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RESEARCH ARTICLE

Molecular design of controllable recombinant adeno-associated virus (AAV) expression systems for enhanced vector production

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Abstract

Recombinant adeno-associated virus (rAAV) is the leading vector for the delivery of gene therapies. However, low viral genome (VG) titers are common and the proportion of "full" capsids containing the therapeutic gene payload can be highly variable. The coordinated molecular design of plasmids encoding viral components and Helper functions remains a major challenge for rAAV manufacturing. Here we present the design of improved Rep/Cap and Helper plasmids for rAAV2/8 production, (i) a Rep/Cap expression vector harboring independently controllable *rep* and *cap* genes and (ii) an improved Helper plasmid harboring E4 gene deletion variants. First, an optimized Rep/Cap vector utilized a truncated p5 promoter, a p5 *cis*-regulatory element at the 3′ end in combination with a heterologous promoter to drive Cap expression and an additional copy of the *rep52/40* gene to overexpress short Rep proteins. We demonstrate that Rep78 is essential for efficient rAAV2/8 production in HEK293 cells, and a higher ratio of short Rep to long Rep proteins enhances genome packaging. Second, we identified regulators and open reading frames within the Helper plasmid that contribute to increased rAAV2/8 production. While L4-33k/22k is integral to optimal production, the use of E4orf6–6/7 subset significantly enhanced VG titer. Together, an optimal combination of engineered Rep/Cap and Helper plasmid variants increased VG titer by 3.1-fold. This study demonstrates that configuring and controlling the expression of the different AAV genetic elements contributes toward high rAAV production and product quality (full/empty capsid ratio).

KEYWORDS
adeno-associated virus, HEK293 cells, promoter, vector design, viral vector production

Abbreviations: Cap, capsid; DBP, DNA binding protein; ddPCR, digital droplet PCR; DoE, design-of-experiment; HEK, human embryonic kidney; ORF/orf, open reading frame; rAAV, recombinant adeno-associated virus; Rep, replication; TF, transcription factor; VCD, viable cell density; VG, viral genome.

Yusuf B. Johari and Thilo H. Pohle contributed equally to this work.

1 | INTRODUCTION

Despite the evidence of notable clinical efficacy, the high cost of manufacture and vector dosages required has limited the economic viability of recombinant adeno-associated virus (rAAV) mediated gene therapies.[1,2] Three plasmid transient transfection of HEK293 cells is currently the most widely utilized method for producing rAAV, where one of the main challenges in creating high-yielding AAV expression systems and generating AAV packaging cell lines is the E1A-mediated transactivation of promoters p5 and p19.[3] The former promoter controls the gene expression of AAV replication proteins Rep78/68 that are known to be cytostatic/cytotoxic[4,5] but are also required for transactivation of promoters p19 (transcribing Rep52/40) and p40 (transcribing capsid (Cap)).[6] On the other hand, overexpression of Cap has been shown to be advantageous for the optimal production of rAAV.[7,8] To this end, Rep78/68 (large Rep), Rep52/40 (small Rep), and Cap expression have to be regulated independently. Furthermore, Rep78 represses adenovirus Helper promoters E1A, E2A, and E4 and therefore needs to be controlled tightly during rAAV production and cell growth.[9] Engineering AAV expression vectors for improved manufacturability using mammalian cell factories remains a highly desirable objective.

In its natural context, AAVs achieve a precise expression stoichiometry of multiple genes within a compact genome (4.7 kb) using a combination of internal (within open-reading frame (ORF/orf)) promoters, overlapping ORFs, differential mRNA splicing, alternative translation start sites (with varying initiation rates) and feedback loops (using transactivators or repressors).[3] Various vector engineering strategies have been utilized to improve rAAV expression in cell hosts. Examples include the use of inducible promoters,[4] intron insertion,[10] Kozak/start codon mutations[11,12] and a four-plasmid system[13] to modulate the *rep* and *cap* gene expression, as well as a hybrid Rep to improve genome packaging efficiency.[14] Further efforts to boost rAAV yields targeted the Helper plasmid via utilization of human bocavirus 1 Helper genes[15] or design-of-experiment (DoE) approach to optimize the Helper, packaging, and transgene plasmid ratios.[16] Despite these improvements, there remain limited reports on the impact of individual (sub)components that need to be considered when designing an AAV vector and how they can be controlled and enhanced.

In this study, we identify the components and regulators of rAAV2/8 transient expression in HEK293 cells by mechanistically dissecting the packaging and Helper plasmids. We systematically determined the impact of p5 *cis*-regulatory element, endogenous and heterologous promoters, introns, removal of ORFs, and up/downregulation of specific genes on rAAV product titer. Using optimized split Rep/Cap and Helper plasmids, we further demonstrate that it is possible to control genome titer and product quality (full/empty capsid ratio) in a transient rAAV expression system.

2 | MATERIALS AND METHODS

2.1 | Plasmid construction

Proprietary Rep/Cap (pAAV2/8) and Helper (Helper 1.0; Figure S1) plasmids were provided by REGENXBIO. The Rep/Cap and Helper plasmid variants were constructed by PCR amplification (Q5 High-Fidelity 2× Master Mix; NEB), site-directed mutagenesis (Q5 Site-Directed Mutagenesis kit; NEB), and/or gene synthesis (Eurofins Genomics). PCR products were purified using QIAquick PCR Purification kit (Qiagen), and gel extraction was performed using QIAquick Gel Extraction kit (Qiagen). Restriction enzymes were obtained from NEB. Ligation was performed using T4 DNA ligase (NEB), and assembly of multiple DNA fragments was performed using NEBuilder HiFi DNA Assembly Master Mix (NEB). The sequence regions of the relevant promoters are detailed in Table S1. The sequence mutations are detailed in Table S2. Rep/Cap plasmids were amplified in DH5α competent cells (Thermo Fisher), and Helper plasmids were amplified in NEB Stable competent cells (NEB). Clonally derived plasmids were purified using QIAprep Spin Miniprep kit (Qiagen) or QIAGEN Plasmid Plus kit (Qiagen). The sequence of all plasmid constructs was confirmed by restriction enzyme analysis and DNA sequencing (Eurofins Genomics).

2.2 | HEK293 cultures

Suspension-adapted HEK293 cells were provided by REGENXBIO and cultured in Dynamis medium (Thermo Fisher) supplemented with L-glutamine (Thermo Fisher). Cells were maintained in Erlenmeyer flasks (Corning) at 37°C, 140 rpm under 5% CO_2, 85% humidity, and were subcultured every 3–4 days by seeding at 3×10^5 viable cells/mL. Cell viability and viable cell density (VCD) were measured using a Vi-CELL XR (Beckman Coulter).

2.3 | PEI-mediated transient vector production

rAAV2/8 production was performed by triple transfection and in shallow-well 24-well plates (Corning) using the Deutz system as previously described.[17,18] Briefly, cells were subcultured in an Erlenmeyer flask and grown to 4×10^6 cells/mL. Prior to transfection, aliquots of 700 µL were added to each well of 24-well plate. Plasmid DNA (weight ratio of 1:2:0.1 for packaging, Helper, transgene plasmids)[18] and PEIpro (Polyplus-transfection) were each prediluted in Dynamis medium, combined and incubated at room temperature for 10 min before being added into the culture. Transfected cells were cultured for 72 h at 37°C, 230 rpm under 5% CO_2, 85% humidity.

2.4 | Quantification of viral genome titer by ddPCR

Intra and extracellular rAAV2/8 titer was quantified by digital droplet PCR (ddPCR) as previously described.[17] Briefly, 10× cell lysis buffer containing 1× cOmplete EDTA-free Protease Inhibitor Cocktail (Roche) was added to cell culture and incubated at 37°C for 1 h. Samples were centrifuged to remove cell debris and the supernatant was treated with DNase I (Roche), followed by dilution in GeneAmp PCR Buffer I (Thermo Fisher) containing 0.02% UltraPure Salmon Sperm DNA Solution (Thermo Fisher) and 0.1% Pluronic F-68 Nonionic Surfactant (Thermo Fisher). Viral genome (VG) titer was quantified using QX200 Droplet Digital PCR system (Bio-Rad) and primers and a probe (Table S3) targeting the poly A sequence of the transgene plasmid harboring a CAG promoter and a GFP gene. The absolute VG titer was determined using the Quantasoft analysis software (Bio-Rad).

2.5 | Measurement of recombinant mRNA copy numbers

3×10^6 viable cells were collected at 72 h post-transfection by centrifugation at 300 × *g* for 5 min. Cell pellets were resuspended in 150 µL of RNAlater (Sigma-Aldrich). Total RNA was extracted using RNeasy Plus Mini kit in combination with QIAshredder homogenizer (Qiagen) according to the manufacturer's instructions. gDNA-free RNA was converted to cDNA and quantified using One-Step RT-ddPCR Advanced Kit (Bio-Rad) and QX200 Droplet Digital PCR system (Bio-Rad) according to the manufacturer's instructions. Primers and probes used are detailed in Table S3. mRNA copy number was determined using the Quantasoft analysis software (Bio-Rad).

2.6 | Measurement of intracellular proteins by Western blotting

Cells were harvested at 72 h post-transfection by centrifugation at 300 × *g* for 5 min and lysed using RIPA buffer supplemented with Halt Protease Inhibitor Cocktail (Thermo Fisher) according to the manufacturer's instructions. Protein concentration of cell lysates was determined by BCA assay (Pierce) and SDS-PAGE was performed using 10% Novex Tris–Glycine gels (Thermo Fisher) loaded with ≈40 µg and ≈10 µg of lysate for Rep and Cap Western blot, respectively. For Rep, proteins were transferred to nitrocellulose membranes using the miniblot module system (Thermo Fisher), blocked with 5% (w/v) milk-PBST for 1 h at room temperature, and then probed using the anti-AAV2 replicase antibody (1:200; 303.9, Progen) in 2% (w/v) milk-PBST at 4°C overnight, followed by anti-mouse IgG HRP antibody (1:2,000; 7076, CST) in 2% (w/v) milk-PBST for 1 h at room temperature. For Cap, proteins were transferred to PVDF membranes, blocked with 5% (w/v) BSA-PBST (Sigma-Aldrich) for 1 h at room temperature, and then probed using the anti-AAV VP1/VP2/VP3 antibody (1:200; B1, Progen) in 2% (w/v) BSA-PBST at 4°C overnight, followed by anti-mouse IgG HRP antibody (1:2,000) in 2% (w/v) BSA-PBST. The anti-vinculin HRP antibody (1:2,000; E1E9V, CST) was used similarly to the other primary antibodies. Membranes were exposed to ECL substrate (Pierce) for imaging by iBright CL1500 (Thermo Fisher).

2.7 | Quantification of intact capsid titer

Total capsid titer quantification was performed using the AAV8 titration ELISA (Progen) according to the manufacturer's instructions. Total cell lysis supernatant diluted in 1× ASSB assay buffer (Progen). OD values at 450 and 650 nm (background absorbance) were measured using a SpectraMax iD5 microplate reader (Molecular Devices).

2.8 | Statistics

Microsoft Excel 2016 (Microsoft) was used to analyze the difference between the means (normalized titers or intact capsids) of a plasmid construct and the control. As there were multiple batches of transfection, titers were normalized to the mean of control from the same batch to correct for possible differences in cell number and growth. Analysis was performed using an unpaired Student's *t*-test with p-value < 0.05 was considered significant.

3 | RESULTS

3.1 | A heterologous promoter and inclusion of Cap intron enable a controllable Rep/Cap plasmid system

Previous studies showed that unregulated overexpression of Rep78/68 inhibited rAAV production, while reduced levels of Rep78/68 enhanced rAAV titers.[12,19–21] Further, promoter p5 acts a *cis*-regulatory element where its deletion was shown to cause downregulation of promoters p19 and p40.[6,22,23] Our standard Rep/Cap plasmid comprised two modifications; (i) truncation of the p5 promoter to attenuate expression of the large Rep proteins, and (ii) introduction of a p5 promoter downstream of the AAV *cap* region to retain the expression of p19 and p40 (Figure 1A).[24] As shown in Figure 1B, the improved Rep/Cap plasmid (Rep/Cap 1.2; Control) displayed a 32% increase in rAAV2/8 titer over the conventional packaging plasmid ($p < 0.01$), reaching 10^{14} VG/L in serum-free media. We note that triple transfection was performed at a weight ratio of 1:2:0.1 (packaging, Helper, transgene plasmids).[18] While it is relatively easy to regulate *rep78/68*, control of *rep52/40* and *cap* expression would involve the complex multimeric gene assembly in which the p19 and p40 promoters are located within the *rep* coding sequences.

In order to enable control of *cap* gene expression, we split the *rep* and *cap* genes by cloning the p40 promoter and *cap* open reading frame (ORF) downstream of the *rep* gene stop codons (Figure 1C). To prevent expression of truncated viral gene products, the TATA box and Initiator (Inr) of the internal p40 promoter as well as the start codon

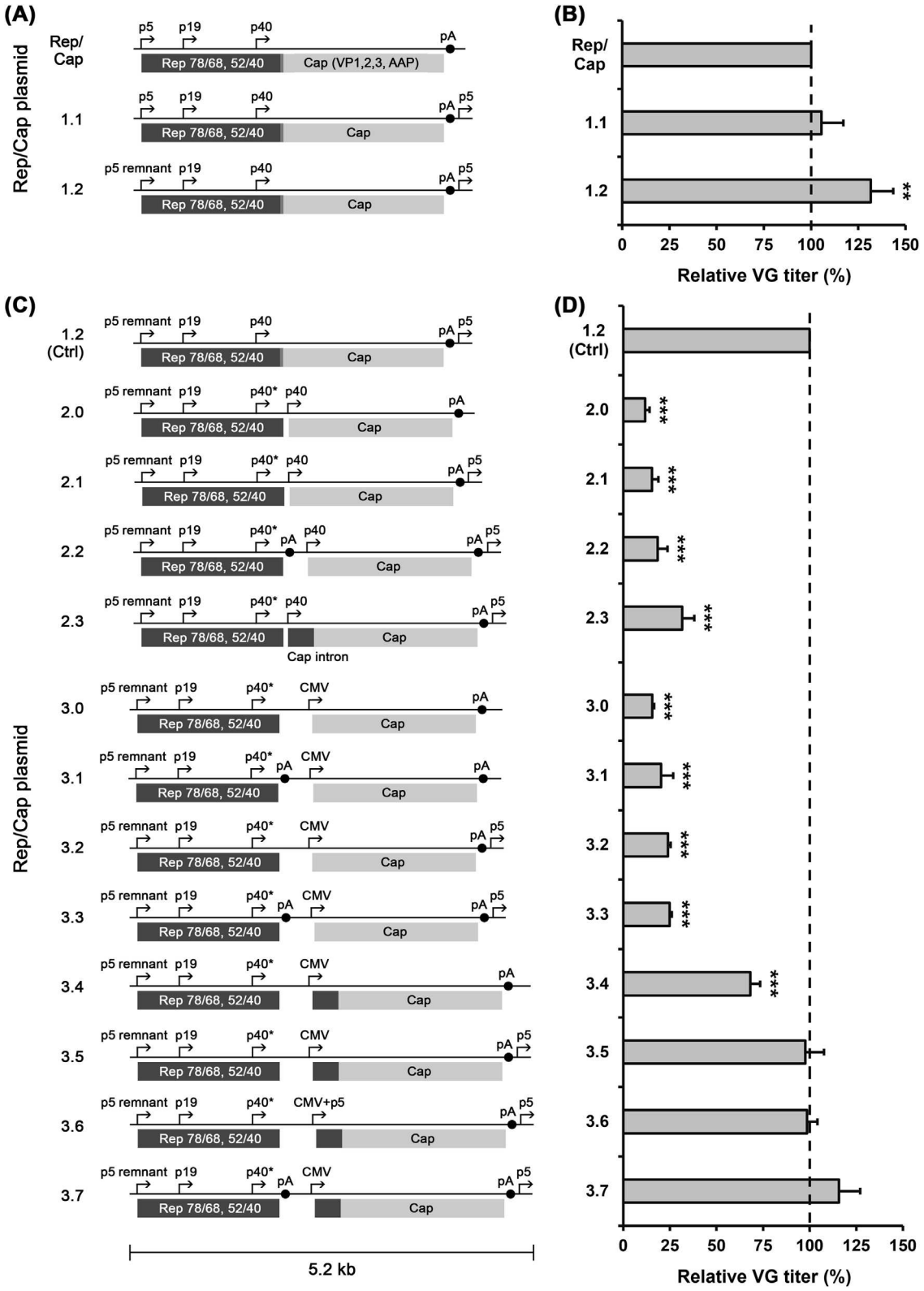

FIGURE 1 Functional evaluation of the p5 *cis*-regulatory element and split Rep/Cap plasmid constructs for rAAV2/8 production. (A, C) Schematic depiction of Rep/Cap plasmid constructs. Replication (Rep) and capsid (Cap) open reading frames are indicated. Arrow denotes a promoter, asterisk denotes sequence mutation, black circle denotes a poly A. All components are drawn to approximate scale. (B, D) HEK293 cells were triple transfected with each Rep/Cap, Helper and transgene plasmid at 1:2:0.1 weight ratio. rAAV2/8 crude viral genome (VG) titers were analyzed 72 h post-transfection, expressed as a percentage compared to the conventional Rep/Cap plasmid (B) or Rep/Cap 1.2 (D). Data shown are the mean ± SD of three independent transfections. Data were analyzed using unpaired Student's *t*-test with respect to the conventional Rep/Cap plasmid or Rep/Cap 1.2. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

of the internal *cap* remnant were inactivated by mutations while preserving the functionality of the Rep proteins (i.e., without altering the encoded amino acid; Table S2). Measurement of rAAV2/8 titer at 72 h post-transfection is shown in Figure 1D. Separation of the *rep* and *cap* genes, either without or with a downstream p5 promoter (Rep/Cap 2.0 and 2.1, respectively) dramatically reduced the rAAV titer to <15% compared to the control. The addition of a poly A for the *rep* ORFs (Rep/Cap 2.2) for independent termination of transcription did not result in a noticeable increase in rAAV titer. While the inclusion of *cap* intron (Rep/Cap 2.3) for efficient post-transcriptional processes increased the titer by two-fold compared to Rep/Cap 2.1 (indicative of a critical element), it was only one-third of the control titer. Accordingly, we surmised that the transcription factor (TF) binding sites within the upstream (inactivated) p40 promoter acted as competing binding elements[25] resulting in reduced *cap* transcriptional activity.

In order to evaluate whether the upstream p40 promoter corresponded to "TF decoy sites," we replaced the downstream p40 with a heterologous promoter, the human CMV.[26] As shown in Figure 1D, employing the CMV promoter to drive *cap* expression without its intron (Rep/Cap 3.0–3.3) resulted in slight increases in rAAV titer compared to the Rep/Cap 2.0–2.2 constructs, with Rep/Cap 3.3 exhibiting 25% of the control titer. The inclusion of the *cap* intron (Rep/Cap 3.4) significantly increased the rAAV production to 70% of the control titer, and the addition of a p5 promoter downstream (Rep/Cap 3.5) restored the titer to the control level—consistent with the enhancer function associated with p5.[22,23] While the addition of a second copy of p5 *cis*-regulatory element directly upstream of the CMV promoter (Rep/Cap 3.6) did not increase the titer relative to the Rep/Cap control, introducing a poly A downstream of the *rep* gene (Rep/Cap 3.7) further enhanced the rAAV yield to 116% (albeit statistically insignificant, $p = 0.075$). ddPCR and Western blot analyses on Rep/Cap 3.7 at 72 h post-transfection demonstrated that *cap* expression was upregulated, with similar VP1–3 stoichiometry compared to the Rep/Cap 1.2 control (Figure 2A,B). The analyses also showed that the codon mutations in the split *cap* system indirectly attenuated long Rep expression at the post-transcriptional level (Figure 2C,D). Combining all observations made above, we inferred that (i) a heterologous promoter is required to drive efficient transcription of the split *cap* gene, (ii) the *cap* intron is a key regulator of Cap expression, and (iii) a poly A can be used to enable independent control of the *rep* genes for improved rAAV production. The split Rep/Cap system will permit attunement of the expression level of both the Rep proteins and the capsids to increase production of rAAV for use in gene therapy, for example, through modification of promoters and Kozak sequences, rearrangement of the genes, as well as codon (de)optimization.

3.2 | Rep78 protein is essential while higher ratio of Rep52/40 to Rep78/68 proteins enhances rAAV2/8 production

Previous studies have suggested that the functions of Rep78 and Rep68 are the same, as reported for Rep52 and Rep40.[27,28] Further, Rep52/40 proteins (in contrast to Rep78/68) were found not to inhibit the growth of primary, transformed, and immortalized cells.[29] Therefore, we hypothesized that rAAV production can be increased via (i) complete ablation of highly cytotoxic replication protein Rep78, and (ii) overexpression of Rep52/40 to enhance the packaging and accumulation of single-stranded viral genome[30] without inducing cytotoxicity. With regard to the latter, the constraint in regulating p19 is due to the position of this promoter which is located within the protein-coding sequence of Rep78/68. To illustrate this, we mutated the weak Kozak sequence (TACATGG, start codon underlined) of Rep/Cap 3.5 to promote the short Rep expression (see Figure S2). Measurement of rAAV2/8 titer after transient transfection of HEK293 cells showed that mutating the TAC (tyrosine) to ATC (isoleucine) within the Kozak diminished the rAAV production by ≈1000-fold (Rep/Cap 4.0; Figure 3). As all Rep/Cap mRNAs and proteins were expressed as expected (Figure 2C,D), the data implies a loss in Rep78/68 functionality.

To test the hypothesis that rAAV2/8 can be produced in HEK293 cells using only one large Rep protein and one small Rep protein, we modified the Rep/Cap 3.5 plasmid (Figure 1C) with deleted alternate splice sites within the *rep* codons to produce only Rep68 and Rep40 proteins (Rep/Cap 4.1; Figure 3A) according to Emmerling et al.[13] Subsequent Western blotting confirmed that only Rep68 and Rep40 proteins were present (Figure 2D). As shown in Figure 3B, the removal of *rep78* and *rep52* markedly reduced rAAV titer by 73% compared to the Rep/Cap 1.2 control. We observed no significant differences in cell viability or VCD between Rep/Cap 3.5 (or 3.7) and Rep/Cap 4.1–4.4 constructs (data not shown) although this was not entirely unexpected due to the very low level of Rep78 protein using the split *cap* system (see Figure 1). To determine whether the absence of rep52 was responsible for the titer reduction, we introduced a rep52/40 gene downstream of cap (Rep/Cap 4.2). To further overexpress the short Rep proteins, we added a second copy of p5 (*cis*-regulatory element for the p19 promoter) upstream of the CMV (Rep/Cap 4.3), or substituted the p19 with p5 promoter and mutated the weak Kozak sequence (Rep/Cap 4.4). We note that the p5 promoter is approximately twice as active as the p19 promoter (Figure S2), while ddPCR and Western blotting confirmed that Rep52 was reintroduced (Figure 2C,D). The results showed that neither reintroduction of *rep52* nor attempts to augment Rep52/40 expression resulted in noticeable improvements in rAAV titer compared to Rep/Cap 4.1. To understand the impact of Rep78 and Rep52 removal on rAAV production, we measured the AAV-associated mRNA and protein levels in Rep/Cap 4.1 and 4.4 (Figure 2). These analyses revealed that while the Cap mRNA levels were comparable to Rep/Cap 3.7, VP expression appeared to be dependent on Rep78 and Rep52 where the former has been reported to augment the splicing of Cap premRNA.[31] Additional studies are needed to determine the mechanism responsible for the reduction of VP expression.

Based on the above observations, we created a library of Rep/Cap plasmids with all four *rep* coding regions including an additional copy of *rep52/40* downstream of *cap* to enhance the packaging and accumulation of single-stranded viral genome. The second copy of *rep52/40* was driven by either promoter p19 or p5 and with or without a mutated Kozak sequence (Rep/Cap 5.0–5.4; Figure 3A). ddPCR and

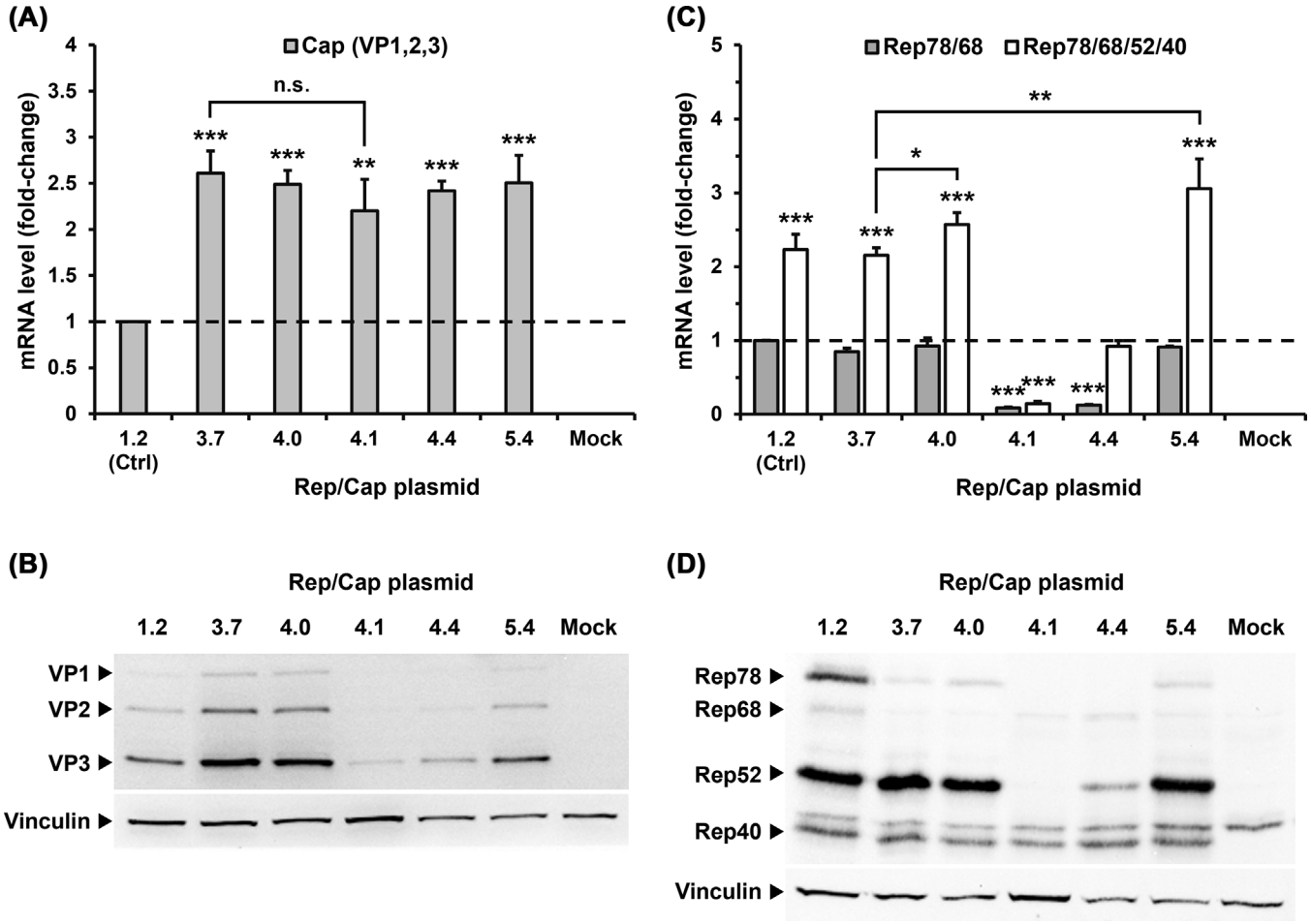

FIGURE 2 Comparative analysis of Rep/Cap mRNAs and proteins during rAAV2/8 production using the split Rep/Cap plasmid. Cells triple-transfected with a subset of Rep/Cap plasmids in Figures 1 and 3, or mock-transfected with no plasmid were harvested at 72 h post-transfection. (A) Cap (VP1, VP2, and VP3) and (C) Rep mRNA transcript levels were analyzed by ddPCR, expressed as a fold-change compared to the Cap or Rep78/68 mRNA of Rep/Cap 1.2 control. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, n.s. not significant. (B) Representative immunoblots of VP and (D) Rep proteins in rAAV-producing cells transfected with the different Rep/Cap plasmids. Vinculin was used as an internal standard.

Western blot analyses indicate that Rep/Cap 5.4 had increased short Rep expression levels compared to Rep/Cap 3.7 (Figure 2C,D). As shown in Figure 3B, analysis of rAAV titer confirmed that p5 promoter at the 3′ end was critical for maximal titers (see Rep/Cap 5.1 and 5.2 vs. 5.0). Importantly, the data demonstrated that the reintroduction of *rep78* reinstated the rAAV production system with two constructs (Rep/Cap 5.3 and 5.4) exceeded the Rep/Cap 1.2 control titer (≈118%; $p < 0.05$). No further increase in rAAV titer was observed when a poly A was added between the long *rep* and *cap* genes (Rep/Cap 5.3 vs. 5.4). In summary, even though either Rep78 or Rep68 alone may be sufficient for AAV DNA replication,[27] our study shows that rAAV production in HEK293 cells is critically regulated by the full-length Rep78—corroborating previous studies suggesting that Rep78 and Rep52 proteins are necessary for efficient viral production.[32,33] We deduce that (low level) Rep78 is required for optimal rAAV DNA replication, and a higher ratio of short Rep (Rep52/40) to long Rep (Rep78/68) proteins may enhance genome packaging without inducing cytotoxicity in transient rAAV expression systems.

3.3 | L4-33k/22k proteins are required for optimal rAAV2/8 production

The Helper plasmid (Helper 1.0; Figure S1) utilized in this study was composed of the E2A (encoding DNA binding protein (DBP)), E4, and VA RNA regions derived from the adenovirus-5 genome. The E2A is transcribed by two promoters, namely E2-early and E2-late (Table S1), with DBP mRNA levels coming from the E2-early promoter being dominant. Additionally, the E2-early promoter/intron sequence encodes the L4-33k/22k proteins on the opposite strand, driven by L4 promoter (Figure 4A).[34,35] Even though the L4-22k/33k proteins have been indicated to play a role in adenovirus assembly, gene expression, and viral DNA packaging,[36,37] their significance in rAAV production is largely undetermined. This element represents a potential engineering target or possibly a redundant motif that could be eliminated from the vector. With regard to the latter, minimizing plasmid size is desirable for enhanced transient production by increased transfection efficiency and copy numbers of required genes per DNA weight.

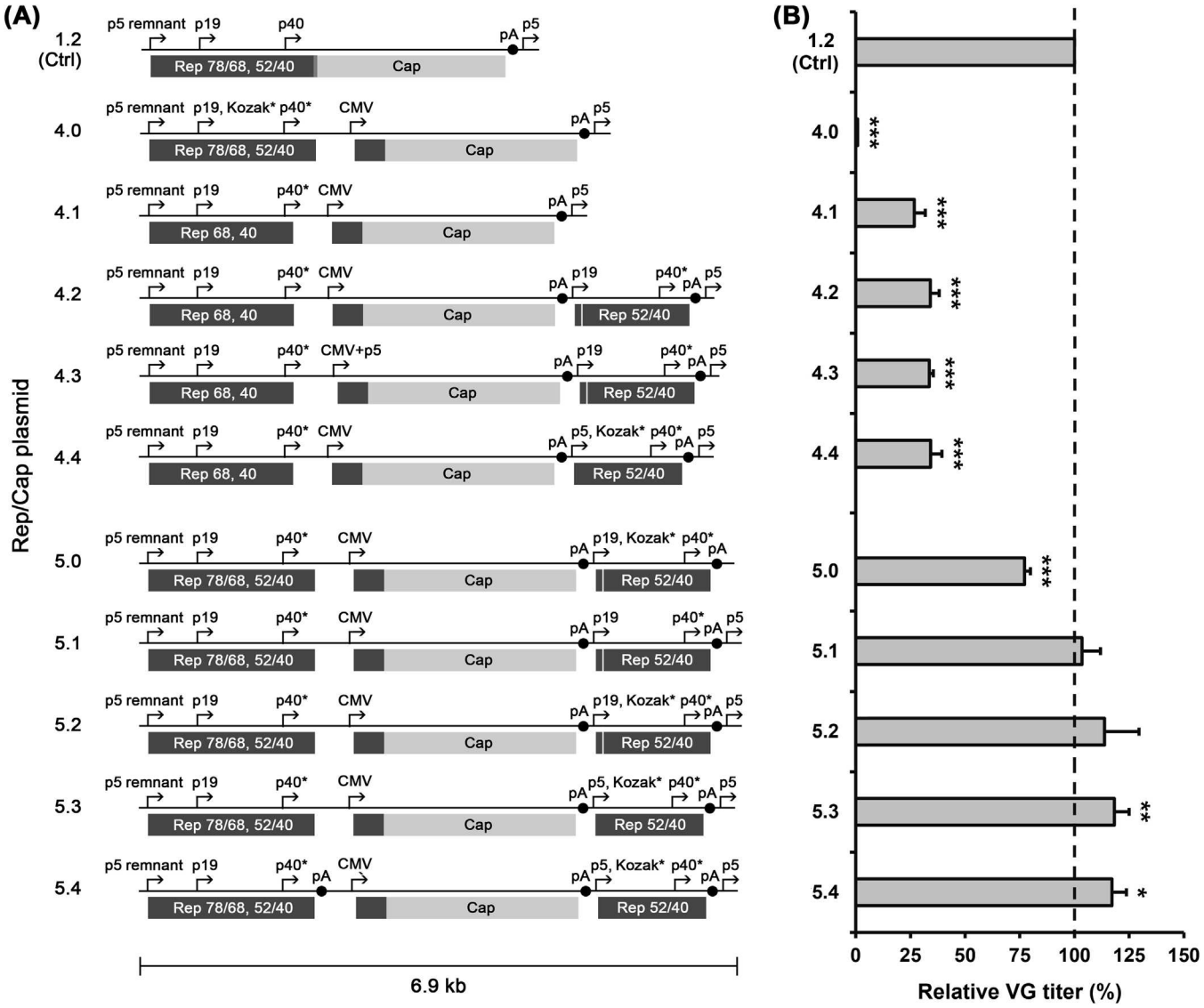

FIGURE 3 Functional evaluation of different Rep proteins within the split Rep/Cap plasmid construct for rAAV2/8 production. (A) Schematic depiction of Rep/Cap plasmid constructs. Replication (Rep) and capsid (Cap) open reading frames are indicated. Rep78 and Rep52 were removed by deleting the alternate splice site within the *rep* codons to produce only Rep68 and Rep40 proteins. The Rep52/40 Kozak sequence was optimized by mutating TACATGG → ATCATGG (start codon underlined). Arrow denotes a promoter, asterisk denotes sequence mutation, black circle denotes a poly A. All components are drawn to approximate scale. (B) HEK293 cells were triple transfected with each Rep/Cap, Helper and transgene plasmid at 1:2:0.1 weight ratio. rAAV2/8 crude viral genome (VG) titers were analyzed 72 h post-transfection, expressed as a percentage compared to the Rep/Cap 1.2. Data shown are the mean ± SD of three independent transfections. Data were analyzed using unpaired Student's *t*-test with respect to the Rep/Cap 1.2. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

In order to evaluate the function of the E2A region, we constructed Helper plasmids containing a partially or fully deleted E2A intron (including a 77 bp exon contained within),[34,35] with the former retaining the E2-late promoter and L4-33k/22k coding sequence ($E2A_{min1}$ and $E2A_{min2}$ Helper; Figure 4A). Triple transfection was performed as described above and rAAV2/8 titer (Figure 4B) and E2A mRNA level (Figure 5A) were measured 72 h post-transfection. These data show that the $E2A_{min1}$ and $E2A_{min2}$ reduced the titer to 82% and 37% ($p < 0.05$) of that deriving from E2A control, respectively, despite the latter exhibiting similar E2A mRNA level. Indeed, the L4-33k/22k ORFs located in the E2A promoter/intron fragment did not allow the conclusion that only the DBP contributed to the rAAV Helper function. To elucidate this, we constructed an L4 promoter-driven plasmid expressing only the L4-33k/22k proteins and co-transfected it with the $E2A_{min2}$ Helper plasmid at an equal molar ratio. This analysis demonstrated that the L4-33k/22k single gene co-expression ($E2A_{min2}$ + L4-33k/22k) resulted in a significant increase in rAAV titer compared to $E2A_{min2}$ (53% increase, $p < 0.05$; Figure 4B) with no significant difference in the E2A mRNA level (Figure 5A). As L4-33k mutant virus has been shown to produce only empty adenoviral capsids,[37] we hypothesized that the L4-33k-deficient rAAV production suffered from a defect in viral DNA packaging resulting in a lower titer. We inferred that the

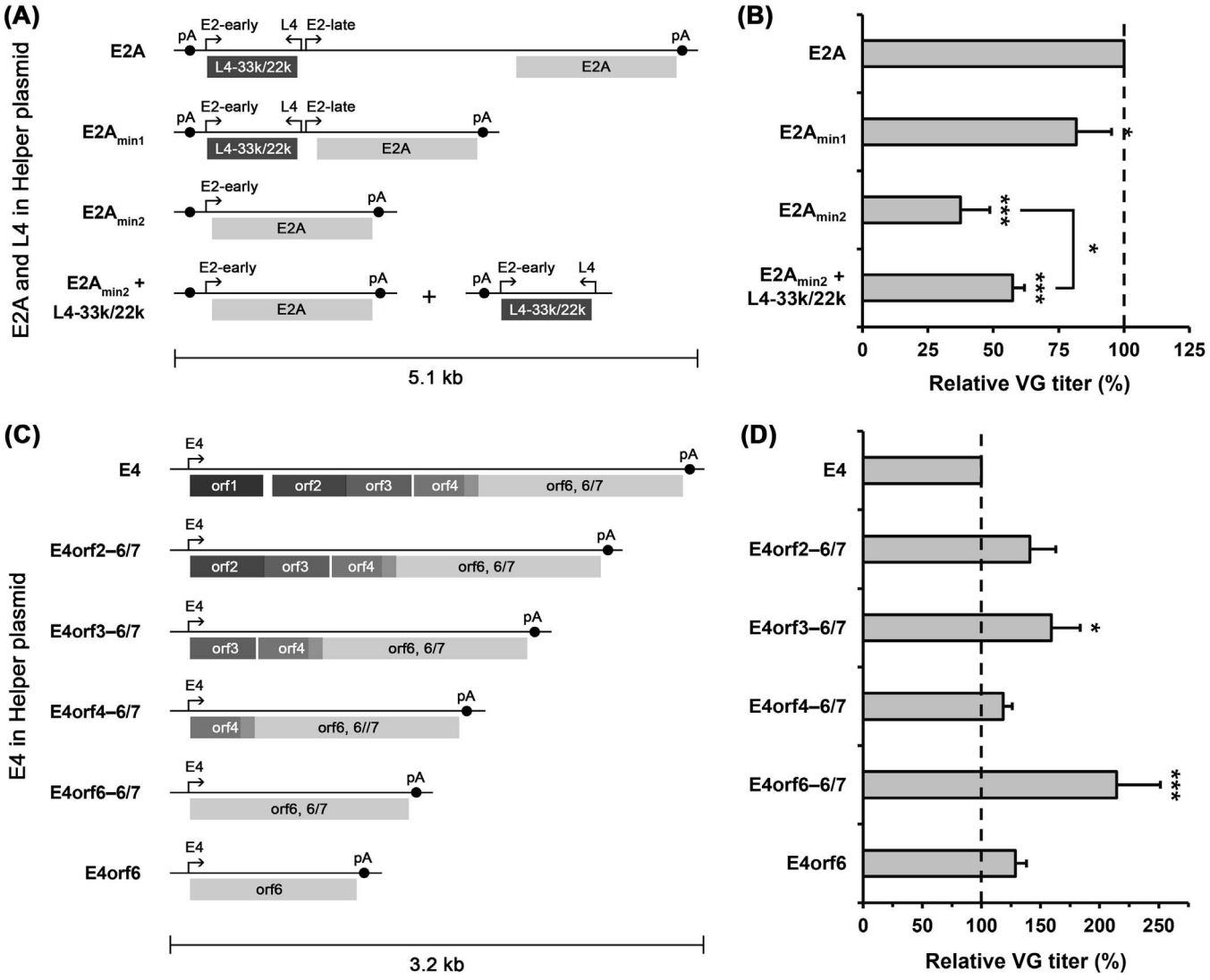

FIGURE 4 Functional evaluation of the E2A, L4-33k/22k and E4 Helper components for rAAV2/8 production. (A) Schematic depiction of the E2A and L4-33k/22k open reading frames within Helper plasmid constructs (E4 and VA RNA are not indicated). A L4 promoter-driven plasmid expressing only the L4-33k/22k protein was also constructed (L4-33k/22k plasmid). Arrow denotes a promoter, black circle denotes a poly A. All components are drawn to approximate scale. (B) HEK293 cells were triple transfected with each Helper, Rep/Cap 1.2 and transgene plasmid at 2:1:0.1 weight ratio. The L4-33k/22k plasmid was spiked at equal molar ratio to the E2A$_{min2}$ Helper plasmid. rAAV2/8 crude viral genome (VG) titers were analyzed 72 h post-transfection, expressed as a percentage compared to the Helper plasmid consisting the complete E2A and L4-33k/22k components. (C) Schematic depiction of the E4 orfs (open reading frames) within the Helper plasmid constructs (E2A, L4-33k/22k, and VA RNA are not indicated). (D) HEK293 cells were triple transfected with each Helper, Rep/Cap 1.2 and transgene plasmid at 2:1:0.1 weight ratio. rAAV2/8 crude VG titers were analyzed 72 h post-transfection, expressed as a percentage compared to the full-length E4 gene. Data shown are the mean ± SD of three independent transfections. Data were analyzed using unpaired Student's *t*-test with respect to the complete E2A or E4 Helper plasmid. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

L4-33k/22k proteins were integral components for optimal, high-yield rAAV2/8 production and that it represents a potential engineering target (e.g., via its overexpression) to enhance rAAV production.

3.4 | Helper plasmid comprising E4 orf6 and 6/7 subset enhances rAAV2/8 production

The E4 gene of adenovirus encodes seven proteins, namely E4 orf (open reading frame) 1, 2, 3, 3/4, 4, 6, and 6/7, each with different functions including promoting viral gene expression and replication as well as modulation of TF activities.[38] Among these, only the E4orf6 protein was thought to contribute to rAAV production and solely employed in a number of Helper plasmid variants.[39–41] Despite the minimal observed effect of other E4orfs on adenovirus growth in cultured cells,[41,42] we hypothesized that rAAV production could be optimized by specific combinations of the E4orf proteins.

In order to specifically determine the functional contribution of different E4orfs, we dissected the E4 gene by constructing Helper plasmids containing different subsets of the orfs (Figure 4C). This set of

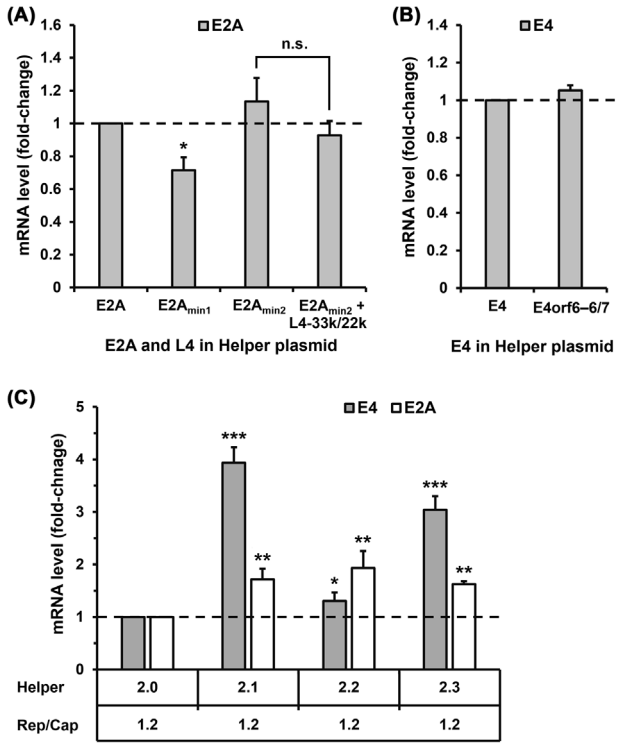

FIGURE 5 Comparative analysis of E2A and E4 mRNAs during rAAV2/8 production using the engineered Helper plasmids. (A, B) Cells triple-transfected with truncated E2A and E4orf6–6/7 Helper plasmids in Figure 4 (all utilizing Rep/Cap 1.2), and (C) CMV-driven Helper plasmids in Figure 6 were harvested at 72 h post-transfection. mRNA transcript levels were analyzed by ddPCR, expressed as a fold-change compared to the complete E2A or E4 Helper, or Helper 2.0 plasmid. Data were analyzed using unpaired Student's *t*-test with respect to the complete E2A or E4 Helper, or Helper 2.0 plasmid. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, n.s., not significant.

six plasmids was then tested for their ability to mediate AAV2/8 vector production. Measurement of rAAV titer after triple transfection of HEK293 cells with each Helper variation is shown in Figure 4D. As anticipated, the E4orf6 was capable of producing rAAV help equivalent to a full-length E4 gene ($p > 0.05$). Moreover, the data show that the removal of orf1 and 2 (i.e., E4orf2–6/7 and E4orf3–6/7 subsets) increased the rAAV titer by 41% and 59% ($p < 0.05$), respectively, compared to the E4 control. In this regard, we conjecture that the deletion of these two redundant orfs (where their functions are largely undefined)[37] increased the abundance of other orf mRNAs spliced from the same precursor mRNA transcript. Deletion of orf1–3 (E4orf4–6/7 subset) decreased the rAAV titer to the control level—this was not unexpected considering that orf3 (similar to orf6) functions in promoting viral gene expression and replication.[41] Importantly, our data shows that further deletion of orf4 while retaining orf6/7 (E4orf6–6/7 subset) significantly enhanced the titer to 214% of the E4 control titer ($p < 0.001$). This result accords with previous studies that identified orf4 as a negative regulator of E1A and E4 transcription[43] while orf6/7 modulates the activity of the cellular transcription factor E2F.[44] Measurement of E4 mRNA at 72 h post-transfection showed that the E4orf6–6/7 construct did not result in a higher overall E4 transcript level (Figure 5B). We conclude that rAAV production in HEK293 cells can be enhanced via removal of redundant E4orfs.

3.5 | Engineered Rep/Cap and Helper plasmids can be used together to control rAAV2/8 gene expression

To evaluate whether the controllable Rep/Cap system could complement the engineered Helper to enable efficient rAAV production, we utilized E4orf6–6/7 Helper plasmid in combination with either Rep/Cap 3.7 or 5.4 plasmid. Additionally, previous studies showed that regulatory loops exist in which E1A, DBP and E4orf6/7 proteins positively or negatively regulate promoters p5, E1A, E2-early, and E4 as well as transcription factor E2F, among others.[44,45] Therefore, we evaluated whether substitution of constitutively active CMV promoter sequences for the E2A and/or E4 regulatory sequences in the E4orf6–6/7 Helper plasmid (Figure 6A) have positive/negative effects on rAAV vector production.

Measurement of rAAV2/8 titer after triple transfection of HEK293 cells with different Helper and Rep/Cap variant combinations is shown in Figure 6B. The result showed that both Rep/Cap 3.7 and 5.4 were incompatible with Helper E4orf6–6/7 (Helper 2.0) where they displayed 42% and 26% reduction in rAAV titer, respectively, relative to the Rep/Cap 1.2 control ($p < 0.01$). In this regard, we postulate that the use of the strong, highly complex CMV promoter in Rep/Cap 3.7 and 5.4 plasmids titrated away the limited pool of available TF molecules from the E4 promoter resulting in E4orf6–6/7 downregulation.[25,26] The use of CMV promoter to drive E4orf6–6/7 transcription (Helper 2.1) restored the rAAV titer comparable to Helper 2.0 with Rep/Cap 1.2 (83%–110%). In contrast, substituting the E2-early promoter with the CMV promoter (Helper 2.2) led to reduced titers especially when used in conjunction with Rep/Cap 1.2 and 5.4. Further decreases in rAAV level were observed with Helper 2.3 plasmid that harbored the CMV promoter to drive both E4orf6–6/7 and E2A expression. Measurement of mRNA levels (Figure 5C) showed that the use of Helper 2.1 resulted in a 3.94-fold increase in E4orf6–6/7 transcript level as well as a 1.72-fold increase in E2A transcript level. This is expected considering that the E4 promoter is inhibited by DBP whereas E4orf6/7 stimulates the activity of E2-early promoter.[44] E4 transcript level was also slightly increased when CMV was used to drive E2A expression, which can be attributed to the CMV acting as a "downstream enhancer" to the E4 promoter.[46] Slightly lower E4orf6–6/7 and E2A transcript levels were observed when CMV was utilized to simultaneously drive E4orf6–6/7 and E2A expression (Helper 2.3) compared to when it was used separately (Helper 2.1 and 2.2)—in general agreement with our view of competing TF binding sites. Nevertheless, our data did not show any correlation between rAAV titer (Figure 6B) and the E4 or E2A transcript level (Figure 5C), thus illustrating the highly complex interactions between various components during the viral production process.

The data in Figure 6B also shows that Rep/Cap 5.4 with Helper 2.0 or 2.1 yielded higher titers compared to Rep/Cap 3.7. To expound this observation, we selected a subpanel of different Rep/Cap and

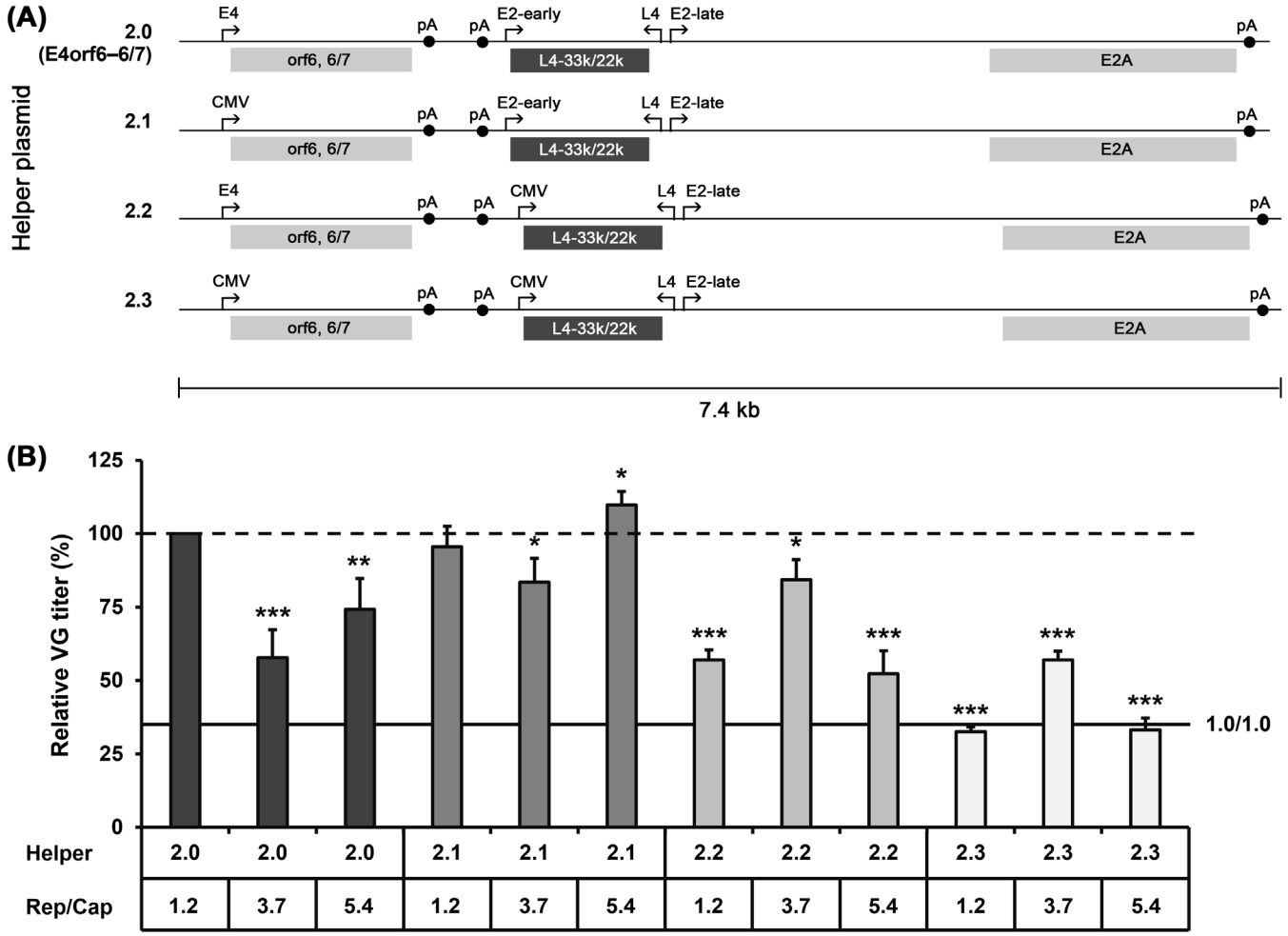

FIGURE 6 Evaluation of engineered Helper and Rep/Cap plasmid combinations for rAAV2/8 production. (A) Schematic depiction of the Helper plasmid constructs (VA RNA is not indicated). The E4orf6–6/7 Helper plasmid (Figure 4A) is denoted as Helper 2.0. Arrow denotes a promoter, black circle denotes a poly A. All components are drawn to approximate scale. (B) HEK293 cells were triple transfected with the Helper, Rep/Cap and transgene plasmids at 2:1:0.1 weight ratio. rAAV2/8 crude viral genome (VG) titers were analyzed 72 h post-transfection, expressed as a percentage compared to the Helper 2.0 and Rep/Cap 1.2 plasmid combination. Solid horizontal line represents the titer level of the conventional Helper 1.0 and Rep/Cap 1.0 (1.0/1.0) in Figure 1B. Data shown are the mean ± SD of three independent transfections. Data were analyzed using unpaired Student's *t*-test with respect to the Helper 2.0 and Rep/Cap 1.2 plasmid combination. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

Helper combinations from Figure 6B as well as the Rep/Cap 1.2 control from Figure 1D (utilizing the original Helper plasmid; Helper 1.0), and quantified fully assembled, intact capsids to determine the ratio of full to empty particles. This analysis indicated that the use of the CMV promoter to drive Cap expression (Rep/Cap 3.7 and 5.4) with either Helper 2.0 or 2.1 boosted total capsids by an average of ≈3-fold compared to Rep/Cap 1.2 (Figure 7A), resulting in full/empty capsid ratio of <9% (Figure 7B). Importantly, the analysis also revealed that all transfections utilizing Rep/Cap 5.4 (overexpressing Rep52/40 proteins) yielded relatively higher full-to-empty capsid ratios compared to Rep/Cap 3.7, indicating a higher rate of packaging and accumulation of single-stranded DNA progeny genomes (see also the accompanied increase in VG titer for Helper 2.0 and 2.1; Figure 7C). Very high full/empty ratios (up to 66%) were achieved using Helper 2.2 and 2.3 although this was largely due to considerable reductions in intact capsid abundance compared to other Helper variants. Taken together, these data demonstrate that it is possible to control both genome and total viral particle titer in a transient rAAV expression system. We anticipate that the novel library of CMV promoter sequences with variable strengths,[26] combined with reoptimization of the triple plasmid ratio for the new vector design,[47] would enable systematic determination of the optimal Cap expression for maximal rAAV product titer and quality.

4 | DISCUSSION

In this study we have characterized the diverse components (ORFs) and regulators (promoters, introns) of the AAV transient triple transfection plasmid system underpinning the biomanufacturing processes, for example, of how the abundance (or absence) of the four Rep proteins affects the efficiency of rAAV production yield. Specifically, our

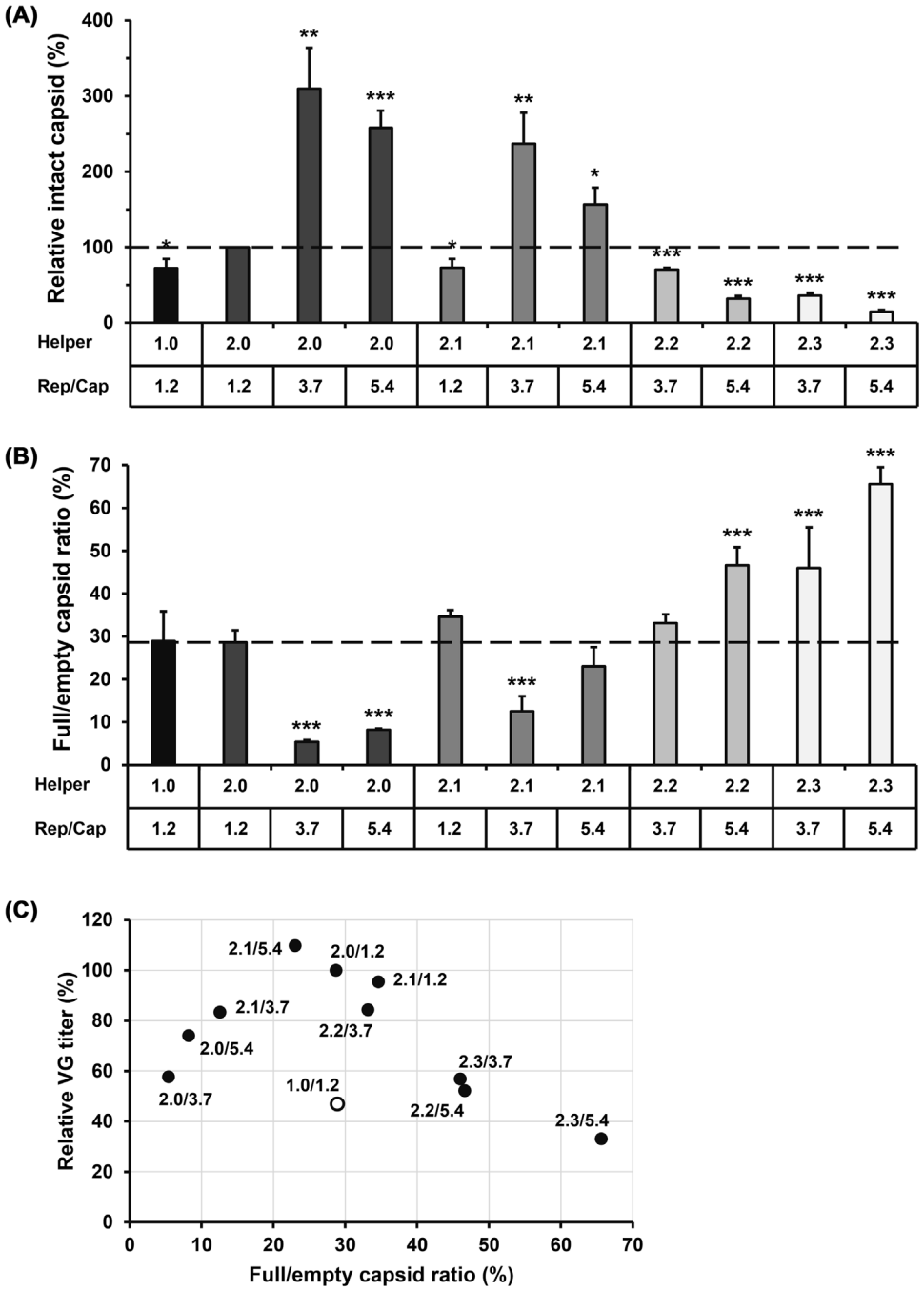

FIGURE 7 Determination of product quality of the engineered Helper and Rep/Cap plasmids for rAAV2/8 production. The original Helper plasmid (Figure S1) is denoted as Helper 1.0. (A) Intact capsids were quantified at 72 h post-transfection using rAAV8-specific capsid ELISA and expressed as a percentage compared to the Helper 2.0 and Rep/Cap 1.2 plasmid combination. (B) The full/empty capsid ratio was calculated from the measured intact capsids in (A) and its viral genome (VG) titer. Data shown are the mean ± SD of three independent transfections. Data were analyzed using unpaired Student's *t*-test with respect to the Helper 2.0 and Rep/Cap 1.2 plasmid combination. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. (C) Scatter plot of VG titer (Figure 6B) and full/empty capsid ratio (B) of the engineered Rep/Cap and Helper plasmids. Number refers to Helper/Rep/Cap constructs. Open circle denotes the Helper 1.0 and Rep/Cap 1.2 control in Figures 1B and 3B.

data indicated a suboptimal rAAV2/8 production state when *rep78* was removed. This finding is in line with the previous reports in which Rep78 was shown to be more efficient than Rep68 in producing infectious Rep-negative AAV,[27] and hence indirectly favors the vector design strategy that omitted *rep68*[48] rather than *rep78*[13] to mitigate Rep toxicity effects. Furthermore, this study identified the L4-33k/22k gene as an integral rAAV component, corroborating a recent study that showed a >20-fold decrease in *rep* and *cap* DNA in HeLa cells transfected with the 33k/22k-targeting siRNA[49]—suggesting a cell engineering opportunity for increased rAAV production, for example,

via overexpression of these adenovirus Helper proteins. More generally, while previous studies demonstrated the need to lower long Rep expression,[12,19–21] further overexpression of short Rep (e.g., by adding a second gene copy) could promote enhancement of rAAV titer and product quality. Overall, the Helper 2.1 and Rep/Cap 5.4 plasmid system presented in this study enabled a ≈3.1-fold increase in titer compared to the conventional Rep/Cap and Helper plasmids system (Figure 6B; we note that similar results are achieved in TubeSpin or Erlenmeyer flask[17]).

Another key finding of our work is that E4 orf1, 2, and 4 are functionally redundant within the AAV expression system, consistent with the existing notion that a single E4orf6 protein is needed to produce rAAV vectors in HEK293 cells.[39–41] Critically, our results reveal that rAAV titer could be enhanced by specific combinations of the E4orf proteins particularly orf6 and 6/7. The removal of redundant orfs also likely improved the expression of other orfs due to reduced splice sites. With regard to the latter protein, E4orf6/7 modulates the activity of the E2-early promoter by forming a direct complex with transcription factor E2F and stabilizing the DNA-bound form.[44] As the E4 promoter is inhibited by the E2A product,[45] a regulatory loop exists in which E4orf6/7 protein increases E2A transcription while DBP negatively regulates E4 transcription. From a mechanistic perspective, we assume that the advantage of this temporal coordination is restricted DBP "toxic" effects[50,51] during the bioproduction process thus ensuring maximal productivity. Replacing the E2-early promoter with the constitutive, highly active promoter CMV could result in uncontrollable E2A gene expression and therefore rapid cellular accumulation of DBP. However, our cell concentration/viability data showed no differences between the endogenous E2-early and CMV promoter-driven E2A constructs (data not shown). We speculate that the detrimental effects of the CMV-driven E2A in HEK293 cells were via negative regulation of specific AAV components rather than direct exertion of cytotoxicity on the host cells.

Even though the heterologous CMV promoter is beneficial for the production of rAAV from the split packaging vector system, product quality analysis showed that most of the capsids generated from these vectors were empty and therefore were unable to provide therapeutic benefits. This remains the case even when a second short *rep* gene was introduced to enhance packaging and accumulation of single-stranded viral genome. Nevertheless, it may be possible to circumvent this drawback by using specific *cis*-regulatory modules within the CMV promoter architecture (i.e., specific strengths) we previously reported[26] for defined capsid expression levels. Moreover, promoter activity in a given cell host is governed by a system-specific combination of interactions between the promoter's constituent TF binding sites and the availability of endogenous TFs.[26] Accordingly, the use of the CMV promoter to drive *cap* gene expression likely resulted in the titration of TFs away from the endogenous E4 promoter affecting the expression level of E4orf proteins (and consequently rAAV titer; Figure 6). Expectedly, further bioinformatic analysis of regulatory elements within these promoters indicated significant (active) TF binding site overlaps between them (Figure S3). In this regard, vectors utilizing synthetic promoters designed de novo using specific TF binding site building blocks[52,53] are likely to be the solution for predictable stoichiometries of different AAV vector components in transient as well as stable systems.[54]

Lastly, our study highlights the complexity of rAAV vector expression systems and that coordinated optimization of a variety of linked dynamic processes (within and between packaging and Helper plasmids) is ultimately necessary to maximize volumetric rAAV product yield and quality. Systematic optimization study could be achieved via DoE-based co-transfection of multiple plasmids each carrying a specific AAV gene(s). This approach explores a large design space and theoretically enables the identification of the "ideal" gene expression stoichiometry for a given system. However, such experimental design discounts the spatial relationship underpinning promoter behavior that may prevent rational improvement or confident prediction of their functionality—thus necessitating testing of each component directly in the final packaging/Helper plasmid constructs. The constraint of the latter method is the difficulty in constructing and screening hundreds of possible vector variants to identify the optimal vector design(s). Nevertheless, given the availability of high-throughput screening techniques,[55] and as gene synthesis costs are becoming cheaper[56] and the emerging technology of DNA-synthesizing enzymes (for long genes and whole vectors) is becoming more efficient,[57] rapid parallel evaluation of rAAV vector designs may indeed be tractable.

AUTHOR CONTRIBUTIONS

Jared Whitehead: Data curation (supporting); formal analysis (supporting); investigation (supporting); methodology (supporting); validation (supporting); writing—review and editing (supporting). Ping Liu: Conceptualization (supporting); funding acquisition (supporting); methodology (supporting); project administration (supporting); resources (supporting); supervision (supporting); validation (supporting); writing—review and editing (supporting). Ayda Mayer: Conceptualization (supporting); funding acquisition (supporting); methodology (supporting); project administration (supporting); resources (supporting); supervision (supporting); validation (supporting); writing—review and editing (supporting).

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CONFLICT OF INTEREST STATEMENT

The authors have patent applications filed based on the work in this paper.

DATA AVAILABILITY STATEMENT

Data is available in the article's supplementary material.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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