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Generation of adenovirus by transfection of plasmids

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ABSTRACT

Biologically active fragments of Adenovirus 5 (Ad5) DNA that span the entire genome have been cloned into plasmids. The covalently attached terminal protein was removed and Eco RI linkers added in a fashion that preserves the Ad5 terminal sequences. When plasmids containing overlapping fragments that represent the entire genome are cotransfected onto 293 cells, infectious virus is obtained. Generation of virus depends upon the release of the 0 or 100 mu Ad5 terminus from pBR322 DNA by Eco RI cleavage. During virus production the modified termini of the transfected fragments are corrected exactly to that of wt viral DNA. The above method for preparing adenovirus recombinants has been used to construct a mutant, Ad5 Δ(78.9-84.3), lacking most of the non-essential EIII transcriptional unit. This mutant is phenotypically wild type with respect to burst size and kinetics of growth. Surprisingly, it inhibits wt viral growth upon mixed infections of HeLa or 293 cells, apparently at the level of DNA replication.

INTRODUCTION

The ability to manipulate the adenovirus (Ad) genome in vitro is important in facilitating biological studies on its organization and expression. Precedence for the invaluable advantages of using cloned viral DNAs to generate infectious particles can be taken from a number of viral systems (for review see ref. 1), most notably for SV40. To date, all Ad recombinants have been generated either by ligation of viral DNA, or by recombination of viral DNA-protein complexes, with subgenomic cloned sequences.

Two different types of defective Ad recombinants have previously been constructed. The first type is complemented for reproduction by coinfection with nondefective virus. Typically, a segment of SV40 DNA encoding the early T antigen is inserted into Ad DNA, rendering the viral DNA defective (2,3). Growth of the Ad-SV40 recombinant is complemented by coinfection of monkey cells with wt Ad. Since early SV40 gene function is required for growth of human Ad in monkey cells, only those cells coinfected with both the defective recombinant and wt virus yield progeny. Such recombinant stocks can yield high levels of SV40 T antigen, but are difficult to manipulate for the cloning

and expression of other genes. The second type of defective Ad recombinant is complemented by growth on 293 cells, a permissive human cell line established by transformation with sheared Ad5 DNA (4). This cell line expresses EIa and EIb gene products from integrated viral sequences and Ad recombinants containing substitutions in EIa and EIb can thus be grown to high titers as homogeneous stocks.

Biochemical manipulation and subsequent screening have been used to generate a number of deletion, insertion and substitution Ad mutants (5,6). However, it has been difficult to apply site-directed mutagenesis to generate Ad mutants because of the large size of the genome and the low efficiency of transfection with viral DNA. Ad DNA is a linear 35 kb duplex with a 55K-protein covalently coupled to each of the 5' termini (7,8). The infectivity of protease-digested DNA (approximately 1000 plaques/ug) is one-tenth that of the viral DNA-protein complex (9). However, this level is sufficient to permit generation of infectious virus from a cloned left-terminal fragment by ligation of plasmid DNA to viral DNA and transfection into human cells (10).

Ad offers excellent potential as a cloning vehicle by which genes of interest can be introduced into mammalian cells and expressed in large amounts. During the late stage of Ad infection, viral protein synthesis can account for over half of the total cellular activity. In addition, the large Ad genome size affords substitution of large fragments. A recombinant genome formed by substituting the EI and the nonessential EIII region with up to 7 kb of foreign sequences could be propagated as a homogeneous virus by infection of the previously mentioned 293 cells.

This paper describes the preparation of cloned, subgenomic Ad5 fragments that span the entire genome. We have demonstrated their biological activity, and have developed a system by which they can be manipulated *in vitro*, and subsequently be moved with ease into the form of virus stocks. Initial steps in adapting Ad as a cloning vehicle are also described.

MATERIALS AND METHODS

Reagents

All restriction endonucleases were purchased from New England Biolabs, with the exception of Bam HI from BRL, and Hpa I from Boehringer Mannheim. The enzymes T4 polynucleotide ligase and T4 polynucleotide kinase were obtained from New England Biolabs, T4 DNA polymerase was from BRL and S1 nuclease and calf intestinal phosphatase (CIP) were from Miles. The unit

definitions were as specified by the vendors. Most radionucleotides were from NEN. [γ-^{32}P]-ATP was also purchased from Amersham.

Preparation of Ad5 recombinants

The 55K-terminal protein covalently attached to Ad at each terminus was removed by the following enzymatic manipulations: proteinase K-digested Ad5 DNA (10 ug) was incubated in 20 ul of 50 mM Tris pH 8, 7 mM $MgCl_2$, 7 mM β-mercaptoethanol, and 100 uM dTTP with 3 units of T4 DNA polymerase for 30' at 11°C. After phenol extraction and ethanol precipitation, DNA was resuspended in 20 µl of 30 mM $NaC_2H_4O_2$ pH 4.5, 1 mM $ZnSO_4$, 5% glycerol, 0.25 M NaCl and 1000 units of S1 nuclease and incubated at 9°C for 30'. The DNA was resuspended in 20 ul of 50 mM Tris pH 7.5, 10 mM $MgCl_2$, 15 mM dithiothreitol, 50 uM ATP, and 50 uM of ^{32}P end-labeled Eco RI-oligonucleotide linkers (Collaborative Research) and incubated with 500 units of T4 polynucleotide ligase for 16 hr at 16°C. The DNA was resolved from linkers by chromatography over Sephadex 2B in 10 mM Tris pH 8, 1 mM EDTA), 10 µg/ml tRNA and then cleaved with Eco RI endonuclease and fractionated on a 1.2% agarose gel. Eco RI fragments A-C were isolated by electroelution, and ligated to Eco RI-cleaved, CIP-treated pBR322 DNA (11). After ligation, DNA was transfected into C600 *E. coli*, and colonies containing Ad5 sequences were detected by colony hybridization (12) and DNA rapid preparation analysis (13).

Recombinant pEcoRIBAd5 (84.3-100) served as a progenitor for the recombinant pBamBAd5 (59.5-100) as follows: pEcoRIBAd5 DNA was grown in a DAM$^-$ *E. coli* strain (14), to avoid methylation of the Xba I recognition site at 84.3 mu. This pEcoRIBAd5 DNA was partially digested with Xba I, and singly-cleaved DNA was isolated after electrophoresis in a 1.2% agarose gel. This DNA was digested with Bam HI and purified by agarose gel electrophoresis. The fragment containing Ad5 sequences from 84.3-100 mu and pBR322 sequences from 375-4361 nucleotides was isolated. This DNA was ligated with a 59.5-84.3 mu (Bam HI and Xba I) gel-purified fragment of Ad5 DNA and transformed into C600 *E. coli*.

A derivative of pBamBAd5 (59.5-100) lacking the Ad5 DNA sequences from 78.9-84.3 mu was prepared by digesting pBamBAd5 (59.5-100) grown in DAM$^-$ *E. coli* with Xba I endonuclease, purification of the large fragment by agarose gel electrophoresis and ligation at a DNA concentration of 5 ug/ml. This DNA was transfected into C600 *E. coli*, and subsequently into DAM$^-$ *E. coli*.

DNA transfections

The calcium phosphate transfection protocol used was similar to that of Graham and van der Eb (15), with the additional glycerol shock (16).

Transfections were done on 293 cells because of their high transfection efficiency. Plasmid or viral DNA at a 10 ug total was mixed with 10 ug salmon sperm DNA (Sigma), and ethanol precipitated. The DNA was resuspended in 0.5 ml of 40 mM Hepes, 2 mM Na_2HPO_4, 10 mM KCl, 300 mM NaCl and 10 mM dextrose at pH 7.05 and an equal volume of 250 mM $CaCl_2$ was added, while aerating the DNA solution. After 20 min at ambient temperature, the cocktail was added directly onto two 60 mm dishes of 293 cells (20-40% confluent), each containing 5 ml of DME and 10% fetal calf serum. Four hours later the cells were incubated for 1 min in 20% glycerol in Tris saline (5 mM KCl, 5 mM Na_2HPO_4, 140 mM NaCl, 25 mM Tris pH 7.9 and 1 mM sucrose), rinsed with 1 ml Tris saline, and either overlaid with media or with agar for plaque detection. Wild type Ad5 DNA (0.2-0.4 ug) was always used as a control, and transfection efficiencies were routinely between 750-1500 plaques/ug DNA. Plaques or CPE could usually be detected within 5 days post-transfection, and virus harvested 10-12 days after transfection was plaque purified twice.

Preparation of viral or cloned DNAs

Viral DNA was prepared from HeLa spinner cultures (S3) infected with virus at multiplicities of 5-20 pfu/cell. The DNA was isolated from equilibrium CsCl-banded virions by proteinase K and RNase treatment (17). For analytical purposes, HeLa monolayers (JW35) or 293 cells infected at a moi of 10 were labeled with $^{32}PO_4$ (10 μCi/ml) from 20-40 hours post infection. The cells were harvested approximately 40 hours after infection, and the DNA was HIRT extracted, proteinase K and RNase-digested, and phenol extracted before ethanol precipitation (18). Recovery of restriction enzyme DNA fragments from gels was either by electroelution or by absorbance to glass beads after NaI treatment (19). The 34 kb piece of Xba I-cleaved 309 DNA from 4-100 mu was isolated by sucrose gradient sedimentation (10). DNA was spun in a 5-20% gradient of sucrose in 0.1 M NaCl, 25 mM Tris pH 8, 1 mM EDTA for 7 hours at 35 K rpm at 20°C in an SW41 rotor.

DNA sequencing

The 0 and 100 mu termini of cloned or viral DNA were sequenced according to the procedure of Maxam and Gilbert (20). pEcoRIAAd5 (0-75.9) and pEcoRIBAd5 (84.3-100) were cleaved with Eco RI endonuclease and labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase by the exchange reaction (21). Viral DNA termini were labeled by incubating protease-digested DNA (2-10 ug) in 20 ul 50 mM Tris pH 8, 7 mM $MgCl_2$, 7 mM β-mercaptoethanol, and a nucleotide combination of 100 uM dTTP, 6 uM [α-^{32}P]dGTP or 200 uM dGTP, 6 uM [α-^{32}P]dTTP with 3 units of T4 DNA polymerase for 30 min at 11°C. These DNAs were

digested with Hae III endonuclease, and labeled fragments were purified by electrophoresis through 8% acrylamide gels. Viral DNAs were, in addition, labeled on the 5' strand using a modification of the protocol of Pettersson et al. (22). To hydrolyze the serine-phosphate bond, protease-digested DNA (4 ug) was incubated in 100 ul of 50 mM NaOH for 30' at 70°C (23), and then neutralized by the addition of an equal volume of 50 mM HCl, 50 mM Tris pH 8. DNA termini were end-labeled with T4 polynucleotide kinase and digested with Hha I endonuclease, which cleaves 72 nucleotides from the 0 and 100 mu termini (24). The DNAs were run directly on an 8% sequencing gel, along with a sequencing ladder.

Analysis of RNA and protein products

293 cells or HeLa cells infected with virus at multiplicities of 5-20 pfu/cell were labeled with [^{35}S]-methionine (at 12 μCi/ml) for 1 hour either 5 or 24 hours post infection. The cells were rapidly chilled, rinsed in cold phosphate buffered saline, lysed and electrophoresed through SDS-polyacrylamide gels (25).

HeLa monolayers or 293 cells infected at a multiplicity of 20 pfu/cell were harvested for RNA either 8 hours or 24 hours post-infection. For early RNA isolation, 20 ug/ml cytosine arabinoside (Sigma) was included in the media added following virus adsorption. Total polyA$^+$ RNA was prepared as described by Seed and Goldberg (26), and the RNA concentrations were quantitated by absorbance at 260 nm.

For Northern blot analysis, generally 100-500 ng of RNA was electrophoresed through 1.2% formaldehyde-agarose gels (26) for 2 hours at 200V. The RNA was transferred to nitrocellulose (27) and subsequently hybridized to appropriate nick-translated probes (28) in dextran sulfate and formamide (29).

RESULTS

Generation and characterization of recombinants spanning the entire Ad5 genome

In order to prepare Ad5 DNA termini for cloning into plasmid DNA, the 55K-protein was removed in such a fashion as to leave the Ad terminal sequences intact. Proteinase K-digested wt Ad5 DNA was incubated with T4 DNA polymerase and dTTP. In the presence of excess dTTP, a single nucleotide from the 3'-strand should be removed (30), generating the product shown in Figure 1. The protruding 5'-dCMP and covalently attached residual peptide were removed by digestion with S1 nuclease. Addition of Eco RI linkers restored the terminal C-G base pair (Fig. 1). Cleavage of linkered Ad5 DNA with Eco RI

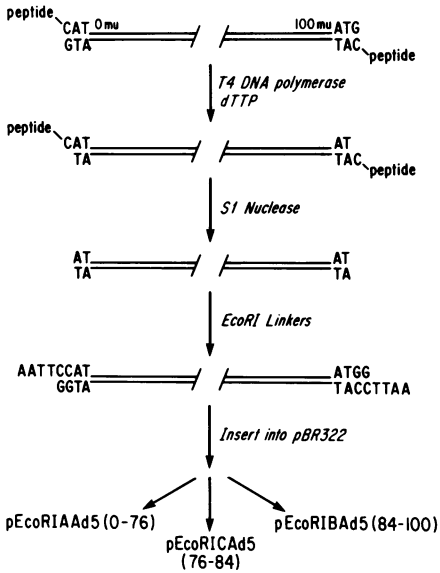


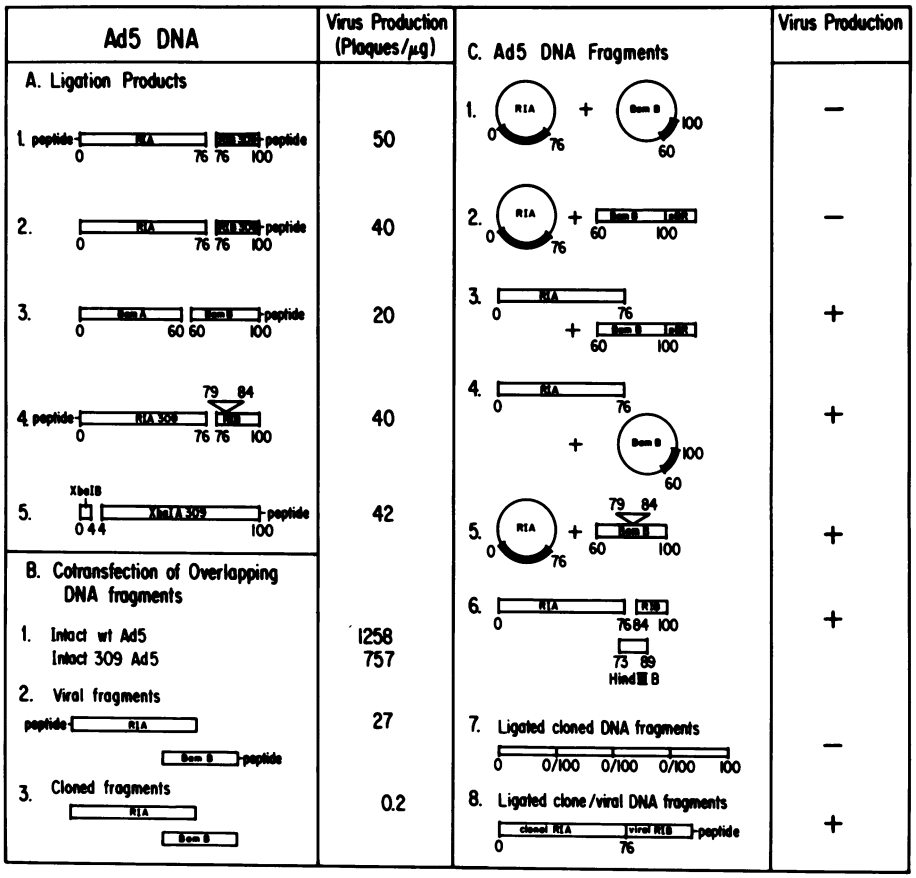
Figure 1: Construction of plasmids containing subgenomic Ad5 fragments that span the entire genome. The individual enzymatic steps are described in detail in Materials and Methods.

endonuclease generated 3 subgenomic Ad5 fragments, which were cloned into the Eco RI site of pBR322 DNA: pEcoRIAAd5 (0-75.9), pEcoRIBAd5 (84.3-100) and EcoRICAd5 (75.9-84.3). Most of the viral DNA termini were adjudged to be freed of the covalently attached peptide, as roughly equal colony numbers of pEcoRIBAd5 (84.3-100) and pEcoRICAd5 (75.9-84.3) were obtained. pEcoRIAAd5 (0-75.9) was detected at about 100-fold lower frequencies, presumably because of its larger size (27 kb). Multiple isolates of pEcoRIAAd5 (0-75.9) and EcoRIBAd5 (84.3-100) were labeled at the 0 or 100 mu terminus, respectively, and sequenced, along with progenitor wt Ad5 DNA termini. Most differed from wt DNA only in having the additional bases attributable to the Eco RI linker (data not shown).

A clone which overlaps with pEcoRIAAd5 (0-75.9): pBamBAd5 (59.5-100), was constructed by inserting a fragment of wt Ad DNA from 59.5 to 84.3 mu into the cloned pEcoRIBAd5 (84.3-100), as described in Materials and Methods. The availability of overlapping cloned subgenomic fragments made it possible to test whether ligation of such subgenomic fragments prior to transfection onto mammalian cells, or recombination of two such fragments in vivo, could generate infectious virus following transfection.

The biological activity of the subgenomic fragments was tested by ligation to pieces of viral DNA that would make genomic-length products (10). Viral DNA from mutant 309 was used, as it differs in restriction enzyme specificity

TABLE I. Infectivity of Ad5 DNA plasmids



A. Viral or recombinant DNA fragments were purified by agarose gel electrophoresis prior to ligation. Fragments were mixed in approximately equimolar concentrations, to a final total concentration of 10 ug/ml and incubated with ligase. The reaction was then transfected as described in Materials and Methods. In control experiments where mixed fragments were not ligated, the transfection efficiencies were at least 10-fold lower. Both Ad5 309 DNA and Ad5Δ(78.9-84.3) DNA (used in number 4) contain only one Eco RI site, at 75.9 mu. The transfection efficiency for Eco RI fragments of Ad5 309 DNA after purification and ligation was 5-fold lower than with non-purified Eco RI fragments ligated directly after Eco RI cleavage.

B. Overlapping fragments of viral or plasmid DNA were mixed and transfected onto 293 cells. Immediately after glycerol shock the cells were overlaid with agar, and plaques were counted from duplicate plates 8-10 days later.

C. Overlapping fragments of viral or plasmid DNAs were mixed or ligated prior to $CaPO_4$ precipitation and transfection onto 293 cells. After 6-10 days, lysates were prepared from transfected cells, and used to infect HeLa and 293 cells. Detection of CPE 2-4 days post-infection was scored as a positive.

from wt DNA (6). These DNAs were transfected onto 293 cells, and quantitated for infectivity by plaque assay. Viral DNAs from progeny virus were analyzed after HIRT extraction by cleaving with different restriction endonucleases. As can be seen in Table I, the cloned fragments of Ad5 were biologically active. Each plasmid-viral DNA transfection generated virus at approximately the same efficiency as a corresponding viral-viral DNA transfection. The efficiency of virus production was roughly equivalent whether cloned DNA was joined to viral DNA at 4 mu (Xba I site), 60 mu (Bam HI site) or 76 mu (Eco RI site) (Table I).

The biological activities of the Ad5 recombinants were ultimately demonstrated by the observation that homogeneous, infectious virus stocks were generated by merely transfecting two overlapping subgenomic clones of Ad5 DNA. Virus could be generated by transfection with 3 subgenomic fragments, as well, and the amount of overlap necessary was as little as 3 mu (Table I). The physical structure of the 0 and 100 termini of the plasmid DNA introduced into cells was important, in that virus production depended upon the release of pBR322 sequences from at least one terminus, prior to transfection. Initial experiments in which supercoiled pEcoRIAAd5 (0-75.9) and pBamBAd5 (59.5-100) were coprecipitated, or where the two were ligated together at the Bam HI site (at 59.5 mu), were negative for virus production after transfection. However, the release of either terminus by Eco RI digestion resulted in infectious virus production after transfection of the DNA. The efficiency of virus production appeared to be at least 100-fold lower when total cloned sequences were used, as compared with overlapping viral DNA fragments (Table I).

Progeny virus derived totally from recombinant pieces of DNA were indistinguishable from wt virus, by a variety of criteria. These include the viral growth cycle, as adjudged by viral burst size and the time course for the infection cycle, and restriction endonuclease mapping (data not shown). In addition, the 0 and 100 mu termini of recombinant-generated viral DNA have been sequenced and found to be identical to bona fide viral DNA ends (Figure 2). Both the pattern of $^{32}PO_4$ incorporation observed with different deoxynucleotide combinations (Fig. 2a) and the sequencing of the termini (Fig. 2b) confirmed the removal of linker sequences from cloned DNAs during virus production. As additional proof, viral DNA termini were also treated with alkali to remove the terminal peptide and end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. After digestion with Hha I endonuclease, which cleaves 73 nucleotides from each terminus, the DNAs were sized by collectrophoresis in an acrylamide gel with a sequencing ladder (data not shown). In all regards,

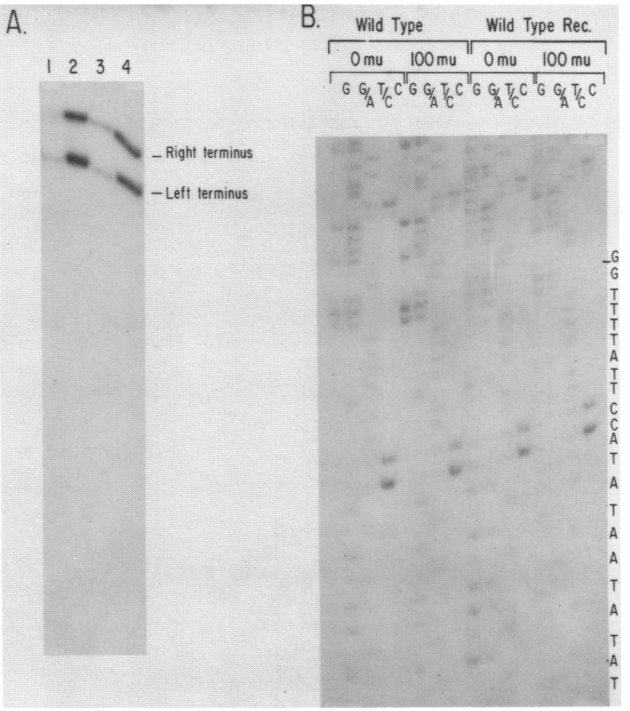


Figure 2: Analysis of termini of viral DNA produced by transfection with plasmids.

A. Protease-digested wt (lanes 1,2) and wt-recombinant (lanes 3,4) viral DNAs were incubated with T4 DNA polymerase and [α^{32}P]dGTP, dTTP (lanes 2,4) or [α^{32}P]dTTP, dGTP (lanes 1,3) at sufficiently high concentrations (see Materials and Methods) to favor net polymerization. T4 DNA polymerase will exchange only with the terminal nucleotide if the deoxynucleotide triphosphate is present in excess concentration (our unpublished observation). Incorporation of label into DNA was measured by cleavage with Hae III endonuclease and resolution of terminal fragments on an acrylamide gel. As predicted from the sequence, substantial $^{32}PO_4$ incorporation into Ad5 DNA termini was only observed when [α^{32}P]dGTP, dTTP were used to label the DNA (lanes 2,4).

B. The Hae III fragments from lanes 2 and 4 were excised, eluted and sequenced. The first 4 nucleotides of each terminus were run off of the gel. The sequence for bona fide viral DNA ($\begin{matrix}CATCTATATAA...\\GTAGATATATT...\end{matrix}$) differs slightly from the sequence reported for presumably a different strain of Ad5 ($\begin{matrix}CATCATCAATAA...\\GTAGTAGTTATT...\end{matrix}$) (24).

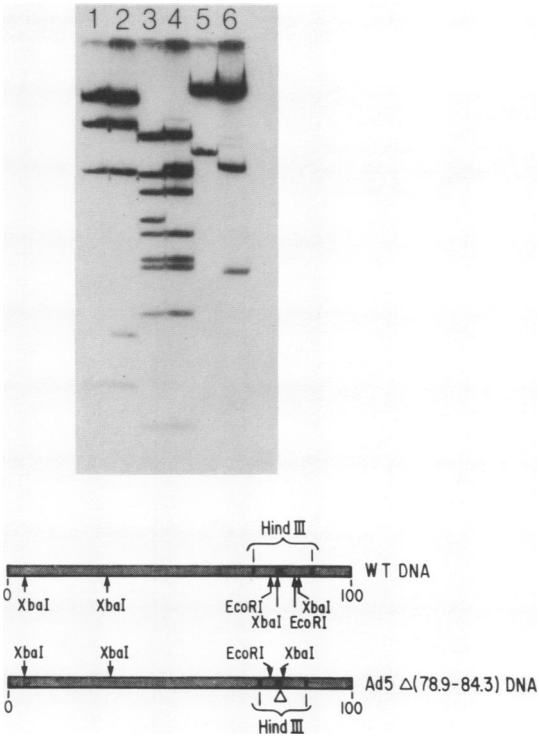


Figure 3: Restriction endonuclease mapping of the Ad5Δ(78.9-84.3) variant. Wildtype Ad5 or Ad5Δ(78.9-84.3)-infected 293 cells were labeled with $^{32}PO_4$ (10 μCi/ml) in phosphate-minus DME + 5% fetal calf serum 20-40 hours post-infection. The DNAs were isolated by HIRT extraction (18) digested with restriction endonucleases and RNase and electrophoresed on 1.4% agarose gels. The DNAs are Ad5Δ(78.9-84.3) (lanes 1, 3, 5) or wt Ad5 (lanes 2, 4, 6) cleaved with Xba I (lanes 1, 2), Hind III (lanes 3, 4) or Eco RI (lanes 5, 6) endonuclease. The restriction maps for both DNAs are illustrated for Eco RI and Xba I endonucleases, and for Hind III endonuclease only in that portion of the genome affected by the deletion.

the recombinant termini were identical to those found on bona fide viral DNA.

Creation of and characterization of Ad5 virus deleted in the Early III transcriptional unit

The EIII region is not essential for Ad lytic growth (6,31,32) and is a possible site for insertion of foreign DNA. Deletions of the EIII region were generated by preparing derivatives of pBamBAd5 (59.5-100) and pHindIIIBAd5 (72.8-89.1), which have been cleaved with Xba I and religated, eliminating the sequences from 78.9 to 84.3 mu (see Materials and Methods). Each of these clones contains a unique Xba I site into which additional sequences can be

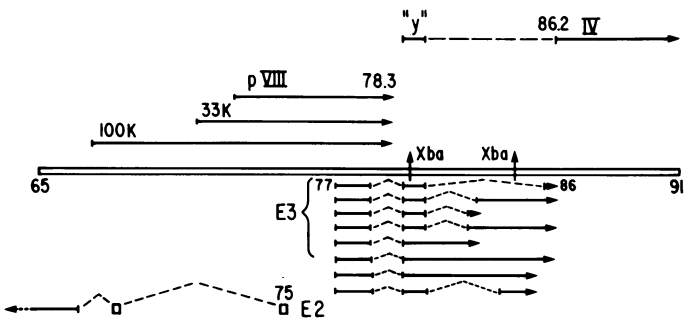


Figure 4: Localization of transcriptional information around the site of deletion in Ad5Δ(78.9-84.3). The eight EIII mRNAs have been mapped by Berk and Sharp (33) and Chow et al. (34), and the sequence for this region has been determined (35,36). The deletion of sequences between the two Xba I sites, at 78.9 and 84.3 mu, removes most of the EIII mRNA coding sequences and the extra Y leader for fiber (IV) mRNA.

inserted. The subgenomic clones were mixed with appropriate overlapping clones, transfected onto 293 cells, and virus harvested within one week. Viral DNAs with deletions of EIII [Ad5Δ(78.9-84.3)] were identified by restriction endonuclease fragmentation (Fig. 3). In addition, heteroduplexes of Ad5Δ(78.9-84.3) and wt Ad5 DNA revealed only one observable difference, mapping at the site of the deletion (data not shown). The average deletion size measured from several heteroduplexes was 6 mu, consistent with the restriction endonucleases data. The sequence of the termini, determined for two EIII-deletion mutants, was found to be identical to that of wt Ad5 DNA.

Deletion of sequences between 78.9 and 84.3 mu removes the bulk of all 8 messages normally detected in the EIII transcriptional unit (Figure 4; 33-35). However, the EIII initiation site, polyadenylation site, and one set of 5' and 3' splice sites should remain intact in the deletion. In order to determine whether these signals were utilized in vivo following infection of 293 or HeLa cells, RNA was analyzed by S1 digestion (33) using an end-labeled probe that would detect both spliced and unspliced messages (Figure 5). The deletion from 78.9 to 84.3 mu should reduce most of the EIII mRNAs to two composite RNAs originating at 76.6 mu, terminating at 86 mu, and differing by the absence or presence of a splice between 77.6 and 78.7 mu (Fig. 5). Hybridization with a probe from Ad5Δ(78.9-84.3) DNA end-labeled at the 85 mu Hpa I site revealed a roughly equal population of the expected spliced and unspliced RNAs (Fig. 5). When RNAs from wt Ad5 and Ad5Δ(78.9-84.3) infected cells were analyzed by S1 digestion using a probe from wt Ad5 DNA labeled at

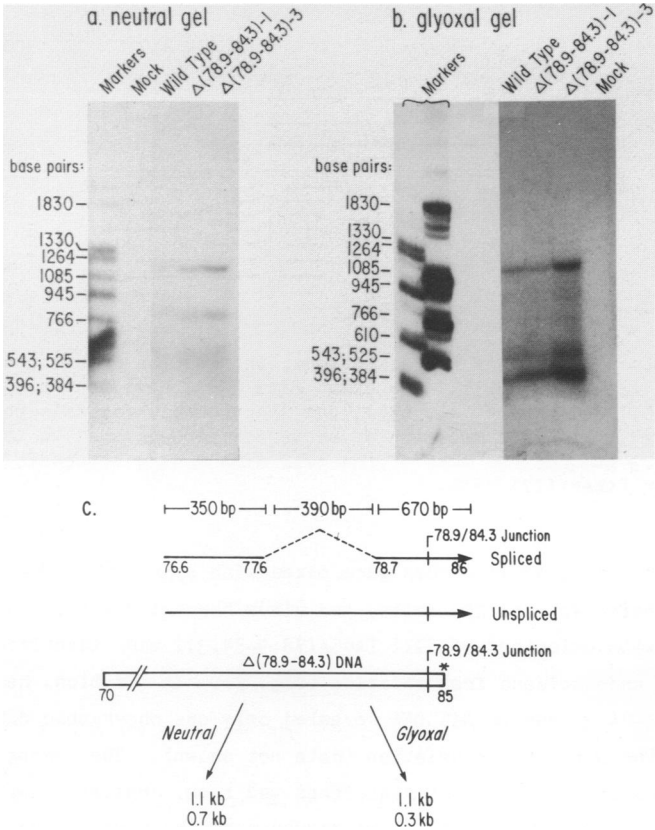


Figure 5: S1 analysis of polyA$^+$ EIII mRNAs from wt or Ad5Δ(78.9-84.3) infected cells. RNA (100 ng) prepared from early infected cells was mixed with 10 ug HeLa cytoplasmic RNA and analyzed by S1 as previously described (36). Hybridization was for 7 hr at 54°C, the predetermined Tm for the probe used, followed by digestion for 30' at 45°C with 4×10^4 units of S1 nuclease. DNAs were resolved either on a neutral agarose gel (a) or glyoxalated and electrophoresed on a denaturing gel (b). Two different isolates of Ad5Δ(78.9-84.3), numbered 1 and 3, were examined.

(c) The 78.9-84.3 deletion reduces most of the EIII messages to two composite mRNA species, as illustrated. The DNA probe, extending from 70-85 mu, was $^{32}PO_4$ end-labeled with T4 polynucleotide kinase at the 85 mu Hpa I site. This DNA fragment is deleted for sequences between 78.9 and 84.3 mu. The probe will detect only those EIII mRNAs terminating at 86 mu.

the same Hpa I site, the expected products for Ad5 were observed, while the bands generated by Ad5Δ(78.9-84.3) RNA were the same as in Fig. 5 (unpublished data).

The deleted region in Ad5Δ(78.9-84.3) DNA juxtaposes the L-4 (100K, 33K

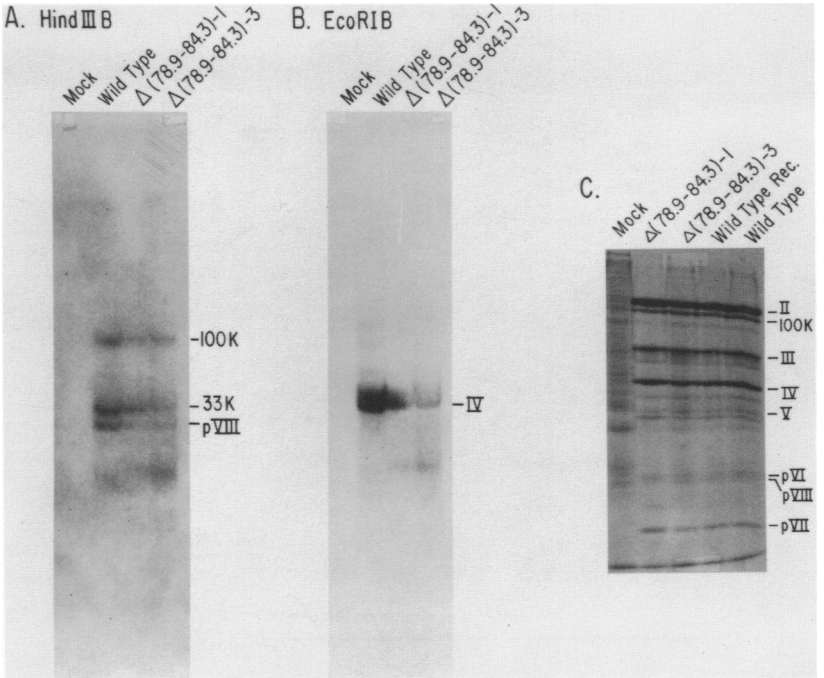


Figure 6: Expression of Ad5 late mRNAs and proteins in wild type and Ad5Δ(78.9-84.3)-infected cells.

A. Total polyA$^+$ mRNA was isolated from late-infected 293 cells, as described in Materials and Methods. 100 ng RNA samples in 20 mM MOPS pH 7.0, 1 mM EDTA, 5 mM Na acetate, 50% formamide, 2.2 M HCHO were heated at 60° C for 5 min and electrophoresed through formaldehyde gels (26), transferred to nitrocellulose (27) and hybridized with nick-translated plasmids containing sequences for 100K (HindIIIBAd5) or fiber (EcoRIBAd5). The quantitative difference in RNA levels for Ad5Δ(78.9-84.3) and wt RNAs seen here, is not always observed.

B. HeLa cells were pulsed with [^{35}S]methionine late in infection, as described in Materials and Methods. After lysis in 10 mM Tris pH 7.5, 1% deoxycholate, 1% Triton X100, 0.1% SDS, 5 mM EDTA, 0.7 M NaCl, aliquots equivalent to about 10^5 cells were electrophoresed through 7.5% SDS-polyacrylamide gels (25). The protein assignments were based on migration relative to BioRad MW standard protein markers from 14K to 94K.

and pVIII) and L-5 (IV) mRNAs (Fig. 4), and deletes part of the "Y" segment found spliced to some fiber (IV) mRNAs. In order to determine whether these transcriptional regions were affected by the 78.9-84.3 deletion, RNA and proteins of wt Ad5 or Ad5Δ(78.9-84.3) infected cells were compared. As is clear from Figure 6, the L-4 or L-5 family of mRNAs and protein is the same for wt or deleted virus.

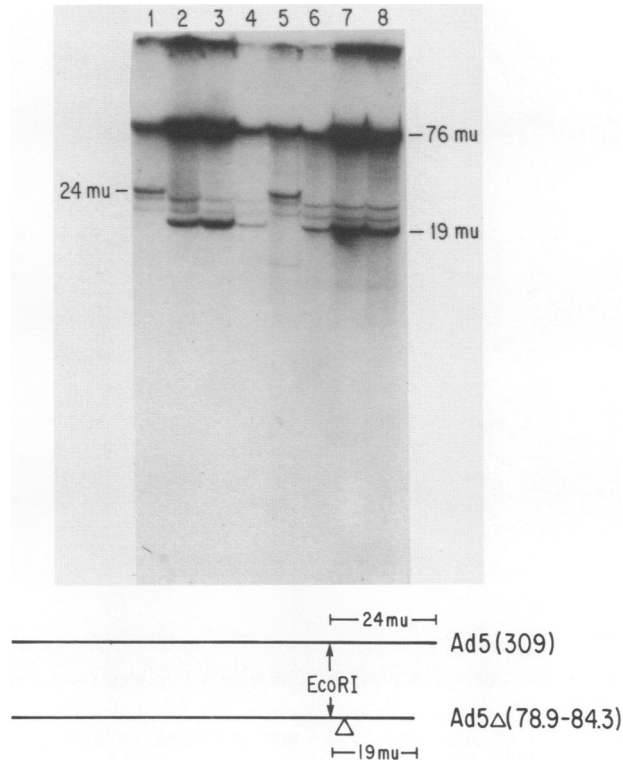


Figure 7: Inhibition of wt Ad5 DNA replication by Ad5Δ(78.9-84.3). 293 cells were coinfected with Ad5Δ(78.9-84.3) and Ad5 (309) at multiplicities of 0:10 (lanes 1,5), 10:1 (lanes 2,6), 10:10 (lanes 3,7) or 10:0 (lanes 4,8), respectively. Infected cells were either labeled with $^{32}PO_4$ at 10 μCi/ml 20-40 post-infection, HIRT-extracted and restricted with Eco RI endonuclease (lanes 1-4), or were harvested 40 hr post-infection, reinfected at a moi of 10, and the DNA analyzed in the same manner (lanes 5-8). The DNA doublet common to all lanes is mitochondrial DNA, and is observed in mock-infected cells as well (data not shown).

Ad5Δ(78.9-84.3) was not temperature sensitive for growth and had the same kinetics of infection as wt Ad5 (data not shown). Surprisingly, Ad5Δ(78.9-84.3) virus demonstrated a decided growth advantage when coinfected onto 293 or HeLa cells with wt Ad5. Cells coinfected with equal multiplicities (of 10 pfu/cell) of Ad5Δ(78.9-84.3) and Ad5 (309) were labeled with $^{32}PO_4$ and viral DNA was prepared by Hirt extraction. Restriction endonuclease analysis of these DNAs revealed preferential replication of Ad5Δ(78.9-84.3) DNA (Fig. 7, lanes 1-4), and this was reflected in the progeny virus. When virus was isolated from cells infected with a mixture of

Ad5Δ(78.9-84.3) and wt virus, and used for a second round of infection, most of the DNA replicating was Ad5Δ(78.9-84.3) DNA (Fig. 7, lanes 5-8). This inhibition by Ad5Δ(78.9-84.3) was observed with wt Ad5 virus, as well (data not shown).

DISCUSSION

We have developed a system for the facile manipulation of Ad5 sequences *in vitro* and subsequent mobilization of these plasmids into the form of homogeneous, encapsidatable stocks of virus. Fragments from both the left and right ends of Ad5 were cloned into pBR322 plasmids with Eco RI linkers, so that the terminal viral sequences were conserved. Ad5 subgenomic recombinants from both the left and right termini were tested for biological activity by ligation with viral fragments that would produce a full-length Ad5 DNA, and transfection onto 293 cells. Plasmids from both termini generated infectious virus. This protocol for incorporating plasmid DNAs into the viral genome had previously been described for the left terminus (10), and is equally as efficient for the right terminus.

When a set of overlapping subgenomic recombinants that represents a complete complement of genome sequences was transfected onto 293 cells, infectious virus was obtained. Generation of recombinant virus by cotransfection of as many as three subgenomic fragments should provide several advantages for cloning in adenovirus. Use of subgenomic fragments will obviate problems associated with manipulating the large viral genome. This approach also eliminates the possibility of contamination with wt viral DNA. Viral DNA-plasmid DNA reconstructions have usually been performed with fragments of viral DNA associated with the 55K-protein, since higher transfection efficiencies are obtained (9). This approach can be hampered by background of non-recombinant virus due to the presence of a small amount of highly infectious uncleaved viral DNA-protein complex.

Generation of infectious virus with a set of plasmid Ad sequences was at least 100-fold less efficient than with viral DNA. This lower infectivity is probably related to the structure of the termini, as viral DNA-plasmid DNA ligations are comparable in transfection efficiencies to viral DNA-viral DNA ligations (Table I). These viral DNA-clone DNA ligation products differ from totally cloned sequences in that they share a common structure, the viral DNA terminus. Recombinant Ad5 termini are modified by the presence of Eco RI linkers, and they lack the residual peptide left after proteolytic digestion of viral DNA. These features might affect either the efficiency of

replication or the stability of the termini, and thus account for the lower infectivity.

Since infectious virus results from transfection with subgenomic plasmids containing both the 0 and 100 mu termini, viral DNA replication must be capable of initiation at modified ends. Generation of infectious virus from plasmid DNAs requires cleavage at either Eco RI site at the left or right terminal sequence. Previous studies have shown that initiation of viral replication in an *in vitro* system requires a terminus freed from plasmid DNA (37). In these studies, a similar linker-modification of cloned left end sequences was a sufficient perturbation to effect some incorrect initiation of replication (37). However, initiation of replication *in vivo* must be highly specific, and relies on more than simply deoxycytidylylation of terminal protein precursor and priming at the first dGMP. In the Eco RI-cleaved plasmids used here, the termini contain a dGMP doublet, yet viral DNA with a terminal sequence identical to wt viral DNA ($\begin{matrix}CAT\ldots\\GTA\ldots\end{matrix}$) was generated. This specificity of initiation is possibly due to interaction of the terminal protein -dCMP and other replication proteins with appropriate internal recognition sequences.

As a demonstration of using plasmids containing overlapping subgenomic fragments to construct virus mutants, a nondefective variant deleted in sequences from 78.9 to 84.3 mu was isolated. The EIII region encodes at least 8 mRNAs (34) and 2 glycoproteins (38) in Ad2, but is deleted in many of the nondefective Ad2-SV40 hybrid viruses (31,32), and in mutated Ad5 (6). The sequences between the Xba I sites at 78.9 and 84.3 mu were deleted from a plasmid which was subsequently used to generate virus. The unique Xba I site joining 78.9 mu to 84.3 mu is bracketed by the transcription initiation site and polyadenylation site of the EIII region, and this short transcriptional unit produced both spliced and unspliced RNAs. Virus with the 78.9 to 84.3 deletion was indistinguishable from wt in growth kinetics, yield of progeny and level of synthesis of various late viral polypeptides. However, one surprising phenotype observed for the Ad5Δ(78.9-84.3) virus was the inhibition of wt viral DNA replication during coinfection of either HeLa or 293 cells. At least ten fold higher levels of replicating deletion mutant DNA were observed following coinfection at equal multiplicities. This inhibition must be the product of a cis effect and is probably not the result of faster replication by a shorter genome, since a similar inhibition is not observed with other deletion mutants (our unpublished observations).

We are presently exploring ways to insert genes into the EIII region for

expression during the growth cycle. Sequences inserted into this region will probably be transcribed during both the early and late phase of infection. During the latter part of the lytic cycle, the inserted fragment might be expressed at high levels, comparable to that of other late viral mRNAs. To attain this high level of expression, the inserted segment would have to have the appropriate 3' splice site and polyadenylation signals. Segments containing these signals would have to be adapted from other late genes.

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