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Cowpox Virus Contains Two Copies of an Early Gene Encoding a Soluble Secreted Form of the Type II TNF Receptor

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The inverted terminal repeats of the DNA of cowpox virus (Brighton Red strain) contain the *crmB* gene, an additional member of a family of viral genes that modify cytokine responses to infection. The *crmB* gene is transcribed from an early promoter. The primary product is a 355-amino-acid protein containing a signal peptide sequence and three potential N-linked glycosylation sites. The mature gene product is a secreted soluble protein that has an apparent molecular mass of 48 kDa. TNFα and TNFβ bind to this protein in a competitive manner, consistent with the sequence of its N-terminal 176 amino acids, which closely resembles the ligand-binding domains of the type II (75-kDa) human TNF receptor. The sequence of the C-terminal 161 amino acids of the CrmB protein is unlike that of human TNF receptors, but overall, the CrmB protein is similar to the T2 proteins of the leporipoxviruses (48% identity) and the predicted product of the G4R/G2R open reading frame of variola virus (85% identity), suggesting that not only the TNF-binding domains but also the C-terminal regions contribute to the functions of these viral proteins. These results show that orthopoxviruses such as cowpox virus encode secreted forms of TNF receptors that can contribute to the modification of TNF-mediated antiviral processes.

INTRODUCTION

Host defenses against viral infections are controlled by a number of immunological mediators, including several cytokines (reviewed by Meagher, 1991). Viruses can inhibit or delay a number of antiviral processes by modifying host cytokine responses mounted during the course of the infection. This strategy is employed by viruses of several types, particularly viruses of the adeno-, herpes-, and poxvirus families, which have genomes large enough to encode modifiers of multiple cytokine responses (reviewed by Gooding, 1992; Pickup, 1994).

Cowpox virus, a member of the orthopoxvirus genus of the Poxviridae, encodes several proteins that modify cytokine-mediated defenses against infection. These cytokine-response modifiers include the CrmA protein, which is an inhibitor of the interleukin-1β converting enzyme (Ray *et al.*, 1992), and the B15R protein, which is a secreted, soluble form of the type II interleukin-1β receptor (Spriggs *et al.*, 1992; Alcami and Smith, 1992). The interleukin-1β-converting enzyme is required for the synthesis of active interleukin-1β (Black *et al.*, 1989; Kostura *et al.*, 1989). This enzyme, or closely related enzymes, may also contribute to apoptosis (Yuan *et al.*, 1993; Miura *et al.*, 1993; Gagliardini *et al.*, 1994). Interleukin 1β, the predominant secreted form of interleukin-1, is a major proinflammatory cytokine and unusually pleiotropic (reviewed by Dinarello, 1992). Its effects include the activation of endothelial cells, the induction of synthesis of other cytokines, and the induction of cyclooxygenase and lipoxygenase gene expression.

Both the CrmA protein and the soluble interleukin-1β receptor can affect the nature of the virus–host interaction. Infection of the chorioallantoic membranes (CAMs) of chick embryos with a cowpox virus lacking a functional *crmA* gene results in a number of events that are not generated upon infection by the wild-type virus. These events are manifested by production of white pocks instead of the characteristic red pocks of the wild-type virus (Pickup *et al.*, 1986). The white pocks are characterized by massive infiltration of heterophils and macrophages, lack of hemorrhage, increased proliferation of ectodermal and endodermal cells, enhanced generation of chemotactic factors, and the alteration of arachidonate metabolism (Downie and Haddock, 1952; Van Tongeren, 1952; Palumbo *et al.*, 1989, 1993; Chua *et al.*, 1990; Fredrickson *et al.*, 1992). Infection of the CAMs of chick embryos with a cowpox virus lacking a functional gene encoding the soluble IL-1 receptor did not markedly affect the form of the pocks produced (Pickup *et al.*, 1986; Ray *et al.*, 1992), but the deletion of the equivalent gene from vaccinia virus did affect the pathogenic properties of the variant vaccinia viruses in mice (Spriggs *et al.*, 1992; Alcami and Smith, 1992). These results are consistent with the expected effects of the viral modification of the action of Interleukin-1β-converting enzyme and interleu-

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kin-1β. However, neither the CrmA protein nor the soluble interleukin-1β receptor, nor a combination of the two, was sufficient to inhibit inflammatory responses to vaccinia virus in the CAM of chick embryos (Ray *et al.*, 1992). These results, taken with the evident inhibition of inflammatory processes by the wild-type cowpox virus, suggested that cowpox virus encodes additional inhibitors of the synthesis or activities of inflammatory mediators.

In addition to Interleukin-1, the tumor necrosis factors, α and β (lymphotoxin), whose biological effects overlap with those of interleukin-1, are proinflammatory cytokines of major importance (reviewed by Dinarello, 1992). The first evidence that viruses could interfere with TNF-mediated processes was provided by Gooding *et al.* (1988), who showed that a 14.7-kDa adenovirus protein could inhibit TNF-mediated lysis of cells. More recently, two additional proteins encoded by adenoviruses have been shown to interfere with TNF-mediated antiviral processes (Gooding *et al.*, 1991; Wold and Gooding, 1991), emphasizing the importance of viral countermeasures to TNF-mediated antiviral processes. Evidence that poxviruses can interfere with TNF-mediated responses was first provided by Smith *et al.* (1990), who noted the similarity between the ligand-binding domain of the human type II TNF receptor (p75) and the protein encoded by T2 open reading frame (orf) of Shope fibroma virus (SFV), a leporipoxvirus. The T2 orf from SFV, when expressed in recombinant form, was shown to encode a soluble secreted protein that binds both TNFα and TNFβ, which compete for binding to the viral receptor (Smith *et al.*, 1991). The T2 gene from myxoma virus, a closely related leporipoxvirus, was subsequently shown to encode a protein that has similar properties. Moreover, the inactivation of the T2 gene of myxoma virus attenuated the virulence of the virus in susceptible rabbits. When eight rabbits were infected with 1×10^3 plaque-forming units of a myxoma virus lacking the T2 gene, only three of the rabbits succumbed to this normally lethal dose; the remaining five rabbits recovered completely (Upton *et al.*, 1991).

Evidence that poxviruses other than the leporipoxviruses might have the capacity to interfere with TNF-mediated responses was first obtained from sequence analyses of the genome of vaccinia virus, an orthopoxvirus, and the prototype of the poxvirus family. The Western Reserve and Copenhagen strains of vaccinia virus were each found to possess two short orfs in addition to the *Sal*I F19R/*Hin*dIII A53R orf, which, if connected in-frame, could encode a protein similar to the ligand-binding portion of the TNF receptors (Howard *et al.*, 1991). This suggested that these orfs are the remnants of a gene that once encoded such a protein. Also, by inspection of the sequence of the genome of the Copenhagen strain of vaccinia virus, Upton *et al.* (1991) identified three short orfs in addition to the *Hin*dIII C22L orf (Goebel *et al.*, 1990), which, if connected in-frame, could encode a protein similar to that encoded by the leporipoxvirus T2 gene. Although neither of these two fragmented genes of vaccinia virus is functional, these data suggested that more virulent orthopoxviruses, such as cowpox virus, might possess intact genes encoding soluble TNF receptors that could contribute to the pathogenic effects associated with these viruses.

In this study we show that cowpox virus possesses two copies of an early gene, designated *crmB* (cytokine-response modifier B), encoding a soluble secreted protein that specifically binds TNFα and TNFβ. This result indicates that cowpox virus seeks to evade antiviral processes mediated by TNF as well as those mediated by interleukin-1.

MATERIALS AND METHODS

Cells and viruses

The Brighton Red strain of cowpox virus and the Western Reserve (WR) strain of vaccinia virus were grown in human osteosarcoma 143 cells cultured as monolayers in minimal essential medium (MEME from Gibco Laboratories, Grand Island, NY) supplemented with 5% fetal calf serum. In addition, to determine the effect of *crmB* gene expression upon pock morphology, viruses were cultured in the CAMs of chick embryos as described (Pickup *et al.*, 1984).

DNA sequence analysis

Overlapping restriction fragments of the segment of cowpox virus DNA shown in Fig. 1 were cloned into the polylinker site of vector pGEM7zf(+) DNA (Promega Corp., Madison, WI) generating the following plasmids: p1463 (containing the *Cla*I fragment spanning nucleotides 4025–4831); p1466 (containing the *Cla*I fragment spanning nucleotides 3263–4025); p1489 (containing the *Msp*I fragment spanning nucleotides 2563–3564); and p1491 (containing the *Xba*I fragment spanning nucleotides 3398–4814). The nucleotide sequences of both strands of this portion of the viral DNA were determined from these cloned DNA fragments by standard procedures as described (Tabor and Richardson, 1990), using chemically synthesized oligonucleotides as primers.

The DNA sequence and predicted protein sequences were analyzed with the aid of software from the Genetics Computer Group (1991).

RNA analyses

Total cellular RNAs were extracted according to the method of Chomczynski and Sacchi (1987). Infected human 143 cells (2×10^7) were washed with phosphate-buffered saline (PBS) and lysed by the addition of 2 ml of 4 *M* guanidium thiocyanate, 0.025 *M* NaOAC (pH 7.0), 0.5% Sarkosyl, and 0.72% β-mercaptoethanol. The lysate was passed through a 22.5-gauge needle five times, after which 0.2 ml of 2 *M* NaOAC (pH 4.0) was added before

phenol–chloroform extraction. The solution was adjusted to 0.3 *M* NaOAc (pH 5.2), 2.5 vol of ethanol was added, and the RNAs were precipitated at −70° for 2 hr. Precipitated RNAs were solubilized in diethyl pyrocarbonate-treated water. Residual DNA was removed by treatment with RQ1 DNase (Promega Corp.). The RNA was then deproteinized, precipitated, and solubilized in diethyl pyrocarbonate-treated water, as described above. Polyadenylated RNAs were recovered by oligo(dT)-cellulose chromatography (Aviv and Leder, 1972).

Primer extension analysis as described (Sambrook *et al.*, 1989) was used to characterize the 5′-ends of the transcripts of the *crmB* gene. Oligonucleotides 5′-CATGAAAGCAGCAATAGC-3′ (nucleotides 4530 to 4547, Fig. 2), corresponding to a region of the DNA template strand at the beginning of the coding region of the *crmB* gene, were 5′-end-labeled with ^{32}P by the method of Weaver and Weissman (1979). The labeled primers were annealed overnight at 37° to 50 μg of unfractionated RNAs. Primer/RNA complexes were precipitated for 1 hr on ice with 2 vol of ethanol and then dissolved in 20 μl reverse transcription buffer (50 m*M* Tris–Cl (pH 7.6), 60 m*M* KCl, 10 m*M* $MgCl_2$, 1 m*M* dATP, 1 m*M* dCTP, 1 m*M* dGTP, 1 m*M* dTTP, 1 m*M* dithiothreitol, 1 unit/μl RNasin, 50 μg/ml actinomycin D). Primers were extended by the addition of 28 units AMV reverse transcriptase (USB, Cleveland, OH) and incubated at 37° for 2 hr. RNAs were removed by digestion with 2 μg/ml RNase A (Boehringer-Mannheim, IN) at 37° for 30 min. The DNA products were then deproteinized by phenol–chloroform extraction. The precipitated DNAs were dissolved in 6 μl of 80% formamide, 10 m*M* EDTA (pH 8.0) and loaded onto a 6% polyacrylamide gel containing 8 *M* urea. The ^{32}P-labeled extension products were visualized by autoradiography of the dried gel.

S1 nuclease protection analysis was used to determine the extent of complementarity between the *crmB* gene and the 5′-ends of its mRNAs. A *Cla*I–*Msp*I restriction fragment (nucleotides 4423–4831, Fig. 2) was 5′-end-labeled with ^{32}P by the method of Weaver and Weissman (1979). This fragment was cleaved with *Xba*I, generating the *Xba*I–*Msp*I fragment of 390 bp (^{32}P labeled at the *Msp*I site) that was used as the hybridization probe. The conditions for S1 nuclease protection assays were as described (Sambrook *et al.*, 1989). The probe and the RNAs were annealed at 41° for 15 hr; residual single-stranded nucleic acids were digested by incubation with 200 units of S1 nuclease (USB) at 23° for 1 hr. The protected products were electrophoresed in a 6% polyacrylamide gel containing 8 *M* urea and visualized by autoradiography of the dried gel.

Construction of recombinant viruses

A recombinant cowpox virus (A500) in which both copies of the *crmB* gene are inactive was constructed according to the method of Falkner and Moss (1988) as follows. The *Ssp*I fragments between nucleotides 2196 and 2405 of plasmid pGEM-7Zf(+) were deleted and replaced with a *Sal*I linker fragment, generating plasmid p1493, which lacks *Ssp*I sites. *Eco*RI linkers were added to the ends of an *Xba*I fragment containing the intact *crmB* gene (nucleotides 3398–4814, Figs. 1 and 2), and then this fragment was inserted into the *Eco*RI site within plasmid p1493, creating plasmid p1491. From this plasmid, the *Ssp*I fragment (nucleotides 3818–4630, Figs. 1 and 2), spanning the 5′-half of the *crmB* gene (but not extending into the coding region upstream) was replaced with a repaired *Eco*RI fragment of the DNA of plasmid pTK61-gpt (Falkner and Moss, 1988), which was kindly provided by Dr. B. Moss (NIH); this *Eco*RI fragment contained the *Escherichia coli* guanosine phosphoribosyltransferase gene (*Eco–gpt* gene) under the control of the vaccinia virus p7.5 promoter. In the resulting plasmid, p1497, most of the *crmB* gene, including its promoter region, has been replaced by the *gpt* gene (Fig. 1).

Plasmid p1497 was used to replace the functional copies of the *crmB* gene in the genome of cowpox virus with the *gpt* gene. The recombinant viruses were generated by infecting human 143 cells (2.5×10^6 cells) with cowpox virus at multiplicities of 0.1 PFU per cell. Five hours after infection the cells were transfected with 10 μg of *Eco*RI-cut p1497 DNA in 50 μg of Lipofectin (Gibco–BRL, Gaithersburg, MD). Virus progeny were harvested 48 hr after infection. Recombinant viruses expressing the *gpt* gene were selected by culturing the virus in cells in the presence of 12 μg/ml mycophenolic acid, 250 μg/ml xanthine, and 15 μg/ml hypoxanthine (Sigma, St. Louis, MO). The recombinant viruses were plaque-purified, and their DNA structures were analyzed by DNA hybridization (Southern, 1975) using DNA fragments containing either the *crmB* gene or the *gpt* gene as hybridization probes. The first recombinants isolated contained one inactivated *crmB* gene and one intact *crmB* gene. These recombinant viruses were passaged further under selection conditions until DNA hybridization analyses showed that the plaque-purified virus contained two copies of the *gpt* gene, one in the place of the *crmB* gene in each of the inverted terminal repeats. This conversion was mediated at a high frequency by a spontaneous process that copies one end of the virus genome to the other, a process that generates identical inverted terminal repeats (McFadden and Dales, 1979; Pickup *et al.*, 1984).

A recombinant vaccinia virus (A495) containing an intact copy of the cowpox virus *crmB* gene was constructed according to the method of Mackett *et al.* (1982, 1984) as follows. Plasmid p1475 was used for the insertion of the cowpox virus *crmB* gene into the thymidine kinase (TK) gene of the genome of vaccinia virus. Plasmid p1475 was constructed by inserting the *Eco*RI (*Xba*I) fragment of plasmid p1491 (this *Eco*RI fragment corresponds to nucleotides 3398–4814 shown in Fig. 1, containing the *crmB* gene and its promoter) into the *Eco*RI site of the

vaccinia virus *Hin*dIIIJ fragment contained in a derivative of plasmid pUC19 (Yanisch Perron *et al.*, 1985) lacking *Eco*RI sites (p1378). The recombinant virus A495 was generated by infecting human 143 cells (2.5×10^6 cells) with vaccinia virus at multiplicities of 0.1 PFU per cell. One hour after infection the cells were transfected with 10 μg of p1475 DNA in 50 μg of Lipofectin (Gibco–BRL). Virus progeny were harvested 48 hr after infection. Viruses that did not express the thymidine kinase gene were selected by plaque purification in TK^- 143 cells cultured in the presence of 25 μg/ml 5-bromodeoxyuridine. DNA hybridization analyses using DNA probes derived from plasmids p1378 and p1463 were used to identify viruses whose genomes contained the inserted *crmB* gene.

Protein analyses

Subconfluent monolayers of human 143 cells in 25-cm^2 flasks were infected with viruses at multiplicities of 10 PFU per cell. After 1 hr adsorption at 37°, 8 ml MEME plus 5% serum was added to each culture. The medium was removed 2 hr later and the cells were washed twice with PBS, after which 2 ml cysteine-free MEME without serum, but supplemented with 50 μCi [^{35}S]cysteine (1300 Ci/mmol, Amersham, Arlington Heights, IL), was added to each culture. The medium was collected 3 hr later; cells in the medium were removed by centrifugation (1000 rpm for 10 min at 4° in a Sorvall RT6000B rotor). The medium was then concentrated 25- to 50-fold by centrifugation (5000 rpm for 8 hr at 4° in a Sorvall SS34 rotor) in a Centricon-10 concentrator (Amicon, Beverly, MA). Proteins were resolved by electrophoresis in 12% polyacrylamide gels as described (Laemmli, 1970). The ^{35}S-labeled proteins were visualized by autoradiography of the dried gel.

Recombinant proteins

Human interleukin-1α, human interleukin-2, murine interleukin-3, human interleukin-4, human interleukin-7, human granulocyte–macrophage colony-stimulating factor (GM-CSF), and human granulocyte colony-stimulating factor (G-CSF) were each expressed from cloned genes and then purified and assayed for biological activity by standard procedures (at Immunex). A soluble fusion protein (Fc/p75) consisting of the entire extracellular portion of the human type II TNF receptor fused to truncated human IgG1 heavy chain (Mohler *et al.*, 1993) was used as a positive control for the binding of murine TNFα to TNF receptor proteins immobilized on nitrocellulose sheets. The purified Fc/p75 protein was a disulfide-linked homodimer with an affinity for murine TNFα of 5×10^{10} M^{-1}. Murine TNFα was a gift from Dr. David Goeddel (Genentech, San Francisco, CA). Human TNFβ, human interleukin-6, human transforming growth factor β (TGFβ, R & D Systems, Minneapolis, MN), murine nerve growth factor (NGF; Bioproducts, Indianapolis, IN), and human interferon-γ (IFN-γ; Genzyme, Boston, MA) were obtained commercially.

TNF-binding assays

Subconfluent monolayers of human 143 cells in 25-cm^2 flasks were infected with viruses at multiplicities of 10 PFU per cell. After 1 hr adsorption at 37°, the virus inocula were removed, the cells were washed with PBS, and finally 2 ml MEME (without serum) was added to each culture. The medium in each culture was harvested 5 hr later. Cells in the medium were removed by centrifugation at 1000 rpm for 10 min at 4° in a Sorvall RT6000B rotor. The medium was then concentrated eightfold by centrifugation (5000 rpm for 4 hr at 4° in a Sorvall SS34 rotor) in a Centricon-10 concentrator (Amicon). Proteins in the medium or purified proteins were dissolved in sample loading buffer (lacking β-mercaptoethanol) and electrophoresed through 8–18% polyacrylamide gels. Proteins were electrophoretically transferred to nitrocellulose sheets (Schleicher & Schuell, Keene, NH), which were then blocked by overnight incubation in binding buffer (PBS, 3% bovine serum albumin, 1% nonfat dried milk). The blots were incubated with or without competitor ligands in binding buffer containing ^{125}I-labeled murine TNFα (1 n*M*; 3×10^{15} cpm/mmol) for 1 hr at 4°. A cocktail of potential competitive inhibitors containing 0.1 μ*M* (each) of interleukins 1–4, interleukin-6, interleukin-7, GM-CSF, G-CSF, TGFβ, NGF, and IFN-γ was also used to test the specificity of binding of the murine TNFα to the CrmB protein. The blots were washed five times in PBS, after which radiolabeled complexes were detected by phosphorimaging (Molecular Devices, Palo Alto, CA). Radiolabeled marker proteins (^{14}C-labeled) were obtained from Amersham.

RESULTS

The inverted terminal repeats of cowpox virus DNA contain a gene encoding a protein similar to the ligand-binding domain of human TNF receptors

The inverted terminal repeats in the DNA of the Copenhagen strain of vaccinia virus contain the *Hin*dIII C22 L orf and adjacent orfs that appear to be the remnants of a gene that once encoded a protein similar to the ligand-binding portions of the human TNF receptors (Upton *et al.*, 1991). A comparison of the nucleotide sequence of the inverted terminal repeats of the DNA of vaccinia virus with restriction maps of the terminal regions of the DNA of cowpox virus (Pickup *et al.*, 1984) indicated that cowpox virus might possess a corresponding coding region about 4 kb from each terminus of the viral genome. In a previous study, the nucleotide sequence of the terminal 2.7 kb of the cowpox virus genome had been determined (Pickup *et al.*, 1982). In the present study, the analysis

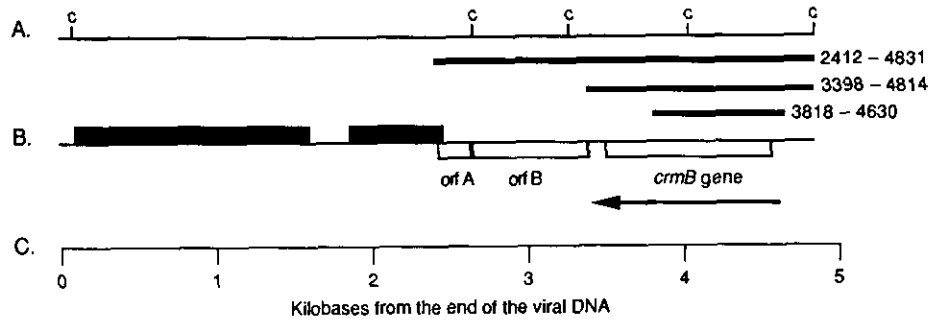

FIG. 1. The position of the *crmB* gene in genome of cowpox virus. (A) A *ClaI* restriction map of the terminal 4.8 kb of the viral DNA. The bars, labeled according to nucleotide number, correspond to fragments of the DNA. Bar 2412–4831 corresponds to the region whose nucleotide sequence is presented in Fig. 2. Bar 3398–4814 corresponds to an *XbaI* fragment containing the intact *crmB* gene; this *XbaI* fragment is contained in plasmids p1475 and p1491. Bar 3818–4630 corresponds to an SspI fragment containing the promoter of the *crmB* gene and the region encoding the N-terminal region of the CrmB protein; this SspI fragment was deleted from each copy of the *crmB* gene in the genome of recombinant cowpox virus A500. (B) The positions of tandemly repeated sequences (black rectangles) and open reading frames (white rectangles) in the telomeric DNA. The arrow indicates the extent and orientation of the *crmB* transcription unit. (C) Scale measured in kilobases from the end of the viral DNA.

of the sequence of the terminal regions of the viral genome was extended to include the region predicted to contain the gene encoding the TNF receptor (Fig. 1). The nucleotide sequence of this region is shown in Fig. 2.

The terminal 4.8-kb region of the viral genome contains three nonoverlapping orfs capable of encoding proteins of at least 65 amino acids (Figs. 1 and 2). Orf A, which has the capacity to encode a protein of 65 amino acids, is not downstream of a recognizable promoter element. Similar but shorter orfs are present in the genomes of vaccinia virus (strains Copenhagen, WR, and Lister) and raccoonpox virus (Venkatesan *et al.*, 1981; Patel *et al.*, 1990; Goebel *et al.*, 1990; Parsons and Pickup, 1987), suggesting that this orf may be derived from the gene of an ancestral orthopoxvirus. However, it is unclear if this short orf is part of a functional gene. Orf B is capable of encoding a 30-kDa protein that possesses a high degree of similarity (81% identity) to the 35-kDa protein encoded by the 35K gene of the Lister strain of vaccinia virus (Patel *et al.*, 1990). In addition, the sequence upstream of orf B is similar to the promoter region of this 35K gene, suggesting that orf B is part of the cowpox virus equivalent of the 35K gene, which is itself the counterpart to the 7.5K gene of the WR strain of vaccinia virus (Patel *et al.*, 1990; Venkatesan *et al.*, 1981). As shown below, the third and innermost orf constitutes the coding region of a functional gene, designated *crmB*, encoding a secreted TNF receptor.

The *crmB* gene is capable of encoding a protein of 355 amino acids (Fig. 2). The sequence immediately upstream of the transcriptional start site is similar to the consensus sequence for early promoters (Davison and Moss, 1989), suggesting that this is part of the promoter of the *crmB* gene. Interestingly, this sequence adjoins a region comprising 13 direct repeats of the sequence 5′-GAAAT-3′ (coding strand), which is a subset of the sequence of the consensus early promoter element. The presence of the sequence 5′-TTTTTAT-3′, which matches the consensus for the early transcriptional termination signal (Rohrmann *et al.*, 1986), at the 3′-end of the coding region of the *crmB* gene is also consistent with the transcription of this gene at early times during infection.

The predicted amino acid sequence of the CrmB protein contains the sequence motifs characteristic of proteins grouped in the TNF receptor family, which includes TNF receptor types I and II (Schall *et al.*, 1990; Smith *et al.*, 1990), low-affinity nerve growth factor receptor (Johnson *et al.*, 1986), FAS antigen (Itoh *et al.*, 1991), human T-cell activation antigen CD27 (Goodwin *et al.*, 1993), OX40 (Mallett *et al.*, 1990), mammalian B-cell activation antigen CD40 (Stamenkovic *et al.*, 1989), Hodgkin's disease-associated antigen CD30 (Smith *et al.*, 1993), and the leporipoxvirus T2 TNF-binding proteins (Smith *et al.*, 1990). Of the various members of this family, the CrmB protein shares the greatest homology with the ligand-binding domain of the human p75 TNF receptor (about 42% identity between the cysteine-rich ligand-binding domains) and the TNF-binding protein encoded by the T2 gene of myxoma virus (about 48% identity overall). The CrmB protein also contains a signal peptide sequence, three potential N-linked glycosylation sites, and a C-terminal element (161 residues), which shows no homology with the cytoplasmic (signal transduction) domains of TNF receptor family members. These features, and the lack of a transmembrane element, are consistent with the soluble secreted nature of the CrmB protein.

The *crmB* gene is transcribed at early times during virus replication

The transcriptional start site of the *crmB* gene was identified by primer extension analysis and S1 nuclease protection analysis of transcripts of the *crmB* gene. The primer extension analysis (Fig. 3) indicated that the 5′-end of the *crmB* early mRNAs corresponded to a G resi-

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     SspI                                  SspI
2412 AATATTTTTGTTACTCCATCAGAAAGAGGTTTAATATTTTAGTGATCATAATCGTATCAC 2471
               *  E  M  L  F  L  N  L  I  K  T  I  M  I  T  D

2472 ATATTGAGACAGAAAAAGAAGAAGTCGCGAGAGGTAACTTTTTGTGAATGTAGTTAAGAA 2531
   C  I  S  V  S  F  S  S  T  A  L  P  L  K  K  H  I  Y  N  L

2532 CATTTTTGTTTTGCAAACCGGAATATAGTGTCCGGTACACTTTTTTTAGTTTGTGGTGTG 2591
   V  N  K  N  Q  L  G  S  Y  L  T  R  Y  V  K  K  L  K  H  H
                                                        ClaI
2592 CCTGAATCGCTCGATTAACCCTACTCATCCAATTTCAGATGAATAGGGTTATCGATTCAG 2651
   A  Q  I  A  R  N  V  R  S  M <orfA

2652 ACACACGCTATAAGTTTTGCTGAATTGATGAGTGAAGTATCATCGGCTGCATCTTCCGAT 2711
     V  C  A  I  L  K  A  S  N  I  L  S  T  D  D  A  A  D  E  S

2712 GCCGATCCGTCGACATACTTGAATCCATCCTTGACCTCAAGTTCAGATGATTCCTTGCAC 2771
     A  S  G  D  V  Y  K  F  G  D  K  V  E  L  E  S  S  E  K  C
                              XbaI
2772 ATGTCTCCTATACGAACGCTAATCTCTAGATTCTTGACGCATTTCGTATCGACGATCGTT 2831
     M  D  G  I  R  V  S  I  E  L  N  K  V  C  K  T  D  V  I  T

2832 GATCCGATGATATCTTCGTAACTCACTTTCTTATGGGAGATGTTAGACCCGAGTACTGGA 2891
     S  G  I  I  D  E  Y  S  V  K  K  H  S  I  N  S  G  L  V  P

2892 TGGGTCTTGATGTTGCTGTCTTTCTCTTCTTCGCTACATTTGATGTTGATAGACATTTCA 2951
     H  T  K  I  N  S  D  K  E  E  E  S  C  K  I  N  I  S  M  E

2952 CAGTCTTTGATCATAGACAGAGCTTCTTCACGAGTGATAGCGGGAGAGTCCTTACCTTGT 3011
     C  D  K  I  M  S  L  A  E  E  R  T  I  A  P  S  D  K  G  Q

3012 CCTGGGGACACGCTAGACAATCTAGCATTCACAGTGTTTCCGTCAGAGTATTCAGAGATG 3071
     G  P  S  V  S  S  L  R  A  N  V  T  N  G  D  S  Y  E  S  I

3072 GATGAAATCTTTGGGCATTTGGTGAATCCAAAGTCCATCGTAAGACCTCCACCGACGACA 3131
     S  S  I  K  P  C  K  T  F  G  F  D  M  T  L  G  G  G  V  V

3132 GTGTAATAAGTGGTGGGATCTCCCTTTACGACTTCTTCTGACTCGTCTTCAGACTCTGTA 3191
     T  Y  Y  T  T  P  D  G  K  V  V  E  E  S  E  D  E  S  E  T

3192 ACTTCGGTTACTGATTGACAAATCTTATCATTGGTCGGTGTTTGGTCTTGCTTGGTGACT 3251
     V  E  T  V  S  Q  C  I  K  D  N  T  P  T  Q  D  Q  K  T  V
               ClaI
3252 TTGATAATAACATCGATTCCCATATGATGTTTGTTTTCTTCTTCCGTACACGAGGATGAG 3311
     K  I  I  V  D  I  G  M  H  H  K  N  E  E  E  T  C  S  S  S

3312 GATGAGAATGATTGCTGAAGACTGGTAGGGATAGCAACTGCCGCCAGGCATATGCATGCC 3371
     S  S  F  S  Q  Q  L  S  T  P  I  A  V  A  A  L  C  I  C  A
                                  XbaI
3372 AGGACGATTTGTTTCATGATTGCTGTTCTAGATGATTCTATTTCCTCACCATACAATAAA 3431
     L  V  I  Q  K  M <orfB

3432 TTAGAATATATTTTCTACTTTTACGAAAAATTAATTATTGTATTTATTTATGGGTTAAAA 3491

3492 ACTTACTATAAAAAGTGGGTGGGATACTGGGAATTGGAATTAGTAATCAGTTTATGTGTA 3551
           *  L  F  H  T  P  Y  Q  S  N  S  N  T  I  L  K  H  T

3552 TCGCACCTACCGGGCATATGGCTATCGACATCGAGAACATTACCCACATGATAAGAGATT 3611
     D  C  R  G  P  M  H  S  D  V  D  L  V  N  G  V  H  Y  S  I

3612 GTATCAGTTTCGTAGTCTTGAGTATTGGTATTACTATATAGTATATAGATGTCGACGCTG 3671
     T  D  T  E  Y  D  Q  T  N  T  N  S  Y  L  I  Y  I  D  V  S
```

FIG. 2. Nucleotide sequence of a segment of cowpox virus DNA containing the *crmB* gene. The nucleotides are numbered in accordance with the previously described sequence of the telomeric region of the cowpox virus DNA (Pickup *et al.*, 1982). The nucleotide sequence of 4.8 kb of the telomeric region of cowpox virus DNA (including the sequence presented in this figure) has been assigned GenBank Accession No. L08906. The predicted amino acid sequences encoded by the open reading frames and the *crmB* gene are indicated beneath the nucleotide sequence. The transcriptional start site of the *crmB* gene is at nucleotide 4608 (see Figs. 3 and 4); a transcriptional termination signal (Rohrmann *et al.*, 1986) is located at nucleotide 3499. The underlined sequence (−27 to −95 relative to the transcriptional start site of the *crmB* gene) comprises 13 tandem repeats of the sequence 5′-GAAAT-3′ and one incomplete repeat, 5′-GAAA-3′ (coding strand). The predicted cleavage site of the leader sequence is indicated by the arrow. Brackets demarcate the cysteine-rich region showing sequence homology to members of the TNF receptor family (see Fig. 7). Potential N-linked glycosylation sites are marked by underlined asparagine residues.

due (coding strand) 44 nucleotides upstream of the first methionine codon. Similar 5′-ends were detectable in RNAs harvested at late times during infection, but late 5′-polyadenylated *crmB* mRNAs were not detected. S1 nuclease protection analyses (Fig. 4) showed that complementarity between the *crmB* mRNAs and the template DNA extended to the position of the C residue (template strand) 44 nucleotides upstream of the first methionine codon, indicating that this residue is the transcriptional start site of the *crmB* gene. The S1 nuclease protection

```
3672 GATAGACAGTCGCCCACTAGAGTTACCGATTCCGAATGCGGCATGATGGAGTCGTCATCA 3731
     S  L  C  D  G  V  L  T  V  S  E  S  H  P  M  I  S  D  D  D

3732 TTCTTTGTTTTCGTTAACTGTTTGGAGGAAGAATAAGAATCTTTGTTATTACATTTAATC 3791
     N  K  T  K  T  L  Q  K  S  S  S  Y  S  D  K  N  N  C  K  I
                              SspI
3792 TCGAAATTCAGAGTGCATACCTTTGAAATATTCTGATATCTATTTTGTCCTGTAAAGAAC 3851
     E  F  N  L  T  C  V  K  S  I  N  Q  Y  R  N  Q  G  T  F  F

3852 CCTGAAGTTGCTACCTCATTAAGAACGGAGAAGTATCCATTACGAAAGACGGGATCGCAG 3911
     G  S  T  A  V  E  N  L  V  S  F  Y  G  N  R  F  V  P  D  C

3912 TCTTTATGATTCATAGTAATCGTTAGTTCCGAAGTTGAGATGGATTCACTGAGACCGGTA 3971
     D  K  H  N  M  T  I  T  L  E  S  T  S  I  S  E  S  L  G  T
                                                          ClaI
3972 GTGGTCGTCCGAGTACACGATGTGTCGTTGACGGGATACAGATTAATTTCCACATCGATA 4031
     T  T  T  R  [T  C  S  T  D  N  V  P  Y  L  N  I  E  V  D  I

4032 TAGTTAAAGGTATTACTGGGTACGGGTTCGCATTTATCTACGGAAGAGACGGTGTGAGAA 4091
     Y  N  F  T  N  S  P  V  P  E  C  K  D  V  S  S  V  T  H  S

4092 TATGTTCCGAGACCACACGGAGAACAGACGACGTCTCCGGTAGGCGTGTGTCCGGATACT 4151
     Y  T  G  L  G  C  P  S  C  V  V  D  G  T  P  T  H  G  S  V

4152 CCGTATCCTATTCCACACTTTGTTTGGGAAACACATGCCTTGCATCCGGATGATCCTTTG 4211
     G  Y  G  I  G  C  K  T  Q  S  V  C  A  K  C  G  S  S  G  K

4212 AGAAAACAATAATATCCGGGAGCACAATCACAGATTCTATTGTGAGTCGTGTTACACGAT 4271
     L  F  C  Y  Y  G  P  A  C  D  C  I  R  N  H  T  T  N  C  S

4272 CGCGTCTCTACCTGATTACTATCGCATCTTCCGTTACAACTTAGACAAGCGGGTAAATGA 4331
     R  T  E  V  Q  N  S  D  C  R  G  N  C  S  L  C  A  P  L  H

4332 TTATTGCGAGACGTAAAGGTGTCCGACGCACACGGCGTACATTGTGTGTTTGTGTTAGTC 4391
     N  N  R  S  T  F  T  D  S  A  C  P  T  C  Q  T  N  T  N  T

4392 TTGCTATCGCATAATCTGGAAGCGTATGTTCCCGGAGGACACGATAAACAACATAGATGA 4551
     K  S  D  C  L  R  S  A  Y  T  G  P  P  C  S  L  C  C  L  H

4452 TGGCGTTTGTATTCGTTGTCTTTACACTTTCCGTTGGATGGTTCATGCGGTGTTATATCG 4511
     H  R  K  Y  E  N  D  K  C  K  G] N  S  P  E  H  P  T  I  D ↑

4512 CTGTTTATTATGATTATACATGAAAGCAGCAATAGCAATATATATGACTTCATGATTTAA 4571
     S  N  I  I  I  I  C  S  L  L  L  L  L  I  Y  S  K  M <crmB

4572 TAATGTAGTAGTAATTATCACCTCGTTCCCGCTCTCCAATCATTATAGATATTTTTTAAA 4631
                                          J transcriptional start-site
     SspI
4632 TATTTTCATTTCATTTCATTTCATTTCATTTCATTTCATTTCATTTCATTTCATT 4691
     ═══════════════════════════════════════════════════════

4692 TCATTTCATTTCTAGTATGGATAATGTTTGTAATGGTTCTTTCCGTACAACATACTTTTT 4751
     ═══════════

4752 AGATGATAGTCGCTTAACTTGGATATGATGCTGCGTATAATTTCTGGAGGCAAATAATAT 4811

       XbaI          ClaI
4812 AGTCTAGATTGACCATCGAT 4831
```

FIG. 2—*Continued*

analysis also detected *crmB* mRNAs at late times, but the extent of complementarity between the 5′-ends of these RNAs and the template DNA was the same as that between the early mRNAs and the DNA. These results were confirmed by the method of Hu and Davidson (1986), which determined the site at which the T4 DNA polymerase-mediated extension of a primer annealed to the DNA template strand was inhibited by the 5′-ends of *crmB* mRNAs annealed to the same DNA (data not shown). Therefore, the results show that the *crmB* gene is an early gene, having a single transcriptional start site, despite the presence of multiple tandem subsets of the early promoter element.

Transcripts of the gene were detectable at late times during infection, but there is no evidence to suggest that this gene contains a functional late promoter. A component of late transcriptional promoters, the sequence 5′-ATTTA-3′ (template strand), is present 1 nucleotide upstream of the initiation codon, but primer extension analysis, S1 nuclease protection analysis, and the T4 DNA polymerase extension assay did not indicate that any late transcription of the *crmB* gene was initiated at this site.

The *crmB* gene encodes a secreted protein

Two recombinant viruses were constructed in order to identify the protein encoded by the *crmB* gene. One

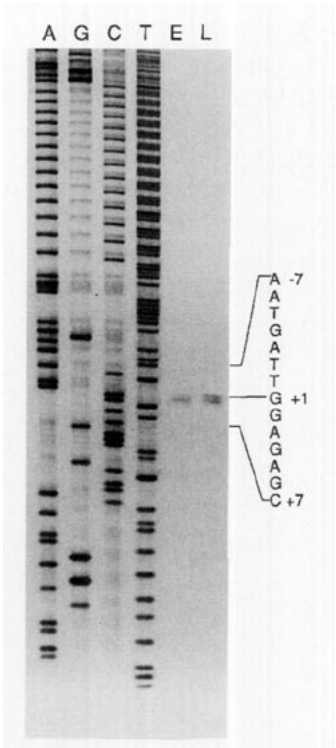

FIG. 3. Primer extension analysis of the 5′-ends of the mRNAs of the *crmB* gene. Oligonucleotides 5′-CATGAAAGCAGCAATAGC-3′ corresponding to the region between nucleotides 4530 and 4547 (Fig. 2) were 5′-end-labeled with ^{32}P. The oligonucleotides were annealed to RNAs extracted from cells infected with cowpox virus, and then the annealed oligonucleotides were extended by transcription with AMV reverse transcriptase. The primer-extended products were resolved by electrophoresis in a 6% polyacrylamide gel containing 8 *M* urea and then visualized by autoradiography. Lanes E and L contain the extended products obtained using RNAs extracted at either 3 hr postinfection (E) or 16 hr postinfection (L). Size markers were provided by coelectrophoresis of the products of dideoxynucleotide chain termination reactions containing dideoxy ATP (A), dideoxy GTP (G), dideoxy CTP (C), and dideoxy TTP (T), using the oligonucleotides described above annealed to a DNA template containing the beginning of the *crmB* gene. Note the repeated sequences upstream of the transcriptional start site. The nucleotide sequence of the coding strand of the DNA is shown.

recombinant, cowpox virus A500, was constructed such that each of the two copies of the *crmB* gene was inactivated by partial substitution with the *E. coli gpt* gene. A second recombinant, vaccinia virus A495, was constructed by inserting a copy of the cowpox virus *crmB* gene into the thymidine kinase gene of vaccinia virus.

Proteins synthesized in cells infected with either of the two recombinant viruses, or with either cowpox virus or vaccinia virus, were metabolically labeled with [^{35}S]-cysteine. Six hours after infection the proteins secreted into the growth medium of each culture were analyzed by polyacrylamide gel electrophoresis (Fig. 5). The media of cells infected with wild-type cowpox virus (lane 2) contained a protein (of about 48 kDa) that was not detectable in the media of cells infected with the recombinant cowpox virus A500 (lane 3). The 48-kDa protein was not detectable in the media of cells infected with wild-type vaccinia virus (lane 4), but a similar protein was present in the media of cells infected with the recombinant vaccinia virus A495, which contains a copy of the cowpox virus *crmB* gene (lane 5). These data suggested that the secreted 48-kDa protein is the product of the *crmB* gene. The apparent molecular mass of this protein is greater than that predicted from the amino acid sequence, consistent with the predicted post-translational modification of this gene product. Similar analyses of viral proteins synthesized 14–17 hr after infection failed to provide any evidence of the synthesis and secretion of nascent CrmB protein at late times after infection (data not shown).

The CrmB protein binds both TNFα and TNFβ

The culture media of cells infected either with recombinant viruses or with wild-type viruses were examined for the presence of proteins capable of binding murine TNFα. Proteins present in the media were first resolved by SDS–gel electrophoresis under nonreducing conditions, after which they were transferred to a nitrocellulose

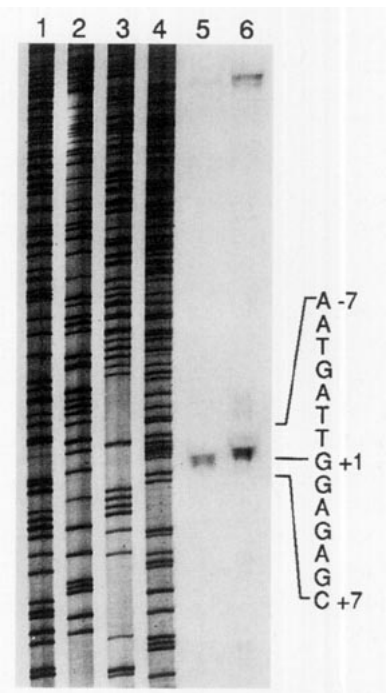

FIG. 4. S1 nuclease protection analysis of the transcriptional start site of the *crmB* gene. *MspI–ClaI* fragments (nucleotides 4423–4831, Fig. 2) were 5′-end-labeled with ^{32}P and hybridized with RNA extracted from cells infected with cowpox virus. Residual single-stranded nucleic acids were digested with S1 nuclease, and the nuclease-resistant products were resolved by electrophoresis in an 6% polyacrylamide gel containing 8 *M* urea. DNA fragments protected from digestion were visualized by autoradiography of the dried gel. The autoradiogram shows: lanes 1–4, size markers consisting of the products of sequence reactions using template DNA of known sequence (plasmid p1489); lanes 5 and 6, nuclease-resistant products remaining after hybridization of the probe with RNA extracted from cells either 3 hr (lane 5) or 16 hr (lane 6) after infection with cowpox virus. The longer nuclease-resistant products present in lanes 5 and 6 are full-length probe DNAs. The nucleotide sequence of the coding strand of the DNA is shown.

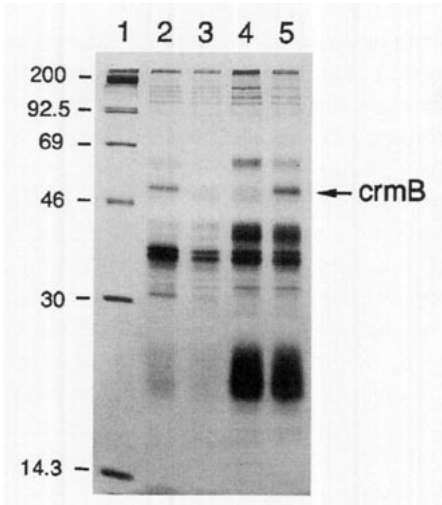

FIG. 5. The *crmB* gene encodes a soluble protein that is secreted from infected cells. Between 2 and 5 hr postinfection, human 143 cells were incubated in cysteine-free MEME supplemented with [^{35}S]-cysteine (1300 Ci/mmol) at 50 μCi/ml. At 5 hr postinfection, the media were removed and concentrated 25-fold. Proteins in the concentrated media were resolved by electrophoresis in a 12% polyacrylamide gel. The radiolabeled proteins were visualized by autoradiography of the dried gel. Lane 1, ^{14}C-labeled proteins that served as molecular mass standards (Amersham, Arlington Heights, IL); the molecular masses of these proteins are indicated in kDa. Lane 2, proteins secreted from cells infected with wild-type cowpox virus. Lane 3, proteins secreted from cells infected with cowpox virus A500, in which both copies of the *crmB* gene have been inactivated. Lane 4, proteins secreted from cells infected with wild-type vaccinia virus. Lane 5, proteins secreted from cells infected with vaccinia virus A495, which contains an intact copy of the cowpox virus *crmB* gene.

sheet and then assayed for their ability to bind ^{125}I-labeled TNFα (Fig. 6). Media from cells infected either with wild-type cowpox virus or with the recombinant vaccinia virus A495, which expresses the cowpox virus *crmB* gene, contained a protein that bound ^{125}I-labeled TNFα (lanes 3 and 4), whereas media from cells infected with wild-type vaccinia virus, which lacks functional *crmB* genes, did not (lane 8). Binding of ^{125}I-labeled TNFα to the CrmB protein was inhibited either by unlabeled murine TNFα (lane 5) or by human TNFβ (lane 7). Binding of the ^{125}I-labeled TNFα to the CrmB protein was not inhibited by a cocktail of cytokines including Interleukins 1–4, Interleukins 6 and 7, GM-CSF, G-CSF, TGFβ, NGF, and human interferon-γ (lane 6), indicating that the CrmB protein, like the human p75 TNF receptor, binds TNFα and TNFβ specifically.

The *crmB* gene is not required for the production of red pocks in the chorioallantoic membrane of the chick embryo

To determine if the *crmB* gene is one of the genes necessary for the formation of the red pocks characteristic of cowpox virus infection of CAMs of chick embryos, the forms of the pocks produced by cowpox virus A500 (containing inactivated *crmB* genes) were examined by means of a standard pock assay. After 3 days incubation, simple visual inspection revealed no marked differences between the majority of the pocks produced by the recombinant virus and those produced by the wild-type virus. Of 474 pocks produced by the A500 virus, 370 (78%) of the pocks were red, 88 (18.6%) were red with a white edge, and 16 (3.4%) were white. By comparison, of 874 pocks produced by the wild-type cowpox virus, 837 (95%) were red, 5 (0.6%) were red with a white edge, and 32 (3.6%) were white. After 4 days incubation, the results were similar. Of 216 pocks produced by the A500 virus, 150 (69.4%) of the pocks were red, 57 (26.4%) were red with a white edge, and 9 (4.2%) were white. Also, of 147 pocks produced by the wild-type cowpox virus, 127 (86.4%) were red, 11 (7.5%) were red with a white edge, and 9 (6.1%) were white. Therefore, these results show that red pocks are formed in the absence of the *crmB* gene product, demonstrating that this gene is not necessary for the production of red pocks. The generation of white pocks probably reflects the spontaneous generation of whitepock variants similar to those generated spontaneously in stocks of wild-type cowpox virus (Downie and Haddock, 1952; Van Tongeren, 1952), an event

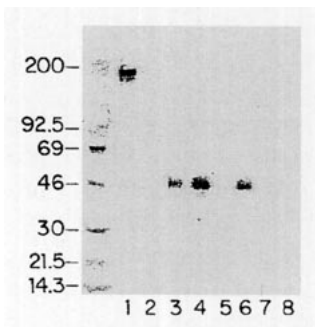

FIG. 6. The *crmB* gene encodes a secreted TNF-binding protein. Serum-free media from virus-infected cells were collected 5 hr after infection. Proteins in the media were concentrated eightfold and, together with purified p75/Fc TNF receptor, were subjected to nonreducing PAGE in 8–18% polyacrylamide gels. The resolved proteins were electrophoretically transferred to a nitrocellulose sheet. Sections of the sheet were then incubated for 1 hr at 4° with ^{125}I-labeled murine TNFα (1 n*M*; 3 × 10^{15} cpm/mmol) in the presence or absence of competitor ligands. The sheets were washed five times in phosphate-buffered saline, after which radiolabeled proteins immobilized on the sheets were detected by a phosphorimager. Lanes 1 and 2, purified recombinant p75/Fc TNF receptor (50 ng) incubated with ^{125}I-labeled murine TNFα in the absence (lane 1) or presence (lane 2) of unlabeled murine TNFα. Lane 3, medium from cells infected with vaccinia virus recombinant A495 (which contains an intact copy of the cowpox virus *crmB* gene) incubated with ^{125}I-labeled murine TNFα in the absence of competitor. Lane 4, media from cells infected with wild-type cowpox virus incubated with ^{125}I-labeled murine TNFα in the absence of competitor. Lanes 5, 6 and 7, media from cells infected with vaccinia virus recombinant A495 incubated with ^{125}I-labeled murine TNFα in the presence of unlabeled murine TNFα (lane 5), a cocktail containing interleukins 1–4, 6, and 7, GM-CSF, G-CSF, TGFβ, NGF, and γIFN, each at 0.1 μ*M* (lane 6), or unlabeled human TNFβ (lane 7). Lane 8, media from cells infected with wild-type vaccinia virus incubated with ^{125}I-labeled murine TNFα in the absence of competitor. The first lane contains ^{14}C-labeled proteins to provide molecular mass markers (indicated in kDa).

unrelated to the inactivation of the *crmB* gene (Archard *et al.*, 1984; Pickup *et al.*, 1984). The generation of pocks manifesting both red and white characteristics may reflect either the inactivation of the *crmB* gene or additional unidentified genetic alterations independent of, or unrelated to, those affecting the *crmB* gene.

Pocks produced in CAMs infected with vaccinia virus A495 (containing an active *crmB* gene) were also examined by visual inspection. After 3 days incubation, no significant differences were detected between the white pocks produced by the recombinant virus and those produced by the wild-type vaccinia virus. These results suggest that although the *crmB* gene may contribute to the viral inhibition of TNF-mediated host defenses, it is neither necessary nor sufficient for the generation of red pocks by cowpox or vaccinia viruses in CAMs during the first 3 to 4 days after infection.

DISCUSSION

The cowpox virus *crmB* gene encodes a soluble secreted TNF-binding protein, consistent with the amino acid sequence of the primary gene product. This protein contains a hydrophobic N-terminal region that could act as a signal peptide, the removal of which would generate an N-terminal region (~176 amino acids in length) structurally and functionally homologous to the ligand-binding portions of both p55 and p75 human TNF receptors (Fig. 7). These homologous regions all display fourfold pseudo-repeats, each containing about 40 residues and six cysteines. A recent crystal structure of a complex between human TNFβ and the extracellular portion of the human 55-kDa TNF receptor clearly establishes these repeats as true domains with highly conserved, intradomain, disulfide linkages, as indicated in Fig. 7 (S–S 1, 2, and 3 for domains 1–3; Banner *et al.*, 1993). The four similar domains make up the TNF-binding portions of these receptors.

The CrmB protein, like the cellular receptors, binds both TNFα and TNFβ, and the ligands cross-compete for binding. CrmB, however, shows considerably greater sequence similarity to the p75 TNF receptor than to the p55 version. This is most clearly seen through pairwise sequence alignments of CrmB with the p75 and p55 TNF receptors, using the Align program of the National Biomedical Research Foundation (Genetics Computer Group, 1991). CrmB and the p75 TNF receptor showed an Align score of 20 standard deviations above the mean score for an ensemble of randomly permuted sequences of the same lengths and amino acid composition. Scores greater than 3.0 are considered significant indicators of common ancestry. CrmB and the p55 TNF receptor, on the other hand, showed an Align score of only 9.0. The cowpox virus CrmB protein, therefore, may be derived from a cellular type II TNF receptor. A copy of part of the gene encoding this receptor may have been acquired from the host by an ancestral poxvirus.

The CrmB protein lacks a transmembrane domain and a cytoplasmic portion, both of which are present in most of the other members of the TNF receptor superfamily of proteins (reviewed by Smith *et al.*, 1994). Consequently, the CrmB protein resembles the soluble forms of many cytokine receptors, including p75 and p55 TNF receptors, which are generated through either alternative RNA splicing pathways or proteolytic release. The soluble versions of the receptors are believed to regulate cytokine activity *in vivo* (reviewed by Tracey and Cerami, 1993). Soluble receptors for TNF might modify the effects of TNF in a number of ways: by preventing the interaction between TNF and TNF receptors on the cell surface; by stabilizing free TNF in circulation, as has been proposed for the effects of soluble human TNF receptors (Mohler *et al.*, 1993); by promoting the removal or degradation of circulating TNF; or by affecting the activity or release of membrane-associated TNF.

The importance of viral inhibition of TNF-mediated antiviral processes is underscored by the conservation of genes encoding secreted TNF receptors in orthopoxviruses, as well as leporipoxviruses. Overall, the amino acid sequence of the cowpox virus CrmB protein shares a significant degree of identity (~48%) with the T2 proteins encoded by Shope fibroma virus and myxoma virus (Smith *et al.*, 1990; Upton *et al.*, 1991). The virulent orthopoxvirus, variola virus (strains India 1987 and Bangladesh), contains an orf (G4R in variola virus strain India and G2R in variola virus strain Bangladesh) predicted to encode a 349-amino-acid protein with 85% identity with the cowpox virus CrmB protein (Shchelkunov *et al.*, 1993; Massung *et al.*, 1993). This high degree of identity suggests that the properties of the putative variola virus TNF receptor are likely to be similar to those of the cowpox virus CrmB protein.

The divergence in the sequences of the TNF receptors of the orthopoxviruses and leporipoxviruses may reflect adaptation to optimize the interactions between a given viral receptor and the TNF of the species in which the virus replicates. Goodwin *et al.* (1991) have demonstrated that the murine p75 TNF receptor shows dramatic differences in affinities between human and murine TNF. The natural hosts of leporipoxviruses are rabbits, whereas the most common natural hosts of cowpox virus are rodents, cats, and humans, and the only natural hosts of variola virus are humans (reviewed by Fenner *et al.*, 1989). The divergence in the sequences of the viral TNF receptors may also reflect the adaptation of the secreted receptors to the differences in immunological surveillance among the various hosts.

Almost half of the CrmB protein, the C-terminal 161 amino acids, does not appear to be directly involved in TNF binding. Interestingly, as the alignment in Fig. 8 shows, this region is nearly as well conserved among the CrmB protein, the Shope fibroma virus T2 protein, and the predicted product of the variola virus G4R orf as

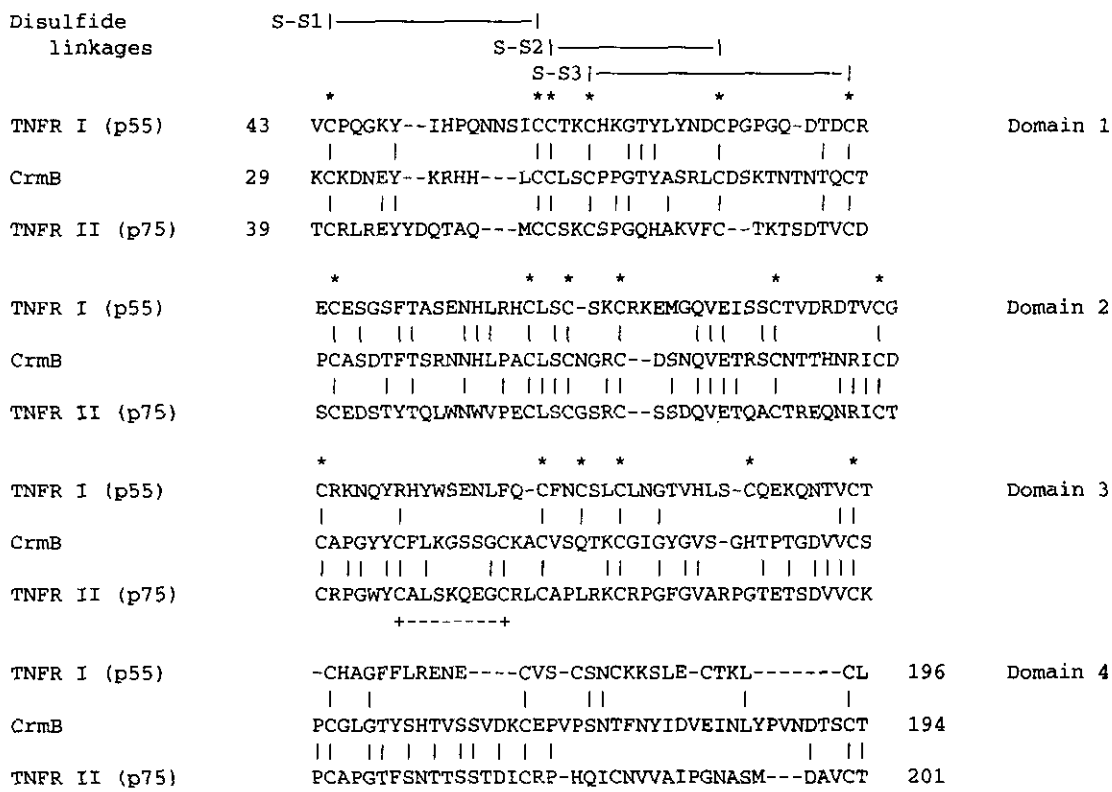

FIG. 7. The N-terminal region of the CrmB protein is similar to the ligand-binding domains of the human TNF receptors (p55 and p75). The amino acid sequences of the four cysteine-rich domains that form the ligand-binding portions of the human TNF receptors are aligned with the predicted ligand-binding domain of the CrmB protein. The disulfide linkages determined for the first three domains of the p55 TNF receptor are indicated (S–S 1–3), as are the conserved cysteines (*) and the predicted additional disulfide linkage (+ --- +) in the p75 TNF receptor, according to the structural determinations of Banner *et al.* (1993). The amino acid sequences of the human TNF receptor proteins are from Schall *et al.* (1990) and Smith *et al.* (1990). Numbers at the NH_2- and COOH-termini refer to residues as cited in the descriptions of the cDNA clones of the mRNAs encoding the human TNF receptors. Gaps, shown as "–", were introduced to optimize the alignment.

the ligand-binding domains. The conservation of this C-terminal region suggests that it may contain additional protein-binding sites that contribute to the activities of the CrmB protein *in vivo*. Attempts to identify proteins that interact with this portion of the receptor are under way.

The cowpox virus *crmB* gene is transcribed during the early phase of virus replication, consistent with the presence of transcriptional control elements that resemble the consensus sequences of both early promoters and early termination signals. The promoter of the *crmB* gene is immediately downstream of a set of tandem direct repeats of the sequence GAAAT, which is a subset of the consensus sequence for an early promoter (Davison and Moss, 1989). Whether this set of repeats influences the efficiency of transcription of the gene has yet to be determined. The corresponding region of the variola virus (India 1967) DNA, including the orf G4R and the predicted leader and promoter of this gene, shares 89% nucleotide sequence identity with the cowpox virus *crmB* gene, but the tandem repeats of the GAAAT sequence are not present in the variola virus DNA (Shchelkunov *et al.*, 1993). The same is true for the G2R orf of the Bangladesh strain of variola virus (Massung *et al.*, 1993). Interestingly, the promoter of the myxoma virus T2 gene, which is also an early gene, is immediately downstream of a set of tandemly repeated sequences (Upton *et al.*, 1991), but these repeats are not similar to those in the cowpox virus DNA, nor do they resemble a subset of the sequence of the early promoter.

The cowpox virus *crmB* gene, like the leporipoxvirus T2 genes, is present in two copies, one in each of the inverted terminal repeats of the viral DNA. This gene duplication may help the virus to generate larger quantities of the soluble TNF receptors than would be possible from a single copy of a gene expressed before viral DNA replication. The quantity of protein produced is likely to be especially important for this protein because after its secretion from the cell, its concentration may be rapidly reduced by both dilution and immune clearance. Similar conclusions have been reached by Mohler *et al.* (1993) with regard to the efficacy of soluble chimeric TNF receptors in clinical contexts. However, it should be noted that even greater quantities of CrmB protein could be produced if the gene were expressed throughout the course of the viral replication cycle. Therefore, the lack

N-terminal region including the signal peptide sequence

```
G4R      1 MKSVLYLYILFLSCIIINGRDAAPYTPPNG 30
CrmB     1 MKS..YILLLLLSCIIIINSDITPHEPSNG 28
T2       1     MLRLIALLVCVVYVYGDDVPYSSNQG 26
```

TNF-binding domains

1

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G4R     31 KCKDTEYKRHNLCCLSCPPGTYASRLCDSK..TNTQCT 66
CrmB    29 KCKDNEYKRHHLCCLSCPPGTYASRLCDSKTNTNTQCT 66
T2      27 KCGGHDYEKDGLCCASCHPGFYASRLCGP..GSNTVCS 62
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2

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G4R     67 PCGSGTFTSRNNHLPACLSCNGRCNSNQVETRSCNTTHNRICE 109
CrmB    67 PCASDTFTSRNNHLPACLSCNGRCDSNQVETRSCNTTHNRICD 109
T2      63 PCEDGTFTASTNHAPACVSCRGPCTGHLSESQPCDRTHDRVCN 105
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3

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G4R     110 CSPGYYCLLKGSSGCKACVSQTKCGIGYGVSGHTSVGDVICS 151
CrmB    110 CAPGYYCFLKGSSGCKACVSQTKCGIGYGVSGHTPTGDVVCS 151
T2      106 CSTGNYCLLKGQNGCRICAPQTKCPAGYGVSGHTRAGDTLCE 147
```

4

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G4R     152 PCGFGTYSHTVSSADKCEPVPNNTFNYIDVEITLYPVNDTSCT 194
CrmB    152 PCGLGTYSHTVSSVDKCEPVPSNTFNYIDVEINLYPVNDTSCT 194
T2      148 KCPPHTYSDSLSPTERC....GTSFNYISVGFNLYPVNETSC. 185
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C-terminal region

```
G4R     195 RTTTTGLSESILTSELTITMNHTDCNPVFREEYFSVLNKVATSGFFTGENRYQN 248
CrmB    195 RTTTTGLSESISTSELTITMNHKDCDPVFRNGYFSVLNEVATSGFFTGQNRYQN 248
T2      186 .TTTAGHNEVIKTKEFTVTLNYTDCDPVFHTEYYATSGKEGAGGFFTGTDIYQN 238

G4R     249 ISKVCTLNFEIKCNNKG..SSFKQLTKAKNDDG.MMSHSETVTLAGDCLSSVDI 299
CrmB    249 ISKVCTLNFEIKCNNKDSYSSSKQLTKTKNDDDSIMPHSESVTLVGDCLSSVDI 302
T2      239 TTKVCTLNVEIQCSEGDDI.....HTLQKTNGGSTMPHSETITVVGSCLSDVNV 287

G4R     300 YILYSNTNAQDYETDTISYRVGNVLDDDSHMPGSCNIHKPITNSK...PTRFL 349
CrmB    303 YILYSNTNTQDYETDTISYHVGNVLDVDSHMPGRCDTHKLITNSNSQYPTHFL 355
T2      288 DIMYSDTNHPGEVDDFVEYHWGTRLRF.FPLPKRCTPVS....... 325
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FIG. 8. The entire sequence of the CrmB protein, including the portion C-terminal to the TNF-binding domains, is conserved among poxvirus soluble TNF receptors. The sequence of the CrmB protein is aligned with the corresponding regions of the Shope fibroma virus T2 protein (Upton *et al.*, 1987; Smith *et al.*, 1990) and the predicted product of the variola virus G4R/G2R orf (Shchelkunov *et al.*, 1993; Massung *et al.*, 1993).

of late synthesis of this protein suggests that this receptor may be advantageous to the virus only during the early phase of the virus replication cycle.

One reason for determining whether cowpox virus encodes a protein similar to a TNF receptor was to see if such a protein might be necessary for the production of the red pocks characteristic of cowpox virus infection of the CAMs of chick embryos. However, red pocks were

produced by cowpox virus A500 even after the inactivation of both copies of the *crmB* gene, and white pocks were produced by vaccinia virus A495 containing the cowpox *crmB* gene. Similar results have been obtained by Palumbo *et al.* (1994), who also found that recombinant cowpox virus A500 predominantly produced red pocks, some of which contained pockets of inflammatory cells. Therefore, although the cowpox virus *crmB* gene may contribute to the viral inhibition of TNF-mediated processes, it appears to be neither necessary nor sufficient for production of red pocks in the CAMs of chick embryos.

The role of the cowpox virus *crmB* gene during the course of infection in mammals has yet to be determined. This gene encodes an early protein which binds TNFα and β. Therefore, by analogy with the proposed actions of soluble versions of cellular TNF receptors and the leporipoxvirus TNF receptors, it is likely that the cowpox virus TNF receptors modify TNF responses to infection. This may occur either by inhibition of the interaction between TNF and TNF receptors on the cell surface or by alteration of either the stability or the distribution of TNF in the infected animal. The demonstration that cowpox virus encodes this soluble TNF receptor provides further evidence that the simultaneous inhibition or attenuation of multiple cytokine responses to infection is advantageous to cowpox virus during replication in its host animals.

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