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Analysis of Simian Virus 40 DNA with the Restriction Enzyme of *Haemophilus aegyptius*, Endonuclease Z

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Limited digestion of simian virus 40 (SV40) DNA from both small- and large-plaque strains with the restriction endonuclease Z from *Haemophilus aegyptius* yielded 10 specific fragments. The number of nucleotide pairs for each fragment, determined by co-electrophoresis with ΦX174 RF fragments produced by endonuclease Z, ranges from 2,050 to 80. The difference in the pattern between the large- and small-plaque strains is the disappearance of one fragment containing approximately 255 nucleotide pairs and the appearance of a new fragment with 145 nucleotide pairs. This finding can be explained either by deletions or insertions totaling 110 nucleotide pairs. Complementary RNA synthesized in vitro from the adeno-SV40 hybrid virus, strain ND-1, hybridized preferentially to four of the fragments of SV40 DNA.

The genetic map of simian virus 40 (SV40) has not been delineated. To relate the steps in viral oncogenesis to the gene functions of SV40 the definition of at least a physical map of the SV40 genome would be helpful. Danna and Nathans (4) used a restriction enzyme from *Haemophilus influenzae* endonuclease R (23) to generate a set of specific SV40 DNA fragments to construct such a map. However, in the strategy for the use of such fragments, an additional restriction enzyme would be of value because it could localize more reference points and complement the results with the first enzyme. Also, smaller fragments produced by double enzyme digestion would be more amenable to DNA sequencing studies.

We describe here the action of a new restriction enzyme, endonuclease Z, isolated from *H. aegyptius* by Middleton et al. (14), on the DNA of small- and large-plaque strains of SV40. Also, we make a preliminary characterization of the SV40 DNA fragments by hybridization with complementary RNA (cRNA) synthesized from the DNA of the adeno-SV40 hybrid viruses, Ad2$^+$ ND-1 and E46$^+$.

MATERIALS AND METHODS

Cell lines and virus strains. SV40 small- (9) and large-plaque strains (19) were plaque purified three times in MA-134, an African green monkey kidney cell line, and propagated either in MA-134 or in primary monkey kidney (AGMK) cells (6) E46$^+$ virus, originally from W. P. Rowe, was propagated in AGMK cells. Ad2$^+$ ND-1, obtained from A. Lewis, was grown in human embryonic kidney cells; for preparation of ND-1 DNA, the virus was propagated in HeLa cells. All cell cultures were grown in Eagle minimal essential medium containing 10% fetal bovine serum.

Labeling of viral DNA. Three microcuries of ^{3}H-thymidine (44.9 Ci/mmol; New England Nuclear) or 0.05 μCi of ^{14}C-thymidine (55.7 mCi/mmol; International Chemical and Nuclear) per ml was added 12 h after infection. For ^{32}P labeling, the medium was changed to phosphate-free medium containing 2% dialyzed fetal calf serum with 10^{-5} M Na_2HPO_4 at 12 h after infection, and 20 μCi of carrier-free ^{32}P-orthophosphate per ml was added.

Purification of labeled SV40 DNA. SV40 was purified by the polyethylene glycol precipitation method, the DNA was extracted with sodium dodecyl sulfate (SDS) and phenol, and DNA component I was separated from component II with ethidium bromide (100 μg/ml) and equilibrium density centrifugation in CsCl as described before (20). Viral DNA was also extracted from cells infected for 48 h as described by Hirt (10). The DNA was extracted twice with phenol, precipitated with salt-saturated alcohol at −20 C, and then dissolved in 0.01 M Tris-hydrochloride (pH 8.0) with 0.01 M EDTA, mixed with ethidium bromide and centrifuged to equilibrium in CsCl (density, 1.56 g/cm^3). The SV40 DNA component I was further sedimented in a neutral 5 to 20% sucrose gradient in Tris-buffered 0.15 M saline solution (TBS). The specific activities of ^{32}P DNA from the small- and large-plaque strains were 4.1×10^5 and 4.0×10^5 counts per min per μg, respectively.

Restriction endonuclease Z. The enzyme

(14) was purified from *H. aegyptius* ATCC 11116 (American Type Culture Collection) by the method of Smith and Wilcox (23), except for the following modifications. (i) The ammonium sulfate differential precipitation preceded the Biogel chromatography step; (ii) glycerol was added to the buffers at a final concentration of 5%. Sonically treated ^{3}H-thymidine-labeled MA-134 DNA (1.8×10^4 counts per min per μg) was used for testing the exonuclease activity of the purified enzyme. Twenty microliters of enzyme (1 unit/ml) and 1 μg of MA-134 DNA in 0.1 ml of TBS, with 0.01 M $MgCl_2$ and 0.005 M β-mercaptoethanol (β-ME), were mixed and incubated at 37 C for 15 h. There was no obvious acid-soluble radioactivity; the result was the same in the control without enzyme.

Enzymatic fragmentation of SV40 DNA. Five microliters of enzyme, 5 μliters of 0.13 M $MgCl_2$, and 5 μliters of 0.05 M β-ME were added to 50 μliters of ^{32}P- or ^{14}C-labeled SV40 DNA in TBS. The digestion was carried out at 37 C for 14 h; then 5 μliters more of the enzyme was added, and the DNA was incubated for 2 more h to assure complete digestion. Twenty microliters of 60% sucrose with 0.5% bromophenol blue and 0.003 M EDTA were added to the mixture before electrophoresis.

Electrophoresis and autoradiography. Three percent, 5%, and 7% acrylamide gels (with T, 20:1) were used for electromobility studies. Agarose at a final concentration of 0.5% was used as a matrix only in the 3% gels. The gels were prepared in Lucite tubes with slots (0.2 by 1.0 by 17.0 cm) as described before (5). The E buffer (1, 5) was used both in the gels and as electrode buffer. Electrophoresis was carried out with 3 mA per tube at about 110 V at room temperature for 5.5 h in 3% gels, and for 12 to 15 h in 5% and 7% gels. Bromophenol blue was used as a tracking dye.

The gels were dried on filter paper with vacuum and heat radiation. Kodak X-ray film (RP/S X-O-Mat) was used for the autoradiographs. The exposure times varied from several hours to several days, depending on the radioactivity of the sample. For ^{14}C-thymidine-labeled DNA, 1 to 2 weeks is required; for ^{32}P-DNA, exposure overnight is enough.

The dried gels were cut into slices 1-mm wide for determination of radioactivity after autoradiography without solubilization.

Recovery of DNA fragments for hybridization. To obtain pure DNA fragments for hybridization studies, the enzymatically digested DNA was applied to long disk gels (35-cm long by 0.6-cm diameter). The electrophoresis was carried out with 5 mA per tube for 12 h. The gels were then fractionated in 0.1 × SSC (0.15 M NaCl plus 0.015 M sodium citrate) with a Maizel Autogeldivider (Savant). Samples were taken for counting the radioactivity. The crushed gel was removed from the solutions of DNA fragments by centrifugation at 3,000 rpm.

Synthesis of SV40, ND-1, and E46$^+$ cRNA. The cRNA was synthesized as described previously (11) with purified *Escherichia coli* DNA-dependent RNA polymerase prepared as described by Burgess (2).

DNA-RNA hybridization. The viral DNA fragments eluted from the gel were diluted in 0.1 × SSC and denatured by heating at 100 C for 15 min. After rapid chilling in an ice bath, the DNA solution was adjusted to 6 × SSC and immobilized on a membrane filter (0.22-μm pore size; Millipore Corp). The DNA-cRNA hybridization was carried out by the method of Gillespie and Spiegelman (7). The DNA filters were immersed in 1 ml of 6 × SSC containing ND-1 or E46$^+$ ^{3}H cRNA, 3.0×10^5 to 7×10^5 counts per min per filter (8×10^6 counts per min per μg), 1 mg of yeast RNA, and 0.1% SDS. The hybridization was carried out at 66 C for 20 h.

RESULTS

Number of SV40 fragments produced by endonuclease Z digestion. The sensitivity of SV40 DNA to digestion by endonuclease Z was first determined by sedimentation analysis in a neutral sucrose gradient; both form I and II were reduced to a 6 to 9 *S* product. Figure 1 shows autoradiograms of ^{32}P-labeled SV40 DNA, digested with endonuclease Z and electrophoresed in acrylamide gels of different porosity. The DNA of the large-plaque strain of SV40 yielded eight distinct bands (ZA through H) in 3% gels (Fig. 1, gel 3). Band ZE is more intense than expected for a band containing a single fragment. Band ZF is resolved as a doublet (ZF_1 and ZF_2) that can be seen more clearly in the 5% gel. Bands ZA and ZB were not resolved in 7% gels.

The autoradiograms of small-plaque SV40 DNA digested with endonuclease Z are also shown in Fig. 1. The pattern of bands is similar to the large-plaque strain; bands ZA to ZH are all found in the small-plaque strain. There are two apparent differences: band ZE is not as intense in the small-plaque strain as it is in the large-plaque strain, and there is an extra band (ZX) between bands ZF and ZG. Band ZF in the small-plaque strain is also resolved as a doublet in 5%, 7%, and even in 3% gels. The new band, ZX, seems to show heterogeneity in the 7% gel.

The relative amount of the labeled DNA in each band in 3% gels (Fig. 1, gels 3 and 4) was determined by cutting the dried and autoradiographed gel into 1-mm segments and counting in toluene scintillation fluid. Figure 2 depicts graphically the radioactivity in the bands from both the small-plaque (A) and large-plaque (B) strains. The results confirm the impression from the autoradiographs that band ZE has relatively higher counts in the large-plaque strain than in the small-plaque strain, and also that the small-

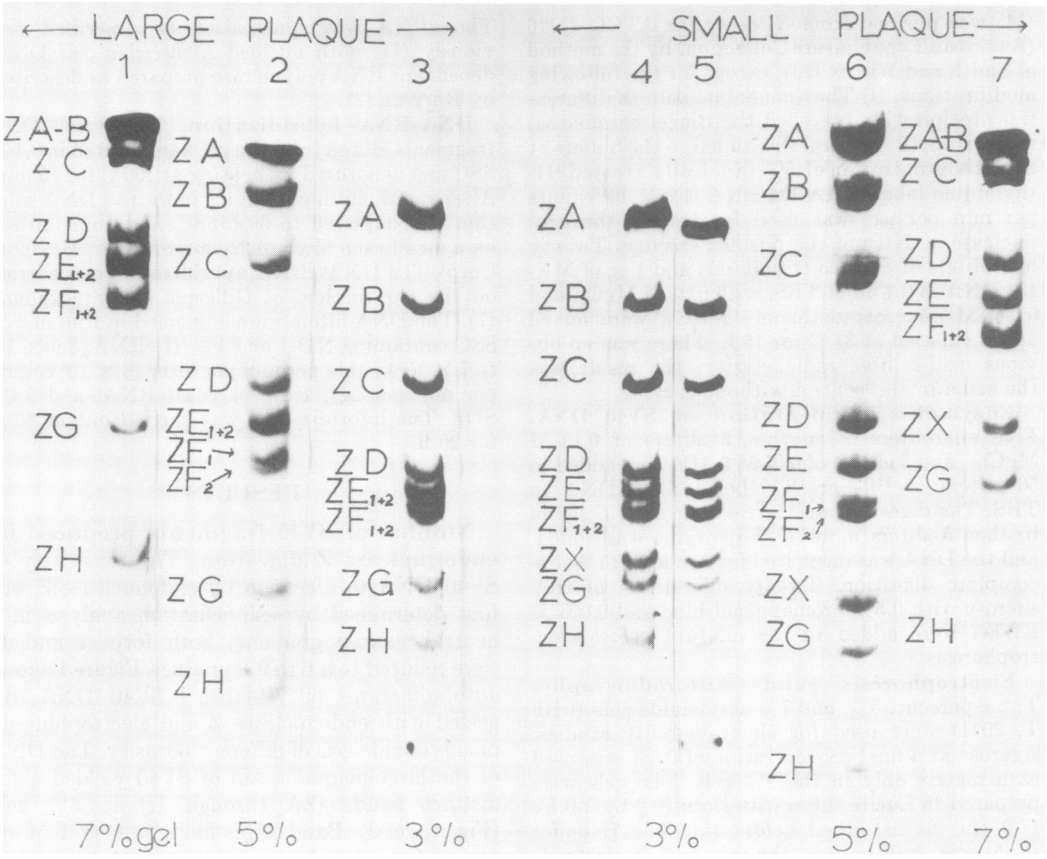

FIG. 1. *Autoradiograms of acrylamide gel pattern of SV40 DNA digested with endonuclease Z. ^{32}P-labeled large-plaque (gels 1 to 3) and small-plaque (gels 4 to 7) SV40 DNA was digested with the enzyme and electrophoresed in various concentrations of gels. Migration is from top to bottom. Only 3% gels were subjected to electrophoresis long enough for the tracking edge to migrate 15.5 cm to the bottom (about 5.5 h). The time of electrophoresis in the 5 and 7% gels varied from 12 to 15 h. For comparison, 3% gels of the large- (gel 3) and the small-plaque strain (gels 4 and 5) are aligned. The spots at the bottom of the 3% gel indicate the position of the tracking dye.*

plaque strain has an extra band, ZX, not found in the large-plaque strain.

^{32}P-labeled DNA of the large-plaque strain and ^{3}H-thymidine-labeled DNA of the small plaque strain were co-digested with endonuclease Z and co-electrophoresed in 3% gels. The corresponding bands of the large- and small-plaque strains migrated at exactly identical positions except for the ZX band, which is not found in the large-plaque strain.

Sizes of the SV40 endonuclease Z fragments. The sizes of ΦX174 RF endonuclease Z fragments have been very well characterized (14). These fragments were used to estimate the sizes of the endonuclease Z fragments of SV40 DNA by co-electrophoresis in 3% gels. Fig. 3 shows the comparison of SV40 and ΦX174 RF endonuclease Z fragments in distances migrated during electrophoresis. The largest SV40 fragment (ZA) is estimated to contain approximately 2,050 nucleotide pairs, and the smallest fragment (ZH) about 80 (Table 1).

Hybridization of Ad2^{+} ND-1 and E46^{+} ^{3}H-cRNA to endonuclease Z fragments of SV40 DNA. Two naturally occurring specific fragments of SV40 DNA are available in the form of adenovirus-SV40 hybrid viruses. The Ad2^{+} ND-1 virus contains a nearly complete Ad2 genome and also 15.9% (22) or 18% (21) of the SV40 genome. The SV40 sequences present in ND-1 are considered to be predominantly early sequences since the RNA transcribed from the ND-1 hybrid virus during its own lytic cycle competes efficiently with early SV40 lytic RNA (18). However, a small portion of late SV40 sequences may also be present; a recent study

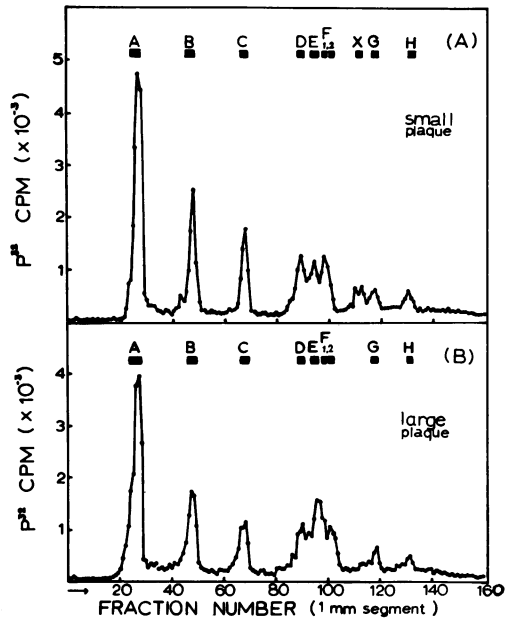

FIG. 2. *The radioactivity of the fragments of SV40 DNA generated with endonuclease Z. The 3% gels shown in Fig. 2, gel 3 and 4, were cut into 1-mm segments, and the radioactivity was counted in toluene scintillation fluid. A, Small-plaque strain, B, large-plaque strain. The bars at the top of each gel indicate the position of each band determined by autoradiograms. Migration is from left to right.*

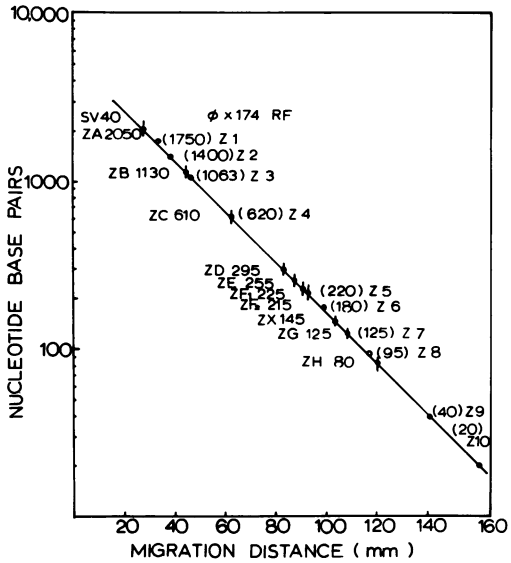

FIG. 3. *Comparison of the sizes of the SV40 and ΦX174 RF DNA fragments produced by endonuclease Z. Fifty microliters of ^{32}P-labeled ΦXRF DNA and 50 μliters of ^{3}H-thymidine small-plaque SV40 DNA I were mixed and digested with 20 μliters of endonuclease Z at 37 C for 24 h. After electrophoresis, the gel was dried and cut into 1-mm segments for counting. Reference lines of the sizes of the fragments versus mobility were plotted according to the migration distances and the number of nucleotide pairs in the ΦX174 RF endonuclease Z fragments (○). The sizes of SV 40 fragments (◆) were then determined by mapping the migration distance of these fragments on ΦX174 RF reference lines.*

reveals that the SV40 late mRNA species hybridize efficiently to ND-1 DNA (Weinberg, Ben-Ishai, and Newbold, submitted for publication). The second virus, E46^{+}, is a hybrid between Ad 7 and SV40 and contains 75% of the SV40 genome (12, 17). Clearly, the in vitro transcripts from the DNA of these two hybrid viruses represent specific subsets of the SV40 genome and are consequently useful probes for preliminary characterization of the SV40 endonuclease Z fragments.

Table 2 shows the results of DNA-RNA hybridization experiments. First, the control experiment with the cRNA transcribed from SV40 DNA component I indicates that each of the specific fragments (ZA to ZH) of small-plaque SV40 DNA yielded a positive hybridization test. The ratio of ^{3}H (cRNA) to ^{14}C (SV40 DNA fragment) in the hybrids is not constant for each of the different fragments, however, but varies by a factor of about 2. The ND-1 ^{3}H-cRNA appears preferentially hybridized to the fragments ZC, ZD, ZG, and ZH; with these fragments the hybridization ratios are significantly greater than the ratios for the other fragments.

These results suggest that ZC, ZD, ZG, and

TABLE 1. *Endonuclease Z fragments of Simian virus 40 DNA*

Electrophoresis band	Large-plaque strain		Small-plaque strain	
	No. of fragments	Estimated nucleotide pairs[a]	No. of fragments	Estimated nucleotide pairs[a]
Z A	1	2,050	1	2,050
Z B	1	1,130	1	1,130
Z C	1	610	1	610
Z D	1	295	1	295
Z E	2	255	1	255
Z F_1	1	225	1	225
F_2	1	215	1	215
Z X			1	145
Z G	1	120	1	120
Z H	1	80	1	80
Total	10	5,235	10	5,125

[a] Obtained from electrophoretic migration compared with migration of endonuclease Z fragments of ΦX174 RF.

TABLE 2. *Hybridization of ND-1 and E46+ ^{3}H-cRNA to endonuclease Z fragments of small-plaque SV 40DNA*[a]

SV40 DNA fragments on filters	ND-1 ^{3}H-cRNA				E46+ ^{3}H-cRNA				SV40 ^{3}H-cRNA	
	Expt 1[b]			Expt 2[c]	Expt 1[b]			Expt 2[c]	Expt 1[b]	Expt 2[c]
	ND-1 ^{3}H-cRNA hybridized (counts/min)	Amt of ^{14}C-DNA on filters (counts/min)	^{3}H/^{14}C	^{3}H/^{14}C	E46+ ^{3}H-cRNA hybridized (counts/min)	Amt of ^{14}C-DNA on filters (counts/min)	^{3}H/^{14}C	^{3}H/^{14}C	^{3}H/^{14}C	^{3}H/^{14}C
ZA	224	137	1.6	0.5	3,405	134	25.4	7.9	52.9	36.1
ZB	265	87	3.0	1.4	259	114	2.3	0.7	75.7	36.2
ZC	2,853	74	38.6	15.8	2,302	85	27.1	6.5	62.3	34.0
ZD	2,114	60	35.2	14.2	1,047	72	14.5	6.6	34.9	18.1
ZE	360	80	4.5	2.7	986	94	10.5	4.0	34.3	16.8
ZF_1[d]	594	75	7.9	5.4	156	38	4.1	4.2	46.8	30.5
ZF_2[d]	455	50	9.1	7.6	258	39	6.6	4.0	49.8	30.0
ZX	546	53	10.3	7.6	599	49	12.2	5.0	35.9	18.9
ZG	847	41	20.6	15.5	1,007	57	17.6	13.6	42.0	24.0
ZH	1,773	50	35.46	19.7	1,730	58	29.8	17.2	50.4	26.0

[a] Background of radioactive counts has been subtracted; in experiment 1, 147 counts/min for ^{3}H-cRNA and 15 for ^{14}C; in experiment 2, 80 for ^{3}H-cRNA and 15 for ^{14}C (average of three filters); with 0.01 μg of calf thymus DNA, there were 168 to 240 counts/min hybridized (without background subtraction).

[b] Input ^{3}H-cRNA for each filter was 7×10^5 counts/min (8×10^6 counts per min per μg); for SV40 ^{3}H-cRNA, the input was 3×10^5.

[c] Input ^{3}H-cRNA for each filter was 3×10^5 cpm; for SV40 ^{3}H-cRNA, the input was 10^5.

[d] Separation of fragments ZF_1 and ZF_2 is not very clear. The leading portion of the band is designated F_2, the trailing portion F_1.

ZH contain some of the early gene sequences of SV40. The sum of the nucleotide pairs in these fragments is 1,105, about 21% of the total genome. Of course, this portion of the SV40 genome does not contain all of the early genes.

The results of hybridization with 7×10^5 counts/min E46+ ^{3}H-cRNA show that the ratio of ^{3}H-cRNA to ^{14}C-DNA is higher in ZA, ZC, ZD, ZG, and ZH than in the others. The hybridization appeared to include fragments ZE and ZX also. The sum of the number of nucleotide pairs in ZA, ZC, ZD, ZG, ZH, ZE, and ZX is about 70% of the total genome, close to the portion of the SV40 genome contained in E46+ (12).

DISCUSSION

In acrylamide gel electrophoresis, DNA migrates inversely as the log of its molecular weight (1). If we assume that the ^{32}P label is evenly distributed throughout the DNA, then the ^{32}P counts in each band should be proportional to the molecular weight of the fragment in the bands containing a single fragment. In Fig. 4 from the plot of the log of ^{32}P counts in each band versus the fragment mobility, we find most of the points located on a straight line corresponding to the predicted line for molecular weight of single fragments versus mobility (reference 5, fragment number, $n = 1$). Bands ZF in both the small- (Fig. 4A) and large-plaque strains (Fig. 4B) fall on the line expected for bands containing two fragments ($n = 2$) as does band ZE of the large-plaque strain. Therefore, the number of the SV40 DNA fragments produced by endonuclease Z digestion is at least 10 in both the large- and small-plaque strains. The number of fragments generated with endonuclease R reported by Danna and Nathans (4) is 11.

From autoradiograms (Fig. 1) and the plot of mass versus migration distances (Fig. 4) we find two distinct differences between the small- and large-plaque strains. First, band ZE in the large-plaque strain contains two fragments very close in molecular weight (255 base pairs), whereas in the small-plaque strain it appears to contain only one fragment. Second, an extra band, ZX, with 145 nucleotide pairs, is found in the small-plaque strain. This band in the more concentrated gels (5% and 7%) appears somewhat hetero-

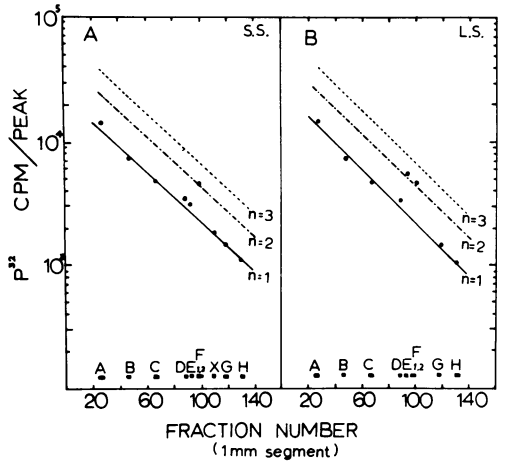

FIG. 4. *Mass determined by radioactivity in each band versus fragment mobility. Integrated radioactive counts of peaks in Fig. 3A and 3B were plotted against the migration distances; most of the points fall on the solid line* (——). *The parallel dashed lines* (----) *represent the predictions for the peaks with two or three times the number of fragments as those points falling on the solid line. A, Small-plaque strain; B, large-plaque strain.*

geneous, but, from the plot of the mass versus migration distance (Fig. 4A), band ZX is thought to contain a single fragment.

The two virus strains used in this study have distinct and independent identity; that is, neither strain was derived immediately from the other and, therefore, one cannot draw conclusions as to their origins and relations. However, the difference between the large- and small-plaque strain as revealed by this analysis can be stated in two ways that are in fact equivalent. A net deletion of 110 nucleotide pairs in one of the ZE fragments (255 base pairs) of the large-plaque DNA would transform the large-plaque fragment pattern into the small-plaque pattern, with the new band ZX (145 base pairs) being itself a specific fragment of one of the ZE fragments. Conversely and equivalently, a net insertion of 110 base pairs into the ZX fragment would produce the second ZE fragment seen only in the large-plaque strain. Experiments are under way to test if in fact there is sequence homology between ZX and the ZE fragments. This kind of variation in fragment patterns of different virus strains was also found by Nathans and Danna with endonuclease R (16).

Recently, Zeiger et al. (24) demonstrated that the mobility of DNA in acrylamide gels is dependent also in part on its base composition. DNA with high adenosine plus thymidine (AT) or low guanine plus cytosine (GC) content has lower electromobility than DNA of the same molecular weight with low AT and high GC content. If some of the endonuclease Z fragments of ΦXRF or SV40 have an unusual base distribution, then the estimation of the size of SV40 endonuclease Z fragments according to electromobility comparisons will vary somewhat. The precise size of these fragments has to be calibrated by electron microscopy and other physical methods. From a comparison of SV40 and ΦX174 RF fragments, the total nucleotide pairs of the SV40 large-plaque strain are 5,235. If we assume each nucleotide pair has a molecular weight of 600, then the total molecular weight for SV40 large-plaque strain DNA is about 3.14×10^6, close to a previous estimate of 3.2×10^6 (3).

The largest and the smallest DNA fragments generated with endonuclease R as described by Danna and Nathans (4) have molecular weights of 6.5×10^5 to 7.2×10^5 and 7.4×10^4: about 1,100 to 1,200 and 125 nucleotide pairs, respectively. The largest endonuclease Z fragment is larger than any of the endonuclease R fragments, and the smallest Z fragment is smaller than the R fragments.

The hybridization data with ND-1 and $E46^+$ ^{3}H-cRNA represent, of course, only a preliminary characterization of the endonuclease Z fragments. The results do demonstrate the specificity of the ^{3}H-cRNA probes and their potential usefulness for identifying and mapping fragments of SV40 DNA. They strongly suggest that fragments ZC, ZD, ZG, and ZH are present at least in part in the ND-1 genome; whereas, in addition to these, in $E46^+$ fragments ZA and perhaps also ZE and ZX are similarly represented.

The SV40 sequences present in ND-1 DNA are regarded as predominantly early in character and represent roughly half of the early gene sequences present in SV40 DNA. They are known to encode at least one early function, the U antigen (13). The $E46^+$ virus, however, contains the entire early SV40 genes, including the nuclear T antigen. Clearly, most of the SV40 sequences present in ND-1 must also be present in $E46^+$; the hybridization data demonstrate this point.

This analysis can be extended to suggest functional assignments to some of the endonuclease Z fragments, e.g., U antigen to fragments ZC, ZD, ZG, and ZH or some portion of them. Also, since the SV40 T antigen is not made in ND-1-infected cells (13), it might be assigned mainly to that portion of the early SV40 sequences present in $E46^+$ but not represented in ND-1, most likely in fragment ZA.

Analysis of SV40 DNA with other specific endonucleases is under way. We have found

that endonuclease R_1 (15) cleaves the ZB fragment (unpublished data, with C. Mulder). A detailed characterization of the cleavage is under investigation.

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