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Genetic Analysis of Adeno-Associated Virus: Properties of Deletion Mutants Constructed In Vitro and Evidence for an Adeno-Associated Virus Replication Function

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Transfection of a pBR322-based, recombinant plasmid, pAV2, containing the entire adeno-associated virus (AAV) type 2 genome into human 293 cells in the presence of helper adenovirus resulted in rescue and replication of AAV to yield infectious particles. We constructed mutants of pAV2 containing deletions within the AAV sequence. We describe here the phenotypes of these AAV deletion mutants. The results can be summarized as follows. Mutants (*cap*$^-$) with deletions between map positions 53 and 85 did not synthesize capsid antigen or progeny single-stranded DNA but accumulated normal levels of duplex replicating form DNA. Mutants (*rep*$^-$) with deletions between map positions 17 and 36 failed to rescue or replicate any AAV DNA. The *rep*$^-$ mutants could be complemented for replicating form DNA synthesis by a *cap*$^-$ mutant. This clearly demonstrates an AAV-coded replication function which is different from the capsid antigen. Other mutants (*inf*$^-$) with deletions in the region between map positions 40 and 52 synthesized abundant amounts of replicating form DNA and capsid antigen but gave a low yield of infectious particles. This suggests that there may be an additional region of AAV, perhaps within the intron, which is required for efficient particle assembly. This work shows that AAV is genetically complex and expresses at least three clearly different functions.

The DNA genome of the defective human parvovirus adeno-associated virus (AAV) is single stranded and linear, but strands of either complementarity are packaged into separate virions (31, 40). After infection of permissive cells in the presence of a helper adenovirus (1, 18) or herpesvirus (3), the AAV single strand is converted to a duplex replicating form (RF) apparently by a self-priming mechanism involving the terminal palindrome sequence (2, 16, 46). Multiple rounds of RF replication then occur, and single strands are generated from RF DNA by strand displacement replication (single-strand replication). Both AAV RF and progeny single-stranded (SS) DNA replication require functions provided by AAV as well as by the helper virus, although the precise nature of these functions is not understood (reviewed in references 5 and 6).

The AAV genome contains overlapping transcription units which yield three families of mRNAs beginning from three different promoters but having a common 3′ terminus (13, 27, 29, 30). The DNA sequence contains two major open reading frames in the left half (orf-1) and right half (orf-2) of the genome, respectively (45). Thus far, the only clearly identified AAV gene products are three capsid polypeptides (4, 40) which contain overlapping sequences (28, 34) and apparently are coded by the right half of the genome (22). From a study of the properties of AAV defective-interfering particles, we previously suggested (5, 25) that an AAV gene product (*rep*) may be required for AAV RF replication. We subsequently showed that the AAV capsid protein was apparently not required for AAV RF replication (37, 38).

To analyze more clearly the possible functions of the open reading frames in AAV DNA, we have used mutant genomes constructed in vitro. The entire AAV genome has been cloned into bacterial plasmids (26, 42, 43a). When such recombinant AAV plasmid DNA molecules are transfected into human 293 cells in the presence of helper adenovirus particles, the AAV genome is rescued from the recombinant plasmid and replicated to produce an efficient yield of infectious progeny particles (26, 42). We have taken advantage of this and performed in vitro mutagenesis on the AAV plasmid recombinant to produce a series of AAV deletion mutants. The phenotype of each of these mutants was examined by transfection into 293 cells in the presence of helper adenovirus. These studies provide evidence for an AAV *rep* function coded by orf-1 and for AAV capsid protein coded by orf-2. The *rep* and capsid proteins are separate functions and represent different complementation groups. An additional AAV function may be required for stable particle assembly.

MATERIALS AND METHODS

Viruses and cells. AAV type 2 (AAV2) and adenovirus 2 were grown in KB spinner cells as described before (7). Production of AAV capsid antigen was assayed by fixing cells at 24 or 30 h after infection or 24 h after transfection, respectively, and staining for AAV capsid protein, using an indirect immunofluorescence assay as described previously (7). Production of infectious AAV was assayed by titrating cell lysates on cells grown in monolayer and using the same AAV immunofluorescence procedure.

Human 293-31 cells, an established line of adenovirus-transformed human embryonic kidney cells (12), were grown at 37°C in monolayer culture in 35-mm plastic dishes in Eagle minimal essential medium supplemented with antibiotics and 10% fetal calf serum.

Growth and preparation of plasmids and plasmid DNA. Plasmids or DNA ligation mixtures were used to transform *Escherichia coli* HB101, using the RbCl procedure (23), and

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ampicillin-resistant colonies were selected by growth on Luria broth agar containing ampicillin (50 μg/ml). Individual colonies were examined for the presence of the appropriate plasmid by growing small-scale cultures in Luria broth medium and lysing according to the rapid boiling procedure (20). Plasmids in the minilysates were analyzed by restriction endonuclease cleavage and electrophoresis in agarose gels. Restriction endonucleases were purchased from New England Biolabs (Beverley, Mass.), Bethesda Research Labs (Gaithersburg, Md.), or Boehringer Mannheim Biochemicals (Indianapolis, Ind.) and used according to the supplier's specifications. DNA fragments were purified from agarose gels according to the procedures of Langridge et al. (24) or Weislander (47). Large-scale preparations of plasmids were obtained by growing the appropriate *E. coli* strain in minimal M9 medium supplemented with Casamino Acids (0.2%), thiamine (1 μg/ml), and ampicillin (20 μg/ml) and amplifying with chloramphenicol (100 μg/ml). Plasmids were then prepared by lysozyme-Triton X-100 lysis and polyethylene glycol precipitation followed by cesium chloride-ethidium bromide equilibrium gradient centrifugation (21, 35). All manipulations with recombinant DNA were performed in accordance with the National Institutes of Health Guidelines.

Construction of individual plasmids. Figure 1 shows the structure of the AAV2 genome, the location of the relevant restriction sites, and the structures of the individual plasmids used in this study. pAV2 contains the entire AAV2 genome inserted into a pBR322-derived plasmid, pA11P.Xba (14). Therefore, pA11P.Xba has the same structure as pAV2 except that the AAV2 insert is deleted. pAVdlHc23 was derived from pAV2 by deletion of the *Hin*cII fragment between map positions 51 and 86 (i.e., between sites Hc2 and Hc3) by partial *Hin*cII cleavage followed by blunt-end ligation.

Some mutants were derived from pAV2 by restriction cleavage and religation at compatible sticky ends, using T4 polynucleotide ligase (Boehringer Mannheim). Thus, pAVdlX12 was derived by cleaving pAV2 with *Xho*I and religating to yield a mutant which is deleted for the *Xho*I-C fragment (186 base pairs [bp]) but contains one *Xho*I site. Similarly, pAVdlK12 was derived by cleaving pAV2 with *Kpn*I.

Several mutants were derived by cleavage of pAV2 at nonsimilar restriction sites followed by blunt-end ligation. In each case pAV2 DNA was cleaved with the required enzymes and then incubated with the *E. coli* DNA polymerase I Klenow fragment (Boehringer Mannheim) to remove 3′ extensions or to fill in recessed 3′ termini. Reactions with the Klenow fragment were performed in a final volume of 20 μl containing 50 mM Tris-hydrochloride (pH 7.2)–10 mM $MgSO_4$–0.1 mM dithiothreitol–50 μg of bovine serum albumin per ml–500 μM each dATP, dCTP, dTTP, and dGTP–3 U of enzyme at 37°C for 30 min. The DNA was then incubated with T4 polynucleotide ligase (Collaborative Research, Waltham, Mass.) under the conditions for blunt-end ligation as specified by the supplier and transfected into *E. coli* HB101. By this procedure, mutants pAVdlK1X2 and pAVdlX1K2 were derived by full cleavage with *Xho*I and partial cleavage with *Kpn*I. Mutants pAVdlHiX2 and pAVdlHiK2 were derived by complete cleavage with *Hin*dIII and either *Xho*I or *Kpn*I, respectively. pAVdlSBc was derived by complete cleavage of pAV2 with *Sst*I and *Bcl*I, and pAVdlBcBs was derived by complete cleavage with *Bcl*I and *Bst*EII. For these latter two constructions, pAV2 was prepared from the methylation-deficient strain *E. coli* GM119 (11) to avoid methylation of the AAV2 *Bcl*I site.

Mutant pAVR1.11 was derived by site-specific mutagenesis at the AAV2 *Eco*RI site at map position 38.1, using endonuclease Bal 31 as will be described elsewhere. Nucleotide sequence analysis (32) showed that pAVdlR1.11 is deleted between AAV nucleotides 1,635 and 1,861 (M. West, unpublished data).

Transfection of cells with recombinant plasmid DNA. All transfection experiments were performed on 293 cells grown in 35-mm plastic dishes (10^6 cells per dish), using either a DEAE-dextran or a $CaPO_4$ procedure.

The DEAE-dextran procedure was used as described before (26, 33, 36) when assaying the transfected recombinant AAV DNA for its ability to be replicated. Mixtures of recombinant DNA (in amounts per dish as specified in individual experiments) in the presence or absence of helper adenovirus particles (5 to 10 PFU per cell) were added to subconfluent cells in the presence of minimal essential medium containing 250 μg of DEAE-dextran per ml (average molecular weight, 500,000) and 50 mM Tris-hydrochloride, pH 7.5, for 30 min. This inoculum was removed and the cells were incubated at 37°C in minimal essential medium containing 10% fetal calf serum. For in vivo labeling of viral DNA, cells were transferred to low-phosphate medium (1% of normal phosphate concentration) at 16 h after transfection and supplemented with H_3PO_4 (10 to 25 μCi/ml).

$CaPO_4$ transfection was used when testing for expression of capsid antigen or for production of virus particles and was performed according to the procedure of Wigler et al. (48). In this procedure, adenovirus particles (5 to 10 PFU per cell) were added to the cells 1 h before transfection with DNA.

Analysis of intracellular viral and plasmid DNA. Viral DNA was selectively extracted from cells at 44 to 48 h after transfection, using a Hirt procedure (17) modified to prevent reannealing of AAV ssDNA and to remove contaminating RNA as described before (9). DNA was electrophoresed in horizontal 1.4% agarose gels (11 by 14 cm) in 10 mM Tris–40 mM sodium acetate–1 mM Na_2EDTA, pH 8.0, at 100 mA per gel. The gels were then dried and autoradiographed directly, using an intensifying screen (Cronex 70, Dupont), or were blotted onto nitrocellulose paper (44) and hybridized with AAV ^{32}P-DNA labeled by in vitro nick translation (39). After hybridization in 6× SSC (SSC = 0.15 M NaCl plus 0.015 M sodium citrate) at 70°C for 16 to 24 h the nitrocellulose paper was washed in 0.1%× SSC–0.05% sodium dodecyl sulfate at 52 or 70°C and autoradiographed.

RESULTS

Structure of the AAV2 genome. The structure of the AAV2 genome is shown in Fig. 1, which identifies the relevant restriction sites, the AAV transcription units, and the open reading frames. The transcription of AAV is reviewed extensively elsewhere (8). AAV RNA synthesis begins from three separate promoters, p_5, p_{19}, and p_{40}, at map positions 5, 19, and 40, respectively, and proceeds rightward to a single polyadenylation site at map position 96 (8, 13, 27, 29, 30, 45). There are two major open reading frames (orf) as determined from the nucleotide sequence (45). The left-hand reading frame (orf-1) apparently can be entered from transcripts originating from either p_5 or p_{19}. Furthermore, splicing of AAV RNA to remove the single intron alters the carboxyl terminus of orf-1. This suggests that the four largest AAV RNAs may enable orf-1 to encode at least four proteins differing in their amino and carboxyl termini. orf-2 is in the right half of the genome and may be accessible from a spliced p_{40} transcript. There are two small reading frames located

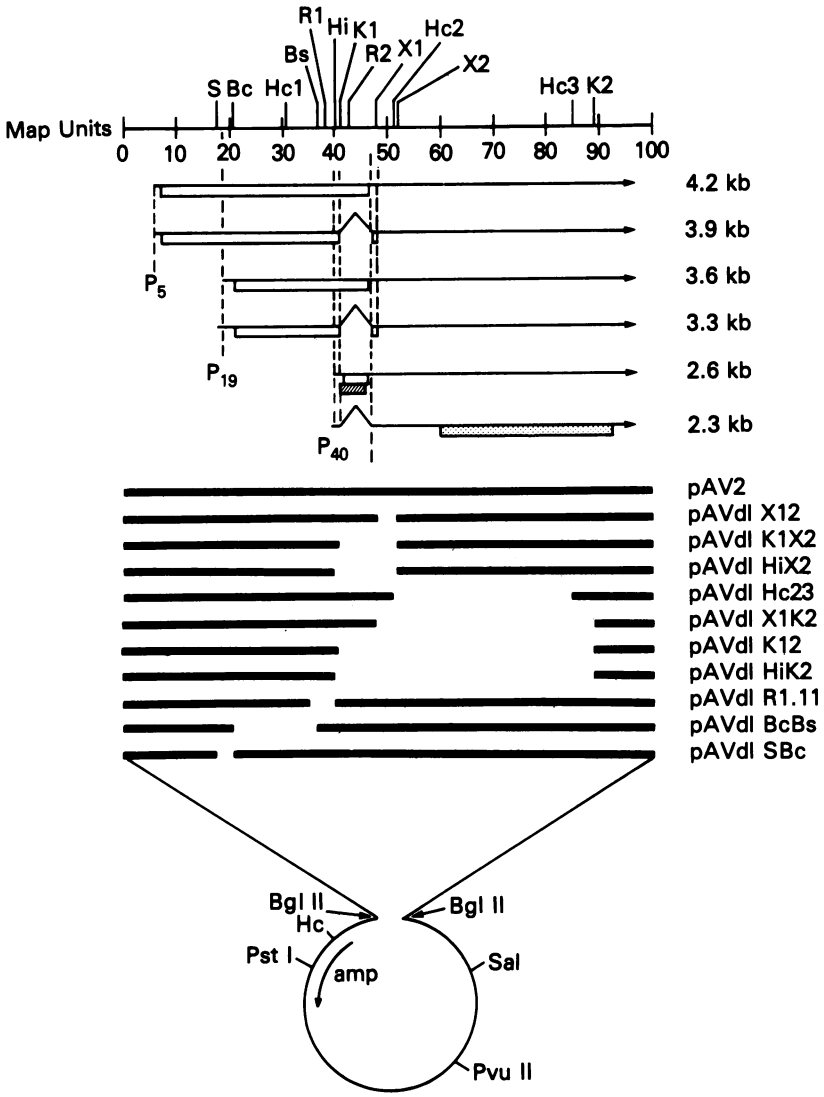

FIG. 1. Structure of the AAV2 DNA and mutant AAV genomes. The upper portion shows the AAV2 DNA on a scale of 100 map units. Restriction endonuclease sites are designated as follows: S, *Sst*; Bc, *Bcl*I; Hc, *Hinc*II; R, *Eco*RI; Hi, *Hin*dIII; K, *Kpn*I; X, *Xho*I. For Hc, R, K, and X, the sites in AAV are designated numerically from the left as indicated by the number following the abbreviated name, e.g., Hc2. Horizontal arrows show the structures of the six mRNAs having the sizes as indicated (in kilobases) at the right. The caret indicates the AAV intron. The arrowhead indicates the polyadenylation site at 96 map units. p_5, p_{19}, and p_{40} indicate the three promoter sites. Based on sequence analysis (45), the potential open reading frames (orf) accessible from each RNA are indicated by the boxes below individual RNAs as follows: orf-1, open box; orf-2, stippled box; orf-3, diagonally shaded box. The lower portion shows the structure of the AAV mutants. The parental AAV plasmid recombinant pAV2 contains the entire AAV2 genome (solid horizontal line) inserted into a pBR322-derived plasmid, pA11P.Xba (open circle), via *Bgl*II linkers. The other plasmids were derived from pAV2 by deleting regions of AAV sequence as indicated by the gaps in the horizontal line. The figure is drawn approximately to scale and several reference restriction sites are shown in the plasmid sequence as well as the location of the ampicillin resistance gene (*amp*). The construction of individual plasmids is described in the text.

within the intron which may be accessible for translation from the 2.6-kilobase (kb) unspliced p_{40} transcript. One of these small reading frames is the same as the carboxyl terminus of orf-1 expected from translation of the spliced 4.2- and 3.6-kb mRNAs: the other, orf-3, is different from but overlaps orf-1 and is contained entirely within the intron. There is an additional open reading frame, different from orf-2, between map positions 85 and 97 (45), but this is not shown in Fig. 1 because this reading frame is not apparently accessible from any currently known AAV mRNA.

Structure of individual mutant AAV genomes. The structures of individual AAV plasmid recombinants are detailed in Fig. 1. For these constructions we describe the entire recombinant plasmid, using the prefix pAV, e.g., pAVcdl23. When referring to the AAV genome contained within, or rescued from, the plasmid, we will not use the prefix, e.g., dlHc23. The AAV mutants fall into several overlapping classes. Restriction site X1 is 6 bp downstream of the intron, and mutant dlX12 deletes 186 bp immediately downstream of this which includes the last six codons of the orf-1 expected from the spliced transcripts. Two mutants, dlHiX2 and dlK1X2, delete the intron as well as the region deleted in dlX12. Mutants dlHc23 and dlX1K2 also overlap mutant dlX12 but extend the deletion to the right to remove nearly all of orf-2. Another pair of mutants, dlK12 and dlHiK2, have deleted both the intron and most of orf-2. All of these

mutants contain deletions which are to the right of the *Hin*dIII site at map position 40. A second class of mutants contained deletions which mapped only to the left of this site. These mutants, dlSBc, dlBcBs, and dlR1.11, have deletions of internal portions of orf-1. The mutation in dlR1.11 also deletes the promoter and the first 23 nucleotides at the 5′ terminus of p_{40} transcripts.

Replication of AAV mutants and accumulation of duplex RF DNA. The replication of the AAV mutants was tested by transfection of 293 cells with recombinant plasmids in the presence of adenovirus. Viral DNA was selectively extracted and analyzed by agarose gel electrophoresis. Several examples of experiments with individual mutants are summarized in Fig. 2. In some experiments, AAV DNA was detected by blotting the gels and hybridizing with an in vitro labeled AAV ^{32}P-DNA probe (Fig. 2A and B). In other experiments when in vivo ^{32}P labeling was used, the DNA was detected by direct autoradiography of the gels (Fig. 2C and D).

The transfections with the wild-type AAV-plasmid pAV2 (Fig. 2, tracks 5, 9, 12) clearly show the three AAV DNA species, including the monomeric and dimeric duplex RF DNAs and the progeny ssDNA. These AAV DNA species, which arise by rescue of the AAV genome from the plasmid followed by replication, have been characterized before (26). In the experiments using in vivo ^{32}P labeling, adenovirus DNA was also labeled, whereas the residual unreplicated input recombinant AAV plasmid DNA was not (Fig. 2C and D). When the experiment was performed by blotting and hybridizing with an AAV ^{32}P-labeled DNA probe (Fig. 2A and B), the adenovirus DNA was not observed but the unreplicated input plasmid DNA was detected (Fig. 2A, track 4). The input plasmid DNA is generally observed as two species comprising the linear and open circular forms. However, the adenovirus DNA and both input plasmid DNA species migrated more slowly than the major AAV DNA species.

Each of the three mutants containing deletions to the left of the *Hin*dIII site (dlSBc, dlBcBs, dlR1.11) failed to accumulate either RF DNA or AAV ssDNA (Fig. 2C, tracks 15 to 17). These three DNA-negative mutants are designated *rep*$^-$. All of the other AAV mutants having deletions only to the right of the *Hin*dIII site were rescued and replicated in transfected cells as judged by the accumulation of duplex RF DNA. Further, each of these mutants accumulated RF DNA about as efficiently as wild-type AAV. Based on the production of RF DNA we describe these mutants as *rep*$^+$. That the mutant RF genomes observed in the transfected cells indeed correspond to those expected to be rescued from the transfecting plasmid was verified by their size and restriction cleavage (not shown).

Accumulation of progeny ssDNA. Although the *rep*$^+$ AAV mutants efficiently accumulated RF DNA, they varied greatly in their ability to accumulate progeny ssDNA (Fig. 2). For

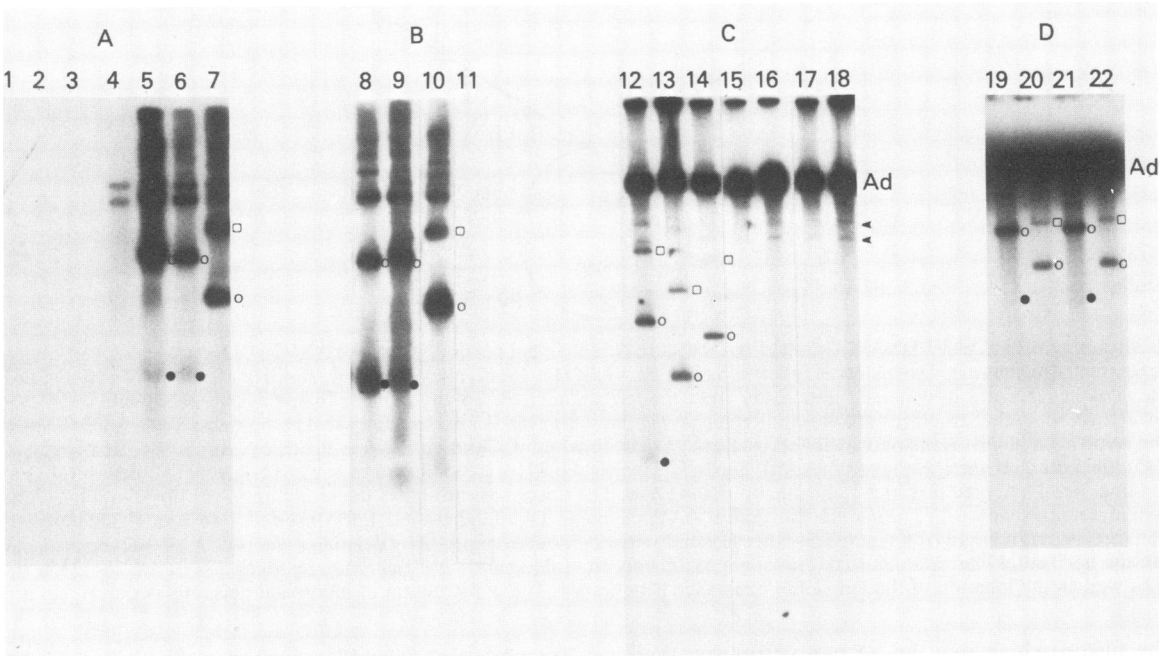

FIG. 2. Effect of deletions on replication of AAV genomes in transfected 293 cells. Cultures of 293 cells were transfected with adenovirus particles and supercoiled AAV plasmid DNA (10 μg). Viral DNA was isolated and electrophoresed in agarose gels. (A), (B), (C), and (D) show separate experiments. In (A) and (B) the DNA was detected after electrophoresis by blotting and hybridizing with a nick-translated AAV ^{32}P-labeled DNA probe. In (C) and (D) the DNA was labeled in vivo and the agarose gel was dried down and autoradiographed directly. Tracks 1 and 11 show pAV2 DNA cleaved with *Bgl*II to serve as a marker. Track 2, DNA from mock-transfected cells in which both adenovirus and DNA were omitted. Track 3, Adenovirus-infected cells. Track 4, Transfected with pAV2 DNA in the absence of adenovirus. All other cultures were transfected with adenovirus and supercoiled plasmid DNA as follows: track 5, pAV2; track 6, pAVdlX12; track 7, pAVdlHc23; track 8, pAVdlX12; track 9, pAV2; track 10, pAVdlXlK2; track 12, pAV2; track 13, pAVdlHc23; track 14, pAVdlK1X2; track 15, pAVdlSBc; track 16, pAVdlBcBs; track 17, pAVdlR1.11; track 18, pA11P.Xba; track 19, pAVdlHiX2; track 20, pAVdlHiK2; track 21, pAVdlK1X2; track 22, pAVdlK12. DNA species for each mutant are denoted by the symbols at the right of each track as follows: (□) duplex dimer RF DNA; (○) duplex monomer RF DNA; (●) ssDNA. Arrows (C) indicate one or two cell DNA species usually seen in in vivo labeling experiments (26). In (C) and (D) the heavy, slowly migrating band is adenovirus DNA. In (A) and (B) the slowly migrating doublet (e.g., track 4) shows input plasmid DNA.

wild-type AAV DNA (i.e., pAV2), the ratio of ssDNA to monomeric RF DNA showed some variation between different experiments (Fig. 2, tracks 5, 9, 12) but was generally similar in a single experiment (Fig. 3, tracks 2, 5, 8, 10). Mutant dlX12 accumulated ssDNA about as efficiently as wild-type AAV (Fig. 2, tracks 6, 8). In contrast, mutant dlHc23 did not accumulate detectable ssDNA (Fig. 2, tracks 7, 13). Similarly, the three mutants containing even larger deletions, dlX1K2 (Fig. 2, track 10), dlHiK2 (Fig. 2, track 20), and dlK12 (Fig. 2, track 22), also did not accumulate detectable ssDNA. It is important to note that it may be difficult to detect very low levels of AAV ssDNA because of a variable background of heterogeneous material which migrated faster than the duplex RF DNA and probably arises from partially replicated molecules. This precludes detection of low levels of ssDNA simply by increased autoradiographic exposure. Nevertheless, with the four mutants dlHc23, dlHiK2, dlK12, and dlX1K2, we have reproducibly failed to observe accumulation of ssDNA. We designate the phenotype of these mutants as *cap*$^-$.

Two mutants, dlK1X2 and dlHiX2, showed a very low level of ssDNA in some experiments (e.g., Fig. 2, tracks 19, 21), whereas in other experiments (Fig. 2, track 14) no ssDNA was observed. As shown below, these two mutants have a phenotype designated *inf*$^-$ which is different from that of the *cap*$^-$ mutants.

Production of capsid antigen and infectious virus. Table 1 summarizes for each of the AAV mutants production of RF and ssDNA as well as accumulation of AAV capsid antigen and infectious particles. Production of capsid antigen was measured by fixing cells 24 to 30 h after transfection in the presence of adenovirus and staining for nuclear fluorescence, using the indirect immunofluorescence assay with antibody directed against the AAV capsid protein. In these experiments 5 to 10% of the cells transfected with wild-type pAV2 showed positive staining. This indicates that the transfection was reasonably efficient (20) since cells infected with AAV particles normally do not show more than 50 to 60% positive staining for capsid protein (7). Mutant dlX12 produced capsid antigen as efficiently as pAV2. The four *cap*$^-$ mutants, dlHc23, dlX1K2, dlK12, and dlHiK2, having deletions in orf-2 failed to synthesize detectable AAV capsid antigen. In contrast, for the two *inf*$^-$ mutants, dlHiX2 and dlK1X2, the number of cells positive for AAV antigen was 25 to 100% of that with pAV2.

The two *rep*$^-$ mutants, dlBcBS and dlSBc, also showed positive AAV antigen staining at a level of about 10 to 30% that of wild-type AAV. The reduced level probably reflects decreased genome copy number in the absence of replication. The *rep*$^-$ mutant dlR1.11 retains all of orf-2 but failed to synthesize capsid antigen presumably because the deletion removes both p_{40} and the 5′ terminus of the 2.3- and 2.6-kb mRNAs. These results are consistent with previous evidence that the AAV capsid protein is coded by the right half of the genome (22). None of the plasmids, including the wild-type AAV, synthesized detectable AAV antigen in the absence of adenovirus. This is consistent with previous evidence that an adenovirus function is required for translation of AAV capsid antigen (6, 22) since transcription of AAV DNA occurs in 293 cells even in the absence of helper (J. Tratschin, M. West, T. Sandbank, and B. Carter, submitted for publication).

Production of infectious particles in transfected cells is also summarized qualitatively in Table 1. The only mutant that produced infectious virus at significant levels was dlX12, which was about 50% as efficient as wild-type AAV. For most of the mutants, the failure to produce infectious virus can be explained by either failure to replicate (*rep*$^-$) or failure to produce capsid antigen (*cap*$^-$). The two *inf*$^-$ mutants, dlHiX2 and dlK1X2, which readily synthesized RF DNA and capsid antigen, produced infectious particles only very inefficiently if at all, as determined by the immunofluorescence assay (Table 1). This correlates with poor accumulation of ssDNA by these *inf*$^-$ mutants. The failure of mutants to make infectious virus is also shown by another assay described below.

Complementation of AAV mutants. Other studies (P. Senapathy, J. Tratschin, and B. Carter, unpublished data) showed that naturally occurring mutants of AAV having much larger deletions removing most of orf-1 and a large part of orf-2 exhibit a *rep*$^-$ phenotype. These mutants could be complemented for RF DNA replication by a wild-type AAV or a *rep*$^+$ mutant. Recombination was not observed by analyzing viral DNA from lysates of the transfected cells and did not interfere with the complementation assay for RF DNA synthesis. Recombination did occur at a very much lower level than complementation but was detected only by successive passaging of cell lysates from the transfected cells (43a).

We examined the *rep*$^-$ mutants described here for complementation for RF DNA replication by *cap*$^-$ mutant dlHc23. The three *rep*$^-$ mutants dlBcBs, dlSBc, and dlR1.11 all failed to replicate when transfected only with adenovirus (Fig. 3, tracks 1, 4, 7). In a mixed transfection with pAV2 or dlHc23, dlBcBs RF DNA genomes were observed (Fig. 3, tracks 2, 3) which shows that the *rep*$^-$ mutant was complemented. For the other two mutants, dlSBc and dlR1.11, an RF molecule of the expected size was observed in the mixed transfections with dlHc23 (Fig. 3, tracks 6, 9). Because the deletion in these two mutants is only 150 and 225 bp, respectively, complementation would be obscured in the mixed transfections with wild-type AAV (Fig. 3, tracks 5, 8). However, at least for dlR1.11, the monomeric R1.11 RF DNA in the complementation with dlHc23 (Fig. 3, track 9) migrated slightly faster than the wild-type RF DNA in the parallel tracks.

Encapsidation of mutant AAV genomes. The *inf*$^-$ mutants such as dlK1X2 readily synthesized DNA and capsid antigen but yielded very little infectivity as judged by immunofluorescent staining of the P1 cultures for capsid antigen (Table 1). Immunofluorescence may be relatively insensitive for measuring low levels of AAV. Therefore, we used an alternative assay to analyze further the *inf*$^-$ phenotype. These experiments were performed in a similar way, but production of infectious particles in the P0 cells was detected by analyzing viral DNA replication in the P1 cells. Deletion mutant dlX12 readily yielded RF and ssDNA in both P0 and P1 cells (Fig. 4A), whereas *cap*$^-$ mutant dlHc23 yielded RF DNA in the P0 cells but no DNA replication in the P1 cells. In the same experiment, the *inf*$^-$ mutant dlK1X2 yielded RF and ssDNA in the P0 cells, but in the P1 cells only a very low level of DNA replication was observed upon prolonged autoradiographic exposure (Fig. 4A, track 8). This again suggested that the *inf*$^-$ mutant produced only very few infectious particles. Further, in the experiment of Fig. 4A, the P0 culture contained appreciable amounts of dlK1X2 ssDNA but this was still relatively much less than for dlX12. In this experiment there is also evident some heterogeneous AAV DNA as noted above.

The *cap*$^-$ mutants did not synthesize any capsid antigen or ssDNA which is consistent with other evidence (see Discussion) that capsids may be required to allow accumulation of

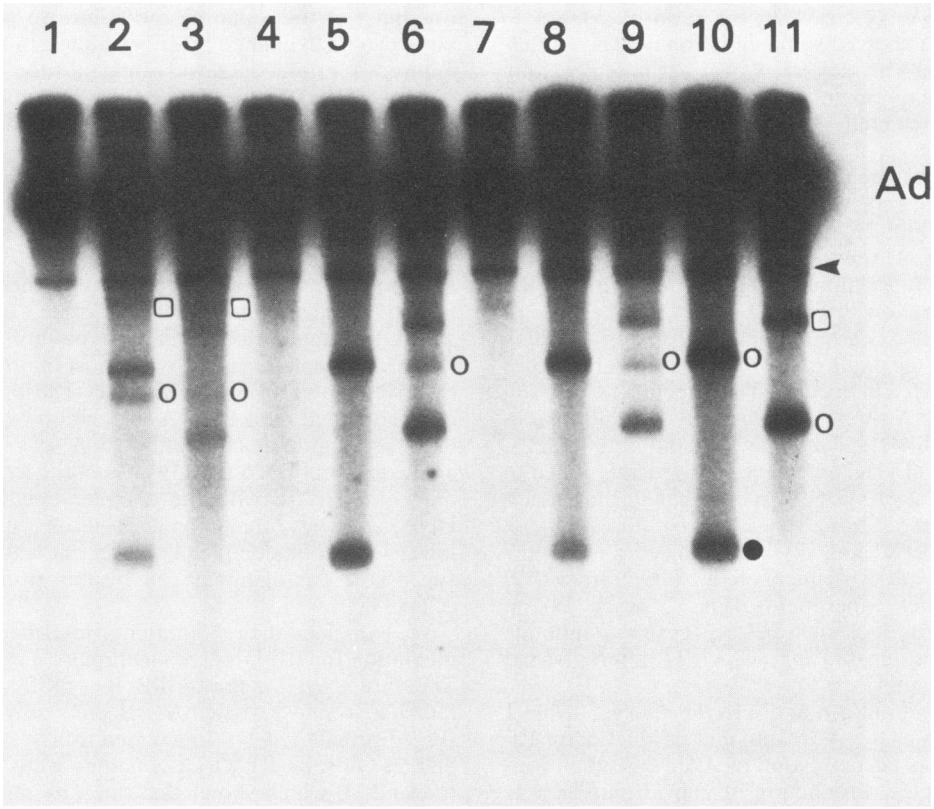

FIG. 3. Complementation of AAV mutants. Adenovirus-infected 293 cells were transfected with supercoiled plasmid DNA by using the DEAE-dextran procedure, and in vivo labeled, viral ^{32}P-DNA was analyzed as in the legend to Fig. 2. As for Fig. 2, DNA species are indicated at the right of each track: (□) AAV duplex dimer RF DNA; (○) AAV duplex monomer RF DNA; (●) AAV progeny ssDNA. Ad, Adenovirus DNA; arrow, cell DNA. For tracks 1 to 9, only mutant AAV DNA species are indicated. Those of AAV wild type or AVdlHc23 are indicated in tracks 10 and 11, respectively. Cells were transfected with 10 μg of pAVdlBcBs (tracks 1 to 3), pAVdlSBc (tracks 4 to 6), pAVdlR1.11 (tracks 7 to 9), pAV2 (track 10), or pAVdlHc23 (track 11). Cells were also complemented with 5 μg of either pAV2 (tracks 2, 5, 8), or pAVdlHc23 (tracks 3, 6, 9).

TABLE 1. Properties of AAV mutants in transfected cells

AAV mutant	Production of:			
	RF DNA[a]	ssDNA[a]	Capsid antigen[b]	Particles[c]
pAV2	+	+	+	+
pAVdlX12	+	+	+	+
pAVdlK1X2	+	(+)	+	(−)
pAVdlHiX2	+	(+)	+	(−)
pAVdlHc23	+	−	−	−
pAVdlX1K2	+	−	−	−
pAVdlK12	+	−	−	−
pAVdlHiK2	+	−	−	−
pAVdlR1.11	−	−	−	−
pAVdlBcBs	−	−	+	−
pAVdlSBc	−	−	+	−

[a] RF or ssDNA synthesis was assayed as shown in Fig. 2 or 3. (+) indicates that ssDNA was detected at low levels or not at all (see text).

[b] Capsid antigen was scored by fixing cells 24 h after transfection in the presence of adenovirus and staining for AAV capsid antigen immunofluorescence.

[c] Production of AAV particles was scored by transfection of cells with the indicated plasmid in the presence of adenovirus. At 48 h after transfection the cells (P0 culture) were lysed, heated at 60°C to inactivate adenovirus, and treated with DNase. The lysates were assayed for AAV infectivity by infection of 293 or KB cells (P1 culture) together with adenovirus, followed either by staining for capsid antigen or analysis of AAV DNA replication. (−) indicates no detection with the capsid antigen assay and very low levels or not at all with the DNA replication assay (see text for details).

single strands. However, it was important to determine whether these mutant genomes could be packaged into virus particles. This was tested for dlHc23 by the same assay as described in Fig. 4A. When the dlHc23 mutant was transfected together with wild-type AAV in P0 cells, significant amounts of replicating dlHc23 DNA, as well as the wild-type DNA, were seen in the second passage (Fig. 4B, track 6). In the absence of wild-type AAV in P0, no replicating dlHc23 DNA was seen in the second passage (Fig. 4B, track 3). We take this to mean that genomes of dlHc23 were packaged into AAV particles when replicated in the presence of wild-type virus. Again dlX12 did not require complementation for encapsidation (Fig. 4B, track 2). Similar experiments (data not shown) with the *inf*$^-$ mutants dlHiX2 and dlK1X2, which produced infectious particles at only very low efficiency, showed that the presence of wild-type AAV increased the efficiency of encapsidation of the mutant genomes.

DISCUSSION

The experiments described here provide an initial genetic analysis of AAV gene expression and show that the two major open reading frames, orf-1 and orf-2, code for a replication function and a capsid protein, respectively. The deletions in the *rep*$^-$ mutants dlSBc, dlBcBs, and dlR1.11 were located entirely to the left of map position 40 and prevented duplex RF replication. This indicates that there is

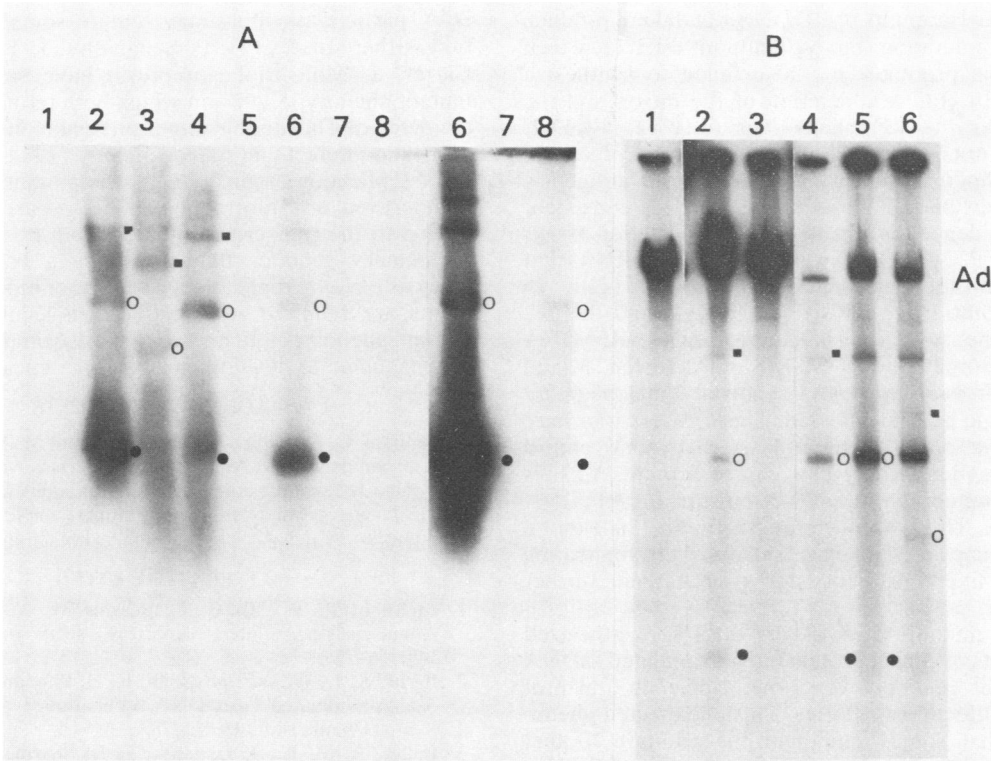

FIG. 4. Encapsidation of mutant AAV genomes. Cultures (P0) of 293 cells were transfected with AAV plasmids in the presence of adenovirus. At 44 h the cells were harvested and part of each culture was used for isolation and analysis of viral DNA. The remainder of each P0 lysate was treated with DNase, heated at 60°C, and used to infect fresh 293 cells (P1) together with adenovirus. At 44 h viral DNA was again isolated from the P1 cultures. DNA from P0 and P1 cultures was analyzed by electrophoresis. In (A) the viral DNA was detected by blotting and hybridization with AAV ^{32}P-labeled DNA. In (B) the DNA was detected by labeling in vivo. (A) Tracks 1 to 4 show DNA from P0 cultures and tracks 5 to 8 show DNA from the corresponding P1 cultures. The P0 cultures were transfected, in the presence of adenovirus, with no plasmid DNA (track 1) or 10 μg of pAVdlX12 (track 2), pAVdlHc23 (track 3), or pAVdlK1X2 (track 4) DNA. Portions of the lysate from the P0 cultures in tracks 1 to 4 were used to infect the P1 cultures in tracks 5 to 8, respectively. The right-hand three tracks show a longer autoradiographic exposure of tracks 6 to 8. (B) All six tracks show DNA isolated from P1 cultures. These cultures were infected with adenovirus and lysates from P0 cells that had been transfected (in the presence of adenovirus) with plasmid DNA as follows: track 1, no DNA; track 2, pAVdlX12 (10 μg); track 3, pAVdlHc23 (10 μg); track 4, pAV2 (10 μg); track 5, pAVdlX12 (10 μg) plus pAV2 (5 μg); track 6, pAVdlHc23 (10 μg) plus pAV2 (5 μg). DNA species are designated at the right of each track: adenovirus (Ad); AAV duplex dimer RF (■); AAV duplex monomer RF (○); AAV single strands (●). In (A) track 5, the mutant and wild-type genomes are too similar in size to be readily resolved in this gel. In (B) tracks 5 and 6, only the mutant DNA species are designated.

an AAV *rep* function coded from orf-1. We had previously postulated the existence of such an AAV *rep* function based upon a consideration of the properties of naturally occurring AAV varient (defective-interfering) particles which contain internally deleted genomes (5, 7). Since orf-1 can apparently be entered for translation from either p_5- or p_{19}-promoted transcripts and mutants dlSBc and dlBcBs affect both types of transcript, it is not known whether one or both transcripts specify the *rep* function. The precise nature of this *rep* function also is not known but it might be required for one or more of several processes which apparently occur during AAV replication (2, 9, 16, 46), including cleavage of covalently closed termini in RF molecules, regeneration of the terminal sequence, and conversion of oligomeric replicating molecules to monomeric units. The *rep* function may also be required to rescue AAV genomes which are covalently integrated into cell chromosomes (10, 15, 19) or from the recombinant plasmid in transfected cells (26, 42, 43). The transfection experiments do not distinguish between involvement of the *rep* function in excision or in subsequent amplification of duplex RF molecules. Indeed, *rep* may be involved in both processes. For instance, the excision of AAV genomes from plasmids may be analogous to the conversion of oligomeric RF DNA to monomeric units.

orf-1 has two possible carboxyl termini depending upon splicing of the mRNA. Several mutants (dlHiX2, dlHiK2, dlK12, and dlK1X2) have deletions extending from map position 40 or 41 through the intron. These deletions remove both types of carboxyl terminus from orf-1. Also, in mutant dlX12, the last six codons from orf-1 downstream of the intron are deleted. Yet all of these mutants have a *rep*$^+$ phenotype. Apparently the putative carboxyl termini of the *rep* gene products are not required for any *rep* functions involved in RF accumulation or excision from the plasmid. However, we note that for another class of AAV mutants having insertions of foreign DNA at the *Hin*dIII site, some, but not all, of these mutants replicate poorly (Tratschin et al., submitted for publication).

AAV particles (41) contain three capsid polypeptides: the major component (by mass) is VP3 (61,000 molecular weight) and there are two minor components, VP1 (85,000 molecular weight) and VP2 (72,000 molecular weight). These polypeptides share extensive sequence homology (28, 34). The *cap*$^-$ mutants (dlHc23, dlX1K2, dlK12, and dlHiK2) all

synthesized normal amounts of RF DNA but did not produce capsid antigen. In addition, the *rep*$^-$ mutant dlR1.11, which is deleted for the promoter p_{40}, also failed to synthesize capsid antigen. In contrast, deletion of the intron and the immediately following 186 nucleotides (dlHiX2, dlK1X2, and dlX12) did not prevent abundant synthesis of capsid antigen. These findings suggest that the capsid antigen is coded by the right half of the genome, which is consistent with previous evidence that, in an in vitro translation assay, proteins VP1, VP2, and VP3 were coded by mRNA that cosedimented with the major AAV 2.3-kb mRNA (22).

For *cap*$^-$ mutants such as dlHc23, dlK12, and dlHiK2 which deleted the AAV capsid coding region, RF DNA synthesis was normal but ssDNA was not detected. Mixed transfection with wild-type AAV showed that the *cap*$^-$ mutant DNA could be encapsidated. These observations are consistent with a previous hypothesis that AAV capsid production and at least the major capsid protein, VP3, is required for accumulation of ssDNA but not for RF DNA synthesis (37, 38). This was interpreted as indicating either a requirement for capsid to displace ssDNA from replicating DNA or, more likely, to sequester ssDNA from further replication (38).

The two *inf*$^-$ mutants dlHiX2 and dlK1X2 synthesized abundant amounts of capsid protein but accumulated ssDNA at a variable, but generally very low, efficiency and produced very few infectious particles. This unexpected phenotype suggests that some component, in addition to that coded by orf-2, may be required for particle assembly. This component could be part of VP1 or VP2 as discussed below or another product such as from the small reading frames in the intron. That this function may involve the intron is supported by the evidence that dlX12 does not exhibit the *inf*$^-$ phenotype, although this mutant has a deletion, immediately following the intron, of 186 bp which corresponds to the 3′ region of the deletions in the two *inf*$^-$ mutants. The *inf*$^-$ phenotype may reflect a lack of any particles containing DNA, synthesis of particles which are stable but noninfectious, or assembly of unstable particles. This last possibility is the most attractive because we previously reported evidence indicating that, after association of an AAV DNA strand with a preformed empty capsid, maturation to yield infectious particles may occur via an unstable intermediate (37). Such unstable intermediates might still allow sequestering and accumulation of progeny ssDNA at low levels as was observed for the *inf*$^-$ mutants.

It has been noted that orf-2, which begins about 640 nucleotides downstream from the 5′ terminus of the 2.3-kb mRNA, may be too small to code entirely for VP1 or perhaps VP2 (45). It was further suggested that these minor proteins could have additional amino-terminal sequences coded from upstream of the putative initiation codon of orf-2 (34). Recent experiments (J. Janik, M. Houston, and J. Rose, unpublished data) show that the VP1 protein apparently is coded from AAV sequences beginning within the intron and that the 186-nucleotide deletion in dlX12 is an in-frame deletion resulting in the synthesis of a truncated VP1 protein. This might require an unexpected translation initiation on the major 2.3-kb mRNA or another minor mRNA with a different structure. These observations are not inconsistent with our results because the immunofluorescence assay probably scores preferentially for VP3.

In summary, this paper describes a genetic analysis of AAV and shows at least two complementation groups for a *rep* function and capsid production, respectively. A third mutant phenotype, *inf*$^-$, was unexpected but suggests that AAV particle assembly may require some additional function. Other studies (43; P. Senapathy, J. Tratschin, and B. Carter, J. Mol. Biol., in press) have revealed a fourth mutant phenotype, *ori*$^-$, in which both terminal palindromes are deleted. This double mutation results in a *cis*$^-$-dominant replication defect and reflects deletion of at least part of the AAV replication origin. Finally, the mutants described here do not reveal any information about the part of orf-1 which is unique to the transcripts initiated from p_5. However, these preliminary genetic studies provide a basis for detailed analysis of AAV replication, and further biochemical experiments are required to define the molecular basis of the mutant phenotypes. This work is also important for other studies aimed at developing AAV as a eucaryotic vector.

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