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Mutational Analysis of the Resolution Sequence of Vaccinia Virus DNA: Essential Sequence Consists of Two Separate AT-Rich Regions Highly Conserved among Poxviruses

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In replicative forms of vaccinia virus DNA, the unit genomes are connected by palindromic junction fragments that are resolved into mature viral genomes with hairpin termini. Bacterial plasmids containing the junction fragment for vaccinia virus or Shope fibroma virus were converted into linear minichromosomes of vector sequence flanked by poxvirus hairpin loops after transfection into infected cells. Analysis of a series of symmetrical deletion mutations demonstrated that in vaccinia virus the presence of the DNA sequence ATTTAGTGTCTAGAAAAAAA on both sides of the apical segment of the concatemer junction is crucial for resolution. To determine the precise architecture of the resolution site, a series of site-directed mutations within this tract of nucleotides were made and the relative contribution of each nucleotide to the efficaciousness of resolution was determined. The nucleotide sequence necessary for the resolution of the vaccinia virus concatemer junction, (A/T)TTT(A/G)N_{7-9}AAAAAAA, is highly conserved among poxviruses and found proximal to the hairpin loop in the genomes of members of the *Leporipoxvirus*, *Avipoxvirus*, and *Capripoxvirus* genera.

Vaccinia virus, the prototypical member of the poxviruses, has a large, linear double-stranded DNA genome of approximately 185,000 base pairs with 10-kilobase-pair inverted terminal repetitions (11, 33). The ends of the genome are joined to form 104-nucleotide terminal hairpin loops (1) that are rich in A and T residues, incompletely base paired, and present in two forms that are complementary and inverted.

Restriction enzyme analysis of replicative forms of vaccinia virus (2, 25), rabbitpox virus (26), and Shope fibroma virus (8) DNA have revealed palindromic forms of the terminal sequence. The vaccinia virus DNA fragment that connects concatemers has been isolated, cloned into a bacterial plasmid, and shown to be a nearly perfect palindrome, with each strand corresponding to one of the sequence conformers of the terminal hairpin from vaccinia virus (20). The plasmid containing the concatemer junction, when transfected into vaccinia virus-infected cells, was resolved into the linear minichromosome, a covalently continuous linear molecule of vector DNA flanked by vaccinia virus hairpin loops (10, 21).

A series of symmetrical insertions, deletions, and site-directed oligonucleotide mutations demonstrated that the presence of a DNA sequence consisting of ATTTAGT GTCTAGAAAAAAA on both sides of the apical segment of the concatemer junction in vaccinia virus is crucial for resolution (23). 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. To determine the precise architecture of the resolution site, a series of site-directed mutations within this tract of nucleotides were made and the relative contribution of each nucleotide to the efficaciousness of resolution was determined. The nucleotide sequence necessary for the resolution of the vaccinia virus concatemer junction was determined to be (A/T)TTT(A/G) N_{7-9}AAAAAAA. This stretch of nucleotides is highly conserved among poxviruses and found proximal to the hairpin loop in the genomes of members of the *Orthopoxvirus*, *Leporipoxvirus*, *Avipoxvirus*, and *Capripoxvirus* genera.

MATERIALS AND METHODS

Plasmids. Plasmids with altered resolution sequences were assembled from derivatives of the plasmids containing halves of the 412-base-pair vaccinia virus *Hin*fI concatemer junction, pEC and pHC (see Fig. 1) (23). Sets of oligonucleotides were annealed to generate double-stranded DNA fragments with defined changes in the resolution sequence and overlapping ends corresponding to *Cla*I, *Xba*I, or *Nco*I sites. The molecules were combined with the portion of pEC and pHC generated by the digestion of each plasmid with *Cla*I and *Xba*I, *Cla*I and *Nco*I, or *Xba*I and *Nco*I and isolation of the larger DNA fragment by electrophoresis through agarose and electroelution (23). The nucleotide sequence of the resolution site for each of the derivatives of pEC and pHC was verified by dideoxynucleotide sequencing (16). Portions of the modified pEC or pHC were combined into a molecule containing the entire palindromic *Hin*fI concatemer junction by following the scheme outlined for the construction of pECHC (23) (Fig. 1). The nucleotide sequence for the resolution site for each plasmid was verified by using dideoxynucleotide sequencing on plasmid DNA that was isolated by alkaline minilysis (18). Both sides of the palindrome were examined by using oligonucleotide primers corresponding to the reverse primer (New England BioLabs and gift of S. Chakrabati) and universal primer (gift of A. J. Davison). Restriction enzymes and reagents to determine the nucleotide sequence were purchased from New England BioLabs and United States Biochemicals, respectively, and used as suggested by the manufacturers.

Transfections. Confluent monolayers of human 293 cells (15) on six-well tissue culture dishes (17.5-mm radius) were infected with vaccinia virus WR at 10 PFU/cell. Between 1 and 2 μg of plasmid DNA per well was transfected, and cytoplasmic DNA was isolated at 24 h posttransfection as

described elsewhere (18). The samples were digested with the restriction enzymes *Dpn*I and *Nde*I, electrophoresed through ME agarose (FMC Corp.), transferred to GeneScreen Plus (Dupont, NEN Research Products), and hybridized with the plasmid vector labeled with ^{32}P by random primer extension (Bethesda Research Laboratories) as suggested by the manufacturer. The radioactive material hybridizing to each fragment was quantitated by analysis on a Betagen Betascope 603. Fluorographs were performed by using Kodak XAR film.

RESULTS

Experimental approach. A series of mutations demonstrated that the presence of a DNA sequence consisting of ATTTAGTGTCTAGAAAAAAA on both sides of the apical segment of the concatemer junction is crucial for resolution (23). This work, as well as other experimental approaches utilizing unidirectional deletion mutants (9), served only to establish the borders of the region required for resolution. To determine the precise architecture of the resolution site, the effects of a series of site-directed mutations within the resolution sequence were examined by the resolution assay. Plasmids containing mutant resolution sequences were constructed from the pair of plasmids comprising halves of the concatemer junction, pEC and pHC. Each of these molecules has the normal resolution sequence containing an *Xba*I site but has been modified so that the resolution sequence is flanked by *Cla*I and *Nco*I sites (23) (Fig. 1). Double-stranded oligonucleotides with either a *Cla*I or an *Xba*I site on one end and a *Xba*I or an *Nco*I site on the other end were inserted into pEC and pHC. Portions of the altered pEC and pHC were combined to form plasmids containing the entire concatemer junction as described for pECHC (23).

The individual plasmids were transfected into 293 cells infected with vaccinia virus. The DNA was isolated 24 h after transfection, digested with *Dpn*I and *Nde*I, and analyzed by Southern blot hybridization using vector sequences labeled with ^{32}P. This resolution assay distinguishes the input methylated plasmid, which is *Dpn*I sensitive, from replicated DNA which is unmethylated and therefore *Dpn*I resistant. Since the input plasmids contain a single *Nde*I site, replicated circular or linear concatemeric molecules will be cleaved into unit-length segments of 3.1 kilobases (Fig. 1). All plasmid DNA, irrespective of origin, replicates in vaccinia virus-infected cells (8, 21, 22); thus, a unit-length 3.1-kilobase *Nde*I-*Dpn*I-resistant band is always observed. The resolved linear minichromosome, however, will be cleaved into two smaller pieces. One fragment of 2.7 kilobases migrates slightly faster than the unit length DNA while the other fragment of 0.4 kilobases migrates off the gel. Therefore, in those samples in which a portion of the replicated plasmid is also converted into linear minichromosomes, a second 2.7-kilobase *Nde*I fragment, diagnostic of resolution, is also detected (23).

After hybridization with labeled vector sequences, the amount of radioactivity was determined for both the 2.7- and 3.1-kilobase fragments by using a Betagen scanner, and the percentage of ^{32}P material in the 2.7-kilobase band was calculated. The value for the percentage of resolved material for each sample was determined by averaging the results from three Southern blots derived with DNA isolated from independent transfections. The plasmid containing the normal resolution sequence of vaccinia virus, pECHC, had approximately 50% of the radioactivity in the 2.7-kilobase fragment. The plasmid pXXD, which contains a 4-base-pair insertion at the *Xba*I sites in each resolution site, was scored as unresolved, as none of the 2.7-kilobase fragment was detected after autoradiographic exposure. However, a weak band and between 5 and 10% of the ^{32}P-labeled material were detected at the 2.7-kilobase position after prolonged survey with the Betagen scanner.

Mutational analysis of ATTTA. Since plasmids containing the concatemer junction of Shope fibroma virus were resolved in vaccinia virus-infected cells (10), the critical sequences for resolution must be shared between these poxviruses. The DNA sequence of the Shope fibroma virus genome and the corresponding resolution sequence in vaccinia virus are identical save for the replacement of nucleotides GTGT with TAACC (10). Thus, it appears the resolution sequence can tolerate some variability in the nucleotide sequence without abrogating resolution. For the purposes of this study, the resolution site was divided into three sections which were analyzed independently: first, the nucleotides ATTTA, which are identical in the two viruses; second, the nucleotides GTGTCTAG, which differ at GTGT in the two viruses; and finally, the stretch of seven A residues maintained in each of the two virus DNAs.

A set of plasmids containing alterations in the nucleotides ATTTA were assembled by using double-stranded oligonucleotides spanning from the *Cla*I to the *Xba*I site. Most changes within the ATTTA had a considerable deleterious effect on resolution (Fig. 2). Any deviation in the central three T residues (plasmids 99, 101, 103, 105, 107, 109, 147, and 149) reduced the percentage of ^{32}P counts in the 2.7-kilobase fragment to less than 10% except for sample 149 in which a faint band with 11% of the counts was detected. Diverse effects on resolution were noted for mutations in either of the flanking A residues. Replacement of the first A with a T, C, or G residue (plasmids 91, 141, and 93, respectively) changed the amount of the 2.7-kilobase material from 49% in pECHC to 45, 26, and 12%, respectively. Thus, resolution can still occur when the A has been replaced by a T residue and, to a lesser degree, when it is replaced by a C residue. Replacement of the second A residue with a G or T residue (plasmids 145 and 143) resulted in 47 and 35% levels of resolved material, respectively, implying that this A can be replaced by a G and, to a lesser extent, by a T. The replacement of both A residues (plasmids 95 and 97) by T or G residues abrogated resolution, dropping the percentage of counts in the 2.7-kilobase fragment to less than 10%.

Mutational analysis of the spacer region. Next, a series of plasmids with mutations in the sequence GTGTCTAG were assembled and tested in the resolution assay as shown in Fig. 3. The plasmids were constructed by using double-stranded oligonucleotides extending from the *Cla*I site to the *Xba*I site (plasmids 65, 67, 69, and 55) or from the *Cla*I site to the *Nco*I site (plasmids 127, 129, 131, 133, 135, and 137). All plasmids containing modifications within this stretch of nucleotides were resolved as efficiently as pECHC. Introduction of single nucleotide changes (plasmids 127, 129, 131, and 133), two nucleotide changes (plasmids 67, 135, and 137), or four nucleotide changes (plasmids 65 or 69) did not alter the efficiency of resolution. The replacement of GTGT by TAACC, the nucleotide sequence in this position for Shope fibroma virus (plasmid 55), did not decrease the efficiency of resolution (Fig. 3), even though this substitution increases the length of this section from 8 to 9 base pairs. Substitution of the 8 base pairs with 11, 12, 15, 18, or 19 base pairs, however, abolished resolution (data not shown).

Mutational analysis of the seven A residues. A set of

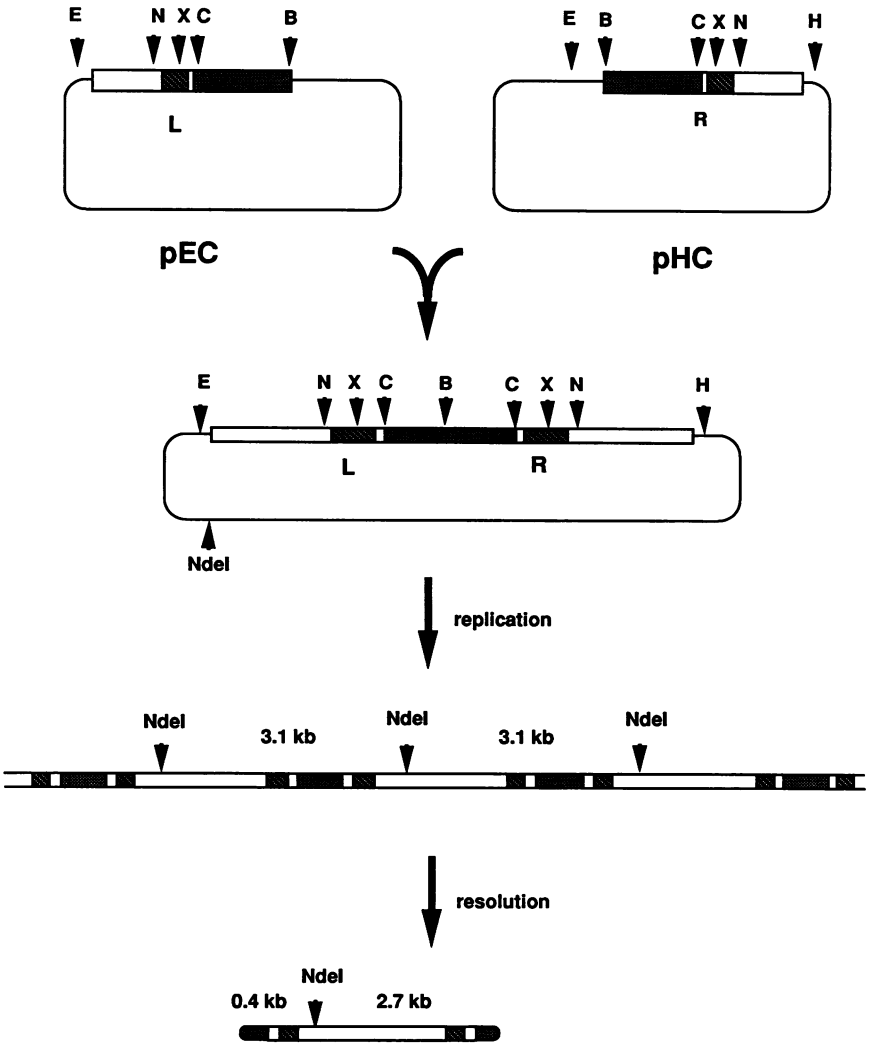

FIG. 1. Resolution of plasmids containing the concatemer junction of vaccinia virus. The top portion of the diagram denotes the plasmids pEC and pHC. ▨, Resolution sequence; ▩, double-stranded copy of the hairpin loop. The sites for the restriction enzymes *Eco*RI (E), *Nco*I (N), *Xba*I (X), *Cla*I (C), *Hin*dIII (H), and *Bst*XI (B) are shown. Each plasmid contains one-half (denoted as L or R) of the intact concatemer junction. The second portion of the figure denotes the plasmids containing the entire concatemer junction derived from the smaller *Eco*RI-*Bst*XI fragment from pEC and the larger *Eco*RI-*Bst*XI fragment from pHC. The site for the restriction enzyme *Nde*I is also indicated. The next portion of the figure denotes the product of replication of plasmids in cells infected with vaccinia virus. The large multimeric molecules contain *Nde*I sites separated by unit length (3.1 kilobases). The lowest portion of the figure denotes the product of resolution of plasmids in cells infected with vaccinia virus, the linear minichromosome. The origin of the 2.7- and 0.4-kilobase fragments is shown.

plasmids with modifications in the seven A residues or in the abutting TG nucleotide stretch were assembled and tested in the resolution assay as shown in Fig. 4. The plasmids were constructed by using double-stranded oligonucleotides spanning from the *Xba*I site to the *Nco*I site in the resolution sequence. The run of TGTGTG proximal to the seven A residues, which influences resolution in vaccinia virus (23), is not present in the Shope fibroma virus genome (10). Replacement of this alternating GT tract with eight G or T residues (plasmids 125 and 123) yielded 43 and 28%, respectively, of the material in the 2.7-kilobase band in comparison to 46% for pECHC. Thus, changes within this stretch alter the efficiency of, but do not abolish, resolution.

Mutations within the stretch of seven A residues were also tested for their effect on resolution. The magnitude of the effect of replacement of a single A with a T residue was dependent on the position of the substituted nucleotide, since replacement of the fourth, third, or second base pair from the inner border of the A tract (plasmids 157, 111, and 155) generated values of 43, 30, and 28% for the resolved fragment. Thus, the efficiency of resolution was directly proportional to the length of the A stretch closest to the spacer. Replacement of the third A in the tract with a G (plasmid 117) gave 25% of the counts in the 2.7-kilobase fragment, similar to the value of 30% reported for the substitution of the same A with a T (plasmid 111). Substitution of more than a single A residue with either T or G nucleotides (plasmids 113, 115, 119, and 121) dropped resolution levels below 10%.

The resolution efficiency was also measured after replacing the A tract with shorter A stretches. Plasmids were constructed containing six, five, or four consecutive A residues while retaining the surrounding nucleotide sequence. The levels of resolution decreased from 48 to 47 to

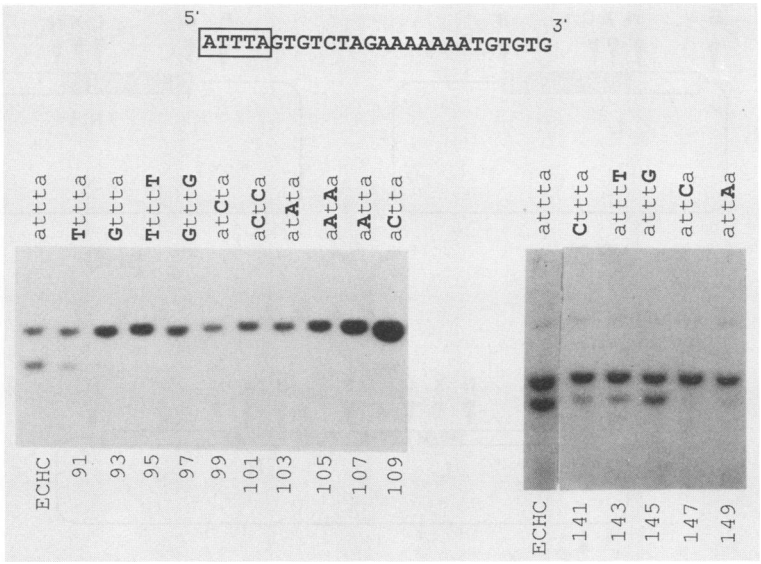

FIG. 2. Mutational analysis of the nucleotide sequence ATTTA. A series of plasmids containing altered resolution sites and the plasmid containing the normal resolution sequence from vaccinia virus (ECHC) were transfected into virus-infected 293 cells. After 24 h, the cytoplasm was isolated and processed as described in Materials and Methods. The DNA was analyzed by digestion with *Nde*I and *Dpn*I, electrophoresis through a 1.5% agarose gel, transfer to a nylon membrane, and Southern blot hybridization using vector DNA labeled with ^{32}P by random primer extension. The filter was analyzed with a Betascope 603 and visualized by autoradiography. The top of the figure illustrates the sequence of the resolution site in vaccinia virus. The boxed region corresponds to the altered region of the resolution sequence shown. The orientation of the mutagenized resolution sequences corresponds to plasmid pHC in Fig. 1. The nucleotide sequence through the boxed region is shown above and the number of each plasmid is shown below the lane for each sample. Upper- and lowercase letters refer to nucleotides different from or identical to those in vaccinia virus, respectively.

28 to 15% for lengths of 7 (pECHC), 6 (159), 5 (161), or 4 (163) A nucleotides, respectively.

A functional resolution sequence is conserved among the poxviruses. The members of the *Orthopoxvirus* genus, vaccinia virus, raccoonpox virus, and cowpox virus, share extensive nucleotide identity near the genomic termini (1, 27, 29). Cowpox virus contains a nucleotide sequence identical to the resolution sequence in vaccinia virus, whereas raccoonpox virus differs from the other two solely at the initial G nucleotide in the spacer region. The sequence required for telomere resolution appears to be retained among the members of the *Orthopoxvirus* genus (23).

The *Leporipoxvirus* Shope fibroma virus is disparate in nucleotide sequence from vaccinia virus, but cells infected with Shope fibroma virus resolve plasmids containing the concatemer junction from either Shope fibroma virus or vaccinia virus (10). Both viral genomes retain the ATTTA and seven A residues present in the vaccinia virus resolution sequence but differ in the length and primary sequence of the spacer region. These results imply that the sole primary nucleotide sequence determinants for resolution lie in the AT-rich regions on either side of the spacer.

The universality of the resolution sequence was investigated by comparing the nucleotide sequence from representatives of other genera of poxviruses with that of vaccinia virus. The nucleotide sequence near the genomic termini for the prototypical poxviruses, *Capripoxvirus* (12) and fowlpox virus (4), an *Avipoxvirus*, have recently been published. Both genomes are dissimilar to that of vaccinia virus but retain a pair of AT-rich regions interrupted by seven to nine nucleotides near the genomic termini. To determine if these regions could function as resolution sites, the sequence from these viruses corresponding most closely to the resolution sequence for vaccinia virus has been substituted for the resolution sequence in vaccinia virus and checked by the resolution assay. Also, a nucleotide sequence derived from the *Iridopoxvirus*, African swine fever virus, which has many properties in common with poxviruses, including the structure of the DNA ends in both replicating and nonreplicating molecules (13), was tested. In each example, double-stranded oligonucleotides spanning the *Cla*I to the *Nco*I sites were substituted for the vaccinia virus resolution sequence, and the plasmids were assayed for resolution as shown in Fig. 5. Plasmids containing the resolution sequence for Shope fibroma virus and vaccinia virus were resolved most efficiently. The putative resolution sequences for both *Capripoxvirus* and fowlpox virus were also resolved, albeit more poorly, in vaccinia virus-infected cells. The putative resolution sequence from African swine fever virus was not resolved in vaccinia virus-infected cells. Therefore, a nucleotide sequence which can serve as a resolution site in vaccinia virus-infected cells is maintained across at least four poxvirus genera.

DISCUSSION

Plasmids containing the DNA fragment that connects concatemers in vaccinia virus are resolved into linear minichromosomes in virus-infected cells (10, 21). This process requires a specific sequence of 20 nucleotides localized near the termini of the genome (23). The limits to this sequence element were previously ascertained by analysis of a series of deletion mutants and, thus, provided no information as to the relative contributions to resolution of each nucleotide within the sequence. This work describes a set of experiments utilizing site-directed mutants within the borders of the previously described region to identify the precise nucleotide sequence which participates in resolution. Compilation of the results presented in this report allows generation of a set of rules governing the constitution of the generic

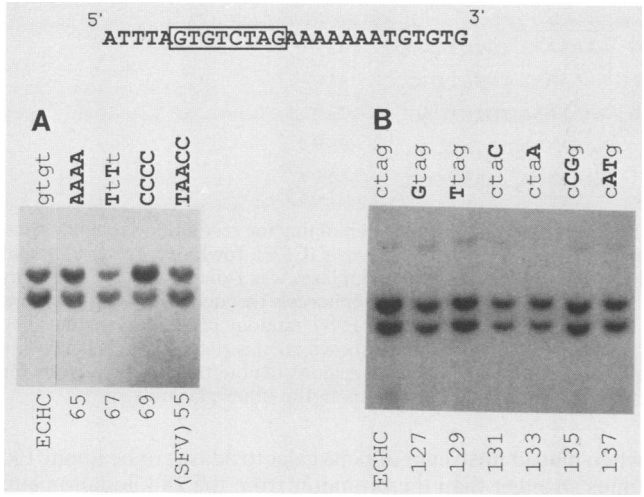

FIG. 3. Mutational analysis of the spacer region. A series of plasmids containing altered resolution sites, the plasmid containing the normal resolution sequence from vaccinia virus (ECHC), and the plasmid containing a nucleotide sequence from Shope fibroma virus (SFV) were transfected into vaccinia virus-infected 293 cells. After 24 h, the cytoplasm was isolated and processed as described in Materials and Methods. The DNA was analyzed by digestion with *Nde*I and *Dpn*I, electrophoresis through a 1.5% agarose gel, transfer to a nylon membrane, and Southern blot hybridization using vector DNA labeled with ^{32}P by random primer extension. The top of the figure illustrates the sequence of the resolution site in vaccinia virus. The boxed region corresponds to the altered region of the resolution sequence shown. The orientation of the mutagenized resolution sequences corresponds to plasmid pHC in Fig. 1. (A) Series of plasmids with mutations in the initial GTGT of the spacer region. The nucleotide sequence which replaces the GTGT is shown above and the number of each plasmid is shown below the lane for each sample; (B) series of plasmids with mutations in the CTAG section of the spacer region. The nucleotide sequence which replaces the CTAG is shown above and the number of each plasmid is shown below the lane for each sample. Upper- and lowercase letters refer to nucleotides different from or identical to those in vaccinia virus, respectively.

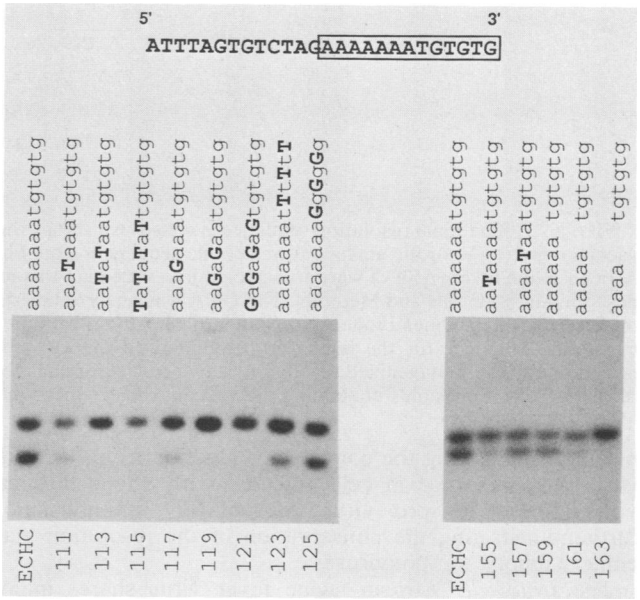

FIG. 4. Mutational analysis of the right-hand side of the resolution sequence. A series of plasmids containing altered resolution sites and the plasmid containing the normal resolution sequence from vaccinia virus (ECHC) were transfected into virus-infected 293 cells. After 24 h, the cytoplasm was isolated and processed as described in Materials and Methods. The DNA was analyzed by digestion with *Nde*I and *Dpn*I, electrophoresis through a 1.5% agarose gel, transfer to a nylon membrane, and Southern blot hybridization using vector DNA labeled with ^{32}P by random primer extension. The filter was analyzed with a Betascope 603 and visualized by autoradiography. The top of the figure illustrates the sequence of the resolution site in vaccinia virus. The boxed region corresponds to the region of the resolution sequence where mutations were introduced in this analysis. The orientation of the mutagenized resolution sequences corresponds to plasmid pHC in Fig. 1. The nucleotide sequence through the boxed region is shown above and the number of each plasmid is show below the lane for each sample. Upper- and lowercase letters refer to nucleotides different from or identical to those in vaccinia virus, respectively.

poxvirus resolution site. Analysis of the ATTTA stretch found at the left end of the resolution site revealed that any alterations within the three T residues abrogated resolution. In contrast, the initial A could be replaced with a T and the second A could be replaced with a G without significantly affecting resolution. Substitution of the first A with a C or the last A with a T reduced, but did not eliminate, resolution. The left-hand region of the resolution sequence can be represented as (A/T)TTT(A/G). The next eight nucleotides, GTGTCTAG, functioned as a spacer region as changes within this stretch had little or no effect on the efficiency of resolution. The region can vary from seven (fowlpox virus) to nine (Shope fibroma virus) nucleotides, although the fowlpox virus resolution site can also be considered an example of an eight-nucleotide spacer followed by six A residues (Fig. 5). Plasmids containing spacers greater than nine nucleotides were not resolved. Thus, this region can be represented as N_{7-9}. The primary determinant for resolution in the third segment of the resolution site, the seven A residues, was the length of the A stretch. Substitution of a single A residue with a T had a polar effect on resolution as a more dramatic reduction in efficiency was detected as the length of the A stretch closest to the spacer was shortened. Also, the resolution efficiency declined steadily as the length of the A stretch was reduced below 6 nucleotides. This portion of the resolution site can be represented as A_{6-7}. Changes in the GT stretch proximal to the seven A residues affected the efficiency of, but did not prevent, resolution. The results from the mutagenesis of each of the sections of the resolution sequence can be used to establish a generic resolution sequence for vaccinia virus as (A/T)TTT(A/G) N_{7-9} A_{6-7}. The resolution sequence is composed of two disparate AT-rich regions separated by a sequence-independent spacer.

A nucleotide sequence which functions as a resolution site in cells infected with vaccinia virus is found proximal to the hairpin loop in the following representatives of the poxvirus genera, *Orthopoxvirus*, *Leporipoxvirus*, *Avipoxvirus*, and *Capripoxvirus*. For each example, the precise nucleotide sequence may differ from that in vaccinia virus; however, the changes are compatible with the generic resolution sequence for vaccinia virus. For the members of the *Orthopoxvirus*, *Leporipoxvirus*, and *Avipoxvirus* genera, the changes reside only in those nucleotides shown to form the sequence-independent spacer. In *Capripoxvirus*, an additional change has led to the replacement of the ATTTA present in vaccinia virus with TTTTA. This variation of the resolution sequence was successfully resolved (Fig. 1). Also,

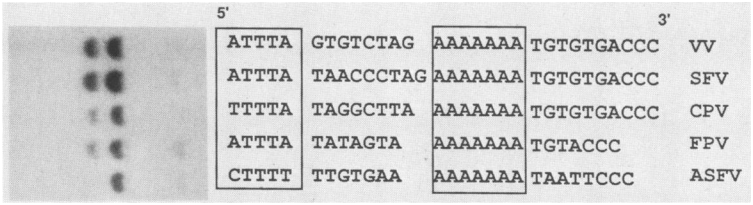

FIG. 5. A functional resolution sequence is conserved among the poxviruses. A series of plasmids containing the resolution sequence from vaccinia virus (VV) or the analogous nucleotide sequences from Shope fibroma virus (SFV), *Capripoxvirus* (CPV), fowlpox virus (FPV), and African swine fever (ASFV) were transfected into vaccinia virus-infected 293 cells. After 24 h, the cytoplasm was isolated and processed as described in Materials and Methods. The DNA was analyzed by digestion with *Nde*I and *Dpn*I, electrophoresis through a 1.5% agarose gel, transfer to a nylon membrane, and Southern blot hybridization using vector DNA labeled with ^{32}P by random primer extension. The nucleotide sequence for the region corresponding to the resolution sequence in vaccinia virus is shown to the right of each lane. The orientation of the mutagenized resolution sequence corresponds to plasmid pHC in Fig. 1. The boxed regions denote the critical regions for resolution of the plasmids containing the vaccinia virus concatemer junction and the analogous regions in the other plasmids.

plasmids containing the concatemer junction from vaccinia virus were resolved in cells infected with Shope fibroma virus (10) or fowlpox virus (Merchlinsky, unpublished), further illustrating the conservation of the resolution machinery among the poxviruses.

The *Iridovirus* African swine fever virus shares many structural similarities to the poxviruses in its mature and replicating forms of DNA (13). A putative resolution sequence derived from African swine fever virus DNA chosen from approximately the same distance from the apex of the terminal loop as the resolution sequence in vaccinia virus was not resolved in vaccinia virus-infected cells. The nucleotide sequence from African swine fever virus was incompatible with the rules derived for the sequence of the resolution site in vaccinia virus. Thus, the sequence required for resolution in African swine fever virus may be present elsewhere in the genome, or conversely, the two virus genera may have diverged so that the apparatuses for resolution are no longer compatible.

The mechanism for conversion of concatemer junctions to mature telomeres is presently under investigation. Experimental evidence implies that resolution occurs via a conservative strand exchange and is dependent on interaction between the pair of resolution sites (19). These observations are consistent with resolution mediated by the formation of a site-specific recombination complex or processing of an extruded cruciform (17). Many site-specific recombination systems are characterized by discontinuous resolution sequences divided into regions utilized for protein binding and nucleotide sequence-independent spacers where strand exchange occurs (5, 30, 32). The role of the different regions of the resolution site in vaccinia virus resolution awaits the purification of the resolution components and characterization of the reaction process.

The critical nucleotides of the resolution site in poxviruses are characterized by the exclusive use of A and T residues. The rightmost critical region for resolution consists of an A tract of greater than 5 residues in length. Adenine tracts of this length have been observed to form unusual conformations within the minor groove that induce DNA bending (3). DNA bending has been implicated in the process of site-specific recombination in lambda (14, 31). The possible role of DNA structure in the formation of intermediates for strand exchange in vaccinia virus resolution is unknown and presently under investigation.

The complementary strand of the resolution sequence, 5′-TTTTTTTN$_{7-9}$TAAAT-3′, is an example of a synthetic strong late promoter. The nucleotide sequence TAAAT, a 6-nucleotide spacer, and a run of T residues were found, when placed upstream of beta-galactosidase, to be about 100 times stronger than the promoter from the 28-kilodalton late gene in vaccinia virus (6). Although RNA transcripts have been observed in the viral telomere region late after infection (28), this putative late promoter, which is directed towards the apex of the hairpin loop, does not contain a proximal ATG or encode a protein. The absence of an open reading frame, however, does not preclude an essential role for the synthesis of an RNA from this sequence late in infection.

The relationship between transcription and resolution can be investigated by comparing the requirements for either process by using the battery of mutants described in this study and those of Davison and Moss (6). The central three A residues from the nucleotide stretch TAAAT were absolutely required for both resolution and late transcription. The mutations in TAAAT with the smallest effect on the activity of the synthetic strong late promoter, the replacement of the initial and last T residues with an A or C (6), had the least detrimental effect on resolution (Fig. 2). The effects of mutations within the TAAAT stretch are comparable for resolution and late transcription.

The correlation between resolution and transcription, however, is not absolute. A great majority of the nucleotide sequences that serve as late promoters (6) do not qualify as resolution sequences (this work). However, the resolution sequence corresponds to nucleotide sequence more active than any natural viral promoter. Therefore, the mutations described by Davison and Moss (6) were checked to determine whether viable resolution sequences always corresponded to late promoters with high levels of activity. Examination of their battery of mutants revealed a strong late promoter that is a poor candidate for a resolution sequence (promoter 496), as well as a relatively weak late promoter that is a good candidate for a resolution sequence (promoter 20). The resolution site does not simply consist of two strong late promoters directed towards each other separated by the double-stranded copy of the hairpin loop. Still, these results do not discount the possibility that an RNA molecule is required in catalytic amounts.

Vaccinia virus concatemers are processed poorly by conditionally lethal viruses deficient in late protein synthesis, implying that resolution is dependent on the synthesis of late gene products (7, 24). Alternatively, these results may reflect a direct interaction of the resolution sequence with a component(s) of late transcription. The description of the primary sequence used for resolution provides a reagent for investigating the regions of the protein-DNA interactions important in the process. Efforts are underway to discriminate among the potential mechanisms for resolution.

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