Adenovirus Type 5 Packaging Domain Is Composed of a Repeated Element That Is Functionally Redundant

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Previous analyses have demonstrated that adenovirus DNA is packaged into virions *in vivo* in a polar, left-to-right fashion. The packaging of viral DNA is dependent on *cis*-acting elements at the left end of the genome. In this report, we describe a genetic analysis of the sequences that are required for efficient packaging of adenovirus type 5 (Ad5) DNA. Our results demonstrate that the Ad5 packaging domain (nucleotides 194 to 358) is composed of at least five distinct elements that are functionally redundant. An AT-rich repeated sequence motif, the A repeat, is located in four of five of these regions; the fifth region is also AT rich. The efficiency of viral packaging depends on the number of individual A repeats that are present in the viral genome. The deletion of the entire packaging domain resulted in the loss of virus viability. A virus that contains a multimerized oligonucleotide corresponding to A repeat II in place of the packaging domain could package viral DNA, although with reduced efficiency compared with that of the wild-type virus. Our results also suggest that the spacing of specific sequences at the left end of the Ad5 genome are important for enhancer region function *in vivo*.

The selective packaging of adenovirus DNA into virions late in infection appears to involve the specific recognition of viral DNA sequences and then condensation and encapsidation of the viral genome into preformed capsids (4, 6, 7, 10, 20, 36). Incomplete particles of adenoviruses in groups A, B, and C contain subgenomic segments of the viral genome that are enriched for left-end sequences, suggesting that the viral genome is packaged in a polar fashion from left to right in a manner that is dependent on a *cis*-acting element(s) (5, 14, 15, 37). Evidence to support this idea has been obtained from the analysis of evolutionary variants of adenovirus types 3 and 16 (Ad3 and Ad16, respectively). With Ad3, the analysis of a group of mutants that contain rearrangements at the left end of the viral genome indicates that the sequences that specify polar packaging lie between nucleotides (nt) 319 and 390 (29). With Ad16, the analysis of variants that contain different amounts of left-end sequences duplicated at the right end of the genome has shown that the Ad16 sequences between nt 290 and 390 are required for efficient polar packaging (14). Because of a *cis* requirement for virus replication, it has not been possible to determine whether sequences within the viral inverted terminal repeat (Fig. 1A) (approximately 100 to 150 nt at each end of the adenovirus genome, depending on the virus type) are also involved in the packaging process.

We previously identified a *cis*-acting packaging domain that is located between nt 194 and 358 at the left end of the Ad5 genome (Fig. 1A) (16, 17). Mutant viruses that lack this region are nonviable, but this defect is complemented by the insertion of the left terminal 355 nt at the right end of the Ad5 genome (17). The Ad5 packaging domain functions at either end of the viral genome, in an inverted orientation, and can be moved within several hundred base pairs of its original location without a reduction in virus yield (16). The Ad5 packaging domain, therefore, shares properties with eucary-

otic transcriptional enhancer elements. A repeated sequence motif, the A repeat, is present in the Ad5 packaging domain and has been implicated in the packaging process (Fig. 1B, AI through AV) (18).

The Ad5 packaging domain is located within the early region 1A (E1A) enhancer region (Fig. 1 and 2) (17, 18). Two distinct enhancer elements in this region that regulate viral gene expression have been defined. Enhancer element I is a repeated sequence that specifically regulates E1A transcription (17, 18); a cellular protein that binds to this motif has been identified (3). Viral element I mutants are efficiently complemented when the E1A products are provided *in trans*. Enhancer element II spans a region of approximately 30 base pairs (bp) (nt 250 to 280) and enhances transcription from each of the viral early transcription units (18). The reduced expression of the early gene products with viral element II mutants results in decreased viral DNA replication and a three- to sevenfold reduction in virus yield compared with that of the wild-type virus (16, 18). The *cis*-acting defect in virus yield with element II mutants is complemented *in trans* in a mixed infection with a wild-type virus in which the wild-type virus provides early proteins required for efficient replication (16, 18).

In this report, we describe a genetic analysis of the sequences that are required for the efficient packaging of Ad5 DNA. Our results demonstrate that the Ad5 packaging domain (nt 194 to 358) is composed of multiple, functionally redundant elements. At least five distinct regions in the Ad5 packaging domain are involved in the packaging process. Four of these regions contain a repeated sequence motif (the A repeat, 5'- $\hat{A}$ AN $\hat{A}$ TTTG-3') (Fig. 2). The efficiency of viral packaging depends on the number of A repeats in the viral genome. A virus that contains a multimerized oligonucleotide of a single A repeat in place of the entire packaging domain could package viral DNA, albeit with reduced efficiency compared to that of the wild-type virus. Finally, we demonstrate that the phenotype of viral enhancer element II

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A.

B.

FIG. 1. (A) Schematic diagram of the left end of the Ad5 genome including the E1A 5' flanking region. Nucleotide numbers relative to the left terminus of the genome are indicated below the line. The inverted terminal repeat (ITR) and the packaging-enhancer region are represented by hatched and open boxes, respectively. The TATA box motif (T/A) is indicated by the stippled circle. The E1A start site (nt 499) and transcription unit are indicated by the arrow. (B) Schematic view of the packaging-enhancer region (nt 194 to 358). The positions of A repeats I through V are shown as hatched boxes. Nucleotide numbers relative to the left terminus are indicated below the line. Components of the enhancer region (elements I and II) are represented by solid bars above the line.

mutants is also observed with viruses that contain mutations outside of the element II region. Our data indicate that the element II phenotype may reflect a spacing requirement between sequences within and outside of the E1A enhancer region.

MATERIALS AND METHODS

Mutant plasmids and viruses. Ad5 *dl309* (21) is a phenotypically wild-type virus that is the parent of all of the

viruses used in this study. *dl309* contains a unique *Xba*I cleavage site at 3.8 map units. Plasmid pE1A-WT contains the left-end *Xba*I fragment from *dl309* (0 to 3.8 map units) cloned into pBR322 (17). All of the mutants analyzed in this study were originally constructed in plasmid pE1A-WT, and the mutations were subsequently rebuilt into intact viruses by the method of Stow (34). Mutant viruses were propagated in cells from cell line 293, a human cell line which expresses the Ad5 E1A products (13).

FIG. 2. Nucleotide sequence of the packaging-enhancer region. Repeats AI through AV are indicated. Numbers relative to the left terminus are indicated below the sequence. Enhancer elements I and II are indicated by solid bars above the sequence. A sequence comparison of the individual A repeats is shown below the packaging-enhancer region sequence. The A repeat consensus sequence is given.

A unique *XhoI* linker was inserted in plasmid pE1A-WT at either the *RsaI* site at nt 194 or the *SstII* site at nt 358. Unidirectional deletion mutations were introduced into these plasmids, progressing from the unique *XhoI* site in either plasmid and extending into the packaging domain. Deletion mutations were generated, using exonuclease III and S1 nuclease, as described previously (25); *XhoI* linkers were introduced at each deletion endpoint. The endpoint of each mutation was determined by nucleotide sequence analysis, using the dideoxy sequencing procedure (30). Deletion mutations *dIA5*, *dI18*, and *dI2* in the packaging domain were described previously (16-18).

Plasmids containing double mutations that combine selective single deletions were generated, using the unique *BalI* site at nt 270 (or the *HaeIII* site at nt 270) and unique *EcoRI* site in pBR322 to interchange left-end DNA fragments. Insertions mutations *dI194/310:in4* and *dI194/316:in4* were generated by linearizing the parental mutant plasmid with *XhoI*, followed by a repair reaction with Klenow DNA polymerase and subsequent ligation.

A 31-bp oligonucleotide that corresponds to A repeat II (nt 252 to 275) and that contains cohesive *XhoI* ends was introduced into a derivative of pE1A-WT that lacks the packaging domain (nt 194 to 358; equivalent to mutant *dI2* previously described [17]) and contains an *XhoI* linker at the deletion junction. The oligonucleotides were inserted in a head-to-tail and a direct orientation with respect to the orientation of A repeat II in the wild-type virus. The sequence of the oligonucleotide is 5'-TCGAGGTAACCGAGTAAGATTTGGCCATTCC-3':5'-TCGAGGAATGGCCAAATCTTACTCGGTTACC-3'.

Cells and determination of virus yields and packaging efficiency. Cell line 293 (13) was maintained in Dulbecco modified minimal essential medium containing 10% calf serum. All virus infections were performed at a multiplicity of infection of 3 to 5 PFU per cell at 37°C for 1 h. After infection, the cells were washed twice with phosphate-buffered saline solution, and fresh medium was added. For the determination of virus yields in single-virus infections, infected cultures were harvested 48 h after infection, the cells were lysed by three cycles of freezing and thawing, and infectious virus yields in cleared lysates were determined by plaque assay on 293 cells. With experiments involving mixed-virus infections, 293 cells were infected with 3 to 5 PFU of each virus per cell, as described above. In all of the experiments described, *dI309* was used as the coinfecting wild-type virus. At 48 h after infection, one-half of the cells were used to isolate high-molecular-weight DNA, and the other half of the cells were used to prepare viral DNA from virions. For the isolation of infected-cell high-molecular-weight DNA, the cells were lysed by the addition of Nonidet P-40 to 0.4%, the nuclei were precipitated, and high-molecular-weight DNA was isolated as described previously (26). For the isolation of viral DNA from virions, the procedure described by Hammarikjold and Winberg (14) was used, with the following modifications. Infected cells were precipitated and suspended in lysis buffer (20 mM Tris [pH 9.0], 0.2% deoxycholate, 10% ethanol). After incubation for 60 min at room temperature, the lysate was cleared at 10,000 × g for 30 min. The supernatant was adjusted to 2 mM CaCl₂ and 2 mM MgCl₂ and was digested with 40 µg of RNase A per ml and 10 µg of DNase I per ml at 37°C for 30 min. The reaction was stopped by the addition of EDTA and EGTA to a final concentration of 5 mM each. Virus particles were lysed by the addition of Sarkosyl to 0.5%, and the samples

were digested with 1 mg of pronase per ml at 37°C for 1 h to several hours. After extensive phenol-chloroform extractions, the viral DNA was precipitated with ethanol. DNAs isolated from nuclei or virions were digested with *ClaI* and *XhoI* and analyzed by Southern hybridization (26). The plasmid pE1A-WT, ³²P labeled by the random primer method (11), was used as a probe in the Southern hybridization analyses. The relative intensities of the bands in autoradiograms were determined by laser densitometry, using blots that were exposed to X-ray film without an intensifying screen. The data presented for virus yields from single infections represent the averages of three independent experiments. The data presented for packaging efficiency based on coinfection experiments represent the averages of three to five independent experiments.

The level of the input viral genomes was analyzed by slot blot analysis as previously described (19). 293 cells were infected with 3 to 5 PFU of each virus per cell, and high-molecular-weight DNA was prepared from isolated nuclei at 6 h after infection. The level of total input viral DNA was quantitated by slot blot analysis, using a ³²P-labeled probe of the Ad5 genome.

RESULTS

Construction and analysis of viruses with mutations in the packaging domain. To further define the sequences that are required in *cis* for the efficient packaging of Ad5 DNA into virions, we constructed a series of viral mutants that contain single- and double-deletion mutations in the previously defined packaging domain (Ad5, nt 194 to 358) (Fig. 1) (16, 17). Unidirectional deletions that progress into the packaging domain that start at either the upstream border (nt 194) or the downstream border (nt 358) of this region were generated. Combinations of specific mutations of interest were also constructed. Viral mutants were propagated and titrated in 293 cells (13). This cell line expresses the E1A gene products and complements the packaging domain mutants that express reduced E1A levels due to mutations that affect the E1A enhancer sequences. A number of the viral mutants produced small plaques. To verify that each of the mutant stocks was titrated accurately, 293 cells were infected with the viral mutants, and viral DNA present in the nucleus at 6 h after infection was analyzed by slot blot analysis. Each of the mutant viruses displayed comparable levels of nuclear DNA (within twofold of each other) at early times after infection (data not shown).

Two independent assays were used to analyze the efficiency of packaging with the viral recombinants. First, the mutant viruses were used in single infections in 293 cells, and the infectious virus yield obtained after 2 days was determined by a plaque assay. As described above, viruses with mutations in enhancer element II synthesize reduced levels of the early gene products and consequently display a three- to sevenfold reduction in virus yield (termed the element II phenotype) (16, 18). This effect is independent of viral packaging. The reduced yield with enhancer element II mutants is complemented in *trans* in a mixed infection with a wild-type virus, since the coinfecting virus provides normal levels of the viral early gene products (16, 18). To determine what portion of a reduction in a virus yield was the result of a packaging defect, we performed a second assay that used a coinfection with a wild-type virus (the parental virus *dI309*). In these experiments, 293 cells were coinfecting with a mutant and wild-type virus. At 2 days after infection, one-half of the infected cells were used to prepare

FIG. 3. Schematic view of viral mutants that carry deletions with a common start site at nt 358. The top of the figure positions A repeats I through V and the enhancer elements. The individual deletion mutant names are given on the left. The nucleotide numbers correspond to the first nucleotides present on either side of the deletion. The deleted sequences are indicated by solid bars. For single-virus infections, 293 cells were infected with the individual mutant viruses, and the infectious virus yield was determined by a plaque assay with cellular extracts prepared 48 h after infection. Mutant virus yields (YIELD) are expressed as the fold reduction in yield relative to that of the wild-type virus (yield wild type/yield mutant virus ratio). NV, Nonviable mutant virus. In coinfection experiments, 293 cells were coinfecting with a wild-type virus (*dl309*) and the individual mutant viruses. At 48 h after infection, high-molecular-weight DNA was prepared from isolated nuclei and encapsidated viral DNA was prepared from virion particles. Wild-type and mutant viral DNAs in each preparation were distinguished by restriction enzyme digestion and Southern hybridization analysis (Fig. 4). Mutant virus packaging efficiency (COINF.) is expressed as the fold reduction in packaged mutant DNA relative to the packaged coinfecting wild-type DNA. These data were normalized to the amount of each viral DNA (mutant and wild type) present in total nuclear DNA. ND, Encapsidated mutant viral DNA was below the level of accurate quantitation; -, not done.

nuclear high-molecular-weight DNA, and the other half of the cells were used to prepare viral DNA from virion particles. The coinfecting input viral genomes were distinguished by restriction endonuclease digestion and Southern hybridization analysis. By comparing the relative amounts of mutant and wild-type viral DNA present in the nucleus of infected cells with the relative amounts of each viral DNA present in intact virions, we could accurately measure the packaging efficiency of mutant viruses, independent of any effect of enhancer element II.

The packaging domain contains functionally redundant elements. The first set of mutant viruses contains unidirectional deletions which progress from a common site at nt 358 towards the upstream border of the packaging domain (Fig. 3); the data obtained with these mutants in viral infections are shown in Fig. 3 and 4. Viruses containing deletions that extend from nt 358 to 293 grew as well as the wild-type virus did when they were assayed in single infections or in coinfections with a wild-type virus (Fig. 3, *dl309-336/358*, *dl309-317/358*, and *dl309-293/358*). The deletion of sequences between nt 293 and 274 resulted in a three- to ninefold decrease in virus yield in a single infection and an approximate sixfold decrease in a coinfection (Fig. 3, *dl309-287/358* and *dl309-274/358*). The deletion of an additional 7 nt beyond the endpoint in mutant *dl309-274/358* resulted in a dramatic decrease in packaging efficiency. Mutant *dl309-267/358* was reduced nearly 100-fold in virus yield in a single infection (Fig. 3), and no detectable signal was observed in packaged virion DNA in a coinfection (Fig. 4). This mutant replicated to the level of the wild-type virus, however, when nuclear DNA was examined, demonstrating that this effect was due to a packaging deficiency (Fig. 4). The deletion of an additional 6 nt beyond the *dl309-267/358* endpoint resulted in a similar defective phenotype (Fig. 3, *dl309-261/358*). The deletion of sequences to nt 238 resulted in the loss of virus viability (Fig. 3, *dl309-238/358*). On the basis of control

titration experiments, we estimate that the coinfection experiments detected mutants with an approximate reduction of up to 25-fold in packaging efficiency. In repeated reconstruction experiments, we found that mutants that displayed an approximate decrease greater than 1,000-fold in virus yield were nonviable. From the analyses with this group of mutants, we conclude that essential sequences for viral packaging are located between nt 238 and 293 and that this region appears to be composed of three distinct elements located between nt 238 and 261, nt 261 and 274, and nt 274 and 293. Two of these subregions correspond to A repeats I and II (Fig. 1 and 2) that have been implicated in the Ad5

FIG. 4. Southern hybridization analysis of nuclear and virion DNAs isolated from 293 cells coinfecting with the wild-type (*dl309*) and individual mutant viruses (Fig. 3). Nuclear high-molecular-weight DNA and virion DNA were digested with *Cla*I and *Xho*I and analyzed by Southern hybridization, using the Ad5 left-end *Xba*I fragment (plasmid pE1A-WT) as a ³²P-labeled probe. The corresponding wild-type (WT) and mutant (DL) left-end DNA fragments are indicated. The mutant viruses tested were *dl309-336/358* (lane 1), *dl309-317/358* (lane 2), *dl309-293/358* (lane 3), *dl309-287/358* (lane 4), *dl309-274/358* (lane 5), *dl309-267/358* (lane 6), and *dl309-261/358* (lane 7).

FIG. 5. Schematic view of viral mutants that carry deletions with a common start site at nt 194. The schematic, mutant names, endpoints of the deletions, and in vivo packaging analyses are as described in the legend to Fig. 3.

packaging process (16). In addition, the region between nt 274 and 293 contributes to efficient viral packaging.

The second set of mutant viruses contain unidirectional deletions which progress from a common site at nt 194 towards the downstream border of the packaging domain (Fig. 5); the data obtained with these mutants in viral infections are shown in Fig. 5 and 6. Mutants carrying deletions of sequences between nt 194 and 254, including A repeat I, grew nearly as well as the wild-type virus in a single infection or coinfection (Fig. 5, *dl309-194/208*, *dl309-194/243*, and *dl309-194/254*). The additional deletion of A repeat II

resulted in a two- to fivefold decrease in yield in a single infection and a four- to ninefold reduction in packaged DNA in a coinfection (Fig. 5, *dl309-194/265* and *dl309-194/273*). Thus the region that was critical for viral packaging on the basis of results obtained with the first set of mutant viruses had only a moderate effect on packaging when the downstream half of the packaging domain was intact. The larger decrease observed in a coinfection relative to a single infection may represent a competition between the coinfecting viruses for a limiting factor(s) required for packaging (see Discussion). The deletion of sequences between nt 273 and 316 had no additional effect on packaging when assayed in a coinfection experiment (Fig. 5, mutants *dl309-194/290* through *dl309-194/316*). However, reproducible fluctuations were observed with these mutants when virus yields in single infections were examined (5-, 16-, 6-, 20-, and 3-fold decreases in yield for mutants *dl309-194/273*, *dl309-194/290*, *dl309-194/295*, *dl309-194/310*, and *dl309-194/316*, respectively) (Fig. 5). This phenotype will be discussed in greater detail below. When nuclear DNA was examined in coinfections, each of these mutants replicated to a level comparable to that of the wild-type virus (Fig. 6). Finally, the deletion of sequences between nt 316 and 342 resulted in the loss of virus viability (Fig. 5, *dl309-194/342*); this region contains A repeats IV and V. We conclude from the analyses of both sets of mutants that the Ad5 packaging domain contains redundant packaging signals.

On the basis of these results, we created two series of double mutants that combine individual deletions from the sets of mutants described above; a schematic view of these mutants is shown in Fig. 7. The first series is composed of mutants which contain a deletion of the downstream half of the packaging domain (nt 274 to 358) in addition to deletions that remove increasing amounts of the upstream portion of this region. The packaging efficiency of these mutant viruses was examined in single infections and coinfections with a wild-type virus. The data obtained from viral infections with these mutants are shown in Fig. 7.

FIG. 6. Southern hybridization analysis of nuclear and virion DNAs isolated from 293 cells coinfecting with the wild-type (*dl309*) and individual mutant viruses (Fig. 5). Southern hybridization analysis of nuclear high-molecular-weight DNA and virion DNA was performed as described in the legend to Fig. 4. The mutant viruses tested were *dl309-194/208* (lane 1), *dl309-194/243* (lane 2), *dl309-194/254* (lane 3), *dl309-194/265* (lane 4), *dl309-194/273* (lane 5), *dl309-194/290* (lane 6), *dl309-194/295* (lane 7), *dl309-194/310* (lane 8), and *dl309-194/316* (lane 9).

FIG. 7. Schematic view of viral mutants that combine individual deletions from the sets of mutants shown in Fig. 3 and 5 or deletions mutants *dl309-A5*, *dl309-18*, and *dl309-9* that were previously described (16–18). The schematic, mutant names, endpoints of the deletions, and in vivo packaging analyses are as described in the legend to Fig. 3.

With double mutants in the first series (*dl309-274/358* background), the deletion of sequences between nt 194 and 223 did not reduce packaging efficiency further than the parental mutant virus did (Fig. 7, *dl309-274/358* versus *dl309-194/208:274/358* and *dl309-194/223:274/358*). However, the deletion of sequences to nt 243, including part of A repeat I, resulted in an approximate 40-fold decrease in virus yield in a single infection (Fig. 7, *dl309-194/243:274/358*). The complete deletion of A repeat I resulted in a similarly defective virus (Fig. 7, *dl309-194/254:274/358*). Neither of the latter two mutants displayed detectable packaged viral DNA in a coinfection assay; both mutants, however, exhibited wild-type levels of nuclear DNA (data not shown). The additional deletion of sequences that include A repeat II resulted in the loss of virus viability (Fig. 7, *dl309-194/265:274/358*). With double mutants in the second series (*dl309-A5* background), the deletion of sequences between nt 336 and 358, including A repeat V, resulted in a 70-fold decrease in virus yield in a single infection compared with the yield from the parental mutant virus (Fig. 7, *dl309-A5* versus *dl309-A5:336/358*). No viral DNA was detectable in virions in a coinfection experiment using this double mutant, although this virus replicated to a level comparable to that of the wild-type virus (not shown). The additional deletion of sequences to nt 317, including A repeat IV, resulted in the loss of virus viability (Fig. 7, *dl309-A5:317/358*).

The phenotype of mutant viruses in the second series of double mutants was attributable, at least in large part, to A repeats I and II in the upstream half of the packaging domain. This conclusion is based on the analysis of an additional double mutant that combines a smaller deletion of A repeats I and II (Fig. 7, *dl18*) with the deletion of A repeat V (*dl309-18:336/358*). Mutant *dl309-18* was reduced fivefold for packaging (Fig. 7). The additional deletion of A repeat V in conjunction with the deletion of A repeats I and II resulted

in a 13-fold decrease in virus yield in a single infection and the lack of detectable packaged DNA in a coinfection experiment (Fig. 7) (data not shown). Each of these mutant viruses replicated to a level comparable to that of the wild-type virus in a coinfection (data not shown).

The results obtained with the double mutants strongly support the idea that the A repeats constitute redundant functional elements of the Ad5 packaging domain. In the absence of A repeats III, IV, and V, A repeats I and II provided sufficient, albeit reduced, packaging functions. This residual packaging efficiency was greatly reduced with the deletion of A repeat I, and viruses that lacked all A repeats were nonviable. Similarly, in the absence of A repeats I and II, residual packaging activity was provided by A repeats IV and V. The deletion of A repeat V resulted in a dramatic decrease in packaging efficiency, and a virus that lacked A repeats I, II, IV, and V was nonviable.

Late viral protein synthesis was examined with a representative set of the mutants described above. Each of the mutant viruses accumulated viral late polypeptides at levels that were within twofold of that of the wild-type virus (data not shown). Since each of these viruses replicated viral DNA at normal levels in coinfection experiments and synthesized normal levels of viral late proteins in single infections, we conclude that the defect observed with these mutants occurs late in the lytic cycle. This conclusion is consistent with a defect in the packaging of viral DNA into virions.

Insertion of an A repeat oligonucleotide in place of the packaging domain. Our model suggests that the A repeats function to enhance viral packaging and have an additive effect on packaging efficiency. To test this model further, we constructed recombinant viruses that contain one or more copies of an oligonucleotide to A repeat II (nt 252 to 275) in place of the entire packaging domain. The data obtained from infections with these recombinant viruses are shown in

FIG. 8. Analysis of viral mutants that contain one or more copies of an oligonucleotide to A repeat II (the sequence is shown below the mutant diagram) in place of the entire packaging domain (mutant *dI309-2* background). The solid bar represents the deletion in mutant *dI309-2*. The hatched boxes represent the A repeat II oligonucleotide(s) present in mutants *dI309-2-AII-1* (1 oligonucleotide), *dI309-2-AII-2* (2 oligonucleotides), *dI309-2-AII-3* (3 oligonucleotides), and *dI309-2-AII-6* (6 oligonucleotides). Infectious virus yields (YIELD) in single infections of 293 cells were determined as described in the legend to Fig. 3.

Fig. 8. The insertion of one or two A repeat oligonucleotides in place of the packaging domain did not restore virus viability. A virus that contained three repeated oligonucleotides was viable but was reduced approximately 200-fold in virus yield in a single infection compared with the wild-type virus. A virus that contained six copies of the A repeat oligonucleotide was also viable and was reduced 28-fold in virus yield compared with the wild-type virus. We could not detect packaged DNA in a coinfection experiment with either viable virus (data not shown). These results demonstrate that a single A repeat (A repeat II), when multimerized, can rescue the packaging defect, although these recombinant viruses were clearly still defective compared with the wild-type virus.

A spacing phenomenon correlates with an enhancer element II phenotype. A number of the mutants had an apparent spacing effect on virus yield in a single infection. This was particularly evident with viruses that contain unidirectional deletions that progress from nt 194 towards the downstream border of the packaging domain. Specifically, a cyclic effect on virus yield was observed with mutants *dI309-194/273* through *dI309-194/316* (Fig. 5). We were intrigued by the fact that all of the mutants that exhibited less severe growth defects contain deletions in which the variable 3' endpoints were separated from each other by nearly integral turns of the helix (*dI309-194/273*, *dI309-194/295*, and *dI309-194/316*). The 3' endpoints of deletions with mutants that displayed greater reductions in virus yields were placed on the opposite face of the DNA helix (*dI309-194/290* and *dI309-194/310*). This result suggested that the fluctuations in virus yield observed with these mutants (similar to the enhancer ele-

ment II phenotype described previously [18]) reflect a specific spacing requirement between one or more elements that are located within or outside of the enhancer region. This possibility was supported by the data obtained in the coinfection experiments in which each of these viruses was reduced in packaging efficiency to a similar degree (Fig. 5). The element II phenotype would be complemented by the coinfecting wild-type virus in these experiments (16, 18).

To test this model, we constructed two additional mutant viruses that alter the spacing in the packaging-enhancer region. By repairing the *XhoI* linker present at the junction of the deletion endpoints, we created two mutants, *dI309-194/310:in4* and *dI309-194/316:in4*, that differ from their respective parental viruses by a 4-bp insertion (Fig. 9). We reasoned that if the higher yield obtained with mutant *dI309-194/316* reflects a favorable spacing arrangement in the enhancer region, the insertion of 4 bp would reduce the virus yield obtained with this mutant. Similarly, if the reduced yield obtained with mutant *dI309-194/310* reflected an unfavorable spacing arrangement in the enhancer region, the insertion of 4 bp may increase the virus yield obtained with this mutant. The data obtained with the insertion mutants in single infections and coinfections are shown in Fig. 9. In single infections, the yields obtained with the parental viruses *dI309-194/310* and *dI309-194/316* were reduced 20- and 3-fold, respectively, compared with yields obtained with the wild-type virus. With mutant *dI309-194/310:in4*, the yield obtained in a single infection was reduced sixfold compared with the yield from the wild-type virus, a threefold increase relative to the parental mutant. Similarly, with mutant *dI309-194/316:in4*, the yield obtained in a single infection was

		YIELD	COINF.
<i>dI309-194/310</i>	194 310	20	6
<i>dI309-194/310:in4</i>	4 bp	6	7
<i>dI309-194/316</i>	316	3	6
<i>dI309-194/316:in4</i>	4 bp	10	8

FIG. 9. Analysis of viral mutants that contain spacer mutations in the packaging-enhancer region. Mutant viruses *dI309-194/310* and *dI309-194/316* are the same as those shown in Fig. 5. The 4-bp spacer mutations were inserted at the deletion endpoints (triangles). The in vivo packaging analyses (YIELD and COINF.) were performed as described in the legend to Fig. 4, except that the isolated DNAs were digested only with *ClaI* for Southern hybridization analysis.

reduced 10-fold compared with the yield from the wild-type virus, a threefold decrease relative to the parental mutant virus. In contrast, each of these mutant viruses displayed a comparable decrease in packaging efficiency when assayed in a coinfection experiment. These results support the notion that the enhancer element II phenotype observed with various mutant viruses in these studies and in previous analyses (18) reflects, at least in part, a spacing constraint of sequences at the left end of the Ad5 genome that influence enhancer region activity. The nature of these sequences is unknown.

DISCUSSION

On the basis of the results obtained with wild-type adenovirus and several temperature-sensitive mutants, it is generally assumed that adenovirus packages its DNA into preformed empty capsids, the proheads (4, 6, 7, 10, 20, 36). The demonstration of polarity in the packaging process (5, 14, 15, 37) and the delimitation of packaging domains in several adenovirus genomes (14, 16, 29) suggest that the process by which viral DNAs are selected from a pool of intracellular DNAs for packaging involves the recognition of specific viral sequences by a packaging protein(s). This was clearly shown for the packaging of bacteriophage lambda DNA in which a phage-encoded protein, terminase, recognizes and binds to a specific viral DNA sequence termed the *cos* site (12, 31). The terminase-lambda DNA complex then binds to the proheads, and packaging ensues (12). In other systems (for example, phages T7, T3, or p22), similar specific DNA-protein interactions are required to ensure proper packaging of the phage chromosome (8). *cis*-Acting packaging regions have also been shown to be present in the genomes of several retroviruses (1, 22, 24, 27, 32, 39) and herpesviruses (33, 35, 38). In these cases, specific proteins that interact with these sites have not been identified.

Here we report the identification of *cis*-acting elements that are required for efficient encapsidation of the Ad5 genome. Our results show that the packaging signal is composed of at least five elements which are located within the packaging domain between nt 238 and 261, nt 261 and 274, nt 274 and 293, nt 317 and 336, and nt 336 and 358. Four of five of these regions contain one copy of a repeated sequence, termed the A repeat (16), with the consensus sequence 5'- $\hat{\text{A}}$ ANA $\hat{\text{T}}$ TTTG-3' (Fig. 2). The region between nt 274 and 293 does not contain any obvious sequence similarity with the repeated element, aside from the fact that it is also very AT rich (Fig. 2). Other AT-rich sequences are located in the packaging domain (nt 208 to 215 and nt 361 to 368); a role for these sequences in packaging has not been determined. Our results strongly suggest that the A repeat represents a bona fide packaging element. Viruses that contained either the region with A repeats I and II (nt 238 to 274) or A repeats IV and V (nt 316 to 358) were reduced approximately sixfold in viral packaging in coinfection experiments (Fig. 3, *dl309-274/358*; Fig. 5, *dl309-194/316*; Fig. 7, *dl309-194/223:274/358* and *dl309-A5*). The deletion of an additional A repeat with any of these mutants greatly reduced packaging efficiency (Fig. 3, *dl309-267/358* and *dl309-261/358*; Fig. 7, *dl309-194/243:274/358*, *dl309-194/254:274/358*, and *dl309-A5:336/358*). The functional redundancy of individual elements in the packaging process is consistent with the role of a repeated sequence in this process. On the basis of these results, the A repeats appear to be redundant and have an additive effect on packaging efficiency. The additive effect of individual A repeats was also evident with

recombinant viruses that contained multiple copies of A repeat II (Fig. 8). The fact that the recombinant virus that carries six copies of A repeat II did not package viral DNA more efficiently may reflect the need for a particular spacing of adjacent A repeat motifs with respect to each other or the possibility that a functional hierarchy exists within the A repeat family. With Ad3, the region between nt 319 and 390 specifies polar packaging (29). The comparable region in Ad5 contains A repeats IV and V (Fig. 2), which are conserved between the Ad3 and Ad5 genomes (23). This suggests that the packaging of other adenovirus serotypes also relies on elements that are similar to the Ad5 A repeat sequence.

On the basis of the results obtained in these studies, we suggest the following model to account for the selectivity of the packaging process. In analogy to the phage lambda system, the individual packaging elements may represent the specific binding sites for a packaging protein(s). The binding of this protein(s) to these sites would allow the viral genome to recognize and position itself properly with a prohead, and packaging may ensue. It is possible that the redundancy of the packaging elements simply increases the number of binding sites in the viral genome to increase the probability of factor binding to viral DNA, as opposed to cellular sequences. This phenomenon has been clearly demonstrated with the repeated 60/81-bp elements in the *Xenopus* ribosomal RNA promoter region and has been termed the sink effect (28). This possibility is consistent with the results obtained with a number of mutants in which a greater decrease in packaging efficiency in coinfection experiments was evident in comparison with the results obtained with the same mutant viruses in single infections. We speculate that the greater reduction observed in the coinfection with these mutants represents a competition between the wild-type and mutant genomes for limiting concentrations of a packaging protein(s). Since the mutant genome contains fewer binding sites, it is a weaker competitor for factor binding. This competitive effect is also reminiscent of results obtained with the *Xenopus* repetitive ribosomal gene array (28).

It is also possible that the repeated packaging elements represent an array of interspersed binding sites whereby the binding of appropriate proteins would result in the formation of a defined DNA-protein structure. Only molecules bearing this structure would be suitable substrates for the packaging process. Both of these models imply that the packaging elements represent the binding sites for a specific protein(s) involved in the packaging process and that the specific DNA-protein interaction provides the molecular basis for the observed packaging efficiency. However, taking into account that the fifth packaging element (nt 274 to 293) does not contain any primary sequence homology to the A repeats aside from its AT-rich character, another model to account for the packaging process can be established. It is known that AT-rich domains alter the overall conformation of a DNA molecule by introducing bends in the helix (40). Restriction fragments that span the packaging-enhancer region show abnormal migration behavior in polyacrylamide gels, a result which is consistent with bent DNA (Gräble and Hearing, unpublished results). The presence of bending sites in the left end of the Ad5 genome was also recently reported (2, 9). Three of the bending loci proposed in this study coincide with A repeats I, IV, and V (2, 9). Therefore, it is conceivable that a packaging protein(s) recognizes the overall structure of the DNA molecule, represented as bent DNA, or that this structure is required for recognition of a prohead.

A number of mutant viruses displayed a complex pheno-

type which showed an apparent contradiction between the results obtained in single infections and those obtained in coinfection experiments (Fig. 5, mutants *dl309-194/273* through *dl309-194/316*). Several of these mutants exhibited packaging defects in coinfections which could account for only a portion of the defect observed in virus yield in a single infection. These results indicated that the introduced deletion, aside from reducing the packaging capacity of the mutant genome, also impaired an additional function required for normal virus replication. Since the presence of a wild-type virus in the coinfection experiment can compensate for this additional defect, the defect is probably the result of the lack of a *trans*-acting factor. At the present time, the nature of this defect is unknown. However, since the phenotype of these mutants is reminiscent of the phenotype displayed by enhancer element II mutants previously described (18), we believe that the additional growth defect with these mutants might be caused by the inactivation of an enhancer element. Enhancer element II was previously mapped to Ad5 nt 250 to 280 (18). Our results, however, indicate that the regulatory element(s) responsible for this effect may be located outside of the E1A enhancer region, since the fluctuation in virus yield in single infections was clearly related to a spacing effect of flanking regions (Fig. 9, *dl309-194/310* versus *dl309-194/310:in4* and *dl309-194/316* versus *dl309-194/316:in4*). This does not exclude a role for the region located between nt 250 and 280, but additional flanking elements are probably involved in this phenomenon. The nature of these elements is unknown.

The organization of the Ad5 packaging-enhancer region is complex, with redundant as well as overlapping transcriptional and packaging regulatory elements. The binding of specific proteins to the packaging sequences at late times after infection may displace or prevent the interaction of transcriptional components with this region. The overlapping organization of enhancer and packaging sequences may have evolved to repress transcription at late times after infection when packaging occurs. This may alter the conformation of viral DNA from an open conformation involved in transcription to a closed conformation to elicit viral DNA condensation by the core and auxiliary proteins. Thus, the packaging process may represent an additional level at which viral expression is temporally regulated.

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