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Enhanced transgene expression from single-stranded AAV vectors in human cells *in vitro* and in murine hepatocytes *in vivo*

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We identified that distal 10 nucleotides in the D-sequence in AAV2 inverted terminal repeat (ITR) share partial sequence homology to 1/2 binding site of glucocorticoid receptor-binding element (GRE). Here, we describe that (1) purified GR binds to AAV2 D-sequence, and the D-sequence competes with GR binding to its cognate binding site; (2) dexamethasone-mediated activation of GR pathway significantly increases the transduction efficiency of AAV2 vectors in human cells; (3) human osteosarcoma cells, U2OS, which lack expression of GR, are poorly transduced by AAV2 vectors, but stable transfection with a GR expression plasmid restores vector-mediated transgene expression; (4) replacement of the distal 10 nucleotides in the D-sequence of the AAV2 ITR with a full-length GRE consensus sequence significantly enhances transgene expression in human cells *in vitro* and in murine hepatocytes *in vivo*; and (5) none of the ITRs in AAV1, AAV3, AAV4, AAV5, and AAV6 genomes contains the GRE 1/2 binding site, and insertion of a full-length GRE consensus sequence in the AAV6-ITR also significantly enhances transgene expression from AAV6 vectors, both *in vitro* and *in vivo*. These novel vectors, termed generation Y AAV vectors, which are serotype, transgene, or promoter agnostic, should be useful in human gene therapy.

INTRODUCTION

Adeno-associated virus (AAV), a non-pathogenic parvovirus, contains a single-stranded (ss) DNA genome,[1] and requires co-infection with a helper virus, such as adenovirus,[2] herpesvirus,[3] or human papillomavirus,[4] for its optimal gene expression and replication. In the absence of co-infection with a helper virus, the wild-type (WT) AAV genome remains transcriptionally inactive, and establishes a latent infection following site-specific integration into human chromosome 19.[5,6] The non-pathogenic nature and the site-specific integration of AAV prompted the development of recombinant AAV vectors for gene transfer and gene therapy.[7] Although recombinant AAV vectors do not integrate site specifically,[8] they have taken center stage in gene therapy of a wide variety of human diseases.[9–11] To date, more than 315 phase 1, 2, and 3 clinical trials with recombinant AAV vectors have been, or are currently, being performed, and the U.S. Food and Drug Administration (FDA) has granted approval of six AAV drugs, namely, Luxturna,[12] Zolgensma,[13] Hemgenix,[14] Elevidys,[15] Roctavian,[16] and Beqvez[17] for gene therapy of Leber congenital amaurosis, spinal muscular atrophy, hemophilia B, Duchenne muscular dystrophy (DMD), hemophilia A and hemophilia B, respectively.

Despite these remarkable achievements, it has also become increasingly clear that the first generation of recombinant AAV vectors are not optimal; in nearly all instances, relative high doses were needed to achieve clinical efficacy, which has been shown to lead to serious adverse events (SAEs) and in deaths of 10 patients to date.[18] Extensive efforts have been undertaken to pursue AAV capsid modifications, which have been reported to significantly improve targeting a given tissue/organ.[19–36] Unfortunately, however, in four separate clinical trials, capsid-modified AAV vectors have also been reported to lead to SAEs.[37–40]

We have posited that, beyond capsid modifications, it is equally important to pursue genome modifications to improve transgene expression from recombinant AAV vectors, since it is the genome, and not the capsid, that expresses the therapeutic transgene.[41] The development of self-complementary (sc) AAV vectors that bypass the requirement for viral second-stranded DNA synthesis has circumvented this problem to some extent and facilitated its use, but the limited packaging

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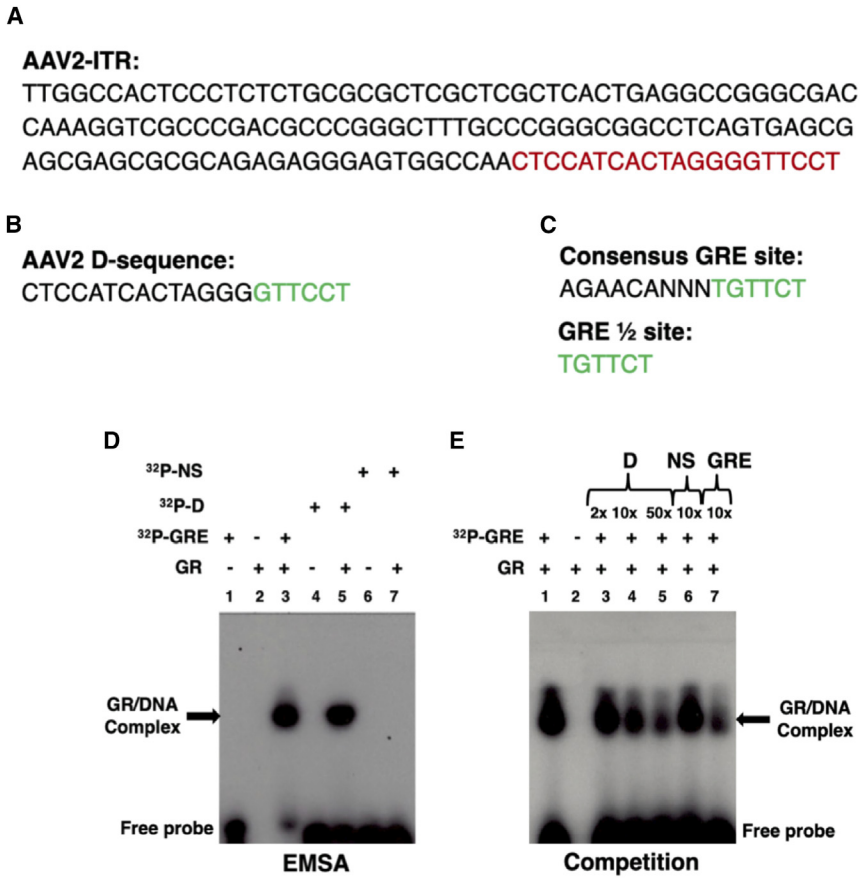

Figure 1. Nucleotide sequences of AAV2- ITR D-sequence and the consensus GRE site

(A–C) The first 125 nucleotides in the AAV ITR are shown in black fonts, and the 20 nucleotides in the D-sequence in red fonts. The distal six nucleotides in the D-sequence, shown in green, share partial homology with the consensus GRE 1/2 site. (D and E). Purified GR protein binds to the AAV D-sequence. EMSAs were performed using ^{32}P-labeled oligonucleotides containing a conventional GRE site (GRE), AAV2 D-sequence (D), or a NS sequence used as probes. Arrows indicate the bound complexes. Competition experiments using various indicated excess amounts of unlabeled oligonucleotides.

capacity of scAAV vectors precludes the use of these vectors for larger genes that exceed the approximately 2.5-kb limit.[42–44] Thus, alternative strategies are warranted to increase the extent of transgene expression from ssAAV vectors carrying larger genes.

In the current study, we describe the identification of a partial sequence homology between the D-sequence in the AAV inverted terminal repeat (ITR) and the halfbinding site of the glucocorticoid receptor-binding element (GRE). This observation prompted us to examine whether AAV2 infection involves the GR signaling pathway. Replacement of the distal 10 nucleotides in the D-sequence of the AAV-ITR with a full-length GRE consensus sequence resulted in significantly enhanced transgene expression from these vectors, termed generation Y (GenY) AAV vectors, in human cells *in vitro* and in a mouse model *in vivo*. The availability of these vectors, which are serotype, transgene, or promoter agnostic, promises to be useful in gene therapy of human diseases caused by aberrations of larger genes that exceed the packiging capacity of scAAV vectors.

RESULTS

Distal 10 nucleotides in the AAV2 D-sequence share partial sequence homology with the GRE 1/2 site

We have previously reported that three cellular proteins, heat shock protein 90 (HSP90),[45] FK506-binding protein (FKBP52),[46] and protein phosphatase 5 (PP5),[47] are involved in modulating transgene expression from recombinant AAV2 vectors. Since the GR is normally present in its inactive hetero-complex form with HSP90, PP5, and FKBP51,[48] is activated by phosphorylation after ligand binding,[49] and activated GR undergoes immunophilin switching by substitution of FKBP51 with FKBP52, enters the nuclear compartment through the FKBP52-dynein network,[50] and binds to the GRE sites (5′-GGTACANNNTGTT/CCT-3′), and the TGTTCT 1/2 site is the essential core element, which is sufficient to relay glucocorticoid signaling.[51] We wished to evaluate whether AAV2 infection also involves GR signaling, since we made a serendipitous observation that the distal 6 nucleotides in the D-sequence, 5′-GGTTCCT-3′, 20 nucleotides in the AAV ITRs, share a partial homology to the consensus GRE 1/2 site. These sequences are illustrated in Figures 1A–1C.

Purified GR protein interacts specifically with the AAV2 D-sequence

To experimentally test the possibility whether purified GR protein could bind to the D-sequence, electrophoretic mobility-shift assays (EMSAs) were carried out using double-stranded D-sequence oligonucleotides (D: 5′-CTCCATCACTAGGGGTTCCT-3′). One of the conventional double-stranded GRE sequences (GRE: 5′-CTAGGCT GTACAGGATGTTCTG CCTAG-3′), and a non-specific (NS) sequence (NS: 5′-TATTAGATCTGATGGCCGCT-3′) were used as appropriate positive and negative controls, respectively. Each of the ^{32}P-labeled oligonucleotide probes was individually incubated with purified GR proteins and analyzed using an EMSA. These results are shown in Figures 1D and 1E. As can be seen, whereas the purified GR protein formed a specific complex with the GRE probe and retarded its mobility (Figure 1D, lane 3), it also formed a similar complex with the D-sequence (Figure 1D, lane 5), but not with the NS probe (Figure 1D, lane 7). Densitometric scanning of autoradiographs revealed that the binding of GR to the conventional GRE probe was slightly more efficient (compare lanes 3 and 5 in Figure 1D) than

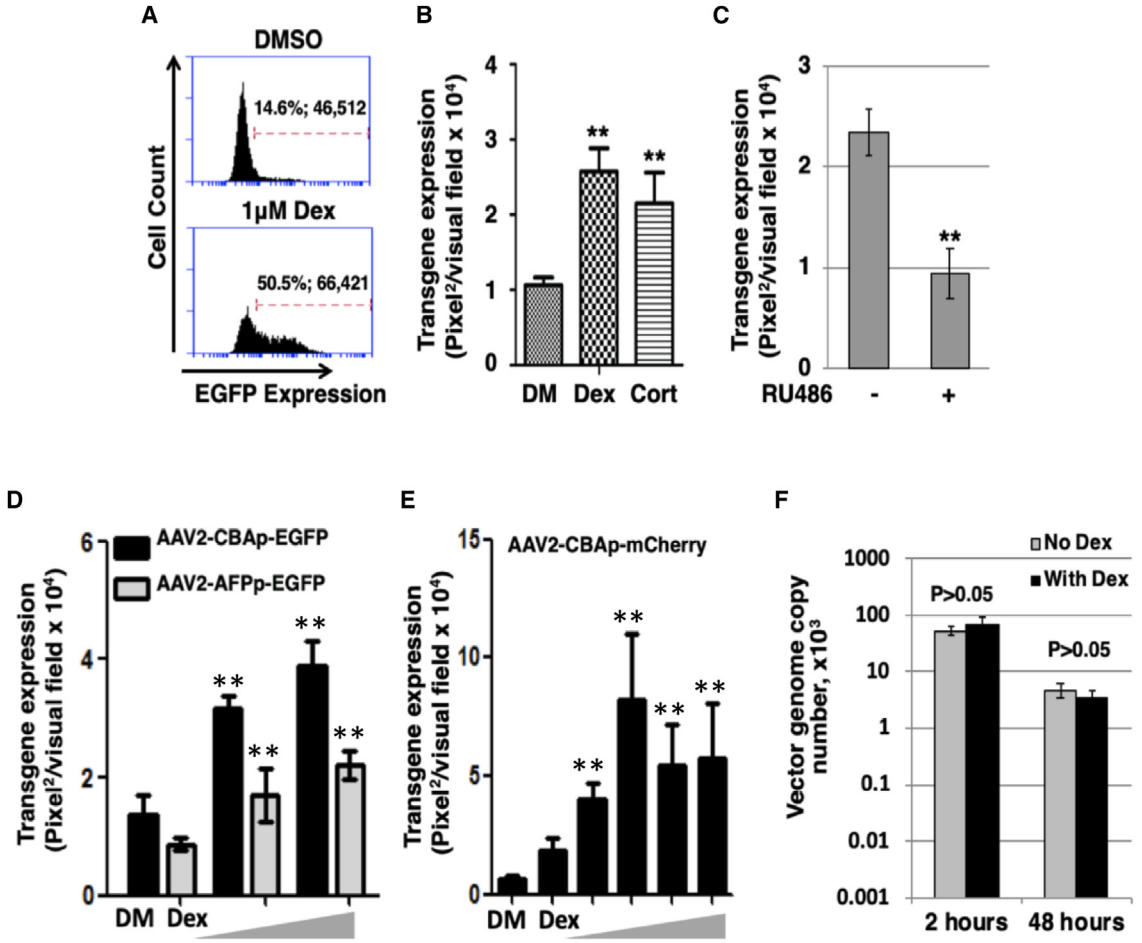

Figure 2. Glucocorticoid-treatment enhances AAV2 vector-mediated transgene expression in human Huh7 cells, and is inhibited by a glucocorticoid antagonist in Huh7 cells

(A–C) Cells were transduced with AAV2-EGFP vectors at 1×10^4 vgs/cell in the presence of either 0.1% DMSO or 1 μM Dex. Transgene expression was determined by flow cytometry 48 h after transduction. Cells were transduced with AAV2-EGFP vectors as above in the presence of either DMSO (DM), or Dex, or cortisol (Cort). Transgene expression was determined by fluorescence microscopy 48 h after transduction. Cells were transduced with AAV2-EGFP vectors as above in the absence or presence of 20 μM RU486, and transgene expression was determined by fluorescence microscopy 48 h after transduction. (D–F) Dex-mediated increased transgene expression from AAV2 vectors is promoter and transgene independent in Huh7 cells. Cells were transduced with 1×10^4 vgs/cell of AAV2-EGFP vectors containing either the CBA promoter, or the human α-fetoprotein (AFP) promoter, in the presence of 0.1% of DM or increasing concentrations of Dex, and transgene expression was determined by fluorescence microscopy 48 h after transduction. Cells were transduced with 1×10^4 vgs/cell of AAV2-CBAp-mCherry vectors in the presence of 0.1% of DM or increasing concentrations of Dex, and transgene expression was determined by fluorescence microscopy 48 h after transduction. Cells were transduced with 1×10^4 vgs/cell of AAV2-CBAp-mCherry vectors as in the absence or presence of 1 μM Dex (**$p < 0.01$). Low-molecular-weight DNA samples were isolated at 2 h and 48 h after transduction, and vg copy numbers were determined by qPCR.

that to the D-sequence probe (data not shown). Correspondingly, the GR binding to the ^{32}P-labeled GRE probe could be competed with a 2- to 50-fold molar excess of unlabeled D-sequence oligonucleotides (Figure 1E, lanes 3–5), and with a 10-fold molar excess of unlabeled GRE sequence oligonucleotides (Figure 1E, lane 7), but not with the unlabeled NS oligonucleotides (Figure 1E, lanes 6). These data establish the specificity of the GR-D-sequence binding, and suggest that the D-sequence potentially functions as a GRE 1/2 site, forms a complex with the GR protein, and influences AAV vector-mediated transgene expression.

Dexamethasone treatment significantly enhanced transgene expression from AAV2 vectors in human cells *in vitro*

Beyond the physical interaction between GR and AAV D-sequence, we next wished to examine whether dexamethasone (Dex), a known activator of the GR pathway, influences the transduction efficiency of AAV2 vectors. Human hepatocarcinoma cells, Huh7, were treated with DMSO or Dex and transduction with AAV2 vectors expressing EGFP driven by chicken β-actin promoter (CBAp). These results are shown in Figure 2A. As can be seen, approximately 3.5-fold increase in transgene expression from AAV2 vectors could be

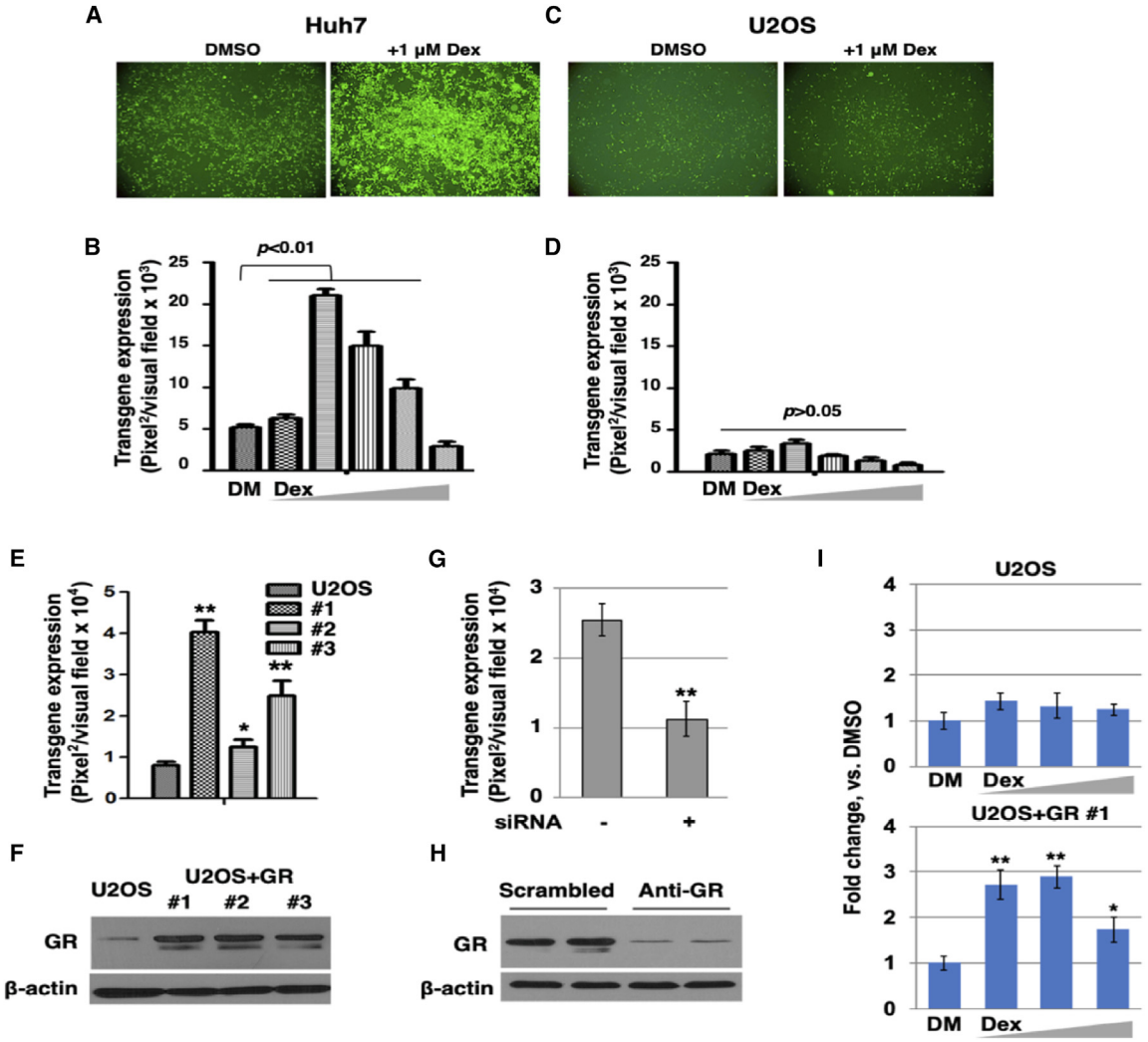

Figure 3. GR is required for Dex-mediated increased transgene expression by AAV2 vectors

(A–D) GR-positive Huh7 cells were transduced with AAV2-EGFP vectors at 1×10^4 vgs/cell cultured in the presence of 0.1% DM or with increasing concentrations of Dex, and transgene expression was determined by fluorescence microscopy 48 h after transduction. Representative images of cells treated with DM and 1 μM Dex are shown. Quantitation of the transgene expression data in Huh7 cells. GR-deficient U2OS cells were transduced with AAV2-EGFP vectors at 1×10^4 vgs/cell cultured in the presence of 0.1% DM or with increasing concentrations of Dex, and transgene expression was determined by fluorescence microscopy 48 h after transduction. Representative images of cells treated with DM and 1 μM Dex are shown. Quantitation of the transgene expression data in U2OS cells. (E–I) Introduction of GR restores AAV2 vector-mediated transgene expression in U2OS cells. U2OS cells were stably transfected with a human GR expression plasmid, and three independently isolated clones were transduced with 1×10^4 vgs/cell of AAV2-EGFP vectors. Transgene expression was determined by fluorescence microscopy 48 h after transduction. U2OS and U2OS + GR cell clone lysates were analyzed on western blots for the presence of GR. Huh7 cells were transfected with either scrambled siRNA (–) or anti-GR siRNA (+) using Lipofectamine 3000, and then transduced with AAV2-EGFP vectors at 1×10^4 vgs/cell. Transgene expression was determined by fluorescence microscopy 48 h after transduction. Western blot analysis of GR expression in Huh7 cells transfected with either scrambled siRNA or anti-GR siRNA performed 24 h after transfection. Parental U2OS cells and U2OS + GR clone #1 cells were transduced with AAV2-EGFP vectors at 1×10^4 vgs/cell, and cultured in the presence of 0.1% DM or with increasing concentrations of Dex, and transgene expression was determined by fluorescence microscopy 48 h after transduction. $^*p < 0.05$; $^{**}p < 0.01$.

achieved following Dex treatment. Dex treatment-mediated increased transgene expression was only seen following ITR-containing plasmid transfection (Figure S1A). Similar results were obtained following treatment with corticosteroids (Figure 2B), as well as with various forms of Dex (Figures S2A, S2B, and 3A). To ascertain the specificity of Dex-mediated activation of GR, and its subsequent effect, we used a drug, RU486, known to inhibit GR activation by competing for the binding sites with its ligand, glucocorticoids.[52] Huh7 cells were treated with RU486, and its effect on the transduction efficiency of AAV2 vectors was evaluated as described above. As can be seen in Figure 2C, RU486 treatment significantly decreased the transduction efficiency of AAV2 vectors, suggesting

that sustained GR activation is required for increased transduction efficiency. Taken together, these results suggest that the GRE-like 1/2 site in the D-sequence is functional and mediates transgene activation.

Dex-mediated enhanced transgene expression from AAV2 vectors is promoter and transgene independent

The classical model for GR function is based on the binding of a hormone-GR complex to the GREs in the regulatory regions of target genes, thereby changing the expression pattern of these genes by up-regulating the expression of anti-inflammatory proteins in the nucleus or repressing the expression of pro-inflammatory proteins in the cytosol, known to be mediated by activation of the GR pathway.[48] Thus, we reasoned that the GRE-like 1/2 site in the D-sequence-mediated increased transgene expression following Dex treatment would be expected to be promoter and transgene independent. This was tested experimentally by using AAV vectors expressing the EGFP reporter gene under the control of either the CBA promoter or the human α-fetoprotein promoter. These results, shown in Figure 2D, document that transgene expression from both promoters was significantly increased after Dex treatment. Similarly, CBA promoter-driven expression of a different reporter gene, mCherry, was also significantly increased after Dex treatment (Figure 2E). It was also important to corroborate that the increased transgene expression was not due to increased uptake of the vectors after Dex treatment. This was tested by determining total vector genome copy numbers (vgs) with and without Dex-treatment at 2 h and 48 h after vector transduction. As can be seen in Figure 2F, at both time points, there were no significant differences in the vgs under either condition.

Dex treatment fails to mediate enhanced transgene expression from AAV2 vectors in GR-deficient human osteosarcoma cells, U2OS

To unequivocally corroborate our contention that GR activation-mediated augmentation of the transduction efficiency of AAV2 vectors is specific, we used a human osteosarcoma cell line, U2OS, which lacks endogenous GR expression.[53] It has previously been reported that U2OS cells are transduced extremely poorly by AAV2 vectors, even at very high multiplicities of infection (MOIs).[54] In our studies, we observed that whereas significantly increased transduction occurred in Huh7 cells after Dex treatment (Figures 3A and 3B), only a modest level of transduction was observed in U2OS cells, and Dex treatment had no effect (Figures 3C and 3D). The possibility that the lower transduction efficiency was due to the lack of expression of cell surface viral receptors in U2OS cells, was tested in experiments in which these cells were treated with heparin, a specific ligand for heparan surface proteoglycan (HSPG), the primary receptor for AAV2,[55] prior to vector transduction. Consistent with previously published studies,[55] treatment with heparin, significantly inhibited AAV2 vector-mediated transduction efficiency in both Huh7 and U2OS cells (data not shown; Figure S3B), suggesting that HSPG functions as the main receptor in U2OS cells as well.

Stable transfection with a GR-expression cassette restores Dex-mediated enhanced transgene expression from AAV2 vectors in U2OS cells

We next wished to examine whether re-introduction of GR into U2OS cells could restore the AAV2 vector transduction efficiency. To this end, U2OS cells were stably transfected with a human GR-expression plasmid, and three independent clones were obtained. The parental U2OS cells as well as those expressing GR (U2OS + GR) cell clones were transduced with AAV2 vectors and the extent of the transgene expression was determined. These results, shown in Figure 3E, clearly show that the transduction efficiency of AAV2 vectors was significantly increased in each of the three U2OS + GR cell clones compared with the parental U2OS cells, which also correlated well with the increased levels of GR expression (Figure 3F). In these experiments, Huh7 cells were included as a positive control, in which transfection with anti-GR small interfering RNA (siRNA) was observed to significantly decrease the transduction efficiency of AAV2 vectors (Figure 3G), which also correlated well with the levels of GR expression in these cells (Figure 3H). Transfection with scrambled siRNA had no effect on transgene expression or on GR protein expression (data not shown). Furthermore, whereas Dex-induced GR activation had no significant effect on vector-mediated transgene expression in the parental U2OS cells (Figure 3I, top), as observed before, the transduction efficiency of AAV2 vectors in a U2OS + GR cell clone was significantly increased after Dex treatment (Figure 3I, bottom). Taken together, these results provide further evidence that GR signaling is involved in AAV2 vector-mediated transgene expression.

AAV vector transduction activates the GR pathway in human cells *in vitro* and in murine hepatocytes *in vivo*

We next wished to examine whether transduction with AAV vectors in and of itself leads to activation of the GR pathway both in human cells *in vitro* and in a mouse model *in vivo*. To this end, two human hepatocellular carcinoma cell lines, Huh7 and HepG2, were either mock-transduced, or transduced with increasing MOIs ranging from 1×10^2 to 1×10^5 vgs/cell. Six hours after transduction, equivalent amounts of cell lysates were analyzed on western blots using S211 antibodies that detect activated forms of GR phosphorylated at serine (S) residues. As is evident in Figure 4A, in both cell types, there was a vector dose-dependent increase in phosphorylated GR, corroborating that transduction with AAV2 vectors leads to activation of the GR pathway. After phosphorylation in the cytoplasm, the activated GR is known to translocate to the nucleus, bind to GREs in upstream regulatory sequences, and induces the expression of anti-inflammatory genes (Figures S1B and S1C). To further examine whether, after transduction with AAV2 vectors, activated GR also translocates to the nucleus, total and cellular, cytoplasmic, and nuclear fractions were analyzed on western blots as described above. Dex-treated cells were used as a positive control. These results, shown in Figures 4C and 4D, further corroborate that both following Dex treatment and following AAV2 vector-mediated

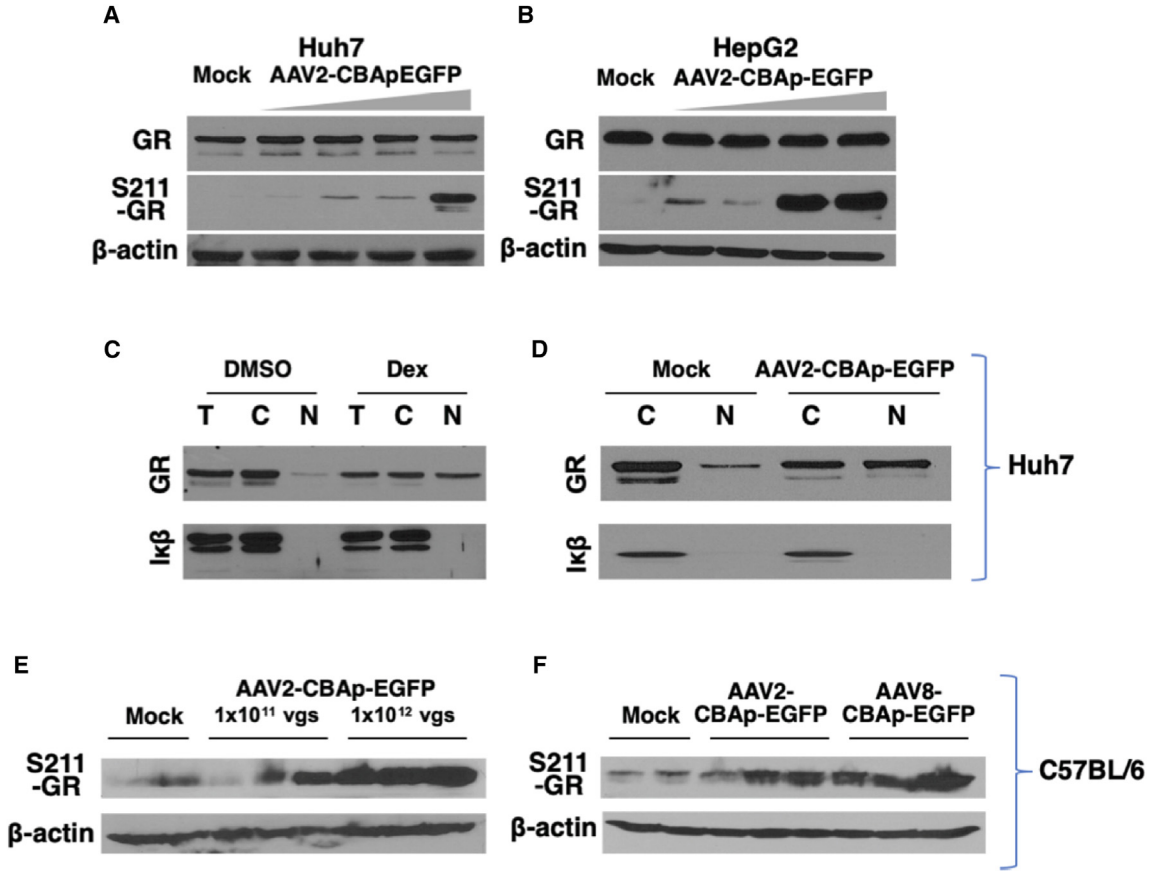

Figure 4. Transduction with AAV vectors activates the GR pathway in human cells *in vitro* and in mouse hepatocytes *in vivo*

(A and B) Huh7 (A) and HepG2 (B) cells, were transduced with increasing amounts AAV2-EGFP vectors, ranging from 1×10^2 vgs/cell to 1×10^5vgs/cell. Western blot assays were performed 6 h after vector transduction using anti-phosphorylated GR antibodies. Mock-transduced cells an anti-β-actin antibodies were used as appropriate controls. (C) Huh7 cells were treated with 0.1% DM or 1 μM Dex, and 6 h after treatments, total (T) and subcellular fractions (C, cytoplasmic; N, nuclear) were isolated and equivalent amounts were analyzed by western blotting. Iκβ was used as cytoplasmic marker. (D) Cytoplasmic and nuclear fractions of mock-transduced and AAV2-EGFP vector-transduced Huh7 cells were also analyzed as described above. (E) C57BL/6 mice were either mock injected or injected with AAV2-EGFP vectors via tail vein at either 1×10^{11} vgs/mouse or 1×10^{12} vgs/mouse ($n = 3$ each). Livers were harvested was 24 h after vector administration, and equivalent amounts of cell lysates were analyzed on western blots as described above. (F) C57BL/6 mice were mock injected or injected with AAV2-EGFP or AAV8-EGFP vectors via tail vein at 1×10^{12} vgs/mouse ($n = 3$ each/group). Livers were harvested was 24 h after vector administrations, and equivalent amounts of cell lysates were analyzed on western blots as described above. β-Actin was used as a loading control.

transduction, the activated GR indeed translocates to the nucleus. Iκβ, a cytoplasmic protein,[56] was used as an appropriate control.

These studies were extended to include similar analyses in a mouse model *in vivo* in which C57BL/6 mice were either mock-injected, or injected with increasing MOIs 1×10^{11} and 1×10^{12} vgs of AAV2-CBA-EGFP vectors via the tail vein ($n = 3$/group). Liver tissues were harvested 24 h after vector administration, and equivalent amounts of tissue lysates were analyzed on western blots using S211 antibodies as described above. As can be seen in Figure 4E, there was a vector dose-dependent increase in phosphorylated GR, corroborating that transduction with AAV2 vectors leads to activation of the GR pathway in mouse hepatocytes *in vivo*. Similar results were observed following intravenous administration of AAV8 vectors as well (Figure 4F).

Replacement of the distal 10 nucleotides in the AAV2 D-sequence with the full-length consensus GRE site leads to significantly enhanced transgene expression in human cells *in vitro* and in murine hepatocytes *in vivo*

We next speculated that by engineering a fully functional GRE sites in the D-sequence in the AAV2-ITR, it might be possible to exploit this feature to generate novel recombinant AAV vectors to achieve improved transgene expression. We have previously reported that the proximal 10-nucleotides in the D-sequence are indispensable, since they serve as the packaging signal for the AAV genome.[57–59] Thus, we replaced the distal 10-nucleotide in the D-sequence with a 15-nucleotide consensus full-length GRE site to generate recombinant AAV2 genome, termed GenY vectors, shown schematically in Figure 5A. WT and GenY AAV2-CMV-hrGFP vectors were used to

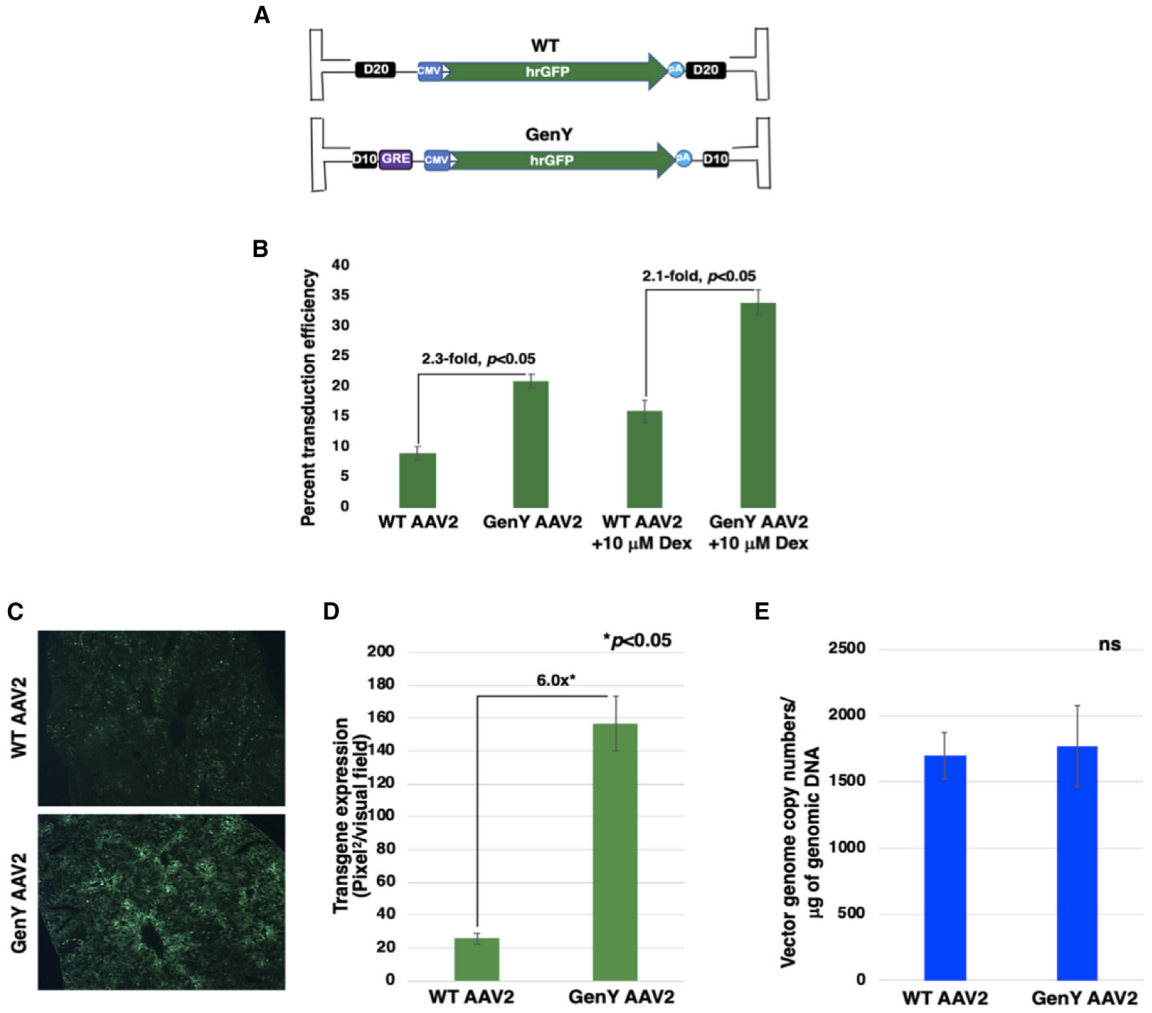

Figure 5. GenY AAV2 vectors mediate increased transgene expression in human cells *in vitro*

(A) Schematic structures of WT and GenY AAV2 vector genomes. (B) HeLa cells with transduced with 1×10^3 vgs/cell of WT- and GenY-AAV2-hrGFP vectors, and in the absence or presence of 10 μM Dex. Transgene expression was determined by flow cytometry 72 h after infection. (C–E) GenY AAV2 vectors mediate increased transgene expression in murine hepatocytes *in vivo*. WT and GenY AAV2-hrGFP vectors were injected at 1×10^{10} vgs/mouse ($n = 6$ each) via the tail vein, and at 4 weeks after vector administrations, livers were harvested and visualized under a fluorescence microscope. Quantitation of the data as determined by ImageJ analysis. *$p < 0.05$. Total genomic DNA samples were isolated from liver tissues, and vg copy numbers were determined by qPCR.

transduce human HeLa cells at 500 vgs/cell and transgene expression was determined using flow cytometry. These results, shown in Figure 5B, document approximately a 2-fold increase in transgene expression from GenY AAV2 vectors, which is further increased by approximately 2-fold after Dex treatment.

The transduction efficiency of WT and GenY AAV2-CMV-hrGFP vectors was also evaluated in a mouse model *in vivo*. To this end, 1×10^{10} vgs of each vector were injected via tail vein in C57BL/6 mice ($n = 6$ each). Four weeks after vector administration, livers were harvested, sectioned, and visualized under a fluorescence microscope (Figure 5C). Quantitation of these data, shown in Figure 5D, documents approximately 6-fold improved transgene expression from GenY AAV2 vectors. The observed increase was not due to increased uptake and delivery of GenY vectors into hepatocytes, since the vgs were not significantly different than that from the WT vectors (Figure 5E).

Insertion of the full-length consensus GRE site in the AAV6 D-sequence leads to significantly enhanced transgene expression from AAV6 vectors in human cells *in vitro* and in murine hepatocytes *in vivo*

During the course of these studies, we also made the observation that the GRE-like 1/2 site is present only in the D-sequence of the AAV2-ITR, and in none of the D-sequences in five other AAV serotype ITRs for which the sequences are available (Figure 6A). It was, therefore, of interest to determine whether insertion of a full GRE

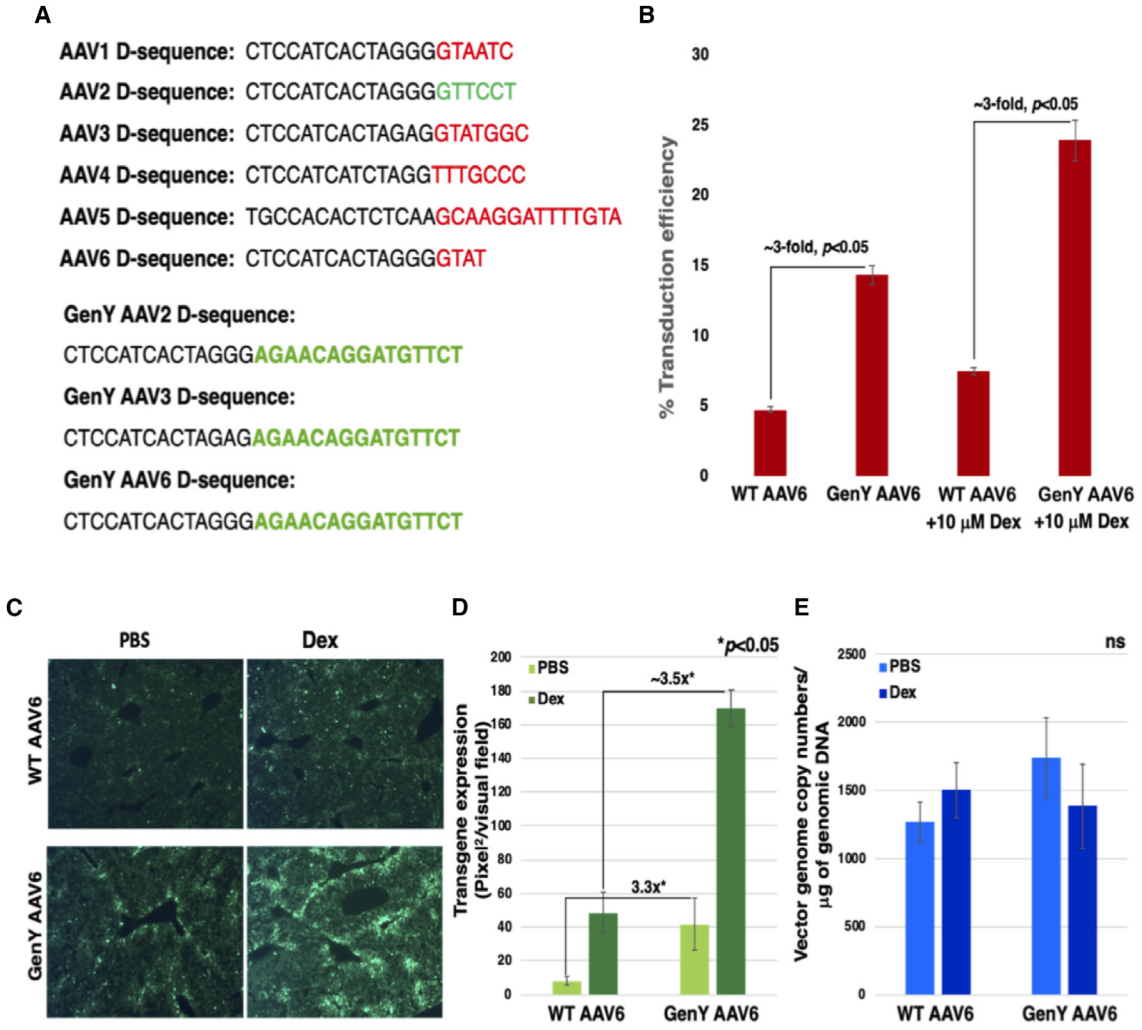

Figure 6. The GRE 1/2 site is present only in the D-sequence of the AAV2-ITR, and insertion of a full-length GRE site in the AAV6-ITR mediates improved transgene expression from AAV6 vectors in HeLa cells *in vitro*

(A–D) Nucleotide sequences of D-sequences in the AAV serotypes 1 through six genomes. The GRE 1/2 site (in green font) is absent in the distal sequences (in red) of the rest of the D-sequences in the AAV serotype genomes. A consensus full-length GRE site was also inserted in the D-sequence of the AAV6-ITR. HeLa cells with transduced with 1×10^3 vgs/cell of WT- and GenY-AAV2-hrGFP vectors, and in the absence or presence of 10 μM Dex. Transgene expression was determined by flow cytometry 72 h after infection. (C–E) GenY AAV6 vectors also mediate increased transgene expression in murine hepatocytes *in vivo*. WT and GenY AAV6-hrGFP vectors were injected at 1×10^{10} vgs/mouse (n = 6 each) via tail-vein. Dex (0.2 mg/mouse/day) or PBS was injected into mice intraperitoneally (i.p.) on 5 consecutive days (days 24–28), as described previously.[77] Four weeks after vector administrations, livers were harvested, sectioned, and visualized under a fluorescence microscope. *$p < 0.05$. Quantitation of the data was performed by ImageJ analysis. Total genomic DNA samples were isolated from liver tissues, and vg copy numbers were determined by qPCR.

site in other D-sequences will also improve transgene expression. Because of our long-term interest in AAV3 and AAV6 vectors for their remarkable tropisms for primary human hepatocytes and hematopoietic stem cells, respectively,[60–63] we also generated GenY AAV3 and GenY AAV6 vectors. Although GenY AAV3 vectors increased the efficiency of transgene expression in human Huh7 cells (data not shown), we were unable to evaluate their efficacy in mice *in vivo* since AAV3 vectors fail to transduce any mouse tissues or organs.[60,61,64] GenY AAV6-CMV-hrGFP vectors were used to transduce human HeLa cells at 500 vgs/cell and transgene expression was determined using flow cytometry. These results, shown in Figure 6B, document an approximately 3-fold increase in transgene expression from GenY AAV6 vectors, which is further increased by approximately 3-fold after Dex treatment.

Similarly, the transduction efficiency of GenY AAV6 vectors was also increased approximately 3-fold compared with that from WT AAV6 vectors in murine hepatocytes *in vivo* (Figure 6C), which was further increased by approximately 3.5-fold after intraperitoneal administration of Dex (Figure 6D). Again, the observed increase was not due to

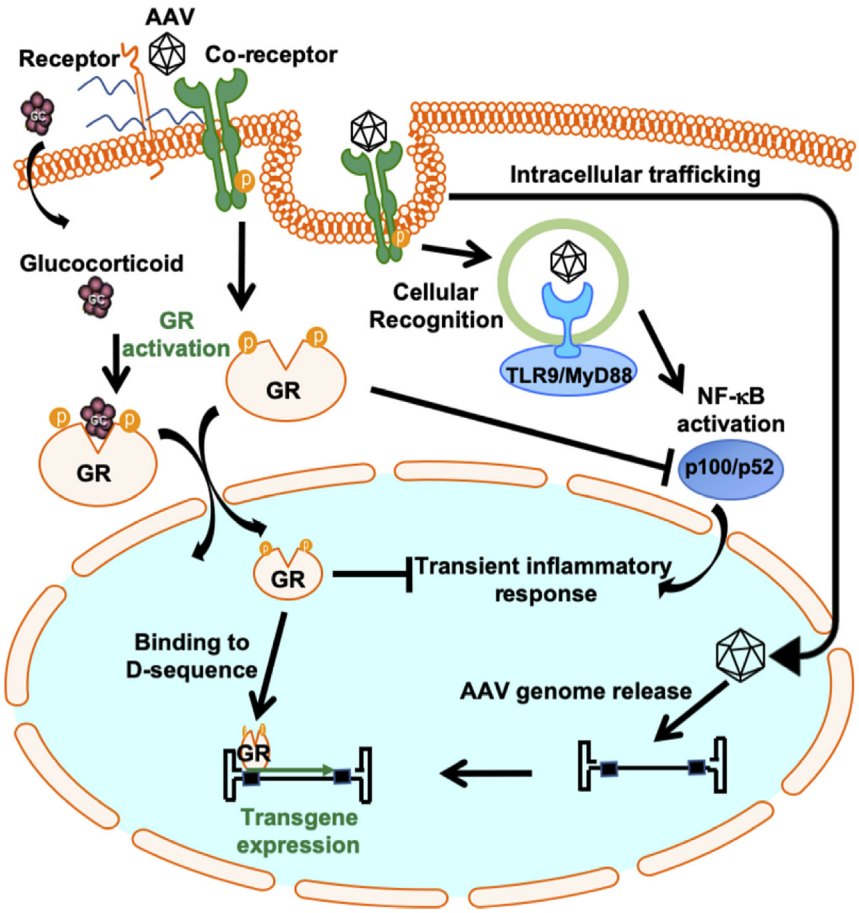

Figure 7. A model for AAV vector-mediated activation of the GR pathway leading to improved transgene expression

Following binding to primary cellular receptors, AAV vectors gain entry into target cell utilizing a cellular co-receptor, which leads to activation of the cellular GR pathway, similar to that induced by treatment with glucocorticoids. Following activation in the cytoplasm, GR translocates to the nucleus, binds to GRE upstream regulatory sequences, and induces expression of anti-inflammatory genes.[48,49] Activated GR also binds to the GRE 1/2 site in the D-sequence of the AAV2-ITR, and increases AAV vector-mediated transgene expression. Replacement of the GRE 1/2 site with a consensus full-length GRE site leads to increased transgene expression from GenY AAV serotype vectors.

increased uptake and delivery of GenY AAV6 vectors into hepatocytes, since the vgs were not significantly different than that from the WT AAV6 vectors (Figure 6E).

DISCUSSION

There is little doubt that AAV evolved as a virus, rather than as a vector for the purposes of delivery of therapeutic genes. The ss nature of the WT AAV genome, flanked by ITRs, is designed to limit transcription of the viral genes. Thus, it has been clear for more than one-quarter of a century that the viral second-strand DNA synthesis is a major rate-limiting step in transgene expression from recombinant AAV vectors *in vitro* as well as *in vivo*,[65–68] since there is no RNA polymerase that can transcribe a ssDNA genome. Although scAAV vectors have been developed that bypass this rate-limiting step,[44] the limited packaging capacity of scAAV vectors precludes therapeutic genes that exceed the approximately 2.5-kb limit. Thus, it has also become increasingly clear that, despite the therapeutic efficacy achieved with the first generation of ssAAV vectors, these vectors are not optimal, and strategies are needed that facilitate vector second-strand DNA synthesis thereby augmenting transgene expression.

For more than three decades, we have pursued a number of strategies to achieve this objective that include the use of pharmacological agents,[47,69,70] enzymatic supplementation,[71–73] and ITR modifications.[74,75] It is during the pursuit of the ITR modifications that we made a serendipitous observation that the D-sequence in the AAV2-ITR shares partial sequence homology with the GRE-like 1/2 site. Despite only partial homology, the GRE-like 1/2 site in the D-sequence turned out to be biologically active, responding to Dex treatment, and leading to improved transgene expression. The observation that transduction by AAV vectors in and of itself leads to activation of the GR pathway, is further corroborated by published reports that Dex administration augments transgene expression from AAV serotype vectors in mice *in vivo*.[76,77] Based on all cumulative data presented in the current studies, we propose a model, which is depicted in Figure 7.

However, a fundamental question remains unanswered: How does AAV infection activate the GR pathway? Although additional studies are warranted to gain insights into the underlying mechanism, the following is a brief account of our working hypothesis based on published data by others. It is known that AAV capsid binds to Toll-like receptor 2 (TLR2) at the cell surface,[78] and TLR2 activation has been shown to increase the phosphorylation of extracellular signal-regulated kinase (ERK).[79] Furthermore, studies have shown that phosphorylation of serine-211 of GR leads to ligand-independent transactivation and nuclear transport, which are impaired during ERK inhibition.[80] Interestingly, in our studies, phosphorylation of serine-211 on GR was increased in the presence of AAV in a dose-dependent manner, both *in vitro* and *in vivo* (Figure 4). Thus, we speculate that AAV infection triggers TLR2-mediated phosphorylation of ERK, which, in turn, phosphorylates GR, leading to its activation, followed by nuclear translocation.

It is also noteworthy that, in two clinical trials with AAV vectors for gene therapy for hemophilia B, a transient increase in clotting factor IX expression was observed in patients who were administered prednisolone to counter capsid-induced immune responses.[81–83] We speculate that this was due to the presence of the GRE-like 1/2 site in the AAV2-ITRs in both AAV8 and AAVS3 vector genomes. Engineering a full-length GRE site in the D-sequence in the AAV2, AAV3, and AAV6 ITRs, it was possible to exploit this feature to generate novel recombinant GenY AAV serotype vectors to achieve significantly improved transgene expression. More recently, we reported that transgene expression from GenY AAVrh74 vectors could also be significantly increased in primary human skeletal muscle cells *in vitro*, as well as in all major muscle tissues in a mouse model *in vivo*.[84] It should be noted that the WT AAVrh74-based vectors (Elevidys) received FDA approval for gene therapy of DMD, but a recent phase 3 clinical trial failed to reach its clinical endpoint. Thus, it is tempting to speculate that the use of GenY vectors might prove to be more efficacious.

Although one fundamental question—why is the GRE-like 1/2 site is present only in the AAV2-ITR—remains unanswered, the presence of a GRE site in the mouse mammary tumor virus long terminal repeat, a retroviral promoter, and the hormonal activation of transcription from this promoter has been described.[85] Thus, it stands to reason that AAV2 vector-mediated transduction, leading to GR activation, is employed to dampen the host cell immune and inflammatory responses by up-regulating the expression of anti-inflammatory proteins in the nucleus or repressing the expression of pro-inflammatory proteins in the cytosol, known to be mediated by activation of the GR pathway.[48] In this context, it is noteworthy that the use of Dex, in combination with bortezomib, a proteasome inhibitor, has been reported to lead to a significant decrease in humoral response to a transgene product expressed from AAV2 and AAV8 vectors,[86] and, more recently, prednisolone has been shown to promote hepatocyte transduction from AAV5 vectors in mice.[87] Thus, we speculate that by engineering a fully functional GRE site in the D-sequence in the AAV2-ITRs, it might be possible to exploit this feature to generate novel recombinant AAV vectors to achieve high-efficiency transgene expression as well as dampened immune response, which has implications in the use of these vectors in human gene therapy. Furthermore, since GR also shares significant homology with other nuclear receptors such as androgen receptors, estrogen receptors, and mineralocorticoid receptors, we have observed that D-sequence also shares a significant homology with the cognate binding elements of these receptors as well (Figure S4). Whether substitution of the D-sequence with these receptor binding elements can be exploited to generate additional hormone-responsive AAV vectors remains an interesting possibility.

In summary, we have documented that the D-sequence in the AAV2-ITR shares partial sequence homology to the GRE site, and that transduction with AAV2 vectors activates the GR pathway. Dex, known to activate the GR pathway, enhances AAV2 vector-mediated transgene expression, which is transgene- and promoter-independent. Replacement of the GRE 1/2 site with the authentic full-length GRE site in AAV2-ITR significantly improves the transduction efficiency of the AAV2 vectors in human cells *in vitro* and in murine hepatocytes *in vivo*. None of the other AAV serotype genomes contains the GRE-like 1/2 site, and insertion of a full-length GRE site in AAV6 D-sequence also leads to increased transgene expression from GenY AAV6 vectors in human cells *in vitro* and in murine hepatocytes *in vivo*. The availability of these GenY AAV serotype vectors has implications for their use in the potential gene therapy of a wide variety of human diseases.

MATERIALS AND METHODS

Cells and cell cultures

Human HEK293, HeLa, Huh7, HepG2, and U2OS cell lines were purchased from the American Type Culture Collection (Old Town Manassas, VA, USA). All cells were maintained in DMEM (Gibco, Waltham, MA, USA) supplemented with 10% fetal bovine serum (Gibco) and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA, USA), as described previously.[81] Cultures were grown in incubators at 37°C and 5% CO_2.

Recombinant AAV vectors

Highly purified stocks of recombinant scAAV2-EGFP, and ssAAV2-hrGFP vectors expressing the reporter genes under the control of a CBAp and cytomegalovirus promoters, respectively, were generated using the triple-transfection method using polyethyleneimine50 (linear, MW 25000; Polysciences Inc., Warrington, PA, USA) followed by purification by iodixanol (Sigma, St. Louis, MO, USA) gradient ultracentrifugation, and ion-exchange chromatography using HiTrap SP/Q HP columns (GE Healthcare, Piscataway, NJ, USA). Vector titers were determined by using a Bio-Rad CFX96 qPCR system as described previously[51–53] Vectors used for *in vivo* experiments were purchased from PackGene (PackGene Biotech, Worcester, MA, USA).

EMSAs

EMSAs were performed essentially as described previously.[45,70] Briefly, ^{32}P-labeled oligonucleotides containing a conventional GRE site (GRE), AAV2 D-sequence (D), and a NS sequence were used as probes and incubated individually with the purified GR protein (Sigma) for 20 min at 25°C and electrophoresed on 6% polyacrylamide gels using 0.5× TBE buffer (pH 8.0) containing 89 mM Tris, 89 mM boric acid, and 1 mM EDTA. Gels were dried *in vacuuo* and subjected to autoradiography using X-ray film (Kodak, Rochester, NY, USA).

AAV vector transduction assay

Cells were seeded in 12-well plates at 1×10^5 cells per well and incubated at 37°C overnight. Growth medium was removed and replaced with serum-free medium containing AAV vectors or mock treatment at MOIs ranging from 5×10^2 to 1×10^4 vgs/cell in triplicates following incubation for 2 h at 37°C. Treatment with various corticoids were performed in triplicates and incubated for 48–72 h. Wells were washed with PBS and imaged with a Leica fluorescence microscope. Total fluorescence was calculated as integral density of GFP fluorescence or arbitrary fluorescence units using NIH ImageJ

software. GFP expression was also determined using flow cytometry (Accuri C6, Beckton Dickinson, Franklin Lakes, NJ, USA), followed by processing with software FCS Express 6 Flow.

qPCR assays

Extrachromosomal DNA, chromosomal DNA, and cDNA samples, and standards were serially diluted and subjected to quantitative PCR utilizing PowerUpTM SybrTM Green 2× Mix (Thermo Fischer Scientific, Waltham, MA, USA), qPCR primers (EuroFins, Luxembourg), and C1000 CFX96 Real-Time System (Bio-Rad, Hercules, CA, USA). Standards were linearized plasmids for the respective gene cassette of known masses as determined by NanoDrop Lite (Thermo Fischer Scientific). Quantitation analysis was conducted using Microsoft Excel (Redmond, WA, USA) to generate standard curves and determine vgs.

Western blotting assay

Huh cells, with and without vector transductions or other treatments, were harvested using RIPA Lysis Buffer (G-Bioscience, St. Louis, MO, USA). Cell lysates were centrifuged at 13,000×*g* for 15 min at 4°C. Protein concentrations were determined using a Pierce bicinchoninic acid (Thermo Fisher Scientific) protein assay kit. Equivalent amounts were electrophoresed on 12% polyacrylamide gels and transferred onto nitrocellulose membranes. Membrane was blocked for 2 h at room temperature using a 5% OmniBlok (AmericanBio, Canton, MA, USA) solution and incubated with anti-GR, or β-actin, or Iκβ antibodies, and incubated overnight at 4°C. The membranes were then washed with tris-buffered saline with Tween 20 (TBST) three times before adding goat anti-rabbit secondary antibodies (American Research Products, Waltham, MA, USA). Membranes were washed three times with TBST and visualized using Thermo Fisher Scientific Chemiluminescent Assay kit.

Animal experiments

Male C57BL/6 mice (6–8 weeks old) were purchased from the Jackson Laboratory and maintained in the University of Florida Animal Care Facility. All experimental protocols involving animals were approved by the Institutional Animal Care and Use Committee. Approximately 1×10^{10} to 1×10^{12} vgs of AAV2, or AAV6, or AAV8 CBA-EGFP vectors were injected in a total volume of 100 μL by tail vein (n = 3–6 mice per group). At 24 hours to 4 weeks after vector administrations, liver tissues were harvested for western blot analyses, or sectioned for transgene expression analyses using a fluorescence microscope. In some experiments, Dex was administered intraperitoneally following vector administrations as described in the legend to Figure 6. The vgs were determined by qPCR using total genomic DNA isolated from liver tissues as described above.

Statistical analysis

Results are presented as means ± SD. Unpaired Student's t test was performed for statistical analysis, and p values of less than 0.05 were considered statistically significant. Statistical significances are indicated as $^{*}p < 0.05$ and $^{**}p < 0.01$.

DATA AND CODE AVAILABILITY

All data will be made available upon reasonable request.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2024.102196.

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AUTHOR CONTRIBUTIONS

Y.L., C.L., J.S., H.Y., A.N., G.D.K., and K.Q. designed and performed the experiments. Y.L., C.L., J.S., H.Y., A.N., G.D.K., K.Q., and A.S. analyzed the data. Y.L., C.L., and A.S. conceived of the idea. Y.L., C.L., and A.S. wrote the manuscript, and all authors read and approved the final version.

DECLARATION OF INTERESTS

A.S. is an inventor on several issued and filed patents on recombinant AAV vectors that have been or are being licensed to various AAV gene therapy companies. All other authors declare that they have no competing interests.

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