

ARTICLE

Recombination–deletion between homologous cassettes in retrovirus is suppressed via a strategy of degenerate codon substitution

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Transduction and expression procedures in gene therapy protocols may optimally transfer more than a single gene to correct a defect and/or transmit new functions to recipient cells or organisms. This may be accomplished by transduction with two (or more) vectors, or, more efficiently, in a single vector. Occasionally, it may be useful to coexpress homologous genes or chimeric proteins with regions of shared homology. Retroviridae include the dominant vector systems for gene transfer (*e.g.*, gamma-retro and lentiviruses) and are capable of such multigene expression. However, these same viruses are known for efficient recombination–deletion when domains are duplicated within the viral genome. This problem can be averted by resorting to two-vector strategies (two-chain two-vector), but at a penalty to cost, convenience, and efficiency. Employing a chimeric antigen receptor system as an example, we confirm that coexpression of two genes with homologous domains in a single gamma-retroviral vector (two-chain single-vector) leads to recombination–deletion between repeated sequences, excising the equivalent of one of the chimeric antigen receptors. Here, we show that a degenerate codon substitution strategy in the two-chain single-vector format efficiently suppressed intravector deletional loss with rescue of balanced gene coexpression by minimizing sequence homology between repeated domains and preserving the final protein sequence.

Molecular Therapy — Methods & Clinical Development (2014) **1**, 14022; doi:10.1038/mtm.2014.22; published online 9 July 2014

INTRODUCTION

Retroviridae, including gamma-retroviral vector (RV) and lentiviral vector (LV), occupy a dominant position in gene transfer strategies for laboratory and clinical applications.^{1–6} In some instances, obtaining the desired characteristics in the target cells may require coordinate transfer of two or more genes to achieve desired functional outcomes and to which these vectors are readily adapted. In a subset of these instances, there may be a rationale for gene components to be repeated between the expression cassettes. Yet, retroviridae are noted for their high frequency recombination between homologous domains during the process of reverse transcription to provirus formation that has been much studied.^{7–9}

During the production of recombinant proteins, such intramolecular homologous recombination within an expression vector results in a loss of one of the transgenes. We previously confirmed this as an obstacle to coordinate expression of homologous genes within the same vector. One means around this obstacle is to prepare two separate vectors, each expressing one of the homologous genes. However, this is accompanied by need to separately prepare and validate each vector, a significant expense for any clinical application, and to accept the binomial combinatorial inefficiencies of dual gene expression when efficiencies of single gene expression can be

low. For example, a vector transduction efficiency of 10% will predict two-vector gene expression in just 1%, with still worse numbers for lower transfer rates. By contrast, the same 10% transduction efficiency for a single vector containing both genes would be 10% for dual expression without deletion, for a 10-fold gain in coexpression.

A need was apparent for means to reduce or eliminate the occurrence of homologous recombination events. This was achieved by a strategy of degenerate nucleotide substitution in codons of the repeated segments, demonstrated here with a gamma-RV system.

RESULTS

Intramolecular deletion

Our initial experience with gene deletion arose during an attempt to engineer coordinate expression of chimeric antigen receptors (CARs) to provide two signals, with antigen activation (T cell receptor (TCR) zeta Signal 1) and with costimulation (CD28 Signal 2) in T cells. For this, we created an expression vector with two cassettes: one with IgZetapc and the second with IgCD28myc (termed two-chain single-vector (tcsv)). IgZetapc is a chimeric molecule that consists of immunoglobulin (Ig) and CD3 zeta chain while IgCD28myc is a chimeric molecule that consists of immunoglobulin and CD28 (Figure 1a). The two Ig segments have the same

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Received 26 December 2013; accepted 14 April 2014

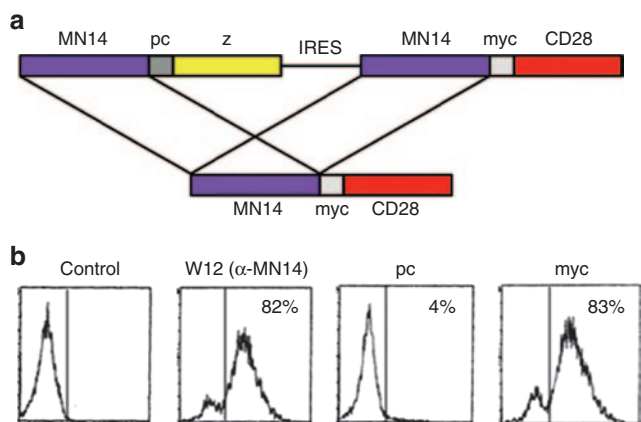


Figure 1 The retroviral deletion phenomenon in two-chain single-vector format. **(a)** Schematic diagram of how two-chain single-vector (tcsv) anti-CEA Ig-chimeric antigen receptor (CAR) results in homologous recombination and deletion. **(b)** Expression of CARs by flow cytometry (FACS). Viral supernatant from transiently transfected E86 cells was used to transduce PA317 packaging cells, then stained individually with W12, anti-pc or anti-myc antibody for flow cytometric analyses. Representative histogram plots are shown. IRES, internal ribosomal entry site.

anti-carcinoembryonic antigen (CEA) immunoglobulin sequences (MN14) in a single-chain Fv format (V_L -linker- V_H). The myc and pc tags were incorporated to follow the separate expression of the two transgenes.

This IgZetapc-internal ribosomal entry site (IRES)-IgCD28myc construct was transferred into the MFG RV. The vector was packaged by usual methods, with plasmid transfection into ecotropic E+86 helper cells, retrovirus harvest, and then infection of amphotropic PA317 helper cells that served as vector producer cells (VPCs) for subsequent human cell transductions. This procedure allows for a single cycle of infection of the PA317 cells; there is no reinfection or infection spread. It is a general feature of VPCs that the high level envelope production by the VPC saturates all receptors and blocks infection of the VPC by virus of the same type as it produces.¹⁰ This is corroborated by the observation that VPCs are stable in their transgene expression and do not increase that expression over time when maintained in culture.

After infection of PA317 with RV, cell sorting was performed with W12 antibody to enrich the transduced cells; W12 is specific for the Ig region and binds both IgZetapc and IgCD28myc. On reculture, 82% of the sorted cells were proven to be transduced by vector with W12 remaining (Figure 1b). When stained with anti-pc or anti-myc antibody, 83% of PA317 cells expressed IgCD28myc, but only 4% of PA317 cells expressed IgZetapc. Thus, the transduced cells were 100% positive for myc (83%/82%) but only 5% positive for Pc (4%/82%). Reanalysis of the 5% pc+ fraction (not shown) proved these cells to be 92% IgCD28myc+ (data not shown), meaning these 5% of cells (myc+ pc+) were the only ones undelivered in the tcsv construct for expression, compatible with a 95% deletion rate overall.

These results are consistent with a homologous recombination between the cassettes, which in effect deletes the equivalent of the upstream cassette (Figure 1a). As a result, most (95%) of the transduced packaging cells expressed only IgCD28 with only a small 5% fraction also expressing IgZeta.

Example with TCR-CAR

When we and others subsequently validated the possibility to express two signals (e.g., CD28 and zeta) in one chimeric molecule

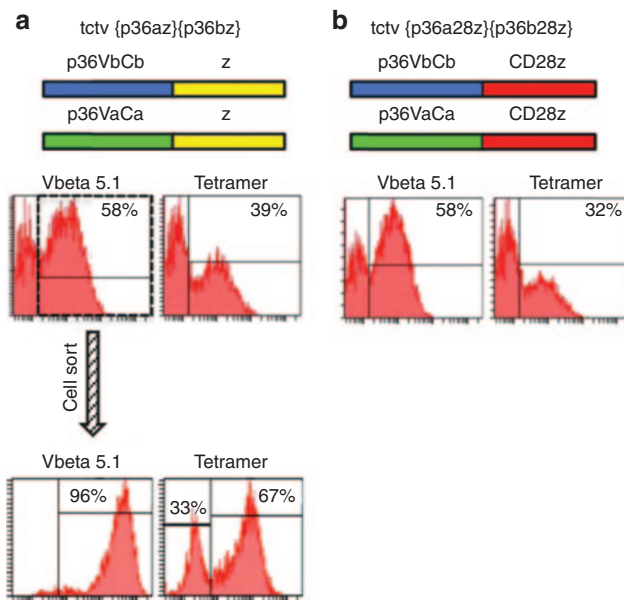


Figure 2 Reduced efficiency of gene co-expression by retrovirus in two-chain two-vector format (tctv). Viral supernatant collected from 293-gp cells transiently co-transfected with indicated two vector constructs ($\{p36az\}$ and $\{p36bz\}$ in **(a)**, or $\{p36a28z\}$ and $\{p36b28z\}$ in **(b)**) were used to transduce PG13 packaging cells. Cells were then stained with either anti-T cell receptor (TCR) Vb5.1 antibody or p36/A2 tetramer for flow cytometric analyses. **(a,b)** Schematic diagram of tctv constructs are shown (top), and the representative example of the expression of CARs by FACS are shown below. In **(a)**, TCRVb5.1 positive cells were enriched by cell sorting and stained again with either anti-TCR Vb5.1 antibody or p36/A2 tetramer.

(e.g., Ig28z),^{11–13} the impetus to overcome the problem of intravector deletion dissipated. However, the matter resurfaced as an obstacle as the field progressed to consider chimeric T cell receptors (TCR-CARs) for immunotherapy applications,¹⁴ which requires two chains for major histocompatibility complex class I peptide antigen recognition. We prepared TCR-CARs against JC virus (JCV) for therapy of progressive multifocal leukoencephalopathy,¹⁵ a deadly demyelinating brain disease that accompanies immunodeficiency and that occurs iatrogenically with anti-VLA4 antibodies that block access of T cells to brain, as applied in multiple sclerosis therapies.¹⁶

The general form of the TCR-CARs was TCR α -z/28/28z (Va) and TCR β -z/28/28z (Vb), in which the signaling domains were now the repeated segments rather than the antigen recognition domains as in Figure 1. This effort was initiated with construction of separate vectors and cotransduction, which we have termed two-chain two-vector (tctv) format (Figure 2). This provided a pattern of single and double transductions, in which only the double transduction yielded functional Va + Vb products.

This format had the expected inefficiencies of a two-vector design, as illustrated in the following. In Figure 2, Vb could be detected with anti-Vb Ab, but Va could not be separately detected because there are no anti-Va Abs available. Va + Vb was detected by tetramer staining. In the examples of Figure 2a,b, we observe 58% staining for Vb in each case, but only 32–39% staining for Va + Vb (Tetramer). In general, if p is the transduction rate for one vector, then p² is the cotransduction rate for two vectors, if they are equal in efficiency (or p₁*p₂ if different in efficiency). The results of Figure 2 are compatible with ~58% of cells transduced with Va and ~58% of cells transduced with Vb, to give 58% × 58% = ~34% with Va + Vb. In contrast, the low predicted

cotransduction rate that results from use of separate vectors can theoretically be remedied by using a nondeleting tcsv format.

Degenerate codon substitution: concept

In concept, the problem of intrachain deletion is mediated by recombination between homologous domains. High recombination rates are a hallmark of retroviruses that have been much-studied,⁷⁻⁹ with all models involving the reverse transcription step (Figure 3). As an approach to bypass this problem, we hypothesized that reducing the homology via substitution of degenerate codons might inhibit the process while preserving the native protein amino acid sequences. Methionine (Met, M) and tryptophan (Trp, W) are uniquely encoded by single codons (ATG and TTG, respectively), but all other amino acid residues are encoded by two or more codons, mostly differing by a single base (17 amino acids), but in two cases

differing in two of the three bases (Leu, L; Arg, R).¹⁷ This degeneracy of the genetic code allows for synthesis of identical polypeptides by nonidentical nucleic acid sequences.

On average, if one base per codon were changed for each amino acid, homology would decrease by one-third, to 66.7%. We redesigned the zeta (z) chain to maximize the mismatch via degenerate nucleotide substitution (dz), and obtained a result of 61.6% homology (Figure 4). For a second generation construct, we engineered a CD28-zeta (28z) conformer with degenerate nucleotides (d28z), with 60.5% homology (not shown). We created tcsv TCR-CARs in alternative formats: Vb.z/28/28z-2A-Va.dz/d28/d28z, in which 2A peptide was employed in place of IRES for an improved dual gene expression.¹⁸

Deletion rates

Because Ab detection was not available for Va chain, Va could be assessed only as Va + Vb by tetramer binding. To assess undeleted versus deleted forms, it was necessary to design the vector with Vb upstream: all transduced products are Vb+: intact two-chain product is Vb+ tetramer+, deleted one-chain product is Vb+ tetramer- (e.g., Figure 6a below). (Va upstream would yield Va-zeta for the deleted form with no opportunity to assess its presence or, thus, to specify the denominator for total transduction.)

As a control, we applied transfections of the tcsv retroviral expression plasmids with wild-type or mutated signaling domains (Figure 5). Cell transfection is a setting where no deletion is expected. We measured Vb and tetramer on seven tcsv constructs, four with wild-type sequence and three with degenerate codon substitution. A comparison of three vector pairs in pair-wise fashion showed differences of 0-1% in the tetramer/Vb ratios, confirming efficient transcription and translation that was undisturbed by the codon substitutions. Across all seven tcsv, the tetramer/Vb ratio was 0.98 ± 0.08 . This ratio is important for showing equivalence of the Vb and tetramer staining that otherwise could be imbalanced at baseline. With this ratio result (0.98) being close to 1.0, no correction was applied in the ensuing tests.

These statistics are used to specify the sensitivity of the flow cytometry assay to detect deletions. From these control tests, the 0.98 ± 0.08 tetramer/Vb ratio can be shown to calculate to an apparent "deletion" rate of $D = 2 \pm 8\%$ where no deletion occurs. This is the flow cytometry assay "noise." An assay sensitivity or limit of detection was set at +1 SD = 10%, above which 16% of undeleted forms may be predicted to be falsely positive (*Methods*).

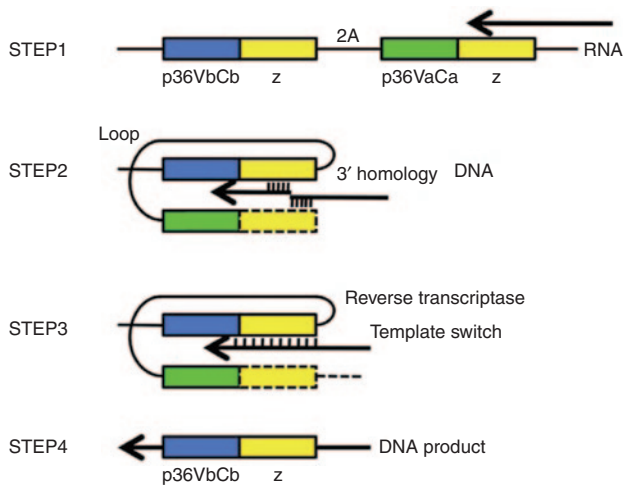


Figure 3 Deletion mechanism. The recombination-deletion is conceived to occur by the following steps. Step 1: Reverse transcription begins at the 3' end of the retrovirus RNA. Step 2: A homologous domain (Z) in the second, upstream (5') cassette interacts with the extending complementary DNA (cDNA) strand to initiate a transfer of the growing cDNA strand to the new site. Step 3: There is a template switch and completion of the transcription of the upstream gene that bypasses the intervening sequence. Step 4: The completed synthetic product is equivalent to a copy of the upstream gene (Vb) with the downstream gene lost. The normal high frequency of retroviral vector RNA fragmentation, along with virion RNase H activity to create cDNA overhangs, is likely integral to this process.

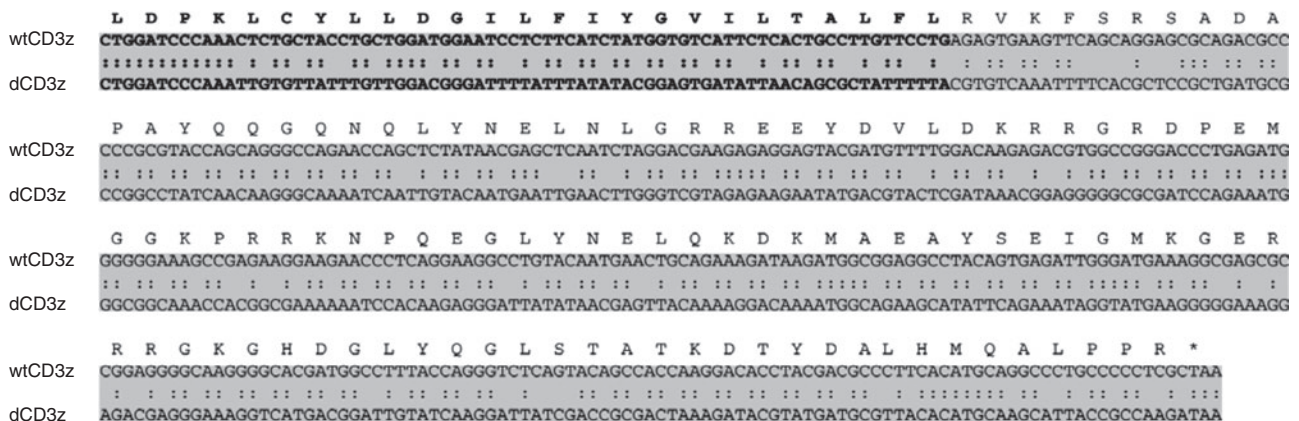


Figure 4 Degenerate nucleotide design. Alignment of wild-type (wtCD3z, top) and degenerate codon substituted zeta (dCD3z, bottom) nucleotide sequences and their amino acid sequences. Bold letters correspond to the transmembrane domain of zeta chain.

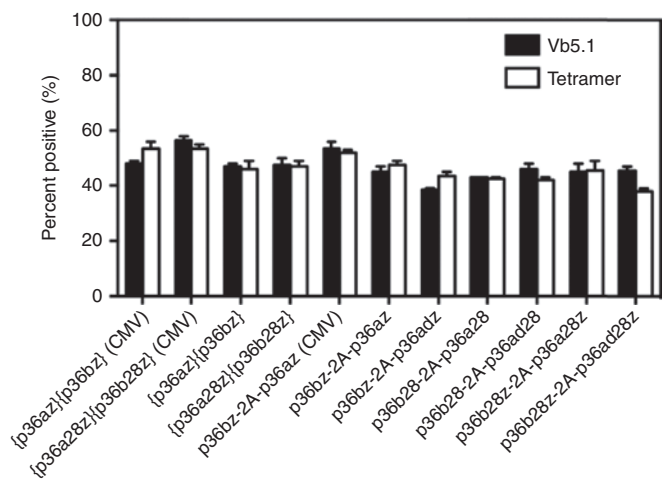


Figure 5 Transfection that avoids reverse transcription results in no deletion. 293-gp cells were transiently transfected with the indicated two-chain two-vector (tctv) or two-chain single-vector (tcsv) constructs. Constructs with cytomegalovirus (CMV) vector were included as a negative control for homologous recombination. Cells were then stained with either anti-TCR Vb5.1 antibody or p36/A2 tetramer for flow cytometric analyses. The black and white bars indicate percentage of TCR Vb5.1 or p36/A2 tetramer positive population, respectively. Error bars represent standard deviation from duplicate assays.

When RV supernatant was collected from these cells, then used to infect gene transfer targets, deletions became apparent (Figure 6). Wildtype and degenerate constructs (Figure 6a) were compared for deletion. Marked deficiencies were evident in tetramer versus Vb in controls, representing deletion of the Va chain (Figure 6b), that were reversed in the degenerate codon-substituted constructs (Figure 6c). Deletion rates were quantified as the loss of Va necessary to cause the under-representation of tetramer in the staining procedure, estimated at 37–72% for one transduction cycle (Table 1; Figure 6d). When measured after degenerate codon substitution, the calculated deletion rate was markedly suppressed (4–9%). The calculated values of 4–9% are all under the 10% threshold specified for sensitivity and therefore not distinguishable from true deletion rates of 0. That is, this strategy may have totally suppressed deletion, or it may leave a small residual rate.

The study was initially conducted by singly staining the cells with either tetramer or Vb antibody with the same fluorophore to ensure no mutual interference during staining that could affect the accurate measurement of the deletion rate. In Figure 7, we applied dual (two-color) staining of cells transduced with either p36bz-2A-p36az or p36bz-2A-p36adz. Cells transduced with wild-type zeta showed a distinct deleted population (Vb + Tetramer-) with a mean deletion rate of 54% (single-positive/(single-positive + double-positive)), while cells transduced with degenerate zeta showed no corresponding population of cells in the single-positive quadrant. By all

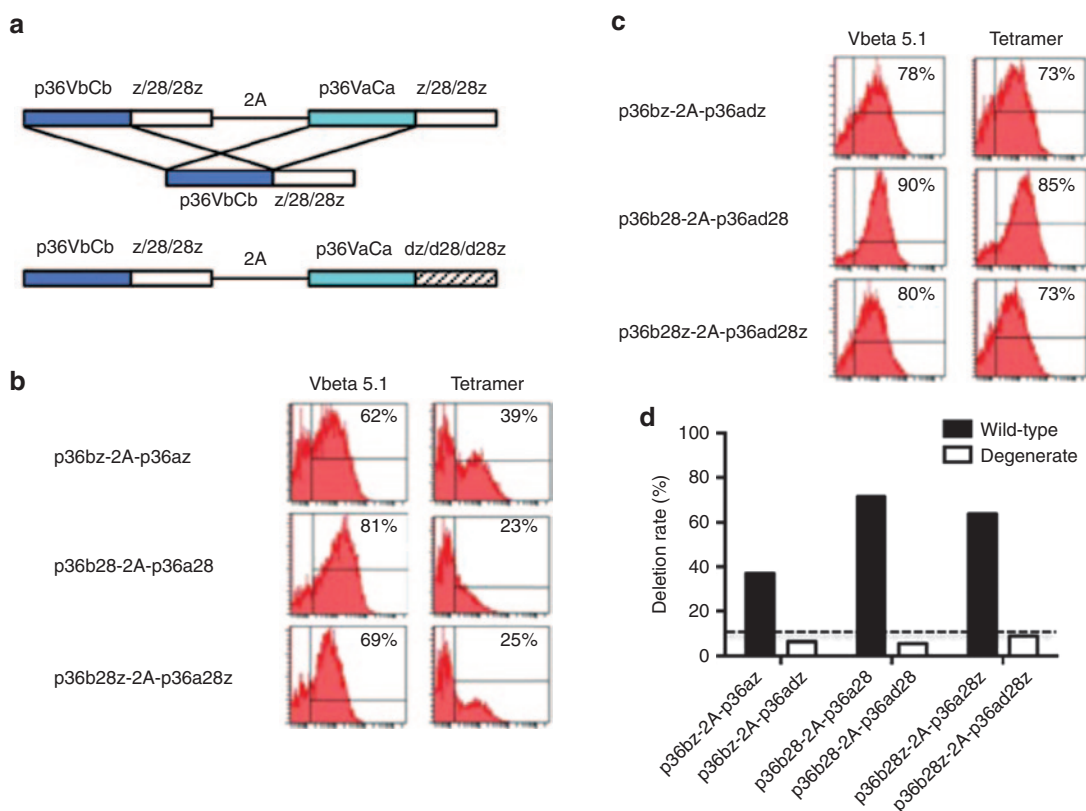


Figure 6 Degenerate codon substitution suppresses deletion in tcsv retroviral vector. (a) Schematic diagram of tcsv chimeric antigen receptor (CAR) constructs resulting in deletion by homologous recombination (p36bz/28/28z-2A-p36az/28/28z, top) or avoiding deletion via degenerate codon substitution (p36bz/28/28z-2A-p36adz/d28/d28z, bottom). The degenerate constructs dz/d28/d28z had a reduction in homology by 61.6%/60.5%/59.7%, respectively. Supernatant from 293-gp cells transiently transfected with the tcsv constructs in (a) were used to transduce PG13 cells to assess the expression level of CAR by FACS. Cells were stained with either anti-TCR Vb5.1 antibody or p36/A2 tetramer. The representative data shown in (b) and (c) are PG13 cells transduced with wild-type or degenerate codon-substituted constructs. (d) Summary of deletion suppression: Results from (b) and (c) was calculated as described in *Methods*. The dotted line at D = 10% represents the lower limit for detecting deletion activity by FACS, with values below this not distinguishable from zero. See text for derivation.

appearances, deletion was fully suppressed. Comparable results were obtained with a recently constructed vector coexpressing MN14CD28z and degenerate MN14CD2 (Figure 8). This construct with degenerate nucleotide coding overcame the deletion problem seen in Figure 1 with an analogous construct (compare myc and V5 in Figure 8b with pc and myc in Figure 1b).

Finally, as a test of the deletion rate with a direct measure of TCR α chain, tctv and tcsv anti-JCV chimeric TCR proviral integrant levels in transduced PG13 cells were analyzed using real-time polymerase chain reaction (PCR) with primers specific to quantitate the ratio of p36VaCa to p36VbCb chains (Figure 9). An approximate equal ratio of Va to Vb was detected in cells in mixed infections with the separate tctv anti-JCV vectors (ratio, 0.89 ± 0.03). The ratio of Va to Vb however was lower in cells transduced with tcsv anti-JCV cTCRs with homologous CD3z domains, indicating deletion of the internal p36VaCa chain (0.57 ± 0.02 with IRES, 0.47 ± 0.04 with P2A). In contrast, an equal ratio of Va to Vb was detected in PG13 cells transduced with tcsv anti-JCV cTCRs engineered with degenerate CD3z nucleotides (1.22 ± 0.04 with IRES, 1.09 ± 0.05 with P2A). Deletion rates of Va with four wild-type tcsv constructs ranged from 50 to 80% in one cycle that appeared fully suppressed with degenerate codons. On comparison of the two methods for deletion detection, there was a general correspondence between the flow and quantitative PCR data for the constructs tested (Table 2).

DISCUSSION

The intramolecular recombination between homologous sequences in retroviral genomes has been amply demonstrated

in prior studies. The vulnerable period for genome instability is during reverse transcription, where the RNA genome is converted to DNA provirus.⁷⁻⁹ Our results indicate that phenomenon is replicated in modern RV systems with deletion of segments between the duplicated elements, ranging from 35 to 95% in one infection cycle versus no deletion by transfection. This means that transfection can create a VPC with balanced expression of genes that will be deleted only when virus produced by this VPC infects the cell targeted for gene transfer. The quantitative differences in deletion rates may be influenced by the composition and length of the repeated sequences, their separation distance in the vector, or even by the host cell and temperature conditions. Where there is sequential infection and reverse transcription, as in so-called ping-pong methods for VPC generation¹⁹ or virus spread in cultures, a complete loss of the gene duplication may be envisioned.

Accordingly, this phenomenon was predicted to constrain protocols for coexpression of homologous genes for transfer of desired cell functions. CARs are created as genetic fusions of antigen recognition domains with intracellular signaling chains.²⁰ In the CAR formats, one could need to express two nonhomologous chains such as TCRs with common signaling domains,^{14,15} or common antigen recognition domains with differing signaling domains (Figure 1). In other situations, the impact of gene duplication to increase cellular protein levels may have its own benefits. This problem of intravector deletion can be circumvented by cumbersome means, with preparation of two (or more) vectors with higher costs, and at sacrifice to efficiency versus a single vector.

To overcome this obstacle, we devised a strategy of interdomain homology minimization via degenerate codon substitution within duplicated domains in a CAR model. This method reduces domain homologies from 100 to ~60%. Under this circumstance, the residual deletion rate was markedly suppressed, as assessed by flow cytometry and confirmed by quantitative PCR, to levels that were indistinguishable from a no-deletion control. As a result, this enabled strategy of degenerate nucleotide substitution could vastly enhance emerging methods for viral and cancer targeting with CAR-modified T cells ("designer T cells") and should work equally in retroviral and lentiviral vector systems. To this point, a lentiviral model to codeliver two highly homologous Zn-finger nuclease monomers for site-specific gene modification recently applied this strategy for improved gene coexpression,²¹ confirming the generality of this approach for two genres of Retroviridae.

Table 1 Summary of deletion rates

	Wild type			Degen codon		
	Vb	Tet	deletion (%)	Vb	Tet	deletion (%)
p36bz-2A-p36az	62	39	37	77	74	(4)
p36b28-2A-p36a28	81	23	72	90	85	(6)
p36b28z-2A-p36a28z	69	25	64	80	73	(9)

Expression levels from Figure 6. Deletion rates calculated as in Methods. () indicate deletion rates <10% that are below the detection threshold and not statistically distinguishable from 0% deletion.

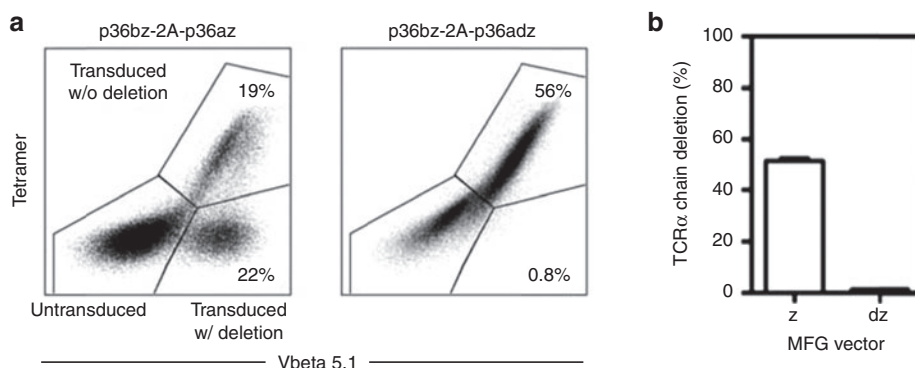


Figure 7 Degenerate codon substitution prevents intravector deletion. **(a)** Expression of CARs by FACS: viral supernatant from transiently transfected 293-gp cells with indicated constructs was used to transduce PG13 packaging cells, then costained with anti-T cell receptor (TCR) Vb5.1-APC antibody and p36/A2 tetramer-PE for flow cytometric analyses. Representative data are shown. **(b)** TCR α chain deletion rate. The deletion rate was calculated as transduced cells with deletion (TCR Vb5.1⁺Tetramer⁻ population)/total transduced cells (TCR Vb5.1⁺ population) \times 100 (%). Error bars represent standard deviation of the mean from four results.

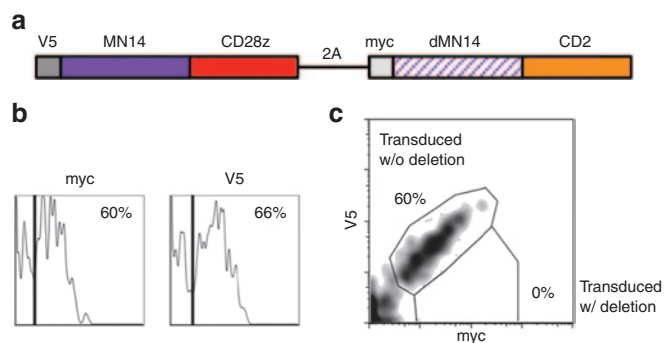


Figure 8 Rescuing of dual antibody recognition domains coexpressed in a bicistronic retroviral vector by degenerate codon substitution. **(a)** Schematic diagram of tcsv chimeric antigen receptor (CAR) construct MN14CD28z_2A_dMN14CD2. Hashed area depicts degenerate codon substitution (dMN14). Note, the different location of tag epitopes (V5, myc) compared to the construct in Figure 1 (pc, myc). In Figure 1, pc is lost in deleted form, whereas here, myc is lost in the deleted form. **(b)** and **(c)** Expression of CARs by FACS: Viral supernatant from transiently transfected 293-gp cells were used to transduce PG13 cells, then costained with anti-V5-Alexa Fluor 647 and anti-myc-fluorescein isothiocyanate antibodies for flow cytometric analyses. Representative data are shown in **(b)** as single-parameter histogram comparable to Figure 1b and in **(c)** as dual-parameter density plot analogous to Figure 7a. The vertical line in **(b)** indicates untransduced controls. Panel **(c)** shows absence of products in deletion region.

Finally, the extent of mismatch required to maintain effective deletion suppression and dual gene expression remains undefined. The possibility exists, therefore, that still higher replications of homology could be undertaken in a single vector. By this, the bases constituting the 40% mismatch are apportioned across not just two domains as in these examples but across three or more homologous regions, with each domain, e.g., 20% different from each of the other repeated domains.

MATERIALS AND METHODS

Antibodies and cell lines

W12 anti-idiotypic antibody specific for hMN14, the antibody portion of the anti-CEA CAR, was a gift of Hans Hansen (Immunomedics, Morris Plain, NJ). Mouse anti-pc, anti-V5, and anti-myc epitope antibodies were purchased from Invitrogen (Grand Island, NY). Phycoerythrin (PE)-conjugated anti-human Vb5.1 antibody (clone IMMUI57) was purchased from Immunotech (Beckman Coulter, Pasadena, CA). JCV-specific p36 peptide (VP1_{p36-44}; SITEVECFLL)²² (Invitrogen) was prepared as PE-conjugated p36/HLA-A*0201 tetramer by the NIH Tetramer Core Facility (Emory University, Atlanta, GA).

Retroviral packaging cell lines 293-gag-pol (gp) and PG 13 were obtained from American Type Culture Collection (ATCC, Manassas, VA). 293T p36 positive control cells were generated by transduction with tctv {p36VaCaz} {p36VbCbz} constructs and sorted to >95% tetramer and Vb5.1 expression using FACSVantage SE (Becton Dickinson, Franklin Lakes, NJ).

Retroviral vector construction

DNA fragments coding IgZetapc and IgCD28myc were inserted into MFG RV (gift of R Mulligan, Children's Hospital, Boston). An IRES fragment was inserted between IgZetapc and IgCD28myc. To construct IgZetapc, PCR was performed to insert a pc epitope sequence at the 3' end of anti-CEA immunoglobulin (MN14) single-chain sequence from a RV that contains anti-CEA IgZeta molecule (termed IgTCR).²³ The newly generated PCR fragment was inserted in place of the original anti-CEA immunoglobulin single-chain sequence in the RV. To construct IgCD28myc, PCR was performed to insert a myc epitope at the 3' end of anti-CEA immunoglobulin single-chain sequence from the same RV and fused with a PCR fragment that was encoding for CD28. The IRES fragment was generated by PCR from plasmid pCITE-4a that contains IRES sequence.

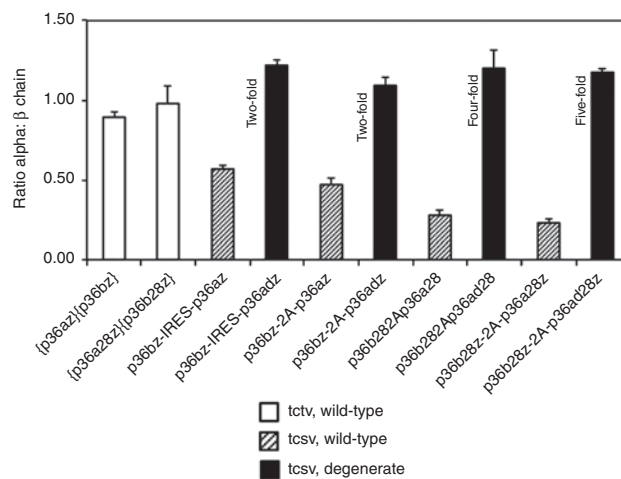


Figure 9 Ratio of TCR α : β chain determined by real-time PCR. 293-gp cells were transiently transfected with indicated constructs, and the viral supernatants were used to transduce PG13 packaging cells. Real-time PCR was then performed using the isolated genomic DNA from transduced cells to measure the deletion rate. The deletion rate was calculated as $D = (Vb - Va)/Vb$. Error bars represent standard error of the mean from triplicate results. tctv, two-chain two-vector; tcsv, two-chain single-vector.

Table 2 Net gain in dual expression via degenerate codon substitution estimated by different methods

Constructs, tcsv	Flow	PCR
	tet:Vb ratio fold-change	
	Degen:Wildtype	Va:Vb ratio fold-change
p36bz-IRES-p36adz	2	2
p36bz-2A-p36adz	2	2
p36b28-2A-p36ad28	3	4
p36b28z-2A-p36ad28z	3	5

PCR, polymerase chain reaction; tcsv, two-chain single-vector.

To construct tctv {p36VaCaz} and {p36VbCbz}, the human CD3 zeta (ζ) (NM_000734.3) transmembrane (tm) to cytoplasmic (cyt) region was PCR amplified with 5'-XhoI-BamHI/NotI-3' restriction sites (primers f1/r1) and cloned into the modified MFG mcs to generate an MFG-CD3z entry vector. The p36VaCa and p36VbCb chains were PCR amplified with 5'-XhoI/BamHI-3' sites (primers f2/r2 and f3/r3) and cloned into the MFG-CD3z vector. cDNA sequences encoding the p36VaCa and p36VbCb chains were described previously.¹⁵ To construct tcsv {p36VbCbz-IRES-p36VaCaz}, p36VbCbz was PCR amplified with NcoI/Clal-NotI sites (primers f4/r4) and cloned into the modified MFG mcs. IRES was PCR amplified with Clal/XhoI-NotI sites (primers f5/r5/r6) for cloning to generate p36VbCbz-IRES, and for subsequent entry of p36VaCaz. To construct tcsv {p36VbCbz-2A-p36VaCaz}, p36VbCbz was PCR amplified with NcoI/Clal sites (primers f4/r7) to remove the stop codon. A peptide motif P2A derived from Foamy virus 2A sequence, was amplified with Clal/XhoI sites (primers f6/r8) and cloned inframe to replace IRES. To construct {p36VbCbz-IRES-p36VaCadz} and {p36VbCbz-2A-p36VaCadz}, a degenerate CD3z sequence was constructed with multiple oligonucleotides (primers d1 to d8) and flanking BamHI/NotI sites (primers df1/dr1) for cloning to replace the wildtype CD3z in the p36VaCaz chain. Primer sequences are shown (Table 3).

Table 3 Oligonucleotide sequences used for constructing retroviral vectors

Two-chain two-vector (tctv) and two-chain single-vector (tcsv) primers	
Primer	Sequence (5'→3')
f1	Gctcgaggctagcccatggctgagatcccaactctgctac
r1	Gcggccgcttagcgaggggcaaagcttg
f2	Cccggccctctgagttatgatgaatccttgagag
r2	Caattgggatccagatccccacaggaacttctgg
f3	Gctcgagaccatgggctccaggctgctctgttggg
r3	Cggggatccagatccccacagtctgctctacccaggc
f4	Gctagccatgggctccaggctgctctgttgg
r4	Ggatccagatccccacagtctgctctacccaggc
f5	Gctaaatcgatgtattttccacatattgccgttttgg
r5	Gcggcctctagacataggaattctcgag
f6	Atcgatagcagcaacagcgaagaagcgtagcac
r6	Catatggaattcctcgagtatcatctgttttcaaagg
r7	Ggtggaagcggcgcaataacatcgatcgagggggcaaag
r8	Gcggcctatataaaactcgagaagcccggttttc
Degenerate CD3 zeta primers	
Primer	Sequence (5'→3')
df1	Ctggatcccaattgtgttatttggtagcggg
d1	Gttatttggtagcgggatttattatatacggagt
d2	Cgggattttatatacggagtattacacagcgc
d3	Gtgatattaacagcgtattttacgtgcaaatccacgctccgctg
d4	Ttcttctcagcccaagtcaattcattgtacaattgttgccttggtagcggcgatcagcggagcgtgaaaattgacacg
d5	Cttgggtcgtagagaagaatagcgtactcagataaacggagggggcgatccagaatggcgcgcaaacaccgcgcaaaaatccacaag
d6	Cccctcaccatttctgaatgcttctgcaatttgcctttgtaactcgttataatacctcttgggatttttgcgctggttgg
d7	Ttcagaaataggtgaagggggaaaggagacgagggaaaggtcatgacggattgtatcaaggattatcgaccgactaaagatacgtatgatg
d8	Ttacttggcgtaagtctgcatgtaacgcacatcgtatcttttagtcg
dr1	Gcggcctctagattatcttggcgtaagtcttgc

Retrovirus production and transduction

RV MFG-IgZetapc-IRES-IgCD28myc was transfected into ecotropic E86 retroviral packaging cells by lipofectamine (Gibco, Carlsbad, CA)-mediated transfection. Supernatant from transfected E86 cells was collected and used to transduce amphotropic PA317 retroviral packaging cells. Transduced PA317 cells were sorted by flow cytometer with WI2 antibody or anti-pc antibody.

For the TCR-CAR, retroviral supernatant was produced using an optimized protocol. 293-gp cells were transiently transfected with MFG RV plasmid and vesicular stomatitis virus envelope G protein plasmid (LipoD293; SigmaGen Laboratories, Rockville, MD). Supernatant was collected and concentrated for subsequent use to transduce PG13 packaging cells.

Flow cytometry

For the TCR-CAR studies, for single antibody staining, cells were incubated at room temperature for 15–30 minutes with either PE-conjugated anti-human Vb5.1 (Affymetrix, Santa Clara, CA) or PE-conjugated p36/HLA-A0201 tetramer (NIH Tetramer Core Facility). Tetramer-stained cells did not undergo washing prior to flow cytometry. For double staining, cells were incubated at room temperature for 15 minutes with p36 tetramer-PE followed by Vb5.1-APC and incubation on ice for 15 minutes. After subsequent washing, cells were analyzed directly using the BD-LSRII (Becton Dickinson).

Real-time PCR

Genomic DNA was isolated from transduced PG13 cells using the DNeasy kit (Qiagen, Valencia, CA). Oligonucleotides were designed using PrimerSelect (DNASar, Madison, WI) specific for the p36 α constant domain (5'-ggcctggagcaacaatct-3'/5'-cagatccccacaggaactt-3', 106 bp) and p36 β constant domain (5'-aaccacttccgctgtcaagt-3'/5'-tccccacagtctgctacc-3', 125 bp). Real-time PCR was performed using the Bio-Rad CFX96 detection system (Bio-Rad, Hercules, CA). Reactions contained Maxima SYBR Green qPCR Master Mix (Fermentas, Pittsburgh, PA), 0.3 μ mol/l each oligonucleotide and 200 ng genomic DNA. Amplifications were at 95 °C for 10 minutes, 40 cycles at 94 °C for 20 seconds, 60 °C for 30 seconds, and 72 °C for 30 seconds. Fluorescence data were acquired at the 72 °C extension phase. Product specificity was confirmed by melt curve analysis. Ratios of p36 α to p36 β in PG13 cells transduced with tcsv p36 constructs were calculated from absolute copy numbers extrapolated from plasmid standard curve dilutions.

Calculations

For assays by flow cytometry, the ratio of tetramer/Vb indicates the fraction of vector that is undeleted and $D = 1 - \text{tetramer}/Vb = (Vb - \text{tetramer})/Vb$ is the rate of deletion. For assays by quantitative PCR, the deletion rate was calculated as $D = (Vb - Va)/Vb$. On the basis of control tests for nondeletion by flow, the tetramer/Vb ratio was 0.98 ± 0.08 ($N = 7$), which calculated to a "deletion rate" of $2 \pm 8\%$; this represents the "noise" of the flow cytometry

assay. From this, a limit of detection (sensitivity) for deletion was set as mean $+1\text{ SD} = 2\% + 8\% = 10\%$. By this criterion, under a normal distribution of values, 16% of non-deleted constructs would be expected to appear as deleting ($D > 10\%$); this is the expected false positive rate for a true negative sample.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENTS

We thank Esther Beaudoin and Kartik Pawar for technical assistance, and Elizabeth Barros for graphics and clerical assistance. This work was supported by grants to R.P.J. from the NIAID and the DOD Breast Cancer Research Program, as well as RWMC research funds to E.J.I.

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