The EMBO Journal vol.3 no.12 pp.2917-2922, 1984

Covalently closed circles of human adenovirus DNA are infectious

F.L.Graham

Departments of Biology and Pathology, McMaster University, Hamilton, Ontario, L8S 4K1 Canada

Communicated by T.Lindahl

Replication of the linear adenovirus DNA molecule is thought to result from semiconservative synthesis off linear templates, starting from origins at either end of the genome. Recently, however, it has been shown that in cells infected with adenovirus type 5 (Ad5) a significant fraction of the ends of viral DNA molecules become joined head-to-tail due at least in part to the formation of covalently closed circles. Circular DNA is not present in virions but joining of the ends of viral DNA is detectable shortly after infection, well before the onset of viral DNA replication. To learn more about the structure and possible function of these circular forms of viral DNA, I have cloned Ad5 circles as plasmids replicating in *Escherichia coli*. Two plasmids have been analyzed in detail and shown to generate infectious virus with an efficiency comparable with that of virion DNA following transfection into human cells. These results suggest that circles are not totally inert or functionless but that, once formed, they are capable of re-entering the pool of replicating molecules to generate linear progeny.

Key words: adenovirus/DNA replication/cloning/infectious plasmids

Introduction

The genome of human adenoviruses is a linear, double-stranded DNA molecule ~36 kb in size (reviewed in Tooze, 1980). The ends of adenovirus DNA molecules are characterized by inverted terminal repeats (ITRs) of ~100 bp, and a terminal protein covalently linked to the 5′ end of each strand. Both these features are thought to play a role in viral DNA replication: the terminal protein, in the form of an 80 000-87 000 dalton precursor, becomes covalently bound to dCMP by a phosphodiester linkage and the deoxycytidine acts as a primer and first nucleotide in *de novo* synthesis which proceeds the length of the molecule and results in displacement of one strand (for reviews, see Winnacker, 1978; Challberg and Kelly, 1982; Stillman, 1983). It has been suggested that duplication of the displaced strand might follow hybridization of the ITRs to generate panhandle structures with ends similar to those of duplex DNA (Tooze, 1980). Thus, it is generally believed that adenovirus DNA replication occurs off linear DNA molecules, a model which is consistent with data obtained from both *in vitro* and *in vivo* studies.

Recently, however, it has been shown that a significant fraction of the ends of adenovirus DNA molecules become joined head-to-tail in cells infected with adenovirus type 5 (Ad5) and that at least part of this head-to-tail joining is due to the formation of covalently closed circles (Ruben *et al.*, 1983). Circular DNA was never found in DNA preparations purified from virions but head-to-tail joining of viral DNA molecules was detected as early as 3 h after infection, well before initiation of viral DNA synthesis. These observations raise obvious questions. For example, what is the precise structure of the joint, what role, if any, do circles have in viral replication and finally might circular intermediates be involved in integration during cell transformation? As a first step toward answering some of these questions I have cloned Ad5 circles as plasmids replicating in *Escherichia coli*. Two plasmids containing virtually the entire Ad5 genome have been characterized in detail and in particular have been shown to generate infectious virus following transfection into human cells.

Results

The strategy for cloning adenovirus circles is described in Figure 1. The first step was the construction of a small (2.2 kb) plasmid, pMX2, containing a single *Xba*I site and encoding β-lactamase or ampicillin (Ap) resistance. This

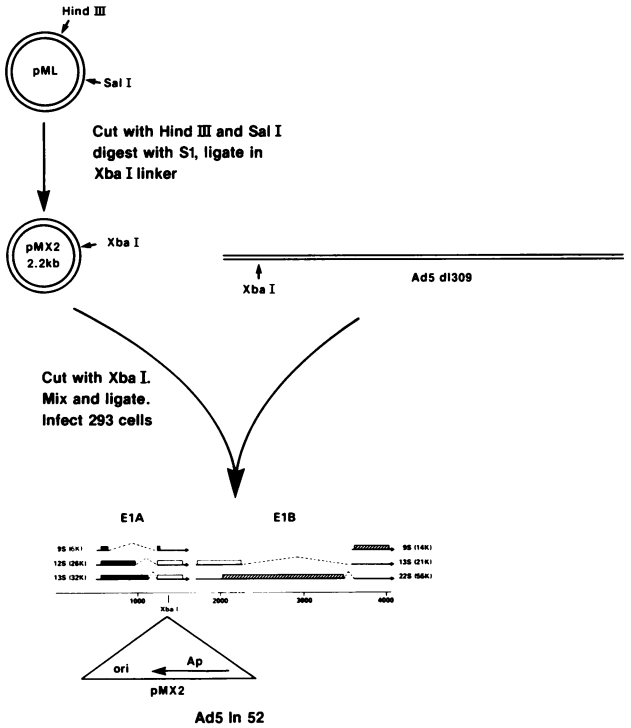

Fig. 1. Strategy for the construction of Ad5 In52 (reproduced from Graham *et al.*, 1984, with permission). Because the maximum size of DNA which can be inserted into Ad5 DNA without exceeding what presumably are packaging constraints is ~2 kb (J.Haj Ahmad and F.L.Graham, unpublished observations), it was necessary first to construct a small plasmid for insertion into Ad5. A 2.2-kb derivative of pBR322 was constructed from pML1 (Lusky and Botchan, 1981) by cleavage with *Hind*III and *Sal*I, digestion with nuclease S1, and insertion of a synthetic *Xba*I linker. The resulting plasmid, pMX2, was cut with *Xba*I, mixed in 20-fold molar excess with *Xba*I-digested DNA from Ad5 dl309 (Jones and Shenk, 1978) and ligated. Infection of 293 cells resulted in 33 plaques of which four contained virus having the desired structure.

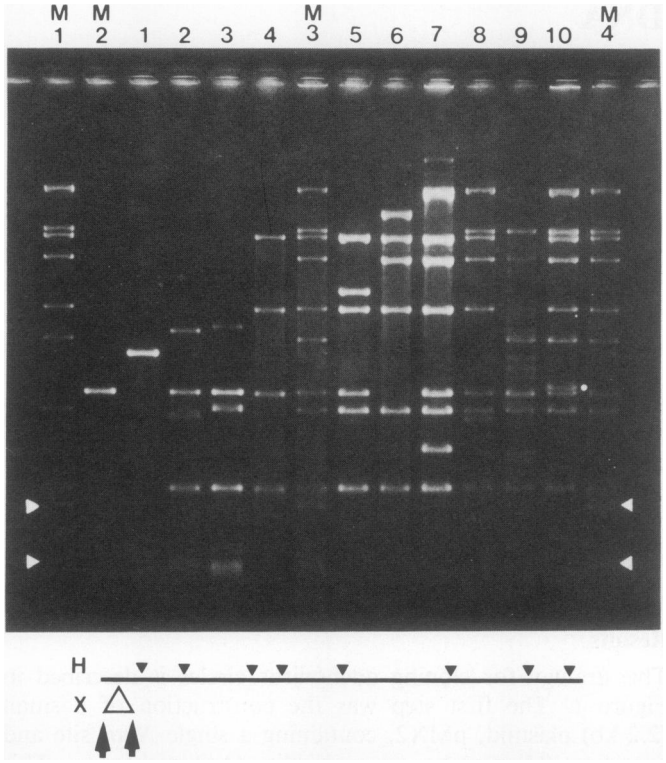

Fig. 2. Structural analysis of In52-derived plasmids. Plasmid DNA prepared according to Birnboim and Doly (1979) was digested with *Hind*III (▼) and *Xba*I (▲) which cut at sites indicated in the map shown below the gel. The insertion of pMX2 in In52 is represented by the triangle. Restricted DNA was electrophoresed through 1.2% agarose. Channels labelled M represent marker DNAs: dl309 DNA in **lane M1**, pMX2 in **lane M2**; and In52 DNA in **lanes M3** and **M4. Lanes 1–10** contain plasmids obtained following transformation of *E. coli* with DNA extracted from In52-infected rat cells. The plasmids are arranged approximately in order of increasing size with pFG140 in **lane 10**. The left and right terminal fragments of In52 DNA are indicated by arrows and in **lane 10** the dot indicates the fragment in pFG140 which has been generated by joining of the ends of In52.

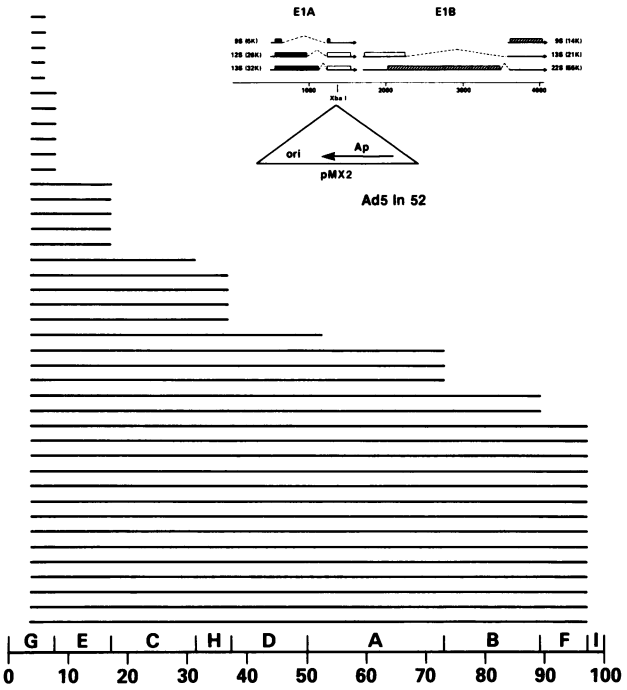

Fig. 3. Schematic representation of the structures of Ad5 In52-derived plasmids. The horizontal lines in the diagram, and the *Hind*III cleavage map below, serve to indicate which internal Ad5 *Hind*III fragments were contained within the plasmids. In addition the plasmids contain sequences from the ends of viral DNA whose structure and extent has not been determined precisely except for two of the plasmids as described in the text and in Figure 4.

plasmid was inserted into the single *Xba*I site at 3.8% in Ad5 d1309 and the resulting insertion mutant, Ad5 In52, was used to infect rat cells under conditions known to result in efficient head-to-tail joining and presumably circle formation (Ruben *et al.*, 1983). Finally, to obtain plasmids containing Ad5 sequences, DNA extracted from In52-infected cells was used to transform *E. coli* strain HB101 or HMS174 to ampicillin resistance. Forty one clones were obtained from two separate experiments and plasmid DNA from the resulting cells was analyzed by restriction endonuclease cleavage and agarose gel electrophoresis. Figure 2 shows the results of analysis of 10 representative plasmids. DNA was digested with *Hind*III and *Xba*I which cut as indicated in the Ad5 map shown beneath the gel. It can be seen that the structures of plasmids containing Ad5 DNA varied considerably from one isolate to another. Most of the plasmids contained a fragment which is recognizable as pMX2 and many contained extensive regions of Ad5 DNA as well, in some cases virtually the entire genome. In particular, pFG140 (channel 10) contained all the internal fragments of In52 plus a fragment representing the junction of the left and right termini of viral DNA.

The interpretation of the patterns obtained from analyses such as that in Figure 2 is shown in Figure 3 which illustrates schematically the structures of 41 clones isolated and analyzed to date. The maps have been determined only approximately by *Hind*III cleavage or by digestion with *Hind*III + *Xba*I and are only meant to indicate the content of internal *Hind*III fragments. The plasmids are represented as linear structures but of course the right and left ends are joined. A few plasmids, such as those run in lanes 8 and 10 of Figure 2 and others not shown, had all of the internal Ad5 DNA fragments. However, further analysis with additional enzymes has shown that of these only two plasmids, pFG140 and pFG141, had no detectable deletions of viral DNA (detection limit ~20–30 bp) in the region of the junction. These two plasmids were chosen for further study and are discussed below.

The distribution of plasmid DNA structures shown in Figures 2 and 3 probably does not reflect pre-existing viral DNA structures in adeno-infected cells but rather results from rearrangements occurring during or subsequent to transformation of *E. coli*. Firstly, if the head-to-tail junctions of Ad5 DNA in virus-infected cells were rearranged as haphazardly as would be required to generate the distribution shown in Figure 3, it would have been impossible to detect head-to-tail joining and circularization of Ad5 DNA by the methods used successfully by Ruben *et al.* (1983). Secondly, there is ample evidence that palindromes of the sort which would be generated by joining of the ITRs of adenovirus DNA are not compatible with replication of plasmids in most strains of *E. coli* (Lilly, 1981; Leach and Stahl, 1983). Thus deletions or rearrangements within the joint would be required to obtain stable plasmids and this, combined with selection for the sequences containing β-lactamase and an origin of plasmid replication, has almost certainly generated the structures shown in Figure 3. It was thus somewhat surprising that two

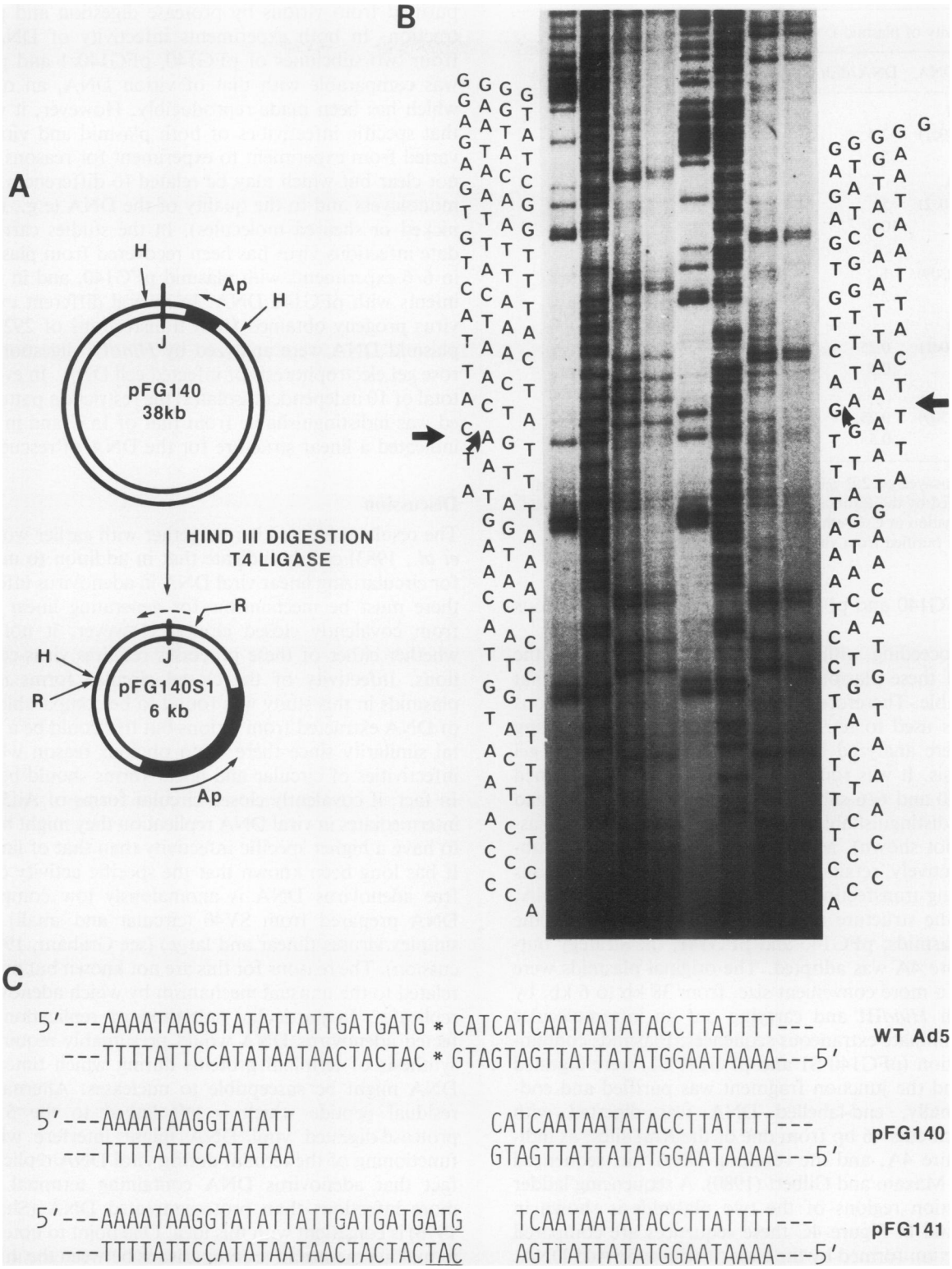

Fig. 4. Sequence analysis across viral joints in cloned circles of Ad5 DNA. The relatively large (38 kb) plasmids, pFG140 and 141, were first reduced to a more manageable size by digesting plasmid DNA with *Hind*III and ligating with T4 DNA ligase as indicated in **A**. (Only relevant *Hind*III sites in pFG140 or relevant *Rsa*I sites in pFG140S1 are shown and for clarity neither plasmids nor restriction sites are drawn strictly to scale.) This step generated plasmids in which the *Hind*III I fragment from the right end of the Ad5 genome and the *Hind*III G fragment from the left end are joined at two sites, one of which represents the junction (J) of the viral DNA termini (indicated by the vertical bar at 12 o'clock) and the other the *Hind*III site (H). The resulting plasmids were then digested with *Rsa*I (R) which cuts at 194 bp from the left end of wild-type Ad5 DNA and at a site 46 bp in the counterclockwise direction from the *Hind*III site in pFG140S1. *Rsa*I-digested DNA was treated with alkaline phosphatase, electrophoresed through 1.2% agarose and fragments containing viral termini were purified off agarose gels and end-labelled with ^{32}P using T4 polynucleotide kinase. After a limit digest with *Hind*III the end-labelled DNA was sequenced by the Maxam and Gilbert procedure (1980). The dashed arrow above pFG140S1 indicates the direction of sequencing across the junction. **B** shows part of a sequence ladder spanning the junctions of pFG140 on the right and pFG141 on the left. Points at which the sequences diverge from the sequence of linear wild-type Ad5 DNA are indicated by the arrows. In **C** the sequences for the junction regions of plasmids pFG140 and pFG141 are compared with the junction which would be formed by perfect blunt end joining of the termini of wt Ad5 DNA.

Table I. Infectivity of plasmid DNA

Expt.	Input DNA	DNA/dish (μg)	Plaques/dish	Plaques/μg DNA
1	plasmid (pFG140c1)	2.5	10,6	3
		5	26,33	6
	plasmid (pFG140c2)	5	10,17	3
		10	10,15	1.3
	virion (Ad5 dl309)	1	0,2	1
		2	8,6	3.5
2	plasmid (pFG140c1)	0.25	3,2	10
		0.5	7,11	18
	virion (Ad5 dl309)	0.25	9,5	28
		0.5	26,38	64

Infectivity was assayed on 203 cells using the calcium technique. Plasmid DNA was purified by the Birnboim and Doly method followed by buoyant density centrifugation in CsCl-ethidium bromide gradients; virion DNA (Ad5 dl309) was purified from virions by pronase-digestion and phenol extraction.

plasmids, pFG140 and pFG141, were nonetheless infectious in 293 cells.

Before proceeding with a more detailed analysis of the properties of these plasmids it was important to show that they were stable. Therefore DNA from each of the infectious plasmids was used to retransform *E. coli* and the resulting subclones were analyzed by cleavage with *Hind*III and gel electrophoresis. It was found that 28/28 subclones obtained from pFG140 and 6/6 subclones derived from pFG141 had structures indistinguishable from that of the parental plasmids (data not shown). More important, 7/7 and 2/2 subclones, respectively, retained the ability to generate infectious virus following transfection of 293 cells with plasmid DNA. To analyze the structure of the head-to-tail junction in the infectious plasmids, pFG140 and pFG141, the strategy outlined in Figure 4A was adopted. The original plasmids were cut down to a more convenient size, from 38 kb to 6 kb, by cleaving with *Hind*III and carrying out an intramolecular ligation to eliminate extraneous sequences. Plasmids containing the junction (pFG140 S1 and pFG141 S1) were digested with *Rsa*I and the junction fragment was purified and end-labelled. Finally, end-labelled DNA was digested with *Hind*III which cuts 46 bp from one of the *Rsa*I sites, as indicated in Figure 4A, and the resulting DNA was sequenced according to Maxam and Gilbert (1980). A sequencing ladder for the junction regions of the two plasmids is shown in Figure 4B, and in Figure 4C these sequences are compared with the junction formed by the ends of wild-type Ad5 DNA. It would appear that plasmid pFG140 has undergone deletion of 13 bp distributed asymmetrically across the junction whereas pFG141 contains a 3-bp insert plus a deletion of 5 bp. The resulting sequences would have the potential to form step and loop structures with stems of 93 and 98 bp and loops of 7 and 8 nucleotides, respectively. These alterations to the palindrome which would be generated by perfect head-to-tail joining are evidently sufficient to stabilize the resulting plasmids without eliminating infectivity.

Table I contains the results of transfections in which the infectivity of plasmid DNA was compared with that of DNA purified from virions by protease digestion and phenol extraction. In both experiments infectivity of DNA isolated from two subclones of pFG140, pFG140c1 and pFG140c2, was comparable with that of virion DNA, an observation which has been made reproducibly. However, it was found that specific infectivities of both plasmid and virion DNAs varied from experiment to experiment for reasons which are not clear but which may be related to differences in the cell monolayers and to the quality of the DNA (e.g., number of nicked or sheared molecules). In the studies carried out to date infectious virus has been recovered from plasmid DNA in 6/6 experiments with plasmid pFG140, and in 4/6 experiments with pFG141 DNA. In several different experiments virus progeny obtained from transfections of 292 cells with plasmid DNA were analyzed by *Hind*III digestion and agarose gel electrophoresis of infected cell DNA. In every case (a total of 10 independent isolates) the restriction pattern obtained was indistinguishable from that of In52 and in particular indicated a linear structure for the DNA of rescued viruses.

Discussion

The results presented here together with earlier work (Ruben *et al.*, 1983) clearly indicate that in addition to mechanisms for circularizing linear viral DNA in adenovirus infected cells, there must be mechanisms for generating linear molecules from covalently closed circles. However, it not yet clear whether either of these processes requires virus-coded functions. Infectivity of the closed circular forms isolated as plasmids in this study was found to be comparable with that of DNA extracted from virions but this could be a coincidental similarity since there is no obvious reason why specific infectivities of circular and linear forms should be identical. In fact, if covalently closed circular forms of Ad5 DNA are intermediates in viral DNA replication they might be expected to have a higher specific infectivity than that of linear DNA. It has long been known that the specific activity of protein-free adenovirus DNA is anomalously low compared with DNA prepared from SV40 (circular and small) or herpes simplex viruses (linear and large) (see Graham, 1977 for discussion). The reasons for this are not known but probably are related to the unusual mechanism by which adenovirus DNA replicates. In particular, initiation of replication of transfected adenovirus DNA would presumably require *de novo* synthesis of terminal proteins during which time the linear DNA might be susceptible to nucleases. Alternatively, the residual peptide which is left linked to the 5′ ends of protease-digested viral DNA might interfere with proper functioning of the termini during viral DNA replication. The fact that adenovirus DNA containing terminal protein is more infectious than protease-treated DNA (Sharp *et al.*, 1976) is consistent with this idea. One point to note, however, in making quantitative comparisons between the infectivity of circular Ad5 DNA and that of linear forms, is that in all cases transfection of mammalian cells with DNA is extraordinarily inefficient, requiring $\sim 10^7 - 10^8$ DNA molecules per successful infection. Also, the junctions in both the infectious plasmids isolated to date are 'imperfect' and consequently these plasmids could have reduced biological activity compared with circles formed *in vivo*.

Recently the entire Ad5 genome has been cloned into a bacterial plasmid, pXf3, as a linear insert in which the ends of viral DNA are joined to plasmid sequences by *Eco*RI linkers (Hanahan and Gluzman, 1984). Interestingly, these plasmids

were non-infectious unless viral DNA was cleaved out of pXf3. In an earlier study (Berkner and Sharp, 1983) virus was recovered from cells infected with mixtures of plasmids containing overlapping segments of Ad5 DNA but only if at least one viral terminus was released from pBR322 DNA. Both these studies indicate that termini linked to plasmid DNA are inert. In agreement with this, most *in vitro* studies on initiation of viral DNA replication seem to support a model in which initiation involves free termini (Tamanoi and Stillman, 1982; Lally *et al.*, 1984). However, Pearson *et al.* (1983) reported that replication can initiate *in vitro* from termini embedded in plasmid DNA, a result which is consistent with the data described in this paper, suggesting that viral DNA termini joined to each other, even if somewhat imperfectly, are capable of serving as origins of replication in infected cells. Some of the discrepancies between various *in vivo* and *in vitro* observations might be reconciled if it is found that termini linked to plasmid DNA serve as origins with only low efficiency. It will be interesting to see how termini joined head-to-tail behave in *in vitro* replication systems.

Apart from a possible role in viral replication there is a good deal of circumstantial evidence suggesting that circles may be intermediates in integration of viral DNA into the host chromosome during cell transformation (reviewed by Graham *et al.*, 1984). Firstly, although only the left ~10% of the adenovirus genome is required for maintenance of transformation there are many examples of transformed cell lines containing virtually the entire genome inserted as a co-linear structure (Green *et al.*, 1976; Dorsch-Hasler *et al.*, 1980; Ibelgaufts *et al.*, 1980; Kuhlman *et al.*, 1982; Fisher *et al.*, 1982; Ruben *et al.*, 1982). This suggests that integration involves some sort of specificity with respect to the ends of the viral DNA and occurs *via* a process which may be most simply described by invoking circular intermediates. Secondly, several examples of transformed cells exist in which the integrated viral DNA is arranged with left and right termini linked together (Sambrook *et al.*, 1979; Stabel *et al.*, 1980; Visser *et al.*, 1981, 1982; van Doren *et al.*, 1984) again implying that head-to-tail joining of viral DNA molecules may precede integration. Finally, the conditions which resulted in most efficient head-to-tail joining of Ad5 DNA molecules (i.e., infection of rat cells with viral mutants in E1A) (Ruben *et al.*, 1983) were also conditions which resulted in highly efficient transformation of primary baby rat kidney cells (Graham *et al.*, 1978) and produced cell lines with co-linear integrates of the entire Ad5 DNA molecule.

In addition to their possible relevance for viral DNA replication and cell transformation, the infectious plasmids isolated and characterized in this study may have practical implications in relation to the use of adenoviruses as cloning vectors in mammalian cells. The plasmids pFG140 and 141 are stable during re-transformation of bacteria and have been introduced into several strains of *E. coli* without noticeable alteration in their structure. In spite of their large size, yields of plasmid DNA are reasonable, with a few hundred μg of purified plasmid DNA being routinely recoverable from a litre of cells. In addition, of course, cloning of plasmids in bacteria requires approximately a tenth of the time needed for plaquing and growing adenoviruses in mammalian cells. Thus it may be convenient to carry out genetic and biochemical manipulation of viral genomes replicating as bacterial plasmids then introduce the altered molecules into appropriate mammalian cells to obtain the desired viral mutants. Furthermore, with the development of strains of *E. coli* capable of supporting the replication of plasmids containing long palindromes (Leach and Stahl, 1983) it may be possible for mutant adenoviruses such as In52 to shuttle back and forth between mammalian cells and bacteria.

Materials and methods

Cells and viruses

The Ad5-transformed human embryo kidney cell line, 293, has been described previously (Graham *et al.*, 1977). Ad5 dl309 (Jones and Shenk, 1978) was obtained from T.Shenk.

Recombinant DNA manipulations

Restriction enzymes, T4 DNA ligase, and polynucleotide kinase were obtained from Boehringer Mannheim Corp. or Bethesda Research Laboratories Inc. and were used according to the manufacturers recommendations. S1 nuclease was purchased from Sigma Chemical Co. For the construction of Ad5 In52 (Figure 1) DNA transfections with ligated DNA were carried out on 293 cells using the calcium technique (Graham and van der Eb, 1973). Plaques were isolated and seeded onto 60 mm dishes of 293 cells and after the appearance of extensive cytopathic effect, infected cell DNA was extracted, digested with *Hind*III, and analyzed on 1.2% agarose gels to identify the desired mutant. To clone circular forms of Ad5 DNA, primary baby rat kidney cells were infected with In52 and total infected cell DNA was purified at 72 h as described previously (Ruben *et al.*, 1983). Infected rat cell DNA was used to transform *E. coli* strains HB101 or HMS174 essentially according to Mandel and Higa (1970) and ampicillin-resistant colonies were isolated. Plasmid DNA was purified by the method of Birnboim and Doly (1979) and analyzed by *Hind*III cleavage and gel electrophoresis. A preliminary report on the construction of Ad5 In52 and the cloning of circular Ad5 DNA has been presented previously (Graham *et al.*, 1984).

Infectivity assays with plasmid DNA (Table I) were carried out on 293 cells again using the calcium technique and using plasmid DNA which had been prepared by a scaled up, slightly modified Birnboim-Doly procedure, and further purified by banding in CsCl-ethidium bromide gradients.

Sequencing

Plasmid DNA fragments to be end-labelled were isolated from agarose gels as described previously (Girvitz *et al.*, 1980) and kinased using polynucleotide kinase according to Maniatis *et al.* (1982). After cutting with *Hind*III to remove one end, labelled DNA was chemically degraded (Maxam and Gilbert, 1980) and run on 6% polyacrylamide gels containing 7 M urea. Electrophoresis was at 3000 V and 70°C on an LKB Macrophor unit.

Acknowledgements

I am grateful to John Rudy and Paul Rotsaert for technical assistance, to Doty Bautista for advice on sequencing, and to Silvia Bacchetti for helpful comments on the manuscript. This research was supported by the National Cancer Institute and Medical Research Council (Canada).

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.
Challberg,M.D. and Kelly,T.J., Jr. (1982) *Annu. Rev. Biochem.*, **51**, 907-934.
Dorsch-Hasler,K., Fisher,P., Weinstein,B. and Ginsberg,H. (1980) *J. Virol.*, **34**, 305-314.
Fisher,P.B., Babiss,L.E., Weinstein,I.B. and Ginsberg,H.S. (1982) *Proc. Natl. Acad. Sci. USA*, **79**, 3527-3531.
Girvitz,S.C., Bacchetti,S., Rainbow,A.J. and Graham,F.L. (1980) *Anal. Biochem.*, **106**, 492-496.
Graham,F.L. (1977) in Klein,G. (ed.), *Advances in Cancer Research*, Academic Press, NY, pp. 1-51.
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.
Graham,F.L., Harrison,T. and Williams,J. (1978) *Virology*, **86**, 10-21.
Graham,F.L., Rowe,D.T., McKinnon,R., Bacchetti,S., Ruben,M. and Branton,P.E. (1984) *J. Cell Physiol. Suppl.*, **3**, 151-163.
Green,M., Chinnadurai,G., Mackey,J. and Green,M. (1976) *Cell*, **7**, 419-428.
Hanahan,D. and Gluzman,Y. (1984) *Mol. Cell. Biol.*, **4**, 302-309.
Ibelgaufts,H., Doerfler,W., Scheidtmann,K.H. and Wechsler,W. (1980) *J. Virol.*, **33**, 423-437.
Jones,N. and Shenk,T. (1978) *Cell*, **13**, 181-188.
Kuhlmann,I., Achten,S., Rudolph,R. and Doerfler,W. (1982) *EMBO J.*, **1**, 79-86.
Lally,C., Dorper,T., Groger,W., Antoine,G. and Winnacker,E. (1984) *EMBO J.*, **3**, 333-337.

Leach,D.R.F. and Stahl,F.W. (1983) *Nature,* **305**, 448-451.
Lilly,D.M.J. (1981) *Nature,* **292**, 380-382.
Lusky,M. and Botchan,M. (1981) *Nature,* **293**, 79-81.
Mandel,M. and Higa,A. (1970) *J. Mol. Biol.,* **53**, 453-471.
Maniatis,T., Fritsch,E.F. and Sambrook,J. (1982) *Molecular Cloning. A Laboratory Manual,* published by Cold Spring Harbor Laboratory Press, NY.
Maxam,A.M. and Gilbert,W. (1980) *Methods Enzymol.,* **65**, 499-560.
Pearson,G.D., Chow,K.-C., Enns,R.E., Ahern,K.G., Corden,J.L. and Harpst,J.A. (1983) *Gene,* **23**, 293-305.
Ruben,M., Bacchetti,S. and Graham,F.L. (1982) *J. Virol.,* **41**, 674-685.
Ruben,M., Bacchetti,S. and Graham,F.L. (1983) *Nature,* **301**, 172-174.
Sambrook,J., Greene,R., Stringer,J., Mitchison,T., Hu,S.-L. and Botchan, M. (1979) *Cold Spring Harbor Symp. Quant. Biol.,* **44**, 569-584.
Sharp,P.A., Moore,C. and Haverty,J.L. (1976) *Virology,* **75**, 442-456.
Stabel,S., Doerfler,W. and Friis,R.R. (1980) *J. Virol.,* **36**, 22-40.
Stillman,B.W. (1983) *Cell,* **35**, 7-9.
Tamanoi,F. and Stillman,B.W. (1982) *Proc. Natl. Acad. Sci. USA,* **79**, 2221-2225.
Tooze,J., ed. (1980) *The Molecular Biology of Tumor Viruses, Pt2,* 2nd ed., published by Cold Spring Harbor Laboratory Press, NY.
van Doren,K., Hanahan,D. and Gluzman,Y. (1984) *J. Virol.,* **50**, 606-614.
Visser,L., Reemst,A.C.M.B., van Mansfeld,A.D.M. and Rozijn,T.H. (1982) *Nucleic Acids Res.,* **10**, 2189-2198.
Visser,L., Wassenaar,A.T.D.C., van Maarschalkerweerd,M.W. and Rozijn, T.H. (1981) *J. Virol.,* **39**, 684-693.
Winnacker,E.L. (1978) *Cell,* **14**, 761-773.

Received on 20 August 1984