The mapping and ordering of fragments of SV40 DNA produced by restriction endonucleases

K.N.Subramanian, J.Pan, S.Zain, and S.M.Weissman

Department of Human Genetics, Yale University School of Medicine, 333 Cedar Street, New Haven, Connecticut 06510, USA

Received 7 March 1974

ABSTRACT

SV40 DNA is cleaved by the Eco RII and Hae restriction endonucleases to give rise to two different sets of 16 fragments each. These fragments have been ordered by analysis of the products of redigestion of one set of fragments with another restriction enzyme. The cleavage sites have been precisely mapped based on length measurements of the products of each cleavage. This provides a convenient group of small DNA fragments suitable for sequence analysis investigation of the transcripts present in infected cells, or construction of deletion substitution variants of the virus.

INTRODUCTION

Recently a great deal of information has been accumulated about DNA replication and transcription, the structural proteins and the genetics of the small transforming virus SV40. Restriction endonucleases which cleave SV40 DNA at specific sites have proved to be a very useful tool in the analysis of the DNA of the virus. The total DNA of the virus has an estimated molecular weight of about 3.3 million daltons. Specific cleavage of this DNA to smaller fragments and ordering them should prove valuable in deriving nucleotide sequence data from this agent.

Our laboratory has been concerned in the last several years with

the nucleotide sequence analysis of SV40 DNA and portions of
Abbreviations used: SV40 - Simian Virus 40. Eco RII: Escherichia coli bearing Resistance Transfer Factor II. Eco RII Escherichia coli bearing Resistance Transfer Factor I. Hin: Haemophilus influenzae. Hae: Haemophilus aegyptius. Hpa: Haemophilus parainfluenzae. These abbreviations, when used alone, refer to the restriction endonucleases isolated from these sources.

the sequence have been published previously^{1, 2}.

Of those restriction endonucleases which fragment SV40 DNA, the Eco RI^{3, 4} and the Hpa II⁵ enzymes cleave the DNA once at unique sites. The Hpa I endonuclease cleaves the DNA at three specific sites^{6, 7, 5}. The Hin restriction enzyme cleaves SV40 DNA into 11 fragments which have been mapped and ordered by Danna et al⁷. The Hae endonuclease is reported to cleave the DNA into 10 fragments⁸.

As part of our work on the nucleotide sequence analysis of SV40 DNA we have studied the cleavage of the DNA by the restriction endonucleases of E. coli RII and Haemophilus aegyptius and mapped and ordered the fragments produced by them. We present here the size estimates and localization on the SV40 genome of the fragments produced by these endonucleases. Using an approach similar to ours, the ten larger Hae fragments of SV40 DNA have also been ordered by Lebowitz et al (manuscript submitted for publication).

MATERIALS AND METHODS

Viral stock of plaque-purified small plaque simian virus 40 strain 776 was kindly provided by Dr. Kathleen Danna. The virus was propagated and grown on BSC-1 cells. Uniformly -(³²P)-labelled form I SV40 DNA was isolated and purified from virus-infected cells essentially as described by Gelb et al⁹.

Eco RII restriction endonuclease was purified according to the procedure of Yoshimori¹⁰. Hpa I restriction enzyme was purified as described by Sharp et al⁵. Hin restriction endonuclease purified by the method of Smith and Wilcox¹¹ was kindly provided by Dr. J. Sklar. Hae restriction enzyme purified by a modification of the method of Middleton et al¹² was a gift of Drs. P. Lebowitz, W. Siegel and J. Sklar. E. coli RNA polymerase holoenzyme made according to Lebowitz et al¹³ was kindly provided by Dr. P. Lebowitz. Eco RI restriction enzyme prepared according to Yoshimori¹⁰ was a gift of Dr. P. Yot. All enzyme preparations used were free of exonuclease and nonspecific endonuclease activities.

The reaction mixture for the digestion of SV40 DNA by Eco-RII or Eco-RI restriction enzymes consisted of 90 mM Tris, pH 7.5, 10 mM MgSO_4 . Hin digestion was done in 6.6 mM Tris, pH 7.5, 10 mM MgCl_2 , 20 mM NaCl, 6.6 mM 2-mercaptoethanol and 0.05% bovine serum albumin. The reaction mixture for the Hae restriction enzyme consisted of 6.6 mM Tris, pH 7.5, 10 mM MgCl_2 , 6 mM 2-mercaptoethanol and 0.05% bovine serum albumin. Digestions by the Hpa I enzyme were carried out in 1 mM Tris, pH 7.5, 10 mM MgCl_2 , 6 mM KCl and 1 mM DTT. All digestions were carried out at 37° C for 6 - 8 hours. To insure complete digestion of DNA, a ratio of 2 units of enzyme activity per μg of DNA was generally used. One unit of enzyme activity is defined as the minimum amount of enzyme necessary for the complete digestion of 1 μg of DNA under these conditions.

Polyacrylamide gel electrophoresis of SV40 DNA fragments was done in vertical 40 cm gel slabs essentially as described by Danna *et al*⁷ in a buffer containing 40 mM Tris, 20 mM sodium acetate, 2 mM sodium EDTA, pH 7.8. To each sample, before application to the gel, a few crystals of sucrose and 2 μl of 1% bromophenol blue were added. The percentage of acrylamide stock used to prepare the gel slab depended on the size of the fragments to be separated. For example, for the separation of the Hpa I fragments of SV40 DNA, a 3% gel was used while a 4% gel was used for the separation of the Hin fragments of SV40 DNA. The production of a few very small fragments was observed after the digestion of SV40 DNA by the Eco RII or Hae restriction enzymes. In order to trap and separate these small fragments (which would have run out of a 4% gel) a composite slab gel consisting of 8% gel to about one fourth to one third of the length from the bottom and 4% gel the rest of the way up to the top was used. A 4% gel with an 8% gel trap, or an uniform 8% gel was routinely used for the separation of fragments obtained by the redigestion of one set of restriction enzyme fragments with another restriction enzyme.

Elution of fragments from gel for redigestion: After electrophoresis, the wet gel was autoradiographed to locate the ^{32}P - bands corresponding to the DNA fragments. The gel segments corresponding to these fragments were then cut out and ground in 0.1 X SSC, pH 7.5 in a Tekmar tissue mixer. The suspension was repeatedly centrifuged in glass conical tubes to remove the acrylamide and the DNA was precipitated by the addition of two volumes of cold ethanol. After storage at -20°C overnight, the DNA precipitate was recovered by centrifugation in polyallomer tubes at 40,000 r.p.m. in an ultracentrifuge for 30 minutes. Whenever redigestion of the fragment with another restriction enzyme was intended, traces of ethanol were removed by drying under nitrogen gas, the DNA precipitate was dissolved in 25–50 μl of 0.01 X SSC, pH 7.5, and then digested with the other restriction enzyme in the appropriate buffer system.

Molecular hybridizations: RNA transcripts of the Eco RII fragments were made with E. coli RNA polymerase and hybridized with DNA from Adeno-SV40 hybrid viruses and some Hin fragments of SV40 DNA essentially as described by Zain et al¹.

RESULTS

Figures 1A and 1B show the electrophoretic pattern of digests of SV40 DNA by the Eco RII restriction endonuclease with the corresponding Hin digest as a reference in a 4% gel and a 4% gel with an 8% gel trap respectively. A total of 14 discrete bands are located by autoradiography. The nine top bands are of comparable mobility to the Hin bands and correspond to RII fragments A through I. The five lower bands correspond to RII fragments much smaller than any of the Hin fragments. Bands 10 and 14 are much darker than the sizes of the respective fragments would warrant. When the percent radioactivity recovered in most bands are plotted on semilogarithmic paper against relative mobilities a straight line is obtained. The radioactivity in bands 10 and 14 is twice that predicted from their mobility (Fig. 2). This shows that bands 10 and 14 actually correspond to two fragments each (denoted J1 and J2, and N and O respectively). Further confirmation of this is obtained from

Figure 1

Electrophoretic pattern of A) *Eco* RII digest of SV40 DNA in a 4% polyacrylamide slab gel; B) *Eco* RII digest run in a 4% gel with an 8% gel trap; and C) *Hae* III digest of SV40 DNA run in a 4% gel with an 8% gel trap. The pattern of the *Hin* fragments of SV40 DNA is included for comparison.

Figure 2

Plot of percent recovery of radioactivity in Eco RII fragments versus their relative electrophoretic mobility in 4% polyacrylamide gel in semilog paper. Percent recovery of radioactivity is the ^{32}P recovered in the fragments from uniformly ^{32}P - labelled SV40 DNA. Relative electrophoretic mobility is mobility relative to that of Eco RII-H ($\frac{\text{mobility of fragment}}{\text{mobility of Eco RII-H}}$).

The mobilities of fragments M, N, & O in 4% gel are estimated values based on their mobility in 8% gel.

the Eco RII cleavage of the Hpa I fragments of SV40 DNA (Fig. 3). Hpa I-A generates one of the Eco RII-J's (J2) and Hpa I-B produces the other J (Eco RII-J1) upon cleavage with Eco RII. Again, Hpa I-A gives rise to Eco RII-O and Hpa I-B produces Eco RII-N following redigestion with Eco RII. Thus, Eco RII digestion of SV40 DNA gives rise to a total of 16 fragments designated A, B, C, D, E, F, G, H, I, J1, J2, K, L, M, N and O.

Huang *et al*⁸ have reported recently that the digestion of small plaque SV40 DNA by the Hae endonuclease results in the production of 10 fragments entitled A, B, C, D, E, F1, F2, X, G and H, with X always being produced in submolar quantities. Lebowitz *et al* (submitted for publication), using a preparation of the Hae endonuclease prepared by a modification of the method of Middleton *et al*¹², including final concentration by ammonium sulfate precipitation, have observed the absence of X and the presence of two fragments banding in the position of F1. By running the Hae digest of SV40 DNA in a 4% gel with an 8% gel trap we observe the production of fragments A through H in agreement with results reported by Lebowitz *et al*. In addition, we also observe the production of 6 new small fragments entitled I, J, K, L, M and N, which are all smaller than the smallest Eco RII fragment of SV40 DNA (Fig. 1C). These six fragments are also observed in the Hae digests of Hpa I fragments of SV40 DNA (Fig. 3). In total, 16 fragments are produced by the digestion of SV40 DNA by the Hae endonuclease. These are designated A, B, C, D, E, F_{1a}, F_{1b}, F₂, G, H, I, J, K, L, M and N.

A reasonably accurate estimate of the sizes of the Hin fragments of SV40 DNA has been made by Danna *et al*⁷. The values for the sizes of the Eco RII and Hae fragments of SV40 DNA observed by the percent recovery of radioactivity in them and estimated by their mobilities relative to

that of the Hin fragments, agree closely with each other. The estimated sizes of the Eco RII and Hae fragments in terms of the percent of total SV40 DNA that they carry and the number of nucleotide base pairs present in them, are presented in Table 1. The values given for the six very small Hae fragments are those calculated on the basis of their mobility in an 8% gel relative to that of the small Eco RII fragments. The values given for Hae fragments A through H are in close agreement with those reported by Lebowitz *et al* (personal communication).

Strategy for the ordering of fragments of SV40 DNA: The first step in the ordering of the fragments is to assign them to specific broad regions of the SV40 genome. This has been done by redigesting the Hpa I fragments with the Eco RII and Hae endonucleases and observing which Eco RII or Hae fragments are produced from which Hpa I fragments. Then, by use of the ordered set of the Hin fragments of SV40 DNA, the Eco RII fragments are mapped and ordered by examining the reciprocal cleavage of the Hin fragments with the Eco RII endonuclease and the Eco RII fragments with the Hin enzyme. The products of each cleavage are separated by gel electrophoresis. Those Hin fragments which give rise to whole RII fragments and those RII fragments which give rise to whole Hin fragments upon redigestion, are identified. More importantly, any RII fragment which spans the junction between two adjacent Hin fragments will not be generated in full upon redigestion of Hin fragments with the Eco RII enzyme. Moreover, such an RII fragment will be cleaved by the Hin enzyme to give rise to two products. In the example described below (Fig. 4), the Eco RII fragment X overlaps the junction between adjacent Hin fragments Y and Z. Since Eco RII-X contains a Hin cleavage site, it will be cleaved by Hin to two fragments which are designated P_1 and P_2 . Hin-Y and Hin-Z each contains an Eco RII cleavage site, and upon cleavage with Eco RII, Hin-Y should yield P_1 as one of the products, and Hin-Z should yield P_2 .

TABLE 1

Eco RII Fragment	Estimated Size*		Hae Fragment	Estimated Size*	
	Percent of Total SV40 DNA	Number of base pairs		Percent of Total SV40 DNA	Number of base pairs
A	20.0	1,000	A	31.0	1,550
B	15.0	750	B	15.5	775
C	13.5	675	C	10.5	525
D	11.0	550	D	7.0	350
E	9.0	450	E	6.5	325
F	7.5	375	F _{1a} +F _{1b}	6.2+6.2	310+310
G	6.2	310	F ₂	6.0	300
H	4.8	240	G	4.5	225
I	4.0	200	H	3.4	170
J ₁ +J ₂	2.5+2.5	125+125	I	0.8	40
K	2.0	100	J	0.8	40
L	1.7	85	K	0.8	40
M	1.2	60	L	0.6	30
N	1.0	50	M	0.6	30
O	1.0	50	N	0.6	30

*Values are based on the observed recovery of radioactivity in the fragments from radioactive SV40 DNA as well as on the electrophoretic mobility of the fragments relative to that of the Hin fragments of SV40 DNA. In case of the Hae fragments I through N the given values are calculated values only.

TABLE 2

THE PRODUCTS OF REDIGESTION OF Hpa I FRAGMENTS
OF SV40 DNA WITH THE Hin, Eco RII AND Hae
RESTRICTION ENZYMES

Hpa I fragment digested	<u>Hin</u> digestion products	<u>Eco RII</u> digestion products	<u>Hae</u> digestion products
A	D, E, F, G, J, and K	A, E, J ₂ , O and two end pieces of 375 and 140 base pairs	B, F ₂ , G, H, I, J, L and two end pieces of 400 and 140 base pairs
B	A, C and H	B, G, I, J ₁ , M, N and two end pieces of 280 and 140 base pairs	E, F _{1a} , K, M and two end pieces of 1,000 and 120 base pairs
C	B and I	H, K, L and two end pieces of 400 and 230 base pairs	D, N and two end pieces of 550 and 130 base pairs

If this happens, then Eco RII-X should partially overlap Hin-Y on the one hand and Hin-Z on the other hand. Conversely, any two Eco RII fragments which, upon redigestion with the Hin enzyme yield two products which are also produced by the Eco RII cleavage of one Hin fragment, should be adjacent to each other on the SV40 genome, and should be overlapped by this Hin fragment. The products P_1 and P_2 are separated by gel electrophoresis, their sizes are estimated based on their electrophoretic mobility relative to that of known fragments and, on the basis of these length measurements, the Eco RII cleavage sites on Hin fragments Y and Z are mapped. In the same manner, the products of redigestion of each Eco RII fragment with the Hin enzyme and each Hin fragment with the Eco RII enzyme are separated by gel electrophoresis, scanned carefully for partial or full overlap, and are then analyzed to order the RII fragments and to map the RII cleavage sites on the SV40 genome. In some cases the placement of the Eco RII fragments on the SV40 genome is further confirmed by making *in vitro* transcripts of these RII fragments and hybridizing to denatured Hin fragments and *vice versa*.

In a similar manner, reciprocal redigestion of Eco RII fragments with the Hae enzyme and Hae fragments with the Eco RII endonuclease has been successfully used to map and order the Hae fragments of SV40 DNA. In many instances (to be described below) the ordering of the Eco RII and Hae fragments have been mutually beneficial especially in the ordering of the small fragments produced by either enzyme. The 10 larger Hae fragments have also been ordered independently by Lebowitz *et al* (personal communication).

Cleavage of Hpa I fragments and the linear DNA generated by Eco RI:

The Eco RII and Hae fragments are assigned to specific broad regions of the SV40 DNA by recleavage experiments done with the Hpa I fragments of the DNA. Hpa I makes three cleavages on SV40 DNA giving rise to three fragments designated Hpa I-A, Hpa I-B and Hpa I-C which carry 41.5, 38.5 and 20 percent of the total SV40 DNA

respectively^{3, 6}. The three Hpa I fragments were redigested with the Eco RII and Hae enzymes and the results are presented in Table 2 and Figure 3. Whole Eco RII fragments generated by the redigestion are as follows: Hpa I-A gives Eco RII-A, E, one of the J's and O; Hpa I-B gives B, G, I, the other J, M, and N; finally Hpa I-C generates H, K, and L. In addition, each Hpa I fragment gives two fragments each which cannot be identified with any whole Eco RII fragments; These therefore, must be part of the three RII fragments which are not produced in full upon recleavage of the Hpa I fragments, viz. C, D and F which should overlap the Hpa I cleavage sites on the SV40 DNA.

Upon redigestion of Hpa I fragments with the Hae enzyme (Fig. 3 and Table 2) Hpa I-A gives Hae B, F2, G, H, I, J and L; Hpa I-B produces Hae E, one of the F1's, K and M; Hpa I-C generates Hae D and N. The three Hae fragments which are not recovered in full are A, C and the other F1 and the end pieces generated by the redigestion of Hpa I fragments with the Hae enzyme, must constitute these Hae fragments.

The Eco RII and Hae fragments which contain the Eco RI cleavage site have been determined. Recleavage of the linear SV40 DNA produced by Eco RI cleavage with the Eco RII endonuclease generates in full all RII fragments except A. Instead of A, two new fragments of about 750 and 250 base pairs appear. Recleavage of the linear DNA by Hae results in the production of all Hae fragments but Hae B; in its place, two new fragments of approximately 500 and 250 base pairs are generated. Thus, Eco RII-A and Hae-B contain the Eco RI cleavage site on SV40 DNA.

Ordering of the Eco RII fragments: The pattern of recleavage of Hin fragments with Eco RII and of Eco RII fragments with Hin are presented in Tables 3 and 4 respectively. Hin A, upon redigestion with Eco RII (Table 3), gives rise to four products corresponding to RII-B, RII-I, a product which is also produced by the

Figure 3

Electrophoretic pattern of the products of redigestion of Hpa I fragments A, B and C with the Eco RII and Hae restriction enzymes in 8% polyacrylamide slab gel. Columns 1, 2, and 3- pattern of redigestion of Hpa I-A, -B, and -C respectively with the Eco RII enzyme; Columns 4, 5, and 6- pattern of cleavage of SV40 DNA with the Eco RII, Hin and Hae endonucleases; Columns 7, 8 and 9- pattern of redigestion of Hpa I-C, B and A with the Hae enzyme.

TABLE 3

REDIGESTION OF HIN FRAGMENTS WITH THE ECO RII ENZYME

Products of redigestion of H _{in} fragments with the <u>Eco</u> RII enzyme	Estimated molecular weight in nucleotide pairs based on relative electrophoretic mobility	Whole or part of <u>Eco</u> RII fragment that the digestion product corresponds to (if any)
1 <u>H_{in}</u> - A1	750	<u>Eco</u> RII-B
<u>H_{in}</u> - A2	200	<u>Eco</u> RII-I
<u>H_{in}</u> - A3	75	<u>H_{in}</u> <u>Eco</u> RII-G2
<u>H_{in}</u> - A4	65	<u>Eco</u> RII-M (?)
2 <u>H_{in}</u> - B1	240	<u>Eco</u> RII-H
<u>H_{in}</u> - B2	225	<u>H_{in}</u> <u>Eco</u> RII-F1
<u>H_{in}</u> - B3	140	<u>H_{in}</u> <u>Eco</u> RII-C3
<u>H_{in}</u> - B4	100	<u>Eco</u> RII-K
<u>H_{in}</u> - B5	85	<u>Eco</u> RII-L
3 <u>H_{in}</u> - C1	230	<u>H_{in}</u> <u>Eco</u> RII-G1
<u>H_{in}</u> - C2	125	<u>Eco</u> RII - J ₁
<u>H_{in}</u> - C3	115	<u>H_{in}</u> <u>Eco</u> RII - D2
<u>H_{in}</u> - C4	50	<u>Eco</u> RII - N
4 <u>H_{in}</u> - D1	380	<u>H_{in}</u> <u>Eco</u> RII - D1
<u>H_{in}</u> - D2	80	<u>H_{in}</u> <u>Eco</u> RII - E2
<u>H_{in}</u> - D3	50	<u>Eco</u> RII - O
5 <u>H_{in}</u> - E1	350	<u>H_{in}</u> <u>Eco</u> RII - E1
<u>H_{in}</u> - E2	80	<u>H_{in}</u> <u>Eco</u> RII - J ₂ 1
6 <u>H_{in}</u> - F not digested	375	Present in <u>Eco</u> RII-A
7 <u>H_{in}</u> - G1	220	<u>H_{in}</u> <u>Eco</u> RII - A3
<u>H_{in}</u> - G2	135	<u>H_{in}</u> <u>Eco</u> RII - F2
8 <u>H_{in}</u> - H not digested	275	Present in <u>Eco</u> - RII-C
9 <u>H_{in}</u> - I not digested	250	Present in <u>Eco</u> RII - C
10 <u>H_{in}</u> - J not digested	225	Present in <u>Eco</u> RII - A
11 <u>H_{in}</u> - K1	160	<u>H_{in}</u> <u>Eco</u> RII - A4
<u>H_{in}</u> - K2	40	<u>H_{in}</u> <u>Eco</u> RII - J ₂ 2

Hin cleavage of Eco RII-G, and finally what is possibly RII-M. Looking at the redigestion of Eco-RII fragments with Hin, it can be seen that RII-B and RII-I are not cleaved by Hin. Therefore Hin-A must contain RII-B, RII-I and possibly RII-M (with their order to be described later), and Eco RII Hin-A3 which should be at one end of Hin-A. Since the last product is also generated by the Hin cleavage of RII-G, this suggests that RII-G partially overlaps Hin-A. The largest product of the cleavage of Hin C by Eco RII is also produced from RII-G by cleavage with Hin (Tables 3 and 4). This indicates that RII-G overlaps both Hin-A and Hin-C. The other products of Eco RII cleavage of Hin-C are one of the RII-J's, RII-N and a product (Eco RII Hin-C3) which is also produced by the Hin cleavage of Eco RII-D (Hin Eco RII-D2) (Table 4). Thus, RII-J (which will be called RII-J1 hereafter), and RII-N should be internal constituents of Hin-C (their order will be described later), and the two terminal parts of Hin-C are partially overlapped by RII-G and RII-D respectively.

Hin-D, which lies to the right of Hin-C, gives three products on digestion with Eco-RII (Table 3). The only RII product generated in full is Eco RII-O. Therefore it must be the internal constituent of Hin-D. The other products are Eco RII Hin-D1 and Eco RII-Hin-D2 which are also produced by Hin cleavage of RII-D (Hin Eco RII-D1) and RII-E (Hin Eco RII-E2) respectively (Table 4). Thus RII-D and RII-E must be overlapping the two ends of Hin-D.

The Eco RII cleavage of Hin-E gives rise to two products, Eco RII-Hin E1 and Eco RII Hin-E2 (Table 3), which are also produced by the Hin cleavage of RII-E (Hin Eco RII-E1) and RII-J2 (Hin Eco RII-J21) respectively (Table 4). Thus, RII-E and RII-J2 partially overlap Hin-E on either side.

Adjacent to Hin-E is Hin-K, and its cleavage pattern indicates that it is overlapped by RII-J2 on the left and by RII-A on the right.

Hin-F and Hin-J which lie to the right of Hin-K in that order are not cleaved by Eco RII. They are actually generated in full by the cleavage of Eco RII-A by Hin. The other product of Hin digestion of Eco-RII-A, viz. Hin Eco RII-A3 (Table 4) is also produced by the Eco RII cleavage of Hin-G, viz. Eco RII Hin-G1 (Table 3). Thus, Eco RII-A overlaps Hin-K on one side, contains Hin-F and Hin-J as internal constituents, and overlaps Hin-G on the other side. The other product of the digestion of Hin-G with Eco RII, viz. Eco RII Hin G-2 is also produced by the Hin digestion of Eco RII-F, viz. Hin Eco RII-F2 (Table 4). Hin Eco RII-F1 is one of the products of digestion of Hin-B with Eco RII.. Hence RII-F overlaps Hin G and Hin-B. Hin-B gives four more products on digestion with Eco RII (Table 3). Three of these are whole RII fragments, viz. RII-H, RII-K, and RII-L; and the other product (Eco RII Hin-B3) is also found to be produced by the cleavage of RII-C by Hin (Hin Eco RII-C3). Thus, the other end of Hin-B has a overlap with RII-C. Eco RII-C gives two other products on digestion with Hin, viz. Hin-I and Hin-H, which lie to the right of Hin-B in that order.

By putting together the results described in the foregoing, the order of the Eco RII fragments of SV40 DNA is found to be: A-F-(H, K, L)-C-(B, I, M)-G-(J1, N)-D-O-E-J2-E. In order to order the fragments appearing within parenthesis we had to resort to recleaving experiments with a third restriction enzyme, the H. aegyptius endonuclease. The results of these experiments and the final ordering of the Eco RII fragments will be described in a later section of this paper.

Ordering and specific placement of the smaller Hae fragments:

The Hae fragments of SV40 DNA have been ordered by making use of the same technique used in ordering the Eco RII fragments. Eco RII fragments are redigested with the Hae endonuclease, and the Hae fragments are redigested with Eco RII, and the products are analyzed carefully. Based on the data presented in Tables 5 and 6, the order of the Hae fragments is derived to be B-C-D-N-

TABLE 4

RESULTS OF REDIGESTION OF ECO RII FRAGMENTS WITH THE HIN ENZYME

Products of Eco RII fragments digested	Estimated size in base pairs	Corresponding whole or part <u>Hin</u> fragment
1 A1	375	<u>Hin</u> - F
A2	225	<u>Hin</u> - J
A3	220	<u>Eco</u> RII <u>Hin</u> - G1
A4	160	<u>Eco</u> RII <u>Hin</u> - K1
2 B not digested	750	Present in <u>Hin</u> - A
3 C1	275	<u>Hin</u> - H
C2	250	<u>Hin</u> - I
C3	140	<u>Eco</u> RII <u>Hin</u> B3
4 D1	380	<u>Eco</u> RII <u>Hin</u> - D1
D2	115	<u>Eco</u> RII <u>Hin</u> - C3
5 E1	350	<u>Eco</u> RII <u>Hin</u> - E1
E2	80	<u>Eco</u> RII <u>Hin</u> - D2
6 F1	225	<u>Eco</u> RII <u>Hin</u> - B2
F2	135	<u>Eco</u> RII <u>Hin</u> - G2
7 G1	230	<u>Eco</u> RII <u>Hin</u> - C1
G2	75	<u>Eco</u> RII <u>Hin</u> - A3
8 H not digested	240	Present in <u>Hin</u> - B
9 I not digested	200	Present in <u>Hin</u> - A
10 J ₁ not digested	125	Present in <u>Hin</u> - C
11 J ₂ ¹	80	<u>Eco</u> RII <u>Hin</u> - E2
J ₂ ²	40	<u>Eco</u> RII <u>Hin</u> - K2
12 K not digested	100	Present in <u>Hin</u> - B
13 L not digested	85	Present in <u>Hin</u> - B
14 M ?	60	Present in <u>Hin</u> - A?
15 N not digested	50	Present in <u>Hin</u> - C
16 O not digested	50	Present in <u>Hin</u> - D

TABLE 5

REDIGESTION OF ECO RII FRAGMENTS WITH THE HAE ENDONUCLEASE

Products of Eco RII fragment digested		Estimated size in base pairs	Corresponding whole or part <u>Hae</u> fragment
1	A1	775	<u>Hae</u> - B
	A2	260	<u>Eco</u> RII <u>Hae</u> - C2
2	B1	720	<u>Eco</u> RII <u>Hae</u> - A1
	B2	30	<u>Eco</u> RII <u>Hae</u> - E3
3	C	675	Present in <u>Hae</u> - A
	not digested		
4	D1	300	<u>Hae</u> - F _{1b}
	D2	150	<u>Eco</u> RII <u>Hae</u> - H1
	D3	40	<u>Hae</u> - I
	D4	30	<u>Hae</u> - L
	D5	10	<u>Eco</u> RII <u>Hae</u> - M2
5	E1	270	<u>Eco</u> RII <u>Hae</u> - F ₂ 1
	E2	170	<u>Eco</u> RII <u>Hae</u> - G1
6	F1	275	<u>Eco</u> RII <u>Hae</u> - C1
	F2	95	<u>Eco</u> RII <u>Hae</u> - D2
7	G1	160	<u>Eco</u> RII <u>Hae</u> - F _{1a} 1
	G2	95	<u>Eco</u> RII <u>Hae</u> - E2
	G3	40	<u>Hae</u> - K
8	H	240	Present in <u>Hae</u> - D
	not digested		
9	I	200	Present in <u>Hae</u> - E
	Not digested		
10	J1(J ₁ 1)	90	<u>Eco</u> RII <u>Hae</u> - F _{1a} 2
	J2(J ₂ 1)	60	<u>Eco</u> RII <u>Hae</u> - G2
	J3(J ₂ 2)	40	<u>Hae</u> - J
	J4(J ₁ 2)	30	<u>Eco</u> RII <u>Hae</u> - M1
11	K1	40	<u>Eco</u> RII <u>Hae</u> - A5
	K2	30	<u>Hae</u> - N
	K3	25	<u>Eco</u> RII <u>Hae</u> - D3
12	L	85	Present in <u>Hae</u> - A
	not digested		
13	M	60	Present in <u>Hae</u> - A(?)
	not digested		
14	N	50	Present in <u>Hae</u> - F _{1a}
	not digested		
15	O1	30	<u>Eco</u> RII <u>Hae</u> - F ₂ 2
	O2	20	<u>Eco</u> RII <u>Hae</u> - H2

TABLE 6**REDIGESTION OF HAE FRAGMENTS WITH THE ECO RII RESTRICTION
ENDONUCLEASE**

Products of <u>Hae</u> fragment digested		Estimated size in base pairs	Corresponding whole or part <u>Eco RII</u> fragment
1	A1	720	<u>Hae Eco RII</u> - B1
	A2	675	<u>Eco RII</u> - C
	A3	85	<u>Eco RII</u> - L
	A4	60	<u>Eco RII</u> - M (?)
	A5	40	<u>Hae Eco RII</u> -K1
2	B not digested	775	Present in <u>Eco RII</u> - A
3	C1	275	<u>Hae Eco RII</u> - F1
	C2	260	<u>Hae Eco RII</u> - A2
4	D1	240	<u>Eco RII</u> - H
	D2	95	<u>Hae Eco RII</u> - F2
	D3	25	<u>Hae Eco RII</u> - K3
5	E1	200	<u>Eco RII</u> - I
	E2	95	<u>Hae Eco RII</u> - G2
	E3	30	<u>Hae Eco RII</u> - B2
6	F _{1a} ¹	160	<u>Hae Eco RII</u> - G1
	F _{1a} ²	90	<u>Hae Eco RII</u> - J ₁ ¹
	F _{1a} ³	50	<u>Eco RII</u> - N
7	F _{1b} not digested	300	Present in <u>Eco RII</u> - D
8	F ₂ ¹	270	<u>Hae Eco RII</u> - E1
	F ₂ ²	30	<u>Hae Eco RII</u> - O1
9	G1	170	<u>Hae Eco RII</u> - E2
	G2	60	<u>Hae Eco RII</u> - J ₂ ¹
10	H1	150	<u>Hae Eco RII</u> - D2
	H2	20	<u>Hae Eco RII</u> - O2

A-E-K-F_{1a}-M-(F_{1b}, I, L)-H-F₂-G-J-B.

The precise placement of the 6 smaller Hae fragments in the genome has been achieved as a result of this work. Hae-N is placed between Hae-D and Hae-A by virtue of its production from Eco RII-K. Hae-K finds itself placed between Hae-E and Hae-F_{1a} because of its production in full from Eco RII-G. The larger and smaller sub-fragments of Hae-M are produced by Hae cleavage of Eco RII-J₁ and Eco RII-D respectively. This evidence, coupled with the fact that Hae-M is generated in full by the Hae cleavage of Hpa I-B (Fig. 3 and Table 2) enables us to place it between Hae-F_{1a} and the (F_{1b}, I, L) group. The fragments within the (F_{1b}, I, L) group have been further ordered to -F_{1b}-(I, L)- by virtue of the observation that the Hae cleavage of Hpa I-A (but not Hpa I-B) yields both Hae-I and -L (Fig. 3 and Table 2), and that F_{1b} is cleaved by Hpa I. Hae-J, by virtue of its production in full from both Eco RII-J₂ and Hpa I-A, has been placed between Hae-G and -B. On the basis of these and other results reported in Tables 5 and 6, the complete order of the Hae fragments is deduced to be B-C-D-N-A-E-K-F_{1a}-M-F_{1b}-(I, L)-H-F₂-G-J-B.

Complete order of the Eco RII fragments: The results of reciprocal redigestion of Eco RII fragments with Hae and the Hae fragments with Eco RII, presented in Tables 5 and 6, have enabled us to remove the three parentheses in the order of the Eco RII fragments presented above and to determine their complete order. Redigestion of Hae-D with Eco RII yields a piece of Eco RII-F, whole of Eco RII-H and a piece of Eco RII-K. The other part of Eco RII-K and the whole of Eco RII-L and Eco RII-C are generated along with other products by the cleavage of Hae-A with Eco RII. This results in the order -F-H-K-L-C- etc. for Eco RII fragments in this region. The other products of the Eco RII cleavage of Hae-A are Eco RII-M(?) and most of Eco RII-B. The remainder of Eco RII-B and the whole Eco RII-I are yielded by the Eco RII digestion of Hae-E. The

resulting order of Eco RII fragments in this region should be -C-M(?) -B-I etc. Finally, redigestion of Hae-F_{1a} with Eco RII generates a large piece of Eco RII-G, the whole of Eco RII-N and a part of Eco RII-J₁. This resolves the order of Eco RII fragments in this region to _G-N-J₁- etc. On the basis of these data we have determined the complete order of Eco RII fragments to be A-F-H-K-L-C-M(?) -B-I-G-N-J₁-D-O-E-J₂-A.

The Eco RII and Hae cleavage sites have been precisely mapped based on the sizes of the products of each cleavage and are shown in Figure 5 along with the complete order of the Eco RII and Hae fragments, with the Hin⁷ and Hpa I^{7, 5} and Eco RI^{3, 4} cleavage sites as reference points. For convenience, the cleavage map, which is actually circular, is presented as a linear one.

Molecular Hybridization Data: The ordering of fragments presented above has been confirmed in a few regions by molecular hybridization. Eco RII fragments are transcribed using E. coli RNA polymerase holo-enzyme with one of the triphosphates labelled with α -³²P. The transcripts are then hybridized to DNA isolated from Adeno-SV40 hybrid viruses ND₁ and ND₄ and also to some of the Hin fragments of SV40 DNA. The results of these hybridizations have confirmed the placement of the Eco RII fragments in the regions having overlaps with Adeno-SV40 hybrid DNA. For example, RII-F, RII-H, RII-K and RII-L hybridize to Ad₂+ND₁ DNA. These and also RII-C, RII-B and RII-I hybridize to Ad₂+ND₄ DNA. RII-F anneals to both Hin-G and Hin-B; RII-G hybridizes to both Hin-A and Hin-C. In the latter two instances the overlaps have been further confirmed by making transcripts of the Eco RII and corresponding Hin and making T₁ RNase fingerprints of the transcripts.

DISCUSSION

The Hin fragments of SV40 DNA have been ordered by Danna *et al*⁷ by obtaining partial Hin digestion products and redigesting them to completion with Hin. On the basis of these results they have constructed a physical map of the SV40 genome, but many of the Hin

fragments are rather large and require further fragmentation to make them amenable for nucleotide sequencing studies. Our approach in ordering the Eco RII and Hae fragments is based on analysis of the products of redigestion of one set of restriction enzyme fragments with another restriction enzyme. A direct consequence of this study has been to cleave SV40 DNA to many small fragments making it amenable to sequencing studies being carried out in this laboratory at present^{1, 2}. Digestion of SV40 DNA with a combination of Hin, Eco RII and Hae endonucleases would result in the production of an ordered set of 42 fragments. All of these (except Hae Eco RII-B1 which constitutes about 720 base pairs) are small and should prove valuable for sequencing work.

The technique used for sequencing segments of SV40 DNA in this laboratory is to get specific transcripts RNA's of fragments of the DNA and to sequence these RNA molecules. By this approach, a good part of the Hin-G fragment which contains a strong promoter for E. coli RNA polymerase and the sites of termination of DNA replication and early and late viral RNA transcription has been sequenced in this laboratory^{1, 2}. The site of initiation of SV40 DNA replication has been located at 0.67 map units on the genome^{14, 15}. This falls within Eco RII-G, which is further cleaved into three sub-fragments with the Hae enzyme. We are at present completing the sequence of this part of the genome. The fragments are also being used to map the termini of early and late transcription products from infected cells and to locate the site of strand switching when changing over from early to late transcription in vivo.

Eco RII makes staggered cuts on DNA giving rise to fragments with pentanucleotide long cohesive ends^{16, 17}. It may be possible to join any two Eco RII fragments covalently with the help of a ligase resulting in the construction of a longer fragment. This technique may be used to construct deletion-substitution variants of the viral DNA.

It can be seen from Fig. 4 that the right half of the SV40 genome contains many more Eco RII and Hae cleavage sites than the left half.

Figure 4

The horizontal lines denote a portion of the hypothetical Eco RII and Hin cleavage maps of SV40 DNA. The solid vertical lines represent the Eco RII cleavage sites in the Eco RII map and the Hin map. The dotted vertical lines in each cleavage map denote sites of recleavage by the other restriction enzyme.

Figure 5

The cleavage map of the SV40 genome based on results reported in this study. Map units are expressed as $\frac{\text{distance from Eco RI site}}{\text{length of SV40 DNA}}$

The Hinf and Hpa I cleavage maps described by Danna et al⁷ is included for reference.

The cleavage map of the ten larger Hae fragments has also been derived by Lebowitz et al (personal communication)

For instance, Hin-C and Hin-D contain a total of 13 cleavage sites for the Eco RII and Hae endonucleases, which is much above the average of one cleavage site per 320 base pairs. The nucleotide sequence at the Eco RII cleavage site is known to be

The preponderance of the Eco RII and Hae cleavage sites in the right half of the genome, especially in the Hin-C, -D region, indicates a higher G + C content than the rest of the genome. This confirms the direct observation by Danna¹⁸ who found that Hin-C and to a lesser extent Hin-D have a higher G + C content than the rest of the DNA.

It is interesting to note that the very small fragment Hae-M which is only about 30 base pairs long contains an Eco RII cleavage site which requires a pentanucleotide specificity.

The fragment Eco RII-M is produced in full by the Eco RII digestion of Hpa I-B. One of the products of the Eco RII digestion of Hin-A and Hae-A appears to have the same electrophoretic mobility as that of Eco RII-M and it could not be identified with any other whole or sub-fragment produced by Eco RII cleavage. The trace of uncertainty in the identification of this fragment as Eco RII-M makes its placement in the cleavage map between Eco RII-C and Eco RII-B a tentative one.

In the course of their studies on the elucidation of the nucleotide sequence at the Eco RII cleavage sites, Boyer *et al*¹⁶ have obtained results which seem to indicate the production of 17 fragments by the Eco RII digestion of SV40 DNA. We observe only 16 fragments in the Eco RII digests of SV40 DNA. However, the production of one or more very small fragments which ran out of the 8% gel we utilize for trapping small fragments, cannot be ruled out at present.

In addition to the 16 Hae fragments described above we have noticed a faint band which moves much faster than the blue dye when a Hae digest of SV40 DNA is electrophoresed in a 10% gel. At present we are not certain if this band represents a real fragment or is an artifact. If it were a real fragment, we estimate that it would be only about 10 base pairs long.

The Eco RII and Hae cleavage maps are currently being utilized in our laboratory in the precise mapping and ordering of fragments of SV40 DNA produced by other restriction endonucleases.

ACKNOWLEDGMENTS

We thank Dr. P. Lebowitz for many helpful discussions and for having permitted reference to his unpublished observations. We are deeply indebted to Dr. Ravi Dhar for many valuable discussions, helpful suggestions and continued encouragement. We are grateful to Drs. H. W. Boyer and S. Goodgal for sending us the E. coli RII and H. para-influenzae strains respectively. We are very grateful to Dr. Jeff Sklar for generously providing the Hin restriction enzyme. We thank Fran DeNoto and Ben Jones for growing the BSC-1 cells used in this study. We also thank Elaine Collins for having typed and edited this manuscript. K. N. S. was supported by USPHS Training Grant No. AM 1003-10.

REFERENCES

1. Zain, B. S., Dhar, R., Weissman, S. M., Lebowitz, P., and Lewis, A. M. Jr. (1973) J. Virol., 11, 682-693
2. Dhar, R., Zain, S., Weissman, S. M., Pan, J., and Subramanian, K. N. (1974) Proc. Nat. Acad. Sci. (U. S. A.) (in press)
3. Mulder, C., and Delius, H. (1972) Proc. Nat. Acad. Sci. (U. S. A.) 69, 3215-3219
4. Morrow, J. F., and Berg, P. (1972) Proc. Nat. Acad. Sci. (U. S. A.) 69, 3365-3369
5. Sharp, P. A., Sugden, B., and Sambrook, J. (1973) Biochemistry, 12, 3055-3063
6. Sack, G. H. Jr. and Nathans, D. (1973) Virology, 51, 517-520
7. Danna, K. J., Sack, G. H. Jr., and Nathans, D. (1973), J. Mol. Biol. 78, 363-376
8. Huang, E. S., Newbold, J. E., and Pagano, J. S. (1973) J. Virol. 11, 508-514
9. Gelb, L. D., Kohne, D. E., and Martin, M. A. (1971) J. Mol. Biol., 57, 129-145
10. Yoshimori, R. N., Ph. D. Thesis, University of California Medical Center, San Francisco, 1973
11. Smith, H. O. and Wilcox, K. W. (1970) J. Mol. Biol., 51, 379-391
12. Middleton, J. H., Edgell, M. H., and Hutchinson, C. A. III (1972) J. Virol., 10, 42-50
13. Lebowitz, P., Weissman, S. M., and Radding, C. (1971) J. Biol. Chem. 246, 1520-1535
14. Fareed, G. C., Garon, C. F., and Salzman, N. P. (1972) J. Virol. 10, 484-491

15. Danna, K., and Nathans, D. (1972) Proc. Nat. Acad. Sci(U.S.A.) 69, 3097-3100
16. Boyer, H.W., Chow, L.T., Dugaiczky, A., Hedgepeth, J., and Goodman, H.M. (1973) Nature New Biol., 244, 40-43
17. Bigger, C.H., Murray, K., and Murray, N.E. (1973) Nature New Biol., 244, 7-10
18. Danna, K. (1972) Ph.D. Thesis, John Hopkins University, Baltimore, Maryland