A 39000 M₁ immunodominant protein of fowlpox virus contains multiple copies of a 12 amino acid repeat sequence

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The nucleotide sequence of an unusual fowlpox virus gene which maps immediately upstream from the fowlpox virus 4b gene has been determined. The 34000 M₁ protein predicted to be encoded by the gene contains 11 copies of a 12 amino acid serine-rich repeat sequence. The seven amino-terminal copies of the repeat sequence are perfectly conserved but variation exists in the four carboxy-terminal copies. Three peptides were synthesized which contained either one copy of the repeat sequence, two copies of the repeat sequence or a hydrophilic amino-terminal region of the protein. All three peptides when injected with adjuvant into rabbits gave rise to antibodies which reacted strongly on Western blots of purified fowlpox virus proteins with a 39000 M₁ protein. When directly compared in Western blots the antipeptide sera were shown to recognize a protein comigrating with one of the two immunodominant proteins recognized by chicken anti-fowlpox virus sera taken 2 weeks post-infection. The virion protein is removed by treatment with sodium deoxycholate suggesting that it is located at or near the surface of the virus.

Introduction

Live attenuated strains of fowlpox virus have been used extensively as vaccines in the poultry industry against virulent strains of fowlpox virus and in general most poxviruses appear to be potent immunogens. Poxviruses induce long-lasting, strong humoral and cellular responses in their hosts although at present the basis of this response is unknown. Recently there has been great interest and debate about the possibilities of using poxviruses as live viral vaccine vectors into which foreign immunogen genes are cloned and expressed. Fowlpox virus was initially viewed as an ideal vector for control of the many diseases that confront the highly intensive poultry industry (Brown, 1984; Binns et al., 1986). More recently it has been demonstrated that fowlpox virus is also a suitable vector for a number of mammalian species (Taylor et al., 1988), possibly including humans. Although the replication of fowlpox virus is limited to avian hosts the virus is able to undergo sufficient gene expression in mammalian cells to be able to induce protective immune responses against foreign antigens cloned into it. It thus could provide an extremely safe vector system having the advantages of both the effectiveness of a live vaccine and the safety of a killed vaccine. If fowlpox virus is to be applied broadly it is of importance to determine which of the viral products modulate the strong immune responses in the host and to see whether these can be manipulated to any advantage in recombinant viruses. In this paper we describe the characterization of one important immunogenic protein of fowlpox virus.

Methods

Cloning and sequencing of fowlpox virus DNA. The cloning of pMB371 has been described previously (Binns et al., 1989). pMB371 was sequenced using the dideoxynucleotide chain termination method (Sanger et al., 1977) using [³²S]dATP and buffer gradient gels (Biggin et al., 1983). Sequence data were analysed using the programs of Staden (1982, 1984) and amino acid comparisons carried out using FASTA (Pearson & Lipman, 1988) with the PIR database (Release 40, September 1989).

Use of peptides to generate specific antisera. Three peptides, NH₂-EGTSSSVGLAPC-CONH₂, NH₂-QGTSSVSGLAPQGTSSSVGLAPC-CONH₂ and NH₂-EKLDEDDSRNEKEC-CONH₂, containing one copy of the repeat sequence, two copies of the repeat sequence or a hydrophilic region from the amino end of the protein, respectively, were prepared by solid-phase synthetic methods (Merrifield et al., 1982). Additional cysteine residues were added to the termini of the peptides to promote the formation of cross-links between peptides with the aim of eliminating the need for a carrier protein. Three adult New Zealand rabbits were inoculated intramuscularly three times with 1 mg of peptide for the first inoculation and 500 µg for the two subsequent inoculations. For the first inoculation the peptides were emulsified with an equal volume (500 µl) of Freund's complete adjuvant. Subsequent booster inoculations were carried out using
Freund's incomplete adjuvant 30 and 50 days after the initial inoculation. The rabbits were bled 20 days after the last inoculation.

**Immunoblotting.** Purified fowlpox virus virions (Mockett et al., 1987) were heated at 100 °C for 5 min in Laemmli's loading buffer with DTT and proteins were separated on 10% polyacrylamide gels. After electrophoresis one lane of fowlpox virus proteins and one of M, markers were stained with Coomassie blue. Proteins in the remainder of the gel were transferred electrophoretically to nitrocellulose at 30 V for 16 h. Antigens were detected as described by Mockett et al. (1987). Briefly, strips of nitrocellulose were incubated with preimmune rabbit sera (1/50 dilution) or antipeptide rabbit sera (1/50 dilution) followed by alkaline phosphatase-conjugated goat anti-rabbit IgG (1/1000 dilution). Further strips of nitrocellulose were incubated with either normal chicken serum (1/100 dilution) or chicken antisera taken 2 weeks after fowlpox virus inoculation (1/500 dilution). These strips were then incubated with alkaline phosphatase-conjugated rabbit antichicken IgG.

Purified fowlpox virus virions were incubated with 0.25 M-NaCl, 0.1 M-Tris—HCl pH 8.5, 10 mM-DTT and 0.2% sodium deoxycholate (Rosel & Moss, 1985) for 30 min at 0 °C. Cores were then pelleted by centrifugation at 35000 r.p.m. for 30 min at 4 °C in an SW40 rotor. The protein pellet was dissolved in sample loading buffer (0.0625 M-Tris-HCl pH 6.9, 4% SDS, 2% mercaptoethanol, 10% glycerine, 0.003% bromophenol blue) and heated at 100 °C for 3 min. Proteins were separated on a 10% polyacrylamide gel and immunoblotted as described above.

**Results**

**Sequence analysis of the gene containing tandem repeats**

During sequencing of pMB371 to identify the 4b core protein late promoter (Binns et al., 1989), sequences which were clearly repetitive in nature were observed. The tandemly repeated sequences consisted of 36 bp and formed a distinctive ladder pattern. The sequence of pMB371 was therefore completed to determine whether the tandem repeats were within a coding region. The sequence of 1305 bp from pMB371 is presented in Fig. 1 together with a translation of the major open reading frames (ORFs) encoded in this region.

Analysis of the sequence showed that the repeated sequences did indeed lie within a coding region and that 11 copies of a 12 amino acid repeat sequence (one of the copies contains only 11 amino acids) were present within a protein with a predicted M, of 34091. When the repeated sequences were examined more closely it could be seen that the first seven copies of the repeated sequence were perfectly conserved whereas the last four copies had diverged extensively so that only two amino acids were completely conserved in all 11 copies of the repeats (see Fig. 2). In addition, the ninth repeat consisted of only 11 amino acids. The sequencing of the repeat region presented problems in that it was not possible to sequence through all the repeats in one sequencing run. We believe that there are 11 copies of the 36 bp repeat for the following reasons. M13 clones starting close to either side of the repeats were sequenced into the repeats. In one case the presence of seven perfect copies followed by divergence in the repeat sequence was noted and in the other case four non-perfect repeats were followed by a series of perfect repeats. In addition, pMB371 was cleaved with EcoRV which cuts on either side of the repeats to yield a fragment with a predicted size of 646 bp. An EcoRV fragment of 656 bp was observed on an agarose gel supporting the proposition that 11 copies of the repeat are present (data not shown).

A hydropathicity plot is shown in Fig. 3 where it can be seen that the first seven copies of the repeats have hydrophilic and hydrophobic regions whereas the last four copies of the repeats are considerably altered. This diagram also reveals that there is no obvious amino-terminal signal sequence. Further analysis of the sequence revealed that there are three potential N-linked glycosylation sites (see Discussion) and no cysteine residues are present in the protein indicating that it cannot form aggregates held together by disulphide bonds. When the predicted amino acid sequence of the 39K protein was compared with the PIR protein sequence database using FASTA, the highest scores were against collagen alpha I (III) chain (optimum score 133) and circumsporozoite protein from Plasmodium berghei (optimum score 113). These proteins both contain repeat units which partially match the repeat units described here. It is not thought that these matches are significant as they do not extend outside the repeat regions of the 39K protein.

**Demonstration that the gene containing tandem repeats is translated**

As no other poxvirus genes containing tandem repeats have been described it was of interest to confirm that the ORF containing the repeats was actually transcribed and translated. The approach taken involved the synthesis of three peptides, one containing one copy of the repeated sequence, one containing two copies of the repeated sequence and the third containing a hydrophilic region near the amino terminus. The peptides were then used in rabbits to raise specific antisera which were used in Western blots to determine whether the gene product was produced. The result of such a Western blot using the antipeptide sera against one copy of the repeat is shown in Fig. 4. All three peptides raised antisera which reacted in a similar manner with a virion protein of M, 39000 showing that the gene containing the repeats is translated. The size of the protein as estimated in 10% polyacrylamide gels is slightly larger than that predicted from translation of the DNA sequence and may reflect either post-translational modification or an aberrant
The 39000 M₆ protein is highly immunogenic

As several proteins containing tandemly repeated amino acid sequences are highly immunogenic (e.g. streptococcal M protein, plasmodium circumsporozoite antigen), we wished to determine whether the 39000 M₆ protein was strongly recognized by chickens during fowlpox virus infection. It had previously been noted (Mockett et al., 1987) that chicken antiserum taken at early times after a primary inoculation with fowlpox virus contains antibodies to a very limited number of virus proteins and...
that the major reaction is against a protein(s) with an $M_r$ of approximately 37000. Using the same antiserum as used in the above work we were able to show that the antipeptide sera recognize a protein of $M_r$ identical to that reacting strongly with chicken anti-fowlpox virus sera when tested using strips of nitrocellulose from the same immunoblot (see Fig. 4). It is therefore probable that the 39K protein, containing the repeated sequences is an immunodominant antigen of fowlpox virus virions.

**Distribution of the 39K protein**

In order to locate the 39K protein within the fowlpox virus virions, viral cores were produced by treating virions with sodium deoxycholate and DTT to remove the viral envelope. The proteins present in the core preparation were examined by polyacrylamide gel electrophoresis followed by Western blotting and incubation with the rabbit antipeptide antisera. The results are shown in Fig. 5 where it can be seen that the majority of the 39K protein has been removed from the core preparation (Fig. 5, lane 1) although trace amounts remain. It can be seen that the amount of 39K protein in approximately the same concentration of untreated virus is much greater (Fig. 5, lane 2). The 39K protein has therefore been stripped from the virion by the detergent.
dominant protein in vaccinia virus to the region between Maa & Esteban (1987) have mapped a 39K immuno-
treatment and is probably located at or near the viral surface.

Discussion

In this paper we report the identification of an immunodominant 39K protein in fowlpox virus which contains 11 copies of a 12 amino acid tandem repeat. No proteins containing tandem repeats have previously been identified in poxviruses although in the herpesvirus, varicella-zoster virus, variable numbers of a 42 bp repeated sequence have been seen within the glycoprotein C homologue (Kinchington et al., 1986).

The 39K protein maps immediately upstream of the fowlpox virus 4b core protein and it is of interest that Maa & Esteban (1987) have mapped a 39K immunodominant protein in vaccinia virus to the region between the 4b and the 4a genes. The immunogenic vaccinia virus protein was identified from within a agt11 expression library using hyperimmune rabbit anti-vaccinia virus serum. The nucleotide sequence of this region in vaccinia virus has not yet been determined so it is not possible to deduce whether the protein reported here is the same as that observed in vaccinia virus. A difference between the reported virion location of the vaccinia virus protein and that determined here for the fowlpox virus protein exists. The vaccinia virus 39K protein was located in the viral core whereas the fowlpox virus 39K protein is located at or near the surface of the virus. We note, however, that different procedures were used to prepare the cores; with fowlpox virus, the weakly ionic detergent sodium deoxycholate was used whereas the non-ionic detergent Triton X-100 was used for the vaccinia virus work. The differing virion locations may therefore just reflect the more efficient stripping of proteins by sodium deoxycholate compared to Triton X-100 rather than a real difference in virion location. Interestingly, the vaccinia virus protein is reported to increase or decrease in size by about 2000 M, during virus passage. This would be consistent with an increase or decrease in copy number of a tandem repeat within the gene and points to the fact that the vaccinia virus and fowlpox virus proteins may be related. The vaccinia virus protein is conserved in different members of the orthopoxviruses although its presence was not detected in Shope fibroma virus, a member of the leporipoxviruses (Maa & Esteban, 1987).

The 39K protein recognized by all three anti-peptide antisera is slightly larger than the size (34K) of the protein predicted by translating the ORF. This difference may indicate that the three potential glycosylation sites present in the sequence are indeed used. Alternatively, it may reflect aberrant migration in polyacrylamide gels possibly resulting from an unusual structure being formed by the repeated sequences. The finding that both antipeptide antisera and anti-fowlpox virus antisera taken 2 weeks post-infection recognize a protein of identical M, using strips from the same immunoblot strongly suggests that the 39000 M, protein containing the repeats is immunodominant. The immunoblots were carried out using purified fowlpox virions as antigens. Such virions have previously been found to contain approximately 30 proteins (Mockett et al., 1987). The possibility remains, however, that the two antisera could be recognizing two different proteins of the same M,. This could be resolved by successive immunoprecipitations with the two antisera.

It will be interesting to determine whether the gene identified here is essential for viral replication and to determine its function. If non-essential it would be important to investigate whether the absence of the 39K protein modulates the immune response to other viral antigens, particularly foreign antigens cloned into poxvirus vectors. In this way it may be possible to stimulate increased humoral responses to foreign antigens.

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References


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