaining noncoding part of exon IV plus 3′ human flanking sequences.

Four of the 13 offspring (Tg6, Tg9, Tg11, and Tg12) were transgenic as determined by Southern blots; the copy num-

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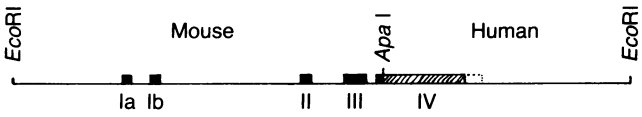

FIG. 1. The mouse Thy-1.1–human Thy-1 recombinant gene. The gene was constructed from the 5′ half of the murine Thy-1.1 gene (to an *Apa* I site in exon IV) and the 3′ half of the human Thy-1 gene. The hybrid gene was injected as a 9.1-kilobase (kb) *Eco*RI fragment.

bers of the transferred gene were 100, 20, 20, and 60, respectively. Of these, Tg6 died early but had the same expression pattern as Tg11 and Tg12 (see below). Tg9 is a slow breeder, and only preliminary results are reported. The experiments are therefore based on mice of the Tg11 and Tg12 lines using, except where otherwise stated, animals that were heterozygotic for the transferred gene.

The Developmental Appearance of Exogenous and Endogenous Thy-1 in the Brain. In Tg12 transgenic mice, the level of endogenous Thy-1.2 antigen and the exogenous Thy-1.1 antigen rose during postnatal development in synchrony and on schedule (24) to the normal adult level by 28 days (Fig. 2). However, by 8 days the level of exogenous Thy-1.1 was 3 times higher than Thy-1.2, and from 28 days, was 7–11 times higher. Three adult homozygous mice showed 12–16 times the normal level.

This developmental rise of both exogenous and endogenous Thy-1 was reflected in the steady-state mRNA levels (Fig. 3) detected in whole brain from 8-day- or 8-week-old animals (at day 0, the mRNA was barely detectable; data not shown). The amount of transferred-gene RNA increased to a level that was 9-fold higher than the level of the endogenous RNA after correction for the 3-fold difference in probe specific activity. In comparison, the level of mouse histone H4 RNA did not increase (Fig. 3 *Inset*).

To examine the cell-type expression, adjacent serial sections of brains of mice ranging from 11 to 84 days old were stained for Thy-1.1 and Thy-1.2. In every case, the pattern of

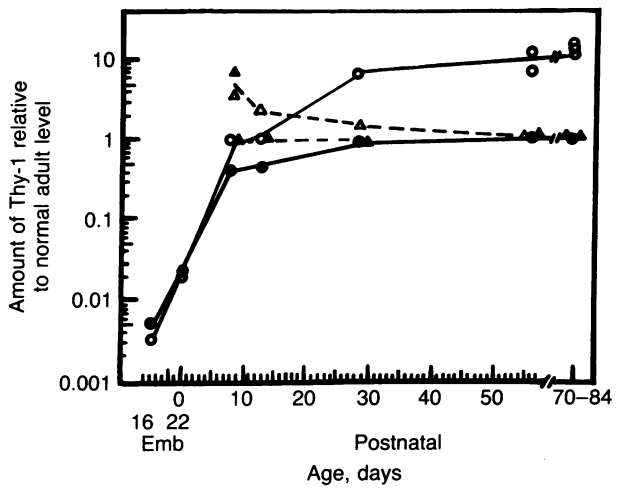

FIG. 2. Relative amount of Thy-1.1 (○, △) and endogenous Thy-1.2 (●, ▲) antigens in the brains (○, ●) and on thymocytes (△, ▲) of Tg12 mice at different embryonic (Emb) and postnatal ages, as measured by radioimmunoassay. For the time points of postnatal day 11 and earlier, the values shown for brain are the mean obtained for individuals in an entire litter (Thy-1.2 values; there was no difference between littermates carrying or lacking the exogenous gene) or those bearing the exogenous gene (Thy-1.1 values). The standard error in each case was in the range of 5–15% of the mean. Points shown for Thy-1.1 values on brain at later ages are for individual animals; the Thy-1.2 level was always identical to that of normal adult mouse brain at these older ages. With thymocytes, points for two individual animals are shown at day 8; later time points are an average.

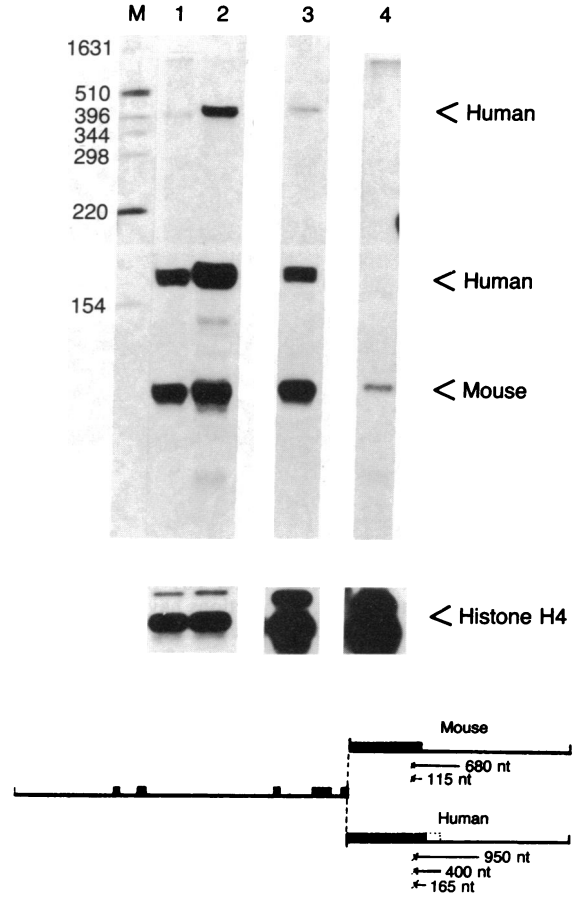

FIG. 3. Steady-state levels of Thy-1 RNA, measured by nuclease S1 protection, in 8-day-old brain (lane 1) and 8-week-old brain (lane 2), thymus (lane 3), and spleen (lane 4) of the transgenic mouse line Tg12. RNA (30 μg) was hybridized at 52°C to a 680-nucleotide (nt) *Bam*HI 3′-end-labeled probe from the 3′ end of the Thy-1.2 gene and a 950-nt *Nco* I–*Bgl* II 3′-end-labeled probe from the 3′ end of the human Thy-1 gene (see diagram below autoradiographs). Normally, the endogenous Thy-1.2 gene produces an mRNA that protects a probe fragment of 115 nt from nuclease S1 digestion. Normal transcription from the mouse–human hybrid gene produces a major mRNA that protects a probe fragment of 165 nt from S1 digestion. A minor band at 400 nt represents a second poly(A)-addition site for the transgenic mRNA. The ratio of specific activity of the mouse Thy-1.2 to that of the human Thy-1 probe was 1:0.3 to avoid problems with overexposure for the endogenous signal. (*Inset*) Control autoradiographic signals obtained from nuclease S1 protection assays of an *Eco*RI–*Tth* III 5′-labeled probe derived from the 5′ end of the mouse histone H4 gene. Lane 4 was exposed longer than the other lanes in order to clearly visualize the spleen Thy-1 RNA (compare the H4 RNA controls). Sizes were estimated from an end-labeled *Hin*fI digest of pBR322 DNA (lane M; lengths of standard fragments given at left in nt).

staining with each antibody was identical, although the anti-Thy-1.1 labeling was considerably more intense. In adult brain, Thy-1.1 immunohistochemistry showed the transgenic molecule to be predominantly on neuronal plasma membranes, although some cytoplasmic staining [of newly synthesized antigen (1)] occurred above the nucleus in large neurons (Fig. 4*a*). Transgenic antigen was not found in the Thy-1-negative axons of the olfactory nerve (Fig. 4*b*) or on non-neuronal cells such as glia or cells of the choroid plexus, vasculature, and meninges. The developmental changes in neuronal Thy-1 expression (1, 25) were also found with the transferred gene. For instance, in the molecular layer of cerebellum in early postnatal life, Thy-1.1 was found on Purkinje neurons and their dendrites but not on the granule

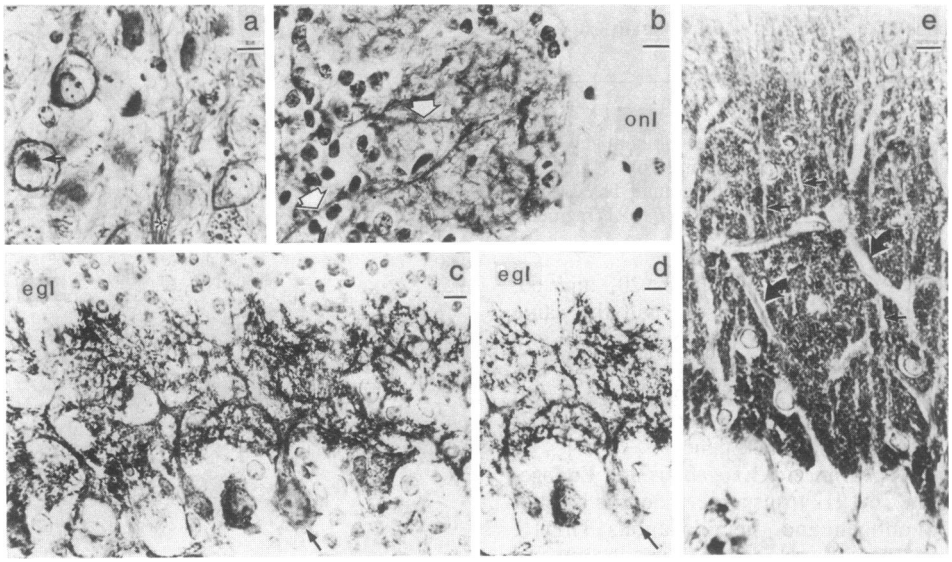

FIG. 4. Immunoperoxidase staining for Thy-1.1 on sections of brain of Tg12 mice. The peroxidase substrate was diaminobenzidine, which gives a brown reaction product, and sections have been counterstained blue with thionin to identify nuclei. (*a*) Heavy immunolabeling of the plasma membrane around three large neurons in the medial nucleus of the trapezoid body of a 28-day-old mouse, with weaker cytoplasmic labeling above one pole of the nucleus (e.g., arrow). Immunolabeled axons can be seen in longitudinal (asterisk) and cross (dots, lower right corner) sections. (*b*) Olfactory bulb, 28-day-old mouse. Two Thy-1.1-labeled dendrites of mitral cells (arrows) branch extensively to form a synaptic glomerulus with the olfactory nerve axons, which occupy the completely Thy-1.1-negative layer (onl, olfactory nerve layer). Thionin-stained nuclei of Thy-1-negative glial cells are in onl. (*c*) Cerebellum, 11-day-old mouse, showing heavy labeling of the Purkinje cells (e.g., arrow) and their dendrites. Above the dendrites are the thionin-stained nuclei of the neuroblasts of the external granule cell layer (egl); these cells migrate to beneath the Purkinje cell bodies (some of these thionin-stained nuclei can be seen), and their axons are at this stage growing between the Purkinje dendrites. (*d*) Same field as part of *c*, photographed using a blue filter to suppress thionin counterstaining to allow absence of immunolabeling of granule cells to be seen. (*e*) Cerebellum, 8-week-old mouse. Three Purkinje cell bodies (bottom) and their dendrites (e.g., curved arrows) lack appreciable antigen, whereas the granule cell axons (cut in cross-section) now stain heavily to provide dense labeling between the Purkinje dendrites. These axons demonstrate the correct (1) gradient in staining intensity, from the more mature (lower) axons to the less mature (upper) ones. The fine vertical Thy-1-negative processes (small arrows) belong to the Bergmann glial cells. (All scale bars = 10 μm.)

cells (interneurons) or their axons (Fig. 4*c*). Later in development, the Purkinje cells lacked detectable Thy-1.1; instead, it appeared on the granule cell axons lying between the Purkinje dendrites (Fig. 4*d*).

Despite a transferred-gene copy number of 20 rather than 60, the brains of adult (56–84 days old) Tg11 mice resembled Tg12 mice in having 7- to 9-fold elevated Thy-1.1 levels, normal Thy-1.2 levels, and a normal cell type distribution of both antigens. Two mice of the Tg9 strain (the same copy number as Tg11) had identical, normal levels of Thy-1.1 and Thy-1.2 in brain. Thus, there was no quantitative correlation between copy number and degree of gene expression, but there was a strict correlation in all tissues between the rate of transcription, the steady-state level of mRNA, and the amount of Thy-1 protein.

Lymphoid Expression of Thy-1. *Thymocytes.* In contrast to the postnatal rise of Thy-1 in brain, the average level of Thy-1 remains constant on thymocytes (3), a phenomenon found with the endogenous Thy-1.2 in the transgenic animals (Fig. 2). However, the level of exogenous Thy-1.1 varied, being higher in younger animals [compare enhanced immunogenicity at this stage (26)] and declining to the normal level by 56 days (Fig. 2). Within the thymus, both antigens showed the appropriate distribution by immunohistochemical analysis (Fig. 5)—i.e., more intense on the majority cortical population than on the medullary thymocytes. Analysis of thymocyte suspensions by immunofluorescence confirmed that essentially all cells carried both Thy-1.1 and Thy-1.2 (data not shown). Thymus suspensions and sections from two adult Tg11 mice showed a level and a distribution of both Thy-1 antigens that were identical to those in the Tg12 mice. In contrast, thymocytes of two Tg9 mice showed normal levels of Thy-1.2 but only 20–25% the normal level of Thy-1.1. Lysis of thymocytes from all strains with detergent prior to assay (21) failed to reveal any additional antigen.

The lower level of Thy-1.1 in adult thymocytes compared to brain was confirmed by the steady-state mRNA levels in

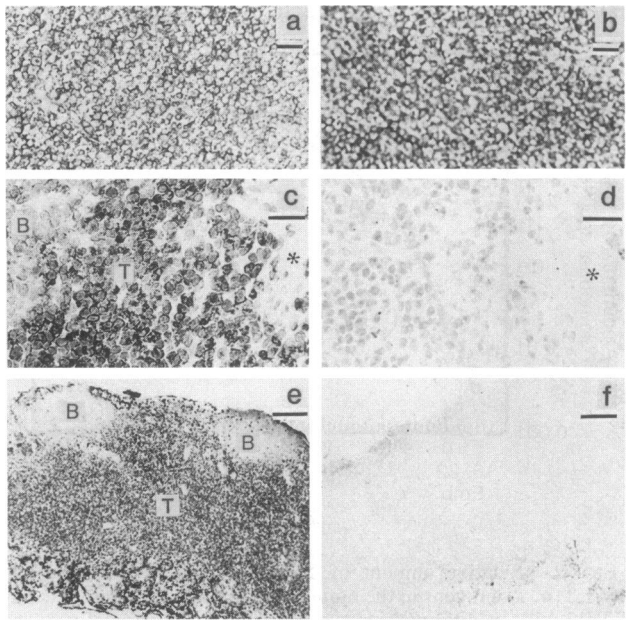

FIG. 5. Immunoperoxidase staining for Thy-1 of adjacent serial sections of thymus (*a* and *b*), spleen (*c* and *d*), and lymph node (*e* and *f*) from an 8-week-old Tg12 mouse labeled with anti-Thy-1.2 (*a*, *c*, and *e*) and anti-Thy-1.1 (*b*, *d*, and *f*) antibodies. Nuclei were counterstained with hematoxylin/eosin. The areas shown of spleen and lymph node span the border between T and B lymphocyte areas (marked T and B), where the T lymphocytes are demonstrated by their reaction with anti-Thy-1.2, but not anti-Thy-1.1, antibodies. Central arteriole is indicated by an asterisk (spleen only). [Scale bars = 20 μm (*a–d*) and 200 μm (*e–f*).]

these two tissues (Fig. 3). Comparable levels of Thy-1.1 and Thy-1.2 RNA were found after correction for probe specific activity. Moreover, the total level of RNA was lower than in the brain when compared to the histone H4 RNA control (*Inset*).

Peripheral T-lymphocytes. Although expression of the exogenous gene on adult thymocytes was reduced compared to brain, on peripheral T lymphocytes expression of transgenic Thy-1.1 was extinguished entirely. Immunofluorescence analysis of spleen cell suspensions showed that in adult Tg12, Tg11, and Tg9 mice, 30–35% of the nucleated spleen cells were Thy-1.2-positive, as in normal littermates. However, essentially no cells (0–4%) were Thy-1.2-positive. As expected, 40–45% of cells (the B lymphocytes) reacted with secondary anti-immunoglobulin antibodies alone.

This was confirmed by immunohistochemistry on sections of spleen and lymph node. Anti-Thy-1.2 antibodies (Fig. 5 *c* and *e*) and anti-immunoglobulin antibodies labeled cells in the T and B areas, respectively. Immunolabeling with antibodies to T-lymphocyte subsets (anti-L3T4, anti-Lyt-2) confirmed the normality of the T-lymphocyte composition in the transgenic mice (data not shown). However, anti-Thy-1.1 antibody failed to stain any cells in these sections (Fig. 5 *d* and *f*). This was also true (data not shown) for two Tg12 mice at 11 days of age, when the thymocyte Thy-1.1 level is still elevated (Fig. 2).

The mRNA levels reflected this absence of Thy-1.1 from spleen cells: mRNA from the endogenous Thy-1.2 gene, but not the Thy-1.1 gene, could be detected (Fig. 3). To assess whether this difference occurred at the level of transcription or RNA stabilization, transcription assays were done on nuclei isolated from several tissues (Fig. 6), and the newly synthesized RNA was hybridized to 3′-end probes specific for the human or murine Thy-1 (see lane F_1 thymus). The results show that the differences between endogenous Thy-1.2 and transgenic Thy-1.1 expression originate from their respective rates of transcription. The ratios between endogenous and transgenic expression in the thymus and brain were the same as observed for the protein or steady-state RNA levels; 8- to 10-fold more radioactive RNA hybridized to the human 3′-end-specific probe than to the mouse 3′-end-specific probe. In contrast, splenic T lymphocytes showed transcription of the endogenous gene but not of the transferred gene. The total level of endogenous Thy-1.2 transcription in the spleen was considerably lower (by a factor of 10) than in the thymus, reflecting the lower percentage of T lymphocytes in spleen (≈30%) and the normal decrease (25%) in surface Thy-1 on peripheral T lymphocytes. Liver, which is at this stage still a hematopoietic tissue, showed a mixed pattern: it contains both early progenitors and late T lymphocytes resulting in a slightly higher level of endogenous expression (early and late cells), compared to the transferred-gene expression (early cells only). By 8 weeks, there was no detectable Thy-1.1 in the liver (data not shown). Intron probes excluded RNA processing as a factor in the observed differences (data not shown).

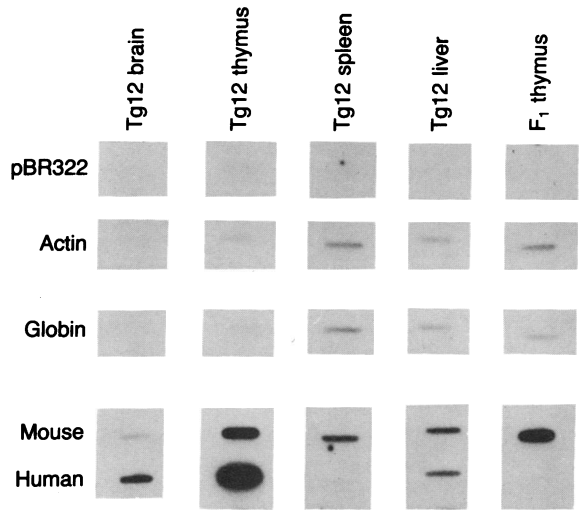

FIG. 6. Estimation of endogenous Thy-1.2 and mouse–human Thy-1 transcription rate by run-on assays in nuclei from tissues of 4-week-old mice (see *Materials and Methods*). Filters for hybridization contained an *Apa* I fragment from the 3′ end of the mouse Thy-1.2 gene; an *Apa* I–*Bgl* II fragment from the 3′ end of the human Thy-1 gene; or, as positive and negative controls, pBR322 sequences (background always negative), globin sequences, and α-actin sequences. Each hybridization was carried out under identical conditions, using 200,000 dpm of each RNA in a 2-ml hybridization volume. Results were quantitated by scanning various exposures of the autoradiograph and were corrected for probe length and base composition.

Finally, the possibility that the Thy-1.1 gene was deleted in peripheral T lymphocytes was investigated. Southern blotting of enriched splenic T lymphocytes from a Tg12 mouse (74% Thy-1.2-positive cells, 8% immunoglobulin-positive, and 0% Thy-1.1-positive by immunofluorescence) showed the continued presence of the Thy-1.1 and Thy-1.2 genes in the T lymphocytes (data not shown). We therefore conclude that the introduced Thy-1.1, but not the endogenous Thy-1.2 gene, has been transcriptionally silenced in peripheral T lymphocytes.

DISCUSSION

The very high expression of the transgenic Thy-1.1 in these brains was the result of enhanced levels of transcription, as evidenced by the mRNA levels and transcriptional run-off experiments. This was probably caused by the high copy number of the injected gene, although there was no strict correlation between copy number and the level of expression. The remarkable feature of the quantitatively enhanced expression of the exogenous gene in brain is the fidelity of developmental, and cell-specific, regulation. This argues strongly that the fragment (Fig. 1) that was used for the injection contains all the regulatory elements necessary for appropriate nervous tissue expression.

In contrast, not all the regulatory elements required in murine lymphocytes are present. In mouse, Thy-1 is found at low levels on some progenitors of thymocytes (21, 27) but reaches maximal levels of expression on cortical thymocytes, where it is present at 10^6 molecules per cell (18) and accounts for about 25% of the cell surface glycoprotein (28). Peripheral T lymphocytes have one-fourth the level of Thy-1 protein (29) and mRNA (Fig. 3) compared to thymocytes, but both remain readily detectable. In humans, Thy-1 appears on lymphoid cells (of both T and B lineage) only at an early phase of their differentiation (30) and is not found on most thymocytes or any peripheral T lymphocytes.

The pattern of lymphoid expression of the transgenic Thy-1.1 has elements of both patterns: the higher expression on thymocytes earlier in development and the extinction of expression at the level of transcription as the cells mature resemble the human situation; the presence on thymocytes follows the mouse pattern. To explain this we suggest two possibilities. First, the human 3′ end of the hybrid gene could contain regulatory elements that might (partially) determine the human pattern of Thy-1 expression in lymphoid tissue. Alternatively, the failure to obtain normal murine lymphoid expression of the transferred gene could be due to the absence of a required murine element from the injected fragment or the replacement of it with human sequences. The recent finding of a site upstream of the *Eco*RI fragment that is specifically hypomethylated only in T-lineage cells lends

indirect support to this second possibility (E.S., unpublished data). Consequently, Thy-1 expression in murine T-lineage cells might be regulated by at least two cis-acting DNA sequences, one (or more) contained within the *Eco*RI fragment and one upstream of this fragment. Expression in the murine thymus might use both these elements to obtain maximal levels of expression. When the cells mature and move to the periphery, only one of the elements (missing from the mouse–human construct) would be used, resulting in the normal drop in the levels of transcription of the endogenous gene. Transcription of the transferred gene would cease altogether because it lacks this element.

The elevated levels of Thy-1.1 on thymocytes in young mice were measured on the cell surface (using viable cells), and the immunohistochemical evidence strongly suggests that in brain also, the 7- to 16-fold elevated Thy-1.1 levels were incorporated on the neuronal surface. Despite this, the mice showed no obvious developmental defects, such as neurological symptoms or abnormalities in the disposition of cells within brain. Thy-1 is thought to play a role in binding on the cell surface (1, 2), in which case the concentration of its natural ligand would be limiting and such a high level of Thy-1 expression would not make any difference to normal neural (or any other) development or function. It should be noted, however, that the line Tg12 does have one physiological abnormality: many mice of this strain develop renal dysfunction in the third month of life. Tg12 mice have a high level of Thy-1.1 antigen on their kidney glomeruli, which might (indirectly) interfere with kidney function. However, Tg11 mice have similarly high levels but do not develop any abnormalities. This raises the possibility that the lethal condition in the Tg12 homozygous strain arises from an insertional mutation.

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