Transcriptional Regulation of Early-Response Genes during Polyomavirus Infection

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Infection of quiescent BALB/c 3T3 cells with polyomavirus leads to the biphasic accumulation of RNA from several early-response genes. The steady-state levels of RNA of *c-fos*, *c-myc*, and *c-jun* were detected at 0 to 1 and 12 to 30 h after infection but not at 6 h postinfection. Infections with mutant viruses suggest that in the context of a virus infection with other tumor (T) antigens present, large T and middle T antigens are dispensable for the effect and small t antigen is important for the regulation, perhaps in conjunction with large T or middle T antigens. These results are in agreement with those of Zullo et al. (Proc. Natl. Acad. Sci. USA 84:1210-1214). We have further found that this regulation occurs in NIH 3T3 cells and primary mouse embryo fibroblasts. DNA synthesis is not required for this effect. Half-lives of *c-fos*, *c-myc*, and *c-jun* were similarly short at both early and late times after infection, as determined by dactinomycin chase. The regulation of expression occurs at the transcriptional level. Nuclear run-on experiments showed increased rates of transcription both early and late after infection. Also, the polyomavirus early region can transactivate the *c-fos* promoter in transient transfection assays.

The mouse polyomavirus is a small DNA tumor virus which undergoes a lytic infection in mouse cells but transforms other rodent cells (40) and can induce tumors in newborn mice. The virus encodes three early proteins, the tumor (T) antigens which regulate various aspects of the virus lytic cycle. Large T (LT) antigen is required for the initiation of viral DNA replication (17) and the integration and excision of the viral genome from host chromosomes (12, 37). In addition, LT antigen can immortalize primary cells in culture (34) and can cooperate with other oncogenes in transforming primary cells (28). In lytic infection, middle T (MT) antigen affects the phosphorylation of VP1, the major viral capsid protein, thereby promoting efficient assembly of virions (19). In nonpermissive cells, MT antigen induces cell transformation, at least in part through its association with and activation of pp60^{c-src} and a phosphatidylinositol kinase, p81 (25, 41, 42). Small T (sT) antigen, although largely dispensable for infection in tissue culture, appears to enhance viral DNA replication in infected cells (3, 19, 31, 39). It can also promote the post-confluent growth of cells (9, 23) and may play a role in MT antigen-dependent tumorigenesis in vivo (1, 2).

Polyomavirus replicates in the nucleus of the host cell and is dependent upon the host cell transcription and DNA replication machinery for its lytic cycle. Therefore, when the virus infects a quiescent cell, it must be able to induce the cell to enter a state permissive for viral replication. Indeed, infection of quiescent cells by polyomavirus induces cellular DNA synthesis and cell division. Thus, it is possible that specific effects of viral genes on the cell cycle, including the expression of early-growth-regulated genes, may play an important role in viral infection.

It is widely appreciated that induction of quiescent cells to enter the cell cycle by a variety of mitogens results in the orderly expression of sets of genes, eventually culminating in DNA synthesis and cell division. Garcea and co-workers showed that infection of quiescent BALB/c 3T3 cells by

polyomavirus results in the biphasic accumulation in the steady-state levels of RNA from the early-response genes *c-fos*, *c-myc*, and *c-jun* (44). They showed that the first peak is due to VP1, the major virus capsid protein, presumably interacting with its receptor, and that the second peak is due to the expression of polyomavirus T antigens. Studies with virus mutants indicated that LT antigen alone is not sufficient to induce the second peak, that MT antigen is dispensable, and that sT antigen, either alone or together with LT antigen, may be the regulator.

In this paper we confirm the observation that polyomavirus infection leads to expression of early-response genes, that LT antigen is not sufficient, and that MT antigen is dispensable. In addition we show that the biphasic regulation occurs in other cells, NIH 3T3 and primary mouse embryo fibroblasts, and with at least one other early-response gene, *c-jun*. We also show that DNA replication is not required for the second accumulation of RNA. We have extended the observations by showing that LT antigen does not have an obligatory role in this regulation. Finally, we show that the regulation occurs at the level of transcription.

MATERIALS AND METHODS

Cells and virus. BALB/c 3T3 cells and NIH 3T3 cells were maintained in Dulbecco modified Eagle media (DME) supplemented with 10% calf serum. Primary mouse embryo fibroblasts were prepared by collagenase treatment of 12- to 14-day-old embryos from outbred NIH Swiss mice and frozen at -70°C. Cells were thawed, passaged once in DME containing 10% calf serum, and then infected. For experiments with quiescent cells, plates at 70 to 80% confluency were washed with Tris-buffered saline, refed with DME supplemented with 0.5% calf serum, and incubated for 24 to 48 h. Cells were then stimulated by addition of 20% calf serum or by infection with polyomavirus. In experiments with metabolic inhibitors, dactinomycin (Boehringer Mannheim Biochemicals, Indianapolis, Ind.) was added to a final concentration of 5 µg/ml; aphidicolin (National Institutes of

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Health; 20 μ g/ml, final concentration) or 1- β -D-arabinofuranosylcytosine (ara-C; Sigma Chemical Co., St. Louis, Mo.; 20 μ g/ml, final concentration) was added at the time of infection and at subsequent 8-h intervals throughout the remainder of the experiment.

Stocks of wild-type polyomavirus and the mutant viruses *ts25* (16), MOP1033 (38), and NG18 (7) were grown on 3T6 cells. Infected cell lysates were prepared by freeze-thawing. Titers of the virus stocks were determined by hemagglutination assay. Infections were carried out at a multiplicity that induced DNA synthesis in 30 to 40% of quiescent BALB/c 3T3 cells as measured by autoradiography. After a 1-h adsorption period, virus was removed and cells were refed with their spent media. The expected T antigens were expressed by the mutant viruses as analyzed by immunoprecipitation.

Plasmids. The plasmid pZ2, kindly provided by J. Atwater, is a genomic clone of the mouse *c-fos* gene and contains the first exon, the first intron, and 5' flanking sequences cloned into an SP6 expression vector (27). After linearization with *Hind*III, a 1,137-nucleotide (nt) cRNA transcript was produced by using SP6 polymerase. This cRNA protected a 289-nt fragment corresponding to the first exon of *c-fos*. pMCMyc54, kindly provided by K. Nishikura, contains a cDNA copy of the murine *c-myc* gene cloned into the *Pst*I site of pBR322 (36). *Pst*I fragments encoding either the first or third exon of the *c-myc* gene were cloned into pGem4 (Promega Biotec, Madison, Wis.); each clone also contained some sequences from the second exon of *c-myc*. The plasmid encoding the first exon was linearized with *Hind*III, and a 500-nt cRNA transcript was generated by using SP6 polymerase; this cRNA produced a protected fragment of approximately 500 nt. The plasmid pIcjb3, kindly provided by W. Lamph, consists of a genomic fragment of the murine *c-jun* gene containing the first exon and 5' flanking sequences cloned into pIBI30 (International Biotechnologies, Inc., New Haven, Conn.). After linearization with *Eco*RI, a 1-kilobase cRNA transcript was produced from this plasmid by using T3 RNA polymerase; this probe protected a 500-nt fragment of *c-jun*. A genomic clone of the murine β_2 -microglobulin gene containing intron 1 and exon 2, kindly provided by J. Parnes, was used in RNase protection experiments. An 800-base-pair *Hind*III-*Eco*RI fragment was obtained from a 4-kilobase *Hind*III genomic clone spanning exons 2 through 4 (34) and subcloned into pGem4. When linearized with *Hind*III, an 800-nt cRNA was produced by using SP6 RNA polymerase; this probe protected a 200-nt fragment of β_2 -microglobulin mRNA. A cDNA clone encoding the entire murine β_2 -microglobulin gene (33), kindly provided by I. Verma, was used in nuclear run-on experiments. pAI, a cDNA clone of the murine β -actin gene (11), was also provided by I. Verma. The plasmid pPyBam consists of the entire polyomavirus genome cloned into the *Bam*HI site of pBR322. FC-2 is a plasmid in which the bacterial chloramphenicol acetyltransferase gene (CAT) is expressed from the murine *c-fos* promoter consisting of sequences 1 kilobase pair upstream of the *c-fos* mRNA start site (13) and was kindly provided by P. Sassone-Corsi. The plasmid pRSVPyE consists of the entire polyomavirus early region cloned into pRSV (14), kindly provided by G. Gonzalez.

RNA extraction. At indicated times after infection or serum stimulation, total cellular RNA was isolated by the method of Chomczynski and Sacchi (10). Briefly, a 10-cm plate of cells was washed with ice-cold TD (137 mM NaCl, 5

mM KCl, 0.7 mM NaHPO₄, 25 mM Trizma base) and lysed by using 2 ml of GTC solution (4 M guanidinium thiocyanate [Eastman Kodak], 25 mM sodium citrate [pH 7], 0.5% sarcosyl, 0.1 M 2-mercaptoethanol). Lysed cells were scraped and placed in a polypropylene tube, and 0.2 ml of 2 M sodium acetate (pH 4), 2 ml of water-saturated phenol, and 0.4 ml of chloroform-isoamyl alcohol (49:1) were added, sequentially, mixing after each addition. Samples were shaken vigorously for 1 min, placed on ice for 15 to 20 min, and centrifuged (10,000 $\times$ g, 20 min, 4°C). The aqueous phase was transferred to a new tube and precipitated with 2 ml of isopropanol at -20°C. RNA was pelleted, resuspended in 0.4 ml of GTC solution, transferred to Eppendorf tubes, and precipitated out of sodium acetate by using 2 volumes of ethanol. After centrifugation, RNA was suspended in water and precipitated a third time. Samples were centrifuged, suspended in diethyl pyrocarbonate-treated water, quantitated by optical density, and stored at -70°C.

RNase protection. Labeled cRNA transcripts were produced and purified according to the method of Melton et al. (32). Reactions were carried out in 11 μ l containing 1 $\times$ transcription buffer (40 mM Tris hydrochloride [pH 7.5], measured at 37°C, 6 mM MgCl₂, 2 mM spermidine, 10 mM NaCl); 10 mM dithiothreitol; 10 U of RNasin (Promega); 0.5 mM each of ATP, GTP, and CTP; 200 μ Ci of [α -³²P]UTP, dried down (Dupont, NEN Research Products, Boston, Mass.; 800 Ci/mmol); 1 μ g of linearized DNA template; and 10 U of SP6 or T7 polymerase (Promega). Reactions were incubated for 60 min at 37°C; 10 U of RQ1 DNase (Promega; 10 U/ μ l) was added, and the mixtures were incubated another 10 min. A 10- μ g portion of yeast tRNA was added, samples were extracted with phenol-chloroform, and ethanol was precipitated out of ammonium acetate. Labeled cRNA transcripts in which T3 RNA polymerase was used (Bethesda Research Laboratories, Inc., Gaithersburg, Md.) were prepared according to the instructions of the supplier, and this procedure was followed by RQ1 DNase treatment, phenol extraction, and ethanol precipitation as described above. cRNA transcripts were purified by electroelution onto Sartorius collodion bags by using an electroeluter (Isco, Lincoln, Neb.), after being fractionated on a 4% polyacrylamide-urea sequencing gel. Yeast tRNA was added to 50 μ g/ml, final concentration, and RNA was precipitated from sodium acetate. RNA was suspended in 100 μ l of hybridization buffer (80% deionized formamide, 40 mM PIPES [piperazine-N,N'-bis(2-ethanesulfonic acid)] [pH 6.7], 0.4 M NaCl, 1 mM EDTA), and 1 μ l was counted by using a liquid scintillation counter (model LS-233; Beckman Instruments, Inc., Fullerton, Calif.).

For each RNase protection, 50 μ g of RNA (10 to 20 μ g of total cellular RNA plus yeast tRNA to 50 μ g) was annealed to 5 $\times$ 10⁵ Cerenkov counts of labeled cRNA overnight at 45°C in a final volume of 30 μ l of 80% formamide hybridization buffer. Samples were then treated with 300 μ l of RNase digestion buffer (10 mM Tris hydrochloride [pH 7.5], 5 mM EDTA, 300 mM NaCl) containing 40 μ g of RNase A (Boehringer-Mannheim; 10 mg/ml of stock) per ml and 700 U of RNase T1 (Boehringer-Mannheim) per ml at 32 to 33°C for 45 min (*c-fos*) or 60 min (all others). Digestion was terminated by the addition of 22.5 μ l of sodium dodecyl sulfate (SDS)-proteinase K solution (7 volumes of 10% SDS and 2 volumes of 10 mg of proteinase K per ml) and incubation at 37°C for 15 min. Samples were extracted with phenol-chloroform and ethanol precipitated by using 30 μ g of glycogen as carrier. Protected RNAs were fractionated by electrophoresis on 6%

polyacrylamide-urea sequencing gels. Gels were dried down, and bands were visualized by using preflashed Fuji X-ray film and Cronex lightning intensifying screens.

[³H]thymidine incorporation. BALB/c 3T3 cells were seeded at 2×10^5 cells per 6-cm dish. When cells were 80% confluent, they were washed three times with Tris-buffered saline and refed with DME containing 0.5% calf serum. After 64 h in low serum, cells were either infected or stimulated with 20% calf serum. Cells were labeled for 8 h (from 21 to 29 h after infection or serum stimulation) in 1 ml of DME containing 0.1% calf serum and 20 μ Ci of [³H]thymidine (Dupont, NEN Research Products; 6.7 Ci/mmol). After being labeled, the cells were washed with ice-cold Tris-buffered saline, scraped, and pelleted. Cell pellets were suspended in 1 ml of 0.3 N NaOH and incubated on ice for 45 min. A 1-ml portion of 20% trichloroacetic acid (TCA) was added, and samples were incubated for 15 min on ice. TCA-precipitable counts were filtered onto GF/C glass filters (Whatman) and washed three times with ice-cold 10% TCA and once with cold 95% ethanol. Filters were counted in Ecolite (WestChem, San Diego, Calif.) by using a liquid scintillation counter (model LS-233; Beckman Instruments).

Transcription in isolated nuclei. Nuclei were prepared from cells by Nonidet P-40 lysis (10 mM Tris hydrochloride [pH 7.4], 10 mM NaCl, 3 mM MgCl₂, 0.5% Nonidet P-40) and suspended at a concentration of 2×10^8 to 3×10^8 nuclei per ml in glycerol buffer (40% glycerol, 50 mM Tris hydrochloride [pH 8.3], 5 mM MgCl₂, 0.1 mM EDTA). The nuclei were frozen in liquid nitrogen until use. Nuclear run-on reactions were performed as described by Groudine and co-workers (21). A 100- μ l portion of nuclei was thawed and immediately mixed with 100 μ l of 2 $\times$ transcription buffer containing nucleotides (10 mM Tris hydrochloride [pH 8.0]–5 mM MgCl₂–300 mM KCl–5 mM dithiothreitol–1 mM each of ATP, GTP, and CTP) and 10 μ l of [α -³²P]UTP (Dupont, NEN Research Products; 760 Ci/mmol, 10 mCi/ml). The reactions were incubated at 30°C for 30 min, after which time 0.6 ml of DNase I (Worthington Diagnostic Systems Inc., Freehold, N.J.; DPFF, 40 μ g/ml in 500 mM NaCl, 50 mM MgCl₂, 2 mM CaCl₂, 10 mM Tris hydrochloride [pH 7.4]) was added, and the mixtures were incubated a further 5 min at 30°C. The reactions were made to 1 $\times$ SET (5 $\times$ is 5% SDS, 500 mM Tris hydrochloride [pH 7.4], 125 mM EDTA), and proteinase K was added to a concentration of 200 μ g/ml. After 30 min at 37°C, samples were extracted with an equal volume of phenol-chloroform; the interface was again extracted with 2 ml of TE (10 mM Tris hydrochloride [pH 8], 1 mM EDTA) and combined with the first aqueous phase. An equal volume of 10% TCA–60 mM sodium pyrophosphate–100 μ g of yeast tRNA was added, and the mixtures were incubated on ice for 30 min. TCA precipitates were filtered through membrane filters (Millipore Corp., type HA) (0.45 μ M), washed with 5% TCA–30 mM pyrophosphate. Filters were transferred to scintillation vials and treated with 1.5 ml of 25 μ g of DNase I (DPFF, in 20 mM HEPES [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid] [pH 7.5], 5 mM MgCl₂, 1 mM CaCl₂) per ml for 30 min at 37°C. Reactions were quenched by the addition of EDTA and SDS to final concentrations of 15 mM and 0.9%, respectively. Labeled RNA was eluted by heating to 65°C, and the supernatant was removed; RNA was eluted twice more with 0.75 ml of 1% SDS–10 mM Tris hydrochloride, pH 7.5–5 mM EDTA, and supernatants were combined. RNA was extracted once with phenol, once with phenol-chloroform, and once with chloroform-isoamyl alcohol; 0.75 ml of 1 N NaOH

was added, and the mixtures were incubated on ice for 10 min and quenched with 1.5 ml 1 M HEPES. RNAs were ethanol precipitated out of sodium acetate.

Precipitated RNAs were suspended in 10 mM TES [N-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid] (pH 7.4)–0.2% SDS–10 mM EDTA at 5×10^5 cpm/ml. This was mixed with an equal volume of 10 mM TES [pH 7.4]–0.2% SDS–10 mM EDTA–600 mM NaCl and hybridized to 10 μ g of plasmid DNAs immobilized on nitrocellulose strips. Hybridizations were carried out for 36 h at 65°C. Filters were washed in several changes of 2 $\times$ SSC (1 $\times$ SSC is 0.15 M NaCl plus 0.015 M sodium citrate) for 2 h at 65°C, incubated at 37°C in 2 $\times$ SSC containing 10 μ g of RNase A per ml for 30 min, and washed again in 2 $\times$ SSC at 37°C. Filters were exposed to Kodak XAR-5 film with a Cronex lightning intensifying screen.

Transfections and CAT assays. Transfections were carried out by using the BES-CaPO₄²⁺ precipitation method as described by Chen and Okayama (8). Plates (10 cm) of BALB/c 3T3 cells were transfected at approximately 50 to 60% confluency with 20 μ g of plasmid DNA. For each transfection, plasmid DNA was mixed with 0.5 ml of 0.25 M CaCl₂ and 0.5 ml of 2 $\times$ BBS (50 mM BES [N,N-bis-(2-hydroxyethyl)-2-aminoethanesulfonic acid] [pH 6.95], 280 mM NaCl, 1.5 mM Na₂HPO₄) was added. The mixture was incubated for 10 to 20 min at room temperature and then added dropwise to the cells. Precipitates were incubated at 37°C in 3% CO₂ overnight. After 14 to 16 h, cells were washed twice with Tris-buffered saline and refed with DME supplemented with 0.5% calf serum. Cells were incubated for 14 h, at which time calf serum was added to control plates (20%, final concentration). Cell extracts were prepared 4 h later by freeze-thawing four times in 100 μ l of 50 mM Tris hydrochloride, pH 8.0. Protein concentrations were determined by using the BCA protein assay reagent (Pierce Chemical Co., Rockford, Ill.). CAT assays were performed by using standard procedures (20), and after thin-layer chromatography, products were visualized by autoradiography with Kodak XAR-5 film.

RESULTS

Polyomavirus infection leads to a biphasic accumulation of early-response RNAs. In agreement with Garcea and co-workers (44), we found that infection of quiescent BALB/c 3T3 cells leads to a biphasic accumulation of *c-fos* and *c-myc* RNA. In addition, the RNA of *c-jun*, another early-response gene, also showed a biphasic accumulation. *c-fos*, *c-myc*, and *c-jun* RNAs were readily detected at both 30 min and 24 h after infection of quiescent BALB/c 3T3 cells by polyomavirus (Fig. 1). In contrast, little if any RNA was detected at 6 h after infection (Fig. 1). The first phase of expression lasted for approximately 1 h after the adsorption period. During the second phase, the RNA was present at a relatively constant level over a prolonged period of time ranging from 12 to 33 h after infection (data not shown). Thus, there are at least four cellular early-serum-response genes (*c-fos*, *c-myc*, *c-jun*, and JE) which exhibit a biphasic accumulation of their RNA after infection of quiescent BALB/c 3T3 cells with polyomavirus. However, not all cellular genes show this biphasic pattern of expression. β_2 -Microglobulin was expressed at a constant level throughout polyomavirus infection (Fig. 1).

The biphasic accumulation of *c-fos* mRNA was observed upon infection of both quiescent NIH 3T3 cells and second-

FIG. 1. Biphasic accumulation of early-response gene RNA during polyomavirus infection. Total cellular RNA (20 μ g; 10 μ g for serum-stimulated cells) was analyzed by RNase protection by using 5×10^5 Cerenkov counts of the indicated cRNA transcript. Shown are autoradiograms of protected RNAs after fractionation on 6% polyacrylamide-urea sequencing gels and exposure to Fuji X-ray film. Exposures are for 6 to 12 h (lanes Q and S and β_2 -M) or 1 to 4 days (lanes 1 through 3). RNAs were isolated from quiescent BALB/c 3T3 cells (lane Q), serum-stimulated cells (15 min for *c-fos*, 90 min for *c-myc*, or 60 min for *c-jun*; lane S), or polyomavirus-infected cells either 30 min (lane 1), 6 h (lane 2), or 24 h postinfection (lane 3).

ary mouse embryo fibroblasts derived from outbred NIH Swiss 3T3 mice (Fig. 2). Both the pattern and level of *c-fos* expression were similar in all three cell types (compare Fig. 1, *c-fos*, and Fig. 2). The biphasic accumulation of *c-fos* also occurred in C127 cells, a mouse mammary epithelial cell line (data not shown). Thus, the regulation of expression of serum early-response genes during polyomavirus infection is not unique to BALB/c 3T3 cells.

Expression of polyomavirus sT antigen correlates with the second peak of *c-fos* mRNA. Infections with polyomavirus mutants were carried out to examine which viral early gene product(s) were required for the second peak of accumulated *c-fos* mRNA. The mutant virus MOP1033, which encodes normal LT and sT antigens but has a truncated form of MT antigen, is nontransforming, and fails to associate with and activate pp60^{c-src} protein tyrosine kinase activity (38), was used to assess the role of MT antigen. Both peaks of *c-fos* mRNA were observed when infections were carried out with MOP1033 (Fig. 3), suggesting that MT antigen is not required for the appearance of the second peak. The host range nontransforming mutant, NG18, which contains a lesion affecting both MT and sT, but not LT antigens (7), was used to assess the requirement for sT and MT antigens. When the NG18 mutant was used to infect BALB/c 3T3 cells, only the first peak of *c-fos* mRNA was seen (Fig. 3). These results are in agreement with those of Zullo et al. (44). We used the mutant virus *ts25*, which has a temperature-sensitive lesion affecting only LT antigen (24), to assess the role of LT

FIG. 2. Analysis of *c-fos* RNA after infection of primary mouse embryo fibroblasts and NIH 3T3 cells. Total cellular RNA (20 μ g) was analyzed by RNase protection as described in the legend to Fig. 1 by using a *c-fos* cRNA probe. (A) Autoradiogram of protected RNAs isolated from primary mouse embryo fibroblasts, either mock-infected (lane M) or infected with polyomavirus for 30 min (lane 1), 6 h (lane 2), or 24 h (lane 3). (B) Autoradiogram of protected RNAs isolated from polyomavirus-infected quiescent NIH 3T3 cells. Lanes are as described above.

antigen. Both peaks of *c-fos* mRNA were observed at both the permissive (32°C) and nonpermissive (39°C) temperatures for virus infection. Other experiments showed that there were no differences in the levels or patterns of *c-fos* mRNA expressed in *ts25*-infected cells at the two temperatures (data not shown). Therefore, LT antigen does not play an obligatory role in the accumulation of the second peak of RNA nor does it appear to be sufficient to induce the second peak of RNA. MT antigen appears to be dispensable for the second peak, whereas sT antigen appears to be important, either alone or in conjunction with LT or MT antigens.

The second peak of *c-fos* RNA does not require DNA synthesis. Because polyomavirus infection induces DNA synthesis in quiescent cells, it was of interest to determine whether viral or cellular DNA replication played any role in the accumulation of the second peak of *c-fos* RNA. Quiescent BALB/c 3T3 cells were infected in the presence of either ara-C or aphidicolin to inhibit DNA synthesis and were analyzed for *c-fos* RNA expression by RNase protection. Both the early (30-min) and late (24-h) peaks of *c-fos* RNA were detected when infections were carried out in the presence of ara-C or aphidicolin (Fig. 4A). Cells were labeled in parallel with [³H]thymidine to measure viral and cellular DNA synthesis. Cells infected with polyomavirus or stimulated by the addition of serum incorporated 35 to 75 times as much [³H]thymidine as mock-treated cells (Fig. 4B). Cells infected by polyomavirus in the presence of either ara-C or aphidicolin incorporated the same amount of [³H]thymidine as mock-treated cells. Thus, both peaks of *c-fos*

FIG. 3. Analysis of *c-fos* expression in polyomavirus mutant-infected BALB/c 3T3 cells. Total cellular RNA (20 μ g) was analyzed by RNase protection by using a *c-fos* cRNA probe as described in the legend to Fig. 1. Quiescent BALB/c 3T3 cells were infected with either *ts25*, MOP1033, or NG18. For *ts25*, adsorption of the virus was carried out at 37°C, virus was removed, and cells were refed with spent media and incubated at either the permissive (32°C) or nonpermissive (39°C) temperatures. Shown are autoradiograms of protected RNA isolated from cells infected for 30 min (lane 1), 6 h (lane 2), or 24 h (lane 3).

RNA were detected (Fig. 4A), even when DNA synthesis was blocked by ara-C or aphidicolin. Therefore, DNA synthesis is not required for the biphasic accumulation of *c-fos* RNA, and the biphasic accumulation is not simply due to expression of *c-fos* as cells progress through multiple cell cycles.

The half-lives of early response RNAs are similar at early and late times after infection. The previous experiments demonstrated a biphasic pattern of expression, as reflected in the steady-state levels of RNA of several early-response genes. The elevated levels could be due to increased transcription, increased RNA stability, or both. We measured the half-lives of RNAs by dactinomycin chase at early and late times after infection and compared them with the half-lives observed after serum stimulation. Similar decay rates were observed for *c-fos* mRNA after addition of dactinomycin to cells stimulated with serum for 15 min or by infection with polyomavirus for either 30 min or 24 h (Fig. 5). The bands were quantified by scanning densitometry, and half-lives were determined (data not shown). A half-life of 35 min was observed for *c-fos* mRNA after serum stimulation; half-lives of 26 and 13 min were obtained for *c-fos* mRNA at early and late times after polyomavirus infection, respectively. Dactinomycin decay rates for *c-myc* RNA were likewise similar in cells stimulated with serum for 90 min or infected with polyomavirus for 30 min or 24 h (Fig. 5). In all three instances, half-lives of 40 to 50 min were observed. Similarly, half-lives of 35 and 18 min were seen for *c-jun* mRNA at early and late times after infection, respectively

FIG. 4. Analysis of *c-fos* expression in cells infected in the presence of inhibitors of DNA synthesis. (A) Total cellular RNA (20 μ g) was analyzed by RNase protection by using a *c-fos* cRNA probe as described in the legend to Fig. 1. Quiescent BALB/c 3T3 cells were infected with polyomavirus in the presence of either ara-C or aphidicolin (20 μ g/ml for each inhibitor added after virus adsorption as well as at subsequent 8-h intervals). Shown is an autoradiogram of protected RNAs isolated from cells infected for 30 min (lane 1), 6 h (lane 2), or 24 h (lane 3). (B) Incorporation of [³H]thymidine into cells in the presence or absence of inhibitors of DNA synthesis. Plates (6 cm) of quiescent BALB/c 3T3 cells were labeled with [³H]thymidine from 18 to 24 h after serum stimulation or virus infection, and TCA-precipitable counts were determined as described in Materials and Methods. Infections in the presence of ara-C or aphidicolin were carried out as described above.

(Fig. 5). Thus, none of these RNAs is stabilized at late times after polyomavirus infection. The half-life of β_2 -microglobulin mRNA was greater than 4 h at both early and late times after polyomavirus infection (Fig. 5) and thus served as a control for both the dactinomycin treatment and RNA isolation. These results indicate that the elevated levels of early-response gene RNAs do not result from increased mRNA stability in polyomavirus-infected cells.

Regulation of early-response genes occurs at the transcriptional level in polyomavirus-infected cells. Transcription rates for *c-fos* and *c-myc* were measured directly by nuclear run on in isolated nuclei prepared at various times after serum stimulation or infection. A 20-fold increase in *c-fos* transcription over that in quiescent cells was observed at 15 min after serum addition (Fig. 6; *c-fos*, lanes 1 and 2). Transcription fell to a relatively low but constant level (two- to threefold above the level in quiescent cells) by 90 min after serum stimulation (Fig. 6; *c-fos*, lanes 3 through 5). Following polyomavirus infection, the rate of *c-fos* transcription increased threefold at 30 min after infection, dropped to a rate

FIG. 5. Analysis of the half-lives of RNAs after serum stimulation or polyomavirus infection by dactinomycin chase. Total cellular RNA (20 μ g) was analyzed by RNase protection by using the indicated cRNA probe as described in the legend to Fig. 1. Shown are autoradiograms of protected RNAs. (A) RNAs isolated after serum stimulation, as described in the legend to Fig. 1; (B) RNAs isolated 30 min after polyomavirus infection; (C) RNAs isolated 24 h after virus infection. For each panel, RNAs were isolated either before addition of dactinomycin (lane 1) or 10 min (lane 2), 20 min (lane 3), 40 min (lane 4), 60 min (lane 5), 120 min (lane 6), or 240 min (lane 7) after addition of 20 μ g of dactinomycin per ml to the cells.

similar to that observed in quiescent cells at 6 h, and then increased again at 24 h to a rate comparable to that seen early after infection (Fig. 6; *c-fos*, lanes 6 through 8). The rates of transcription of the first and third exons of *c-myc* increased threefold and sevenfold, respectively, by 90 min after serum stimulation (Fig. 6; *c-myc* first and third exons, lane 3). Transcription across both exons fell to background levels by 24 h after stimulation (Fig. 6; *c-myc* first and third exons, lanes 4 and 5). Transcription rates of *c-myc* followed the biphasic pattern seen with *c-fos* after polyomavirus infection (Fig. 6; *c-myc* first and third exons, lanes 6 through 8). Transcription of both the first and third exons increased

about threefold at 30 min after infection, dropped to a rate similar to that observed in quiescent cells at 6 h, and then increased again at 24 h to a rate comparable to that seen early after infection (Fig. 6; *c-myc* first and third exons, lanes 6 through 8). Transcription of β_2 -microglobulin, which served as an internal control, did not increase after either serum addition or polyomavirus infection (Fig. 6; β_2 -microglobulin, lanes 1 through 8). The rate of transcription of actin increased transiently (approximately fivefold) after serum stimulation (Fig. 6; actin, lanes 1 through 5) but did not appear to change after polyomavirus infection (Fig. 6; actin, lanes 6 through 8); however, an increase might have been obscured by the high basal level of actin transcription in the quiescent cells (Fig. 6; actin, lane 1). As expected, transcription from the polyomavirus genome was observed only at 24 h after infection (Fig. 6; polyomavirus, lane 8). These results indicate that the transcription rates of *c-fos* and *c-myc* remain relatively elevated at late times after polyomavirus infection; thus, it appears that the regulation of the early response genes by polyomavirus occurs at the transcriptional level.

To corroborate the results of the nuclear run-on experiment, we tested whether the polyomavirus early region could transactivate the murine *c-fos* promoter in transient assays. A very low level of CAT activity was observed when cells were transfected with FC-2, a plasmid consisting of the murine *c-fos* promoter driving expression of the bacterial CAT gene, and then incubated in 0.5% calf serum as described in Materials and Methods (Fig. 7, lanes A). The amount of CAT activity was increased in cells stimulated by the addition of calf serum to 20% during the last 4 h before extracts were prepared (Fig. 7, lanes B). A similar elevated level of CAT activity was observed in cells cotransfected with either pPyBam or pRSVPyE, plasmids which encode all three polyomavirus T antigens, and maintained in 0.5% calf serum throughout the transfection period (Fig. 7, lanes C and D, respectively). Thus, in agreement with the effects of polyomavirus infection on the endogenous early-response genes, the polyomavirus early region can transactivate the *c-fos* promoter in transient transfection assays and thus mimic the second accumulation of *c-fos* RNA.

DISCUSSION

In agreement with the work of Garcea and co-workers (44), we have shown that *c-fos* and *c-myc* RNA accumulate in a biphasic manner after infection of quiescent BALB/c 3T3 cells with polyomavirus. In addition, we found that the

FIG. 6. Analysis of transcription rates of various genes after serum stimulation or polyomavirus infection by nuclear run on. Transcription was carried out in nuclei isolated at various times after serum addition or virus infection by using [α - 32 P]UTP. Labeled RNAs were isolated and hybridized to 10 μ g of the indicated plasmid DNAs immobilized on nitrocellulose strips. Shown is an autoradiogram of hybridized RNA. Transcription was carried out in nuclei isolated from quiescent cells (lane 1), cells stimulated by the addition of serum for 15 min (lane 2), 90 min (lane 3), 6 h (lane 4), or 24 h (lane 5), or cells infected with polyomavirus for 30 min (lane 6), 6 h (lane 7), or 24 h (lane 8).

FIG. 7. Transactivation of the *c-fos* promoter by polyomavirus T antigens. Plates (10 cm) of BALB/c 3T3 cells were transfected by BES- CaPO_4^{2+} precipitation with plasmid DNAs when cells were at 50 to 60% confluency. Precipitates were allowed to form overnight in an incubator containing 3% CO_2 ; cells were then washed and fed with DME supplemented with 0.5% calf serum. Extracts were prepared 18 h later, CAT assays were performed by using standard procedures, and products were separated by thin-layer chromatography. One plate of cells was stimulated by the addition of calf serum to 20% 4 h before extracts were prepared. Shown is an autoradiogram of a thin-layer chromatography plate. CAT assays were performed on cells transfected with 2 μg of FC-2 maintained in 0.5% serum (lanes A) or serum stimulated (lanes B) or cells cotransfected with 2 μg of FC-2 and either 5 μg of pPyBam (lanes C) or 5 μg of pRSVPyE (lanes D); in both cases, they were maintained in 0.5% serum. All transfections were brought up to 20 μg of plasmid DNA by using pGem4.

c-jun protooncogene is regulated in a similar manner. Thus, at least four cellular early-response genes, *c-fos*, *c-jun*, *c-myc*, and JE, show a biphasic pattern of expression following infection by polyomavirus. The early peak, detected from 0 to 1 h postadsorption of the virus, was shown to be due to VP1, the major virus capsid protein, presumably interacting with its receptor (44). The later peak, present from 12 to 33 h postinfection, results from early viral gene expression. Experiments by Garcea and co-workers (44) suggested that sT antigen, either alone or in conjunction with LT antigen, is responsible for the second peak of RNA. Our work supports this conclusion and suggests that the *ts-a* function of LT antigen, as defined by the temperature-sensitive mutant *ts25*, is not required for the second phase of *c-fos* expression. Also, this mutant does not undergo viral DNA replication or late-gene expression at the nonpermissive temperature, ruling out a role for VP1 in the accumulation of RNA late in infection. However, while neither LT nor MT antigen appears to be obligatory for the second peak of *c-fos* RNA synthesis in the context of a virus infection when other T antigens are present, it is possible that LT or MT antigen might individually transactivate the *c-fos* promoter. Polyomavirus LT antigen can transactivate some promoters, most notably the hsp70 promoter (26). It was recently shown that v-*src* can transactivate the *c-fos* promoter (18); therefore, by analogy, MT antigen may transactivate the *c-fos* promoter through association with pp60^{c-src}. Finally, simian virus 40 sT antigen is capable of transactivating the adenovirus E2 and VA promoters (30). Thus, it is possible that polyoma sT antigen may also transactivate the *c-fos* promoter.

In our experiments, infection of quiescent cells by polyomavirus induces expression of the early-response genes to a lesser extent (10- to 20-fold) than serum. This difference may

result in part from fewer cells being stimulated after virus infection, since approximately 30% of the number of nuclei were labeled with [^3H]thymidine in polyomavirus-infected cells as compared with serum-stimulated cells when visualized by autoradiography (data not shown).

We also showed that the biphasic accumulation of *c-fos* RNA is not unique to BALB/c 3T3 cells. It should be noted that simian virus 40 sT antigen facilitates transformation of BALB/c 3T3 CIA31 cells, under conditions of limiting LT antigen (4, 5). This suggests that BALB/c 3T3 cells may have a unique requirement for a function of simian virus 40 sT antigen, perhaps in the regulation of expression of early-response genes. However, the biphasic accumulation of *c-fos* RNA following polyomavirus infection also occurs in other fibroblast cells (NIH 3T3 and primary mouse embryo fibroblasts), as well as in epithelial cells (C127). Thus, this regulation is a general consequence of polyomavirus infection of quiescent cells rather than a unique requirement for sT antigen in BALB/c 3T3 cells.

DNA replication, either viral or cellular, is not required for the second peak of accumulated *c-fos* mRNA, since it was still observed when infections were carried out in the presence of inhibitors of DNA synthesis. This suggests that the prolonged second peak of *c-fos* expression is not simply due to a low level of expression as cells undergo multiple rounds of DNA synthesis. Moreover, it implicates viral T antigens as being responsible for the second peak of *c-fos* RNA.

There is a notable difference in the regulation of early-response genes between serum-stimulated and polyomavirus-infected cells. When cells are stimulated to enter the cell cycle by the addition of serum, there is a transient induction of transcription followed by a repression of transcription. Similarly, in polyomavirus-infected cells, the first peak of early-response gene RNA is transient in nature. In contrast, the second accumulation of RNA expressed is prolonged, lasting from 12 to 30 h postinfection. This suggests that polyoma T antigens chronically activate transcription of the early-response genes and perhaps interfere with the subsequent repression of these genes. A similar prolonged expression was observed for *c-jun*, but not *c-fos*, when primary human fibroblasts were stimulated with tumor necrosis factor- α (6).

Other viruses also affect the expression of cell growth-regulated genes. Infection of quiescent rodent cells with adenovirus stimulates cellular DNA synthesis. Adenovirus activates the expression of several cell cycle-regulated genes (15, 29, 43), in particular those expressed in late G1/S phase of the cell cycle. Notably, several genes expressed in early G1 phase do not appear to be activated by adenovirus infection (29), even though the *c-fos* and *c-myc* promoters are transactivated by E1A in transient cotransfection experiments (35). In contrast, infection of quiescent cells with polyomavirus activates the expression of several early-response genes (*c-fos*, *c-myc*, *c-jun*, and JE). In both cases, activation is due to the expression of early-viral-gene products. The adenovirus E1A gene product(s) activates expression of thymidylate synthetase, PCNA (43), and p34/cdc2 (15), most likely by distinct mechanisms, since different conserved domains of E1A are required for the activation of the various genes. Also, the E2 gene product of bovine papillomavirus can transactivate several lymphokine promoters in transient transfection experiments (22). Therefore, activation of growth-related cellular genes appears to be important for infection by a variety of DNA tumor viruses.

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