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Enhancement of Simian Virus 40 Infection in Simian and Human Cells by Permissive Cell Extracts (38238)

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Evidence has accumulated indicating that deoxyribonucleic acid (DNA) of simian virus 40 (SV40) is integrated into the host DNA of cultured cells that are infected and transformed by SV40 (1). Usually, SV40 virions are not recovered from transformed cells. In some cases, SV40 production occurs after cocultivation and fusion of SV40-transformed cells with uninfected whole (2–4) or enucleated (5) cells that are permissive for SV40 replication. Also, it has been reported that infectious SV40-DNA can be recovered from transformed cells after addition of extracts of permissive simian host cells (6) or by transfer of DNA, isolated from transformed cells, to permissive simian cell cultures (7). These results suggest that permissive cells or extracts of these cells provide factors required for release and replication of the viral genome.

The present report describes a transferable, subcellular component of uninfected simian cells that enhances SV40 titers in less susceptible simian and human cell cultures and increases frequency of SV40-induced transformation in a line of human diploid fibroblasts. This enhancer was detected by procedures used previously for isolation and characterization of another enhancer for an enterovirus (8–10). Some of the properties of the SV40 and enterovirus enhancers are compared.

Materials and Methods. Cell cultures. Permanent simian (LLC-MK_2, CV-1, Vero) and human (WISH) cell lines were obtained from the American Type Culture Collection and Child Research Center of Michigan. Starter cultures of Vero cells were also purchased from Grand Island Biological Company. A line of human diploid fibroblasts that was derived from a skin biopsy from a normal individual (Detroit 549) was kindly provided by C. S. Stulberg, Child Research Center of Michigan.

CV-1 (CCL-70) and Vero (CCL-81) lines of African green monkey kidney cells were grown in Eagle's minimum essential medium (MEM) containing nonessential amino acids (0.5%) and 10% fetal bovine serum. LLC-MK_2 (CCL-7) cells were propagated in Hanks salts supplemented with 0.45% lactalbumin hydrolysate (HLH) and 2% calf serum, and WISH (CCL-25) cells were grown in HLH containing 15% calf serum. Detroit 549 cells were used at the seventh serial transfer in MEM supplemented with nonessential amino acids (0.5%), sodium pyruvate (1.0 mmol/liter), lactalbumin hydrolysate (0.1%) and fetal bovine serum (10%). All cell cultures were maintained with MEM containing 5% fetal bovine serum and were free of mycoplasma.

Cell homogenates and cellular extracts. Cell monolayers were washed (3 times) with Dulbecco's balanced salt solution buffered with .001 *M* phosphate at pH 7.2 (BSS). Homogenates of cells were prepared from cell suspensions (10^7 cells/ml) in BSS as described previously (10). Cell-free extracts were obtained by incubation of washed cell monolayers with BSS (2.5 ml/75 cm^2 Falcon flask) for 5 hr at 37°. The extracellular fluids were decanted and clari-

fied by centrifugation (20,000*g*, 1 hr, 5°) in order to remove host receptors and other viral inhibitory substances (9).

Virus preparations and assay. Plaque-purified preparations of the small plaque mutant of SV40 (12) and the *m*+ variant of echovirus 6 (13) were passaged serially at low input multiplicities of infection (.01–0.1 PFU/cell) and harvested from CV-1 and WISH cells, respectively. Virus assays were performed by determination of tissue culture infectious doses ($TCID_{50}$) and plaque forming units (PFU) after inoculation of serial 10-fold dilutions on each of 3 or 4 monolayers. For some experiments, virus preparations were purified by equilibrium centrifugation in CsCl density gradients as described previously (10).

Transformation assays. Transformation studies were performed with Detroit 549 cells as described by Aaronson and Todaro (14). Cells in logarithmic growth phase were inoculated with SV40 at an average input multiplicity of 130 PFU/cell. After incubation for 3 hr at 37°, cells were washed (3 times) and reincubated for 18–24 hr in MEM containing 5% fetal bovine serum and a concentration of rabbit anti-SV40 serum (0.5%) that neutralized 2×10^5 PFU of SV40 in 1 hr. Cells were rewashed, harvested by trypsin treatment and subcultured at a density of 10^5 cells/petri dish. Media were supplemented with SV40 antiserum and changed at 4–5 day intervals. Cell cultures (10 per sample) were fixed with Bouin's reagent and stained with Harris hematoxylin after an incubation period of 20–26 days and examined microscopically for transformed foci and chromosomal aberrations.

Enhancer assays. Procedures used previously for assay of enhancer activity for echovirus 6 were employed (8–11). Homogenates and extracts of permissive host cells (1.8 ml) were incubated with 0.2 ml of appropriate dilutions of virus for 20 min at 23° before the mixtures were inoculated on cell monolayers. A unit of enhancer activity was expressed as the amount of cellular preparation that increased the virus titers by two-fold in semipermissive host cells.

Protein assays. Protein concentrations were determined with the Folin-phenol reagent by the method of Lowry *et al.* (15). Crystalline bovine serum albumin was used as a standard.

Results. Differential susceptibility of cultured simian cells to SV40 infection. Lines of African green monkey kidney cells differed in their sensitivity to SV40 infection. Plaque and $TCID_{50}$ titers of SV40 were 45–100-fold higher in CV-1 cells than in Vero cells. The same virus preparation titered 3.7×10^8 and 4.4×10^8 PFU/ml and $10^{8.5}$ $TCID_{50}$/ml in 2 different lots of CV-1 cells, but only 2.8×10^6, 5.7×10^6 and 7.9×10^6 PFU/ml and $10^{6.0}$–$10^{6.5}$ $TCID_{50}$/ml in 3 different stock cultures of Vero cells. The virus titers were reproducible after serial transfers (12–16) of each of the stock cultures of CV-1 and Vero cell lines.

Enhancement of the susceptibility of semipermissive cell cultures to SV40. The possibility that uninfected CV-1 cells may contain a substance that increases the plating efficiency of SV40 in less susceptible Vero cells was examined. Extracts of CV-1 cells were incubated with appropriate dilutions of SV40 for 20 min at 23° before assay on monolayers of Vero and CV-1 cells. Vero cell extracts were included for comparison. Virus preparations that were diluted and incubated in BSS served as controls. As shown in Table I, extracts of CV-1 cells increased the SV40 titer in Vero cells by 19-fold (from 5.7×10^6 to 10.7×10^7 PFU/ml), but did not alter the virus titer in CV-1 cells (3.8×10^8 PFU/ml). In contrast, extracts of Vero cells did not

TABLE I. Enhanced Plating Efficiency of SV40 in Semipermissive Simian Cells by Extracts of Permissive Simian Cells.

Virus treatment[a]	Virus titer[b]		Plating efficiency
	Vero	CV-1	CV-1/Vero
None	5.7	387	68
CV-1 extract	107.0	377	3
Vero extract	5.4	365	67

[a] Virus (0.2 ml) was incubated with 1.8 ml of BSS or cellular extracts for 20 min at 23° before assay in Vero and CV-1 cells.

[b] PFU/ml $\times 10^{-6}$.

TABLE II. Effect of Enhancer on Production of SV40 by Human Fibroblast Cells (Detroit 549).

Incubation time[a] (Days)	Virus titer[b]	
	SV40	SV40 + Enhancer[c]
16	4.3	5.3
21	4.8	6.0
26	5.3	6.8

[a] Medium recovered from cell monolayers at indicated times at 37°, and medium and cells harvested at 26 days after inoculation.

[b] $\log_{10}$ $TCID_{50}$/ml assayed in CV-1 cells.

[c] CV-1 extracts incubated with virus for 20 min at 23° before inoculation.

change SV40 titers in either Vero or CV-1 cells. Thus, extracts of CV-1 cells contained an enhancer that reduced the virus titer ratio of permissive (CV-1) to semipermissive (Vero) cells from 68 to 3. Comparable enhancement (10–40-fold) of $TCID_{50}$ and plaque titers of SV40 in three different stock cultures of Vero cell lines was demonstrated when virus was incubated with homogenates or extracts of uninfected CV-1 cells prior to inoculation on Vero cells. However, CV-1 extracts did not permit replication of SV40 in nonpermissive simian ($LLC\text{-}MK_2$) and human (WISH) cell lines.

The effect of CV-1 enhancer preparations on the production of SV40 by a line of human diploid fibroblasts (Detroit 549) was also examined. CV-1 extracts, containing 20 units enhancer activity, were incubated (20 min, 23°) with virus before inoculation. Controls consisted of cells inoculated with either virus, BSS, or CV-1 extracts. Medium, that had been in contact with cell monolayers for 5 days, was collected at 16 and 21 days and cells and medium were harvested at 26 days after inoculation. All samples were assayed for SV40 on CV-1 cells (Table II). Maximal virus titers were attained between 21 and 26 days. Treatment of virus with CV-1 extracts increased SV40 production by approximately 30-fold from $10^{5.3}$ to $10^{6.8}$ $TCID_{50}$/ml.

Enhancement of frequency of SV40-induced transformation. Investigations were undertaken to determine if CV-1 extracts increased the efficiency of SV40-induced transformation as well as SV40 production in Detroit 549 cell cultures. Cell monolayers were inoculated with preincubated mixtures of SV40 (130 PFU/cell) and CV-1 extracts (10 units enhancer activity). Controls consisted of cells inoculated with SV40 incubated in BSS and mock-infected cells inoculated with either BSS or CV-1 extracts. After a virus adsorption period of 3 hr at 37°, SV40 antiserum was included in media throughout the incubation period (20–26 days).

Transformed foci first appeared in infected cultures within 2 weeks after virus inoculation. The foci consisted of epithelial-like cells that exhibited loss of contact inhibition and nuclear alterations. Foci were not detected in control cell cultures that were inoculated with either BSS or CV-1 extracts. The results of 3 experiments (Table III) indicated that the average number of transformed foci per plate was increased approximately 3-fold by the addition of CV-1 extracts to virus before

TABLE III. Enhancement of SV40-induced Transformation of Cultured Human Cells by Extracts of Uninfected Permissive Simian Cells.

Experiment	Transformed foci[a]		Fold-enhancement of transformation efficiency
	virus alone	virus + enhancer[b]	
1	6.0 ± 0.6	16.1 ± 0.7	2.7
2	6.6 ± 1.2	23.6 ± 2.5	3.6
3	6.2 ± 0.5	16.3 ± 1.1	2.6

[a] Each count represents mean ± standard error of number of transformed colonies/10^5 cells determined from 10 cultures per sample.

[b] CV-1 extracts that enhanced SV40 titers in Detroit 549 and Vero cells by 20- to 30-fold were combined with virus before inoculation (20 min, 23°).

inoculation. In each experiment, a minimum of 60 foci were scored in monolayers inoculated with untreated virus and 160 foci in monolayers inoculated with enhancer-treated virus.

Despite the presence of SV40 antiserum, virus was detected in the medium at 12 days after inoculation. The virus titers did not increase thereafter in samples collected at 15, 19 and 26 days after infection. SV40 titers recovered from cells that were inoculated with untreated virus were 1.6×10^2 PFU/ml and $10^{2.3}$ $TCID_{50}$/ml. Presence of CV-1 extracts in the initial virus inoculum resulted in recovery of approximately 10-fold higher virus titers (1.2×10^3 PFU/ml and $10^{3.3}$ $TCID_{50}$/ml).

Specificity of SV40 enhancer. The procedures used for extraction of CV-1 cells and detection of enhancer activity for SV40 in these cell extracts were comparable to methods employed previously (9) for extraction and concentration of an echovirus 6 enhancer from a line of human heteroploid cells (WISH). However, the SV40 enhancer differed from the enhancer for echovirus 6 in its viral and host specificity. CV-1 extracts, that increased SV40 titers in Vero cells by 30-fold (from $10^{6.5}$ to $10^{8.0}$ $TCID_{50}$/ml), did not alter the titers of echovirus 6 ($10^{4.5}$ $TCID_{50}$/ml) in either CV-1 or Vero cells. In contrast, WISH cell extracts increased echovirus 6 titers in both CV-1 and Vero cells to the titers attained in WISH cells ($10^{6.5}$ $TCID_{50}$/ml), but did not alter SV40 titers in either of the simian cell lines. Thus, enhancer activity was detected only in host cells that were permissive for the specific virus studied.

Interaction between SV40 and enhancer. Incubation of CV-1 extracts with virus before inoculation (20 min, 23°) resulted in formation of a complex that was not dissociated by dilution (Table IV). Mixtures containing undiluted preparations of CV-1 extracts (1.8 ml) and virus (0.2 ml) were incubated and then diluted (10^{-6}) before assay on Vero cells. The diluted preparations increased SV40 titers by 14-fold (from 7.9×10^6 to 10.8×10^7 PFU/ml). Comparable enhancement (12-fold) occurred when preincubated mixtures of undiluted extracts and diluted virus (10^{-6}) were assayed on Vero cells without further dilution. The enhanced virus titer was 9.6×10^7 PFU/ml. Enhancement of SV40 titers in Vero cells did not occur if CV-1 extracts were diluted 10-fold or greater prior to incubation with virus.

Properties of SV40 enhancer. Enhancer activity in extracts of CV-1 cells remained in the supernatant fraction after centrifugation at 20,000*g* for 1 hr. The enhancer was not dialysable and was stable for 3 hr at 23° and for 2 weeks at 5°. Dialyzed CV-1 extracts have been stored at −20° for at least 6 mo without loss of enhancer activity.

A direct correlation between enhancer activity and protein concentration of dialysed CV-1 extracts was observed. The SV40 titer in Vero cells (4×10^6 PFU/ml) was increased 6.5-fold (2.6×10^7 PFU/ml), 12-fold (4.8×10^7 PFU/ml) and 18-fold (7.3×10^7 PFU/ml) by different lots of extracts (1 ml) containing 30 μg/ml, 50 μg/ml and 70 μg/ml protein, respectively. Most (65%) of the enhancer activity and approximately 10% of the pro-

TABLE IV. Irreversibility of Reaction Between Enhancer With Virus.

Reaction mixture dilutions[a]		Dilution after incubation	SV40 Titer in Vero cells[b]	SV40 titer increase
Virus	Enhancer			
10^0	—	10^{-5}	7.9	—
10^{-6}	10^0	None	96.0	12
10^0	10^0	10^{-6}	108.0	14
10^{-5}	10^{-1}	None	7.7	1

[a] The indicated dilutions of virus and enhancer (CV-1 extracts) were combined and incubated for 20 min at 23°.

[b] PFU/ml × 10^{-6}.

tein in dialysed CV-1 extracts were precipitated by addition of ammonium sulfate (33% final concentration). The precipitate fraction, which was dissolved in BSS and dialysed against BSS, contained 36 enhancer units and 0.18 mg protein. Pretreatment of precipitate fractions (2 ml) with 200 μg pronase (Calbiochem) for 1 hr at 23° abolished enhancer activity (10 units/ml). Since SV40 was not inactivated by pronase, the loss of enhancer activity could be detected by incubation of untreated and pronase-treated enhancer preparations with undiluted virus. Before assay the mixture was diluted beyond the toxic endpoint (10^{-4}) of pronase for Vero and CV-1 cells.

Discussion. The results indicate that a permissive line of simian cell cultures (CV-1) contains an enhancer of SV40 infection. Addition of homogenates or extracts of uninfected CV-1 cells to virus before inoculation of the mixture results in 10 to 40-fold higher plating efficiency of SV40 in semipermissive simian cells (Vero). In contrast, extracts of Vero cells and nonpermissive WISH cells do not increase plating efficiency of SV40 in simian cells. Increased SV40 production (10–30-fold) also occurs after inoculation of human cell cultures (Detroit 549) with virus and enhancer. Thus, extracts of permissive cells remove or decrease the restriction exhibited by less susceptible host cells for SV40 replication.

Crude virus preparations harvested from CV-1 cells do not enhance SV40 titers in Vero cells. This observation suggests that detectable quantities of enhancer are not available in these preparations. It is also possible that cellular substances in crude virus preparations may prevent detection of enhancer activity (9). The inability of clarified and concentrated CV-1 extracts to increase SV40 titers in CV-1 cells suggests that permissive cells are saturated with enhancer and cannot respond further to addition of exogenous enhancer preparations. However, as might be expected, enhancer does not appear to be the only factor required for SV40 infection. Extracts of CV-1 cells do not permit replication of SV40 virions in nonsusceptible simian (LLC-MK_2) or human (WISH) cells. Expression of enhancement appears to be limited to cells that are semipermissive for SV40 infection.

Enhancer preparations increase frequency of SV40-induced transformation in Detroit 549 cells. Although the concentration of SV40 antiserum employed should have been sufficient to neutralize released virus, some virus production (1×10^2 PFU/ml) occurs in cell cultures containing transformed foci. These observations may be due to inadequate quantities of antiserum or incomplete neutralization of the virus by the available antiserum. The presence of transformed foci and virus progeny in the same cell cultures may reflect a heterogenous cell population. Some of the cells may be stimulated to replicate virus, while others become transformed. Alternatively, certain cells may undergo a transient stage of transformation and then revert to production of virions. In either case, virus replication would cause cellular destruction and might be responsible for the smaller increase of transformed foci (3-fold) than the increase in virus production (10-fold) observed after inoculation of mixtures of CV-1 extracts and virus. Use of more concentrated enhancer preparations along with non-permissive cell lines or virus preparations that are capable of inducing transformation without replication may permit higher efficiency of transformation.

Enhancement of virus production and virus-induced transformation by CV-1 extracts may occur at a stage in virus infection that is required for both processes. Increased frequency of transformation is expressed in cells that are washed, trypsinized and subcultured 24 hr after inoculation of virus-enhancer mixtures. Since exogenous enhancer is inactivated by pronase and was presumably destroyed by the trypsin treatment, it is possible that enhancement is initiated prior to subculture of inoculated cells. Enhancer-virus complexes in the inocula may promote stability of viral infectivity and/or permit efficient attachment of virions to surfaces of cultured cells and thus enable subsequent events leading to either transformation or viral replication. Alternatively, enhancer may penetrate cells and facilitate events that occur beyond the attachment

stage of virus infection. Suarez *et al.* (6) were able to recover infectious viral DNA from SV40-transformed cells after addition of extracts of uninfected cells that are fully permissive for SV40 replication. The protein "activator" in their system may be analogous to the SV40 enhancer described in this paper. Poly-L-ornithine is required for activation of the SV40 genome in transformed cells by permissive cell extracts. In the present study, addition of basic polymers is not necessary for enhancement of SV40 infection of semipermissive host cells. It remains to be determined whether the activation and/or release of the SV40 genome and the enhancement of efficiency of SV40 infection by cell extracts occurs by the same mechanism.

Several of the properties of the enhancer for SV40 are similar to those described previously for an enhancer of echovirus 6 (8–11). Enhancer activity for each of the viruses remains in the supernatant fraction after centrifugation of permissive host cell extracts (20,000*g*, 1 hr, 5°). SV40 enhancer activity in CV-1 cells as well as echovirus 6 enhancer activity in human cell lines is precipitated by ammonium sulfate (33%) and inactivated after treatment with pronase. These observations along with the direct correlation between enhancer activity and protein concentration in cell extracts indicate that enhancer activity is associated with protein. Also, in both the SV40 and echovirus 6 systems, the enhancer binds to virus at 23° and the resultant complex is not dissociated by dilution. Despite these similarities, the cell source and viral specificity of the two enhancers are different. The specificity of the enhancers suggests that permissive host cells contain viral-specific reactive sites in addition to viral receptors. These cellular components may be necessary for maximal virus production and are transferable to semipermissive systems. It is possible that specific enhancers exist for other viruses and can be isolated from permissive host cells. Purification of viral enhancers and elucidation of their mode of action will increase our understanding of host-controlled factors that determine susceptibility of cells to infection and transformation by viruses.

Summary. The sensitivity of semipermissive cell cultures to SV40 infection can be enhanced by a component of uninfected, simian cells (CV-1) that are permissive for SV40 infection. Enhancement is demonstrated by 10- to 40-fold higher plating efficiency in simian cells, 10- to 30-fold increase in virus production by human cells and 3-fold increase in frequency of transformation in a line of human diploid fibroblasts after inoculation of mixtures containing CV-1 extracts and virus. The enhancer is recovered from cultured cells by a mild extraction procedure and is not sedimented by centrifugation at 20,000*g* for 1 hr. The active component of the supernate appears to be protein. Incubation of virus with enhancer preparations for 20 min at 23° results in the formation of a complex that is not dissociated by dilution. Although these properties of the SV40 enhancer are similar to those described previously for an enhancer of an enterovirus, the SV40 enhancer differs from the enterovirus enhancer in its host and viral specificity. These observations suggest that analogous enhancement systems may exist for other viruses.

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