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Polyomavirus Large and Small T Antigens Cooperate in Induction of the S Phase in Serum-Starved 3T3 Mouse Fibroblasts

EGON OGRIS, INGRID MUDRAK, AND ERHARD WINTERSBERGER*
Institut für Molekularbiologie, Universität Wien, Wasagasse 9, A-1090 Wien, Austria

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The induction of an S phase in the host cell is a prerequisite for the lytic replication cycle of polyomavirus. This function was attributed to proteins coded for by the early region of the viral DNA, the T antigens. A consideration of the role of the T antigens in the initiation of a mitogenic response of the host cell has to take into account the recent discovery that virus adsorption is sufficient to induce the synthesis of proteins which are known to appear early after quiescent cells are stimulated by the addition of serum, namely *fos*, *jun*, and *myc* (J. Zullo, C. D. Stiles, and R. L. Garcea, Proc. Natl. Acad. Sci. USA 84:1210–1214, 1987; G. M. Glenn and W. Eckhart, J. Virol. 64:2193–2201, 1990). This induction is followed by an initiation of DNA synthesis. It is therefore important to dissociate the effects of the T antigens on the host cell from those of virus adsorption. To do so, we used dexamethasone-regulated versions of the large and small T antigens of polyomavirus stably integrated into the genome of Swiss 3T3 cells to study their function in S-phase induction. When the production of the large or small T antigen in serum-starved 3T3 mouse fibroblasts was activated, only a small fraction of cells was able to leave G_0/G_1 despite the synthesis of considerable amounts of the respective T antigen. Activation of both T antigens within the same cell, on the other hand, resulted in S-phase induction in a notable percentage of cells, suggesting that the two proteins cooperate in this activity. Polyomavirus T antigens appear to bypass the pathway of growth regulation involving the activation of c-*fos*. These results are discussed in relation to other known functions of the two virally coded proteins.

The region of the genome of polyomavirus which is expressed early after infection encodes three proteins, referred to as large, middle, and small tumor antigens (LT, MT, and ST antigens; for reviews, see references 18, 21, and 57). LT antigen is a multifunctional nuclear protein which is required for viral DNA replication and takes part in the control of viral gene expression. It is capable of immortalizing certain cell types (11, 25, 47, 55; reviewed in references 34 and 54) and is reported to reduce the serum requirement of immortalized cells (47, 55), thus perhaps playing an important role in growth induction. MT antigen is responsible for the transforming activity of polyomavirus (45, 46), while ST appears to enhance the infectivity of the virus (37, 40, 56, 58), possibly by allowing more-extensive replication of viral DNA (2). ST antigen may also promote cell growth and cooperate with MT antigen in cell transformation (10, 41). It was found to form a complex with two cellular proteins which were recently shown to be the catalytic subunit and a regulatory subunit of protein phosphatase 2A (42). Phosphatase 2A was observed to be one of the factors stimulating simian virus 40 (SV40) DNA replication in vitro (32, 59).

It is well known that an infection of resting mouse cells with polyomavirus results in the onset of DNA synthesis in the host cell, which is a prerequisite for and occurs together with viral DNA replication (57). It was assumed that T antigens are responsible for the induction of the S phase in infected cells, but how these viral gene products cause cells to start DNA synthesis is unknown. Recent studies on early events following an infection of permissive cells with polyomavirus have resulted in the unexpected observation that virus adsorption suffices, independent of the expression of any viral gene product, to cause a rapid expression of genes known to be induced early after serum stimulation of resting cells. The induction of c-*fos*, c-*jun*, and c-*myc* (20, 64) is followed by an initiation of S phase and can even be caused by an addition to quiescent fibroblasts of purified viral capsid protein VP1 (64). Hence, the binding of the virus to the cell membrane sets in motion a series of reactions known to follow the stimulation of resting cells by growth factors. Very similar observations were made with cytomegalovirus (7, 8). A second wave of induction of *fos*, *jun*, and *myc* occurs later and appears to depend on viral gene products. By using a polyomavirus mutant coding for a temperature-sensitive (*ts*) LT antigen (*ts*25), it was deduced that ST antigen plays an important role in the second wave of induction (20). However, it cannot be ruled out that LT antigen also plays a part in this reaction, because *ts*25 is a point mutation selected by its temperature sensitivity with regard to virus replication, and cellular function of the protein may not be equally affected.

These results make it necessary to uncouple early mitogenic effects on cells, which are induced by virus adsorption, from those caused by viral gene products. This may be possible by conditionally expressing T antigens in cells stably transfected with the genetic information for LT or ST antigen or both. The use of *ts* mutants for this purpose has several disadvantages. Aside from the fact that most of these mutants were selected because they are defective in virus replication (which does not mean that other properties of the multifunctional protein are influenced in the same way), one disadvantage of *ts* mutants is that under normal growth conditions of transfected cells, the viral protein is functional. A reverse situation in which particular conditions have to be fulfilled in order to switch on the production of viral T antigen rather than to inactivate it would be more useful.

We have therefore used an alternative approach. The genetic information for polyomavirus LT or ST antigen was placed under the control of the dexamethasone-inducible

* Corresponding author.

FIG. 1. Structure of the pMMTV-Py plasmids carrying the information for polyomavirus LT or ST antigen joined to the dexamethasone-inducible MMTV promoter. Py, intron-free gene for either LT or ST antigen which was isolated from plasmid pPyLT1 or pPyST1 (63), respectively. The rest of the plasmids stems from pMSG (Pharmacia), except that the *gpt* gene and adjoining SV40 sequences were removed. The remaining SV40 sequences come from the 3′ part of the SV40 late region and have no function in our plasmid.

mammary tumor virus (MMTV) promoter, and mouse 3T3 fibroblasts were transfected with these constructs together with a gene, allowing selection of transfected cells with Geneticin or hygromycin. Cell clones which produce the viral gene product(s) after induction with dexamethasone were isolated. Using these cells, we found that neither LT nor ST antigen alone was capable of driving a significant fraction of serum-starved 3T3 cells into S phase, while both T antigens expressed at the same time within one cell did so.

MATERIALS AND METHODS

Construction of plasmids. Plasmids pMMTV-PyLT and pMMTV-PyST were constructed as follows. The sequence coding for LT or ST antigen was isolated from pPyLT1 or pPyST1 (63), respectively, by cleavage with *Bst*XI (at nucleotide 173 of the polyoma genome) and *Hin*dII (nucleotide 2962). This was cloned into the *Nhe*I site of the polylinker, which is situated at the 3′ end of the MMTV promoter in plasmid pMSG (Pharmacia). The *gpt* gene plus SV40 sequences present in pMSG but not required for our purpose were then removed by digestion with *Xho*I and *Bam*HI. The final constructs start with the ATG of the early viral gene directly linked to the MMTV promoter and completely lack viral control sequences (Fig. 1).

Cell culture. Cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal calf serum. One day before transfections, cells were trypsinized and seeded at a density of 10^6/10-cm dish. At 4 h prior to transfection, the medium was changed. Cotransfections were done by the calcium phosphate precipitation method as modified by Wigler et al. (62), with 18 μg of the plasmid carrying polyomavirus information plus 2 μg of the plasmid used for selection (pSV2-*neo* or pSV2-*hygro*). All plasmids were linearized at suitable sites before transfection. At 20 h after transfection, a glycerol shock was applied (44). Cells were split 24 h later and then received Geneticin (600 μg/ml) or hygromycin (200 μg/ml). Medium was changed every 3 to 4 days. After about 10 days, clones were visible. They were isolated and expanded to mass cultures.

To arrest cells, the cells were seeded at a density of 5×10^5/10-cm dish in medium containing 10% serum. After 24 h, cells were washed twice with medium without serum and then covered with medium containing 0.2% serum. They were used 3 days later.

For labeling of DNA by tritiated thymidine, the radioactive compound (0.5 μCi/ml) was added to the medium. After 30 min, medium was removed, cells were washed extensively with phosphate-buffered saline, and acid-insoluble radioactivity was determined.

Incorporation of bromodeoxyuridine was done by adding the compound at 0.1 mM final concentration to the medium. After 30 min, cells were washed and fixed with acetone-methanol (1:1). For immunofluorescence detection of incorporated bromodeoxyuridine, we used a mouse monoclonal antibromodeoxyuridine antibody (Dakopatts) followed by rhodamine-conjugated goat anti-mouse immunoglobulin (Accurate).

For flow cytometry, cells were trypsinized, washed, and fixed in 70% ethanol. The fixed cell pellet was suspended in a few drops of 0.05% pepsin, stained with 4′,6-diamidino-2-phenylindole (DAPI; Merck) and sulforhodamine 101 (Sigma), and analyzed in a Partec PAS-2 cytofluorometer.

Analysis of T antigen by immunological methods. For induction of T antigens, dexamethasone was added to the medium to a final concentration of 1 μmol/liter.

Immunofluorescence analysis of LT and/or ST antigen was done by washing cells twice with phosphate-buffered saline and then fixing them with acetone-methanol (1:1) at room temperature. The antibodies used were rat monoclonal antibody αPyC4 (13) followed by fluorescein-conjugated goat anti-rat immunoglobulin (Pierce).

Protein extracts for Western immunoblotting were prepared by directly dissolving cells previously washed with phosphate-buffered saline on the petri dish in 100 μl of sample buffer (prewarmed to 85°C) by using a rubber policeman. Extracts were heated at 100°C for 10 min, sonified for 30 s, and centrifuged for 10 min in an Eppendorf centrifuge. Protein concentration was then determined by using the BioRad assay, and defined amounts of protein (up to 300 μg per lane) were applied to 7.5% (for LT antigen), 15% (for ST antigen), or 10% (for LT plus ST antigens) polyacrylamide gels by the method of Laemmli (29).

Protein transfer for Western blotting was carried out as described elsewhere (50). Blots were first incubated overnight at 4°C with the monoclonal antibody αPyC4 (13), which recognizes polyomavirus LT and ST antigens. After blots were washed, they were incubated for 2 h at room temperature with rabbit anti-rat immunoglobulin (Nordic Immunology) and finally for another 2 h with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin (Sigma). The substrate for alkaline phosphatase was 5-bromo-4-chloro-3-indolyl phosphate (Boehringer), and Nitro Blue Tetrazolium (Boehringer) was used for staining.

Replication assay. Cells were seeded at a density of 2.5×10^5/5-cm dish 1 day before transfection. The plasmid pGEMPyA3ori, which contains the control region of polyomavirus strain A3 from the *Bcl*I site (nucleotide 5021) to the *Nar*I site (nucleotide 84) (including the enhancer, the origin core region, and the strong T-antigen binding site A cloned into the *Bam*HI site of plasmid pGEM3Zf [Promega]) was transfected by using the DEAE-dextran method (19). Five hundred nanograms of the plasmid dissolved in 800 μl of Tris-buffered saline containing 400 μg of DEAE-dextran per ml was placed on top of the cell layer for 30 min; cells were then washed and covered with fresh medium (10% fetal bovine serum). Transfection of pPyLT1 into an ST-antigen-expressing cell line was done similarly. When dexamethasone (1 μmol/liter, final concentration) was added, this was done 4 h later. Incubation was continued overnight for pGEMPyA3ori or for 40 h for pPyLT1. Plasmid DNA was then extracted by the Hirt method (24), purified, digested with *Dpn*I (which cleaves unreplicated but not replicated DNA) and (in the case of pGEMPyA3ori only) *Eco*RI for linearization, and separated on 1% agarose gels. The Southern blots were hybridized with the polyomavirus control region.

Other methods. DNA and RNA were isolated by using

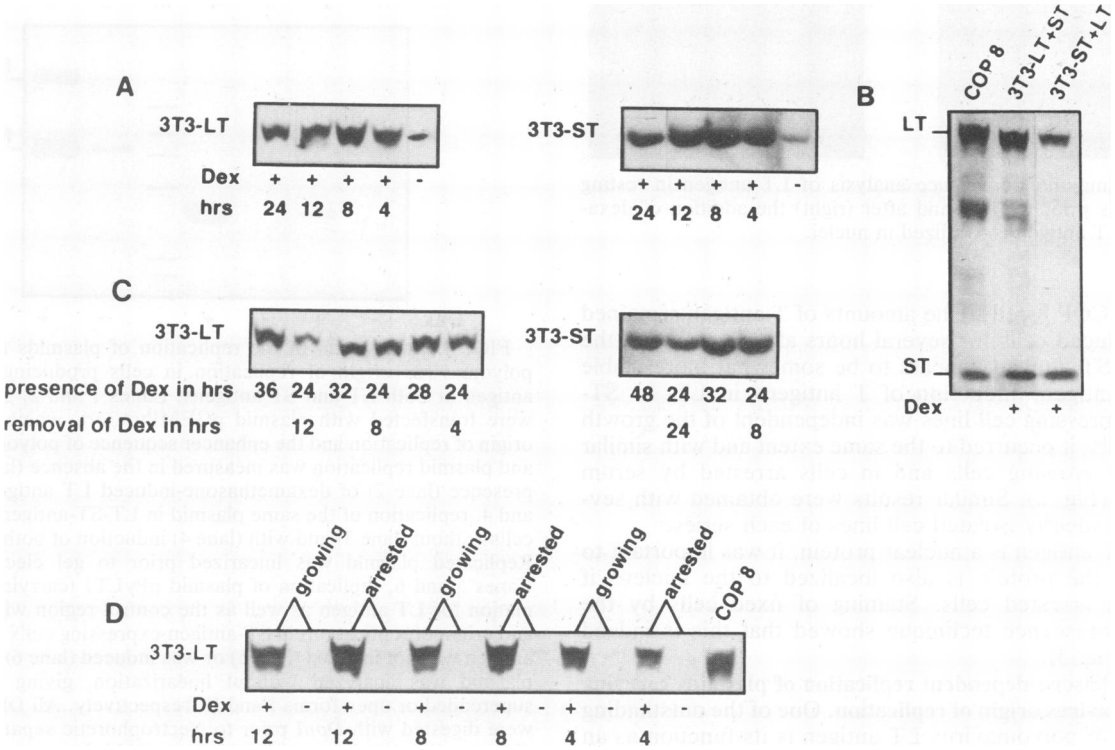

FIG. 2. Induction of T antigens as determined by Western blotting. 3T3 cells carrying the information for LT or ST antigen or both were analyzed. (A) Induction of LT or ST antigen in a 3T3-LT or 3T3-ST cell line, respectively. Dexamethasone (1 μmol/liter) was added to logarithmically growing cells at time zero, and cell extracts were analyzed as described in Materials and Methods at the times indicated. The rightmost lane shows T antigen present in uninduced cells. (B) Induction of LT and ST antigens in cell lines stably transfected with both genes. 3T3-LT+ST was constructed by secondary transfection of 3T3-LT cells with pMMTV-PyST, while 3T3-ST+LT was obtained by secondary transfection of 3T3-ST cells with pMMTV-PyLT. Cells were arrested for 3 days in 0.2% serum, T antigens were then induced by the addition of dexamethasone, and extracts were analyzed 16 h later. The same amount of protein from an extract of COP 8 cells was applied for comparison. In the absence of dexamethasone, only very small amounts of both T antigens were seen, just like those present in uninduced cells carrying information for only a single T protein (panel A). (C) Stability of LT or ST antigen in induced cells after removal of dexamethasone. T antigen was induced for 24 h. Dexamethasone was then removed from every other dish but remained on the cells in the other dishes as a control for continuous induction. Cells from which the glucocorticoid was removed were analyzed at 4, 8, and 12 h thereafter (3T3-LT) and at 8 and 24 h thereafter (3T3-ST). (D) Comparison of induction of LT antigen in logarithmically growing 3T3-LT cells with that in cells arrested for 3 days. Cell extracts were analyzed at 4, 8, or 12 h after addition of the hormone.

protocols described elsewhere (50). For Northern (RNA) analysis, 7 μg of RNA was separated in formaldehyde-agarose gels and blotted onto nitrocellulose. Prior to hybridization, a mouse c-*myc* probe and a v-*fos* probe were labeled with ^{32}P by the random primer method (17).

RESULTS

Construction of cell lines conditionally expressing polyomavirus T antigens. Plasmids pMMTV-PyLT and pMMTV-PyST were constructed such that the final constructs carry the polyomavirus early regions starting at the ATG (*Bst*XI site) linked to the MMTV long terminal repeat (Fig. 1). In order to affirm the intactness of the initiation codon, the correct linkage was verified by sequencing.

Either one of these plasmids was cotransfected with plasmid pSV2-*neo* (51) into Swiss 3T3 mouse fibroblasts, and stably transfected cells were selected by growth in the presence of Geneticin and tested for the induction of the respective T antigen by Western blotting. Southern analysis (not shown) on DNA of some of the 3T3-LT or 3T3-ST cell lines indicated the presence of one to five copies of the viral gene per genome. LT-ST double transfectants were produced by a second transfection either of 3T3-LT lines with pMMTV-PyST or of 3T3-ST lines with MMTV-PyLT. We made use of the hygromycin B phosphotransferase as an alternative selectable marker (4). Cells producing both T antigens after the addition of dexamethasone were again found by the Western blotting technique.

Dexamethasone-induced synthesis of T antigens in transfected cell lines. Selected cell lines conditionally producing LT or ST antigen or both were used to examine the kinetics of induction of the T antigen and its stability. Typical results of such experiments are shown in Fig. 2. All cell lines exhibit a low degree of leakiness; small amounts of the viral protein are synthesized even in the absence of dexamethasone. However, about 4 h after addition of the hormone (to a final concentration of 1 μmol/liter), there was a strong increase in the quantity of the T antigen produced; maximal amounts were reached between 8 and 12 h. To evaluate the levels of T antigens induced, they were compared with those present in extracts of COP 8 cells, a C127 mouse cell line transformed with multiple copies of a polyomavirus mutant lacking a functional origin of replication but constitutively expressing large quantities of all three T antigens. In some of our cell lines, T antigens reached levels close to those

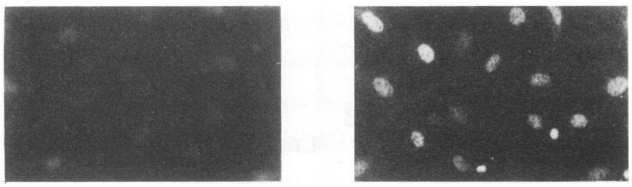

FIG. 3. Immunofluorescence analysis of LT antigen in resting 3T3-LT cells prior to (left) and after (right) the addition of dexamethasone. T antigen is localized in nuclei.

present in COP 8 cells. The amounts of T antigen remained high in induced cells for several hours after removal of the hormone; ST antigen appears to be somewhat more stable than LT antigen. Induction of T antigens in LT- or ST-antigen-expressing cell lines was independent of the growth state of cells; it occurred to the same extent and with similar kinetics in growing cells and in cells arrested by serum starvation (Fig. 2). Similar results were obtained with several independently isolated cell lines of each series.

Since LT antigen is a nuclear protein, it was important to show that the protein is also localized to the nucleus if induced in arrested cells. Staining of fixed cells by the immunofluorescence technique showed that this is indeed the case (Fig. 3).

Dexamethasone-dependent replication of plasmids carrying the polyomavirus origin of replication. One of the outstanding properties of polyomavirus LT antigen is its function as an origin-binding protein in the replication of polyomavirus genomes (reviewed in references 18 and 21). In order to prove that cell lines conditionally expressing LT antigen can carry out this function, we transfected growing cells with a plasmid (pGEMPyA3ori) carrying the polyomavirus origin of replication and tested for replication of this plasmid. As seen in Fig. 4 (lanes 1 and 2), the plasmid replicates in an LT-antigen-expressing cell line in the presence of dexamethasone but not in its absence, as expected.

ST antigen was observed to enhance the infectivity of polyomavirus in mouse cells, probably by indirectly stimulating the replication of the viral genome (2, 37, 40, 56, 58). To test for this activity in an ST-antigen-expressing cell line, the cell line was transfected with a plasmid (pPyLT1) which contains the total control region, including the origin of replication, and the sequence coding for LT antigen. This plasmid therefore provides LT antigen for its replication and was earlier found to replicate more efficiently when cotransfected with another plasmid from which ST antigen was expressed (2). In agreement with these results, we found (Fig. 4, lanes 5 and 6) that pPyLT1 replicated more efficiently in ST-antigen-expressing cells when the viral protein was induced by addition of dexamethasone. In addition, the replication efficiency of pGEMPyA3ori was significantly higher in LT-ST-antigen-expressing cell lines than in those synthesizing only LT antigen (Fig. 4, lanes 3 and 4).

Efficient induction of S phase in serum-starved 3T3 cells requires the action of both LT and ST antigens. The main purpose of this work was to see if expression of either LT or ST antigen alone from within the cell can drive serum-starved 3T3 cells into S phase. To this end, cells growing in full medium in the absence of dexamethasone were arrested by reducing the serum concentration to 0.2% and continuing the incubation for 3 days. Dexamethasone was then added to such cultures, and the serum concentration was kept low. Induction of DNA synthesis was initially measured by the incorporation of labeled thymidine (followed by counting of

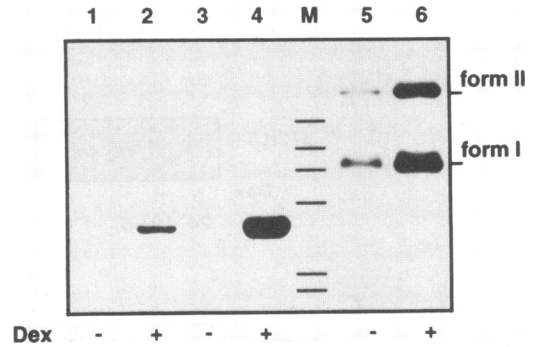

FIG. 4. Stimulation of the replication of plasmids carrying the polyomavirus origin of replication in cells producing LT or ST antigen or both LT and ST antigens. Lanes 1 and 2, 3T3-LT cells were transfected with plasmid pGEMPyA3ori, which carries the origin of replication and the enhancer sequence of polyomavirus A3, and plasmid replication was measured in the absence (lane 1) or the presence (lane 2) of dexamethasone-induced LT antigen. Lanes 3 and 4, replication of the same plasmid in LT-ST-antigen-expressing cells without (lane 3) and with (lane 4) induction of both T antigens. Replicated plasmid was linearized prior to gel electrophoresis. Lanes 5 and 6, replication of plasmid pPyLT1 (carrying the information for LT antigen as well as the control region with enhancer and origin of replication) in ST-antigen-expressing cells in which ST antigen was not induced (lane 5) or was induced (lane 6). Replicated plasmid was analyzed without linearization, giving rise to the supercoiled or open forms I and II, respectively. All DNA samples were digested with *Dpn*I prior to electrophoretic separation. *Dpn*I digests methylated, unreplicated input DNA but not the unmethylated replicated plasmids. The digestion products accumulate near the bottom of the gel; they are not shown in the figure. Lane M, size marker in *Hin*dIII digest of phage lambda DNA.

radioactivity incorporated into DNA) or bromodeoxyuridine (followed by immunological visualization of the incorporated precursor). In control experiments, S phase was induced by increasing the concentration of serum to 10% either in the absence or in the presence of dexamethasone. In addition, normal 3T3 cells lacking polyomavirus information were subjected to the same procedures.

The preliminary results (Table 1) indicated that in the presence of low concentrations in serum, expression of LT or ST antigen alone stimulated DNA synthesis only 3- to 4-fold, while simultaneous expression of both antigens stimulated cellular DNA synthesis 12-fold. However, because of uncertainties inherent in experiments involving measurements of the incorporation of DNA precursors, a more-

TABLE 1. Stimulation of DNA synthesis as measured by thymidine incorporation

Cell line	cpm of [^{3}H]thymidine incorporated into DNA[a]			
	0.2% serum, no DEX	0.2% serum + DEX	10% serum, no DEX	10% serum + DEX
3T3	3,195	2,384	42,136	56,710
3T3-LT	3,785	11,384	41,620	54,644
3T3-ST	3,101	10,221	47,756	51,464
3T3-LT+ST	2,416	30,933	61,593	63,291

[a] Experiments involved 10^6 cells. Values are from an extensive kinetic study. Time points were chosen when the rate of incorporation reached a maximum. This was 28 to 32 h after addition of dexamethasone (DEX) to quiescent cells in experiments in which serum was kept at 0.2% and 16 to 20 h after stimulation of resting cells with 10% serum in the absence or presence of dexamethasone.

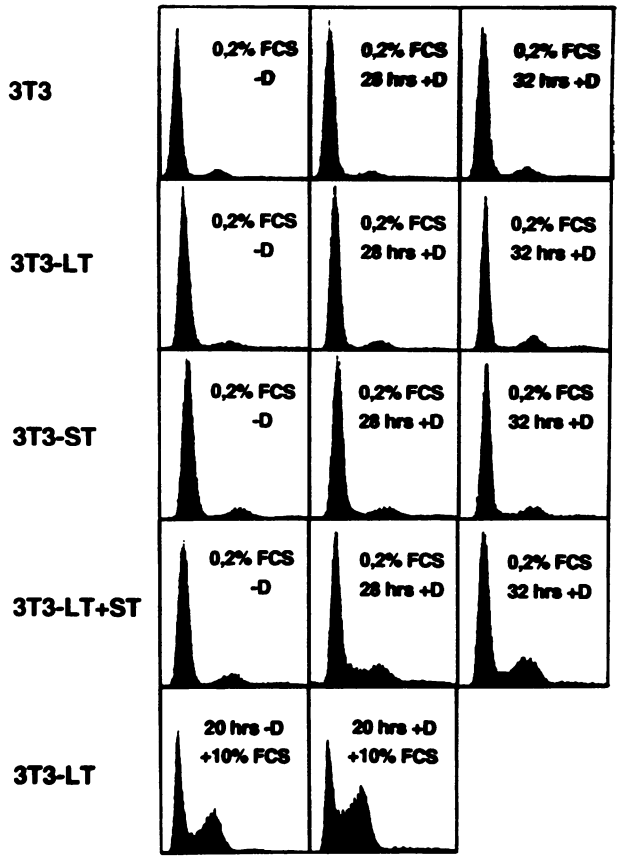

FIG. 5. Examples of a flow cytometric analysis of various 3T3 cell lines producing no T antigen, LT antigen, ST antigen, or both. Cells were made quiescent by keeping them for 3 days in 0.2% fetal calf serum (FCS). Dexamethasone was then added to the medium to a final concentration of 1 μmol/liter, and cells were prepared for fluorescence-activated cell sorting analysis 28 and 32 h later. The bottom row shows an example of the result obtained when cells were stimulated by addition of serum in the absence and presence of dexamethasone. A shorter time was chosen in this experiment because preliminary studies, in which induction of DNA synthesis was measured by incorporation of DNA precursors, indicated that S phase starts earlier if stimulated by addition of serum (Table 1). Very similar results were obtained when 3T3 cells, 3T3-ST cells, or 3T3-LT+ST cells were stimulated by addition of serum.

detailed analysis was carried out with flow cytometry to measure the fraction of cells moving from G_0/G_1 into S and G_2. Results obtained with this approach are shown in Fig. 5. Induction of LT antigen in 3T3-LT cell lines caused only a very small fraction of cells to move into S phase (6% in the experiment shown in Fig. 5). Similar results were obtained with several different such cell lines which varied in the amount of LT antigen produced after full induction. In particular, the fraction of cells initiating S phase did not seem to correlate with the quantity of LT antigen produced in these cells. Cell lines expressing ST antigen after stimulation by dexamethasone at a low concentration of serum also entered S phase at a low percentage (about 9%) only. Significantly, however, cells producing both T antigens at the same time entered S phase to a considerable extent (25 to 30% of the cells). Several independently isolated cell lines capable of conditional synthesis of LT and ST antigens gave similar results. Control experiments proved that the addition

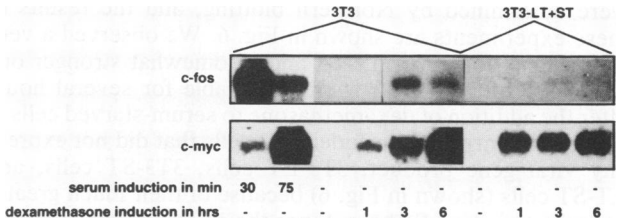

FIG. 6. Analysis by Northern blotting of the production of c-*fos* and c-*myc* mRNA in 3T3 cells and in 3T3 cells conditionally expressing LT and ST. Serum-starved cells were induced by the addition of serum to 10% or by dexamethasone-dependent production of LT and ST antigens. RNA was isolated at the times indicated, separated by electrophoresis, and probed as described in Materials and Methods.

of dexamethasone to resting 3T3 cells did not cause the entry into S phase of a measurable fraction of cells, whereas addition of the hormone to cells whose growth had been stimulated by addition of serum enhanced the initiation of S phase, if anything, but certainly did not retard it. In a further control experiment (not shown), we found that the addition of dexamethasone does not negatively influence the infection cycle of polyomavirus in 3T3 cells kept at low concentrations of serum. This confirms earlier results which showed that dexamethasone has a positive effect on polyomavirus replication as measured by plaque formation on 3T3 cells (39). We conclude that expression of both T antigens is necessary to allow serum-starved 3T3 mouse fibroblasts to proceed from G_0/G_1 into the S phase of the cell cycle.

Are early-response genes expressed after dexamethasone-induced production of LT and ST antigens? Stimulation of serum-starved fibroblasts by addition of serum results in the expression of a variety of genes which starts very early and independent of ongoing protein synthesis (for a review, see reference 43). Prominent among these early-response genes are c-*fos* and c-*myc*. While c-*fos* is one of the earliest activated genes in growth-stimulated 3T3 cells (its expression reaches high levels within 30 min after serum addition and declines after 1 h), c-*myc* expression starts after about 1 h and increases for up to 4 h after provision of serum.

It is also known that both LT and ST antigens of SV40 and polyomavirus are able to transactivate certain genes. While it is well established that LT antigen influences transcription from the viral early and late promoters in a negative and positive manner, respectively (21, 26), it was also recently found to transactivate cellular promoters, notably the human *hsp70* promoter (27). ST antigen of SV40 was also reported to transactivate several cellular promoters (35). Hence, it is not unreasonable to speculate that the T antigens exert their cooperative mitogenic effect via the transactivation of cellular genes involved in growth control. We have therefore investigated whether induction of LT and ST antigens in the absence of serum leads to a stimulation of the expression of the c-*fos* and c-*myc* genes. The time course of transcription of these genes in cells whose growth was stimulated by induction of LT and ST antigens may differ considerably from that seen in serum-stimulated cells, because some time is required after the addition of dexamethasone before sufficiently high levels of LT and ST antigens accumulate to drive cells out of G_0/G_1. Time points chosen for these experiments were therefore later than those chosen for the serum-stimulated control. Levels of c-*fos* and c-*myc* mRNAs

were determined by Northern blotting, and the results of these experiments are shown in Fig. 6. We observed a very weak signal for c-*fos* mRNA and a somewhat stronger one for c-*myc* mRNA. Both were detectable for several hours after the addition of dexamethasone to serum-starved cells in all experiment which included 3T3 cells that did not express any viral gene product, 3T3-LT cells, 3T3-ST cells, and LT-ST cells (shown in Fig. 6) because of their much greater capacity to induce S phase. Since the 3T3 cells failed to enter S phase to a measurable extent, as determined by flow cytometry (Fig. 5), we conclude that the minor degree of expression of c-*fos* and c-*myc* is due to the addition of dexamethasone and is not related to the presence of polyomavirus T antigens. These results indicate that the mitogenic stimulation of fibroblasts by the cooperative action of polyomavirus LT and ST antigens may not involve the pathway which depends on the expression of c-*fos*.

DISCUSSION

The early-gene products of polyomavirus have a variety of effects on the host cell. Most of these are only poorly understood so far. MT antigen, the transforming protein of polyomavirus, associates with and modulates the tyrosine kinase activity of cellular proto-oncogene products (see, e.g., reference 9 and references therein). In its N-terminal part, MT antigen is identical with ST antigen and shares with ST antigen the interaction with protein phosphatase 2A (42, 60). LT antigen, possibly aided by ST antigen, has been ascribed a role in the growth stimulation of resting cells, an important reaction which is a prerequisite for efficient virus replication during a lytic-infection cycle. The recent discovery that the process of virus infection itself leads to an induction of S phase, without the need for activity by any early viral gene product (64), necessitated a reexamination of the potential roles of LT and ST antigens in this process. We have attempted to avoid interference with effects induced by virus adsorption and have produced cell lines expressing the viral gene product(s) from a hormone-inducible promoter. This allowed us to analyze the effect of induction of T antigen in serum-starved cells on their capacity to enter S phase. Our results show that LT or ST antigen alone, although produced in quantities comparable to those in infected cells or in cells transformed by polyomavirus, resulted in a shift into S phase of only a very low fraction of cells. Expression of both T antigens within the same cell, on the other hand, caused a significant percentage of cell to leave G_0/G_1 and enter S and G_2.

According to our data, LT antigen by itself (as synthesized in serum-starved cells) cannot be considered a mitogen for mouse fibroblasts. This statement has to be discussed in the light of earlier views on the function of this protein in the lytic- or abortive-infection cycle and of current ideas about the role of LT antigen in the immortalization of certain cell types. Although the assumption is not well supported by experimental evidence, LT antigen has often been assumed to be responsible for the induction of cellular DNA synthesis in infected cells. This idea rests strongly on the effect of mutations in the gene coding for LT antigen, which give rise to a temperature-sensitive protein (for a review, see reference 21). Although these mutants (*tsa* mutants such as *tsa*-a or *ts*25) were isolated by their temperature sensitivity in virus replication, they were often found to be defective in the stimulation of host cell DNA synthesis. However, sequence analyses of several of these mutants revealed a change in the C-terminal part of the protein, a region which is indeed important for virus replication (21), whereas many of the cellular functions of LT antigen seem to reside in the N-terminal half of the molecule (1, 47). The relation of these mutations to the effect on the host cell, therefore, is not at all clear. Another type of polyoma mutants, *hrt* mutants, are nontransforming host range mutants which lack the information for intact MT and ST antigens but produce normal LT antigen. Although LT antigen is the only viral protein necessary for viral DNA replication, these mutants do not grow in normal mouse 3T3 cells, but they do grow in 3T3 cells transformed by polyomavirus and thereby supplying MT and ST antigens. Several properties of viral mutants, therefore, support the idea that LT antigen may not be the only viral gene product required for the induction of an S phase in the host cell.

Another point which at first appears to be at variance with our observations relates to the fact that expression of LT antigen in primary cell cultures seems sufficient to immortalize cells (11, 25, 47, 55). Results obtained with rat cells immortalized by a *tsa* mutant (47) or with human cells immortalized by polyomavirus LT antigen under an inducible promoter (55) furthermore suggest that continued expression of LT antigen is necessary to keep cells immortal. Moreover, both rat and human cells immortalized by polyomavirus LT antigen show a reduced serum requirement. It should be noted, however, that there is a great difference in our experimental system and that used to study immortalization. We have used an already immortal cell line to investigate the capacity of LT antigen to drive arrested cells into S phase. In the immortalization assay, replicating primary cells are supplied with LT-antigen information, and as a consequence, a very low percentage of the cells survives "crisis" and becomes immortal. Such cells are under a strong selection pressure for many generations. It should also be remembered that immortal cells arising by transfection with viral genetic information usually exhibit a variety of chromosomal aberrations which may be intimately related to their capability to escape senescence. In fact, a mutagenic and/or recombinogenic activity of papovavirus LT antigens might be important for the immortalizing function of these proteins (48, 52, 53).

How might LT and ST antigens cooperate in moving cells from G_0/G_1 into S phase? Two principal possibilities can be envisaged. First, either protein by itself has a low activity in this reaction by interacting with different pathways of the network of reactions leading to S phase. Expression of both proteins within one cell should, then, have an additive effect. Although we cannot entirely exclude this possibility, we consider it unlikely, as most of our LT-ST-antigen-expressing cell lines allow more cells to enter S phase than would be expected from an additive effect of LT and ST antigens. The second, more likely possibility is that ST antigen somehow aids LT antigen in its stimulating effect, for instance by interference with the protein phosphorylation-dephosphorylation system. It is not known whether the activity of LT antigen which we investigated in this work requires the known interaction of this protein with the product of the retinoblastoma susceptibility gene, *RB* (14). Future work will have to address this question, as it was shown in a recent report that LT mutants affected in binding the RB protein are defective in immortalization (31). It is also interesting that the interaction of RB with the LT antigen from SV40 (12, 38, 61) proved to be influenced by the phosphorylation status of the RB protein (22, 36). This association may also depend on the presence of a threshold amount of a subspecies of

polyomavirus LT antigen showing a particular phosphorylation pattern (5, 6, 23). It is therefore not unreasonable to assume that ST antigen might influence the phosphorylation status of either protein and thereby their interaction. Moreover, SV40 ST antigen was shown to transactivate some promoters for RNA polymerase II or III (35). If polyomavirus ST antigen has a similar activity, this could likewise contribute to the S phase-inducing activity of the LT-ST-antigen combination.

Our experiments show that expression of c-*fos* is probably not a prerequisite for T-antigen-mediated stimulation of S phase in 3T3 cells. This result differs from those reported by others for polyomavirus-infected cells, in which T-antigen-dependent induction of c-*fos* was observed (20, 64). On the other hand, conclusions similar to ours were recently drawn from studies on functions of SV40 T antigen (49). These studies and our findings may indicate that the viral proteins circumvent the c-*fos*-dependent signal transduction cascade. A bypassing of the c-*fos*-dependent passway (which is used if cells are stimulated by serum, growth factors, virus infection, or simple binding of the polyomavirus protein VP1) could explain why cells respond in a rather asynchronous mode when S phase is induced from within the cell by T antigens if the concentrations of the cellular components interacting with the T antigens vary in individual cells. This might also explain why only a fraction of cells seems to respond to T antigen. Certainly, this important question requires more attention, and our cell lines may be helpful for such studies.

Despite the possible similarity of polyomavirus and SV40 T antigens with respect to the capability of overriding the c-*fos*-dependent transduction pathway, it is worthwhile to point out significant differences between the two proteins. Whereas the transforming activity of polyomavirus resides in the MT antigen, such activity in SV40 is connected with LT antigen, and while both proteins share the capacity for interaction with RB, SV40 but not polyomavirus LT antigen also forms a complex with the products of another tumor suppressor gene, *p53* (for reviews, see references 30 and 33). It is therefore possible that SV40 LT antigen suffices for S-phase induction but that polyomavirus LT antigen does not. On the other hand, it has already been shown that SV40 ST antigen enhances the transforming activity of SV40 LT antigen in BALB/c 3T3 cells (3).

Finally, we point out that the cell lines we have constructed may be helpful for studies of a variety of questions related to the functions of LT or ST antigen. The only rodent cell line previously available which conditionally expresses a polyomavirus T antigen is one in which the MT antigen is under the control of an MMTV promoter (45). In other rodent cell lines carrying information for a single polyomavirus T antigen, MT antigen is under the control of its own promoter (46) or of a strong promoter causing constitutive, unregulated expression of the protein (10, 11, 25). Recently, attempts have been made to produce cell lines which conditionally express another growth- or cell cycle-regulated (or regulating) protein, namely, c-*myc* (15). Such cell lines have already yielded interesting information on the function of this protein (16). For similar purposes, a mammalian regulatory system using the *Escherichia coli lac* repressor was created for inducible expression of the p53 protein (28). The use of these cell lines will shed new light on the mechanisms by which important regulatory proteins influence the cell cycle and growth. Our cell lines may help dissect the roles of polyomavirus T antigens in these reactions.

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