Research Report

BiZyme: A Novel Fusion Protein-Mediating Selection of Vaccinia Virus Recombinants by Fluorescence and Antibiotic Resistance

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ABSTRACT

Recombinant vaccinia virus is a useful and powerful tool for the expression and study of foreign genes. Methods that are currently available for the selection of vaccinia virus recombinants include the restoration of viral plaque-forming phenotype, the replication of viral DNA in the presence of BUdR or mycophenolic acid, and the maturation and propagation of virus under antibiotic selection. Though effective, each of these methods requires several weeks of concerted effort to isolate, purify, and amplify a potential recombinant virus. Here we report the development of a bifunctional enzyme (BiZyme) to simplify and expedite the isolation and purification of vaccinia virus recombinants. This novel selection marker is composed of an in-frame fusion between the genes encoding gfp and the neomycin phosphotransferase enzyme (neo). Remarkably, expression of the chimeric gfp-neo cassette in the presence of G418 confers both viability and fluorescence to transfected or recombinant virus-infected cells, indicating that both activities are retained within the fusion protein. Therefore, BiZyme was incorporated into a recombination plasmid (pGMR) to enable the concomitant insertion of a foreign gene of interest. Here we demonstrate that this selection/amplification process requires a minimum of 11 days to produce the desired vaccinia virus recombinants. Furthermore, recombinants produced in this

fashion have been shown to express both biologically active enzymes and antigenically authentic foreign antigens. In addition to its use in the vaccinia virus vector system, the BiZyme bifunctional selection scheme should be applicable to other eukaryotic and prokaryotic expression systems, simply by coupling it to the appropriate host-specific transcription regulatory signals.

INTRODUCTION

Vaccinia virus has played an important role in history as the first successful vaccine developed to combat a human disease. Global immunization efforts with vaccinia virus resulted in the eradication of smallpox in the late 1970s, thereby terminating further need for this viral vaccine. However, the utility of vaccinia virus persists because it is now widely used as a valuable research tool. Specifically, vaccinia virus is being used as a eukaryotic cloning and expression vector for the study of various gene systems, to overexpress proteins of interest, and for the development of new vaccines and immunotherapeutics (3,23,24).

There are several aspects of the vaccinia virus replication cycle that make it particularly amenable to laboratory manipulation. First, the availability of the entire genome sequence (13,18) provides a quick means of comparison of predicted vaccinia virus genes to similar viral, prokaryotic, or even eukaryotic sequences for the elucidation of gene function and provides the basis for designing effective gene insertion strategies. Second, analysis of the vac-

cinia virus genome has shown that many vaccinia virus proteins are dedicated to nucleic acid metabolism and biosynthesis (8,19,20,25,27), a feature that contributes to the ability of vaccinia virus to replicate entirely within the host cell cytoplasm. The cytoplasmic replication mode employed by vaccinia virus facilitates the homogeneous recombination of plasmid-borne foreign genes. Third, proteins expressed by vaccinia virus in the cytoplasm are subjected to a wide range of cellular posttranslational modifications including N- and O-linked glycosylation, phosphorylation, acylation, proteolytic cleavage, polarized membrane, and nuclear transport and secretion. Therefore, expressed foreign gene products are usually indistinguishable in structure, location, and activity from the bona fide protein (29). In addition, because of the extranuclear vaccinia virus replication strategy, the expression of a foreign gene from the context of the vaccinia virus genome does not require splice junction enhancers or RNA targeting signals. Recombinants expressing such diverse foreign proteins as streptococcal M protein (17), HIV gp120 envelope glycoprotein, and a membrane-bound tyrosine kinase (23) have all been produced.

The large vaccinia virus genome allows insertions of up to 26000 bp foreign DNA (22), and there are number of nonessential loci that have been targeted for homologous recombination, such as sites within the *HindIII* fragments C, F, and K and the hemagglutinin and thymidine kinase genes (1). Recombination vectors can be con-

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structed where a gene of interest is abutted to a native vaccinia virus promoter and is flanked by sequences homologous to a specific region of the vaccinia virus genome. These homologous sequences are able to catalyze a double reciprocal recombination event that results in the generation of a recombinant virus (2,4). While vaccinia virus recombination occurs at a very high fidelity with at least 99% accuracy, the event occurs at a very low frequency, resulting in recombination in approximately 0.1% of the progeny virus genomes (1,2). This makes selection and amplification essential to generate recombinants. Most of the techniques that are currently available rely on a specific phenotype of the recombinant virus, such as antibiotic resistance or altered plaque formation.

Though effective, the recombination systems have a major disadvantage because of the time required to isolate pure vaccinia virus recombinants. Even at a low MOI, several viruses can co-infect the same cell, resulting in the rescue of wild-type virus by recombinants and heterogeneous viral populations. Because of the low frequency of the recombination, the titer of recombinant virus must be significantly increased for the isolation of pure recombinant stocks. This requires numerous passages of the virus by transferring the total cell extract of one viral-infected culture to a new cell monolayer in the presence of a selective agent such as G418. Although this process will eventually dilute out the wild-type virus, it takes several weeks before the recombinant virus can be isolated by plaque purification. Therefore, current methods are not well suited for the rapid generation of recombinants required for a variety of purposes in molecular biology.

In this contribution, we describe a new selection methodology that was developed to overcome these limitations. The genes encoding GFP (*gfp*) and neomycin phosphotransferase (*neo*) have been fused together to generate a single protein that retains both biological activities. This bifunctional protein, BiZyme, serves to greatly reduce the time required to isolate and purify recombinant virus by coupling fluorescence to antibiotic selection. We base this technique on previous reports that

the selectable marker *gpt* can be fused to the *gus* gene for the selection of recombinant poxviruses (6) and that vaccinia virus is capable of expressing the *gfp* with no toxicity (10). However, the technique described here has some additional advantages. A homologous recombination vector, pGNR, was constructed to quickly produce purified recombinants by insertional inactivation of the vaccinia virus thymidine kinase locus with the BiZyme gene. The versatility of the pGNR selection system was demonstrated by the production of recombinant virus capable of expressing CAT and the chlamydial protein *IncA*. These results suggest that the vaccinia virus recombinants generated using pGNR can be effective tools for protein expression and a means for the rapid generation of recombinant viral vaccines.

MATERIALS AND METHODS

Construction of Vectors

The *gfp* gene was amplified from the vector pGFP-C2 (9) with primers GFP1, 5'-GGAATTCATATGGTGA GCAAGGCGAG-3', and GFP2, 5'-CCGGAATTCCTTGTACAGCTCGTC-3', which introduced the restriction sites *NdeI* and *EcoRI*, respectively. The *neo* gene was also amplified separately from pGFP-C2 with the primers NEO1, 5'-CCGGAATTCATGATTGAACAAG-3', which introduced an *EcoRI* site, and NEO2, 5'-CGCGGATCCTCAGAAG AACTCGTC-3', which included a *BamHI* site. The expression vector pSH2.0 (15) containing vaccinia virus synthetic early/late promoter (7) was then used as the cloning vector. First, *gfp* was cloned into the *NdeI* and *EcoRI* sites of pSH2.0, followed by the cloning of *neo* between the *EcoRI* and *BamHI* sites. The structure and sequence of the vector, pNEOGreen, was confirmed by sequence analysis.

The GFP-NEO recombination vector, pGNR, was derived from the pUC118 cloning vector, with the *HindIII* restriction site deleted and replaced with a *NheI* site. An approximate 600-bp region flanking the upstream portion of the J2R open reading frame (encoding for thymidine kinase) of vaccinia virus western reserve (WR)

was PCR-amplified using the primer TKUp1 to introduce a 5' *SacI* restriction site, 5'-TCCGAGCTCATACCCGATCCTATTG-3', and TKUp2 to introduce 3' sites *BamHI* and *Eco47III*, 5'-CGCGGATCCAGCGCTGATGAC-AATAAAGAAT-3'. The J2R upstream fragment was ligated into pUC118-*HindIII* between the *SacI* and *BamHI* restriction sites, generating pTKUp. The downstream flanking portion of the J2R open reading frame (TKDown) was then amplified with primers TKDown1 and TKDown2, which introduced 5' *BamHI*, *StuI*, *ClaI* sites, and a 3' *NheI* site, 5'-CGCGGATCCAGG CTTATTATTATTTTATCTAA-3' and 5'-CTAGCTAGCGATCCATTGATCTGGAAAC-3'. This segment was inserted into the *BamHI* and *NheI* sites of pTKUp to generate pTKUp/Down. The GFP-NEO fusion was then isolated from pNEOGreen by restriction with *BamHI* and *HindIII*. Sticky ends from the digest were then filled with T4 DNA polymerase to generate blunt ends for insertion into the *Eco47III* site of pTKUp/Down. The resulting pGFP-NEO clones were screened for GFP-NEO orientation complementary to the thymidine kinase flanking sequences. The 7.5 synthetic early/late vaccinia virus promoter was then isolated from the expression vector pDG4.0 as previously described (14), with oligonucleotide primers 7.5/1 and 7.5/2, which introduced an upstream *BamHI* site and a downstream *StuI* site, 5'-CGCGGATCCGGTTCGACCTGCAGATATAC-3' and 5'-AAAAGGCCTGTCACTGTTCTTTATGATTTC-3', respectively. The segment was then inserted into the *BamHI* and *StuI* sites of pGFP-NEO, generating pGFP-NEOPro. A multiple cloning site was designed and synthesized as two complementary primers, 5'-AAAAGGCCTACTAGTGTTAACCTTAAGCCCGGGATCGATGGG-3' and 5'-CCCATCGATCCCGGGCTTAAGGTAACTAGTAGGCCTTT-3', respectively. The primers were annealed and then inserted into the *StuI* and *ClaI* sites of pGFP-NeoPro, which produced a polylinker of six unique restriction sites, *StuI*, *SpeI*, *HpaI*, *AflIII*, *SmaI/XmaI*, and *ClaI* in the completed plasmid, pGNR. All intermediate plasmids were initially confirmed by restriction analysis and agarose gel elec-

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trophoresis. Final confirmation was obtained by dideoxy DNA sequencing.

Two recombination vectors containing foreign genes for insertion into the thymidine kinase locus of vaccinia virus were derived from pGNR. The CAT gene (*cat*) was amplified by PCR from pVV5.1:CAT (16) using the primers *cat1* and *cat2* which introduced the *SpeI* and *SmaI* restriction sites, 5'-GACTAGTATGGAGAAAAAATC-3' and 5'-TCCCCCGGGTTACGCCC-CGCCCTG-3', respectively. Additionally, chlamydial *IncA* was amplified from pRB21-*IncA* (26) using primers *incA1*, which introduced a *SpeI* site, and *incA2*, which introduced an *SmaI* site, 5'-GGACTAGTATGACAGTATC-CACA-3' and 5'-TCCCCCGGGTTA-ACTATCTTTATG-3'. The PCR products *cat* and *IncA* were then inserted into separate pGNR plasmids between the *SpeI* and *SmaI* sites of the multiple cloning site, generating the two recom-

bination/expression plasmids pGNR.CAT and pGNR.*IncA*. The correct plasmid sequence was confirmed by restriction analysis and DNA sequencing.

Cells and Virus

African green monkey kidney cells (BSC₄₀) were cultured in MEM (Mediatech, Herndon, VA, USA) supplemented with 10% (v/v) heat-inactivated FBS (BioWhittaker, Walkersville, MD, USA), 2 mM L-glutamine, and 10 µg/mL gentamicin sulfate. The cells were incubated at 37°C, 95% humidity, and 5% carbon dioxide. Vaccinia virus WR strain was grown and titered as previously described (21).

Engineering of Recombinant Virus

The plasmids described earlier, pGNR, pGNR.CAT, and pGNR.*IncA*, were transfected into BSC₄₀ cells that

were simultaneously infected with vaccinia virus. Ten micrograms of each plasmid were added to 1 mL infection media in separate polystyrene conical tubes. Twenty-five microliters of liposome transfection reagent DMRIE-C (Invitrogen, Carlsbad, CA, USA) were added to each tube. The tubes were vortex mixed and incubated at room temperature for 15 min. Sufficient virus was then added to each tube to achieve an MOI of 1. MEM was aspirated from cell monolayers prepared in 35-mm wells and replaced by plasmid/liposome/virus inoculum.

Recombinant virus was selected by adding 200 mg/mL (11) G418 to BSC₄₀ monolayers 24 hpi. Infections were allowed to continue for another 24 h before the cells were harvested into a 1.5-mL microcentrifuge tube by washing with culture medium. The virus and cells were pelleted by microcentrifugation at 15 000× g at 4°C for 10 min.

Following the aspiration of the supernatant, 80 μ L ice-cold PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4 , 1.4 mM KH_2PO_4 , 1 mM MgCl_2) were used to resuspend the pellet. The virus was released from the cells by a freeze-thaw method. This process was repeated three times, and the solution was subjected to centrifugation at $2000\times g$ at 4°C to pellet cellular debris. A dilution of the isolated supernatant was used to infect new cell monolayers.

For plaque purification experiments, 40 μ L viral suspension were added to 1 mL infection media. The inoculum was diluted to $10^3/\text{L}$ and then added to confluent BSC₄₀ monolayers in 35-mm well plates in serial dilutions to $10^8/\text{L}$.

A 1.2% agarose overlay supplemented with MEM was prepared by combining 1.2 g SeaPlaque™ agarose (FMC Bio-Products, Rockland, ME, USA) and 50 mL double-distilled water, autoclaving the solution, and adding 50 mL MEM after the solution cooled to 37°C . The infection inoculum was removed from the cells at 4 hpi and replaced with 1 mL prepared MEM agarose overlay. The infections were allowed to continue until plaques could be visualized and then viewed with an inverted fluorescent microscope (Leica DMIL; Bartels and Stout, Bellvue, WA, USA) to identify fluorescent plaques.

Agarose plugs containing the recombinant virus were isolated by stab-

bing fluorescent plaques with Pasteur pipets. Following resuspension in 80 μ L PBS, the recombinant virus was added to new monolayers for further amplification, large-scale preparation, and analysis.

SDS-PAGE and Immunoblotting

BSC₄₀ monolayers in 35-mm wells were infected with positive control vCAT, previously described (16), vGNR, and vGNR.IncA at an MOI of 10, or transfected with pNeoGreen, pGNR, and pRB21:IncA. The cells and virus were harvested at 12 hpi. Equal volume (80 μ L) $2\times$ SDS-PAGE sample-reducing buffer was added, and the samples were boiled for 3 min. The proteins were then resolved by discontinuous gel electrophoresis as described previously using 12.5% polyacrylamide and transferred from gels to nitrocellulose (28). The proteins were detected using antibodies directed against the vaccinia virus antigen thymidine kinase and foreign antigens IncA and GFP. Thymidine kinase and IncA antibodies obtained from polyclonal antisera produced in rabbits were used as previously described (11,26). Following the primary antibody incubation, secondary goat anti-rabbit antibody conjugated to HRP was used to facilitate detection. Polyclonal GFP antiserum was obtained from Invitrogen and used according to manufacturer's instructions. Immunoblots were developed by incubation with SuperSignal® chemiluminescent substrate solutions (Pierce Chemical, Rockford, IL, USA), followed by exposure to Biomax® MR-2 film (Eastman Kodak, Rochester, NY, USA).

CAT Assays

Monolayers of BSC₄₀ cells were grown to confluency on 35-mm plates and infected with vCAT, transfected with pGNR.CAT, or infected with vGNR. At 24 hpi, the cells were lysed by the freeze-thaw method described earlier. Pelleted total cell extracts containing cells and virus were resuspended in 80 μ L PBS and subjected to CAT assays as previously described (16). The results were quantified by relative fluorescence using percent conversion.

Figure 1. Plasmid map of pGNR. The recombination vector pGNR was designed to facilitate homologous recombination directed at the vaccinia virus thymidine kinase locus. As a derivative of the pUC family of expression vectors, pGNR contains both bacterial and phage origins of replication and an ampicillin resistance marker for ease in selection during cloning. In addition, the vector contains sequences homologous to the upstream and downstream flanks of the vaccinia virus J2R open reading frame, the gene coding for thymidine kinase.

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RESULTS

Construction of Recombination Vectors

Insertional inactivation of the vaccinia virus thymidine kinase open reading frame (J2R) provides a site within the vaccinia virus genome for the introduction of foreign genes. Previous studies have shown that the inactivation or deletion of this locus does not affect virus growth in vitro and has an attenuating effect in vivo (5) that can be considered advantageous when constructing a candidate vaccine. These considerations led to the design and construction of a recombination vector using thymidine kinase gene flanks and the bifunctional enzyme GFP-NEO, hereafter referred to as BiZyme.

BiZyme was constructed by abutting the gene sequences encoding *gfp* and *neo* within the context of a standard vaccinia virus expression vector. The stop codon of *gfp* was removed during the PCR amplification of the gene, which allowed the direct translation of *gfp* and *neo* as one continuous open reading frame, with termination at the stop codon of *neo*. *Gfp* and *neo* have open reading frames of 797 and 794 bp and are predicted to code for proteins of 30 and 29 kDa, respectively. Translation of the complete fusion cassette should result in a single protein of 530 amino acids with a predicted molecular weight of 60 kDa. The fusion gene was cloned downstream of a vaccinia virus synthetic early/late promoter to produce a complete cassette capable of expression during all stages of infection.

A recombination vector was constructed by the PCR amplification of the upstream and downstream flanks of the thymidine kinase gene and inserting them into a pUC-derived expression vector. To facilitate selection of recombinants by fluorescence and antibiotic selection, the BiZyme construct was then subcloned into the recombination vector. The pGNR vector was completed with further addition of the native 7.5 vaccinia virus promoter and six unique restriction endonuclease sites for the insertion of a foreign gene (Figure 1). Care was taken to place the synthetic early/late promoter that drives BiZyme expression and the 7.5 promot-

er in opposite orientation to ensure the optimal expression of BiZyme and the putative gene of interest. Note that different promoters were used for the two cassettes to prevent the possible deletion or "looping out" of intervening sequences during the DNA replication in viral-infected cells.

Recombination Vectors Containing Foreign Genes

Results of the fluorescent microscopy of the transfected plasmids indicated that BiZyme retained both the fluorescence and antibiotic resistance functions of its constituent proteins (data not shown). The possibility that this enzyme could be a potent selectable marker for recombinants suggested that the vector should be further analyzed for use in the selection of recombinant virus. To this end, the recombination vectors pGNR.CAT and pGNR.IncA were constructed (data not

shown). CAT, a common antibiotic resistance gene found in bacterial species such as *E. coli*, was chosen for its readily detectable and assayable activity. A vector expressing the foreign antigen *IncA*, a protein isolated from *Chlamydia* species, was also constructed to demonstrate the versatility of the recombination system in generating potential recombinant viral vaccines.

Selection of Vaccinia Virus Recombinants

Next, the ability of the pGNR vector to mediate homologous recombination and to facilitate recombinant selection was evaluated. The vectors pGNR, pGNR.CAT, and pGNR.IncA were transfected into vaccinia virus-infected BSC₄₀ cells. At 24 hpi, the appropriate amount of G418 was added to begin selection for the recombinants. After an additional 24 h, total cell extracts containing both recombinant and non-

Figure 2. Selectable plaques formed by vGNR. Following the transfection of BSC₄₀ cells with wild-type vaccinia virus and pGNR, total cell extracts were used to infect new cell monolayers. At 48 hpi, plaques identified by light microscopy (panels A and C) were also analyzed for green fluorescence. Plaques formed from wild-type vaccinia virus (panel B) showed no fluorescence, while plaques formed by recombinant vGNR (panel D) were visibly fluorescent and therefore selectable.

recombinant virus were used to infect new BSC₄₀ monolayers. Following sufficient time for plaque formation to occur, the cells were analyzed by inverted light microscopy (Figure 2, A and C) to identify plaques. To specifically isolate recombinants from the heterogeneous population, the plaques were then viewed by fluorescence microscopy to note those generated by non-recombinant (Figure 2B) or recombinant virus (Figure 2D). Fluorescent plaques from recombinant viruses vGNR (as shown in Figure 2), vGNR.CAT, and vGNR.IncA were recorded and picked for further amplification, purification,

and analysis. After purification, the virus was analyzed by PCR to test for purity. PCR data demonstrated that there was no contamination of wild-type virus genes (data not shown).

Immunoblot Analysis of vGNR Recombinant Gene Expression

Recombination mediated by the pGNR vector is intended to target the thymidine kinase locus of vaccinia virus. To test for the correct insertion of BiZyme into the viral genome and the consequent inactivation of the thymidine kinase locus, immunoblot analysis

was used to test for the presence of BiZyme and the absence of the thymidine kinase protein in vGNR-infected cells. Fluorescent plaques containing vGNR recombinants isolated as described earlier were subjected to two rounds of amplification under G418 antibiotic selection. Total cell extracts that had been harvested from the final infection were subjected to protein separation by SDS-PAGE and visualized by immunoblotting with anti-GFP and anti-thymidine kinase polyclonal antibodies separately, as shown in Figure 3.

Figure 3A, lanes 1–6, show proteins probed with α -GFP. Controls from the cells alone, vaccinia virus-infected cells, and vCAT showed no protein bands. Lysates from pNEOGreen- and pGNR-transfected and vGNR-infected all show a band of approximately 56 kDa. The predicted molecular weight of the GFP-Neo fusion protein is 60 kDa, as calculated by VectorNTI™ (North Bethesda, MD, USA) from the amino acid sequence. In Figure 3B, lanes 1 and 3 serve as negative controls because the cells do not express vaccinia virus thymidine kinase and vCAT is a known recombinant virus with the thymidine kinase gene replaced by CAT. These lanes showed no bands near the 20-kDa molecular weight of vaccinia virus thymidine kinase. Positive controls in lanes 2, 4, and 5 consisted of WR-infected cells; the pNEOGreen- and pGNR-transfected cells did show reactive bands near 20 kDa, as seen in previous experiments (12). Finally, lane 6, containing lysates from purified vGNR recombinant virus-infected cells, did not contain the 20-kDa thymidine kinase band. In summary, GFP and thymidine kinase immunoblots showed expression of BiZyme and no expression of vaccinia virus thymidine kinase in vGNR-infected cells, which indicated insertional inactivation of thymidine kinase by the BiZyme gene.

Analysis of vGNR.CAT Recombinants by CAT Assay

Having demonstrated the ability of pGNR to target and insertionally inactivate the thymidine kinase locus, it was of interest to evaluate the viability and applicability of the recombination system in expressing an enzymatically

Figure 3. Immunoblot analysis of GFP and thymidine kinase expression in recombinant infected cells. Total cell extracts were obtained from uninfected and wild-type vaccinia virus-infected cells for control lanes 1 and 2. The cells were also infected with a known and titered recombinant, vCAT, which has thymidine kinase insertionally inactivated by the CAT gene (lane 3). Lanes 4 and 5 contained extracts from the cells transfected with the expression vector pNeoGreen and the pGNR recombination vector, respectively. Lane 6 contains proteins from vGNR-infected cells. (A) Immunoblot probed with anti-GFP. (B) Immunoblot probed with anti-thymidine kinase.

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active protein. To facilitate this, the pGNR.CAT vector was transfected into WR-infected cells, and recombinants were selected and purified as described for vGNR. Total cell extracts from recombinant vGNR.CAT-infected and pGNR.CAT-transfected cells were then compared for enzyme activity to vCAT (a previously constructed recombinant expressing CAT) (16) and CAT derived from *E. coli*.

Acetylation of the 3-hydroxyl group of fluorescent BODIPY® 1-deoxy-chloramphenicol was assayed using catalysis in vitro by the above enzymes. Following the TLC separation of the reaction products, fluorometry was used to determine relative amounts of the acetylated reaction products (3-acetylated 1-deoxy-chloramphenicol) and unreacted substrate (Figure 4). The comparison of enzymatic activity by percent conversion of substrate to product showed similar activity of vGNR.CAT and vCAT after a 45-min reaction, 62.2% and 54.9%, respectively. Negative controls consisting of whole cell lysates from BSC₄₀ cells alone (1.4% conversion), WR-infected cells (0.7%), and a vGNR-infected monolayer (0.1%) showed little to no enzymatic activity. As positive controls, *E. coli* CAT displayed 28.6% conversion, while pGNR.CAT-transfected cells had 94.8% conversion.

Immunoblot Analysis of vGNR.IncA Recombinants

Once the ability of this vector to generate recombinant virus capable of producing a biologically active enzyme was confirmed, further applications of the system were explored. Specifically, the ability to create recombinant virus expressing a foreign antigen for potential use as a live vaccine was of interest. The expression of *IncA* was evaluated in this system. Total cell extracts harvested from infected cells were analyzed for *IncA* protein expression by SDS-PAGE and immunoblotting with anti-*IncA* antibody (data not shown). Consistent with previous studies, the *IncA* protein was observed to migrate as multiple bands with an electrophoretic mobility in SDS-PAGE ranging from 39 to 42 kDa (26). These data indicate that the isolated virus expresses immunoreactive foreign antigen.

DISCUSSION

The power and utility of recombinant vaccinia virus as an expression system and potential vaccine would be greatly facilitated by a more rapid and reliable selection system. To address this need, the pGNR vector containing gene flanks to the J2R open reading frame of vaccinia virus and a bifunctional protein was constructed. J2R was chosen as the target of pGNR because of the attenuating effect insertional inactivation of vaccinia virus thymidine kinase gene on the virus. This makes the system suitable for vaccine production. The fusion of the GFP and NEO gene products was chosen to allow for the selection of recombinant virus under G418, with the additional advantage of simultaneous detection based on fluorescent phenotype. The acquisition of both phenotypes virtually ensures that each and every plaque selected will be a bona fide recombinant, without any background noise caused by spontaneous mutants.

However, the use of BiZyme allows a quick and efficient means of circumventing a lengthy selection process. Visualization of fluorescent plaques can occur very soon after the initial transfection and recombination. The amplification of a presumably homogeneous virus population under antibiotic selection is the only subsequent requirement for the isolation of pure recombinant virus stocks.

To demonstrate the effectiveness of this system, the pGNR vector was transfected into vaccinia virus-infected cells. Fluorescent microscopy showed the expression of GFP in the presence of G418, a known inhibitor of protein synthesis (data not shown). This evidence suggested that the two phenotypes could be simultaneously exploited and that BiZyme could be a potent means of selection for vaccinia virus recombinants. To test the applicability of this selection process, additional recombination vectors were constructed. One such vector was designed to produce recombinant virus vGNR.CAT, which expresses the bacterial CAT enzyme. Successful generation of these recombinants was intended to demonstrate the ability to generate virus that expresses a biologically functional enzyme. The second construct enabled the isolation of vGNR.IncA, a potentially immunogenic virus expressing the *IncA* bacterial antigen from the *Chlamydia* species (26) (data not shown).

The examination of plaques formed shortly after transfection and recombination showed that potential recombinants could be quickly and easily identified by fluorescent microscopy (Figure 2). By picking specific fluorescent plaques, purified recombinants were obtained in as few as 9–11 days and used for analysis or the preparation of large-scale virus stocks. Immunoblot analysis of recombinant vGNR generated in this fashion

Figure 4. Quantitative analysis of CAT assay. Acetylated and unreacted substrates were collected from the TLC plate and resuspended in methanol. Following extraction, samples were diluted 1:100 and analyzed by fluorimetry to obtain a fluorescent intensity (FI, data table). CAT activity was measured as percent conversion of the BODIPY 1-deoxychloramphenicol to the 3-acetylated enzymatic products.

showed that BiZyme was expressed from nontransfected cells and migrated in SDS-PAGE analysis at approximately the same molecular weight as predicted by its amino acid sequence (Figure 3A). Insertional inactivation of the J2R locus was also demonstrated by immunoblot, in which recombinant vGMR-infected cells lacked expression of thymidine kinase, in contrast to cells transfected with BiZyme containing plasmids or infected with wild-type vaccinia virus (Figure 3B). These data demonstrate that pGMR specifically targets and inactivates the thymidine kinase gene by insertion of BiZyme into that locus. Furthermore, the selectable characteristics of G418 resistance and green fluorescence were successfully conferred to the resulting recombinants. Most importantly, the absence of any thymidine kinase protein demonstrates that purified virus stocks were obtained within 11 days using this method. The inactivation of thymidine kinase by BiZyme also indicates that other vaccinia virus genes could be targeted for insertional inactivation, making this an important tool for the study of vaccinia virus gene function.

This study describes the utility of BiZyme in the construction of recombinant vaccinia virus. However, using the BiZyme technology in other viral, eukaryotic, and prokaryotic systems will accelerate the selection of recombinant clones. The use of the FACS[®] (BD Biosciences, San Jose, CA, USA) could enhance the ease of selection and isolation. FACS would enable individual cells, virally infected cells, and bacteria expressing BiZyme to be isolated early during the selection process, further reducing the isolation time.

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