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High-Level Expression of a Cloned HLA Heavy Chain Gene Introduced into Mouse Cells on a Bovine Papillomavirus Vector

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A gene encoding the heavy chain of an HLA human histocompatibility antigen was isolated from a library of human DNA by recombination and selection in vivo. After insertion into a bovine papillomavirus (BPV) DNA expression vector, the gene was introduced into cultured mouse cells. Cells transformed with the HLA-BPV plasmids did not appear to contain extrachromosomal viral DNA, whereas BPV recombinants usually replicated as plasmids in transformed cell lines. Large amounts of HLA RNA were produced by the transformed cells, and the rate of synthesis of human heavy chain was several-fold higher than in the JY cell line, a well-characterized human lymphoblastoid cell line which expresses high levels of surface HLA antigen. Substantial amounts of human heavy chain accumulated in the transformed cells, and HLA antigen was present at the cell surface. These observations establish the feasibility of using BPV vectors to study the structure and function of HLA antigens and the expression of cloned HLA genes.

The major histocompatibility complex codes for cell surface glycoproteins that play a role in a number of cell recognition phenomena (for a comprehensive review, see reference 11). The HLA-A, HLA-B, and HLA-C antigens in humans and their counterparts in other species, e.g., the H-2K, H-2D, and H-2L antigens in mice, are expressed in almost all cell types and are involved in restricting the activity of cytotoxic T lymphocytes and in tissue rejection (6, 8, 10, 18, 24, 59, 61). These class I antigens consist of a 43,000- to 45,000-dalton heavy chain polypeptide noncovalently associated with the 12,000-dalton light chain, also known as β_2-microglobulin. β_2-Microglobulin and most of the heavy chain molecule are located at the external surface of the plasma membrane, and the entire complex is anchored to the cell by the transmembrane domain of the heavy chain (53).

These antigens show an exceptional degree of polymorphism which forms the basis for specific recognition of allogeneic cells and for major histocompatibility complex restriction of cytotoxic T lymphocytes. Structural studies of HLA antigens demonstrated that this polymorphism resides exclusively in the amino acid sequence of the heavy chain (42, 52) and that there are numerous amino acid substitutions between different allelic variants (32). It has not been determined which amino acid differences are required for the specificity of the cellular recognition and which, if any, are solely a consequence of the mechanism used to generate the necessary polymorphism.

The study of individual HLA proteins has been hampered by the limited availability of monospecific antisera and cell lines. This difficulty has been overcome to a certain extent by the development of monoclonal antibodies against single antigenic specificities and by the use of apparently homozygous cell lines derived from offspring of consanguinous marriages (2, 4, 38, 43, 44). An alternative method of generating cell lines that express single, defined HLA antigens is to isolate HLA genes and introduce them into cells in culture that do not otherwise express HLA antigens. This approach has been used to identify and analyze transferred HLA genes (1, 69) and to detect the presence of active HLA genes in cosmid clones and in total genomic DNA (23, 31). Similar experiments have identified specific cloned murine and porcine transplantation antigen genes (16, 60). In these experiments, the genes were transferred into mutant mouse and hamster cells by biochemical selection for the cotransferred herpes simplex virus thymidine kinase or *Escherichia coli* guanine phosphoribosyltransferase gene. Use of this technique often leads to gross rearrangement of the transferred DNA and to low or variable levels of expression (37, 47, 48, 68). We decided to test the feasibility of using bovine papillomavirus (BPV) DNA vectors to transfer histocompatibility genes into mouse C127 cells because a wide variety of foreign genes are expressed at a high level in cells transformed by BPV recombinant molecules (9, 39, 46, 57, 70). Morever, because BPV DNA replicates as an extrachromosomal element in transformed mouse cells (28, 29), it is often possible to isolate cell lines with no or minimal rearrangement of the foreign DNA (9, 27, 56).

In this report, we describe the isolation of an HLA heavy chain gene and its introduction into mouse cells on a BPV DNA-derived cloning vector. Analysis of its transcriptional activity and the production of its primary protein product demonstrates that the transferred gene is expressed at a high level. However, contrary to our expectations, the foreign DNA is apparently integrated into mouse chromosomal DNA.

MATERIALS AND METHODS

Recombination screening and analysis of phage DNA. A 365-base-pair *Pst*I to *Pvu*II fragment of the cDNA insert in pHLA-1 (see Fig. 1) was purified by gel electrophoresis and ligated to the *Pst*I to *Cla*I large fragment of plasmid πVX (58), after the *Cla*I terminus was converted into a blunt end with the large fragment of DNA polymerase I and deoxyribonucleoside triphosphates. *E. coli* w3110 (p3) was transformed with the ligation mixture, and plasmid DNA from individual ampicillin-resistant (Amp^r) and tetracycline-resistant (Tet^r) colonies was analyzed. The structure of πHLA-1 (Fig. 1) was confirmed by restriction analysis and DNA blotting experiments.

We performed a recombination screen (58) to isolate

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phage containing DNA homologous to the HLA region in πHLA-1. Phage stocks were prepared by infecting a fresh, saturated, 200-μl culture of bacteria carrying πHLA-1 with 2 × 10^7 PFU of a library of human DNA cloned in Charon 4A (30). Phage able to form plaques on w3110 (Su^-) bacteria were present in the lysate at a frequency of 3 × 10^{-8} and were designated λ HB-1 to λ HB-20. After three rounds of plaque purification on Su^- cells, phage DNA was prepared from plates of w3110 cells infected with individual plaque suspensions (36).

Restriction maps (see Fig. 3) were generated by digesting DNA preparations with the indicated enzymes singly and in combination. After agarose gel electrophoresis, bands were visualized by staining with ethidium bromide and identified by hybridization (63) to the following specific probes labeled by nick-translation (55): πVX, πHLA-1, *Pst*I insert fragment from pHLA-1 (3′ HLA coding and nontranslated region), *Pvu*II small fragment from pHLA-1 (3′ HLA coding region and pBR322 Amp^r gene), and pHLA-2 (entire HLA coding region in pBR322 [62]).

To identify probes for a second round of recombination screening (see below), we first used an EDTA enrichment procedure (45) to isolate phage that had deleted the πHLA-1 sequences. *E. coli* LE392 (Su^+ $RecA^+$) was infected with λ HB-1 and λ HB-4 phage. A total of 3 × 10^8 PFU/ml of each of the resulting phage stocks in 10 mM Tris-hydrochloride–10 mM EDTA was heated at 45°C for 20 to 30 min and then cooled to 0°C after the addition of $MgSO_4$ to 20 mM (45). Phage which had deletions of the suppressor tRNA gene were identified by their ability to lyse Su^+ but not Su^- bacteria and were plaque purified on Su^+ bacteria. Restriction analysis of phage DNA preparations identified phage, such as 4d-5 and 8d-5, in which the πHLA-1 segment seemed to have been precisely excised by homologous recombination between the direct repeats present in the parental phage. In these deleted phage, the loss of πVX sequences with the retention of sequences homologous to the recombination probe in πHLA-1 was confirmed by DNA blotting and by the ability of the deleted phage to recombine with πHLA-1 but not with πVX.

We constructed a collection of miniplasmids containing inserts from the cloned HLA region by ligating *Sau*3A fragments of phage 8d-5 DNA (digested to about one-half completion) to the calf intestinal alkaline phosphatase-treated *Bgl*II fragment of πVX. Strain w3110 (p3) cells were transformed to Amp^r Tet^r with the ligation mixture, and individual, transformed colonies were spotted onto selective plates. After overnight growth of each culture, a large scoop of bacteria (about 5 to 10 mm^3 of cells) was dispersed in 50 μl of SM (36). To identify bacterial isolates that carried a miniplasmid containing a nonrepetitive insert from the HLA gene region, approximately 10^6 4d-5 or library phage were incubated with the bacteria for 20 min at 37°C, and then 200 μl of a fresh, saturated culture of w3110 (Su^-) was added immediately before plating the mixture in top agar on L plates. During the 37°C incubation, the phage adsorb to the miniplasmid-containing cells and initiate infection. In the absence of recombination, the released phage cannot successfully infect Su^- bacteria. If recombination does occur between a phage and the resident miniplasmid, a burst of *supF*-containing (am^+) phage is released which can successfully infect the excess Su^- bacteria. Therefore, when the cells in the preincubation carry πHLA-1, library infection results in a lawn with predominantly small, mottled plaques due to the phage growth in the Su^+ bacteria in the lawn. In contrast, infection of πHLA-1-containing bacteria with 4d-5 results in the appearance of many large, clear plaques due to growth of the abundant recombined phage in both bacterial cell types. Similarly, three independent bacteria isolates generated by transformation by the library of 8d-5 DNA fragments in πVX released abundant am^+ phage after infection with 4d-5 but not after infection with the genomic library.

A standard genomic library recombination screen was performed on isolates, B17, B28, and B30, and the relative yield of am^+ phage was similar to that for bacteria carrying πHLA-1. The absolute frequencies of phage recovery from the three isolates was between 3 × 10^{-9} and 7 × 10^{-9}. DNA of plaque-purified phage was analyzed by digestion with restriction endonucleases to locate the inserted miniplasmid and to determine its orientation.

Transfer of an HLA gene into mouse cells. *Hin*dIII-digested pBPV-H11 (9) was treated with calf intestinal alkaline phosphatase and ligated to a mixture of *Hin*dIII restriction fragments of phage B28-14 DNA. *E. coli* DH-1 (19) was transformed to Amp^r with the ligation mixture, and a plasmid containing the inserted HLA gene was isolated. This plasmid was partially digested with *Hin*dIII, and full-length linear molecules were purified by gel electrophoresis, treated with calf intestinal alkaline phosphatase, and ligated to the purified 7.6-kilobase pair (kb) *Hin*dIII fragment from the human β-globin gene cluster. Plasmids pBPV-βHLA-1 and pBPV-βHLA-3 (see Fig. 1) were isolated from DH-1 bacteria transformed with the ligation mixture; the structures shown in Fig. 1 were determined by restriction endonuclease analysis.

Mouse C127 cells were transformed with 1.5 to 2.5 μg of pBPV-βHLA-1 or pBPV-βHLA-3 as described previously (9), except carrier DNA was omitted and a 20% dimethyl sulfoxide shock was performed. Individual foci, which arose at a frequency of about 150 per μg of BPV DNA, were picked and expanded into cell lines. Analysis of preparations of low-molecular-weight DNA (20) demonstrated that in most cell lines, DNA hybridizing to the input plasmid comigrated with contaminating high-molecular-weight DNA. DNA from cell lines βH1.2, βH1.8, and βH3.1, among others, contained all the *Hin*dIII plus *Bam*HI fragments present in the input plasmids, and these cell lines were cloned by limiting dilution and selection of morphologically transformed cells.

Analysis of DNA and RNA in transformed mouse cells. Total cellular DNA was prepared by standard procedures (36). DNA (5 μg per lane) was mildly mechanically sheared, digested with *Sal*I, or digested with *Hin*dIII plus *Bam*HI. After agarose gel electrophoresis, DNA samples were partially depurinated (66) and transferred to nitrocellulose (63). Specific DNA fragments were detected by hybridization to nick-translated pBPV-βHLA-3 and autoradiography.

Cytoplasmic RNA was prepared as described previously (13). RNA (20 μg per lane) was subjected to electrophoresis in agarose gels containing 0.31 M formaldehyde and was transferred to nitrocellulose (15). HLA-specific RNA was detected by hybridization to nick-translated pHLA-2 (entire HLA coding region) or the 1-kb *Sst*I fragment of the cloned, unrearranged gene (3′ end of the HLA gene).

Analysis of proteins. Subconfluent, adherent mouse cells or human JY cells in suspension were labeled at 37°C with [^{35}S]methionine (New England Nuclear Corp.) in minimal essential medium (for mouse cells) or RPMI 1640 medium (for JY cells). For pulse-labeling, cells were rinsed once with methionine-free medium (2% fetal calf serum), incubated for 20 min in the same medium, and labeled for 30 min in the

medium supplemented with 100 μCi of [^{35}S]methionine per ml. For long-term labeling, cells were incubated for 24 to 26 h with medium containing 25 μCi of [^{35}S]methionine (total methionine concentration, 50 μM) per ml and 5% fetal calf serum. After labeling, cells were rinsed twice with ice-cold phosphate-buffered saline, lysed with ice-cold binding buffer (20 mM Tris-hydrochloride [pH 7.5], 25 mM NaCl, 5 mM $MgCl_2$, 0.5% Nonidet P-40 [NP-40], 150 μg of phenylmethylsulfonyl fluoride per ml, 1 mM dithiothreitol), and incubated for 40 min at 0°C with occasional mixing. Cell debris was removed by centrifugation, and the supernatant was assayed for trichloroacetic acid-insoluble radioactivity and stored at −20 or −70°C.

Equal amounts of incorporated [^{35}S]methionine (generally 0.3×10^5 to 3×10^5 cpm) were included in each set of samples for immunoprecipitation. To quantitate total human heavy chain, 120-μl samples in binding buffer containing 0.8% sodium dodecyl sulfate (SDS) were boiled for 3 to 5 min to dissociate any heavy chain β_2-microglobulin complexes. After cooling to room temperature, 480 μl of binding buffer supplemented with 1% NP-40 was added. After addition of saturating amounts of anti-heavy chain antibody, the mixture was incubated at 0°C for 1 h. Fixed *Staphylococcus aureus* cells were then added before an additional 1 h of incubation at 4°C with constant gentle agitation. Precipitated samples were washed at 0°C four times with 50 mM Tris-hydrochloride (pH 7.5), 0.65 M NaCl, 5 mM EDTA, 0.5% NP-40, 1 mg of bovine serum alubumin per ml, 150 μg of phenylmethylsulfonyl fluoride per ml, and 1 mM dithiothreitol, twice with the above buffer with 0.15 M NaCl, and once with the buffer with 0.15 M NaCl without NP-40. Precipitated proteins were visualized by SDS-polyacrylamide gel electrophoresis, fluorography, and autoradiography by standard procedures.

RESULTS

Isolation of an HLA gene by recombination screening. We used the in vivo recombination screening procedure developed by Seed (58) to isolate phage containing heavy chain HLA sequences from a bacteriophage lambda library of human genomic DNA. In this procedure a "recombination probe" is inserted into the miniplasmid πVX which carries a synthetic suppressor tRNA gene, *supF*. When bacteria bearing the miniplasmid recombinant are infected with a phage library, homologous recombination can occur between the probe sequence on the miniplasmid and a corresponding sequence in individual phage in the genomic library. This reciprocal recombination event results in the insertion of the miniplasmid into phage bearing the homologous sequence, with duplication of the probe sequence. These phage can then be purified from the vast excess of nonrecombinant phage by selection for the *supF* gene on the inserted miniplasmid. We used a recombination probe derived from the 3′ nontranslated region of pHLA-1, a cDNA clone of an HLA class I heavy chain mRNA (51). A 365-base-pair fragment of DNA extending from 13 base pairs 3′ of the translational termination codon to about 50 base pairs 5′ of the end of the transcript was inserted into the polylinker segment of πVX (58). Bacteria containing the resulting plasmid, πHLA-1 (Fig. 1), were used to screen a library of human DNA of unknown HLA type (30), and phage able to grow on Su^- bacteria were isolated.

DNA isolated from plaque-purified phage was analyzed by restriction mapping and gel blotting techniques. In addition to vector fragments and a number of fragments specific for individual isolates, most of the phage DNA inserts contain a common set of *Eco*RI restriction fragments of 4.1, 2.5, 1.9, and 1.8 kb (data not shown). Isolates that did not contain these common fragments were not further analyzed. Insertion of the probe-containing plasmid into the phage DNA by reciprocal, homologous recombination generates a duplication of the homologous segment separated by the inserted πVX sequences. The cellular *Eco*RI DNA fragment homologous to the recombination probe is thus disrupted by the *Eco*RI sites in the inserted miniplasmid, and upon digestion with *Eco*RI, it gives rise to two DNA fragments which should hybridize to the HLA cDNA fragment used as the recombination probe (which contains no *Eco*RI cleavage sites). In all of the phage recombinants examined, the entire pHLA-1 cDNA insert hybridizes to a 4.1-kb *Eco*RI fragment and to a second fragment characteristic of each independent recombinant phage (Fig. 2A). The existence of a variable-sized, hybridizing *Eco*RI fragment probably indicates that the insertion of πHLA-1 occurred near one of the endpoints of the cellular DNA segments which were joined to the phage vector by means of synthetic *Eco*RI sites when the library was constructed (30).

These results suggest that the inserts in the majority of the phage were derived from a single genomic region or from several closely related genomic regions, that the common *Eco*RI fragments represent the region of overlap present in all the isolates, and that the recombination event occurred near one end of the cellular DNA insert. Mapping studies with a number of other restriction endonucleases confirmed these conclusions and allowed the construction of the restriction map of the genomic region shown in Fig. 3A. The transcriptional orientation of the gene was determined by hybridization of an *Eco*RI digest of λ HB-3 DNA with

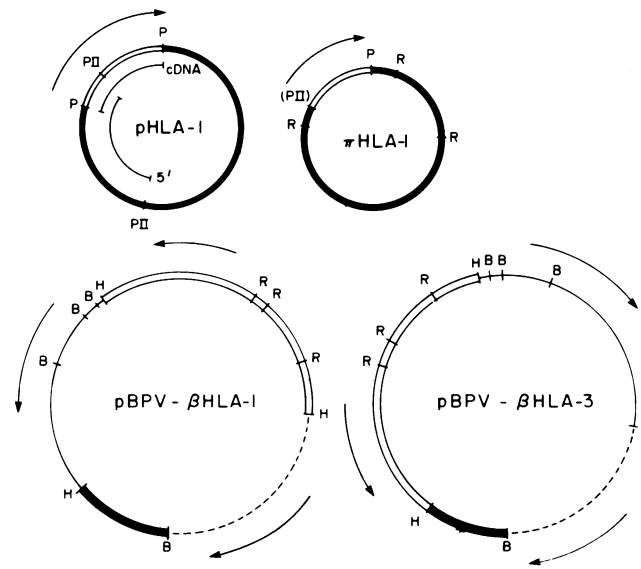

FIG. 1. Schematic diagrams of BPV and HLA plasmids used in this study. Symbols: Thin lines, human globin DNA segment; thick lines, bacterial plasmid sequences; double lines, HLA segment; dashed lines, BPV 69% fragment; arrows indicate the direction of transcription of the various segments. Restriction sites: P, *Pst*I; PII, *Pvu*II; R, *Eco*RI; H, *Hin*dIII; B, *Bam*HI. The *Pvu*II site in the HLA segment in pHLA-1 is 13 base pairs in the 3′ direction to the translational termination codon (51). The arcs inside the map of pHLA-1 indicate the segments used as specific probes for the entire cDNA insert or its 5′ portion. The *Pvu*II site in πHLA-1 was destroyed during construction of this plasmid. The *Eco*RI sites in the HLA segment are the only ones indicated in the maps of pBPV-βHLA-1 and pBPV-βHLA-3.

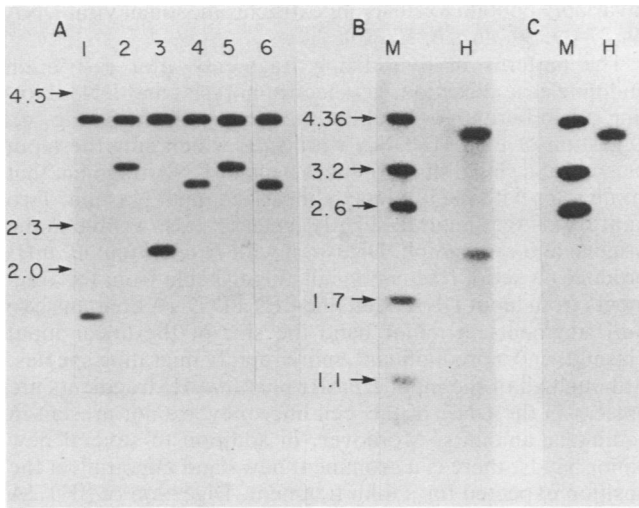

FIG. 2. Hybridization analysis of recombinant HLA phage. (A) DNA from the individual phage isolates λ HB-1 through λ HB-6, as indicated at the top of the figure, was digested with *Eco*RI, electrophoresed through a 1.2% agarose gel, and transferred to nitrocellulose. The filter was hybridized to nick-translated *Pst*I HLA fragment from pHLA-1, washed at 68°C in 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and 0.1% SDS, and autoradiographed. The faint band at 1.75 kb in some of the lanes was not reproducibly observed and is presumably due to a contaminant in these digests. (B) λ HB-3 DNA was treated as in (A). (C) λ HB-3 DNA was treated as in (A), except that the small *Pvu*II fragment from pHLA-1 was used as the probe. Sizes (in kb) and positions of marker fragments are as indicated. M, Marker; H, λ HB-3.

specific probes derived from pHLA-1 (Fig. 1). As already noted, the cDNA insert probe hybridizes to two *Eco*RI fragments because it is homologous to the direct repeats of the recombination-probe sequences which are separated by *Eco*RI sites (Fig. 2A and B). In contrast, the 5′ *Pvu*II fragment hybridizes only to the 4.1-kb fragment (Fig. 2C), indicating that transcription proceeds from the 4.1-kb fragment through the region homologous to the recombination probe, i.e., left to right as the map is drawn in Fig. 3A. This 5′ to 3′ orientation is also consistent with the orientation of the πVX sequences in the recovered phage DNA, as determined by restriction endonuclease mapping (data not shown). Since none of the human sequences in the *Pvu*II fragment are present in πHLA-1, hybridization of phage λ HB-3 DNA to this fragment also indicates that the cloned cellular DNA is homologous to HLA coding sequences and not just to the 3′ nontranslated region of the mRNA used for the genomic screen. pHLA-2, a heavy chain cDNA clone that extends further toward the 5′ end of the message than does pHLA-1 (62), does not hybridize to *Eco*RI fragments other than those detected with the 3′ nontranslated region probe (data not shown). By this test, the genomic region cloned does not contain additional HLA-like genes.

Isolation of an unrearranged HLA gene and transfer into mouse cells. Because the HLA gene initially cloned is disrupted in its 3′ nontranslated region by an inserted miniplasmid, it was necessary to isolate an unrearranged version of the gene for introduction into mammalian cells. For this purpose, we used a nonrepetitive sequence flanking the cloned HLA gene as the probe for a second recombination screen of the genomic library. To identify such a flanking sequence, we made the assumption that the frequency with which a segment of DNA recombined with phage in the genomic library would roughly correspond to its genomic reiteration frequency. As described above, a rapid, double-plating technique was used to identify three bacterial isolates that by this criterion contained recombinant miniplasmids with nonrepetitive inserts from the HLA region.

We performed a standard library screen on each of these three isolates. The structure of the DNA of the phage

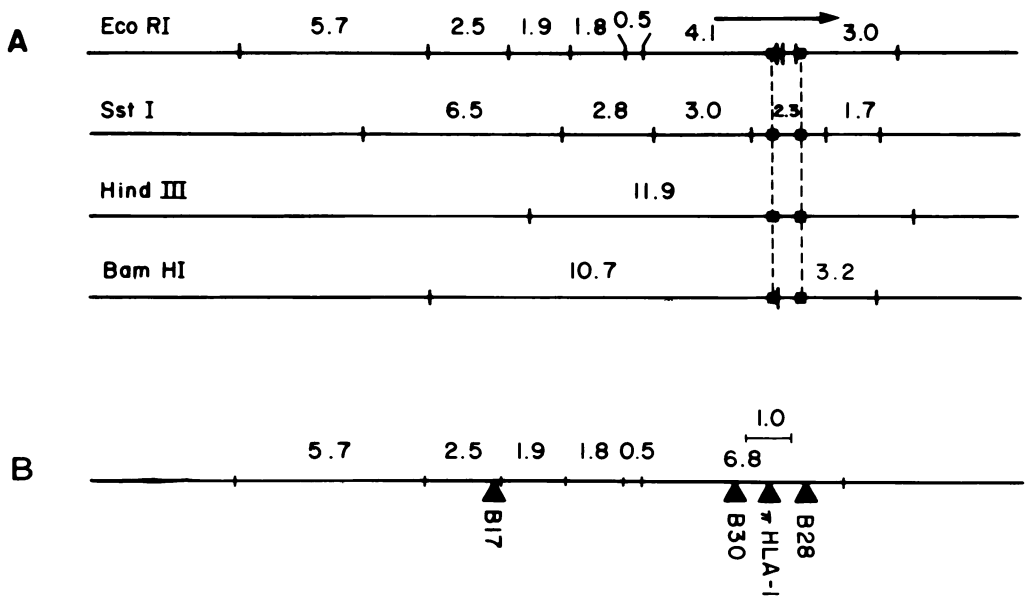

FIG. 3. Restriction maps of the cloned HLA region. (A) Map of the consensus genomic insert. The dotted lines delimit the inserted πHLA-1 sequences, the boxes represent the direct repeat generated by insertion of the miniplasmid, and the arrow indicates the approximate location and the direction of transcription of the cloned HLA gene. Sizes of DNA fragments are in kb. No sites were mapped to the left of the 5.7-kb *Eco*RI fragment or to the right of the 11.9-kb *Hin*dIII fragment. (B) Location of miniplasmid inserts. The thin line represents the *Eco*RI map of the consensus insert from which πHLA-1 was deleted (1.2-kb deletion). The positions of inserted miniplasmids are indicated by triangles. The bar labeled 1.0 indicates the 1.0-kb *Sst*I fragment used as a probe in the experiments shown in Fig. 5.

recovered in this screen was consistent with the insertion of a miniplasmid at new locations in the unrearranged consensus genomic insert (the structure of which was deduced from the restriction analysis of phage recovered by recombination with πHLA-1) (Fig. 3B). Phage B28-14 was selected as the source of the unrearranged gene for transfer because restriction analysis indicated it contained a uninterrupted stretch of cellular DNA large enough to contain an intact class I gene. The 1-kb *Sst*I fragment encompassing the 3′ end of the unrearranged HLA gene was used as a hybridization probe to examine homologous sequences in the human genomic DNA preparation from which the genomic library was constructed. The most intensely hybridizing bands generated by *Sst*I or *Eco*RI digestion of genomic DNA were the same size as the fragments in the cloned, unrearranged gene, but additional, minor bands, probably derived from genes related to the one cloned, were also detected (data not shown).

The unrearranged HLA gene was inserted into a BPV vector for transfer into mouse cells in culture. The maps of the plasmids pBPV-βHLA-1, and pBPV-βHLA-3 used for transformation are shown in Fig. 1. Both contain an 8.5-kb fragment of HLA DNA extending from the *Hin*dIII site in the 1.9-kb *Eco*RI fragment to the *Hin*dIII site present in the inserted miniplasmid polylinker in B28-14. They also contain the 69% subgenomic fragment of BPV DNA necessary to morphologically transform mouse cells (33), a 7.6-kb segment of human DNA from the β-globin region that stimulates transformation by the 69% fragment (9), and pBRd, a selectable bacterial plasmid replicon (9). Unlike the 7.6-kb β-globin DNA fragment, the 8.5-kb HLA gene fragment does not stimulate transformation by the 69% fragment of BPV DNA (data not shown). We used only plasmids with the orientations shown in Fig. 1 because we have previously found that when the BPV genes and a foreign gene are transcribed from the same DNA strand, there is a considerable amount of aberrant read-through transcription of the foreign gene (9; K. Zinn personal communication). Mouse C127 cells were transfected by these plasmids, and foci of morphologically transformed cells arose at an efficiency of about 150 foci per μg of BPV DNA. Individual foci were expanded into cell lines, and preliminary analysis of the recombinant DNA in these lines indicated that in many cases the DNA had undergone rearrangement or deletion or both. The cell lines analyzed below were derived from single cells of three transformed lines that showed minimal DNA rearrangement. Cell lines βH1.2A and βH1.8D were cloned from foci generated by pBPV-βHLA-1 and cell line βH3.1D from a focus generated by pBPV-βHLA-3.

State of the BPV-HLA DNA in transformed cell lines. We used DNA blotting experiments to analyze total DNA extracted from the cloned cell lines generated by transformation with the BPV-HLA plasmids. In the absence of restriction endonuclease digestion, DNA homologous to the input plasmid migrated exclusively with the high-molecular-weight cellular DNA from the three cell lines (Fig. 4). We did not detect DNA migrating at the position of the supercoiled or nicked input plasmid, and we were unable to resolve more than one component by CsCl-ethidium bromide density gradient centrifugation (data not shown). The absence of a very slowly migrating, heterogeneous smear of DNA suggested that catenated plasmids were not present (29); furthermore, incomplete digestion with pancreatic DNase did not release full-length linear molecules (data not shown). These results were unexpected because mouse C127 cells transformed by BPV or BPV recombinant plasmids almost invariably contain exclusively extrachromosomal viral DNA (9, 27–29, 39, 46, 56, 57, 70).

The patterns of hybridizing fragments after restriction endonuclease digestion, gel electrophoresis, and DNA blotting are different for each of the three cell lines (Fig. 4). Digestion of βH3.1D DNA with *Sal*I, which cuts the input plasmid at a single site, produces a major DNA fragment that comigrates with the full-length linearized input plasmid. Two faint bands that migrate slightly faster are also visible in the original autoradiograph. Digestion with *Hin*dIII plus *Bam*HI produces a set of fragments indistinguishable from the fragments from input DNA. Cell line βH1.8D DNA contains two *Sal*I fragments, a minor band the size of the linear input plasmid, and a predominant, more rapidly migrating species. Although all of the input *Hin*dIII plus *Bam*HI fragments are present in the DNA of this cell line, they are not present in equimolar amounts. Moreover, in addition to several new minor bands, there is a prominent new band migrating at the position expected for a 6-kb fragment. Digestion of βH1.2A DNA with either *Sal*I or *Hin*dIII plus *Bam*HI produces multiple DNA fragments, most of which do not migrate with DNA fragments derived from the input DNA. The DNA rearrangements in cell lines βH1.8D and βH1.2A have not been mapped in detail. From comparison with lanes containing known amounts of DNA, the approximate copy number of the transferred DNA in cell lines βH3.1D, βH1.8D, and βH1.2A is 10, 25, and 50 copies per diploid mouse genome equivalent, respectively.

The results of the analysis of recombinant DNA from cell line βH1.2A are most consistent with multiple sites of integration into cellular DNA. The data for βH3.1D and βH1.8D are consistent with the existence of the recombinant DNA as either an integrated head-to-tail tandem duplication

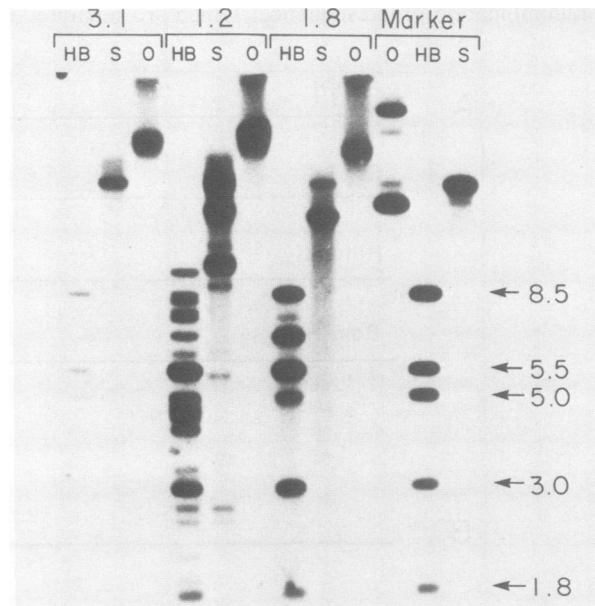

FIG. 4. BPV-HLA DNA in transformed mouse cell lines. Cellular DNA (5 μg) was sheared by passage through a 22-gauge needle or digested with a fivefold excess of *Sal*I or *Hin*dIII plus *Bam*HI. Samples were electrophoresed through a 0.7% agarose gel, transferred to nitrocellulose after partial acid depurination, and hybridized to nick-translated pBPV-βHLA-3. Samples: 3.1, βH3.1D DNA; 1.2, βH1.2A; 1.8, βH1.8D DNA; marker, 120 pg of pBPV-βHLA-3; 0, uncut; HB, *Hin*dIII plus *Bam*HI-digested; S, *Sal*I-digested.

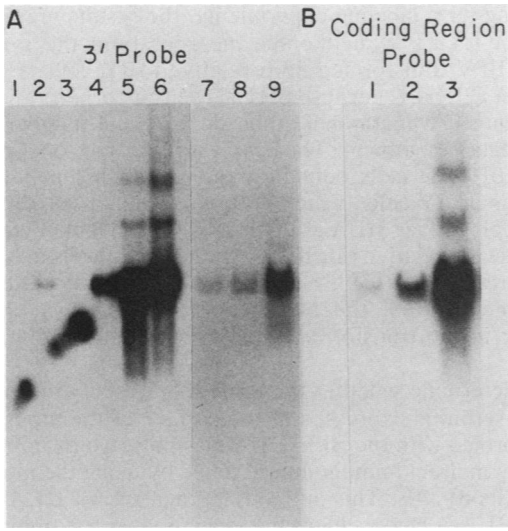

FIG. 5. Analysis of HLA RNA in BPV-HLA-transformed cell lines. Cytoplasmic RNA (20 μg in A, lanes 1 through 6, and in B, lanes 1 through 3; 2 μg in panel A, lanes 7 through 9) from the indicated cell lines was electrophoresed for 6 h at 60 V/cm in a 1% agarose gel containing 0.31 M formaldehyde (15). After electrophoresis, transfer to nitrocellulose, and hybridization to specific probes, filters were washed at 68°C in 2× SSC and 0.1% SDS for about 1.5 h and in 0.2× SSC and 0.1% SDS for 40 min. RNA was detected by autoradiography. (A) Probe was the subcloned 1-kb *Sst*I fragment from the unrearranged HLA gene. (B) Probe was pHLA-2. Source of RNA in (A): lane 1, C127 cells; lanes 2 and 7, HeLa cells; lane 3, β5A cells; lane 4, βH1.2A; lanes 5 and 9, βH1.8D; lane 6, βH3.1B; lane 8, JY cells. Source of RNA in (B): lane 1, HeLa cells; lane 2, JY cells; lane 3, βH1.8D cells.

of the input plasmid (or a deleted version in the case of βH1.8D) or a tandemly arranged oligomeric plasmid. These experiments are probably not sufficiently sensitive to detect single-copy, exogenous-cellular DNA junction fragments. Attempts to transform bacterial cells to ampicillin resistance with DNA preparations from these cell lines have failed, even though βH3.1D and βH1.8D clearly contain an intact pBRd segment (Fig. 4).

Transcription of the HLA gene. RNA blotting experiments indicate that the transferred HLA gene is transcribed in mouse cells. Cytoplasmic RNA was purified from a number of the BPV-HLA cell lines, as well as from a BPV-transformed cell line, β5A, which does not contain an HLA gene (9). RNA was also prepared from the JY cell line, an Epstein-Barr virus-transformed human lymphoblastoid cell line that expresses high levels of cell-surface HLA antigen (43, 65). After electrophoresis and transfer to nitrocellulose, HLA RNA was detected by hybridization to the 1.0-kb *Sst*I fragment that comprises the 3′ one-third of the transcript. We know this probe does not cross-hybridize with mouse heavy chain transcripts under the stringent washing conditions we used, since we do not detect RNA in mouse C127 cells (Fig. 5A, lanes 1 and 3). Moreover, this probe does not detect discrete bands in restriction endonuclease-digested mouse C127 cell DNA (data not shown). Substantial amounts of HLA RNA of the correct size were detected in all three BPV-transformed cell lines containing the HLA gene (Fig. 5A, lanes 4 to 6 and 9). The hybridization signal for βH1.8D RNA was considerably greater than that for JY human lymphoblastoid cell RNA (Fig. 5A, lane 8). The lower hybridization signal in JY RNA is not due to limited homology between the cloned HLA gene probe and the genes expressed in JY cells, because this probe hybridizes to pHLA-2 (an almost full-length cDNA clone of an allele expressed in JY cells [62]) under stringent hybridization conditions (data not shown). The relatively high levels of HLA transcripts in βH1.8D were also demonstrated by using pHLA-2 DNA as a hybridization probe (Fig. 5B). Hybridization of these same filters to an actin cDNA probe demonstrated that RNA from all cell lines was intact (data not shown).

HLA heavy chain production in transformed mouse cells. Analysis of [^{35}S]methionine-labeled proteins from the BPV-HLA-transformed cell lines revealed the presence of HLA heavy chain protein. NP-40 lysates were prepared from labeled cell lines transformed by BPV plasmids with or without the HLA gene. A rabbit immune serum raised against denatured HLA heavy chain protein (α-heavy) (25) immunoprecipitated a prominent protein from extracts of transformed cells containing the transferred HLA gene (Fig. 6, lane 2) but not from BPV-transformed mouse cells without the gene (Fig. 6, lane 1). This protein comigrates on SDS-polyacrylamide gel electrophoresis with an HLA heavy chain marker, and addition of excess, unlabeled purified HLA protein (A2 specificity) inhibited immunoprecipitation of this protein (Fig. 6, lane 3). No [^{35}S]methionine-labeled proteins were immunoprecipitated from these lysates by control serum (Fig. 6, lanes 4 and 5). Cell lines βH1.2A and βH3.1D also contain a protein with similar electrophoretic mobility that was precipitated with α-heavy serum (data not shown). These results demonstrate that human heavy chain is synthesized in cells transformed by BPV vectors containing a cloned HLA heavy chain gene.

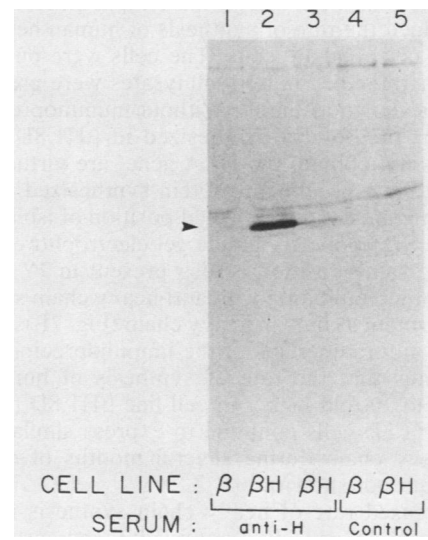

FIG. 6. Identification of human heavy chain protein in transformed mouse cells. [^{35}S]methionine-labeled (for 40 min) detergent lysates of βH1.8D or β5A cells were boiled in SDS before immunoprecipitation with rabbit anti-rat serum (lanes 4 and 5) or with α-heavy serum (lanes 1 through 3). Samples were loaded on a 9% polyacrylamide and 2.25% bis-acrylamide gel and electrophoresed at 120 V until the bromophenol blue reached the bottom of the gel. The arrow indicates the position of purified, HLA-A2 heavy chain. Lane 1, β5A lysate; lane 2, βH1.8D lysate; lane 3, βH1.8D lysate, 6 μg of unlabeled HLA-A2 was added before boiling; lane 4, β5A lysate; lane 5, βH1.8D lysate.

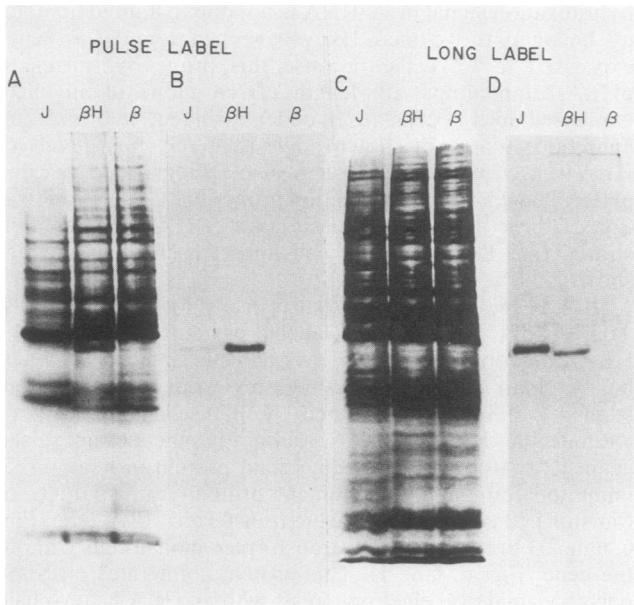

FIG. 7. Comparison of the levels of HLA heavy chain in different cell lines. Cells were labeled with [^{35}S]methionine for 20 min (pulse) or 26 h (long). Total detergent lysates (A and C) or α-heavy immunoprecipitates (B and D) were electrophoresed as described in the legend to Fig. 6, except 1.13% bis-acrylamide was used for the gel shown in (D). With this lower concentration of bis-acrylamide, the heavy chain detected after a pulse- or long-term label of βH1.8D cells migrates more rapidly than does the heavy chain from JY cells. Source of lysates: J, JY cells; βH, βH1.8D cells; β, β5A cells.

To compare the level of HLA expression in BPV-HLA transformed cells with that in the lymphoblastoid cell line, we determined the rate of synthesis of human heavy chains in βH1.8D cells and JY cells. The cells were pulse-labeled with [^{35}S]methionine, and NP-40 lysates were prepared and analyzed as shown in Fig. 7. Without immunoprecipitation, the patterns of proteins synthesized in βH1.8D and β5A, which does not contain the HLA gene, are virtually identical, except for a prominent protein synthesized in βH1.8D cells that migrates at the expected position of isolated heavy chain upon SDS-polyacrylamide gel electrophoresis; a faint band at the same position is also present in JY cells (Fig. 7A). Immunoprecipitation with anti-heavy chain serum identified this protein as human heavy chain (Fig. 7B). Densitometry of the autoradiograms of the immunoprecipitated samples indicates that the rate of synthesis of human heavy chain is 5- to 10-fold higher in cell line βH1.8D than in cell line JY. βH1.8D cells continue to express similar levels of human heavy chain during several months of continuous passage (data not shown).

The increased rate of heavy chain synthesis in βH1.8D compared with JY cells does not result in increased accumulation of the protein. When cells are labeled for 26 h, no novel band migrating at the position of the heavy chain is discernible in extracts of βH1.8D cells (Fig. 7C). Immunoprecipitation with α-heavy serum demonstrates that the steady-state level of human heavy chain in extracts of βH1.8D cells appears to be two- to threefold lower than in JY cell extracts (Fig. 7D). The apparent difference in the mobility of human heavy chain from βH1.8D cells after short or long labeling periods is a consequence of the conditions of gel electrophoresis (see legend to Fig. 7). In both the pulse- and long-term labeling experiments, the results are normalized for total [^{35}S]methionine incorporation; the synthesis in the BPV-transformed lines relative to JY cells is greater on a per cell basis because βH1.8D cells incorporate severalfold more [^{35}S]methionine than do an equal number of JY cells (data not shown). Thus, we estimate that, on a per cell basis, βH1.8D cells contain about as much human heavy chain as do JY cells, which in turn contain about 10-fold as much cell surface HLA as do normal human leukocytes (43). Because the relative rate of heavy chain synthesis in βH1.8D compared with JY cells is greater than its steady-state level, we conclude that the half-life of human heavy chain is shorter in the transformed mouse cells than in human JY cells.

To determine whether the protein detected with the anti-heavy serum is expressed at the surface of the mouse cells transformed with the BPV-HLA plasmids, we examined the cells by indirect immunofluorescence by using the monoclonal antibody, 4E. This antibody recognizes all HLA-B and some HLA-A heavy chains in association with β_2-microglobulin (B. DuPont, personal communication). β5A cells did not react with this antibody (data not shown). Figure 8 shows that this antibody reacts with an antigen with a cell surface distribution on βH1.8D cells. Although there is considerable variation in the fluorescence intensity of individual cells, essentially all cells reacted with this antibody to some extent. This result confirms that the transferred gene is an active HLA allele and indicates that some of the human

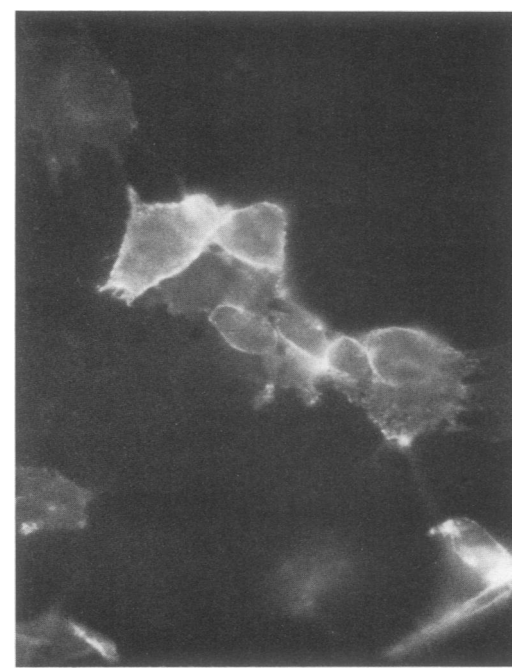

FIG. 8. Localization of HLA antigens in βH1.8D cells by indirect immunofluorescence. Mouse ascitic fluid containing monoclonal antibody 4E was diluted into phosphate-buffered saline containing 1% bovine serum albumin and incubated for 45 min at 0°C with cells growing on cover slips. Cells were washed with the phosphate-buffered saline–bovine serum albumin solution and incubated at 22°C for 30 min with fluoresceinated F(ab')$_2$ fragment of goat anti-mouse immunoglobulin G. Cells were washed extensively and then fixed in 95% ethanol. After excitation at 440 to 490 nm, cells were photographed with a Canon 35-mm camera mounted on a Zeiss fluorescence microscope.

heavy chain in the transformed cells associates with mouse β_2-microglobulin at the cell surface.

DISCUSSION

In this paper we report the isolation of an active HLA heavy chain gene and its introduction into mouse cells with a BPV DNA-derived eucaryotic expression vector. In the transformed cells, the transferred DNA is maintained in a high-molecular-weight form, and the gene is actively transcribed. Substantial amounts of human heavy chain are produced in these cells and heavy chain β_2-microglobulin complexes are present at their surface.

The recombination probe used to screen the genomic library was derived from pHLA-1, which probably corresponds to a cDNA copy of a B allele transcript (51). Unlike the 3′ nontranslated region of some H-2 mRNAs, the recombination probe does not contain highly repeated sequences (5, 64). The restriction map of the cloned gene is consistent with the results of our genomic blotting experiments and corresponds very closely to the maps of two other active HLA alleles, B7 and B40, which were cloned independently (A. Biro, personal communication). The gene we recovered is clearly different from an apparently inactive HLA-like gene cloned from the same genomic library with an H-2 cDNA hybridization probe (21, 35).

The use of a 3′ noncoding sequence as a recombination probe should provide a generally useful approach for isolating specific genes that are members of large gene families. Two considerations suggest that this procedure would be especially useful in identifying expressed genes in gene families with large numbers of pseudogenes. First, comparison of the DNA sequences of different members of the same gene family reveals that their 3′ noncoding sequences are often considerably more divergent than the 5′ noncoding or protein-coding sequences (for example, see Efstradiatis et al. [12]). A 3′ noncoding sequence probe is therefore likely to pick out the gene that encodes the mRNA from which the cDNA clone was prepared. Second, the frequency of recombination in the πVX system decreases substantially with base mismatching between the probe sequence and the corresponding sequence in the phage. Seed has shown that the frequency of recombination between sequences that differ by 10% is 100 times less than that of perfectly matched sequences (58). Thus, a high proportion of phage that are recovered by recombination will be nearly identical to the cDNA used as the source of the recombination probe. In the experiments reported here, 15 of 20 of the clones we recovered contain the same HLA gene, even though there are many closely related class I HLA-like genes in the human genome (3, 41).

A disadvantage of the recombination screening technique is the insertion of the miniplasmid into the recovered gene. This problem may be circumvented by using a recombination probe comprised of the 3′ nontranslated region including all the signals (such as AAUAAA [54]) required for production of the correct 3′ end of the transcript. Alternatively, in favorable cases, an unrearranged gene can be reconstructed in vitro from purified DNA fragments (70), or as we have shown (see above), the miniplasmid can be deleted by homologous recombination between the duplicated probe sequences, by using EDTA enrichment for deleted phage (45). Although deletion in vivo could result in sequence changes at the site of recombination, it is unlikely that sequences within the 3′ noncoding sequence will affect transcription or processing. We chose to perform a second round of recombination screening with a nonrepetitive flanking sequence probe to isolate an unrearranged HLA gene. To identify bacteria carrying miniplasmids containing suitable probes, we developed a method that does not require purification of individual plasmid subclones (see above); using this approach, we isolated a gene with the miniplasmid inserted outside of the transcriptional unit. A similar approach could be used to identify flanking recombination or hybridization probes useful for isolating large contiguous regions of genomic DNA ("genomic walking"). Phage with inserts extending further to the 3′ side of the HLA gene were not recovered in either cycle of recombination screening; this may be a consequence of underrepresentation of the corresponding parental phage in the genomic library.

The cloned, intact HLA gene was inserted into BPV vectors, and the recombinants were used to transform mouse C127 cells. All cell lines derived from morphologically transformed foci contained the transferred DNA. Multiple copies of the transferred DNA were maintained in a high-molecular-weight form, in contrast to the usual situation with BPV-transformed mouse C127 cells (9, 27–29, 39, 56, 57, 70). For βH1.8D and βH3.1D DNA, the data do not distinguish between tandemly arranged integrated sequences and oligomeric plasmids. Oligomeric viral plasmids (as well as integrated DNA) have been observed in rabbit carcinomas induced by cottontail rabbit papillomavirus (67), and high-molecular-weight viral DNA was the only detected species in mouse L cells biochemically transformed with BPV-thymidine kinase recombinant molecules (34). Moreover, imposition of mycophenolic acid selection (40) on C127 cells containing certain *E. coli gpt* gene-BPV recombinant plasmids appears to lead to integration of the BPV DNA (unpublished data; M.-F. Law and P. Howley, personal communication). We do not know whether the integrated state of the BPV-HLA plasmids is a consequence of their large size, of the arrangement of their composite segments, or of their homologous recombination with H-2 genes. The latter possibility is suggested by a report of reconstitution of H-2 antigens after the transfer of truncated H-2 genes into cultured mouse cells (17). We are currently testing these alternatives.

High levels of human heavy chain RNA of the correct size are detected in BPV-HLA-transformed mouse cells, suggesting that the gene is accurately transcribed and that the primary transcripts are faithfully processed. To determine the amount of human heavy chain protein in these cells, we used a rabbit antiserum that recognizes naturally occurring and mutant HLA-A, HLA-B, and HLA-C heavy chains and related class I proteins (7, 25). Because this serum only recognizes heavy chain when it is not associated with β_2-microglobulin, we denatured the proteins before immunoprecipitation. The results from these experiments indicate that the human heavy chain is synthesized at a high rate in the transformed mouse cells. Although βH1.8D cells contain large amounts of human heavy chain, some of which is at the cell surface in association with mouse β_2-microglobulin, the steady-state level of this heavy chain is not commensurate with its rate of synthesis. Thus, the amount of cell surface expression of such a protein in gene transfer experiments is not necessarily a valid measure of gene activity. The results of Karmack et al. (22) suggest that the expression of class I histocompatibility antigens at the surface of hybrid mouse-human fibroblasts is limited by the amount of β_2-microglobulin in these cells. Similarly, if the human protein is synthesized in molar excess over mouse β_2-microglobulin in the BPV-HLA cell lines, the presence of a large pool of

free heavy chain may account for its relative instability in these cells. In fact, heavy chains that have not yet associated with β_2-microglobulin or that have been physically dissociated from it are more sensitive to proteolysis in vitro (26, 42).

Because of the different assay systems used and the small number of cell lines examined, we did not directly compare the level of expression of the BPV-linked heavy chain gene to that of heavy chain genes after cotransfer with a biochemically selectable gene. Recent experiments designed to quantitate the level of HLA antigen at the surface of the cells transformed with BPV-HLA DNA indicate that βH1.8D and JY cells contain comparable amounts of cell surface HLA-B antigen (T. Sieck, personal communication). In contrast, Barbosa et al. (1) reported that each mouse cell stably transformed with a cloned HLA gene and HSV thymidine kinase expresses at most 1/20 of the cell surface HLA antigen as do JY cells, even though the transformants contain up to 100 copies of the transferred gene. The high level of expression we observed may be, in part, the consequence of the recipient C127 mouse cells or of the multiple copies of the transferred gene, but the effects of the BPV transcriptional enhancer (34) or other regulatory signals may also contribute.

One of our initial objectives for using BPV vectors to study the expression of cloned histocompatibility genes was to establish a procedure for selecting and rescuing mutant genes, based on the high level of expression of BPV-linked genes and on the ability of BPV plasmids to replicate as extrachromosomal DNA in both bacteria and mammalian cells (9, 27, 56). Cells with mutant HLA antigens have been isolated by selecting against mutagenized human cells that express wild-type antigens (14, 49, 50). Similarly, it should be possible to mutagenize BPV-HLA plasmids in vitro, transform mouse cells with the pool of mutagenized DNA, and select cells expressing a variant cell surface antigen by cell sorting or other immunological methods. Although detailed structural characterization of the mutant antigens can be carried out (26), thorough genetic analysis of the basis of the defect requires the isolation of the mutant gene. If BPV-HLA recombinants could be propagated as plasmids in mouse cells, plasmids carrying the mutant gene could be recovered by transformation of bacteria. Although the apparently integrated state of the BPV-HLA plasmids reported in this study does not allow the final step of this approach, it may be possible to construct similar plasmids with the requisite properties.

In summary, the results reported here demonstrate the feasibility of using BPV vectors to establish cell lines producing large amounts of a single HLA protein. Detailed analysis of these cell lines and similar ones generated with other normal and mutant HLA genes may yield information on the synthesis, structure, and function of these important cell surface glycoproteins.

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