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Protein IX, a minor component of the human adenovirus capsid, is essential for the packaging of full length genomes

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Human adenovirus type 5 (Ad5) contains a 36-kb double-stranded DNA molecule in an icosahedral capsid. Attempts to construct Ad5 insertion mutants containing DNA of more than about 105% of the genome size resulted in viral progeny in which deletions had occurred suggesting the existence of severe constraints on the size of packageable DNA molecules. To partially circumvent these constraints we used an adenovirus vector, Ad5dlE1,3, with deletions in early regions 1 (E1) and 3 for a total net reduction in genome size of 5349 bp and an expected capacity for inserts of >7 kb. To use this vector efficiently we generated a circular form of dlE1,3 DNA which could be propagated as an infectious bacterial plasmid. When this plasmid was used as a recipient for inserts of various sizes it was found that its capacity was much less than expected and that dlE1,3 virion capsids could not even package DNA as large as the wt genome. Because the E1 deletion of dlE1,3 extends into the coding sequences for protein IX, a minor capsid component known to affect the heat stability of adenovirions, the possibility that absence of this polypeptide might also affect the DNA capacity of the virion was investigated. It was found that when the coding sequences for protein IX were restored the packaging capacity of the vector was also restored to that of wt virions. Thus protein IX is an essential constituent of virion capsids dispensable only for virions containing DNA of less than genomic size.
Key words: adenovirus/cloning vectors/packaging constraints/protein IX

Introduction

Human adenoviruses contain a linear double-stranded 36-kb DNA molecule packaged in an icosahedral protein shell. For some time circumstantial evidence has existed suggesting that, like most viruses with this type of capsid architecture, the wild-type adenovirus genome size is very close to the maximum amount of DNA which can be contained within the capsid. For example a variety of SV40-adenovirus hybrid viruses exist in which recombination between the two genomes has resulted in the insertion of part of the SV40 genome into the adenovirus DNA molecule (see reviews by Grodzicker, 1980; Klessig, 1984). In every case a presumably compensatory deletion of adenovirus sequences has occurred such that the final size of the hybrid genome is less than that of the wt adenovirus parent or is increased by not more than ~ 1000 bp. In contrast a variety of defective viral mutants exist with genome sizes ranging in a virtual continuum from nearly empty capsids to genomes of wt length suggesting that there is no lower limit to the amount of DNA which can be stably packaged in virions.

Because of the potential utility of adenoviruses as eukaryotic cloning vectors the exact capacity of the virion for inserts of foreign DNA was of some practical interest. We therefore set out firstly to define this limit by rescuing into the Ad5 genome a series of insertion mutations of various sizes and secondly to construct vectors with increased capacity. In the course of this work we discovered that protein IX, a minor constituent of the virion previously thought to be dispensable, was in fact essential for packaging of full-length viral DNA molecules. At the same time we have developed a methodology with the potential to facilitate the rescue of mutations made in cloned viral sequences anywhere into the viral genome.

Results

Rescue of E1A insertion mutants

To systematically examine the capacity of Ad5 for inserts of foreign DNA we constructed a series of insertion mutations at a single locus in E1A sequences cloned in the bacterial plasmid, pBR322 (Bolivar *et al.*, 1977). We then rescued these into infectious virus by the method of Stow (1981). The mutants were all constructed from a single plasmid, pHE1, containing the left 4.5% of Ad5 and either insertions of sequences derived from the bacterial transposable element, Tn5 (McKinnon *et al.*, 1982), or an insert consisting of the herpes simplex I thymidine kinase gene flanked by DNA from the inverted repeat sequences of Tn5. Insert sizes ranged from 650 bp to 3.3 kb (Figure 1A). Figure 1B illustrates the general procedure used to rescue these mutations into infectious virus. Briefly, Ad5 dl309 DNA (Jones and Shenk, 1979) was digested with *Cla*I and *Xba*I, which cut at 2.6% (917 bp) and 3.7% (1339 bp) respectively from the left end of the genome, and ligated to *Xba*I-digested pHEa,b,c or d DNA. Ligated DNA was then used to transfect 293 cells and the resulting plaques were isolated, expanded, and viral DNA was restricted with a variety of enzymes and analyzed on agarose gels to identify and characterize resulting insertion mutants. An example of such an analysis for an experiment in which we rescued the pHEa mutant is shown in Figure 2. In this case the 650-bp insert contained a diagnostic *Xho*I restriction site resulting in a characteristic pattern for insertion mutants compared with parental dl309 viral DNA (reduction of the *Xho*I C fragment to C′ co-migrating with *Xho*I D, and generation of a new fragment F′ migrating just ahead of F). *Xho*I analysis (Figure 2) as well as analyses with additional enzymes indicated that nine out of nine mutants analyzed had the predicted structure and that no rearrangements had occurred during ligation or transfection. This was not the case when attempts were made to rescue larger insertions. Figure 3 shows the results of *Hin*dIII analysis of a series of mutants derived from plasmids pHEa (lane 2-5), pHEc (lanes 6-9), pHEb (lanes 10-13), and pHEd (lanes 14-19). Plaque isolates obtained from rescue of the pHEa insertion all contained a left end *Hin*dIII fragment (closed arrow) which was increased

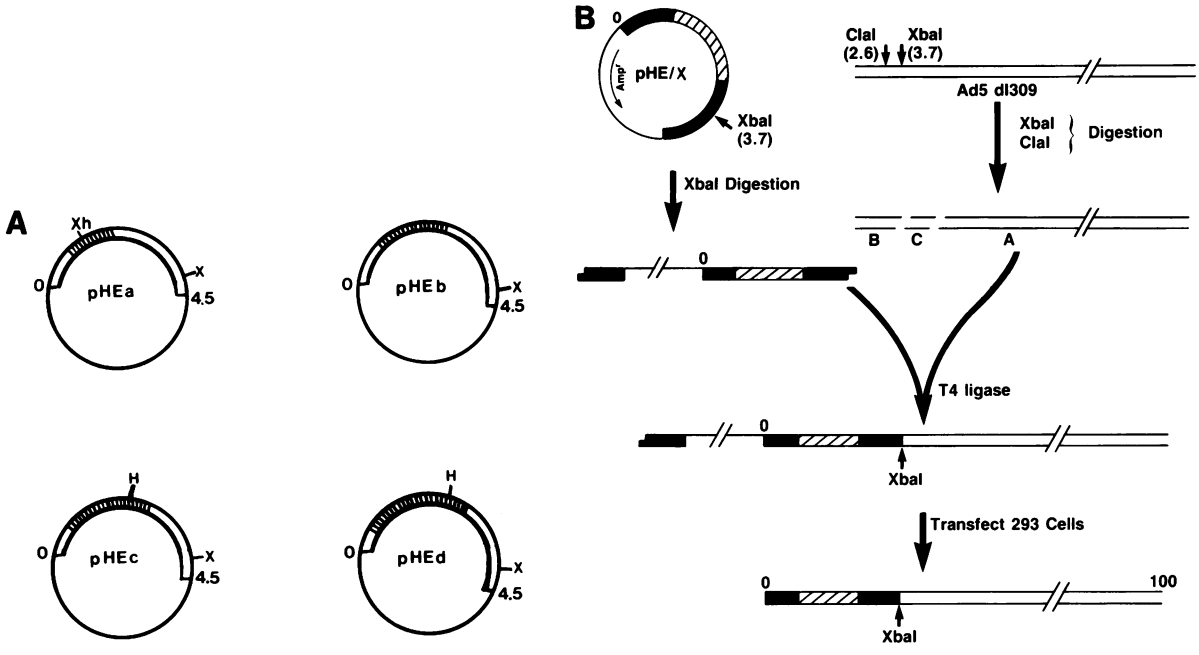

Fig. 1. (**A**) Schematic diagrams of E1A plasmids used to rescue insertion mutations. All plasmids are derived from pHE1 (McKinnon *et al.*, 1982) which contains the leftmost *Hpa*I fragment (4.5%) of the Ad5 genome cloned in pBR322. The open bar represents Ad5 sequences, the hatched bars represent various insertions: 650 bp, 3.1 or 3.3 kb of Tn5 DNA in pHEa, pHEc and pHEd, respectively, and 2.4 kb of DNA containing the herpes simplex 1 thymidine kinase gene in pHEb. Restriction enzyme sites H, X, and Xh indicate recognition sites of *Hin*dIII, *Xba*I and *Xho*I, respectively. (**B**) Protocol for rescue of E1A mutants after Stow (1981). Solid bars represent cloned Ad5 sequences, hatched bars represent insertions. PHEa,b,c or d DNA was cut with *Xba*I and ligated with Ad5dl309 DNA which had been digested with *Xba*I and *Cla*I (the latter enzyme used to reduce the frequency of regeneration of dl309). Ligated DNA was used to transfect 293 cells and plaque isolates were expanded and analyzed by restriction and agarose gel electrophoresis.

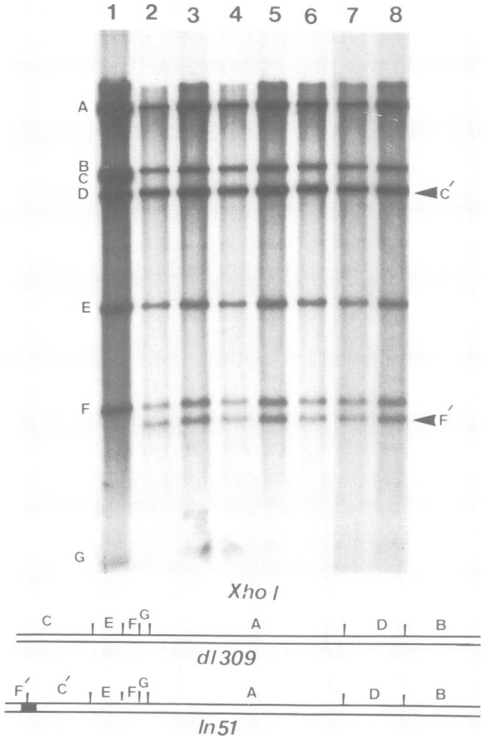

Fig. 2. *Xho*I restriction enzyme analysis of seven insertion mutants rescued using plasmid pHEa (650-bp insert). Viral DNA was labeled with ^{32}P, purified, digested with *Xho*I, and electrophoresed on 1% agarose gel. **Lane 1** contains marker dl309 DNA, and **lanes 2–8** contain DNA from seven different mutant isolates. The *Xho*I restriction map for dl309 and the predicted map for insertion mutants are shown below the autoradiogram. Because the insert contained an *Xho*I site, DNA of mutants containing the insert has lost the *Xho*I C fragment and contains two new fragments, C′ and F′ indicated on the gel by arrows.

in size by ~650 bp whereas isolates obtained following attempts to rescue larger insertions (2.4–3.3 kb) were heterogeneous. No matter which mutant was used in the ligation and transfection the largest net insert which was obtained was ~1.7 kb. The results of a number of experiments in which viral DNA was analyzed with at least two restriction enzymes are summarized in Table I.

It is not clear at what stage rearrangements occur, whether during the initial cleavage and ligation reactions or after transfection during the initial rounds of replication in the host cell. In either case the results in Figure 3 and Table I suggest that there is a strong selection for variants which contain net insertions of < ~2 kb. Once established, however, these insertion mutants seemed to be quite stable: when a mutant with an insertion of 1.7 kb was passaged five times successively in 293 cells and replaqued, 24 out of 24 plaque isolates analyzed had a structure which was identical to that of the parental mutant (unpublished observations). In other studies we have succeeded in constructing a stable insertion mutant, Ad5in52, with 2.2 kb of inserted DNA (Graham, 1984) but we are not aware of any larger insertions which have been introduced into the Ad5 genome in the absence of compensatory deletions.

Viral and plasmid cloning vectors with deletions of viral DNA

To increase the cloning capacity of Ad5 and thereby extend its utility as a vector, we developed a mutant with extensive deletions in early region 1 (E1) and E3 (Haj-Ahmad and Graham, 1986). This vector has a deletion of 3470 bp from 354 to 3824 bp removing all of the E1 coding sequences and the N-terminal half of the protein IX gene. The E3 deletion (78.5–84.7%) removes 1880 bp for a total deletion of 5350 bp. E3 appears to be non-essential for adenovirus replication in cultured cells, and E1 deletion mutants can be propagated in 293 cells, a human cell line containing and expressing E1 (Graham *et al.*, 1977). The absence

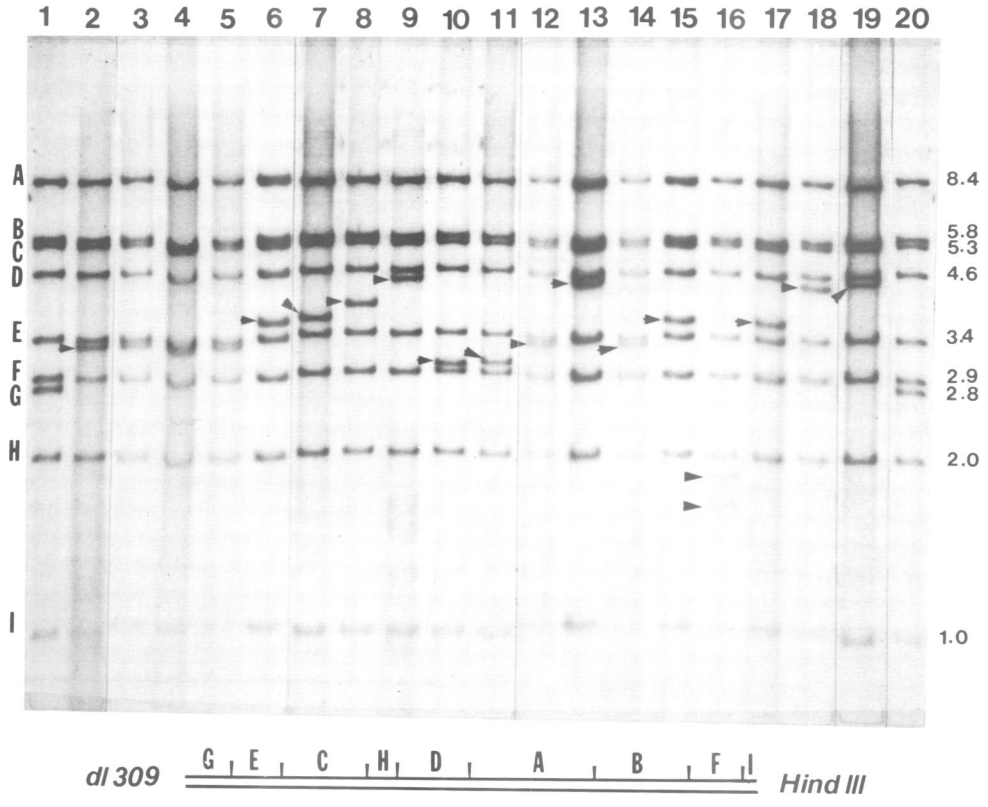

Fig. 3. *Hin*dIII restriction enzyme analysis of 18 insertion mutants rescued using plasmids pHEa (**lanes 2–5**), pHEb (**lanes 6–9**), pHEc (**lanes 10–13**) or pHEd (**lanes 14–19**). **Lanes 1** and **20** contain *Hin*dIII-digested dl309 DNA. The *Hin*dIII restriction map for dl309 DNA is shown below the autoradiogram. Novel terminal fragments are indicated by arrows.

Table I. Summary of Results from E1A Mutant Rescue

Plasmid	Expected insert size (in kb)	No. of plaque isolates analyzed	Observed insert size (in kb)	No. of mutants in each size range[a]
pHEa	0.65	12	0.65	9
pHEb	2.4	14	−0.3	1
			0.4	1
			0.6	1
			0.7	1
			1.2	3
			1.3	1
			1.6	1
pHEc	3.1	11	−0.2	1
			0.3	1
			0.7	1
			1.2	2
			1.6	1
			1.7	2
pHEd	3.3	12	−0.2	1
			0.6	2
			1.0	1
			1.3	2
			1.4	1
			1.7	1

The indicated plasmids were rescued as described in Figure 1B. Plaques were picked at 7–8 days post transfection, expanded, and progeny viral DNA was analyzed on agarose gels for the presence and structure of inserts. The restriction enzyme patterns of some mutants are shown in Figures 2 and 3. A negative sign indicates a net deletion.
[a]The difference between the sum of numbers in the last column and in the 3rd column corresponds to the number of wt isolates.

of protein IX, which is not expressed in 293 cells, renders dlE1,3 virions more heat labile than wt virions (Haj-Ahmad and Graham, 1986), an observation which has been made previously (Colby and Shenk, 1981). Other than this, dlE1,3 appeared to replicate well in 293 cells and was therefore considered to be a useful vector with a theoretical capacity for inserts 7–7.5 kb in size.

To simplify manipulations of the dlE1,3 genome and facilitate the rapid cloning of DNA inserts, we used methods described previously (Ruben *et al.*, 1983; Graham, 1984) to convert the viral genome to a series of bacterial plasmids able to regenerate infectious virus following transfection. The development and use of the resulting plasmids has been described in detail elsewhere (Ghosh-Choudhury *et al.*, 1986). Figure 4 illustrates one such plasmid and outlines the kinds of procedures used to introduce insertions. The plasmid pGGC145 represents a circularized dlE1,3 genome with ends joined at the position indicated by 'J', and with the left-to-right direction of the viral genome oriented clockwise. pPB3 is a 4.3-kb bacterial plasmid which encodes neomycin (kanamycin) resistance and confers on the circular dlE1,3 molecule the ability to be propagated in *Escherichia coli*. As described in Figure 4 and elsewhere (Ghosh-Choudhury *et al.*, 1986) substitution of pPB3 for other plasmids linked to DNA fragments to be cloned, followed by transfection of the resulting dlE1,3 based plasmid into 293 cells, provides a simple method for introducing foreign DNA into the Ad5 genome.

When this procedure was used to insert a variety of different DNA segments, such as the gene coding for dihydrofolate reductase, as in Figure 4, it was found that many of the resulting plasmids had reduced infectivity and generated small plaques, or else were non-infectious. We found that this phenomenon cor-

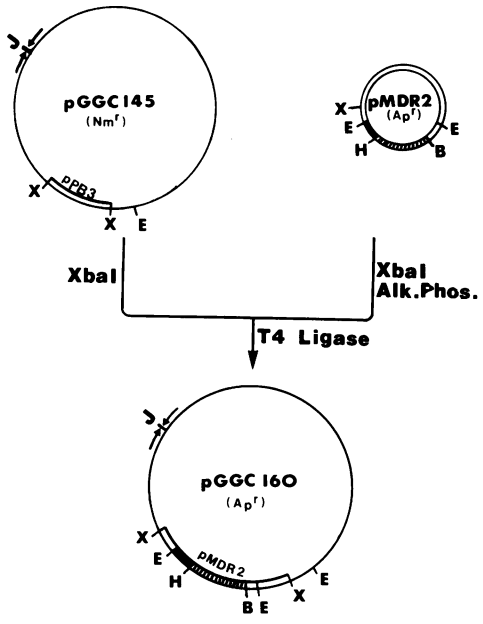

Fig. 4. Cloning foreign DNA into infectious circular molecules of Ad5 DNA. The construction and manipulation of plasmids such as pGGC145 has been described elsewhere (Ghosh-Choudhury *et al.*, 1986). It consists of circularized Ad5dlE1,3 DNA with an insert in E3 of a 4.3-kb plasmid, pPB3, encoding neomycin resistance (Nm^r). The joined ends of the viral genome are indicated by 'J' and B, E, H and X indicate *Bam*HI, *Eco*RI, *Hin*dIII and *Xba*I recognition sites. The construction of pMDR2 will be described in detail elsewhere. Briefly, this plasmid consists of a dihydrofolate reductase gene driven by the SV40 early promoter and linked to the β-lactamase gene (Ap^r) and bacterial origin of replication of pBR322. Cloning into circular viral DNA was accomplished by switching one antibiotic resistance marker for another as shown above. PGGC145 DNA was cut with *Xba*I and ligated with *Xba*I-digested, alkaline-phosphatase-treated pMDR2 DNA. Ligated DNA was used to transform competent bacteria and ampicilin-resistant colonies were screened for plasmids containing pMDR2 inserted in dlE1,3.

Table II. Rescue efficiency for dlE1,3 based plasmids carrying various sized inserts

Plasmid	Insert size (kb)	Infectivity	Approximate plaque size[a]
pFG144	2.2	+++	6 mm
pFL155	3.6	+++	6 mm
pGGC145	4.3	++	1 mm
pGGC160	5.4	+/−[b]	−
pGGC161	6.0	−	−

[a]Transfected 293 cell cultures were fixed and stained at 10 days post transfection. Plaque size is an average of 10−20 plaques.
[b]Minute areas of apparent cpe were visible in the microscope in unstained cultures at approximately 2 wks post transfection.

related with net size of the DNA insert (Table II) and that inserts > ~4 kb seemed to have a deleterious effect on infectivity. Since this was less than the amount of DNA which had been removed from the Ad5 genome in the construction of dlE1,3, and much less than the predicted cloning capacity of 7 kb, these observations were unexpected. In particular, pGGC160, with an insert of 5.4 kb, was almost totally non-infectious resulting in minute plaques after long incubations and generating viral progeny which grew much slower than wt virus (results not shown). Furthermore, when three plaque isolates obtained from infection with pGGC160 DNA were analyzed (Figure 5), it was found that all three had deletions of various sizes in and around the insert which

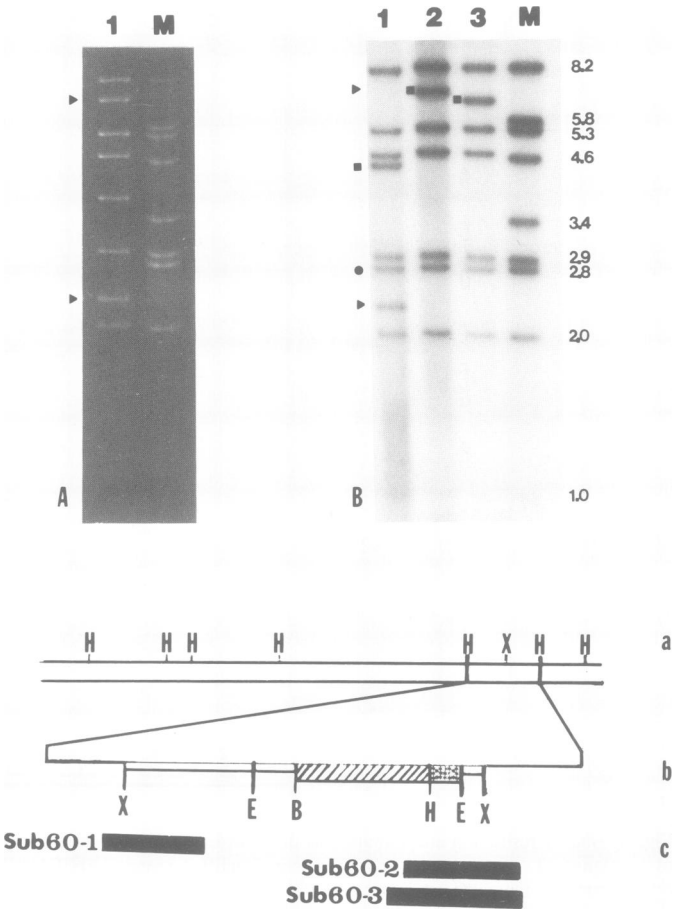

Fig. 5. Analysis of viral progeny obtained from transfections with pGGC160 DNA. The *Hin*dIII cleavage pattern of pGGC160 DNA is shown in **panel A, lane 1,** and the pattern for three independently isolated viral mutants is shown in **panel B, lanes 1, 2** and **3**, *Hin*dIII-digested Ad5 marker DNA was run in **lanes M.** Solid arrows indicate the positions of pGGC160 *Hin*dIII fragments containing pMDR2 DNA or, in the case of viral DNA, the expected positions for viral DNA fragments containing pMDR2 if no rearrangements had occurred. Solid squares indicate the actual positions of fragments containing pMDR2 sequences in viral progeny DNA. The closed circle in **panel b** indicates the position of the left terminal *Hin*dIII fragment for dlE1,3. Restriction maps for dlE1,3 (**a**) or the pMDR2 insert at the *Xba*I site (**b**) are shown below the gels. Solid bars (**c**) indicate the sequences deleted from pMDR2 and flanking viral DNA in rescued progeny as determined by restriction analysis using *Hin*dIII (H), *Bam*HI (B), *Eco*RI (E) *Xba*I (X) and combinations of those enzymes.

reduced the net amount of DNA rescued into the dlE1,3 genome to ~2.8−3.4 kb. This observation was similar to the results of studies described earlier in which we had attempted to rescue E1 insertion mutants which presumably exceeded the packaging constraints of the virion. Thus we concluded that dlE1,3 only had a capacity for inserts of foreign DNA of up to ~4.3 kb representing a total of ~35 kb or 3 kb less than the capacity of wt virions.

The importance of protein IX

The only known difference in structure between wt capsids and those of dlE1,3 was the absence of protein IX in the latter. Since it was known that protein IX affected the stability of the virion as measured by heat lability (Colby and Shenk, 1981), and since the only mutants of protein IX reported to date have been deletion mutants, it seemed possible that the reduced DNA capacity of dlE1,3 virions might be due to the absence of protein IX. To test this we carried out an experiment involving transfection of

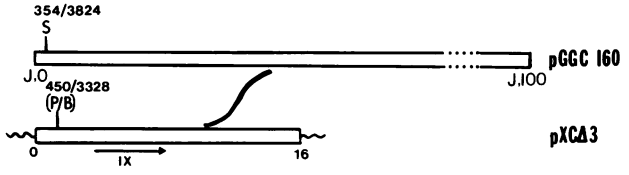

Fig. 6. A possible mechanism for rescue of protein IX coding sequence by *in vivo* recombination. Co-transfected plasmids pGGC160 and pXCΔ3 are represented linearly though in actuality the left (0) and right (100) ends of viral DNA in pGGC160 are joined as in Figure 4, and pXCΔ3 is circularized by pBR322 sequence (squiggly lines). 'S' represents the deletion of E1 sequences in dlE1,3 between *Sac*II sites at 354 and 3824 bp, and 'P/B' represents a deletion of sequences between a *Pvu*II site at 450 bp and a *Bgl*II site at 3328 bp which leaves the protein IX coding sequences (3609−4029 bp) intact in pXCΔ3. In the simplest model a single recombination, indicated by the solid line, results in an exchange of pXCΔ3 sequences for the left-end sequences of dlE1,3. It is assumed that this occurs after pGGC160 DNA has linearized and that the pBR322 sequences linked to the left end of viral DNA in pXCΔ3 are removed by a kind of repair process similar to the events which occur when mutants are rescued by the method described in Figure 1B.

Table III. Recovery of infectious virus by co-transfection[a]

Plasmid(s)	Insert size (kb)	Input (μg DNA/dish)	Plaques/dish
pFG144	2.2	5	23,35
pGGC145	4.3	5	5,3
pGGC160	5.4	10	0,0,0,0[b]
pGGC161	6.0	10	0,0[c]
pXCΔ3	–	5	0,0,0,0
pGGC160+pXCΔ3		10+5	1,2,0,2
pGGC161+pXCΔ3[d]		10+5	2,1,2,2
pGGC161+pXCΔ3		5+2.5	3,4

[a]Infectivity was measured in 60 mm Petri dishes of 293 cells transfected by the calcium phosphate coprecipitation technique. Assays of pGGC161 or pGGC161+pXCΔ3 DNA were done in a separate experiment from that used to assay the other plasmid DNA preparations. Plaques were counted at 7−8 days post transfection.
[b]This plasmid gave a few minute plaques after very prolonged incubation time (two weeks) and generated virus progeny which had undergone DNA rearrangements reducing the size of the E3 insert (see Figure 5).
[c]This plasmid appeared to be totally non-infectious in single transfection assays.
[d]This pXCΔ3 DNA preparation was digested with *Stu*I.

293 cells with a mixture of two plasmids: pGGC160 with the 5.4-kb insert which rendered the plasmid almost totally non-infectious, and pXCΔ3, a plasmid containing the left 16% of the Ad5 genome and having a deletion of E1 sequences which left the protein IX gene intact. Assuming that absence of protein IX was responsible for the replication defect of pGGC160, and provided the two plasmids could undergo a recombination such as that illustrated in Figure 6 it should be possible to rescue the protein IX coding sequences back into the dlE1,3 genome and select for viable virus. The results of several experiments, one of which is shown in Table III, indicated that viable progeny were readily obtained from such mixed transfections. Furthermore, no difference in rescue efficiency was seen whether pXCΔ3 was uncut, or restricted with *Stu*I which cleaves once in pXCΔ3 at the extreme right end of the Ad5 DNA insert. To ensure that the viral progeny obtained from mixed transfections had indeed arisen from recombination we picked and expanded three independent plaque isolates, and analysed the DNA structure of the resulting viruses. The results of analysis with *Hin*dIII are shown in Figure 7. All three viral mutants had precisely the structure expected if recombination had occurred as shown in Figure 6: the left end of the genome was derived from pXCΔ3, resulting in a left terminal *Hin*dIII fragment (closed circle) 590 bp larger than the corresponding fragment from dlE1,3 (closed circle in Figure 5), and the rest of the viral DNA was derived from pGGC160 and contained the 5.4-kb insert in E3 with no detectable deletions or rearrangements. Furthermore, virions obtained from these cotransfections contained protein IX as determined by SDS−polyacrylamide gel electrophoretic analysis of labeled, purified virions (results not shown). Similar results have been obtained with a plasmid, pGGC161, carrying an insert of 6.0 kb at the E3 cloning site and again the structure of the resulting progeny viruses was as predicted by the model in Figure 6 (Table III and data not shown).

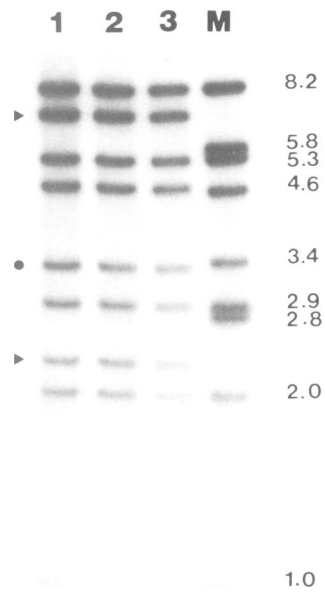

Fig. 7. Structure of virus obtained from co-transfections. **Lanes 1, 2** and **3** contain *Hin*dIII digests of DNA from progeny viruses isolated following co-transfection of 293 cells with pGGC160 and pXCΔ3 DNA. **Lane M** contains marker Ad5 DNA. Closed arrows indicate the expected (and actual) positions of fragments containing pMDR2 DNA (see Figure 5) and the closed circle indicates the position of the novel terminal fragment generated by recombination as shown in Figure 6 (cf. Figure 5).

Discussion

The earliest indications that the DNA content of the adenovirus capsid was probably rather severely limited at a maximum of not much more than the wt genome came from studies on the structure of Ad2-SV40 hybrid viruses (reviewed by Grodzicker, 1980; Klessig, 1984). The non-defective hybrids were found to contain various insertions of SV40 DNA in the Ad2 E3 region, ranging in size up to as much as 2.7 kb. However, these insertion mutants all have compensatory deletions of E3 sequences such that in no case is the net size of the adenovirus genome increased by $> \sim 1$ kb. These observations suggested that the wt genome may be very close in size to the limit of DNA molecules that can be packaged, and also indicated that the E3 region may be dispensable for virus growth in cell culture, a suggestion later verified by isolation and characterization of mutants with deletions in E3 (Challberg and Ketner, 1981; Berkner and Sharp, 1983). Of the many Ad2 and Ad5 insertion mutations which have been made, those with the largest net increase

in genome size are Ad5in307 with an insert in the E3 region of ~1.7 kb (Jones and Shenk, 1978) and Ad5in52 with an insert in E1A of 2.2 kb (Graham, 1984). The results presented here on rescue, or attempted rescue, of various E1A insertion mutations into infectious virus suggest that the 2.2-kb insertion in mutant In52 must be very close to the absolute limit since we failed in attempts to rescue a mutation bearing a 2.4-kb insert in E1A although several mutants with inserts of ~1.7 kb were isolated.

To be able to clone inserts of >2 kb into adenovirus we constructed a vector, dlE1,3, with deletions which reduced the genome size by 5350 bp and which should have increased the capacity of Ad5 to ~7.0–7.5 kb of foreign DNA (Haj Ahmad and Graham, 1986). Our previous work using dlE1,3 employed conventional methods to insert DNA segments of ~2 kb into the E3 region but attempts to insert fragments > ~4 kb had been unsuccessful (unpublished observations) for reasons we did not understand until now. The results obtained in the present study suggest that this failure was due to the fact that the E1 deletion in dlE1,3 extends into the coding sequences for protein IX.

Polypeptide IX has been shown to be associated with the hexons that constitute 'groups of nine' (GON) within the facets of the icosahedron (Maizel *et al.*, 1968; Everitt *et al.*, 1973; Boulanger *et al.*, 1979). The stoichiometric relationship of the Ad2 capsid proteins has recently been determined to a high degree of accuracy and a model has been proposed for the association of protein IX with hexons (Burnett, 1984; van Oostrum and Burnett, 1985). There are 240 molecules of polypeptide IX per virion, 12 per 'GON' hexons and it has been suggested that polypeptide IX molecules are inserted as trimers within the large cavities between adjacent hexons in GONs. Other than affecting the thermal stability of virions, protein IX was previously thought to be dispensable for adenovirus replication (Colby and Shenk, 1981). The results presented here suggest that the synthesis of protein IX is essential for packaging of full length DNA. To date the only mutations of protein IX coding sequences, which have been made, have been deletion mutants such as Ad5d1313 [having a deletion of 2307 bp extending into protein IX gene (Jones and Shenk, 1979, Colby and Shenk, 1981)], or dlE1,3. From our observations we would predict that point mutations or small deletions which inactivate protein IX should be non-viable unless they are rescued into a viral background containing deletions elsewhere in the genome.

The deletion of E1 sequences in pXCΔ3 combined with the deletion of E3 sequences represents a net loss of 4760 bp of viral DNA. This should permit the insertion of 6.5–7.0 kb of foreign DNA into the genome or ~2 kb more than the maximum of 4.3 kb which we have been able to insert into dlE1,3. To date, the largest fragment we have inserted using the procedure outlined in Figure 6 was 6.0 kb. We have also inserted a 4.0-kb fragment into E3 of a viral mutant having the 1.9-kb E3 deletion but containing an intact E1 region (see below) resulting in a net increase in virion DNA of 2.1 kb.

There are several methods available for inserting foreign DNA into adenovirus vectors or for rescuing mutations constructed *in vitro*. One of the simplest, dependent on the presence of unique restriction enzyme recognition sites is the method first developed by Stow (1981) and which we used to rescue E1A insertion mutations as outlined in Figure 1B. A second approach involves cotransfection with overlapping fragments and selection for *in vivo* recombination events which generate infectious virus. A third approach involves insertion of DNA fragments between left and right arms of the viral genome by trimolecular ligation. Any of these methods will give satisfactory results under most circumstances. However, all involve the use of infectious viral DNA which must be restricted, and sometimes fractionated on gels, or treated with alkaline phosphatase to reduce the frequency of occurrence of parental virus, and all result in an unavoidable background of undesirable progeny which must be screened to obtain the desired mutant. Often this is not a serious problem, but it is clear that if the desired mutant happens to be one which replicates poorly (point mutations in protein IX would be an excellent example) then much time and effort can be expended in isolating and analyzing viral progeny which turn out to be consistently parental.

The success of the experiment outlined in Figure 6 suggests a fourth approach to rescuing mutations into the viral genome. If a plasmid such as pGGC145 were rendered completely non-infectious by introduction of a lethal insertion (or deletion) in the region which is to be mutagenized then infectious progeny could be generated by recombination with an overlapping fragment containing a mutation or insertion to be rescued into the viral genome. The advantages of this approach are that it is less dependent on the existence of convenient restriction enzyme recognition sites since the cotransfecting plasmids need not be cleaved, and requires only DNA which can be propagated easily as bacterial plasmids. In preliminary studies we have used this approach to clone foreign DNA into E3 and are currently attempting to optimize the procedure and modify it so that it can be used to rescue E1 mutations. The method should be sufficiently general to allow the rescue of mutations anywhere in the viral genome under conditions where the background of parental virus is zero.

Materials and methods

Construction of plasmids and bacterial transformation

Restriction endonucleases and other DNA modifying enzymes were purchased from Bethesda Research Laboratories or Boerhinger Mannheim Inc. and were used according to the vendor's recommendations. All plasmids were constructed by standard protocols (Maniatis *et al.*, 1982) except for plasmids containing circularized Ad5 DNA which were generated according to methods described elsewhere (Ruben *et al.*, 1983; Graham, 1984; Ghosh-Choudhury *et al.*, 1986). Bacterial strains (LE392 and HMS174) were made competent for transformation according to Mandel and Higa (1970) or Hanahan (1983). Plasmid DNA was prepared by the alkaline lysis method of Birnboim and Doly (1979) for both small- and large-scale preparations. Large-scale preparations were further purified by CsCl density gradient centrifugation.

Generation and analysis of viral mutants

Ad5dl309 (Jones and Shenk, 1979) was grown in KB cells and titrated on 293 cell monolayers (Graham *et al.*, 1977). All mutant viruses were propagated in 293 cells growing in monolayer, or in suspension culture (Graham, 1987). Transfections with viral or plasmid DNA were carried out by the method of Graham and van der Eb (1973), and plaques were isolated, expanded and analyzed as described previously (Haj Ahmad and Graham, 1986).

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References

Berkner,K.L. and Sharp,P.A. (1983) *Nucleic Acids Res.*, **11**, 6003–6020.
Birnboim,H.C. and Doly,J. (1979) *Nucleic Acids Res.*, **7**, 1513–1523.
Bolivar,F., Rodriquez,R.L., Green,P.J., Betlach,M.C., Heyneker,H.L., Boyer,H.W., Crosa,J.H. and Falkow,S. (1977) *Gene*, **2**, 95–113.
Boulanger,P., Lemay,P., Blair,G.E. and Russell,W.C. (1979) *J. Gen. Virol.*, **44**, 783–800.
Burnett,R.M. (1984) In McPherson,A. and Jurnak,F.A. (eds), *Biological Macromolecules and Assemblies*, Vol. 1. *Virus Structures*. John Wiley and Sons, New York, pp. 337–385.
Challberg,S.S. and Ketner,G. (1981) *Virology*, **114**, 196–209.
Colby,W.W. and Shenk,T. (1981) *J. Virol.*, **39**, 977–980.

Everitt,E., Sundquist,B., Petterson,U. and Philipson,L. (1973) *Virology*, **52**, 130–147.
Ghosh-Choudhury,G., Haj Ahmad,Y., Brinkley,P., Rudy,J. and Graham,F.L. (1986) *Gene*, **50**, 161–171.
Graham,F.L. (1984) *EMBO J.*, **3**, 2917–2922.
Graham,F.L. (1987) *J. Gen. Virol.*, **68**, 937–940.
Graham,F.L. and van der Eb,A.J. (1973) *Virology*, **52**, 456–467.
Graham,F.L., Smiley,J., Russell,W.C. and Nairn,R. (1977) *J. Gen. Virol.*, **36**, 59–72.
Grodzicker,T. (1980) In Tooze,J. (ed.), *The Molecular Biology of Tumor Viruses*. Part 2. *DNA Tumor Viruses*. Cold Spring Harbor Laboratory Press, New York, pp. 577–614.
Haj-Ahmad,Y. and Graham,F.L. (1986) *J. Virol.*, **57**, 267–274.
Hanahan,D. (1983) *J. Mol. Biol.*, **166**, 557–580.
Jones,N. and Shenk,T. (1978) *Cell*, **13**, 181–188.
Jones,N. and Shenk,T. (1979) *Cell*, **16**, 683–689.
Klessig,D.F. (1984) In Ginsberg,H.S. (ed.), *The Adenoviruses*. Plenum Press, New York, pp. 399–449.
Maizel,J.V., Jr, White,D.O. and Scharff,M.D. (1968) *Virology*, **36**, 126–136.
Mandel,M. and Higa,A. (1970) *J. Mol. Biol.*, **53**, 453–471.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning*. Cold Spring Harbor Laboratory Press, New York.
McKinnon,R.D., Bacchetti,S. and Graham,F.L. (1982) *Gene*, **19**, 33–42.
Ruben,M., Bacchetti,S. and Graham,F.L. (1983) *Nature*, **301**, 172–174.
Stow,N.D. (1981) *J. Virol.*, **37**, 171–180.
van Oostrum,J. and Burnett,R.M. (1985) *J. Virol.*, **56**, 439–448.

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