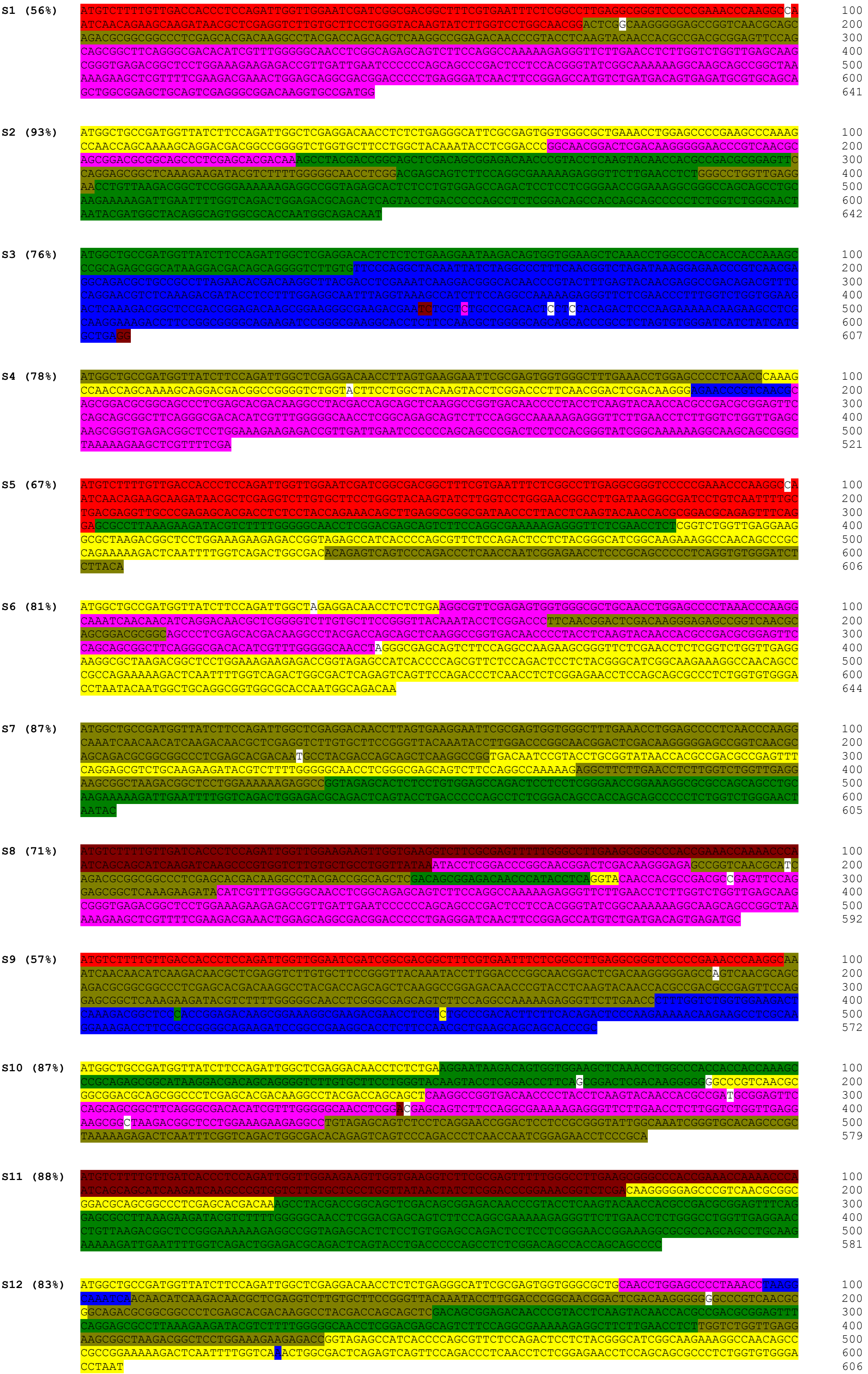
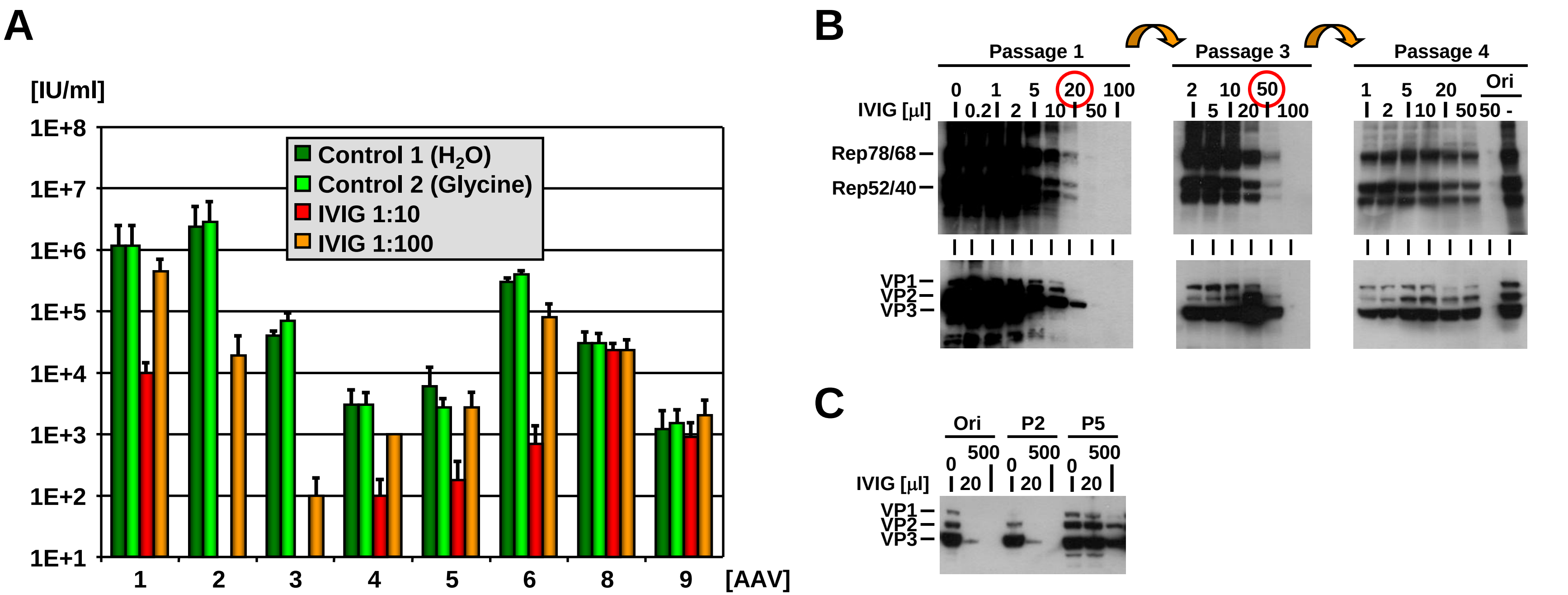
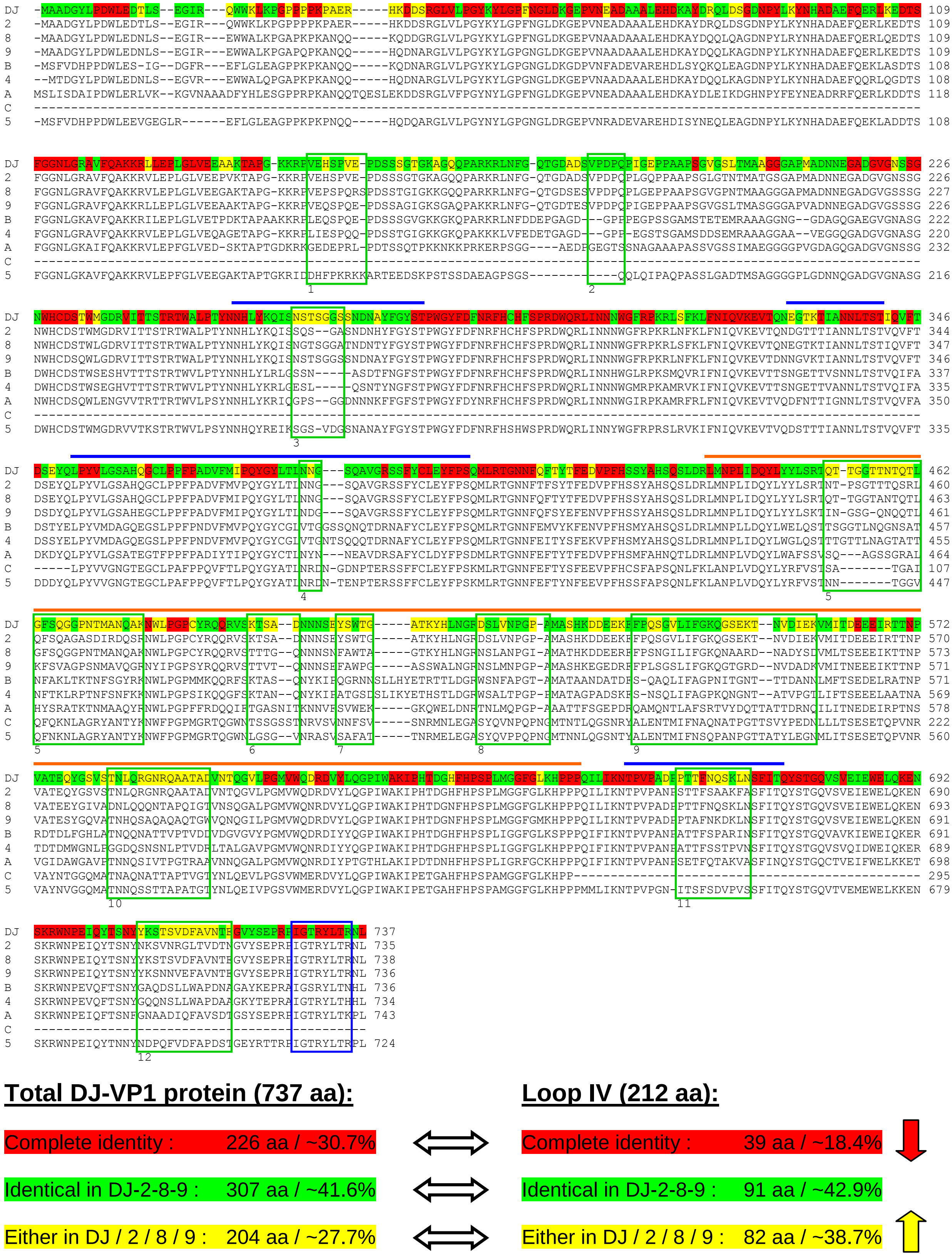


SUPPL. FIG 1. Nucleotide sequences of 12 randomly chosen clones from the unselected library (5' ends). The sequences were aligned to the 8 parental viruses and color-coded according to sequence identity. The colors are similar to Fig. 1: green, AAV-2; pink, AAV-4; brown, AAV-5; yellow, AAV-8; olive AAV-9 (orange in Fig. 1); blue, AAAV; red, BAAV. White background indicates point mutations. CAAV is not found in the 5' end, as only a downstream subfragment was used for shuffling. Numbers in parentheses indicate sequence identity with the AAV-2 prototype; in the shown examples, they ranged from 56 to 93%. Note that this is very similar to the range found for naturally occurring wildtype serotypes (compare Table 1).

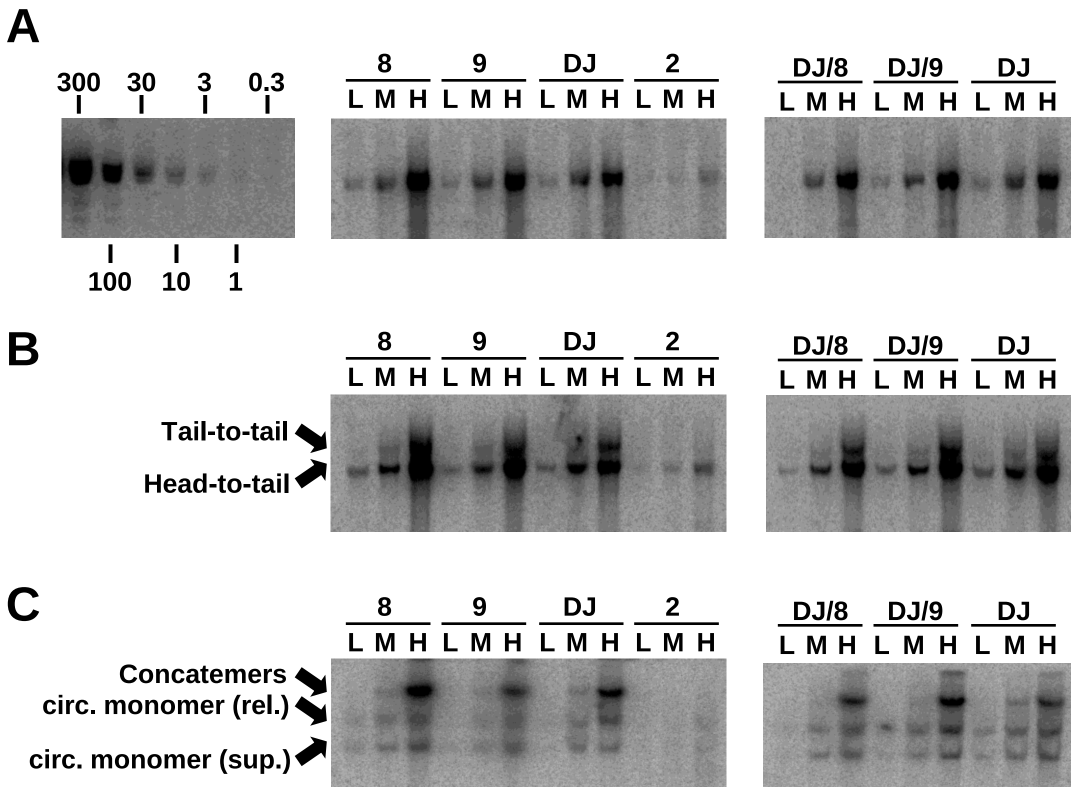
SUPPL. FIG. 2. Nucleotide sequences of 12 randomly chosen clones from the unselected library (3' ends). The clones, labels and colors are identical to Suppl. Fig. 1. The 3' ends of all clones are shown here. Only clone S8 contained the AAV-2-derived HBD (the triplets encoding the two crucial arginines are shown white on black).



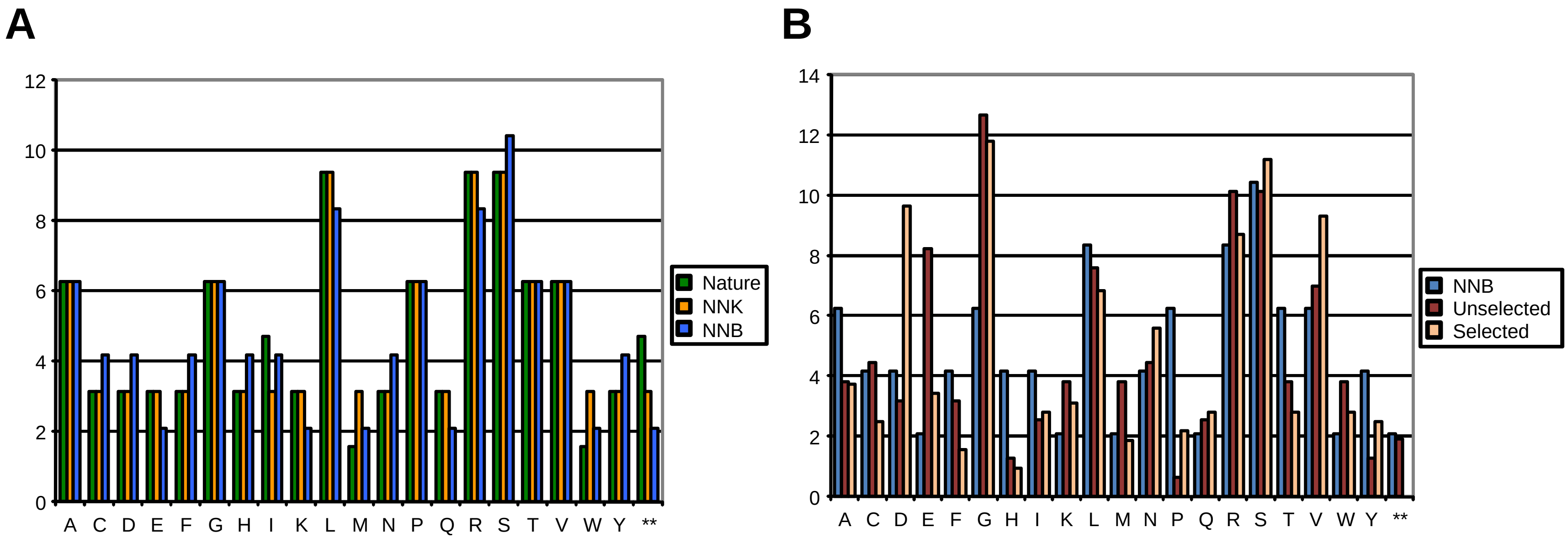
SUPPL. FIG. 3. Selection of shuffled AAV capsids with human immunoglobulin (IVIG). (A) To analyse the neutralizing acitivity of the particular IVIG batch, recombinant *gfp*-expressing AAVs of the shown serotypes were incubated for 1 hour at 37°C with the shown agents, and then titered on 293 cells (all virus stocks were normalized to $2x10^9$ particles per ml). IVIG had the strongest neutralizing effect on serotypes 2 and 3, followed by 6, 1, 4, and 5. AAV-8 or -9 were only inhibited (~10x) with undiluted IVIG (not shown). (B) AAV library amplification on Huh-7 cells under IVIG pressure. For passage 1, 20 µl of the library were incubated for 1 hour at 37°C with the shown amounts of IVIG, and then left on the cells overnight. The next day, the cells were washed and super-infected with helper Adenovirus. The cells were lysed three days later, and 20 µl from the supernatant showing minimal AAV protein expression (circled in red) were processed as before. Shown is expression of AAV replication (Rep, top) and capsid (VP) proteins (bottom). Ori, original library. The blot in (C) documents the increasing resistance of the amplified particles to high IVIG doses over the various passages.



SUPPL. FIG. 4. Protein sequence alignment of AAV-DJ and the 8 parental viruses. The 9 shown protein sequences (full-length VP1 proteins) were aligned using the ClustalW tool. Sequence identities between individual proteins are highlighted, using AAV-DJ as a reference. Individual amino acids are color-coded: red, identical between all 9 AAVs; green; identical between AAV-DJ and serotypes 2, 8 and 9; yellow, not conserved between serotypes 2, 8 and 9. The bars highlight the amino acids constituting the five exposed capsid loops (the largest and most divergent loop IV is highlighted by the orange bar). Green boxes depict 12 hypervariable regions in AAV capsid genes. Note the good correlation with amino acids highlighted in yellow, indicative of a high degree of evolution in these regions. The blue box indicates the B1 antibody epitope (fully conserved in AAV-DJ, -2, -5, -8, -9). Shown at the bottom are comparisons of amino acid identities between the full-length VP1 protein and the major loop IV. Remarkable is the decrease in complete sequence identity in this loop, and the concurrent increase in diversity between AAV-DJ and the other parental serotypes. This suggests the strongest selection pressure was exerted on this loop, resulting in the highest degree of evolution.



SUPPL. FIG. 5. Analyses of liver vector DNA. Mice were injected as described in Fig. 7, with hFIX-expressing AAV-2, -8, -9 or -DJ at low (L, $5x10^{10}$), medium (M, $2x10^{11}$), or high (H, $1x10^{12}$) particle doses. Six weeks later, total liver DNA was extracted and digested with Bam HI and Xho I (A), to determine vector copy numbers, or either Bam HI (single cutter, B) or Nco I (non-cutter, C), to analyze vector DNA forms. There were no differences between vector DNAs delivered by AAV-8, -9, or -DJ (including the HBD mutants) at any dose. All vectors mainly persisted as circular monomers (relaxed or supercoiled), or concatemers at higher doses, in agreement with previous reports for AAV-8 and -9.



SUPPL. FIG. 6. Amino acid frequencies in AAV peptide display libraries before and after selection. (A) Shown are natural frequencies of amino acids based on the genetic code ("Nature") versus theoretical frequencies in an NNK or NNB (this paper) library. Note that the frequency of unwanted stop codons ("**") is lowest with the NNB design. (B) Comparison of overall amino acid frequencies in the unselected AAV-DJ-based library versus that in 46 peptides cloned after *in vivo* biopanning in mouse lungs. Note that the frequency of stop codons ("**") is even slightly below the theoretical prediction in the unselected library. Importantly, stop codons were no longer detected after *in vivo* selection, arguing for a 100% coupling of viral genomes and capsids.