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Analysis of the Interaction of the Adenovirus L1 52/55-Kilodalton and IVa2 Proteins with the Packaging Sequence In Vivo and In Vitro

Pilar Perez-Romero, Ryan E. Tyler, Johanna R. Abend, Monica Dus,† and Michael J. Imperiale*

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

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We previously showed that the adenovirus IVa2 and L1 52/55-kDa proteins interact in infected cells and the IVa2 protein is part of two virus-specific complexes (x and y) formed in vitro with repeated elements of the packaging sequence called the A1-A2 repeats. Here we demonstrate that both the IVa2 and L1 52/55-kDa proteins bind in vivo to the packaging sequence and that each protein-DNA interaction is independent of the other. There is a strong and direct interaction of the IVa2 protein with DNA in vitro. This interaction is observed when probes containing the A1-A2 or A4-A5 repeats are used, but it is not found by using an A5-A6 probe. Furthermore, we show that complex x is likely a heterodimer of IVa2 and an unknown viral protein, while complex y is a monomer or multimer of IVa2. No in vitro interaction of purified L1 52/55-kDa protein with the packaging sequence was found, suggesting that the L1 52/55-kDa protein–DNA interaction may be mediated by an intermediate protein. Results support roles for both the L1 52/55-kDa and IVa2 proteins in DNA encapsidation.

Assembly of adenovirus particles is a multistep process that occurs in the nucleus, where the replication of viral DNA coupled with the accumulation of large quantities of viral structural polypeptides sets the stage for virus assembly (2, 39). It has been well established that packaging of the adenovirus genome involves the specific recognition of the viral DNA packaging sequence. The packaging sequence contains at least seven functional units called A repeats (12, 21, 26, 35). Recent studies have focused on characterizing cellular and viral components that interact with the packaging sequence. A group of cellular *trans*-acting factors, called the P complex, was described to have a strong affinity for the A1 and A5-A7 repeats (38), which correlate with maximum packaging activity in vivo (37). Recently one of the functional components of the P complex was identified as CCAAT displacement protein (CDP) (10). CDP is a highly conserved cellular protein that is generally found to function as a transcriptional repressor (7, 30); it is a 200-kDa protein that is cleaved in S phase into a 110-kDa fragment, which has four DNA binding domains, and an 85-kDa fragment (22).

The IVa2 protein is a viral product that was first reported to be a transcriptional activator of the adenovirus major late promoter (31, 32, 40). More recently, the IVa2 protein has been shown to bind to the A repeats in the packaging domain in vitro (42). Characterization of a IVa2 mutant virus, *pm*8002, demonstrated that no viral capsids or particles were formed during infection of 293 cells (43). These results support the idea that IVa2 plays a role in DNA packaging. The IVa2 protein was also shown by use of the yeast two-hybrid system to interact with the viral L1 52/55-kDa protein (20). In addition, the IVa2 protein is able to interact with a glutathione *S*-transferase (GST)–L1 52/55-kDa fusion protein in vitro and with the L1 52/55-kDa protein in infected cells (20).

The L1 52/55-kDa protein is a nuclear phosphoprotein that is present in empty capsids, assembly intermediates, and young virions but is not found in mature virions (24), suggesting a scaffolding role for this protein. Immunoelectron microscopy studies determined that the L1 52/55-kDa protein is located in infected nuclei at sites associated with virion assembly intermediates that are distinct from viral replication centers (24). The role of the L1 52/55-kDa protein in DNA packaging has been well established. An L1 52/55-kDa temperature-sensitive mutant, Ad5*ts*369, produces empty capsids associated with the left end of the viral genome at the nonpermissive temperature (25). Infection with a mutant virus, *pm*8001, which is unable to produce the L1 52/55-kDa protein, resulted in normal early and late viral gene expression and DNA replication but production of only empty capsids (19). However, no evidence of binding of the L1 52/55-kDa protein to DNA was found in vitro, suggesting that the IVa2-L1 52/55-kDa protein interaction may not occur while the IVa2 protein is bound to the viral DNA (42).

The traditional model for adenovirus assembly has been proposed to begin with the formation of empty capsids followed by the insertion of viral DNA. This model is based on pulse-chase analyses and the study of temperature-sensitive viral mutants (4, 5, 9). However, more recent results obtained with the IVa2 mutant virus, as well as with mutants in the gene encoding the L4 33-kDa protein, point to a model in which capsids are assembled around the DNA (11, 43). In this model, DNA binding proteins bind to viral DNA, triggering the subsequent events that lead to capsid formation around the DNA.

In this report we describe the in vivo interaction of the IVa2 and L1 52/55-kDa proteins with the packaging sequence as determined by chromatin immunoprecipitation (ChIP) analysis. The in vivo interaction of the IVa2 protein with the pack-

* 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: Cold Spring Harbor Laboratory, Cold Spring Harbor, NY 11724.

aging sequence is independent of the L1 52/55-kDa protein, and conversely, the L1 52/55-kDa protein interaction with the packaging sequence is independent of the IVa2 protein. In vitro results reveal a strong and direct interaction of the IVa2 protein with the DNA, while for purified L1 52/55-kDa protein, no direct interaction with the DNA is detected. The results presented here support a role for the interaction of both the L1 52/55-kDa and IVa2 proteins with the viral packaging sequence in DNA encapsidation.

MATERIALS AND METHODS

Cells and viruses. 293 cells are adenovirus-transformed human embryonic kidney cells (15). 293 cells that stably express the L1 52/55-kDa protein (293-L1 cells) were used as a helper cell line to grow *pm*8001 virus, which fails to express the L1 52/55-kDa protein (19). 293 cells that stably express the IVa2 protein (293-IVa2 cells) were used as a helper cell line to grow *pm*8002, which fails to express the IVa2 protein (43). A549 cells are human lung carcinoma cells, and 3T3 cells are derived from fibroblasts of disaggregated Swiss mouse embryos (29). All cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum. 293-L1 and 293-IVa2 cells were grown under selection with 500 μg of G418/ml. Wild-type Ad5 virus was propagated as previously described (14, 42). *Spodoptera frugiperda* (Sf21) insect cells were grown in SF-900 SFM medium (Invitrogen/Gibco) at 27°C.

Purified proteins and nuclear extracts. A GST fusion protein with the full-length L1 52/55-kDa protein was obtained by cloning the L1 52/55-kDa protein open reading frame (ORF) into the pGEX-3X vector and transforming the DNA into *Escherichia coli* BL21 cells. Induction of GST-L1 52/55-kDa protein expression was performed by adding 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) to exponential cultures for 2 h at 37°C. The expressed protein was purified using glutathione-agarose (Sigma) and ammonium sulfate precipitation. The hexahistidine-tagged IVa2 expression baculovirus was generated using the Bac-to-Bac baculovirus expression system from Invitrogen (Carlsbad, Calif.) according to the manufacturer's protocol. For protein purification, Sf21 insect cells were infected with the baculovirus at a multiplicity of infection of 5 PFU/cell. Fifty-milliliter aliquots of cells were harvested by centrifugation 96 h postinfection, and cell pellets were lysed in 5 ml of lysis buffer (50 mM NaH_2PO_4 [pH 8.0], 300 mM NaCl, 10 mM imidazole, 1% [vol/vol] NP-40). Cell debris was centrifuged at 10,000 × *g* at 4°C for 10 min, and the supernatant was bound to 0.3 ml of an 80% slurry of Ni-nitrilotriacetic acid agarose beads (QIAGEN). The bead complexes were collected by centrifugation and washed twice with wash buffer (50 mM NaH_2PO_4 [pH 8.0], 300 mM NaCl, 20 mM imidazole). The protein was eluted in 0.3 ml of elution buffer (50 mM NaH_2PO_4 [pH 8.0], 300 mM NaCl) for 15 min at 4°C with increasing concentrations of imidazole (100 to 300 mM) to decrease the copurification of nonspecific proteins. Proteins were dialyzed overnight. The purity of the isolated proteins was >90% as evidenced by Coomassie staining.

The cells used to prepare nuclear protein extracts were mock-infected 293 cells, 293-L1 cells, 293-IVa2 cells, or 293 cells infected with Ad5, *pm*8001, or *pm*8002 at a multiplicity of infection of 5 PFU/cell for 24 h. Nuclear extracts were prepared as described previously (6, 42).

Preparation of anti-IVa2 antiserum. Anti-IVa2 goat antiserum was generated by immunizing a goat with a GST-IVa2 fusion protein purified from bacteria. The pGEX-5X-3 GST fusion vector containing the IVa2 cDNA was described previously (43). The induction of the expression of the GST-IVa2 fusion protein was performed by adding 0.1 mM IPTG to exponential bacterial cultures for 3 h at 16°C. The protein was then purified by glutathione Sepharose-4B affinity. Immunization of the goat was performed by Bethyl Laboratories, Inc. (Montgomery, Tex.).

EMSA. DNA binding assays using nuclear extracts were performed as described (42). The probes were oligonucleotides of double-stranded DNA within a range of 42 to 45 bp, each spanning two consecutive A repeats of the Ad5 packaging sequence. A1-A2 repeats were nucleotides 236 to 278, A4-A5 repeats were nucleotides 315 to 358, and A5-A6 repeats were nucleotides 335 to 380. The full-length packaging sequence probe was prepared by PCR amplification using Ad5 genomic DNA as a template and primers designed to amplify a DNA fragment from nucleotide 200 to nucleotide 370, which includes the A1 through A7 repeats. For purified proteins, electrophoretic mobility shift assays (EMSAs) were performed as described previously (1), with the following modifications. Radiolabeled probe was mixed with the protein of interest at the indicated concentrations in binding buffer (10 mM HEPES [pH 7.9], 20 mM KCl, 3 mM EDTA, 12% glycerol, 115 μg of bovine serum albumin per ml, 5 μg of dI-dC/ml, 1 mM dithiothreitol) for 30 min at 4, 25, or 30°C. Binding reactions were resolved on 5% nondenaturing polyacrylamide gels in 0.5× Tris-borate-EDTA buffer at 200 V for ~2 h at 4°C. For supershift assays, proteins were incubated with antibody on ice for 15 min and then added to the probe. The antibodies used for supershifts were polyclonal rabbit anti-IVa2, polyclonal goat anti-IVa2, polyclonal rabbit anti-L1 (20), monoclonal mouse anti-SV-40 large T antigen (TAg) (PAb416) (23), and polyclonal goat anti-CDP (C-20; Santa Cruz Biotechnology, Inc).

ChIP. Chromatin immunoprecipitation experiments were performed using the protocol described by Orlando et al. (33). Briefly, cells in 15-cm-diameter plates were cross-linked by addition of 1% formaldehyde in phosphate-buffered saline for 10 min at room temperature. Cross-linking was stopped by adding 125 mM glycine for 5 min. Cells were washed twice with phosphate-buffered saline and protease inhibitors (1 μM phenylmethylsulfonyl fluoride, 5 μg of aprotinin/ml, 5 μg of leupeptin/ml), collected, resuspended in 1 ml of hypotonic buffer (20 mM HEPES [pH 7.9], 1 mM EDTA, 1 mM EGTA), transferred to a glass Dounce homogenizer, and lysed with 25 strokes of a tight-fitting pestle. The nuclei were centrifuged at 10,000 × *g* for 1 min at 4°C and resuspended in 400 μl of lysis buffer (1% sodium dodecyl sulfate [SDS], 10 mM EDTA, 50 mM Tris-HCl [pH 8.1], protease inhibitors). Lysates were sonicated at 20% power for four pulses of 10 s each with a Sonicator 550 sonic dismembrator (Fisher Scientific) and centrifuged for 10 min at 10,000 × *g* at 4°C to remove debris.

Analysis of input chromatin DNA and input proteins was performed by analyzing 50 μl of the lysates. For immunoprecipitations, 100 μl of lysates was diluted 10 times with ChIP dilution buffer (1% Triton X-100, 2 mM EDTA, 150 mM NaCl, 20 mM Tris-HCl [pH 8.1], protease inhibitors) and precleared by adding 40 μl of protein G beads (50% slurry; Amersham) and 2 μg of sonicated salmon sperm DNA. The precleared lysates were then immunoprecipitated by using 4 μg of antibodies at 4°C overnight with rotation. Immunocomplexes were collected by adding 50 μl of protein G beads (Amersham); washed once each with TSEI buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.1], 150 mM NaCl), TSEII buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl [pH 8.1], 500 mM NaCl), and wash buffer (0.25 M LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris-HCl [pH 8.1]); and washed twice with TE buffer (10 mM Tris-HCl [pH 8], 1 mM EDTA). Immunocomplexes were eluted from the beads by incubating twice with 250 μl of elution buffer (1% SDS, 0.1 M $NaHCO_3$) for 10 min at room temperature. Cross-links were reversed by heating at 65°C in the presence of 200 mM NaCl followed by proteinase K treatment (10 mM EDTA, 40 mM Tris-HCl [pH 6.5], 20 μg of proteinase K) for 1 h at 45°C. The DNA was recovered by phenol-chloroform extraction followed by ethanol precipitation. The resulting DNA (ChIP DNA) was resuspended into 30 μl of distilled water. The ChIP DNA was used as template for PCR amplification using the following oligonucleotides: PS (packaging sequence) (forward, GGAAGTGACAATTTTCGCGCGGTT; reverse, GGTCAAAGTCCCCGCGGCCCTA), MLP (forward, TCGTCCTCAC TCCTCTTCCGCAT; reverse, CGTTTGCCACCAAGCTTGACAA), and L1 (forward, GCGGATCCTACATCCGGTGCTGCGGCAGAT; reverse, CGGA ATTCTACTCTCCCTCGCGGTCGCGAA).

Western blot analysis. Approximately 10 μg of nuclear or 40 μg of total cell extracts were boiled in 2× SDS sample buffer and separated on an SDS–10% polyacrylamide gel. Proteins were transferred to nitrocellulose and probed with a 1:10,000 dilution of rabbit polyclonal anti-IVa2 or anti-L1 antibodies. Proteins were visualized by using a secondary horseradish peroxidase-conjugated antibody and chemiluminescence as recommended by the manufacturer (Amersham).

RESULTS

L1 52/55-kDa and IVa2 proteins bind to the packaging sequence. Previous results from EMSA analyses demonstrated that the IVa2 protein interacts with the packaging sequence: two virus-specific complexes, x and y, were detected using a probe containing the A1-A2 repeats of the packaging sequence and nuclear extracts from infected cells (42). However, no evidence that the L1 52/55-kDa protein was part of the x or y complexes was found. For example, extracts from cells infected with the L1 null virus, *pm*8001, formed the same two complexes. However, this in vitro approach has some limitations, and as a result DNA-protein complexes that form in vivo might be missed in these assays.

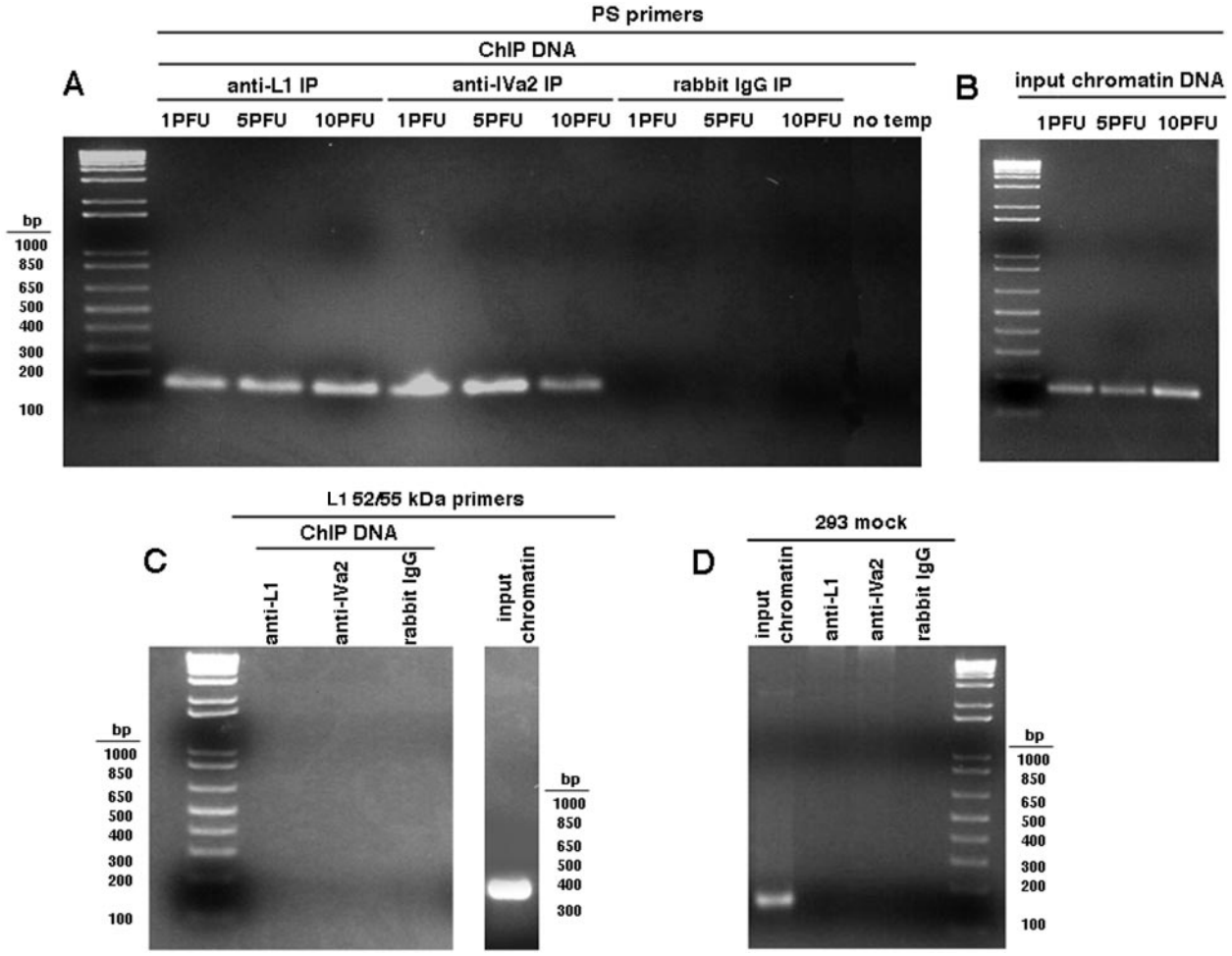

FIG. 1. The L1 52/55-kDa and IVa2 proteins interact in vivo with the packaging sequence (PS). Chromatin immunoprecipitation assays were performed on 293 cells infected with wild-type Ad5 at 1, 5, and 10 PFU. (A) Cross-linked nuclear lysates were used in immunoprecipitations with antibodies to L1 or IVa2 or with immunoglobulin G (IgG). After immunoprecipitation, the DNA (ChIP DNA) was isolated and used as template for PCR using primers to amplify the packaging sequence. No template (no temp) was used as a negative control for the PCR. (B) Input chromatin DNA was used as a control to detect the presence of the packaging sequence by PCR. (C) ChIP DNA and input chromatin DNA were amplified with primers specific for a 315-bp fragment of the L1 52/55-kDa protein ORF (52/55-kDa primers). (D) ChIP DNA and input chromatin DNA from mock-infected 293 cells were used to amplify the packaging sequence.

To continue studying IVa2 and L1 52/55-kDa protein–DNA interactions, we took advantage of the ChIP assay. This technique allows the identification of in vivo interactions of proteins of interest and their DNA binding sites (33). We performed ChIP analysis on cross-linked nuclear lysates from 293 cells infected with Ad5 at 1, 5, or 10 PFU/cell. PCR amplifications were performed using primers designed to amplify the entire packaging sequence, as well as a DNA fragment of 315 bp from the L1 52/55-kDa ORF, which was used as a control for specificity of binding. The results demonstrate that both the IVa2 and L1 52/55-kDa proteins interact with the packaging sequence, regardless of the multiplicity of infection (Fig. 1A). No PCR product was detected using immunoprecipitations with rabbit immunoglobulin G as a negative control (Fig. 1A) or using primers to the L1 region of the genome (Fig. 1C). Input chromatin DNA was used as a template to verify the presence of both the packaging sequence and L1 52/55-kDa sequences in the starting DNA (Fig. 1B and C). Using mock-infected 293 cells, we detected the packaging sequence in the starting DNA (these cells contain an integrated copy of the packaging sequence), but no PCR product was detected after immunoprecipitation (Fig. 1D). The same analysis was performed using Ad5-infected A549 cells, and the results were identical (data not shown).

Next, we were interested in determining whether binding of the L1 52/55-kDa and IVa2 proteins to the packaging sequence were interdependent. To test this hypothesis, additional ChIP assays were performed. 293 cells were infected at 10 PFU/cell with wild-type Ad5, *pm*8001, or *pm*8002, or they were mock infected. Input proteins were analyzed by Western blotting to detect expression of the L1 52/55-kDa and IVa2 proteins in the cells. As expected, no L1 52/55-kDa protein was detected in cell lysates from mock-infected or *pm*8001-infected cells, and no IVa2 protein was found in cell lysates from mock-infected or *pm*8002-infected cells (Fig. 2E). PCR using packaging sequence primers and ChIP DNAs as templates, obtained after immunoprecipitation with antibodies to the L1 and IVa2 proteins, indicated that binding of IVa2 protein to the packaging

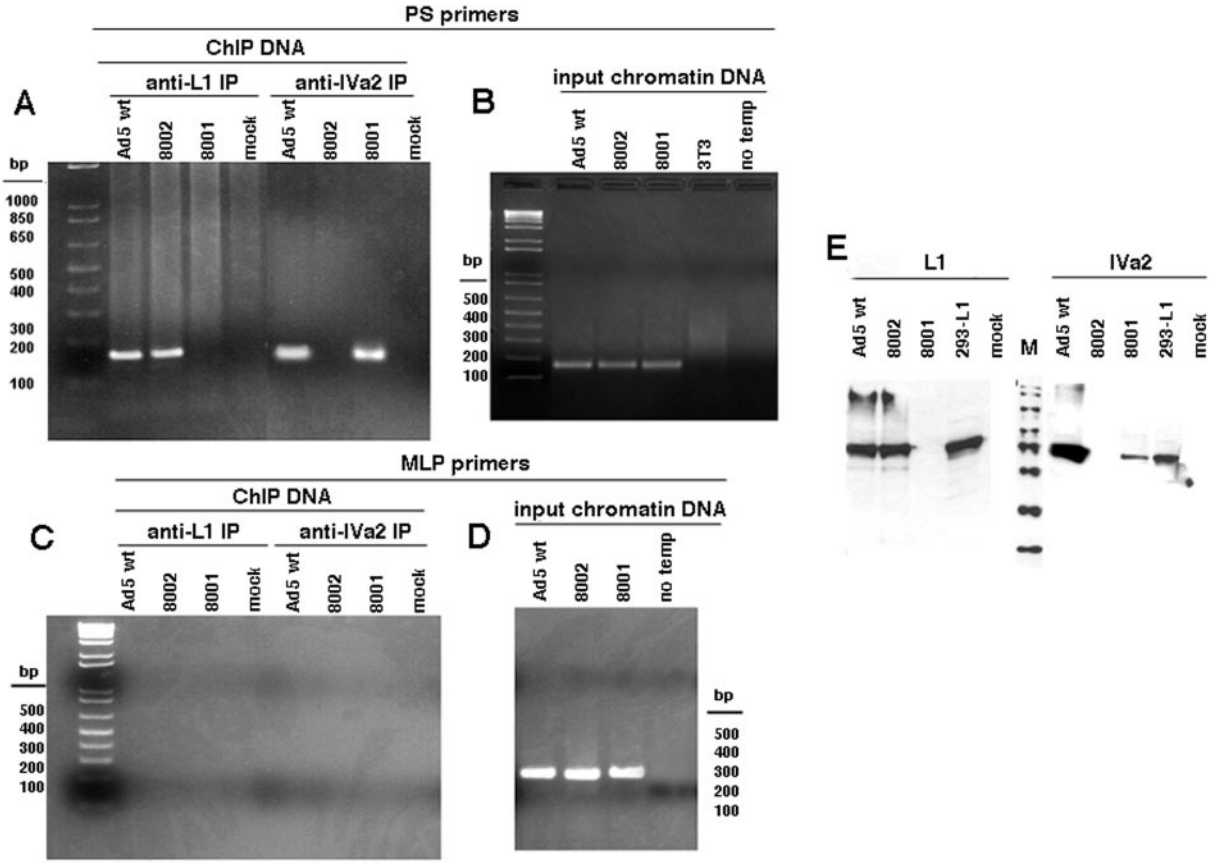

FIG. 2. The L1 52/55-kDa and IVa2 proteins are independent in their interaction with the packaging sequence (PS). Cross-linked nuclear lysates from 293 cells infected with wild-type Ad5 (Ad5 wt), *pm*8001 (8001), or *pm*8002 (8002) or mock-infected cells (mock) were used for ChIP assays with antibodies to L1 52/55-kDa and IVa2 proteins. (A) ChIP DNA was used as a template to amplify the packaging sequence by PCR. (B) Input chromatin DNA was used as a control to detect the packaging sequence. 3T3 cells were used as a negative control for the packaging sequence and no template (no temp) as a negative control for the PCR. (C) ChIP DNA was used to amplify the major late promoter (MLP) as a negative control. (D) Presence of the major late promoter sequence was tested by PCR amplification of the input chromatin DNA. (E) Input proteins were tested by Western blotting to detect expression of L1 52/55-kDa and IVa2 proteins. As a positive control, cell lysate from 293-L1 cells was used in the L1 blot, and 293-IVa2 cell lysate was used in the IVa2 blot. The MagicMark protein ladder (Invitrogen) (M) is shown as the protein standard.

sequence occurs in the absence of the L1 52/55-kDa protein, as shown in the *pm*8001 infections, and conversely, L1 52/55-kDa protein binding to the packaging sequence occurs in the absence of the IVa2 protein as shown in the *pm*8002 infections (Fig. 2A). Control amplifications using major late promoter primers showed no amplification products using the same ChIP DNAs as templates (Fig. 2C). Analysis of input chromatin DNA by PCR showed the presence of intact sequences for the packaging sequence and the major late promoter in all infected samples (Fig. 2B and D). These results suggest that the interaction of the IVa2 and L1 52/55-kDa proteins we previously described (20) is not required for DNA binding.

The IVa2 protein binds directly to the A1-A2 repeats of the packaging sequence. We next investigated whether purified IVa2 and L1 52/55-kDa proteins can bind directly to the packaging sequence. Using nuclear extracts from Ad5-infected cells in an EMSA analysis, we previously demonstrated that the IVa2 protein binds to A repeats in the packaging sequence (42). However, whether this was a direct interaction could not be determined because of the presence of all the other viral and cellular proteins in the nuclear extract. Thus, we performed EMSAs using purified IVa2 and L1 52/55-kDa proteins and a ^{32}P-labeled A1-A2 probe. We detected an interaction of the IVa2 protein alone (Fig. 3A, lanes 1 to 7) under all of the conditions assayed; relatively small amounts of IVa2 protein were sufficient to detect this interaction, being detected using as little as 0.6 nM purified IVa2 protein and 1.9 nM DNA. Furthermore, this binding is specific because a cold homologous probe used as a competitor completely blocked the binding (data not shown). When purified IVa2 protein was mixed with a polyclonal anti-IVa2 serum, the complex shifted to a higher molecular weight band, but no supershifts were detected when anti-L1 or anti-TAg antibodies were used in parallel (Fig. 3B). These results suggest that the IVa2 protein has a strong interaction with the packaging sequence, binding directly to the A1-A2 repeats in the absence of other viral proteins.

For the L1 52/55-kDa protein, no interaction with the A1-A2 probe was observed even at 320 nM protein (Fig. 3A, lanes 8 to 12). Taken together with the results from the ChIP assays, the results suggested that an intermediate protein, but not IVa2, may mediate the L1 52/55-kDa protein interaction with the DNA, or that the L1 52/55-kDa protein binds to the packaging sequence at different sites. When both the IVa2 and L1 52/55-kDa proteins were combined with the A1-A2 probe, we

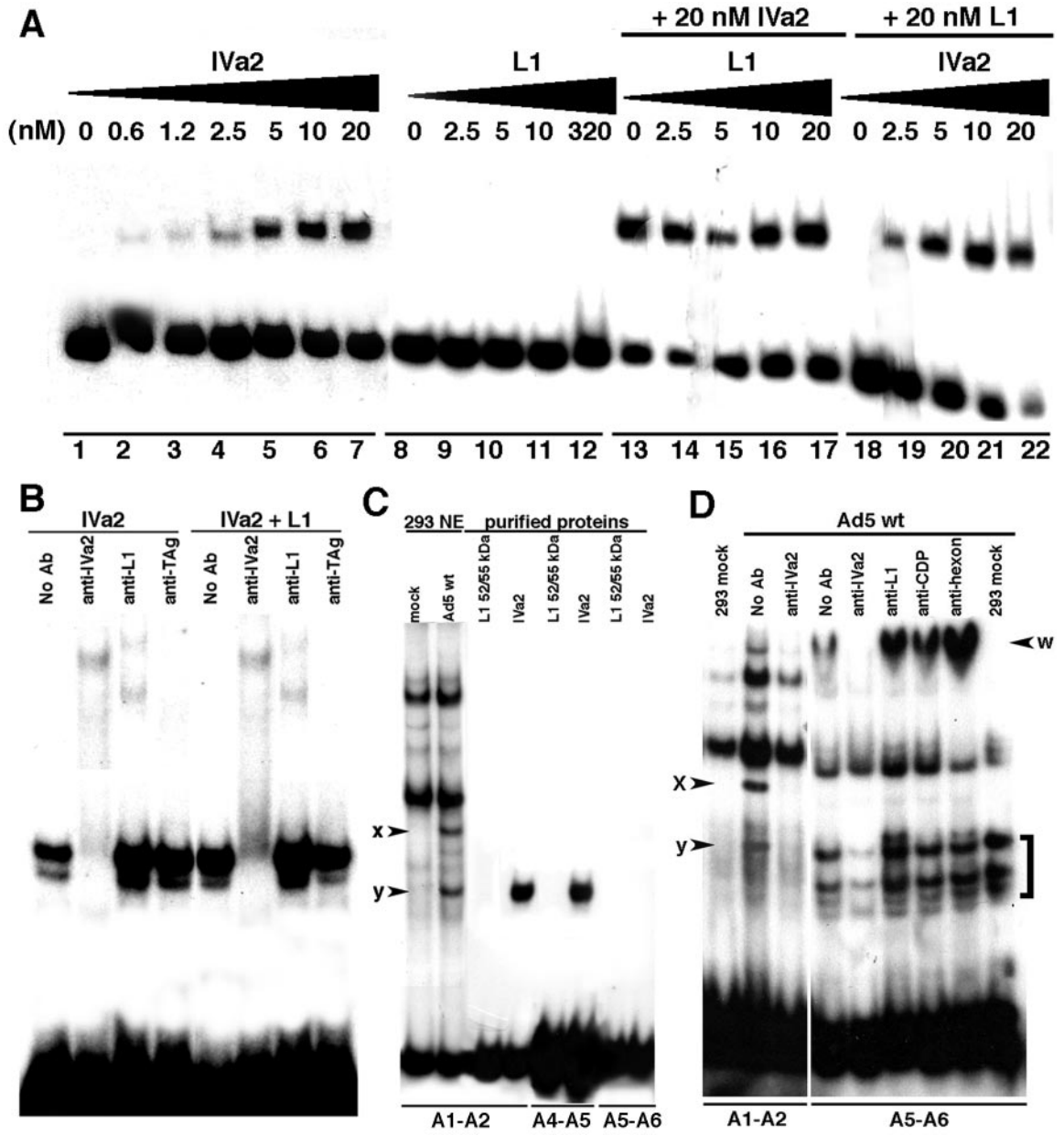

FIG. 3. Purified IV2 protein binds directly to A1-A2 and A4-A5 repeats in the packaging sequence. Electrophoretic mobility shift assays were performed using purified hexahistidine-tagged IVa2 and GST-L1 52/55-kDa proteins. (A) Titration of the proteins at the indicated concentrations using a ^{32}P labeled A1-A2 probe. Combinations of L1 52/55-kDa and IVa2 proteins were performed by maintaining one of the proteins at a constant concentration of 20 nM and increasing the concentration of the second protein from 0 to 20 nM. (B) Supershift assays were performed with purified IVa2 protein alone (IVa2) or in combination with L1 52/55-kDa protein (IVa2 + L1) using antibodies (Ab) to IVa2 (rabbit), L1, or TAg proteins, and a ^{32}P-labeled A1-A2 probe. (C) Electrophoretic mobility shift assays were performed using ^{32}P-labeled probes containing A1-A2, A4-A5, or A5-A6 repeats (as indicated at the bottom) and either nuclear extract (293 NE) prepared from mock-infected (mock) or Ad5-infected (Ad5 wt) 293 cells or purified IVa2 or L1 52/55-kDa proteins. Complexes x and y are labeled with arrows. (D) EMSAs were performed with nuclear extract from mock-infected 293 cells or Ad5-infected 293 cells and ^{32}P-labeled A1-A2 or A5-A6 probes. Supershifts were performed using antibodies to IVa2 (antipeptide), L1 52/55-kDa, CDP, and hexon proteins, as indicated. The bracketed portion indicates cellular complexes with the A5-A6 probes. w, virus-specific complex with A5-A6 probe.

detected the IVa2-DNA interaction, but no L1 52/55-kDa protein binding was observed (Fig. 3A, lanes 13 to 22). This confirms that there is no apparent ternary complex of the L1 52/55-kDa and IVa2 proteins with the DNA.

The IVa2 protein interacts with the A4-A5 but not the A5-A6 repeats of the packaging sequence. We next wanted to analyze interactions with the A4-A5 and A5-A6 repeats of the packaging sequence, to determine whether the two viral proteins preferentially bind different domains, potentially explaining why we did not detect an L1 52/55-kDa protein interaction with the A1-A2 repeats. To test this, EMSA experiments were performed using the conditions described above and ^{32}P-labeled A1-A2, A4-A5, and A5-A6 probes (Fig. 3C). The results suggest a preferential binding of the IVa2 protein to A1-A2 and

A4-A5. Surprisingly, no detectable protein-DNA complex was seen when an A5-A6 probe was used in the experiment. Nuclear extracts from Ad5-infected 293 cells were used as a positive control to detect complexes x and y. The IVa2-DNA complex obtained using purified IVa2 protein and either A1-A2 or A4-A5 probes was of a size similar to that of complex y obtained from the Ad5-infected nuclear extracts using the A1-A2 probe. The A5-A6 probe was used in shift assays with nuclear extracts from Ad5-infected cells to test the functionality of this probe (Fig. 3D). We detected protein-DNA complexes although they were different from those observed using the A1-A2 or A4-A5 probe. One of the differences observed was that the A5-A6 repeats can bind to cellular components that are distinct from those detected when the A1-A2 probe was used, as shown when nuclear extracts from mock-infected 293 cells were used. No x or y complexes were detected using nuclear extracts from Ad5-infected cells when the A5-A6 repeats were used. However, a virus-specific complex of high molecular weight was detected in nuclear extracts from Ad5-infected 293 cells, and it contains IVa2 since it was blocked after addition of antibody to IVa2 but not after addition of antibodies to L1 52/55-kDa, CDP, or hexon proteins. This result confirmed that the probe containing the A5-A6 repeats is functional. The L1 52/55-kDa protein did not bind to any of the probes used, supporting the idea that either an additional factor(s) or a longer probe encompassing the whole packaging sequence is required for this interaction. To test the possibility that the full length of the packaging sequence is necessary for the DNA-L1 52/55-kDa protein interaction, an additional EMSA was performed using purified IVa2 and L1 52/55-kDa proteins and a ^{32}P-labeled full-length packaging sequence probe. The IVa2 protein was detected in complexes with the packaging sequence, but no interaction was observed for the L1 52/55-kDa protein (data not shown), supporting the conclusion that other components are necessary for the DNA-L1 52/55-kDa protein interaction detected in vivo.

Analysis of IVa2 and L1 binding to the packaging sequence using nuclear extracts. Previously we described the formation of two complexes, x and y, that contain the IVa2 protein, as detected by EMSA using nuclear extracts from 293 cells infected with wild-type Ad5 and *pm*8001 (42). To extend this analysis, nuclear extracts were prepared from 293 cells infected with wild-type Ad5, *pm*8001, or *pm*8002 or from mock-infected cells, as well as from cell lines that stably express the IVa2 and L1 52/55-kDa proteins, 293-IVa2 and 293-L1, respectively. The protein lysates were analyzed by Western blotting to detect expression of the L1 52/55-kDa and IVa2 proteins (Fig. 4B). As expected, the IVa2 protein was detected in nuclear lysates from 293-IVa2 cells as well as from wild-type and *pm*8001 infections, and the L1 52/55-kDa protein was found in nuclear extracts from 293-L1 cells and cells infected with *pm*8002 and wild-type viruses. The extracts were used in supershift assays using antibodies to IVa2, L1 52/55-kDa, TAg, and CDP proteins and a ^{32}P-labeled A1-A2 probe (Fig. 4A). When nuclear extracts from mock-infected 293 cells were used, no x and y complexes were detected (Fig. 4A, lanes 1 to 5); however, as shown previously, we detected at least two cellular components that can bind to the A repeats (42). Neither of these was affected by the anti-CDP serum (Fig. 4A, lane 5). As shown in a previous report (42), the x and y complexes were detected in both wild-type (Fig. 4A, lanes 6 and 8 to 10) and *pm*8001-infected nuclear extracts (lanes 11 and 13 to 15), and both disappeared after being treated with antibody to IVa2 (lanes 7 and 12) but not with antibodies to the L1 52/55-kDa, TAg, or CDP proteins (lanes 9, 10, 13, and 14). Using nuclear extracts from *pm*8002-infected cells, neither the x nor the y complex was seen (Fig. 4A, lanes 16 to 20), confirming the conclusion that IVa2 is present in both complexes. However, two new complexes that migrate faster than the x and y complexes, x2 and y2, were detected. We previously described the detection of a smaller form of the IVa2 protein 24 h postinfection in *pm*8002-infected cells (43), and a recent report also identified a 40-kDa form of the protein due to the use of an internal methionine start codon (34). It is likely that the 40-kDa form of the IVa2 protein is present in the x2 and y2 complexes. While incubation with an antibody raised against a peptide in the N terminus of the IVa2 protein, which is missing in the 40-kDa protein, did not have any effect on the x2 and y2 complexes (Fig. 4A, lane 17), an antiserum prepared against the full-length IVa2 protein fused to GST, which does recognize the 40-kDa form (data not shown), supershifts complexes x and y from Ad5-infected 293 cells as well as complexes x2 and y2 from *pm*8002-infected 293 cells (Fig. 5). Interestingly, in extracts from 293 cells that constitutively express IVa2, only complex y is detected (Fig. 4A, lanes 15 to 21), and it is lost after incubation with antibody to IVa2 (lane 22). This is consistent with the finding that the same band was detected when EMSAs were performed with purified IVa2 protein. Nuclear extracts of 293 cells expressing the L1 52/55-kDa protein, in contrast to mock-infected cells, showed no specific bands (Fig. 4A, lanes 26 to 29). However, an examination of Fig. 4A, lanes 26 to 29, indicates that the higher molecular weight cellular complex (C-1) observed in mock, wild-type, *pm*8001 and *pm*8002-infected cells is missing. It seems that in the presence of the L1 52/55-kDa protein and absence of the IVa2 protein, the interaction of this cellular component with the packaging sequence is disrupted.

DISCUSSION

Adenovirus DNA encapsidation is dependent on *cis*-acting sequences located at the left end of the genome, between nucleotides 194 and 380 in Ad5. Seven repeats, which are called A repeats because of their A/T-rich content, have been identified within this domain. A1, A2, A5, and A6 have been described as the most important repeats for packaging activity (12, 26, 37). Although these are redundant elements, they are not functionally equivalent (12, 13).

Using EMSA analysis, we previously described the binding of the adenovirus IVa2 protein but not the L1 52/55-kDa protein to the packaging sequence in vitro (42). Experiments presented here show that in vivo, both the IVa2 and L1 52/55-kDa proteins are bound to the packaging sequence. In addition, we demonstrate that purified IVa2 protein binds directly to the packaging sequence in vitro, preferentially to the A1-A2 and A4-A5 repeats and not to the A5-A6 repeats. We detected an interaction of the IVa2 protein with A5-A6 repeats only when nuclear extracts were used, and the DNA-protein complexes detected were distinct from those observed when A1-A2 repeats were used. These results suggest that the IVa2 protein

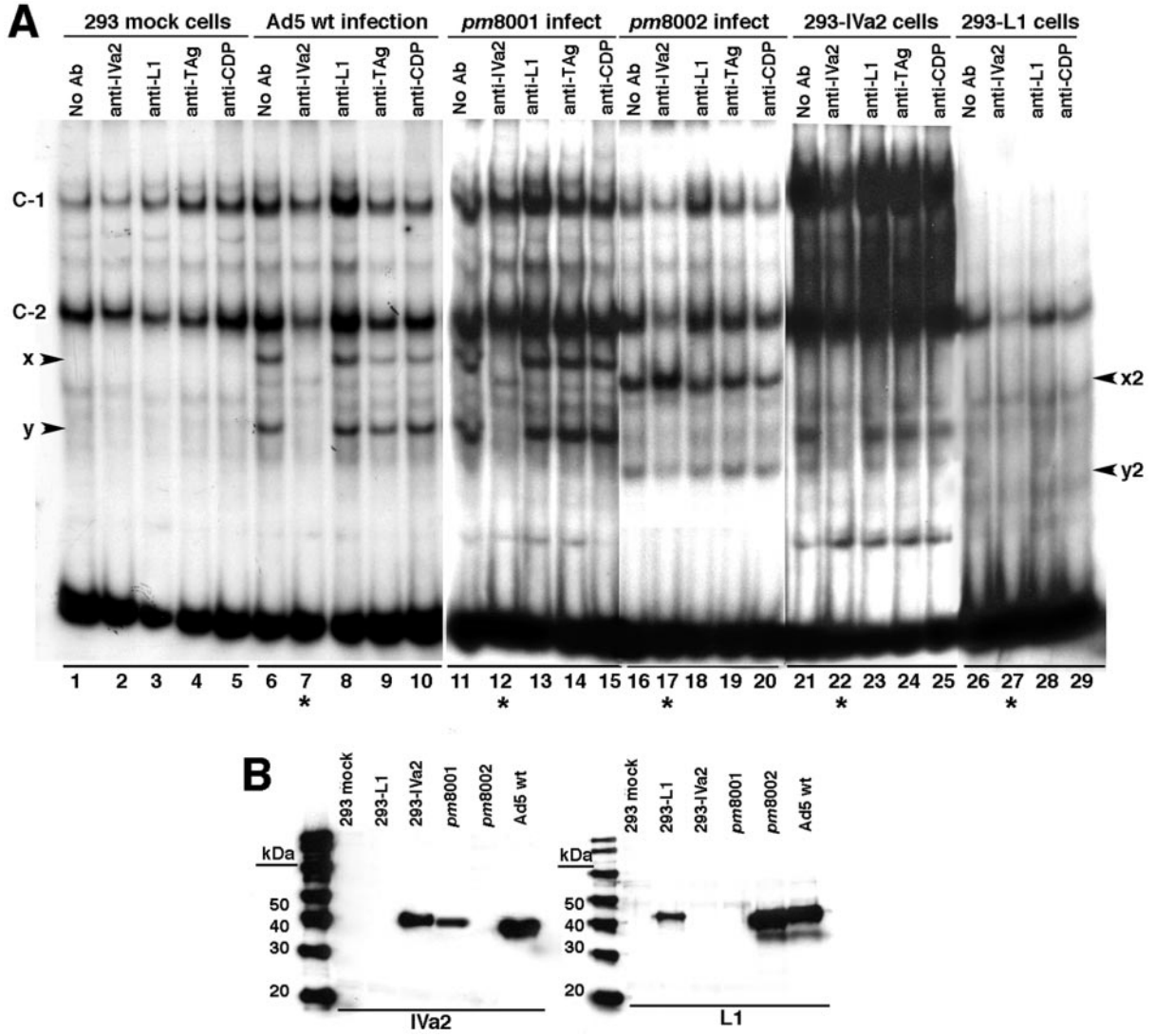

FIG. 4. IVa2 and L1 binding to the packaging sequence using nuclear extracts. (A) Electrophoretic mobility shift assays were performed with nuclear extracts prepared from mock-infected 293 (293 mock) cells, wild-type Ad5 (Ad5 wt)-infected 293 cells, *pm*8001-infected cells, *pm*8002-infected cells, mock-infected 293-IVa2 cells, or mock-infected 293-L1 cells and a ^{32}P-labeled A1-A2 probe. Supershifts were performed using antibodies (Ab) to IVa2 (antipeptide, lanes labeled with asterisks), L1 52/55 kDa, or CDP proteins. Complexes x, y, x2, and y2 are labeled with arrows. Cellular DNA-protein complexes are labeled as C-1 and C-2. (B) Western blots using nuclear extracts from mock-infected 293 (293 mock), 293-IVa2, and 293-L1 cells and 293 cells infected with wild-type Ad5 (Ad5 wt), *pm*8001, or *pm*8002 and probed with rabbit polyclonal antibodies to IVa2 and L1 52/55-kDa proteins.

interaction with A5-A6 repeats is mediated by or dependent on the presence of other cellular or viral proteins. In previous studies we showed that the IVa2 protein is present in two complexes, x and y, when nuclear extracts from infected cells are used in EMSA assays with the A1-A2 probe (42). Binding studies in this report indicate that complex x is a heterodimer consisting of the IVa2 protein and a second, unknown viral protein or virally induced factor, since purified IVa2 protein does not form this complex. Furthermore, we can conclude that the missing protein is not the L1 52/55-kDa protein since we detect the same x and y complexes in extracts from cells infected with *pm*8001, which does not express the L1 52/55-kDa protein (42). In contrast, complex y contains either a monomeric or multimeric form of IVa2 protein, since it is detected using either 293 cells expressing IVa2 alone or purified IVa2 protein in the shift assays, and it is specifically supershifted by incubation with anti-IVa2 antibody. This is consistent with a previous report that demonstrated that the IVa2 protein binds DNA in the major late promoter as a dimer (31).

Previously we detected a smaller form of the IVa2 protein in *pm*8002-infected A549 cells (43), and more recently a 40-kDa IVa2 protein has been described as the result of initiation of translation at Met75 of the open reading frame (34). When we used nuclear extracts from 293 cells infected with *pm*8002 virus in EMSA assays, we detected two complexes migrating faster than the x and y complexes from a wild-type infection (x2 and y2). Since no wild-type IVa2 protein is detected in the *pm*8002-infected cell nuclear extracts, and complexes x2 and y2 disappeared upon incubation with an anti-IVa2 antiserum that recognizes the smaller form, we conclude that these two faster

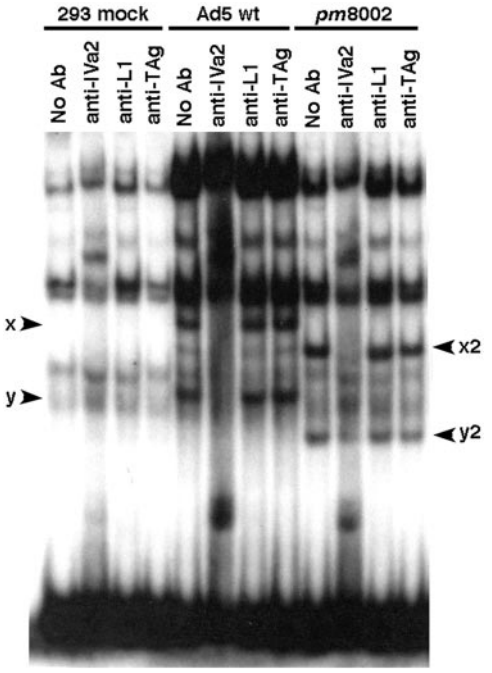

FIG. 5. Binding of the 40-kDa form of IVa2 protein to the packaging sequence. Electrophoretic mobility shift assays were performed with nuclear extracts prepared from mock-infected 293 cells (293 mock), wild-type Ad5-infected 293 cells (Ad5 wt), or *pm*8002-infected cells and the ^{32}P-labeled A1-A2 probe. Supershifts were performed using goat polyclonal anti-IVa2, anti-L1 52/55-kDa protein, or anti-CDP antibodies (Ab). Complexes x, y, x2, and y2 are labeled with arrows.

complexes contain the 40-kDa form of the IVa2 protein. In a *pm*8002 infection, no virus progeny are produced, suggesting that the 40-kDa protein is not functional for virus assembly. However, the recent report from Pardo-Mateos and Young suggested that the 40-kDa form of the IVa2 protein is functional for virus replication and for binding to the packaging sequence (34). The difference in virus viability may be explained by differences in expression levels of the 40-kDa IVa2 protein in the two mutant viruses.

Previously, we were unable to detect an interaction of the L1 52/55-kDa protein with the packaging sequence using EMSA analyses (42). In this report, we demonstrate that the packaging sequence can be specifically coimmunoprecipitated from infected cells with the L1 52/55-kDa protein. This finding does not necessarily contradict the lack of binding obtained in vitro using purified protein or nuclear extracts. In the in vivo approach (i.e., Ad5 infection), all the viral and cellular proteins plus the entire packaging sequence are available for interactions. In the in vitro experiments, fragments of the packaging sequence of approximately 45 bp, each containing two consecutive A repeats, were used as probes. One possible explanation for this discrepancy is that the probes used in vitro do not contain the minimal DNA sequences required for binding of the L1 52/55-kDa protein to the packaging sequence. However, the lack of binding does not appear to be due to such a requirement, since no DNA–L1 52/55-kDa protein complex was detected using the entire packaging sequence as a probe for shift assays. In addition, we were unable to detect any such complexes using various other binding and electrophoresis conditions. Further studies will determine whether the interaction of the L1 52/55-kDa protein with the packaging sequence, detected in vivo by ChIP assays, is mediated by other proteins. It is clear, however, that the L1 52/55-kDa protein can bind to the packaging sequence in the absence of the IVa2 protein and vice versa. The detection of the 40-kDa form of the IVa2 protein in *pm*8002-infected cells suggested the possibility that the interaction of the L1 52/55-kDa protein with the DNA detected by the ChIP assay of *pm*8002-infected cells may be mediated by this 40-kDa form of IVa2. To address this question, coimmunoprecipitations of 293 cell lysates from wild-type Ad5-, *pm*8002-, or mock-infected cells, using goat polyclonal anti-IVa2 and anti-L1 52/55-kDa sera, were performed. No interaction of the 40-kDa and L1 52/55-kDa proteins was detected (data not shown), supporting the conclusion that the L1 52/55-kDa protein interaction with the DNA is IVa2 independent.

DNA encapsidation in bacteriophages has been extensively studied (8, 28). These viruses have some parallels with the adenovirus system. For example, bacteriophage ϕ29 is a double-stranded DNA phage that contains a packaging signal located at one end of the genome and a terminal protein (gp3) which is covalently bound to the 5′ end of the genome (16, 17), similar to adenovirus. In phage ϕ29 a stable capsid shell, called the procapsid, is produced first, and then the viral DNA is inserted (18). The mechanism by which this occurs has been extensively studied. Less is known about the molecular mechanism for adenovirus, however. Several viral and cellular protein components have been reported to play a role in DNA packaging. By analyzing infections with the *pm*8001 and *pm*8002 mutants, we demonstrated the essential role of the L1 52/55-kDa and IVa2 proteins, respectively, in this process (19, 43). The absence of the L1 52/55-kDa protein leads to empty capsid accumulation, while the lack of the IVa2 protein results in the absence of any kind of viral particles. Although it is clear that the L1 52/55-kDa and IVa2 proteins are involved in DNA encapsidation, such a difference in phenotype when either of the proteins is missing argues against a completely overlapping function of both proteins during DNA packaging. CDP has been characterized as a cellular *trans*-acting factor that binds to the packaging sequence and is present in virions. Interestingly, this protein preferentially binds to the A5-A7 repeats (10), while we demonstrate here that the IVa2 protein binds directly to both of the A1-A2 and A4-A5 repeats. Thus, the binding sites for those two proteins may not overlap. However, CDP demonstrates a weak transient interaction with the DNA that might require stabilization by other components (10). Unfortunately, our assay conditions did not allow us to identify which complexes contain CDP.

Two possible mechanisms for adenovirus encapsidation have been proposed. Studies using pulse-chase analysis and temperature-sensitive viral mutants suggest the traditional model in which capsid shells are formed first, followed by DNA insertion (3–5, 9). In pulse-chase studies, light assembly intermediates were found to be associated with small fragments of the viral genome, followed by the detection of particles associated with the entire viral genome (3). Cells infected with temperature-sensitive mutants at the nonpermissive temperature accumulate light assembly intermediates, and these particles become mature, including the addition of DNA, after shifting to the permissive temperature (5, 9). Recently, however, the L4 33-

kDa protein has been proposed to initiate encapsidation by recruiting proteins involved in a DNA-protein interaction that leads to capsid assembly (11). Furthermore, the absence of IVa2 expression in *pm*8002 infection leads to no assembly of virus particles, including empty capsids (43). The interaction of the IVa2 protein with the packaging sequence may suggest that assembly of capsids requires recognition of the packaging sequence by the IVa2 protein and possibly other viral proteins. The formation of empty capsids in the absence of the L1 52/55-kDa protein during *pm*8001 infection may be due to accumulation of dead-end products, which have also been detected in poliovirus-infected cells (41). This is consistent with the smaller number of capsids observed in a *pm*8001 infection (19). Together, these results suggest that the packaging sequence might nucleate protein binding, subsequently leading to formation of mature virus particles. This notion is supported by the finding that viral DNA containing no packaging sequence can be packaged into virions, albeit very inefficiently (27, 36). Whatever the assembly mechanism is, it is clear that the IVa2 protein-DNA and L1 52/55-kDa protein–DNA interactions are critical for efficient packaging. Additional study will be required to assess whether these DNA-protein complexes promote capsid assembly or whether they are inserted into capsid shells.

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