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Vaccinia Virus Vectors: New Strategies for Producing Recombinant Vaccines

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INTRODUCTION

Although in the ongoing battle against infectious diseases it is sometimes difficult to determine which side is winning, a number of new weapons have recently become available which promise to tilt the scales in our favor. These include the development of new candidate antiviral drugs, the site-specific targeting of designer immunotoxins, the synthesis of milligram quantities of epitope-specific synthetic peptide antigens, and, most notably, the ability to construct genetically engineered recombinant viral vaccines. Since there are a considerable number of historical precedents for using viruses as effective vaccines (44), this last approach in particular holds considerable promise because a large number of different types of viruses can be adapted to serve as efficient and effective vectors (107). Although each virus has its own set of unique advantages, which may prove useful in laboratory studies, the system that seems to hold the greatest immediate potential for human medicine is the use of infectious genetically engineered poxvirus recombinants. Several reviews have addressed the general methodology and the specific applications of the system for particular subdisciplines (14, 67, 73, 94, 102, 109, 110, 126, 142, 146, 147, 166). However, because of the high level of research activity in this area, it is rapidly and constantly changing. The purpose of this review is to provide an updated overview of the use of poxvirus expression vectors with an emphasis on new methods that have recently become available, to consider both the advantages and disadvantages of this system, and finally, to preview what the future is likely to hold for poxvirus vectors.

VV BIOLOGY

VV Replication Cycle

Although a number of different poxviruses (e.g., fowlpox, cowpox, and raccoonpox) are now being used as vectors, the following remarks pertain primarily to vaccinia virus (VV), the prototype member of the poxvirus family which has been the focus of most of the efforts to develop poxvirus-based vector systems. VV is a large DNA-containing animal virus that replicates within the cytoplasm of susceptible host cells (69, 71). The VV virion consists of a biconcave core particle, containing the viral genomic DNA molecule, which is flanked by two lateral bodies. The entire structure is enclosed within a lipid envelope. The basic major features of the VV replicative cycle are diagrammed in Fig. 1.

The virus adsorbs to susceptible host cells and makes its way into the interior of the cell by either direct penetration or viropexis. Once inside, the viral envelope is stripped away by host enzymes (uncoating I) and expression of the

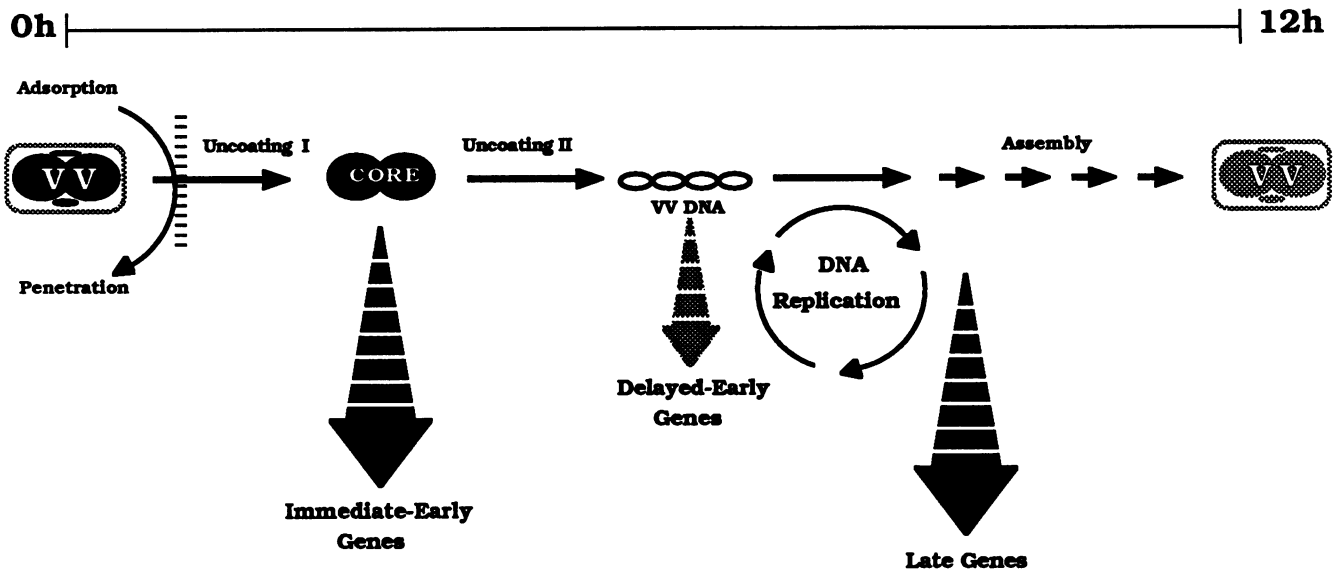


FIG. 1. VV replication. The major events in the growth cycle of VV are indicated schematically.

viral immediate-early genes commences. This phase of transcription is carried out under the influence of viral enzymes which have been packaged into the virion (77). These include VV DNA-dependent RNA polymerase, poly(A) polymerase, capping and methylating activities, and *trans*-acting transcription factors (108). The immediate-early genes correspond to about one-half of the viral genetic potential, i.e., about 100 gene products (10, 118). Included among the VV immediate-early gene products are enzymes required for viral DNA replication such as DNA polymerase (76), thymidine kinase (68), and ribonucleotide reductase (141, 163), as well as virus-encoded enzymes necessary to catalyze the breakdown of the core particle (uncoating II) and release of the viral DNA molecule. Subsequent to core breakdown, but prior to DNA replication, a minor class of VV genes are transcribed (known as delayed-early genes) whose expression is essential for the replication of VV DNA (108). About 3 h postinfection, viral DNA begins to be replicated by viral enzymes in the cytoplasm of infected cells. A pronounced shift in the pattern of VV transcription and translation occurs concomitantly with the onset of viral DNA replication. That is, the transcription of VV early genes and the translation of the resulting early transcripts are attenuated, whereas the transcription and expression of the late genes are initiated. The molecular mechanisms responsible for this pronounced regulatory switch are as yet unclear (177; J. N. Miner and D. E. Hruby, *Advances in Life Sciences: Molecular Genetics*, in press). There are some VV genes whose expression does not follow these rules, being expressed at both early and late times during infection. These genes, such as the VV 7.5-kilodalton (kDa) gene, are termed constitutive, and although the same open reading frame is expressed, it is usually from alternative transcriptional start sites used prior to or after the onset of DNA replication (26). The VV late transcripts are novel in that they do not terminate at a distinct site and hence are heterogeneous in length and have a 3′-poly(A) tail and a 5′-poly(A) head which is added to the VV late transcripts by an unknown mechanism, although polymerase stuttering appears to be the most likely explanation (182). At late times during infection, transcripts derived from the entire VV genome are present in the cytoplasm of infected cells due to transcriptional readthrough, although most likely only the late open reading frames present at the 5′ end of these transcripts are actually translated into protein. Thus far, there is no evidence to suggest that VV translates the internal cistrons from either late or early polycistronic viral transcripts into protein (11).

The replicating viral DNA forms large inclusion bodies which are termed viroplasm or viral factories. The viral factories are the site of assembly of progeny virion particles. This process begins about 5 h postinfection with the appearance of membrane crescents which begin to form and envelop a portion of the viroplasm. The immature particle thus formed then migrates away from the virus factory and undergoes a complex series of morphogenetic condensation steps leading to the formation of a mature virion particle (30). During the maturation process the virus appears to package both viral and nonviral polypeptides (53). This entire complicated process is well regulated and is remarkable in that only about 10 to 12 h is required to complete a single cycle of infection. VV produces 2,000 to 3,000 particles per infected cell, with a particle/infectious PFU ratio of approximately 1:10 to 1:20 (69). Although VV does not transform cells, it is not lytic in the classical sense as infected cells do not lyse. Rather, the virus is able to move from the infected cell to the neighboring uninfected ones by cell fusion and syncytium formation, thereby causing a plaque, but most of the virus stays cell associated, thereby providing a convenient method for partial purification of virus, that is, collection of infected cells by centrifugation.

Advantages as a Vector

Because of the unique cytoplasmic replication mode outlined above, VV not only provides an excellent experimental model system for molecular genetic investigations of the *cis* and *trans* factors required for replication and expression of genetic information, but also is uniquely suited to serve as a

eucaryotic expression vector. The advantages of VV as a vector are outlined below.

Cytoplasmic replication. The cytoplasmic replication site (71) facilitates introduction of foreign genes into the viral genome by marker transfer and also the radiolabeling, detection, and isolation of proteins expressed by recombinant viruses. Furthermore, since the virus remains within the cytoplasmic compartment of infected cells and is apparently strictly lytic, potential complications of integration into the host cell genome or phenotypic transformation are not encountered. These are important considerations when a nuclear virus such as simian virus 40 is used as a vector (129).

Broad host range. VV replicates in both primary cell cultures (e.g., chicken embryo fibroblasts) and many different cell culture lines (L929, CV-1, and HeLa) isolated from virtually any animal species (ranging from mosquitos to humans [52]). VV will also grow in species of experimental animals (mice, rabbits, and guinea pigs) commonly used in the laboratory (72). Thus, the virus can be used essentially as a eucaryotic shuttle vector with which to express a heterologous foreign protein of interest in a different cell type, primary cell culture, or even a whole animal.

Large viral genome. The 185-kilobase genome of VV readily tolerates both large insertions of foreign DNA (≥25 kilobases) (145) and deletions of viral sequences (≥20 kilobases) (116). As opposed to smaller DNA viruses (107), there seem to be no strict requirements or constraints on the absolute size of the VV DNA molecule required to be efficiently packaged or replicated. The ability to insert a large amount of foreign genetic information and its expression by the virus are important factors to consider when VV is used to construct a polyvalent vaccine containing antigens from several different pathogens (50, 125, 147) or perhaps antigens from several different serotypes of the same pathogen.

Viral transcriptional apparatus. VV transcribes its genome by using unique viral enzymes, viral transcription signals, and ancillary transcription factors (103, 104, 183). From an operational standpoint, this means that, if one abuts a foreign gene directly to a VV promoter element, one can be reasonably sure that the foreign sequences will be actively transcribed during the course of the viral infection. Foreign transcripts will be capped and polyadenylated by VV enzymes and will serve as efficient messages for the translation of relatively high levels of the foreign protein within the infected cell. Evidence which has accumulated thus far indicates that the foreign proteins can be expected to be posttranslationally modified in an authentic manner, transported to the correct subcellular compartment, and in most cases to exhibit completely functional activity, be it enzymatic or structural. This is a distinct advantage over using bacterial, yeast, or nonmammalian virus (e.g., baculovirus) vectors to express mammalian proteins, as these systems often fail to modify the expressed proteins in the proper manner for full activity or antigenicity (107).

Virus gene architecture. To date, no VV genes have been reported which contain intervening sequences; i.e., VV does not splice its primary transcripts. Thus, any contiguous coding sequence can be expressed from the context of the VV genome without concern about providing consensus splice signals in the correct locations. Likewise, since VV carries out its replication cycle in the cytoplasm, the requirement for incorporating the genetic information necessary to ensure the transport of foreign transcripts from the nucleus to the cytoplasm is obviated. These last considerations are of particular importance when attempting to express DNA sequences from bacteria or cDNA derived from cytoplasmic RNA viral templates, because these sequences have evolved for expression in non-nonuclear (i.e., cytoplasmic) environments. As such, not only do they lack in the correct location the necessary splice and transport signals for proper expression, but also they may fortuitously contain homologous sequences at inappropriate sites which greatly interfere with their expression, e.g., illegitimate splicing within the open reading frame.

Safety. VV was the first viral vaccine used by Edward Jenner to prevent smallpox. Thus, we might consider that it has been in clinical trials since 1798 (44). Although complications such as postvaccination encephalitis or progressive VV infections can occur, overall VV is quite safe and effective, as evidenced by its successful use to eradicate smallpox from the human population globally.

Cost. Because of its broad host range, VV can be grown to high titer in a variety of cell lines or animal hosts. In fact, much of the vaccine that was grown for human use was propagated by scarification of bovine hosts (44). Currently, better and more aseptic methods for producing vaccines for use in humans are undoubtedly available. A dose of VV vaccine can be produced for literally pennies. Because recombinant VV is a live replicating vaccine, the quantity that must be given to elicit a protective immune response is reduced.

Stability. The VV virion is very stable, maintaining infectious titer while frozen for many years. Furthermore, the virus can be dried down, transported to distant locations, rehydrated, and inoculated with only minimal losses in infectivity. This characteristic allows VV-derived vaccines to be used for large-scale immunizations under conditions in which refrigeration may or may not be routinely available.

Ease of administration. Administering a VV-based vaccine does not require the same level of medical training as an intravenous injection. Rather, the classic method for inoculating VV is intradermally with the aid of the bifurcated needle. The virus can also be administered with an air gun, thus enabling a large number of individuals to be vaccinated under field conditions. When viewed together, these are considerable scientific and practical reasons why VV has lent itself to being developed as an effective vaccine vector.

Limitations as a Vector

Despite the many advantages of the VV vectors, the system does have some inherent limitations that need to be remembered. First, as described above, VV is a live infectious lytic virus which kills the cells that it infects. Thus, VV vectors can be used to express foreign proteins only transiently in infected cells during the course of the virus life cycle (12 to 24 h). VV vectors are not suitable for large-scale, long-term expression of foreign proteins in continuous cell cultures, although through the use of metabolic inhibitors (68) or conditional-lethal viral mutants (27, 66), the time frame of VV protein expression can be extended considerably. Likewise, since VV apparently replicates entirely within the cytoplasmic compartment, at present it is of little use to engineer nuclear gene replacements. Second, although they are rare, postvaccinial encephalitis and progressive VV complications are known to occur after vaccination with VV (44). The latter syndrome is most prevalent in individuals whose cell-mediated immunological mechanisms are impaired; this becomes a particularly important issue to consider in light of the increasing incidence of individuals suffering from acquired immunodeficiency syndrome.

Attempts are under way to make VV expression vectors safer and more acceptable for use in humans (158). One line of research is directed towards identifying viral genes, such as thymidine kinase (17) or ribonucleotide reductase (24a), whose expression is not required in cell culture but which are required for optimal virus replication in vivo. Insertional inactivation of these loci results in the apparent attenuation of recombinant viruses. Other investigators are exploring the possibility of using alternative poxvirus vectors, such as fowlpox, which replicate only to a limited extent in human hosts yet apparently are still capable of expressing sufficient foreign protein to induce a vigorous immune response (160–162).

Ultimately, whether VV vectors will be used in the human population may be decided by the question of risk versus benefit. In the event that the disease under consideration is non-life-threatening (e.g., common cold) or is controllable through the use of more traditional vaccines (e.g., poliovirus), then perhaps the use of VV-based vaccines may be contraindicated. This is especially true in view of potential cosmetic considerations such as the scarring associated with a primary poxvirus inoculation. On the other hand, if the diseases under discussion are serious and often fatal (such as malaria or hepatitis) or diseases for which there are no alternative therapeutic strategies, then the use of VV-based vaccines may well be worth the associated risk, especially in developing countries where routine access to medical treatment is limited (131, 171).

MARKER TRANSFER METHODOLOGY

Marker Transfer

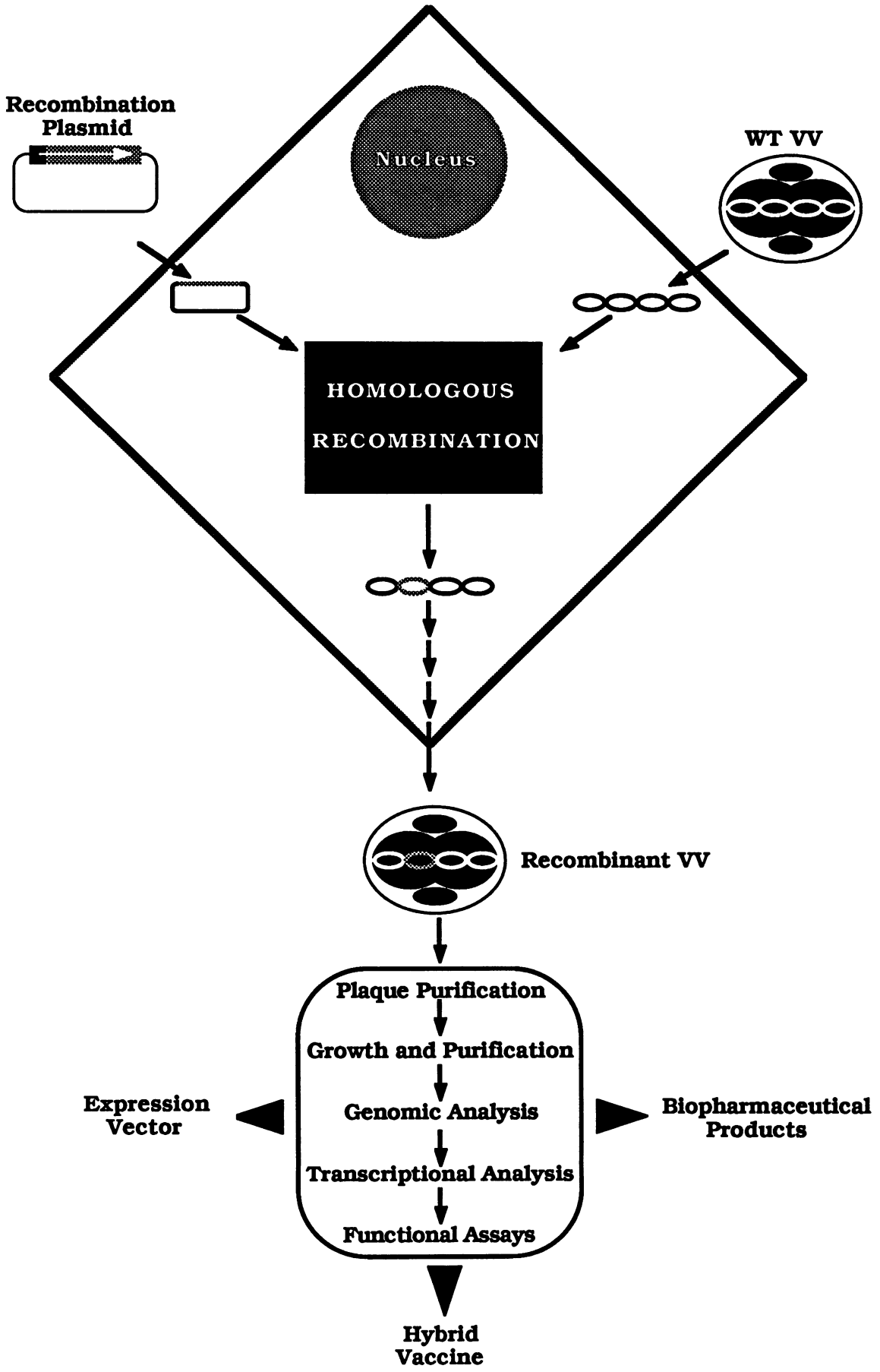
The general method used to construct VV recombinants is diagrammed in Fig. 2 (112). A recombination plasmid is constructed which consists of several elements. (i) One element is the foreign gene of interest abutted in the correct orientation to a VV promoter element. As noted above, the only constraint in this regard is that the insert must not contain any intervening sequences, i.e., be a genomic DNA lacking introns or a cDNA molecule. Aside from lacking introns, most other aspects (e.g., spatial relationships) of the chimeric VV-foreign gene construction appear to be relatively permissive. For optimal levels of expression, however, it is necessary to position the initiator ATG of the foreign gene's open reading frame in close proximity to the VV promoter, in a "good" ribosomal binding context (87), with no alternative start codons available between the initiator ATG and the 5′ end of the transcript. (ii) Another element is VV flanking DNA sequences which are necessary to catalyze homologous recombination and target the foreign gene insertion to a nonessential location in the viral genome. (iii) A third element is bacterial plasmid backbone containing an origin of replication and an antibiotic resistance locus to allow the plasmid to be grown and selected in *Escherichia coli*. Recombination plasmids, which have been purified by equilibrium centrifugation in cesium chloride gradients, are introduced into the cytoplasmic compartment of suitable host cells (usually either Ltk^- or BSC-40 cells) by either a calcium phosphate-mediated transfection (63) or an electroporation procedure. The transfected cells are then superinfected with VV which initiates the viral replication cycle, including liberation and replication of the viral genome. If so desired, the order of infection and transfection can be reversed with little loss of marker transfer efficiency. In any case, although the precise mechanisms are not yet well understood, it is likely that, under the direction of virus-encoded enzymes, the flanking VV DNA sequences of the recombination plasmid interact with homologous sequences present in the replicating viral DNA and catalyze insertion of the foreign sequences by a double-reciprocal crossover (homologous recombination). Evidence has been obtained to indicate that poxvirus DNA is subject to a relatively high rate of recombination during the normal course of infection (31), which undoubtedly facilitates this process. Provided that the insertion event has not inactivated an essential VV gene, such as DNA polymerase, the recombinant genome containing the foreign gene should be amplified by replication and packaged into infectious progeny recombinant virions.

Even in the absence of selection, recombinant VV production is remarkably efficient. In a typical marker transfer experiment, about 1 to 2% recombinants are produced in the progeny (although this number can be reduced several orders of magnitude depending on the size of the insert and the nature of the encoded protein). Recombinants can usually be found simply by using either a plaque hybridization (174) or an immunoblotting procedure. To facilitate this process, a number of methods to improve both the detection and the isolation of the desired recombinant have been developed, and these will be discussed in some detail later. Regardless of the detection scheme used, recombinant plaques of interest are picked and subjected to several rounds of plaque purification to ensure a pure population. The recombinant viruses are then ready for several levels of molecular analyses to ensure genomic authenticity and to determine whether the gene product of interest is being actively expressed.

Molecular Genetic Analyses of Recombinants

DNA from recombinant virions is extracted and purified and digested with appropriate restriction endonucleases; the resulting DNA fragments are separated by agarose gel electrophoresis, and the gel is analyzed by Southern blotting procedures, using radioactive probes which are specific for either the region containing the insertion site or the foreign gene which was inserted (130). These procedures are done to ensure, at least at this level of resolution, that an insert of the correct size and identity has been recombined into the anticipated location within the viral genome and that no other gross genomic alterations (rearrangements, second-site insertions, or deletions) have occurred. Although illegitimate recombination occurs only rarely, because viral recombinants may be used as vaccines, genomic maps must be verified prior to use. To obtain more detailed information concerning the inserted DNA, recently developed polymerase chain reaction techniques (152) can be used together with synthetic oligonucleotide primers to amplify and sequence the chimeric gene from the recombinant viral genome to

FIG. 2. Construction of recombinant VV. Marker transfer techniques were used to introduce foreign genes into the VV genome by homologous recombination. Recombinant DNA molecules were packaged into infectious viral particles which could be identified by plaque hybridization or a variety of selection/detection procedures. The recombinant viruses were then isolated, purified, and subjected to molecular analyses at a number of levels. Once characterized, the recombinants are available for a variety of experimental uses. WT, Wild type.


Recombination Plasmid
Nucleus
WT VV
HOMOLOGOUS RECOMBINATION
Recombinant VV
Plaque Purification
Growth and Purification
Genomic Analysis
Transcriptional Analysis
Functional Assays
Expression Vector
Biopharmaceutical Products
Hybrid Vaccine

determine whether any sequence alterations, i.e., missense or nonsense mutations, have occurred.

Once the structural or sequence analyses of the recombinant viral genome have been completed, the next step is to verify that the foreign DNA insert is actively and correctly transcribed from within the context of the VV genome. This is usually done at two levels. First, cytoplasmic RNA from recombinant VV-infected cells is analyzed by denaturing gel electrophoresis and Northern (RNA) blot hybridization analyses to determine whether a transcript of the predicted size is present (130, 167). Second, the 5′ and 3′ ends of the transcript are determined by a combination of nuclease S1 mapping and primer extension procedures to ensure that the chimeric RNA is initiated and terminated in the expected locations. These transcriptional analyses are important in the event that the foreign insert fortuitously contains cryptic VV transcriptional start or stop signals which will greatly attenuate the expression of the foreign gene product. If such problems do occur, they can be easily overcome by using site-directed mutagenesis (187) to introduce translationally silent mutations into the foreign insert, which will preserve the amino acid sequence of the derived gene product while destroying the cryptic VV transcriptional signals.

Assuming that the foreign DNA sequences are correctly transcribed, the final step in the characterization process is to determine whether the transcripts are actively translated within infected cells, whether the foreign proteins are stable, whether they are correctly transported and posttranslationally modified, and whether they are biologically active. In general, VV has been used in three ways as an expression vector: as a recombinant vaccine, to produce biopharmaceutical products, and as an expression vector for basic research. Obviously, the nature of the expressed protein dictates the types of protein assays that will be used to monitor the expression of the foreign gene product. For example, in the case of an antigen, antibody-directed reactions such as Western blotting of infected cell extracts or immunofluorescence of intact infected cells are used (130). If the encoded protein is an enzyme, such as chloramphenicol acetyltransferase (120) or neomycin phosphotransferase (54, 55, 98), then the infected cell extracts can simply be assayed for enzymatic activity as a measure of gene expression. Finally, if the encoded protein is not an enzyme, then alternative functional assays can be designed. An example of this approach might be the demonstration that Sindbis virus coat proteins expressed by a VV recombinant are able to become associated with infectious Sindbis virions during viral coinfections (130). In any case, with very few exceptions and regardless of the assay procedures used, one can reasonably expect that the recombinant VV will express microgram quantities of biologically active protein for use in the intended experiments.

Once the DNA, RNA, and protein analyses of recombinant viruses have been completed, a large purified stock of the recombinant is grown and purified which should be sufficient for a large number of experiments (usual titers of purified VV recombinants of $\geq 10^{10}$). Before moving on to several illustrations of how recombinant VV vectors have been used, several practical aspects of the marker transfer scheme require some amplification for those who anticipate using VV vectors in their laboratories.

Recombination Plasmids

The first generation of VV insertion plasmids were monoexpression vectors of the type diagrammed in Fig. 3A (13). Within a bacterial plasmid backbone, a single VV promoter is abutted to a polylinker region and flanked by genomic VV DNA targeting sequences. A number of different promoter elements have been used successfully in these constructions, including the F promoter (DNA sequences immediately upstream of the unique *Bam*HI site of the VV *Hin*dIII F fragment) (119), the 11-kDa promoter from the *Hin*dIII A fragment (40), and the 7.5-kDa promoter found within the inverted terminal repeats present at both ends of the VV genomic DNA molecule (172). These three promoters are each capable of directing efficient high-level expression of downstream foreign genes; however, they differ in the kinetics of expression. The F promoter is expressed as an immediate-early gene (prior to viral DNA replication), the 11-kDa promoter is expressed as a late gene (after viral DNA replication), and the 7.5-kDa promoter is expressed as a constitutive gene (throughout the replication cycle). It is likely that, as additional regions of the poxvirus genome are sequenced, new promoter elements will be described which will be equally useful for directing efficient or perhaps even regulated expression of inserted foreign genes. Along those lines, it is of interest to note that in recent studies the regulatory elements of the *lac* operon have successfully been transposed into the poxvirus genome to allow the inducible expression of inserted foreign genes (57).

In any case, regardless of the promoter used, in the monoexpression vector system, foreign DNA sequences are inserted in a colinear orientation downstream of the VV promoter element to form a recombination vector. This type of plasmid has been used in concert with marker transfer techniques to construct the vast majority of VV recombinants that have been reported to date in the literature. Although effective, this approach is somewhat limiting when attempting to recombine large foreign inserts into the VV genome (which occurs at a low frequency) or when attempting to insert foreign DNA (such as a protease gene) whose expression is somewhat detrimental to viral replication. Under these conditions it is difficult, if not impossible, to find the recombinant virus of interest. To circumvent this problem, several dual coexpression insertion vector systems have been developed. In these cases, a VV promoter-polylinker expression cassette is abutted to a second VV promoter which drives either a selectable (e.g., *neo* [54, 55, 98]) or an easily detectable (e.g., β-galactosidase [19, 75, 117]) reporter gene, with the entire tandem array flanked by VV targeting DNA sequences (170). Although the two cassettes are usually oriented in opposite directions (Fig. 3B), there appears to be little transcriptional interference between neighboring VV genes so that alternative gene arrangements are equally active (55). Using coexpression plasmids, the foreign gene is inserted into the plasmid, marker transfer is carried out, and recombinants are detected by expression of the reporter gene. In most, if not all, of the VV recombinants that express the reporter gene, the foreign gene is also convected into the viral genome and actively expressed.

All of the insertion vectors discussed thus far are constructed with bacterial plasmid vectors. It is also possible to design VV insertion vectors based on single-stranded M13 bacteriophage DNA (181). These single-stranded insertion vectors have some experimental advantages, including ease of propagation and higher marker transfer frequency. Furthermore, the single-stranded DNA template is a suitable substrate for both DNA sequencing and site-directed mutagenic procedures, making this the system of choice when VV

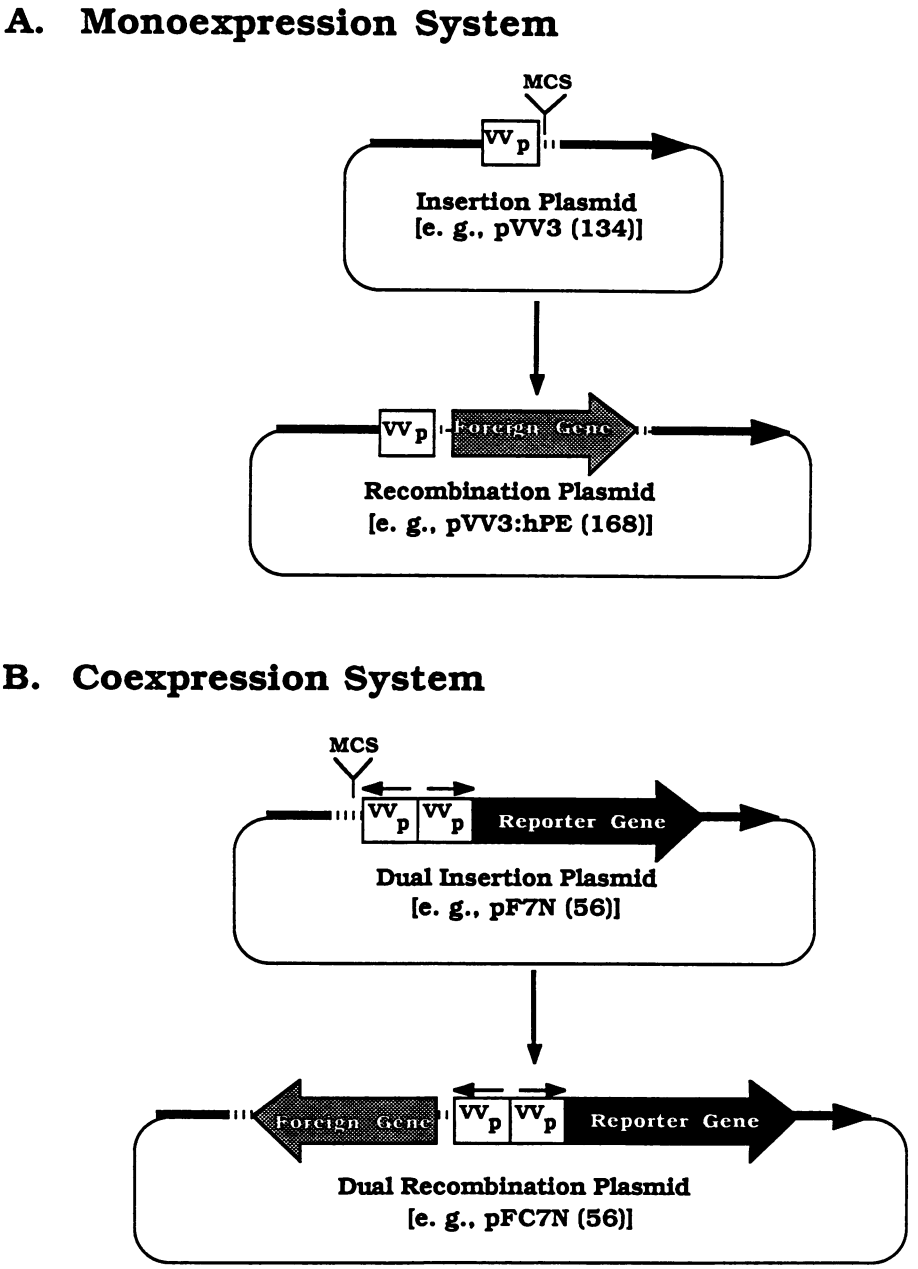


FIG. 3. General types of VV insertion vectors. (A) Monoexpression system in which a chimeric VV promoter-foreign gene is inserted into the middle of a nonessential VV gene. (B) Coexpression system in which the chimeric VV promoter-foreign gene is linked in tandem to a second cassette containing a gene which will facilitate the detection or selection or both of the derived recombinant virus.

vectors are used to carry out structure-function investigations of foreign proteins.

Insertion Sites

It is believed that VV carries a large amount of genetic information which is apparently dispensable for growth in tissue culture. This notion is based on several lines of evidence, including (i) the isolation of spontaneous VV mutants that contain large (≥20-kilobase) insertions or deletions of genomic DNA (116); (ii) the relatively small number of complementation groups to which the large number of temperature-sensitive mutants can be assigned (27); and (iii) random mutagenesis studies carried out on several regions of the VV genome which demonstrate that many VV genes can be inactivated with little apparent effect on viral replication (12, 124). Taken together, these results suggest that a number of different nonessential loci within the VV genome are available for insertion sites for foreign DNA.

Thus far, VV recombinants have been constructed by using a relatively limited number of insertion sites (Fig. 4). These include the thymidine kinase (tk) gene (95), the *Bam*HI site of the VV *Hin*dIII-F DNA fragment (119), the VV growth factor gene located within both terminal repeats (16), the N2 and M1 genes on the left side of the genome (159), the M1 subunit of the ribonucleotide reductase gene in the VV *Hin*dIII-I DNA fragment (24a), and the VV hemag-

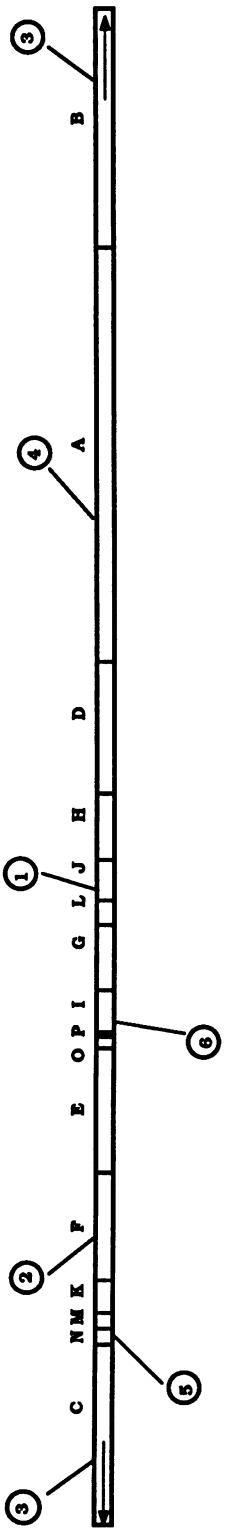


FIG. 4. Viable insertion sites in VV DNA. Physical map of the VV genome with the *Hin*dIII restriction sites and inverted terminal repeats (arrows) indicated. Numbers correspond to insertion sites which have been used to insert foreign genes and produce viable recombinants. (1) Thymidine kinase gene; (2) *Bam*HI site of the *Hin*dIII F fragment; (3) VV growth factor gene; (4) HA gene; (5) N2 and M1 genes; (6) gene encoding large M1 subunit of VV ribonucleotide reductase; (7) 14-kDa fusion protein gene.

glutinin (HA) (139, 140) and 14-kDa fusion protein genes, which both map to the large VV *Hin*dIII-A DNA fragment (132, 133). Insertion into the indicated positions within the VV *Hin*dIII M, N, or F fragment, while producing viable recombinants, has no other special attributes. In contrast, insertions into the VV tk or HA gene are convenient because they confer either a selectable or a detectable phenotype on the recombinants. In the case of the tk gene, recombinant viruses become tk^- by virtue of insertional inactivation and can be selected by growth in the presence of 5′-bromodeoxyuridine. Likewise, insertional inactivation of the HA gene renders the recombinant viruses HA^- so that plaques arising from the HA^- recombinants can be recognized by virtue of their inability to bind erythrocytes.

Of particular relevance to using VV as a vaccine is the observation that insertional inactivation of the VV tk, VV growth factor, M1 subunit of the ribonucleotide reductase, or 14-kDa fusion protein gene apparently attenuates the in vivo replication of these recombinant viruses without harming their ability to replicate in cell culture (16, 17, 24a, 132). One important point to note is that, considering the size and complexity of the VV genome (≥200 genes), only a relatively few insertion sites have been utilized thus far. Although not yet recognized, some context-specific effects may be encountered that may affect the expression of the inserted foreign gene. Therefore, many laboratories are assessing the use of additional insertion sites within the VV genome.

Detection and Selection of Recombinants

As noted above, under the best conditions, only a small percentage (0.01 to 1.0%) of the progeny virions isolated from a typical marker transfer experiment can be expected to contain the inserted foreign gene. Furthermore, when the recombinational insertion of large, multiple, or semitoxic sequences (e.g., protease genes) is attempted, this frequency can be substantially lower. To circumvent the experimental equivalent of "looking for a needle in the haystack," several approaches (described below) have been used to facilitate the isolation, detection, and/or selection of the desired recombinant VV.

Several laboratories have taken advantage of available temperature-sensitive VV mutant collections, and information concerning their map locations, to develop procedures to amplify the apparent recombination frequency (43, 81). In this approach, recombination plasmids are cotransfected into cells together with a cloned VV DNA fragment. The assumption is that any cell which is competent to take up the cloned genomic VV DNA fragment will also take up the recombination plasmid. The cotransfected cells are then superinfected with a temperature-sensitive VV mutant whose lesion resides within the confines of the cloned fragment. The culture is then grown at the nonpermissive temperature of 40°C. The parental virus cannot grow under these conditions, whereas recombinants in which the cloned gene has rescued the temperature-sensitive lesion (and which hopefully also have recombined the foreign insert into the correct targeted location) can. In practice, screening the progeny virus from the 40°C passage reveals that a high percentage (10 to 25%) also contain and express the second marker.

In theory, 5′-bromodeoxyuridine selection can be used to permit the selective growth of insertionally inactivated VV tk^- recombinants (95). While this procedure is useful as an amplification scheme, a considerable background of spontaneous VV tk^- mutants complicates the situation, requiring

plaque hybridization as a necessary final purification step. Using the coexpression recombination plasmids, selection can be achieved with reporter genes (such as luciferase [134] or β-galactosidase [19]) whose expression is easily detectable. In the latter case, the plaques arising from double recombinants expressing β-galactosidase can be easily identified among the background of wild-type plaques because they stain blue when the chromogenic substrate X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) is included in the agar overlay. This detection scheme can also be used in the opposite manner by recombining foreign genes into a β-galactosidase gene present in the genome of the superinfecting virus. In this case, the recombinants of interest are those that do not stain blue, whereas the plaques arising from the input virus will appear blue.

The coexpression system can also be used together with a reporter gene to encode a biochemically selectable activity. The two systems that have proved most useful in this regard are neomycin phosphotransferase-G418 (55, 98) and guanosine phosphoribosyltransferase-mycophenolic acid (42). These selection schemes are particularly powerful in that only recombinant viruses grow and there is little or no possibility that the parental virus can spontaneously revert to drug resistance. With these selection schemes, if the initial marker transfer is carried out in the presence of drug, followed by one or two additional passages to rid the population of any wild-type virus replicating in the same cells as a recombinant virion, then virtually the entire progeny population will correspond to the desired recombinants. Individual plaques can then be picked and analyzed without any need for plaque hybridization techniques. The availability of such powerful selection schemes is especially important when maximum efficiency is needed, e.g., in attempting to produce large numbers of different recombinants or when inserting cDNA libraries into VV. In the future, as additional selection schemes are developed for other procaryotic and eucaryotic vectors, they will be adapted for use in concert with VV insertion plasmids.

APPLICATIONS OF VV VECTORS

Prophylactic Vaccines

The most common use of and interest in VV as a vector is as a potential recombinant vaccine. The premise is to insert a gene encoding an antigen from a heterologous pathogen into VV and then use the recombinant virus as a live infectious vaccine with which to induce antigen-specific immune responses in recipient animals (51, 60, 78, 96). In this way, immunity to the pathogenic agent is achieved without the risk of exposure to the pathogen. This approach has been used to construct candidate vaccines directed against viral (e.g., hepatitis) (111), protozoal (e.g., malaria) (89), bacterial (e.g., streptococcal pharyngitis and rheumatic fever) (46), and even oncogenic (175) diseases of both humans and domestic animals. Most of the VV recombinants used to immunize animals are highly effective at inducing both humoral and cellular immunity. As described, live VV recombinants have a number of pronounced advantages over synthetic peptide antigens and traditional killed or attenuated vaccine (91). At the time this review was prepared, however, neither the U.S. Department of Agriculture nor the Federal Drug Administration has allowed VV-based vaccines to be field tested in any extensive manner because of concerns about safety issues. This delay, however, is not likely to be permanent; rather, as experimentalists deal with the safety issues in a careful and considered manner, recombinant vaccine use should become accepted, especially in the field of veterinary medicine in which a related poxvirus, fowlpox, is already used as a vaccine (160). Table 1 is a partial list of the foreign antigens that have been expressed with VV vectors. This list changes almost daily as the basic VV vector technology becomes available in an ever increasing number of laboratories. The basic rule of thumb at present is that, if a contiguous DNA sequence encoding a protein of biological interest exists, someone in the academic or private sector is likely to have put it into a VV vector.

As an example of how VV vectors can provide new approaches to old disease problems, consider some recent results obtained by expressing the M protein from the bacterial pathogen *Streptococcus pyogenes*, using a VV recombinant. Group A streptococci are important human pathogens that are responsible for a variety of serious diseases, including acute pharyngitis, rheumatic fever, and glomerulonephritis (64, 88). It has long been recognized that the primary virulence factor of these organisms is the serotypic determinant M protein which is found on the surface of the bacterium and apparently enables it to avoid phagocytosis (45). Although type-specific antibodies directed against the M protein are known to be protective, attempts to develop an effective antistreptococcal vaccine against these pathogens have been hindered by the fact that there are more than 80 different serotypes of *S. pyogenes* (88). As a further complication, under normal conditions it appears that the immunodominant epitopes responsible for eliciting protective immunity are located in the carboxyl half of the M protein, which contains several hypervariable domains (65). As an alternative approach to the vaccine problem, a VV recombinant was constructed which contained and expressed the entire M-protein gene from serotype 6 *S. pyogenes*. This VV recombinant expressed high levels of immunoreactive M protein in recombinant-infected cell cultures. Surprisingly, the bacterial protein was quite stable and not subject to any apparent aberrant posttranslational processing events (46). Although this recombinant proved immunogenic in animals, it was not suitable for protection experiments. Because of the inclusion of the bacterial sequences encoding the immunodominant epitopes, the induced immunity was predicted to be directed against only the M6 serotype. Therefore, as a step towards developing a broadly protective antistreptococcal vaccine, genetic engineering techniques were used to construct a second-generation VV–M-protein recombinant which expressed only the conserved carboxy-terminal region of the structural gene encoding the M6 molecule (VV:M6′). This portion of the molecule is not usually antigenic when expressed in the context of the bacterial cell. In animal trials, mice immunized intranasally with the VV:M6′ virus showed markedly reduced pharyngeal colonization by streptococci after intranasal challenge. Similarly, M-protein-specific serum immunoglobulin G was significantly elevated in vaccinated animals and absent in controls. Most important, cross-protection could be demonstrated in the immunized mice when they were challenged with a heterologous serotype, M12 (46). Thus, the use of VV–M-protein recombinants has allowed the identification of a previously unrecognized cross-protective conserved M-protein epitope which should be effective in inducing antistreptococcal immunity. A similar approach, using VV vectors, should allow the identification of protective determinants present on other bacterial and viral pathogens for which no vaccine currently exists.

TABLE 1. Summary of foreign proteins expressed by recombinant VV

Genes	Protein	Vector[a]	Reference(s)
Viral	Rabies virus G	RPV, VV	38, 136, 137, 168
	Rabies virus G	FPV	160
	Avian influenza virus hemagglutinin	FPV	161
	Influenza virus PB1, PB2, & PA	VV	7
	Influenza virus HA	VV	3, 21, 28, 148
	Influenza virus PB1, PB2, PA, HA, NP, M1, & NS1	VV	2, 6, 144
	Respiratory syncytial virus F & G	VV	5, 36, 114, 115, 153, 154, 178
	Respiratory syncytial virus N	VV	82
	Venezuelan equine encephalitis virus C, E2, & E1	VV	83, 84
	Sindbis virus C, E2, & E1	VV	130
	Tobacco etch virus capsid	VV	33
	Lassa fever virus G	VV	4, 47
	Human leukemia virus 1 *env*	VV	20, 139
	Human immunodeficiency virus gp160	VV	157
	Human immunodeficiency virus gp120	VV	180
	Human immunodeficiency virus RT	VV	48, 176
	Human immunodeficiency virus *tat*	VV	41
	Lymphadenopathy-associated virus gp41 & gp110	VV	185
	Friend Leukemia virus gp85	VV	35
	Friend Leukemia virus gp70	VV	59
	Vesicular stomatitis virus G & N	VV	184
	Vesicular stomatitis virus M	VV	92
	Polyomavirus LT, MT, & ST	VV	90
	Polyomavirus VP1 & VP2	VV	151
	SA 11 rotavirus VP7	VV	1
	Epstein-Barr virus gp340	VV	93
	Hepatitis B virus LS	VV	24
	Hepatitis B virus surface antigen	VV	119
	Hepatitis B virus MS	VV	23
	Paramyxovirus simian virus 5 F & HN	VV	121
	Herpes simplex virus 1 gB protein	VV	18, 101
	Herpes simplex virus 1 gD	VV	99, 100, 135
	Herpes simplex virus 1 gG protein	VV	156
	Dengue virus NS, NS_{2A}, C, M, & E	VV	32, 186
	Pseudorabies virus gp50	VV	97
	Lymphocytic choriomeningitis virus GP & NP	VV	179
	Rift Valley Fever virus M	VV	80
	Cytomegalovirus gp89	VV	79
	Infectious bronchitis virus spike	VV	169
	Parainfluenza virus type 3 F & HN	VV	149, 150
	Hantaan virus M	VV	123
	Measles virus HA & F	VV	34
	Adenovirus DNA polymerase, pTP	VV	155
	Papillomavirus type 16 L1	VV	15
	Bovine leukemia virus gp51	VV	113
Bacterial	Chloramphenicol acetyltransferase	CPV	120
	S. pyogenes M protein	VV	70
	β-Galactosidase	VV	19
	Neomycin phosphotransferase	VV	55
	Guanosine ribosylphosphotransferase	VV	42
Protozoan	*Plasmodium falcipirum* CS	VV	61, 62
	P. knowlesi sporozoite antigen	VV	143
	P. falcipirum S antigen	VV	89
Cellular	Preproenkephalin	VV	164
	Mouse preproopiomelanocortin	VV	165
	Atrial natriuretic peptic clearance receptor	VV	127
	neu oncogene	VV	9
	Human melanoma antigen p97	VV	39, 74
	CD4 antigen	VV	105
	H-2k^d	VV	29
	Factor VIII	VV	25, 122
	Interleukin-2	VV	49, 128
	Firefly luciferase	VV	134
Yeast	KX2 protease	VV	165
Bacteriophage	T7 RNA polymerase	VV	56, 58

[a] RPV, Raccoonpox virus; FPV, fowlpox virus; CPV, cowpox virus.

Cell Biology

Possibly one of the most rewarding and promising areas in which VV vectors have been used is in the field of cell biology. VV recombinants have been used to express neuropeptides, growth factors, and receptor proteins (164). The advantages of this approach are (i) appreciable (microgram) quantities of these proteins, which usually are found in only low concentrations, are expressed in a biologically active form; (ii) immunological reagents can be prepared with which to follow the synthesis and modification of the proteins within the cell; (iii) the expression of these proteins in heterologous cell types and in cells from different species is facilitated; and (iv) perhaps most important, directed genetics can be used to dissect structure-function relationships of cellular proteins rapidly.

Perhaps the most well-studied example of using VV vectors to investigate cellular processes is in the field of neurobiology, in which the technique was used as a novel approach to study neuropeptide maturation (73, 164–166). Biologically active opioid peptide hormones are produced in a tissue-specific manner by differential proteolysis of larger protein precursors. Little is known about how these reactions are regulated, what enzymes are responsible for the cleavage reactions, or what features of the precursor molecules are essential for correct processing to occur. To address these questions, full-length cDNA inserts encoding human preproenkephalin (hPE) and mouse preproopiomelanocortin (mPOMC) precursors have been recombined into the VV genome. When used to infect nonsecretory cells such as BSC-40 monkey kidney lines, the VV:hPE and VV:mPOMC recombinants express uncleaved precursors, whereas infection of a secretory-competent cell line such as mouse AtT-20 cells results in the expression of fully processed peptides. This system now provides the ability to generate a series of site-specific VV:hPE and VV:mPOMC recombinants in which individual amino acid residues of the precursor molecules have been altered. The effects of these alterations on the posttranslational modification, proteolytic processing, and intracellular trafficking of neuropeptide precursors can then be assessed.

Recently, the gene encoding the yeast α-mating-factor protease (KEX2) was recombined in a fully functional form into VV. Surprisingly, it was possible to demonstrate that coinfection of BSC-40 cells with VV:POMC and VV:KEX2 results in the complete restoration of the authentic neuropeptide processing pathway. Having demonstrated that this reconstitution or *trans*-complementation approach will work with the yeast enzyme, it will now be of interest to extend this approach to identify the cognate cellular proteases that are usually uninvolved in catalyzing these reactions. Similar experiments are under way in a number of laboratories to use VV to express and study a variety of interesting and important biologically active mammalian proteins, including, most notably, transcriptional *trans*-acting activators and repressors.

Immunology

Because of its infectious nature and broad host range, VV has provided a new dimension to study the immunological interactions between a pathogen and the target host organism. Pathogens such as viruses are not a single molecular entity; rather, they are complex structures that present an antigenic mosaic to the host immune system. Thus, it is often difficult (if not impossible) to determine which of the antigens are relevant to the induction of immunity and, hence, are candidates for vaccine development and which are inconsequential. This problem can be greatly simplified by using VV vectors to present a single antigen or epitope to the immune system to dissect out what role each protein may or may not play in immunity (8, 22, 85, 86, 106, 173). For example, in the case of acquired immunodeficiency syndrome caused by type 1 human immunodeficiency virus, recombinant VV strains have been constructed which individually express each of the individual type 1 human immunodeficiency virus proteins, gp160, gp120, *tat*, and reverse transcriptase (Table 1). These recombinants are being used to determine whether the viral proteins are immunogenic and, if so, whether the antibodies induced can neutralize the infectious virus. Likewise, by using these recombinant viruses to infect whole animals, the relative contributions and interactions of the humoral and cellular immune systems can be examined since VV is effective at priming both T- and B-cell-mediated responses. VV vectors together with this "dissection" approach to immunity are also being used to study a number of different virus-cell interactions, including those of type 1 herpes simplex virus (99) and lymphocytic choriomeningitis virus (97).

Basic Research

VV vectors can be used to express and manipulate any DNA sequences of interest, whether they encode antigens, enzymes, or structural proteins. Since the cloned sequences within the recombination vectors are suitable substrates for site-directed mutagenesis procedures, it is a relatively straightforward procedure to prepare a collection of VV recombinants expressing different genetically engineered forms of the same protein to address structure-function relationships. In this regard, it is of interest to note that there are two approaches to using VV vectors (Fig. 5). First, a recombinant virus can be constructed in the manner discussed here and used to infect cells and express the foreign insert. This method has the advantage of ensuring that the foreign insert is efficiently expressed in every cell in a synchronous fashion. It has the disadvantage of requiring the construction and isolation of a different recombinant virus for each gene or gene derivative to be tested. An alternative approach is to use transient expression procedures (25, 138). Foreign genes abutted to a VV promoter are transfected into cells. The cells are then superinfected with VV, which supplies the necessary transcription factors and RNA polymerase required to express the plasmid-borne gene. Sufficient levels of the foreign gene product are expressed to be defected by either functional or immunological assays. Furthermore, the level of expression in the transient assays can be substantially boosted by incorporating a heterologous promoter and polymerase from bacteriophage T7 into the procedure (37). The transient expression approach provides the ability to test quickly a large number of different constructions. It has the limitation that only a small percentage of the cells in a given population, i.e., those taking up the transfected plasmid DNA, will be expressing the gene of interest. Which of these approaches is chosen depends on the nature of the experiment being undertaken.

CONCLUDING REMARKS

VV is a remarkably powerful experimental vector system. One can easily insert and express any foreign DNA from within the context of the viral genome and either use the

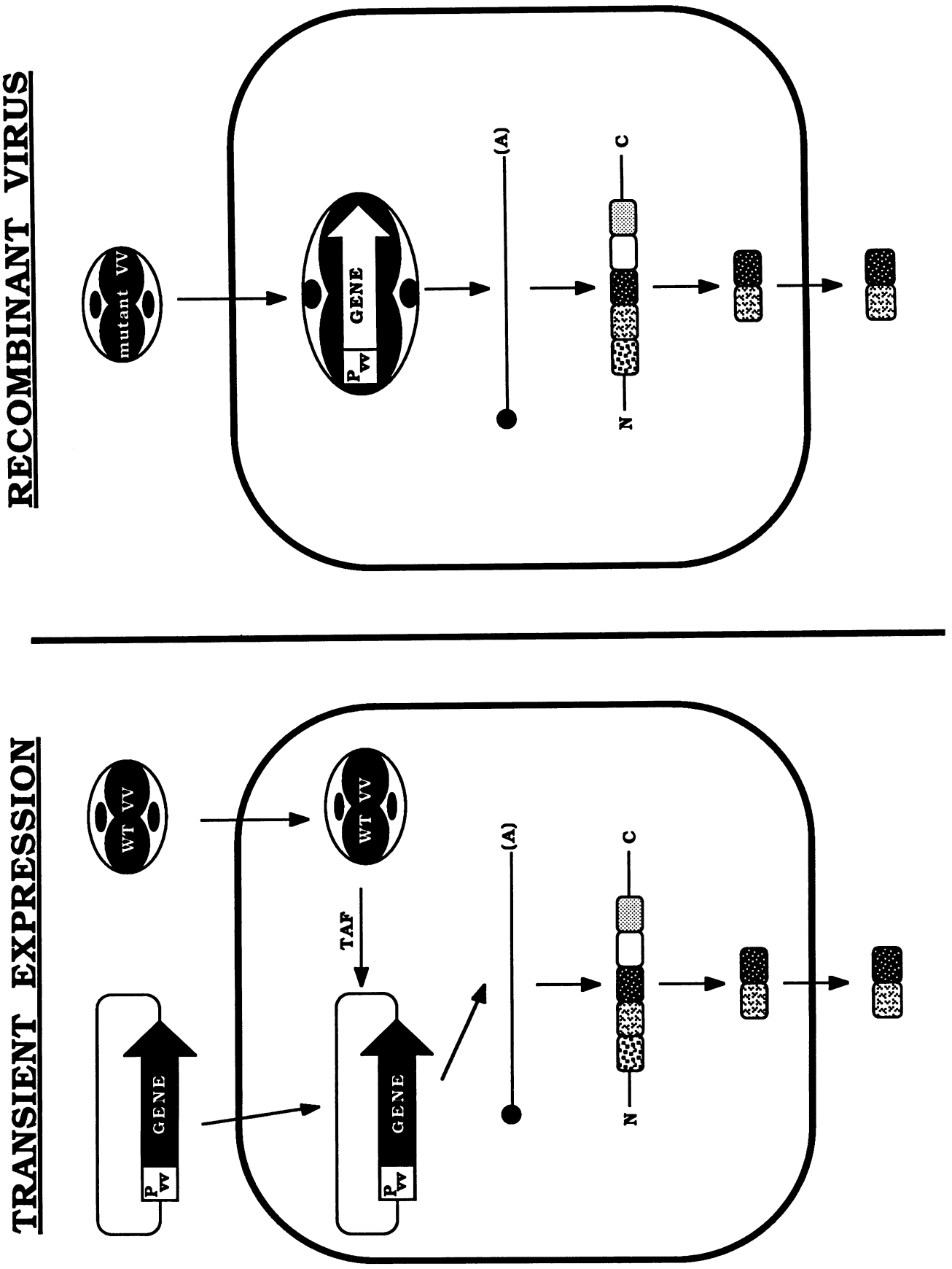


FIG. 5. Comparison of using transient expression and recombinant virus approaches with VV vectors. WT, Wild type.

derived recombinants in the basic research laboratory to conduct structure-function studies of the encoded protein or use the recombinant virus as a live vaccine. While the system's use is limited (e.g., it cannot be used to catalyze gene replacement) and some safety issues are yet to be resolved, this vector offers the most rapid approach for producing appreciable amounts of biologically active protein in mammalian cell culture systems, determining whether an antigen can induce a protective response in animals, assessing what the biological function of a protein is, and identifying which structural determinants contribute to its function. Whether or not we eventually use these vectors in their present form as vaccines in the clinical setting remains to be debated and experimentally tested. However, it is certain that the information generated by using these vectors in the laboratory will prove invaluable when formulating practical solutions to disease problems. Finally, it is likely that the potential of VV vectors is just beginning to be tapped. As additional information concerning the structure, function, and regulation of poxvirus genetic information becomes known, it is likely that new generations of VV vector systems will be developed. Therefore, rather than considering this contribution as a review, perhaps it would be more appropriate to view it as a progress report.

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