Analysis of the fowlpox virus genome region corresponding to the vaccinia virus D6 to A1 region: location of, and variation in, non-essential genes in poxviruses

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The DNA sequence of the fowlpox virus genome corresponding to the vaccinia virus D6 to A1 region has been determined. Translation of this sequence reveals fowlpoxvirus gene homologues corresponding to the D6, D7, D9, D10, D11, D12, D13 and A1 genes of vaccinia virus. In contrast, no gene homologue for the non-essential vaccinia virus D8 gene was present in fowlpox virus. Instead, a gene transcribed from the opposite strand to the vaccinia virus D8 gene showing no homology to any previously sequenced poxvirus gene was present. The amino terminus of the fowlpox virus D9 homologue had undergone substantial changes, including frameshifts which would be predicted to inactivate the gene. Insertion of a gene cartridge composed of the vaccinia virus p7.5 promoter and the lacZ gene into the fowlpox virus D8, D9 and D10 genes in vitro, followed by recombination into fowlpox virus, was carried out. Stable insertion mutants with the correct genotype were obtained for D8 and D9 which, when tested in chickens did not appear to have been attenuated. No stable insertion mutants were obtained for D10, indicating that this gene probably encodes a function which is essential for virus replication. The D8 and D9 genes of fowlpox virus represent useful insertion sites for the construction of recombinant fowlpox virus vaccines.

Introduction

The HindIII D fragment of the orthopoxvirus vaccinia is one of the best characterized regions of the vaccinia virus genome. The fragment has been completely sequenced and found to contain 13 genes, D1 to D13 (Niles et al., 1986; Weinrich & Hruby, 1986). Transcriptional analysis (Lee-Chen & Niles, 1988a) has located the initiation and termination sequences of the seven early genes in this region (Lee-Chen et al., 1988) and the 5' ends of eight mRNAs synthesized from the HindIII D late genes have been mapped (Lee-Chen & Niles, 1988b; Weinrich & Hruby, 1986). A molecular dissection of the promoter elements for the D11, D12, D13 and A1 genes has also been carried out (Miner et al., 1988; Miner & Hruby, 1989). The functions of several genes within the vaccinia virus HindIII D fragment have been determined. These include D11, which is a nucleic acid-dependent ATPase (Rodriguez et al., 1986; Broyles & Moss, 1987), and D12, which is the small subunit of the guanylyl-transferase mRNA capping enzyme (Niles et al., 1989). It is likely that the D7 gene encodes the 21K subunit of RNA polymerase (Jones et al., 1987) and the D13 gene is the rifampicin resistance locus of vaccinia virus (Tartaglia & Paoletti, 1985; Tartaglia et al., 1986; Baldick & Moss, 1987), although the normal viral function of this protein has not been determined. The vaccinia virus D8 gene encodes a non-essential virion transmembrane protein (Niles & Seto, 1988) which shows 36% identity to carbonic anhydrase (Niles et al., 1986).

In addition to the functional assignments described above, temperature-sensitive (ts) mutants have been mapped to seven genes within the vaccinia virus HindIII D fragment, D2, D3, D5, D6, D7, D11 and D13 (Seto et al., 1987). This demonstrates that these genes encode essential viral functions. When the phenotypes of these ts mutants were examined at the non-permissive temperature, mutations in D7 and D11 resulted in defective late protein synthesis, whereas a D5 mutant exhibited a DNA-minus phenotype (Seto et al., 1987). It has recently been determined that D12 is also an essential gene (Niles et al., 1989).

As part of our interest in using fowlpox virus as a vaccine vector for birds and mammals we have been carrying out an extensive analysis of the fowlpox virus genome (Binns et al., 1987, 1988, 1989; Tomley et al., 1988; Campbell et al., 1989). We are also interested in determining the extent to which gene organization has

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been conserved in different poxvirus genera because, at approximately 280 kb, the fowlpox virus genome is considerably larger than that of vaccinia virus, which is about 185 kb. We are particularly interested in locating non-essential fowlpox virus genes which could serve as sites for the insertion of foreign genes, and in the location of strong early and late fowlpox virus promoters with which to drive the expression of foreign genes.

A search for non-essential genes was carried out within a region of the fowlpox virus genome corresponding to the right half of the vaccinia virus HindIII D fragment. Such an approach, as well as identifying non-essential genes, will also allow a detailed comparison of the transcriptional regulatory signals of members of two genera of poxviruses to be carried out in the future.

Methods

Viral DNA isolation and cloning. Fowlpox virus DNA was isolated from strain HP444 as previously described (Binns et al., 1987). Viral DNA (5 µg) was restricted with either EcoRI or DraI and ligated into pUC13 vector DNA which had been cleaved with either EcoRI or Smal. After transformation into Escherichia coli TGI (Hanahan, 1983), recombinant clones were identified as white colonies on X-gal plates. An M13 clone, MFP142, showing homology to the vaccinia virus D9 gene was identified within a random fowlpox virus library, the construction, sequencing and computer analysis of which has been described (Binns et al., 1987, 1988, 1989). A prime-cut probe (Farrell et al., 1983) was generated from the fowlpox virus insert present in MFP142 and used to probe the pUC13 EcoRI and DraI libraries by colony hybridization (Grunstein & Hogs, 1975). An EcoRI clone, pMB379, and a DraI clone, pMB389, showing positive hybridization to the MFP142 probe were subjected to further analysis and subcloning as described later.

DNA sequencing and computer analysis. Random subclones of pMB379 and pMB389 were generated by sonication (Deininger, 1983), followed by end-repair, size selection and ligation into Smal-cleaved, phosphatase-treated M13mp10 vector (Amersham). M13 clones containing fowlpox virus inserts were identified by probing with 32P-labelled inserts from pMB379 and pMB389, labelled using a random-primer procedure (Pharmacia). Positive clones were then sequenced by the deoxyribonucleotide method using [35S]dATP and buffer gradient gels (Biggin et al., 1983). Sequencing data were analysed on a VAX 11/750 using the programs of Staden (1982, 1984). Comparison of fowlpoxvirus sequences with a database containing all the published D10 genes respectively is shown in Fig. 5. D8 cloning involved the use of FASTP (Lipman & Pearson, 1985). Fowlpox virus homologues of genes corresponding to the vaccinia virus D6, D7, D10, D11, D12, D13 and A1 genes were identified. These showed a level of amino acid identity of 59% for D7, 40% for D10, 62% for D11, 53% for D12, 55% for D13 and 49% for A1. In addition, a homologue of the vaccinia virus D9 gene was present in fowlpox virus but had undergone three frameshifts in the sequence. A transla-

Results and Discussion

Analysis of the sequence and open reading frames (ORFs) encoded by pMB379 and pMB389

The nucleotide sequences of plasmids pMB379 and pMB389 was determined as described in Methods. The sequences were assembled and are presented in Fig. 1 along with a translation of the major ORFs. The arrangement of these ORFs within pMB379 and pMB389 is shown in Fig. 2. These ORFs were compared with our database of vaccinia virus genes using FASTP (Lipman & Pearson, 1985). Fowlpox virus homologues of genes corresponding to the vaccinia virus D6, D7, D10, D11, D12, D13 and A1 genes were identified. These showed a level of amino acid identity of 59% for D7, 40% for D10, 62% for D11, 53% for D12, 55% for D13 and 49% for A1. In addition, a homologue of the vaccinia virus D9 gene was present in fowlpox virus but had undergone three frameshifts in the sequence. A translation of the fowlpox virus D9 gene showing these frameshifts is shown in Fig. 3. The origins of the frameshifts are unknown; the sequence determined comes from a fowlpox virus isolate that has been highly passaged in vitro and it would be interesting to determine the sequence of D9 in a recently isolated strain of fowlpox virus. The frameshifts could in principle result from cloning or sequencing artefacts. The presence of the
Fig. 1. Assembled nucleotide sequence of the pMB379 and pMB389 plasmids with translation of the major ORFs using the single-letter amino acid code. The sequence for pMB389 extends from 1 to 3474 nucleotides and that of pMB379 from 2004 to 8358 nucleotides. Restriction sites used in the clonings described in the text are underlined (DraI, BgII, AsuII, EcoRI, BcII). Also underlined are early poxvirus termination signals (TTTTTNT) and the repeat sequence surrounding GAATTC.

Fig. 2. Arrangement of ORFs D6 to A1 within the plasmids pMB379 and pMB389. Also shown are the positions of the restriction sites used in the construction of the D8, D9 and D10 insertion mutants.
frameshift around position 2188 bp in both pMB379 and pMB389 suggests that the sequence at this point is present in the viral genome. The sequence around the frameshift at 1958 bp has been determined in both directions, from three single-strand templates, making a sequencing error at this position unlikely. In contrast, the frameshift at 2014 bp has only been determined to exist in poxviruses (see Howard & Smith, 1989 for examples), although as yet the function of D9 and D10 is not known. In contrast to all the other gene families so far identified, the D9 and D10 genes are adjacent to each other. The finding that D9 and D10 are related to each other is of relevance in fowlpox virus where the D9 gene requires one of these genes to be maintained intact.'
The organization of the fowlpox virus genes within the sequence is very similar to that in vaccinia virus with two exceptions (in addition to the frameshifts in the D9 gene noted above). Firstly, in fowlpox virus the D6 and D7 genes overlap by 14 nucleotides (see Fig. 1), whereas in vaccinia virus these genes are separated by 27 nucleotides. Interestingly, the homology between the fowlpox virus and vaccinia virus D6 and D7 proteins extends to the carboxy terminus of D6 and begins at the amino terminus of D7. Therefore fowlpox virus encodes these two regions in one DNA sequence whereas they are encoded by two DNA sequences in vaccinia virus. The D7 transcription initiation site in vaccinia virus has been mapped to the 27 nucleotide gap which is missing in fowlpox virus and this region therefore probably contains the early promoter sequence. D7 is thought to encode a small subunit of RNA polymerase and is an essential gene, which in turn suggests that the fowlpox virus gene must be transcribed and translated. It would be of interest to map the 5' end of the fowlpox virus D7 mRNA, which presumably lies within the coding region of the D6 gene, and to compare the DNA sequence at this point with that in vaccinia virus.

The second region in which fowlpox virus gene organization differs from that in vaccinia virus concerns the sequences between the 3' end of the D7 gene and the 5' end of the D9 gene. In vaccinia virus, the D8 gene is encoded on the opposite strand to the D7 and D9 genes, the gene being initiated 41 bp upstream from the D9 initiation codon and overlapping with the 3' end of the D7 gene. The D8 gene encodes a virion transmembrane protein of 304 amino acids, which shares homology to horse carbonic anhydrase. The D8 gene has been shown to be non-essential for virus propagation in tissue culture. In fowlpox virus no homologue of the vaccinia virus D8 gene is present in the equivalent position. Instead a protein of 275 amino acids is encoded on the same DNA strand as the D7 and D9 genes; unusually large non-coding sequences flank the fowlpox virus gene at the 5' (163 bp) and 3' (249 bp) ends. Over the rest of the sequence between D6 and A1, only 131 bp are non-coding, which is more typical of the tightly packed gene arrangement seen in poxviruses. The fowlpox virus D8 protein shows no homology to any other poxvirus protein in our database of all published poxvirus sequences. When compared against the PIR database (Release 40, September 1989) using FASTA, the fowlpox virus protein shows limited identity with the Neisseria gonorrhoeae IgA protease precursor. The match involves 17.1% identity and 50% similarity over a 111 amino acid stretch, with only one pad in either sequence, and gives an optimized score of 116. The IgA protease protein is composed of three domains, signal sequence, protease and helper, and although the region of identity lies within the protease domain it does not extend to the proposed active site of the protease (Pohner et al., 1987). In vaccinia and cowpox viruses, genes which are thought to interfere with the complement system and with coagulation have already been identified (Kotwal & Moss, 1988; Pickup et al., 1986). We speculate that the gene may be transcribed at early times post-infection as it contains no early poxvirus termination signals (TTTTNTT) (Yuen & Moss, 1987) within the coding region but has such a signal, TTTTTCT, 55 bp beyond the translation termination codon for the gene. When the codon usage of the fowlpox virus D8 gene is examined it conforms well with other fowlpox virus genes. When the hydrophilicity plot of the fowlpox virus D8 protein is compared to one of the vaccinia virus D8 protein no similarity can be seen and, in particular, there is no obvious carboxy-terminal membrane anchor as is seen in the vaccinia virus protein.

It is possible that the frameshifts present in the fowlpox virus D9 gene were the result of the insertion of the D8 gene and that the disrupted 5' end of the D9 gene was not homologous to the vaccinia virus D9 gene. Fig. 3 clearly shows however that amino acids conserved between the fowlpox virus and vaccinia virus D9 proteins are present before the frameshifts, indicating that the frameshifts are not directly related to the insertion of the D8 sequence. The mechanism whereby the D8 genes have been changed in vaccinia virus and fowlpox virus is unknown. We note however the presence of direct repeats sharing 14 of 15 nucleotides which are present in the fowlpox virus sequence at the 3' end of the D7 gene and just 5' of the D9 gene (see Fig. 1). These would be consistent with a retrotransposon mechanism of translocation and we note that short 9 bp direct repeats flank a copy of a retroviral protease-like gene in vaccinia virus (Slabaugh & Roseman, 1989), suggesting that poxviruses may acquire genes in this manner.

Sites have been underlined in the D11 and D13 protein sequences in Fig. 1. These correspond to the consensus nucleotide binding sites in D11 and to two sites in D13 to which mutations to rifampicin resistance in vaccinia virus have been mapped. In the first of these positions, fowlpox virus retains the wild-type vaccinia virus amino acid glutamine. At the second site, which in vaccinia virus involves the loss of a potential glycosylation site in the rifampicin-resistant mutant, the fowlpox virus sequence differs from wild-type vaccinia virus. The HP444 fowlpox virus strain is resistant to rifampicin (data not shown) and it is tempting to speculate that the sequence at this second site may be responsible for the observed resistance. It may alternatively be due to any of the other differences that exist between the vaccinia virus and fowlpox virus D13 proteins.
The fowlpox virus D10, D11, D12, D13 and A1 genes all have sequences closely related to the consensus sequence $\mathrm{A}^3\mathrm{TAAAT}\mathrm{A}^3$ determined for vaccinia virus late promoters (Hanggi et al., 1986) in a position close to or including their ATG translation initiation codons. Recently it was found that the vaccinia virus DNA ligase gene, which also possesses a consensus late promoter sequence in this position, is predominantly transcribed early during infection (Smith et al., 1989), with the 5' end of the mRNA mapping within the late consensus sequence. This means that, although the D10, D11, D13 and A1 genes are all transcribed late during infection, in vaccinia virus we cannot assume that the presence of the late motif indicates that the fowlpox virus homologues are also expressed late during infection. However, the presence of early transcription termination signals within the D11 and D12 genes strongly suggests that these are expressed late in fowlpox virus.

Location of non-essential genes

In order to construct recombinant fowlpox viruses for use as prototype multivalent vaccines it is necessary to locate non-essential viral genes into which foreign genes can be inserted without disrupting essential viral functions. Several such genes have been located in vaccinia virus, many of which result in some level of attenuation of the virus, e.g. inserts into the thymidine kinase and virus growth factor genes (Buller et al., 1985, 1988). Plasmids were therefore constructed for recombination into fowlpox virus which contained insertions into the fowlpox virus D8, D9 and D10 genes to test the non-essential nature of these genes. The insertion consisted of a gene cartridge with the lacZ gene under the control of the vaccinia virus p7.5 promoter. The strategies used to construct the relevant plasmids are shown in Fig. 5. Once constructed, the plasmids were transfected into fowlpox virus-infected cells for recombination to take place. Virus was then harvested and replaqued under an overlay containing X-gal, at which time recombinant virus could be identified as blue plaques. Such virus was subjected to three rounds of plaque purification, after which a stock of virus was grown and DNA was isolated. This DNA was cleaved with BclI (which generates a fragment containing all the insertion sites and which does not cleave within the lacZ gene cartridge), run on a 0.8% agarose gel, Southern-blotted and probed with a 1.3 kb EcoRI fragment from pMB389 which hybridizes to the DraI fragment containing the D8, D9 and D10 insertion sites. The blot was also probed with a lacZ probe and the results of both experiments are shown in Fig. 6. The results show that for the D8 and D9 insertions both probes hybridize to a single fragment of about 6850 bp and 7500 bp respectively (the D8 fragment is slightly smaller than the D9 fragment due to the removal of the 583 bp BclI fragment during the construction of pMB447, see Fig. 5). Wild-type fowlpox virus DNA showed one fragment of about 3500 bp when probed with the fowlpox virus probe and did not show any bands with the lacZ probe. The situation with the D10 inserts is more complicated and is typical of an unstable insertion. A band corresponding to that in wild-type virus is present when probed with the fowlpox virus probe, in addition to three other bands, one of which corresponds to the size expected for the correct insertion (faint band of the same size as the adjacent D9 insert in Fig. 6). When probed with the lacZ probe, four bands are again observed, three of which correspond to those observed with the fowlpox virus probe. In addition, a small fragment of about 2.6 kb is observed. These numerous bands in both probings probably result from a single crossover recombination event such that a complete copy of the pMB443 plasmid has been inserted into the fowlpox virus genome. This results in the maintenance of a complete copy of the D10 gene (which would be

![Fig. 5. Schematic representation of the cloning stages involved in the construction of lacZ insertions into the D8, D9 and D10 genes of fowlpox virus. For the D8 insertion, the BclI fragment was removed from pMB389 to give pMB441 which contains a unique BclI site. For D10, the EcoRI fragment containing D10 was cloned from pMB389 into EcoRI-cleaved pUC13 to give pMB439 which contains a unique BgII site in the D10 gene. Cloning of the p7.5/lacZ BamHI cartridge into pMB441 and pMB439 was carried out directly as BamHI, BclI and BgII all generate compatible sticky ends. For the D9 insertion, pMB389 was cleaved with AsuII, end-repaired and ligated with the end-repaired lacZ cartridge.](image)
Fig. 6. Southern blot of DraI digests showing the genomic arrangement of the D8 (lanes 2), D9 (lanes 3) and D10 (lanes 4) insertion mutants and wild-type fowlpox virus (lanes 5). Panel (a) was probed with an EcoR1 probe derived from pMB389 which hybridizes to the DraI fragment which contains all three insertion sites. Panel (b) was probed with a lacZ probe. Lanes 1 contain lambda HindIII fragment size markers.

required if the gene is essential) and the presence of additional sequences, including a copy of the D10 gene containing the lacZ cartridge. On passage the D10 insertion mutant generated some white plaques which probably resulted from further recombination events which can generate a wild-type virus structure. Five blue plaques from two independent transfections were purified for the D10 insertions, all of which showed a similar genome structure, strongly suggesting that the D10 gene is essential.

In order to assess whether the insertions into the fowlpox virus D8 and D9 genes attenuate the virus, insertions identical to those described above were introduced into the Poxine strain of fowlpox virus. This was necessary as HP440 is attenuated to the extent that it does not produce lesions in chickens. In contrast, Poxine strain virus produces lesions near the site of wing-web inoculation in young chickens. The insertions into the Poxine strain virus were checked at the DNA level and found to be identical to those described above for HP440. The results of assessing lesion scores in 2-week-old chickens given different doses of the Poxine strain virus and the Poxine strain recombinants by the wing-web route are shown in Table 1. The results show that the insertion mutants do not appear to have been attenuated.

The results presented above demonstrate that genes within the HindIII D fragment of vaccinia virus (an orthopoxvirus) are largely conserved in another genus of poxvirus, the avipoxviruses. In addition to the D6, D7, D9, D10, D12 and D13 genes described here, we have also noted the presence of homologues to the D1 and D5 genes in a random library of fowlpox virus M13 clones (data not shown). Fowlpox virus also contains homologues of the vaccinia virus genes I3, I5, I6, I7, I8, G1, G2, G3, G5, L1, L2, L3, L4, L5, J1, J3, J4, J6, H1, H2, H3, H4, H5, H6, H7 and H8 (M. M. Binns, unpublished observations), suggesting that a large central portion of the vaccinia virus genome is conserved across different genera. This supports the idea that the central region of poxviruses encodes functions important to viral replication and morphogenesis whereas the more terminal regions encode virus-specific functions such as host-range genes and genes involved in virulence.

The replacement of the non-essential D8 gene in vaccinia virus with a different but non-essential gene in fowlpox virus is reminiscent of the organization of the fowlpox virus genes in the equivalent of the vaccinia virus HindIII L and J regions, where gene collinearity exists with the exception of the non-essential thymidine kinase gene which is translocated elsewhere (Drillien et al., 1987; Binns et al., 1988). It would appear that some of the non-essential genes have far greater mobility than many of the essential genes, whose organization is strictly conserved.

The finding that the D10 gene appears to encode an essential function is of interest, particularly as D9 to which it is related is non-essential. It could be predicted that it would be possible to make insertions into the D9 gene because the fowlpox virus gene had been disrupted by three frameshift mutations which render the gene non-functional. In vaccinia virus it has been determined that the D9 gene is transcribed early after infection,
whereas the D10 gene is transcribed at late times. It will be interesting to see whether either or both genes are non-essential in vaccinia virus.

The demonstration that stable insertions can be made into the fowlpoxvirus D8 and D9 genes indicates that these should provide useful non-essential genes for use in the construction of recombinant vaccines. The fact that insertion into these sites does not appear to attenuate the virus means that these sites may be particularly useful in the construction of multivalent vaccines where a number of foreign genes are inserted into several different non-essential genes. If most of these insertions resulted in attenuation it is possible that the virus containing several foreign genes could be attenuated to such an extent that it would no longer be able to stimulate effective immune responses in the host. The location of the apparently neutral insertion sites in the D8 and D9 genes should therefore facilitate the development of recombinant multivalent fowlpox virus vaccines.

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References


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