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Construction and Applications of a Highly Transmissible Murine Retrovirus Shuttle Vector

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Summary

We develop a murine retrovirus shuttle vector system for the rapid transfer of cloned genes into mammalian cells and recovery of the inserted sequences as molecular clones. Three protocols allow rapid recovery of vector DNA sequences from mammalian cells. Two of the methods rely on SV40 T-antigen-mediated replication of the vector sequences and yield thousands of bacterial transformants per 5×10^6 mammalian cells. The majority of plasmids recovered by all three protocols exhibited the proper structure and were as active as the parental vector in the generation of transmissible retrovirus genomes upon transfection of mammalian cells. One of the rescue methods, which relies on "onion skin" replication and excision of an integrated provirus from the host chromosome, enables facile recovery of the chromosomal site of proviral integration. The system was also used to generate, and then efficiently recover, a cDNA version of a genomic insert from the adenovirus E1A region.

Introduction

The development of mammalian transducing vectors has been motivated by a variety of interests. Most of these interests relate to the prospect of reintroducing cloned genes into particular cell types in order to study either the functional domains of proteins (Gething and Sambrook, 1981; Rose and Bergmann, 1982), the interactions among different polypeptides (Land et al., 1983; Ruley, 1983), or the regulatory elements that affect the expression of particular genes (McKnight and Kingsbury, 1982; Chao et al., 1983; Green et al., 1983). In addition, with the recognition that a number of biologically and commerically important proteins required postranslational modifications unique to animal cells, there has been considerable interest in the use of mammalian vectors to simply overproduce gene products in animal cells.

To meet such varied applications, a mammalian gene transfer system must fulfill a number of requirements. First, the system should permit the introduction of genes into a wide range of recipients, both in vitro and in vivo. This is particularly important for studies of the regulated expression of cloned genes in which the use of specialized recipients or whole animals is often crucial. In addition, a highly efficient gene transfer system is also important, both for introducing genes into a minor component of a heterogenous cell population (e.g., hematopoietic stem cells in bone marrow tissue) and for studies in which gene transfer is used as a basis for gene isolation (Perucho et al., 1980; Okayama and Berg, 1983). Finally, it is often advantageous to be able to rapidly recover sequences introduced into mammalian cells as molecular clones, both to analyze the structure of the transduced sequences and to recover the chromosomal DNA sequences flanking the site of insertion.

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et al., 1982) is particularly useful.

Here we describe the generation and characterization of a murine retrovirus vector system that meets these requirements. The system allows for the efficient conversion of vector DNA into highly transmissible virus capable of infecting many cell types both in vitro and in vivo. The vectors make use of the strong retrovirus transcriptional signals to promote the expression of both dominant selectable markers and nonselectable cDNA sequences. The vectors also allow for the rapid recovery of integrated proviral sequences from mammalian cells as bacterial plasmids. This latter feature has been successfully applied towards the isolation of cellular sequences flanking provirus integration sites and the isolation of cDNA sequences generated directly by passage of genomic DNA sequences as part of a recombinant retrovirus genome.

Results

Vector Design and Transmissibility

The pZIP-Neo SV(X)1 vector (hereafter termed SVX, shown in Figure 1), consists of a Moloney murine leukemia virus (M-MuLV) transcriptional unit derived from an integrated M-MuLV provirus (Hoffman et al., 1982), and pBR322 sequences necessary for the propagation of the vector DNA in E. coli. The specific M-MuLV sequences retained in the vector include the long terminal repeats (LTRs) necessary for the initiation of viral transcription and the polyadenylation of viral transcripts, as well as for integration; sequences necessary for the reverse transcription of the viral genome; sequences for the encapsidation of viral RNA (Mann et al., 1983); and 5′ and 3′ splicing signals involved in the generation of the subgenomic viral *env* RNA. In place of the retroviral sequences encoding the *gag-pol* and *env* polypeptides, two unique restriction endonuclease cleavage sites (Bam HI and Xho I) were inserted, to permit the expression of sequences introduced into the vector from either the full-length or spliced retroviral transcripts (Weiss et al., 1982). DNA sequences derived from the transposon Tn5, which encode G418 resistance in mammalian cells (Davies and Jimenez, 1980; Colbère-Garapin et al., 1981; Southern and Berg, 1982) and kanamycin resistance in E. coli (Jorgensen et al., 1979), as well as sequences encoding the SV40 and pBR322 origins of

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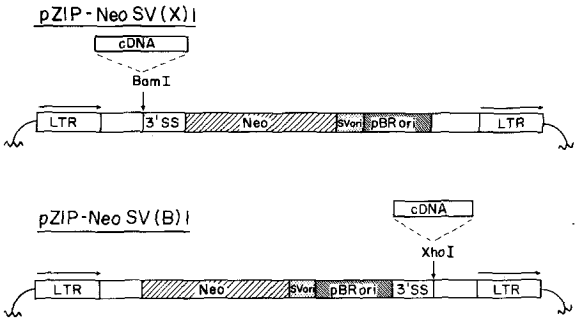

Figure 1. Structure of the ZIPNeo Shuttle Vectors

The pZIP provirus clone of Hoffmann et al. (1982) was used as the source of all M-MuLV sequences. From the 5′ LTR to the Bam HI site was formally the 0–1.0 kb fragment of pZIP (the Pst I site at nucleotide 566 [Shinnick et al., 1981] was converted to a Bam HI site by the addition of a linker). The 3′ SS fragment extends from the M-MuLV Bgl II site at nucleotide 5409 to the Xba I site at nucleotide 5768, and encodes a 3′ splice acceptor sequence utilized in the formation of the subgenomic *env* RNA. The neo fragment was originally the 1.4 kb Hind III–Sal I fragment from Tn5 (Jorgensen et al., 1979; Southern and Berg, 1982). Sequences labeled SVori were derived from pSVOD (Mellon et al., 1981) and extend from SV40 nucleotide 160 to nucleotide 5171 (Tooze, 1981). The pBRori sequences extend from nucleotide 2521 to nucleotide 3102 (Sutcliffe, 1978) (the orientation of these sequences has not been established). The 3′ end of the vector was taken from pZIP, from the Hpa I site at nucleotide 7197 to the 3′ end of the pZIP provirus. The restriction sites that were created during the construction are as shown. For reference of pZIP sites, the standard M-MuLV map can be consulted (Shinnick et al., 1981; Weiss et al., 1982).

replication, have been introduced into the Xho I site. These sequences allow for the selection of mammalian cells harboring the SVX provirus and the rapid recovery of free or integrated proviral genomes as bacterial clones (see below). In a related vector, SVB (Figure 1), the Tn5, SV40, and pBR322 sequences were inserted into the Bam HI site, rather than the Xho I site. Although little data will be [redacted]

To generate highly transmissible virus carrying the SVX genome, SVX DNA was transfected into ψ2 cells. The ψ2 cell line contains integrated copies of an M-MuLV provirus genome that provides all the *trans* functions necessary for the encapsidation of a recombinant genome, yet is defective in the ability to encapsidate its own RNA (Mann et al., 1983). Eighteen hours after transfection, the ψ2 culture fluid was harvested and assayed for the presence of virions capable of conferring G418 resistance (G418^R) upon NIH3T3 cells. A titer of 1–5 × 10^3 CFU/ml was observed on 3T3 cells. To generate permanent cell lines that produce high titers of the SVX virus, transfected ψ2 cells were directly selected in G418 media. Several 42-SVX lines generated in this manner yielded viral titers ranging from 10^4 to over 10^6 cfu/ml.

The structure of the SVX genome that is transferred to recipient cells via virus infection was characterized by Southern blot analysis of DNA obtained from a random G418 resistant cell line, termed SVX 2B (Figure 3C). Digestion with Sac I (lane 2), as well as other restriction enzymes (Aha III, lane 7) confirmed the presence of a single proviral genome of the expected structure. Similar analyses of DNA from a variety of G418 resistant colonies confirmed these findings (data not shown). The transcriptional activity of the integrated SVX genome was demonstrated in several ways. Northern analysis of the RNAs produced in SVX transformants indicated that two viral RNAs, corresponding in size to the expected genomic and spliced transcripts, were produced in equal abundance (R. Cone, unpublished data). Furthermore, superinfection of SVX transformants with wild-type M-MuLV resulted in cell lines that produced titers of SVX virus of up to 10^7 cfu/ml.

Recovery of Integrated Proviral Genomes as Molecular Clones

Three protocols were developed for the facile recovery of integrated SVX proviruses as bacterial plasmids.

Nonamplified Plasmid Rescue

This method relies upon the generation of free circular proviral forms prior to retrovirus integration (Weiss et al., 1982). NIH3T3 cells were cocultivated with the SVX 2B producer cell line for 20 hr, and Hirt DNA (Hirt, 1967) containing putative closed circular proviral molecules was isolated and used to transform E. coli. Transformants were selected on media containing kanamycin sulfate and quick plasmid DNA preps were prepared from individual colonies to characterize the structure of the rescued viral genomes. As summarized in Table 1, a majority of the plasmids exhibited the proper structure by restriction endonuclease analysis. Furthermore, upon retransfection of the rescued genomes into ψ2 cells, all of the plasmids yielded at least as much SVX virus as the parental plasmid (Table 1).

Amplified Plasmid Rescue Following Infection of Cos Cells

Although the nonamplified plasmid rescue procedure was effective, it was inefficient and necessitated the use of highly competent E. coli for transformation. A method was [redacted] closed circles produced immediately following retrovirus infection. Plasmids that contain an SV40 origin replicate to high copy number upon transfection of *cos* cells (Mellon et al., 1981), a monkey cell line which expresses the SV40 T antigen (Gluzman, 1981). Experiments were designed to determine if the SVX viral genome, which encodes an SV40 origin, could be introduced into *cos* cells and if the covalently closed circular products of reverse transcription could be successfully amplified via SV40 T-antigen-mediated replication. For these experiments, a control plasmid, ZIPNeo, which lacked the SV40 origin but was identical to SVX in all other respects, was constructed and used to generate a cell line (designated ZIPNeo 1A) via transient rescue from transfected ψ2 cells. Both the SVX 2B and ZIPNeo 1A cell lines were superinfected with a mixture of the amphotropic helper virus 1504A (Hartley and Rowe, 1976), and the ecotropic virus, M-MuLV. The 1504A virus has been shown to infect a variety of mammalian cell lines, including mouse, monkey, rat, and human (Hartley and Rowe, 1976). The culture supernatant from SVX 2B was titered on both *cos* and CV-1 cells, and was found to have a titer of 2 × 10^3 G418^R cfu/ml and 3 × 10^5 G418^R cfu/

Table 1. Summary of Rescue Efficiency and Activity

Rescue Method	Helper Virus	Vector	Efficiency of Recovery[a]	No. of Clones: Predicted Structure	Deletions	Insertions	Activity in Transient Rescue[b]
3T3 Infect.	M-MuLV	SVX	0–3	7	0	1	≥100%
cos Fusion	–	SVX	$2–5 \times 10^3$	14	2	2	≥100%
	–	E1a(SVX)	$10^2–10^3$	28	2	4	ND[c]
	–	SVB	5×10^3	14	2	2	ND
cos Infect.	1504A	SVX	$10^4–10^5$	26	1	2	≥100%
	1504A	SVB	10^4	17	0	0	ND
	1504A + M-MuLV	SVX	$10^2–10^3$	6	35	0	≥100%

[a] Number of bacterial colonies (HB101 LM1035) recovered from the Hirt supernatant prepared from $5 \times 10^6–10^7$ mammalian cells, assuming that the competent bacteria have a transformation efficiency of 5×10^7 colonies/μg plasmid (SVX) DNA.
[b] Rescued plasmids and parental SVX (recircularized, single LTR plasmid) were transfected onto 42 and the transiently produced virus was harvested at 18 hr postglycerol shock. The supernatants were titered for G418 resistance on NIH 3T3 cells and the titer of the rescued plasmid was compared with the titer of the parental SVX. At least three individual rescued plasmids were tested in each case.
[c] ND–Not determined.

ml, respectively. The culture supernatant from ZIPNeo 1A was found to exhibit titers of 3×10^4 G418^R cfu/ml on *cos* cells and 9×10^5 cfu/ml on CV-1 cells. Both viral genomes could therefore be successfully pseudotyped and introduced into *cos* and CV-1 cells, where they were also capable of neo gene expression. Examination of the integration status of SVX in several independent *cos* lines indicated that SVX was not integrated and varied in copy number, from approximately 2 to 100 copies per cell (data not shown). The ZIPNeo virus was found to be integrated as a single copy provirus in several *cos* lines, as expected.

The superinfected SVX 2B and ZIPNeo 1A lines, producers of virus of both amphotropic and ecotropic host range, were then cocultivated with *cos* cells for up to 4 days. Hirt supernatants were prepared and examined for the level of SVX or ZIPNeo plasmid by Southern blot analysis and by transformation of E. coli HB101. A high level of SVX plasmid (5.0 ng/5×10^6 *cos* cells) was observed in the Hirt supernatant after 3 days of cocultivation of SVX 2B and *cos* cells (data not shown). However, the majority of the plasmids suffered deletions (see below). Other *cos* cell infections were then set up, using as a source of virus clonal cell lines generated by infection of SVX nonproducers with the amphotropic helper virus 1504A. When the Hirt supernatant was examined from one of these infections, 10-fold more plasmid was obtained, and very few molecules were seen to be deleted. (Figure 2, lanes 5–7). Preliminary experiments indicated that the level of SVX plasmid in *cos* cells reached a plateau after 3 days of cocultivation. No ZIPNeo plasmid was observed in the Hirt supernatant from cocultivation of *cos* and ZIPNeo 1A (which produced virus that did not contain the SV40 origin of replication) (Figure 2, lane 8). Bgl II digestion of the Hirt DNA obtained after cocultivation of the SVX producer cells and *cos* cells revealed bands of 4.1 and 4.7 kb (Figure 2, lane 6). The 4.1 kb band comigrated with the single LTR-SVX control plasmid, shown in lane 2, and the higher MW band of 4.7 kb migrated at the position expected for a plasmid with two LTRS. After digestion of the

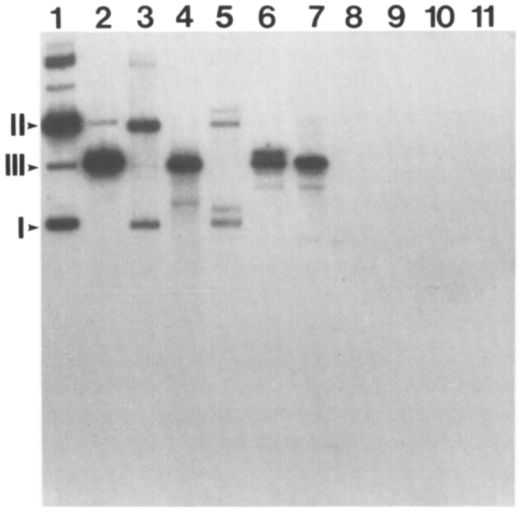

Figure 2. Analysis of SVX Sequences Amplified during *cos* Cell-Mediated Rescue

The DNAs indicated below were run on a 1.0% agarose gel, transferred to Zetabind paper (AMF/Cuno), and hybridized with a nick-translated neo probe according to standard procedures. A marker plasmid of SVX was prepared by cutting the original SVX vector with Sac I (which cuts once in each LTR) and recircularizing the viral sequences by incubation with T4 DNA ligase prior to transformation of E. coli HB101. The cloned plasmid, named SVX recirc, thus contained only one LTR and no proviral flanking cellular sequences or pBR322 sequences outside of those shown in Figure 1. (1) 50 pg of undigested SVX recirc; (2) 50 pg of SVX recirc digested with Bgl II; (3) 1.0% of the Hirt supernatant from a 10 cm dish of fused SVX 2B and *cos* cells, undigested; (4) Same as lane 3 but digested with Bgl II; (5) 0.1% of the Hirt supernatant from a 10 cm dish of *cos* cells infected with SVX, undigested; (6) Same as lane 5 but digested with Bgl II; (7) Same as lane 5 but digested with Sac I; (8) 1.0% of the Hirt supernatant from a 10 cm dish of *cos* cells infected with ZIPNeo 1A producers, undigested; (9) 1.0% of the Hirt supernatant from a 10 cm dish of *cos* cells fused with ZIPNeo 1A; (10) 1.0% of the Hirt supernatant from a 10 cm dish of unfused, uninfected, *cos* cells; (11) 1.0% of the Hirt supernatant from a 10 cm dish of unfused SVX 2B cells.

Hirt DNA with Sac I, which cuts once in each LTR, only one band of 4.1 kb was observed, suggesting that the larger 4.7 kb DNA contained two LTRs (lane 7). Undigested

Hirt DNA electrophoresed as form I and form II molecules containing one or two LTRs, thus demonstrating that the amplified SVX plasmids did not exist as concatemers (lane 5).

Transformation of E. coli HB101 with Hirt DNA obtained from cocultivation of 5×10^6 *cos* cells with SVX producer cells (superinfected with 1504A alone) yielded up to 10^5 kanamycin sulfate-resistant colonies. The structure of plasmids isolated from these colonies was analyzed by digestion with restriction enzymes, with the results summarized in Table 1. The majority of plasmids examined exhibited the predicted SVX structure (see Table 1). However, if plasmids were isolated from SVX producers that were superinfected with both M-MuLV and 1504A, 85% of the rescued plasmids contained deletions between the 3′ end of the LTR and the unique Bam HI site (data not shown). Interestingly, this region was also a popular site for insertions. Restriction mapping of three randomly chosen plasmids that contained insertions (including one plasmid which was rescued via NIH3T3 cell infection) revealed that insertion, as well as some deletion, occurred between the 3′ end of the LTR and the Bam HI site. The reason for this phenomenon is presently unclear.

Amplified Plasmid Rescue Following Fusion of SVX Nonproducer and Cos *Cells*

Botchan et al. (1979, 1980) have shown that the fusion of rodent cells carrying integrated copies of the SV40 genome with monkey CV-1 cells leads to activation of the SV40 origin of replication in situ and the eventual excision and amplification of closed circular SV40 DNA. In cells containing multiple tandem copies of the SV40 genome, excision appears to occur via homologous recombinational events, since the majority of recovered viral genomes are unit length and infectious. Based on these studies, we determined whether the SVX proviral genome in SVX 2B cells could be similarly excised and amplified via fusion of the SVX 2B line with *cos* cells. Fusion between SVX 2B cells and *cos* cells, or ZIPNeo 1A cells and *cos* cells, was accomplished by a brief exposure to polyethylene glycol 1000 (Davidson et al., 1976). After incubation of the fused cells for 1, 2, or 3 days at 37°C, Hirt DNA was prepared and analyzed by Southern analysis. As shown in Figure 2, the SVX 2B–*cos* cell combination resulted in 5 ng of predominately unit length closed circular SVX plasmid per 5×10^6 *cos* cells. In contrast, the ZIPNeo 1A line, which does not contain an SV40 origin, yielded no plasmid when fused with *cos* cells (lane 9). After digestion of the SVX Hirt DNA with Bgl II (which cuts once in the neo sequences of SVX), a band of 4.1 kb, which corresponds with the control single LTR-SVX plasmid, was observed (lane 4). Thus the predominant recombination event that freed the SVX plasmid from the flanking cellular DNA likely occurred within the 600 bp direct repeat sequences of the LTR. Alternatively, molecules that arose from recombination events that occurred outside of the LTRs in the flanking cellular sequences (see below) efficiently recombined within the LTRs.

Transformation of E. coli HB101 with the Hirt supernatant from *cos*–SV2B fusion yielded up to 5×10^3 colonies from 5×10^6 *cos* cells. The structure of the majority of these plasmids was as predicted (single LTR-SVX molecules) and the rescued plasmids were again active in a transient rescue assay (Table 1). Occasionally, plasmids that contained insertions were isolated. Restriction mapping of several of these plasmids indicated that these molecules contained one LTR, were deleted at either the 5′ or 3′ end of the SVX genome, and contained cellular sequences. This structure is consistent with occasional recombination events that occurred between flanking cellular sequences and SVX sequences.

Isolation of Cellular Sequences Flanking Recombinant Proviruses

The studies of SV40 excision from rodent cells (Botchan et al., 1979, 1980) also indicated that in cell lines containing either a single SV40 genome or simply segments of the SV40 genome containing the viral origin of DNA replication, fusion with CV-1 cells led to the excision and amplification of a heterogenous population of molecules containing variable amounts of cellular DNA sequences adjacent to the site of integration of viral sequences. These observations suggested that the site of recombinant retrovirus integrations might be similarly obtained from the pool of free SVX molecules found in the Hirt supernatant following PEG-mediated *cos* cell fusion. Southern analysis of the Hirt DNA from SVX 2B–*cos* cell fusions indicated that 10%–15% of the excised plasmids contained putative inserts. To enrich for these molecules, Hirt DNA was fractionated on a 1% agarose gel and the DNA isolated from the region at the top of the gel was used to transform E. coli. Restriction analysis of plasmids rescued by this method indicated that 60% of the DNAs contained inserts ranging from 5–8 kb and approximately half of the DNAs with inserts contained two LTRs. Moreover, the site of insertions in the two LTR plasmids mapped between the LTRs, as expected for cellular sequences adjacent to the proviral site of integration (data not shown).

Restriction mapping of five of these inserts indicated that the inserts were highly related, although not identical. One clone, Fx1 1, was further mapped with restriction enzymes and several fragments that contained exclusively cellular sequences were nick translated and used as probes for a Southern blot of uninfected NIH3T3 DNA. Four of six fragments tested in this manner contained repetitive DNA. One Aha III fragment of 500 bp was identified as containing only single copy sequences, and was therefore used as a diagnostic probe to analyze the other two LTR-containing inserted plasmids (designated Fx1 plasmids) as well as DNA of NIH3T3 cells and the SVX 2B cell line. As shown in Figure 3A, all four of the Fx1 plasmids not only contained sequences homologous to the putative integration site probe of clone Fx1 1, but also retained the same Aha III fragment that was used as a probe (lanes 2–5). As a control, SVX plasmid DNA was also examined and found to be negative, as expected (lane SVX).

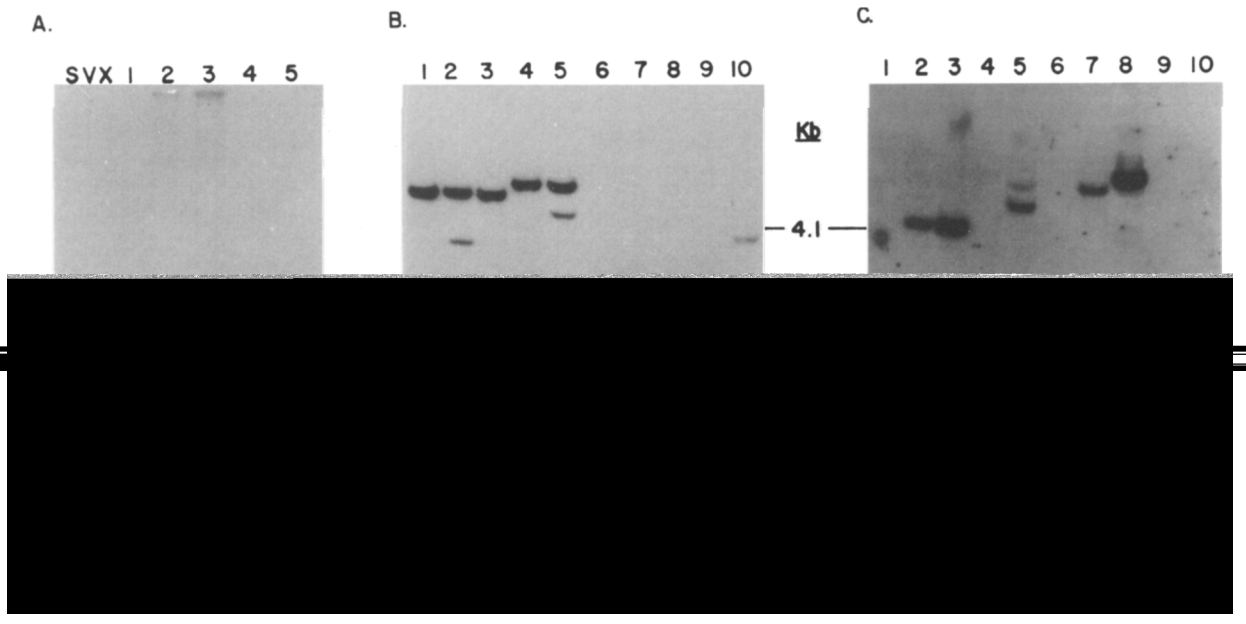

Figure 3. Recovery of Cellular Sequences Flanking the Site of SVX Integration via the *cos* Cell-Fusion Technique

The *cos* fusion technique was used to generate plasmids which were screened for the presence of insertions outside the LTRS (see Experimental Procedures). These plasmids, termed clones Fx1 1–5, were analyzed by Southern blot analysis to determine if the inserted sequences were from the site of virus integration in the line SVX 2B. Plasmid Fx1 1 was digested with Aha III and a 500 bp fragment of entirely cellular sequence was nick translated and used to probe Southern blots of SVX recirc, Fx1 1–5, and cellular DNA from uninfected NIH3T3 and SVX 2B cells (A and B). (A) Analysis of Fx1 plasmids cut with Aha III. (1) SVX DNA cut with Aha III. (2–5) Fx1 plasmids cut with Aha III. (B) Analysis of uninfected NIH3T3 and SVX 2B cellular DNAs using the Aha III probe. (1) NIH3T3 DNA digested with Sac I; (2) SVX 2B DNA digested with Sac I; (3) NIH3T3 DNA plus 10 pg of SVX recirc DNA digested with Sac I; (4) NIH3T3 DNA digested with Hind III; (5) SVX 2B DNA digested with Hind III; (6) NIH3T3 DNA digested with Aha III; (7) SVX 2B DNA digested with Aha III; (8) NIH3T3 DNA plus 50 pg Fx1 1 digested with Aha III; (9) NIH3T3 DNA digested with Pst I; (10) SVX 2B DNA digested with Pst I. (C) Analysis of uninfected NIH3T3 and SVX 2B cell DNAs using Neo probe. The blot shown in (B) was washed with NaOH (according to manufacturer's specifications) and reprobed with a virus-specific probe (the 1.4 kb neo fragment of SVX).

To further characterize the putative site of proviral integration in SVX 2B cells, DNA from uninfected 3T3 cells and the SVX 2B cell line was cleaved with a variety of enzymes, transferred to Zetabind paper, and probed with the Fx1 1 Aha III fragment. If the probe was indeed from cellular DNA flanking the SVX provirus, a different Southern band pattern would be expected for line SVX 2B as compared to NIH3T3 cells. As shown in Figure 3B, lanes 1, 2, 4, 5, 9, and 10, digestion of SVX 2B DNA with Sac I, Hind III, and Pst I yielded the expected heterozygous pattern. (Heterozygous patterns were also obtained with Xba I and Bgl II, data not shown.) The blot shown in Figure 3B was washed and reprobed with neo sequences to confirm that the "new" bands in the SVX 2B line, and absent in NIH 3T3 DNA, contained the viral sequences. As shown in Figure 3B, the Hind III digest yielded positive hybridization with neo where expected. As Sac I cleaved in the LTRs, the Sac I digest of SVX 2B simply liberated the intact SVX virus, which hybridized with neo, and comigrated with positive control plasmid added to NIH3T3 DNA (lanes 2 and 3). As shown in Figure 3B (lanes 6 and 7), hybridization of Aha III digested NIH3T3 and SVX 2B DNA with the cellular probe yielded the expected patterns of a single band of 500 bp, indicating that the probe was indeed entirely of cellular origin. If Fx1 1 plasmid DNA was added to NIH3T3 DNA and cut with Aha III (lane 8), it can be seen that the recovered Aha III probe of Fx1 1 was in fact identical in size to the homologous fragment in uninfected NIH 3T3 DNA. This confirms that the ligation of flanking sequences did not occur within this fragment.

Generation of cDNA Copies of Genomic DNA by Passage in the SVX Vector

Passage of recombinant retrovirus genomes carrying DNA fragments which contained introns have been shown by two other groups to result in the precise deletion of the intervening sequences, presumably via splicing of the retrovirus genome prior to encapsidation (Shimotohno and Temin, 1982; Sorge and Hughes, 1982). Since the SVX vector affords a rapid means of recovering retrovirus genomes as bacterial plasmids, we explored the use of the vector to rapidly isolate cDNA copies of genomic DNA sequences. As a test case, the adenovirus 5 E1A region was chosen. This region is of interest in that it represents a complex transcriptional unit which encodes several interesting functions implicated in viral and cellular transcriptional control (Berk et al., 1979; Jones and Shenk, 1980; Nevins, 1982; Schrier et al., 1983). In a typical lytic infection of Hela cells, three distinct E1A mRNAs, designated 13S, 12S, and 9S, are generated from a single transcript that is processed by utilizing three different 5′ splice sites and one common 3′ splice site (Berk and Sharp, 1978; Chow

et al., 1979; Spector et al., 1978). Therefore, it was conceivable that passage of the E1A sequences in SVX would result in the generation of three distinct retrovirus genomes encoding the individual E1A functions. The particular adenovirus DNA fragment used for these experiments ex-

site at nucleotide 1574 (Tooze, 1981). The fragment therefore contained the E1A promoter (but not enhancer) and the entire coding region for the 9, 12, and 13S RNA encoded proteins, but lacked the E1A polyadenylation site. After introduction of the E1A containing fragment into the Bam HI site of SVX, the recombinant DNA was transfected into the packaging line, $\psi 2$; a transient harvest was taken 18 hr later, and the supernatant used to infect NIH3T3 cells. G418^R colonies were selected, isolated, and ex-

and Xba I digests of DNA from several independent colonies indicated that the E1A insert in each case had undergone a specific deletion that corresponded in size to that expected for the 13S E1A mRNA (data not shown). No E1A inserts corresponding to unspliced E1A or the two other known E1A splices were found. In order to confirm the results of the Southern blot analysis, several independent colonies were fused with *cos* cells as described above and Hirt DNA was isolated and used to transform competent E. coli HB101. Digestion of several rescued plasmid genomes with the diagnostic enzymes Sma I, Bam HI, Xba

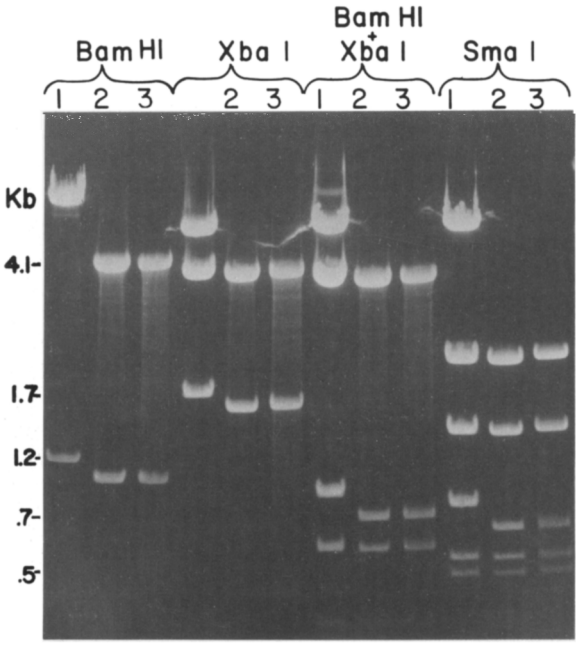

Figure 4. Restriction Enzyme Analysis of E1A-SVX Proviral DNAs Recovered from Fusion of E1A-SVX-Infected NIH3T3 Clonal Lines and *cos* Cells

Lane 1 contains DNA from the starting plasmid; lanes 2 and 3 contain DNA from two representative rescued plasmids isolated from fusion of two independent NIH3T3 colonies.

As Bam HI was the unique site into which E1A was inserted, digestion of the original E1A-SVX plasmid with Bam HI resulted in a 1.2 kb E1A fragment and a 10 kb vector backbone fragment (Figure 4, lane 1, Bam HI digest). The splice donor for a 13S splice is located at adenovirus nucleotide number 1111, while the 12S and 9S donors are located at nucleotides 973 and 636, respectively (Svensson et al., 1983). The common 3′ acceptor is at nucleotide number 1226. Therefore digestion of rescued plasmids with Bam HI should yield a 1.1 kb, .96 kb, or .71 kb E1A-containing fragment if a 13S, 12S, or 9S splicing event has occurred. As shown in lanes 2 and 3 (Bam HI digest), the expected 1.1 kb E1A fragment diagnostic of the 13S cDNA was observed. The fragment of 4.1 kb was derived from the vector backbone. Xba I cuts twice in the E1A-SVX proviral DNA, once in the middle of the LTR and once in E1A sequences at adenovirus nucleotide number 1340. The original E1A-SVX construct contained two LTRs and therefore yielded three fragments of 6.0 kb, 1.7 kb, and 3.5 kb upon Xba I digestion (Figure 4, lane 1, Xba I). The rescued plasmids, which contained a single LTR and a 13S cDNA, yielded fragments of 3.5 kb (containing primarily vector sequences) and 1.6 kb. Since the 1.7 kb Xba I fragment of the original E1A-SVX contained the 13S splice donor, the reduction in size to 1.6 kb was as expected for a 13S cDNA. Digestion of the original E1A-SVX plasmid with Bam HI plus Xba I yielded fragments of 6 kb, .98 kb, and .23 kb (Figure 4, lane 1, Bam HI and reduce the size of this fragment to .87 kb, .73 kb, or .47 kb, respectively. As shown in Figure 4, lanes 2 and 3, the expected band of .87 kb was observed. One further digest, Sma I, confirmed the 13S structure of the rescued plasmids. Sma I cuts once in the LTR, once at the 5′ end of the inserted E1A fragment (an artifact of the construction method), once within E1A at adenovirus nucleotide number 1010, once near the 3′ end of the Bam–Xho fragment of SVX which encodes the 3′ splice site of M-MuLV, and once near the 3′ end of the neo gene. Digestion of the original E1A-SVX with Sma I resulted in fragments of 6 kb, 0.5 kb, 0.15 kb, 0.92 kb, 1.3 kb, and 1.9 kb. As the 0.92 kb fragment contains the 5′ donor sequence for a 13S splice, this fragment should be reduced to .80 kb after Sma I digestion (Figure 4, lanes 2 and 3, Sma I digest). A 12S or 9S splice would eliminate the Sma I site at 1010 nucleotides, and thus eliminate both the .92 kb and .65 kb fragment.

Several rescued plasmids were subsequently sequenced and found to contain precise DNA copies of the 13S E1A mRNA. Retrovirus genomes containing DNA copies of the 12S and 9S mRNAs have been similarly obtained by passage of the unspliced E1A virus through Hela cells (B. E. Roberts et al., unpublished data).

Discussion

The SVX shuttle vector described here provides a powerful system for both the introduction of genes into mammalian

cells and the whole animal and eventual recovery of the transferred sequences as molecular clones. In construction of the vector, all of the *cis*-acting sequences necessary for the efficient transmission of the viral genome have been retained. Generation of highly transmissible virus carrying the appropriate recombinant genome is made possible by transfection of the SVX DNA into cells that provide essential virus gene products (Mann et al., 1983). Expression of sequences inserted into SVX is achieved through the use of the normal retrovirus transcriptional signals, which give rise to both genomic length and spliced viral transcripts.

To date, we have introduced over a dozen sequences (both cDNA and genomic) into the vector and have demonstrated both transcriptional and translational expression in a majority of cases. Although the size constraints upon the inserted sequences have not yet been rigorously defined, we have introduced sequences as large at 4.4 kb into SVX without perturbation of transmission or splicing. Based on the occurrence of variant M-MuLV genomes as large as 11 kb (S. P. Goff, personal communication), we suspect that 6.0 kb will represent the upper limit of insert sizes. However, since the size limitation applies only to the encapsidated genome, it is likely that large genomic inserts (in the 20 kb size range) could be introduced into the vector for the purpose of intron deletions as long as the truncated insert is less than 6 kb in size. Experiments to test this prediction are in progress.

In addition to size, there do appear to be several other factors that can affect transmission of a recombinant genome. Shimotohono and Temin (1981) reported that the presence of polyadenylation sites within an insert can drastically reduce transmissibility, presumably because of the generation of prematurely polyadenylated transcripts that lack the capacity to be properly replicated. In the one instance in which we have inserted a sequence with a poly(A) site (the polyoma large T cDNA sequence), no reduction in G418 titer from that of SVX itself was detected (C. Cepko, P. Jat, P. Sharp, and R. Mulligan, unpublished observations). Since the rules that govern the preferential utilization of a particular poly(A) site when situated adjacent to several additional poly(A) sites have not yet been defined (Ucarowitz et al., 1982), the effects of various poly(A) likely be variable.

We have also found that the insertion of a few sequences into SVX appears to affect drastically the generation of the subgenomic viral RNA that encodes G418 resistance. In such cases, the few G418^R colonies that can be isolated contain rearranged proviral genomes (A. Korman and R. C. Mulligan, unpublished data). To alleviate this problem, we have constructed vectors that lack the retroviral 5′ and 3′ splice sites and contain independent transcriptional signals for promotion of selectable marker expression.

Similar strategies for exploiting retroviruses as vectors have been reported by a number of laboratories (Shimotohno and Temin, 1981; Wei et al., 1981; Tabin et al., 1982; Miller et al., 1983; Joyner and Bernstein, 1983). However, one important difference between our protocol for generating virus from vector DNA and previously reported strategies is that we have exploited the high transient expression of the vector shortly after transfection to increase the representation of cloned DNAs as infectious virus. Although the SVX vector per se yields only approximately 10^3 G418^r Cfu/ml, we have previously shown that the incorporation of a plasmid backbone containing the polyoma virus early region permits the rescue of over 10^5 cfu/ml (Mulligan, 1983). This efficiency of rescue will be crucial for the eventual use of the system for gene isolation studies.

All of the rescue protocols described here result in the rescue of proviral genomes exhibiting both the proper structure and high biological activity upon retransfection. The most facile means of rescuing a recombinant genome is probably the *cos* fusion method. This method does not rely on the superinfection of the transformant of interest with virus, and results in thousands of bacterial transformants harboring the desired proviral DNA. Nevertheless, the transformant prior to fusion. For certain applications, particularly in cases where creation of cDNAs from genomic DNA sequences is desired, it may be possible to shorten the process significantly. For example, we are currently testing whether it is possible to rescue proviral genomes by simply infecting *cos* cells with the small amount of virus harvested from a transient-rescue assay.

The ability to recover recombinant retroviral genomes and adjacent chromosomal sequences rapidly has implications beyond application to gene isolation schemes. We are particularly interested in applying the system towards studies of the effects of chromosomal position upon the expression and regulation of genes transferred into cells in vitro and in vivo via retrovirus-mediated transduction. The ability to rapidly isolate sequences flanking proviral integration sites will provide a source of probes for examining adjacent chromosomal sequences in a number of ways (e.g., transcriptional activity, DNA methylation, DNAase hypersensitivity, etc.). In addition, as retroviruses have been observed to behave as insertional mutagens et al., 1981; Payne et al., 1982), SVX integrations that create a selectable alteration in the expression of nearby sequences can provide flanking sequence probes for the gene under study. Another interest is to use the shuttle features of the vector to characterize the spectrum of mutations induced as a consequence of retrovirus replication.

The most useful practical application of the system at present, however, is probably the rapid isolation of cDNA sequences from genomic DNAs containing intervening sequences. In particular, in cases where the site of synthesis of an mRNA is not known, a corresponding genomic DNA sequence can be isolated, or in the case of genes encoding large mRNAs, the shuttle rescue procedure may offer the most rapid means of generating full-length cDNA

sequences. We have already utilized the vector to isolate cDNA copies of a variety of genomic inserts, including the adenovirus E1A, adenovirus E1B, and SV40 T-antigen-coding regions (in collaboration with P. Jat and P. A. Sharp, M.I.T.) and human DR sequences (A. Korman et al., unpublished data). Similar success in isolating cDNA sequences has been obtained in M. Botchan's laboratory (University of California at Berkeley). Our recent studies of adenovirus E1A function which have utilizted the SVX vector for the isolation of individual E1A cDNAs suggest that the system may also be generally useful for dissecting complex ✓transcriptional ✓ units ✓encoding ✓differentially spliced, overlapping transcripts (B. E. Roberts et al., unpublished data).

The generality of the method for isolating cDNA se-

inserts. In particular, the finding that only 13S cDNA-SVX genomes are generated after transfection of ψ2 cells with the E1A-SVX recombinant DNA, yet all three cDNA species are generated after transfection of Hela cells, suggests that an added complexity exists in the system. Although the results may reflect a species or cell type difference in the efficiency of the various splicing reactions, we have recently shown that the infection of 3T3 cells with Ad5 virus results in the production of both 13S and 12S mRNAs. It is therefore likely that the differences observed using the retrovirus constructs in part reflect inherent differences in either the structure of chimeric retrovirus-E1A primary transcripts or the kinetics of transport of unspliced recombinant retrovirus RNA versus normally spliced Ad5 RNA. Experiments to test a variety of additional genomic inserts, as well as to resolve the different patterns of cDNA recovery observed in mouse and human cells, are underway.

Experimental Procedures

Cell Culture, DNA Transfections, and Rescue of Recombinant Virus

NIH3T3 cells, obtained from G. Cooper (Dana-Farber Cancer Institute), CV-1 cells, and ψ2 cells (Mann et al., 1983) were maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% calf serum. M-MuLV virus was obtained from the clone 1 cell line (Fan and Paskind, 1974), and 1504A virus (Hartley and Rowe, 1976) was a gift from Nancy Hopkins (M.I.T.). A subclone of *cos* cells, *cos* M6, isolated by M. Horowitz (Horowitz et al., 1983) from cos-1 cells of Y. Gluzman (1981) (Cold Spring Harbor) were maintained in DMEM and 10% fetal calf serum. Transfections were via the $CaPO_4$ technique of Graham and van der Eb, as modified by Parker and Stark (1979). To rescue recombinant SVX virus, approximately 5×10^5 ψ2 cells (seeded 24 hr earlier) were transfected with 10 μg SVX DNA. Eighteen hours after the glycerol shock, culture media was collected and filtered through a .45μ Nalgene filter. To infect cells, 1 ml of the supernatant was added to 5×10^5 NIH3T3 cells in a 100 cm^2 dish and incubated for 2 hr at 37°C in the presence of 8 μg/ml polybrene (Aldrich). Complete media was added, the cultures were incubated for 2–3 days, and were then split into complete media containing 1 mg/ml G418 (Gibco) (Davies and Jimenez, 1980).

Plasmid Construction and Analysis of Cellular and Plasmid DNAs

Plasmid constructions, Southern blotting procedure, and agarose gel electrophoresis were performed according to standard procedures (Maniatis et al., 1982). Enzymes were obtained from New England Biolabs and were used according to manufacturer's specifications.

Protocols for Rescue of Proviral DNA

For rescue of nonamplified SVX, producer cell lines were cocultivated with uninfected NIH3T3 cells at a ratio of 1:4 (approximately $1–5 \times 10^6$ cells/100 cm^2 dish) in the presence of 2 μg/ml polybrene for 20 hr. A Hirt supernatant (Hirt, 1967) was then prepared, extracted once with phenol:chloroform (1:1 ratio) and once with chloroform. The aqueous phase was precipitated with isopropanol at −20°C at least twice. The final pellet was resuspended in a minimum volume of 10 mM Tris (pH 7.4) and 1 mM EDTA. E. coli HB101 (strain LM 1035, a gift from Y. Nagamine, Rockefeller University) were then transformed directly with this preparation (Lederberg and Cohen, 1974) or the preparation was digested for analysis by the Southern blotting procedure. In preparation of competent E. coli HB101 for transformation, the bacteria were harvested at $OD_{600} = 0.2$ and the heat shock step was carried out at 37°C, per Y. Nagamine (personal communication). Previously frozen (Morrison, 1979) competent LM 1035 cells yielded $5 \times 10^7–10^8$ transformants per μg of pBR322.

For rescue via *cos* cell infection, a line of SVX-infected cells was superinfected with 1504A virus. After allowing 1–2 weeks for spread of

serum, and 2 μg/ml polybrene. Media was changed after 1 and 2 days. A Hirt extract was then prepared as described above.

For fusion rescue, SVX 2B and *cos* cells were plated at a 1:1 ratio by splitting both cell types from newly confluent dishes at a 1:2 ratio into Dulbecco's Modified Eagle's medium (DMEM) containing 10% fetal calf serum and 10% calf serum. When the cells were very confluent (12–48 hr after plating), they were washed twice with DMEM and exposed to a 50% polyethylene glycol 1000 (Baker) solution in DMEM (50% v/v), for 60 sec at room temperature (Davidson et al., 1976). The cells were then quickly washed twice with DMEM and twice with DMEM plus 10% calf serum. The fused cells were incubated in DMEM containing 10% calf serum and 10% fetal calf serum for 3 days, with media changes every day. Hirt DNA was then prepared and used to transform HB101 as described above.

For recovery of flanking sequences at the site of virus integration, *cos* cells were fused with line SVX 2B as described above. The resulting Hirt supernatant from a 100 cm^2 dish of fused cells was fractionated by electrophoresis on a 14 cm 1.0% agarose gel. After running the bromphenol blue dye to the bottom of the gel, a 1–2 cm wide slice of the gel just below the sample well was removed (high molecular weight DNA of the isolated slice ran just ahead of the selected slice). The DNA in the slice was isolated by the glass bead procedure (Vogelstein and Gillespie, 1979) and used to transform E. coli.

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