JOURNAL OF VIROLOGY, May 1995, p. 2842–2849 Vol. 69, No. 5
0022-538X/95/$04.00+0

Zinc-Binding and Protein-Protein Interactions Mediated by the Polyomavirus Large T Antigen Zinc Finger

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Received 17 October 1994/Accepted 31 January 1995

Polyomavirus large tumor antigen (LT) contains a potential C_2H_2 zinc binding element between residues 452 and 472. LT also contains a third histidine in this region, conserved among the polyomavirus LTs. Synthetic peptides of this region bound a single atom of zinc, as determined by spectroscopic analysis. Blotting experiments also showed that fusion proteins containing the element, as well as full-length LT, bound ^{65}Zn. Polyomavirus middle T and small T antigens also bound zinc in the blotting assay. Site-directed mutagenesis showed the importance of this element in LT. Point mutations in four of the conserved residues (C-452, C-455, H-465, and H-469) blocked the ability of LT to function in viral DNA replication, while mutation of H-472→L decreased replication to 1/30th that of the wild type. Point mutations in intervening residues tested had little effect on replication. Mutants resulting from mutations in the conserved cysteine or histidine residues retained the ability to bind origin DNA. However, they did show a defect in self-association. Because double-hexamer formation is involved in DNA replication, this deficiency is sufficient to explain the defect in replication. Mutants created by point mutations of the coordinating residues were also deficient in replication-associated phosphorylations.

Murine polyomavirus large T antigen (LT) acts directly on the viral DNA and indirectly on the host cell. LT functions in initiation of DNA replication (16). It has properties expected for this function. LT binds 5′-GAGGC-3′ sequences in the viral origin (11, 12, 39, 45). LT has ATPase and helicase activities (19, 49, 61). It self-associates to form oligomers (60). In transformation, LT is involved in integration of viral DNA (13). The host cell effects of LT include immortalization of primary cells (40, 41) and blocking differentiation of myoblasts (32). Both of these situations are characterized by continued cellular replication. LT indeed induces cellular DNA synthesis (47). In the host cell, LT can promote DNA recombination (51).

LT has multiple domains. One boundary occurs around amino acid 260. An N-terminal domain of residues 1 to 259 is sufficient for immortalization (24). This region of LT contains the binding site for retinoblastoma gene family members (14, 24, 26). This binding is critical for both immortalization and blocking differentiation (18, 26, 32, 33). This domain also stimulates cellular DNA replication in resting 3T3 cells in a manner that does not require retinoblastoma gene family member binding (20). A C-terminal domain (residues 264 to 785) is sufficient for the replication of viral DNA in growing cells (20). The ability to bind GAGGC has been associated with amino acids 282 to 398 (53). The ATP binding site is also found in the C-terminal domain (8).

Within the C-terminal domain is a potential zinc binding element (1). Zinc fingers occur as several different types (10, 57). The element in LT is of the C_2H_2 type. All polyomavirus LTs contain a third conserved histidine, so that the element would contain cysteine residues 452 and 455 as well as two of three histidines (residues 465, 469, and 472). Although zinc fingers have often been implicated in nucleic acid binding, there are proteins that have such elements but do not bind DNA. In two such cases, protein kinase C (25) and adenovirus E1A (62), this region may be involved in protein-lipid or protein-protein contacts. Detailed analysis of simian virus 40 (SV40) LT has shown that analogous sequences are important for DNA replication and self-association, but zinc binding has not been addressed (28, 29).

This work was directed toward examining the role of the zinc finger element of polyomavirus LT. Both analysis of model peptides and protein blotting with ^{65}Zn suggest that the element binds zinc. Site-directed mutagenesis was used to show the importance of this element to viral DNA replication. Biochemical analysis showed defects in both self-association and phosphorylation.

MATERIALS AND METHODS

Cells, plasmids, and viruses. NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% calf serum. Stable cell lines were made from NIH 3T3 cells by cotransfection of pLTR880 derivatives and pRSV neo followed by selection with G418.

pLTR880 (a gift from T. Roberts, Dana-Farber Cancer Institute) expresses LT from a cDNA. The vector consists of pBR322 sequences, the Harvey sarcoma-virus long terminal repeat for transcription initiation, and an LT cDNA from pTR880 (44). pCMV-LT has been described previously (20). For generation of baculovirus expressing mutant LTs, the plasmid pBLT was constructed by ligation of the *Bam*HI-*Bcl*I fragment of the LT cDNA from pTR880 with *Bam*HI-cleaved baculovirus transfer vector pVL941 (30). *Eco*RI sites (not in LT sequences) of both pBLT and pLTR880 were deleted by filling in of partially *Eco*RI-digested DNAs and ligation of filled-in blunt ends. pUCori (20) contains the origin of replication of polyomavirus, from the *Bcl*I site at nucleotide 5024 to the *Sph*I site at nucleotide 163, cloned into the *Eco*RI site of pUC12.

For mutagenesis of LT, polyomavirus sequences from the *Eco*RI site at 1560 to the *Hin*CII site at 2962 were cloned into M13mp11, yielding the construct M13mp11 EH2. After mutagenesis, LT sequences were recovered by digestion with *Eco*RI and *Kpn*I. This fragment containing the mutation was ligated into pLTR880 cleaved with *Eco*RI and partially cleaved with *Kpn*I.

Baculoviruses were prepared by standard techniques (52). However, recombinant virus was screened for by direct immunoblotting of plaques. In some virus constructions, Baculogold viral genomic DNA (Pharmingen) was used.

Mutagenesis. Site-directed mutagenesis was carried out on polyomavirus LT

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cDNA (bp 1560 to 2962) cloned into the M13 phage. Mutagenesis was done by the methods of Eckstein et al. (54, 55). The mutagenic oligonucleotides were synthesized with mixed wild-type and mutation-coding nucleotides at multiple positions as follows, with degeneracies underlined: Cys-452→Arg, Cys-455→Arg, 5′-TTCCTTCAT/CGCATAAAGT/CGTAGCAAAG-3′; Lys-457→Asn, Glu-458→Gln, Glu-459→Gln, 5′-AAAGTGTAGCAAC/AC/GAGC/GAAACCCGCCTC-3′; His-465→Asp, Lys-467→Glu, 5′-CCTCCAAATAG/CATTGGG/AAAAACCATAG-3′; and His-469→Leu, His-472→Leu, 5′-GGAAAAACCA/TTAGAAAGCA/TTGCAGAG-3′.

Mutants which contained single, double, or triple point mutations in combinations of nine sites were obtained with four oligonucleotides. Because yields were greater than 90%, mutant screening was by DNA sequencing. A segment of the LT cDNA in pLTR880 from the *Eco*RI site at 1560 to the *Kpn*I site at 2176 was then replaced with the corresponding mutant fragment by standard techniques of DNA manipulation (43). For production of baculovirus transfer vectors, *Eco*RI 1560-to-*Bam*HI 4630 fragments were transferred from pLTR880 to pVL941-LT. For production of pCMV-LT constructs, the mutations were transferred as *Esp*I 1078-to-*Bam*HI 4630 fragments from pLTR880 to pCMV-LT.

Metabolic labeling and T antigen immunoprecipitation. In vivo labeling was carried out by using published procedures (24). Briefly, cells were rinsed with phosphate-free media for $^{32}P_i$ labeling or with Hanks' salts for [^{35}S]methionine labeling, and then labeling medium was added for 2 h. Typically, 25 to 100 μCi of [^{35}S]methionine (Dupont-NEN Express label) or 200 to 1,000 μCi of $^{32}P_i$ (Dupont-NEN) was used in a volume of 1.5 to 2 ml to label a 100-mm-diameter dish of cells. Labeled proteins were detected by fluorography (27) or autoradiography.

Immunoprecipitations were done as described previously (24). T antigens were extracted with TEB (0.137 M NaCl, 0.020 M Tris [pH 9.0], 0.00092 M $CaCl_2$, 0.00049 M $MgCl_2$, 1% [vol/vol] Nonidet P-40, 10% [vol/vol] glycerol). After 20 min at 4°C, the lysate was spun for 15 min at 10,000 × *g*. After 30 min at 4°C with mixing, the beads were washed twice with phosphate-buffered saline (PBS), twice with 0.5 M LiCl–0.1 M Tris (pH 8.0), and once with distilled water at 5 ml for each wash.

DNA binding. DNA binding assays were done as described by McKay (36), with modifications (11, 22). Briefly, 100 μl of Nonidet P-40 extracts expressing LT was incubated in 900 μl of binding buffer (0.010 M $NaPO_4$ [pH 7.0], 0.002 M dithiothreitol, 0.01% bovine serum albumin [BSA], 0.001 M EDTA, 0.05% Nonidet P-40, 3% dimethyl sulfoxide, 0.1 M NaCl, 5 μg of sonicated salmon sperm DNA per ml) with 10 to 20 ng of end-labeled *Eco*RI-*Dde*I restriction fragments from pUCori. After 1 h at 4°C, anti-T antigen serum and protein A-Sepharose were added, and incubation continued for another hour at 4°C. The immunoprecipitates were washed four times with 1 ml of wash buffer (0.020 M Tris-HCl [pH 8.0], 0.002 M dithiothreitol, 0.01% BSA, 0.5% Nonidet P-40, 0.001 M EDTA, 0.1 M NaCl, 10 μg of sonicated salmon sperm DNA per ml), and the DNA fragments were eluted with 100 μl of 100 mM ammonium chloride (pH 9.0), phenol-chloroform extracted, and ethanol precipitated before electrophoresis. For separation of origin sites from high-affinity sites A, B, and C, a triple-restriction enzyme digest (*Nar*I, *Ear*I, *Stu*I) of pUCori was used, which separated the origin palindrome as a 99-bp fragment from the other LT binding sites. This set of restriction fragments was filled in with the Klenow fragment and deoxynucleoside triphosphates and end labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase.

Replication assay. Replication assays were done as modifications of published procedures (37, 63). NIH 3T3 cells were plated at (4 to 5) × 10^5 cells per 60-mm-diameter dish. Twelve to 24 h later, the cells were transfected with a total of 10 μg of DNA per 60-mm-diameter dish by a modified $CaPO_4$ procedure (9). Equal amounts of LT vector and pUCori were cotransfected. Forty-eight to 72 h later, low-molecular-weight DNA was isolated by a modified Hirt procedure (23). After digestion with *Dpn*I and *Hinc*II, the DNA obtained from approximately 1/6th to 1/20th of a 100-mm-diameter dish was examined by Southern blotting. The DNA was separated on a 1% agarose gel in 1/2× Tris-borate-EDTA buffer, transferred to a nylon membrane by capillary blotting overnight, and probed with a ^{32}P-labeled random hexamer-primed 454-bp restriction fragment from pUCori containing the entire polyomavirus origin. The washed blots were used to expose Molecular Dynamics PhosphorImager screens. Images from the PhosphorImager screens were obtained with ImageQuant software. The images were lettered and resized with Adobe Photoshop software.

Sucrose gradients. Nonidet P-40 extracts (200 μl) from cell lines expressing LT were layered over 5-ml 5 to 20% (wt/vol) linear sucrose gradients and spun for 3 to 4 h at 53,000 rpm in a Beckman SW55 rotor. Twenty fractions of 250 μl each were collected and boiled in sodium dodecyl sulfate (SDS) dissociation buffer. Aliquots were separated by SDS-polyacrylamide gel electrophoresis (PAGE) (7.5% polyacrylamide), electroblotted to nitrocellulose, and probed with antiserum to LT with alkaline phosphatase for detection. Alternatively, the blots were probed with a monoclonal antibody against LT (PN116) (24), with a second antibody conjugated to horseradish peroxidase and developed by chemiluminescence (ECL; Amersham). The films were scanned, and the images were lettered and resized with Adobe Photoshop software.

Zinc binding experiments. (i) Peptides. The following LT zinc finger peptides were synthesized by the Tufts Protein Facility: LTZF, AKEVPS**C**IK**C**SKEETRLQI**H**WKN**H**RK**H**AEN; LTZF-465, AKEVPS**C**IK**C**SKEETRLQIDWKN**H**RK**H**AEN; LTZF-469, AKEVPS**C**IK**C**SKEETRLQI**H**WKNLRK**H**AEN; and LTZF-472, AKEVPS**C**IK**C**SKEETRLQI**H**WKN**H**RKLAEN. These peptides were purified by high-performance liquid chromatography (HPLC) on a C_{18} column for metal binding studies.

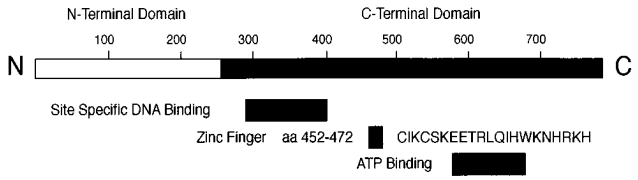

FIG. 1. Diagram of polyomavirus LT. The landmarks on LT have been previously described. The N-terminal domain (24) is represented by an open box, the C-terminal domain (20) is represented by the large solid box, and the DNA binding domain (53), ATP binding region (8), and zinc finger (1) are represented by smaller solid boxes. aa, amino acids.

(ii) GST fusions. PCR was used to construct glutathione *S*-transferase (GST) fusions containing the zinc finger region (residues 446 to 475). The oligonucleotides for PCR of the wild-type and mutant LT zinc fingers were BZF-5′ (5′-GGCTGGGATCCCTGCCAAAGAGGTT-3′) and EZF-3′ (5′-GCGACCGAATTCGTCTGCATTCTCT-3′). The PCR product was purified, cleaved with *Bam*HI and *Eco*RI, and inserted into pGEX-3X (Pharmacia).

^{65}Zn(II) binding assayed by blotting. SDS gels of T antigens or GST-zinc finger fusions were electroblotted to nitrocellulose. The blots were then treated with 6 M guanidine-HCl and renatured by serial dilution of the guanidine-HCl (58) as follows. The blots were incubated twice for 10 min each in 250 ml of 6 M guanidine-HCl–0.1 M Tris-HCl (pH 6.8)–0.05 M NaCl–0.005 M dithiothreitol. Five serial dilutions of 50% into solution A (0.1 M Tris-HCl [pH 6.8], 0.05 M NaCl, 0.001 M dithiothreitol) were made, and the blot was incubated for 10 min in each. The blots were then incubated twice for 10 min each in solution A with no guanidine. The blots were probed by the methods of Schiff et al. (46) with 100 μCi of $^{65}ZnCl_2$ in 30 ml for 1 h in a Seal-a-Meal bag and washed twice for 8 min each in 250 ml before being used to expose Molecular Dynamics Phosphor Imager screens.

RESULTS

Point mutations in putative zinc-coordinating residues create mutants defective in viral DNA replication. Cysteine residues 452 and 455 and histidine residues 465, 469, and 472 represent a potential C_2H_2 zinc finger (Fig. 1). To test the importance of this element and to decide which histidines might be involved, point mutations were created by site-directed mutagenesis. As one test, mutations (positions 452, 455, 469, and 472) were reconstructed into polyomavirus genomes. However, no virus was recovered (data not shown), suggesting that the residues were critical for LT function.

To test the mutants more directly, expression vectors were prepared in which the mutants were expressed from either the Harvey murine sarcomavirus long terminal repeat or the human cytomegalovirus immediate-early promoter. The mutant LTs were expressed in 3T3 cells as stable cell lines or by transient transfection. In all cases, the LTs were nuclear, giving a pattern and intensity of nuclear immunofluorescence indistinguishable from those of wild-type LT (not shown). In [^{35}S] methionine labeling and Western blots (immunoblots) from stable cell lines and transient transfections, similar amounts of LT were observed.

Viral DNA replication was measured in a transient assay in which a plasmid containing the polyomavirus origin of DNA replication was cotransfected with an LT expression vector. DNA replication was measured by acquisition of *Dpn*I resistance. Figure 2 shows that wild-type LT supported DNA replication, but mutants carrying mutations at residues C-452→R, C-455→R, H-465→D, and H-469→L did not. The failure of these mutants to support viral DNA replication was consistent with their inability to produce virus. The LT mutant with a mutation at position 472 was not completely inactive in DNA replication but was substantially defective (Fig. 2, lane 12). For comparison, mutations were made in additional residues (po-

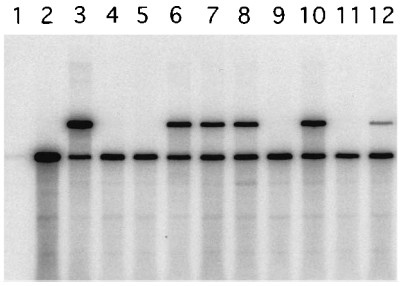

FIG. 2. In vivo replication assay of polyomavirus LT zinc finger mutants. Southern blot of low-molecular-weight DNA from 3T3 cells transfected with polyomavirus origin plasmid (pUCori) and LT expression vectors (pCMV-LT). Extracted DNA was cut with *Hin*cII and *Dpn*I. The blot was probed with ^{32}P-labeled polyomavirus origin (bp 5024 to 163). Lane 1 is a control transfected with pCMV-LT but no pUCori. Lane 2 is a control with pUCori but no LT. All subsequent lanes were from cells transfected with pUCori plus wild-type LT (lane 3), plus C-452→R LT (lane 4), plus C-455→R LT (lane 5), plus K-457→N LT (lane 6), plus E-458→Q LT (lane 7), plus E-459→Q LT (lane 8), plus H-465→D LT (lane 9), plus K-467→E LT (lane 10), plus H-469→L LT (lane 11), and plus H-472→L LT (lane 12). The arrowhead indicates the *Dpn*I-resistant linear pUCori indicative of DNA replication.

sitions 457, 458, 459, and 467) in the same region of LT. All supported DNA replication at an efficiency reduced only two- to threefold from that of the wild type (compare lanes 6, 7, 8, and 10 with lane 3 of Fig. 2).

Mutants defective in DNA replication retain the ability to bind specifically to DNA. Several lines of evidence argue against overall unfolding of the protein structure in the mutants. For example, these mutants retain the ability to inhibit myoblast differentiation (32). The mutants also retain the ability to activate some cellular promoters, such as the hsp70 promoter (data not shown). Immunofluorescence of cells expressing either wild-type or mutant LT gives identical patterns of nuclear staining with nucleolar exclusion (not shown). Pulse labeling with [^{35}S]methionine followed by a chase with unlabeled methionine gives similar estimates of the half-lives for wild-type and mutant protein on the order of 1 h (not shown). Also, Western blots showed similar amounts of mutant and wild-type LT.

Of particular interest for DNA replication is the ability to bind polyomavirus DNA. LT binds specifically to 5′-GAGGC-3′-related sequences in the origin (11, 12, 39, 45). DNA fragment immunoprecipitation assays can be used to measure specific DNA binding by LT. Figure 3A is an assay of wild-type and mutant LT with the entire polyomavirus origin used as a probe. Baculovirus-expressed LT was used to bind DNA consisting of an *Eco*RI-*Dde*I double digest of pUCori. The digest yields eight fragments, including a 454-bp fragment containing the polyomavirus origin region. Clearly, the mutant and wild-type proteins bind the polyomavirus origin equally well. To look more closely at individual sites, an *Ear*I-*Nar*I-*Stu*I triple digest was used. This digest divides the origin into four fragments, three of which contain LT binding sites. These are shown in Fig. 3B. Figure 3C shows a McKay assay done with these fragments as a probe. The 96-bp fragment contains the origin palindrome. Site A is in the 41-bp fragment, which labels very poorly for unknown reasons. Site B and most of site C are found in the 60-bp fragment. The 96-bp origin fragment as well as the 60-bp fragment containing binding sites B and C was efficiently bound by wild-type and mutant LTs.

Mutants defective in replication are defective in self-association. The formation of self-associated complexes is thought to be fundamental to initiation of polyomavirus and SV40 DNA replication. For SV40, these structures have been shown to be

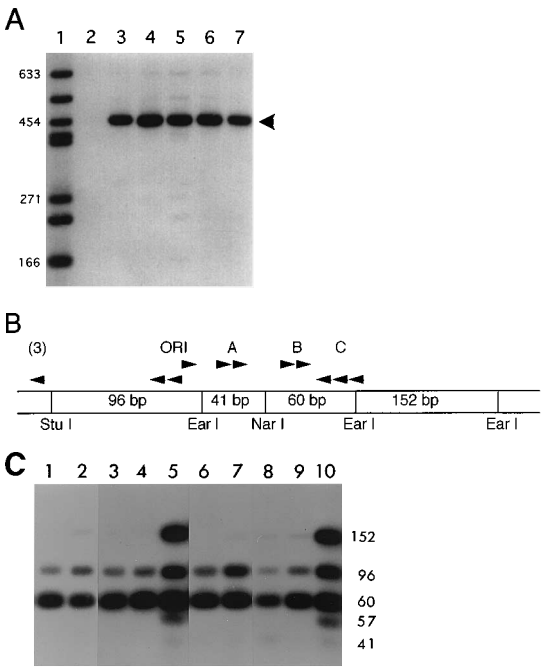

FIG. 3. Mutations in coordinating residues do not abolish DNA binding. (A) McKay assay showing DNA binding to the origin region. LT extracts from *Sf*9 cells infected with recombinant baculoviruses were mixed with labeled DNA fragments and immunoprecipitated with polyclonal anti-T antigen serum. The input DNA was a ^{32}P end-labeled *Eco*RI-*Dde*I double digest of pUCori. Lanes: 1, 1/50th of the input DNA; 2, nonrecombinant baculovirus (no LT); 3, wild-type LT; 4, C-452→R LT; 5, C-455→R LT; 6, H-469→L LT; 7, H-472→L LT. The arrowhead marks the position of the 454-bp band containing the entire origin of replication. The sizes of the fragments are indicated on the left (in kilodaltons). (B) Diagram of polyomavirus origin (ORI) of DNA replication showing fragments generated from a triple digest (*Ear*I, *Nar*I, *Stu*I). Arrowheads represent 5′-GAGGC-3′ and related repeats. (C) McKay assay with the origin fragments described in panel B as a ^{32}P end-labeled probe. The pairs of lanes were from lysates containing approximately 5 and 10 μg of LT, respectively. Lanes: 1 and 2, wild-type LT; 3 and 4, C-455→R LT; 5, marker equal to 1/50th of the input DNA; 6 and 7, H-472→L LT; 8 and 9, triple point mutation mutant C-452→R–H-469→L–H-472→L LT; 10, same as lane 5. The sizes of the fragments are indicated on the right (in base pairs).

double hexamers (35). To test this ability, sucrose gradients of wild-type and mutant LTs were compared. An immunoblot of fractions from a sucrose gradient of wild-type LT gives a characteristic pattern of two major peaks, as shown in Fig. 4A. In contrast, LT C-452→R (Fig. 4B) shows predominantly unassociated protein cosedimenting near the peak of alcohol dehy-

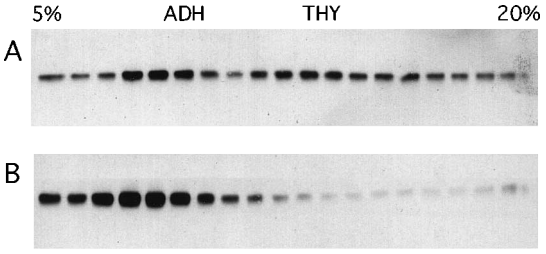

FIG. 4. Sucrose gradients of LT. Nonidet P-40 extracts of LT from insect cells were subjected to centrifugation on sucrose gradients. Twenty fractions were collected and analyzed by immunoblotting of 7.5% SDS gels. The molecular mass markers alcohol dehydrogenase (ADH [150 kDa]) and thyroglobulin (THY [669 kDa]) were run on a separate gradient and analyzed by Coomassie staining of polyacrylamide gels; their positions are noted above panel A. (A) Wild-type LT. (B) C-452→R LT.

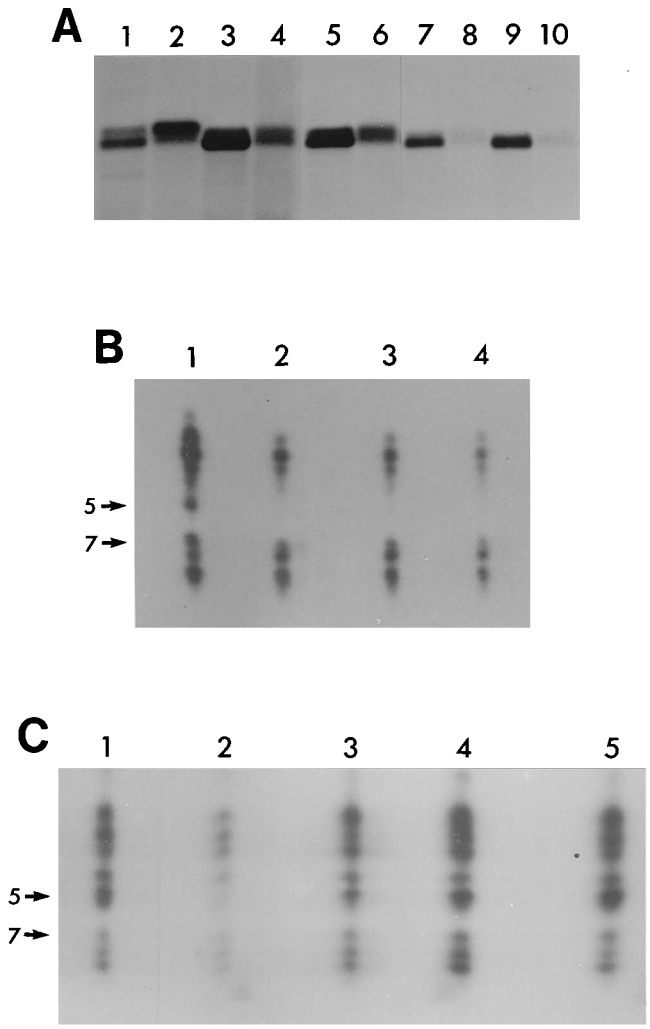

FIG. 5. Phosphorylation of zinc finger mutant LTs. (A) Specific activity of LT zinc finger mutants. Parallel 10-cm-diameter plates of stable cell lines were labeled with either [^{35}S]methionine or $^{32}P_i$, extracted with TEB, and immunoprecipitated with polyclonal anti-T antigen serum. Each lane contains the LT immunoprecipitated from a 10-cm-diameter dish of cells. Odd-numbered lanes contain [^{35}S]methionine-labeled samples. Even-numbered lanes contain $^{32}P_i$-labeled samples. Lanes: 1 and 2, wild-type LT; 3 and 4, C-455→R LT; 5 and 6, C-452→R LT; 7 and 8, H-469→L LT; 9 and 10, H-472→L LT. (B) V8 protease mapping of LTs from NIH 3T3 cells. $^{32}P_i$-labeled LT was immunoprecipitated from mouse cell extracts, separated on cylinder gels, and subjected to *S. aureus* V8 protease digestion during electrophoresis on a second-dimension slab gel. The arrowheads indicate the positions of phosphopeptides 5 and 7 in wild-type LT. Lanes: 1, wild-type LT; 2, C-452→R LT; 3, C-455→R LT; 4, H-469→L LT. (C) V8 protease mapping of LT phosphopeptides from *Sf*9 cells infected with recombinant baculoviruses. The procedure was performed as described for panel B. The arrowheads mark the positions of V8 phosphopeptides 5 and 7. Lanes: 1, wild-type LT; 2, C-452→R LT; 3, C-455→R LT; 4, H-469→L LT; 5, H-472→L LT.

drogenase at 150 kDa. This result points to a role for the C_2H_2 element in self-association of LT. Some C-terminal mutants of SV40 LT are known to be *trans*-dominant repressors of DNA replication, presumably because mixed oligomers are inactive (15). Experiments looking for a dominant lethal effect of polyomavirus C_2H_2 were negative. This is consistent, because mutants that fail to associate would not form mixed oligomers.

Mutants defective in replication are underphosphorylated, especially on peptides associated with DNA replication, in mouse cells but not in insect cells. Figure 5A shows a comparison of wild-type and mutant LTs immunoprecipitated from Nonidet P-40 extracts of cells labeled with $^{32}P_i$ or [^{35}S]methionine. The $^{35}S/^{32}P$ ratios showed a striking underphosphorylation of mutant LT. Wild-type LT shows more labeling with ^{32}P, concentrated in a more slowly migrating form of LT, than do the mutants (compare lane 2 with lanes 4, 6, 8, and 10). To examine the phosphorylation more closely, ^{32}P-labeled LT was subjected to partial proteolysis with *Staphylococcus aureus* V8 protease. Figure 5B shows two-dimensional partial V8 protease maps of the immunoprecipitated proteins. Phosphorylation of peptides 5 and 7 has been associated with the ability of LT to function in DNA replication (6, 7). These phosphopeptides are marked with arrowheads for wild-type LT (lane 1). They are largely absent from the mutant patterns (Fig. 5B, lanes 2, 3, and 4). Interestingly, a different pattern was observed when mutant LTs from baculovirus-infected *Sf*9 cells were examined. When isolated from *Sf*9 cells, the mutants incorporate label into those peptides (Fig. 5C). These phosphorylations occur on peptides arising from the junction of the N- and C-terminal domains, and they consist of multiple phosphorylations (5). Whether the mutants are phosphorylated on all possible sites when expressed in insect cells is not known.

Spectroscopy shows that the C_2H_2 element binds one equivalent of zinc as a peptide. Wild-type and mutant synthetic peptides were used to examine the specificity and stoichiometry of zinc binding by spectroscopy. Because the assignment of the histidine ligands was ambiguous, peptides consisting of the 30 amino acids from amino acids 446 to 475 of LT and including the wild type and mutants H-465→D, H-469→L, and H-472→L were synthesized. Spectroscopic methods have been used to examine binding of zinc and cobalt by C_2H_2 peptides from TFIIIA (17). Cobalt(II) has characteristic absorption spectra from 300 to 800 nm when complexed with model compounds of different coordination numbers and ligand types (4). Zinc(II) complexes do not absorb significantly in this range. When a 30-residue peptide of the wild-type LT zinc finger was mixed with excess cobalt(II), a characteristic spectrum was obtained (Fig. 6A). This absorption spectrum allows identification of interactions between Co(II) and cysteine and histidine ligands. Absorption shoulders at 310 and 340 nm are marked with arrowheads in Fig. 6A. These metal-to-sulfur charge-transfer bands are due to interactions between the sulfur of cysteines and Co(II) (4). Tetrahedrally coordinated Co(II) gives a characteristic absorption spectrum which is described as a series of intense absorption bands in the region from 500 nm to 700 nm with extinction coefficients on the order of 400 M^{-1} cm^{-1} (4). The wild-type peptide gave an extinction coefficient of 375 M^{-1} cm^{-1} at 635 nm when saturated with Co(II) chloride. The absence of strong absorption bands between 700 and 800 nm argues against complexes with more than two cysteines. There was a 1:1 stoichiometry between the peptide and Co(II), because addition of cobalt in excess had no effect. If a site occupied by cobalt has a higher affinity for zinc, then zinc will replace the cobalt by competition. Figure 6B shows the titration of the wild-type peptide complexed with excess cobalt(II) with zinc(II) chloride. When equimolar zinc is added, the spectrum reverts instantaneously to one similar to that of free peptide. The equilibrium toward a complex of peptide with Zn(II) is greatly favored, because a 10-fold molar excess of Co(II) over Zn(II) shows no maxima characteristic of the cobalt complexes. The important conclusion is that the wild-type zinc finger sequence, as an isolated peptide, binds one equivalent of zinc with high affinity.

Mutant peptides at each of the potential coordinating histidines were examined for binding to cobalt. Titration allows estimation of the dissociation constants of the peptide-cobalt

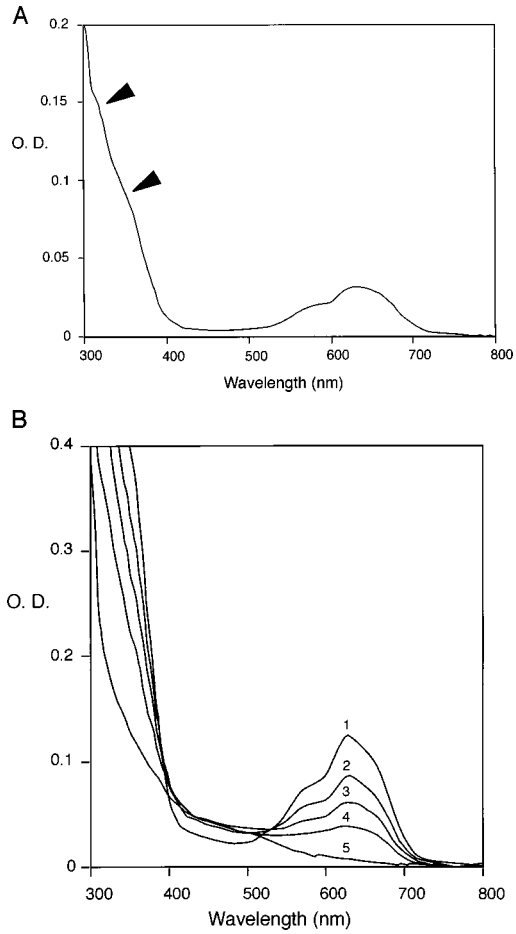

FIG. 6. Optical absorption spectra of wild-type LT zinc finger peptide complexes with metal. (A) Peptide purified by C_{18} HPLC was dissolved at 150 μM in 10 mM Tris (pH 7.0). The peptide concentration was estimated by the method of Mach et al. (31). Excess cobalt (300 μM) was added, and the absorption spectrum was recorded. The arrowheads point to the shoulders seen at approximately 310 and 340 nm, which are indicative of charge transfer between the metal and sulfur ligands. (B) Optical absorption spectra of zinc-cobalt competition for an LT zinc finger peptide. Peptide at 300 μM in 10 mM Tris (pH 7.0)–1.3 mM $CoCl_2$ was mixed with increasing amounts of $ZnCl_2$. Traces: 1, no added zinc; 2, 90 μM $ZnCl_2$; 3, 180 μM $ZnCl_2$; 4, 270 μM $ZnCl_2$; 5, 300 μM $ZnCl_2$. O.D., optical density.

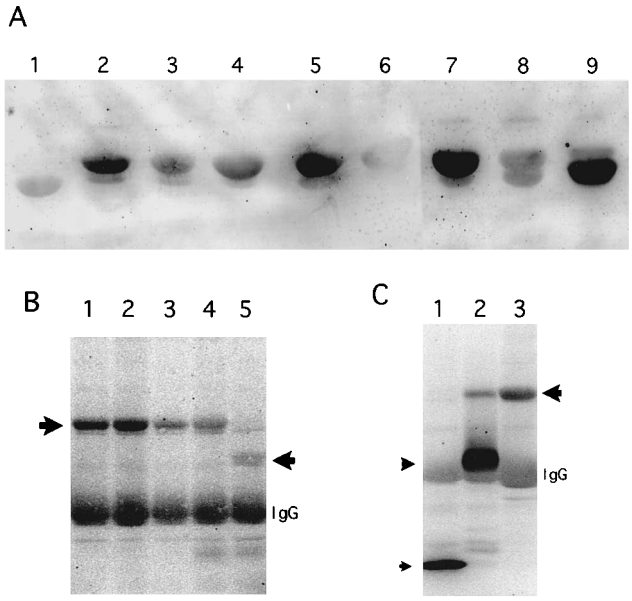

FIG. 7. ^{65}Zn binding. (A) GST-zinc finger fusions. GST fused to wild-type or mutant LT residues 446 to 475 was prepared in *Escherichia coli* JM109 cells and purified by reduced glutathione-agarose chromatography. Purified proteins were separated on a 10% polyacrylamide gel, blotted to nitrocellulose, and probed with ^{65}Zn. Lanes: 1, GST unfused; 2, GST-wild type; 3, GST–C-452→R; 4, GST–C-455→R; 5, GST–K-457→N; 6, GST–H-465→D; 7, GST–K-467→E; 8, GST–H-469→L; 9, GST–H-472→L. ^{65}Zn signals were quantitated and expressed as PhosphorImager counts (10^{-6}): GST, 6.9; GST–wild-type zinc finger, 34.3; GST–C-452→R, 17.9; GST–C-455→R, 23.8; GST–K-457→N, 61.0; GST–H-465→D, 19.2; GST–K-467→E, 48.2; GST–H-469→L, 13.5; GST–H-472→L, 41.2. (B) Full-length LT. Anti-T antigen immunoprecipitates were prepared with extracts of baculovirus-infected *Sf*9 cells. Lanes: 1, wild-type LT; 2, H-472→L LT; 3, C-452→R–C-455→R LT; 4, C-452→R–H-469→L–H-472→L LT; 5, LT with deletion of positions 330 to 530—this deletes the entire zinc finger. The arrowheads mark full-length or deleted LT. The position of immunoglobulin G (IgG) heavy chain from the immunoprecipitation is noted. ^{65}Zn signals were quantitated and expressed as PhosphorImager counts (10^{-5}): wild-type LT, 0.70; H-472→L LT, 0.75; C-452→R–C-455→R LT, 0.27; C-452→R–H-469→L–H-472→L LT, 0.32; and LT with deletion of positions 330 to 530, 0.26. (C) Small T, middle T, and LT antigens. Immunoprecipitations of small T, middle T, and LT antigens produced by recombinant baculoviruses were separated on a 5 to 20% acrylamide gradient gel and processed as described above. Lanes: 1, small T antigen produced from a polyomavirus early region baculovirus; 2, middle T antigen; 3, wild-type LT. The band at the position of LT in the middle T antigen lane (C, lane 2) is likely to be a middle T antigen dimer resistant to reduction. The large arrowhead notes the position of LT. Middle T antigen is noted by the middle-sized arrowhead, and small T antigen is noted by the small arrowhead. ^{65}Zn signals were quantitated and expressed as PhosphorImager counts (10^{-5}): small T antigen, 39.0; middle T antigen, >10; LT, 0.70.

complex (2). Wild-type peptide had a K_d for cobalt of $(1.9 \pm 1.8) \times 10^{-7}$ M. Mutant H-469→L was very defective in cobalt binding, giving an affinity for cobalt of 0.2% that of the wild-type peptide [K_d of $(9.5 \pm 2.9) \times 10^{-5}$ M]. Peptide H-465→D had a K_d for cobalt of $(9.1 \pm 2.0) \times 10^{-7}$ M (approximately 20% that of wild-type). Mutant H-472→L had a K_d for cobalt of $(3.3 \pm 2.0) \times 10^{-6}$ M, with an affinity 6% of that of wild-type peptide. This suggests that H-469 is a required metal ligand while either H-465 or H-472 could also participate in the cobalt-peptide complex.

^{65}Zn blotting shows that not only the C_2H_2 element of LT but also the middle T and small T antigens bind zinc. Some zinc binding proteins can be blotted and probed with ^{65}Zn to demonstrate zinc binding (46). GST gene fusions including LT sequences from amino acids 446 to 475 were tested first in this assay. Equal amounts of protein were blotted to nitrocellulose. After denaturation with guanidine and renaturation, the blots were probed with ^{65}Zn. Figure 7A shows that the wild-type zinc finger fused to GST gave a strong ^{65}Zn signal. It bound approximately five times as much zinc as GST alone. Fusions with mutations at cysteine 452 or 455 and histidine 465 or 469 showed greatly reduced binding. However, the GST–zinc finger H-472→L mutant bound zinc as well as the wild type in this assay.

The blotting assay was also used to test full-length LT and the other polyomavirus early gene products. Wild-type LT gave a clear signal, as shown in lane 1 of Fig. 7B. A larger deletion (residues 330 to 530) removing the entire zinc finger was significantly defective in zinc binding (lane 5). A mutant created by a double point mutation in both cysteines (lane 3) and a mutant created by a triple point mutation (C-452→R, H-469→L, and H-472→L) (lane 4) showed reduced binding. The effects are less striking than those seen with the deletion. H-472→L bound zinc as well as the wild type in this assay, similar to its behavior as a fusion protein. Middle and small T antigens were also tested in this assay (Fig. 7C). Both gave

strong signals in this assay. These proteins share an identical sequence from cysteine 120 to cysteine 153 which resembles known cysteine zinc binding motifs. A homologous sequence in the closely related SV40 small T antigen has been shown to bind two atoms of zinc (56). Comparison of the blotting intensities with protein staining suggests that middle and small T antigens have a higher specific binding activity than LT.

DISCUSSION

Genetic and physical studies have been used to investigate a putative zinc finger in polyomavirus LT. These studies suggest that the element does bind zinc. If the peptides accurately reflect the intact protein, there is unit stoichiometry. Our inability to reconstruct viable viruses containing mutants in coordinating residues points to the importance of this element to LT function.

The element between positions 452 and 472 is critical for the function of LT in viral DNA replication. While mutations in four of the conserved residues abolished the ability to replicate viral DNA, mutation of position 472 only decreased it substantially. Direct measurement by bromodeoxyuridine labeling in serum-starved 3T3 cells showed that zinc finger mutants retained the ability to induce cellular DNA synthesis (not shown). This is consistent with previous data showing that these mutants retain the ability to block myoblast differentiation (32); this block is characterized by continuing cellular DNA replication. It is also consistent with data showing that the N-terminal domain of LT, comprising residues 1 to 259, is sufficient to drive S phase in 3T3 cells (20).

A priori, the failure of zinc finger mutants to replicate viral DNA could arise at different levels. Most zinc finger proteins of the C_2H_2 type are DNA binding transcription factors in which the zinc finger is part of the DNA binding element (10). However, these LT mutants reveal no defect in DNA binding, consistent with genetic analysis arguing that residues 280 to 398 are sufficient for DNA binding (53). Others have reported that a mutant created by a point mutation (C-452→S) was defective in origin DNA binding (3); in our hands, all of the mutants created by point mutations in potentially coordinating residues, including C-452→R, were capable of binding origin sequences.

Failure in DNA replication appears to be connected to a failure to oligomerize. For SV40, LT has been shown to form double hexamers at the replication origin. This assembly is thought to be critical to replication. Polyomavirus LT is also known to undergo oligomerization. Earlier data showed that *tsA* mutants defective in replication failed to oligomerize (15). The data here show little evidence of self-association for mutants with mutations in the putative metal-coordinating residues.

How the zinc finger functions in oligomerization is not clear. At least two models for the contact sites can be envisioned. The zinc finger could form a contact site which associates with the zinc finger on another LT molecule. This seems unlikely, because native electrophoresis of GST fusions containing the zinc finger element did not show any indication of forming oligomeric structures (not shown). A more likely model is that the element makes contact with another region of LT. The N-terminal 259 amino acids cannot be involved in this interaction, because the C-terminal domain expressed alone forms higher-order structures (21). If the zinc finger contacts another region of LT, it must be in the C-terminal 521 amino acids. A group of point mutations in the SV40 LT DNA binding domain can block oligomerization (50, 64). These could identify a contact site for the LT zinc finger. Sequence comparison suggests another possibility, namely, that the zinc finger organizes a larger contiguous region. While the noncoordinating residues between the cysteines and histidines are weakly conserved, the regions on either side of the zinc finger are more conserved between all polyomavirus LTs (38). For example, 12 residues C terminal from the last histidine (polyomavirus residue 472) are very highly conserved (10 out of 12 residues of polyomavirus and SV40 are identical). A conserved hydrophobic cluster is located six residues N terminal to the first cysteine at residue 452. It may be that these conserved regions represent a contact site or sites organized by the zinc binding element.

LT is not the only protein in which zinc is involved in mediating associations other than that with nucleic acid. In protein kinase C, zinc is involved in organizing phorbol ester binding (25). The zinc finger of adenovirus E1A is thought to be important for protein-protein interactions (62). There may already be a precedent for participation of such elements in both homotypic and heterotypic interactions. An acetylcholine receptor binding protein binds zinc, self-associates, and can associate with the acetylcholine receptor (48).

Besides the defect in oligomerization, mutants in coordinating residues are also deficient in phosphorylation in mouse cells. This is significant because a connection between phosphorylation and DNA replication has been noted (7, 59). The correlation between phosphorylation and self-association could have a physical connection. Phosphorylation at specific residues might be necessary for self-association. Alternatively, self-associated LT could be a substrate for a specific phosphorylation. For SV40 LT, bacterially expressed protein which should be unmodified can form hexamers (42); this argues against the first possibility. Experiments with polyomavirus LT-expressing baculoviruses from *Sf*9 cells also argue against such direct connections. The mutants appear to be phosphorylated like the wild type even though they still show defects in association. However, this result is not definitive, because it remains possible that some of the nine known LT phosphorylations could be different in insect cells.

Studies with synthetic peptides, fusion proteins, and full-length proteins were carried out to test zinc binding. Cobalt binding and zinc displacement indicated a 1:1 stoichiometry of zinc to peptide and a coordination with both cysteine and histidine. One goal in all of these studies was to resolve which two of the three histidine residues were critical. These experiments were not entirely successful. Mutation of residue 469 reduced metal affinity by 2 orders of magnitude; reduced but significant binding was observed for both mutant H-465→D and mutant H-472→L when assayed spectroscopically. ^{65}Zn binding experiments confirmed the binding of zinc for LT. When assayed by ^{65}Zn blotting, H-465→D was very defective, but H-472→L was not. These different results may be explained by the nature of the assays. The zinc blotting is not quantitative, so both H-465→D and H-472→L might have been expected to be positive, given their reduced but significant binding. However, not all zinc binding proteins work in such assays; perhaps H-465→D as a GST-peptide fusion fails to renature on nitrocellulose to make the 6% affinity seen in the spectroscopic assays evident. Because the LT mutant with a mutation at H-465 was more defective in replication, oligomerization, and zinc blotting than the mutant with a mutation at H-472, it is tempting to suggest that H-465, along with H-469, is a coordinating residue. Another interesting possibility is that there could be a switch in which the coordination changes in response to LT function.

The final important point determined from the zinc blotting is the zinc binding by both the middle and small T antigens. Zinc binding by polyomavirus small T antigen is expected on

the basis of what is known about the SV40 small T antigen. That molecule binds two equivalents of zinc in a cysteine-rich region. Middle T antigen binds zinc, and this observation is not unexpected, because middle T antigen shares the cysteine motif with small T antigen. This region is important for the ability of middle T antigen to transform (34). A direct test of the role of zinc binding in middle T antigen function should be of considerable interest.

ACKNOWLEDGMENT

This work was supported by National Institutes of Health grant CA34722.

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