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SHORT COMMUNICATIONS

Sequences of the Raccoon Poxvirus Hemagglutinin Protein[1]

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Primers based on sequences flanking the vaccinia virus (VV) strain IHD hemagglutinin protein (HA) open reading frame (ORF) enabled amplification of HA DNA segments from the genome of raccoon poxvirus (RCN) and VV strain WR. The amplified segments produced unequal cross-hybridization signal intensities against each other, indicating sequence differences between the HA of RCN (in *Hin*dIII-G) and that of VV-WR (in *Hin*dIII-A). About 1.5 kb of sequences in the HA region were then determined from clones pRCN/*Hin*dIII-G and pVV/*Bam*HI-32, a subclone of VV-WR *Hin*dIII-A. Pairwise analyses of the RCN and VV-WR HA nucleotide sequences showed 82, 66, and 86% homology, respectively, between the putative promoter, ORF, and transcript terminator regions and 53% homology between the deduced amino acid sequences of the HA of RCN (310 residues) and those of VV-WR (314 residues). The deduced HA amino acid sequences showed a putative signal peptide and transmembrane-anchor moiety of 70 and 62% homology and a rather distinct central domain (residues 146 to 254) of 32% homology. Additional hybridizations with the amplified segments described above enabled location of the HA gene in the *Hin*dIII-A fragment of the orthopoxviruses volepox virus (VPX) and skunk poxvirus (SKP); however, amplified DNA of either the entire HA ORF of RCN or that of VV-WR, or a portion, from the center to right end, did not hybridize with VPX or SKP, suggesting that the HA of RCN, VV, VPX, and SKP are rather diverged from each other. The VV HA was found to be closely related to that of ectromelia and variola viruses. The data are consistent with reports of hemagglutination-inhibition partial cross-reactivity between RCN, VPX, VV, and other orthopoxviruses and might lead to an explanation of the basis of syncytia formation by RCN, VPX, and SKP.

Studies, mainly with vaccinia virus (VV), have indicated that hemagglutinin preparations are composed of modified infected-cell membranes that contain a complex of host-cell and virus-encoded components, including a hemagglutinin protein (HA) that is required for the hemadsorption (HAD) and hemagglutination of chicken erythrocytes (reviewed in Ref. *1*). Recently, transcription of the VV HA was shown to be regulated by two promoters, functional at late and at early and late times postinfection, producing an 85-kDa major glycoprotein that is phosphorylated and sulfated. The 85-kDa species and several subspecies were detected mainly late in infection (*2, 3*). Modified cell membranes show altered biologic and antigenic properties which consequently affect the envelope of extracellular enveloped virions (*4*). The mechanisms involved and the interactions between the HA and other components of infected cells are unclear. However, studies (*1, 5, 6*) have indicated that monolayer cells form polykaryons after infection with VV strains with a deleted or altered HA, such as VV-IHDW and the VV subspecies rabbitpox virus (RPV, Utrecht strain), which show a truncated HA open reading frame (ORF). Recently, a VV serine protease inhibitor homologue, ORF K2L, has also been implicated as a cell fusion inhibitor (*7, 8*). Curiously, cells infected with variola major virus isolates usually had been HAD^+ and cells infected with Alastrim virus isolates often had been HAD^- (*9*), but both variola types induced hyperplastic foci of nonfused infected cells (reviewed in Ref. *10*). In contrast, many cell lines infected with raccoon poxvirus (RCN), volepox virus (VPX), or skunk poxvirus (SKP), and other orthopoxviruses which are HAD^+, form large syncytia (*11, 12*), suggesting that conformationally distinct, functional HAs influence in polykaryocytosis.

The suggestion that VV, RCN, and VPX have distinctive HAs is supported by serologic data. RCN, isolated

[1] Sequence data from this article have been deposited with the EMBL/GenBank Data Libraries under Accession Nos. M94169 and M93956, which include 70 bases upstream of the sequences shown in Fig. 2.

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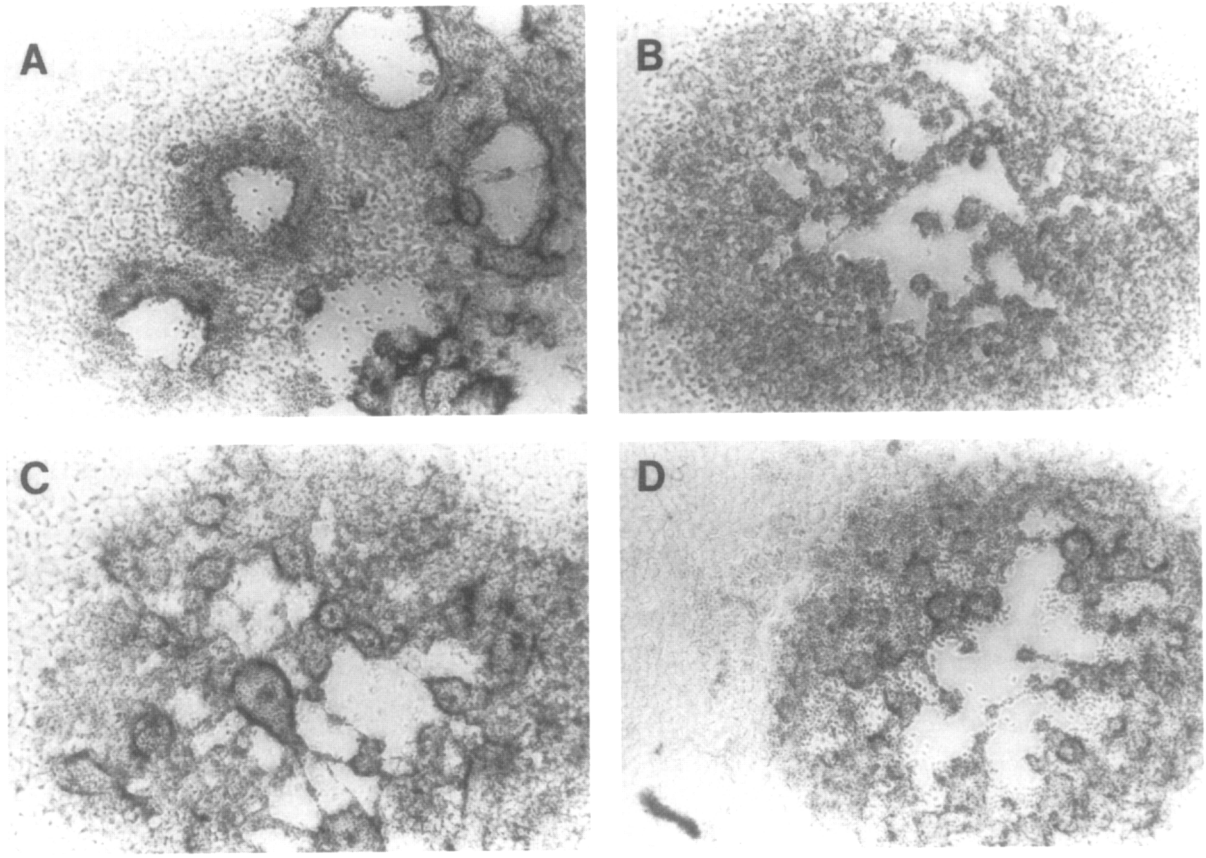

FIG. 1. Hemadsorption of chicken erythrocytes to Vero cell monolayers infected with (A) RCN, (B) VV-WR, (C) VPX, and (D) SKP. Cell monolayers were infected with 10^2 PFU (50-mm petri dishes) of virus and at 48 hr p.i. The medium was removed and the monolayers were washed twice with PBS. Monolayers were overlaid with 0.5% suspension of PBS-washed erythrocytes and after incubation at 37° for 1 hr, unadsorbed erythrocytes were rinsed gently from the monolayers. Hemadsorption was observed by phase contrast microscopy. SKP was isolated in 1978 from lung tissues of a striped skunk shot in Colfax, WA, and was identified as an orthopoxvirus based on results of morphologic, antigenic, and DNA cross-hybridization assays (D. Burger and J. Esposito, data not shown).

from 2 of 92 raccoons trapped in Maryland, was proposed to be an orthopoxvirus when it was noted that a RCN hemagglutinin preparation reacted with VV hyperimmune rabbit serum (*13*). However, it was later reported (*14*) that sera from 22 of the raccoons reacted highly with a RCN hemagglutinin preparation, but hemagglutinin preparations of VV or monkeypox virus (MPV) showed little or no cross-reactivity. Thomas *et al.* (*15*) further noted that RCN hemagglutinin preparations were partly inhibited by VV or cowpox virus (CPV) hyperimmune rabbit sera, and VV or CPV preparations were partly inhibited by RCN hyperimmune rabbit or raccoon sera. Regnery (*16*) later noted that sera from VPX-infected voles showed greater reactivity with a VPX hemagglutinin preparation than with RCN or VV preparations.

To gain further insight into the biologic and antigenic relationships of the HA of RCN and other orthopoxviruses, the present study shows DNA sequences encoding the HAs of RCN and VV strain WR and describes results which indicate that the HAs of VV, RCN, VPX, and SKP are rather diverged. Initially, we established that a functional hemagglutinin was produced by the virus stocks that we used for preparing DNA of the orthopoxviruses used in this study by showing chicken erythrocytes circumscribing plaques formed in Vero monkey kidney cell monolayers infected with RCN, VV-WR, VPX, or SKP (Fig. 1). Large syncytia containing RCN-, VPX-, or SKP-infected cells at the periphery of the plaques were seen prior to the hemadsorption of erythrocytes (see also *11*).

Next, Southern blot hybridizations between polymerase chain reaction (PCR) DNA amplified from the genome of VV-WR and RCN and *Hin*dIII digests of genome DNA of VV-WR, RCN, VPX, and SKP enabled us to locate the HA gene in the *Hin*dIII-G DNA fragment of RCN and in the *Hin*dIII-A fragments, of VV-WR, VPX, and SKP, although weak autoradiograph signals were observed between the heterologous virus DNA digests (data not shown). Amplified DNAs were made with

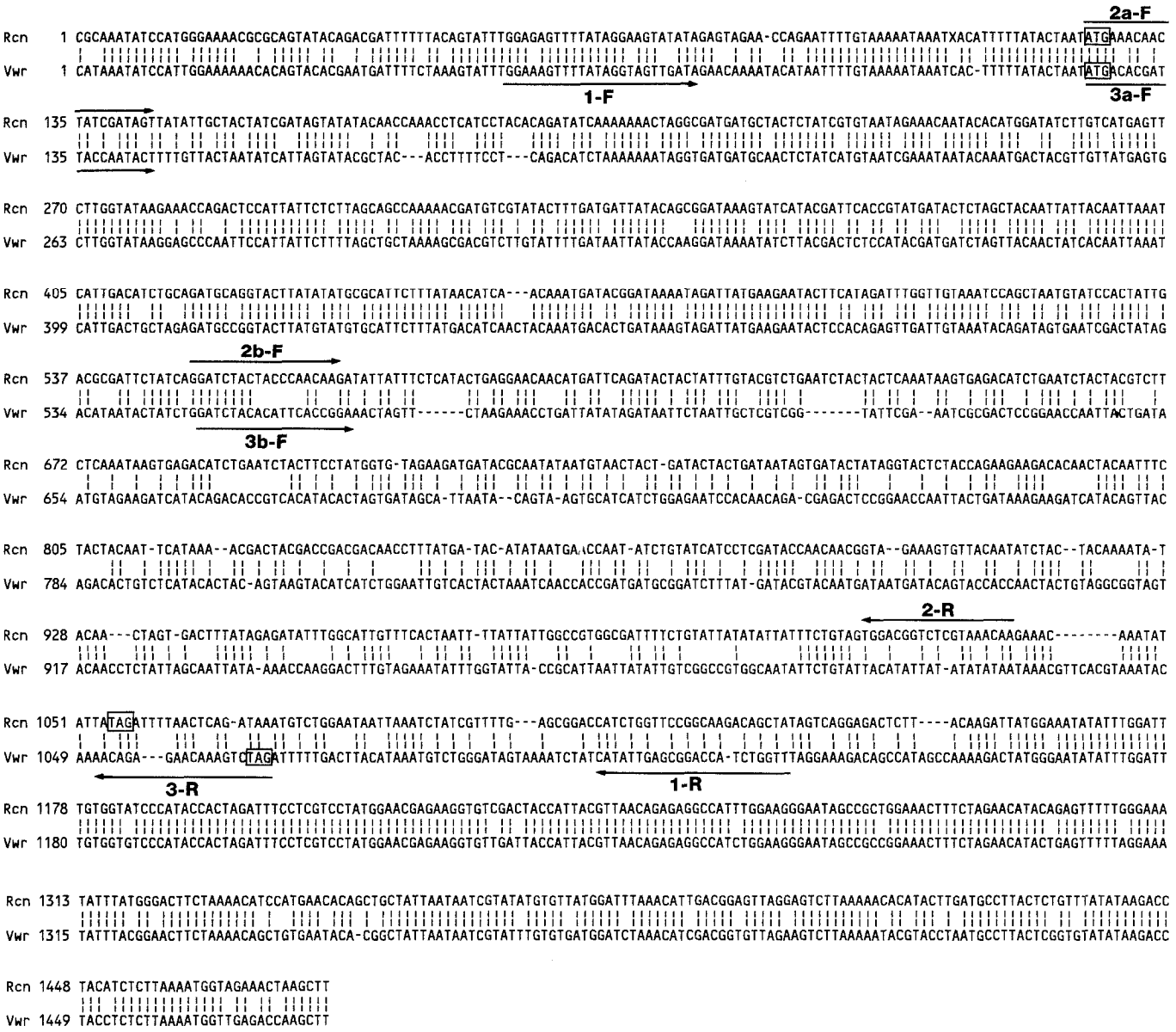

FIG. 2. RCN and VV-WR HA nucleotide sequences displayed in FastA (*19*) pairwise analysis format. Sequences of the HA region of RCN and VV-WR were determined from plasmids pRCN/*Hin*dIII-G and pVV/*Bam*HI-32 by "primer walking" and dideoxy chain termination sequencing reactions (*27*), beginning with primers 1-F and 1-R that were based on VV-IHDJ HA sequences (*17*). Sequences were determined in twofold redundancy from each DNA strand. Translation of the sequences showed a major ORF of 930 bases in RCN DNA and 942 bases in VV-WR DNA; the positions of the start and stop codons are enclosed in boxes. Also indicated are the nucleotides used as a basis for synthesizing primers 2a-F, 2b-F, 2-R for amplifying from the RCN genome and primers 3a-F, 3b-F, and 3-R for amplifying from the VV-WR genome to produce type-specific hybridization probes described in the text.

primers 1-F and 1-R, indicated in Fig. 2., which were designed from sequences flanking the HA ORF reported for VV-IHDJ (*17*).

Having located the HA region in RCN and VV-WR DNA, primers 1-F and 1-R were then used to begin determination of the HA base sequences in plasmids pRCN/*Hin*dIII-G and pVV/*Bam*HI-32, that contains a 6.3-kb segment of the VV-WR *Hin*dIII-A fragment (*18*). Altogether, sequences of about 50 bases upstream of 1-F and 350 bases downstream of 1-R were determined. Figure 2 depicts the determined sequences in FastA pairwise analysis format (*19*); aligning 1478 bases of the RCN HA region with 1479 bases of the VV-WR HA region indicated that the sequences were 69% homologous. During our study, 42 kb of VV-WR, including sequences of its HA, were reported by Smith *et al.* (*20*). Compared with those sequences, residue 1003 of our HA sequences was A, not T (amino acid

unchanged), and the reported sequences included G between bases 1348 and 1349.

A major ORF was found in the sequences (Fig. 2) that FastA pairing indicated were 66% homologous. Interestingly, the nucleotide region between bases 550 and 900 composed a rather dissimilar domain of 59% homology. Further FastA analyses indicated that the sequences flanking the dissimilar domain became gradually more homologous: pairing of a 125-base region upstream of each ORF (putative early–late promoter region) showed 82% homology; pairing the first 435 bases of each ORF showed 78% homology; pairing the last 150 bases of each ORF showed 74% homology; and pairing 400 bases down from the stop codon of each ORF (putative transcript terminator region) showed 86% homology.

Comparison of the deduced amino acid sequences of the RCN HA (310 residues) and VV-WR HA (314 residues) by FastA indicated 53% amino acid homology. Saliently, pairing of portions of the HA amino acid sequences of RCN and VV-WR showed a rather dissimilar domain of 32% homology between residues 146 and 254, and greater homologies between amino acids 1 and 145 (71%) and from residue 255 to the carboxyterminal residue (62%) were noted, consistent with the base sequence homologies.

Further insight into viral phylogenetic relationships was gained by GENALIGN (*21*) multiple alignment of the HA amino acid sequences. Figure 3 shows the RCN and VV-WR HA sequences aligned with sequences deduced from reported HA base sequences of the HAD^+ strains VV-IHDJ, VV-Copenhagen (Merieux variant), and VV-Tian-Tan and of the HAD^+ revertants of rabbitpox virus (RPV) strain Utrecht, RPVu23 and RPVrev (*6, 17, 22, 23*). Amino acids of the putative truncated HAs of the HAD^- viruses, RPV and VV-IHDW (*6*), are also aligned. Differences were noted between the HAD^+ VV and RPV strains at residue positions 2, 16, 19, 110, 152, 155, 156, 160, 198, 259, 264, and 302; glutamic acid was deleted at position 215 of the VV-WR HA. Separate FastA analyses that paired the HA of the HAD^+ strains of VV and RPV against each other indicated that these viruses were 98% homologous in HA base sequence and of about 97% homologous in amino acid sequence. The "hotspot" of differences between the HAD^+ strains of VV and RPV was from residue 150 to 160.

The putative structural relationship of the RCN and VV HAs was predicted by comparisons for various amino acid motifs using Genetics Computer Group software (*24*), including potential glycosylation sites, hydropathies, and secondary structure. Accordingly, four sites in the RCN HA (residues 39–41, 113–115, 133–135, and 203–205) and five sites in the VV-WR HA (residues 37–39, 69–71, 112–114, 161–163, and 253–255) were predicted to be asparagine-linked glycosylation sites (*25*). The 5 VV-WR sites were conserved among all HAD^+ VV strains and RPV HAD^+ revertants. Two of the four N-linkage sites in the RCN HA (residues 39–41 and 113–115) aligned with two of the five sites in HAD^+ VV and RPV strains. Calculation of the mole percentage of amino acids indicated that RCN and VV-WR had similar amounts of individual amino acids, including relatively abundant residues, serine (mole % = 12) and threonine (mole % = 17), which are potential sites for O-linked glycosylation (*25*). Reported studies (*1*) on HAD^- mutants and HAD^+ revertants of VV-IHDJ and on glycosylation inhibition have suggested that glutamic acid at position 121 of the VV-IHDJ HA likely plays a role in forming an active center of HAD activity and that O-linked glycosylation is required for hemagglutination and HAD activities of infected cell membranes. In the present study, we noted that glutamic acid at position 121 of the VV-WR HA aligned with the glutamic acid at position 122 of the RCN HA (Fig. 3), suggesting analogy of the residue function. Comparison of the VV-WR and RCN HA deduced amino acid sequences indicated that about 50% of the serine and threonine residues were at conserved sites.

Studies with HAD^- mutants and HAD^+ revertants of VV-IHD have also indicated that the VV HA inhibits syncytia formation (*1*). Cysteine at position 103 of the VV-IHDJ HA was implicated to be required for protein conformation that enables fusion inhibition (*5*). Interestingly, RCN (HAD^+, $fusion^+$) shows cysteine at position 105 aligning with cysteine at position 103 in the HAD^+ VV and RPV revertants (Fig. 3). Moreover, cysteine at positions 34, 162, and 296 of these viruses (295 in VV-WR) aligned with cysteine at positions 36, 165, and 292 in the RCN HA.

Hydropathy analyses (not shown) of the deduced HA sequences indicated that the amino terminals of the RCN and VV-WR HAs contain a rather similar putative hydrophobic signal peptide and that the carboxyterminals contain similar putative hydrophobic transmembrane and hydrophilic anchor domains. The hydropathic indicise from residues 146 to 254 (33% homology domain) suggested that the amino acid sequences of the RCN HA are slightly more hydrophilic than in the VV-WR HA. Of interest, the Chou–Fasman algorithm predicted conformational differences: three α-helices were predicted in the VV-WR HA, whereas seven were predicted in the RCN HA. One α-helix in the VV-WR HA, between residues 146 and 254, aligned with one of three α-helices in the RCN HA.

The nature of the predicted protein conformation differences and whether these differences or other fac-

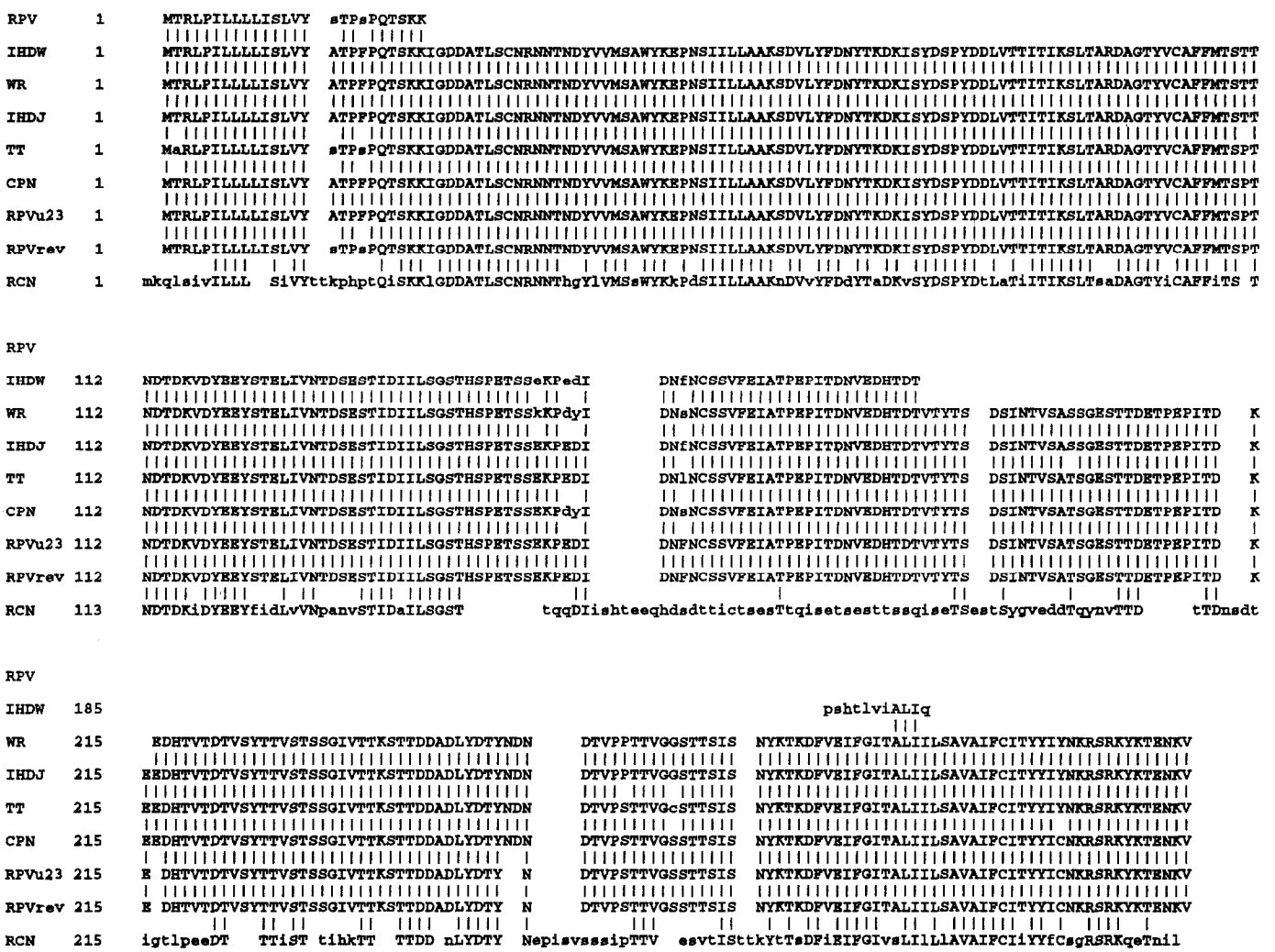

FIG. 3. GENALIGN "region" alignment (*21*) of the deduced amino acids of the HA of RCN, VV-WR, VV-IHDJ, VV-Copenhagen, VV-Tian Tan, RPV HA$^+$ revertants RPVu23 and RPVrev, and the putative truncated HA proteins of RPV Utrecht and VV-IHDW. Upper case letters represent homologies and lower case letters represent differences.

tors are responsible for the innate fusion of infected cells by RCN (and VPX and SKP) must yet be determined. In another study (*11*), we showed that RCN produces an 18-kDa analogue, and VPX produces a 20-kDa analogue of the VV-WR 14-kDa fusion protein that appears to be a major factor in polykaryocytosis, at least when VV-WR-infected cells are adjusted to pH 5.5 (*26*). The RCN HA and fusion protein may be folded in such a way that they are not interactive, or there might be a lack or malfunction of a K2L analogue. On the other hand, the RCN HA protein may interact with other viral and cell membrane elements for polykaryocytosis.

As mentioned above, VV and RCN PCR DNA segments produced with primers 1-F and 1-R (Fig. 2) showed faint Southern blot autoradiograph signals with the *Hin*dIII-A fragment of VPX and SKP DNA. After determining the sequences, to further examine viral HA relationships, we amplified segments of either the entire HA ORF of RCN and VV-WR or a portion of the ORF from the center to right end with primers 2a-F and 2-R, 3aF and 2-R, 2b-F and 3-R, or 3b-F and 3-R, as depicted in Fig. 2. Each segment annealed with homologous virus DNA but neither segment annealed with DNA of VPX or SKP. On the other hand, the VV-WR amplified segments annealed strongly with DNA of ectromelia (ECT), variola (VAR), and various other orthopoxviruses not known to be indigenous to the Americas (data not shown). These cross-hybridizations suggested that the HA of RCN, VPX, and SKP are separately diverged and that the HA of VV, ECT, VAR, and most other orthopoxviruses are closely related. However, the hybridizations also indicated that orthopoxvirus regulatory sequences flanking the HA ORF

are rather conserved. We have been successful at amplifying various other genes from orthopoxviruses with PCR primers based on such sequences.

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