Restriction Enzyme Mapping of Vaccinia Virus DNA

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The cleavage sites for the restriction enzymes *Bgl*II, *Hind*III, *Kpn*I, *Sal*I, *Sma*I, and *Xho*I were located, from primary data, on the DNA isolated from the WR strain of vaccinia virus. *Bgl*II and *Sma*I divide the DNA into five segments which can be isolated by sucrose gradient centrifugation. These large segments provide a convenient means to group segments produced by other enzymes. The construction of physical maps was initiated by identifying the segments at each end of the DNA and then finding segments which were adjacent to these terminal sections. This was accomplished by isolating large shear fragments which contained the covalently linked termini of the DNA. Most of the data needed to derive the maps were obtained by isolating segments produced by one enzyme and then cleaving these individual segments with a second enzyme.

Many of the most widely used restriction enzymes can be expected to cleave the 122×10^6 -dalton DNA from vaccinia virus (strain WR) into a large number of segments. *Eco*RI enzyme cleaves the related rabbit poxvirus (strain Utrecht) DNA at about 50 sites (20). The construction of physical maps under these conditions is tedious. The location of cleavage sites for enzymes such as *Hind*III which generate a relatively small number of large segments is more readily achieved. However, ordering large segments has the disadvantage that old and new methods of analysis of partial digestion products (6, 22) cannot be used to a significant extent, since very large segments cannot be resolved on standard agarose gels. Wittek et al. (29) derived the *Hind*III and *Sst*I maps for rabbit poxvirus and vaccinia virus (strain Elstree) by analyzing overlapping segments. Then other workers (2, 13) used these maps to develop new maps by hybridizing their restriction segments to the DNA mapped by Wittek et al. (29).

This paper presents several maps of the WR strain of vaccinia virus DNA derived independently from those described above. The arrangement of map segments was determined mostly from data on the overlap of segments produced by two enzymes (15) and the hybridization of strands from segments produced by two enzymes. The *Sal*I map orders the 23 segments produced by that enzyme. *Bgl*II generates four segments which can be used to group the larger number of segments usually found in other digests. Digestion with *Sma*I, which cleaves the DNA at one site, and *Bgl*II divides the DNA into five large segments, all of which can be separated by sucrose gradient centrifugation. Data dem-

onstrating the usefulness of the *Bgl*I segments in defining the *Xho*I and *Kpn*I maps are presented.

The location of the *Hind*III and *Xho*I sites has been derived from hybridization data (13), so the results reported here include information from special techniques which help to determine vaccinia DNA maps. Since the linear vaccinia virus DNA is covalently closed at the ends (11), the DNA can be sheared, and fragments containing the end link can be selected (8). Fragments up to one-fourth of the intact DNA length can easily be generated and efficiently recovered after selection. Digests of a series of these fragments indicate which segments are closer to the termini of the DNA.

The ability to produce and recover large pieces of the vaccinia genome not only aids in the location of smaller segments but also should allow the entire viral DNA to be cloned in a small number of large hybrid plasmids (3-5).

MATERIALS AND METHODS

DNA. Vaccinia virus (strain WR) DNA was isolated from purified virions as previously described (8). In early experiments, DNA was isolated from virus particles after infection with uncloned virus, but in most cases, DNA was isolated from virus particles after infection with plaque-purified virus. Unless stated otherwise, the results are from DNA obtained under the latter conditions. DNA labeled with [14 C]thymidine was prepared as described (8) and contained 15,000 cpm/ μ g. DNA was stored at 4°C in K buffer (10 mM KCl, 1 mM sodium EDTA, 10 mM Tris-hydrochloride [pH 8.3]).

Enzyme digests. All concentrations are reported as the final concentration in the solution. Endonuclease *Sal*I was prepared from sporulating *Streptomyces albus* by standard procedures (7, 23) with additional

steps (H. Boyer, personal communication) or was purchased from Miles Laboratories, Inc., as was S1 nuclease. *Bgl*I was purchased from Bethesda Research Laboratories or from New England BioLabs. DNA polymerase I was from Boehringer Mannheim Corp. All other enzymes were from New England BioLabs.

All restriction digests contained 10 mM MgCl_2 , 10 mM Tris-hydrochloride, 100 μg of bovine serum albumin per ml, and 5 mM dithiothreitol. The salt concentration and pH used for individual enzymes were: *Bgl*I, 67 mM KCl (7.4); *Hind*III, 60 mM NaCl (7.4); *Kpn*I, 6 mM NaCl (7.5); *Sal*I, 140 mM NaCl (7.6); *Sma*I, 15 mM KCl (8.5); *Xho*I, 140 mM NaCl (7.4). Particular partial digestion products were produced by digesting 10 μg of DNA with 80 U of *Hind*III in the presence of 25 μg of actinomycin D (Boehringer Mannheim).

Restriction digests were for 4 h at 37°C. Reactions were terminated with 40 mM sodium EDTA and 0.5% sodium dodecyl sulfate. After phenol extraction, either the aqueous phase was dialyzed against K buffer or the DNA was ethanol precipitated and dissolved in K buffer. The DNA from the actinomycin-*Hind*III digest was extracted with phenol, and the aqueous phase was mixed with ether four times.

S1 nuclease digests were at 45°C for 30 min in solution N (250 mM NaCl, 3 mM ZnCl_2 , 30 mM sodium acetate [pH 4.5]), using 50 U (Miles Laboratories, Inc.) per μg of DNA. The reaction was terminated with sodium EDTA, and the DNA was precipitated and dissolved in K buffer.

Centrifugation. Linear gradients from 10 to 30% sucrose contained 1 M NaCl, 10 mM sodium EDTA, 20 mM Tris-hydrochloride, and 0.5% Sarkosyl NL-97 (Geigy, Inc.) (pH 8.3). Centrifugation was in an SW41 rotor (Beckman Instruments, Inc.) at 20°C. The four *Bgl*I segments were separated by centrifugation at 27,000 rpm for 12.5 h. *Hind*III and *Sal*I segments were partially separated by centrifugation at 30,000 rpm for 15 h and 34,000 rpm for 15.5 h, respectively. Gradient profiles were obtained by either sedimenting [^{14}C]DNA or electrophoresis of every third gradient fraction. Fractions with particular segments were pooled, and the DNA was precipitated and dissolved in K buffer.

Gel electrophoresis. Electrophoresis was through 0.6% agarose (SeaKem, FMC Corp.) gels formed in E buffer (5 mM sodium acetate, 1 mM sodium EDTA, 40 mM Tris-hydrochloride [pH 7.95]). Tube gels were prepared as described (8), and flat gels were formed as gel slabs (25 by 20 by 0.5 cm) (15). DNA samples were loaded in one-half-strength E buffer with 5% glycerol. Electrophoresis was at 2 V/cm of gel at 24°C for 24 to 48 h. Gels were photographed with a 365-nm source (C50 transilluminator; Ultraviolet Products, Inc.) after 30 min of immersion in ethidium bromide solution (1 $\mu\text{g}/\text{ml}$).

DNA transfer and recovery. Strands of restriction segments of [^{14}C]thymidine-labeled DNA were transferred from gels to nitrocellulose sheets (BA-85; Schleicher & Schuell Co.) by the Southern procedure (24). Generally, about 25 strips were cut from a single nitrocellulose sheet after transfer of the DNA from a preparative size gel. Strips from the same transfer produced radioautographic patterns with very similar intensities. If possible, a group of strips from the same transfer were used to hybridize to a group of related

^{32}P -labeled DNA segments. Film exposed to the nitrocellulose sheet for 72 h at 24°C showed a well-defined imprint of the original gel pattern. Film was exposed at -70°C to samples of the nitrocellulose before hybridization with strands of segments of ^{32}P -labeled DNA to provide a measure of the ^{14}C background which would be added to the hybridization signal. This double-label system conveniently located hybridized bands, with their enhanced signals, in the patterns of vaccinia DNA digests. Radiation damage of [^{14}C]DNA also increased the amount of transfer of large restriction segments to nitrocellulose.

DNA was recovered from agarose by dissolving the gel in G buffer (5.4 M NaI, 7 mM sodium EDTA, 7 mM Tris-hydrochloride [pH 7.0]) and collecting the DNA on glass beads which were pelleted by centrifugation at $900 \times g$ for 15 min at 4°C (26). After washing the bead-DNA complex at 4°C with G buffer, the DNA was eluted with R buffer (50 mM NaCl, 0.5 mM sodium EDTA, 5 mM Tris-hydrochloride, 50% formamide [pH 8.0]) (D. Gillespie, personal communication) containing 50 μg of sRNA, and the beads were pelleted at $12,000 \times g$ for 20 min. Sodium acetate was added to the supernatant (0.04 M), and the DNA was precipitated with ethanol and dissolved in K buffer.

Labeling with ^{32}P . Restriction segments were labeled by nick translation, using the procedure of Rigby et al. (18). Reactions contained 5 mM MgCl_2 , 2.5 mM dithiothreitol, 50 μg of bovine serum albumin per ml, 0.5 μM each dCTP and dGTP, 1 μM dATP, 50 μCi of [α - ^{32}P]TTP (410 Ci/mmol; Amersham Corp.), 0.1 to 10 μg of DNA, 2 U of DNA polymerase I, and 50 mM potassium phosphate (pH 7.4). One hundred-microliter reactions were at 16°C for 4 h. The reaction was stopped with sodium EDTA (20 mM) and Sarkosyl (0.5%), and the solution was passed through a 2-ml column of Bio-Gel A-1.5 m (Bio-Rad) in K buffer. Sodium dodecyl sulfate (0.5%) was added to DNA fractions which were mixed with phenol. After ether extraction, the DNA was precipitated and dissolved in K buffer.

Restriction segments were labeled at their termini with the large fragment of DNA polymerase I (31). After restriction nuclease digestion, the DNA was passed through a 2-ml Bio-Gel A-1.5 m column equilibrated with one-third-strength K buffer, and the DNA fractions were concentrated threefold. About 1 μg of DNA was added to a reaction which contained 70 mM NaCl, 70 mM potassium phosphate, 10 mM MgCl_2 , 10 mM dithiothreitol, 100 μg of bovine serum albumin per ml, 10 μCi of [α - ^{32}P]TTP, and 2 U of polymerase. After 60 min at 24°C, the reaction was stopped, and the DNA was treated as in the nick-translation procedure.

Hybridization. [^{32}P]DNA (10^6 cpm) strands of a segment produced by one restriction enzyme were hybridized to strands of segments generated by another enzyme, which were immobilized on nitrocellulose. Hybridization was at 60°C for 36 h in 0.5 ml of solution H (1 M NaCl, 10 mM sodium EDTA, 0.02% each of bovine serum albumin, Ficoll [Pharmacia Fine Chemicals, Inc.], and polyvinylpyrrolidone [Sigma Chemical Co.], 0.5% sodium dodecyl sulfate, and 20 mM PIPES [piperazine-*N,N'*-bis(2-ethanesulfonic acid)] [pH 6.9]). The filters were washed with solution W (0.5 M NaCl, 10 mM sodium EDTA, 0.5% sodium dodecyl sulfate, 10 mM PIPES [pH 6.9]) at 60°C for 6 h and then with 10 mM Tris-hydrochloride (pH 9.1) at 24°C

for 15 min. The nitrocellulose was air dried, and radioautographs were produced at -70°C with an intensifying screen.

Shear degradation and isolation of CLC fragments. Freshly prepared nonlabeled or [^{14}C]DNA was added to N_2 -saturated solution S (1 M NaCl, 1 mM sodium EDTA, 10 mM Tris-hydrochloride [pH 8.0]) to final concentrations of from 10 to 40 $\mu\text{g}/\text{ml}$. One milliliter was stirred with a micropropeller (The VirTis Co., Inc.) at a constant speed, using a GT-21 motor controller (G. K. Heller Corp.) for 40 min at 0°C . Speeds from 2,400 to 7,500 rpm were used to shear DNA to fragments having mean lengths of from 1/4 to 1/16 of the intact length (12, 17) as measured by centrifugation. The sheared DNA solution was mixed at 0°C with an equal volume of 0.2 M sodium phosphate (pH 11.7), and the DNA was denatured by keeping the solution at 50°C for 5 min (1). After cooling at 0°C , one-half volume of solution R (0.3 M NaH_2PO_4 , 2 M NaCl) was added, and the mixture was passed through a 1-ml column of benzoylated-naphthoylated DEAE-cellulose (BND; Serva-Technik GmbH & Co.) equilibrated with solution S. The flow-through contained duplex molecules, most of which were covalently linked complementary (CLC) fragments. This DNA was precipitated and dissolved in K buffer.

For S1 digestion, the sheared DNA was precipitated and dissolved in 50 μl of K buffer. Ten minutes after adding 25 μl of 1 N NaOH (24°C), the solution was mixed with 25 μl of 1 M Tris-hydrochloride (pH 7.6) and 25 μl of 1 N HCl. The DNA was passed through a 1-ml column of Bio-Gel A-1.5 m in K buffer to exchange solutions. The DNA fraction was mixed with concentrated S1 buffer and digested with S1 nuclease.

The above procedures were also used to isolate CLC segments after restriction nuclease digestion.

RESULTS

The following results are representative of those which were most useful in obtaining maps. All maps were derived from original data and not from published maps (2, 13, 29).

Gel patterns. Typical patterns for stained gels are shown in Fig. 1. Every segment was assigned a separate letter, so that bands with several segments are labeled with consecutive letters. Segments in patterns of digests by two or more enzymes are identified when necessary by numbers, and bands with several segments are denoted by consecutive numbers. When referring to segments, the number used to identify an enzyme is dropped, so that *Sal*I segment A is designated *Sal*-A, and double-digest segments are designated *Bgl* + *Sal*-2. The masses of the segments are shown in Table 1.

Centrifuge patterns. *Bgl*I digestion produced four segments which were separated by sucrose gradient centrifugation (Fig. 2). Segments from 30 μg of DNA could be resolved in a single centrifuge tube. *Bgl*-A could be cleaved at the single *Sma*I site found in vaccinia DNA to produce two segments which were resolved by centrifugation. The larger segment from *Bgl*-A is

designated *Sma* + *Bgl*-1, and the smaller is designated *Sma* + *Bgl*-3.

The three largest *Hind*III segments could also be separated after centrifugation of up to 25 μg of digest. *Sal*-A and -B could be resolved for digests with less than 10 μg of DNA, but extensive contamination could be avoided only by using the heavier side of the *Sal*-A peak and the lighter side of the *Sal*-B peak.

Isolation of end segments. Vaccinia DNA is a linear duplex molecule with ends that are covalently closed by single-stranded DNA which is less than 100 nucleotides long (11). If the DNA is cleaved by a restriction enzyme, then the strands of most of the duplex segments separate after denaturation. The CLC end segments rapidly form duplex structures after the denaturing condition is removed (8). Duplex segments were separated from single-strand DNA by chromatography on BND cellulose or by digestion with S1 nuclease (19, 27). Figure 3, lanes 1 and 2, shows a *Sal*I digest and the *Sal* end segments isolated by BND chromatography. The presence of faint bands at other positions indicated that a small amount of nonlinked DNA also formed duplex structures. These faint bands served to align the tube gels. Lane 3 contains a total *Xho*I digest of DNA from uncloned virus, and lane 4

FIG. 1. Agarose slab gel electrophoresis of vaccinia virus DNA segments. The enzyme used for digestion is given with the lane showing the digest pattern. Lane 1, *Bgl*I; lane 2, *Hind*III; lane 3, *Sal*I; lane 4, *Kpn*I; lane 5, *Xho*I.

TABLE 1. Molecular weights of vaccinia DNA segments^a

<i>Bgl</i> II		<i>Hind</i> III		<i>Sal</i> I		<i>Xho</i> I		<i>Kpn</i> I		<i>Sma</i> I	
Segment	Mol wt × 10 ⁶	Segment	Mol wt × 10 ⁶	Segment	Mol wt × 10 ⁶	Segment	Mol wt × 10 ⁶	Segment	Mol wt × 10 ⁶	Segment	Mol wt × 10 ⁶
BA	72.4									SMA	98.5
BB	28.0	HA	32.8			XA	27.0			SMB	23.5
		HB	18.8	SA	17.0	XB	22.2	KA	22.5		
				SB	14.9			KB	14.9		
BC	13.8	HC	13.8			XC, D	14.5				
		HD	10.7					KC	13.2		
		HE	10.2					KD, E, F	10.3		
				SC	9.8			KG, H	9.3		
		HF	9.0	SD, E	9.25						
				SF	8.65	XE	7.48				
BD	7.9			SG	8.05						
				SH	6.7	XF	6.22				
				SI	6.3						
		HG	5.7	SJ	6.05						
		HH	5.5	SK	5.78	XG	4.71				
		HI	4.0			XH, I	3.94				
				SL	3.75			KI	3.73		
				SM	3.55	XJ	3.29	KJ, K	3.21		
						XK	3.18				
		HJ	2.98	SN	2.8						
		HK	2.62			XL, M	2.49				
		HL	2.40			XN	2.46	KL	2.45		
				SO, P	2.02			KM	2.1		
				SQ	1.76	XO	2.03	KN, O	2.03		
				SR	1.45			KP	1.68		
		HM	1.35					KQ	1.22		
		HN	1.0								
		HO	0.9								
				SS, T, U, V	0.62						
				SW	0.56						

^a The molecular weights of vaccinia virus DNA segments were determined by electrophoresis with λ cI857 and adenovirus 2 DNA segments as standards. Segments with molecular weights greater than 30×10^6 were also measured by sucrose gradient centrifugation with standard DNAs, including intact vaccinia virus DNA.

contains the CLC ends from an *Xho*I digest of cloned virus DNA. These CLC segments migrated to form a sharp band at the same position as a diffuse band in the complete digest. This broad band is produced by digesting uncloned DNA

which has terminal segments of heterogeneous length (8, 30). Shorter terminal segments produced by cleaving uncloned DNA with enzymes such as *Sal* and *Xho* may be identified by their broad bands in gel patterns of total digests.

FIG. 2. Centrifugation of *Bgl*I digest segments. Digestion of vaccinia virus DNA was terminated with sodium EDTA and 0.5% Sarkosyl, and a 120- μ l sample containing 10 μ g of DNA was loaded on an 11-ml sucrose gradient. Centrifugation was from right to left. Fractions were collected and counted after addition of scintillation fluid.

Lanes 5 and 6 identify the ends in a *Kpn* digest. The terminal segments in this and other digests form a band with increased fluorescence compared with adjacent bands, owing to the presence of segments of equal size on each end of the molecule (10, 28).

*Hind*III segments B and C are the CLC segments which define the ends in maps for this enzyme (10; data not shown). The *Hind* terminal segments are large, so they form compact gel bands even when they are generated from digestion of uncloned DNA.

Digestion of renatured shear fragments. The procedures used to isolate CLC restriction segments were used to obtain CLC fragments from sheared DNA. Hershey et al. used controlled stirring of solutions to shear λ DNA (12). The size of the fragments was an inverse function of the stirring speed, which was regulated to generate molecules with mean lengths of one-half or one-fourth, etc., of that of the intact DNA.

After shearing vaccinia DNA, the sizes of the fragments were determined by centrifugation with [14 C]DNA, which was included in the stirred solution. Although the spread of the peaks for the fragments was broader than that for the intact DNA, all of the curves were symmetrical (21; data not shown). Stirring at 2,500 rpm produced 1/4-length molecules, whereas stirring at 7,500 rpm produced fragments equal to or less than 1/16 of the length of the intact DNA. Fragments of the desired size were collected, denatured, and renatured, and the CLC fragments were isolated. The same

enzyme was used to digest a series of CLC fragments of different sizes. Digests of smaller fragments identified restriction segments at the end of the molecule, whereas larger fragments yielded additional segments located adjacent to the termini. This procedure does not distinguish one end of the DNA from the other, but it does determine whether a segment is located near the end or the interior of the DNA.

CLC fragments which were generated by stirring at 5,000 rpm were digested by *Sal*I (Fig. 3, lane 8) or *Xho*I (lane 10), and the patterns were compared with those for the complete digests. The five brightest bands in lane 8 contain those *Sal* segments most likely to survive shearing. The bottom band represents the very end segments, whereas the upper bands must contain DNA adjacent to these segments. These bands are labeled H, I, K, N, O, and P in the diagram of Fig. 1. Band G, which is not as bright as H, survives to a lesser degree and is found interior to segments I and P. Since segments A and B do not appear, they are either located in the interior or are long enough to be sheared even if they are near the termini. A and B cannot be between the segments seen in lane 2 and the termini. Segments L, M, Q, and R, which are small, must not be near the ends, since they are not detected.

The *Xho* segments which survived shearing (Fig. 3, lane 10) are G, H, I, J, and M (Fig. 1), with H and I representing the terminal segments.

Map construction. Patterns for and maps of *Hind*III digests of vaccinia DNA have been reported (2, 9, 14, 29). Particular partial diges-

FIG. 3. Electrophoretic identification of segments at or near the termini of vaccinia virus DNA. Lanes 1 through 6 identify end segments produced by restriction enzyme digestion. CLC segments were isolated by BND cellulose chromatography and were identified by an electrophoretic pattern of the total digest. Lane 1, *SalI* digest; lane 2, *Sal* CLC segments; lane 3, *XhoI* digest of DNA from uncloned virus; lane 4, *Xho* CLC segments from cloned virus DNA; lane 5, *KpnI* digest; lane 6, *KpnI* CLC segments. Lanes 7 through 10 identify segments from a restriction digest of CLC fragments of sheared DNA. DNA was sheared at 5,000 rpm, and CLC fragments were isolated by BND chromatography. Lane 7, *SalI* digest; lane 8, *SalI* digest of CLC fragments; lane 9, *XhoI* digest; lane 10, *XhoI* digest of CLC fragments. Lane S, diagram of *Sal* segments A through R identified in Fig. 1; lane X, diagram of *Xho* segments A through O identified in Fig. 1.

tion products are formed when actinomycin D is present during *HindIII* digestion of DNA (16). With vaccinia DNA, one combination of drug and enzyme generated a 68×10^6 -dalton partial product which was isolated by centrifugation. A limit digest of this product without the drug yielded *HindIII* segments A, B, D, and H (data not shown). Since B is a terminal segment, the order of A, D, H was determined in part by *HindIII* digestion of sheared one-fourth-length CLC fragments. Only segment B survived the shearing, which places A between B and D, H

(data not shown). The order of D, H was determined by digesting DNA with *HindIII* with actinomycin for 6 h and then isolating the heaviest DNA from this digest by centrifugation. Segments A, B, and D were recovered from a limit digest of this DNA to a greater extent than was H, so the segment order was assumed to be H, D, A, B from left to right on the right end. Since digests of one-fourth-length CLC fragments also produced segments C, F, and K, these last two segments were placed to the right of the terminal C segment on the left end (data not shown).

The 15 segments from a *HindIII* digest were recovered from 0.6% agarose preparative gels. Fig. 4 shows the digestion of *Hind*-A, -B, and -C by *SalI* (lanes 2, 5, and 6). Although several faint bands are present, the brightest bands identify the *Sal* digestion products as *Sal*-A, -F, -L, and -R from *Hind*-A. Similarly, *Sal*-I and -N are from *Hind*-B, but the band at the top of lane 5, at the level of *Hind* + *Sal*-4 (lane 4), is an overlapping segment derived from *Sal*-G, as shown by other data. *Sal*-K is from *Hind*-C, whereas the bright band below contains segments which can be shown to be from *Sal*-H. *Sal* segments T and V from *Hind*-B and *Sal*-S and -U from *Hind*-C are too small to be seen in this gel. Although *Hind*-B and -C are terminal segments, they were produced from DNA from uncloned virus, so that the small *Sal* end segments O and P derived from these *Hind* segments had different lengths and therefore did not form discrete bands in these patterns. Most of the *HindIII* and *SalI* segments were isolated from preparative gels and digested with the second enzyme to form maps by the method of overlaps (15).

Fig. 5 shows the *SalI* digest of each of the four *BglI* segments. The double digest, *Bgl* + *Sal* (lane 2), is flanked by the *Bgl* (lane 1) and *Sal* (lane 3) digests. *Sal*-C, -D, and -H are not present in lane 2, since they contain *Bgl* sites. *Bgl* cleaved two (C and D) of the three *Sal* segments which are poorly resolved in gels. *Bgl*-A contained *Sal*-A, -E, -F, -G, -I, -J, -L, -M, -N, -P, -Q, and -R and one new segment, which turned out to be the right side of segment D (lane 4). Since *Bgl*-A contained the end segment *Sal*-P, it must be at the right end of the vaccinia map. Lanes 6, 8, and 10 show *SalI* digests of *Bgl*-B, -C, and -D. *Bgl*-C is at the left end since it contains the terminus, *Sal*-O. Several experiments, including hybridization of *Sal*-H to both *Bgl*-C and -D and shear degradation to produce a CLC fragment containing *Bgl*-C and -D, placed *Bgl*-D between *Bgl*-C and -B and determined the *Bgl* map.

The pattern for the *Bgl*-C digest has bands at the levels of *Sal*-K and -S and a new segment not found in the original *Sal* digest. Although this

FIG. 4. Electrophoretic patterns of *SalI* digests of *HindIII* segments. Segments A, B, and C were isolated from a preparative *HindIII* digest of DNA from uncloned virus and were digested with *SalI*. Lane 1, total *Hind* digest; lane 2, *Sal* digest of *Hind*-A; lane 3, total *Sal* digest; lane 4, total *Sal* + *Hind* digest; lane 5, *Sal* digest of *Hind*-B; lane 6, *Sal* digest of *Hind*-C; lane H, diagram of segments A through L of a total *Hind* digest; lane S, diagram of segments A through R of a total *SalI* digest.

observation indicated that either *Sal*-K or -S is next to *Sal*-O, there are actually two small segments of identical size, S and U, located between *Sal*-O and -K. The three segments, O, S, and U, at the left end are represented at the right end by segments of the same size, where they are labeled, from the end in, P, T, and V. The location of *Sal*-S next to *Sal*-O was determined by finding segments S and T in *Sal* digests of the terminal *XhoI* segments H and I. The presence of four small bands of identical size, S, T, U, and V, was determined by slicing and counting a gel with labeled segments. Segments

produced by *Sal* digestion were end labeled with the large fragment of DNA polymerase. The number of counts included in the region of *Sal*-S was about four times that found for *Sal*-R. When *Sal* digests of *Bgl*-C were run for short times, the band at the S level was brighter than expected, indicating occupation by two segments. The two small segments S and U, on the left, are adjacent and repeated on the right end of the genome. The presence of S and U has been reported by Venkatesan and Moss (25), who examined a *Sal* digest of an *EcoRI* segment at the end of the genome.

SmaI cleaved *Bgl*-A within the *Sal*-F segment to produce *Sma* + *Bgl*-1 and -3. These segments were isolated from sucrose gradients in such a way that segment 1 contained less contamination with 3 than the converse. Under this condition, examination of the patterns of *Sal* digests of the two parts of *Bgl*-A should begin with Fig. 6, lane 2, which shows the *Sal* digest of *Sma* + *Bgl*-1. *Sal*-A, -E, -J, -L, -M, -Q, and -R are

FIG. 5. Electrophoretic patterns of *SalI* digests of *BglI* segments. Lane 1, total *BglI* digest; lanes 2 and 12, total *Sal* + *Bgl* digest; lanes 3, 5, 7, 9, and 11, total *Sal* digest; lanes 4, 6, 8, and 10, *Sal* digests of *Bgl*-A, -B, -C, and -D, respectively; lane S, diagram of segments A through W of total *Sal* digest (Fig. 1).

FIG. 6. Patterns for *SalI* digests of the two segments produced by *SmaI* digestion of *Bgl-A*. Lanes 1 and 4, total *SalI* digest; lane 2, *Sal* digest of *Sma* + *Bgl-1*; lanes 3 and 6, total *Sal* + *Bgl* digest; lane 5, *Sal* digest of *Sma* + *Bgl-3*; lane S, diagram of segments A through R of a total *Sal* digest.

present as valid *Sal* segments. The segment located at the *Sal-H* position is not found in the complete double digest *Sal* + *Bgl* (lane 3) and is formed by the *Sma* digest of *Sal-F*. Lane 5 contains the *Sal* digest of *Sma* + *Bgl-3*, which is at the right end of the *Sal* map. *Sal-G*, -I, -N, and -P are present as bands not found in lane 2. The other less bright bands, such as *Sal-E*, -L, -M, etc., are contaminants from the larger *Bgl-A* subsegment. A new segment located below *Sal-(S, T)*, which was not found in the complete

Sal + *Bgl* digest, was derived from the right end of *Sal-F*.

The patterns for *XhoI* digests of isolated *BglII* segments are shown in Fig. 7. The double digest (lane 2) indicated *Bgl* sites in *Xho-A* and -K. *Xho-B* was not cut by *Bgl* (lane 2), so the new segment *Xho* + *Bgl-2* (lane 2) must be from *Xho-A*, which is the only segment large enough to generate it. This new segment was also seen to produce the top band in the *Xho* digest of *Bgl-B* (lane 5), so that *Xho* + *Bgl-2* is an overlap of *Xho-A* and *Bgl-B*. The band between *Xho-K* and -L is from K. *Bgl-D* (lane 10) is included in *Xho-A*, whereas *Bgl-C* (lane 8) contains the three left-end segments *Xho-H*, -J, and -G. Segment I, which corresponds to H, is at the right end of the *Xho* map and is seen in lane 4 with the *Xho* digest of *Bgl-A*. *Xho-L*, which is at the right end of *Bgl-B*, is seen in lane 5. This segment is in *Sal-D*, which was also cleaved by *BglII*. Since the sites which define *Xho-L* reduce the ambiguity of the closely spaced segments *Sal-C*, -D, and -E, it is important to locate *Xho-L*.

SmaI cut *Xho-B*. *Xho* digests of *Sma* + *Bgl-1* and -3 had patterns (data not shown) that showed *Xho-C*, -D, -E, -N, and -O in *Sma* +

FIG. 7. Patterns of *XhoI* digests of *BglII* segments. Lane 1, total *Bgl* digest; lanes 2 and 7, total *Xho* + *Bgl* digest; lanes 3, 6, and 9, total *Xho* digest; lanes 4, 5, 8, and 10, *Xho* digests of *Bgl-A*, -B, -C, and -D, respectively; lane X, diagram of segments A through O of a total *Xho* digest.

FIG. 8. Patterns for *KpnI* digests of *BglI* segments. Lane 1, total *BglI* digest; lane 2, total *KpnI* + *BglI* digest; lanes 3, 5, 7, and 9, total *KpnI* digest; lanes 4, 6, 8, and 10, *KpnI* digests of *BglI*-A, -B, -C, and -D, respectively; lane K, diagram of segments A through Q of a total *KpnI* digest.

BglI-1 and *Xho*-M and -I in *Sma* + *BglI*-3. *Xho*-B contained *Sal*-F and -G and most of *Sal*-I, and *Xho*-A contained *Sal*-C and -W and a large part of the left end of *Sal*-B and the right end of *Sal*-H (data not shown). The pattern of a *Sal* digest of *Xho*-C and -D, which run together, indicated that these segments include *Sal*-E and -Q (*Xho*-C) and most of *Sal*-R (*Xho*-D). The left end of *Sal*-A was overlapped by almost the entire length of *Xho*-D.

KpnI segments D, E, F, and G, H migrated together, as shown in Fig. 8, where *KpnI* digests of *BglI*-A, -B, -C, and -D are shown. *BglI* cut two segments in the D, E, F group and one in the G, H group (lane 2). *BglI*-A (lane 4) contained *KpnI*-A, -B, -C, -H, -K, -O, -P, and -Q and a new band slightly below *KpnI*-I (*KpnI* + *BglI*-10). *BglI*-B contained the one intact segment of the D, E, F group, which turned out to be *KpnI*-E and *KpnI*-I, -L and two new segments between *KpnI*-H and -I (lane 6). *BglI*-C contained *KpnI*-J, -M, and -N and one new segment found between *KpnI*-H and -I. *BglI*-D contained two new segments. *Xho*-A spanned *BglI*-D and the left end of *BglI*-B. Other

experiments showed that *Xho*-A includes one intact segment from *KpnI*-D, -E, -F and one intact segment from *KpnI*-G, -H. Since the entire *Xho*-A segment was to the left of *BglI*-A, it must contain *KpnI*-G, which was cut by *BglI*. *Xho*-A was not large enough to contain two segments from the *KpnI*-D, -E, -F group and *KpnI*-G. Therefore, one segment from the *KpnI*-D, -E, -F group must be to the left of *KpnI*-G and the single *KpnI*-D, -E, -F segment included in *Xho*-A, and the remaining *KpnI*-D, -E, -F segment must be to the right of *KpnI*-G and the single *KpnI*-D, -E, -F segment included in *Xho*-A. The segment to the right of those contained in *Xho*-A was *KpnI*-F, which was cleaved at the *BglI*-B, -A junction, the only *BglI* site to the right of *Xho*-A. This leaves *KpnI*-D as the segment to the left of the *KpnI* segments in *Xho*-A. Since *KpnI*-D and -G were both cleaved by *BglI*, *KpnI*-D must be cut at the *BglI*-C, -D junction, which was to the left of the *BglI*-D, -B junction, which cut *KpnI*-G. *KpnI*-E was the segment included in both *Xho*-A and *BglI*-B.

SmaI cut *KpnI*-A. A *KpnI* digest of *Sma* + *BglI*-1 placed *KpnI*-B, -C, -H, and -Q in the large *BglI*-A subsegment. The *KpnI* digest of *Sma* + *BglI*-3 had a pattern that showed *KpnI* bands K, O, and P to be included in the smaller *BglI*-A subsegment (data not shown).

Hybridization of strands of segments produced by one restriction enzyme to immobilized strands of segments produced by another enzyme generated much of the early data for map construction. Strands of segments separated on gels were immobilized on nitrocellulose. Usually, these strands were labeled with [^{14}C]thymidine to produce a radioautographic imprint of the original gel pattern on the film. Strands from segments generated by a second enzyme, labeled with ^{32}P by nick translation, enhanced the film blackening of those bands which contained homologous sequences.

Fig. 9 shows the results of hybridizing strands of *Sal*-A, -B, -C, and *Sal*-D, -E to immobilized *HindIII* strands. *Sal*-A (lane 1) mainly enhanced the blackening of *Hind*-A, although there was some additional signal at band E. *Sal*-B enhanced the signal on *Hind*-E and -I and, to a lesser extent, on *Hind*-A. The signal on *Hind*-E with *Sal*-A as the designated input strand was due to contamination with strands from the adjacent segment *Sal*-B, and the signal on *Hind*-A with *Sal*-B as the designated input was due to contamination with *Sal*-A. Cross-contamination in hybridizations with Southern blots is known (29). The ability to detect bands which are validly enhanced, such as band I in lane 2, depends on comparing that band with the same band in a radioautogram produced by exposure to ^{14}C alone in the absence of hybridization.

FIG. 9. Hybridization of individual *Sal*I segment strands to immobilized *Hind*III strands. ^{14}C -labeled DNA was digested with *Hind*III, and segment strands were transferred to nitrocellulose. Strands of isolated *Sal* segments labeled with ^{32}P were hybridized to the *Hind* strands. Lanes 1, 2, 3, and 4, Results of hybridization of designated input strands from *Sal*-A, -B, -C, and -D plus -E, respectively, to the *Hind* segment strands; lane H, diagram of segments A through L of a total *Hind* digest.

Hybridization to *Sal*-C (lane 3) produced a signal in *Hind*-D, -F, -G, -H, -K, and -L. This result was produced by *Sal*-C, which overlapped *Hind*-K and -F, and by the contaminants *Sal*-D and -E, which hybridized to *Hind*-G, -L and *Hind*-D, -H, respectively. As previously stated, *Sal*-C and *Sal*-D, -E were not well resolved. This analysis was consistent with the result in lane 4, where *Sal*-D + E hybridized to *Hind*-D, -G, and -H but not to *Hind*-K or -F. If a *Hind* segment hybridizes to *Sal*-C, -D, or -E, it will blacken the region of the two bands containing these three segments to an extent that will make it difficult to detect specific hybridization. *Sal*-C, however, could be detected by shearing experiments, since it was located closer to the left end of the DNA than were segments D and E, which were near the center of the molecule.

*Xho*I cut *Sal*-D but not *Sal*-C, -E, -F, and -G. Bands C and E could be resolved on a combined *Sal* + *Xho* digest which was immobilized on nitrocellulose (Fig. 10). The first and second bands are parts of *Sal*-B and -A, which were both cut by *Xho*I. The next four bands are *Sal*-C, -E, -F, and -G, as determined by hybridization to the original *Sal* segments and by the following results. Hybridization to ^{32}P *Hind*-G did not produce a signal in bands C, E, F, or G but did blacken two smaller segments (lane 4). *Hind*-H hybridized to *Sal*-E (lane 6), although there was also a signal at the two bands reacting with *Hind*-G. At least part of the signal in the two lower bands in lane 6 was caused by con-

FIG. 10. Hybridization patterns of individual *Hind*III segment strands to immobilized strands from segments produced by digestion with *Sal*I and *Xho*I. ^{14}C -labeled DNA was digested with *Sal* + *Xho*, and segment strands were transferred to nitrocellulose. Strands of isolated *Hind* segments labeled with ^{32}P were hybridized to the *Sal* + *Xho* strands. The designated input *Hind* segments are: lane 1, K; lane 3, F; lane 4, G; lane 6, H; lane 8, E. Lanes 2, 5, and 7 contain the ^{14}C radioautographic imprint of the electrophoretic pattern of the *Sal* + *Xho* digest. Lanes labeled d are diagrams of the original *Sal*I segments C, E, F, and G, which remain intact after digestion with *Xho*I.

tamination by *Hind*-G, although the bottom band probably has a legitimate contribution from *Hind*-H. It is known that both *Hind*-G and -H hybridize to *Sal*-D and -E, so the smaller *Xho* + *Sal* segments which responded to *Hind*-G must be derived from *Xho* cleavage of *Sal*-D. *Hind*-G was not cleaved by *Sal*, so *Sal*-D must contain *Hind*-G. Lanes 3 and 1 show that *Hind*-F and -K blackened *Sal*-C, so both *Sal*-C and -E could be correlated with *Hind* positions.

Xho-C and -D migrated together, complicating the construction of *Xho* maps. *Xho*-C was cleaved by *Hind*III, whereas *Xho*-D was in *Hind*-A, so one of these bands is easily delineated by *Hind*III. The three left-hand *Xho* segments, H, J, and G, could be isolated from *Bgl*-C or *Hind*-C, whereas the two right-hand segments could be isolated from *Hind*-B. *Xho*-A included *Hind*-N, -M, -K, -F, -E, and -O, and *Xho*-B was cleaved by *Hind*III to form two new bands, one of which migrated between *Hind*-C and -D and one of which comigrated with *Hind*-D. The *Sma*I site was in *Xho*-B. *Hind*-L was the only other segment not cleaved by *Xho* that has been found in *Xho*-K. The location of other *Xho* segments was determined by *Bgl* digestion and hybridization to *Hind*III segments.

*Hind*III segments A through H were hybridized to *Kpn* strips (Fig. 11). *Hind*-A spanned *Kpn*-A, -B, and -C (lane 1). *Hind*-B spanned *Kpn*-A, -P, -O, and -K (lane 2). Part of the signal in *Kpn*-A and all of the signal in *Kpn*-B and -C were caused by contamination from *Hind*-A. *Hind*-C (lane 3) blackened the left-end segments *Kpn*-J, -N, -M, and -D. The signal in D confirmed my earlier placement of the *Bgl*-C, -D junction in that segment. *Hind*-D covered *Kpn*-B and -Q (lane 4), whereas *Hind*-E covered *Kpn*-E and -I (lane 5). *Hind*-F covered *Kpn*-G, placing it near *Kpn*-G, as shown earlier in the analysis of the *Bgl* cuts (lane 6). *Hind*-G (lane 7) hybridized to *Kpn*-F, which placed it near the *Bgl*-B, -A junction. *Hind*-H (lane 8) covered *Kpn*-H, although there was some signal on *Kpn*-F caused by *Hind*-G.

The cleavage sites of the enzymes which have been discussed are shown in the diagram of Fig. 12.

DISCUSSION

The initial step in establishing a map for vaccinia DNA was to identify the CLC terminal segments. Denaturation of cleaved DNA with pH 11.7 phosphate buffer (1), followed by BND chromatography (19), gave higher recoveries (greater than 60%) of terminal segments than do procedures in which higher pH or temperature is used, followed by either hydroxyapatite chromatography or S1 digestion (8, 27). In some cases, the end segments could be identified simply by digesting DNA from uncloned and

cloned virus and observing which gel band was diffuse. The variation in the length of the DNA in uncloned virus is due to the variation in the length of the terminal segments (30). This variation did not extend beyond the shortest terminal segments, *Sal*-O and -P, reported in this paper. Since the relative variation in length of the terminal segments derived from uncloned DNA decreased with increased average size, bands containing these segments appeared to be more compact as the segment size increased. Results obtained with uncloned DNA were checked by using cloned DNA, and all map assignments were from results with cloned DNA. Enzymes such as *Sal*II, which generate small segments at

FIG. 11. Hybridization of individual *Hind*III segment strands to immobilized *Kpn*I segment strands. 14 C-labeled DNA was digested with *Kpn* and segment strands were transferred to nitrocellulose. Strands of isolated *Hind* segments labeled with 32 P were hybridized to the *Kpn* strands. Lanes 1, 2, 3, 4, 5, 6, 7, and 8, hybridization of designated input strands from *Hind*-A, -B, -C, -D, -E, -F, -G and -H, respectively; lane K, diagram of *Kpn* segments.

FIG. 12. Location of vaccinia virus DNA restriction sites. The tick below each line marks the single *SmaI* site.

or near the termini, demonstrate the inverted terminal repetition structure of vaccinia DNA (10, 28), since *Sal*-O, -S, and -U at one end of the molecule are matched by segments P, T, and V at the other end.

DNA was sheared by procedures that minimize production of single-stranded tails (17) to separate CLC duplex fragments from single-stranded interior fragments on BND cellulose. Restriction of a series of CLC fragments of increasing mass produced a series of segments whose genomic positions could be correlated with their distances from the termini. The inclusion of a small segment, produced by one enzyme, in a particular set of CLC fragments suggested where that segment is included in a second series of segments produced by another enzyme for which the map is known. The closed termini of vaccinia DNA precluded the routine end-labeling which Smith and Birnstiel used in their partial digestion procedure for obtaining restriction maps (22). Since this strategy is designed for electrophoretically resolvable segments of less than 10^7 daltons (20, 22), it is not useful for general mapping of vaccinia DNA, but it can confirm the existence of adjacent small segments such as *Sal*-S and -U (R. Wittek, personal communication).

Stirring vaccinia DNA at 4,000 to 5,000 rpm produces shear fragments that are about one-eighth of the intact DNA length that should be the proper size for inserts in cosmid vectors (3-5). A relatively small number of hybrid plasmids, constructed from either these fragments or

sheared *Bgl*-A or -B, would represent the entire viral genome. The availability of large segments or fragments which span specific regions is important for deriving more detailed restriction maps, as was reported by Schumperli et al. in their presentation of the *EcoRI* maps for two poxvirus DNAs (20). Data have been presented to demonstrate the utility of the *Bgl* + *Sma* segments in forming the *SalI*, *KpnI*, and *XhoI* maps. The restriction enzyme systems which are now known for vaccinia virus DNA should allow a systematic selection for any region of the genome.

The interpretation of patterns containing restriction digests of individual segments produced by another enzyme was determined by the purity of the isolated segments. When several segments were close in a preparative gel, one segment was isolated that was as free of contaminating segments as possible, whereas adjacent segments were collected from the remainder of the sample. The pattern of the digest of the purest segments was analyzed first, since the patterns for the adjacent segments were contaminated with bands from the purer segment. Only those bands in the patterns of the adjacent segments which were more prominent than those found in the first pattern were assigned to the designated isolated segment.

When an enzyme produced segments with nearly identical electrophoretic mobilities, a second enzyme was used which cleaved some of the segments that were similar in size. *XhoI* cleaved *Sal*-D but not *Sal*-E or -C, all of which had

similar sizes. *Bgl*I cleaved *Sal*-C and -D but not *Sal*-E. *Bgl*I also cleaved three of the five *Kpn*I segments which were grouped in two gel bands. *Hind*III cleaved *Xho*-C but not *Xho*-D, which allowed a simplification of that pattern.

Initially, the locations of the *Sal*I and *Hind*III segments were determined by isolating the *Hind* end segments B and C, and also A, and finding what *Sal* segments were spanned by these *Hind* pieces. *Hind*III digestion in the presence of actinomycin D gave the linkage group *Hind*-H, -D, -A, -B, which gave the right side of the *Hind* map, and showed what *Sal* segments were included in the right end. Digestion of CLC fragments produced by shear with *Hind*III showed segments F and K (small segments such as N and M were not considered in the original attempts to arrange segments) to be near the termini. Since the right side of the *Hind* map was established, F and K were placed near the left terminal segment C. Initially, K was placed to the left of F, since CLC fragments which contained K but not F were obtained. Later experiments confirmed this arrangement, since *Hind*-K was included in *Bgl*-D (data not shown). *Sal* digests of CLC fragments showed which segments were near the terminal segments O and P. Hybridization and reciprocal double digests showed that *Hind*-B overlapped *Sal*-G and that this *Sal* segment spanned the *Hind*-A, -B junction. The same techniques also showed that *Sal*-H overlapped the right end of *Hind*-C. Whenever possible, the largest segments from *Xho*I, *Kpn*I, or *Bgl*I digests were used to determine linkage groups and overlaps in *Sal* or *Hind* maps. *Kpn*-A included *Sal*-F and -G, and *Kpn*-B contained *Sal*-Q, -J, and -R and overlapped the left end of *Sal*-A and *Hind*-A (data not shown). Since *Hind*-A included *Sal*-A, -F, -L, and -R, this placed *Sal*-L between *Sal*-A and -F. *Xho*-A included *Sal*-C and -W and overlapped the left end of *Sal*-B and also contained *Hind*-N, -M, -K, -F, -E, and -O (data not shown). The *Bgl*-A, -B junction cleaved *Sal*-D and *Hind*-L, which located these segments. *Hind*-D hybridized to a segment in the *Sal*-C, -D, -E group which must be *Sal*-E, since only *Sal*-E was included in *Bgl*-A, which also contained *Hind*-D. *Sal*-M was included in the large subsegment of *Bgl*-A (*Bgl* + *Sma*-1) generated by cleavage with *Sma* and was the last segment contained in *Bgl* + *Sma*-1 to be placed on the map. *Sal*-M was not contained in *Hind*-A, -D, -H, which overlapped all but the left end of *Sma* + *Bgl*-1 and was not in *Kpn*-B or *Xho*-C, -D. These considerations placed *Sal*-M as the first segment to be contained in the left end of *Bgl*-A. This result was indicated by the hybridization of *Hind*-J to *Sal*-M. Although all of the data needed to locate a particular segment have not been presented or discussed, the con-

siderations above indicate the process by which the maps were constructed. Reciprocal double-digest and hybridization data were used to fill in the maps.

Since the resolution on gels of *Hind*III segments was greater than that of segments of other digests except for *Bgl*I, the *Hind* segments were nick translated most frequently and hybridized to a digest of a second enzyme. The *Bgl*I grouping plus this hybridization data provided most of the data for the *Xho*I and *Kpn*I maps. Finally, although the information is not presented, some of the smaller segments, such as *Hind*-N, -M, and -K, which are included in a large segment, such as *Xho*-A, were ordered by progressively digesting the ends of the large segment with λ exonuclease and noting which segments could still be formed by *Hind*III digestion of the exonuclease-treated DNA. Segments which survive exonuclease digestion must be recessed from the ends of the large segment (15).

The *Xho*I and *Hind*III maps presented here confirmed in most details the published maps for the WR strain of vaccinia virus DNA (13). The *Kpn*I map is similar to the published maps for rabbit poxvirus and the Elstree strain of vaccinia virus (20), except for segments located near the termini. It has been shown that different poxvirus DNAs have variable terminal regions and large internal regions (75 megadaltons or more) which appear to be very similar since they contain highly conserved restriction sites (13, 20). My results confirm this view.

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