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Cleavage of Adenovirus Type 2 DNA into Six Unique Fragments by Endonuclease R·RI

(restriction enzyme/electron microscopy/DNA gel electrophoresis/DNA sequence complexity)

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ABSTRACT **The DNA of adenovirus type 2 was cleaved by restriction endonuclease R·RI into six fragments. These fragments were separated by electrophoresis on composite agarose–polyacrylamide gels. Their molecular weights ranged from 1.1 × 10^6 to 13.6 × 10^6, as measured by electron microscopy. Each fragment represented a unique segment of adenovirus type 2 DNA since: (*i*) the fragments were obtained in equimolar amounts; (*ii*) the sum of their molecular weights was equal to the molecular weight of complete adenovirus DNA; and (*iii*) each fragment exhibited a rate of renaturation that was inversely proportional to its size.**

Danna and Nathans (1) reported that a restriction endonuclease from *Hemophilus influenzae* Rd cleaves DNA of simian virus 40 (SV40) into 11 fragments. Such fragments are of great value for mapping of specific loci on the viral chromosome. In order to cleave the DNA of adenovirus type 2 (Ad2), we have used a restriction endonuclease from *Escherichia coli* carrying the drug resistance transfer factor RTF-1 (endonuclease R·RI). This endonuclease cuts circular SV40 DNA at one unique site (2, 3); it also cleaves duplex DNA by making single-stranded scissions in positions that are four base pairs apart, thereby generating cohesive ends (4, 5). Endonuclease R·RI has a molecular weight of about 80,000 and requires only magnesium as a cofactor (6).

Adenoviruses contain linear duplex DNA with a molecular weight of about 23 × 10^6 (7). Adenovirus DNA is not circularly permuted, but each of its strands contains complementary sequences at its ends that are inverted with respect to each other (8, 9). Adenovirus type 2, the most extensively studied serotype, belongs to the group of "nononcogenic" adenoviruses. These adenoviruses are capable of transforming cells *in vitro*, but are not known to cause tumors in animals (10). The adenoviruses are useful for investigation of cell transformation and for studies of regulatory mechanisms in eukaryotes since, during lytic adenovirus infection, host cell macromolecular synthesis is suppressed so that DNA, RNA, and protein synthesis is almost entirely controlled by the virus. Specific fragments of Ad2 DNA should be valuable for studying of gene functions expressed during the lytic cycle and in transformed cells.

MATERIALS AND METHODS

Virus and Cell Cultures. Adenovirus type 2, originally obtained from Dr. J. Rose, was propagated at an input multiplicity of about 1500 particles per cell in suspension cultures of KB-cells (11). The identity of the virus was established by immunodiffusion of antigens released from purified virus after treatment with 10% pyridine (12) against specific antisera. The virus was purified by the method of Green and Piña (13) as modified by Lonberg-Holm and Philipson (14), and the DNA was extracted from the virus as described by Pettersson and Sambrook (15). ^{32}P-labeled virus was obtained by including 10 μCi of [^{32}P]phosphoric acid/ml in a phosphate-free medium (Gibco "F13" without phosphate) from 6–40 hr after infection. The labeled DNA usually had a specific activity of about 5 × 10^5 cpm/μg. Although our virus stock had been grown at high multiplicities for many generations, the DNA was not heterogeneous when analyzed by electronmicroscopy after denaturation and renaturation (P. Sharp, unpublished results).

The Origin and Purification of Endonuclease R·RI was described by Mulder and Delius (3).

Enzyme Incubations. The standard incubation mixture contained 10 mM $MgCl_2$ and 90 mM Tris·HCl pH 7.9, 0.3 μg of protein per μg of DNA, and was incubated at 37° for 30 min. Digestion was stopped by addition of EDTA.

Rate Zonal Centrifugation. Samples (50–100 μl) were analyzed on linear gradients of 5–20% sucrose containing 50 mM Tris·HCl (pH 7.9)–1 M NaCl 1 mM EDTA in a Beckman/Spinco SW56 rotor. After 2 hr of centrifugation at 55,000 rpm and 20°, the gradients were fractionated by collection of drops from the bottom of the tube. Sedimentation coefficients were calculated in relation to that of intact adenovirus 2 DNA, which was assumed to be 31 S (16).

Agarose–Polyacrylamide Gel Electrophoresis. Samples were adjusted to 0.1 M NaCl, extracted with an equal volume of chloroform–isoamyl alcohol (24:1), and precipitated with 2 volumes of ethanol. The precipitate was dissolved in a 1:5 dilution of the electrophoresis buffer (see below). Composite gels containing 2.2% acrylamide (Bio-Rad), 0.11% *N',N'*-methylene-bis-acrylamide (Eastman Kodak), and 0.7% agarose (Sigma) were used essentially as described by Peacock and Dingman (17). The gels were polymerized in cylindrical glass tubes (0.6 cm internal diameter × 14 cm) by addition of 0.5 μl of *N,N,N', N'*-tetramethylethylenediamine and 5 μl of 10% ammonium persulfate per ml of agarose–acrylamide solution at 45°. The electrophoresis buffer contained 40 mM Tris·HCl–5 mM sodium acetate–1 mM EDTA (pH 7.8). After addition of sucrose to a concentration of about 10%, 100 μl of

Abbreviations: Ad2, Adenovirus type 2; SV40, Simian virus 40; SDS, sodium dodecyl sulfate: $P_T t_{1/2}$, the product of the DNA concentration P_T and the time taken for 50% of the DNA sequences to reassociate, expressed as mol of nucleotide × sec/liter.

sample was layered onto each gel. An initial voltage of 1 V/cm was applied for 30 min, after which the voltage was increased to 4 V/cm. The gels were removed from their tubes and cut into 1-mm slices with razors. The amount of radioactivity was measured as Čerenkov radiation in a liquid scintillation counter or in 4 ml of Aquasol (New England Nuclear). After counting, the DNA was recovered as follows: One or two slices were placed inside a glass tube (0.5 cm internal diameter × 5 cm) that had a constriction at one end. The constricted end was covered with dialysis tubing (1–2 cm). The tube was filled with electrophoresis buffer and placed in an apparatus for gel electrophoresis (Hoeffer Scientific Instruments). The constricted end was immersed in the buffer chamber with the anode, and a potential of 100 V was applied across the tube. After 2 hr of electrophoresis, 70–100% of the radioactivity was recovered from the buffer in the dialysis tubing.

Determination of the Rate of DNA Reassociation. Samples containing 200 μl of ^{32}P-labeled DNA were mixed with 50 μl of 1 M NaOH and boiled for 30 min to melt and fragment the DNA to uniform size. The size of the DNA after this treatment was about 500 nucleotides, as determined by sedimentation through alkaline sucrose gradients with marker fragments of SV40 DNA obtained by digestion with a restriction endonuclease from *H. influenzae* Rd (J. Sambrook, personal communication). The samples were neutralized by addition of 0.5 ml of 0.28 M phosphate buffer (pH 6.8) (containing equimolar amounts of Na_2HPO_4 and NaH_2PO_4) in 0.8% sodium dodecyl sulfate (SDS). The volume was then adjusted to 1 ml with water, and samples were overlayed with mineral oil before they were incubated at 68°. Aliquots (50–100 μl) were withdrawn at intervals and immediately diluted 10-fold with 0.14 M phosphate buffer containing 0.4% SDS. The amount of double-stranded DNA in each sample was determined by chromatography on hydroxyapatite (Biogel HTP) (18).

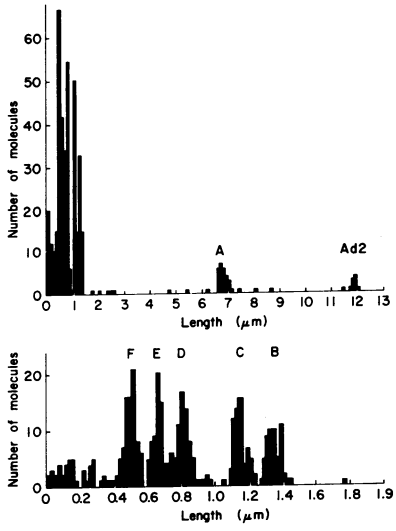

FIG. 1. Histograms of length measurements of Ad2 DNA fragments. Ad2 DNA was incubated with endonuclease R·RI under standard conditions. After digestion had been stopped by addition of EDTA to 0.05 M, Ad2 DNA was added as a marker. The mixture was spread for electronmicroscopic length measurement without further treatment. The absolute length was determined by calibration with 200 lines per diffraction grating. Small molecules were preferentially scored because some large molecules were entangled and, therefore, impossible to measure.

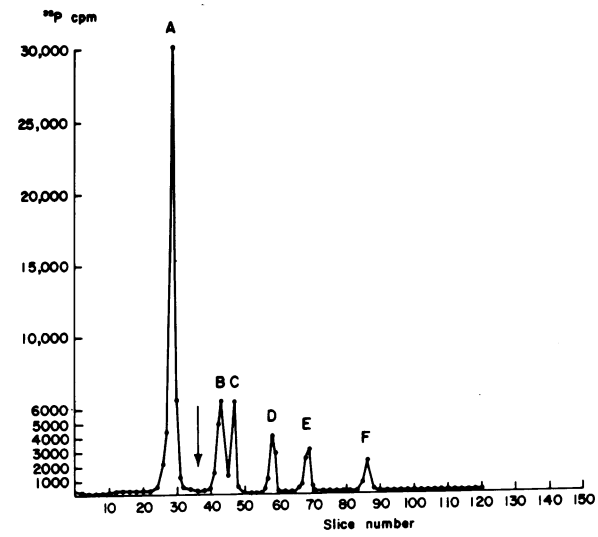

FIG. 2. Gel electrophoresis of ^{32}P-labeled fragments of Ad2 DNA. ^{32}P-labeled Ad2 DNA was incubated with endonuclease R·RI under standard conditions. The sample was extracted with chloroform–isoamyl alcohol; after ethanol precipitation, the digested DNA was subjected to electrophoresis on 2.2% acrylamide–0.7% agarose gels. After electrophoresis at 4 V/cm for 20 hr at 20°, the gels were cut into 1-mm slices. Radioactivity was measured as Čerenkov radiation. The anode is towards the *right*. Six peaks designated *A–F* were resolved. The *arrow* marks the position of linear SV40 DNA, as determined on a separate gel containing fragments *A–F*.

Electron Microscopy. DNA was mounted by the basic protein film technique for observations in an electron microscope (3, 19). Circular DNA from bacteriophage PM 2 (20) was sometimes added as an internal length standard.

RESULTS

Digestion of Ad2 DNA with Endonuclease R·RI. ^{32}P-labeled Ad2 DNA was digested with endonuclease R·RI and analyzed on neutral sucrose gradients with ^{3}H-labeled intact Ad2 DNA as a marker. The digested Ad2 DNA was resolved into three peaks: one major peak had a sedimentation coefficient of about 26 S and contained about 60% of the DNA; two minor, slower-sedimenting, broad peaks apparently contained DNA of various sizes (not shown). The digested Ad2 DNA was also examined by electron microscopy; no detectable amounts of intact DNA (<1%) remained after digestion. The lengths of the digested fragments were measured with intact Ad2 DNA as a standard; the digested DNA comprised six distinct size classes. The average length of the DNA in each of these classes, measured in relation to that of the marker DNA, was 0.57, 0.11, 0.10, 0.07, 0.06, and 0.04 (Fig. 1). A heterogeneous population of small DNA segments (0.004–0.03 of Ad2 DNA) was also observed (Fig. 1); as will be discussed below, we do not consider these to be cleavage products of Ad2 DNA.

Analysis of Digested Ad2 DNA by Gel Electrophoresis. ^{32}P-labeled Ad2 DNA, cleaved by endonuclease R·RI, was analyzed by electrophoresis on composite agarose–polyacrylamide

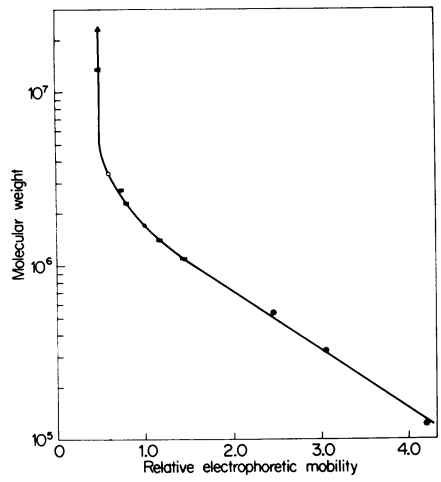

FIG. 3. Relationship between molecular weight and electrophoretic mobility of fragments *A–F*. Electrophoresis was done on 12-cm gels at 4 V/cm for 4, 20, and 25 hr. Relative electrophoretic mobility was defined as: (distance migrated by a fragment)/(distance migrated by fragment *D*). The following DNAs were used as markers: (▲) Intact Ad2 DNA (23×10^6), (○) linear SV40 DNA (3.6×10^6), and (*) fragments SV40-B (5.4×10^5), SV40-D (3.2×10^5), and SV40-H (1.2×10^5) obtained by treatment of SV40 DNA with a restriction endonuclease from *H. influenzae* Rd (1). Fragment *D* migrated with an electrophoretic mobility of 1.13×10^5 cm²/V × sec (17).

gels. All separations were performed at 20° to prevent joining of the cohesive ends that are generated by endonuclease R·RI (4, 5). Six peaks, designated *A–F*, were resolved by this method (Fig. 2). No radioactivity was recovered from the top of the gel, and more than 90% of the input radioactivity was recovered after electrophoresis. A separation time of 20 hr was required for good resolution. No additional peaks were observed when the electrophoresis time was decreased to 4 hr, which would allow a segment of DNA of molecular weight below 100,000 to remain on the gel (Fig. 3). For calibration of the gel system, marker DNAs were added to the digested samples before electrophoresis (Fig. 3). For this purpose we used intact Ad2 DNA (23×10^6), linear SV40 DNA (3.6×10^6), and fragments SV40-B (5.4×10^5), SV40-D (3.2×10^5), and SV40-H (1.2×10^5 daltons) obtained by digestion of SV40 DNA with a restriction endonuclease from *H. influenzae* Rd (1). All markers were resolved from the six fragments of Ad2 DNA, except intact Ad2 DNA, which migrated with fragment *A*. The mobilities of the five marker DNAs in relation to fragment *D* of adenovirus DNA were 0.5, 0.6, 2.5, 3.1, and 4.2 (Fig. 3).

In order to establish that digestion of Ad2 DNA was complete, we analyzed samples of ^{32}P-labeled Ad2 DNA that were digested for 5–120 min. The sum of the radioactivity in peaks *A–F* and the fraction of label recovered from each peak was determined. Analysis of samples after digestion for 5, 30, 60, and 120 min revealed no difference in distribution of radioactivity among the six peaks (Table 1).

Determination of Molecular Weight of Isolated Fragments. Ad2 fragments that migrated as peaks *A–F* were collected after electrophoresis and examined in an electron microscope with circular DNA from bacteriophage PM 2 as a standard. Each peak contained DNA of a unique size class as judged by the standard deviation in lengths (Table 2). If a molecular weight of 6.40×10^6 is assigned to PM 2 DNA, the molecular weights ($\times 10^{-6}$) of the fragments are: 13.6 (*A*), 2.7 (*B*), 2.3 (*C*), 1.7 (*D*), 1.4 (*E*), and 1.1 (*F*) (Table 2). The sum of the molecular weights of DNA fragments from peaks *A–F* ($22.8 \pm 0.8 \times 10^6$) was in good agreement with that of intact Ad2 DNA ($22.9 \pm 0.5 \times 10^6$) (Table 2); the experimental error did not exclude that one of the small peaks (*E* or *F*) contained two different segments of DNA of identical size. If all peaks contained segments of DNA of one kind there should be a correlation between the amount of DNA recovered in each peak and its molecular weight. Therefore, we determined the fraction of the total radioactivity that was recovered in peaks *A–F* after electrophoresis of seven separate samples of ^{32}P-labeled digested Ad2 DNA. As is shown in Table 2, there is a good correlation between the fraction of radioactivity and the fractional length of the DNA recovered from each of the six peaks.

Determination of the Complexity of DNA from Complete Ad2 DNA and Separated Fragments. To further establish that peaks *A–F* contained unique fragments of DNA, we examined the reassociation kinetics of each fragment and of complete Ad2 DNA (18). Before analysis, all DNAs were partially hydrolyzed to an average size of 500 nucleotides by being boiled in alkali (J. Sambrook, personal communication) to eliminate the effect of size on the rate of duplex formation (21). The time ($t_{1/2}$) required for 50% of the sequences to reassociate at a given DNA concentration (P_T) was calculated for fragments *A–F* and complete adenovirus DNA (Fig. 4; Table 2). The product ($P_T t_{1/2}$) for renaturation of two different DNAs will primarily depend upon their complexities, provided they have been degraded to identical size. Complexity is defined as the number of base pairs in nonrepeating sequences in a given DNA (21). DNA segments that do not contain reiterated sequences should have complexities that are proportional to their lengths (21, 22). Thus, the complexity of fragments *A–F* may be predicted from their molecular weights, provided each is unique. A 20% variation from this prediction must be allowed

TABLE 1. *Distribution of DNA in peaks A–F after incubation of Ad2 DNA with endonuclease R·RI for different periods of time*

Min of incubation	Distribution of ^{32}P (%)				
	A	*B + C*	*D*	*E*	*F*
5	58.4	23.5	7.3	5.8	5.1
30	59.2	21.1	7.5	6.8	5.4
60	59.8	22.7	7.3	6.2	4.0
120	59.4	19.8	7.8	6.1	6.7
Average of 4 separate 30-min digestions*	58.1 ± 1.1	22.6 ± 1.3	7.6 ± 0.3	6.6 ± 0.2	5.1 ± 0.3

Gels were sliced into 1-mm slices, and radioactivity was determined as Čerenkov radiation. Peaks *B* and *C* were not completely resolved on all electropherograms and, therefore, the number of counts in both peaks were added together. In all experiments, peaks *B* and *C* were of similar magnitude. The enzyme concentration was increased from 3 μg/ml to 6 μg/ml after 30 min and to 9 μg/ml after 60 min.

* ±SD.

TABLE 2. *Properties of fragments of Ad2 DNA after separation by gel electrophoresis*

Fragment	Length (μm)	Molecular weight ($\times 10^{-6}$)	Fractional length (%)	Distribution of radioactivity* (%)	$P_T t_{1/2}$ (mol $\times$ sec/liter) $\times 10^3$
A	7.54 ± 0.10	13.6 ± 0.2	58.3	58.5 ± 1.1	10.7 ± 0.5
B	1.54 ± 0.05	2.7 ± 0.1	11.9	22.4 ± 1.5†	2.28 ± 0.14
C	1.28 ± 0.05	2.3 ± 0.1	9.9		2.64 ± 0.50
D	0.924 ± 0.05	1.7 ± 0.1	7.2	7.5 ± 0.3	1.46 ± 0.09
E	0.747 ± 0.03	1.4 ± 0.1	5.8	6.3 ± 0.4	1.73 ± 0.22
F	0.584 ± 0.03	1.1 ± 0.1	4.5	5.2 ± 0.8	1.14 ± 0.09
Intact Ad2 DNA	12.9 ± 0.3	22.9 ± 0.5	(100)	—	20.2 ± 0.2

Samples were mixed with nicked circular PM 2 DNA before electron microscopic analysis. The molecular weight of PM 2 DNA was calculated to be 6.40 $\times$ 10^6 from measurements that were standardized with T7 DNA (25.2 $\times$ 10^6; ref. 23). $P_T t_{1/2}$ was calculated as described in the legend to Fig. 4.

* Average of seven experiments. Background radioactivity was measured from fractions between peaks and was subtracted. Fragments *B* and *C* were not entirely resolved on all electropherograms and, therefore, the number of counts in both peaks were added together. In all experiments peaks *B* and *C* were of similar magnitude.

† For fragment *B* plus fragment *C*.

because of the effect of differences in base composition on the rate of renaturation (21). We found a good correlation between the complexity of each of the six fragments and its molecular weight, with a maximum deviation of 35% from the predicted value for $P_T t_{1/2}$ calculated from the results for complete Ad2 DNA (Table 2). From this we conclude that none of the fractions *A–F* contained more than one kind of DNA segment. The presence of two kinds of DNA segments in one peak would cause a 2-fold increase above the predicted value for $P_T t_{1/2}$.

Since fragment *A* was not resolved by gel electrophoresis from segments of DNA larger than 5 $\times$ 10^6 (Fig. 3), we wanted to ascertain that fragment *A* was not contaminated with large DNA fragments generated because of incomplete digestion. Such fragments could arise if Ad2 DNA was being modified by a contaminating methylase during digestion or if Ad2 DNA was heterogeneous with respect to cleavage sites for endonuclease R·RI. Electronmicroscopic length measurements of purified samples of fragment *A* showed that less than 1% of the molecules had a length corresponding to the sum of fragments *A* and *B* or *A* and *C* (data not shown). A significant contamination of molecules having a molecular weight of about 1 $\times$ 10^6 above the average could not be excluded however. Therefore, we estimated the amount of contaminating *D*, *E*, and *F* fragments in purified samples of fragment *A* by the following method: ^{32}P-labeled samples of fragments *D*, *E*, and *F* were mixed with a 200- to 1000-fold excess of either unlabeled fragment *A* or unlabeled intact Ad2 DNA. The samples were denatured and partially hydrolyzed, and the rate of renaturation was measured. Fig. 5 shows the results when 2 ng of ^{32}P-labeled fragment *F* was mixed with 1 μg of unlabeled fragment *A* or 1.7 μg of unlabeled Ad2 DNA. As expected, Ad2 DNA caused about a 40-fold increase in the rate of renaturation of the labeled DNA, whereas fragment *A* had an insignificant effect. Since contamination of 2 ng would have caused a 2-fold increase in the rate of renaturation, we conclude that purified samples of fragment *A* were contaminated with less than 0.2% by mass of fragment *F*. In a similar way, we determined that the corresponding amounts of contamination for fragments *E* and *D* were <0.3% and <0.8%, respectively.

DISCUSSION

We have identified, by gel electrophoresis, six fragments from Ad2 DNA after digestion with endonuclease R·RI. The cleavage was complete and the six fragments (fragments *A–F*) were demonstrated to be unique segments of the Ad2 DNA by the following observations: (*i*) The sum of the molecular weights of the six fragments equaled that of intact adenovirus type 2 DNA. (*ii*) All six fragments were generated in equimolar amounts. (*iii*) Each fragment had a renaturation

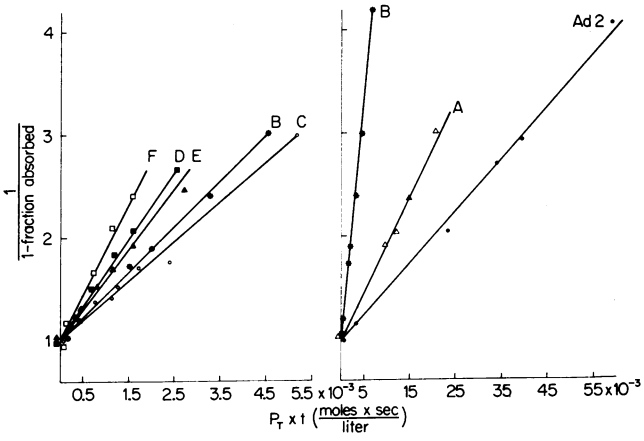

FIG. 4. Kinetics of reassociation of denatured ^{32}P-labeled samples of fragments *A–F* and complete Ad2 DNA. Samples in 0.14 M phosphate buffer (18) and 0.4% SDS were incubated at 68°. Aliquots (100–200 μl) were withdrawn at intervals and assayed by chromatography on hydroxylapatite. The time required for 50% of the sequences to renature ($t_{1/2}$) was calculated from $t_{1/2} = [t(1 - f_{DS})/f_{DS}]$; ($t$ = time of incubation; f_{DS} = fraction of double-stranded DNA in a sample at a given time, t, as determined by chromatography on hydroxylapatite). Each line represents the theoretical curve computed from the average $t_{1/2}$; in all cases, $t_{1/2}$ was calculated from three or more samples containing between 10 and 65% renatured DNA. The fraction of double-stranded DNA was calculated from the fraction of ^{32}P-counts that adsorbed to hydroxylapatite in 0.14 M phosphate buffer. No corrections were made for zero- or infinite-time renaturation.

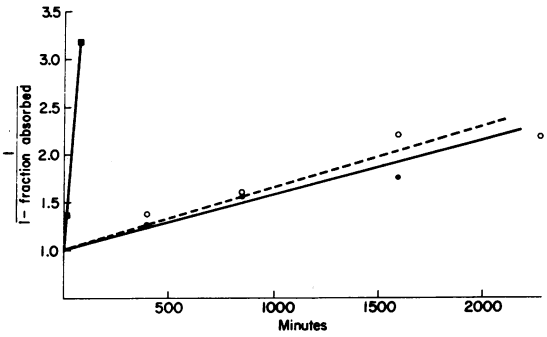

FIG. 5. Kinetics of reassociation of denatured ^{32}P-labeled fragment *F* in the presence of excessive amounts of unlabeled fragment *A* or Ad2 DNA. Reaction mixtures contained 2 ng of ^{32}P-labeled fragment *F* and 1 μg of fragment *A* (○) or 1.7 μg of Ad2 DNA (■) per ml. The rate of reassociation was also estimated for fragment *F* alone under identical conditions (●). The fraction of duplex DNA in the samples was estimated as described in the legend to Fig. 4, after different times of incubation at 68° in 0.14 M phosphate buffer (18) and 0.4% SDS.

complexity that is proportional to its molecular weight. (*iv*) The fraction of the total DNA migrating in each of the six peaks remained constant during prolonged digestion.

Electron microscopy showed, in addition, a heterogeneous population of fragments with molecular weights in the range 200,000–600,000 (Fig. 1). These fragments were apparently not derived from Ad2 DNA since (*i*) they were not present in equimolar amounts (Fig. 1) and (*ii*) gel electrophoresis of digested samples of ^{32}P-labeled Ad2 DNA containing 5×10^5 cpm (under conditions where DNA of molecular weight of 100,000 or less would be resolved) revealed no labeled material smaller than fragment *F*. Electron microscopy of our enzyme preparation showed some DNA-like material, and it is likely that this was the origin of the small fragments.

Electrophoresis on composite agarose–polyacrylamide gels (17) was a useful method for separation of the six fragments of Ad2 DNA. All fragments were clearly resolved from each other, although some of the fragments differ by as little as 15% in length. This method would not, however, resolve fragment *A* from DNA segments larger than 5×10^6 daltons (Fig. 3). The homogeneity of fragment *A* was therefore ascertained by electron microscopy and by estimates of the rate of DNA renaturation of fragments *D–F* in the presence of a vast excess of fragment *A*.

In view of its specificity, endonuclease R·RI should be of general importance for obtaining defined fragments of DNA from large mammalian DNA viruses. The fragments that were generated from Ad2 DNA with this enzyme should facilitate mapping of the adenovirus chromosome.

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