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Hemorrhage in lesions caused by cowpox virus is induced by a viral protein that is related to plasma protein inhibitors of serine proteases

(DNA rearrangements/protein sequence/blood coagulation)

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ABSTRACT **Several recombinant cowpox viruses were constructed and used to identify a viral gene that controls the production of hemorrhage in lesions caused by the Brighton Red strain of cowpox virus (CPV-BR). This gene is located in the *Kpn*D fragment of CPV-BR DNA, between 31 and 32 kilobases from the end of the genome. This position corresponds well with that predicted from analyses of the DNA structures of spontaneously generated deletion mutants. The gene responsible for hemorrhage encodes a 38-kDa protein that is one of the most abundant early gene products. The 11-base-pair sequence GAAAATATATT present 84 base pairs upstream of its coding region is also present upstream of three other early genes of vaccinia virus; therefore, this sequence may be involved in the regulation of transcription. There is extensive similarity between the predicted amino acid sequence of the 38-kDa protein and the amino acid sequences of several plasma proteins that are inhibitors of various serine proteases involved in blood coagulation pathways. This suggests that the viral protein may possess a similar biological activity, which may enable it to effect hemorrhage by inhibiting one or more of the serine proteases involved in the host's normal processes of blood coagulation and wound containment.**

The inoculation of cowpox virus (CPV) into the skin of various mammals (guinea pigs, rabbits, humans) results in lesions that exhibit edema, hypertrophy of the epidermis, and hemorrhage. Similar effects are produced in CPV lesions (pocks) in the chorioallantoic membrane of developing chicken embryos; there is extensive proliferation of ectodermal and mesodermal cells, edema, and localized hemorrhage that gives the pocks a deep red color (1).

CPV variants that do not produce hemorrhage have been isolated from the white pocks that usually comprise up to 1% of the pocks present on chorioallantoic membranes infected with wild-type CPV (2, 3). The existence of these white-pock variants demonstrates that a viral function controls the production of the hemorrhage.

Studies of the structures of the DNAs of these white-pock variants have shown that their genomes are generated from the genome of the wild-type virus by large deletions and duplications of sequences (4, 5). Similar results have been obtained from studies of the DNAs of white-pock variants of rabbitpox virus and monkeypox virus (6–8). The rearrangements of DNA that generate the white-pock variants of CPV typically involve the replacement of up to 39 kilobases (kb) of one end of the genome with an inverted copy of up to 50 kb of DNA from the other end (4, 5). The mechanisms by which these rearrangements proceed are unknown; but it is clear that there are no identifiable homologous sequence elements at the sites of the duplication/deletion end points (5). This result suggests that it is the location of essential genes rather than DNA sequence content that limits where rearrangements may occur. The position of genes that encode markers, such as production of hemorrhage, will place a further constraint on the possible genome structures of variants selected for loss of that marker.

The objectives of this study were 2-fold: first, to identify the location of the gene responsible for the production of hemorrhage, in order to determine whether the end points of the deletions that generated the variant genomes can be correlated with the position of this gene; and second, to characterize this gene and its product, in order to gain a better understanding of the molecular mechanisms involved in the virus–host interaction that results in hemorrhage.

MATERIALS AND METHODS

Virus Strains and Cells. CPV strain Brighton Red (CPV-BR) and a white-pock variant (CPV-W2), were isolated and cultured as described (5).

Production of Recombinant CPV. These were constructed according to the methods described by Mackett *et al.* (9). An insertion vector plasmid (p101) was made by inserting the *Hin*dIII J fragment of the DNA of vaccinia virus (strain WR) into a pBR322 vector whose *Eco*RI site had been deleted. Various restriction fragments from the DNA of wild-type CPV-BR were inserted into the *Eco*RI site within the coding region of the thymidine kinase (*tk*) gene in the *Hin*dIII J fragment. The p101 plasmids containing inserts were transfected into tk^- 143 cells 5 hr after the cells had been infected with tk^+ CPV-W2 (0.01–0.05 pfu per cell). The cells were harvested 48 hr after infection. The total tk^- progeny of each transfection/infection experiment were screened for the ability to produce hemorrhage in pocks on the chorioallantoic membranes of 11-day-old chicken embryos. DNA hybridization experiments were done to confirm that the genomes of the viruses isolated from the various pocks were the appropriate recombinant constructions. Recombinants that did not produce hemorrhage were examined to confirm that this deficiency was unrelated to the orientation of the inserted DNA.

Protein Analysis. Infection of 143 cells, labeling of proteins with [^{35}S]methionine, immunoprecipitation, and PAGE of proteins were done as described (10, 11). Antisera against viral proteins (both structural and nonstructural) were obtained from BALB/c mice that had been inoculated with infectious virus (either CPV-BR or CPV-W2) or a CPV-W2 recombinant of the A series. Hybridization–selection of mRNAs and their *in vitro* translation were done by standard methods (12–14).

Nucleotide Sequence Analysis. The nucleotide sequences of both strands of the DNA were determined by standard

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Abbreviations: CPV, cowpox virus; bp, base pair(s); kb, kilobase(s).

procedures (15, 16). The protein sequence database, and also the programs SEARCH and DOTMATRIX (17–19), were provided by the Protein Identification Resource of the National Biomedical Research Foundation (Georgetown University, Washington, DC).

RESULTS

Mapping the Gene Responsible for the Production of Hemorrhage. The innermost end points of the deletions (Fig. 1, horizontal lines a–j) that produced 10 different white-pock variants (W1–W10) of CPV were all located between 32 and 39 kb from one end of the genome of the wild-type virus (5).

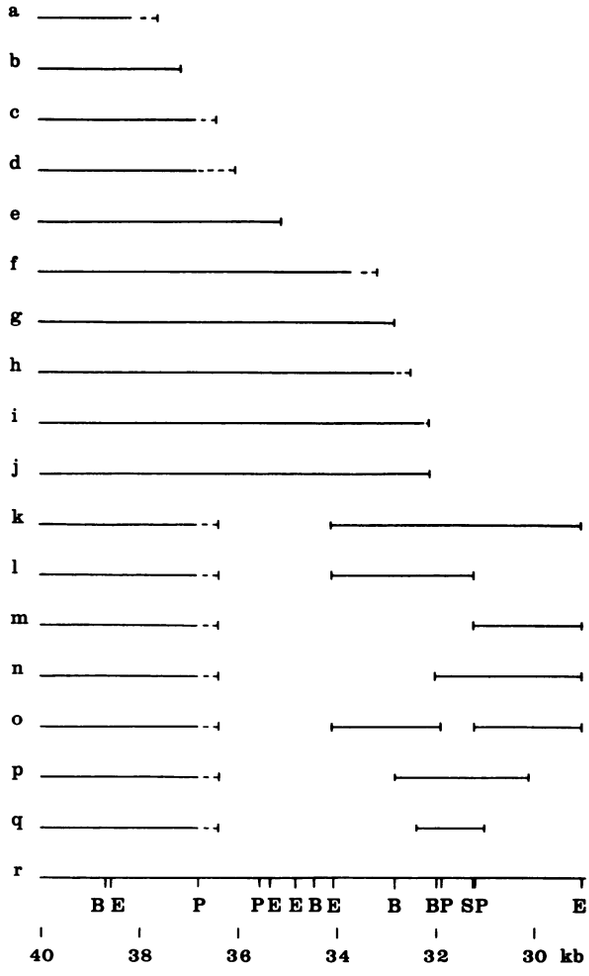

FIG. 1. The inner end points of the DNA deletions that generated the white-pock variants W1–W10 and the DNA fragments that were inserted into the *tk* gene of the white-pock variant CPV-W2. Map coordinates are in kbp from the end of the CPV-BR DNA farther from the internal *Sma* I sites (5). Horizontal bars on the left correspond to the DNA that is present in the genome of CPV variant: a, W1; b, W6; c, W2; d, W7; e, W5; f, W3; g, W10; h, W4; i, W8; j, W9; k–q, W2. Broken lines indicate the limits between which the end point of the deletion is located. The corresponding genome region of wild-type CPV (CPV-BR) is shown in line r. The horizontal bars on the right (lines k–q) correspond to the fragments of DNA that were inserted into the CPV-W2 genome: k, 5.2-kb *Eco*RI fragment (A series recombinants); l, 2.9-kb *Eco*RI/*Sal* I fragment (B series recombinants); m, 2.3-kb *Sal* I/*Eco*RI fragment (C series recombinants); n, 3.0-kb *Bam*HI/*Eco*RI fragment (D series recombinants); o, 5.2-kb *Eco*RI fragment lacking its internal 700-bp *Pst* I fragment (E series recombinants); p, 2.7-kb *Bgl* II fragment (F series recombinants); q, 1465-bp *Nco* I/*Hae* III fragment (G series recombinants). Restriction sites are abbreviated as follows: B, *Bam*HI; E, *Eco*RI; P, *Pst* I; and S, *Sal* I.

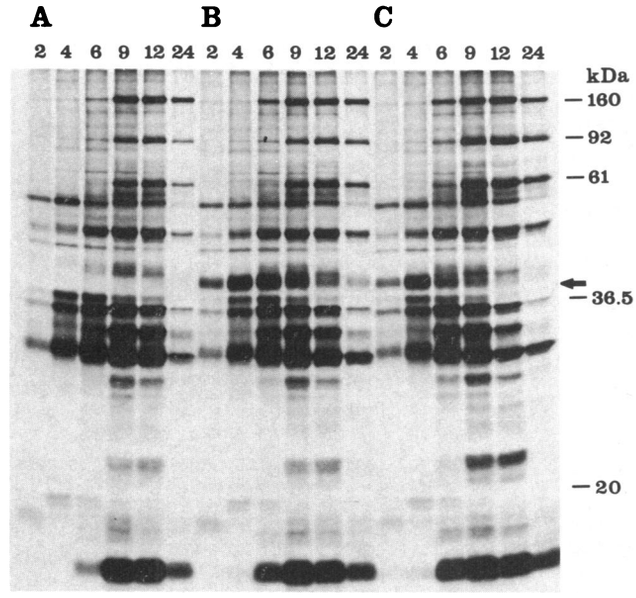

FIG. 2. Proteins synthesized in human 143 cells infected with CPV-W2 (*A*), a CPV-W2 recombinant (G series) containing the 1465-bp *Nco* I/*Hae* III fragment (*B*), and the wild-type virus CPV-BR (*C*). Numbers above lanes indicate the various times (in hr) after infection when the cells were labeled with [^{35}S]methionine for 45 min and then harvested. The viral proteins were immunoprecipitated with antiserum raised against the proteins of a CPV-W2 recombinant of the A series. The immunoprecipitated proteins were solubilized, denatured, and resolved by PAGE in a 12% polyacrylamide gel. Labeled proteins were visualized by autoradiography. In these infections, viral DNA synthesis was initiated between 4 and 6 hr after infection. Arrow indicates the relatively abundant protein species that is synthesized in cells infected with either the wild-type virus (*C*) or the recombinant CPV-W2 (of the G series) that produces hemorrhage (*B*), but is not synthesized in cells infected with the white-pock variant CPV-W2 (*A*).

This suggested that the gene controlling the production of hemorrhage is located ≈32 kb from the end of the viral DNA.

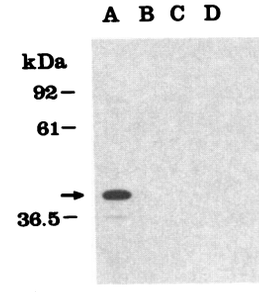

FIG. 3. Immunoprecipitation of the protein encoded by a gene within the 1465-bp *Nco* I/*Hae* III fragment. This DNA fragment was used in hybridization–selection of mRNA synthesized early in cells infected with the wild-type virus CPV-BR. The selected RNA was translated *in vitro* in a rabbit reticulocyte lysate containing [^{35}S]methionine, and the proteins in the lysate were then immunoprecipitated with antiserum raised against the proteins of a CPV-W2 recombinant of the A series. These proteins were resolved by PAGE in a 12% polyacrylamide gel and visualized by autoradiography. The figure shows the immunoprecipitated products of mRNA selected with filters containing DNA of a plasmid that contains the 1465-bp *Nco* I/*Hae* III fragment (lane A), DNA of the plasmid without this inserted fragment (lane B), or no DNA (lane C). Lane D shows the results of immunoprecipitation of a lysate to which RNA had not been added. The *Nco* I/*Hae* III fragment selected an early mRNA that encoded a protein (arrow) with the same relative electrophoretic mobility as the early protein identified (Fig. 2) as being synthesized only in cells infected with virus that produced hemorrhage.

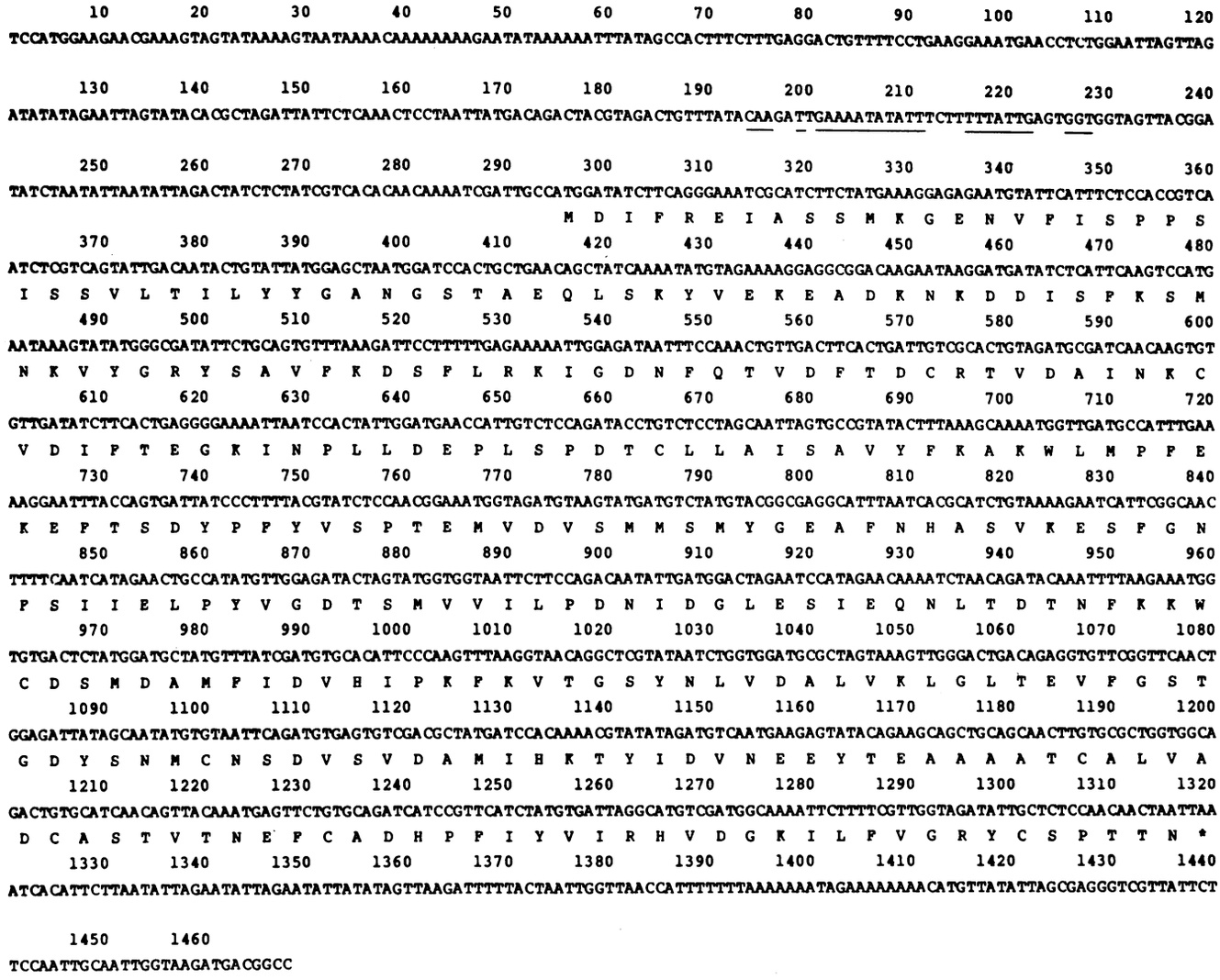

FIG. 4. Nucleotide sequence of the *Nco* I/*Hae* III fragment and predicted amino acid sequence (single-letter code) of the protein controlling production of hemorrhage. The open reading frame beginning at nucleotide 295 encodes a 38,077-Da protein. The underlined nucleotide sequences are identical to those present at the RNA start site of the early/late 7.5-kDa gene of vaccinia virus (20).

To test this hypothesis, the following reconstruction experiments were done. One of the white-pock variants (CPV-W2) was used as a cloning vector, and fragments of the DNA of the wild-type CPV were inserted into its *tk* gene. The fragment that was inserted first was a 5.2-kb *Eco*RI fragment containing the region between 29 and 34.2 kb from the end of the genome of the wild-type virus (Fig. 1, line k). Recombinants (A series) that contain this *Eco*RI fragment produced hemorrhage in pocks. This indicated that a gene necessary for this effect is located within the inserted fragment; however, the hemorrhage produced by these recombinants was not as extensive as that produced by the wild-type virus. This indicated that, although the genetic information in this fragment was sufficient to produce hemorrhage, the magnitude of the effect might be dependent on additional functions.

Several recombinant viruses that contained subfragments of the 5.2-kb *Eco*RI fragment were constructed to identify the exact location of the gene responsible for hemorrhage production. The initial set of recombinants included those of the following series: B series (line l), C series (line m), D series (line n), and E series (line o). None of these recombinants produced hemorrhage, which indicated that the gene responsible for this function spans the *Bam*HI, *Pst* I, and *Sal* I restriction enzyme cleavage sites that are located between 32 and 31 kb from the end of the genome. The next set of recombinants that were constructed included the F series (line p) and the G series, CPV-W2 containing a 1465-base-pair (bp) *Nco* I/*Hae* III fragment (line q). These recombinants produced hemorrhage similar to that produced by the A series recombinants.

Collectively, these data show that viral genetic information that is sufficient to control the production of hemorrhage is contained within this 1465-bp region of the genome. Furthermore, as predicted, this information is located ≈32 kb from the end of the genome.

Comparison of the Proteins of Wild-Type CPV, the White-Pock Variant CPV-W2, and the CPV-W2 Recombinants. At various times after infection, nascent proteins of infected cells were labeled with [^{35}S]methionine, and subsequently these proteins were immunoprecipitated with antiserum raised against the proteins of a CPV-W2 recombinant of the A series. Fig. 2 shows that the white-pock variant CPV-W2 has lost the ability to direct the synthesis of a protein that appears to be one of the most abundant of the early gene products of wild-type virus. The electrophoretic mobility of this protein indicated that its molecular mass was ≈40 kDa. Similar analyses of the proteins synthesized in cells infected either with the other white-pock variants or with the various CPV-W2 recombinants showed a correlation between the ability of virus to direct the synthesis of this protein and the ability of that virus to produce

hemorrhage. Fig. 2*B* shows the proteins synthesized in cells infected with a hemorrhage-producing CPV-W2 recombinant of the G series. The genome of this recombinant was identical to that of CPV-W2, except that it contained the 1465-bp *Nco* I/*Hae* III fragment (depicted in Fig. 1, line q). This fragment provided sufficient information to direct the synthesis of the hemorrhage-specific protein.

Hybridization–selection experiments (Fig. 3) demonstrated that this fragment contained the gene that encoded this protein. The 2.9-kb *Eco*RI/*Sal* I fragment (depicted in Fig. 1, line l), and the 2.3-kb *Sal* I/*Eco*RI fragment (depicted in Fig. 1, line m) each selected the mRNA encoding this protein (data not shown). These results indicated that the mRNA was transcribed from a gene spanning the *Sal* I site in the *Nco* I/*Hae* III fragment.

Nucleotide Sequence Analysis. The nucleotide sequence of this fragment is shown in Fig. 4. It contains only one open reading frame capable of encoding a polypeptide chain longer than 66 amino acids, and this spans the *Bam*HI, *Pst* I, and *Sal* I sites. Furthermore, this open reading frame could encode a protein that, unmodified, would have a molecular weight of 38,077; this is in good agreement with the estimated size of the hemorrhage-specific protein identified by the immunoprecipitation and hybridization–selection experiments.

The predicted amino acid sequence of this protein is also shown in Fig. 4. It is not unusually rich in methionine codons (13 in a total of 341 codons). Therefore, the relative intensities of the bands containing the 38-kDa protein in the autoradiograms shown in Fig. 2 *B* and *C* probably reflect the actual abundance of this protein in cell lysates.

The 38-kDa gene is expressed early in the replication cycle; therefore, cis-acting signals controlling its transcription might be expected to resemble those of some of the early genes of vaccinia virus. Cochran *et al.* (21) demonstrated that a 31-bp sequence immediately upstream of the early RNA start site of the 7.5-kDa gene of vaccinia virus is sufficient to control the early transcription of this gene. The sequence (underlined in Fig. 4) of a region upstream of the open reading frame of the CPV 38-kDa gene is identical to part of the putative promoter region of the 7.5-kDa gene.

Comparison of the Predicted Amino Acid Sequence of the CPV Hemorrhage-Specific Protein and the Amino Acid Sequences of Other Proteins. The SEARCH program (17) was used to compare the predicted amino acid sequence of the CPV hemorrhage-specific protein with the amino acid sequences of proteins in the database provided by the National Biomedical Research Foundation. The proteins that showed the greatest similarity to the 38-kDa protein were those belonging to a proposed superfamily of proteins, each related to the extent of ≈30% sequence identity. Several members of this family are plasma proteins that are inhibitors of serine proteases; these include human antithrombin III (22), human α_1-antichymotrypsin (23), mouse and human α_1-proteinase inhibitor (22, 24), human heparin cofactor II (25), baboon α_1-antitrypsin (26), and mouse contrapsin (24). Fig. 5 shows graphic matrix plots of the similarity between the amino acid sequences of the CPV 38-kDa protein and the corresponding sequences of human antithrombin III (*A*), and the region at the carboxyl terminus of the mouse contrapsin protein (*B*).

Proteins of this superfamily are thought to have similar structures in which their reactive center (interacting with the serine protease) is located on a strained exposed part of the polypeptide chain near their COOH-terminus (27). The specificity of the inhibitor is largely determined by the amino acids

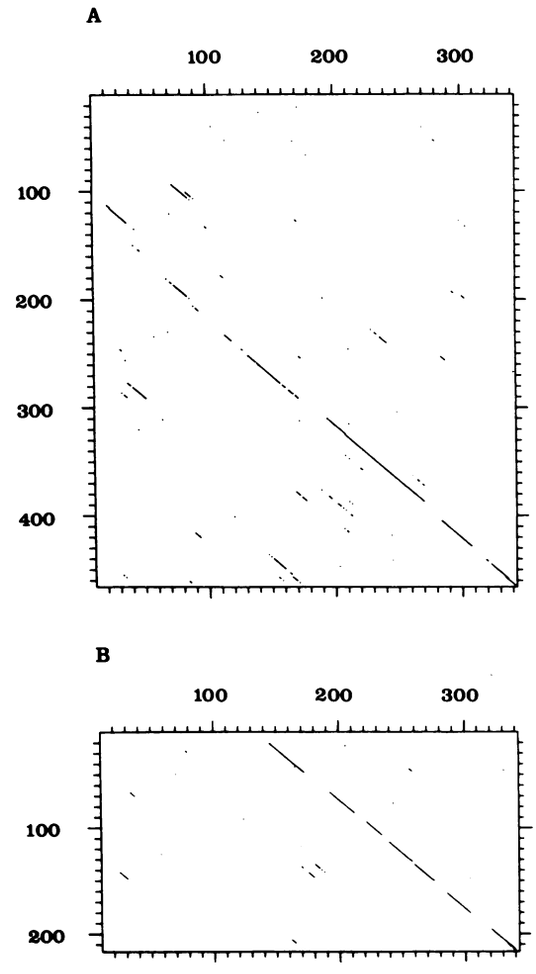

FIG. 5. Graphic display of the similarities between the amino acid sequence of the CPV 38-kDa protein (341 residues) and the amino acid sequences of (*A*) human antithrombin III (464 residues) and (*B*) the carboxyl terminus of mouse contrapsin (215 residues). Similarity was scored by use of a pairwise amino acid scoring matrix using a mutation data matrix for 250 accepted point mutations per 100 residues (18, 19). Segments 20 residues long were compared to other 20-residue-long segments; a minimum score of 20 was required to produce a dot in the graphic display.

contained within this region. Thus, the most highly conserved regions of these proteins can be expected to preserve the strained structure, whereas the regions containing the active-site determinants are less likely to be highly conserved, because they may have different substrate specificities. Fig. 6 shows a comparison between the amino acid sequences at the carboxyl terminus of the CPV 38-kDa protein and the carboxyl terminus of contrapsin. Allowing only a one-residue gap in each sequence, there is identity between 23 of the 66 amino acids. The reactive site of the contrapsin protein is at the lysine residue in the center of the region that is dissimilar to the amino acid sequence of the 38-kDa protein.

DISCUSSION

The viral gene responsible for the production of hemorrhage by CPV is located between 31 and 32 kb from one end of the

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38kDa protein (cowpox virus):   SVDAMIHKTYIDVNEEYTEAAAATCALVADCASTVTNEFCADHPFIYVIRHVDGK-ILFVGRYCSPTTN
Mouse contrapsin:               SVSQVVHKAVLDVAETGTEAAAATGVIGGIRKAILPAVHFNR-PFLFVIYHTSAQSILFMAKVNNPK
Common residues:                SV    HK   DV E  TEAAAAT                  PF  VI H     ILF      P
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FIG. 6. Similarity between the amino acid sequences (single-letter code) at the carboxyl termini of the CPV 38-kDa protein and the mouse contrapsin protein (24). The reactive site of contrapsin is at the lysine residue (underlined) in the center of the dissimilar region.

genome of CPV-BR. The position of this gene provides one explanation for the restricted distribution of the end points of the deletions that generate the genomes of the white-pock variants. To produce a white-pock variant, a deletion must extend into, if not through, this gene. All the white-pock variants CPV-W2 to CPV-W10 were generated by deletions that extend at least 32 kb from the end of the CPV-BR genome (5). The 11 CPV white-pock variants described by Archard *et al.* (4) appear to have been generated in a similar manner.

The gene responsible for the production of hemorrhage encodes a 38-kDa protein that is one of the most abundantly expressed early CPV genes.

The early expression of the 38-kDa gene in cells infected with the CPV-W2 recombinants containing the 1465-bp *Nco* I/*Hae* III fragment is similar to its expression in cells infected with wild-type virus. This indicates that the transcriptional control elements of the 38-kDa gene are also contained within this fragment; however, these elements have not yet been identified. The presence of the 11-bp sequence GAAAATATATT 84 bp upstream of the coding region is interesting because this exact sequence is also present 8 bp upstream of the RNA start site of the 7.5-kDa gene of vaccinia virus (20), 10 bp upstream of the RNA start site of the DNA polymerase gene of vaccinia virus (28), and 22 bp upstream of an open reading frame present in the *Hin*dIII L fragment of the DNA of vaccinia virus (29). Therefore, this conserved sequence may be one of the elements involved in the transcriptional control of at least a subset of early genes.

The recombinants do not produce as much hemorrhage as the wild-type virus. Conceivably, the production of hemorrhage may be enhanced by other gene products that are encoded only by wild-type virus.

In the context of a lytic infection within vascularized tissue, viruses that express the 38-kDa gene cause the production of hemorrhage. The molecular basis for this virus–host interaction is not clear; however, the similarity between the amino acid sequence of the CPV 38-kDa protein and the amino acid sequences of certain inhibitors of serine proteases suggests one possible mechanism. The 38-kDa protein may have biological activities similar to those of its plasma protein relatives, and so it too may be capable of inhibiting one or more serine proteases. Most of the enzymes involved in the blood coagulation pathway are serine proteases—most notably thrombin, whose major physiological inhibitor is antithrombin III. Thus, if the virus produces a lytic infection within vascularized tissue, then the processes of blood coagulation will be activated as part of the host's normal wound-containment response. This response will tend to prevent local hemorrhage. However, if the virus produces a protein that can inhibit the enzymes involved in blood coagulation, then this will interfere with normal blood clotting, and local hemorrhage may ensue.

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