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Efficient Transformation of Human Fibroblasts by Adenovirus-Simian Virus 40 Recombinants

KEVIN VAN DOREN* AND YAKOV GLUZMAN
Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724

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The origin-defective simian virus 40 (SV40) mutant 6-1 has been useful in transforming human cells (Small et al., Nature [London] 296:671–672, 1982; Nagata et al., Nature [London] 306:597–599, 1983). However, the low efficiency of transformation achieved by DNA transfection is a major drawback of the system. To increase the efficiency of SV40-induced transformation of human fibroblasts, we used recombinant adenovirus-SV40 virions which contain a complete SV40 early region including either a wild-type or defective (6-1) origin of replication. The SV40 DNA was cloned into the adenovirus vector in place of early region 1. Cell lines transformed by viruses containing a functional origin of replication produced free SV40 DNA. These cell lines were subcloned, and some of the subclones lost the ability to produce free viral DNA. Subclones that failed to produce free viral DNA were found to possess a mutated T antigen. Cell lines transformed by viruses containing origin-defective SV40 mutants did not produce any free DNA. Because of the high efficiency of transformation, we suggest that the origin-defective chimeric virus is a convenient system for establishing SV40-transformed cell lines from any human cell type that is susceptible to infection by adenovirus type 5.

Cells range from fully permissive to fully nonpermissive for simian virus 40 (SV40) infection. Permissivity results in full expression of the viral genome, replication of viral DNA, and production of infectious virions. Nonpermissive infection results in expression of the early viral genes, but no viral DNA is synthesized or virions produced. Nonpermissive cells can be transformed, and the viral genome can be integrated into the host cell genome. Human cells are semipermissive for SV40. Virus production occurs to ca. 1% the level produced by permissive monkey kidney cells (12). The majority of viral DNA replication occurs in ca. 1 to 2% of the cells, where it reaches levels equivalent to wild-type infection (18). A portion of the population can become transformed and integrate viral DNA into the host cell genome (18); however, the efficiency is quite low. The frequency of transformation can be enhanced by transfection of SV40 origin-defective mutants (13). Because DNA transfection is an inefficient process, the frequency of transformation is still not very high. We wanted to determine whether we could increase the efficiency of SV40-induced tranformation after infection with recombinant viruses. We were also interested in determining whether having a functional versus a defective SV40 origin of replication would affect the frequency of transformation by recombinant viruses.

The chimeric viruses contain the SV40 early region which encodes T antigen (*Hpa*II-*Bam*HI fragment) (16) cloned into the helper independent adenovirus vector, ΔE1/X (17). The SV40 origin of replication is either wild type or the *ori*$^-$ mutant 6-1, having six nucleotides deleted at the *Bgl*I site (4, 5). The SV40 DNA is cloned into the adenovirus vector in place of early regions 1a and 1b. Early region 1 encodes the functions responsible for adenovirus-induced transformation and for the enhanced expression of the remainder of the viral transcription units (8, 11). The vector has no transforming activity which could interfere with the SV40-induced transformation.

Primary human fibroblasts (HS74BM cells) (14) were used to test the transforming potential of the recombinant viruses. Figure 1 shows the morphology of the transformed foci. It is apparent from Fig. 1 that the chimeric viruses transform more efficiently than wild-type SV40 at the same multiplicity of infection (MOI). The transformed cells form large foci which are easily distinguished from the background monolayer. Table 1 shows the results of experiments testing the *ori*$^+$ and *ori*$^-$ chimeric viruses at various MOIs. Wild-type SV40 was included as a control. *ori*$^+$ and *ori*$^-$ chimeric viruses produce about 20-fold more foci than wild-type SV40 at a 4-fold lower MOI. At a high MOI (>200 PFU per cell), individual foci cannot be counted. The efficiencies of transformation for the two chimeric viruses are approximately equal and are independent of the orientation of the SV40 early region in the vector DNA (data not shown).

Transformed cell lines were cloned, and both low-molecular-weight (7) and high-molecular-weight (17) DNAs were prepared. Low-molecular-weight DNA from cell lines derived from transformations with an *ori*$^+$ chimeric virus contained free viral DNA which hybridized to ^{32}P-labeled SV40 DNA (data not shown). The size of the viral DNA molecules was very heterogeneous. Previous work had shown that such heterogeneous viral DNA molecules are produced by excision from the SV40-transformed cell line 14B (1, 2). The viral DNA integrated in the host genome of 14B cells does not contain any SV40 sequence duplications which could be used as points for excision via homologous recombination. The heterogeneity in the size of the molecules was probably due to random excision.

Subclones of the transformed lines were isolated from single cells. Analysis of the low-molecular-weight DNA (7) revealed that in two of three cases, the subclones had lost the ability to produce free viral DNA (Fig. 2). These subclones can produce free viral DNA when wild-type T antigen is produced through fusion with COS-1 cells (3) (Fig. 2). Fusion with CV1 cells did not result in the excision of SV40 DNA, which indicated that it was the T antigen and not a permissive factor(s) that had been altered in the subcloned cell lines. These data indicated that the subclones contained a mutated T antigen which could not support viral DNA replication. Two SV40-transformed human cell lines which

* Corresponding author.

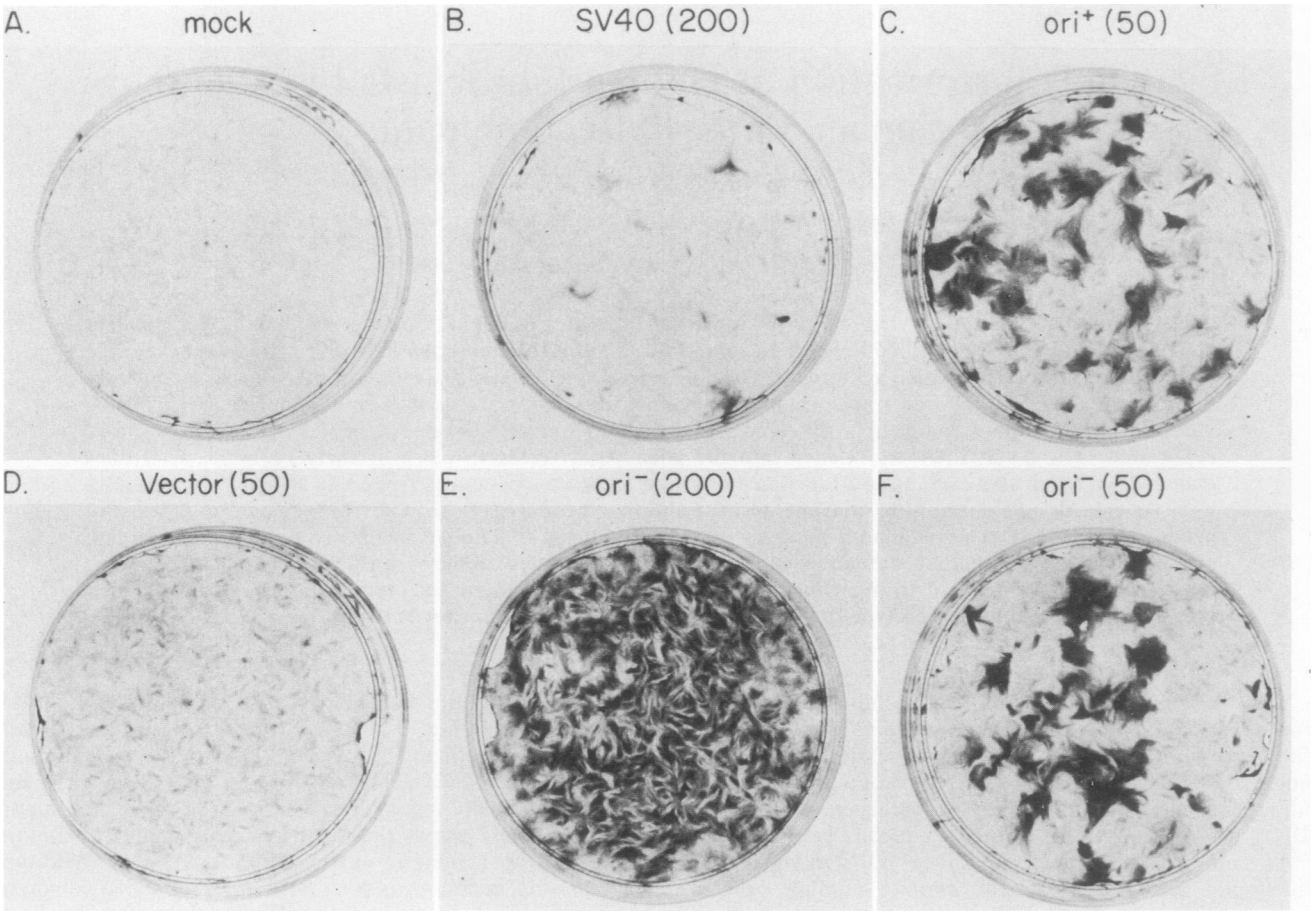

FIG. 1. Morphology of SV40-transformed HS74BM foci. Cells were infected as described in Table 1, fixed with methanol and acetone (1:1), and stained with 10% Giemsa stain. (A) Background monolayer of human fibroblasts (HS74BM) after mock infection. (D) Cells infected with ΔE1/X at an MOI of 50. (B and E) Foci from cells infected with wild-type SV40 and the origin-defective chimeric virus, respectively, at an MOI of 200. (C and F) Foci from cells infected with the ori^+ and ori^- chimeric viruses, respectively, at an MOI of 50.

TABLE 1. Transformation efficiency of HS74BM cells by chimeric adenovirus-SV40 virions

Virus	MOI	In situ foci[a]	24-h subcultivation[b]
Mock		0, 0	0
ΔE1/X	50	0, 0	0
ori^+	0.5	0, 1	0
	5	15, 16	1.5 ± 0.5
	50	85, 78	17 ± 3
ori^-	0.5	1, 0	0
	5	8	1 ± 1
	50	56, 82	17 ± 3
	200	Many	ND[c]
	1,000	Many	ND
SV40	200	3, 5	ND

[a] The number of foci scored at 21 days postinfection on plates which contained 2×10^5 cells per plate at the time of infection.

[b] The average number of foci per plate from five plates of cells infected at 2×10^5 cells per plate and split 1 to 5 at 24 h postinfection. The foci were scored at 21 days postinfection.

[c] ND, Values not determined.

possess a mutated T antigen have been described (SV80 and Gm638; W. R. Gish and M. Botchan, personal communication). Our results indicate that such mutations arise at high frequency. The apparent frequency of mutation indicates that there is a very strong selective pressure against a functional SV40 replication system in human cells.

High-molecular-weight DNA extracted from ori^- SV40-transformed cell lines was analyzed by Southern transfer experiments (15) after cleavage with various restriction endonucleases. Viral DNA sequences were integrated into the host cell chromosomes (Fig. 3). The SV40 DNA located in the left-end *Sst*I fragment was displaced to a higher molecular weight (Fig. 3A). This indicates an association with other DNA sequences. The right-end *Sst*I fragment was also displaced relative to the marker virion DNA fragments (Fig. 3B). Because the internal viral DNA fragments comigrated with the virion DNA markers, we conclude that most or all of the viral DNA is integrated colinearly with the viral genome. The ori^- SV40-tranformed human cell lines established in this study contained one to two independently integrated viral DNA molecules per cell. A similar pattern of integration was previously observed in Rat2 and CV1 cell lines transformed by recombinant adenoviruses carrying the gene for neomycin resistance (17). It has not been possible to

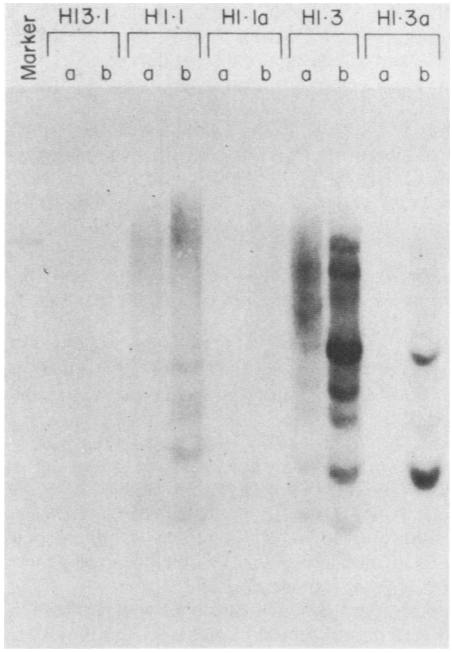

FIG. 2. Analysis of low-molecular-weight DNA in SV40-transformed HS74BM cell lines. SV40-transformed human cells (5×10^6) were fused to an equal number of either CV1 or COS-1 cells (3). Cells were incubated in Dulbecco modified Eagle medium–10% fetal bovine serum at 37°C for 48 to 50 h. Viral DNA was prepared (7), extracted with phenol and chloroform, ethanol precipitated, and resuspended in 10 mM Tris (pH 7.6)–1 mM EDTA. DNAs were digested with *Bgl*I, separated on a 1.0% agarose gel, transferred to nitrocellulose, and hybrized to ^{32}P-labeled SV40 DNA, and autoradiograms were established as described previously (6). H13.1 is a cell line transformed by the *ori*$^-$ chimeric virus; H1.1 and H1.3 are cell lines transformed by the *ori*$^+$ chimeric virus. H1.1a and H1.3a are subclones of the above cell lines transformed by the *ori*$^+$ chimeric virus. Lanes a, DNA from fusions to CV1 cells; lanes b, DNA from fusions to COS-1 cells. The marker is linear SV40 DNA (form III).

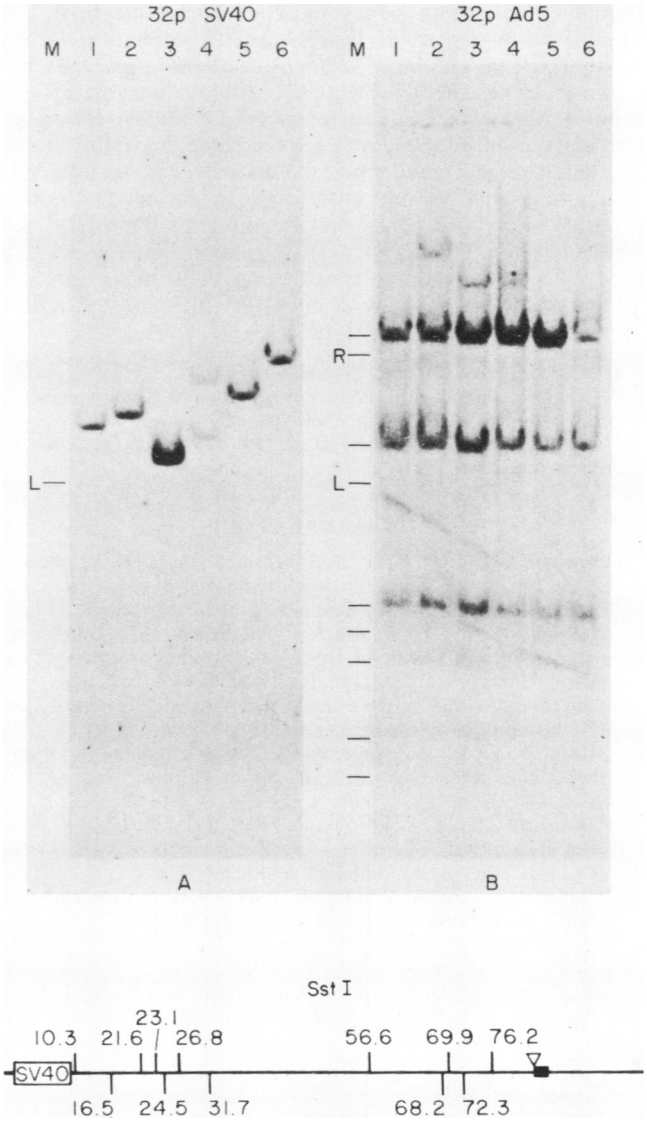

FIG. 3. Analysis of integrated viral DNA in *ori*$^-$-transformed cell lines. High-molecular-weight DNA (17) was extracted from 10 confluent 10-cm plates of the *ori*$^-$-transformed cell lines. DNA was digested with restriction endonuclease *Sst*I, and the fragments were separated on a 0.7% agarose gel and transferred to nitrocellulose (15). The DNA on the filter was hybridized to ^{32}P-labeled SV40 DNA, which was removed after autoradiograms were established, and then the filter was subsequently hybridized to ^{32}P-labeled adenovirus DNA. (A) The pattern of hybridization obtained with SV40 DNA as a probe. (B) The pattern obtained when the same filter was rehybridized to an adenovirus DNA probe. Lane M is a schematic representation of the authentic viral fragments. Lanes 1 through 6 are DNAs from transformed cell lines H13.1, H13.2, H13.3, H13.5, H13.6, and H13.8, respectively. L and R are the left and right ends of the viral genome, respectively. Below the panels is a restriction map of the recombinant viruses for the restriction endonuclease *Sst*I. The open triangle and filled rectangle represent the deleted adenovirus DNA and inserted foreign DNA sequences of the adenovirus type 5 mutant *dl309*, respectively (cited in reference 8).

determine the integration patterns of the *ori*$^+$-transformed cell lines owing to the presence of the free viral DNA.

Analysis of high-molecular-weight DNA from the two subcloned cell lines that lost the ability to produce free viral DNA revealed that the integration pattern in these cells is similar to that observed in the cell lines transformed by recombinant viruses containing a defective SV40 origin of replication.

There is growing interest in establishing human SV40-transformed cell lines for investigating various naturally occurring disorders and malignancies. Furthermore, there is great interest in establishing SV40-transformed cell lines from cell types which normally do not grow in culture. Recently, a small plaque mutant of SV40 and the *ori*$^-$ mutant 6-1 have been used to transform human skeletal muscle (9) and monocyte and macrophage cells (10), respectively.

In related experiments, we have used the chimeric viruses to transform primary cultures of mouse embryo fibroblasts and baby rat kidney cells (unpublished observations). These viruses could be used as tools to efficiently establish transformed cell lines from primary cells which are difficult to grow and have a limited life span in culture. We have constructed a double deletion adenovirus vector by creating a deletion in early region 3 of the adenovirus vector, ΔE1/X. This construction makes it possible to clone a second gene into the early region 3 deletion while retaining the SV40 DNA in the early region 1 deletion. The results of our studies indicate that a high percentage of transformed cell lines

should contain both genes integrated into the host cell genome with more than 20 kilobases of adenovirus DNA between them. The large segment of adenovirus DNA between the genes should obviate the problem of transcriptional interference which has been observed when two transcription units are introduced into cells in close proximity to one another. This will make it possible to deliver genes into cells which normally do not grow well in culture with high certainty that both genes will be integrated into the cell genome. The efficiency of transformation and the relative ease of infecting versus transfecting cells make this the system of choice for use with any cells which are susceptible to infection by adenovirus type 5.

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