

Enhancement of DNA-mediated gene transfer by high- M_r carrier DNA in synchronized CV-1 cells

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Synchronized CV-1 cells were transfected with SV40 (simian virus 40) DNA–calcium phosphate co-precipitates. In the presence of carrier DNA, the transfection efficiency of SV40 DNA was decreased 5-fold in S-phase cells and was increased 4-fold in preparations of mitotically enriched cells as compared with asynchronous controls. No difference was observed when carrier DNA was omitted, when cells had progressed through S-phase and into G₂-phase, or when the infectivity of cells to intact SV40 virus was tested. These results highlight the importance of cell-cycle-dependent factors on DNA-mediated gene transfer.

The transfection of DNA into mammalian cells by calcium phosphate precipitates is now an extensively used technique and has led to many advances in our understanding of the control of eukaryotic gene transcription and of cellular transformation (Scangos & Ruddle, 1981; Spandidos & Wilkie, 1984). Surprisingly little is known, however, of the conditions within the cytoplasm and nucleus which influence the stability and hence expression of DNA taken up by cells. Factors that influence the stability of exogenous DNA within cells are of interest for two reasons. Firstly they may constitute a mechanism whereby cells are protected against indiscriminate entry of 'foreign' DNA, for example after viral infection. Secondly, study of the intracellular mechanisms concerned with degradation of 'foreign' DNA may shed light on the means whereby endogenous genomic DNA is conserved. We have previously reported that, although as much as 5–10% of calcium phosphate-transfected DNA is internalized by recipient cells and about half reaches the nucleus, most is rapidly degraded by intracellular endonucleases (Strain & Wyllie, 1984). Here we report the results of experiments with synchronized cells to determine whether the cell cycle plays a role in the efficiency whereby transfected

DNA is taken up and expressed by mammalian cells.

Methods

Preparation of SV40 DNA and calcium phosphate transfection of CV-1 cells was as previously described (Strain & Wyllie, 1984). Where cells were transfected in suspension, up to 10⁶ cells suspended in 5 ml of Dulbecco's modified Eagle's minimum essential medium +10% newborn-bovine serum were plated into bacteriological Petri dishes and 0.5 ml of calcium phosphate suspension was added. As preliminary experiments had shown that agitation resulted in lower transfection efficiencies, the dishes were left undisturbed during transfection at 37°C in a humidified 5% CO₂/air incubator. After 2 h, cells were harvested by centrifugation, washed and plated into secondary 5 cm tissue-culture dishes together with 5 × 10⁵ control cells, and viral plaque assays were performed as described by Strain & Wyllie (1984). The viability of cells, as assessed by Trypan Blue dye exclusion, was always greater than 95% throughout the incubation in suspension. Cells were synchronized at the G₁/S-phase boundary by a double-block treatment with hydroxyurea (Pfeiffer & Tolmach, 1967). Briefly, 0.5 mM-hydroxyurea was added to dishes for 24 h and then removed by washing three times in warm phosphate-buffered saline (140 mM-NaCl/2.7 mM-KCl/8 mM-Na₂HPO₄/1.5 mM-NaH₂PO₄, pH 7.4). The cells were re-fed with fresh Dulbecco's

Abbreviation used: SV40, simian virus 40.

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medium + 10% serum to release the arrested cells. After 12h, 0.5mM-hydroxyurea was added for a further 24h. The degree of synchrony after release from the second hydroxyurea block was assessed by autoradiography after a 30min pulse with 5 μ Ci of [³H]thymidine/ml. Cells were shown to be reversibly arrested and to begin DNA synthesis immediately on removal of hydroxyurea.

Mitotic cells were prepared by the technique of selective detachment described by Terasima & Tolmach (1963), modified to increase cell yield by incorporating a 'cold shock' period (Sinclair & Morton, 1963) and exposure to low-Ca²⁺-containing medium (Robbins & Marcus, 1964). Subconfluent monolayers of CV-1 cells plated in 175cm² tissue-culture flasks were fed with 50ml of Dulbecco's medium + 10% serum, with a final Ca²⁺ concentration of 1mM. Flasks were maintained for 1h at 4°C and then at 37°C for a further 4h. Cells were then harvested by shaking each flask vigorously five times, collected by centrifugation and transfected in suspension as described above. In some experiments, mitotic cells received a brief exposure to trypsin before transfection, as the suspensions of control cells were prepared by trypsin treatment of asynchronous monolayers. 'Cold shock' and low-Ca²⁺ treatment of asynchronous monolayers were not found to influence the subsequent response of cells to transfection with purified SV40 DNA or to infectivity with intact virus. The mitotic index of harvested cells was determined by light-microscopy of slides prepared in a cytospin centrifuge, fixed in 70% (v/v) ethanol for 1h and stained with aq. 1% (v/v) Giemsa.

Results

Transfection of S-phase cells

Cells were transfected for 2h with calcium phosphate-SV40-DNA co-precipitates prepared

in the absence or presence of an excess (40 μ g/ml) of sheared high-*M_r* calf thymus DNA. The results indicate that, in the presence of carrier DNA, immediately after release from the second 24h hydroxyurea block, the transfection efficiency of control asynchronous cells was 5-fold higher than that of S-phase cells (Table 1a). When cells were transfected in the absence of carrier DNA the transfection efficiency was, as expected, much lower, but no difference was observed between S-phase and asynchronous cells. The labelling index of cultures (percentage of cells incorporating [³H]-thymidine as determined by autoradiography) immediately after release was almost 90% (Table 1a) and, although this was found to decrease slowly, within the 2h transfection period over 70% of cells remained in S-phase (Fig. 1). Immediately after release from hydroxyurea treatment, there was no difference in the growth rate of treated cells compared with controls (doubling time approx. 24h). The plating efficiency was also unchanged (controls, 36.0 \pm 10.3%; S-phase, 30.6 \pm 4.9%).

We next determined the transfection efficiency of cells allowed to progress through the cell cycle for 12h after removal of hydroxyurea, when the labelling index had fallen to 7% (Fig. 1). Under these conditions, no difference was detected in the transfection efficiency of SV40 DNA in either the absence or the presence of carrier DNA (Table 1b). As the first mitotic cells were not detected until about 10h after release, we concluded that these cultures comprised cells mainly in the G₂-phase of the cell cycle.

Transfection of mitotic cells

Cells synchronized by hydroxyurea treatment were found to lose synchrony relatively quickly, and sufficiently enriched populations of mitotic cells were not obtainable by this method. We therefore prepared populations of cells in mitosis by the technique of selective mitotic-cell detach-

Table 1. *Transfection efficiency of SV40 DNA in cells treated with hydroxyurea*

CV1-cell monolayers were synchronized by a double-block treatment with 0.5mM-hydroxyurea. They were then transfected for 2h with calcium phosphate-SV40-DNA co-precipitates immediately (a) or 12h (b) after release from hydroxyurea treatment. Results are expressed as means \pm s.d. for the numbers of determinations given in parentheses. Abbreviation: PFU, plaque-forming units.

	10 ⁻³ \times Transfection efficiency (PFU/ μ g of SV40 DNA)		Labelling index (%)
	SV40 DNA	SV40 DNA + carrier DNA	
(a) Control	5.75 \pm 2.63 (9)	461.5 \pm 61.5 (6)	32.1 \pm 5.4 (8)
Hydroxyurea (0h)	6.04 \pm 1.56 (9)	88.4 \pm 23.5 (9)	88.9 \pm 5.9 (8)
(b) Control	9.40 \pm 1.81 (6)	903.3 \pm 249.7 (6)	22.3 \pm 7.8 (11)
Hydroxyurea (12h)	22.01 \pm 1.22 (6)	723.3 \pm 66.6 (6)	7.2 \pm 4.4 (5)

ment. These cells were then transfected in suspension, resulting in transfection efficiencies which were consistent but considerably lower than for cells transfected in monolayer (cf. Tables 1 and 2). The yield of mitotic cells varied between 20% and 55% in different preparations, representing an average enrichment of approx. 20-fold.

It is clear that the transfection efficiency of SV40 DNA in the presence of carrier DNA was almost 4-fold higher in mitotically enriched populations than in control cells (Table 2). As before, however, no significant difference was noted in the absence of carrier DNA (Table 2). Similar results were obtained when mitotic cells received an exposure of trypsin for 30s before the transfection period (results not shown). Although cells were transfected for a total of 2h, which is longer than the average duration of mitosis, estimated at 1.25h for

CV-1 cells (Zucker *et al.*, 1979). We were able to show, by morphological criteria, that these mitotic cells were arrested while in suspension during exposure to DNA co-precipitates. They were found to progress through the remainder of mitosis and into G₁-phase only on re-attachment to tissue-culture flasks. As with S-phase cells, there was no difference in the growth rate and plating efficiency of mitotic cells, when compared with asynchronous controls (results not shown).

Viral infection of S-phase and mitotic cells

Infectivity of cells to intact virus was lower in the cell suspensions than in monolayers (Fig. 2), but there was no significant difference in the response of S-phase cells (Fig. 2a) or of mitotically enriched cells (Fig. 2b) when compared with asynchronous controls.

Discussion

The results presented in this study demonstrate that there is cell-cycle-phase dependence in the efficiency with which SV40 DNA, co-precipitated with calcium phosphate and carrier DNA, initiates lytic infection in permissive cells. An enhancement in transfection efficiency of around 20-fold was observed for populations enriched in mitotic cells, compared with populations synchronized early in S-phase. In view of the incomplete purification of mitotic cells in these experiments, it is possible that the true enhancement in transfection efficiency at this phase of the cycle is higher still.

This cycle-dependence cannot be attributed to events related directly to viral replication, as it was not observed in cells transfected with purified DNA in the absence of carrier, or infected with intact SV40 virions, although in both these conditions viral replication and assembly clearly took place. Furthermore, as the hydroxyurea block was shown by autoradiography to be fully reversible and the growth rate and plating

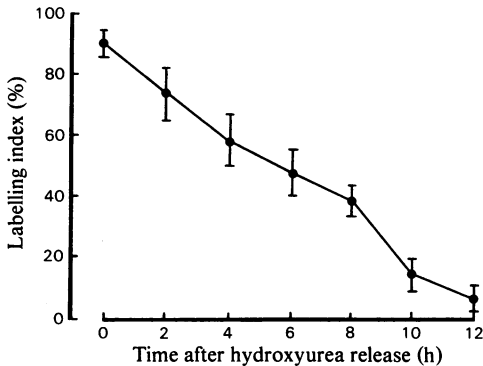


Fig. 1. Labelling index of CV-1 cells after hydroxyurea treatment

Cultures were pulse-labelled for 30 min with 5 μ Ci of [³H]thymidine/ml at various intervals after release from a double-block treatment with 0.5mM-hydroxyurea. They were fixed in methanol/acetic acid (3:1, v/v) and processed for autoradiography. Each point represents the means \pm s.d. for six cultures.

Table 2. Transfection efficiency of SV40-DNA co-precipitates in enriched populations of mitotic cells

Control and mitotically enriched cells were transfected for 2h in suspension with SV40 DNA, harvested, and viral-plaque assays performed as described in the Methods section. Results are expressed as means \pm s.d. for the numbers of determinations given in parentheses.

	$10^{-3} \times$ Transfection efficiency (PFU/ μ g of SV40 DNA)		Mitotic index (%)
	SV40 DNA	SV40 DNA + carrier DNA	
Control	1.65 \pm 0.07 (6)	4.16 \pm 0.94 (6)	1.7 \pm 0.3 (6)
Mitotic	1.70 \pm 0.09 (6)	16.13 \pm 2.31 (6)	29.6 \pm 8.6 (5)

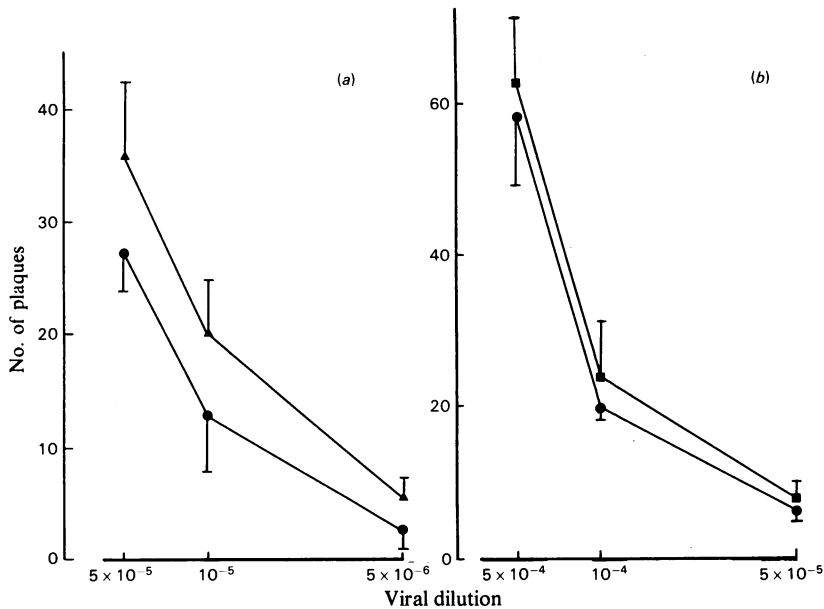


Fig. 2. SV40 viral infectivity of CV-1 cells

The infectivity to intact SV40 virus of asynchronous control cells (●) was compared (a) in monolayer with cells immediately after release from hydroxyurea treatment (▲) and (b) in suspension with cells enriched in mitosis (■). Each point represents the mean \pm S.D. for five cultures.

efficiency of both S-phase and mitotic cells were similar to those of asynchronous cells, the cycle-dependence is unlikely to be an artefact generated by the methods employed to achieve cell synchrony. These results therefore suggest that the conditions within mitotic cells preferentially enable transfected DNA to acquire a stable state (and perhaps site) where it is capable of replication.

Our findings appear to conflict with those reported by Giulotto & Israel (1984), who found enhanced expression of the herpes-simplex-virus thymidine kinase (*tk*) gene, when transfected during S-phase as compared with other phases of the cycle. The reason for this discrepancy is not clear, but may arise because stable transformation of the recipient *tk*-negative cells involves integration into the host genome, usually in the form of tandemly linked concatamers including flanking sequences derived from carrier DNA (Perucho *et al.*, 1980). Neither the formation of concatamers nor integration is required during the infection of permissive cells by SV40 under the conditions of the present study. The data presented here may also be compared with those obtained by direct micro-injection of mammalian cells. Almost 100% of interphase cells injected with DNA from papova and herpes viruses expressed early antigens (Graessmann *et al.*, 1976, 1980). In those

experiments, however, the possibility of transient expression from an unstable DNA template was not excluded. In addition, it has been suggested that the high efficiency of micro-injection derives from the fact that DNA can be placed directly into the nucleus (Graessmann *et al.*, 1981).

Our results are in accord, however, with those obtained from experiments in which SV40 DNA was introduced by micro-injection into amphibian oocytes and fertilized eggs (Wyllie *et al.*, 1977). Those experiments suggested the existence of nuclear factors which confer stability on DNA and are dispersed throughout the cell on breakdown of the nuclear membrane, either physiologically or as a result of experimentally induced trauma. The present data support the proposition that similar factors may exist in mammalian cells and may contribute to the stability of endogenous chromosomal DNA during mitosis, despite the substantial endonucleolytic activity of the cytoplasm. Changes in many cellular components are known to occur in mitosis, including the phosphorylation of several proteins (Henry & Hodge, 1983; Laskey, 1983) which could regulate the activity of nuclease enzymes.

The mechanism whereby carrier DNA enhances gene transfer by calcium phosphate is not known. Our previous results have shown that carrier DNA does not increase the cellular uptake of the SV40

DNA (Strain & Wyllie, 1984), nor does it, in our hands, directly protect SV40 DNA from degradation by nucleases. Rather it appears to interact with cellular sites involved in DNA processing, since presentation of carrier DNA to transfected cells at times close to but not coincident with the SV40 DNA still enhances transfection efficiency (A. J. Strain & A. H. Wyllie, unpublished work). Evidence in favour of the view that these sites might include the endosome/lysosome compartment is supported by the fact that treatment of cells with lysosomotropic agents can result in the enhancement of liposome-mediated SV40-DNA transfection (Fraleley *et al.*, 1981) and of calcium phosphate-mediated transfection of polyoma DNA (Luthman *et al.*, 1983). These agents are known to raise the intralysosomal pH and hence inhibit the activity of lysosomal degradative enzymes (de Duve *et al.*, 1974). Thus the major role of carrier DNA may be to 'saturate' this nuclease-rich compartment.

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