Cell Cycle Control of Polyomavirus-Induced Transformation

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The cell cycle dependence of polyomavirus transformation was analyzed in infections of nonpermissive Fischer rat (FR3T3) cells released from G₀. A 5- to 100-fold (average, ca. 20-fold) difference in relative frequency of transformation was found for cells infected in the early G₁ phase of the cell cycle compared with cells infected in G₂. Differences in the relative level of early viral gene expression in those two cell populations were equivalent to those obtained for transformation frequencies. The difference in transformation potential was accounted for only in part by a cell cycle control of viral adsorption (2- to 15-fold effect). Furthermore, in cells infected in the early G₁ phase, viral gene expression was induced as a big synchronous burst of large transcripts of variable sizes, delayed till the G₁ phase of the cell cycle after that in which infection took place. Thus, the results demonstrate that the abortive infection cycle of G₀-released FR3T3 cells is cell cycle regulated at least at two steps: adsorption and another early step, nuclear transport, decapsidation, up to or including the transcription of the viral early genes. The cell cycle regulation of these steps results in a similar regulation of the abortive and stable transformation processes, although it is more pronounced for the latter. A model implicating *c-fos* and *c-jun* is proposed.

The ability of polyomavirus and simian virus 40 (SV40) to induce the cell cycle and to exert their mitogenic effects is thought to be intimately linked to the neoplastic potential of these viruses and has been the focus of intense research. This property is mediated, at least in part, by the action of the T antigens, which can induce the expression of highly regulated cellular genes such as *c-myc* and *c-fos* (6, 28) or which interact with cellular proteins that are themselves cell cycle regulated (such as the retinoblastoma gene product [14]). In contrast, the study of the regulation of the viral infection process during the cell cycle has received little attention. Early studies focused on differences in rates of neoplastic transformation. A twofold difference was found for cycling hamster cells infected by polyomavirus in the G₂ compared with the G₁ phase (3). Multiplicity-dependent differences between the infection of quiescent and growing cells were also described. At a multiplicity of infection of 10 PFU per cell, a 20-fold-higher rate was observed for the transformation of quiescent cells by both polyomavirus and SV40 (15, 22), a differential which was not seen at higher multiplicities. Cell-cycle-mediated differences in the timing of the steps of the infectious lytic cycle have been reported as well: the onset of SV40 DNA synthesis took place during the first S phase in cells infected in G₁ but was delayed to the next S phase in cells infected during S or G₂ (17). A less careful study suggested a similar pattern in polyomavirus infection (13). In these early experiments, the step at which cell cycle control might be exerted was not determined. It might be expected that viruses with very limited genetic information such as polyomavirus and SV40, which therefore rely heavily on the host cell synthetic capacity, would be subjected to cell cycle regulation at multiple steps of the infection process.

In this study we have reexamined the cell cycle control of various steps in the infection of nonpermissive Fischer rat

3T3 (FR3T3) cells by polyomavirus, with a special accent on stable neoplastic transformation. Our results show large variations in transformation frequency for cells infected at various times during the cell cycle following release from G₀, with a maximum for cells infected early in the G₁ phase. Cell cycle control of virus uptake and an additional step, an early genome-processing step and/or the transcription of the viral early genes, appear to contribute to the cell cycle regulation of transformation.

MATERIALS AND METHODS

Virus and cells. Polyomavirus A2 (wild type) was used (9). FR3T3 cells (20) were maintained in Dulbecco's modified Eagle medium (DMEM; GIBCO) supplemented with 5% heat-inactivated newborn calf serum (Hazleton), hereafter designated normal calf serum. For synchronization, cells were grown to confluency; 1 day later, the medium was changed to DMEM supplemented with 0.2% gamma globulin-free calf serum (GIBCO). After 1 day, cells were released from G₀, both by trypsinization and by addition of fresh DMEM supplemented with 10% normal calf serum (time zero). Reattachment of cells to the culture plates occurred within 3 h. Cells were infected at various times starting at 3 h, counted as 3 h after release from G₀. This protocol is referred to below as protocol 1.

Measurement of DNA synthesis during the cell cycle: Incorporation of [³H]thymidine ([³H]TdR) (1 μCi/ml; ICN Biomedicals Inc.) was carried out for 1 h. After incubation, cells were washed with phosphate-buffered saline (PBS; pH 7.4) and the nucleic acids were precipitated with 10% trichloroacetic acid and trapped onto Whatman 2.4-cm fiberglass filters. Incorporation of [³H]TdR was determined by scintillation counting.

Transformation assays. FR3T3 cells were arrested in G₀ and trypsinized by following protocol 1 described above, and they were seeded at 10⁵ cells per 35-mm tissue culture plate and infected at various times after release from G₀ at a multiplicity of infection of 1 PFU per cell unless otherwise indicated. Adsorption was carried out for 1 h, after which

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nonattached virus was removed and DMEM supplemented with normal calf serum was added (protocol 1). Cells were fed twice a week with DMEM supplemented with normal calf serum: 10% and 2% for subconfluent and confluent monolayers, respectively. In most experiments, transformation frequencies were determined by counting the number of transformed foci overgrowing the monolayer (protocol 1). Foci became apparent by 7 days postinfection, reached a steady-state level by 2 weeks, and were monitored for approximately 3 weeks. In other experiments (as indicated in the text), transformation was assayed by anchorage-independent growth in semisolid medium containing 10% fetal calf serum. For this purpose, infection was carried out by protocol 1 except that infected cells were trypsinized and transferred to semisolid medium (10^5 cells per 60-mm dish), approximately one cell cycle (i.e., 24 h) postinfection. Transformants were scored as macroscopic colonies 2 to 3 weeks postinfection. Abortive transformation was determined for cells suspended in semisolid medium by counting cell clumps larger than those seen in the mock-infected plates (more than three or four cells) under a microscope. Abortive transformation was scored at 1 and 2 weeks postinfection.

Isolation of nuclei. Infected cells were washed and incubated on ice for 5 min in lysis buffer consisting of 5 mM $MgCl_2$ and 0.5% Nonidet P-40 in $1\times$ PBS. The nuclei were pelleted by centrifugation at 3,000 rpm at $4^\circ C$. Cell debris were removed by washing twice with PBS. DNA was extracted as described below.

Preparation and analysis of DNA. Infected cells or nuclei were washed and lysed in 10 mM Tris–10 mM EDTA–0.2% sodium dodecyl sulfate (SDS) (pH 7.6) supplemented with 50 μg of protease K (Sigma) per ml. DNA was extracted with phenol-chloroform by standard procedures (1). DNA recovery was quantitated by spectrophotometer reading. To measure virus uptake, we performed extraction at 4 or 20 h postinfection, i.e., prior to de novo viral DNA synthesis. To assess viral DNA replication, we generally extracted DNA 48 h postinfection or as indicated. DNA (5- μg samples) was digested with *EcoRI* (GIBCO BRL), a restriction enzyme that cuts the polyomavirus genome once, electrophoresed in 0.7% agarose, stained with ethidium bromide to confirm sample equivalence, and blotted onto nylon membranes (21). Hybridization was carried out at $65^\circ C$ in $1\times$ Denhardt's solution– $2\times$ SSC ($1\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) by standard procedures (1) with a ^{32}P -radiolabeled probe containing the entire polyomavirus DNA. Hybridization probes were labeled to a specific activity of 1×10^9 to 2×10^9 cpm/ μg with [^{32}P]dCTP (3,000 $\mu Ci/mm$ ol; New England Nuclear) with a multiprime DNA-labeling kit (Amersham). The hybridized blots were washed under stringent conditions. Band intensities were determined by densitometer scanning. In some cases, the amount of radioactive probe in each band was determined by β -scanning of the membrane (AMBIS). The blots were then exposed to X-ray film (Kodak) for 1 to 7 days at $-70^\circ C$ with an intensifying screen.

Preparation and analysis of RNA. Total RNA was extracted 48 h postinfection by the method of Chirgwin et al. (5). Briefly, cells were lysed in guanidine thiocyanate solution (4.0 M guanidine thiocyanate, 25 mM sodium citrate [pH 7.0], 0.5% Sarkosyl, 0.1 M β -mercaptoethanol), the lysates were placed on top of a cushion of cesium chloride (5.7 M CsCl in 0.1 M EDTA [pH 7.0]), and the RNA was pelleted (7). The RNA pellet was resuspended, and the recovery of RNA was quantitated by spectrophotometer reading. For Northern (RNA) blots, 20- μg samples were electrophoresed

in a 1% agarose gel containing 2.2 M formaldehyde and transferred to nytran membranes (Schleicher & Schuell) in a $10\times$ SSC solution (1). For dot blot analysis, RNA samples were directly deposited on nytran membranes. Probes corresponding to the entire polyomavirus genome or to a segment of the glyceraldehyde phosphate dehydrogenase (GapDH) gene were prepared by multiprime labeling with [^{32}P]dCTP and used to quantitate viral and cellular transcripts, respectively. Two single-stranded riboprobes were made from pG3PyH3 containing fragment 3 of a *HpaII* polyomavirus digest, encoding a fragment of the late genes, cloned between the T7 and SP6 promoters of a pGem vector. To detect anti-late mRNA, a riboprobe was made from the T7 promoter of pG3PyH3 with T7 polymerase, and to detect late mRNA, a riboprobe was made from the SP6 promoter of pG3PyH3 with SP6 polymerase. Filters were prehybridized for 2 to 4 h and hybridized for 12 h at $42^\circ C$ in 0.05 M sodium phosphate buffer (pH 7)–1 M NaCl–50% formamide–1% SDS–5% dextran sulfate–100 μg of salmon sperm DNA per ml (1). The filters were washed under stringent conditions. The blot was then exposed to Kodak X-ray film for 2 to 8 days at $-70^\circ C$ with an intensifying screen.

DNase treatment of RNA. RNA samples (10 μg) were resuspended in 400 μl of TE buffer (10 mM Tris [pH 7.6], 1 mM EDTA). Then 20 U of RNasin (Boehringer Mannheim Biomedicals), 10 mM $MgCl_2$, and 1 μl of RNase-free DNase (1 mg/ml) were added, and the reaction was carried out at $37^\circ C$ for 30 min (1). The reaction was stopped with EDTA (final concentration, 20 mM) and 1/10 volume of 3 M sodium acetate (pH 5.2), and the RNA was reextracted and precipitated.

Protein analysis. Infected FR3T3 cells were lysed 48 h postinfection with sample buffer (5% SDS, 0.03% bromophenol blue, 20% glycerol, 5% β -mercaptoethanol) and boiled at $100^\circ C$ for 5 min. One-quarter aliquots of the lysates were electrophoresed in 10% polyacrylamide and electroblotted onto nitrocellulose membranes (4). Rabbit anti-small T-antigen serum (a gift of B. S. Schaffhausen), which can detect large, middle, and small T antigens via their co-N-terminal 79 amino acids, was used as the primary antibody. Goat anti-rabbit immunoglobulin G coupled to alkaline phosphatase was used as the secondary antibody. For detection, both bromochloroindolyl phosphate and Nitro Blue Tetrazolium were used as the developer for the color reaction.

RESULTS

Cell cycle of FR3T3 cells. Fibroblastic cell lines derived from Fischer rats have been commonly used in recent years to study neoplastic transformation by polyomavirus and SV40. Infections of these cells are characterized by the usual landmarks of the abortive infection of nonpermissive cells by papovaviruses. With polyomavirus, between 1 and 10% of infected cells undergo abortive transformation and between 0.05 and 1% undergo stable transformation associated with the integration of the viral genome into host cellular DNA (26). We have examined the cell cycle regulation of polyomavirus infection in one specific Fischer rat cell line, FR3T3 (20). FR3T3 cells were brought to a quiescent G_0 state by contact inhibition and serum starvation and then released from G_0 by treatment with trypsin and by replating at subconfluent density (approximately 10%) in medium containing a high serum concentration as described in Materials and Methods (protocol 1). These cells were either not treated further or infected as monolayers 3 h after release from G_0 (protocol 1), i.e., at the earliest time of stable

FIG. 1. Phases of the cell cycle and determination of transformation frequencies during the cell cycle in FR3T3 cells released from G₀. FR3T3 cells were brought to G₀ by contact inhibition and serum starvation as described in Materials and Methods (protocol 1). At time zero, cells were trypsinized, and replated at a subconfluent density (approximately 10%) in serum-supplemented medium (10% serum). Cells were either not treated further (open symbols) or infected (solid symbols) at 3 h postrelease (panels A and B) or at the times shown (panels C and D). Cell DNA synthesis (panel A), cell division (panel B), and transformation (panel C) were monitored in the same experiment. (A) DNA synthesis. Cells released from G₀ uninfected (○) or infected 3 h postrelease (●) were allowed to incorporate [³H]TdR (1 μCi/ml) during pulses of 1 h at various times after release from G₀, as shown on the x axis. Cells were then extracted, the DNA was precipitated with trichloroacetic acid, and the [³H]TdR incorporation was determined by scintillation counting as described in Materials and Methods. (B) Cell number. Numbers of both uninfected (○) and infected (●) cells were determined with a hemacytometer. (C). Transformation. G₀-released cells (10⁵ cells per 35-mm plate) were infected with polyomavirus A2 at a multiplicity of infection of 10 at the times shown. The formation of transformed foci over the monolayer was monitored for 10 to 14 days postinfection and scored. (D). Transformation. The conditions were the same as for panel C, including infections in the second cell cycle. Transformed foci (●) and cell numbers (▲) are shown.

attachment to the plates. The timing of the cell cycle was determined by measuring DNA synthesis by [³H]TdR incorporation as described in Materials and Methods. Results of a typical experiment are shown in Fig. 1A. Low levels of [³H]TdR incorporation were observed between 0 and 9 h after release from G₀, marking one border of the first G₁ phase. The S phase was broad, taking place between 9 and 23 h, as indicated by a peak of incorporation of [³H]TdR during this period. An increase in mitoses was observed (by microscope examination) at 23 h after release from G₀, and the cell number had doubled by 26 h after release from G₀ (Fig. 1B), indicating the first cell division and the end of the

first cycle, in agreement with the [³H]TdR incorporation data (compare Fig. 1A and B). These results defined the synchrony of FR3T3 cells following release from both contact inhibition and serum starvation and showed that infection with polyomavirus at 3 h after release from G₀ did not disturb the first cell cycle. The second cell cycle occurred between 26 and 39 h after release from G₀ with a much shorter G₁ phase. A second round of cell doubling was usually detected by 39 h. In the infected population, the cell number doubled at a higher rate following the first division, suggesting a shortening of the cell cycle. Similar results were obtained in multiple experiments. As shown below, the

FIG. 1—Continued.

synchronous pattern of appearance of the viral mRNA in the second cycle suggests that some synchrony was maintained until that time. The length of the cell cycle varied from experiment to experiment, and cell cycle kinetics were determined in all experiments.

Cell cycle effect on transformation rates. FR3T3 cells, arrested in G_0 and then released as described above (protocol 1), were infected with polyomavirus A2 (wild type) at various times after release from G_0 ; transformation was assayed by counting transformed foci overgrowing the monolayer (protocol 1) and was found to reach a plateau between 10 and 14 days postinfection. The number of foci was determined from duplicate plates and averaged. As shown for a typical experiment in Fig. 1C, a peak in transformation frequency was observed for cells infected at the earliest possible time following cell attachment, i.e., early in the G_1 phase. Similar results were obtained at low and intermediate multiplicities of infection (1 and 10, respectively [data not shown]). The difference between the minimal

and maximal transformation frequencies was 5- to 100-fold. A G_1 peak of relative susceptibility was also observed with slight modifications of protocol 1 such as trypsinization before infection and infection in suspension, monolayer infection and transfer to semisolid medium 24 h after infection, treatment with serum alone (for 15 min prior to infection) without trypsinization, and serum starvation at low cell density and infection without trypsin treatment (data not shown). Thus, the result did not depend on the means by which cells were brought into G_0 or on the treatment with trypsin. Despite variations in the length of the first cell cycle (from 24 h to 30 h) observed from experiment to experiment following protocol 1, a peak of transformation for cells infected around 3 h after release from G_0 was observed in all experiments and was followed by a rapid decrease. The minimum was usually reached by the beginning of the S phase and was maintained for the remainder of the cycle. Infection in the G_1 phase of the second cell cycle (exponential growth) resulted in a minor increase in transformation

TABLE 1. Abortive and stable transformation during the cell cycle^a

Time ^b	Transformation at MOI ^c of:			
	1		0.2	
	Abortive ^d	Stable ^e	Abortive ^d	Stable ^e
3	6	1.0	4	0.5
6	1.5	0.75	2.5	0.3
9	3.1	0.33	1.3	0.06
24	0.7	0.05	0.6	0.01
Mock	0	0	0	0
Amplitude ^f	8.2	20	6.6	40

^a G₀-released FR3T3 cells were infected at the multiplicities of infection shown and at the times shown as described in Materials and Methods and in the legend to Fig. 1. Infected or mock-infected cells were resuspended in semisolid medium 24 h postinfection, and abortive and stable transformation were scored at 2 weeks postinfection as described in Materials and Methods.

^b Time of infection given in hours after release from G₀.

^c MOI, multiplicity of infection.

^d Percentage of infected cells based on counts of cell clumps larger than those seen in mock-infected cells.

^e Percentage of infected cells based on counts of macroscopic colonies.

^f Ratio of the number of transformed colonies at the peak (3 h) over that at the minimum (24 h).

(Fig. 1D), suggesting that the G₁ phase following release from G₀, rather than the G₁ phase of normally cycling cells, provides an environment for maximal susceptibility to polyomavirus transformation. Varying the multiplicity of infection between 0.2 and 20 did not alter the cell-cycle-dependent transformation profile. Protocol 1 was adopted for all experiments, except where mentioned. As described below, specific parameters of infection were examined. The cell cycle dependence of each infection step was tested in duplicate samples in at least three individual experiments. In the assays shown in Fig. 3, 5, and 6 (below), all parameters were assayed in one and the same experiment.

Abortive transformation. Infection of nonpermissive cells by polyomavirus results in two types of events: abortive transformation and stable transformation. The former is thought to be mediated by unintegrated genomes, whereas the latter requires the stable integration of the viral genome into the host genome (26). The effect of the cell cycle on these two types of events was analyzed at two multiplicities of infection. For this purpose, protocol 1 was followed, except that cells were trypsinized 24 h (i.e., approximately one cell cycle) postinfection and resuspended in semisolid medium. The results are presented in Table 1. The frequencies of both abortive and stable transformation peaked in parallel for G₁-infected cells. The amplitude of the differential in stable transformation frequencies was larger (19.5- and 39.5-fold) than that in abortive transformation (8.2- and 6.6-fold) (multiplicities of 1.0 and 0.2, respectively). The maximum stable transformation frequencies were 1 and 0.5% for multiplicities of infection of 1 and 0.2, respectively, whereas the equivalent maximal abortive transformation frequencies were 6 and 4%, respectively.

Virus uptake. To assay whether differences in virus uptake at different phases of the cell cycle account for the differences in transformation, we infected cells at various times after release from G₀, following protocol 1, and extracted DNA at 4 h postinfection; we detected viral DNA by hybridization and quantitated it by scanning of hybridized membranes or their autoradiographs. As shown in Fig. 2A, a peak in virus uptake was observed for cells infected early in

FIG. 2. Analysis of virus uptake in intact cells and in nuclei. Cells were released from G₀ as described in the legend to Fig. 1 and in Materials and Methods, and duplicate plates were infected at 3, 6, 9, and 24 h after release from G₀ as shown. Total (A and C) or nuclear (B) DNA was extracted at 4 and 48 h (A) or 20 h (B and C) postinfection (hrpi) digested with *Eco*RI, electrophoresed on a 0.7% agarose gel, and transferred to a membrane. Viral DNA was detected by hybridization with a ³²P-labeled probe containing the entire polyomavirus genome. The 5.3-kb position of the polyomavirus genome is shown.

the G₁ phase. A 2- to 15-fold ratio was observed in different experiments between cells infected at 3 and 24 h after release from G₀. These assays detect viral genomes specifically or nonspecifically bound to the cell surface, as well as internalized genomes. Thus, the difference in virus uptake at different phases of the cell cycle could reflect a difference in attachment to the virus receptor or in penetration, or possibly in both. We will refer to this difference as a difference in adsorption.

We also compared cellular and nuclear uptake. In contrast to cellular uptake, which is complete by 4 h postinfection (data not shown), very little nuclear uptake has taken place by that time; by 20 h postinfection the bulk of the virus signal (over 60%) has become associated with the nucleus (data presented below demonstrate that replication begins around 36 h postinfection). Thus, total or nuclear DNA was extracted at 20 h postinfection, as described in Materials and Methods. As seen in Fig. 2C, a peak of virus signal in the nuclei was also observed in cells infected in early G₁, with a 30-fold ratio between cells infected 3 and 24 h after release from G₀, compared with a 14-fold differential for the whole-cell-associated signal (Fig. 2B). The difference between the amplitudes of the virus uptake differential and that for nuclear uptake in the same experiment is compatible with

FIG. 3. Analysis of viral transcripts. FR3T3 cells were treated and infected as described in Materials and Methods (protocol 1) and in the legend to Fig. 2. Times of infection after release from G_0 are indicated. Samples of total RNA (20 μ g), extracted from cells 48 h postinfection, were electrophoresed, blotted, and hybridized with a 32 P-labeled probe containing either the entire polyomavirus DNA (top panel) or the GapDH DNA (bottom panel) (rehybridization of the top panel) as described in Materials and Methods. The positions of 28S, 19S, 18S, and GapDH RNA are shown. The positions of the 28S and 18S RNA species were determined by staining the gel, and that of the GapDH was obtained by superposition of the GapDH hybridization pattern onto the polyomavirus pattern. The position of the 19S band is deduced.

the existence of a cell-cycle-dependent step between viral adsorption and entry into the nucleus. By itself, this result should not be given too much weight, since total and nuclear virus uptakes were compared directly in only a single experiment. Nevertheless, the same conclusion is also suggested by the difference between the adsorption and the transcription differentials observed multiple times (see below). However, this hypothesis relies on an accurate measure of the adsorption differential. This may be problematic if, for example, the minimal signal (i.e., for 24 h after release from G_0) represents mostly background, consisting of viral genomes that are nonspecifically associated with the cell membrane and that almost never enter the infectious cycle. This problem would lead to an underestimation of the adsorption differential. It is also possible that virus uptake (and hence transformation) observed in G_2 infections is mediated by a minor uptake pathway(s) that is not regulated or is differently regulated during the cell cycle.

Viral gene expression. Viral gene expression was monitored in cells infected at different phases of the cell cycle, following protocol 1. For this purpose, total RNA was extracted at 48 h postinfection, blotted, and hybridized as described in Materials and Methods. The results are shown in Fig. 3. A large difference in the number of viral transcripts (40-fold in the experiment shown) was observed in cells infected 3 and 24 h after release from G_0 . A similar (50-fold) difference was found in a dot blot analysis of the same RNA. Rehybridization of the same blot with a GapDH probe shows that equal amounts of RNA were loaded in each lane.

The size of the polyomavirus transcripts was very variable and included very large molecules (Fig. 3), corresponding to species of 4 to 5 times the unit length of the polyomavirus genome. The compactness of the 2.4-kb GapDH mRNA band in each lane and the very large size of these transcripts

FIG. 4. Detection of anti-late mRNA. RNA samples from the same experiment as that shown in Fig. 3, isolated from cells 48 h postinfection, were processed for Northern blot analysis as described in Materials and Methods and in the legend to Fig. 3. Hybridization was to a 32 P-labeled single-strand riboprobe specific for the detection of polyomavirus anti-late mRNA. The positions of 28S and 18S RNA are shown (see the legend to Fig. 3).

suggest that the heterogeneity was not due to degradation. The heterogeneity in the size of the polyomavirus signal was not caused by contamination with DNA, since treatment with DNase did not alter the RNA pattern whereas an added plasmid DNA control was completely digested (data not shown). Poly(A)-containing mRNA species displayed the same heterogeneity in size as the total transcripts did (data not shown). Most of the mature viral transcripts in infected rat cells would be expected to be early transcripts, whose sizes are 18S and 19S (11, 12). A possible explanation for the high-molecular-weight polyomavirus transcripts is run through transcription, obviating the early poly(A) addition signal. Runthrough transcription has been documented for the late transcripts produced in polyomavirus-infected mouse cells (27). Runthrough transcription of early mRNA would lead to the production of anti-late mRNA. To test this possibility, we performed Northern blot hybridization to a single-strand riboprobe of appropriate polarity. Abundant anti-late transcripts were found (Fig. 4). In contrast, true late transcripts were not detected (not shown). We conclude that the heterogeneous size of polyomavirus RNA transcripts represents early as well as anti-late mRNAs derived by runthrough transcription.

Viral early-protein expression. The expression of large T antigen was analyzed at 48 h postinfection by Western immunoblotting as described in Materials and Methods in the same experiment as that for which the transcripts are shown in Fig. 3. A polyclonal rabbit serum against polyomavirus small T antigen which theoretically should detect large, middle, and small T antigens, was used. As shown in Fig. 5, only large T antigen was detected. Its expression paralleled that of viral transcripts: a peak was observed in cells infected early in the G_1 phase, with a 10-fold increase in cells infected at 3 h after release from G_0 compared with those infected at 24 h. The absence of middle and small T antigens is likely to reflect the lower abundance of these two proteins and/or a detection problem, since the assay was optimized for large T-antigen detection.

Viral DNA replication. To determine whether viral DNA synthesis parallels viral gene expression, we extracted total

FIG. 5. Western blot analysis of large T antigen. Cells were infected as described in Materials and Methods and in the legend to Fig. 3 (protocol 1). Cell lysates were harvested from infected cells 48 h postinfection. Aliquots of the cell lysates were analyzed in 10% polyacrylamide and electroblotted onto nitrocellulose filter paper. The filter paper was incubated with rabbit polyclonal antibody directed against small T antigen. Bound antibody was detected as described in Materials and Methods. Positions of stained molecular weight markers are shown (in thousands). The position of large T antigen (LT-Ag) (derived from a cutout sample of a lytic infection) is also shown.

DNA following the onset of viral DNA replication, e.g., 48 h postinfection (see below) from the same experiment as that for which viral transcripts and large T-antigen profiles are shown in Fig. 3 and 5, respectively. Viral DNA was assayed by hybridization. As shown in Fig. 6, the maximal viral DNA signal was also seen in cells infected in early G_1 phase, with a fivefold-larger yield in cells infected at 3 h than in those infected at 9 h. A comparison of the profile of viral genome levels at 4 and 48 h postinfection was also shown for a different experiment in Fig. 2A. Thus, the cell-cycle-related differential in viral genomes at 48 h is assumed to reflect differences in adsorption. As perhaps expected for nonpermissive cells (26), the amplification of the viral genome is small despite the presence of clearly detectable levels of large T antigen. Although the levels of genome per cell is not significantly increased at 48 h in the experiment shown in Fig. 2A, it represents at least a sixfold amplification, since the cell number has increased this much. We previously

FIG. 6. Analysis of viral DNA replication. Cells were infected as described in Materials and Methods and in the legend to Fig. 3. Total DNA was prepared from infected cells 48 h postinfection. A 10- μ g sample of total DNA was digested with *Eco*RI, electrophoresed on a 0.7% agarose gel, and probed to a 32 P-labeled probe containing the entire polyomavirus sequence.

FIG. 7. Kinetics of viral gene expression and viral DNA replication in FR3T3 cells infected 3 h after release from G_0 . (A) Transcription kinetics. Cells were infected 3 h after release from G_0 following protocol 1. Total RNA was extracted from the infected cells at 12, 24, 36, 48, and 60 h postinfection. Samples of total RNA (20 μ g) were analyzed in 1% formaldehyde agarose, hybridized to a 32 P-labeled probe containing the entire polyomavirus DNA, stripped, and rehybridized with a GapDH probe, as described in Materials and Methods and in the legend to Fig. 2. (B) Detailed transcription kinetics. Cells were infected 3 h after release from G_0 following protocol 1. Total RNA was prepared from the infected cells 12, 18, 24, and 27 h postinfection. Samples of total RNA (5 μ g) were deposited on membranes and hybridized to 32 P-labeled probe containing the entire polyomavirus DNA. (C) Kinetics of viral DNA replication. In the same experiment as that for which the transcription kinetics is shown in panel A, total DNA was extracted from cells at 12, 24, 36, 48, and 60 h postinfection. Samples (10 μ g) were digested with *Eco*RI and analyzed as described in Materials and Methods and in the legend to Fig. 6. For hybridization, a 32 P-labeled probe containing the complete polyomavirus DNA was used.

found that in polyomavirus infection of nonsynchronized FR3T3 cells, viral DNA replication is delayed and highly heterogeneous, with some cells synthesizing large numbers of genomes and others synthesizing undetectable levels (10).

Kinetics of viral gene expression and viral DNA replication. To further examine the regulation of viral gene expression during the cell cycle, we examined the kinetics of viral transcription and DNA replication under conditions which maximize transformation, i.e., in cells infected 3 h after release from G_0 (protocol 1). As shown in Fig. 7A, viral transcripts were not detected until between 24 and 36 h postinfection, when a dramatic increase took place. The timing of the cell cycle in the same infection was determined by monitoring the incorporation of [3 H]TdR and cell multiplication. The first cell cycle took place between 0 and 26 h after release from G_0 , and the second took place between 27 and 40 h after release from G_0 . Thus, these results show that although virus entered the cells in early G_1 of the first cell cycle, substantial viral gene expression occurred only in the

next cell cycle. The transcripts remained at a high, apparently constant level until at least 60 h into the infection process. To better pinpoint the onset and the synchrony of viral transcription, we carried out a more detailed kinetic study. In this experiment, incorporation of [3 H]TdR and cell number counting showed that the first cell cycle ended at 23 h. A large synchronous burst of expression was observed (Fig. 7B) within a 6-h period, corresponding to the early G₁ phase of the second cell cycle in the infection. The time of cell doubling and the onset of viral transcription coincided in three independent experiments.

The kinetics of viral DNA synthesis was established in parallel with the transcription kinetics shown in Fig. 7A in the same experiment; the results are shown in Fig. 7C. Genome amplification closely paralleled the transcription pattern. There appeared to be no delay between transcription and genome amplification. However, in contrast to transcription, the level of DNA replication was modest and continued to increase up to 60 h into the infection.

DISCUSSION

In this study we present evidence for at least two levels of cell cycle control in the abortive infectious cycle of polyomavirus in nonpermissive FR3T3 cells released from G₀. For the low to moderate range of multiplicities used (0.2 to 20), the transformation frequency, coinciding in both abortive and stable processes, peaked for cells infected early in G₁ phase. Frequencies decreased for late G₁ infection and reached a minimum for early S-phase infection; there was not much further decrease for infections in the remainder of the cell cycle. The total differential between the highest and lowest stable transformation frequencies varied between 5 and 100 from experiment to experiment and averaged around 20. The peak frequencies represent the highest frequencies reported for these cells, and minimal frequencies were somewhat below those obtained for infections of exponentially growing cells. Our results are not directly comparable to relevant previous data. Only two such reports are available to our knowledge. (i) In hamster cells infected during exponential growth and synchronized postinfection by periodic shakeoff of mitotic cells, a maximum in neoplastic transformation was found for cells infected at the S/G₂ boundary, representing a modest twofold increase over frequencies obtained for cells infected in other phases (3). (ii) In comparisons of the effect of various growth conditions on transformation by polyomavirus and SV40 of another independently derived fibroblast Fischer rat cell line (3Y1), transformation was reduced when cells were in a proliferating state compared with a quiescent state at the time of virus inoculation.

To understand the factors associated with the variations in transformation, we analyzed virus uptake, viral gene expression (mRNA and proteins), and viral DNA replication in cells infected at different phases of the cell cycle. A 2- to 15-fold early-G₁-versus-G₂ differential was found in virus uptake (assayed as the total viral DNA entering into host cells) between cells infected at 3 h after release from G₀ and those infected later than 9 h after release from G₀. (As discussed in the Results section, this differential may have been underestimated.) Thus, these data suggest that an early step of the infection is cell cycle regulated and maximal in G₁ (at least during the G₁ phase following release from G₀), perhaps the appearance of the polyomavirus receptors on the cell surface. Differential rates of viral adsorption have been described previously for SV40 on the basis of binding

of [3 H]TdR-labeled virus particles to CV1 cells. These results showed a two- to threefold increase in virion binding in G₁ compared with G₂ or M cells (15, 17). However, recent experiments show an increase in virion binding of about threefold in S and G₂ M-phase cells compared with G₁ phase cells (2). Little and dated information is available for polyomavirus, whose receptor remains unidentified. No cell cycle variations were reported for infections of BHK cells (3), whereas a threefold increase in relative rates of virus binding was observed in G₁ compared with G₂ phase cells in infections of BALB/c-3T3 mouse cells following a release from G₀ (24). Tracking with maximal adsorption for G₁ infection in the experiments described above, other parameters—nuclear uptake, viral transcripts levels, and large T-antigen levels—were also maximized in early G₁ infections. Comparisons are not available for these parameters; dated short reports suggest threefold increases in relative yields of infection for mouse cells infected in G₁ compared with those infected in G₂ following release from G₀ (13, 25). In terms of their amplitudes, the G₀ versus G₂ differentials in nuclear uptake, viral transcripts levels, and large T-antigen levels were larger than the adsorption differential but were of the same order of magnitude (20- to 50-fold) as the differential in stable transformation. This result may reflect a simple linear relationship between these parameters, at least for the first three mentioned; e.g., more adsorption leads to more transcripts leads to more large T antigen leads to more transformation (in the low to moderate range of multiplicities used in these experiments).

The comaximization of the parameters tested suggests that transformation is controlled by a single major rate-limiting step or by multiple steps which are all maximized by infection in the G₁ phase following release from G₀, which step(s) needs not be occurring in G₁ phase. The data are perhaps more compatible with multiple steps, since the amplitude of the differential in virus uptake only partially accounts for the differential found for subsequent steps, especially when comparing viral uptake and viral transcription. This and other observations discussed below suggest the existence of at least one other rate-limiting step in the abortive-infection cycle between adsorption and gene expression which is also regulated during the cell cycle. This step could be penetration, decapsidation, nuclear transport, integration, or another yet to be determined cell-cycle-regulated step(s) in the viral infection. The coincidence between the G₀ and G₂ differentials for abortive and stable transformation suggests that integration is either cell cycle insensitive or also maximized in infection of G₀-released cells. Preliminary results on integration patterns support the latter hypothesis, as is also suggested by a larger differential in stable than abortive transformation.

In addition to the virus uptake step, a second level of cell cycle control was clearly demonstrated when the kinetics of viral gene expression was analyzed. An essentially synchronous and large burst of early viral transcripts was observed, coinciding with the G₁ phase of the cell cycle following that in which infection took place. Thus, it appears that viral genomes inoculated 3 h into the first G₁ phase of the cell cycle have penetrated inside the cells by 4 h postinfection (7 h after G₀ release), have migrated to the nucleus between 4 and 20 h postinfection (23 h after G₀ release), and are transcribed only in the next G₁ phase. This lengthy delay in viral gene expression is reminiscent of a similarly long delay in SV40 DNA synthesis demonstrated in G₁, S, or G₂ infection of permissive cells synchronized by double-thymidine block (17) and also suggested in polyomavirus infec-

tions of G₀-released mouse cells infected in the S phase (13). Two possible explanations might account for this result: early G₁ regulation of the viral early promoter, or late G₂ (or very early G₁) regulation of a step between nuclear uptake and transcription. In the first case, viral genomes entering in early G₁ are not processed in time to catch the transcription competence step and must wait a full cycle to become transcribed. In the second case, viral genomes become transcription competent (decapsidated, transported to the nucleus, etc.) at a specific time in the cell cycle and transcription itself is unrestricted. The experiments performed do not allow us to distinguish between these two possibilities, which are in fact not mutually exclusive. The fact that the level of transcripts remained high in the remainder of the experiment, e.g., over a complete cell cycle following the first burst of transcription, is compatible with the second hypothesis, as well as with a long half-life of these transcripts or with the predictable loss of synchrony in the infected cells.

As is always the case when studying neoplastic transformation by polyomavirus or SV40, only circumstantial conclusions can in principle be drawn between the events observed in the population as a whole and those occurring in the population undergoing abortive or stable transformation, since the last two represent a minority of the total population (6 and 1%, respectively, in this particular case). However, there is no a priori reason to doubt that the cell-cycle-regulated events which are taking place in the whole population are at least in part responsible for the documented cell cycle regulation of the induction of the abortive and stable transformation phenotype.

In conclusion, these experiments demonstrate multiple levels of cell cycle regulation in the infection of FR3T3 cells released from G₀. These include at least a very early step in adsorption and a later step(s) also maximized by infection in G₁, which is either the recognition of the early promoter or a step between adsorption and transcription, or both. Altogether, this regulation results in a large differential in transformation between G₁ and G₂ infection in cells released from G₀. As the results appear specific for cells released from G₀, this phenomenon may be akin, or causally related, to the large G₁ burst of expression of cellular early response genes, such as *c-fos*, *c-jun*, and *c-myc*, observed in fibroblasts released from G₀ by serum factors (8, 19) but not in the normal G₁ phase of cycling cells (23). The important function in polyomavirus early-gene expression (18), viral DNA replication (16), and possibly viral genome integration (5a) of two of these cellular genes (*c-jun* and *c-fos*) suggests a possible causal relationship between these observations. Although the interaction of the viral capsid with the polyomavirus receptor in G₀-arrested cells also triggers the expression of the same early-response genes (*c-fos* and *c-myc*) (28), it is not likely to play a role at the low multiplicities of infection used in the present experiments.

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