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Generation of Hybrid Genes and Proteins by Vaccinia Virus-Mediated Recombination: Application to Human Immunodeficiency Virus Type 1 *env*

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The ability of poxviruses to undergo intramolecular recombination within tandemly arranged homologous sequences can be used to generate chimeric genes and proteins. Genes containing regions of nucleotide homology will recombine to yield a single sequence composed of portions of both original genes. A recombinant virus containing two genes with a number of conserved regions will yield a population of recombinant viruses containing a spectrum of hybrid sequences derived by recombination between the original genes. This scheme has been used to generate hybrid human immunodeficiency virus type 1 *env* genes. Recombinant vaccinia viruses that contain two divergent *env* genes in tandem array have been constructed. In the absence of selective pressure to maintain both genes, recombination between conserved homologous regions in these genes generated a wide range of progeny, each of which expressed a novel variant polypeptide encoded by the newly created hybrid *env* gene. Poxvirus-mediated recombination may be applied to map type-specific epitopes, to create novel pharmaceuticals such as hybrid interferons, to study receptor-binding or enzyme substrate specificities, or to mimic the antigenic diversity found in numerous pathogens.

Intramolecular recombination between homologous sequences has been documented in a number of animal viruses, including picornaviruses (25, 54), influenza virus (13), adenovirus (6), and poxviruses (2, 49). In vaccinia virus, for example, it has been shown that tandemly duplicated sequences are genetically unstable (2, 38, 49). Intramolecular recombination between these identical sequences results in a condensation of the duplicated structure to yield a single copy of the previously duplicated sequences.

Although intramolecular recombination between tandemly arranged, identical DNA sequences has been documented, there has been no examination of intramolecular recombination between partially homologous DNA sequences. It is possible that virus-mediated recombination can be used to generate hybrid genes from two related genes that share regions of homology.

In this study, the feasibility of using vaccinia virus-mediated recombination to produce a population of progeny containing a spectrum of variant genes and proteins was demonstrated by using two divergent human immunodeficiency virus type 1 (HIV-1) viral envelope (*env*) genes. The occurrence of genomic and antigenic variation in HIV, the causative agent of acquired immunodeficiency syndrome, has been demonstrated by a number of researchers (19, 51). These studies have revealed that hypervariable regions are found primarily in the *env* gene, interspersed with regions of relatively conserved nucleotide sequence. A major component of the antibody response to HIV is directed against envelope proteins (4, 14).

The antigenic diversity exhibited by HIV and by other pathogenic organisms presents obstacles to the formulation of efficacious vaccines. A vaccine that protects against one variant or subtype may not be effective against other strains. The predominant neutralizing antibodies, for example, are directed against HIV envelope and have been shown to be type specific (32, 37, 56). To develop successful vaccination protocols for these pathogens, it will be necessary to elicit immune responses capable of recognizing the many variant epitopes present in the family of diverse but related proteins expressed by the pathogens. To achieve this, it may be necessary to immunize with a vaccine formulation in which many, if not all, of these variant epitopes are represented.

To generate a large number of variant HIV *env* genes, two divergent *env* genes were introduced in tandem into the vaccinia virus genome. Intramolecular recombination between conserved sequences in the two *env* genes yielded a single hybrid sequence composed of portions of the two original genes. As the recombination event could occur between any homologous regions shared by the two genes, each of the progeny viruses contained a different hybrid gene resulting from the condensation of the original DNA sequences. Thus, a spectrum of hybrid *env* genes was generated from two related genes that contain regions of DNA sequence homology.

MATERIALS AND METHODS

Cells, virus, and plasmids. *Escherichia coli* MC1061 (9) was used as the host for the isolation and growth of all plasmids. The monkey kidney cell line BSC-40 (7), the rabbit kidney cell line RK_{13} (5), or the thymidine kinase-deficient (tk^-) human cell line Hu143TK$^-$ (1) was used for vaccinia virus infections and transfections.

Vaccinia virus strain New York City Board of Health (NYCBH; ATCC VR-325), WRΔ417, a tk^- strain (49), or vAbT33, a 29K$^-$ *lacZ*$^+$ strain (K. Smith et al., unpublished data) was used as the parental virus for construction of vaccinia virus recombinants.

Plasmid pSV-Henv, containing the *env* gene from HIV-1 strain BH10 (42), and plasmid pRF3HS, containing the *env*

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gene from HIV-1 strain RF (51), were obtained from Blair Ferguson (E. I. du Pont de Nemours and Co.).

Preparation of vaccinia virus recombinants. Virus infection, transfection, plaque purification, and virus amplification were performed essentially as described previously (49). tk^+ recombinant plaques were selected on Hu143TK$^-$ cells in the presence of 5 μM methotrexate, using WRΔ417 as the parental tk^- virus. 29K$^+$ recombinants were selected and purified on RK_{13} cells (Smith et al., unpublished data). The starting virus was vAbT33, a derivative of the NYCBH strain, which contains a deletion in the *Hin*dIII-M region, which includes the 3′ end of the 29K host range gene (16). The deletion therefore does not allow replication of vAbT33 in RK_{13} cells. However, it does not affect replication in BSC-40 cells. The *E. coli lacZ* gene, under the control of a vaccinia virus promoter, is inserted in place of the deleted sequences in *Hin*dIII-M. vAbT33 thus expresses β-galactosidase and forms blue plaques on BSC-40 cells in the presence of BluoGal (Bethesda Research Laboratories). Recombination with a plasmid vector containing *Hin*dIII-M sequences flanking the foreign gene of interest results in restoration of 29K host range function concurrent with insertion of the foreign gene and deletion of the *lacZ* gene. The resulting recombinants can be selected by their ability to grow on RK_{13} cells and form clear plaques in the presence of BluoGal. Recombinant viruses are then plaque purified on RK_{13} cells in the presence of BluoGal.

Mycophenolic acid-resistant recombinants containing the *E. coli gpt* gene were obtained essentially as described previously (12). β-Galactosidase production was visualized by using 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) as described previously (49).

Vaccinia virus genomic analysis. DNA was extracted from vaccinia virus-infected cells as described previously (11) and analyzed by restriction enzyme digestions and Southern hybridization as described elsewhere (30). Vaccinia virus genomic DNA was subcloned into bacteriophage m13mp18 (New England BioLabs) and sequenced by the chain termination method (46). Polymerase chain reaction (PCR) was performed by using a GeneAmp kit (Perkin Elmer Cetus) as instructed by the manufacturer.

Protein analysis. Black plaque assay, an in situ enzyme-based immunoassay performed on vaccinia virus plaques, was performed essentially as described previously (29), using sera from vaccinia virus-seronegative, HIV-seropositive humans, obtained from John L. Sullivan (University of Massachusetts Medical School, Worcester).

Immunoprecipitation analysis (24) was performed on vaccinia virus-infected BSC-40 cells labeled with [^{3}H]leucine, using antisera from HIV-infected humans. BSC-40 cells were infected for 1 h with recombinant vaccinia virus at a multiplicity of infection of 10 and were then incubated in medium containing [^{3}H]leucine. After 16 h at 37°C, the medium was removed, and the cell monolayer was washed with phosphate-buffered saline (13 mM NaCl, 2.7 mM KCl, 8.1 mM Na_2HPO_4, 1.5 mM KH_2PO_4) and lysed with 1 ml of immunoprecipitation buffer (10 mM Tris hydrochloride [pH 7.2], 500 mM NaCl, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 5 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 0.1 mg of soybean trypsin inhibitor per ml). HIV-positive antiserum was added to a sample of cell lysate or clarified cell culture medium and incubated overnight at 4°C. Protein A-Sepharose suspended in immunoprecipitation buffer was added, and the samples were incubated with rotation for 90 min at room temperature. The samples were washed four times with immunoprecipitation buffer and once with phosphate-buffered saline. Sepharose pellets were dried and were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Labeled proteins were visualized by autoradiography.

Nucleotide sequence accession numbers. The sequences of vAbT168-1 and vAbT168-2 have been submitted to GenBank and assigned accession numbers M38707 and M38708, respectively.

RESULTS

Insertion of related genes into vaccinia virus to produce hybrid genes. Tandemly arranged, related genes can be inserted into the vaccinia virus genome in a number of ways. In one approach (Fig. 1A), two partially homologous genes are inserted into the vaccinia virus genome in a single recombination event by means of a plasmid vector containing the two genes. These genes are separated in the plasmid and in the resultant recombinant virus by a gene encoding a selectable marker. The plasmid vector also contains vaccinia virus DNA sequences (L and R in Fig. 1A) flanking the two genes, the flanking segments being homologous to a region of the viral genome into which the tandem genes are to be inserted.

In an alternative approach (Fig. 1B), tandemly arranged, related genes are inserted into the viral genome via two sequential recombination events. The first gene is inserted by conventional homologous recombination into the viral genome, and the recombinant virus is isolated. The second gene, together with a gene encoding a selectable marker, is then inserted into the recombinant virus containing the first gene. This insertion occurs via a single recombination event between homologous sequences in the first gene, contained within the viral genome, and the second gene, contained on a circular plasmid vector. This recombination event inserts the entire plasmid into the vaccinia virus genome, generating a virus that contains two hybrid genes, separated by the gene encoding the selectable marker and the rest of the plasmid vector, including the bacterial replicon and bacterial drug resistance marker (for example, ampicillin resistance).

The gene encoding the selectable marker is required for maintenance of the tandemly duplicated structures during viral replication. Examples of selectable markers include the gene encoding thymidine kinase (*tk*; 8), the vaccinia virus 29K host range gene (16, 40; Smith et al., unpublished data), and genes that confer antibiotic or chemical resistance, such as the *E. coli neo* (15) or *gpt* gene (12). Both approaches (Fig. 1) will yield recombinant viruses in which the selectable marker resides between the two genes. This arrangement permits the isolation and propagation of viruses containing the genetically unstable tandem gene arrangement.

Removal of selective conditions permits the growth of progeny that have undergone intramolecular recombination, excising the marker gene. In viruses generated by approach 1 (Fig. 1A), intramolecular recombination between tandemly arranged, partially homologous genes will yield a final hybrid gene that contains a single fusion junction; the 5′ portion of the hybrid gene will be derived from the 5′ region of one gene, while the 3′ portion of the hybrid gene will be derived from the 3′ region of the second of the two genes. Each individual hybrid gene will contain a unique fusion junction, generated by recombination between different homologous regions shared by the two original genes.

In recombinant viruses generated by approach 2 (Fig. 1B), each of the tandemly arranged genes is itself a hybrid gene, formed by recombination between the first gene located in

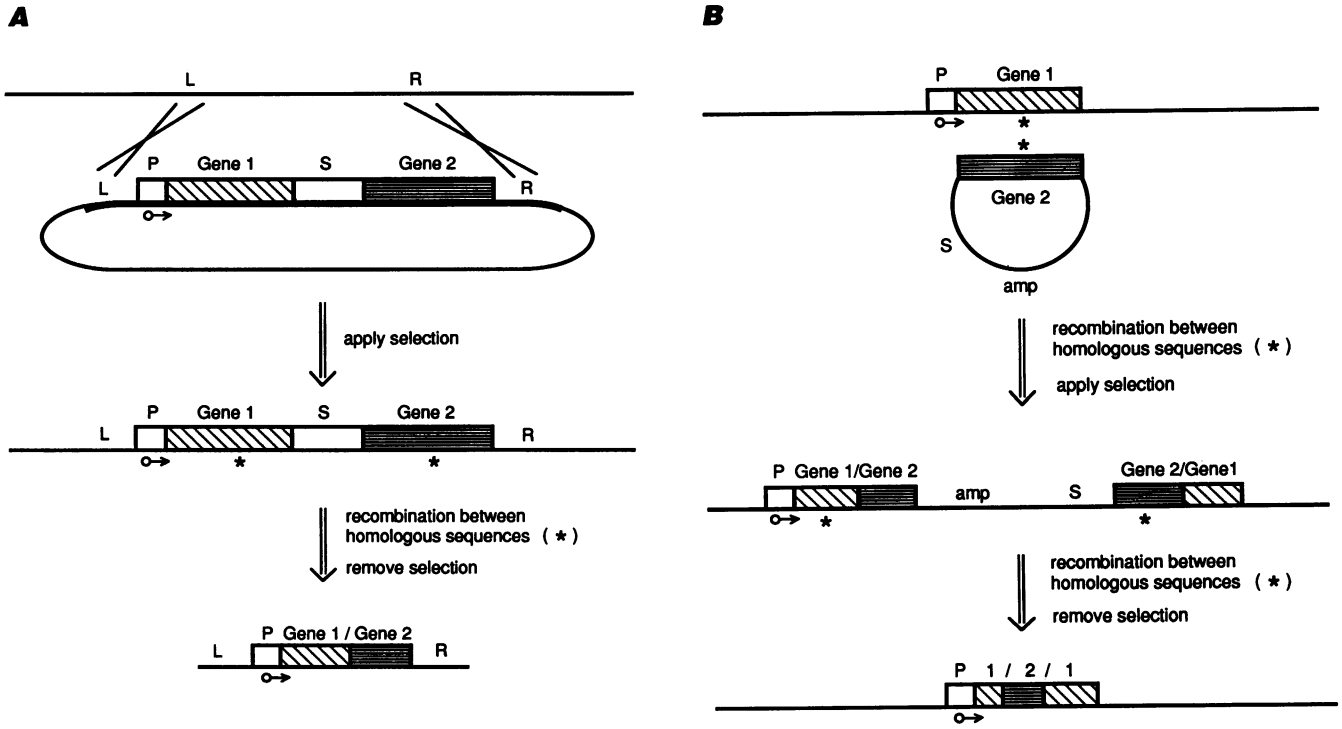

FIG. 1. Generation of chimeric genes in vaccinia virus. (A) Approach 1: simultaneous insertion of both genes, separated by a selectable marker. (B) Approach 2: step-wise insertion of each gene, separated by a selectable marker. In both approaches, selective conditions are removed to allow the propagation of recombinant progeny which have undergone intramolecular recombination between genes 1 and 2 to form hybrid gene 1-gene 2 (as in panel A) or hybrid gene 1-gene 2-gene 1 (as in panel B). P, Promoter; S, selectable marker; L (left) and R (right), vaccinia virus DNA sequences homologous to the vaccinia virus genomic insertion site; amp, ampicillin resistance gene.

the viral genome and the second gene contained in the plasmid vector. Subsequent intramolecular recombination between this pair of hybrid genes will result in the formation of a single hybrid gene that contains two separate junctions. Thus, the final hybrid gene will contain 5′ and 3′ segments from one of the two genes flanking a center segment derived from the second of the two genes.

Demonstration of vaccinia virus-mediated recombination between divergent HIV genes. To test whether hybrid genes could be formed by vaccinia virus-mediated recombination between related genes, two divergent *env* genes, from HIV-1 strains BH10 and RF, were used. Strains BH10 and RF are positioned at opposite ends of a phylogenetic tree of North American HIV-1 isolates (48). Their *env* genes differ by 14% in the nucleotide sequence and by 21% in the predicted amino acid sequence of their exterior envelope proteins (51). However, there are a number of conserved regions spanning the *env* genes, and a vaccinia virus recombinant containing the two different *env* genes (Fig. 2A) should generate a diverse set of chimeric *env* genes. To restrict homologous recombination to the gp120 portion of the *env* genes and avoid recombination within gp41-encoding sequences, which are well conserved among *env* genes, only RF sequences through nucleotide 1612 (numbered as in the EMBL sequence; accession number M12508) were included. These sequences contain all of gp120 and 31 bp of the 5′ end of gp41 (ending at a restriction endonuclease *Sty*I site) derived from the RF *env* gene. The *E. coli lacZ* gene, fused in frame to the BH10 *env* gene at a *Hin*dIII site at nucleotide 7723 (numbering as in reference 42) in gp41, was included as an intramolecular recombination marker; plaques resulting from the growth of virus containing both *env* genes should be colorless in the presence of X-Gal because there is no promoter to express *lacZ* (Fig. 2A), whereas progeny virus containing a single hybrid *env* gene fused in frame to *lacZ* should give rise to blue plaques (Fig. 2B). *lacZ* therefore serves as a marker for the fidelity of recombination resulting in an in-frame chimera.

Approach 1 (Fig. 1A) was used to construct a vaccinia virus recombinant containing the *tk* gene flanked by the HIV-1 BH10 and RF genes (Fig. 2A). tk^+ recombinants were selected in the presence of methotrexate, which selects

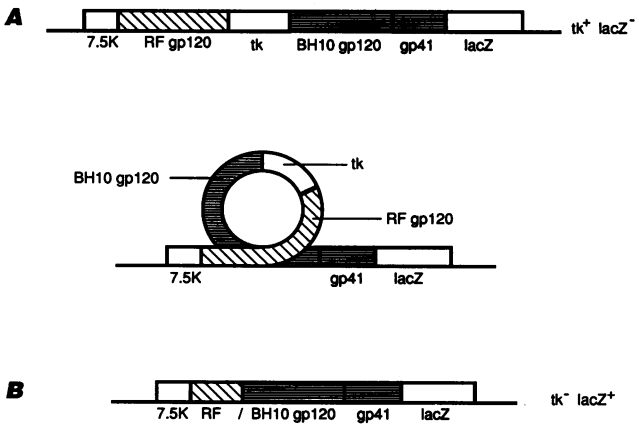

FIG. 2. Generation of chimeric HIV *env-lacZ* genes in vaccinia virus. (A) Intermediate recombinant containing the RF and BH10 gp120 genes separated by the *tk* gene. (B) Intramolecular recombination between homologous sequences in the RF and BH10 gp120 genes, resulting in a hybrid RF-BH10 *env-lacZ* gene. *lacZ* serves as a marker for the in-frame, intramolecular recombination event.

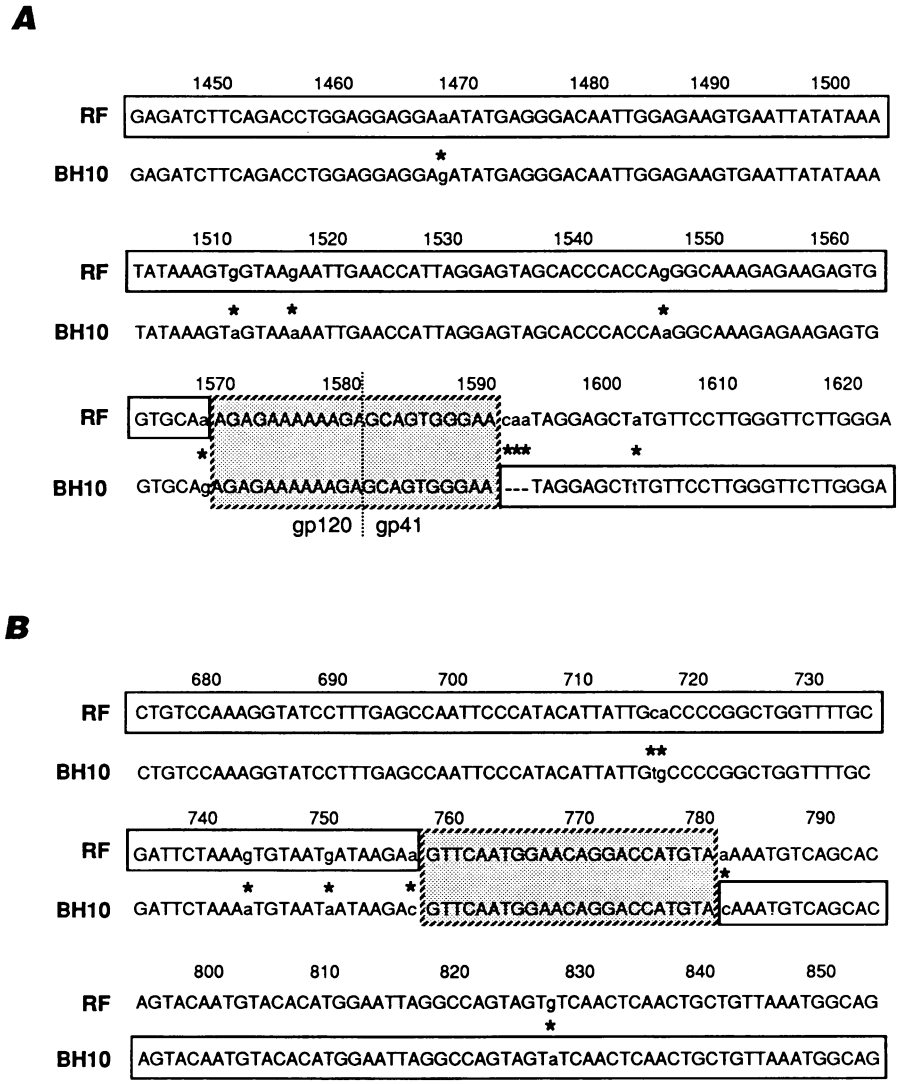

FIG. 3. Partial nucleotide sequences of HIV strains RF and BH10 and of two chimeric *env* genes. The recombination sites in two putative hybrid *env* genes were mapped by RFLP analysis and then were sequenced to define the exact site of homologous recombination. (A) Chimeric *env* gene from vAbT168-1. (B) Chimeric *env* gene from vAbT168-2. Symbols: □, sequence of the chimeric *env* gene; ▨, hybrid junction in the chimeric *env* gene, indicating the homologous region of recombination between RF and BH10 *env* genes; *, nucleotide difference between RF and BH10 *env* genes (there are several discrepancies between the RF published sequence [51; numbering as in EMBL accession number M12508, locus REHIVRFE] and the RF sequence presented here: T at position 1457, G at position 1524, G at position 1536, and T at position 1545 in the published RF sequence are C, A, A, and C, respectively, in the sequence shown here).

against the parental tk^- virus. When the pool of recombinant virus was plated in the presence of methotrexate and overlaid with X-Gal, only colorless (*lacZ*-negative) plaques were observed. When the same pool of recombinants was plated in the absence of methotrexate and overlaid with X-Gal, both blue plaques and colorless plaques were observed, indicating that recombination had occurred between the two *env* genes to form a hybrid RF-BH10 *env* gene fused to *lacZ* (Fig. 2B). Two independently isolated blue recombinants, designated vAbT168-1 and vAbT168-2, were plaque purified in the absence of methotrexate and amplified.

A 3-kb *Hin*dIII fragment containing the putative chimeric *env* gene was subcloned from vAbT168-1 for further analysis. The hybrid *env* gene was mapped by using restriction fragment length polymorphisms (RFLPs) in the RF and BH10 *env* genes (data not shown). The results indicated that the 5′ end of the hybrid *env* gene was derived from the RF *env* gene and that the 3′ end was derived from the BH10 *env* gene, as expected. The RFLP analysis localized the RF-BH10 junction to a 118-bp region spanning the junction between gp120 and gp41. This region was sequenced to exactly define the RF-BH10 junction. The corresponding regions of the parental RF and BH10 *env* genes were also sequenced. The relevant sequences of the RF, BH10, and vAbT168-1 hybrid *env* genes are shown in Fig. 3A. The RF-BH10 condensation event occurred within a conserved 22-bp region common to both the RF and BH10 *env* genes, spanning the gp120-gp41 junction between nucleotides 1569 and 1592. The recombination site contains gp41 sequences, as the RF sequences in Fig. 2A extended beyond gp120 to a convenient restriction site, *Sty*I, at nucleotide 1612 near the 5′ end of gp41.

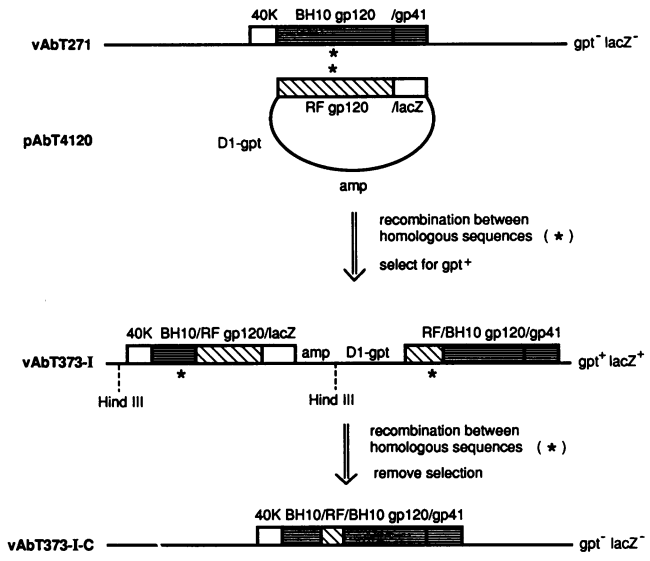

FIG. 4. Generation of chimeric HIV *env* genes in vaccinia virus. Approach 2 (Fig. 1B) was used to generate a population of vaccinia virus containing a spectrum of hybrid HIV *env* genes. Plasmid pAbT4120 was transfected into vAbT271-infected cells. Recombination between homologous regions of the BH10 *env* gene in vAbT271 and the RF *env* gene in pAbT4120 inserted the entire plasmid into vAbT271. Selection for gpt^+ progeny resulted in a collection of intermediate vaccinia virus recombinants, designated vAbT373-I, each recombinant containing two hybrid *env* genes and forming blue plaques in the presence of X-Gal, as a result of the presence of *lacZ* fused in frame to the first hybrid *env* gene. A number of intermediate recombinants containing different hybrid *env* genes were generated, defined by the site of homologous recombination between the *env* genes. *gpt*-selective conditions were then removed to allow the propagation of progeny viruses, each containing a single hybrid, full-length *env* gene resulting from intramolecular recombination between the two hybrid *env* genes in the intermediate recombinant. Loss of the *lacZ* gene served as a marker for this recombination event, resulting in colorless plaques in the presence of X-Gal. Amp, Ampicillin resistance gene.

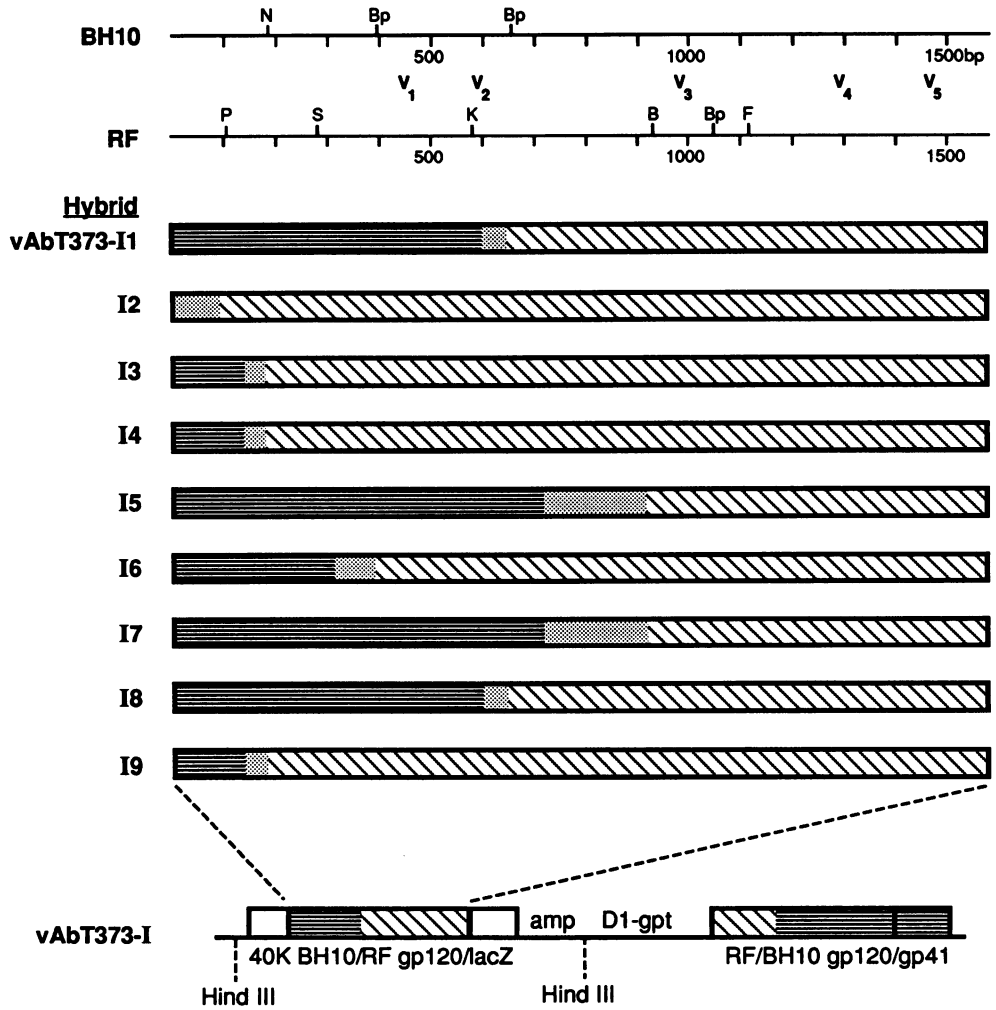

FIG. 5. Structures of hybrid BH10-RF *env* genes in intermediate recombinants. The *Hin*dIII fragment from nine intermediate vAbT373-I vaccinia virus recombinants was subcloned into *E. coli* for restriction mapping of the hybrid BH10-RF *env* gene. The positions of restriction sites unique to BH10 or RF and hypervariable regions V_1 to V_5 are shown. Restriction sites: N, *Nde*I; Bp, *Bsp*1286; P, *Pst*I; S, *Sca*I; K, *Kpn*I; B, *Bsm*I; F, *Fok*I. Symbols: ▤, BH10 sequences; ▨, RF sequences; ▥, hybrid junction in the chimeric *env* gene, indicating the homologous region of recombination between RF and BH10 *env* genes; the actual homologous region is within these maximum boundaries, as determined by restriction mapping.

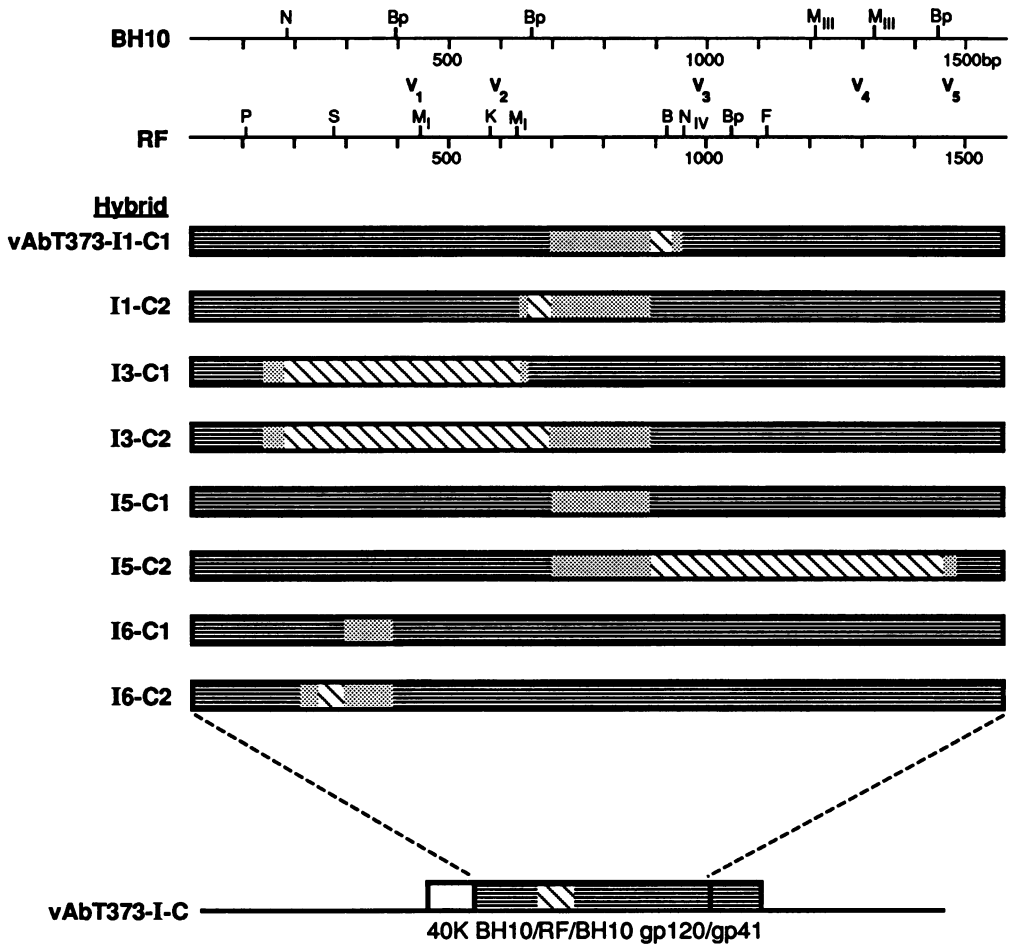

FIG. 6. Structures of hybrid BH10-RF-BH10 *env* genes in condensed recombinants. The hybrid *env* sequences in vaccinia virus genomic DNA were amplified by PCR for restriction mapping of the hybrid BH10-RF-BH10 *env* gene in eight individual vAbT373-I-C recombinants. The positions of restriction sites unique to BH10 or RF and hypervariable regions V_1 to V_5 are shown. Restriction sites: N, *Nde*I; Bp, *Bsp*1286; M_{III}, *Mae*III; P, *Pst*I; S, *Sca*I; M_I, *Mae*I; K, *Kpn*I; B, *Bsm*I; N_{IV}, *Nla*IV; F, *Fok*I. Symbols: ▤, BH10 sequences; ▨, RF sequences; ▥, hybrid junction in the chimeric *env* gene, indicating the homologous region of recombination between RF and BH10 *env* genes; the actual homologous region is within these maximum boundaries, as determined by restriction mapping.

Vaccinia virus genomic DNA was prepared from recombinant vAbT168-2, and RFLPs were analyzed by Southern hybridization. The results indicated that the RF-BH10 junction in vAbT168-2 was within a 360-bp sequence in the central region of gp120, between hypervariable regions V_2 and V_3 (data not shown). This region of vAbT168-2 was sequenced; a portion is shown in Fig. 3B. In vAbT168-2, the RF-BH10 condensation event occurred within a conserved 23-bp sequence located between nucleotides 758 and 782. vAbT168-1 and vAbT168-2 are thus two recombinants containing different hybrid *env* genes (fused to *lacZ*) derived by vaccinia virus-mediated recombination between adjacent RF and BH10 *env* sequences.

Generation of hybrid *env* genes. Having demonstrated the feasibility of using vaccinia virus-mediated recombination to derive hybrid *env-lacZ* genes, we proceeded to generate populations of recombinant vaccinia virus containing full-length, chimeric *env* genes. Approach 2 (Fig. 1B and 4) was used in this set of experiments. First, the HIV-1 BH10 *env* gene was inserted, under the control of the vaccinia virus 40K promoter (designated H6 in reference 44), into the *Hin*dIII-M region of the vaccinia virus genome, using 29K host range selection as described in Materials and Methods; this recombinant virus was designated vAbT271. We then constructed plasmid vector pAbT4120, containing a portion of the HIV-1 RF *env* gene fused at nucleotide 1449 (immediately following hypervariable region V_5 in gp120) to the *lacZ* gene. This vector also contained the *E. coli gpt* gene, under the control of the vaccinia virus D1 promoter (27), for selection of recombinants. Cells were infected with vAbT271 and then were transfected with pAbT4120. A recombination event between homologous regions of the BH10 *env* gene in vAbT271 and the RF *env* gene in pAbT4120 inserted the entire plasmid into vAbT271, resulting in a collection of intermediate vaccinia virus recombinants designated vAbT373-I. Each recombinant (Fig. 4) contained two hybrid *env* genes separated by the entire plasmid vector, including the *gpt* selectable marker, the ampicillin resistance gene, and bacterial replicon. Since the initial recombination event could have occurred at any of a number of homologous regions in the *env* genes, a collection of vAbT373-I recombinants should contain a variety of tandemly arranged, hybrid *env* genes. Nine different vAbT373-I recombinants were isolated and purified (using

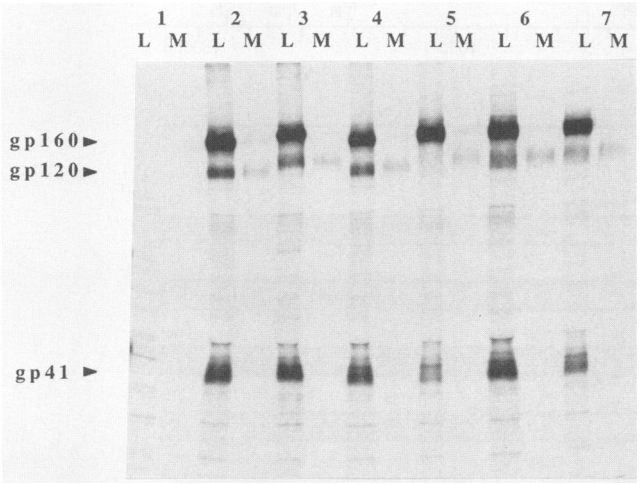

FIG. 7. Immunoprecipitation of chimeric envelope proteins. BSC-40 cells were infected with the indicated vaccinia virus recombinant, labeled with [^{3}H]leucine, and subjected to immunoprecipitation, using HIV-positive serum as described in Materials and Methods. L, Cell lysate; M, clarified cell culture medium. Lanes: 1, NYCBH; 2, vAbT271 (BH10 *env*); 3, vAbT272 (RF *env*); 4, population vAbT373-I1-C; 5, population vAbT373-I3-C; 6, population vAbT373-I5-C; 7, population vAbT373-I6-C.

gpt and *lacZ* selection), and one putative hybrid gene from each recombinant was characterized. The presence of a bacterial replicon and ampicillin resistance gene facilitated the subcloning of this genomic region. Genomic DNA preparations were digested with *Hin*dIII and were self-ligated, and the resulting plasmids were used to transform *E. coli* to ampicillin resistance. Restriction mapping of these plasmids revealed a number of different hybrid junctions in these intermediate recombinants (Fig. 5).

In four different vAbT373-I recombinants (recombinants I1, I3, I5, and I6; Fig. 5), *gpt*-selective conditions were removed to allow the propagation of progeny virus containing a single hybrid, full-length *env* gene. The *lacZ* gene served as a marker to distinguish virus containing a single hybrid *env* gene (colorless plaques) from intermediate virus containing both *env* genes (blue plaques) (Fig. 4). After four serial passages of virus under nonselective conditions, approximately 90% of the progeny plaques from each of the four populations were colorless, indicating that nearly all of the viral genomes had condensed to contain a single *env* gene. Analysis of genomic DNA, amplified by PCR, from each of the four populations of progeny containing single hybrid genes revealed each RFLP expected in a population containing a spectrum of hybrid genes (data not shown).

The two junction regions in each of the putative hybrid genes from eight individual recombinants (two from each population) which formed colorless plaques were located by restriction mapping of genomic DNA amplified by PCR; restriction maps are shown in Fig. 6. Several interesting points emerge from these results. At least seven of the eight viruses contained a unique, hybrid *env* gene. The hybrids designated I5-C1 and I6-C1 were indistinguishable from each other and resembled the parental BH10 *env* gene at the level of restriction mapping. Since each gene was derived from a different intermediate set of hybrid *env* genes (vAbT373-I5 and I6 in Fig. 5), the hybrid junctions are depicted as shown in Fig. 6. These two hybrid genes may contain a portion of the RF *env* gene, distinguishable by sequence analysis but not by restriction analysis. Alternatively, the second recombination event in each case may have occurred at the same region of homology in which the first recombination event occurred, resulting in an *env* gene identical to the BH10 *env* gene.

The restriction map of the hybrid designated I1-C1 reveals an unexpected hybrid junction. Hybrids I1-C1 and I1-C2 are derived from intermediate vAbT373-I1 and should share a hybrid junction defined by the *Kpn*I and *Bsp*1286 sites shown in Fig. 5. I1-C2 contains this junction, but I1-C1 does not. This apparent discrepancy can be explained either by an error in the derivation of this recombinant (for example, derivation from intermediate vAbT373-I5 instead of -I1) or, more intriguingly, by an additional recombination event between the *Kpn*I and *Bsp*1286 sites, undetectable in this case by restriction mapping or sequence analysis. Such additional recombination events could occur as a result of intermolecular recombination between two hybrid *env* genes.

Production of chimeric envelope protein. Black plaque assay, an in situ immunoassay performed on viral plaques, confirmed the expression of envelope protein in 100% of the plaques in each of the four populations of vaccinia virus containing chimeric *env* genes (data not shown).

To determine whether full-length envelope proteins were being expressed by these hybrid genes, immunoprecipitation analysis was performed (Fig. 7) on the vaccinia virus recombinants expressing the parental BH10 or RF *env* gene and on the four populations of recombinant virus in which at least 90% of the progeny contained genomes with a single hybrid *env* gene. Analysis was carried out by using infected cell lysates or cell culture medium to assess proper processing and secretion of envelope protein. The parental BH10 and RF *env* genes expressed gp160 precursor protein and the cleavage products gp120 and gp41 (Fig. 7). Of these, only gp120 was found in the culture medium. The apparent electrophoretic mobilities of the gp160, gp120, and gp41 envelope proteins were different in strains BH10 and RF, most likely because of differences in glycosylation of the BH10 and RF proteins. The gp41 proteins in each of the four populations containing hybrid *env* genes all resembled BH10 gp41, as expected, since gp41 should be derived from BH10 in these hybrids. Interestingly, the gp120 patterns varied in the four populations. vAbT373-I5-C and -I6-C (Fig. 7, lanes 6 and 7) behaved as expected; that is, the gp120 bands were more heterogeneous than the parental BH10 or RF band. These broader protein bands encompassed the RF band and most of the BH10 band, indicating a wide distribution of chimeric proteins. In population vAbT373-I1-C (lane 4), however, the pattern more closely resembled that of BH10, indicating a less random distribution of hybrid envelope proteins in this population. In population vAbT373-I3-C (lane 5), the gp120 band was heterogeneous but was greatly decreased in abundance in the cell lysate. However, normal levels of gp120 were present in the culture medium of cells infected with this population.

Finally, only full-length envelope proteins were observed. There was no evidence of truncated proteins in the infected cells or in the cell culture medium. Thus, the recombination events which created the chimeric genes occurred with a high degree of fidelity, maintaining the appropriate reading frame encoding full-length, chimeric envelope proteins which are glycosylated, cleaved, and transported similarly to the parental BH10 and RF proteins.

DISCUSSION

Conventional recombinant DNA techniques, such as restriction endonuclease cleavage and ligation or oligonucleotide-directed mutagenesis, have been used to construct hybrid DNA sequences to study a variety of structural, functional, antigenic, or pathogenic properties of the encoded polypeptides. These studies include analysis of poliovirus neurovirulence (26), substrate specificity among UDP glucuronosyltransferases (28), binding specificity of antibodies against human chorionic gonadotropin (35), type specificity of antibodies against glycoproteins of vesicular stomatitis virus serotypes (22), ligand-binding-site specificity in β-adrenergic receptor subtypes (31), alterations responsible for the transforming activation of the *neu* oncogene (3), and novel antiviral properties of hybrid interferons (52, 55). However, construction of hybrid DNA sequences by conventional techniques is laborious, requiring individual construction of each hybrid in vitro, insertion of each hybrid into an expression system, and finally analysis of the properties of interest in the hybrid product.

This study shows that intramolecular recombination can be used to facilitate the generation of large numbers of hybrid sequences in recombinant vaccinia virus and should be applicable to other organisms in which duplicated DNA sequences are unstable. In addition to readily generating a number of different hybrids, vaccinia virus provides an immediate expression system for the polypeptide encoded by the hybrid gene. The expression of hybrid sequences by vaccinia virus may also be exploited for the direct characterization of the encoded polypeptide.

In this study, vaccinia virus-mediated homologous recombination was applied to the generation of hybrid *env* genes from the divergent HIV-1 strains BH10 and RF. The collection of chimeric envelope polypeptides encoded by these hybrid genes may mimic the antigenic variation among strains of HIV, which is encoded primarily by the *env* genes (51). Such variation among native HIV strains presents a major challenge in the development of an HIV vaccine, as a vaccine effective against one variant may fail to protect against divergent strains.

The successful use of this approach for the generation of a broadly protective vaccine against HIV faces several potential obstacles. Although homologous recombination may be a contributing factor to the observed sequence diversity among HIV strains (50), mutations introduced by reverse transcriptase during replication of the viral genome are thought to play a major role (41, 43, 53). In addition, since recombination occurs only between homologous (that is, relatively conserved) regions, new hypervariable regions will not be generated by this procedure. Such hypervariable regions (34), including in particular the V_3 region, which has been identified as the predominant, linear, neutralizing epitope, are primarily responsible for the type specificity of the immune response to divergent HIV strains (17, 21, 45).

The formation of new discontinuous epitopes or alterations in the conformation, and thus the immunogenicity, of linear epitopes is anticipated in the generation of hybrid envelope proteins. Discontinuous neutralizing epitopes have been well documented in other antigens, including herpes simplex virus glycoproteins B (39) and D (36), poliovirus VP1 (57) and VP2 (58), and lymphocytic choriomeningitis virus GP-1 (60). Discontinuous epitopes in HIV have not yet been defined, as most approaches to date have used synthetic peptides which limit the analysis to linear epitopes (10, 20, 23, 47). However, the analysis of neutralizing antibodies against envelope that do not bind to any linear envelope peptide (59) and neutralization-resistant mutants that map outside hypervariable region V_3 (18, 33) may reveal discontinuous epitopes. As such mutants are relatively common, identification of these putative discontinuous epitopes may be critical to the success of a vaccine against acquired immunodeficiency syndrome (18). Such epitopes cannot be mapped by using linear, synthetic peptides. Vaccinia virus-mediated recombination can be applied to mapping type-specific, discontinuous epitopes. For example, a population of vaccinia virus containing a spectrum of hybrid genes encoding chimeric antigens can be directly evaluated with respect to the ability of a type-specific monoclonal antibody to bind hybrid proteins expressed by individual recombinants, using a live black plaque assay. Virus plaques presenting hybrid polypeptides with the desired characteristics can be selected and propagated for genomic analysis. Restriction endonuclease mapping or DNA sequence analysis of hybrid genes of interest will identify sequences that define the type-specific epitope. This approach can be applied to the elucidation of linear or discontinuous epitopes.

The major advantage of this recombination system is the ease with which large numbers of hybrid DNA sequences can be generated. There are 20 regions in the *env* gp120 genes of HIV-1 strains BH10 and RF that contain 25 bp or more of exact homology. By using approach 2 (Fig. 1B and 4), in which two hybrid junctions are generated in each chimeric gene, a total of 190 different hybrid combinations could potentially be generated (that is, $19 + 18 + 17 + 16 + \ldots + 1$) in addition to the parental BH10 and RF genes. The frequency of recombination at each of the 20 homologous sites will not be completely random and may depend on the size and sequence of the homologous regions. An immunization dose of 10^7 PFU of a vaccinia virus population containing a spectrum of hybrid *env* genes will thus contain, on average, 5×10^4 copies of each hybrid gene, which should be sufficient to generate an immune response against each encoded hybrid protein. Studies are in progress to determine whether antibodies elicited by such populations of recombinant vaccinia virus in vivo are effective for the neutralization not only of the parental HIV-1 BH10 and RF strains but also of other HIV-1 strains which are not effectively neutralized by antibodies directed against BH10 and RF envelope glycoproteins.

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LITERATURE CITED

1. **Bacchetti, S., and F. L. Graham.** 1977. Transfer of the gene for thymidine kinase to thymidine kinase-deficient human cells by purified herpes simplex viral DNA. Proc. Natl. Acad. Sci. USA **74**:1590–1594.
2. **Ball, L. A.** 1987. High-frequency homologous recombination in vaccinia virus DNA J. Virol. **61**:1788–1795.
3. **Bargmann, C. I., M.-C. Hung, and R. A. Weinberg.** 1986. Multiple independent activations of the neu oncogene by a point mutation altering the transmembrane domain of p185. Cell **45**:649–657.

4. **Barin, F., M. F. McLane, J. S. Alan, T. H. Lee, J. E. Groopman, and M. Essex.** 1985. Virus envelope protein of HTLV-III represents major target antigen for antibodies in AIDS patients. Science **228**:1094–1096.
5. **Beale, A. J., G. C. Christofinis, and I. G. S. Furminger.** 1963. Rabbit cells susceptible to rubella virus. Lancet **ii**:640–641.
6. **Boursnell, M. E. G., and V. Mautner.** 1981. Recombination in adenovirus: crossover sites in intertypic recombinants are located in regions of homology. II Virology **112**:198–209.
7. **Brockman, W. W., and D. Nathans.** 1974. The isolation of simian virus 40 variants with specifically altered genomes. Proc. Natl. Acad. Sci. USA **71**:942–946.
8. **Campione-Piccardo, J., W. E. Rawls, and S. Bacchetti.** 1979. Selective assay for herpes simplex viruses expressing thymidine kinase. Virology **31**:281–287.
9. **Casadaban, M. J., and S. N. Cohen.** 1980. Analysis of gene control signals by DNA fusion and cloning in *Escherichia coli*. J. Mol. Biol. **138**:179–207.
10. **Chanh, T. C., G. R. Dreesman, P. Kanda, G. P. Linette, J. T. Sparrow, D. D. Ho, and R. C. Kennedy.** 1986. Induction of anti-HIV neutralizing antibodies by synthetic peptides. EMBO J. **5**:3065–3071.
11. **Esposito, J., R. Condit, and J. Obijeski.** 1981. The preparation of orthopoxvirus DNA. J. Virol. Methods **2**:175–179.
12. **Falkner, F. G., and B. Moss.** 1988. *Escherichia coli gpt* gene provides dominant selection for vaccinia virus open reading frame expression vectors. J. Virol. **62**:1849–1854.
13. **Fields, S., and G. Winter.** 1982. Nucleotide sequence of influenza virus segments 1 and 3 reveal mosaic structure of a small viral RNA segment. Cell **28**:303–313.
14. **Franchini, G., M. Robert-Guroff, A. Aldovini, N. C. Kan, and F. Wong-Staal.** 1987. Spectrum of natural antibodies against five HTLV-III antigens in infected individuals: correlation of antibody prevalence with clinical status. Blood **69**:437–441.
15. **Franke, C. A., C. M. Rice, J. H. Strauss, and D. E. Hruby.** 1985. Neomycin resistance as a dominant selectable marker for selection and isolation of vaccinia virus recombinants. Mol. Cell. Biol. **5**:1918–1924.
16. **Gillard, S., D. Spehner, R. Drillien, and A. Kirn.** 1986. Localization and sequence of a vaccinia virus gene required for multiplication in human cells. Proc. Natl. Acad. Sci. USA **83**:5573–5577.
17. **Goudsmit, J., C. Debouck, R. H. Meloen, L. Smit, M. Bakker, D. M. Asher, A. V. Wolff, C. J. Gibbs, Jr., and D. C. Gajdusek.** 1988. Human immunodeficiency virus type 1 neutralization epitope with conserved architecture elicits type-specific antibodies in experimentally infected chimpanzees. Proc. Natl. Acad. Sci. USA **85**:4478–4482.
18. **Goudsmit, J., C. L. Kuiken, and P. L. Nara.** 1989. Linear versus conformational variation of V3 neutralization domains of HIV-1 during experimental and natural infection. AIDS **3**(Suppl. 1): S119–S123.
19. **Hahn, B. H., G. M. Shaw, M. E. Taylor, R. R. Redfield, P. D. Markham, S. Z. Salahuddin, F. Wong-Staal, R. C. Gallo, E. S. Parks, and W. P. Parks.** 1986. Genetic variation in HTLV-III/LAV over time in patients with AIDS or at risk for AIDS. Science **232**:1548–1553.
20. **Ho, D. D., M. G. Sarngadharan, M. S. Hirsch, R. T. Schooley, T. R. Rota, R. C. Kennedy, T. C. Chanh, and V. L. Sato.** 1987. Human immunodeficiency virus neutralizing antibodies recognize several conserved domains on the envelope glycoproteins. J. Virol. **61**:2024–2028.
21. **Javaherian, K., A. J. Langlois, C. McDanal, K. L. Ross, L. I. Eckler, C. L. Jellis, A. T. Profy, J. R. Rusche, D. P. Bolognesi, S. D. Putney, and T. J. Matthews.** 1989. Principal neutralizing domain of the human immunodeficiency virus type 1 envelope protein. Proc. Natl. Acad. Sci. USA **86**:6768–6772.
22. **Keil, W., and R. R. Wagner.** 1989. Epitope mapping by deletion mutants and chimeras of two vesicular stomatitis virus glycoprotein genes expressed by a vaccinia virus vector. Virology **170**:392–407.
23. **Kennedy, R. C., G. R. Dreesman, T. C. Chanh, R. N. Boswell, J. S. Allan, T. H. Lee, M. Essex, J. T. Sparrow, D. D. Ho, and P. Kanda.** 1987. Use of a resin-bound synthetic peptide for identifying a neutralizing antigenic determinant associated with the human immunodeficiency virus envelope. J. Biol. Chem. **262**:5769–5774.
24. **Kessler, S. W.** 1981. Use of protein-A-bearing staphylococci for the immune precipitation and isolation of antigens from cells. Methods Enzymol. **73**:442–454.
25. **King, A. M., W. R. Slade, J. W. I. Neuman, and D. McCahon.** 1980. Temperature sensitive mutants of foot-and-mouth disease virus with altered structural polypeptides. II. Comparison of recombination and biochemical maps. J. Virol. **34**:67–72.
26. **La Monica, N., C. Meriam, and V. R. Racaniello.** 1986. Mapping of sequences required for mouse neurovirulence of poliovirus type 2 Lansing. J. Virol. **57**:515–525.
27. **Lee-Chen, G.-J., N. Bourgeois, K. Davidson, R. C. Condit, and E. G. Niles.** 1988. Structure of the transcription initiation and termination sequences of seven early genes in the vaccinia virus HindIII D fragment. Virology **163**:64–79.
28. **Mackenzie, P. I.** 1990. Expression of chimeric cDNAs in cell culture defines a region of UDP glucuronosyltransferase involved in substrate selection. J. Biol. Chem. **265**:3432–3435.
29. **Mackett, M., and J. R. Arrand.** 1985. Recombinant vaccinia virus induces neutralising antibodies in rabbits against Epstein-Barr virus membrane antigen gp340. EMBO J. **4**:3229–3234.
30. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual, p. 382–389. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
31. **Marullo, S., L. J. Emorine, A. D. Strosberg, and C. Delavier-Klutchko.** 1990. Selective binding of ligands to B1, B2 or chimeric B1/B2-adrenergic receptors involves multiple subsites. EMBO J. **9**:1471–1476.
32. **Matthews, T. J., A. J. Langlois, W. G. Robey, N. T. Chang, R. C. Gallo, P. J. Fischinger, and D. P. Bolognesi.** 1986. Restricted neutralization of divergent human T-lymphotropic virus type III isolates by antibodies to the major envelope glycoprotein. Proc. Natl. Acad. Sci. USA **83**:9709–9713.
33. **McKeating, J. A., and R. L. Willey.** 1989. Structure and function of the HIV envelope. AIDS **3**(Suppl. 1):S35–S41.
34. **Modrow, S., B. H. Hahn, G. M. Shaw, R. C. Gallo, F. Wong-Staal, and H. Wolf.** 1987. Computer-assisted analysis of envelope protein sequences of seven human immunodeficiency virus isolates: prediction of antigenic epitopes in conserved and variable regions. J. Virol. **61**:570–578.
35. **Moyle, W. R., M. M. Matzuk, R. K. Campbell, E. Cogliani, D. M. Dean-Emig, A. Krichevsky, R. W. Barnett, and I. Boime.** 1990. Localization of residues that confer antibody binding specificity using human chorionic gonadotropin/luteinizing hormone B subunit chimeras and mutants. J. Biol. Chem. **265**:8511–8518.
36. **Muggeridge, M. I., T. T. Wu, D. C. Johnson, J. C. Glorioso, R. J. Eisenberg, and G. H. Cohen.** 1990. Antigenic and functional analysis of a neutralization site of HSV-1 glycoprotein D. Virology **174**:375–387.
37. **Nara, P. L., W. G. Robey, S. W. Pyle, W. C. Hatch, N. M. Dunlop, J. W. Bess, Jr., J. C. Kelliher, L. O. Arthur, and P. J. Fischinger.** 1988. Purified envelope glycoproteins from human immunodeficiency virus type 1 variants induce individual, type-specific neutralizing antibodies. J. Virol. **62**:2622–2628.
38. **Panicali, D., and E. Paoletti.** 1982. Construction of poxviruses as cloning vectors: insertion of the thymidine kinase gene from herpes simplex virus into the DNA of infectious vaccinia virus. Proc. Natl. Acad. Sci. USA **79**:4927–4931.
39. **Pereira, L., M. Ali, K. Kousoulas, B. Huo, and T. Banks.** 1989. Domain structure of herpes simplex virus 1 glycoprotein B: neutralizing epitopes map in regions of continuous and discontinuous residues. Virology **172**:11–24.
40. **Perkus, M. E., K. Limbach, and E. Paoletti.** 1989. Cloning and expression of foreign genes in vaccinia virus, using a host range selection system. J. Virol. **63**:3829–3836.
41. **Preston, B. D., B. J. Poiesz, and L. A. Loeb.** 1988. Fidelity of HIV-1 reverse transcriptase. Science **242**:1168–1171.
42. **Ratner, L., W. Haseltine, R. Patarca, K. J. Livak, B. Starcich, S. F. Josephs, E. R. Doran, J. A. Rafalski, E. A. Whitehorn, K.**

Baumeister, L. Ivanoff, S. R. Petteway, Jr., M. L. Pearson, J. A. Lautenberger, T. S. Papas, J. Ghrayeb, N. T. Chang, R. C. Gallo, and F. Wong-Staal. 1985. Complete nucleotide sequence of the AIDS virus, HTLV-III. Nature (London) **313**:277–284.

43. **Roberts, J. D., K. Bebenek, and T. A. Kunkel.** 1988. The accuracy of reverse transcriptase from HIV-1. Science **242**: 1171–1173.
44. **Rosel, J. L., P. L. Earl, J. P. Weir, and B. Moss.** 1986. Conserved TAAATG sequence at the transcriptional and translational initiation sites of vaccinia virus late genes deduced by structural and functional analysis of the *Hin*dIII H genome fragment. J. Virol. **60**:436–449.
45. **Rusche, J. R., K. Javaherian, C. McDanal, J. Petro, D L. Lynn, R. Grimaila, A. Langlois, R. C. Gallo, L. O. Arthur, P. J. Fischinger, D. P. Bolognesi, S. D. Putney, and T. J. Matthews.** 1988. Antibodies that inhibit fusion of human immunodeficiency virus-infected cells bind a 24-amino acid sequence of the viral envelope, gp120. Proc. Natl. Acad. Sci. USA **85**:3198–3202.
46. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74**:5463–5467.
47. **Schreier, R. D., J. W. Gnann, Jr., A. J. Langlois, K. Shriver, J. A. Nelson, and M. B. A. Oldstone.** 1988. B- and T-lymphocyte responses to an immunodominant epitope of human immunodeficiency virus. J. Virol. **62**:2531–2536.
48. **Smith, T. F., M. Marcus, and G. Myers.** 1988. Phylogenetic analysis of HIV-1 and HIV-2, p. 317–321. *In* H. Ginsberg, F. Brown, R. A. Lerner, and R. M. Chanock (ed.), Vaccines 88. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
49. **Spyropoulos, D. D:, B. E. Roberts, D. L. Panicali, and L. K. Cohen.** 1988. Delineation of the viral products of recombination in vaccinia virus-infected cells. J. Virol. **62**:1046–1054.
50. **Srinivasan, A., D. York, R. Jannoun-Nasr, S. Kalyanaraman, D. Swan, J. Benson, C. Bohan, P. A. Luciw, S. Schnoll, R. A. Robinson, S. M. Desai, and S. G. Devare.** 1989. Generation of hybrid immunodeficiency virus by homologous recombination. Proc. Natl. Acad. Sci. USA **86**:6388–6392.
51. **Starcich, B. R., B. H. Hahn, G. M. Shaw, P. D. McNeely, S. Modrow, H. Wolf, E. S. Parks, W. P. Parks, S. F. Josephs, R. C. Gallo, and F. Wong-Staal.** 1986. Identification and characterization of conserved and variable regions in the envelope gene of HTLV-III/LAV, the retrovirus of AIDS. Cell **45**:637–648.
52. **Streuli, M., A. Hall, W. Boll, W. E. Stewart II, S. Nagata, and C. Weissmann.** 1981. Target cell specificity of two species of human interferon produced in *Escherichia coli* and of hybrid molecules derived from them. Proc. Natl. Acad. Sci. USA **78**:2848–2852.
53. **Takeuchi, Y., T. Nagumo, and H. Hoshino.** 1988. Low fidelity of cell-free DNA synthesis by reverse transcriptase of human immunodeficiency virus. J. Virol. **62**:3900–3902.
54. **Tolskaya, E. A., L. A. Romanova, M. S. Kolesnikova, and V. I. Agol.** 1983. Intertypic recombination in poliovirus: genetic and biochemical studies. Virology **124**:121–132.
55. **Weck, P. K., S. S. Apperson, N. Stebbing, P. W. Gray, D. Leung, H. M. Shepard, and D. V. Goeddel.** 1981. Antiviral activities of hybrids of two major human leukocyte interferons. Nucleic Acids Res. **9**:6153–6166.
56. **Weiss, R. A., P. R. Clapham, J. N. Weber, A. G. Dalgleish, L. A. Lasky, and P. W. Berman.** 1986. Variable and conserved neutralization antigens of human immunodeficiency virus. Nature (London) **324**:572–575.
57. **Wiegers, K., H. Uhlig, and R. Dernick.** 1989. N-AglB of poliovirus type 1: a discontinuous epitope formed by two loops of VP1 comprising residues 96-104 and 141-152. Virology **170**:583–586.
58. **Wiegers, K. J., K. Wetz, and R. Dernick.** 1990. Molecular basis for linkage of a continuous and discontinuous neutralization epitope on the structural polypeptide VP2 of poliovirus type 1. J. Virol. **64**:1283–1289.
59. **World Health Organization Global Programme on AIDS.** 1988. Report of a WHO workshop on the measurement and significance of neutralizing antibody to HIV and SIV. AIDS **4**:269–275.
60. **Wright, K. E., M. S. Salvato, and M. J. Buchmeier.** 1989. Neutralizing epitopes of lymphocytic choriomeningitis virus are conformational and require both glycosylation and disulfide bonds for expression. Virology **171**:417–426.