Analysis of Simian Virus 40-induced Transformation of Hamster Kidney Tissue In Vitro

V. Variability of Virus Recovery from Cell Clones Inducible with Mitomycin C and Cell Fusion

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Clones of simian virus 40-transformed hamster kidney cells from which infectious virus could be recovered and clones which did not yield virus by the overlay technique were subjected to chemical (mitomycin C) induction and to co-cultivation and fusion studies with indicator cells. The results of these studies indicate that the complete viral genome is present in all clones tested but that considerable heterogeneity with respect to inducibility exists among the clones. It is suggested that differences either in the number of viral genomes per transformed cell or in the status of the viral genome in transformed cells exist among the various clones. Furthermore, the inducibility of the clones may be correlated with their malignant potential.

The question of the persistence of the complete viral genome without production of infectious virus in cells transformed by the oncogenic deoxyribonucleic acid (DNA) virus, simian virus 40 (SV40), has been extensively examined in the recent past. In previous studies, 11 of 12 clones derived from a line of SV40-transformed hamster kidney cells by establishing single cells in micro-drop cultures were found to yield infectious virus when the viable cells were overlaid upon indicator cells (1). Since a carrier state was essentially ruled out by the cloning procedure, these studies indicated that the complete SV40 genome was present in the vast majority of these clones. However, a clone derived from an inducible clone, and 10 clones subsequently derived from it, did not yield virus when tested by this technique. It was not clear from these findings whether a noninducible clone had been isolated from an inducible one because of partial or complete loss of the viral genome or because of a change in the relationship of the viral and host-cell genomes.

The present studies were undertaken in an attempt to compare the inducibility of both overlay-positive and overlay-negative clones by utilizing additional techniques. These included chemical induction (3), co-cultivation of tumor and indicator cells (a modification of the overlay technique), and heterokaryon formation from tumor and indicator cells by using Sendai fusion factor (FF; 4, 7, 15). The results indicate that infectious SV40 can be recovered from all clones,

but that heterogeneity with respect to inducibility exists among the various clones.

MATERIALS AND METHODS

Cell cultures. The origin and history of both the transformed hamster kidney (THK-1) cell line and the clones derived from it have been described (1). The clones utilized in the present studies were as follows: T-6 and T-8 were clones established from THK-1 at the 75th passage level and yielded virus only when overlaid on indicator cells. T-1-1 represents a second cloning from the parent cell line, and T-1-1-5 and T-1-1-10 were subsequently cloned from T-1-1 eight passages later. The latter three clones did not yield virus by the overlay procedure employed at that time. All clones were grown in Eagle's minimal essential medium (MEM) with four times the usual concentration of amino acids and vitamins, 10% fetal calf serum, and antibiotics. The SV40 susceptible cells utilized were from the MA 134 cell line derived from African green monkey kidneys (obtained from Microbiological Associates, Inc., Bethesda, Md.); they were maintained in MEM with 10% fetal calf serum.

Mitomycin C treatment. Confluent roller tube cultures of transformed clones maintained in MEM with four times the usual concentration of amino acids and vitamins, and 5% inactivated (56 C, 30 min) fetal calf serum were exposed to various concentrations of mitomycin C for 1, 8, or 20 hr, whereupon the mitomycin C was removed, the monolayers were washed, and fresh medium was added. The cultures were maintained in the dark in a humidified atmosphere containing 5% CO₂ in air. At various time intervals after exposure to mitomycin C, two or three replicate cultures were frozen and thawed three times and treated in a Bran-

son Sonifier (3 amp, setting at 5) for 8 sec. No cells were observed upon microscopic examination of these extracts, and attempts to grow cells from these preparations were unsuccessful.

Cell fusion. Cell fusion studies were carried out with Sendai virus obtained from J. F. Enders. The virus was inoculated into the allantoic cavities of 10-day-old chick embryos. The allantoic fluid was harvested 3 days later, concentrated, and inactivated with 0.0625% β -propiolactone, as described by Neff and Enders (10). The preparation utilized in these studies had a hemagglutination titer of 24,000 units per ml. For cell fusion, equal volumes of 5×10^6 to 10×10^6 THK cells per ml and 10×10^6 to 15×10^6 MA 134 cells per ml in Hank's solution without glucose were mixed with an equivalent volume of Sendai FF containing 12,000 hemagglutination (HA) units per ml, as described by Okada (11) and Harris and Watkins (5). The mixture was diluted in MEM with 4 $\times$ amino acids and vitamins and 10% inactivated agammaglobulinic calf serum (IACS); then 10^6 cells were seeded in roller tubes and plastic petri dishes containing cover slips. The degree of cell fusion was determined from the fixed cover-slip preparations, stained with hematoxylin and eosin. With the conditions described, over 60% of the nuclei were contained within multinucleated giant cells at 2 to 4 days after fusion. At 2, 3, 5, and 8 days after seeding, two to three replicate cultures were frozen, and cell extracts were obtained as described above.

Co-cultivation studies. Mixtures of THK cells and MA 134 cells for co-cultivation studies were prepared as for cell fusion, with the exception that Hank's solution was used in place of Sendai FF.

Assay and identification of SV40. The cell-free extracts were tested for virus content by inoculating 0.3 ml of extract into three drained, confluent, roller-tube cultures of MA 134 cells. After adsorption for 90 min, MEM with 2% IACS was added. Subsequent changes of medium, which contained 7% IACS, were carried out every 3 to 4 days; all cultures were observed for a period of 4 weeks. The end-point dilution method was used to titrate the quantity of infectious SV40 in the extracts. Reconstruction experiments demonstrated that 1 to 5 tissue culture infectious doses 50% (TCID₅₀) of SV40 could be recovered when they were mixed with 10^6 cells and extracts were obtained as described.

All isolates produced a characteristic SV40 cytopathogenic effect on MA 134 cells. Confirmatory evidence for the identity of the isolates as SV40 was obtained from positive complement-fixation (CF) studies with hyperimmune SV40 monkey antiserum. The isolates were negative in CF tests when tested against polyoma viral and a pool of human adenovirus convalescent serum.

RESULTS

Mitomycin C induction of clones. Preliminary experiments revealed that similar quantities of virus were obtained from induced cultures 3 days after the start of treatment with mitomycin C and for at least the subsequent 3 days. Therefore, cultures were harvested 3 or 4 days after the beginning of mitomycin C treatment. Table 1

TABLE 1. *Mitomycin C induction of transformed hamster kidney cells^a*

Concn of mitomycin C	Time of exposure	Clone					WHK ^b
		T-6	T-8	T-1-1	T-1-1-5	T-1-1-10	
$\mu\text{g/ml}$	hr						
0		—	—	—	—	—	—
0.1	8	—	—	—	—	—	
	20	—	—	—	—	—	
0.3	8	—	—	—	—	—	
	20	+	+	—	—	—	—
0.5	8	+	—	—	—	—	—
	20	+	—	—	—	—	—
1.0	8	+	+	—	—	—	—
	20	+	—	—	—	—	—
2.0	8	+	—	—	—	—	—
	20	—	—	—	—	—	—
10.0	1	+	—	—	—	—	—
	8	—	—	—	—	—	—
	20	—	—	—	—	—	—
50.0	8	—	—	—	—	—	—
	20	—	—	—	—	—	—

^a Symbols: +, infectious SV40 recovered (30 to 300 TCID₅₀/ml); —, no infectious SV40 recovered.

^b Primary weanling hamster kidney cultures.

shows the results of mitomycin C treatment of 5 THK clonal lines.

Virus was recovered from two of the five clones tested when 0.3 to 10.0 μg of mitomycin C per ml was used and exposure lasted for 1, 8, or 20 hr. It is noteworthy that the two inducible clones were those from which virus had been recovered by the overlay technique, whereas overlay-negative clones have consistently failed to yield virus after mitomycin C treatment. The amount of infectious virus present in the induced cell extracts varied from 30 to 300 TCID₅₀ per ml. In general, there was no correlation between the amount of virus present in induced cell extracts and the concentration of mitomycin C utilized. Primary weanling hamster kidney cells yielded no virus after mitomycin C treatment.

Evidence has been presented that a metabolite of mitomycin C is the active agent within mitomycin-treated cells which is responsible for the linking of complementary DNA strands (9, 13), and the possibility remains that cellular factors affecting the permeability to or metabolism of mitomycin C might account for differences in inducibility. The activity of mitomycin C responsible for induction is not known and therefore cannot be assayed at present. However, as judged by cellular toxicity, all clones were similarly affected by mitomycin C.

Recovery of SV40 from all clones by fusion or co-cultivation with MA 134. The results of the

experiments utilizing Sendai FF appear in Table 2. Virus was present in all clones 3 days after fusion, including clones which were negative when tested by the overlay technique and mitomycin C treatment. In all the clones, virus appeared earlier in cultures with Sendai FF than in cultures without FF. When the cells of each clone and of MA 134 were fused by themselves, no virus was recovered. Similar quantities of virus were recovered from cultures of overlay-positive and overlay-negative clones after fusion with MA 134 cells.

During these fusion experiments, infectious virus was obtained from unfused clones which were co-cultivated with indicator cells and from which no virus had been recovered when tested by the overlay technique (1; see Table 2). This was probably due to the use of a higher proportion of indicator cells and the fact that tumor and indicator cells were mixed prior to plating, allowing for more intimate contact between the two cell types and the increased probability of heterokaryon formation. Recent evidence indicates that a greater percentage of heterokaryons produce infectious virus when the proportion of indicator to tumor cells is high (15).

DISCUSSION

The results of the present studies, summarized in Table 3, indicate that infectious virus can be obtained from all the clonal lines tested, even those previously regarded as negative when tested by the overlay technique. These results also suggest that probably all of the clones derived from the THK-1 cell line contain the complete SV40 genome. The experiments with mitomycin C confirm and extend those described by Gerber (3), since the use of clones in the present studies eliminates the possibility of a

TABLE 3. *Summary of induction of infectious SV40 virus from transformed hamster kidney cells by various methods*

Clone	Passage no. ^a	Virus recovery by			
		Overlay	Mito-mycin C	Co-cultivation	Sendai FF
T-6	6-10	+	+	+	+
T-8	6, 7	+	+	+	+
T-1-1	9-14	-	-	+	+
T-1-1-5	5, 6	-	-	+	+
T-1-1-10	4, 9	-	-	+	+

^a For passage history prior to cloning see reference 1.

carrier state (1). In several other reports, attempts to induce virus from SV40-transformed hamster cell lines with mitomycin C failed (8, 14).

The use of Sendai FF proved to be the most sensitive technique for the recovery of SV40 virus. It has previously been demonstrated that cell contact is essential in the induction of virus from tumor cells by indicator cells (4), and Sendai FF brings about the most intimate contact between cells by first forming intercellular bridges and later heterokaryons (5, 11). These processes also occur to a limited degree when cells are co-cultivated and to a lesser extent in the overlay cultures; the extent to which intercellular bridges and heterokaryon formation occur probably accounts for the variable success of these techniques in isolating virus from SV40-transformed cells.

The factors responsible for the induction of virus from tumor cells by indicator cells are not known at present. The indicator cell may derepress the SV40 genome or supply some enzyme(s) needed for viral maturation. At present it is difficult to conclude from cell fusion studies which of these factors is operative in allowing full expression of the viral genome. The results from mitomycin C induction indicate, however, that the viral genome in the transformed cell may direct the synthesis of infectious virus without introduction of a factor(s) from the indicator cells.

The studies presented here indicate considerable heterogeneity among the clones with respect to the inducibility of the viral genome. This may be due to varying numbers of viral genomes per transformed cell in the several clones. Nucleic acid hybridization techniques, however, have demonstrated no significant difference in the number of viral genomes per transformed cell between clones which produced virus and clones which did not produce virus

TABLE 2. *Virus recovery from transformed hamster kidney cells fused and co-cultivated with MA 134 cells^a*

Clone	With fusion factor				Without fusion factor			
	2 days ^b	3 days	5 days	8 days	2 days	3 days	5 days	8 days
T-6.....	-	+	+	+	-	-	+	+
T-8.....	+	+	+	+	-	-	+	+
T-1-1.....	-	+	+	+	-	-	+	+
T-1-1-5.....	-	+	+	+	-	-	-	+
T-1-1-10.....	+	+	+	+	-	-	+	+

^a Symbols: +, infectious SV40 recovered (10^3 to 10^5 TCID₅₀ per ml); -, no infectious SV40 recovered.

^b Day culture was harvested.

when overlaid on indicator cells; however, variations within a clone cannot be ruled out with this technique (12). Another possible explanation for this heterogeneity may be differences in the status of the viral genome in the transformed clones. This may be related to the type of association the viral genome establishes with the host DNA (i.e., integrated, extrachromosomal, or episomal), to possible interactions between the viral genome and nucleoproteins, or to the site(s) of insertion of the viral DNA if integrated.

In previous studies, considerable heterogeneity in the transplantability to hamsters of the various clones was found (2). It is of interest that the transplantability of the clones correlates with their inducibility, as determined in the present study, in that the more inducible clones were more readily transplanted to hamsters. It has recently been reported that the malignant potential of clones of SV40-transformed hamster cells increases with *in vivo* passage (J. F. Enders and G. Diamondopoulos, *in press*). In accord with this observation, clones used in the present study show a marked increase in malignant potential after one *in vivo* passage; the latent period prior to tumor development of clones tested decreased from 8 weeks to 2 weeks, and fewer cells were required for tumor formation (*unpublished data*). Preliminary studies indicate an increased inducibility after one *in vivo* passage of at least one clone (T-8); approximately 10- to 100-fold more virus was recovered with mitomycin C induction after one *in vivo* passage. Whether a correlation between inducibility and malignant potential of SV40-transformed cells will prove to be a general finding remains to be seen. Also, whether the inducibility of the SV40 viral genome is causally related to the malignant potential of the transformed cell is unknown at present.

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