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A Novel 165-Base-Pair Terminal Repeat Sequence Is the Sole *cis* Requirement for the Adeno-Associated Virus Life Cycle

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Adeno-associated virus (AAV) replication is dependent on two copies of a 145-bp inverted terminal repeat (ITR) that flank the AAV genome. This is the primary *cis*-acting element required for productive infection and the generation of recombinant AAV (rAAV) vectors. We have engineered a plasmid (pDD-2) containing only 165 bp of AAV sequence: two copies of the D element, a unique sequence adjacent to the AAV nicking site, flanking a single ITR. When assayed in vivo, this modified hairpin was sufficient for the replication of the plasmid vector when Rep and adenovirus (Ad) helper functions were supplied in *trans*. pDD-2 replication intermediates were characteristic of the AAV replication scheme in which linear monomer, dimer, and other higher-molecular-weight replicative intermediates are generated. Compared to infectious AAV clones for replication, the modified hairpin vector replicated more efficiently independent of size. Further analysis demonstrated conversion of the input circular plasmid to a linear substrate with AAV terminal repeat elements at either end as an initial step for replication. This conversion was independent of both Rep and Ad helper genes, suggesting the role of host factors in the production of these molecules. The generation of these substrates suggested resolution of the modified terminal repeat through a Holliday-like structure rather than replication as a mechanism for rescue. Production of replicative intermediates via this plasmid substrate were competent not only for AAV DNA replication but also for encapsidation, infection, integration, and subsequent rescue from the chromosome when superinfected with Ad and wild-type AAV. These studies demonstrate that this novel 165-bp ITR substrate is sufficient in *cis* for the AAV life cycle and should provide a valuable reagent for further dissecting the *cis* sequences involved in AAV replication, packaging, and integration. In addition, this novel plasmid vector can be used as a substrate for both rAAV vector production and synthetic plasmid vector delivery.

Adeno-associated virus (AAV) is a nonpathogenic defective parvovirus which contains a single-stranded DNA genome of 4,680 nucleotides. The defective nature of the virus is characterized by its dependence on a helper virus, e.g., adenovirus (Ad) or herpes simplex virus, for lytic infection and production of progeny. In the absence of the helper virus, AAV establishes a latent infection by integrating into the host genome. Cells that have been latently infected with AAV can reenter the lytic pathway after superinfection with a helper virus (for reviews, see references 1, 2, and 22).

Genetic analysis of the AAV genome has identified three major components: the two *trans*-acting Rep and Cap genes that are required for viral DNA replication and virion production; and *cis*-acting inverted terminal repeats (ITRs) required for replication, packaging, integration, and rescue of the AAV genome (1, 22, 29). The ITR is a 145-bp element in which the first 125 nucleotides contain palindromic sequence elements in the order A, B, B′, C, C′ A′ (see Fig. 2). These sequences are over 80% GC rich and are capable of forming a T-shaped secondary structure when the viral genome is in a single-stranded form (Fig. 1 and 2). After infection, the 3′ end of the T-shaped ITR serves as a primer for DNA replication, converting the single-stranded genome into a double-stranded template with a covalently closed hairpin at one end (Fig. 1A, construct a). Between the palindromic A sequence and unique D sequence of the terminal repeat (TR) resides the terminal resolution site (*trs*) recognized by the Rep proteins (Fig. 1A, construct b). Completion of the replication scheme requires resolution of the covalently closed hairpin structure by Rep-dependent nicking, which results in the transfer of this sequence to the progeny DNA. The nicking reaction generates a new 3′ OH on the parental strand, providing the substrate for copying back the transferred ITR sequences (13, 15 for a review, see reference 22).

In the presence of helper viruses or when infected cells are stressed by genotoxic treatment (44–46), AAV will enter the replication cycle via the terminal transfer model (3, 22). During AAV replication, DNA intermediates containing head-to-head and tail-to-tail dimeric molecules are generated from reinitiation of replication on monomer substrates (Fig. 1A, construct c) (12, 36). These intermediates are novel in that a single ITR flanked by unique D and D′ sequences forms a bridge between the two AAV molecules (Fig. 1A, construct d). Resolution of these molecules back to the monomer pool ensures the production of wild-type virus. Similar dimer intermediates have been found in AAV proviral structures, suggesting the importance of this structure in the AAV life cycle (Fig. 1). These results suggest that our understanding of these critical sequences is still incomplete, although a model relying on the dimer intermediates has been proposed for successful rescue from the integrated state (25).

Previous studies have demonstrated that both ITRs seem to be required for AAV viability. Infectious clones containing the AAV genome, when transfected into Ad-infected cells, will rescue from the plasmid backbone and replicate to produce wild-type AAV virions (29, 32). Furthermore, if the AAV coding region is replaced by foreign DNA, these recombinant

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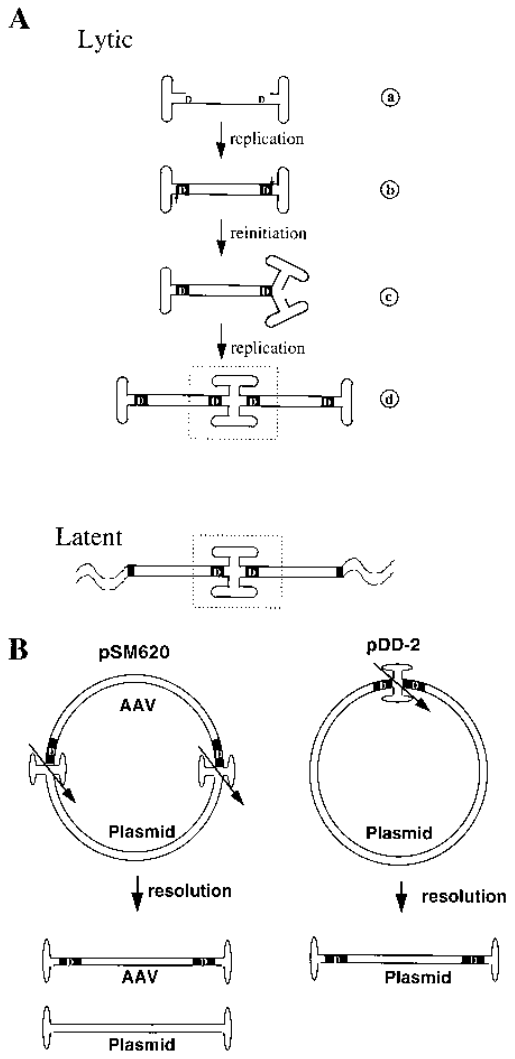

FIG. 1. (A) Generation of DD ITR intermediates in the AAV life cycle. Lytic constructs: a, single-stranded AAV molecule with TRs in the hairpin conformation; b, product generated after the first round of replication (small arrows denote the AAV nicking site positioned between the A and D palindromes); c, reinitiation of replication off of a linear duplex substrate; d, dimer replicative intermediate (the boxed portion illustrates DD ITR intermediate generated during AAV replication). Latent, diagram illustrating the same DD ITR intermediate (boxed) as it exists in proviral state. Wavy lines represent chromosomal DNA. (B) Model for AAV rescue. Plasmid pSM620 is a wild-type infectious AAV construct containing the entire AAV-2 genome (top half of the plasmid) including two ITRs with only one copy of the D elements (denoted by the cruciform structures and the black box labeled D within the circular construct). Plasmid pDD-2 contains only one modified ITR sequence (drawn as a cruciform structure flanked by two copies of the D element). The ITRs in the plasmids are drawn to show the cruciform structure that they can form due to palindromic sequences within the ITRs. Diagonal arrows show potential sites of resolution. Linear molecules with covalently closed T-shaped ITRs at the termini are the products of resolution. Note that the resolved linear AAV and pDD-2 molecules contain covalently closed ITRs with D sequences, in contrast to the linear plasmid portion from pSM620, which contains only convalently closed ITRs.

AAV plasmids can still rescue and replicate, as long as the two ITRs are present in *cis* and the Rep and Cap gene products are supplied in *trans* (9, 27, 37). However, when one of the two ITRs is completely deleted or partially deleted in such a manner that it cannot be repaired by gene conversion, the substrate is not viable for virus production (29, 32). Recently, a report has demonstrated that a cryptic *trs* in the AAV p5 promoter can partially substitute for replication if the left end ITR is completely deleted (39, 40).

Efforts to further delineate the *cis*-acting DNA sequences necessary for AAV viability have determined that the 20-bp D sequence of the ITR plays a critical role in viral DNA rescue and replication. Deletion of the D sequence from both ITRs severely impairs AAV DNA replication (39, 40, 43). Furthermore, when infectious wild-type AAV plasmids are transfected into Ad-infected cells, only the AAV portion which contains the ITR and D sequence of the plasmid is capable of replication after rescue. This same phenomenon was also found in an in vitro rescue and replication experiment (11). According to the AAV rescue model (41), two linear DNA fragments consisting of AAV and plasmid backbone are generated from the infectious clone by excision through the ITRs (Fig. 1B). Both

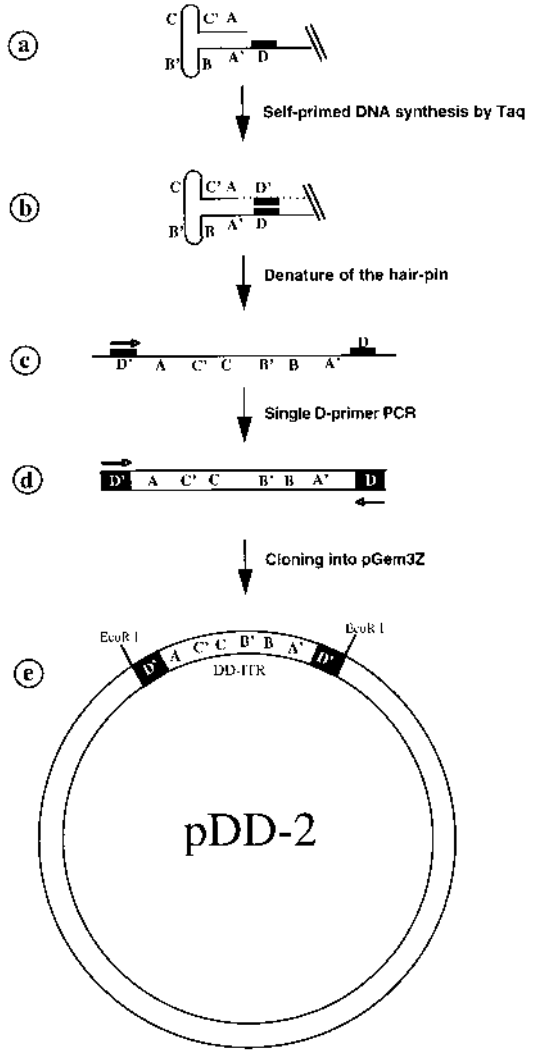

FIG. 2. Construction of plasmid pDD-2. a, diagram showing the 3′ hairpin structure formed after heat denaturation of linear AAV virus (A, A′, B, B′, C, C′, and D represent palindromic sequences within the ITR). b, Product generated after the first round of PCR in the absence of primer. Note that this step relies on the self-priming mechanism of the AAV ITRs. c, Template used for primer-specific PCR (primer denoted by the horizontal arrow). Note that after the first round of PCR, the template primer contains two copies of the D element flanking the ITR palindromic sequences. d, Final product generated from single-primer PCR. e, Cloning of the modified DD ITR via *Eco*RI restriction sites into a pGEM-3Z plasmid vector.

linear molecules generated contain covalently closed T-shaped ITR structures on each end. However, only the AAV molecules containing D or D′ sequences, preserving the *trs*, go on to replicate (Fig. 1B). AAV vectors are identical to wild-type clones in that after rescue, only vector sequences flanked by ITRs with D elements go on to replicate (26, 29). From these data, we hypothesized that any plasmid substrate containing a single modified ITR flanked by D and D′ sequences should rescue (Fig. 1B) and replicate via the AAV replication scheme.

To test this hypothesis and better elucidate the role of the D sequence, we have constructed a novel modified TR structure by using PCR (Fig. 2). This 165-bp ITR has flanking D and D′ sequences which when transfected in *cis* into Rep-expressing human cells confers rescue and replication to plasmid substrates regardless of size. Replication intermediates generated with this substrate (monomer, dimer, etc.) are produced via the AAV replication scheme and appear to be very efficient compared to infectious clones containing two wild-type ITRs. Rescue experiments suggest that resolution occurs through the modified ITR via a Holliday-like structure, generating linear molecules with covalently closed hairpins at either end. Interestingly, this resolution process can take place in the absence of both Ad and AAV gene expression, suggesting a role for host functions in this initial step. Replicating molecules generated from this ITR substrate are capable of completing the entire AAV life cycle, including packaging, infection, integration, and subsequent rescue after helper and wild-type superinfection. These studies have determined that this modified ITR is sufficient in *cis* for all steps of the AAV life cycle and should be an important substrate for further characterization of *cis* elements required for AAV replication, packaging, and integration. In addition, this novel ITR provides a unique substrate for the study of synthetic AAV plasmid vectors in vivo.

MATERIALS AND METHODS

Cell lines and general procedures. Human Detroit 6 and 293 cells were maintained in Dulbecco's modified Eagle medium (DMEM; GIBCO-BRL) supplemented with 10% fetal calf serum (FCS; HyClone) at 37°C in CO_2-controlled environments. Low-molecular-weight DNA from transfected cells was extracted as described by Hirt (10). Any general molecular biological techniques used in this study were performed according to standard procedures described previously (24) unless otherwise noted.

DNA transfection and construction of rAAV stocks. Transfection of plasmid DNA was performed by using Lipofectin as described by the manufacturer (GIBCO-BRL). Subconfluent 293 cells in a 6-cm-diameter dish were washed twice with DMEM. Five micrograms of plasmid DNA was incubated with 50 μl of Lipofectin at room temperature for 10 min, mixed with 2 ml of Opti-MEM (GIBCO), and added to the cells. After 12 h, the medium was replaced with 3 ml of fresh DMEM containing 10% FCS with or without Ad type 5 (Ad5) at a multiplicity of infection of 5. The cells were incubated for an additional 48 h. To produce recombinant AAV (rAAV), the vector plasmid and the helper plasmid pAAV/Ad (27) (in a 1:3 ratio) were cotransfected into the cells as described above. At 48 h after Ad5 infection, the cells were harvested and resuspended in 1 ml of Opti-MEM. After three freeze-thaw cycles, the cellular debris was removed by low-speed centrifugation. The virus lysate was heated at 56°C for 30 min to inactivate Ad and then stored at −20°C.

Southern hybridization. Restriction enzyme-digested DNA subject to Southern analysis was separated on agarose gels and then transferred onto Genescreen Plus nylon membranes (Du Pont). Southern hybridizations using [α-^{32}P]dCTP random primer-labeled plasmids were performed as recommended by the manufacturer (Du Pont). Southern hybridizations using [γ-^{32}P]ATP-end-labeled ITR oligonucleotide probe A-1 (5′TTGGCCACTCCCTCTCTGCG3′, derived from the A region of the ITR) were carried out as follows. DNA-bound nylon membranes were prehybridized in 5× SSC (1× SSC is 0.15 M NaCl plus 0.15 M sodium citrate)–10× Denhardt's solution–10% dextran sulfate–5% sodium dodecyl sulfate at 60°C for at least 1 h. Twenty-five nanograms of ^{32}P-end-labeled probe and 200 μg of heat-denatured salmon sperm DNA in 0.5 ml of H_2O were then added, and hybridization was carried out at 60°C overnight. The membranes were then washed twice in 3× SSC–5% sodium dodecyl sulfate at 60°C for 30 min and once in 0.2× SSC at room temperature for 10 min. Probe hybridization was visualized by exposure of hybridized membranes to X-ray film (Du Pont).

PCR and construction of ITR plasmids. Low-molecular-weight DNA from AAV/Ad5-infected cells was used as the template for the generation of the double-D (DD) TR, using a single primer (TR-1) derived from the D sequence of AAV. PCRs were performed at 95°C for 1 min, 55°C for 30 s, and 72°C for 1 min for 35 cycles in a 50-μl volume containing 20 mM Tris-HCl (pH 8.8), 1.5 mM $MgCl_2$, 50 mM KCl, 2.5% formamide, 100 μM dATP, dTTP, and dCTP, 75 μM 7-deaza-dGTP, 25 μM dGTP, 2.5 U of AmpliTaq (Perkin-Elmer Cetus), 1 ng of AAV DNA, and 100 pmol of primer TR-1 (5′GGAATTCAGGAACCCCTAG TGATGG3′). The PCR product was isolated by agarose gel electrophoresis, cut with *Eco*RI, and ligated with an *Eco*RI-digested and dephosphorylated plasmid, pGEM-3Z (Promega). The ligated plasmid was transformed into *Escherichia coli* Sure cells (Stratagene). Positive clones were screened for the presence of the DD TR by colony hybridization and confirmed by dideoxy sequencing with 7-deaza-dGTP substituted for dGTP (31). The selected DD clone was named pDD-2. Plasmid pDD-neo was constructed by using a *neo* gene cassette excised with *Xho*I and *Sal*I from plasmid pMC1-neo polyA (Stratagene) and cloned into the *Sal*I site of pDD-2. Plasmid pDD-AAV was constructed by inserting the 4.3-kb *Xba*I fragment from psub201 (28) into the *Xba*I site of pDD-2.

Cloning of Neo^r cell lines. AAV stocks prepared from pDD-neo as described above were used to infect (multiplicity of infection of 10) 80% confluent Detroit 6 cells maintained in DMEM with 10% FCS plus antibiotics. At 24 h postinfection, neomycin-resistant (Neo^r) cells were selected in the presence of geneticin G418 (GIBCO) at 400 μg/ml. Single cell clones were isolated and expanded under G418 selection.

RESULTS

Construction of a modified AAV ITR with DD sequences. PCR was used to create the ITR with flanking D elements (Fig. 2). The rationale is based on the T-shaped structure of the AAV ITR. Upon denaturation, this single-stranded DNA can serve as a template for self-primed PCR based on the inverted symmetry of the A-A′, B-B′, C-C′, D sequences in the ITR (Fig. 2, construct a). Viral DNA was isolated, denatured to promote self-priming hairpin structures, and extended with *Taq* polymerase in the first round of the PCR without the addition of primers. The self-primed elongated material will produce a T-shaped hairpin structure containing flanking D and D′ sequences (Fig. 2, construct b). Following elongation, a single primer homologous to the D sequence with *Eco*RI adapter sequences was used (Fig. 2, construct c) to amplify the desired hairpin product (Fig. 2, construct d). Due to the high GC content and the strong palindromic structure in the ITR region, incorporation of 7-deaza-dGTP, 2.5% formamide, and a high concentration of primer were used (see Materials and Methods). PCR product generated from these conditions was cleaved by *Eco*RI and cloned into the polylinker of pGEM-3Z (Fig. 2, construct e). To avoid rearrangement and instability of the ITR in bacteria, the recombinant plasmid was transformed into the *E. coli* recombination-deficient SURE strain (Stratagene). Using the strategy described above, we obtained positive clones, which were confirmed by restriction digestion and sequencing (data not shown). While many clones lost either the A or the A′ element during the PCR, two clones contained an intact ITR consisting of D′A B B′C C′A′D regions at the *Eco*RI site of the pGEM-3Z. One plasmid, pDD-2 (Fig. 2, construct e), was used in in vivo transfection experiments and assayed for AAV replication.

The DD ITR is sufficient for AAV replication and dependent on Rep in *trans*. To determine if the DD ITR was sufficient for AAV-like replication, plasmid pDD-2 was transfected into Ad5-infected 293 cells with or without cotransfection of an AAV helper plasmid, pAAV/Ad (27). The AAV helper plasmid pAAV/Ad supplies Rep and Cap proteins in *trans* but is incapable of replicating itself due to the absence of AAV ITRs (27). Low-molecular-weight DNA was isolated 48 h posttransfection and fractionated by electrophoresis on an agarose gel with or without *Dpn*I digestion followed by Southern blot analysis (Fig. 3). *Dpn*I distinguishes between newly replicated DNA and input plasmid DNA based on resistance to digestion due to loss of methylation. In the absence of the AAV helper plasmid (Fig. 3, lanes 1 and 2), pDD-2 DNA was completely *Dpn*I

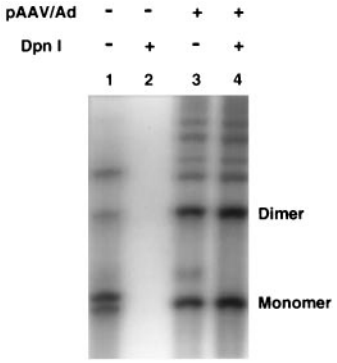

FIG. 3. Replication assay for the plasmid containing the DD ITR. Plasmid pDD-2 was transfected into Ad5-infected 293 cells with (+) or without (−) cotransfection of the helper plasmid pAAV/Ad. Low-molecular-weight DNA was extracted 48 h postinfection and separated on an 1% agarose gel with (+) or without (−) *Dpn*I digestion. Southern blot hybridization used ^{32}P-labeled plasmid pGEM-3Z as a probe. Positions of replicated intermediates are denoted by monomer and dimer.

sensitive (Fig. 3, lane 2). A signal migrating with linear monomer appeared at higher levels than normal in the helper-minus, *Dpn*I-minus lane (Fig. 3, lane 1), suggesting resolution of the plasmid (Fig. 1, construct b). Detailed analysis of this linear *Dpn*I-sensitive product is presented below in Fig. 5. However, in the presence of helper plasmid (Fig. 3, lanes 3 and 4), pDD-2 replicated very efficiently and exhibited a typical AAV replication pattern of monomer, dimer, and other replicated intermediates (Fig. 3, lanes 3 and 4). All of the replication intermediates were resistant to *Dpn*I digestion (Fig. 3, lane 4), supporting the role of this *cis* element in replication. The cloning vector pGEM-3Z did not replicate under the same conditions, and a plasmid containing only the Rep genes was positive for complementing pDD-2 replication (data not shown). These experiments demonstrate that pDD-2 replication is dependent on two factors: the DD ITR in *cis* and AAV Rep gene products in *trans.*

DD ITR replication is efficient and independent of plasmid size. Since the replication of pDD-2 with one modified ITR appeared very efficient, a comparison was made between pDD-2 and two infectious AAV plasmids, psub201 (28) and pSM620 (26). These constructs contain two ITRs as well as Rep and Cap genes. Plasmid pDD-2 was cotransfected into Ad5-infected cells with equal amounts of either pAAV/Ad helper or infectious clones psub201 and pSM620 DNA. Low-molecular-weight DNA was isolated digested with *Dpn*I and analyzed as described above. In this experiment, a labeled oligonucleotide probe derived from the A region of the ITR was used to detect all replicated DNAs containing AAV *cis*-acting sequences. As illustrated in Fig. 4A, all three plasmids containing AAV coding genes complemented pDD-2 for replication. In addition, infectious clone pSM620 replicated to a level similar to that of pDD-2 (26), while infectious clone psub201 replicated at a much lower level (Fig. 4A, lanes 4 and 3, respectively). Although psub201 replicated less efficiently, it complemented pDD-2 replication as effectively as the other AAV helper constructs (Fig. 4A; compare lane 3 with lanes 2 and 4). Inefficient replication of psub201 is attributed to the 13-bp deletion in the A region of these ITRs, resulting in impaired rescue and much delayed replication (28). From this analysis, pDD-2 appeared to replicate as efficiently as if not better than the infectious clones containing two wild-type AAV ITRs.

To determine whether the efficient replication of pDD-2 observed above was due to the modified DD ITR or simply due to the smaller size of the plasmid (2.9 kb), an 1.2-kb Neo^r gene fragment was inserted into the *Sal*I site of pDD-2 polylinker. The new plasmid, pDD-neo, is 4.1 kb in size, similar to wild-type AAV (4.6 kb). In addition, another variant pDD-2 was made by inserting the 4.3-kb AAV *Xba*I fragment from psub201 into the *Xba*I site of pDD-2, generating a 7.3-kb plasmid called pDD-AAV. As shown in Fig. 4B, the three DD constructs replicated to similar levels regardless of size. These results demonstrate that the 163-bp modified DD ITR is an efficient *cis*-acting substrate for Rep-dependent replication, and these plasmid substrates are not size dependent.

Rescue involves resolution of the ITR. The ITRs are the AAV rescue substrates and replication origins (39–41). Since the DD plasmids contain only a single modified ITR and replicated via an AAV mechanism in the presence of Rep proteins, we tested this substrate for the ability to rescue in vivo. Previous studies using a plasmid substrate with a deleted ITR (psub201) demonstrated that the rescue event was a rate-limiting step in viral replication (28). Digestion of a restriction site engineered to release the plasmid backbone from the viral sequences of psub201 prior to transfection into Ad-infected 293 cells demonstrated a 10-fold increase in replicating AAV molecules. Data supporting rescue have been extended by in vitro studies using wild-type infectious clones (41). Based on the above studies, and the appearance of a putative rescue fragment in Fig. 3, lane 1, pDD-2 DNA was assayed for rescue by transfection into uninfected and Ad5-infected 293 cells (Fig. 5B, lanes 3 and 6 and lanes 1, 2, 4, and 5, respectively), with and without cotransfection of AAV helper plasmid (Fig. 5B, lanes 1 and 4 and lanes 2, 3, 5, and 6, respectively). Resolution of pDD-2 should result in a linear molecule with ITR sequences at both ends as diagrammed in Fig. 1B. To distinguish between input and resolved molecules, low-molecular-weight DNA was recovered 24 h posttransfection and subjected to restriction analysis by single-cut enzymes *Ssp*I and *Sca*I, respectively (Fig. 5A). pDD-2 sequences were detected after Southern blot analysis using an ITR-specific probe. The results from this analysis are shown in Fig. 5B. After *Ssp*I or *Sca*I

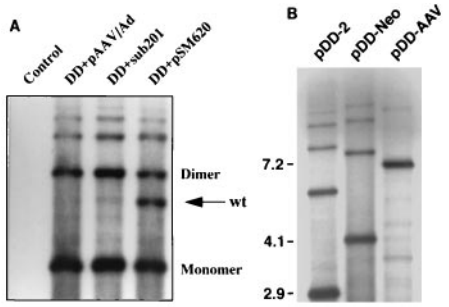

FIG. 4. (A) Comparison of replication of pDD-2 with psub201 and pSM620. Plasmid pDD-2 was transfected alone (lane 1) or cotransfected with an equal amount of either pAAV/Ad (lane 2), psub201 (lane 3), or pSM620 (lane 4) into Ad5-infected 293 cells. Low-molecular-weight DNA was extracted, digested with *Dpn*I and analyzed on an 1% agarose gel by Southern blot hybridization using a ^{32}P-labeled ITR oligonucleotide probe. Monomer and dimer represent the replicated pDD-2 molecules. The arrow indicates the position of the monomer band expected for wild-type (wt) AAV. (B) Replication assay for DD plasmids of different sizes. pDD-2, pDD-neo, or pDD-AAV was cotransfected into Ad5-infected 293 cells with (lanes 1 and 2) or without (lane 3) helper plasmid pAAV/Ad. Low-molecular-weight DNA was extracted and digested with *Dpn*I and analyzed on an 1% agarose gel by Southern blot hybridization using a ^{32}P-labeled plasmid pGEM-3Z probe. The molecular weight markers indicate the position of monomer pDD-2 (2.9 kb), pDD-neo (4.1 kb), and pDD-AAV (7.2 kb).

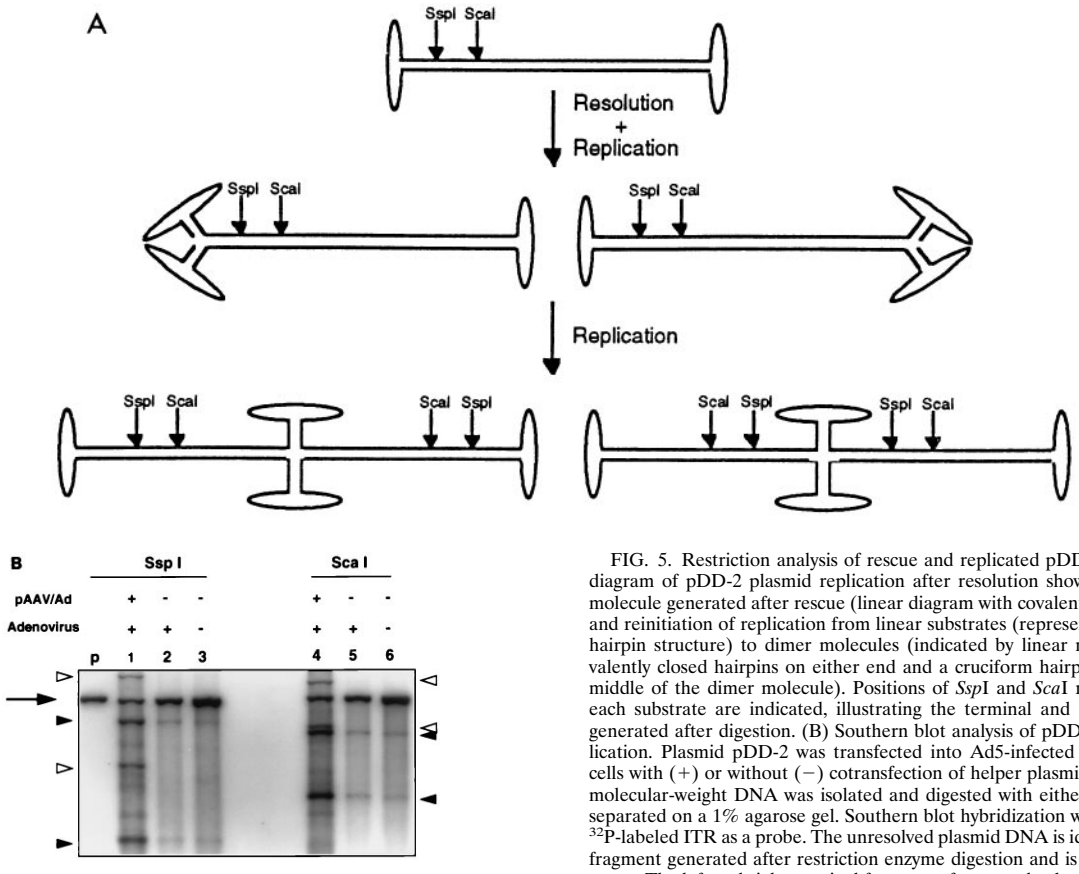

FIG. 5. Restriction analysis of rescue and replicated pDD-2. (A) Schematic diagram of pDD-2 plasmid replication after resolution showing the monomer molecule generated after rescue (linear diagram with covalently closed hairpins) and reinitiation of replication from linear substrates (represented by the double hairpin structure) to dimer molecules (indicated by linear molecules with covalently closed hairpins on either end and a cruciform hairpin structure in the middle of the dimer molecule). Positions of *Ssp*I and *Sca*I restriction sites for each substrate are indicated, illustrating the terminal and internal fragments generated after digestion. (B) Southern blot analysis of pDD-2 rescue and replication. Plasmid pDD-2 was transfected into Ad5-infected or uninfected 293 cells with (+) or without (−) cotransfection of helper plasmid pAAV/Ad. Low-molecular-weight DNA was isolated and digested with either *Ssp*I or *Sca*I and separated on a 1% agarose gel. Southern blot hybridization was performed with ^{32}P-labeled ITR as a probe. The unresolved plasmid DNA is identified as a linear fragment generated after restriction enzyme digestion and is marked by a large arrow. The left and right terminal fragments from resolved monomer and dimer molecules are identified by the solid arrowheads. The head-to-head and tail-to-tail dimer junction fragments generated after digestion are indicated by the open arrowheads. Lane p, digested plasmid control.

digestion, a 2.9-kb unit-length band can be observed in all lanes migrating with predigested plasmid control (Fig. 5B). This fragment is generated after *Ssp*I or *Sca*I digestion, which will linearize unresolved input circular plasmid DNA. Plasmid DNA resolved in vivo would generate two fragments smaller than linearized input after restriction digestion (Fig. 5A). Resolved products of predicted sizes were observed under all conditions (Fig. 5B). Use of the ITR-specific probe supported TR sequences at both ends of the resolved molecules. Although resolution appeared more efficient when AAV and Ad genes were present (compare lanes 1 and 4 to lanes 2, 3, 5, and 6), Ad alone did not augment resolution (Fig. 5B; compare lanes 2 and 5 to lanes 3 and 6). In fact, resolution of the plasmid substrate occurred in noninfected cells, suggesting a role for host factors in the rescue step (Fig. 5B, lanes 3 and 6). The additional bands observed in the Ad5-plus-AAV helper plasmid lanes (Fig. 5B, lanes 1 and 4) are expected products generated from AAV replicated intermediates as shown in Fig. 5A. The two darker bands in lanes 1 and 4, which are common fragment in all lanes containing resolved species, are generated by the digestion of linear monomer and dimer AAV molecules (Fig. 5B, solid arrowheads), while the two lighter bands represent products from internal head-to-head or tail-to-tail dimer junction fragments (Fig. 5B, lanes 1 and 4, open arrowheads). Fine restriction mapping confirmed the presence of ITR sequences at either end of the resolved molecules (data not shown). The results from this experiment support rescue of the input circular plasmid to a linear molecule with ITR sequences at either end. Resolution of the DD plasmid in the absence of AAV gene products is in agreement with in vitro rescue results (41). Based on the previous experiment (Fig. 3), the rescued DNA molecules were *Dpn*I sensitive and did not replicate in the absence of Rep (Fig. 3, lanes 2 and 4). These results in combination with those of Fig. 5 support rescue using host factors by resolution via the ITR which is independent of DNA replication.

The DD ITR in *cis* is sufficient for the complete AAV life cycle. Since plasmids with the modified DD ITR could efficiently replicate in the AAV replication scheme, we addressed the remaining steps of the AAV life cycle, primarily packaging, infection, integration, and rescue after Ad and wild-type AAV superinfection. First, we assayed if plasmid pDD-neo could be efficiently packaged into the AAV particles compared with rAAV-*neo* plasmid pTR-UF2, derived from wild-type infectious clone pSM620. pTR-UF2 contains an identical thymidine kinase-*neo* gene cassette along with a green fluorescent protein gene flanked by two ITRs (50). The two vector plasmids pDD-neo and pTR-UF2 should produce virus particles similar in size (4.1 and 4.3 kb, respectively). The two constructs generated very similar titers (2.3×10^{10} pDD-neo and 4.0×10^{10} pTR-UF2 particles) when assayed by the viral DNA dot blot method using a *neo*-specific probe (data not shown). pDD-neo virus produced was assayed for transduction and integration. Infection of human Detroit 6 cells with the DD viral stock resulted in numerous Neo^r clones which were isolated for further characterization. Genomic DNA from a subset of the clones was analyzed by Southern blot hybridization after single-cut restriction enzyme digestion (Fig. 6). Analysis of the digestion pattern supported independent integration in each

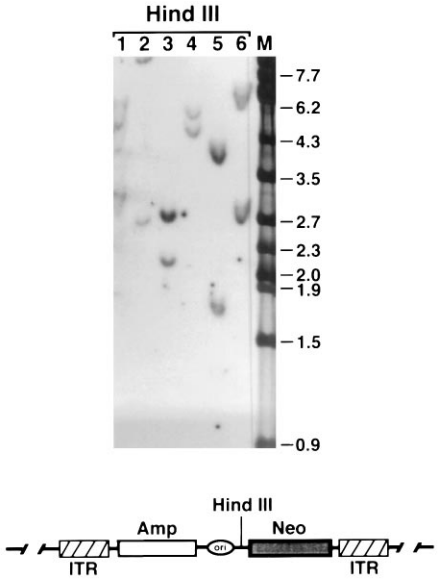

FIG. 6. Genomic Southern analysis of the DD-*neo* AAV provirus structure in latently infected Detroit 6 cell clones. Genomic DNA from various latent cells was digested with restriction enzyme *Hin*dIII, a single cutter for vector sequences. DNA was fractionated on a 1% agarose gel and assayed for vector sequences by Southern hybridization using a *neo*-specific probe. Lane M represents molecular weight markers; lanes 1 to 6 represent individual genomic clones. The diagram illustrates the provirus structure as a linear molecule with ITRs (hatched boxes) forming junctions with chromosome DNA (−//−). Ampicillin- and G418-resistant genes of the plasmid vector are denoted by Amp and Neo, respectively. ori is the pUC plasmid origin of replication (theta structure), and the position of the *Hin*dIII restriction site is indicated. Sizes are indicated in kilobases.

clone (Fig. 6). Chromosome 19 PCR supported lack of targeted integration (data not shown) consistent with previously reported AAV vector integration studies. By these criteria, virus generated by using pDD-2 as a starting substrate was indistinguishable from that produced from conventional vectors (27, 38) for both virus transduction and integration. We assayed for rescue and replication of pDD-2 by superinfection with wild-type AAV-2 and Ad5. After superinfection, low-molecular-weight DNA was extracted, fractionated on an agarose gel, and probed with a *neo*-specific fragment for rescued and replicated vector DNA. In this assay, 6 of 22 Neo^r cell clones tested rescued (Fig. 7). These results demonstrate that the 165-bp DD ITR *cis* sequence is a sufficient substrate after resolution for all steps of the AAV life cycle, including DNA replication, virus encapsidation, integration, and rescue.

DISCUSSION

We have constructed and cloned a novel modified AAV ITR of 165 bp containing flanking D and D′ sequences. The single DD ITR is a very effective AAV replication substrate in *cis* when Rep is provided in *trans*. Analysis of the potential secondary structure formed by this modified ITR suggest why this substrate may work for both plasmid rescue and replication. In the unresolved circular plasmid, the DD ITR is an exact replica of an AAV dimer junction generated during viral DNA replication (Fig. 1A) (12, 36). These intermediates are naturally resolved from dimers to monomers as part of the AAV replication scheme (Fig. 1). The availability of these substrates immediately after transfection may facilitate their resolution into Rep-dependent linear replication substrates (see below). In addition, in a supercoiled plasmid substrate, this modified ITR sequence has the potential to form a T-shaped hairpin structure with contiguous D-A elements, the natural substrate for binding and nicking of AAV Rep proteins (Fig. 1B) (13, 14, 23, 33). This contiguous D-A stem structure, which is present in the hairpin structure of AAV replication monomer intermediates, as well as the resolved plasmid product described above (Fig. 1B), may enter the AAV replication scheme more efficiently (12, 36). Therefore, it appears that this *cis* element that we constructed may generate AAV replication substrates by two mechanisms: resolution and Rep-dependent nicking.

Resolution of DD plasmid. Our rescue assay determined that the input circular plasmid was converted to a linear molecule with covalently closed hairpin ITR structures at either end (Fig. 5). This product is identical to the no-end substrate synthesized in vitro and used in AAV Rep nicking assays (35) as well as the type of molecule generated in an in vitro rescue experiment previously described (41). From our analysis, it appears that resolution takes place through a Holliday-like structure which can form due to the secondary structure of the ITR sequences, which is replication independent (Fig. 1B, 3, and 5). While this is an intriguing observation, more important is the fact that resolution could take place in noninfected cells, suggesting a role for host enzymes. The fact that the DD plasmid can be resolved in noninfected cells, and the fact that numerous latently AAV-infected cell lines contain provirus in the form of dimers (see Fig. 1) or multimers (4, 19, 21, 25) carrying DD-like ITR structures between adjacent AAV molecules, is very interesting (6, 49). Previous studies have indicated that AAV can rescue from integrated proviral structures in the absence of Ad superinfection (21). In addition, none of the virus-cell junctions isolated to date contain intact ITRs (16, 30, 42). These two observations suggest that assurance of rescue from the proviral state requires preservation of at least one intact ITR sequence after integration. Dimer integrants containing DD ITR sequences as diagrammed in Fig. 1 achieve this objective. This structure not only provides a means for rescue but supplies an intact ITR for gene conversion of deleted ITR-cell junctions (6, 29). Since these substrates can resolve in the presence of host enzymes, this may be a common mechanism for rescue of all latent AAV genomes. Our data demonstrate rescue via resolution of DD ITR sequences and support a model which has previously been proposed utilizing DD ITR intermediates as critical components of AAV integration and rescue (25).

DD replication is efficient and not dependent on size. Our replication assays demonstrated that this *cis*-acting substrate was not dependent on plasmid size (Fig. 4B) and replicated very efficiently compared to wild-type clones (Fig. 4A). The effective replication and encapsidation of the DD construct indicates the crucial role that the D sequence plays in these processes. This is consistent with observations that deletion of D sequences abolishes AAV replication and packaging (39, 40, 43). In addition, in vitro Rep68 binding and nicking assays are affected by mutant substrates that altered the *trs* sequence or

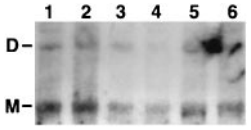

FIG. 7. Rescue assay of DD-*neo* proviruses. Latently DD-*neo* virus-infected Detroit 6 cell clones were superinfected with wild-type AAV and Ad5. Low-molecular-weight DNA was extracted and separated on 1% agarose gel. Southern blot hybridization was performed with ^{32}P-labeled *neo* as a probe. M and D represent the rescued and replicated provirus monomer and dimer molecules.

spacing between A and D elements (23, 34). In our replication assay, plasmid pDD-2 rescued and replicated much better than infectious clones psub201 and at least as well as pSM620 (Fig. 4A). The difference in replication between psub201 and pSM620 may be due to the respective ITR sequences. In psub201, there is a terminal 13-bp deletion of both ITRs which has been shown to impair rescue (28). In contrast, pSM620 contains two wild-type ITRs which were cloned by GC tailing. The GC tails in this plasmid have been shown to be a substrate for a cellular endonuclease (7). As a result, pSM620 may rescue more effectively through a cellular endonuclease that cleaves the GC tail as well as resolution through the ITR sequences. In spite of the two wild-type ITRs and GC tails in pSM620, our data support just as efficient rescue and replication of pDD-2 (Fig. 4A). Since only one copy of the D element is present in wild-type ITR clones, the potential to form the extended D-A stem structure in pDD-2 is not possible for plasmids pSM620 and psub201, suggesting that this may be the most effective natural substrate for rescue and replication. Moreover, the 13-bp deletion in the A region of psub201 further prevents the ability to form this structure and most likely is the reason for the inefficient rescue and consequently delayed replication of this substrate (28).

AAV replicating plasmids. Recently Yalkinoglu et al. have shown that a plasmid named pA2Y1, containing 1 kb of the extreme left half of AAV with a single ITR, could replicate in cells treated with genotoxic reagents in a Rep-independent manner (47). In our experiments, pDD-2 replication was completely dependent on Rep gene expression. In trying to explain these observations, differences between constructs, such as the modified ITR of pDD-2 compared to the single ITR with additional AAV sequences (p5 and p19 promoters) of pA2Y1, should not be overlooked (5, 8). In addition, the cellular environment for pDD-2 replication was AAV lytic, while pA2Y1 was assayed in cells treated with genotoxic reagents. Most interestingly, the DD plasmids rescued and replicated as linear molecules via the AAV replication scheme (12, 36), whereas pA2Y1 replicated bidirectionally as a circular molecule (47). Bidirectional replication of pA2Y1 is supportive of a carcinogen-inducible DNA amplification mechanism (47). Since both assays utilized the ITRs as their origins, it will be interesting to test if pDD-2 can also replicate as a circular molecule in cells treated by genotoxic reagents.

The DD construct as a plasmid vector. One important characteristic of wild-type AAV is its preferential integration into human chromosome 19 (16–18, 30). Although targeted integration appears mediated by the ITRs in the presence of Rep proteins (20, 25), this characteristic is apparently lost with rAAV in the absence of Rep gene product (25, 38). Since the DD ITR is functional in *cis*, reduces the TR requirement from 300 to 165 bp, and is cloned in a polylinker, this substrate should facilitate further manipulation and mutational studies aimed at understanding the role of the ITRs in replication, packaging, and integration. In fact, preliminary studies with this construct have supported this premise. For example, plasmids containing the DD ITR transfected in the presence of Rep have been shown to preferentially integrate into chromosome 19 (42). This observation suggests that DD ITR plasmids have potential as nonviral targeted vectors. The ability to circumvent the size limitation (5 kb) of AAV vectors and retain targeted integration is of significant interest. Virus generated from DD ITR plasmids was capable of integration and subsequent rescue, supporting the role of this sequence in all steps of the AAV life cycle (Fig. 6 and 7). The inclusion of bacterial origin and ampicillin-resistant sequences in these vectors has facilitated the cloning and characterization of integrated proviruses (48) compared to conventional genomic library methods (16, 30). Finally, as with the initial AAV infectious clone, this study demonstrates the importance of AAV plasmid substrates for dissecting the complex life cycle of this nonpathogenic defective parvovirus and suggests that these plasmid vectors may be useful substrates for human gene therapy.

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