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Genetic Analysis of Polyomavirus Large T Nuclear Localization: Nuclear Localization Is Required for Productive Association with pRb Family Members

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Polyomavirus large T antigen (LT) is a multifunctional nuclear protein. LT has two nuclear localization signals (NLSs), one spanning residues 189 to 195 (NLS1) and another spanning residues 280 to 286 (NLS2). Site-directed mutagenesis showed that each signal contains at least two critical residues. The possibility of connections between NLSs and adjacent phosphorylations has attracted much attention. Cytoplasmic LT (CyT) mutants were underphosphorylated, particularly at sites adjacent to NLS2. However, since a nuclear LT bearing an inactivated NLS2 was phosphorylated normally at adjacent sites, the signal was not directly required for phosphorylation. Conversely, LT could be translocated to the nucleus via NLS2 even when the adjacent phosphorylation sites were deleted. CyT was examined to probe the importance of LT localization. CyT was unable to perform LT functions related to interactions with retinoblastoma susceptibility gene (pRb) family members. Hence, CyT was unable to immortalize primary cells or to transactivate an E2F-responsive promoter. Consistent with these findings, CyT, though capable of binding pRb in vitro, did not cause relocalization of pRb in cells. Assays of transactivation of the simian virus 40 late promoter and of the human c-*fos* promoter showed that defects of CyT were not limited to functions dependent on pRb interactions.

Polyomavirus large T antigen (PyLT) is a nuclear protein with a variety of biological activities connected to virus growth and transformation. Many of these can be directly connected to effects on DNA. LT is important for integration (13) and excision (5) of the viral genome during transformation. It can promote recombination (63). It is responsible for initiation of viral DNA synthesis (17) and can stimulate host DNA synthesis as well (19, 45, 59). LT transactivates cellular promoters (27, 28, 42, 46) and can affect cellular growth control. It immortalizes primary cells (51) and blocks myoblast differentiation (38). Both of these functions depend upon the ability of LT to associate with the antioncogene pRb or related proteins (15, 18, 21, 33, 39). The pRb interaction is also important for some transactivation pathways (42, 46).

LT is organized into separable domains. The N-terminal domain (NT) comprises approximately the first 259 residues. NT contains the pRb-binding sequence and has immortalization activity (21). The C-terminal domain (CT; residues 264 to 785) contains the DNA binding domain, zinc finger, and ATP-binding elements. In growing cells, CT is sufficient for viral DNA replication (19). In resting cells, CT requires complementation by NT or other gene products that promote S phase.

PyLT is localized to the nucleus by two nuclear localization signals (NLSs) (Fig. 1). PyLT NLS1 spans residues VSRK-192RPR, and NLS2 spans residues PKK-282ARED (52). Either one of these signals, in the absence of the other, is sufficient to localize LT to the nucleus. When both are deleted, the molecule is cytoplasmic (52). Their positions are such that NT and CT each contain a signal. As expected, each domain is localized in the nucleus when expressed individually (19). PyLT is homologous to simian virus 40 (SV40) LT (62). This homology includes the SV40 LT NLS, PKK-128KRKV, which is similar to PyLT NLS2. The PyLT NLS1 signal and surrounding sequences are not found in SV40 LT.

Phosphorylation at sites adjacent to an NLS have been suggested to modulate transport (1, 12, 23, 25, 35, 41, 50, 53, 54). This is of interest for at least two reasons. First, PyLT is phosphorylated immediately upstream of both NLSs (6) (Fig. 1). Second, there is a connection between phosphorylation and activity. The phosphorylations are induced by LT itself (7) and, like SV40 LT (40), are important for DNA replication (70). Furthermore, these phosphorylations have been connected to the oligomerization of LT (55).

The purposes of this study were severalfold. Site-directed mutagenesis was used to identify critical residues in each NLS. The connection between NLS2 and the adjacent phosphorylation sites was also examined. Finally, the function of cytoplasmic PyLT (CyT) was studied. CyT was found to be deficient in activities connected to interactions with pRb family members. It failed to transactivate an E2F-responsive promoter and could not immortalize primary cells. Both results could be explained by the inability of CyT to associate with pRb in vivo. CyT was also deficient in pRb-independent transactivation, suggesting that nuclear localization was required for non-pRb-related functions as well. Differences between these data and those obtained previously for SV40 LT are discussed.

MATERIALS AND METHODS

Plasmids and mutagenesis. pPRB (19, 21), which contains LT cDNA under control of the Harvey long terminal repeat in pIBI24, was used to express LTs in cells. The Δ2208 deletion of residues 191 to 209 was generated previously (43). The 1401 mutation was made with a thionucleotide-enriched site-directed mutagenesis kit from Amersham with the oligonucleotide 5′-CCACCTAAGGAG GCTAGG-3′, which mutated Lys-282 to Glu, and with PR3, a single-stranded template consisting of a *Pst*I-*Eco*RI LT cDNA fragment cloned into M13-mp8. The 1401 mutation was then transferred to pLTR880 (55) as an *Ava*I-*Eco*RI fragment. The Δ2208 and 1401 mutations were reconstructed in pPRB as *Esp*I-

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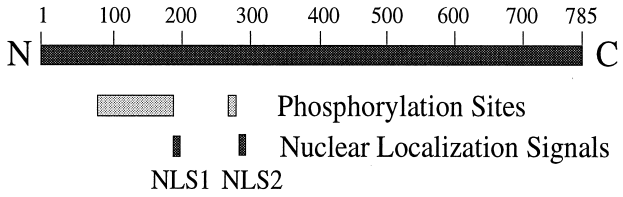

FIG. 1. Schematic diagram of PyLT antigen.

*Nco*I and *Nco*I-*Eco*RI fragments, respectively. The LT construct containing both mutations, termed CyT, was previously derived from a pLTR880 parent vector.

Single-stranded DNA was produced from pPRB/1401 or pPRB/Δ2208 parent vectors by growth in XL1-Blue cells (Stratagene) in the presence of helper phage M13KO7 (56). Degenerate oligonucleotides were used to introduce conservative substitutions into each residue of the NLSs (see Results) with the Amersham kit. All mutant DNAs were verified by the Sequenase version 2.0 (United States Biochemicals) Sanger-dideoxynucleotide method (56). Transformation, sequencing, and DNA preparation were carried out by standard techniques (56).

LT and CyT cDNAs were also reconstructed into expression vectors bearing the human cytomegalovirus immediate early promoter. A full-length LT coding sequence was amplified from pLBE$^-$ (55) by PCR. LT4, 5′-GCGCGCGCTAG CTGATCATGGATAGAGTTCTGAGCAGAG-3′, was complementary to the noncoding strand at the LT start codon (LT nucleotide 1 is pLBE$^-$ nucleotide 3767) and was used as the upstream primer. LTC.Bcl, 5′-GCGCGCTGATCA CGGGGGACCCTGATATGACGCGC-3′, complementary to the coding strand beginning at nucleotide 6207 in the pLBE$^-$ sequence, was used as the downstream primer. The LT coding sequence spans nucleotides 3767 to 6129 in pLBE$^-$, so the PCR product included a portion of the 3′ untranslated region. PCR (4) was carried out with Vent polymerase in a Perkin-Elmer thermal cycler for 25 cycles of 1 min at 96°C, 1 min at 55°C, and 7 min at 72°C. After *Bcl*I digestion, the fragment was ligated into the *Bam*HI site of the pCMV parent vector to obtain pCMV-LT. pLTR-CyT was digested with *Esp*I and *Eco*RI, and the 482-bp fragment containing the Δ2208 and 1401 mutations was recovered. This fragment was ligated into a pCMV-LT backbone generated by an *Eco*RI-*Esp*I digest to give pCMV-CyT. pCMV-LT.Rb$^-$ was produced by the subcloning of a PCR fragment containing mutations of Leu-142 to Val and Glu-146 to Gln, which abolish pRb binding (39), into the *Bam*HI site of the parent vector (20a).

The pA10-E2F-CAT plasmid (kindly provided by A. Yee) consisted of the −85-to-−30 adenovirus E2 promoter sequence and a minimal SV40 promoter fused to a chloramphenicol acetyltransferase (CAT) gene (37). The pLS16n-CAT plasmid containing a basal SV40 late promoter lacking a 2-kb *Hin*dIII fragment downstream of the transcriptional start site, the bacterial CAT gene, and a nonreplicative SV40 viral origin was a gift from J. Alwine (9). The p*fos*-CAT plasmid containing the human c-*fos* promoter was a gift from W.-B. Wang (71).

Cell lines and transfections. LT-expressing cell lines were derived from NIH 3T3 cells grown in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% calf serum (Hyclone). The myeloma cell line NS1 and C2-7, an undifferentiated muscle cell line, were maintained in DMEM supplemented with 20% fetal calf serum (Gibco).

Stable NLS mutant cell lines were derived by HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid)-buffered saline $CaPO_4$ coprecipitation of double-CsCl-purified plasmid DNA and pRSVneo, a plasmid carrying a neomycin resistance gene, and subsequent selection in DMEM plus 10% calf serum and 800 μg of G418 (Sigma) per ml. Colonies were screened for the expression of LT variants by indirect immunofluorescence. All LT-expressing cell lines were maintained in DMEM plus 10% calf serum and 200 μg of G418 per ml.

For transient expression, BES-buffered saline $CaPO_4$ transfection (4, 56) of NIH 3T3 cells was employed. Cells were utilized 48 h posttransfection.

Immunofluorescence. Glass coverslips with 70 to 80% confluent, LT-expressing cells were washed with phosphate-buffered saline (PBS) and fixed in 3.7% (vol/vol) formaldehyde (Sigma) in PBS for 20 min at 37°C. Cells were washed twice in PBS and permeabilized directly by treatment with 1:1 acetone-water for 1 min, acetone for 5 min, and 1:1 acetone-water again for 1 min. Permeabilization was carried out on ice with 4°C acetone solutions. Treated coverslips were washed twice with PBS and incubated with blocking solution (100 mM Tris-Cl [pH 7.5], 0.01% [vol/vol] Triton X-100, 1% [wt/vol] bovine serum albumin, 0.2% [wt/vol] milk powder, in PBS) in a humidity chamber at 37°C for 10 min. Coverslips were then washed in PBS and incubated with a 1:100 dilution of polyclonal anti-T serum at 37°C in the humidity chamber for 30 min. Coverslips were washed again and incubated with a 1:50 dilution of anti-rabbit fluorescein isothiocyanate-conjugated antibody (Kirkegaard & Perry Laboratories) at 37°C in the humidity chamber for 30 min. Immunofluorescence was detected with a Zeiss microscope and a Neofluor lens. Photographs were taken on a Nikon microscope with an oil immersion lens.

Immortalization assays. Primary rat embryo fibroblasts were prepared (2) and transfected via HEPES-buffered saline-mediated $CaPO_4$ precipitation (56) with 6 μg of pPRB, pLTR880-CyT, or pPRB with a stop codon at position 1 for a negative control and 2 μg of pRSVneo. Cells were then passaged and grown in selective medium, and the number of surviving immortal cell lines was determined as previously described (21).

CAT assays. NIH 3T3 cells were transfected at a 15 to 25% confluence with 2 μg of pCMV-based LT plasmids and 2 μg of pA10-E2F-CAT, p*fos*-CAT, or pLS16n-CAT reporter plasmid by BES-buffered saline-mediated $CaPO_4$ precipitation in a total volume of 1.5 ml. One milliliter of each precipitate was added to a 100-mm-diameter dish for CAT assay; the remainder was added to a 60-mm-diameter dish for quantification of protein. Cells were harvested 48 h posttransfection.

CAT activity was measured by standard chromatographic techniques (4). Thin-layer chromatography plates were quantitated with ImageQuant software (Molecular Dynamics) to determine the percentage of total ^{14}C in acetylated forms of chloramphenicol versus that in all forms. This was expressed as fold induction over the percent conversion obtained for a pCMV-negative control.

The 60-mm-diameter dishes were washed twice and scraped into 2 ml of PBS$^+$. Cells were pelleted and boiled for 10 min in 100 μl of dissociation buffer (62.5 mM Tris-Cl [pH 6.8], 5% [wt/vol] sodium dodecyl sulfate [SDS], 25% [vol/vol] glycerol, 0.0075% [wt/vol] bromophenol blue, 50 μl of β-mercaptoethanol per ml) and subjected to SDS-polyacrylamide gel electrophoresis (PAGE) (29) on a 6% acrylamide gel. Protein was detected by enhanced chemiluminescence-Western blot (immunoblot) analysis.

LT extraction and phosphorylation analysis. Labeling, extraction, and immunoprecipitation of LT have been previously described (6). Briefly, cell lines were labeled metabolically with 200 μCi to 1 mCi of $^{32}P_i$ (New England Nuclear) per ml. LT was extracted in T extraction buffer (137 mM NaCl, 10 mM Tris-Cl [pH 9.0], 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10% [vol/vol] glycerol, 1% [vol/vol] Nonidet P-40) supplemented with 20 mM NaF for 20 min at 4°C. Cleared extracts were incubated with polyclonal anti-T and protein A-Sepharose (Pharmacia) for 1 h. Washing and reimmunoprecipitation were carried out as described previously (10).

Phosphorylation analysis of LT variants was carried out by SDS-PAGE followed by blotting onto nitrocellulose and Western analysis (68). The blot was then blocked in Tris-buffered saline-Tween (50 mM Tris-Cl [pH 7.5], 0.15 M NaCl, 0.05% [vol/vol] Tween 20) containing 5% (wt/vol) dried milk (Carnation) for 1 h at room temperature. The blot was incubated with 1:50 PN-116 monoclonal anti-LT antibody and then with 1:5,000 horseradish peroxidase-conjugated anti-mouse antibody (Amersham). Protein was detected by enhanced chemiluminescence (Amersham) and compared with phosphate incorporation as measured by PhosphorImager detection and with ImageQuant software (Molecular Dynamics).

The procedure for one- and two-dimensional phosphate analysis of LT has been detailed previously (6). For one-dimensional analysis, samples were electrophoresed on a 7.5% acrylamide cylindrical gel which was then placed on a 12.5% acrylamide gel, overlaid with 2 ml of enzymatic digestion solution (125 mM Tris-Cl [pH 6.8], 10 mM EDTA, 20% [vol/vol] glycerol, 0.015% [wt/vol] bromophenol blue, 100 μg of *Staphylococcus aureus* V8 protease [United States Biochemicals] per ml) and electrophoresed at 50 V. For two-dimensional analysis, samples were incubated with 2 M hydroxylamine (HA; Sigma) in 200 mM K_2CO_3 (pH 9.0) for 1 h at 45°C prior to electrophoresis in the cylindrical gel. The resolved HA fragments were electrophoresed in the presence of V8 protease.

For differential extraction of LT from the nucleus and cytoplasm, 100-mm-diameter dishes of 80 to 90% confluent cells were washed twice in PBS$^+$ and extracted in 800 μl of modified Penman cytosol extraction buffer {10 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); pH 6.8], 10 mM KCl, 300 mM sucrose, 2.5 mM $MgCl_2$, 100 U of aprotinin per ml, 1 μg of leupeptin per ml, 1% [vol/vol] Nonidet P-40} (48) for 60 s at 4°C. The cytosolic extract was recovered by aspiration, and the remaining material was scraped into 2 ml of PBS$^+$ and pelleted for 2 min at 2,000 rpm in a Beckman GPR centrifuge. The recovered pellet, which was predominantly nuclei and insoluble cellular material, was boiled in 200 μl of Sol buffer (10), and LT protein was renatured and immunoprecipitated. LT protein was directly immunoprecipitated from the cytosolic extract.

Analysis of pRb and LT in cytosolic versus nuclear extracts was done with a modified differential extraction protocol. Ten unlabeled 80 to 90% confluent 100-mm-diameter dishes of LT- or CyT-expressing cells were used. The Penman cytosol extraction buffer extraction time was increased to 90 s, and the cytosolic extract was recovered as described above and pooled. After the remaining nuclear material was scraped into PBS$^+$ and pelleted, it was extracted in T extraction buffer for 20 min on ice. Extracts were pooled and pelleted in a refrigerated microcentrifuge for 5 min; the supernatant was retained as the soluble nuclear extract. The soluble extracts were boiled for 2 min after the addition of dissociation buffer. The inextractable material in the pellet was boiled in dissociation buffer for 10 min. Samples were electrophoresed on an SDS–6% acrylamide gel and blotted onto nitrocellulose. LT detection by Western analysis was performed with a 1:50 dilution of PN-116. pRb was detected with a 1:10 dilution of a monoclonal anti-human pRb (245; PharMingen) and visualized by enhanced chemiluminescence.

Sucrose gradient analysis. Extracts of LT cell lines were analyzed on 5 to 20% sucrose gradients as previously described (55). Samples were boiled in 100 μl of dissociation buffer for 2 min, and 150-μl aliquots were run on 7.5% acrylamide

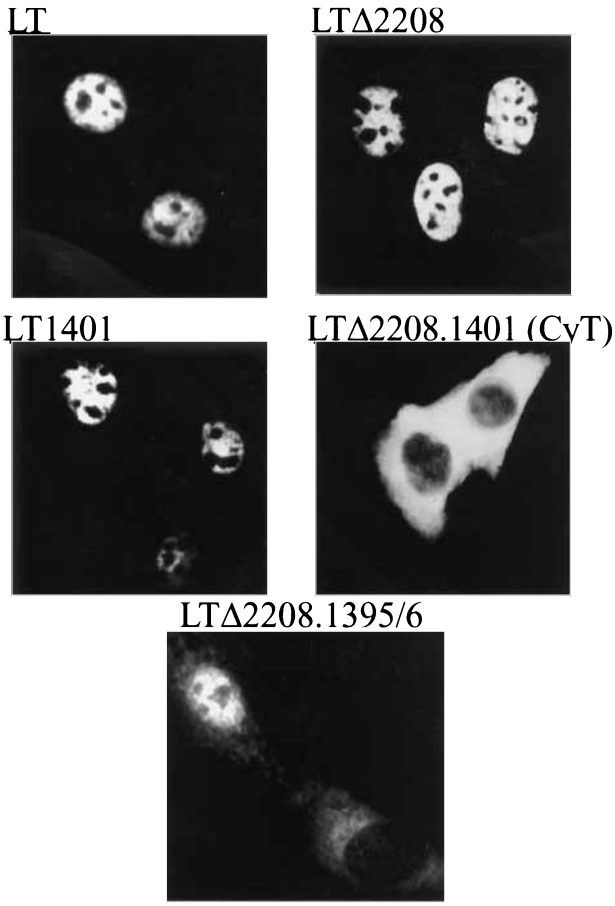

FIG. 2. Immunofluorescence detection of LT. NIH 3T3 cells expressing wild-type LT, LTΔ2208, LT1401, LTΔ2208.1401 (CyT), and LTΔ2208.1395/6 (Val-280) were stained by indirect immunofluorescence as described in Materials and Methods.

gels. After blotting to nitrocellulose, the protein was then visualized with anti-T serum by enhanced chemiluminescence.

RESULTS

Identification of critical residues in each PyLT NLS. Site-directed mutagenesis was employed to generate mutants within the NLS sequences of LT. Since it was necessary to examine mutants of each NLS in a background where the other signal was nonfunctional, two parent vectors were generated for the mutagenesis. The deletion Δ2208 (43), which removes residues 191 to 209, was constructed into pPRB to use for mutagenesis of the downstream signal. To test the upstream NLS, the mutant 1401 changing Lys-282 to Glu was constructed. Lys-282 of NLS2 was chosen as a target because it is homologous to the critical Lys-128 of the SV40 LT NLS. These mutants, as well as a construct bearing both mutations, pLTR880Δ2208.1401 (CyT), were stably expressed in NIH 3T3 cells. Figure 2 shows the localization of these molecules as determined by indirect immunofluorescence. Neither the deletion of NLS1 in Δ2208 nor the mutation of NLS2 in 1401 by itself affected the nuclear localization of LT. The double-NLS mutant, CyT, however was cytoplasmic. Each mutation therefore inactivated one NLS, providing a suitable negative background for mutagenesis of the other NLS. Site-directed mutagenesis was then performed on each parent with degenerate oligonucleotides. For NLS1, the single-stranded template pPRB.1401 was used with three oligonucleotides: (i) 1123, 5′-AGAACTCTCCTGT/CAT/GCCAGAAAGCG-3′, with the mutations Val-189 to Ala and Ser-190 to Ala; (ii) 1129, 5′-CTGTATCCAG/CAAAG/CCG/AACCAAGACC-3′, with the mutations Arg-191 to Thr, Lys-192 to Asn, and Arg-193 to Gln; and (iii) 1137, 5′-AGAAAGCGAC/AC/TAAG/CACCA GCTGG-3′, with the mutations Pro-194 to Ile, Thr, or Leu and Arg-195 to Thr. For NLS2, pPRBΔ2208 was used as a template with two oligonucleotides: (i) 1395, 5′-ACGCCAC/GC/TAA GAAGG/TCTAGGG/CAGGACC-3′, with the mutations Pro-280 to Val, Ala, or Leu, Ala-283 to Val, and Glu-285 to Gln and (ii) 1400, 5′-CGCCACCTAAG/CAAG/CGCTAG/CGGA GGACCC-3′, with the mutations Lys-281 to Asn, Lys-282 to Asn, and Arg-284 to Thr. After confirmation of the mutations by DNA sequencing, stable cell lines were derived by cotransfection of mutant DNA with pRSVneo and then by G418 selection. Subcellular localization of mutant LT in the cell lines was determined by indirect immunofluorescence with polyclonal anti-T serum. Similar results were obtained with transiently expressed mutants assayed 48 h posttransfection (data not shown).

The mutants and their localizations are shown in Table 1. The mutations of two basic residues in NLS1, Lys-192 and Arg-193, rendered the molecule cytoplasmic in a 1401 background. The mutation of another basic residue, Arg-193, had no effect on localization. For NLS2, the mutation of Lys-282 to an asparagine as well as the original mutation to a glutamate inactivated the signal. This is similar to the result obtained with Lys-128 of SV40 LT (11, 31). However, the mutations of Lys-281 and Arg-284 in NLS2 also gave a cytoplasmic phenotype in a Δ2208 background. The mutation of Pro-280 to Val pro-

TABLE 1. Localizations of mutations in PyLT NLSs

Mutant	Mutation in NLS1[a] at position:							Localization[b]	Mutant	Mutation in NLS2[c] at position:							Localization
	Val-189	Ser-190	Arg-191	Lys-192	Arg-193	Pro-194	Arg-195			Pro-280	Lys-281	Lys-282	Ala-283	Arg-284	Glu-285	Asp-286	
1123	Ala							N	1395/6	Val							N and C
1123/5	Ala	Ala						N	1400		Asn						C
1125		Ala						N	1401			Glu					C
1129			Thr					N	1403			Asn					C
1133				Asn				C	1404				Val				N
1135					Gln			C	1408					Thr			C
1137						Thr		N	1404/10				Val		Gln		N
									8N142							Asn	N

[a] All NLS1 mutants were derived in pPRB.1401. Data represent differences from the wild-type sequence.
[b] Localization was determined by indirect immunofluorescence in NIH 3T3 cells. N, nucleus; C, cytoplasm.
[c] All NLS2 mutants were derived in pPRB.Δ2208.

TABLE 2. Immortalization assay

Construct[a]	No. of surviving colonies/ no. of colonies passed[b]	Immortalization efficiency (%)[c]
LTR-LT	11/70	16
	14/83	17
LTR-CyT	0/82	0
	0/54	0
LTR-control	0/86	0
	0/42	0

[a] LTR, long terminal repeat.
[b] The number of surviving colonies are represented as lines surviving all passages over colonies passed into 96-well plates. The results of two experiments are shown and indicate that CyT does not immortalize rat embryo fibroblast cells.
[c] Surviving colony data expressed as a ratio.

duced a surprising result. The mutant was seen in the nucleus, the cytoplasm, or in both compartments of the cell (Table 1 and Fig. 2).

Cytoplasmic LT fails in pRb-binding-dependent immortalization. The next experiments tested the properties of cytoplasmic PyLT. Properties such as initiation of viral DNA replication would be expected to depend on nuclear localization. As expected, CyT did not support viral DNA replication (not shown). For other activities, expectations were less clear. Cytoplasmic SV40 LT, for example, could transform NIH 3T3 cells (65).

Immortalization of primary embryo fibroblasts can be achieved with the N-terminal domain (21, 51) in a manner dependent on binding pRb family members (18, 33). Table 2 shows the results of two immortalization experiments in which CyT was unable to immortalize primary rat embryo fibroblast cells. The result did not arise from a lower expression level, since Western blots showed similar amounts of protein 48 h after transfection (not shown).

Cytoplasmic LT is defective not only in pRb-binding-dependent transactivation but also in pRb-independent transactivation. Since immortalization by LT is dependent on interaction with pRb (18, 33), the behavior of CyT in other pRb-dependent processes was examined. Transactivation of E2F-responsive promoters is mediated by LT's interaction with pRb (42, 46). To investigate transactivation, wild-type and CyT cDNAs were reconstructed into a vector carrying the cytomegalovirus immediate early promoter. These constructs were then assayed for transactivation in CAT assays. pA10-E2F-CAT, containing an SV40 early minimal promoter and two E2F sites from the adenovirus E2 enhancer, was used as the target. Wild-type LT increased CAT activity three- to fourfold. pCMV-LT.Rb$^-$ bears mutations that inactivate the pRb-p107 binding function of LT (39); as expected, this construct did not transactivate pA10-E2F-CAT. CyT also did not transactivate (Fig. 3) even though CyT and LT were comparably expressed (data not shown).

Interestingly, CyT was also unable to transactivate promoters in experiments in which wild-type function did not depend on pRb binding. Activation of the promoter of c-*fos* (Fig. 3) is associated with LT's stimulation of the cell cycle (44). The role of pRb-p107 binding in this transactivation has not been previously demonstrated for LT. Figure 3 shows that pCMV-LT.Rb$^-$ can transactivate p*fos*-CAT as efficiently as the wild type. This shows that LT transactivation of c-*fos* is independent of pRb-p107 binding but dependent on localization. Similarly, transactivation of the SV40 late promoter was independent of pRb binding (34). CyT is deficient in this transactivation as well (Fig. 3).

CyT fails to relocalize pRb in cells. The failure of CyT in activities mediated by pRb family interactions was initially surprising since CyT has an intact pRb binding site. In fact, pRb could be coprecipitated with CyT from whole-cell Nonidet P-40 extracts of CyT cell lines with anti-LT serum (data not shown). Since LT produced in baculovirus-infected SF9 cells could also be used to precipitate 3T3 pRb under the same conditions, such interactions probably occurred after extraction. To try to get around this problem, a modification of the differential extraction procedure developed by Penman and colleagues (48) that required only a 60-s extraction was used. Extracts were directly subjected to SDS-PAGE, and LT and pRb were detected by Western analysis with monoclonal antibodies. The distribution of LT is shown in the top panel of Fig. 4, and the distribution of pRb in the same cells is shown in the bottom panel. As expected, much of the extractable wild-type LT was found in the soluble nuclear fractions (Fig. 4, top panel, LT lanes labeled Nuc Sol). CyT was not detectable in the soluble nuclear fractions but was abundant in the cytoplasm (Fig. 4, top panel, CyT lanes labeled Cyto and Nuc Sol). However, pRb was found only in the soluble nuclear fraction of both wild-type LT and CyT cells (Fig. 4, bottom panel, lanes labeled Nuc Sol). Therefore, in CyT-expressing cells, pRb was able to localize to the nucleus normally despite the presence of a high concentration of CyT in the cytoplasm (Fig. 4, bottom panel, compare CyT lanes labeled Cyto and Nuc Sol with similarly labeled CyT lanes in the top panel).

Nuclear localization, but not the NLS signals themselves, is important for LT phosphorylation. Considerable attention has been given to possible connections between phosphorylation and the NLS for SV40 LT (23, 24, 53, 54). However, PyLT, which has two signals, allows the separation of localization from the function of a particular NLS. This separation permits direct analysis of the function of a signal and the phosphorylation adjacent to it. The patterns of LT phosphorylation are intrinsically interesting because they can be a reflection of, and important to, LT activity (7, 70).

The level of phosphorylation of CyT mutants was compared with that of the wild type to determine whether phosphorylation was dependent on localization. Figure 5 shows that cyto-

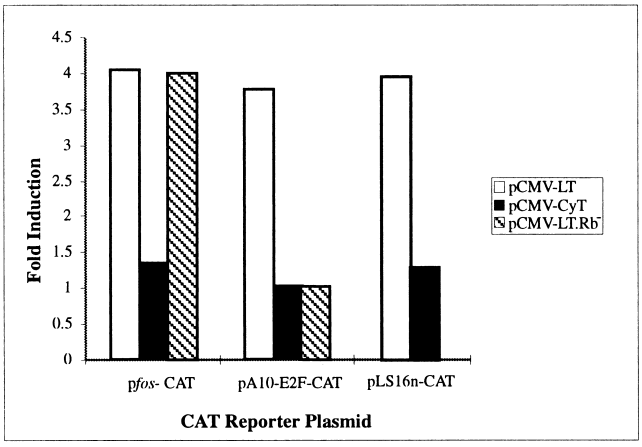

FIG. 3. CAT assays. The indicated pCMV-LT, pCMV-LT.Rb$^-$, and pCMV-CyT expression vectors were cotransfected with either an E2F-responsive promoter (pA10-E2F-CAT), a human c-*fos* promoter (p*fos*-CAT), or SV40 late promoter (pLS16n-CAT) reporter plasmid, and CAT activities were assayed as described in Materials and Methods. Data represent the fold inductions of CAT activities over that of a pCMV-negative control. Each value was the average of two experiments, and each experiment used two separate transfections for each LT-pCMV construct.

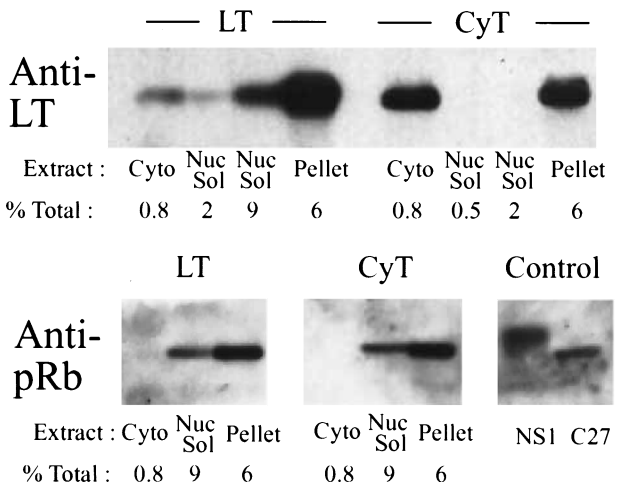

FIG. 4. Differential extraction of LT and pRb from wild-type LT and CyT cell lines. Wild-type LT and CyT cell lines were differentially extracted as described in Materials and Methods. Cytoplasmic (Cyto) and soluble nuclear (Nuc Sol) extracts and the remaining pellet were loaded in the indicated lanes. % Total refers to the percentage, by volume, of the appropriate extract that was loaded onto the gel. The top panel was blotted and probed with PN-116 to detect LT, and the bottom panel was blotted and probed with anti-human pRb to detect pRb. NS1 is a whole-cell extract of a myeloma cell line which served as a marker for phosphorylated pRb. C2-7 is an extract from an undifferentiated myoblast line and provided a marker for unphosphorylated pRb. The images in this figure and in Fig. 5 to 7 were produced with Adobe Photoshop and Aldus Freehand.

plasmic mutants are less phosphorylated than nuclear LT. The decrease in specific activity is on the order of 10-fold. In previous mappings, a distinction has frequently been noted between downstream sites at the boundary of the two domains and those upstream within NT. These differences can be resolved with HA, which cleaves at residue 210, and then by V8 digestion. Figure 6 shows HA-V8 protease maps of LT from cell lines expressing the wild-type LT and LTΔ2208, LT1401, and cytoplasmic LTΔ2208.1400, variants of LT. Clearly the effects on the downstream sites are much greater for CyT. That it is localization that is critical to the phosphorylation was supported by analysis of a Δ2208.1395/6 cell line, the LT mutant cell line with mixed localization. Phosphopeptides 5 and 7 were shown to be absent in the cytoplasmic fraction but present in the nuclear fraction of Δ2208.1395/6 (not shown). This data supported the observations made with the CyT variants and strongly suggested that phosphorylation adjacent to NLS2 at phosphopeptides 5 and 7 was correlated with the presence of LT in the nuclear compartment.

The Lys-282-to-Glu mutation in LT1401 inactivates NLS2, yet phosphopeptides 5 and 7 are present in amounts similar to those of the wild type (Fig. 6). This result indicates that mutation of NLS2 does not directly affect phosphorylation at adjacent positions.

LT mutants that cannot oligomerize properly are not phosphorylated at sites adjacent to NLS2 (55). *ts*-a mutants also show similar behavior at the nonpermissive temperature (7). The underphosphorylated phenotype of CyT could result from an inability to self-associate. To examine this, extracts of LT cell lines were analyzed by sucrose gradient ultracentrifugation. Gradient fractions were analyzed by Western blotting. Figure 7 shows the results for wild-type LT, the cytoplasmic mutant LTΔ2208.1401, and the mutant LT-452, which bears a mutation in a zinc finger motif and is oligomerization negative. Each blot showed a peak corresponding to monomeric LT running towards the bottom of the gradient and a broader peak corresponding to the multimeric forms. LT could therefore oligomerize in the cytoplasm, so the absence of the phosphorylation of phosphopeptides 5 and 7 did not result from failure to oligomerize.

DISCUSSION

Given the homology between SV40 and PyLT seen both in functions and in sequences, it is useful to compare issues of nuclear localization for the two proteins. While there are many similarities, there are also important differences.

Basic residues appear critical in each NLS of PyLT. This is consistent with a substantial body of data on other proteins (8). Both polyomavirus NLSs appeared more sensitive to perturbation than the NLS of SV40 LT. In SV40, only mutation at one position, Lys-128, can abolish NLS activity (11, 26, 31, 61). In contrast, no residue in either NLS was uniquely critical for PyLT nuclear localization. Mutations at residue Lys-192 or Arg-193 of NLS1 as well as at either Lys-281, Lys-282, or Arg-284 of NLS2 inactivated the signal. Mutations analogous to those of Lys-281 or Arg-284 do not abolish nuclear localization in SV40 LT. Since NLS2 is homologous to the SV40 NLS, this similarity might not have been expected. However, this region of LT is important for functions other than that of nuclear localization. Residue 286, for example, is critical in origin recognition (64). The mutation of Pro-280 to Val, however, caused a mixed phenotype. Unfortunately, efforts to de-

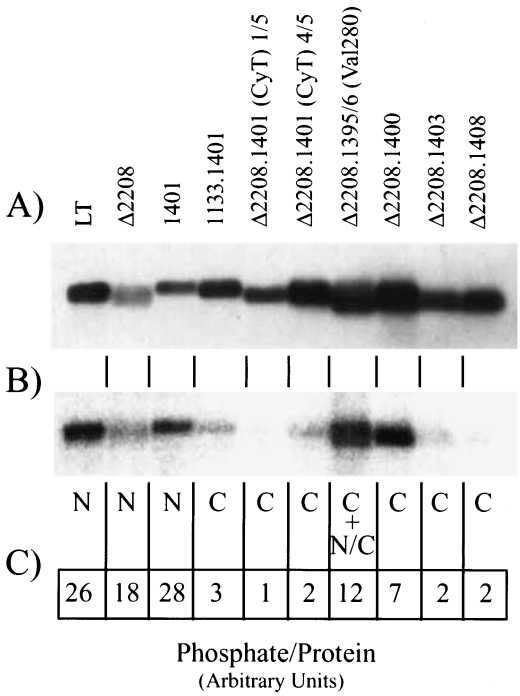

FIG. 5. Phosphorylation of LT NLS mutants. NIH 3T3 cell lines expressing the indicated LT variant were metabolically labeled with ^{32}P-O_4 and immunoprecipitated as described in Materials and Methods. (A) Western blot showing the amount of protein expressed in each cell line. (B) PhosphorImager scan of the Western blot shown in panel A. Band intensity is indicative of ^{32}P incorporation into the LT variants. Localization of the variants is shown as N for nuclear, C for cytoplasmic, and C+N/C for cytoplasmic and nuclear plus cytoplasmic in different cells. (C) Data labeled Phosphate/Protein are arbitrary ratios expressing the ^{32}P counts obtained from the bands in panel B with ImageQuant software divided by the integrated optical density of the bands in panel A obtained with a Millipore optical scanner and software with a Sun systems SPARC station 2. The Δ2208.1401 (CyT) samples labeled 1/5 and 4/5 indicate the fractions of the CyT sample loaded in the respective lanes.

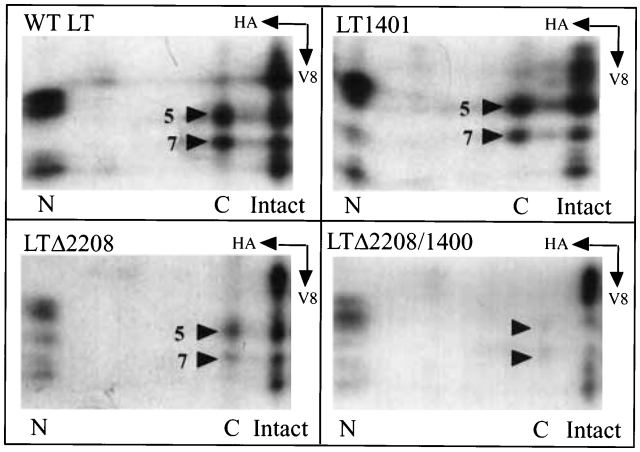

FIG. 6. Two-dimensional phosphopeptide mapping of LT NLS mutants. ^{32}P-O_4-labeled immunoprecipitates of LT cell lines were treated with HA and electrophoresed on 7.5% acrylamide cylinders in the direction indicated by the HA arrows. Cylinders were then placed horizontally on top of 12.5% acrylamide slab gels and electrophoresed in the presence of 100 μg of V8 protease. N and C denote the positions of the amino- and carboxy-terminal LT fragments generated by the HA cleavage. Intact denotes LT that was not cut by HA. The positions of V8 phosphopeptides 5 and 7 are indicated by 5 and 7. The absence of these phosphopeptides is indicated with unlabeled arrowheads.

termine the basis for this phenomenon were not successful. Synchronization experiments, for example, did not reveal a dependence of this localization on the cell cycle (21a).

Cytoplasmic mutants were generally underphosphorylated. This has also been seen for SV40 mutants (58). The loss of phosphate at sites adjacent to NLS2 was particularly striking. However, isoelectric focusing of N-terminal fragments confirmed decreased phosphorylation upstream of NLS1 as well (21a). Although a previous study noted that defects in oligomerization were associated with underphosphorylation (55), this phenotype did not result from failure of CyT to self-associate. A likely explanation is that the kinases responsible are compartmentalized in the nucleus. Consistent with this explanation, mutants that lack NLS2 but that are nuclear because of the presence of NLS1 show normal levels of phosphorylation. Another possibility that could be considered is that CyT fails to induce the appropriate kinases. However, a previous study showed that only in resting cells did LT need to induce kinase activities; in growing cells the activities appeared to be constitutive (7).

Previous work has shown that functional correlations can exist between an NLS and its adjacent phosphorylation cluster (23, 25, 53). LT1401 bearing only an inactivating mutation in NLS2 is nuclear because of NLS1. It is also fully phosphorylated in the V8 peptides coming from sequences adjacent to NLS2. This shows that a functional signal is not important per se to the adjacent modification. Neither is phosphorylation at the sites adjacent to NLS2 required for nuclear localization of LT. LT97 lacks the adjacent phosphorylations because of a deletion of residues 271 to 280 (2, 3). Nonetheless, in the absence of NLS1, this construct is predominantly nuclear (21b). Some cells showed cytoplasmic staining, so it remains possible that phosphorylation could have a modulatory effect.

A number of LT functions have been associated with the ability to bind pRb family members. PyLT mutants incapable of binding pRb are deficient in immortalization (33) and fail to block differentiation of myoblasts (39). Transactivation of the thymidine kinase promoter by PyLT has been shown to require intact E2F sites in the promoter, as well as an intact pRb binding site in LT (46). The pRb binding LT mutant was also unable to induce three E2F-responsive gene products, thymidine kinase, dihydrofolate reductase, and DNA polymerase α, in growth-arrested cells (42).

CyT is defective in immortalization and transactivation requiring association with members of the pRb family. This is true even though CyT has an intact pRb binding site and can associate with pRb in vitro. In vivo the two products do not seem to be associated, since CyT and pRb existed in different cellular compartments. It is possible that the kinetics of pRb localization to the nucleus are sufficiently rapid to preclude its interaction with CyT in the cytoplasm. Alternately, it might be imagined that another cytoplasmic protein interacts with either CyT or pRb to preclude their interaction with each other. At least for CyT, ^{35}S immunoprecipitates failed to show such a protein (not shown).

The dependence of immortalization and transactivation activities of PyLT on nuclear compartmentalization stands in contrast to the results for SV40 LT (69, 72). When cytoplasmic, SV40 LT retains its ability to immortalize primary cells (65, 69), initiate transformation (16, 32, 65), and transactivate E2F-responsive promoters (73). However, the extent to which these capabilities are connected to pRb binding is unclear. For example, SV40 LT K1, which is deficient in pRb binding, is still capable of transactivating E2F-responsive promoters (36). It is possible that cytoplasmic SV40 large T could affect pRb function. Another possibility is a form of leakiness. SV40 LT associates with p53 through sequences in the C terminus of the molecule (30). p53 can be localized to the nucleus by virtue of three NLSs (60). A small amount of cytoplasmic SV40 LT could associate with p53, become localized to the nucleus by "piggyback" transport, and function there. Alternatively, a p53-SV40 LT complex that formed in the cytoplasm might be a better trap for pRb family members than large T alone. PyLT has not been shown to interact with p53.

A more likely explanation for the differences is that SV40 LT possesses additional activities that PyLT does not. While PyLT requires the presence of additional oncogenic proteins for transformation, SV40 LT is capable of fully transforming cells by itself (57). p53 binding has been connected to SV40 immortalization (66, 67) and the stimulation of DNA synthesis (14, 20, 49) and is involved in repression of the human cytomega-

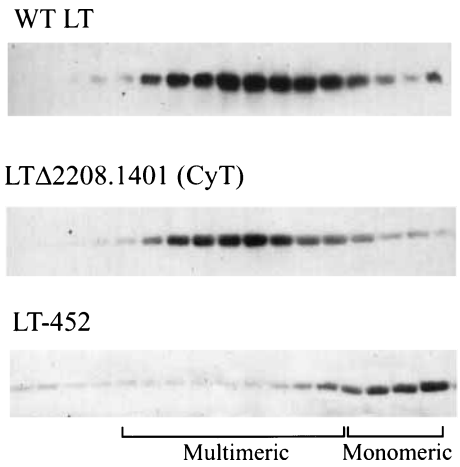

FIG. 7. Sucrose gradient analysis of LT variants. Western blots of sucrose gradients of wild-type LT, LTΔ2208.1401, and LT452 are shown. Fractions were taken from the bottom of the gradient, and the relative positions of the monomeric and oligomeric forms are indicated at the bottom of the figure.

lovirus, Rous sarcoma virus, and SV40 early promoters (22). The activity of cytoplasmic SV40 LT could be related to overlapping functions of p53 and pRb.

A final point is worth making. The defects seen with CyT extend beyond functions related simply to the binding of pRb family members. Previous work has established that the ability of PyLT to transactivate the SV40 late promoter does not depend on pRb (34). Yet CyT is defective in this activity. This suggests that there must be at least two pathways dependent on localization. LT is well known for its multifunctional nature. The recent demonstration of an additional function in NT for driving S phase is just the latest example (19). It is likely that CyT will be of further use in dissecting LT pathways.

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