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Activation of the Viral Genome in Simian Virus 40-Transformed Nonpermissive Cells by Permissive Cell Extracts

(capsid antigens/protein activator/rescue)

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ABSTRACT Simian virus 40-transformed nonpermissive cells in which neither infectious viral DNA nor virions had previously been detected reproducibly yield low levels of infectious simian virus-40 DNA after treatment with extracts of normal permissive cells. Virions were obtained in one out of 37 experiments. Activation of the viral genome is detected only with normal permissive cell extracts and only in simian virus 40-transformed cell lines that are rescuable by heterokaryon formation or by treatment with chemicals. The activating factor(s) is insensitive to deoxyribonuclease and ribonuclease, but is sensitive to heat at 56° and to proteolytic enzymes.

The interaction of animal cells with Simian Virus 40 (SV40) has been extensively studied (1, 3, 8, 11, 16, 17, 22, 23, 25). Infection by SV40 can be either productive (leading to virus replication and death of the host cell) or abortive (resulting in blockage of virus replication at an intermediate stage and survival of the host cell). Cells in which infection is usually productive (e.g., monkey and human) are called permissive, while those in which infection is usually abortive (e.g., hamster, mouse, and rat) are termed nonpermissive. Cells may be either highly permissive (monkey) or semipermissive (human). The basis of cell permissiveness is unknown.

Under suitable conditions, nonpermissive (and, more rarely, permissive) cells infected with SV40 may be transformed. Nonpermissive, transformed cell lines are fully resistant to superinfection by SV40 virus and DNA, while permissive transformed lines are variably susceptible. With the exception of some transformed permissive cell lines, cells transformed by SV40 do not produce infectious virus or viral DNA but contain functioning viral genes which, at least in the case of nonpermissive cells, are integrated in the chromosomal DNA.

That some transformed cell lines that do not produce virus contain a complete viral genome is demonstrated by the fact that they can synthesize infectious virus upon fusion with permissive cells (heterokaryon formation) and, more rarely, upon treatment with chemicals. This phenomenon has been given several names: induction, activation, reactivation, rescue, or detection of transforming virus. Its mechanism remains to be determined.

So far, attempts to activate the synthesis of infectious virus or viral DNA in SV40-transformed cells that do not produce virus by treatment with extracts of permissive cells have failed (9, 20). In the present work we show that it is possible to activate the viral genome upon treatment with extracts in some of these cell lines. Furthermore, the factor(s) responsible for activation, to which we give the general name "activator," is detectable only in normal permissive cells and is at least in part a protein.

MATERIALS AND METHODS

Cell cultures

Normal Cells. BSC-1 (13), Vero (27), and CV-1 (15) are established lines of African green monkey kidney cells. MA-104 is an established line of Rhesus monkey kidney cells, developed by M. M. Vincent, Microbiological Associates, Inc., Bethesda, Md. KB (6) is an established cell line derived from a human epidermoid oral carcinoma. HE are Syrian hamster embryo cells in primary culture. EHB (2) is an established line of Syrian hamster cells. ME are mouse embryo (C3H) cells in primary culture. L (7) is an established line of mouse cells.

SV40-Transformed Cells. BSC-SV clone (Cl) 1.1 (23) is an established line of SV40-transformed cells of African green monkey kidney (BSC-1). W 98 VaH, W 98 VaD (10) are established lines of SV40-transformed human skin cells. TSV-5 Cl2, TSV-11, EHSVi (24) are established lines of SV40-transformed Syrian hamster cells. mKS-BU 100 Cl 1.1 (5) is an established line of SV40-transformed mouse (Balb/C) cells. Upon fusion with SV40 permissive cells or treatment with chemicals, infectious SV40 virus can be rescued from TSV-5 Cl2, EHSVi (ref. 24, and unpublished data), or mKS-BU 100 Cl 1.1 (5), but not from BSC-SV Cl 1.1 (23), W 98 VaH, W 98 VaD (19) or TSV-11 (24). Normal and SV40-transformed cells were cultivated in modified Eagle's medium supplemented with 10% calf serum (24). All cell lines were free from pleuropneumonia-like organisms (PPLO).

Preparation of cell extracts

The cells were suspended in cold Tris·HCl buffer (0.01 M, pH 8.1) at a density of 5×10^7 cells per ml, and sonicated for 3–6 min at low frequency in the cold (Siduse sonic oscillator, model US 77-5, Paris). Complete cell disintegration was monitored by microscopic examination. The sonicated material was centrifuged for 1 hr at 37,000 rpm and 4° (Beckman SW-65 rotor), and the clear supernatant (crude extract with an average protein content of 8 mg/ml), which in some experiments was filtered through 0.22-μm Millipore membranes, was used immediately after preparation. The absence of SV40 virions in crude extracts was confirmed by plaque

formation (24) on monolayer cultures of CV-1 indicator cells (0.2 ml of sample per dish; 5–10 dishes per sample).

Preparation of poly(L-ornithine) solution

Each 1 mg of poly(L-ornithine) (mol. wt. 90,000, Sigma, St. Louis, Mo.) stock solution was stored at −20° in 1 ml of medium without serum.

Assay for activation

A solution of cold poly(L-ornithine), a basic polymer that increases the cellular uptake of proteins (21), was added to the crude cell extract to a final concentration of 10 μg of polymer per ml. Extract dilutions were made in poly(L-ornithine) solution (10 μg/ml of serum-free medium) and were kept in ice.

Cells were washed with serum-free medium, pelleted in siliconized 50-ml centrifuge tubes, and resuspended in dilutions of the extract at a concentration of 1×10^6 cells per ml. Control cultures were treated with 10 μg/ml of poly(L-ornithine) solution. After a 30-min incubation at 37° with shaking (100 strokes per min), the cells were centrifuged and resuspended in complete growth medium at a concentration of 1.4×10^5 cells per ml. For each sample, 5-ml aliquots were seeded in two plastic dishes (6 cm in diameter) and in a plastic flask (25 cm^2). Occasionally, Leighton tubes containing glass cover slips (12 × 32 mm) were seeded with 5×10^4 cells per tube (three tubes per sample). Cell cultures were incubated at 37° for 72 hr (except where noted); dishes were kept in air–5% CO_2. The search for infectious SV40 DNA, SV40 capsid (V) antigens, and SV40 virions was performed as follows:

(a) *Infectious SV40 DNA.* DNA, extracted from cultures by the selective procedure devised by Hirt (12), was sterilized with phenol (0.05 volume of 90% redistilled phenol) and dialyzed extensively against cold SSC (0.15 M NaCl–0.015 M sodium citrate, pH 7.2). DNA infectivity was determined, as described by Kit *et al.* (18), by plaque titration on monolayer cultures of CV-1 cells (0.2 ml of sample per dish; 10 dishes per sample). Confirmation that plaques obtained were caused by SV40 DNA was obtained by the immunofluorescence technique described below.

(b) *SV40 Capsid (V) Antigens.* Cell cultures on glass cover slips were withdrawn, washed three times with phosphate-buffered saline (pH 7.2), fixed with acetone for 8 min, and air-dried. The presence of SV40-specific capsid (V) antigens was determined by staining of the cells with fluorescein–isothiocyanate-conjugated antibodies. Serum for staining of V antigens was obtained from rabbits immunized against SV40 virus (26).

(c) *SV40 Virions.* Cell cultures in plastic flasks were frozen and thawed twice (−70° to 37°). The cell homogenates were sonicated for 5 min at low frequency in the cold. Presence of SV40 virions was assayed by plaque titration on CV-1 monolayer cultures (0.4 ml of sample per dish; 10 dishes per sample). Confirmation that plaques obtained were caused by SV40 virus was by the immunofluorescence technique described above.

For each cell line tested for activating ability, at least two separate extract preparations were assayed.

Sensitivity of the activator(s) to various treatments

Heat Treatment. Comparable aliquots of extract were incubated in a water bath at 56° or kept in ice (control) for 30 min. Thereafter, poly(L-ornithine) was added, dilutions were made, and activating ability was assayed as described above.

Enzyme Treatment. Enzyme stock solutions were prepared at a concentration of 1 mg per ml as follows:

(a) Trypsin (Nutritional Biochemicals Co., Cleveland, Ohio) in phosphate-buffered saline. For inactivation of trypsin, Iniprol (Choay, Paris) was used at a final concentration of 167,000 peptidase inhibitory units per ml of extract.
(b) Pronase (Calbiochem, Los Angeles, Calif.) in SSC was heated for 10 min at 80°.
(c) Deoxyribonuclease I (Worthington Biochemical Co., Freehold, N.J.) in 0.05 M HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid, Calbiochem)–15 mM $MgSO_4$ (pH 6.9).
(d) Ribonuclease A (Calbiochem) in SSC, heated for 10 min at 80°.

Crude extracts were incubated for 30 min at 37° either with enzyme (200 μg/ml) or with the respective buffer (control); activating capability was then assayed as described above.

RESULTS

The data summarized in Table 1 show that, after treatment with an extract of BSC-1 cells, infectious SV40 DNA is extractable from SV40-transformed hamster cells (TSV-5 Cl2). The effect is reproducible, but does not seem to depend on the

TABLE 1. *Activation of infectious viral DNA in SV40-transformed hamster cells (TSV-5 Cl2) by normal monkey-cell extracts (BSC-1)*

Exp. no.	SV40 DNA, PFU/10^6 TSV-5 Cl2 cells — BSC-1 extract concentration (cell equivalent per treated cell): 0	0.02	0.05	0.1	0.2	0.4	0.8	1.6	3.1	6.2	12.5
1	0	n.d.*	1	0	1	1	0	3	0	1	0
2	0	1	0	0	0	1	0	0	0	6	9
3	0	3	0	0	1	1	0	7	0	0	6
4	0	3	11	11	5	0	2	3	0	1	3
5	0	3	1	3	3	3	0	0	3	4	13
6	0	0	7	0	0	0	0	0	0	3	0
7	0	9	0	7	0	4	0	0	0	0	0

* Not done.
PFU, plaque-forming unit.

TABLE 2. *Effect of various normal cell extracts on activation of infectious viral DNA in SV40-transformed hamster cells*

Cell extracts	Treated cells	Activation
Monkey (BSC-1, CV-1, Vero, MA-104)	TSV-5 Cl2, EHSVi	+*
Human (KB)	TSV-5 Cl2	+*
Hamster (HE, EHB)	TSV-5 Cl2	−
Mouse (ME, L)	TSV-5 Cl2, EHSVi	−

* Yields of infectious SV40 DNA were comparable to those quoted in Table 1.

concentration of the extract. Untreated TSV-5 Cl2 cells do not shed SV40 virions spontaneously (24), nor can infectious viral DNA be extracted from them (4, 18). No infectious SV40 DNA was ever found in cells treated with poly(L-ornithine) alone, with dilutions of Tris·HCl buffer in poly(L-ornithine), or with deoxyribonuclease in poly(L-ornithine), nor in the controls with any other extract. Thus, the viral DNA detected in TSV-5 Cl2 cells that were treated with extracts of BSC-1 cells is related to a factor(s) present in BSC-1 cells.

The ability of various normal-cell extracts to activate infectious SV40 DNA in TSV-5 Cl2 and EHSVi cells was tested (Table 2). Activation occurred with all normal permissive (monkey and human) cell extracts, and in all cases yields were comparable to those quoted in Table 1. On the other hand, infectious viral DNA was never detected in transformed cells treated with extracts of any normal nonpermissive cells (hamster and mouse).

The capacity of BSC-1 cell extracts to activate the viral genome in various SV40-transformed cell lines known to be rescuable or nonrescuable by either fusion with permissive cells or by treatment with chemicals (see *Methods*) was investigated (Table 3). Upon treatment with extract, infectious SV40 DNA is detectable only in SV40-transformed cell lines known to be rescuable.

Unlike transformed nonpermissive cells, transformed permissive cells are able, upon fusion, to activate the SV40 genome in transformed nonpermissive cells (14, 19). Neither class of transformed cell extracts is able to activate, in detectable amounts, the SV40 genome in TSV-5 Cl2 or EHSVi cells.

In a time-course study, where TSV-5 Cl2 cells were treated with BSC-1 cell extracts, it was found that viral DNA—not detectable immediately after treatment—could be extracted after 6–120 hr of incubation of the cultures. No clear relation was found between the duration of incubation and the extractability of infectious viral DNA. The number of plaques obtained was of the same order of magnitude as that described in Table 1. The highest yield was observed 72 hr after incubation.

The presence of SV40 virions and infectious viral DNA was monitored for each cell extract [10 extract concentrations, varied between 0.02 and 12.5 cell equivalents (one "cell equivalent" corresponds to the quantity of extract given by 1 cell) per treated cell were used]. In 37 experiments with permissive cell extracts, in all of which viral DNA was detected,

TABLE 3. *Effect of BSC-1 cell extracts on activation of infectious viral DNA in various SV40-transformed cell lines*

Treated cells		Activation
Hamster	TSV-5 Cl2, EHSVi	+*
	TSV-11	−
Mouse	mKS-BU 100 Cl 1.1	+*
Monkey	BSC-SV Cl 1.1	−
Human	W 98 VaD	−

* Yields of infectious SV40 DNA were comparable to those quoted in Table 1.

virions were found only once. The titer of positive samples ranged from 1 to 13 plaque-forming units (PFU) per 10^6 cells. In two other experiments, fluorescence studies to detect SV40 capsid (V) antigens were negative.

The chemical nature of the activator(s) was investigated by treating crude BSC-1 cell extracts with either heat or various enzymes (Table 4). The activator(s) is insensitive to DNase and RNase, but sensitive to heat and proteolytic enzymes.

DISCUSSION

Some SV40-transformed cell lines that do not produce virus yield virions upon fusion with permissive cells (3, 8, 11, 16). Under these conditions, infectious virus is first detected in the nucleus of the transformed cell (25). It was therefore of interest to see if extracts of permissive cells were also able to activate the viral genome, in order to use this assay to obtain more information about the factor(s) involved in SV40 rescue and about the basis of cell permissiveness to viral infection.

As shown in the present work, upon treatment with permissive cell extracts, SV40-transformed cells, in which neither infectious viral DNA nor virions can normally be detected, reproducibly yield infectious SV40 DNA. In previous work from this (unpublished data) and other laboratories (9, 20), activation of the viral genome was measured either by virion production or incorporation of [^{3}H]thymidine into viral DNA. In view of the low yield of SV40 DNA and of the

TABLE 4. *Sensitivity of the activator(s) present in BSC-1 cell extracts to various treatments*

Treatment	Activation of infectious SV40 DNA in TSV-5 Cl2 cells
−Trypsin	+*
+Trypsin	−
−Pronase	+*
+Pronase	−
−DNase	+*
+DNase	+*
−RNase	+*
+RNase	+*
Unheated	+*
Heated (56° for 30 min)	−

* Yields of infectious SV40 DNA were comparable to those quoted in Table 1.

rarity of SV40 virions observed in the present work, previous negative results are understandable. We have also found that, in the absence of poly(L-ornithine), extracts rarely activated detectable quantities of SV40 DNA.

Activation was successful only with extracts of permissive cells and only in those cell lines in which the viral genome can be rescued by other techniques (heterokaryon formation or induction by chemicals). To this extent, this new assay appears analogous to the fusion system, with the exception that extracts of permissive transformed cells do not activate detectable amounts of SV40 DNA. Another important difference between these two assays is that treatment with extracts of normal permissive cells did not reproducibly yield measurable quantities of infectious virus. The significance of these differences, however, awaits a better understanding of the process by which the activating factor(s) rescues the viral genome.

The percentage of cells activated by the extracts also remains to be determined. If virions become regularly detectable, this question will be studied by the infectious center technique (23).

It is important to know if the rescued SV40 DNA is the product of excision and circularization, or whether it reflects vegetative replication of the excised viral genome in the cells treated with extract. Since in one experiment SV40 virions were detected, it seems probable that vegetative replication can occur.

We have begun purification and have found that the activator(s) is nondialyzable, stable for several days at 4°, and precipitable with 40% ammonium sulfate. In a preliminary experiment, column chromatography on diethylaminoethyl (DEAE)-cellulose yielded several-fold purification of the activating factor(s). With this more purified material, it has already been possible to increase the rescue of SV40 DNA by tenfold.

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