

8. Because of the initial presence of late successional organisms, the ecological succession was not a case of facilitation. It therefore would be classified under the neutral (tolerance) or inhibition models of J. H. Connell and R. O. Slatyer [*Am. Nat.* 111, 1119 (1977)]. M. J. Keough [*Ecology* 65, 423 (1984)] showed that recruitment was a much more important process than interference competition on small isolated patches, so that the neutral model of succession is most plausible here.
9. T. J. Palmer and C. D. Palmer [*Lethaia* 10, 197 (1977)] discussed the morphological and evolu-

tionary effects of scour on a Middle Ordovician hardground community. M. A. Wilson [*ibid.* 15, 263 (1982)] described a Pennsylvanian brachiopod-bryozoan ecosystem in which diversity was gradually enhanced by the accumulation of biogenic debris.

10. I thank G. M. Wilson, W. P. Sousa, M. J. James, and G. G. Whitney for reviewing this manuscript, and J. W. Bartley for bryozoan identification. Supported by grants from Sigma Xi and College of Wooster.

15 October 1984; accepted 14 February 1985

Expression of a Microinjected Porcine Class I Major Histocompatibility Complex Gene in Transgenic Mice

Abstract. A porcine class I major histocompatibility complex (SLA) gene has been introduced into the genome of a C57BL/10 mouse. This transgenic mouse expressed SLA antigen on its cell surfaces and transmitted the gene to offspring, in which the gene is also expressed. Skin grafts of such transgenic mice were rejected by normal C57BL/10 mice, suggesting that the foreign SLA antigen expressed in the transgenic mice is recognized as a functional transplantation antigen.

The introduction of isolated class I major histocompatibility complex (MHC) genes into cultured mammalian cells has been used to study their expression, antigenicity, and antigen-presenting capacity (1). This approach allows the introduction of genes not normally expressed in the recipient cell, such as foreign, mutant, or hybrid MHC genes constructed *in vitro* (2, 3). In addition, the expression of individual members of a multigene family can be studied in isolation.

However, such studies have been generally limited to a small number of cell types, such as L cells, and the questions addressed have been limited to those related to the expression of the introduced gene in that particular cellular environment. It has not been possible to address developmental issues such as the patterns of tissue-specific expression of individual class I MHC genes or the influence of newly introduced genes on the induction of self tolerance and the maturation of the immune response. To study these unanswered questions, we microinjected a cloned xenogeneic class I MHC gene into the fertilized eggs of mice. We now report the identification of a transgenic mouse that expresses a porcine class I MHC gene that can be transmitted to progeny, in which it is also expressed.

Earlier we showed that the introduction of PD1, a porcine genomic clone containing a class I MHC (SLA) gene, into mouse L cells by DNA-mediated gene transfer resulted in the cell-surface expression of SLA antigen (3), as detected by antibody-mediated, complement-dependent cytotoxicity, cell-surface immunofluorescence, and immunoprecip-

itation. The latter studies also revealed that the SLA heavy chain associates with the endogenous murine β_2 -microglobulin of the L cell. Further studies indicated that the expression of the swine MHC gene was actively regulated (4). In addition, the swine DNA and RNA sequences as well as the protein products were readily distinguishable from the endogenous murine sequences. Therefore, this gene seemed to be an ideal candidate for microinjection experiments.

Male pronuclei of one-cell C57BL/10SCN (B10) fertilized eggs were injected either with approximately 500 copies of a 9-kilobase (kb) Hind III fragment derived directly from the PD1 clone or with about 2000 copies of a 5.6-kb Hind III-Bam HI fragment, subcloned into pBR322 and linearized with either Hind III or Eco RI (Fig. 1). These fragments of swine DNA each contain the entire SLA gene and have been shown to direct the synthesis of SLA antigen in mouse L cells (5). Of 171 eggs injected with the 5.6-kb Hind III-Bam HI DNA fragment, 100 were transferred to pseudopregnant Sim:SW females, and there were 63 offspring. Twenty-seven eggs were injected with the 9-kb Hind III DNA fragment,

and 20 were transferred to a single foster mother; there were five offspring.

In testing for the swine DNA sequences, DNA was extracted from a segment of tail of each of the mice and hybridized with an SLA DNA probe (Fig. 1) under conditions in which only SLA DNA sequences, but not endogenous H-2 sequences, would hybridize. A single SLA-positive mouse (3931), which had been injected as an embryo with the swine 9-kb Hind III DNA fragment, was identified by dot blot analysis. For further verification of the SLA gene in animal 3931, total tail DNA was digested with the restriction enzyme Bgl II. This enzyme releases three SLA coding-sequence DNA fragments of 2.9, 1.8, and 0.9 kb from the intact gene (Fig. 1). Therefore, it was expected that all three of these bands would be present in the DNA of animal 3931 but not in the DNA of its negative littermates. The SLA probe hybridized specifically to appropriately sized DNA fragments from animal 3931 and pig liver but not to those from negative littermates or normal B10 animals. This confirms the presence of the SLA gene in animal 3931.

The ability of the SLA gene to be expressed in the transgenic mouse was assessed by cell-surface labeling of peripheral blood lymphocytes (PBL's) with a monoclonal antibody specific for SLA determinants (6), which does not cross-react with murine class I MHC antigens. This monoclonal antibody has been shown to react specifically with L cells transfected with either the whole PD1 DNA or the Hind III 9-kb DNA fragment but not with control L cells (4, 5). PBL's from animal 3931 and normal B10 mice were stained with biotin-conjugated antibody to SLA. Binding of the monoclonal antibody was assessed with fluoresceinated avidin, which binds to biotinylated antibody on the cell surface. The antibody to SLA reacted specifically with PBL from animal 3931 (Fig. 2a). In contrast, lymphocytes from normal B10 animals were not stained above background levels by this reagent (Fig. 2b).

In efforts to examine the stability of expression and heritability of the SLA

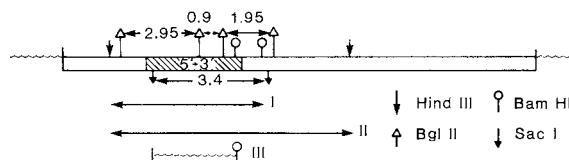


Fig. 1. Partial restriction map of PD1, which contains a functional SLA gene (shaded area) (3). The 5.6-kb swine DNA fragment subcloned into pBR322 and used in microinjection (I), the 9-kb Hind III swine DNA fragment used in microinjection (II), the 2.5-kb Sac I-Bam HI DNA fragment used as an SLA-specific probe in hybridization experiments, and the origins of the hybridizing 3.4-kb Sac I fragment and of the 2.95-, 1.95-, and 0.9-kb Bgl II fragments are noted (see Fig. 3).

gene and to estimate the number of loci into which the SLA gene integrated, animal 3931 was mated to B10 females. Initially, 30 offspring were tested for the presence of the SLA gene by hybridization of tail DNA with the SLA-specific probe and for gene expression by cell-surface staining of PBL with SLA-specific antibodies. All mice scored as posi-

tive for SLA DNA by hybridization expressed SLA antigen. Furthermore, mice that did not contain SLA DNA sequences did not express SLA antigen.

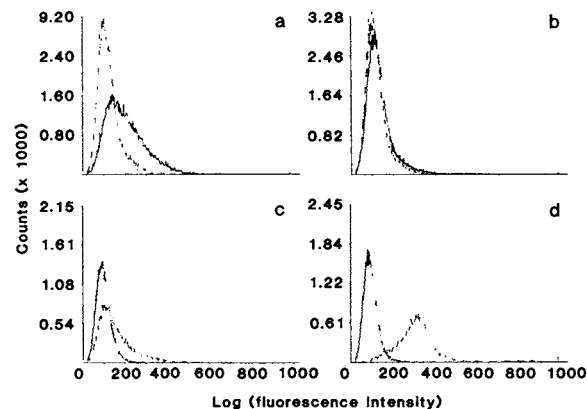
Representative cell-surface staining profiles from a pool of five positive animals (Fig. 2, c and d) were obtained by using two different staining reagents: the monoclonal antibody to SLA^d previous-

ly used to characterize animal 3931 (Fig. 2c) and a swine alloantiserum, haplotype ac antibodies to haplotype dd, which is specific for MHC antigens of the relevant SLA^d haplotype (7) and from which the PD1 clone was derived (Fig. 2d). Both reagents specifically stained transgenic lymphocytes. The specificity of staining was confirmed by inhibition studies; the staining of the transgenic mouse lymphocytes by the biotin-conjugated monoclonal antibody to SLA could be blocked by unlabeled antibody to SLA but not by monoclonal antibody to H-2K (8), which binds to the H-2K^b molecule expressed on B10 PBL's. Reciprocally, the binding of biotinylated antibody to H-2K^b could not be inhibited by antibody to SLA but was completely blocked by the unlabeled antibody to H-2K^b. Therefore, both the SLA gene and the ability to express that gene were transmitted to the progeny.

Because the results were concordant between DNA analysis and cell-surface expression, subsequent litters were tested only by cell-surface staining. Of 65 progeny of animal 3931 examined, 33 (50.7 percent) expressed the SLA gene. This frequency of transmission suggests that a single genetic linkage group containing the SLA gene is integrated into the mouse genome. Because both male and female positive offspring were identified, integration of the SLA gene into either the X or Y chromosome is unlikely.

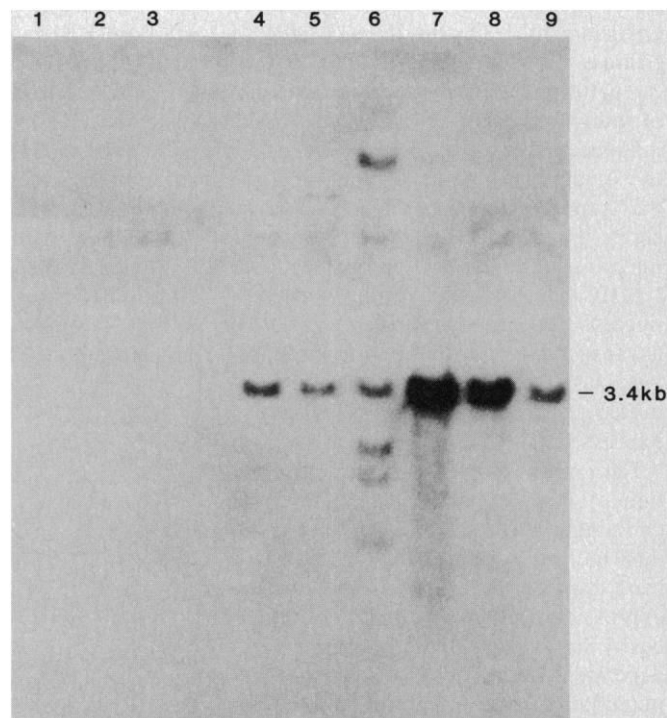
The copy number and stability of the

Fig. 2. Cell-surface expression of SLA antigen in a transgenic mouse and its offspring. (a) PBL's from transgenic animal 3931 examined for cell-surface expression of SLA antigen by flow microfluorometry (11). PBL's were incubated for 45 minutes with biotin-conjugated, protein A-purified (12) monoclonal antibody to SLA (74-11-10) (6), counter-stained with fluorescein isothiocyanate-conjugated avidin (FITC-avidin), and analyzed in a fluorescence-activated cell sorter (FACS II; Becton Dickinson). Fluorescence data were collected on 2×10^4 to 5×10^4



viable cells and displayed at a 1000-channel cell frequency histogram, in which log (fluorescence intensity) was plotted against cell number. The solid line represents the binding of biotinylated antibody 74-11-10; the broken line represents the binding of FITC-avidin alone. (b) Staining of PBL's from normal B10 animals as in (a). (c) Staining of a pool of PBL's from five progeny of animal 3931 with the biotinylated antibody 74-11-10 and counterstaining as in (a). (d) Staining of a pool of PBL's from five progeny of animal 3931 with an alloantiserum, 1920 (7), produced by immunizing an SLA^{ac} pig with lymphocytes from an SLA^{ad} animal. This serum was purified by affinity column chromatography on protein A-Sepharose, absorbed extensively with B10 spleen cells to remove cross-reactive antibodies to mouse H-2, and directly conjugated with FITC (13). PBL's were incubated for 45 minutes and analyzed as in (a). The solid line represents the fluorescence of cells incubated with the FITC-labeled alloantiserum, and the broken line represents the fluorescence of unlabeled cells. The results are displayed on a log scale, where every 100 units represents approximately a twofold increase in the level of expression.

Fig. 3. Identification of transgenic mice by genomic hybridization and determination of SLA gene stability and copy number. DNA samples (10 μ g) from two progeny of animal 3931 that were positive for the SLA gene as determined by dot hybridization (lanes 4 and 5) and from three animals that were negative (lanes 1, 2, and 3) were digested with the restriction enzyme Sac I at a ratio of 15 U per microgram. Total pig DNA (10 μ g) was similarly digested (lane 6). Amounts of PD1 DNA corresponding to 1 (0.2 ng, lane 9), 10 (2 ng, lane 8), and 50 (10 ng, lane 7) copies of SLA gene per genome were added to 10 μ g of normal B10 DNA and digested with Sac I under the same conditions. DNA fragments were resolved by electrophoresis on 0.8 percent agarose gels in TPE [0.08M tris-phosphate, 8 mM EDTA (pH 7.6)] for 24 hours at 30 V. DNA was transferred to nitrocellulose by the method of Southern (14). Hybridization was carried out with the SLA-specific probe, labeled by nick-translation (15), in 40 percent formamide, 10 percent dextran sulfate, 4 \times standard saline citrate (SSC), 1 \times Denhart solution, 20 μ g of salmon sperm DNA per milliliter, and 10 μ g of *Escherichia coli* DNA for 24 hours at 42 $^{\circ}$ C. The filter was washed two times in 2 \times SSC at room temperature for 30 minutes each, and two times in 0.1 \times SSC at 65 $^{\circ}$ C for 1 hour each. The position of the hybridizing 3.4-kb Sac I fragment is noted.



SLA gene were assessed further by analyzing restriction endonuclease digests of liver DNA from two representative SLA-positive mice compared with three negative littermates. After digestion with the enzyme Sac I, DNA samples were subjected to electrophoresis on agarose gels in parallel with various amounts of Sac I-digested PD1 DNA, corresponding to 1, 10, and 50 copies per haploid genome, as well as Sac I-digested total pig DNA. After transfer to nitrocellulose paper, the samples were hybridized with the SLA-specific probe (Fig. 1). Genomic DNA from both positive transgenic mice and pig contained a single hybridizing band with the same mobility as the PD1 band (Fig 3); no hybridizing bands were observed in the DNA samples of the negative littermates. These data suggest that there are no major rearrangements of the SLA genes in the progeny. By comparing the intensity of hybridization of the probe to the transgenic mouse DNA with that of the various PD1 DNA samples, we estimated that there are one or a few copies of the SLA gene in each of the positive progeny. Taken together with the transmission frequency of SLA-antigen expression, we conclude that, in the transgenic mice, the SLA gene is in a single integration site and is transmitted in accordance with Mendelian inheritance.

The expression of the SLA gene in various lymphoid tissues was examined next. Previous studies have indicated that expression of the PD1 SLA gene in mouse L cells appears to be actively regulated (4). Initial studies suggest that the PD1 SLA gene is differentially expressed in vivo in the pig (9). The pattern of SLA gene expression in the transgenic mice was analyzed by cell-surface staining with the monoclonal antibody to SLA^d. For comparison, expression of the endogenous H-2K^b antigen was determined in parallel with a monoclonal antibody specific for H-2K^b (8). The results indicated that the levels of expression of SLA antigen varied among different tissues in the transgenic mice (Fig. 4, a, c, and e). Namely, splenocytes expressed less surface SLA antigen than lymph node cells, which expressed less than bone marrow cells. In particular, two distinct subpopulations of cells were observed in the bone marrow with different levels of cell-surface antigen expression. Similar analyses of thymocytes revealed a weak level of SLA antigen on these cells as well. The expression of H-2K^b also varied somewhat between the tissues examined (Fig. 4, b, d, and f), although there did not appear to be a

direct correlation with SLA expression. The fluorescence intensity of the H-2K^b antibody staining was consistently greater than that of the SLA antibody. It is not clear whether the differences in intensity of staining of the two antibodies are due to quantitative differences between SLA and H-2K^b or to a lower affinity of the SLA antibody for SLA than of the H-2K^b antibody for H-2K^b. Staining of pig PBL's with the SLA antibody gave a fluorescence intensity similar to that observed with the H-2K^b antibody staining of mouse cells. Initial analysis of total RNA from various tissues revealed tissue-specific variations in the levels of SLA RNA expression. These data suggest that SLA expression in transgenic mice may be regulated.

Because antigenic determinants resident on the SLA gene product are expressed on the surface of transgenic mouse cells, it was next of interest to determine whether the expressed SLA

antigen can be recognized by T cells as a classic transplantation antigen.

Rejection of skin graft is one feature of T cell-mediated MHC recognition. The ability of the SLA antigen to be recognized as a transplantation antigen was assessed by engrafting normal B10 animals with tail skin from transgenic and normal B10 mice. Rejection was scored as complete when no viable graft could be detected. None of the recipients ($n = 10$) rejected skin from normal B10 animals; however, all the recipients ($n = 8$) rejected skin from transgenic mice, with a median survival time of 18 days (Fig. 5). This rejection time is not significantly different from the rejection time of an allogeneic class I difference (10).

It should now be possible to determine the relation between those T cells specific for xenogeneic MHC products and the T cells specific for syngeneic and allogeneic murine MHC determinants. In addi-

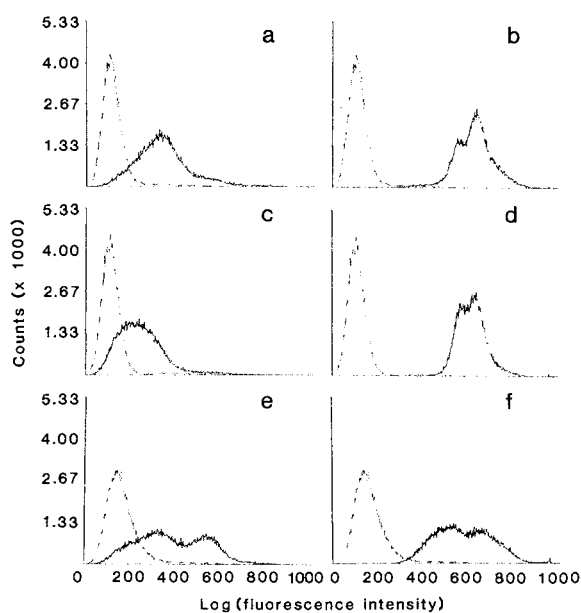


Fig. 4. Tissue distribution of SLA and H-2 antigens in transgenic mice. Cells from spleen (a and b), lymph node (c and d), and bone marrow (e and f) of a single animal, 3815, were stained with either a monoclonal antibody to SLA (74-11-10) (a, c, and e) or monoclonal antibody to H-2K^b (28-13-3) (b, d, and f) and analyzed by flow microfluorometry as described in the legend to Fig. 2. Solid lines represent binding of the biotinylated antibody, and broken lines represent binding of the FITC-avidin counterstain alone.

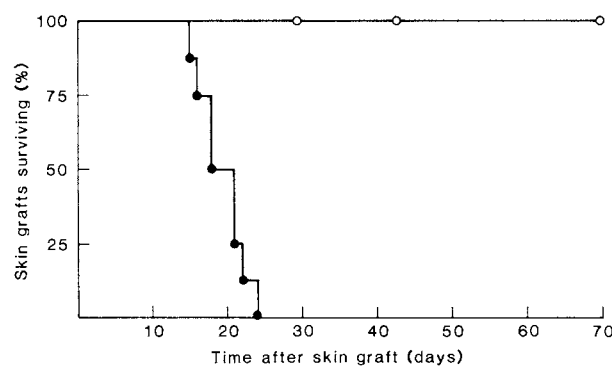


Fig. 5. Survival of skin grafts from transgenic and normal B10 mice grafted onto normal B10 mice. Skin grafts of full thickness were harvested from the tails of three transgenic mice and three normal B10 mice. Heterotopic grafting was performed as described (16); minimum follow-up time was 29 days. Graft survivals were calculated by the life-table method (17). The calculated median survival time of transgenic grafts (●) was 18 days; that of normal B10 skin (○) was greater than 70 days.

tion, the mechanisms mediating murine self-tolerance to the normally foreign SLA antigens can be compared with those involved in conventional self-tolerance to murine H-2 products. Finally, it will be of interest to determine the degree to which the murine T cell repertoire can utilize a transfected SLA gene product as a restricting element for antigen recognition and physiologic cell interactions.

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18. We thank M. Pescovitz, R. Germain, D. Margulies, and A. Singer for helpful discussions and critical review of the manuscript, M. Pescovitz for providing the biotin-conjugated SLA antibodies, and David Stephany for FACS analysis. All experiments were done in accordance with the NIH Guidelines for Recombinant DNA Research. Supported by NIH grant GM 2116B (W.I.F.) and NIH training grant GM 07825 (M.R.C.).

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