Proc. Nat. Acad. Sci. USA
Vol. 68, No. 12, pp. 2913–2917, December 1971

Specific Cleavage of Simian Virus 40 DNA by Restriction Endonuclease of Hemophilus Influenzae*

(gel electrophoresis/electron microscopy/DNA mapping/DNA fragments/tumor virus)

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Communicated by Albert L. Lehninger, September 22, 1971

ABSTRACT A bacterial restriction endonuclease has been used to produce specific fragments of SV40 DNA. Digestion of DNA from plaque-purified stocks of SV40 with the restriction endonuclease from *Hemophilus influenzae* gave 11 fragments resolvable by polyacrylamide gel electrophoresis, eight of which were equimolar with the original DNA. The fragments ranged from about 6.5 × 10^5 to 7.4 × 10^4 daltons, as determined by electron microscopy, DNA content, or electrophoretic mobility.

The small oncogenic virus, SV40, contains double-stranded, covalently closed-circular DNA (1) of molecular weight about 3 × 10^6 (2). Because of the small size of its genome, and because of its ability to transform cells in culture to a state resembling that of cancer cells, SV40 is currently under intensive study. We have been interested in preparing unique linear fragments of SV40 DNA that might be used to establish reference points on this circular genome for the mapping of such functions as initiation of DNA replication and transcription, and for the localization of genes. Unique fragments might also be helpful in nucleotide sequence determination.

Our general strategy for obtaining specific fragments has been to cleave the DNA with bacterial restriction enzymes, a group of endonucleases that make double-strand breaks in DNA at specific sites (3), and to separate the products by gel electrophoresis. Although this group of enzymes has been studied primarily with natural DNA substrates, we have found that most of those tested also cleave SV40 DNA (4). In the present paper, we present our results with the restriction endonuclease from *Hemophilus influenzae* (5, 6).

MATERIALS AND METHODS

Cell Lines and Virus. Two lines of African green monkey kidney cells, CV-1 and BSC-1, were obtained from M. A. Martin and Microbiological Associates, respectively. They were grown in Eagle's minimal essential medium containing 10% fetal-bovine serum, penicillin, and streptomycin. Small-plaque SV40 (from strain 776), kindly supplied by K. K. Takemoto, was plaque purified on CV-1 cells and propagated in BSC-1 cells to obtain stocks of about 10^8 plaque-forming units (PFU)/ml.

Preparation of Radioactive SV40 DNA from Virions. Confluent BSC-1 monolayers in 100-mm plastic Petri dishes were infected with SV40 at a multiplicity of about 10 PFU/cell. For ^{32}P-labeling, 0.15 mCi of carrier-free [^{32}P]orthophosphate was added to each dish, at 24 hr after infection, in 6 ml of phosphate-free medium containing 4% dialyzed fetal-bovine serum. For labeling with [^{3}H]thymidine of high specific-activity the medium was made 10^{-4}M in FdU 24 hr after infection. 3 hr later, 6 ml of fresh medium, which contained 4% dialyzed serum, 50 μM FdU, and 0.1 mCi of [^{3}H]thymidine (7 Ci/mmol), was added. At about 90 hr, the cells and medium were frozen, the virus was purified, and DNA was extracted from the CsCl-purified, complete virus as described by Martin (7). After centrifugation in CsCl–ethidium bromide to isolate DNA I (8), the [^{32}P]DNA had a specific activity of 2 × 10^5 cpm/μg, and the [^{3}H]DNA, had a specific activity of 1.3 × 10^5 cpm/μg.

Isolation of SV40 DNA by the Hirt Procedure. Infection and labeling procedures were the same as for the preparation of virion DNA, except that FdU was generally omitted. Where [^{14}C]thymidine was used, 2 μCi per dish was added (44 Ci/mol). At about 60 hr after infection, the cells were lysed (9). The supernatant was extracted once with redistilled phenol [saturated with 0.1 M Tris (pH 8.0)], the aqueous solution was made 2% in potassium acetate (pH 6.0), and the DNA was precipitated with two volumes of ethyl alcohol at −20°C. The precipitate was dissolved in SSC-0.01 M Tris (pH 7.5), and the DNA was centrifuged to equilibrium in CsCl–ethidium bromide. Before phenol treatment, ^{32}P-labeled preparations were incubated for 1 hr at 25°C with 10 μg/ml of heated (10) ribonuclease A. Form I DNA was sedimented in a neutral 5–20% (w/v) sucrose gradient, and the fractions of the peak at 21 S were pooled and dialyzed. Unless stated otherwise, DNA used in all the experiments reported was prepared by this method.

H. influenzae Restriction Endonuclease. The enzyme was purified by the method of Smith and Wilcox (5), except that the phosphocellulose fraction was concentrated with Lyphogel (Gelman Instrument Co.) and stored at −20°C in 0.15 M KCl–0.02 M Tris (pH 7.5)–50% glycerol at 1.3 units/ml. The enzyme was tested for exonuclease activity by incubation of 0.1 μg of sonicated T7 [^{32}P]DNA (4 × 10^5 cpm/μg), a kind gift from C. A. Milcarek, with 0.01 unit of enzyme in 0.05 ml TMSH–50 mM NaCl for 90 min at 37°C. Less than 0.2% of the DNA was acid soluble; a control without enzyme gave

Abbreviations: SSC, 0.15 M NaCl–0.015 M sodium citrate; TMSH, 6.6 mM Tris·HCl (pH 7.45)–6.6 mM $MgCl_2$–6.6 mM 2-mercaptoethanol.

* This is paper no. 1 in a series: "Studies of SV40 DNA."

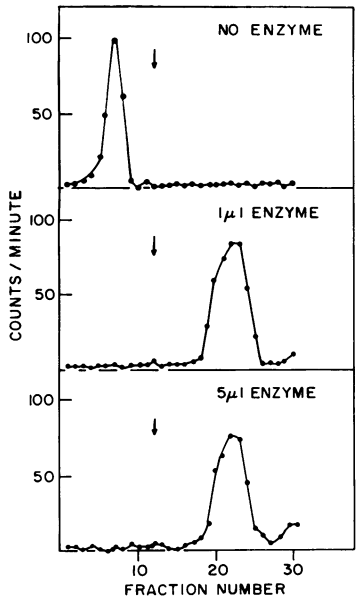

FIG. 1. Sucrose-gradient sedimentation of SV40 DNA digested with *H. influenzae* restriction endonuclease. 27 ng of SV40 [^{3}H]DNA I (3.9×10^4 cpm/μg) was incubated at 37°C for 30 min in 50 μl of TMSH–40 mM NaCl, either with no enzyme, with 1 μl (0.016 units) of enzyme, or with 5 μl of enzyme. After termination of the reactions by the addition of EDTA, SV40 [^{14}C]DNA II (16 S) was added to each sample as a marker. Samples were then sedimented in 5-ml linear 5–20% sucrose gradients containing SSC–0.05 M Tris (pH 8.0), for 4 hr at 49,000 rpm and 4°C in an SW50 Spinco rotor. 10-drop fractions were collected into scintillation vials and the samples were counted in Triton X-100–toluene scintillation fluid. For each gradient, the position of the 16S marker is indicated by an *arrow*.

the same result. *H. influenzae* DNA and unlabeled T7 DNA, used in viscometry assays, were kindly supplied by H. O. Smith; P22 DNA was supplied by B. Weiss.

Polyacrylamide Gel Electrophoresis. Electrophoresis of DNA in 0.6 × 13 cm polyacrylamide gels was performed by the method of Loening (11), but with 0.2% sodium dodecyl sulfate included in the gels and buffer (12). Samples were electrophoresed at 25°C, at a constant voltage of 60 V. Under these conditions, bromphenol blue moved about 13 cm in 8 hr. To quantitate radiolabeled bands, the gels were frozen and sliced into 1.2-mm segments. Each segment was dissolved in 0.2 ml of 30% H_2O_2 (13) and counted with Triton X-100–toluene scintillation fluid. In some experiments, the radioautographic technique of Fairbanks *et al.* (14) was used to visualize radioactive DNA fragments; gels were fixed in 7.5% acetic acid at 0–4°C for 30 min before slicing. DNA was eluted from gel segments by two successive incubations in 0.3 ml of electrophoresis buffer for 24 hr at room temperature. Dodecyl sulfate and gel debris were removed by centrifugation, after chilling of the pooled eluants at 0°C for 1 hr in the presence of 1 M NaCl. The supernatant fluids were concentrated and dialyzed against 0.1 × SSC–0.03 M NaCl.

Electron Microscopy. Samples of purified DNA fragments, with open-circular SV40 DNA II added as a reference, were mounted as described by Davis *et al.* (15) on parlodion-coated, 200-mesh, copper grids, then stained with uranyl acetate or shadowed with Pt–Pd 80:20, and examined with an AEI EM6B microscope. Micrographs were projected to a final magnification of 3×10^5 and the DNA contour lengths were measured with a K & E map reader. Results are expressed as

$$\frac{\text{Length of linear DNA fragment}}{\text{Length of DNA II in the same field}} \times 100.$$

RESULTS

Digestion of SV40 DNA by restriction endonuclease of *H. influenzae*

An initial survey by Adler and Nathans (4) of the activity of bacterial restriction enzymes on closed-circular SV40 DNA (DNA I) indicated that the restriction endonuclease of *H. influenzae* reduced the sedimentation rate of the DNA from 21 S to about 7.5 S, as shown by centrifugation in neutral sucrose (Fig. 1). The product was heterogeneous, but there was no further change in its sedimentation profile with the addition of more enzyme. These results indicated that the restriction endonuclease made several double-strand breaks in SV40 DNA.

To resolve the products into component DNA fragments, the digest was electrophoresed in polyacrylamide gels of different acrylamide concentration. Gels with 5% acrylamide gave the best overall resolution. A typical result is illustrated in Fig. 2. As seen in the figure, when the gel was sliced and counted, nine distinct peaks were observed. Under conditions that would permit the detection of other, smaller fragments, none were found. No change in this pattern was observed as a result of sequential addition of more enzyme or prolongation of the incubation beyond the conditions given in the legend of Fig. 2. Since electrophoresis in polyacrylamide gel separates according to polynucleotide chain length, one would expect a correlation between electrophoretic mobility and the DNA content of each peak. However, as seen in Fig. 2, two of the peaks (at segments 39 and 45) contained more than the expected quantities of [^{32}P]DNA in relation to their electrophoretic mobility, which suggested

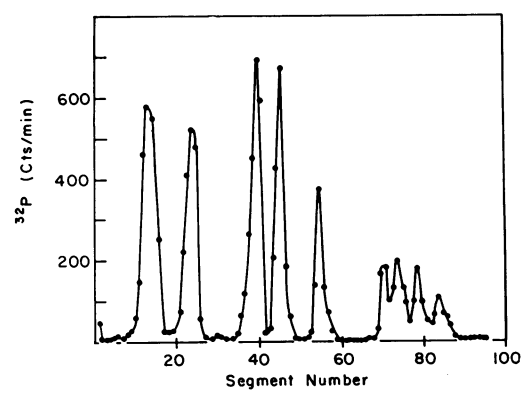

FIG. 2. Electrophoresis of SV40 DNA digested with *H. influenzae* restriction endonuclease. 2 μg of SV40 [^{32}P]DNA I (8200 cpm/μg) in 0.09 ml of TMSH–50 mM NaCl was digested at 35°C. Enzyme (0.003 units) was added at 0 time, at 2 hr, and at 3 hr. The reaction was terminated at 16 hr by the addition of EDTA to 0.01 M. After a 2.5-fold concentration by evaporation, the sample was made 1% in SDS and 10% in sucrose, incubated for 15 min at 35°C, and electrophoresed for 10.5 hr.

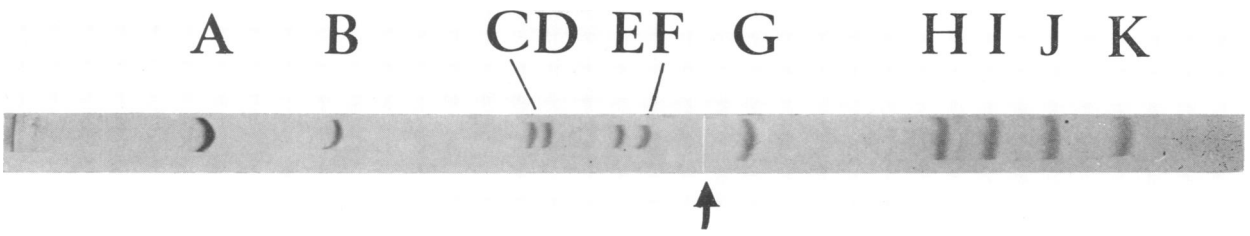

FIG. 3. Radioautographic analysis of SV40 DNA digested with *H. influenzae* restriction endonuclease. 1 μg of SV40 [^{14}C]-DNA I (3×10^4 cpm/μg) was digested (see Fig. 2) for 6 hr in a volume of 55 μl; 0.0015 unit of enzyme was added at 0 time and at 1, 2, 3, 4, and 5 hr. 20 μl of sample was electrophoresed for 12.3 hr and the radioautogram was prepared as described in *Methods*. The origin is at the *left*. The *arrow* below the radioautogram indicates a transverse cut made in the gel prior to slicing.

that they were heterogeneous. Each of these peaks was subsequently shown to consist of two fragments by radioautography (Fig. 3). As can be seen in Fig. 3, there are eleven resolvable fragments of DNA, labeled *A* through *K*, after exhaustive digestion with the endonuclease.

A comparison of different DNA preparations

Smith and Wilcox (5) found an average of one double-strand break per 1000 base pairs of T7 DNA with the *H. influenzae* restriction endonuclease. Since our results with SV40 DNA suggested that about 11 susceptible sites were present in this DNA, which contains about 5000 base pairs, we wondered whether the starting DNA was heterogeneous. Such heterogeneity might be due to the use of the Hirt procedure to prepare SV40 DNA, or due to heterogeneity of the virus population. We therefore compared a digest of SV40 DNA I prepared by the Hirt procedure, and purified as described in *Methods*, with a digest of DNA I extracted from virions that banded in the position of complete particles in CsCl. No difference was detectable by electrophoresis and radioautography. To investigate the possibility that the virus stock used to make labeled DNA was grossly heterogeneous, we prepared labeled SV40 DNA I from cells infected with fresh virus stocks made from each of three individual plaques. In all cases, the electrophoretic profiles of DNA digests were identical to that obtained with DNA prepared from the original virus stock. Neither of these experiments, therefore, gave evidence for heterogeneity of the DNA preparations.

Molecular weights of DNA fragments

The molecular weights of the larger DNA fragments were determined by measurement of their lengths, relative to that of open circular SV40 DNA (DNA II) on the same grid, by electron microscopy. In addition, molecular weight estimates were made on the basis of the percentage of total DNA (i.e., radioactivity) present in each fragment, and on the basis of sedimentation in neutral sucrose gradients.

Electron microscopy and length measurements of an un-

TABLE 1. *Molecular weights of SV40 DNA fragments produced by cleavage with* H. influenzae *restriction endonuclease*

	Electron microscopy		Distribution of label			Sedimentation analysis	
Product	% length ± 1 SD	Molecular weight ($\times 10^{-5}$)	%	Molecular weight ($\times 10^{-5}$)	s	Molecular weight $\left[\frac{S2}{S1} = \left(\frac{M2}{M1}\right)^{0.255}\right]$ ($\times 10^{-5}$)	Molecular weight $\left[\frac{S2}{S1} = \left(\frac{M2}{M1}\right)^{0.35}\right]$ ($\times 10^{-5}$)
A	21.8 ± 1.6	6.5	24	7.2	10.1	6.1	9.4
					9.8		
					9.7		
					9.4		
B	13.9 ± 1.4	4.2	18	5.4	9.2	4.6	7.5
					8.9		
					8.9		
C	10.6 ± 0.7*	3.2*	10.5*	3.2*			
D	10.6 ± 0.7*	3.2*	10.5*	3.2*	8.2	3.2	5.9
					8.2		
E	7.7 ± 1.4	2.3	7.5†	2.3†	7.6	2.4	4.7
F			7.5†	2.3†			
G			7	2.1	7.3	2.0	4.2
H			3.9	1.2	7.0	1.7	3.6
I			5.3	1.0‡			
J			4.1	0.87‡			
K			3.6	0.74‡			

* These values were obtained with a mixture of *C* and *D*. Percent distribution of label was divided by 2.

† These values were obtained with a mixture of *E* and *F*. Percent distribution of label was divided by 2.

‡ Molecular weights were estimated from mobilities of the products in a 5% polyacrylamide gel, with *A* through *H* as standards (see Fig. 5).

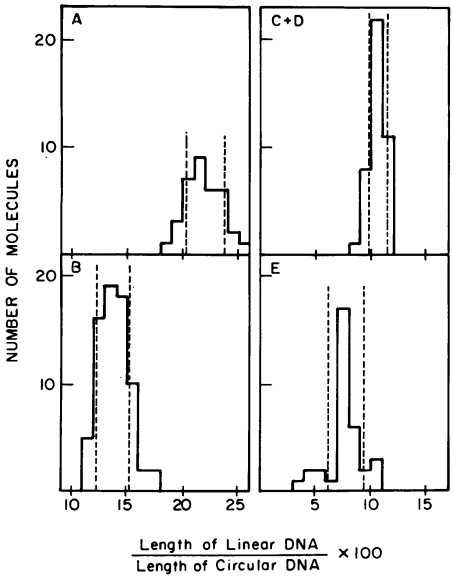

FIG. 4. Histograms of lengths of digestion products A, B, $C + D$, and E, expressed as the percentage of the length of SV40 DNA II on the same grid. *Vertical dotted lines* indicate ± 1 SD from the mean.

fractionated digest of SV40 DNA gave discrete length distributions for fragments corresponding to A through $E+F$, but not for fragments smaller than F. The lengths of fragments A, B, $C+D$, and E were then measured on purified preparations; length distributions are shown in Fig. 4, and the mean values and molecular weights derived are presented in Table 1. Table 1 also indicates the percentage of the total DNA present in each peak. A comparison of the molecular weights of fragments A through E determined by length measurements and those estimated by percentage of total

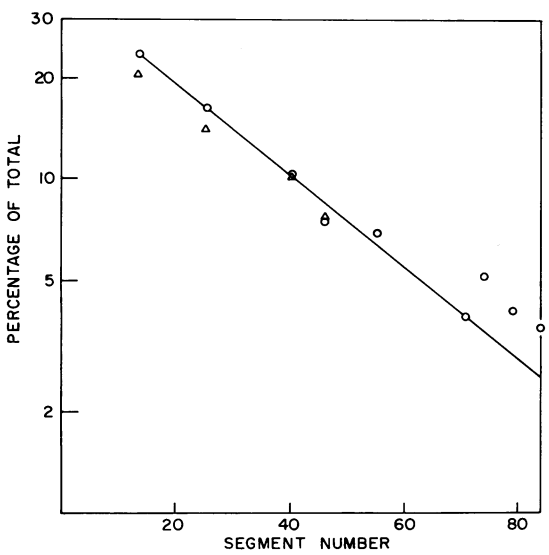

FIG. 5. Relationship between size and electrophoretic mobility for SV40 DNA fragments produced by cleavage with *H. influenzae* restriction endouncelease. —O—, percentage of total radioactivity in each peak of the electropherogram of Fig. 2. —Δ—, percentage by length as determined by electron microsocpy of purified fragments. The points representing radioactivity of $C + D$ and $E + F$ were each divided by two.

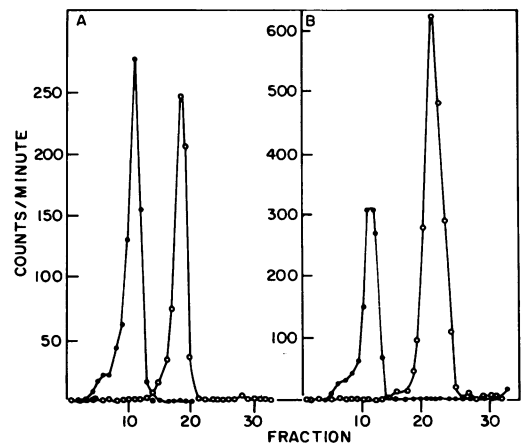

FIG. 6. Sucrose-gradient sedimentation analysis of purified digestion products A and D. 80-μl samples of purified products eluted from gels were sedimented for 4.5 hr in 5–20% neutral sucrose gradients a described in the legend of Fig. 1. Each contained SV40 [^{3}H]DNA III B (14.5 S) as marker. (A) 0.01 μg of ^{32}P-labeled fragment A (1×10^5 cpm/μg). (B) 0.02 μg of ^{32}P-labeled fragment D (1×10^5 cpm/μg). —●—, ^{3}H; —O—, ^{32}P. The linearity of the gradients was verified by making gradients in which bromophenol blue was included in the 20% sucrose stock solution and measuring the concentration of dye in each fraction. Under the same conditions of centrifugation, DNA III B sedimented at 14.5 S, compared to 16 S for DNA II.

DNA in a given fragment shows generally good agreement, from which we conclude that every molecule of SV40 DNA yields one of each of these fragments.

The molecular weights of those fragments that appear to be too small to measure accurately by electron microscopy can be deduced from the relationship between molecular weight and electrophoretic mobility. As shown in Fig. 5, the logarithm of the mass (expressed as percentage of the total DNA in a fragment) is inversely proportional to the mobility for those fragments shown to be present in unit amount. From this relationship, we have estimated the molecular weights of fragments G through K, as indicated in Table 1. A comparison of these values with the percentage of total DNA in each fragment (seen graphically in Fig. 5) suggests that fragments G and H are also equimolar with the original SV40 DNA, whereas fragments I, J, and K are not.

Sedimentation rates and calculated molecular weights for DNA fragments

Originally, we attempted to calculate the molecular weights of DNA fragments by the sucrose gradient method of Burgi and Hershey (16), using as a reference linear molecules of SV40 DNA (DNA III B) prepared by cleavage of DNA I with *Escherichia coli* B restriction endonuclease (ref. 4, and unpublished observations). DNA III B is equal in length to open-circular DNA II (Adler and Nathans, unpublished observations) and sediments at 14.5 S in 5–20% (w/v) sucrose gradients, when 16 S is used for the value of DNA II and 21 S for the value of DNA I (2). Fig. 6 shows typical sucrose gradient patterns of specific fragments mixed with DNA III B, and all the sedimentation data are collected in Table 1.

When the s values were used to calculate molecular weights by the formula of Burgi and Hershey, or the modification

suggested by Freifelder (17), the results were uniformly high in comparison with the values derived from length measurements or DNA content (Table 1). When the exponent relating the ratio of sedimentation rates to the ratio of molecular weights was changed to a value of 0.255, as indicated in Table 1, rather good agreement was obtained for nearly all the fragments. It appears, therefore, that Hershey's original formula should be further modified for DNA molecules of less than 1×10^6 in molecular weight.

DISCUSSION

The availability of pieces of SV40 DNA from specific sites in the molecule should be helpful for analysis of the function of the SV40 genome. For example, when the order of fragments in the genome is known, it should be possible to map "early" and "late" genes and those genes that function in all transformed cells. It may also be possible to localize specific genes by testing for biological activity, e.g., T-antigen production or abortive transformation. If specific deletion mutants become available, the analysis of restriction enzyme digests may supplement denaturation mapping of such mutants. Comparison of restriction endonuclease digests by polyacrylamide gel electrophoresis has also provided a new method for detecting differences in DNA. By this means, we have found that the DNA of small-plaque, large-plaque, and minute-plaque SV40 strains show specific differences in the mobility of particular DNA fragments (unpublished observations).

Restriction endonuclease from *H. influenzae* has been shown by Kelly and Smith (6) to cleave double-stranded DNA at specific hexanucleotide sequences. Although the nucleotide sequence specificity of other enzymes of this type has not been determined, on the basis of their biologic and enzymatic functions (3, 18–20), many appear to have even greater specificity than does the *H. influenzae* enzyme. Therefore, the restriction endonuclease should prove of general usefulness in the analysis of DNA, much as highly specific proteolytic enzymes have been used in the analysis of proteins. We have found, for example, that polyoma DNA is also cleaved by most of the restriction endonucleases tested (Adler and Nathans, unpublished observations). Since several restriction enzymes have been described, and a general method is available to detect new enzymes of this type (5), it should be possible to produce sets of overlapping fragments and, by appropriate sequential digestion, to obtain quite small, specific fragments useful for the determination of nucleotide sequence.

We are grateful to our colleagues, H. O. Smith, T. J. Kelly, M. A. Martin, and S. P. Adler for their generous help. This research was supported by National Cancer Institute grant CA 11895 and by an institutional grant from the USPHS. Kathleen Danna is a predoctoral fellow of the USPHS.

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