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AAV2 *X* increases AAV6 *rep*/*cap*-driven rAAV production

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ABSTRACT

We have recently identified a new gene, involved in DNA replication, at the far 3′ end of the adeno-associated virus type 2 (AAV2) genome. The AAV type 6 (AAV6) genome has a disrupted *X* open reading frame (ORF) whose two halves, when combined, have full-length homology and comparable size to AAV2 X. Hypothesizing that AAV6 *X* is inactive, we assessed if AAV2 *X* augments recombinant (r)AAV2 DNA replication and virion production, but with *rep* and *cap* trans-functions of AAV6. Using AAV2 *X* expressing HEK293 cell lines we show AAV2 *X* significantly boosts rAAV DNA replication/virion production, driven by AAV6 *rep/cap* as it does the AAV2 *rep/cap* system. Protein BLAST search for homology between AAV2 X and various AAV Rep78 proteins suggests that *X* might be AAV8 Rep 78-derived and have some of its activities. These data suggest that AAV2 *X*, and the corresponding *X* genes of other AAV types/clades, warrant further study.

Introduction

Now over 100 adeno-associated virus (AAV) types have been isolated. While AAV type 2 (AAV2) was the first AAV type used for gene transfer (Hermonat, 1984, 2014; Hermonat and Muzyczka, 1984; Tratschin et al., 1984a), over time more and more AAV types, each with its own somewhat different cellular tropisms, are coming into use. In general these other AAV types have the same genomic structure as AAV2 (Gao et al., 2005; Srivastava et al., 1983). Analysis of the first cloned adeno-associated virus AAV type 2 (AAV2) genome showed that there were two main open reading frames (ORFs) and mutation within the identified ORFs indicated three *trans* phenotypes were present (Hermonat et al., 1984, Tratschin et al., 1984b). Mutations in the left half of the genome were defective in DNA replication and transcription and given the *rep* phenotype. This region encodes replication / transcription factor proteins Rep78, Rep68, Rep52, and Rep40. Mutations within the right half of the genome were defective in wild type virion production, but the region had two phenotypes. One was given the name *lip* for the production of viral particles of low infectivity (missing VP1)(also described as *inf*), while the *cap* phenotype did not produce any viral particles at all (encoding the major structural protein, VP3) (Hermonat et al., 1984; Tratschin et al., 1984b). In addition, recently, a new fourth *trans* phenotype, involved in virion maturation, has been identified by Jurgen Kleinschmidt and called the *AAP* gene (Sonntag et al., 2010).

Recently we discovered a fifth phenotype, a new gene we called X (GenBank KM186843.1), within the AAV2 genome (Cao et al., 2014). The X gene is located at the carboxy-end of the *cap* gene but in a different translational frame. We have shown that X is needed for maximal wt AAV2 and rAAV2 DNA replication and virion production by several methods. The X gene also has a dedicated promoter located just upstream, called p81 (at map unit 81) (Hermonat et al., 1999). However, the question arises is AAV2 *X* activity only specific for helping/augmenting AAV2, or is it capable of helping other AAV types? Most other AAV clades also have members with an open reading frame (ORF) in the same position as AAV2 X, but these potential genes are usually smaller than AAV2 X. Other AAVs may have mutated X genes, such as in AAV6 there are two ORFs, divided by a few bases, which take up the position analogous to where the AAV2 *X* gene is. Here we observed that AAV2 X is able to augment or boost an rAAV production system based exclusively on the AAV6 *rep* and *cap*, trans sequences and we find that X is capable of increasing rAAV2 DNA replication and virion production

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when driven by the AAV6 *rep* and *cap* genes. In addition, we hypothesize that AAV2 *X* may be derived from a 5′ region of the AAV Rep78/NS1 gene.

Results

AAV6 genome contains an X gene but which is divided into two abutting ORFs

If one observes the open reading frames of the prototype AAV6 genome (Genbank AF028704) it is observed that there are two ORFs, which we refer to as Xa and Xb, which take up the position analogous to where the AAV2 *X* gene is. There is a small gap between the stop codon of Xa and the initiation codon of Xb. However, analyzing two other AAV6 sequences, specifically Genbank EU368909 and EU36910, there is an even smaller gap between Xa and Xb of only 13 nucleotides, and the Xb ORF encodes a further 22 amino acids (aa) at its amino terminus. Fig. 1A shows the gene/ORF organization of AAV6 using largely the AF028704 prototype sequences, but with the X region of EU368909 replacing the analogous sequences of the prototype. Figs. 1B and C show the DNA and amino acid sequences of Xa and Xb. Fig. 2 is a homology analysis by standard NCBI Protein BLAST of the amino acid sequence of AAV2 X versus those of the fused Xa and Xb aa of EU368909. As can be seen there is significant homology between the two X sequences across their length. This extensive homology suggests that AAV6 Xa-b is a homolog of AAV2 X and it has either evolved or mutated at some point in time. Presently, it is unknown if AAV6 Xa and Xb represent two potentially functional proteins or are fully inactive and "broken". In any case AAV6 appears to have or have had a very AAV2 "X"-like protein(s).

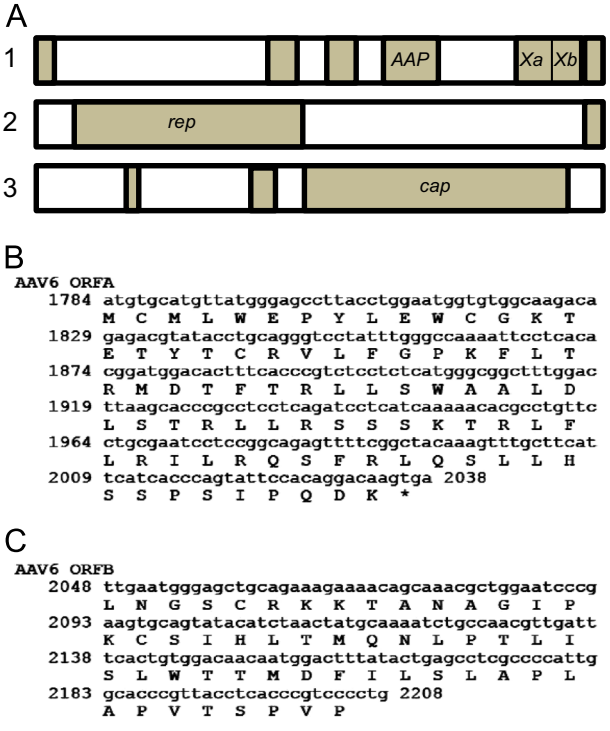

Fig. 1. The AAV6 genome showing *Xa* and *Xb*. Shown in A are the ORFs of AAV6 by NIH ORF finder and derived from the Genbank AF028704, but with the AAV6 *X* region replaced with sequences from EU368909. Note that there are two open reading frames, *Xa* and *Xb*, present in the position occupied by AAV2 *X*. B shows the DNA and amino acid sequences of *Xa*. **C** shows the DNA and amino acid sequences of *Xb*.

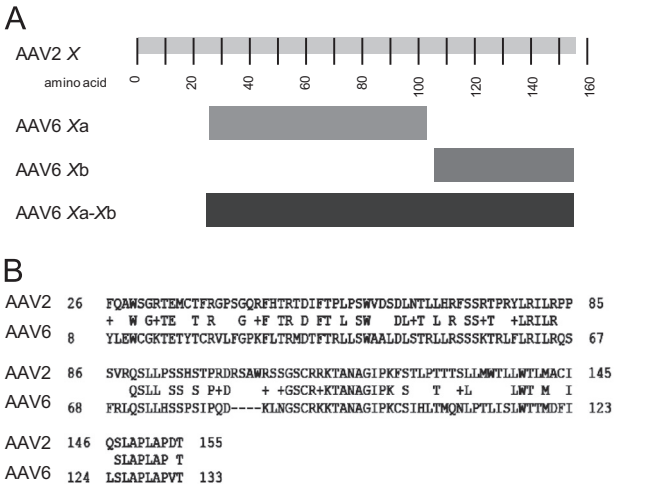

Fig. 2. Homology of AAV2 *X* with AAV6 *Xa* and *Xb*. Shown in A are a more detailed caricature of the *X* ORFs of AAV6 by NIH ORF finder derived from the Genbank AF028704 plus EU368909. In B is shown an NCBI Protein BLAST analysis of the artificially fused AAV6 *Xa-Xb* aa sequence (normally two separate ORFs) with that of AAV2 *X*. Note that the homology of the two *X* sequences extends the length of fused AAV6 *Xa-Xb*.

AAV2 X helps rAAV2/6-eGFP DNA replication and virion production

As we know that AAV2 *X* increases rAAV2 yield, and AAV6 X may be non-functional, we investigated whether AAV2 *X* might complement AAV6 *rep*/*cap* driven rAAV production. Previously we generated HEK293 cell lines containing chromosomal AAV2 X (293-X-B and 293-X-K) and we compared them to parental HEK293 cells for supporting rAAV2 DNA replication and virus production. Shown in Fig. 3A is a Southern blot of rAAV2/eGFP DNA replication (probed with ^{32}P-eGFP DNA) by transfecting the vector plasmid with AAV6-repcap and pHelper (Ad5 helper genes) plasmids. Fig. 3B shows a dot blot of DNaseI-resistant virion DNA which shows higher rAAV production in X-positive 293-X-B and 293-X-K than in unaltered 293 cells. Moreover the higher virion production mirrors the higher vector DNA replication levels. As can be seen, the presence of the AAV2 *X* gene within the B and K cell lines was able to boost rAAV production in based on the AAV6 *rep* and *cap* proteins as it did for rAAV based on AAV2 *rep* and *cap* driving vector production.

AAV2 X helps rAAV2/6-Foxp3 DNA replication and virion production

Similar experiments were done with the vector rAAV2/Foxp3 in place of AAV2/eGFP. Fig. 4A shows a Southern blot of rAAV2/Foxp3 DNA replication (probed with ^{32}P-Foxp3 DNA) by transfecting the vector plasmid with AAV6repcap and pHelper (Ad5 helper genes) plasmids. Fig. 4B then shows a dot blot of DNaseI-resistant virion DNA which shows higher rAAV production in X-positive 293-X-B and 293-X-K than in unaltered 293 cells. Again, as with the AAV2/eGFP vector, the presence of AAV2 X in the 293 cells, in conjunction with AAV6 *rep* and *cap* proteins, boosted vector rAAV2/Foxp3 DNA replication and virion production as it did for rAAV driven by AAV2 *rep* and *cap*.

AAV2 X has homology to the Rep78 proteins of various AAVs

It was noticed during various NCBI Protein Blast searches that *X* showed homology with Rep78/NS1 of AAV2, but also other AAVs as well. Therefore in Fig. 5A, B, C, and D we show the results of homology analyses with AAV2, AAV4, AAV8 and Go.1. The largest region of homology is seen between AAV8 Rep78 and AAV2 *X*. It can be seen that most homology with *X* lies in a region from about