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Tissue-specific control elements of the Thy-1 gene

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We have exploited the structural homology, but different patterns of expression of the murine and human Thy-1 genes to map a number of tissue-specific enhancer elements in the genes. All of these are located downstream from the site of transcriptional initiation. The human gene contains separate elements which direct expression to the kidney or spleen epithelium. The murine gene lacks these elements but instead contains a thymocyte specific enhancer in the third intron. Developmentally-regulated expression in nerve cells is directed (at least in part) by an atypical element in the first intron. The latter is active on heterologous promoters, but is position and distance dependent.

Key words: neuronal element/Thy-1/thymocyte enhancer/tissue specificity

Introduction

The Thy-1 gene is a member of the immunoglobulin superfamily of genes and codes for a 112 amino acid surface glycoprotein that is anchored on the membrane through a phospholipid tail (Williams and Gagnon, 1982; Low and Kincade, 1985; Tse *et al.*, 1985). Certain members of the family are expressed in both the immune and the nervous system (e.g. Thy-1, OX2, CD4) or solely in the nervous system of several eukaryotes (e.g. L1, N-CAM, fasciclin-II, MAG, PO, amalgam) (Dodd and Jessel, 1988; Harrelson and Goodman, 1988; Williams and Barclay, 1988). It was, therefore, proposed that the immune system is evolutionarily related to the nervous system and that Thy-1 possibly represents the primordial domain of the immunoglobulin superfamily (Williams and Gagnon, 1982).

The Thy-1 gene is expressed in a wide variety of tissues during development and the pattern varies considerably between species (for review see Morris, 1985; Cooper and Mansour, 1989). Typically, it appears in mammals on the surface of all neurons except those of the olfactory mucosa (Morris, 1985). Expression in the nervous system is under tight developmental control and represents a terminal differentiation marker (Morris and Grosveld, 1989).

The mouse protein is also expressed in thymocytes (Reif and Allen, 1963), peripheral T cells and, to a lesser extent, in several other tissues (e.g. muscle), but it is not present in kidney. In contrast, human Thy-1 appears in adult human spleen and kidney epithelium but is absent from thymocytes and T cells (McKenzie and Fabre, 1981).

The function of the protein remains to be determined, but its conserved pattern of expression in the nervous system of most species implies a possible role in cell–cell recognition and interaction during morphogenesis (Cohen *et al.*, 1981).

By virtue of its developmental expression in certain tissues, Thy-1 represents an excellent system to study mechanisms that underlie tissue-specific regulation of genes.

The promoter of the Thy-1 gene (Spanopoulou *et al.*, 1988) is located in a CpG-rich island (Bird, 1986) and lacks a TATA box which is characteristic of housekeeping gene promoters (Dynan, 1986). Initiation of transcription occurs from multiple sites and, interestingly, the distribution of these sites is different in murine brain and thymus (Spanopoulou *et al.*, 1988). Previous studies from several laboratories (Chen *et al.*, 1987; Gordon *et al.*, 1987; Kollias *et al.*, 1987a,b) have shown that an 8.2 kb fragment containing the human or murine gene contained sufficient sequences to direct expression of the gene(s) to the appropriate tissues in transgenic mice with the exception of peripheral T cells. Taken together with the presence of a number of DNase I hypersensitive sites downstream from the promoter (Spanopoulou *et al.*, 1988), these data indicated that at least some regulatory regions might be situated within the body of the gene. A number of hybrid and deletion constructs were generated to identify the sequences that dictate tissue specificity in the Thy-1 transcriptional unit. These were analysed in transgenic mice to allow the study of the developmental regulation of the transgenes in several tissues. In this paper we describe the localization of several of the elements in the mouse and human genes that are responsible for the expression of the Thy-1 gene in thymus, kidney, spleen and brain.

Results

Expression of mouse and human Thy-1 transgenes and generation of mouse–human hybrid constructs

As a first step in identifying *cis*-acting regulatory sequences of the Thy-1 gene, we decided to exploit the differences in tissue-specific expression between the murine and human genes by studying the expression of chimaeric mouse–human genes in transgenic mice. As the control we generated transgenic mice carrying the complete mouse and human Thy-1 genes (Figure 1, m and h) to establish their normal expression patterns. We restricted our study (in all constructs) to five tissues, i.e. brain, thymus, kidney, liver and spleen, using S1 protection analysis to assay for transcripts and immunostaining and radioimmunoassay for protein. The latter is possible by exploiting the difference between the transgenic Thy-1 (human Thy-1 or murine Thy-1.1) and the endogenous Thy-1.2 gene. The results obtained (Figures 1 and 2; protein data not shown) are in agreement with the normal patterns of expression of Thy-1

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genes in mouse and man (Morris, 1985), with the exception of the lack of expression of the murine gene in peripheral T cells, as represented by the spleen. This confirmed a previous report on the expression of murine and human Thy-1 genes in transgenic mice (Gordon *et al.*, 1987). Thus, both genes were expressed in brain with the expected developmental appearance on different neuronal cell types. The murine, but not the human gene, was expressed in thymus. The low level of human Thy-1 transcripts in thymus contrasts with its complete absence from isolated thymocytes (data not shown) and may reflect the normal expression of the human gene in thymic stromal cells (Ritter *et al.*, 1981). As expected, only the human gene was expressed in kidney and spleen epithelium (data not shown). The pattern of expression was not altered when the upstream sequences of the murine Thy-1 promoter were deleted to leave only 270 bp (construct 5′ Δ, Figures 1 and 2), indicating that either a short promoter fragment and/or downstream sequences were important for murine Thy-1 expression in the brain or thymus.

We then constructed a number of hybrid genes based on the structural homology of both genes (Giguere *et al.*, 1985; Seki *et al.*, 1985) keeping the transcriptional unit intact.

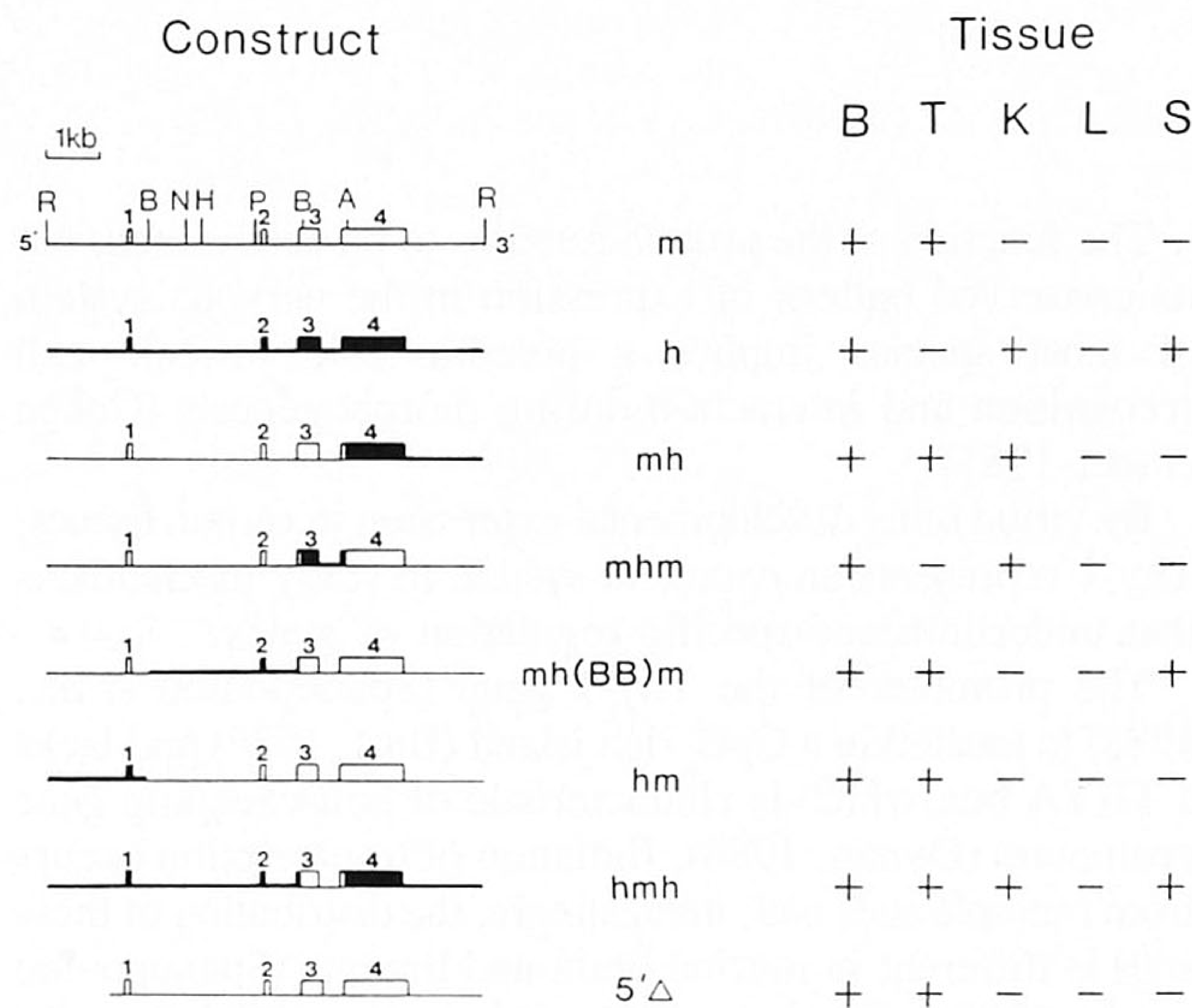

Fig. 1. Summary of murine, human and mouse−human hybrid Thy-1 transgenes and their respective pattern of expression. Open and filled lines indicate the mouse (m) and human (h) genes, respectively. Restriction sites are indicated, *Eco*RI (R), *Bst*EII (B), *Nru*I (N), *Hin*dIII (H), *Pvu*II (P), *Apa*I (A). Replacement of the mouse sequences with the human equivalent, was based on the presence of common restriction sites between the two genes. The mh hybrid contains the 3′ untranslated part of the human gene as an *Apa*I−*Eco*RI 3.3 kb fragment. In the mhm construct the *Bst*EII−*Apa*I 800 bp fragment was replaced by the equivalent human one (980 bp), thus coding for the human protein. mh (BB)m contains a *Bst*EII 2.3 kb human fragment replacing introns 1 and 2 and exon 2 of the mouse gene. Construct hm contains 5′ flanking sequences of the human gene as an *Eco*RI−*Bst*EII 1.6 kb promoter fragment. Hybrid hmh represents the human gene now containing a substitution of the *Bst*EII−*APa*I fragment for the mouse equivalent. Finally in the 5′ construct, promoter sequences were eliminated up to −270 bp from the CAP site (*Sma*I site). All of the constructs were analysed by S1 protection analysis and protein assays in brain (B), thymus (T), kidney (K), liver (L) and spleen (S).

Expression analysis of mouse−human hybrid genes

Each of the four segments corresponding to different hypersensitive sites in the murine gene (Spanopoulou *et al.*, 1988) was substituted by its human counterpart (Figure 1, mh, mhm, mh (BB)m and hm) and in one case the converse construct was also prepared on a human gene (Figure 1, hmh).

Construct mh contains the 3′ end of the human gene, whereas in mhm part of the coding sequences (exons 3 and 4) and intron 3 were replaced with the corresponding human part, generating a molecule which now codes for the human protein. In hybrids mh(BB)m and hm the central part or the 5′ end of the gene were substituted with the human counterpart, respectively. Finally, hmh is the opposite of mhm, i.e. a human gene in which the coding sequences (exons 3 and 4) and intron 3 were replaced with the corresponding mouse sequences.

All hybrid constructs expressed in brain as judged by S1 analysis (Figures 1 and 2) and immunohistochemistry (data

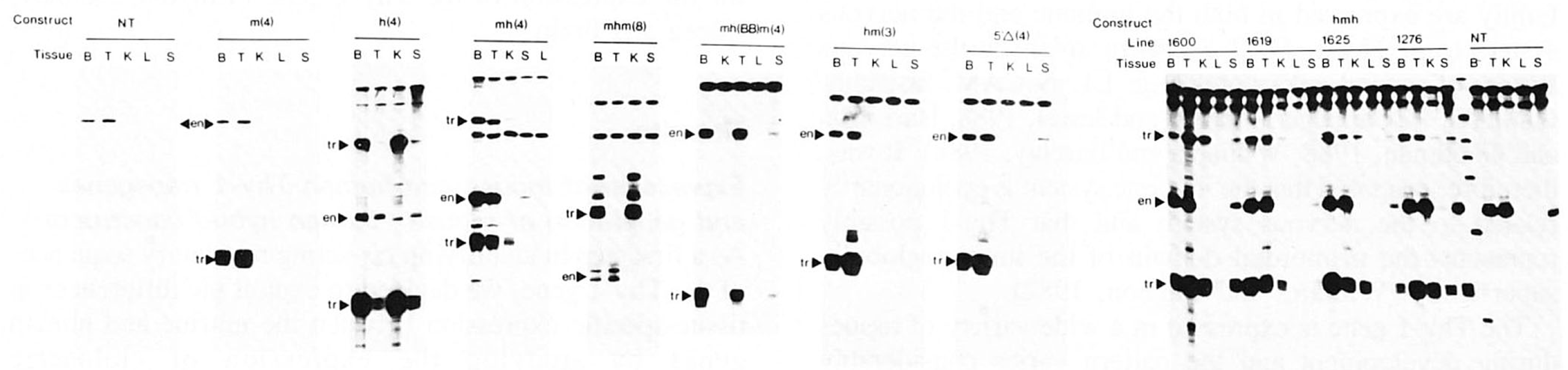

Fig. 2. Steady-state mRNA levels of murine, human and mouse−human Thy-1 transgenes. Constructs are illustrated in Figure 1. RNA was prepared from brain (B), thymus (T), kidney (K), liver (L) or spleen (S), and assayed by S1 protection analysis for endogenous (en) and transgenic (tr) transcripts. One representative example of each construct is shown and the number of independent transgenic lines analysed per construct is indicated in parenthesis above each panel. For hmh each line is shown. S1 probes used were as follows. Constructs m, mh(BB)m, hm and 5′Δ were marked for RNA analysis by deleting a 300 bp fragment from the untranslated region of the last exon (Chen *et al.*, 1987). A 360 bp *Sph*I−*Ava*II probe (derived from the wild-type fragment and cloned in pUC 18), was labelled at the 5′ end (*Ava*II site), thus protecting both the endogenous and a shorter fragment for the exogenous transcripts (360 and 160 bp, respectively). RNA from transgenic lines: h, mh and hmh was hybridized to a 3′ labelled *Nco*I−*Bgl*II 950 bp human probe which detects the poly(A) sites of the foreign mRNA (protected fragments: 165 and 390 bp). The presence of two poly(A) sites in the human gene has been reported previously (Kollias *et al.*, 1987a). mhm transcripts were assayed with a 3′ labelled *Bst*EII−*Apa*I 980 bp human probe detecting the splicing junction between exon 3−intron 3 (protected fragment: 320 bp). Endogenous mouse Thy-1 mRNA was detected by a 5′ end-labelled *Sac*I−*Tth*llII 680 bp fragment which protects part of the fourth exon (protected fragment: 244 bp) except in panel mhm, where a *Pvu*II−*Ava*II 563 bp probe was used to detect the 5′ end of the third exon (231 bp protected fragment). 10−30 μg of total RNA was analysed per reaction. NT are non-transgenic littermates. The bands without a tr or en label and present in all lanes are reannealed input probe signals.

not shown). This indicates that the sequences required for proper expression in the central nervous system are present in the homologous regions of the two genes, since all five combinations of mouse–human sequences are efficiently expressed in neuronal cells. Moreover, in agreement with earlier results (Kollias *et al.*, 1987a,b), the transgenes followed the endogenous developmental pattern of neuronal expression.

Expression in the early thymocytes of transgenic animals was similar between the different hybrids, with the exception of the mhm construct (Figures 1 and 2). In agreement with earlier data (Kollias *et al.*, 1987a) this expression was confined to a decreasing number of thymocytes with increasing age of the animals (not shown). No mRNA or human Thy-1 protein was found in the thymus of mhm mice, consistent with the absence of Thy-1 protein from human thymocytes *in vivo*. Thymus expression was obtained in the opposite construct (hmh) where exon 3–intron 3 were represented by the mouse sequences (Figure 2). These results strongly indicated the presence of thymic regulatory sequences within this region.

Two types of constructs expressed in the mouse kidney (Kollias *et al.*, 1987a,b); constructs that contained human exon 3 and intron 3 resulted in expression in the proximal tubular epithelium (mhm) and it is known (Kollias *et al.*, 1987b; Grosveld and Kollias, 1988) that this element is capable of directing the expression of a heterologous promoter (SV40). In contrast, all hybrid constructs containing the 3′ end of the human gene (e.g. mh, hmh), but not the complete human gene, expressed in the glomeruli (podocytes). This enhancing activity was also independent of the Thy-1 promoter (see below). We therefore conclude that the human gene contains two separate *cis*-acting sequences; one in the third exon/intron responsible for the expression in proximal tubuli in kidney and a second in the 3′ end of the gene, which in combination with the third exon/intron of the murine gene results in a novel expression site in glomeruli.

Interestingly, the element responsible for expression in the tubular epithelium (exon and intron 3) is different from the element required for expression in the spleen connective tissue (Foon *et al.*, 1984). Analysis of the different hybrids [mh, mhm, mh (BB)m, hm and hmh; Figures 1 and 2] showed that this latter activity is located in the 2.7 kb *Bst*EII fragment spanning most of intron 1, exon 2 and intron 2 [construct mh(BB)m]. We have not tested this element on a separate promoter, but presumably this contains a similar Thy-1 promoter independent element as the kidney and the thymus (see below) specific elements. In conclusion, the mouse–human hybrid constructs exhibited a proper pattern of expression in brain; indicated the presence of thymic specific sequences within the region exon 3–intron 3– part of exon 4, and revealed the presence of kidney and spleen specific elements within the human gene. Although all hybrids mirrored the mouse or human pattern of expression, they failed to restore expression in peripheral T lymphocytes.

Because we are mainly interested in the developmental regulation of murine Thy-1 in thymus and brain, we decided to focus on the *cis*-acting elements for these tissues. We therefore constructed a number of deletion mutants placed downstream from the principal transcriptional initiation site of the Thy-1 promoter, or combined Thy-1 sequences with a number of heterologous promoters (Figure 3).

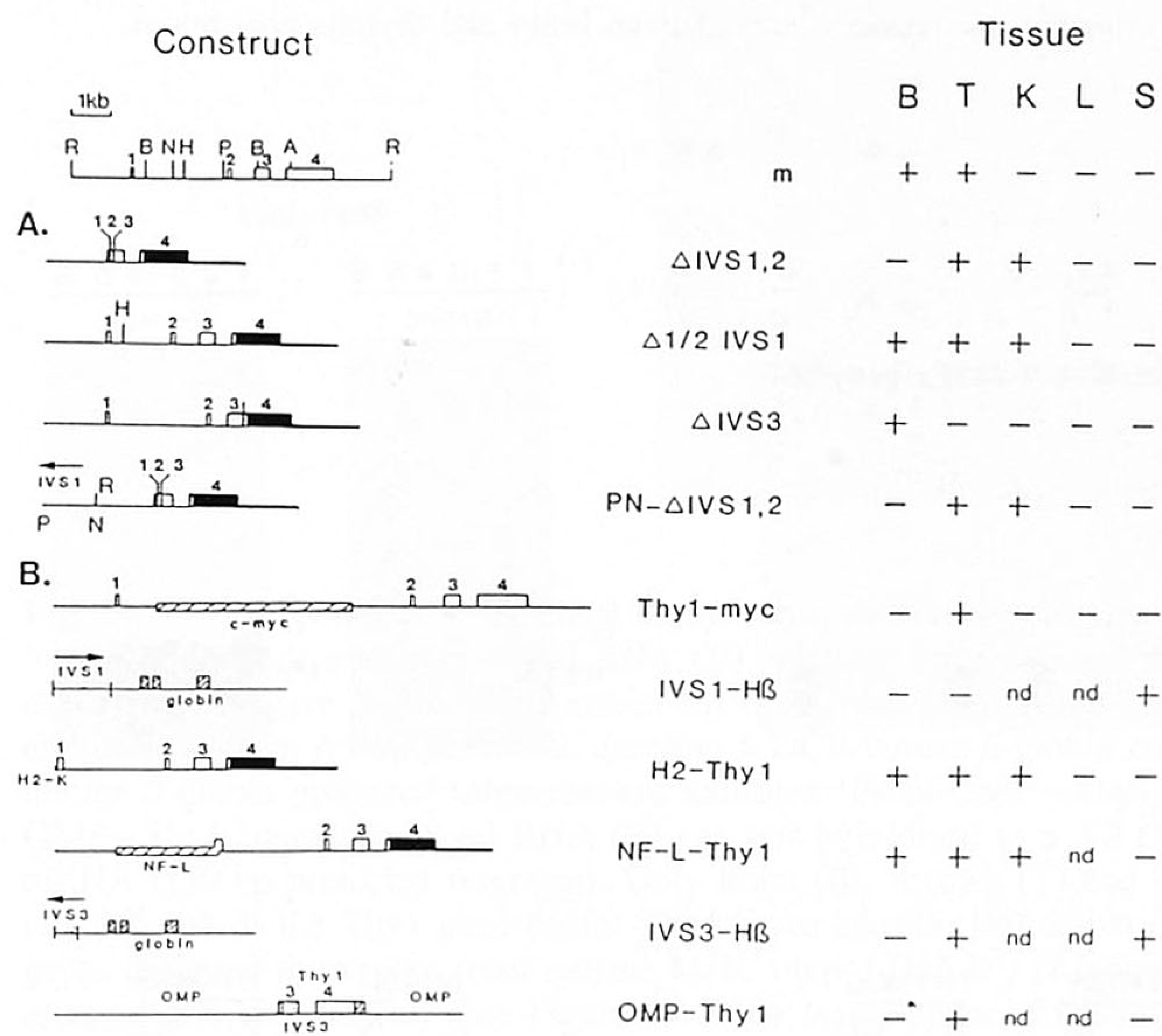

Fig. 3. (**A**) Internal deletions in the murine Thy-1 gene. All of the deletion mutants were based on the previously described mh hybrid (Figure 1). Open and filled lines represent the mouse and human parts, respectively. In ΔIVS1,2 the *Pst*I(exon1)–*Bst*EII (exon3) genomic fragment was replaced by the corresponding cDNA sequences, therefore eliminating introns 1 and 2. Only five out of eight lines are shown. Construct PN–ΔIVS1,2 is identical to ΔIVS1,2 but it now contains part of the first intron (*Nru*I–*Pvu*II, 1.3 kb fragment) at the 5′ end of the gene, immediately upstream of the *Eco*RI site. Δ1/2IVS1 is an internal deletion within the first intron of the mh hybrid where a *Bst*EII–*Hind*III 1.1kb fragment was deleted. ΔIVS3 deletion mutant lacks intron 3 since the *Sac*I–*Apa*I genomic fragment was replaced by the corresponding cDNA sequences. (**B**) Heterologous Thy-1 fusion genes. Hatched boxes indicate foreign sequences. Thy-1–*myc* is a murine Thy-1 gene in which a 4.8 kb genomic fragment of the mouse c-*myc* gene containing exons 2 and 3, was inserted in the *Nru*I site of the first intron (Spanopoulou *et al.*, 1989). In the IVS1–Hβ gene, the second half of mouse Thy-1 intron 1 was cloned as an *Nru*I–*Pvu*II fragment upstream (−700 bp) of the human β-globin gene. H2–Thy-1 construct was based on the mh hybrid in which the 5′ flanking sequences of Thy-1 were replaced by a minimal promoter (100 bp) from the murine H2-K^b gene (Kimura *et al.*, 1986). In NF-L–Thy1, a 2.5 kb fragment containing 5′ flanking and promoter sequences of the human neurofilament light chain gene (NF-L), was inserted in the first exon of the mh hybrid. In IVS3–Hβ, the third intron of the mouse Thy-1 gene was cloned upstream of the human β-globin (−700). Finally, OMP–Thy-1 hybrid represents the rat OMP gene in which coding sequences were replaced by exon 3– intron 3 and part of exon 4 of the mouse Thy-1 gene (Danciger *et al.*, 1989). The (*) indicates expression of the transgene in the olfactory nerve (lane B). As in Figure 1, we analysed brain (B), thymus (T), kidney (K), liver (L) and spleen (S). Non-determined (nd).

Murine Thy-1 brain expression

To verify a previous observation (Chen *et al.*, 1987) and to address the role of introns 1 and 2 in the tissue specific expression of Thy-1, construct ΔIVS1,2 was prepared by substituting genomic sequences with the corresponding cDNA. This deletion of introns 1 and 2 (ΔIVS1,2, Figures 3 and 4) resulted to very low levels of expression in the brain of eight different transgenic lines (only five are shown), while thymocyte and kidney expression was high in these animals. This indicates the presence of a functional regulatory element influencing brain expression in the intron 1 and 2 region of the gene. Deletion of the 5′ half of intron 1 (Δ1/2IVS1, Figures 3 and 4) or deletion of intron 3 (ΔIVS3, Figures 3 and 4) had no effect on brain specific expression. These results are in agreement with those obtained for a small

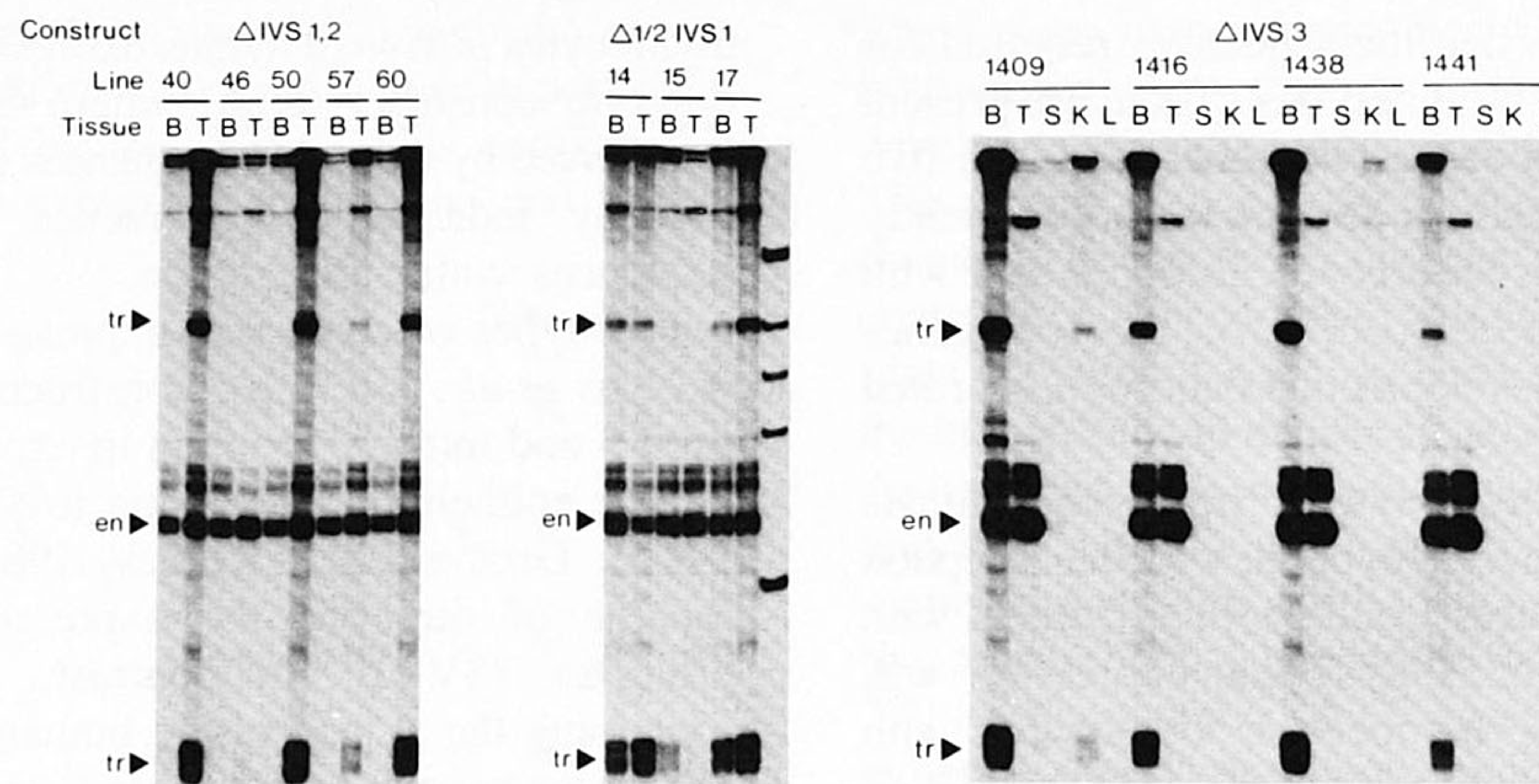

Fig. 4. Steady-state mRNA levels of transgenic lines carrying Thy-1 internal deletion mutants. Total RNA, (30 μg) from each sample was assayed in S1 protection analysis for the presence of transgenic (tr) and endogenous (en) RNA. Trancripts of all three transgenes were probed with an *Nco*I–*Bgl*II 950 bp human fragment described in Figure 2. Only five representative lines are shown for ΔIVS1,2 (out of nine analysed in total). Constructs, ΔIVS1,2 and Δ1/2IVS1 were analysed in all five tissues (Figure 3) but only the results obtained from brain and thymus are shown.

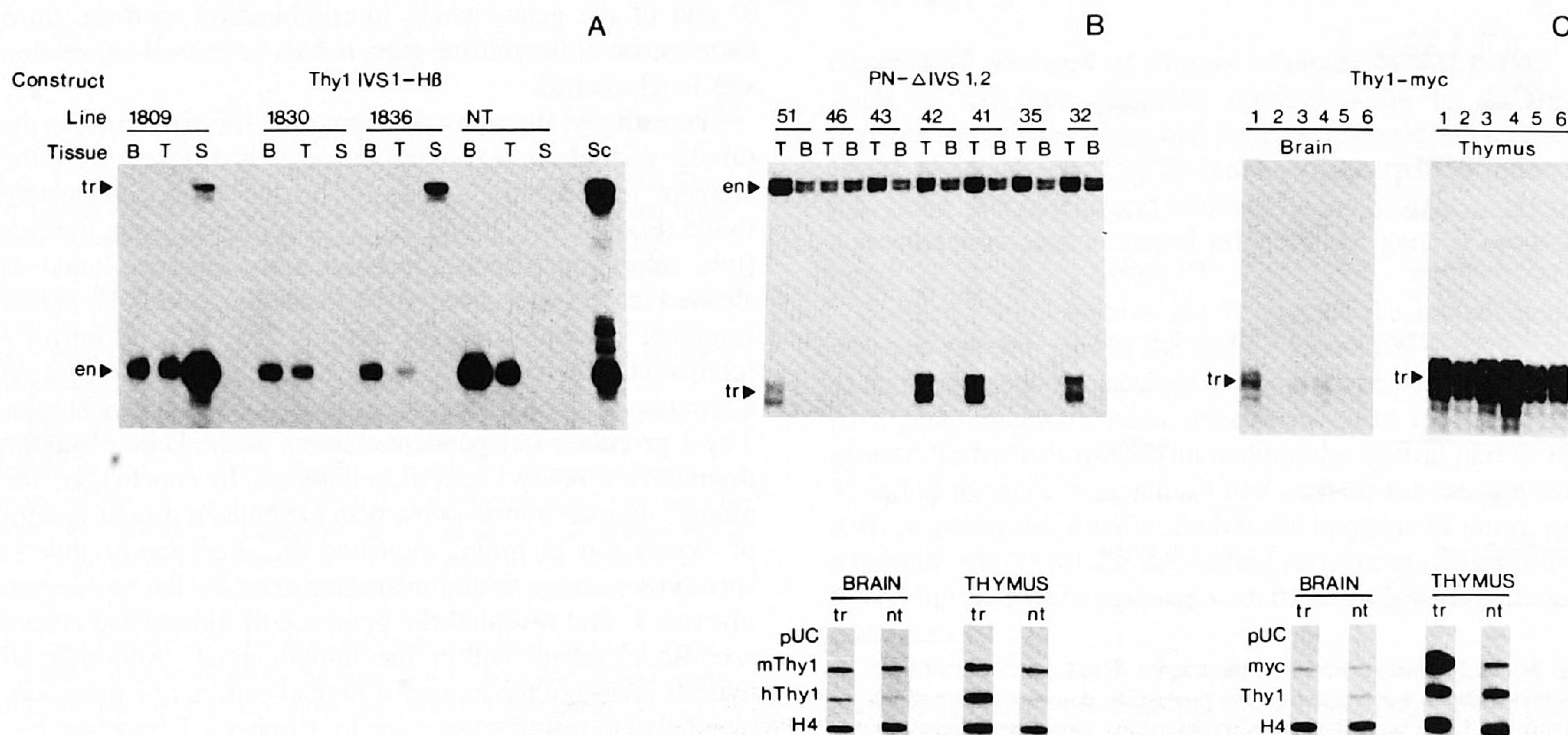

Fig. 5. Transgenic expression in brain. The second half of the first Thy-1 intron (*Nru*I–*Pvu*II 1.3 kb fragment) was tested for brain-specific enhancer activity either in front of the human β-globin gene (**A**) or a Thy-1 deletion (**B**) lacking introns 1 and 2 (ΔIVS1,2). (**A**) Total RNA (30 μg) from brain (B), thymus (T) or spleen (S) was assayed by S1 protection analysis for the presence of endogenous and transgenic β-globin transcripts. Probes were: a 5′ human β-globin fragment (525 bp *Acc*I fragment which covers part of the cDNA and protects 160 bp) and a second exon mouse β maj.-globin fragment (730 bp *Nco*I–*Hin*dIII fragment protecting 100 bp) (Grosveld *et al.*, 1987). Note that splenic RNA from lines 1830 and 1836, and the non-transgenic mice was not probed for endogenous levels. Lane Sc represents 1 μg of splenic RNA derived from transgenic mice carrying a human β-globin gene under the control of the β-globin DCR sequences (Grosveld *et al.*, 1987). (**B**) The upper panel illustrates steady-state RNA levels of the PN–ΔIVS1,2 mutant described in Figure 3. S1 probes detecting the endogenous (en) and transgenic (tr) Thy-1 transcripts are described in Figure 2. Although only seven transgenic lines are shown, 12 were tested in total. The lower panel depicts run-on analysis performed on transgenic line 41. ^{32}P labelled nascent RNA was hybridized to: pUC vector sequences as a negative control, a mouse Thy-1 probe (mThy1) covering most of exon 4 (*Apa*I 1.35 kb fragment) for the endogenous transcripts, a human Thy-1 (hThy1) *Apa*I–*Bgl*II 2.0 kb fragment covering part of the fourth human exon and detecting the transgenic transcripts and finally histone H4 sequences as a positive control. (**C**) The upper panel illustrates steady-state RNA levels of the Thy-1–myc transgene in six different transgenic lines as detected by RNase protection analysis. A *Sac*I–*Pst*I 200 bp fragment from the mouse c-*myc* cDNA covering part of exons 1 and 2 was used as a probe (Spanopoulou *et al.*, 1989). The probe protects 145 bp of the exongenous transcripts (tr). The lower panel shows the results of run-on assays performed on nuclei isolated from the Thy-1–myc transgenic line 6. Probes were: pUC vector sequences, murine *myc* sequences (*myc*) covering exons 2 and 3 which detect both transgenic and endogenous myc transcripts, mouse Thy-1 sequences (Thy-1) from the 3′ untranslated part of the gene (*Apa*I 1.35 kb fragment) and finally histone H4 sequences.

deletion in the 3′ half of IVS1 in one transgenic mouse which showed greatly reduced levels of RNA in brain when compared to the thymus (Chen *et al.*, 1987). We therefore tested directly whether this region can restore brain activity in the mutant Thy-1 and also confer brain specific expression on a heterologous gene, by using the 1.3 kb *Nru*I–*Pvu*II fragment from IVS1 cloned 5′ to a homologous (Thy-1) or heterologous (β-globin) promoter. In both cases (PN-ΔIVS1,2 and IVS1-Hβ, Figures 3 and 5) the transgenic construct failed to express in high levels in brain, while it was active in other tissues, i.e. thymocytes of the Thy-1 construct and spleen for the β-globin construct. To exclude

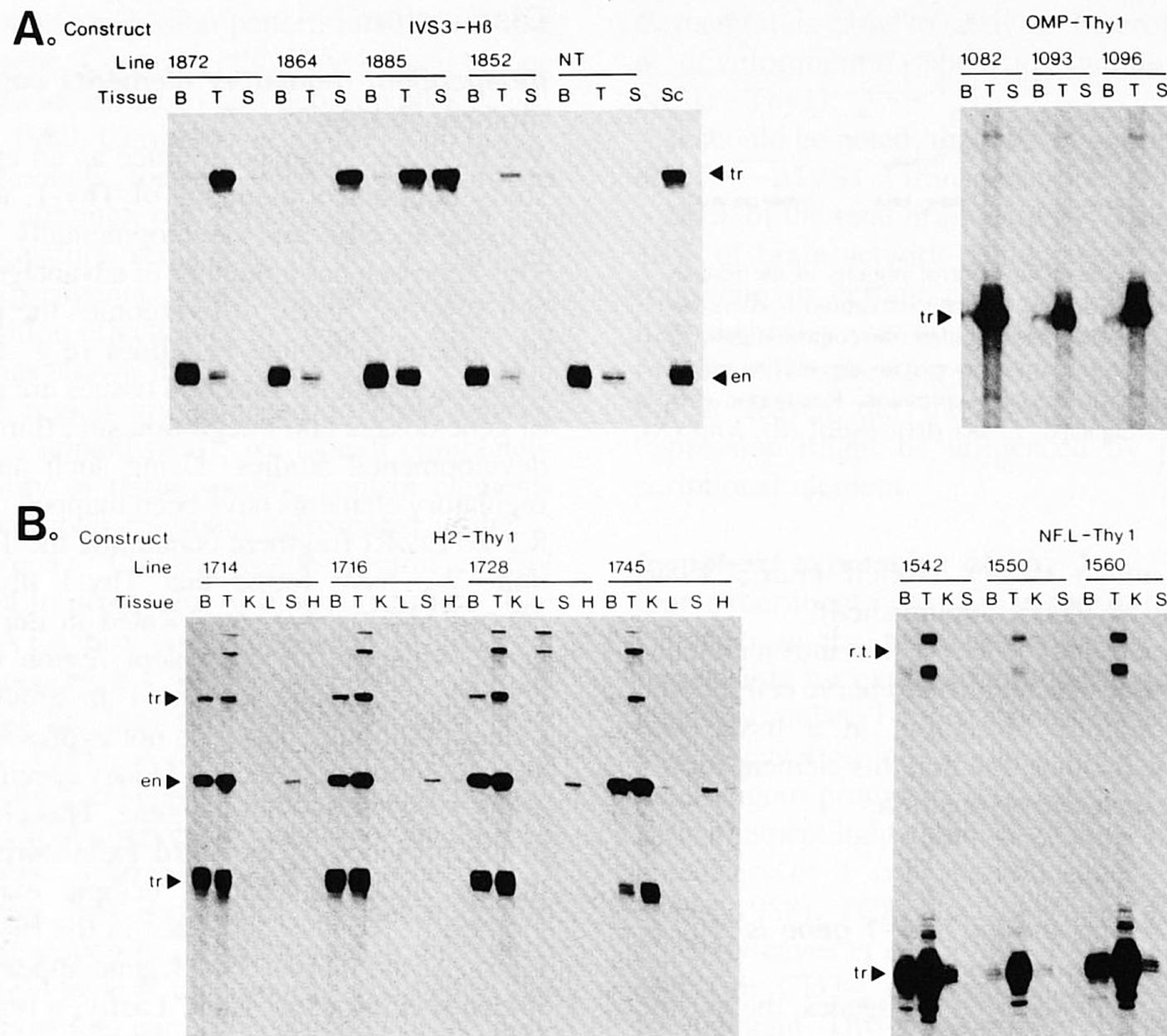

Fig. 6. (**A**) Co-operation of the third intron enhancer with heterologous promoters. Panel IVS3−Hβ shows steady-state mRNA in four transgenic lines carrying that construct. Total RNA (30 μg) from brain (B) and thymus (T) was hybridized to mouse βmaj. and human β-globin probes described in Figure 5. However, spleen (S) RNA was assayed only for transgenic transcripts. Sc is splenic RNA (1 μg) used as a positive control and derived from a transgenic line carrying a DCR/human-β-globin construct (Grosveld *et al.*, 1987). Endogenous (en) mouse and transgenic (tr) human β-globin protected fragments are indicated. Panel OMP−Thy1 illustrates the expression pattern of three different transgenic lines with an OMP−Thy1 transgene. Total RNA (30 μg) was hybridized to a 3.2 kb *Bam*HI−*Eco*RI fragment which detects the poly(A) site of the transgenic mRNA (150 bp protected fragment). Only brain (B), thymus (T) and spleen (S) samples are shown. (**B**) Sequences downstream of the transcription initiaton site of the Thy1 gene confer Thy1 tissue-specific expression on heterologous promoters. Constructs H2−Thy1 and NF-L−Thy1 are fusion genes designed to express from murine MHC class I (H2-K^b) (Kimura *et al.*, 1986) or human neurofilament light chain (NF-L) promoters (Julien *et al.*, 1987), respectively (see Figure 3). RNA was prepared from brain (B), thymus (T), liver (L), spleen (S) and heart (H), and assayed for transcripts by S1 protection analysis. Probes specific for the endogenous (en) and transgenic (Thy1) of the H2−Thy1 construct are described in Figure 2. S1 analysis of NF-L−Thy1 lines detects only transgenic transcripts with a 620 bp *Bss*HI−*Hin*fI probe (a fragment derived from the NF-L−Thy1 construct) specific for initiation of transcription (112 bp protected fragment). r.t. are read-through transcripts. NF-L−Thy1 line 1550 is an established line and therefore heterogeneity in expression cannot be due to mosaicism.

the possibility that the element may at least in part be within intron 2, we also tested a larger 2.1 kb *Pst*I fragment which includes exon 2−intron 2 sequences. However, this construct also failed to express in the brain, in four transgenic lines tested (data not shown). To investigate whether the brain specific sequences would be functional if placed further downstream, we inserted a 4.8 kb fragment from a constitutively expressed gene (c-*myc*) into the *Nru*I site of the first intron of Thy-1 (Thy1−*myc*). This construct which leaves out the *myc* first exon and attenuation sequences (Bentley and Groudine, 1986; Nepveu and Marcu, 1986) results in high expression in the thymus where it causes large thymomas by ~2 months of age (Spanopoulou *et al.*, 1989). However, it fails to express in the brain (Figure 5), even though the c-*myc* was placed within the part of the intron not required for expression (see above Δ1/2IVS1, Figures 1 and 4). This suggests that the brain specific regulation may take place at the level of RNA stabilization and we therefore carried out transcriptional run-on analysis with the constructs Thy1−*myc* or PN−ΔIVS1,2. The results (Figure 5, bottom) showed that, with both constructs, transcriptional activity in brain and thymus was in agreement with the steady state levels of RNA as detected by S1 analysis and RNase protection (Figure 5, top panels). This suggests that the lack of expression in brain by the interruption or deletion of intron 1 is not due to RNA instability, but to the loss of a transcription element.

To test whether brain-specific sequences could be functional in their natural context, the Thy-1 promoter sequences were replaced by heterologous promoters, maintaining the body of the Thy-1 gene intact. In the first construct, 2.5 kb fragment of the neurofilament light chain gene promoter was used to drive expression of Thy-1 (NF-L−Thy1, Figure 3); in the second, the Thy-1 promoter was substituted with a minimal promoter (−100 bp) from the MHC class I H2-K gene (Kimura *et al.*, 1986) (H2−Thy1, Figure 3). Both constructs expressed at high levels in the brain (Figure 6). Moreover, when the NF-L−Thy1 lines were analysed for expression in brain of neonate animals, no transgenic transcripts were detected, thus mimicking the developmental expression pattern of the Thy-1 gene (Kollias *et al.*, 1987a) rather than that of the neurofilament gene

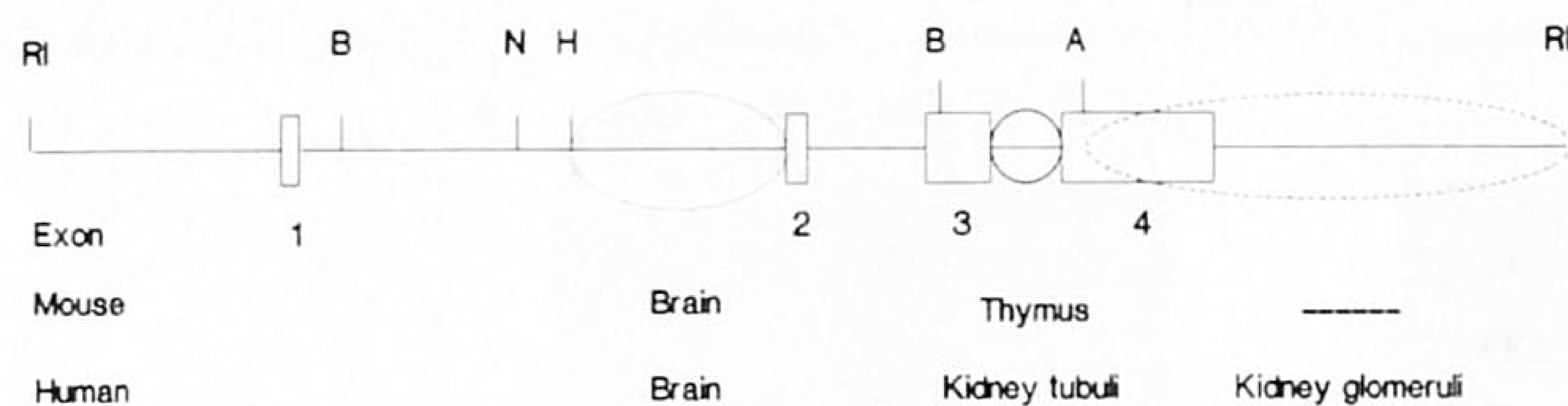

Fig. 7. Schematic representation of the control regions in the mouse and human Thy-1 genes. Dotted lines indicate maximum borders of the different control regions. The kidney glomeruli control region of the human gene is only active in combination with the murine exon 3–intron 3 region and results in ectopic expression. Restriction sites in the murine Thy-1 gene are depicted, *Eco*RI (RI), *Bst*EII (B), *Nru*I (N), *Hin*dIII (H) and *Apa*I (A).

which is activated much earlier (Julien *et al.*, 1987; K.Yazdanbakhsh and M.Vidal, unpublished).

We therefore conclude that the Thy-1 first intron contains at least part of an element which enhances nerve cell specific expression of heterologous promoters in a tissue and developmental specific manner, but that this element is only active in a particular context or position.

Thymic expression of the mouse Thy-1 gene is dependent on intronic sequences

During the analysis of mhm and hmh transgenes, the murine third intron emerged as a strong candidate for a *cis*-acting element in the thymic environment (see Figure 2). This was confirmed by deletion of intron 3 (ΔIVS3, Figures 3 and 4). Four transgenic lines were analysed and found to be negative for expression of the transgene in the thymus in contrast to simultaneous high levels of expression in the brain (Figure 4). Thus, intron 3 might be a 'classical' enhancer element or, alternatively, a *cis*-acting element affecting Thy-1 expression only when located in its natural context. We therefore tested if intron 3 could direct the expression of heterologous promoters to the thymus and if it was independent of its position in the transcriptional unit. Three heterologous promoters were tested in three different transgenic line series, i.e. the NF-L–Thy1 and H2–Thy1 hybrids (see above), as well as an olfactory marker protein (OMP)–Thy1 fusion gene (Figure 3). All three different transgenes were highly expressed in the thymus demonstrating the ability of Thy-1 thymic specific sequences to activate heterologous promoters in the thymus (Figure 6).

These sequences were also able to activate early thymic expression of the Thy1–myc fusion construct, even when placed 5.0 kb further downstream to the Thy-1 promoter (Figure 5, S1 and run-off analysis). To test whether intron 3 could activate transcription of heterologous promoters autonomously (like 'classical' enhancers), IVS3 was cloned in front of the human β-globin gene (IVS3–Hβ construct, Figure 3). With the exception of line 1864, all mice showed human β-globin transcripts in the thymus at levels at least as high as seen in spleen (Figures 3 and 6); the comparison of the levels of endogenous β-globin found in brain and thymus exclude that transgenic globin transcripts in thymus came from contaminating blood. We therefore conclude that expression of the murine Thy-1 gene in thymus is controlled by an element in the third intron with the properties of a classical enhancer.

Discussion

Independent regulatory elements control Thy-1 in multiple tissues

We have used transgenic mice as an expression system to study the control elements of Thy-1, a gene expressed in a tissue-specific and developmentally controlled manner. This approach has a number of advantages over tissue culture cell systems; firstly, it overcomes the problem of the lack of particular suitable cell lines (e.g. neurons); secondly, expression data for different tissues are comparable in terms of gene dosage and integration site; thirdly, it allows proper developmental studies. Using such an approach, several regulatory elements have been mapped (Figure 7) within the 8.2 kb *Eco*RI fragment containing the Thy-1 transcriptional unit. We have found that Thy-1 thymus expression is controlled by sequences located in intron 3 of the murine gene, whereas the equivalent region in the human gene controls expression of Thy-1 in tubular epithelia of the kidney. Although Thy-1 is not expressed *in vivo* in human kidney glomeruli, a second kidney specific element is located at the 3′ end of the human gene. This element is only active in the presence of the third exon/intron sequences of the murine gene and causes ectopic expression in kidney glomeruli. Another sequence in the first and second intron region of the human Thy-1 gene appears to be involved in spleen specific expression. Lastly, a region at the 3′ end of intron 1 is required for expression in brain. All these elements appear to act separately because expression in different tissues can be affected independently (Figures 1 and 2). For example, deletion of introns 1 and 2 abolish brain expression with no effect on thymus expression and, conversely, deletion of intron 3 allows the gene to be expressed in brain but not in thymus. The fact that multiple regulatory elements work independently of each other has also been observed for other 'single' promoter genes,e.g. *Drosophila*, yp1 (Garabedian *et al.*, 1985), *Fushi tarazu* (Ftz) (Hiromi *et al.*, 1985) or *Ultrabithorax* (Ubx) (Bienz *et al.*, 1988), and human apolipropotein A1 (Sastry *et al.*, 1988), rat PEPCK (Benvenisty *et al.*, 1989) or the extensively characterized chicken lysozyme gene (Sippel and Renkawitz, 1989). This type of regulation must be present in many genes and perhaps even ubiquitously expressed (housekeeping) genes are regulated by a number of different enhancer elements. *Cis*-acting elements have also been found to be located downstream of the transcriptional initiation site and within the body of genes. The immunoglobulin genes were the first examples of this class (for review see Atchison, 1988) and an increasing number of genes are now known to possess *cis*-acting elements downstream of their RNA leader sequence, including the Ubx. (Bienz *et al.*, 1988) and yellow locus (Geyer and Corces, 1987) in *Drosophila*. Troponin I (Yutzey *et al.*, 1989), α2(I) collagen (Rossi and de Crombrugghe, 1987), α1(I) collagen (Bornstein *et al.*, 1988), yeast PGK (Mellor *et al.*, 1987), 4F2HC (Gottesdiener *et al.*, 1988), ADA (Aronow *et al.*, 1989) and rat II collagen (Horton *et al.*, 1987) are other examples of RNA pol.II transcribed genes which contain activating regulatory sequences within the body of the gene. In Thy-1 all the control elements appear to be located downstream of the cap site. This is best illustrated by H2–Thy1 (Fig. 3) which is transcribed from a minimal (100 bp) H2–k^b

promoter and shows an expression pattern identical to that of the intact Thy-1 gene. Consequently, Thy-1 could be one of the very rare genes identified so far (Georgopoulos *et al.*, 1988; Basler *et al.*, 1989; Clevers *et al.*, 1989) with tissue-specific control elements located only downstream to promoter elements, although our experiments did not test specifically for regulatory sequences in the 5′ flanking sequences. Such experiments, which are made difficult to interpret due to position effects, are in progress. Although promoter analysis has shown that certain sequences within the −100 to +10 region are required for full activity (Spanopoulou *et al.*, unpublished), we cannot completely dismiss the possibility of tissue-specific control elements within the 270bp promoter fragment represented in the 5′ Δ construct (Figure 1). Clearly, tissue-specific control elements are present in promoters of other genes that, like Thy-1, have no TATA box and are present in GC rich hypomethylated sequences, e.g. the human HMG CoA gene (Luskey, 1987) and the human 4F2 heavy chain gene (Gottesdiener *et al.*, 1988). A different question relates to the suppression of Thy-1 expression in the negative tissues. This could be the result of a repressor function or the absence of activating factors. Elimination of different Thy-1 sequences [a strategy that sometimes has succeeded in identifying repressor sequences, as in the case of the human retinol binding protein gene (Colantuoni *et al.*, 1987)] did not result in a reproducible gain of expression in the non-expressing tissues (liver, spleen and, in the case of H2−Thy1, also heart).

Brain expression

Analysis of the expression of deleted Thy-1 genes showed that the second half of intron 1 is required for expression in brain (this paper and Chen *et al.*, 1987). However, the mechanism by which this element works is unclear. When cloned upstream of a Thy-1 gene lacking that sequence (PN−ΔIVS1,2, Figure 1), or upstream of a β-globin gene (Figure 3), it was inactive in brain. Run-on analysis showed that the very low levels of transcripts are not a consequence of cytoplasmic instability of the RNAs or of the absence of sequences which would stabilize the nuclear hnRNA. The results rather suggest a transcriptional role for that element, similar to an element identified in the first intron of the mouse ribosomal protein gene rp132 (Chung and Perry, 1989), of α1(I) collagen (Bornstein *et al.*, 1988) and also that of the human β-globin gene (Collis *et al.*, 1989). Deletion of these Thy-1 intron sequences has no effect on thymus expression which suggests a tissue-specific role for that element, rather than the general one deduced for introns in the expression of transgenes (Brinster *et al.*, 1988). Furthermore, insertion of a 4.8kb genomic fragment of the c-*myc* gene (Figure 3) between the promoter and the brain element diminishes the expression in the brain but not thymus, indicating that the brain element is very sensitive to distance effects.

An alternative explanation for these results might be that we have discovered an element which is necessary, but not sufficient, for brain expression. (The analysis of our data leaves room for species specific complementary elements only in the 3′ end of the gene). This was shown to be the case for the *Drosophila* dopa decarboxylase gene (Ddc) (Johnson *et al.*, 1989). Whatever the mechanism, the brain element(s) is able to activate heterologous promoters in a developmental specific manner (H2−Thy1 and NF-L−Thy1).

It should be noted, that loss of expression in the ΔIVS1,2 and PN−ΔIVS1,2 transgenic lines (Figure 3) accounts for ~90% of the total brain activity. This implies that the rest 10% of brain activity could reflect the expression of the transgene in certain neuronal subtypes. Since immunohistochemistry was not performed on these lines, the type of cells still expressing the transgene have not been defined, but it leaves the interesting possibility that neuronal expression might be influenced by more than one transcriptional element.

Lymphoid expression of Thy-1

Our experiments clearly demonstrate the presence of an enhancer in the third intron of the murine Thy-1 gene responsible for expression in the thymus. This enhancer is able to stimulate T cell restricted transcription in a distance- and orientation-independent manner and can activate heterologous promoters (H2, olfactory marker protein gene, neurofilament light chain, β-globin). However, unlike other enhancers of T cell specific genes, TCRβ (Krimpenfort *et al.*, 1988), TCRα locus (Winoto and Baltimore, 1989), CD2 (Greaves *et al.*, 1989), CD3-δ (Georgopoulos *et al.*, 1988) or CD3-ε (Clevers *et al.*, 1989), it is insufficient to maintain Thy-1 expression in the final part of the differentiation pathway towards mature T cells. Whereas the endogenous Thy-1 undergoes a down-regulation (~25% on T cells compared to thymocytes), the transgene is only expressed in part of thymocytes in older animals and not at all in peripheral T cells (Kollias *et al.*, 1987a and this paper). This suggests that mature T cells use a different regulatory mechanism to control Thy-1 expression. The simplest model would imply the inactivation or absence of a thymic factor(s) interacting with the enhancer in intron 3 and new (or already present factors) may then bind to a separate (as yet unknown) control element for expression in T cells. The microinjection of a cosmid clone with ~30 kb of 5′ flanking sequences failed to express in splenic T cells (data not shown), which would point to the 3′ end of the gene as a candidate region for the putative Thy-1 T cell element. Work is in progress to identify these sequences and ultimately to understand which factors modulate expression in the differentiation of the lymphoid system.

Materials and methods

Materials

Restriction enzymes, T4 DNA ligase, reverse transcriptase, T4 polynucleotide kinase, proteinase K, S1 nuclease and T7 polymerase, were purchased from Boehringer, New England Biolabs and Anglian Biotechnology. Nitrocellulose filters were from Schleicher & Schuell.

Construction of transgenes

Construction of the mouse−human hybrid series was performed by employing the available common restriction sites to exchange the homologous parts of the two genes. Restriction fragments used are described in Figure 1. The ΔIVS1,2 deletion mutant was prepared by replacing the *Bst*EII 2.7kb genomic fragment of the mh hybrid with a 135 bp *Bst*EII cDNA fragment derived from the corresponding mouse Thy-1 cDNA (Ingraham and Evans, 1986). A *Hin*dIII linker was then introduced in the new construct in the *Pst*I site of the first exon. This second construct was linearized with *Hin*dIII and the *Pst*I and *Bst*EII sites were fused by means of the *Hin*dIII linkers.

In PN−ΔIVS1,2 the *Nru*I−*Pvu*II 1.3 kb fragment derived from mouse Thy-1 first intron was inserted in the *Bam*HI site of the polylinker (in front of the 5′ *Eco*RI site) as a *Bam*HI fragment after the addition of *Bam*HI linkers. Construction strategy followed for the rest of the constructs is described in the corresponding figure legends.

DNA fragment preparation, microinjection and analysis of transgenic animals

DNA fragments were isolated free of vector sequences from a low-melting agarose gel (SeaPlaque) after phenol extraction. Fertilized oocytes from (CBA×C57-BL/10)F1 mice were isolated, and pronuclei were injected as described previously (Kollias *et al.*, 1987a). Transgenic mice were identified by Southern blot analysis of 10 μg of genomic DNA (Kollias *et al.*, 1987a). Tissues from mice were homogenized in 3 M LiCl−6 M Urea and RNA was isolated according to Auffray and Rougeon (1980). Total RNA was analysed by S1 protection analysis or RNase protection assays as described previously (Spanopoulou *et al.*, 1988, 1989). Probes were labelled with either T4 polynucleotide kinase or reverse transcriptase and restriction fragments used as probes are described in the corresponding figure legends. Conditions for run-on transcription reactions were indentical to those described previously (Spanopoulou *et al.*, 1988). However, hybridization was now performed at 42°C for 36 h in a hybridization buffer containing: 50% formamide, 5×SSC, 2×Denhardts, 2 mM sodium phosphate, 0.2% SDS, 250 μg/ml salmon sperm DNA, 10 μg/ml polyA,polyC and 10 μg/ml mouse genomic DNA. Immunohistochemistry, radioimmunoassay and FACS analysis were done as previously described (Kollias *et al.*, 1987a).

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