

## Anti-Thy-1 antibody responses evoked by Thy-1 antigen expressed in transfected mouse mastocytoma cells and rat fibroblast

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Accepted for publication 4 July 1985

**Summary.** The mouse genomic Thy-1.1 gene was isolated from a phage library constructed from AKR/J (Thy-1.1) mouse DNA. Partial nucleotide sequence analysis of the coding region showed that it has only a single nucleotide difference from the Thy-1.2 gene, namely that amino acid 89 reads CGA (Arg) in Thy-1.1 and CAA (Glu) in Thy-1.2, corresponding to the amino acid substitutions previously identified. It was subcloned into an SV-40 derived vector for transfection. Transient transfection into HeLa cells gave 2% positive staining by immunofluorescence. The gene in this vector was also co-transfected into L cells and mastocytoma cells (both of Thy-1.2 strain origin) together with the Agpt gene. L-cell clones selected for transformation proved almost negative for Thy-1.1 expression, and any positive clones gradually lost Thy-1.1 antigen expression in culture. On the contrary, all clones of mastocytoma transformants gave a high level of expression after more than 3 months in culture.

Abbreviations: PFC, plaque-forming cell; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; PIHTR, highly transformable subline of the P815 mastocytoma; RaMB, rabbit anti-mouse brain antiserum; PIHTR.C.13.1, PIHTR-transformant Clone 13.1; Agpt, aminoglycosyl 3'-phosphotransferase; G418, Geneticin G-418 sulphate; FITC, fluorescein isothiocyanate; FACS, fluorescent activated cell sorter.

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The mastocytoma transformants were used to study the immunogenicity of Thy-1.1 molecules expressed on transfected cells. They evoked clear anti-Thy-1.1 plaque-forming cell (PFC) responses both *in vivo* and *in vitro*. The mastocytoma transformants also proved able to induce a T-dependent anti-Thy-1.1 antibody response in a cell transfer experiment. The immunogenicity of Thy-1.2 molecules on rat fibroblasts was also studied after transfection with a Thy-1.2 gene cosmid. Although Thy-1.2 expression was very low, these transfectants elicited a clear anti-Thy-1.2 PFC response from AKR spleen cells hyperimmunized against CBA thymocytes.

### INTRODUCTION

The Thy-1 molecule is of interest as an immunogen for several reasons. Structurally it is a single domain glycoprotein homologous to the multidomain immunoglobulin (Campbell *et al.*, 1981; Cohen *et al.*, 1981), major histocompatibility complex (Cushley & Owen, 1983) and T-cell receptor (Patten *et al.*, 1985) glycoproteins; jointly with  $\beta$ 2-microglobulin (Peterson *et al.*, 1972) it therefore provides the simplest example of this group of molecules.

In the mouse, where it is a major cell surface antigen on thymocytes, T cells, and brain cells (Reif & Allen, 1964), it occurs in two allelic forms which differ by a single amino acid substitution at position 89, Arg



(Thy-1.1)/Glu (Thy-1.2) (Williams & Gagnon, 1982). This is sufficient to generate a strong alloantibody response, which under different conditions can be either T-cell dependent or independent, and can be obtained both *in vivo* and *in vitro* (Isobe *et al.*, 1984a).

Brain Thy-1, for unknown reasons, does not elicit this alloimmune response (Lake & Mitchison, 1977; Isobe *et al.*, 1983). *In vivo* T cell Thy-1 is an excellent immunogen, in part because it can be helped by other non-H-2 alloantigens (Lake & Douglas, 1978; Zaleski & Klein, 1978); but, again for unknown reasons, Thy-1 on intact T cells inhibits rather than stimulates the *in vitro* T-independent response (Lake, 1975; Isobe *et al.*, 1984a). Whether or not Thy-1 itself can generate a T-cell response is unknown. Furthermore, the Thy-1 allo-response is subject to strong competitive interference from MHC-incompatibility (Zaleski & Gorzynski, 1979; Clark, Lake & Favila-Castillo, 1981). These are all problems which can perhaps best be pursued by DNA-mediated gene transfer.

To this end, the Thy-1.1 gene was isolated from an AKR/J mouse genomic library and obtained stable transformants of cultured cells derived from Thy-1.2 mice by DNA-mediated gene transfer. This is the first report to show that such cells can evoke an anti-Thy-1.1 antibody response. Also we show that rat fibroblast transfected with the Thy-1.2 gene can evoke an anti-Thy-1.2 PFC response in AKR spleen cells *in vitro*.

## MATERIALS AND METHODS

### *Isolation of the Thy-1.1 gene*

Whole DNA from AKR/J mice was cut with EcoRI, and the 8 Kb fraction extracted from low-melting agarose, since fragments of that size yielded a single band on southern blotting using the coding region of the Thy-1.2 genomic gene as a probe (Giguère, Isobe & Grosveld, 1985). The probe has been ligated in the EcoRI site of a  $\lambda$ 641 vector (Scherer *et al.*, 1981). The 8 Kb fraction was packaged *in vitro* and a library constructed.

### *Cell culture and DNA-mediated gene transfer*

BW5147 thymoma, EL-4 thymoma and HeLa cells were maintained in RPMI 1640 medium supplemented with 10% fetal calf serum (FCS) and antibiotics. Mouse L cells and rat fibroblast R2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS and anti-

biotics. PIHTR is highly transformable subline of the P815 mastocytoma (Van Pel, De Plaen & Boon, 1985) and maintained in RPMI-1640 medium with BUdR (100  $\mu$ g/ml), 10% FCS and antibiotics. PIHTR was cultured without BUdR for three days before transfection. Cells were transfected with the genomic Thy-1 gene or co-transfected with this gene together with PTCF (Grosveld *et al.*, 1982a), a vector incorporating the animoglycosyl 3'-phosphotransferase (Agpt) gene, using the calcium phosphate/DNA coprecipitation procedure of Wigler *et al.* (1979). Transformants were selected in medium containing 1 mg/ml of the antibiotic G418 (Gibco, Paisley, Renfrewshire).

The antibodies that were used for detection of cell surface Thy-1 antigens are as follows; rabbit anti-mouse brain antiserum (RaMB) kindly donated by Dr Alan Williams (Sir William Dunn School of Pathology, Oxford); mouse monoclonal anti-Thy-1.1 antibody HO-22.1 (Marshak *et al.*, 1979) and mouse monoclonal anti-Thy-1.2 antibody F7D5 (Lake *et al.*, 1979) both used as hybridoma culture supernatants. Target cells of ( $5 \times 10^5$ ) were incubated with 50  $\mu$ l of the first antibody (anti-Thy-1) for 30 min at 4°. After three washings cells were resuspended in 50  $\mu$ l of the second antibody for 30 min at 4°. In the case of adherent cells, the cells were detached by trypsin plus versine 3 hr before assay. For immunofluorescence, FITC-labelled goat anti-rabbit Ig (Nordic Immunology) was used as the second antibody following RaMB; FITC-rabbit anti-mouse Ig (Nordic Immunology, Tilburg, The Netherlands) was used following the mouse monoclonal antibodies. After three washings, stained cells were counted, or quantified by flow microfluorimetry. For radioimmunoassay,  $^{125}$ I-sheep anti-mouse Ig was used as the second antibody.

### *PFC assay for anti-Thy-1 responses*

For the *in vivo* response, thymocytes and transformants were injected i.p. in mice. For the *in vitro* response, spleen cells from mice were cultured with frozen and thawed thymocytes or transformants in RPMI medium with 10% FCS plus antibiotics (Isobe *et al.*, 1984a). After 7 days (*in vivo* primary response), or after 4 days (*in vivo* secondary or *in vitro* primary), direct (IgM) anti-Thy-1 PFC were determined (Fuji, Zaleski & Miligrom, 1971; Isobe *et al.*, 1984a).

### *Adoptive secondary antibody responses*

Spleen cells from CBA mice hyperimmunized (eight weekly injections i.p. of  $10^7$  AKR thymocytes) against

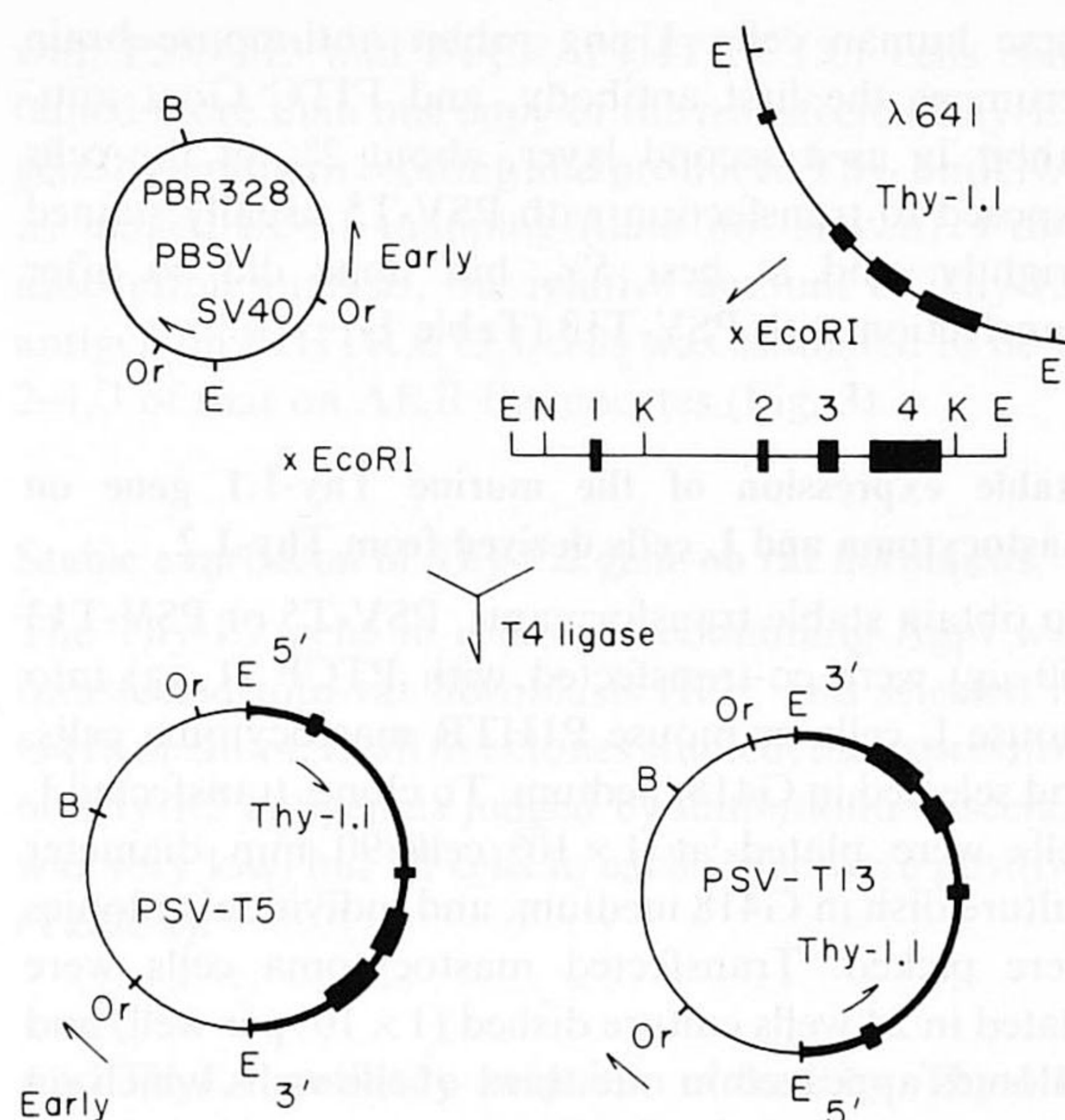


Thy-1.1 were pooled, trypsinized, and transferred i.p. into irradiated (600 rads) syngeneic hosts (Lake *et al.*, 1979). They were boosted by i.p. injection of simultaneously irradiated (2000 rads) cells, and serum antibody titres of anti-Thy-1.1 antibody were determined 9 days later by complement-mediated  $^{51}\text{Cr}$  release. Immunoglobulin G titres were determined by treatment of sera with 0.1 M 2-mercaptoethanol. Geometric means and standard errors were calculated for the 50% lysis endpoints (Mitchison, 1984).

## RESULTS

### Isolation of Thy-1.1 gene

Using the coding region of the genomic Thy-1.2 gene as a probe, an AKR/J (Thy-1.1) mouse  $\lambda$ 641 library was screened and an 8.1 Kb fragment encoding Thy-1.1 selected. Restriction enzyme mapping in comparison with the Thy-1.2 gene, and partial DNA sequence analysis by the Maxam & Gilbert method (1977) confirmed that the recombinant clone contained the Thy-1.1 gene. In particular the DNA sequence corresponding to amino acid 89 residue was CGA (Thy-1.1) in place of CAA (Thy-1.2) (data not shown). For transfection experiments, the Thy-1.1 gene was subcloned into the PBSV vector, a construct of pBR328 and SV40 (Grosveld *et al.*, 1982b). The Thy-1.1 gene was inserted in both orientation into PBSV, and the constructs named PSV-T5 and PSV-T13 (Fig. 1). In some experiments the Thy-1.2 gene was used in a cosmid which already contained the Agpt gene. (For more detailed study of Thy-1.1 and Thy-1.2 gene structure, see Giguère *et al.*, 1985).



**Figure 1.**  $\lambda$ 641 containing the Thy-1.1 gene was digested with EcoRI, and 8.1kb fragments were cut out from 0.6% low melting agarose gel. The purified Thy-1.1 fragment was ligated with EcoRI-digested PBSV and transfected into *Escherichia coli* HB101. From ampicillin-resistant colonies, PBSV-Thy-1.1 recombinants containing both orientations of the Thy-1.1 gene were isolated, and designates PSV-T5 and PSV-T13. Exons are depicted as boxes; 1~4. Enzymes used: E; EcoRI, N; NcoI, K; KpnI.

### Transient expression of murine Thy-1 gene on HeLa cells

PSV-T5 or PSV-T13 were transfected into HeLa cells using  $\text{CaPO}_4$ -precipitated DNA. After 40 hr the expression of mouse Thy-1 antigen was examined on

**Table 1.** Transient Thy-1.1 antigen expression on transfected HeLa cells

Source of DNA	Amount of DNA transfected ( $\mu\text{g}$ )	% of positive staining*	
		RaMB (1:100)	NRS (1:100)
(1) PSV-T5	0	0	0
PSV-T5	1	0	0
PSV-T5	10	0	0
PSV-T5	50	1.5	0
(2) PSV-T5	30	2.5	0
PSV-T13	30	0	0

\* Two hundred cells were counted by immunofluorescence. Results are means of two dishes. Positively stained cells were bright and easily discriminated from the background.



these human cells. Using rabbit anti-mouse brain serum as the first antibody, and FITC Goat anti-rabbit Ig as a second layer, about 2% of the cells exposed to transfection with PSV-T5 usually stained brightly, and at best 5%, but none did so after transfection with PSV-T13 (Table 1).

### Stable expression of the murine Thy-1.1 gene on mastocytoma and L cells derived from Thy-1.2

To obtain stable transformants, PSV-T5 or PSV-T13 (50 µg) were co-transfected with PTCF (1 µg) into mouse L cells or mouse PIHTR mastocytoma cells, and selected in G418 medium. To clone, transfected L cells were plated at  $1 \times 10^6$  cells/90 mm diameter culture dish in G418 medium, and individual colonies were picked. Transfected mastocytoma cells were plated in 24 wells culture dish (1 × 10<sup>6</sup> per well) and colonies appeared in one third of the wells which on microscopic examination were confirmed as single. From two independent transfection into L cells, of 20

clones picked and examined for Thy-1.1 antigen expression by immunofluorescence and radioimmunoassay, only two clones expressed Thy-1.1 antigens (one from PSV-T5, another from PSV-T13). Although at first examination after 1 month of transfection both clones had about 50% positive staining, the number of positive cells decreased gradually and stabilized at around 4% positive cells in each clone (Table 2). From four independent transfections into PIHTR mastocytoma cells, 25 stable clones were selected. All clones from both the PSV-T5 and PSV-T13 transformants stained as brightly with RaMB serum and monoclonal anti Thy-1.1 (HO-22.1) as did BW5147 thymoma cells, but did not stain with monoclonal anti-Thy-1.2 antibody (F7D5) using EL-4 thymoma cells as a control, as shown in Table 2 and Fig. 2. After over more than 3 months in culture without G418, the level of Thy-1.1 antigen expression on PIHTR-transformant lines did not change. For further study a clone designated PIHTR.C13.1 was chosen, which has been derived from transformation of a PIHTR mastocytoma cell

**Table 2.** Expression of Thy-1 antigens on mastocytoma, L cells and R2 cells transfected with Thy-1 gene

Exp.	Cell lines	Radioimmunoassay (c.p.m.) <sup>‡</sup> HO-22.1 (Thy1.1)	Immunofluorescence (%) <sup>§</sup>		
			HO-22.1	F7D5(Thy-1.2)	
(1)	PIHTR	97	0	+ (-)	
	PIHTR.	C5.1	732	100	+++
		C5.2	938	100	+++
		C5.3	1078	100	+++
		C5.4	1582	100	+++
		C13.1	1325	100	+++
		C13.4	714	100	+++
	BW5147	1320	100	+++	
EL-4			100	+++	
(2)	L cells	45	0		
	L cell	C5.1*		40	++
		C5.1†	57	4	++
		C5.2	46	0	
		C5.3		0	
		C13.1		0	
		C13.4*	315	30	++
C13.4†	68	4	++		
(3)	R2 cells			0	
	R2 trans. C1-C5			100 +	

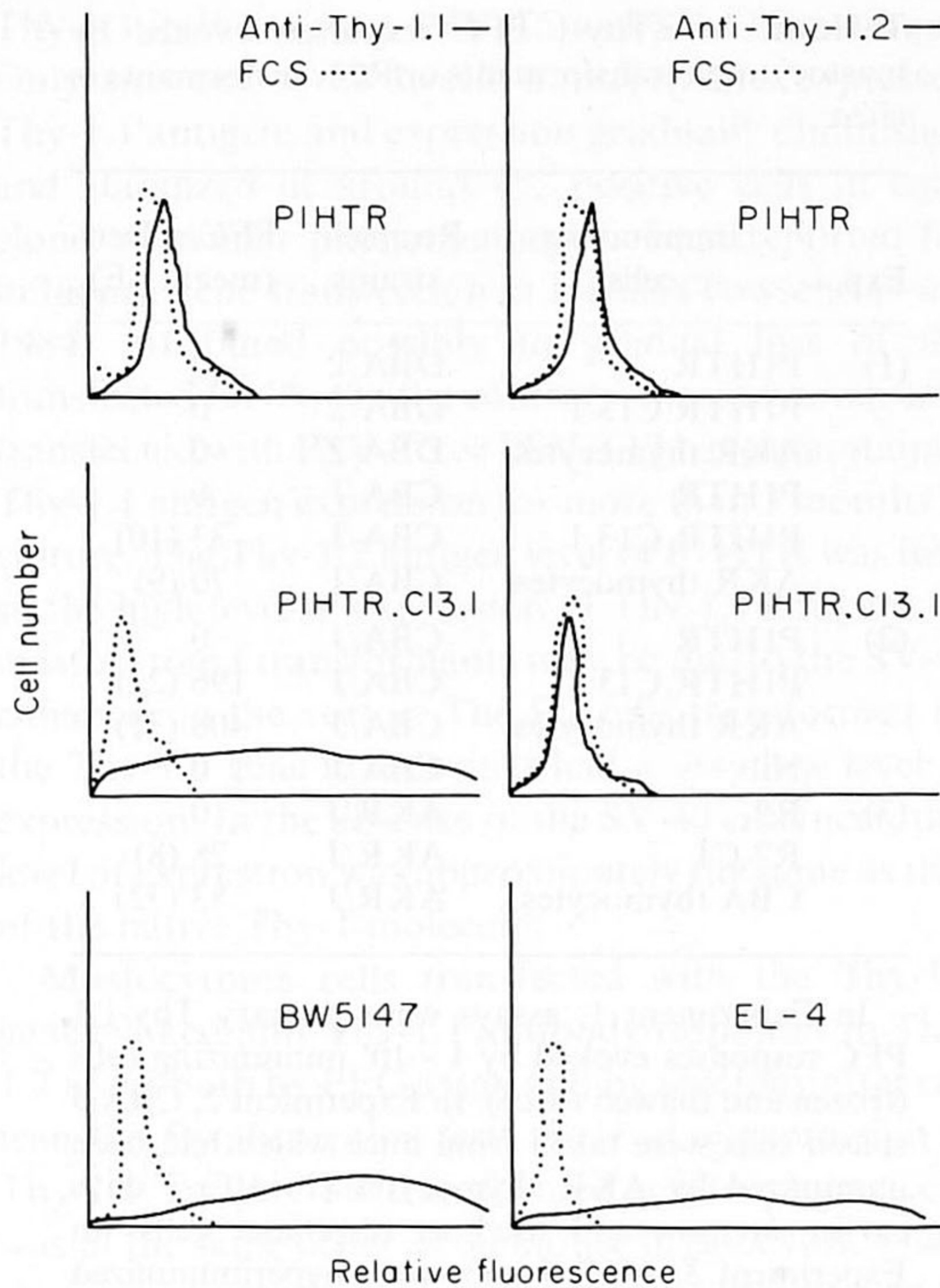
\* L-cell transformant assayed at 1 month after transfection.

† L-cell transformant assayed at 3 months after transfection.

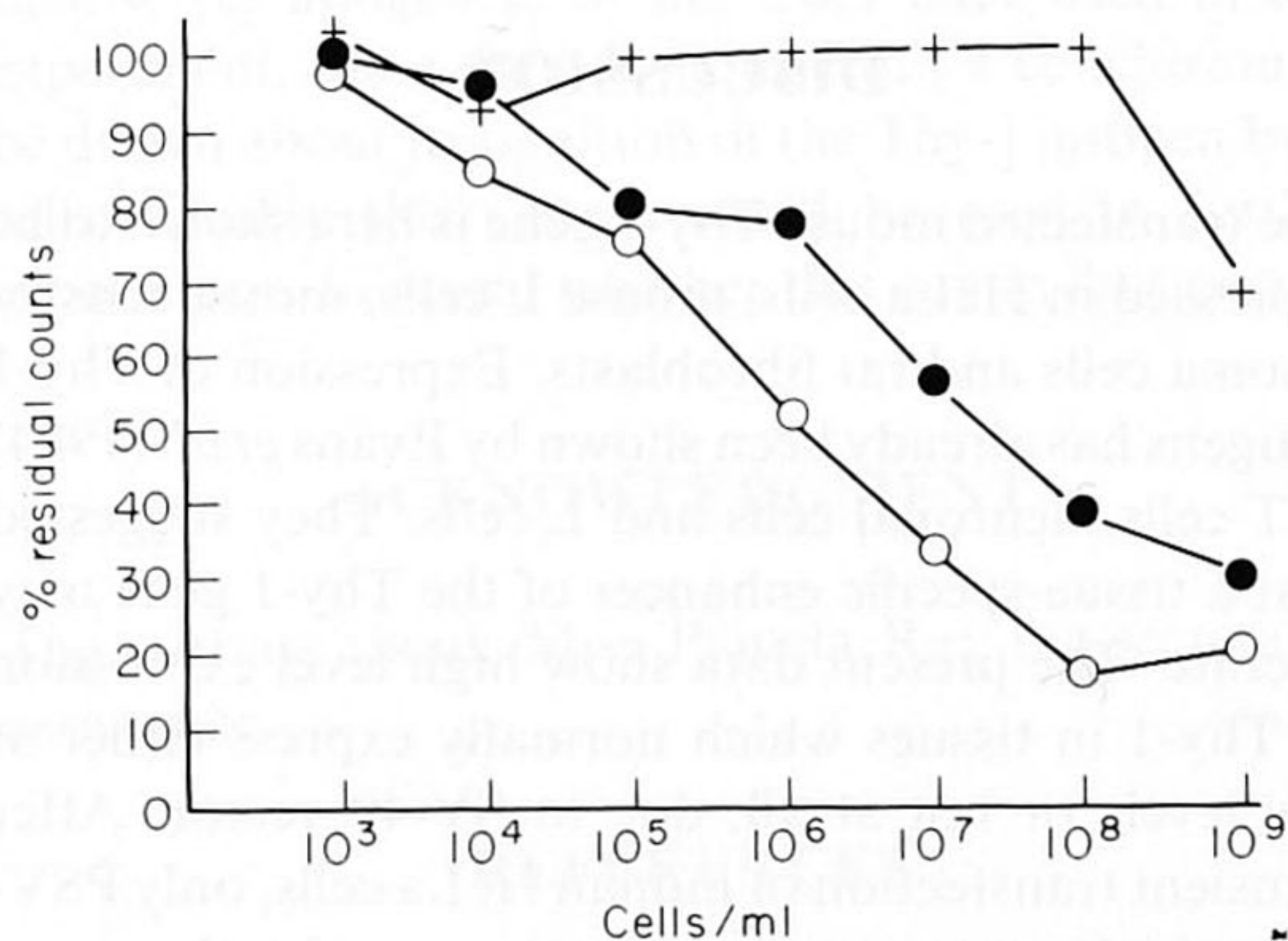
‡ The results are an average of two independent assays and expressed as c.p.m. after subtraction of control (5% FCS). The background counts were 20 c.p.m.

§ Two hundred cells were counted by immunofluorescence. + + +, bright, + +, medium; +, weak staining.





**Figure 2.** Expression of Thy-1.1 antigen on a PIHTR transformant clone line. PIHTR, PIHTR.C13.1, BW5147 and EL-4 cells were exposed to anti-Thy-1.1 monoclonal antibody (HO22.1), anti-Thy-1.2 monoclonal antibody (F7D5) or medium alone. Bound antibody was detected with FITC-rabbit anti-mouse Ig and analysed by FACS.



**Figure 3.** Absorption assay of Thy-1.1 antigen on PIHTR.C13.1 cells using radioimmunoassay. Fifty  $\mu$ l of monoclonal anti-Thy-1.1 antibody (HO-22.1) was mixed with 50  $\mu$ l of various dilutions of cells of PIHTR, PIHTR.C13.1 and AKR thymocytes. Binding was assayed in radioimmunoassay for Thy-1.1 antigen. Results are the mean of two separate experiments. PIHTR (+); PIHTR.C13.1 (●); AKR thymocytes (○).

with PSV-T13 and PTCF. PIHTR.C13.1 cells contained more than one copy of the transfected Thy-1.1 gene by southern blotting and produced Thy-1 mRNA as judged by S1 mapping (data not shown). From absorption analysis, the relative amount of Thy-1.1 antigen on PIHTR.C13.1 cells was estimated to be 1/2–1/3 of that on AKR thymocytes (Fig. 3).

#### Stable expression of Thy-1.2 gene on rat fibroblasts

The Thy-1.2 gene in a cosmid containing Agpt was transfected into rat fibroblasts (R2), and selected in G418 medium. In all five clones studied, the expression of Thy-1.2 antigen as judged by immunofluorescence was very low, but all cells in each clone were positive (Table 2).

#### Anti-Thy1.1 antibody response evoked by Thy-1.1-transfected mastocytoma cells

PIHTR.C13.1 ( $5 \times 10^7$ ) cells were injected into DBA/2 mice i.p. Although the parental PIHTR mastocytoma grew progressively in DBA/2 mice, the PIHTR.C13.1 cells were rejected by DBA/2 mice (data not shown). At 7 days after injection, the primary anti-Thy-1.1 antibody response of spleen cells was measured by PFC assay. Both PIHTR.C13.1 cells and AKR thymocytes evoked relatively low levels of this response in DBA/2 mice (Table 3), but the plaques were clear and the backgrounds extremely low. After repeated injection of PIHTR.C13.1 cells the level of the response

**Table 3.** Anti-Thy-1.1 PFC responses evoked by mastocytoma transformants *in vivo*

Immunizing cells	Recipient strain of mice	No. of injection	PFC/spleen Mean (SE)
PIHTR.C13.1	DBA/2	1	120 (21)
PIHTR.C13.1	DBA/2	2	720 (108)
PIHTR.C13.1	DBA/2	3	2460 (678)
PIHTR.C13.1	BALB/c	2	600 (60)
PIHTR	DBA/2	1	0
AKR thymocytes	DBA/2	1	390 (4)
AKR thymocytes	DBA/2	2	3690 (438)
AKR thymocytes	CBA/J	1	5100 (876)
Nil	DBA/2	—	0

Intact PIHTR.C13.1 cells ( $5 \times 10^7$ ) PIHTR cells ( $5 \times 10^7$ ) or AKR thymocytes ( $2.5 \times 10^7$ ) were injected into mice i.p. Anti-Thy-1.1- PFC assays were done after 7 days (for primary) or after 4 days (for secondary). Number of PFC is the mean of four mice in each group.

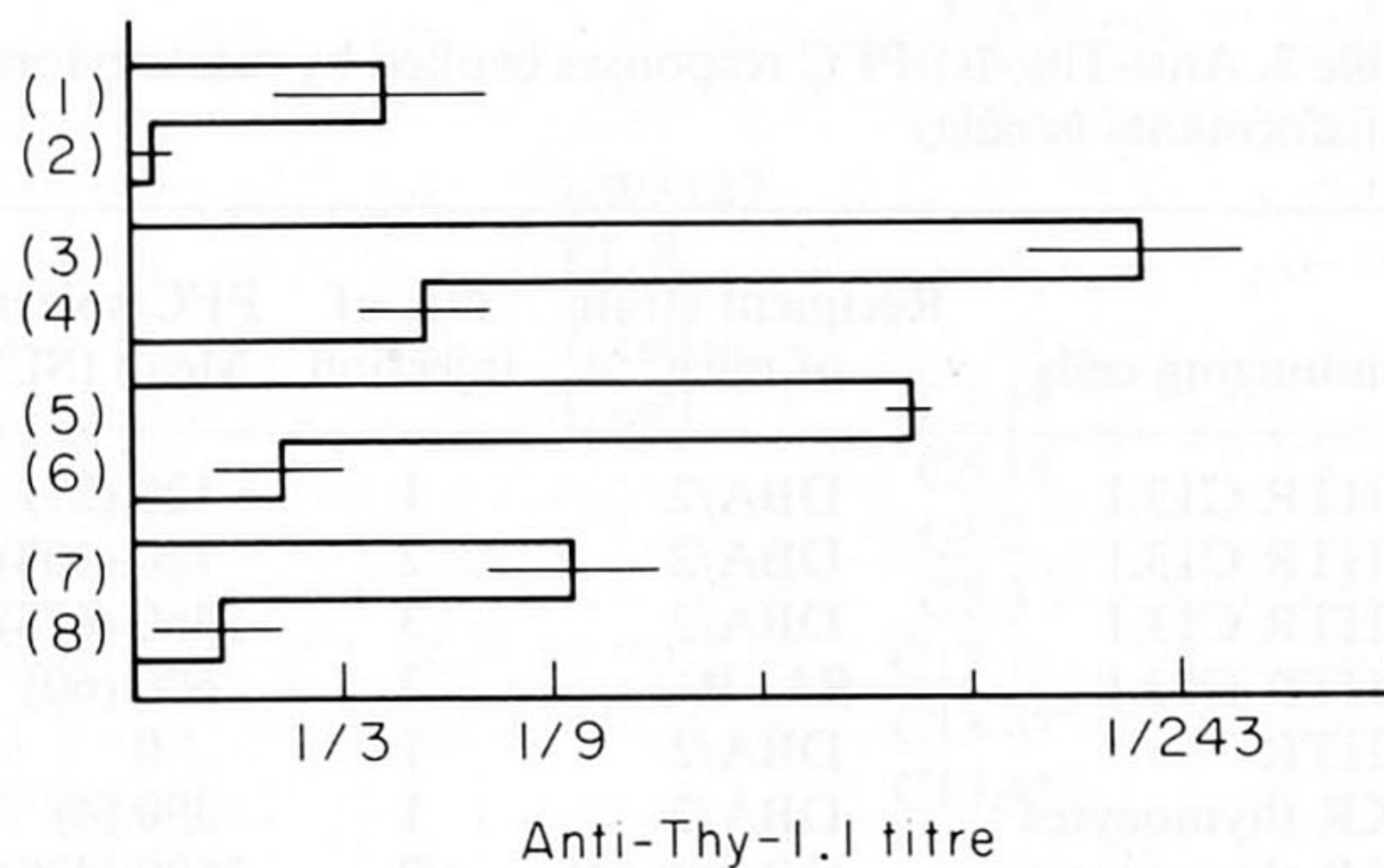


rose. Primary PFC responses were also measured *in vitro*. DBA/2 spleen cells did not generate a detectable response, no doubt because of the low responsiveness of this strain (Isobe *et al.*, 1984b). But PIHTR.C13.1 cells did evoke a primary anti-Thy-1.1 response in CBA spleen cells *in vitro* (Table 4).

In order to evaluate their T-cell dependent immunogenic activity, PIHTR.C13.1 cells were tested in an adoptive secondary response (Fig. 4). AKR thymocytes and the PIHTR.C13.1 cells both evoked a significant ( $< 0.05$ ) response, (3),(5) versus (1). To our surprise, non-transfected PIHTR cells also evoked a small response, but this was significantly less than that of the transfected cells, (7) versus (5). Depletion of T cells from the primed population by means of anti-Thy-1.2 antibody plus complement significantly diminished the evoked responses, (3) versus (4), (5) versus (6), but still left responses which were themselves significantly greater than background (2). The transformants thus proved able to evoke a T cell dependent response.

#### Anti-Thy-1.2 antibody responses evoked by Thy-1.2 gene-transfected rat fibroblasts

Probably because the Thy-1.2 antigen level on rat fibroblast transformants was very low, repeated injection of R2-transformants did not evoke anti-Thy-1.2 PFC responses in rats or AKR mice. Spleen cells from AKR mice repeatedly injected with CBA thymocytes, taken 7 days after their last injection and cultured with



**Figure 4.** Detection of the transfected Thy-1 gene in an adoptive secondary response. Responses of groups of mice receiving hyperimmunized CBA anti-AKR (anti-Thy-1.1) lymphocytes. (1),(2),(5),(7) complete spleen cells (T+B); (2),(4),(6),(8) B cells isolated by anti-Thy-1.2+C treatment. (1),(2) No booster antigen; (3),(4) boosted with  $10^7$  AKR thymocytes; (5),(6) boosted with  $10^7$  Thy-1.1 transfected PIHTR cells; (7),(8) boosted with  $10^7$  non-transfected PIHTR cells.

**Table 4.** Anti-Thy-1 PFC responses evoked by mastocytoma transformants or R2 transformants *in vitro*

Exp.	Immunizing cells	Recipient strains	PFC/spleen (mean, SE)
(1)	PIHTR	DBA/2	0
	PIHTR.C13.1	DBA/2	0
	AKR thymocytes	DBA/2	0
	PIHTR	CBA/J	0
	PIHTR.C13.1	CBA/J	33 (10)
	AKR thymocytes	CBA/J	70 (9)
(2)	PIHTR	CBA/J	0
	PIHTR.C13.1	CBA/J	196 (22)
	AKR thymocytes	CBA/J	408 (31)
	Nil	CBA/J	0
(3)	R2	AKR/J	0
	R2-C1	AKR/J	28 (8)
	CBA thymocytes	AKR/J	83 (32)

In Experiment 1, assays were primary Thy-1.1 PFC responses evoked by  $1 \times 10^6$  immunizing cells (frozen and thawed twice). In Experiment 2, CBA/J spleen cells were taken from mice which had been immunized by AKR thymocytes ( $1 \times 10^7$ ) 7 days earlier *in vivo*, and used as responder cells. In Experiment 3, AKR/J mice were hyperimmunized by CBA thymocytes before providing responder spleen cells. Assays were done in four wells in each group, and results are means of four wells.

frozen and thawed R2 transformants, did however yield detectable anti-Thy-1.2 PFC responses (Table 4).

## DISCUSSION

The transfected mouse Thy-1 gene is here shown to be expressed in HeLa cells, mouse L cells, mouse mastocytoma cells and rat fibroblasts. Expression of Thy-1 antigens has already been shown by Evans *et al.* (1984) in T cells, neuronal cells and L cells. They suggested that a tissue-specific enhancer of the Thy-1 gene may operate. The present data show high level expression of Thy-1 in tissues which normally express either at low levels or not at all, due to SV-40 vector. After transient transfection of human HeLa cells, only PSV-T5, which contains the Thy-1 gene in the same transcriptional direction as the SV-40 early promoter, produced expression after 40 hr. Positively stained cells were as bright as BW5147 thymoma cells at various dilution of RaMB serum (data not shown). Mouse L cells and mouse mastocytoma cells (derived from Thy-1.2 mice) were cotransfected with the genes



Thy-1.1 in the vectors PSV-T5 or PSV-13, and Agpt. Only two out of 20 L cell transformants expressed Thy-1.1 antigen, and expression gradually diminished and stabilized at around 4% positive cells in each clone. A similar phenomenon has been reported for influenza gene transfection in L cells (Towsend *et al.*, 1984) attributed possibly to gradual loss of the transfected DNA. On the contrary, mastocytoma cells transfected with PSV-T5 or PSV-T13 have maintained Thy-1.1 antigen expression for more than 3 months in culture. The Thy-1.2 antigen level of P1HTR was low, so the high level of expression of Thy-1.1 antigens on mastocytoma transformants may be due to the SV-40 enhancer in the vector. The R2 cells transformed by the Thy-1.2 gene in a cosmid had a very low level of expression. In the absence of the SV-40 enhancer, this level of expression was approximately the same as that of the native Thy-1 molecule.

Mastocytoma cells transfected with the Thy-1.1 gene evoked anti-Thy-1.1 antibody responses in Thy-1.2 mice, both by PFC assay and by serology after cell transfer. By absorption tests the level of expression of Thy-1.1 antigen on transformed mastocytoma cells was of the same order as on mouse thymocytes, which explains why the level of antibody responses evoked by the transformants was so high. The data exclude the possibility that only Thy-1 on thymocytes or T-cell membranes has the capacity to induce an antibody response. In addition, the cell transfer data indicate that transferred mastocytoma cells can evoke a T-dependent response. As the mastocytoma cells are themselves allogeneic to the CBA mice used in this experiment, these data do not permit a conclusion to be drawn about recognition of the Thy-1 antigen by T cells. Clearly this system could be used in further experiments to reveal whether this can in fact occur.

#### ACKNOWLEDGMENT

The authors thank Miss Pamela Ray for secretarial assistance.

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