Int. J. Dev. Biol. 33: 369-378 (1989)

Original Article

The Thy-1 glycoprotein is expressed in mouse embryonal carcinoma cells P19

PETR DRÁBER[1]*, PETR MALÝ[1], PETR HAUSNER[2] and ZORA POKORNÁ[1]

[1]*Institute of Molecular Genetics, Czechoslovak Academy of Sciences, Prague,* and
[2]*Oncological Research Laboratory, Faculty of General Medicine, Charles University, Prague, Czechoslovakia.*

ABSTRACT Thy-1 is a well characterized glycoprotein known to be variably expressed on the surface of different cell types. Serological analysis of a limited number of teratocarcinoma-derived cell lines suggested that mouse embryonal carcinoma cells do not express Thy-1 and that its expression is associated with the appearance of differentiated cells. In this report we show that monoclonal antibody 1aG4, recognizing Thy-1.2 epitope, binds specifically to P19 embryonal carcinoma cells and their undifferentiated subclones. A number of control experiments confirmed that 1aG4 antibody binds to the Thy-1.2 glycoprotein expressed on the surface of P19 embryonal carcinoma cells and not to the antigen expressed on differentiated derivatives of these cells or to a cross-reactive epitope. Transcriptional activity of the Thy-1 gene in undifferentiated P19 cells was shown by transfection experiments in which transfer of the Thy-1.1 gene into P19 cells resulted in stable expression of the Thy-1.1 antigen on the surface of recipient cells. Direct evidence for the presence of Thy1 mRNA in P19 cells was obtained by Northern blot analysis with a Thy-1-specific cDNA probe. Treatment of P19 cells with retinoic acid resulted in a decrease in the expression of Thy-1 antigen which preceded changes in morphology of the cells. These data indicate that Thy-1 is a developmentally regulated surface marker of P19 embryonal carcinoma cells which is amenable to direct genetic analysis.

KEY WORDS: *teratocarcinoma stem cells, retinoic acid, Thy-1 antigen, differentiation, cell surface*

Introduction

Embryonal carcinoma (EC) cells, the stem cells of teratocarcinomas, provide a model system for studying the molecular mechanisms underlying differentiation and early embryonic development (Martin, 1980). These cells can be maintained in the undifferentiated state or can be induced to differentiate depending on the local cell density and/or addition of various chemical stimuli. The process of cell differentiation is marked by dramatic morphological changes that are accompanied by a decrease in the growth rate and DNA synthesis, and by changes in the protein synthesis. One of the surface structures whose expression has been found to be modulated during EC cell differentiation is the Thy-1 antigen. This is a glycoprotein with apparent molecular weight of 25,000 which is bound to the cell surface through a phosphatidylinositol-containing membrane binding domain (Williams and Gagnon, 1982; Low and Kincade, 1985; Tse *et al.*, 1985). The Thy-1 antigen has been extensively characterized and its gene has been isolated and sequenced (Giguere *et al.*, 1985; Seki *et al.*, 1985; Ingraham *et al.*, 1986). Thy-1 is similar in structure to a variable region domain of the immunoglobulin superfamily of cell surface and secreted glycoproteins (Williams and Barclay, 1988). In the mouse, the Thy-1 glycoprotein occurs in two allelic forms, Thy-1.1 and Thy-1.2, which were originally named by Reif and Allen (1964) θ_{AKR} and θ_{C3H}, respectively. They differ by a single amino acid substitution at residue 89 (arginine in Thy-1.1, glutamine in Thy-1.2: Williams and Gagnon,

Abbreviations used in this paper: BSA, bovine serum albumin; EC, embryonal carcinoma; H-MEMd, Eagle's minimal essential medium supplemented with nonessential amino acids, 3 mM L-glutamine and 1 mM pyruvate sodium; PAGE, polyacrylamide gel electrophoresis; PBS,10 mM sodium phosphate, 150 mM NaCl, pH 7.2; RIA, radioantibody binding assay; SDS, sodium dodecyl sulfate.

***Address for reprints**: Institute of Molecular Genetics, Czechoslovak Academy of Sciences, Vídeňská 1083, 142 20 Prague 4, Czechoslovakia.

0214-6282/89

TABLE 1

REACTIVITY OF 1aG4 ANTIBODY WITH CELL LINES

Cell line	Description (origin)	Reference	^{125}I-labeled antibody bound (%cpm in cell pellet)[b]	
			1aG4	TEC-01
P19X1[a]	EC (C3H/He)	McBurney and Rogers (1982)	4.1	30.0
P19XT.1.1	EC (from P19X1)	Dráber and Malý (1987)	5.2	<0.2
P19S1801A1	EC (from P19)	McBurney *et al.*(1982)	5.6	29.0
P19ST.1.3	EC (from P19S1801A1)	Dráber and Vojtíšková(1984)	7.1	<0.2
P19ST.1.5	EC (from P19S1801A1)	Dráber and Vojtíšková(1984)	6.4	<0.2
RAC65	EC (P19S18RAC6)	Jones-Villeneuve *et al.* (1983)	6.6	24.5
F9	EC (from OTT 6050)	Bernstine *et al.* (1973)	1.3	34.0
OTF9-63	EC (from F9)	Rosenstraus *et al.* (1980)	<0.2	35.0
PCC4/Aza	EC (from OTT 6050)	Jakob *et al.* (1973)	1.0	19.0
PYS-2	Parietal endoderm	Lehman *et al.* (1974)	<0.2	<0.2

[a] A subclone isolated in this laboratory from a solid tumor of P19 cells growing in a C3H/Di mouse immunosuppressed with anti-Thy-1.2 antibody.

[b] Cells (2.5 x 10^5) were incubated with ^{125}I-labeled 1aG4 antibody or control TEC-01 antibody (10^5 cpm/tube) and the percentage of cpm bound was determined as described in Materials and Methods. Average values of triplicate measurements from two to four independent experiments are shown.

1982). Thy-1 is expressed in mouse thymus and T cells, in neuronal cells, skin fibroblasts and several other cell types (Morris, 1985).

Using conventional alloantibodies, Stern *et al.* (1975) found that the Thy-1 antigen was also expressed in a population of heterogeneous teratocarcinoma cells SIKR. Detailed serological analysis indicated that Thy-1 is expressed in cells with morphology of fibroblast-like cells, whereas EC cells were Thy-1 negative. These results were confirmed by analysis of RNA isolated from F9 EC cells (Giguere *et al.*, 1985). The Thy-1 mRNA was undetectable in undifferentiated F9 cells but switched on after differentiation induced with retinoic acid. On the other hand, in human teratocarcinoma-derived cells the Thy-1 antigen was expressed on the majority of EC cells and its expression was not modulated during differentiation *in vitro* (Andrews *et al.*, 1983).

In an attempt to understand the regulation of Thy-1 antigen expression in EC cells, we analyzed its expression in mouse P19 cells. P19 are multipotent EC cells as demonstrated by their ability to form different tissues in chimeric mice (McBurney and Rogers, 1982; Rossant and McBurney, 1982). In culture, P19 EC cells can differentiate into tissues of different embryonic origin. When monolayer cultures are treated with retinoic acid, virtually all cells differentiate into fibroblasts-like cells (Jones-Villeneuve *et al.*, 1982). On the other hand when the cells are aggregated and treated with the same drug, neurons, glia and fibroblasts appear (Jones-Villeneuve *et al.*, 1982, 1983). The experiments presented below show that undifferentiated P19 cells, in contrast to some other mouse EC cells, express small amounts of surface Thy-1 antigen which disappears during retinoic acid-induced differentiation. Thus, Thy-1 is a well characterized surface marker of the P19 EC cells whose expression is developmentally regulated.

Results

Expression of Thy-1 epitope in EC cell lines

It has been previously shown that conventional anti-Thy-1.2 antibodies do not react with certain EC cell lines (Stern *et al.*, 1975). To determine the expression of Thy-1.2 epitope in other EC cell lines, a monoclonal anti-Thy-1.2 antibody (clone 1aG4) was employed. The data presented in Table 1 indicate that the antibody reacted with all EC cell lines derived from P19 EC cells, including the embryoglycan-defective P19XT.1.1, P19ST.1.3,

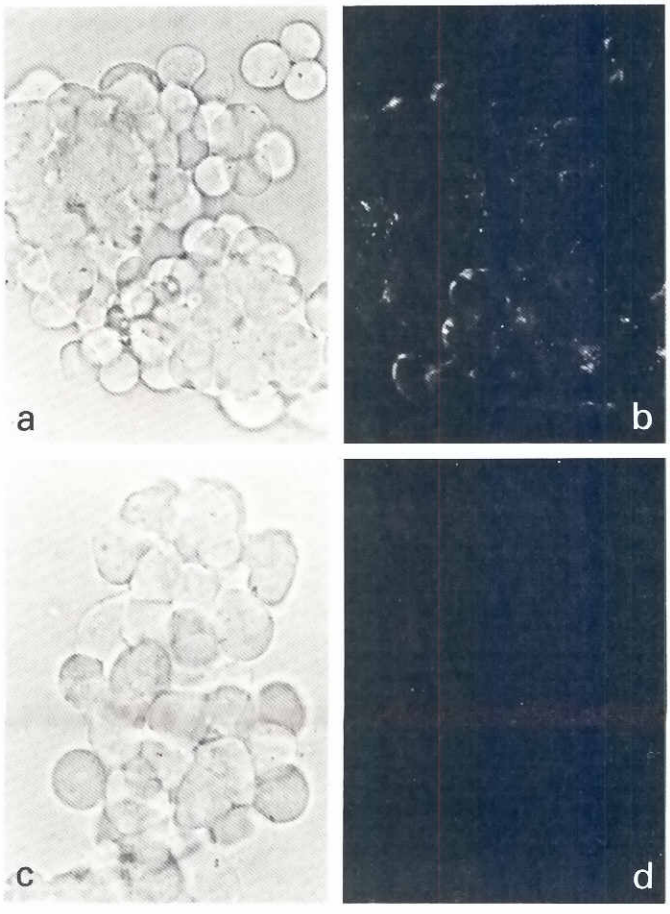

Fig. 1. Expression of Thy-1.2 antigen on P19S1801A1 cells as demonstrated by indirect immunofluorescence. *The cells were released from tissue culture dish by 0.02% EDTA in PBS and incubated with anti-Thy-1.2 antibody, clone 1aG4* **(a, b)**, *or anti-Thy-1.1 antibody, clone MRC OX 7* **(c, d)** *followed by fluorescein isothiocyanate-conjugated swine anti-mouse IgG. Photomicrographs were taken using bright-field illumination* **(a, c)** *or ultra-violet illumination* **(b, d)**. *Magnification x 260.*

TABLE 2

BINDING PROPERTIES OF ANTI-Thy-1 MONOCLONAL ANTIBODIES

Cells (origin)	^{125}I-labeled antibody bound (% cpm in cell pellet)*		
	anti-Thy-1.2 1aG4	anti-Thy-1.2 F7 D5	anti-Thy-1.1 MRC OX 7
P19S1801A1 (from P19)	5.6	0.3	<0.2
F9 (from OTT 6050)	1.3	<0.2	<0.2
Thymus (A/Ph)	25.7	27.4	<0.2
Thymus (A.Thy-1.1)	<0.2	<0.2	19.3
Spleen (A/Ph)	16.2	4.2	<0.2
Spleen (A.Thy-1.1)	<0.2	<0.2	9.2

* EC cells (2.5 x 10^5) or thymus or spleen cells (1 x 10^6) were incubated with the monoclonal antibodies (10^5 cpm/tube) and the percentage of cpm bound was determined as described in Materials and Methods. Average values of triplicate measurements are shown.

and P19ST.1.5 mutants and retinoic acid-resistant RAC65 cells. 1aG4 antibody reacted weakly with F9 and PCC4/Aza cells and did not react with OTF9-63 and PYS-2 cells. There was no correlation between the expression of Thy-1.2 antigen and SSEA-1 recognized by TEC-01 antibody. Negative control antibody MRC 0X 7 did not bind to any EC cell line tested (binding <0.2%).

When another anti-Thy-1.2 antibody (clone F7 D5) was used, very weak binding to P19S1801A1 EC cells was observed and there was no binding to F9 cells (Table 2). Because the purity and specific activity of both antibodies was similar, it appeared possible that the observed differences reflected lower affinity of F7 D5 antibody for cells expressing small amounts of Thy-1.2 antigen. In further experiments we therefore tested binding of the antibodies to thymus and spleen cells. Although both antibodies bound at comparable levels to Thy-1.2$^+$ A/Ph thymocytes, F7 D5 antibody bound less efficiently to spleen cells, which express a smaller amount of Thy-1 antigen (Raff, 1971). The data in Table 2 also demonstrate the specificity of the anti-Thy-1 antibodies used.

The conclusion that Thy-1.2 antigen is expressed on P19 EC cells and not on their differentiated derivatives was arrived at through immunofluorescence assays. 1aG4 antibody reacted with approximately 85% P19X1 cells and 90% P19S1801A1cells (Fig. 1a, b). Because more than 90% of P19S1801A1 cells were also stained with TEC-01 antibody, the majority of the cells must express both Thy-1 and SSEA-1 antigen. There was no staining with anti-Thy-1.1 antibody (Fig. 1c, d).

Preimplantation-stage mouse embryos express several stage-specific embryonic antigens that are also expressed by EC cells. To determine whether the epitope detected by 1aG4 antibody is also expressed on embryonic cells, unfertilized and fertilized eggs and embryos at the 2 cell, 4 cell, 8 cell, morula and early and late blastocyst stages were analyzed in indirect immunofluorescence tests. Such analyses showed that all the samples were Thy-1.2 negative.

The finding that P19 EC cells express Thy-1 antigen was surprising and required further specificity controls. The anti-Thy-1.2 specificity of ^{125}I-labeled 1aG4 antibody was confirmed by absorption with A/Ph (Thy-1.2$^+$) or A.Thy-1.1 (Thy-1.2$^-$) thymus and brain cells. Data presented in Fig.2 show that absorption with 0.5 x 10^6 A/Ph thymus cells decreased the binding of the remaining antibody to about 20%, whereas there was no change in the reactivity after absorption with 10^7 A/Thy-1.1 thymus cells. Similar data were obtained when brain cells were used. A small decrease in the binding after absorption with A.Thy-1.1 brain cells probably reflects the effect of dilution of the antibodies.

In another set of experiments tissue culture supernatants from cultures of 1aG4 hybridoma cells or myeloma parental cells P3-NSI/1-Ag4-1 were incubated with EC cells and the binding was determined in indirect RIA. Preincubation of P19S1801A1 cells with the supernatant of 1aG4 cells or myeloma cells resulted in 1740 cpm bound and 280 cpm bound, respectively. This excluded the possibility that natural anti-EC cell antibodies contaminating 1aG4 antibody preparation isolated from ascites fluid are responsible for the observed immunobinding. The binding of 1aG4 antibody to P19 cells was not the result of nonspecific binding of the IgG_3 subclass antibody to P19 cells, because ^{125}I-labeled control IgG3 antibody, MEM-37, did not bind either to P19S1801A1 cells or to P19ST.1.3 cells.

Analysis of 1aG4 antibody binding

The data presented in Tables 1 and 2 indicated that P19-derived cells bound 1aG4 antibody less efficiently than thymus cells. However, the experiments were performed with relatively low concentrations of the antibody and they did not allow conclusions to be made concerning the complexity of the binding and number of binding sites. In further experiments were therefore analyzed the binding of ^{125}I-labeled 1aG4 antibody to the P19S1801A1 and thymus cells over a 10^4-fold range of antibody concentrations. The data presented in Fig. 3 are plotted as B/F versus $\log_{10}$ B where B is the amount of 1aG4 antibody bound and F is the amount of free antibody. This modification of the Scatchard plot was found to provide a better overall description of the binding data (Stanley *et al.*, 1980). Thymus cells showed a weak positively cooperative binding at the lowest concentrations of 1aG4 antibody and approached saturation at the highest concentrations used. The positively cooperative binding was not observed with P19S1801A1 cells. At saturation both thymus and EC cells exhibited linear and nearly parallel descending

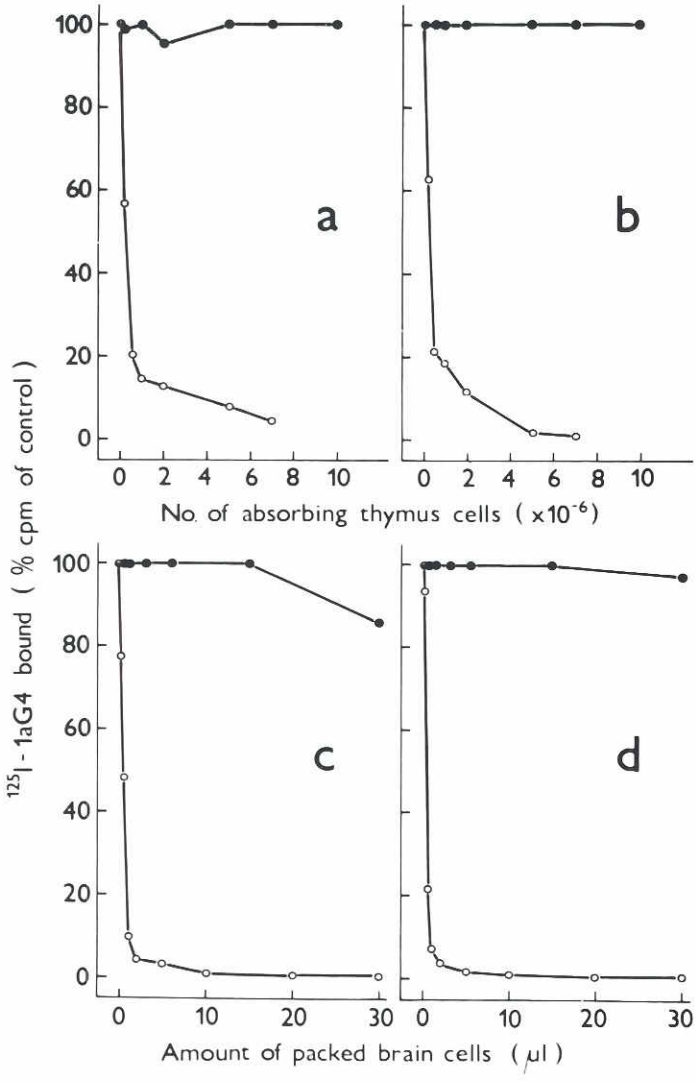

Fig. 2. Absorption analysis of 1aG4 antibody. *^{125}I-labeled 1aG4 antibody (30 ng in 350 µl) was absorbed with various numbers of thymus cells* **(a, b)** *or various amounts of packed brain cells* **(c, d)** *from A/Ph (○) or A.Thy-1.1 (●) mice. The residual biding activity was tested in direct RIA using 2.5 x 10^5 P19S1801A1 cells* **(a, c)** *or P19ST1.3 cells* **(b, d).**

lines. This indicated that the association constant (K_A) is similar for both cell types. From the data in Fig. 3 we calculated that there are approximately 10-fold fewer binding sites on P19S1801A1 cells compared to thymus cells.

Identification of Thy-1 glycoprotein in EC cells

It has been previously found that anti-Thy-1-specific antibodies may cross-react with some cell structures. Dulbecco *et al.* (1981) found that anti-Thy-1.1 antibody, clone T11A9e, cross-reacts with vimentin, and Dales *et al.* (1983) found cross-reactivity of some anti-Thy-1.2 antibodies with actin. To identify the cell surface glycoprotein recognized by 1aG4 antibody, we analyzed the material immunoprecipitated from metabolically labeled P19X1 cells. The data presented in Fig. 4d show that 1aG4 antibody precipitated specifically a [D-6-^{3}H]galactose-labeled glycoprotein of apparent molecular weight 25,000 which corresponds to the molecular weight of Thy-1 antigen. Other bands were nonspecificities bound not only to 1aG4 but also to MRC OX 7 antibody (Fig. 4c) and *S. aureus* armed with rabbit anti-mouse IgG (Fig. 4b).

Transcription of Thy-1 antigen in P19 EC cells

Evidence that P19 cells have the capability to initiate transcription from the Thy-1 gene promoter came from serological analysis of the surface expression of Thy-1.1 antigen in P19 EC cell lines transfected with the Thy-1.1 gene. As shown in Table 2, P19S1801A1 cells do not bind anti-Thy-1.1 antibody. When the cells are transfected with the Thy-1.1 gene, they stably express both endogenous and transfected Thy-1 gene products (Table 3). Similar results were obtained when the Thy-1.1 gene was transfected into P19XT.1.1 cells (Table 3). Direct evidence for the presence of Thy-1 mRNA in P19 EC cells was obtained by Northern blot analysis with a Thy-1 specific cDNA probe. Fig. 5 shows that BW5147 cells express large amounts of Thy-1 mRNA which are detectable in isolates of total RNA. The Thy-1 mRNA of the same size was detected in undifferentiated P19 cells. However, to be able to detect Thy-1 mRNA in the latter cells, poly $(A)^+$ RNA isolated from 1 mg of total RNA had to be used for such analysis. Estimating that P19 poly $(A)^+$ RNA bound half of the radiolabeled probe, compared to BW5147 total RNA, we can calculate that P19 cells possess approximately two order lower amount of Thy-1 mRNA than BW5147 cells.

Effect of retinoic acid on Thy-1 antigen expression

Data presented in Fig. 6 show that P19S1801A1 or P19ST.1.3 cells exposed to progressively higher concentrations of retinoic acid (all-trans) exhibit a progressive decline in the binding of ^{125}I-labeled 1aG4 antibody. At a concentration of 10^{-6} M there was a 90% decrease in the binding. Significant reduction in the number of Thy-1.2^+ cells was observed after 24 h incubation in the presence of 5 x 10^{-7} M retinoic acid, and after three days there were no Thy-1.2 positive cells detectable at the level of indirect immunofluorescence assay using a Leitz fluorescence microscope (Fig. 7) or fluorescence-activated cell sorter (Fig. 8).

Discussion

In this study we have shown that undifferentiated P19 cells bind monoclonal antibody 1aG4. Several lines of evidence indicated that the antibody bound to Thy-1 antigen expressed on P19 EC cells and not to a cross-reactive epitope or to the differentiated derivatives of these cells. First, the antibody bound to several P19-

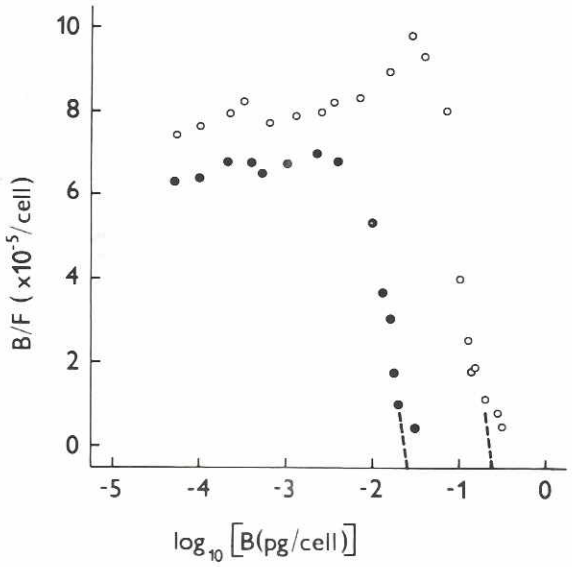

Fig. 3. Analysis of the binding of ^{125}I-labeled 1aG4 antibody to P19S1801A1 cells (●) and to mouse A/Ph thymocytes (○). *The data were plotted according to the method of Scatchard (1949) using log$_{10}$ scale for bound antibody (B). F represents amount of free antibody.*

derived EC cell lines, including retinoic acid-resistant mutant cells RAC65, with impaired ability to differentiate *in vitro*. The cells expressing Thy-1 antigen had properties of EC cells such as typical morphology and the ability to differentiate *in vitro*, and they expressed SSEA-1. Second, using congenic mouse strains, it was confirmed that the antibody is specific for Thy-1.2 antigen. In addition, the binding of the radiolabeled 1aG4 antibody to P19 EC cells was removed by absorption with Thy-1.2$^+$ brain and thymus cells but not with the Thy-1.1 cells. As determined by saturation analysis, the antibody bound similarly to both Thy-1.2$^+$ thymocytes and P19-derived EC cells. However, although the latter cells had a roughly 5-fold larger surface area than thymocytes, they expressed one order fewer Thy-1 epitopes than thymus cells. The low density of Thy-1 antigen in P19 cells may favor univalent binding and this may explain why some anti-Thy-1.2 antibodies with a relatively low association constant do not bind effectively to P19 EC cell lines. Certain other EC cell lines exhibited weak binding of 1aG4 antibody in direct RIA. Because this binding was below the sensitivity of indirect immunofluorescence assay, we cannot exclude the possibility that the binding to other cell lines was due to the presence of differentiated cells. This would agree with previous data on the expression of Thy-1 on cells derived from Thy-1$^-$ teratocarcinoma stem cells (Stern *et al.*, 1975). Third, in the direct RIA and indirect immunofluorescence assay the antibody reacted with the majority of P19 cells. When these cells were treated with retinoic acid, they differentiated into Thy-1.2$^-$ fibroblast-like cells. Fourth, the 1aG4 antibody precipitated specifically [D-6-^{3}H]galactose-labeled glycoprotein of apparent molecular weight 25,000, which corresponds to the apparent molecular weight of Thy-1 glycoprotein (Williams and Gagnon, 1982). Finally, after transfection of the cloned Thy-1 gene into P19 cells they expressed products of both endogenous and transfected genes. This confirmed that P19 cells are able to initiate transcription from the Thy-1 gene promoter. Direct evidence for transcriptional activity of the Thy-1 gene came from Northern blot analysis with Thy-1 specific cDNA probe.

The differences in the expression of Thy-1 antigen among various EC cell lines may reflect different developmental stages at which early embryo-like EC cells are immortalized. Another explanation is that immorta-

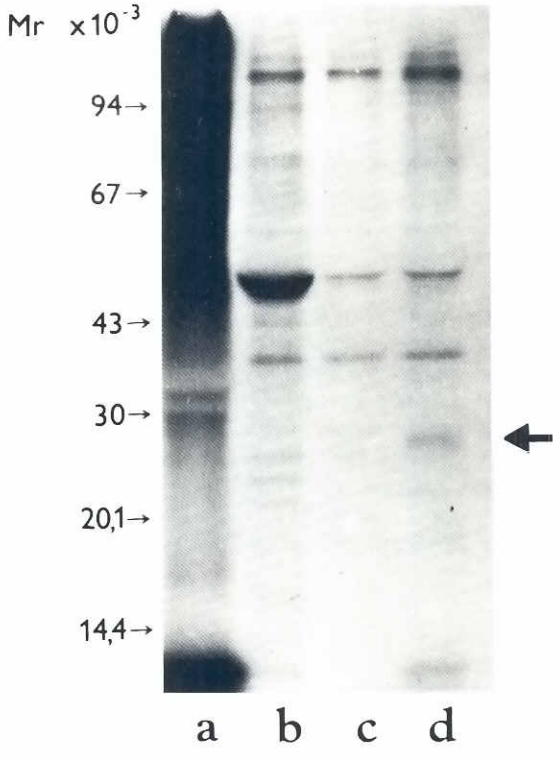

Fig. 4. Immunoprecipitation of Thy-1.2 antigen from P19S1801A1 cells. *The cells were metabolically labeled with [D-6-^{3}H]galactose and solubilized in lysis buffer. Cell extract was precleared with* S. aureus *loaded with rabbit anti-mouse IgG. The precleared extract was incubated with* S. aureus *armed with anti-Thy-1.1 or anti-Thy-1.2 antibody. Bound material was released by boiling in SDS sample buffer and analyzed by SDS-PAGE on a 10% gel under reducing conditions, followed by fluorography. Lane a: cell extract (50,000 cpm); lane b: material bound to* S. aureus *loaded with rabbit anti-mouse IgG (4600 cpm); lane c: material immunoprecipitated with anti-Thy-1.1 antibody, clone MRC OX 7 (1200 cpm); lane d: material immunoprecipitated with anti-Thy-1.2 antibody, clone 1aG4 (3275 cpm). The position of molecular weight markers is shown: phosphorylase b (94,000), BSA (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), and α-lactalbumin (14,400). Big arrow indicates the position of Thy-1 antigen of apparent molecular weight 25,000.*

TABLE 3

REACTIVITY OF ANTI-Thy-1 ANTIBODIES WITH EC CELL LINES TRANSFECTED WITH CLONED Thy-1.1 GENE

Cell line	^{125}I-labeled antibody bound (% cpm in cell pellet)[a]	
	1aG4	MRC OX 7
P19S1801A1-derivatives		
P190-gT1.1/T2.A4	6.6	11.7
P190-gT1.1/T2.A5	3.7	11.5
P190-gT1.1/T2.C6	3.2	9.5
P19XT.1.1-derivatives		
P19XT-gT1.1/T4.B2	1.9	8.2
P19XT-gT1.1/T4.B3	2.0	7.6
P19XT-gT1.1/T4.B5	1.6	6.4

[a] Transfected cells (2.5 x 10^5) were incubated with the anti-Thy-1 antibodies and the percentage of cpm bound was determined in direct RIA. Average values of triplicate measurements from two to four experiments are shown.

lization of P19 cells was accompanied by a modulation of the transcription factor level which led to transcription from the Thy-1 gene promoter. However, this transcription, compared to e.g. BW5147 cells, seems to be relatively inefficient (see Fig.5).

The finding that Thy-1 is expressed on P19 EC cells and embryoglycan-negative mutants derived from these cells by immunoselection (Dráber and Maly, 1987) is also important for interpretation of the developmental stage of these mutants. Although unlikely, the possibility remained that the embryoglycan-defective phenotype might reflect the phenotype of differentiated cells rather than a mutation in developmentally regulated gene(s). The expression of Thy-1 on the surface of the mutants makes this interpretation very unlikely, because Thy-1 is a marker of undifferentiated P19 cells. Moreover, our data indicate that significant changes in the expression of glycosyltransferases and other enzymes involved in building up of embryoglycan do not affect the expression of Thy-1 antigen.

Combined data indicate that Thy-1 extends the number of cell surface markers that are modulated during EC cell differentiation. A number of the well characterized markers are poorly expressed in the stem cells but efficient transcription occurs after differentiation. These include H-2 (Croce *et al.*, 1981), plasminogen activator (Strickland *et al.*, 1980; Rickles *et al.*, 1989), collagen type IV and laminin (Hogan, 1980; Strickland *et al.*, 1980), and α-fetoprotein (Young and Tilghman, 1984). Some other markers, such as SSEA-1 (Solter and Knowles, 1978), ECMA 2 and ECMA 3 (Kemler *et al.*, 1979), and TEC-5 (Dráber *et al.*, 1988) are expressed in the stem cells and disappear during EC cell differentiation. However, these markers are located in the carbohydrate moiety of the surface glycoconjugates and are not amenable to direct genetic analysis. The finding that Thy-1 glycoprotein disappears early during retinoic acid-induced differentiation of P19 EC cells is,

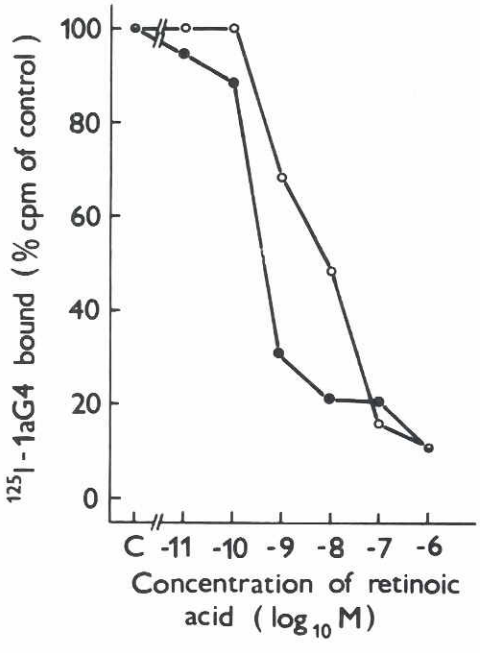

Fig. 6. Relationship between retinoic acid concentration and expression of Thy-1.2 antigen. *P19S1801A1 cells (●) and P19ST.1.3 cells (○) were cultured in the absence of (C) or in the presence of the indicated concentrations of retinoic acid for 52 h. The cells were harvested and the binding of ^{125}I-labeled 1aG4 antibody to the cells was determined in direct RIA. The binding to control cells, cultured in the absence of retinoic acid, was taken as 100%.*

Fig. 5. Northern blot analysis of Thy-1 mRNA. *Lane a: 20 μg total BW5147 cell RNA; lane b: 10μg poly (A)$^+$ RNA obtained from P19S1801A1 EC cells. A* Pst1 *fragment from Thy-1 cDNA, clone TM8, was used as a probe. Markers on the left are 18S and 28S ribosomal RNAs.*

therefore, important for analysis of the mechanisms behind down-regulation of genes expressed in EC cells. Evidence exists that DNA methylation of 5' sequences in the Thy-1 gene can play a central role in regulating expression of this gene in murine T cell lymphoma EL-4 (Sneller and Gunter, 1987). Interestingly, the Thy-1 gene promoter lacks TATA box sequences, is located within a methylation-free island and exhibits tissue-specific DNase I-hypersensitve sites, which seems to be associated with Thy-1 gene transcription (Sneller and Gunter, 1987; Spanopoulou *et al.*, 1988). It is possible that retinoic acid exerts its effect on Thy-1 gene expression via retinoic acid receptor (Evans, 1988) which may function in the case of Thy-1 gene in P19 EC cells as a transcriptional suppressing factor. Alternatively, the retinoic acid-receptor complex may induce the activity of other transcriptional factors.

In conclusion, in P19-derived stem cells the expression of Thy-1 is associated with maintaining pluripotency and rapid growth of the cells. During retinoic acid-induced differentiation, the Thy-1 antigen disappears early, before the onset of morphological differentiation. Identification of the nucleotide sequence which is recognized by the retinoic acid-receptor complex may be the key for elucidating the mechanism of modulation of Thy-1 antigen expression in differentiating EC cells at the molecular level.

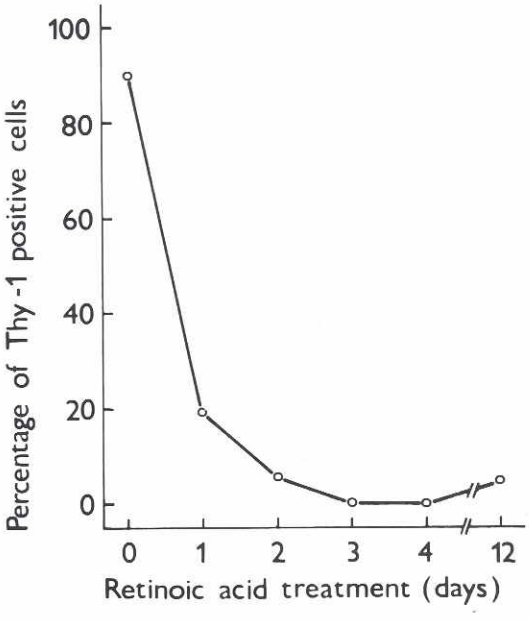

Fig. 7. Changes in the expression of Thy-1.2 antigen on the surface of P19S1801A1 cells during retinoic acid-induced differentiation. *Monolayer cultures were incubated with 0.5 μM retinoic acid for the number of days indicated. The cells were harvested and the percentage of Thy-1.2 (1aG4) positive cells was determined by an indirect immunofluorescence assay.*

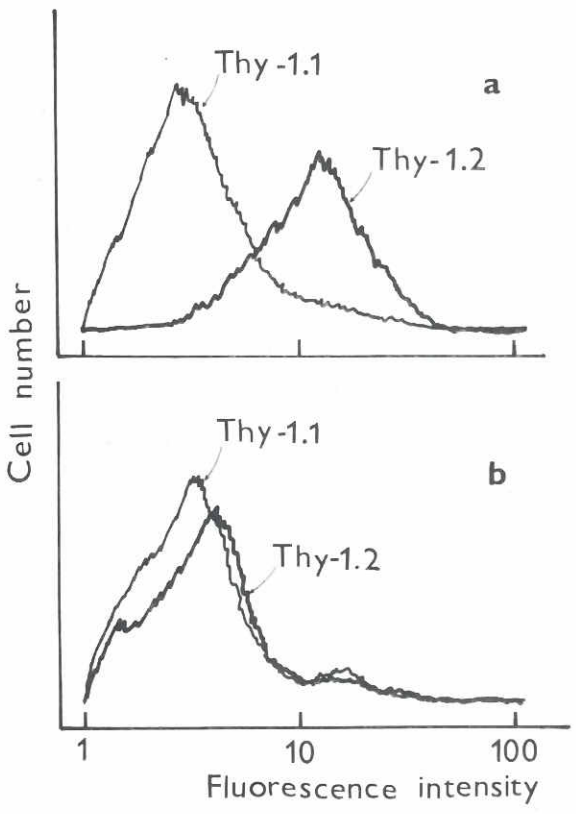

Fig. 8. Flow cytofluorimetric analysis of the expression of Thy-1.2 antigen in untreated and retinoic acid-treated P19S1801A1 cells. *Normal untreated cells* **(a)** *or retinoic acid-treated (0.5 μM: 3 days) cells* **(b)** *were harvested and labeled with anti-Thy-1.1 antibody (MRC OX 7) or anti-Thy-1.2 antibody (1aG4).*

Materials and Methods

Cells and culture techniques

Cell lines used in these experiments and their origins are described in Table 1. EC cell lines expressing both Thy-1.2 and Thy-1.1 genes were obtained after transfection of an 8.2-kb *EcoRI* fragment containing the Thy-1.1 gene (Giguere *et al.*, 1985) into P19S1801A1 cells (clones P190-gT1.1/T2.A4, P190-gT1.1/T2.A5, and P190-gT1.1/T2.C6) or into embryoglycan-defective P19XT.1.1 cells (clones P19XT-gT1.1/T4.B2, P19XT-gT1.1/T4.B3, and P19XT-gT1.1/T4.B5). The isolation of these transfected cell lines and their properties will be described elsewhere. The cultures were grown in 1:1 mixture of Eagle's minimal essential medium, supplemented with nonessential amino acids, 3 mM L-glutamine and 1 mM pyruvate sodium, (H-MEMd) and RPMI-1640 medium supplemented with penicillin (100 units/ml), streptomycin (100 μg/ml), extra glucose (2.5 mg/ml) and 10% (v/v) heat-inactivated fetal calf serum. All cultures were maintained at 37°C in an atmosphere of 5% CO_2 in air. Cell cultures were Mycoplasma-free as judged by the Hoechst staining method (Chen, 1977). To induce differentiation, the cells were released by treatment with 10 mM sodium phosphatase, 150 mM NaCl, pH 7.2 (PBS) containing 0.02% EDTA and 0.01% trypsin, harvested, washed, and plated into tissue culture dishes in culture medium supplemented with all-trans retinoic acid (Sigma). Thymus, spleen, and brain cells were isolated from females of A/Ph or A.Thy-1.1 mouse

strains from the breeding colony of the Institute of Molecular Genetics, Prague.

Monoclonal antibodies

Monoclonal anti-Thy-1.2 antibody of the IgG_3 subclass, clone 1aG4, was obtained as hybridoma supernatant or ascites fluid as described previously (Dráber *et al.*, 1980). Another hybridoma cell line, clone F7 D5, producing anti-Thy-1.2 antibody of the IgM class (Lake *et al.*, 1979) was kindly provided by Dr. I. Hilgert from the Institute of Molecular Genetics. Hybridoma cell line, MRC OX 7, producing anti-Thy-1.1 antibody of the IgG_1 subclass (Mason and Williams, 1980), was obtained from Dr. P. Thorpe, Imperial Cancer Research Fund, London, UK. Control IgG_3 antibody, HL-37, directed against a determinant present on human DQ1+DQ3 molecules (Hořejší *et al.*, 1986), was kindly provided by Dr. V. Hořejší from the Institute of Molecular Genetics. Hybridoma cell line producing TEC-01 antibody has been described earlier (Dráber and Pokorná, 1984). This antibody recognizes the same oligosaccharide sequence Gal(β1-->4)- [Fuc(α1-->3)] GlcNAc as anti-SSEA-1 antibody (Gooi *et al.*, 1981). The antibodies were purified from ascites fluid by 50% saturated ammonium sulfate precipitation followed by gel filtration through Sephacryl S-300 (Pharmacia Fine Chemicals, Uppsala, Sweden). The final product was more than 80% pure immunoglobulin by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE).

Radioantibody binding assay

Thirty-μg portions of immunoglobulins were iodinated by the chloramine T method as described (Stanley and Carver, 1977). The specific activities of ^{125}I-labeled antibodies were 1.2 - 2.3 x 10^7 cpm/μg. To measure binding to cells, the ^{125}I-labeled antibody was mixed with 2.5 x 10^5 or 1 x 10^6 cells in H-MEMd supplemented with 1% bovine serum albumin (BSA) in a final volume of 100 μl. After 1 h at 4°C, the cells were separated from unbound antibody by centrifugation (1200 x g for 10 min at 4°C) through a layer of 12% BSA in H-MEMd. Then the tubes were frozen, the tips cut, and the radioactivity bound to the cell pellet and supernantant was counted separately in a gamma counter. Binding of antibodies over 4 logs of concentrations was analysed as described (Dráber and Stanley, 1984). Indirect radioantibody binding assay (RIA) using ^{125}I-labeled affinity purified rabbit anti-mouse IgG was performed as described (Dráber *et al.*, 1985).

Indirect immunofluorescence

Cells were harvested with 0.02% EDTA in PBS, washed twice in tissue culture medium and sequentially reacted with monoclonal antibody (40 μg/ml) and fluorescein isothiocyanate-conjugated swine anti-mouse IgG (Institute of Sera and Vaccines, Prague) diluted 1:10 in PBS. Cells were examined by flow cytometry using FACS 440 (Becton Dickinson). Microphotographs were obtained using a Leitz fluorescence microscope equipped with epi-illumination. Binding of antibodies to preimplantation-stage embryos was determined as described (Dráber *et al.*, 1988).

Quantitative absorption analysis

Thymuses and brains were removed from A/Ph or A.Thy-1.1 mice and minced thoroughly in ice-cold H-MEMd-1% BSA. The cells were washed twice in H-MEMd-1% BSA, and various amounts of the cells were transferred to 1.5 ml polypropylene Eppendorf tubes. After centrifugation the cells were resuspended in 350 μl of H-MEMd-1% BSA containing ^{125}I-labeled 1aG4 antibody (5 x 10^5 cpm, 30 ng). Absorption was performed for 1 h at 4°C with shaking every 10 min. After centrifugation 70-μl aliquots of the absorbed supernantant were taken and mixed with 30 μl of H-MEMd-1% BSA containing 2.5 x 10^5 reference cells and the binding was analyzed as described above.

Cell labeling and immunoprecipitation

P19X1 cells were labeled metabolically with [D-6-^{3}H] galactose (2MBq/ml; 851 GBq/mmol: Amersham, UK) for 11 h as described (Dráber, 1987). The cells were harvested with EDTA in PBS, washed three times in PBS and 20 x 10^8 cells were lysed for 30 min in 1 ml of ice-cold lysis buffer containing 1% Nonidet P-40, 2 mM phenylmethylsulfonyl fluoride in PBS. The nuclei were removed by centrifugation at 600 x g for 15 min at 4°C and the supernatant centrifuged at 16,000 x g for 10 min. Immunoprecipitation was carried out using *Staphylococcus aureus* Cowan strain (Sigma) armed with monoclonal antibodies. *S. aureus* was washed three times in lysis buffer and incubated 2 h at room temperature with affinity purified rabbit anti-mouse IgG (100 μg/ml in PBS). After washing, part of the armed *S. aureus* was incubated with purified anti-Thy-1.2 antibody (clone 1aG4) or anti-Thy-1.1 antibody (clone MRC OX 7) at a concentration of 200 μg/ml in PBS). The cell lysate (0.8 ml) containing [D-6-^{3}H]galactose-labeled material was precleared with 100 μl of packed *S. aureus* loaded with rabbit anti-mouse IgG. The precleared supernatant was divided in two parts, each incubated 6 h with 30 μl of *S. aureus* armed with the analyzed monoclonal antibody. Bacteria were then washed twice in washing buffer containing 0.05% Nonidet P-40 and 0.1% SDS in PBS and the absorbed proteins were dissociated by boiling for 5 min in 100 μl of SDS sample buffer containing 10 mM Tris-HCl, pH 7.6, 150 mM NaCl, 2% (w/v) SDS, and 2-mercaptoethanol. After centrifugation, the supernatant was collected and analyzed on SDS-PAGE according to Laemmli (1970). Slab gels were fluorographed according to the method of Bonner and Laskey (1974) and exposed to Medix Rapid X-ray film (Foma, Hradec Králové, Czechoslovakia).

Isolation of RNA and Northern blot analysis

Total RNA was isolated from BW5147 cells and undifferentiated P19S1801A1 cells with 3 M LiCl-6 M urea by the method of Auffray and Rougeon (1980). Poly $(A)^+$ RNA was prepared by oligo (dT) cellulose chromatography (Edmonds *et al.*, 1971). RNA was electrophoresed on 1% formaldehyde agarose gels and blotted onto nylon filter (Hybond N, Amersham). Hybridization was performed according to Church and Gilbert (1984), using [^{32}P] dATP (6000 Ci/mmol: Amersham)-labeled *Pst*1 fragment of Thy-1.2 cDNA (clone TM8: Hedrick *et al.*, 1984), in 50% formamide for 20 h at 42°C. The filter was washed four times (each washing for 10 min) in 200 ml of 2 x SSC (1 x SSC is 150 mM NaCl and 15 mM sodium citrate), containing 0.1% SDS, at room temperature and then once for 30 min with 200 ml of 0.1 x SSC plus 0.1% SDS at room temperature, and once for 30 min with the same buffer at 50°C, followed by autoradiography.

Acknowledgments

We wish to thank Dr. Frank Grosveld for providing us with the cloned Thy-1.1 genomic DNA, Dr. Stephen Hedrick for Thy-1 cDNA, clone TM8, our colleagues for monoclonal antibodies, Miluše Aulická and Jan Vodák for their excellent technical assistance, and Dr. Marta Rauscherová for her help in

preparing the manuscript. We also thank Dr. František Franěk and Dr. Miloš Němec for their critical reading of the manuscript.

References

ANDREWS, P.W., GOODFELLOW, P.N. and BRONSON, D.L. (1983). Cell-surface characteristics and other markers of differentiation of human teratocarcinoma cells in culture. In *Teratocarcinoma Stem Cells* (Eds. L.M. Silver, G.R. Martin and S. Strickland). Cold Spring Harbor Conferences on Cell Proliferation Vol. 10, Cold Spring Harbor Laboratory, Cold Spring Harbor, pp. 579-590.

AUFFRAY, C. and ROUGEON, F. (1980). Purification of mouse immunoglobulin heavy-chain messenger RNAs from total myeloma tumor RNA. *Eur. J. Biochem. 107*: 303-314.

BERNSTINE, E.G., HOOPER, M.L., GRANDCHAMP, S. and EPHRUSSI, B. (1973). Alkaline phosphatase activity in mouse teratoma. *Proc. Natl. Acad. Sci. USA 70*: 3899-3903.

BONNER, W.M. and LASKEY, R.A. (1974). A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem. 46* : 83-88.

CHEN, T.R. (1977). *In situ* detection of Mycoplasma contamination in cell cultures by fluorescent Hoechst 33258 stain. *Exp. Cell Res. 104:* 255-262.

CHURCH, G.M. and GILBERT, W. (1984). Genomic sequencing. *Proc. Natl. Acad. Sci USA 81:* 1991-1995.

CROCE, C.M., LINNENBACH, A., HUEBNER, K., PARNES, J.R., MARGULIES, D.H., APPELLA, E. and SEIDMAN, J.G. (1981). Control of expression of histocompatibility antigens (H-2) and β_2-microglobulin in F9 teratocarcinoma stem cells. *Proc. Natl. Acad. Sci. USA 78:* 5754-5758.

DALES, S., FUJINAMI, R.S. and OLDSTONE, M.B.A. (1983). Serologic relatedness between Thy-1.2 and actin revealed by monoclonal antibody. *J. Immunol. 131*: 1332-1338.

DRÁBER, P. (1987). The epitope of mouse embryonic antigen(s) recognized by monoclonal antibody TEC-02 is a carbohydrate carried by high-molecular-weight glycoconjugates. *Cell Differ. 21*: 119-130.

DRÁBER, P. and MALY, P. (1987). Mutants of embryonal carcinoma cells defective in the expression of embryoglycan. *Proc. Natl. Acad. Sci. USA 84*: 5798-5802.

DRÁBER, P. and POKORNÁ, Z. (1984). Differentiation antigens of mouse teratocarcinoma stem cells defined by monoclonal antibodies. *Cell Differ. 15*: 109-113.

DRÁBER, P., POKORNÁ, Z., NOSEK, J. and HINZOVÁ, E. (1988). Inhibition of adhesion of F9 embryonal carcinoma cells to substratum by a novel monoclonal antibody, TEC-05, reactive with a developmentally regulated carbohydrate epitope. *Differentiation 37*: 205-214.

DRÁBER, P., POKORNÁ, Z. and VOJTÍŠKOVÁ, M. (1985). Antibodies against surface antigens common to spermatogenic and F9 teratocarcinoma cells in mice immunized with blood group substances from human saliva. *Exp. Clin. Immunogenet. 2*: 174-182.

DRÁBER, P. and STANLEY, P. (1984). Cytotoxicity of plant lectins for mouse embryonal carcinoma cells. *Somatic Cell Mol. Genet. 10*: 435-443.

DRÁBER, P. and VOJTÍŠKOVÁ, M. (1984). Developmentally regulated surface structures of teratocarcinoma stem cells studied by mutant cell lines. *Cell Differ. 15*: 249-253.

DRÁBER, P., ZIKÁN, J. and VOJTÍŠKOVÁ, M. (1980). Establishment and characterization of permanent murine hybridomas secreting monoclonal anti-Thy-1 antibodies. *J. Immunogenet. 7*: 455-474.

DULBECCO, R., UNGER, M., BOLOGNA, M., BATTIFORA, H., SYKA, P. and OKADA, S. (1981). Cross-reactivity between Thy-1 and a component of intermediate filaments demonstrated using a monoclonal antibody. *Nature (London) 292*: 772-774.

EDMONDS, M., VAUGHAN Jr., M.H. and NAKAZATO, H. (1971). Polyadenylic acid sequences in the heterogeneous nuclear RNA and rapidly-labeled polyribosomal RNA of HeLa cells: possible evidence for a precursor relationship. *Proc. Natl. Acad. Sci. USA 68*: 1336-1340.

EVANS, R.M. (1988). The steroid and thyroid hormone receptor superfamily. *Science 240*: 889-895.

GIGUERE, V., ISOBE, K. and GROSVELD, F. (1985). Structure of murine Thy-1 gene. *EMBO J. 4*: 2017-2024.

GOOI, H.C., FEIZI, T., KAPADIA, A., KNOWLES, B.B., SOLTER, D. and EVANS, M.J. (1981). Stage-specific embryonic antigen involves $\alpha 1 \rightarrow 3$ fucosylated type 2 blood group chains. *Nature (London) 292*: 156-158.

HEDRICK, S.M., COHEN, D.I., NIELSEN, E.A. and DAVIS, M.M. (1984). Isolation of cDNA clones encoding T cell-specific membrane-associated proteins. *Nature (London) 308*: 149-153.

HOGAN, B.L.M. (1980). High molecular weight extracellular proteins synthesized by endoderm cells derived from mouse teratocarcinoma cells and normal extraembryonic membranes. *Dev. Biol. 76*: 275-285.

HOŘEJŠÍ, V., NĚMEC, M., ANGELISOVÁ, P., KRIŠTOFOVÁ, H., GORGA, J.C. and HILGERT, I. (1986). Characterization of seven new monoclonal antibodies against human DR, DR + DP and DQ1 + DQ3 antigens. *Tissue Antigens 28*: 288-291.

INGRAHAM, H.A., LAWLESS, G.M. and EVANS, G.A. (1986). The mouse Thy-1.2 glycoprotein gene: complete sequence and identification of an unusual promoter. *J. Immunol. 136*: 1482-1489.

JAKOB, H., BOON, T., GAILLARD, J., NICOLAS, J.-F. and JACOB, F. (1973). Tératocarcinome de la souris: isolement, culture et propriétés de cellules á potentialités multiples. *Ann. Microbiol. (Inst. Pasteur) 124 B*: 269-282.

JONES-VILLENEUVE, E.M.V., McBURNEY, M.W., ROGERS, K.A. and KALNINS, V.I. (1982). Retinoic acid induces embryonal carcinoma cells to differentiate into neurons and glial cells. *J. Cell Biol. 94*: 253-262.

JONES-VILLENEUVE, E.M.V., RUDNICKI, M.A., HARRIS, J.F. and McBURNEY, M.W. (1983). Retinoic acid-induced neural differentiation of embryonal carcinoma cells. *Mol. Cell. Biol. 3*: 2271-2279.

KEMLER, R., MORELLO, D. and JACOB, F. (1979). Properties of some monoclonal antibodies raised against mouse embryonal carcinoma cells. In *Cell Lineage, Stem Cells and Cell Determination* (Ed. N. LeDouarin). INSERM Symposium No.10. Elsevier/North-Holland Biomedical Press, Amsterdam, pp. 101-113.

LAEMMLI, U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (London) 227*: 680-685.

LAKE, P., CLARK, E.A., KHORSHIDI, M. and SUNSHINE, G.H. (1979). Production and characterization of cytotoxic Thy-1 antibody-secreting hybrid cell lines. Detection of T cell subsets. *Eur. J. Immunol. 9*: 875-886.

LEHMAN, J.M., SPEERS, W.C., SWARTZENDRUBER, D.E. and PIERCE, G.B. (1974). Neoplastic differentiation: characteristics of cell lines derived from a murine teratocarcinoma. *J. Cell. Physiol. 84*: 13-28.

LOW, M.G. and KINCADE, P.W. (1985). Phosphatidylinositol is the membrane-anchoring domain of the Thy-1 glycoprotein. *Nature (London) 318*: 62-64.

MARTIN, G.R. (1980). Teratocarcinomas and mammalian embryogenesis. *Science 209*: 768-776.

MASON D.W. and WILLIAMS, A.F. (1980). The kinetics of antibody binding to membrane antigens in solution and at the cell surface. *Biochem. J. 187*: 1-20.

McBURNEY, M.W., JONES-VILLENEUVE, E.M.V., EDWARDS, M.K.S. and ANDERSON, P.J. (1982). Control of muscle and neuronal differentiation in a cultured embryonal carcinoma cell line. *Nature (London) 299*: 165-167.

McBURNEY, M.W. and ROGERS, B.J. (1982). Isolation of male embryonal carcinoma cells and their chromosome replication patterns. *Dev. Biol. 89*: 503-508.

MORRIS, R. (1985). Thy-1 in developing nervous tissue. *Dev. Neurosci. 7*: 133-160.

RAFF, M.C. (1971). Surface antigenic markers for distinguishing T and B lymphocytes in mice. *Transplant. Rev. 6*: 52-80.

REIF, A.E. and ALLEN, J.M. (1964). The AKR thymic antigen and its distribution in leukemias and nervous tissues. *J. Exp. Med. 120*: 413-433.

RICKLES, R.J., DARROW, A.L. and STRICKLAND, S. (1989). Differentiation-responsive elements in the 5' region of the mouse tissue plasminogen activator gene confer two-stage regulation by retinoic acid and cyclic AMP in teratocarcinoma cells. *Mol. Cell. Biol. 9*: 1691-1704.

ROSENSTRAUS, M.J., BALINT, R.F. and LEVINE, A.J. (1980). Pluripotency of somatic cell hybrids between nullipotent and pluripotent embryonal carcinoma cells. *Somatic Cell Genet. 6*: 555-565.

ROSSANT, J. and McBURNEY, M.W. (1982). The developmental potential of a euploid male teratocarcinoma cell line after blastocyst injection. *J. Embryol. Exp. Morphol. 70*: 99-112.

SCATCHARD, G. (1949). The attractions of proteins for small molecules and ions. *Ann. NY Acad. Sci. 51*: 660-672.

SEKI, T., SPURR, N., OBATA, F., GOYERT, S., GOODFELLOW, P. and SILVER, J. (1985). The human Thy-1 gene: structure and chromosomal location. *Proc. Natl. Acad. Sci. USA 82*: 6657-6661.

SNELLER, M.C. and GUNTER, K.C. (1987). DNA methylation alters chromatin structure and regulates Thy-1 expression in EL-4 T cells. *J. Immunol. 138*: 3505-3512.

SOLTER, D. and KNOWLES, B.B. (1978). Monoclonal antibody defining a stage-specific mouse embryonic antigen (SSEA-1). *Proc. Natl. Acad. Sci. USA 75:* 5565-5569.

SPANOPOULOU, E., GIGUERE, V. and GROSVELD, F. (1988). Transcriptional unit of the murine Thy-1 gene: different distribution of transcription initiation sites in brain. *Mol. Cell. Biol. 8*: 3847-3856.

STANLEY, P. and CARVER, J.P. (1977). Selective loss of wheat germ agglutinin binding to agglutinin-resistant mutants of Chinese hamster ovary cells. *Proc. Natl. Acad. Sci. USA 74:* 5056-5059.

STANLEY, P., SUDO, T. and CARVER, J.P. (1980). Differential involvement of cell surface sialic acid residues in wheat germ agglutinin binding to parental and wheat germ agglutinin-resistant Chinese hamster ovary cells. *J. Cell Biol. 85*: 60-69.

STERN, P.L., MARTIN, G.R. and EVANS, M.J. (1975). Cell surface antigens of clonal teratocarcinoma cells at various stages of differentiation. *Cell 6*: 455-465.

STRICKLAND, S., SMITH, K.K. and MAROTTI, K.R. (1980). Hormonal induction of differentiation in teratocarcinoma stem cells: generation of parietal endoderm by retinoic acid and dibutyryl cAMP. *Cell 21*: 347-355.

TSE, A.G.D., BARCLAY, A.N., WATTS, A. and WILLIAMS, A.F. (1985). A glycophospholipid tail at the carboxyl-terminus of the Thy-1 glycoprotein of neurons and thymocytes. *Science 230*: 1003-1008.

WILLIAMS, A.F. and BARCLAY, A.N. (1988). The immunoglobulin superfamily domains for cell surface recognition. *Annu. Rev. Immunol. 6:* 381-405.

WILLIAMS, A.F. and GAGNON, J. (1982). Neuronal cell Thy-1 glycoprotein: homology with immunoglobulin. *Science 216*: 696-703.

YOUNG, P.R. and TILGHMAN, S.M. (1984). Induction of α-fetoprotein synthesis in differentiating F9 teratocarcinoma cells is accompanied by a genome-wide loss of DNA methylation. *Mol. Cell. Biol. 4*: 898-907.

Accepted for publication: August 1989