Kinetic Analysis of the Steps of the Polyomavirus Lytic Cycle

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Received 13 December 2000/Accepted 13 June 2001

Kinetic studies of the accumulation of early and late transcripts, early and late proteins, genomes, and live virus, during the lytic cycle of murine polyomavirus wild-type A2, were carried out in synchronized NIH 3T3 cells released from G_0 by the addition of serum after infection. This first-time simultaneous analysis of all parameters of the virus life cycle led to new insights concerning the transcriptional control at the early-to-late transition. During the early phase, early transcripts were synthesized at very low levels, detectable only by reverse transcription-PCR, from 6 h postinfection (hpi). Large T protein could be detected by 8 hpi (while infected cells were in the G_1 phase). The level of expression of the middle T and small T proteins was lower than that of large T at all times, due, at least in part, to a splicing preference for the large-T 5' splice site at nucleotide 411. A large increase in the level of both early and late transcripts coincided closely with the detection in mid-S phase of viral genome amplification. Thereafter, both classes of transcripts continued to further accumulate up to the end of the experiments (48 hpi). In addition, during the late phase, "giant" multigenomic transcripts were synthesized from the early as well as the late promoter. Thus, a major type of transcriptional control appears to be applied similarly to the transcription of both early and late genes. This view differs from that in the literature, which highlights the enhancement of late transcription and the repression of early transcription. However, despite this parallel transcriptional control, additional regulations are applied which result in higher levels of late compared to early transcripts, as previously described. In the accompanying article, a key role for middle T and/or small T in this late-phase enhancement of early and late transcription is demonstrated (16). Other novel findings, e.g., the synthesis of a very abundant short early promoter proximal RNA, are also described.

Polyomaviruses have served as important model systems for the understanding of various aspects of cellular organization and function, such as chromatin structure, the role of enhancers in gene expression, splicing mechanism, and the composition of the DNA replication machinery, to name a few. This is in part a consequence of the DNA nature and small size of the genomes of this virus family, which render viral replication highly dependent on, and hence compatible with, cellular processes. As a result, and with the added interest in their capacity to induce neoplastic transformation, these viruses have been the subjects of extensive studies.

Surprisingly at this late stage of analysis, there has been no comprehensive study of the temporal relationships between all the various steps of the lytic cycle. Current references to the timing of these steps invariably quote the 1981 review by Acheson (2), which offers a thorough integration of the studies available at the time. However, these studies were done at different times, in different laboratories, with different viral strains, and with different primary cells or cell lines. Furthermore, little information is available about the timing of these lytic events in the function of the cell cycle.

In the present study, an integrated kinetic and quantitative analysis of the transcripts, proteins, genomes, and virus produced during murine polyomavirus lytic infection is described for the first time in relation to phases of the cell cycle. The major rationale for these experiments was to define the timing

of synthesis of the middle and small T proteins with respect to other events of the lytic cycle to further our understanding of the role of these proteins in lytic growth. The experiments were carried out in cells synchronized and infected in the G_0 phase of the cell cycle. Because G_0 -released cells maintain a good synchrony up to the beginning of the S phase and since viral DNA replication cannot be initiated prior to reaching the S phase, this strategy had the advantage of producing a long early phase and a very sharp early-to-late transition. This allowed the detailed examination of this transition. Detectable effects of middle and small T proteins were found to be delayed to the S phase, coinciding with the onset of viral DNA replication and a major transcriptional switch that affected both the early and the late promoter. Further studies, described in the accompanying study (16), demonstrate a causal rather than a coincidental relationship.

MATERIALS AND METHODS

Virus, cells, and infections. Polyomavirus wild-type strain A2 (WTA2) was used (23). An NIH 3T3 cell subline, originally obtained from T. Benjamin, was used for the study as well as to grow virus stocks. This line has a functional p53 function, since it induces the expression of p21 in response to treatment with actinomycin D; however, it is negative for p16 expression and has an unregulated cyclin D1 expression. Prior to infection, cells were brought into synchrony in the G_0 -state as follows. Cells were plated at 3×10^4 cells per 60-mm culture plate in Dulbecco modified Eagle medium (Gibco-BRL) supplemented with 10% newborn calf serum (Gibco-BRL) (abbreviated as "serum" throughout). After 3 days, the cells reached about 25% confluency, and the serum supplement was lowered to 0.5% for another 24-h incubation period. The exit out of the cell cycle into the G_0 state prior to infection was confirmed by fluorescence-activated cell sorting (FACS) analysis. The medium was removed, and virus was added in 0.5 ml of $1 \times$ phosphate-buffered saline ($1 \times$ PBS) supplemented with 0.5% serum. A multiplicity of infection of 10 PFU was used. After 1 h, the unadsorbed virus was

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removed; cells were washed once with $1\times$ PBS and refed with medium supplemented with 10% serum. Analysis of the proportion of genomes that entered the cells compared to the level in the "input" suggests that the actual multiplicity may be less than 10 (data not shown). The times given throughout (in hours postinfection [hpi]) also correspond to times post-release from G_0 .

FACS analysis. Cells were harvested at the times shown (hours post-release from G_0 = hpi), washed twice, resuspended in 1 ml of cold $1\times$ PBS containing 2% serum, and fixed by rapid injection into 10 volumes of ice-cold 80% ethanol. Cells were pelleted by centrifugation, washed in $1\times$ PBS and incubated in 300 μ l of PI reagent (50 mg of propidium iodide per ml, 0.1% Triton X-100, 100 mM EDTA, and 50 mg of RNase A per ml in $1\times$ PBS; pH 7.4) in the dark for at least 30 min at room temperature. The cell cycle stage of the cell population was determined with a fluorescence-activated cell sorter (Becton Dickinson FACS Vantage model) using Cell Quest. The analysis of the cell cycle was carried out using the Multiprime or Wincycle programs.

Preparation and analysis of RNA. Infected cells were lysed in Trizol solution (Gibco-BRL) at various times. Total RNA was extracted with chloroform and precipitated with isopropanol. For Northern blots, RNA samples were electrophoresed in a 1% agarose gel containing 2.2 M formaldehyde and transferred to a nylon membrane (Amersham). Probes were labeled either with radioactive [α - 32 P]dCTP or with digoxigenin as indicated in the figure legends.

To simultaneously detect all transcripts and quantitate RNA, a 32 P-substituted double-stranded genomic DNA probe was used. Additionally, specific *MspI* DNA fragments introduced in the pUC19 plasmid were used to identify specific regions in transcripts. The genomic and fragment DNA probes were labeled with [α - 32 P]dCTP (3,000 mCi/mmol; New England Nuclear) with a multiprime DNA-labeling kit (Amersham). Hybridization was performed at 42°C in 0.05 M sodium phosphate buffer (pH 7.0), 1 M NaCl, 50% formamide, 1% sodium dodecyl sulfate (SDS), 5% dextran sulfate, and 100 mg of salmon sperm DNA per ml. Membranes were washed, and exposed to X-Ray film for a few days at -70°C with an intensifying screen. Transcript levels were quantitated, by counting with a PhosphorImager (Molecular Dynamics).

The following probes were used to detect the six specific PYV early and late mRNA species. To detect early transcripts, a pGEM1-based plasmid, pG3PyH4, was used that contains PYV *MspI* fragment 4 (nucleotides [nt] 399 to 1101), cloned between the *HindIII* and *EcoRI* sites. The plasmid was cleaved with *HindIII* and the T7 RNA polymerase was used to synthesize the early-specific probe. This probe spans the three overlapping early introns and can detect all three early mRNAs, i.e., the 19S middle T and small T mRNAs and the 18S large T mRNA, as well as the unspliced precursor and multigenomic early RNAs. To score for late RNAs, sequences spanning nt 3918 to 2928 were inserted between the *HindIII* and *BamHI* sites of pSPT18 (Roche Molecular Biochemicals). The plasmid was cleaved with *HindIII* and the T7 RNA polymerase was used to synthesize RNA. This probe detects all major late RNAs, the major 16S VP1 mRNA, and the minor VP2 and VP3 mRNAs since it spans the 3' terminus of the late RNAs, spliced and unspliced. This probe also detects multigenomic late RNAs.

The strand-specific RNA probes were labeled with digoxigenin (unless otherwise noted) using the kit from Roche Molecular Biochemicals, according to the instructions from the manufacturer. The hybridizations with digoxigenin-substituted probes were carried out as follows. Blots were prehybridized for 2 h at 68°C in 50% formamide, 5 $\times$ SSC, 0.02% SDS, 0.1% *N*-laurylsarcosine, and 2% blocking reagent (Roche Molecular Biochemicals) and then hybridized overnight at 68°C in a hybridization mixture containing digoxigenin-labeled RNA probe. The membranes were washed twice with 2 $\times$ SSC and 0.1% SDS at room temperature for 15 min each time and twice with 0.5 $\times$ SSC and 0.1% SDS at 68°C for 15 min each time. The membranes were treated with blocking agent solution for 1 h and then with the anti-digoxigenin-alkaline phosphatase, diluted 10,000-fold in blocking buffer for 30 min. After an extensive washing, the chemiluminescent phosphatase substrate detection reagent CSPD was applied for 1 min, and the membranes were exposed to X-ray films.

For the reverse transcription-PCR (RT-PCR) analysis, RNAs were partially purified with polyAT Tract mRNA Isolation System Kit (Promega) and used as templates. The position of the primers used to detect the early RNAs is shown Fig. 2; the primers span the three overlapping early introns. The downstream primer (nt 875 to 856) was added first for RT by the Moloney murine leukemia virus reverse transcriptase for 1 h at 42°C. The upstream primer (nt 386 to 405) was then added, and the PCR cycling was started (95°C for 45 s, 58°C for 45 s, 68°C for 1 min; 20 or 30 cycles as specified in the text). The products were separated by gel electrophoresis and stained with ethidium bromide or transferred to a membrane and hybridized with a genomic polyomavirus probe.

Protein analysis. Cells were lysed at various times with protein sample buffer (5% SDS; 0.03% bromophenol blue; 20% glycerol; 5% β -mercaptoethanol; 0.5

M Tris-HCl, pH 6.8) and boiled for 5 min. One-third aliquots of the lysates were electrophoresed in 10% polyacrylamide and electroblotted onto polyvinylidene difluoride membranes (Amersham). A polyclonal rat antitumor serum, harvested as ascites fluid, was used as the primary antibody. This antibody recognizes all three early proteins: large, middle, and small T. It also reacts with a few cellular proteins which can serve as an internal loading control. Goat anti-rat horseradish peroxidase (HRP; Pierce) was used as the secondary antibody. Two mouse monoclonal antibodies to the common amino termini of large, middle, and small T antigens, PN116 and PAb762 (gifts of B. Schaffhausen and S. Dilworth, respectively), were used to estimate the relative level of the three early proteins, combined with goat anti-mouse HRP (Pierce) as the second antibody. A rabbit anti-capsid antibody, a gift from R. Garcea, was used to for the detection of the VP1 capsid protein, with goat anti-rabbit HRP as the second antibody.

Middle-T-associated kinase assay. The procedure of Schaffhausen and Benjamin was used (40). Infected NIH 3T3 cells (2×10^6 cells) were lysed with sample buffer (50 mM Tris-HCl, pH 7.5; 150 mM NaCl; 1 mM EDTA, pH 8.0; 100 mM NaF; 10% glycerol; 1 mM MgCl₂; 1% NP-40; 1 mM phenylmethylsulfonyl fluoride, 10 μ g of leupeptin per ml; 5 μ g of aprotinin per ml; 1 mM sodium orthovanadate) and incubated on ice for 30 min. Lysates were centrifuged at 12,000 rpm for 10 min at 4°C. The pellet was removed. The antibodies were added to the supernatant and then incubated at 4°C for 2 h with shaking. Both the polyclonal and MAb762 monoclonal serum were used. A total of 50 μ l of Sepharose-protein A was then added to the supernatant and incubated for 1 h at 4°C. The beads were washed twice with 0.5 M LiCl–0.1 M Tris-HCl (pH 6.8) and finally 0.2 M Tris-HCl (pH 7.5)–5 mM MgCl₂. The immune complexes were suspended in 0.2 ml of kinase buffer (20 mM Tris-HCl, pH 7.5; 5 mM MgCl₂; 20 μ M of [γ - 32 P]ATP) at 37°C for 15 min. The beads were pelleted and washed in 1 $\times$ PBS and then twice in 0.5 M LiCl–0.1 M Tris-HCl (pH 6.8) and once in 0.1 M sodium pyrophosphate (pH 6.8) and were finally suspended in 1 $\times$ PBS. The proteins were dissociated from the beads by boiling in 1 $\times$ protein loading buffer. The supernatant was loaded in a SDS-polyacrylamide gel; the gel was dried and exposed to X-ray film.

Preparation and analysis of DNA. Infected cells were lysed in 10 mM Tris-HCl–10 mM EDTA–0.2% SDS (pH 7.6) supplemented with 0.1 μ g of proteinase K (Sigma) per ml. DNA was extracted with phenol-chloroform. DNA was digested with the restriction endonuclease *EcoRI* (Gibco-BRL), which has a single recognition site in the polyomavirus genome and linearizes it. Digested DNA was electrophoresed in 1% agarose, stained with ethidium bromide, and blotted onto nylon membranes (Amersham Pharmacia Biotech). The hybridization was carried out at 65°C in 1 $\times$ Denhardt solution, 2 $\times$ SSPE (1 $\times$ SSPE is 0.18 M NaCl, 10 mM NaH₂PO₄, and 1 mM EDTA [pH 7.7]) by standard procedures with a 32 P-radiolabeled probe containing the whole polyomavirus genomic DNA. Hybridization probes were labeled with [α - 32 P]dCTP as described above. The hybridized blots were washed under stringent conditions. The blots were exposed to X-ray film (Kodak) for a few days at -70°C with an intensifying screen. To quantitate the level of viral genomes, membranes were scanned with the PhosphorImager.

Plaque assays. NIH 3T3 cells were grown to 80% confluence and infected with virus dilutions for 1 hr at 37°C. Infected cells were refed with medium containing 5% serum and 0.9% agar and then incubated for 7 days at 37°C. The plates were stained with neutral red. The plaques were counted after >4 h of incubation at 37°C.

RESULTS

Progression of the infected cells through the cell cycle. NIH 3T3 cells were maintained in a subconfluent quiescent state for over 24 h, infected at a multiplicity of 10 for 1 h with a wild-type strain of polyomavirus (WTA2), and then released from G_0 by refeeding in medium supplemented with 10% serum as described in Materials and Methods. In order to time the events in the lytic infection in relation to the progression of the host cell cycle, a cell cycle analysis was carried out by FACS analysis. The progressions of uninfected cells and infected cells through the cell cycle were essentially identical (data not shown). As is usually the case for cells released from G_0 , the first G_1 phase was long (12 h). The first S phase lasted about 10 h, the first G_2 phase was short, and the first cell cycle was completed in 26 h. Both uninfected and infected cells divided

FIG. 1. Northern analysis of the viral transcripts. Cells were infected as described in Materials and Methods. Total RNA was extracted at the times shown (HPI = hpi), electrophoresed, and transferred onto a membrane. The blot was hybridized to a ^{32}P -labeled probe for the cellular GAPDH gene (shown at the bottom), stripped, and rehybridized with a genomic ^{32}P -labeled polyomavirus DNA probe. The positions of the 28S and 18S RNAs derived from the ethidium bromide staining are shown, as well as that of the remnant of the GAPDH signal. The phase of cell cycle of the largest fraction of the cell population at the time of sampling was defined by FACS analysis and is shown at the top. The time scale is not linear.

and proceeded into a second less synchronous cycle and, yet later, a third cycle. The cell cycle profiles of NIH 3T3 cells synchronized with the protocol described showed very small variations from experiment to experiment, depending upon initial conditions. Cell cycle phases were determined in most experiments and, where appropriate, are indicated in the figures.

Samples were taken at various times to assay transcripts, proteins, viral genomes, and live virus. Most experiments were stopped at 48 hpi, when cytopathic effects became visible. Various aspects of this analysis were repeated in multiple experiments, and all conclusions are based on at least two experiments. The data shown in Fig. 1, 2, 3, 5, 7, and 8 were generated in the same comprehensive experiment.

Experiments carried out in parallel with or without serum stimulation showed similar results with regard to the kinetics of the first cycle (data not shown). The major difference observed was a delay of approximately 2 h in the detection of early proteins, and a delay in entry into S (also about 2 h) in the absence of serum. All data presented in this report were obtained in cells that were serum stimulated at the end of the adsorption period.

Analysis of the early viral transcripts. (i) Northern blot analysis. The analysis of viral transcripts was carried out by Northern blotting of total RNA, probed successively with (glyceraldehyde-3-phosphate dehydrogenase) and genomic polyomavirus DNA. Since the pattern of polyomavirus transcripts is very complex, with multiple 5' ends, overlapping transcripts, and overlapping introns, this is a helpful strategy that allows the simultaneous tracking of all transcripts and the changes in their abundance. There are, however, two major disadvantages, namely, the blocking and band-like distortions around the rRNAs and the "fuzziness" of the bands. The results of one experiment are shown in Fig. 1. Viral transcripts

were below the detection level of Northern blotting until ca. 16 to 18 hpi. This occurs despite the use of a probe with high specific activity and an $\sim 3\text{-kb}$ -long hybridization template. Thus, the early transcripts are synthesized at surprisingly low levels during the early phase of infection in NIH 3T3 cells. A sharp increase in transcript levels was observed by between 20 and 24 hpi: in this 4-h interval, the polyomavirus signal increased 63-fold, according to phosphorimaging determination of the blot as shown in Fig. 1. As discussed below, this switch involves both early and late transcripts. From 20 hpi, the total transcripts (immature plus mature) were of very diverse sizes and included giant transcripts. Hybridization with the GAPDH probe indicates that close to equal amounts of RNA were loaded in each lane and that the diverse sizes of the polyomavirus RNAs was not caused by degradation. A slight progressive increase in the level of GAPDH transcripts was observed which might reflect progression through the S phase and the duplication of the cell genome. The remnant of the GAPDH signal on the polyomavirus blot demonstrates that most of the polyomavirus transcripts are larger than the 1.2-kb GAPDH transcript. A detailed analysis of the early transcripts during the early-to-late transition using strand-specific probes and including considerations of the size of the transcripts is presented below (Fig. 6).

(ii) RT-PCR analysis. To increase the sensitivity of detection of the transcripts at early times, partially purified poly(A)⁺ RNA was subjected to RT-PCR analysis using primers flanking the large T, middle T, and small T introns (Fig. 2). This strategy allows the detection of the four possible RNAs in each sample: the unspliced RNA and the three early transcripts. The sizes expected for the PCR products corresponding to these four RNAs are shown. Since a single pair of primers was used, the relative abundance of the four products should be directly proportional to that of the four transcripts. All four

FIG. 2. RT-PCR analysis of the early viral transcripts. (A) The positions of the two primers with respect to the early-region introns are shown. The positions of the two 5' (nt 411 and 748) and the two 3' (nt 797 and 811) splice sites are shown on the bottom line. The size of the four resulting RT-PCR products for the large T (LT), middle T (MT), small T (ST), and unspliced transcripts are shown on the right. (B) RNA isolated at the times shown (HPI = hpi) and purified as described in Materials and Methods was subjected to one round of RT and various cycles of PCR amplification, using primers P1 and P2. A control for each transcript using viral DNA (M1), small T cDNA (M2), middle T cDNA (M3), and large T cDNA (M4) was carried out in the same reaction. The experimental and control products were separated by gel electrophoresis, transferred to a membrane, hybridized, and exposed to X-ray films. A darker exposure of the film region corresponding to the large T product is shown. The counts in the bands were determined by scanning the membrane with a PhosphorImager. The ratio of the counts in the large T band to those the middle T-small T band are shown. The ratio in the relative level of transcripts is corrected for the difference in size.

products were detected from 12 hpi by Southern blot hybridization of the 20-cycle RT-PCR products (Fig. 2) and by ethidium bromide staining following 30 cycles of amplification (data not shown). The abundance of the large T mRNA, assayed in the 20 cycle PCR products, increased at each time point, resulting in a >400-fold difference by between 6 and 36 hpi. Only the large T band was detected in the 6-hpi sample. A more sensitive hybridization with a bias to the detection of middle T-small T sequences also detected the large T product in the 6-hpi sample and also failed to detect middle T-small T bands at 6 hpi (not shown). The counts contained in the large T and middle T-small T bands and their ratios are shown. The level of the large T transcript was the highest of all four RNAs at all times tested. After the 4-fold correction for the size difference of the bands, the ratio of large T to middle T-small T bands at 12, 18, 24, and 36 hpi varied between 4- and 16-fold (or 3.5- to 7.5-fold in the 30 cycle products; data not shown). Thus, if the splicing of the middle T and small T transcripts is equally efficient, the levels of large T to middle T transcripts varied between 8- and 32-fold. The level of unspliced transcripts was low at all times.

Expression of the early proteins. The expression of the viral early proteins was analyzed by Western blotting, as described in Materials and Methods. The blots were probed alternatively with a polyclonal, and two monoclonal antibodies that recognize an epitope common to the amino termini of large, middle, and small T proteins. Using the more sensitive polyclonal antibody (Fig. 3A), large T antigen was detected from 8 hpi, i.e., 2 h postdetection of its transcript by RT-PCR and 8 h prior to its detection by Northern blotting. The detection of large T preceded that of middle and small T by at least 6 h (see Fig. 4 for another example of delayed middle T and small T synthesis). There was a continuous, quasimonotonous increase in the levels of the three T proteins during the early phase of infection and throughout the time course analyzed, up to 48 hpi. The level of large T remained higher than that of middle T and small T at all times. Use of two monoclonal antibodies gave very similar results, including the delayed detection of middle T-small T, and the higher levels of large relative to middle T-small T at all times (data not shown or Fig. 3B). Lower levels of expression of middle T and small T relative to large T were also observed in infections of NIH 3T3 cl7, a subline characterized in C. Scherr's laboratory, which has a regulated cyclin D1 expression (data not shown). Furthermore, lower levels of middle T and small T relative to large T expression were also observed in a different genetic background (e.g., WTB2; see reference 16). Experiments using a multiplicity of infection in a range between 0.1 and 30 showed that the multiplicity of 10 used throughout is still within an apparently linear response range (data not shown). Nearly 100% of the cells expressed the large T protein when assayed by immunofluorescence at 32 hpi (data not shown).

Middle-T-associated kinase activity. Since middle T exerts its function by associating with and activating the c-Src family kinases, a kinetic analysis of middle-T-associated kinase function was carried out. At the times shown, protein samples from 10^6 cells were immunoprecipitated with the polyclonal anti-tumor serum, and a kinase assay was performed (Fig. 4). Middle-T-associated kinase activity representing an increase over the background could be detected at 14 hpi and increased throughout the time course tested (up to 36 hpi). The pattern of phosphorylated proteins did not change until 24 hpi, when novel bands appeared. A Western blot of extracts from 5×10^4 cells from the same experiment is shown for comparison. Middle T protein was not detected at 14 hpi in the Western blot. Similar results were found using the MAb762 monoclonal antibody, and in repeat experiments kinase activity was detected from 12 hpi.

Viral DNA replication. The accumulation of viral genomes was assayed by Southern blotting. Total DNA was isolated at the times shown and analyzed with a probe for polyomavirus genomic DNA. The results are shown in Fig. 5. The level of viral genomes remained constant or showed a slight drop from the beginning of the infection (4 hpi or input genomes) throughout the G_1 phase. An increase became detectable between 16 and 18 hpi, approximately in mid-S phase, with a 6-h delay with respect to the entry of cells into the S phase. This apparent delay between the initiation of viral and cell DNA synthesis was observed in many experiments. The counts hybridized per band were quantitated by a PhosphorImager and are shown under the bands. The increase in the levels of ge-

FIG. 3. Analysis of the early viral proteins. Cells were infected as described in the legend to Fig. 1. Proteins were extracted at the times shown (HPI = hpi). The first lane represents an uninfected control. (A) The Western blot was probed with a polyclonal antiserum. The three early polyomavirus proteins are identified on the right as large (LT), middle (MT), and small (ST) T antigens. The phase of the cell cycle, for the majority of the infected cells, at the time of harvest is shown on the top. (B) The blot shown in panel A was stripped and probed with a monoclonal antiserum (PN116) directed against the amino terminus common to the three viral proteins.

nome during the first S phase was at least 100-fold. An increase in the number of counts in each 2-h interval was observed throughout the experiment, even when the host cells were out of S phase and until the cytopathic effects became visible. The

total increase at the last time point at 48 hpi reached 8,000-fold. As cells have divided twice (not shown), the increase per cell is likely to be fourfold smaller. Since this increase is relative to an input multiplicity of 10, the number of genomes

FIG. 4. Middle-T-associated kinase activity. Cells were infected as described in the legend to Fig. 1 and extracted at the times shown. (A) Kinase assay. A total of 2×10^6 cells were immunoprecipitated with the polyclonal serum at the times shown, and a kinase assay was performed as described in Materials and Methods. Controls with preimmune serum were carried out at the 24- and 36-hpi time points. An uninfected cell control precipitated with immune serum was carried out at 24 hpi. The band representing middle T phosphorylated in tyrosine residue(s) is marked with a star. (B) A Western blot of extracts of the same infected cells (10^5 cells). This blot was probed with a polyclonal serum. The three early polyomavirus proteins are identified on the right: large (LT), middle (MT), and small (ST) T. This antiserum detects a cellular protein (identified with an arrow), which serves as a loading control. HPI = hpi.

FIG. 5. Southern blot analysis of the amplification of the viral genomes. Cells were infected as described in the legend to Fig. 1, and total DNA was extracted at the times shown, digested with *Eco*RI which linearizes the viral genome, processed for Southern blotting, and hybridized to a 32 P-labeled genomic probe as described in Materials and Methods. Samples from 4, 12, and 16 hpi were undiluted, while those from 18 to 48 hpi were diluted eightfold as shown. The cell cycle phases are shown above the lanes. Hybridized counts were determined with a PhosphorImager and corrected for dilution, and the increase relative to the input is shown for each time under each lane. HPI = hpi.

produced per cell might be above 20,000. However, this number may be smaller since not all the viruses enter the cell. The fraction of genomes entering the cell was not determined in this particular experiment.

Transcriptional transition from the early to the late phase.

The analysis of transcripts (Fig. 1) showed a dramatic increase in the transcript levels by between 20 and 24 hpi following the onset of viral DNA replication. To better understand the relationship between the onset of replication and transcription, this phase of the infection was analyzed in greater detail in two separate experiments, and one set of results is shown in Fig. 6. A period from before the detectable increase in viral genome amplification (14 hpi) to beyond the end of the first cell cycle (28 hpi) was examined in 2-h intervals. Protein (not shown), DNA, and RNA samples were collected. In the experiment shown, the S phase started before 14 hpi, a slight increase (1.14-fold) in viral genomes was observed between 14 and 16 hpi, and a 5.2-fold increase was observed between 16 and 18 hpi, at the peak of the S phase (Fig. 6, top). As described in Fig. 1, no transcripts were detected before 18 hpi.

To distinguish between early and late transcripts, the blot was hybridized successively to one early- and two late-specific RNA probes that were substituted with digoxigenin. These probes are short (see Materials and Methods) and detect true early and late region transcripts (but not the anti-early and anti-late sequences). With the early-specific probe the detection of transcripts began at 18 hpi, following the onset of viral DNA replication. This "band" (which can sometimes be resolved into a doublet: see Fig. 3 of reference 16) appears to be larger than the 19S value attributed to the early transcripts (reviewed in references 2 and 39). It increased in intensity by between 18 and 22 hpi and became broader, perhaps reflecting the presence of the three early mRNAs. The level of these early transcripts continued to increase in function of time at all other time points tested. A second transition in early transcription became apparent by between 24 and 26 hpi, with the appearance of large ("giant") heterogeneous RNAs.

Use of the late-specific probes showed that late transcription followed a very similar pattern, with the following differences. The detection of the late RNA was delayed by approximately 2 to 4 h compared to that of the early RNA. Late RNA consisted of two major bands, one larger than the early tran-

script (thus also clearly larger than the size assigned by sedimentation studies [reviewed in references 2 and 39]), presumably representing the VP2 and/or VP3 transcripts, and a more abundant band, shorter than 18S RNA, presumably representing the 16S VP1 transcript. The appearance of this "16S" band is strongly distorted by the 18S rRNA. The abundance of the late transcripts also increased continuously in function of time. The switch to large heterogeneous size transcripts was also apparent in the late transcripts and coincided in time with the appearance of similarly large early transcripts. The two late RNA-specific probes, which hybridize to VP1 or VP2-VP3 coding sequences, showed the same overall patterns (VP2 and VP3 data not shown). Optical scans of the films showed increases in intensity of 83- and 230-fold, respectively, for the early and late transcripts, by between 14 and 48 hpi. Both showed that the shorter "16S" RNA is much more abundant than the large RNA.

Results from the hybridization with strand-specific probes were consistent with those derived with a genomic 32 P-labeled probe. This probe detects all transcripts, including the anti-early and anti-late sequences, present in giant transcripts and their processed products. The distinct sizes of the early and late major transcripts allowed for a comparative kinetic and quantitative analysis in the blot hybridized to the genomic probe. The latter confirmed the earlier detection of the early transcripts and demonstrated that early transcripts remained the most abundant species for 4 h after the detection of the late transcripts (18 to 22 hpi). At later times, the 16S late RNA was clearly the most abundant species. As presented in Table 1, the increase in total transcript abundance, deduced from the PhosphorImager counting of the blots, was in the range of 3,000-fold at between 14 and 48 hpi.

To examine whether the increase in transcript levels could be accounted for solely by the increase in the level of viral genomes, the ratio of RNA to genomes was computed, as a PhosphorImager estimate of the 32 P counts hybridized in genomes over those hybridized in transcripts. These results show that if the RNA/DNA ratio is set at 1 at 18 hpi (when the transcripts become clearly detectable), this ratio increased to 13 by 28 hpi (Table 1). Very similar results were obtained in two separate experiments, showing that the increase in tran-

TABLE 1. Increase in viral genomes and transcripts during the early to late transition

Time (hpi)	DNA ^a	Transcripts ^b	RNA/DNA ^c	Relative RNA/DNA ^d
14	3.6	0	0	0
16	4.1	3	0.73	5.2
18	21.5	3	0.14	1.0
20	34.1	6	0.18	1.3
22	52	20	0.4	2.9
24	103	84.2	0.82	5.9
26	181	255.5	1.4	10
28	287	504.4	1.8	13
48	2,892	2,978	1.03	7.4

^a Relative counts obtained by scanning Southern blot shown in Fig. 6.^b Relative counts obtained by scanning the Northern blot shown in Fig. 6.^c Ratio of RNA counts to DNA counts.^d Relative ratio, setting a ratio of 1 for the 18-hpi time point.

scripts reaches an at-least-10-fold excess over the increase in genomes.

The ³²P-labeled genomic probe also detected a small RNA of disperse size (see arrow in Fig. 6), which had run out in short

gels (e.g., Fig. 1). This RNA hybridized neither to the early (nt 399 to 1101)- nor to the late (nt 3918 to 2928)-specific RNA probes (Fig. 6). Use of DNA probes for various regions determined that this RNA maps in the promoter proximal early region (*Msp*I fragment 5 [nt 40 to 400]), can be detected from 18 hpi, and is produced in very high abundance (data not shown).

Capsid protein expression. In order to examine the expression of the major late protein VP1, Western blots were probed with an antibody directed against VP1. For the data presented in Fig. 7, the blot shown in Fig. 3 was stripped and exposed to the VP1-directed antibody. The VP1 protein became detectable from 22 hpi, that is, following the appearance of the 16S late transcript with a 2- to 4-h delay. The level of VP1 protein increased continuously up to 48 hpi.

Production of live virus progeny. Intracellular (i.e., cell-associated) and extracellular live virus particles released in the medium were quantitated by plaque assays at the times shown in Fig. 8. Virus infectivity was lost very rapidly at the onset of infection, reaching a minimum during the 1-h adsorption period. A residual level of cell-associated virus (0.5%) remained

FIG. 6. Transcriptional analysis of the early-to-late switch period. Cells were infected as described in the legend to Fig. 1. Samples were taken at the times shown at the top of the lanes. Cell cycle phases were determined by FACS analysis, and the percentages of the cells in S phase are shown above the genome amplification data. The DNA amplification data was analyzed as described in Fig. 5. The sample taken at 48 hpi was diluted fourfold. Total RNA was extracted, processed for Northern blotting, sequentially hybridized, and stripped with probes for the early, late, or all transcripts. For early transcripts, the blot was hybridized with a digoxigenin-substituted RNA probe (*Msp*I fragment 4 [nt 399 to 1101]), which detects all early RNAs. For late transcripts, the blot was stripped and rehybridized with a digoxigenin-substituted RNA probe spanning nt 3918 to 2928, which detects all late RNAs. For total transcripts, the blot was hybridized to a genomic ³²P-labeled DNA probe; the position of the early (■ ■) and late (■ ■) transcripts deduced by superposing the X-ray films is shown. The arrow on the right points to the small RNA. HPI = hpi.

FIG. 7. VP1 capsid protein expression. The Western blot shown in Fig. 3 was stripped and reprobed with a monoclonal antibody directed against the VP1 capsid protein. The phase of cell cycle for the largest fraction of the cell population at the time of sample collection is given. HPI = hpi.

alive through the eclipse period. An increase in the level of intracellular virus was detected from 22 hpi, rapidly following the increase in the level of VP1 capsid protein. Most of the live virus was found to be cell associated until the end of the experiment (at 48 hpi) when cytopathic effects became visible in the culture. The average virus yield at 48 hpi approximated 200 PFU/cell for intracellular virus and 2 PFU/cell for extracellular virus. Other experiments showed that the yield of virus has not reached its maximum by 48 hpi.

DISCUSSION

We have carried out an integrated analysis of all the steps of the wild-type polyomavirus lytic cycle (strain A2) in NIH 3T3 cells infected in G_0 . The A2 strain is widely used. Its tumor profile has been defined and characterized as a highly tumor inducing strain (20). At the multiplicity of infection of 10 used, infection of serum-stimulated cells did not appear to alter the first two cell cycles, including the capacity of cells to divide. Since viral DNA replication cannot start before the infected cells have entered the S phase, the early phase lasted through the long G_1 phase of the first cell cycle (reliably 12 h) and the beginning of the first S phase. Low-level transcription of the viral early gene(s) and synthesis of the large T viral protein characterized the early phase and, at a lower level and hence detected with a delay, that of middle T and small T. The transition from the early to the late phase was very sharp. The late phase started within the first S phase (at approximately 16 hpi) and lasted at least through two cell cycles. Cytopathic

effects became evident in the third cell cycle. The late phase showed a continued increase in the level of the early proteins, amplification of viral genomes, a marked increase in transcription (including the appearance of the late transcripts), the synthesis of the late proteins, and virus maturation. All viral products—early and late transcripts, early and late proteins, and genomes—continued to increase from the time of their first detection up to the last time point taken. The results are integrated in a summary form in Fig. 9.

It should be noted that some of the results presented here might not be generalizable to other host cells or even to other sublines. This stems from the high dependence of the virus lytic cycle upon the host, in particular, the activation of the early promoter prior to the synthesis of viral proteins. Differences between host cells have been noted before; for example, infection of 3T6 cells results in higher yields of viral RNA and live virus than infection of 3T3 or NIH 3T3 cells. However, NIH 3T3 has been one of the major cell lines used in the study of polyomavirus gene expression. The particular, the NIH 3T3 subline used was subcloned in the Benjamin lab. It was shown to have a normal p53 response (induction of p21 following actinomycin D treatment) and does not express p16 (J. Dahl and T. Benjamin [unpublished results] and also data not shown). Similarly, the specific “wild-type” virus strain used and host used to grow the virus may affect the outcome of some events.

Expression of the early viral proteins. The large T protein could be detected from 8 hpi on, that is, with a 2-h delay after the first detection of large T transcripts. Given the low level of transcripts synthesized during the early phase, the ability to detect large T suggests that translation from the early initiation site must be very efficient. The level of large T increased continuously throughout the course of infection. The rate of increase of the proteins appeared quasimonotonous and is discussed further below. A large T to multiplicity dose-response curve in a range of between 0.1 to 30 showed a proportional response between the two parameters (data not shown). Thus, the multiplicity of 10 used in the present set of experiments does not represent a limit, either for transcription or for protein expression.

Levels of middle T and small T were lower than those of large T at all times. Thus, the detection of middle T and small T proteins was delayed for 4 to 8 h compared to large T. This differential expression was paralleled by different levels of transcripts (discussed below). The middle-T-associated kinase activity paralleled the middle T expression pattern. Similarly to the pattern of large T expression, the levels of middle T and

FIG. 8. Live virus production. At the times shown (hpi), the production of live virus was assayed by plaque assays in the cell-associated fraction and in the cell supernatant as described in Materials and Methods. The yield of live viruses per cell was calculated. The theoretical number of viruses/cell (multiplicity) at the beginning of infection (-2 hpi) is indicated by a star. Symbols: $\blacklozenge$, intracellular live virus particles; $\bullet$, live virus particles released in the cell supernatant. HPI = hpi.

FIG. 9. Schematic representation of the polyomavirus life cycle. The events and viral products in the lytic cycle of PYV are presented in a schematic manner. The three phases of the first cycle are shown: G₁, S, and G₂. The line in the center of S marks the detection of an increase in viral genomes and the transition between the early and the late phases. The following aspects are highlighted. For transcripts, the delay and lower abundance of the middle T and small T compared to the large T transcripts, the enhancement of transcription of early genes following the onset of DNA replication, the high accumulation of late transcripts (especially VP1), and the continuous increase in transcript abundance is shown. For proteins, the lower level of middle T-small T compared with large T, the delay of appearance of middle T-small T until the S phase, the appearance of VP1 following the onset of DNA replication, and the continuous increase in the abundance of all proteins is shown. Since different antibodies were used to assay VP1 and the early proteins, the relative abundance of these proteins is theoretical. For viral DNA, the delay of amplification of viral genomes to mid-S phase is highlighted.

small T proteins continued to increase during the time course of infection, as did their transcripts.

In the accompanying article (16), we show that middle T (and/or small T) plays a major role in a transcriptional switch, further discussed below, that takes place at the early-to-late transition, i.e., from 18 hpi on, as well as in DNA replication. These events occur with a considerable delay from the time when middle T and its kinase activity are first detected.

Viral DNA replication. As expected, the initiation of viral DNA replication was tied to the progression of the infected host cells into the S phase. An increase in viral genomes became detectable when cells were in mid-S phase. However, based on preliminary experiments analyzing nascent DNA strands, it is likely that this apparent delay reflects the limitation of Southern blotting in detecting the first replicating ge-

nomes against the background of the persisting "input" genomes. Once replication started, it proceeded regardless of the phase of the cell cycle. As can be seen in Fig. 5, a continuous increase in genome accumulation was observed until the end of the experiment. The overall increase in genomes during the first S phase was in the 100-fold range, while the overall amplification was 8,000-fold at the last time point assayed (48 hpi). The data provide evidence that viral DNA replication proceeds as cells exit the S phase. However, changes in the rate and mode of replication were observed in connection with the cells' exit from the S phase (preliminary data).

The encapsidation of genomes into live virus was initiated shortly after the detection of viral capsid proteins. At the last assay point (48 hpi), the increase in viral genomes was 8,000-fold. In contrast, the yield of live virus per cell was 200 PFU. As noted in the Results section, the latter may be an underestimate, since the yield of virus continued to increase after the first detection of cytopathic effects (48 hpi). However, it has long been noted that a large fraction of the viral genomes fails to become encapsidated (reviewed in reference 2). Furthermore, it is widely believed that the ratio of physical particles to PFU is generally in the order of 100:1. These yield values are in general agreement with those obtained repeatedly for titers of virus stocks produced in the same cells.

Transcription. The majority of studies on the viral transcripts during the lytic infection reported in the literature have been carried out at two time points, one early and one late, with techniques that included mostly Cot curve analyses and S1 mapping. The sharp early-to-late transition, observed in the present experiments, allowed a detailed kinetic analysis of the transition using Northern blot analysis in which transcripts can be identified by their sizes, as well as by their sequence composition. The experiments have generated information on the timing of appearance of various viral RNAs, their identification, changes in their abundance in function of time postinfection, and the relative abundance of the various RNA species at specific times, including a straightforward comparison of the early and late transcripts. Whether the observed changes are taking place at a transcriptional or posttranscriptional level was not determined by this analysis.

(i) **Activation of the early promoter.** A large fraction of the input virus became noninfectious within the 1-h adsorption period, the bulk of the genomes reached the nucleus within 1 hpi and, starting at 2 hpi, the viral chromatin became sensitive to nucleases (data not shown). This suggests that the promoters might become rapidly available for transcription. However, the earliest transcripts detected using RT-PCR appeared at 6 hpi, and the earliest detection of large T protein was at 8 hpi. This expression kinetics suggests that the PYV early transcripts are controlled similarly to "delayed early" cellular genes, such as cyclin D1 (49). This is compatible with the fact that the polyomavirus early promoter (19, 27, 35, 36, 37, 43) and the cyclin D1 promoter (6, 12) are both controlled by the serum-activated PEA1/AP1 and PEA3 transcription factors (45, 46, 47). Unfortunately, this could not be verified directly, since the NIH 3T3 subline used in these experiments expresses cyclin D1 constitutively (data not shown).

Examination of the RT-PCR products shows that the bulk of the RNA was spliced. The quantitation of these products indicates a 6-fold increase in transcript abundance by between 6

and 12 hpi and another 6- to 10-fold increase by between 12 and 18 hpi, resulting in a total increase of between 40- and 60-fold during the early phase of infection. This is a soft number since the RT-PCR reactions were not carried out under quantitative conditions. However, despite this large increase, the level of transcripts still remained below the threshold of detection by Northern blotting until approximately 14 hpi. It is common knowledge that very low levels of polyomavirus early transcripts are synthesized during the early phase of infection (reviewed in references 2 and 39), although the level is clearly multiplicity dependent and also cell- and virus strain specific.

(ii) Splicing disadvantage of the middle T and small T transcripts. An interesting new finding is the preferential splicing of the large T intron compared to that of middle T and small T transcripts, i.e., a higher 5' splicing efficiency at nt 411 compared to that at nt 748. Protein levels reflected the levels of transcripts. A quantitative difference in the relative levels of these transcripts (with a 4- to 16-fold advantage for the large T mRNA) persisted into the late phase of infection, at least up to 36 hpi, the last sampling time. The level of middle T and small T transcripts appeared of equal abundance throughout. To our knowledge this has not been reported previously. However, it has been shown that, in an *in vitro* splicing reaction using simian virus 40 (SV40) as a template, the concentration of the Sf2/Asf factor differentially affects the level of large-versus small-T intron splicing (24). Splice site preferences are also seen in the generation of the late mRNAs (see below). In both cases, the splice site choice leads to an excess of the mRNA with the larger intron. However, in contrast to the case of the early RNA, the choice for the late RNA involves the 3' rather than the 5' splice site (7).

(iii) Late enhancement of synthesis of the early transcripts. Following the onset of DNA replication, a large increase in the level of transcripts was observed. For example, in the experiment shown in Fig. 1, a 63-fold increase was observed in a 4-h interval. Similar results were observed in multiple experiments. The large amplitude of this early-to-late transition-specific "burst" contrasts with the more progressive increases observed both before and after the transition. A detailed analysis revealed the following new findings. The increase in the level of transcripts involved the early as well as the late transcripts. However, the increase in late transcripts, described below, was delayed by 2 to 6 h. In two experiments, the early transcripts became detectable from 14 to 16 hpi on and represented the most abundant species for 4 to 8 h. A peak in the rate of increase was observed between 18 and 20 or between 20 and 22 hpi in two experiments (see Table 1). A continuous further increase in transcript levels was seen until the end of the time course, and the transcripts accumulated to a high level. For example, an ~80-fold increase in early transcript levels between 18 and 48 hpi was estimated from the optical scan of the data in Fig. 6 (early transcripts). The increase in the level of early RNA late in infection has been briefly noted previously (see, for example, reference 2 for a review); however, the kinetics of the increase and the amplitude of the increase were either not characterized or assayed with less-sensitive methods. This has led to the notion that the increase is negligible, which is clearly not the case. In the accompanying article (16), a role for middle T-small T in the enhancement of early transcription at and beyond the early-to-late switch is demonstrated. That

study also shows that the enhancement of early transcription is diminished but not abolished by blocking genome amplification.

Mechanisms to repress or degrade the early transcripts during the late phase have been previously documented (see below) (17, 21, 22, 31). The present results demonstrate that the existence of these mechanisms does not result in a leveling off of the early transcripts at late times.

The increase in early transcript levels was paralleled by a continuous increase in the level of early proteins. As judged by visual inspection of the Western blots, the rate of increase appeared quasimonotonous and did not directly reflect the large burst in transcript levels seen at the early-to-late switch. This could be related to inefficient posttranscriptional modifications and the nuclear export of these viral RNAs late in infection. However, the continued increase in the level of early proteins until the end of the cycle is likely to reflect a parallel increase in the levels of mature early mRNAs exported out of the nucleus.

(iv) Synthesis of the late transcripts. Following the onset of DNA replication (from 18 to 20 hpi on), late transcripts were synthesized to a level detectable on Northern blots. This was followed within 2 h or less by the synthesis of VP1 capsid proteins. Following their first detection, the increase in late transcripts was rapid, very large, and continued to the end of the lytic cycle. Within 6 h after their first detection, the late transcripts became more abundant than the early transcripts. Most of these aspects of late transcription have been observed previously, and the results presented here are compatible with previous results (as reviewed by Acheson [2] and Salzman et al. [39]). The new data concern the comparison of the induction of transcription of the late genes and the enhancement of transcription of the early genes. As was the case for the early genes, the increase in late transcript accumulation was accompanied by a continuous increase in the level of VP1 capsid protein. The roles of middle T and/or small T and DNA replication in the activation of the late promoter are discussed in detail in the reference 16.

(v) Splicing of the late transcripts. As observed with the early RNAs, splicing was very efficient, as judged from the abundance of the various RNA species. The spliced 16S late transcript encoding the VP1 protein was the first transcript to be detected and was by far the most abundant throughout the late phase, confirming previous results. The 3' splicing site preference to generate the 16S VP1 transcript from the late RNA precursor has been examined previously (7). Similar results have also been observed for SV40 late RNA (as reviewed by Salzman et al. [39]).

(vi) Synthesis of "giant" multigenomic transcripts. From 26 hpi on, very large RNAs were detected, among both the early and the late transcripts. From the time of their detection, both early and late giant transcripts increased in abundance throughout the course infection. Giant late transcripts were also observed in the absence of middle T and small T proteins (see reference 16). The presence of late RNAs with large heterogeneous sizes late in infection has been previously described. They are thought to be generated by continuous transcription of the circular genome template, without termination, generating head-to-tail tandem repeats of the entire viral genome (1, 38). However, little information has been generated

in the case of similar early giant transcripts. Their existence has been implied from the presence of so called "LE" early transcripts seen in the late phase of infection. LE transcripts contain uncapped 5' ends that map upstream of the 5' ends of the dominant early "EE" transcripts synthesized during the early phase. It has been suggested that LE RNAs are produced by splicing of early giant RNAs (18, 29).

Later studies of the late giant transcripts showed that these RNAs are inefficiently polyadenylated and confined to the nucleus. A polyadenylated subset is processed by leader to leader splicing, generating mature transcripts with a single mRNA body sequence, and multiple copies of a 57-nt leader (3, 5, 10, 13, 28, 32, 34, 38, 44). Leader-to-leader splicing, and hence the synthesis of giant late transcripts, plays an important role in the generation of a stable mature 16S VP1 mRNA (4, 5). The exact fate and role of the early giant RNA remain to be determined.

The oligomeric RNAs begin to accumulate simultaneously on both the late and early strands as the cells exit the S phase. This timing coincides with a switch in the DNA replication mode (data not shown). Various direct and indirect evidence shows that viral DNA replication switches from a uniquely theta form to include rolling-circle replication intermediates (to be described elsewhere). Rolling-circle intermediates might be suitable templates for high-level transcription, allowing the DNA and RNA polymerases to move in the same direction on a given template. Thus, an important role of DNA replication may be to create a new type of template, in addition to increasing their number and/or diluting a repressor in the case of late transcription (14, 33, 48).

The synthesis of complete genomic RNA leads to the generation of antisense sequences and sense-antisense double-stranded RNA hybrids. The late strand-specific anti-early sequences have been implicated in an antisense-mediated mechanism of degradation of the early mRNA (31). Because genomic early RNAs had not been specifically identified previously, the situation with the late giant RNA contrasting with the short early RNA appeared asymmetric. It had then been hypothesized that the sense-antisense inactivation mechanism specifically targets the early mRNA. However, the existence of symmetric complete genomic late- and early-strand RNAs suggests that the late protein coding sequences are not protected from forming double-stranded-RNA hybrids.

(vii) Small abortive RNAs. Closely following the onset of the late phase of infection (from 18 hpi), a new species of RNA was produced. This RNA is of very short but disperse sizes, it is very abundant, and it represents sequence proximal to the early promoter (between nt 40 and 400). Its polarity (early versus anti-early) remains to be determined. A small aborted RNA, encoding the agnoprotein, has been reported for the late strand of SV40 (25). No such RNA has been previously been reported in the case of polyomavirus. What has been demonstrated, however, is that RNA polymerase II stalls at many sites on both the early and the late strands (8, 9, 11, 26, 41, 42), especially in regions that contain a large-T-antigen-binding site. Thus, this RNA may be a result of stalling polymerases. Alternatively, this RNA may be a relatively stable degradation product of the genomic late (anti-early) transcripts or an abortive early RNA.

(viii) Differential fates of the early and late promoters. The present and accompanying studies demonstrate a strong parallel in the control of both early and late transcripts, at the time of transition from the early to the late phase of infection. The following results are observed for both classes of transcripts: (i) a substantial increase in the levels of transcripts; (ii) the dependence of this activation on the presence of middle T and/or small T proteins (16); (iii) the requirement for DNA replication (16); and (iv), with some delay, the generation of giant transcripts. In the accompanying study (16), the role of middle T in transcription is discussed in relation to the activation of transcription factors that are activated by the middle T downstream signaling and that are known to control both the early and the late promoters (16).

Despite this parallel, differences were also seen, prior to and following the early-to-late switch. These include (i) the complete absence of late transcripts during the early phase, as previously noted; (ii) a differential effect of blocking DNA replication on early and late transcription (16); and (iii) the overabundance of late compared to early transcripts at late times, also previously noted. These differential fates of the early and late promoters despite their control by the same enhancer region have been the subject of much research for both polyomavirus and SV40. The absence of late gene expression during the early phase may be caused by a repressor binding on the late side of the origin and repressing late transcription (14, 33, 48) and/or by the assembly of TFIID on the early TATA box occluding the access to the late TATA-less Inr site (50). Both of these problems would be alleviated by genome amplification. The differential effect of DNA replication on early and late transcripts is compatible with these models. Furthermore, it has also been shown that the late promoter is "on" at early times, with RNA polymerase II stalled at various sites (26). Mechanisms to explain the overabundance of late over early transcripts late in infection have been proposed. They include the repression of early transcription by the large T protein (17, 21, 22), degradation of the early RNA sequestered in double-stranded RNA (31), and disputed induction by large T (30, 50). Thus, much remains to be resolved in understanding the steps involved in the early-to-late transition. However, the view presented here of parallel effects on early and late transcription and the involvement of middle T in the process does not contradict previous data and models.

In summary, the detailed analysis of the lytic cycle has revealed a major novel control step in the infection. As a summary, a picture of the events in the lytic cycle is presented in graphic form in Fig. 9.

ACKNOWLEDGMENTS

The help of Louis King with the FACS analysis is gratefully acknowledged. We also thank Nicholas Acheson for helpful discussions.

This work was supported by grant R01-CA29270 from the National Cancer Institute.

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