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Mapping of Restriction Fragments and Transforming Ability of Adenovirus 31

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SUMMARY

Human adenovirus (Ad) 31 DNA (strain 1315) was cleaved into nine, eleven, nineteen, four and five specific fragments by the restriction endonucleases *Eco*RI, *Bam*HI, *Hin*dIII, *Sal*I and *Sma*I, respectively. The size of each fragment was estimated from the relative mobility in agarose gel electrophoresis. These specific fragments were mapped on the Ad31 genome by analyzing partial digestion products and double digestion products and by Southern blot hybridization. The *Hin*dIII-G fragment, which was mapped on one end of the genome, was shown to have transforming ability of Ad31 by cell transformation experiments.

INTRODUCTION

Of the human adenoviruses, highly oncogenic subgroup A (Ad12, Ad18, and Ad31) and weakly oncogenic subgroup B (Ad3, Ad7, Ad14, Ad16, and Ad21) transform rodent cells and induce tumors in newborn hamsters (7). Although human adenoviruses in subgroup C (Ad1, Ad2, Ad5, and Ad6) can transform rodent cells, they fail to induce tumors in animals (7). Among the three members of highly oncogenic subgroup A, Ad12 is the most extensively studied on the basis of DNA nucleotide sequence (2, 9, 19), mRNA mapping (12), and analyses of complementary DNA's of mRNA's (11). Comparison of the organization and expression of the transforming genes of Ad12 with those of nononcogenic Ad5 and Ad2 (1, 21) has revealed some characteristic features of Ad12, though they shared the common basic conformation of the genes (2, 9). For elucidation of the characteristics specific to highly oncogenic subgroup A it is necessary to extend studies to the other members of the subgroup. In this study, we constructed cleavage maps of Ad31 DNA with five different restriction endonucleases and localized the transforming ability on the genome.

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MATERIALS AND METHODS

Viral DNA

The strain 1315 of Ad31, obtained from Dr. Hiroto Shimojo of Tokyo University, was propagated in KB cells. Viral DNA was extracted from purified virions, and DNA fragments were purified after restriction endonuclease digestion and agarose gel electrophoresis.

Restriction endonuclease digestion and gel electrophoresis

*Eco*RI, *Bam*HI, *Sma*I were purchased from Takara Shuzo Co. Ltd. *Hin*dIII and *Sal*I were prepared according to the procedure described elsewhere (10, 20). *Bam*NI (16), an isoscyzomer of *Bam*HI, was used at an early stage of this study, and the results were summarized in the *Bam*HI mapping data. Reaction buffers for the enzymes used were described previously (13). One unit of these enzymes was defined as the minimum amount which was able to digest 1.0 μg of Ad12 DNA at 37°C for 1 hr. Partial digestion was achieved by reducing the amount of enzymes and shortening the reaction period. DNA fragments produced were separated by agarose gel electrophoresis and were visualized under UV light after staining with ethidium bromide (0.5 μg/m*l*) or detected by autoradiography. The size of each DNA fragment was calculated from the relative mobility using Ad12 DNA fragments as markers (13).

Labeling of DNA

Ad31 DNA and its fragments were labeled at both ends of molecules using T4 DNA polymerase (3). Nick translation reaction (14) was used to prepare highly radioactive DNA fragments as probes for Southern blot hybridization (4).

Transformation of rat cells

Transformation of rat 3Y1 cells (clone B-1-6), established from a Fischer rat embryo (8) was carried out according to the calcium technique (6) as described in (22). Focus formation was examined microscopically for 3 weeks.

RESULTS AND DISCUSSION

Ad31 DNA was digested with five different restriction endonucleases, *Eco*RI, *Bam*HI, *Hin*dIII, *Sal*I and *Sma*I, and the resulting specific fragments were identified after resolution by agarose gel electrophoresis (Fig. 1). The size of each DNA fragment was calculated from the relative mobility in agarose gel electrophoresis, and summarized in Table 1. Most of these values were confirmed by analysis of recleavage products (see Table 4).

Ad31 DNA was labeled at both ends by T4 DNA polymerase and digested with *Eco*RI, *Bam*HI and *Hin*dIII. After agarose gel electrophoresis and autoradiography, two fragments retaining the radioactivity were identified among the

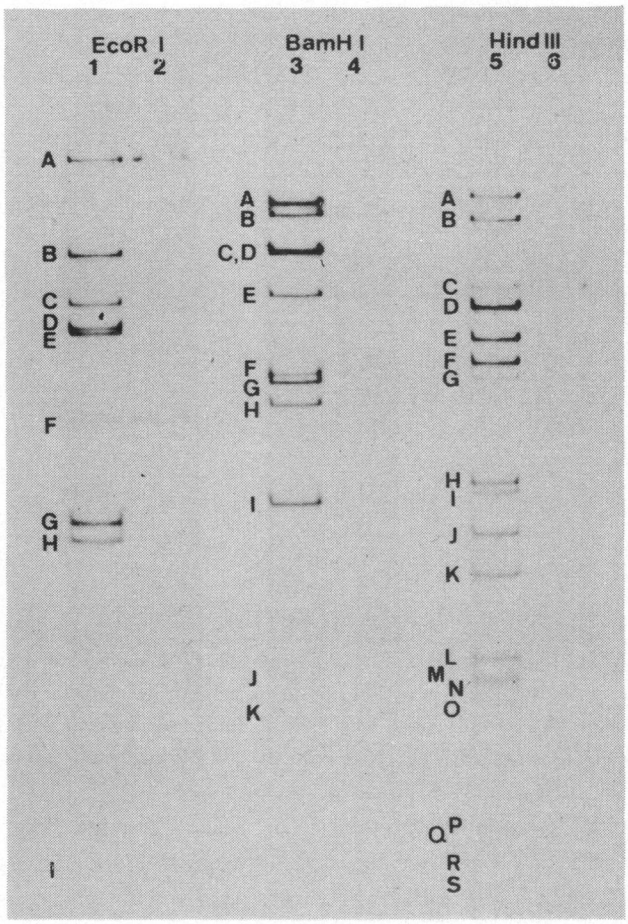

Fig. 1. Identification of Ad31 DNA fragments cleaved by three different restriction endonucleases.

Restriction fragments of Ad31 DNA produced with *Eco*RI (1), *Bam*HI (3), and *Hin*dIII (5) were labeled by the T4 DNA polymerase reaction, and separated by 1.4% agarose gel electrophoresis. Ad31 DNA labeled on both ends was digested and electrophoresed in parallel to identify the terminal fragments among the multiple fragments produced by digestion with each enzyme (2, 3 and 5).

Table 1. *Sizes of Ad31 DNA Fragments Cleaved by Five Different Restriction Endonucleases*

*Eco*RI	*Bam*HI	*Hin*dIII	*Sal*I	*Sma*I
A15,480(46.5)	A 6,500(19.5)	A 6,850(20.6)	A15,320(46.0)	A14,250(42.8)
B 4,540(13.6)	B 5,780(17.4)	B 5,500(16.5)	B14,870(44.7)	B 8,820(26.5)
C 3,300(9.9)	C 4,480(13.5)	C 3,520(10.6)	C 2,350(7.1)	C 5,560(16.7)
D 2,950(8.9)	D 4,430(13.3)	D 3,180(9.5)	D 760(2.3)	D 4,300(12.9)
E 2,860(8.6)	E 3,440(10.3)	E 2,700(8.1)		E 370(1.1)
F 1,880(5.6)	F 2,300(6.9)	F 2,380(7.1)		
G 1,160(3.5)	G 2,190(6.6)	G 2,220(6.7)		
H 1,070(3.2)	H 1,990(6.0)	H 1,380(4.1)		
I 60(0.2)	I 1,240(3.7)	I 1,300(3.9)		
	J 530(1.6)	J 1,070(3.2)		
	K 420(1.3)	K 880(2.6)		
		L 580(1.7)		
		M 520(1.6)		
		N 490(1.5)		
		O 430(1.3)		
		P 120(0.4)		
		Q 90(0.3)		
		R 60(0.2)		
		S 30(0.1)		

The Size of each fragment was estimated as described in the text, and presented in nucleotide pairs. The value in percent genome of each fragment was also shown in parenthesis. The size of Ad31 genome was estimated to be 33,300 nucleotide pairs with an error limit of ± 1,000 nucleotide pairs.

multiple specific fragments produced by each enzyme (Fig. 1). Thus, the fragments A and F of *Eco*RI digests, the fragments B and E of *Bam*HI digests and the fragments C and G of *Hin*dIII digests were localized at the ends of Ad31 genome, respectively.

To construct a cleavage map with *Eco*RI, partial digestion products were labeled by T4 DNA polymerase and separated by agarose gel electrophoresis. Each partial digestion product recovered from gel was recleaved with *Eco*RI and examined for fragments carrying the radioactivity. If the size of a partial digestion product was significantly larger than the summed size of detected fragments, one or more fragments of reasonable size were estimated as additional components as summarized in Table 2. The order of the *Eco*RI DNA fragments along the viral genome was determined to be ADIGBHCEF by overlapping the estimated components in each partial digestion product.

Table 2. *Cleavage of Partial Digestion Products with EcoRI*

Partial digestion product	Complete cleavage product detected[a]	Candidate for inside fragment[b]	Linkage order[c]								
a	D			D	(I)						
b	C, H							H	C		
c	E, F									E	F
d[d]	B, G, H					G	B				
							B	H			
e	C, E								C	E	
f	G, H	B				G	(B)	H			
g	E, H	C						H	(C)	E	
i[d]	B, C	H					B	(H)	C		
	B, D	G, I		D	(I)	(G)	B				
	F, H	C, E						H	(C)	(E)	F
j	C, G	B, H				G	(B)	(H)	C		
k[d]	A, D		A	D							
	A, G	D, I	A	(D)	(I)	G					
α[e]					(I)	(G)					
β[e]				(D)	(I)	(G)					
			A	D	I	G	B	H	C	E	F

Ad31 DNA (46 μg) was partially digested with 20 units of *Eco*RI at 37°C for 15 min. The digestion products were labeled as described in the text and electrophoresed in a 0.9% agarose slab gel. Each partially digested fragment was extracted from gel and redigested with 3- to 5-fold excess amount of *Eco*RI. The digestion products were resolved by electrophoresis in a 1.4% agarose slab gel, and detected by autoradiography.

a Two fragments on the labeled terminals were detected in principle.

b When the partially digested products were composed of three or more fragments, internal fragments were not detected on the autoradiograms and estimated on the basis of size.

c Fragments not detected on autoradiograms are represented in parentheses.

d Partially digested products d, i and k were composed of two or more products, which were not resolved with each other because of their similar sizes.

e Partially digested products α and β were identified in an agarose gel, but not recovered for redigestion because of their small amounts. Their component fragments were estimated on the basis of their sizes.

A series of partial digestion products containing one end of Ad31 DNA were detected by partial digestion of the viral DNA labeled on both ends with *Bam*HI and *Hind*III (Table 3). Some of the partial digestion products were also detected by

limited digestion of the *Eco*RI-A fragment labeled on one end (Table 3). It was also shown that the *Bam*HI-B, the *Hin*dIII-G and the *Eco*RI-A fragment shared a common end of the genome. These partial digestion fragments were characterized

Table 3. *Serial terminal fragments produced by partial digestion of Ad31 DNA labeled on both ends with BamHI and HindIII*

fragment containing labeled *Bam*HI-B	size (% of Ad3l DNA)	order of component fragment	fragment containing labeled *Bam*HI-E	size (% of Ad3l DNA)	order of component fragment
0	17.4	B	0	10.3	E
1	23.4	BH	1	11.9	JE
2	30.3	BHF	2	13.1	KJE
3	33.9	BHFI	3	26.4	CKJE
4	47.1	BHFID	4	33.0	GCKJE
			5	52.6	AGCKJE

overall linkage : BHFIDAGCKJE

fragment containing labeled *Hin*dIII-G	size (% of Ad3l DNA)	order of component fragment	fragment containing labeled *Hin*dIII-C	size (% of Ad3l DNA)	order of component fragment
0	6.7	G	0	10.6	C
1	10.8	GH	1−8[a]	11.0−17.6	(L-S)[a]C
2	20.6	GHD	9	20.4	K(L-S)C
3	23.7	GHDJ	10	41.1	AK(L-S)C
4	32.1	GHDJE	11	57.1	BAK(L-S)C
5	36.0	GHDJEI			
6	43.5	GHDJEIF			

overall linkage : GHDJEIFBAK(L-S)C

Ad3l DNA was labeled on both ends as described in the text, and an aliquot was digested with *Eco*RI to obtain the *Eco*RI-A fragment labeled on one end. Two series of labeled partial digestion products containing *Bam*HI-B and *Hin*dIII-G were produced by limited digestion of the *Eco*RI-A fragment labeled on one end with *Bam*HI and *Hin*dIII, respectively. Those containing *Bam*HI-E and *Hin*dIII-C were produced by limited digestion of Ad3l DNA labeled on both ends, and identified as products not deduced from the *Eco*RI-A fragment. Fragments of similar sizes were distinguished based on the data of double digestion and Southern hybridization experiments.

a Eight bands were detected closely with each other on autoradiograms, indicating that small fragments L to S were localized in sequence. The linkage order of these fragments were not determined exactly.

sequentially from small ones to larger ones based on the difference of their sizes. The order of the *Bam*HI and the *Hin*dIII DNA fragments was thus estimated along the genome. The results of double digestion experiments summarized in Table 4 were used to distinguish a fragment from others of similar sizes. 1. *Sal*I cleaved *Bam*HI-D but not *Bam*HI-C. 2. *Eco*RI cleaved *Bam*HI-G but not *Bam*HI-F nor *Bam*HI-H. 3. *Bam*HI cleaved *Hin*dIII-I and *Hin*dIII-J but not *Hin*dIII-H nor *Hin*dIII-K. Southern blot hybridization experiments provided further confirmation of the localization of these fragments. 1. *Hin*dIII-K hybridized *Bam*HI-C and *Eco*RI-C. 2. *Hin*dIII-I hybridized *Bam*HI-D, *Bam*HI-I and *Eco*RI-A. 3. *Hin*dIII

Table 4. *Double Digestion of Ad31 DNA*

Fragment recleaved	Restriction endonuclease for recleavage	Size of recleavage products (nucleotide pairs)
*Bam*HI-B	*Sma*I	4,300, 1,450
*Bam*HI-F		2,100, 200
*Bam*HI-A		2,950, 3,550
*Bam*HI-E		3,050, 370
*Bam*HI-D	*Sal*I	4,000, 420
*Bam*HI-A		320, 2,400, 3,800
*Sma*I-B		5,400, 760, 2,400, 240
*Hin*dIII-B	*Eco*RI	1,250, 2,980, 1,170
*Hin*dIII-A		4,480, 1,070, 1,310
*Hin*dIII-C		1,630, 1,890
*Bam*HI-D		4,150
*Bam*HI-A		2,730, 1,170, 2,600
*Bam*HI-G		1,940
*Bam*HI-C		820, 3,300
*Bam*HI-E		1,550, 1,890
*Hin*dIII-D	*Bam*HI	2,180, 980
*Hin*dIII-J		980
*Hin*dIII-E		2,180
*Hin*dIII-I		700, 500
*Hin*dIII-B		1,490, 4,000
*Hin*dIII-A		2,510, 2,190, 2,140

Double digestion experiments were carried out by digestion of intact DNA with two different restriction endonucleases in sequence or at the same time. In some cases the first digestion products were labeled as described in the text before the second digestion. The size of each fragment was estimated as described in the text. Some double digestion products were not detected in these experiments because of their small sizes or inefficient labeling.

-J hybridized *Bam*HI-H. 4. *Hin*dIII-H hybridized *Bam*HI-B. Based on the above results, the *Bam*HI and *Hin*dIII cleavage maps of Ad31 DNA were constructed as shown in Fig. 2. In the *Bam*HI cleavage map, eight small fragments L to S were not localized exactly, because they were not distinguished from each other in the experiment summarized in Table 3.

*Sal*I cleaved Ad31 DNA into four fragments (figure not shown). By analyzing the double digestion products summarized in Table 4, *Sal*I sites were located at the map positions 46.0, 48.3, 55.3 from the end on which *Hin*dIII-G was located. The order of the fragments was thus determined as ADCB (Fig. 2). Five *Sma*I fragments were also aligned along the genome in the order of DCBAE from the *Hin*dIII-G end by the above results (Fig. 2).

The size of Ad31 DNA was estimated to be 33,300±1,000 nucleotide pairs as the summation of the sizes of specific DNA fragments. Sizes of fragments larger than 3,000 nucleotide pairs were based on the summation of those of recleavage products with other enzymes. On the basis of the fragment-size so obtained we constructed the cleavage maps of Ad31 DNA as accurately as possible. When these were compared to the maps of Ad12 DNA (13), some similarity was found between maps of respective enzymes, but the extent seemed lower than that of similarity found between Ad2 and Ad5 or between Ad3 and Ad7 (23).

The transforming ability of human adenoviruses examined so far resides in a limited region of about 7 to 8% in size and localized on one end of the viral genome (5, 15, 17). So the terminal fragments *Hin*dIII-G and *Hin*dIII-C of Ad31 DNA were examined for transforming ability. Each of *Hin*dIII-G (0.5 μg/dish) and

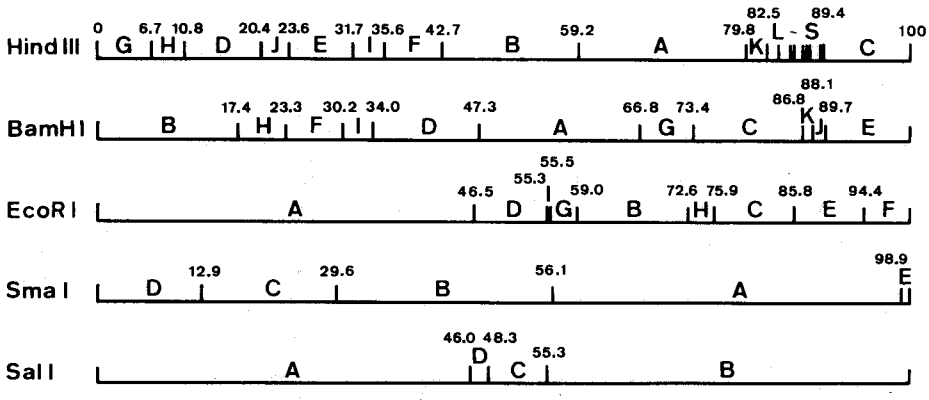

Fig. 2. Cleavage maps of Ad31 DNA with *Hin*dIII, *Bam*HI, *Eco*RI, *Sma*I and *Sal*I.

Fragments are located on the Ad31 genome represented by horizontal lines. Cleavage sites are marked in map units (percent genome length from the left end) over vertical lines.

*Hin*dIII-C (0.5 μg/dish) coprecipitated with calcium phosphate was transfected to subconfluent monolayers of 3Y1 cells in 60 mm dishes. Transformed foci (>10 foci/dish) became visible to the naked eye 3 weeks after transfection of the *Hin*dIII -G fragment. Each focus consisted of small piled-up cells surrounded by untransformed cells (Fig. 3). No focus was formed in 3Y1 cells transfected with the *Hin*dIII-C fragment or mock-transfected cells. Thus the transforming ability of Ad31 was localized at the end of the genome where *Hin*dIII-G was mapped. This end was placed to the left as shown in Fig. 2.

All the transformed cell lines (31GY cells) established from the foci could

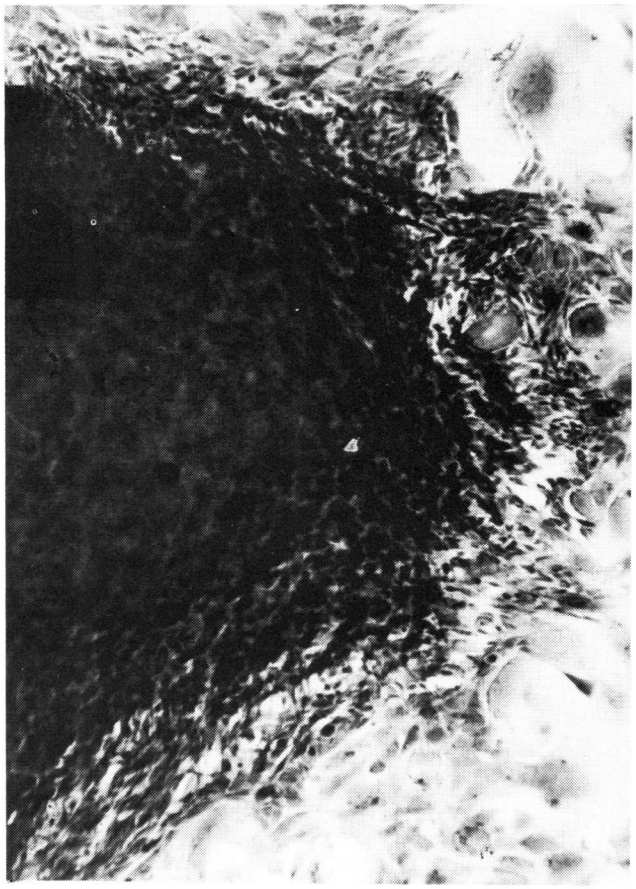

Fig. 3. A microscopic picture of a transformed focus.
A focus of 3Y1 cells transfected with the *Hin*dIII-G fragment of Ad31 DNA (31GY) was stained with Giemsa and examined under a microscope. A focus consisted of small piled-up cells (transformed cells) surrounded by untransformed cells.

form small tiny colonies with a diameter of less than 0.1 mm in soft agar, which were detectable under a light microscope (T. Yamashita, unpublished observation). This seems a remarkable feature of 3lGY cells, because GY cells, which were transformed by the *Hin*dIII-G fragment (leftmost 0-6.8%) of Ad12 DNA, formed visible colonies to the naked eye under the same conditions (17). 3lGY cells are rather similar to HY cells, which were incompletely transformed by the *Acc*I-H fragment (leftmost 0-4.7%) of Ad12 DNA (18). It is important to know which portion of transforming genes is integrated and expressed in 3lGY cells for comparative studies of the functions of transforming genes of highly oncogenic adenoviruses.

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