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Regulation of c-*myc* and c-*fos* mRNA levels by polyomavirus: Distinct roles for the capsid protein VP_1 and the viral early proteins

(tumor antigens/virus receptor/cell cycle genes)

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ABSTRACT **The levels of c-*myc*, c-*fos*, and *JE* mRNAs accumulate in a biphasic pattern following infection of quiescent BALB/c 3T3 mouse cells with polyomavirus. Maximal levels of c-*myc* and c-*fos* mRNAs were seen within 1 hr and were nearly undetectable at 6 hr after infection. At 12 hr after infection mRNA levels were again maximal and remained elevated thereafter. Empty virions (capsids) and recombinant VP_1 protein, purified from *Escherichia coli*, induced the early but not the late phase of mRNA accumulation. Virions, capsids, and recombinant VP_1 protein stimulated [^{3}H]thymidine nuclear labeling and c-*myc* mRNA accumulation in a dose-responsive manner paralleling their affinity for the cell receptor for polyoma. The second phase of mRNA accumulation is regulated by the viral early gene products, as shown by polyomavirus early gene mutants and by a transfected cell line (336a) expressing middle tumor antigen upon glucocorticoid addition. These results suggest that polyomavirus interacts with the cell membrane at the onset of infection to increase the levels of mRNA for cellular genes associated with cell competence for DNA replication, and subsequently these levels are maintained by the action of the early viral proteins.**

In contrast to the acutely transforming retroviruses, the "oncogenes" of DNA tumor viruses appear to have an integral role in the virus life cycle. For polyomavirus, mutations in middle and small tumor antigens (T antigens) (the host-range nontransforming, hr-t, mutant class) directly link tumorigenicity and cell transformation to normal lytic viral growth (1, 2). One approach to the function of the polyomavirus oncogenes is therefore the study of virus production during hr-t mutant infection. Initial studies have identified at least two defects in the lytic cycle of hr-t mutant viruses when grown on nonpermissive NIH 3T3 mouse cells: (*i*) a decrease in viral DNA accumulation by a factor of 3 and (*ii*) a defect in encapsidating the viral minichromosome, associated with decreased phosphorylation of the major capsid protein VP_1 (3, 4). The current investigation is an attempt to examine the defective hr-t mutant viral DNA accumulation in the context of current knowledge of the regulation of cell division.

Early studies of cell growth found that simian virus 40 (SV40) could induce quiescent BALB/c 3T3 cells to proceed through the cell cycle in a manner suggesting that the viral genes could substitute for serum factors (5, 6). Schlegel and Benjamin (7) found that polyoma wild-type (wt) and ts-a mutant viruses could stimulate multiple rounds of cell DNA synthesis in confluent rat F-111 cells, whereas hr-t mutants promoted only a single round of replication. These results suggested that the early genes of SV40 or polyoma might induce cellular genes associated with the entry of cells into S phase.

By using growth factors, the commitment of 3T3 cells to enter the growth cycle may be resolved into two stages: (*i*) competence, which is regulated by platelet-derived growth factor (PDGF), and (*ii*) progression, regulated by epidermal growth factor and the insulin-like growth factors (8). The cloning of gene sequences regulated by PDGF and the identification of c-*myc* and c-*fos* as two of these genes permit evaluation of the production of these gene products after a growth stimulus (9-14). Assuming that cellular genes associated with the commitment to enter the cell cycle might also be important in the replicative cycle of a DNA virus, we measured the levels of c-*myc*, c-*fos*, and *JE* (another PDGF-inducible gene) mRNAs after lytic polyomavirus infection of quiescent BALB/c 3T3 cells. We found these mRNAs temporally accumulated in a distinctive biphasic pattern, with the virion capsid proteins responsible for the first phase and the viral early proteins responsible for the subsequent sustained accumulation of these mRNAs. The increased accumulation of c-*myc*, c-*fos*, and *JE* mRNAs effected by polyomavirus is an example of recruiting cellular protooncogenes by polyoma oncogenes and biologically integrating the virus life cycle with normal cell pathways.

METHODS

Cells and Viruses. BALB/c 3T3 cells (clone A31) were maintained as described (8). Stock cultures were discarded after five or six passages, and new stocks were obtained from liquid nitrogen storage. Prior to infection cells were plated at 3×10^5 cells per 100-mm (diameter) culture dish in 10% serum. After 3 days, the 10% calf serum medium was replaced with Dulbecco's modified Eagle's medium (DMEM) supplemented with 0.5% platelet-poor plasma for 12 hr prior to infection. Infections were standardly performed at a multiplicity of infection of 10 for a period of 1 hr, the virus was removed, and the cells were maintained in 5% platelet-poor plasma. 336a cells were obtained from Leda Raptis and were selected as described (15).

Polyomavirus mutants were obtained from T. Benjamin (Harvard Medical School). CsCl-purified virions and empty capsids were prepared by an initial velocity sedimentation banding in a CsCl cushion (16); this was followed by equilibrium banding in a second CsCl gradient. The virion and capsid preparation were dialyzed into phosphate-buffered saline before infection. Hemagglutinin (HA) assays were performed as described (17).

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Abbreviations: HA, hemagglutinin; T antigen, tumor antigen; wt, wild-type; β_2m, β_2-microglobulin; PDGF, platelet-derived growth factor.

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Recombinant VP_1. The polyoma capsid protein VP_1 was purified after high-level expression of the cloned gene in *Escherichia coli* RB791 as described (18). The protein obtained from the phosphocellulose column step was dialyzed into phosphate-buffered saline before use. This preparation appears by electron microscopy to contain capsomere and capsid-like structures (19) and is a single predominant isoelectric subspecies (18). The VP_1 preparation was assayed for HA activity and protein concentration (Bio-Rad Bradford assay). The HA-to-protein ratio is similar for CsCl-purified capsids and the purified recombinant VP_1 (10 ng of protein per ml equals 1 ± 0.5 HA unit). The control bacterial lysate was made from *E. coli* RB791 in an identical manner to the purification of VP_1. The flow-through from the DEAE-cellulose column step (18) was used as the control lysate.

RNA Extraction and Modified S1 Nuclease Analysis. Total RNA was isolated from cells by the method of Chirgwin *et al.* (20) utilizing the CsCl gradient modification. RNA pellets were washed twice by reprecipitation in ethanol and quantitated by absorbance at 260 nm. Modified S1 nuclease analysis was performed as described (21). The c-*myc* probe was derived from a *Pst* I fragment of plasmid pMcmyc 54 (22) representing the first exon of c-*myc*. This fragment was cloned into plasmid pSP65 (Promega Biotec, Madison, WI) and the DNA was linearized with *Hin*dIII before synthesizing a 150-base mRNA probe using SP6 polymerase (23). The *JE* probe was derived from a *Pst* I fragment of the entire *JE* cDNA (9) cloned into SP65 and linearized with *Bam*HI. The c-*fos* probe was a gift of Tom Curran (Hoffman LaRouche, Nutley, NJ) and represents the entire coding region of the mouse c-*fos* gene cloned into SP65 as an *Eco*RI–*Sal* I fragment and linearized with *Pst* I to give a 200-base mRNA probe. The actin probe was derived from a *Pst* I fragment of the mouse β-actin gene (24) cloned in SP65 and linearized with *Hin*dIII to yield a 660-base probe. The β_2-microglobulin (β_2m) probe was derived from an *Eco*RI to *Hin*dIII fragment representing a portion of the second exon (11). This fragment was cloned into SP64 and linearized with *Eco*RI to yield a 203-base probe.

Mitogen Response Assays. BALB/c 3T3 cells were grown to the confluent monolayer stage in microtiter culture wells as described (8). The mitogenic response of 3T3 cells to wild-type polyomavirus, polyoma capsids, and recombinant VP_1 protein was assayed under conditions similar to virus infection. The spent culture medium was removed and replaced with either virus, capsids, or VP_1 protein in a volume of calcium-free phosphate-buffered saline that was just sufficient to cover the monolayer. After 1 hr at 37°C, unadsorbed materials were removed by aspiration. The cells were then incubated in DMEM supplemented with 5% platelet-poor plasma and 5 μCi of [^{3}H]thymidine per ml (1 Ci = 37 GBq). After 24 hr the cell monolayers were fixed and processed for autoradiography.

RESULTS

Biphasic Induction of c-*myc* and c-*fos* mRNAs During Polyomavirus Infection. Fig. 1*A* demonstrates the temporal expression of c-*myc* mRNA following polyomavirus infection of quiescent BALB/c 3T3 mouse cells with a crude wt virus lysate. Maximal accumulation was seen 1 hr after the adsorption period but the mRNA levels fell to nearly undetectable levels at 6 hr after infection. At 24 hr after infection the abundance level of c-*myc* mRNA was again elevated. Prior to infection, c-*myc* mRNA levels were nearly undetectable in the quiescent or mock-infected cells. To detect possible contaminants in the virus preparation that might affect c-*myc* mRNA accumulation, several control experiments were performed. First, the crude virus lysate was treated with

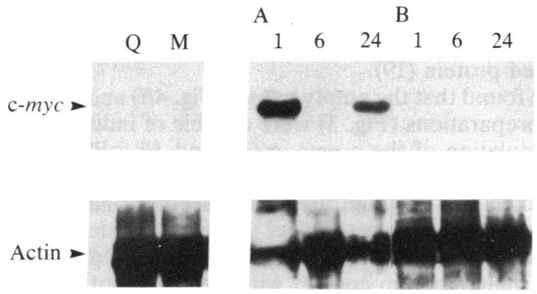

FIG. 1. Polyomavirus infection of quiescent BALB/c 3T3 cells induces c-*myc* mRNA. Total cellular mRNA was isolated from quiescent (Q), mock-infected (M), or wt virus (59RA)-infected cells (*A* and *B*) at 1, 6, and 24 hr after infection. Specific mRNAs for c-*myc* and actin were measured by an S1 nuclease assay. The virus lysate used for infection in *A* was adsorbed with sheep erythrocytes before infecting the cells assayed in *B*.

sheep erythrocytes prior to infection. Polyomavirus agglutinates erythrocytes and is cleared from the lysate by this treatment, and Fig. 1*B* shows that this treatment eliminated stimulation of c-*myc* mRNA accumulation.

As a second control for contaminating inducers of c-*myc*, virions were purified by banding twice in CsCl (see *Methods*). Fig. 2 shows the accumulation pattern of c-*fos* and *JE* mRNAs in addition to c-*myc* mRNA after infection of quiescent cells using the highly purified virus preparation. The extended time course demonstrates that maximal mRNA levels were achieved within 0.5 hr after the adsorption period and rose from undetectable levels at 12 hr after infection. The levels of c-*myc* and c-*fos* mRNAs remained elevated through 36 hr, at which time cell lysis began (not shown).

We found two conditions that disrupted the biphasic accumulation pattern for c-*myc* and c-*fos* mRNAs resulting in their sustained accumulation: (*i*) continuous contact of the cells with the virus after the initial infection period and (*ii*) treatment of the cells with cycloheximide at 10 μg/ml from 0.5 hr prior to, during, and after infection (not shown).

Virus Capsids and Recombinant VP_1 Induce the First Phase of c-*myc* and c-*fos* mRNA Accumulation. Quiescent BALB/c cells were treated with either CsCl banded empty virions or with a recombinant preparation of the major polyoma capsid protein VP_1 purified from *E. coli*. The recombinant VP_1

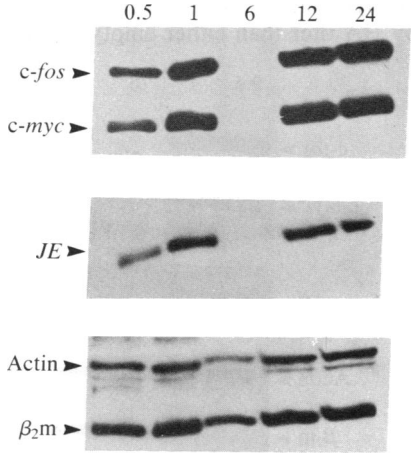

FIG. 2. Time course of mRNA accumulation after infection with CsCl-purified virus. Total cellular mRNA was isolated between 0.5 and 24 hr after infection of quiescent BALB/c 3T3 cells with CsCl-purified wt virus, and specific mRNAs were assayed by an S1 nuclease assay. Controls for extraction efficiency were mRNAs for actin and β_2m. In separate experiments c-*myc* mRNA remains detectable up to 36 hr after infection.

preparation was predominantly in capsid forms arising from spontaneous *in vitro* self-assembly of the bacterially expressed protein (19).

We found that the empty capsid (Fig. 4*B*) and recombinant VP_1 preparations (Fig. 3) were capable of inducing maximal accumulation of the c-*myc*, c-*fos*, and *JE* mRNAs immediately after "infection." After 1 hr, however, mRNA levels remained low, in contrast to the second phase of mRNA accumulation seen with intact virions. Persistent high levels of mRNA were seen, however, if the capsid preparations were maintained in contact with the cells (not shown).

Control experiments using sheep erythrocytes to adsorb capsids from the preparation demonstrated (Fig. 4*C*) that, as seen for the intact virus, the mRNA accumulation was again eliminated. The recombinant VP_1 preparation was free of contaminating bacterial stimulants of c-*myc* as shown by the lack of mRNA induction using a crude bacterial lysate from *E. coli* not expressing the VP_1 gene (Fig. 5*A*).

Virus Capsids Are Mitogenic in a Dose-Responsive Manner. Empty virus capsids and recombinant VP_1 do not efficiently compete with intact virus for infection (25). For this reason we considered that the accumulation of c-*myc* and c-*fos* mRNAs induced by capsids or recombinant VP_1 would also be less efficient. Each preparation may be normalized by its HA ability, since the HA titer reflects the nonspecific receptor interactions of each preparation, and virus and capsids assay equivalently (10 ng of capsid protein per ml equals 1 ± 0.5 HA unit). Dose–response analysis of c-*myc* mRNA accumulation by the three preparations demonstrated maximal levels at 1 hr after stimulus with 8–16 HA units of intact virus, 400 HA units of empty capsids, and 1600–8000 HA units of recombinant VP_1 (data not shown).

Assuming that the initial high-level accumulation of c-*myc* and c-*fos* mRNAs was analogous to that induced by PDGF, we tested whether this induction was followed by a mitogenic response. Density-arrested BALB/c 3T3 cells in microtiter wells were treated with virus, capsids, and recombinant VP_1 capsids at increasing dilutions. [^{3}H]Thymidine uptake into cell nuclei was determined by autoradiography 24 hr after the stimulus. A control bacterial lysate was also used at the same protein concentration as the recombinant VP_1 capsid preparation. Fig. 5 shows a dose–response analysis for virions, capsids, and recombinant VP_1 for induction of [^{3}H]thymidine incorporation into cell nuclei measured at 24 hr after the stimulus. As shown, intact virus was capable of maximally inducing [^{3}H]thymidine labeling at a 10^2 lower concentration measured by HA titer than either empty capsids or recombinant VP_1 preparations. This differential corresponds to that seen for the induction of c-*myc* and c-*fos* mRNAs and for the inhibition of virus infection by these preparations (25).

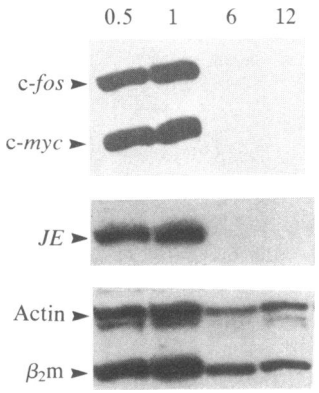

FIG. 3. The early phase of c-*myc* and c-*fos* mRNA accumulation is mediated by virus capsids. Cells were treated with recombinant VP_1 capsids in a manner similar to infection with virus. Total mRNA was isolated at times between 0.5 and 12 hr after the stimulus. No further c-*myc*, c-*fos*, or *JE* mRNA could be detected from 6 hr after stimulation. The pattern of mRNA accumulation was identical for capsids derived from virions (Fig. 4*B*).

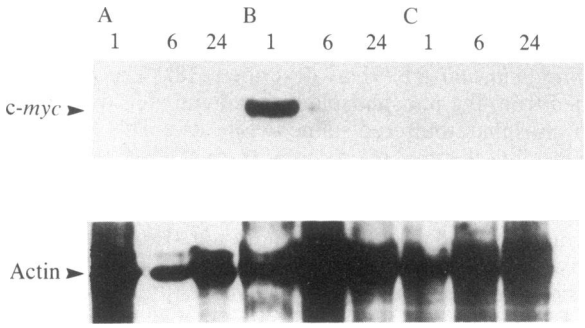

FIG. 4. The early phase of c-*myc* mRNA accumulation is specific for capsids. As controls for possible contaminants that induce c-*myc*, cells were treated with a crude bacterial lysate from *E. coli* not expressing the recombinant VP_1 (*A*) or CsCl-purified viral capsids (*B*) that had been preadsorbed with sheep erythrocytes (*C*). No c-*myc* mRNA was detected with either control preparation (*A* and *C*).

Viral Early Proteins Are Responsible for the Second Phase of c-*myc* and c-*fos* mRNA Induction. We investigated the second phase of mRNA induction using viruses with early gene mutations. As shown in Fig. 6, the prototypic hr-t mutants NG18 and NG59 (26) triggered the first phase of mRNA accumulation, but they were unable to mediate the second phase. We also investigated two site-directed early gene mutants: (*i*) 1387T, which terminates middle T antigen prematurely, thus deleting the hydrophobic carboxyl terminus (27), and (*ii*) 808A, which alters the middle T antigen splice acceptor site such that only large T and small T antigens are made (28). Both of these mutant viruses are defective in cell transformation and in the *in vitro* middle T antigen kinase assay. Fig. 7 shows that both mutants were capable of inducing the late phase of c-*myc* and c-*fos* mRNA accumulation, in contrast to NG18 and NG59, which are completely defective in both small T and middle T antigens.

The possible role of middle T antigen alone in regulating c-*myc* mRNA levels was investigated with a NIH 3T3 cell line, 336a, which has been stably transfected with the middle T antigen cDNA under the transcriptional control of the mouse mammary tumor virus long terminal repeat (15). As

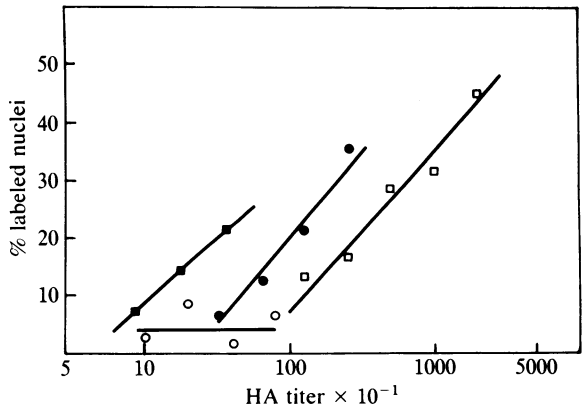

FIG. 5. Virus, capsids, and recombinant VP_1 stimulate mitogenesis in a dose-responsive manner. Cells were stimulated with either CsCl-purified virus (■), empty capsids (●), or recombinant VP_1 (□), and [^{3}H]thymidine nuclear incorporation was measured by autoradiography 24 hr after the stimulus. The preparations are compared by their HA titer measured with sheep erythrocytes. An *E. coli* bacterial lysate (○) (μg/ml) was used as a control for potential mitogens that may be present in the recombinant VP_1 preparation.

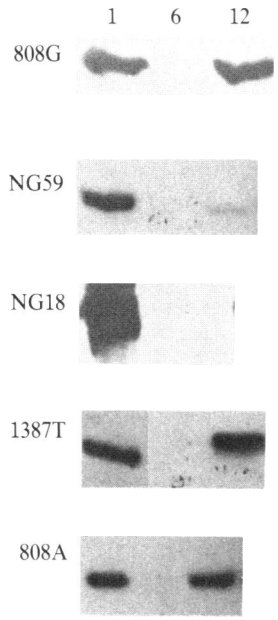

FIG. 6. The second phase of c-*myc* mRNA accumulation is a consequence of the viral early proteins. Cells were infected with crude lysates of polyomavirus early gene mutants and assayed for c-*myc* mRNAs at 1, 6, and 12 hr after infection. 808G is a wt virus (marker-rescued from 808A). NG59 and NG18 are prototypical hr-t mutants defective in middle and small T antigens. 1387T and 808A have intact large and small T antigens but are defective in middle T antigen. c-*fos* and *JE* mRNAs are affected similarly to c-*myc* mRNA levels, and controls for β_2m and actin mRNAs demonstrate approximately equivalent levels in all lanes (not shown).

measured by an *in vitro* kinase assay (29), constitutive middle T antigen expression in 336a cells was ≈5% that of a polyomavirus-transformed cell line (Py NIH 3T3) and was induced ≈20-fold upon the addition of dexamethasone (data not shown). As seen in Fig. 7, c-*myc* mRNA was induced 3.5-fold at 12 hr after dexamethasone addition to the 336a cells. Addition of dexamethasone to the quiescent BALB/c 3T3 cells had no effect on c-*myc* mRNA levels. The induction of middle T antigen kinase-associated activity and c-*myc* mRNA may not be directly comparable due to relatively high constitutive c-*myc* mRNA expression in 336a cells without added dexamethasone.

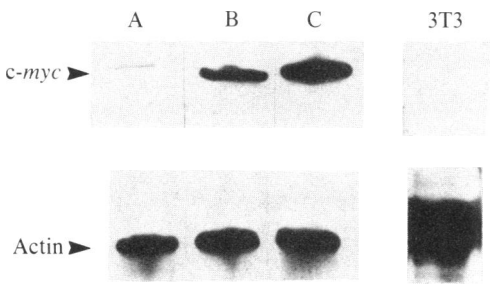

FIG. 7. Middle T antigen alone regulates c-*myc* mRNA levels. The cell line 336a expressing middle T antigen under control of the mouse mammary tumor virus long terminal repeat was growth-arrested at 50% confluence by serum starvation. c-*myc* mRNA was assayed before addition of dexamethasone (lane A), 12 hr after addition (lane B), or 3 hr after addition of PDGF (lane C). Quiescent BALB/c cells (3T3) treated with dexamethasone show no c-*myc* induction. Middle T antigen kinase activity is induced ≈20-fold at 12 hr after dexamethasone addition to 336a cells (data not shown). Densitometry using the actin control to normalize mRNA levels yields at least a 3.5-fold induction of c-*myc* mRNA between lanes A and B.

DISCUSSION

Our experiments demonstrate that the levels of c-*myc* and c-*fos* mRNAs accumulate in a biphasic manner after polyomavirus infection of BALB/c 3T3 cells. We have not determined whether the increased mRNA levels are regulated at a transcriptional level or by altering the stability of the mRNAs. The induction of c-*myc* and c-*fos* mRNAs is not due to contaminants in the preparations because (*i*) the induction is eliminated by preadsorption of the virus or capsids with sheep erythrocytes, (*ii*) CsCl-purified virions and capsids yield distinct responses, (*iii*) capsids prepared from a recombinant VP_1 protein preparation induce the mRNAs similar to virus capsids derived from virions, and (*iv*) mutant viruses have a distinct response from wt.

The initial phase of mRNA accumulation is rapid, does not require protein synthesis, and is effected by virions, capsids, or recombinant VP_1 capsids in a dose-responsive manner paralleling their affinity for the virus receptor. Thus, we believe that the initial high levels of c-*myc* and c-*fos* mRNAs may result from virus (or capsid) attachment to a cell membrane receptor. Although the polyomavirus receptor has not yet been characterized, the virion determinants that recognize the receptor have been partially elucidated. Bolen *et al.* (30) found that a particular VP_1 subspecies that is phosphorylated and present in virions but not capsids appears to account in part for the 100- to 1000-fold increase in binding affinity of the virus for its receptor. A number of cell surface proteins have been identified that may interact with viruses (31–35). Although in most cases it is unclear whether these same cell-surface proteins are used for the actual entry of the virus into the cell, it is teleologically consistent that important membrane molecules have been chosen by pathogens for their entry. For polyomavirus, the physiological consequences of inducing cellular genes associated with the commitment to cell division is obviously advantageous to the entering virus since, once uncoated, the virus has entered a metabolic milieu primed for its immediate replication.

The second phase of c-*myc* and c-*fos* mRNA accumulation is associated with the expression of the viral early proteins—i.e., the polyoma T antigens. The pattern of mRNA accumulation shown by the NG59 and NG18 hr-t mutant viruses indicates that the first phase, resulting from a virion–cell-surface interaction, proceeds normally but that the lack of middle T and small T antigen expression results in a failure to accumulate mRNAs during the second phase. These mutants demonstrate that the expression of large T antigen alone is not sufficient for the regulation of c-*myc* and c-*fos* mRNA levels. The 808A mutant virus, which expresses only large T and small T antigens, is capable of inducing the second phase of mRNA accumulation, thereby demonstrating that middle T antigen expression is dispensable for induction and that small T antigen may be the regulator, either alone or in combination with large T antigen. The second phase of mRNA accumulation by the 1387T mutant virus is consistent with the 808A result, since this mutant also makes wt large and small T antigen proteins. The induction of c-*myc* mRNA in 336a cells suggests that the second phase may be independently regulated by middle T antigen alone.

Previously identified roles for polyomavirus small T antigen in lytic infection, cell transformation, or tumorigenicity are consistent with its ability to induce genes associated with the commitment to cell division. The initial impetus for this investigation was that polyoma hr-t mutants accumulated less viral DNA (by a factor of 3–10) during nonpermissive infections (4, 36), and infection using DNA transfection of large T antigen-only viruses could be complemented for viral DNA synthesis using small T/middle T antigen viruses (37). Thus, lytic growth of mutant viruses suggests small T antigen plays a role in viral DNA accumulation. The 808A mutant

virus (R.L.G. and T. Benjamin, unpublished data) accumulates wt levels of viral DNA but fails to encapsidate the viral DNA into virions, thus separating the roles of middle T and small T antigens in the lytic cycle. Our data suggest that the defect in hr-t mutant virus DNA accumulation is associated with a failure to induce the sustained second phase of c-*myc* and c-*fos* mRNA accumulation. The viral DNA accumulation defect appears only as a 3-fold difference in comparison to wt, presumably because the first phase of induction by the capsid protein proceeds normally during mutant infection.

In contrast to acute transforming retroviruses, the oncogenes of DNA tumor viruses are not closely related to cellular homologs. However, the association of polyoma middle T antigen with $pp60^{c\text{-}src}$ (38) and the regulation of c-*myc* and c-*fos* mRNA levels by the viral early proteins suggest that polyoma has evolved to recruit several cellular protooncogenes during infection. This strategy may be distinct from retroviruses because the polyoma oncogenes are essential in the virus life cycle (2) and must therefore carefully integrate virus production with normal cell functions.

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