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Analysis of 15 Adenovirus Hexon Proteins Reveals the Location and Structure of Seven Hypervariable Regions Containing Serotype-Specific Residues

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The first full-length hexon protein DNA and deduced amino acid sequences of a subgenus D adenovirus (AV) were determined from candidate AV48 (85-0844). Comprehensive comparison of this sequence with hexon protein sequences from human subgenera A, B, C, D, F, bovine AV3, and mouse AV1 revealed seven discrete hypervariable regions (HVRs) among the 250 variable residues in loops 1 and 2. These regions differed in length between serotypes, from 2 to 38 residues, and contained >99% of hexon serotype-specific residues among human serotypes. Alignment with the published crystal structure of AV2 established the location and structure of the type-specific regions. Five HVRs were shown to be part of linear loops on the exposed surfaces of the protein, analogous to the serotype-specific loops or "puffs" in picornavirus capsid proteins. The HVRs were supported by a common framework of conserved residues, of which 68 to 75% were hydrophobic. Unique sequences were limited to the seven HVRs, so that one or more of these regions contain the type-specific neutralization epitopes. A neutralizing AV48 hexon-specific antiserum recognized linear peptides that corresponded to six HVRs by enzyme immunoassay. Affinity-purification removal of all peptide-reactive antibodies did not significantly decrease the neutralization titer. Eluted peptide-reactive antibodies did not neutralize. Human antisera that neutralized AV48 did not recognize linear peptides. Purified trimeric native hexon inhibited neutralization, but monomeric heat-denatured hexon did not. We conclude that the AV48 neutralization epitope(s) is complex and conformational.

The adenoviruses (AVs) comprise a large family of double-stranded DNA viruses found in mammals, amphibians, and birds which have a common genome organization and nonenveloped icosahedral capsid structure (48). The capsid is made up of two types of capsomeres containing three proteins: 12 vertex capsomeres composed of fiber attachment protein and its penton base, and 240 hexons (10). Each hexon capsomere is a homotrimer of the hexon protein, a complex protein of >900 residues (42). Electron microscopy and X-ray crystallography of human AV2 hexon revealed a dense pedestal base composed of two eight-stranded, antiparallel beta barrels stabilized by an internal loop. A tower region projects away from the surface of the virion formed by three loops, L1, L2, and L4. The L2 and L4 loops coil to interact on either side with the tower regions of the other two copies of the protein in the trimer. This interdigitation of the loops, combined with adjacent pedestal interactions, gives the trimer extreme structural stability. The L1 loop is the longest and most complex, folding back on itself several times and projecting furthest into the solvent, providing maximal interaction with the environment (1, 31, 42).

The human AVs now number 49 serotypes (16, 36) divided into six subgenera (A through F) on the basis of nucleic acid differences, fiber protein characteristics, and biological properties (50). Neutralization is type specific (15) and is directed against an epitope(s), historically designated eta, on the hexon protein (22). Studies in which the protein sequences of several AV hexon proteins have been compared have noted that while most of the pedestal regions are conserved, variable regions exist in L1 and L2 (19, 21, 28, 40, 41, 46).

Until recently, complete protein sequence information available on human AVs has been limited to subgenera C and F. Much less is known about the subgenus D AVs. First isolated in the 1950s from epidemic conjunctivitis (20), they are currently recognized as significant pathogens in immunocompromised populations (13, 14, 49). They are the largest group, containing 30 of the 49 known serotypes, and the group which appears to be changing the fastest, with the appearance of multiple intermediates and intertypic strains (14, 49). The seven most recent human AVs to be characterized (AV43 to AV49) were all subgenus D serotypes isolated primarily from AIDS patients (13, 16, 36). AV48 first appeared in 1985, isolated from AIDS patients in the San Francisco Bay area. Among isolates from subsequent years, only one was not associated with concurrent human immunodeficiency virus infection (5, 36). As part of ongoing investigations into the source and evolution of AIDS-associated AVs, we examined a part of the genome undergoing rapid evolution, the major capsid protein.

The purpose of this study was to obtain the first full-length subgenus D protein sequence and perform a comprehensive comparison of hexon protein sequences for fine-mapping of conserved and variable residues in order to define those residues which are responsible for type specificity. We found seven discrete regions which contained unique sequences that accounted for >99% of type-specific variation. These regions also varied in length between serotypes, sometimes dramatically. Overlapping peptides corresponding to the entire L1 and L2 regions of AV48 were synthesized to define the type-spe-

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cific neutralization epitopes. We found that the neutralization epitopes of AV48 were nonlinear and conformational.

MATERIALS AND METHODS

PCR and direct DNA sequencing. The complete hexon gene of AV48 (85-0844), including part of the upstream pVI core protein and the hexon 5′ noncoding region, was sequenced by generating overlapping PCR products and direct cycle sequencing. A set of 10 pairs of primers were designed and synthesized from the published sequences of AV2 (32), AV5 (21), AV40 (41), AV41 (40), and BAV3 (19). Deoxyinosine was used in codons with ambiguity. All products were sequenced from both directions with internal and template primers. A pair of primers consisted of the rightward coding sequence primer and the leftward complement of that sequence. Rightward coding primers were (AV48 numbering): UP, 5′-AACAGCATIGTGGGTITGGGIGTG-3′ (pVI); OR, 5′-ATGGCIACCCCITCGATGATGCCG-3′ (codons 1 to 8); 1R, 5′-TACTTTGA CATCCGCGGCGTGCTGGA-3′ (98 to 106); 2Rm, 5′-CCITGCTATGGITCIT TTGC-3′ (220 to 226); 3R, 5′-ATGTGGAAICAGGCIGTIGACAG-3′ (381 to 388); 4R, 5′-TTTGCCATGGAIATIAAICT-3′ (453 to 459); 5R, 5′-ATGGACA AIGTIAAICCITTIAAICACCACCG-3′ (523 to 533); 6R, 5′-GCIGCIAACAT GCTITAICCIATICC-3′ (643 to 650); 7Rm, 5′-ATGTAITCCTTITTICGIAAC TTCCAICCIATG-3′ (785 to 795); and 8R, 5′-TTCTCIGCCGGIAACGCIACI ACATAA-3′ (938 to 946). Full-length AV48 DNA template was prepared by Hirt extraction of infected A549 cells (17). Standard PCR conditions (35) of 100/pmol of each primer, 50 mM KCl, 10 mM Tris, 1.5 mM $MgCl_2$, 0.01% gelatin, 2.5 U of *Tfl* heat-stable DNA polymerase (Epicenter Technologies, Madison, Wis.), 250 μM each of the four deoxynucleoside triphosphates (Pharmacia, Piscataway, N.J.), and 1 to 5 μg of template DNA in a 100-μl reaction volume were supplemented with 5 to 7% glycerol and 1 μg of single-stranded DNA binding protein (Bind-Aid; US Biochemical, Cleveland, Ohio). An initial denaturation of 94°C for 2 min was followed by 30 cycles of 94°C for 1 min, annealing for 1 min, and extension at 72°C for 2 min. The annealing temperature varied with the primer pair, from 45 to 55°C. PCR products were isolated directly or from agarose gels by adsorption to silica (GeneClean; Bio 101, La Jolla, Calif.). Cycle sequencing with the "fmol" DNA Sequencing System (Promega, Madison, Wis.) was carried out with 10 pmol of primer and 1 to 6 μl of PCR product (30).

Immune serum preparation. Enriched AV48 hexon protein, prepared as described before (2), was purified by electrophoresis in 4% high-resolution agarose (ProSieve; FMC Corp, Rockland, Maine). A band containing 100 μg of 300-kDa hexon trimer was cut from the gel, homogenized with an equal volume of complete Freund's adjuvant, and injected intraperitoneally into New Zealand White rabbits. Two more 100-μg injections with incomplete Freund's adjuvant were given at 1-month intervals.

Peptides and ELISAs. A set of 45 biotinylated 15-mer peptides with an overlap of 5 were synthesized for the entire AV48 variable regions of L1 and L2, from residues 121 to 322 and 409 to 454, with an SGSG linker between the biotin and peptide moieties (Chiron Mimotopes Ltd., Victoria, Australia). Peptide enzyme-linked immunosorbent assays (ELISAs) were performed as described before (47), using streptavidin to coat the plates and bind biotin with casein blocker (45), with the AV48 hexon-specific antiserum and with two human sera shown previously to contain neutralizing activity against AV48 (5). Affinity purification of antipeptide antibodies was carried out as reported before (12) with biotinylated acrylic beads which were coated with avidin, followed by the biotinylated peptides, and blocked with casein. Five peptides from hypervariable regions (HVRs) 1, 2, 3, 4, and 7 were used individually and in combination to remove antipeptide antibodies from the serum. The peptide core sequences were as follows: peptide 8, AAMGGIEITAKGLQI (HVR 1); peptide 11, GIDATKEED NGKEIY (HVR 2); peptide 16, IGEENWQDSDNYYGG (HVR 3); peptide 26, GEEPKELDIDLNFFD (HVR 4); and peptide 42, GVKVKTTNNTEWEKD (HVR 7).

Neutralization assays and hexon protein competition. Neutralization was assessed in a microtiter colorimetric assay with A549 human lung carcinoma cells (6). Native hexon protein was purified as described before (2), and monomeric unfolded hexon polypeptide was obtained by boiling an aliquot for 3 min. Hexon protein competition for neutralizating antibodies was determined by incubating undiluted AV48 hexon-specific antiserum for 1 h with increasing concentrations of native trimeric hexon protein or heat-denatured monomeric hexon protein. Protein-antiserum mixtures were assayed for neutralization as described above. Controls included virus-protein mixtures to rule out protein inhibition of virus infectivity.

Nucleotide sequence accession number. The complete hexon protein DNA sequence from subgenus D candidate AV48 (85-0844) was determined, and the amino acid sequence of 946 residues was deduced. The nucleotide sequences of 2,935 bp of AV48 hexon protein, the hexon 5′ noncoding region, and the 3′ terminus of the pVI core protein have been entered in the GenBank database under accession number U20821.

RESULTS

Deduced protein sequence. The deduced AV48 protein sequence was aligned and compared with that of 14 other AVs for which sequence data are available (Fig. 1). Partial sequences for AV9 (26), AV15 (9), and AV31 (29) were omitted from Fig. 1 but were included in the analysis and conform to the structural alignments made here. Secondary structural designations below the sequences were taken from the published coordinates published for AV2 hexon (1). Conservation throughout the protein was greatest within a subgenus (88%) and was reduced between human subgenera (79 to 81%) and between animal serotypes (66 to 68%). Shaded areas represent complete and functional conservation between serotypes within the variable regions from residues 131 to 331 (AV2 numbering) of L1 and residues 427 to 475 of L2. Functional conservation is defined as substitution with amino acids similar in size and charge (basic, R and K; aliphatic, A, C, G, I, L, M, and V; aromatic, F, Y, W, and H; hydrophilic, E, Q, N, and D; hydroxyl, S and T; P has no functional equivalent) (7). Among the 250 residues in variable regions, 82 residues were conserved among human serotypes, 72 were conserved within bovine AV3 (BAV3), and 61 were conserved in mouse AV1 (MAV1). Of these, hydrophobic residues accounted for 68, 71, and 75%, respectively.

Alignment of conserved residues defined seven HVRs which differed in both length and sequence. Table 1 summarizes these differences. With a few exceptions, the composition and length in each HVR showed subgenus clustering. Serotypes within subgenera A and F were the most closely related to each other, differing in length by at most two residues in all HVRs. The greatest variability was in HVR 1, as has been noted previously (19, 28, 40, 41). Subgenus C serotypes and AV8 (subgenus D) contained an insertion of more than 30 residues in this region. The AV8 insertion was slightly larger, with a more basic and hydrophobic chemical composition than that of the subgenus C serotypes.

HVR 4, which forms the exterior side surface of the L1 tower, had two distinct conformations. Subgenus A and the animal serotypes contained an abbreviated 5 to 7 residues, making them the smallest hexons, while all others had 13 to 17 residues in this region. HVR 5 exhibited the same type of dichotomy, although not as extreme: subgenera A, B, and F and the animal serotypes contained a shorter version (9 to 12 residues), and subgenera C and D (other than AV8) contained longer stretches (16 to 19 residues).

More than 99% of serotype-specific variability was contained in the seven HVRs among human subgenera A, C, D, and F. When the sequences were aligned with the published crystal structure of the AV2 hexon (1), five of the HVRs (2, 3, 4, 5, and 7) occurred on the external surface of the protein (Fig. 2). For the most part, they were shown to be loops lacking secondary structure, but a single beta strand was seen in HVR 4. HVR 5 contained a region which remains unresolved crystallographically. HVRs 1 and 6 were internal loops beneath HVRs 2, 3, 4, and 5.

Peptide ELISAs and serum neutralization. Figure 3 and Table 2 summarize the lack of correlation between neutralization and linear peptide reactivity. AV48 hexon-specific antiserum, with a neutralization titer of 800, reacted with peptides that corresponded to residues in the five surface HVRs and part of HVR 1 (Fig. 3a). These residues are underlined in the AV48 sequence in Fig. 2. A broad peak from peptides 26 to 28 recognized residues from both HVR 4 and HVR 5. After affinity removal of all peptide reactivity from the serum by adsorption with five peptides, the neutralization titer was 400

```
aa 1                                                                                                  100
 2 ATPSMMPQWS YMHISGQDAS EYLSPGLVQF ARATETYFSL NNKFRNPTVA PTHDVTTDRS QRLTLRFIPV DREDTAYSYK ARFTLAVGDN RVLDMASTYF
 5 ********** ********** ********** ********** ********** ********** ********** ********** ********** **********
40 ********** ****A***** ********** ****D***** G********* ********** *******V** ***E****** V********* **********
41 ********** ****A***** ********** ****D***** G********* ********** *******V** ********** V********* **********
12 ********** ****A***** ********** ****D***T* G********* ********** *******V** *****T**** ********** *******S**
16 *********A ****A***** ********** ****D****M G********* ********** ***M***V** ****NT**** V*Y******* ********F*
48 *********A ****A***** ********** ****D***** G********* ********** *******V** *****T**** ********** **********
B3 *****L**** ****A***** ********** *Q***S**NI G********* *******E** ***Q***V** *****Q**** T**Q****** **********
M1 T****Q***A F***A***** Q*****V*** *A**D***** G********* ********** ******IV** ****SQ*T** T**Q****** **********
  NH2<[--------Ln--undefined--------]--------->                                                       <-----

                                   131
                                    ¦                                       HVR1                         191
 2 DIRGVLDRGP TFKPYSGTAY NALAPKGAPN SCEWEQTEDS GRAVAEDEEE ---EDEDEEE EEE-EQNARD QATKKTHVYA QAPLSGETIT K-----SGLQ
 5 ********** ********** ********** P***DEAATA LEINL*E*DD ---DN***VD *QA-**Q--- ----****FG ***Y**IN** *-----E*I*
 1                    ** ********** ******E*PT QEMAG*L*D* --E*A*E**A ***A*APQAG *LV******* ****A*GK** A-----N***
 6                    ** ********** ******N*TA QVDAQ*LD** ---*N*AN*A QAR-**EQA- ---*****F* ******GK** *-----E***
40 ********** S********* *S******** PSQ*TNQN-- ---------- ---------- ---------- ----**NSFG ***YI*QK** N-----G*V*
41 ********** S********* *S****T*** P***KDNN-- ---------- ---------- ---------- ----*IK*RG ***FI*TN*N *D----N*I*
12 ********** S********* *S******** ASQ*SDNA-- ---------- ---------- ---------- ----*LNTF* ***YLSD*** AA----D*IK
16 ********** S********* *S******** T*Q*KDSDSK ---------- ---------- ---------- -----M*TFG V*AMP*V*GK *IEA--D**P
 8 ********** S********* *S******** PSQ***AKTG RGVDQNQK*T RTYGVAHWRY NITKTGAGV* *NQ*E*RT*G E-*-T*D-** LQKKAKE***
48 ********** S********* *S******** PSQ**EKKNG *--------- ---------- -------GS* ANQMQ**TFG V*AMG*IE** A-----K***
B3 ****T****A S********* *SF***S*** NTQFR*ANNG ---------- ---------- ---------- ---HPAQTI* **SYVA-**G GA--NND-**
M1 ****R***** S********* *S***RA*** NLMFKTGA** ---------- ---------- ---------- ----**MTI* **NIV*S**D Q-----D**K
   betaP1B->    <--betaP1C-->  <----betaP1D-------><----------------------L1------------------------------

         HVR2                           HVR3                                                   HVR4       281
 2 IGSDNAE--T QAKPVYADP- SYQPEPQIGE SQWNE----A DANAAGGRVL KKTTPMKPCY GSYARPTNPF GGQSVLVPDE KGVPLPK--- VDLQFFSNTT
 5 **VEGQT--- ---*K***-K TF******** ***Y*----T EI*H*A**** ********** ****K***EN ***GI**KQQ N*KLESQ--- *EM****T*E
 1 ****TQ---* EGN**F***- *******V** *****----* E*T*I***** ********** ********KN ***GI**ANN Q*ALES*--- IEM***APSG
 6 **TAD*TVAG AGTEIF***- *F*****V** *****----* ********** ********** ********SN ***G**W-LT N*KLESQ--- *EM****TS*
40 V*L*SNN--- --RD*F**-K T******V*Q T***I----N PMQN*A**I* *Q****Q*** ********EK ***AK**--K NDDNQTTTTN *G*N**TTA*
41 **T*TTN--- --Q*I***-K T******V*Q T***SEV--G A*QKVA**** *D****L*** ****K***EK ***AS*I--T N*TDQTLTSD *N****ALPS
12 V*T*T*Q--- AGAA***N-K T******V*P *E**TSI--- ENVK****A* *Q**A*Q*** ********EH ****-----K DDN------- IE*K**DSAN
16 **I*STSG-- TDTVI***-K TF*****V*N AS*VDANG-- TEEKY***A* *D**K***** **F*K***KE ***AN*KDS* TAATT*NY-D I**A**D*KN
 8 **I*ETKED- -QTTKFMQIK TF******** NN*QDTV--- ---FY***A* **E*K***** **F*****KK ***AKVLTT* D*Q*TEN-FD I**A**DIPQ
48 **I*ATKEED NG*EI***-K TF******** EN*QDS---- -D*YY***AI **E*K***** **F*****EK ***AKFKTP* *EGEE**ELD I**N**DIPS
B3 M*V*ERQ--- --L*****N-T T******L*I EG*TAG-SM* VIDQ****** RNP*QT-*** ****K***EH **------IT *ANTQ----- *EKKYYRTGD
M1 *-NNV----- -----ALN*N AGE*AV*E*L EN*M*ELQIG ADKKFAS*** *ADQ*VL*N* ****Y***IN *T*------T *DGTT----- **K-VYMCSK
   -----------------------------------L1-----------------------------------<>--< >------><bL1C>--[---undefined--
                                                                  331        bL1A bL1B
     HVR5                                       HVR6                ¦                                     375
 2 SLNDR--QGN ATKPKVVLYS EDVNMETPDT HLSY-KPG-- KGDENSKAML -GQQSMPNRP NYIAFRDNFI GLMYYNSTGN MGVLAGQASQ LNAVVDLQDR
 5 ATAG---N*D NLT******* ***DI***** *I**-M*T-- IKEG**RELM -********* ********** ********** ********** **********
 1 TAMNER---* *VQ*SI**** ********** *I**-**S-- *T******** -***A***** V********* ********** **C******* **********
 6 NATNE---V* NIQ*T***** ********** ****-F*F-- M**F*AFVS* -***A***** ********** ********** **C******* **********
40 ETAN------ -FS******* ****L*A*** **VF-**D-- VNGTSAELL* -***AA**** ***G****** ********** ********** **********
41 TP*E------ ---**A***A *N*SI*A*** **V*-**DVA Q*TIS*ADL* -T**AA**** ***G****** ********** ********** **********
12 NAAN------ --TAQ**F*T ****L*M*** **VF-**TVT N*TIA*ESL* -***AA***A ********** ********** ********** **********
16 IAA------- NYD*DI*M*T *N*DLQ**** *IV*-***-- TEDTS*ESN* -***AA**** ***G****** ********** ********** **********
 8 A--GGNG--- --D*TMI**A *N**L***** *VV*-***-- *D*AS*A*N* -T******** ***G*****V ********** ********** **********
48 TGTGGNGTNV NF**DMIM*A *N**L***** *IV*-***-- *E*AS*ESN* -T******** ***G*****V ********** ********** **********
B3 NG*------- ---*ET*F*T *EADVL**** **VHAV*--- AA*RAKVEG* -S*HAA**** *F*G***C*V *******G** L******S** **********
M1 GA*------- VQN*D***** *E**LQA*** **L-DG*--- -*AD*P*DRV -AFCAA**** ***G****** *******N** Q********* **********
   -undefined--]><betaL1D>--L1---<betaL1E>-------L1-------<betaL1F>--><--------betaP1E-------> <--betaP1F->
                                                          425
                                                           ¦                          HVR7                473
 2 NTELSYQLLL DSIGDRTRYF SMWNQAVDSY DPDVRIIENH GTEDELPNYC FPLGGIGVTD TYQAIKANGN GSGDNGDTTW TKDET-FA-T RNEIGVGNNF
 5 ********** ********** ********** ********** ********** *****VIN*E *LTKV*PKTG Q-----ENG* E**A*E*S-D K***R*****
 1 ********** ********** ********** ********** *****W**** ********** ***G**S*** *NPQ*----* **NDD-**-A **********
 6 ********** ********** ********** ********T* ********** *******I** *F***V*TTAA N-**Q*N*** Q**S*-*S-E **********
40 ********M* *AL***S*** ********** ********** *V******** ***N*Q*ISN S**GV*TDNG -------*N* SQNN*DVS-S N***SI**V*
41 ********M* *AL***S*** ********** ********** *V******** *****SAA** **SG*****Q --------** *A*DN-Y*DR GA**ES**I*
12 ********M* *AL******* *L**S***** *****V**** *V******** ***SAV*EIK N*KG**PDNG *G*G-----* *A*N*-VS-E A*H**I**IA
16 ********** **L******* ********** ********** *V******** ***N*V*F** ***GV*VKTD AVAGTSG*Q* D**D*TVS-* A***HG**P*
 8 ********** **L******* ****S***** ********** *V******** ***D*T*TNA ***GVEPDNA QGQNDK---* K***KVA*-- Q*Q*CK**IY
48 ********** **L******* ****S***** ********** *V******** ***D*A*TNA V**GV*VKTT NN-----*E* E**-*AVS-E H*Q*CK**VY
B3 *******M** ANTT**S*** ******M*** **E**V*D*V *V***M**** ***S*VQIGN RSHEVQR*QQ Q--------* -QN-VANS-D N*Y**K**LP
M1 *******F** **LY**S*** *L****I*** **R******D *V**DITSF* *D*R***D-L P*KQVQTHNG N--------- -QQSANTT-D TCY**K**MA
      <-alphaP1A-->                                     <bL2A><----<bL2B>------------------------------><bL2C>  <
```

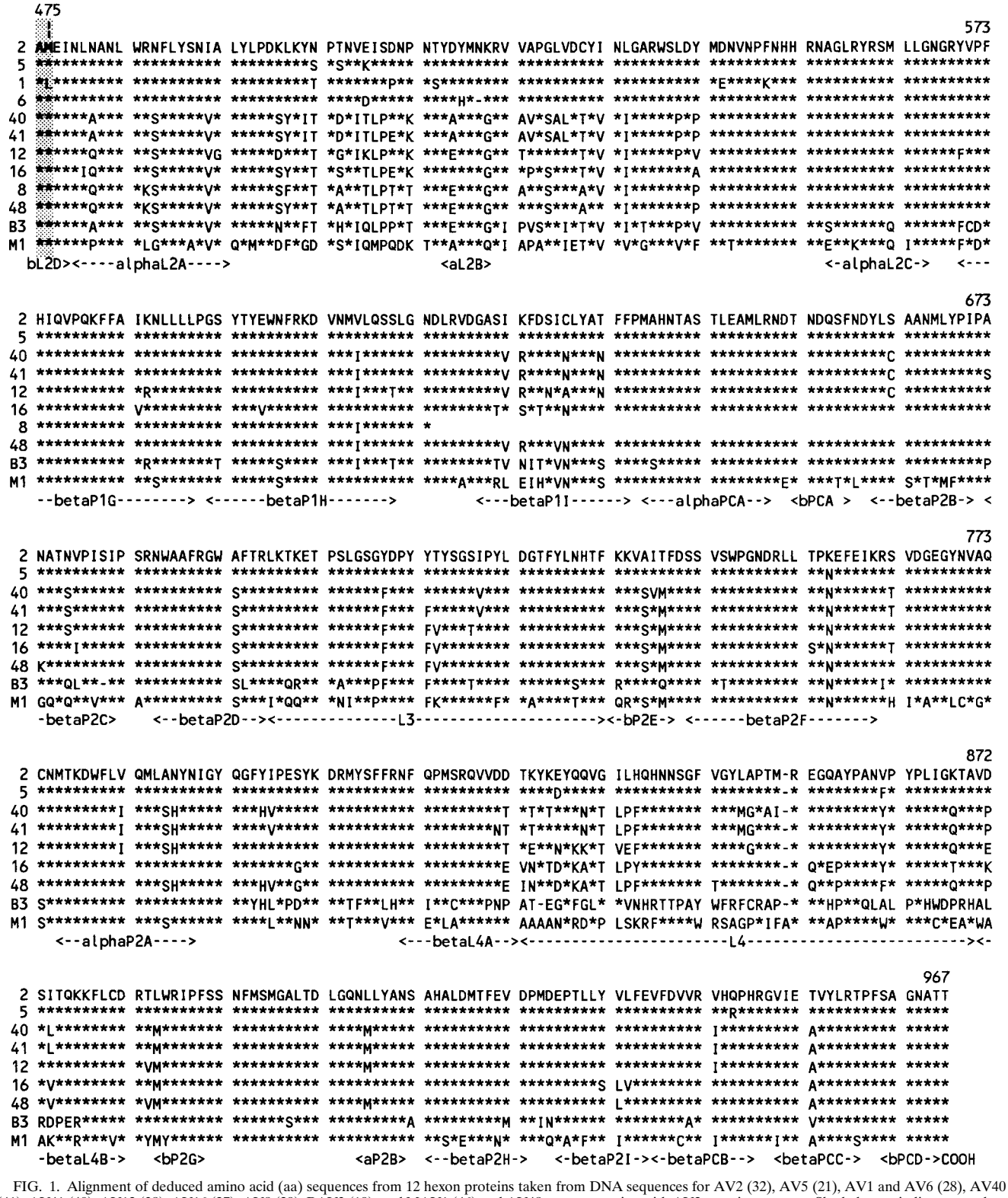

FIG. 1. Alignment of deduced amino acid (aa) sequences from 12 hexon proteins taken from DNA sequences for AV2 (32), AV5 (21), AV1 and AV6 (28), AV40 (41), AV41 (40), AV12 (38), AV16 (27), AV8 (29), BAV3 (19), and MAV1 (46) and AV48. *, conservation with AV2 protein sequence. Shaded areas indicate complete and functional conservation within the variable regions of L1 and L2. Underlined residues in the AV48 sequence correspond to peptide-reactive epitopes in HVRs. Labeling beneath each line indicates secondary-structure designations (1). Residue numbering is from the AV2 sequence.

TABLE 1. Hexon protein amino acid conservation and variation among human, bovine, and mouse AVs

Type and subgenus	Serotype or subgroup	Total no. of aa[a]	No. (%) of aa completely conserved[b]	No. of aa varying outside HVRs	Size (no. of residues[c])							
					L1 variable region (aa 131–331)	HVR 1 (aa 137–188)	HVR 2 (aa 194–204)	HVR 3 (aa 222–229)	HVR 4 (aa 258–271)	HVR 5 (aa 278–294)	HVR 6 (aa 316–327)	L2 (aa 423–477), HVR 7 (aa 433–465)
Human												
C	2	967		2	201	52	11	8	14	17	12	34
	5	951	838 (88)	3	189	45	7	8	14	16	12	30
	1	963			201	54	10	8	14	16	12	30
	6	961			196	48	13	8	13	16	12	33
F	40	922	735 (80)	4	162	20	8	8	15	12	12	28
	41	924	747 (81)	1	165	21	8	10	15	10	14	27
A	12	918	727 (79)	5	159	21	10	9	5	11	14	29
	31	918			162	23	10	10	6	12	14	26
B	16	939	729 (78)	13	172	24	11	10	16	12	10	34
D	8	966			203	58	11	6	16	11	12	31
	9	924			172	22	11	7	17	16	12	33
	15	926			173	25	11	7	14	17	12	33
	48	946	745 (79)	3	185	30	13	7	17	19	12	29
BAV3	I	910	617 (68)	104	155	25	8	11	7	9	11	25
MAV1		908	596 (66)	112	150	22	3	12	6	12	10	24
Difference range		59			46	38	10	6	12	10	4	10

[a] Hexon sizes for AV1, AV6, AV8, AV9, AV15, and AV31 are estimates based on available data for the HVRs and the consensus lengths of other subgenus A, C, and D serotypes in the conserved regions.
[b] Compared with the AV2 sequence.
[c] Residue numbering is for the AV2 sequence.

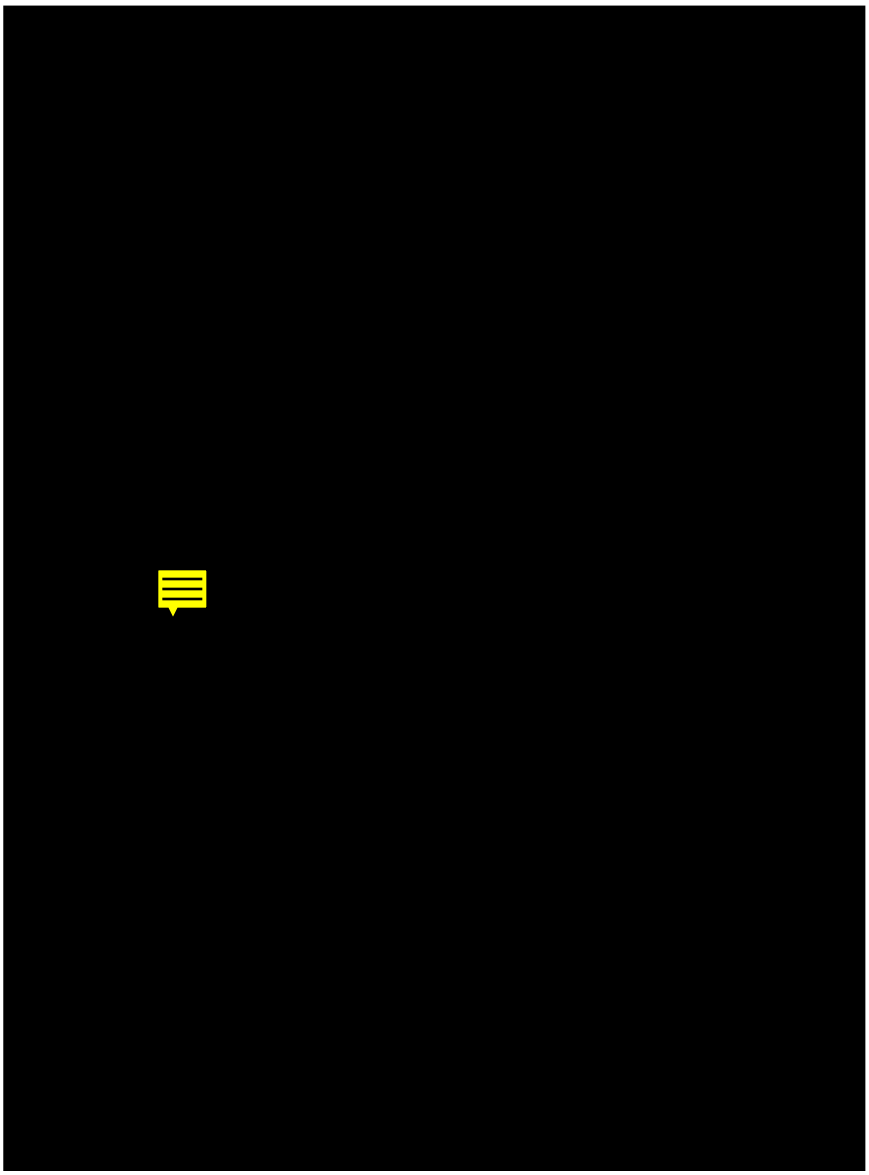

FIG. 2. Ribbon diagram of hexon protein main chain, indicating HVRs containing serotype-specific residues. A single monomer is represented, viewed from the internal cavity at the center of the trimer. The bottoms of the densely packed pedestal regions (P1 and P2) form the interior surface of the capsid, through which the N-terminal loop (Ln) is threaded. The exterior surface of the capsid is formed by L1, L2, and L4. Six HVRs are seen in the L1 loop: red, HVR 1, residues 137 to 188 (AV2 numbering); orange, HVR 2, residues 194 to 204; blue, HVR 3, residues 222 to 229; yellow, HVR 4, residues 258 to 271; brown, HVR 5, residues 278 to 294; green, HVR 6, residues 316 to 327. HVR7 encompasses most of L2, residues 433 to 465, shown in magenta. Dashed lines indicate disordered regions that are not defined in the crystallographic model. Produced with MOLSCRIPT from coordinates furnished by R. M. Burnett (1).

(Fig. 3b). The single-peptide adsorptions gave comparable titers (data not shown). Serum adsorbed with an unrelated control peptide also neutralized at a titer of 400 while retaining its AV48 peptide reactivity. Eluted antipeptide antibodies reacted with the corresponding peptides but were not able to neutralize AV48. Human sera already shown to possess neutralizing activity against AV48 did not recognize any of the linear peptides.

Hexon protein competition. Hexon protein in solution occurred only in trimeric and multimeric forms (Fig. 4, lane 2). Monomeric hexon obtained by boiling was denatured so that it did not reassociate into the trimer (Fig. 4, lane 3). Endpoint neutralization titers for each native and denatured protein-antiserum mixture were determined. Neutralization was eliminated by adsorption with native trimeric protein but was completely unaffected by up to 18 μg of heat-denatured monomeric protein (Table 2).

DISCUSSION

Our sequence analysis of a large number of AV hexon proteins showed that unique sequences were limited to seven discrete HVRs that varied in length as well as sequence. HVR 1 to 6 were found in L1, and HVR 7 was found at the tip of L2, in regions previously shown to vary in serotype comparisons (19, 21, 28, 29, 40, 41, 46). That five of the seven HVRs were found to lie on the surface of the molecule was not unexpected. It has been shown with the picornavirus capsid proteins that

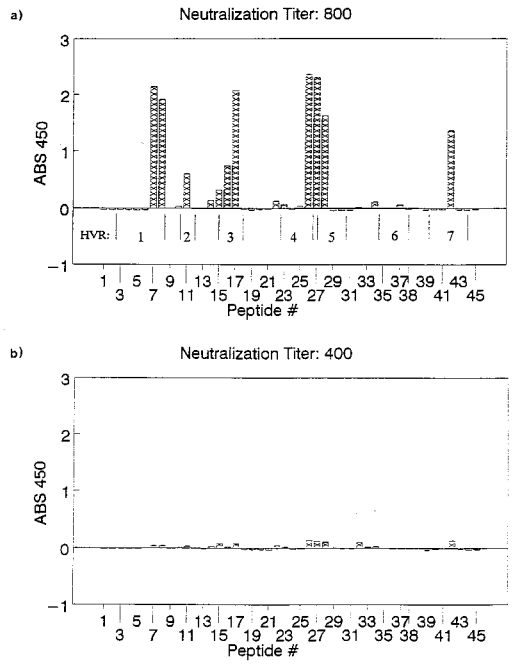

FIG. 3. Peptide ELISAs of AV48 hexon-specific rabbit antiserum tested with 45 overlapping linear peptides. (a) Serum adsorbed with biotinylated beads only. The neutralization titer is given as the reciprocal of the highest dilution giving complete neutralization of the virus. (b) Serum adsorbed with five reactive linear peptides, showing insignificant reduction of neutralization titer.

type-specific variability is limited to loops that arise on the surface of conserved beta barrel structures. Poliovirus type 1 has three distinct neutralization epitopes (25), and human rhinovirus type 14 has four (33) that are excursions from the conserved beta barrels of the three external capsid proteins. These surface loops can accommodate variability in length and sequence without disrupting the underlying structure while giving each picornavirus its unique type specificity (34). The same appears to be true of AVs.

Conserved residues form the framework for the HVRs in L1 and L2. The folding of the L1 loop is complex, and the conservation of residues may represent a conservation of interactions between adjacent, noncontiguous chains that give the hexon its unique structure. The fact that more than two-thirds of the conserved residues are hydrophobic strengthens the assertion that this matrix has a structural function (7). Of particular note is the conservation of 12 bulky aromatic residues (histidine, tyrosine, tryptophan, and phenylalanine), 7 proline residues, and 8 glycine residues. Proline and glycine are important for chain folding and reverse-turn conformations (7, 31).

TABLE 2. Neutralization and ELISA titers[a] of rabbit and human sera against AV48

Antiserum	Neutralization titer	ELISA titer
AV48 antihexon		
Adsorbed with beads only	800	>15,000
Adsorbed with control peptide	400	15,000
Adsorbed with peptides 8, 11, 16, 26, and 42	400	<100
Eluate of peptides 8, 11, 16, 26, and 42	<80	10,000
Adsorbed with native hexon trimer	<10	
Adsorbed with monomeric hexon	800	
50951-14 (human)	320	<100
51048-11 (human)	640	<100

[a] Reciprocal of the highest dilution giving (i) complete neutralization of 100 50% tissue culture infective doses or (ii) greater than 2:1 signal-to-noise ratio in the ELISA.

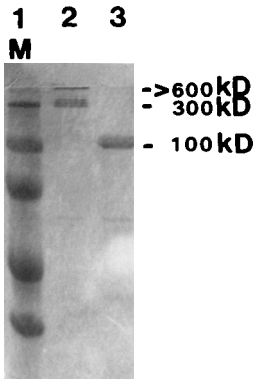

FIG. 4. SDS-PAGE of enriched hexon protein solution stained with Coomassie brilliant blue (Sigma). Lane M, size standards. Lane 2, native protein solution with an equal volume of SDS loading buffer. Protein appears only in trimeric and concatemeric forms. Lane 3, same as lane 2 but boiled for 3 min. Protein appears in monomeric form.

HVR 1 and HVR 6 are internal structures that support the L1 portion of the tower. Variability in these areas may reflect context effects, which are the correctional or compensatory changes or mutations in a protein structure required by mutations in the structures that surround them. This topological clustering of mutations during protein evolution has been observed for dihydrofolate reductase genes in gram-negative bacteria (11).

It has been noted previously that subgenus F, BAV3, and MAV1 viruses lack the long stretch of acidic residues in HVR 1 seen in the subgenus C serotypes (19, 40, 41, 46), leading to speculation regarding the possible role of this region in tissue tropism for the respiratory subgenus C viruses (46). AV8 contains an even longer insertion in this region, but the chemical composition is very different, somewhat basic, with three times the number of hydrophobic residues as AV2. None of the other AV serotypes studied to date contain this large insertion. Much of what we know about the three-dimensional structure of the hexon is based on AV2, which may not be representative of the AVs as a whole. The absence of 20 to 30 residues in HVR 1 may mean that the tertiary structure of the L1 loop may be significantly different for other AV subgenera.

The variation in length of HVR 4, the primary external support region of the L1 portion of the tower, and HVR 5, the tip of the tower, may indicate that there are significant differences between AVs in the height of the tower, which is determined by these two regions. The nonhuman and subgenus A serotypes are the shortest in this region (16 to 18 combined residues), followed by subgenera B and F (25 to 28), subgenus C (29 to 31), and subgenus D other than AV8 (31 to 36). The newest AV serotype studied (AV48) contains the longest HVR 4 and HVR 5 regions, suggesting an evolutionary mechanism that involves insertions in these regions.

BAV3 and MAV1 are surprisingly closely related to the human AVs, 66 to 68% of all residues being completely conserved compared with AV2 and AV48. These similarities indicate a constant relationship to human serotypes. The greatest variability between human AVs and nonhuman serotypes comes in the L4 loop. This region is conserved within human

subgenera, but the mouse and bovine serotypes are almost completely different from the human ones and from each other. As more information on other animal serotypes becomes available, it will be interesting to learn if the L4 loop contains species-specific residues.

Neutralization of AVs by antihexon antisera is thought to involve prevention of a low-pH-induced conformational change of the capsid in an endocytic vesicle (44, 51). It has been stated that a single antibody molecule per virion is required for neutralization (51). The L1 and L2 loops appeared to be good candidates for linear neutralization epitopes because of their lack of extensive secondary structure (31) and their location on the surface of the virion (23). This has been observed for the V3 loop of the human immunodeficiency virus envelope glycoprotein (37) and the NIM 1A region of rhinovirus type 14 (33). We optimized antibody recognition of peptides by using biotinylated peptides with a long, flexible Ser-Gly-Ser-Gly linker. Theoretically, this provides the least antigen-binding constraint and maximum surface contact with antibody, enhancing peptide recognition by antibody made to native antigens (3).

On the other hand, it has been estimated that only 5 to 10% or less of all antibodies directed against native antigens bind to linear epitopes (4, 43). Complex native antibody epitopes on influenza A virus neuraminidase that contain residues from as many as five discontinuous sites on four separate loops have been identified (24). The folding of the hexon tower region brings several discontinuous peptide chains into close proximity, within the 20 by 25 Å (1 Å = 0.1 nm) (43) to 25 by 30 Å (8) range required for an antibody-binding surface. The neutralization epitope(s) may consist of two or more surface HVRs, with antibody bridging adjacent strands. Our results demonstrate that the AV48 neutralization epitopes are conformational, not linear. An antibody that bridges several chains and anchors them together would be more efficient in resisting conformational change than an antibody attached to a single linear epitope.

One study has reported that linear peptides corresponding to 12 residues in HVR 5 of AV2 were able to elicit neutralizing antibody in two of two rabbits, and 15 residues of HVR 7 elicited antibody in one of two rabbits. These peptides were recognized in an ELISA by a human serum with neutralizing activity to AV2 (39). Peptides that do not correspond to native or surface epitopes have been shown to elicit neutralizing antibody when used as immunogens by cross-reaction with part of the native epitope (43). Such a response might be seen if HVR 5 and HVR 7 were part of complex, discontinuous neutralization epitopes. Recognition of AV2 linear peptides by human serum may reflect either (i) significant differences in the structure of HVR 5 in the AV2 hexon versus AV48 or (ii) the problems inherent in the use of human sera in peptide ELISAs with high signal-to-noise ratios and multiple nonspecific cross-reactivities with synthetic peptides (18).

For the most part, our analysis is consistent with the X-ray crystal structure. It is striking that the mobile segments that were the most difficult to resolve in the X-ray crystal structure of AV2 (1) correspond to three of the HVRs, although the significance is unclear. Sequence analysis does reveal a few small ambiguities in these regions. Between HVR 4 and HVR 5, which is near the tip of the loop that projects the furthest away from the virion, is a hydrophobic node between residues 272 and 277 (AV2 numbering), consisting of two completely conserved phenylalanines in the human types and tyrosines in BAV3 and two functionally conserved hydrophobic residues that alternate with hydrophilic residues. Phenylalanine is large and extremely hydrophobic, the aromatic ring comparable to that of organic solvents (7), and two of them in position near the apex of the most exposed loop is unusual. The lack of electron density in this region indicates that the residues are disordered, appearing in different locations in different molecules of the protein. The complete conservation of these hydrophobic residues argues that they are vital. If they are conserved for structural reasons, as in the hydrophobic framework regions, we would expect them to be ordered. Another possibility is that there is maximum mobility in this region to facilitate virus-environment interactions in this exposed position and that these residues are conserved for host cell receptor binding or fusion. Further investigation into the mechanism of AV penetration and uncoating may reveal their function.

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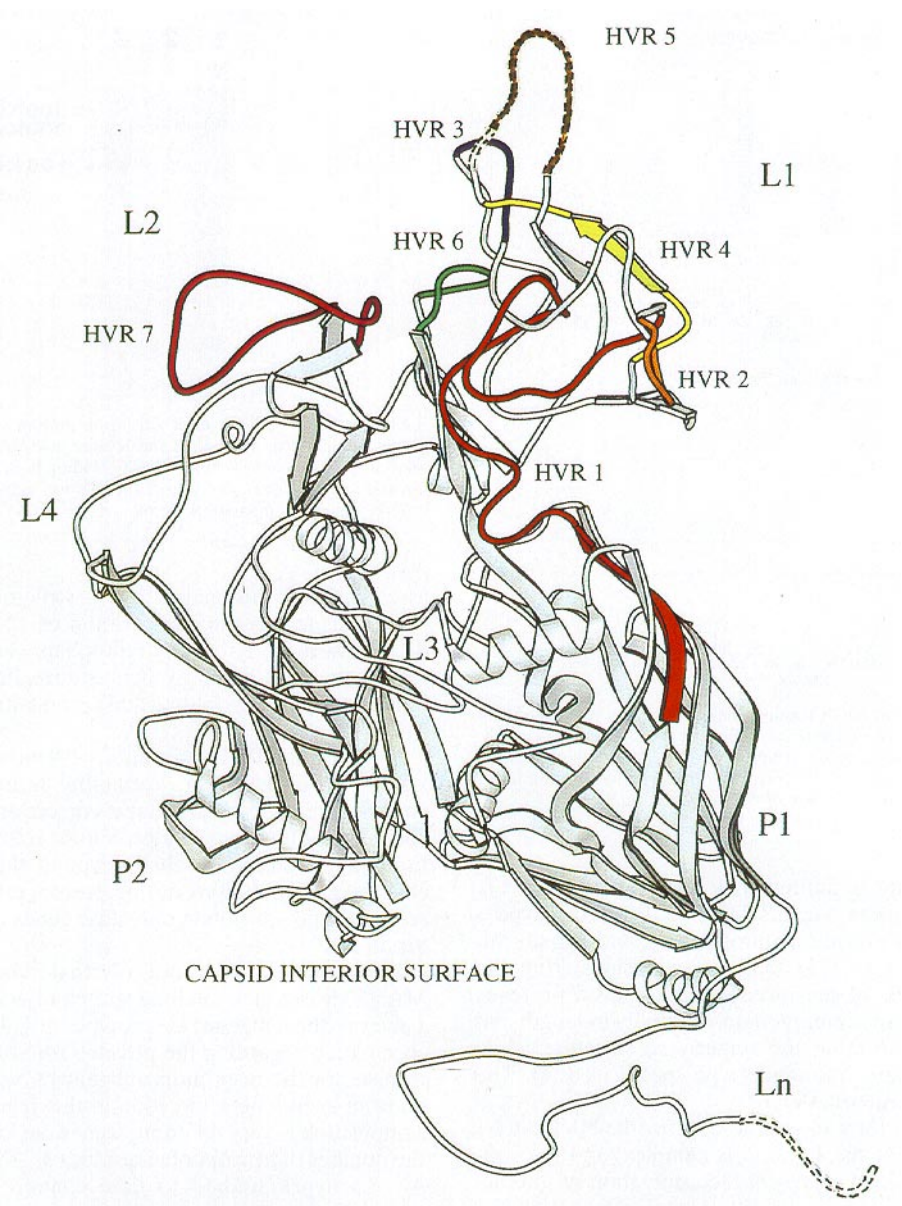
HVR 5
HVR 3
L1
L2
HVR 6
HVR 4
HVR 7
HVR 2
HVR 1
L4
L3
P1
P2
CAPSID INTERIOR SURFACE
Ln