Transfer of Cloned Human Class I Major Histocompatibility Complex Genes into HLA Mutant Human Lymphoblastoid Cells†

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Three new kinds of recombinant DNA constructs were used to transfer cloned human class I HLA genes (A2 and B8) into unique HLA mutant lymphoblastoid cells: (i) pHeBo(x); a class I gene, "x," in plasmid vector pHeBo, which contains a hygromycin resistance gene and Epstein-Barr virus oriP element that sustains extrachromosomal replication; (ii) pHPT(x); gene x in a vector with a hypoxanthine-guanine phosphoribosyltransferase (HPRT) gene; (iii) pHPTe(x); gene x in a vector with the HPRT gene and oriP element. Cell surface class I antigen expression was strong in transferents made with class I-deficient lymphoblastoid cell line mutants .144 (A-null), .53 (B-null), and .184 (A-null, B-null). Transferents expressing HLA-A2 were recognized specifically by HLA-A2-specific cytotoxic T lymphocytes. When introduced on either of the vectors with the Epstein-Barr virus oriP element, the class I gene replicated extrachromosomally and was lost at rates of 0.2 to 0.3 per cell division. When introduced with vector pHPT (lacking Epstein-Barr virus oriP), the B8 gene was inserted at different chromosomal locations. Introduction of the HLA-B8 gene failed to restore antigen expression by HLA-B-null mutant .174, providing evidence that, unlike mutants exemplified by .53, .144, and .184, some HLA antigen loss mutants are deficient in a *trans*-acting function needed for class I antigen expression. Of more general interest, the results obtained with HLA class I genes in vectors that replicate extrachromosomally suggest ways of relating genic expression to chromatin structure and function and of attempting to clone functional human centromeres.

We describe below the combined use of unique human HLA mutant cells and new recombinant DNA molecules for studying expression of human class I histocompatibility genes in human lymphoblastoid cells by means of gene transfer. Our presentation of data has two foci. The immediate focus is on the expression of transferred HLA genes. Since the expression of cloned human class I major histocompatibility complex (MHC) genes after transfer into human lymphoid cells has not been described previously, we illustrate the versatility of the cell-gene-vector combinations by presenting data with different genes, vectors and mutant recipient cells.

In humans the class I antigens HLA-A, -B, and -C are critically important in the recognition of cells as antigenically foreign by most cytolytic T-lymphocytes (CTL). Genetically different forms of the class I molecules are stimuli that trigger CTL response against allogeneic cells. CTL recognition of other antigens (e.g., viral) on autologous cells requires concurrent recognition of autologous class I molecules (46). The critical role of class I molecules in such surveillance by the immune system is emphasized by the increasing number of reports of deficient class I antigen expression on tumor cells. Furthermore, individuals who have a severe form of the "bare lymphocyte" syndrome, which is characterized by deficient class I antigen expression on blood cells, have combined immunodeficiency disease

and die young of infections (37, 41). Therefore, knowledge about control of class I antigen expression has considerable basic biological and medical importance.

Class I antigens (reviewed in reference 27) are cell surface glycoproteins that consist of an α chain (M_r 44,000) noncovalently associated with β_2 microglobulin (β_2m ; M_r 12,000). The α chains are encoded by members of the class I gene family, which is located in the distal part of the MHC on the short arm of chromosome 6; β_2m is encoded on chromosome 15. Recent studies with recombinant DNA probes indicate the presence of at least 17 class I loci in one human MHC haplotype (Koller et al., unpublished data).

Transfer of human class I genes into mouse cells has been an important means of identifying the serologically defined determinants encoded by the genes (3, 21, 23, 24). However, the transferents were not always lysed by CTL known to recognize the antigen on human cells (e.g., see references 3, 9, 17). Failure of CTL attack did not result from association of the human class I α chain with mouse β_2m , since CTL did not lyse mouse cells that expressed a human class I α chain and human β_2m (4). Mouse cell transferents expressed relatively little human class I antigen detectable with antibodies, but this paucity alone could not explain the failure of specific lysis by CTL; some transferents made by introducing the same genes into human cells also expressed relatively little antigen but were lysed by antigen-specific CTL (2, 42). In these experiments the expression of the transferred gene was superimposed on the endogenous class I antigen expression of the recipient cells, which sometimes resulted in significant background lysis of recipient cells (2). This background lysis and the evidence for species-specific accessory factors that are needed for class I-mediated cytotoxicity can

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confound functional studies with transferred human class I genes. The study of human class I genes and antigens by means of gene transfer would be expedited if the cloned genes were transferred into human cells and achieved quantitatively large expression against a background of minimal expression of endogenous class I antigens. Toward this end, we used as recipient cells immunoselected mutant human B-lymphoblastoid cells that no longer expressed class I antigens as a result of gamma ray-induced mutations. Cloned HLA-A2 and -B8 genes were reintroduced into these mutant recipient cells by means of electroporation, and their expression was monitored by antibody binding and CTL lysis. The amounts of cell surface class I antigen encoded by the transferred class I genes at least equalled those of normal lymphoblastoid cell lines (LCLs). Expression of the transferred genes was also manifested by efficient lysis of transferents by antigen-specific CTL.

A second focus of this paper is on possible extrapolations of some of the results with HLA genes to studies of more general interest. We describe the maintenance of human genomic genes on plasmids that replicate extrachromosomally and the gradual loss of such plasmids from cell populations grown in the absence of selection. These results suggest interesting ways of studying the relation between gene expression and chromatin structure or composition and of attempting to clone functional human centromeres (see Discussion).

MATERIALS AND METHODS

Cells and culture media. LCL 721 is a cloned Epstein-Barr virus (EBV)-transformed B-LCL (19). All of its derivative HLA antigen loss mutants referred to in this report (see Fig. 1) were derived by immunoselection with complement and appropriate cytotoxic antibody following gamma irradiation (300 rads) of parental cells (Fig. 1) following the basic protocols described in reference 19.

Cells were maintained in medium RPMI 1640 (85 volumes; GIBCO Laboratories) supplemented with heat-inactivated fetal bovine serum (15 volumes; HYCLONE Sterile Systems, Inc.). Hypoxanthine-guanine phosphoribosyltransferase-deficient (HPRT⁻) mutants (.53TG⁴, .174TG¹, and .184TG⁶ below) were isolated by gamma irradiation (300 rads) of parental cells and, after a 5-day post-irradiation interval, selection in medium supplemented with 5×10^{-5} M 6-thioguanine. Selection for HPRT⁺ transferents (see below) was imposed by supplementing medium with 10^{-6} M azaserine and 10^{-4} M hypoxanthine.

Gene transfer by electroporation (26, 33). Cultures were maintained in the log phase of growth by being diluted to about 3×10^5 cells per ml with fresh medium on each of the 2 days prior to electroporation. Cells were collected by centrifugation ($1,500 \times g$ for 5 min), washed once with Ca-Mg-free phosphate-buffered saline (14), and suspended in phosphate-buffered saline at 10^7 cells per ml. Cells were kept on ice until used. The DNA to be transferred (10 μ g in ≤ 20 μ l) was added to 0.5 ml of cell suspension, which was then placed in an electroporation cuvette in an ice bath. Electroporation was performed as described previously (40). The cells were transferred to culture medium and 1-ml samples containing varied numbers of cells were transferred to 16-mm-diameter wells of 24-well plates (Nunc no. 143982). If selection for HPRT⁺ transferents was to be imposed, the wells contained gamma-irradiated feeder layers of human HPRT⁺ L144 fibroblasts (19). After 5 days, selections were begun by adding fresh medium to result in a final

hygromycin (Calbiochem) concentration of 300 μ g/ml (prepared as in reference 40) or in azaserine-hypoxanthine medium (see above). Thereafter, 1.5- to 2.0-ml volumes of selection medium were maintained by replacing 0.75- to 1.0-ml volumes with fresh selection medium once or twice per week. Resistant cells were evident within 10 days. Isolation of transferents began during week 4 of selection. Independent transferents were isolated from separate wells.

Plasmid construction. The plasmid vector pHPTe was constructed by digesting plasmid pHPT32 (6) with *Sph*I and *Sst*II and isolating the 6.8-kilobase (kb) fragment out of an agarose gel by a modification of the technique of Dretzen et al. (13). Briefly, the DNA fragment was electrophoresed onto a strip of DEAE membrane paper inserted into the gel. The membrane containing the fragment was washed four times with water and once for 5 min with 0.05 M NaCl-5 mM EDTA-20 mM Tris (pH 7.5) and then was placed in a 1.5-ml Eppendorf tube containing 500 μ l of 1.5 M NaCl-5 mM EDTA-20 mM Tris (pH 7.5) at 37°C overnight. The membrane was removed, the solution was centrifuged in a Microfuge (Beckman Instruments, Inc.) for 15 min, and the supernatant was transferred to a new tube. The solution was extracted three times with 2 volumes of water-saturated *n*-butanol. The fragment was then ethanol precipitated, dried, and suspended in DNA dialysis buffer (10 mM Tris, 1 mM EDTA, 1 mM NaCl) at a concentration of 0.5 μ g/ μ l.

Plasmid 127.3 (45) was similarly digested with *Sph*I and *Sst*II, and the 2.2-kb fragment of EBV DNA containing orIP was isolated as described above. This fragment was ligated to the isolated fragment of pHPT32 DNA with T4 ligase according to the specifications of the manufacturer, and the ligation mixture was then used to transform *Escherichia coli* C600 as described previously (15). The structure of the resulting recombinant plasmid, pHPTe, was verified by restriction enzyme mapping.

Recombinant plasmids containing HLA genes were created by treating the plasmid vector DNA cleaved with the appropriate restriction enzyme with calf intestinal phosphatase and ligating in the DNA fragment containing the HLA class I gene by cohesive end ligation. The ligation mixture was then used to transform C600, and the resulting recombinant plasmids used in gene transfer experiments were extensively analyzed with restriction enzyme mapping.

The HLA-A2 gene was isolated by cleaving plasmid pHLA-2a (22) with *Hind*III and isolating the 5.1-kb fragment from a gel as described above. The HLA-B8 gene was isolated by cleaving cosmid 50.2 (Koller, unpublished results) with *Eco*RI and isolating the 6.2-kb fragment containing a class I sequence from a gel.

DNA analysis. High-molecular-weight DNA was isolated as described before (29) and DNA of low molecular weight was isolated by the selective extraction technique of Hirt (18). Restriction endonuclease digestions were executed with 12 μ g of total cell DNA or 5 to 7 μ g of Hirt extract cell DNA for 3 h at 37°C. Southern blotting analysis was then performed with ³²P-labeled nick translation probes as previously described (29). Nonspecific probing for class I loci was performed with the nearly-full length class I HLA-B7 cDNA clone (39). Class I locus-specific probing was performed with probes pHLA-2a.1 (A locus) and pHLA-1.1 (B-locus), using stringencies described in reference 22.

Analysis of antigen expression. Cell surface antigens were assayed by using flow cytometers to quantify binding of anti-class I monoclonal antibodies (Mabs). A FACS IV flow cytometer (Becton Dickinson and Co.) and the HLA-A2-specific MAb BB7.2 (32) were used to analyze pHeBo(A2)

FIG. 1. Origins of mutants used in these experiments. Shaded squares indicate reduced or absent antigen expression. All mutants were isolated after gamma irradiation (300 rads) of their parental cells and negative immunoselection with complement and the antibodies shown. The basic procedures are described in reference 19, as are the origins of LCL 721 and mutants .19 and .45. Mutant .127 is described in references 10 and 28. Large microscopically visible deletions involving the short arm of one chromosome, no. 6, are possessed by mutants .19 (19), .45 (20), and .127 (unpublished data). Mutant .144 has a homozygous deletion of the HLA-A locus (28) and .184 has, in addition, a homozygous deletion of the HLA-B locus (12). Mutant .174 was isolated as described before (11); while the HLA-A2 and -B5 loci are transcribed, the corresponding surface antigens are absent (HLA-B5) or greatly reduced (HLA-A2) (11, 12). An abnormality in the HLA-B5 gene of .53 has not yet been found but HLA-B transcripts have not been detected (unpublished data). The HPRT⁻ derivatives .53TG⁴, .174TG¹, and .184TG⁶ used in some of the experiments were derived as described in Materials and Methods. moab, Monoclonal antibody.

transferrants. pHPTe(B8) and pHPT(B8) transferrants were analyzed with an EPICS C clinical flow cytometer (Coulter Electronics) and MAb B1.23.2. B1.23.2 was originally described as a general anti-class I MAb (34) but appears to be directed primarily against HLA-B antigens in the LCL 721

cell system (11; see below). Cells were prepared for analysis with cell sorters as described previously (12).

The specificity of antigen expression by HLA-B8 transferrants was also determined with complement-dependent cytotoxicity tests performed by the University of

FIG. 2. Construction of pHeBo(A2). Plasmid pHeBo(A2) was created by inserting a 5.1-kb *Hind*III-generated fragment containing the HLA-A2 gene isolated from plasmid pHLA.2a (21) into the *Hind*III site of vector pHeBo by cohesive end ligation. Plasmid pHeBo (40) contains a 2.2-fragment of EBV DNA (thickest arc) that includes the oriP element (in black), the *E. coli hph* gene (thick black arc) that confers resistance to hygromycin and that is flanked by the herpes simplex virus type 1 TK gene promoter, and polyadenylate addition signals (thick open arcs). Thin lines represent plasmid pBR322. Restriction endonuclease cut sites shown are *Hind*III (H), *Cla*I (C), *Eco*RI (E), *Bam*HI (B), *Sal*I (S), *Sma*I (Sma), and *Eco*RV (ERV). C.I.P., Calf intestinal phosphatase.

Wisconsin Tissue Typing Laboratory, using coded cell samples and a panel of 72 HLA class I typing alloantisera.

Cell-mediated cytotoxicity. HLA-A2 specific cytotoxic effector cell (CTL) populations were generated in secondary mixed lymphocyte culture between two combinations of donors in which sensitization would be expected to occur to only a single HLA class I molecule, HLA-A2. FR4 α W5 (5) was raised by priming FR4 cells (A1,1, B8,8 Cw-, DR1,4, DQw1,3, DPw4) against W5 cells (A2, B8, Cw-, DR3, DQw2, DPw2,4) and B12 α FC4 (W. E. Biddison and S. Shaw, unpublished data) was raised by B12 cells (A1, B7,8, Cw-, DR2,3, DQw1,2, DPw4) against FC4 cells (A2,2, B7,7 Cw-, DR2,2, DQw1,1, DPw4,5). The routine conditions for allogeneic sensitizations have been described previously (38). Briefly, 9×10^6 peripheral blood lymphocytes from the responder were cultured with 3×10^6 irradiated (2,000 rads) peripheral blood cells from the stimulator donor in 8 ml of RPMI 1640 with penicillin, streptomycin, and 5% pooled heparinized plasma from normal males. After 10 days in culture, the primed cells were restimulated at 3×10^6 responders and 9×10^6 irradiated stimulator cells in 6 ml of medium with 10% heat-inactivated plasma for an additional 4 days. A standard short-term ^{51}Cr release assay was performed as described before (38). Cold target competition studies were performed by addition to the microtiter wells of a five-fold excess of the unlabeled competitor cell (relative to the number of targets to be added) and preincubation with effectors for 20 min at 37°C before addition of labeled target cells.

RESULTS

Choice of vectors and recipient cells. Anticipating the usefulness of being able to transfer two physically separate genes into the same cells, we used vectors that had different selectable markers. The pHeBo vector (40) (Fig. 2) was chosen because it includes a gene for hygromycin resistance that is dominantly expressed in LCL cells. Furthermore, the presence of the EBV oriP element in pHeBo results in a high frequency of transferents and in persistent, efficient replication of the vector as an extrachromosomal plasmid in EBV-transformed LCL. For some purposes it is useful to be able to study expression of a transferred gene without complications that can result from insertion of the gene into an

abnormal chromosomal location. Growth of pHeBo transferents in the absence of hygromycin selection had revealed spontaneous loss of the vector at rates of at least several percent per cell division (40, 45). One can take advantage of such spontaneous loss to study the relation between genic expressions that occur after DNA transfer and presence of the transferred gene.

The pHPT32 vector (6) (Fig. 3) was used because it contained a functional human HPRT cDNA gene, which would permit convenient isolation of HPRT⁺ cells after transfer of pHPT32 recombinant plasmids into HPRT⁻ recipient cells. Preliminary studies showed that pHPT32 inserted into chromosomes of mutant derivatives of LCL 721, thereby permitting the establishment of mitotically stable transferents. HPRT⁻ derivatives should then permit transfer of a second pHPT32-linked gene into the same transferent.

Three considerations governed the choice of recipient cells (Fig. 1). First, it was important to study the expression of transferred HLA genes in human lymphoid cells. Second, we wished to observe the expression of transferred class I genes in cells in which endogenous class I expressions were minimal. Mutant .144 was chosen because it has a homozygous deletion of the HLA-A locus (22, 28), while mutant .184 was chosen because it has homozygous deletions of both the HLA-A and -B loci (12). Third, we wished to determine if deficiencies of *trans*-active factors were responsible for reduced expressions of class I antigens in some of our mutants. We thought that if such deficiencies existed transfer of appropriate class I genes into such mutants would not be followed by expression of the antigen. Mutants .53, .174, and .184 were chosen to study this question for reasons given in the legend to Fig. 1.

Transfer of the HLA-A2 gene. The HLA-A2 genomic gene is located on a 5.1-kb *Hind*III-generated DNA fragment that has been cloned in recombinant plasmid pHLA-2a (21). The 5.1-kb fragment was isolated from pHLA-2a with *Hind*III and inserted into the *Hind*III-cut site of the pHeBo vector to create recombinant plasmid pHeBo(A2) (Fig. 2).

pHeBo(A2) was transferred into mutants .144 and .184 by means of electroporation, and transferents were isolated by selection with hygromycin (300 $\mu\text{g}/\text{ml}$). DNA isolated from recipient cells and transferents was subjected to Southern

FIG. 4. Southern blotting analysis of hygromycin-resistant transferents of mutants .184 and .144 resulting from transfer of the pHeBo(A2) plasmid. Whole-cell DNA and Hirt extracts were isolated from pHeBo(A2)→.184 transferent (Tr) 1 (A) and from pooled populations of pHeBo(A2)→.144 transferents (B and C). DNA was digested with *Hind*III, electrophoresed on a 0.7% agarose gel, transferred to nitrocellulose, and hybridized to the HLA-A locus-specific probe pHLA-2a.1 (A and B) or to the general class I HLA-B7 cDNA probe (C). Arrows indicate the 5.1-kb fragment containing the 3' untranslated region of the HLA-A2 gene and the 4.7-kb fragment containing the 3' untranslated region of the HLA-A1 gene. Lanes: (1, 7, and 11) LCL 721; (2) .127; (3, 8, and 12) .45; (4) .184; (5) pHeBo(A2)→.184 Tr1 whole-cell DNA; (6) Tr1 Hirt extract; (9 and 13) .144; (10) pHeBo(A2)→.144 Tr whole-cell DNA; (14) pHeBo(A2)→.144 Tr Hirt extract.

blotting analysis (Fig. 4). Both mutants have homozygous deletions of the HLA-A locus and lack the 5.1-kb *Hind*III-generated DNA fragment that includes the HLA-A2 gene (Fig. 4A, lane 4, and B, lane 9). In contrast, total cell DNA of hygromycin-resistant transferents resulting from transfer of pHeBo(A2) contained a 5.1-kb *Hind*III-generated class I DNA fragment (Fig. 4A, lane 5, and B, lane 10).

FACS-assayed binding of HLA-A2-specific MA b BB7.2 showed that presence of the 5.1-kb fragment was associated with cell surface expression of HLA-A2 antigen (Fig. 5A and B). The peak amounts of BB7.2 antibody bound to different .144 (Fig. 5A) and .184 (Fig. 5B) transferents was between one and two times the amount bound to the HLA-A2⁺ control mutant .45, which expresses one copy of the HLA-A2 gene.

pHeBo(A2) transferents are lysed by HLA-A2-specific CTL. Two bulk populations of HLA-A2-specific human CTL were used to characterize functional expression of the HLA-A2 product on the transferents. CTL population FR4 anti-W5 derived from donor FR4 and directed against the A2 antigen of cells from individual W5 has been described previously (5); the other population (B12 anti-FC4) has also been carefully characterized as A2 specific (Biddison and Shaw, unpublished observations).

Table 1 shows that lysis of HLA-A null mutant .144 by these CTL populations was minimal, while the lysis of two independent pHeBo(A2)→.144 transferents at least equalled that of the HLA-A2⁺ control line .45. Further evidence for specific recognition of HLA-A2 on the transferents by the CTLs was obtained by showing that the unlabeled transferent cells blocked the lysis of radioactively labeled HLA-A2⁺ target cells by the antigen-specific CTLs as efficiently as did the HLA-A2⁺ .45 control cells (Table 1). Therefore, assays of antigen by means of specific antibody binding and specific CTL recognition showed that the surface display of HLA-A2 antigen by pHeBo(A2) transferents of human HLA-A-null LCL mutants was quantitatively and functionally comparable to that of HLA-A2⁺ control LCL cells.

pHeBo(A2) replicates as a mitotically unstable plasmid in transferents. The location of the pHeBo(A2) molecules in transferents was determined by probing Hirt extracts (18). Figure 4C shows that the characteristic *Hind*III-generated 5.1-kb class I DNA fragment was present in the Hirt-extracted DNA of pHeBo(A2)→.144 transferents. Use of HLA-A-specific probe pHLA-2a.1 showed that the fragment found in the Hirt extracts was the HLA-A2 gene; i.e., as expected (40, 45), pHeBo(A2) was replicating as an extrachromosomal plasmid in transferent cells. This sug-

FIG. 3. Construction of pHPT(B8) and pHPTe(B8). Cosmid 50.2 was digested with *Eco*RI, and the 6.2-kb fragment containing the HLA-B8 gene was isolated and ligated by cohesive end ligation into the *Eco*RI site of vectors pHPT32 and pHPTe (see Materials and Methods) to create the recombinant plasmids pHPT(B8) and pHPTe(B8), respectively. Plasmids pHPT32 and pHPTe contain a human HPRT cDNA gene (stippled arc) flanked by retrovirus sequence (thick open arc). The thick black arc within the retrovirus sequence represents the 5' long terminal repeat of Friend spleen focus-forming virus. In addition, pHPTe contains the 2.2-kb fragment of EBV DNA containing the oriP element (thickest block) derived from plasmid 127.3 (45). Thin lines represent pBR322 DNA. Details of the construction of pHPTe are described in the text. Restriction endonuclease cut sites shown are *Eco*RI (E), *Hind*III (H), *Bam*HI (B), *Bgl*II (Bg), *Pvu*II (P), *Sma*I (Sma), *Eco*RV (ERV), *Sph*I, *Pst*I, and *Sst*II.

FIG. 5. FACS assayed binding of HLA-A2-specific MAb BB7.2 to hygromycin-resistant pHeBo(A2)→.144 (A) and pHeBo(A2)→.184 (B) transfers. Nontransferent .45 cells express one copy of the HLA-A2 gene, while mutants .144 and .184 have homozygous deletions of the HLA-A locus. The .144 transfers (Tr) shown (A) had been maintained in the presence of 100 μ g of hygromycin per ml prior to analysis. The HLA-A2⁻ fraction of the populations was not evident when the same transfers were maintained at hygromycin concentrations of 300 to 1,000 μ g/ml; e.g., the .184 transfers shown in (B) were grown with 300 μ g of hygromycin per ml.

gested that the rate of loss of the plasmids could be estimated by removing the cells from hygromycin and subsequently following the proportion of HLA-A2⁺ cells.

Several pHeBo(A2)→.144 transfers that had been maintained for about 3 months in medium containing hygromycin (300 μ g/ml) were then passaged in hygromycin-free medium. At the beginning of nonselective growth and at

intervals thereafter samples were removed for FACS determination of the proportion of cells that bound MAb BB7.2. Figure 6 shows that the proportion of HLA-A2⁺ cells declined at fairly constant exponential rates of about 10% (Tr11) and 15% (Tr9) per doubling. These values correspond to loss of the pHeBo(A2) plasmid at rates of about 20% (Tr11) and 30% (Tr9) per doubling. Declines in the proportion of HLA-A2⁺ cells were not observed when the transfers were maintained continuously in the presence of hygromycin (300 μ g/ml). We would expect the appearance of transfers in which the HLA-A2 gene had become stabilized by insertion into a chromosome to be manifested by flattening of the decay curves, but such cells have not yet been observed. Therefore, the probability of insertion was $<10^{-2}$ in Tr9.

Transfer of the HLA-B8 gene. Observations with deletion mutants suggested that gamma ray-induced loss of HLA-B8 expression in LCL 721 is often associated with the loss of a 4.2-kb *Pvu*II-generated DNA fragment (29, 30). As part of the characterization of the complete class I gene family in LCL 721, a cosmid library has been constructed from the genomic DNA of this LCL (D. Geraghty and B. Koller, unpublished data). The library was screened with a class I cDNA clone (39). One cosmid clone, 50.2, was thought to encode the HLA-B8 heavy chain because it contained the 4.2-kb *Pvu*II-generated class I DNA fragment associated with HLA-B8 expression. Further analysis showed that the 4.2-kb fragment spanned the 3' end but not the 5' end of the class I gene in cosmid 50.2. We then determined that a 6.2-kb *Eco*RI-generated fragment in cosmid 50.2 overlapped the 5' end of the 4.2-kb *Pvu*II-generated fragment and apparently contained a complete class I gene. This 6.2-kb *Eco*RI-generated fragment was excised from cosmid 50.2 with *Eco*RI and inserted into the single *Eco*RI site in vector pHPT32 (Fig. 3). The resultant pHPT(B8) plasmid was transferred into HPRT⁻, HLA-B null mutant .53TG⁴ (Fig. 1; see Materials and Methods) by means of electroporation. Several independent transfers were analyzed.

Mutant .53TG⁴ was used as a recipient for pHPT(B8) because it is phenotypically HLA-B null and HPRT deficient. The nature of the mutation that is responsible for the HLA-B null mutant phenotype is not known. HLA-B transcripts have not been detected in .53 by Northern blotting analysis with a locus-specific probe for part of the 3'-untranslated region of the HLA-B8 gene (22), but Southern blotting analysis with probes for the coding sequences, 3'-flanking DNA, and 5'-flanking DNA has, so far, not revealed an abnormality associated with the HLA-B5 gene (H. T. Orr, unpublished observations). We reasoned that if the B-null phenotype of .53 results from a mutation in the antigen-encoding gene or in a *cis*-acting regulatory DNA sequence, introduction of pHPT(B8) into the mutant cells should be followed by HLA-B8 antigen expression. Alternatively, if .53 is deficient in a *trans*-acting function needed for expression of the HLA-B antigen, introduction of the pHPT(B8) plasmid would not result in antigen expression.

Expression of HLA-B antigen was assayed as binding of MAb B1.23.2. The preference of MAb B1.23.2 for the HLA-B antigen was confirmed by antibody-binding studies with an EPICS C flow cytometer (data not shown). Figure 7 shows that pHPT(B8)→.53TG⁴ transfers bound at least as much B1.23.2 antibody as did mutant .127 cells, which express one copy of the HLA-B8 gene.

Since we did not have a MAb that was specific for the HLA-B8 antigen, the specificity of the antigen expressed on pHPT(B8)→.53TG⁴ transfers was determined with com-

TABLE 1. Restoration of HLA-A2-specific recognition by T cells by transfer of the pHeBo(A2) plasmid into HLA-A null mutant .144

Priming combination	Mean % specific lysis of targets ^a				Mean % lysis of A2 ⁺ targets when blocked by ^a :			
	.45	.144	Tr3 ^b	Tr4 ^b	Nothing	.45	.144	Tr3 ^b
FR4 against W5	48.0	10.6	56.5	45.3	48.0	6.5	31.5	2.9
B12 against FC4	27.0	7.6	26.9	22.1	27.0	5.0	18.4	1.3

^a Calculated from triplicate values. Standard errors for all data were <10% of the mean.

^b Tr: pHeBo(A2)→.144 transferents.

plement-dependent cytotoxicity tests, using a panel of 72 typing alloantisera (see Materials and Methods). Two independent transferents were specifically and completely lysed only by alloantisera that recognized HLA-B8 or the HLA-Bw6 group specificity, which is shared by several members of the HLA-B series, including HLA-B8 (data not shown). This evidence for the HLA-B8 specificity of the antigen

expressed on pHPT(B8)→.53TG^r4 transferents was also confirmed by showing that the transferents did not bind MAb 4D12, which is specific for the HLA-B5 antigen (16) in LCL 721 and its derivatives (data not shown). Therefore, according to the serological criterion used to define the HLA-B8 antigen, this gene transfer study indicates that the class I gene on the 6.2-kb *Eco*RI-generated DNA fragment present in LCL 721 and cosmid 50.2 encodes an antigen that is indistinguishable from HLA-B8.

The transferred HLA-B8 gene is associated with chromosomes in pHPT(B8) transferents. DNA isolated from pHPT(B8)→.53TG^r4 transferents was digested with *Bgl*III

FIG. 6. Spontaneous loss of pHeBo(A2) from hygromycin-resistant pHPT(B8)→.53TG^r4 transferents during growth in the absence of hygromycin. Paired cultures of each transferent (Tr) were maintained in nonselective medium or in medium supplemented with hygromycin (300 μ g/ml). Three times per week one-half of each culture was discarded and replaced with an equal volume of fresh medium. At intervals the proportion of HLA-A2⁺ cells in each population was determined by using a FACS IV to count cells binding the HLA-A2-specific antibody BB7.2. For each transferent, the percentage of HLA-A2-positive cells was determined at the initial time point (doublings = 0) and was assigned a value of 1.00. At each subsequent measurement, the proportion of HLA-A2⁺ cells was divided by the proportion of A2⁺ cells present at the time growth without hygromycin was initiated to estimate the residual fraction of A2⁺ cells plotted on the vertical axis.

FIG. 7. Binding of MAb B1.23.2 to HPRT⁺ pHPT(B8)→.53TG^r4 transferents (Tr). Nontransferent .127 cells express one copy of the HLA-B8 gene, which has been deleted in .53. Binding of MAb B1.23.2 was assayed with an EPICS flow cytometer. Overlapping areas of histograms are darkly shaded.

FIG. 8. Southern blotting analysis of HPRT⁺ transferents (Tr) of mutant .53TG'4 resulting from transfer of pHPT(B8). Whole-cell DNA and Hirt extracts were digested with *Bgl*II, and the resulting Southern blots were hybridized to the HLA-B locus-specific probe pHLA-1.1. Arrows indicate the 7.0-kb fragment containing the 3' untranslated region of the HLA-B8 gene and the 5.8-kb fragment containing the 3' untranslated region of the HLA-B5 gene. Transferents 2 to 6 are HPRT⁺ transferents resulting from transfer of pHPT(B8), while transferent 1 is an HPRT⁺ transferent resulting from transfer of pHPT32, which contains the human HPRT cDNA gene but no HLA genes. Lanes: (1) LCL 721; (2) .127; (3) .45; (4) .53TG'4; (5) Tr4 whole-cell DNA; (6) Tr5 whole-cell DNA; (7) Tr6 whole-cell DNA; (8) Tr1 whole-cell DNA; (9) Tr2 whole-cell DNA; (10) Tr3 whole-cell DNA; (11 to 13) Hirt extracts of Tr1, -2, and -3, respectively.

and analyzed with Southern blotting and probe pHLA-1.1 (22), which is specific for the 3' untranslated region of the HLA-B gene (Fig. 8). While .53TG'4 contained only the 5.8-kb *Bgl*II-generated fragment that is associated with the HLA-B5 allele (22) (Fig. 8, lanes 4), the transferents contained additional DNA fragments that reacted with the probe (Fig. 8, lanes 5 to 7, 9, and 10). These additional fragments differed in size but none was 7.0 kb in length, the size of the pHLA-1.1-reactive fragment that *Bgl*II liberates from DNA of the HLA-B8-containing haplotype in LCL 721 (22) (Fig. 8, lanes 2). Figure 8B (lanes 11 to 13) also shows that the Hirt extracts of pHPT(B8) transferents did not contain DNA that reacted with class I probes. Therefore, in contrast to the pHeBo(A2) plasmids that have the EBV oriP element (Fig. 4), the pHPT(B8) plasmids were associated with chromosomes.

The origin of the diverse pHLA-1.1 reactive *Bgl*II-generated DNA fragments in pHPT(B8) transferents is not known. The 6.2-kb *Eco*RI fragment that was inserted into pHPT32 contains two *Bgl*II cut sites within the HLA-B8 gene, but the pHLA-1.1 probe reacts with only one of them. The differently sized pHLA-1.1-reactive fragments in pHPT(B8)→.53TG'4 transferents could have resulted from post-transfer rearrangements, e.g., nonhomologous exchanges between plasmids as an accompaniment of concatamer formation, which has not yet been demonstrated in these cells. Alternatively, different copies of the plasmid could have been inserted into different chromosomal locations by means of exchanges involving different sites in the plasmid. Combinations of these two processes could also have occurred. Regardless of the specific type of event, the number of different pHLA-1.1-reactive DNA fragments is a lower-limit estimate of the number of copies of the plasmid that were inserted into chromosomal DNA. This number of differently sized fragments varied between 1 and 3 (Fig. 8) and agrees, at least approximately, with copy number estimates based on the intensity of the 6.2-kb *Eco*RI-generated

band in Southern blot autoradiographs of the transferents (not shown).

pHPTe: a vector that combines a selectable HPRT gene with the EBV oriP element. We combined the convenience of selecting for the HPRT⁺ phenotype with the plasmid-maintaining properties of the oriP element. This was accomplished by transferring the oriP element from plasmid 127.3 (45) into pHPT32 (Fig. 3). The functionality of the resultant pHPTe vector was then evaluated by inserting the 6.2-kb *Eco*RI-generated HLA-B8-containing fragment into the *Eco*RI site to make pHPTe(B8) (Fig. 3) and then transferring this recombinant plasmid into HLA-B null mutants .53TG'4, .174TG'1, and .184TG'6.

Mutants .53TG'4 and .184TG'6 were used as recipients for reasons given above. Mutant .174TG'1 was used as a recipient because there was evidence that, although expression of HLA-A2 antigen was greatly reduced and that of HLA-B5 antigen was essentially nil (11), the corresponding genes were present and transcribed. Furthermore, the expressions of HLA-A2 and -B5 antigens were fully restored in cell hybrids made by fusing .174 with .127 (12), indicating that .174 is deficient in a *trans*-active function needed for surface expression of class I antigens. Therefore, we expected that, unlike the results obtained by transferring the pHPT(B8) plasmid into mutant .53TG'4 (see above), transfer of the HLA-B8 gene into .174TG'1 would not restore expression of the B8 antigen. HLA-B8 expression was fully restored in pHPTe(B8) transferents made with .53TG'4 (not shown) and .184TG'6 (Fig. 9B). In contrast, expression of the transferred HLA-B8 gene was barely above background in transferents made with .174TG'1 (Fig. 9A).

Southern blotting analysis of the transferents showed that failure of pHPTe(B8)→.174TG'1 transferents to express HLA-B8 antigen did not result from absence of the HLA-B8 gene (Fig. 10). Since there are no *Bgl*II cut sites in the pHPTe vector and two cut sites in the HLA-B8-containing insert, cleavage of pHPTe(B8) with *Bgl*II is expected to

generate a 12.7-kb DNA fragment that reacts with the pHLA-1.1 probe. Figure 10A (lanes 6, 7) shows that a 12.7-kb fragment was liberated from pHPTe(B8)→.174TG^r1 transferents 1 and 2, in addition to the resident 5.8-kb HLA-B5-associated fragment. The autoradiographic intensity of the 12.7-kb autoradiographic band from transferent 1 was about 10 times that corresponding to a single copy per cell when total cell DNA of .127 and of transferent 1 cells were compared. Two additional fragments giving autoradiographic band intensities suggesting multiple copies per cell were also present in this transferent. Unexpected DNA fragments, at least one of which was multicopy, were also present in transferent 2. All of the transferred class I DNA that was detected was in the Hirt extracts rather than integrated into chromosomes (Fig. 10A, lanes 8 and 9). This was expected because of the presence of the EBV oriP element in pHPTe(B8). Digestion of DNA from both pHPTe(B8)→.174TG^r1 transferents with *Eco*RI liberated the 6.2-kb DNA fragment that contains the functional HLA-B8 gene (Fig. 10B, lanes 20 to 23). Thus, the transferred HLA-B8 gene is present in the pHPTe(B8)→.174TG^r1 transferents but the HLA-B8 antigen is not expressed on the cell surface.

The pattern of Southern blot observations obtained with DNA from pHPTe(B8)→.174TG^r1 transferents was also obtained with the HLA-B8 antigen-expressing transferents made with pHPTe(B8) and .184TG^r6 (Fig. 10A and B): (i) unexpected *Bgl*II-generated pHLA-1.1-reactive DNA fragments were observed in addition to the expected 12.7-kb fragment; (ii) some of the unexpected fragments occurred in multiple copies per cell; (iii) all of the observed transferred class I DNA was extrachromosomal.

DISCUSSION

Three kinds of vectors described above were used to introduce human MHC genes into human EBV-transformed LCLs. The MHC genes were linked to selectable markers in the recombinant constructs. Hygromycin resistance is dominantly expressed in virtually any kind of mammalian cell. The selection protocol that is most often applied in transfer studies is the use of thymidine kinase-deficient (TK⁻) recipient cells and the isolation of MHC gene transferents by means of co-transformation with a plasmid bearing a TK⁺ gene. It is difficult to obtain phenotypically TK⁻ mutants from many kinds of cells because TK⁻ mutations are recessive and autosomal; i.e., at least two mutations are required in diploid cells. In contrast, it is usually easy to obtain X-chromosomal HPRT⁻ mutants from diploid mammalian cells; one mutation on the single active X chromosome suffices because the HPRT alleles on the other, inactive, X chromosomes are normally not phenotypically expressed (25, 36). In these studies, we exploited the convenience of isolating HPRT⁻ mutants from three human LCLs that had two X chromosomes per cell to introduce an HLA gene into the cells by means of transfer with HPRT⁺-bearing vectors.

By providing an additional selectable marker, HPRT⁺ vectors increase the opportunities for introducing at least two genes. Furthermore, 6-thioguanine selection alone can be used for this purpose. 6-Thioguanine selection against transferents that have the HPRT⁺ phenotype can be used to isolate HPRT⁻ derivatives that continue to express the previously co-transferred MHC gene. This permits introduction of additional genes by selection for the HPRT⁺ phenotype a second time.

Transfer of class I HLA genes with the vectors pHeBo and pHPTe, both of which contain the EBV oriP element,

FIG. 9. Binding of MAb B.1.23.2 to HPRT⁺ pHPTe(B8)→.174TG^r1 (A) and pHPTe(B8)→.184TG^r6 (B) transferents. Nontransferent .127 cells express one copy of the HLA-B8 gene, while .184TG^r6 cells have a homozygous deletion of the HLA-B locus. Mutant .174TG^r1 transcribes the HLA-A2 and HLA-B5 genes but surface expression of the antigens is absent (HLA-B5) or deficient (HLA-A2). An EPICS C flow cytometer was used to assay the binding of antibody B1.23.2.

resulted in persistent replication of the recombinant DNA molecules as extrachromosomal plasmids in all 10 transferents studied so far. With both oriP-containing vectors, the transferred class I gene and vector DNA were detected exclusively in the Hirt extracts containing small, extrachromosomal DNA molecules in transferents (Fig. 4

FIG. 10. Southern blotting analysis of HPRT⁺ transferents (Tr) of mutants .174TG'1 and .184TG'6 resulting from transfer of pHPTe(B8). Whole-cell DNA and Hirt extracts were digested with *Bgl*II (A) or *Eco*RI (B), and the resulting Southern blots were hybridized to the HLA-B locus-specific probe pHLA-1.1. Arrows indicate the 7.0-kb HLA-B8-specific fragment and the 5.8-kb HLA-B5-specific fragment (A), the 12.7-kb fragment representing the linearized pHPTe(B8) plasmid (A), and the 6.2-kb *Eco*RI-generated fragment specific for both HLA-B8 and -B5 (B). Lanes: (1 and 15) LCL 721; (2 and 16) .127; (3 and 17) .45; (4 and 18) .174TG'1 Hirt extract; (5 and 19) .174TG'1 whole-cell DNA; (6 and 20) pHPTe(B8)→.174TG'1 Tr1 whole-cell DNA; (7 and 21) .174TG'1 Tr2 whole-cell DNA; (8 and 22) .174TG'1 Tr1 Hirt extract; (9 and 23) .174TG'1 Tr2 Hirt extract; (10 and 24) .184TG'6; (11 and 25) pHPTe(B8)→.184TG'6 Tr1 whole-cell DNA; (12 and 26) .184TG'6 Tr3 whole-cell DNA; (13 and 27) .184TG'6 Tr1 Hirt extract; (14 and 28) .184TG'6 Tr3 Hirt extract.

and 10; not shown). This observation accorded with the evidence that HLA-A2⁻ cells spontaneously appeared at rates of about 0.10 to 0.15 per cell division during hygromycin-free growth of transferents containing one to two copies of pHeBo(A2) (Fig. 6).

HLA-A2⁻ cells could result from unequal segregation of plasmid replicas during cell division, a process that could also generate cells with additional copies of the plasmid. HLA-A2⁺ pHeBo(A2)→.144 transferents grown at 200 μ g of hygromycin per ml had an average of one to two copies of the plasmid in comparison to the 5 to 10 copies per cell present in the same transferent grown at 1,000 μ g/ml (not shown). However, varying the concentration at which pHeBo(A2)→.144 transferents were maintained between 100 and 1,000 μ g/ml did not significantly affect peak HLA-A2 antigen expression as determined with FACS analysis of MAb BB7.2 binding; it merely resulted in sharpened distributions about the same peak as the hygromycin concentration was increased (e.g. see Fig. 5A). At least three transferents of pHeBo(A2)→.144 and →.184 grown in the

presence of 300 μ g of hygromycin per ml had one to three copies of the plasmid per cell and symmetrical antigen expression distributions.

These observations regarding gene copy number and quantity of antigen suggest that EBV oriP-bearing plasmids could be amplified in the cells we used but that factors other than gene copy number limited the expression of the HLA-A2 surface antigen. This disparity between gene copy number and antigen expression has been observed in other transfer studies (1, 43) and was not limited to HLA-A2 in our work; expression of the HLA-B8 antigen corresponded to 1 to 2 copies per cell (Fig. 7 and 9) in transferents that had 4 to 20 copies of the HLA-B8 gene, regardless of whether the gene was integrated into a chromosome (Fig. 8) or was present on extrachromosomal plasmids (Fig. 10). Northern blotting and immunoprecipitation studies are being performed to determine the factors that limit surface antigen expression in transferents.

Transfer of the HLA-B8 gene with the pHPT32 vector resulted in introduction of the gene into chromosomal DNA:

the HLA-B8 gene was not detected in Hirt extracts and digestion of transferent cell DNA with *Bgl*III showed that the gene had acquired different flanking DNA in different pHPT(B8)→.53TG⁴ transferents (Fig. 8). The presence of a single *Bgl*III-generated band of approximately unit autoradiographic intensity indicated that some transferents contained a single copy of the pHPT(B8) plasmid (transferents 4 and 6, Fig. 8). Multiple *Bgl*III-generated autoradiograph bands in other transferents were evidence for the presence of multiple copies of the plasmid. This evidence for multiplicity (one to five per cell) was supported by dot blot analysis, in which the B8-specific DNA autoradiographic dot intensity corresponding to known numbers of cell genomes was compared with that of known numbers of copies of B8-bearing plasmids (not shown). Since some bands corresponding to a given insertion site of pHPT(B8) were of distinctly greater intensity than that expected for one copy per cell (e.g., transferent 5, Fig. 8, lane 6), there is evidence for amplification of the HLA-B8 gene inserted at some chromosomal sites. Observations made with some pHeBo(A2)→.184 and pHPTe(B8)→.184 transferents suggest that such amplification could have occurred before integration of plasmid DNA into a chromosome. In both cases, autoradiograph bands that correspond to multiple copies of a rearranged plasmid are present in Hirt extracts (Fig. 4 and 10). We do not yet know if rearranged plasmids are physically associated.

The results obtained with transfer of the HLA-B8 gene into mutants .53TG⁴, .174TG¹, and .184TG⁶ exemplify the use of gene transfer for analyzing HLA antigen loss mutants. LCL mutants 721.61, -.134, and -.174 are phenotypically HLA-B null and have greatly reduced expression of HLA-A2 (11). However, the HLA-A2 and -B5 genes and cytoplasmic transcripts are present, and fusion of these class I antigen-deficient mutants with mutant 721.127 results in full cell surface expression of the HLA-A2 and -B5 antigens (12). This result was confirmed in another laboratory with mutant .174 (35). The evidence suggesting that reduced cell surface expression of class I HLA antigens by mutant .174 results from deficiency of a *trans*-acting function is supported by the gene transfer experiments described above. The introduction of pHPTe(B8) into .174 did not result in the cell surface expression of HLA-B8 antigen (Fig. 9A), even though the pHPTe(B8) DNA was found in the transferents (Fig. 10). Thus, the *trans*-acting function that is deficient in .174 is required for the expression of a class I antigen, whether it is encoded by a resident gene (HLA-B5) or by a gene introduced by DNA transfer (HLA-B8). Available evidence suggests that a locus responsible for the class I-deficient phenotype of mutant .174 is located between the complement gene cluster and the class II region in the MHC (44; H. Erlich, J. S. Lee, J. L. Petersen, and R. DeMars, Hum. Immunol., in press; unpublished data).

The evidence summarized above suggests that deficient cell surface expression of a class I HLA antigen by a B-LCL may sometimes result from changes at an unsuspected locus within the MHC. Such mutants could not be used as recipients for class I genes when the objective is expression of the corresponding antigen. The gene transfer experiments described here distinguished between a mutant (.174) that could not be used and mutants (.53 and .184) that readily express transferred class I genes. Therefore, the mutations present in mutants .53 and .184 do not affect *trans*-acting functions necessary for normal class I antigen expression. Southern blotting analysis of mutant .53 has not revealed a structural alternation of the HLA-B5 gene that is still present, but cytoplasmic HLA-B transcripts have not been

detectable with a probe specific for part of the B locus 3' untranslated region (unpublished data). Mutant .184 still expresses the HLA-C antigen. We may recently have increased the usefulness of .184 as a recipient for class I genes by isolating from it a gamma ray-induced mutant that appears to be completely class I null; i.e., it does not bind the anti-class I MAb W6/32 (31) or the anti- β_2m MAb BBM.1 (7), although it has normal amounts of β_2m mRNA (unpublished data).

The extrachromosomal replication of genomic HLA genes in vectors that contain EBV oriP suggests two kinds of interesting experiments. First, the cloned genes can be subjected to *in vitro* alterations, such as site-directed mutagenesis or methylation in sequences that regulate transcription. While methylation alterations would not be replicated in bacterial hosts, all alterations would be replicated in LCL transferents. The effects of alterations on genic expression can be determined. The isolation of the extrachromosomal plasmids as minichromosomes would then also permit attempts to identify effects of the DNA alterations on chromatin structure and composition.

Second, the spontaneous loss of EBV oriP-containing plasmids (Fig. 6) probably results from unequal distribution of replicas into sister cells during mitosis. If a plasmid, such as pHeBo(A2), also contained an insert of genomic DNA that had centromere function, sister replicas of the plasmid might regularly segregate into sister cells, thereby mitotically stabilizing the plasmid. The principle of mitotic stabilization of extrachromosomal plasmids was used in cloning functional yeast centromeres (8). Our work suggests how the same principle might be used in attempts to clone functional human centromeres.

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