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Characterization of Flat Revertant Cells Isolated from Simian Virus 40-Transformed Mouse and Rat Cells Which Contain Multiple Copies of Viral Genomes

KAZUO MARUYAMA, TAKAKI HIWASA, AND KIN-ICHIRO ODA*

Department of Tumor Virus Research, Institute of Medical Science, University of Tokyo, 4-6-1, Shirokanedai, Minato-ku Tokyo, 108, Japan

Eight clones of flat revertants were isolated by negative selection from simian virus 40 (SV40)-transformed mouse and rat cell lines in which two and six viral genome equivalents per cell were integrated, respectively. These revertants showed either a normal cell phenotype or a phenotype intermediate between normal and transformed cells as to cellular morphology and saturation density and were unable to grow in soft agar medium. One revertant derived from SV40-transformed mouse cells was T antigen positive, whereas the other seven revertants were T antigen negative. SV40 could be rescued only from the T-antigen-positive revertant by fusion with permissive monkey cells. The susceptibility of the revertants to retransformation by wild-type SV40 was variable among these revertants. T-antigen-negative revertants from SV40-transformed mouse cells were retransformed at a frequency of 3 to 10 times higher than their grandparental untransformed cells. In contrast, T-antigen-negative revertants from SV40-transformed rat cells could not be retransformed. The arrangement of viral genomes was analyzed by digestion of cellular DNA with restriction enzymes of different specificity, followed by detection of DNA fragments containing a viral sequence by blot hybridization. The integrated viral genomes in the transformed mouse and rat cells were serially arranged within the length of about 30 kilobases, with at least two intervening cellular sequences. A head-to-tail tandem array of unit-length viral genomes was present in at least one insertion site in the transformed rat cells. All of the revertants had undergone a deletion(s), and only a part of the viral genome was retained in T-antigen-negative revertants. A relatively high frequency of reversion in the transformed rat cells suggests that reversion occurs by homologous recombination between the integrated viral genomes.

Cells transformed by tumor virus have a variety of growth properties which differ from those of untransformed normal cells and variable capacities to form tumors after transplantation into susceptible animals. Most of these altered growth properties in vivo and in vitro are regulated by a function of the viral transforming gene, which is integrated into the cellular chromosome. In simian virus 40 (SV40)-transformed cells, it is known that T antigen encoded by early gene *A* governs these altered growth properties, since cells transformed by temperature-sensitive mutants of gene *A* show normal growth properties in cultures at the restrictive temperature (8, 22, 23, 34). T antigen is localized in nuclei and is involved both in the initiation of viral DNA replication (33) and in the control of viral mRNA synthesis (1, 19) in productively infected monkey cells. This pleiotropic action of T antigen suggests that T antigen interacts with cellular regulatory elements in a way that is not yet clear.

Revertant cells having the growth behavior of normal cells have been isolated from virus-transformed cells. These revertants may provide a useful system in which the mechanisms of virus-host interactions can be analyzed. In general, the revertants can be classified into the following four categories in terms of the defective step in the expression of the transformed phenotype. First, the revertants arise either by a loss of the viral genome generating cured cells or by a mutation or a deletion in the viral transforming gene. Second, the revertants contain a complete viral genome but are unable to synthesize stable mRNA owing to a defect(s) in cellular functions. Third, the revertants are somehow altered in their translational machinery so that viral mRNA is translated inefficiently. Fourth, the revertants are capable of synthesizing a transforming protein(s) but are unable to express a completely transformed phenotype.

Revertant cells have been isolated from SV40-transformed mouse cells by selective killing of

transformed cells with fluorodeoxyuridine (FUdR) (11, 25), bromodeoxyuridine (BUdR) (35), concanavalin A (10, 24), and colchicine (15, 37) under conditions in which only transformed cells could grow. Most of these revertants showed a growth behavior similar to that of normal cells despite the expression of T antigen and were insusceptible to retransformation by SV40 (11, 36, 37). A common feature of these revertants is an increase in chromosome number to subtetraploid (10, 15, 16, 26, 36). The inability of these revertants to express the transformed phenotype may, therefore, be ascribed to an altered interaction between T antigen and a cellular regulatory protein(s) due to the change in chromosome complements.

The isolation of revertants from SV40-transformed cells, in which the arrangement of viral genomes has been well analyzed, was first performed by Steinberg et al. (31). Assuming that revertants from transformed cells with multiple copies of dominant viral genes must arise by mutations in cellular genes, they isolated revertants from an SV40-transformed rat cell line with only one copy of the viral genome (5). These revertants were all T antigen negative, and many of them had undergone a deletion(s) in the viral genome.

We isolated revertants from SV40-transformed mouse and rat cells which integrated two and six viral genomes, respectively. Contrary to our expectations, all of the revertants had undergone a deletion(s) in the viral genome, and only a part of the viral genome was retained in T-antigen-negative revertants. These revertants showed variable susceptibility to retransformation by SV40. The mechanisms of integration of multiple copies of SV40 genomes and their excision will be discussed.

MATERIALS AND METHODS

Cells. The C3H2K-C4 cell line is a clonal line of C3H2K cells derived from C3H/He mouse kidney (39). The 3Y1-B cell line, clone 1-6, is a clonal line of Fischer rat embryo fibroblasts (20). The transformed cell lines W-2K-11 and W-3Y-23 were established by N. Yamaguchi and T. Kuchino by infecting C3H2K-C4 and 3Y1-B clone 1-6 cells with SV40 strain 777, respectively. These established cell lines were cultivated at 37°C in Eagle medium with 10% calf or fetal calf serum.

Isolation of flat revertants. Revertants from W-2K-11 cells were isolated by selective killing of transformed cells which incorporated BUdR in suspension cultures (32, 38). W-2K-11 cells were inoculated into 90-mm petri dishes. After 3 days of growth, when the cells were in an exponential phase, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine was added to the medium at a final concentration of 2.0 μg/ml. After 3 h of exposure, the cells were replated at an appropriate dilution and incubated for 5 to 6 days to fix the mutation. The cells were then plated in 1.28% methylcellulose medium overlaid on a preset base of 0.9% agar medium and incubated at 37°C for 48 h. BUdR was then added at a final concentration of 5×10^{-6} M, and incubation was continued for 48 h. The methylcellulose medium containing BUdR-treated cells was diluted fourfold with cold liquid medium, and the cells were pelleted by centrifugation. The cells were plated in liquid medium containing 10% calf serum at 1×10^5 to 2×10^5 cells per 90-mm plastic petri dish. After 6 to 12 h, the cells were exposed to visible light for 1.5 h through the cover of a petri dish at 8 cm from a Hitachi 40-W white lamp. The cells were incubated for 1 to 2 weeks, and flat colonies were isolated with sterile cloning rings. The revertants thus isolated were cloned three times and established as cell lines.

Revertants from W-3Y-23 cells were isolated by selective killing of transformed cells with FUdR at confluence (25). W-3Y-23 cells were inoculated in 90-mm petri dishes to yield confluent cultures in 3 to 4 days. The cells were then exposed to 10 μg of FUdR per ml for 3 days in the presence of a 10-fold-excess concentration of uridine. Cells adhering to the dishes were washed twice with phosphate-buffered saline, dispersed with phosphate-buffered saline containing 0.05% trypsin and 5×10^{-4} M EDTA, and replated at approximately 10^4 cells per 90-mm petri dish. Flat colonies were isolated as described above after cultivation for 7 to 10 days.

T-antigen assay. Cells grown on cover slips were washed twice with phosphate-buffered saline and fixed with acetone at −20°C for 10 min. The cells were treated with anti-T hamster serum at 37°C for 1 h. After washing three times with phosphate-buffered saline, T-antigen-positive cells were stained with fluorescein-conjugated rabbit anti-hamster immunoglobulin G. Cells were examined through a Nikon UV microscope.

Virus rescue by cell fusion. Transformed cells (4×10^5 cells) were mixed with African green monkey kidney cells, clone GC 7 (2×10^6 cells), in 0.4 ml of BSS (10 mM Tris-hydrochloride [pH 7.6], 0.114 M NaCl, 54 mM KCl, 0.34 mM Na_2HPO_4, 0.44 mM KH_2PO_4, 1 mM $CaCl_2$) at 0°C, and an equal volume of UV light-irradiated Sendai virus (2,000 hemagglutination units per ml) was added. After standing at 0°C for 10 min, the cells were gently shaken at 37°C for 20 min. The cells (6×10^5 cells) were then cultivated in 2-ounce (ca. 60-ml) plaque bottles at 37°C for 6, 10, and 14 days. The cell extract was prepared together with medium by freezing and thawing five times, followed by centrifugation at $2,000 \times g$ for 5 min. The virus was titrated by plaque formation on GC 7 cells.

Cell transformation. Transformation of revertant cells by SV40 was carried out either by colony formation in soft agar medium or by focus formation in liquid medium. Cells in a late exponential phase were infected with SV40 at 1,000 PFU per cell and incubated at 37°C for 24 h. Infected cells were replated in 0.4% agar medium at 4×10^4 cells per 60-mm petri dish, and 4 ml of the agar medium was overlaid every week. After 1 month, the number of colonies larger than 0.1 mm in diameter was counted. In the other method, 10^4 infected cells were plated in a 60-mm petri dish in

liquid medium, and the medium was changed every 4 days. The foci were counted at 3 weeks after plating by Giemsa staining.

Preparation of viral and cellular DNAs. Purified SV40 virions were treated with 125 μg of papain per ml at 37°C for 3 to 4 min and solubilized by the addition of 0.5% sodium dodecyl sulfate at 45°C. DNA was extracted twice with water-saturated phenol (pH 8.0) and twice with chloroform-isoamyl alcohol (24:1). Form I DNA was isolated by sedimenting the DNA in a 5 to 20% sucrose gradient in 20 mM Tris-hydrochloride (pH 8.0)–0.1 M NaCl–10 mM EDTA and concentrated by precipitation with 2 volumes of ethanol.

For the preparation of cellular DNA, cells were washed twice with phosphate-buffered saline, suspended in isotonic buffer (10 mM Tris-hydrochloride [pH 7.4], 0.14 M NaCl, 1.5 mM $MgCl_2$) containing 0.5% Nonidet P-40 at 2×10^7 cells per ml, and kept at 0°C for 20 min. Nuclei were pelleted by centrifugation and washed once with isotonic buffer. Nuclei were suspended in 0.14 M NaCl–0.1 M EDTA with a Dounce homogenizer and dissolved by the addition of SDS (final concentration, 0.5%) at 45°C for 10 min. DNA was extracted twice with water-saturated phenol and dissolved in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate; ca. 2×10^7 to 3×10^7 nuclei per ml) after precipitation with 2 volumes of ethanol. The extract was treated with 50 μg of pancreatic RNase per ml at 37°C for 1 h and then with 50 μg of proteinase K per ml for 2 h. DNA was extracted twice with water-saturated phenol, precipitated with ethanol, and dissolved in 0.1× SSC. After the addition of 0.11 volume of 3 M sodium acetate–1 mM EDTA (pH 7.0), DNA was precipitated by the addition of 0.54 volume of isopropanol. DNA was finally dissolved in 0.1× SSC containing 1 mM EDTA.

Nick translation. ^{32}P-labeled SV40 DNA with a specific activity of 2×10^8 cpm/μg was prepared by nick translation (29). The reaction mixture contained 15 μg of SV40 DNA per ml, 20 μM [α-^{32}P]dCTP (400 Ci/mmol), 15 μM each of unlabeled dTTP, dATP, and dGTP, 50 mM potassium phosphate buffer (pH 7.4), and 5 mM $MgCl_2$. Activated DNase I was first added to the reaction mixture at a final concentration of 10 μg/ml and incubated at room temperature for 2 min. DNA polymerase I (50 U/1 μg of SV40 DNA) was then added, and the reaction was carried out at 14°C for several hours. The reaction was stopped by the addition of EDTA at a final concentration of 75 μM and heated at 68°C for 10 min. DNA was extracted twice with phenol and separated from nucleotides by gel filtration in a Sephadex G-50 (fine) column with 10 mM Tris-hydrochloride (pH 7.4)–10 mM NaCl–2 mM EDTA. Labeled DNA was concentrated by ethanol precipitation after adjusting the NaCl concentration to 1 M.

DNA-DNA reassociation kinetics. ^{32}P-labeled SV40 DNA (ca. 10^8 cpm/μg) was heat denatured. Cellular DNA was denatured and fragmented to a length of about 400 bases by boiling in 0.3 N NaOH. These DNAs were mixed at final concentrations of 1.0×10^{-3} μg/ml and 1.3 mg/ml, respectively, and incubated in 0.2 M sodium phosphate buffer (pH 6.8)–1 M NaCl–0.1% sodium dodecyl sulfate at 68°C in siliconized disposable pipettes (50 μl, Micropet) sealed at both ends. The reaction was stopped by cooling the samples in ice at various times after incubation, and the fraction of ^{32}P-labeled SV40 DNA hybridized was determined by hydroxyapatite column chromatography (7, 13).

Spot hybridization. DNA prepared from SV40-transformed cells and revertants was heat denatured in 0.1× SSC at a concentration of 500 μg/ml at 100°C for 20 min, followed by rapid cooling. The concentration of SSC was adjusted to 4× by the addition of 20× SSC, and 20 μg of denatured DNA in 50 μl was spotted onto a circular area (6 mm in diameter) marked on a Millipore membrane filter (pore size, 0.45 μm) under gentle suction (12). Reconstruction spots were made by mixing salmon sperm DNA with SV40 DNA; e.g., the reconstruction of one copy of SV40 DNA per cell was made by mixing 20 μg of salmon sperm DNA with 1.5×10^{-5} μg of SV40 DNA. The filter was air dried and immobilized at 80°C for 2 h in vacuo. Hybridization of immobilized DNA with ^{32}P-labeled SV40 DNA was performed as described below.

Blot hybridization. High-molecular-weight cellular DNA was digested with restriction endonuclease *Eco*RI, *Xba*I, *Kpn*I, or *Hin*fI and electrophoresed on a 1.0 or 1.2% agarose slab gel as described in the figure legends. After alkali denaturation, DNA was transferred from the gel to a nitrocellulose filter by the method of Southern (30). The filter was rinsed in 2× SSC and dried at 80°C for 2 h in a vacuum oven.

Before hybridization, the filter was rinsed in 3× SSC at room temperature for 30 min and incubated in 3× SSC–0.1% sodium dodecyl sulfate–10× Denhardt solution (1× Denhardt solution contains 0.02% each bovine serum albumin, Ficoll, and polyvinylpyrrolidone 360) at 65°C for 30 min. The filter was then incubated in hybridization buffer (3× SSC, 10× Denhardt solution, 50 μg of heat-denatured salmon sperm DNA per ml, 10 μg of yeast RNA per ml, 0.1% sodium dodecyl sulfate) at 68°C for 2 h. Hybridization was started by the addition of 2×10^6 cpm of denatured ^{32}P-labeled SV40 DNA per ml at 68°C. At 48 h after incubation, the filter was washed by gentle shaking in 2× SSC at room temperature for 15 min and five times in 0.1× SSC containing 0.1% sodium dodecyl sulfate at 68°C for 30 min. The filter was then rinsed two to three times in 0.1× SSC at room temperature for 1 min, dried, and exposed against Fuji RX-S film at −80°C for 2 to 3 weeks with an intensifying screen.

RESULTS

Viral DNA in SV40-transformed cell lines. Flat revertant cells were isolated from the SV40-transformed rat cell line W-3Y-23 and the SV40-transformed mouse cell line W-2K-11. SV40 DNA sequences in these transformed cell lines were analyzed by reassociation kinetics of ^{32}P-labeled SV40 DNA obtained by nick translation. The number of SV40 genome equivalents per diploid cell was estimated by the acceleration in the reassociation rate in the presence of transformed cell DNA (Fig. 1). The results indicate that the W-3Y-23 and W-2K-11 cell lines contained about six and two SV40 genome

equivalents per diploid quantity of cell DNA, respectively. As shown below, infectious SV40 could be recovered from both W-3Y-23 and W-2K-11 cells by fusion with permissive monkey cells.

Isolation of flat revertants from SV40-transformed cells. Flat revertant cells were isolated by negative selection with either FUdR or BUdR. Confluent cultures of SV40-transformed W-3Y-23 rat cells were exposed to FUdR for 72 h at a concentration of 10 μg/ml in the presence of a 10-fold-excess concentration of uridine. After washing, cells were plated at a two-fold dilution and incubated at 37°C until surviving cells could grow into distinct colonies. Under these conditions, the fraction of cells capable of forming colonies was 2×10^{-5} to 4×10^{-5}, and approximately 20% of the colonies showed the flat morphology similar to normal cells. The fraction of spontaneous flat revertants in W-3Y-23 cell cultures was, therefore, 4×10^{-6} to 8×10^{-6}. Four flat revertants were isolated and cloned three times. No flat revertants could be isolated from SV40-transformed W-2K-11 mouse cells by a similar procedure, partly owing to their relative resistance to FUdR.

The isolation of revertants from W-2K-11 cells was performed after mutagenesis by exposing the cells to 2 μg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine per ml for 3 h. The selective killing of transformed cells was carried out by growing the cells in methylcellulose medium in the presence of BUdR, followed by irradiation with visible light. Four flat revertants were isolated and cloned three times.

Properties of revertants. The morphology of flat revertants from SV40-transformed W-3Y-23 rat cells and from SV40-transformed W-2K-11 mouse cells is shown in Fig. 2 and 3, respectively. The photographs were taken when the cells reached confluence. Both W-3Y-23 and W-2K-11 cells (Fig. 2A and 3A) formed dense multilayers, with cells piling up at many sites. Grandparental untransformed 3Y1-B and C3H2K-C4 cells (Fig. 2B and 3B) formed monolayers, with cells having an elongated shape typical of fibroblasts. Revertants R3-3, R4, R6-5, and R10-1 (Fig. 2C, D, E, and F) derived from W-3Y-23 cells formed monolayers, with cells having an elongated shape, but the morphologies and orientations of the cells differed from each other. Revertants M5, M18, M31, and M42 (Fig. 3C, D, E, and F) derived from W-2K-11 cells formed monolayers, but the morphology of the cells was converted from an elongated shape to a rather round one when they reached confluence. The morphologies of these revertants also differed from each other, and the size of an M5 cell (Fig. 3C) was smaller than those of others.

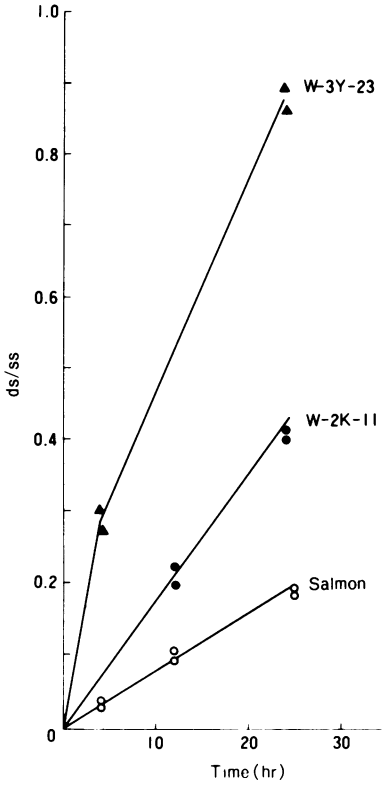

FIG. 1. *Kinetics of reassociation of ^{32}P-labeled SV40 DNA in the presence of DNA extracted from SV40-transformed cells. ^{32}P-labeled SV40 DNA (10^8 cpm/μg, 1.0×10^{-3} μg/ml) was incubated in the presence of 1.3 mg of salmon sperm DNA, W-2K-11 cell DNA, or W-3Y-23 cell DNA per ml at 68°C after denaturation and fragmentation as described in the text. Samples were withdrawn at the times indicated. The ratio of renatured double-stranded (ds) to single-stranded (ss) ^{32}P-labeled SV40 DNA in the sample was determined by chromatography on hydroxyapatite.*

The saturation density of these revertants ranged from the normal cell type to a type intermediate between normal and transformed cells (Table 1). The saturation density of R4, R6-5, and M18 was lower than that of their grandparental untransformed cells. The presence of T antigen in the revertants was examined by indirect immunofluorescence. The cells were stained at the late exponential phase. Only revertant M5 was T antigen positive and showed the bright fluorescence similar to parental W-2K-11 cells. The other seven revertants were all T antigen negative. All revertants from W-2K-11 and W-3Y-23 cells were unable to grow in soft agar medium, irrespective of the saturation density and the expression of T antigen.

The ability of the revertants to produce infec-

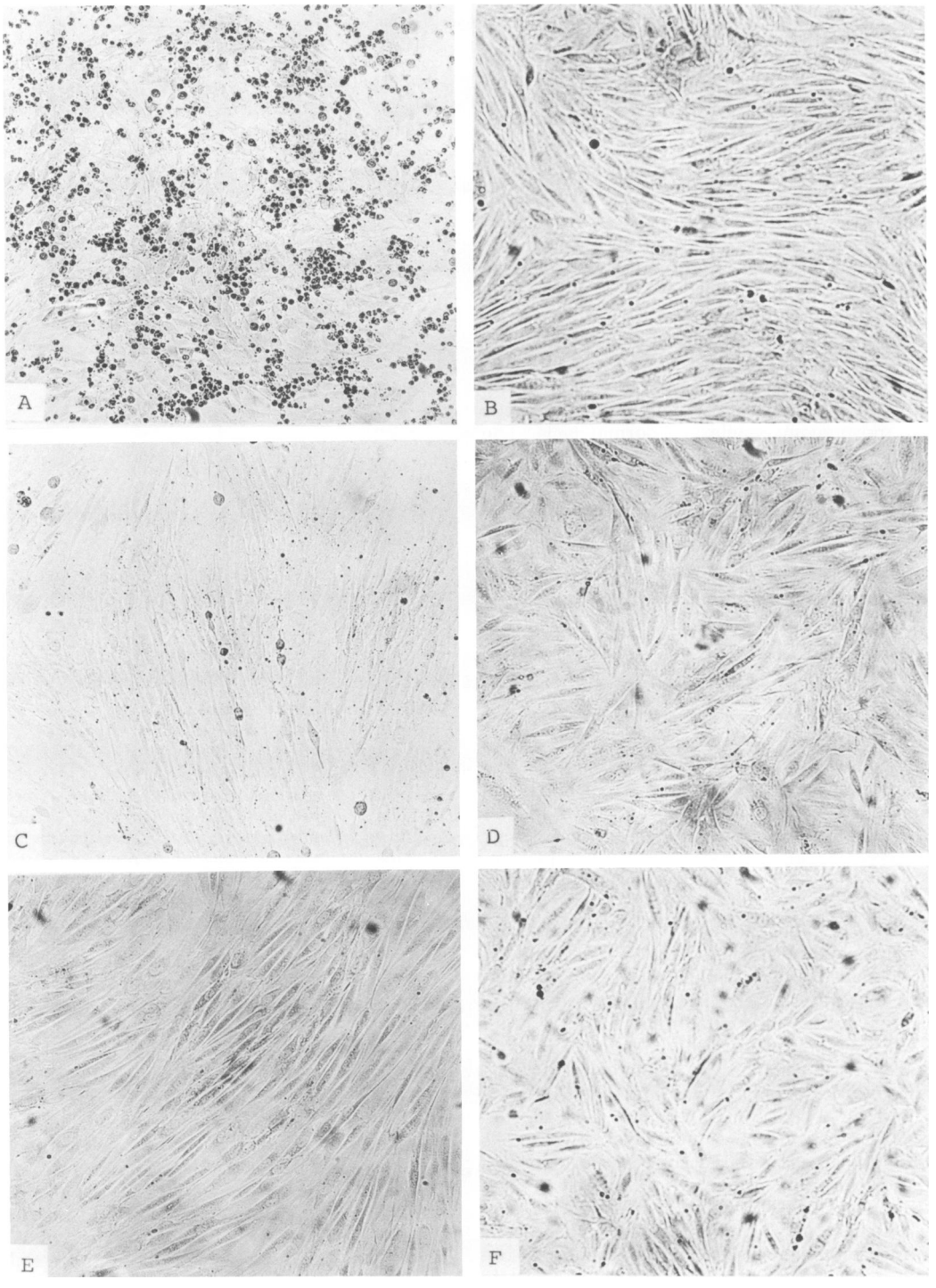

FIG. 2. *Morphology of revertant cells from SV40-transformed rat cells. (A) W-3Y-23; (B) 3Y1-B; (C) R3-3; (D) R4; (E) R6-5; (F) R10-1. Photographs were taken when the cells had nearly reached confluence.*

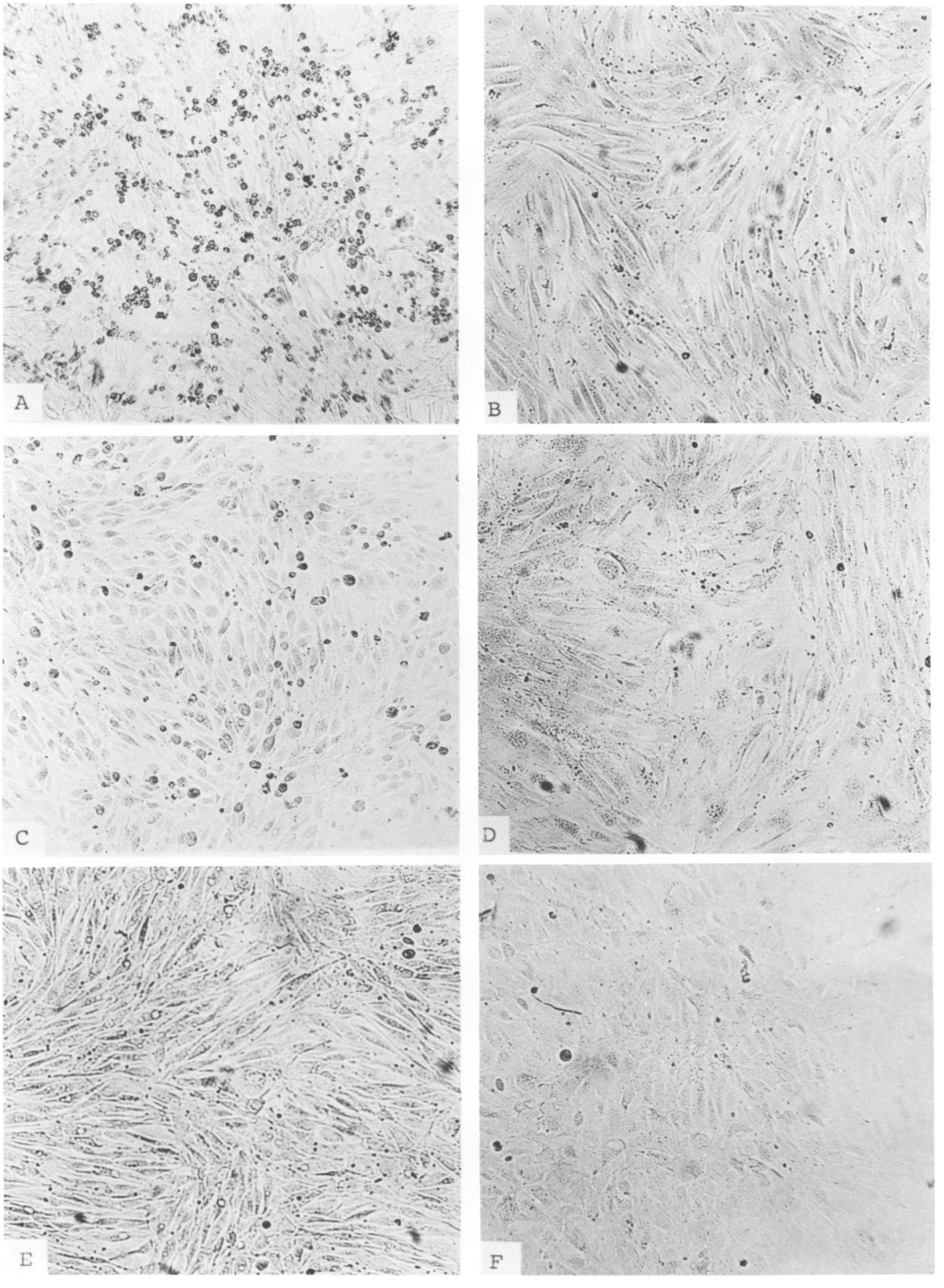

FIG. 3. *Morphology of revertant cells from SV40-transformed mouse cells. (A) W-2K-11; (B) C3H2K-C4; (C) M5; (D) M18; (E) M31; (F) M42. Photographs were taken as described in the legend to Fig. 2.*

tious SV40 upon fusion with permissive monkey cells was also examined. At 10 to 14 days after fusion, the cells were frozen and thawed five times, and the virus was titrated on monkey cell monolayers after clarification of the lysate by centrifugation. Virus was produced only from the T-antigen-positive revertant M5, as well as from W-3Y-23 and W-2K-11 cells. No virus was rescued from the other seven revertants. The yield of virus from M5 was 7.5×10^5 PFU/ml and was about 10 times lower than that rescued from parental W-2K-11 cells. Rescued viruses were propagated in GC 7 cells, and DNA from progeny viruses (SVM5) was compared with that of wild-type SV40 after cleavage by several restriction endonucleases. The cleavage patterns of SVM5 DNA by *Eco*RI, *Hinc*II, *Hin*dIII, and *Hin*fI were identical to those of wild-type SV40 DNA, although a small deletion may not be detected. SVM5 could transform C3H2K-C4 cells at a frequency slightly lower than that by wild-type SV40 (data not shown).

Retransformation of revertants by SV40. The susceptibility of revertants to retransformation by superinfection with SV40 was examined by colony formation in soft agar medium. The frequency of transformation of the T-antigen-negative revertants M18, M31, and M42 from W-2K-11 cells was 3 to 10 times higher than that of grandparental C3H2K-C4 cells (Table 2). This high frequency did not result from an increase in uptake of superinfecting SV40 since the proportion of T-antigen-positive cells in these revertants by 48 h after superinfection was 10 to 20% and even lower than that in C3H2K-C4 cells (40 to 50%). The T-antigen-positive revertant M5 could not be retransformed.

In contrast to the above results, the T-antigen-negative revertants R3-3, R4, R6-5, and R10-1 from W-3Y-23 cells could not be retransformed to a measurable extent. Insusceptibility of these revertants to retransformation was not due to an inefficient uptake of superinfecting SV40 since 10 to 30% of the cells became T antigen positive at 48 h postinfection. This value was not significantly different from that of grandparental 3Y1-B cells.

SV40 DNA in revertants. To determine whether the T-antigen-negative revertants arose by a loss of all or part of the integrated viral genome, the amount of viral DNA present in the revertants was roughly estimated by spot hybridization. High-molecular-weight DNA extracted from cells was spotted onto a circular area marked on a nitrocellulose filter after heat

TABLE 1. *Properties of flat revertants isolated from SV40-transformed cells*

Species	Negative selection with:	Cell line	Saturation density ($\times 10^{-4}$/cm^2)	T antigen	Virus rescue by fusion	Growth in agar medium	Transformation by SV40
Fischer rat	FUdR	W-3Y-23	13.8	+	+	+	
		3Y1-B	3.5	−	−	−	+
		R3-3	4.2	−	−	−	−
		R4	2.7	−	−	−	−
		R6-5	2.8	−	−	−	−
		R10-1	5.8	−	−	−	−
C3H/He mouse	BUdR	W-2K-11	15.8	+	+	+	
		C3H2K-C4	5.4	−	−	−	+
		M5	7.5	+	+	−	−
		M18	4.2	−	−	−	+
		M31	5.2	−	−	−	+
		M42	8.5	−	−	−	+

TABLE 2. *Retransformation of revertants by SV40*

Cell line	No. of colonies		Frequency of transformation	
	Infected	Mock infected	Infected	Mock infected
C3H2K-C4	23	0	5.7×10^{-4}	0
M5	2.5	2	6.3×10^{-5}	5×10^{-5}
M18	77	0	1.9×10^{-3}	0
M31	213	2	5.3×10^{-3}	5×10^{-5}
M42	190	1	4.8×10^{-3}	2.5×10^{-5}

denaturation. Hybridization was carried out by incubating the filter with nick-translated ^{32}P-labeled SV40 DNA, and the amount of viral DNA integrated into cellular DNA was estimated by comparing the intensity of autoradiograms with that of reconstruction spots. Reconstruction spots were made by mixing salmon sperm DNA with 0.5 to 10 copies of SV40 DNA per cell (Fig. 4). The numbers of viral genomes in W-2K-11 and W-3Y-23 cells were estimated to be approximately two and seven copies per diploid cell. These numbers coincided with those obtained by reassociation kinetics described above. The intensity of autoradiograms of revertant M18, M31, and M42 DNAs was weaker than that of W-2K-11 DNA, indicating a partial loss of integrated viral genomes. The intensity of the autoradiogram of T-antigen-positive revertant M5 DNA was also weaker than that of W-2K-11 DNA. Revertants R3-3 and R4 from W-3Y-23 cells lost a majority of viral genomes. R6-5 showed no radioactivity, indicating that the viral genome was almost completely lost. R10-1 retained more than one copy of viral genome equivalent.

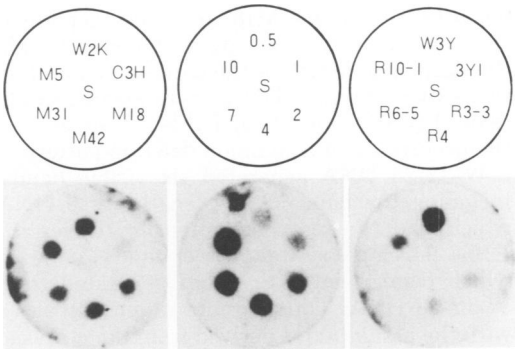

FIG. 4. *Spot hybridization of revertant cell DNA with ^{32}P-labeled SV40 DNA. DNAs extracted from SV40-transformed cells (W3Y and W2K), untransformed parental cells (3Y1 and C3H), and revertant cells were heat denatured, and samples of 20 μg of DNA were spotted onto the filter. Salmon sperm DNA (S) was spotted onto the center of the filter as the control DNA. Hybridization was performed with denatured ^{32}P-labeled SV40 DNA (10^8 cpm/μg, 5×10^6 cpm/ml) as described in the text. Reconstruction spots are shown in the center circle.*

Arrangement of integrated SV40 DNA sequences in transformed and revertant cells. The arrangement of integrated SV40 DNA sequences in transformed and revertant cells was analyzed by Southern blot hybridization (30). Cellular DNA was digested extensively with various restriction endonucleases and electrophoresed on an agarose gel. DNA fragments were blotted onto a nitrocellulose filter and hybridized with nick-translated ^{32}P-labeled SV40 DNA. DNA fragments containing the viral DNA sequence were detected by autoradiography. Radioactive bands visualized were schematically represented on the side of an autoradiogram, although the thickness of the bands does not necessarily reflect the quantity of viral sequences. Figure 5 shows the results obtained with restriction enzyme *Eco*RI, which cuts SV40 DNA once. W-2K-11 DNA yielded four bands with lengths of approximately 9.0, 6.5, 5.2, and 3.0 kilobases (kb), indicating that the DNA contains at least two insertions of SV40 DNA. Among these bands, the intensity of two bands of 6.5 and 3.0 kb was stronger than that of the other two bands. DNA from the T-antigen-positive revertant M5 generated two bands which migrated slightly faster than the 9.0- and 6.5-kb bands generated from W-2K-11 DNA. DNAs from the T-antigen-negative revertants M18, M31, and M42 each generated only one band of 5.3, 4.0, and 5.3 kb, respectively, suggesting the absence of the complete viral genome in these cell lines.

W-3Y-23 DNA generated seven bands; one of the bands with the strongest intensity comigrated with unit-length SV40 DNA. The results suggest that the insertion of SV40 DNA had occurred at at least three different sites, and in at least one of the integration sites, the viral genomes were tandemly repeated in a head-to-tail arrangement. Among four T-antigen-negative revertants, only R10-1 generated visible bands of approximately 3.5, 3.0, and 2.5 kb. Judging from the intensity, the 2.5-kb band may be the same as the 2.5-kb band generated from W-3Y-23 DNA. No visible band was detected with R3-3, R-4, and R6-5 DNAs (data not shown) owing to a loss of almost all of the viral genomes.

To confirm the integration and deletion patterns of SV40 DNA, DNA from transformed and revertant cells was also digested with *Kpn*I, which cuts SV40 DNA once at 0.71 map unit, and analyzed similarly (Fig. 6). Four bands of about 9.0, 7.0, 5.7, and 3.2 kb were generated from W-2K-11 DNA. The 5.7-kb band with a strong intensity might consist of two different bands of similar lengths. T-antigen-positive revertant M5 DNA generated four bands of about 9.0, 5.7, 2.0, and 1.0 kb. The 9.0- and 5.7-kb bands may not be identical to the 9.0- and 5.7-kb bands from W-2K-11 DNA since the relative intensity was different between these bands. The results suggest that, in revertant M5, a small viral DNA segment was retained in addition to a complete set of the viral genome. DNA from T-antigen-

negative revertants M18 and M42 generated only one band with a length of about 1.3 kb. Similarities in length of the bands generated by these two revertant DNAs were also observed upon digestion with *Eco*RI (Fig. 5), suggesting the occurrence of a common deletion pattern.

W-3Y-23 DNA generated six visible bands, including the band of unit-length SV40 DNA which had the strongest intensity. Revertant R10-1 DNA generated three bands, none of which comigrated with bands generated from W-3Y-23 DNA. These results essentially agree with the assumptions derived from the cleavage patterns obtained with *Eco*RI.

DNA from transformed and revertant cells was then cleaved with *Xba*I, which does not cleave SV40 DNA. The fragment containing the viral DNA sequence was analyzed similarly (Fig. 7). DNAs from W-2K-11 and W-3Y-23 cells generated one broad band. The length was estimated to be approximately 30 kb from that of adenovirus type 12 DNA simultaneously run as a size marker, although an accurate estimation of the length is difficult at the top region of the gel. No band was detected at the positions of SV40 form I and II DNAs. Revertant M5 and R10-1 DNAs generated a band with a similar length. Revertant M18, M31, and M42 DNAs generated a band of 20 to 25 kb. These results, together with those obtained with *Eco*RI and

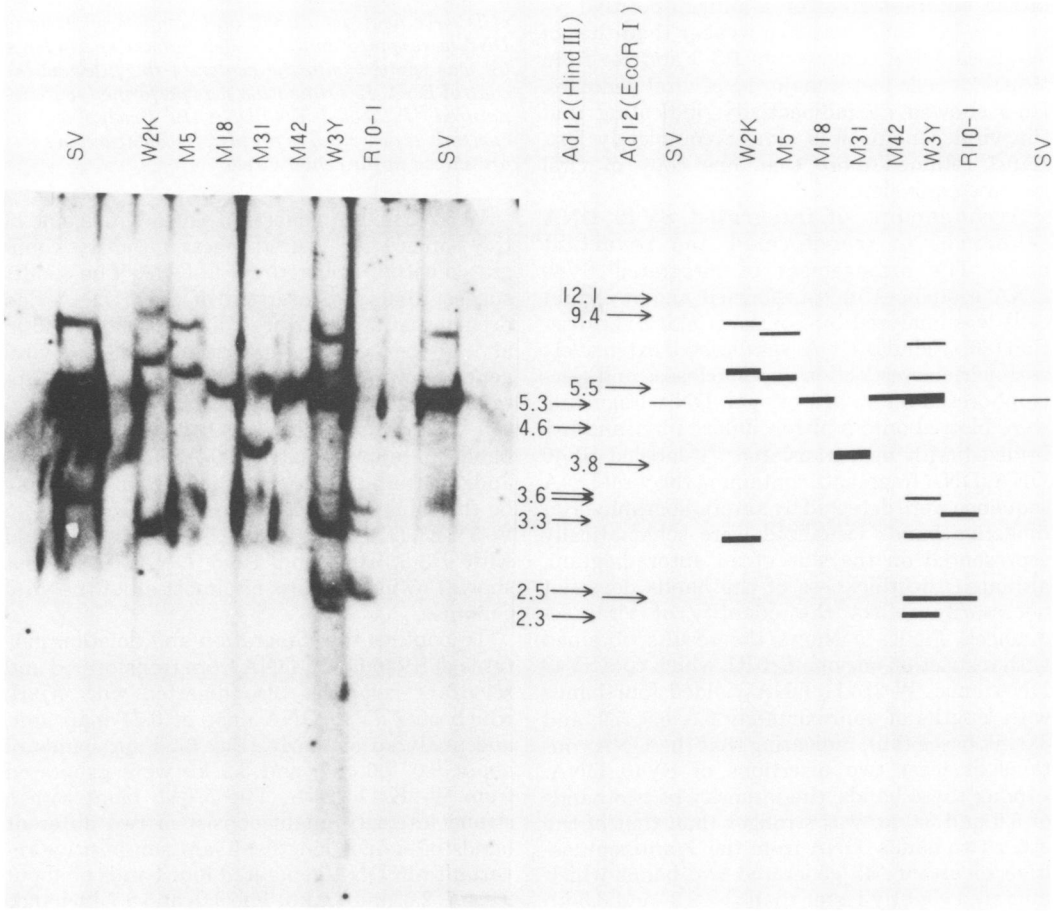

FIG. 5. *Analysis of SV40 DNA sequences in transformed and revertant cell DNAs digested with restriction endonuclease EcoRI. DNA (20 μg) extracted from SV40-transformed cells, untransformed parental cells, and revertant cells was digested with 200 U of EcoRI (Boehringer) in 0.1 M Tris-hydrochloride (pH 7.5)-50 mM NaCl-10 mM $MgCl_2$ at 37°C for 24 h. The DNA was electrophoresed on a 1.2% agarose slab gel at 50 V for 13 h. The SV40 DNA sequence was analyzed by blot hybridization as described in the text. Adenovirus type 12 (Ad12) DNA digested with EcoRI and HindIII was run as a size marker. Positions and lengths of fragments (kb) are indicated by arrows. SV, SV40; W2K, W-2K-11; W3Y, W-3Y-23.*

*Kpn*I, suggest that SV40 DNAs are serially arranged, with intervening cellular DNA between the viral DNA sequences in a limited portion of the host chromosome.

The pattern of integration and deletion of viral DNA was also analyzed by digesting DNA with *Hin*fI, which cleaves SV40 DNA at multiple sites (Fig. 8a). W-2K-11 DNA generated four bands which migrated at rates similar to those No off-size fragment could be detected. Revertant M5 DNA generated five bands, three of which migrated at rates similar to *Hin*fI fragments A, C, and DE. One off-size fragment migrated between fragments A and B, and the other migrated slightly slower than fragment B. Hybridization was also carried out with ^{32}P-labeled *Hin*fI fragment A (0.64 to 0.99 map unit) as a probe (Fig. 8b). This fragment spans about half the late region, and one of the ends is very near the *Eco*RI site. This result indicated that the off-size fragment that migrated between fragments A and B contains the *Hin*fI-A sequence. The revertants M18 and M42 generated only one band, which did not comigrate with any *Hin*fI DNA fragments, but contains the *Hin*fI-A sequence. The mobilities of these fragments were again similar, as has been observed with *Eco*RI and *Kpn*I digestions. Revertant M31 DNA generated one band, which comigrated with *Hin*fI fragment A.

W-2Y-23 DNA generated all of the *Hin*fI DNA fragments. In addition, three off-size DNA fragments were detected. Revertant R10-1 DNA contained *Hin*fI fragments B and DE but lacked

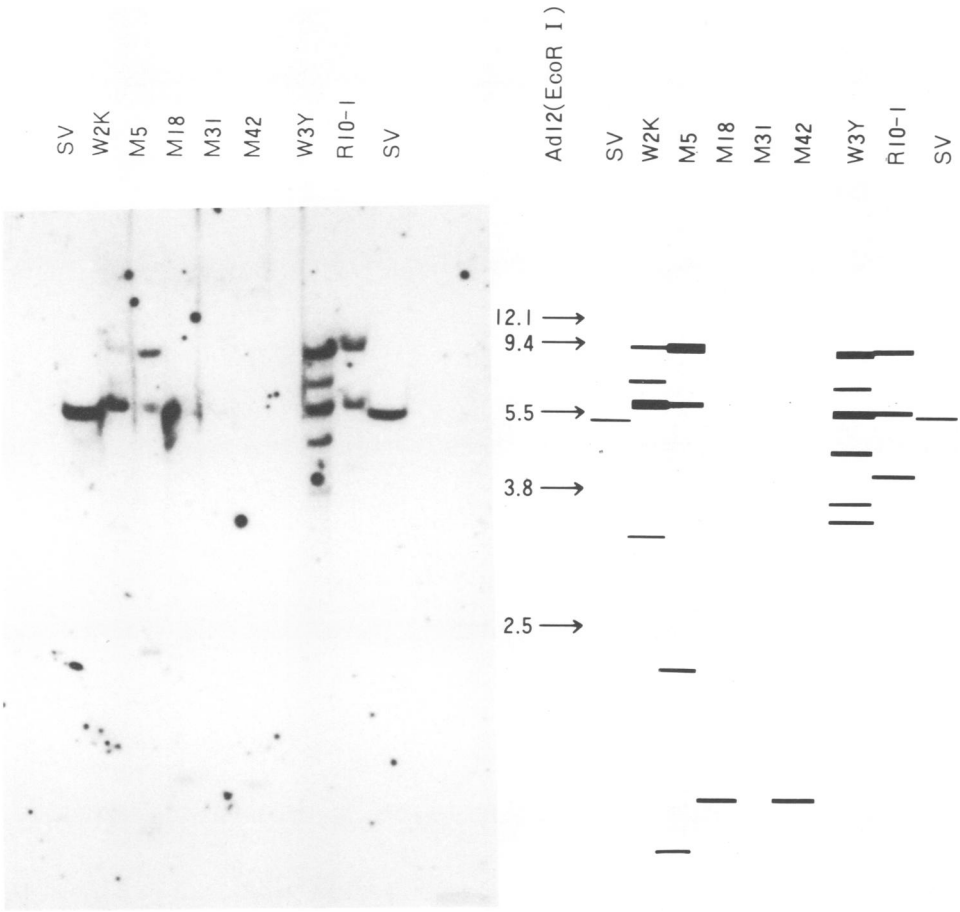

FIG. 6. *Analysis of SV40 DNA sequences in transformed and revertant cell DNAs digested with restriction endonuclease KpnI. DNA was digested with KpnI (Bethesda Research Laboratories) at 5 U/μg of DNA in 6 mM each Tris-hydrochloride (pH 7.5)-NaCl-$MgCl_2$-2-mercaptoethanol at 37°C for 24 h. The DNA was electrophoresed on a 1.2% agarose slab gel at 50 V for 14 h. The SV40 DNA sequence was analyzed as described in the legend to Fig. 5.*

fragments A and C. The absence of fragment C coincides with the inability of R10-1 cells to synthesize T antigen since fragment C is located in the early region. Two off-size DNA bands were generated, both of which may be identical to those generated by W-3Y-23 DNA. One off-size fragment that migrated between fragments C and DE contains the *Hin*fI-A sequence.

For a further analysis of integration sites, *Eco*RI-digested DNA from W-2K-11 and its revertants was also hybridized with ^{32}P-labeled *Hin*fI fragment A after blotting to the filter (Fig. 9). The patterns of the radioactive bands detected by autoradiography were essentially the same as those obtained with whole SV40 DNA as the probe (compare Fig. 9 with 5), with the exception of the absence of the 5.2-kb band generated from W-2K-11 DNA. Hybridization of three of the four bands generated from W-2K-11 DNA by *Eco*RI suggests that two copy equivalents of SV40 DNA were integrated into three sites in W-2K-11 DNA since one of the ends of *Hin*fI fragment A (0.99 map unit) is very near the *Eco*RI site and no off-size DNA fragment containing the *Hin*fI-A sequence was detected after digestion of W-2K-11 DNA with *Hin*fI. A possible integration model is shown in Fig. 10.

DISCUSSION

Arrangement of viral DNA. SV40-transformed mouse W-2K-11 and rat W-3Y-23 cell lines integrated approximately two and six ge-

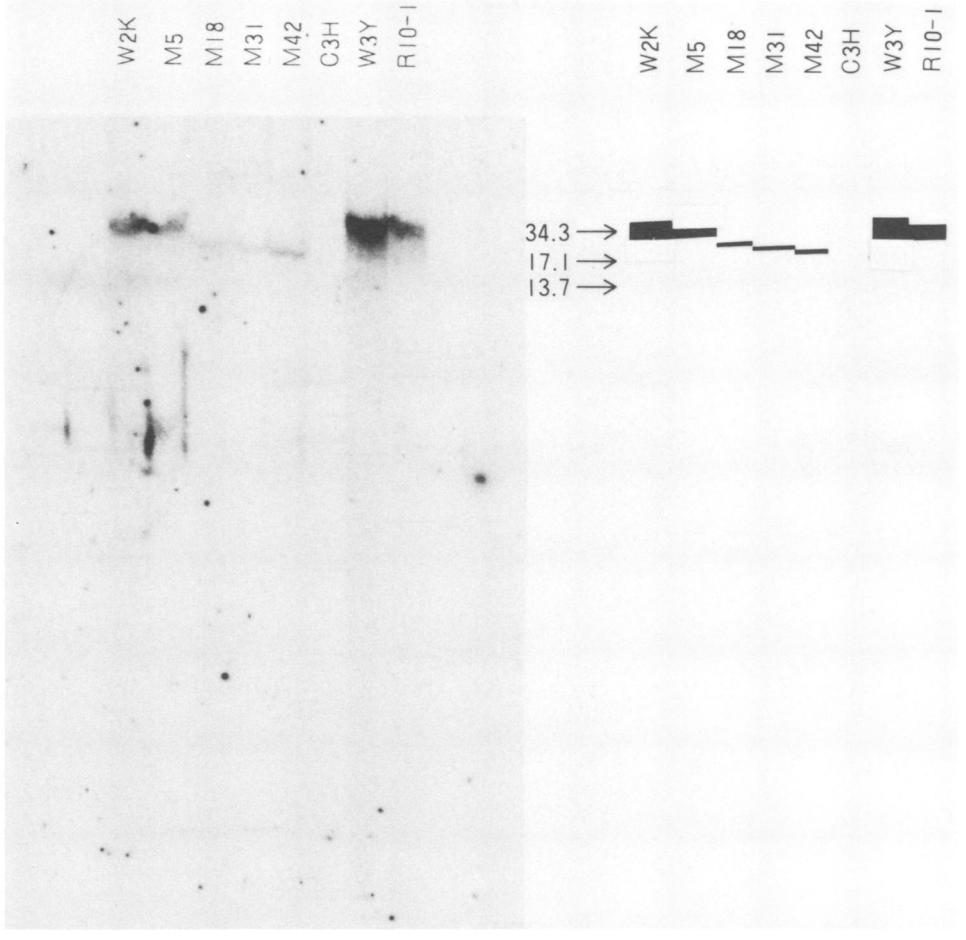

FIG. 7. *Analysis of SV40 DNA sequences in transformed and revertant cell DNAs digested with restriction endonuclease XbaI. DNA was digested with XbaI (Bethesda Research Laboratories) at 5 U/μg of DNA in 6 mM Tris-hydrochloride (pH 7.4)-100 mM NaCl-6 mM $MgCl_2$ at 37°C for 24 h. The DNA was electrophoresed on a 1.0% agarose slab gel at 50 V for 14 h. The SV40 DNA sequence was analyzed as described in the legend to Fig. 5. Adenovirus type 12 DNA (34.3 kb) and its fragments (17.1 and 13.7 kb) generated by SalI were run as size markers.*

nome equivalents of viral DNA per cell, respectively, judging from the results obtained by reassociation kinetics, spot hybridization, and Southern blot hybridization. These viral DNAs seemed to be serially arranged within the length of about 30 kb, with at least two intervening cellular DNA sequences between the viral DNAs as schematically shown in Fig. 10. A relative broadness of the band generated from W-3Y-23 DNA by digestion with *XbaI*, which does not cleave SV40 DNA, raises the possibility that the viral genomes were separately integrated into two or more different fragments of similar length. However, this possibility seems unlikely

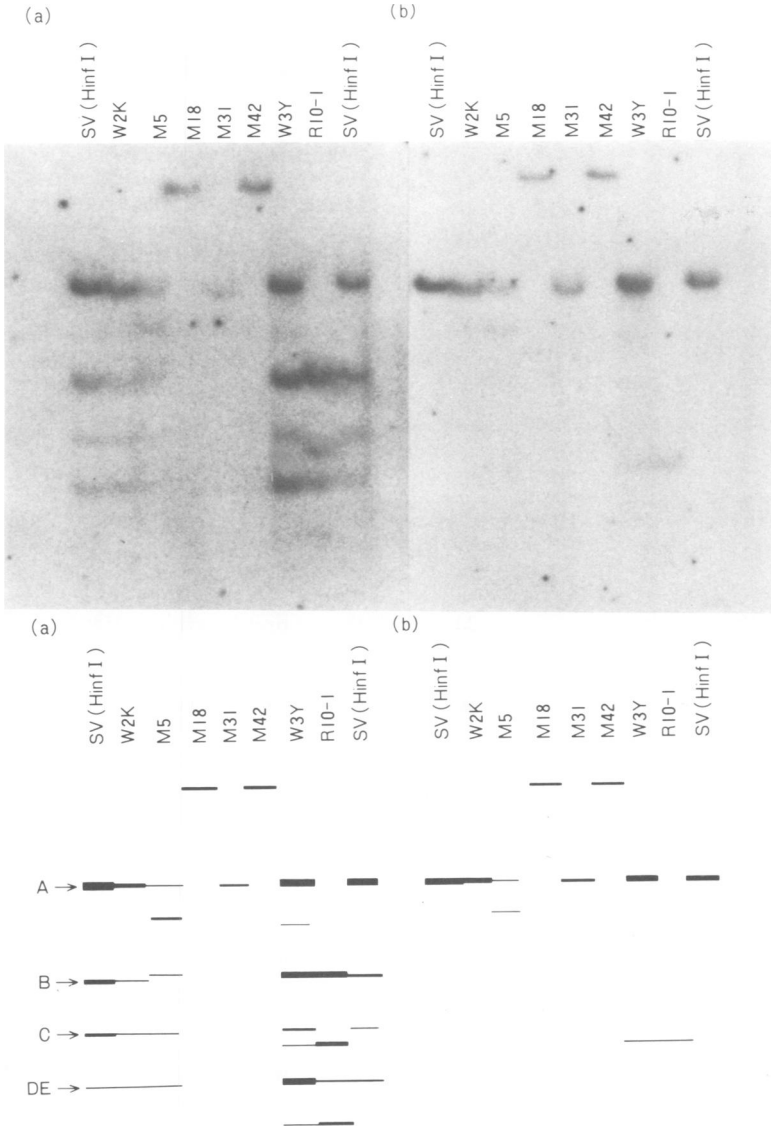

FIG. 8. *Analysis of SV40 DNA sequences in transformed and revertant cell DNAs digested with restriction endonuclease HinfI. DNA was digested with HinfI (Bethesda Research Laboratories) at 5 U/μg of DNA in 6 mM Tris-hydrochloride (pH 7.5)-6 mM $MgCl_2$-100 mM NaCl-6 mM 2-mercaptoethanol at 37°C for 24 h. The DNA was electrophoresed on a 1.2% agarose slab gel at 32 V for 14 h. The SV40 DNA sequence was analyzed by hybridization with either whole SV40 DNA (a) or SV40 HinfI fragment A (b) as a probe. HinfI fragment A was eluted from the gel slice by electrophoresis. Arrows A, B, C, and DE indicate the positions of the SV40 DNA fragments generated by digestion with HinfI.*

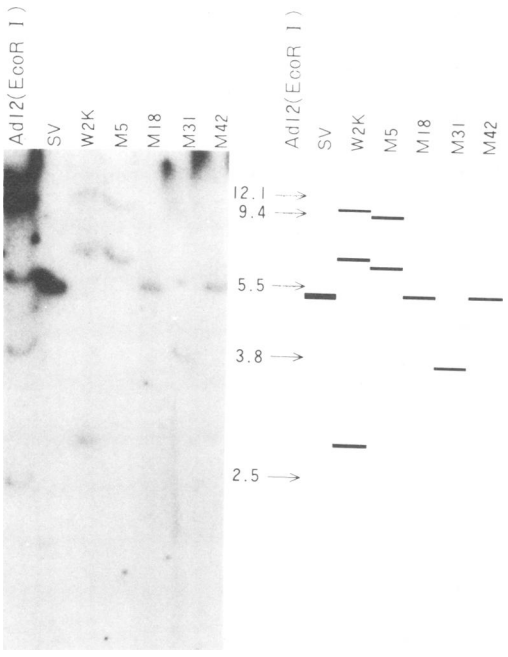

FIG. 9. *Analysis of SV40 DNA sequences in transformed and revertant cell DNAs digested with EcoRI, with HinfI fragment A as the probe. DNA was digested and electrophoresed as described in the legend to Fig. 5. Adenovirus type 12 DNA digested with EcoRI was run as a size marker.*

since spontaneous reversion in W-3Y-23 cells occurred at a fairly high frequency and only a small amount of viral DNA was retained in most of the revertants. This type of reversion, accompanying the loss of a majority of viral genomes, could not have occurred at a high frequency if the viral genomes were integrated into three or more distantly separate sites. The distribution of some radioactivity in the leading side of the band suggests the presence of a subpopulation in the transformed cells, as will be discussed below.

A possible arrangement of viral DNA sequences in W-2K-11 DNA (Fig. 10) was constructed based on the following reasons. (i) Among four *Eco*RI DNA fragments containing viral sequences, three fragments hybridized with *Hin*fI fragment A. If the viral sequence is integrated into two sites, two of the fragments containing the *Hin*fI-A sequence must be generated from both ends of the integrated viral sequence and should have the off size. However, no off-size fragments were detected after digestion of W-2K-11 DNA with *Hin*fI. These results suggest that the viral sequence was integrated into at least three sites. (ii) One of the viral sequences must contain the complete viral genome since infectious SV40 could be rescued upon fusion with monkey cells. The other two viral sequences must be the defective ones since these sequences do not contain the *Eco*RI site. (iii) Integration of the complete viral genome seems to have occurred outside the *Hin*fI-B region (0.992 to 0.199 map unit) near the one end (0.199 map unit) since intact *Hin*fI fragment B could be generated by digestion with *Hin*fI. This site is located at the 3′ end of gene *A*. To synthesize intact T antigen and to excise infectious SV40 precisely, the region containing the integration site may be partially duplicated tandemly, resulting in the presence of homologous sequences at both ends of the integrated sequence. The requirement of such a tandem duplication for precise excision has been suggested previously (3, 6, 18). (iv) Revertant M5, from which infectious SV40 could be rescued, generated an off-size fragment that migrated slightly slower than authentic *Hin*fI fragment B. This fragment may have been generated by a deletion spanning the *Hin*fI site near the integration site. The deletion did not affect the infectivity of the viral genome, presumably by the presence of tandem duplication at the other integration site.

Judging from the size of the *Xba*I fragment containing the viral sequence and the amount of the viral sequence integrated, the total length of the intervening cellular DNA sequence in W-3Y-23 cells (Fig. 10) may not exceed much more than a unit length of SV40 DNA, and in at least one integration site, the viral genomes must be arranged in a tandem array. This integration pattern of SV40 DNA is unique since SV40-transformed nonpermissive rodent cells are known not to contain a tandem repeat of more than two copies (5). A head-to-tail tandem repeat of viral DNA has been observed in polyoma virus-transformed rat cells (3, 4) and in mouse-human somatic cell hybrids made by fusion of SV40-transformed human cells with mouse peritoneal macrophages (9). These cell lines are semipermissive to viral infection.

The following mechanisms can be considered for generation of serially integrated viral genomes in the host chromosome. First, SV40 DNA might be able to replicate to a limited extent to form an oligomer in cells which have been considered nonpermissive (27). Illegitimate recombination between oligomeric viral DNA and cellular DNA at several sites within oligomeric viral DNA may result in the formation of serially integrated viral DNA sequences, with an insertion of the host DNA sequence between the viral DNAs. Oligomeric SV40 DNA of a size 2 to 10 times larger than the monomer has been

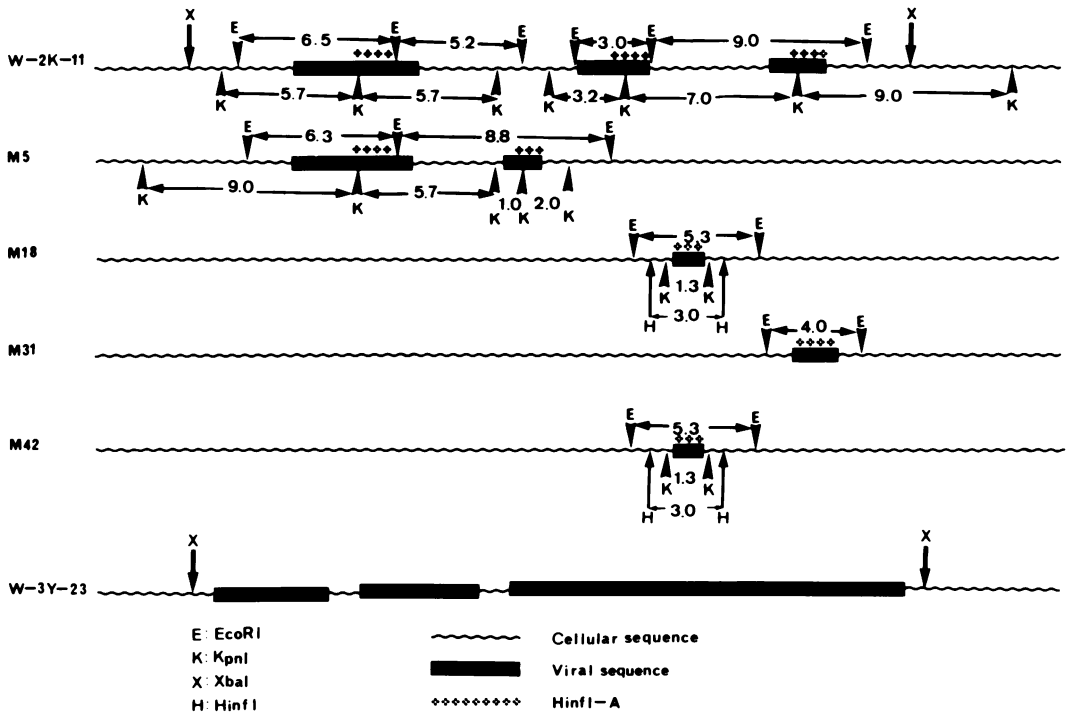

FIG. 10. *Maps of viral DNA sequences integrated into the host chromosome in SV40-transformed mouse and rat cells and in revertant cells. The maps represent a possible sequence arrangement that was constructed based on the results.*

found in monkey cells in the late phase of infection (14, 17, 21, 28). Alternatively, the serial arrangement of viral genomes might be generated by amplification after the initial integration event. Such an amplification may occur by legitimate or illegitimate unequal recombination between daughter chromosomes, as suggested by Birg et al. (4), based on the model proposed for amplification of genes in bacteria and bacteriophages (2). An exact mechanism for the generation of the complicated arrangement may be clarified by an analysis of base sequences at various virus-host junctions.

Excision of viral DNA. Excision of integrated viral DNA sequences may occur more frequently in cells containing a serial arrangement of viral genomes through homologous recombination than in the cells which contain only one genome. The frequency of occurrence of revertants in W-3Y-23 cells was estimated to be approximately 10^{-5} after negative selection with FUdR. This frequency was several times higher than that reported in SV40-transformed Fischer rat cells containing one copy of the viral genome (31), although the data cannot be compared directly since the number of passages of the cell culture may be different between these two cell lines. In four revertants from W-3Y-23 cells, the amount of viral DNA retained after the deletion was small, and a complete viral genome was not detected. The deletion may not be a result of one recombination event but may occur through successive homologous recombination events, ultimately leading the cells to a normal phenotype. The DNA band containing the viral sequence generated by digestion of W-3Y-23 DNA with *Xba*I, which does not cleave SV40 DNA, was broad toward the leading side, suggesting the presence of shorter fragments of heterogeneous sizes in the digest. These shorter fragments may have derived from the cells in which a portion of the viral genome had been deleted. These cells might retain the transformed phenotype until they lose the entire set of complete viral genomes. The presence of these precursor cells can be demonstrated by an analysis of integrated viral genomes in subclones of transformed cells. During these successive homologous recombinations, there would be a common deletion pattern since the size of the fragment containing a viral sequence generated from revertant M18 and M42 DNAs upon digestion with *Eco*RI, *Kpn*I, and *Hin*fI was quite similar although these revertants were isolated inde-

pendently and have different morphologies (Fig. 10). Revertant M5, which could produce infectious SV40 upon fusion with monkey cells, may also have suffered a deletion in the viral sequence, although the cells still retained a complete set of viral genome. The deletion pattern must be complicated since none of the fragments generated by digestion with *Eco*RI and *Kpn*I was identical to the fragments generated from W-2K-11 DNA. The deletion may have occurred by homologous recombination between two serially arranged viral sequences spanning the deletion mainly in the flanking cellular sequence. The frequency of spontaneous reversion, however, could also be determined by various cellular factors. Using the similar procedure of negative selection with FUdR, we could not isolate a spontaneous revertant from SV40-transformed mouse W-2K-11 cells, although this cell line was relatively resistant to FUdR.

Retransformation. T-antigen-negative revertants isolated from W-2K-11 and W-3Y-23 cells showed marked differences in their susceptibility to retransformation by SV40. All revertants from W-3Y-23 cells were unable to be retransformed by SV40 to a measurable extent. In contrast, all T-antigen-negative revertants from W-2K-11 cells were retransformed by SV40 at a frequency of 3 to 10 times higher than that of their grandparental C3H2K-C4 cells. This difference was not due to a difference in the efficiency of virus uptake since a fraction of the cells which became T antigen positive after infection of SV40 was not significantly different between revertants and their grandparental cells. Both types of revertant could be transformed by murine sarcoma virus. The reason as to why the response of revertant cells to superinfection of SV40 is so different between these two cell lines is presently unclear. One possible reason may be ascribed to the difference in isolation methods. Revertants from W-3Y-23 cells were isolated by negative selection with FUdR, whereas revertants from W-2K-11 cells were isolated with BUdR. The former method selects a revertant by its inability to grow at confluence, whereas the latter method selects a revertant by its inability of anchorage-independent growth. This possibility can be proven by the isolation of revertants by a reciprocal method. However, three additional revertants isolated so far from W-3Y-23 cells by negative selection with BUdR are all T antigen positive: Another possible approach for explaining this difference may be an analysis of the fate of the superinfecting viral genome. For instance, it can be asked whether the high frequency of transformation in revertants from W-2K-11 cells is caused by a preferential integration of the superinfecting viral genome into the preexisting viral DNA fragment by analysis of integrated viral genomes in retransformed cells. Although the situation is different, Botchan et al. could not find such an integration in SV40 *tsA*-transformed cells by superinfection of wild-type SV40 at the restrictive temperative (6).

T-antigen-positive revertant M5 from W-2K-11 cells could not be retransformed by SV40; none of the superinfected cells formed colonies in soft agar medium. Insusceptibility of M5 cells to retransformation is not due to an inefficient uptake of superinfecting SV40 since the radioactivity incorporated into nuclei after infection with ^{3}H-labeled SV40 was not significantly different between M5 and its grandparental C3H2K-C4 cells. Since revertant M5 was isolated after mutagenesis of W-2K-11 cells with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, the reversion may be caused by a mutation in a cellular gene(s). Karyotype analysis of M5 cells, however, revealed an increase in the chromosome number compared with parental transformed W-2K-11 cells, as has been observed in most of the T-antigen-positive, untransformable revertants (36, 37). Whether the inability of M5 cells to express a completely transformed phenotype is due to some defect in a cellular function is an important problem for the understanding of a cellular site(s) with which T antigen would interact.

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