Cleavage Maps of Adenovirus Type 12 Transforming Segment by Restriction Endonucleases *Acc* I, *Hae* III, *Hha* I, *Hind* II, *Hinf* I and *Hph* I

Takao KIMURA, Yukiharu SAWADA, Seiichiro UESUGI and Kei FUJINAGA[1]

Department of Molecular Biology, Cancer Research Institute Sapporo Medical College, Sapporo 060, Japan

SUMMARY

Detailed cleavage maps of the left part of adenovirus type 12 DNA by restriction endonucleases *Acc* I, *Hae* III, *Hha* I, *Hind* II, *Hinf* I and *Hph* I were constructed by analyzing redigestion products and partial digestion products and by the DNA-DNA hybridization.

INTRODUCTION

The transforming genes of human adenovirus (Ad) 12 reside in a small region of the left end of the viral genome, and restriction fragments derived from the left end can transform cells (1, 2). The nucleotide sequence of the transforming *Hind* III-G fragment of Ad12 DNA, the left 6.8% of the genome, has been determined, providing highly detailed cleavage maps by various restriction endonucleases (3, 4). However, a more progressive mapping of the right region next to the *Hind* III-G, is required to analize the structure and the function of the entire early region E1 and to characterize the viral genes integrated into tumor and transformed cells induced by the Ad12 DNA and Ad12 DNA fragments (3).

In this paper, detailed cleavage maps of the left part of Ad12 DNA with various restriction endonucleases (EndoR) were constructed by analyzing redigestion products and partial digestion products and by the DNA-DNA hybridization.

MATERIALS AND METHODS

Virus and viral DNAs

The Huie strain of Ad12 was grown in KB cells and viral DNA was

[1]: Author to whom reprint requests should be addressed.

isolated from purified virions as described by Green and Pina (5, 6). To prepare ^{32}P-labeled Ad12 DNA, KB cells were infected in phosphate-free Eagle's minimum essential medium containing 100 μCi/ml [^{32}P]-orthophosphate and 5% dialyzed calf serum, as described previously (7). The specific activity of Ad12 [^{32}P]-DNA was $1\times10^6-2\times10^6$ cpm/μg.

Restriction endonuclease digestion and gel electrophoreses

Eco RI and *Hind* II were purchased from Boehringer Mannheim. *Acc* I, *Hae* III, *Hha* I and *Hinf* I were kindly provided by Dr. Mituru Takanami, Kyoto University. *Hind* III and *Hph* I were purified from *Hemophilus influenzae* Rd strain (8) and *Haemophilus parahaemolyticus* (9), respectively.

Reaction buffers for enzyme digestion were as follows: *Acc* I [10 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 50 mM KCl, 5 mM β-mercaptoethanol]; *Hae* III and *Hha* I [50 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 0.5 mM dithiothreitol]; *Hind* II [10 mM Tris-HCl (pH 7.9), 6.6 mM $MgCl_2$, 60 mM NaCl, 1 mM dithiothreitol]; *Hind* III [20 mM Tris-HCl (pH 7.6), 7 mM $MgCl_2$, 60 mM NaCl, 7 mM β-mercaptoethanol]; *Hinf* I [10 mM Tris-HCl (pH 7.5), 5 mM $MgCl_2$, 5 mM β-mercaptoethanol]; *Hph* I [10 mM Tris-HCl (pH 7.4), 10 mM $MgCl_2$, 10 mM β-mercaptoethanol]; *Eco* RI [0.1 M Tris-HCl (pH 7.5), 10 mM $MgCl_2$, 50 mM NaCl].

Digestion of Ad12 DNA and purification of DNA fragments were carried out as described (10). Agarose gel and polyacrylamide gel electrophoresis were carried out as described previously (11).

Agarose gel was dried over steam heat on a Toyo 514 paper as described by Maizel (12), and then autoradiographed on a sheet of Kodak X-ray film. Saranwrap-covered polyacrylamide gel was placed facing a sheet of Kodak X-ray film, sandwiched between two glass plates, and exposed at 4°C.

End labeling of Restriction Fragments

Termini of resulting fragments were labeled with ^{32}P either by polynucleotide kinase reaction for 5′ ends (13) or by T4 DNA polymerase reaction for 3′ ends (14) as described by Sugisaki *et al.* (4).

Southern's Hybridization

Southern's hybridization was performed as follows: After gel electrophoresis, *Hind* II fragments of Ad12 DNA were transferred to a nitrocellulose membrane filter by Southern's technique (15), and hybridized with ^{32}P-labeled probes in 2 ml of hybridization buffer containing 0.2 M Tris-HCl (pH 7.9), 0.6 M NaCl, 0.02 M EDTA, 0.5% SDS and 50% formamide at 37°C for 40 hr (16, 17). The filter was washed, air-dried and autoradiographed. ^{32}P-labeled probes were prepared by *in vitro* nick-translation (18) as described (17).

RESULTS AND DISCUSSION

Cleavage maps of Ad12 DNA with *Eco* RI (19) and *Hind* III (20) have been determined, and those of the left end 18.2% of Ad12 DNA are summarized in Fig. 1. These are useful for detailed mapping of the transforming gene.

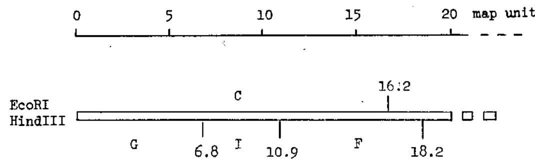

Fig. 1. *Cleavage Maps of the Left end of Ad 12 DNA with Eco RI and Hind III.*

The location of *Hph* I cleavage sites in Ad12 *Hind* III-I and -F fragments were determined by analyzing the partial digestion of labeled fragments with ^{32}P at one end. A partial digestion in which all of the partial products are adequately represented is essential for the site mapping. The conditions for this are determined by analyzing a number of early time points in a reaction, so constituted as to reach completion. An example of optimal condition is shown in Fig. 2.

For reaction site mapping analysis the *Eco* RI-C fragment now labeled at only left of its 3′ termini partially digested with *Hph* I and products were electrophoresed. Reading from the labeled terminus there were eight *Hph* I sites at 2.0, 3.2, 3.5, 3.7, 4.9 and 6.5 —these six map positions agreed perfectly with the sequencing data of Ad12 *Hind* III-G fragment, and then 11.5 and 11.9— these two map positions were located within the *Hind* III-F (Fig. 2 a). Indeed, Ad12 *Hind* III-I fragment was not cleaved with *Hph* I (data not shown).

The *Hind* III-F labeled with ^{32}P at both 3′ termini was cleaved with *Eco* RI for analyzing detailed cleavage sites within the *Hind* III-F. Sizes of partial products from 10.9 to 16.2 map position were 0.6, 1.0, 1.3, 1.6, 4.1, 4.3 and 5.3 map units (m. u.) (Fig. 2 b). Also sizes of partial products from 16.2 to 18.2 map position were 1.0 and 2.0 m. u. (Fig. 2 b). Therefore, the above results showed that there were seven *Hph* I sites at 11.5, 11.9, 12.2, 12.5, 15.0, 15.2, and 17.2 map positions.

Hind II digestion of Ad12 DNA yielded fifteen fragments and their molecular sizes are summarized in Table 1. The order of Ad12 *Hind* II fragements along the viral genome was determined by filter hybridization (Table 2). Ad12 *Hind* III fragments G, I, F and B were labeled *in vitro*

by nicktranslation and hybridized with Ad12 *Hind* II fragments and *Hind* II-*Hind* III double digestion fragments immobilized on nitrocellulose membrane filter. Ad12 *Hind* II fragments E and C hybridized with *Hind* III fragments (G+I) and (I+F+B), respectively. Thus, the order of the *Hind* II fragments was determined as EC. Ad12 *Hind* II fragments C, D, N and 0

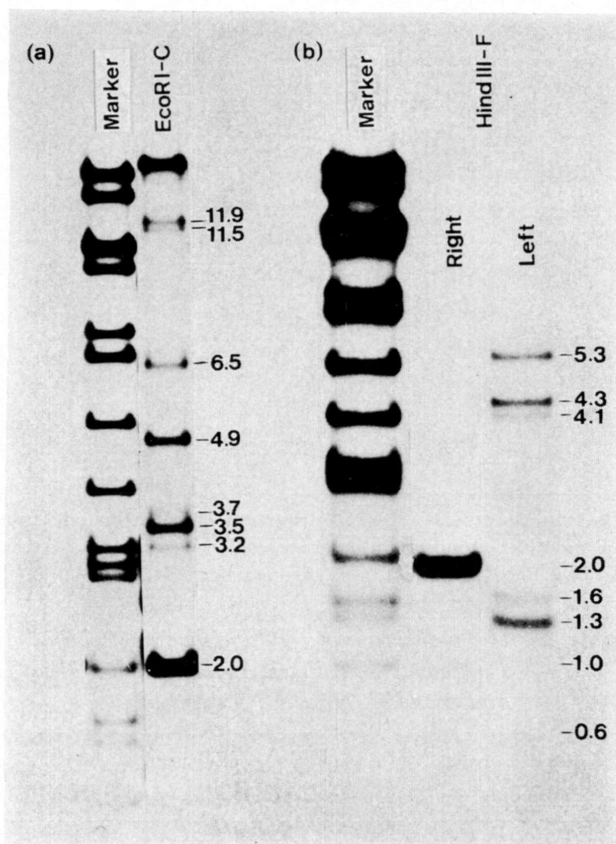

Fig. 2. *Partial digestions of end-labeled Ad 12 DNA fragments by Hph I.*

(a) End-labeled Ad 12 [^{32}P] DNA (1 μg) was entirely cleaved with *Eco* RI. End-labeled *Eco* RI-C fragments were isolated by a 1.4% agarose gel, and was partially cleaved with 0.08 units of *Hph* I at 37°C for 10 min. The digestion products were electrophoresed in a 1.4% agarose slab gel (0.3×16×22 cm) at 45 V for 16 hr. Marker fragments were Ad 12 *Hind* III fragments.

(b) End-labeled Ad 12 [^{32}P] *Hind* III-F fragment (1 μg) was completely cleaved with *Eco* RI. The left and right fragments of *Hind* III-F were isolated by a 1.4% agarose gel, and were partially cleaved with 1.14 units and 0.36 units of *Hph* I for 10 min at 37°C, respectively. Each digestion product was electrophoresed in a 1.4% agarose slab gel (0.2×16×22 cm) at 40 V for 14 hr.

Table 1. *Relative Size of Ad 12 DNA Fragments Produced by Cleavage with Endo R Hind II*

Products	Relative size (m. u.)
A	18.0
B	12.0
C	11.5
D	10.3
E	9.8
F	6.7
G	6.6
H	5.4
I	4.9
J	3.8
K	3.3
L	3.1
M	2.1
N	1.9
O	0.8

The molecular size of each fragment was estimated from the relative mobility in agarose gel electrophoresis using Ad 12 *Hind* III fragments as size markers.

Table 2. *Hybridization of ^{32}P-labeled Ad 12 Hind III Fragments G, I, F and B with Ad 12 Hind II and Hind II–Hind III Fragments*

^{32}P-labeled probe Fragments	Hybrized with *Hind* II	Hybrized with *Hind* II–*Hind* III
Hind III–G	E	
–I	E, C	
–F	C	
–B	C, D, (N, O)[a]	N, O
	E C (N, O) D	

Nick-translated Ad 12 *Hind* III fragments G, I, F and B were annealed to Ad 12 *Hind* II and *Hind* II–*Hind* III fragments on the nitrocellulose filter, and hybrids were detected by autoradiography. a: These two fragments could be barely detected for the high background.

hybridized with the *Hind* III-B. The order of C, D, N and O was determined by hybridization of the *Hind* III-B with Ad12 *Hind* II-*Hind* III fragments. The *Hind* III-B hybridized with two *Hind* II-*Hind* III fragments, corresponded to the *Hind* II-N and -O, indicating that the *Hind* II-N and -O were located within the *Hind* III-B. Therefore, the order of Ad12 *Hind* II fragments was determined to be EC (N, O) D.

The location of *Acc* I cleavage sites in A d12 *Hind* III fragments I and F were determined by analyzing double digestion products and partial diges-

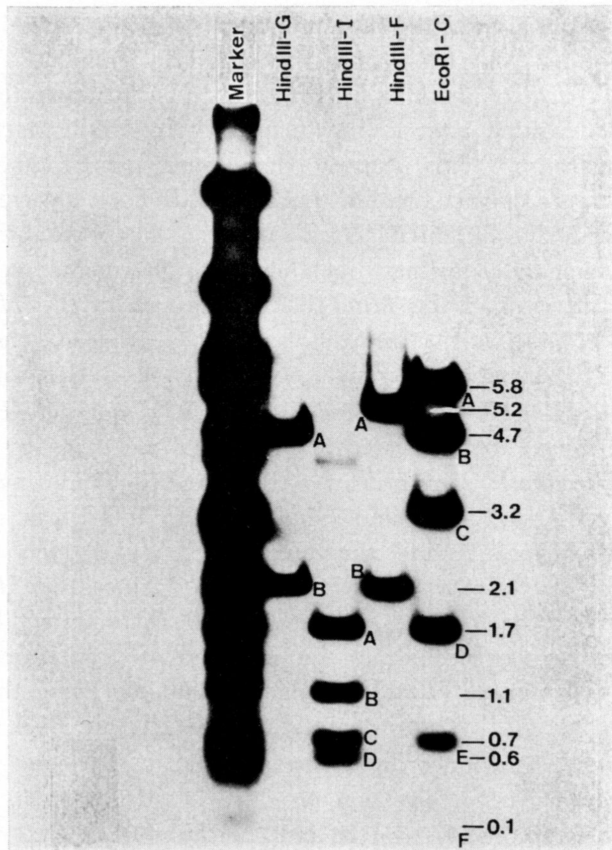

Fig. 3. *Gel electrophoretic patterns of digests at Ad 12 Eco RI fragment C and Hind III fragments G, I and F.*

In vivo labeled Ad 12 [^{32}P] DNA (1.85×10 cpm/μg) was digested completely with *Eco* RI and *Hind* III, and then each fragment was purified. *In vivo* labeled Ad 12 *Eco* RI fragment C and *Hind* III fragments G, I and F were cleaved with 2.0 units of *Acc* I at 37°C for 5 hr. The digestion products were separated by a 1.4% agarose gel electrophoresis for 13 hr at 40 V. Bands were labeled as A, B, C, D, E and F for complete digestion fragments.

tion products. Ad12 *Eco* RI fragment C and *Hind* III fragments G, I and F were redigested by *Acc* I, and separated by agarose gel electrophoresis (Fig. 3). *Acc* I cleaved the *Hind* III-G into two fragments, *Hind* III-G/*Acc* I-A (4.7 m. u.) and *Hind* III-G/*Acc* I-B (2. 1 m. u.). The sequence of Ad12 *Hind* III-G showed that *Acc* I cleavage site in the *Hind* III-G was located at 4.7 map position, and this was consistent with the above results.

Acc I cleaved the *Eco* RI-C into six fragments, *Eco* RI-C/*Acc* I-A (5. 8 m. u.), *Eco* RI-C/*Acc* I-B (4.7 m. u.), *Eco* RI-C/*Acc* I-C (3.2 m. u.), *Eco* RI-C/*Acc* I-D (1.7 m. u.), *Eco* RI-C/*Acc* I-E (0.7 m. u.) and *Eco* RI-C/*Acc* I-F (0.1 m. u.). *Acc* I cleaved the *Hind* III-I into four fragments, *Hind* III-I/*Acc* I-A (1.7 m. u.), *Hind* III-I/*Acc* I-B (1.1 m. u.), *Hind* III-I/*Acc* I-C (0.7 m. u) and *Hind* III-I/*Acc* I-D (0.6 m. u.). The *Hind* III-I/*Acc* I-A and the *Hind* III-I/*Acc* I-C had the same size as the *Eco* RI-C/*Acc* I-D and the *Eco* RI-C/*Acc* I-E, respectively. This showed that these two fragments (1.7 m. u. and 0.7 m. u.) were located within the *Hind* III-I. However, the *Hind* III-I/*Acc* I-B and the *Hind* III-I/*Acc* I-D did not have the same size as *Eco* RI-C/*Acc* I fragments, showing that these two fragments were located at the right and left ends of the *Hind* III-I. The sum of the *Hind* III-G/*Acc* I-B and the *Hind* III-I/*Acc* I-B was 3.2 m. u. and agreed with the size of the *Eco* RI-C/*Acc* I-C. Since the *Hind* III-G/*Acc* I-B is adjacent to the left end of the *Hind* III-I, the *Hind* III-I/*Acc* I-D and the *Hind* III-I/*Acc* I-B were determined to be located at the right and the left ends of the *Hind* III-I, respectively. *Acc* I cleaved the *Hind* III-F into two fragments, *Hind* III-F/*Acc* I-A (5. 2 m. u.) and *Hind* III-F/*Acc* I-B (2.1 m. u.). The sum of the *Hind* III-I/*Acc* I-D and the *Hind* III-F/*Acc* I-A was 5.8 m. u. and agreed with the size of the *Eco* RI-C/*Acc* I-A. Since the *Hind* III-I/*Acc* I-D is adjacent to the left end of the *Hind* III-F, the *Hind* III-F/*Acc* I-A was determined to be located at the left end of the *Hind* III-F. This showed that the order of *Hind* III-F/*Acc* I fragments along the *Hind* III-F was determined as AB. The order of the *Hind* III-I/*Acc* I-A and the *Hind* III-I/*Acc* I-C was determined by analyzing partial digestion products (Fig. 4 a). The size of partial digestion product No. 1 (2.3 m. u.) agreed with the sum of the *Hind* III-I/*Acc* I-A and the *Hind* III-I/*Acc* I-D. Also the size of partial digestion product No. 2 (1.8 m. u.) agreed with sum of the *Hind* III-I/*Acc* I-B and the *Hind* III-I/*Acc* I-C. This showed that the *Hind* III-I/*Acc* I-A and the *Hind* III-I/*Acc* I-C were adjacent to the *Hind* III-I/*Acc* I-D and the *Hind* III-I/*Acc* I-B, respectively. Therefore, the order of *Hind* III-I/*Acc* I fragments along the *Hind* III-I was determined as BCAD.

Hinf I cleaved the *Hind* III-I into three fragments, *Hind* III-I/*Hind*f I-A (1.9 m. u.), *Hind* III-I/*Hinf* I-B (1.4m. u.) and *Hind* III-I/*Hinf* I-C (0.8 m. u.).

The order of these three *Hind* III–I/*Hinf* I fragments was determined by analyzing partial digestion profucts (Fig. 4 b). The size of partial digestion product No. 1 (4.1 m. u.) agreed with the sum of A, B and C. And the size of partial digestion product No. 2 (3.3 m. u.) and No. 3 (2.7 m. u.) agreed with the sum of A and C, and the sum of A and B, respectively. This showed that A was located between B and C. *Acc* I cleaved the *Hind* III–I/ *Hinf* I–B into two fragments, but did not cleave the *Hind* III–I/*Hinf* I–C (data not shown). This showed that the *Hind* III–I/*Hinf* I–C was located

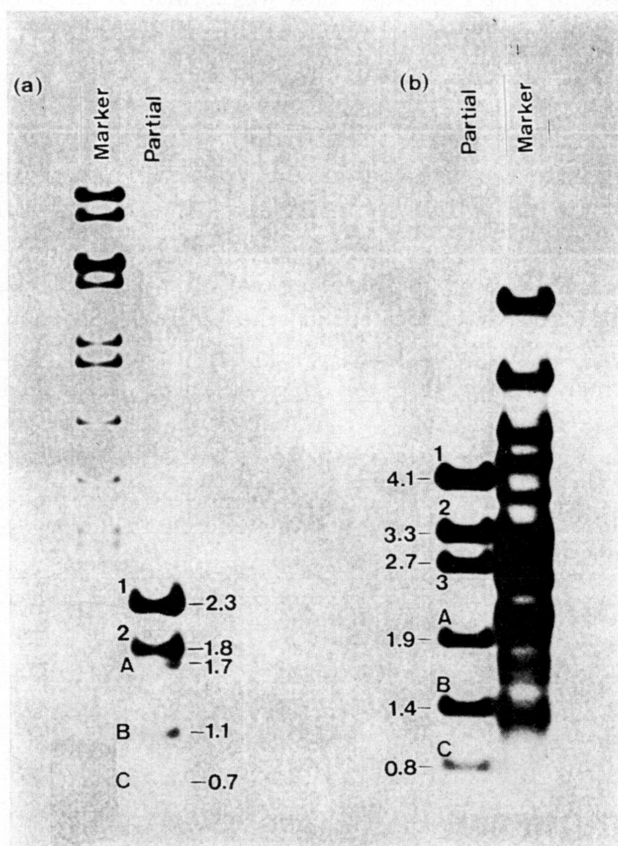

Fig. 4. *Partial digestion of Ad 12 Hind III fragment I by Acc I and Hinf I.*

In vivo labeled Ad 12 [^{32}P] *Hind* III fragment I (0.1 μg) was partially cleaved with 0.01 unit of *Acc* I or 0.01 unit of *Hinf* I at 37°C for 10 min. The partial digestion products were eletrophoresed in a 1.4% agarose gel for 13 hr at 40 V. Bands were labeled as A, B and C for complete digestion fragments and as 1 to 3 for the partial digestion products.

(a) *Acc* I partial digestion (b) *Hinf* I partial digestion

at the left end of the *Hind* III-I, determining the order of the *Hind* III-I/*Hinf* I fragments to be CAB.

Cleavage map of *Hha* I in the *Hind* III-I was determined by analyzing digestion products of the *Hind* III-I/*Acc* I and *Hind* III-I/*Hinf* I fragments labeled at its 5′ end with [γ^{32}-P]-ATP by means of T4 polynucleotide kinase by *Hha* I (Table 3).

Hha I cleaved the *Hind* III-I/*Acc* I-B and the *Hind* III-I/*Hinf* I-C at one site, yielding a common DNA fragment with a size of 0.2 m. u.. This showed that one of *Hha* I cleavage sites was located at the position 0.2 m. u. immediately to the left end of the *Hind* III-I. *Hha* I cleaved the *Hind* III-I/*Hinf* I-A into two fragments with the size of 1.1 and 0.8 m. u., and cleaved the *Hind* III-I/*Acc* I-A into two fragments with the size of 0.3 and 0.1 m. u.. However, this did not cleave the *Hind* III-I/*Acc* I-C. This showed that *Hha* I cleavage site was located at the position 1.9 m. u. immediately to the left end of the *Hind* III-I. *Hha* I cleaved the *Hind* III-I/*Hinf* I-B into two fragments with the size of 0.9 and 0.5 m. u., but did not cleave the *Hind* III-I/*Acc* I-D. This indicated that one of two *Hha* I cleavage sites in the *Hind* III-I/*Acc* I-A was located at the position 3.2 m. u. immediately to the left end of the *Hind* III-I.

Cleavage map of *Hae* III in the *Hind* III-I was determined by analyzing

Table 3. *Double Digestions of Ad 12 Hind III Fragment I*

Fragment used for re-cleavage	Fragment size (map unit)	Size of end fragments re-cleaved by restriction endonuclease (mapunit)	
		Hha I	*Hae* III
Hind III-I/*Acc* I-A	1.7	0.3, 0.1	0.1
-B	1.1	0.9, 0.2	0.6, 0.1
-C	0.7	0.7	0.7
-D	0.6	0.6	0.3, 0.3
Hind III-I/*Hinf* I-A	1.9	1.1, 0.8	
-B	1.4	0.9, 0.5	
-C	0.3	0.6, 0.2	
Hind III-I/*Hha* I-A	1.7		0.3, 0.1
-B	1.3		1.3
-C	0.9		0.3, 0.2
-D	0.2		0.1, 0.1

Each DNA fragment (5.0 pmol) for recleavage was labeled with ^{32}P-ATP in polynucleotide Kinase reaction for 5′ ends, and then was entirely cleaved by *Hha* I or *Hae* III. The digestion products were separated by 5% polyacrylamide gel electrophoresis for 2hr at 200 V.

digestion products of the *Hind* III-I/*Acc* I and *Hind* III-I/*Hha* I fragments labeled at its 5′ end by *Hae* III (Table 3).

Hae III cleaved the *Hind* III-I/*Hha* I-D and the *Hind* III-I/*Acc* I-B yielding a common DNA fragment with the size of 0.1 m. u.. This showed that one of *Hae* III cleavage sites in the *Hind* III-I was located at the position 0.1 m. u. immediately to the left end of the *Hind* III-I. The other fragment of the *Hind* III-I/*Acc* I-B cleaved by *Hae* III had a size of 0.6 m. u., and one of two *Hae* III fragments of the *Hind* III-I/*Hha* I-A had a size of 0.3 m. u.. This showed that the other *Hae* III cleavage site in the *Hind* III-I/*Acc* I-B was located at the position 0.5 m. u. immediately to the left end of the *Hind* III-I. Another *Hae* III fragment of the *Hind* III-I/*Hha* I-A had a size of 0.1 m. u., and *Hae* III did not cleave the *Hind* III-I/*Acc* I-C, indicating that the other *Hae* III cleavage site in the *Hind* III-I/*Hha* I-A was located at the position 1.8 m. u. immediately to the left end of the *Hind* III-I. *Hae* III cleaved the *Hind* III-I/*Hha* I-C into two fragments with the size of 0.3 and 0.2 m. u., and cleaved the *Hind* III-I/*Acc* I-D into two fragments with the size of 0.3 m. u.. This showed that there were two *Hae* III cleavage sites in the *Hind* III-I/*Hha* I-C, and were located at the positions 3.4 and 3.8 m. u. immediately to the left end of the *Hind* III-I, respectivel. *Hae* III did not cleaved the *Hind* III-I/*Hha* I-B.

Cleavage maps were constructed from the above results and are shown

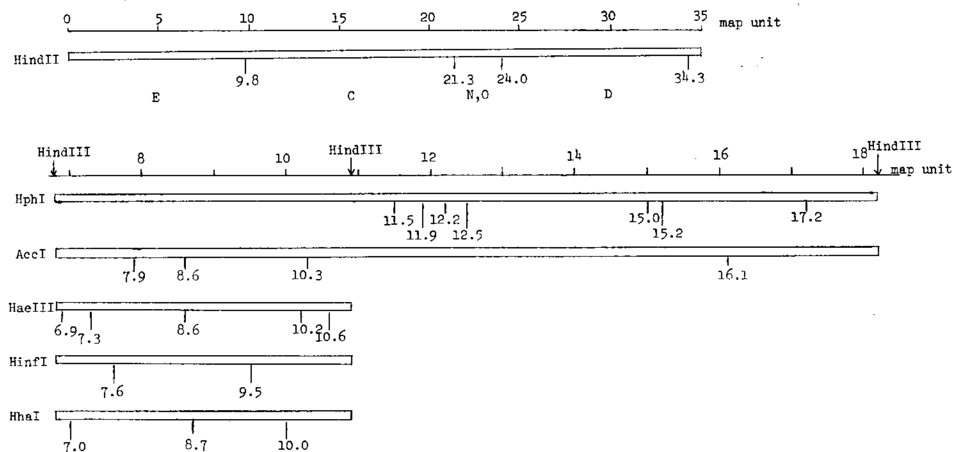

Fig. 5. *Detailed cleavage maps of the Ad 12 transforming segment by Acc I, Hae III, Hha I Hind III, Hinf I and Hph I.*

The transforming segment of Ad 12 is shown by six thickbars, and the genome is marked in map units. Arrows and numbers under thick bars indicate the cleavage sites of *Acc* I, *Hae* III, *Hha* I, *Hind* II, *Hinf* I and *Hph* I. Letters between bars indicate the nomenclature of the *Hind* II fragments.

in Fig. 5. These detailed mapping of transforming segment will be useful for sequencing DNA stretch of Ad12 and analyzing the structure and the function of the entire early region E1 and characterizing the integrated viral genome in transformed cells. Nucleotide sequence datermination of the remainder of the entire early region E1 of Ad12 DNA are now in progress.

ACKNOWLEDGEMENTS

This work was supported in part by Grants-in-Aid for Cancer Research and for Scientific Research from the Ministry of Education, Science and Culture, Japan.

We are grateful to Dr. M. Takanami, Kyoto University, for donations of restriction endonucleases.

REFERENCES

1. SHIROKI, K., HANDA, H., SHIMOJO, H., YANO, S., OJIMA, S. and FUJINAGA, K.: Virology **82**, 462–471 (1977).
2. YANO, S., OJIMA, S., FUJINAGA, K., SHIROKI, K. and SHIMOJO, H.: Virology **82**, 214–220 (1977).
3. FUJINAGA, K., SAWADA, Y., UEMIZU, Y., YAMASHITA, T., SHIMOJO, H., SHIROKI, K., SUGASAKI, H., SUGIMOTO, K. and TAKANAMI, M.: Cold Spring Harbor Symp. Quant. Biol. **44**, 519–532 (1979).
4. SUGISAKI, H., SUGIMOTO, K., TAKANAMI, M., SHIROKI, K., SAITO, I., SHIMOJO, H., SAWADA, Y., UEMIZU, Y., UESUGI, S. and FUJINAGA, K.: Cell **20**, 777–786 (1980).
5. GREEN, M. and PINA, M.: Virology **20**, 199–207 (1963).
6. GREEN, M. and PINA, M.: Proc. Natl. Acad. Sci. USA **51**, 1251–1259 (1964).
7. SEKIKAWA, K. and FUJINAGA, K.: Virology **82**, 509–512 (1977).
8. LAI, C. J. and NATHANS, D.: J. Mol. Biol. **89**, 179–193 (1974).
9. KLEID, D., HUMAYUN, Z., JEFFREY, A. and PTASHNE, M.: Proc. Natl. Acad. Sci. USA **73**, 293–297 (1976).
10. SEKIKAWA, K., SHIROKI, K., SHIMOJO, H., OJIMA, S. and FUJINAGA, K.: Virology **88**, 1–7 (1978).
11. YAEGASHI, T., YOSHIDA, K., UEMIZU, Y. and FUJINAGA, K.: Tumor Res. **14**, 9–15 (1979).
12. MAIZEL, J. V., Jr., *In* "Methods in Virology" (K. MARAMOROSH and H. KOPROWSKI, eds.), Vol. 5. Academic Press, New York and London, 1971.
13. MAXAM, A. M. and GILBERT, W.: Proc. Natl. Acad. Sci. USA **74**, 560–564 (1977).
14. ENGLUND, P. T.: J. Mol. Biol. **66**, 209–224 (1972).
15. SOUTHERN, E. M.: J. Mol. Biol. **98**, 503–517 (1975).
16. FUJINAGA, K., SAWADA, Y., SEKIKAWA, K.: Virology **93**, 578–581 (1979).
17. SAWADA, Y., OJIMA, S., SHIMOJO, H., SHIROKI, K. and FUJINAGA, K.: J. Virol. **32**, 379–385 (1979).
18. KELLEY, R. B., CAZZARELLI, N. R., DEUTSCHER, M. P., LEHMAN, I. R. and

KORNBERG, A.: J. Biol. Chem. **245**, 39–45 (1970).

19. ORTIN, J., SCHEIDTMAN, K. H., GREENBERG, R., WESTPHAL, M. and DOERFLER, W.: J. Virol. **20**, 355–372 (1976).
20. SAWADA, Y., HONDA, E., YANO, S. and FUJINAGA, K.: Tumor Res. **15**, 35–43 (1980).