Structural analysis of unstable intermediate and stable forms of recombinant fowlpox virus

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The stability and structure of the products of recombinant fowlpox virus (FPV) systems using the thymidine kinase (TK) gene as the insertion site were examined. A 4.6 kb chimeric DNA fragment from the pUV1 expression vector, containing the bacterial lacZ gene and the vaccinia virus P7-5 promoter, was ligated into the XbaI site of the FPV TK gene. The resulting vector, pFTKlacZb, was transfected into chicken embryo fibroblast cultures infected with FPV at an m.o.i. of 0-1. Recombinants were screened for the expression of β-galactosidase. Five recombinants were isolated and plaque-purified to 80 to 90% for expression of β-glucosidase. Serial cell culture passage of the recombinants led to the gradual reappearance of the non-recombinant parental phenotype. Southern hybridization analysis of EcoRI fragments from all five recombinants indicated that a single cross-over homologous recombination had occurred between either the 5' or the 3' end fragments of the TK gene, generating unstable intermediate recombinants incorporating the entire pFTKlacZb vector. Secondary intermolecular or intramolecular recombination of intergenic repetitive sequences within the intermediate recombinants appears to have resulted in frequent regeneration of the parental genotype and an infrequent generation of more stable recombinants. A method was developed to select stable recombinants by passage of the intermediate recombinants in chicken embryo fibroblast cultures treated with 5-bromo-2'-deoxyuridine.

Homologous recombination has been used to introduce foreign genes into the genome of poxviruses. A foreign gene, downstream from an appropriate promoter, is inserted into a non-essential cloned region of viral DNA. The resulting expression vector is introduced into virus-infected cells by various methods of transfection. The recombinants are isolated using appropriate selection or screening methods. Vaccinia virus has been extensively used for construction of recombinants expressing genes from many human and animal viruses for the purpose of developing recombinant vaccines (Belsham et al., 1990; Chakrabarti et al., 1986; Elango et al., 1986; Moss et al., 1984; Paoletti et al., 1984). Although both single cross-over and double cross-over recombination events may generate recombinants, the former is more likely to occur. Such single cross-over events may occur within the 5' or the 3' end fragments of the non-essential viral sequences and result in the initial creation of unstable intermediate recombinants which contain one or more copies of the entire expression vector (Spyropoulos et al., 1988). A second intermolecular recombination may result in deletion of the plasmid sequences and generation of a more stable recombinant. Such stable recombinants may also be generated, along with non-recombinant parental virus, as a result of intramolecular homologous recombination between the inverted repeat regions (Falkner & Moss, 1990).

Fowlpox virus (FPV), a member of the poxvirus group, has also been used for the construction of recombinants expressing foreign genes (Boyle & Coupar, 1988; Taylor et al., 1988a, b, 1990). The thymidine kinase (TK) gene and other randomly selected non-essential regions of FPV have been used to generate these recombinants. Stable FPV recombinants that express genes related to immunity against other viral diseases of poultry (Boyle & Coupar, 1988; Taylor et al., 1988a, b, 1990) as well as mammalian viruses (Taylor et al., 1988b) have been reported. The stability and structural processes involved in arriving at these stable recombinants are not well understood. In this report we have used the expression of β-galactosidase to study the stability of FPV recombinants with the lacZ gene inserted into the TK region of the virus and have analysed the structure of the intermediate and stable recombinants.

The FPV TK is located within the 5-7 kb EcoRI Q fragment (Beisel & Nazerian, 1987). Our plasmid

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The resulting plasmid pFTK1 had a unique TK gene cloned in pUC19. This plasmid was further modified so that multiple cloning site inserted downstream from the vaccinia virus P7.5 promoter. MCS, pCEB104 contains an internal insert an plasmid contains all the sites present in the pUC19 polylinker, it was further modified by removing an EcoRI-AccI fragment from pUVI which carried the E. coli lacZ gene inserted downstream from the vaccinia virus P7.5 promoter.

Fig. 1. Construction of pFTKlacZb. The plasmid pCEB104 contains an AccI segment from the 5.7 kb EcoRI FPV fragment containing the TK gene cloned in pUC19. This plasmid was further modified so that the resulting plasmid pFTK1 had a unique XbaI site that was utilized to insert an XbaI fragment from pUVI which carried the E. coli lacZ gene inserted downstream from the vaccinia virus P7.5 promoter. MCS, multiple cloning site.

pCEB104 contains an internal AccI fragment (carrying the TK gene) cloned into the AccI site of pUC19 (C. Beisel & K. Nazerian, unpublished data). Since this plasmid contains all the sites present in the pUC19 polylinker, it was further modified by removing an EcoRI-AccI fragment. The resulting plasmid, pFTK1, is shown in Fig. 1. The 4.6 kb XbaI fragment from the pUVI plasmid (Falkner et al., 1987) contains the Escherichia coli lacZ gene regulated by the early/late promoter of the vaccinia virus 7.5K gene (P7.5), as well as a small segment of the vaccinia virus TK gene. This fragment was inserted in the unique XbaI site of pFTK1 and the resulting plasmid was designated pFTKlacZb (Fig. 1).

Chicken embryo fibroblast (CEF) cultures in 60 mm tissue culture Petri dishes were infected for 2 h with 0.1 m.o.i. of a large plaque phenotype virus (Nazerian et al., 1989) derived from FPV vaccine (CEVA Laboratories) and then were transfected with 10 μg of purified pFTKlacZb by the calcium phosphate procedure and incubated for 2 days. Progeny virus was harvested and screened for expression of β-galactosidase in the presence of Blu-o-gal. Briefly, 0.5 ml of a 5 μg/ml solution of Blu-o-gal was added to the agar overlay and 24 h later cultures were examined for the appearance of blue plaques. A rather low recombination efficiency of 1/10000 was observed. Five blue plaques were picked and virus from them was independently plaque-purified.

After four plaque purification steps, 80 to 90% purity was achieved among the five recombinants, and further plaque purification steps did not improve on this. Thus, the progeny virus isolated from each blue plaque resulted in the emergence of white plaques at a frequency of 10 to 15% in each subsequent purification step. This suggests that the five recombinants are inherently unstable. Two of the five recombinants were serially passaged in CEF cultures and were scored for expression of the lacZ gene in the presence of Blu-o-gal after each passage. The results given in Fig. 2 indicate that the recombinant viruses were unstable and the non-recombinant parental FPV had re-emerged.

After the failure to obtain stable recombinants through plaque purification, all intermediate recombinants and the non-recombinant parental FPV were separately grown in secondary CEF cultures treated 24 h prior to infection with medium containing 40 μg/ml 5-bromo-2'-deoxyuridine (BudR) and maintained in the same medium throughout the infection. Parallel untreated CEF cultures were also infected with the same viruses and showed c.p.e. typical of FPV cultures by 48 h post-infection. Cultures treated with BudR showed no c.p.e. when infected with the parental FPV, but developed limited c.p.e. after 96 to 120 h when infected with all five recombinants. The recombinants and the parental virus were serially passaged three times in the presence of BudR. The parental virus failed to grow in medium containing BudR, whereas all five recombinants grew well and produced 100% blue plaques in the presence of Blu-o-gal. Further passage of these pure recombinants in medium without BudR showed that the progeny virus from all five recombinants stably expressed β-galactosidase.

DNA from the five FPV intermediate (unstable) recombinants and BudR-selected stable recombinants...
and from the non-recombinant parental FPV was digested with EcoRI and analysed by electrophoresis in a 0.9% agarose gel and Southern hybridization on a Zeta-Probe membrane (Bio-Rad) according to the manufacturer's instructions. The results are presented in Fig. 3. When the agarose gel was stained with ethidium bromide and photographed (Fig. 3c) the profile of DNA fragments from all five recombinants was similar to that of the parental virus. No obvious shift in the size of the DNA fragments due to recombination was observed, possibly because the newly generated fragments overlapped previously existing fragments of similar size. However, when the same blot was hybridized with 32P-labelled FPV TK (EcoRI–HindIII fragment of pFTK1) obvious structural changes were observed between DNA from recombinants and from the parental virus (Fig. 3b). As expected, a 5.7 kb fragment from the parental virus (W), corresponding to the EcoRI Q fragment, hybridized to the FPV TK probe. Additional fragments of 10.3, 9.7, 9.1, 5.1, 4.5 and 3.9 kb from FPV recombinants hybridized to the FPV TK probe. The 10.3 kb fragment corresponds to the lacZ–P7.5 (4.6 kb) fusion inserted in the XbaI site of FPV TK (5.7 kb) and did not hybridize with sequences of the base plasmid (data not shown). A schematic representation of the likely recombination events giving rise to the observed EcoRI fragments is shown in Fig. 5.

Since FPV has a large genome, replacement of non-essential regions by homologous recombination has been the only approach utilized for construction of recombinant expression vectors. Taylor et al. (1988a) used a 5.5 kb PvuII segment as an insertion site for the construction of FPV recombinants. Studies on the stability of recombinants in this region and recombination frequencies were not reported. In order to construct recombinant FPV, we used the cloned TK region of FPV as an insertion site and the E. coli lacZ gene as a marker. Boyle et al. (1987) also used the FPV TK for insertion of foreign DNA. However, their analysis included only a selected stable recombinant that most likely had resulted from a double cross-over event. Spehner et al. (1990) used a 30 bp intergenic region for insertion and found both stable and unstable recombinants.
As in the case of the unstable vaccinia virus recombinants reported by Spehner et al. (1990), recombinants isolated during this study did not yield 100% blue plaques even after several rounds of plaque purification. This observation points to an inherent instability in these recombinants which may be due to either the presence of lacZ sequences within the pUC19 plasmid portion of pFTKlacZb or to the duplication of TK sequences.

DNA analysis of all five FPV recombinants prior to selection in BUdR showed similar patterns, suggesting that in all cases the original recombination event led to integration of the entire plasmid by a single cross-over event at a homologous region of the FPV TK gene. A schematic diagram of possible recombination events resulting in the insertion of the entire plasmid at the 5' end or the 3' end of the TK fragment is shown in Fig. 5. The presence of various bands in the same isolate (Fig. 3) suggests that the initial event leads to the integration of the entire plasmid. Owing to the presence of multiple copies of the TK gene, these recombinants are unstable and during each round of replication frequent inter- and intramolecular recombination occurs. Since the virus goes through several rounds of replication in the process of plaque formation, a variety of recombinants are generated within the same plaque. Thus, each plaque isolate remains a mixture of several recombinants generated by secondary events. In addition, the pFTKlacZb plasmid sequences generated following recombination of pFTKlacZb with PFV DNA may replicate in CEF cells independently of FPV replication and give rise to the 3.9 kb fragment that hybridizes both to the TK probe and the lacZ probe.

When grown in BUdR-treated CEF cultures, intermediate recombinants containing multiple copies of the TK gene and the TK+ parental FPV failed to replicate and only recombinants containing one interrupted copy of the TK gene were recovered and remained stable. These observations demonstrated that the screening procedure for expression of lacZ was useful for initial isolation of recombinants, but it did not differentiate between the intermediate and stable recombinants. Selection in the presence of BUdR served to isolate and purify such recombinants.

The products of recombination in vaccinia virus-infected cells have been thoroughly investigated (Spyropoulos et al., 1988) and it has been shown (Falkner & Moss, 1990) that the most frequently generated initial recombinants occur as a result of a single cross-over event leading to the integration of the entire plasmid. Our study is the only one on the analysis of random recombination events involving the TK region of FPV. It appears that, as with vaccinia virus, the frequency of reciprocal exchange of plasmid and virus DNA mediated by a double cross-over is significantly lower than plasmid integration resulting from a single cross-over event. Although the calcium phosphate transfection procedure used in these experiments yields a high number of transiently expressing cells (Dhawale et al., 1990), the frequency of recombination was only 0.01%. This is in agreement with the frequencies reported by Spehner et al. (1990) and may explain why stable
recombinants were not directly obtained by selection for expression of β-galactosidase. The number of initial recombinants generated in this study may not have been high enough to allow detection of a double cross-over event. Transfection of FPV-infected cells with linearized plasmid may increase the chance of such an exchange and consequently result in generation of stable recombinants, although the overall efficiency of recombination may be lower than when circularized plasmid is used for transfection.

Studies on random recombinants using other plasmids are in progress and may provide further insights into recombination events in FPV-infected cells. Procedures developed in this study for selection of TK- recombinants in TK+ cells in the presence of BUdR may greatly facilitate isolation of similar TK- stable recombinants where inserts used are those other than the lacZ gene.

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References


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