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Simian Virus 40 and Polyoma Virus Gene Expression Explored by the Microinjection Technique

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1 Introduction

The transfer of genetic material through parasexual processes (transformation, transduction, conjugation) has contributed enormously to our understanding of bacterial genetics. Since the transformation experiments with prokaryotic cells reported by *Avery* et al. (1944), numerous techniques have also been developed for assay of the biological activity of heterologous and homologous nucleic acids in mammalian cells. Most of these techniques are based on the use of divalent metal cations, polycations, hyper- or hypotonic shocks (for reviews, see *Sarkar*, 1967; *Pagano*, 1969; *Bhargava* and *Shanmugam*, 1971; *Graham*, 1977). All these procedures may enhance the uptake of macromolecules into mammalian cells, but reproducible results have been obtained mainly with RNA or DNA from various oncogenic or nononcogenic viruses. However, the infectivity of viral nucleic acids assayed was always significantly lower than that of full virus particles. A more promising technique in this regard involves fusion of loaded erythrocyte ghosts or liposomes with mammalian cells (*Loyter* et al., 1975).

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A totally different approach allows microinjection of cell organelles (*Graessmann*, 1968) or macromolecules (*Graessmann* and *Graessmann*, 1971) into mammalian tissue culture cells without affecting them. The transfer is performed under a phase-contrast microscope with small glass capillaries (Fig. 1). By microinjection of RNA isolated from Harding-Passey mouse melanoma tumor cells into rat myotubes, we found that even

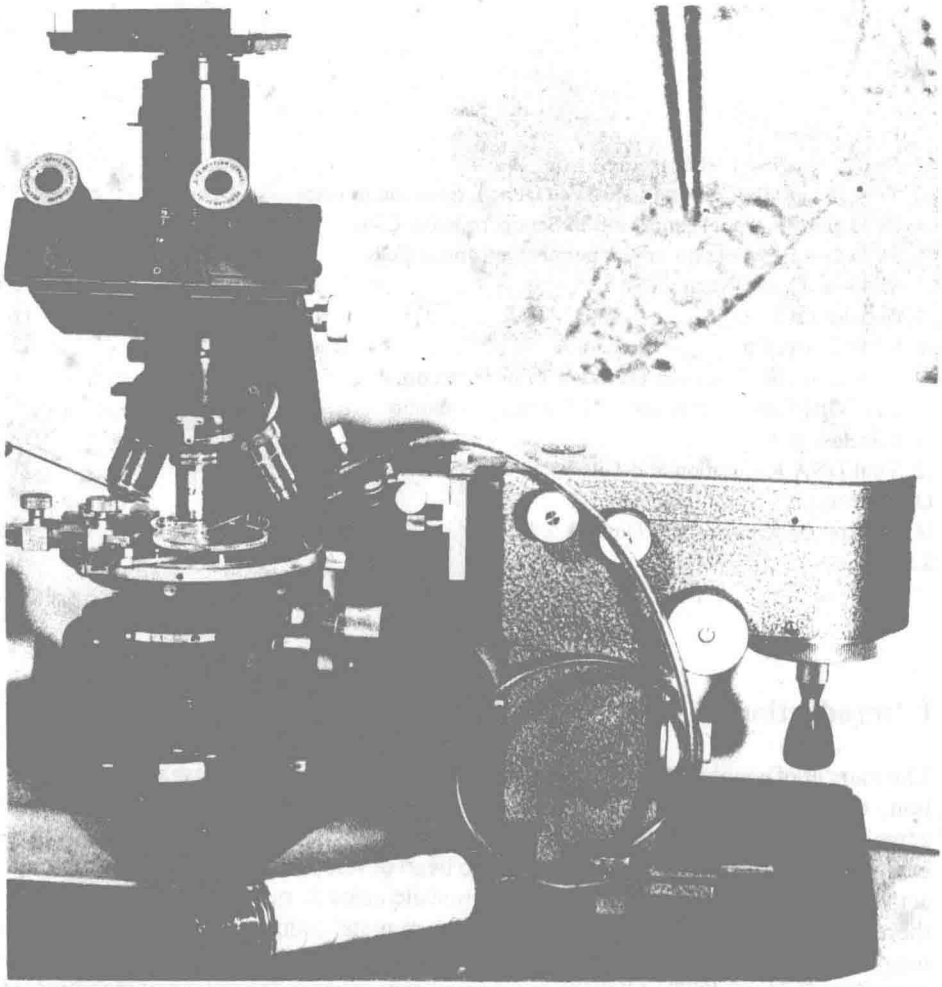

Fig. 1. Assembled instruments for microinjection. A glass capillary, drawn form a glass tube (outer diameter 1.5 mm) to a tip diameter of 0.5–1 µm, is inserted in the instrument holder of the micromanipulator (Leitz, Wetzlar) and connected to a 50-ml syringe. The capillary is filled from the tip by negative pressure exerted with the syringe. This process and the microinjection procedure are performed under a phase-contrast microscope (Ortholux, Leitz, Wetzlar). Cells grown on glass slide (imprinted with squares of 1 mm^2) are brought into focus and injected by moving the capillary tip into a target cell. An injection volume of about 2×10^{-11} ml per cell is transferred by gentle pressure exerted by the syringe. A stream of CO_2 (glass tube from the left) maintains the pH of the medium throughout the procedure. Up to 1,000 cells can be microinjected per hour. The insert shows the injection step at the cellular level

these terminally differentiated mammalian cells are able to translate foreign mRNA (*Graessmann* and *Graessmann*, 1971). Subsequently, translation of rabbit hemoglobin mRNA was demonstrated in the frog oocyte microinjection system (*Lane* et al., 1971; *Gurdon* et al., 1971).

During recent years microinjection of tissue culture cells has turned out to be a sensitive tool yielding reproducible results for the assay of viral and nonviral macromolecules (*Graessmann* and *Graessmann*, 1976; *Graessmann* et al., 1976a; *Stacey* and *Allfrey*, 1976; *Graessmann* et al., 1977a; *Stacey* et al., 1977; *Celis*, 1977; *Tjian* et al., 1978). The usefulness of the microinjection technique is well demonstrated in this review of our studies on simian virus 40 (SV40) and polyoma virus (PV) gene expression in permissive and nonpermissive cells.

2 Simian Virus 40 and Polyoma Virus

SV40 and PV are small oncogenic DNA viruses of the papova virus group (for review, see *Tooze*, 1973). The manner of viral gene expression is highly cell-dependent. Only cells of the native host (monkey for SV40, mouse for PV) support virus multiplication with high efficiency. In these cells viral gene expression proceeds in two phases. Fig. 2 shows a simplified scheme of the SV40 or PV infection cycle.

After virus adsorption, penetration, and intracellular release, the first virus-specific products synthesized are the 19S early mRNAs transcribed from the early viral DNA strand (Fig. 3). These mRNAs are then translated into tumor (T) antigens. Intranuclear T

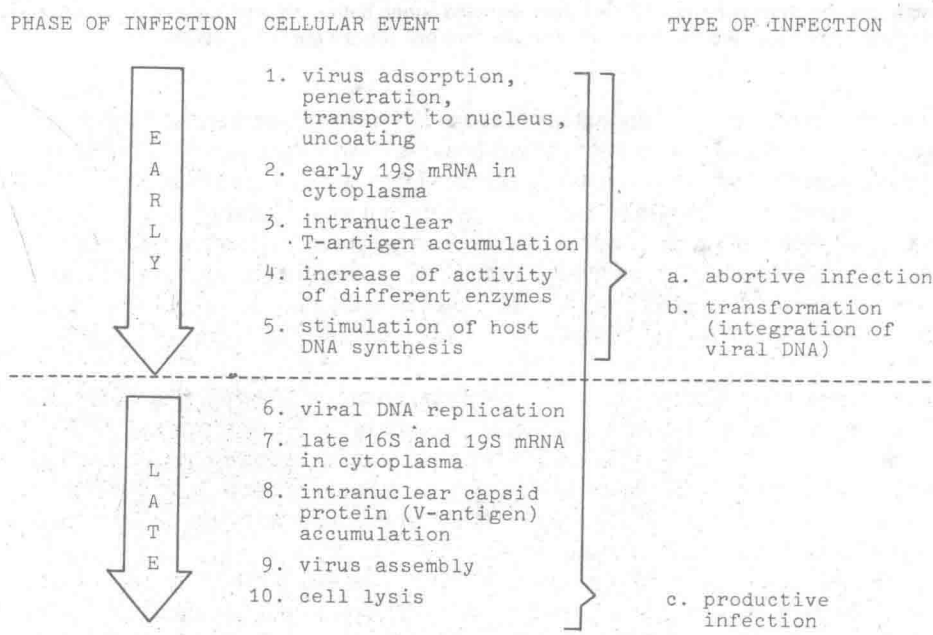

Fig. 2. A simplified scheme of the SV40 or PV infection cycle. Reproduced with permission from *Graessmann* et al. (1977c)

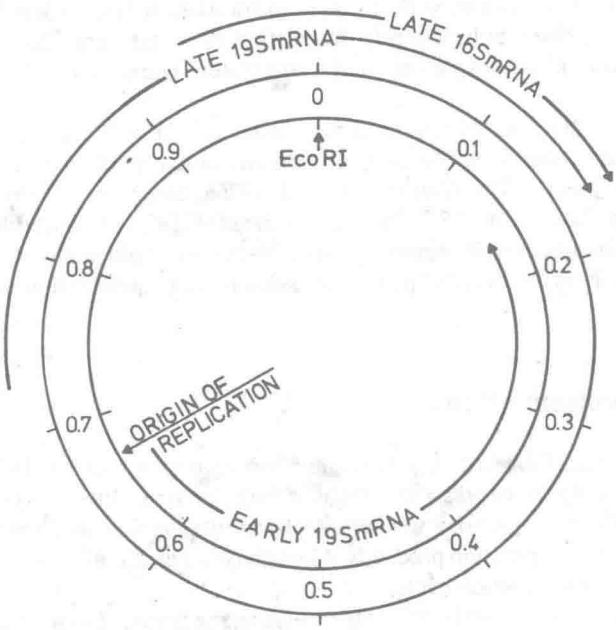

Fig. 3. Transcription map of SV40. By convention, the cleavage site of restriction endonuclease EcoRI serves as reference point 0 on the physical map of SV40 DNA. About one-half of the total viral genome (5,200 base pairs) makes up the early region, the other half the late region. The transcriptional pattern is more complex than depicted, since both early and late primary transcripts undergo "splicing." Reproduced with permission from *Graessmann* et al. (1977c)

antigen accumulation and induction of cellular DNA synthesis are specific events of the early phase of the infection cycle. The late phase starts with the onset of viral DNA replication, which mediates transcription of the late DNA strand. Late 19S and 16S mRNAs are synthesized in addition to the early species, but in significantly larger quantities (*Acheson*, 1976). Intranuclear capsid protein (V antigen) accumulation is a marker of late viral gene expression at the single cell level. After virus maturation, progeny viruses are released through cell lysis. The average yield per cell is 10^2-10^3 plaque forming units (PFU) for SV40 and 10^3-10^4 PFU for PV (1 PFU is equivalent to about 100 virus particles).

Cells supporting early and late viral gene expression are called permissive cells; they undergo a productive infection. Nonpermissive cells support early but not late viral gene expression; these abortively infected cells synthesize T antigen, but not V antigen. Semipermissive cells are infected with low frequencies and generate poor yields of virus, and most of those infected cells respond in an abortive manner. Cells of other species may be totally resistant to virus infection.

Elucidation of the regulatory mechanisms responsible for the particular mode of viral gene expression in different cell types is a central problem in the molecular biology of SV40 and PV. It is of special importance for the understanding of viral cell transformation, since SV40 or PV transformed cell lines in general only support early viral gene expression, although many of them contain the entire viral genome covalently integrated

into the cellular DNA. Three important regulatory steps in the SV40 or PV infection cycle will be discussed in later sections:

1. Very early events in the viral infection: virus adsorption, penetration, intracellular release
2. Efficient synthesis of T antigen in terms of quality and quantity
3. Viral DNA replication and late gene transcription.

3 Time Course of Early and Late SV40 Gene Expression in Permissive Cells

Only cells of the native host efficiently support virus multiplication. In these cells early and late viral gene expression is demonstrable even after infection with 1 virus particle (0.01 PFU) or after microinjection of 1–2 viral DNA molecules per cell (*Graessmann* et al., 1976a). The time course of SV40-specific antigen accumulation is correlated with the virus input per cell, as shown in Figs. 4 and 5. With multiplicities of infection (MOI) of 100 PFU per cell or more, intranuclear T or V antigen is first demonstrable 12 or 16 h, respectively, after inoculation.

To determine the temporal sequence in infected cells of a) virus adsorption, penetration, and intracellular release; b) intranuclear accumulation of viral template DNA; c) transcription of early mRNAs in appropriate quantities; and d) transport of T antigen from cytoplasma to nucleus, the following substances were microinjected into TC7 cells: a) full virus particles into nuclei; b) SV40 DNA I (superhelical form) into nuclei; c) early SV40 RNA into the cytoplasm; and d) purified T antigen into the cytoplasm. Following microinjection, cells were fixed at appropriate time intervals and stained for T and V antigen. Fig. 6 correlates times required for detectable expression of viral antigens after microinjection or after conventional virus adsorption at a MOI of 100 PFU per cell.

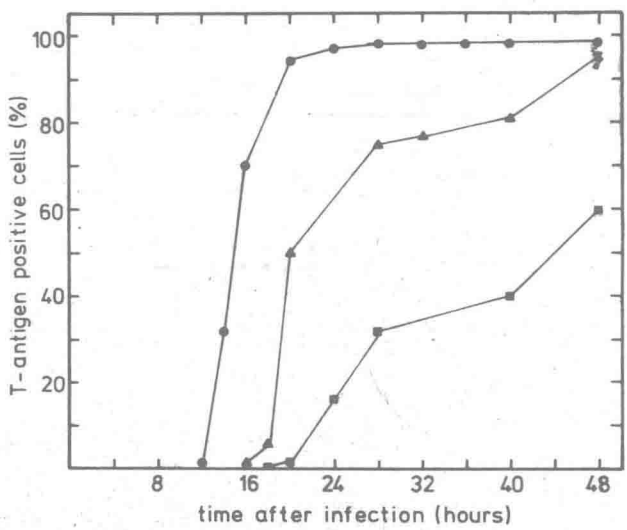

Fig. 4. Time course of SV40 T antigen synthesis in CV1 monkey cells infected with 100 (●), 10 (▲), or 1 (■) PFU per cell. Reproduced with permission from *Graessmann* et al. (1978)

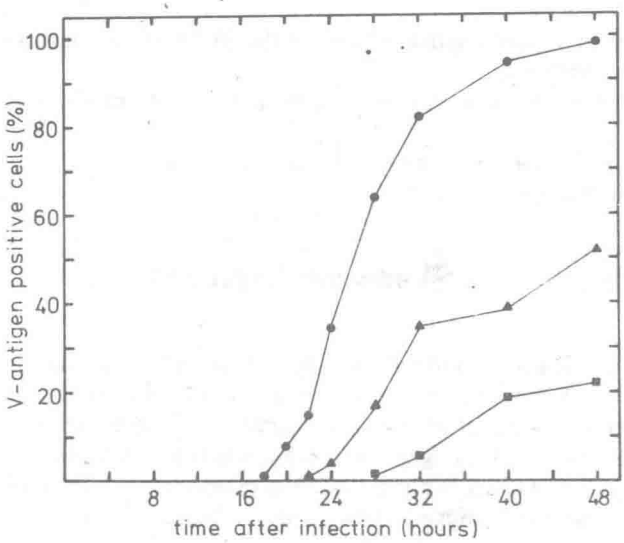

Fig. 5. Time course of SV40 V antigen synthesis in CV1 monkey cells infected with 100 (●), 10 (▲), or 1 (■) PFU per cell. Reproduced with permission from *Graessmann* et al. (1978)

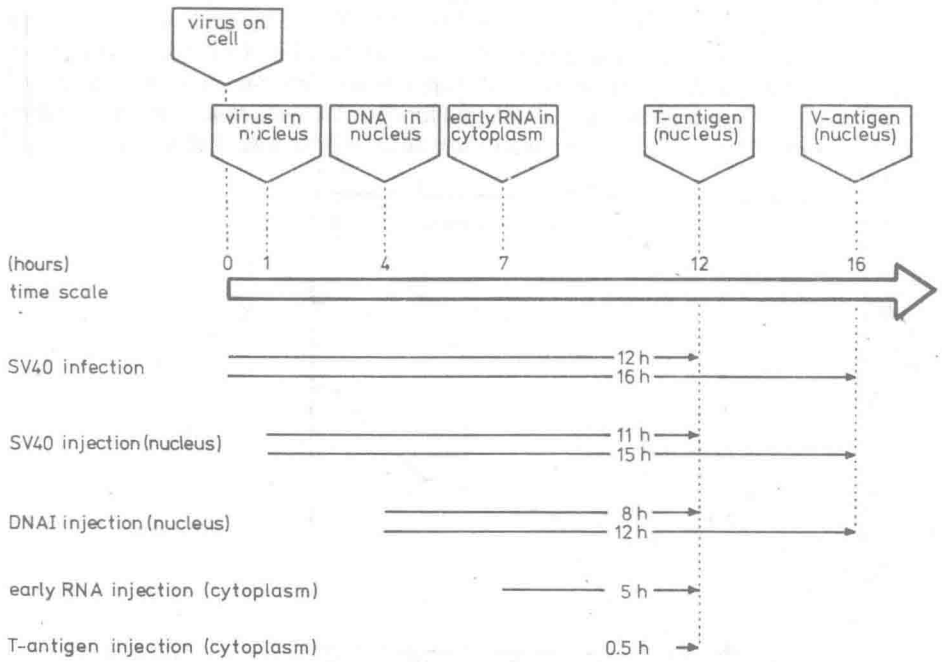

Fig. 6. Temporal sequence of SV40-specific events in TC7 monkey cells. Times of first appearance of intranuclear SV40-specific antigens after inoculation of virus, viral DNA, viral RNA or T antigen are given below the time scale. The temporal sequence depicted above the time scale is deduced from these experimental observations

Table 1. Synthesis of SV40- or PV-specific antigens in semipermissive cells at different times after infection

Cells derived from	Percentage of SV40 antigen-positive cells at (hours after infection):						Percentage of PV antigen-positive cells at (hours after infection):					
	24		48		96		24		48		96	
	T	V	T	V	T	V	T	V	T	V	T	V
Hamster [a]	0	0	0.01	0	0	0	0.01	0	0.5	0	2	0
Rat [b]	0	0	5	<0.001	ND [d]	ND [d]	1	0	2	0.01	5	0.1
Human [c]	0.5	0	8	0.5	10	2	0	0	0	0	<0.001	0

[a] Baby hamster kidney cells, passage 10
[b] Embryonic rat cells (Wistar strain), passages 3–6
[c] Human Wi38 cells
[d] Not done

4 SV40 and PV Gene Expression in Semipermissive Cells

Table 1 is a summary of data showing that cells known to be semipermissive for SV40 or PV (*Tooze*, 1973) synthesize little or no virus-specific protein at various times after infection with MOIs of 100 PFU per cell: 2 h after infection, cells were washed with PBS and incubated in Dulbecco's MEM supplemented with 10% FCS at 37° C until fixation. This nonsusceptibility of the majority of semipermissive cells may be caused either by failure of a) very early event(s) of the infection cycle (adsorption, penetration, intracellular release) or b) by a subsequent regulatory step. To discriminate between these possibilities, SV40 or PV DNA I was microinjected into cells of different semipermissive lines and found to induce synthesis of viral T antigens with high efficiencies even at multiplicities of microinjection (MOM) as low as 20–40 DNA I molecules per cell. In contrast to conventionally infected semipermissive cells, cells microinjected with viral DNA strongly supported late viral gene expression (V antigen synthesis) within 24 h after transfer (Tables 2 and 3).

Table 2. Synthesis of SV40-specific antigens in semipermissive cells fixed and stained 24 h after microinjection with SV40 DNA I

Cells derived from	Percentage of SV40 antigen-positive cells after transfer of (no. of DNA I molecules injected per cell):					
	2.000–4.000		200–400		20–40	
	T	V	T	V	T	V
Hamster[a]	99	12	78	3	20	0
Rat[b]	82	12	76	2	25	0
Human[c]	99	55	99	25	99	1

[a] Baby hamster kidney cells, passage 10
[b] Embryonic rat cells (Wistar strain), passages 3–6
[c] Human Wi38 cells

Table 3. Synthesis of PV-specific antigens in semipermissive cells fixed and stained 24 h after microinjection with PV DNA I

Cells derived from	Percentage of PV antigen-positive cells after transfer of (no. of DNA I molecules injected per cell):					
	2.000–4.000		200–400		20–40	
	T	V	T	V	T	V
Hamster[a]	80	32	80	2	30	0
Rat[b]	99	50	99	40	45	10
Human[c]	95	10	80	0	ND	ND

[a] Baby hamster kidney cells, passage 10
[b] Embryonic rat cells (Wistar strain), passages 3–6
[c] Human Wi38 cells

5 SV40 Gene Expression in Nonpermissive Mouse Cells

Mouse cells are nonpermissive for SV40, supporting early but not late viral gene expression. Up to 95% of primary mouse kidney cells or 3T3 cells (a continuous mouse cell line) infected with 500 PFU per cell accumulate T antigen during the first 64 h after infection and are stimulated for cellular DNA synthesis, but capsid protein synthesis is not demonstrable in these cells (*Graessmann* et al., 1978). However, late SV40 gene expression occurred in mouse cells microinjected with either virus particles or SV40 DNA I. At least 125–250 SV40 genome equivalents were required for late viral gene expression and efficient virus production (*Graessmann* et al., 1976a; *Graessmann* et al., in preparation). With this MOM, about 4% of the recipient cells synthesized V antigen in detectable amounts. After microinjection of 2,000–4,000 DNA I molecules per cell, 32% of them stained positively for V antigen 48 h later (Table 4).

To exclude the possibility that this gene-dose effect was due to an unspecific degradation of the injected DNA, SV40 DNA was mixed with PV DNA in proportions ensuring that the absolute amount of DNA transferred remained constant. Under these conditions, the percentage of V antigen-positive mouse cells again correlated directly with the number of SV40 DNA molecules injected. The implications of this gene-dose dependence will be discussed in Section 10.

Table 4. SV40 V antigen formation in 3T3 cells microinjected with SV40 DNA or with a mixture of SV40 DNA and PV DNA[a]

Injection of SV40 DNA I			Injection of a mixture of SV40 and PV DNA I			
Conc. of SV40 DNA (μg/ml)	Ave. no. of injected DNA molecules/cell	V antigen %	Conc. of each DNA (μg/ml)		Ave. no. of injected DNA molecules/cell	V antigen %
			SV40	PV		
1,000	2,000–4,000	38	1,000	0	2,000–4,000	38
500	1,000–2,000	25	500	500	2,000–4,000	27
250	500–1,000	12	250	750	2,000–4,000	ND[b]
120	250–500	4	120	870	2,000–4,000	4
60	125–250	0	60	940	2,000–4,000	0

[a] Cells were fixed and stained 48 h after microinjection. Data reproduced with permission from *Graessmann* et al. (1976a)
[b] Not done

6 SV40- or PV-Resistant Cells

Resistance against virus infection can be a constitutional species-specific quality; acquired during the process of cell differentiation; or a property of virus-transformed cells.

Human cells are not susceptible to PV infection. Primary or secondary human embryonic cells infected with up to 1,000 PFU per cell do not synthesize any virus-specific protein and are not stimulated for DNA synthesis. A permanent line of human cells (Wi38), however, synthesized T but not V antigen after PV infection, although with a

very low frequency (Table 1). In contrast, human cells microinjected with either PV particles or viral DNA I supported T antigen synthesis with high efficiency. Moreover, 10% of the injected cells exhibited V antigen formation at an MOM of 2,000–4,000 DNA molecules per cell. At a lower input ratio only T antigen synthesis was detectable (Table 3). Stimulation of cell DNA synthesis as another event in the early phase of viral infection was tested in secondary human cells following microinjection of DNA I isolated from an early temperature-sensitive PV mutant, SP2. Table 5 shows that stimulation of DNA synthesis was clearly demonstrable even at the nonpermissive temperature of 41.5° C, at which viral DNA replication is blocked.

Table 5. Stimulation of DNA synthesis in secondary human embryonic cells at 37° C or 41.5° C after microinjection of SP2 DNA I

Cells incubated at[a]	Mode of inoculation	Percentage of T antigen-positive cells	Percentage of T antigen-positive cells stimulated for DNA synthesis
37° C	SP2 infection (100 PFU/cell)	0	0
	SP2 DNA I injection (2,000–4,000 molecules/cell)	95	88
	Mock injection (PBS)	0	0
41.5° C	SP2 infection (100 PFU/cell)	0	0
	SP2 DNA I injection (2,000–4,000 molecules/cell)	95	73
	Mock injection (PBS)	0	0

[a] After infection/microinjection, cells were incubated in serum-free medium containing 0.5 μCi ^{3}H-thymidine/ml for 24 h, then fixed, stained, and processed for autoradiography. Data corrected against ^{3}H-thymidine background incorporation of 4% at 37° C and 1% at 41.5°C

Multinucleated muscle cells (myotubes) are formed in vivo and in tissue culture by fusion of mononucleated myoblasts. These postmitotic cells do not synthesize DNA at all. Although rat myoblasts are susceptible to SV40 or PV infection, they acquire resistance during the process of differentiation and cell fusion (*Fogel* and *Defendi*, 1967). Microinjection experiments unequivocally demonstrated that this resistance to infection is caused by a step before intracellular virus decapsidation, since SV40 or PV T antigens were efficiently synthesized in rat myotubes after microinjection of full virus particles or of viral DNA I molecules, as shown for PV in Table 6. In contrast to the "PV-resistant" human cells, stimulation of cell DNA synthesis or late viral gene expression was not obtained in microinjected rat myotubes. However, when virus inoculation had occurred at an earlier stage of muscle cell differentiation, at the myoblast state, terminally differentiated myotubes derived from these precursor cells showed cellular DNA synthesis and mitotic activity (*Graessmann* et al., 1973).

SV40- and PV-transformed cells are generally resistant to superinfection by the transforming virus. The mechanism of this resistance is not understood. The restriction

is again overcome upon microinjection of full particles of the transforming virus or of its DNA. During recent years we have tested ten different SV40- or PV-transformed cell lines and found them to support late viral gene expression upon microinjection, as shown for three SV40-transformed cell lines in Table 7.

Table 6. Synthesis of PV-specific antigens in rat myotubes [a] after microinjection [b] of full PV particles or PV DNA I

Full PV particles				PV DNA I			
Virus titer (PFU/ml)	No. of particles injected per cell	Percentage of PV antigen-positive cells		DNA I concentration (mg/ml)	No. of DNA molecules injected per cell	Percentage of PV antigen-positive cells	
		T	V			T	V
10^{11}	100–200	63	0	0.1	100–200	80	0
10^{10}	10–20	24	ND	0.01	10–20	71	ND [c]
10^{9}	1–2	0.1	ND	0.001	1–2	2	ND [c]

[a] Rat myotubes were obtained from 16- to 18-day rat embryos as described elsewhere (*Graessmann* et al., 1973)
[b] Cells were fixed and stained 48 h after microinjection
[c] Not done

Table 7. Late SV40 gene expression in SV40-transformed cell lines after microinjection of SV40 DNA I

Cell line (origin)	Percentage of SV40 V antigen-positive cells after [a]			
	Infection with SV40 (500 PFU/cell)	Microinjection of SV40 DNA I (no. of DNA I molecules injected per cell)		
		2.000–4.000	200–400	20–40
SVT2 (mouse)	0	12	7	0
SV80 (human)	0	18	2	ND [b]
14-B (rat)	0	6	ND [b]	ND [b]

[a] Cells were fixed and stained 48 h after infection or microinjection
[b] Not done

7 Conclusion 1

The data summarized in the preceding sections clearly demonstrate that one or more of the very early infectious events preceding virus uncoating: adsorption, penetration, intracellular release of virus, play(s) a significant role in the mode of response to SV40 or PV infection in different cell types.

8 SV40 T Antigen

SV40 and PV T antigens (large T antigens) both have an apparent molecular weight of about 90 kilodaltons. Subclasses of T antigens sharing sequences with the large species (medium, small) have recently been isolated and characterized in different laboratories (*Prives* et al., 1977; *Prives* and *Beck*, 1977; *Paucha* et al., 1977; *Tuerler* and *Salomon*, 1977; *Ito* et al., 1977), but in this review we will refer mainly to the functions of the large SV40 T antigen. The first experimental evidence that this protein is indeed virus-coded was obtained in microinjection experiments with early SV40-specific RNA transcribed in vitro from SV40 DNA I with *E. coli* DNA-dependent RNA polymerase. Cells of the native host (TC7) and nonpermissive primary mouse kidney cells efficiently synthesized SV40-specific T antigen even in the presence of actinomycin D upon microinjection of this RNA preparation (Table 8). These results, together with the results obtained in appropriate controls, clearly proved that SV40 T antigen is a virus-coded protein (*Graessmann* et al., 1974; *Graessmann* and *Graessmann*, 1976). Comparable results were obtained with PV (*Graessmann* et al., 1976b). Our data were further confirmed in other laboratories by in vitro translation of early viral RNA (*Prives* et al., 1977; *Greenblatt* et al., 1976; for review, see *Fried* and *Griffin*, 1977).

Table 8. SV40 T antigen formation in primary mouse kidney cells[a]

Injection of	% Cells with T antigen formation
SV40 cRNA (0.5 mg/ml)	41
SV40 cRNA (0.5 mg/ml) in actinomycin D-treated cells	41
SV40 cRNA (0.5 mg/ml) in cycloheximide-treated cells	0
SV40 DNA I (1 mg/ml) in actinomycin D-treated cells	0
SV40 DNA I (0.1 mg/ml) TC7 cell RNA (1 mg/ml) in actinomycin D-treated cells	0
SV40 cRNA, RNase treated	0
TC7 cell RNA	0

[a] Cells were fixed and stained 15 h after microinjection. Data reproduced with permission from *Graessmann* and *Graessmann* (1976)

SV40 T antigen is a multifunctional protein (*Weil* et al., 1974). Several functions can be attributed to this protein, although mechanistic details are still obscure:

- Stimulation of host-cell DNA synthesis (*Graessmann* and *Graessmann*, 1976; *Tjian* et al., 1978)
- Induction of viral DNA replication, and thus mediation of late viral gene expression (*Tegtmeyer*, 1972; *Cowan* et al., 1973; *Graessmann* et al., 1977a)

- Helper function for adenovirus 2 in monkey cells (*Grodzicker* et al., 1974, 1976; *Tjian* et al., 1978)
- Initiation and maintenance of the transformed state (*Tegtmeyer*, 1975; *Martin* and *Chou*, 1975; *Osborn* and *Weber*, 1975; *Brugge* and *Butel*, 1975).

In addition, SV40 T antigen contains another antigenic determinant, U antigen (*Kelly* and *Lewis*, 1973), and may regulate its own production (*Reed* et al., 1976).

9 Correlation of T Antigen Quality with its Function

SV40 T antigen induces viral DNA replication, a process needed for late viral gene expression. This was demonstrated in experiments involving infection of permissive cells with early temperature-sensitive (tsA) mutants at the nonpermissive temperature. These cells are stimulated for cellular DNA synthesis but viral DNA replication or late SV40 gene expression is not demonstrable (*Tegtmeyer*, 1972; *Cowan* et al., 1973; *Martin* and *Chou*, 1975). This result suggests a correlation of T antigen quality to its proper function for viral DNA replication.

To test whether this and other T antigen-specific functions can be assigned to defined regions of the early viral genome, we prepared various early SV40 DNA fragments by digestion of DNA I with appropriate restriction endonucleases, as depicted in Fig. 7. We tested TC7 cells microinjected with the purified fragments for the following

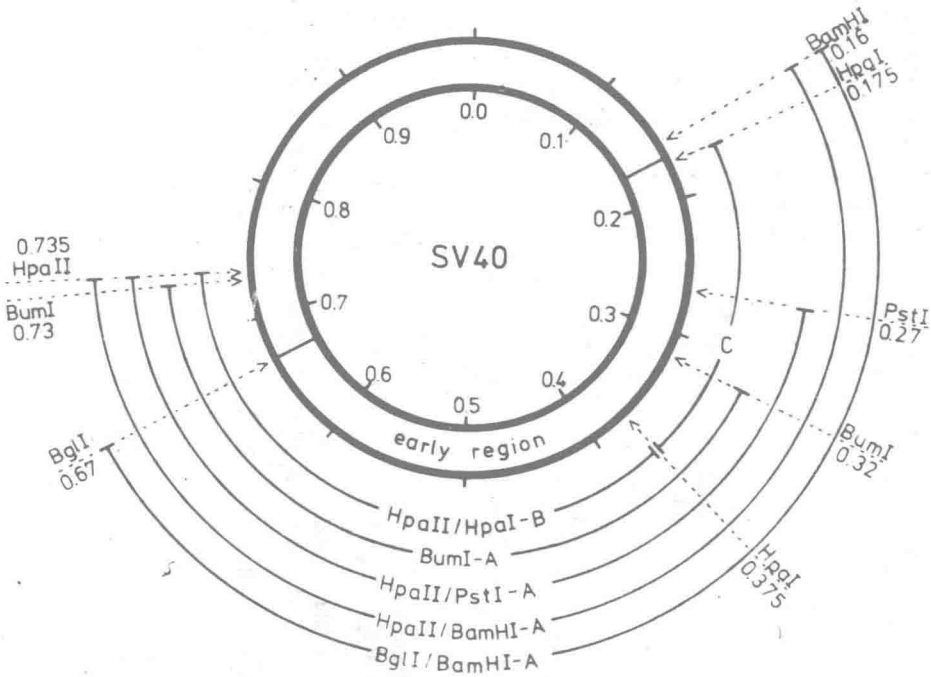

Fig. 7. Assignment of DNA fragments used for microinjection (Table 9) to the physical map of the SV40 genome. Fragments were purified by twofold agarose gel electrophoresis. Reproduced with permission from *Müller* et al. (1978)

Table 9. Early virus-specific functions in TC7 cells injected with various SV40 nucleic acids[a]

SV40 Nucleic acid injected			Percentage of Antigen-positive injected cells			Injected cells positive for		
Type	Concentration mg/ml	Percentage of early genome	T	U	V	T antigen-positive cells incorporating ^{3}H-thymidine[b]	Complementation of tsA7 or tsA58 at 41.5°C[c]	Helper function for Ad2 virus[d]
DNA I	0.1	100	100	91	100	94	ND	85
cRNA	0.5		72	64	0	93	45	ND
HpaII/HpaI-B	0.5	58	61	0	0	3	0	0
BumI-A	0.5	69	63	0	0	34	0	0
HpaII/PstI-A	0.5	80	65	0	0	84	0	0
HpaII/BamHI-A	0.5	100	64	55	0	91	39	52
BglI/BamHI-A	0.5	100	0	0	0	0	0	0
cRNA (BglI/BamHI-A)	0.5	-	36	17	0	89	ND	ND
HpaII/HpaI-C	0.5	42	0	0	0	0	0	ND

[a] Data reproduced with permission from *Müller* et al. (1978). Cells were fixed and processed for evaluation 24 h after microinjection
[b] Identical results were obtained in primary mouse kidney cells. Values are corrected against a background incorporation of 3%
[c] Percentage of cells positive for SV40 V antigen
[d] Percentage of cells positive for Ad2 fiber protein