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Kinetic studies on the cleavage of adenovirus DNA by restriction endonuclease Eco RI

Sven Forsblom, Rudolf Rigler, Måns Ehrenberg, Ulf Pettersson* and Lennart Philipson*

Department of Medical Biophysics, Karolinska Institute, S-104 01 Stockholm 60, Sweden

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ABSTRACT

The kinetics of cleavage of DNA from Adenovirus Type 1 (Ad1), Type 5 (Ad5) and Type 6 (Ad6) by restriction endonuclease Eco RI was investigated by quantitative evaluation of the fluorescence from ethidium stained DNA fragments separated on agarose gels. The apparent rate constants of cleavage at different cleavage sites have been determined and large differences in the cleavage rates of the individual sites within one type of DNA were found. From the kinetics of cleavage information on the sequence of the DNA fragments can be obtained. The order of the fragments A, B, C, D of Ad6 DNA obtained after complete cleavage by restriction endonuclease Eco RI was found to be A-D-C-B; the order of the corresponding fragments A, B, C of Ad1 and Ad5 DNA was found to be A-C-B.

INTRODUCTION

Kinetic studies are of importance in order to elucidate the reaction mechanism of restriction endonucleases. Based on a new technique for quantitative evaluation of the fluorescence of ethidium bromide stained DNA fragments separated on agarose gels we have investigated the kinetics of cleavage of DNA from Adenovirus Type 1 (Ad1), Type 5 (Ad5) and Type 6 (Ad6) by restriction endonuclease Eco RI. From the time dependent variation of the relative number of molecules of final and intermediary fragments of these DNAs the rate constants of cleavage for each site and its dependence on enzyme concentration and temperature have been determined. The kinetics of cleavage also yields information on the sequence of the fragments in the intact DNA and the order of the fragments of Ad1, Ad5 and Ad6 DNA has been determined from fluorometric data. The sequence of these fragments was confirmed by hybridization of the fragments with those of the related Adenovirus Type 2 (Ad2), the order of which is already known (1,2).

MATERIALS AND METHODS

Eco RI endonuclease: The Eco RI restriction endonuclease was purified according to Polisky et al. (3) except that the last purification step over Sephadex G 100 was omitted. The enzyme was stored at a concentration of 0.2 mg protein/ml in 10 mM K-phosphate, 0.2 M NaCl, 0.1 mM EDTA, 7 mM mercaptoethanol, 0.2% Triton and 58% glycerol at -20°C.

DNA from Adenovirus, Type 1, Type 2, Type 5 and Type 6 was isolated as previously described (4).

Endonuclease cleavage: The reaction was carried out in 20 µl of a mixture which contained 0.2 µg DNA and various concentrations of Eco RI endonuclease. The mixtures were incubated for varying time periods in a buffer containing 0.2 M Tris-HCl, 0.05 M NaCl, 10 mM $MgCl_2$, 0.5 mM EDTA, pH 7.5. The reaction was stopped by addition of 5 µl 0.16 M EDTA. 10 µl of the reaction mixture was used for electrophoresis on agarose gels and two gels were prepared for each sample. Due to the high glycerol content of the enzyme stock solution varying amounts of glycerol were present in the incubation mixture as indicated in the text (Table II).

Liquid phase hybridization: ^{32}P-labelled fragments of Ad2 DNA were prepared (5) and the individual fragments were strand-separated (6). Unlabelled Eco RI fragments of Ad1 or Ad6 DNA were denatured and degraded to an approximate size of 350 nucleotides by boiling for 20 min in 0.3 M NaOH (7) and then mixed with approximately 150 cpm of h-strand DNA from various ^{32}P-labelled fragments of Ad2 DNA. The mixture was neutralized with HCl and the NaCl concentration was adjusted to 1M. The samples were analyzed by chromatography on hydroxylapatite (8) after incubation at 65°C for 48 hours.

Agarose gels: Agarose (Seakem, MCI Biomedicals, Rockland, Maine, USA) was dissolved in a buffer containing 0.04 M Tris, 0.005 M Na-ac, 0.02 M EDTA, pH 7.8 (E-buffer), to yield a final concentration of 1-1.2% (w/v) and was stirred for 5 hours in a water bath at 95°C. For stirring a rotating glas rod immerged in an Erlenmeyer flask was used. A tight connection between glas rod and the Erlenmeyer flask prevented evaporation of the solvent. The hot gel was poured into capillary tubes (2.7 mm inner Ø,

8 mm outer Ø, 90 mm length) with their bottom covered by Parafilm.

Gel electrophoresis: 16 capillary tubes were mounted vertically in an electrophoresis apparatus Type GE-4 (Pharmacia, Uppsala, Sweden) operated at 50V with a DC power supply Type EPS 500/400 (Pharmacia, Uppsala, Sweden). Electrophoresis was carried out for 150 min in E-buffer at room temperature.

Staining of the gels: The gels were stained with ethidium bromide (a gift of Boots Pure Drug Co., England) at a concentration of 0.4 µg/ml in E-buffer. For this purpose the gels were pressed out of the capillary tubes by applying gentle pressure at the top of the capillary tubes such that 4/5 of the gels protruded from the lower part of the capillary tube and hang freely in the staining solution. After 120 min the gels were sucked gently into the capillary tubes. During this staining time complete equilibration of the gels with ethidium was reached as judged from the fluorescence intensity of the gels.

Measurement of gel fluorescence: The beam of an argon-ion laser operated at 4880 Å (Spectra Physics, Model 165) was focussed at the center of the capillary tube. The fluorescence passing a small horizontal slit of 0.2 mm width and a OG 590 cut off filter (Schott & Gen., Mainz, W-Germany) was detected at right angle to the exciting beam by a photomultiplier designed for photon counting (RCA Quantacon 8850).

Gel scanning was provided by moving the capillary tube through the laser beam at a speed of about 1 mm/sec. Simultaneously with the gel scanning the photomultiplier pulses proportional to the current gel fluorescence were stored in successive channels of the core memory of a ND 812 computer. The distribution of the fluorescent bands in the agarose gel was displayed on an CRT-screen for inspection and data evaluation. The total scan width of about 40 mm was resolved into 600 data channels. A detailed account of the experimental set up and the conditions required for the quantitative evaluation of the gel fluorescence will be given elsewhere.

RESULTS

The order of the cleavage fragments

DNA from Ad1, Ad5 and Ad6 was incubated for various time

periods with Eco RI restriction endonuclease. After electrophoretic separation of the resulting DNA fragments and staining with ethidium bromide the fluorescence profiles were determined (Fig. 1 a, b). After complete digestion of Ad1 DNA, three peaks are visible which correspond to the large fragment A and the smaller fragments B and C (Fig. 1 a). With short incubation times an intermediary fragment turns up with an electrophoretic mobility between that of fragments B and A. From the possible combinations AC, AB and BC only BC remains as a candidate for this peak since fragments AC and AB must be located under peak A or even further towards the cathode. We also note that peak C increases simultaneously with the decrease in peak BC. Ad5 DNA

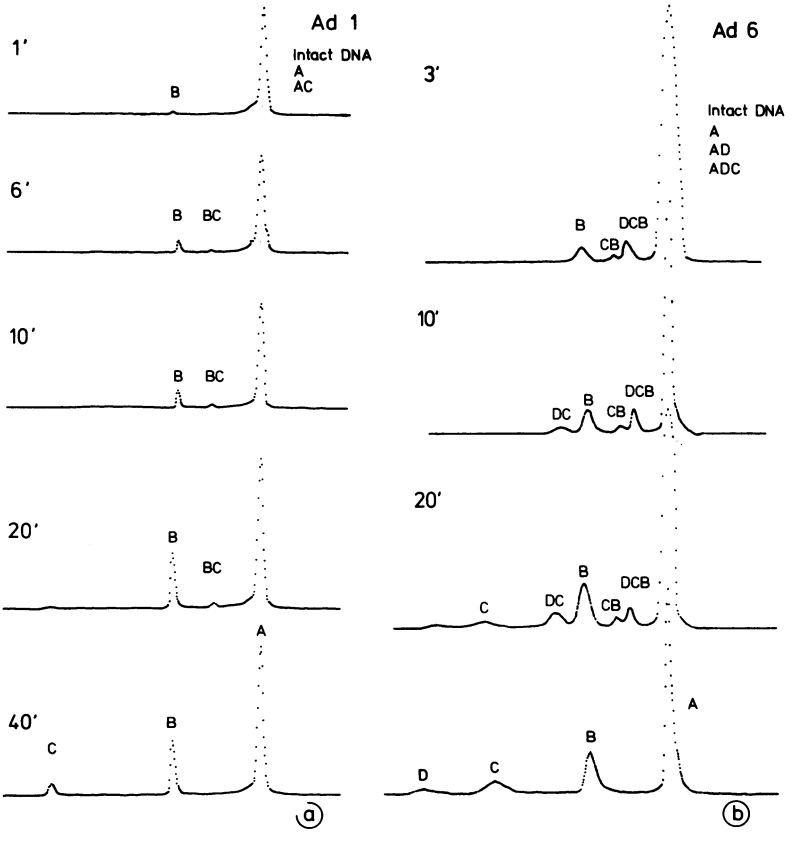

Figure 1. Fluorescence profiles of agarose gels containing separated fragments obtained by cleavage of 0.1 µg DNA with Eco RI restriction endonuclease (dilution 1:10) for various times at 37°C. a) Adenovirus Type 1; b) Adenovirus Type 6.

(not shown) showed an identical cleavage pattern as Ad1 DNA. Cleavage of Ad6 DNA (Fig. 1 b) yields fragments A, B, C and D after complete digestion with peaks A, B and C at the same locations as the corresponding peaks of Ad1 and Ad5 DNA, whereas fragment D has a faster electrophoretic mobility than any of the Ad1 fragments. Out of 5 possible intermediary fragments only 3 can be separated. Thus there exist two intermediary fragments which contain the large fragment A. From this fact it follows that A must be a terminal fragment. From the relative length of fragments B, C and D we further conclude that the intermediary fragment left of B must be DC and the fragment close to the peak containing all intermediates of fragment A must be DCB. The fragment immediately to the right of peak B must then contain either CB or DB. From its electrophoretic mobility compared with that of the fragments of Ad2 and Ad5 we identify this peak as containing the intermediary fragment CB.

With this analysis we are left with the following alternatives for the order of the Ad1 fragments: ABC and ACB and for the Ad6: ABCD and ADCB. The order is uniquely determined if we can decide whether B or C is a terminal fragment of Ad1 and whether D or B is a terminal fragment of Ad6. The kinetics of appearance of these fragments was investigated to decide on this issue. As outlined in Appendix II we expect the terminal fragment to grow exponentially and internal fragments to grow with an initial delay. The result (Fig. 3a, 3b) shows that in the case of Ad1 and Ad5 (not shown) fragment B increases exponentially while fragment C is delayed and in the case of Ad6, fragment B increases exponentially while fragment D is delayed (as well as fragment C). The sequence of the fragments is now determined and reads A-C-B for Ad1 and Ad5 and A-D-C-B for Ad6. The sequence for Ad5 agrees with previous results (2). For Ad1 and Ad6 the sequence has not been determined before. Therefore the order of the Ad1 and Ad6 fragments was confirmed by an independent method.

Since Ad1, Ad2 and Ad6 belong to the same Adenovirus subgroup, their DNAs share considerable sequence homology (9,10). Several sets of restriction enzyme fragments of Ad2 DNA have been mapped on the viral genome. Therefore it should be possible to order fragments of Ad1 or Ad6 DNA by hybridization to fragments of

Ad2 DNA with a known location. In order to confirm the positions of the Eco RI fragments of Ad1 or Ad6 DNA, fragments of ^{32}P-labelled Ad2 DNA were prepared by cleavage with restriction endonucleases Eco RI and Bam HI. Single stranded probes of the ^{32}P-labelled fragments were prepared (6) and hybridized to denatured unlabelled Eco RI fragments of Ad1 or Ad6 DNA. The results which are summarized in Table I show that fragment Eco RI-C of Ad1 DNA shares homology with fragment Eco RI-D of Ad2 DNA.

TABLE I

Per cent of ^{32}P-labelled Ad2 fragment in duplex DNA[a)]

Eco RI fragments	Bam-A	Bam-B	Bam-C	Bam-D	Eco RI-B	Eco RI-C	Eco RI-D	Eco RI-E	Eco RI-F
Ad 1-A	23	47	49	53	35	0	0	0	41
-B	20	0	0	0	0	43	0	16[b)]	0
-C	13	0	0	0	0	0	32	0	0
Ad 6-A	12	55	45	56	63	0	0	0	0
-B	15	0	0	0	0	51	0	28[b)]	0
-C	17	0	0	0	0	0	59	0	0
-D	8	0	0	0	0	0	0	0	44

a) ^{32}P-labelled h-strand DNA from various fragments of Ad2 DNA was used as a probe for hybridization. Per cent DNA in duplex was determined by chromatography on hydroxylapatite. Approximately a tenfold molar excess of unlabelled DNA was used in the hybridization mixtures.

b) The region of the Adenovirus genome, which is represented by fragment Ad2-Eco RI-E, corresponds to a region which shows pronounced sequence heterology between Adenovirus serotypes. Therefore a comparativley small fraction of this fragment is recovered as duplex after hybridization.

Fragment Eco RI-B of Ad1 DNA, on the other hand, hybridizes to both fragments Eco RI-C and -E of Ad2 DNA. Since the order of the Eco RI fragments of Ad2 DNA is A-B-F-D-E-C (2), we conclude that the order of the Eco RI fragments of Ad1 DNA is A-C-B (Fig. 2).

It is noteworthy that the electrophoretic mobility of fragments Ad1- Eco RI-C and Ad2- Eco RI-D is identical whereas the mobility of fragment Ad1- Eco RI-B corresponds to a fragment of the combined length of fragments Ad2- Eco RI-C and -E (not shown). Table I also shows that fragments Eco RI-C and -D of Ad6 DNA hybridize to fragments Ad2- Eco RI-D and -F respectively. Fragment Ad1- Eco RI-C hybridizes to both fragments Ad2- Eco RI-C and

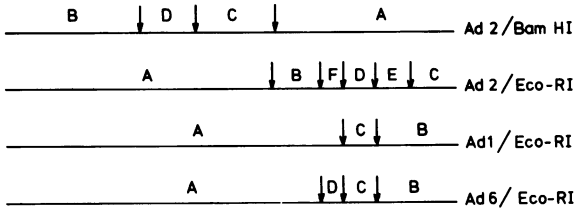

Figure 2. A map showing the locations of various restriction enzyme fragments of Ad1, Ad2 and Ad6 DNA. The positions of the cleavage sites for endonucleases Eco RI and Bam HI on the Ad2 DNA has been reported previously (11).

-E. Thus we conclude that the order of the Eco RI fragments of Ad6 DNA is A-D-C-B in agreement with the kinetic analysis (Fig. 2).

Kinetics of cleavage

Since differences in the rate of hydrolysis at the cleavage sites for Eco RI restriction endonuclease on λ-DNA have been reported (12) it was of interest to determine the rate constants of cleavage for the three Adenovirus DNAs (Ad1, Ad5, Ad6). The rate constants have been obtained (see Appendix II) by fitting the experimentally determined normalized number of molecules of each fragment (R(t)), identified on gels. (Fig. 1 a, b) for Ad1 (Ad5) DNA and Ad6 DNA respectively (Fig. 3a and 3b). It was found that at 37°C the rate constant for cleavage of the site between fragments A and C(k_{AC}) of Ad1 and Ad5 DNA was about one third of the rate constant for cleavage at the site between fragments C and B (k_{CB}). This difference became more pronounced at 20°C (Table II). Similar observations were made for Ad6 DNA. In this case the cleavage sites between fragments D and C and between fragments C and B are located at the same positions as the cleavage sites between fragments A and C and between fragment C and B of Ad1 or Ad5 DNA (Fig. 2). The additional cleavage site in Ad6 DNA between fragments A and D has the same rate constant (k_{AD}) as the site between fragments C and B (k_{CB}). For Ad6 the three rate constants k_{AD}, k_{DC}, k_{CB} have also been determined as a function of the enzyme concentration (Table II). Their absolute values increase with increasing concentration of enzyme.

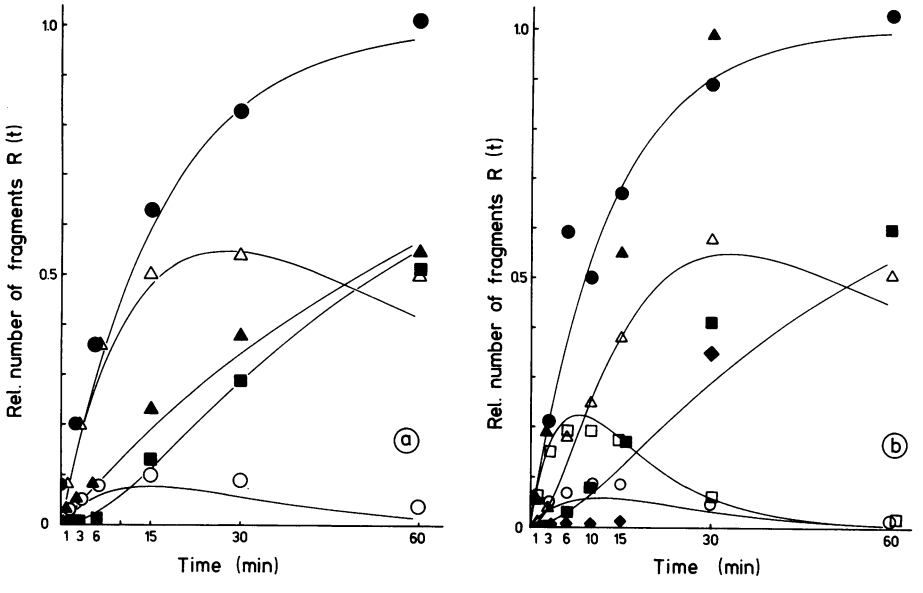

Figure 3. Relative number of fragments R(t) as a function of incubation time with Eco RI restriction endonuclease (dilution 1:10). a) Adenovirus Type 1: fragment A (▲), fragment B (●), fragment C (■), fragment AC (△), fragment BC (○). b) Adenovirus Type 6: fragment A (▲), fragment B (●), fragment C (■), fragment D (◆), fragment DC (△), fragment CB (○), fragment BCD (□). The solid lines are simulated progress curves using the rate constants of Table II.

DISCUSSION

We have shown that by analysis of the cleavage kinetics the terminal fragments can be identified through their characteristic growth curve. Although fragment A cannot be resolved because of its size, information about its initial growth rate can be obtained. When the sequence is known the stoichiometric relationship between fragment A and the other fragments gives for Ad1 (Ad5) that $R_A(t)=R_C(t)+R_{CB}(t)$ and for Ad6 that $R_A(t)=R_D(t)+R_{DC}(t)+R_{DCB}(t)$. Similar relationships can be found for all A containing fragments. As expected, fragment A, which is a terminal fragment for Ad1 (Ad5) and Ad6 DNA has no initial delay. This confirms the sequence analysis. We thus have demonstrated that the kinetics of cleavage is a useful complement to conventional sequence determination.

The cleavage rate constants determined in this study are

TABLE II

Rate constants of cleavage of Adenovirus Type 1, Type 5 and Type 6 by Eco RI restriction endonuclease

	Enzyme dilution	Temp. [°C]	k_{AC} [$\times 10^5 sec^{-1}$]	S.E. [$\times 10^5$]	k_{CB} [$\times 10^5 sec^{-1}$]	S.E. [$\times 10^5$]
Ad1	1:10	37	23	2.3	100	3.8
	1:10	20	3.3	0.3	27	3.8
Ad5	1:10	37	62	13	160	15
	1:10	20	4.8	1.4	50	3.2

			k_{AD} [$\times 10^5 sec^{-1}$]	S.E. [$\times 10^5$]	k_{DC} [$\times 10^5 sec^{-1}$]	S.E. [$\times 10^5$]	k_{CB} [$\times 10^5 sec^{-1}$]	S.E. [$\times 10^5$]
Ad6	1:10	37	141	13	20	4.1	143	5.8
	1:10	20	30	0.9	1.4	0.6	25	0.8
	undiluted†	37	500	97	220	53	530	68
	1:10†	37	52	5.7	23	4.3	48	4.7
	1:20†	37	23	2.2	9.3	1.5	18	1.5
	1:30†	37	11	1.4	4.5	1.2	11	1.4

† In these experiments the glycerol concentration in the incubation mixture amounted to 14.5%. In all other experiments the glycerol concentration was 1.5%.

phenomenological and can be related to the parameters of a more detailed model of the cleavage process. A minimal scheme consists of a binding step, where the endonuclease E is attached to its specific site S, and a catalytic cleaving step leading to a product (P):

$$E + S \underset{k_d}{\overset{k_r}{\rightleftharpoons}} ES \xrightarrow{k_c} P \qquad (I)$$

If the concentration of free endonuclease [E] remains constant during the reaction the scheme leads to two time constants obtainable from the determinant equation:

$$\lambda^2 + (k_r[E] + k_d + k_c)\,\lambda + k_r[E] \cdot k_c = 0, \qquad [1]$$

which gives the eigenvalues λ_1 and λ_2. By assuming a rapid equilibration of the first step in (I) the experimentally determined cleavage rate k is given by:

$$k = -\lambda_1 = \frac{k_r[E]\cdot k_c}{k_r[E]+k_d+k_c} = \frac{[E]\cdot k_c}{[E]+K_M}, \qquad [2]$$

with $K_M = \frac{k_d+k_c}{k_r}$ and $|\lambda_1| << |\lambda_2|$.

Thus, from the overall rate constant as a function of the free endonuclease concentration the catalytic constant k_c as well as K_M can be determined. A plot of 1/k against 1/[E] gives a straight line as can be seen from equ. [2]. The intercept yields $1/k_c$ and the slope is K_M/k_c. The data of Table II has been used to plot 1/k against $1/E_o$ where E_o is the relative input concentration of Eco RI restriction endonuclease (Fig. 4). E_o can replace [E] in equ. |2| if the dissociation constant for the interaction between Eco RI restriction endonuclease and Adenovirus DNA is larger than the concentration of all binding sites on the Adenovirus DNA as well as of E_o. Deviations from linearity in Fig.4 indicate that this equivalence does not hold over the whole concentration range of E_o. Fig. 4 suggests that the main difference in the values of the rate constants k for the three cleavage sites of Ad6 DNA are apparently due to differences in the K_M values rather than to differences in the respective k_c

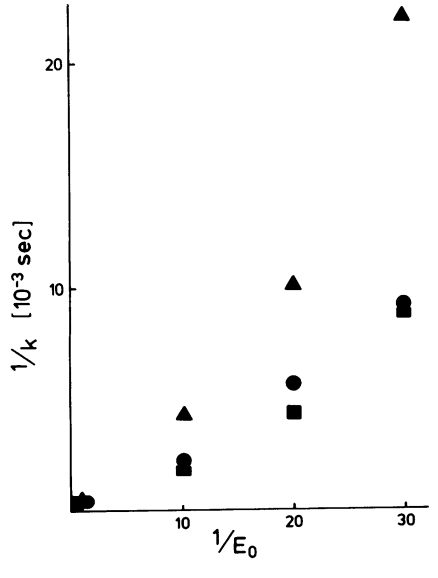

Figure 4. Dependence of the reciprocal rate constants of cleavage of Adenovirus Type 6 on the reciprocal concentration of Eco RI restriction endonuclease. Data taken from Table II. k_{AD} (■), k_{DC} (▲), k_{CB} (●).

values. Preliminary evaluation of Fig. 4 indicates that k_c for all cleavage sites ($\geq 10^{-2} sec^{-1}$) is of the same order of magnitude as the value obtained for the cleavage of SV_{40} DNA (13).

In the case of the binding of lac-repressor to its operator the bulk of the non-operator DNA, which is in large excess over the operator, also influences the binding of the lac-repressor considerably (14). For ionic conditions, similar to ours in the case of the endonuclease-DNA reaction, a binding constant of approximately $10^6 M^{-1}$ was determined (14). Greene et al. (13) report a K_M value between 1.6×10^{-5}M and 6×10^{-8}M for the cleavage of the recognition sequence pTGAATTCA alone and imbedded in the bulk DNA of SV_{40} (13). We therefore conclude that in the case of the binding of endonuclease Eco RI to DNA containing the recognition sequence, the binding of the enzyme is mainly determined by the bulk of unspecific DNA which for adenovirus DNA is in 10^4 fold excess over the recognition sites calculated on a nucleotide basis. The free concentration of endonuclease is therefore likely to be constant during catalysis.

The difference in K_M values suggests structural differences at the cleavage site affecting the affinity for, or the accessibility of the site for the Eco RI restriction endonuclease. This also is supported by the pronounced temperature sensitivity of the rate constants k_{AC} (Ad1, Ad5) and k_{DC} (Ad6), (Table II). We finally point out the similarity of the values for the rate constants related to equivalent cleavage sites (Table II, Fig. 2). This is consistent with the fact that these three virus types are very closely related and differ by the presence or absence of certain cleavage sites for Eco RI restriction endonuclease.

APPENDIX I

Data evaluation and calculation of the concentration of DNA fragments resolved on agarose gels: The fluorescence profile of the gel was stored in 600 channels of a ND 812 computer. The total fluorescence of stained DNA F_i of peak i containing fragment i was experimentally determined from

$$F_i = \sum_{k=n_i}^{m_i} (S_k - B_k);\ i=1,2....M. \qquad [3]$$

S is the number of counts in channel k and B_k the estimated background originating primarily from ethidium bromide not bound to DNA. Peak i is contained between channels n_i and m_i and M is the maximum number of peaks in a gel. The total fluorescence of peak i is proportional to its amount of DNA, i.e. to the number of fragment molecules times the relative fragment length l_i. The quantity $R_i(t)$, where

$$R_i(t) = F_i(t)/(l_i \sum_{j=1}^{M} F_j(t));\ i=1,2....M, \qquad [4]$$

is thus a normalized measure of the number of fragment molecules in peak i as a function of time. $R_i(t)$ then is varying between 0 and 1 during the time course of cleavage. The relative lengths of the end products can be determined after complete cleavage from the relation [5]

$$l_i = F_i / \sum_{j=1}^{N} F_j;\ i=1,2....N. \qquad [5]$$

In this special case M=N, where N is the number of final fragments.

Determination of the relative lengths of DNA fragments: The fluorescence intensity profiles of the final cleavage products of DNA from Ad2 and Ad5 after incubation with Eco RI were used to investigate if the fluorescence was proportional to the length of the stained DNA fragments. From the fluorescence intensity of each peak the relative lengths of the fragments were determined according to equ. [3]. Since the relative lengths of both virus DNAs have been determined by the electron microscope (1,2), a control of the fluorometrically determined values of the relative lengths l_i is available. In Fig. 5 the relative length of various fragments of Ad2 and Ad5 estimated by both methods was compared. The values obtained by the two methods are in good agreement. The linearity extends from a fragment size of 1.1×10^6 dalton for fragment F of Ad2 of 1.8×10^7 dalton for fragment A of Ad5.

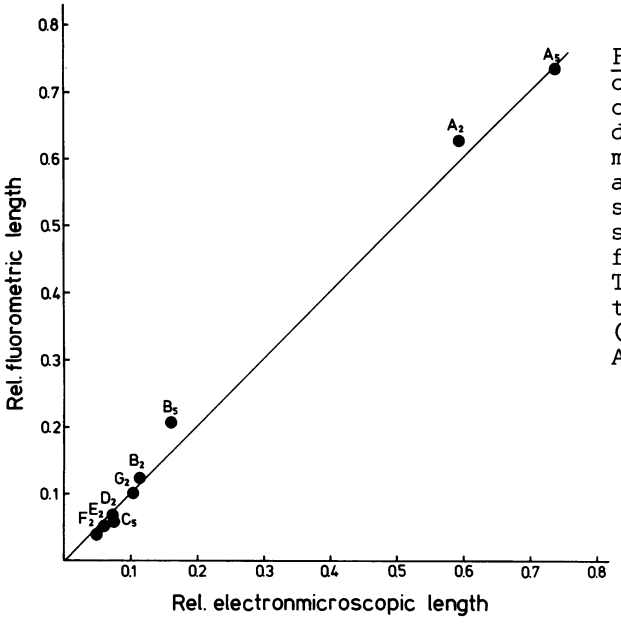

Figure 5. Comparison of rel. length (length of intact DNA = 1) as determined by fluorometric gel scanning and by electronmicroscopy (1,2). The straight line is drawn for a 1:1 relationship. The subscripts indicate the source of fragments (Adenovirus Type 2; Adenovirus Type 5).

APPENDIX II

Time dependence of the concentration of DNA fragments

Ad1 and Ad5 yields 3 final and 2 intermediary fragments while Ad6 yields 4 final and 5 intermediary fragments. We have analyzed the time dependence of the normalized number of molecules of each fragment ($R_i(t)$) assuming independent cleavage of all sites. Given a constant concentration of enzyme during catalysis the irreverisble cleavage of DNA is described by first order rate equations. The following results were obtained for Ad1 and Ad5:

$$R_1(t)=1-e^{-k_1t} \qquad R_{12}(t)=e^{-k_1t}(1-e^{-k_2t})$$

$$R_2(t)=1-e^{-k_2t} \qquad R_{23}(t)=e^{-k_2t}(1-e^{-k_1t}) \qquad [6]$$

$$R_3(t)=1-e^{-k_1t}-e^{-k_2t}+e^{-(k_1+k_2)t} \qquad R_{123}(t)=e^{-(k_1+k_2)t}$$

R_1 and R_2 refer to the terminal fragments while R_3 refers to the internal fragment. R_{12} and R_{23} refer to the two possible intermediates and R_{123} refers to the intact DNA. k_1 and k_2 are the

rate constants of cleavage between fragments 1 and 2 and between fragments 2 and 3 respectively.

Similar results were obtained for Ad6:

$$R_1(t)=1-e^{-k_1 t}$$

$$R_2(t)=1-e^{-k_1 t}-e^{-k_2 t}+e^{-(k_1+k_2)t}$$

$$R_3(t)=1-e^{-k_2 t}-e^{-k_3 t}+e^{-(k_2+k_3)t}$$

$$R_4(t)=1-e^{-k_3 t}$$

$$R_{12}(t)=(1-e^{-k_2 t})\cdot e^{-k_1 t} \qquad [7]$$

$$R_{123}(t)=(1-e^{-k_3 t})\cdot e^{-(k_1+k_2)t}$$

$$R_{23}(t)=(1-e^{-k_1 t}-e^{-k_3 t}+e^{-(k_1+k_3)t})\cdot e^{-k_2 t}$$

$$R_{234}(t)=(1-e^{-k_1 t})\cdot e^{-(k_2+k_3)t}$$

$$R_{34}(t)=(1-e^{-k_2 t})\cdot e^{-k_3 t}$$

$$R_{1234}(t)=e^{-(k_1+k_2+k_3)t}$$

In this case R_1 and R_4 refer to the terminal fragments while R_2 and R_3 refer to internal fragments. R_{1234} refers to the intact DNA and the other variables to the 5 intermediary fragments. k_1, k_2 and k_3 are the rate constants of cleavage between fragments 1 and 2, fragments 2 and 3 and between fragments 3 and 4 respectively.

The relative concentration function of a fragment which needs two cleavages for its creation is characterized by a zero time derivative at time zero whereas fragments needing only one cleavage have a positive slope at zero time. This makes it possible to identify fragments and to determine the sequence of final fragments.

To obtain the rate constants k_j ($j=1,2....N_c$) where N_c is the number of cleavage sites, we have fitted the theoretical expressions from equs. [6] or [7] to the experimentally determined quantities $R_i(t)$ (equ. [4]). The parameter fitting was done with a computer program for non linear regression (15,16). The consistency of the estimates for the rate constants

was checked by comparing the estimates evaluated from the time course of one fragment at a time with the corresponding estimates obtained from a simultaneous evaluation of the time course of all observable fragments.

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***Department of Microbiology, Biomedical Center,Box 570, S-751 23 Uppsala,Sweden**

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