The molecular mechanism for packaging of the adenovirus (Ad) genome into the capsid is likely similar to that of DNA bacteriophages and herpesviruses—the insertion of viral DNA through a portal structure into a preformed prohead driven by an ATP-hydrolyzing molecular machine. It is speculated that the IVa2 protein of adenovirus is the ATPase providing the power stroke of the packaging machinery. Purified IVa2 binds ATP in vitro and, along with a second Ad protein, the L4 22-kilodalton protein (L4-22K), binds specifically to sequences in the Ad genome that are essential for packaging. The efficiency of binding of these proteins in vitro was correlated with the efficiency of packaging in vivo. By utilizing a virus unable to express IVa2, pm8002, it was reported that IVa2 plays a role in assembly of the empty virion. We wanted to address the question of whether the ATP binding, and hence the putative ATPase activity, of IVa2 was required for its role in virus assembly. Our results show that ATPase activity was not required for the assembly of empty virus particles. In addition, we present evidence that particles were assembled in the absence of IVa2 by using two viruses null for IVa2—a deletion mutant virus, ΔIVa2, and the previously described mutant virus, pm8002. Empty virus particles produced by these IVa2 mutant viruses did not contain detectable viral DNA. We conclude that the major role of IVa2 is in viral DNA packaging. A characterization of the empty particles obtained from the IVa2 mutant viruses compared to wild-type empty particles is presented.

The double-stranded DNA genome of adenovirus (Ad) is packaged with at least 12 different viral proteins; 7 of these proteins, hexon, penton, fiber, IIIa, VI, VIII, and IX, associate to form the protein capsid. Proteins V, VII, and mu are associated with the DNA forming the core (38, 44). These proteins are believed to condense the Ad DNA and mediate interactions between the core and capsid. Terminal protein is covalently linked to the 5′ ends of the genome and is the primer for replication; Ad protease is essential for maturation of the assembled particle with the Ad DNA (14, 18). The most abundant protein of the capsid is the trimer of polypeptide II, referred to as hexon. Hexons comprise about 60% of the protein of the virion. The 240 hexons, assembled 12 per facet of the icosahedral structure, have remarkable molecular flexibility, allowing the hexons to occupy four different environments. Hexons are associated with pentons at the 12 vertices; penton bases associate with a trimer of polypeptide IV called fiber. Additional minor components, IIIa, VI, VIII, and IX, contribute to capsid structure and stability (4, 21, 36, 37).

The molecular mechanism for the encapsidation process with Ad is unknown. However, there is evidence to suggest that the process in Ad is likely similar to what is observed for herpesviruses and DNA bacteriophages (reviewed in references 35 and 40). That is, the DNA genome of the virus is inserted into a preformed procapsid through a portal assembly at a unique vertex of the capsid. The movement of the DNA into the capsid is facilitated by an ATPase-driven molecular motor. In the case of Ad, particles devoid of viral DNA, empty particles, can be isolated and separated from mature particles by virtue of their different densities in cesium chloride gradients: 1.29 to 1.3 g/cm³ and 1.34 g/cm³, respectively (19, 22, 41). It has not been determined whether these empty particles are dead end products or assembly intermediates. However, they do contain several Ad capsid proteins in their precursor forms, pV1, pVIII, and IIIa, and maturation of these proteins by Ad protease occurs as a post-DNA-packaging event (6). Light intermediate particles band in cesium chloride gradients at a density of ~1.30 g/cm³; it is not clear how they differ from empty virus particles. Furthermore, incomplete particles found in a range of cesium chloride densities between that of the empty and mature particles contain Ad DNA of increasing lengths. The sizes of the DNA increase with the increasing density of the particle and are due to extensions from the left end (5, 42). These observations are consistent with polar packaging of the Ad genome, with the initiation of packaging occurring at the left end. In support of this is the observation that sequences at the left end of the Ad type 5 (Ad5) genome, between nucleotides (nt) 200 and 400, are absolutely required for genome packaging (10, 16, 33). These packaging sequences can function at the right end of the Ad genome as well (13). A reverse in the packaging polarity is seen with virus with a functional domain at the right end of the DNA and not at the left end.

Several Ad5 proteins have been shown to bind to the packaging domain. Ad5 proteins IVa2 and L4 22-kilodalton protein (L4-22K) bind specifically to DNA motifs within the packaging
As a first step in unraveling the role of IVa2 in packaging and assembly, we set out to determine if ATP binding, and hence the putative ATPase activity, is required for both packaging and assembly. Toward this end, we generated several Ad virus genomes with mutations in the IVa2 gene that either eliminated the expression of IVa2 or specifically affected the ATP binding/hydrolisis domain. In order to propagate these mutant viruses, we generated a cell line that inductively expresses the Ad5 IVa2 protein. In contrast to the previously published report (49), we find that Ad5 mutants that do not express the IVa2 protein produce empty virus particles. The ATPase motif mutant virus also produced empty virus particles but was defective for packaging of viral DNA. Empty virus particles produced by these IVa2 mutant viruses did not contain detectable viral DNA. We conclude that the IVa2 protein is not required for empty virus particle assembly and that the IVa2 ATPase domain is required for viral DNA packaging. We present a characterization of the empty particles made in these infections.

MATERIALS AND METHODS

Cells, viruses, and infections. The permissive cell line, N52.E6-Cre, was a gift from G. Schiedner and S. Kochanek (University of Ulm). N52.E6-Cre expresses the adenovirus E1 gene and Cre recombinase (39); Cre recombinase was not important for these experiments. Cells were maintained in Dulbecco’s modified Eagle medium supplemented with 10% Fetalclone III serum (HyClone), penicillin, and streptomycin. An N52.E6-Cre subclone, Tet-C4-IVa2, that expresses IVa2 under the control of a Tet-inducible promoter was generated; the new cell line is termed N52.E6-Cre-IVa2. N52.E6-Cre–IVa2 cells were maintained in Dulbecco’s modified Eagle medium supplemented with 10% Fetalclone III serum, penicillin, streptomycin, 200 μg/ml Geneticin, 200 μg/ml hygromycin B, 10 μg/ml blasticidin S, and 0.1 μg/ml doxycycline. Doxycycline was withdrawn from the culture medium to induce IVa2 expression. ΔIVa2, a IVa2 B box mutant, and pm8802 viruses (27, 49) (Fig. 1) were made by isolating single plaques following transfection of PacI-digested Ad5 infectious clones (2) carrying the respective mutations (pTG3602-ΔIVa2, -Bbox, and pm8802) into the IVa2-inducible cell line. Viruses were amplified by two additional passages, and the titers of final stock lysates were determined by a plaque assay, as described previously (29). The purity of these virus stocks was confirmed by Southern blot analyses of viral extracted DNA from infected cells by the method described by Hirt (17). Southern analyses were done using an Amersham AlkPhos system (GE Healthcare Life Sciences), as described previously (30). The plasmid pTG3602-ΔIVa2 (49) was a gift from M. Imperiale (University of Michigan). The ΔIVa2 and B box mutants were recombined into pTG3602 by following the methods described by Evans and Hearing (7). The B box mutant contained two amino acid changes, amino acid 280 D to N and amino acid 281 E to D, and the introduction of a silent mutation in the IVa2 protein to introduce a BspHI restriction enzyme site for screening purposes.

N52.E6-Cre cells (2 × 10⁶ to 3 × 10⁷) were infected with wild-type Ad5 and the Ad5 mutant viruses at a multiplicity of infection of 5 PFU/cell, as previously described (29). At 72 h postinfection, cells and media were collected, and the cells were pelleted, resuspended in 7.5 ml TD buffer (25 mM Tris [pH 7.4], 137 mM NaCl, 5 mM KCl, 0.7 mM Na₂HPO₄), and frozen and thawed at room temperature for 3 cycles. The lysates were cleared by centrifugation at 3,162 × g at 4°C for 30 min. The cleared supernatants were applied to step gradients of 1.25 g/cm³ and 1.40 g/cm³ cesium chloride in TD buffer and spun at 175,117 × g at 16°C. Visible bands were collected from the equilibrium gradient, the refractive indices of the fractions were measured using a Carl Zeiss refractometer, and results were converted to densities.

Electron microscopy. Particles from 25-μl droplets from fractions of the CsCl equilibrium gradients were adhered to 300 mesh, Formvar/carbon-supported nickel grids (Electron Microscopy Sciences, Hatfield, PA) for 2 to 5 min at room temperature. Grids were floated on 400-μl droplets of the following solutions, particle side down, for the indicated times at room temperature: three times for 2 min each on 0.5× TNE buffer (10 mM Tris [pH 7.5], 250 mM NaCl, and 0.5 mM EDTA); two times for 2.5 min each on 5% glutaraldehyde (electron mi-
FIG. 1. Schematic diagram of the Ad5 IVa2 gene and viral mutants. The viral genome is represented by the thin line, shown in a right to left direction with Ad5 nucleotide (nt) numbers following a left-to-right convention. Hash marks indicate discontinuous representation of the Ad5 genome. The coding region of IVa2 is represented by a thick line, and the white boxes represent the Walker A and B boxes (letters A and B). The deletion that includes Ad5 nt 5186 to nt 1340. BGH polyA refers to the insertion of the bovine growth hormone gene poly(A) signal, and the arrow shows the 5′-to-3′ direction of the insertion. For pm8002 (49), Ad5 nt 5390 to 5384 are shown, with the wild-type sequence above the mutated sequence and substitutions indicated by vertical lines. Amino acid sequences are indicated by single letters for wild-type and mutant proteins above and below the DNA sequences, respectively. Asterisks indicate stop codons. The introduction of diagnostic BspH site (solid line) and AflII (dotted line) sites is shown below the mutant sequence.

RESULTS

In order to examine the role of ATP binding, and hence the putative ATPase activity of the IVa2 protein in virus assembly, we generated two mutant viruses utilizing the Ad5 infectious genomic clone, pTG3602 (2) (Fig. 1). pTG3602-Bbox− has point mutations in the Walker B box domain that result in the substitutions of aspartic acid with asparagine and the neighboring glutamic acid with an aspartic acid. By homology to known ATPases, these amino acids are implicated in the binding and hydrolysis of ATP (46), and purified IVa2 protein with these mutations was shown to be markedly reduced for ATP binding in vitro (27). The other Ad5 mutant genome was deleted for IVa2 sequences; pTG3602-ΔIVa2 lacks Ad nt 1339 to 5186. This deletion removes a large C-terminal segment of the IVa2 reading frame as well as region E1B and part of region E1A. The poly(A) sequences from the bovine growth hormone gene were inserted in order to ensure expression of the Ad polymerase and terminal protein. This was necessary since the polyadenylation site for the Ad polymerase and terminal protein transcripts overlaps the IVa2 gene region that was deleted. Propagation of the resulting ΔIVa2 virus is dependent upon the expression of the IVa2 and the E1 genes using a complementing cell line. The deletion also removed the coding region of protein IX. However, this did not affect the viability of ΔIVa2 virus as was anticipated from previous results (4, 9). If a stable mRNA was made and spliced, it would encode a protein of 112 amino acids that includes the first 84 amino acids of the IVa2 protein. The advantage of this mutant virus is that it is unable to undergo homologous recombination with the IVa2 gene in the complementing cell to generate a rever-
tant virus that restores IVa2 expression. This is not the case with the IVa2 mutant pm8002, for which mutant virus stocks had to be carefully screened for the presence of revertants. Two stop codons introduced into this mutant genome substitute for the 17th and 19th amino acids of the IVa2 protein (49). IVa2 retains the first 252 nucleotides of the IVa2 gene, including the intron and splice sites. Stocks of the mutant viruses were obtained by transfection of the genomic clones, followed by plaque isolation and amplification using a newly developed IVa2 complementing cell line. This cell line expresses the Ad5 E1 (N52.E6-Cre) (38) and IVa2 gene products (called N52.E6-Cre–IVa2). We characterized the properties of this new cell line in comparison to the 293–IVa2 cell line previously described (49) (Fig. 2). The two cell lines produced similar levels of the Ad5 IVa2 protein as found in infections of noncomplementing cells (N52.E6) with wild-type Ad5 (following doxycycline removal with N52.E6-Cre–IVa2 cells) (Fig. 2A, lanes 2 to 4). The N52.E6-Cre–IVa2 cell line displayed elevated levels of IVa2 protein when infected with Ad5 in comparison to 293–IVa2 cells as well as several minor larger and smaller IVa2 species (Fig. 2A, lanes 5 and 6). The nature of these products is not known, although a smaller IVa2 protein that was initiated at an internal ATG codon has been described (32). We analyzed the total virus yield of the IVa2 mutant virus pm8002 (49) produced by each complementing cell line as well as the particle-to-PFU ratio (P/PFU) of the purified, mature mutant virus particles (Fig. 2B). The N52.E6-Cre–IVa2 cell line produced ~14-fold more mature pm8002 virus particles per cell than the 293–IVa2 cell line, with a P/PFU ratio of 125 compared to a P/PFU ratio of 300 for 293–IVa2 cells. Thus, the new IVa2 complementing cell line N52.E6-Cre–IVa2 has several useful features for IVa2 mutant virus production.

The noncomplementing parental cell line N52.E6-Cre was infected with B box mutant virus, and virus production was analyzed using cesium chloride gradients. Figure 3A shows the results of the CsCl equilibrium gradient with lysates from cells infected with the B box mutant virus. Two bands that migrated too close together for separation were observed; therefore, the bands were pooled for further analysis. The ratio of the two bands varied from experiment to experiment. The material banded at a density of 1.29 to 1.3 g/cm³ and will be referred to as empty virus particles. Empty particles, devoid of Ad DNA, from infection with wild-type virus have been shown to band at this density (22). No bands were observed lower in the gradient, where mature virus would be expected (1.34 g/cm³). EM images of negatively stained material from the gradient confirmed that virus particles had been assembled. The empty particles from the B box mutant resembled empty particles obtained with wild-type Ad5 (compare Fig. 3A and D). Southern blot analyses of DNA from B box mutant-infected cells showed that the replicated genomes retained the introduced mutations (data not shown). These results showed that ATP binding by the IVa2 protein, and the putative ATPase activity of IVa2, were not required for assembly of an empty virus.
particle. However, these activities are required to package viral DNA into virions.

A surprising finding, however, was the observation that the IVa2 deletion mutant also produced empty virus particles. Similar to the results with the B box mutant, the mutant virus /H9004 IVa2 produced empty virus particles that banded in CsCl gradients at a density of 1.29 to 1.3 g/cm³ (Fig. 3B). No other material was seen lower in the gradient at the density of mature virus. EM images of negatively stained material showed that the /H9004 IVa2 mutant assembled empty virus particles (Fig. 3B). Southern blot analyses of the DNA from /H9004 IVa2-infected cells showed that the replicated genomes retained the introduced deletion (data not shown). These results raised the possibility that the remaining part of the IVa2 gene in /H9004 IVa2 (the N-terminal 84 amino acids) could play a role in assembly of empty virus particles. The Walker A and B box domains are located downstream of this region (Fig. 1) and would not be present if this truncated protein was made. A pm8002 virus stock was generated and used to infect N52.E6-Cre cells. As was seen with the B box and ΔIVa2 mutant viruses, material was observed in the equilibrium gradient banding at a density of 1.29 to 1.3 g/cm³, consistent with empty virus particles (Fig. 3C). This result was surprising, since pm8002 previously was reported not to produce empty virus particles (49). Virus particles were present in this material, as determined by EM (Fig. 3C). Southern blot analyses of DNA from the pm8002-infected cells showed that the replicated genomes retained the introduced deletions (data not shown).

In all cases in the EM images, some of the empty particles appeared to be degraded as exemplified by the results with the B box mutant particles shown in Fig. 4. It is unclear whether the particles were degraded before EM or had degraded during the process of fixing and staining the particles. These results suggest that the empty virus particles are more fragile than mature particles containing DNA, since images from negative staining and EM of mature particles appeared more uniform (data not shown).

The virus particles obtained from CsCl equilibrium gradients were further characterized by SDS-polyacrylamide gel electrophoresis followed by silver staining and Western blot analysis and for viral DNA content. The results are shown in Fig. 5 and 6. Protein profiles of the empty particles from the three mutant viruses were compared to the profiles from Ad5 mature and empty particles. Figure 5, lane 5, shows the silver stain profile of mature Ad5 virus particles isolated from CsCl gradients. The identities of viral proteins based on molecular weight are indicated, including the major structural proteins: polypeptide II (hexon), III (penton), IV (fiber), and IIIa. Also identifiable are the core proteins, V and VII, the latter being the processed form of pVII. Figure 5, lane 4, shows the profile of the empty particles from wild-type Ad5 isolated from CsCl gradients. The major polypeptides are visible and, as expected, the empty particles lack proteins V and VII and its precursor pVII (6). The empty particles from ΔIVa2, pm8002, and the B box mutant (Fig. 4, lanes 1, 2, and 3, respectively) had profiles very similar to that of the empty particles of wild-type Ad5 with respect to the major polypeptides. The presence of the precursor proteins, pVI and pVIII, were observed with all of the empty particles. The presence of the precursor proteins is consistent with the particles being immature, and the density of the particles is consistent with their being empty virus particles. Subtle differences of unknown origin were evident with the different empty virus particles.

Virus particles were examined for the presence of the IVa2, L1-52/55K, and L4-22K packaging proteins. It had been shown previously that IVa2 is found in mature virus particles (49) but that the L1-52/55K protein is not (11, 15). The proteins from wild-type and mutant infections were separated on SDS-poly-
IVa2 null viruses did not contain the L4-22K protein, whereas the IVa2 B box mutant empty particles and wild-type Ad5 empty particles did contain the L4-22K protein (Fig. 6D). Further, like the L1-52/55K protein, the L4-22K protein was absent from wild-type Ad5 mature virus particles.

Finally, we determined by Southern blotting whether empty virus particles produced by these IVa2 mutant viruses contain viral DNA. As a positive control, we isolated empty virus particles from cells infected at the restrictive temperature with the temperature-sensitive mutant virus ts369. ts369 produces a temperature-sensitive L1-52/55K protein, and empty virus particles isolated from cells infected at the restrictive temperature with this mutant contain Ad5 left-end DNA sequences that correspond to the packaging domain and adjacent sequences (15). Small viral DNA was readily apparent in ts369 empty virus particles; in contrast, no detectable viral DNA was evident in empty virus particles produced by each of the IVa2 mutant viruses (data not shown). These results indicate that the particle heterogeneity observed with the IVa2 mutant viruses on CsCl gradients (Fig. 3) does not represent the presence or absence of left-end viral DNA sequences.

**DISCUSSION**

The Ad5 IVa2 protein is essential for the packaging of the Ad genome into its capsid. We have demonstrated that the IVa2 protein is not required for assembly of an empty viral capsid. This result is opposite to what was previously published (49). In our experiments, empty particles were obtained from infections with mutant viruses containing either a deletion of a significant portion of the IVa2 coding region or containing stop codons that disrupted the expression of the full-length protein (Fig. 3). The replicated genomes that accumulated during these infections were pure mutant genomes as determined by Southern blot analyses; therefore, the accumulation of empty particles was not due to reversion of the mutations. A question arises: why is there a difference between our results and those in the previously published report? The appearance of empty particles is not due to the difference in cell lines used in the present report compared with those used in the previous report. N52.E6-Cre cells were used for the experiments presented here, but infections of 293 cells, the cell type previously used (49) yielded the same results (293 cells maintained in the Hearing laboratory as well as the Imperiale laboratory were tested; data not shown). We believe that the most likely explanation for these differences is the way the virus particles were processed. The Imperiale laboratory lysed infected cells by three cycles of freezing and thawing as well as by sonication (M. Imperiale, personal communication). In our studies, no sonication step was employed. As suggested by the images in Fig. 3 and 4, we believe that the empty virus particles produced by IVa2 mutant viruses are fragile, perhaps disrupted by sonication. We also examined virus production at a later time point (72 h) than in the previously published report (48 h) (49).

Finally, we note that the Imperiale laboratory was able to detect empty particles in N52.8002 infections when our IVa2 complementing cell line was used to produce the mutant virus (M. Imperiale, personal communication). Perhaps this reflects the improved virus yield and P/PFU ratio using IVa2 mutant viruses produced using the N52.E6-Cre–IVa2 cell line.
Utilizing the B box mutant virus, we have determined that preventing ATP binding by the IVa2 protein, and likely inhibiting the putative IVa2 ATPase activity, had no detrimental effect on the assembly of empty Ad virus particles (Fig. 3). Although we did not quantify the amount of empty particles/cell obtained in these infections, the results from the CsCl equilibrium gradients showed that a significant amount of empty particles accumulated. Relatively small amounts of empty particles compared to those of mature particles accumulated during the course of a wild-type Ad5 infection. Previous data implied a precursor/product relationship between the empty and mature capsids (41). If the empty particles we observed are dead-end products rather than procapsids in packaging, this would not negate the fact that assembly occurred in the absence of IVa2 protein. The major effect of preventing ATP binding by the IVa2 protein was the inability of the mutant virus to package DNA. Therefore, it seems likely that IVa2 is an ATPase and that this activity is essential for the packaging of the Ad genome into the capsid. This is consistent with previously published results that localize the IVa2 protein at a unique capsid vertex (3), a hallmark of bacteriophage packaging motors (35).

It is interesting that the IVa2 protein is found in association with the empty particles isolated from infections with wild-type Ad5 and with the B box mutant virus (Fig. 6). This association may be mediated by the L1-52/55K protein which is found in empty Ad particles, since it was previously shown that these proteins interact (12) and L1-52/55K plays a important role in packaging (11, 15). There is no significant difference in the relative amounts of IVa2 protein found in mature Ad5 virus particles and the amounts in the empty particles from the B box mutant virus. At this level of analysis, we could not rule out small changes that might be significant. However, if indeed the empty particles observed are procapsids, then either there are different populations of IVa2, one on the particle and one on Ad DNA-packaging sequences, or the specific selection of Ad DNA by IVa2 occurs at the entry site for the DNA on the procapsid. That empty particles assemble in the absence of IVa2 suggests the possibility that IVa2 associates with capsids and viral DNA late in the assembly process. The empty virus particles lacking IVa2 contained the L1-52/55K protein but not the L4-22K protein (Fig. 6). The IVa2 protein is required for the L4-22K protein to bind to packaging sequences in vitro (8, 26), and the current results suggest that an interaction between these two proteins also is required for the L4-22K protein to enter empty capsids. In contrast, IVa2 B box mutant empty capsids did contain the L4-22K protein, suggesting that ATP binding by IVa2 is not required for such an interaction to occur. Interestingly, wild-type Ad5 mature virus particles lacked the L4-22K protein as previously described for the L1-52/55K protein (11, 15). Ad5 mature virus particles contained the IVa2 protein, as previously reported (49). That the L1-52/55K and L4-22K proteins appear to exit the particle coordinately during capsid maturation may reflect their direct interaction. The L1-52/55K protein associates with the packaging domain in vivo as measured by chromatin immunoprecipitation assays (31, 34), but the IVa2 protein is not required for this interaction (34). Perhaps the L1-52/55K protein is tethered to the packaging domain via interaction with the L4-22K protein.

Detailed analyses of the motor function in the packaging process in bacteriophage phi29 has shown that coordinated, sequential hydrolyses of four ATPs by four adjacent ATPases leads to the movement of 10 bp of DNA into the procapsid (35). Addition of nonhydrolyzable ATP stalls the motor for the duration of the substrate binding, and stalling occurs even if only one ATPase subunit was inhibited for hydrolysis (24, 47). In contrast to these results, the protease ClpX is a hexameric ATPase that is part of a protease and does not appear to function by a sequential mechanism (23). It was suggested that ATP hydrolysis by ClpX hexamer is probabilistic and that this flexibility may be related to its interaction with a substrate that is not uniform in structure. The fact that we were able to propagate the Ad5 B box mutant in an IVa2 complementing cell line shows that the mutant protein did not act as a dominant negative effector. If the IVa2 protein is an ATPase, then we suggest that the packaging motor may not function in a sequential manner. Interestingly, the molecular mechanism of packaging of the Ad genome may be different from that of the bacteriophage, since Ad DNA is found associated with the core proteins pVII, V, and Mu. It is still unknown whether the appearance of these core proteins in an Ad particle occurs concomitantly or by packaging of viral DNA. In either case, the packaging ATPase for Ad may require more flexibility in providing a power stroke for moving substrates that are not just a double helix of DNA but also have bound protein. It is worth noting that the IVa2 protein has been classified as a member of the p loop, ASCE ATPases by sequence comparisons, but within this large group, it is distinct from the bacteriophage packaging motors and more similar to the ATP-binding cassette (ABC) transporter ATPases (1). Mechanistic questions regarding the role of IVa2 in Ad packaging can be approached using the complementation system that we have developed.

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