

Dependence of the Encapsidation Function of the Adenovirus L1 52/55-Kilodalton Protein on Its Ability To Bind the Packaging Sequence

Pilar Perez-Romero, Kurt E. Gustin,[†] and Michael J. Imperiale*

Department of Microbiology and Immunology and Comprehensive Cancer Center, University of Michigan Medical School, Ann Arbor, Michigan 48109-0942

Received 10 October 2005/Accepted 20 November 2005

The adenovirus IVa2 and L1 52/55-kDa proteins are involved in the assembly of new virus particles. Both proteins bind to the packaging sequence of the viral chromosome, and the lack of expression of either protein results in no virus progeny: the absence of the L1 52/55-kDa protein leads to formation of only empty capsids, and the absence of the IVa2 protein results in no capsid assembly. Furthermore, the IVa2 and L1 52/55-kDa proteins interact with each other during adenovirus infection. However, what is not yet clear is when and how this interaction occurs during the course of the viral infection. We defined the domains of the L1 52/55-kDa protein required for interaction with the IVa2 protein, DNA binding, and virus replication by constructing L1 52/55-kDa protein truncations. We found that the N-terminal 173 amino acids of the L1 52/55-kDa protein are essential for interaction with the IVa2 protein. However, for both DNA binding and complementation of the *pm8001* mutant virus, which does not express the L1 52/55-kDa protein, the amino-terminal 331 amino acids of the L1 52/55-kDa protein are necessary. These results suggest that the production of infectious virus particles depends on the ability of the L1 52/55-kDa protein to bind to DNA.

Adenovirus DNA encapsidation is a process that remains poorly understood. Several studies have shown that packaging of the adenovirus genome is dependent on *cis*-acting DNA elements comprising the packaging sequence, located at the left end of the genome between nucleotides (nt) 194 and 380 in adenovirus type 5 (Ad5) (3, 9, 13, 21). More recently, studies have focused on characterizing viral components involved in packaging that specifically interact with the packaging sequence as well as other factors that interact with these components. The packaging sequence contains at least seven functional units, called A repeats (3, 4, 13, 22). At least two viral proteins, L1 52/55-kDa and IVa2, are involved in packaging sequence interactions (19, 20, 30, 32).

The IVa2 protein is a viral product that was first reported to be a transcriptional activator of the adenovirus major late promoter (16, 17, 26). More recently, the IVa2 protein was found to bind directly to the A repeats in the packaging domain *in vitro* and *in vivo* (19, 20, 30). The IVa2 protein is present in assembly intermediates and mature virions (7, 11, 28). Characterization of a IVa2 mutant virus, *pm8002*, demonstrated that no viral particles were formed during infection although gene expression and viral DNA replication were not affected (31). These results support an essential role for the IVa2 protein in virus assembly and suggest that it is involved in DNA packaging.

The L1 52/55-kDa protein is a nonstructural nuclear phosphoprotein that migrates as a doublet on sodium dodecyl sul-

fate (SDS)-polyacrylamide gels. It is detected in empty capsids and assembly intermediates but is not found in mature virions, which suggests a scaffolding role for this protein (11). In infections with the temperature-sensitive mutant virus H5ts369, which produces a nonfunctional L1 52/55-kDa protein at the nonpermissive temperature, empty capsids associated with the left end of the viral genome form at the nonpermissive temperature (12). A more pronounced phenotype occurs in infections with the *pm8001* mutant virus, which is unable to produce the L1 52/55-kDa protein. This virus has normal early and late viral gene expression and DNA replication but produces empty capsids with no associated viral DNA (7).

Recently, we and others demonstrated that the L1 52/55-kDa protein binds to the packaging sequence *in vivo* by using chromatin immunoprecipitation (ChIP) assays (19, 20). This interaction might be mediated by another protein, since purified L1 52/55-kDa protein did not bind to the packaging sequence *in vitro* even in the presence of the IVa2 protein, which is known to bind to the packaging sequence (20). Although the IVa2 and L1 proteins interact in infected cells (8), the interaction is not required for binding of either protein to the DNA since the packaging sequence can be immunoprecipitated from *pm8001*-infected cells with antibody against the IVa2 protein or from *pm8002*-infected cells with antibody against the L1 protein (20).

To further investigate the role of the L1 52/55-kDa protein in adenovirus assembly, we have constructed L1 52/55-kDa proteins containing truncations of the N terminus and the C terminus. These proteins were expressed *in vitro* and *in vivo* and analyzed for functionality in terms of interaction with the IVa2 protein, DNA binding, and the ability to complement the *pm8001* mutant virus *in trans*. We found that the N-terminal 173 amino acids (aa) of the L1 52/55-kDa protein were essential for interaction with the IVa2 protein. However, the amino-

* Corresponding author. Mailing address: University of Michigan Medical School, 6304 Cancer Center, 1500 E. Medical Center Dr., Ann Arbor, MI 48109-0942. Phone: (734) 763-9162. Fax: (734) 615-6560. E-mail: imperial@umich.edu.

[†] Present address: Department of Microbiology, Molecular Biology, and Biochemistry, University of Idaho, Moscow, ID 83844.

TABLE 1. Primer sequences

Primer	Sequence ^a
5'Ad5(aa1) BamHI	5'GCGGATCCATATGCATCCGGTGTCT CGGGCAGAT3'
3'Ad5(aa52) EcoRI.....	5'CGGAATTCCTACGGGGTTCGTAA TCACC3'
3'Ad5(aa105) EcoRI.....	5'GCGAATTCCTACTCTCCCTCGGGTCT3'
5'trip NheI	5'GGGCTAGCTTCATCATCAATA TACCTTATTTT3'
3'trip EcoRI.....	5'GGGAATTCCTACTTGGCGACTGTGAC TGTTAGA3'
5'Ad5(aa1) EcoRI.....	5'CGGAATTCCTATGCATCCGGTGTCT CGGGCAGAT3'
5'Ad5(aa174) EcoRI.....	5'CGGAATTCCTAACCAACCAGTGGC TACGCTT3'
5'Ad5(aa331) EcoRI.....	5'CGGAATTCCTTCGGCGGCGGAGCT CAGCGA3'
3'Ad5(aa52) XbaI	5'CGTCTAGATTTCGGGGTTCGTAAT CACCAT3'
3'Ad5(aa105) XbaI	5'CGTCTAGATTCTCTCCCTCGGGT CGCGAA3'
3'Ad5(aa173) XbaI	5'CGTCTAGATTGCTTTTTTTGAAAGT TAATCTCTGGTT3'
3'Ad5(aa331) XbaI	5'CGTCTAGATTTACGGCTCAGCTC ACGGCCTT3'
3'Ad5(aa416) XbaI	5'CGTCTAGATTTAGTACTCGCCGT CCTCTGGCT3'

^a The positions of the endonuclease recognition sites are underlined. Adenovirus genome sequences are in boldface type.

terminal 331 aa were necessary for both DNA binding and *pm8001* complementation. These results support the idea that DNA binding and the production of infectious virus particles are related functions of the L1 52/55-kDa protein.

MATERIALS AND METHODS

Cells and viruses. 293 cells are adenovirus-transformed human embryonic kidney cells (6). 293 cells that stably express the L1 52/55-kDa protein (293-L1) were used as a helper cell line to grow the *pm8001* mutant virus, which fails to express the L1 52/55-kDa protein (7). 293 cells that stably express the IVa2 protein (293-IVa2) were used as a helper cell line to grow the *pm8002* mutant virus, which fails to express the IVa2 protein (31). Wild-type Ad5 virus was propagated as previously described (5, 30). The L1 52/55-kDa temperature-sensitive mutant virus H5ts369 was propagated in 293 cells at the permissive temperature (32°C) (12). All cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and 5% CO₂. 293-L1 and 293-IVa2 cells were grown under selection with 500 µg/ml Geneticin G418 (Gibco BRL).

Plasmid constructs. We used pGEX-L1 as previously described (8) for expression of the full-length glutathione transferase (GST)-L1 52/55-kDa fusion protein (aa 2 to 416). pGEX-L1(2-173) (numbers in parentheses indicate amino acid numbers) was generated by digesting pGEX-L1 with HindIII (Ad5 nt 11565), filling in the ends with Klenow fragment, digesting with SmaI, and religating the vector. pGEX-L1(2-331) was created by digesting pGEX-L1 with NaeI (nt 12041) and SmaI and religating the vector. pGEX-L1(174-416) and pGEX-L1(331-416) were generated by isolating the HindIII-SmaI (nt 11565 to 13065) or NaeI (nt 12041 to 12590) fragments from pGEX-L1 and cloning them into the SmaI site of pGEX-3X or pGEX-5X, respectively. pGEX-L1(1-52) and pGEX-L1(1-105) were created by PCR amplification of fragments encoding amino acids 1 to 52 and 1 to 105 by using 5'Ad5(aa1) BamHI as the 5' primer and either 3'Ad5(aa52) EcoRI or 3'Ad5(aa105) EcoRI as the 3' primer (Table 1). Amplified fragments were digested with BamHI and EcoRI and ligated into the corresponding sites of pGEX-3X. All of these constructs were sequenced to confirm their composition.

For expression of the truncated L1 52/55-kDa proteins in mammalian cells, we first constructed a vector containing the Ad5 major late tripartite leader. Primers were designed to amplify the tripartite sequence by using pBK-tripIVa2 as a template (32). NheI and EcoRI restriction sites were introduced in the 5' and 3' ends, respectively, of the oligonucleotides (Table 1). The PCR product was digested and inserted in the corresponding sites of pCI (Promega) to obtain pCI-trip. Primers were designed to amplify the full-length L1 52/55-kDa protein and the truncation mutants containing amino acids 2 to 331, 2 to 173, 2 to 105, 1 to 52, 174 to 416, and

331 to 416 (Table 1). The 5' and the 3' primers contained EcoRI and XbaI restriction sites, respectively. PCR products were digested with EcoRI and XbaI and inserted in the corresponding sites of pCI-trip.

Preparation of antisera. Anti-IVa2 rabbit antiserum was generated by immunizing a rabbit with a synthetic peptide that spans amino acids 21 to 37. The anti-L1 52/55-kDa sheep antiserum was generated by immunizing a sheep with a synthetic peptide that spans amino acids 100 to 122. Immunizations were performed by Bethyl Laboratories, Inc. (TX). The anti-L1 monoclonal antibody was generated by immunizing mice with the GST-L1 fusion protein, which was purified by glutathione Sepharose-4B affinity chromatography as previously described (8, 20). The purified protein was injected into three mice. Sera from all three mice tested positive for antibodies to the L1 52/55-kDa protein by Western blot analysis of adenovirus-infected 293 cell lysates. The mouse with the highest anti-L1 titer was chosen for fusion and generation of the hybridoma cell lines. Twenty-three cell lines with supernatants that reacted with the GST-L1 fusion protein but not the GST protein were expanded. When reassayed by enzyme-linked immunosorbent assay, 13 of the expanded cell lines were positive for antibodies to the L1 52/55-kDa protein. Ten of the cell lines were cloned by limiting dilution and retested as described above. This resulted in the isolation of two hybridoma cell lines that secreted antibodies specific to the L1 52/55-kDa protein. The hybridoma termed 4A8 was used for these studies. To map the antibody epitope, several of the deleted GST-L1 fusion proteins were tested by Western blotting with the 4A8 antibody. The antibody reacted with all fusion proteins containing the N terminus of the L1 52/55-kDa protein (amino acids 1 to 52, 1 to 105, 2 to 173, or 2 to 331) but not with constructs lacking it (amino acids 174 to 416 or 331 to 416). To further map the epitope, a GST fusion protein, containing the L1 open reading frame (ORF) from *pm8001* and therefore expressing only the first 17 amino acids of the L1 52/55-kDa protein, was tested by Western blotting. The 4A8 antibody reacted with this protein, suggesting that the epitope was located within the extreme N terminus of the protein.

Expression of GST fusion proteins. *Escherichia coli* BL21 cells transformed with the pGEX-L1 constructs were induced as described previously (8). The expressed protein was purified using glutathione Sepharose-4B beads (Pharmacia Biotech) and 30% ammonium sulfate precipitation.

Preparation of whole-cell or nuclear extracts from mammalian cells. To prepare whole-cell lysates, 293 cells in 10-cm dishes were mock infected or infected with Ad5, *pm8001*, or *pm8002* at a multiplicity of infection of 10 PFU/cell for 24 h. Cells were washed twice with 5 ml of phosphate-buffered saline solution and lysed by addition of 500 µl of E1A lysis buffer (10) and incubation for 30 min at 4°C. Cell debris was removed by centrifugation at 10,000 × g at 4°C for 10 min. Nuclear protein extracts were prepared as described previously (2, 30).

Binding assays. To determine if the GST-L1 proteins could interact with the IVa2 protein produced from adenovirus, *in vitro* binding assays were performed as described previously (8). Briefly, 15 ml of induced bacterial cultures expressing pGEX-L1 constructs was lysed by sonication in phosphate-buffered saline containing 0.1% β-mercaptoethanol and 2 mM EDTA. Cleared extracts were incubated for 1.5 h at 4°C with E1A lysis buffer-equilibrated glutathione Sepharose-4B (Pharmacia Biotech) and mixed with 200 µg Ad5-infected or mock-infected lysates. Mixtures were incubated for 1 h at 4°C and washed three times with E1A buffer. For Western blot analysis, samples were resuspended in SDS sample buffer and separated on a 10% SDS-polyacrylamide gel. Proteins were transferred to nitrocellulose and probed for the L1 52/55-kDa protein with rabbit anti-L1 antibodies (8) and for the IVa2 protein with rabbit anti-IVa2 antibodies (31). Proteins were visualized using a secondary horseradish peroxidase-conjugated antibody and chemiluminescence as recommended by the manufacturer (Amersham Pharmacia Biotech).

Mutant virus complementation assays. Dishes (10 cm) of 50%-confluent 293 cells were transfected using calcium phosphate with 10 µg of the pCI-trip-L1 constructs. Cells were infected 48 h later with 10 PFU/cell of the *pm8001* mutant virus for an additional 48 h. One-third of the cells were subjected to three rounds of freezing and thawing and sonication to prepare viral lysates. Plaque assays with 293-L1 cells were performed to determine the amount of infectious virus produced. To examine possible contamination with revertant wild-type virus, plaque assays were simultaneously performed with 293 cells. The second fraction of cells was used to prepare whole-cell protein lysates to detect expression of the L1 52/55-kDa and IVa2 proteins by Western blotting. The final third of the cell suspension was used to isolate viral genomic DNA to test for possible recombination events during the course of the transfection-infection.

Recombination assays. Viral genomic DNA was isolated from transfected-infected cells by using a modification of the procedure described by Hirt (7, 14) and used as a template for PCR with primers upstream and downstream of the L1 ORF (7). These primers specifically amplify an adenovirus genomic DNA fragment of approximately 2 kb and do not amplify the transfected plasmids. PCR products were digested with SpeI, which recognizes a site introduced into

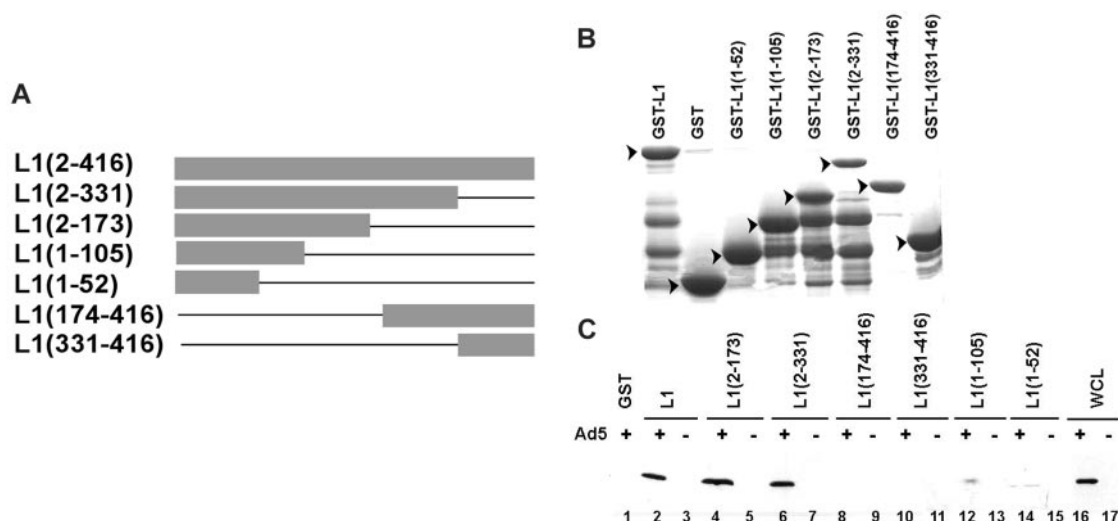


FIG. 1. In vitro binding of L1 52/55-kDa and IVa2 proteins. (A) Schematic representation of the full-length (L1) and N-terminally and C-terminally truncated L1 52/55-kDa proteins (amino acids 2 to 331, 2 to 173, 1 to 105, 1 to 52, 174 to 416, and 331 to 416). The deletions are represented by the thin black lines. (B) Coomassie blue staining of the GST fusion proteins purified by glutathione Sepharose-4B affinity chromatography. The arrowheads point to the intact proteins. (C) Western blot analysis of IVa2-L1 52/55-kDa protein interaction. The purified GST-L1 52/55-kDa fusion proteins were mixed with protein extracts from either Ad5-infected (+) or mock-infected (-) 293 cells and analyzed for binding. The IVa2 protein was detected using polyclonal anti-IVa2 antibody. Lanes 16 and 17 contain 10 μ g of whole-cell lysates (WCL) used as positive or negative controls, respectively.

the ORF at amino acids 19 to 21 of the L1 52/55-kDa protein during the mutagenesis process to generate the *pm8001* mutant (7). Therefore, any revertant due to recombination between the transfected plasmid and the *pm8001* chromosome will not be digested.

Immunoprecipitations and Western blotting. Dishes (10 cm) of 50%-confluent 293 cells were transfected for 48 h as described above and infected at 10 PFU/cell with the *pm8001* virus for 24 h. For radiolabeling, 50 μ Ci/ml of [³⁵S]methionine-cysteine (Amersham Pharmacia Biotech) was added to the media 24 h postinfection and the cells were incubated for an additional 6 h before harvest. Cells were harvested and lysed in E1A lysis buffer as previously described (10).

For immunoprecipitations, 100 μ g of radiolabeled or unlabeled protein lysates was mixed with 4 μ g of the rabbit polyclonal antibodies to L1 52/55-kDa (8) and IVa2 proteins or rabbit immunoglobulin G (IgG) as a control and incubated overnight at 4°C. Immunocomplexes were collected by incubation with 25 μ l protein G-Sepharose beads (50% slurry; Amersham Pharmacia Biotech) for 2 h at 4°C and washed three times with 1 ml of E1A lysis buffer. Radiolabeled proteins were resolved in a 10% SDS-polyacrylamide gel and fixed using a fixing solution (isopropanol:water:acetic acid, 25:65:10) for 30 min. Gels were soaked in Amplify fluorographic reagent (Amersham Pharmacia Biotech) for 30 min, dried under vacuum at 80°C, and exposed to film (Kodak) at -80°C. Nonradiolabeled immunoprecipitated complexes were boiled in SDS sample buffer, separated by 10% SDS-polyacrylamide gel electrophoresis, and analyzed by Western blotting (10). Full-length L1 52/55-kDa and L1(2-331) (numbers in parentheses indicate amino acid numbers) proteins were assayed using the sheep anti-L1 antibody at a 1:10,000 dilution. L1(2-173), L1(1-105), and L1(1-52) proteins were assayed using the mouse anti-L1 antibody at a 1:2,000 dilution. L1(174-416) and L1(331-416) proteins were probed using the rabbit anti-L1 antibody at a 1:10,000 dilution.

ChIP. Chromatin immunoprecipitation experiments, in which protein-DNA interactions in intact cells are measured by cross-linking the cells, coimmunoprecipitating the protein and its cognate DNA, and analyzing the precipitated DNA using PCR, were performed as previously described (20). The indicated polyclonal antibodies and rabbit IgG were used for immunoprecipitations. The resulting immunoprecipitated DNA (ChIP DNA) was used as a template for PCR amplification by use of primers to amplify the packaging sequence or a portion of the L1 gene as a control.

RESULTS

Previous results showed that the IVa2 and L1 52/55-kDa proteins interact in vitro and in vivo (8). Furthermore, both proteins bind to the Ad5 packaging sequence located at the left

end of the genome (19, 20, 30). We examined the domains of the L1 52/55-kDa protein required for the interaction with the IVa2 protein and the DNA and the importance of those interactions for the encapsidation function of the L1 52/55-kDa protein. To do this, we constructed L1 52/55-kDa protein N-terminal and C-terminal truncation mutants (Fig. 1A) and analyzed them in vitro by GST pulldown assays in combination with lysates from *pm8001*-infected cells and in vivo for their ability to coprecipitate the IVa2 protein, to complement the *pm8001* virus in *trans*, and to interact with the packaging sequence.

The N-terminal 173 amino acids of the L1 52/55-kDa protein are necessary and sufficient for interaction with the IVa2 protein. We took advantage of our ability to detect interactions in vitro using a GST-L1 52/55-kDa fusion protein (8). GST proteins were immobilized on glutathione beads and visualized by Coomassie blue staining (Fig. 1B). The smaller proteins that appear in some of the lanes were confirmed as breakdown products by Western blotting (data not shown). We tested the ability of equal amounts of the intact purified GST fusion proteins containing the N- or C-terminal deletions to bind to the IVa2 protein compared to the full-length L1 52/55-kDa protein (Fig. 1C). Neither of the truncations missing the N terminus of the protein was able to interact with the IVa2 protein, nor was GST alone. Results using constructs containing various lengths of the N terminus of the L1 52/55-kDa protein indicated that L1(2-331) and L1(2-173) interacted as well as the full-length construct. Deletion of an additional 68 amino acids from the N terminus [L1(1-105)] impaired binding ability and further deletion eliminated binding, suggesting that the N-terminal 173 amino acids of the L1 52/55-kDa protein are necessary and sufficient to support an interaction with the IVa2 protein in vitro.

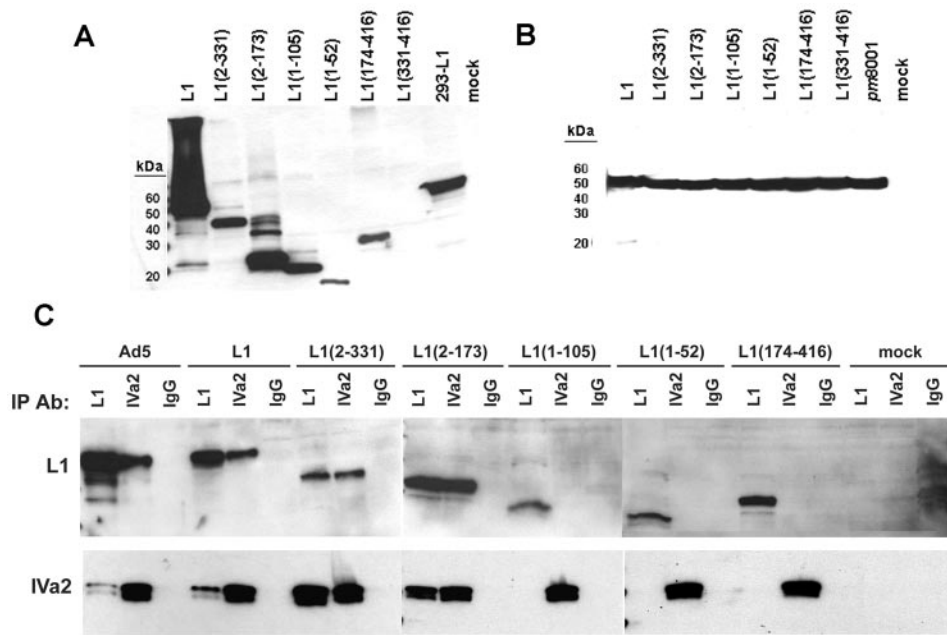


FIG. 2. Interaction of L1 52/55-kDa and IVa2 proteins in vivo. 293 cells were transfected with the indicated L1 52/55-kDa constructs or empty vector for 48 h followed by infection with *pm8001* mutant virus. Cells were lysed 48 h later, and 20 μ g whole-cell lysates was analyzed by Western blotting to detect expression of the (A) L1 or (B) IVa2 proteins. (C) Immunoprecipitations were performed with the indicated polyclonal antibodies (IP Ab), followed by Western blotting with antibodies against L1 52/55-kDa (top panel) or IVa2 (bottom panel) proteins. Wild-type Ad5-infected lysates (Ad5) or mock-infected lysates (mock) were used as a positive control and a negative control, respectively.

To confirm in vivo that the IVa2-L1 52/55-kDa protein interaction was not an artifact of the in vitro approach, we constructed eukaryotic expression vectors containing the adenovirus major late tripartite leader sequence followed by the N- or C-terminal deletions of the 52/55-kDa ORF. We used the tripartite leader to ensure efficient translation in infected cells (15). The L1 constructs were transfected into 293 cells followed by infection with the *pm8001* mutant virus, which fails to express the L1 52/55-kDa protein. Whole-cell lysates were tested for the expression of the L1 52/55-kDa and IVa2 proteins by Western blotting. We used lysates from mock-infected 293 cells as a negative control, from 293-L1 cells as a positive control for L1 52/55-kDa protein expression, or from 293 cells infected with the *pm8001* mutant to confirm the lack of expression of the L1 52/55-kDa protein. We detected the full-length L1 52/55-kDa protein and all truncations with the exception of the L1(331-416) protein (Fig. 2A); it is possible that this small protein is not stable. As expected, we did not detect L1 52/55-kDa protein expression from either mock- or *pm8001*-infected cells, and we detected the L1 52/55-kDa protein in 293-L1 cells. Similar levels of the IVa2 protein were detected in all samples except the mock-infected lysates (Fig. 2B).

Protein lysates were used for coimmunoprecipitation assays with antibodies to either the L1 52/55-kDa or the IVa2 protein. Radiolabeled lysates were also prepared to confirm expression of the mutant proteins and to demonstrate that the antibody used for the coimmunoprecipitations recognized all of the truncated proteins. All of the truncated proteins were immunoprecipitated (data not shown). Western blot results from the coimmunoprecipitation experiments were consistent with those obtained in vitro in the GST pulldown experiments (Fig.

2C). The full-length L1 52/55-kDa protein from Ad5 infection or pCI-trip-L1 transfection, as well as the truncation mutants expressing amino acids 2 to 331 and 2 to 173, was able to coprecipitate with the IVa2 protein. These results confirmed that the 173 amino acids of the N terminus of the L1 52/55-kDa protein were sufficient to interact with the IVa2 protein.

The N-terminal 331 amino acids are necessary to complement the *pm8001* mutant virus in trans. The *pm8001* mutant virus is able to be propagated only in the 293-L1 complementing cell line, which provides the L1 52/55-kDa protein in trans. Using a transient virus complementation assay with 293 cells, we determined whether the transfected truncated forms of the L1 52/55-kDa protein were able to complement the *pm8001* virus infection in trans. 293 cells were transfected with the plasmids expressing the various L1 mutants and subsequently infected with the *pm8001* mutant virus as described above. Virus progeny from these transfection-infections were collected, and titers were determined with 293 and 293-L1 cells. We expected infectious virus progeny if the L1 52/55-kDa protein expressed in trans could complement the *pm8001* mutation. The progeny would be phenotypically infectious but genotypically mutant and unable to produce de novo L1 52/55-kDa protein upon subsequent infection. Therefore, the complemented virus would replicate only in 293-L1 cells, which provide the wild-type L1 52/55-kDa protein in trans. The results from two representative experiments are shown in Table 2 and indicate that only the full-length L1 52/55-kDa protein and the L1(2-331) truncation mutant were able to complement the *pm8001* mutant. The yield obtained with the expression of the L1(2-173) protein was reduced by a factor of 10^3 compared with results for the full-length L1 52/55-kDa protein or the L1(2-331) trun-

TABLE 2. Complementation assay titers

Transfected construct	Result (PFU/ml) for:			
	Expt 1		Expt 2	
	293-L1 cells	293 cells	293-L1 cells	293 cells
L1	4.0×10^6	3.0×10^1	2.1×10^6	7.0×10^1
L1(2-331)	1.0×10^6	1.0×10^1	7.0×10^5	2.0×10^1
L1(2-173)	3.0×10^3	4.0×10^1	2.0×10^3	2.0×10^1
L1(2-105)	1.0×10^1	2.5×10^2	2.0×10^1	6.0×10^1
L1(2-52)	2.5×10^2	2.0×10^1	4.0×10^2	3.0×10^1
L1(174-416)	1.6×10^1	2.0×10^1	4.0×10^1	4.0×10^1
L1(331-416)	2.0×10^1	6.0×10^1	1.5×10^1	4.0×10^1
Empty vector	1.0×10^1	1.0×10^2	2.0×10^1	

cation. Titration of virus in 293 cells served as a control for generation of wild-type virus due to marker rescue and showed no such rescue as measured by the lack of plaque formation after 2 weeks.

To further rule out the possibility of recombination events, viral genomic DNA was isolated from transfected-infected cells and used as a template for PCR with primers that bind upstream and downstream of the L1 ORF and specifically amplify the adenovirus genomic DNA (7). These primers do not amplify the L1 cDNA from the transfected expression plasmids. An SpeI site was introduced in the L1 52/55-kDa protein ORF during the process of generating the *pm8001* mutant virus and therefore is a marker for the presence of the mutations. The PCR products from all of the experimental samples were completely digested, confirming the presence of the mutations in the viral genomic DNA (Fig. 3). As controls, viral genomic DNA from wild-type Ad5- or *pm8001*-infected cells was isolated and amplified. As expected, these PCR products were undigested or digested, respectively. No amplification product was detected when a plasmid containing the L1 52/55-kDa ORF was used as a control template, demonstrating the specificity of the primers for viral genomic DNA. These results confirmed that no recombination events occurred during the experiment, supporting the conclusion that we were observing complementation in *trans*. Our results demonstrate that the mutant L1 52/55-kDa protein containing the N-terminal 331 amino acids was able to complement the *pm8001* virus.

Analysis of L1 52/55-kDa protein binding to the packaging sequence. In vitro electrophoretic mobility shift assays using purified proteins or infected cell nuclear extracts showed no interaction of the L1 52/55-kDa protein and the packaging sequence (20, 23, 30). However, an in vivo interaction of the L1 52/55-kDa protein with the packaging sequence has been reported (19, 20). In order to determine if the mutant L1 52/55-kDa proteins had the ability to bind to the packaging sequence, we performed ChIP assays on cross-linked nuclear lysates from 293 cells that were transfected-infected as described above. PCR amplifications of the ChIP samples were performed using primers designed to amplify the packaging sequence or control primers to amplify a fragment of the L1 ORF (20). Only the full-length L1 protein and the L1(2-331) mutant were able to bind to the packaging sequence (Fig. 4A). No amplification product of the L1 sequence was detected, indicating specificity of the immunoprecipitation for the packaging sequence (Fig. 4B). PCR amplification controls confirmed the presence of

both DNA sequence targets in the input DNA (Fig. 4C), and no PCR product was detected from immunoprecipitations using rabbit IgG (Fig. 4A).

DISCUSSION

The L1 52/55-kDa protein is known to interact with the IVa2 protein and the viral packaging sequence and to play a key role in adenovirus DNA packaging, but very little is known about the mechanism involved. We constructed N-terminal and C-terminal L1 52/55-kDa protein truncations to define the functional domains of the protein. We examined the L1 52/55-kDa-IVa2 protein interaction, binding to the packaging sequence, and the ability of these proteins to complement the *pm8001* mutant virus, which fails to express the L1 52/55-kDa protein.

The ability to detect binding in vitro between the GST-L1 52/55-kDa fusion proteins and the IVa2 protein presented a relatively simple assay to examine the domains within the L1 52/55-kDa protein responsible for this interaction. This analysis showed that the N-terminal 173 amino acids were necessary and sufficient to support an interaction equivalent to that seen for the full-length L1 52/55-kDa protein. Furthermore, the constructs lacking the N terminus of the L1 52/55-kDa protein, L1(174-416) and L1(331-416), were completely incapable of interacting with IVa2. The in vivo coimmunoprecipitation results are consistent with the GST pulldown results and confirm that the L1 52/55-kDa proteins containing the N-terminal 173 or 331 amino acids are able to bind the IVa2 protein; this interaction is detectable using antibodies recognizing either of the proteins for the immunoprecipitation. Binding to the IVa2 protein likely involves sequences at the extreme N terminus of the L1 52/55-kDa protein, since a GST fusion protein containing amino acids 2 to 105 was able to bind in vitro although with much-reduced ability compared to the full-length protein. The region extending to amino acid 173 might be involved in stabilizing this interaction, in providing additional contacts, or in the proper folding of the amino-terminal end of the L1 52/55-kDa protein.

The N-terminal 331 amino acids of the L1 52/55-kDa protein were required for both binding to the packaging sequence and complementation of the *pm8001* mutant virus in *trans*. This suggests that the ability of the virus to complete assembly

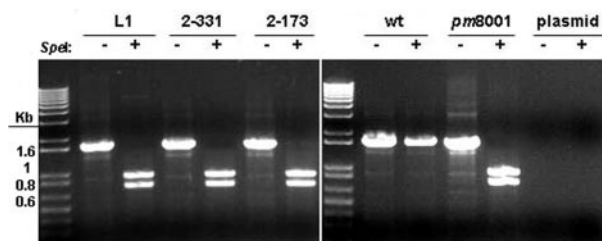


FIG. 3. Recombination assays. Low-molecular-weight DNA was isolated from transfected-infected cells and used as a template for PCR to amplify a DNA fragment containing the L1 ORF. PCR products from transfections using the three functional constructs, full-length, L1(2-331), and L1(2-173), were not digested (-) or digested (+) with SpeI. Controls used for PCR were DNA isolated from wild-type (wt) or *pm8001* infections and pCI-trip-L1 plasmid DNA (plasmid). The 1-kb Plus DNA ladder (Gibco BRL) is shown as the DNA standard.

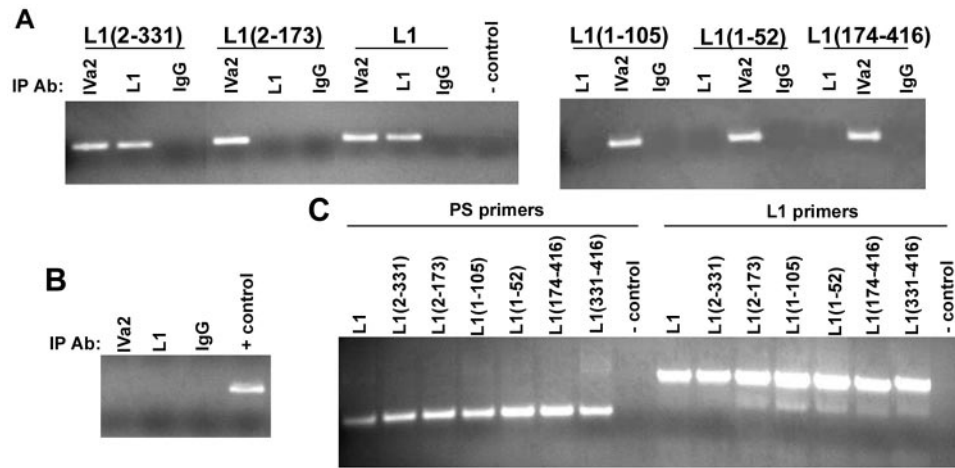


FIG. 4. ChIP assays. 293 cells were transfected-infected with the indicated constructs. Cross-linked nuclear extracts were used for immunoprecipitations with the indicated antibodies (IP Ab) to the L1 52/55-kDa or IVa2 proteins or rabbit IgG control. (A) After immunoprecipitation, ChIP DNA was used as a template for PCR to amplify the packaging sequence. No template (– control) was used as a negative control for the PCR. (B) The immunoprecipitates from the sample which was transfected with the wild-type L1 protein were amplified using primers that recognize the L1 ORF. The same lack of amplification of the L1 ORF was obtained with all constructs (data not shown). The control used for the L1 PCR was the wild-type L1 input chromatin DNA (+ control). (C) Input chromatin DNA was used as a template control for PCR to detect the presence of the PS and L1 DNA targets before immunoprecipitation.

requires the binding of the L1 52/55-kDa protein to the packaging sequence and possibly to other *trans*-acting factors. Furthermore, amino acids between 173 and 331 are essential for the interaction with the packaging sequence, although amino acids 1 to 173 are also required since the L1(173-416) mutant does not bind. Our mutant proteins do not allow us to determine whether binding to the IVa2 protein is required for complementation, since all of the mutants that bind the packaging sequence also bind the IVa2 protein. We constructed two additional mutants, a truncation containing amino acids 105 to 416 and a deletion mutant lacking amino acids 105 to 173, but neither construct was expressed to detectable levels in cells after transfection.

Alignment of the protein sequences from several different human adenovirus serotypes (Ad2, Ad5, Ad7, Ad10, Ad12, Ad17, and Ad40) as well as from other species, such as sheep, mouse, and monkey, indicates that the L1 52/55-kDa protein is highly conserved, with approximately 78% overall sequence identity. However, the first 60 amino acids (N terminus) and the last 50 amino acids (C terminus) of the protein have a lower degree of identity, which might argue against an important role for these sequences during the adenovirus life cycle. However, the variability in both ends might be the reason for the serotype specificity of the L1 52/55-kDa protein: the L1 52/55-kDa protein from adenovirus subgroups A, B, and D cannot complement the defect of the Ad5 *pm8001* mutant virus (subgroup C) (32).

It is likely that the mutation in the H5ts369 virus affects the conformation of the protein at the nonpermissive temperature. Analysis of the ability of the L1 52/55-kDa protein from this virus to bind the packaging sequence or the IVa2 protein when grown at the nonpermissive temperature indicated no defect in either activity, however (data not shown). This suggests that a third, unknown function is affected. A recent report identified three forms of the L1 52/55-kDa protein (25). The authors

proposed that the processed products of the L1 52/55-kDa protein might participate in later stages of the virus maturation process, such as DNA packaging and maturation of virions, the last steps in the formation of virions during adenovirus life cycle. The same three forms of the L1 52/55-kDa protein were previously reported as degradation or cleavage products (11). The identification of a consensus recognition site in the L1 52/55-kDa protein for the 23K viral protease suggests that a 47K protein might be produced as a result of the protease activity after amino acid position 351 (27). We found this position to be identical in all serotypes compared, suggesting that cleavage is important.

Although the L1 52/55-kDa protein is encoded in the major late transcription unit, it is also expressed during the early phase of adenovirus infection (1, 18, 24), suggesting that it might play additional roles during adenovirus infection. While the *pm8001* mutant does not exhibit any early phenotypes in culture (8), it is possible that such an early role exists during a natural infection. The L1 52/55-kDa protein was first reported to have a scaffolding role in adenovirus assembly, since it is not found in mature virions but is detected in empty capsids, assembly intermediates, and young virions (11). The fact that empty capsids are formed in the absence of the L1 52/55-kDa protein expression upon cell infection with *pm8001* virus suggests that the L1 52/55-kDa protein might not be involved in the formation of the capsid shells (7). However, the role of the L1 52/55-kDa protein in packaging of the viral genome is well established, since cells infected with H5ts369 at the nonpermissive temperature accumulate empty capsids associated with only the left end of the genome (12). Furthermore, in the absence of the L1 52/55-kDa protein, empty capsids are formed with no viral genome associated, supporting this conclusion (7). More recently, the L1 52/55-kDa protein has been shown to bind *in vivo* to the packaging sequence (19, 20), a sequence absolutely required for encapsidation of the adenovirus genome (3, 4, 13,

22). Another recent report used *in vitro* pulldown assays, in which biotin-labeled A1-A2 repeats of the packaging sequence were used as a bait to identify DNA binding proteins from Ad5 infected cell nuclear extracts (29). The authors suggested that the L1 52/55-kDa protein binds to the DNA nonspecifically and speculated this is mediated by the IVa2 protein (29). However, based on ChIP assays with Ad5 wild-type-, *pm8001*-, or *pm8002*-infected cells, we and others have demonstrated that the IVa2 and L1 52/55-kDa proteins bind independently to the packaging sequence *in vivo* and that L1 52/55-kDa protein binding is sequence specific (19, 20). Although both the IVa2 and L1 52/55-kDa proteins play a critical role in adenovirus assembly, the interaction between these proteins might be unrelated to packaging sequence binding. Consistent with this hypothesis, we show that the L1(1-173) protein is impaired in its ability to bind to the packaging sequence and to assemble mature virions even though it binds the IVa2 protein. The L1 52/55-kDa and IVa2 proteins have also been reported to bind to pVII (29), the major core protein, suggesting these proteins might form a higher-order complex that plays a role in encapsidation. Further experimentation will be required to assess whether the L1 52/55-kDa-IVa2 interaction is required for DNA packaging, however. Taken together, these results suggest that the L1 52/55-kDa-IVa2 protein interaction does not depend on binding to the packaging sequence and that, furthermore, there might be two different populations of the L1 52/55-kDa protein involved in the two functions, IVa2 interaction and DNA binding.

In summary, we find that the L1 52/55-kDa protein interacts with the IVa2 protein by using the N-terminal 173 amino acids of the protein, while 331 amino-terminal residues are essential for the interaction with the packaging sequence. Furthermore, the ability of the virus to encapsidate its genome correlates with binding of the L1 52/55-kDa protein to the packaging sequence. While the exact biochemical function of the L1 52/55-kDa protein remains elusive, a continued dissection of its roles in assembly should lead us to such an understanding.

ACKNOWLEDGMENTS

We thank the members of the Imperiale laboratory for help with this work and discussions, Kathy Spindler and Dave Miller for critical reading of the manuscript, Tom Shenk for the H5ts369 virus, and the Hybridoma Core of the University of Michigan.

This work was supported by an award from the American Heart Association to P.P.-R., by R01 AI52150 from the NIH to M.J.I., and (in part) by the NIH through a University of Michigan Cancer Center Support Grant (5 P30 CA46592).

REFERENCES

1. Akusjarvi, G., and H. Persson. 1981. Controls of RNA splicing and termination in the major late adenovirus transcription unit. *Nature* **292**:420-426.
2. Dignam, J. D., R. M. Lebovitz, and R. G. Roeder. 1983. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* **11**:1475-1489.
3. Grable, M., and P. Hearing. 1990. Adenovirus type 5 packaging domain is composed of a repeated element that is functionally redundant. *J. Virol.* **64**:2047-2056.
4. Grable, M., and P. Hearing. 1992. *cis* and *trans* requirements for the selective packaging of adenovirus type 5 DNA. *J. Virol.* **66**:723-731.
5. Graham, F. L., and L. Prevec. 1991. Manipulation of adenovirus vectors. *Methods Mol. Biol.* **197**:109-128.
6. Graham, F. L., J. Smiley, W. C. Russell, and R. Nairn. 1977. Characteristics of a human cell line transformed by DNA from human adenovirus type 5. *J. Gen. Virol.* **36**:59-74.
7. Gustin, K. E., and M. J. Imperiale. 1998. Encapsidation of viral DNA requires the adenovirus L1 52/55-kilodalton protein. *J. Virol.* **72**:7860-7870.
8. Gustin, K. E., P. Lutz, and M. J. Imperiale. 1996. Interaction of the adenovirus L1 52/55-kilodalton protein with the IVa2 gene product during infection. *J. Virol.* **70**:6463-6467.
9. Hammarskjold, M. L., and G. Winberg. 1980. Encapsidation of adenovirus 16 DNA is directed by a small DNA sequence at the left end of the genome. *Cell* **20**:787-795.
10. Harris, K. F., J. B. Christensen, and M. J. Imperiale. 1996. BK virus large T antigen: interactions with the retinoblastoma family of tumor suppressor proteins and effects on cellular growth control. *J. Virol.* **70**:2378-2386.
11. Hasson, T. B., D. A. Ornelles, and T. Shenk. 1992. Adenovirus L1 52- and 55-kilodalton proteins are present within assembling virions and colocalize with nuclear structures distinct from replication centers. *J. Virol.* **66**:6133-6142.
12. Hasson, T. B., P. D. Soloway, D. A. Ornelles, W. Doerfler, and T. Shenk. 1989. Adenovirus L1 52- and 55-kilodalton proteins are required for assembly of virions. *J. Virol.* **63**:3612-3621.
13. Hearing, P., R. J. Samulski, W. L. Wishart, and T. Shenk. 1987. Identification of a repeated sequence element required for efficient encapsidation of the adenovirus type 5 chromosome. *J. Virol.* **61**:2555-2558.
14. Hirt, B. 1967. Selective extraction of polyoma DNA from infected mouse cell cultures. *J. Mol. Biol.* **26**:365-369.
15. Logan, J., and T. Shenk. 1984. Adenovirus tripartite leader sequence enhances translation of mRNAs late after infection. *Proc. Natl. Acad. Sci. USA* **81**:3655-3659.
16. Lutz, P., and C. Keding. 1996. Properties of the adenovirus IVa2 gene product, an effector of late-phase-dependent activation of the major late promoter. *J. Virol.* **70**:1396-1405.
17. Lutz, P., F. Puvion-Dutilleul, Y. Lutz, and C. Keding. 1996. Nucleoplasmic and nucleolar distribution of the adenovirus IVa2 gene product. *J. Virol.* **70**:3449-3460.
18. Nevins, J. R., and M. C. Wilson. 1981. Regulation of adenovirus-2 gene expression at the level of transcriptional termination and RNA processing. *Nature* **290**:113-118.
19. Ostapchuk, P., J. Yang, E. Auffarth, and P. Hearing. 2005. Functional interaction of the adenovirus IVa2 protein with adenovirus type 5 packaging sequences. *J. Virol.* **79**:2831-2838.
20. Perez-Romero, P., R. E. Tyler, J. R. Abend, M. Dus, and M. J. Imperiale. 2005. Analysis of the interaction of the adenovirus L1 52/55-kilodalton and IVa2 proteins with the packaging sequence *in vivo* and *in vitro*. *J. Virol.* **79**:2366-2374.
21. Robinson, C. C., and C. Tibbetts. 1984. Polar encapsidation of adenovirus DNA: evolutionary variants reveal dispensable sequences near the left ends of Ad3 genomes. *Virology* **137**:276-286.
22. Schmid, S. I., and P. Hearing. 1997. Bipartite structure and functional independence of adenovirus type 5 packaging elements. *J. Virol.* **71**:3375-3384.
23. Schmid, S. I., and P. Hearing. 1998. Cellular components interact with adenovirus type 5 minimal DNA packaging domains. *J. Virol.* **72**:6339-6347.
24. Shaw, A. R., and E. B. Ziff. 1980. Transcripts from the adenovirus-2 major late promoter yield a single early family of 3' coterminal mRNAs and five late families. *Cell* **22**:905-916.
25. Sutjipto, S., S. Ravindran, D. Cornell, Y. H. Liu, M. Horn, T. Schlupe, B. Hutchins, and G. Vellekamp. 2005. Characterization of empty capsids from a conditionally replicating adenovirus for gene therapy. *Hum. Gene Ther.* **16**:109-125.
26. Tribouley, C., P. Lutz, A. Staub, and C. Keding. 1994. The product of the adenovirus intermediate gene IVa2 is a transcriptional activator of the major late promoter. *J. Virol.* **68**:4450-4457.
27. Webster, A., S. Russell, P. Talbot, W. C. Russell, and G. D. Kemp. 1989. Characterization of the adenovirus proteinase: substrate specificity. *J. Gen. Virol.* **70**:3225-3234.
28. Winter, N., and J. C. D'Halluin. 1991. Regulation of the biosynthesis of subgroup C adenovirus protein IVa2. *J. Virol.* **65**:5250-5259.
29. Zhang, W., and R. Arcos. 2005. Interaction of the adenovirus major core protein precursor, pVII, with the viral DNA packaging machinery. *Virology* **334**:194-202.
30. Zhang, W., and M. J. Imperiale. 2000. Interaction of the adenovirus IVa2 protein with viral packaging sequences. *J. Virol.* **74**:2687-2693.
31. Zhang, W., and M. J. Imperiale. 2003. Requirement of the adenovirus IVa2 protein for virus assembly. *J. Virol.* **77**:3586-3594.
32. Zhang, W., J. A. Low, J. B. Christensen, and M. J. Imperiale. 2001. Role for the adenovirus IVa2 protein in packaging of viral DNA. *J. Virol.* **75**:10446-10454.