

genomes provide new strategies to manipulate viral genome conversion products and direct intermolecular recombination events required for efficient dual AAV vector reconstitution of transgene.

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### 13. Hot Spots for rAAV2 Vector Integration in Mice

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Recent reports on severe adverse effects induced by retroviral integration into the LMO2 proto-oncogene in humans and the demonstration of preferential AAV serotype 2 (rAAV2) vector integration into genes in our previous study with a limited number of integration sites in mice<sup>1</sup>, have raised a possible concern about rAAV-mediated insertional mutagenesis. In order to further address this issue, we have expanded our previous study and performed a high-throughput analysis of rAAV2 integration sites isolated from *in vivo* selected hereditary tyrosinemia type I (HTI) mouse hepatocytes transduced with a human fumaryl acetoacetate hydrolase (FAH)-expressing rAAV2 shuttle vector, AAV-EF1 $\alpha$ -hFAH.AOS. Briefly, total liver DNA was isolated from 4 HTI mice that underwent a 7-month *in vivo* selection of transplanted hepatocytes isolated from the donor HTI mice having received  $3.0 \times 10^{11}$  vector genomes of AAV-EF1 $\alpha$ -hFAH.AOS via the portal vein and undergone an 8-week *in vivo* selection. The whole proviral rAAV2 vector genome and flanking genomic DNA sequences were isolated as a plasmid from the liver DNA by a plasmid rescue technique as previously described<sup>1</sup>. All four mice had a different donor HTI mouse, therefore, any integrations found in different mice should be considered as independent integration events. To date, we have characterized 307 independent integration events and found: 1) 191 of 307 (62%) of integrations occurred in genes; 2) there was a strong bias toward integrating into host genomes around transcription start sites, as has been observed in murine leukemia virus integrations (approximately a quarter of the total rAAV2 integrations occurred within  $\pm 1$  kb from transcription start sites); 3) rAAV integration involved large genomic deletions of over 1 kb in 15% of the cases, suggesting that such large deletions may not be rare events associated with rAAV2 integration. The most striking finding was that among the 307 independent integration events, at least 4 integrations from 3 different mice (1.5% of total integrations and 2.1% of the genes targeted by rAAV) were found within a 30-kb region in the mouse ubiquitin C gene, and at least 4 integrations from 4 different mice fell on another sequence stretch of approximately 2.5 kb. Our preliminary analysis by restriction enzyme mapping and sequencing over a thousand base pairs has indicated that the hot spot of 2.5 kb in length likely resides in a proto-oncogene, the Evi-1 gene. It should be noted that murine leukemia found in a retroviral gene marking study was induced by insertional mutagenesis at this locus. Although the oncogenic potential of this gene in the liver is not known, overexpression of this gene can transform not only hematopoietic cells but also other cell types. Thus, the preliminary results in our high-throughput rAAV2 integration site analysis further emphasize the importance of pursuing the study on the elucidation of the mechanisms of rAAV vector integration and its consequences in the host.

1. Nakai, H. et al. AAV serotype 2 vectors preferentially integrate into active genes in mice. *Nat. Genet.* 34, 297-302 (2003).

### 14. Absence of T-Shaped Structure and Deletions of B and C Hairpins Have Minimal Effects on Essential Functions of AAV Inverted Terminal Repeats

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The inverted terminal repeats (ITRs) of Adeno-associated virus (AAV) play essential roles in viral DNA replication, packaging and integration. Flanking a coding region, the two ITRs are the only viral elements left in recombinant AAV (rAAV) vectors for gene delivery. The objective of this work is to dissect ITR elements to understand further how ITRs function, which will also help us to design better AAV vectors. Each 145 nt AAV ITR is composed of six sub-regions (A-B-B'-C-C'-A') forming a T-shaped self-base paired structure. To characterize the role of each ITR sub-region in viral DNA replication, packaging and integration, we generated a series of ITR deletion mutants from a pDD plasmid which has a single ITR flanked by two D regions containing the terminal resolution site. Deletions of B-B', C-C', or both does not affect viral DNA replication or packaging into infectious virus. Even when B-B', C-C', plus a part of the A region which does not include the Rep-binding site are all deleted, this ITR mutant can produce functional virus at an efficiency comparable to the parental plasmid containing a complete ITR. As expected, further deletion into the Rep-binding site abolishes viral DNA replication. Our quantitative analysis also shows that deletions of B-B', C-C', and A region not including Rep-binding site do not affect viral DNA integration into host chromosomes. These results suggest that the T-shaped secondary structure is dispensable.

### 15. Trans-Splicing Adeno-Associated Viral Vector-Mediated Gene Therapy Is Limited by Transcription across the ITR Junction but Not by Dual Vector Co-Infection Efficiency

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Adeno-associated virus (AAV) is one of the smallest DNA viruses. Therapeutic application of recombinant AAV has been limited by its small carrying capacity. Many large genes, such as the 6kb mini-dystrophin gene for Duchenne muscular dystrophy (DMD), are traditionally excluded from rAAV gene therapy. To overcome the size limitation, we have recently developed trans-splicing AAV vectors. This approach takes advantage of AAV inverted terminal repeat (ITR)-mediated intermolecular recombination and the eukaryotic splicing machinery. A large gene is split into two parts and engineered with splicing signals. Following packaging and co-infection, the transgene is expressed from the reconstituted genome. Despite the success of the proof-of-principle studies, the overall transduction efficiency of trans-splicing vectors is considerably lower than that of a single intact vector in skeletal muscle. To improve trans-splicing vectors for DMD gene therapy, we examined whether co-infection efficiency is a rate-limiting factor for skeletal muscle in a mouse model for DMD (mdx mouse). Two different AAV viruses carrying the gene either for alkaline phosphatase or nuclear-localized LacZ were delivered to mdx anterior tibialis muscle. Consistent with previous reports in normal muscle, co-infection efficiency

reached  $89.2 \pm 2.0\%$  in the diseased muscle. This result suggests that co-infection efficiency is not a hurdle for trans-splicing vector-mediated gene therapy in dystrophic muscle.

A key step in trans-splicing method is the successful transcription and splicing across the ITR junction in the reconstituted genome. Head-to-tail recombination between AAV viral genomes results in an unusual double-D ITR structure. To test whether this irregular DNA structure represents an obstacle for trans-splicing approach, we systematically evaluated the transcription, splicing and translation in a synthetic LacZ construct that mimicked the reconstituted genome. Inserting an intron in the LacZ gene had no effect on its expression ( $2.5 \pm 0.2$  vs.  $2.3 \pm 0.1$ ). However, inclusion of a double-D ITR in the intron significantly reduced expression to a relative level of  $1.2 \pm 0.1$ . To further clarify the molecular mechanism, we directly measured the transcription and splicing by RNase protection assay. Consistent with our expression data, there was no significant difference in mRNA production with or without intron ( $109.4 \pm 7.0$  vs.  $105.0 \pm 11.4$ ). However, in the construct containing the double-D ITR, mRNA level was significantly reduced to a relative level of  $49.1 \pm 10.1$ . Surprisingly, there was a much bigger drop in the level of heterogeneous RNA from intron-containing construct ( $8.7 \pm 1.4$ ) to intron-double-D ITR-containing construct ( $1.7 \pm 0.5$ ). Additional studies suggest that double-D ITR did not diminish, but rather, enhanced splicing efficiency. The ratio of spliced to unspliced transcript was increased from  $12.6 \pm 2.1$  (no ITR) to  $30.1 \pm 4.7$  (with ITR). Taken together, our data suggest that transcription elongation across the ITR junction is a rate-limiting factor in trans-splicing vector-mediated gene therapy.

## 16. Visualization of the Foci of Recombinant AAV Replication and Their Relationship with Cellular Proteins Involved in the Recognition and Repair of Double-Stranded DNA Breaks

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Several properties of AAV are very attractive for human gene therapy; however, many aspects of its life cycle and, in particular, the molecular determinants of cellular permissivity to these vectors, are still largely unexplored. In cultured cells, increased efficiency of wt AAV or rAAV transduction can be obtained by treating cells with agents that affect genomic DNA integrity or metabolism. This effect correlates with an improved conversion of the vector genome into dsDNA. This observation led to the possibility that permissivity for AAV transduction could be linked to the induction of DNA damage checkpoints or of DNA repair mechanisms that mediate replication of ss DNA genomes. A few years ago, we have indeed observed that proteins involved in dsDNA break (DSB) repair bind to the incoming rAAV genomes and regulate vector replication.

In order to get further insights into the role of DSB repair proteins in the processing of rAAV genomes, we developed a strategy to visualize the sites of dsDNA AAV inside the cells. We constructed a rAAV (AAV-LacO) containing 112 LacI binding sites (lacO) between the viral ITRs and obtained stable HeLa cell clones expressing LacI fused to EGFP. In these cells, the sites of rAAV ssDNA to dsDNA conversion are visualized by binding of the EGFP-LacI fusion protein.

After transduction with AAV-LacO and cell treatment with hydroxyurea (HU), dsDNA replication intermediates were visible as small dots within the nucleus appearing as early as after 4 h; at longer time points these dots grew larger in size. Next we examined the localization of dsDNA AAV-LacO in respect to some important proteins of the DSB repair pathway, including Rad51, Rad50 and

histone H2AX. In response to DNA damage, these proteins are rapidly relocalized and concentrated into subnuclear complexes that are detected as foci. Rad51 promotes homologous pairing and strand exchange. We observed colocalization of the Rad51 foci and AAV-LacO only at early times post infection. After 24 h, the Rad51 foci appeared juxtaposed to, but clearly separated from, the AAV-LacO dots. In contrast, the vast majority of Rad50 foci were found to colocalize with AAV-LacO in HU treated cells at both 8 h and 24 h post infection. Rad50 is member of Mre11-Rad50-Nbs1 complex that binds ss and dsDNA and has a pivotal role in the processing of DSBs. Finally, the AAV-LacO foci were found to be distinct from those formed by the accumulation of  $\gamma$ -H2AX, a member of the histone H2A family that is phosphorylated following DSBs ( $\gamma$ -H2AX) and relocalizes into nuclear foci, which form shortly after DNA damage and colocalize with several DSB repair proteins.

Taken together these results suggest that the Rad51 and Rad50 proteins, which have a role in processing DSBs, may facilitate conversion of the rAAV genomes from single strand to double strand and that they interact with these genomes with different dynamics. In contrast,  $\gamma$ -H2AX, which may act by promoting the concentration of proteins in the vicinity of the DNA lesions, apparently does not participate in the maturation of the AAV genomes. We are currently inhibiting these and other DSB proteins by the siRNA technology to further corroborate this model.

## 17. Role of Vp2 in AAV Packaging

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Adeno-associated virus (AAV) is a member of the parvovirus family. A majority of the parvovirus genomes encode for two capsid proteins (Vp1 and Vp2) where the N-terminus of the latter is exposed on the surface of the capsid and subsequently processed into Vp3 after formation of the virion. However, AAV encodes all three capsid proteins which are incorporated into the capsid at a 1:1:10 ratio with Vp3 as the most abundant. The N-terminus of Vp2 has been suggested to be located within the capsid and not exposed on the surface of AAV capsids. Based on the premise that the N-terminus of Vp2 is within the capsid occupying space, we set out to characterize the packaging capacity of AAV serotypes 1-5 with and without Vp2. Mutagenesis of the Vp2 ACG start site codon to ACA was sufficient to inhibit its expression and incorporation into the virion. We utilized the packaging cassettes (4.4 kb through 6 kb), acquired from Dong et al 1996, which contain the CMV-driven chloramphenicol acetyl transferase gene. rAAV serotypes 1 through 5 with and without Vp2 were able to replicate, package and transduce cells with cassettes up to 6019 bp in size. To show that the cassettes packaged within the virions were the correct size, DNA was extracted from the rAAV serotypes after their isolation from 293 cells via freeze/thaw lysis or CsCl purification and assayed on alkaline agarose gels. Small-scale virus productions resulted in Dot blot titers ranging from  $10^6$  to  $10^7/\mu\text{L}$  for each packaging cassette for all 5 serotypes. Transduction of cells with rAAV packaging cassettes of sizes 5.3 and 6 kb was 2-5 fold lower than rAAV packaging the 4.7kb cassette. However, rAAV lacking Vp2 displayed different transduction profiles exhibiting a dramatic drop in transduction with cassettes larger than 4.76 kb, unlike those for viruses with Vp2. These results suggest that rAAV vectors can successfully package and transduce cells with the above transgene cassettes up to 6 kb in size, but Vp2 may play a role in efficient transduction above 4.7 kb. In summary, this study warrants further testing of transgene cassettes of interest that exceed 4.7 kb in length.