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Supplemental Information

"D" matters in recombinant AAV DNA packaging

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Supplementary results

Utilizing the scheme depicted in supplementary Figure 1, we used self-complimentary vectors and systematically analyzed the D-element requirement for rAAV vector encapsidation. Since there are two copies of ITR in a typical AAV vector along with two identical D-elements at each end of the AAV genomes, it is a challenge to replace the two D-sequences in the AAV genomes simultaneously. As shown in Figure 1B, native AAV ITR in scAAV is replicated and duplicated in replication and packaging process. This allowed us to make a plasmid vector library with randomized D -element. After such vector DNA libraries were introduced into AAV production cells, for those molecules that were competent for AAV packaging, the ITRs with mutation were duplicated and restored to the normal dual ITR configuration during the rescue and replication process. Therefore, replacing the single D sequence in scAAV vector had the same biological effects as replacing the two ITRs in a regular AAV vector. The obtained vectors were used to infect the test cell line GM16095 cells and the recovered vector DNA in the test cells were sequenced and the newly identified nucleotides in the D-sequence region of ITR were compared to wild type D sequences. The results shown in Figure 1C clearly demonstrated wtAAV ITR D sequences were only recovered at around 25% or so, which shows the lax requirement for D sequences in rAAV packaging.

To answer the issue of plasmid backbone packaging in rAAV production, we tested the compatibility of the junction sequence next to ITR in the common AAV vector for supporting AAV packaging. The results are shown in the supplementary figure 2. Although wt-D is 10 times more efficient than those non-canonical D-sequences, this backbone “pseudo-D” still allows approximately 10% packaging level as that of the wt-D element.

Experiment Material and methods:

Cell lines

HEK 293 cells and GM16095 cells (a human fibroblast cell line purchased from the Coriell Institute) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 100 μg of penicillin/ml, and 100 units/ml of streptomycin (Invitrogen, Carlsbad, CA). All cells were maintained in a humidified 37 °C incubator with 5% CO_2.

rAAV production and purification

The transgene plasmids packaged in the AAV vectors were described previously[1]. AAV vectors were generated using the triple plasmid co-transfection method[1,2]. Briefly, pAAV-Rep-Cap (serotypes 2 and 8), pAd helper, and the transgene plasmids were co-transfected into HEK293 cells and cultured in roller bottles at a ratio of 1:1:1. The vectors from transfected cells and medium were harvested 72 hours post transfection and purified by two rounds of CsCl gradient ultracentrifugation and extensively dialyzed against PBS containing 5% D-sorbitol. Viral genomes (vg) were quantified by real-time PCR and vector titers are expressed as vg/ml.

D-sequence library and D-variant construction

The plasmid pdsAAV-CB-GFP containing one 145-nt ITR and 88-nt mutant ITR was used as backbone. The 145-nt ITR was replaced with 131-nt wild type ITR by PvuII and Mscl restriction enzymes. New constructed pdsAAV-CB-GFP plasmid was digested with XbaI and Bbsl restriction and ligated with 8-nt, 18-nt, 24-nt random D sequence library respectively, as shown in Figure 1. Thus, Core sequences of the terminal resolution sites (trs) site were replaced with randomized 8, 18, and 24 oligonucleotides. D sequence library was referred to as pseudo “D” sequence lib.

The pseudo “D” lib-CB-GPF plasmid was co-transfected with pFΔ6 and ph22 into HEK293 cells at the ratio of 1:1:1. Cell culture medium was collected at 48 hours after transfection and was used to infect GM16095 cells for rAAV vector evaluation. Cells were lysed, and DNA was extracted at 12 hours following infection. Amplification of pseudo “D” lib was performed using 5 μl of the extracted DNA as input to a 50 μl AccuTaq LA (Sigma) reaction mixture, according to the manufacturer’s instructions, using 1 μl Forward primer (5’-CTTCTAGTTGCCAGCCATC-3’) and 1 μl Reverse primer(5′-GGCCTCAGTGAGCGAGCGAG-3′) with PCR cycling conditions of 98°C for 30 s, 30 cycles of 94°C for 15 s, 50°C for 20 s, and 68°C for 2 min, followed by 68°C for 10 min. TA cloning of PCR products were performed using pCR™2.1 cloning vector (The TA Cloning® Kit with pCR™2.1 vector, Thermos fisher Scientific, US), according to the manufacturer’s instructions. Positive colonies were verified by restriction digestion and sequenced by Sanger sequencing. These verified D-sequences were separately inserted into pdsAAV-CB-GFP plasmid containing wild type ITR by XbaI and Bbsl restriction enzymes, thus specific D-variant vector was constructed.

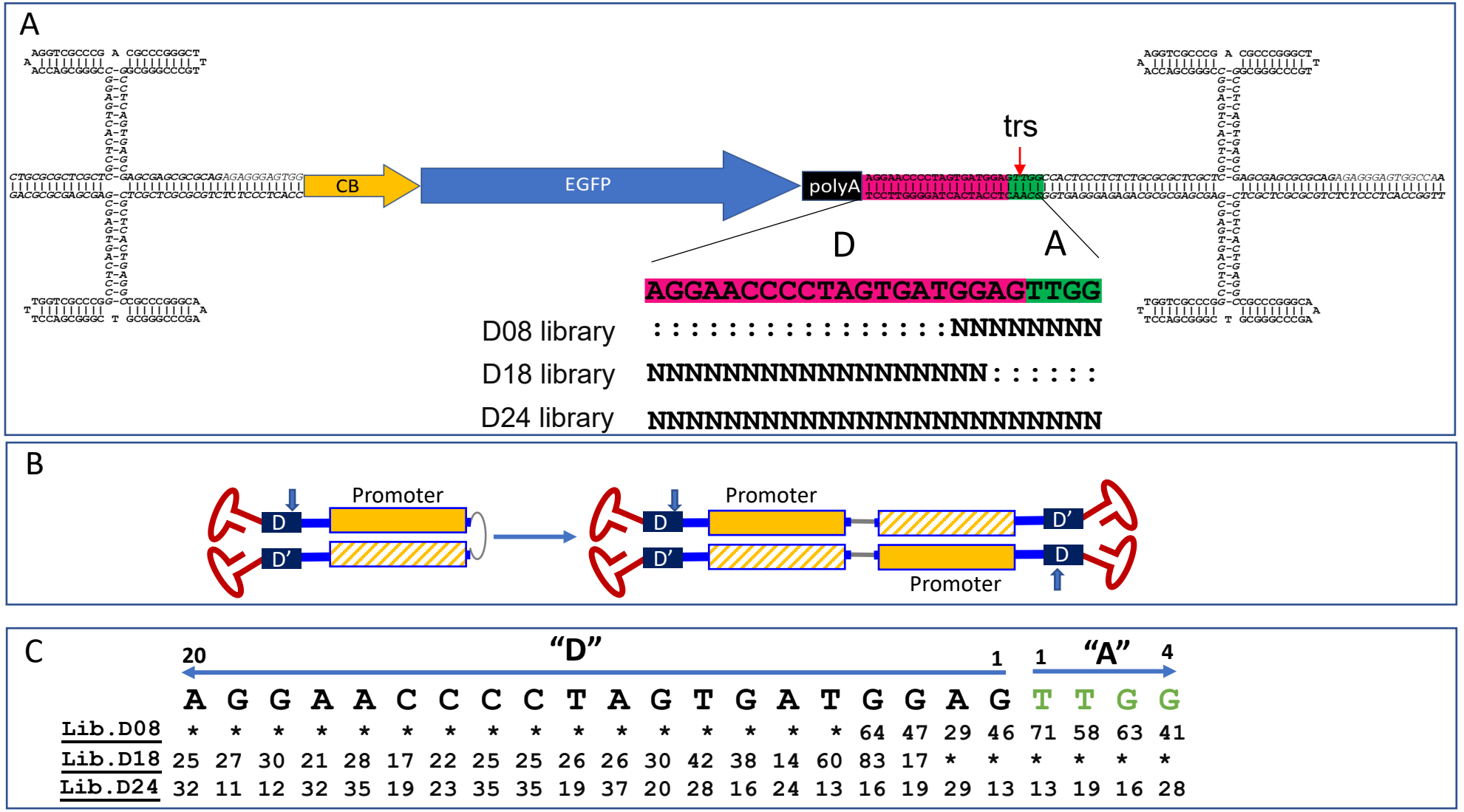
Figure Legend:

Supplementary Figure 1. A. Schematic illustration of D element library construction and screening diagram. The ITR related sequences are shown in the scAAV vector with D-element and terminal resolution region highlighted. The region randomized in the D08, D18 and D24 library are shown as N in the alignment with wild type ITR sequences. B. Illustration of the duplication of D sequence in scAAV replication. C. The summary of the percentage of recovered wild type AAV nucleotide in D sequences.

Supplementary Figure 2. Nucleotides adjacent to ITR in rAAV vector plasmid serve the functionality of D-element in rAAV packaging. A. Illustration of the source of plasmid backbone nucleotides tested in this study. The top construct is a self-complementary AAV vector with a wt D sequences. The lower three vector plasmids have the D-sequences replaced by the nucleotides next to ITR in the plasmid backbone moiety. BB1 was derived from pscAAV-CB-GFP, BB2 was derived from pssAAV-CB-lacZ, BB3 was derived from pssAAV-hFIX. B. Alignment of BB1, BB2 and BB3 to the wt-D element. C. The vectors plasmid shown in A. were used to produce rAAV vector. The results showed BB1, BB2 and BB3 element all supported rAAV production.

References:

1. Wang, Q.*, et al.* High-Density Recombinant Adeno-Associated Viral Particles are Competent Vectors for In Vivo Transduction. *Human gene therapy* **27**, 971-981 (2016).
2. Wang, Q.*, et al.* Efficient production of dual recombinant adeno-associated viral vectors for factor VIII delivery. *Human gene therapy methods* **25**, 261-268 (2014).



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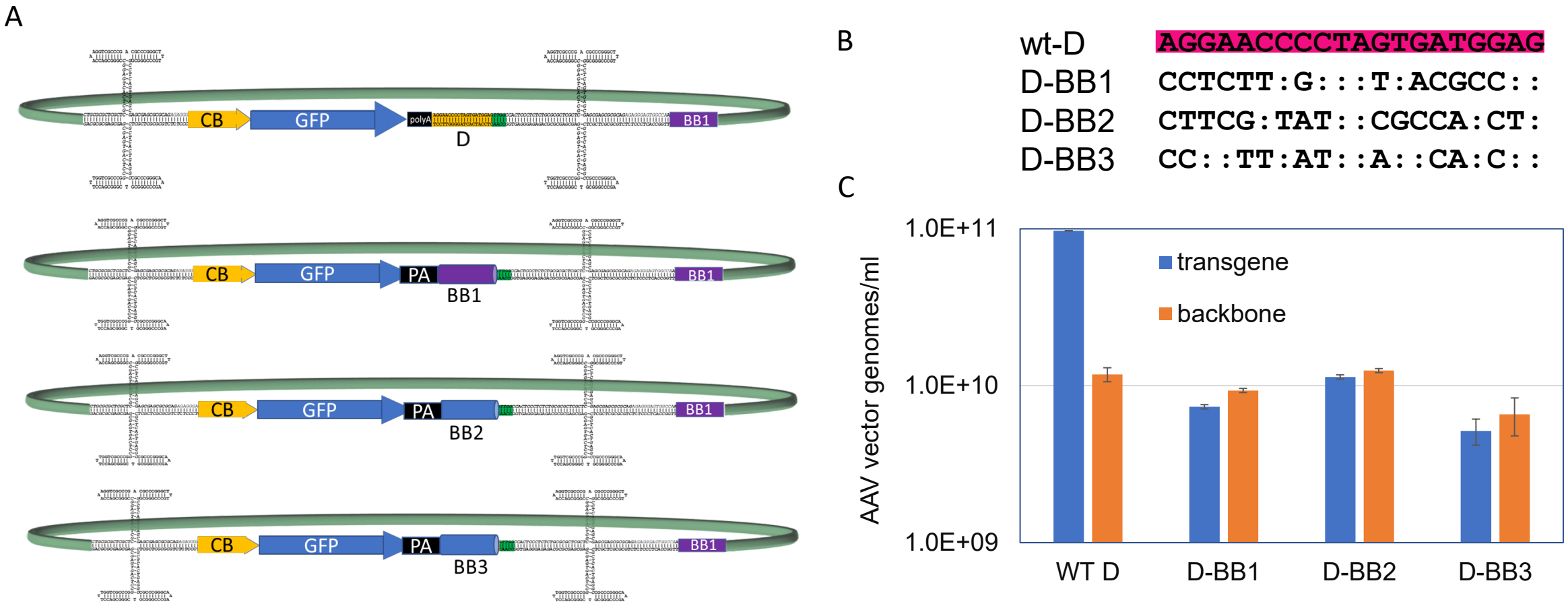

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