A novel vaccinia virus expression system allowing construction of recombinants without the need for selection markers, plasmids and bacterial hosts

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Vaccinia virus is one of the most widely applied expression systems for use in eukaryotes in molecular biology. Expression of heterologous genes in the vaccinia virus system, however, requires integration of the foreign DNA into the vaccinia virus genome by means of homologous recombination or by direct molecular cloning. In both cases, plasmid vector constructs are required that contain the gene of interest and, usually, a marker gene, both of which are controlled by suitable promoter sequences. In order to simplify the construction of recombinants and to eliminate the need for a marker gene we have developed a modified vaccinia virus genome that allows the direct targeted insertion of DNA fragments downstream of a strong vaccinia virus promoter without any further cloning steps. The gene of interest is amplified by PCR using oligonucleotide primers that provide an SfiI site at the 5' end and an RsrII site at the 3' end of the PCR product. Following digestion with these restriction enzymes, the PCR product is operationally linked to a synthetic early/late promoter within the viral genomic DNA via the unique SfiI/RsrII sites of the modified vaccinia virus genome. Using this approach, intermediate plasmid constructs and bacterial hosts are not required and time consuming screening steps can be omitted, because 90% of the virus progeny carry the foreign DNA.

Introduction

Vaccinia virus is one of the most versatile expression systems for use in eukaryotes, with a wide range of applications in molecular biology (for review see Moss, 1991). Since its initial development, the vaccinia virus system has undergone a variety of modifications and improvements and powerful homologous (Chakrabarti et al., 1985; Falkner & Moss, 1988; Patel et al., 1988) and heterologous (Fuerst et al., 1987; Elroy-Stein et al., 1989; Moss et al., 1990) promoter systems have been incorporated that direct the expression of the gene of interest. A basic requirement for the generation of recombinant vaccinia viruses is a plasmid vector that allows cloning of the foreign gene next to the promoter unit. Integration of the foreign DNA into the vaccinia virus genome occurs by homologous recombination of sequences present on the cloning vector and the genome (Panicalli & Paoletti, 1982; Mackett et al., 1982). Alternatively, a recently developed approach allows the in vitro ligation of a gene cassette containing the foreign DNA with the vaccinia virus genome (Scheifflinger et al., 1992; Merchlinsky & Moss, 1992). Selection of recombinant viruses is usually mediated by the expression of a marker gene that cointegrates into the viral genome (Chakrabarti et al., 1985; Falkner & Moss, 1988; Boyle & Coupar, 1988). Although the above methods are widely established, problems may arise from cloning the DNA to be expressed into the vaccinia virus insertion vector. Eukaryotic or prokaryotic genes of interest may be toxic for bacteria, i.e. the propagation in bacteria of plasmid constructs containing those genes may be difficult if not impossible (Gibbs & Cross, 1988; Berish et al., 1992). Furthermore, the presence of a marker gene for the selection of recombinants is often not desired, as in the case when attenuated recombinant vaccinia viruses are constructed for vaccine purposes. The only foreign genetic information contained in these viruses should be the gene coding for the desired antigen.

For these purposes, we have developed a modified vaccinia virus genome that allows the forced ligation of foreign DNA downstream of an endogenous strong vaccinia virus early/late promoter (Davison & Moss, 1990). This genome contains unique SfiI and RsrII sites adjacent to the promoter sequence; these sites permit the directed insertion of DNA fragments having compatible ends. Since naked vaccinia virus genomic DNA is not infectious, recovery of viable recombinant virus is mediated by a fowlpox helper virus in a mammalian cell system that is restrictive for fowlpox virus replication (Scheifflinger et al., 1992; Mayr & Malicki, 1966).
**SfiI/RsrII cleaved vaccinia virus genome is unable to relegate; thus the outcome virus is predominantly recombinant and the need for a marker gene is eliminated. By direct insertion of the PCR amplified ORF of the human blood clotting FIX gene, we demonstrate that 90% of the virus progeny can express the foreign genetic information.

This novel approach allows the integration and efficient expression of foreign DNA, such as PCR products or cDNAs, into the vaccinia virus genome without further cloning steps involving plasmid DNA or bacterial vectors.

**Methods**

**Construction of the modified vaccinia virus vDPC.** The chimeric vaccinia virus vDPC contains a strong synthetic promoter the early/late promoter genomic DNA of wild-type vaccinia virus strain WR. This cassette is without further cloning steps involving plasmid DNA or products or cDNAs, into the vaccinia virus genome recombinant, and the need for a marker gene is

**Recovery of the chimeric vaccinia virus vDPC containing PCR products as foreign DNA.** The complete ligation mixture was coprecipitated with calcium phosphate according to standard protocols and transfected into CV-1 cells in a 10 cm culture dish. The cells were at 80% confluence and had previously been infected at an m.o.i. of 0.05 p.f.u. per cell with the Fowlpox virus strain HP 1-441 (Mayr & Malicki, 1966); 10 days p.i., when the vaccinia virus specific CPE was complete, infected cells were harvested, resuspended in 1 ml PBS and sonicated for 2 min to release the virus. Single virus plaques, purified twice on CV-1 cells, were used for the preparation of small and large chimeric vDPC virus stocks.

**Expression of recombinant protein in cells infected with a chimeric vDPC vaccinia virus.** For the analysis of secreted proteins, 5 x 10° CV-1 cells grown to confluency in 10 cm Petri dishes were infected with a single vDPC plaque isolate that had been purified twice on CV-1 cells; 3-7 days p.i., when the CPE was complete, cells were harvested and resuspended in 1 ml PBS. Cell suspension (450 μl) was incubated overnight in lysis buffer (10 mM-Tris–HCl pH 8.0, 10 mM-NaCl, 10 mM-EDTA) containing 500 μg/ml Proteinase K (Boehringer Mannheim). Genomic DNA was purified with phenol, phenol–chloroform (1:1) and chloroform, precipitated with ethanol and redissolved in water to a final concentration of 1 μg/μl. Purified genomic DNA (10 μg) was used for restriction enzyme analysis. Cleaved DNA fragments were separated by field reversed gradient electrophoresis (FIGE). Fragments were separated at 4 °C on a 1 % agarose gel in a Tris–acetate–EDTA buffer using a microcomputer controlled power supply. Four programme steps were run successively setting the voltage at 7 V/cm; forward (F) and reverse (R) pulses were followed by a pause of 1 s. The following pulse programmes were used: (1) 5 h F6 R3; (2) 5 h F4 R2; (3) 5 h F2 R1; (4) 5 h F8 R4.
Results

Characterization of the modified vaccinia viruses vDPC-1 and vDPC-2

The direct promoter cloning (DPC) approach requires a modified vaccinia virus genome, preferably with a strong vaccinia virus promoter fused to unique restriction endonuclease cleavage sites, allowing the direct insertion of foreign DNA with compatible ends. Fig. 1 illustrates the construction steps from wild-type vaccinia virus to a recombinant vDPC genome.

Hygromycin resistant virus clones resulting from an infection/transfection experiment were selected and analysed for the complete and correct insertion of the NotI gene cassette. Depending on the orientation of the NotI gene cassette within the viral genome, two modified virus species, vDPC1 and vDPC2 (Fig. 1 c), were selected.

The nucleotide sequence of the NotI gene cassette and the genomic context of this cassette within the vDPC1 and vDPC2 genome have been submitted to the EMBL nucleotide sequence database and assigned the accession numbers X89856 (vDPC1) and X89857 (vDPC2).

To verify the presence of the SfiI and RsrII sites, an SfiI/RsrII digest was performed with purified genomic DNA from vDPC1 and vDPC2. Two fragments of 467 bp and 632 bp should hybridize to a NotI gene cassette specific probe. Southern blot analysis (Fig. 2 b) confirmed the predicted fragment sizes. The larger hybridizing fragment with a size of 1.1 kb represents the hygromycin resistance gene with an uncleaved internal RsrII site. In the agarose gel shown in Fig. 2(a), the SfiI/RsrII cleaved genomic DNAs from vDPC1, vDPC2 and wild-type vaccinia virus are compared. Only the

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Fig. 1. Construction of the modified vaccinia virus vDPC. Purified, NotI cleaved genomic DNA from wild-type vaccinia virus (a) is ligated with a NotI gene cassette (b) introducing the selP promoter and the elpI/hph gene cassette framed by unique SfiI and RsrII restriction sites. Purified genomic DNA from hygromycin resistant vDPC1 or vDPC2 virus (c), containing the NotI gene cassette in the two possible orientations, is cleaved with SfiI and RsrII (d) and ligated with a compatible gene cassette (e) placing the gene of interest downstream of the selP promoter (f). Construction steps (d) to (f) involve only the vDPC1 genome. The analogous situation using vDPC2 is not shown. The arrowheads indicate the direction of transcription from the promoters.
modified viruses vDPC1 and vDPC2 are cut into two SfiI/RsrII fragments, representing the small left arm (45 kb) of the viral genome and the large right arm (145 kb). The modified vaccinia virus vDPC2 was selected for all further experiments.

Expression of the human blood clotting factor IX ORF encoded by a PCR product

To demonstrate the efficiency of the vaccinia virus forced cloning approach, the gene coding for the human blood clotting factor IX (huFIX) (Kurachi & Davie, 1982) was amplified by PCR using oligonucleotide primers that introduce an SfiI restriction site at the 5' end of the FIX open reading frame and an RsrII site at its 3' end. Progeny vaccinia virus clones, purified twice on CV-1 cells, were assayed for their ability to produce recombinant human FIX. Western blot analysis of ten different chimeric vDPC2 virus clones (vDPC2-FIX#1.2-vDPC2-FIX#10.2) is presented in Fig. 3(a). Nine of the ten vDPC2 virus clones were chimeric, as indicated by the strong signal specific for FIX. The recombinant human FIX has identical electrophoretic and immunological properties compared to the plasma derived material. To further analyse the identity of the vDPC2-FIX clones, an SfiI/RsrII digest of genomic DNA from CV-1 cells infected with these virus clones was performed. Fig. 3(b) demonstrates that the genomic DNA of all vDPC2
clones that produce recombinant FIX also contains the FIX specific 1-41 kb SfiI/RsrII fragment. This fragment has the same size as the PCR product used for ligation with the vDPC2 genomic DNA. The vDPC2 clone 3.2 that does not express recombinant FIX (Fig. 3a, lane 9) shows no hybridization signal (Fig. 3b, lane 4). It may represent an illegitimate ligation product, or an un-cleaved vDPC2 genome present in the vector arm preparation. Fig. 3(c) documents the size (1410 bp) of the FIX specific SfiI/RsrII cleaved PCR product and its position and orientation within the vDPC2 genome. This experiment confirmed the usefulness of the direct promoter cloning approach.

**Discussion**

We have developed a novel approach to generate recombinant vaccinia viruses based on a forced cloning step that allows the *in vitro* ligation of a cleaved vaccinia virus genome with a PCR product containing compatible ends. The method described has two major advantages compared to conventional strategies. First, there is no need for the selection marker gene usually required for the construction of recombinants. Second, time consuming cloning steps in bacterial or plasmid vectors have been eliminated. This latter point is of particular importance because genes of interest may be toxic for the bacterial host, usually *E. coli*. Consequently, conventional vaccinia virus cloning vectors containing these genes may be difficult or impossible to passage in *E. coli*. This problem may arise when strong vaccinia virus promoters are used that are also active in *E. coli* (M. Pfleiderer, unpublished observation).

Insertion of PCR products into the vaccinia virus genome has been described previously (Turner & Moyer, 1992). However, this recombinant PCR approach was based on the coamplification of a promoter/marker gene cassette and of sequences that allow the targeting of the PCR product into the vaccinia virus genome via homologous recombination, i.e. into the thymidine kinase gene locus. Although this method permits the insertion of PCR products amplified from virtually any source of template, it depends on the use of a marker gene for the selection of recombinants or, alternatively, on time consuming screening steps, because homologous recombination is a very rare event. In contrast, with the directed method described in this paper, recombinants can easily be found without any selection or screening. Generally, 90% of the vaccinia virus progeny is recombinant, a proportion normally achieved when a dominant marker is used for selection. In addition, genes of interest are expressed at high levels because the starting virus, vDPC, contains one of the strongest homologous promoter systems so far described, a synthetic early/late promoter. These features significantly increase the versatility of the vaccinia virus direct molecular cloning approach developed by our group (Scheiflinger et al., 1992).

Direct molecular cloning has been shown for other DNA viruses. Using either a modified herpes simplex virus type 1 genome (Rixon & McLauchlan, 1990) or a modified baculovirus genome (Ernst et al., 1994), insertion of foreign DNA into unique sites of a non-essential region of the viral genome was demonstrated. However, since herpesviruses replicate in the nucleus of an infected cell, extensive cloning steps were required to place the gene of interest into the correct genetic context of a splicing signal, a polyadenylation signal and a suitable promoter. Therefore, direct cloning and expression of PCR products might be difficult with this system, unless engineered herpesvirus strains are used. Moreover, the promoter used in this work was an immediate-early promoter, not active throughout the complete infection cycle. This is in contrast to the vaccinia virus *selP* promoter, which has strong early/late activity, a feature that is important for the intended high level expression of recombinant proteins. For both the herpesvirus and the baculovirus *in vitro* molecular cloning systems, expression of the *E. coli* β-galactosidase gene, a marker gene that allows visual screening of the recombinants was used to emphasize the efficiency of these approaches. Non-selectable genes have not been cloned *in vitro* using these systems. In addition, baculoviruses have a very restricted host range, permitting the infection of only a small set of insect cells. This feature is particularly disadvantageous when complicated eukaryotic proteins such as human plasma factors are expressed. These proteins depend on multiple post-translational processing and modification steps such as glycosylation, carboxylation, hydroxylation and sulphatation to be fully functional. These modifications are only performed in specific cell types. Due to the broad host cell range of vaccinia virus these limitations can easily be overcome.

We have expressed human FIX encoded by a PCR product in CV-1 cells to a final concentration of approximately 4 μg/10⁶ cells/ml tissue culture supernatant. The recombinant product has full biological activity upon purification and identical electrophoretic and immunological properties compared to the plasma derived product. The described approach using the vDPC virus (for the targeted direct molecular cloning of genes of interest) may be adapted to other strains of the poxvirus family, such as potential vaccine strains. These live vaccines should contain exclusively the gene of interest as foreign genetic information.

In summary, direct *in vitro* cloning of recombinant vaccinia viruses offers many advantages compared to the conventional systems. Since compatible PCR products
are directly ligated with the viral genome, cumbersome manipulations such as cloning into expression vectors and propagation and amplification of recombinant plasmid constructs in bacteria are omitted. Continuous improvement of PCR technology has made possible the amplification of very long templates. Thus, contiguous ORFs from virtually all template sources, including various tissues, can be used for PCR amplification of the genetic information needed for expression. Vaccinia virus tolerates the uptake of at least 25 kb of foreign DNA without losing its genetic stability (Moss, 1991) and large quantities of genomic vaccinia virus DNA can easily be prepared. These features facilitate direct insertion of genetic information into the viral genome and allow rapid functional analysis of expressed products and virus recombinants. In addition, direct promoter cloning of vaccinia virus recombinants may decrease the risk of unwanted intra- and intermolecular recombination, a problem encountered when insertion of large fragments of foreign DNA into the vaccinia virus genome is intended by means of homologous recombination. Therefore, our novel system will extend the range of applicability of recombinant vaccinia viruses and will be helpful for the design of live recombinant vaccines.

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References


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