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Mapping and Nucleotide Sequence of the Vaccinia Virus Gene That Encodes a 14-Kilodalton Fusion Protein

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A library of rabbit poxvirus DNA fragments contained in the expression cloning vector λgt11 was screened with monoclonal antibodies that react specifically against a 14-kilodalton envelope protein of vaccinia virus and rabbit poxvirus. The 14-kilodalton protein appears to play an important role in virus penetration at the level of cell fusion; it also elicits neutralizing antibodies, and it forms covalently linked trimers on the surface of virions and in infected cells (Rodriguez et al., J. Virol. 56:482–488, 1985; Rodriguez et al., J. Virol. 61:395–404, 1987). Two recombinant bacteriophages expressing β-galactosidase fusion proteins were isolated. Restriction enzyme analysis and hybridization studies mapped the 14-kilodalton encoding sequences in the middle of vaccinia virus *Hin*dIII A DNA fragment. Nucleotide sequence analysis revealed an open reading frame (ATG) preceded by a characteristic TAA sequence of late genes. The sequence spans 330 nucleotides and codes for a protein with a molecular weight of 12,500 and an isoelectric point of 6.3. There are two small hydrophobic regions, one at the C terminus (11 amino acids) and the other at the N terminus (5 amino acids). The protein contains two cysteines for oligomer formation and one glycosylation site. Inspection of the deduced amino acid sequence of the 14-kilodalton protein revealed consensus sites with the hemagglutinin precursor of influenza A virus and with adenylate kinase and cytochrome *c* of various species.

The molecular mechanisms involved in penetration of poxviruses in animal cells and the primary structure of the viral proteins involved in this event have not yet been elucidated. We have previously identified a 14-kilodalton (kDa) vaccinia virus envelope protein that is highly conserved in members of the orthopoxvirus group. This protein appears to play a major role in virus penetration, acting at a step subsequent to virus attachment, i.e., in cell fusion (7, 9). Because we have specific monoclonal antibodies (MAbs) that react with the 14-kDa protein of vaccinia virus and rabbit poxvirus and because several vaccinia virus genes such as RNA polymerase subunits (5) and DNA-dependent ATPase I (8) have been isolated from a rabbit poxvirus DNA library contained within the expression vector λgt11, we sought to clone the vaccinia virus gene encoding the 14-kDa protein from this library. In this report we provided the mapping, nucleotide sequence, and deduced amino acid sequence of the 14-kDa envelope protein of vaccinia virus.

MATERIALS AND METHODS

Generation of MAbs. MAbs against structural proteins of vaccinia virus were generated as described by Rodriguez et al. (7). MAbC3 and MAbB11 react against a 14-kDa envelope protein of orthopoxvirus (7). This protein forms covalently linked trimers on the surface of virions and in infected cells (9). MAbC3 prevents virus uncoating and blocks virus-induced cell fusion but does not prevent virus attachment to cells (7).

Antibody screening of λgt11 library. A library prepared from randomly generated rabbit poxvirus DNA fragments contained within the expression vector λgt11 (5) was kindly provided by R. W. Moyer (Vanderbilt University, Memphis, Tenn.). The library was screened by the procedure of Young and Davis (12). Bacteriophages (1.2×10^6) were allowed to infect an overnight culture of *Escherichia coli* Y1090 for 15 min at 37°C, plated in 150-mm dishes at 2×10^4 per plate in 0.7% soft agar, and incubated at 42°C for 3 h. Plates were overlaid with nitrocellulose paper presoaked in 10 mM isopropyl-β-D-thiogalactoside and incubated at 37°C for 18 h. Thereafter, filters were washed at room temperature in phosphate-buffered saline and blocked in phosphate-buffered saline containing 0.5% bovine serum albumin and 15% fetal calf serum for 1 h. Filters were washed twice for 10 min with phosphate-buffered saline and incubated overnight with a 1:200 dilution of a mixture of MAbC3 and MAbB11 as ascites. Before its use in screening, the diluted antiserum was preadsorbed with filters containing *E. coli* Y1090 cells infected with wild-type λgt11 phages.

Immunopositive plaques were detected by secondary incubation with ^{125}I-labeled goat anti-mouse immunoglobulin G (Amersham Corp.). Positive plaques were picked, plaque purified, and screened until homogeneously positive. High-titer stocks of each positive phage were prepared by making plate lysates (2) on *E. coli* Y1090.

Fusion proteins and immunoblotting. The selected phage clones (K8 and K10) were used to obtain the corresponding β-galactosidase fusion proteins by infecting the bacterial strain Y1089 as previously described (5). Briefly, an overnight culture of Y1089 was used to inoculate fresh L broth, and this culture was incubated at 30°C to an absorbance at 550 nm of 0.5. The cells were then pelleted, suspended in 1/100 of the original volume in lambda buffer (10 mM Tris hydrochloride [pH 8.0], 10 mM $MgCl_2$), and infected with 5 PFU of phage K8 or K10 per cell. After 15 min at 30°C, the volume was brought to the original by adding fresh L broth containing 10 mM isopropyl-β-D-thiogalactoside. Thereafter, cultures were incubated for 5 min at 42°C and for 2 h at 37°C. Cells were harvested by centrifugation (5,000 × *g* for 15 min), suspended in 100 μl of DNase buffer (50 mM Tris hydrochloride [pH 8.0], 0.2 mM EDTA, 2.5 mM $MgCl_2$, 0.15 M NaCl, 10% glycerol, 4 mM phenylmethylsulfonyl fluo-

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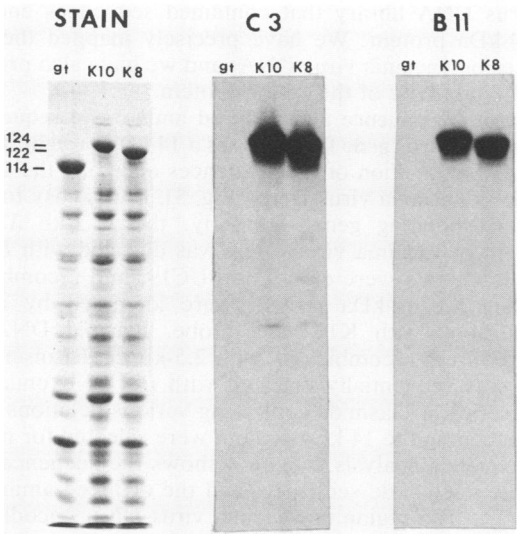

FIG. 1. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of *E. coli* extracts containing the 14-kDa β-galactosidase fusion proteins. The first three lanes on the left contained samples of extracts prepared from phage-infected *E. coli* cells and stained with Coomassie blue. The three lanes in the center are immunoblots from the same cell extracts reacted with mAbC3 followed by treatment with ^{125}I-labeled goat anti-mouse immunoglobulin G. The three lanes to the right are immunoblots from cell extracts reacted with mAbB11 and ^{125}I-labeled immunoglobin. The phages used to prepare the extracts are indicated on top of the gel. Molecular masses in kDa are on the left.

ride), and treated with 1 μl of DNase (10 mg/ml) for 10 min at 37°C. Total cell-free proteins (40 μg) from induced infected Y1089 cells were fractionated on 8% sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The fusion proteins were visualized by Coomassie blue staining and by immunoblotting. After fractionation, the gel was briefly soaked in 25 mM Tris hydrochloride (pH 8.37)–192 mM glycine–2% (vol/vol) methanol–0.1% sodium dodecyl sulfate, and polypeptides were transferred to nitrocellulose (0.45 μm). The conditions of the transfer were 4 h at room temperature with a current of 40 V and 0.25 A. The paper was soaked for 2 h at room temperature with 5% blotto (nonfat dry milk) in phosphate-buffered saline and then incubated overnight with 1:200 dilution of MAbC3 and MAbB11 as ascites in blotto. Thereafter the paper was washed with phosphate-buffered saline four times for 15 min each and then incubated for 2 h at room temperature with ^{125}I-labeled goat anti-mouse immunoglobulin G.

Cloning, mapping, and DNA sequence analysis. Isolation of phage, plasmid, and vaccinia virus DNAs was carried out by standard procedures (1a, 3). Restriction site mapping was carried out with different enzymes according to the instructions of the manufacturer. DNA fragments were resolved by electrophoresis in agarose gels. The gels were photographed, blotted onto nitrocellulose, and hybridized with appropriate nick-translated DNA fragments as probes (3). DNA fragments obtained by digestion of viral and phage DNA were inserted into the plasmid pUC19, cut with the appropriate endonuclease, ligated, and employed for the transformation of *E. coli* JM83. Deletions of the plasmid pE17 were obtained after linearizing the plasmid by restriction with the endonuclease *Cla*I, followed by treatment with the exonuclease *Bal* 31 (3). After *Bal* 31 digestion, DNA was phenol-chloroform extracted twice, cut with *Sma*I to generate a blunt end, ligated, and used to transform *E. coli* JM83. DNA sequencing of different fragments was carried out by the chemical degradation method (4) after end labeling with the Klenow fragment or polynucleotide kinase. Both strands of DNA were sequenced.

RESULTS

Identification of 14-kDa encoding sequences from the rabbit poxvirus DNA library in λgt11. We have previously described MAbs that recognize a 14-kDa envelope protein in vaccinia virus and in rabbit poxvirus (7). Therefore, we sought to clone the 14-kDa gene from the rabbit poxvirus DNA library contained within the expression vector λgt11 by using a mixture of MAbC3 and MAbB11. After the library was screened two positive bacteriophage plaques were picked, cloned, and rescreened several times, and cloned phages K8 and K10 were isolated and used for detailed study. These phages were used to infect competent *E. coli* Y1089 cells; after thermal induction total cell proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and β-galactosidase fusion protein was identified by staining and by immunoblotting with ^{125}I-labeled goat anti-mouse immunoglobulin G (Fig. 1). Although there was no reactivity with proteins from lambda wild-type infected cells, there was strong reactivity in proteins induced by the two recombinant phages. Clones K8 and K10 produced immunoreactive polypeptides of 124 and 122 kDa, respectively. Since the sizes of these polypeptides are greater than the 114-kDa β-galactosidase, the immunoreactive polypeptides of Fig. 1 must represent fusion proteins. Due to the specificity of both MAbs (7), we conclude that we have cloned sequences encoding the 14-kDa gene.

Mapping and direction of transcription of the vaccinia virus gene encoding the 14-kDa protein. DNA was prepared from purified phages, labeled with ^{32}P by nick translation, and used to probe blots of electrophoretically separated restriction fragments of vaccinia virus DNA. When vaccinia virus DNA was digested with *Hin*dIII, *Xho*I, *Kpn*I, and *Sal*I, the phage K10 DNA hybridized with the single fragments *Hin*dIII-A (data not shown) and *Xho*I-E, *Kpn*I-C, and *Sal*I-A (Fig. 2A). The map assignment of restriction fragment is taken from De Filippes (1). A similar pattern of hybridization was obtained with phage K8 DNA as the probe (data not shown). When DNA from the phage K10 was hybridized to *Eco*RI digests of DNAs from clones K8 and K10 the probe hybridized to digestion products of 1.40 kilobases (kb) (clone K8) and 1.40 and 0.4 kb (clone K10). Note an extra band of about 1.8 kb on K10 due to partial DNA digestion. The large hybridization fragment is phage DNA (Fig. 2B). To obtain a more precise mapping, the *Xho*I E fragment was digested with *Eco*RI, blotted, and hybridized independently with three different probes: the insert from phage K8 and the large and small *Eco*RI fragments from phage K10 insert. The results allowed us to map the 14-kDa gene within a 2.5-kb *Eco*RI fragment located in the center of the *Xho*I E fragment (Fig. 3). The mapping and the fusion protein sizes from phages K10 and K8, respectively, indicated that the only possible orientation for the 14-kDa gene was from right to left within the 2.5-kb *Eco*RI fragment. To confirm this assumption, the ends of both K8 and K10 inserts were sequenced after *Eco*RI digestion and terminal labeling. As expected the right ends of these two inserts were in the same reading frame with respect to the β-galactosidase gene. The end corresponding to K10 is 30 base pairs larger than K8; this difference is in perfect agreement with the 2-kDa differ-

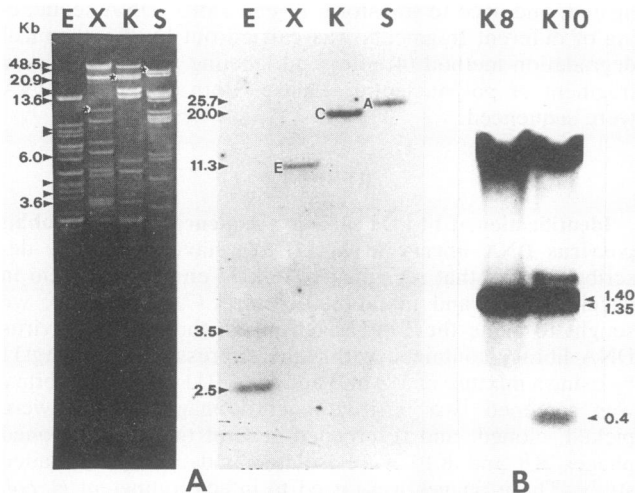

FIG. 2. Mapping and direction of transcription of the gene encoding the 14-kDa protein from recombinant phages. (A) Ethidium bromide staining and Southern blots of total vaccinia virus DNA digested with various restriction enzymes and hybridized with labeled DNA from recombinant phage K10. Molecular sizes in kb are those of *Hin*dIII restriction fragments of vaccinia virus DNA. (B) Southern blot of DNA from recombinant phages digested with *Eco*RI and hybridized with labeled K10 probe.

ence observed in the molecular mass of the fusion proteins encoded by the two recombinant phages. These results were later confirmed by sequencing the *Cla*I-*Eco*RI fragment from vaccinia virus DNA, in which an open reading frame of the expected length was found. The reading frames of K10 and K8 were also coincidental with the reading frame found within the vaccinia virus fragment. We conclude that we have isolated two overlapping inserts from the rabbit poxvirus DNA library that contained sequences encoding the 14-kDa protein. We have precisely mapped these sequences on vaccinia virus DNA, and we have also provided the reading frame of the fusion protein.

Nucleotide sequence and deduced amino acid sequence of the vaccinia virus gene that encodes a 14-kDa protein. Knowing the map position of the sequences encoding the 14-kDa protein in vaccinia virus DNA (Fig. 3), it was easy to clone the corresponding gene. Basically, the 11.3-kb *Xho*I E fragment of vaccinia virus DNA was digested with *Eco*RI, DNA fragments were cloned in pUC19, and recombinants encoding the 14-kDa protein were identified by colony hybridization with K10 as a probe. Plasmid DNA was prepared from recombinants with 2.5-kb *Eco*RI insert. The insert was sequentially digested with *Bal* 31 exonuclease, and a series of plasmids containing various deletions of the gene encoding the 14-kDa protein were selected for nucleotide sequence analysis. Figure 4 shows the sequence strategy, the nucleotide sequence, and the deduced amino acid sequence. The region of vaccinia virus DNA encoding the 14-kDa protein contains 110 amino acids, giving a molecular mass of 12.5 kDa. It contains two contiguous cysteines at positions 71 and 72; hence disulfide bonds are possible. It also contains a putative glycosylation site at positions 60 through 62 and two possible thiol protease cleavage sites at positions 32 through 33 and 104 through 105. This proteolytic cleavage could generate the polypeptide of about 11 kDa that appears during prolonged virus storage (7). The predicted secondary structure deduced by computer analyses is shown in Fig. 5. The protein is hydrophilic. It has two small hydrophobic domains, one at the C terminus (11 amino acids) and the other at the N terminus (5 amino acids). A computer search (The International Biotechnology sequence analysis programs) based on comparison of the amino acid sequence with other known proteins reveals consensus sites with the hemagglutinin precursor of influenza A virus and

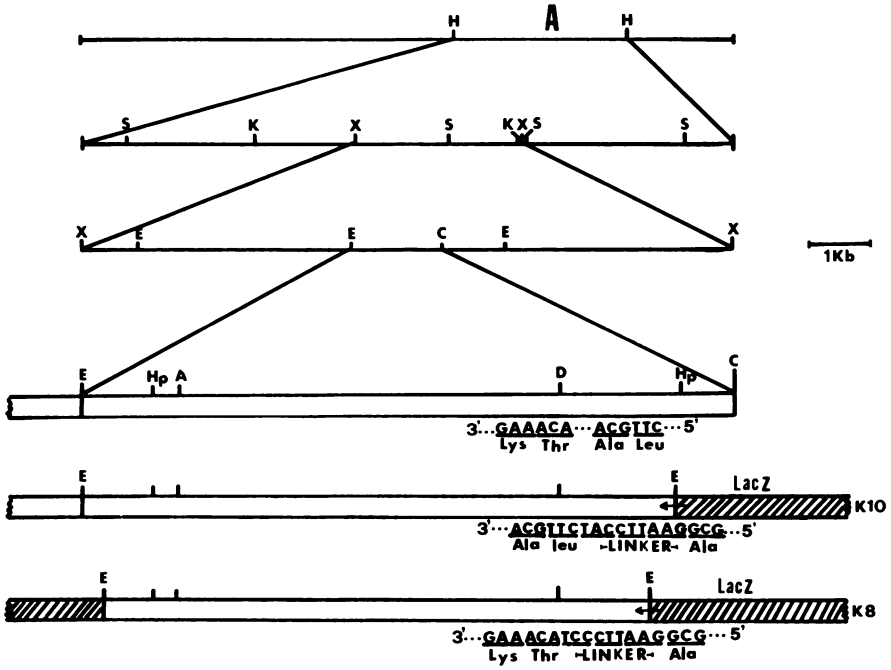

FIG. 3. Physical map of 14-kDa gene on vaccinia virus DNA and nucleotide sequence of the boundary region between the *LacZ* gene, inserts from K10 and K8, and the open reading frame present in the *Cla*I-*Eco*RI region of vaccinia virus DNA. Restriction enzymes: *Hin*dIII (H), *Sal*I (S), *Kpn*I (K), *Xho*I (X), *Eco*RI (E), *Cla*I (C), *Hpa*II (Hp), *Alu*I (A), *Dde*I (D).

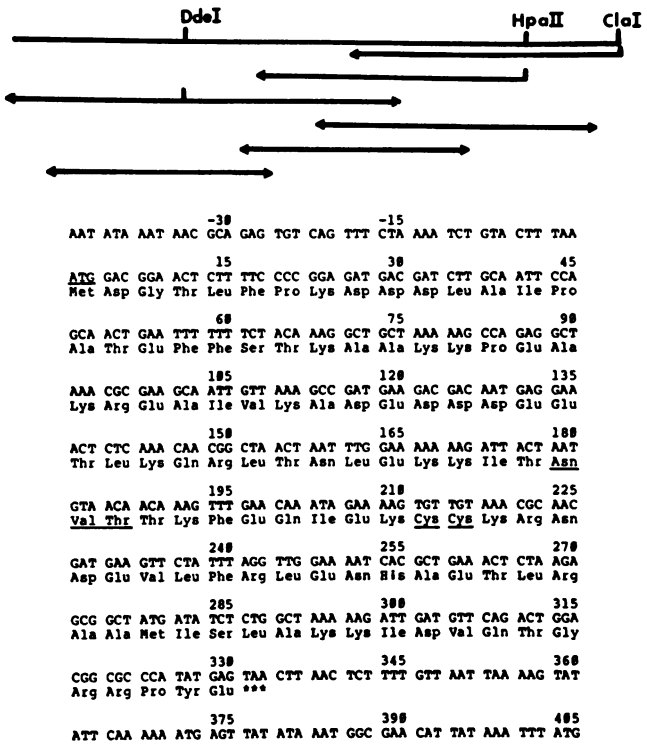

FIG. 4. Nucleotide sequence and deduced amino acid sequence of vaccinia virus gene encoding the 14-kDa protein. The sequence strategy is indicated in the upper part of the figure. Maxam-Gilbert sequence analysis (4) was carried out after labeling in different restriction sites present in the insert (*Cla*I, *Hpa*II, and *Dde*I) and also from overlapping *Bal* 31 deletions generated from the *Cla*I site by labeling in the *Hin*dIII site located in the polylinker region of pUC19. The nucleotides are numbered above the sequence in the 5'-to-3' direction; nucleotide 1 is the A of the ATG codon for the initiator methionine.

with adenylate kinase and cytochrome *c* of various species (Fig. 6).

DISCUSSION

Little is known about the mode of penetration of vaccinia virus in animal cells. In this investigation we have cloned, mapped, and sequenced the virus gene that encodes a 14-kDa structural protein involved in cell fusion and with an important role in virus penetration (7, 9). By using specific MAbs reactive against a 14-kDa structural protein of vaccinia virus and rabbit poxvirus we isolated from a rabbit poxvirus DNA library contained within the expression vector λgt11 recombinants that contained sequences of the 14-kDa gene. We have precisely mapped these sequences in the center region of vaccinia virus *Hin*dIII-A DNA, and we have isolated the corresponding gene from wild-type vaccinia virus DNA. Nucleotide sequence analysis revealed an open reading frame that exist in a region spanning nucleotides 1 (first ATG) to 330 and codes for a polypeptide of 12,500 Da. To confirm that the open reading frame actually corresponded to the gene encoding this polypeptide, sequence analyses of overlapping sequences from cloned inserts were carried out. Both the region of vaccinia virus DNA coding for the 12,500-Da protein and the rabbit poxvirus DNA in the expression vector were in the same reading frame (Fig. 3). Computer analysis of the hydrophobicity plot of the 14-kDa protein showed the existence of two hydrophobic regions, one at the C terminus (11 amino acids) and the other at the N terminus (5 amino acids). The amino acids at the COOH terminus could provide a stop-transfer sequence by binding tightly to the fatty acid core of the membrane. The hydrophobic region in the N terminal of the 14-kDa protein might have anchor sequences together with uncleaved signal peptide regions as in the influenza virus neuraminidase (11).

The 14-kDa protein is very hydrophilic, with an isoelectric point of 6.3. The calculated 12,500-Da molecular mass is lower than the 14-kDa molecular mass estimated from sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The difference in size may be due to protein modifications such as carbohydrate addition due to the potential glycosylation site, Asp-Val-Thr, at positions 60 through 62, to fatty acid incorporation or both. The 14-kDa protein forms covalently linked trimers both on the surface of virions and on infected cells (9). Since the protein contains two contiguous cysteine residues, trimers could be formed through disulfied bridges. A search of the protein sequence data bank found consensus sites with protein domains that are not apparently involved in cell fusion. The significance of consensus sites with these proteins (Fig. 6) is not known.

The 14-kDa protein is expressed at late times postinfection (9). A conserved feature of late genes is the TAA sequence that immediately precedes the ATG translation initiation codon (10) and the vaccinia virus 14-kDa gene does have this conversed TAAATG sequence.

The role of the 14-kDa protein on virus multiplication is not known. We have isolated from Friend erythroleukemia

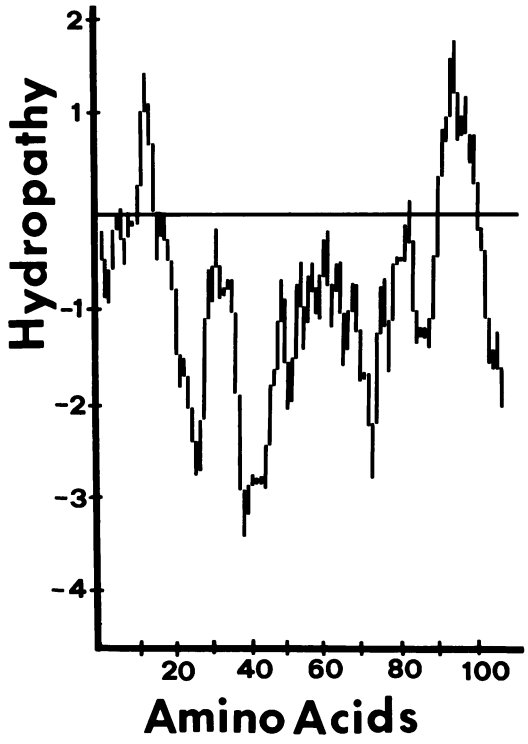

FIG. 5. Hydropathy plot of the 14-kDa envelope protein of vaccinia virus. Hydropathy analyses and homology comparisons were performed by using the International Biotechnologies sequence analyses procedure of Kyte and Doolittle (2). Hydrophobic regions are above the axis, and hydrophilic regions are below it.

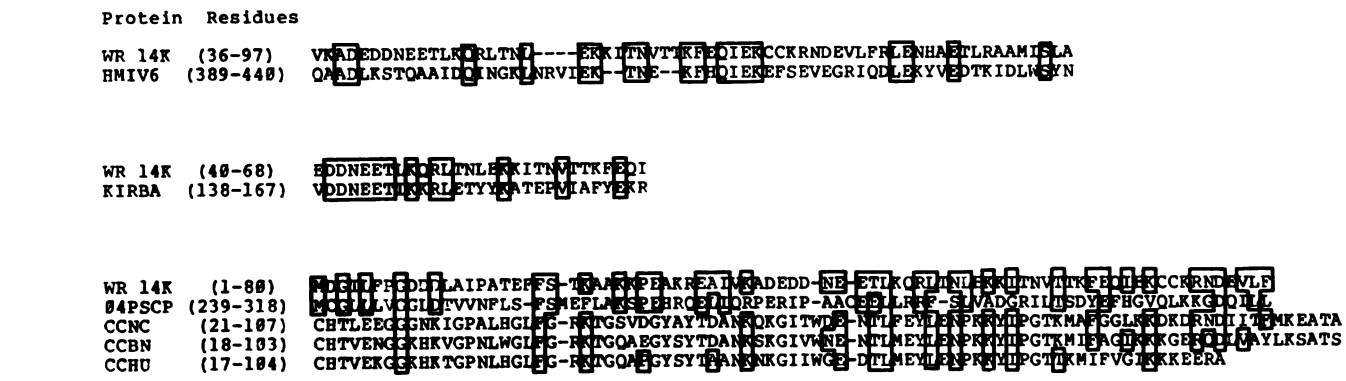

FIG. 6. Consensus sites between the vaccinia virus 14-kDa protein and proteins of different origins. Homologous sequences are aligned with the precursor of hemagglutinin of influenza A virus (HMIV6), with adenylate kinase (KIRBA) of rabbits (bovine, pig, and human adenylate kinase are similar), and with cytochrome *c* P450-CAM of *Pseudomonas putida* (04PSCP), *Neurospora crassa* (CCNC), tuna (CCBN), and humans (CCHU).

cells, persistently infected with vaccinia virus, spontaneous mutants that have reduced plaque size and have increased the size of the 14-kDa protein (6, 6a). The reduced plaque size and protein modifications of the mutants were rescued with the gene that encodes the 14-kDa protein of wild-type virus (1). These findings suggest that the 14-kDa protein is essential for efficient virus multiplication. Because of the ability of the wild-type gene to rescue the small plaque size phenotype of mutant viruses to a large plaque size phenotype and because the rescued viruses grew faster in culture than mutant viruses, this gene potentially could be used to generate novel-expression vaccinia virus vectors where selection and amplification of recombinants can be carried out in a single step. In addition, since the 14-kDa protein is transported and anchored on the cell surface during virus infection (9), the signal sequence and transmembrane domains of this protein could be used to generate recombinants of vaccinia virus in which foreign genes could be efficiently targeted to the cell membrane. If protein domains are now exposed on the cell surface they might evoke a strong immune response. Experiments are in progress to test these possibilities.

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