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cis and *trans* Requirements for the Selective Packaging of Adenovirus Type 5 DNA

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Polar packaging of adenovirus DNA into virions is dependent on the presence of *cis*-acting sequences at the left end of the viral genome. Our previous analyses demonstrated that the adenovirus type 5 (Ad5) packaging domain (nucleotides 194 to 358) is composed of at least five elements that are functionally redundant. A repeated sequence, termed the A repeat, was associated with packaging function. Here we report a more detailed analysis of the requirements for the selective packaging of Ad5 DNA. By introducing site-directed point mutations into specific A repeat sequences, we demonstrate that the A repeats represent *cis*-acting functional components of the packaging signal. Additional elements, located outside the originally defined packaging domain boundaries and that resemble the A repeat consensus sequence, also are capable of promoting the packaging of viral DNA. The *cis*-acting components of the packaging signal appear to be subject to certain spatial constraints for function, possibly reflecting a necessity for the coordinate binding of packaging proteins to these sites. In agreement with this idea, we present evidence that the interaction of a limiting *trans*-acting factor(s) with the packaging domain in vivo is required for efficient encapsidation of the Ad5 genome.

Very little is known about the mechanism that allows selective packaging of the adenovirus (Ad) genome into viral capsids late in the infectious cycle. This event presumably involves the specific recognition of *cis*-acting viral DNA sequences followed by condensation and encapsidation of the viral genome (2, 6–8, 12, 13, 33). It has been shown for adenoviruses in subgroups B (Ad3, Ad7, and Ad16 [3, 19, 25, 30, 34]) and C (Ad5 [3, 20]) that the viral DNA is inserted into preformed empty capsids in a polar fashion from left to right, suggesting the existence of *cis*-acting packaging elements in the left end of the genome. Incomplete particles formed by Ad3 and Ad7 contain subgenomic DNA molecules that are enriched in left-end sequences even though both ends of the viral genome are represented in equivalent quantities in the nuclear pool of subgenomic DNAs (3, 4, 25, 30, 34). Analysis of Ad16 variant viruses that contain different amounts of left-end sequences duplicated at the right end of the genome identified left-end sequences important for selective packaging located between nucleotides (nt) 290 and 390 (19).

We have 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) (17, 21, 22). Mutants lacking this region are nonviable but can be rescued by insertion of the left-terminal 355 nt at the right end of the genome (22). The Ad5 packaging domain shares properties with eukaryotic transcriptional enhancer elements since the packaging signal functions at either end of the viral genome, in an inverted orientation, and can be moved within several hundred base pairs from its original location without a reduction in virus yield (21). Because of a *cis* requirement for virus replication, it has not been possible to determine whether sequences within the inverted terminal repeats (ITR) are also required for packaging.

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The Ad5 packaging domain consists of at least five elements that are functionally redundant (17). Four of the five regions contain an AT-rich repeated sequence motif termed the A repeat (Fig. 1). The fifth region does not contain any obvious primary sequence similarity to the A repeat aside the fact that it is also AT rich. Our results indicated that the A repeats function to enhance viral packaging and that the efficiency of the packaging process is determined by the number of individual elements that are present in the viral genome. On the basis of these results, several models accounting for the selectivity of the encapsidation process can be proposed. It is possible that the individual repeats represent the binding sites for a packaging protein and, considering the repetitive structure of the packaging signal, that the repeated elements form an array of interspersed binding sites whereby the binding of packaging proteins to all the sites would result in the formation of a defined protein-DNA structure. However, given the AT-rich character of the A repeats, it is also conceivable that the function of these elements is to alter the structural conformation of the DNA helix through the introduction of bends (35). Depending on the spatial arrangement of the bending sites, the change in conformation either could be restricted to a confined region such as the AT-rich sequences themselves or could affect the conformation of the left-end portion of the DNA molecule as a whole. In this case, the *cis*-acting recognition signal would be represented by a structural rather than a sequence-specific feature of the viral genome.

The Ad5 packaging domain is located within the early region 1A (E1A) enhancer region (Fig. 1A) (17, 21–23). The E1A enhancer is composed of two functionally distinct enhancer elements that regulate viral gene expression. Enhancer element I is repeated and specifically regulates transcription of the E1A gene (22, 23). Element I mutants are efficiently complemented when the E1A gene products are provided in *trans*. Enhancer element II is located between these repeated sequences and regulates transcription in *cis* of all early regions on the viral chromosome (23). The reduction in viral early gene expression observed with

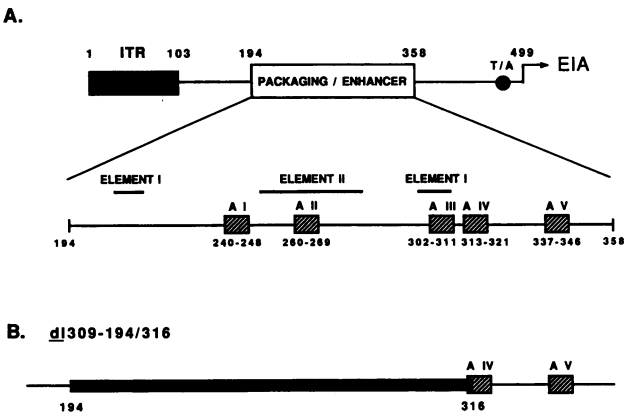

FIG. 1. (A) Schematic diagram of the left end of the Ad5 genome including the E1A 5′ flanking region. Numbers at the top indicate nucleotide positions relative to the left terminus of the genome. The ITR and the packaging/enhancer domain are represented by stippled and open boxes, respectively. The E1A TATA box motif (T/A) is indicated by a black circle. E1A transcription is initiated at nt 499, as indicated by the arrow. An enlarged view of the packaging/enhancer region (nt 194 to 358) is shown below. A repeats AI through AV are represented by the hatched boxes. Their nucleotide positions relative to the left terminus of the genome are indicated by the numbers below the line. Components of the enhancer region (elements I and II [23]) are represented by solid bars above the line. (B) Schematic view of mutant virus *dl*309-194/316. This virus was used as the parental virus for construction of the LS and insertion mutants. The nucleotide numbers correspond to the first nucleotides present on either side of the deletion. The deleted sequences are indicated by the stippled box.

element II mutants results in decreased DNA replication and a corresponding three- to sevenfold reduction in virus yield compared with that of a wild-type virus (21, 23). The *cis*-acting defect observed with element II mutants can be complemented in mixed infections with a wild-type virus in which viral proteins required for efficient propagation are provided in *trans* (17, 21, 23). Our recent data suggest that the originally defined enhancer element II domain (nt 250 to 280) is not sufficient to provide full enhancer activity but that additional flanking sequences are required to ensure proper enhancer function (17). The nature of these elements is still unknown.

Here we describe a more detailed analysis of the *cis* and *trans* requirements for the selective packaging of Ad5 DNA into virions. By introducing site-directed mutations in specific A repeat sequences, we clearly demonstrate that the A repeats represent functional components of the Ad5 packaging signal. Our analysis also identifies additional packaging elements located outside the originally defined packaging domain which resemble the A repeat consensus sequence. Our results suggest that a particular spacing of adjacent packaging elements with respect to each other is required to ensure packaging activity. It is likely that this observed spacing constraint reflects the necessity for the coordinate binding of packaging proteins to these sites. Finally, we present evidence that a limiting *trans*-acting factor(s) interacts with the packaging domain in vivo.

MATERIALS AND METHODS

Mutant viruses and plasmids. Ad5 *dl*309 is a phenotypically wild-type virus that contains a unique *Xba*I cleavage site at 3.8 map units (24). *dl*309-194/316 (Fig. 1B [17]) is a *dl*309 variant that carries a deletion of sequences between nt 194 and 316. Ad5 *dl*10/28 was previously described as *dl*309-194/243:274/358 (17). This double mutant contains a deletion between nt 194 to 243 and between nt 274 to 358 in a *dl*309 background. The linker scanning (LS) mutants (*dl*309-LS) and the spacer mutant *dl*309-380:in6 were originally constructed in plasmid pKS-194/316. This plasmid contains the left-end *Xba*I fragment from *dl*309-194/316 (0 to 3.8 map units) cloned into the *Eco*RI and *Xba*I restriction sites in the polylinker region of the pBS-KS (+) Bluescript vector (pKS; Stratagene). The spacer mutants derived from *dl*10/28 were constructed in plasmid pE1A-10/28 (17), which contains the left-end *Xba*I fragment from *dl*309-194/243:274/358 cloned into pBR322. All mutations were subsequently rebuilt into intact viruses by the method of Stow (32). Mutant viruses were propagated and titered on 293 cells, a human embryonic kidney cell line that constitutively expresses the Ad5 E1A and E1B gene products (18).

A series of LS mutations and an insertion mutation were made by using synthetic oligonucleotides approximately 30 bp in length and complementary to a minimum of 10 bases on each side of the substitution. The names of the LS and insertion mutations and the sequences of the oligonucleotides used to construct them are shown in Table 1. The mutant plasmids were constructed by the method of Kunkel (27). Insertion mutants *dl*10/28-194:in4 and *dl*10/28-358:in4 were generated by partial linearization of the parental plasmid pE1A-10/28 with *Xho*I, followed by a repair reaction with Klenow DNA polymerase and subsequent ligation. The authenticity of each mutation was verified by nucleotide sequence analysis using the dideoxy procedure (31).

Determination of virus yield and packaging efficiency. The 293 cell line (18) was propagated in monolayer cultures in Dulbecco modified Eagle medium supplemented with 10% calf serum. Viral infections of 293 cells were performed at a multiplicity of infection of 5 PFU per cell at 37°C for 1 h. After infection, cells were washed two times with phosphate-buffered saline solution and fresh medium was added.

TABLE 1. Nucleotide sequences of site-directed LS and insertion mutants[a]

Oligonucleotide	Sequence
LS1	5′-GATTTTGTGTTAC**GTCGAC**CGCGTAATATTTG-3′ (328–333)
LS4	5′-GTCCTCGAGG**GTCGAC**TGTTACTCATAGC-3′ (316–321)
LS5	5′-CATAGCGCGTA**GTCGAC**GTCTAGGGCC-3′ (340–345)
LS6	5′-GGGCCGCGGGGA**GTCGAC**CCGTTTACGTGG-3′ (363–368)
LS7	5′-CGGGGACTTTGAC**GTCGAC**CGTGGAGACTCGCC-3′ (370–375)
380:in6	5′-CCGTTTACGTGG**GTCGAC**AGACTCGCCC-3′ (380)

[a] The nucleotide sequence flanking each site-directed mutation is shown; each sequence listed represents the oligonucleotide used for mutagenesis. The 6-bp substitutions with the LS mutants and the insertion mutation described in the text are underlined. Numbers in parentheses indicate the location (nucleotides) of the substitution in the Ad5 genome.

For determination of virus yield in a single infection, the infected cell cultures were harvested 48 h postinfection and lysed by three cycles of freezing and thawing. The amount of infectious virus present in these lysates was determined by plaque assays on 293 cells. To determine the packaging efficiency of the mutant viruses, 293 cells were coinfected with 5 PFU of both a mutant virus and wild-type virus *dl*309. At 48 h after infection, one half of the cells was used to isolate total nuclear DNA and the other half was used to prepare viral DNA from virions. For the isolation of infected cell total nuclear DNA, the cells were lysed by the addition of Nonidet P-40 to 0.4%, the nuclei were precipitated, and total nuclear DNA was isolated as described previously (17, 21). For the isolation of viral DNA from virions, the procedure described by Hammarskjold and Winberg (19) was used, with the following modifications (17). Infected cells were precipitated and resuspended 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 $CaCl_2$ and 2 mM $MgCl_2$ 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 ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) to a final concentration of 5 mM each. Virus particles were lysed by the addition of Sarcosyl 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. DNA isolated from nuclei or virions was digested with *Cla*I and analyzed by Southern hybridization (28) using plasmid pE1A-WT, ^{32}P-labeled by the random primer method (14), as a probe. 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 yield in the single infections and the data for packaging efficiency based on coinfection experiments represent the averages of four to five independent experiments.

Cotransfection experiment. Monolayer cultures of COS1 cells (16) were grown in Dulbecco modified Eagle medium containing 10% fetal bovine serum. Plasmid pUC-WT-ENH contains the wild-type packaging/enhancer region from Ad5 nt 189 to 355 cloned into the *Sma*I site in the polylinker region of pUC9 (1). Plasmid pOR4 (9) contains simian virus 40 (SV40) sequences 5171 to 160 between the SV40 *Hin*dIII and *Bst*NI sites, which encompass the SV40 origin of replication, and an *Eco*RI linker at the *Bst*NI site. The fragment spanning the origin of DNA replication was excised from vector sequences by *Eco*RI digestion followed by a repair reaction using Klenow DNA polymerase and a second digestion with *Hin*dIII. This fragment was then inserted between the Klenow-repaired *Pst*I site and *Hin*dIII site of the vector plasmid pUC-WT-ENH. The resulting plasmid is referred to as pOR4-PAC$^+$. As a control, we inserted the pOR4-derived *Eco*RI-*Hin*dIII fragment into the same location of a pUC19 plasmid that does not contain the packaging/enhancer region. This control plasmid is referred to as pOR4-PAC$^-$. The authenticity of each plasmid construct was verified by nucleotide sequence analysis (31). DNA transfections of COS1 cells were performed by the calcium phosphate precipitation method (36). At approximately 65% confluency, the cells were transfected with 5 μg of *dl*309 DNA and 15 μg of either pOR4-PAC$^+$, pOR4-PAC$^-$, or salmon sperm carrier DNA per 100-mm diameter dish. After incubation for 12 h with the calcium phosphate precipitate, the cells were washed with Tris-buffered saline solution (TBS), TBS containing 3 mM EGTA, and TBS. Fresh medium was added, and cultures were harvested 36 h later. The cells were lysed by three cycles of freezing and thawing, and infectious virus yields were determined by plaque assay on 293 cells. An aliquot of each culture was used to determine total levels of viral DNA and cytoplasmic late mRNA. Total nuclear DNA was isolated from purified nuclei as described above. The level of total viral DNA was quantitated by slot blot analysis, using a ^{32}P-labeled probe of the Ad5 genome. Total RNA was extracted from cell lysates with guanidinium thiocyanate followed by centrifugation over cesium chloride (28). The level of viral late mRNAs were quantitated by slot blot analysis using a ^{32}P-labeled probe corresponding to Ad5 late region L3 (52.5 to 58.5 map units).

RESULTS

Construction and analysis of viruses containing LS mutations in the packaging domain. To further test the involvement of the A repeats in the encapsidation process, we constructed a series of viral mutants in which individual A repeats were specifically inactivated by the introduction of a 6-bp LS mutation. In view of the functional redundancy of the packaging elements, the mutations were introduced into a mutant virus background (*dl*309-194/316; Fig. 1B) (17) containing a deletion of A repeats I, II, and III. Recombinant viruses (Fig. 2) were constructed that contain LS mutations in A repeat IV (*dl*309-LS4, nt 316 to 321), in A repeat V (*dl*309-LS5, nt 340 to 345), and in the spacer region between A repeats IV and V (*dl*309-LS1, nt 328 to 333). Two independent assays were used to test the efficiency of packaging with the mutant viruses. First, overall virus growth was determined in single infections of 293 cells. As described above, viruses containing mutations that affect enhancer element II function show reduced expression of the early gene products which results in decreased viral DNA replication and a corresponding reduction in virus yield (23). This effect, referred to as the element II phenotype, is independent of viral packaging and can be efficiently complemented when viral gene products required for replication are provided in *trans* by a coinfecting wild-type virus (17, 21). To determine what portion of the observed reduction in virus yield was due to a packaging defect, mutant viruses were analyzed in a coinfection experiment with the wild-type parental virus *dl*309. In these experiments, 293 cells were coinfected with a mutant and a wild-type virus. Two days after infection, one half of the cells was used to isolate total nuclear DNA from purified nuclei and the other half was used to isolate encapsidated viral DNA from purified virus particles. Both coinfecting genomes were distinguished by restriction enzyme digestion followed by Southern hybridization analysis. By comparing the relative amounts of mutant and wild-type DNAs in the nuclei of infected cells with the amount of each viral DNA that was actually present in intact virus particles, the packaging efficiency of the mutant genome could be accurately measured independent of an enhancer element II effect.

The parental mutant virus *dl*309-194/316 showed a threefold decrease in virus yield in a single infection and a sixfold reduction in the amount of packaged DNA compared with the wild-type virus in a coinfection (17) (Fig. 2). The data obtained with the LS mutants in A repeats IV and V in single infections and coinfections are shown in Fig. 2 and 3. Mutant viruses *dl*309-LS4 and *dl*309-LS1 grew as well as the parental mutant virus when assayed in single infections or in coinfec-

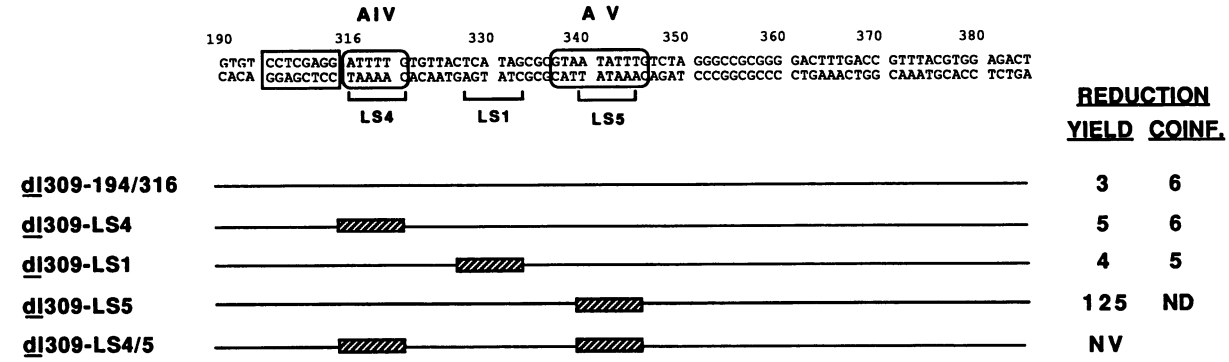

FIG. 2. (A) Viral mutants with LS mutations in A repeats IV and V. At the top is shown the nucleotide sequence of the parent virus *dl*309-194/316 between nt 190 and 385. An *Xho*I linker is present at the deletion junction and is indicated by an open box. Nucleotide numbers relative to the left end are indicated above the line. A repeats IV and V are encircled. The positions and names of the LS mutants are indicated by brackets below the sequence (each LS mutation replaced the wild-type sequence with the sequence GTCGAC). The corresponding single- and double-mutant viruses are listed below, and the LS mutations are indicated by hatched boxes. For single-virus infections, 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 ratio). The total virus yield in an infection with the wild-type virus (*dl*309) was approximately 10,000 PFU per infected cell. In coinfection experiments, 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; NV, mutant virus was nonviable in repeated reconstruction experiments.

tions with a wild-type virus. Mutation of A repeat V (*dl*309-LS5), however, resulted in an additional 40-fold decrease in virus yield relative to the parental mutant virus in a single infection, and no detectable signal was observed in packaged virion DNA in a coinfection. This mutant virus replicated to a level comparable to that of the wild-type virus (Fig. 3), indicating that the reduction in packaged DNA was due to a *cis*-acting packaging defect. When LS mutations in A repeats IV and V were coupled (*dl*309-LS4/5), the resulting virus was nonviable. This result demonstrated that while A repeat IV lacked detectable function when A repeat V was intact, A repeat IV was essential for virus viability in the absence of A repeat V.

Additional packaging elements are located outside the originally defined packaging domain. Our previous studies demonstrated that at least two A repeats were required for virus viability (17). On the basis of this observation, the results described above with the single LS mutations in A repeats IV and V indicated that an additional packaging element(s) may lie outside the originally described packaging domain (nt 194 to 358) that may be required for virus viability in the *dl*309-194/316 mutant background. Our previous results (17) and those described below suggested that there are spatial constraints on the location and function of individual packaging elements. Therefore, a mutant virus (*dl*309-380:in6; Fig. 4) that contains a 6-bp insertion at nt 380 was constructed in a *dl*309-194/316 background. This mutant was constructed on the basis of the hypothesis that a 6-bp insertion between two critical packaging elements would significantly impair virus growth. This insertion, however, reduced packaging efficiency only three- to fourfold compared with the parental virus (Fig. 4). A similar decrease in packaging efficiency was observed when this insertion was coupled with the LS mutation in A repeat IV (*dl*309-LS4/380:in6; Fig. 4), a mutant virus in which A repeat V is critical for viability. These results suggested that if an additional packaging element(s) existed outside the nt 194 to 358 packaging domain, it likely would be located to the left of nt 380.

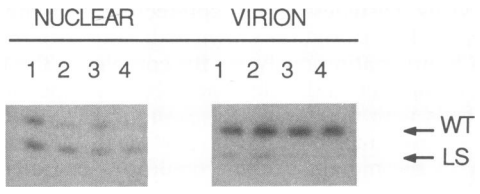

FIG. 3. Southern hybridization analysis of nuclear and virion DNAs isolated from 293 cells coinfected with the wild-type virus and mutants depicted in Fig. 2. Total nuclear DNA and virion DNA were digested with *Cla*I and analyzed by Southern hybridization, using an Ad5 left-end fragment as a ^{32}P-labeled probe. The corresponding wild-type (WT) and LS mutant (LS) left-end DNA fragments are indicated. The mutant viruses tested were *dl*309-194/316 (lanes 1), *dl*309-LS4 (lanes 2), *dl*309-LS1 (lanes 3), and *dl*309-LS5 (lanes 4).

Two sequences which resemble A repeats by virtue of their AT-rich character are located between nt 358 and 380 (Fig. 4). One element, termed A repeat VI, is located between nt 363 and 368, and a second element, A repeat VII, is positioned between nt 370 and 375. We constructed mutant viruses which contain LS mutations in these putative packaging elements in the *dl*309-194/316 mutant virus background. The data obtained with these mutants in single infections and coinfections with a wild-type virus are shown in Fig. 4 and 5. Mutation of A repeat VI (*dl*309-LS6, nt 363 to 368) resulted in the loss of virus viability. Mutation of A repeat VII (*dl*309-LS7, nt 370 to 375) led to a three- to fivefold decrease in virus yield relative to the parental mutant. In the coinfection, the amount of packaged mutant DNA was reduced correspondingly. The double mutation of A repeats IV and VII, *dl*309-LS4/7, resulted in a comparable decrease in packaging efficiency. These results demonstrate that A repeats VI and VII constitute functional components of the Ad5 packaging signal. Since deletion of these elements in a wild-type virus background had no effect on the packaging ability of the mutant genome (22), these results lend further support to the model that the packaging signal is composed of functionally redundant elements (17, 21). From these analyses, we conclude that additional packaging ele-

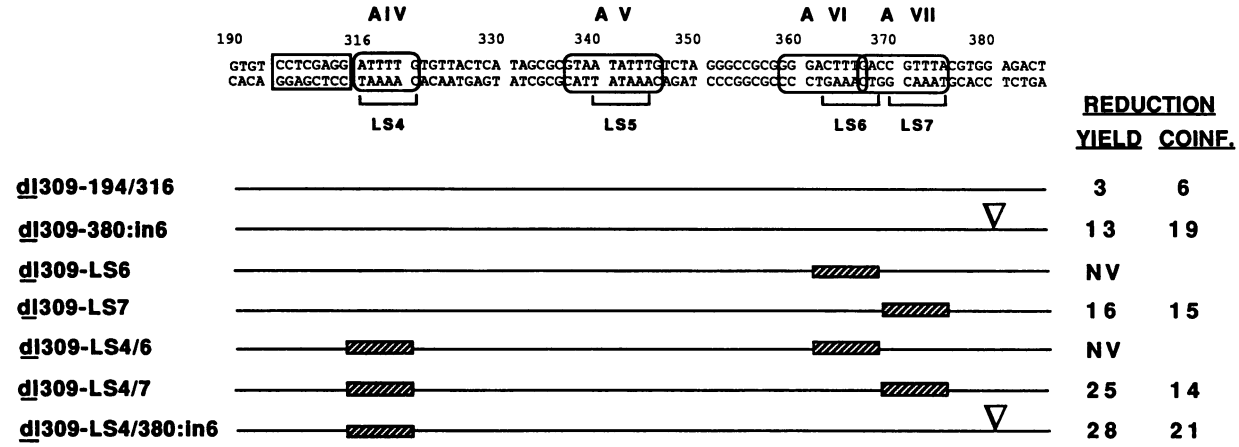

FIG. 4. Viral mutants with LS mutations in A repeats VI and VII and an insertion at nt 380. The schematic, mutant names, and packaging analyses are as described in the legend to Fig. 2. The positions and names of the LS mutants are indicated by brackets below the sequence (each LS mutation replaced the wild-type sequence with the sequence GTCGAC). The insertion mutations contains insertions of the sequence GTCGAC. The corresponding single- and double-mutant viruses are listed below; the LS mutations are indicated by hatched boxes, and the insertions are indicated by inverted triangles.

ments are located outside the originally defined boundaries of the packaging domain. In the absence of A repeats I, II, and III, A repeats V and VI play a key role in the packaging process, since inactivation of either element led to a drastic decrease in virus viability (Fig. 3, *dl*309-LS5; Fig. 5, *dl*309-LS6). A repeats IV and VII and possibly an element to the right of nt 380 help to optimize the packaging efficiency, perhaps by facilitating formation of a packaging-competent complex, but are not absolutely essential for packaging function.

A spatial requirement correlates with packaging efficiency. Our previous studies indicated that there are spacial constraints on the *cis*-acting elements involved in Ad5 packaging (17, 21). To further test the possibility that efficient assembly of a packaging-competent complex requires functional interactions between multiple packaging elements, we constructed two mutant viruses in which the spacing between a truncated packaging domain and flanking leftward and rightward sequences was altered. The parent virus used to construct these variants was a double-mutant virus, *dl*10/28 (Fig. 6) (17), that contains A repeats I, II, VI, and VII. This virus displayed a 143-fold decrease in virus yield in a single infection (17) (Fig. 6). Even though this mutant virus replicated to a level comparable to that of the wild-type virus in a coinfection, no mutant DNA was detectable in virions, indicating that the observed growth defect was largely due to

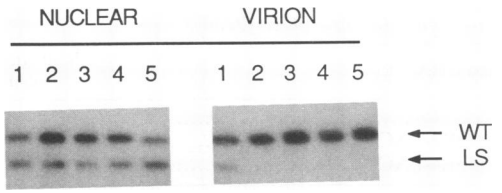

FIG. 5. Southern hybridization analysis of nuclear and virion DNAs isolated from 293 cells coinfected with the wild-type virus and mutant viruses depicted in Fig. 4. Southern hybridization analysis of total nuclear DNA and virion DNA was performed as described in the legend to Fig. 3. The mutant viruses tested were *dl*309-194/316 (lanes 1), *dl*309-LS7 (lanes 2), *dl*309-LS4/7 (lanes 3), *dl*309-380:in6 (lanes 4), and *dl*309-LS4/380:in6 (lanes 5).

a packaging deficiency of the mutant genome (17). Our previous results demonstrated that the residual packaging activity seen with this mutant is due, at least in part, to the presence of A repeats I and II (17). To test whether these two elements are sufficient to allow packaging of the mutant genome or whether functional interactions with adjacent elements are required to generate a packaging-competent substrate, two spacer mutants (*dl*10/28-194:in4 and *dl*10/28-358:in4; Fig. 6) that differ from the parental virus by a 4-bp insertion at the indicated positions (nt 194 or 358) were constructed. The results of single infections and coinfections with these mutant viruses are shown in Fig. 6.

A 4-bp insertion at the leftward deletion endpoint (*dl*10/28-194:in4) resulted in a sevenfold increase in yield in a single infection relative to the parental mutant virus. In a coinfection, the amount of packaged mutant DNA was reduced approximately 9-fold compared with the wild-type virus, while packaged DNA in the coinfection with the parental mutant was not detectable (>50-fold decrease). In contrast, the insertion of 4 bp at the rightward deletion endpoint (*dl*10/28-358:in4) resulted in the loss of virus viability. With both spacer mutants, the relationship of sequences flanking both sides of the truncated packaging domain remained unchanged with respect to each other, whereas their position relative to the intervening packaging region varied according to the site of the insertion. That these results do not reflect the phenotype of enhancer element II mutants was evident by the altered packaging efficiency in the coinfection experiment. We therefore conclude that the particular spacing of adjacent *cis*-acting elements with respect to each other is important in order to develop functional interactions between individual components of the packaging signal. In the absence of A repeats III, IV, and V, A repeats I and II function in conjunction with sequences flanking the originally defined packaging domain. In view of the results obtained with the LS mutants (Fig. 3 and 5), it is likely that A repeats VI and VII represent such interaction sites in the rightward direction. We have not yet identified the leftward sequences that participate in the formation of the packaging-specific complex. It is possible

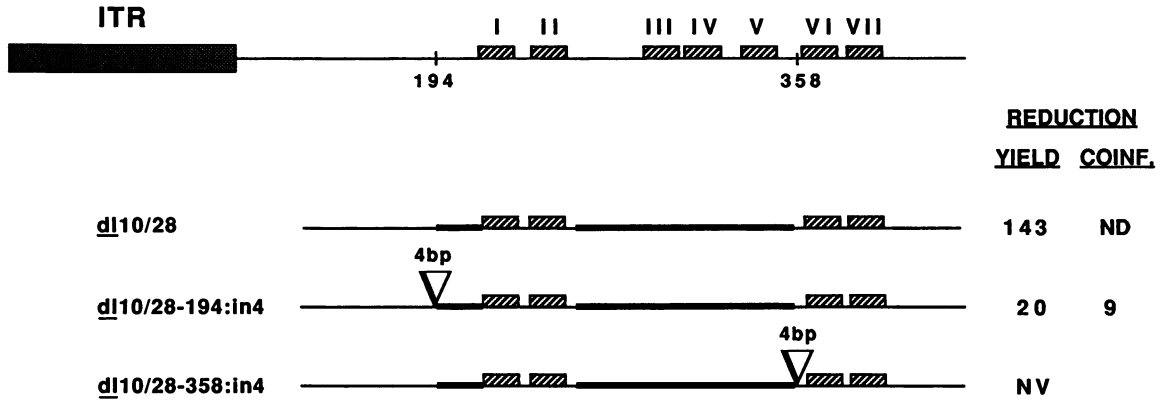

FIG. 6. Viral mutants with insertions at the leftward and rightward deletion endpoints of a minimal packaging domain. At the top are shown the positions of A repeats I through VII, depicted by shaded boxes, relative to the left terminus of the viral genome. The parental mutant virus *dl*10/28 and the spacer mutants described in the text are shown below. The deleted sequences in *dl*10/28 are indicated by solid bars; shaded boxes represent A repeats present in the mutant genome; 4-bp insertions are indicated by open triangles. The nucleotide number in each mutant virus name corresponds to the site of the 4-bp insertion. The packaging analysis of the mutant viruses was performed as described for Fig. 2.

that sequences in the ITR play a role in this process (see Discussion).

A titratable factor binds to the packaging domain. To determine whether the packaging elements represent binding sites for *trans*-acting factors, we inserted the packaging domain (nt 194 to 358; Fig. 1A) into a plasmid vector (pOR4 [9]) containing the SV40 origin of replication (pOR4-PAC$^+$). This plasmid replicates to high copy number in COS1 cells, a monkey cell line that constitutively expresses SV40 large T antigen (16). If a limiting *trans*-acting factor(s) interacts with the packaging signal in vivo, the presence of an excess of unlinked binding sites could compete for factor binding and thus prevent the formation of a functional packaging complex on the Ad5 genome. This possibility was supported by results obtained from the analysis of a number of mutant viruses described previously (17). These mutants showed a greater decrease in packaging efficiency in a coinfection with a wild-type virus relative to the reduction in yield observed in a single infection, a phenotype which could reflect a competition between the coinfecting wild-type and mutant viruses for a limiting packaging factor(s). COS1 cells were cotransfected with pOR4-PAC$^+$ plasmid DNA and wild-type *dl*309 DNA, and the infectious virus yield was determined 36 h after transfection. As controls, *dl*309 DNA was transfected alone or with the vector plasmid pOR4 lacking the packaging domain (pOR4-PAC$^-$). Additionally, total nuclear DNA was examined for the accumulation of replicated Ad5 DNA and total cellular RNA was examined for the accumulation of transcribed late viral mRNAs.

These results (Table 2) demonstrated that high levels of unlinked packaging domain sequences inhibited viral growth. Cotransfection of wild-type *dl*309 DNA with pOR4-PAC$^+$ plasmid DNA resulted in a greater than 600-fold reduction in virus yield, whereas transfection of viral DNA in the presence of an equivalent amount of vector sequences (pOR4-PAC$^-$) reduced virus yield only fivefold. Comparable levels of total viral DNA (within twofold of each other) were present in transfected cells (Fig. 7A), indicating that equal numbers of viral templates were available for packaging, and comparable levels of viral late mRNAs were observed (Fig. 7B). Therefore, the defect responsible for the reduction in virus yield must have occurred at a late step in the infectious cycle, which is consistent with a defect in the packaging of viral DNA. We conclude that the unlinked Ad5 packaging domain present in the transfected plasmid DNA likely competed for the binding of a limiting *trans*-acting component(s) required for the efficient packaging of Ad5 DNA in vivo.

DISCUSSION

In the case of bacteriophage lambda, it has clearly been shown that the process by which phage DNA is selected from a pool of intracellular DNA for packaging involves the recognition of specific viral sequences, termed *cos* sites, by a phage-encoded protein, the terminase (10, 15). Binding of the terminase to its recognition sites results in the formation of a specific nucleoprotein complex which is required for recognition and further interactions of the phage DNA molecule with the empty proheads (10, 15). Experimental evidence obtained from the analysis of several temperature-sensitive mutants suggests that Ad packages its DNA into preformed empty capsids, the proheads (2, 6–8, 12, 13, 33). The identification of packaging domains in several Ad genomes and the demonstration of polarity in the encapsidation process (3, 17, 19–21, 25, 30, 34) suggests that the specificity of the encapsidation process depends on recogni-

TABLE 2. Virus yields generated in COS1 cells transfected with *dl*309 DNA, *dl*309 DNA plus pOR4-PAC$^+$, and *dl*309 DNA plus pOR4-PAC$^-$[a]

Transfected DNA	Reduction in virus yield (fold)
*dl*309	1
*dl*309 + pOR4-PAC$^+$	>600
*dl*309 + pOR4-PAC$^-$	5

[a] Cells were transfected with the indicated DNAs, and infectious virus yields were determined by a plaque assay with cellular extracts prepared 36 h after transfection. Virus yields are expressed as fold reduction relative to the yield obtained by transfection of *dl*309 DNA alone. The total virus yield in a virus infection of COS1 cells with the wild-type virus (*dl*309) was approximately 2,000 PFU per infected cell. The infectious virus yield in a transfection with wild-type viral DNA was reduced approximately 10^4, presumably because of the inefficient nature of the transfection procedure.

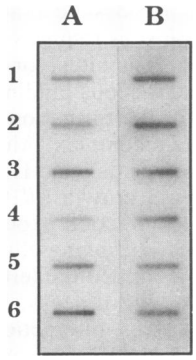

FIG. 7. Viral DNA and RNA accumulation in transfected COS1 cells. COS1 cells were transfected with *dl*309 DNA (rows 1 and 2), *dl*309 plus pOR4-PAC$^+$ (rows 3 and 4), or *dl*309 plus pOR4-PAC$^-$ (rows 5 and 6). Total nuclear DNA (lane A) and total RNA (lane B) were isolated 36 h after transfection and applied to nitrocellulose filters. Viral DNA (lane A) was probed with uniformly labeled Ad5 genomic DNA. Viral RNA (lane B) was probed with a cloned DNA fragment corresponding to late region 3.

tion of specific viral sequences present in the left end of the genome by a packaging protein(s).

In a recent report, we demonstrated that the Ad5 packaging domain is composed of five functionally redundant elements which have an additive effect on the packaging efficiency of the viral genome (17). Four of the five elements contain a repeated sequence motif, the A repeat, with the consensus sequence 5′-GT(N_{3-4})TTTG-3′ (Fig. 8). On the basis of these results, we proposed three models to account for the selectivity of the packaging process. First, the individual packaging elements might represent specific binding sites for packaging proteins. Binding of a packaging protein(s) to its recognition site(s) would allow the viral genome to recognize and position itself with an empty prohead, and packaging could ensue. Second, individual packaging elements might represent an array of interspersed binding sites such that coordinate binding of the appropriate packaging proteins to all of these sites would result in the formation of a structurally defined nucleoprotein complex at the left end of the viral genome. Only molecules bearing this structure would be recognized as bona fide packaging substrates. It is possible that the formation of this complex requires further interactions with factors bound to the inverted terminal repeat sequences (ITR). This assumption is based on our previous results which demonstrated that the packaging domain (nt 194 to 358) can function independent of position and orientation but must be located close to the terminus of the viral genome in order to maintain packaging activity (21). Additional data in support of this model have been obtained from the analysis of several temperature-sensitive mutants, which suggested that factors implicated in the formation of the DNA synthesis initiation complex may play a role in viral DNA encapsidation (5). Third, as a consequence of their AT-rich character, the A repeats could also represent functional bending loci (35). This model implies that the packaging specificity is determined by a structural rather than a sequence-specific feature of the viral genome. It is conceivable that a packaging protein recognizes the overall conformation of a DNA molecule repre-

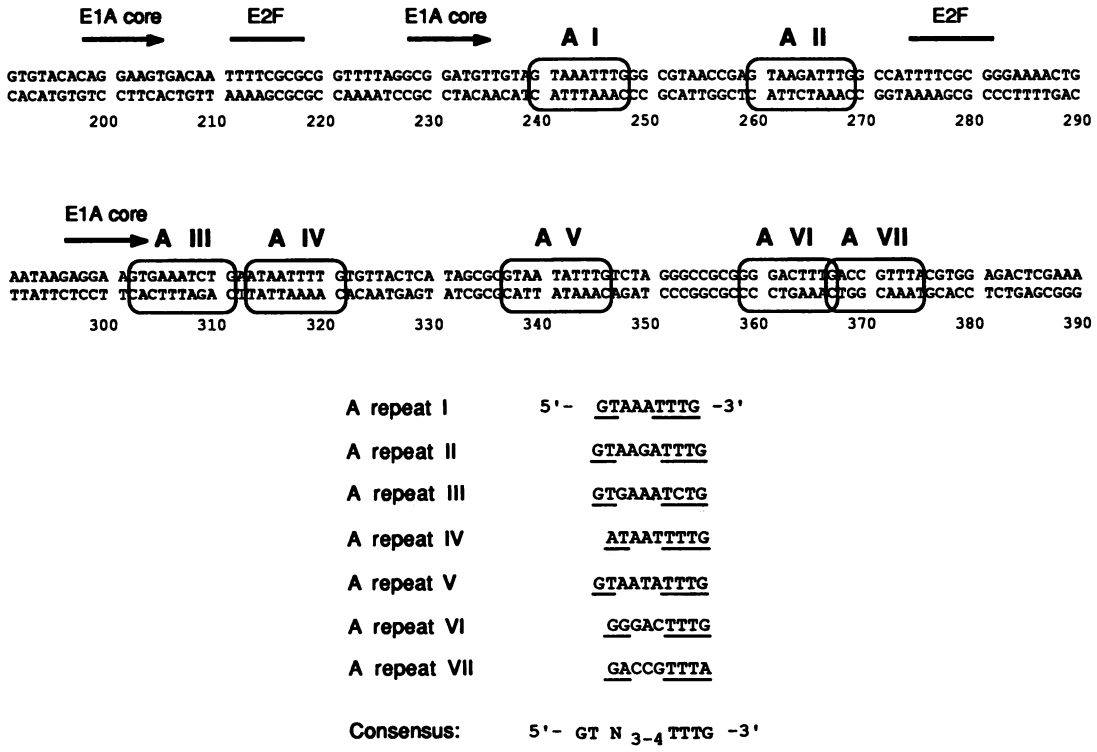

FIG. 8. Nucleotide sequence of the Ad5 genome between nt 191 and 390. Numbers below the sequence correspond to nucleotides relative to the left terminus. A repeats I through VII are encircled. Binding sites in this region for transcription factors are shown above the sequence (E1A core enhancer element I shown by arrows and E2F binding sites shown by dark bars) (1, 23, 26). The nucleotide sequences of the individual A repeats are shown below, along with a consensus sequence drawn from a comparison of these sites.

sented as bent DNA or that this structure is required for recognition of a prohead. To examine the possibility that the A repeats represent functional bending sites, we analyzed the electrophoretic migration of restriction fragments spanning the various LS mutations relative to the electrophoretic mobility displayed by the corresponding parental restriction fragment. While the left end of the viral genome displayed aberrant electrophoretic mobility consistent with bent DNA (data not shown; 11, 29) and particular LS mutations altered the mobility of mutant DNA fragments, our analysis did not reveal any obvious correlation between the packaging efficiency of a mutant virus and the molecular conformation of its mutant genome, determined as bent DNA (data not shown).

In this report, we provide further evidence for the proposal that the A repeats constitute functionally redundant packaging elements. Specific mutation of A repeat V, in the absence of A repeats I, II, and III (Fig. 2), greatly reduced the packaging efficiency of the mutant genome (Fig. 3), clearly demonstrating a functional involvement of this repeat in the packaging process. Analysis of additional LS mutant viruses has shown that additional elements (A repeats VI and VII; Fig. 4) are required for efficient packaging of the viral genome. These elements correspond to sequences which strongly resemble the A repeat consensus sequence (Fig. 8). The fact that inactivation of individual repeats impaired the packaging efficiency of the mutant viruses to different extents (compare *dl*309-LS5, *dl*309-LS4, *dl*309-LS6, and *dl*309-LS7 in Fig. 2 and 4) is indicative of a functional hierarchy between individual members of the A repeat family. The different activities displayed by the packaging elements might reflect differences in affinity of the individual binding sites for factor binding. In the absence of A repeats I, II, and III, A repeats V and VI play a key role in the packaging process, since inactivation of either element had a drastic effect on virus viability (Fig. 2 and 4). In the presence of A repeats V and VI, the remaining elements, A repeats IV and VII and possibly an element to the right of nt 380, were not absolutely required for viral growth but helped to optimize the packaging efficiency of the mutant genome (Fig. 4).

From the results obtained from the analysis of spacer mutants (*dl*10/28-194:in4 and *dl*10/28-358:in4; Fig. 6), we propose that a particular spacing of adjacent A repeat motifs with respect to each other is required to promote efficient packaging. This likely reflects the need for the coordinate binding of packaging proteins to multiple sites. In the absence of A repeats IV and V, A repeats I and II are required but not sufficient to allow packaging of the mutant genome (Fig. 6). The fact that the introduction of approximately one half of a helical turn at the rightward border of the deletion (*dl*10/28-358:in4) resulted in the loss of virus viability suggests that the residual packaging efficiency seen with *dl*10/28 requires functional interactions between A repeats I and II and additional elements located rightward of nt 358. In view of the results obtained with the LS mutants described above, it is likely that these elements are represented by A repeats VI and VII. In this context, it is interesting to note that the most highly conserved tetranucleotide (5′-TTTG-3′) portion of the A repeat consensus sequence is separated by exactly two turns of the DNA helix (21 bp) between A repeats I and II and between A repeats V and VI, the most important repeats based on functional assays (described above and in reference 17). The spatial constraint observed between the packaging elements strongly suggests that protein-protein interactions are important for the generation of a functional packaging complex. It is possible that stable binding of a protein to its recognition sites requires cooperation between proteins bound to different sites, or as proposed in the second model, the simultaneous binding of proteins to multiple sites is required for the formation of a defined packaging-specific nucleoprotein complex. Alteration of the spatial relationship between A repeats I and II and leftward flanking sequences improved viral growth (*dl*10/28-194:in4; Fig. 6). As was evident in the coinfection experiment, this increase was due to an increased packaging efficiency of the mutant genome. We have not identified the leftward sequences that participate in the packaging process, but it is conceivable that this complex involves interactions between factors bound to the left-terminal ITR and proteins bound to the packaging domain.

In our previous analysis of Ad5 packaging mutants, we noted that a number of mutants showed a greater decrease in packaging efficiency in coinfection experiments than was expected from results obtained with the same mutant viruses in a single infection (17). We speculated that the greater reduction observed in the coinfection with these mutants represented a competition between the wild-type and mutant genomes for limiting concentrations of a packaging protein(s). Here we provide further evidence that suggests that the A repeats represent sequence-specific binding sites for a *trans*-acting titratable factor(s). In the cotransfection experiment, the presence of an excess of unlinked packaging domain sequences efficiently interfered with normal viral growth (Table 2). Since viral DNA replication and late gene transcription were unaffected, we believe that the reduction in viral yield reflects a successful competition of the packaging sequences contained in the plasmid with those of the viral genome for a limiting *trans*-acting factor required for packaging of the viral DNA molecule in vivo. These results also demonstrated that the originally defined packaging sequences, encompassing nt 194 to 358, are sufficient for factor binding. However, they do not exclude the possibility that additional protein-protein interactions are required to create a packaging-competent viral DNA substrate.

In conclusion, our observations support the model which proposes that the *cis*-acting packaging elements represent recognition sites for proteins involved in the packaging process and that specific protein-DNA interactions provide the molecular basis for the observed packaging specificity. The generation of the specialized nucleoprotein structure required for the selective encapsidation of the Ad genome appears to require multiple DNA-protein and protein-protein interactions.

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