Two Major DNA Variants Present in Serially Propagated Stocks of the WR Strain of Vaccinia Virus

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Two major DNA variants have been isolated from serially propagated stocks of the WR strain of vaccinia virus. Restriction enzyme mapping of the two variants with *HindIII*, *AvaI*, *XhoI*, *SstII*, and *SmaI* revealed a 6.3-megadalton deletion in the smaller DNA variant. The deletion was mapped at ca. 6.8 megadaltons in from the left terminus, just beyond the inverted terminal repeat. The additional DNA present in the larger variant was found to represent unique viral sequences that were transcribed both in vitro and in vivo. One-step growth curves in HeLa cells revealed no difference in rate of replication or burst size when progeny was scored on CV-1 monolayers.

The members of the poxvirus group are perhaps the largest and most complicated animal viruses known. They are distinguished by their large size, cytoplasmic site of replication, and inclusion of a large number of enzymatic activities within the purified virion (12). Their complex nature has hindered a rapid understanding of their biology and replication strategy. Recently, however, due chiefly to the efforts of Wittek and his collaborators (22-24), significant strides have been made in understanding the organizational and functional structure of the poxvirus genome.

With vaccinia virus as a prototype, the following features of the DNA are noteworthy. (i) The size of the DNA is ca. 120 megadaltons as defined by electron microscopy, restriction enzyme analysis, and reassociation kinetics (5, 6, 23). (ii) The nonpermuted linear double-stranded DNA molecule is terminally cross-linked, such that a single-stranded circle is formed upon denaturation (5), but the nature of the terminal cross-link remains unknown. (iii) Approximately 10 kilobase pairs at the terminus are present as an inverted terminal repetition (4, 22). (iv) The presence of repeated sequences has been described elsewhere (6, 15). (v) Length heterogeneity in the most distal portions of the termini are found in serially passaged virus which can be eliminated upon plaque purification of the virus (24).

A number of physical maps generated by restriction enzyme analysis are currently available (8, 18, 23).

When serially passaged vaccinia virus (strain WR) was analyzed with a variety of restriction endonucleases, we observed an additional type of genomic variation besides that found at the termini. Two major DNA variants differing by

6.3 megadaltons were observed, and their analysis is the subject of this communication.

MATERIALS AND METHODS

Cells and virus. Vaccinia virus (strain WR) was originally obtained from B. Moss of the National Institutes of Health, and stocks have been maintained in our laboratory by serial passage in HeLa cells. Purified virus was prepared essentially as described by Joklik (7). Virus was plaque purified by selection of well-isolated plaques on monolayers of CV-1 and amplified in HeLa cells after the second cycle of plaque isolation.

DNA purification. When virus was to be used for isolation of DNA, all steps in the purification schedule were performed without intermediate freezing. Purified virions were lysed and DNA was extracted as previously described (14).

Restriction endonuclease digestions. *HindIII* was purchased from Boehringer Mannheim Corp.; *SmaI*, *SstII*, *AvaI*, and *XhoI* were purchased from Bethesda Research Laboratories. Analytical digestions were performed in 0.05-ml reactions containing 0.5 to 0.8 μ g of purified DNA in the buffers recommended by the manufacturers, 2 to 4 U of enzyme were used routinely, and incubations were at 37°C for 2 to 5 h. Reactions were stopped by adding 10 μ l of a solution containing 2.5% agarose, 40% glycerol, 5% sodium dodecyl sulfate (SDS), and 0.25% bromophenol blue. Samples were layered at 65°C and allowed to harden before electrophoresis. Preparative reactions for isolation of fragments or transfer to nitrocellulose were in 0.6 ml and contained from 15 to 30 μ g of DNA.

Gel electrophoresis. DNA fragments were separated on 0.5 to 1.0% agarose gradient gels (30 by 14.5 by 0.3 cm) formed by pumping at 65°C a solution of 0.5% agarose-5% sucrose into a solution of 1.0% agarose-20% sucrose in electrophoresis buffer (36 mM Tris-hydrochloride [pH 7.8]-30 mM NaH₂PO₄-1 mM EDTA) into a warmed vertical slab gel electrophoresis apparatus (Bio-Rad Laboratories). Electrophoresis was at 4°C for 42 h at 50 V. Gels were stained with

ethidium bromide and photographed with Polaroid type 55 PN film with UV light and a Wratten 23A filter (19).

Isolation of DNA fragments. DNA fragments were isolated from agarose gels with glass powder as described by Vogelstein and Gillespie (21).

DNA and RNA hybridizations. Approximately 15 μ g of *Ava*I fragments were separated on single-slot preparative agarose gels. DNA was transferred overnight to a nitrocellulose sheet (Schleicher and Schuell, BA85) essentially as described by Southern (20). Hybridization of 32 P-labeled RNA to blotted DNA was done as previously described (14). Hybridization of nick-translated DNA was carried out using nitrocellulose strips that were preincubated for 6 h at 60°C in 6 $\times$ SSC (0.15 M NaCl-0.015 M sodium citrate) (pH 7.2), 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 50 μ g of denatured, sheared salmon sperm DNA per ml, and 0.1 mM EDTA. Strips were then transferred to vials containing the nick-translated DNA probe in 2 $\times$ SSC (pH 7.2), 0.02% Ficoll, 0.02% polyvinylpyrrolidone, 0.02% bovine serum albumin, 50 μ g of denatured, sheared salmon sperm DNA per ml, 0.1% SDS, and 0.1 mM EDTA. Hybridization was at 60°C for 18 h. Strips were then washed 10 times in 2 $\times$ SSC containing 0.5% SDS at 60°C (11). All hybridization strips were flattened, dried on a gel dryer (Bio-Rad), and autoradiographed on Kodak X-Omat R film.

Preparation of labeled RNA and DNA. 32 P-labeled RNA was synthesized *in vitro* by purified vaccinia virus as previously described (14). Immediate early, early, and late viral RNAs were extracted from the cytoplasm of infected cells. Approximately 5×10^7 HeLa cells were infected with vaccinia at 25 PFU/cell in 10 ml of phosphate-free medium containing 5% dialyzed horse serum with either 100 μ g of cyclohexamide per ml, an inhibitor of protein synthesis, for isolation of immediate early RNA, 40 μ g of cytosine arabinoside per ml, an inhibitor of DNA replication, for early RNA or without any additions for isolation of late RNA or for mock-infected cells. After 30 min, cells were diluted to 100 ml with the appropriate media and 32 P phosphoric acid added to 50 μ Ci/ml. Immediate early and early RNAs were isolated at 2 h postinfection (p.i.), and late RNA was isolated at 7 h p.i. as follows. Infected cells or mock-infected cells were washed twice with phosphate-buffered saline and suspended in 10 mM NaCl, 10 mM Tris-hydrochloride (pH 7.25), and 1.5 mM MgCl₂, allowed to swell on ice for ca. 10 min, and broken by Dounce homogenization. Nuclei were pelleted at 1,000 $\times$ g for 3 min, and the cytoplasm was adjusted to 0.01 M sodium acetate (pH 6.0), 0.1 M NaCl, 15 mM EDTA, and 0.5% SDS and extracted with phenol-chloroform (1:1) (16). RNA was precipitated with 2 volumes of ethanol and resuspended in 0.01 M Tris-hydrochloride (pH 7.4), 0.1 M NaCl, and 0.01 M MgCl₂ and passed over a column of Sephadex G50 equilibrated in the same buffer. The excluded material was then treated with 50 μ g of RNase-free DNase (Worthington) per ml for 30 min at 37°C. SDS was added to 0.5%, and the RNA was phenol extracted and ethanol precipitated as described above.

Fragments of vaccinia DNA were labeled by nick translation by the method of Rigby et al. (17). Reac-

tion mixtures contained, in 0.1 ml, 50 mM sodium phosphate (pH 7.6); 5 mM MgCl₂; 20 μ M dCTP, dATP, and dGTP; 14 μ M [α - 32 P]dTTP (128 Ci/mmol; New England Nuclear Corp.); 1 ng of DNase I; 100 U of DNA polymerase I (Boehringer Mannheim Corp.); and 0.1 to 1.0 μ g of DNA fragments. Reactions were incubated at 14°C for 4 h and terminated by adding 50 μ l of 0.5 M Na₃EDTA and heating to 68°C for 10 min. Unincorporated triphosphates were removed by gel filtration on Sephadex G50.

Terminal fragment analysis. Terminal restriction fragments were determined by denaturing DNA fragments with 0.1 M NaOH at 37°C for 15 min and then neutralizing with 0.1 M HCl in 20 mM Tris-hydrochloride (pH 7.6) (24). Under these conditions the cross-linked ends of vaccinia DNA reanneal, whereas un-cross-linked fragments do not. Denatured and reannealed fragments were run in parallel with undenatured fragments on agarose gels.

RESULTS

Restriction endonuclease cleavage of serially propagated and cloned vaccinia virus (strain WR). Figure 1 shows the restriction patterns observed when serially propagated virus was digested with restriction endonucleases *Hind*III, *Ava*I, and *Xho*I. The *Hind*III pattern for the virus is similar to that shown by McCarron et al. (9). Densitometer scanning of the photographic negatives revealed that *Hind*III fragments C and N were submolar, whereas fragment F was greater than 1 M. Similarly *Ava*I fragments H, J, L, and P and *Xho*I fragments F and H were present in submolar amounts. Fragments *Hind*III-C, *Ava*I-M and -N, and *Xho*I-G appeared as diffuse bands, suggesting that they are terminal fragments containing length heterogeneity as described by Wittek et al. (24).

The presence of submolar fragments in the vaccinia virus genome suggested that the serially propagated virus consisted of a mixed population of genomes. To determine the origin of the submolar fragments, we cloned serially propagated vaccinia virus by two cycles of plaque purification on CV-1 monolayers. Several clones were selected, and the DNA was extracted and analyzed with *Hind*III (Fig. 2). Two main variants were observed. In clones 2 and 3, fragments C and N were absent and fragment F was present at a 2 M concentration. Clones 4 to 7 had the same pattern as the serially propagate virus; however, all fragments were present at a 1 M ratio. The *Hind*III pattern obtained with clones 4 to 7 was similar to that shown by Gangemi and Sharp (3) and McCarron et al. (9) for cloned virus. Clones 2 and 7 were chosen for further analysis, clone 2 as representative of the small DNA variant (S) and clone 7 as the representative of the large DNA variant (L).

Determination of the termini in serially propagated virus and the cloned S and L

FIG. 1. Electrophoresis of restriction fragments of DNA from serially propagated vaccinia. DNA from serially propagated virus was digested with either *HindIII*, *AvaI*, or *XhoI* in analytical reactions containing 0.8 μ g of DNA. The resulting fragments were analyzed on 0.5 to 1.0% agarose gradient gels as described in the text. Gels were stained with ethidium bromide and photographed over a UV light through a Wratten 23A filter (Kodak) with Polaroid P/N 55 film.

variants. Since the termini of the linear vaccinia genome are cross-linked, the terminal fragments generated by restriction endonucleases can be readily detected by taking advantage of

their ability to rapidly reanneal after neutralization of the denatured DNA. Fragments derived from internal locations reanneal at a much slower rate (2). Terminal fragment analysis of the S and L variants (Fig. 3, lanes 4 and 6) indicated that fragments B and F in the S variant and fragments B and C in the L variant were the termini. In the serially propagated virus, the B fragment was present in molar quantities, whereas fragments C and F were approximately 0.5 M (Fig. 3, lane 2). Lanes 1, 3, and 5 of Fig. 3 show the *HindIII* profile of serially propagated S- and L-variant DNA before denaturation and reannealing.

The *HindIII* pattern obtained with serially propagated, S- and L-variant DNA (Fig. 2) and the terminal analysis of the *HindIII* digests of these DNAs (Fig. 3) suggested that the S variant was smaller by virtue of a deletion within the C

FIG. 2. *HindIII* analysis of cloned virus. DNAs (0.5 to 1.0 μ g) from serially propagated virus (stock) or from six clones of twice-plaque-purified virus (lanes 2 to 7) were digested with *HindIII*, and the fragments were analyzed on agarose gels as described in text.

FIG. 3. Determination of the *Hind*III terminal fragments of serially propagated, S- and L-variant DNAs. Terminal *Hind*III fragments were determined by denaturing *Hind*III fragments in alkali followed by rapid renaturation as described in the text. Fragments that reannealed were then analyzed on agarose gels. Undenatured *Hind*III fragments were analyzed in lanes 1, 3, and 5. Renatured fragments were analyzed in lanes 2, 4, and 6. Serially propagated viral DNA was in lanes 1 and 2, S-variant DNA was in lanes 3 and 4, and L-variant DNA was in lanes 5 and 6.

fragment and furthermore that the deletion was internal since fragment N was also absent (Fig. 2).

Endonuclease digestion of S- and L-variant DNA. The DNAs derived from the S and L variants were compared by *Hind*III, *Ava*I, and *Xho*I endonuclease digestion. *Ava*I fragments J,

H, L, and P were found to be submolar in serially propagated virus (Fig. 1). In cloned virus, however, *Ava*I fragments, J, H, and L were found only in the L-variant DNA, whereas *Ava*I fragment P was found only in the S-variant DNA (Fig. 4). It should also be noted that *Ava*I digestion of S-variant DNA generated an M fragment present at ca. a 2.5-fold higher concentration than that present in the L-variant DNA. Similarly *Ava*I generated an N fragment from the L-variant DNA at a threefold-greater concentration than the N fragment in the S-variant DNA (Fig. 4).

*Xho*I digestion of serially propagated virus DNA gave submolar fragments F and H (Fig. 1). *Xho*I digestion of L-variant DNA, however, gave both F and H fragments at unimolar concentrations (Fig. 4). Additionally, there appears to be a greater concentration of the J fragment upon *Xho*I cleavage of S-variant DNA than L-variant DNA (Fig. 4).

The molecular weights and molarities of the *Hind*III, *Ava*I, and *Xho*I digestion products of the S- and L-variant DNA are presented in

FIG. 4. *Hind*III, *Ava*I, and *Xho*I analysis of S- and L-variant DNA. S- and L-variant DNAs (0.8 μ g) were digested with either *Hind*III, *Ava*I, or *Xho*I in analytical reactions as described in the text. Fragments were analyzed by electrophoresis in agarose gels. *Hind*III digests of lambda DNA were run on the same gels as molecular weight markers.

Table 1. The average molecular weight of the L-variant DNA derived from such analysis is 123.7 megadaltons and that for the S-variant DNA is 117.4 megadaltons, a difference of ca. 6.3 megadaltons.

The procedure for terminal analysis of the *Ava*I and *Xho*I fragments was similar to that described for *Hind*III in Fig. 3. By such criteria, *Ava*I fragments M and N were determined to be the termini in the S- and L-variant DNAs, respectively; *Xho*I fragment G was determined as the terminal fragment on both S- and L-variant DNA (data not presented). It should be noted, however, that the G fragment from the L-variant DNA is smaller than the G fragment from the S-variant DNA (Fig. 4).

Analysis of all of the clones described in Fig. 2 with *Ava*I and *Xho*I showed that in addition to and independent of the major 6.3-megadalton deletion in the S-variant DNA, the terminal fragments of clones 2, 3, 4, and 7 varied in size (data not presented). This variation was expected, considering the heterogeneity present in the termini of uncloned virus as reported by Wittek et al. (24).

Mapping *Sma*I, *Sst*II, *Ava*I, and *Xho*I cleavage sites on the S and L vaccinia variants. Wittek et al. (23) have presented a *Hind*III restriction map for the Elstree strain of vaccinia. The *Hind*III pattern for the WR strain is similar, except for the terminal fragments B and C as shown by McCarron et al. (9) and Mackett and Archard (8). To compare the L- and S-variant DNAs, we chose to map the variants with *Sma*I, *Sst*II, *Ava*I, and *Xho*I. *Sma*I and *Sst*II were chosen because they cleaved vaccinia DNA infrequently. *Sma*I cleaves at one site, and *Sst*II cleaves at four sites. *Ava*I and *Xho*I were chosen for the following reasons: (i) they cleave more frequently than *Hind*III (*Ava*I, 21 to 22 times; *Xho*I, 15 to 16 times), (ii) cleavage sites present in the L variant which are not present in the S variant were detected with these endonucleases, and (iii) *Ava*I cleaves at the sequence CPYCGPuG, whereas *Sma*I cleaves at CCCGGG and *Xho*I cleaves at CTCGAG; therefore *Sma*I and *Xho*I produce subsets of *Ava*I fragments which facilitated mapping.

Figure 5 shows the *Sma*I, *Sst*II, and *Sma*I-*Sst*II double digestion patterns for both the S-

TABLE 1. Molecular weights of restriction fragments from the large and small vaccinia WR strain variants^a

Fragment	Mol wt ($\times 10^{-6}$)					
	<i>Hind</i> III		<i>Ava</i> I		<i>Xho</i> I	
	L	S	L	S	L	S
A	33.8	33.8	25	25	28	27
B	19.8 ^b	19.8 ^b	16	16	23.5	23.5
C	13.9 ^b		14.4	14.4	14.4 (2)	14.4 (2)
D	10.2	10.2	10.5	10.5	7.2	7.2
E	9.6	9.6	7.5	7.5	6.2	6.2
F	8.6	8.6 ^b (2)	7.2	7.2	4.5	
G	5.7	5.7	6.2	6.2	4.2 ^b (2)	4.25 ^b (2)
H	5.5	5.5	4.5		3.4	
I	4.1	4.1	3.8	3.8	3.3	3.3
J	3.1	3.1	3.4		2.6 (2)	2.6 (3)
K	2.8	2.8	3.3	3.3	2.55	2.55
L	2.6	2.6	3.0		2.2	2.2
M	1.6	1.6	2.6 (2)	2.6 ^b (5)	~ 0.4	~ 0.4
N	1.1		2.55 ^b (3)	2.55 (1)		
O	0.95	0.95	2.2	2.2		
P				1.9		
Q			1.65 (2)	1.65 (2)		
R			~ 0.5	~ 0.5		
S			~ 0.4	~ 0.4		
Total	123.35	116.95	124.05	117.75	123.65	117.45
L - S ^c		6.4		6.3		6.2

^a The molecular weights of vaccinia DNA fragments were estimated by comparison with the mobilities of lambda DNA and lambda *Hind*III fragments. The larger fragments were digested with a second enzyme, and the molecular weight was determined by summing the molecular weights of the resulting smaller fragments. Numbers in parenthesis indicate the molarities of each fragment determined by scanning photographic negatives with a Gelman scanning densitometer and mapping.

^b Terminal fragments were determined by alkaline denaturation of restricted DNA and analysis on agarose gels as described in the text.

^c Molecular weight difference between the large and small variant DNA.

and L-variant DNAs. *Sst*II digestion of the S and L variants generated five fragments. *Sst*II fragment B was present at a 2 M concentration and represented the termini as defined by the terminal analysis described in Materials and Methods (data not presented). The *Sma*I-*Sst*II double digestion of both the S and L variants generated six fragments (Fig. 5). Terminal analysis indicated fragments B and C as the termini (data not presented).

The *Sma*I and *Sst*II cleavage sites were mapped by double digestions with *Hind*III. *Sma*I-*Hind*III digestion of S-variant DNA (Fig. 6) indicated that the *Sma*I site was in the *Hind*III A fragment, reducing the size of *Hind*III A to ca. 30 megadaltons and producing a new fragment which comigrated with *Hind*III fragment J (ca. 3.1 megadaltons). The *Sma*I cleavage site was confirmed by digestion of the *Sma*I-B fragment with *Hind*III, which yielded *Hind*III-B and a 3-megadalton fragment (data not presented). This indicated that the terminal

FIG. 5. Restriction endonuclease digestion of S- and L-variant DNAs with *Sma*I and *Sst*II. S- or L-variant DNA (0.4 to 0.8 μ g) was digested with either *Sma*I, *Sst*II, or *Sma*I-*Sst*II and analyzed on agarose gels as described in the text. Lambda DNA and lambda *Hind*III fragments were run in parallel as molecular weight markers.

FIG. 6. *Hind*III-*Sma*I or *Hind*III-*Sst*II double digestion patterns of S-variant DNA. S-variant DNA (~ 1.0 μ g) was double digested with *Hind*III-*Sma*I or *Hind*III-*Sst*II in analytical reactions as described in the text. The resulting fragments were then analyzed on agarose gels as described previously. Lane 1, *Hind*III-*Sma*I; lane 2, *Hind*III; lane 3, *Hind*III-*Sst*II. Lambda *Hind*III fragments were run in parallel as molecular weight markers.

*Hind*III-B fragment was adjacent to *Hind*III-A, in confirmation of the *Hind*III map of the WR strain presented by Mackett and Archard (8).

*Sst*II cleavage sites were determined in a similar fashion. The *Sst*II-*Hind*III double digestion of S-variant DNA (Fig. 6) yielded *Sst*II cleavage sites in *Hind*III fragments A, G, I, and L. Since terminal analysis of the *Sst*II restriction fragments indicated that both B fragments were terminal, the *Sst*II cleavage sites in *Hind*III-A and -I could be mapped. The remaining two *Sst*II cleavage sites were determined from the molecular weights of the double-digestion products (Table 2). Isolation and subsequent digestion of individual *Sma*I-*Sst*II fragments with *Hind*III confirmed the *Sst*II cleavage sites (data not presented).

TABLE 2. Molecular weights of double digestion fragments of S-variant DNA with *Hind*III, *Sst*II, and *Sma*I restriction enzymes^a

Fragment	Mol wt ($\times 10^{-6}$)					
	<i>Sma</i> I ^c	<i>Sst</i> II	<i>Sst</i> II- <i>Sma</i> I	<i>Hind</i> III	<i>Hind</i> III- <i>Sma</i> I	<i>Hind</i> III- <i>Sst</i> II
A	94	40.7	40.7	33.8	~ 30.0	19.5 ^b (2)
B	22.9	32.8 ^b (2)	32.8 ^b	19.8 ^b	19.8 ^b	13.8
C		9.0	22.5 ^b	10.2	10.2	10.2
D		1.1	10.3	9.6	9.6	9.6
E			9.0	8.6 ^b (2)	8.6 ^b (2)	8.6 ^b (2)
F			1.1	5.7	5.7	5.5
G				5.5	5.5	5.3
H				4.1	4.1	3.6
I				3.1	3.1 (2)	3.1
J				2.8	2.8	2.8
K				2.6	2.6	2.0
L				1.6	1.6	1.6
M				0.95	0.95	0.95
N						~ 0.6
O						~ 0.5
P						~ 0.4

^a Molecular weights were determined by comparison to lambda DNA *Hind*III fragments. Restriction digests were carried out as described in the text. Where two enzymes were used for digestion, the more complete enzyme buffer was used. Numbers in parentheses indicate the molarity of the fragments if greater than one.

^b Terminal fragments determined as described in the text.

^c Molecular weights were determined from *Hind*III-*Sma*I double digestion.

The molecular weights and molarities of the S-variant fragments generated by *Sma*I, *Sst*II, and the *Hind*III double digestions are presented in Table 2. *Sma*I-A fragment and the *Sst*II-B fragment generated from the L-variant DNA were larger by ca. 6.3 megadaltons, and no differences, other than in the size of the *Hind*III left terminal fragment, were detected in the *Hind*III double digestion.

*Ava*I and *Xho*I cleavage sites were determined by digestion of isolated *Hind*III or *Sma*I-*Sst*II fragments. Due to the large number of individual fragments digested, the results are presented in Table 3 rather than in figures. By determining the *Ava*I and *Xho*I fragments, including partial fragments found in overlapping *Hind*III or *Sma*I-*Sst*II fragments along with the *Ava*I digestion of *Xho*I fragments, it was possible to order most of the *Ava*I cleavage sites in both the S and L variants. Two pairs of *Ava*I fragments could not be ordered in this way: fragments D and I and fragments J and H. The order of J and H was subsequently determined by hybridization data (see below). The order of *Ava*I fragments D and I was determined by other studies with additional enzymes (data not presented). The two smallest *Ava*I fragments, R and S, and *Xho*I-M were too small to detect by ethidium bromide staining in the double digests and therefore were not mapped. The restriction maps of *Sma*I, *Sst*II, *Hind*III, *Ava*I, and *Xho*I for the S and L variants of the WR strain of vaccinia are presented in Fig. 7.

Hybridization of isolated *Ava*I fragments to Southern blots. The restriction endonuclease maps of both the L- and S-variant DNAs indicated that they were identical except for the 6.3-megadalton deletion in the S variant. To determine whether the region deleted consisted of unique sequences, isolated *Ava*I fragments H, J, and L were nick translated and hybridized to *Ava*I fragments of both the L- and S-variant DNAs immobilized on nitrocellulose. As noted in Fig. 8, the *Ava*I-H fragment hybridized to its homologous DNA fragment from the L variant (lane 4) but not at all to the S variant DNA (lane 5), indicating that the majority of sequences deleted from the S variant are unique and are not repeated elsewhere in the genome. Additionally, the hybridization data indicated that at least some of the *Ava*I-J sequences fell within the terminally repeated region of the vaccinia genome since the probe hybridized not only to its homologous DNA fragment (Fig. 8, lane 3) as expected but also to *Ava*I-M in both the S- and L-variant DNA blots (Fig. 8, lanes 3 and 6). Furthermore, *Ava*I fragment L hybridized to its own homologous fragment and to *Ava*I-P in the S-variant DNA blot (Fig. 8, lanes 2 and 7), indicating that *Ava*I-P lies to the right of the deletion.

Hybridization of RNA synthesized in vivo or in vitro to *Ava*I restricted S- and L-variant vaccinia DNA. To determine whether the additional sequences present in the L variant were transcribed, we hybridized RNA synthe-

TABLE 3. *Redigestion of isolated fragments with *Ava*I and *Xho*I*

Fragment ^a di- gested	Var- iant	Second enzyme				Fragment ^a di- gested	Var- iant	Second enzyme			
		<i>Ava</i> I		<i>xho</i> I				<i>Ava</i> I		<i>xho</i> I	
		Intact frag- ments	Partial frag- ments (mol wt)	Intact frag- ments	Partial frag- ments (mol wt)			Intact frag- ments	Partial frag- ments (mol wt)	Intact frag- ments	Partial frag- ments (mol wt)
<i>Sst</i> II- <i>Sma</i> I-A+B	L	C D H I J L N N O Q	25 4.0 3.1			<i>Hind</i> III-C	L	H J N Q	1.9	F G H	1.8
						<i>Hind</i> III-D	S, L		9.2 0.9		9.2 0.9
						<i>Hind</i> III-G+H	S		5.4 3.4 2.2 0.2		
<i>Sst</i> II- <i>Sma</i> I-A	S	C D I N O	4.0 3.1	C C K L	4.0 3.1	<i>Hind</i> III-I	S		2.6 1.7		
						<i>Hind</i> III-J	S		1.9 1.1		
<i>Sst</i> II- <i>Sma</i> I-B	S	M M P Q	24			<i>Hind</i> III-L	S		2.2 0.4		
<i>Sst</i> II- <i>Sma</i> I-C	S, L	B M M or N Q		G J	16	<i>Xho</i> I-G	L	N Q			
						<i>Xho</i> I-G	S	M Q			
<i>Sst</i> II- <i>Sma</i> I-D	L	E	3.0		7.5 3.0	<i>Xho</i> I-C (2)	S, L	C D I			
<i>Sst</i> II- <i>Sma</i> I-E	L	G	2.0 0.9	E	1.9 0.9	<i>Xho</i> I-B	S, L	B E			
<i>Hind</i> III-A	S, L	D E F I	3.0 1.7	C D	12.0 1.0	<i>Xho</i> I-A	L	A L			
<i>Hind</i> III-B	S, L	M M or N Q	13	G J	13	<i>Xho</i> I-A	S	A P			
<i>Hind</i> III-F (2)	S	M M Q	1.8 8.6 ^b								

^a Individual restriction fragments were isolated from preparative agarose gels with glass powder and then digested with a second enzyme in standard analytical reactions and analyzed on agarose gels. *Ava*I, *Xho*I, and *Hind*III digests were run for comparison of fragments. Vaccinia *Hind*III fragments were used as molecular weight standards to determine the molecular weight of partial ($\times 10^{-6}$) fragments.

^b Internal *Hind*III-F fragment which was uncleaved by *Ava*I.

sized in vitro or in vivo to Southern blots of *Ava*I fragments. RNA transcribed in vitro by L-variant virus hybridized to a number of *Ava*I fragments, including *Ava*I fragments H, J, and L (Fig. 9A, lane 2); RNA synthesized in vitro by S-variant virus hybridized to a number of *Ava*I fragments but significantly did not hybridize to

L-variant *Ava*I fragment H (Fig. 9A, lane 3); it did, however, hybridize to L-variant *Ava*I fragments J and L (Fig. 9A, lane 3).

To demonstrate whether the unique sequences present in the L-variant DNA were expressed in vivo, we purified ³²P-labeled RNA from L-variant virus-infected HeLa cells and

FIG. 7. *Sma*I, *Sst*II, *Hind*III, *Xho*I, and *Ava*I restriction maps of the S- and L-variant vaccinia DNAs. The *Sma*I and *Hind*III cleavage maps of the L-variant DNA are the same as those described by Mackett and Archard (8). Dotted lines represent the DNA sequences deleted from the S-variant DNA. *Sma*I and *Xho*I cleavage sites represent a subset of the *Ava*I sites.

hybridized the RNA to *Ava*I fragments from the L-variant DNA fixed to nitrocellulose. As demonstrated in Fig. 9B, *in vivo* RNA hybridized to the unique *Ava*I-H sequences. The unique *Ava*I-H sequences were thus shown to be transcribed *in vivo* in the presence of either cycloheximide or cytosine arabinoside (Fig. 9B, lanes 1 and 2, respectively), thus defining the region as an immediate early-early transcriptional unit.

DISCUSSION

In addition to the minor size differences present at the termini of vaccinia virus DNA reported by Wittek et al. (24) and also observed in our studies, we have described an additional major difference in the genomes of serially propagated vaccinia virus (strain WR). This major difference was shown to reside in a 6.3-megadalton deletion of unique DNA sequences occurring in the S variant. This deletion was mapped at ca. 6.85 megadaltons from the left terminus of the vaccinia genome. The deletion, therefore, probably falls outside of the inverted terminal repetition of ca. 6.8 megadaltons (4); however, additional fine mapping of this region is required to determine more precisely the location of the deletion.

The terminal heterogeneity observed in unclosed virus by Wittek et al. (24) and in the studies reported here apparently occurs within the last 2.6 megadaltons of the genome since endonuclease digestion of several clones with *Ava*I indicated only the very terminal *Ava*I-M fragments varied in size. The differences in the termini ranged from approximately 50 to 300 base pairs in size (unpublished data). This is similar in size to the deletions found in the

terminally repeated region of the IDH-W strain of vaccinia reported by McFadden and Dales (10).

The 6.3-megadalton deletion represents approximately 5% of the L-variant genome. This region was shown to be transcribed both *in vitro* and *in vivo*. The deletion, however, had no effect on the replication of the S variant in HeLa cells. One-step growth curves of the S and L variants in HeLa cells gave similar kinetics of replication and similar yields when titrated on CV-1 monolayers (S. Mercer, unpublished data). It will be interesting to determine whether differences in structural or nonstructural protein compositions between these two variants can be detected.

The *Hind*III and *Sma*I maps presented here for the L variant of the WR strain are identical to the maps presented for the same vaccinia strain by Mackett and Archard (8). The *Xho*I map for the L variants is also similar to that of Mackett and Archard except for a minor difference in the region adjacent to the left terminal repeated sequence where the deletion occurs in our S variant. This may reflect additional differences between our vaccinia strain and the one analyzed by Mackett and Archard. In addition, we have presented an *Ava*I and *Sst*II map of both S and L variants of the WR strain which should be useful for further physical and transcriptional mapping.

*Hind*III and *Xho*I maps of a variety of vaccinia strains presented by Mackett and Archard (8) indicate that the central regions of strains WR, DIE, HI, and Elstree are highly conserved. Shümperli et al. (18) have recently compared rabbit poxvirus (Utrecht strain) and the Elstree strain of vaccinia by detailed restriction map-

FIG. 8. Hybridization of isolated *AvaI* L-variant fragments H, J, and L to S- and L-variant *AvaI* fragments bound to nitrocellulose. *AvaI* fragments H, J, and L were isolated from a preparative gel with glass powder as described in the text. The isolated fragments were labeled with ^{32}P by nick translation and then hybridized to Southern blots of S- or L-variant *AvaI* fragments bound to nitrocellulose under the conditions described in the text. L-variant *AvaI* fragments (lanes 1 to 4); S-variant *AvaI* fragments (lanes 5 to 8). Hybridization of ^{32}P -labeled fragments was done at a constant radioactivity of 6×10^6 cpm/ml. *AvaI*-L fragment (lanes 2 and 7); *AvaI*-J fragment (lanes 3 and 6); and *AvaI*-H fragment (lanes 4 and 5). Total ^{32}P -labeled L- and S-variant DNA was hybridized to strips 1 and 8, respectively, at 2×10^6 cpm/ml. All nitrocellulose strips were washed extensively after hybridization as described in the text, dried, and exposed to Kodak X-Omat R film.

ping, and their results indicated that the detectable differences occurred entirely at the termini. These differences included some of the terminal repeated sequences as well as adjacent unique sequences. Gangemi and Sharp (3) and McCarron et al. (9) have reported differences between the WR and CV-1 strains of vaccinia. The main difference appeared to be in the size of the

FIG. 9. Hybridization of in vitro- and in vivo-labeled vaccinia RNA to *AvaI* fragments bound to nitrocellulose. (A) S- or L-variant vaccinia RNA synthesized in vitro was hybridized to *AvaI* blots of S- or L-variant *AvaI* fragments. Hybridization was in $2\times$ SSC (pH 7.4) mixed with an equal volume of phenol-saturated $2\times$ SSC at a constant radioactivity of 3×10^6 cpm/ml. Hybridization was at 60°C for 18 h. L-variant *AvaI* fragments (lanes 1 to 3), S-variant *AvaI* fragments (lanes 4 to 6). L-variant RNA was hybridized to lanes 2 and 4, and S-variant RNA was hybridized to lanes 3 and 5. Ethidium bromide-stained *AvaI* fragments of the respective L and S variants are shown in lanes 1 and 6. (B) RNA synthesized in vivo by L-variant vaccinia virus either in the presence of cycloheximide (immediate early RNA) or cytosine arabinoside (early RNA) or in the absence of drugs (late total RNA) was hybridized to L variant *AvaI* fragments as described above at a constant radioactivity of 6×10^6 cpm/ml. Lane 1, immediate early RNA; lane 2, early RNA; lane 3, late RNA. All hybridization strips were exposed to Kodak X-Omat R film with a Cronex lightening plus intensifying screen at -70°C .

HindIII-C fragment. However, it does not appear to be related to the deletion found in the S variant reported here.

Deletion mutants have been found in other poxviruses. Archard and Mackett (1) have reported a white pock mutant of red cowpox which contains a large deletion (approximately 12% of the genome) near one terminus. Similarly, white

pock mutants of rabbit poxvirus which contain large deletions near the termini which affect host range have been described by Moyer and Rothe (13). It would be interesting to see whether the deletion in the S variant reported here results in host range restrictions.

The deletion of unique sequence DNA described for the S variant in this report most likely represents the loss of unique vaccinia-specific sequences. It cannot be ruled out, however, that the additional sequences present in the L variant may represent a stable insert of cellular DNA acquired by the virus at an earlier time. Deletions such as the one described here or stable insertions could account for some of the differences observed between the various strains of vaccinia and other poxviruses. It would not be surprising if further analysis of our serially propagated virus yielded additional genomic variants.

Hybridization of vaccinia RNA transcribed *in vivo* to *AvaI* restriction fragments of both the S and L variants reported here suggests that it might be a useful means of obtaining information on the temporal control of transcription from different regions of the genome. For example, *AvaI* fragments D, G, K, and N (Fig. 9) appear to be transcribed preferentially after viral DNA replication, whereas transcription from fragments B and E appears to be turned off late in infection. We are currently conducting more detailed transcriptional analysis of the S and L variants using various *HindIII*, *SstII*, and *AvaI* fragments.

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LITERATURE CITED

1. Archard, L. C., and M. Mackett. 1979. Restriction endonuclease analysis of red cowpox virus and its white pock variant. *J. Gen. Virol.* 45:51-63.
2. DeFilippes, F. M. 1976. Restriction enzyme digests of rapidly renaturing fragments of vaccinia virus DNA. *J. Virol.* 17:227-238.
3. Gangemi, J. D., and D. G. Sharp. 1976. Use of a restriction endonuclease in analyzing the genomes of two different strains of vaccinia virus. *J. Virol.* 20:319-323.
4. Garon, C. F., E. Barbosa, and B. Moss. 1978. Visualization of an inverted terminal repetition in vaccinia virus DNA. *Proc. Natl. Acad. Sci. U.S.A.* 75:4863-4867.
5. Geshelein, P., and K. I. Berns. 1974. Characterization and localization of the naturally occurring cross-links in vaccinia virus DNA. *J. Mol. Biol.* 88:785-796.
6. Grady, L. J., and E. Paoletti. 1977. Molecular complexity of vaccinia DNA and the presence of reiterated sequences in the genome. *Virology* 70:337-341.
7. Joklik, W. K. 1962. The purification of four strains of poxvirus. *Virology* 18:9-18.
8. Mackett, M., and L. C. Archard. 1979. Conservation and variation in orthopoxvirus genome structure. *J. Gen. Virol.* 45:683-701.
9. McCarron, R. J., C. V. Cabrera, M. Esteban, W. T. McAllister, and J. A. Holowczak. 1978. Structure of vaccinia DNA: analysis of the viral genome by restriction endonucleases. *Virology* 86:88-101.
10. McFadden, G., and S. Dales. 1979. Biogenesis of poxviruses: mirror-image deletions in vaccinia virus DNA. *Cell* 18:101-108.
11. Mears, J. G., F. Ramirez, D. Liebowitz, F. Nakamura, A. Bloom, F. Konotey-Ahulu, and A. Bank. 1978. Changes in restricted human cellular DNA fragments containing globin gene sequences in thalassemias and related disorders. *Proc. Natl. Acad. Sci. U.S.A.* 75:1222-1226.
12. Moss, B. 1974. Reproduction of poxviruses, p. 405-474. In H. Fraenkel-Conrat and R. R. Wagner (ed.), *Comprehensive virology*, vol. 3. Plenum Press, New York.
13. Moyer, R. W., and C. T. Rothe. 1980. The white pock mutants of rabbit poxvirus. I. Spontaneous host range mutants contain deletions. *Virology* 102:119-132.
14. Paoletti, E., B. R. Lipinskas, and D. Panicali. 1980. Capped and polyadenylated low-molecular-weight RNA synthesized by vaccinia virus *in vitro*. *J. Virol.* 33:208-219.
15. Pedrolli-Noy, G., and A. Weissbach. 1977. Evidence of a repetitive sequence in vaccinia virus DNA. *J. Virol.* 24:406-407.
16. Perry, R. P., J. Latorre, D. E. Kelly, and J. R. Greenberg. 1972. On the lability of poly(A) sequences during extraction of messenger RNA from polyribosomes. *Biochim. Biophys. Acta* 262:220-226.
17. Rigby, P. W. J., M. Dieckmann, C. Rhodes, and P. Berg. 1977. Labeling deoxyribonucleic acid to high specific activity by nick translation with DNA polymerase I. *J. Mol. Biol.* 113:237-251.
18. Schümperli, D., A. Menna, F. Schwendimann, R. Wittek, and R. Wyler. 1980. Symmetrical arrangement of the heterologous regions of rabbit poxvirus and vaccinia virus DNA. *J. Gen. Virol.* 47:385-398.
19. Sharp, P. A., B. Sugden, and J. Sambrook. 1973. Detection of two restriction endonuclease activities in *Haemophilus parainfluenzae* using analytical agarose-ethidium bromide electrophoresis. *Biochemistry* 12:3055-3063.
20. Southern, E. M. 1975. Detection of specific sequences among DNA fragments separated by gel electrophoresis. *J. Mol. Biol.* 98:503-517.
21. Vogelstein, B., and D. Gillespie. 1979. Preparative and analytical purification of DNA from agarose gels. *Proc. Natl. Acad. Sci. U.S.A.* 76:615-619.
22. Wittek, R., A. Menna, H. K. Müller, D. Schümperli, P. G. Boseley, and R. Wyler. 1978. Inverted terminal repeats in rabbit poxvirus and vaccinia virus DNA. *J. Virol.* 28:171-181.
23. Wittek, R., A. Menna, D. Schümperli, S. Stoffel, H. K. Müller, and R. Wyler. 1977. *HindIII* and *SstII* restriction sites mapped on rabbit poxvirus and vaccinia virus DNA. *J. Virol.* 23:669-678.
24. Wittek, R., H. K. Müller, A. Menna, and R. Wyler. 1978. Length heterogeneity in the DNA of vaccinia virus is eliminated on cloning the virus. *FEBS Lett.* 90:41-46.