Generation of RCAS Vectors Useful for Functional Genomic Analyses

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Abstract

Avian leukosis type A virus-derived retroviral vectors have been used to introduce genes into cells expressing the corresponding avian receptor tv-a. This includes the use of Replication-Competent Avian sarcoma-leukosis virus (ASLV) long terminal repeat (LTR) with Splice acceptor (RCAS) vectors in the analysis of avian development, human and murine cell cultures, murine cell lineage studies and cancer biology. Previously, cloning of genes into this virus was difficult due to the large size of the vector and sparse cloning sites. To overcome some of the disadvantages of traditional cloning using the RCASBP-Y vector, we have modified the RCASBP-Y to incorporate “Gateway” site-specific recombination cloning of genes into the construct, either with or without HA epitope tags. We have found the repetitive “att” sequences, which are the targets for site-specific recombination, do not impair the production of infectious viral particles or the expression of the gene of interest. This is the first instance of site-specific recombination being used to generate retroviral gene constructs. These viral constructs will allow for the efficient transfer and expression of cDNAs needed for functional genomic analyses.

Key words: RCAS; tv-a; retrovirus; recombination; Gateway; gene expression

† Replication-Competent Avian sarcoma-leukosis Virus (ASLV) long terminal repeat (LTR) with Splice acceptor (RCAS) vectors1,2 are a useful tool for the introduction and expression of genes in both cell culture and animal model systems. The ability of RCAS vectors to infect cells relies on the cell expressing the avian receptor tv-a3,4 and on active cell division.5–7 The RCAS-TVA system has been used to express genes in avian cells, in addition to mammalian cells and transgenic mice into which tv-a has been introduced under the control of a cell lineage-specific promoter (http://rex.nci.nih.gov/RESEARCH/basic/varmus/tva-web/tva2.html). These animals are extremely useful for experiments involving cell lineage-directed infection by RCAS virus8–10 and for generating murine models of specific cancers.11,12

As the entire repertoire of genes is being identified in the mammalian genome, and clues to cellular gene functions are being proposed by expression studies, it will be useful to test gene function by cell type-directed over-expression in chicken embryos or tv-a transgenic mice. To take full advantage of the RCAS-TVA system as a high-throughput screening tool, it is necessary to efficiently and accurately clone genes into the RCAS vectors. However, previous work with the RCASBP-Y vector and traditional restriction enzyme cloning proved to be a time-intensive process due to the dependence on gene restriction enzyme sites. This was further complicated by the large size of the RCASBP-Y (∼11.6 kb) and the availability of only three restriction enzyme sites for gene insertion. Thus, an independent cloning strategy and a multiple cloning site shuttle vector were required to insert the gene of interest correctly into RCASBP-Y. We sought to modify this vector using a recombinational cloning strategy to allow efficient introduction of genes while simultaneously creating epitope tag fusions.13

In this paper, we describe three RCAS vectors that have been adapted to allow the transfer of genes via recombinational cloning. Use of these vectors overcomes many of the problems associated with sparse cloning sites and large vector size. These vectors can be used for the cloning and evaluation of many genes simultaneously by
taking advantage of the full-length cDNA libraries built in recombinational cloning of compatible vectors.\textsuperscript{14–16}

1. Results and Discussion

Retroviral vectors have provided a useful tool for the introduction and expression of genes in cells. To overcome some of the disadvantages of traditional cloning using the RCASBP-Y vector, we modified the RCASBP-Y vector construct using the Gateway recombinational cloning system. Recombinational cloning involves the introduction of a site-specific recombination cassette and takes advantage of \( \lambda \) integrase recombinase proteins, integrase (INT), integration host factor (IHF) and excisionase (XIS) to transfer DNA sequences flanked by recombination \( \text{att} \) sites between vectors.\textsuperscript{17} Cloning via PCR products is accomplished by the incorporation of \( \text{att} \) site linkers attached at the 5' end to gene-specific PCR primers. Orientation of the PCR product is maintained during the recombination process by modification of the linkers \((\text{att}B1 5' \text{ gene primer}, \text{att}B2 3' \text{ gene primer})\). These \( \text{att} \)B-linked PCR products can then be cloned using donor vectors containing \( \text{att}P1 \) and \( \text{att}P2 \) sites using INT and IHF. Following the recombination between the \( \text{att}B1 \) and \( \text{att}P1 \) sites, and \( \text{att}B2 \) and \( \text{att}P2 \) sites, sequences for \( \text{att}L1 \) and \( \text{att}L2 \) are generated, and the resulting plasmid clone is termed an entry vector. Final transfer of the gene of interest from the entry clone (containing \( \text{att}L \) sites) to any of the final destination vectors can be accomplished by incubation with INT, IHF, and XIS. The definition of a destination vector is a DNA cassette containing the \( \text{ccdB} \) gene flanked by \( \text{attR1} \) and \( \text{attR2} \) sites.\textsuperscript{13}

To develop a generation of RCAS vectors into which genes could be transferred rapidly and efficiently, we modified the RCASBP-Y vector for Gateway recombinational cloning. Three different RCASBP-Y destination vectors were generated (Fig. 1). The first destination vector, RCASBP-Y DV, was created by insertion of a Gateway reading frame cassette (RFC) into the \( \text{LTR} \) site downstream of splice acceptor sequences (Fig. 1A). Genes inserted here would be under the expression control of the upstream retroviral LTR and would be the prominent mRNA splice product when transferred to mammalian cells.\textsuperscript{2} The inserted RFC contained both the \( \text{ccdB} \) gene and the chloramphenicol resistance gene flanked by \( \text{attR1} \) and \( \text{attR2} \) recombination sequence sites. The amino-terminus and carboxy-terminus HA epitope tag destination vector constructs (RCASBP-Y NHA and RCASBP-Y CHA, respectively) were generated incorporating an in-frame oligonucleotide encoding an HA epitope tag in addition to the Gateway RFC (Fig. 1B,1C). This provided for an in-frame fusion of an amino-terminus HA epitope-\( \text{att}B1 \) site linker (23 amino acids) or \( \text{att}B2 \) site linker-carboxy-terminus HA epitope (20 amino acids) fused to expressed proteins, respec-

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\caption{RCAS Gateway retroviral destination vector constructs. The three destination vectors were made using the RCASBP-Y vector. RCASBP-Y DV carries the blunt-ended reading frame cassette (\( \text{att}R1 \)-chloramphenicol resistance gene-\( \text{ccdB} \) gene-\( \text{att}R2 \)) (Invitrogen, Carlsbad, CA) inserted into the RCASBP-Y \( \text{Pme I} \) site and provides for gene expression without any extraneous sequence tags. RCASBP-Y NHA (amino HA tag) and RCASBP-Y CHA (carboxy HA tag) destination vector constructs were generated by digesting the RCASBP-Y vector with \( \text{Not I} \) and \( \text{Swa I} \) followed by dephosphorylation. Oligos were synthesized corresponding to: \( \text{Not I-ATG-HA-Pme I-Swa I} \) (amino HA tag) and \( \text{Not I-Pme I-HA-TGA-Swa I} \) (carboxy HA tag) and allowed to anneal. Double-stranded oligos were ligated into linearized vector. Once confirmed by sequence analysis, vectors were digested with \( \text{Pme I} \) and the proper reading frame cassette was ligated into the linearized vector (Invitrogen, Carlsbad CA). All destination vectors were propagated in \( \text{DB3.1} \) cells that carry a compensatory mutation, \( \text{gyrA}^4 \) to allow for cell growth in the presence of the \( \text{ccdB} \) gene product.}
\end{figure}
Figure 2. HA Epitope fusion sequences. Amino acid sequence of epitope fusion for A) RCASBP-Y NHA and B) RCASBP-Y CHA gene expression constructs. The over line indicates the attB1 and attB2 coding sequences. HA epitopes are indicated by boxed sequences. For RCASBP-Y NHA the fusion translation start ATG is indicated by an arrow. Shaded boxes indicate the position of gene-specific nucleotide sequences required for the proper reading frame of fusion constructs to be maintained. Dashes represent the amino acids that will be determined by analogous gene-specific sequences.

respectively (Fig. 2). Each of the three destination vectors was tested by recombinational cloning of Enhanced Green Fluorescent Protein (EGFP). This allowed us to assess whether the 25-bp attB sequences, which remain flanking the insertion sequence, would hinder the production of infectious particles, be prone to deletion due to the repetitive nature of the sequences, or hinder expression of the desired gene products.

PCR primers were designed to EGFP to allow for direct cloning of an EGFP PCR product. For RCASBP-Y DV and RCASBP-Y NHA, the PCR primers were designed as follows. The 5′ forward primer sequence contained a 25-bp attB1 linker adjacent the initiator methionine of EGFP (attB1-EGFP5′-GGGGACAAGTTTGTACAAAAAAGCAGCCTCTACATGAGCAAGGAGGCGAG), while the 3′ reverse primer sequence was designed to C-terminal EGFP sequence including a stop codon with a 25-bp attB2 linker (attB2-EGFP3′-STP-GGGGACCACTTTGTACAAGAAAGCTGGGTTCTTGTACAGCTCGTCCATG), thus allowing for read-through and fusion to the C-terminal epitope tag.

The EGFP gene products that were introduced in the three RCASBP-Y destination vectors using recombinational cloning yielded a total of 24 retroviral expression clones, with the correct clone being obtained in 76% of the colonies examined (data not shown). To date, eight genes have been introduced into these three vectors yielding a total of 24 retroviral expression clones, with the correct clone being obtained in 76% of the colonies examined (data not shown). All three clones were evaluated to determine if a functional virus could be produced or if the repetitive sequences would result in a loss of gene expression due to deletion of the insert sequences. Retroviral EGFP constructs were transfected into DF1 chicken fibroblasts. For all three constructs, EGFP signal was present after 24 hr in ∼20% of the cells. After 7 days in culture, EGFP expression was seen in 99% of the cells for all three constructs. Viral supernatant was collected and used to transduce mouse melanocyte cells expressing tv-a (melan-a TVA). Cells were transduced with virus, and EGFP signal was detected by 3 days (data not shown). Cells were fixed after 10 days and visualized for HA epitope tags (Fig. 3). All three constructs generated EGFP signal indicating that the gene products could be expressed from the modified RCASBP-Y vectors (Fig. 3). For the RCASBP-Y NHAEGFP and RCASBP-Y CHAEGFP constructs, HA signal and EGFP fluorescence were found within the same cells, indicating the HA epitope was expressed.

The recombinational cloning procedure rapidly and efficiently yielded inserted cDNA clones in the correct orientation due to negative selection (ccdB gene), positive...
Figure 3. EGFP expression in Melan-a TVA cells. All three RCAS Destination vectors (A) RCASBP-Y EGFP (B,D) RCASBP-Y CHA EGFP (C,E) RCASBP-Y NHA EGFP produce infectious particles capable of expressing EGFP as seen by (A–C) EGFP signal. HA Ab staining indicates fusion epitopes are maintained in frame for (D) RCASBP-Y CHA EGFP and, (E) RCASBP-Y NHA EGFP following recombinational cloning. White bar equals 50 µM. Melan-a cells were grown as previously described.

Melan-a cells expressing the avian receptor TVA950 were obtained by transfection of StuI-linearized TVA950 in pcDNA6/V5- plasmid using SuperFect Transfection Reagent (Qiagen, Valencia CA) according to the manufacturer’s instructions. Stably transfected cells were selected using m-box Blasticidin S HCl (Invitrogen, Carlsbad CA) at 5 µg/ml in the growth media. Single clones were isolated, and their ability to be transduced was confirmed using RCAS-beta-gal, a recombinant avian retrovirus expressing the beta-galactosidase reporter (data not shown). All three EGFP constructs were transfected into DF1 chicken fibroblasts using a 1:5 ratio (ug DNA/ul SuperFect) (Qiagen, Valencia CA) for 2 hr. Production and transduction of infectious virus was performed as described in Dunn et al., 2000. The HA epitope was visualized using HAAb (BabCo) and peroxidase staining kit (Vector labs, Burlingame, CA) according to the manufacturer’s instructions.

selection (antibiotic) and sequence specificity of flanking att sites. Modification of the RCASBP-Y vector with the Gateway RFC eliminates the need to redesign a unique cloning strategy for each gene or to be dependent on restriction enzyme sequences within the gene or vector. Upon completion of the recombinational cloning procedure, each RCASBP-Y destination vector contains two 25-bp recognition sequences (attB1 and attB2) flanking the inserted gene sequence. These sequences did not adversely affect the ability of infectious virus to be produced or the ability of the inserted gene to be expressed. The two HA epitope vectors will also allow for rapid determination of cellular localization for uncharacterized genes, utilizing a standardized cloning strategy.

It should be noted that there is a potential limitation on the size of the insert (∼2.5 kb) for producing viable infectious viral particles using the RCAS-TVA system (http://www.ncifcrf.gov/hivdrp/RCAS/overview.html). However, this may not be a significant drawback as 86% of the current annotated full-length coding sequences (CDS) in the human genome are less than or equal to 2.5 kb. This calculation was obtained from analyzing the annotation of GenBank records for human mRNA entries using the Locus Link database from June 1, 2001. From the 15,950 entries annotated in regards to CDS, 14,177 were annotated to represent complete CDS with a mean size of 1507.86 bp. Of the 14,177 entries, 12,238 had a CDS less than or equal to 2.5 kb. It should be noted that inserts greater than 2.5 kb may produce non-replication-competent virus and thus lower titers.

Future cloning of genes into the RCAS viruses will be a uniform process that can be performed either by gene-specific PCR or full-length library-entry clones. Many full-length cDNA libraries are currently being constructed using Gateway-compatible vector systems. The identification of full-length cDNAs from such libraries will allow the direct transfer of the genes of interest by recombinational cloning without PCR amplification from the library clones. With this in mind, it
may be possible to use RCASBP-Y DV vector to generate full-length retroviral cDNA expression libraries. This would allow for functional screens to be carried out on a genome-wide scale.

Use of these reagents in functional screens may prove very useful in conjunction with exploiting differences between how the ASLV functions in chicken and mammalian cells. Avian cells, which endogenously express the retoviral receptor tv-a, allow for expression of viral genes and the cloned genes of interest, resulting in replication of the virus to high titers and the spread of infection to adjacent cells.\(^{3,18,21}\) In mammalian cells transfected with the tv-a gene infected with RCAS virus, integration of the cloned gene of interest into the mammalian genome is followed by transcription and translation using the viral LTR as a promoter. However, unlike the avian cells, mammalian cells do not produce infectious viral particles.\(^{22}\) Thus, the mammalian system has the advantage of allowing for lineage-directed evaluation of genes, and also the potential for multiple rounds of infection into the same cell, allowing analysis of combinatorial gene actions.\(^{12}\) Development of a RCASBP based vector cDNA expression libraries would allow for functional screens that could reveal such combinatorial gene interactions. These viral destination vectors will have a wide application to multiple fields of investigation, including avian and mammalian model systems, to address functioning of genes throughout development and tumorigenesis.

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References

1. Hughes, S. H. and Kosik, E. 1984, Mutagenesis of the region between evnv and src of the SR-A strain of Rous Sarcoma virus for the purpose of constructing helper-independent vectors, virusolgy, 136, 89–99.
2. Hughes, S. H., Greenhouse, J. J., Petropoulos, C. J., and Sutrave, P. 1987, Adaptor Plasmids Simplify the Insertion of Foreign DNA into Helper-Independent Retroviral Insertion, J. Virol., 61, 3004–3012.
3. Bates, P., Young, J. A., and Varmus, H. E. 1993, A receptor for subgroup A Rous sarcoma virus is related to the low density lipoprotein receptor, Cell, 74, 1043–1051.
4. Bates, P., Roug, L., Varmus, H. E., Young, J. A. T., and Crittenden, L. B. 1998, Genetic mapping of the cloned subgroup A avian sarcoma and leukemia virus receptor gene to the TVA locus, J. Virol., 72, 2505–2508.
5. Fritsch, E. F. and Temin, H. M. 1977, Inhibition of Viral DNA Synthesis in Stationary Chicken Embryo Fibroblasts Infected with Avian Retroviruses, J. Virol., 24, 461–469.
6. Varmus, H. E., Padgett, T., Heasley, S., Simon, G., and Bishop, J. M. 1977, Cellular functions are required for the synthesis and integration of avian sarcoma virus-specific DNA, Cell, 11, 307–319.
7. Humphries, E. H., Glover, C., and Reichmann, M. E. 1981, Rous sarcoma virus infection of synchronized cells establishes provirus integration during S-phase DNA synthesis prior to cellular division, Microbiology, 78, 2601–2605.
8. Gaur, M., Murphy, G. J., deSauvage, F. J., and Leavitt, A. D. 2001, Characterization of Mpl mutants using primary megakaryocyte-lineage cells from mpl(−/−) mice: a new system for Mpl structure-function studies, Blood, 97, 1653–1661.
9. Dunn, K. J., Williams, B. O., Li, Y., and Pavan, W. J. 2000, Neural crest-directed gene transfer demonstrates Wnt1 role in melanocyte expansion and differentiation during mouse development, Proc. Natl. Acad. Sci. USA, 97, 10050–10055.
10. Dunn, K. J., Incao, A., Watkins-Chow, D., Li, Y., and Pavan, W. J. 2001, In utero complementation of a neural crest-derived melanocyte defect using cell directed gene transfer, Genesis, 30, 70–76.
11. Holland, E. C. and Varmus, H. E. 1998, Basic fibroblast growth factor induces cell migration and proliferation after glia-specific gene transfer in mice, Proc. Natl. Acad. Sci. USA, 95, 1218–1223.
12. Fisher, G. H., Orsulic, S., Holland, E. et al. 1999, Development of a flexible and specific gene delivery system for production of murine tumor models, Oncogene, 18, 5253–5260.
13. Hartley, J. L., Temple, G. F., and Brasch, M. A. 2000, DNA cloning using in vitro site-specific recombination, Genome Res., 10, 1788–1795.
14. Walhout, A. J., Temple, G. F., Brasch, M. A. et al. 2000, GATEWAY recombinational cloning: application to the cloning of large numbers of open reading frames or ORFs, Methods Enzymol., 328, 575–592.
15. Reboul, J., Vaglio, P. Tzellas, N. et al. 2001, Open-reading-frame sequence tags (OSTs) support the existence of at least 17,300 genes in C. elegans, Nat. Genet., 27, 332–336.
16. Carninci, P., Shibata, Y., Hayatsu, N. et al. 2001, Balanced-sized and Long-sized cloning if fulllength, Captrapped cDNAs into vectors of the novel λ-FLC family allows enhanced gene discovery rate and functional analysis, Genomics, In Press.
17. Landy, A., 1989, Dynamic, structural, and regulatory aspects of lambda site-specific recombination, Annu. Rev. Biochem., 58, 913–949.
18. Boerkoel, C. F., Federspiel, M. J., Salter, D. W. et al. 1993 A New Defective Retroviral System Based on the Bryan Strain of Rous Sarcoma Virus, Virology, 195, 669–679.
19. Federspiel, M. J., Swing, D. A., Eagleson, B., Reid, S. W., and Hughes, S. H. 1996, Expression of transduced genes in mice generated by infecting blastocysts with avian leukosis virus-based retroviral vectors, Proc. Natl. Acad. Sci. USA, 93, 4931–4936.
20. Himly, M., Foster, D. N., Bottoli, I., Iacovoni, J. S., and Vogt, P. K. 1998, The DF-1 chicken fibroblast cell line: transformation induced by diverse oncogenes and cell
death resulting from infection by avian leukemia viruses, *Virology*, 248, 295–304.

21. Schaefer-Klein, J., Givol, I., Barsov, E. V. et al. 1998, The EV-O-derived cell line DF-1 supports the efficient replication of avian leukemia-sarcoma viruses and vectors, *Virology*, 248, 305–311.

22. Federspiel, M. J., Bates, P., Young, J. A., Varmus, H. E., and Hughes, S. H. 1994, A system for tissue-specific gene targeting: transgenic mice susceptible to subgroup A avian leukemia virus-based retroviral vectors, *Proc. Natl. Acad. Sci. USA*, 91, 11241-11245.

23. Bennett, D. C., Cooper, P. J., and Hart, I. R. 1987, A line of non-tumorigenic mouse melanocytes syngeneic with the B16 melanoma and requiring a tumour promoter for growth, *Int. J. Cancer*, 39, 414-418.