Avian retrovirus integrase-enhanced transgene integration into mammalian cell DNA in vivo

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Systems for introducing DNA genes-of-interest into mammalian cellular genomes have ranged from the use of different physical techniques to viruses including retroviruses. We have developed a microinjection method for an efficient and permanent integration of a DNA transgene into the cell genome by use of the retrovirus integrase. A 3.0-kb linear DNA fragment containing an internal herpes simplex virus thymidine kinase gene (tk) with flanking avian retrovirus U5 and U3 terminal attachment sites (U5-pgk/tk-U3) recognized by the integrase was constructed. The other donor, a 3.3-kb linear DNA fragment containing the same gene (pgk/tk) flanked by ApaI restriction sites not recognized by integrase, was also produced. After assembly of integrase-transgene complexes on ice, the complexes were microinjected into the nucleus of human fibroblast cells (143Btk⁻) containing a defective thymidine kinase. The number of hypoxanthine/aminopterin/thymidine (HAT)-resistant colonies produced upon microinjection of either naked DNA or the independently assembled integrase-transgene complexes were determined. Our data suggests that enhanced integration of U5-pgk/tk-U3 required the DNA attachment sites and co-delivery of integrase. The data was consistent with a direct role for both of these elements in producing an approximate 4-fold increase in the number of HAT-resistant colonies observed over microinjection of just naked U5-pgk/tk-U3 ($P < 0.0001$).

INTRODUCTION

Retroviruses have the capability of inserting their linear DNA genomes into host chromosomes. Upon reverse transcription of the viral RNA genome, the viral DNA along with viral proteins form large molecular weight nucleoprotein machines termed preintegration complexes (PIC) (1,2). The two ends of the viral DNA are held together by a protein bridge presumably assisted by the viral integrase (IN) (2). IN removes two nucleotides from the blunt-ended viral genome, and subsequently, the PIC is transported into the nucleus (1). The 3' OH recessed viral DNA termini in the PIC are inserted by IN in a concerted fashion into the host genome producing proviral DNA. Insertion of the retrovirus genomes into the host chromosomes appears random although preferentially into active genes (3,4).

Avian retroviruses integrate their DNA efficiently into mammalian chromosomes (5,6). We investigated whether in vitro-assembled nucleo-

protein complexes containing avian myeloblastosis virus (AMV) IN and a linear DNA fragment possessing a selectable gene-of-interest could be integrated directly into mammalian host chromosomes. The linear DNA donor contained avian retrovirus terminal attachment (att) site sequences that are recognized by IN for integration (7,8). The delivery method for the assembled IN-DNA complexes was direct microinjection of the complexes into the cell nucleus. We produced a construct containing the herpes simplex virus (HSV) thymidine kinase (*tk*) gene with terminal att sites (U5-pgk/tk-U3, 3.0 kb) (Figure 1). For an additional naked DNA background control, the same pgk/tk construct lacking terminal att sequences was produced (pgk/tk, 3.3 kb) (Figure 1). Human fibroblast cells (143Btk⁻) containing a defective thymidine kinase were used as the host. We tested a variety of experimental protocols to examine whether both the att sites and IN were required to produce a significant increase in the

number of hypoxanthine/aminopterin/thymidine (HAT)-resistant colonies upon microinjection of assembled IN-DNA complexes. We present data demonstrating that co-delivery of IN with U5-pgk/tk-U3 results in a significant 3.9-fold increase in stable transduction ($P < 0.0001$) and that this significant increase is dependent on both att sites and IN.

MATERIALS AND METHODS

Plasmid Constructs

A plasmid construct containing the *tk* gene driven by the phosphoglycerate kinase (pgk) promoter and flanked by att sequences was produced as follows. The expression vector (pCMV β ; BD Biosciences Clontech, Palo Alto, CA, USA) contained the *lacZ* gene driven by the cytomegalovirus (CMV) promoter. The *EcoRI*, *HindIII*, *SalI*, and *PvuII* sites of pCMV β were converted to *PacI*, *SfiI*, *AscI*, and *FseI* sites, re-

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spectively, to produce pCMV9 β . The *lacZ* gene in pCMV9 β was replaced with the HSV *tk* gene from pIC19/MCI-TK (provided by M.-J. Fackler, Johns Hopkins University School of Medicine, Baltimore, MD), and the CMV promoter sequences were replaced with the pgk promoter sequences (Ppgk) from pPGK-neo-bpA (provided by J. Ceci, University of Texas Medical Branch, Galveston, TX). *Apa*I digestion of this pgk/tk construct produced a linear 3.3-kb fragment lacking att sites (Figure 1). Complementary 51-bp oligonucleotides corresponding to avian U5 (italicized)-*Nde*I (bolded)-U3 (underlined) (5'-GCACCTGCATGAA-GCAGAAGGCTTCATATGTAGTCTT-ATGCAATACTCTTG-3') and (5'-CA-AGAGTATTGCATAAGACTACA-TATGAAGCCTTCTGCTTCATGCAG-GTGC-3') were synthesized to flank the Ppgk/tk sequences with att site sequences for IN recognition. The Ppgk/tk transgene was removed from the original pgk/tk construct to produce a blunt-ended fragment that was ligated with the annealed oligonucleotides, digested with *Nde*I, and subsequently cloned into the *Nde*I site of pUC19. The pgk/tk transgene was released from pUC19 by *Nde*I digestion and was designated U5-pgk/tk-U3 (3.0 kb in length) (Figure 1). *Nde*I digestion produces linear DNA containing 3' OH recessed att sites necessary for strand transfer activity (8). DNA was isolated by 1% agarose gel electrophoresis and electro-eluted into dialysis tubing. The DNA was subsequently purified

through a 0.1- μ m filter, extracted with phenol-chloroform, precipitated by ethanol, and dissolved into sterile TE (10 mM Tris-HCl, pH 7.2, 0.1 mM EDTA). The DNA concentration was determined by absorbance at 280 nm.

Purified IN

AMV IN was purified from virions to near homogeneity (9).

Microinjection

The 143Btk⁻ human fibroblast cell line was obtained from ATCC (Manassas, VA, USA) and maintained in minimal essential medium containing 10% fetal bovine serum, penicillin/streptomycin, and 15 μ g/mL bromodeoxyuridine. Approximately 10⁴ 143Btk⁻ cells were plated into 35-mm dishes that were etched on the bottom surface to contain a grid pattern of 25 squares. Just prior to microinjection, cells were washed with media and fed with 2 mL of fresh media. Only one cell per square was injected with 50–100 cells injected per experiment.

The U5-pgk/tk-U3 DNA transgene was assembled with IN for approximately 15 min on ice in 3 mM potassium phosphate-buffered solution (pH 7.2) containing 114 mM KCl. No Mg²⁺ was present in the assembly buffer to help prevent integration of the DNA termini by IN into the same DNA molecule or adjacent DNA molecules prior to deliver into the nucleus. The IN dimer to DNA donor end molar ratio

was generally kept between 10:1 and 44:1, respectively, unless otherwise indicated. The assembled IN-U5-pgk/tk-U3 complexes or corresponding naked DNA were microinjected with similar volumes (approximately 10 fL/cell) directly into the nuclei of cells. At 24–72 h postinjection, the media was replaced with fresh HAT-containing medium, and the cells were incubated with new HAT medium every 3 days. At 10–15 days postinjection, HAT-resistant colonies (approximately 5 $\times$ 10² to 10⁴ cells) in the individual squares were clearly identifiable and scored. There were no HAT-resistant colonies in the non-injected plates or in regions of the plate outside of the grid regions. For analysis of individual colonies or stable transductants, individual HAT-resistant colonies isolated by a cloning ring were trypsinized and expanded in HAT-containing medium for isolation of high molecular weight (HMW) genomic DNA.

Statistical Analysis

Data are presented as the mean of the sample populations $\pm$ the standard deviation ($\bar{x} \pm$ SD). To test for significant difference between means, the Student's *t*-test (two tail) was employed. *P* values were considered to be statistically significant if <0.01.

Genomic DNA Analysis

Individual isolated stable transductant cells were washed with phosphate-buffered saline (PBS) and suspended in extraction buffer (10 mM Tris-HCl, pH 8.0, 100 mM EDTA) at 0.5 $\times$ 10⁶ cell/mL. Sodium dodecyl sulfate (SDS) and RNaseA were added to a final concentration of 0.5% and 20 μ g/mL, respectively, for 1 h incubation at 37°C. The samples were further digested with proteinase K (for 3 h at 56°C), extracted several times with phenol-chloroform, precipitated by ethanol, and dissolved in TE buffer. Twenty micrograms of genomic DNA from each sample was digested with 200 U of *Hind*III. Digestion of genomic DNA with *Hind*III (not cutting within U5-pgk/tk-U3) yields fragments corresponding to individual integration events. The samples were subjected to electrophoresis in 1% aga-

Figure 1. Diagram of the linear U5-pgk/tk-U3 and pgk/tk transgenes. (A) The HSV *tk* gene driven by the pgk promoter is flanked by U5 and U3 att sites. Digestion of the pUC19 construct by *Nde*I site produced the appropriate 3' OH recessed termini for integration activity. The pA is the poly(A) tail. (B) Digestion of the original construct containing the pgk/tk gene by *Apa*LI produces the same transgene without the terminal att sites necessary for integration activity.

rose gels using a horizontal apparatus and transferred to Hybond[®]N⁺ membrane (Amersham Biosciences, Piscataway, NHJ, USA). After UV cross-linking, the membrane was hybridized with a ³²P-labeled *tk* gene probe. After processing, the membrane was exposed to X-ray film and developed.

Inverse PCR Analysis

The inverse PCR method by Nolte et al. (10) was applied to identify the host site 6-bp duplications observed upon concerted integration of the avian retrovirus genome in vivo (1). The reaction conditions, the sequence of the primers, and the DNA sequence of the approximately 230-bp PCR fragment containing the 6-bp host site duplication (AATTTG) identified in the host chromosome upon concerted insertion of U5-pgk/tk-U3 DNA by IN are available upon request.

RESULTS

AMV IN Enhances Stable Transduction of Human 143Btk⁻ Fibroblasts with att Site-Flanked Transgene

In order to investigate AMV IN-facilitated integration of DNA transgenes in vivo, we produced a linear 3.0-kb DNA fragment containing an internal HSV *tk* gene flanked by terminal U5 and U3 att sequences recognized by IN (Figure 1A). Stable transduction frequencies were determined for cells injected with U5-pgk/tk-U3 either in the absence (average 4.9% $\pm$ 2% stable transduction frequency) or presence (average 18.5% $\pm$ 3.9%) of AMV IN (Table 1). Inclusion of AMV IN with the DNA yielded a significant 3.8-fold (average) increase in transduction ($P < 0.0001$). The significance of this result is evident when the stable transduction frequency is normalized to the average survival frequency (47%) of control 143Btk⁻ cells injected with Oregon Green-coupled dextran (four experiments with a total of 250 cells injected) (11). Thus, taking this control survival factor into consideration, approximately 40% of cells surviving injection were stably transduced when AMV IN was co-delivered with U5-pgk/tk-U3.

We examined the role of the terminal att site sequences in IN-facilitated transgene delivery. The 3.0-kb U5-pgk/tk-U3 donor (Figure 1A) (Table 1) was compared to the similar 3.3-kb pgk/tk donor (Figure 1B) containing terminal *Apa*L1 sites. The U5-pgk/tk-U3 donor was delivered without (-IN) or with IN (+IN) (Table 2, left). The stable transduction frequency was 5% $\pm$ 2% without IN in comparison to 20% $\pm$ 5.8% with IN, a significant 4-fold (average) increase ($P = 0.0007$) similar to results shown in Table 1. For the pgk/tk donor lacking the terminal att sites, the stable transduction frequencies were 5.8% $\pm$ 1.5% and 5.6% $\pm$ 3% without and with IN, respectively (Table 2, right). Comparison of U5-pgk/tk-U3 delivered with IN to pgk/tk was also significant ($P = 0.0013$) (Table 2). There was no significant difference in stable transduction frequency for naked U5-pgk/tk-U3 in comparison to naked pgk/tk indicating that the att site sequences do not affect the stable transduction frequency. A significant difference in stable transduction frequency between the two DNA fragments was only apparent with IN present. The enhancement of stable transduction frequency requires both the att site sequences and IN, which is consistent with a direct role of both of these elements. The specific requirement for att sequences suggests that IN did not increase the stable transduction frequency for injected DNAs by simple coating of the DNA nonspecifically, thereby protecting it from nuclease degradation within the nuclei of injected cells.

We proceeded to evaluate the effect of DNA concentration on stable transduction frequency, both in the absence and presence of AMV IN. We utilized the U5-pgk/tk-U3 donor and an IN dimer to DNA end molar ratio of 20:1,

Table 1. Co-delivery of AMV IN with U5-pgk/tk-U3 Enhances Stable Transduction of 143Btk⁻ Cells

Experiment (No.)	Stable Transduction Frequency ^a		
	-IN (%)	+IN (%)	+IN/-IN
1 ^b	2	20	10×
2 ^{b,c}	8	22	2.8×
3 ^b	4	14	3.5×
4 ^d	5.3	21	4.0×
5 ^b	4	13	3.3×
6 ^{b,c}	6	21	3.5×
Average	4.9 $\pm$ 2.0 ^e	18.5 $\pm$ 3.9 ^e	3.8 ^f

^a Total number of HAT-resistant colonies as % of total injected cells.
^b DNA concentration = 12 ng/ μ L.
^c The injected buffer included 4% polyethylene glycol.
^d DNA concentration = 1 ng/ μ L.
^e $\bar{x} \pm$ SD.
^f Average fold increase was calculated as the ratio of the average frequency +IN (18.5) to the average frequency -IN (4.9).
 AMV, avian myeloblastosis virus; HAT, hypoxanthine/aminopterin/thymidine; IN, viral integrase.

respectively (see Figure 2). At the DNA concentrations examined (1, 4, and 12 ng/ μ L), co-delivery of IN with U5-pgk/tk-U3 DNA significantly increased the stable transduction frequency over that observed when just injecting U5-pgk/tk-U3 ($P < 0.0001$) (Tables 1 and 2). There was not a significant difference in transduction frequencies observed for the different naked DNA concentrations used for these experiments.

Dependence of Enhanced Stable Transduction Frequency as a Function of IN Concentration

To further investigate the enhanced transduction frequency of U5-pgk/tk-U3 in the presence of IN, we examined the stable transduction frequency as a function of IN concentration, while maintaining a constant concentration of DNA (4 ng/ μ L or 2 nM). The stable transduction frequency was at a maximum for ratios of IN dimer per DNA at molar ratios greater than 8:1, respectively (Figure 2). Enhancement of concerted

Table 2. Co-delivery of AMV IN With U5-pgk/tk-U3 but Not With pgk/tk Enhances Stable Transduction of 143Btk⁻ Cells

Experiment (No.)	U5-pgk/tk-U3 ^a			pgk/tk ^a		
	-IN (%)	+IN (%)	+IN/-IN	-IN (%)	+IN (%)	+IN/-IN
1	4	18	4.5×	6	10	1.7×
2	7	13.3	1.9×	5	6	1.2×
3	6	26	4.3×	4	2	0.5×
4	6	16	2.7×	8	4	0.5×
5	2	26	13×	6	6	1.0×
Average ^b	5.0 ± 2.0 ^c	20.0 ± 5.8 ^c	4.0×	5.8 ± 1.5 ^c	5.6 ± 3.0 ^c	1.0×

^a Total number of HAT-resistant colonies as % of total injected cell number.
^b Average fold increase was calculated as the ratio of the average frequency +IN (20.0) to the average frequency -IN (5.0).
^c $\bar{x} \pm \text{SD}$. The concentrations of DNAs in experiments 1–5 were 1, 1, 6, 6, and 5 ng/μL, respectively, and the DNAs were assembled with IN under normal assay conditions.
AMV, avian myeloblastosis virus; HAT, hypoxanthine/aminopterin/thymidine; IN, viral integrase.

DNA integration events at similar AMV IN dimer to att DNA end molar ratios has been observed in vitro (12–14).

Analysis of IN Mediated Integration of Transgene

We examined individual HAT-resistant colonies for the number of integration events, the total number of gene copies integrated, and the organization

of the integrated transgenes. HMW DNA isolated from individual expanded colonies was subjected to *Hind*III digestion, gel electrophoresis, Southern blot transfer, and hybridization with a ³²P-labeled HSV *tk* probe. All hybridizing bands should be >3.0 kb (the size of U5-pgk/tk-U3), provided that there was no rearrangement of transgene sequences. A simple pattern of integration events is reflected in the *Hind*III

digestion of DNA upon Southern blot analysis (Figure 3A) when the experiment was performed with U5-pgk/tk-U3 at 1 ng/μL, corresponding to approximately three DNA copies delivered per injected cell (Table 1, experiment no. 4). *Hind*III analysis revealed an increase in the number of integration events per stable colony of cells in comparison to stable colony of cells without IN (Figure 3A). At 1 ng/μL DNA with IN, the number of transgene copies (approximately 1–3 copies of a specific internal transgene 1.6-kb *Bam*HI fragment) observed in most stable colony sets (Figure 3B) were significantly

Figure 2. Enhanced transduction frequency is a function of the viral integrase (IN) concentration at a constant DNA concentration. IN was assembled with U5-pgk/tk-U3 at various IN dimers-to-donor end ratios. The DNA concentration was held constant for assembly at 4 ng/μL or 2 nM. The experiments were performed twice with the error bars indicating the repeated concentrations analyzed. Stable transduction frequency was determined as described in Table 1.

lower than the number of transgene copies observed using 12 ng/ μ L DNA with IN (Figure 3D). *Hind*III analysis of the HMW DNA isolated from cells transduced at 12 ng/ μ L DNA with IN (Figure 3C) also revealed more complex patterns of integration sites than observed at 1 ng/ μ L DNA with IN (Figure 3A). In addition, we further analyzed each of the clones for the presence of head-to-tail concatamers by *Nco*I digestion. Since *Nco*I cuts only once within the transgene, head-to-tail concatamers yield a diagnostic, *tk*-hybridizing 3.0-kb fragment. At 12 ng/ μ L DNA with IN, significantly more integrated copies of U5-pgk/*tk*-U3 were also evident in all five colonies, either as single-copy genes or transgene concatamers, than at 1 ng/ μ L DNA with IN (data not shown). Thus, at the lower DNA concentration, IN-DNA complexes appear to form the desired structures mimicking the integration of viral DNA upon infection of cells by retroviruses.

Concerted integration of the avian retrovirus genome into the host genome results in a characteristic 6-bp duplication of host chromosomal DNA se-

quences at the site of insertion (1). The duplication of host sequences is also observed upon concerted integration of similar DNA fragments containing terminal att site sequences by purified IN into target DNA in vitro (12–16). We evaluated the chromosomal integration sites for some of integration events utilizing inverse PCR (10). Although we were successful in individually cloning either upstream or downstream cellular flanking sequences adjacent to the inserted terminal att site for several individual integration events, we were only successful with one integration fragment in cloning both the upstream and downstream flanking sequences. The stable transductant for this one analysis was from the original HMW DNA used for *Hind*III analysis shown in Figure 3A, lane 5. Two individual *Hind*III integrated U5-pgk/*tk*-U3 bands (approximately 6.5 and 8.5 kb) were identified. Nested inverse PCR yielded two products total: one upstream product of approximately 270 bp (corresponding to the U5 sequence) and one downstream product of approximately 1000 bp (corresponding to the U3 sequence). If the inverse PCR

method had been completely successful, we would have expected a total of four DNA products: two upstream and two downstream. Sequencing of both the upstream and downstream product revealed a common “AATTG” sequence flanking both U5 and U3. This suggested that both the upstream and downstream sequences flanked the same integrated DNA fragment and that the integration event occurred via an enzymatically active IN-U5-pgk/*tk*-U3 complex. In order to confirm that the upstream and downstream flanking sequences from this genomic DNA sample corresponded to the same integration event, oligonucleotides corresponding to the upstream and downstream chromosomal sequences were synthesized. These primers were used to amplify normal chromosomal DNA of the expected size (approximately 230 bp) from the original 143Btk⁻ cell line. Sequencing of this normal chromosomal DNA revealed the cellular sequence of 5'-TTATGTGTGAATTGATCCTGTCGT-3' (the bolded nucleotides represent the adjacent cellular DNA sequences upstream and downstream

Figure 3. Viral integrase (IN)-facilitated integration of U5-pgk/*tk*-U3 at low and high DNA concentrations: analysis by *Hind*III digestion/Southern blot analysis and copy number determinations. (A) The DNA concentration for assembly with IN was 1 ng/ μ L (left). Each lane shows the *Hind*III/Southern blot analysis of high molecular weight (HMW) DNA isolated from individually expanded colonies. There are no *Hind*III sites within U5-pgk/*tk*-U3, and therefore, each hybridizing band reveals a separate integration event. Lanes 1–3 contain individual samples minus IN (-IN), and lanes 4–10 contain individual samples plus IN (+IN) present upon microinjection. The size markers indicated on the right were derived by *Hind*III digestion of λ DNA. (B) For copy number determination, the same cellular DNA samples (lanes 1–10) were digested with *Bam*HI, which releases a specific, internal 1.68-kb *tk*-hybridizing fragment from U5-pgk/*tk*-U3. PhosphorImagerTM (Amersham Biosciences) analysis was performed on the 1.68-kb band, and an X-ray film exposure is shown. There is approximately 1 transgene copy in lanes 1, 3, 4, 6, and 7; approximately 2 copies in lane 5; approximately 2.5 copies in lane 8; approximately 3–5 copies in lanes 2 and 9; and >8 copies in lane 10. A set of the 1.68-kb fragments of known copy numbers are shown on the left. (C) The DNA concentration for assembly with IN was 12 ng/ μ L (left). *Hind*III/Southern blot analysis of HMW DNA isolated from individually expanded colonies minus IN (-IN) (lanes 1–3) and plus IN (+IN) (lanes 4–8) are shown. (D) The same copy number analysis using the *Bam*HI 1.68-kb band described above was performed on these samples. The fifth lane from the left had accidental spill over of the 1.68-kb standard. The copy number of transgenes in all the samples were significantly >8 copies except lanes 1, 3, and 6.

of the duplicated host site identified as AATTTG). This duplication of target chromosomal sequences demonstrated that the AMV IN delivered via microinjection was, in fact, enzymatically active and acted as expected for the insertion of viral DNA normally observed in virus-infected cells. In addition, the two other independently sequenced clones derived from a different colony set, each containing a different downstream viral-host junction, had the inserted terminal att CA dinucleotide joined to cellular sequences as well as the removal of the two unpaired nucleotides (AT) located at the 5' ends of the att sites.

DISCUSSION

This is the first report that has successfully shown microinjected in vitro assembled IN-DNA transgene complexes being integrated into the host chromosomes. The microinjection data suggest that enhanced transgene integration was dependent upon both the att sites and IN, which is consistent with a direct role for both of these elements in producing the approximately 4-fold increase in the number of HAT-resistant colonies observed over microinjection of naked DNA only (Tables 1 and 2). Lipid vesicle-mediated DNA transfections of a linear U5-NeoR-U3 transgene (1 µg) with a glutathione S-transferase (GST)-fusion avian retrovirus IN (1 µg) into CHO-K1 cells produced a several-fold enhancement of stable transduction efficiency for G418 resistant cells (17). Our results differ from these prior results in several significant ways. Mizuarai et al. (17) observed enhanced transduction with IN when the NeoR construct was not flanked by att sites; suggesting, that their observed transduction effect may not be related to enzymatically active IN mediated integration. In contrast, the enhanced integration presently reported would only occur when flanking att sites were present arguing for specific interactions between IN and the att sites, which are necessary to mediate concerted DNA integration (Table 2). This specificity was confirmed by observing the 6-bp host site duplication mediated by specific host repair enzymes, which is a hallmark characteristic of enzymatically active retroviral integrase. Taken together, our

results are consistent with the expected role of both att sites and IN in facilitating the increased integration rate. Further experiments are necessary to determine if there is a limitation with the length of the transgene using this approach. The integration of any transgene containing flanking att site sequences by co-delivery with IN into the male pronucleus of a fertilized egg may allow for more efficient production of animals containing low copy number of stable transgenes in their chromosomes (18,19).

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