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Genetics

Gene transfer in lymphoid cells: Expression of the Thy-1.2 antigen by Thy-1.1 BW5147 lymphoma cells transfected with unfractionated cellular DNA

(positive immunoselection)

JOAN W. BERMAN, ROSS S. BASCH, AND ANGEL PELLICER

Department of Pathology and The Stanley Kaplan Cancer Center, New York University Medical Center, 550 First Avenue, New York, NY 10016

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ABSTRACT **We have transferred the gene coding for the Thy-1.2 alloantigen into a Thy-1.1-bearing T-cell lymphoma. BALB/c thymocyte DNA, precipitated with calcium phosphate, was used to effect the transfer. We report the stable transformation of lymphoid cells by total cellular DNA. To our knowledge, this has not been previously reported. Transient expression of the transfected gene could be detected by flow cytofluorometry, and 5% (range, 1.5%–11%) of the recipient cells had Thy-1.2 antigen detectable on their surface 48 hr after transfer. "Stable" transformants were isolated by repeatedly selecting for cells expressing the Thy-1.2 antigen, by use of fluorescence-activated cell sorting or "panning." No metabolic selection was required. The transferred gene, detected by Southern blotting, encoded a product that is indistinguishable from the normal antigen by immunoprecipitation and NaDodSO$_4$/PAGE.**

The recent development of techniques to transfer DNA into tissue culture cell lines permits the study of the physical state, regulation, and function of heterologous genes in new cellular environments. Since the introduction of the calcium phosphate-DNA coprecipitation technique (1) for transfer of genes from viruses of low DNA complexity, subsequent improvements have permitted the transfer of single copy genes using total mammalian cell DNA as donor (2, 3). More recently, the combination of recombinant DNA technology with high efficiency gene transfer has led to the isolation of some eukaryotic genes (4–6).

Transient expression of single copy genes after transfer of cellular DNA has been reported in both Daudi and L cells with efficiencies of 0.5%–1.0% (5, 7). Most experiments that achieved stable transfers were with mouse fibroblast lines L or 3T3 and required an appropriate selection system because the frequency of transfer of single copy genes was low ($\approx 10^{-5}$) (2, 3).

We now report the stable incorporation and expression of the gene encoding the murine T-cell alloantigen, Thy-1.2, into a Thy-1.1-bearing mouse T-cell lymphoma, BW5147, using DNA extracted from BALB/c thymocytes (Thy-1.2-expressing cells) and the calcium phosphate precipitation method of DNA-mediated gene transfer. Positive transformants expressing the surface marker are selected after 48 hr either by cell sorting or by "panning," and selective media are not required.

MATERIALS AND METHODS

Cell Lines. BW5147 is a ouabain-resistant, HPRT$^-$ AKR thymoma (8). It is maintained in Iscove's minimum essential medium supplemented with 10% fetal calf serum/1% penicillin/streptomycin.

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Transformation. High molecular weight DNA was obtained from BALB/c thymocytes and from Thy-1.2-positive clones as described (9).

Cells are transformed by a modification of the $CaPO_4$ precipitation method (1, 2). Prior to transformation, BW5147 cells were resuspended in Dulbecco's modified Eagle's medium with 10% fetal calf serum and 1% antibiotics to a concentration of 5×10^5 cells per ml. Ten milliliters of precipitate containing 500 μg of BALB/c thymus DNA was added to 100 ml of cell suspension in a 500-ml roller bottle. Bottles were gently rolled at 37°C for 8 hr, after which the cells were pelleted and resuspended in Iscove's medium with fetal calf serum.

Cells used as controls were treated with calf-thymus DNA.

Southern Blotting. Southern blotting (10) was performed with the mouse Thy-1 cDNA clone, TM8, generously provided by Mark Davis. This clone hybridizes with the two Thy-1 alleles. Prior to nick-translation, the Thy-1 insert was removed from its vector. Appropriate DNAs were digested for 4 hr at 37°C with *Pvu* II (New England Biolabs) at a concentration of 3 units of enzyme per μg of DNA with the recommended buffer. Electrophoresis, blotting, hybridization, and washes were done as described (11).

Immunofluorescence. *Single label experiments.* Forty-eight hours after transformation, 10^6 cells were resuspended in 50 μl of a 1:50 dilution of biotinylated monoclonal rat anti-mouse Thy-1.2 antibody (Becton Dickinson clone 30H12) in phosphate-buffered saline (P_i/NaCl)/1% bovine serum albumin/0.1% NaN_3 for 30 min at 4°C. Cells were washed 3 times and treated with 100 μl of a 1:100 dilution of fluoresceinated avidin for 10 min in the cold. After three washes, cells were pelleted and fixed in 100 μl of 4% formaldehyde and kept cold until examined either with a Leitz fluorescence microscope or by analysis in an Ortho 50H cytofluorograph. Cells treated with only fluoresceinated avidin served as an additional control. For flow cytometry, 20,000 cells were analyzed.

Double label studies. Cells were also examined for surface expression of both Thy-1.1 and Thy-1.2. Ten million cells were incubated with 10 μl of a 1:50 dilution of monoclonal mouse anti-Thy-1.1 ascites (New England Nuclear) in 100 μl of P_i/NaCl azide for 30 min at 4°C. After three washes, they were incubated with a 1:100 dilution of rhodaminated anti-mouse IgM (absorbed to remove reactivity with rat Ig) and 10 μl of biotinylated monoclonal rat anti-Thy-1.2 and incubated at 25°C for 30 min. The cells were again washed and treated with fluoresceinated avidin (1:100 dilution) for 10 min. The last two incubations were at room temperature to permit "capping' (12, 13), which greatly facilitates identification of double-labeled cells. Cells were washed and, after fixation with 4% formaldehyde, examined in a Leitz Ortholux microscope using epi-illumination with a Hg lamp. To detect rhodamine-labeled cells, the samples were illuminated using a 530–560 nm BP filter for excitation and an LP580 nm

filter in the emitted light. To detect fluorescein-labeled cells, illumination using a 450–490 nm BP filter was used and viewed through a 525/20 narrow BP filter. This filter combination is highly specific for fluorescein.

Iodination of Cells. Ten million BW5147, BALB/c thymocytes, control cells, and BALB/c-transformed BW5147 cells were surface-labeled with ^{125}I Iodogen (1,3,4,6-tetrachloro-3α,6α-diphenylglycouril) (Pierce) as described (14, 15).

Immunoprecipitation. Radiolabeled cells (10^7) were lysed with 0.5% Nonidet P-40 containing 100 units of Trasylol per ml and their nuclei were removed by centrifugation at 10,000 rpm for 10 min. The lysates were treated with 10 μl of monoclonal rat anti-Thy-1.2 at 4°C for 1 hr. Fifty microliters of goat anti-rat Ig were added and, after overnight incubation at 4°C, the samples were centrifuged 5 min at 10,000 rpm. The pellets were saved and the supernates were reprecipitated as described above, using either monoclonal anti-Thy-1.1 or Thy-1.2 antibody and goat anti-mouse Ig.

Cell Sorting and "Panning." For cell sorting, 10^8 cells were stained with biotinylated anti-Thy-1.2 and fluoresceinated avidin as described above, but they were not fixed. Ten million cells per ml in P_i/NaCl/1% bovine serum albumin/0.1% NaN_3 were sorted for Thy-1.2-positive cells (2×10^7 cells per hr). Positive cells were immediately re-sorted and collected directly into 50-mm tissue culture dishes containing 1 ml of medium, or single cells were deposited in a 96-well flat-bottomed microtiter plate containing 100 μl of medium. The cells were allowed to grow and were analyzed 2 weeks later for expression of surface Thy-1.2.

For panning (16, 17), 100-mm plastic Petri dishes were filled with 5 ml of avidin (100 μg/ml) in P_i/NaCl and stored at 4°C for at least 48 hr. The avidin solution was decanted and the plates were washed to remove unbound avidin. The cells to be panned (10^8) were first treated with biotinylated anti-Thy-1.2 Ig as described above, washed, and plated (4 ml; 5×10^7 cells total) on the coated dishes. After 2 hr at 4°C, the nonadherent cells were washed from the plates, fresh medium was added to the adherent cells, and the plates were incubated at 37°C.

RESULTS

Expression of Surface Thy-1.2 Antigen on Transformed BW5147 Cells. Thy-1.2 has been transferred into a Thy-1.1-bearing mouse T-cell lymphoma, BW5147, using DNA extracted from BALB/c thymocytes (Thy-1.2-expressing cells) and the calcium phosphate precipitation method of DNA-mediated gene transfer. Control cells received calf thymus DNA. Forty-eight hours after transfer, the cells were assayed for transient surface Thy-1.2 expression in a fluorescence assay using biotinylated anti-Thy-1.2 monoclonal antibody and fluoresceinated avidin. As seen in Table 1, in two experiments, one analyzed with a fluorescence microscope and the other with an Ortho 50H cytofluorograph, 8% and 7% of the cells expressed the Thy-1.2 antigen. Cells binding biotinylated anti-Thy-1.2 were then selected either by panning on avidin-coated Petri dishes or by two successive rounds of cell sorting. Two weeks later, they were re-analyzed for the number of "stable" transformants expressing Thy-1.2. Of the panned populations, 9% and 6% of the cells were positive; of the sorted population, 3% were positive. The positive population present at 2 weeks in experiment 1 was resorted to yield a population of ≈90% Thy-1.2-positive, and it was subsequently cultured. After one month in culture, 28% of the cells remained Thy-1.2 positive (Fig. 1). Transformants have also been isolated by repeatedly panning the DNA-transfected cells. The first selection was made 48 hr after transfection (when transient expression is at or near maximal) and was repeated at ≈10-day intervals. After five such cycles, 35% of the cells were Thy-1.2-positive. The stable transformants were cloned by sorting the Thy-1.2-positive cells with aid of the cytofluorograph and a single cell deposition device. Although positive cells were sorted, only 18/72 clones that could be grown in mass culture continued to express Thy-1.2.

Table 1. Expression of Thy-1.2 antigen

	% positive cells	
	Exp. 1	Exp. 2
Transient expression		
Thy-1.2 DNA transferred	13	13
Calf thymus DNA transferred	5	6
Net	8	7
Expression at 2 weeks in positively selected transient expressors*		
Thy-1.2 DNA	12	8 (5)
Calf thymus DNA	3	2 (2)
Net	9	6 (3)
Expression at 4 weeks (from cells sorted at 2 weeks)		
Thy-1.2 DNA (sorted positive at 2 weeks)	28	
Thy-1.2 DNA (sorted negative at 2 weeks)	2	
Net	26	

In experiments analyzed using the cytofluorograph, positive cells were defined as those in which fluorescence intensity exceeded the mean by 1.67 times. In experiment 1, transient expression was determined by conventional fluorescence microscopy. Cells transformed with calf thymus DNA do not differ in their staining from untransformed cells. Their staining thus represents background.
*Cells obtained by either panning or sorting the transient population for Thy-1.2 expressing cells. Numbers in parentheses are the values obtained with sorted cells.

Immunoprecipitation of the Thy-1.2 Antigen from Positive Transformants. The molecule expressed by these BW5147 transformants is Thy-1.2, as shown by polyacrylamide gel electrophoresis of the immunoprecipitates obtained (Fig. 2). Positive transformants and control cells were radiolabeled with ^{125}I, lysed, and treated with anti-Thy-1.2 antibody and goat anti-rat IgG. The precipitates were saved and the supernates were reprecipitated with anti-Thy-1.1 and secondary antibody. Both Thy-1.2 and Thy-1.1 were precipitated from the surface of BW5147-transformed cells, whereas only Thy-1.1 was precipitated from the BW5147 parent and control cells. BALB/c thymocytes, as expected, gave a Thy-1.2 band.

Double-Label Immunofluorescence Studies. To prove that a rare contaminating Thy-1.2-bearing cell had not been erroneously selected from among an otherwise Thy-1.1 population, the sorted positive cells and the controls were analyzed in a double-label fluorescence assay with biotinylated monoclonal anti-Thy-1.2 antibody (rat IgG) and fluoresceinated avidin, as well as a monoclonal anti-Thy-1.1 antibody (mouse IgM) and a rhodaminated anti-mouse IgM, which does not cross-react with rat Ig. As seen in Fig. 3, several cells are

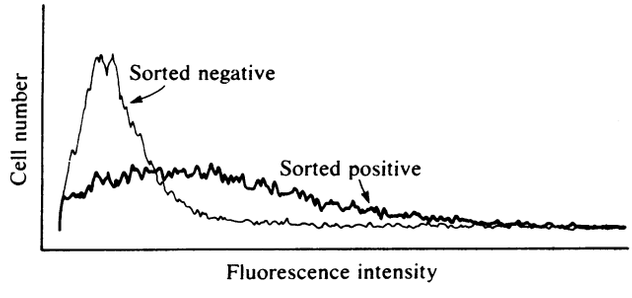

FIG. 1. Fluorescence histogram of BW5147-transformed cells (experiment 1, Table 1). Positive or negative cells were selected 2 weeks after transformation. After 1 month in culture, they were re-analyzed, giving the histograms shown.

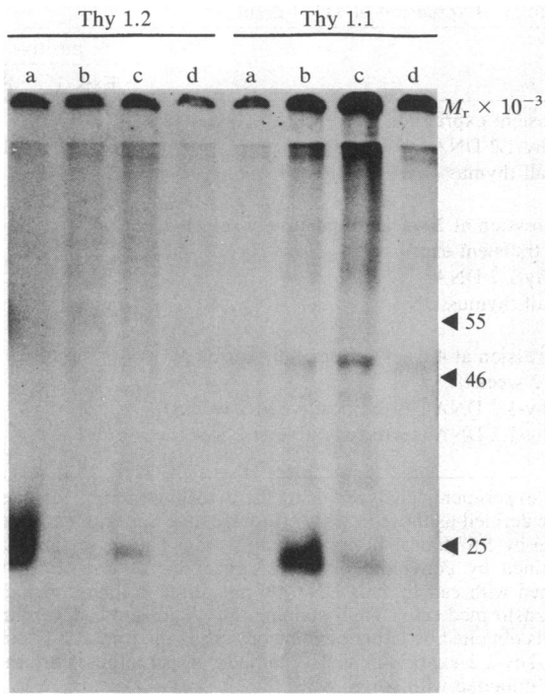

FIG. 2. Immunoprecipitation of Thy-1 antigen from the surface of BW5147-transformed cells. $NaDodSO_4$/10% polyacrylamide gel run under reducing conditions. Lanes a, BALB/c thymocytes; b, BW5147; c, transformed cells; d, reprecipitation to demonstrate completeness of immunoprecipitation of lane c (transformed cells). The Thy-1 band migrates with an apparent M_r of ≈25,000.

expressing both Thy-1.1 and Thy-1.2 antigens, thus eliminating the possibility that the population analyzed was comprised of some Thy-1.1-bearing cells and others that were expressing Thy-1.2. Control cells were found to express Thy-1.1 only.

Molecular Genetic Evidence for the Presence of Both Genes. Molecular evidence for the presence of both genes (Thy-1.1 and Thy-1.2) is shown in Fig. 4. Restriction mapping with *Pvu* II shows a single high molecular weight band hybridizing with the Thy-1 probe in DNAs isolated from either BW5147 cells or BALB/c thymus. These bands are electrophoretically distinguishable (18). The transformed cells (clone t.29) show a distinct doublet in this region, indicating the presence of both genes. The additional bands in t.29 migrating faster than the wild-type Thy-1 alleles presumably result from multiple integration sites or rearrangements that frequently occur during DNA-mediated gene transfer.

Thus, the BW5147 cells are excellent recipients for this gene, and the efficiency of long-term transformation in these experiments is high (≈10^{-4}; see *Discussion*). Although no co-selection system is used, many cells appear to be transformed, expressing the Thy-1.2 antigen.

DISCUSSION

With total cellular DNA as a donor in transfection, the single copy gene encoding the Thy-1.2 alloantigen, characteristic of BALB/c mice, has been stably incorporated into the genome of a normally Thy-1.1 murine T-cell tumor. Both alloantigens are expressed on the surface of the recipient cells, and both genes can be demonstrated in the DNA extracted from transformed cells. Since only cells expressing the new alloantigen 48 hr after transformation are selected for further culture, these results provide experimental evidence for the long-held belief that long-term transformed cells are derived from cells that express the transferred genes promptly.

The cells expressing the transferred gene were obtained by positive immunoselective techniques, which depend only on the expression of the desired antigen on the cell surface. The transfection was performed without the cotransfer of a metabolically selectable vector. Both the recipient line and the transformants grow equally well in the medium used. In the absence of continuous selection, the stability of the positive population is not very high, but long-term transformants are obtainable. It is clear from Fig. 3 that there is heterogeneity in the cell population with respect to this expression. That has been reported before for other genes and cells (19) and may have to do with either the special configuration of the large concatemeric DNA aggregate formed during transformation or its location in the chromosome.

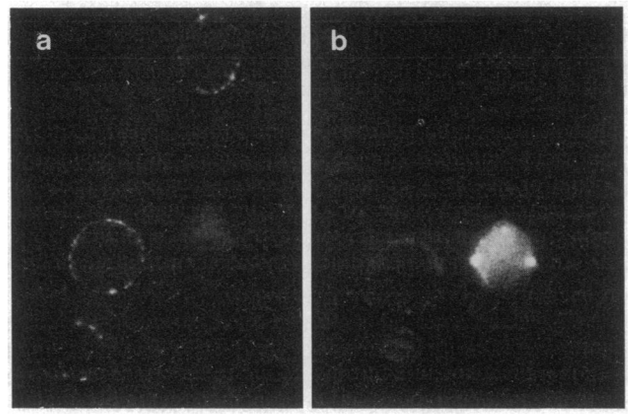

FIG. 3. Immunofluorescent staining of BW5147 cells expressing the transfected Thy-1.2 gene. (*a*) Thy-1.1 stained cells. (*b*) Thy-1.2 stained cells. (×460.) Aliquots were stained with monoclonal mouse anti-Thy-1.1, washed, and stained with rhodaminated goat anti-mouse Ig and biotinylated monoclonal rat anti-Thy-1.2. After further washing, they were stained with fluoresceinated avidin, fixed, and examined. All of the cells stain for Thy-1.1, and the staining is relatively homogeneous. Both sides of the figure show the same field. As expected from the histogram in Fig. 1, the staining is very heterogeneous. Some cells are very bright, but several others are so dim that they cannot be identified in the photomicrograph.

A gene normally expressed by cells of the T lineage has been transferred to another cell of the same lineage. The recipient cell, a T-cell lymphoma, grows without attachment to the surface of the culture vessel and expresses numerous T-cell markers (8). Most of the early gene transfer studies were

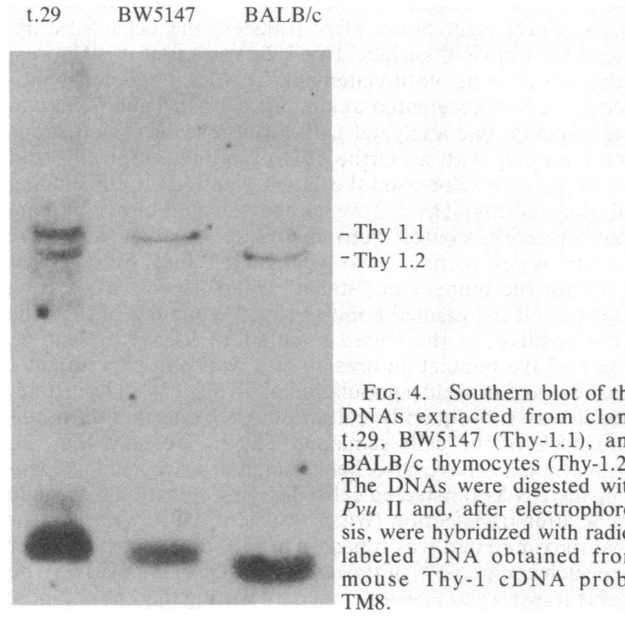

FIG. 4. Southern blot of the DNAs extracted from clone t.29, BW5147 (Thy-1.1), and BALB/c thymocytes (Thy-1.2). The DNAs were digested with *Pvu* II and, after electrophoresis, were hybridized with radiolabeled DNA obtained from mouse Thy-1 cDNA probe TM8.

done with mouse fibroblasts as recipients. The genes, which were expressed at near normal levels, were generally those coding for "housekeeping" functions (thymidine kinase, adenine phosphoribosyltransferase, hypoxanthine-guanine phosphoribosyltransferase, etc.). While these have provided new and exciting means of studying the regulation of eukaryotic gene expression, they are not well-suited for the study of the complex interactions characteristic of differentiating systems.

L cells have been obtained that express the human lymphoid antigen Leu-2 by transfecting with total human DNA and a purified metabolically selectable gene (20). L-cell transformants expressing the human transferrin receptor have been obtained similarly (21).

Other cell types have been used in transfection systems and specialized genes have been introduced into developmentally appropriate cells. For example, globin genes have been transferred into Friend erythroleukemia cells (22), and several reports have appeared demonstrating stable transfer and expression of purified immunoglobulin genes into cells of the lymphoid system (23, 24). Homologous transfers, as used in these experiments, offer an opportunity to study genes whose expression requires the presence of other elements in a developmental pathway as well as those whose product(s) influence the subsequent differentiation of the lineage.

Finally, the efficiency of transfection in some of the experiments we report is extraordinarily high. In five separate experiments, an average of 6.5% of the recipient cells transiently express the new Thy-1.2 alloantigen (range, 2.2%–12%). Since total thymus DNA has been used to effect the transfer, even assuming that all the cells are equally competent to incorporate and express DNA, at least 5% of a genome must have been incorporated by the cells to reach this efficiency.

Calculation of the frequency of long-term transformed cells is somewhat more difficult, as the number is dependent on what time frame is chosen, after which cells are considered to be stable transformants. Two weeks after transfection, a small proportion (3%–5%) of the transiently expressing cells is still antigen-positive, indicating that there was gene replication. If one selects this time point as defining cells as stable, our efficiencies are extraordinarily high. In the absence of further selection, the number of positive cells decreases. At 4 weeks, only one-quarter of the cells are still positive. Twenty-five percent of the clones selected as positive after 1 month remain positive 3 months later. The final transformation frequency is thus $0.065 \times 0.03 \times 0.025 \times 0.025 = 1 \times 10^{-4}$. The value usually reported for fibroblasts is 10^{-5}.

The mechanisms that produce this high efficiency transformation are not readily apparent. It is possible that our recipient line is uniquely competent in transfection. It is well known that fibroblast lines vary greatly in their transformability and that even subclones from the same cell line can have very different transformation efficiencies (26). At least part of the explanation may lie in the fact that this is a homologous transfer. Cells of the T lineage are being asked to synthesize a product characteristic of the lineage. The complex enzymatic and transport systems required for the expression of the Thy-1 antigen system (27) are already in place in the recipient BW5147 cells. In addition, tissue- or organ-specific enhancers are known (24, 25) and these would enhance the efficiency of homologous transfers. Presumably, the same factors that promote homologous expression in transfection are operative in cell fusion experiments. B cells fused to myelomas, and T cells fused to thymomas, produce cells that retain characteristic function, while heterologous fusions (B–T) extinguish differentiated function in the progeny (28, 29).

The transfer and stable expression of a single copy gene into cells of the lymphoid system with total cellular DNA as donor without metabolic co-selection has not been previously reported. The ability of this T-cell line to express transfected genes coding for specialized products of the T lineage could offer an effective way of cloning these genes.

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