Cloning of DNA Fragments from the Left End of the Adenovirus Type 12 Genome: Transformation by Cloned Early Region 1

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SUMMARY

The human adenovirus serotype 12 (Ad-12) EcoRI-C DNA fragment (0 to 16.5 map units) was cloned in the plasmid vector pAT153. This cloned Ad-12 EcoRI-C DNA fragment was subcloned generating recombinant plasmids which contained the Ad-12 SauI-C fragment (0 to 10.3 map units), the Ad-12 HindIII-G fragment (0 to 6.8 map units) and the Ad-12 AccI-H fragment (0 to 4.7 map units). Thus, we constructed recombinant plasmids which contain Ad-12 DNA sequences which represent all or part of the virus transforming gene region. The capacity of the cloned Ad-12 EcoRI-C DNA fragment to transform rat cells in vitro was assessed using the focus assay on primary cultures of rat cells. The specific transforming activity of this recombinant plasmid was in the same range as that found for intact Ad-12 DNA. Transformed foci which were induced by the cloned Ad-12 EcoRI-C DNA fragment were established as cell lines and the presence of Ad-12 DNA in these lines was demonstrated using the Southern blotting technique.

INTRODUCTION

The genes responsible for the morphological transformation of rodent cells in vitro by the subgroup A human adenovirus serotype 12 (Ad-12) have been mapped near the left terminus of the virus genome (Shiroki et al., 1977; Yano et al., 1977). This E1 region is transcribed early during lytic infection (Ortin et al., 1976) and is subdivided into two transcription units Ela and Elb which lie within 0 to 4.5 map units and 4.5 to 11.2 map units respectively (Fujinaga et al., 1980).

The roles of the Ela and Elb regions in transformation have been investigated by transf ecting left terminal Ad-12 DNA fragments into rat cells. A fragment of DNA representing the left terminal 16.5% of the virus genome (the EcoRI-C fragment), which contains both the Ela and Elb regions, effects classical morphological transformation (Yano et al., 1977; Shiroki et al., 1979). The AccI-H fragment (0 to 4.7 map units), which contains the Ela region only, does not effect complete transformation (Shiroki et al., 1979). The HindIII-G fragment (0 to 6.8 map units), which contains the Ela region and part of the Elb region, is considered to be the smallest fragment having the ability to induce complete transformation (Shiroki et al., 1979). It has been suggested that the Ela region is responsible for initiating transformation whilst part of the Elb region between 4.7 and 6.8 map units is necessary for the maintenance of transformation (Shiroki et al., 1979). This hypothesis cannot be tested presently because no Ad-12 mutants have been mapped to the E1 region.

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We have cloned a left terminal Ad-12 DNA fragment that contains the E1 region, thus enabling the preparation of large amounts of pure Ad-12 transforming genes. In this paper we describe the cloning in Escherichia coli of the Ad-12 EcoRI-C DNA fragment and the subcloning from this recombinant plasmid of the Ad-12 SalI-C (0 to 10.3 map units), HindIII-G and AccI-H fragments. The sequence of the left terminus of the cloned Ad-12 EcoRI-C fragment has been determined and we present data which show that our sequence, with the exception of the first six base pairs, is identical to previously published Ad-12 DNA sequences (Shinagawa & Padmanabhan, 1980; Sugisaki et al., 1980; Tolun et al., 1979). Finally, we report on the transforming activity of the cloned Ad-12 EcoRI-C fragment and demonstrate that homologous sequences can be found in rat cells transformed by this fragment.

METHODS

Cells, viruses and virus DNA. Human embryo kidney (HEK) cells were grown in Ham's F10 medium supplemented with 10% foetal calf serum (FCS). The Huie strain of Ad-12 was passaged three times in HEK cells at 5 to 10 p.f.u./cell and the virus was purified by three cycles of caesium chloride density gradient centrifugation (Russell et al., 1967). Ad-12 DNA was prepared from purified virions by a modification of the method described by Levine & Ginsberg (1968). Primary cultures of baby rat kidney (BRK) cells were prepared from 7- to 10-day-old Hooded Lister (HL) or AS rats. These cells were grown in Dulbecco's modified Eagle's medium (DME) supplemented with 8% FCS. Primary cultures of HL rat embryo brain (REB) cells were obtained from 18-day-old embryos and grown in DME supplemented with 5% FCS.

Ligation of EcoRI molecular linkers to the termini of Ad-12 DNA molecules. Ad-12 DNA molecules with flush ends were generated from DNA isolated from virions by digestion with endonuclease S1 in 30 mM-sodium acetate, 250 mM-NaCl, 4.5 mM-ZnSO₄ pH 4.6 (Shenk et al., 1975). Three 50 μl reactions were used, each containing 10 μg Ad-12 DNA together with 5, 50 or 500 units endonuclease S1. Digestions which were performed on ice for 1 h were terminated by phenol extraction. The DNA was then precipitated and the precipitates were washed extensively with 70% (v/v) ethanol. Five μg EcoRI linkers (sequence GGGAAATTCCC; Uniscience, Cambridge, U.K.) were phosphorylated using 2 units T4 polynucleotide kinase in ligation buffer [70 mM-tris–HCl pH 7.5, 10 mM-MgCl₂, 15 mM-dithiothreitol (DTT), 1 mM-ATP] for 1 h at 37 °C. A 1-25 μg amount of phosphorylated linker was ligated to 10 μg S1-treated Ad-12 DNA in 50 μl ligation buffer at room temperature for 5 h using a predetermined excess of T4 DNA ligase (Murray et al., 1979). The ligation reactions were checked by electrophoresing 5 to 10 μl of each mixture on a 12% polyacrylamide gel; ladders of ligated oligonucleotide linkers were observed. The Ad-12 DNA was separated from polymerized linkers by gel filtration on 1.5 ml Bioigel A150 columns equilibrated and run in 10 mM-tris–HCl pH 7.5, 100 mM-NaCl, 1 mM-EDTA.

Preparation of cellular DNA and plasmid DNA. High molecular weight cellular DNA was prepared according to the procedure of Gross-Bellard et al. (1973). The integrity of cellular DNA was checked on 0.4% agarose gels, using intact Ad-12 DNA as a molecular weight marker, before carrying out Southern blotting analysis (Southern, 1975). The plasmid vectors pBR322 (Bolivar et al., 1977) and pAT153 (Twigg & Sherratt, 1980) were used in the construction of recombinant plasmids. The plasmid vectors and recombinant plasmids were purified from cultures of E. coli strain HB101 (Boyer & Roulland-Dussoix, 1969) as described by Wensink et al. (1974).

Construction and propagation of recombinant DNA molecules. Linear pBR322 DNA, produced by digestion with EcoRI, was treated with a predetermined excess of calf intestinal phosphatase in 10 mM-tris–HCl pH 8, 1 mM-EDTA at 37 °C for 1 h in order to reduce the
Transformation by cloned Ad-12 E1 region

vector background. Ad-12 DNA, to which EcoRI linkers had been attached as described above, was digested to completion with EcoRI. The digested DNA was ligated to linear pBR322 at a total DNA concentration of 50 µg/ml (vector :target ratio of 1:4) using an excess of T4 DNA ligase at 12 °C for 15 h. The resulting hybrid plasmid molecules were introduced into E. coli HB101 by a transformation procedure developed by M. R. D. Scott (personal communication) from published protocols (Morrison, 1977; Norgard et al., 1978). Ampicillin-resistant colonies were selected and screened by the procedure of Hanahan & Meselson (1980). Subsequent subcloning experiments, described in the text, used essentially the same procedures. All recombinant DNA work was carried out under Category I containment conditions, in accordance with advice received from the Genetic Manipulation Advisory Group.

Labelling of nucleic acids. DNAs to be used in hybridization reactions were labelled with ³²P in vitro by nick translation (Rigby et al., 1977). Radiolabelled fragments for DNA sequencing were prepared by using reverse transcriptase to fill in the cohesive ends produced by HindIII digestion. Fifty µg pAsc 2 DNA were digested with HindIII and labelled in a 20 µl reaction containing 20 mM-tris–HCl pH 7-4, 40 mM-NaCl, 3-5 mM-MgCl₂, 10 mM-DTT, 0-2 mM each of dATP, dCTP and dGTP, and 20 µCi [α-³²P]TTP (3000 Ci/mmol, Amersham International) and 14 units reverse transcriptase for 1 h at 37 °C.

DNA sequencing. DNA sequencing was performed as described by Maxam & Gilbert (1980).

Enzymes. T4 polynucleotide kinase was purchased from Uniscience. Restriction endonucleases were purchased from Uniscience or CP laboratories (Bishop's Stortford, Herts., U.K.). Endonuclease S1 was purchased from Sigma (type III). T4 DNA ligase was purified from induced cultures of E. coli strain 1100 (NM989) and was the generous gift of L. Woods, Department of Biochemistry, Imperial College, London, U.K. AMV reverse transcriptase was obtained from J. W. Beard, Life Sciences Inc., St. Petersburg, Fla., U.S.A. E. coli DNA polymerase I, purified by the procedure of Jovin et al. (1969), was the generous gift of A. Atkinson, C.A.M.R., Porton Down, Wilts., U.K.

Gel electrophoresis. Agarose gels were cast and run in either 40 mM-tris, 5 mM-sodium acetate, 1 mM-EDTA adjusted to pH 7-8 with acetic acid, or in 89 mM-tris, 89 mM-H₃BO₃, 2.5 mM-EDTA pH 8.2. Twelve percent polyacrylamide gels were prepared and run as described by Maniatis et al. (1975). DNA-sequencing gels were as described by Sanger & Coulson (1978).

Assay of the biological activity of cloned virus DNA sequences. The ability of cloned virus sequences to transform rat cells was assayed by focus formation on primary cultures of REB or BRK cells. Cells at densities between 4 × 10⁵ and 8 × 10⁵ cells/5 cm dish were transfected with recombinant plasmid DNA using the calcium phosphate technique (Graham & van der Eb, 1973 a, b). However, no carrier DNA was used in the transfecting DNA solution. Cultures were treated with 20% glycerol in DME medium (without serum) for 2 min, 4 h after transfection (Frost & Williams, 1978). The cultures were washed three times in DME after removal of the glycerol and subsequently grown in DME supplemented with 5 to 8% foetal calf serum. Single transformed foci were picked from separate dishes and established as cell lines.

Detection of virus DNA in transformed cells. Five µg samples of high molecular weight cellular DNA were digested with restriction endonucleases and the resulting fragments were fractionated in 0-8% agarose gels. The sizes of specific fragments were determined relative to restriction endonuclease-digested Ad-12 DNA or pAsc 2 recombinant plasmid DNA. The cellular DNA fragments which were resolved in agarose gels were depurinated using 0-25 M-HCl (Wahl et al., 1979) and transferred to nitrocellulose filters using the procedure of Southern (1975). After transfer the filters were pretreated, hybridized and washed using a
modification of the procedure of Jeffreys & Flavell (1977), as described by Kidd & Glover (1980). Ten percent (w/v) dextran sulphate was included in all hybridization solutions (Wahl et al., 1979). After air-drying the filters were exposed for 17 to 96 h at −70 °C to preflashed Fuji RX X-ray film with a Fuji Mach II intensifying screen (Laskey & Mills, 1977).

Preparation of specific fragments of Ad-12 DNA and plasmid vector DNA. Restriction endonuclease-digested Ad-12 DNA, plasmid vector DNA or recombinant plasmid DNA was fractionated by electrophoresis in horizontal, low-gelling-temperature, agarose gels and specific DNA fragments were recovered essentially as described by Wieslander (1979).

RESULTS

Construction of recombinant plasmids containing segments from the transforming region of Ad-12 DNA

Adenovirus DNA cannot be radiolabelled by T4 polynucleotide kinase because a virus-coded protein binds to and blocks the 5′ termini (Carusi, 1977; Tolun et al., 1979). This blockage is not relieved by extensive protease treatment of the virus DNA because some amino acids of the terminal protein remain covalently bound to the termini (Rekosh et al., 1977; Tolun et al., 1979). Although the genomes of the human adenoviruses, serotypes 2 and 5 (Ad-2 and Ad-5), are flush-ended (Arrand & Roberts, 1979; Shinagawa & Padmanabhan, 1980), Ariga et al. (1979) reported that endonuclease S1 specifically cleaved the terminal protein away from the 5′ termini. There is uncertainty about the structure of the termini of the Ad-12 genome because Shinagawa & Padmanabhan (1980) have reported that they are flush-ended while Sugisaki et al. (1980) have suggested that the 5′ ends are extended by five nucleotides beyond the 3′ ends. However, even if the Ad-12 genome is flush-ended it is likely that some molecules in preparations of purified Ad-12 DNA contain short single-stranded stretches at the 5′ termini which would be substrates for endonuclease S1. This is suggested because Arrand & Roberts (1979) reported that some preparations of Ad-2 DNA contained molecules which lacked the 3′ terminal G residue, thus exposing the 5′ terminal C residue.

As the first step in cloning left-end fragments of the Ad-12 genome, we generated unblocked, flush-ended virus genomes by digesting protease-treated Ad-12 DNA with endonuclease S1 using conditions which minimized strand separation at the termini. EcoRI oligonucleotide linkers were then ligated to the flush-ended Ad-12 DNA molecules which resulted from endonuclease S1 digestion and excess linkers were removed by gel filtration. The Ad-12 DNA was digested to completion with EcoRI and the resulting fragments were ligated to EcoRI-cleaved pBR322 that had been treated with calf intestinal phosphatase. The resulting hybrid plasmids were used to transform E. coli HB101 and approx. 3000 ampicillin-resistant colonies were obtained from 1 μg ligated Ad-12 DNA. Clones containing the EcoRI-C fragment were identified by colony hybridization using gel-purified 32P-labelled Ad-12 EcoRI-C fragment as probe. Twenty positive colonies were obtained and plasmid DNA prepared from two of these was analysed by restriction endonuclease mapping. An Ad-12 DNA fragment indistinguishable from the authentic Ad-12 EcoRI-C fragment was found in the recombinant plasmids isolated from both colonies. The EcoRI-C fragment of the clone derived from the Ad-12 DNA that had been subjected to the least rigorous endonuclease S1 digestion was purified by gel electrophoresis and cloned in both orientations in pAT153 (see Fig. 1 b). These recombinants are named pAsc 1 and pAsc 2.

In pAsc 1 the left end of intact Ad-12 DNA is distal to the single HindIII site of pAT153. pAsc 1 was digested to completion with either HindIII or SalI and the resulting fragments were recircularized by ligation at a DNA concentration of 5 μg/ml. In this way, recombinants containing the left terminal 6.8% (pAsc 6.8) and 10.3% (pAsc 10.3) of the Ad-12 genome were obtained (Fig. 1 c).
Transformation by cloned Ad-12 E1 region

Fig. 1. Construction of recombinant plasmids containing Ad-12 DNA sequences derived from the E1 region. (a) Diagrammatic representation of the Ad-12 genome. (i) The numbers above the line refer to the conventional adenovirus map which is divided into 100 units starting at the left end; the EcoRI restriction endonuclease map of Ad-12 DNA is depicted below the line. (ii) Enlargement of the Ad-12 EcoRI-C fragment showing the Sall, HindIII and AccI cleavage sites that are nearest to the left end; other sites for AccI and HindIII have been omitted for clarity. The letters H, G and C refer to the Ad-12 AccI-H fragment, the Ad-12 HindIII-G fragment and the Ad-12 Sall-C fragment respectively. (b) Structure of pAsc 1 and pAsc 2 showing the different orientations of the virus sequences in the two plasmids. (c) Structure of pAsc 6.8 and pAsc 10.3 which contain the Ad-12 HindIII-G fragment (0 to 6.8 map units) and the Ad-12 Sall-C fragment (0 to 10.3 map units) respectively. (d) Structure of pAsc 4.7 which contains the Ad-12 AccI-H fragment (0 to 4.7 map units). In (b to d) the black regions of the recombinant plasmids represent the virus sequences. Unfilled regions in (b) and (c) represent pAT153 DNA sequences while the stippled region in (d) represents the EcoRI-AccI-A DNA fragment of pBR322. The double arrows between (a) and (b) indicate that the Ad-12 EcoRI-C fragment was initially cloned in pBR322 and subsequently subcloned (b) in pAT153. The plasmids pAsc 6.8 and pAsc 10.3 were subcloned from pAsc 1 while pAsc 4.7 was subcloned from pAsc 6.8.

In order to clone the left terminal 4.7% of the virus genome, pAsc 6.8 was digested to completion with EcoRI and AccI and the fragment corresponding to the authentic Ad-12 AccI-H DNA fragment was purified by electrophoresis in a low-gelling-temperature agarose gel. The largest AccI–EcoRI fragment of pBR322 was purified in the same way and then treated with calf intestinal phosphatase. The gel-purified virus DNA fragments and plasmid DNA fragments were ligated and the resulting hybrid plasmid molecules were used to transform E. coli HB101 to ampicillin resistance. This procedure generated recombinant plasmids containing the left terminal 4.7% (pAsc 4.7) of the Ad-12 genome (Fig. 1d).
Fig. 2. Restriction endonuclease analysis of recombinant plasmids which contain Ad-12 DNA sequences. Plasmid DNA and Ad-12 DNA were digested with restriction endonucleases and subjected to electrophoresis through a 0.8% agarose gel. Tracks 1, 3, 5 and 7 contain Ad-12 DNA digested with EcoRI, SalI, HindIII and AccI respectively; tracks 2, 4, 6 and 8 contain EcoRI-cleaved pAsc 2 DNA, EcoRI-SalI-cleaved pAsc 10-3 DNA, EcoRI-HindIII-cleaved pAsc 6-8 DNA and EcoRI-AccI-cleaved pAsc 4-7 DNA respectively; track 9, pAT153 DNA cleaved by EcoRI; track 10, pAT153 DNA forms I and II. The markers on the left of the gel refer to the sizes in kilobase pairs (kb) of the Ad-12 EcoRI DNA fragments; the Ad-12 EcoRI-F fragment (0.7 kb) was run off the end of this gel.

The virus sequences which are released from the recombinant plasmids co-migrate with their Ad-12 genomic counterparts; the cloned Ad-12 DNA fragments are identified on the right of the gel.

Fig. 2 shows that digestion of pAsc 10-3, pAsc 6-8 and pAsc 4-7 with EcoRI in combination with SalI, HindIII and AccI respectively, released fragments which co-migrated with the expected Ad-12 DNA restriction endonuclease fragments.

_Determination of the sequence of the cloned left terminal fragment of Ad-12 DNA_

DNA sequencing was used to determine exactly how close to the left terminus of Ad-12 DNA our clones extend. The sequencing strategy is illustrated diagrammatically in Fig. 3 (a). By sequencing from the vector HindIII site of pAsc 2 we were able to read firstly the sequence of a segment of pAT153 DNA, then that of the synthetic linker and finally the sequence of 55 nucleotides of the left end of the cloned Ad-12 DNA fragment. The sequence we obtained, excluding the sequence of pAT153, is shown in Fig. 3 (b). The complete sequence we obtained is shown alongside the sequencing gel in Fig. 4. In Fig. 3 (b) we have compared the Ad-12 sequence, which we have determined, with those that have been published previously (Shinagawa & Padmanabhan, 1980; Sugisaki et al., 1980; Tolun et al., 1979). Our Ad-12
sequence is identical to the published sequences from nucleotide 9 to nucleotide 57. The Ad-12 DNA sequences which were determined by Shinagawa & Padmanabhan (1980) and Sugisaki et al. (1980) are different between nucleotides 1 and 8 and we have been unable to match completely their sequences with our own in this region.

Assay of the transforming activity of the cloned Ad-12 EcoRI-C fragment

The capacity of the Ad-12 EcoRI-C recombinant plasmid to transform rat cells was assessed using the focus assay with primary cultures of REB cells or BRK cells. The results of four typical experiments which were performed on cultures of REB cells are presented in Table 1. These experiments were performed on cell cultures which originated from the
Table 1. *Analysis of the transforming activity of pAsc 2 on primary cultures of Hooded Lister rat embryo brain cells*

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*The cells were at a density of 4 × 10⁵ cells/50 mm dish.
† Calculated from the fraction of the plasmid which the virus sequences constitute and the fraction of the Ad-12 genome which these sequences represent (Dijkema et al., 1979).
‡ The Ad-12 EcoRI-C fragment was gel-purified from EcoRI-cut pAsc 2.
§ pAsc 2 was cut with EcoRI but the virus sequences and plasmid sequences were not separated.

Hooded Lister strain of rat; we have observed no tissue difference or strain differences with regard to transformation by Ad-12 DNA. The transforming activity of pAsc 2 is presented in terms of microgram genome equivalents so that the efficiency of transformation by intact Ad-12 genomes can be compared directly to that of the recombinant plasmid. The fact that the virus sequences represent only 60% of the sequences of the recombinant plasmid has been taken into account in these calculations.

Experiment 1 in Table 1 demonstrates that the specific transforming activity of the cloned Ad-12 EcoRI-C fragment is similar to that of intact Ad-12 DNA. The suggestion that the transforming activity of the cloned virus sequences was enhanced when they were cleaved away from or purified away from the plasmid sequences (Table 1, experiments 1 and 2) was not confirmed by experiment 3 in Table 1. The transforming activity of pAsc 2 averaged 0.7 foci/µg genome equivalent/4 × 10⁵ cells (Table 1, experiment 3) regardless of whether the plasmid was intact or digested with EcoRI prior to transfection.

It is well established that the specific transforming activity of transfecting DNA decreases with increasing DNA concentration (Graham & van der Eb, 1973b). We have observed this phenomenon with pAsc 2 (Table 1, experiment 3) but, as demonstrated in experiment 4 of Table 1, we have not found it to be a consistent feature of every experiment in which we have titrated the transforming activity of this recombinant plasmid.

Fig. 4. DNA sequence of the left terminus of the cloned Ad-12 EcoRI-C fragment. The virus–vector junction containing the left terminus of the cloned Ad-12 EcoRI-C fragment was sequenced using the chemical DNA sequencing method described by Maxam & Gilbert (1980). The letters T, C, A and G above the tracks indicate the particular base-specific cleavage. Samples were subjected to electrophoresis at a constant current of 30 mA on an 8% polyacrylamide gel. The four samples in the right half of the gel were electrophoresed for 90 min at which time the samples in the left half were loaded and electrophoresis continued for a further 90 min. An autoradiograph of the gel was obtained after electrophoresis, by exposing X-ray film to the gel at -70 °C. The expected sequence of pAT153, which is identical to pBR322 in this region (Sutcliffe, 1979), is followed by the synthetic linker and the sequence of the cloned fragment of Ad-12 DNA.
Fig. 5. The edge of a pAsc 2 DNA-transformed BRK cell focus. The background of normal BRK cells is apparent in the bottom right of the field. The cells were fixed, and stained with Giemsa. Bar marker represents 40 μm.

Properties of rat cells transformed by cloned Ad-12 EcoRI-C DNA fragments

Foci of Ad-12 EcoRI-C-transformed cells usually became visible to the naked eye 17 to 21 days after transfection. Each focus was multi-layered and contained small epithelioid cells (Fig. 5). Ad-12 EcoRI-C induced foci established into cell lines with no difficulty and no 'crisis' was observed. All Ad-12 EcoRI-C cell lines, tested by the indirect immunofluorescence technique for the presence of Ad-12 T antigen, showed the nuclear fluorescence characteristic of Ad-12-transformed rat cells. Generally the properties of the Ad-12 EcoRI-C-transformed rat cell lines were indistinguishable from Ad-12 virion-transformed rat cells (Gallimore & Paraskeva, 1980), as reported by van der Eb et al. (1980).

Some cell lines which were established from Ad-12 EcoRI-C-induced transformed foci have been cultured continuously for more than a year and the morphology of the cells and their T antigen-staining patterns have not altered.

Detection in Ad-12 EcoRI-C-transformed rat cell lines of sequences homologous to the left terminal 16.5% of the virus genome

Five Ad-12 EcoRI-C recombinant plasmid DNA-transformed rat cell lines, designated pAsc 2 HLBRK1, pAsc 2 HLBRK2, pAsc 2 ASBRK1, pAsc 2 HLREB1 and pAsc 2 HLREB2, were established from single foci picked off separate dishes, to ensure that each cell line was derived from a unique transformation event. These cell lines were analysed by the Southern blotting molecular hybridization technique using intact Ad-12 DNA and gel-purified Ad-12 EcoRI-C DNA fragment as radiolabelled probes.

Total cell DNA from the five cell lines named above was digested with the restriction endonucleases SstI or BgII; neither enzyme cleaves within the virus sequences or plasmid sequences of pAsc 2. The five lines analysed all contained sequences which were homologous to the Ad-12 EcoRI-C probe and which were integrated into high molecular weight SstI or BgII DNA fragments (Fig. 6, tracks 1 to 5 and 7 to 11). Both pAsc 2 ASBRK1 and pAsc 2 HLREB1 appeared to contain only one insert of virus sequences (Fig. 6, tracks 3 and 9, and 4 and 10 respectively) which resided in the high molecular weight DNA fragments mentioned above. The remaining three lines each contained additional inserts of virus
Transformation by cloned Ad-12 E1 region

Fig. 6. Detection of Ad-12 DNA in five lines of pAsc 2-transformed rat cell lines using the Southern blotting technique. Tracks 1, 2, 3, 4 and 5, BglII digestion of DNA from pAsc 2 HLBRK1, pAsc 2 HLBRK2, pAsc 2 ASBRK1, pAsc 2 HLREB1 and pAsc 2 HLREB2 respectively; tracks 7, 8, 9, 10 and 11, as for tracks 1 to 5 except that the cellular DNA was digested with SstI; track 6, SstI-digested normal rat cell DNA; tracks 12, 13, 14 and 15, SstI-digested normal rat cell DNA mixed with 20 pg undigested pAsc 2 DNA, SstI-digested pAsc 2 DNA, BamHI-cleaved pAsc 2 DNA and EcoRI-cleaved pAsc 2 DNA respectively; tracks 16 and 17, SstI-digested normal rat cell DNA containing 20 pg EcoRI-cleaved Ad-12 DNA and 20 pg SstI-cleaved Ad-12 DNA respectively. Tracks 1 to 11, 12 to 15, and 16 and 17 were hybridized with 32P-labelled Ad-12 EcoRI-C DNA fragment, pAsc 2 DNA and genomic Ad-12 DNA respectively. The sizes of the Ad-12 EcoRI DNA fragments are given as markers.

sequences which were detected in smaller DNA fragments (Fig. 6, tracks 1, 2, 5 and 7). The intensities of the bands in the autoradiograms suggested that each cell line contained multiple copies of virus sequences.

Since the five lines analysed were all derived from cells that had been transfected with EcoRI-digested pAsc 2 plasmid, it was of interest to determine whether the recognition sequence of this enzyme had been reconstructed in any of the virus inserts. No fragments were found to co-migrate with the Ad-12 EcoRI-C fragment following EcoRI digestion of transformed cell DNA (Fig. 7, lanes 1, 3, 9, 11 and 13). Therefore, none of the lines examined contained polymeric virus inserts, with respect to the EcoRI recognition sequence.

No evidence was found that any of the cell lines analysed carried unintegrated, monomeric recombinant plasmids (Fig. 7, lanes 2, 4, 10, 12 and 14). The failure to detect any fragments equal in size to the Ad-12 EcoRI-C fragment excluded the possibility that the high molecular weight fragments, which hybridized with the probe DNA after SstI or BglII digestion of transformed cell DNA, were oligomers of the pAsc 2 plasmid.

DISCUSSION

This paper describes the construction of recombinant plasmids that contain Ad-12 sequences which represent part or all of the left terminal 16.5% of the virus genome. The cloning procedure was designed to obviate difficulties during ligation of the virus and vector DNA which would have arisen due to blockage of the left terminal 5' end of the virus genome by the terminal protein. While this work was in progress Stenlund et al. (1980) and Stow
Fig. 7. Detection of Ad-12 DNA sequences in five pAsc 2-transformed rat cell lines using the Southern blotting technique; analysis of undigested or EcoRI-digested cellular DNA. Tracks 1, 3, 9, 11 and 13, EcoRI digestion of DNA from pAsc 2 HLREB2, pAsc 2 HLREB1, pAsc 2 ASBRK1, pAsc 2 HLBRK2 and pAsc 2 HLBRK1 respectively; the same order was repeated in tracks 2, 4, 10, 12 and 14, but the cellular DNA was undigested in these tracks; tracks 5, 6 and 7 contain EcoRI-digested normal rat cell DNA mixed with 20 pg undigested pAsc 2 DNA, BamHI-cleaved pAsc 2 DNA and EcoRI-cleaved pAsc 2 DNA respectively; track 8, EcoRI digestion of a mixture of normal rat cell DNA and 20 pg Ad-12 DNA; 20 pg pAsc 2 DNA and 20 pg complete Ad-12 genomes are equivalent to 2.4 copies/rat cell genome and 0.64 copies/rat cell genome respectively. Track 15, normal rat cell DNA only. All the tracks were hybridized with $^{32}$P-labelled Ad-12 DNA.

(1981) described different procedures for cloning terminal adenovirus DNA fragments which circumvented problems resulting from the terminal protein.

By sequencing from the vector HindIII site of pAsc 2 we were able to determine the sequence of 55 nucleotides of the left end of the cloned Ad-12 DNA fragment. We attempted to align our sequence with the published Ad-12 sequences (Shinagawa & Padmanabhan, 1980; Sugisaki et al., 1980) even though they themselves do not match at the left end between positions 1 and 8. The match was imperfect unless gaps were inserted in one or other sequence to maximize homology, no matter which position was assumed as the start of our sequence. These differences in our sequence might indicate that our cloned Ad-12 DNA was derived from a variant molecule or that sequence changes occurred during the cloning procedure. Beyond this region of imperfect homology we found that 49 nucleotides of our sequence were identical to a stretch of the published sequences which starts at position 9 and ends at position 57. These 49 base pairs include the 14 base pair region of homology which extends from position 9 to 22 at the left end of the DNA of all serotypes (Tolun et al., 1979; van Ormondt et al., 1980). This highly conserved sequence is thought to be important in the replication of adenoviruses. While we have no evidence to suggest that adenovirus DNA molecules with alterations in the left terminal 8 base pairs would be capable of replicating, the sequence differences which we found in the left terminus of our cloned Ad-12 EcoRI-C fragment did not impair the transformation capacity of this fragment. This is consistent with the discovery that the left terminal 1% of the adenovirus genome is dispensable for transformation (Graham et al., 1974).
Transformation by cloned Ad-12 E1 region

The specific transforming activity of the pAsc 2 plasmid, regardless of whether it had been digested with EcoRI, was in the same range as that found for intact Ad-12 DNA. Equivalent transforming activity for intact virus DNA molecules and subgenomic fragments which represent all or part of the E1 region have been noted for other adenovirus serotypes (van der Eb et al., 1977; Dijkema et al., 1979). The values we obtained for the specific transforming activity of the cloned Ad-12 EcoRI-C fragment agree closely with the average value of 0.5 foci/microgram genome equivalent for this fragment which was reported by van der Eb et al. (1980). The low transforming activity of adenovirus DNA does not result from inhibition of ligation of the virus DNA to the host genome (by residues of the terminal protein), since our cloned Ad-12-transforming gene region is equally inefficient at morphologically transforming rat cells.

Southern blotting molecular hybridization analysis performed on five pAsc 2-transformed rat cell lines demonstrated that each line contained sequences homologous to the virus DNA fragment. The analysis failed to determine the number of inserts of Ad-12 DNA in these lines, but indicated that none of the lines contained the intact, tandemly repeated Ad-12 EcoRI-C fragment. A more detailed analysis is underway and we hope that by using other enzymes which cleave pAsc 2 once only, we will be able to decide conclusively whether these cell lines contain polymeric virus inserts.

Although this paper presents evidence of the biological activity of the cloned Ad-12 EcoRI-C fragment only, we have succeeded in transforming primary rat cells with pAsc 10-3, pAsc 6-8 and pAsc 4-7. Our preliminary results indicate that the specific transforming activities of pAsc 6-8 and pAsc 4-7 are much lower than that of pAsc 2; van der Eb et al. (1980) reported previously that the transforming activity of the Ad-12 HindIII-G fragment was reduced relative to that of the Ad-12 EcoRI-C fragment or intact Ad-12 DNA. The plasmid pAsc 2 has also been used to transfect cultures of human cells and we have isolated several human embryo kidney cell lines and human embryo retinal cell lines following transformation by the cloned Ad-12 EcoRI-C fragment. The results of analyses which have been performed on all the rat cell lines and the human cell lines will be reported elsewhere.

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