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Distinct positive and negative elements control the limited hepatocyte and choroid plexus expression of transthyretin in transgenic mice

Chen Yan, Robert H.Costa[1,2], James E.Darnell,Jr[1], Jiandong Chen and Terry A.Van Dyke

Department of Biological Sciences, University of Pittsburgh, Pittsburgh, PA 15260 and [1]The Rockefeller University, New York, NY 10021, USA

[2]Present address: Department of Biological Chemistry, University of Illinois, College of Medicine, Chicago, IL 60612, USA

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Transthyretin (TTR) is a thyroid hormone transport protein that is secreted by hepatocytes into the serum and by the choroid plexus epithelium into the cerebral spinal fluid. The protein is not made elsewhere in adult animals in significant amounts. We find that the start site for mRNA synthesis is the same in both cell types. The sequences required for mouse TTR expression in cultured hepatocytes include an enhancer at −1.86 to −1.96 kbp and a promoter-proximal region at −70 to −200 bp relative to the mRNA cap site. We demonstrate that in transgenic mice these regulatory regions (~300 bp) are sufficient for quantitatively normal expression of a TTR minigene in hepatocytes, but not for restricted expression in the choroid plexus cells of the brain. Instead, they direct aberrant widespread expression in regions of the brain outside the choroid plexus. With 3 kbp of upstream sequence the TTR minigene is expressed specifically in the choroid plexus as well as in the liver, demonstrating the normal cell type specificity for TTR. These results suggest that 3 kbp of upstream sequence contains positive element(s) required for choroid plexus expression which are distinct from those utilized in the hepatocyte, and may also contain negative element(s) that function to suppress transcription in other brain cell types.

Key words: choroid plexus/gene expression/liver/transgenic mice/transthyretin

Introduction

The mechanisms of gene regulation that elicit cell type-specific expression in higher organisms are central to the problem of cellular differentiation. Liver-specific genes have provided a useful model for transcriptional regulation because a large number of genes are expressed specifically and coordinately in hepatocytes and many are regulated by environmental stimuli such as inflammation, trauma and starvation. A subset of these genes is also expressed in a limited number of additional cell types which are unique to each particular gene. For example, the gene for transthyretin (TTR), the subject of the present study, is expressed to high levels in the choroid plexus epithelium in addition to hepatocytes (Soprano *et al.*, 1985; Dickson *et al.*, 1986, 1987; Herbert *et al.*, 1986; Kato *et al.*, 1986; Stauder *et al.*, 1986).

Transient assays in cultured hepatoma cells have identified *cis*-regulatory regions which may play a role in liver-specific expression of a number of genes (e.g. Ott *et al.*, 1984; Costa *et al.*, 1986, 1988a; DeSimone *et al.*, 1987; Godbout *et al.*, 1988; Reue *et al.*, 1988). Some of these genes have been introduced into the mouse germline with the result that liver specificity was maintained. These include α-fetoprotein (Hammer *et al.*, 1987), α_1-antitrypsin (Kelsey *et al.*, 1987), albumin (Pinkert *et al.*, 1987) and α_1-acid glycoprotein (Dente *et al.*, 1988).

In the experiments described in this report we used transgenic mice to determine the ability of TTR regulatory sequences identified in cultured cells to direct cell-specific transcription in the animal. TTR is an abundant serum and cerebral spinal fluid transport protein synthesized primarily by hepatocytes and the choroid plexus epithelium (reviewed by Schreiber, 1987). Mutations in the human TTR gene which result in specific single amino acid substitutions correlate with the hereditary disease, familial amyloidotic polyneuropathy (FAP; for review, see Saraiva *et al.*, 1988). Thus, in addition to investigating the basic mechanisms of TTR regulation in two distinct cell types, *in vivo* experiments may provide insight into this disease and serve as a prelude for the production of an animal model.

Previous analysis of sequences upstream of the mouse gene identified two regions that direct RNA expression in Hep G2 (hepatoma) cells but not in HeLa cells: a promoter-proximal region at −70 to −202 bp relative to the cap site and a 100 bp enhancer element at −1.86 to −1.96 kbp (Costa *et al.*, 1986, 1988b). These DNA segments contain several binding sites for nuclear protein factors present in cells of hepatic origin. Six protein binding sites have been localized to the region between −180 and −60, and four sites to the upstream enhancer region (Costa *et al.*, 1988a, 1989; Grayson *et al.*, 1988). The signals which control TTR expression in the choroid plexus have not previously been investigated.

One objective of the current studies was to test whether the minimal hepatoma-specific DNA elements were sufficient for quantitatively accurate hepatocyte expression in animals. The second objective was to determine whether these regulatory sequences would also function in choroid plexus, since the TTR transcriptional initiation site is the same in both tissues. For this purpose we produced and analyzed transgenic mice carrying recombinant TTR minigenes under the control of either the minimal hepatoma-specific elements or 3 kbp of TTR upstream sequence. Quantitatively normal expression indeed occurred in liver with only the minimal elements. However, brain expression limited to the choroid plexus required the 3 kbp upstream region.

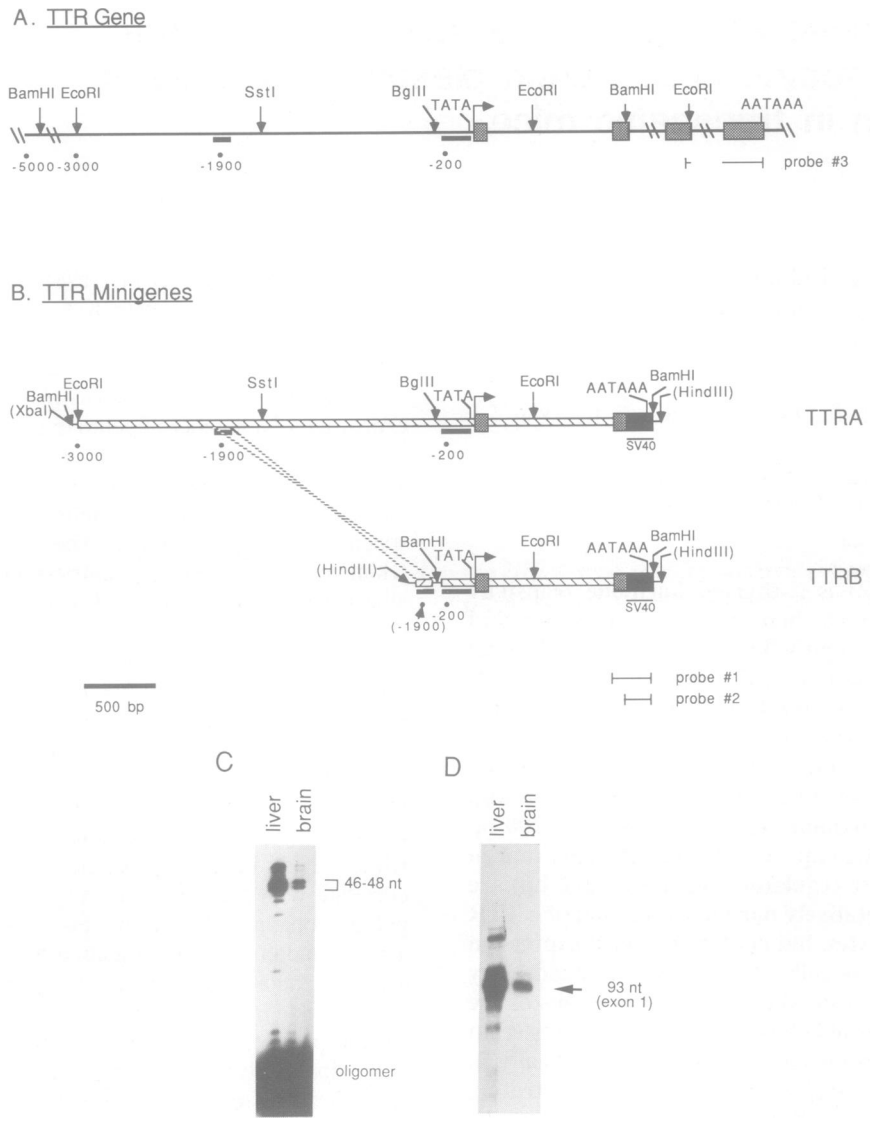

Fig. 1. The mouse transthyretin gene (**A**) and minigene fragments (**B**). TTR exons are represented as stippled boxes on the linear map. SV40 sequences which carry the early polyadenylation signal are indicated by a black box. The RNA start site utilized in both the liver and choroid plexus is indicated by a horizontal arrow. This was determined by primer extension analysis (**C**) and RNase protection analysis (**D**). Black lines below the maps indicate DNA sequences which are important for expression in Hep G2 cells (see text). Numbers refer to the position in base pairs relative to the cap site in the genomic configuration. The extents of homology of three RNA probes to transcripts encoded by these genes are indicated by lines below pertinent maps.

Results

TTR transgenic mouse lines

Transgenic mice were produced using two TTR minigene constructs that differed in the amount of TTR 5′ flanking region which they contained (diagrammed in Figure 1). TTRA contained 3 kb of upstream sequence, while TTRB carried only the minimal enhancer (−1.96 to −1.86) and promoter (cap site to −202) elements shown to be necessary for expression in cultured hepatoma cells. The reporter minigene consisted of two of the four exons of the mouse TTR gene and a portion of SV40 DNA containing the polyadenylation signal (Figure 1 and Costa *et al.*, 1986). We demonstrated that the promoter present in these constructs was indeed used for the endogenous gene in both the brain (choroid plexus) and liver. Using an oligonucleotide complementary to the first exon of TTR, primer extension analysis of poly(A)$^+$ mRNA from each tissue was carried out and yielded the same major 46 and 48 base fragments (Figure 1C). Confirmation that the same start site was used in both tissues was obtained in an RNase protection analysis in which the same 5′ end fragments were protected (Figure 1D).

Eight transgenic founder mice whose pedigrees are shown in Figure 2 were chosen to generate animals for analysis. The presence of intact transgenes in the founder mice was indicated by the appearance of the appropriate *Bam*HI fragment upon Southern hybridization analysis (Figure 3A). An intact fragment from TTRA was 4.2 kbp, from TTRB 1.6 kbp and from the endogenous gene 5.3 kbp. The number of transgenes present in founder animals and their offspring (Table I) was estimated by densitometry of autoradiograms similar to the one shown in Figure 3. TTRB was used as the probe for quantification because all sequences contained in TTRB were also present in the other two fragments. Two founder mice, TA36 and TB94, carried transgenes that segregated in the F1 generation (shown for TB94 in

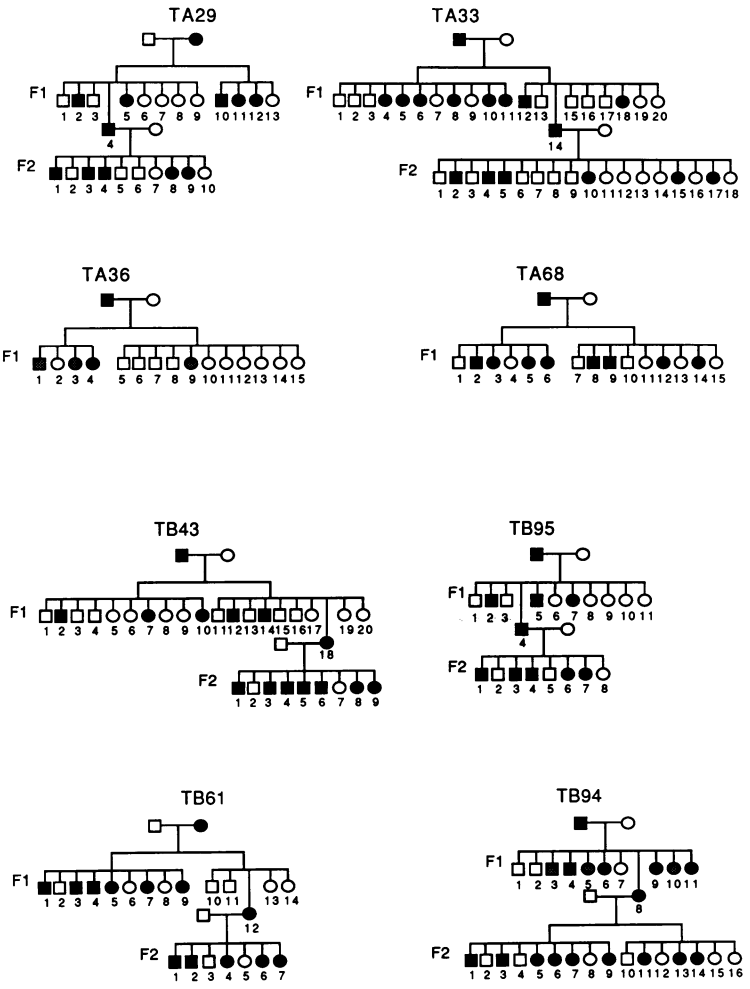

Fig. 2. Early pedigrees of transgenic mouse lines carrying TTR minigenes. Females are indicated by circles, males by squares and transgenic mice by solid or stippled circles and squares. Stippled symbols represent mice carrying lower transgene numbers than their siblings in lines where two transgene integrations segregated (TA36 and TB94). The prefix TA or TB indicates whether the founder mouse carried the TTRA or the TTRB (Figure 1) transgene, respectively. In each cross a TTR^+ transgenic mouse was bred with a non-transgenic B6D2F1 mouse.

Figure 3B) indicating integration on at least two different chromosomes. In addition, TA36 was probably mosaic since the number of transgenes carried by the founder was less than the sum of that carried by two different offspring with the transgene. Non-Mendelian transmission to the TA36 F1 offspring was consistent with this idea (see the pedigrees in Figure 2).

300 bp of 5′-flanking sequence from the mouse TTR gene is sufficient for hepatic expression

Total liver RNA from several mice in each transgenic line was analyzed for the presence of TTR transgene-specific and endogenous transcripts using an RNase protection assay. (The analysis of founder animals was avoided due to the possibility of mosaicism.) In each case a 1 kb anti-sense RNA probe was used which spanned the TTR second exon and SV40 3′ end region (probe 1, Figure 1). RNA from the endogenous gene protected fragments of ~120 bases and RNA from the transgene protected 310 bases. The expected transgenic and endogenous TTR fragments were present in all transgenic liver RNAs examined (Figure 4A and data not shown). RNA from livers of normal animals contained only the fragments at ~120 bases that represent the endogenous TTR RNA. (The heterogeneity in these protected fragments probably represents an artifact of RNase T2 digestion since the use of RNases T1 and A reduced the number of fragments while leaving the 120 nucleotide fragment intact; data not shown.)

By tracing the autoradiograms the amount of RNA from transgenes in hepatic tissue was estimated and compared to the amount of RNA from the endogenous TTR gene in the same sample (see histogram in Figure 4B). These values were corrected for the difference in size between the protected transgenic and endogenous fragments (Materials and methods). Prolonged hybridization was performed using excess amounts of probe to ensure that the majority of the RNA of each species was indeed protected. For some samples with an abundance of transgenic transcript (e.g. mouse TB94-6, Figure 4) it was necessary to repeat the analysis using less sample RNA or more probe before an accurate calculation could be made. Mice in all lines, with the exception of TA68 and TB95, showed levels of TTR transgene expression equal to or greater than that of the endogenous TTR gene (Figure 4B). In lines where hepatic transgene expression was lowest, the line still averaged 45% (TA68) and 35% (TB95), that of the endogenous gene (Figure 4B). Even a few copies of either the TTR-A or B constructs (i.e. 3 kb of upstream sequence or only the promoter and enhancer regions required in hepatoma cells)

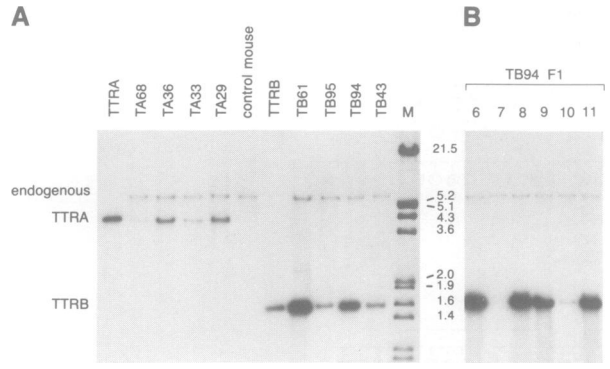

Fig. 3. Southern analysis of TTR transgenes. Liver DNA of founder animals (**A**) or tail DNA of TB94 offspring (**B**) was digested with *Bam*HI and analysed by the method of Southern (1975) using ^{32}P-labeled TTRB fragment as a probe. The numbers at the top of each lane indicate the mouse from which the DNA was isolated. The sizes of *Hin*dIII + *Eco*RI lambda DNA fragments (M) are indicated. The positions of specific fragments from the endogenous TTR gene and the TTRA and TTRB transgenes are also indicated.

Table I. Summary of transgenic mouse lines

Gene	Line	Transgene copy no.[b]			Expression[c] (% endogenous)	
		F_0	F_1		Liver	Brain
TTRA	TA29	6	6		150	33
	TA33	1	1		120	0.6
	TA36[a]	4	1–2	(L)	165	2
			9	(H)	80[d]	20[d]
	TA68	<1	1		45	4
TTRB	TB43	8	8		197	37
	TB95	5	5		35	7
	TB94[a]	33	1	(L)	60	0.7
			31	(H)	285	315
	TB61	38	38		225	405

[a]The founder animal contained transgenes on two segregating chromosomes. Based on transmission frequency TA36 appeared to be mosaic.
[b]The number of transgenes per haploid genome was calculated by Southern hybridization analysis of genomic liver or tail DNA and densitometry. The endogenous TTR signal in the same lane served as the standard (single copy per haploid genome). Numbers shown were rounded to the nearest whole integer.
[c]An average of values shown in Figures 4B and 6B.
[d]Only one mouse was analyzed, not an average.

supported quantitatively normal transcription (see line TA33, TA36-1 and -3 and TB94-3, Figure 4). In some mice with 5-to 20-fold more transgene copies there was at most a 2-to 3-fold increase in mRNA levels. Thus, as is the case with most transgenic mice, there was no absolute correlation between the transgene copy number and the level of transgene expression in the liver (Table I).

Expression in some non-hepatic tissues is affected by transgene copy number

To determine if transgene expression showed the tissue distribution characteristic of normal TTR expression (i.e. mostly in choroid plexus epithelium and liver), we examined the RNA from a variety of tissues. We again carried out RNase protection analyses that distinguished transgenic and endogenous expression in each organ. Shown in Figure 5 is a sample of those experiments which are indicative of the

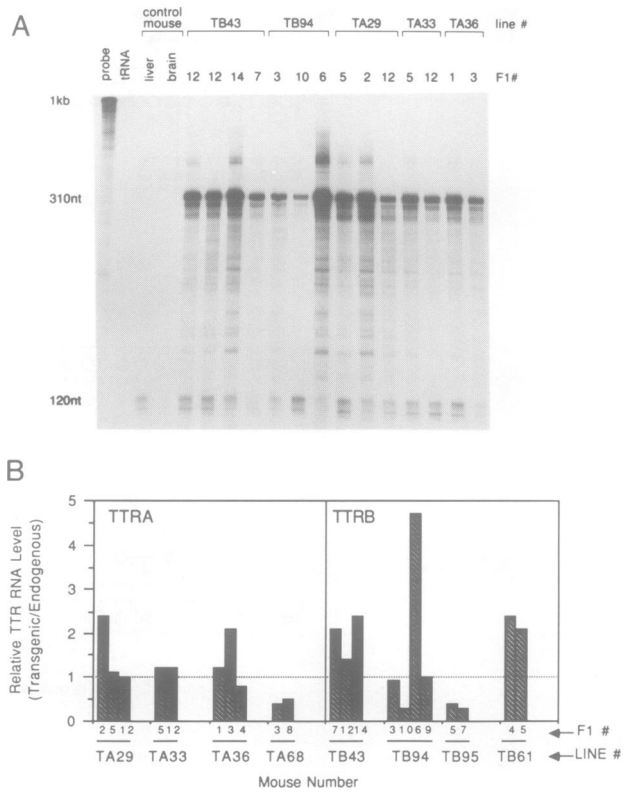

Fig. 4. Hepatic expression of TTR minigenes. (**A**) Total liver RNA (5 μg) from indicated mice and brain RNA (5 μg) from a control mouse was analysed using an RNase T2 protection assay as described in the text. Probe 1 (Figure 1) was used for each sample. The positions of protected fragments from transgenic (310 nt) and endogenous (120 nt) transcripts are indicated. Liver RNA from mouse TB43-1-12 was analyzed in two separate samples, the second of which was incubated with twice the amount of probe to determine if the probe was in excess. (**B**) A histogram shows the amount of transgenic RNA expression in the liver relative to the endogenous level of TTR transcript. Values were determined by densitometric tracing of autoradiograms similar to the one shown at the top as described (Materials and methods). In cases where endogenous fragments were not easily detectable and transgenic fragments were in abundance, the analysis was repeated using increasing quantities of probe before quantitation. The dotted line represents the level of endogenous TTR transcript in the liver of each mouse (1 ×).

overall results we obtained. The endogenous TTR gene was consistently expressed only in liver and brain. (Endogenous brain expression was routinely ~10–20% that observed in the liver.) Mice carrying only 1–2 copies of the transgene, which was sufficient for high level hepatic expression, showed very low, nearly undetectable transgene expression in the brain (Table I, Figure 5A and C, Figure 6). In these animals expression was not apparent in the spleen, salivary gland, intestine, heart, lung, striated muscle and gonads. In some mice with only a few copies of the transgene low expression was detected in the kidney. (Occasional non-transgenic and transgenic mice also showed weak endogenous expression in this tissue.) The same result was observed for TTRA and TTRB (for example, TA36-1-3 and TB94-1-3, Figure 5).

Mice carrying higher numbers of transgenes (six or more per haploid genome) consistently expressed easily detectable levels of transgenic RNA in the brain (Figure 5, TB43-1-7, TB94-1-6; Figure 6 and Table I). Expression in some non-hepatic secretory organs such as kidney (e.g. TB94-1-6),

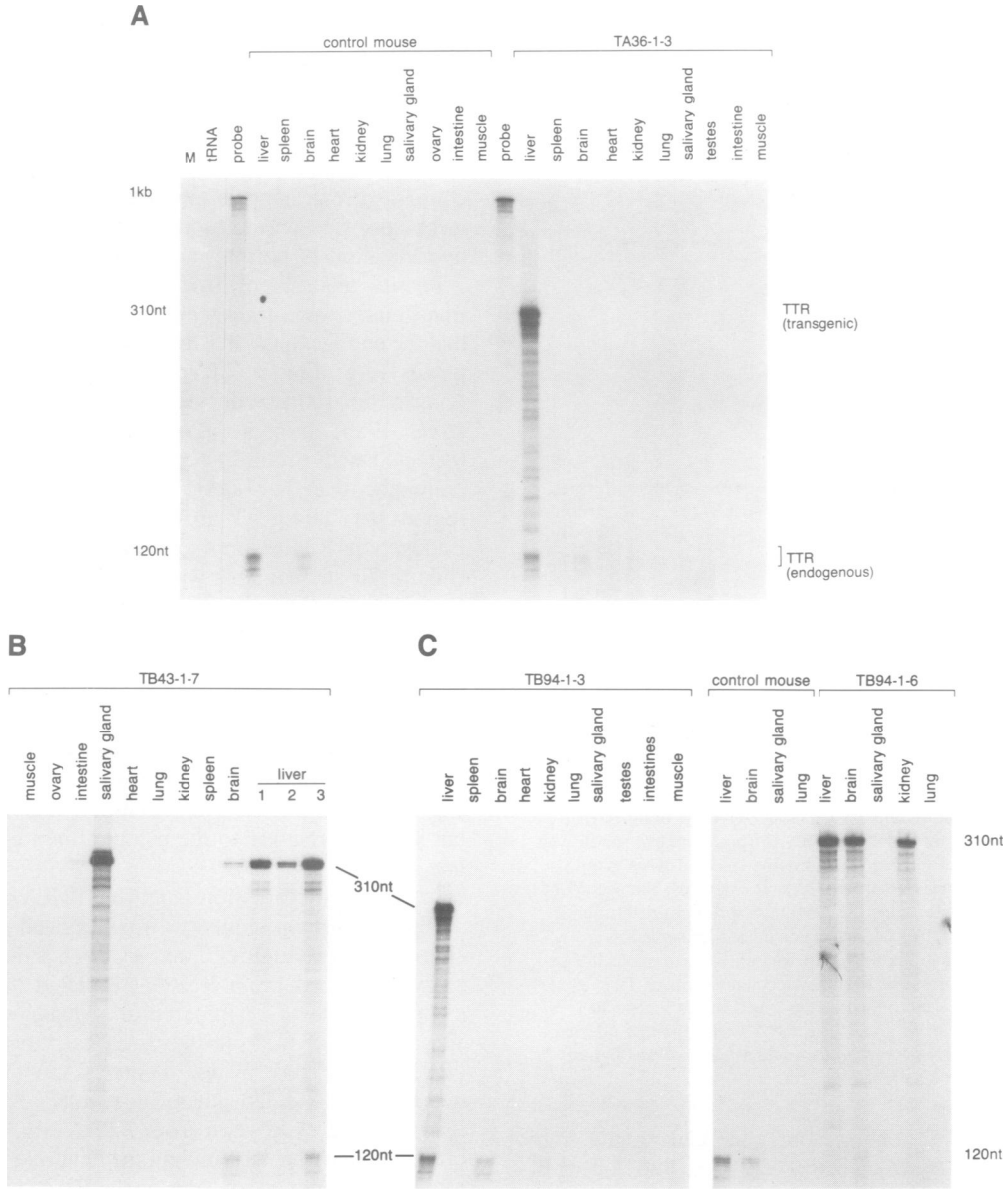

Fig. 5. Tissue distribution of transgene expression. Total RNA (10 μg) from indicated tissues was analysed for the presence of transgenic and endogenous transcripts using a T2 RNase protection assay as described in the legend to Figure 4. Mice shown are as follows: (**A**) control non-transgenic mouse and a TA36 F1 offspring that carried 1–2 copies of the TTRA transgene. (**B**) A TB43 F1 offspring that carried ~8 copies of the TTRB transgene. (**C**) Two TB94 F1 offspring and a control non-transgenic mouse. The TB94 line demonstrated a segregation of transgenes (Figure 2); TB94-1-3 carried 1–2 copies of TTRB, while TB94-1-6 carried ~31 copies.

salivary gland (e.g. TB43-1-7) and intestine (e.g. TB43-1-7) was often also pronounced. The level of brain expression was similar between animals of the same line (discussed further below) while the level and distribution of transgenic RNA among the other non-hepatic organs was sporadic, even within a line (Figure 5B and C and data not shown). Northern blotting analysis indicated that the size of the minigene transcript in all tissues was ~400 bases, consistent with the use of the correct promoter in each case (data not shown). In none of the animals was there significant inhibition of endogenous TTR expression resulting from overexpression of the transgene in liver or brain, as determined by comparing the level of endogenous TTR RNA in the transgenic mice to that present in non-transgenic animals (Figures 4, 5 and 6 and data not shown).

Since TTR is normally expressed specifically in the choroid plexus portion of the brain we measured the level of total brain-specific transgene expression compared to the signal from the endogenous gene as had been done for the liver. In addition we performed *in situ* RNA hybridization on brain sections to determine which cells expressed the transgenic RNA (see below). A representative RNase protection experiment is shown in Figure 6(A) and the results for total brain RNA are summarized in the histogram of Figure 6(B) and in Table I. In general, expression of the transgenic transcript in the brain increased with the presence of increasing numbers of transgenes (Figure 6, summarized in Table I), although RNA levels were not precisely proportional to DNA copy number. Expression in most of the mice (those carrying 1–10 copies of the transgene) was

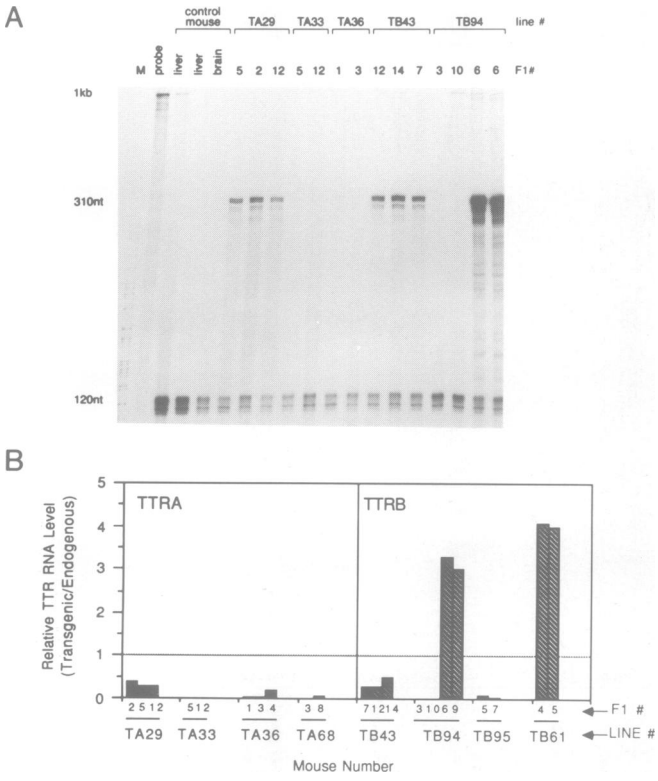

Fig. 6. Brain expression of miniTTR genes. (**A**) Total brain RNA (10 μg) from indicated mice and liver RNA from a control mouse was analysed as described in the legend to Figure 4. Brain RNA from mouse TB94-1-6 was analysed in two separate samples, the second of which was incubated with twice the amount of probe to determine if the probe was in excess. An M13 sequencing ladder served as a marker (M). (**B**) A histogram shows the amount of transgenic RNA expression in the brain relative to the endogenous level of TTR transcript. Values were determined as described in the legend to Figure 4. In cases where the transgenic RNA was difficult to detect, increased amounts of RNA were used in additional analyses before quantification.

lower than that of the endogenous gene and ranged from 0.4 to 50% (Figure 6). Mice in the TB94 and TB61 lineages, which carried >30 copies of transgene TTRB, expressed levels of transgenic RNA in the brain which were 3- to 4-fold higher than the endogenous level (Figure 6). Two lines of mice nicely demonstrated the effect of copy number on expression in the brain. The founder animals TA36 and TB94 contained transgenes on two separate chromosomes which segregated in their F1 offspring (Figure 2). In each case this resulted in independent offspring carrying different numbers of transgenes; either 1−2 or nine copies in offspring from TA36; and one or 31 copies in offspring from TB94 (Figure 3B and Table I). The level of brain-specific transgene expression in these offspring was also higher when more transgene copies were present (Figure 6, compare TA36 F1 nos 1 and 3 with no 4; TB94 F1 nos 3 and 10 with nos 6 and 9). Table I contains a summary of these results.

Choroid plexus specificity is conferred with 3 kbp of 5′-flanking sequence but not with the minimal liver-specific elements

To determine what cells in the brain expressed transgene mRNA, *in situ* RNA hybridization analysis was carried out (Materials and methods). Two different anti-sense RNA probes were utilized for this study. Probe 2 (Figure 1) contained only SV40-specific sequences and thus hybridized to the transgenic TTRA and B transcripts but not to the endogenous TTR RNA. Probe 3 (Figure 1) was specific for the 4th and part of the 3rd exons of the endogenous TTR transcript and did not hybridize with the minigene transcripts. Hybridization of adjacent sections with a TTR sense probe confirmed that signals obtained with probes 2 and 3 were specific. Furthermore probe 2 showed no specific hybridization to normal mouse brain (data not shown).

In situ analysis of frozen brain sections from either transgenic or non-transgenic mice using probe 3 indicated that the endogenous TTR RNA was localized to the choroid plexus (e.g. Figure 7b and c), as has previously been reported for rat (Stauder *et al.*, 1986) and human (Herbert *et al.*, 1986). Expression of the minimal transgene TTRB (detected with probe 2) occurred mainly in regions of the brain other than the choroid plexus. Lateral and 4th ventricle regions of TB43-2-3 are shown in Figure 7d and e. Endogenous TTR expression in the same brain was normal (Figure 7b and c). This was true for all three of the lines examined that carried TTRB regardless of the level of transgene expression in total brain (data for TB94H and TB61 not shown). The major sites of brain expression outside the choroid plexus were consistent in all three lines, indicating that this phenomenon was not line specific (that is, the distribution of abnormal expression was not dictated by the chromosomal integration site). In one line, TB94H, expression in some cells of the choroid plexus was detected, but was low relative to the ectopic brain expression (data not shown).

In contrast, expression from the TTRA transgene (with 3 kbp of upstream sequence) was localized primarily to the choroid plexus (Figure 7f and g), even though quantitative analysis of total brain RNA determined that the level of expression was low compared to endogenous (~33%). *In situ* analysis of mice in two lines carrying TTRA, TA29 (TA29-2-1, Figure 7f and g) and TA36 (TA36-1-4; data not shown), yielded identical results. A low level of inappropriate expression from TTRA was detected in the brain parenchyma surrounding the ventricles (Figure 7f and g), but the amount of transgenic RNA in these regions was significantly lower than that in the choroid plexus. Expression from TTRA was not detected in other ectopic regions of the brain which abundantly expressed the TTRB transgene (compare panel d and f; e and g in Figure 7). Note that the TA29 and TB43 mice compared in Figure 7 carried similar numbers of transgenes and expressed about the same level of transgenic RNA in total brain samples (Table I).

Discussion

Transient transfection assays for cell-specific gene expression in cultured, partially differentiated cells have proven valuable for the initial identification of *cis*-regulatory elements and the cognate proteins which may regulate the genes in the animal (e.g. Banerji *et al.*, 1983; Gillies *et al.*, 1983; Queen and Baltimore, 1983; Ott *et al.*, 1984; Ciliberto *et al.*, 1985; Edlund *et al.*, 1985; Serfling *et al.*, 1985). This approach has been particularly useful in identifying positive acting proteins that are present only in specialized cells. However, sequences involved in cell-specific repression of transcription in many different cell types of an animal may not be revealed by such transfection experiments. Further it is likely

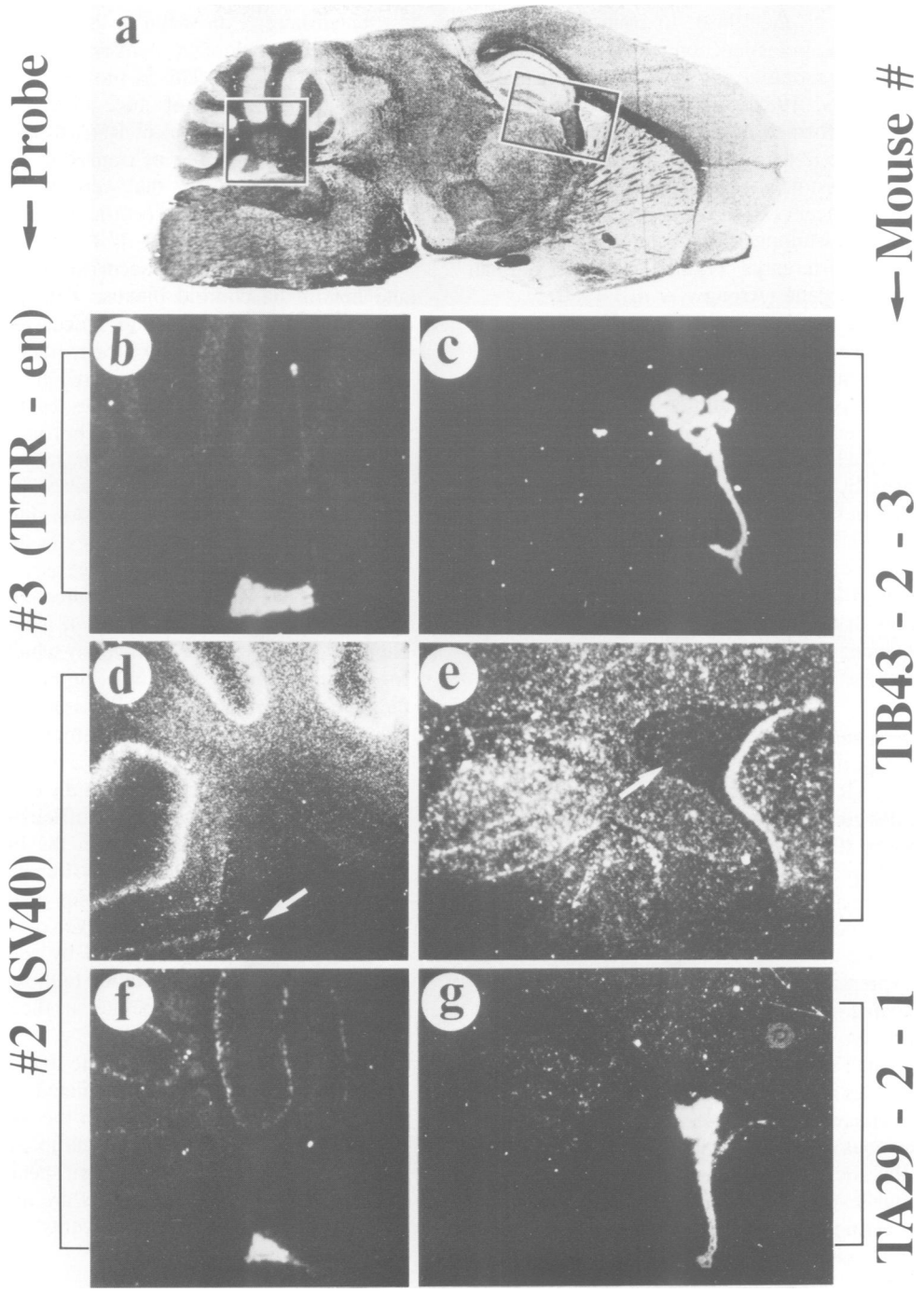

Fig. 7. *In situ* localization of brain-specific transgene expression. The bright field image of a mouse brain stained with hematoxylin and eosin is shown in (**a**) for orientation. The location of regions present in the dark field images of b–g, which include the fourth ventricle choroid plexus (**b**, **d**, **f**) and lateral ventricle choroid plexus (**c**, **e**, **g**), are indicated approximately with boxes in (a). Sections from mouse TB43-2-3 (**b**–**e**) were hybridized either with probe 3 (**b** and **c**; exposure = 17 h) which detected only endogenous TTR transcripts, or with probe 2 (**d** and **e**; exposure 8 days) which detected only transgenic minigene transcripts. Sections from mouse TA29-2-1 (**f** and **g**; exposure 3 days) were also hybridized with probe 2 to detect transgene transcripts. Arrows in **d** and **e** indicate the location of the choroid plexus.

that the regulation of genes in response to a variety of physiological conditions in the body would not be accessible to study in cultured cells. Thus it has been useful to test and extend the results of transfection experiments by germline transformation of animals. A number of cell type-specific regulatory elements have now been confirmed and a number of new elements identified with the use of transgenic fruit flies (reviewed by Spradling, 1986; Rubin, 1988) and mice (reviewed by Palmiter and Brinster, 1986; Jaenisch, 1988).

The mechanism by which a gene is regulated from a single promoter in multiple specific cell types has been most extensively explored in *Drosophila*. In most cases distinct *cis*-acting elements appear to be required for expression in various cell types (Garabedian *et al.*, 1985, 1986; Levis *et*

al., 1985; Pirotta et al., 1985; Scholnick et al., 1986; Beall and Hirsh, 1987; Geyer and Corces, 1987; Hiromi and Gehring, 1987; Goto et al., 1989). In contrast, common *cis*-regulatory elements, in conjunction with a tissue-specific promoter, drive the *Drosophila Adh* gene in multiple tissues (Fischer and Maniatis, 1988). Relatively fewer detailed studies have been performed in higher eukaryotes. *In vivo* analyses of the mouse α-fetoprotein gene indicate that the diversity of its expression is regulated by the differential activity of three enhancers (Hammer et al., 1987; Camper and Tilghman, 1989). Multiple distinct elements also appear to play a role in differential regulation of the human adenosine deaminase gene (Aronow et al., 1989).

Our studies on the regulation of TTR gene expression in transgenic mice have led to three main conclusions. First, the enhancer and promoter-proximal sequences (~300 bp) required for TTR expression in cultured hepatoma cells are indeed sufficient, even in low copy number, to direct normal transcription of a TTR minigene in the liver. Second, these limited sequences by themselves do not facilitate normal regulation of TTR in the brain, but rather induce widespread brain expression. Third, the correct specificity of brain expression, which is limited to cells of the choroid plexus, is conferred with 3 kbp of upstream sequence. Thus the experiments show that positive acting elements, probably in conjunction with negative element(s), operate to facilitate proper TTR expression in the animal.

Hepatocyte expression

Although the reporter minigene in these experiments contained some TTR coding and intron sequences which could theoretically contain liver-specific signals, we believe that the upstream 300 bp is sufficient for two reasons. First the minimal upstream regions are both necessary and sufficient for optimal expression of a reporter gene in cultured hepatoma cells (Costa et al., 1989). Second we have used the same upstream region to drive high level, liver-specific expression of a heterologous gene (creatine kinase) in transgenic mice (Koretsky et al., 1990). Furthermore, Yamamura et al. (1987) found that transgenic mice carrying a human TTR gene which contained only 500 upstream nucleotides and lacked the region of homology to the mouse distal enhancer was expressed in the liver at only 10% the endogenous level. Together with our results this would suggest that the distal enhancer region is required for obtaining optimal levels of transcription in the liver.

The positive acting liver-specific elements in the TTR promoter-proximal and enhancer regions are likely to include some or all of the 10 protein binding sites identified so far by *in vitro* binding assays and transfection assays in cultured hepatoma cells (Costa et al., 1989). Having shown that as little as 300 bp of upstream sequence directs normal hepatic expression, we are now in a position to test the importance of each of these sites in hepatocyte expression *in vivo*.

Choroid plexus expression

Our data clearly show that the liver-specific elements alone are insufficient for expression in the choroid plexus, perhaps because these cells lack the corresponding binding proteins. Consistent with this possibility is our observation that some hepatocyte nuclear factors known to bind in this region (Courtois et al., 1988; Costa et al., 1989) were not detectable in choroid plexus nuclear protein extracts under conditions that detected common activities such as AP1 (Costa, Van Dyke and Darnell, unpublished results). In the experiments presented here, expression in the choroid plexus required one or more *cis* elements contained in minigene TTRA, but absent from TTRB. This is most clearly demonstrated by comparing two lines of mice (TA29 and TB43) which expressed the same level of RNA in the brain and carried about the same number of transgenes, but differed in the TTR regulatory regions that were present. TB43, the line with the minimal liver-specific elements, expressed the transgene in the brain at ~37% the endogenous level but expression was aberrant, occurring in widespread regions and not in the choroid plexus. This was not a result of chromosomal location because it occurred in three independent transgenic lines. In contrast, mice in the TA29 line, which carried the 3 kbp upstream segment, expressed the minigene at ~33% the endogenous level and expression was localized to the correct tissue, choroid plexus.

Thus it appears that this region may contain element(s) that function to repress extensive brain expression while leaving choroid plexus expression intact. Proof that a silencer-like element in fact exists in this stretch of DNA will require further studies, since new brain specificities could also have fortuitously been created by juxtaposing the liver-specific elements in TTRB. It is clear, however, that choroid plexus-specific element(s) which are not required for expression in the liver must lie in this region. Whether they function in the choroid plexus in addition to, or instead of, the liver-specific elements remains to be investigated.

Although we have identified a segment that mediates qualitatively correct expression in the choroid plexus, 3 kbp of 5′ flanking sequence was insufficient to obtain normal levels of minigene expression in the brain. Rather, near normal levels of transgene expression in the choroid plexus were achieved only if many copies of the transgene were present. Thus the appropriate level of TTR expression in the brain must also be regulated by additional signal(s)—either distinct from or in addition to those required for liver expression—that could operate at the transcriptional or post-transcriptional level.

In summary our studies indicate that expression of TTR in two distinct cell types is regulated by multiple distinct *cis*-acting signals that operate on the same transcriptional start site. Within each cell type multiple elements appear to play a role in directing the proper specificity and level of expression. These *in vivo* studies are an important step in understanding the molecular events responsible for the complex regulation of TTR.

Materials and methods

Plasmids and probes

Two pGEM(Promega)-based plasmids, pTTR1 and pTTR2, containing TTR minigenes with different portions of upstream sequence (Costa et al., 1986, 1988a) were used to generate DNA fragments for microinjection. The reporter minigene in each case consisted of the first exon, first intron and most of the second exon (to the *Bam*HI site, Figure 1) of the mouse TTR gene. The second exon was fused to the *Sau*3A−*Bam*HI fragment of SV40 which contains the early region polyadenylation signal, thus creating a hybrid exon. Upstream of the minigene, pTTRA carries 3 kb of mouse TTR DNA sequence 5′ to the cap site and pTTRB contains the segment of TTR enhancer DNA from −1.96 to −1.86 fused, in the reverse orientation relative to the endogenous gene, to the promoter-proximal sequences at −202. The construction of these plasmids has been described (Costa et al., 1986, 1988b). The fragments used for microinjection (TTRA from plasmids pTTR1, and TTRB from plasmid pTTR2) are diagrammed in Figure 1.

Anti-sense RNA probes were generated *in vitro* using phage RNA polymerases and linear plasmid DNA templates according to the method of Melton *et al.* (1984). The extent of homology of three separate probes to TTR transcripts is indicated in Figure 1. Probe 1 (~1 kb long), which was used in RNase protection studies, hybridized to the second exons of endogenous and transgenic TTR RNAs. It was generated by SP6 transcription of *Eco*RI-digested pTTR1. Probe 2, 809 bases long, hybridized only to the SV40-specific sequence in the second exon of the transgenic TTR RNA; it was generated from pSVT using SP6 polymerase. Plasmid pSVT carries the *Stu*I to *Bam*HI early region fragment of SV40 DNA. Probe 2 which was used for *in situ* hybridization was first treated with alkali as described by Ingham *et al.* (1985) to generate fragments of ~300 bases (Cox *et al.*, 1984). Probe 3 (also used for *in situ* hybridization) was 275 bases long and hybridized to the 4th and part of the 3rd exons of endogenous TTR RNA. It did not hybridize to the transgenic TTR minitranscript. A TTR cDNA clone, pTTR8, linearized at the *Eco*RI site in the third exon served as a template for T7 polymerase to generate this probe. The TTRA or TTRB fragment, radiolabeled by the random priming method (Feinberg and Vogelstein, 1983), served as a DNA probe for the analyses of genomic DNA.

Production and screening of transgenic mice

Plasmid pTTR1 was digested with *Xba*I and *Hin*dIII to generate the 4.2 kb TTRA fragment. The TTRB fragment was generated by *Hin*dIII digestion of pTTR2. Both fragments were isolated by electrophoresis in LMP agarose and purified by NACS (BRL) chromatography as previously described (Chen *et al.*, 1989). Before microinjection, DNA fragments were dialyzed against injection buffer [5 mM Tris−HCl (pH 7.4), 5 mM NaCl, 0.1 mM EDTA], using a Millipore VM 0.05 μM filter. The DNA concentration (estimated by comparison to standards of known concentration after ethidium bromide staining of agarose gels) was adjusted with injection buffer to give an approximate concentration of 2 ng/μl. About 2 pl of the DNA solution was microinjected into one of the two pronuclei of fertilized mouse eggs that were obtained from a cross between B6D2F1 (C57B16/J × DBA2, The Jackson Laboratory) parents. Embryos that survived injection were implanted the same day into the oviducts of Swiss Webster (Taconic) pseudopregnant recipients as described by Hogan *et al.* (1986). Transgenic offspring were identified by Southern hybridization (Southern, 1975) of *Bam*HI-digested tail DNA using TTRA as a probe. In subsequent generations the mice were often screened by the polymerase chain reaction (PCR) assay (Saiki *et al.*, 1988) on tail DNA using oligonucleotides specific for the transgenes.

DNA isolation and Southern hybridization

Isolation of tissue DNA was carried out as previously described (Chen *et al.*, 1989). DNA was analyzed for the presence of transgenes by the method of Southern (1975) as described previously (Chen *et al.*, 1989) except that pre-hybridization and hybridization were carried out at 65°C. ^{32}P-Labeled TTRA or TTRB fragment (10^8-10^9 c.p.m./μg, $1-5 \times 10^5$ c.p.m./ml) was used as a probe. End-labeled *Hin*dIII and *Eco*RI fragments of lambda DNA served as size markers.

RNA isolation and analysis

Total cellular RNA was isolated from tissues using the guanidinium/cesium chloride method of Chirgwin *et al.* (1979) as described in Maniatis *et al.* (1982) or by the RNAzolTM method as suggested by the supplier (Cinna/Biotecx, Friendswood, TX). Primer extension analysis and RNase protection analysis to determine the 5′ end of the endogenous TTR transcript in brain and liver was carried out as described previously (Costa *et al.*, 1986). For nuclease protection analysis of transgenic mice, 10 ng ^{32}P-labeled RNA probe 1 ($2-5 \times 10^9$ c.p.m./μg) was co-precipitated in 70% ethanol with 10 μg RNA from various tissues. The RNA pellet was resuspended in 30 μl of hybridization buffer [80% formamide, 40 mM PIPES (pH 6.4), 400 mM NaCl, 1 mM EDTA], heated to 85°C for 10 min, and then incubated overnight at 55°C. Samples were chilled on ice, and 330 μl of a solution containing 50 mM NaOAc (pH 5.0), 100 mM NaCl, 2 mM EDTA, and 10 units of T2 RNAse (BRL) were added. After incubation at 32−33°C for 2 h, 10 μg carrier tRNA was added, the RNA extracted once with phenol/chloroform and then precipitated in 70% ethanol. The pellet was resuspended in 6 μl 98% formamide, 1% bromophenol blue, 1% xylene cyanol, heated at 100°C for 2 min, chilled on ice and electrophoresed in a 5% polyacrylamide gel containing 7 M urea and TBE [0.1 M Tris−HCl, 0.083 M borate (pH 8.3), 0.001 M EDTA]. The M13 DNA sequence ladder which served as a marker was synthesized as described by Sanger *et al.* (1977). The gels were dried and exposed to Kodak XAR5 film for 12−24 h without an intensifying screen.

Autoradiograms were scanned using a microdensitometer (EC Apparatus Corp.). The area within the peak representing the major band of 310 bases along with a faster migrating shoulder, was taken as a measure of TTR RNA derived from the transgene. Two peaks routinely appeared from the bands in the 120 base region of the gel representing the endogenous TTR RNA. They were quantified and summed to obtain a value for this transcript. Before calculating the amount of TTR transgenic RNA relative to endogenous TTR RNA the values were corrected for the 2.6-fold difference in size.

In situ RNA hybridization

Frozen 8 μm sections were thaw-mounted on polylysine-treated slides and air-dried for at least 20 min. The sections were fixed in 4% paraformaldehyde [in phosphate-buffered saline (PBS)] for 20 min, washed in PBS with three changes of buffer and dehydrated with a series of washes of increasing EtOH concentration (from 30 to 100%). The slides were incubated in 0.2 N HCl for 20 min, rinsed in water and incubated in 2 × SSC at 70°C for 30 min. Sections were treated with 125 μg/ml pronase (Calbiochem.) in 50 mM Tris−Cl (pH 7.5), 5 mM EDTA at room temperature for 10 min, incubated briefly with 2 mg/ml glycine in PBS and then washed twice with PBS. Following a second treatment with 4% paraformaldehyde, the sections were incubated for 10 min in 0.1 M triethanolamine (pH 8.0), 0.25% acetic anhydride. They were then washed twice in PBS, dehydrated as described above and air dried.

Hybridizations were performed at 50°C in 10−15 μl of a solution containing 0.15 ng/μl ^{35}S-labeled RNA probe ($2-5 \times 10^9$ c.p.m./μg), 50% formamide, 10% dextran sulfate, 0.3 M NaCl, 0.01 M Tris−Cl (pH 8.0), 5 mM EDTA, 10 mM $NaPO_4$ (pH 6.8), 1 × Denhardt's reagent, 10 mM dithiothreitol (DTT) and 1 mg/ml tRNA. The slides were covered with siliconized coverslips and incubated in moist chambers for 12−16 h. Coverslips were removed and the slides were washed twice for 30 min in 2 × SSC, 10 mM DTT at 55°C, treated with 20 μg/ml RNase A and 1 U/ml RNase T1 in 2 × SSC at 37°C for 30 min, rinsed with 2 × SSC 3 to 5 times, and washed twice with 2 × SSC, 10 mM DTT at 55°C for 30 min. Dehydration with EtOH was repeated. For autoradiography slides were dipped in 50% (v/v) NTB-2 liquid emulsion (Kodak) at 45°C in the dark and placed with desiccant in light-tight boxes for 1−10 days at 4°C. The emulsion was developed as suggested by Kodak using D-19 developer. The sections were stained with 5% (v/v) Giemsa in 10 mM $NaPO_4$ (pH 6.8) and rinsed in dH_2O.

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References

Aronow,B., Lattier,D., Silbiger,R., Dusing,M., Hutton,J., Jones,G., Stock,J., McNeish,J., Potter,S., Witte,D. and Wiginton,D. (1989) *Genes Dev.*, **3**, 1384−1400.

Banerji,J., Olson,L. and Schaffner,W. (1983) *Cell*, **33**, 729−740.

Beall,C.J. and Hirsh,J. (1987) *Genes Dev.*, **1**, 510−520.

Camper,S.A. and Tilghman,S.M. (1989) *Genes Dev.*, **3**, 537−546.

Chen,J., Nielson,K. and Van Dyke,T.A. (1989) *J. Virol.*, **63**, 2204−2214.

Chirgwin,J.M., Przbyla,A.E., MacDonald,R.J. and Rutter,W.J. (1979) *Biochemistry*, **18**, 5294−5299.

Ciliberto,G., Dente,L. and Cortese,R. (1985) *Cell*, **41**, 531−540.

Costa,R.H., Lai,E. and Darnell,J.E., Jr (1986) *Mol. Cell. Biol.*, **6**, 4697−4708.

Costa,R.H., Grayson,D.R., Xanthopoulos,K.G. and Darnell,J.E. (1988a) *Proc. Natl. Acad. Sci. USA*, **85**, 3840−3844.

Costa,R.H., Lai,E., Grayson,D.R. and Darnell,J.E., Jr (1988b) *Mol. Cell. Biol.*, **8**, 81−90.

Costa,R.H., Grayson,D.R. and Darnell,J., Jr (1989) *Mol. Cell. Biol.*, **9**, 1415−1425.

Courtois,G., Baumhueter,S. and Crabtree,G.R. (1988) *Proc. Natl. Acad. Sci. USA*, **85**, 7937−7941.

Cox,K.H., DeLeon,D.V., Angerer,L.M. and Angerer,R.C. (1984) *Dev. Biol.*, **101**, 485−502.

Dente,L., Ruther,U., Tripodi,M., Wagner,E.F. and Cortese,R. (1988) *Genes Dev.*, **2**, 259−266.

DeSimone,V., Ciliberto,G., Hardon,E., Paonessa,G. and Palla,F. (1987) *EMBO J.*, **6**, 2759−2766.

Dickson,P.W., Aldred,A.R., Marley,P.D., Banniseter,D. and Schreiber,G. (1986) *J. Biol. Chem.*, **261**, 3475−3478.

Dickson,P.W., Aldred,A.R., Menting,J.G.T., Marley,P., Sawyer,W.H.

and Schreiber,G. (1987) *J. Biol. Chem.*, **262**, 13907–13915.
Edlund,T., Walker,M.D., Barr,P.J. and Rutter,W.J. (1985) *Science*, **230**, 912–916.
Feinberg,A.P. and Vogelstein,B. (1983) *Anal. Biochem.*, **132**, 6–13; amended (1984) *Anal. Biochem.*, **137**, 266–267.
Fischer,J.A. and Maniatis,T. (1988) Cell, **53**, 451–461.
Garabedian,M.J., Hung,M.-C. and Wensink,P.C. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 1396–1400.
Garabedian,M.J., Shepherd,B.M. and Wensink,P.C. (1986) *Cell*, **45**, 859–867.
Geyer,P.K. and Corces,V.G. (1987) *Genes Dev.*, **1**, 996–1004.
Gillies,S.D., Morrison,S.L., Oi,V.T. and Tonegawa,S. (1983) *Cell*, **33**, 717–728.
Godbout,R., Ingram,R. and Tilghman,S.M. (1988) *Mol. Cell. Biol.*, **8**, 1169–1178.
Goto,T., Macdonald,P. and Maniatis,T. (1989) *Cell*, **57**, 413–422.
Grayson,D.R., Costa,R.H., Xanthopoulos,K.G. and Darnell,J.E., Jr (1988) *Science*, **239**, 786–788.
Hammer,R.E., Krumlauf,R., Camper,S.A., Brinster,R.L. and Tilghman,S.M. (1987) *Science*, **235**, 53–58.
Herbert,J., Wilcox,J.N., Pham,K.-T.C., Fremeau,R.T., Zeviani,M., Dwork,A., Soprano,D.R., Makover,A., Goodman,D.S., Zimmerman,E.A., Roberts,J.L. and Schon,E.A. (1986) *Neurology*, **36**, 900–911.
Hiromi,Y. and Gehring,W.J. (1987) *Cell*, **50**, 963–974.
Hogan,B., Costantini,F. and Lacy,E. (1986) *Manipulating the Mouse Embryo: A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Ingham,P.W., Howard,K.R. and Ish-Horowicz,D. (1985) *Nature*, **318**, 439–445.
Jaenisch,R. (1988) *Science*, **240**, 1468–1474.
Kato,M., Soprano,D.R., Makover,A., Kato,K., Herbert,J. and Goodman,D.S. (1986) *Differentiation*, **31**, 228–235.
Kelsey,G.D., Povey,S., Bygrave,A.E. and Lovell-Badge,R.H. (1987) *Genes Dev.*, **1**, 161–171.
Koretsky,A.P., Brosnan,M.J., Chen,L., Chen,J., and T.A.Van Dyke (1990) *Proc. Natl. Acad. Sci. USA*, in press.
Levis,R., Hazelrigg,T. and Rubin,G.M. (1985) *EMBO J.*, **4**, 3489–3499.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual.* Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
Melton,D.A., Krieg,P.A., Rebagliati,M.R., Maniatis,T., Zinn,K. and Green,M.R. (1984) *Nucleic Acids Res.*, **12**, 7035–7056.
Otto,M.O., Sperling,L., Herbomel,P., Yaniv,M. and Weiss,M.C. (1984) *EMBO J.*, **3**, 2505–2510.
Palmiter,.D. and Brinster,R.L. (1986) *Annu. Rev. Genet.*, **20**, 465–499.
Pinkert,C.A., Ornitz,D.M., Brinster,R.L. and Palmiter,R.D. (1987) *Genes Dev.*, **1**, 268–276.
Pirrotta,V., Steller,H. and Bozzetti,M.P. (1985) *EMBO J.*, **4**, 3501–3508.
Queen,C. and Baltimore,D. (1983) *Cell*, **33**, 741–748.
Reue,K., Leff,T. and Breslow,J.L. (1988) *J. Biol. Chem.*, **263**, 6857–6864.
Rubin,G.M. (1988) *Science*, **240**, 1453–1459.
Saiki,R.K., Gelfand,D.H., Stoffel,S., Scharf,S.J., Hiiguchi,R., Horn,G.T., Mullis,K.B. and Erlich,H.A. (1988) *Science*, **239**, 487–491.
Sanger,F., Nicklen,S. and Coulson,A. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.
Saraiva,M.J.M., Costa,P.P. and Goodman,D.S. (1988) In DiDonato,S. *et al.* (eds), *Advances in Neurology, Vol. 48: Molecular Genetics of Neurological and Neuromuscular Disease.* Raven Press, NY, pp. 189–200.
Scholnick,S.B., Bray,S.J., Morgan,B.A., McCormick,C.A. and Hirsh,J. (1986) *Science*, **234**, 998–1002.
Schreiber,G. (1987) In Putnam,F.W. (ed.), *The Plasma Proteins, Structure, Function and Genetic Control.* Academic Press, NY, Vol. 5, pp. 293–363.
Serfling,E., Jasin,M. and Schaffner,W. (1985) *Trends Genet.*, **1**, 224–230.
Soprano,D.R., Herbert,J., Soprano,K.J., Schon,E.A. and Goodman,D.S. (1985) *J. Biol. Chem.*, **260**, 11793–11798.
Southern,E. (1975) *J. Mol. Biol.*, **98**, 503–517.
Spradling,A.C. (1986) In Roberts,D.B. (ed.), *Drosophila—A Practical Approach.* IRL Press, Oxford and Washington, DC, pp. .....
Stauder,A.J., Dickson,P.W., Aldred,A.R., Schreiber,G., Mendelsohn,F.A.O. and Hudson,P. (1986) *J. Histochem. Cytochem.*, **34**, 949–952.
Yamamura,S., Kenich,S., Wakasugi,S., Maeda,S., Inomoto,T., Iwanaga,T., Uehira,M., Araki,K., Miyazaki,J. and Shimada,K. (1987) *Dev. Genet.*, **8**, 195–205.

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