Note added in proof: After this manuscript was submitted, Vale *et al.* (*20*) described the isolation and the structure of a peptide with corticotropin-releasing factor activity. Although this peptide might be the long-sought corticotropin-releasing factor, its exact physiological role remains to be established.

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5. The same medium is used for washing and further incubations of the cells, except that the fetal bovine serum is replaced by bovine serum albumin (0.5 mg/ml) and Trasylol (20 μg/ml).
6. The synthetic α-, β-, γ_1-, γ_2-, and γ_3-MSH were kindly supplied by N. Ling. Synthetic arginine-vasopressin was purchased from Peninsula Laboratories Inc. Pitressin was a gift of Parke-Davis.
7. The peptides were added in a final concentration of 10 μg/ml and incubated with 4.8×10^5 cells. The Dunnett statistical procedure was used to test the difference between control incubations and the incubations with the different MSH peptides.
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Identification of a BALB/c H-2L^d Gene by DNA-Mediated Gene Transfer

Abstract. *Gene transfer and immunoselection were used in the identification of a BALB/c genomic clone containing an H-2L^d gene (clone 27.5). Transformation of thymidine kinase–negative C3H mouse L cells with the cloned 27.5 DNA together with the herpes simplex virus tk gene produced transformants expressing L^d molecules detected by radioimmune assay with monoclonal hybridoma antibodies to L^d antigens. The foreign L^d gene products expressed by cloned mouse L cell transformants were shown to be virtually indistinguishable from BALB/c spleen L^d molecules by two-dimensional electrophoretic analysis of H-2L^d immunoprecipitates. These results indicate that the genomic clone 27.5 contains a functional BALB/c H-2L^d gene and demonstrate the usefulness of this approach for identifying the gene products encoded by cloned genes which are members of a multigene family. Furthermore, the ability to place cell-surface recognition molecules on the surfaces of foreign cells provides a powerful opportunity for functional analyses of these molecules.*

Transplantation antigens were first defined on the basis of their ability to mediate graft rejection (*1, 2*). These cell-surface molecules, which are associated with β_2-microglobulin, a 12,000-dalton polypeptide, are encoded by multiple genes of the murine major histocompatibility complex or H-2 complex (*3*). In the inbred BALB/c mouse, the H-2 complex of chromosome 17 appears to encode at least four transplantation antigens, denoted K, D, L, and R. Transplantation or class I antigens constitute one of the most polymorphic systems studied in eukaryotes. Virtually every inbred strain of mice has a distinct constellation or haplotype of class I genes. Because transplantation antigens play a fundamental role in T cell and target cell interactions (*4*), they represent an excellent model system for analyzing the genetic and molecular basis of cell-surface recognition phenomena.

Several laboratories have cloned complementary DNA (cDNA) probes for transplantation antigens (*5–8*). Class I cDNA probes were used to isolate 30 to 40 different genomic clones from a BALB/c sperm library constructed in Charon 4A lambda bacteriophage (*9*). One of these clones, designated 27.1, contained a gene that was found on restriction enzyme fragments of different sizes in differing strains of inbred mice. This restriction polymorphism was used to map this gene to the Qa-2,3 region of the Tla complex, a region that is adjacent to the H-2 complex and that encodes various hematopoietic (Qa) and T cell (TL) differentiation antigens (*9*). The DNA sequence analysis of clone 27.1, presumably a Qa-2,3 pseudogene, demonstrates that 27.1 is closely related to the classical transplantation antigens. Therefore, the 30 to 40 genomic class I clones may encode differentiation antigens in the Tla complex as well as the classical transplantation antigens of the H-2 complex. This complexity demonstrates a problem inherent in analyzing all multigene families; namely, the difficulty in correlating numerous genomic clones with their corresponding gene products, especially in view of the paucity of available amino acid sequence data.

DNA-mediated gene transfer of BALB/c class I clones into mouse L cells was used to identify the transferred BALB/c genes. Monoclonal antibodies readily distinguish the foreign BALB/c class I molecules (H-2^d haplotype) from the recipient mouse L cell transplantation antigens (H-2^k haplotype). We now report the application of this approach to the identification of a genomic clone containing the BALB/c L^d gene (clone 27.5). The nucleic acid sequence of this gene (*10*) confirms the assignment and demonstrates the usefulness of this approach in determining the coding function of cloned class I genes.

Several genomic clones were chosen for DNA-mediated gene transfer on the basis of their intense hybridization to the cDNA probes for transplantation antigens. These class I genomic clones were used independently to transform thymidine kinase–negative C3H mouse L cells (Ltk$^-$), together with the herpes simplex virus (HSV) tk gene (*11*). Transformants derived from transfections with each of these cloned genes were selected in hypoxanthine, aminopterin, thymidine

0036-8075/82/0205-0677$01.00/0

(HAT) medium and either cloned directly or tested as mixed populations for the expression of the class I antigens, with specific monoclonal antibodies.

One positive cloned transformant (8-5) reacted with two different monoclonal hybridoma antibodies to L^d molecules (Fig. 1A). Reagents specific for D^d antigens did not react with 8-5 cells (Fig. 1A). Transformants derived from transfections with other genes or the HSV tk gene alone (tk^+ transformants) also failed to react with the antibodies to L^d molecules (Fig. 1B). A positive correlation between the transfection with the 27.5 gene and the expression of L^d molecules was established as follows. The DNA from clone 27.5 was digested either with restriction enzymes Bam HI or Eco RI, and the sticky ends of the resulting DNA fragments were made blunt-ended with T4 polymerase. Bam HI cuts within the 27.5 gene (*10*), whereas Eco RI effectively excises the gene from the λ vector. The digested DNA's were then used to transform mouse Ltk^- cells, which were subsequently tested for the expression of L^d molecules. Positive L^d transformants were not obtained from transfections with Bam HI–digested DNA (Fig. 1C). Eco RI digestion had little effect on the transformation results as compared to the control transformation with undigest-

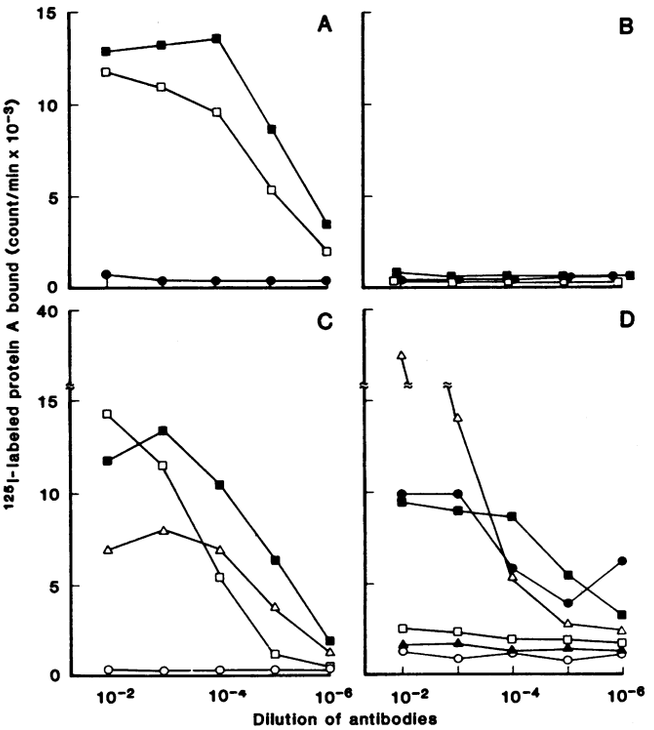

Fig. 1. Radioimmune assay of L^d transformants. Mouse Ltk^- cells were transformed with clone 27.5 DNA together with the cloned HSV-1 tk gene in pBR322 (ptk5) by a modification of the method of Wigler *et al.* (*11*). Transfection required 250 to 500 ng of 27.5 DNA, 5 ng of ptk5 DNA, and 5 μg of mouse Ltk^- carrier DNA per 10^6 cells. Calcium phosphate–DNA precipitates were applied to cell monolayers for 10 minutes, diluted tenfold with growth medium, and incubated for 6 hours at 37°C, followed by dimethyl sulfoxide (DMSO) shock (10 percent DMSO, 30 minutes, 37°C). Medium was replaced and HAT selection was initiated 36 hours later. Individual colonies, selected in HAT medium, were isolated by means of cloning cylinders. The transformants derived from transfections with digested 27.5 DNA's were trypsin-dispersed after HAT selection and passaged as mixed populations of tk^+ co-transformants. Cells were tested for the expression of class I molecules as follows. Fifty microliters of a single cell suspension of transformants or spleen cells (1×10^7 cells per milliliter in RPMI 1640 with 10 percent fetal calf serum and 10 m*M* Hepes) were mixed with 50 μl of dilutions of monoclonal hybridoma antibodies to class I molecules in flexible microtiter plates (Dynatech). After 3 to 4 hours of incubation at 4°C, the cells were washed twice in medium and resuspended in 50 μl of medium containing approximately 75×10^3 count/min (1 ng) of ^{125}I-labeled protein A (Amersham). The cells were incubated overnight at 4°C and washed twice before counting in a Searle gamma counter. (A) Cloned 8-5 transformants assayed with monoclonal hybridoma antibodies (□) 28-14-8 and (■) 30-5-7 to L^d molecules (*14*) and (●) antibodies to D^d molecules (*15*) (ascites fluids). (B) Assay of mouse L cell tk^+ transformants with antibodies (■) 30-5-7 and (□) 28-14-8 to L^d and (●) 34-2-12 to D^d molecules. (C) Assay of transformants derived from transfection with 27.5 DNA with 30-5-7 monoclonal hybridoma antibodies (ascites fluids) to L^d molecules. Transformants derived from transfections with (■) uncut DNA, (○) Bam HI-digested 27.5 DNA, and (△) Eco RI–digested 27.5 DNA. (□) Assay of 27.5 transformants with purified (Becton-Dickinson) 11-4.1 monoclonal hybridoma antibodies to K^k molecules (*16*). (D) Assay of spleen cells for class I molecules. BALB/c spleen cells assayed with monoclonal hybridoma antibodies to (▲) K^k, (●) D^d, and (■) L^d molecules. C3H spleen cells assayed with antibodies to (△) K^k and (□) L^d molecules, and (○) CBA spleen cells with antibodies to D^d.

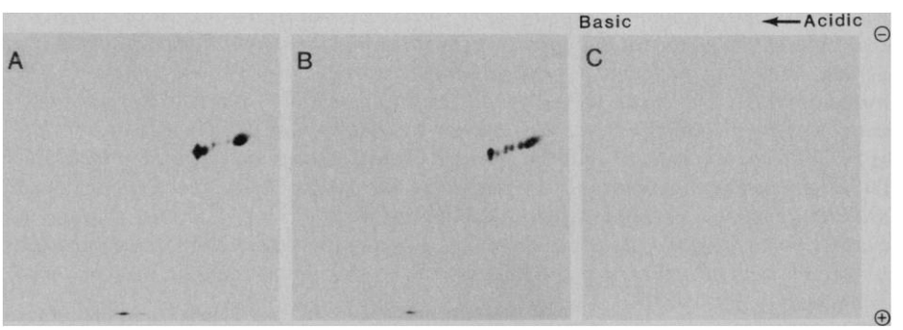

Fig. 2. Fluorographs of two-dimensional gels of H-$2L^d$ molecules immunoprecipitated from cell lysates of (A) 8-5 H-$2L^d$ transformants, (B) BALB/cJ spleen lymphocytes, and (C) tk^+ transformants. Transplantation antigens were biosynthetically radiolabeled (*17*). About 1.25×10^7 8-5 and tk^+ cells were cultured in 1.25 ml of medium containing HAT (*10*) and 1.25 mCi of [^{3}H]phenylalanine (New England Nuclear), and 6.2×10^6 splenic lymphocytes were incubated in 0.25 ml of medium containing 0.5 mCi of [^{3}H]phenylalanine. After 6 hours, each sample of cells was lysed with 0.25 ml of buffer containing 0.01*M* tris, 0.15*M* NaCl (*p*H 7.3), 0.5 percent Triton X-100, and 1 m*M* phenylmethylsulfonylfluoride. The resulting cell lysates were then subjected to immunoprecipitation with Formalin-fixed *Staphylococcus aureus* Cowan I strain (SAC) (*18*) modified as follows: 25 μl of normal mouse serum (Cappel), 10 μl of 10 percent (weight to volume) sodium dodecyl sulfate (SDS), and 250 μl of 10 percent (weight to volume) suspension of SAC were added to each cell lysate. After 30 minutes, the SAC was pelleted and discarded. Monoclonal antibodies to L^d (supernatant) derived from the hybridoma cell line 30-5-7 (*14*) were added to the cell lysate and, after 60 minutes, antibody-antigen complexes were isolated in the usual way with SAC. After the SAC was washed three times with a buffer containing 0.01*M* tris, 0.15*M* NaCl (*p*H 7.3), 0.5 percent Triton X-100, and 0.1 percent SDS, the radiolabeled H-$2L^d$ molecules were eluted from the SAC with O'Farrell's lysis buffer [9.5*M* urea, 2 percent Triton X-100, 1.6 percent LKB Ampholine (*p*H 5 to 7), 0.4 percent Ampholine (*p*H 3.5 to 10), and 5 percent 2-mercaptoethanol]. Each sample was then analyzed by two-dimensional gel electrophoresis with the methods of O'Farrell (*19*, *20*) as modified by McMillan (*17*). The gels were prepared for fluorography using Enhance (New England Nuclear) and were then dried and exposed to preflashed XAR-5 film (Kodak) (A) for 4 days and (B and C) for 14 days.

ed DNA. Since ligation of the digested fragments is not possible, these results demonstrate that λ vector sequences are not required for the expression of this gene. Furthermore, Southern blot analysis demonstrated that the L^d-transformed mouse L cells contain vector sequences, presumably flanking the L^d gene. It is not possible to directly identify the L^d gene in these transformed cells with cDNA probes because of the cross-hybridization of multiple H-2^k haplotype class I genes of the recipient mouse L cells.

The level of L^d expression in mouse L cells is comparable to that of K^k for the transformants (Fig. 1C) and approximately equal to that of L^d for BALB/c spleen cells (Fig. 1D). In contrast, the relative level of L^d expression by normal BALB/c fibroblast and spleen cells is lower than the level of K^d expression as determined by fluorescent labeling and analysis by the fluorescence-activated cell sorter (*12*). Therefore, relative to K molecules, the L^d gene products are expressed at a higher level on the transformed cells than on normal fibroblasts. Transformants expressing L^d molecules at an even higher level than that of 8-5 cells have been isolated, an indication that expression of the transferred L^d gene at a higher level than for K^k molecules is not a lethal event. Moreover, the expression of L^d molecules by the cloned 8-5 transformants is a stable phenomenon. The initial level of L^d expression by these cells has remained constant throughout the maintenance of the cell line in culture for over 49 passages.

To compare the charge and size distributions of the L^d polypeptides synthesized in transformed and normal cells, we analyzed L^d molecules immunoprecipitated from BALB/c spleen and 8-5 cells by two-dimensional gel electrophoresis (Fig. 2). The size and charge heterogeneity of the L^d antigens appear virtually identical for polypeptides isolated from BALB/c lymphocytes (Fig. 2A) and the 8-5 cloned transformed L cells (Fig. 2B). In general the two-dimensional gel patterns of these antigens are characteristic of transplantation antigens isolated by immunoprecipitation (*13*). Mouse Ltk^+ transformants analyzed in a similar manner do not express L^d polypeptides (Fig. 2C). These results demonstrate that the 8-5 cells express L^d antigens that are virtually indistinguishable from L^d antigens of normal spleen cells. The tryptic peptides of these proteins are presently being analyzed by high-performance liquid chromatography to confirm these structural similarities.

Gene transfer and immunoselection provide a powerful approach for identifying and characterizing the genes encoding transplantation antigens. The correlation between gene 27.5 and its serologically defined L^d gene products made on the basis of transformation has been substantiated by the DNA sequence of this cloned L^d gene (*10*). DNA-mediated gene transfer thus provides a general approach to both the phenotypic identification of individual members of a multigene family and the analysis of the functions of cell-surface recognition molecules.

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DNA Sequence of a Gene Encoding a BALB/c Mouse L^d Transplantation Antigen

Abstract. *The sequence of a gene, denoted 27.5, encoding a transplantation antigen for the BALB/c mouse has been determined. Gene transfer studies and comparison of the translated sequence with the partial amino acid sequence of the L^d transplantation antigen establish that gene 27.5 encodes an L^d polypeptide. A comparison of the gene 27.5 sequence with several complementary DNA sequences suggests that the BALB/c mouse may contain a number of closely related L-like genes. Gene 27.5 has eight exons that correlate with the structural domains of the transplantation antigen.*

The transplantation or class I antigens of the major histocompatibility complex (MHC) are present on the surface of all mammalian somatic cells and play a key role in T cell immunosurveillance (*1, 2*). In the inbred BALB/c mouse, three genes for transplantation antigens, D, L, and R, are closely linked and these are separated from a fourth class I gene, K, by approximately 0.3 centimorgan (*3*). We cloned 30 to 40 genes of the BALB/c mouse (H-2^d haplotype) that are homologous to complementary DNA (cDNA) probes for transplantation antigens (*4*) and have demonstrated by gene transfer experiments that one of these clones (27.5) encodes an L^d transplantation antigen (*5*). We have determined the nucleotide sequence of the L^d gene present in clone 27.5.

The genomic clone 27.5 was isolated as previously described (*4*) from an amplified library of BALB/c sperm DNA cloned in the λ vector Charon 4A. The nucleotide sequence of gene 27.5 was determined by the dideoxy sequencing technique with M13mp2 as cloning vector (*4, 6, 7*). The sequencing strategy is shown in Fig. 1. The exons of gene 27.5 were defined on the basis of homology to available amino acid sequences for transplantation antigens (*8, 9*), to several DNA sequences of cDNA's encoding transplantation antigens (*10, 11*), and to a genomic class I clone 27.1, which bears a pseudogene mapping to the Qa-2,3 re-

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