Arthrobacter luteus Restriction Endonuclease Recognition Sequence and Its Cleavage Map of SV40 DNA†

Ernest Jay‡ and Ray Wu*

ABSTRACT: The nucleotide sequence at the cleavage site of the restriction endonuclease isolated from *Arthrobacter luteus* (*Alu*) has been determined. The endonuclease cleaves at the center of a palindromic tetranucleotide sequence to give even-ended duplex DNA fragments phosphorylated at the 5′-end. The endonuclease cleaves SV40 form I DNA into 32 fragments. The order and sizes of these fragments have been determined to provide an *Alu* cleavage map of the SV40 genome.

A restriction endonuclease from *Arthrobacter luteus* (ATCC 21606) has been isolated and purified by R. J. Roberts (1974), personal communication). It has been shown to be different from other restriction enzymes by comparing the digestion patterns of DNA[1] from different sources. To confirm this and to compare its recognition sequence with those of other restriction enzymes, we have determined the nucleotide sequence of the DNA at the cleavage sites produced by *Alu*. Two different methods were used for 5′- and 3′-terminal labeling of the fragments produced by digestion of SV40 form I DNA with *Alu*. DNA sequence analysis of the terminal regions of these fragments was carried out by several mapping techniques (Wu et al., 1976).

One of the unusual aspects of this restriction endonuclease is that it cleaves SV40 form I DNA into 32 fragments. Most of the other restriction enzymes give relatively few fragments except *Eco* RII and *Hae* III which gave 16 and 18 fragments, respectively (Subramanian et al., 1974; Yang et al., 1976a). Thus, *Alu* will be most useful for digesting SV40 DNA, or fragments of SV40 DNA produced by cleavage with other restriction endonucleases, into smaller fragments for sequence analysis.

The order of the 32 fragments produced by digestion of SV40 form I DNA with *Alu* has now been arranged to provide a physical cleavage map of the SV40 genome as shown in Figure 1. This was accomplished by (a) complete digestion of each of the uniformly or terminally labeled SV40 DNA *Hind* III fragments with *Alu*, (b) partial digestion of individual *Hind* III fragments with *Alu*, and (c) partial digestion of SV40 DNA with *Alu*. Each of the DNA fragments obtained from the partial digestions were isolated and completely digested with *Alu*, and their polyacrylamide gel electrophoresis patterns were compared to allow overlap and ordering of the 32 fragments.

Experimental Procedure

Materials

SV40 DNA was purified based on several procedures (Hirt, 1967; Danna and Nathans, 1971; Sharp and Sambrook, 1974, personal communication). Two lines of African green monkey kidney cells CV-1 and TC-7 were supplied by J. Sambrook and J. A. Robb, respectively. Plaque purified SV40 was kindly given to us by D. Nathans. SV40 was grown on either cell line by the procedure of Danna and Nathans (1971). Unlabeled and uniformly ^{32}P-labeled SV40 form I DNA were purified as previously described (Wu et al., 1976).

Enzymes

Arthrobacter luteus restriction enzyme (*Alu*) was a generous gift of Dr. R. J. Roberts. Subsequent isolation of the enzyme in our laboratory was accomplished according to the procedure provided by R. J. Roberts. *Hind* was purified according to Smith and Wilcox (1971), except that a linear gradient was used instead of a step gradient for the phosphocellulose column. The *Hind* III was separated from the *Hind* II by a final DE-52 cellulose column. Bacterial alkaline phosphatase (BAPF) and pancreatic DNase were purchased from Worthington Biochemical Co.; polynucleotide kinase was purchased from Biogenics Research Corp.; calf thymus deoxynucleotidyl terminal transferase was a gift of R. Roychoudhury; DNA polymerase I was purified as described earlier (Jovin et al., 1969).

Chemicals

[α-^{32}P]NTP and [α-^{32}P]dNTP (60–120 Ci/mmol) were purchased from New England Nuclear Co.; [γ-^{32}P]ATP and ^{32}P$_i$ were purchased from ICN (Irvine, California). Cellogel was purchased from Reeve Angel. DEAE-cellulose thin-layer plates and homomixtures for homochromatography (using limited amounts of KOH) were prepared as described by Jay

† From the Department of Biochemistry, Molecular and Cell Biology, Cornell University, Ithaca, New York 14853. *Received February 17, 1976.* This work was supported by research Grant CA-14989, awarded by the National Cancer Institute, Department of Health, Education and Welfare, and BMS 73-01859 A01 from the National Science Foundation. This is paper XXVIII in a series on Nucleotide Sequence Analysis of DNA. Paper XXVII is by Jay, Roychoudhury, and Wu (1976), *Biochem. Biophys. Res. Commun. 69,* 678–686.

‡ Present address: Department of Chemistry, The University of New Brunswick, Fredericton, N.B., Canada E3B 5A3.

[1] Abbreviations used: *Alu*, restriction endonuclease from *Arthrobacter luteus;* similarly, *Hind, H. influenzae* type d; *Hind* is a mixture of *Hind* II and *Hind* III; *Hae* III, *H. aegyptius; Eco* R II, *E. coli* RTF II; *Alu* fragment, DNA fragment produced by digesting SV40 DNA with *Alu;* p*Alu*, partial digestion of DNA with *Alu;* BAPF, bacterial alkaline phosphatase; 2-D homochromatogram, two-dimensional fractionation (see legend to Figure 2); DNA, deoxyribonucleic acid; DEAE, diethylaminoethyl; NTP, nucleoside triphosphate; dNTP, deoxynucleoside triphosphate; ATP, adenosine triphosphate; P$_i$, inorganic phosphate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid, GTP, guanosine triphosphate; CTP, cytidine triphosphate; uv, ultraviolet; GMP, guanosine monophosphate. The symbols A, G, T and C are used throughout to stand for deoxynucleosides; N is an unspecified deoxynucleoside.

et al. (1974).

Methods

Restriction Enzyme Digestions. The superhelical SV40 form I DNA was digested with *Alu* (activity, 1 μg of SV40 DNA per 3 μl of *Alu* per 30 μl of incubation volume per 6 h) into 32 fragments in 6.6 mM Tris-HCl, pH 7.5, 6.6 mM $MgCl_2$, and 6.6 mM mercaptoethanol. The reaction was stopped by adjusting to 10 mM EDTA. Samples for further terminal labeling experiments were extracted twice with phenol and four times with ether, and the DNA fragments were precipitated in 70% ethanol at −20 °C. Fragments that were to be labeled with [γ-^{32}P]ATP and kinase were treated with BAPF before the phenol step.

For the production of the six SV40 DNA *Hind* III fragments (for redigestion with *Alu*), SV40 form I DNA was digested with *Hind* III (activity, 5 μg of DNA per 0.5 μl of *Hind* III per 50 μl of incubation volume per 5 h) in 6.6 mM Tris-HCl, pH 7.5, 6.6 mM $MgCl_2$, 6.6 mM merceptoethanol, and 50 mM NaCl. The reaction was stopped by adjusting to 10 mM EDTA, and the six *Hind* III fragments were separated on a 20 × 40 cm step polyacrylamide gel (3.5–6%).

Terminal Labeling of DNA Fragments. Both for sequence analysis of the terminal regions of the SV40 DNA fragments generated by digestion with *Alu* and for ordering of the fragments produced by *Alu*, the 5′ ends were labeled with ^{32}P using [γ-^{32}P]ATP and polynucleotide kinase. The 3′ ends were labeled with ^{32}P using [α-^{32}P]CTP or [α-^{32}P]GTP and terminal transferase as described recently (Roychoudhury et al., 1976).

Partial Digestion of Labeled Fragments for Sequence Analysis. The terminally labeled fragments were purified (to remove the excess triphosphates) and desalted through a Sephadex G-50 column in 0.05 M triethylamine bicarbonate. Salmon sperm DNA was added as carrier to give a total of 50 μg of DNA per sample. The sample was concentrated and the triethylamine bicarbonate removed by adding 100 μl of water and again dried in a desiccator. The labeled fragments were dissolved in 80 μl of 100 mM NH_4HCO_3, pH 7.8, 5 mM $MgCl_2$. The mixture was divided into four equal aliquots (20 μl each) and pancreatic DNase was added (0.2, 0.5, 1.0, and 2.0 μg, respectively). Digestion was allowed to proceed for 3 h and the reaction stopped by adjusting to 5 mM EDTA and boiling for 3 min. The four aliquots were combined, desalted by repeated evaporation, and finally allowed to evaporate down to 5 μl. A portion of the digest (2 μl) was applied on cellulose acetate strip (cellogel) for electrophoresis.

Polyacrylamide Gel Electrophoresis. The 20 × 40 cm polyacrylamide slab gels were prepared by a method similar to that described by Maizel (1971) and Studier (1973). The polyacrylamide gels were prepared and electrophoresed in 40 mM Tris-HCl, pH 7.5, 5 mM NaOAc, and 1 mM EDTA at 4 V/cm (50–60 mA) at room temperature for 16–18 h. For ordering the 32 fragments produced by digestion of SV40 form I DNA with *Alu*, linear gradient gels of 5 to 15% polyacrylamide were used (Jeppesen, 1974). Our modification for the preparation of the gradient gel has been described (Wu et al., 1976). This type of 40-cm long gradient polyacrylamide gel has proven most useful in separating complex mixtures of DNA fragments ranging from sizes 20 to 1000 base pairs long. The DNA bands, particularly for the smaller fragments, are much sharper compared with those fractionated on uniform gels. The gels after electrophoresis can be stained with an ethidium bromide solution (0.5 μg/ml) for the visualization of the DNA bands under uv light (if sufficient DNA is present), or radioautographed (if DNA is labeled with ^{32}P). The DNA bands were extracted and ethanol precipitated as described elsewhere (Wu et al., 1976).

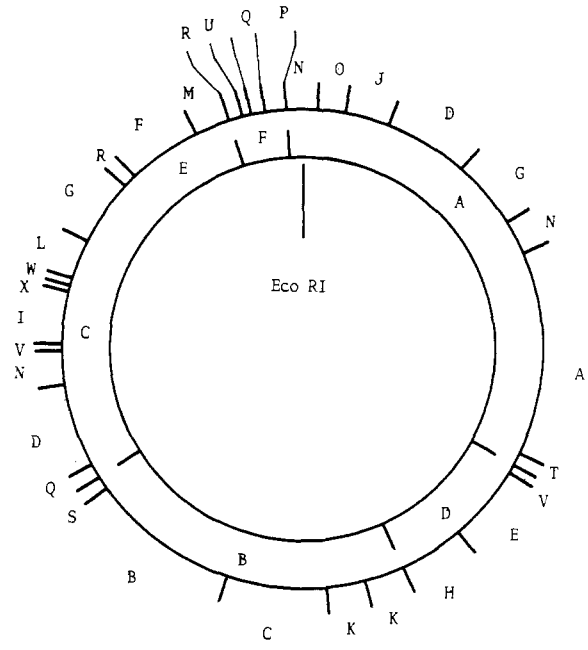

FIGURE 1: A restriction endonuclease *Alu* and *Hind* III cleavage map of SV40 form 1 DNA. (outer circle) *Alu* map; (inner circle) *Hind* III map.

TABLE I: Terminal Analysis of SV40 DNA Fragments Produced by Digestion with *Alu*.

5′-End Analysis of SV40-*Alu* Fragments			
p*A	p*G	p*T	p*C
1.8%	0.2%	3%	95%
3′-End Nearest Neighbors Analysis of SV40-*Alu* Fragments			
Ap*	Gp*	Tp*	Cp*
7%	86%	2%	5%

Results and Discussion

Sequence Analysis of the 5′-Ends of the SV40 DNA-Alu Fragments. SV40 form I DNA was digested with *Alu*. The mixture of digestion products, after treatment with the phosphatase BAPF, was labeled with polynucleotide kinase and [γ-^{32}P]ATP. Attempted 5′ labeling without prior phosphatase treatment gave essentially no ^{32}P incorporation, indicating that the *Alu* digestion products are phosphorylated at the 5′ termini. A sample of the mixture of labeled fragments was exhaustively digested with pancreatic DNase and venom phosphodiesterase for 5′ terminal analysis (Wu and Taylor, 1971). Table I (upper panel) shows a distribution of ^{32}P label for the four 5′, ^{32}P-labeled mononucleotides, illustrating that all the fragments were 5′-pC terminated.

The remaining sample was partially degraded with different levels of pancreatic DNase to produce 5′-terminally labeled fragments of increasing chain lengths. The partial products were subjected to sequence analysis by mapping on two-dimensional homochromatography (Brownlee and Sanger, 1969; Jay et al., 1974) as shown in Figure 2. From the mobilities of the nucleotides shown in Figure 2a and reproduced on Figure

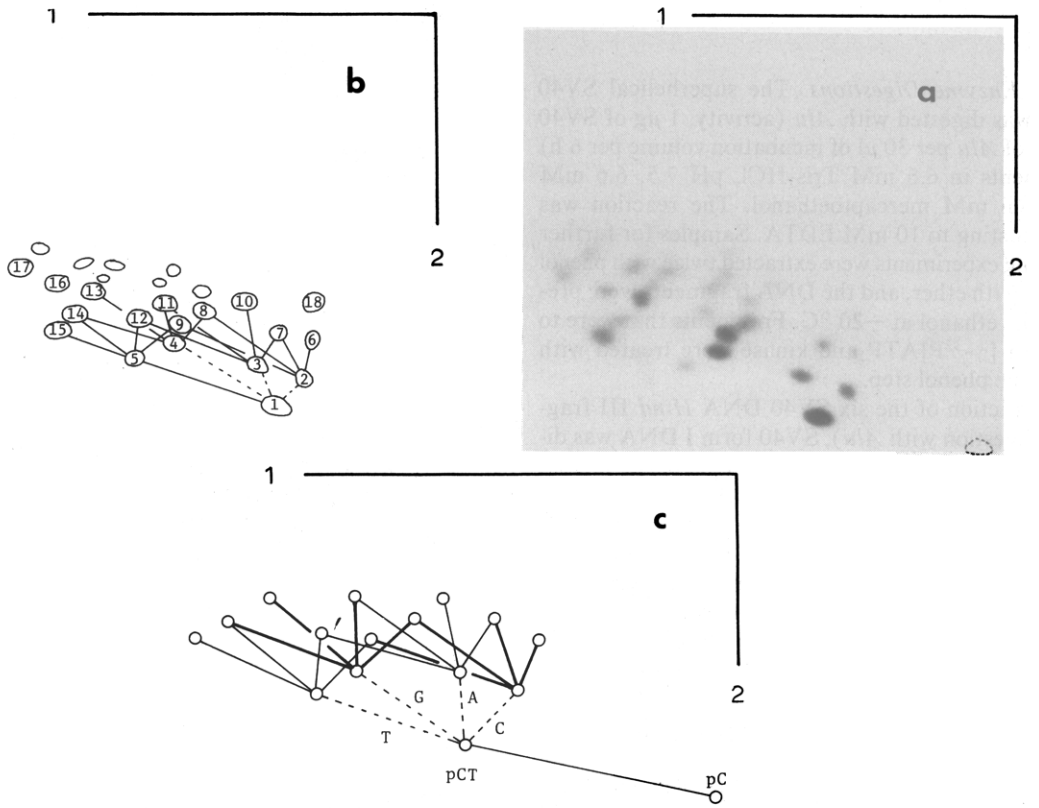

FIGURE 2: Two-dimensional homochromatogram of the partial pancreatic DNase digest of the 5′-^{32}P-labeled SV40 DNA–*Alu* fragments. (Dimension 1) Electrophoresis on cellulose acetate strip in pyridine acetate at pH 3.5; (dimension 2) homochromatography on DEAE-cellulose thin-layer plate in homomixture IV. Figure 2a shows the autoradiogram of the fingerprint; Figure 2b shows the tracing of the autoradiogram. The dotted circle on Figure 2a shows the position of standard [^{14}C]pC. Spot 1 was shown to be d(pC-T) by mobility calculations (Tu et al., 1976) and further analysis. The sequence of the tri- and tetranucleotides can be deduced from the parent dinucleotide following the characteristic mobility shifts for the addition of each of the four mononucleotides (Tu et al., 1976). Figure 2c shows the theoretical fingerprint calculated for the partial digest of pC-T-N-N using the mobility formulas developed in our laboratory. The sequence of the oligomers (in b) can also be obtained by direct comparison with this theoretical fingerprint.

TABLE II: The Nucleotide Sequences of Oligomers Shown in Figure 2.[a]

Oligomers	Sequence	Oligomers	Sequence
1	pC-T	10	pC-T-A-A
2	pC-T-C	11	pC-T-G-A
3	pC-T-A	12	pC-T-A-T, pC-T-T-A
4	pC-T-G	13	pC-T-G-G
5	pC-T-T	14	pC-T-G-T, pC-T-T-G
6	pC-T-C-C	15	pC-T-T-T
7	pC-T-C-A, pC-T-A-C	16	pC-T-T-A-T, pC-T-A-T-T
8	pC-T-C-G, pC-T-G-C	17	pC-T-T-G-G
9	pC-T-C-T, pC-T-T-C	18	pC-T-C-C-C

[a]The oligomers were isolated from the homochromatogram, partially digested with venom phosphodiesterase, and analyzed on one-dimensional electrophoresis on DEAE-cellulose paper at both pH 1.9 and 3.5.

2b, it is suggested that only the first two nucleotides at the 5′ ends of the *Alu* fragments are unique (5′ pC-T), and that only the third and succeeding nucleotides exhibit differences. This is supported by comparison with a two-dimensional homochromatogram (Figure 2c) expected to be of the sequence 5′ pC-T-N··· constructed by using the method for calculating electrophoretic mobilities of oligonucleotides, introduced in our laboratory (Bambara et al., 1974; Tu et al., 1976). The sequences of each of the oligomers indicated in Figure 2 and Table II were confirmed after isolation of the oligomers from the homochromatogram. Each oligomer was partially digested with venom phosphodiesterase to obtain fragments of varying lengths and subjected to electrophoresis on DEAE-cellulose paper at both pH 1.9 and 3.5. The sequence of the oligonucleotides was deduced by using the characteristic *m* values introduced by Sanger et al. (1965). This confirmed the sequence 5′-pC-T-N- for the 5′ ends of the fragments produced by *Alu* cleavage.

Sequence Analysis of the 3′ Ends of the SV40 DNA–Alu Fragments. The *Alu* fragments were labeled at the 3′ end using terminal transferase and [α-^{32}P]GTP (Roychoudhury et al., 1976). The labeled fragments were treated with alkali and phosphatase to remove diaddition products, thus leaving a [^{32}P]GMP residue at the 3′ end of the *Alu* fragments. The resulting singly 3′-terminally labeled fragments were subjected to sequence analysis as described above. Table I (lower panel) shows the nucleotides at the 3′ ends of the fragments, determined by the nearest neighbor analysis. Essentially all the fragments were 3′-G terminated. Figure 3 shows the two-dimensional homochromatogram for the partial pancreatic DNase digest of the *Alu* fragments labeled at the 3′ termini with a single [^{32}P]GMP. The sequence deduced for the 3′ ends of the *Alu* fragments is thus 5′···N-A-G.

Cleavage Site for Alu. From the results of the two terminal labeling experiments followed by sequence analysis by two-

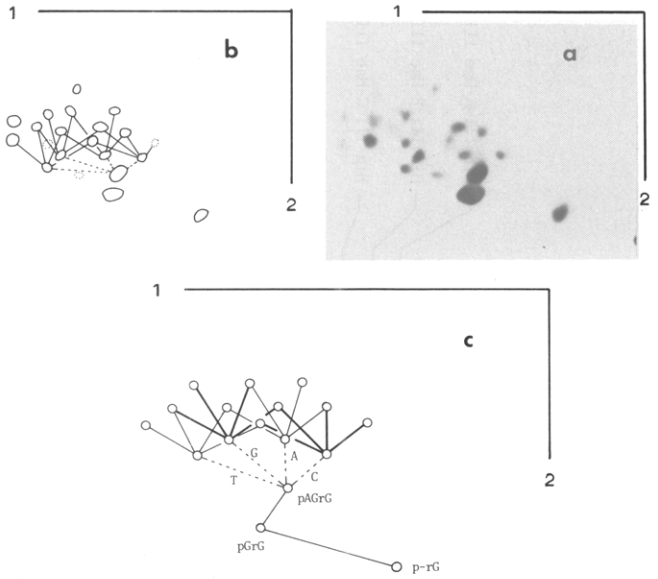

FIGURE 3: Two-dimensional homochromatogram of the partial pancreatic DNase digest of the *Alu* fragments labeled at the 3′ ends with a single [^{32}P]rGMP. Figure 3a shows the autoradiogram of the homochromatogram; Figure 3b shows the tracing of the homochromatogram; Figure 3c shows the theoretical fingerprint for the partial digest of pN-A-G-rG. The sequence of the tetranucleotides can be deduced from the parent trinucleotide pA-G-rG following the characteristic mobility shifts for the addition of each of the four mononucleotides.

dimensional mapping, a unique sequence for the cleavage site of *Alu* can be deduced as shown below.

$$5'\text{——NpApG}\downarrow\text{pCpTpN——}3'$$
$$3'\text{——NpTpCp}\uparrow\text{GpApN——}5'$$

It contains a complementary palindrome of only four nucleotides with the cleavage site at the center to produce 5′ phosphorylated, even-ended fragments. This tetranucleotide sequence lies within the cleavage site of *Hind* III which consists of a hexanucleotide sequence 5′-A-A-G-C-T-T- (Murray and Old, 1974). This was confirmed by comparing the polyacrylamide gel pattern of the fragments produced by digestion of SV40 DNA with *Alu* alone, and with *Alu* plus *Hind* III (data not shown).

Ordering of the SV40 DNA Fragments Produced by Digestion of SV40 Form I DNA with Alu. SV40 DNA on exhaustive digestion with *Alu* gave 32 fragments varying in length from 750 to 30 base pairs (Figure 4), which are fractionated into 24 distinct bands in a 5–15% linear polyacrylamide gradient gel. The lengths of these fragments approximate those reported by Yang et al. (1976b). The orders of these 32 fragments were determined by (a) digesting SV40 DNA with *Hind* III into six fragments. Each of these six fragments were individually partially digested with *Alu*. Each of these six *Hind* III fragments and their partial *Alu* digestion products were exhaustively digested with *Alu* and subjected to 5–15% polyacrylamide gradient gel electrophoresis for comparison and overlap. (b) The orientation of the six *Hind* III and thus the *Alu* fragments within each *Hind* III fragment was determined by a new, end-labeling method. In this method, the unlabeled *Hind* III fragments were labeled with ^{32}P at their 3′ ends. Each of these fragments was digested with *Hae* III into two, end-labeled *Hind* III–*Hae* fragments. These fragments, which are of known location in a *Hind* III–*Hae* cleavage map, were separated and then digested with *Alu*. The resulting labeled *Alu* fragment generated from each *Hind* III–*Hae* III digestion was compared with the gel pattern of an exhaustive *Alu* digestion mixture, thus allowing identification of the terminal *Alu* fragments from each *Hind* III fragment and revealing their orientation with respect to one another. Another advantage of using terminally labeled DNA fragments for mapping (as compared with uniformly labeled DNA) is that the small fragments are as highly labeled as the large fragments. Thus, the small fragments can be readily detected on

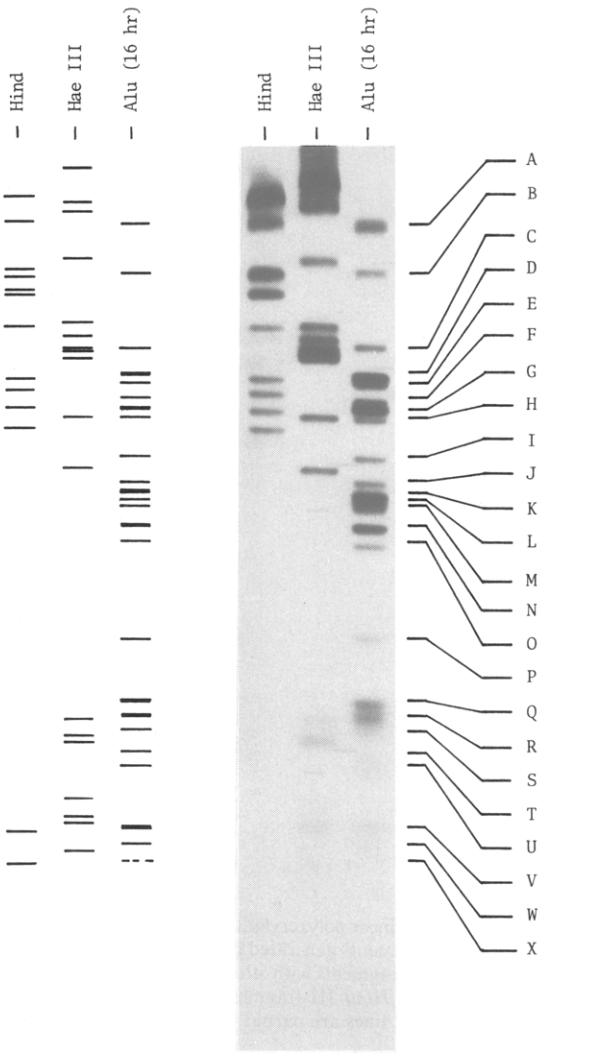

FIGURE 4: Polyacrylamide gel electrophoresis pattern of the SV40 DNA–*Alu* digest. Uniformly ^{32}P-labeled SV40 form I DNA was digested with restriction enzymes *Hind*, *Hae* III, and *Alu*, and their digests were separated on a 5–15% linear polyacrylamide gradient gel for 24 h.

TABLE III: Summary of the SV40 DNA-*Alu* Fragments at the Termini of Each of the Six *Hind* III Fragments.

Hind III		*Alu*	
Fragment	Base Pairs	Fragments	Sum of Base Pairs
A	1761–1937	A, D, G, J, N, N, O, T	1976
B	1057–1163	B, C, K, K, S	1190
C	1063–1165	D, G, I, L, N, Q, V, W, X	1096
D	500–550	E, H, V	531
E	425–468	F, M, R	432
F	195–215	P, Q, R, U	186

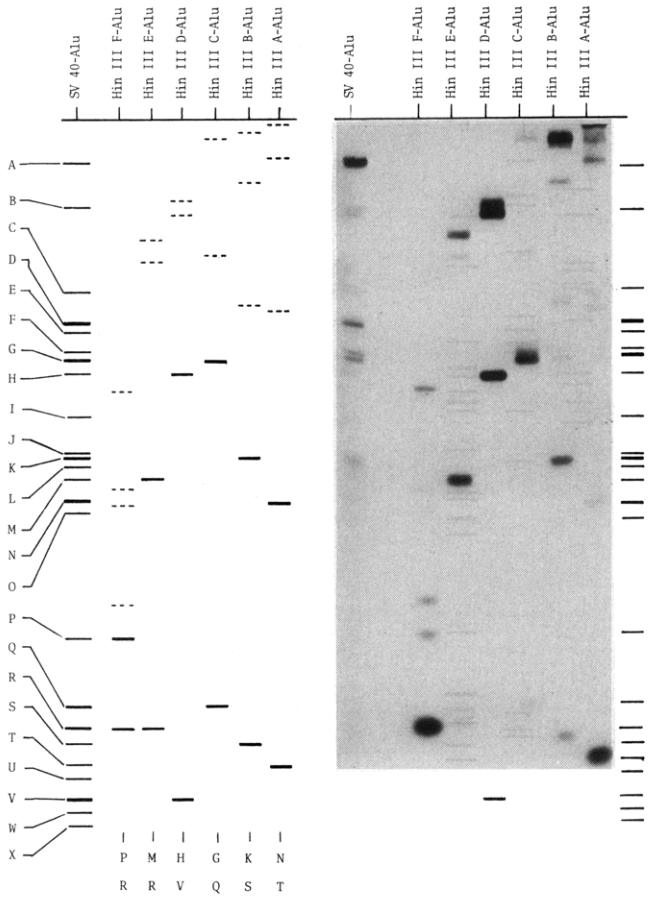

FIGURE 5: A 5–15% linear polyacrylamide gradient gel separation of the SV40 DNA–*Alu* fragments generated by digestion of the six terminally ^{32}P-labeled *Hind* III fragments with *Alu* to identify the two terminal *Alu* fragments from each *Hind* III fragment. The DNA bands on the gel represented by dotted lines are partial products due to incomplete *Alu* digestion.

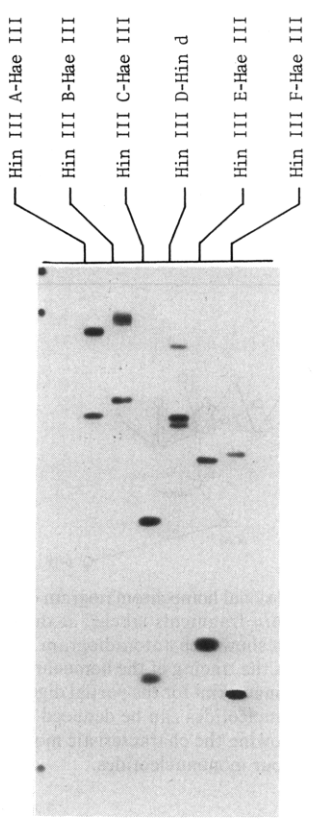

FIGURE 6: A 3.5–8% step polyacrylamide gradient gel separation of the two terminally ^{32}P-labeled ends of the six SV40 DNA–*Hind* III fragment after cleavage with *Hae* III. *Hind* III D fragment does not contain any *Hae* III cleavage site and the two ends were thus separated by cleavage of *Hind* III D with *Hind*.

gel by radioautography after a short period of exposure of the x-ray film. (c) Intact SV40 form I DNA was partially digested with *Alu*. Each of these partial *Alu* fragments was exhaustively digested with *Alu* and subjected to analysis on a 5–15% linear polyacrylamide gradient gel. The resulting *Alu* fragments from each partial *Alu* fragment were compared with an exhaustive *Alu* digest for overlap.

Identification of Alu Fragments Produced from Each of the Six Uniformly Labeled Hind III Fragments and Their Relative Orientation. [^{32}P]SV40 form I DNA on digestion with *Hind* III gives six fragments of the order A-D-B-C-E-F (Danna et al., 1973). Their sizes are shown in Table III. The six fragments were isolated from a 3.5–6% step polyacrylamide gel, eluted, and precipitated. On digestion of each of these six *Hind* III fragments with *Alu*, the corresponding *Alu* fragments are produced and identified as shown in Table III. The sum of the *Alu* fragments agrees well with the size of the corresponding *Hind* III fragments. Thus, a partial sequence of the SV40 DNA *Alu* fragments can be deduced and represented by:

SV40 DNA–*Hind* III fragment

A	D	B	C	E	F
(A,D,G,J,N,N,O,T)	(E,H,V)	(B,C,K,K,S)	(D,G,I,L,N,Q,V,W,X)	(F,M,R)	(P,Q,R,U)

SV40 DNA–*Alu* fragment

Identification of the Terminal Alu Fragments Generated from Each Hind III Fragment and Their Relative Orientation. SV40 DNA (25 μg) was digested with *Hind* III. The digest was extracted with phenol and the six DNA fragments were precipitated in 70% ethanol. The mixture of *Hind* III fragments was terminally labeled at the 3′ end with [α-^{32}P]CTP and deoxynucleotidyl terminal transferase. Labeling at the 3′ end with terminal transferase proved to be much more efficient than 5′-end labeling using the more laborious technique of removing the 5′ phosphoryl group by BAPF followed by the use of the polynucleotide kinase and [γ-^{32}P]ATP (Roychoudhury et al., 1976). After removal of excess [α-^{32}P]CTP on a Sephadex G-50 column, the six 3′-terminally labeled *Hind* III fragments were separated on a 3.5–6% step polyacrylamide gel and precipitated as described earlier. Each of these six DNA bands was exhaustively digested with *Alu* and the digests were fractionated on a 5–15% gradient polyacrylamide gel together with a complete [^{32}P]SV40 DNA *Alu* digest for comparison (Figure 5). The results indicate that the terminal *Alu* fragments N and T are generated from *Hind* III A, fragments K and S from *Hind* III B, fragments G and Q from *Hind* III C, fragments H and V from *Hind* III D, fragments M and R from *Hind* III E, and fragments P and R from *Hind* III F.

The next step was to orient these terminally labeled *Alu* fragments generated from the six *Hind* III fragments with respect to one another. This was done by first digesting each of the six terminally labeled *Hind* III fragments with another restriction enzyme (*Hae* III) whose cleavage pattern had previously been elucidated (Subramanian et al., 1974). This allowed us to distinguish the two ends of the *Hind* III fragments. Digestion of each of the terminally labeled *Hind* III

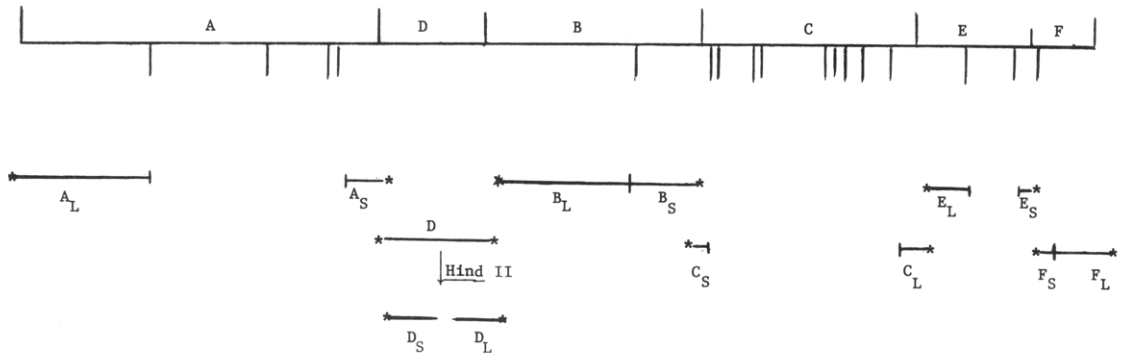

FIGURE 7: A map showing the two labeled fragments generated from each of the terminally ^{32}P-labeled SV40 DNA–*Hind* III fragments after redigestion with *Hae* III. The *Hind* III D fragment which does not contain a *Hae* III cleavage site is resistant. Separation of the two ends of *Hind* III D into *Hind*-H (D_L) and *Hind*-I (D_S) was accomplished by digestion with *Hind* II.

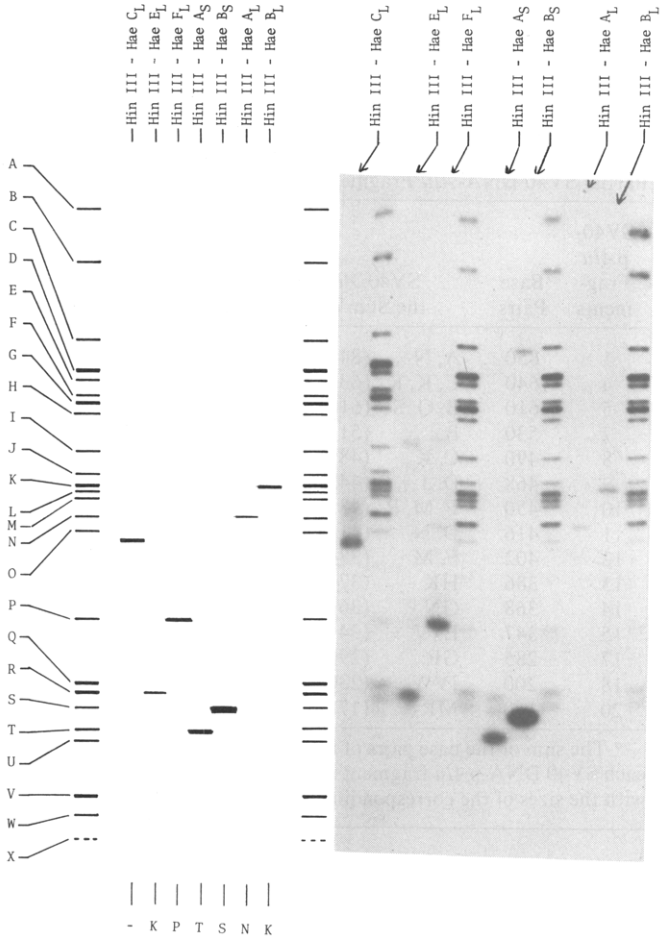

FIGURE 8: A 5–15% linear polyacrylamide gradient gel separation of the SV40 DNA–*Alu* fragments generated from 7 out of the 12 singly end-labeled *Hind* III–*Hae* fragments isolated from the polyacrylamide gel shown in Figure 6. Only one end-labeled fragment was expected and found in each sample. The four lanes with multiple bands represent complete SV40 DNA–*Alu* digest used as markers.

TABLE IV: SV40 DNA-*Alu* Fragments from the Two Ends of Terminally Labeled *Hind* III Fragments.

Hind III Fragments (Terminally Labeled)	*Alu* Fragments from Digesting *Hind* III Fragment in Column 1	*Hae* III Fragments from Digesting *Hind* III Fragment in Column 1	Corresponding *Alu* Fragment
A	N, T	A_L	N
		A_S	T
B	K, S	B_L	K
		B_S	S
C	G, Q	C_L	G[a]
		C_S	
D	H, V	D *Hind* II → D_L = *Hind* H	H
		D_S = *Hind* I	V
E	M, R	E_L	R
		E_S	
F	P, R	F_L	P
		F_S	

[a] Terminally labeled *Hind* III C on digestion with *Alu* gave terminally labeled *Alu* fragments G (243 base pairs) and Q (52 base pairs). Terminally labeled *Hind* III C on digestion with *Hae* III gave terminally labeled fragments C_S and C_L (115 base pairs). C_L showed no further digestion with *Alu* indicating that the *Alu* fragments at this end of *Hind* III C must be *Alu* G and not K since *Alu* G is larger than C_L.

fragments with *Hae* III gave a large (designated by L) and a smaller (designated by S) labeled fragment (Figure 6), except for *Hind* III D which does not contain the cleavage site for *Hae* III. In this case, the two ends were separated by digestion with *Hind* II or *Hind* which cleaves it into fragment D_L (equivalent to *Hind* H) and D_S (equivalent to *Hind* I). Thus, by comparing the redigestion map of *Hind* III with *Hae* III, the orientation of the labeled large and small *Hind* III–*Hae* fragments from each *Hind* III fragment can be determined as shown in Figure 7. Each of these terminally labeled *Hind* III–*Hae* fragments of known location was digested with *Alu* to determine the [^{32}P]*Alu* fragments produced. Figure 8 shows the autoradiogram of the 5–15% gradient polyacrylamide gel pattern for redigestion of the larger *Hind* III–Hae fragments with *Alu*. From these results (Table IV), the following partial sequence can be derived for the *Alu* cleavage map.

(a) SV40 DNA–*Hind* III fragment; (b) *Hind* III–*Hae* fragment; (c) SV40 DNA–*Alu* fragment

(a)	A			D			B			C			E		F	
(b)	A_L		A_S	D_S		D_L	B_L		B_S	C_S		C_L	E_L	E_S	F_S	F_L
(c)	N(A,D, G,J,N, O)		T	V	(E)	H	K(B,C, K)		S	Q(D,I,L, N,Q,V, W,X)G			R	(F)	M	R(Q,U)P

Partial Digestion of Hind III Fragments with Alu for Overlapping the Alu Fragments. To determine the order of the

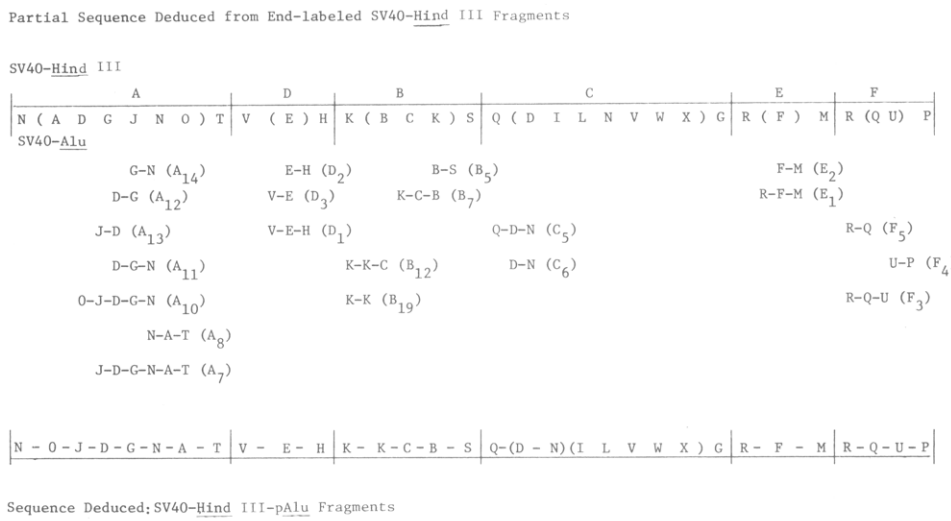

FIGURE 9: Overlapping the SV40 DNA-*Alu* fragments generated from the partial *Alu* bands of each of the six *Hind* III fragments to produce a partial *Alu* cleavage map. The numbers (subscript) in brackets refer to the *Hind* III-p*Alu* bands isolated from the polyacrylamide gel.

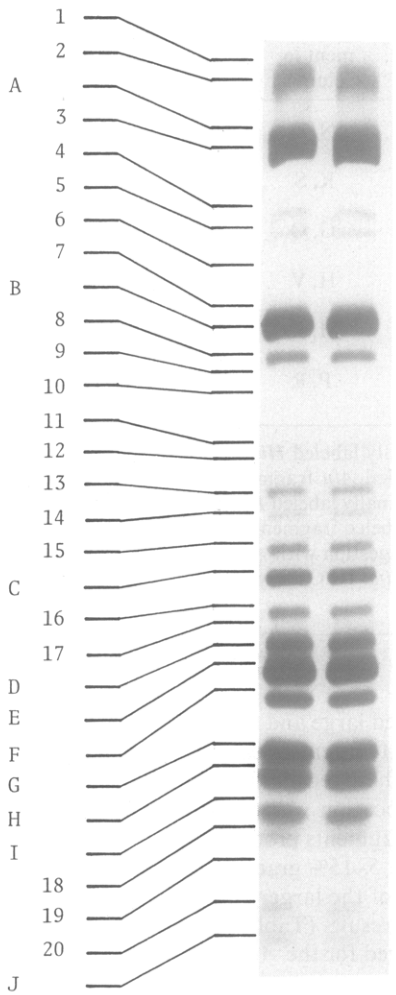

FIGURE 10: A 4% polyacrylamide gel separation of the partial SV40 DNA-*Alu* fragments generated by partial digestion of intact SV40 with *Alu*.

internal *Alu* fragments within each *Hind* III fragment, each uniformly ^{32}P-labeled *Hind* III fragment was partially digested with *Alu*. The partial *Hind* III-p*Alu* fragments were purified and isolated from polyacrylamide gel. Each partial *Hind* III-p*Alu* fragment was then exhaustively digested with *Alu* and the digests were electrophoresed on a 5–15% gradient polyacrylamide gel for comparison with the complete *Alu* digestion products. In this way, the *Alu* fragments generated from each *Hind* III-p*Alu* fragment can be identified to allow overlap and ordering of the *Alu* fragments. A summary of the overlapping sequences deduced from this method is represented in Figure 9.

TABLE V: The SV40 DNA-*Alu* Fragments Generated from the Partial SV40 DNA-*Alu* Fragments for Overlap.[a]

SV40-p*Alu* Fragments	Base Pairs	SV40-*Alu* Fragment Generated and the Sum of the Number of Base Pairs		
1	850	A, N	(848)	B, C (860)
4	640	C, K, K	(633)	
5	610	B, Q, S	(610)	
7	530	B	(515)	HKK (515)
8	490	C, K	(489)	
9	468	D, J	(443)	
10	450	F, M, R	(433)	
11	416	D, N	(413)	
12	402	F, M	(386)	G, L (381)
13	386	HK	(371)	
14	368	GN	(361)	ILW (342) or ILWX (363)
15	347	ETV	(341)	INV (324)
17	285	GR	(290)	JO (254)
18	200	IVW	(230) or	IVWX (251)
20	160	MR	(178)	

[a] The sum of the base pairs of the SV40 DNA-*Alu* fragments for each SV40 DNA-p*Alu* fragment is shown in brackets for comparison with the sizes of the corresponding SV40 DNA-p*Alu* fragments.

Partial Digestion of SV40 DNA with Alu for Overlapping Alu Fragments. Intact SV40 form I DNA was partially digested with *Alu* and the digest was fractionated on a 4% gel (Figure 10). Each partial *Alu* fragment was then completely digested with *Alu* and compared with the *Alu* fragments generated from intact SV40 DNA, shown in Figure 11. Results from the redigestion patterns on Figure 11 are summarized in Figure 12. Note that a few of the p*Alu* bands (Figure 11) in fact contain two partial products. However, on exhaustive digestion with *Alu*, they gave two series of *Alu* bands of distinctly

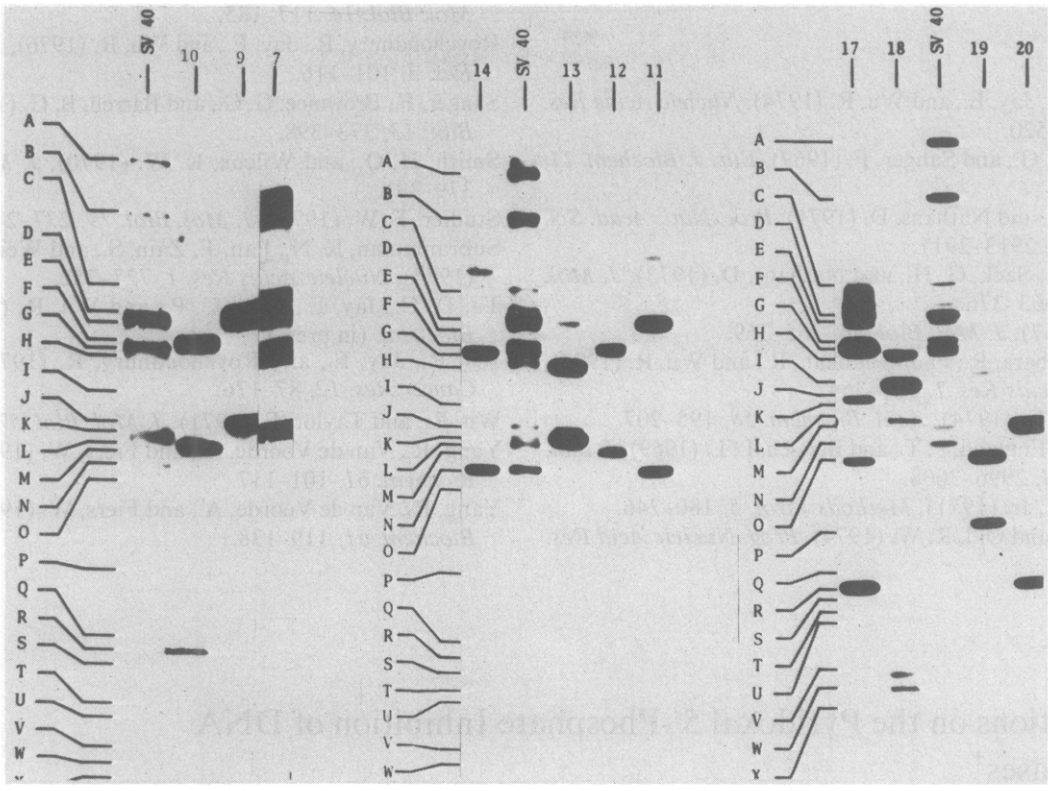

FIGURE 11: A 5–15% polyacrylamide gel separation of the SV40 DNA–*Alu* fragments produced from the p*Alu* fragments isolated from polyacrylamide gel shown in Figure 10 and exhaustively digested with *Alu*. The lanes labeled as SV40 represent complete SV40 DNA–*Alu* digest used as markers.

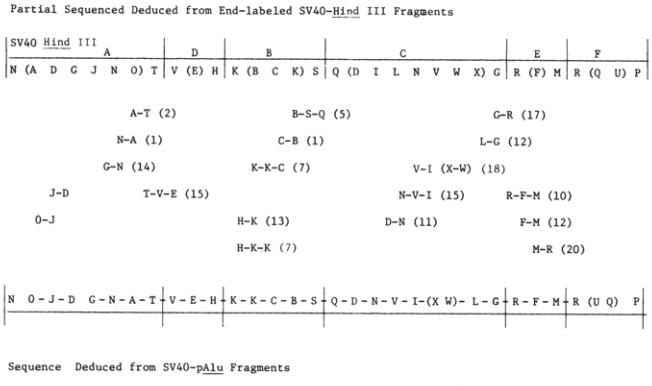

FIGURE 12: Overlapping the SV40 DNA–*Alu* fragments generated from the SV40 DNA–p*Alu* fragments in Figure 10 to produce a partial *Alu* cleavage map. The numbers in brackets refer to the p*Alu* bands in Figures 10 and 11.

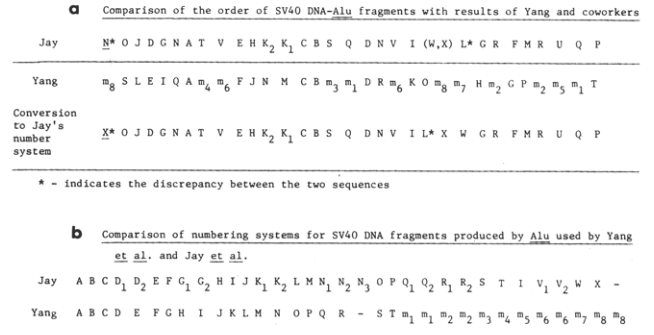

FIGURE 13: (a) A comparison of the order deduced for the SV40 DNA–*Alu* fragments with results independently obtained by Yang et al. (1976b). (b) A comparison of the numbering system for the SV40 DNA–*Alu* fragments used by Yang et al. (1976b) and by us.

different intensity. In addition, they are further checked by comparing the sizes of the partial p*Alu* fragments with the sum of sizes of its complete *Alu* digestion products as indicated in Table V.

From the results of these two partial digestion patterns of each *Hind* III and intact SV40 DNA for overlapping the *Alu* fragments, the order for the 32 SV40 DNA–*Alu* fragments can be deduced as represented in Figure 13a. Other supporting evidence (not shown here) was also obtained by digesting SV40 DNA with *Hind*, and then exhaustively digesting the largest 11 of the 13 *Hind* fragments with *Alu* to identify the *Alu* fragments generated. Useful information (not shown) which allowed some preliminary insight into the orders of the *Alu* fragments within each *Hind* III fragment was obtained by partially digesting each terminally labeled *Hind* III–*Hae* fragment and determining the sizes of these terminally labeled partial fragments. Knowing the sizes of the *Alu* fragments in each *Hind* III fragment, the orders of the *Alu* fragments in the *Hind* III D, E, and F and some in fragments A, B, and C can be deduced.

After completion of this work in our own laboratory, in August 1975, Yang et al. (1976b) communicated to us their results for the order of the 32 fragments obtained with a completely different approach. Figure 13b (lower panel) shows the comparison of the two numbering systems used for the 32 fragments. The orders are in complete agreement except for the beginning of the *Hind* III A fragment next to the *Hind* F fragment and the location of fragment L. These minor discrepancies could be due to a difference in the origin of the SV40 used for the investigation.

Acknowledgment

We thank William Broaddus for assistance in part of the work.

References

Bambara, R., Jay, E., and Wu, R. (1974), *Nucleic Acids Res. 1,* 1503–1520.
Brownlee, G. G., and Sanger, F. (1969), *Eur. J. Biochem. 11,* 395–399.
Danna, K. J., and Nathans, D. (1971), *Proc. Natl. Acad. Sci. U.S.A. 68,* 2913–2917.
Danna, K. J., Sack, G. H., and Nathans, D. (1973), *J. Mol. Biol. 78,* 363–376.
Hirt, B. (1967), *J. Mol. Biol. 26,* 365–369.
Jay, E., Bambara, R., Padmanabhan, R., and Wu, R. (1974), *Nucleic Acids Res. 1,* 331–354.
Jeppesen, P. G. (1974), *Anal. Biochem. 58,* 195–207.
Jovin, T. M., Englund, P. T., and Bertsch, L. L. (1969), *J. Biol. Chem. 244,* 2996–3008.
Maizel, J. V., Jr. (1971), *Methods Virol. 5,* 180–246.
Murray, K., and Old, R. W. (1974), *Prog. Nucleic Acid Res. Mol. Biol. 14,* 117–185.
Roychoudhury, R., Jay, E., and Wu, R. (1976), *Nucleic Acids Res. 3,* 101–116.
Sanger, F., Brownlee, G. G., and Barrell, B. G. (1965), *J. Mol. Biol. 13,* 373–398.
Smith, H. O., and Wilcox, K. W. (1970), *J. Mol. Biol. 51,* 379–391.
Studier, F. W. (1973), *J. Mol. Biol. 79,* 237–248.
Subramanian, K. N., Pan, J., Zain, S., and Weissman, S. M. (1974), *Nucleic Acids Res. 1,* 727–752.
Tu, D. C., Jay, E., Bahl, C. P., and Wu, R. (1976), *Anal. Biochem.* (in press).
Wu, R., Jay, E., and Roychoudhury, R. (1976), *Methods Cancer Res. 12,* 87–176.
Wu, R., and Taylor, E. (1971), *J. Mol. Biol. 57,* 491–511.
Yang, R., Van de Voorde, A., and Fiers, W. (1976a), *Eur. J. Biochem. 61,* 101–117.
Yang, R., Van de Voorde, A., and Fiers, W. (1976b), *Eur. J. Biochem. 61,* 119–138.

Observations on the Pyridoxal 5′-Phosphate Inhibition of DNA Polymerases†

Mukund J. Modak

ABSTRACT: Pyridoxal 5′-phosphate at concentrations >0.5 mM inhibits polymerization of deoxynucleoside triphosphate catalyzed by a variety of DNA polymerases. The requirement for a phosphate as well as aldehyde moiety of pyridoxal phosphate for inhibition to occur is clearly shown by the fact that neither pyridoxal nor pyridoxamine phosphate are effective inhibitors. Since the addition of nonenzyme protein or increasing the amount of template primer exerted no protective effect, there appears to be specific affinity between pyridoxal phosphate and polymerase protein. The deoxynucleoside triphosphates, however, could reverse the inhibition. The binding of pyridoxal 5′-phosphate to enzyme appears to be mediated through classical Schiff base formation between the pyridoxal phosphate and the free amino group(s) present at the active site of the polymerase protein. Kinetic studies indicate that inhibition by pyridoxal phosphate is competitive with respect to substrate deoxynucleoside triphosphate(s).

DNA polymerases from a wide variety of sources, ranging from viruses to eukaryotes, appear to share several basic characteristics with respect to requirements and mechanism of polymerization (Kornberg, 1974; Loeb, 1974; Saxinger et al., 1975). However, even though catalytic activities, functions, and to some degree structure of several of these enzymes is known, the exact enzyme mechanisms involved in catalysis and the actual structure of the active site of these enzymes are poorly understood. We have found that a naturally occurring coenzyme pyridoxal 5′-phosphate is a specific competitive inhibitor that offers the possibility of labeling the active site. The effect of pyridoxal phosphate was discovered during our search for oncornaviral reverse transcriptase specific inhibitors. Earlier, we had observed that inorganic phosphate may specifically inhibit DNA synthesis catalyzed by variety of mammalian C-type viral reverse transcriptases (Modak and Marcus, submitted for publication) and hence the examination of several phosphate-containing compounds was carried out. However, the studies with pyridoxal phosphate indicated a lack of specificity toward a particular class of DNA-polymerizing enzymes, since DNA polymerases from both B- and C-type oncornaviruses, as well as prokaryotic and eukaryotic cell enzymes, are equally susceptible to inhibition. We now demonstrate that inhibition by pyridoxal phosphate is competitive with nucleotide substrates and is expressed via the formation of a Schiff base. This offers the prospect of labeling the active site of DNA polymerase and deciphering the geometry of the active site on these enzymes.

Materials and Methods

Materials. All radioactive deoxyribonucleoside triphosphates were obtained from Amersham Searle, Inc. Unlabeled triphosphates and template primers were products of P. L. Biochemicals, Inc. The molar ratio of template to primer was 1:1 in the case of poly(rA)·$(dT)_{10}$ and 10:1 in the case of poly(rC)·$(dG)_{12-18}$ and poly(dC)·$(dG)_{12-18}$. Activated DNA was

† From the Memorial Sloan-Kettering Cancer Center, New York, New York 10021. *Received March 8, 1976.* This research is supported by National Cancer Institute Grant CA 08748.