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Infection of Eucaryotic Cells by Helper-Independent Recombinant Adenoviruses: Early Region 1 Is Not Obligatory for Integration of Viral DNA

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Recombinant viral genomes carrying a selectable drug resistance marker have been constructed by insertion of a hybrid gene for neomycin resistance into the helper-independent adenovirus vector, ΔE1/X. The hybrid gene consists of sequences coding for the aminoglycoside 3′-phosphotransferase II from Tn*5*, under the control of the simian virus 40 early promoter, and renders mammalian cells resistant to the neomycin analog, G-418. Most of adenovirus early region 1 is deleted from ΔE1/X (nucleotides 455 to 3330), and recombinant viral genomes carry the hybrid gene in its place. The large and small *Xba*I fragments of ΔE1/X were ligated to the hybrid gene, and the mixture was transfected into 293 cells. Single plaques were isolated and subsequently passaged in 293 cells to produce virus stocks. The recombinant viruses efficiently rendered cultured rat (Rat2) and simian (CV1) cells resistant to G-418. Cloned cell lines selected for resistance to G-418 contained viral DNA integrated into the host cell genome, demonstrating that early region 1 is not essential for integration of the viral genome. Southern transfer experiments revealed that (i) the sites of integration in the host genome were not unique; (ii) in general, transformed CV1 cell lines contained single-copy, full-length viral genomes, colinear with the infecting virus; (iii) transformed Rat2 cell lines generally contained one to several copies of full-length viral genomes integrated colinearly with the infecting viral DNA; and (iv) three of these five lines of transformed Rat2 cell lines contained tandemly repeated viral DNA sequences in which the right and left ends of the viral genome were joined to each other.

Rodent cells transformed in vitro by adenovirus type 2 or 5 (Ad2 or Ad5) typically retain only portions of the adenovirus genome integrated into the host cell genome. The viral sequences extending from the left end to 12% of the viral genome are always present in transformed cells, although other viral sequences can be detected (41). DNA transfection experiments show that the leftmost 8% of the viral genome is sufficient to produce a transformed phenotype (7, 14, 18, 43, 44).

Several transformed lines have been analyzed in which the right and left ends of the viral genome were linked (8, 33, 34, 46–49). The ends of the viral DNA in these cell lines were either interspersed with short regions of cellular DNA or linked directly to each other. In one case, the viral DNA sequences in the linked ends were found to be inverted (34), whereas the other cell lines contained viral DNA sequences colinear with the viral genome (8, 46–49). These data lead to the suggestion that circular intermediates may be involved in the integration of adenovirus DNA. Such circular forms of adenovirus have indeed been found in infected cells (32).

Infection with adenoviruses under conditions in which expression of functions leading to viral DNA synthesis is suppressed allows the isolation of transformants that contain most or all of the viral DNA sequences. For example, cells infected at the nonpermissive temperature with adenovirus mutants that code for a thermosensitive 72,000-molecular-weight DNA binding protein (H5ts125 and H5ts107) yield transformants that have incorporated most of the sequences of the viral genome (10, 27). Similarly, in cells that are nonpermissive for adenovirus DNA replication (for example, hamster cells infected with adenovirus 12), transformation is characterized by integration of full-length viral genomes (reviewed in reference 8). Finally, group I host range mutants, which map in early region 1A, induce an abnormal transformation of BRK (baby rat kidney) cells (15, 31). Most or all of the viral genome becomes integrated into the host DNA of cells transformed by early region 1A mutants (1, 31).

Group II host range mutants, which map in early region 1B, fail to transform rodent cells infected in culture (15). However, mutants in early region 1B can induce a fully transformed phenotype when purified viral DNA is transfected into rodent cells (30). This suggests that the early region 1B 58,000-molecular-weight protein may have a role in some initial step of transformation by virus particles which can be circumvented when purified viral DNA is introduced into cells (30).

Expression of early region 1A, in addition to the functions described above, results in alteration of the normal cell growth cycle (3). Changes include the stimulation of DNA synthesis in quiescent cells, the shortening of the G1 phase, DNA synthesis being uncoupled from rRNA and polyamines, and cells prematurely reinitiating successive rounds of cellular DNA synthesis, resulting in polyploidy or aneuploidy and chromosome aberrations (4, 5, 39, 50). What, if any, role these changes play in transformation is unclear.

Helper-independent adenovirus vectors (Y. Gluzman, D. Solnick, H. Reichl, and J. Harper, manuscript in preparation) carrying a selectable marker for drug resistance in place of early region 1 were used in this study to examine the integration of viral DNA into the host genome in the absence of early region 1. This allowed us to examine whether any of the functions described above or additonal functions were essential for integration of adenovirus DNA during nonlytic infection. A dominant selection for resistance to the antibiotic G-418, which has been used effectively in a variety of eucaryotic cells (6, 37), was chosen for this study.

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MATERIALS AND METHODS

Cells. CV1 cells and Rat2 cells (42) were cultured in plastic dishes in the Dulbecco modification of Eagle medium (DME) as described previously (13).

Construction of recombinant viruses. The neomycin resistance gene from the bacterial transposable element Tn*5* (2) was cloned under the control of the simian virus 40 (SV40) early promoter as follows. The SV40 origin of replication and the early promoter were isolated with restriction endonucleases *Pvu*II and *Hin*dIII. A 95-base-pair fragment of the *lac* UV5 promoter (20) was also isolated by an *Eco*RI-*Alu*I double digestion. These two DNAs were ligated in a trimolecular reaction containing pBR322 digested with *Eco*RI and *Hin*dIII. An intron was provided by isolating the SV40 *Mbo*II fragment from nucleotides 4713 to 4098 (41) (this includes the small t-intron), the ends were filled by using the Klenow fragment of DNA polymerase I, and *Hin*dIII linkers (dodecamer) were ligated and cut back, using *Hin*dIII. The DNA was then subjected to a partial *Hin*fI digestion, and the fragments were separated on a gel. The fragment from nucleotides 4713 to 4566 (41) was removed from the gel, electroeluted, passed through a DEAE-sephacel column, and ethanol precipitated. A *Hin*fI-*Bam*HI fragment from nucleotides 2825 to 2536 (41) which includes the SV40 polyadenylic acid addition site was isolated as described above. The plasmid described above, containing the SV40 early promoter, was cleaved with *Hin*dIII and *Bam*HI and purified from a gel as described above. A three-part ligation resulted in the production of pko (see Fig. 1), a plasmid containing the SV40 early promoter, followed by a *Hin*dIII site, intron, and terminator.

The neomycin structural gene cloned in a plasmid was a gift from Dan DiMaio. This plasmid contained the *Hin*dIII-*Bam*HI fragment of Tn*5* which covers the entire neomycin gene (2). Plasmid DNA was digested with *Bgl*II, which cuts in the 5′ untranslated region, and *Xho*I, whose site resides past the 3′ end of the gene (2). The fragment was isolated by gel electrophoresis and the ends filled with the Klenow fragment of DNA polymerase I, and *Hin*dIII linkers were added and then cut back. The fragment was then cloned into pko (cleaved by *Hin*dIII), and the colonies which resulted after transformation were analyzed for the correct orientation creating pko-neo.

pko-neo DNA was digested with *Bgl*I and *Bam*HI, and the fragment containing the neomycin resistance gene was cloned into a plasmid containing the early region sequence of SV40 digested with *Bgl*I and *Bam*HI. The *Eco*RI and *Bam*HI sites of this plasmid had *Xba*I linkers added in such a way as to preserve the original sites (see Fig. 1). This allowed the entire fragment containing the SV40 early promoter, neomycin resistance gene, SV40 small t-intron, SV40 terminator, and 25 nucleotides of pBR322 from the *Cla*I to *Eco*RI sites to be excised, using *Xba*I, and cloned to the adenovirus vector ΔE1/X, after digestion with *Xba*I. Ligation mixtures were transfected into 293 cells, and single plaques were purified. Virus stocks were prepared by infection of 293 cells in plastic dishes (30 to 40 by 90 mm). After 28 h at 37°C, cells were scraped from the plates and concentrated by centrifugation. Virus particles were isolated as described previously (23) and then purified by CsCl bouyant density centrifugation in 10 mM Tris (pH 7.9). The virus band was removed from the gradient, mixed with an equal volume of glycerol–10 mM Tris (pH 7.9), and stored at −20°C. A portion of this was used to prepare viral DNA and verify the orientation.

Selection of G418-resistant cells. Rat2 or CV1 cells were plated at 1×10^5 to 2×10^5 cells per 60-mm dish and infected 18 to 24 h later. Cell densities at the time of infection were 2×10^5 to 5×10^5 cells per 60-mm dish. Recombinant viruses carrying the neomycin resistance gene described above were used at multiplicities of infection (MOI) between 0.2 and 200 PFU per cell. Cells were infected with 1 ml of virus in DME at 37°C. Each MOI was used to infect a set of five plates. Plates were agitated once every 15 min after the addition of virus. Virus was removed after 1 h, and 10 ml of fresh DME plus 10% calf serum was added. At 24 h postinfection, the medium was removed and replaced with fresh DME and 10% calf serum with 200 μg of G-418 per ml for Rat2 cells or 300 μg of G-418 per ml for CV1 cells. The medium was changed twice per week while cells were selected which were resistant to the drug. Mock infected cells were killed 3 to 7 days after the addition of G-418. Rat2 cells proved more susceptible to the action of the drug than did CV1 cells. The background monolayer of cells on infected plates died 7 to 14 days postinfection, and resistant colonies were apparent 14 to 21 days postinfection. Cells were cloned 21 to 28 days postinfection by picking isolated colonies, treating them briefly with trypsin, and plating them in DME with 10% calf serum on 25-mm plates. Cell lines were established by subsequently passaging the isolated clones in the presence of G-418.

To determine the maximum number of cells in a population which could be transformed to G-418 resistance, Rat2 or CV1 cells were infected as described above with a MOI of 200. At 24 h postinfection, cells were trypsinized and replated at 10^2, 10^3, or 10^4 cells per 60-mm dish. Cells infected with virus were plated in the presence or absence of 5×10^4 uninfected cells and subsequently exposed to G-418 at the appropriate concentrations. As a control experiment, mock infected cells were treated as described above but plated without the addition of 5×10^4 uninfected cells and maintained without G-418.

Isolation of cellular DNA. Ten confluent, 90-mm plates of cells were lysed with 1 ml of lysis buffer (0.6% sodium dodecyl sulfate, 10 mM EDTA) per plate and scraped into a 30-ml plastic tube, pronase was added to 1 mg/ml, RNase was added to 20 μg/ml, and the entire mixture was digested at 37°C for 6 to 8 h. DNA was extracted with phenol, followed by chloroform-isoamyl alcohol (24:1), and ethanol precipitated. High-molecular-weight DNA was then pulled from the mixture with a Pasteur pipette, allowed to air dry, and resuspended in 10 ml of sterile TE (10 mM Tris [ph 7.6], 1 mM EDTA). The above steps were repeated, and DNAs were resuspended in 2 ml of TE after the ethanol precipitation.

Restriction enzyme analysis. DNAs were digested with restriction endonucleases *Sst*I or *Bgl*II (New England Biolabs or Bethesda Research Laboratories), separated on horizontal 0.7% agarose gels, and transferred to nitrocellulose by the method of Southern (36). Filters were hybridized to ^{32}P-labeled DNA probes and prepared by nick translation, using four ^{32}P-labeled deoxynucleoside triphosphates (26) under previously described conditions (13). Autoradiographs were established by standard procedures.

RESULTS

Construction of Ad5-neoR1 and Ad5-neoR2. Recombinant viral genomes were constructed to deliver a selectable marker by viral infection. A hybrid gene providing a dominant selection for drug resistance was constructed, utilizing a eucaryotic transcription unit comprised of the SV40 early promoter, the small t-intron, and terminator-polyadenylic

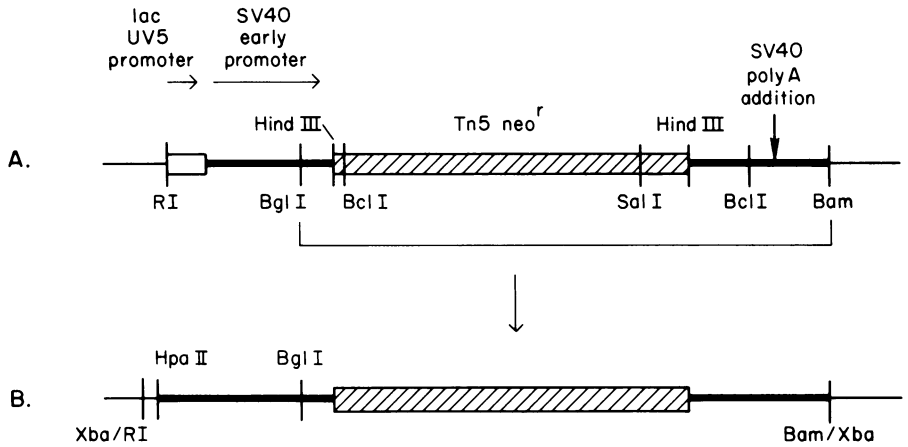

FIG. 1. Description of pko-neo. Panel A depicts pko-neo, the plasmid containing the neomycin resistance gene from bacterial transposable element Tn*5* (2), under the control of the SV40 early promoter. The *Bgl*I-*Bam*HI fragment of pko-neo was excised and ligated to a plasmid, pACSE2, which contains the SV40 early region (panel B). The *Xba*I fragment from pACSE2-neo was ligated to the *Xba*I fragments of ΔE1/X to produce the recombinant viruses Ad5-neoR1 and Ad5-neoR2. Hatched boxes, neomycin resistance gene; open boxes, *lac* UV5 promoter; thick lines, SV40 DNA; and thin lines, pBR322 sequences.

acid addition signal of SV40. The neomycin phosphotransferase coding sequences from Tn*5* (2) were cloned under the control of the SV40 early promoter producing pko-neo (Fig. 1A). pko-neo, when introduced into mammalian cells, produces the bacterial protein and renders cells resistant to G-418 (Y. Gluzman and D. Hanahan, unpublished data; D. Kurtz, personal communication; M. Wigler, personal communication). The hybrid gene in pko-neo was recloned into a plasmid so that it was flanked by *Xba*I sites (Fig. 1B). The hybrid gene was excised from the plasmid with *Xba*I and ligated to the large and small *Xba*I fragments of the vector ΔE1/X. This vector was constructed from a *d1309* genome (21) by deletion of the region between nucleotides 455 to 3330 (*Pvu*II-*Bgl*II) (24, 25, 45). An *Xba*I linker has been inserted at the site of the deletion, providing a single *Xba*I site into which the gene of interest can be inserted. The mixture resulting from ligation of the *Xba*I fragments of ΔE1/X and the hybrid gene was transfected into 293 cells, single plaques were isolated, and viral DNA was analyzed for orientation by cleavage with restriction endonucleases. Two recombinant virus stocks were chosen for propagation in 293 cells (16). In one virus, the direction of transcription of the hybrid neomycin gene proceeded from left to right on the viral genome (the same orientation as early region 1) (Ad5-neoR1), and a second derivative carries the hybrid neomycin gene in the opposite orientation (Ad5-neoR2).

Transformation of Rat2 and CV1 cells to G-418 resistance. Colonies of cells resistant to the drug G-418 developed and were counted 21 days after infection with Ad5-neoR1 or Ad5-neoR2 and selection in media containing 200 or 300 μg of G-418 per ml for Rat2 and CV1 cells, respectively (Table 1). The transcriptional orientation of the neomycin gene in the recombinant viruses did not affect the frequency of transformation to G-418 resistance. More CV1 cells per PFU are converted to drug resistance than are Rat2 cells, and the transformation frequency is roughly linear with the input of virus (Table 1).

Fewer G-418-resistant colonies develop when cells are plated at low densities ($<10^4$ cells per 60-mm plate) after infection and trypsinization (Table 2). A 10-fold reduction in the number of cells plated results in a 30- to 40-fold decrease in the number of colonies observed. Two- to threefold more G-418-resistant colonies are established when the infected cells are plated together with 5×10^4 uninfected cells. This suggests that there may be some form of cross feeding or cross protection between the cells. From a population of 10^4 cells infected with a multiplicity of 200 PFU per cell, the transformation efficiency is 0.4% for Rat2 and 0.75% for CV1 cells (Table 2). Due to the very inefficient plating (5%) observed for Rat2 cells in control experiments, the actual efficiency may be as much as 20 times higher. Since CV1

TABLE 1. G418-resistant colonies established after infection with Ad5-neoR1 or Ad5-neoR2[a]

Cell type	Virus	MOI	No. of foci[b]
Rat2	Ad5-neoR1	20	85 ± 3
		2	2 ± 1
		0.2	0
	Ad5-neoR2	20	87 ± 13
		2	2 ± 1
		0.2	0
CV1	Ad5-neoR1	10	Conf.
		1	163 ± 2
		0.1	35 ± 10
	Ad5-neoR2	10	Conf.
		1	175 ± 18
		0.1	36 ± 10

[a] CV1 and Rat2 cells were infected at MOIs of 0.2, 2, and 20 and selected in medium containing 300 or 200 μg of G-418 per ml, respectively. The medium was changed twice per week, and cells were fixed 21 days postinfection, using methanol-acetone (1:1). Plates were stained with Giemsa, and colonies were counted. The data represent an average from three plates. The transformation efficiencies of Ad5-neoR1 were: Rat2, 2×10^{-6} to 8.5×10^{-6} cells transformed per PFU; CV1, 5×10^{-5} to 2×10^{-4} cells transformed per PFU.

[b] The number of foci (mean ± standard deviation) per 5×10^5 cells (Rat2) or per 10^6 cells (CV1) was determined. Conf., Plates contained a monolayer of cells, and individual colonies were not discernible.

TABLE 2. Proportion of cells converted to G-418 resistance after infection at a high MOI[a]

Cells	Virus	MOI	No. of cells plated	No. of uninfected cells plated	No.[b] of colonies
Rat2	Ad5-neoR1	200	10^4	5×10^4	40 ± 8
				0	12 ± 3
			10^3	5×10^4	0
				0	0
CV1	Ad5-neoR1	200	10^4	5×10^4	75 ± 6
				0	35 ± 4
			10^3	5×10^4	2 ± 1
				0	0

[a] Cells infected with Ad5-neoR1 (MOI of 200) were replated at densities of 10^2, 10^3, or 10^4 infected cells per 60-mm plate, in the presence or absence of 5×10^4 uninfected cells, at 36 h postinfection. Colonies were selected by growth in medium containing 200 or 300 μg of G418 per ml for Rat 2 and CV1 cells, respectively. Uninfected cells of each type were plated at 10^2, 10^3, or 10^4 cells per 6-cm plate to check the efficiency of plating of these cells. The data represent the average from three plates. The plating efficiencies of the control cells were: Rat2, 5%; CV1, 50%.

[b] Mean ± standard deviation.

cells plated very efficiently (50%) in control experiments, the efficiency of transformation would not be markedly different (Table 2).

Viral DNA sequences in CV1-transformed cells. Drug-resistant cell lines were isolated by picking single colonies and subcloning them, such that each line was derived from a single cell. These cell lines were examined for the presence and organization of viral DNA. Cellular DNA was extracted, digested with restriction endonucleases, separated on agarose gels, transferred to nitrocellulose, and hybridized with ^{32}P-labeled DNAs as described above. With one exception, DNAs extracted from transformed CV1 cell lines and digested with *Bgl*II or *Sst*I contained a single fragment which hybridized to a ^{32}P-labeled pko-neo DNA. One exceptional DNA (extracted from cell line CN5) yielded two hybridizing fragments (Fig. 2A and C). These fragments migrated more slowly through the gel than did the authentic fragment (Fig. 2, lanes R). These data suggested that these fragments were covalently linked to cellular DNA sequences. To display the drug-resistant phenotype, the transformed cells must contain at least a single functional neomycin resistance gene. Integration in these cell lines would have had to occur within the left-end 500 nucleotides of the viral DNA to preserve a functional neomycin resistance gene because of the location of the gene in the recombinant viruses.

To facilitate the analysis of adenovirus DNA integrated in these cell lines, the ^{32}P-labeled pko-neo DNA was removed from the filters (13) used to establish the autoradiographs shown in Fig. 2A and C; then the filters were rehybridized to ^{32}P-labeled Ad5 DNA (Fig. 2B and D). These data show that digested DNA from cell lines CN2, CN3, and CN5 contain most or all of the authentic internal adenovirus restriction fragments observed in the reconstruction experiment. DNA fragments containing the hybrid neomycin gene hybridized very weakly to ^{32}P-labeled Ad5 DNA because these fragments contain only ca. 900 nucleotides of adenovirus DNA.

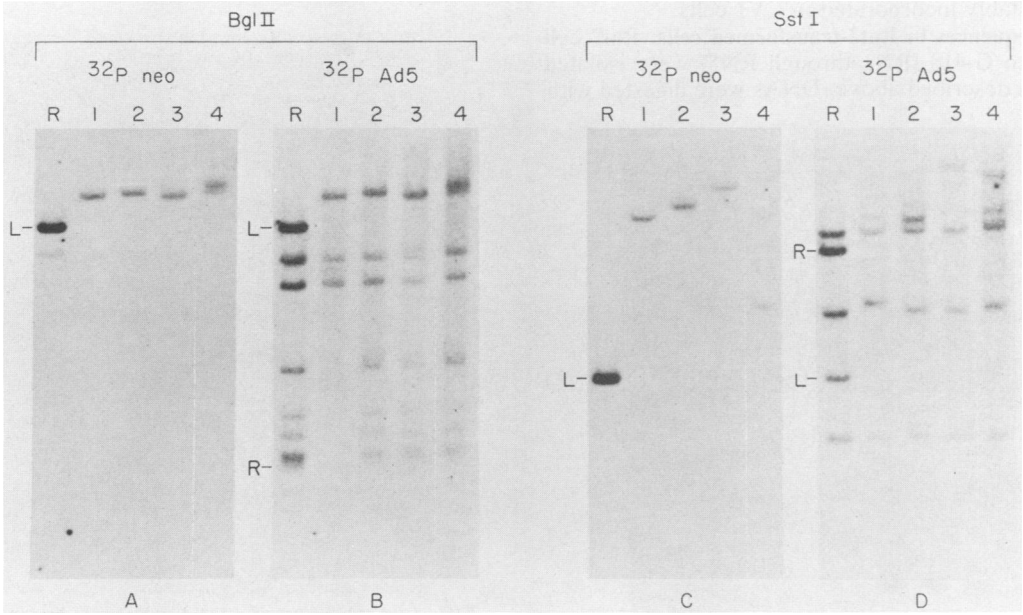

FIG. 2. Restriction analysis of CV1 G-418-resistant cell lines. DNA from G-418-resistant CV1 cell lines was digested with restriction endonucleases *Bgl*II or *Sst*I, separated on 0.7% agarose gels, transferred to nitrocellulose, and hybridized to ^{32}P-labeled nick-translated DNAs. Panel A shows the pattern of hybridization of *Bgl*II-digested DNAs hybridized to ^{32}P-labeled pko-neo DNA. Panel B is the same filter after the removal of the pko-neo probe and hybridization to a ^{32}P-labeled Ad5 DNA probe. Panels C and D represent sequential hybridizations of *Sst*I digestions, using ^{32}P-labeled pko-neo and Ad5 DNA, respectively. Restriction maps of the recombinant neomycin viruses are shown in the lower portions of Fig. 6 and 7. Lanes R represent a reconstruction, using five genome equivalents of virion DNA per genome equivalent of CV1 DNA (35 pg/mg). Lanes 1 through 4 represent cell lines CN1, CN2, CN3, and CN5, respectively. Cellular DNA (5 μg) was loaded onto each lane of the gel. CV1 DNA (5 μg) was used in the reconstruction. L and R denote the native left and right ends of the viral DNA, respectively.

DNA fragments which contain the right-end viral DNA sequences integrated into cellular DNA migrate through the gel more slowly than did the authentic *Sst*I fragment A (Fig. 2D). To verify that the right ends of the viral DNA were linked to cellular sequences, *Bgl*II- and *Sst*I-digested DNAs were separated on a gel, transferred to nitrocellulose, and hybridized to ^{32}P-labeled pE4 DNA (Fig. 3). pE4 is a plasmid which contains sequences between 89% and the right end of the Ad5 genome. All DNA from CN2, CN3, and CN5 cells digested with *Bgl*II contained an authentic DNA fragment, as well as a fragment which migrated more slowly than an authentic DNA fragment (CN5 has two such fragments). This is consistent with the linkage of these fragments to cellular DNA sequences. Supporting this interpretation, *Sst*I fragments which hybridized to ^{32}P-labeled pE4 DNA also migrated through gels more slowly than did the authentic DNA fragment.

Digested DNA from CN1 lacks portions of the viral genome, and from the data, we conclude that the junction between viral and cellular DNA is located between positions 60.2 and 68.2% of the adenovirus genome. DNA from CN1 cells digested with *Bgl*II did not contain any sequences which hybridized to pE4, as expected from the previous data. A schematic representation of the viral sequences contained in the seven CV1 neomycin-resistant cell lines analyzed is shown in Fig. 4, although data for only four of these clones are presented in Fig. 2 and 3.

Cloned lines of G-418-resistant CV1 cells were isolated after infection at high multiplicity (200 PFU per cell) as described above. Analysis of viral DNA sequences in DNA extracted from these cells revealed integration patterns very similar to those observed in cell lines transformed after infection at low multiplicity (Fig. 5). These data indicate that the MOI does not affect the number or integration pattern of viral genomes stably incorporated in CV1 cells.

Viral DNA sequences in Rat2-transformed cells. Rat2 cell lines resistant to G-418 (RN1 through RN5) were isolated and analyzed as described above. DNAs were digested with

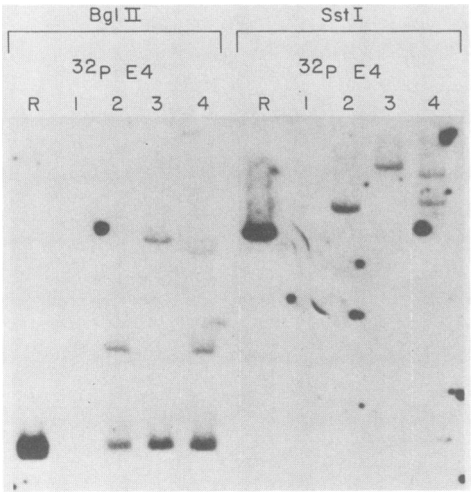

FIG. 3. Analysis of viral right-end sequences integrated in CV1 G-418-resistant cell lines. Cellular DNAs were treated as described in the legend for Fig. 2 but were hybridized to ^{32}P-labeled pE4 DNA, a plasmid containing the sequences from 89% to the right end of Ad5 DNA. Lanes R are a reconstruction experiment containing five viral genome equivalents per cellular genome equivalent. Cellular DNA (5 μg) was loaded onto each lane. Lanes 1 through 4 correspond to cell lines CN1, CN2, CN3, and CN5, respectively.

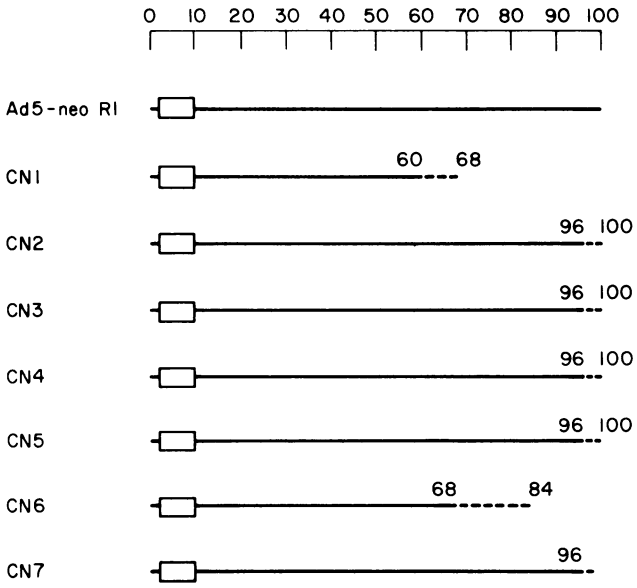

FIG. 4. Viral DNA sequences integrated in G-418-resistant CV1 cell lines. This is a schematic representation of the viral DNA sequences present in G-418-resistant CV1 cell lines. Ad5-neoR1 depicts the original recombinant viral genome. Open boxes represent the hybrid neomycin gene. Solid lines represent the adenovirus sequences we know to be present, whereas dotted lines represent the maximum to which these sequences may extend because the precise point of integration has not been determined. The scale above Ad5-neoR1, as well as the numbers above the clones, is in Ad5 map units.

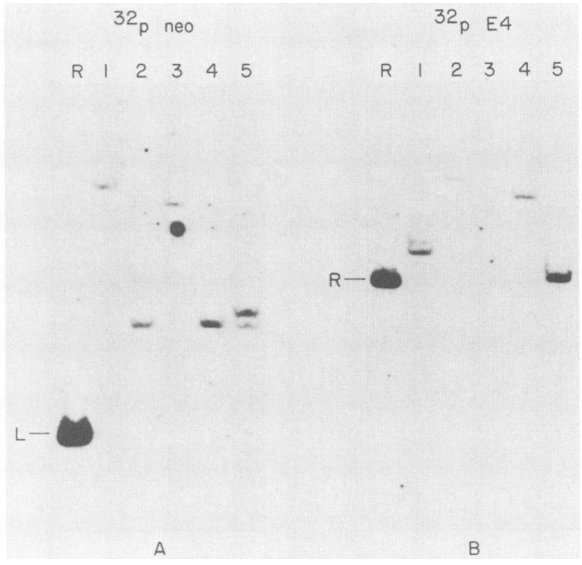

FIG. 5. Analysis of viral DNA sequences integrated into G-418-resistant CV1 cells infected with a high MOI. CV1 cell lines were isolated from an infection at an MOI of 200, as described above. DNA was prepared, digested with *Sst*I, separated on a 0.7% agarose gel, transferred to nitrocellulose, and hybridized to either ^{32}P-labeled pko-neo (A) or pE4 (B) probes. Lanes R depict a reconstruction experiment at five genome equivalents per cellular genome equivalent. Lanes 1 through 5 represent cell lines CN8, CN12, CN26, CN27, and CN28, respectively. L and R denote the authentic left and right ends of the viral genome.

*Sst*I, separated on agarose gels, transferred to nitrocellulose, and hybridized to ^{32}P-labeled pko-neo DNA. Digested DNAs from RN1 and RN4 contained one and three prominent hybridizing fragments, respectively, whereas digested DNA from RN2 contained two hybridizing fragments which migrated very close together (Fig. 6A). The fragments which hybridized to the ^{32}P-labeled pko-neo DNA were larger than the authentic fragment. This was consistent with their being linked to other DNA sequences. One exceptional fragment which hybridized to the ^{32}P-labeled pko-neo DNA migrated more rapidly than the authentic fragment. This suggests that integration of one of the viral genomes contained in cell line RN4 occurred within the neomycin gene, such that digestion of the DNA with *Sst*I produced a fragment smaller than that in the reconstruction track. One fragment from *Sst*I-digested DNA, which hybridized to the neomycin probe, appeared to migrate to the same or very nearly the same position in all three cell lines (indicated by arrow in Fig. 6). This situation could be explained three ways. First, this could represent an example of site-specific integration into the host cell genome. Second, it could simply be fortuitous, and upon digestion with another restriction endonuclease, comigrating fragments would not be present. Third, the right and left ends of the viral genome could have become linked to each other before integration into the host cell DNA, and this represented an "internal" fragment of the viral DNA through the preservation of the restriction sites. To test this possibility, the ^{32}P-labeled pko-neo DNA was removed from the filter, which was subsequently hybridized to ^{32}P-labeled pE4 DNA (Fig. 6C). The fragments in *Sst*I digests of RN1, RN2, and RN4 DNAs which hybridized to ^{32}P-labeled pko-neo DNA also hybridized to sequences derived from the right end of adenovirus DNA (Fig. 6, arrow). This supports the proposal that the left and right ends of the viral genome are directly linked to each other. The size of this fragment is roughly equal to the sum of the right- plus left-end fragments. Figure 6B shows the same filter rehybridized to ^{32}P-labeled Ad5 DNA. It is apparent that the internal viral fragments detected in the reconstruction experiment were also present in the DNA extracted from the neomycin-resistant cell lines. These data indicate that most or all of the viral genome is present in the G-418-resistant Rat2 cell lines and is integrated in a colinear fashion.

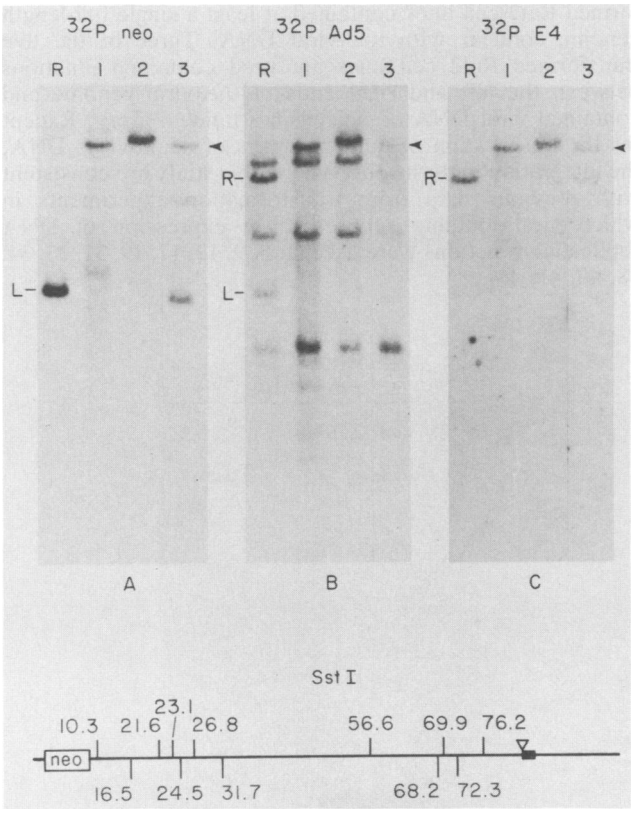

FIG. 6. Restriction analysis of Rat2 G-418-resistant cell lines, using *Sst*I. DNAs from Rat2 neomycin-resistant cell lines were digested with *Sst*I, separated on a 0.7% agarose gel, and transferred to nitrocellulose. The nitrocellulose filter was then sequentially hybridized to three ^{32}P-labeled DNA probes, with the previous probe being removed before rehybridization. Panel A shows the pattern obtained when pko-neo DNA was used as a probe. Panels B and C are the patterns for Ad5 and pE4, respectively. Cellular DNA (5 μg) was loaded onto each track. Lanes R are a reconstruction. Lanes 1, 2, and 3 represent cell lines RN1, RN2, and RN4, respectively. The arrow denotes the fragment containing the joined left and right viral ends. Below the figure is a schematic representation of the restriction map of the recombinant virus.

DNAs digested with *Bgl*II and hybridized to ^{32}P-labeled pko-neo DNA revealed that DNA from RN1 contained a single prominent hybridizing fragment, whereas DNA from RN2 and RN4 contained two and three hybridizing fragments, respectively (Fig. 7). These fragments were all larger than the authentic virion fragment, which indicated that they are linked to additional DNA sequences. Furthermore, it can be seen that DNA from each cell line contained one fragment that migrated to the same position or very close to the same position (indicated by arrow in Fig. 7). This fragment also hybridized when the ^{32}P-labeled pko-neo DNA was removed and the filter was rehybridized to ^{32}P-labeled pE4 DNA (data not shown). These data further support the conclusion that the right and left ends of the viral genome are linked to each other. Each cell line contained one additional right-end fragment integrated into cellular DNA (see below). Figure 7B represents the same filter from which the ^{32}P-labeled pE4 DNA has been removed; this same filter was subsequently hybridized to a ^{32}P-labeled Ad5 DNA. DNA from the three cell lines contained all or most of the authentic viral DNA fragments (Fig. 7, lanes R). These data indicated that most or all of the viral sequences were present in the transformed cells colinear with the viral DNA.

From the data obtained when using *Bgl*II and *Sst*I, it was not possible to determine whether the fragment containing the linked left and right ends of adenovirus was from a single genome or whether it was formed by a tandem repeat. To answer this question, DNAs from the cell lines were digested with *Xba*I (Fig. 8). Since Ad5-neoR1 was constructed by inserting the hybrid neomycin gene into the single *Xba*I site of the ΔE1/X, digestion with this endonuclease should excise the neomycin gene. If the cell lines contained a tandem repeat, digestion would liberate a fragment that contains sequences stretching from nucleotide 3330 through the right end of the adenovirus genome to nucleotide 455 of the left end—precisely the length of ΔE1/X DNA (Fig. 8). DNAs from each cell line contain a fragment which (i) hybridized to both a right-end probe (pE4) and to an Ad5 DNA probe and (ii) comigrated with the band in the reconstruction experiment (Fig. 8, lanes R). The fragment in the reconstruction experiment should be 455 nucleotides smaller than the fragment in the G-418-resistant cell lines if the linkage between the left and right viral ends occurred without deleting any nucleotides. Since these fragments are greater

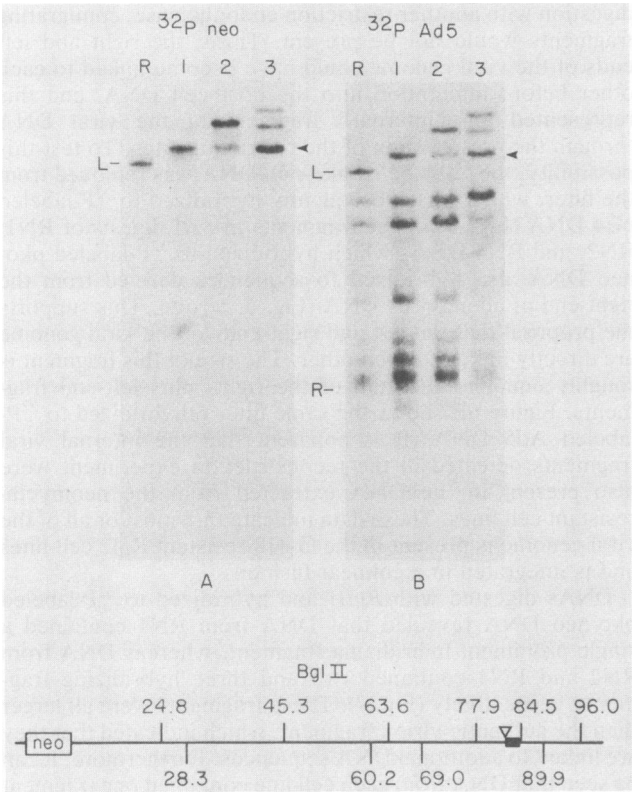

FIG. 7. Restriction analysis of Rat2 G-418-resistant cell lines, using *Bgl*II. DNAs were treated as described in the legend to Fig. 6. Panels A and B are the patterns obtained after hybridization to ^{32}P-labeled pko-neo and Ad5 DNA, respectively. Below the panels is a schematic representation of the *Bgl*II restriction map of the virus. The arrow denotes the fragment containing the joined left and right ends of the virus. Lanes R are a reconstruction. Lanes 1, 2, and 3 denote cell lines RN1, RN2, and RN4, respectively.

than 32 kilobases, such a difference would not be resolved in this gel system. These data show that the transformed cell lines must contain a tandem repeat of the adenovirus genome. Data obtained, using *Bam*HI, support this conclusion (data not shown).

The amount of viral DNA added to the reconstruction experiments was equivalent to a single copy of the viral genome per cell. The intensity with which the viral probe hybridizes to transformed cell DNA suggests that there are between one and three copies of viral DNA in each of the transformed Rat2 cell lines used in this work.

DISCUSSION

To assess whether early region 1 functions were essential for the integration of viral DNA into host cell chromosomes, recombinant viruses were constructed that lacked this region of the genome. A dominant biochemical selection was provided by a hybrid gene comprised of the structural sequences of the neomycin resistance gene from Tn5 (2), under the control of the SV40 early promoter. Cells which permanently acquired the gene through incorporation of viral DNA were selected by growth in medium containing the neomycin analog G-418.

Transformation studies in which Ad5 was used are typically performed in primary rat cells. Because our recombinant viruses do not carry the functions necessary for the establishment of primary cells in culture (18), we used the established Rat2 cell line in this study. In addition, we used the CV1 cell line which is completely permissive for wild-type adenovirus DNA replication. Although our recombinant viruses are negative for DNA replication as determined by biochemical assays, we used CV1 cells to test the possible involvement of abortive DNA replication on the efficiency of transformation and the pattern of integrated viral DNA.

Recombinant viruses efficiently transformed Rat2 and CV1 cells to G-418 resistance. The efficiency of transformation after infection of the recombinant viruses is higher than that achieved by DNA transfection. The high efficiency of transformation, together with the relative ease of virion infection as opposed to DNA transfection, makes this an attractive system for use with any selectable marker. Recombinant viruses which carry a selectable marker can be used to transform any cell type that is susceptible to adenovirus infection.

Transformed cell lines contained integrated viral DNA, which illustrates that after infection adenovirus DNA can integrate into the host cell genome in the absence of early region 1. Transformed CV1 cell lines integrated 60 to 100% of the viral genome colinear with viral DNA. Most transformed Rat2 cell lines contained at least a single full-length genome colinear with the viral DNA. Three of the five transformed Rat2 cell lines analyzed contained junctions between the left and right ends of the viral genome and contained viral DNA organized in a tandem array. Except for the observation of tandem arrays of adenovirus DNA, the integration patterns observed in this study are consistent with previous data from transformation experiments in which viral mutants suppressed in expression of DNA replication functions were used (1, 8, 9, 12, 17, 19, 31, 33, 34, 38, 40, 41, 46).

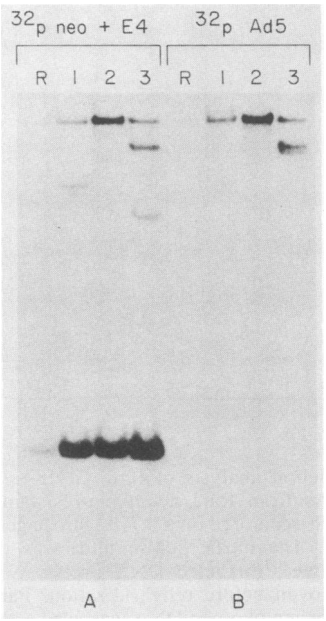

FIG. 8. Restriction analysis of Rat2 G-418-resistant cell lines, using *Xba*I. DNAs were treated as described in the legend to Fig. 6. Panel A is the pattern obtained after hybridization to ^{32}P-labeled pko-neo DNA in conjunction with ^{32}P-labeled pE4 DNA. Panel B is the Ad5 hybridization pattern. Lanes R are a reconstruction. Lanes 1, 2, and 3 represent cell lines RN1, RN2, and RN4, respectively.

Linkage of the right and left ends of adenovirus has been observed in several other transformed cell lines (34, 46–49). Some linkages may have been interrupted by short segments of cellular DNA, whereas others have been shown to be direct. Visser et al. (47) have sequenced the linked ends of one such line, 5RK-20. The linkage was found to be direct, linking nucleotide 63 of the left end to nucleotide 108 of the right end of the viral genome. The mechanism by which such junctions are created is not known, but the observation that circular forms of adenovirus exist in infected rat cells suggests that circular forms may play a role in the integration process (32).

Although we cannot unambiguously determine the integration pattern of the viral genomes present in the transformed Rat2 cell lines due to their complexity, we think that the simplest interpretation would be the organization of viral DNA into tandem repeats comprised of two viral genomes. However, it is not possible to exclude the possibility of tandem insertions of the adenovirus genomes interspersed by short stretches of cellular DNA.

Linkage of the left and right ends of adenovirus DNA was observed in Rat2 cells but not in CV1 cells. This phenomenon is not due merely to the difference in the MOI used to isolate the transformed cell lines. Transformed CV1 cell lines isolated from an infection at a multiplicity of 200 revealed an integration pattern identical to the cell lines isolated from the low multiplicity infections (0.1 PFU per cell). This suggests that there may be a cellular factor, which is present in one of the two cell types, involved in either bringing the ends of the viral DNA together or preventing this from occurring.

In experiments to be reported elsewhere, primary human and mouse cells were transformed by infection of recombinant adenoviruses carrying the early region of SV40. The organization of viral DNA has been determined by restriction enzyme analysis of cellular DNA. Cell lines contained viral DNA integrated into high-molecular-weight DNA. The organization was very similar to that described in this report, in that full-length viral genomes were integrated at one to several copies per cell. In addition, two of eight transformed mouse cell lines may contain the left and right ends of the viral genome linked to each other, as observed in the Rat2 neomycin-resistant cell lines (unpublished data).

In previous reports, linear DNA molecules were observed to integrate from their ends, with the subsequent loss of a small number of nucleotides (8, 11, 22). This has been observed in cells after viral infection or microinjection (pressure or iontophoretic) of DNA. Although the precise point of integration of the viral genome in the transformed CV1 cell lines is not known, restriction enzyme analysis suggested that the integration had resulted in the loss of at most a few percent of the viral DNA. Furthermore, linear DNA molecules have been reported integrated as head-to-tail tandems in transformed cells after viral infection or microinjection (pressure) of DNA (8, 11, 34, 46–49). Homologous recombination has been suggested to be responsible for the generation of head-to-tail tandem arrays (11). Homologous recombination has also been shown to restore functional genes from two altered genes after transfection of these genes into eucaryotic cells (28, 29, 35). The mechanism which is responsible for this homologous recombination may be similar to the mechanism which generated the tandem arrays in the transformed Rat2 cell lines.

From these data, it can be seen that there are at least three main factors which affect the integration of DNA in eucaryotic cells. First, the mode of delivery of DNA into the cell clearly affects integration into the cellular genome. The second factor is the degree of expression of viral functions after delivery into cells. This is in turn affected by factors including (i) the permissivity of the cell or the ability to allow the virus to go through the normal life cycle and (ii) the competence of the virus to express its own genome. The third main factor is the involvement of cellular factors which might promote or hinder interaction between viral genomes or the ends of a viral DNA molecule.

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