

Molecular Cloning and Sequence of the Concatemer Junction from Vaccinia Virus Replicative DNA

Viral Nuclease Cleavage Sites in Cruciform Structures

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The concatemer junction from replicative forms of vaccinia virus DNA was cloned into plasmid vectors and shown to be a precise duplex copy of the viral terminal hairpin structure, with each strand corresponding to one of the alternative sequence isomers. The plasmids were relaxed circles with extruded cruciforms representing two copies of the vaccinia telomere hairpin structure. Head-to-head dimers containing two copies of the vaccinia virus concatemer junction were observed to contain only one set of stem-loop structures per molecule, suggesting that the initial formation of a small cruciform, and not branch migration, was the rate-limiting step in cruciform formation.

The plasmids containing the concatemer junction were converted into nicked circular, linear and cross-linked linear molecules by a nuclease isolated from vaccinia virions. The region-specific cleavage near the border of the hairpin loop and the formation of DNA cross-links in some of the molecules is consistent with the nuclease acting as a nicking-closing enzyme that participates in the resolution of mature termini from replicative concatemer intermediates.

1. Introduction

Several strategies have evolved for replicating the 5' ends of linear genomes in the absence of a primer-independent DNA polymerase (Kornberg, 1981). Some genomes rely on circular or concatemer replicative intermediate to copy their ends while others use protein primers to initiate replication at the 5' terminus. Another solution utilizes the ability of terminal palindromes to fold back on themselves to form hairpins. The 5' end of the genome can be replicated by a site-specific nick and hairpin transfer or by site-specific nicking, branch migration and religation to form hairpin ends (Bateman, 1975). These processes generate two daughter molecules with terminal hairpins that are complementary and inverted. In vaccinia virus, the prototypical member of the poxvirus family, the mature 185,000 base-pair (bp†) genome has cross-

linked ends (Geshelin & Berns, 1974), which were shown to be 104-nucleotide hairpin structures (Baroudy *et al.*, 1982). The hairpin structures are extremely rich in adenylate and thymidylate residues, cannot completely base-pair, and exist in two forms that are inverted and complementary.

The exact mechanism for the replication of vaccinia virus DNA is poorly understood; however, a series of studies has shown that nicks are apparently introduced near the ends of parental DNA molecules early after infection and the nicks are sealed as cross-links in daughter molecules at late times (Pogo, 1977, 1980; Pogo & O'Shea, 1978; Esteban & Holowczak, 1977a,b; Pogo *et al.*, 1984). Furthermore, during viral replication the terminal sequences were detected in concatemeric forms by restriction digests, of intracellular forms of rabbit pox (Moyer & Graves, 1981) and vaccinia (Baroudy *et al.*, 1983; Moss *et al.*, 1983) virus DNA. These observations are consistent with models for DNA replication of both poxviruses (Moyer &

† Abbreviations used: bp, base-pair; kb, 10³ base-pairs.

Graves, 1981; Baroudy *et al.*, 1982) and parvoviruses (Berns & Hauswirth, 1979; Astell *et al.*, 1985), which involve site-specific nicking and religation of concatemers proximal to mismatched nucleotides of the hairpin in order to generate sequence conformers. The putative enzymes responsible for these activities could have similarities to the gene A protein of ϕ X174 (Eisenberg *et al.*, 1977) or topoisomerase type I (Gellert, 1981) with regard to an ability to break and rejoin DNA strands transiently. Recently, Lakritz *et al.* (1985) demonstrated that a vaccinia nuclease can cleave supercoiled plasmid and simian virus 40 (SV40) DNA and cross-link the DNA strands.

The purpose of the present study was to clone molecularly the concatemer junction of replicating vaccinia virus DNA in order to confirm its predicted structure and to prepare substrates for analyzing the activities of viral proteins *in vitro*. The concatemer junction was shown to be a precise duplex of the vaccinia virus terminal hairpin structure, with each strand corresponding to one of the alternative sequence isomers. Analysis using nucleases, chemical reagents and electron microscopy demonstrated that the plasmids were relaxed circles with extruded cruciforms corresponding to two copies of the mature vaccinia telomere. We have also studied the *in vitro* interaction of a nuclease purified from virions with the concatemer junction. The enzyme was shown to cleave primarily near the border of the hairpin loop and in some cases cross-link the ends of the linear molecules. These activities are consistent with a nicking-closing enzyme that would resolve concatemers to form hairpin termini. We have demonstrated (Merchlinsky & Moss, 1986) that these recombinant plasmids are resolved into linear vaccinia minichromosomes when they are transfected into cells that are infected with vaccinia virus.

2. Materials and Methods

(a) Cloning of concatemer junction fragments

Replicating viral DNA was prepared from 4 l of HeLa cells infected at a multiplicity of 30 plaque-forming units per cell with vaccinia virus strain WR. The cells were harvested 3.5 h post-infection and protein-nucleic acid complexes containing replicative intermediates were isolated by centrifuging the infected cell cytoplasm through a discontinuous sucrose gradient (Moyer & Graves, 1981). This viosome fraction was brought to 1% sodium dodecyl sulfate (SDS) and treated with 4 mg proteinase K/ml at 37°C overnight. The samples were gently extracted with phenol, phenol/chloroform, and chloroform before precipitation with ethanol. The pellet was resuspended in TE buffer (10 mM-Tris·HCl (pH 8.0), 1 mM-EDTA), digested with *Bst*EII, and the DNA fragments were resolved by agarose gel electrophoresis. A fragment of 2.6 kb corresponding to the concatemer junction was recovered by dissolving the gel slice in 6 M-sodium perchlorate (Yang & Wu, 1979) followed by extraction with glass beads (Vogelstein & Gillespie, 1979). The junction fragment was digested with *Pvu*I and ligated to *Pvu*I-digested, dephosphorylated pBR322. The

ligated DNA was used to transform *Escherichia coli* LE392 or HB101 and the ampicillin-sensitive, tetracycline-resistant colonies were screened by colony hybridization with vaccinia DNA labeled with 32 P by nick-translation (Rigby *et al.*, 1977). Two recombinants containing the *Pvu*I concatemer junction fragment were designated pPD3 and pPD4.

The *Bst*EII concatemer junction fragment was directly cloned into plasmid vectors. The gel-purified *Bst*EII junction fragment was treated with *E. coli* DNA polymerase I, Klenow fragment, in order to convert the 5' overhang to a blunt end (Maniatis *et al.*, 1982), and ligated to *Bam*HI linkers. After digestion with *Bam*HI, the concatemer junction fragment was purified from an agarose gel and ligated to *Bam*HI-digested, dephosphorylated pUC13 or pBR322. Ampicillin-resistant colonies were screened with vaccinia DNA labeled with 32 P by nick-translation, and recombinants pBD6 and pBD22 containing the *Bst*EII concatemer junction in pUC13 and pBR322, respectively, were isolated.

The plasmid containing the *Sal*I concatemer bridge fragment was constructed from recombinant DNA. The plasmid pAG5 (Baroudy *et al.*, 1982), which contains a 3.5 kb insert including the viral sequences 0.1 to 3.6 kb from the terminus, was digested with *Bst*EII and *Sal*I. The *Bst*EII-*Sal*I fragment corresponding to the viral DNA 1.3 to 3.6 kb from the terminus was purified and ligated to *Sal*I-digested, dephosphorylated pUC13. The ligation mixture was digested with *Bst*EII and the 7.3 kb fragment containing the *Bst*EII-*Sal*I fragment attached to both ends of the linearized vector was agarose-gel-purified and ligated to the *Bst*EII concatemer junction fragment from pBD22. A recombinant containing the *Sal*I concatemer junction fragment was detected and designated pSD1.

(b) Isolation of DNA

DNA was routinely prepared from pPD or pBD by alkaline lysis followed by banding in isopycnic CsCl gradients (Maniatis *et al.*, 1982). In order to prepare DNA from pSD1, chloramphenicol-amplified cultures were pelleted by low-speed centrifugation, resuspended in 20 ml of 25 mM-Tris·HCl (pH 8.0), 10 mM-EDTA, 50 mM-glucose per liter, placed on ice and treated sequentially with 50 mg of lysozyme, 5 ml of 0.5 M-EDTA and 0.3 ml of 10% Triton X-100. The bacterial chromosome was pelleted, the supernatant was expanded to 30 ml and 31 g of CsCl was added. The samples were clarified by a low-speed centrifugation and the DNA was banded by overnight centrifugation. The supercoiled DNA was removed by side-puncture, extracted with 1-butanol, dialyzed against TE buffer, and precipitated with ethanol.

(c) DNA sequencing

The recombinants pPD3 and pPD4 were digested with *Ava*II and *Hinc*II, and the fragment containing the vaccinia DNA insert was gel purified. The fragments were labeled at the *Ava*II site with [α - 32 P]dGTP (Maniatis *et al.*, 1982) and the sequence was determined using the method described by Maxam & Gilbert (1980). The reactions used were G, G+A, C, C+T and A>C, and the reaction products were separated on 6% or 8% polyacrylamide/8 M-urea gels.

(d) Purification of vaccinia nuclease

Soluble extracts of vaccinia virions were prepared by lysis in deoxycholate as described by Rohrmann & Moss

(1985). The enzyme was purified from crude extracts by sequential elution over hydroxyapatite (Rosemond-Hornbeck *et al.*, 1974), heparin-agarose, and Affi-gel blue agarose (all from Bio-Rad). Proteins in the peak in nuclease fractions from the Affi-gel blue column were dissociated with β -mercaptoethanol and SDS, and analyzed by polyacrylamide gel electrophoresis; 2 polypeptides with approximate molecular weights of 20,000 (20K) and 50,000 (50K) were detected by silver staining. The 50K polypeptide appears to be the vaccinia nuclease, since it co-eluted with the peak of enzyme activity during other purification schemes whereas the 20K polypeptide did not. Evidence correlating the 50K polypeptide with endonuclease activity was obtained by Rosemond-Hornbeck *et al.* (1974) and Lakritz *et al.* (1985).

(e) Nucleases

DNA was incubated with vaccinia nuclease as described by Lakritz *et al.* (1985). The reactions were typically 20 μ l, containing DNA, 10 mM-Mes [2-(*N*-morpholino)-ethanesulfonic acid, pH 6.5], 1 mM-EDTA, 100 μ g bovine serum albumin/ml and vaccinia nuclease at 37°C or 55°C for 2 h. The reaction was stopped by addition of 0.1 vol. of dye buffer (0.3% xylene cyanol FF, 0.3% bromophenol blue, 1 M-urea, 50 mM-Tris·HCl (pH 8.0), 20 mM-EDTA, 0.2% Sarkosyl, 30% Ficoll 400) and loaded directly onto an agarose gel or stored on ice.

S_1 nuclease was obtained from Pharmacia. Incubations included 7.5 μ g DNA in 100 μ l with 0.2 M-NaCl, 30 mM-sodium acetate (pH 4.6), 1 mM-ZnSO₄ at 37°C for 30 min and were stopped by addition of 0.1 vol. of dye buffer and loaded onto agarose gels.

Bacteriophage T4 endonuclease VII was a gift from K. Mizuuchi and was used as described by Mizuuchi *et al.* (1982a) or Lilley & Kemper (1984). Incubations were performed using 20 μ g DNA in 50 μ l of 50 mM-Tris·HCl (pH 8.0), 10 mM-MgCl₂, 10 mM- β -mercaptoethanol, and 100 μ g bovine serum albumin/ml with 600 units of T4 endonuclease VII for 90 min at 37°C. The reaction was stopped by the addition of dye buffer and directly loaded onto an agarose gel.

Restriction enzymes were obtained from New England Biolabs and Boehringer-Mannheim Biochemicals, and used as directed by the manufacturers.

(f) DEPC reaction

Diethyl pyrocarbonate (DEPC) was obtained from Sigma and used as described by Scholten & Nordheim (1986). Incubations included 15 μ g pHd in 200 μ l of 10 mM-Tris·HCl (pH 8.0), 60 mM-NaCl, 10 mM-MgCl₂, 1 mM-EDTA at 37°C for 5 min with 3 μ l DEPC. The reaction was stopped by precipitation with ethanol, resuspended in 100 μ l TE buffer and centrifuged through a spun column (Maniatis *et al.*, 1982). The sample was digested with AccI, labeled at the 5' or 3' end, digested with EcoRI and the fragment containing the vaccinia DNA insert was purified by electro-elution onto DEAE-cellulose (Whatman DEAE 81), elution with 1.25 M-NaCl and precipitation with ethanol. The purified fragment was treated with piperidine (see Maxam & Gilbert, 1980) prior to electrophoresis.

(g) DNA labeling

DNA containing 5' protruding ends were labeled at the 5' end with [γ -³²P]ATP and polynucleotide kinase or at the 3' end with [α -³²P]dNTP and DNA polymerase, Klenow fragment as described by Maniatis *et al.* (1982).

DNA with 3' protruding ends were labeled with [α -³²P]dideoxyadenosine and terminal transferase as described by Yousaf *et al.* (1984). Incubations were performed in 50 μ l of 140 mM-sodium cacodylate (pH 7.2), 1 mM-CoCl₂, 0.1 mM-dithiothreitol at 37°C for 1 h and terminated by adding 5 μ l of 0.5 M-EDTA. In each case unincorporated nucleotide was removed by centrifugation through a spun column.

(h) Cross-linked material

Supercoiled monomers were purified from a plasmid preparation of pHd by electrophoresis on a preparative agarose gel, electro-elution onto DEAE paper, elution with 1.25 M-NaCl, and precipitation with ethanol. The supercoils were incubated with vaccinia nuclease at 55°C and analyzed by electrophoresis on a preparative 1% alkaline agarose gel. The cross-linked material (mobility of 6000 nucleotides) was purified using DEAE paper, resuspended in TE buffer (mobility was now 3000 bp), digested with AccI and labeled at the 3' end with [α -³²P]dCTP and DNA polymerase, Klenow fragment. The sample was electrophoresed on a 10% polyacrylamide gel and the gel slab containing the 240 bp fragment was excised, chopped into small fragments and incubated with 0.4 ml of 0.5 M-ammonium acetate, 1 mM-EDTA at 37°C overnight and precipitated with ethanol. The sample was resuspended in dye buffer (0.1% xylene cyanol FF, 0.1% bromophenol blue, 80% formamide) before electrophoresis on a 6% polyacrylamide sequencing gel.

(i) Electron microscopy

DNA was mounted for electron microscopy using the aqueous technique as described (Garon, 1981). Grids were examined in a JEOL 100B electron microscope at 40 kV accelerating voltage. Electron micrographs were taken on Kodak Electron Image plates at a magnification of 7000 \times . The magnification was calibrated for each set of plates with a grating replica (E. F. Fullam, catalog no. 1000) and contour lengths were measured with a Numonics Graphics calculator.

3. Results

(a) Molecular cloning and structure of the joint connecting unit length genomes

Previous studies showed that rapidly sedimenting nucleoprotein complexes from rabbit pox virus (Moyer & Graves, 1981) and vaccinia virus (Baroudy *et al.*, 1983; Moss *et al.*, 1983) infected cells contain concatemers of poxvirus DNA. The concatemers were identified by sucrose gradient sedimentation and restriction enzyme analysis. Unique junction fragments that were twice the size of corresponding terminal hairpin fragments of mature DNA were resolved. The limited amount of concatemeric DNA, however, prevented a detailed analysis of the joint. In order to overcome this problem, the region was amplified by molecular cloning in a bacterial plasmid.

Discontinuous sucrose gradients were used preparatively to isolate rapidly sedimenting nucleoprotein complexes from the cytoplasm of HeLa cells that had been infected 3.5 hours earlier with vaccinia virus. The purified DNA was digested with

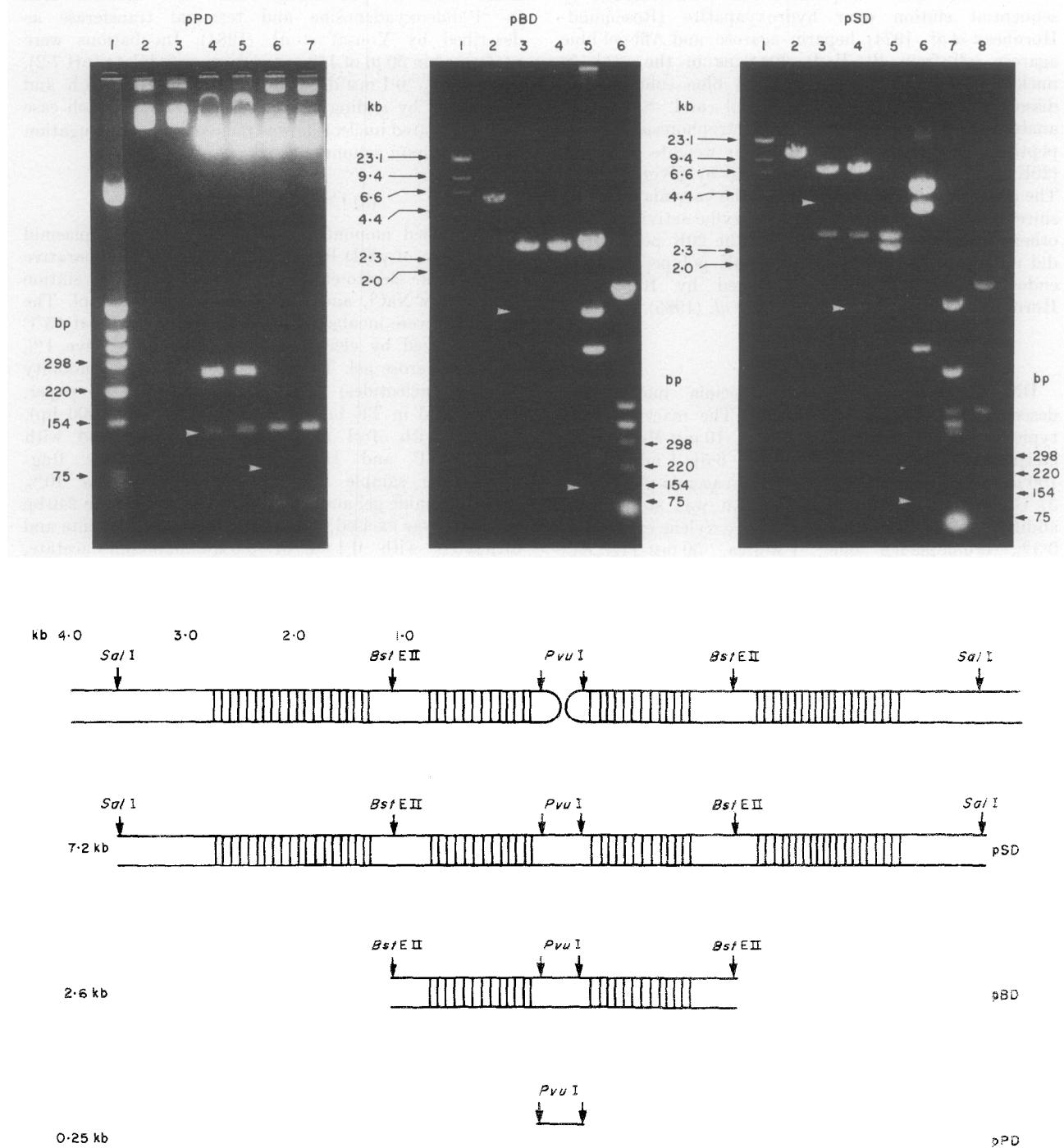


Figure 1. Analysis of recombinant plasmid containing the joint connecting vaccinia virus DNA concatemers. The leftmost panel (pPD) shows pPD3 (lanes 2, 4 and 6) and pPD4 (lanes 3, 5 and 7), which contain the *Pvu*I concatemer joint fragment cloned in pBR322, digested with no enzyme (lanes 2 and 3), with *Pvu*I (lanes 4 and 5) or *Xba*I (lanes 6 and 7), electrophoresed next to pBR322 digested with *Hinf*I (lane 1). The bands indicated by the large arrowheads correspond to the reannealed hairpins formed after denaturation of the symmetrical duplex. The center panel (pBD) shows pBD6, which contains the *Bst*ECII concatemer joint fragment cloned into pUC13, digested with the restriction enzymes *Eco*RI (lane 2), *Bam*HI (lane 3), *Bst*ECII (lane 4), *Pvu*I (lane 5) and *Hinf*I (lane 6), and electrophoresed next to phage λ digested with *Hind*III (lane 1). The rightmost panel (pSD) shows pSD1, which contains the *Sal*I concatemer joint fragment cloned into pUC13, digested with the restriction enzymes *Eco*RI (lane 2), *Sal*I (lane 3), *Bst*ECII (lane 4), *Sal*I and *Bst*ECII (lane 5), *Pvu*I (lane 6) and *Hinf*I (lane 7), and electrophoresed next to λ digested with *Hind*III (lane 1) and pBR322 digested with *Hinf*I (lane 8). The small arrowhead refers to the duplex concatemer junction fragment generated by *Pvu*I digestion. The lower portion of the Figure illustrates the relationship between the mature ends of the vaccinia virus genome and the cloned concatemer junction fragments. The bars refer to the 70 bp direct repeats that contain a *Hinf*I restriction enzyme site (Baroudy *et al.*, 1982).

(a)

*Xba*I

5 ATCGATCTTCTTACACTCTAGAGTTCCATACAGTCATGGGTACACATTCTAGACACTAAATAAAAATTTAAATAT
3 TAGCTAGAAGAATGTGAGATCTAAAGGATGTCAGTACCCAGTGTGAAAAAAAGATCTGTGATTATTTATAAATTTATA

S

AATATTAATGTAATAAACTTATATATTATTAATTCTAACTAAAGTTAGTAAATTATATATAATTATATAATTAAATT
TTATAATTACATGATTGAAATATATAATAATTAAATAGATTGATTCAATCATTAAATATATAATTAAATATTAAATTAA

F

* *Xba*I * *Xba*I *

TAATTTACTAATTTATTTAGTGTCTAGAAAAAAATGTGTGACCCATGACTGTAGGAACCTAGAGGTAAAGAAAGATCGAT
ATTTAAATGATTAAATAATCACAGATCTTTTACACACTGGGTACTGACATCCTTGAGATCTCACATTCTTAGCTA

5

(b)

S

AAGTTAG· TAAATT· AT· ATATATAA· TTTTA· TA· ATTAAT· TTA· ATTTTA· T· ATTTTATTTAGTGT
ATCAATC· ATTTAA· TA· TATATATT· AAAAT· AT· TAATTA· AAT· TAAAT· A· TAAATAAATCACA

F

A A A G G C A T AA *
TAGTTAG· TAAATT· AT· ATATATAA· TTTTA· TA· ATTAAT· TTA· ATTTTA· T· ATTTTATTTAGTGT
ATCAATC· ATTTAA· TA· TATATATT· AAAAT· AT· TAATTA· AAT· TAAAT· A· TAAATAAATCACA

Figure 2. Nucleotide sequence and hairpin loop structure of the vaccinia concatemeric junction in pPD. (a) Nucleotide sequence of the vaccinia insert in pPD. The borders of the hairpin loop are marked by asterisks. (b) Hairpin loop structures of each strand of the junction fragments. The loops are arranged to maximize base-pairing (Baroudy *et al.*, 1982).

*Bst*EII, which cuts 1.3 kb from the ends of mature vaccinia virus DNA (Baroudy *et al.*, 1982), and the fragments were separated by agarose gel electrophoresis. A faint 2.6 kb band corresponding to the predicted concatemer junction fragment was visualized by staining with ethidium bromide. The DNA was eluted from the gel and digested with *Pvu*I, which produces a 126 bp terminal fragment from the ends of mature vaccinia virus DNA, and ligated to *Pvu*I-digested pBR322. *E. coli* were transformed and two recombinant plasmids designated pPD3 and pPD4 were further analyzed. In each case a 250 bp fragment (twice the size of the mature ends) was liberated upon digestion with *Pvu*I (Fig. 1, pPD, lanes 4 and 5). Since two *Xba*I sites are located 66 and 103 bp from the ends of the mature vaccinia virus genome, the release of fragments of about 130 bp ($2 \times 66 = 132$) and 40 bp ($103 - 66 = 37$) after digestion of pPD with *Xba*I was consistent with the predicted palindrome structure (Fig. 1, pPD, lanes 6 and 7). Faint bands (indicated by arrowheads) that migrated faster than the putative *Pvu*I and *Xba*I junction fragments were noted. We suspected that these faint bands with half of the apparent molecular weight of the junction fragments represented hairpins formed by inadvertent denaturation of duplex fragments and renaturation of the self-

complementary single-strand hairpins. This idea was confirmed by quantitatively converting the 250 bp *Pvu*I fragment to the more rapidly migrating form by heating and quick cooling (not shown).

The structures of the cloned *Pvu*I fragments of the two isolates were determined by DNA sequencing and were found to be identical. Inspection of the sequence (Fig. 2(a)) indicates that the central 104 bp forms an imperfect palindrome, each strand of which is an exact copy of one of the alternative hairpin structures (Fig. 2(b)) present at the ends of mature genomes (Baroudy *et al.*, 1982).

(b) Molecular cloning and structure of longer concatemer junction fragments

Two sets of 70 bp tandem repeats occur proximal to the hairpin structure at each end of the vaccinia virus genome (Fig. 1). Since these repeated sequences might represent important protein binding sites or have other functions in replication, we wished to clone larger concatemer junction fragments that included this region. First, the 2.6 kb *Bst*EII fragment that contains only one set of repeats on either side of the apex (Fig. 1) was cloned by adding *Bam*HI linkers to the gel-purified *Bst*EII fragment and ligating the product to

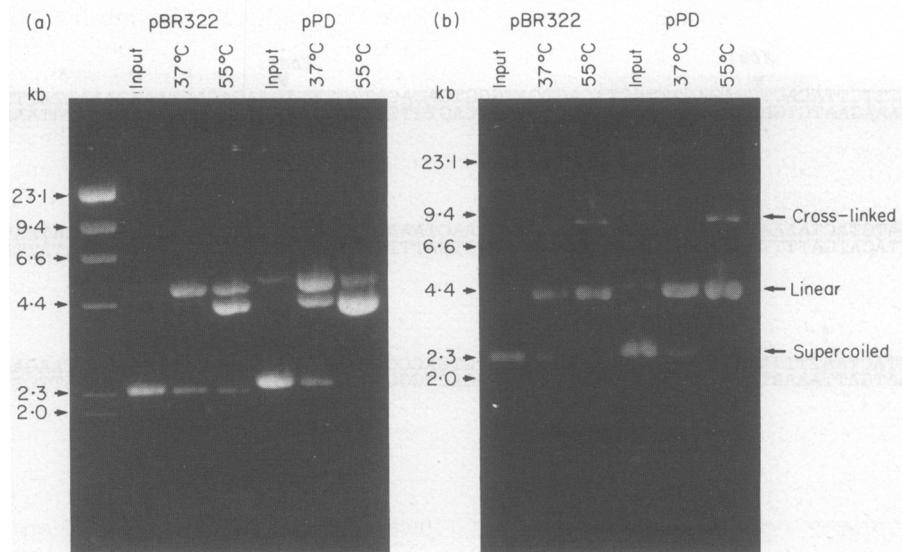


Figure 3. Vaccinia nuclease cleavage and crosslinking of DNA. The plasmids pBR322 and pPD were incubated with the vaccinia nuclease at 37°C or 55°C and each sample was analyzed by electrophoresis on (a) 1% neutral agarose or (b) 1% alkaline agarose gel. (a) The neutral gel contains (left to right): λ digested with *Hind*III; pBR322; pBR322 incubated with vaccinia nuclease at 37°C and 55°C, respectively; pPD; and pPD reacted with vaccinia nuclease at 37°C and 55°C, respectively. (b) The alkaline agarose gel contains (left to right): pBR322; pBR322 incubated with vaccinia nuclease at 37°C and 55°C, respectively; pPD; and pPD incubated with vaccinia nuclease at 37°C and 55°C, respectively. The positions of the supercoiled, linear and cross-linked forms are indicated.

*Bam*HI-cleaved pUC13 and pBR322. Bacterial recombinants were detected by colony hybridization with 32 P-labeled vaccinia virus DNA and the plasmids designated pBD were purified and analyzed. After digestion with *Bam*HI, the predicted 2.6 kb DNA fragment was liberated (Fig. 1, pBD, lanes 3 and 4) as well as faint 1.3 kb bands (indicated by arrowheads) corresponding to spontaneously formed hairpin structures. A 250 bp fragment was released upon digestion of pBD with *Pvu*I, which was the same size as the insert of pPD (Fig. 1, pBD, lane 5), demonstrating that the center of the junction fragment was conserved. Again, the faint denatured band is indicated by an arrowhead. The multimolar fragment of about 70 bp obtained upon digestion with *Hinf*I (Fig. 1, pBD, lane 6) confirmed that the plasmids contained a set of tandem repeats (Baroudy *et al.*, 1982).

Next pSD, which has a 7.2 kb palindrome with both sets of repeats as well as the entire non-coding terminal region of the vaccinia virus genome (Fig. 1), was constructed. Rather than restarting with viral concatemer DNA, pSD was assembled by sandwiching the *Bst*EII fragment from pBD with two copies of the *Sall*-*Bst*EII fragment from cloned genomic DNA (Baroudy *et al.*, 1982). The structure of this plasmid was confirmed by digestion with *Sall*, *Bst*EII, *Pvu*I and *Hinf*I (Fig. 1, pSD). Even though the recombinant plasmids contained nearly perfect palindromes and sets of direct repeats, they were stably propagated in both *recA*⁺ (LE392) and *recA*⁻ (HB101) hosts.

Special care was required in the preparation of pSD, however. When alkaline lysis of transformed *E. coli* was tried, the DNA migrated as a smear on agarose gels, apparently because of denaturation

and imperfect reannealing. We suspect annealing between direct repeats out of register leads to many partially renatured molecules. Accordingly, denaturation of DNA was subsequently avoided by using Triton X-100 cell lysis.

(c) Cleavage of recombinant plasmids with vaccinia nuclease

Deoxyribonuclease activities that are active on single stranded and supercoiled DNA have been isolated from vaccinia virions (Pogo & Dales, 1969; Rosemond-Hornbeak *et al.*, 1974; Rosemond-Hornbeak & Moss, 1974; Pogo & O'Shea, 1977). Lakritz *et al.* (1985) have shown that when highly purified preparations of the nuclease were incubated with supercoiled plasmid or SV40 DNA, nicked circles and then linear molecules formed. Cleavage occurred at specific regions and 10 to 20% of the linear molecules were cross-linked at one end. They proposed that this multifunctional viral enzyme might be involved in the processing of replicative intermediates into progeny genome-length molecules that are cross-linked at both ends. The enzyme was not tested, however, on DNA derived from vaccinia virus.

In preliminary experiments, we confirmed that purified preparations of vaccinia nuclease converted supercoiled pBR322 into a mixture of nicked circular and linear molecules (Fig. 3(a)). Furthermore, under denaturing conditions some of the DNA migrated as expected for molecules that were terminally cross-linked (Fig. 3(b)). Even more rapid conversion to linear forms occurred with plasmids containing vaccinia concatemer junctions. Data obtained with pPD are also presented in Figure 3.

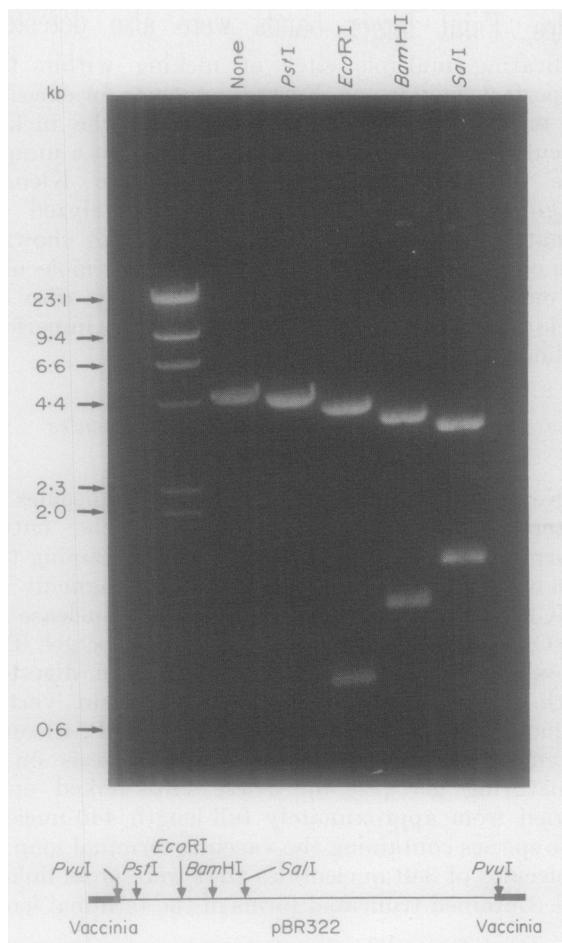


Figure 4. Site-specific cleavage of pPD by vaccinia nuclease. Plasmid pPD was treated with vaccinia nuclease at 37°C and the linear molecules purified by preparative agarose gel electrophoresis. The linear molecules were digested with restriction enzymes and electrophoresed on a 1% agarose gel. The gel contained (left to right): λ digested with *Hind*III; pPD treated with vaccinia nuclease and digested with nothing; *Pst*I; *Eco*RI; *Bam*HI; and *Sal*I, respectively. The lower portion represents a map of linear pPD generated by vaccinia nuclease consistent with restriction enzyme digestion products. The plasmid vector sequence is indicated by the thin line and the vaccinia DNA is represented by the thick line.

In agreement with Lakritz *et al.* (1985), we found that the enzyme was most active in low salt solution, at neutral pH and temperatures of about 55°C, and that no more than 10 to 20% of the molecules appeared to become cross-linked. Neither ATP nor Mg²⁺ was required for cleavage or cross-linking.

(d) Determination of vaccinia nuclease cleavage sites

The linear molecules generated by vaccinia nuclease could arise from random or specific cleavage within the vector or vaccinia DNA sequences. In order to determine the sites of nuclease susceptibility, linear molecules of pBD

were electrophoretically purified and samples were digested with restriction endonucleases that cut at one or two sites within the vector. When analyzed by agarose gel electrophoresis, discrete bands were seen (Fig. 4), indicating that the vaccinia nuclease cleaved in a unique region. From the size of the digestion products, we deduced that the nuclease had cleaved pBD near the center of the vaccinia insert and generated the linear molecule shown at the bottom of Figure 4. Similar results, i.e. cleavage near the center of the vaccinia insert, were also observed when pPD and pSD were used as substrates for vaccinia nuclease, and when the vector used to clone the vaccinia palindrome was either pBR322 or pUC13 (data not shown). With pBR322 alone as substrate, cleavage occurred at approximately nucleotide 3250. Thus, in spite of the fact that pBR322 nuclease sites are present in pPD and pBD, cleavage was only observed within the vaccinia concatemer junction.

Further analyses were done to determine more precisely the sites of cleavage within the vaccinia DNA. The purified linear molecules derived by incubating pPD with vaccinia nuclease at 37°C and 55°C were digested with *Pvu*I, which cuts at the border of vaccinia and vector DNA, and then labeled by adding [α -³²P]dideoxyadenosine to the 3' hydroxyl end with terminal transferase. The labeled material was then applied to a polyacrylamide gel and the precise sizes of the fragments were determined by electrophoresis alongside a Maxam-Gilbert sequence ladder (Fig. 5). The autoradiographic bands indicated that at both temperatures the ends created by nuclease digestion were derived from the edges of the 104 bp imperfect palindrome indicated by asterisks in Figures 2 and 5. At 55°C, the majority of ends mapped outside the imperfect palindrome, whereas at 37°C they mapped just inside and outside. A similar pattern of bands was also obtained when the linearized DNA was labeled at the nuclease cleavage sites with [γ -³²P]ATP and polynucleotide kinase prior to *Pvu*I digestion (data not shown).

The linear molecules generated by the vaccinia nuclease could occur by cutting one or both DNA strands at each edge of the imperfect palindrome. In the former case resolution into linear molecules would involve branch migration and the hairpin structures would be preserved. In the latter case the hairpins would be excised. One can distinguish between these two possibilities by isolating the linear molecules, cleaving them at a unique *Acc*I site in the vector DNA, and labeling the restriction site at the 5' end with polynucleotide kinase or the 3' end with [α -³²P]dCTP and the Klenow fragment of DNA polymerase. When analyzed by polyacrylamide gel electrophoresis under denaturing conditions, we found that the same pattern and sizes of bands (similar to Fig. 5) were obtained with either 5' or 3' end-labeling. Since linear molecules with hairpin ends would contain strands of unequal length, both DNA strands were cut and the hairpins were removed from the majority of linear mole-

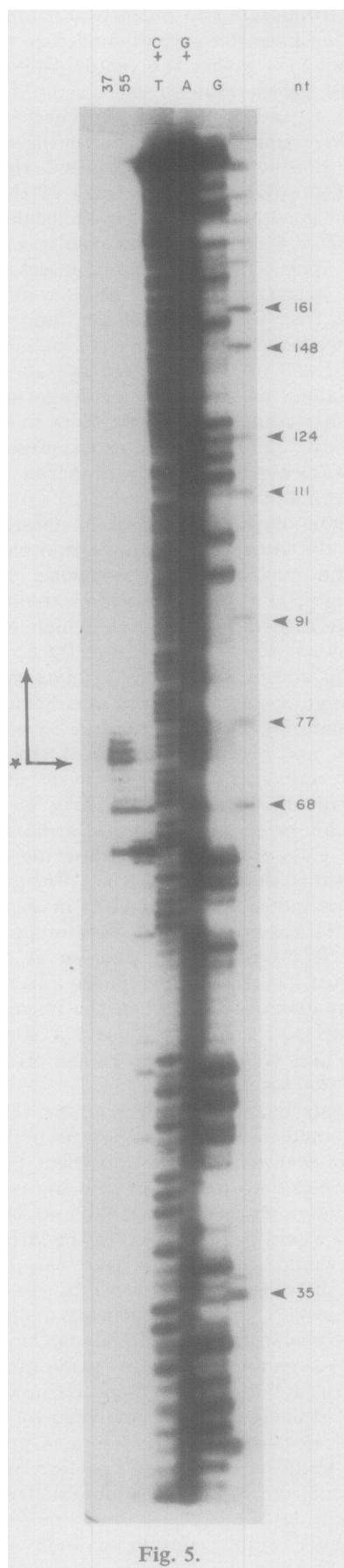


Fig. 5.

cules. Faint larger bands were also detected, indicating multiple sites of nicking within the imperfect palindrome. Further evidence for excision at multiple sites was obtained when the nicked circular products were purified, cleaved at a unique site in the vector, labeled with the Klenow fragment of DNA polymerase and analyzed on denaturing polyacrylamide gels (data not shown). We concluded that the majority of linear molecules do not have hairpin ends and that they arise by nicking at multiple sites within the imperfect palindrome.

(e) *Identification of cross-linked molecules formed by vaccinia nuclease*

Next, we examined the cross-linked molecules to determine whether they contained the entire hairpin terminus. The plasmid pH containing the 416 bp *HinfI* concatemer junction fragment in pUC13 was treated with the vaccinia nuclease at 55°C, and analyzed on an alkaline agarose gel. The cross-linked DNA was eluted, and then digested with *AccI*, which cuts uniquely within vector sequence. After labeling with [α -³²P]dCTP, the end fragment was analyzed by electrophoresis on a denaturing gel (Fig. 6). These cross-linked ends varied from approximately full-length 440-nucleotide species containing the vaccinia terminal loop to molecules of 340 nucleotides that were cross-linked but contained truncated forms of the terminal loop.

(f) *Vaccinia nuclease cleavage sites occur within single-stranded regions*

The nicked circular and linear molecules might arise by the single-stranded endonuclease activity of the vaccinia enzyme. To evaluate this possibility, the vaccinia, S₁ nuclease and mung bean nuclease cleavage sites on pPD, pBD, pSD and pBR322 were compared. In each case a mixture of nicked circular and linear molecules was formed. When the linears were gel-purified and digested with a series of restriction enzymes as in Figure 4, a similar pattern of bands was obtained, indicating that the three nucleases recognized the same region in the DNA molecules.

Figure 5. Phosphodiester bonds cleaved in pPD by vaccinia nuclease. Plasmid pPD was treated with vaccinia nuclease at 37°C or 55°C and the linear molecules were purified by preparative agarose gel electrophoresis, digested with *Pvu*I, and labeled with [α -³²P]dideoxyadenosine using terminal transferase. These samples were electrophoresed on a 6% polyacrylamide sequencing gel with the Maxam-Gilbert C+T, G+A and G reactions on the pPD concatemer junction insert labeled with [α -³²P]dideoxyadenosine at the *Pvu*I site. The rightmost lane contains pBR322 digested with *Msp*I and labeled with [α -³²P]dCTP and DNA polymerase, Klenow fragment. The asterisk indicates the border and the arrow points in the direction of the remainder of the hairpin loop. nt, nucleotides.

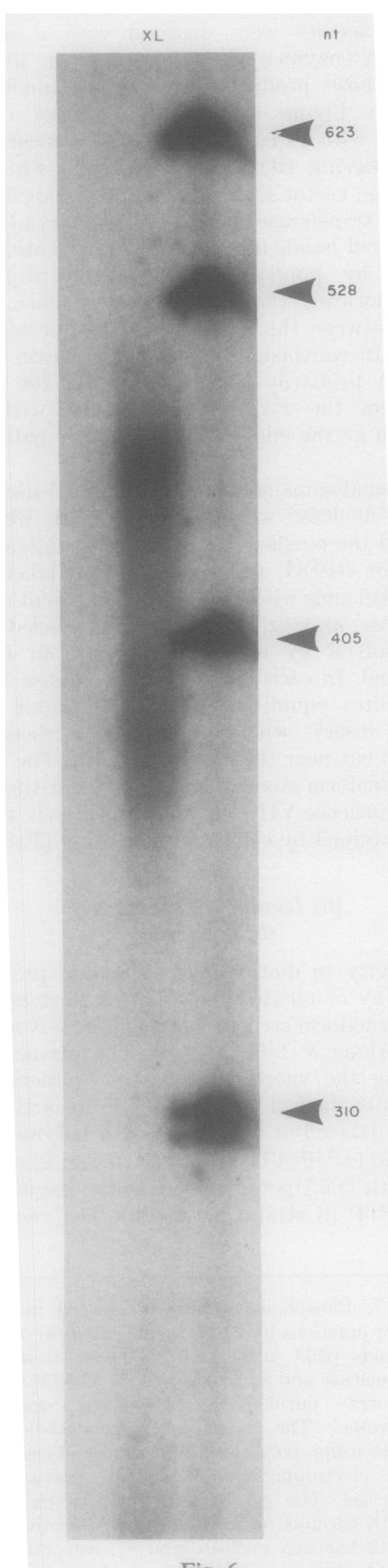


Fig. 6.

To compare more precisely the S_1 and vaccinia nuclease cleavage sites, the gel-purified linear pPD, pBD and pSD products were labeled with polynucleotide kinase and digested with *Pvu*I. Similar electrophoretic patterns were obtained in each case, despite the fact that the vaccinia inserts varied from 0.25 to 7.2 kbp (Fig. 7). The series of bands produced by nuclease S_1 were about ten nucleotides shorter than those formed by vaccinia nuclease. Thus, the imperfect palindrome and sites of vaccinia nuclease action fall within the S_1 -sensitive regions of the supercoiled plasmids.

(g) *Vaccinia nuclease cleavage sites are within cruciform structures*

If the imperfect palindromes were extruded from the supercoiled plasmids as cruciforms, the structures would resemble the hairpin termini of mature linear vaccinia genomes, and because of incomplete base-pairing might be susceptible to single-stranded nucleases. Although isolated as supercoiled forms by banding in CsCl in the presence of ethidium bromide, the plasmids were then routinely extracted with butanol, dialyzed and precipitated with ethanol. Electron micrographs of representative spreads of pUC13, pBD, pSD and pSV9 are shown in Figure 8. Most of the molecules containing the vaccinia concatemer junctions, including pPD (data not shown) appeared as relaxed circles. In the case of pBD and pSD, two protrusions of equal length were readily visualized. The lengths of the stems were proportional to the total size of the plasmids (Table 1). Another plasmid, pSV9, also was spread in the same manner. This plasmid is a head-to-head dimer of pBD and was isolated from *E. coli* that had been transformed with a linear form of pBD generated in vaccinia virus-infected CV-1 cells (Merchlinsky & Moss, 1986). Although pSV9 contains two concatemer junctions and could potentially produce two cruciforms, only one was observed per molecule (Fig. 8). Presumably, nucleation of one cruciform immediately relaxes the DNA and precludes extrusion of the second.

The enzyme T4 endonuclease VII was used to map the location of the cruciform. This enzyme produces nicks in the four-strand base of cruciforms or Holliday structures (Lilley & Kemper, 1984; Mizuuchi *et al.*, 1982a). The plasmids pPD, pBD

Figure 6. The cross-linked (XL) ends of pHD formed by vaccinia nuclease. Plasmid pHD was treated with vaccinia nuclease at 55 °C and the cross-linked material was purified by preparative alkaline agarose electrophoresis. The sample was digested with *Acc*I, which cut uniquely in the vector sequence, labeled with [α -³²P]dCTP and DNA polymerase, Klenow fragment, and the approx. 240-nucleotide terminal fragment was isolated on a preparative polyacrylamide sequencing gel next to pBR322 digested with *Msp*I and labeled with [α -³²P]dCTP and DNA polymerase, Klenow fragment. nt, nucleotides.

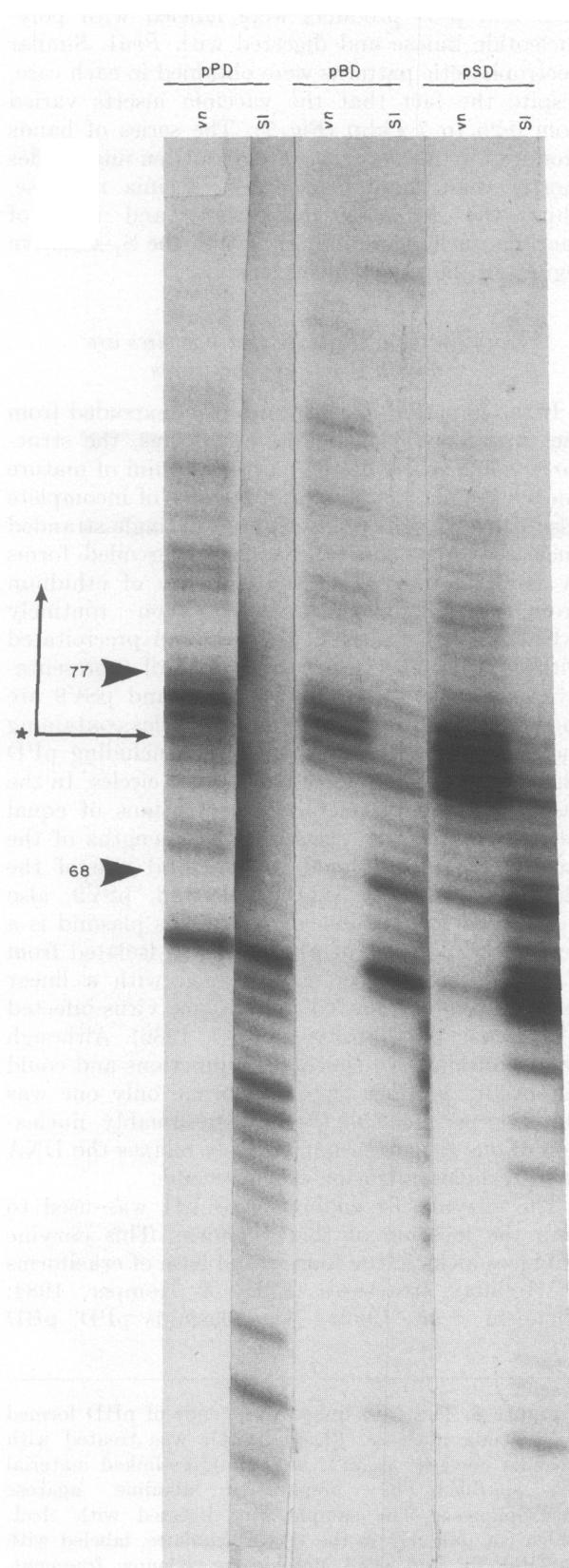


Fig. 7.

and pSD were all susceptible to T4 endonuclease VII and linear molecules were formed. When the linear molecules were digested with a series of restriction enzymes, the products were similar in size to those produced by vaccinia nuclease as shown in Figure 4. Further analysis of pPD linearized with T4 endonuclease VII was carried out by subcleaving the DNA with *Pst*I, which cuts uniquely in vector sequences, and then labeling with terminal transferase and [α -³²P]dideoxyadenosine. Two labeled bands of 125 and 380 nucleotides were resolved by polyacrylamide gel electrophoresis, thereby locating the base of the cruciform at the border between the vaccinia and vector sequences (Fig. 9). In contrast, the vaccinia nuclease cleaved the DNA primarily between 190 and 200 nucleotides from the *Pst*I site, placing it within the cruciform at the edge of the imperfect palindrome (Fig. 9).

In an analogous manner, T4 endonuclease VII or vaccinia nuclease were incubated with pBD and pSD, and the purified linear products digested with *Bam*H I or *Bst*EII, respectively. After labeling the 3' hydroxyl ends with the Klenow fragment of DNA polymerase and [α -³²P]dGTP, the labeled bands were resolved by electrophoresis on an alkaline agarose gel. In each case, T4 endonuclease VII cut at two sites equidistant from the center of the vaccinia insert, whereas vaccinia nuclease continued to cut near the center (Fig. 10). The lengths of the cruciform stems determined from the site of T4 endonuclease VII cleavage agreed well with the values obtained by electron microscopy (Table 1).

(h) Location of the apex of the cruciform

Reactivity to diethyl pyrocarbonate provides a precise way of identifying unpaired purines in the apex of cruciform structures (Scholten & Nordheim, 1986; Furlong & Lilley, 1986). The plasmid pH D containing the vaccinia concatemer junction was treated with diethyl pyrocarbonate, linearized with *Acc*I and labeled at the 5' end with polynucleotide kinase and [γ -³²P]ATP (F strand in Fig. 2) or at the 3' end with DNA polymerase Klenow fragment and [α -³²P]dCTP (S strand in Fig. 2). The vector was

Figure 7. Phosphodiester bonds cleaved in vaccinia concatemer junctions by vaccinia nuclease (vn) or S₁ (S₁). The plasmids pPD, pBD and pSD were treated with vaccinia nuclease and S₁ nuclease at 37°C, and the linear products were purified by preparative agarose gel electrophoresis. The samples were labeled with [γ -³²P]ATP using polynucleotide kinase, digested with *Pvu*I and electrophoresed on a 6% polyacrylamide sequencing gel. The gel contains (left to right): pPD treated with vaccinia nuclease and S₁, respectively; pBD treated with vaccinia nuclease and S₁, respectively; and pSD treated with vaccinia nuclease and S₁, respectively. The asterisk marks the border and the arrow points in the direction of the remainder of the incompletely base-paired hairpin loop. nt, nucleotides.

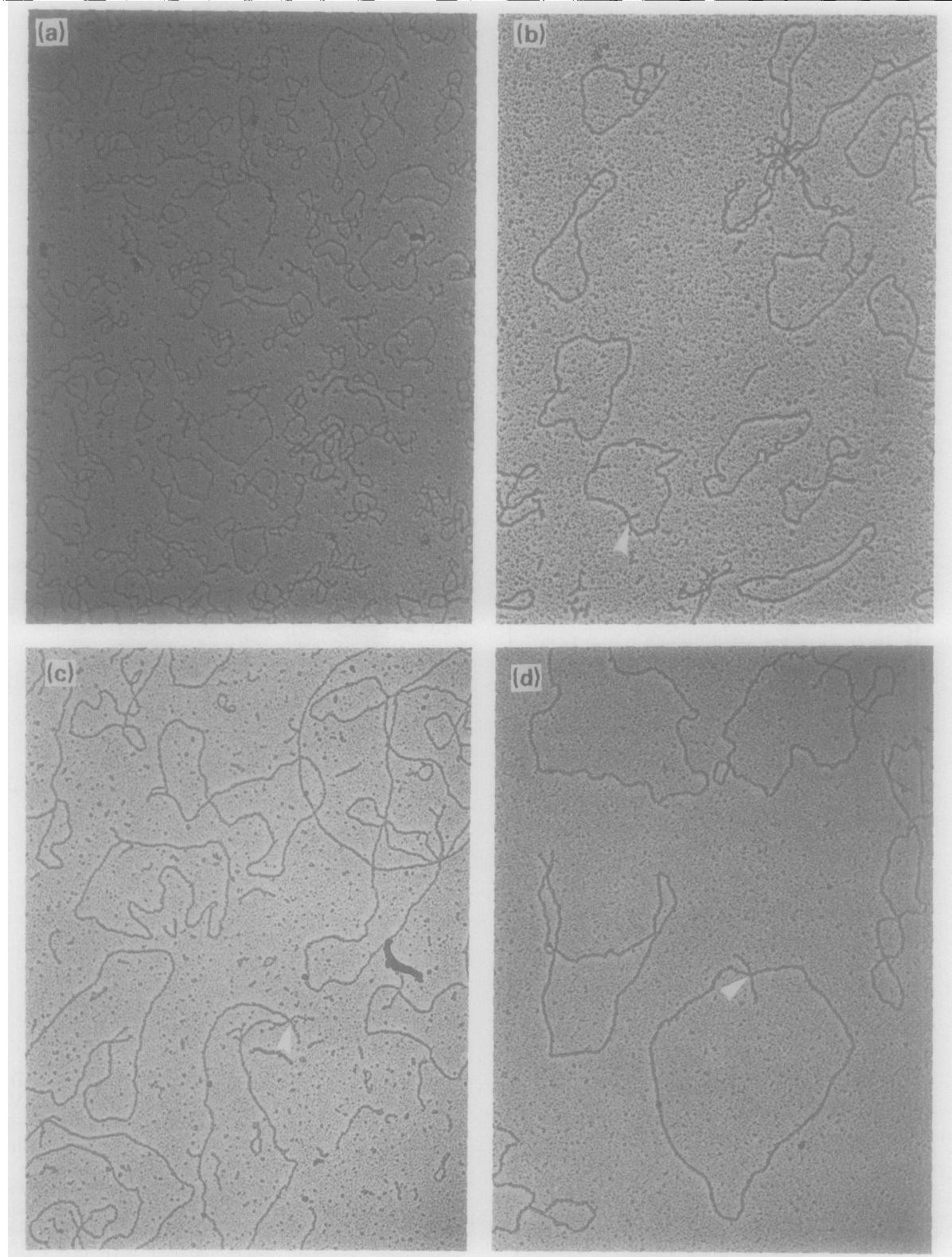


Figure 8. Plasmids containing the vaccinia concatemer junction are relaxed circles with extruded cruciforms *in vitro*. Samples of (a) pUC13, (b) pBD, (c) pSD and (d) pSV9 were mounted aqueously and visualized under the electron microscope. Arrowheads indicate examples of extruded stem structures.

cut with *Eco*RI and the vaccinia fragment was gel-purified, treated with piperidine, and run on a denaturing gel (Fig. 11). In the S strand the primary sites of carbethoxylation mapped to the three adenine nucleotides at the apex of the imperfectly paired hairpin (Fig. 2(b)), as well as the edges

of the imperfect palindrome (marked by the asterisks). In the F strand the primary sites of carbethoxylation mapped to the region distal to the center of the hairpin loop (Fig. 2(b)). Therefore, the extruded cruciforms are not wholly single-stranded but adopt the structure predicted in Figure 2(b)

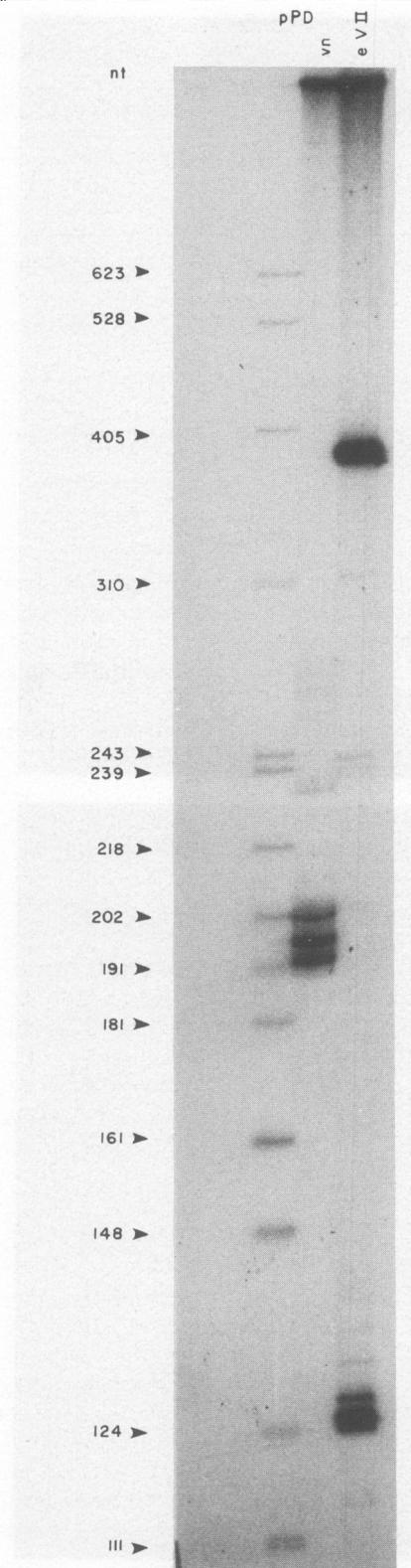


Figure 9. Phosphodiester bonds cleaved in pPD by vaccinia nuclease and T4 endonuclease VII. Plasmid pPD was treated with vaccinia nuclease and T4 endonuclease VII at 37°C, and the linear products were purified by preparative agarose gel electrophoresis, digested with *Pst*I, and labeled with [α - 32 P]dideoxyadenosine and terminal transferase. The vaccinia nuclease (vn) and T4 endonuclease VII (e VII) products were electrophoresed on a 6% polyacrylamide sequencing gel containing pBR322 digested with *Msp*I and labeled with [α - 32 P]dCTP and DNA polymerase, Klenow fragment.

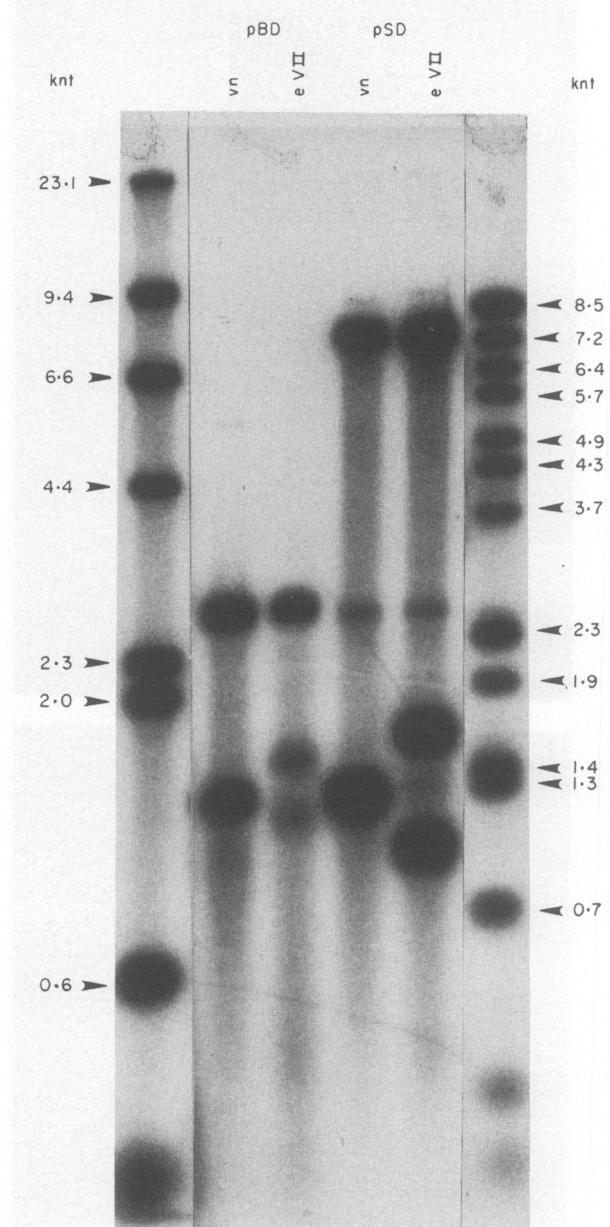


Figure 10. Site-specific cleavage of pBD and pSD by vaccinia nuclease (vn) and T4 endonuclease VII (VII). Plasmids pBD and pSD were treated with vaccinia nuclease and T4 endonuclease VII at 37°C, and the linear products were purified by preparative agarose gel electrophoresis. The samples were digested with *Bam*HI (pBD) or *Bst*EII (pSD), which cleaved each plasmid about 1.3 kb on both sides of the center of the vaccinia concatemer junction insert, labeled with [α - 32 P]dGTP and DNA polymerase, Klenow fragment, and electrophoresed on a 1% alkaline agarose gel. The gel contains (left to right): lambda DNA digested with *Hind*III; pBD treated with vaccinia nuclease and T4 endonuclease VII, respectively; pSD treated with vaccinia nuclease and T4 endonuclease VII, respectively; and lambda DNA digested with *Bst*EII. knt, 10^3 nucleotides.

Table 1
Length of cruciform relative to total size of the plasmid

	Plasmid length (kb)	Cruciform stem length (kb)
pBD	5.2	0.20
pSD	9.8	0.31
pSV9	10.4	0.40

The lengths of the cruciform stems were determined by contour length measurements of molecules (20 to 30 of each) visualized under the electron microscope.

(Baroudy *et al.*, 1982) with a small single-stranded loop at the apex and a majority of the nucleotides base-paired with the unpaired nucleotides on one side of the hairpin.

4. Discussion

The first objective of this study was to determine the precise structure of the junction between unit-length vaccinia virus genomes in concatemeric replicative intermediates. To accomplish this, we cloned restriction endonuclease fragments containing the junction and various lengths of flanking DNA into bacterial plasmids. All of the inverted repeat structures, including one 7200 bp long, were stably maintained and propagated in *E. coli*. Although perfect inverted repeats are very unstable in *E. coli* (Collins, 1980; Lilley, 1981; Collins *et al.*, 1982; Mizuuchi *et al.*, 1982b), the predicted structure of the joint in the vaccinia inserts is not perfectly symmetrical, since the central portion of the insert corresponds to the incompletely base-paired hairpin loop. This feature was confirmed by the nucleotide sequence of pPD. The concatemer junction was shown to be a precise duplex copy of the hairpin structure found at the end of the genome. Moreover, each strand corresponded to the alternative inverted and complementary forms of the polynucleotide loop (Baroudy *et al.*, 1982). The conversion of this structure into hairpin ends provides a structural basis for the observed sequence isomers. We have (Merchlinsky & Moss, 1986) demonstrated that the plasmids containing the concatemer junction are resolved into linear minichromosomes when they are transfected into cells infected with vaccinia virus. Similar transfection results have been noted by DeLange *et al.* (1986) with plasmids that contained palindromes derived from the hairpin ends of

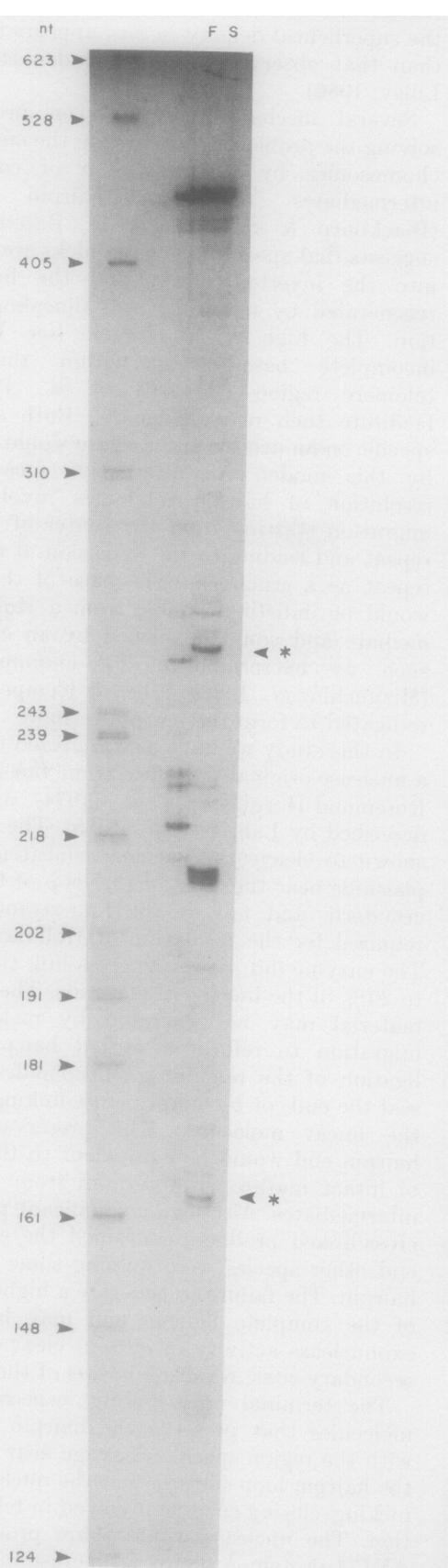


Fig. 11.

Figure 11. DEPC modification of pHd. Plasmid pHd was reacted with DEPC at 37°C, digested with *Acc*I, and labeled with [α - 32 P]dCTP and DNA polymerase, Klenow fragment, and [γ - 32 P]ATP and polynucleotide kinase. The *Acc*I-EcoRI fragment containing the vaccinia DNA insert was purified by preparative agarose gel electrophoresis and cleaved with piperidine. The samples labeled at the 5' (F) or 3' (S) ends were electrophoresed on a 6% polyacrylamide sequencing gel next to pBR322 digested with *Msp*I. The asterisks indicate the border of the terminal hairpin loop. nt, nucleotides.

mature Shope fibroma virus and vaccinia virus genomic DNA.

In this work we demonstrated that the isolated plasmids containing the concatemer junction of vaccinia are relaxed circles with extruded cruciforms. Although the molecules were isolated as supercoiled plasmids in the presence of ethidium bromide by isopycnic banding in cesium chloride, most of the molecules are visualized as relaxed circles with easily detected extruded hairpins following removal of the intercalating agent. Analysis of the structure with diethyl pyrocarbonate or S_1 nuclease, which react with the molecules in single-stranded regions such as the apex of the cruciform loop, and T4 endonuclease VII, which cleaved the DNA at the base of the cruciform stem, indicated that the cruciform was formed by the extrusion of the vaccinia insert into a stem-loop structure in which the apex of the cruciform corresponds to the hairpin loop of mature vaccinia DNA. Selective reactivity to diethyl pyrocarbonate indicated that the extruded cruciform adopts a structure that is mostly double-stranded, with the unpaired nucleotides primarily on one side of the hairpin. This arrangement is consistent with the maximum base-paired loops proposed by Baroudy *et al.* (1982). Thus, the structure of these plasmids *in vitro* is equivalent to relaxed circles with two extended copies of the vaccinia telomere.

The formation of cruciforms from supercoiled DNA is thought to occur either by the denaturation and snap-back of an inverted repeat region to form the entire cruciform in one step or by the formation of a localized unpaired region creating a small cruciform that branch migrates to extrude the complete cruciform (Lilley, 1985). The first model would require large unpaired regions of 0.4, 0.6 or 0.8 kb in pBD, pSD or pSV9 for the formation of the cruciform in one step. It seems more likely that the plasmids undergo local denaturation in the highly A+T-rich region near the apex of the hairpin loop and the full-length cruciform is formed by branch migration. The size of the extruded cruciforms for each of the completely relaxed plasmids is proportional to the size of the plasmid. Cruciform formation utilizes the energy stored in superhelicity so that the fractional length of the hairpin matches the fractional deficit in the linking number of the DNA (Mizuuchi *et al.*, 1982b). For pBD, pSD and pSV9 a specific linking difference of -0.07 or -0.08 was determined using the values in Table 1 (Mizuuchi *et al.*, 1982b). The plasmid pSV9, which contains two possible regions of cruciform formation, was only observed to contain one pair of stem-loop structures *in vitro*. Thus, the rate-limiting step for cruciform formation is the initial denaturation and formation of the small cruciform. Once this structure is formed, branch migration relaxes the molecule using the energy that would be required to form a second stem-loop. These cruciforms that are seen *in vitro* would most probably be deleterious if formed in the bacterial

cell (Lilley, 1981); however, it is likely that these cruciforms will not be extruded in bacteria, since the superhelical density *in vivo* appears to be lower than that observed *in vitro* (Sinden *et al.*, 1983; Lilley, 1986).

Several mechanisms have been proposed for solving the problem of replicating the ends of linear chromosomes by the resolution of concatemeric intermediates to form hairpin structures (Blackburn & Szostak, 1984). Bateman (1975) suggests that specific staggered nicks are introduced into the inverted repeat and the hairpins are regenerated by unfolding, self-annealing and ligation. The high A+T content (see Fig. 2) and incomplete base-pairing within the vaccinia telomere region (Baroudy *et al.*, 1982) could facilitate such rearrangements. Both a sequence-specific endonuclease and a ligase would be required by this model. An alternative means for the resolution of hairpin telomeres involves branch migration starting from the center of an inverted repeat and leading to the extrusion of the inverted repeat as a cruciform. The base of this structure would be indistinguishable from a Holliday intermediate and could be nicked by an endonuclease such as bacteriophage T4 endonuclease VII (Mizuuchi *et al.*, 1982a; Lilley & Kemper, 1984) and re-ligated to form the hairpin termini.

In this study we have also analyzed the action of a nuclease originally purified from vaccinia virus by Rosemond-Hornbeck *et al.* (1974) and recently described by Lakritz *et al.* (1985). The enzyme was shown to cleave the vaccinia concatemer junction plasmids near the apex of the loop of the extruded cruciform and not at the four-stranded base as required for the resolution of Holliday structures. The enzyme did, however, cross-link the ends of 10 to 20% of the linearized plasmids. The cross-linked material may be generated by nicking, branch migration to reform a nicked hairpin end, and ligation of the remaining phosphodiester bond to seal the end, or by simply cross-linking the ends of the linear molecules. The preservation of the hairpin end would be equivalent to the generation of intact mature viral termini from concatemeric intermediates. Although a significant portion of the cross-linked products contained the entire hairpin end other species were missing some or all of the hairpin. The failure to generate a higher percentage of the complete hairpin end may be caused by exonuclease activity *in vitro* or cleavage at several secondary sites, resulting in loss of the hairpin.

The terminal cross-linking, especially in those molecules that preserve the hairpin end, coupled with the region-specific cleavage near the border of the hairpin loop suggest that the nuclease may be a nicking-closing enzyme involved in telomere resolution. The nuclease would share properties of the ϕ X174 protein A or topoisomerase in that it could introduce and reseal nicks in DNA. We have observed that the vaccinia nuclease cleavage site is readily labeled with $[\gamma-^{32}\text{P}]$ ATP and polynucleotide kinase without prior dephosphorylation whereas the

3' side of the nick is very poorly labeled with Klenow fragment and deoxynucleotide triphosphates or with terminal transferase and [α -³²P]dideoxyadenosine triphosphate. This is consistent with termini that retain a 3' phosphate, such as those observed after a protein linkage with eucaryotic topoisomerase I. Further efforts are being made to define the role this enzyme plays in vaccinia replication.

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