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Genetics

Transfer of genes into hematopoietic cells using recombinant DNA viruses

(DNA transfer/viral vectors/hematopoietic cell lines/simian virus 40/adenovirus)

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ABSTRACT **The ability of recombinant DNA viruses to transfer genes into hematopoietic cells has been explored. A recombinant simian virus 40 (SV40) in which the early region had been replaced with the chloramphenicol acetyltransferase (*CAT*) gene driven by the promoter from Rous sarcoma virus (RSV), was constructed. This virus transferred the *CAT* gene more efficiently into mouse and human bone marrow cells and into the K562, MEL, and WEHI hematopoietic tissue culture cell lines, than the classical calcium phosphate DNA transfer procedure, as shown by assay for CAT activity 48 hr after infection. Recombinant SV40 virions were also shown to be capable of stably transforming Chinese hamster ovary cells by use of an early region recombinant containing the methotrexate-resistant dihydrofolate reductase (*DHFR*) gene driven by the RSV promoter. The entire DHFR transcriptional unit could be detected in the genome of transformed cells that were also shown to be resistant to methotrexate. A recombinant adenovirus stock containing the neomycin-resistance gene driven by the SV40 early promoter was used to infect the K562 and MEL hematopoietic cell lines to resistance to the antibiotic G418. Transformation frequency was 10- to 100-fold higher than that obtained with calcium phosphate-precipitated DNA. Most or all of the recombinant adenovirus genome was integrated as 1–3 copies in the transformed cells. These studies show the feasibility of using DNA viruses for introduction of new genetic material into hematopoietic cells.**

Recombinant viral vectors may offer several advantages for transfer of genetic information into animal cells compared to the most frequently used method that utilizes calcium phosphate ($CaPO_4$)-precipitated DNA (1). Among these advantages are a higher efficiency of gene transfer, particularly for suspension cells, possible specificity for certain cell types, and the potential for introducing only one or two copies of new genetic information.

Retroviral vectors have been used to introduce genes into enzyme-deficient cells (2–5). Recently, transfer of new genetic information into hematopoietic cells of mice has been achieved with a recombinant retrovirus (6). Rearrangement of intron-containing genes (7) and the inhibiting effects of promoter and polyadenylylation (3, 6) signals on the propagation of recombinant retroviral genomes may limit the usefulness of this approach. Recombinant simian virus 40 (SV40) viruses have been useful mainly for study of gene expression in short-term assays (8, 9), an application largely superseded by the ability to study gene expression after $CaPO_4$-mediated transfer of recombinant plasmid DNA into tissue culture cells (10, 11). Cell transformation due to integration of portions of the SV40 genome after infection by SV40 is a well described phenomenon and suggests that appropriate recombinant SV40 virions could be used for gene transfer (12). The papilloma virus has the advantage that it replicates as an episome (13). Recombinant adenoviruses have recently been used as vehicles for high efficiency transfer of either the SV40 early region (14) or the gene that confers neomycin resistance (15) into tissue culture cells.

Recombinant virus stocks can be obtained by use of defective helper viruses or by use of a permissive cell line into which a complementing portion of viral genome has been integrated. Such cell lines are already available for preparation of recombinant retroviral stocks (16). The Cos cell lines (17) can replicate molecules containing a SV40 origin (18) but do not express T antigen at a level sufficient to allow preparation of high titer SV40 viral stocks. Deletion mutants of adenovirus lacking the E1A region are complemented by E1A protein production in the human kidney cell line 293 (19). The 293 cell line can be used to prepare helper-free adenovirus stocks containing up to 6 kilobases (kb) of inserted foreign DNA (14, 20).

Our interest is the introduction of eukaryotic genes containing introns into hematopoietic cells. Recombinant SV40 vectors were shown to be efficient for achieving transient gene expression in hematopoietic cells. However, the propensity of the complementing SV40 molecules present in viral preparations to undergo recombination with generation of wild-type SV40 prevented use of these vectors for stable gene transfer. Hence, we explored the use of adenoviruses to achieve this purpose.

METHODS

Cell Culture. African green monkey kidney (GMK) cells were purchased from Flow Laboratories and grown in Iscove's modified Eagle's medium (IME medium) containing 2% (vol/vol) fetal calf serum. LTK^- cells, HeLa cells, and mouse erythroleukemia (MEL) cells that grow in suspension and a MEL $aprt^-$ subclone that grows in a monolayer (21) were cultured in IME medium supplemented with 10% fetal calf serum. HL-60, WEHI (22), and K562 suspension cells and a subclone of K562 that grows in a monolayer (provided by M. Fordis) were cultured in RPMI-1640 medium containing 10% fetal calf serum. Chinese hamster ovary (CHO) DG 21 cells that lack both dihydrofolate reductase (*DHFR*) alleles (23) were obtained from L. Chasin and were grown in IME medium supplemented with 10% fetal calf serum; hypoxanthine/glycine/thymidine and proline were also added. Selective medium for CHO DG 21 cells lacks hypoxanthine/glycine/thymidine and contains dialyzed fetal calf serum. Bone marrow cells were grown in Iscove's modified Dulbecco's medium containing 20% fetal calf serum. Penicillin (100 μg/ml) and streptomycin (100 units/ml) were added to all tissue culture media.

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Abbreviations: SV40, simian virus 40; CAT, chloramphenicol acetyltransferase; RSV, Rous sarcoma virus; DHFR, dihydrofolate reductase; kb, kilobase(s); bp, base pair(s).

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Construction of SV40 Recombinants. Fig. 1 shows a map of a SV40 recombinant containing the chloramphenicol acetyltransferase gene (SV-CAT) and the methotrexate-resistant DHFR gene (SV-DHFR^{MtxR}). The plasmids pUSV-CAT and pUSV-DHFR^{MtxR} containing the recombinant SV40 genomes were each made from three DNA fragments, a SV40 piece, a linearized pUC9 plasmid, and the inserted gene. To construct SV-CAT, pRSV-CAT (24, 25) (obtained from B. Howard and C. Gorman) was used. The plasmid pUSV-CAT was identified; restriction of plasmid DNA with *Bam*HI released a 5.2-kb fragment that could be cyclized by ligation at a low DNA concentration (1 μg/ml). SV-DHFR^{MtxR} was created by an identical strategy except the DHFR^{MtxR} coding sequence, isolated from pFR 400-12 (26) (obtained from C. Simonsen), was used for the construction of pUSV-DHFR^{MtxR}, from which the SV-DHFR^{MtxR} genome was prepared.

Preparation of the viral stocks SV-CAT and SV-DHFR^{MtxR} required a complementing helper virus with an intact early region. We used a viral genome (SV-STN DHFR) in which the SV40 late region sequences 1483 (*Hin*dIII) to 2533 (*Bam*HI) had been replaced with normal mouse DHFR coding sequences derived from pDHFR26 (8, 27). GMK cells were transfected with an equal molar mixture of early region and late region recombinant viral genomes in DEAE-dextran (0.67 mg/ml) using standard techniques. Hirt extracts of low molecular weight DNA, prepared using standard techniques (28), were analyzed by restriction endonuclease mapping. The SV-CAT stocks contained <5% SV-CAT and 95% wild type SV40; the SV-DHFR^{MtxR} stock contained 15%–25% SV-DHFR^{MtxR} and 75%–85% wild type SV40. The complementing viral genome SV-STN DHFR was negligible in both lysates.

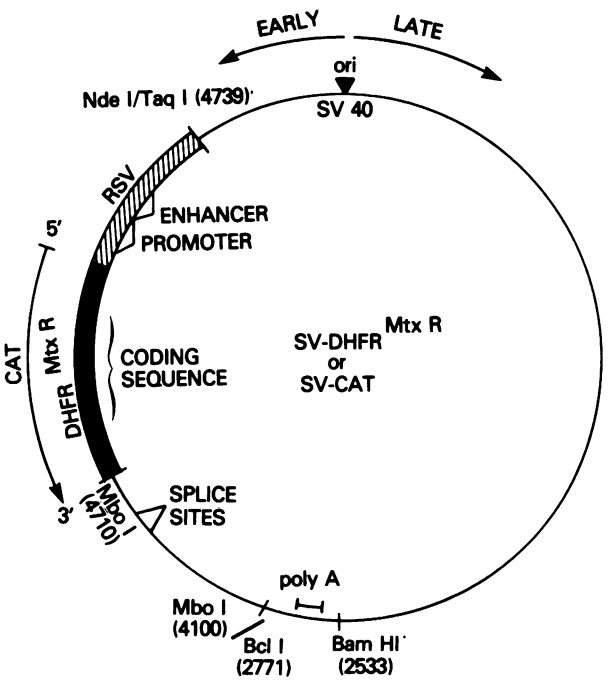

FIG. 1. Maps of the recombinant SV40 viruses SV-DHFR^{MtxR} and SV-CAT. Both coding sequences (*CAT* or *DHFR^{MtxR}*) are inserted into the early region of SV40 and are driven by the RSV long terminal repeat promoter. Splice sites for the small t antigen and the poly(A) site and signal from SV40 are placed downstream from the coding sequence. SV40 origin of replication (ori) together with the entire late region is intact in both recombinants. Thin line indicates SV40 sequences; thick solid line indicates coding sequence of the inserted gene; thick hatched line indicates the RSV long terminal repeat. Numbers in brackets indicate SV40 sequence number.

Preparation of the recombinant adenovirus 5 containing the neomycin resistance (*Neo^R*) gene in human embryonic kidney 293 cells that produce the E1A protein (19) has already been described in detail (15, 20). The viral lysate used in these experiments has a titer of 10^9 plaque-forming units on 293 cells.

Cell Transformation. Cells were infected with SV-CAT by exposure of 10^6 (cell lines) or 2–10 $\times$ 10^8 (bone marrow) cells to 1 ml of secondary viral lysate for 1 hr at 37°C. An equal number of cells in a 50-cm^2 Petri dish were exposed to a precipitate containing 10 μg of pUSV-CAT, 10 μg of pSTN 9a-DHFR (complementing helper), and 20 μg of salmon sperm DNA. Cells were harvested for the CAT assay after 48 hr (24).

CHO DG 21 cells were infected with SV-DHFR^{MtxR} by exposure of 5 $\times$ 10^5 cells to 1 ml of secondary viral lysate for 4 hr. Subsequently, the cells were grown in medium lacking hypoxanthine/glycine/thymidine. Transformation of the adherent and floating MEL cell lines with the SV-DHFR^{MtxR} stock was attempted by similar methods, although selection was in 0.4 μM methotrexate (Lederle Laboratories, Pearl River, NY). Adherent and "floating" K562 and MEL cells were infected with adenovirus-Neo^R (15); 10^6 cells in logarithmic phase were exposed to virus at a multiplicity of infection of 400 plaque-forming units per cell in 1 ml of appropriate medium in a shaking air bath at 37°C for 1 hr. The cells were washed and resuspended in medium for 24 hr and then 10^2–10^4 cells were plated in individual microtiter wells in medium containing G418 (GIBCO) at a concentration of 1 mg/ml. DNA samples prepared by standard methods from cell clones were analyzed by the Southern blotting method.

RESULTS

Infection of Adherent and Hematopoietic Cells with SV-CAT. Fig. 2 shows data from seven cell lines tested, three adherent cell lines that grow as a monolayer (*a*) and four hematopoietic cell lines that grow in suspension (*b*). CAT activity was much higher in permissive GMK cells infected with SV-CAT than in GMK cells exposed to $CaPO_4$ DNA precipitate containing pUSV-CAT (and pSTN 9a-DHFR). Both semipermissive HeLa and nonpermissive L-cells expressed more CAT activity after exposure of $CaPO_4$-precipitated DNA than after exposure to virus. In contrast, SV-CAT was much more effective in transferring CAT activity into each of the hematopoietic cell lines than was the $CaPO_4$ DNA precipitate. Identical results were obtained in three separate experiments. The two methods for transferring the CAT transcriptional unit were also compared on mouse and human bone marrow cells (Fig. 3). SV-CAT infection gave readily detectable enzyme activity after 48 hr but exposure of marrow cells to $CaPO_4$-precipitated plasmid DNA gave little activity in mouse cells and no activity in human cells.

Transformation of CHO DG 21 and MEL Cells with SV-DHFR^{MtxR} Lysate. Viral lysate containing SV-DHFR^{MtxR} was made as described above using a complementing recombinant viral genome having the normal DHFR coding sequences in the late region. The plasmid of SV-DHFR^{MtxR}, pUSV-DHFR^{MtxR}, could transform L-cells and CHO cells to methotrexate resistance, whereas the plasmid containing the late region replacement, pSTN 9a-DHFR, could not. Infection of DHFR$^-$ CHO DG 21 cells with SV-DHFR^{MtxR} lysate followed by selection in medium lacking hypoxanthine/glycine/thymidine, yielded multiple colonies whereas no colonies were detected in the uninfected control plates. The transformation frequency was 0.1% in two separate experiments. Colonies that formed from infection of 5 $\times$ 10^5 infected cells ($\approx$500) were harvested and grown for prepara-

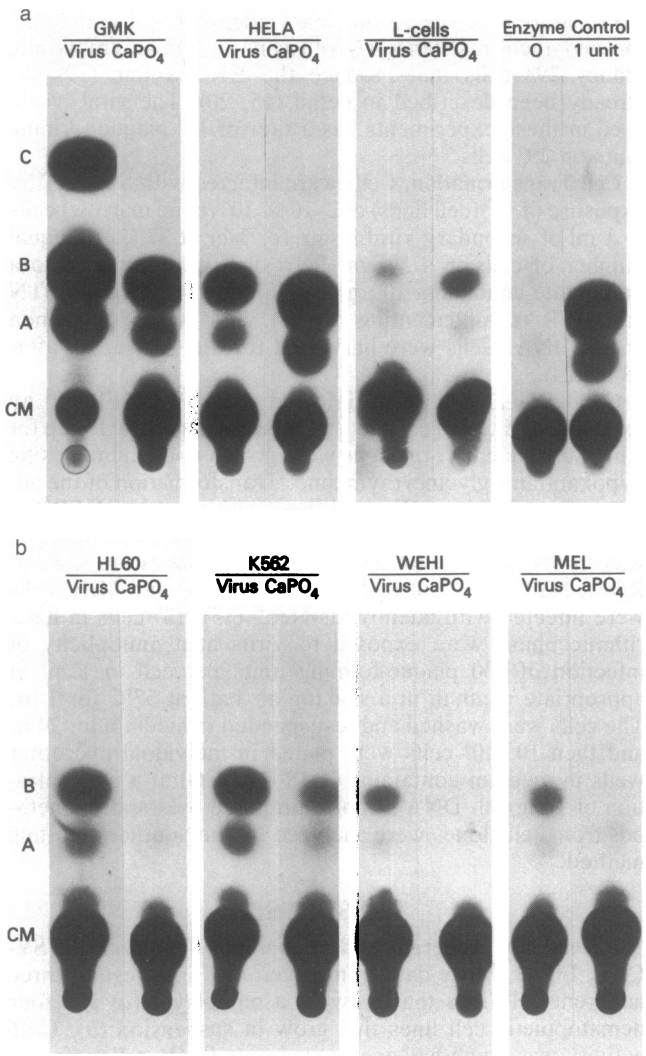

FIG. 2. Autoradiograms of thin-layer chromatography plates showing CAT activity after gene transfer in various cell lines tested. CM indicates [^{14}C]chloramphenicol, and A and B indicate its conversion to monoacetate forms. C indicates the diacetate form. GMK, African green monkey kidney cells; virus, infection with SV-CAT viral lysate; $CaPO_4$, transfection with pUSV-CAT and pSTN-DHFR DNA obtained with $CaPO_4$. (*a*) Enzyme control and results obtained with cells that grow in a monolayer. (*b*) Results obtained with cells that grow in suspension.

tion of DNA. Southern blot analysis revealed a 1.0-kb *Hin*dIII band detected with a probe containing the RSV promoter sequences and a 1.5-kb *Hin*dIII band detected with a probe for DHFR coding sequences (Fig. 4). Restriction with *Bam*HI yielded a 5.0-kb band that hybridized to both the RSV and DHFR probes (data not shown). The polyclonal population of SV-DHFR^{MtxR} cells were grown in various concentrations of methotrexate. Colonies formed in methotrexate concentrations up to 25.6 μM, a concentration that is $\approx$500 times that required to kill CHO cells that contain the normal endogenous *DHFR* gene.

MEL cells were also infected with SV-DHFR^{MtxR} lysate. A population of cells that grew in methotrexate concentrations up to 1.6 μM was obtained; growth of noninfected cells was completely inhibited with 0.4 μM methotrexate. Southern blots of DNA from the methotrexate-resistant MEL cells were probed with the RSV and DHFR probes and a nick-translated SV40 genome. No or very weakly hybridizing fragments were detected with the RSV and DHFR probes; the strongly hybridizing *Hin*dIII bands detected with the SV40 probe indicated that the majority of cells in this mixed

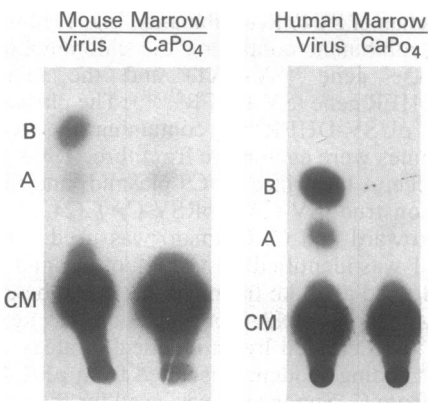

FIG. 3. CAT activity in mouse and human bone marrow cells after infection with SV-CAT secondary viral lysate (Virus) and transfection of pUSV-CAT using the $CaPO_4$ DNA transfer procedure. CM indicates [^{14}C]chloramphenicol; A and B indicate its two monoacetate forms.

clonal population had integrated a single copy of wild-type SV40 genome (data not shown).

Transformation of K562 and MEL Cells Using Adenovirus-Neo^R. Both adherent and suspension K562 and MEL cells were readily transformed to G418 resistance with adenovirus-Neo^R. For comparison, transfections were also carried out using $CaPO_4$-precipitated pSV2neo DNA, which contains a similar Neo^R transcriptional unit to the one in the recombinant adenovirus. In two separate experiments, the transformation frequency with the recombinant adenovirus was 10- to 100-fold greater than that obtained by $CaPO_4$-mediated DNA transfer. DNA derived from G418-resistant colonies

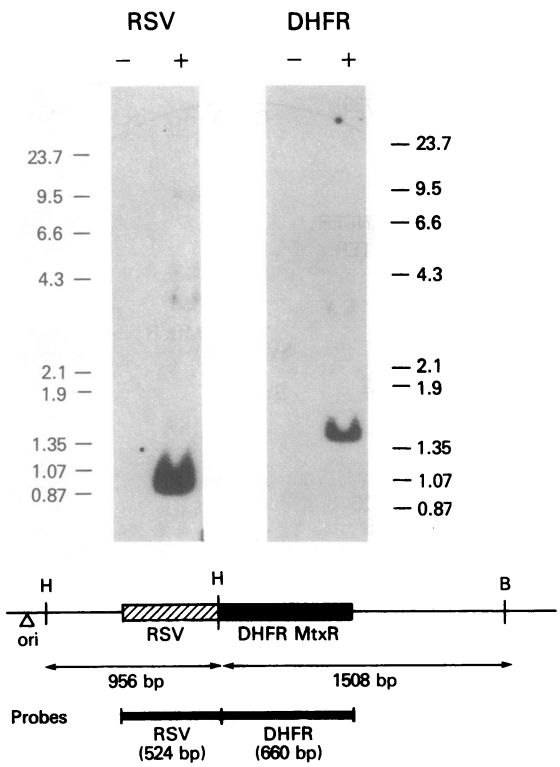

FIG. 4. A Southern blot of DNA from CHO DG 21 cells that have (+) or have not (−) been infected with the SV-DHFR^{MtxR} viral lysate. The DNA was cut with *Hin*dIII and probed with the Rous sarcoma virus long terminal repeat (RSV) or the DHFR^{MtxR} coding sequence (DHFR). Numbers on left and right indicate mobility of molecular weight markers (in kb). Lower part of figure shows a scheme of the whole transcription unit of the insert (RSV-DHFR^{MtxR}), indicating cutting sites for *Hin*dIII (H) and *Bam*HI (B).

was analyzed by Southern blotting using total adenovirus 5 genome or the 762-bp *Pvu* II fragment from the Neo^R gene coding sequence as probes (Fig. 5). The *Hin*dIII and *Xba* I fragments detected with the Neo^R probe were those expected based on the strategy used to construct the recombinant adenovirus genome. *Bam*HI yielded a single large fragment that differed in size between the four individual K562 or MEL cell clones analyzed.

The same filters were annealed to the adenovirus DNA probe. The K562 cell lines were found to contain adenovirus DNA sequences prior to infection that hybridized with the adenovirus probe (Fig. 5*A*). A reconstitution experiment was performed in which one or three genome equivalents of the adenovirus-Neo^R DNA was mixed with DNA from uninfected K562 cells, cut with *Hin*dIII or *Sst* I and probed with adenovirus 5 DNA. The restriction endonuclease pattern of the recombinant viral DNA was compared with that of the two transformed K562 clones (Fig. 5*A*). Both clones contain all the internal *Hin*dIII and *Sst* I fragments, indicating that the whole genome of the adenovirus-Neo^R has integrated into the chromosomes of the transformed cells. The intensity of the hybridizing bands implies integration of one to three copies per chromosome.

The DNA from two transformed MEL clones showed the expected hybridization pattern with the Neo probe (Fig. 5*B*), but the pattern detected with the adenovirus 5 probe was more complex (data not shown). One MEL clone had most of the adenoviral recombinant integrated, whereas the other had only a part of the viral genome integrated, suggesting significant deletion and/or rearrangement of the vector genome during integration. In contrast to the K562 uninfected control, no bands were detected in the control MEL DNA using the adenovirus probe.

DISCUSSION

Our studies imply that the recombinant DNA viruses used here offer a dramatic improvement in the efficiency of gene transfer into the hematopoietic cells used here compared to $CaPO_4$-mediated DNA transfer. Both transient expression of the *CAT* gene with recombinant SV40 virus and stable transformation to neomycin (G-418) resistance with recombinant adenovirus was readily achieved on infection of hematopoietic cells. We do not know, however, which cell types in the mixed population of bone marrow cells became infected with SV-CAT and actively expressed the *CAT* gene. Apparent recombination of the viral genome containing *CAT* or $DHFR^{MtxR}$ in the early region and the recombinant SV40 genome containing *DHFR* in the late region led to contamination of the recombinant SV40 stocks with wild-type SV40. Attempts to transform cells containing a normal *DHFR* gene to methotrexate-resistance with stocks containing SV-$DHFR^{MtxR}$ resulted in stable integration of wild-type SV40 rather than SV-$DHFR^{MtxR}$. In contrast, pure recombinant adenovirus stocks were highly efficient in transforming hematopoietic cells to neomycin resistance.

A higher level of expression of the *CAT* gene in hematopoietic cells transformed with SV-CAT compared to those in which the *CAT* gene was introduced by DNA-mediated transfer may reflect more efficient transfer of the viral genome across the cell membrane, more effective delivery of virally introduced DNA to the cell nucleus, or intranuclear amplification of the viral genome. Human cells are semipermissive for SV40 (29, 30) and therefore the presence of wild-type SV40 capable of generating T antigen in the recombinant stocks may allow amplification of SV-CAT in human cells, thereby contributing to the high levels of enzyme activity. For comparison, both pUSV-CAT and a recombinant genome containing an intact early region were introduced into hematopoietic cells to control for the ability of T antigen to amplify circular DNA molecules containing a SV40 early region (18). Also, mouse cells are generally considered to be nonpermissive for SV40 infection (31) and yet these cells exhibited higher levels of CAT activity after infection with SV40 compared to levels observed after DNA-mediated gene transfer. Poison sequences that may inhibit replication of plasmid molecules containing a SV40

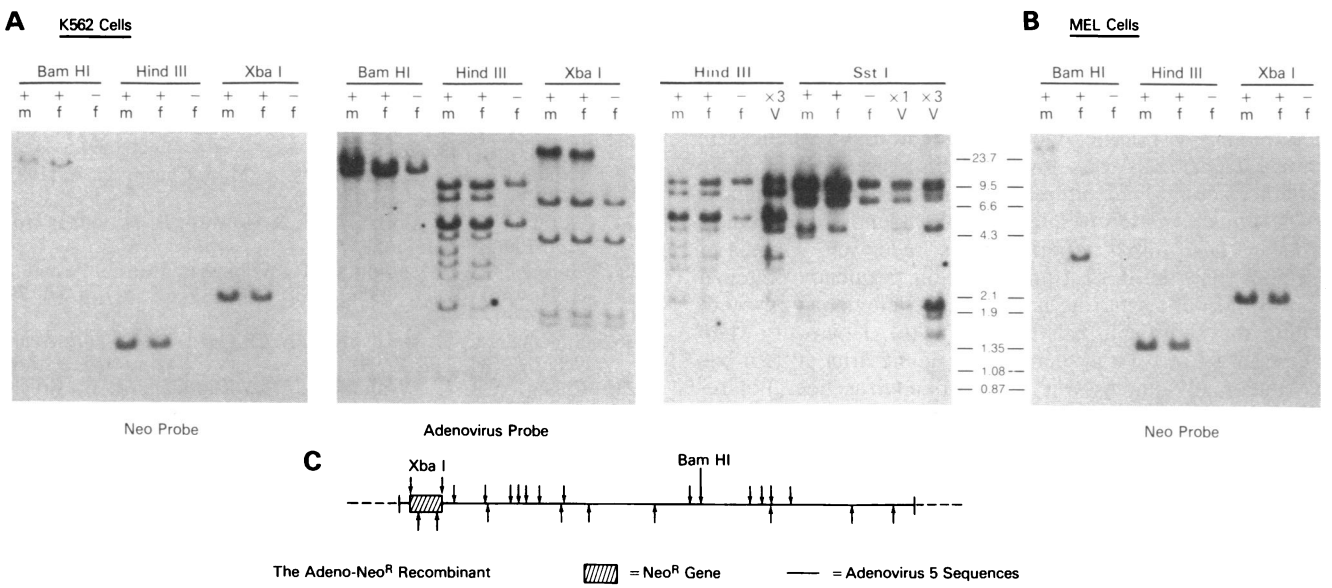

FIG. 5. Southern blot analysis of K562 (*A*) and MEL cells (*B*) transformed with the adeno-Neo^R recombinant virus. DNA samples were cut with the restriction enzymes indicated. (+), DNA from cells that had been infected with adeno-Neo^R and successfully transformed to G418 resistance; (−), DNA from uninfected control cells; m, subclone that grows in a monolayer; f, cells that grow in suspension. Migration of molecular weight markers ($\times 10^{-3}$) is indicated by numbers (in kb). Filter to the left in *A* was first probed with the Neo probe (see text) and, after washing, was reprobed with the adenovirus 5 DNA (Adeno probe). *A* also shows a reconstitution experiment in which the adenovirus-Neo^R recombinant DNA is mixed with K562 DNA (1 genome equivalent virion DNA per genome of K562 DNA is 7 pg/μg), cut with *Hin*dIII and *Sst* I, probed with the adeno probe, and compared to the pattern of the transformed clones. V, recombinant virus; x1 and x3, number of genome equivalents. (*C*) Restriction map of the adeno-Neo^R recombinant virus. *Sst* I sites are shown above, *Hin*dIII are shown below, and the two *Xba* I sites and the single *Bam*HI site are shown above.

origin (32) have been partially removed from the pUC vectors (33). Comparison of data obtained in semipermissive human and nonpermissive mouse cells suggest that poison sequences are not important. Our results appear to imply that recombinant viruses introduced DNA more efficiently through the cell membrane or enhanced delivery to the nucleus in the suspension cells tested.

The lack of a cell line capable of supporting propagation of helper-free recombinant SV40 stocks complicated our attempts to use this vector for gene transfer. Although the SV-DHFR^{MtxR} stock was capable of introducing the *DHFR^{MtxR}* gene into enzyme-deficient CHO cells lacking DHFR activity, transformation of DHFR$^+$ MEL cells by wild-type SV40 present in the SV-DHFR^{MtxR} stock apparently rendered these cells relatively insensitive to methotrexate by a complex and poorly understood mechanism (12).

Another complication encountered in our attempts to use SV40 for gene transfer was the inability to propagate a recombinant viral genome containing the neomycin-resistant gene. Several attempts, using a complimenting late region replacement helper viral genome, only resulted in a generation of wild-type SV40. Apparently some property of the neomycin-resistance coding region inhibits successful preparation of SV40 virions containing this sequence. However, we have shown that SV40 recombinants are potentially useful as viral vectors to transfer genes (2 kb or less) into various cells if a system could be developed to allow replication of pure recombinant viruses.

The recombinant adenovirus containing the neomycin-resistance marker has been successfully used to transform CV1 monkey kidney cells and Rat2 cells to G418 resistance (15). Integration of one to three copies of linear intact adenoviral genome was observed in these cell lines. Similar transformation and integration of the recombinant adenoviral genome into both human and mouse hematopoietic cell lines was readily achieved, suggesting recombinant adenoviral stocks may be capable of high efficiency gene transfer into cells of diverse origin. Several modifications could enhance gene transfer efficiency. No attempt was made in these initial experiments to achieve an optimal multiplicity of infection. Use of other promoters, for example the Rous long terminal repeat promoter, may also enhance transformation efficiency, as has already been shown in experiments that utilized the DNA transfection methodology (34). The finding of adenoviral sequences in control K562 cells was unexpected; expression of these sequences could have altered the transformation frequency.

The transformation frequency of the adenovirus recombinants in these initial experiments is lower (by a factor of 100–500) than the 0.3% transformation frequency obtained with the Moloney murine leukemia recombinant virus containing the neomycin-resistance gene (6). However, when compared to retroviral vectors, the adenovirus system has two major advantages. First, the adenovirus does not remove intron sequences and no inhibition effects of promoter sequences or polyadenylylation signals have been reported to date using adenovirus recombinants. This should allow the adenovirus to accommodate inserts of eukaryotic genes with their own promoter, intervening, and polyadenylylation sequences. Second, two transcriptional units (a total of 6 kb) may potentially be inserted into the E1 and E3 region of the adenovirus and will thus be separated by ≈20 kb of relatively inactive DNA. A selectable marker and a test gene could therefore in theory be inserted into the same adenovirus recombinant to study tissue-specific regulation of the test gene—for example, a globin gene in hematopoietic cells.

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