Differential expression of the mouse and human *Thy-1* gene in embryonal carcinoma cells

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**ABSTRACT** Mouse P19 embryonal carcinoma (EC) cells express on their surfaces a *Thy-1* glycoprotein. The expression of *Thy-1* at the mRNA and protein levels is down-regulated during differentiation induced by retinoic acid (RA). *Thy-1* is also expressed in human NTERA-2 EC cells, but its expression is not down-regulated during RA-induced differentiation. As a first step towards understanding differential regulation of the mouse and human *Thy-1* gene in EC cells, we have introduced genomic DNA fragments encompassing the mouse or human *Thy-1* gene into NTERA-2 and P19-derived cells and analyzed surface properties of the transfectants. In the transient transfection assay, both mouse and human *Thy-1* genes were expressed on cell surfaces at comparable levels. P19-derived stable transfectants exhibited great clonal variations in the expressions of the transfected *Thy-1* gene products, which in part reflected copy numbers. There was no simple correlation between the expression of the transfected *Thy-1* gene and two stem cell surface markers, TEC-1 and TEC-4. In the course of differentiation induced by RA several clones with a surface phenotype of EC cells exhibited a significant decrease in the expression of the transfected mouse *Thy-1*, whereas expression of the human *Thy-1* was less efficiently down-regulated. The results suggest the presence of multiple cis- and trans-acting elements controlling expression of the mouse and human *Thy-1* genes in P19 EC cells and their differentiated derivatives.

**KEY WORDS:** *Thy-1*, retinoic acid, cell differentiation, embryonal carcinoma

Embryonal carcinoma (EC) cells are multipotent stem cells of teratocarcinomas whose developmental potential closely resembles that of early embryonic cells. EC cells express on their surfaces several antigenic determinants which disappear during differentiation in vitro (for review see Muramatsu, 1988). The epitopes, defined by monoclonal antibodies, are carbohydrates and are carried predominantly by a high molecular weight glycoconjugate, embryoglycan (Muramatsu, 1988). Recently we have shown that one of our previously prepared monoclonal antibodies, 1aG4, reacts with P19 EC cells but not with their differentiated derivatives (Dráber et al., 1989a). This antibody recognizes the *Thy-1* antigen, which has been extensively characterized, and the corresponding gene has been cloned and sequenced (Giguère et al., 1985; Seki et al., 1985). The *Thy-1* gene is a member of the immunoglobulin superfamily of genes and codes for a 112 amino acid surface glycoprotein that is anchored to the membrane through a phosphatidylinositol-containing membrane domain (Williams and Gagnon, 1982; Low and Kincade, 1985; Tse et al., 1985).

The expression of *Thy-1* on teratocarcinoma-derived cells was first reported by Stern et al. (1975). These authors found that a fraction of cells of the heterogeneous teratocarcinoma cell line SIKR bound *Thy-1*-specific antibody, whereas homogeneous SIKR-derived EC cells were *Thy-1* negative. *Thy-1* antigen was found on differentiated derivatives of the EC cells after their long-term culturing in vitro. These results were confirmed by analyzing the mRNA isolated from F9 cells. Undifferentiated F9 cells had no detectable *Thy-1* mRNA, which was switched on after differentiation induced by RA (Giguère et al., 1985). The reverse pattern of *Thy-1* antigen expression was observed in P19 EC cells; undifferentiated P19 cells were *Thy-1*+ whereas their differentiated derivatives were *Thy-1* (Dráber et al., 1989a). In human EC cells, the *Thy-1* antigen was found to be expressed in both the undifferentiated and differentiated cells (Andrews et al., 1983). Thus, expression of *Thy-1* glycoprotein in EC cells depends on the origin of the corresponding gene (human versus mouse) and on the cell type.

As a first step towards understanding the differential regulation of the mouse and human *Thy-1* genes in EC cells, we have introduced an 8.2 kilobase (kb) fragment encompassing the mouse or human *Thy-1* gene into P19 EC cells. Previous studies from several

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Abbreviations used in this paper: DMSO, dimethylsulfoxide; EC, embryonal carcinoma; RA, retinoic acid.
mRNA, as detected by Northern blot analysis, correlated with changes in the expression of surface complex of RA with its receptor(-s) may be directly involved in the inhibition of down-regulation of laboratories. Chang et al. (1987) and Gordon et al. (1987) have shown that the expression of Thy-1 gene is dependent on the activity of transcriptional factors. In this paper, we describe properties of P19-derived cell lines with the transfected mouse or human Thy-1 gene and changes in Thy-1 gene expression during RA-induced differentiation.

To determine whether the decrease in the surface expression of Thy-1 in RA-treated P19 cells reflects changes at the mRNA level, we have analyzed Thy-1 mRNA in cells cultured for different periods of time with RA. Northern blot analysis indicated that Thy-1 mRNA is a sensitive indicator of RA-induced differentiation (Fig. 1). Rapid down-regulation of Thy-1 mRNA in RA-treated cells suggests that the complex of RA with its receptor(s) may be directly involved in the inhibition of Thy-1 gene transcription. Alternatively, the complex of RA with its receptor(s) may activate other genes whose products (transcriptional factors) may regulate Thy-1 gene expression in a negative manner. Because changes in the expression of Thy-1 mRNA, as detected by Northern blot analysis, correlated with changes in the expression of surface Thy-1 antigen, as detected by 1aG4 antibody binding (Dräber et al., 1989a), we employed a simple direct radioantibody binding assay to analyze the expression of both endogenous and exogenous Thy-1 genes in transfected cells.

To determine whether the 8.2 kb Eco RI genomic fragments encompassing the human or mouse Thy-1 gene can be expressed in mouse and human EC cells, we evaluated the expression of the transfected genes in transient expression assays (Table 1). Transfer of human Thy-1 or mouse Thy-1 gene into P19S1801A1 and NTERA-2 cells resulted in significant binding of the corresponding antibodies to the transfected cells. Antibodies recognizing the transfected gene products bound to the transfected cells at equal or greater levels than antibodies to the endogenous Thy-1 gene products. These data indicated that the transfected DNA fragments carried all regulatory sequences necessary for expression of the Thy-1 genes in EC cells and, therefore, we prepared stable cell lines expressing the transfected gene products.

In the first series of experiments, circular plasmids or isolated linear Eco RI fragments containing the mouse or human Thy-1 gene were introduced into P19S1801A1 cells by co-transfection with a vector, pSTneoB, transferring resistance to G418 and the transfected cells were selected in culture medium supplemented with G418. Individually growing colonies, visible after approximately two weeks, were expanded and analyzed for the surface expression of the transfected gene products (Fig. 2). The majority of colonies (19 of 22) isolated after transfection with the isolated Eco RI fragment of human Thy-1 gene expressed hThy-1 antigen detectable by radioantibody binding assay. Thy-1 antigen expression varied greatly. When the hThy-1 gene in the form of a circular plasmid was transfected into P19S1801A1 cells, the corresponding Thy-1 antigen was found on fewer colonies (13 of 48) and the antibodies bound to the positive cells less efficiently. A significant difference between linearized and circular form of human or mouse Thy-1 genes was also observed in cell lines with the transfected mouse Thy-1.1. These results were confirmed in a second series of experiments, in which the linearized or circular form of human or mouse Thy-1 genes were transfected into P19XT.1.1 cells, which are embryoglycan-defective derivatives of P19X1 cells (Dräber and Maly, 1987). After cloning and selection of the cell lines with stable expression of the transfected Thy-1 genes, we analyzed copy numbers of the genes transfected. Southern blotting analysis revealed considerable variation in the number of copies of the transfected genes (Table 2). There was no simple relationship between the gene copy number and surface antigen expression.

Stable cell lines expressing the transfected gene products were analyzed in detail for expression of TEC-1 and TEC-4. We have

Table 1

<table>
<thead>
<tr>
<th>Cells</th>
<th>Gene transfected</th>
<th>αThy-1.2</th>
<th>αThy-1.1</th>
<th>αmThy-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>P19S1801A1</td>
<td>None</td>
<td>6.4</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>P19S1801A1</td>
<td>Thy-1.1</td>
<td>6.4</td>
<td>14.0</td>
<td>0.2</td>
</tr>
<tr>
<td>P19S1801A1</td>
<td>hThy-1</td>
<td>7.2</td>
<td>0.3</td>
<td>13.5</td>
</tr>
<tr>
<td>NTERA-2</td>
<td>None</td>
<td>0.2</td>
<td>0.2</td>
<td>3.7</td>
</tr>
<tr>
<td>NTERA-2</td>
<td>Thy-1.1</td>
<td>0.2</td>
<td>12.1</td>
<td>3.4</td>
</tr>
</tbody>
</table>

*The cells were analyzed for expression of surface antigens using a direct radioantibody binding assay as described in Experimental Procedures. Numbers represent percentage of radioactivity bound to cells. Mean values of at least two experiments performed in triplicates are shown.
previously shown that TEC-4 is a sensitive marker of EC cell differentiation and that its decrease precedes the decrease in the expression of TEC-1 (Draber et al., 1989a,b). According to the expression of these markers, the transfected cell lines presented in Table 2 can be divided into several groups. It should be noted that because P19S1801A1- and P19XT.1.1-derived cell lines behaved similarly in the tests employed, we did not divide the transfected cell lines into subgroups according to their origin and combined them only according to their differentiation state. Among the P19S1801A1-derived cell lines with the transfected human Thy-1 gene, we have found only one, HLP-1, with the surface phenotype of undifferentiated EC cells. Several other cell lines expressing hThy-1 and with the morphology of EC cells exhibited a significant decrease in the expression of TEC-1 epitope (clones 5-9). Surprisingly, these cells showed a relatively high level of TEC-4 epitope, compared to TEC-1 epitope. Only in HLP-10 cells was the TEC-1 more strongly expressed than TEC-4, suggesting an early stage of differentiation. All P19XT.1.1-derived cell lines (clones 2-4) had the morphology and antigenic makeup of undifferentiated EC cells except for the absence of TEC-1, which was removed genetically (Draber and Maly, 1987). Three cell lines (11-13) with the morphology of differentiated fibroblast-like cells and with a relatively high level of the exogenous Thy-1 had very weak expression of both TEC-1 and TEC-4 epitopes.

Cell lines with an exogenous Thy-1.1 gene exhibited weaker expression of the Thy-1 antigen compared to cells with transfected human Thy-1 and, again, most of them, although having the morphology of EC cells, exhibited relatively low expression of TEC-1 compared to TEC-4 expression. MLP-14 was the only cell line with high expression of both TEC-1 and TEC-4 epitopes. The MLP-20 cells on the other hand had the morphology of differentiated cells and exhibited decreased expression of both stem cell markers.

To determine whether mouse and human Thy-1 genes will respond to changes induced by RA, the transfected cells were cultured in the presence of 1 μM RA for 4 days and their surface antigenic phenotype was determined. Again, the expression of surface antigens varied considerably among individual clones after differentiation. In EC cells expressing human Thy-1 gene (clones 1-9), treatment with RA induced a marked decrease in the expression of TEC-4; if expression of TEC-4 in untreated cells is taken as 100%, then the mean decrease was by 94%. In the same cells the mean decrease in hThy-1 antigen expression was only by 14%.

Similar analysis of cell lines with the integrated mouse Thy-1.1 gene (clones 14-19) showed a decrease in TEC-4 antigen expression (mean decrease by 92%); mean decrease in expression of the transfected Thy-1.1 gene product was by 57%.

In several cell lines with low expression of TEC-4, different results were obtained. In the TEC-1* cell line (HLP-10), there was only a weak down-regulation of both the endogenous and transfected Thy-1 gene products. However, these cells were able to respond to RA as determined by pronounced changes in the expression of TEC-1 and TEC-4 epitopes. RA had no significant effect on the expression of Thy-1 genes in cells with morphology of differentiated cells and expressing low amounts of the TEC-1/TEC-4 epitopes (clones 11-13 and 20). HLP-11 cells resembled 3T3 fibroblasts in their relatively high levels of the endogenous Thy-1 which were slightly up modulated after treatment with RA. Other clones of differentiated cells expressed low levels of the endogenous Thy-1 and relatively high levels of the transfected Thy-1 gene product and responded to RA treatment weakly or not at all.

The results reported here indicate that down-regulation of the Thy-1 antigen expression on the surface of RA-treated P19 EC cells reflects changes at the Thy-1 mRNA level. This implies that this
regulation occurs at the level of Thy-1 gene expression or Thy-1 mRNA processing. Comparison of the expression of the Thy-1 gene products in undifferentiated EC cells and their RA-induced differentiated derivatives showed that both exogenous Thy-1 gene products of mouse Thy-1.1 and human Thy-1 are less efficiently downregulated compared to endogenous Thy-1.2 and that mouse Thy-1 is more efficiently downregulated than human Thy-1. The combined data indicate different regulation of the mouse and human Thy-1 genes in differentiating mouse EC cells. Recently Vidal et al. (1990) have described that tissue-specific enhancer elements in the Thy-1 genes are located downstream from the site of transcriptional initiation and that these elements are different in human and mouse Thy-1. At present we do not know which of these elements are important for the expression of Thy-1 in EC cells. However, significant heterogeneity in Thy-1 gene expression in EC cells and their differentiated derivatives suggests that other more distant elements are involved and that the site of integration is important for the expression of the transfected genes.

### Experimental Procedures

#### Cell lines

The origin and properties of the mouse EC cell lines (P19X1, P19Cl.801A1 and P19X1-derived embryogonfatic defective P19XT1.1), human EC cell line (NTERA-2), mouse fibroblast cell line (3T3) and culture conditions have been described (Dräber, 1987; Dräber et al., 1989). To induce differentiation, the cells were harvested, washed and plated into tissue culture dishes in culture medium supplemented with all-trans retinoic acid (RA; Sigma Chemical Co., St. Louis, MO).

#### Monoclonal antibodies

Antibodies specific for mouse Thy-1.1 (clone MRCOX7) and human Thy-1 (clone F15-42-1-5) have been described (Dalchau and Fabre, 1979; Dräber et al., 1980; Mason and Williams, 1980). TEC-01 antibody (Dräber and Pokorná 1984) recognizes the same carbohydrate epitope, Ga1[3-4]Fuc[1-3]GalNAc, as does SSEA-1 specific antibody (Gooi et al., 1981), whereas TEC-04 antibody recognizes an EC cell surface glycoprotein epitope (Dörr et al., 1989). The antibodies were isolated from ascites fluid by precipitation at 50% ammonium sulfate saturation and purified by ion-exchange chromatography on DEAE-Sephadex A-50 (Pharmacia Fine Biochemicals, Uppsala, Sweden).

#### Plasmids

The 8.2 kb Eco RI genomic fragments encompassing the mouse Thy-1.1 or human Thy-1 gene were obtained as pBSV-derived plasmids pBST1 (Giguere et al., 1985) and pBSHT (van Rijs et al., 1989), respectively, from Dr. F. Grosveld. A plasmid containing a pCDI fragment of mouse Thy-1.2 cDNA, clone TM8 (Hedrick et al., 1984), was obtained from Dr. S. Hedrick. The pCD-29-5A clone (Hiraki et al., 1986), in which Thy-1.2 cDNA is under the control of SV40 promoter, was a gift from R.L. Coffman. For selection of transfected cells we used the pStneoB vector, in which the bacterial aminoglycoside 3’ phosphotransferase gene is under the control of the neomycin analog G418 on mammalian cells, was provided by K. Katoh.

#### Transfection and selection of stable clones

DNA transfection was performed by a modification of the method of Chen and Okayama (1987). EC cells (5 x 10⁵) were seeded into a 60-mm culture dish, incubated in culture medium for one day, washed and then 500 μl of DNA mixture was added to 5 ml of incubation media on the plate. DNA mixture containing 9 μg of DNA of mouse Thy-1.1 or human Thy-1 Eco RI genomic fragments encompassing the respective genes (in the plasmid vectors or excised from the plasmid by Eco RI and electrophoretically purified) and 1 μg of pStTcB plasmid DNA in 0.5 ml transfection buffer containing 125 mM CaCl₂, 25 mM N,N-bis(hydroxyethyl)-2-aminoethane-sulfonic acid (pH 7.0), 140 mM NaCl, and 0.75 mM NaH₂PO₄, After 4 h of incubation at 37°C and 3% CO₂ in air, the medium was removed and 2 ml of 20% dimethylsulfoxide (DMSO) in transfection buffer was added. After 4 min of incubation, DMSO was replaced with culture medium. Next, the cells were harvested and transferred into a 90-mm tissue culture dish in selective medium containing 500 μg/ml of G418 antibiotic (Genetocin, Sigma). Surviving clones were picked out after 14 days and expanded for further analysis. Transient expression assay was performed in the same way except that pStTcB DNA was omitted from the DNA mix. Two days after transfection, the cells were harvested and analyzed by a direct radioantibody binding assay.

#### Northern blot analysis

Total RNA was isolated by a modified method of Auffray and Rougeon (1980) using B Murae-3 M UCl. Twenty μg of total RNA was electrophoretically separated on a 1% formaldehyde agarose gel and blotted onto nylon membranes (Hybond N, Amersham, England). A 570 basepair (bp) Pat I
fragment of mouse Thy-1 cDNA or a 161.7 bp fragment of rat α-Tubulin cDNA (Lemischka et al., 1981) labeled by α-32P-deoxyadenosine 5'-triphosphate (3000 C/cm mol, Du Pont de Nemours, Bad Homburg, Germany) was used for hybridization. Hybridization was performed at 42°C in 50% formamide using Denhardt's solution with 10% dextran sulfate. The filters were washed four times for 10 min at room temperature in 100 ml of 2x standard sodium citrate (SSC), 0.1% sodium dodecyl sulfate (SDS), followed by two washes for 30 min at room temperature and then at 60°C in 0.1x SSC, 0.1% SDS. The filters were dried and exposed to X-ray films for 1-5 days.

Radioantibody binding assay and indirect immunofluorescence

These have been described (Dräber et al., 1989a). The specificity of the 125I-labeled antibodies was approximately 2x10^7 cpm/ug. The antibody bound specifically to the target cells as has been previously described (Dräber and Malý, 1987).

Southern blot analysis

Southern blot analysis was performed according to Maniatis et al., 1982.

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