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# Nucleotide Sequence Required for Resolution of the Concatemer Junction of Vaccinia Virus DNA

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**The mature form of the vaccinia virus genome consists of a linear, 185,000-base-pair (bp) DNA molecule with a 10,000-bp inverted terminal repetition and incompletely base-paired 104-nucleotide hairpin loops connecting the two strands at each end. In concatemeric forms of intracellular vaccinia virus DNA, the inverted terminal repetitions of adjacent genomes form an imperfect palindrome. The apex of this palindrome corresponds in sequence to the double-stranded form of the hairpin loop. Circular plasmids containing palindromic concatemer junction fragments of 250 bp or longer are converted into linear minichromosomes with hairpin ends when they are transfected into vaccinia virus-infected cells, providing a model system with which to study the resolution process. To distinguish between sequence-specific and structural requirements for resolution, plasmids with symmetrical insertions, deletions, and oligonucleotide-directed mutations within the concatemer junction were constructed. A sequence (ATTTAGTGTCTAGAAAAAAA) located on both sides of the apex segment was found to be critical for resolution. Resolution was more efficient when additional nucleotides, TGTG, followed the run of A residues. Both the location and sequence of the proposed resolution signal are highly conserved among poxviruses.**

Vaccinia virus, the prototypical member of the poxvirus family, contains a linear double-stranded DNA genome of approximately 185 kilobase pairs (kbp) with a 10,000-bp inverted terminal repetition (32) and cross-links at each end (12). The hairpin loops connecting the two DNA strands are incompletely base paired, 104 nucleotides long, and exist in two sequence conformers that are inverted and complementary with respect to each other (1). Restriction enzyme analysis of intracellular forms of vaccinia virus (2, 27) and rabbitpox virus (28) DNA revealed the presence of palindromic forms of the terminal sequence. DNA fragments containing the palindromes were isolated and cloned into bacterial plasmids. The apical segment of the palindrome was shown to be a precise duplex copy of the mature hairpin loop, with each strand corresponding to one of the alternative sequence conformers (22). The transient existence of the palindromic sequence and its association with high-molecular-weight vaccinia virus DNA suggested that it forms the junction between unit-length genomes in replicative intermediates. To test this hypothesis, bacterial plasmids containing the concatemer junction from vaccinia virus DNA (23) or the equivalent sequence from Shope fibroma virus (8) were transfected into poxvirus-infected cells. The plasmids replicated and were converted into linear minichromosomes with hairpin ends. Efficient conversion was observed for plasmids containing junction fragments larger than 240 to 250 bp (7, 23).

The size dependence for conversion of the plasmids could signify a requirement for either a specific DNA sequence, a minimum-length palindromic structure, or both. Plasmids containing the palindromic concatemer junction were shown by electron microscopy to extrude cruciforms in vitro (8, 22) and could be converted into linear molecules by cleavage within the viral DNA with endonuclease VII or I from bacteriophage T4 (22) or T7 (10), respectively. These well-characterized enzymes recognize structural properties of DNA and cleave irrespective of the primary nucleotide sequence (9, 11, 18, 26). An enzyme from vaccinia virions is able to cut supercoiled plasmids and also to cross-link the ends in a non-sequence-specific manner (17, 22, 31). The role of this enzyme in vivo, however, is unknown.

To discriminate between sequence-specific and purely structural requirements for resolution, plasmids containing symmetrical insertions or deletions were prepared and transfected into virus-infected cells. Preliminary results demonstrated that plasmids with palindromic inserts larger than 250 bp but with an altered nucleotide sequence were not resolved in infected cells, implying sequence specificity (24). The purpose of the present study was to determine the precise boundaries of the putative specific nucleotide sequence utilized for resolution. A series of symmetrical insertions, deletions, and site-directed oligonucleotide mutations demonstrated that the presence of a DNA sequence consisting of the bases ATTTAGTGTCTAGAAAAAAA on both sides of the apical segment of the concatemer junction is crucial for resolution. Conservation of this sequence near the ends of the genomes of other poxviruses suggests that it has a general role in the resolution of concatemers.

## MATERIALS AND METHODS

**Construction of plasmids.** The plasmid pNCO was generated from pHD, a plasmid containing the *Hin*fI fragment derived from the concatemer junction of vaccinia virus DNA (24). pHD was digested with *Xba*I and ligated to a 37-bp synthetic DNA fragment containing nucleotides 192 through 228 (and nucleotides 19 through 55) except for changes A to G at position 216 and T to A at position 228 (and corresponding changes of T to C at position 35 and A to T at position 23) as marked by the asterisks in Fig. 1. These changes created *Nco*I sites at positions 35 and 211 and destroyed the *Xba*I sites originally present at positions 18 and 228. When the central 132-base-pair *Xba*I fragment that includes the double-stranded form of the hairpin loop was added, the con-

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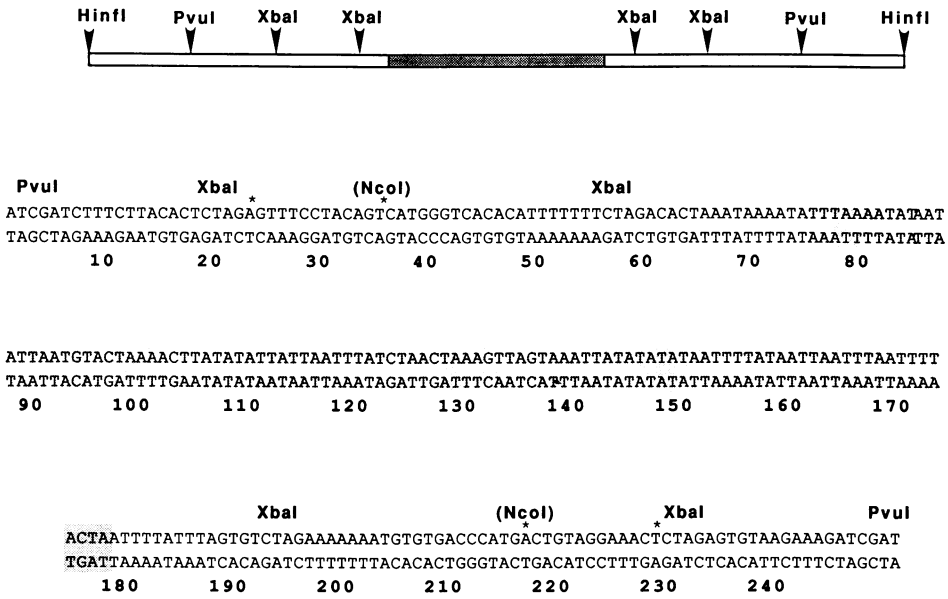


FIG. 1. Schematic representation and nucleotide sequence of the vaccinia virus concatemer junction. The upper portion of the figure denotes the restriction enzyme map of the concatemer junction fragment present in pHD. The lower portion of the figure shows the nucleotide sequence between the *Pvu*I sites in pHD. The shaded area is the double-stranded form of the hairpin loop which forms an imperfect palindrome at the apex of the concatemer junction. Nucleotides changed to construct pNCO are indicated (*) (see Materials and Methods for details). The *Nco*I sites created to form pNCO are indicated in parentheses.

catemer junction, except for the changes at the outer *Xba*I sites and the new *Nco*I sites, was recreated.

A series of plasmids containing deletions between the *Xba*I site at position 55 and the *Nco*I site at position 35 (as well as the *Xba*I site at position 191 and the *Nco*I site at position 211) were built by following the strategy outlined in Fig. 2. The pNCO was digested with *Nco*I and joined to double-stranded oligonucleotides phosphorylated with T4 kinase (19). The mixture was digested with *Xba*I and subjected to agarose gel electrophoresis. The plasmid DNA was electrophoretically transferred to DEAE 81 paper (Whatman) and eluted with 1.25 M NaCl–7.5 mM Tris hydrochloride, pH 7.5–0.75 mM EDTA. After ethanol precipitation, the pellet was suspended in 10 mM Tris hydrochloride, pH 8.0–1 mM EDTA. The 132-bp fragment containing the double-stranded form of the hairpin loop was then added, regenerating a plasmid containing the concatemer junction.

The nucleotide sequence of each of the inserts was verified by using dideoxynucleotide sequencing on plasmid DNA (15) that was isolated by boiling (16) or alkaline minilysis (3). Both sides of the palindrome were examined by using oligonucleotide primers corresponding to the reverse primer (New England BioLabs) and the universal primer (a gift of A. J. Davidson).

The plasmids pEC and pHC were constructed from pNCO. pNCO was digested with *Xba*I and ligated to the double-stranded oligonucleotide 5′-CGATATTTTATTTAG TGT-3′ which replaced nucleotides 178 through 191 (as well as 60 through 73) in Fig. 1 and generated a *Cla*I restriction enzyme site at the border of the hairpin loop. The molecule was digested with *Eco*RI and *Hin*dIII, which cleaved the DNA into three fragments: a large one containing solely pUC13 and two corresponding to the halves of the vaccinia concatemer junction from the *Hin*fI site (and including the polylinker of pUC13 to the *Eco*RI or *Hin*dIII sites) to the *Cla*I site at the border of the hairpin loop. The mixture was ligated to the large *Eco*RI-*Cla*I and *Hin*dIII-*Cla*I fragments of pBR322. The *Eco*RI-*Hin*dIII fragments from the two pBR322 clones containing the concatemer junction were purified after separation from the vector DNA by agarose gel electrophoresis and ligated to *Eco*RI- and *Hin*dIII-digested pUC13. Two plasmids were generated: one containing the left side (pHCE) and the other containing the right side (pECH) of the vaccinia virus concatemer junction from the *Hin*fI site to the *Cla*I site at the border of the hairpin loop. Next, the plasmids were digested with *Cla*I and either *Hin*dIII (pECH) or *Eco*RI (pHCE) and ligated to the double-stranded oligonucleotide 5′-CGATATTTAAAATATAATA TTAATGTACTAAAACTTATATATTATTAATTTATCCA CTAAAGTGGA-3′ (pECH) or 5′-AATTCATATCCACTA AAGTGGTAAATTATATATATAATTTTATAATTAATT TAATTTTACTAAT-3′ (pHCE), generating pEC and pHC. The restriction maps of these two plasmids are illustrated in Fig. 3. pHC and pEC each contain half of the concatemer junction with a *Bst*XI site at the apex of the region corresponding to the duplex form of the hairpin loop. The nucleotide sequence of each plasmid was verified by dideoxynucleotide sequencing.

The plasmid pECHC was constructed from pEC and pHC. Both plasmids were digested with *Eco*RI and *Bst*XI, and the large fragment from pHC and the smaller DNA fragment from pEC were purified by agarose gel electrophoresis and ligated to form pECHC, a plasmid containing a synthetic concatemer junction from vaccinia virus with a 102-bp region corresponding to the hairpin loop.

A series of plasmids containing deletions or site-directed mutations between the border of the hairpin loop and the inner *Xba*I site were constructed from pEC and pHC by following the strategy outlined in Fig. 3. The plasmids were digested with *Xba*I and *Cla*I, and the large DNA fragment from each was purified by agarose gel electrophoresis and ligated to double-stranded oligonucleotides. The structures of the inserts were verified by dideoxynucleotide sequencing of plasmids that had been isolated by a boiling minilysis

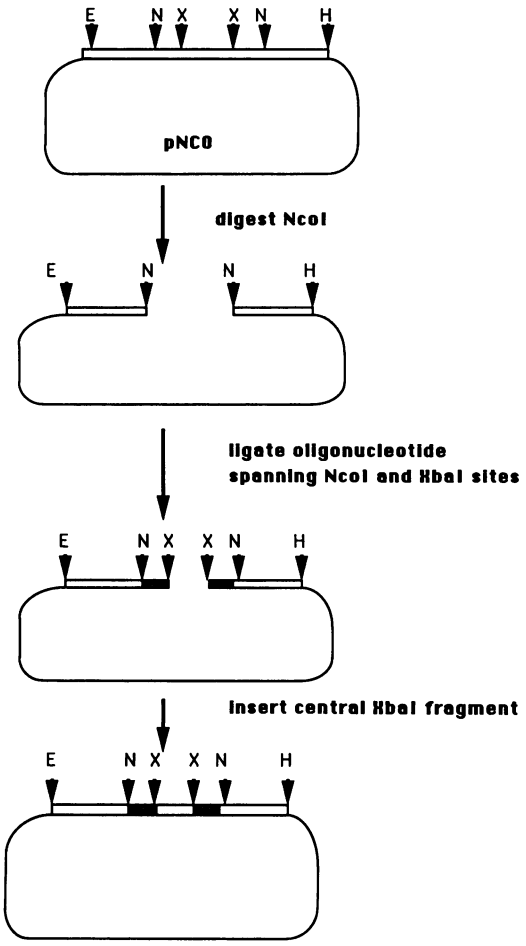


FIG. 2. Strategy for constructing plasmids to determine the outer boundary of the resolution sequence. The starting plasmid, pNCO, was digested with *Nco*I to remove the central portion of the concatemer junction. The latter was rebuilt in two steps with synthetic oligonucleotides and the *Xba*I fragment. ▨, Region corresponding to the double-stranded oligonucleotide containing the appropriate mutation. E, *Eco*RI; N, *Nco*I; H, *Hin*dIII; X, *Xba*I.

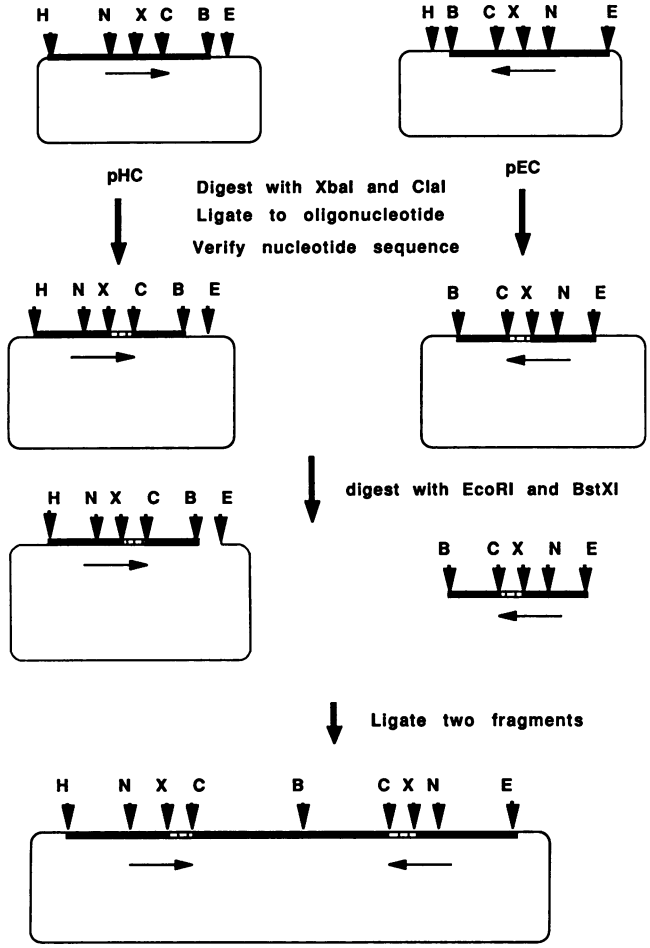


FIG. 3. Strategy for constructing plasmids to determine the inner boundary of the resolution sequence. The plasmids pEC and pHC were constructed from pNCO as described in Materials and Methods. The vaccinia virus DNA (▬), plasmid DNA (——), and the double-stranded oligonucleotide containing the appropriate mutation (▥) are indicated. The horizontal arrows denote the nucleotide sequence of the vaccinia virus concatemer junction from the *Hin*fI site towards the hairpin loop. Restriction sites are indicated as follows: H, *Hin*dIII; N, *Nco*I; X, *Xba*I; C, *Cla*I; B, *Bst*XI; and E, *Eco*RI.

procedure (16). Each plasmid was digested with *Eco*RI and *Bst*XI, and the large fragment from the pHC-derived plasmid and the small fragment from the pEC-derived plasmid were purified by agarose gel electrophoresis and joined with T4 DNA ligase to form plasmids containing modified concatemer junctions. The structures of the inserts were verified by dideoxynucleotide sequencing. All restriction endonucleases were purchased from New England BioLabs and used as suggested by the manufacturer.

**Transfections.** Confluent monolayers of human 293 cells (14) on six-well tissue culture dishes (17.5-mm radius) were infected with vaccinia virus WR at 10 PFU per cell. Between 1 and 2 μg of plasmid DNA was transfected per well without carrier DNA as reported elsewhere (21). Cytoplasmic DNA was isolated at 24 h posttransfection (21) and digested with the restriction enzymes *Dpn*I and *Nde*I. After electrophoresis through ME agarose (FMC Corp.), the DNA was transferred to GeneScreen Plus (Du Pont Co.) and hybridized with the plasmid vector labeled with $^{32}P$ by nick translation. Fluorographs were performed by using Kodak XAR film.

## RESULTS

**Determination of the outer border of the resolution sequence.** In concatemeric forms of vaccinia virus DNA, the unit genomes are connected by very long palindromes derived from the 10,000-bp inverted terminal repetition. The nucleotide sequence of the central portion of the concatemer junction of vaccinia virus DNA is shown in Fig. 1. The shaded 104-bp segment (74 to 177) derived from the incompletely base-paired hairpin loop is an imperfect palindrome. The left- and right-flanking sequences are identical but opposite in orientation. Previously, it was shown that a plasmid called pHD, containing the 412-bp junction fragment produced by *Hin*fI cleavage, was replicated and resolved into a linear minichromosome when transfected into vaccinia

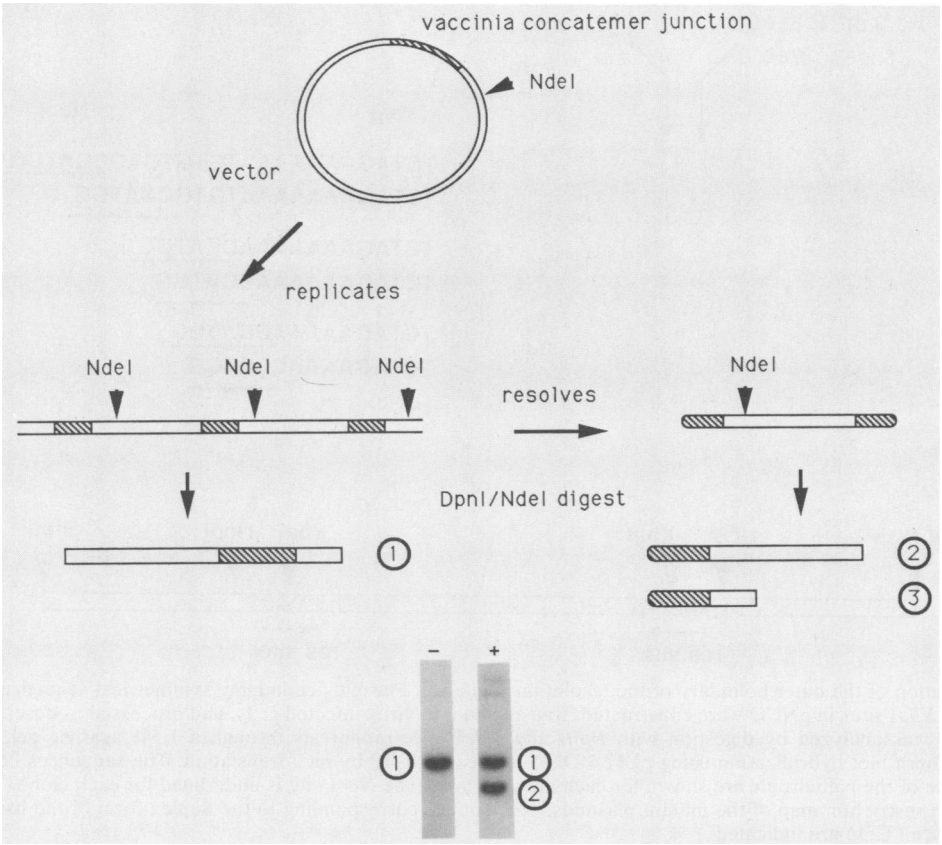


FIG. 4. Protocol used to detect replication and resolution of input plasmid. Plasmids containing the vaccinia virus concatemer junction were transfected into virus-infected cells and processed as described in Materials and Methods. DNA was analyzed by digestion with *Dpn*I and *Nde*I, electrophoresis through agarose gels, transfer to nylon membranes, and Southern blot hybridization using vector DNA labeled with $^{32}P$ by nick translation. A single unit-length DNA segment was produced from replicated circular or concatemeric DNA, whereas two smaller bands were produced from resolved linear molecules. Under the electrophoretic conditions used, the smaller of the two segments from the resolved DNA migrated off the bottom of the gel. The autoradiograph on the left shows the single segment of replicated DNA (segment 1), and the one on the right shows both the segment of replicated DNA and the larger of the two segments of resolved DNA (segments 1 and 2, respectively).

virus-infected cells (24). This transfection assay (Fig. 4) distinguishes the input methylated plasmid, which is *Dpn*I sensitive, from replicated DNA, which is unmethylated and therefore *Dpn*I resistant. Furthermore, replicated circular or linear concatemeric molecules are cleaved into unit-length segments by the *Nde*I restriction enzyme; resolved molecules, however, are cleaved into two smaller pieces. After agarose gel electrophoresis, the DNA fragments were transferred to a nylon membrane and hybridized to a $^{32}P$-labeled vector plasmid probe containing no vaccinia virus DNA sequences. Under the conditions of electrophoresis, the unit-length fragment migrated most slowly; the larger fragment from the resolved DNA migrates slightly faster, and the smaller one ran off the bottom of the gel. In Fig. 4, the gel at the bottom left shows the products of DNA that was replicated, whereas the gel on the right shows the products of a mixture of replicated and resolved DNA.

We were interested in determining whether a specific DNA sequence within the concatemer junction fragment was required for resolution and, if so, in locating its boundaries. As a preliminary experiment, we demonstrated that the inversion of both 37-base-pair fragments bounded by *Xba*I sites in Fig. 1 was sufficient to abolish resolution, even though the size of the palindromic insert was unchanged (24). Thus, we assumed that at least part of the sequence required for resolution would lie between the two *Xba*I sites. In order to facilitate mutagenesis of this region, two of the four *Xba*I sites of pHD were altered and two convenient *Nco*I sites were inserted as described in Materials and Methods. The resulting plasmid, pNCO, contained a 412-bp concatemer junction differing from the wild-type sequences only at nucleotides 23 and 228 (destroying the *Xba*I site on each side of the palindrome) and at nucleotides 35 and 216 (creating a *Nco*I site on each side of the palindrome) as shown in Fig. 1. The new plasmid, when transfected into infected cells, was resolved into linear minichromosomes as efficiently as the original one (data not shown).

Next, we wished to determine whether the DNA proximal to the *Nco*I site was required for resolution. Accordingly, the *Nco*I fragment containing the concatemer junction from pNCO was purified, and the ends were filled in with DNA polymerase. The blunt fragment was then ligated to the large *Xba*I fragment of pHD whose ends also had been filled in with DNA polymerase. Although the resulting plasmid contained a symmetrical deletion between the *Nco*I site and the outer *Xba*I site (positions 23 to 35 and 216 to 228 in Fig. 1), it was resolved as efficiently as pHD or pNCO (data not shown). This result implied that the outer boundary of the resolution site lies between the *Nco*I site and the border of the hairpin loop.

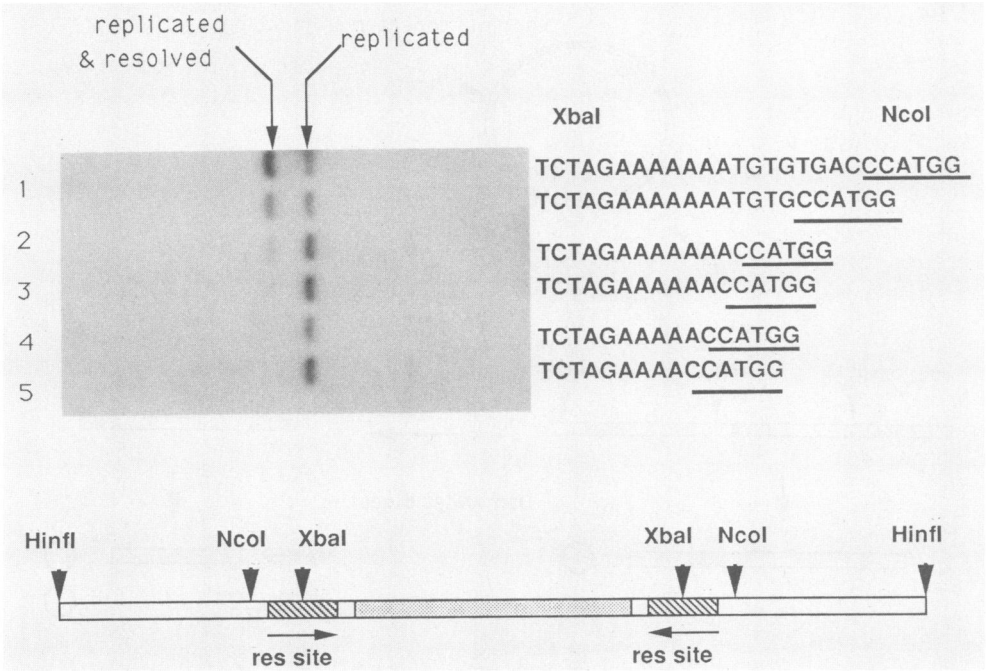


FIG. 5. Determination of the outer boundary of the resolution sequence. Plasmids containing symmetrical sequence alterations between each set of *Nco*I and *Xba*I sites in pNCO were constructed, transfected into virus-infected cells, and processed as described in Materials and Methods. The DNA was analyzed by digestion with *Dpn*I and *Nde*I, electrophoresis through a 1.5% agarose gel, transfer to a nylon membrane, and Southern blot hybridization using pUC13 DNA labeled with $^{32}P$ by nick translation. The sequences between the *Xba*I and *Nco*I sites on one side of the palindrome are shown for each mutant clone. The *Nco*I site is underlined for each clone. The lower portion of the figure denotes the restriction map of the mutant plasmids. Nucleotides corresponding to the duplex form of the hairpin loop ( ▥ ) and the resolution sequence ( ▨ ) are indicated.

To precisely determine the outer boundary of the resolution site, a series of plasmids was constructed by using synthetic oligonucleotides spanning the *Nco*I-to-*Xba*I sites at positions 40 to 56 and 191 to 211 as depicted in Fig. 1. The strategy for fabricating this set of mutants is outlined in Fig. 2. In each case, the plasmid contained a symmetrical concatemer junction with two identical copies of the mutant sequence in inverted orientation. The resolution assay for the set of mutants used to define the outer boundary of the resolution site is shown in Fig. 5. Since all plasmids, irrespective of sequence, are replicated in vaccinia virus-infected cells (6, 24), a *Dpn*I-resistant band corresponding to unit-length monomer was detected in all samples. After digestion with *Dpn*I and *Nde*I, two fragments were detected for pNCO (Fig. 5, lane 1). The faster-migrating band, derived from the resolved linear minichromosome, constituted more than half of the total *Dpn*I-resistant material. The percentage of the material found in the band arising from resolved linear minichromosomes decreased as the deletions progressed from the *Nco*I site towards the *Xba*I site. When only 3 bp were deleted (Fig. 5, lane 2), resolution occurred at nearly wild-type levels, while less (but detectable) resolution was observed when 7 nucleotides, including TGTG, were removed (lane 3). Little or no resolution was observed when an additional A was deleted (lane 4), even though the plasmid retained a symmetrical 396-bp insert. Thus, the last of the seven A residues and the adjacent TGTG nucleotides mark the outer boundary of the resolution sequence.

**Determination of the inner border of the resolution sequence.** We suspected that the inner border of the sequence required for resolution would not extend into the central 104-bp imperfect palindromic region that forms the hairpin loop. In order to facilitate analysis of this region, a set of vectors that could be manipulated more easily than the wild-type concatemer junction was constructed. Two plasmids containing opposing halves of the concatemer junction, pEC and pHC, were constructed. A *Bst*XI site was placed at the apex of the junction, and a *Cla*I site was placed at the outer border of the imperfect palindrome. When the segments of pEC and pHC were combined, they made a complete symmetrical concatemer junction (Fig. 3), with three changes at each border of the imperfect palindrome in order to introduce the pair of *Cla*I sites and 5 nucleotide changes within the imperfect palindrome in order to accommodate the *Bst*XI site. Plasmid pECHC, containing nucleotides identical to those of the wild-type virus save for the changes required to introduce the *Cla*I and *Bst*XI sites, was replicated and resolved (Fig. 6, lane 1) indistinguishably from pNCO or pHD.

A series of plasmid derivatives of pECHC was constructed, using pEC and pHC as intermediates and using the strategy outlined in Fig. 3. Each plasmid contained a concatemer junction with a symmetrical deletion between the natural *Xba*I sites at positions 56 and 191 in Fig. 1 and the newly introduced *Cla*I sites at the edges of the imperfect palindrome. Removal of 5 bp between the *Cla*I and *Xba*I sites had little or no effect on resolution (Fig. 6, lane 2). Deletion of a single additional nucleotide, however, resulted in a dramatic reduction in the resolution of the plasmid (lane 3). The inner boundary of the resolution site was sharply defined at the A or T residues at positions 68 or 69 and 183 or 184 in Fig. 1.

On the basis of the two sets of deletion mutants, we deduced that two inverted copies of the sequence ATTTAG TGTCTAGAAAAAAATGTG separated by the duplex form of the hairpin loop is sufficient for resolution. This prediction

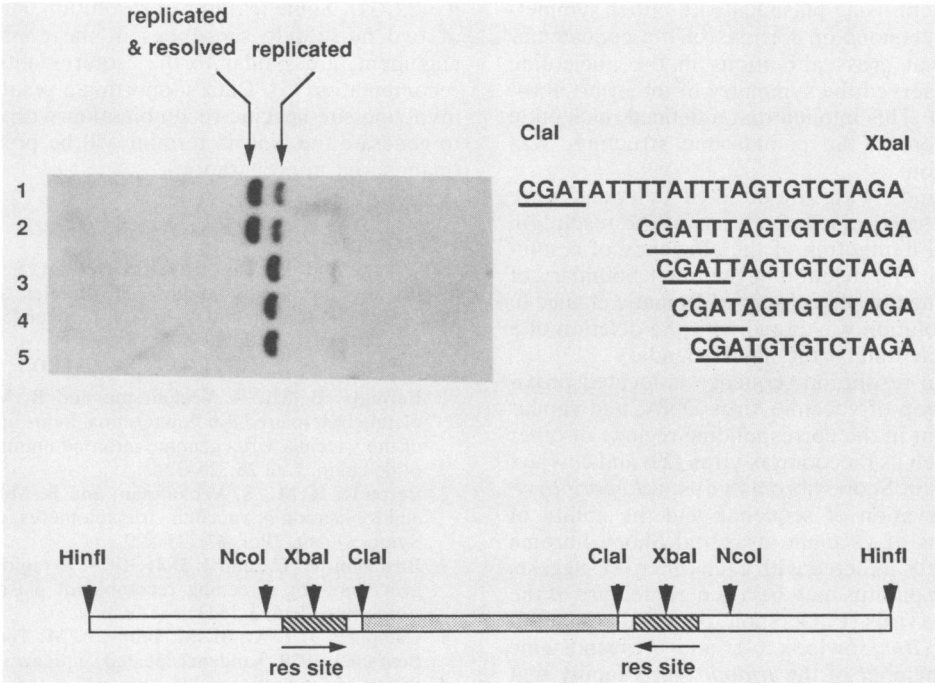


FIG. 6. Determination of the inner boundary of the resolution sequence. Plasmids containing symmetrical sequence alterations between the *Cla*I site at the border of the hairpin loop and the *Xba*I site on both sides of the palindrome were constructed, transfected into virus-infected cells, and processed as described in Materials and Methods. The DNA was analyzed by digestion with *Dpn*I and *Nde*I, electrophoresis through a 1.5% agarose gel, transfer to a nylon membrane, and Southern blot hybridization using pUC13 DNA labeled with $^{32}P$ by nick translation. The sequences between the *Cla*I and *Xba*I sites on one side of the palindrome are shown for each mutant clone. The *Cla*I site at the border of the sequences corresponding to the duplex form of the hairpin loop is underlined. The lower portion of the figure denotes the restriction enzyme map of the mutant plasmids. Sequences corresponding to the duplex form of the hairpin loop ( ▥ ) and the resolution sequences ( ▨ ) are indicated.

was confirmed by the construction of a plasmid by using pEC and pHC containing ATTTAGTGTCTAGAAAAAAT GTG between the *Cla*I and *Nco*I sites. This plasmid resolved as efficiently as plasmids containing the complete wild-type concatemer junction (data not shown).

**Conservation of the resolution sequence among poxviruses.** The sequence shown to be required for the resolution of plasmids containing the vaccinia virus concatemer junction was compared with the region of the genome proximal to the hairpin loops of the *Orthopoxviruses* raccoonpox virus and cowpox virus and of a *Leporipoxvirus*, Shope fibroma virus (Fig. 7). In each viral genome, a stretch of nucleotides closely corresponding to the sequence required for resolution in vaccinia virus was observed. Two regions (boxed shaded regions in Fig. 7) were identical, while the intervening sequence was different in both length and nucleotide composition.


FIG. 7. Alignment of the nucleotide sequence proximal to the hairpin loop. The nucleotide sequences from the terminal regions of the *Orthopoxviruses* vaccinia virus (1), raccoonpox virus (29), and cowpox virus (30) and the *Leporipoxvirus* Shope fibroma virus (8) were aligned with respect to the sequence required for resolution for vaccinia virus. The leftmost nucleotide shown for vaccinia virus is adjacent to the hairpin loop, whereas the corresponding nucleotide for Shope fibroma virus lies 10 bases from the hairpin loop. The boxed shaded regions are conserved.

## DISCUSSION

The aim of this investigation was the elucidation of the boundaries of the nucleotide sequence required for the conversion of the vaccinia virus concatemer junction to mature hairpin termini. Advantage was taken of the previous demonstration that plasmids containing such sequences were converted into linear molecules with hairpin termini after transfection of virus-infected cells (8, 23). Since neither replication nor resolution occurs in uninfected cells, the assumption is that these processes are carried out by viral factors. Those earlier studies demonstrated efficient resolution of plasmids containing palindromic inserts larger than 240 bp. Further investigation with Shope fibroma virus has identified an approximately 60- to 70-bp region as being important for resolution (7). In the latter experiments, mutations were generated by progressive deletions resulting in a series of smaller palindromic inserts. Thus, this approach could not discriminate between the size of the palindromic insert and the nucleotide sequence present within the concatemer junction as the determinant responsible for resolution. To divorce structural effects from those due to nucleotide sequence, a series of plasmids containing symmetrical insertions, deletions, and site-directed oligonucleotide mutations were tested for their abilities to be resolved. This method of construction preserved the palindromic structure of the insert, allowing one to ascribe any changes in resolution to alterations in the primary nucleotide sequence. A

preliminary experiment using plasmids with either symmetrical deletions or inversions of portions of the concatemer junction revealed that gross alterations in the nucleotide sequence which conserved the symmetry of the insert abrogated resolution (24). This implied that a defined nucleotide sequence, in addition to the palindromic structure, was required for resolution.

A series of plasmids with site-directed deletions were made to determine the precise boundaries of the resolution sequence. A gradual diminution of the efficiency of resolution occurred over a 5-bp deletion at the outer boundary of the resolution sequence, whereas a more dramatic change in the efficiency of resolution was noted with the deletion of a single additional nucleotide at the inner boundary.

The 20-bp minimal resolution sequence is located proximal to the hairpin loop of vaccinia virus DNA, and similar sequences are present in the corresponding regions of other *Orthopoxviruses*, such as raccoonpox virus (29) and cowpox virus (30), as well as in Shope fibroma virus, a *Leporipoxvirus* (8). This conservation of sequence and the ability of concatemer junctions of vaccinia virus and Shope fibroma virus to resolve in cells infected with each other (8) suggests that the resolution apparatus may be a general feature of the poxviruses. Capripoxvirus (P. Gershon, personal communication), the *Avipoxvirus* fowlpox (4), and African swine fever virus (13), a member of the *Iridopoxvirus* family that shares many of the properties of the poxviruses, also contain consecutive T-rich and A-rich sequences proximal to the hairpin loop. Functional studies will be required to determine whether these sequences are also required for resolution.

Although the present study has localized the boundaries of the resolution sequence, the importance of each nucleotide within it remains to be determined. Interestingly, the 4 nucleotides GTGT found between the group of A and T residues in vaccinia virus are the 5 nucleotides TAACC in Shope fibroma virus. Since plasmids containing the Shope fibroma virus resolution site can be resolved in cells infected with vaccinia virus (8), the precise sequence required for resolution may be discontinuous. Studies are under way to determine the precise sequence requirements for the resolution site.

Resolution does not solely depend on the presence of two inverted copies of the 20-nucleotide sequence. Resolution is not observed when the region corresponding to the hairpin loop is replaced by nonpalindromic DNA for both vaccinia virus (data not shown) and Shope fibroma virus (20). Evidently, hairpin loops can only be formed if the region between the resolution sequences can be organized into a self-complementary structure. Resolution was not observed when the sequences were placed farther than 200 nucleotides apart, even when they were separated by a palindromic center (data not shown). A similar reduction in resolution with an increase in length of the palindromic central region has also been noted for Shope fibroma virus (20). Resolution, therefore, depends on the interplay of sequence-specific elements within the context of a definite structure.

The mechanism for conversion of the concatemer junction to mature hairpins remains unknown. Resolution is contingent on viral late gene expression, as temperature-sensitive viral mutants defective in this step process their terminal sequences poorly at the nonpermissive temperature (25). Moreover, resolution is not simply a product of replication or homologous recombination, as those processes only require early gene expression (21, 25). One candidate protein involved in resolution is a DNase with nicking-joining activity (22, 31). Some features of resolution, such as the position of two nucleotide sequences in the context of a specific alignment, are similar to the requirements for site-specific recombination (5). Data supporting a resolution mechanism involving site-specific recombination with branch migration to generate the hairpin termini will be presented elsewhere (manuscript in preparation).

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