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SHORT COMMUNICATION

to Rescue Modified Viruses

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The genome of ovine adenovirus OAV287 has an arrangement which is unique among known adenoviruses. To facilitate further experimentation on the structure and function of this genome, plasmids containing a complete clone of the genome were constructed. The cloned viral genome was released from plasmids by restriction enzyme digestion as an intact linear molecule with authentic 5′ termini. Transfection of the linear DNA into cells which supported replication produced infectious virus. Mutation of a unique *Sal*I site at the right-hand end of the genome disrupted reading frames of unknown function without affecting virus rescue, identifying this region as nonessential for replication *in vitro.* A 20-bp oligonucleotide was also inserted into the short intergenic region between the pVIII and the fiber sequences, identifying a second site for gene insertion. These studies will facilitate the development of OAV as a gene transfer vector.

Human adenoviruses (Ad) have been studied for many years. They are well characterized and have provided important insights into gene regulation and virus replication (reviewed in *10*). This has led to their adaptation as vectors for vaccine and gene therapy applications (*1, 7*). In contrast, adenoviruses from animal and avian species are less well studied. Partial characterization of some viruses has been reported and some structural protein homologues have been described (*3–5, 12, 16, 19, 21*). Avian Ads perhaps represent the most distinct virus group studied to date but, in general, the genome arrangement in the animal Ads is similar to that of the human Ads. Homologies can be detected between most animal Ad proteins and their human Ad counterparts. Similarly, the group of reading frames comprising the E3 region is always located between the pVIII and the fiber genes although its size and complexity varies in animal Ads compared with human Ads. These sequences are of interest because in human Ads the E3 region is nonessential for replication *in vitro* (*14*) and can therefore be replaced by foreign DNA (*11, 17*). The identification of similar nonessential sequences in ovine adenovirus (OAV) might therefore assist the development of this virus as a vector.

The determination of the complete nucleotide sequence of OAV287 was recently completed (*22–24*). This revealed that the OAV E3 sequences were not present between the pVIII and the fiber genes. However, two other groups of open reading frames which showed little or no detectable homology to other Ad genomes were located at the right-hand end of the genome on the right-to-left strand (*23*). To begin to investigate the structure and function of these regions and to facilitate further development of OAV as a vector we constructed a plasmid carrying a full length, infectious clone of the OAV genome. Mutagenesis was used to identify two potential sites for the insertion of gene expression cassettes.

Procedures for the growth of OAV in CSL503 cells, virus purification, and isolation of the linear viral DNA were described previously (*2, 18*). OAV genomic DNA was cloned as *Bam*HI fragments (*2*) which occur from left to right in the genome in the order D, B, C, E, F, and A. OAV genomic fragments were modified using phosphorylated synthetic oligonucleotides of the desired sequence and mutagenesis kits (Muta-Gene Phagemid, Bio-Rad Labs; Altered Sites II, Promega Corp., Madison, WI). The terminal *Bam*HI A and D fragments (cloned in the *Bam*HI/*Hin*cII sites of Bluescribe M13$^+$ (Stratagene, La Jolla, CA) were each modified at the 5′ terminus of the inverted terminal repeat (ITR) to restore two nucleotides (CT) lost during cloning (*2*) and to create a *Kpn*I site, producing plasmids pAK and pDK. The genomic *Bam*HI B fragment was modified by mutagenesis (GCATGC to GCATCC) to remove the *Sph*I site at position 8287 producing pBM. This modified *Bam*HI B fragment

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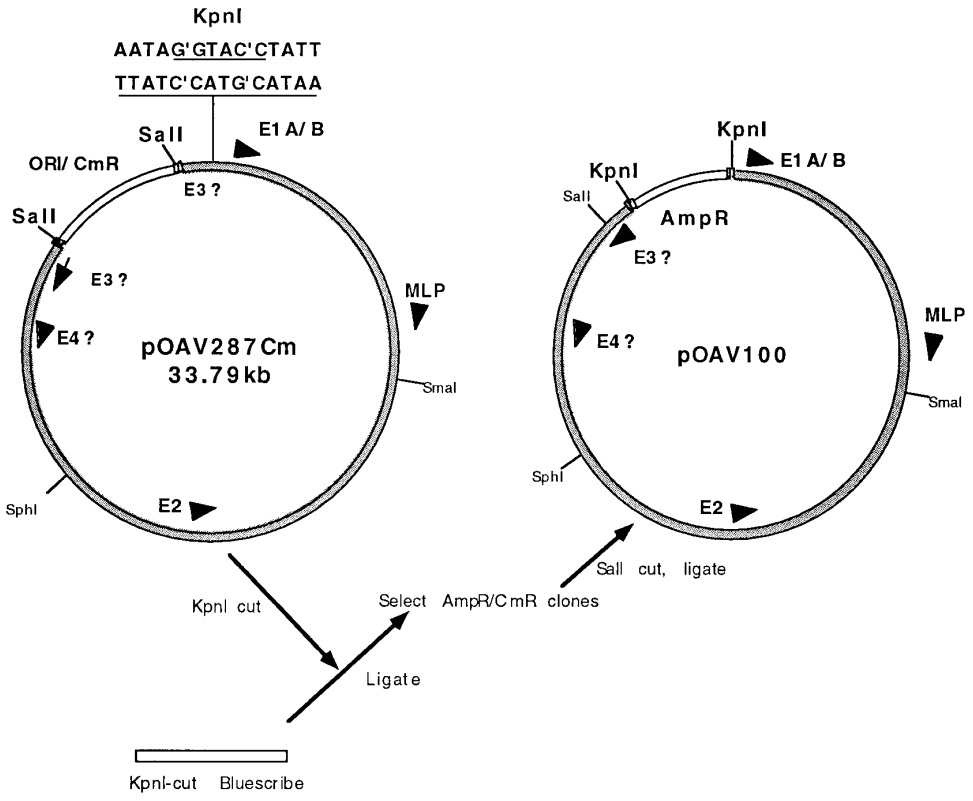

FIG. 1. Construction of plasmids containing a full length clone of the OAV genome. Plasmid pOAV287Cm was constructed from partial genomic clones and pOAV100 was derived from it as described in the text. Open sequences in pOAV287Cm are from pACYC184. In pOAV100 they are derived from modified Bluescribe M13$^-$.

was linked to the terminal D fragment in plasmid pDBM. The *Sph*I A fragment (~13 kb) was cloned into pSELECT (Promega) to form pSESPH. This fragment had a *Sma*I site in common with pDBM near its left-hand end. The *Kpn*I/*Sma*I fragment from pDBM was subcloned into pSESPH to produce pSELLH which now contained the left-hand ~21.5 kb of the OAV genome. Plasmid pAK contained the right-hand *Bam*HI A fragment of ~8.6 kb which overlapped the *Sph*I A fragment in pSELLH. pAK was cut at a unique *Sal*I site and ligated with *Sal*I-cut pACYC184 (*20*) (4.24 kb) to form pACm. pACm was cut with *Sph*I and *Kpn*I to produce the right-hand genomic fragment incorporating the pACYC184 sequences. This was ligated with the left-hand *Kpn*I/*Sph*I fragment of ~21.5 kb prepared from pSELLH to produce the final plasmid pOAV287Cm (Fig. 1). This plasmid replicated stably in *Escherichia coli* strain JM109. Yields were improved by the growing cultures in the presence of 200 μg/ml ampicillin.

The presence of pACYC184 sequences in the *Sal*I site of pOAV287Cm disrupted two open reading frames (*23*) of unknown function. Therefore pOAV287Cm was modified further. Plasmid Bluescribe M13$^-$ (Stratagene) was cut with *Hin*dIII and end filled, then cut with *Sma*I, blunt-end ligated, and transformed. The resulting plasmid lacked *Sal*I and *Sph*I sites, but retained a unique *Kpn*I site. After digestion with *Kpn*I this plasmid was ligated with *Kpn*I-cut pOAV287Cm. Plasmids which were Amp^R/Cm^R were selected and grown. One of these was cut with *Sal*I to release the pACYC184 sequences, religated, and transformed. The resulting plasmid, pOAV100, carried the Amp^R gene and an origin of replication in the *Kpn*I site between the genomic ITRs (Fig. 1). This plasmid replicated stably in *E. coli* strain JM109. Digestion of pOAV100 with *Kpn*I released potentially infectious, linear viral DNA which had the correct 5′ nucleotides.

Twenty-four hours prior to transfection, sheep fetal lung CSL503 cells (*18*) were seeded at a density of 3.5 $\times$ 10^5 per 55-mm dish with EMEM (5 ml) (Multicel, Trace Scientific, Clayton, Victoria) containing 10% FCS. Linear DNA purified from OAV by proteinase K digestion (*2*) or cloned genomic DNA released from pOAV100 or related plasmids by *Kpn*I digestion (3–6 μg/dish) was transfected into cells using Lipofectamine (10 μl) (Life Technologies, Inc., Gaithersburg, MD) using procedures recommended by the supplier, except that serum-free EMEM used during the transfection also contained fresh glutamine (2 m*M*), HEPES (10 m*M*), penicillin (50 units/ml), and streptomycin (50 μg/ml). Following transfection the cells were incubated for 5 hr in EMEM lacking FCS. The medium was then changed and cells were incubated in EMEM plus 10% FCS until a cytopathic effect (CPE)

was visible (7–20 days). When CPE was complete, virus was harvested and a stock was grown by reinfecting fresh CSL503 cells (*8*). DNA was extracted (*8*) and characterized by restriction enzyme digestion to confirm that the recombinant had the expected genome structure.

To carry out structure/function studies on the OAV genome and to adapt it as a vector it was necessary to be able to introduce viral DNA into cells and recover it as the corresponding infectious virus. Initially, naked linear DNA was prepared from purified virus as described (*2*) and transfected into CSL503 cells. Wild-type virus was recovered from the transfected cells, confirming that the viral genomic DNA was infectious. Thus, our strategy to modify the OAV genome was to construct a plasmid from which a linear copy of the genome with the authentic 5′ nucleotides could be derived, and then to show that it was infectious. This was achieved in two steps. First, pOAV287Cm was constructed (Fig. 1). In this plasmid a prokaryotic origin of replication and a gene encoding Cm^R (contained in pACYC184) were cloned into the unique *Sal*I site of the genome and the ITRs were linked back to back by a GTAC linker which, together with the 3′ and 5′ nucleotides of the genome, created a unique *Kpn*I site. Nucleotide sequencing of the two ITR-containing fragments showed that these sequences remained intact after replication of the plasmid in *E. coli,* even though ITR sequences linked directly in other Ad plasmids initially underwent rearrangements in prokaryotes (*6*). As it was anticipated that a substitution in the *Sal*I site would disrupt the reading frame(s) in the genome, pACYC184 sequences were later excised and a modified Bluescribe plasmid (Amp^R) was cloned into the *Kpn*I site, forming pOAV100 (Fig. 1). This plasmid differed from the wild-type genome by one silent point mutation

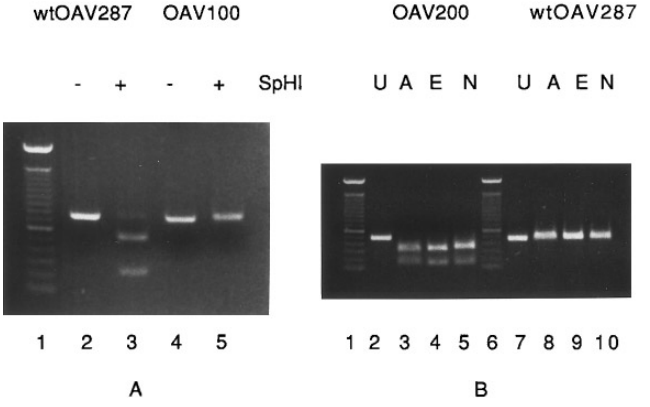

FIG. 2. Analysis of rescued viral genomes. Portions of the OAV287 and modified OAV100 and OAV200 genomes were amplified by PCR using primer pairs flanking (A) the *Sph*I site or (B) the site I insertion point between the pVIII and the fiber genes. A portion of each PCR product was left undigested (lanes A2, A4, B2, and B7) or was digested with the appropriate restriction enzyme as indicated (lanes A3, A5, B3–5, and B8–10). U, undigested; A, *Apa*I; E, *Eco*RV; N, *Not*I. Lanes A1, B1, and B6 contain marker DNA fragments.

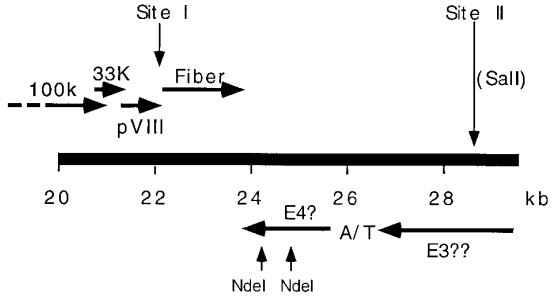

FIG. 3. Organization of the OAV genome according to (*23*) showing the location of site I and II modifications and insertions.

in the terminal protein gene which removed the *Sph*I site at base 8287. pOAV100 was digested with *Kpn*I and the linear DNA was transfected into CSL503 cells. A virus was rescued and its genomic DNA subjected to restriction analysis. This confirmed that its overall structure was correct. Portions of this genome, which contained the *Sph*I site mutation, and wild-type genomic DNA were then amplified by PCR and digested with *Sph*I. As the wild-type, but not the OAV100, DNA fragment was cut by *Sph*I (Fig. 2A, lanes 3 vs 5), this showed that a modified genome had been rescued (as distinct from contamination by a wild-type virus) and confirmed that the assembled genomic clone was infectious.

To determine whether there were any nonessential regions in the genome (such as the E3 region in other Ads) pOAV100 was mutated and virus rescue was attempted. Sequences in the two groups of reading frames near the right-hand end of the genome (Fig. 3) were targeted. The *Bam*HI A fragment of the genome was subcloned into a plasmid so that sequences between the *Nde*I sites at nucleotides 24,048 and 24,887 could be excised. Deletion of this 739-bp fragment disrupted three potential reading frames including a 30.8-kDa ORF which showed homology to the E4 34-kDa protein of other Ads (*23*). Subsequently, the modified *Sph*I/*Sal*I fragment lacking these sequences was subcloned into *Sph*I/*Sal*I-cut pOAV100. In addition, the unique *Sal*I site at the right-hand end of the genome (Fig. 3) was destroyed by end-filling and ligation, disrupting open reading frames of 185 (22.3 kDa, Ref. *23*) and 62 residues. Genomic plasmids lacking the *Nde*I fragment or the *Sal*I site were transfected to determine whether viruses could be recovered. Despite several attempts, no virus lacking the *Nde*I fragment was rescued, suggesting those sequences within the proposed E4 region (*23*) were not dispensable for replication *in vitro.* However, a virus lacking the *Sal*I site was rescued. Digestion with *Bam*HI and *Sal*I produced fragments of 8.7, 6.7, 5.2, 4.3, 3.5, and 1.1 kb, which reflected the expected genome structure. Thus, at least one significant open reading frame at the right-hand end of the genome was dispensable for replication *in vitro* and could be used for insertion of foreign DNA.

As the intergenic region between the OAV pVIII and

fiber genes was 197 bp and the location of RNA processing signals was known (*22*), we investigated whether a small insertion could be introduced into this region. A 20-bp sequence incorporating sites for the restriction enzymes *Apa*I, *Eco*RV, and *Not*I was inserted 5′ to base 26,645 by mutagenesis. The appropriate virus was rescued following transfection, as shown by PCR amplification and digestion of the relevant portion of the genome (Fig. 2B, lanes 3–5). Thus, a second site for gene insertion was identified.

The unusual genome structure of OAV 287 (*23*) raised numerous questions as to the role of certain regions in replication and confounded the task of adapting the virus as a vector based on comparisons with other Ads. Thus, we sought to test directly whether some sites in the genome could be modified. This was achieved previously for other Ads by ligating together (modified) DNA fragments which reconstituted the genome prior to transfection or by transfecting noninfectious, overlapping fragments of the genome which subsequently underwent recombination in the cell to produce infectious particles (reviewed in *8*). However, these methods necessitated plaque purification of recombinant viruses, as parental viruses were often obtained. Creation of a plasmid carrying a genome that was too large to package further improved the process of recombinant virus production by allowing the use of plasmids that could recombine in the transfected cell (*15*). In the present work, the introduction of purified plasmid-derived genomes into cells eliminated the problem of contaminating parental virus. In this and other work in progress, we have observed faithful recovery of the expected virus without rearrangement of the genome.

Plasmids such as pOAV287Cm also propagated stably in *E. coli* without rearrangement of the 46-nucleotide-long ITR sequences. This is significant as it is known that perfect palindromes longer than 30 bp are often unstable in *E. coli* (*13*). Infectious Ad5 plasmids such as pFG140 that were similar in structure to pOAV287Cm had rearranged ITRs after propagation in *E. coli,* although these subsequently replicated stably (*6*). There has only been one other report where a plasmid containing a copy of the Ad2 genome was used to rescue virus after transfection. In this case release of the genome by prior digestion was necessary (*9*). We are investigating whether *Kpn*I digestion is always necessary for virus rescue from OAV plasmids.

Mutagenesis of plasmid pOAV100 has identified two sites, I and II (Fig. 3), at which foreign DNA might be inserted. Site I is located at the point where the E3 region exists in other Ads. Site II is the *Sal*I site near the right-hand end of the genome. A significant reading frame which is disrupted by modification of this site is clearly nonessential for replication *in vitro.* However, neither this reading frame nor others nearby show detectable homology to any E3 sequences in other Ads (*23*). Their function in replication is therefore unclear.

In conclusion, the work described here lays the foundation for the development of this virus as a vector and for further structure and function studies on this unusual viral genome.

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