The uptake and stability of Simian-virus-40 DNA after calcium phosphate transfection of CV-1 cells

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The uptake and fate of purified SV40 (Simian virus 40) DNA, transfected into permissive CV-1 cells by calcium phosphate precipitates, was examined. By using a viral plaque assay, optimum conditions for transfection were established and transfection efficiencies of up to 10^6 plaque-forming units/ μ g of SV40 DNA were obtained. After a 2 h exposure to ^3H -labelled SV40 DNA–calcium phosphate co-precipitates under basal conditions, up to 7% of the input DNA became cell-associated, with approx. 4% reaching the nuclear fraction. This value was never exceeded, even under conditions known to enhance significantly the ultimate transfection efficiency, such as increased exposure time, addition of carrier DNA or treatment with DMSO (dimethyl sulphoxide) or glycerol. Substantial degradation of this SV40 DNA occurred within a further 4 h, apparently in both nucleus and cytoplasm. Degradation of form-II and form-III SV40 DNA, which have lower transfection efficiencies than form-I DNA, was no more rapid than degradation of form-I DNA. The results indicate that less than 0.5% of the transfected DNA which reached the nucleus is protected from nuclease attack. The mechanism of action of agents such as glycerol, DMSO or carrier DNA remains obscure, but they may be involved in conferring greater stability to the intracellular SV40 DNA rather than merely affecting its rate of entry into the cell.

It is now well-established that DNA that enters mammalian cells with the aid of facilitators such as DEAE-dextran or calcium phosphate can be stably integrated into the host-cell genome and expressed at both the transcriptional and translational levels (Pellicer *et al.*, 1980; Scangos & Ruddle, 1981). The overall transformation efficiency of these assays, however, is extremely low, and the precise reason for this is unclear, notably because little is known of the events after internalization but preceding integration and expression.

Mammalian cells do contain many nuclease

enzymes capable of degrading nucleic acids (Sierakowska & Shugar, 1977; Nakamura *et al.*, 1981), but their physiological role is poorly understood. It is therefore of some interest to determine to what extent these enzymes can destroy 'foreign' DNA after its facilitated entry into cells and whether there are structural features of such transfected DNA which favour intracellular stability.

The uptake and immediate fate of radioactively labelled DNA in mammalian cells have been examined (Howard *et al.*, 1971; Groneberg *et al.*, 1975; Nicolau & Sene, 1982), but in most studies the ultimate stability and expression of the conserved DNA has not been monitored. Loyer *et al.* (1982) have investigated the uptake of whole genomic DNA into mouse L-cells, by the calcium phosphate transfection technique. That study yielded some useful information regarding the mechanism of uptake of such transfers, but again the system did not permit an assessment of the stability and expression of that DNA.

Here we have examined the stability of purified SV40 DNA after calcium phosphate transfection of permissive African-Green-monkey kidney (CV-

Abbreviations used: SV40, Simian virus 40; DMSO, dimethyl sulphoxide; DMEM, Dulbecco's modified Eagle's minimal essential medium; SDS, sodium dodecyl sulphate; Hepes-buffered saline, 5 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid]/280 mM-NaCl/1.5 mM- Na_2HPO_4 , pH 7.15; phosphate-buffered saline, 140 mM-NaCl/2.7 mM-KCl/8 mM- Na_2HPO_4 /1.5 mM- NaH_2PO_4 , pH 7.4; Hewish–Burgoyne Buffer A, 20 mM-NaCl/60 mM-KCl/0.5 mM-EDTA/0.1 mM-EGTA/0.5 mM-spermidine/0.15 mM-spermine/15 mM-Tris/HCl, pH 7.4; PFU, plaque-forming units; DNAase, deoxyribonuclease.

1) cells. The immediate fate of DNA was followed by radioisotope tracer techniques and ultimate stability assessed by the ability of the DNA to initiate lytic cycles of infection in the recipient cells.

Experimental

Cells

CV-1 cells [derived from African-Green-monkey (*Ceropithecus aethiops*) kidney] were cultured in DMEM, supplemented with 10% (v/v) heat-inactivated newborn-bovine serum (both from Gibco, Paisley, Renfrewshire, Scotland, U.K.). Normally cells were plated in 5cm Petri dishes at a concentration of 4×10^5 cells/5ml, 48 h before each experiment, and were 'refed' with fresh medium 1–2h before addition of calcium phosphate precipitates.

Preparation of SV40 DNA

CV-1 cells were infected with wild-type plaque-purified SV40 virus at a low multiplicity of infection (0.01) on day 1, and DNA was labelled with [^3H]thymidine (5 $\mu\text{Ci/ml}$; The Radiochemical Centre, Amersham, Bucks., U.K.) between day 7 and day 12. Monolayers were then lysed in 1% SDS/20mM-Tris/HCl/10mM-EDTA/1M-NaCl (Hirt, 1967) and form-I SV40 DNA harvested and purified by CsCl-buoyant-density centrifugation (Bauer & Vinograd, 1968). The specific radioactivity of the recovered SV40 DNA was up to 10⁵ c.p.m./ μg . Closed circular supercoiled (form-I) DNA was separated from nicked circular relaxed (form-II) and linear (form-III) DNA by centrifugation through 10–30% (w/v) neutral sucrose gradients (16h at 150000g, 4°C, Beckman SW 41 Ti rotor). SV40 DNA preparations and restriction-enzyme digests were run on 1.4% (w/v) agarose gels and purity assessed by fluorography after impregnation of gels with diphenyloxazole (Laskey *et al.*, 1977). Samples for analysis on denaturing gradients were pretreated for 15min in 0.5M-NaOH at 4°C and were then loaded on to 5–25% sucrose gradients (1M-NaCl/0.25M-NaOH/1mM-EDTA). They were centrifuged at 270000g for 90min at 20°C in a Beckman SW60 Ti rotor.

Calcium phosphate transfection

Transfection of cells was carried out by the technique originally described by Graham & Van der Eb (1973) and modified by Wigler *et al.* (1978). SV40 DNA dissolved in 10mM-Tris/HCl/0.1mM-EDTA, pH 7.9, was adjusted to 250mM-CaCl₂ and was added dropwise to an equal volume of 2 × Hepes-buffered saline. After 45min, the resulting co-precipitate was added to cell monolayers (0.5ml

to 5ml of medium) and incubated at 37°C. When carrier DNA was required, sheared high-*M*_r salmon spermatozoa or calf thymus DNA (both from Sigma, Poole, Dorset, U.K.) was added to the SV40 DNA buffer at a concentration of 40 $\mu\text{g/ml}$. Cells were refed with fresh medium at the appropriate time and plaque assays carried out as described by Mertz & Berg (1974). Where indicated, immediately after exposure to calcium phosphate, cells were washed once with phosphate-buffered saline and were then treated at room temperature for 2min with 2ml of 25% (v/v) DMSO in serum-free DMEM (Stow & Wilkie, 1976) or for 4min with 25% (v/v) glycerol (Frost & Williams, 1978). Monolayers were then carefully washed a further three times with phosphate-buffered saline, fresh medium added and plaque assays carried out.

Recovery of ^3H -labelled SV40 DNA

After exposure to ^3H -labelled SV40 DNA-calcium phosphate co-precipitates, cells plated in 10cm-diameter tissue-culture dishes were washed three times with phosphate-buffered saline and were then treated with DNAase I (5 $\mu\text{g/ml}$; Boehringer Mannheim, Lewes, Sussex, U.K.), dissolved in phosphate-buffered saline supplemented with 1mM-MgCl₂, at 37°C for 30min. After careful washing in phosphate-buffered saline, the cells were lysed in 2ml of Hewish-Burgoyne (1973) Buffer A (Johnson *et al.*, 1980). In some experiments this was further supplemented with sheared calf thymus DNA (25 $\mu\text{g/ml}$) to reduce non-specific adsorption of [^3H]DNA to cellular structures. Cells were lysed by gentle rocking and the supernatant (*S*₁) containing soluble membranes and cytoplasm was removed (Johnson *et al.*, 1980). Electron microscopy of the residual monolayer showed it to consist of nuclei lacking the outer membrane laminae. Although there was also some fibrillar material of presumed cytoskeletal origin (Savion *et al.*, 1981), recognizable cytoplasmic organelles were not observed. No improvement in the purity of this nuclear fraction was obtained by further centrifugation through sucrose cushions (Savion *et al.*, 1981) and this step was not included routinely. After careful washing (three times) in Hewish-Burgoyne Buffer A, the residual monolayer was solubilized in 10mM-EDTA/1% SDS/0.5mM-NaCl/20mM-Tris/HCl, pH 7.4 (Hirt, 1967). Samples were collected in silicone-treated glass centrifuge tubes, left overnight at 4°C and then centrifuged at 11000g for 30min. The supernatant (*S*₂), containing free nuclear SV40 DNA, was removed and the pellet (*P*) of host chromatin taken up in 10mM-Tris/1mM-EDTA/1% SDS, pH 7.4. SV40 DNA samples were concentrated by precipitation in 67% (v/v) ethanol/0.13M-NaCl overnight at 4°C.

Fig. 1. Transfection efficiency time course of SV40-DNA-calcium phosphate co-precipitates

Precipitates were prepared as described in the Experimental section and were added to cultures after 45 min (0.5 ml to 5 ml of medium). Cells were incubated at 37°C for the times indicated and were then refed with fresh culture medium. They were agar-overlaid the following day and lytic viral plaques were scored 14 days later. Each point represents the mean $\pm$ S.D. for six cultures.

Results

Optimum conditions for calcium phosphate transfection of SV40 DNA

The aim of our initial experiments was to establish optimum conditions for transfection of purified SV40 DNA into permissive CV-1 cells. Transfection efficiency was found to rise progressively when the exposure time of cells to calcium phosphate was increased (Fig. 1). Although in low-efficiency transformation assays overnight incubation is often described (Graham *et al.*, 1980; Davies *et al.*, 1982), for most experiments here a relatively short exposure time of 2 h was chosen to facilitate subsequent studies of the fate of internalized DNA.

The transfection efficiency of SV40 DNA under a variety of treatments is shown in Table 1. In contrast with the original findings of Graham & Van der Eb (1973) the addition of carrier DNA was not required for the successful transfection of SV40 DNA with calcium phosphate. Indeed, in our hands, transfection efficiencies of up to 10^4 PFU/ μ g (approx. 10 plaques/5 cm dish per ng of SV40 DNA) were achieved. When an excess (20 μ g) of carrier DNA was included during co-precipitation, however, the transfection efficiency was consistently enhanced (Table 1). Exposure of cells to glycerol or DMSO after the incubation period further increased the transfection efficiency to around 8×10^5 PFU/ μ g. Treatment with these agents, however, increased the risk of detach-

Table 1. Transfection efficiency of SV40 DNA-calcium phosphate co-precipitates after various treatments

Form-I SV40 DNA-calcium phosphate co-precipitates were prepared with or without sheared calf thymus or salmon spermatozoa DNA as carrier (final concn. 40 μ g/ml). Precipitates were added to cell monolayers and after a 2 h incubation period, cells were washed with phosphate-buffered saline and treated with 2 ml of serum-free DMEM alone or DMEM containing 25% DMSO or 25% glycerol. Dishes were again washed in phosphate-buffered saline, refed with medium and incubated for a further 14 days for viral-plaque assays. Results are expressed as means $\pm$ S.D. for six cultures.

Post-transfection treatment	10 ⁻³ × Transfection efficiency (PFU/ μ g of SV40 DNA)	
	SV40 DNA	SV40 DNA + carrier DNA
None	8.6 $\pm$ 0.3	52.7 $\pm$ 2.0
DMSO (25%)	154.2 $\pm$ 14.7	786.7 $\pm$ 4.7
Glycerol (25%)	202.5 $\pm$ 7.9	820.0 $\pm$ 29.4

ment of the monolayer from the dish. These values are considerably higher than previously described for calcium phosphate transfection of SV40 DNA (Graham & Van der Eb, 1973) and compare favourably with the DEAE-dextran technique (Mertz & Berg, 1974; McCutchan & Pagano, 1968; Mantel *et al.*, 1975).

Transfection efficiency of DNA in different configurations

We next compared the transfection efficiency of SV40 DNA in different configurations (Table 2). Form-I DNA was three times more effective than form-II and 30 times more effective than form-III DNA. Carrier DNA was noted to enhance efficiency of all DNA configurations (Table 2), as were further treatments with glycerol and DMSO (results not shown). The sensitivity of the method used to monitor purity of DNA preparations was sufficient to exclude the possibility that lytic plaques observed after transfection of form-II or form-III DNA were due to incomplete digestion and contamination by form-I DNA.

Fate of ³H-labelled SV40 DNA after uptake

The recovery of [³H]DNA from cytoplasmic (S₁) and nuclear (S₂) fractions after calcium phosphate transfection is shown in Fig. 2(a). After a 2 h exposure, up to 7% of the input DNA became cell-associated, with 4.5% reaching the nuclear fraction. In cells mock-transfected with microgram quantities of ³H-labelled SV40 DNA in the absence of calcium phosphate, previously shown to be ineffective in initiating lytic infections (A. J. Strain, un-

Table 2. *Effects of SV40 DNA configuration on calcium phosphate-mediated-transfection efficiency*
Form-I and form-II SV40 DNA were separated by neutral-sucrose-gradient centrifugation and form-III DNA was prepared by digestion of form-I DNA with restriction endonucleases *Eco*R1 or *Bam*HI (37°C for 120 min). Calcium phosphate-DNA co-precipitates were incubated with cells for 2 h. Results are expressed as means $\pm$ s.d. for six cultures. Abbreviations: ND, not determined.

DNA configuration	Carrier DNA ...	$10^{-3} \times$ Transfection efficiency (PFU/ μ g of SV40 DNA)			
		Expt. 1		Expt. 2	
		-	+	-	+
Form I		14.60 $\pm$ 0.87	81.50 $\pm$ 0.87	ND	ND
Form II		5.05 $\pm$ 0.63	29.67 $\pm$ 0.18	ND	ND
Form III					
<i>Eco</i> R1		0.49 $\pm$ 0.09	2.05 $\pm$ 0.29	0.17 $\pm$ 0.04	2.13 $\pm$ 0.20
<i>Bam</i> HI		ND	ND	0.23 $\pm$ 0.02	2.57 $\pm$ 0.51

Fig. 2. *Recovery of 3 H-labelled SV40 DNA from CV-1 cells after calcium phosphate transfection*
(a) Monolayers were transfected with 3 H-labelled SV40 DNA for 2 h. They were then washed and lysed in two stages, immediately or after a further 2 h or 4 h, to yield a cytoplasmic fraction (S_1), a nuclear fraction (S_2) and an insoluble host chromatin pellet (P). (b) and (c) Monolayers transfected for 4 h or 6 h respectively and lysed immediately thereafter. Results are expressed as a percentage of total input DNA and represent means $\pm$ s.d. for three experiments.

published work; Graham & Van der Eb, 1973), [3 H]DNA uptake never exceeded the background level (result not shown).

To ensure that labelled DNA recovered from S_1 represented cytoplasmic internalized DNA and not simply DNA adsorbed on to the outer membrane of the cell, monolayers were treated with DNAase I for 30 min before lysis. In view of the recent report that calcium phosphate affords HeLa cell DNA some degree of protection from nuclease attack (Loyter *et al.*, 1982), we tested the ability of DNAase I to digest SV40 DNA in the presence of calcium phosphate precipitates (Figs. 3a-3c). Although the degradation products of 3 H-labelled SV40 DNA under these conditions remained

ethanol-precipitable, it is clear from the alkaline-sucrose-gradient profile that SV40 form-I and form-II DNA were substantially degraded to smaller DNA fragments that were able to enter only the top portion of the gradient (Fig. 3c). In addition, [3 H]DNA added to cells at the end of the 2 h exposure to calcium phosphate, but before DNAase I treatment, was almost completely degraded, with less than 1% of the label recovered in the S_1 fraction (results not shown). Treatment with EDTA and co-digestion with micrococcal nuclease and DNAase I, as employed by Loyter *et al.* (1982), was no more effective. To exclude the possibility that radioactivity found in the nuclear fraction (S_2) resulted from non-specific adsorption

Fig. 3. Alkaline-sucrose-gradient profiles of ${}^3\text{H}$ -labelled SV40 DNA

Input SV40 DNA showing a large form-I peak and a smaller peak including form-II and form-III DNA (a). Recovery profiles of calcium phosphate-precipitated ${}^3\text{H}$ -labelled SV40 DNA after incubation in serum-free medium in the absence (b) or presence (c) of DNAase I ($5\text{ }\mu\text{g/ml}$) for 30 min at 37°C . After incubation, calcium phosphate was dispersed by addition of 10 mM -EDTA, and SV40 DNA was recovered from the supernatants by precipitation with ethanol. Arrows indicate the positions run in the gradient by form-I and form-II SV40 DNA.

Table 3. Recovery of form-I ${}^3\text{H}$ -labelled SV40 DNA from cells after various treatments

Cultures were transfected for 2 h with ${}^3\text{H}$ -labelled SV40 DNA with or without sheared calf thymus or salmon spermatozoa DNA as carrier and were then treated with DMEM alone, or DMEM containing 25% DMSO or 25% glycerol. They were then washed and lysed in two stages to yield a cytoplasmic fraction (S_1), a nuclear fraction (S_2) and an insoluble host chromatin pellet (P).

Uptake of ${}^3\text{H}$ -labelled SV40 DNA (%)

Fraction	Treatment ...	None	Glycerol	DMSO	Carrier DNA
S_1		1.75 ± 0.30	1.72 ± 0.10	1.28 ± 0.02	0.38 ± 0.05
S_2		1.96 ± 0.13	2.66 ± 0.16	2.77 ± 0.11	0.26 ± 0.02
P		0.27 ± 0.01	0.33 ± 0.03	0.36 ± 0.04	0.05 ± 0.01

of cytoplasmic [${}^3\text{H}$]DNA during extraction, ${}^3\text{H}$ -labelled SV40 DNA was added with or without excess calf thymus DNA at the time of cytoplasmic lysis. Under these conditions, less than 0.05% of the applied DNA was subsequently recovered in S_2 .

Cells treated with [${}^3\text{H}$]DNA and calcium phosphate for 2 h and then washed, progressively lost [${}^3\text{H}$]DNA from both S_1 and S_2 fractions (Fig. 2a). Indeed, loss of [${}^3\text{H}$]DNA from these fractions appeared to occur throughout the transfection period also, since cells exposed to the co-precipitate continuously for 4 or 6 h contained less SV40 DNA than did cells exposed for only 2 h (Figs. 2b and 2c), despite the fact that transfection efficiency increased with exposure time (Fig. 1).

The uptake of ${}^3\text{H}$ -labelled SV40 DNA in cells treated with glycerol or DMSO or in the presence of carrier DNA is shown in Table 3. Despite the substantial enhancing effect (up to 24-fold) of the agents on transfection efficiency (Tables 1 and 2), only a small increase (30–40%) in uptake was noted in the nuclear fraction (S_2) of the glycerol- and DMSO-treated groups, and it was actually decreased with carrier DNA. Furthermore, carrier DNA did not arrest the loss of [${}^3\text{H}$]DNA from the cellular fractions with time after transfection (results not shown). Finally, although associated with considerably decreased transfection efficiencies (Table 2), the uptake of form-II and form-III SV40 DNA was the same or even slightly higher than that of form-I DNA (Table 4).

Configuration of [^3H]SV40 DNA after transfection

To examine the configuration of DNA in fractions recovered from transfected monolayers, samples were concentrated by ethanol precipitation and analysed on alkaline sucrose gradients (Fig. 4). Input SV40 DNA used in these experiments was predominantly supercoiled form-I DNA, but with a small proportion of nicked circular form-II DNA (see Fig. 4c). Immediately after the 2h transfection period, intact supercoiled SV40 DNA was identified in both nuclear and cytoplasmic fractions (Fig. 4a). Comparison with the input material, however, clearly indicated extensive de-

gradation of form-I to form-II and linear DNA. The rate of degradation appeared to be slightly less rapid in the nuclear fraction (S_2) than in the cytoplasmic (S_1). This difference was not large, but did indicate that the configuration of DNA in S_1 and S_2 fractions was qualitatively different and hence could not merely represent adsorption of a pool of degraded DNA to different cellular constituents during extraction. Over the next 4h, form-I DNA became undetectable in both nuclear and cytoplasmic fractions, being replaced by forms II, III and heterogeneous small fragments (Fig. 4b). Agarose-gel electrophoresis confirmed that these fragments represented progressive digestion of SV40 DNA rather than fixed-length linear molecules.

During the extraction procedures monolayers were exposed to exogenous DNAase I. It was therefore important to show that the observed degradation was not due to residual nuclease activity incompletely removed after the treatment. This was demonstrated in parallel experiments in which monolayers were exposed to calcium phosphate alone, treated with DNAase I, washed and finally lysed in Hewish-Burgoyne Buffer A which contained ^3H -labelled SV40 DNA. After extraction, the added ^3H -labelled SV40 DNA retained the

Table 4. Recovery of SV40 DNA in different configurations

Cultures were transfected for 2h. Results are expressed as a percentage of total input DNA and represent the mean results for three dishes.

Fraction	SV40 DNA (form) ...	Uptake of ^3H -labelled SV40 DNA (%)		
		I	II	III
S_1		2.7	4.8	2.6
S_2		3.9	5.2	4.6
P		0.2	0.4	0.2

Fig. 4. Alkaline-sucrose-gradient profiles of ^3H -labelled SV40 DNA recovered from CV-1 cells after calcium phosphate transfection

Monolayers were lysed in two stages to give a cytoplasmic fraction S_1 (○) and a nuclear fraction S_2 (●) immediately after (a), or 4h after (b), the 2h transfection period. (c) ^3H -labelled SV-40 DNA was added to cells transfected with calcium phosphate alone at the time of the first iso-osmotic-lysis step and was subsequently recovered in the S_1 fraction. The profile shown here was identical with that obtained with the input ^3H -labelled SV40 DNA used in this experiment (results not shown).

original proportions of form I and form II as revealed by alkaline-sucrose-gradient analysis (Fig. 4c).

Discussion

It is clear from these studies that very large quantities of DNA are taken up by cells relative to the number of infective units which are ultimately detected by the viral-plaque assay. Our results are in agreement with those of Loyer *et al.* (1982), who reported that 7.5% of labelled DNA was taken up by cells transfected with calcium phosphate, and of Groneberg *et al.* (1975), who found, in addition, that 70% of adenovirus type 2 DNA (that is 4–5% of the total input) reached the nucleus of human cells within 2 h. These workers also found high levels of DNA uptake (7%) in the absence of facilitators such as DEAE-dextran or calcium phosphate. Under these conditions in the present study (mock transfection) we found DNA uptake to be negligible, and the discrepancy may reflect differences in the mechanism of DNA uptake in the two cell types. Although DNA transfection procedures clearly subject cells to non-physiological conditions, it is notable that a similar proportion of papova-virus DNA is recovered from the nucleus of cells soon after infection with intact virus (Barbanti-Brodano *et al.*, 1970; Frost & Bourgaux, 1975), when ultimately only about 1% of papova virions initiate replicative cycles of infection (Crawford *et al.*, 1962; Black *et al.*, 1964).

Cell-associated transfected DNA is clearly rapidly degraded. When the specific radioactivity of ^3H -labelled SV40 DNA and the sensitivity of the alkaline-gradient detection technique are taken into account, we have calculated that this method is capable of detecting approx. 10^2 molecules of supercoiled form-I SV40 DNA per cell. From our results presented in Fig. 2, it can be estimated that each nucleus would receive on average 0.5×10^5 copies of SV40 DNA. There is, thus, at least a 500-fold decrease in the number of DNA molecules present within a few hours of transfection, demonstrating that DNA introduced in this way is highly unstable even if it reaches the nucleus. Moreover, supercoiled DNA is degraded as rapidly as nicked circular or linear DNA.

The cellular site of DNA degradation is unknown. It is probable, however, that co-precipitates of DNA–calcium phosphate, like other particulate material ingested by cells (Goldstein *et al.*, 1979; Willingham & Pastan, 1980; Hopkins *et al.*, 1981) is initially sequestered within the endosome compartment. That intralysosomal digestion then ensues is suggested by the action of 'lysosomotropic' agents, which, by raising the intralysosomal pH, render lysosomal enzymes less effective (de

Duve *et al.*, 1974) and, at the same time, increase the efficiency of transfection (Fraley *et al.*, 1981; Luthman & Magnusson, 1983). Nonetheless, our data indicate that there is also degradation of transfected DNA which appeared to have arrived in the nucleus and was at any rate structurally distinguishable from DNA recovered from the rest of the cell.

Although this degradation is not surprising in view of the presence of nucleases within the nucleus of mammalian cells (Ishida *et al.*, 1974; Lambert & Studzinski, 1979; Nakamura *et al.*, 1981), it does indicate that most foreign DNA is susceptible to digestion whereas host genomic DNA remains unaffected by nuclease activity. The small proportion of transfected DNA which clearly is conserved may be more adequately protected from nuclease attack; alternatively it may be in a different configuration, or be distributed to different subcellular compartments.

Calcium phosphate precipitates appear to afford DNA some protection against nuclease digestion *in vitro* (Loyer *et al.*, 1982), but it is clear from our data that within cells this protection is largely ineffective. It is not known how long after internalization the DNA–calcium phosphate complex remains intact, and it is possible that the DNA which is conserved remains associated with the calcium phosphate crystals. If protection is due to binding of DNA to the surface of calcium phosphate crystals, however, one might expect carrier DNA in large excess to displace SV40 DNA from the putative protected sites, whereas carrier has the effect of significantly enhancing transfection efficiency. Different conformations of DNA, supercoiled, relaxed circular or linear, do show differing transfection efficiencies. By using the much more efficient method of micro-injection, Graessmann *et al.* (1976, 1981) have obtained similar results suggesting that DNA conformation has some role to play in determining intracellular stability. It is also evident, however, that most of the calcium phosphate-transfected DNA in all three configurations is rapidly degraded.

The possibility remains that a fraction of transfected DNA is conserved because it is located in a unique intracellular site. Such privileged sites might be present in all cells, but reached more readily as a result of treatment with agents such as glycerol or DMSO, which although enhancing transfection efficiency up to 20-fold, do not significantly increase the overall amount of DNA taken up into the cell. They might also be reached more readily after saturation of a nuclease-rich compartment, hence the effect of carrier DNA. Alternatively a small subclass of cells may be uniquely accessible to transfected DNA. Recent work has suggested that the latter possibility may be correct

(Sompayrac & Danna, 1981; Nicolau & Sene, 1982), but the evidence is far from conclusive. In view of the well-known requirement that cells be in the exponential ('log') phase of growth for optimum transformation efficiency (Todaro & Green, 1966), the possibility that the cells susceptible to transfected DNA represent a population in a particular phase of the cell cycle requires further investigation.

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