Isolation of DNA from agarose gels using DEAE-paper. Application to restriction site mapping of adenovirus type 16 DNA

Gösta Winberg and Marie-Louise Hammarskjöld

Department of Virology, Karolinska Institutet, c/o SBL, S 105 21 Stockholm, Sweden

ABSTRACT

A new method for isolating DNA from agarose gels is described. The method involves the simultaneous transfer of all DNA-fragments from an agarose slab gel onto DEAE-cellulose paper and the elution of the individual fragments from the paper with 1 M NaCl. DNA isolated from agarose gels in this way is susceptible to cleavage with several restriction endonucleases, and can be labeled in vitro with E coli DNA-polymerase I, T_4 DNA-polymerase and T_4 polynucleotide kinase.

We have used the method to construct restriction endonuclease maps of adenovirus type 16 DNA.

INTRODUCTION

Isolation of DNA-fragments is a prerequisite for mapping of genetic material with restriction endonucleases, for DNA sequencing, DNA-hybridization studies and recombinant DNA techniques. Fragments ranging in size from about 0.15 Kb (kilo-base pairs) up to more than 40 Kb may be separated by electrophoresis in agarose gels, and several methods have been described for extracting fragments from the agarose gels for secondary cleavage with restriction endonucleases or other experimental procedures (1-12).

The present method is designed to suit experiments where many DNA-fragments have to be isolated simultaneously from an agarose gel. Technically, we have modified the transfer method of Southern (13) by substituting DEAE-cellulose paper for nitrocellulose filters. Transfer of DNA-fragments to DEAE-paper sheets combines extraction and purification into one preparatory step. Since this procedure is performed simultaneously on all fragments in a gel, the amount of work involved is significantly reduced. We have applied the method to the construction of several restriction endonuclease maps of adenovirus type 16, and for studying the genome structure of subgenomic DNA in incomplete adenovirus particles and infected cells.

MATERIALS AND METHODS

Virus and nucleic acid. The adenovirus type 16 strain used was plaque purified four times from the prototype strain Chang 79. Virus was purified as described (14) except that 10% ethanol was added to the 0.2% sodium deoxy-cholate lysis buffer to prevent the formation of deoxycholate micelles with a too high aggregation number. DNA was extracted from the purified virions with pronase, Sarcosyl (Ciba) and phenol as described (14). In vivo label-ing of virus with $^{32}PO_4$ was done as follows; 1655 cm^2 roller bottles of HeLa cells (J.F. Williams) were infected with 50 pfu/cell. At 1½ h. p.i. the medium was changed to 300 ml Eagle MEM -phosphate +0.5% FCS and 370 MBq of ^{32}P-orthophosphate (Amersham, PBS. 41) was added. Harvest was at 60 h. p.i. In vitro labeling of 5'-termini of restriction fragments was done essen-tially as described by Maxam and Gilbert (15). Labeling of 3'-ends followed the method of Englund (16), and "nick translation" was done according to Maniatis et. al. (17).

Enzymes. Restriction endonucleases Eco R I, Bam H I and Taq I were puri-fied as described (19,20,21). Sma I, Hae III and Hin f were gifts from Gunilla Isaksson, Elisabeth Ljungquist and Göran Akusjärvi respectively. Alu I and Hpa I were purchased from New England Biolabs and Hind III and Bgl I were from Bethesda Research Labs. Two standard buffers were used for incubation mixtures; standard buffer I (SB I) was 20 mM Tris-HCl, pH 7.4, 15 mM $MgCl_2$, standard buffer II (SB II) was 10 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM 2-mercaptoethanol. Incubation conditions for the individual enzymes were as follows; Eco R I: SB I + 100 mM NaCl, Bam H I: SB I, Hae III: SB I or SB II + 5 mM NaCl, Hin f, Hind III and Alu I: SB II + 50 mM NaCl, Hpa I: SB II + 20 mM KCl, Bgl I: SB II + 66 mM KCl, Sma I: 30 mM glycine-KOH, pH 9.2, 3 mM $MgCl_2$, 20 mM KCl, Taq I: 10 mM Tris-HCl, pH 7.5, 2 mM $MgCl_2$, 50 mM NaCl (pH at 70°C). All enzymes were incubated at 37°C except Taq I which was incubated at 70°C. Polynucleotide kinase and T_4 DNA-polymerase were purified from E. coli B cells infected with T_4 am N 82 according to a modification of the method of Richardson (22, 23). E. coli DNA-polymerase I was a gift from Lambert Skoog.

Preparation of adenovirus DNA-protein complex. To 1 ml of virus suspen-sion (ca 5 OD_{260}/ml) in CsCl, density 1.333 g/cm^3, 20 mM Tris-HCl, pH 8.0, 2 mM EDTA-Na$_2$ was added 0.4 g urea (Schwarz/Mann, Ultrapure), to give a final volume of 1.34 ml of 1.94 M CsCl and 5 M urea. The solution was kept for 30 min. at 0°C and was then applied to a 10 ml column of Sepharose CL-2B (Pharmacia) in 5 M urea, 2 M NaCl, 20 mM Tris-HCl, pH 8.0, 0.2 mM

EDTA-Na$_2$. The void volume fractions were collected, and dialyzed for 30 min.
at 23°C in incubation buffer (see above) in the following way; to 45 μl of
the void volume fraction was added 5 μl 10x concentrate of the incubation
buffer and the 50 μl drop was placed on a 13 mm ϕ. Nucleopore polycarbonate
membrane (0.03 μm pore size, Nucleopore Corp., Pleasanton, Ca 94566) floating
on 5 ml 1x incubation buffer in a 35 mm Petri dish. After 30 min. the dia-
lysis was complete, and the DNA was transferred to an Eppendorff tube, re-
striction enzyme was added and digestion was carried out for the necessary
time. The DNA-protein complex (DPC) prepared in this way had an OD$_{260}$/OD$_{280}$
ratio of 1.8-1.87, was free from urea and salt and was susceptible to
digestion with all restriction endonucleases used in this study.

Agarose electrophoresis of DNA. After digestion with restriction enzymes,
one tenth volume of a solution of 40% w/v Ficoll 400 (Pharmacia), 50 mM EDTA
and 0.3% bromophenol blue was added to each sample. The digests were sepa-
rated on 0.8-1.5% vertical agarose slab gels, 14x17x0.4 cm, or, 0.35-1.2%
horizontal agarose gels, 21x27x0.5-1 cm. The maximum load of DNA was 0.1
μg/mm^2 slot area, and the gels were run at 4 V/cm at 4°C. The gels were pre-
pared and run in 89 mM Tris, 89 mM boric acid, 2.5 mM EDTA, pH ca 8.3. After
staining in the same buffer with 0.5 μg/ml ethidium bromide, the gels were
photographed under short wave UV-light through a Wratten no. 29 filter on
Kodak Plus-X film. Autoradiography of gels or DEAE-filters was done on
Kodak XR-1 film.

RESULTS

Transfer of DNA from agarose gels to DEAE-paper. For use in the DNA trans-
fer technique the DEAE paper (DE-81, Whatman) is precycled as recommended by
the manufacturer for dry DEAE-cellulose. This step is rather difficult to
perform due to the low wet strength of the paper, but we consider it necessary
for two reasons. First, only after precycling is the full adsorbtion capacity
of the ion-exchanger available. Second, since DNA can be irreversibly adsorbed
to cellulose by drying (24), precycling is used to ensure complete hydration
the cellulose fibers. 20-40 quarter sheets of DE-81 are precycled at a time,
and the sheets are rinsed two times for 2 h. in a container with about 100L
of deionized water after the acid and the alkali treatments. During precyc-
ling, the papers should be transferred between the liquid containers in a
thick stack, since single sheets tear easily. When precycled, the papers
are placed in 1xSSC over night to equilibrate and then sandwiched indivi-
dually between polythene plastic sheets. The DE-81 filters may be stored

wet in this way, and retain the adsorbtion capacity for DNA for at least 6 months when kept at 4^OC. All subsequent handling of the DE-81 paper is done with the moist paper adhering to a sheet of polythene plastic.

For the DNA transfer, a 5 cm thick foam plastic sponge is soaked in a trough containing 1xSSC (0.15 M NaCl, 0.015 M Na-citrate, pH 7.0), taking care to press the air out completely with a roller. A sheet of Whatman 540 filter paper is soaked in 1xSSC and placed on the sponge, and the agarose gel is laid on top. Spacers are placed along all four sides. The polythene plastic sheet on one side of the DE-81 paper is peeled away, leaving the remaining sheet to act as a support for the fragile DE-81 paper. The paper is now lowered onto the surface of the gel by holding the corners of the plastic support.

After removing the plastic support, 3 sheets of moist Whatman 3MM paper are placed on the DE-81 paper and a stack of tissue paper is placed on top of them. A light weight (e.g. a 4 mm glass plate) is put on the tissue pa- per stack so that the solvent flow will not be too fast. It is important to flood the gel surface with 1xSSC before covering it with DE-81 paper, to ensure that no air bubbles are trapped. The trough is filled about 2.5 cm deep with 1xSSC and the transfer is allowed to proceed for about 24 hours. After the transfer is completed, the Whatman 3MM papers are removed and the DE-81 sheet is flooded for a few minutes with 1xSSC to avoid sticking of DE-81 fibres to the gel surface. The DE-81 paper is then covered with a sheet of polythene plastic, excess buffer is pressed away, and the paper and plastic backing are peeled away together beginning from one corner. The entire sheet of DE-81 paper can thus be lifted without being torn. The paper is then transferred to a trough with 1xSSC and washed for 15-20 min.

Detection and preparation of individual DNA fragments. Both unlabeled and radioactive DNA fragments can be effectively detected on the DE-81 papers. For detection of unlabeled fragments, the paper is lifted out of the washing trough on a plastic sheet, a few ml of 1xSSC with 1 μg/ml of ethidium bromide are pipetted onto the paper, and a second plastic sheet is laid on top. The stain is distributed over the paper, which is now ready for viewing under UV-light. Using a short wave UV-light source, the limit of visual detection is 20-30 ng of DNA in a band 11x1.5 mm, or about 1-1.5 ng/mm^2 (fig. 1). The position of the different bands is then simply marked on the plastic with a marking pen, and the corresponding paper strips are cut out of the DE-81 sheet with a scalpel as shown in
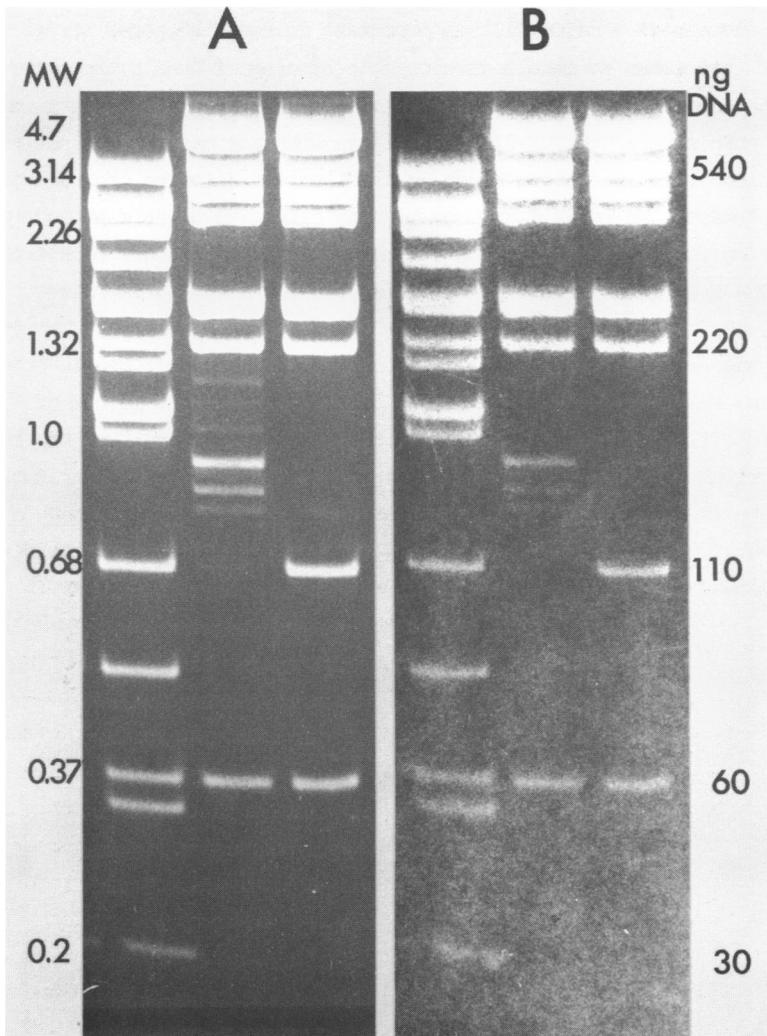
Figure 1. Panel A shows an ethidium bromide-stained 1.4% agarose gel. The mol.wt. figures on the left side are in megadaltons. The three lanes show the cleavage pattern of two different strains of Ad 16, after cleavage with either Bam HI+Sma I or Sma I only. Panel B shows the Ethidium bromide stained DE-81 paper to which the gel in A was transferred. The figures on the right side show the amount of DNA in the corresponding bands.

figure 2. The paper strips are placed in Eppendorff tubes for sub-
sequent elution (see below). It is important to keep the paper strips
moist at all times to reduce irreversible binding of DNA to the paper.

Detection of radioactive fragments is performed by placing one X-ray
film on each side of the DE-81/plastic sandwich and marking the position
of the films relative to the paper by stapling the films to the sandwich.
The staplemarks are readily visible on both films and paper sandwich, and
by aligning the staple-holes, the fragments can be accurately localized.

The DNA-fragments are eluted by adding 100-200 μl of 40 mM Tris-
CH_3COOH, pH 7.8, 10 mM Na-acetate, 1 mM EDTA, 1 M NaCl. The paper strip
is then mascerated in the Eppendorff tube with a micropipet tip in order
to suspend the paper fibres. A hole is pierced through the bottom of the
tube (lid off), it is inserted into another Eppendorff tube, and the two
are centrifuged together for a few minutes in the Eppendorff centrifuge.
The eluate is now freed from residual DEAE-fibres by passage through a
micropipet tip plugged with siliconized glass wool. The DNA is precipi-
tated by the addition of 5-10 μg t-RNA and 2.5 vol. ethanol. After 15
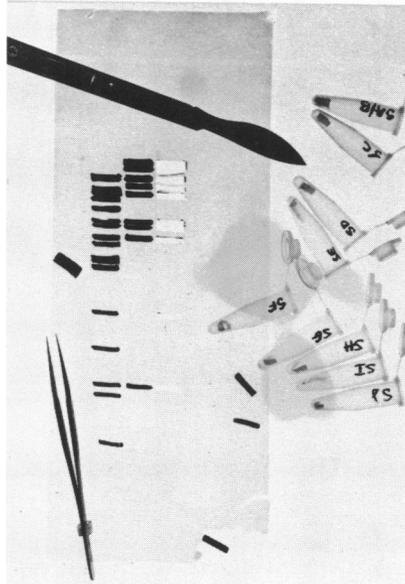

Figure 2. The flourescent bands on the DE-81 paper in figure 1, panel B have
been marked on the plastic covering the paper, and some fragments have been
cut out for counting the radioactive tracer DNA.

min at -70°C, the DNA is pelleted at 20,000 rpm for 30 min in the
Sorvall SS-34 rotor (holder No 00381 fits Eppendorff tubes).

Efficiency of transfer to and elution from DEAE-paper. Conditions
that influence the kinetics of transfer of DNA from the agarose gels to
DEAE-paper were investigated. The efficiency of transfer was tested
using uniformly ^{32}P-labeled adenovirus DNA or 5'-^{32}P labeled restriction
digests of viral DNA. The dependence on time of transfer, gel concentra-
tion and size of the DNA fragments is shown in figure 3. For fragments with
a size of 6×10^6 daltons or less, the optimal transfer time is about 24 h.
Such fragments are transferred and bound to the paper in equimolar quan-
tities and in more than 90% yield (panel A). In contrast, after a 6 h
transfer only fragments below 3×10^6 daltons are transferred in good
yield (panel B). For DNA the size of the adenovirus genome (23×10^6
daltons), no more than about 40% is transferred.

The binding of DNA to DEAE-paper is quantitative at a salt concentra-
tion equal to 1xSSC, and elution from the DEAE-paper begins at about
3xSSC and is nearly complete at 4xSSC. The binding capacity of the
DE-81 paper was tested in two ways. When a one cm^2 square of DE-81 is
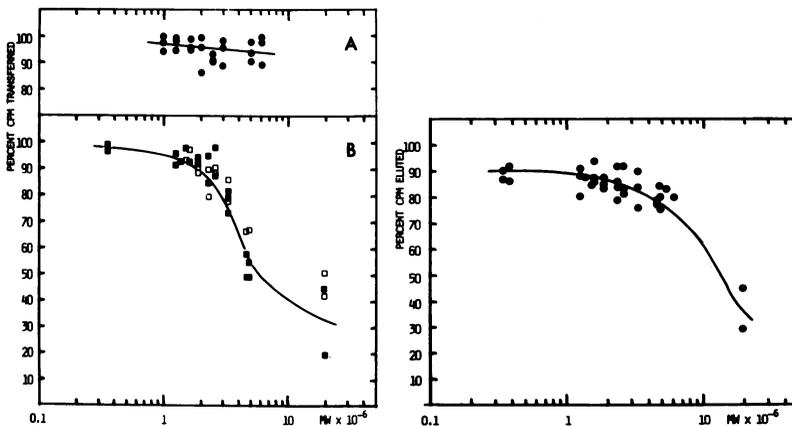charged with a small volume of 1xSSC containing varying amounts of DNA

Figure 3 (left). Panel A shows the efficiency of transfer of DNA-fragments
from a 1.5% agarose gel to DE-81 paper after 24h at room temperature.
Panel B shows the transfer after 6h. Filled squares are values obtained
with a 1.5% gel, and unfilled squares represent a 0.5% gel.

Figure 4 (right). The efficiency of elution of DNA from DE-81 paper
strips.

and a trace amount of radioactive DNA, the binding is quantitative for amounts below 1 μg, and the binding curve levels off above 15 μg. This experiment does not fully reflect the experimental conditions of the transfer, but indicates a capacity of about 15 $\mu g/cm^2$. From direct transfer experiments one can calculate that 3-5 $\mu g/cm^2$ can be bound without leakage of radioactive DNA through a single layer of DE-81 paper. The capacity is at least sufficient to bind quantitatively the fragments produced by electrophoresis of a Bam H I digest of 100 μg Ad 16 DNA on a 1 cm thick gel if a maximum load of 0.1 $\mu g/mm^2$ is used.

The recovery of DNA after salt elution of the DE-81 paper is presented in figure 4. For fragments of less than 6×10^6 daltons, the recovery is more than 75%. Since the efficiency of transfer after 24 h averages 90%, the total yield is at least 60% for DNA-fragments less than 6×10^6 daltons. For large DNA molecules, i.e. adenovirus DNA (23×10^6 daltons), the total yield is limited to 10-20%.

Use of DEAE-cellulose slurry for DNA-fragment preparation. The DE-81 paper method is useful on an analytical scale, when small strips of DE-81 paper can be handled in Eppendorff tubes. To overcome the practical problems encountered when trying to scale up the preparations by this method, we tried electrophoresis of DNA-fragments into microgranular DEAE-cellulose (DE-52, Whatman), in a fashion similar to the hydroxyapatite method described by Tabak and Flavell (7). A narrow trough in the agarose gel was filled with DE-52 slurry and the DNA was electrophoresed into the DE-52. The slurry was removed and packed into a small column and the DNA was eluted with 1 M NaCl in the buffer described above. The purity and yield of DNA by this method were as described for the DE-81 method. It was found necessary to place a "basket" of fine nylon net (10) in the trough before pouring in the DE-52 slurry, since otherwise, the DE-52 granules would stick hard to the wall of the trough (on the cathode side) due to their positive charge.

Application to restriction site mapping of adenovirus type 16 DNA. Maps were constructed for the enzymes Sma I, Hind III and Bgl I. ^{32}P-labeled adenovirus 16 DNA was partially cleaved. The partial digests were separated on 1.2% and 0.4% horizontal agarose gels and the fragments in each gel were transferred to a DE-81 paper as described. The partial fragments were identified by autoradiography and were isolated as described. Each partial fragment was then recut with at least a 2-fold excess of the same enzyme and the digest was separated on a second agarose gel. The restric-
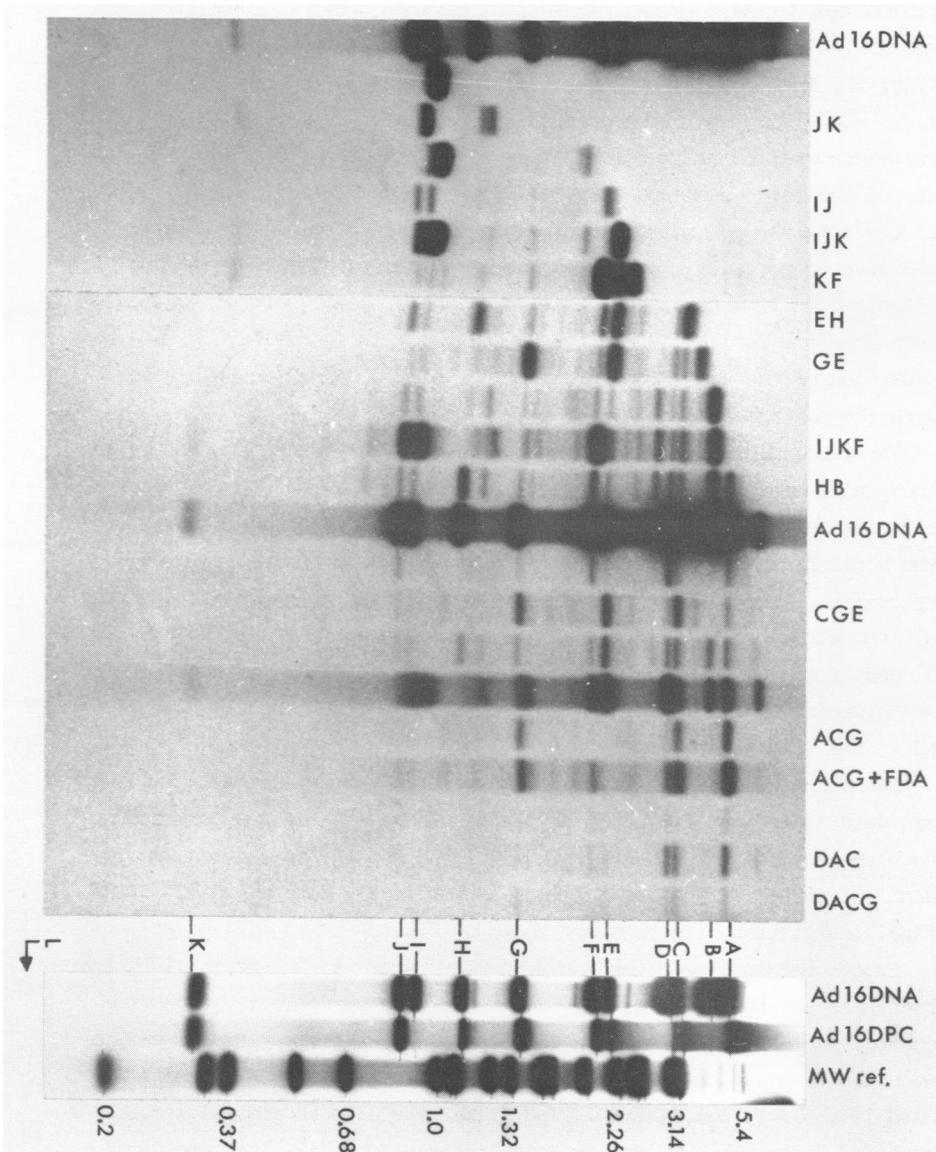
Figure 5. Partial Hind III fragments of Ad 16 DNA were isolated by the DE-81 paper method and were recleaved with Hind III. The gel on the left side shows the recleaved partial fragments and the letters at the top refer to the corresponding restriction fragments of the viral DNA (compare with the Hind III map in fig.6). The gel on the right hand side shows the determination of the Hind III terminal fragments by cleavage of the DNA-protein complex (DPC). The mol. wt. reference is Ad 16 DNA cleaved with Bam H I plus Sma I. Mol. wt's are in megadaltons.

tion fragments constituting a specific partial fragment could now be identified and the sequence of restriction fragments could be deduced (fig. 5). The partial fragments isolated cannot be expected to be very pure, since they are often present in small amounts compared to other fragments near them. Therefore it is necessary to control that the mol. wt. of the major bands that appear after recleaving add up to the size of the original partial fragment. This makes it possible to identify comigrating partials, such as in the lane labeled ACG+FDA in figure 5. Often a band remains at the position of the original partial fragment, even when the recleavage has been complete. This is due to the fact that a portion of the background smear of ^{32}P-nicked DNA is cut out along with the partial fragment (fig. 5).

The terminal restriction fragments of the DNA were determined by an independent method. We made use of the observation that the terminal restriction fragments are retained at the start of the agarose gel if the viral DNA has been extracted without the use of proteolytic en- zymes (25). The electrophoretic mobility of these fragments is apparently altered due to the presence of a protein, covalently linked to the 5'-end of the genome (26). Figure 5 shows the cleavage pattern of the DNA-protein complex (DPC) compared to that of conventionally purified Ad 16 DNA (right hand panel).

The orientation of the restriction site maps constructed in this way was determined, and the position of the cleavage sites was corrected by double cleavage of the DNA with Bam H I and one of the enzymes Sma I, Hind III or Bgl I. The Bam H I map was previously determined (27). The final restriction site maps are shown in figure 6.

Functional purity of the isolated DNA-fragments. DNA-fragments prepared both with the DE-81 and the DE-52 methods were sufficiently free from inhibiting substances to allow complete cleavage without excess enzyme with the restriction enzymes Alu I, Bam H I, Bgl I, Eco R I, Hae III, Hind III, Hin f, Hpa I, Sma I, Taq I. We were also successful in labeling purified fragments in vitro by "nick translation" (17, 18), and in labeling 3' and dephosphorylated 5' ends with T_4-polymerase (16) and T_4-polynucleotide kinase (15) respectively.

DISCUSSION

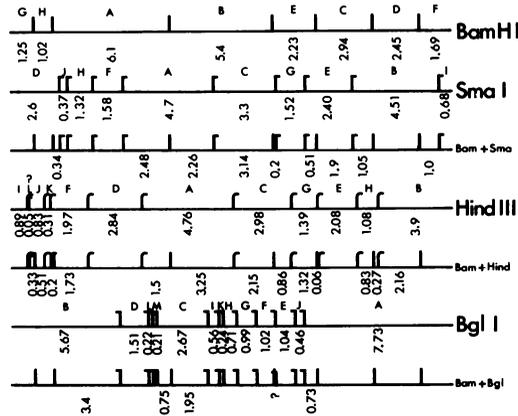Experimental designs, which involve the preparation of many DNA-fragments

Figure 6. Restriction site maps of Ad 16 DNA obtained with the DE-81 paper method. The proper position of the cut sites and the orientation of the maps was derived by double cleavage with Bam H I. The mol. wt's of the fragments are in megadaltons. The G-C rich end is to the left and the A-T rich end to the right. The mol. wt. of Ad 16 DNA was taken as 23.06 megadaltons + 2.3% (6.47 Øx 174 RF II units by E.M.).

from an agarose gel for secondary analysis, are very laborious if any of the more complex methods for fragment isolation (5-10) have to be used in order to ensure a sufficient purity of the fragments. Our studies of the DNA sequences present in subgenomic DNA in incomplete adenovirus particles and infected cells (M-L. H. and G.W., (1979), submitted) required a method by which many accurately sized subgenomic DNA-fragments could be isolated from an agarose gel and recleaved with different restriction enzymes. The method described greatly simplified this work. In addition, it proved to be convenient for constructing restriction enzyme maps of the viral genome.

Like the "northern" blotting technique of Alwine et.al. (28) and the protein transfer procedure of Towbin et.al. (29), our method extends the use of Southerns original transfer technique (13). Although the DEAE-paper method can not claim any advantage over existing procedures as regards the overall yield of DNA or the size of DNA that can be effective-ly recovered, the combination of simultaneous extraction and purifica-tion of DNA-fragments from agarose will yet be of use in many applications where isolation of DNA-fragments is required.

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