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RESTRICTION SITE MAPS OF THE HUMAN ADENOVIRUS TYPE 8 DNA

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Summary. — Restriction site maps of human adenovirus 8 (Ad h 8) were constructed with *Bam*HI, *Hin*dIII, *Pst*I, *Sal*I and *Kpn*I endonucleases. The genome size was found to be 22.1 to 22.3×10^6 M_r. Comparison of the results with the data available on h Ad subgenera A, B, C showed that the *Sal*I enzyme revealed subgenus-specific differences in the genomes. Similar patterns of the *Sal*I fragments in both type 8 and 10 suggest that the differences were specific for the subgenus D of h Ad.

Key words: *human adenovirus type 8*; *subgenus D*; *restriction site map*; *genome size*

Introduction

Genome of the members of the family *Adenoviridae* is formed by linear double stranded DNA, the M_r of those of genus *Mastadenovirus* being $20—25 \times 10^6$ (Matthews, 1982). The discovery of the restriction enzymes highly contributed to the recognition of the characteristics of the DNA genome, which may be decomposed with their help to several fragments. Each of these fragments can be separated, purified and studied when knowing the specific recognition sites of the enzyme (Roberts, 1976). Earlier examination performed with electronmicroscopic hetero-duplex analysis (Garon *et al.*, 1973) proved that the adenovirus genomes were all organized in an identical way. Physical maps provided means to reveal successfully the differences not only between the individual types but also between strains of the same type (Wadell and Varsányi, 1978; Wadell *et al.*, 1981*a*; Wadell *et al.*, 1981*b*). Several data were published concerning Ad h type 12 belonging to subgenus A, human Ad types 3, 7 and 16 belonging to subgenus B as well as Ad h 1, Ad h 2, Ad h 5 and Ad h 6 species from subgenus C (Berencsi *et al.*, 1978; Doerfler *et al.*, 1979; Green *et al.*, 1979; Medveczky *et al.*, 1981; Mulder *et al.*, 1974; Naroditsky *et al.*, 1980; Tibetts, 1977; Tooze, 1980; Varsányi *et al.*, 1977; Wadell and Varsányi, 1978; Wadell *et al.*, 1981*a*; Winberg and Hammarskjöld, 1980); in contrast, relatively few authors were dealing with types belonging to the largest subgenus D (Hierholzer *et al.*, 1982; Wadell *et al.*, 1980; Wadell *et al.*, 1981*b*). Finally, data concerning Ad h 8 are lacking at all. In present paper, therefore, we aimed at physical mapping of Ad h 8 DNA and compared the results with those obtained on the DNA of other adenovirus species.

Materials and Methods

Viruses. Virus strain Ad h 8 used in earlier experiments (Lengyel and Nász, 1970) was isolated by our group during epidemic keratoconjunctivitis outbreak; all other adenoviruses were prototype strains. The Ad h 8, 9, 10, 27 strains were propagated on HEp-2, the Ad bos 4 virus on primary calf testis monolayer cell cultures, the Ad h 1, 4, 6, 7 (Gomen) 11, and 12 strains in HEp-2 suspension cultures.

High-salt-neutral detergent extraction of virions. Infected and sedimented cells were subjected to one cycle of freezing-thawing in 20 mmol/l sodium phosphate buffer pH 7.0 containing 0.5 mol/l NaCl and 5 mg/ml Triton × 100 (final concentration) and centrifuged for 20 min at + 4 °C with 5000 × g.

Isolation of virus DNA. The virions were purified by CsCl equilibrium gradient centrifugation (Berencsi *et al.*, 1978; Medveczky *et al.*, 1981; Green *et al.*, 1967). Complete virions were dialyzed against DNA buffer (100 mmol/l NaCl, 50 mmol/l, Tris-HCl pH 7.5) and digested for 1 hr at 37 °C with 1 mg/ml pronase (Calbiochem, San Diego, Calif.) in the presence of sodium dodecyl-sulphate (SDS) of 10 mg/ml. The material was subsequently shaken with phenol which had been pre-extracted several times with identical volume of DNA buffer. Chloroform-isoamylalcohol (24 : 1) was added to the suspension (Palmiter, 1974) in a vol identical with the original quantity of the material and further shaken for 15 min. Then the mixture was centrifuged at 4 °C for 30 min with 3000 rev/min. The upper aqueous phase of the two-phase-system was drained off and adjusted to 0.2 mol/l with Na-acetate. The DNA was precipitated with 2.5-fold vol of ethanol at − 70 °C. The precipitate was centrifuged under the above conditions for 20 min and washed with DNA buffer: ethanol (1 : 2). The DNA was dissolved in a buffer containing 10 mmol/l Tris-HCl and 10 mmol/l KCl. The DNA concentration was determined by the difference between the absorptions of the solution measured at 260 and 300 nm in a Pye-Unicam SP8-100 spectrophotometer and adjusted to 100 μg/ml with DNA buffer.

Digestion of DNA with restriction endonucleases. Samples containing 0.5 μg adenovirus DNA were digested according to the standard protocols of the Bethesda Research Laboratories (Bethesda, Md. U.S.A.) (Roberts, 1976) with 5 different restriction enzymes: *Bam*HI, *Hin*dIII, *Pst*I, *Sal*I, and *Kpn*I. The last was obtained from the laboratory of C. Mulder (Worchester, Mass. U.S.A.), the others were prepared in our Institute. After addition of 0.1 mg/ml bromphenolblue, 20 mg/ml SDS and 80 mg/ml final concentration of sucrose, the fragments were separated with horizontal slab gel electrophoresis (Forsblom *et al.*, 1976).

Slab gel electrophoresis. The electrophoresis was performed in Saekem $(ME)^{++}$ agarose (Marine Colloids, Rockland, Maine, U.S.A.) gels of 6—14 mg/ml concentration at room temperature, at 1—2 V/cm for 14—18 hr. The buffer (Helling *et al.*, 1974) contained ethidium bromide at 1 μg/ml final concentration. The gels were photographed in ultraviolet light on ORWO DK5 film, using orange transmission and ultraviolet closing filters. Under the conditions used, DNA fragments larger than 0.5×10^6 M_r could be regularly detected.

Isolation of DNA fragments. The fluorescent DNA bands were localized using photographic films and cut out from the gel. The DNA fragments were extracted either by electromicrodialysis (Geck, unpublished data) or by heat treatment in the presence of $NaClO_4$ followed by hydroxylapatite chromatography and dialysis.

Determination of the denaturation pattern of DNA fragments. For the conventional determination of the physical map (Sharp, 1977), the virus DNA was digested by the restriction enzyme in usual way. The reaction was stopped with 0.1 vol of 100 mg/ml SDS. After the addition of identical quantity of formamide, the mixture was incubated for 15 min at the appropriate temperature and quenched into an ice bath. The material was kept at 0 °C prior to electrophoresis.

Results

Direct comparison of the virion DNAs

The DNA of Ad h 8 has been compared with the genomes of other Ad species in horizontal slab-gel electrophoresis. These examinations provided means for the control of the homogeneity of the DNA preparations and for

Table 1. M_r of restriction fragments of Ad h 8 DNA

Fragment	Restriction enzymes				
	*Bam*HI	*Kpn*I	*Sal*I	*Hin*dIII	*Pst*I
A	11.8*	8.7	8.2	3.6	4.4
B	4.6	5.4	6.1	3.1	3.8
C	3.4	3.6	2.15	3.05	2.75
D	1.38	2.92	1.82	3.00	2.40
E	0.76	1.45	1.72	2.45	2.15
F			1.50	2.05	1.82
G			0.60	1.47	1.24
H				1.03	1.20
I				0.93	1.10
J				0.76	0.65
K				0.50	0.60
L					0.50
Total	22.14	22.07	22.09	21.94	22.61

* $= M_r \times 10^{-6}$

the determination of the M_r of the DNA studied by means of DNAs of known M_r. The results obtained could be compared with the M_r values calculated from the experiments with restriction enzymes. Fig. 1 demonstrates an experiment in which besides of the DNA of Ad h 8 those of the Ad h 1, Ad h 6, Ad bos 4 as well as the *Eco*RI-A fragment of Ad h 1 of M_r 17.5×10^6 were studied. The Ad h 1 and 6 DNAs of M_r 23.0×10^6 appeared as homogeneous bands. The DNA of Ad h 8 seemed to be slightly smaller and a more rapidly migrating band, thus indicating that the presence of a DNA population of lower M_r could also be revealed on the original photograph. In other experiments the Ad h 8 DNA was found to be reproducibly heterogeneous while the DNA of other human strains (4, 7, 9, 10, 11, 12 and 27) showed homogeneity. The Ad bos 4 genome showed also heterogeneity consisting, however, of five different populations.

Table 2. Partial denaturation of the *Hin*dIII fragments of Ad h 8 DNA

Fragment	Incubation temperature (°C)
—	58.5
—	59.5
E, I, K,	60.5
A, J, (B, C, D)*	61.5
H	62.5
F, G, (B, C, D)*	62.5

* One or two of the BCD cluster.

Table 3. Results of combined digestion of Ad h 8 DNA with restriction endonucleases

*Bam*HI + *Sal*I		*Kpn*I + *Sal*I		*Hind*III + *Sal*I		*Hind*III + *Kpn*I	
*Bam*HI-B	4.8*	*Sal*I-B	6.1	*Hind*III-A	3.6	*Hind*III-A	3.6
*Bam*HI-C	3.4	*Kpn*I-B	5.4	*Hind*III-B	3.1	*Hind*III-B	3.1[b]
I.	2.7	*Sal*I-C	2.15	*Hind*III-E	2.45	*Hind*III-C	3.0[b]
*Sal*I-C	2.15	*Sal*I-E	1.72	I.	1.9	*Hind*III-D	3.0[b]
*Sal*I-D	1.82	*Sal*I-F	1.53	II.	1.32	I.	1.7
*Sal*I-E	1.72	*Kpn*I-E	1.43	III.	1.28	II.	1.6
*Sal*I-F	1.53	I.—II.	1.32	IV.	0.97[a]	*Hind*III-G	1.45[b]
*Bam*HI-D	1.35	*Sal*I-G	0.60	*Hind*III-I	0.93[a]	*Kpn*I-E	1.43[b]
II.	1.1			V.	0.92[a]	III.	1.05
*Bam*HI-E	0.85			VI.	0.86	*Hind*III-H	1.02
				*Hind*III-J	0.76	IV.	0.80
				*Sal*I-G	0.60	*Hind*III-J	0.76
				*Hind*III-K	0.50	V.	0.60

* $= M_r \times 10^{-6}$

[a] The number of fragments of about 10^6 M_r is uncertain.

[b] The uncleaved fragment could not be specified within the overlapping band clusters.

Digestion of Ad h 8 DNA with restriction endonucleases

Results of digestion of Ad h 8 DNA with 5 different endonucleases and subsequent electrophoresis are presented in Fig. 2. Digestion with *Sal*I produced 7 fragments of a M_r between 8.2 and 0.6×10^6, *Bam*HI 5 fragments of M_r 11.8 to 0.76×10^6, *Hin*dIII digestion resulted in 11 fragments of M_r 3.6 to 0.5×10^6, *Kpn*I in 5 fragments of M_r 8.7 to 1.45×10^6 and *Pst*I produced 12 fragments with M_r 4.4 to 0.5×10^6 (Table 1). The M_r of the Ad h 8 genome was accordingly between 21.96 and 22.61×10^6. On Fig. 2, a few submolar

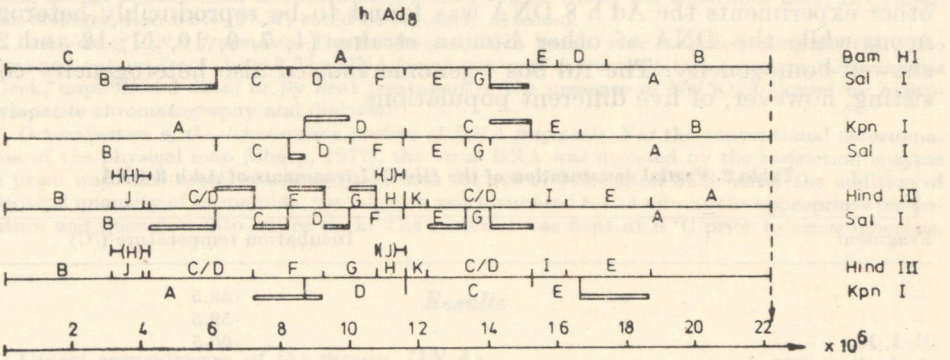

Fig. 4.

Restriction site maps of Ad h 8 DNA

Open bars indicate fragments between the specific sites of two different enzymes.

fragments are visible between the *Sal*I-A and -B as well as between *Kpn*I-B and -C fragments. Since the digestion has been complete, the appearance of submolar fragments indicates the presence of different populations of defective genomes in a part of the virions.

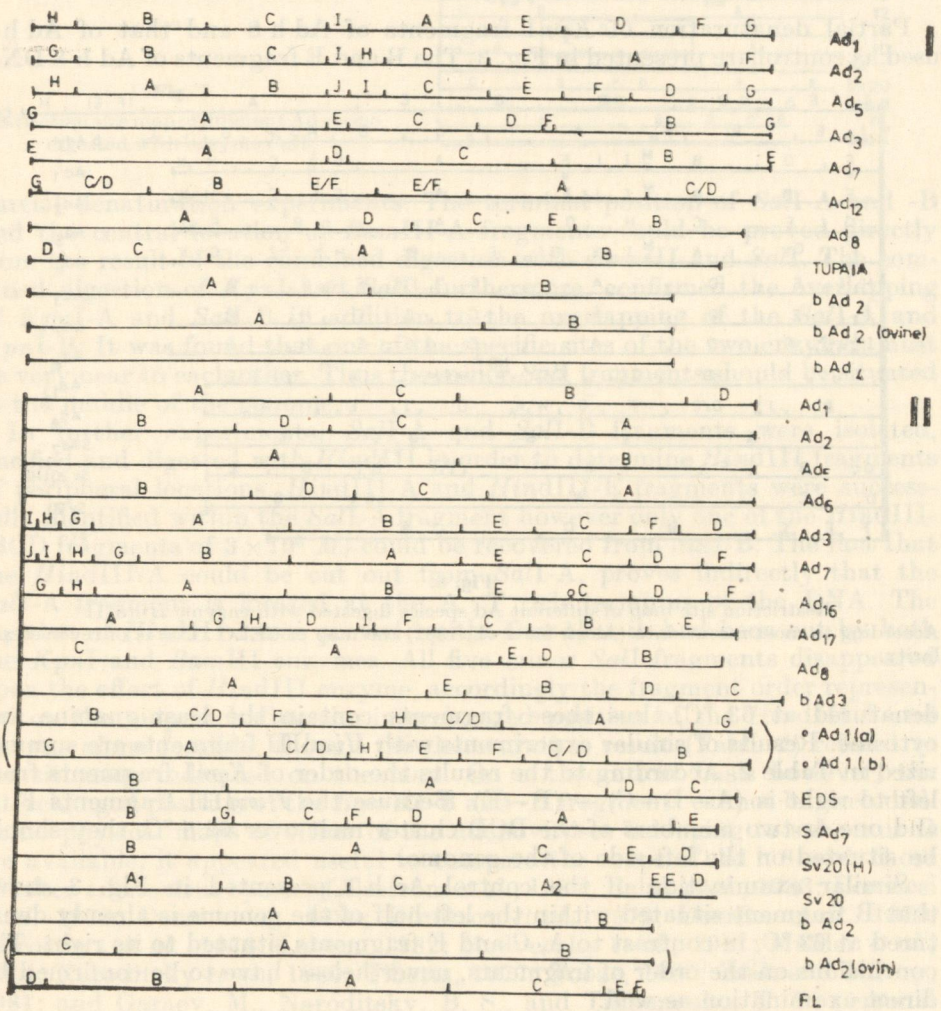

Fig. 5.

Restriction site maps of different Ad species

Enzymes: *Kpn*I (I) and *Bam*HI (II).

Capital letters indicate the individual restriction fragments. The absence of labelling indicates DNA regions where the exact order of fragments has not been determined (Ad h 10, Ad h 12). Distances are proportional with the M_r of the genomes, taking the size of Ad h 1 DNA 23×10^6 M_r or 36,500 base pairs.

The *Hin*dIII-BCD fragments (Fig. 2) could not be separated even in case of longer running distance, their possible M_r might have been between 3.0 and 3.1×10^6.

Partial denaturation of Ad h 8 fragments

Partial denaturation of *Kpn*I fragments of Ad h 8 and that of Ad h 1 used as control are presented in Fig. 3. The B and E fragments of Ad h 8 DNA

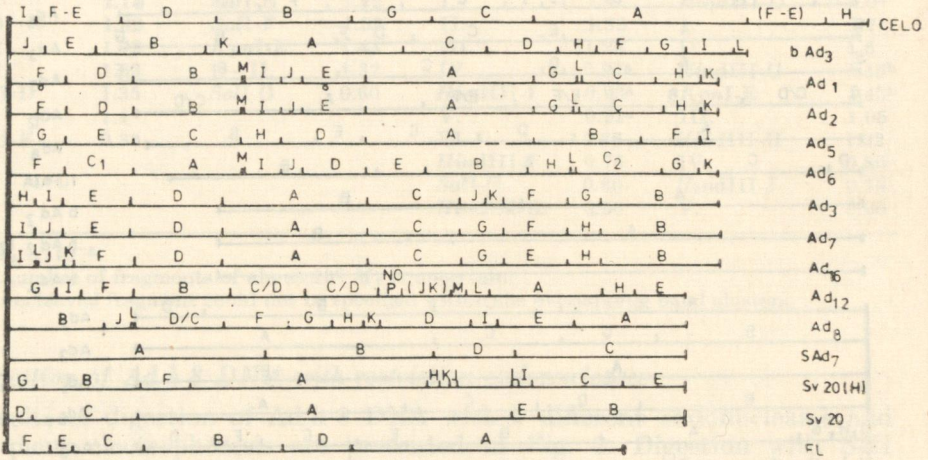

Fig. 6.

Restriction site map of different Ad species digested with enzyme *Hin*dIII According to most recent data (Alestrom *et al.*, 1982) the map of CELO DNA is of reversed polarity.

denatured at 63 °C, thus these fragments contain the least guanine and cytosine. Results of similar experiments with *Hin*dIII fragments are summarized in Table 2. According to the results the order of *Kpn*I fragments from left to right is (A—D—C) — (B—E). Because the *Hin*dIII fragments F, G and one or two members of the BCD cluster melt over 62.5 °C, they should be situated on the left side of the genome.

Similar examination of the control Ad h 1 presented in Fig. 3 shows that B fragment situated within the left half of the genome is already denatured at 63 °C, in contrast to A, C and E fragments situated to its right. The conclusions on the order of fragments, nevertheless, have to be confirmed by direct examination as well.

Combined digestion of Ad h 8 DNA with restriction endonucleases

The relative molecular weights of fragments obtained by double digestion of the Ad h 8 genome are summarized in Table 3. Based on these results, the physical map of the Ad h 8 DNA has been drawn as presented in Fig. 4. The fragments between the specific sites of two different enzymes have been designated by open bars. The polarity has been determined on the basis of

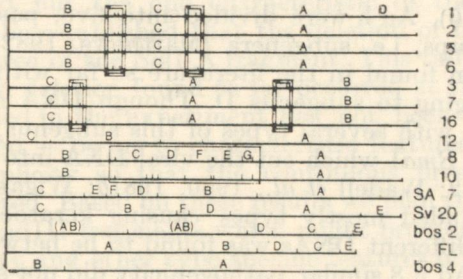

Fig. 7.
Restriction size map of different Ad species digested with enzyme *Sal*I

partial denaturation experiments. The terminal position of *Sal*I-A and -B and the central location of *Bam*HI-A fragments could be proved directly from the result of the combined digestion with *Bam*HI and *Sal*I. The combined digestion of *Kpn*I and *Sal*I, furthermore, confirmed the overlapping of *Kpn*I-A and *Sal*I-B in addition to the overlapping of the *Sal*I-A and *Kpn*I-B. It was found that one of the specific sites of the two enzymes must be very near to each other. Thus the minor *Sal*I fragments should be situated in the middle of the genome.

In further experiments, *Sal*I-A and *Sal*I-B fragments were isolated, purified and digested with *Hin*dIII in order to determine *Hin*dIII fragments of peripheral locations. *Hin*dIII-A and *Hin*dIII-E fragments were successfully identified within the *Sal*I-A fragment however only one of the *Hin*dIII--BCD fragments of 3×10^6 M_r could be recovered from *Sal*I-B. The fact that the *Hin*dIII-A could be cut out from *Sal*I-A, proves indirectly that the *Sal*I-A fragment is located at the A : T rich terminus of the DNA. The position of *Hin*dIII-I was proved by the fact that it had been cut by both the *Kpn*I and *Bam*HI enzymes. All five minor *Sal*I fragments disappeared upon the effect of *Hin*dIII enzyme, accordingly the fragment order represented on the map including the interchanged position of the *Hin*dIII-J and -H was alone feasible, though the denaturation result (Table 2) refered to the situation indicated on the map. On a theoretical basis however, the interchanged position of the *Bam*HI-B and -D fragments cannot be excluded. As no physical maps of either of the adenoviruses belonging to subgenus D are available, it appeared useful to compare the maps of Ad h 8 with those of other adenovirus species (Aleström *et al.*, 1982; Belák *et al.*, 1983; Berencsi *et al.* 1978; Darai *et al.*, 1980; Dimitrov *et al.*, 1979; Doerfler *et al.*, 1979, Kurokowa *et al.*, 1978; Larsen, 1982; Medveczky *et al.*, 1981; Mulder *et al.*, 1974; Naroditsky *et al.*, 1980; Tibetts, 1977; Tooze, 1980; Zsák and Kisary, 1981; and Garaev, M., Naroditsky, B. S., and Tikhonenko, T. I., personal communication). The results of this comparison are demonstrated in Figs 5-I, 5-II, 6 and 7.

Discussion

Based on the sequence homology of the genomes as well as on structural, biological, immunological and biochemical characteristics (Fujinaga *et al.*, 1979; Garon *et al.*, 1973; Green *et al.*, 1979; Mackey *et al.*, 1979; Wadell *et al.*,

1980), Ad h were divided into five, possibly six (Wadell *et al.*, 1980) subgroups, i.e. subgenera (Matthews, 1982). Data of physical maps have not been found in the literature so far with of the large number of species belonging to subgenus D. Though DNA restriction experiments were carried out with several types of this subgenus, only one enzyme has been used — the *Sma*I which cut the viral DNA into 14—17 fragments (Hierholzer *et al.*, 1982; Wadell *et al.*, 1980, 1981*b*; Wigand *et al.*, 1980). These examinations included mostly types causing keratoconjunctivitis; the molecular weight of different DNAs was found to be between 22.28 and 23.14×10^6. The DNA of Ad h 8 similar pathogenicity did not show significant deviation from these values (Table 1). Taking into consideration the fragments above M_r 0.5×10^6, the values were between $21.96—22.61 \times 10^6$. The constructed physical map (Fig. 4) allowed a more precise calculation of the molecular weight of the genome and it was found to be $22.1—22.3 \times 10^6$. The Ad h 8 strain examined is not the prototype strain, thus strain-specific differences demonstrated with other species (Wadell and Varsányi, 1978; Wadell *et al.*, 1981*a*) must also be taken into consideration. The physical maps described so far (Figs 5 to 7) fail to reveal essential differences among the species within the adenovirus subgenera. The specific sites of certain restriction enzymes may disappear or reappear in consequence of mutation or dislocate due to insertions, deletions and recombinations and may cause displacement of their specific sites (Medveczky *et al.*, 1981; Tibetts, 1977; Wadell and Varsányi, 1978). Deviations may develop mainly in the right or central thirds of the genome. No relationship was observed between the different subgenera on the basis of the physical maps. Comparing these maps with the restriction maps of Ad h 8 belonging to subgenus D (Figs. 5, 6, 7), it could be established that they differ from every finding published so far.

As no other physical map of Ad belonging to subgenus D has been available so far we do not know which part of the Ad h 8 map could be considered as subgenus-specific. The *Sal*I enzyme-digestion of Ad h 10 DNA (Fig. 7) indicate that the specific sites are to be found in the central third of the genome which might be characteristic for the subgenus specificity.

It is striking in this comparison that in the adenovirus species studied so far — with one exception — the *Hin*dIII sites could always be found in the vicinity of the sites corresponding to M_r 4×10^6 and 11.6×10^6 apart the left terminus of the genome. It appears feasible that these sites are situated within highly conserved regulatory sequences characteristic of the DNA of human adenoviruses, they may be shifted slightly but do not disappear from the genome.

It was considered as characteristic for virus strains propagated in cell cultures that DNA fragments up to 0.15 map units may be deleted or replaced occasionally by host cell sequences in the DNA of virions (Kurokawa *et al.*, 1978; Tooze, 1980). The electrophoresis of the complete Ad h 8 genome refers similarly to this phenomenon. Two populations were found in the *Sal*I digestion mixture and one in the *Kpn*I which developed assumably in consequence of deletion. In the course of cross-digestion with these two enzymes

the *Sal*I fragments disappeared but the one characteristic of the *Kpn*I enzyme remained. Thus it can be established that the deletion of both defective DNA-s occurred in the area of the *Sal*I-A fragment. This tallies with the earlier observation that each deletion found so far occurred at the right side of the DNA. The Ad h 8 used in the experiment has not been plaque--purified and could be propagated — owing to characteristic features of the type — only with high multiplicity so that the continuous presence of defective genomes is to be expected. Based on these results we plan the molecular cloning of the *Pst*I and *Hin*dIII fragments which might provide means for the study of the pathogenicity and other type specific features of Ad h 8.

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Explanation of Micrographs (Plates XXXIII—XXXV):

Fig. 1. Direct comparison of viral genomes. Agarose slab-gel electrophoresis run at 1.2 V/cm for 40 hr, at gel concentration 0.6%. Different concentrations of Ad h 1 (1, 2, 12, 13), Ad h 1 *Eco*RI-A fragment (3, 4), Ad h 6 (5, 6, 7) Ad bos 4 (8, 9), and Ad h 8 (10, 11).

Fig. 2. Electrophoretic pattern of Ad h 8 DNA fragments. Ad h 8 DNA was digested with restriction endonucleases *Sal*I (1), *Bam*HI (2), *Hin*dIII (4) and *Kpn*I (5); the *Hin*dIII digested fragments of Ad h 1 DNA were used as controls (3 and 6) at 1.2 V/cm for 16 hr. Gel concentration in the left 0.6% in the right 1%. The corresponding bands in controls are connected with horizontal lines.

Fig. 3. Electrophoretic pattern of *Kpn*I fragments of Ad h 1 (1—3) and Ad h 8 (5—7) DNA after partial denaturation at 63 °C (1 and 5) and 58 °C (2 and 6) compared with unheated fragments (3 and 7) and unheated *Hin*dIII fragments of Ad h 1 DNA (4 and 8) as control (M_r of the control: Medveczky *et al.*, 1981). Electrophoresis at 1.2 V/cm for 16 hr, gel concentration 1%. Arrows indicate the fragments melted at 63 °C.