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Infection of mouse preimplantation embryos with simian virus 40 and polyoma virus

(papova viruses/T and V antigens/α-amanitin/virus-membrane interactions/permissiveness)

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ABSTRACT **Mouse two-cell embryos, morulae, and blastocysts were killed when infected *in vitro* with simian virus 40 (SV40) at high multiplicities of infection. Polyoma virus was not deleterious for preimplantation embryos, even at a very high multiplicity of infection; however, the outgrowths of polyoma-infected blastocysts disintegrated after several days of culture. Indirect immunofluorescence tests revealed the presence of SV40 T and V antigens and polyoma virus V antigen in the nuclei of trophoblastic cells. Virus-specific antigens were not found in the nuclei of cells forming inner cell masses of blastocysts or in inner cell mass-derived cells in blastocyst outgrowths. The appearance of SV40 T and V antigens in the nuclei was inhibited by α-amanitin, a RNA polymerase II inhibitor. The amount of infectious virus recovered from cultures of morulae or blastocysts on subsequent days after infection with SV40 initially declined but later increased. These points of evidence indicate that some cells of early mouse embryos are permissive for the expression of early and late functions of SV40 genome and that susceptibility to infection with polyoma virus and/or permissiveness for the expression of polyoma virus late functions develop gradually between the two-cell and blastocyst stages. Electron microscope observations showed the presence of specific complexes of membranes and virions in the cytoplasm of trophoblastic cells. Single viral particles could be found in the nuclei and also in mitochondria.**

Reports concerning the interaction of preimplantation mouse embryos and DNA tumor viruses are not in agreement. Exposure of preimplantation embryos to simian virus 40 (SV40) or SV40 DNA has been reported either to have no effect on subsequent development (1–3) or to seriously affect or inhibit development (4, 5). Evidence indicating that SV40 replicates in preimplantation embryos was suggested by electron microscope studies (4) and by the isolation of infectious virus from embryos after exposure to SV40 DNA (1). However, SV40 DNA replication was not detectable in embryos exposed to virus and labeled with [^{3}H]thymidine (3).

In this report we present evidence that both early and late genes of SV40 and late genes of polyoma virus are expressed in preimplantation mouse embryos and that this expression is probably restricted to differentiated trophoblastic cells. In addition, exposure to high multiplicities of SV40 inhibits further development of preimplantation mouse embryos *in vitro*, and the effect is virus-specific.

MATERIALS AND METHODS

Isolation and Culture of Embryos. Two-cell embryos and morulae were isolated from the oviducts and blastocysts were isolated from the uteri of ICR mice. After removal of zonae pellucidae with 0.2% Pronase, the embryos were rinsed three times and cultured in droplets of Whitten's medium (6) (2-cell and 8–16-cell embryos) or minimal essential medium supplemented with 10% fetal calf serum (morulae and blastocysts). Two-cell embryos developed into blastocysts within 3 days of culture, 8–16-cell embryos within 2 days, and morulae within 1 day.

For further culture, blastocysts were transferred into cloning rings on glass coverslips. Within 3 days of culture, blastocysts attached and formed outgrowths consisting of a spread layer of trophoblastic cells and a clump of inner cell mass (ICM)-derived cells. After 6 days of culture, outgrowths gradually degenerated, as indicated by a decrease in the number of cells.

ICMs of blastocysts were isolated by immunosurgery (7) and cultured similarly to blastocysts.

Viruses. SV40 was used either as a crude pool [lysate of a culture of infected TC-7 monkey cells (8)] or after purification (9). The original virus used to prepare these two stocks was plaque-purified large-plaque virus of the RH 911 strain. The titer of the "crude" pool was 6×10^7 infectious units (IU)/ml, and the titer of purified virus was 4×10^{10} IU/ml. Titrations were performed by means of the end-point dilution microtest (10) in which infected TC-7 cells were detected by staining for V antigen.

The "crude" pool of polyoma virus was produced according to Winocour (11) from a wild-type polyoma seed obtained from Roger Weil. The titer, assayed by means of an agar plaque test on primary cultures of National Institutes of Health embryo fibroblasts, was 7×10^9 plaque-forming units (PFU)/ml.

Infection of Embryos. Embryos were infected by placement in 1-μl (unless otherwise stated) droplets of SV40 or polyoma virus diluted with an appropriate culture medium: 2-cell embryos, for 3 days; 8–16-cell embryos, for 2 days; and morulae or blastocysts for 1 day. After exposure to virus, embryos were washed four times and cultured in virus-free medium.

Indirect Immunofluorescence (IIF). Cleavage stage embryos cannot be examined by this technique because they are destroyed during fixation with acetone. Blastocysts were placed on a glass slide in a small volume of medium, air-dried, and immersed in acetone for 5–10 min. Trophoblast outgrowths were fixed with acetone without drying.

Embryos were exposed to rabbit anti-SV40 capsid (V) and anti-polyoma V sera diluted 1:200–1:400 with phosphate-buffered saline for 1 hr at 37°, washed three times with phosphate-buffered saline, and stained with fluorescein isothiocyanate-tagged goat anti-rabbit IgG serum (Cappel Labs, Inc.). Hamster anti-SV40 tumor (T) serum was diluted 1:30 with phosphate-buffered saline; fluorescein-tagged rabbit anti-hamster IgG serum (Cappel Labs, Inc.) was used as the second

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Abbreviations: SV40, simian virus 40; ICM, inner cell mass; IU, infectious units; PFU, plaque-forming units; VMS, virus-containing membranous structures.

Table 1. Development of mouse two-cell embryos in the presence of SV40 or polyoma virus (Py)

MOI, IU or PFU/embryo	% embryos attaining blastocyst stage*		
	SV40		Py
	Purified	"Crude" pool	"Crude" pool
0	85(240)	—	84(51)
2.5×10^2	—	86(55)	—
1.0×10^3	61(145)	73(55)	—
2.0×10^3	39(208)	—	—
4.0×10^3	2(82)	36(53)	—
4.0×10^5	—	—	78(45)
4.0×10^6	—	—	73(45)

* Number of embryos cultured is shown in parentheses; —, not done.

reagent. Neither noninfected controls nor infected embryos stained when normal rabbit or hamster serum was used as the first reagent.

α-Amanitin. Blastocysts that developed from morulae cultured for 24 hr in the presence of SV40 (4×10^4 PFU per embryo) were washed four times and placed in cloning rings containing 0.2 ml of minimal essential medium and α-amanitin (Sigma Chemical Co.) at 2 μg/ml. Fifteen to 20 blastocysts were placed in each cloning ring. Twenty-four or 48 hr later, blastocysts were fixed and stained for the presence of SV40 T- and V-positive nuclei. In parallel controls, the drug was omitted.

Titration of SV40 in Embryo Cultures. Embryos were removed from the virus after 24 hr of exposure and pooled, washed four times in minimal essential medium, and distributed into several groups. Each group was placed in one 6-mm well of a multidish Dispositray (Linbro Chemical Co., Inc.) in 0.2 ml of minimal essential medium and cultured for up to 5 more days. For estimation of the amount of infectious virus released in the culture supernatant, medium was carefully removed from the wells on a given day of culture and stored at −20°. For estimation of the amount of infectious virus in the entire culture, each tray was frozen and stored until the end of the experiment. After the last day of the experiment, all trays were thawed and frozen three times and continuously shaken on a Micro-Shaker (Cooke Laboratory Products) during each thawing. After the last thawing, the contents of the wells were transferred to plastic tubes and sonified for 1 min with a 150-W Branson sonifier while being chilled every 15 sec. The titer of SV40 in each well was estimated by the end-point dilution microtest (10), and the results were presented as numbers of recovered IU per embryo.

Electron Microscopy. Infected mouse embryos were processed for electron microscopy as described (4).

For ultrastructural visualization of SV40 V antigen, the embryos were fixed for 3 hr in periodate/lysine/paraformaldehyde solution (12), washed in 0.1 M phosphate buffer plus 7% sucrose overnight, exposed to rabbit anti-SV40 V serum (diluted 1:40) for 8 hr at room temperature (22°), washed several times in phosphate-buffered saline for 5 hr, exposed for 10 hr to goat antirabbit serum conjugated with peroxidase (Cappel Labs, Inc.) diluted 1:40, fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) for 3 hr, and washed again in phosphate-buffered saline for 3 hr. Peroxidase was detected by using the diaminobenzidine method (13).

Two controls were used: (*i*) embryos treated as above except the first exposure was to normal rabbit serum, and (*ii*) embryos exposed only to diaminobenzidine for visualization of endogenous peroxidase. Ultrathin sections were examined either without or after staining with lead citrate.

RESULTS

Embryonic Development after Infection with SV40 or Polyoma Virus. SV40 exerted a dose-dependent deleterious effect on the development of two-cell embryos (Table 1); most embryos infected at the multiplicity of infection (MOI) of 4×10^3 IU per embryo were destroyed after two or three cell divisions.

Eight- to 16-cell embryos or morulae exposed to SV40 at 4×10^4 IU per embryo formed blastocysts but failed to develop further and disintegrated. Infection of blastocysts under similar conditions also usually resulted in the inhibition of further development. Polyoma virus did not significantly affect the development of two-cell embryos, even when present at MOI of 4×10^6 PFU per embryo. Two-cell embryos exposed, at 4×10^3 IU per embryo, to purified SV40 neutralized with rabbit anti-SV40 serum did not degenerate.

Expression of SV40 T and V Antigens in Trophoblastic Nuclei after Infection of Embryos. Blastocysts developed from two-cell embryos that survived infection with SV40 at 1×10^3 or 4×10^3 IU per embryo occasionally showed the presence of T-positive but not of V-positive nuclei. When such blastocysts were cultured further, some T- and V-positive nuclei were found in trophoblastic cells. After exposure of morulae or blastocysts to 4×10^4 IU of virus per embryo in 1-μl droplets, SV40 T- and V-positive nuclei appeared in the trophoblastic cells of blastocysts 1–2 days after infection (Table 2; Fig. 1 *A* and *B*). During subsequent culture, such blastocysts disintegrated without forming outgrowths.

It was possible, however, to obtain outgrowth cultures from blastocysts exposed to SV40 at 4×10^4 IU per embryo suspended in 4-μl droplets. In such cultures, giant trophoblast nuclei

Table 2. Expression of SV40 T and V and polyoma (Py) V antigens in nuclei of trophoblastic cells of blastocysts after infection of morulae or blastocysts

Days after infection	Mean positive nuclei/embryo* after infection with:							
	SV40 (4×10^4 IU/embryo)				Py (4×10^4 PFU/embryo)		Py (4×10^6 PFU/embryo)	
	Morula		Blastocyst		Morula,	Blastocyst,	Morula,	Blastocyst,
	T	V	T	V	V	V	V	V
1	0.6	0.03	4.5	1.4	0	—	—	0.2
2	13.2	4.7	41.0	2.5	0	0	0	1.0
3	11.1	7.3	18.0	7.3	—	0.2	—	5.3
4	2.9	4.1	6.4	3.5	0.2	4.6	0.2	2.5
6	1.7	1.4	3.9	1.9	0.3	5.5	1.4	0.3
8	—	—	—	—	0.3	1.5	1.0	—

* All cultured embryos taken into account; —, not done. Each value is based on the examination of at least 15 embryos.

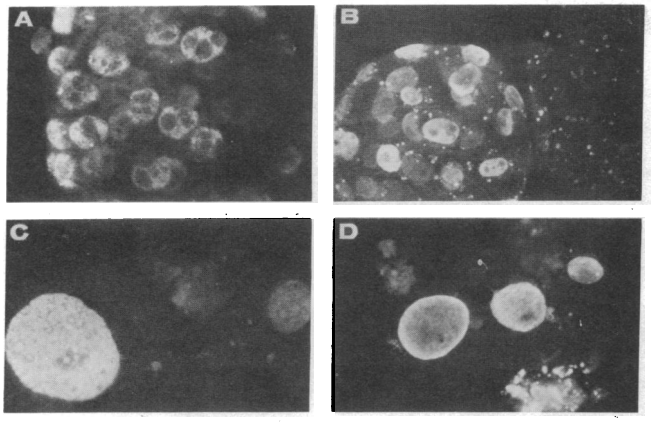

FIG. 1. SV40- and polyoma virus-specific antigens in infected mouse embryos as shown by indirect immunofluorescence technique. (*A*) SV40 T antigen in nuclei of blastocyst, 48 hr after infection at morulae stage. (×150.) (*B*) SV40 V antigen in nuclei of one of two blastocysts, 48 hr after infection at morulae stage. Cytoplasmic V-positive granules were present in both blastocysts. (×99.) (*C*) SV40 V antigen in giant trophoblastic nuclei of a blastocyst outgrowth 8 days after infection. (×150.) (*D*) Polyoma V antigen in giant trophoblastic nuclei of a blastocyst outgrowth 6 days after infection. (×150.)

stained positively for T and V antigens (Fig. 1*C*); ICM-derived cells did not contain either T- or V-positive nuclei but, because of cell clumping, these tests were difficult to perform. However, when immunosurgically isolated ICMs that had been exposed to SV40 at 4×10^4 IU per ICM formed cell monolayers, which could be easily analyzed, no T- or V-positive nuclei were observed.

Inhibition of SV40 T and V Antigens by α-Amanitin. Morulae were exposed to α-amanitin 24 hr after infection with SV40 4×10^4 IU per embryo. The embryos were kept in the presence of α-amanitin during the duration of the experiments without apparent deleterious effect. α-Amanitin was more effective in inhibiting the appearance of V-positive than that of T-positive nuclei in trophoblastic cells of blastocysts (Table 3). This difference in inhibition may be attributed to the fact that the production of T antigen was already advanced at the time (24 hr after infection) the embryos were exposed to α-amanitin.

Expression of Polyoma V Antigen in Trophoblastic Nuclei after Infection of Embryos. When morulae were infected with polyoma virus at 4×10^4 or 4×10^6 PFU per embryo, the number of V-positive trophoblastic nuclei was considerably lower than that found when morulae were infected with SV40 (Table 2). However, infection of blastocysts with polyoma virus resulted in the formation of V-positive trophoblastic nuclei (Fig. 1*D*) in numbers similar to those found after infection with SV40 (Table 2).

Table 3. Inhibition of the expression of SV40 T and V antigens by α-amanitin

Days after infection	Mean positive nuclei/embryo			
	T-positive		V-positive	
	No α-amanitin	α-Amanitin	No α-amanitin	α-Amanitin
2	13.7 (17)	8.9 (15)	5.0 (38)	0.3 (18)
3	11.1 (14)	5.4 (11)	5.5 (13)	0.0 (16)

α-Amanitin (2 μg/ml) applied 24 hr after infection of morulae with SV40 at 4×10^4 IU per embryo. Number in parentheses is number of embryos examined.

Table 4. Infectivity recovered from cultures of embryos after infection with SV40

Stage at infection and MOI	Exp. no.	Infectivity (IU/embryo) recovered after infection: At day 1	At day 2	At day 4	At day 6
2-cell	1*	2666	222	—	22
(1×10^4 IU/embryo)	2*	162	7	16	—
Morula	1*	1818	176	182	X
(4×10^4 IU/embryo)	2*	131	20	66	101
	3†	—	50	33	110
Blastocyst	1*	5455	76	1440	2222
(4×10^4 IU/embryo)	2*	760	4	8	87
	3†	—	17	44	400

* Infectivity assayed in cells and supernatant; —, not done; X, lost.
† Infectivity assayed in supernatant only.

Formation of V-Positive Cytoplasmic Granules after Infection of Morulae or Blastocysts with SV40 or Polyoma Virus. One day after infection of morulae or blastocysts with SV40 at 4×10^4 IU, bright, V-positive granules with sharp, regular outlines appeared in the cytoplasm of trophoblastic cells (Fig. 1*B*). The granules were most distinct 2 days after infection, when they reached a diameter of 3–5 μm. Later, their number seemed to decrease. Polyoma V-positive granules were smaller and not as regular and distinct.

Infectivity of Embryo Cultures Infected with SV40. Amounts of infectious SV40 recovered from embryo cultures on different days after infection are presented in Table 4. At 2 days after infection, the amount of infectivity recovered had dropped markedly in comparison to the infectivity recovered on day 1. Subsequently, however, infectivity recovered from the supernatants or entire cultures of morulae and blastocysts increased. In cultures of two-cell embryos the amount of infectious virus recovered did not increase significantly after the initial decrease.

Electron Microscopy. During the first 24 hr after infection of morulae or blastocysts with SV40, most virus particles were either free in the cytoplasm or in large phagocytic vacuoles where they showed signs of disintegration. At the same time we observed the development of cytoplasmic structures composed of smooth-surfaced membranes forming tubular sacs and containing virus particles [virus-containing membranous structures (VMS)]. The membranes of VMS showed continuity with either the nuclear envelope or the rough endoplasmic reticulum (Fig. 2 *A–C*). Cell nuclei were free of virus particles.

After 48 hr of infection with SV40, the number and size of VMS increased, whereas those of phagocytic vacuoles decreased. The VMS contained, in addition to virus particles, amorphous material that was denser than the surrounding cytoplasm (Fig. 2*G*). Occasionally, a few membrane-free virus particles appeared in the perinuclear space(s) budding into the nucleus (Fig. 2*E*). Nevertheless, only in a few nuclei were the structures resembling single, membrane-free virus particles of 35-nm diameter noted. Single naked or enveloped virus particles were also observed in some mitochondria (Fig. 2*F*). Mitochondria, some of which appeared "empty," were frequently lying in close proximity to VMS (Fig. 2*D*); the same was true for the clusters of endogenous crystalloid material observed in almost all cells examined (Fig. 2*B*). In almost all VMS, a single or very few A type particles were also observed.

Immunohistochemical reaction indicating the presence of SV40 V antigen in VMS was more pronounced at 48 hr than at 24 hr after infection. The massive coating of the majority of

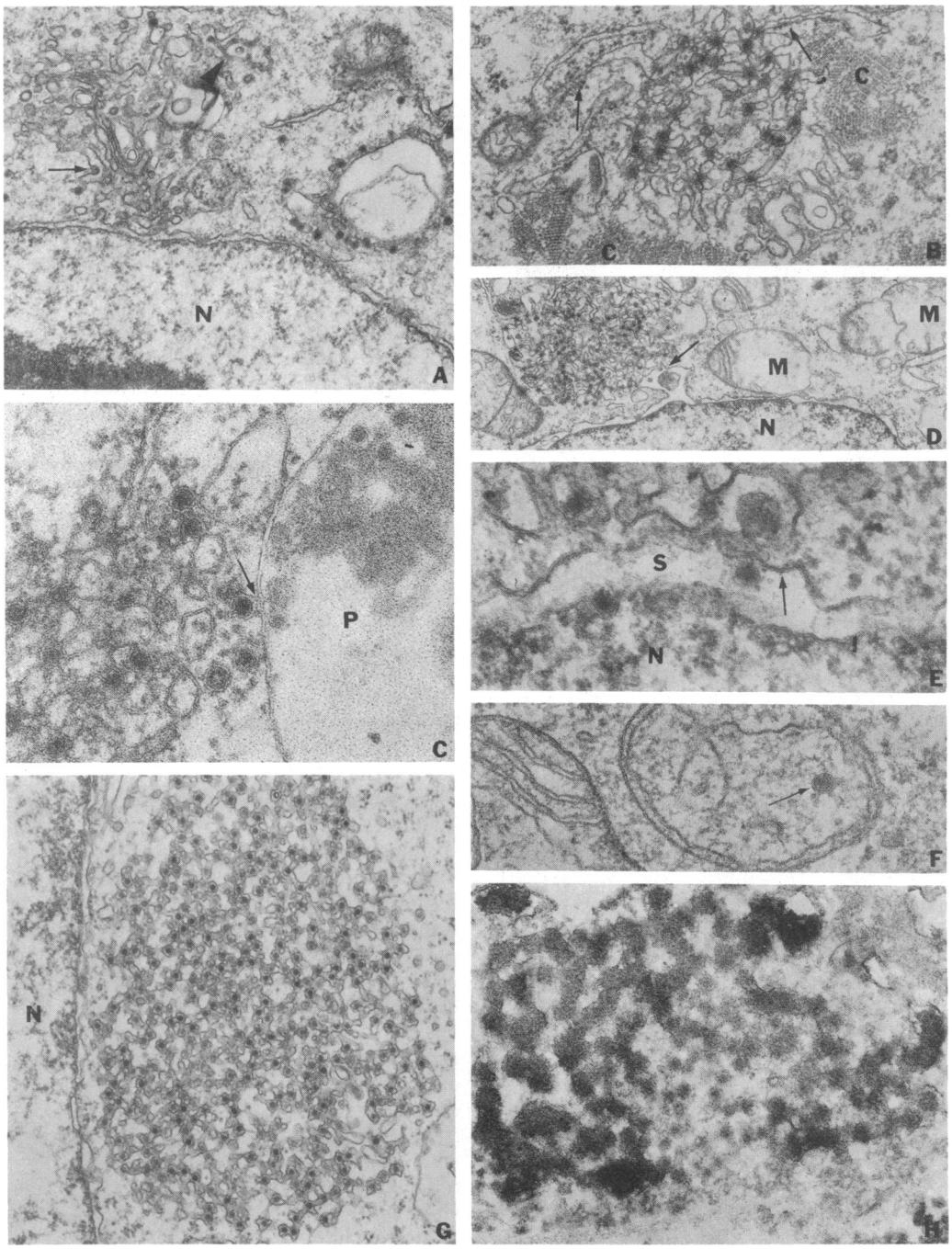

FIG. 2. Electron micrographs of mouse morulae and blastocysts examined at indicated times after beginning of exposure to SV40. (*A*) Morula after 4.5-hr exposure to SV40, showing outgrowth of the outer leaflet of the nuclear envelope. Arrow points to a virus particle budding into elongated cisternae of early VMS; arrowhead indicates tubuloreticular membraneous structure; N, nucleus. (×24,640.) (*B*) Morula after 14-hr exposure to SV40, showing early formation of VMS containing several virus particles. Arrow points to rough endoplasmic reticulum; C, crystalloid. (×30,800.) (*C*) Blastocyst after 24-hr exposure to SV40, showing close contact (arrow) of membranes of VMS and phagocytic vacuole (P). (×77,000.) (*D–H*) Blastocysts after 48-hr exposure to SV40. (*D*) Close structural relationship between outer leaflet of the nuclear membrane (arrow) and VMS. Numerous mitochondria (M), some of them "empty," are in proximity to VMS; N, nucleus. (×11,550.) (*E*) Two marked virus particles in the space(s) between two leaflets of the nuclear envelope; the outer leaflet (arrow) shows close structural relationship to the membranes of VMS. (×77,000.) (*F*) Enveloped virus particle (arrow) in the mitochondrial matrix. (×57,750.) (*G*) Large cytoplasmic VMS close to the nucleus (N). (×24,640.) (*H*) The localization of V antigen in VMS. Most of the virus particles are coated with antibodies, obscuring the fine structural details. (×38,500.)

virus particles with peroxidase-conjugated antibodies obscured the fine structure of VMS. However, small groups of virus particles not showing positive reaction were also present in many VMS (Fig. 2*H*).

In two-cell and four-cell mouse embryos infected with SV40, the formation of VMS was not observed, though the virus particles were endocytosed and collected in large phagocytic vacuoles.

Examination of embryos fixed 48 and 72 hr after infection of blastocysts with 4×10^6 PFU of polyoma virus showed the presence of many cytoplasmic vacuoles filled with viral particles frequently arranged in crystalloid-like formations. The struc-

tures resembling VMS were found only occasionally and, as in the case of SV40, their membranes were in close proximity to mitochondria and showed continuity with rough endoplasmic reticulum membranes. Only in some nuclei were single or a few viral particles seen.

DISCUSSION

In contrast to the conclusions of Jaenisch and Berns (3) that preimplantation mouse embryos are not permissive for the expression of "late" functions of SV40 and polyoma virus genomes after infection, we have observed the appearance of SV40 T and V antigens and polyoma V antigen in the nuclei of trophoblastic cells after infection of mouse embryos at high MOI.

The detection, by immunofluorescence, of SV40 V antigen in the cytoplasm and nuclei of cells of infected embryos does not conclusively demonstrate that viral capsid protein is synthesized in these cells. However, inhibition of SV40 V antigen by exposure of embryos to α-amanitin which blocks polymerase II (14) seems to present a strong argument for the synthesis of viral capsid protein in mouse embryos. Transcription of "late" regions of SV40 genome after infection of mouse cells has been observed by others (15), but the synthesis of viral capsid proteins has never been detected (16).

The recovery of infectious SV40 in media and embryos might be accounted for by the reisolation of input virus. Therefore, these experiments do not provide proof of viral synthesis but rather demonstrate late viral gene expression. The mechanism of destruction of two-cell embryos after exposure to SV40 is not known, but the following evidence strongly suggests that it is a virus-specific event: rabbit anti-SV40 serum prevented the destruction but normal rabbit serum did not; and embryos at the same stage exposed to polyoma virus remained perfectly viable. Even microinjection of 6–7 PFU of polyoma virus per blastomere of two-cell embryos did not impair their subsequent development. Again, our observations on the survival of preimplantation embryos infected with high MOI of SV40 are not in agreement with those of Jaenisch and Berns (3) who did not observe a deleterious effect of SV40 infection on the subsequent development of eight-cell mouse embryos.

It is interesting to note that, although the amount of SV40 deleterious for a two-cell embryo (4×10^3 IU) is lower than that deleterious for a morula or blastocyst (4×10^4 IU), the MOI of both two-cell embryos and blastocyst cells exposed directly to the virus is approximately the same—i.e., 2×10^3 IU per cell. A comparable number of SV40 DNA molecules has recently been shown to be required to induce V antigen formation in the nucleus of a microinjected mouse kidney cell (17).

Electron microscopic observations did not reveal the presence of many virions in the nuclei of trophoblastic cells of infected embryos; virions were observed almost exclusively in the cytoplasm in VMS. Membraneous components of VMS often showed connections with the outer leaflet of the nuclear envelope, with rough endoplasmic reticulum, and with mitochondria. It is not clear whether the presence of single viral particles in mitochondria has any biological significance. The other striking observation was the localization of endogenous crystalloids close to VMS. These crystalloids are probably of proteinaceous nature and may be a kind of reserve material utilized later in development (18). Whether, on the basis of these observations, we can postulate that both SV40 DNA replication and virus assembly occur in the cytoplasm is still uncertain, but further experiments with metabolic inhibitors may provide a definite answer.

Observation of preimplantation embryos after infection with polyoma virus gave quite different results. Two-cell embryos were completely resistant to polyoma virus and morulae were only slightly more susceptible for production of polyoma V antigen. It is only at the stage of the blastocyst that the number of cells containing polyoma V-positive nuclei equalled the number containing SV40 V-positive nuclei. It is thus possible that, at this stage of development, embryos become susceptible to polyoma virus infection.

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