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Conditional splicing system for tight control of viral overlapping genes

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ABSTRACT Viral genomes frequently harbor overlapping genes, complicating the development of virus-vectored vaccines and gene therapies. This study introduces a novel conditional splicing system to precisely control the expression of such overlapping genes through recombinase-mediated conditional splicing. We refined site-specific recombinase (SSR) conditional splicing systems and explored their mechanisms. The systems demonstrated exceptional inducibility (116,700-fold increase) with negligible background expression, facilitating the conditional expression of overlapping genes in adenovirus-associated virus (AAV) and human immunodeficiency virus type 1. Notably, this approach enabled the establishment of stable AAV producer cell lines, encapsulating all necessary packaging genes. Our findings underscore the potential of the SSR-conditional splicing system to significantly advance vector engineering, enhancing the efficacy and scalability of viral-vector-based therapies and vaccines.

IMPORTANCE Regulating overlapping genes is vital for gene therapy and vaccine development using viral vectors. The regulation of overlapping genes presents challenges, including cytotoxicity and impacts on vector capacity and genome stability, which restrict stable packaging cell line development and broad application. To address these challenges, we present a "loxp-splice-loxp"-based conditional splicing system, offering a novel solution for conditional expression of overlapping genes and stable cell line establishment. This system may also regulate other cytotoxic genes, representing a significant advancement in cell engineering and gene therapy as well as biomass production.

KEYWORDS overlapping genes, regulation system, viral genome engineering, conditional gene expression, conditional splicing, loxp-splice-loxp

Overlapping genes, defined by their shared nucleotides across two or more coding sequences, are prevalent in both viral and eukaryotic genomes (1). Studies suggest that approximately 26% of human protein-coding genes and over half of viruses feature overlapping genes (2–4). Historically understudied, the significance of these genes is now being rigorously explored due to their potential in vaccine and viral vector development (5–7). However, the intrinsic complexity of overlapping genes introduces challenges, particularly in their application to gene therapy and vaccine production.

One primary challenge is the potential cytotoxicity of overlapping genes, which complicates the development of stable viral packaging cell lines. Traditional methods, such as incorporating these genes into viral vectors or plasmid transfection, are limited by reduced vector capacity, compromised genome stability, and possible immune responses against the gene products (8). These limitations not only impact the efficacy of gene therapies and vaccines but also escalate production costs, especially for vectors like adenovirus-associated virus (AAV) and lentivirus (9–11).

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Current gene regulation systems, heavily reliant on engineered inducible promoters, fall short in managing overlapping genes due to the difficulty in integrating additional regulatory elements within their coding sequences. While RNA riboswitches and site-specific recombinase (SSR) systems present alternative solutions, they have their drawbacks. RNA riboswitches, though innovative, tend to exhibit leaky expression (13–15), undermining their regulatory precision. The SSR systems, notably the "loxp-stop-loxp" strategy introduced in the early 1990s (12–14), utilize artificial intron harboring a stop cassette to block gene expression, which is lifted by recombinase action to restore gene function. Despite its promise, this system's efficiency is compromised by limitations such as ineffective transcription termination and potential for DNA recombination (15, 16), as observed in strategies like the "dual splicing switch" (17, 18).

Addressing these shortcomings, our study introduces a refined approach to regulate overlapping genes using SSR-mediated conditional splicing. This method significantly reduces leaky expression compared to existing SSR strategies and allows for precise control over the expression of multiple overlapping genes. By tackling the inherent challenges of overlapping gene regulation, this novel strategy aims to enhance the development and scalability of gene therapies and vaccine production, marking a significant advancement in the field.

RESULTS

Development of conditional splicing systems

In our exploration of regulating viral overlapping genes through cis-acting element-mediated alternative splicing, we posited that introducing a highly efficient splicing site within an intron could disrupt standard splicing processes, enabling the selective expression of these genes. Our initial step involved crafting a synthetic intron that mimicked the fundamental attributes of human introns. This intron was enhanced with several intronic splicing enhancers (ISEs) to boost its splicing efficiency (19, 20), assessed through luciferase Gluc reporter assays. The enhancements observed with ISE groups A&C and B&D were significant, amplifying Gluc expression by 41 and 45 times, respectively, achieving a splicing efficiency near 99.9% (Fig. S1).

Further testing of the "dual splicing switch" concept involved integrating a "loxp-poly A-loxp" cassette within our synthetic intron. Interestingly, the inclusion of one to three tandem SV40 poly A sequences modestly affected Gluc expression (Fig. S1). Detailed mRNA abundance analyses revealed that a single copy of the SV40 poly A sequence marginally decreased Gluc mRNA levels by approximately 1.5-fold (Fig. S1).

Advancing our system, we delineated three intron variants—PT, PS, and PST—each incorporating a single SV40 poly A sequence. Distinctively, we embedded a single copy of the human immunodeficiency virus (HIV) TAR sequence into the PT and PST introns to explore its potential to induce transcription stalling, given its known capacity to significantly impede RNA transcription. Furthermore, to facilitate conditional splicing, we inserted a splicing acceptor (SA1) into the PS and PST introns (Fig. 1A).

Upon transfecting these constructs into HEK293T cells for Gluc expression analysis, we observed nuanced effects. Without Cre recombinase, both the PT intron and the "1x poly A" intron led to a slight reduction in Gluc expression when compared to the control (Fig. 1B). In stark contrast, the PS intron drastically lowered the Gluc expression by 3146-fold, and the inclusion of TAR in the PST intron further diminished expression by 50-fold (Fig. 1B). Crucially, the simultaneous expression of Cre recombinase reversed this suppression, fully restoring the Gluc expression (Fig. 1B).

RT-qPCR analysis of the spliced products post-Cre co-expression indicated the elimination of RNA products spliced between SD and SA1, with a notable increase in RNA spliced between SD and SA2 in the PST intron (Fig. 1C). This finding, coupled with twofold enhancement in Gluc expression from the PST intron compared to the PT intron post-Cre-mediated loxp recombination, underscored the TAR sequence's dual role in enforcing specific splicing events and augmenting Cre-mediated DNA recombination.

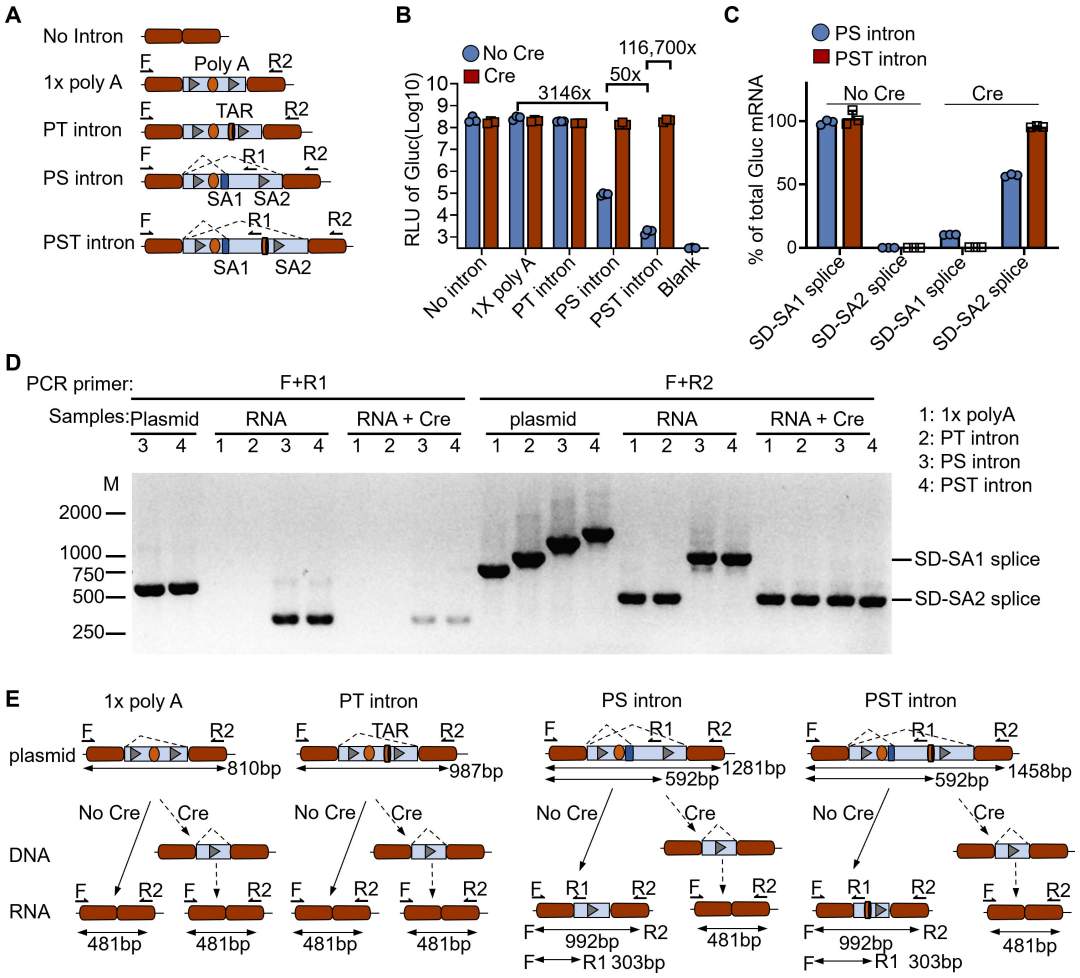

FIG 1 Development and validation of a conditional splicing intron for gene regulation (A) Illustration of a synthetic intron engineered with various regulatory elements. This intron includes an SV40 poly A sequence, the HIV TAR sequence, and a splicing trap sequence. The diagram identifies the locations of primers "F," "R1," and "R2" used in RT-PCR to verify spliced RNA products. Alternative splicing acceptor sites within the PS and PST introns are marked as "SA1" and "SA2." (B) Assessment of introns engineered for conditional expression of the reporter gene Gluc. Equal quantities of plasmids were transfected independently or in combination with a Cre overexpression plasmid into HEK293T cells, and the secretion of Gluc into the cell culture medium was measured 48 hours later. (C) Quantitative analysis of RNA splicing. The proportion of spliced versus unspliced RNA products was determined through RT-qPCR, targeting the regions spanning the splice donor site or the reconstituted region post-splicing. Labels "SD-SA1" and "SD-SA2 splice" refer to mRNA spliced at SA1 or SA2, respectively. (D) Gel electrophoresis analysis of RNA from PS and PST introns. Following total RNA extraction and cDNA synthesis, PCR was conducted over 25 or 30 thermal cycles to amplify either unspliced RNA or the SD-SA2 spliced product using primer pairs "F + R1" or "F + R2." (E) Schematic overview of RNA splicing across four introns, with numerical annotations indicating the expected PCR product sizes.

Electrophoresis analysis of RNA splicing patterns, conducted after 25 or 30 thermal cycles of RT-PCR with designated primer pairs, corroborated our designs (Fig. 1E). Specifically, RNA from the PS and PST introns underwent specific splicing at SA1 (992 bp) in the absence of Cre, while Cre co-expression redirected splicing to SA2 (481 bp), demonstrating the precision and efficiency of our conditional splicing system (Fig. 1D).

Dual splicing and transcription stalling induced by HIV TAR in the PST intron

Our exploration into the TAR element's role within the PST intron focused on its potential to either inhibit or enhance specific RNA splicing events. The PST intron, when compared to the PS intron, presented a decreased proportion of RNA spliced between the second splicing donor (SD) and the second splicing acceptor (SA2) (Fig. 2A and B). This suggested that TAR might act as an intronic splicing silencer (ISS) or RNA hairpin to diminish splicing between SD and SA2 or as an exonic splicing enhancer (ESE), promoting splicing

between SD and SA1. The distinction between these roles was investigated by orienting TAR on the complementary strand within the intron (Fig. 2C). Our analyses showed that the insertion of TAR did not result in a significant alteration in the efficiency or pattern of splicing. This suggests its negligible influence when included separately, as illustrated in Fig. 2D through F.

Interestingly, the formation of TAR-induced hairpin structures, regardless of their orientations, had a minimal effect on splicing efficiency (Fig. 2E). However, a significant transcriptional slowdown was observed with the TAR and reverse-TAR (R-TAR) introns, reducing the Gluc expression by approximately twofold (Fig. 2D). This reduction alone could not fully account for the substantial TAR impact noted in the PST intron. Sequencing of the RNA from the PST intron unveiled an unexpected splicing between TAR and SA2, leading to a notable ~200 nt truncation, revealing TAR's role as a splice donor site and significantly reducing the likelihood of undesired splicing events between SD and SA2 (Fig. 2G).

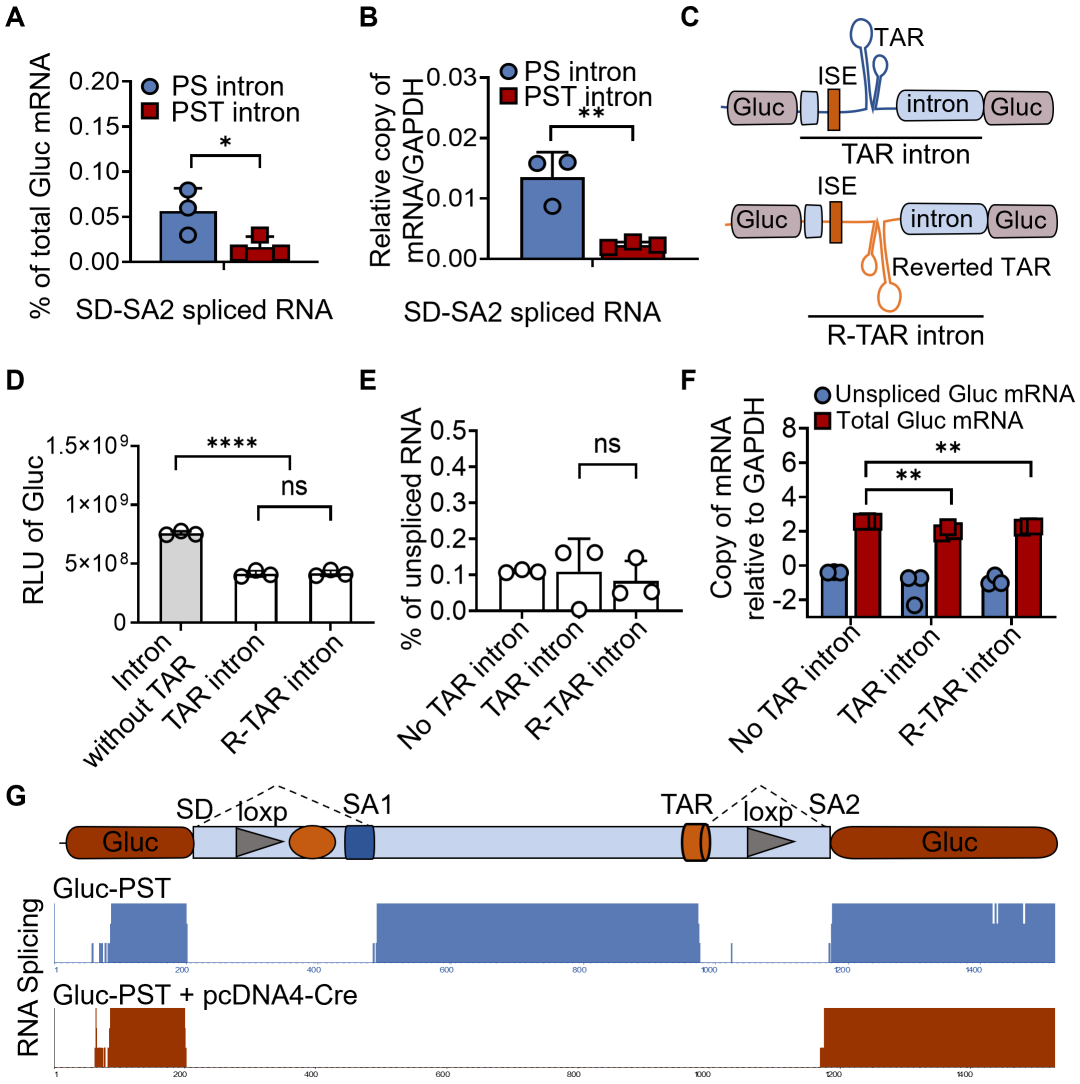

FIG 2 Examining the Cis-acting effect of HIV TAR on RNA splicing. (A & B) Detailed examination of the proportion and quantification of SD-SA2 spliced mRNA products. RT-qPCR utilized a primer pair specific to the reconstituted region post-splicing between SD and SA2. (C) Diagrammatic representation of Gluc reporter genes featuring TAR in either the sense (TAR intron) or complementary strand (R-TAR intron). (D) Assessment of Gluc expression from plasmids embedded with TAR or R-TAR introns. (E-F) Quantitative analysis of spliced RNA products from TAR and R-TAR introns, with primer pairs targeting the intron region or encoding region. Data represent mean ± SD from three biological replicates, analyzed via unpaired *t*-tests. (G) Sanger sequencing analysis of RNA splicing within the PST intron, following transfection of HEK293T cells with the Gluc-PST plasmid, with or without pcDNA4-Cre.

Conditional expression of AAV Cap and HIV genes

For the conditional expression of overlapping genes, the PST intron was strategically integrated into the AAV cap gene at four specific locations (Fig. 3A). Independent of the helper vector, the absence of Cre led to dual splicing at the PST intron, effectively

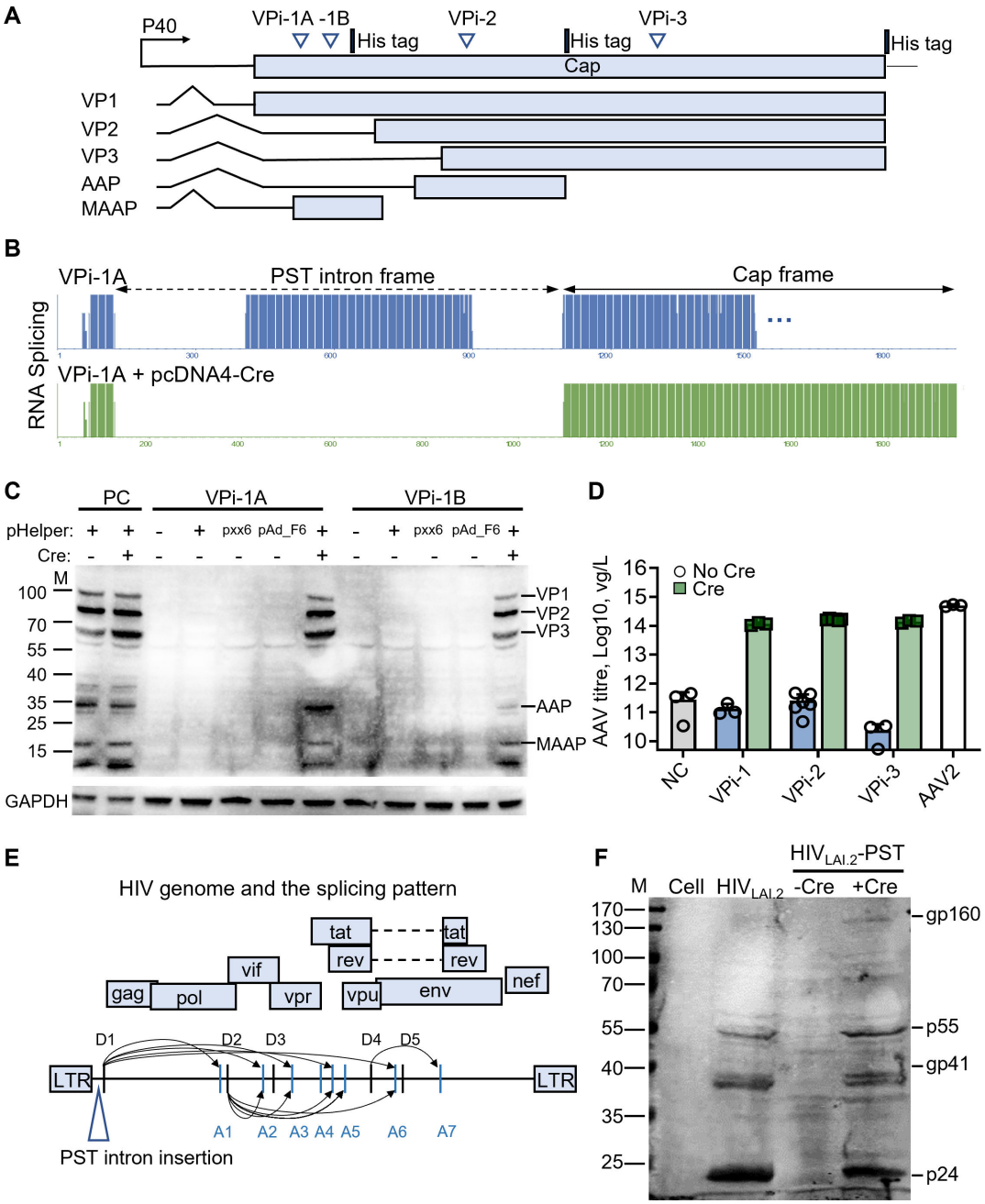

FIG 3 Conditional expression of AAV2 Cap and HIV genes through conditional splicing. (A) Structural depiction of the AAV2 cap gene, which employs two alternative splicing sites to produce a minimum of five proteins. The PST intron was inserted into specified VPi-1 to VPi-3 sites. Three His tags were inserted into different coding frames to label different proteins simultaneously. (B) RNA splicing analysis for VPi-1A, involving co-transfection in HEK293T cells with various helper plasmids and pcDNA4-Cre, followed by Sanger sequencing to determine the RNA splicing pattern. (C) Western blot assessment of Cap protein expression from VPi-1A and VPi-1B plasmids, with positive control (PC without PST intron) included for comparison. Detection was performed using an anti-His antibody. (D) Quantification of DNase I-resistant particles from VPi-1 to VPi-3 plasmids, co-transfected with helper and pAAV vectors, analyzed by qPCR. (E) Overview of the HIV genome's splicing pattern, facilitating the production of over 10 viral proteins *via* alternative splicing. (F) Conditional HIV protein expression analysis *via* Western blot by comparing plasmid HIVLAI.2 and HIVLAI.2-PST, with serum from chronically infected HIV patients serving as the primary antibody.

silencing gene expression (Fig. 3B). Cre's co-expression not only removed this blockage but also reinstated the capsid protein production from various plasmids, emphasizing the controlled expression's efficiency (Fig. 3B and C). This precise regulation was crucial for generating DNase I-resistant rAAV particles, which relied entirely on Cre's presence (Fig. 3D).

Extending our approach to the HIV genome, integrating the PST intron upstream of splice donor 1 (SD1) drastically altered the HIV protein expression landscape (Fig. 3E). Utilizing sera from chronically infected HIV patients for detection, we observed a complete suppression of HIV protein expression in the absence of Cre, which was reversed upon Cre's co-expression, illustrating the intron's potential to finely control gene expression in complex viral contexts (Fig. 3F).

Conditional expression of AAV Rep

Diverging from the AAV cap and HIV genes, the AAV rep gene's expression is governed by multiple promoters and alternative splicing sites. The insertion of the PST intron into the rep gene at specific sequences (CAG/N or AAG/N) validated our conditional splicing system's effectiveness through both Western blot and qPCR analyses (Fig. 4B). Notably, the NPST plasmids yielded AAV levels comparable to the wild-type AAV2 plasmid upon the Cre activation (Fig. 4C). The inducible expression of Cre, triggered by doxycycline, further exemplified the system's utility in controlled AAV packaging, showcasing a scalable and efficient approach to viral vector production (Fig. 4D).

Establishment of rAAV packaging cell lines utilizing the PST intron

The establishment of efficient and reliable recombinant adeno-associated virus (rAAV) packaging cell lines presents significant challenges, largely due to the need to

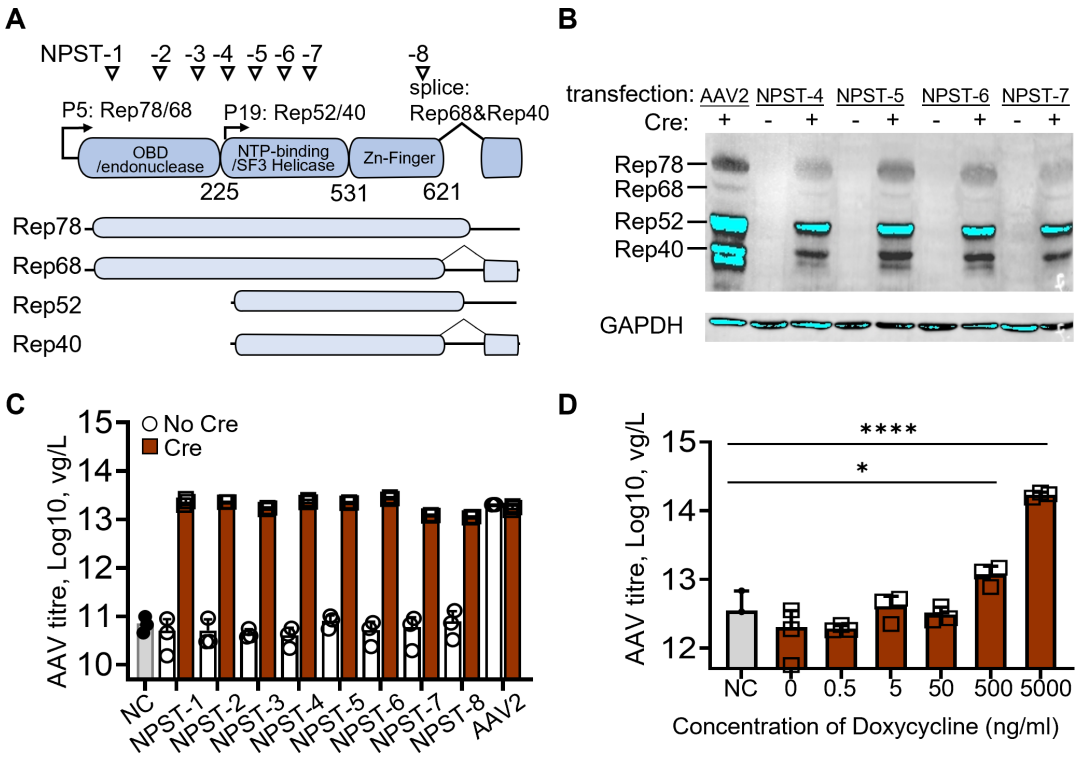

FIG 4 Conditional expression of AAV Rep through conditional splicing. (A) Gene map of AAV rep, with NPST1 to PST8 indicating PST intron insertion sites. (B) Western blot analysis of AAV Rep expression, following transfection of HEK293T cells with pcDNA4-Cre and/or NPST plasmids. (C) Conditional AAV particle production measured post-transfection of NPST plasmids, phelper, and pAAV vectors, with or without pcDNA4-Cre. (D) Induction of AAV2 production *via* doxycycline in cells transfected with NPST-4, pHelper, pAAV, and a Cre-inducible plasmid, demonstrating the system's inducibility and effectiveness.

incorporate multiple AAV packaging genes and the cytotoxic effects associated with the overlapping gene rep and the adenoviral E1 gene. To address these obstacles, we designed an integrated rAAV packaging system named HCN2, detailed in Fig. 5A and 6. The HCN2 system integrates essential components for cell line development, including constitutively expressed selection markers (GFP and puromycin) and regulatory proteins (Tet3G and tTR-KRAB). The expression of Cre recombinase is controlled by the inducible promoter TRE, which is regulated by tTR-KRAB but can be activated in the presence of Tet3G and doxycycline (Dox). Furthermore, the adenovirus helper genes E2A and E4 are also under the control of Tet3G and tTR-KRAB, mediated through TetO7 operon sequences, with Dox acting to release tTR-KRAB's inhibitory effects. This elaborate expression cassette is flanked by PiggyBac ITRs, enabling genome integration *via* transposase (21, 22).

To establish stable cell lines, HEK293T, 293F, and HeLa cells were co-transfected with the HCN2 plasmid and a transposase expression vector, followed by selection using puromycin. The effectiveness of these cell lines as AAV producers was gauged by assessing AAV replication following the administration of Dox and supplemental AAV. Our findings indicated a direct correlation between Dox concentration and AAV titers in the culture supernatant (Fig. 5B). Notably, AAV production significantly increased when

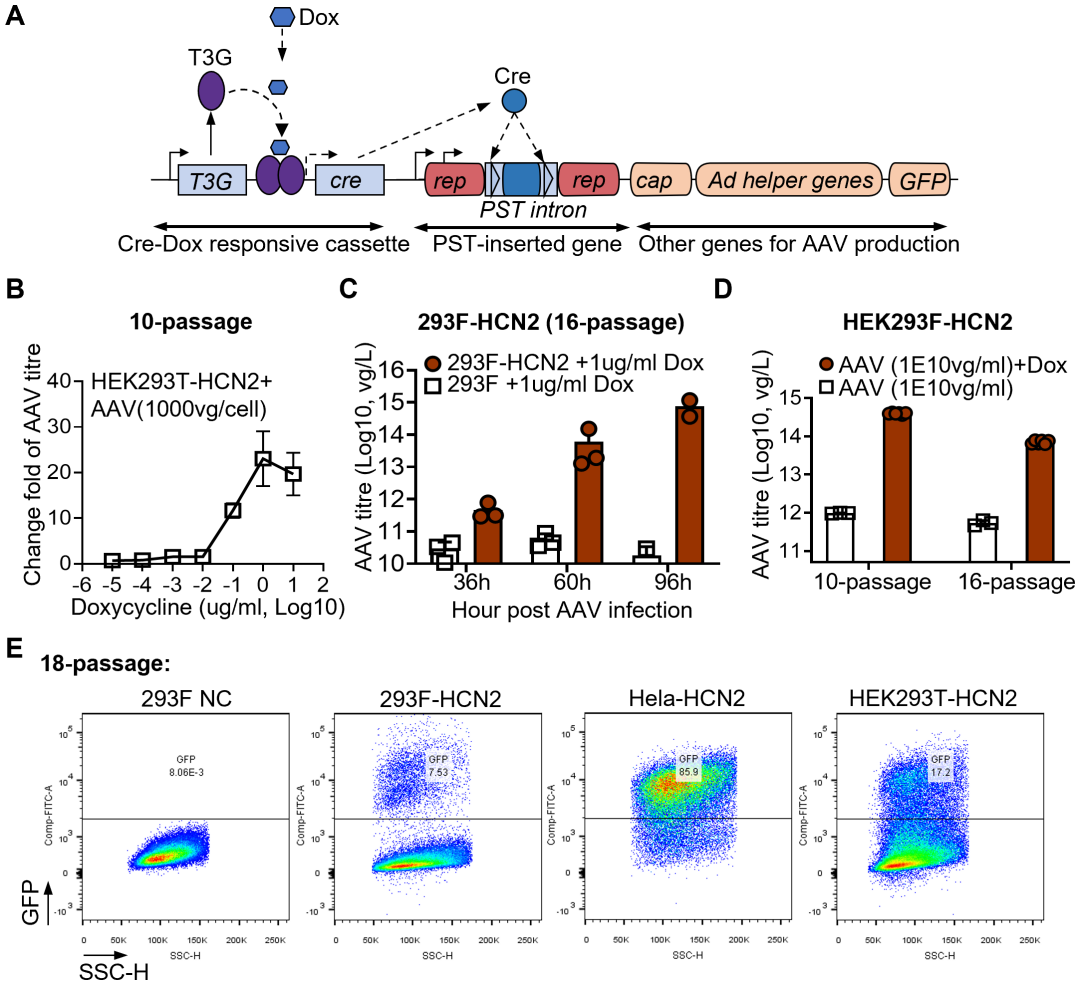

FIG 5 Establishing inducible AAV packaging cell lines with complete packaging genes. (A) Design of the HCN2 shuttle plasmid for inducible AAV packaging cells, featuring regulatory proteins, selection markers, and the PST-inserted gene cassette. (B–D) Evaluation of AAV replication induced by Dox in HEK293T-HCN2 cells, comparison of AAV yields at different cell passages, and analysis of AAV production efficiency in HEK293F-derived packaging cells. (E) GFP expression assessment across various AAV packaging cell lines at the 18th passage *via* flow cytometry, highlighting the system's stable expression capabilities.

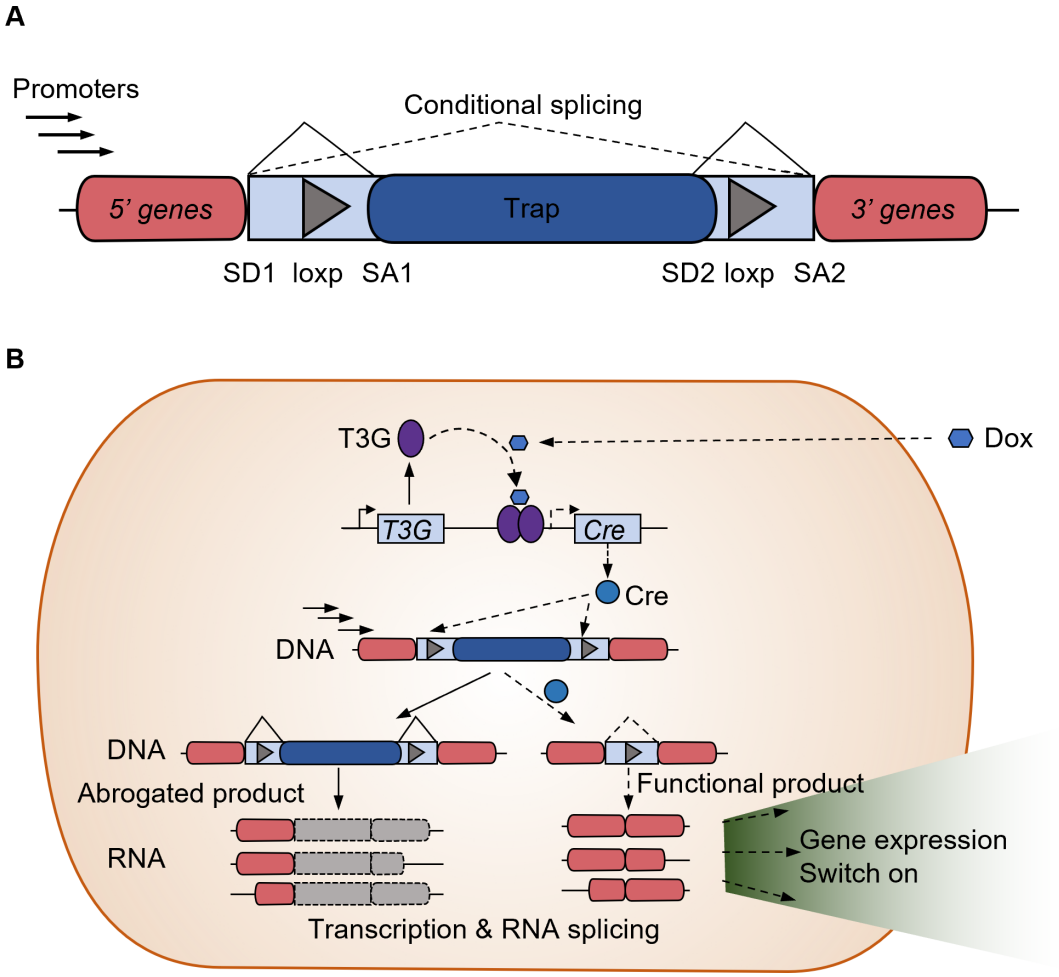

FIG 6 Conceptual framework of the conditional splicing system for overlapping genes. (A) The basic architecture of the conditional splicing system, incorporating artificial introns with recombinase recognition sites to facilitate precise gene regulation. (B) Depiction of a small molecule-inducible regulatory system for overlapping genes, leveraging conditional splicing and Tet-ON inducible mechanisms to control gene expression dynamically, exemplified by the induction of Cre recombinase with doxycycline. The black lines represent the condition without Dox, while the dashed lines represent the condition treated with Dox.

Dox concentrations surpassed 0.1 µg/mL, highlighting the inducible system's efficacy in boosting AAV production over time (Fig. 5C).

However, a comparative analysis between cell lines revealed a decline in AAV yield in HEK293T-HCN2 cells after 16 passages, suggesting a potential loss of packaging gene integrity over prolonged culture periods (Fig. 5D). Flow cytometry analysis of GFP expression further supported these observations, with a majority of HeLa-HCN2 cells retaining GFP positivity, whereas a reduced percentage of 293F-HCN2 and HEK293T-HCN2 cells maintained GFP expression (Fig. 5E). This reduction implicates the detrimental effects of adenovirus E1 overexpression on the stability of integrated HCN2 constructs within HEK293 cells. Hence, to augment the durability and efficacy of AAV packaging cell lines, we propose the concurrent regulation of the adenoviral E1 gene and rep through the PST intron.

DISCUSSION

In this research, we have invented a regulatory system specifically designed for the control of overlapping genes through RNA conditional splicing (Fig. 6). This system was rigorously tested for its efficacy in selectively expressing various genes, including Gluc, AAV rep, AAV cap, and HIV genes. Moreover, we successfully utilized this system to

create highly stable and productive all-in-one AAV packaging cell lines, demonstrating its substantial utility in scenarios requiring the conditional expression of overlapping or cytotoxic genes.

Our system underwent thorough optimization and characterization, leading to several key insights that could inform the future development of conditional splicing systems. A pivotal finding was the critical role of a highly efficient splicing site within the PST intron, which dramatically reduced the background expression. The inclusion of 12 bp ISE elements in intron significantly augmented the splicing efficiency by over 40-fold, leading to a 99.9% spliced RNA fraction (Fig. S1). Efficient splicing between SD and SA1 leads to the consumption of SA1; failure to do so may result in leaky splicing between SD and SA2, leading to inadvertent and incomplete expression.

Another significant advancement was the use of the HIV TAR element, which not only facilitated transcription stalling (Fig. 2D and F) but also unexpectedly enhanced the Cre-mediated DNA recombination and induced specific RNA splicing patterns (Fig. 1B and C). To the best of our knowledge, this marks the first observation that a specific DNA sequence near the loxp sites could enhance the Cre-mediated recombination, leading to nearly complete DNA recombination within the PST intron (Fig. 1C). The exact mechanisms behind this phenomenon warrant further investigation, potentially offering insights to refine recombinase-mediated recombination strategies across various applications.

Furthermore, our findings underscore the utility of TAR as a splice donor site, showcasing its ability to fine-tune the transcription termination and RNA splicing (23, 24). By strategically removing SD and SA2 from RNA through SA1- and TAR-mediated dual splicing, we significantly enhanced the conditional splicing system's performance (Fig. 6). This strategy mirrors the Flexon system's approach (25), which similarly utilizes a recombinase-controlled dual splicing intron. The elegance of this approach resides in its ability to transform the linear induction expression mechanism into a pattern of gene expression characterized by two states within a probability distribution framework. Such a transformation substantially mitigates the issue of leaky expression within regulation systems.

Looking ahead, this study lays the groundwork for further enhancements to the conditional splicing system. By integrating concise cis-acting splicing regulatory elements and optimizing their placement (20), we can tailor gene expression levels more precisely to meet specific research or therapeutic needs.

This innovative system proves invaluable in situations where the presence of overlapping genes complicates vector design. For example, adenoviral vectors have restricted packaging capacity, and the elimination of structural overlapping genes from the adenoviral vector genome generates helper-dependent adenovirus (HDAd), allowing for a larger cloning capacity of up to 37 kb (26–28). A similar constraint applies to other viral vectors such as AAV and lentivirus (29, 30). However, the production of these vectors typically involves the use of helper viruses or helper plasmids, presenting a significant obstacle to vector production and hindering their application in preclinical and clinical studies. By facilitating the establishment of producer cells, our approach holds the promise of overcoming these challenges.

Despite its advantages, the conditional splicing system has some limitations. The dependency on recombinase expression, the irreversibility of splicing events, and tissue-specific splicing variability (31) highlight areas for further refinement. Moreover, the inclusion of virus-derived elements, like HIV TAR, may pose potential challenges for certain applications.

To advance the utility and applicability of conditional splicing systems, future research may focus on elucidating the dynamics between cis-acting elements and trans-acting factors across cell types. The development of chemical-induced conditional splicing systems, independent of external inducible systems, and the design of more refined introns with specific splicing elements will enhance the precision and efficacy

of gene regulation. These advancements promise to broaden the scope of genetic engineering, offering new possibilities for gene therapy and beyond.

MATERIALS AND METHODS

Experimental design

The primary objective of our research was to design a sophisticated conditional splicing system that leverages synthetic introns for the targeted expression of viral overlapping genes. This entailed a meticulous evaluation of various regulatory cis-acting elements within synthetic introns to assess their impact on the conditional expression of the reporter gene Gluc. Following these assessments, the synthetic intron demonstrating the highest efficiency was chosen for further applications in the conditional expression of overlapping genes.

Reagents

For this study, key components such as the Gluc reporter gene, synthetic introns, the tetracycline response element (TRE) promoter, T3G, and Cre recombinase were synthesized by BGI. Essential reagents like DNase I and doxycycline were sourced from Sigma Aldrich. Viral vectors, including AAV2/2, AAV2/8, and the helper vectors were procured from Addgene. Transfection reagents were provided by Promega, with FuGENE HD used for cell transfection, and the luminescent substrate coelenterazine obtained from GeneCopoeia and Promega. The plasmid vector pB513 and the transposase expression vector, critical for our experiments, were kindly provided by Huibin Lv. It is noteworthy that *E. coli* harboring the PST plasmid required cultivation at a controlled temperature of less than 30°C to ensure optimal conditions for plasmid stability.

Transfection and Gluc expression detection

HEK293T cells underwent transfection using the FuGENE HD reagent and plasmid DNA at a 3:1 ratio, with a total DNA concentration of 1 µg/mL of cell culture media. The plasmid DNA was initially diluted in OptiMEM to a specific concentration before being mixed with the FuGENE HD reagent. After a brief incubation, the mixture was evenly distributed across the cell culture media. Gluc expression was monitored 48 hours post-transfection by transferring a small volume of the cell culture to a luminescent assay plate, followed by the addition of coelenterazine substrate. Luminescence readings were captured using a Varioskan LUX Multimode Microplate Reader from Thermo Fisher.

Western blot analysis for AAV Rep and Cap proteins

After 2 days of transfection, HEK293T cells, seeded in 6-well plates, were processed for Western blot analysis. Cells were lysed, and proteins were extracted, quantified, and subjected to SDS-PAGE, followed by transfer to PVDF membranes. These membranes were then probed with specific antibodies against AAV Rep (anti-Rep 303.9 mouse monoclonal antibody), Cap proteins (anti-VP B1 mouse monoclonal antibody from Progen), and mouse anti-His tag primary antibody. The primary antibody was diluted in 5% BSA-PBST at a dilution factor of 1:200. Following incubation with HRP-conjugated secondary antibodies, protein bands were visualized using the Pierce ECL Western Blotting substrate.

AAV packaging and quantification

For AAV packaging, the cell culture medium was replaced with a specialized DMEM formulation prior to transfection. Three days post-transfection, the media was treated with DNase I (100 U/mL) for 30 min to remove any unencapsulated DNA, followed by heat inactivation at 70°C for 10 min. AAV titers were quantified by qPCR, employing

a primer pair specific to the Gluc gene (Gluc-qF4: 5′-GGAGCCCATGGAGCAGTTCA-3′ and Gluc-qR4: 5′-AGGCAAAGGTGGCACATCTCTG-3′). A standard curve, generated from dilutions of a known plasmid, facilitated the accurate determination of AAV genome copy numbers in the sample.

Analysis of RNA splicing

For RNA splicing analysis, HEK293T cells transfected with the relevant plasmids underwent total RNA extraction using the RNeasy Mini Kit (Qiagen), complemented by DNA and residual plasmid digestion using the RNase-Free DNase Set (Qiagen, Cat. 79254) as per the manufacturer's protocols. The purified RNA was then reverse transcribed into cDNA using the PrimeScript RT Reagent Kit (Takara), setting the stage for quantitative PCR (qPCR) and gel-based analyses to quantify and visualize spliced versus unspliced mRNA forms.

qPCR was conducted to discern the proportions and absolute quantities of spliced and unspliced mRNA variants, utilizing primer pairs designed for specific detection against a GAPDH reference. The primer sequences employed are as follows:

- GAPDH (Reference Gene): Forward: ACGAATTTGGCTACAGCAACAGGG, Reverse: TCTACATGGCAACTGTGAGGAGG
- Total Gluc mRNA: Forward: GGAGCCCATGGAGCAGTTCA, Reverse: AGGCAAAGGTGGCACATCTCTG
- Unspliced mRNA (SD-SA1): Forward: GGAGGCCAATGCCAGGAAATGACC, Reverse: GGGCGCCTTTGGTGCTCT
- Spliced mRNA (SD-SA2): Forward: GGAGGCCAATGCCAGGAAGGCT, Reverse: CTCGCCAATGCCGCCCTG

For gel-based RNA splicing visualization, PCR amplification was performed using the specified primer pairs, with the resulting products subjected to electrophoresis on TAE gels to confirm the predicted sizes of spliced and unspliced RNA fragments.

Establishment of rAAV packaging cell lines and single-cell cloning

The development of rAAV packaging cell lines entailed co-transfecting HEK293T, 293F, or HeLa cells with the HCN2 and transposase expression vectors. Post-transfection, cells were selected and maintained in DMEM supplemented with FBS, penicillin/streptomycin, GlutaMax, and puromycin. Single-cell cloning was executed by diluting the cells to a specific concentration, followed by seeding in 96-well plates to isolate individual clones, which were further expanded and characterized.

Statistical analysis

Data comparisons were conducted using unpaired *t*-tests, setting significance thresholds at $*P < 0.05$, $**P < 0.01$, and $****P < 0.0001$.

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AUTHOR CONTRIBUTIONS

Qing Yang, Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Project administration, Validation, Visualization, Writing – original draft, Writing – review and editing | Jinlin Wang, Validation | Zhiwei Chen, Supervision, Writing – review and editing, Conceptualization

DATA AVAILABILITY

All data and materials supporting the findings of this study are contained within the article and its supplemental material. Further information will be made available upon request.

ADDITIONAL FILES

The following material is available online.

Supplemental Material

Supplemental figures (JVI00242-24-S0001.docx). Figures S1 and S2.

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