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# Recombinant Vaccinia Viruses as New Live Vaccines

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## Introduction

Recombinant DNA technology has enabled the identification and cloning of genes that code for specific proteins. Much research has focused on genes that code for important surface antigens of infectious agents. By genetic engineering these genes may be expressed in a variety of vector systems that have been designed to synthesize large amounts of a particular protein. Expression vectors from bacteria and yeast (Beggs, 1981; Hinnen and Meyhack, 1982; Maniatis, Fritsch and Sambrook, 1982) and viruses of several different types (Brammar, 1982; Rigby, 1983) have been described. Antigens produced in these expression vector systems can be purified and in some cases used directly as subunit vaccines (McAleer *et al.*, 1984; *see also* Chapter 1 of this volume). However, the necessary purification of subunit vaccines makes them relatively expensive and usually these vaccines need to be administered more than once to elicit an effective immune response.

As an alternative approach to vaccine development, genetic engineering can also be used to construct live recombinant viruses that express foreign antigens and that have potential as live vaccines. Live vaccines are generally cheaper to produce, easier to administer, evoke both humoral antibody and cell-mediated immune responses and give longer-lasting immunity against infectious diseases. The effectiveness of live vaccines is attested to by the successful use of vaccinia virus to eradicate smallpox (WHO, 1980). Undesirable side-effects of live vaccines may also be minimized or eliminated by utilization of recombinant DNA techniques to attenuate the virus in a suitable manner. Several types of animal DNA viruses with large genomes have been used as cloning vectors, including adenovirus (Solnick, 1981; Thummel, Tjian and Grodzicker, 1981,

Abbreviations: CAT, chloramphenicol acetyltransferase; HA, haemagglutinin; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; HSV, herpes simplex virus; TK, thymidine kinase; VSV, vesicular stomatitis virus.

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1982), herpes simplex virus (Post and Roizman, 1981; Spaete and Frenkel, 1982) and vaccinia virus (Mackett, Smith and Moss, 1982; Panicali and Paoletti, 1982). Only vaccinia virus recombinants have expressed foreign genes while retaining complete infectivity and, therefore, these viruses have greatest potential as live vaccines. The enormous experience already gained with the use of vaccinia virus as a live vaccine, its wide host range, large capacity for foreign DNA (Smith and Moss, 1983) and inability to induce oncogenic transformation, are all features enhancing the potential of vaccinia virus recombinants as live vaccines. Here the methods of constructing recombinant vaccinia viruses and their potential use as live vaccines will be discussed.

## Use of vaccinia virus as a smallpox vaccine

Vaccinia virus is most widely known for its role as the live vaccine used for the immunoprophylaxis of smallpox. This practice originated from the remarkable observation of Edward Jenner in 1796 that milkmaids who frequently had contact with cows infected with cowpox were somehow protected from smallpox (Jenner, 1798). Jenner then vaccinated other people with lymph taken from the pustules of patients infected with cowpox and showed that those individuals became immune to direct challenge with infectious smallpox material. We now understand that such immunization is a consequence of the close genetic and serological relationships of cowpox virus and variola virus, the causative agent of smallpox. The word vaccination derives from the latin word *vacca* for cow.

Jenner's introduction of vaccination as a means of preventing smallpox replaced the existing practice of 'variolation' or 'inoculation'. This involved the transfer of smallpox virus taken directly from the pustules of infected patients to other individuals. Those individuals developed a smallpox infection that gave a considerably lower mortality rate than that resulting from a natural infection. This procedure had been widely used in Asia but was not introduced into the West until 1721. Lady Wortley Montagu, who became convinced of the effectiveness of variolation while in Constantinople, Turkey, directed the variolation of her child and was subsequently influential in the expansion of the practice in England. Variolation prevented subsequent natural smallpox infection and the associated high mortality rate but was not without considerable danger. Numerous deaths and small epidemics of smallpox were started by this practice. Despite these dangers, variolation was widely used in Europe and North America during the eighteenth century and provided the best available means of protecting against smallpox until Jenner's discovery.

Vaccination rapidly replaced variolation during the early nineteenth century and was effective in reducing the incidence of smallpox throughout Europe and North America. However, the widespread use of the vaccine in less accessible regions of the world was hampered by the lack of an easily transportable, stable vaccine. Because arm-to-arm transfer was the only effective way known, the Spanish, in attempting to transfer the vaccine to Mexico, resorted to transporting shiploads of orphan children and vaccinating groups of them at regular intervals *en route*. Despite the effectiveness of arm-to-arm transfer this

method had the disadvantages that the availability of the vaccine was sometimes limited and that other human pathogens including measles and syphilis were also spread. The development of calf lymph vaccine in 1860 produced a safer vaccine that was also available in much greater quantity. Even with this advance it was not until 1950 that a freeze-dried vaccine stable without refrigeration became available (Collier, 1954, 1955). Mass vaccinations in tropical countries where smallpox was endemic were then possible.

In 1959 the World Health Organization adopted a resolution put forward by the Soviet Union to bring about the global eradication of smallpox. Subsequent mass-vaccination campaigns, extensive surveillance programmes and containment of fresh outbreaks resulted in smallpox disappearing from the West by 1971 and from Asia by 1975. The last naturally occurring case of smallpox was recorded in Somalia, East Africa in October 1977.

Several properties of vaccinia virus were of importance for the success of the campaign to eradicate smallpox. First, it was possible easily and cheaply to manufacture millions of doses of freeze-dried vaccine that could be transported in tropical countries without refrigeration or loss of potency. The potency of the live vaccine was sufficient for a single vaccination to elicit long-lasting immunity against smallpox. In addition, the vaccine could be easily administered on a mass scale by medically unskilled personnel under non-sterile conditions using a simple bifurcated needle or jet gun. These features, together with the vigour with which the World Health Organization conducted the vaccination, surveillance and containment campaigns, all contributed to the successful eradication of smallpox. Most of these features of vaccinia seem likely to be retained by recombinant vaccinia viruses expressing antigens from unrelated pathogens.

Despite the widespread use of vaccinia virus as a live vaccine, the origins of the virus remain obscure. The possibilities of vaccinia virus being a derivative of either cowpox virus or variola virus or a poxvirus of independent origin have not been resolved even by comparative restriction endonuclease mapping of genomic DNAs (Mackett and Archard, 1979). Whatever its origin, vaccinia virus possesses sufficient serological relatedness to variola virus to make it an effective vaccine against smallpox.

## Biology of vaccinia virus

Detailed reviews of poxvirus biology and replication contain extensive background information (Moss, 1974, 1978, 1984; Dales and Pogo, 1981). Here the biology of vaccinia virus will be described with emphasis on some of the unique biological features relevant to the use of vaccinia virus as a cloning vector.

### VIRUS STRUCTURE

Vaccinia virus is the prototype for the orthopoxvirus genera of the poxvirus family. Distinguishing features of this family include the large size and complex morphology of the virus particle and the cytoplasmic site of transcription and

replication. Poxviruses, of which vaccinia virus is by far the most thoroughly characterized member, are the largest of all animal viruses. Vaccinia virus particles purified from the cytoplasm of infected cells are brick-shaped or oval with dimensions of 200 nm by 300 nm and have a lipoprotein envelope surrounding the virus core. The virus core is biconcave with lateral bodies of unknown function located in the concavities. Within the core, the virus double-stranded DNA genome is associated with several proteins to form a coiled nucleoprotein fibre. The complexity of vaccinia virus is illustrated by the presence of more than 100 polypeptides that can be resolved by two-dimensional polyacrylamide gel electrophoresis of solubilized virus cores (Essani and Dales, 1979; Oie and Ichihashi, 1981). Many enzymes are also located within the virus particle. These include a complete transcriptional system able to synthesize, cap, methylate and polyadenylate functional messenger RNA (mRNA) (Kates and McAuslan, 1967; Munyon, Paoletti and Grace, 1967; Kates and Beeson, 1970; Wei and Moss, 1975; Cooper and Moss, 1978). The RNA polymerase (Nevins and Joklik, 1977; Baroudy and Moss, 1980; Spencer, Shuman and Hurwitz, 1980) and many of the other enzymes have been purified from infected cells or virus particles. The capping and methylating enzymes have been widely used as tools in molecular biology (Moss, 1981).

## VIRUS GENOME

The vaccinia genome is a large double-stranded DNA molecule of 187 000 base pairs (Geshelin and Berns, 1974). Isolated virus DNA is not infectious, consistent with the presence of virus-specific enzymes within the virus particle that are necessary for transcription of the genomic DNA. At both termini the two DNA strands are covalently linked by a partially base-paired hairpin loop into a single polynucleotide chain (Geshelin and Berns, 1974; Baroudy, Venkatesan and Moss, 1982). This hairpin loop is extremely A:T rich and possesses a 'flip-flop' orientation also found at the ends of parvovirus genomes (Straus, Sebring and Rose, 1976; Tattersall and Ward, 1976). Mechanisms of DNA replication based upon this structure have been proposed. The genome also possesses large inverted terminal repetitions of 10 000 base pairs that contain multiple copies of 54, 70 and 125 base-pair repeats (Baroudy and Moss, 1982). Comparison of the genomes of several orthopoxviruses by restriction endonuclease mapping (Esposito, Obijeski and Nakano, 1978; Mackett and Archard, 1979) have indicated extensive homologies within the central region of the genome with most variation located near or within the long inverted terminal repeats.

The vaccinia genome is large enough to code for up to 200 polypeptides, but to date only two proteins of known function have been accurately mapped: these are the thymidine kinase (TK) (EC 2.7.1.21) (Hruby and Ball, 1982; Weir, Bajzsar and Moss, 1982; Weir and Moss, 1983) and DNA polymerase (Jones and Moss, 1984; Traktman *et al.*, 1984). Several regions of the genome have been shown to be non-essential for virus replication in tissue culture. Deletions of DNA in these regions (Moss, Winters and Cooper, 1981a; Panicali

*et al.*, 1981), or interruptions of contiguous DNA sequences by insertion of additional DNA (Mackett, Smith and Moss, 1982; Panicali and Paoletti, 1982) do not prevent virus replication in tissue culture. However, it is possible that these alterations may change the pathogenicity of the virus in man or animals. There is considerable flexibility in the size of DNA genome that can be packaged into vaccinia virus and other poxviruses (Archard and Mackett, 1979; Moyer and Rothe, 1980). Vaccinia virus mutants with deletions of 9 kilobases (kb) (Moss, Winters and Cooper, 1981b; Panicali *et al.*, 1981) or recombinants with an additional 25 kb of foreign DNA (Smith and Moss, 1983) both replicate to high titre in tissue cells and are stable on serial passage. This size flexibility contrasts with genomes of viruses that possess icosahedral symmetry, which have more rigid genomic packaging constraints.

## VIRUS REPLICATION

The most notable feature of poxvirus replication is that it is cytoplasmic. The virus also has a broad host range both *in vivo* and *in vitro*. The ability of a large eukaryotic DNA virus to replicate in the cytoplasm is unusual, and is accounted for by the possession of many virus-coded enzymes involved in nucleic acid metabolism. As mentioned above, the virus packages a complete transcriptional enzyme system within the virus particle. This enables functional virus mRNAs to be synthesized *in vivo* within minutes of infection in the absence of host macromolecular synthesis. None of the virus mRNAs analysed so far are spliced and nucleotide sequencing of several early genes (Venkatesan, Baroudy and Moss, 1981; Venkatesan, Gershowitz and Moss, 1982; Weir and Moss, 1983) and one late gene (Weir and Moss, 1984) indicate the absence of introns. This is not surprising for a virus transcribing and replicating in the cytoplasm, but the possibility that some virus mRNAs are spliced cannot be ruled out at present.

The replication cycle of vaccinia is outlined in *Figure 1* and begins with the penetration of the virus particle into the host cell by either membrane fusion or phagocytosis. There follows a partial uncoating that enables the synthesis of early virus mRNAs. The early phase of virus replication is defined as that occurring prior to DNA replication: most early virus transcription occurs immediately following infection and does not require protein synthesis. Early virus gene products, including the DNA polymerase and presumably other proteins, release the virus DNA from the core into a form that may be replicated. DNA replication occurs 2–5 hours after infection, with a peak at about 3 hours.

After the onset of DNA replication there is a marked change in the pattern of virus transcription. An unidentified transcriptional control mechanism turns off the expression of early genes and turns on that of late virus genes (Belle Isle, Venkatesan and Moss, 1981). Late virus mRNAs are extremely heterogeneous in length and hybridize to at least 90% of the virus genome (Oda and Joklik, 1967; Paoletti and Grady, 1977; Boone and Moss, 1978). This complexity and heterogeneity result from the lack of discrete transcriptional termination. Late mRNAs do, however, have discrete 5′ initiation sites (Cooper, Wittek and

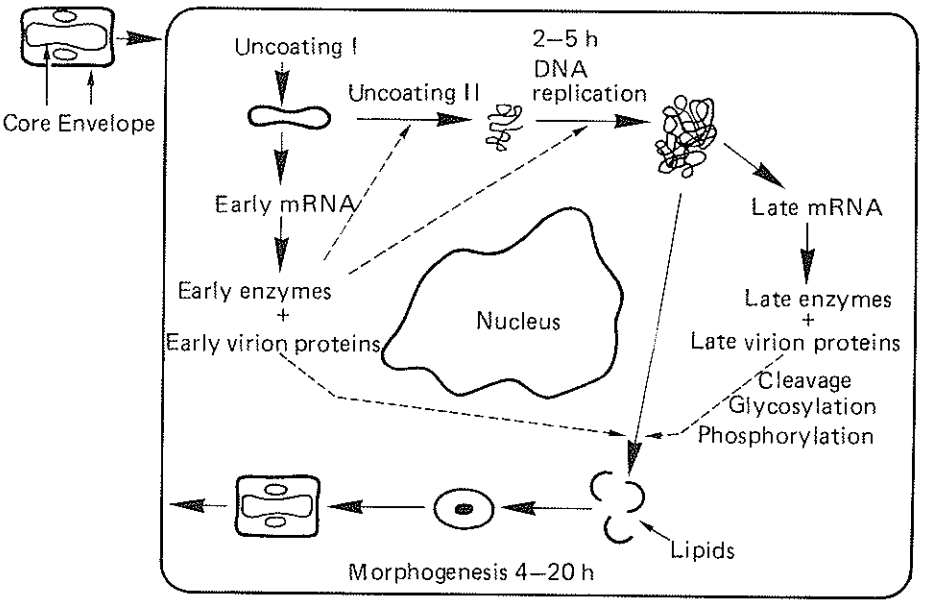


**Figure 1.** Scheme of vaccinia virus replication. Redrawn from Moss, B. (1984). Principles of virus replication: poxviruses. In *Human Viral Diseases* (Eds Fields, B.N., Melnick, J.L., Chanock, R., Shope, R.E. and Roizman, B.). Courtesy of Raven Press, Publishers, New York.

Moss, 1981; Weir and Moss, 1984). Late mRNAs code mostly for structural polypeptides and some enzymes involved in virus maturation. Virus polypeptides undergo several types of modification including proteolytic processing, glycosylation and phosphorylation (Sarov and Joklik, 1972; Moss and Rosenblum, 1973; Payne, 1978). Electron microscopy has revealed several distinct stages of virus maturation as illustrated in *Figure 1*. These include the formation of lipid crescents complexed with virus proteins, the association of nucleoprotein with these crescents and the formation of morphologically distinct structures such as the biconcave core and lateral bodies (Dales and Pogo, 1981).

Infectious virus progeny appear by 5 hours and maximum yields are produced by 15–20 hours. The majority of vaccinia virus remains cell-associated but a small fraction is released and acquires an additional lipid envelope (Payne, 1978, 1979). The presence in this envelope of additional virus glycoproteins that are absent from intracellular virus, explains the observation that antibodies raised against inactivated intracellular virus fail to neutralize the infectivity of extracellular virus either *in vitro* or in animals (Appleyard, Hapel and Boulter, 1971). The extracellular virus may be of importance for the mechanisms of virus spread and pathogenicity in natural infections (Boulter and Appleyard, 1973; Payne, 1980).

## VIRUS TRANSCRIPTIONAL CONTROL

The mechanisms controlling the temporal expression of vaccinia genes are under intensive investigation. Nucleotide sequence data are currently available

for several early vaccinia genes (Venkatesan, Baroudy and Moss, 1981; Venkatesan, Gershowitz and Moss, 1982; Weir and Moss, 1983) and one late vaccinia gene (Weir and Moss, 1984). These data show that the transcriptional control regions (promoters) of vaccinia genes are very A:T-rich for 60 base pairs upstream from the transcriptional initiation site and do not share any extensive homology with either prokaryotic or eukaryotic transcriptional consensus sequences (Nevins, 1983).

An *in vitro* transcription system specific for vaccinia genes has been developed recently (Puckett and Moss, 1983). Using this system it has been shown that vaccinia RNA polymerase will not recognize promoters normally transcribed by eukaryotic RNA polymerase II, and vice versa. This transcription system, a transient expression system for vaccinia genes (M.A. Cochran, M. Mackett and B. Moss, unpublished work) and the ability to reintroduce mutated vaccinia virus promoters back into infectious virus have demonstrated the presence of *cis*-acting regulatory sequences upstream of early and late vaccinia genes.

Specific nucleotide sequences presumably are also involved in defining the transcriptional termination sites of early vaccinia genes. Nucleotide sequences around these termination sites do not possess the consensus sequence TTATTT found about 20 base pairs upstream from the termination sites recognized by eukaryotic RNA polymerase II (Nevins, 1983). Why transcription of late vaccinia genes does not terminate specifically is not clear. Either the nucleotide sequences signalling termination of early genes are absent from late genes or these sequences may no longer be recognized because of modification of the vaccinia RNA polymerase. The latter seems more likely as late mRNAs run through the termination sites of early genes (Bajzsar *et al.*, 1983; Weir and Moss, 1984).

## Construction of recombinant viruses

Three properties of vaccinia virus are important when considering the construction of recombinant viruses. First, the double-stranded DNA genome is very large so that *in vitro* manipulation of the whole genomic DNA is extremely difficult. Second, isolated virus DNA is not infectious so that recombinant virus would not be formed following introduction of genomic DNA into cells. Third, vaccinia virus possesses an RNA polymerase that recognizes its own transcriptional control signals but which will not recognize eukaryotic RNA polymerase II promoters. The strategy for construction of recombinant viruses had therefore to overcome the problems of how to insert foreign DNA into infectious virus, how to ensure that the DNA would be expressed and how to select the recombinant virus.

### STRATEGY

In this laboratory the following strategy was adopted (Mackett, Smith and Moss, 1982, 1984a,b; Moss, Smith and Mackett, 1983) (*Figure 2*). We reasoned that, because of the unique nature of the vaccinia transcriptional regulatory sequences and the existence of a virus-coded RNA polymerase, efficient

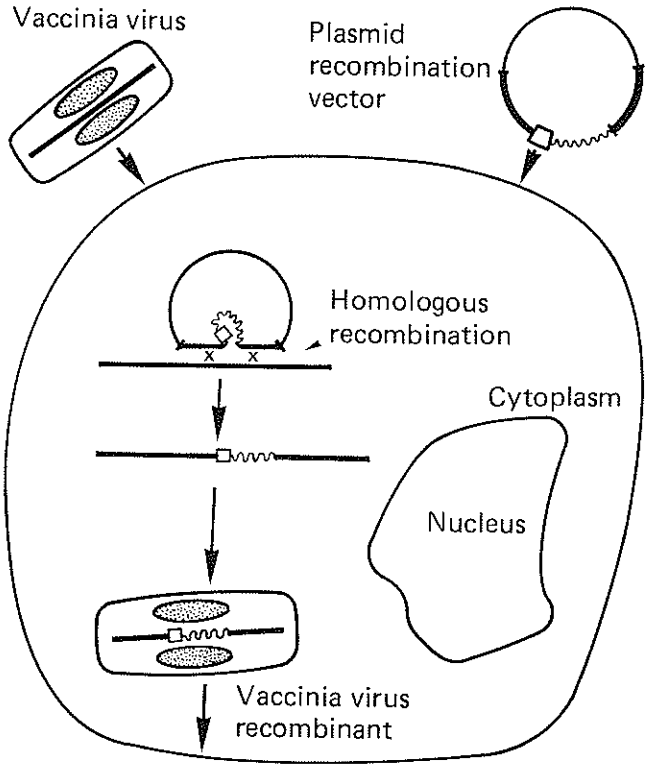


**Figure 2.** Formation of vaccinia virus recombinants. Cells are infected with vaccinia virus and transfected with a plasmid recombination vector. These plasmids contain a foreign protein-coding sequence (∿∿∿) fused to a correctly orientated vaccinia promoter (–□–) and flanked by non-essential vaccinia virus DNA (heavy line). Within these infected cells homologous recombination between the virus genome and plasmid DNA results in insertion of the foreign gene into the vaccinia virus genome. The recombinant genome is replicated and packaged into infectious progeny virus. Total progeny virus from transfected cells is screened for virus recombinants as described in the text.

expression of foreign genes in vaccinia virus might be obtained only if the foreign protein-coding sequences were positioned adjacent to a vaccinia promoter. Consequently, a chimeric gene consisting of a defined vaccinia virus promoter and the protein-coding sequences of the foreign gene is assembled in a plasmid vector. To overcome the difficulty in manipulating whole vaccinia virus DNA, cloned restriction fragments are used. The chimeric gene is flanked by a cloned vaccinia virus DNA fragment, taken from a non-essential region of the virus genome, and then inserted into infectious virus by homologous recombination *in vivo*. This method is based on two observations: first, specific deletions or transpositions of herpes simplex virus (HSV) DNA can occur in cells infected with HSV and transfected with plasmids containing HSV DNA sequences (Post and Roizman, 1981); second, marker rescue can occur in poxvirus infected cells that are transfected with either subgenomic virus DNA

or plasmids containing virus DNA (Sam and Dumbell, 1981; Nakano, Panicali and Paoletti, 1982; Weir, Bajzsar and Moss, 1982). By flanking the foreign gene with vaccinia DNA and introducing this by transfection into cells infected with vaccinia virus, homologous recombination occurs between the vaccinia DNA sequences of the plasmid and virus genome. This results in site-specific insertion of the foreign gene into the vaccinia genome. The flanking vaccinia virus DNA sequences are taken from a non-essential region of the virus genome so that insertion of foreign DNA at this site does not destroy virus infectivity. Within the infected cells, recombinant virus genomes are replicated and packaged into infectious virus. This procedure results in the production of recombinant virus at a frequency usually less than 0·5% of total progeny. Consequently, selective procedures are used to distinguish recombinant from wild-type virus. Either genetic, immunological or DNA:DNA hybridization techniques are used for this purpose (*see below*).

### PLASMID INSERTION VECTORS

To facilitate the routine insertion of foreign genes into vaccinia virus, a series of specially designed plasmids called insertion vectors were constructed (*Figure 3*) (Mackett, Smith and Moss, 1984a). These vectors contain a defined vaccinia promoter that includes the transcriptional but not translational initiation site. Just downstream from the RNA start site of the vaccinia promoter, multiple unique restriction endonuclease sites are positioned for insertion of foreign protein-coding sequences. Importantly, the DNA between the transcriptional start site and the downstream restriction sites does not contain any translational initiation site. This is provided by the foreign gene, ensuring that fusion polypeptides with unpredictable properties are not produced and problems with improper reading frames are avoided. The foreign gene also provides its own translation termination site so that authentic polypeptides should be produced. Foreign DNA has been inserted into several sites on the vaccinia virus genome that are non-essential for virus replication in tissue culture (Mackett, Smith and Moss, 1982; Panicali and Paoletti, 1982). The site of insertion is determined by the flanking vaccinia virus DNA of the plasmid insertion vector. Commonly, the vaccinia *TK* gene is used because recombinant viruses are then selectable as $TK^-$ mutants (*see below*). Several insertion vectors that are generally applicable for the expression of any continuous protein-coding sequences in vaccinia have been constructed (*Figure 3*). Using these vectors, only one cloning step is usually required to produce a plasmid that can be used directly to insert genes into vaccinia.

### TRANSFECTION

Once a chimeric gene flanked by non-essential vaccinia DNA has been assembled within a plasmid vector it can be inserted into the virus genome by homologous recombination *in vivo*. Such plasmids, called recombination vectors, are introduced into vaccinia virus-infected cells by transfection.

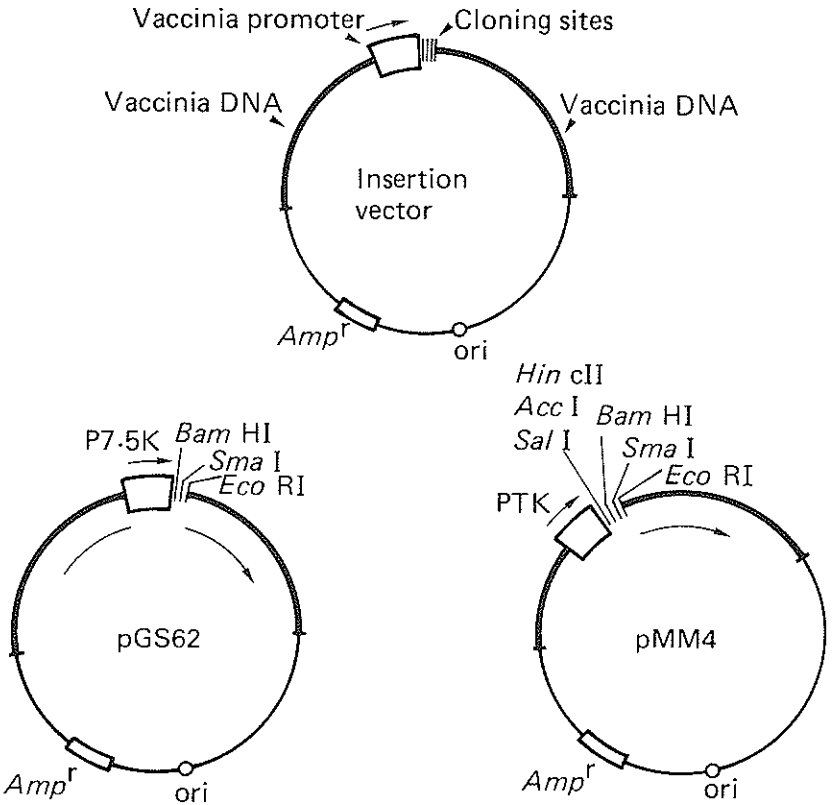


**Figure 3.** Plasmid insertion vector. A generalized plasmid insertion vector is illustrated (top). Essential features include a vaccinia virus promoter upstream from unique cloning sites for insertion of foreign genes. The promoter and cloning sites are flanked by vaccinia virus DNA taken from a non-essential region of the virus genome. The ampicillin-resistance gene (*Amp*$^r$) and bacterial origin of replication (ori) enable the vector to be selectively amplified in *E. coli*. Plasmids pGS62 and pMM4 are specific examples of plasmid insertion vectors. Plasmid pGS62 contains a vaccinia virus promoter taken from upstream of the early gene coding for a 7·5 kilodalton polypeptide. This promoter has been inserted into the coding sequences of the vaccinia virus thymidine kinase gene (indicated by internal arrow) and unique *Bam*HI, *Sma*I and *Eco*RI restriction endonuclease sites have been engineered immediately downstream from the promoter. Plasmid pMM4 has unique *Hin*cII, *Sal*I, *Acc*I, *Sma*I, *Bam*HI and *Eco*RI sites immediately downstream from the TK gene promoter. The amino-terminal half of the *TK* gene including the translational initiation codon has been deleted as previously described (Mackett, Smith and Moss, 1984a).

Calcium phosphate precipitates of plasmid DNA (2 μg), wild-type vaccinia DNA (1 μg) and carrier DNA (17 μg) mixed at 20 μg/ml, are used to transfect cells infected with wild-type vaccinia virus at 0·05 plaque-forming units per cell. The presence of wild-type vaccinia DNA at 1 μg/ml increases the frequency at which recombinant viruses are produced. After 30 minutes at room temperature, tissue-culture medium (9 volumes) is added to the cells and incubation continued at 37°C for 4 hours. Fresh tissue-culture medium is then added and the virus left to replicate for two days. Progeny virus present within the infected

cells is released by freeze/thawing and plaqued on to new cell monolayers. Treatment of transfected cells with either glycerol or dimethyl sulphoxide shock has not increased the frequency at which vaccinia virus recombinants are produced.

## SELECTION OF RECOMBINANTS

The method used to distinguish recombinant viruses from wild type depends upon the site of insertion of the foreign gene and the nature of the foreign gene product. For simplicity, genetic means of selection are preferred wherever possible. In preliminary experiments the foreign gene inserted into vaccinia was itself a genetically selectable marker, so that recombinant viruses could easily be distinguished by expression of that gene. The gene chosen was that for the herpes simplex virus pyrimidine kinase (Enquist *et al.*, 1979). Insertion and expression of this gene in $TK^-$ mutants of vaccinia virus enabled recombinants to be selected by plaque assay in $TK^-$ cells in the presence of methotrexate (Mackett, Smith and Moss, 1982; Panicali and Paoletti, 1982).

A more generally applicable genetic method of selecting recombinant viruses uses the vaccinia virus *TK* gene. By inserting foreign genes within the coding sequences of the *TK* gene, recombinant viruses are made $TK^-$, a phenotype selectable in $TK^-$ cells in the presence of 5-bromodeoxyuridine (BUdR). This method has the advantage that selection is obtained independent of the nature of the foreign gene or whether that gene is expressed. Several genes have been inserted into vaccinia at this locus (Mackett, Smith and Moss, 1982; Smith, Mackett and Moss, 1983a; Smith, Murphy and Moss, 1983; Smith *et al.*, 1984b).

DNA:DNA hybridization methods can also be used to identify recombinant viruses. *In situ* plaque hybridization, used initially for identification of SV40 recombinants (Villareal and Berg, 1977), has also been used to select vaccinia virus recombinants (Nakano, Panicali and Paoletti, 1982; Panicali *et al.*, 1983; Smith and Moss, 1983). The total virus progeny from a transfection is plaqued on to fresh cell monolayers. Once virus plaques have been produced, the whole cell monolayer is transferred to a nitrocellulose membrane. DNA within the cell monolayer is denatured and fixed to the membrane and then probed with $^{32}P$-labelled DNA specific for the foreign gene. After washing and autoradiography of the membrane, recombinant virus plaques can be recovered from the agarose overlay corresponding to the positions of dark spots on the autoradiograph. Recombinant virus alternatively may be recovered from a second nitrocellulose membrane that had been pressed against the primary membrane containing the cell monolayer (Panicali *et al.*, 1983). Dot blot DNA:DNA hybridization has also been used in conjunction with genetic selection methods (Mackett, Smith and Moss, 1982) to distinguish true recombinants from spontaneous mutants.

Antisera specific for the foreign gene product can also be used to identify recombinant viruses. Plaques formed by the progeny of a transfection experiment are screened for the expression of the foreign gene by incubation

with antibodies raised against the foreign gene product. After unbound antibodies have been washed away, bound antibody is detected by incubation with $^{125}$I-labelled staphylococcal A protein (Smith, Murphy and Moss, 1983). Alternatively, a second antibody (raised against the primary antibody) labelled with $^{125}$I or coupled to an enzyme can be used. Autoradiography of washed and dried cell monolayers reveals dark spots that represent the positions of recombinant virus plaques. As with *in situ* plaque hybridization, virus can be recovered from the original agarose overlay.

After the initial identification of recombinant viruses, additional plaque-purification steps are routinely performed before stocks of the recombinant virus are grown up. Where possible, the further plaque purification and initial stages of amplification are performed under conditions selective for the recombinant virus. The purity of the recombinant virus stocks can be checked in several ways. $TK^-$ recombinant viruses can be screened for the presence of significant amounts of $TK^+$ wild-type virus by plaque assay on $TK^-$ cells in the presence of BUdR. Additionally, analyses of genomic DNAs by Southern blotting (Southern, 1975) of restriction endonuclease digests followed by probing with $^{32}$P-labelled DNA fragments can also be used to test for virus purity. A mobility difference should be observed in the DNA fragment corresponding to the position at which the foreign DNA was integrated and there should be an absence of submolar bands. DNA:DNA hybridization and antibody-binding to plaques formed by the recombinant virus can be used to demonstrate that every plaque both contains the foreign DNA and expresses the foreign gene.

### Expression of foreign genes

The concept that efficient expression of foreign genes would be obtained only if the foreign gene were fused in a defined manner to a vaccinia promoter was confirmed in preliminary experiments with the herpes simplex virus pyrimidine kinase gene. A DNA fragment containing the translational initiation site and entire protein-coding sequence of the herpes gene (Enquist *et al.*, 1979) was inserted adjacent to a vaccinia promoter taken from the upstream region of an early gene that codes for a 7·5 kilodalton polypeptide (Venkatesan, Baroudy and Moss, 1981). The promoter contains the normal transcriptional initiation site of that gene together with 235 base pairs upstream and 30 base pairs downstream. The resultant chimeric gene possessed no ATG between the transcriptional start site of the vaccinia promoter and the normal translational start site of the herpes gene. This chimeric gene was inserted into a non-essential region of a $TK^-$ vaccinia virus and recombinant viruses were selected in the presence of methotrexate (Mackett, Smith and Moss, 1982). A yield of 5200 $TK^+$ plaques was obtained per μg of plasmid DNA indicating that the herpes gene was being expressed. Importantly, when plasmids containing the vaccinia virus promoter in the incorrect orientation with respect to the herpes gene, or plasmids lacking the vaccinia virus promoter, were used in parallel transfections, no $TK^+$ recombinant viruses were produced. This study

demonstrated that vaccinia virus promoters were essential for expression of foreign genes within vaccinia virus and also provided the first functional evidence of the nucleotide sequences that constitute a vaccinia promoter.

In a separate study, a DNA fragment containing the herpes pyrimidine kinase gene and flanking DNA sequences was inserted into the *Bam* HI site of the vaccinia *Hin*d III F fragment (Panicali and Paoletti, 1982). Expression of the herpes gene was obtained despite the absence of a specifically engineered vaccinia promoter at the 5′ end of the herpes gene. However, consistent with the requirement for a vaccinia promoter, expression of the gene was obtained only when inserted in one orientation with respect to flanking vaccinia DNA. This unidentified vaccinia promoter must have been at least 850 base pairs from the protein-coding sequences of the herpes gene.

Confirmation of the expression of the herpes gene was obtained in both studies by utilizing the unique ability of the herpes pyrimidine kinase to phosphorylate $^{125}$I-labelled deoxycytidine (Summers and Summers, 1977; Smiley *et al.*, 1980).

After a vaccinia virus DNA sequence of 265 base pairs had been identified, that functioned as a promoter *in vivo*, a series of plasmid insertion vectors were constructed based on this and other promoters (*see above*). These enable foreign protein-coding sequences to be rapidly cloned under the control of vaccinia promoters and then inserted into infectious vaccinia virus. Using these vectors, several genes have been expressed in vaccinia virus including those for hepatitis B virus surface antigen (Smith, Mackett and Moss, 1983a,b), influenza haemagglutinin (Smith, Murphy and Moss, 1983), chloramphenicol acetyltransferase (CAT) (Mackett, Smith and Moss, 1984a), vesicular stomatitis virus glycoprotein and nucleoprotein (M. Mackett, personal communication), herpes simplex virus glycoprotein D (M. Mackett, personal communication) and *Plasmodium knowlesi* sporozoite surface antigen (Smith *et al.*, 1984a). Because, in all cases, the translational initiation and termination sites of the foreign gene were used, authentic polypeptides were produced that could be identified by immunoprecipitation and polyacrylamide gel electrophoresis.

Influenza virus haemagglutinin and herpes simplex virus pyrimidine kinase have also been expressed in vaccinia virus without the use of the insertion vectors described (Panicali and Paoletti, 1982; Panicali *et al.*, 1983). However, neither the basis for expression nor the nature of the polypeptide synthesized was reported in these cases.

Detection of foreign gene expression in cells infected with vaccinia virus recombinants has been accomplished in several ways. Where the foreign gene codes for an enzyme, the infected cell extracts can be assayed for this enzymic activity. This has been done with herpes simplex virus pyrimidine kinase (Mackett, Smith and Moss, 1982; Panicali and Paoletti, 1982) and CAT (Mackett, Smith and Moss, 1984a). As these enzymes both have activities not found in mammalian cells, detection of their expression is sensitive. *CAT* gene expression is also quantitative and makes the use of this gene particularly suitable for studying gene expression in vaccinia virus (Mackett, Smith and

Moss, 1984a; Weir and Moss, 1984) and other systems (Gorman, Moffat and Howard, 1982). Other methods of detecting foreign gene expression in vaccinia-infected cells are mostly immunological. Conventionally, pulse-labelling of infected cells with radioactive amino acids followed by immunoprecipitation and polyacrylamide gel electrophoresis is a common way of identifying and analysing specific polypeptides. Hepatitis B virus surface antigen (Smith, Mackett and Moss, 1983a,b), influenza virus haemagglutinin (Smith, Murphy and Moss, 1983), vesicular stomatitis virus glycoprotein and nucleoprotein (M. Mackett, personal communication), herpes simplex virus glycoprotein D (M. Mackett, personal communication) and *Plasmodium knowlesi* circumsporozoite surface antigen (Smith *et al.*, 1984a) have all been detected in this manner. Radioimmunoassay, Western blotting (Towbin, Staehelin and Gordon, 1979) and binding of antibody directly to plaques formed by the recombinant virus have also been used to detect expression of foreign genes by recombinant vaccinia viruses.

## Vaccination of animals

The potential of vaccinia virus recombinants as new live vaccines has been demonstrated by the vaccination of animals. Several recombinant vaccinia viruses expressing different foreign genes have been shown to induce an immunological response against the foreign gene product in vaccinated animals. Moreover, these animals can be protected against challenge with the heterologous pathogen from which the foreign gene was derived. Examples are given below.

### HEPATITIS B VIRUS SURFACE ANTIGEN (HBsAg)

Vaccinia virus recombinants expressing HBsAg have been constructed (Smith, Mackett and Moss, 1983a,b). Rabbits vaccinated by intradermal injection of the recombinant virus rapidly developed antibodies against HBsAg (anti-HBs). After a primary response, peaking at about 10 days, the levels of anti-HBs decreased before subsequently increasing to persistent high levels. Anti-HBs levels of 10 milli international units (mIU) per ml are sufficient to protect humans against hepatitis B virus (HBV) (Courouce-Pauty *et al.*, 1978). The vaccinated rabbits developed anti-HBs levels of up to one thousandfold higher (10 IU/ml) whereas a control rabbit vaccinated with wild-type vaccinia virus developed no anti-HBs.

HBV infects primates only; therefore, to test for protection against HBV, chimpanzees were vaccinated with the recombinant virus and then challenged with HBV (B. Moss, G.L. Smith, J.L. Gerin and R.H. Purcell, unpublished work). Two chimpanzees were vaccinated with the virus recombinant and one with wild-type virus. After vaccination, no anti-HBs was detected in the serum of any animal with the exception of one weakly positive sample 8 weeks after vaccination of one chimpanzee with the recombinant virus. This poor immunological response of chimpanzees vaccinated with the same virus that stimulated a good response in rabbits is consistent with the poor response of

primates to a single dose of HBsAg in the absence of adjuvants. Fourteen weeks after vaccination the chimpanzees were challenged with an intravenous injection of HBV. The control animal (vaccinated with wild-type virus) then developed typical hepatitis B. Elevated levels of liver enzyme alanine aminotransferase, large amounts of HBsAg and antibodies against the HBV core antigen (anti-HBc) appeared in the serum. By 20 weeks after challenge the HBsAg had disappeared from the serum concomitant with rising levels of anti-HBs. This classic hepatitis B contrasted sharply with the response of the two chimpanzees vaccinated with the vaccinia virus recombinant. Four to seven weeks after HBV challenge, high levels of anti-HBs appeared in both animals and persisted for the duration of the experiment (more than 6 months). Additionally, no HBsAg or elevated levels of liver enzymes were detectable. These animals did, however, seroconvert to anti-HBc at low levels, indicating that a subclinical infection had been initiated. Nevertheless, these animals were protected against liver disease caused by HBV.

The most likely first application of recombinant vaccinia viruses in man is against HBV. Currently over 200 million people are chronically infected with HBV, mostly in Third World countries. HBV causes fulminant hepatitis, liver cirrhosis and hepatocarcinoma resulting in hundreds of thousands of deaths every year. Vaccine development against HBV has been hampered by the inability to propagate HBV in tissue culture. However, a subunit vaccine composed of HBsAg purified from the plasma of infected patients has been developed and licensed (Szmuness *et al.*, 1980; Crosnier *et al.*, 1981). This vaccine is effective after three inoculations but is very expensive and is limited in availability. Additionally, concern over the safety of plasma-derived vaccines resulting from an epidemic of acquired immune deficiency syndrome (AIDS) continues, despite the proven safety record. This has decreased the usage of the vaccine. Recombinant DNA technology has been used to identify and sequence the gene coding for HBsAg. Expression of this gene in heterologous systems has led to an alternative source of HBsAg that might be used as a subunit vaccine (Dubois *et al.*, 1980; Moriarty *et al.*, 1981; Valenzuela *et al.*, 1982; Gough and Murray, 1982; Smith, Mackett and Moss, 1983a). However, at the present time it is not clear whether recombinant HBV subunit vaccines would be cheap enough for utilization in those Third World countries where their need is greatest.

The DNA sequence of the HBsAg gene and deduced amino-acid sequence made possible the chemical synthesis of peptides representing different regions of the HBsAg protein. Some of these are immunogenic when administered together with adjuvants or linked to carrier proteins (Lerner *et al.*, 1981; Dressman *et al.*, 1982) and can sometimes protect against HBV (Gerin *et al.*, 1983). Peptide vaccines would be inexpensive and free from safety questions associated with the use of infectious agents, but an effective and acceptable method of administration needs to be found for human application.

Recombinant vaccinia viruses expressing HBsAg will provide an inexpensive, easy-to-administer vaccine available in large quantities. Unlike peptide or subunit HBV vaccines, a single administration of the vaccine will be effective and, because of the live nature of the vaccine, both cell-mediated and humoral

antibody responses may be evoked. The many advantages of this type of vaccine seem to outweigh the problems of low-frequency side-effects to vaccination (*see below*), particularly in regions of the world where HBV is endemic and the risk of HBV infection is very high.

## INFLUENZA VIRUS HAEMAGGLUTININ

Two studies have reported the expression of influenza virus haemagglutinin (HA) in vaccinia virus recombinants (Panicali *et al.*, 1983; Smith, Murphy and Moss, 1983). Panicali and co-workers described the expression of influenza HA derived from influenza virus A/PR/8/34. Synthesis of the HA was detected by the binding of antibodies against HA to plaques formed by the virus recombinant. Although the nature of the HA polypeptide was not reported, the protein was immunogenic in rabbits immunized by intravenous injection of the virus recombinant. Antibodies from these immunized animals inhibited the agglutination of erythrocytes by influenza virus and neutralized influenza virus infectivity *in vitro*.

In another study the HA derived from influenza virus A/Japan/305/57 was expressed in a vaccinia virus recombinant (Smith, Murphy and Moss, 1983). The HA polypeptide was identified by immunoprecipitation and polyacrylamide gel electrophoresis and was shown to be authentic. Additionally, the polypeptide was glycosylated and transported to the cell surface where it was detectable by indirect immunofluorescence. Rabbits vaccinated by a single intradermal injection of the virus developed antibodies against the haemagglutinin (anti-HA) that inhibited haemagglutination by influenza virus. A hamster model system was used to test if the virus recombinant could induce protective immunity against influenza virus. Groups of 10 hamsters were vaccinated intradermally with either wild-type vaccinia virus or vaccinia virus recombinant expressing influenza HA, or were inoculated intranasally with influenza virus A/Japan/305/57. All animals that received the vaccinia virus recombinant or influenza virus developed similar levels of anti-HA, whereas animals that received wild-type vaccinia virus developed no anti-HA. Hamsters were challenged intranasally with influenza virus A/Japan/305/57, 40 days after vaccination. Animals that initially received influenza virus or vaccinia virus recombinant expressing HA were shown to be protected against influenza virus, compared with the animals that initially received wild-type vaccinia virus. This protection against a respiratory infection following intradermal vaccination on the back, suggests that vaccinia virus recombinants expressing antigens from other respiratory pathogens might protect man or animals against those pathogens also.

The vaccinia virus recombinant expressing the influenza HA from A/Japan/305/57 has also been used to demonstrate that cytotoxic T cells that recognize this haemagglutinin are produced following vaccination of mice (J.R. Bennink, J.W. Yewdell, G.L. Smith, C. Moller and B. Moss, unpublished work). This illustrates an important advantage of live vaccines over killed vaccines, namely that both humoral antibody and cell-mediated immune responses are elicited in the vaccinated host.

### MALARIAL SPOROZOITE SURFACE ANTIGEN

The circumsporozoite surface antigen of *Plasmodium knowlesi* has been expressed in vaccinia virus (Smith *et al.*, 1984a). This is the first expression of a cloned malarial gene in mammalian cells. The protein is antigenic, as shown by immunoprecipitation with specific monoclonal antibody, radioimmunoassay and Western blotting. Rabbits vaccinated with the recombinant virus produced antibodies that specifically bound to sporozoites. Whether vaccinia virus recombinants expressing only sporozoite antigens will constitute effective malarial vaccines, remains to be determined. The great capacity of vaccinia virus for foreign DNA will enable the simultaneous expression of malarial genes from different life stages.

### HERPES SIMPLEX VIRUS I GLYCOPROTEIN D

Vaccinia virus recombinants that express herpes simplex virus glycoprotein D have been constructed. These viruses, when administered to mice intradermally or intraperitoneally, induce immunity that protects against challenge with a lethal dose of herpes simplex virus (K. Cremer, M. Mackett, A.L. Notkins and B. Moss, unpublished work).

### VESICULAR STOMATITIS VIRUS GLYCOPROTEIN

The wide host range of vaccinia virus *in vivo* enables vaccinia virus recombinants to have potential as veterinary as well as medical vaccines. The first potential veterinary vaccine of this type is against vesicular stomatitis virus (VSV). Mice and cattle vaccinated intradermally with a vaccinia virus recombinant expressing VSV glycoprotein develop antibodies that neutralize VSV infectivity *in vitro* (M. Mackett, J.K. Rose, T. Yilma and B. Moss, unpublished work). Experiments have been initiated to determine if cattle are protected against VSV challenge (*see also* Chapter 1 of this volume).

Collectively, these data indicate that vaccinia virus recombinants expressing foreign antigens have potential as new live vaccines in both man and animals. However, there are two obstacles facing the extensive use of such vaccines in man. First, a large percentage of the world's population possesses some degree of immunity to vaccinia virus, because of extensive smallpox vaccination. This immunity will restrict the replication of vaccinia viruses and thereby reduce the amount of antigen synthesized and the resultant immune response. Now that smallpox vaccination has been discontinued in civilian populations this problem will become less significant with time. However, attempts are being made to produce vaccinia viruses that express foreign antigens at sufficiently high levels so that secondary vaccinations in partially immune hosts will still induce protective immunity. Additionally, there already exists an ever-increasing population of non-vaccinated children. For many diseases, such as hepatitis B and malaria, it is in the first few years of life that immunization is most required and effective. The second problem is that of safety and is considered below. These potential problems for man should not be obstacles for veterinary use.

## Safety

Several types of new vaccines are being developed, including subunit vaccines, peptide vaccines and live recombinant vaccines, of which vaccinia recombinants are an example (*see* Chanock and Lerner, 1984). A common goal of these approaches is to produce a vaccine that is effective in preventing disease, inexpensive to manufacture on a mass scale, easy to administer, sufficiently potent as a single inoculation and safe. In all these criteria, except absolute safety, vaccinia virus (and presumably recombinants thereof) have the desired features. In addition, the stability of lyophilized vaccinia virus is a useful feature that enables transportation and storage in tropical countries without refrigeration.

Long before smallpox was eradicated it was recognized that there were side-effects of vaccination that occurred at low frequency. These fell into four major categories: post-vaccinial encephalitis, progressive vaccinia, generalized vaccinia and eczema vaccinatum. The frequency of these complications has varied considerably in different studies but the most recent and comprehensive study occurred in the USA in 1968 (Lane *et al.*, 1969). In this study there were 14·2 million vaccinations and nine deaths. Four of these were attributable to post-vaccinial encephalitis, four to progressive vaccinia and one to eczema vaccinatum. The frequency of eczema vaccinatum and progressive vaccinia were host dependent and not affected by the strain of vaccinia virus used. In contrast, the frequency of post-vaccinial encephalitis was primarily dependent on the strain of virus. The Wyeth strain of vaccinia gave the lowest frequency of post-vaccinial encephalitis and the data of Lane and co-workers are based upon the use of this virus. Other vaccinia-virus strains gave much higher frequencies of post-vaccinial encephalitis. Replacement of the Bern strain by the Lister strain in Austria and Switzerland in the 1960s resulted in a dramatic reduction in the incidence of post-vaccinial encephalitis (Berger and Heinrich, 1973). Progressive vaccinia and eczema vaccinatum occurred in patients who possessed immunological deficiencies or who suffered from eczema, respectively. These conditions are considered contra-indications to vaccination and screening of potential vaccinees for these conditions was routinely performed.

In addition to careful choice of the vaccine strain and screening of individuals for eczema and immunodeficiencies, the safety of vaccinia as a vaccine may be improved by genetic engineering. In this regard, there is already evidence that $TK^-$ vaccinia-virus recombinants have markedly reduced pathogenicity in animals (R.M.L. Buller, G.L. Smith and B. Moss, unpublished work). The dose of $TK^-$ recombinant virus that is lethal by intracerebral injection is ten-thousandfold higher than the lethal dose of $TK^+$ wild-type virus. Other routes of administration of these viruses, such as intraperitoneal injection or scarification, also give differences in pathogenicity and host antibody response. Primates (squirrel monkeys and chimpanzees) also suffer less severe infections by $TK^-$ recombinants than by $TK^+$ wild-type virus (Moss *et al.*, 1984). Although attenuation of the virus makes the vaccine safer, it will also reduce its effectiveness due to lower levels of virus replication and consequential weaker immune response. Attenuation of virus recombinants coupled with high levels of expression of foreign genes should provide effective and safe vaccines.

**Other uses of vaccinia virus recombinants**

Although the most exciting use of vaccinia recombinants is as new vaccines, there are several other areas in which these viruses are applicable. One of these is for studies of vaccinia virus gene expression and regulation. Now that specific vaccinia virus DNA sequences that contain functional transcriptional elements have been isolated, it is possible to mutate these *in vitro* and then reintroduce them into vaccinia virus coupled to foreign genes such as *CAT* (Weir and Moss, 1984). An analysis of the levels and temporal control of *CAT* expression by these mutated promoters *in vivo* will enable more precise determination of the nucleotide sequences controlling transcription. Differences in nucleotide sequence of early and late vaccinia virus promoters may then be correlated with function *in vivo*.

Another application of recombinant vaccinia viruses is as a eukaryotic cloning vector of large DNA fragments. To date, vaccinia virus is the only described eukaryotic cloning vector with capacity for large DNA fragments (more than 25 kilobases) that retains infectivity. Simian virus 40 vectors and retrovirus vectors have only a small capacity for foreign DNA and, in addition, need helper viruses or cell lines expressing virus genes for replication. This large capacity for foreign DNA will permit the simultaneous expression of several foreign genes in vaccinia virus.

A big advantage of vaccinia virus vectors over other vectors concerns their use in animals. As complete infectivity is retained it is possible to study immunological responses to live infections in vaccinated animals. It has already been demonstrated that vaccination of mice with a vaccinia virus recombinant expressing influenza virus haemagglutinin induces the formation of cytotoxic lymphocytes that recognize the influenza HA (J.R. Bennick, J.W. Yewdell, G.L. Smith, C. Moller and B. Moss, unpublished work). Moreover, this system can be used to ask specific questions, such as which influenza virus protein is responsible for the induction of cytotoxic lymphocytes that cross react among different influenza subtypes. The *in vivo* immune response to *in vitro* mutated proteins can also be studied, providing a direct correlation between amino acid sequence and immunogenicity.

**Summary and future prospects**

The development of vaccinia virus for cloning and expressing foreign genes is a technological advance that has occurred since 1982. Since that time, protocols have been developed and refined to facilitate the rapid construction of vaccinia virus recombinants that express foreign genes. Utilization of vaccinia virus transcriptional regulatory sequences has been shown to be essential for expression of foreign genes. Authentic foreign proteins are produced by engineering chimeric genes to ensure utilization of the translational initiation and termination codons of the foreign gene. The expression of foreign proteins in tissue-culture cells infected with the recombinant vaccinia viruses has been detected by enzymatic or immunological methods. Animals vaccinated with virus recombinants can produce humoral antibody and cell-mediated immune responses against the foreign protein. These immunological responses have

protected vaccinated animals against challenge with hepatitis B, influenza and herpes simplex virus. In addition to being effective vaccines, these recombinant viruses are attenuated, thereby making them safer vaccines.

These achievements have been accomplished using the first few vaccinia virus promoters that have been identified and sequenced. Undoubtedly, higher levels of expression of foreign genes may be obtained by using stronger vaccinia virus promoters that code for the major late structural virus proteins. Higher levels of expression coupled with desirable degrees of attenuation are goals for the construction of future vaccinia virus recombinants. Another major goal is the simultaneous expression of several foreign genes in one virus: because of the large capacity of vaccinia virus for exogenous DNA, this should be relatively straightforward. In this manner, polyvalent vaccines may be constructed that are designed to vaccinate against different combinations of pathogens found in different geographical areas.

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