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Cell cycle-dependent expression of early viral genes in one group of simian virus 40-transformed rat cells

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SV40-transformed FR 3T3 rat cells were previously shown to exhibit different patterns of accumulation of the virus-coded T-antigen. One group of transformants accumulates T-antigen throughout the cell cycle, whereas in another group, only the cells in the G2 phase of the cell cycle are stained by immunofluorescence with anti-T antigen antibodies. We investigated the mechanism involved by determining the amounts of early SV40 RNA during the cell cycle. Cells in the various phases of the cell cycle were sorted from an asynchronously growing population using a flow cytofluorimeter. Determination of the amounts of viral RNA in the different nuclear and cytoplasmic RNA fractions showed that in transformants with a G2-restricted accumulation of T-antigen, viral RNA was present in G2, to some extent in S, but could not be detected in cells in G1. In contrast, equivalent amounts of viral RNA were detected in all the phases of the cell cycle in the other group of transformants. Cell sorting, performed after pulse-labeling the cells for 2 h with [^{35}S]methionine, confirmed that translation of the viral mRNAs occurred only in G2 in the first group of transformants, and throughout the cell cycle in the second group.

Key words: cytofluorimetry/cell sorting/T-antigen/transcription

Introduction

Maintenance of the transformed phenotype in SV40-transformed cells requires the continuous expression of at least one viral gene product, the large-T polypeptide, product of the *A* gene (for review, see Tooze, 1981). However, cells transformed with temperature-sensitive SV40 *A* mutants do not always exhibit the expected reversion to a normal phenotype at the restrictive temperature (Rassoulzadegan *et al.*, 1978; Fluck and Benjamin, 1979; Seif and Martin, 1979). Both temperature-sensitive (called type N) and temperature-insensitive (type A) transformed cell lines (Rassoulzadegan *et al.*, 1978) were also obtained upon infection of FR 3T3 rat cells (Seif and Cuzin, 1977) with an early temperature-sensitive mutant of SV40, *tsA30* (Tegtmeyer and Ozer, 1971). The result of the infection, that is the production of a transformed cell of the A or the N type, is not random, but appears to depend at least on two parameters, the multiplicity of infection and the physiological state of the cell at the time of infection (Rassoulzadegan *et al.*, 1980). A and N transformants, by definition, differ by their behaviour at the restrictive temperature (Rassoulzadegan *et al.*, 1978; Gaudray *et al.*, 1978), though they share common phenotypic properties at the permissive temperature (Perbal and Rassoulzadegan, 1980; Rassoulzadegan *et al.*, 1980). We have, however, recently shown that A and N FR 3T3-SV40 transformants differ, at the permissive temperature, with respect to their patterns of immunofluorescent staining for the nuclear T-antigen: T-antigen was accumulated in all the cells of an exponentially growing population of A cells, whereas only a fraction of the nuclei of a similar population of N cells was labeled (Rassoulzadegan *et al.*, 1978; Imbert *et al.*, 1983a). This heterogeneous staining pattern reflects the fact that only the N cells in the G2 phase of the cell cycle accumulate T-antigen, as demonstrated by the analysis, in a flow cytofluorimeter, of suitably stained cells (Imbert *et al.*, 1983a). On the other hand, A cells in all the phases of the cell cycle (cells in G1, S and G2) accumulated T-antigen. Accumulation of T-antigen restricted to the G2 phase of the cell cycle was true for two SV-*tsA30* transformants of the N type, and also for a wild-type (WT) SV40 transformant isolated under the conditions which gave rise to N transformants upon infection with the mutant virus (Rassoulzadegan *et al.*, 1978; Imbert *et al.*, 1983a). On the other hand, two SV-*tsA30* transformants of the A type, together with a WT SV40 transformant isolated under conditions which gave rise to A transformants, exhibited T-antigen positive cells in all the phases of the cell cycle. The fact that WT SV40-transformed cells with the two phenotypes can be isolated, strongly argues that the G2-restricted accumulation of T-antigen does not result from peculiar properties of the mutated large-T protein during the cell cycle but, rather, that the A and N types may represent two different modes of interaction between the transforming virus and the host cell.

We have asked whether this cycle-dependent accumulation of T-antigen results from a regulation at either a pre- or a post-translational level, by determining the steady-state levels of viral mRNA during the cell cycle. This requires either purifying cells in the various phases of the cell cycle, or obtaining synchronously growing cells. Though a variety of methods are available for synchronizing cells, most of them involve either drug treatment or deprivation of essential growth factors, and lead to the arrest of cells at specific positions in the cell cycle (Lloyd *et al.*, 1982). Cells then resume cycling in a more or less synchronous manner upon reversal of the inhibitory conditions. However, most of these techniques introduce perturbations in the cell cycle (Lloyd *et al.*, 1982). We have therefore adopted the alternative approach, and used the cell sorter to prepare pure fractions of cells in the G1, S and G2 phases of the cell cycle.

Results

Sorting cells as a function of their stage in the cell cycle

Actively growing cells were labeled *in vivo* with the fluorescent dye, Hoechst 33342, as described in Materials and methods, and an aliquot was analyzed (Figure 1A). Cells of this population were then sorted as a function of their DNA content into G1, S and G2 fractions, by setting the appropriate windows (Figure 1b-d). The sorting conditions were optimized so as to obtain fractions of at least 10^6 cells in each

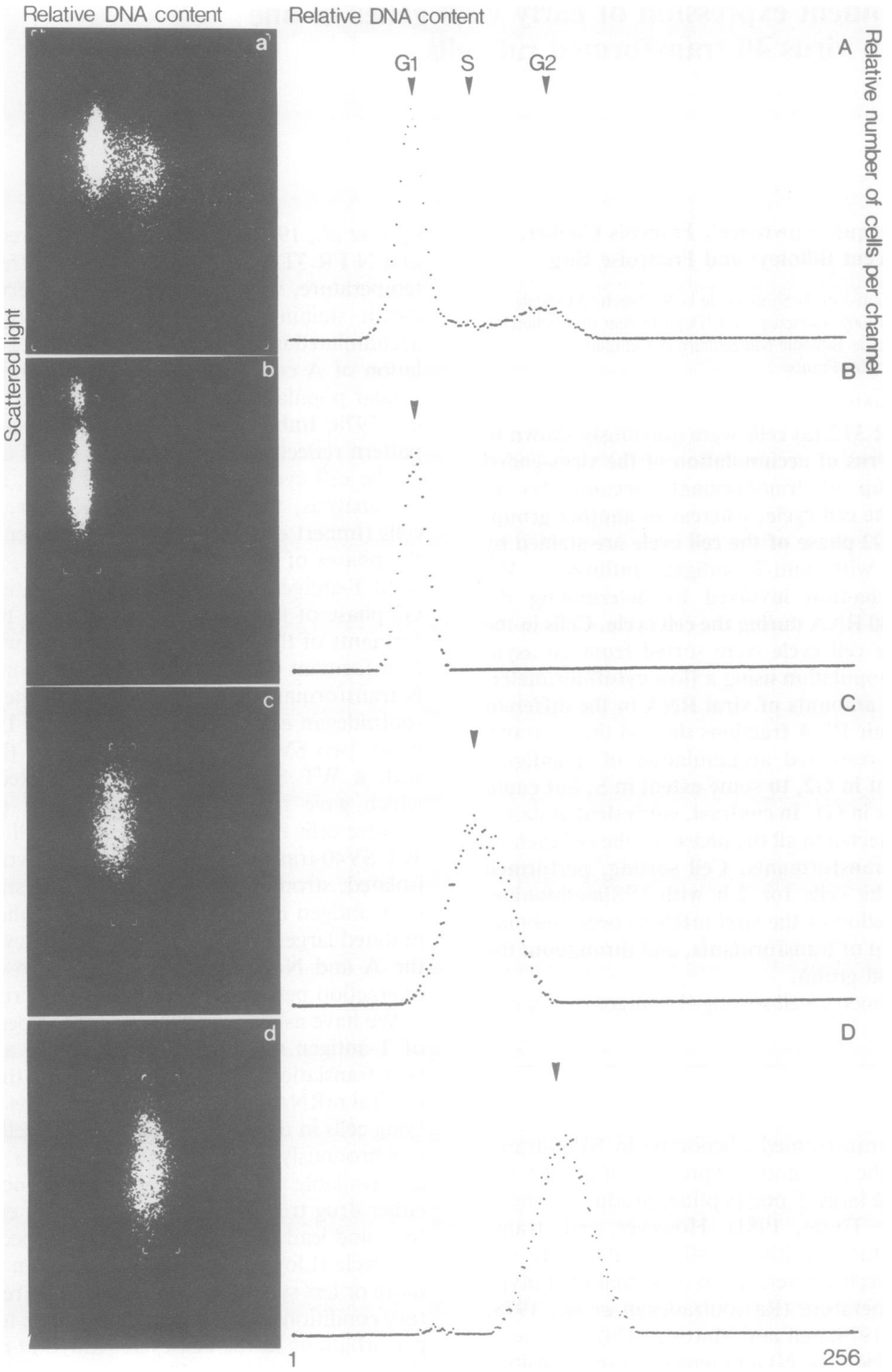

Fig. 1. Sorting cells as a function of their stage in the cell cycle. Cells of an SV-*tsA30*-N1 exponentially growing population (**a**, dot plot representation, **A**, histogram representation of the same population), labeled *in vivo* with Hoechst 33342 as described in Materials and methods, were sorted as a function of their DNA content into G1, S and G2 fractions. Aliquots of these fractions were submitted to purity controls: **bB**, **cC** and **dD** represent the cell fractions with DNA contents corresponding to the G1, S and G2 phases of the cell cycle, respectively. The positions of the three peaks are indicated for the population in exponential growth (**A**). The histograms of the purified fractions were lined up on **A**. Brackets on the dot plot representations indicate the positions of the windows used in the cell sorting.

phase of the cell cycle. Aliquots of these fractions were then checked for their purities (Figure 1B–D); these were better than 90%. Immediately after the purity controls, the nuclear and cytoplasmic RNAs were purified from each fraction, in parallel with a control fraction of 10^6 cells of the exponentially growing population (E). Three independent cell sortings were performed for each transformed cell line analyzed.

Viral transcripts in the different phases of the cell cycle

The transformed cell lines used in these experiments synthesize only the two early viral mRNAs encoding the large-T and small-T proteins (Imbert *et al.*, 1983b). Three transformants showing a G2-restricted accumulation of T-antigen (SV-WT-N1, SV-*tsA30*-N1 and SV-*tsA30*-N2) and two transformants showing an unrestricted accumulation of T-antigen

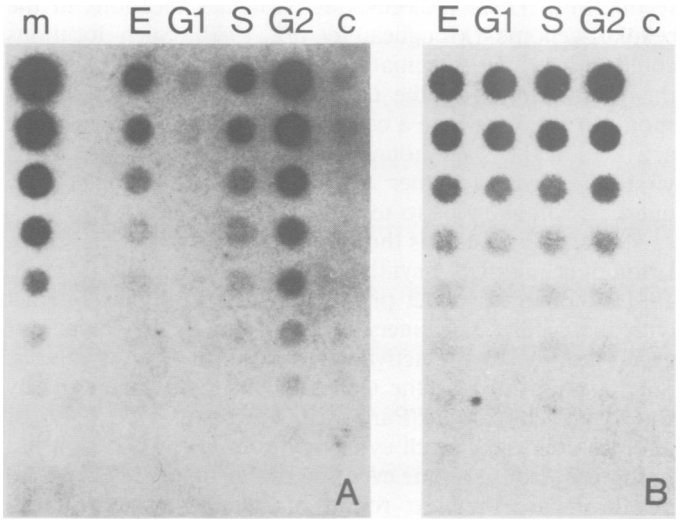

Fig. 2. SV40 RNA in transformed cells in the different stages of the cell cycle. Total cytoplasmic RNA, extracted from control exponentially growing cells (E), and the G1, S and G2 fractions of **(A)** SV-*tsA30*-N1 and **(B)** SV-WT-A1 cells were submitted to dot-blot hybridization as described in Materials and methods. Lane 'M' contained purified SV40 DNA, serially diluted from 5 to 0.04 pg. Lane 'C' contained cytoplasmic RNA extracted from 10^6 exponentially growing FR 3T3 cells. For each fraction, a vertical lane corresponds to serial 2-fold dilutions, starting from 5 x 10^5 cells down to 4 x 10^3 cells. The film was exposed for 10 days.

(SV-WT-A1 and SV-*tsA30*-An3) were submitted to cell sorting as described, and the cytoplasmic RNA fractions were analyzed for their respective contents of viral RNA by dot-blot hybridization (see Materials and methods). These experiments are illustrated in Figure 2, for two cell lines representative of the two groups of transformants, SV-WT-A1 and SV-*tsA30*-N1. Similar amounts of viral RNA were detected in the three cytoplasmic RNA samples prepared from SV-WT-A1 cells (Figure 2B); in contrast, viral RNA was detected preferentially in the G2 phase, and to some extent in the S phase, in the case of SV-*tsA30*-N1 cells (Figure 2A). A slight contamination of the S fraction by G2 cells cannot be excluded, as the technique cannot resolve cells in the end of S from G2 cells. The windows were however set so as to avoid such a contamination.

The distribution of viral RNA in the nuclear RNA fractions was similar to that of the cytoplasmic RNAs of the two transformants, respectively (data not shown).

Quantification of the amounts of viral RNA in cells in the various phases of the cell cycle

A lane containing known amounts of viral DNA was introduced in each dot-blot. Scanning of the viral DNA lanes on the various autoradiograms, and integration of the peaks thus obtained, showed that the spot intensities were proportional to the amounts of DNA in the range 0.2−5 pg, using the hybridization conditions described in Materials and methods. Furthermore, the values obtained for the control DNA lanes of several blots hybridized with the same probe were all distributed along the same linear regression (data not shown), making it possible to estimate the relative amounts of viral RNA in each sample. The lanes corresponding to the transformed cell RNAs were scanned, the peaks were integrated, and equivalent amounts of viral RNA per cell were calculated. The results of several experiments on four of the transformed cell lines are summarized in Table I. Similar numbers of viral RNA copies per cell were obtained for cells in G1, S and G2 in the case of the two transformants with an unrestricted accumulation of T-antigen (SV-WT-A1 and SV-*tsA30*-An3), whereas the number of viral RNA copies per cell varied from zero in G1 to four in G2 for the transformants with a G2-restricted accumulation of T-antigen (SV-*tsA30*-N1 and SV-*tsA30*-N2). These values are minimum estimates, assuming similar efficiencies of binding of denatured DNA and RNA to nitrocellulose.

Table I. Amounts of early viral cytoplasmic RNA in the two types of transformants during the cell cycle

Cell line	Viral RNA[a]	Exponentially growing cells	Phase of the cell cycle		
			G1	S	G2
SV-*tsA30*-N1	Amounts[b]	0.6 ± 0.2[c]	n.d.[d]	0.9 ± 0.4	4.2 ± 1.8
	Copies/cell[e]	0.4	−	0.6	2−4
SV-*tsA30*-N2	Amounts	0.4 ± 0.1	n.d.	0.25 ± 0.1	1.4 ± 0.1
	Copies/cell	0.3	−	0.2	0.5−1.5
SV-*tsA30*-An3	Amounts	3.5 ± 1	3 ± 1	4.4 ± 1	4 ± 1
	Copies/cell	1.5−4	1−3	2−4	2−4
SV-WT-A1	Amounts	6.6 ± 0.6	7.3 ± 0.9	8.7 ± 1.8	9.0 ± 1.3
	Copies/cell	4−5	4.5−6	5−7.5	5.5−7.5

[a]Autoradiograms were scanned and the peaks were integrated as described in Materials and methods and in the text.
[b]Amounts of viral nucleic acids (in pg x 10^6) per cell.
[c]Mean ± confidence interval (α = 0.05). The number of determinations varied from 4 to 6.
[d]Not detectable.
[e]Equivalent number of copies of early viral RNA/cell:

$$\frac{\text{amounts of nucleic acids (pg)}}{\text{weight of one viral DNA molecule}} \times 4 .$$

The correction factor of 4 was used to take into account the fact that both the standard DNA and the probe correspond to complete copies of the double-stranded SV40 DNA molecule.

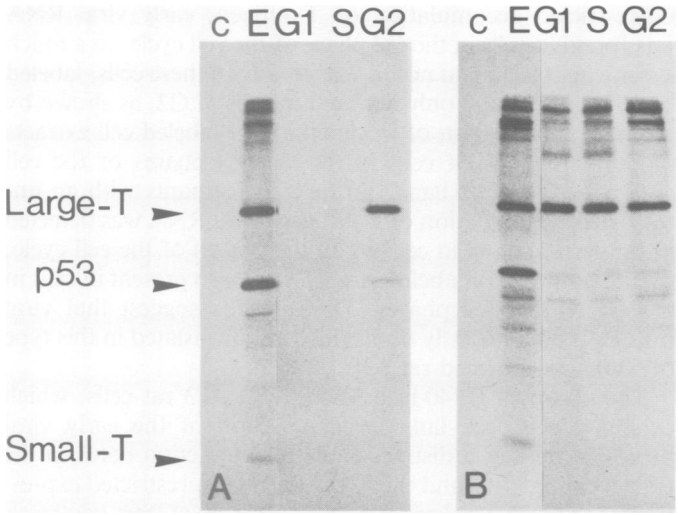

Fig. 3. Synthesis of the early viral polypeptides along the cell cycle. [^{35}S]methionine-labeled SV-WT-N1 and SV-*tsA30*-An3 cells (**panels A** and **B**, respectively) were sorted as a function of their DNA content. The purified fractions, standardized to 2 x 10^5 cells, were sequentially immune precipitated with anti-mouse p53 monoclonal antibodies and with a polyclonal serum from tumor-bearing hamsters as described in Materials and methods. Control samples of 2 x 10^5 exponentially growing cells were immune precipitated either with the polyclonal hamster serum (lanes 'E') or with normal hamster serum (lanes 'C'). The film was exposed for 15 days using fluorographic conditions.

Synthesis of the large-T protein along the cell cycle

To determine when the viral mRNAs were translated during

the cell cycle, exponentially growing cells were labeled with [^{35}S]methionine for 2 h (i.e., for a length of time shorter than the shortest period of the cell cycle), incubated with Hoechst 33342, and then sorted as a function of their DNA content into G1, S and G2 fractions. The labeled cell extracts were immune precipitated with anti-SV40 T-antigen antibodies, and analyzed by PAGE. In the transformants which contain T-antigen in all the phases of the cell cycle, labeled large-T antigen was detected in cells in G1, S and G2 (Figure 3B). In contrast, only the cells in the G2 phase of the cell cycle contained large-T antigen in the case of the second group of transformants (Figure 3A). An overexposure of the gel shown in Figure 3A caused a faint large-T band to appear in the S fraction. These results were confirmed by similar analyses performed on two other transformants with a G2-restricted accumulation of T-antigen (SV-*tsA30*-N1 and SV-*tsA30*-N2) and another unrestricted transformant (SV-WT-A1) (data not shown).

Discussion

One group of SV40-transformed rat cells, derived by infection of FR 3T3 cells with either WT SV40 or the early mutant *tsA30*, exhibits an accumulation of the nuclear virus-coded T-antigen restricted to the G2 phase of the cell cycle (Imbert *et al.*, 1983a). A second group of transformants, continuously exhibit T-antigen. In all the cell lines which exhibit a G2-restricted accumulation of T-antigen, early viral RNA was found in cells in the G2 phase of the cell cycle, to a much lesser extent in S, and not in cells in G1. In these cells, labeled large-T protein was only detected in cells in G2, as shown by immune precipitation of [^{35}S]methionine-labeled cell extracts of pure fractions of cells in the various phases of the cell cycle. On the other hand, in the transformants with an unrestricted accumulation of T-antigen, viral RNA was detected in similar amounts in cells in all the phases of the cell cycle; similar amounts of labeled large-T were also present in cells in the G1, S and G2 phases. These results suggest that viral mRNA is continuously transcribed and translated in this type of SV40-transformed rat cells.

The group of SV40-transformed FR 3T3 rat cells, which exhibit a cell cycle-linked accumulation of the early viral products, defines a distinct mode of interaction between the transforming virus and the host cell. Such a restricted expression of the early viral genes may result from at least two levels of control. The viral mRNAs may be unstable: this possibility is suggested by the very sharp decrease in their steady-state amounts between the G2 and the G1 periods. Thus the rate of viral mRNA degradation may control T-antigen levels in these cells, especially since the large-T polypeptide in FR 3T3-SV40 transformants exhibits an atypically short half-life (Edwards *et al.*, 1979; Imbert *et al.*, 1983b). In addition, the integrated early viral region must be newly transcribed at each G2 phase of the cell cycle; to our knowledge, this may be the first example of a gene expressed in this phase of the cell cycle, and whose expression could be regulated at the transcriptional level (Pardee *et al.*, 1978; Lloyd *et al.*, 1982). Several working hypotheses can be formulated to account for such a transcriptional control. Viral transcription may be regulated through punctual modifications of the early viral region or its control sequences, such as those involved in the regulation processes of active genes (altered chromatin configuration, methylation) (Felsenfeld and McGhee, 1982; Weisbrod, 1982): these modifications should occur in a periodic manner. Alternatively, it could result from the integration of the infecting viral DNA molecules at particular locations in the cellular genome (Mougneau *et al.*, 1980). Such locations could either have chromatin configurations which exclude their transcription in the G1 phase, or alternatively correspond to regions where a coordinate set of cellular genes are activated at the S/G2 boundaries. Availability of the cloned viral insertions, together with their flanking cellular sequences, will allow us to test these hypotheses.

The large-T protein is thought to be involved in the stimulation of cellular DNA synthesis in transformed cells (Martin, 1981), though no direct proof is available. It may interact with the cellular machinery at any point in the cascade of events leading to the activation of cellular DNA synthesis. Such a point may be the G1 restriction point suggested by Pardee and co-workers (Pardee, 1974; Rossov *et al.*, 1979), or another point in the cell cycle, for example in G2, as it has been proposed that some events occurring in the S/G2 period could prepare the next round of cellular DNA synthesis (Cooper, 1979). Whatever the case, it appears that the presence of detectable amounts of the SV40 large-T protein, restricted to the G2 phase of the cell cycle, is sufficient to carry out the required function, and to confer on the cell a transformed phenotype. This cell cycle-dependent expression of large-T may, however, have other effects on the large-T protein. We have recently shown that the large-T polypeptide is apparently stabilized at the permissive as well as at the restrictive temperatures in type A cells only (Imbert *et al.*, 1983b). In SV40-transformed rodent cells, large-T antigen is associated with a protein of cellular origin, p53 (for review, see Klein, 1982; Levine, 1982). p53 has been implicated in the regulation of cellular growth (Milner and Milner, 1981; Mercer *et al.*, 1982; Reich and Levine, 1984). Experiments are in progress to determine the relationship of p53 expression to the cell cycle in normal and transformed FR 3T3 cells, as well as the consequences on the association, along the cell cycle, between large-T antigen and p53 in the transformed cell lines.

Materials and methods

Cell lines and culture conditions

These have been described previously (Seif and Cuzin, 1977, Rassoulzadegan *et al.*, 1978).

Cellular DNA labeling

This was performed on living cells using a modification of a procedure described previously (Lydon *et al.*, 1980). Cells in culture were labeled for 1 h at 33°C with 10 μM Hoechst 33342. They were then harvested with trypsin-EDTA as described (Imbert *et al.*, 1983a), rinsed and stored in phosphate-buffered saline (PBS) containing 0.1% glucose and 1 μM Hoechst 33342 at 0°C before sorting. When the cells were labeled with [^{35}S]methionine to analyze the synthesis of the viral proteins (see below), labeling of the cellular DNA was performed by adding the dye to the cells 1 h before the end of the pulse. Cells were then harvested and stored as described above.

Cell sorting

The Hoechst 33342-labeled cells were analyzed in a cytofluorimeter (FACS IV, Becton Dickinson) using a 5 W argon laser equipped with u.v. mirrors. Cells were excited at 340–360 nm, and the emitted fluorescence was measured after passage through a 440–460 nm band pass filter (Oriel, no. 70706). Data were recorded and stored as described previously (Chabanas *et al.*, 1983). Cells were sorted as a function of their DNA content by setting appropriate windows (see Results). In practice, two successive runs were required: in the first, the window settings were G1/G2, and in the second, they were G2/S. The two G2 populations were pooled. The purity of each fraction was then checked by rerunning aliquots of the samples in the cell sorter (see Results).

Purification of nuclear and cytoplasmic RNAs, quantification of the amounts of early viral RNA

For each cell line, four fractions standardized to 10^6 cells were prepared: the G1, S and G2 fractions, together with a control of exponentially growing (E)

cells. Cells were lysed, and the nuclear and cytoplasmic RNAs were prepared as described previously (Favaloro *et al.*, 1979). RNAs, resuspended in 50 μl of 10 mM Tris-HCl buffer pH 7.4 containing 1 mM EDTA, were denatured by heating at 50°C for 15 min in the presence of 30 μl of 20 x SSC (1 x SSC is 0.15 M NaCl, 0.015 M sodium citrate) and 20 μl of a deionized 30% formaldehyde solution (Fluka) as described previously (White and Bancroft, 1982). The RNA samples were then serially diluted, using 96 wells microtitration plates, and filtered onto nitrocellulose sheets (BA 85, Schleicher and Schull), using a Minifold apparatus (Schleicher and Schull). Viral RNA was detected by hybridization of the dot blots to a [^{32}P]SV40 DNA probe (usual sp. act: 3 x 10^8 c.p.m./μg), obtained by *in vitro* labeling of purified form I SV40 DNA by nick translation with DNA polymerase I (Rigby *et al.*, 1977). Blots were hybridized for 72 h at 68°C in 40 ml of 6 x SSC containing 0.1% SDS, 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin (Sigma), 50 μg/ml of denatured salmon sperm DNA (Sigma), and 10^6 c.p.m./ml of denatured [^{32}P]SV40 DNA. After repeated washes in 2 x SSC containing 0.1% SDS at 68°C, the dot-blots were exposed to Fuji RX films using Dupont Lighting plus intensifying screens. Amounts of viral RNA were quantified after scanning the autoradiograms with a Vernon densitometer.

Synthesis of the early viral polypeptides

Cells in exponential growth were labeled with [^{35}S]methionine (100 μCi/90 mm dish in 1 ml, 1500 Ci/mol, Radiochemical Centre, Amersham) for 2 h after a 1 h methionine deprivation in Dulbecco-modified Eagle medium minus methionine, supplemented with 2% serum substitute (L'Industrie Biologique Française). Labeling of the cellular DNA and sorting of the cells as a function of their DNA content were then performed as described above. Cells were lysed and the various cell extracts were first immune precipitated with monoclonal antibodies from PAb 421-5 cells (Harlow *et al.*, 1981), directed against the mouse p53 protein, using antibody amounts and conditions which precipitated all of the p53 protein but no large-T complexed to p53; this allowed us to analyze the synthesis of p53 along the cell cycle on the same samples as those used for large-T (results not shown). In a second step, the samples were immune precipitated with anti-SV40 T-antigen antibodies from tumor-bearing hamsters as described previously (Gaudray *et al.*, 1978; Ito, 1979). Control samples of exponentially growing cells (E) were immune precipitated either with normal hamster serum or with the anti-SV40 T-antigen hamster serum. The immune precipitated proteins were analyzed by polyacrylamide gel electrophoresis (Laemmli, 1970), and the dried gels were exposed to Fuji RX films and intensifying screens after fluorographic treatment (Bonner and Laskey, 1974).

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